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# Genetics, mycotoxins and diagnostics of *Fusarium* species involved in Fusarium Head Blight of cereals, with particular attention to *Fusarium graminearum*, for a higher quality and safer cereal production.

**Academic Supervisor**

Prof. Salvatore Frisullo

**ISPA-CNR Supervisor**

Dr. Antonio Moretti

**Coordinator**

Prof. Francesco Contò

**PhD candidate**

Dr. Veronica Ghionna

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# SUMMARY

On wheat, *Fusarium* species can cause the so-colled Fusarium Head Blight (FHB), that is one of the most widespread and damaging diseases of cereals and can cause dramatic losses of production. The main agent of FHB is *Fusarium graminearum,* re-classified in the last decade as species complex (FGSC) composed by 16 phylogenetic entities. The MTX profile of each of these species can vary and different chemotypes co-exist. Toxigenic *Fusarium* species may have significant difference in the profile of MTX produced, in pathogenicity and geographical distribution. The worldwide continuous changes of weather conditions, including Italy, are leading to an ever-changing profile of the typical dominant species on cereals which can result also in a dramatic variability of the contaminant MTX associated to the different *Fusarium* species. Therefore, it is important to carry out a correct identification of the *Fusarium* species that alternate or associate in the attacks of the grains, in order to perform a proper mycotoxicological risk assessment associated with them and the subsequent development of control strategies taking into account differing biochemical and genetic traits of these species and also their different ecological and environmental preferences.

The main objective of the project was to contribute to a wide, comprehensive, reliable and statistically significant knowledge on identification of the main species of *Fusarium* contaminating cereals in Italy in comparison with some key areas in Europe (Austria and Germany) and in the world (China). A total of 569 samples of wheat, barley, maize and other cereals as rye, oat, sorghum, and triticalefrom Austria, Germany, China and Italy were analyzed for the multi-mycotoxin occurrence by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and fungal contamination. The *Fusarium* colonies were morphologically identified at species level; representative strains were selected for the molecular confirmation, conducted by a multi-locus housekeeping gene-sequencing (calmodulin, translation elongation factor 1-α and β-tubulin) approach. The fungal strains identified as species of the FGSC were also analyzed to assess their molecular and chemical chemotype, including the new NX-2 recently described.

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# CHAPTER 1

## General introduction

## 1.1 Mycotoxins

Among the emerging issues in food safety, the increase of plant diseases associated to the occurrence of toxigenic fungal species and their secondary metabolites is of major importance. These fungi can synthesize hundreds of different secondary metabolites, most of whose function is completely unknown. Among these metabolites, the mycotoxins (MTX), characterized by low-molecular weight, may have toxic activity to several human and animal physiological functions (Richard, 2007). Therefore, these pathogenic fungi cause considerable yield losses for crops because MTX can be accumulated in the final crop products and many products of a-gro-food interest. Moreover, many of them can also be toxic towards the plants inducing a wide range of symptoms (Logrieco et al., 2002). This contamination can occur both in the field, until the harvest stage, and in the grain storage. MTX are among the most important food contaminants to control, in order to protect public health around the world. According with Kuiper-Goodman (Kuiper-Goodman, 1994), MTX are the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues. According with a Food and Agriculture Organization (FAO) study, approximately 25% of the global food and feed crop output is affected by MTX (FAO, 2004). Due to their broad range of biological activities, many of them discovered in the recent decades, the consumption of MTX-contaminated foods became a main issue in food safety worldwide. This is particularly so since a large proportion of the world's population consumes, as a staple food, cereals. MTX are produced on the plants before the harvest due to toxigenic fungal contamination in the field and also at the postharvest stage, encompassing stages of the food chain (i.e., storage, processing, and transportation). Moreover, MTX can also be accumulated in animal by-products, due to a carry-over effect, as a consequence of the use of highly contaminated feed. The MTX

identified up to now show, even in low concentration, carcinogenic, mutagenic, teratogenic, immuno-, hepato, nephro- and neurotoxic properties (WHO, 2002). According to the International Agency for Research on Cancer, MTX may be a serious concern for both human and animal health because of their wide range of harmful effects, i.e. mycotoxicoses (IARC, 1993). Typically, health effects, associated with MTX exposures, affect populations in low-income nations, where dietary staples are frequently contaminated and control measures are scarce. MTX are very stable and are hardly destroyed by processing or boiling of food. They are mainly problematic due to their chronic effects. Public awareness concerning health risks caused by long term exposured MTX is poor or even does not exist. Some MTX are now under regulation in several countries, while the risk related to emerging problems and/or new discovered MTX requires urgent and wide investigations. Main MTX-producing genera are primarily: *Aspergillus*, *Fusarium* and *Penicillium* (Marasas et al., 2008). However, also the genus *Alternaria* includes several mycotoxigenic species (Logrieco et al., 2009). Most of the species can produce more than a single MTX, but a given MTX can also be produced by species that belong to different genera.

Although toxigenic fungi can produce hundreds of toxic metabolites (currently, approximately 400 compounds described as MTX are known), only few of them represent a serious concern for human and animal health worldwide: aflatoxins, produced by species of *Aspergillus* genus; fumonisins, produced mainly by species of *Fusarium*, but also belonging to *Aspergillus* genus; ochratoxin A, produced by species of *Aspergillus* and *Penicillium* genera; patulin, produced by *Penicillium* species; and the MTX produced by *Fusarium* species, such as trichothecenes (mainly T-2 and HT-2 toxins for trichothecenes type A; deoxynivalenol, nivalenol and related derivatives for trichothecenes type B), and ZEA (Marasas et al., 2008) (Table 1.1).

**Table 1.1** Mycotoxins of public health concern, associated fungi, and food/feed crops at risk of contamination.

| MYCOTOXINS | PRODUCING FUNGI | ASSOCIATED FOOD/ FEED CROPS |
|---|---|---|
| Aflatoxins | *Aspergillus flavus*<br>*A. parasiticus* | Maize, peanuts, tree nuts, copra, spices, cottonseed |
| Fumonisins | *Fusarium verticillioides*<br>*F. proliferatum*<br>*A. niger* | Maize |
| Trichothecenes | *F. graminearum*<br>*F. culmorum* | Maize, wheat, barley, oats |
| Ochratoxin A | *Penicillium verrucosum*<br>*A. ochraceus*<br>*A. carbonarius*<br>*A. niger* | Maize, wheat, barley, oats, dried meats and fruits, coffee, wine |

Due to their toxicity, a tolerable daily intake (TDI) has been established for the most dangerous MTX, that estimates the quantity of a given MTX to which someone can be exposed to daily over a lifetime without it posing a significant risk to health. Aflatoxins, are the most toxic MTX and have been shown to be genotoxic, i.e. can damage DNA and cause cancer in animal species and there is also evidence that they can cause liver cancer in humans (IARC, 1993). Because aflatoxin contamination is one of the most important risk factors for one of the deadliest cancers worldwide, liver cancer, its eradication in the food supply is critical. It is responsible for up to 172,000 liver cancer cases per year, most of which would result in mortality within several months of diagnosis (Wu et al., 2008). For other agriculturally important MTX such as fumonisins, trichothecenes, and ochratoxin A, proofs that link the exposure to specific human health effects is relatively lower. The role of fumonisins in esophageal cancer is evident, although it may be contributory rather than causal. Trichothecenes have been implicated in acute toxicities and gastrointestinal disorders, and other more long-term adverse effects may be caused by trichothecene exposures. With ochratoxin A, impacts to human populations are limited; however, animal studies suggest possible contributions to toxic effects. The potential for decreased food security, should such foods become less available to a growing human world population, must counterbalance the assessment of human health risks and removal of MTX-contaminated foods from the human food supply. A variety

of methods exists by which to mitigate the risks associated with MTX in the diet. Interventions into preharvest, postharvest, dietary, and clinical methods of reducing the risks of MTX to human health, either through direct reduction of MTX levels in crops or reducing their adverse effects in the human body have been set up (Kabak et al., 2006). Preharvest interventions include good agricultural practices, breeding, insect pest damage or fungal infection, and biocontrol. Postharvest interventions focus largely on proper sorting, drying, and storage of food crops to reduce the risk of fungal growth and subsequent MTX accumulation. Dietary interventions include the addition of toxin-adsorbing agents into the diet, or increasing dietary diversity where possible. Finally, the MTX exposure in human populations could be added to the effects caused by other factors such as interaction with nutrients or other diet contaminants or environmental conditions. Therefore, MTX can be also merely increasing factors for health risks. This is particularly true in vulnerable categories such as young people or pregnant women or populations living in poor/degraded areas. In these situations, MTX exposure may cause even greater damage to human health than previously supposed when evaluated separately. Conversely, reducing MTX exposure in high-risk populations may result in even greater health benefits than may have been previously supposed.

Finally, to date, based on the toxigenicity of several MTX, regulatory levels have been set by many national governments and adopted for use in national and international food trade. Internationally, the Codex Alimentarius Commission (CAC), the EU, and other regional organizations have issued maximum levels in foods and feeds of some selected MTX according to the provisional maximum TDI, used as guideline for controlling contamination by MTX, and preventing and reducing toxin contamination for the safety of consumers.

## 1.2 The *Fusarium:* a toxigenic and phytopathogenic genus

The genus *Fusarium* comprises a high number of fungal species that can be plant-pathogenic, causing diseases in several agriculturally important crops including cereals, and also can be harmful for humans and animals.

Many of them produce a wide range of biologically active secondary metabolites (e.g. MTX) with an extraordinary chemical diversity. The biological activity of *Fusarium* MTX can be detrimental to plants, and associated with cancer and other diseases in humans and domesticated animals.

The combined effect of *Fusarium* species infecting several crops and producing MTX in the field is the contamination of cereal grains and other plant-based foods. With many pathogenic and opportunistic species of the genus colonizing plants as part of a complex of *Fusarium* species, it provides an interesting example of biodiversity as well as a consequence of the different environmental conditions that exist in the various agro-ecosystems in which crops are cultivated. These conditions can also influence the fungal-plant interactions of the single species and their capability to produce MTX.

Moreover, the ability of various *Fusarium* species within the complexes to produce different classes of secondary metabolites combined with their ability to coexist in the same host or/and occur in quick succession have allowed these complexes to become "invincible armadas" against many plants. Plant infections by *Fusarium* can occur at all developmental stages, from germinating seeds to mature vegetative tissues, depending on the host plant and *Fusarium* species involved.

Therefore, since most *Fusarium* species have specific MTX profiles, early and accurate identification of the *Fusarium* species occurring in the plants at every step of their growth

is critical to predict the potential toxicological risk to which plants are exposed and to prevent toxins entering the food chain.

## 1.3 Fusarium mycotoxins

Fungal species belonging to *Fusarium* genus are well known pathogens of cereals and produce a wide range of MTX. Among the *Fusarium* MTX occurring on wheat worldwide, the regulated MTX are trichothecenes, zearalenones, and fumonisins, which are considered the most toxic MTX. However also other minor MTX such as beauvericin, enniatins, fusaproliferin, and moniliformin, have also been found in naturally infected kernels and, although they are not regulated yet, are considered as emerging toxicological problems (Desjardins, 2006).

### 1.3.1 Trichothecenes

The trichothecenes are a unique family of over 200 structurally related secondary metabolites that are very stable and persist in contaminated products even long after the death of the fungus, and is possible to find them frequently in food and feed derived from cereals. In fact, the normal production processes of the food industry are not able to remove or destroy the MTX, if the products at the beginning are contaminated.

Trichothecenes are prevalent grain contaminants that can represent a major threat to food safety and animal health. They occur in wheat, maize, barley, rye, oats, and rice following fungal infection in the field and/or as part of postharvest spoilage. The worldwide incidence of *Fusarium* cereal infection, with concomitant trichothecene contamination, has increased because of climate change, increased use of no-till farming to prevent soil erosion, use of susceptible high-yielding cereal cultivars, non optimal crop rotation, and inadequate fungicide application.

Trichothecenes have significant potential for evoking pathophysiological effects in humans and animals because they interfere with protein synthesis, induce phosphokinase-mediated stress pathways, aberrantly activate proinflammatory gene expression, disrupt gastrointestinal function, interfere with growth hormone action, and cause cell death (Pestka, 2010). Acute exposures to high trichothecene dosages in experimental animals induce anorexia, diarrhea, and vomiting; moreover, at extremely high doses, additional effects can include gastrointestinal hemorrhage, leukocytosis, circulatory shock, reduced cardiac output, and ultimately death. Chronic exposure of animals to moderate doses of trichothecenes impairs food intake, reduces weight gain, disrupts immune function, and can cause developmental effects. Trichothecenes can also act as virulence factors and facilitate tissue colonization in some plant hosts (Jansen et al., 2005).Trichothecenes are esters of sesquiterpenoid alcohols positioned around a trichothecane tricyclic ring that is characterized by a double bond at C9-C10 and an epoxide at C12-C13 (Pestka, 2010).

In *Fusarium* two types (A and B) of trichothecenes are produced. Type A trichothecenes are produced by several *Fusarium* species involved in Fusarium Head Blight (FHB) such as *Fusarium langsethiae*, *F. poae*, and *F. sporotrichiodes* and include T-2 and HT-2 toxin, which are among the most toxic members of this MTX family.

Type B trichothecenes are distinguished from type A by the presence of a keto group at C-8 (Fig. 1.1) (Kimura et al., 2007), and include the important trichothecenes deoxynivalenol (DON) and its two acetylated precursors, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as nivalenol (NIV) and its acetylated precursor 4-acetyl-NIV (fusarenon X [FX]).

The principal toxins produced by the *Fusarium graminearum* species complex (FGSC) and other phylogenetically related species causing FHB (*F. culmorum* and *F. crookwellense*) are the type B trichothecenes (O'Donnell et al., 2000; Ward et al., 2008).

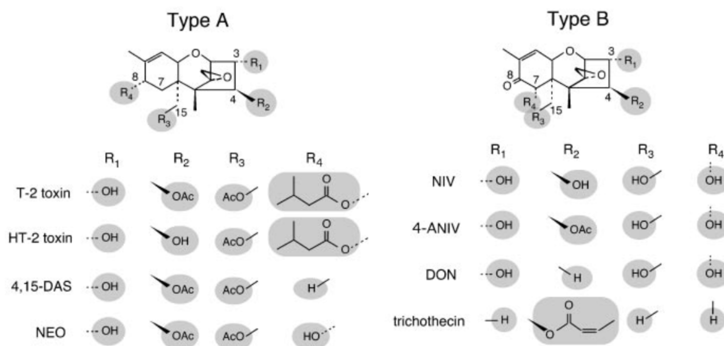


**Figure 1.1** Chemical Structure of Type A and Type B trichothecenes.
In the trichothecene skeleton, there are five positions at which side chains can be added (C-3, C-4, C-15, C-7, and C-8). The trichothecenes are collectively divided into type A (a single bond at C-8) and type B (keto at C-8) on the basis of chemical structure (Kimura et at., 2007).

### 1.3.2 Zearalenones

Zearalenone (ZEA), also known as RAL and F-2 MTX, is a potent estrogenic metabolite produced by several *Fusarium* species, among which the species pathogen *F. graminearum*, *F. culmorum* and *F. crookwellense* (Hagler et al., 2001).

Contamination is especially prevalent in temperate climates when relatively cool temperatures and high humidity coincide with flowering and early kernel filling stages of the grain (CAST, 2003). ZEA is heat-stable and is found worldwide in a number of cereal crops, such as maize, barley, oats, wheat, rice, and sorghum. Given the potential health effects of ZEA and related molecules in humans and domestic animals, food and feed have been, and continue to be frequently tested for these compounds.

ZEA is a resorcylic acid lactone containing an unsaturated bond at C1-C2 and a ketone function at position C6. Either the double bond or the ketone, or both, can be reduced yielding a series of congeners and stereoisomers.

The reductions are important because they affect the biological activity, as well as the physical properties of the molecule. Reduction is also important because it represents a mechanism whereby organisms can biotransform ZEA. The biotransformation for ZEA in animals involves the formation of two metabolites alpha-zearalenol (alpha-ZEA) and beta-zearalenol (beta-ZEA) which are subsequently conjugated with glucuronic acid.

Several animal studies have demonstrated that ZEA and its metabolites have strong estrogenic and anabolic activities. The effects of hyperestrogenism are well known in human diseases, as regards not only alterations of the reproduction cycle but also cancer promotion. In fact, some effects of altered estrogen regulation have been implicated in mammary cancer, liver and colon cancer, desmoid formation and cancer of the uterus (Desjardins, 2006).

**1.3.3 Fumonisins**

Although fumonisins (FUM) are MTX produced by species usually not contaminating small cereals, such as *F. verticillioides* and *F. proliferatum*, recently this group of MTX has been often reported as contaminant of wheat worldwide, including Italy (Palacios et al., 2011; Stankovic et al., 2012; Amato et al., 2015).

FUM are a group of MTX structurally similar to the sphingolipid intermediates sphinganine and sphingosine (Wang et al., 1991). This similarity of FUM with the sphinganine molecule has led some investigators to believe that the mechanism of action of these toxins is related to the inhibition of sphingolipid mechanism, with serious consequences for the cell.

There are at least 28 different forms of FUM, most designated as A-series, B-series, C-series, and P-series, including FUM B1 (FB1), B2 (FB2) and B3 (FB3) that are naturally produced. FB1 is the most important of the group, accounting for 70% of all FUM found in culture and innaturally contaminated maize (Rheeder et al., 2002).

FUM are produced by some species of *Fusarium fujikuroi* species complex (*syn. Gibberella fujikuroi* species complex) (Desjardins et al., 2004). The species of this complex colonize a series of agriculturally important plants and thus pose a serious risk to food safety. The taxonomy of this complex is extremely controversial and some species can not be differentiated morphologically (Leslie and Summerell, 2006). An alternative

taxonomic system species (mating populations) is based on the sexual compatibility. *F. verticillioides* (syn. *F. moniliforme, fujikuroi* mating population A of *Gibberella moniliformis,*) and *F. proliferatum* (syn. *Gibberella intermedia*, *G. fujikuroi* mating population D) are the main source of FUM in food and feed products, especially in maize. *F. verticillioides* is a main pathogen of maize, where it causes ear and stalk rot. This fungus is found in the soil and can be transferred systemically into the plant through the roots, can enter the ear through the silks by air or rainfall, or it can enter through insect damage. Environmental conditions such as drought, high temperatures, and insect feeding can cause stress to the corn plant and can increase the likelihood that species of *Fusarium* will infect the corn kernel.

Conversely, *F. proliferatum* can be a harmful pathogen of a wide range of hosts in addition to maize (Desjardins et al., 2007), and its occurrence in dietary crops such as wheat and barley is gaining recognition (Palacios et al., 2011; Stankovic et al., 2012; Amato et al., 2015).

The primary health concerns associated with FUM are acute (single exposure) toxic effects in horses and swine, and carcinogenic properties. Horses that are poisoned with FUM may develop a fatal disease known as equine leukoencephalomalacia.

Swine that are poisoned with FUM may experience reduced feed intake and weight gain, liver damage, and can develop a fatal disease known as pulmonary edema. Finally, FUM are carcinogenic to laboratory animals, and, in humans, consumption of FUM-contaminated maize is associated with higher rates of esophageal cancer and neural tube birth defects (Desjardins, 2006).

### 1.3.4 Minor *Fusarium* mycotoxins

*Fusarium* species are also able of producing other bioactive compounds considered of ‘‘minor” toxicity. This group includes beauvericin, enniatins, fusaproliferin and moniliformin.

Beauvericin (BEA) is a cyclic lactone trimer containing an alternating sequence of three N-methyl L-phenylalanyl and three D-a-hydroxyisovaleryl residues. BEA was first reported to be produced by some entomopathogenic fungi such as *Beauveria bassiana* (Gupta et al., 1995) and then found in cultures of strains of *F. subglutinans* and *F. proliferatum*, isolated from maize and maize-based feed for swine (Moretti et al., 1995). In addition, BEA was found in cultures of several other *Fusarium* species (Logrieco et al., 1998).

Enniatins (ENNs) are cyclic hexadepsipeptides produced by strains of several *Fusarium* species (Nicholson et al., 2004). ENNs are of high interest because of their wide range of biological activity. They are able to cause ionic imbalance in cells, leading to DNA fragmentation and apoptosis (Kamyar et al., 2004). Also, they inhibit the enzyme acyl-CoA: cholesterol acyl transferase (ACAT), are known as phytotoxins and finally they are associated with plant diseases characterized by wilt and necrosis.

Fusaproliferin (FUS) is a bicyclic sesterterpene consisting of five isoprenic units, which was originally isolated from a pure culture of *F. proliferatum*. FUS is also produced by *F. subglutinans* (Logrieco et al., 1996). FUS is produced through the isoprenoid pathway via common terpene intermediates originating from acetyl-CoA subunits (Meca et al., 2009).

Finally, moniliformin (MON) is an inhibitor of pyruvate dehydrogenase and other thiamine pyrophosphate depending enzymes (Uhlig et al., 2007). It is widespread on cereals in all the world but are its effects on human health are still poorly investigated.

However, in some studies, MON is reported to reduce immune function and cause molecular weakness and acidosis in poultry (Li et al., 2000). Diets containing culture material naturally contaminated with MON, or amended with purified MON, were responsible for reduced performance, haematological disorders, myocardial hypertrophy, and mortality in rodents, chicks, ducklings, and pigs. Severe infections of *F. avenaceum*, *F. tricinctum*, *F. proliferatum* and to a lesser extent *F. subglutinans*, from central to north-east European countries, have been reported as responsible for MON occurrence in scabby grains.

## 1.4 Regulation of mycotoxins

To date, among the countries in the world, the European Union is the first to have the most comprehensive legislative regulations of the MTX.

For this reason, international (FAO, WHO and the European Union) and national (Ministry of Health) institutions, have imposed the introduction of the supervision of all phases of the production chain, to achieve the highest quality standards and to ensure greater security for humans and animals. They report the maximum permissible limits of toxins in food products for trichothecenes, ZEA and FUM in Commission Regulation EC No 401/2006, updated in EC Regulation 1126/2007.

The different trichothecenes have different toxicological properties. NIV is more toxic than DON to humans and domestic animals and a stricter limit for the temporary tolerable daily intake of NIV (0.7 µg kg–1 body weight) has been issued by the European Scientific Committee for Food (Schothorst and van Egmond, 2004; EFSA CONTAM Panel 2013). European Union legal limits for DON introduced in 2007, included 1250 µg/kg for unprocessed cereals intended for human consumption (except durum wheat, maize and oats which have a limit of 1750 µg/kg) (CE, 2007).

Because of their toxic potential, the European Food Safety Authority (EFSA) established a tolerable daily intake (TDI) value of 100 ng/kg body weight per day for the sum of T2 and HT2 (EFSA 2011). Furthermore, the European Commission Recommendation 2013/165/EU provides indicative levels for the sum of these two toxins in cereals and cereal products ranging from 15 μg/kg for cereal-based foods for infants and young children up to 2000 μg/kg for oat milling products (Commission Recommendation 2013/165/EU, 2013).

Several nations have established guidance levels or maximum permissible levels for ZEA in foods, ranging from 50 to 1000 μg/kg (ppb) (FAO, 2004), and regulations relating to MTX in food were recently reviewed (Van Egmond et al., 2007).

The EC No 1126/2007 discipline also the occurrence of FUM toxin, accordingly with the health risk associated with consumption of contaminated commodities. Due to the widespread occurrence of the FUM in maize, a dietary staple in many countries, the carcinogenic risk of FUM humans was evaluated by the International Agency for Research on Cancer. The toxins produced by *F. verticillioides* were evaluated as “Group 2B carcinogens,” i.e., probably carcinogenic to humans (IARC 1993).

In 1999, the Food and Drug Administration published suggested regulations concerning the levels of FUM it would allow in grain. These levels are 5 ppm for human or equine consumption, 10 ppm for swine, and 50 ppm for cattle and poultry. Based on testing of grain samples from fields in eastern North Carolina that showed FUM levels over 20 ppm it quickly became clear that these regulations would pose serious problems for corn growers.

## 1.5 Cereals

Cereals are the main source of food for both humans and animals and represent about 2/3 of the total worldwide nutritional requirements. Their importance derives from some features such as the high-energy, and protein, lipid, mineral and vitamin contents that make them particularly suitable as staple-food.

Cereals are generally of the gramineous family and, in the FAO concept, refer to crops harvested for dry grain only.

For international trade classifications, fresh cereals (other than sweet corn), whether or not suitable for use as fresh vegetables, are classified as cereals.

Production data of cereals are reported in terms of clean, dry weight of grains (12-14 percent moisture).

The nutritional value associated with food derived from cereals is part of our culture and the focus of attention in research programs, oriented to the definition of a correct and healthy diet, is continuously growing. Moulds capable of producing MTX are common widespread contaminants of food and agricultural products, including cereals and by-products. Growth and toxin production can occur in both fields and stock.

Several *Fusarium* species are widespread pathogens on small-grain cereals (soft and durum wheat, barley, oats, rye and triticale) around the world, including all European cereal-growing areas. They can cause root, stem and ear rot diseases, resulting in severe reductions in crop yield, often estimated between 10% and 40%. In addition, several *Fusarium* strains are capable of producing MTX which can be formed in pre-harvest infected plants in the fields, or in stored grain (Bottalico, 2002).

### 1.5.1 Durum wheat

Wheat is one of the oldest and most important of cereal crops. It has become the second largest cereal crop worldwide after rice and is used as a main source of protein and starch for human consumption, animal feed, industrial raw materials, and biofuels.

Wheat is rich in catalytic elements, mineral salts, calcium, magnesium, potassium, sulfur, chlorine, arsenic, silicon, manganese, zinc, iodide, copper, vitamin B, and vitamin E. This wheat wealth of nutrients is why it is often used as a cultural basis or foundation of nourishment.

Among the thousands of varieties known, the most important are common wheat (*Triticum aestivum*), used to make bread, and durum wheat (*T. turgidum ssp. durum*), used for making pasta (alimentary pastes). Common wheat and durum wheat belong to two separate species that form part of the Gramineae family.

Durum wheat is mainly concentrated in the Mediterranean Basin (Central and South Europe, North Africa and western Asia) and North-America. Indeed durum wheat is the main raw material for the production of pasta, bread and couscous in countries bordering the Mediterranean, and in other countries the same cereal is used to produce a wide range of products and often the main food source (Flagella, 2006).

In the last decade has been observed an increase in the consumption of such products, considered a group of important food healthful, balanced and nutritious.

The role that the durum wheat exerts in the feeding is determined by the set of nutritional constituents and by the versatility with which it can be used for the production of widely disseminated that made them particularly appreciated by consumers foods.

The qualitative characteristics of durum wheat grains are important in determining the market designation of the product and also in affecting directly the quality of processed products.

The "long" chain of durum wheat is made up of several components (seed companies, stackers, mills, bakeries and pasta makers) each of which helps to define and influence the "quality" of raw materials and finished products. There are many critical issues that may interfere with the quality and act on the nutritional value associated to the finished product, climatic factors, crop choices, storage operations, processing and packaging processes.

In the last decade, particularly in Italy, many programs aimed at the increasing quality of the grain of durum wheat have been developed, basically directed to effectively respond to the needs expressed by the processing industry in terms of quality standards.

Europe's leading producer of durum wheat is Italy which in 2012 has destined 1.3 million hectares to wheat, with a production of 4 Mt (ISTAT data). Durum wheat certainly plays a fundamental role in the Italian agri-food system, not only from a nutritional point of view, but also from economic, environmental and cultural one.

Community contributions, much higher than ones for soft wheat, have stimulated the expansion of crops of durum wheat from regions where before it was limited exclusively (Sicily, Sardinia, Puglia, Basilicata, Lazio and Tuscany), to others regions of North Italy. The last crop year recorded an increase in sowings of 35.7% while the cultivated areas have increased by 20.1%. The regions with the highest production growth have been Emilia Romagna (+ 113%), Sicily (+ 56%), Basilicata (+ 42%) and Tuscany (+ 41%).

Common wheat, also known as bread wheat or soft wheat (soft because the grain breaks easily), is a cultivated wheat species.

### 1.5.2 Common wheat

About 95% of the wheat produced is common wheat, which is the most widely grown of all crops, and the cereal with the highest monetary yield. It includes different varieties

and has a wider crop extension compared with the other wheat because, for its good cold resistance, is the only in cultivation in the northern countries.

Common wheat is one of the world's three main cereal crops, along with rice and maize.It is a cultigen (a plant that has been altered by humans through a process of selective breeding) and as such is only known in cultivation. First domesticated at least 9,000 years ago, its origins have been the subject of intensive botanical and genetic research. It is a member of the Gramineae family (Poaceae), which includes cereals such as rice (Oryza sativa), maize (Zea mays) and oat (Avena sativa), and ornamentals such as bamboos.

There are about 5,000 cultivars of common wheat in current use. Historically, about 35,000 cultivars have been developed, but the vast majority of these are no longer cultivated on a commercial scale. It is the world's most widely planted crop, occupying 225 million hectares in 2009 (compared with 161 million ha of rice and 159 million ha of maize in that same year). China and India are the largest producers of common wheat, nearly all of which is destined for their domestic markets.

When grains are milled, the outer husk or bran that encloses them becomes a by-product. Wheat bran contains starch, protein, vitamins and minerals, and its dietary soluble fibres are good for the digestive system. It is used to enrich bread and breakfast cereals. Common wheat is a major ingredient in foods such as breads, crackers, biscuits, pancakes, pies, pastries, cakes, sauces, muesli and breakfast cereals.Wheat is used to produce boza (a fermented beverage, produced mainly in the Balkans and Turkey) and wheat beer (production of which is minor compared to beer made using barley). It is also distilled to produce vodka and other spirits.

### 1.5.3 Wheat plant fungal diseases

Generally, most of cereal diseases is caused by classical biotic agents (mainly fungi and virus). All this pathogen can affect different parts of the plant, attacking base of the stem,

roots, leaves, ear and seeds. These diseases can cause damage both in terms of quantity (grain yield) and quality (nutrient quality and the presence of MTX).

Fungal diseases can cause symptoms on all parts of the plants. Among the diseases that cause damage to roots and stems, the most important are the "Take all", caused by *Gaeumannomyces graminis f. sp. tritici syn. Ophiobolus graminis*, important fungus causing rotting of the roots and lower stems, the "Crown and Root Rot" of winter wheat, caused by the infection of roots and crowns by Bipolaris sorokiniana and several species of *Fusarium*, and the so-called syndrome of "*Fusarium* stem rot", characterized by rot and reduced tillering.

Also, leaf diseases of wheat can reduce dramatically the harvest of wheat worldwide, although this appear with different incidence and severity from year to year and from area to area. The main diseases are powdery mildew, septoria and rusts (yellow, brown and black).

Finally, also ears can be attacked by several fungus with devasting effects on the harvest that can be deeply reduced. Among these diseases, the FHB of cereals is reason of wide concern.

## 1.6 Fusarium Head Blight

### 1.6.1 Symptomatology

FHB or scab of wheat and other small cereal grains is an economically devastating disease worldwide (Windels et al., 2000).

The symptoms of FHB are similar in all the affected cereals (Parry et al., 1995). The first symptom, water-soaked lesions of 2-3 mm in length, appears within 2-4 days after infection under favorable conditions, mostly at the base of the middle spikelets in the middle of the head (Stack, 2003). In water-soaked lesions in the glumes, the veins have a

darker olive-green color than the area between veins. Soon after the water soaking appears, symptoms spread to the rachis. Through the rachis the fungus can rapidly spread up, down and horizontally in the spike.

Frequently salmon to pink colored fungal growth and orange colored sporodochia can be seen at the base of the spikelets or along the edge of glumes. Under humid conditions water-soaked lesions may turn brown to purplish-brown with or without a bleached center.

Similarly, early infected spikelets give a 'scabbed' appearance due to formation of blue-black perithecia under prolonged moist and humid conditions. If the environment is dry, the water-soaked symptoms turn the typical color of ripe head. In most of the cases, in susceptible cultivars of wheat, fungal growth in the rachis causes vascular occlusion cutting off the nutrient and water supply to spikelets above the point of infection (Bai and Shaner, 1996). This results in healthy spikelets above the infection point drying out and turning to the color of mature heads.

Such dried spikelets shrink and oppress the rachis. Grains do not form or do not develop fully on such spikelets depending upon the stages of grain at which vascular tissues become dysfunctional. This phenomenon is more evident in susceptible cultivars of wheat. In some cases one or a few vascular bundles remain uninfected continuing the nutrient supply to the spikelets above the infected spikelets allowing the formation of normal grain.

In addition to the floret, characteristic signs and symptoms of FHB also develop in kernels. Symptom development in kernel depends on the time of infection. In severely infected and early infected spikelets, kernels do not develop. If kernels develop, they are smaller and lightweight with a shriveled and chalky appearance and are referred to 'tombstones' or 'Fusarium damaged kernels' (FDK) or scabby kernels (Parry et al.,

1995). If the infection occurs towards late stage of the kernel development, kernels may achieve normal size and weight, but may look discolored with pinkish areas (Atanasoff, 1920).

The frequency and severity of the disease are closely related to the amount of inoculum present, the presence of the stem of plants that have contracted the infection early and/or crop residues left in the soil.

Environmental conditions characterized by rainy or hot humid periods can also promote the development of the disease. The moment of maximum sensitivity of the plant coincides with the beginning of flowering. Several other factors also contribute to increase the virulence of the infection such as early and deep sowing, no tillage high seeding density and the lack of rotations. The development of these pathogens in the period between the heading and maturation, and in particular during the anthesis, may induce the occurrence of serious epidemics with consequent loss of production in terms of yield, quality and health of the grains. The quantitative damage involving a reduction in the specific weight of the grain and a reduction in production that can even reach 70%. Qualitative damage include the production of infected seed, seed production with a reduced content of proteins and the production of grain with presence of harmful MTX for human and animal health. Indeed, several *Fusarium* species can produce a range of MTX in infected grains (Gutleb et al., 2002), making them unsuitable for animal and human consumption.

### 1.6.2 FHB: a species complex

The aetiology of the disease is complex and only partly understood (Xu, 2003). The environment is known to play an important role in FHB development. The distribution and predominance of a *Fusarium* species in a country seem to be determined by climatic factors, particularly rain, humid conditions and temperature at flowering stage. The

species profile of FHB is also due to agronomic practices, such as cropping sequence, soil tillage, sowing on untilled soil, use of nitrogen fertilizers, fungicides and host genotype, as well as to competition from the *Fusarium* species (Doohan et al., 2003). Understanding the population structure of FHB-pathogens is important for several reasons. First, FHB pathogens may respond differentially to control methods; second, the environmental requirement for infection and subsequent colonization may differ among FHB species; third, each *Fusarium* species can have its MTX profile.

Thus, understanding community structure is important for accurate prediction of disease, MTX risk and effective control of FHB. The *Fusarium* species predominantly found associated with FHB in wheat and other small-grain cereals all over Europe are *F. graminearum sensu stricto* (s. s.), *F. avenaceum* and *F. culmorum* (Nicolaisen et al., 2009). Among the less frequently encountered species, there are several others which are less pathogenic or opportunistic, but species that can be also toxigenic. These include *F. poae, F. crookwellense, F. equiseti, F. sporotrichioides, F. tricinctum, F. langsethiae* (Ioos et al., 2004; Xu et al., 2005; Xu and Nicholson, 2009; Somma et al., 2014) and, to a lesser extent, *F. acuminatum, F. subglutinans, F. solani, F. oxysporum, F. verticillioides, F. semitectum* and *F. proliferatum* (Stankovic et al., 2012). In general, *F. graminearum* and *F. culmorum* are the most aggressive pathogens of wheat and barley (Brennan et al., 2003). Species such as *F. poae* can encourage the colonization of other mycetes more aggressive, involved in FHB. *F. culmorum* seems to prefer cooler weather and therefore is prevalent in England, Northern Europe and Canada, where it appears to be the main agent of FHB species. *F.graminearum s. s.*, which is considered the most widespread species in the world (USA, Australia, South America, Asia and Central Europe) is most common in temperate regions (Pasquali et al., 2016) and currently it

seems to have replaced other species (eg. *F. culmorum*), due to short-term climatic variations, ecological factors and aspects of the agronomic practices (Xu et al., 2005).

## 1.7 The main FHB agent: *Fusarium graminearum*, a toxigenic and phytopathogenic fungal species

### 1.7.1 Pathogen biology

The increasing occurrence of FHB in the last decades along with its effect on yield and quality grain (Alvarez et al., 2010), and the grain contamination with MTX make this pathogen one of the most intensively studied fungal plant pathogens. Pathogenicity and aggressiveness are two important characteristics of *F. graminearum* (Von der Ohe et al., 2010). These two words have distinct meanings. Pathogenicity is a qualitative measurementthat reflects the ability of a fungus to cause the disease, while aggressiveness is a quantitative measurement of the level of the disease provoked by the pathogen. Aggressiveness is a fundamental aspect which is considered important to understand the interactionbetween host-pathogen in FHB-wheat system (Wu et al., 2005).

*F. graminearum* is a pathogen of more than 26 plant genera, including economic crops such as wheat (Triticum), barley (Hordeum), rice (Oryza), oats (Avena) and maize (Zea) (Goswami and Kistler, 2004) and is also able to cause disease in several plant parts (i.e. root, stem and spike) (Akinsanmi et al., 2004; Fernandez and Chen, 2005). This low specificity makes its aggressiveness and spread in plant tissues variable and greatly influenced by environmental conditions, thus a precise and accurate assay to quantify the levels of aggressiveness of *F. graminearum* is needed.

*F. graminearum* belongs to the fungal phylum *Ascomycota*. The fungus has both anamorph (asexual stage), during which macroconidia are produced, and teleomorph (sexual stage, previously known as *Gibberella zeae (Schwein) Petch*; syn. *G. roseum*), during which the fungus produces ascospores (sexual spores) in dark purple perithecia,

which appear black. The ascospores are forcibly discharged from the perithecium through a single small opening known as an ostiole.

The majority of the isolates of *F. graminearum* are homothallic, meaning that they are able to sexually reproduce without a partner. Heterothallic isolates, which require a compatible partner for sexual reproduction, are less common. Laboratory studies, however, have demonstrated that many of the homothallic isolates have the ability to outcross with other compatible isolates. The degree to which this occurs in the field under natural conditions is unclear. When *F. graminearum* grows on potato dextrose agar media, white mycelial colonies grow fast and develop a characteristic colour of pink to dark purple (Samuels et al., 2001). Often *F. graminearum* produce chlamydospores, a thick walled structure formed from macroconidia and/or mycelium, when they are in contact with soil. Chlamydospores are globose to oval in shape, 8-12 μm in diameter and pale brown in color. They are generally produced in pairs or short chains. Though the function of chlamydospores in other microorganisms is for survival, its function in *F. graminearum* is largely unknown.

*F. graminearum* overwinters on infested crop residues (corn stalks, wheat straw, and other host plants). On infested residues, the fungus produces macroconidia which are dispersed to plants and other plant debris by rain-splash or wind. When conditions are warm, humid and wet, the sexual stage of the fungus develops on the infested plant debris. Bluish-black perithecia form on the surface of these residues, and forcibly discharge ascospores into the air. The ascospores are picked up by turbulent wind currents and may travel great distances in the air (Fig. 1.2).

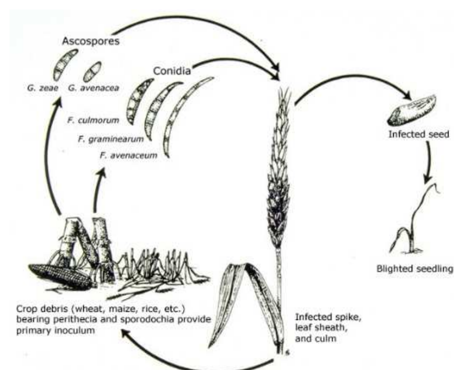


**Figure 1.2** Disease Cycle and Epidemiology.

Infection occurs when the ascospores (and also macroconidia) land on susceptible wheat heads. Extruded anthers during wheat anthesis (flowering) are thought to be the site of primary infection. If the anthers are infected just after their emergence, the fungus will colonize and kill the florets and kernels will not develop. Florets that are infected later will produce diseased kernels that are shriveled and wilted, or "tombstone" in appearance. Kernels that are colonized by the pathogen during late kernel development may not appear to be affected.

Infected kernels may be used as seed for a subsequent wheat crop. These infected seeds, if left untreated, may give rise to blighted seedlings. The degree to which this occurs in the field depends on the percentage of infected seed and soil conditions

At first, *F. graminearum* was thought to be a single potentially panmictic species spanning six continents until genealogical concordance phylogenetic species recognition (GCPSR, Taylor et al., 2000) was used to investigate species limits using a global collection of FHB strains (O'Donnell et al., 2000).

In the last decade, *F. graminearum* has been re-classified as species complex (FGSC) composed by at least 16 recognized phylogenetic entities (O'Donnell et al., 2000, 2004, 2008; Starkey et al., 2007; Yli Mattila et al., 2009; Sarver et al., 2011).

Originally the members of this complex were referred to as lineages within *F. graminearum*, but most have now been formally described as separate species that cannot be differentiated by morphology. The FGSC is a very dynamic complex of morphologically related species that occur in all cereal-growing regions around the globe. Different members show varying levels of host preference, namely *F. asiaticum* on rice, *F. boothii* and *F. meridionale* on maize, whereas *F. graminearum* is encountered on wheat, barley as well as maize.

*F. graminearum* isolates around the world show a geographic substructure. Even populations that are geographically close can show distinct substructuring, meaning that *F. graminearum* is not a single monophyletic species but it should be considered as a meta-population consisting of many relatively independently developing populations.

*F. graminearum s. s.* is the dominant species of FGSC associated with head blight in North America (Ward et al., 2008), whereas *F. asiaticum* appears to be the major species in temperate regions of Asia (Yang et al., 2008).

Species of the FGSC are capable of producing MTX harmful, trichothecenes and ZEA, in planta and each of them has a specific MTX profile.

**1.7.2 Chemotypes and their diffusion in the world**

Among type B trichothecenes, beside DON and NIV, the species of FGSC and other phylogenetically related species causing FHB (e.g. *F. culmorum* and *F. crookwellense*) (O'Donnell et al., 2000; Ward et al., 2008) can produced DON acetylated precursors, 3-ADON and 15-ADON, and NIV acetylated precursor, 4-acetyl-NIV [fusarenon X (FX)]. In particular, the populations of *F. graminearum s. s.* are differentiated according to the production of different types of type B trichothecenes, which define specific toxigenic profiles called chemotypes.

Chemotype is the production profile of chemical molecules and genotype defines the genetic constitution or its DNA sequence (Desjardins, 2008).

Trichothecene chemotype variation has received considerable attention in analyses of populations of *Fusarium* species that cause FHB (Miedaner et al., 2008; Starkey et al., 2007; Xu and Nicholson, 2009).

*F. graminearum* may be divided into two chemotaxonomic groups based on production of 8-ketotrichothecenes (Ichinoe at al., 1983): DON and NIV chemotypes.

The DON chemotype produces DON and acetyl-DONs such as 3-ADON and 15-ADON.

These trichothecenes differ by the presence and absence of acetyl functions at C-3 and C15; 3-ADON has a C-3 acetyl but lacks a C-15 acetyl, whereas 15-ADON has a C-15 acetyl but lacks a C-3 acetyl. DNA sequence polymorphisms in the regions of the core TRI cluster genes TRI3 and TRI12 are correlated with the 3-ADON and 15-ADON chemotypes and have been used as genetic markers to distinguish between the chemotypes in strains of the FGSC (Starkey et al., 2007).

Even though strains tend to produce predominantly 3-ADON or 15-ADON, the nonacetylated trichothecene DON often accumulates in wheat grain (Burlakoti et al., 2008), presumably because wheat or fungal esterases (deacetylases) remove the acetyl units from 3-ADON or 15-ADON.

The NIV chemotype produces NIV and 4-ANIV (also known as fusarenon-X).

The two chemotypes appear to differ in geographic distribution. From different surveys carried out worldwide on the presence of trichothecenes in wheat kernels infected by *F. graminearum*, the DON chemotypes predominate in North America, South America, and Europe, while the NIV chemotypes are rare in North and South America and more frequent in Europe (O'Donnell et al., 2000; Rasmussen et al., 2003). In the United States, *F. graminearum* mainly produces the type B trichothecenes, DON and its acetylated derivatives, and to a lesser extent, the related trichothecene NIV (Liang et al., 2014).

Surveys indicate displacement of the existing 15-ADON population by the 3-ADON population in some regions of North America and Canada (Van der Lee et al., 2015).

Analyses revealed that these chemotypes represent genetically different populations that coincide with different phenotypic attributes: 3-ADON populations produce more and larger conidia, grow faster and accumulate more trichothecene than do 15-ADON producers. When these features are also expressed under field conditions, this can account for enhanced fitness. In turn this can lead to rapid displacement of the 15-ADON

chemotype, in turn leading to a more toxic population in North America (Ward et al., 2008).

Similar results were obtained in the upper Midwest of the United States, where besides a predominate presence of 15-ADON producers (approximately 95%) some isolates with 3-ADON chemotype also were observed (Gale et al., 2007).

The dominance of *F. graminearum* was observed in European countries, as France (Waalwijk et al., 2008), Germany (Talas et al., 2011) and Italy (Prodi et al., 2009, 2011). Boutigny et al. (2014) identified *F. graminearum* as the dominant FGSC species on wheat, barley as well as maize in France. These populations were predominantly 15-ADON, but some NIV types were observed.

In Italy, the 15-ADON genotype was predominantly found in regions with a cool winter climate (Prodi et al., 2009, 2011). These findings were confirmed in another study that showed mostly 15-ADON-producing *F. graminearum* in Italy (Somma et al., 2014).

DON and NIV are frequently found in cereals harvested in some Asian countries, e.g., Korea, China, Nepal and Japan (Desjardins, 2006). NIV is present at higher levels than DON in cereals from these countries (Yoshizawa at al., 1995; Van der Lee et al., 2015).

From the point of view of pathogenicity, chemotypes 3-ADON and 15-ADON are more aggressive towards wheat, compared with NIV chemotypes, probably reflecting a selective advantage to this type of strains (Desjardins, 2006).

To date, the analysis of the distribution of genetically determined chemotypes confirms the dominance of the 15-ADON chemotype in Western, Southern and Central Europe (Pasquali and Migheli, 2014), the dominance of the 3-ADON chemotype in Northern Europe (Yli-Mattila et al., 2013) and NIV chemotype was preferentially found in Western Europe.

## 1.8 Objectives of thesis

Samples of cereals worldwide and from several Italian areas were analyzed, in order to understand the level of fungal contamination and *Fusarium* colonies were isolated for further analyses.

Main objectives of this PhD thesis were:

- Identification of the main species of *Fusarium* contaminating cereals in a multi-year screening.
- Analyses of MTX levels (by multi-mycotoxin liquid chromatography-tandem mass spectrometry) contaminating the cereals samples.
- Identification of the chemotype of the strains belonging to the FGSC, including the new NX-2, in a worldwide collection.
- To evaluate the pathogenicity pathway of *F. graminearum* during the wheat infection process.

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# CHAPTER 2

## IDENTIFICATION OF *FUSARIUM* SPECIES OCCURRING ON CONTAMINATED CEREALS SAMPLED WORLDWIDE

### 2.1 ABSTRACT

Cereals represent the major staple food for many people at worldwide level. Among the diseases that affect these crops, the occurrence of *Fusarium* species is related to the highest risk for the consumers since many *Fusarium* can produce a wide range of harmful mycotoxins (MTX) that can be accumulated in the cereal kernels. In particular, Fusarium Head Blight (FHB) of wheat and other minor cereals is caused by a complex of species, each provided of specific MTX profile. Moreover, the main species can vary in the different geographic areas because they can be influenced from the changing environmental conditions. Therefore, a reliable identification of the most occurring species is important for the correct evaluation of the potential toxicological risk of contaminated kernels. For this reason, screenings are often carried out in order to evaluate the real contamination associated to *Fusarium* species on geographic areas which suffers of particular environmental conditions leading to FHB. In our study, 361 samples of cereals (mainly wheat, barley, maize, and at less extent sorghum, rye, oat and triticale) were collected from Austria, Germany and China during a period of three years (2011-2013) and analyzed for the multi-mycotoxin by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and related *Fusarium* contamination.

Among the samples collected in China, the most frequently occurring MTX was enniatin B1 (94% of contaminated). The average concentration of this MTX in positive samples was 10 (ppb). On the other hand the most common deoxynivalenol (DON), zearalenone (ZEA) and nivalenol (NIV) were found in 85%, 36% and 6% of all the samples, with the positive samples showing an average contamination level of 840, 39 and 53 (ppb).

In Germany and Austria, the average of MTX detected in wheat and barley was high, being beauvericin (BEA), enniatin (ENNs), DON and deoxynivalenol-3-glucoside (D3G) the most detected MTX. This wide contamination of the samples was reflected by the wide variability of *Fusarium* species isolated and identified. Fungal strains were first identified based on their morphological features and therefore confirmed by sequencing calmodulin, translation elongation factor 1α and ß-tubulin genes. In wheat collected in China, *Fusarium graminearum sensu stricto* (s. s.) was the most frequently isolated species. In Germany and Austria, in both barley and wheat, *F. graminearum s. s., F. poae* and species belonging to *F. tricinctum* clade were the most occurring species identified.

Finally, on maize samples collected in Austria and Germany, fungal strains morphologically identified as *F. subglutinans* and *F. temperatum* were the most occurring species, followed by *F. verticillioides* and *F. graminearum.*

## 2.2 Introduction

Mycotoxins (MTX) are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly belonging to *Alternaria spp., Aspergillus spp., Fusarium spp.* and *Penicillium spp.*

The contamination of cereals, that constitute the main staple-food at global level, is causing great concern, because of the increasing level of mycotoxigenic fungi contamination of food products, worldwide. In particular, *Fusarium* MTX are the most frequently detected in the worldwide routine standard screenings, aimed to investigate the MTX associated to cereals. Many species of *Fusarium* can be involved in the colonization of kernels and can cause both loss of harvest, reduction of the kernel quality, and accumulation of MTX on cereals. Moreover, since each of these species have their own pathogenicity capability, ecological niche, and mycotoxin profile (Bottalico et al., 2002), a correct identification is a key issue for both management of disease in the field and risk assessment. According with geographical areas and environmental conditions, the *Fusarium* species associated to cereals can dramatically change, therefore a wide variability can be detected in terms of *Fusarium* species and MTX associated, worldwide. Among the main *Fusarium* MTX, trichothecenes and zearalenones (ZEA) are the most important on wheat and minor cereals since the *Fusarium* species that produce them, frequently occur on these crops. On the other hand, fumonisins have a wide geographic distribution on maize. Beyond to the most common *Fusarium* toxins, other emerging MTX have been reported in huge quantities as cereal contaminant. Among these, beauvericin (BEA), enniatins (ENNs), and moniliformin (MON) have been often detected in wheat, maize and other minor cereals and therefore have been considered as a potential health threat. According with O'Donnell et al. (2013), species of *Fusarium* can be

grouped phylogenetically to species complexes that better reflect their genetic complexity.

While the species related to trichothecenes belong to the *Fusarium sambucinum* and *F. incarnatum-equiseti* species complexes (O'Donnell et al., 2013), the species producing fumonisins belong to *F. fujikuroi* species complex and those that produce BEA and ENNs mainly belong to *F. fujikuroi* and *F. tricinctum* species complexes.

In *Fusarium*, trichothecenes are grouped into two classes, A and B, based on the presence (type B) vs. absence (type A) of a keto group at the C-8 position (Kristensen et al., 2005). Type B trichothecenes are produced by the FGSC species, mainly *F. graminearum s. s.* and species phylogenetically related among which *F. culmorum* is a very common pathogen of cereals. Moreover, also species of the *F. incarnatum-equiseti* complex can produce type B trichothecenes. The level of these MTX have a significant impact on food and feed safety due to their toxicity and the wide natural dissemination in the field at global level (Wu et al., 2014). Both species, *F. graminearum s. s.* and *F. culmorum* are worldwide disseminated (Boutigny et al., 2014, McMullen et al., 2012, Puri et al., 2012), however, in the last decade, *F. graminearum* is increasing its geographical spreading, colonizing also the cooler regions of Europe (Yli-Mattila et al., 2008; Fredlund et al., 2013).

The type A trichothecenes include T-2 and HT-2, provided of acute toxicity (Desjardins, 2006) and mainly produced by strains of *F. langsethiae* (Imathiu et al., 2013), and *F. sporotrichioides*, two common fungal pathogens of cereals (Desjardins, 2006). Occurrence of T-2 and HT-2 have been reported since decades mainly in Northern-Europe in small grain cereals (e.g. oat and rye) (Langseth et al., 1999; Edwards et al., 2009). However, more recently, both MTX have been detected in barley and wheat in

France and Italy (Covarelli et al., 2012; Lattanzio et al., 2012), while their occurrence in maize is rarely described.

Finally, the fumonisins are commonly reported on maize worldwide (Desjardins, 2006), being *F. verticillioides* and *F. proliferatum* almost-universal inhabitant of maize, and in certain climatic and environmental conditions causing Fusarium ear rot (Madania et al., 2013). However, recently, the occurrence of fumonisins in wheat has been reported in several geographical areas such as USA, Argentina, Serbia and also Italy, therefore lot of care must be addressed to the possibility that these MTX can become a common health risk for the consumption of contaminated wheat.

Therefore, the main objects of this part of thesis were: i) to sample some key geographical areas where cereal fusariosis is endemic (Austria/Germany) or poorly investigated for the *Fusarium* genetic variability (China) in order to isolate *Fusarium* populations to compare with those to be isolated from Italian samples; ii) to analyze MTX levels in cereals samples; iii) to identify the main species of *Fusarium* contaminating cereals in a multi-year screening.

## 2.3 Materials and methods

### 2.3.1 Collection of samples and isolation of *Fusarium* species.

A set of 361 samples of cereals (mainly wheat, barley and maize, and at less extent sorghum, rye, oat and triticale) was collected from Austria, Germany and China during a period of three years (2011-2013).

Samples were washed in sterile water, disinfected in a 2% sodium hypochlorite solution for 2 min, rinsed twice in sterile water to eliminate any hypochlorite residue and dried on sterile filter paper. After that, one hundred surface-disinfected kernels for each sample were placed in Petri dishes 90-mm diameter containing a *Fusarium spp*. selective media,

PDA (potato dextrose agar) amended by PCNB (pentachloronitrobenzene), and 0.1 g L-1 streptomycin and 0.05 g L-1 neomycin sulphate antibiotics enriched (Nelson et al., 1983).

Thus, only fungi from internal infestations were studied. After 5 days of incubation at 25 °C, seeds developing colonies of the species of *Alternaria, Aspergillus, Fusarium* and *Penicillium* genera were counted and data of contamination (expressed in%) were collected. A representative colony of each morphotype has been isolated for the morphological identification.

A plug of mycelium from each *Fusarium* colony was placed in sterile water, subjected to several dilutions and drops of the last suspension were plated with a loop on Petri dishes containing water agar. After 48 hours of incubation at 25 °C, the germinated spores were transferred to PDA in order to obtain single-spore cultures.

A total of 544 pure cultures of *Fusarium* species were transferred on SNA (synthetic nutrient-poor agar) and incubated at 25 °C for 5/6 days to produce conidia of uniform size and form, which were identified to species level by their colony characteristics and conidial morphology (Nelson *et al.,* 1983; Leslie and Summerell, 2006).

Morphological identification has been supplemented with molecular approaches, to ensure that morphological similarities do not mask significant genetic differences. A subset of representative strains (n = 80) has been submitted to DNA sequencing (3 genes) (see Table 2.1).

Strains were isolated from Austria (27 strains, from 214 samples), Germany (26 strains, from 47 samples) and China (27 strains, from 100 samples).

Dendrograms were generated for each gene, comparing sequences of 17 *Fusarium* reference strains and of 80 *Fusarium* strains selected following two criteria: first, the selection was made among the strains that showed ambiguous morphological features in

order to confirm unambiguous their identity; second, the strains identified as *F. graminearum s. s.* were selected in order to assign them to a single species of the FGSC. The fungal strains were deposited in the ITEM fungal collection of ISPA (http://www.ispa.cnr.it/Collection).

**Table 2.1** Set of representative *Fusarium* isolates used in this study. Geographical origin and host plant are shown.

| | COLLECTION NUMBER | ORIGIN | HOST PLANT |
|---|---|---|---|
| 1 | 16505 | Austria | Barley |
| 2 | 16506 | Austria | Barley |
| 3 | 16508 | Austria | Barley |
| 4 | 16513 | Austria | Barley |
| 5 | 16515 | Austria | Barley |
| 6 | 16517 | Austria | Barley |
| 7 | 16518 | Austria | Barley |
| 8 | 16519 | Austria | Barley |
| 9 | 16520 | Austria | Wheat |
| 10 | 16521 | Austria | Barley |
| 11 | 16523 | Austria | Wheat |
| 12 | 16524 | Austria | Wheat |
| 13 | 16527 | Austria | Wheat |
| 14 | 16528 | Austria | Barley |
| 15 | 16531 | Austria | Wheat |
| 16 | 16532 | Austria | Wheat |
| 17 | 16533 | Austria | Wheat |
| 18 | 16534 | Austria | Wheat |
| 19 | 16535 | Austria | Barley |
| 20 | 16536 | Austria | Triticale |
| 21 | 16539 | Germany | Barley |
| 22 | 16542 | Germany | Wheat |
| 23 | 16544 | Germany | Wheat |
| 24 | 16545 | Germany | Barley |
| 25 | 16546 | Germany | Wheat |
| 26 | 16547 | Germany | Wheat |
| 27 | 16551 | Germany | Wheat |
| 28 | 16552 | Germany | Wheat |
| 29 | 16555 | Germany | Barley |
| 30 | 16566 | Germany | Barley |
| 31 | 16579 | Germany | Barley |
| 32 | 16583 | Germany | Barley |
| 33 | 16585 | Germany | Barley |
| 34 | 16587 | Germany | Wheat |
| 35 | 16588 | Germany | Wheat |
| 36 | 16589 | Germany | Oat |
| 37 | 16592 | Germany | Barley |
| 38 | 16595 | Germany | Wheat |
| 39 | 16597 | Germany | Barley |
| 40 | 16600 | Germany | Oat |

| | COLLECTION NUMBER | ORIGIN | HOST PLANT |
|---|---|---|---|
| 41 | 16615 | Germany | Barley |
| 42 | 16618 | Germany | Barley |
| 43 | 16619 | Germany | Wheat |
| 44 | 16621 | Germany | Wheat |
| 45 | 16622 | Germany | Barley |
| 46 | 16624 | Germany | Barley |
| 47 | 16628 | Austria | Maize |
| 48 | 16630 | Austria | Maize |
| 49 | 16631 | Austria | Maize |
| 50 | 16652 | Austria | Maize |
| 51 | 16655 | Austria | Maize |
| 52 | 16656 | Austria | Maize |
| 53 | 16697 | Austria | Barley |
| 54 | 17052 | China | Wheat |
| 55 | 17053 | China | Wheat |
| 56 | 17054 | China | Wheat |
| 57 | 17055 | China | Wheat |
| 58 | 17056 | China | Wheat |
| 59 | 17057 | China | Wheat |
| 60 | 17058 | China | Wheat |
| 61 | 17059 | China | Wheat |
| 62 | 17060 | China | Wheat |
| 63 | 17061 | China | Wheat |
| 64 | 17062 | China | Wheat |
| 65 | 17063 | China | Wheat |
| 66 | 17064 | China | Wheat |
| 67 | 17065 | China | Wheat |
| 68 | 17066 | China | Wheat |
| 69 | 17067 | China | Wheat |
| 70 | 17068 | China | Wheat |
| 71 | 17069 | China | Wheat |
| 72 | 17070 | China | Wheat |
| 73 | 17071 | China | Wheat |
| 74 | 17072 | China | Wheat |
| 75 | 17073 | China | Wheat |
| 76 | 17074 | China | Wheat |
| 77 | 17075 | China | Wheat |
| 78 | 17076 | China | Wheat |
| 79 | 17077 | China | Wheat |
| 80 | 17079 | China | Wheat |

### 2.3.2 Fungal DNA isolation

DNA was extracted from mycelium harvested from 7-day-old single-spore cultures grown on PDA. Fungal liquid cultures of the selected isolates were obtained inoculating under sterile conditions the mycelium in flasks containing the Wickerham liquid medium (40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and water up to 1 L). After 48 hours of growth in stirring at 25 °C they were filtered, using a vacuum pump, after evaluation of absence of bacteria and yeasts through microscope observation in ambiguous cultures.

The filtrated mycelium was frozen at -20 °C and subsequently lyophilized. The DNA extraction was conducted using Wizard® Magnetic DNA Purification System kit for Food (Promega), starting from about 10 mg of dryed mycelium and following the manufacturer's instructions. The use of magnetic beads allowed to perform a large part of the protocol in an automated way, with the help of the liquid handling station epMotion 5075 (Eppendorf). The quality and integrity of extracted DNA, as well as absence of RNA, were assessed by electrophoresis in 0.8% agarose gel, using a transilluminator with ultraviolet light. The accurate estimation of DNA quantity was obtained with spectrophotometer “NanoDrop ND-1000” (Thermo Fisher Scientific) and the purity was evaluated by the ratio A260/A280 (wavelength to which absorb nucleic acids and both nucleic acids and proteins, respectively), considering "pure" the DNA sample with the ratio around 1.8.

### 2.3.3 Polymerase chain reaction

For representative isolates, gene-based identification approach was performed to confirm the morphological identification.

DNA extracted from all isolates of this study was used to amplify 3 housekeeping genes by PCR with primers listed in Table 2.2.

**Table 2.2** The primer sequences and sizes of the PCR fragments used to identify the *Fusarium* isolates.

| PRIMER NAME | SEQUENCE (5′ TO 3′) | SIZE (bp) | REFERENCES |
|---|---|---|---|
| CL1 | GARTWCAAGGAGGCCTTCTC | ca.700 | O'Donnell et al., 2000 |
| CL2 | TTTTTGCATCATGAGTTGGAC | | |
| EF1 | ATGGGTAAGGARGACAAGAC | ca.700 | O'Donnell et al., 1998 |
| EF2 | GGARGTACCAGTSATCATGTT | | |
| Bt2a | GGTAACCAAATCGGTGCTGCTTTC | ca.400 | Glass and Donaldson, 1995 |
| Bt2b | ACCCTCAGTGTAGTGACCCTTGGC | | |

Amplifications were performed in 15 µl volume of reactions, set up with 1× PCR buffer, 200 µM dNTP's, 300 nM each primer, 0.375  U of Taq DNA Polymerase and 25 ng of genomic DNA, according to the following profiles:

- CL1/CL2 primer pair: denaturation at 95 °C for 2 min; 40 cycles of amplification with denaturation at 95 °□ for 30 s, annealing at 55 °□ for 30 s, extension at 72 °□ for 50 s; final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples.
- EF1/EF2 primer pair: denaturation at 94 °C for 2 min; 35 cycles of amplification with denaturation at 94 °□ for 50 s, annealing at 59 °□ for 50 s, extension at 72 °□ for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples.
- Bt2a/ Bt2b primer pair: denaturation at 94 °C for 2 min; 35 cycles of amplification with denaturation at 94 °□ for 30 s, annealing at 60°□ for 30 s, extension at 72 °□ for 50 s; final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples.

Size of PCR amplicons were verified by 1.5% agarose gel electrophoresis using a molecular weight marker of DNA (Ladder 100bp).

After amplification, PCR products were purified by enzymatic method based on the use of the FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (ThermoScientific) mixture that allows the removal of unincorporated primers.

When multiple products were obtained, the amplicon of interest was purified by gel electrophoresis, excising DNA band extraction from agarose gel and purifying DNA with NucleoSpin® gel and PCR Clean-up.

### 2.3.4 DNA sequencing

Amplicons were sequenced according to the modified "Sanger method" which involves the use of fluorescent markers linked to dideoxynucleotide triphosphates (ddNTP), using the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) for both strands, according to the manufacturer's instructions. All of the sequencing reactions were purified by gel filtration through mini-columns containing Sephadex G-50 (Sigma-Aldrich, Milan, Italy) and denatured for 5 minute at 95 °C and separated by capillary electrophoresis on ABI3130, a 48 capillary sequencer.

### 2.3.5 Sequence analysis

Fragment sequences were obtained aligning both strands with Bionumerics software (Applied Maths). Sequences homologies were searched against GenBank database using Basic Local Alignment Search Tool (BLAST) and the maximum scoring was considered for species identification (≥ 99 in similarity). In the phylogenetic analysis, the sequences of the three genes of 17 reference strains selected among the fungal ITEM collection (http://www.ispa.cnr.it/Collection) to represent the main *Fusarium* species occurring on cereals, were included (Table 2.3).

**Table 2.3** List of reference strains of the *Fusarium* species considered.

| ITEM | SPECIES |
|---|---|
| ***5356*** | ***Fusarium graminearum s. s.*** |
| ***6748*** | ***Fusarium semitectum*** |
| ***7547*** | ***Fusarium incarnatum*** |
| ***7582*** | ***Fusarium subglutinans*** |
| ***7618*** | ***Fusarium oxysporum*** |
| ***7626*** | ***Fusarium culmorum*** |
| ***7630*** | ***Fusarium avenaceum*** |
| ***7632*** | ***Fusarium crookwellense*** |
| ***7633*** | ***Fusarium equiseti*** |
| ***7634*** | ***Fusarium poae*** |
| ***7637*** | ***Fusarium sporotrichioides*** |
| ***7641*** | ***Fusarium tricinctum*** |
| ***15511*** | ***Fusarium equiseti*** |
| ***15523*** | ***Fusarium langsethiae*** |
| ***15555*** | ***Fusarium sambucinum*** |
| ***16196*** | ***Fusarium temperatum*** |
| ***16895*** | ***Fusarium acuminatum*** |

Each locus was first aligned separately and then concatenated in a super-gene alignment used to infer the phylogeny at *Fusarium* genus.

Phylogenetic analyses were performed for each genomic region and for the combined 3-genes dataset by using MEGA 5.2 (Tamura et al., 2011). Maximum Parsimony (MP) and maximum likelihood (ML) methods were also performed for all data; branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). A tree possessing only branches that received bootstrap properties ≥ 70% was chosen to represent each of the two loci.

### 2.3.6 Chemical analyses

All the samples were investigated with multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Vishwanath et al. (2009).

All samples were analyzed for the occurrence and concentrations of ZEA and DON.

Moreover, samples from Austria, Germany and China isolated during 2012 and 2013 were analyzed by the same method liquid chromatography-mass spectrometry (LC-

MS/MS), with a different approach for a multi-MTX screening which makes possible to reveal the presence of DON, ZEA and MTX minors, such as ENNs, BEA and others at the same time.

The potential of a group of *Fusarium* isolates to produce MTX was tested. Strains were grown firstly on PDA medium, then, after 4 days pieces of cultures were transferred to 30 gr of autoclaved rice previously kept to 45% of moisture for one night and sterilized at 121°C for 15 minutes. Cultures were incubated at 26°C without light for 3 weeks, then dried at 55 °C for 36 h and ground to a fine powder and used for chemical analyses. Control rice was treated in the same way, except fungal inoculation.
The analysis for the production of trichothecenes type A (T-2 and HT-2 toxins; Diacetoxyscirpenol) and type B (Nivalenol, Deoxynivalenol) in vitro was evaluated by ultra-performance liquid chromatography coupled with a photodiode array and Acquity QDa mass detector (UPLC-PDA-QDa). The analysis for the production of BEA toxin was evaluated by using the analytical method, slightly modified, suggested by Prosperini et al. in 2012 (Prosperini et al., 2012).

## 2.4 Results

### 2.4.1 Morphological analyses

The analyses of fungal contamination aimed firstly to have an overview at genus level of main toxigenic fungi occurring on the cereals samples collected in Austria, Germany and China.
For each geographical area the average values of the contamination expressed as a percentage (fraction of the contaminated seeds /total number of the seed) were calculated for the main toxigenic fungal genera: *Alternaria, Aspergillus, Fusarium, and Penicillium.*

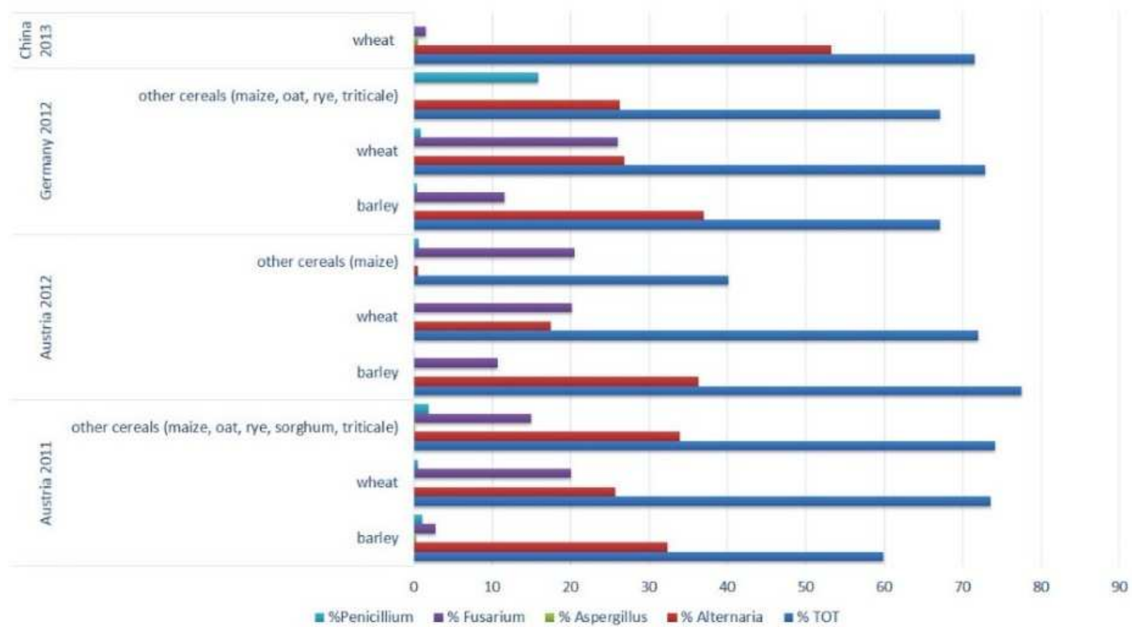


**Figure 2.1** Fungal contamination of 361 cereal samples analyzed, in Austria and Germany (2011-2012) and in China (2013).

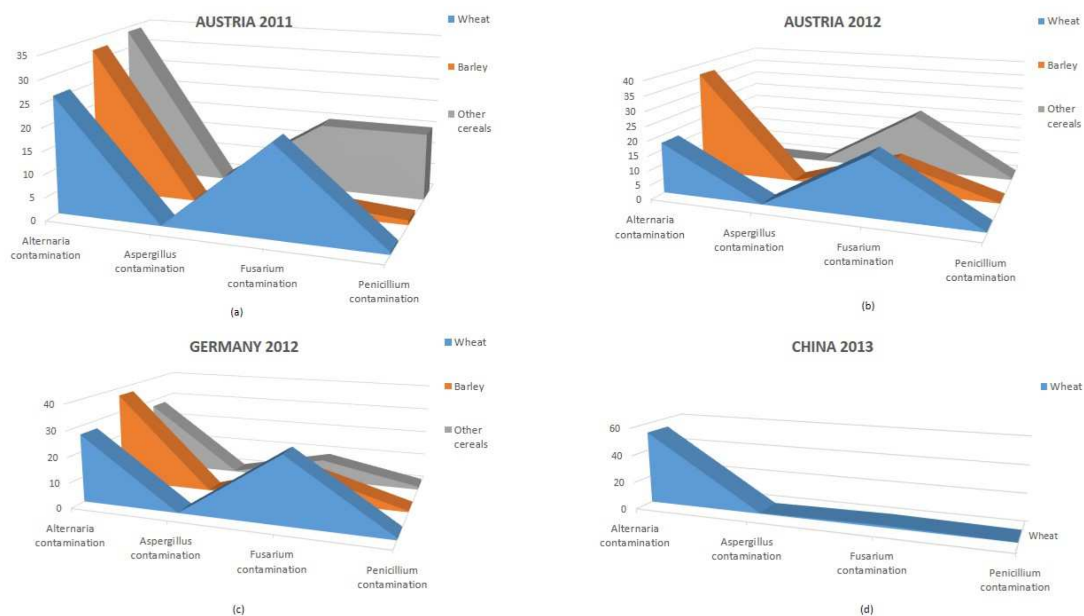


**Figure 2.2** Presence (%) of the main toxigenic fungi occurring on the cereals samples collected in Austria during 2011 (a) and 2012 (b), in Germany during 2012 (c) and in China during 2013 (d).

The average of the total contamination was very high for all the cereals samples. At genus level, no sample was contaminated by *Aspergillus* and *Penicillium* genera, while *Alternaria* showed a higher occurrence than *Fusarium* in all three areas except for maize isolated in Austria during 2012 (0,5 % versus 20,53%).

As regard samples collected in Austria and Germany, *Alternaria* species occurred on barley three times more than *Fusarium* species, whereas, on the other cereals, the level of the species of both genera contamination is similar (in a range from 20% to 26%).

The *Fusarium* species mostly detected in wheat samples collected during 2011 and 2012 belonged to FGSC, *F. poae* and *F. tricinctum* species complex, on barley the occurrence of the two main species was reversed, with *F. poae* more occurred than *F. graminearum.* Among the 30 samples of maize collected in Austria during 2012, strains morphologically identified as *F. subglutinans* and *F. temperatum* were the highest occurring species, followed by *F. verticillioides* and species of FGSC.

Among the samples of wheat collected in China, 43 out of 100 samples were contaminated by *Fusarium* with most of them, 42 samples, contaminated in a range of 1-9%. In these samples the species largely predominant was *F. graminearum.* On the other hand, a single sample was heavily contaminated (68%) only by strains of *F. verticillioides*, a species rarely reported as wheat colonizer.

### 2.4.2 Molecular analyses

To analyze phylogenetic relationships we amplified and sequenced the approximately 600-bp of calmodulin (CaM), 500 bp of translation elongation factor-1α (TEF-1α), and approximately 400-bp for ß-tubulin (ß-TUB) gene.

We conducted phylogenetic analyses of portions of CaM, TEF-1α and ß-TUB genes and a 3-gene combined data set using ML method (Figure 2.3).

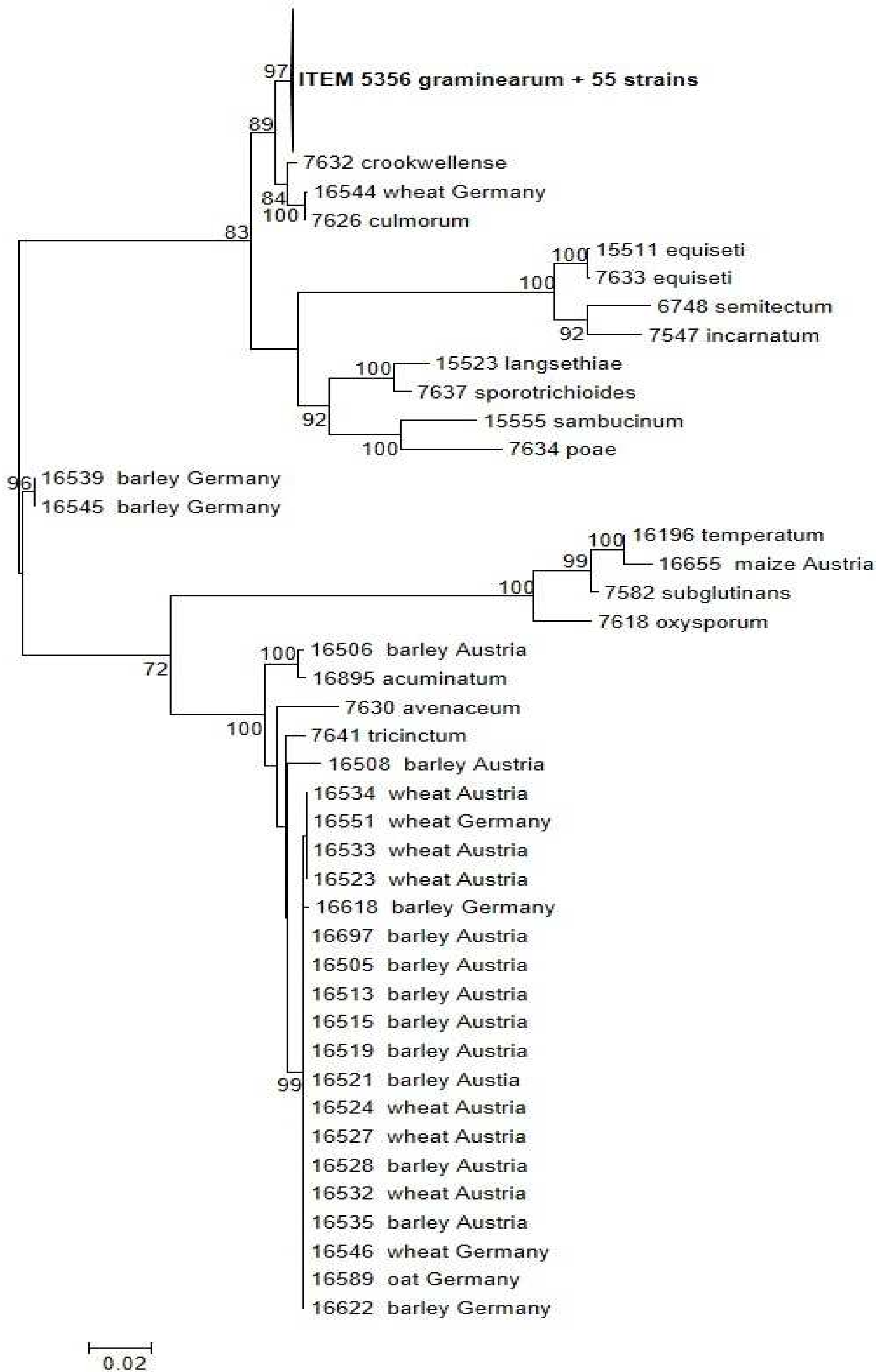


**Figure 2.3** Dendrogram obtained from the ß-TUB, CaM and TEF-1α sequences of 80 *Fusarium* strains and 17 reference strains, using the Maximum Likelihood clustering method. Item number refer to ITEM collection accession numbers. Next to the ITEM number, the host plant and the geographical origin are reported.

In Figure 2.3 the tree with the highest log likelihood (-7374.2653) is shown. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei (Tamura et al., 1993) model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 97 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1478 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.2 (Tamura et al., 2011).

Furthermore, the most parsimonious tree with length = 286 was determined. The evolutionary history was inferred using the MP method (see supplementary material Fig. S2.1).

Both ML and MP phylogenies showed similar topology in dendrograms resolving a big group of 55 strains belonging to *F. graminearum s. s.* (listed in Table S2.1) into a well-supported branch (97% and 99% bootstrap value, respectively) within the *F. graminearum* complex. Single gene trees and combined ß-TUB, TEF-1α and CL tree, including 16 phylogenetically distinct reference species belonging to FGSC (Sarver et al., 2011) clustered all the 55 strains to the species *F. gramineaum s. s.*

A total of 37 strains identified as *F. graminearum s. s.* were isolated from wheat, followed by barley (11 strains), maize (5 strains), oat (1 strain) and triticale (1 strain). Among the *F. graminearum* strains isolated from different geographical areas, a similarity of 100% well-supported by bootstrap analysis (97%) was shown in both of the trees.

3-genes (ß-TUB, TEF-1α and CL) based dendrograms showed that a group of 19 strains (supported by 99% bootstrap value) clusterized closely related to ITEM 7641, the *F. tricinctum* reference strain, and to ITEM 7630, the *F. avenaceum* reference strain.

Through the analysis of the combined genes, strain ITEM 16508 was assigned to the species *F. tricinctum* strain, ITEM 16506 to *F. acuminatum* and strain ITEM 16655 to *F. temperatum.*

Strains ITEM 16539 and ITEM 16545 are located in the dendrogram in an ambiguous position between the branch grouping the trichothecene producing species and the branch grouping the non trichothecene producing species.

Moreover, these strains were not univocally assigned to any species based on sequences analysis. This show that unknown genetical entities do exist also within *Fusarium* populations isolated from cereals collected in geographical area well investigated.

### 2.4.3 Mycotoxins analyses

The 150 samples isolated in Austria in 2011 were analyzed to obtain information on the occurrence of ZEA and DON by LC-MS/MS. The 64 samples isolated in Austria and the 47 samples in Germany in 2012 were investigated with multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The 100 samples collected in China were analyzed for the occurrence and concentrations of DON, 3-ADON and D3G, NIV, ZEA and ZEA-4S, ENN-B1 by LC-MS/MS according to Vishwanath et al. (2009). Figure 2.4 shows the number of samples contaminated by MTX and the maximum level of contamination expressed in μg/kg for each of them.

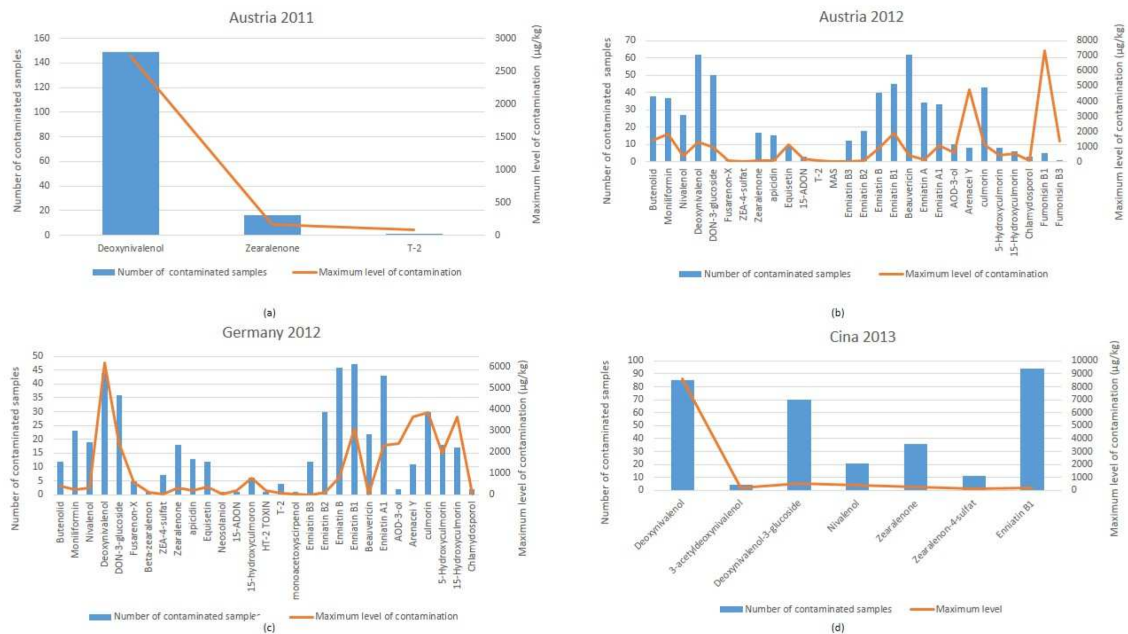


**Figure 2.4** Number of contaminated samples and maxim level of MTX revealed on the cereals samples collected in Austria during 2011 (a) and 2012 (b), in Germany during 2012 (c) and in China during 2013 (d).

Almost all samples (211 out of 214) collected in Austria in both years, were contaminated by DON up to maximum level detected of around 2750 µg/kg (Fig. 2.4, a and b).

While BEA was not analysed in 2011 samples, samples collected in 2012 (62 out of 64) showed contamination by both DON and BEA. However BEA concentration resulted relatively lower to DON one.

Also in Germany, the concentration of DON in samples collected in 2012 was high with 44 positive samples out of 47 contaminated samples ranging from 7 up to around 6200 µg/kg. Moreover, notably, also D3G occurred highly in the samples with 36 out of 47 contaminated samples in a range up to around 2400 µg/kg (Fig. 2.4, c).

Finally, the contamination of ENNs was also very common in the samples from Germany, being all samples contaminated up to a maximum level of around 3100 µg/kg.

The LC-MS/MS analysis carried out on the 100 wheat samples collected in China revealed high concentration levels of *Fusarium* MTX (Fig. 2.4, d).

The most common MTX, DON, ZEA and NIV were found in 85 %, 36 % and 21 % of all the samples, with the positive samples showing a maximum contamination level of

around 8650, 230 and 430 µg/kg, respectively. The incidence of ENN1 was 94% of contaminated samples, with an average of contamination of 10 µg/kg. The masked DON, deoxynivalenol-3-glucoside (D3G), was present in 70 % of all tested samples, which was almost the same level of occurrence as DON, while the masked ZEA, zearalenone-sulfate (Z4S), contaminated 11 % of samples.

A set of 19 *Fusarium* isolates, closely related to the *F. tricinctum* and to the *F. avenaceum* reference strains in molecular analysis (see dendrogram of Figure 2.3), were analyzed by UPLC-PDA-QDa method for their production of MTX. All of them were negative for trichothecenes and showed the production of BEA toxin (see table 2.4).

**Table 2.4** BEA toxin production of a group of *Fusarium* strains isolated from cereal samples.

| N. | ITEM | Origin | Host | Year | Beauvericin (µg/g) |
|---|---|---|---|---|---|
| 1 | 16505 | Austria | barley | 2011 | 7.40 |
| 2 | 16513 | Austria | barley | 2011 | 13.80 |
| 3 | 16515 | Austria | barley | 2011 | 20.60 |
| 4 | 16519 | Austria | barley | 2011 | 6.80 |
| 5 | 16521 | Austria | barley | 2011 | 6.60 |
| 6 | 16523 | Austria | wheat | 2011 | 11.45 |
| 7 | 16524 | Austria | wheat | 2011 | 12.85 |
| 8 | 16527 | Austria | wheat | 2011 | 3.90 |
| 9 | 16528 | Austria | barley | 2011 | 9.70 |
| 10 | 16532 | Austria | wheat | 2011 | 16.70 |
| 11 | 16533 | Austria | wheat | 2011 | 4.80 |
| 12 | 16534 | Austria | wheat | 2011 | 12.20 |
| 13 | 16535 | Austria | barley | 2011 | 11.95 |
| 14 | 16546 | Germany | wheat | 2012 | 7.65 |
| 15 | 16551 | Germany | wheat | 2012 | 13.20 |
| 16 | 16589 | Germany | oat | 2012 | 10.80 |
| 17 | 16618 | Germany | barley | 2012 | 4.60 |
| 18 | 16622 | Germany | barley | 2012 | 66.30 |
| 19 | 16697 | Austria | barley | 2012 | 29.90 |

## 2.5 Discussion

The present study reports the results of a survey on the occurence of mycotoxigenic *Fusarium* species and related MTX in cereals grain samples harvested in two different

geographic areas: Austria/Germany, for two years (2011-2012) and China for one year (2013).

Samples collected during 2011 in Austria showed a high level of contamination for wheat and a low level for barley. In 2012, the *Fusarium* contamination of wheat samples remained substantially unchanged while that of barley increased considerably at the same level of wheat, also including the samples from Germany. With respect to MTX contamination, a high level of DON was recorded in both years in samples collected in Austria/Germany in wheat, with several samples over the maximum tolerance level established by EU (1750 µg/kg) for unprocessed wheat kernels. Moreover, the high level of the masked DON, D3G, is extremely worrisome since this metabolite, which toxicity is lower compared DON, can be hydrolyzed again once ingested by both humans and animals, and therefore DON can become bioavailable and represent a further health risk if occurring in this masked form. Also the common occurrence in this area of the minor MTX ENNs and BEA is interesting since this shows an increased risk related to wheat and pose serious questions about the possible additive, if not synergistic, effects of the multi-MTX occurrence. Both ENNs and BEA are considered emerging MTX and could represent a potential health treat to investigate with deeper care (Ferrigo et al., 2016). Indeed, European Food Safety Agency (EFSA) is currently working on a scientific opinion about these minor toxins as a further concern for food and feed. ENNs occurrence is typically high in Northern and Eastern Europe on barley and wheat where often their incidence was reported to be up to 100% of samples collected and analyzed (Lindblad et al., 2013; Bolechová et al., 2015). However, the data here reported show that also in Central Europe the contamination of cereals by ENNs can be reason of concern.

Also in the investigation carried out on the 100 sample of wheat in China, the level of *Fusarium* MTX detected was high. The most frequently occurring MTX was ENN B1

(94% of sample contaminated), although the average of contamination was low, being 10 μg/kg. On the other hand, the most toxic MTX DON, ZEA and NIV were also found, although at a highly variable incidence, being the DON the MTX with the highest incidence and the highest average of concentration. Remarkably, masked DON, D3G, occurred in 70% of samples, which was almost the same level of presence as DON. Moreover, the masked ZEA, zearalenone-sulfate (Z4S) contaminated 11% of samples. The co-occurrence of a high number of *Fusarium* MTX and the high level of their incidence show that also the area of China investigated is exposed to a high risk for the health of consumers and that wider screening are needed for a more precise risk assessment.

The wide contamination by MTX recorded in all areas investigated was also reflected in the wide variability of *Fusarium* species isolated and identified.

A big group of 55 strains was identified as *F. graminearum s. s.,* the main species of FGSC, known to be an important producer of the type B trichothecene DON (Wang et al., 2011).

In both barley and wheat, *F. graminearum s. s.* was the most occurring species, followed by *F. poae,* species described as type A and B trichothecene and BEA producer (Somma et al., 2010).

Of extreme interest is the molecular analysis of some strains, ITEM 16539 and ITEM 16545 which are located in the dendrogram in an ambiguous position between the branch grouping the trichothecene producing species and the branch grouping the non trichothecene producing species. Moreover, these strains were not univocally assigned to any species based on sequences analyzed. This show that unknown genetical entities do exist also within *Fusarium* populations isolated from cereals collected in geographical area well investigated. Moreover, since each *Fusarium* species or clade has a specific

profile, it is also of great importance to assess also for these unidentified *Fusarium* strains their capability of producing MTX for both to use a poli-phasic approach for identification and eventually evaluate the risk related to the new genetic entities isolated from cereals commonly used for human consumption.

Finally, on maize, strains morphologically identified as *F. subglutinans/F. temperatum* were the most occurred, followed by *F. verticillioides* and *F. graminearum s. s.*

The coexistence of different *Fusarium spp.* in the field is an usual situation and although the number of detectable species can be high (Logrieco et al., 2007), only some of them are pathogenic, especially under suitable climatic conditions. The composition of species involved in the *Fusarium* disease complex is dynamic (Köhl et al., 2007). The species comprising a *Fusarium* community associated with each other and this cohabitation is particularly affected by climatic factors such as temperature and moisture. Moreover, evidences indicates that the environmental conditions that favour the infection process can differ from those that affect colonization; therefore, the relationship among *Fusarium* species may change over time during the infection process.

Therefore, it is important to carry out a correct identification of the *Fusarium* species that alternate or associate in the attacks to the grain, in order to carry out a proper mycotoxicological risk assessment associated with them.

## 2.6 Supplementary material

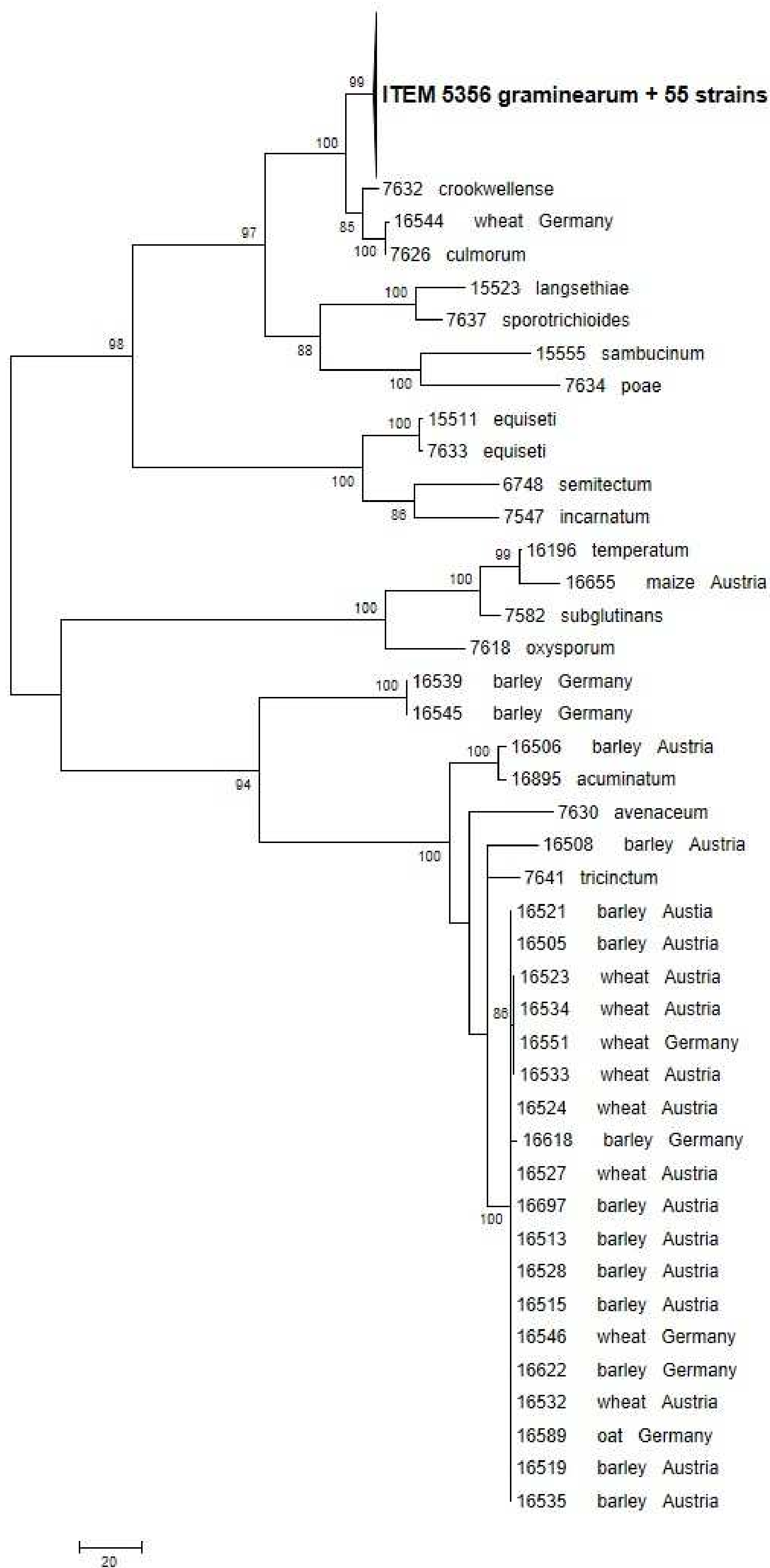


**Figure S2.1.** Dendrogram obtained from the ß-TUB, CaM and TEF-1α sequences of 80 *Fusarium* strains and 17 reference strains, using the Maximum Parsimony clustering method. Next to the ITEM number, the host plant and the geographical origin are reported.

**Table S2.1** Strains identified as *Fusarium graminearum sensu stricto* by molecular analysis.

| ITEM | ORIGIN | HOST PLANT/ YEAR OF SAMPLING |
|---|---|---|
| 16517 | Barley | Austria 2011 |
| 16518 | Barley | Austria 2011 |
| 16520 | Wheat | Austria 2011 |
| 16531 | Wheat | Austria 2011 |
| 16536 | Triticale | Austria 2011 |
| 16542 | Wheat | Germany 2012 |
| 16547 | Wheat | Germany 2012 |
| 16552 | Wheat | Germany 2012 |
| 16555 | Barley | Germany 2012 |
| 16566 | Barley | Germany 2012 |
| 16579 | Barley | Germany 2012 |
| 16583 | Barley | Germany 2012 |
| 16585 | Barley | Germany 2012 |
| 16587 | Wheat | Germany 2012 |
| 16588 | Wheat | Germany 2012 |
| 16592 | Barley | Germany 2012 |
| 16595 | Wheat | Germany 2012 |
| 16597 | Barley | Germany 2012 |
| 16600 | Oat | Germany 2012 |
| 16615 | Barley | Germany 2012 |
| 16619 | Wheat | Germany 2012 |
| 16621 | Wheat | Germany 2012 |
| 16624 | Barley | Germany 2012 |
| 16628 | Maize | Austria 2012 |
| 16630 | Maize | Austria 2012 |
| 16631 | Maize | Austria 2012 |
| 16652 | Maize | Austria 2012 |
| 16656 | Maize | Austria 2012 |
| 17052 | Wheat | China 2013 |
| 17053 | Wheat | China 2013 |
| 17054 | Wheat | China 2013 |
| 17055 | Wheat | China 2013 |
| 17056 | Wheat | China 2013 |
| 17057 | Wheat | China 2013 |
| 17058 | Wheat | China 2013 |
| 17059 | Wheat | China 2013 |
| 17060 | Wheat | China 2013 |
| 17061 | Wheat | China 2013 |
| 17062 | Wheat | China 2013 |
| 17063 | Wheat | China 2013 |
| 17064 | Wheat | China 2013 |
| 17065 | Wheat | China 2013 |
| 17066 | Wheat | China 2013 |
| 17067 | Wheat | China 2013 |
| 17068 | Wheat | China 2013 |
| 17069 | Wheat | China 2013 |
| 17070 | Wheat | China 2013 |
| 17071 | Wheat | China 2013 |
| 17072 | Wheat | China 2013 |
| 17073 | Wheat | China 2013 |
| 17074 | Wheat | China 2013 |
| 17075 | Wheat | China 2013 |
| 17076 | Wheat | China 2013 |
| 17077 | Wheat | China 2013 |
| 17079 | Wheat | China 2013 |

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# CHAPTER 3

## IDENTIFICATION OF *FUSARIUM* SPECIES OCCURRING ON MYCOTOXINS CONTAMINATED ITALIAN WHEAT

### 3.1 Introduction

Mycotoxins (MTX) are toxic secondary metabolites produced by filamentous fungi, mainly belonging to *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* genera.

According to the International Agency for Research on Cancer (IARC), some MTX contamination may be a serious concern for both human and animal health (Richard et al., 2007).

Over the past two decades, Fusarium Head Blight (FHB) epidemics have resulted in multibillion-dollar losses to world agriculture. Outbreaks and epidemics of FHB of wheat, barley and other small grain cereals have been reported in major production areas throughout the world. FHB can affect both quantity, in terms of yield and quality, due to infected grains containing large amounts of MTX (Alvarez et al., 2010).

The distribution of the pathogens causing FHB is also related to climatic factors such as temperature and moisture (Xu et al., 2009).

Among the *Fusarium* species, the primary etiological agent of FHB worldwide is *Fusarium graminearum* (O'Donnell et al., 2008), a species widespread in all the world which mainly produces deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) and which is been re-classified in the last decade as species complex (FGSC) composed by 16 phylogenetic entities.

Together with *F. graminearum*, *F. culmorum* has been reported as one of the main pathogens of wheat worldwide (Wang et al., 2006). Yield and quality losses are particularly important when *F. culmorum* induces FHB, which develops from infection

at anthesis and spreads until grain harvest, causing grain contamination with MTX, such as type B trichothecenes, ZEA and fusarins (Hope et al., 2005).

Other frequently detected species are *F. poae, F. avenaceum, F. langsethiae, F. tricinctum, F. sporotrichioides* (Xu end Nicholson., 2009; Somma et al., 2014) and the non-toxigenic species *Microdochium nivale* and *M. majus* (Glynn et al., 2005).

*F. poae* is a pathogen of increasingly importance as a cause of FHB in countries such as Argentina, Canada, Finland, Belgium, Germany, Switzerland, Hungary, Slovakia, Italy, Ireland and the United Kingdom (Audenaert et al., 2009). The prevalence of *F. poae* in wheat reported in the last years is surprising because the species was believed to be less aggressive than other FHB pathogens (Audenaert et al., 2009; Xu end Nicholson., 2009).

*F. avenaceum* is dominant throughout the regions with cool and wet climate, such as Northern, Central Europe and Canada (Bottalico and Perrone, 2002); however this species is often occurring on cereals isolated in warm areas (Spanic et al., 2010).

The closest relatives of *F. avenaceum, F. tricinctum* and *F. acuminatum,* are usually less widespread and relatively weak pathogens than *F. avenaceum*, and they occur more frequently in regions with cool or temperate climate (Marín et al., 2012).

Non-ambiguous taxonomic identification of *Fusarium* species using morphological and physiological characters is often very difficult because of their high similarity. For example, *F. avenaceum* is often confused with *F. acuminatum* (Harrow et al., 2010; Leslie and Summerell, 2006), *F. tricinctum* can be misidentified as *F. sporotrichioides*, *F. poae*, or *F. chlamydosporum* (Leslie and Summerell, 2006; Stakheev et al., 2013) and *F. culmorum* is similar to *F. sambucinum* (Gagkaeva et al., 2012).

*Fusarium* species may have significant difference in the profile of MTX produced, in pathogenicity and geographical distribution.

Moreover, the worldwide continuous changes of weather conditions, including Italy, are leading to an ever-changing profile of the typical dominant species on cereals which can result also in a dramatic variability of the MTX associated to *Fusarium* species. Therefore, it is important to carry out a correct identification of the *Fusarium* species that alternate or associate in the attacks to the grain, in order to carry out a proper mycotoxicological risk assessment related to them and the subsequent development of control strategies taking into account differing biochemical and genetic traits of these species and also their different ecological and environmental preferences.

The aim of this work was to assess level of *Fusarium* contamination on wheat collected in Italy and to analyze the occurrence of *Fusarium* toxins contaminating the cereals samples. The toxicity of these compounds lead to understand the importance of this study for food safety.

## 3.2 Materials and methods

### 3.2.1 Collection of samples and isolation of *Fusarium* species.

A total of 208 samples (Fig. 3.1) of wheat cereals were collected from several areas of Italy during a period of three years (2013-2015).

| Area | Cereal samples (n=208) | | |
|---|---|---|---|
| | 2013 | 2014 | 2015 |
| 10 | 6 | 5 | 6 |
| 9 | 4 | 5 | 1 |
| 8 | 5 | 6 | 1 |
| 7 | 5 | 6 | 3 |
| 6 | - | 2 | - |
| 5 | - | 28 | 20 |
| 4 | 41 | 31 | 15 |
| 3 | - | 6 | 3 |
| 2 | - | 6 | - |
| 1 | - | - | 3 |
| Total | 61 | 95 | 52 |

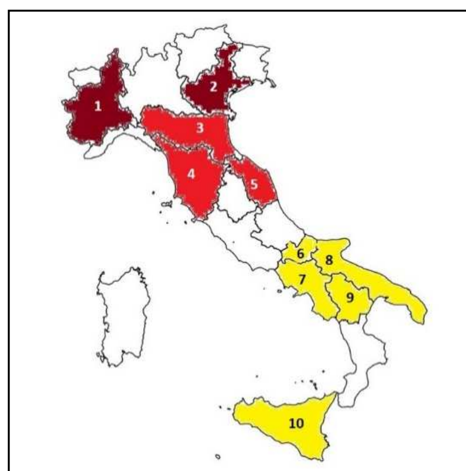


**Figure 3.1** Wheat samples collected in several areas of Italy during three years of study.

Samples were washed in sterile water, disinfected in a 2% sodium hypochlorite solution for 2 min, rinsed twice in sterile water to eliminate any hypochlorite residue and dried on sterile filter paper. After that, one hundred surface-disinfected seeds for each sample were placed in Petri dishes 90-mm diameter containing a *Fusarium spp*. selective media, PDA (potato dextrose agar) amended by PCNB (pentachloronitrobenzene), and 0.1 g $L^{-1}$ streptomycin and 0.05 g $L^{-1}$ neomycin sulphate antibiotics enriched (Nelson et al., 1983). Thus, only fungi from internal infestations were studied. After 5 days of incubation at 25°C, seeds developing colonies of the species of *Alternaria, Aspergillus* and *Fusarium* genera were counted and data of contamination (expressed in%) were collected.

A representative colony of each morphotype has been transferred on SNA (synthetic nutrient-poor agar) for the morphological identification at species level (Nelson et al., 1983; Leslie and Summerell, 2006) and single-spored on PDA substrate. Morphological identification has been supplemented with molecular techniques, to ensure that morphological similarities do not mask significant genetic differences. A subset of strains (n = 72), including all the isolates identified as *F. graminearum*, has been submitted to molecular analyses.

The fungal strains were deposited in the ITEM fungal collection of ISPA (http://www.ispa.cnr.it/Collection).

**3.2.2 Fungal strains and DNA extraction**

The selected strains were cultured in Petri dishes on the surface of a cellophane membrane laid over the PDA medium. After growth of 4 days at 25 °C, the mycelium was collected, frozen at -20 °C and subsequently lyophilized. The DNA was extracted using Wizard® Magnetic DNA Purification System kit for Food (Promega) from about 10 mg of dryed mycelium and following the manufacturer's instructions.

Portions of the calmodulin (CaM), translation elongation factor 1α (TEF-1α), and β-tubulin (β-TUB) genes were amplified and sequenced using primers listed in Table 3.1, respectively.

**Table 3.1** The primer sequences and the sizes of the PCR fragments used to identify the *Fusarium* isolates.

| PRIMER NAME | SEQUENCE (5′ TO 3′) | SIZE (bp) | REFERENCES |
|---|---|---|---|
| CL1 | GARTWCAAGGAGGCCTTCTC | ca.700 | (O'Donnell et al., 2000) |
| CL2 | TTTTTGCATCATGAGTTGGAC | | |
| EF1 | ATGGGTAAGGARGACAAGAC | ca.700 | (O'Donnell et al., 1998) |
| EF2 | GGARGTACCAGTSATCATGTT | | |
| BT2a | GGTAACCAAATCGGTGCTGCTTTC | ca.400 | (Glass and Donaldson, 1995) |
| BT2b | ACCCTCAGTGTAGTGACCCTTGGC | | |

Amplifications were performed in 15 µl volume reactions set up with 1× PCR buffer, 200 µM dNTP's, 300 nM each primer, 0.375 U of Taq DNA Polymerase and 25 ng of genomic DNA, according to the following profiles:

- CL1/CL2 primer pair: 95 °C-2 min; 40 cycles: 95 °□-30 s, 55 °C-30 s, 72 °C-50 s; 72 °C-5 min.

- EF1/EF2 primer pair: 94 °C-2 min; 35 cycles: 94 °□-50 s, 59 °C-50 s; 72 °□-1 min.

- Bt2a/ Bt2b primer pair: 94 °C-2 min; 35 cycles: 94 °□-30 s, 60°C-30 s, 72 °C-50 s; 72 °C-5 min.

The size of PCR amplicons were verified by 1.5% agarose gel electrophoresis using a molecular weight marker of DNA (Ladder 100bp).

### 3.2.3 Sequencing analysis

After amplification, PCR products were purified by enzymatic method (FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I) to remove the unincorporated primers and to degradate the dNTPs residues. The amplicons of interest were purified with a kit for DNA extraction from agarose gel (NucleoSpin® gel and PCR Clean-up) after gel electrophoresis and sequenced according to the modified "Sanger method" which involves the use of fluorescent markers linked to dideoxynucleotide triphosphates

(ddNTP), using the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) for both strands, according to the manufacturer's instructions. All of the sequencing reactions were purified by gel filtration through mini-columns containing Sephadex G-50 (Sigma-Aldrich, Milan, Italy), denatured for 5 minute at 95 °C and separated by capillary electrophoresis on ABI3130, a 48 capillary sequencer.

Fragment sequences of three genes were obtained aligning both strands with Bionumerics software (Applied Maths) and were compared to ones deposited in GenBank by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Alignments were performed by ClustalW and all positions containing gaps were excluded from analysis. Phylogenetic relationships based on individual or combined data sets of three genes were analyzed and corresponding trees were constructed in MEGA 5.2V software (Tamura et al., 2011) using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. The robustness of the topology for each tree was supported by bootstrap analysis with 500 replications. Trees possessing only branches that received bootstrap properties ≥ 70% were chosen to represent each of the loci.

In the phylogenetic analysis, the sequences of 17 reference strains selected among the fungal ITEM collection (http://www.ispa.cnr.it/Collection) to represent the main *Fusarium* species occurring on cereals, were included (Table 3.2).

**Table 3.2** List of reference strains of the *Fusarium* species considered in the phylogenetic analyses**.**

| ITEM | SPECIES | ITEM | SPECIES |
|---|---|---|---|
| **5356** | ***Fusarium graminearum*** | **7634** | ***Fusarium poae*** |
| **6748** | ***Fusarium semitectum*** | **7637** | ***Fusarium sporotrichioides*** |
| **7547** | ***Fusarium incarnatum*** | **7641** | ***Fusarium tricinctum*** |
| **7582** | ***Fusarium subglutinans*** | **15511** | ***Fusarium equiseti*** |
| **7618** | ***Fusarium oxysporum*** | **15523** | ***Fusarium langsethiae*** |
| **7626** | ***Fusarium culmorum*** | **15555** | ***Fusarium sambucinum*** |
| **7630** | ***Fusarium avenaceum*** | **16196** | ***Fusarium temperatum*** |
| **7632** | ***Fusarium crookwellense*** | **16895** | ***Fusarium acuminatum*** |
| **7633** | ***Fusarium equiseti*** | | |

### 3.2.4 Chemical analysis

Analysis of T-2, HT-2, DON and NIV toxins were performed by UPLC-PDA method at CNR-ISPA (Bari). For type A trichothecenes, wheat samples were extracted with methanol/water (90:10, v/v) and diluted extracts were cleaned up through immunoaffinity columns. T-2 and HT-2 toxins were separated and quantified by Ultra Performance Liquid Chromatography (UPLC) with photodiode array (PDA) detector (λ=202 nm), by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions. The limit of detection (LOD) of the method was 8 µg/kg for both toxins (signal to noise 3: 1) (Pascale et al., 2012).

For DON and NIV detection, wheat samples was extracted with water and the filtred extract was cleaned up through an immunoaffinity column containing a monoclonal antibody specific for DON and NIV. Toxins were separated and quantified by UPLC with PDA detector (λ=202 nm) and comparing them with a calibration curve obtained with standard solutions. The LOD of the method was 20 µg/kg for NIV and DON (signal to noise 3: 1) (Pascale et al., 2014).

For ZEA, wheat samples were extracted with acetonitrile-water (90:10, v/v) and the extract was diluted with water (1:10, v/v) and applied to immunoaffinity column. The column was washed with water and ZEA was eluted with methanol and quantified by reversed-phase HPLC with fluorometric detection ($\lambda_{ex}$ =274 nm, $\lambda_{em}$= 440 nm). ZEA was quantified by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions. The LOD of the method was 3 µg/kg for ZEA (signal to noise 3:1) (Visconti *et al.*, 1998).

## 3.3 Results

### 3.3.1 Morphological and chemical analyses of wheat samples

Morphological analyses of 208 wheat samples collected in Italy during 3 years (2013-2014-2015) aimed firstly to have an overview at genus level of main toxigenic fungi occurring on this cereal.

For each geographical area and for each year, the average values of the contamination expressed as a percentage (fraction of the contaminated seeds /total seeds number) were calculated for the main toxigenic fungal genera: *Alternaria, Aspergillus and Fusarium* (Fig. 3.2).

The average values of total contamination were very high in all Italian areas. No sample was contaminated by *Aspergillus* genus. During the first and the second year of study, samples collected in Northern Italy were contaminated by *Alternaria* species 4 times less than samples collected in Southern Italy. During the last year, *Alternaria* genus showed an occurrence of about 20%, in all studied areas.

*Fusarium spp.* were detected by the combination of visual and stereomicroscope observation after 5 days of incubation in the analysed wheat samples during the three years. *Fusarium spp.* were isolated from kernels with incidences higher in samples collected from Northern compared to Central and Southern Italy, particularly during 2015 (67% Piemonte and 34% Emilia-Romagna).

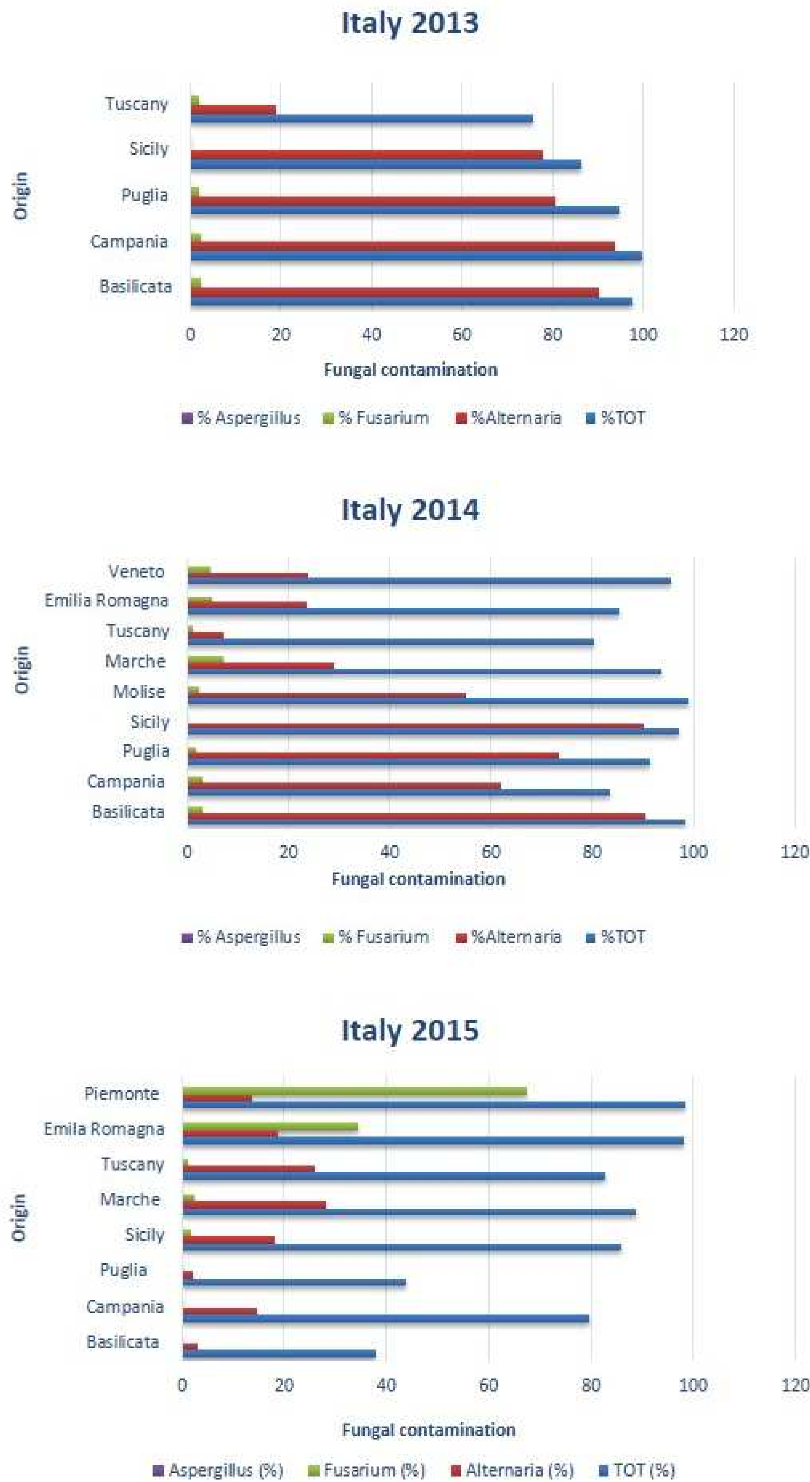


**Figure 3.2** Fungal contamination of wheat samples collected in Italy during years 2013-2014-2015.

In North-Central Italy *F. poae* and *F. graminearum* predominated. In Southern Italy, *F. poae* was the predominant species recovered, and to a lesser extent, *F. tricinctum, F. avenaceum* and *F. langsethiae* were detected.

Fig. 3.3 shows data of MTX production, expressed as percentage of samples contaminated. Chemical analyses revealed ZEA toxin contamination in most of the samples; type B trichothecenes (DON and NIV) were detected in samples collected in Piemonte, Emilia-Romagna, Marche, Tuscany and Puglia, and type A trichothecenes (T-2 and HT-2) were detected mainly in Center-South Italy, in particular Basilicata, Campania, Sicily and Puglia.

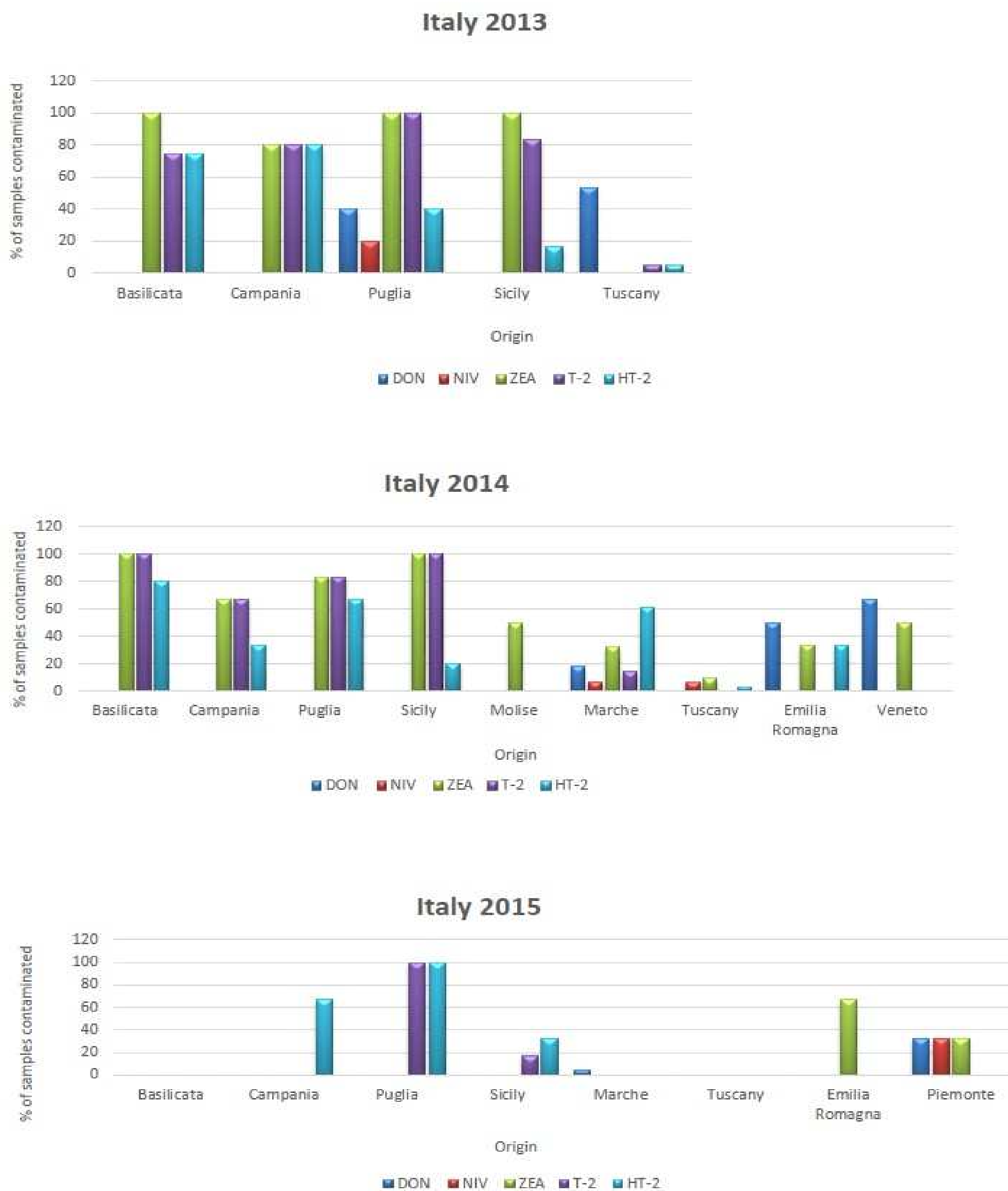


**Figure 3.3** Mycotoxins contamination of wheat samples collected in Italy during years 2013-2014-2015.

### 3.3.2 Molecular analysis

All the 72 strains isolated from Italian wheat samples were analysed by sequencing. Phylogenetic analyses were performed for each locus, ß-TUB, CaM, and TEF-1α (data not shown) and for the combined 3-genes dataset by using MEGA 5.2 version with MP (Supplementary Fig. S3.1) and ML (Fig. 3.4) methods.

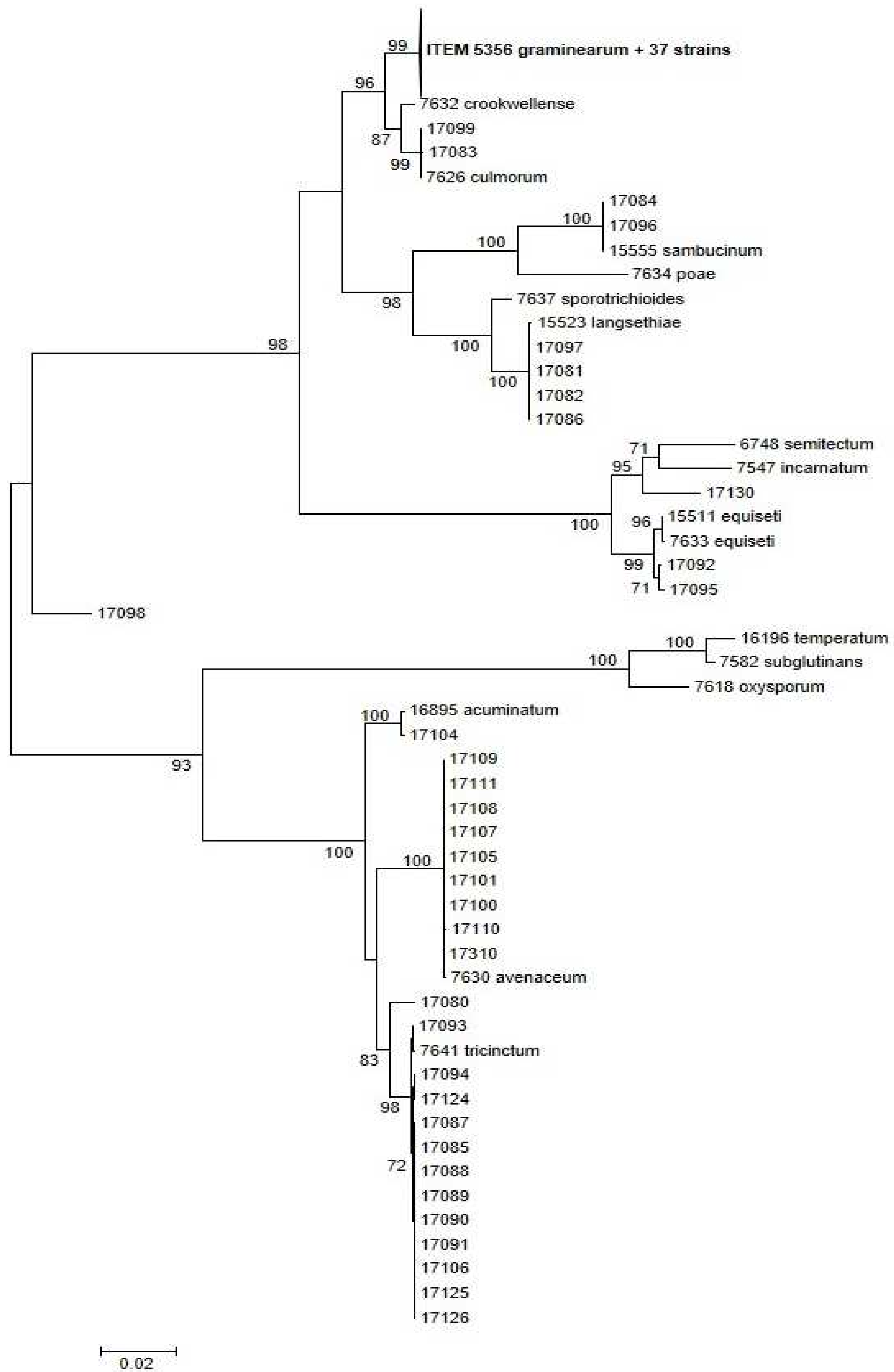


**Figure 3.4** Phylogenetic tree generated from concatenated nucleotide sequences of *ß-TUB, CaM and TEF-1α* fragments from 72 *Fusarium* strains of studied population and 17 reference strains, using the Maximum Likelihood clustering method. For references strains, the identification code is shown.

The evolutionary history was inferred by using the ML method based on the Tamura-Nei model (Tamura et al., 1993). The tree with the highest log likelihood is shown in Fig. 3.4. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the ML approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 89 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1516 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.2 (Tamura et al., 2011).

Both ML and MP analyses resolved a big group of 37 phylogenetically similar species into a well-supported branch (99% and 100% bootstrap values, respectively) within the *F. graminearum sensu stricto* reference. Two strains of the set, ITEM 17099 and ITEM 17083, clustered with *F. culmorum* reference stain; isolates with ITEM 17084 and ITEM 17096 clustered with *F. sambucinum* reference strain; 4 isolates showed a similarity of 100% supported by a bootstrap value of 100% with *F. langsethiae* reference for both MP and ML analyses.

The phylogenetic dendrogram showed the formation of a group with two isolates similar to both reference strains of *F. equiseti*. ITEM 17098 and ITEM 17130 formed no clusters. Dendrograms resulting from ß-TUB, CaM and TEF-1α analyses showed that a set of 12 strains were on the same branch (data supported by high bootstrap value) and were related to the strain ITEM 7641, selected as *F. tricinctum* reference. A single isolate, ITEM 17080, appeared close to this set. A similarity of 100% well-supported by bootstrap analysis (100%) was shown in both of the trees between a group of 9 strains and *F.*

*avenaceum* reference strain. A single isolate, ITEM 17104, clustered with *F. acuminatum* reference strain.

## 3.4 Discussion

This work has provided an accurate assessment of the species composition of *Fusarium* strains isolated from wheat samples harvested in Italy during the years 2013, 2014 and 2015.

It is important to identify and characterize the main species involved in FHB, in order to set up control strategies that will contain the disease and the levels of MTX in food and animal feed.

The DNA-based approach was used to confirm the morphological identification of representative isolates because often the species are morphologically indistinguishable or nearly and, in the past, the use of DNA-based techniques for the taxonomic studies has led to establishment of a number of new *Fusarium* species which were difficult or impossible to identify morphologically (O'Donnell et al., 2008; Yli-Mattila et al., 2010). Therefore, we used sequences of ß-TUB, CaM and TEF-1α genes which revealed to be powerful tools to distinguish species in *Fusarium* genus.

*Fusarium poae* was the most common species associated to FHB in wheat analyzed in this study. However, a noticeable difference in species composition in the three years examinated was recorded. Indeed, in samples collected in South Italy, *F. poae* was the most frequent pathogen in 2013, followed by *F. acuminatum;* in 2014 a lower incidence of *F. poae* compared the previous year and an increase of *F. acuminatum* occurrence were observed; in 2015 *F. poae* has been the most common species detected in Southern Italy, as in 2013.

In Northern-Central Italian areas, during 2013, *F. avenaceum* prevailed among the *Fuasarium* species. This species has not shown any ability to produce trichothecenes MTX up to date (Desjardins and Proctor, 2007).

During the years 2014 and 2015, the presence of *F. poae,* in samples collected in Northern-central Italy, was higher than *F. graminearum* occurrence, comparing previous data reported by Bottalico and Perrone (2002).

In general, the results obtained in the present survey agree with those reported by Xu and Nicholson (2009) that stated the high frequency of *F. poae* and a reduced occurrence of *F. graminearum* distribution along the years in Europe, probably due to changing environmental conditions.

Indeed, distribution and predominance of a *Fusarium* species in a given geographical area usually seems to be determined by climatic factors (temperature, relative humidity, rain, etc.), agronomic practices (cropping sequence, soil tillage, sowing on untilled soil, use of nitrogen fertilizers, etc.), as well as by competition among *Fusarium* species (Parry et al., 1995; Doohan et al., 2003). For this reason and also because a single *Fusarium* strain can produce multiple MTX, none of the *Fusarium* species can be considered of secondary importance. The big occurrence of *F. poae* species on Italian samples and its capability to produce both A and B trihothecenes is reason of concern.

Finally, the chemical analyses carried out in this study on the kernels samples, showed the prevalence of type B trichothecenes, DON and NIV, in Northern Italy, while high levels of type A trichothecenes, T-2 and HT-2, were detected mostly in Southern Italy samples. This indicates a possible negative correlation between the two main MTX DON and T-2 in their distribution on wheat along the different geographical areas.

To this respect, the occurrence of high levels of T-2 and HT-2 in samples collected in Southern Italy is of extremely worring due to the toxicity of these MTX.

T-2 and HT-2 are not only inhibit protein synthesis and cell proliferation in plants, but also cause acute or chronic intoxication of humans and animals.

Moreover, given the absence of T-2 and HT-2 toxins-producing species, mainly *F. sporotrichioides* and *F. langsethiae*, and the occurrence of others *Fusarium* species on the samples, a further investigation of the origin of this poor correlation between high level of T-2 and HT-2 and non-producing species is needed.

# 3 .5 Supplementary material

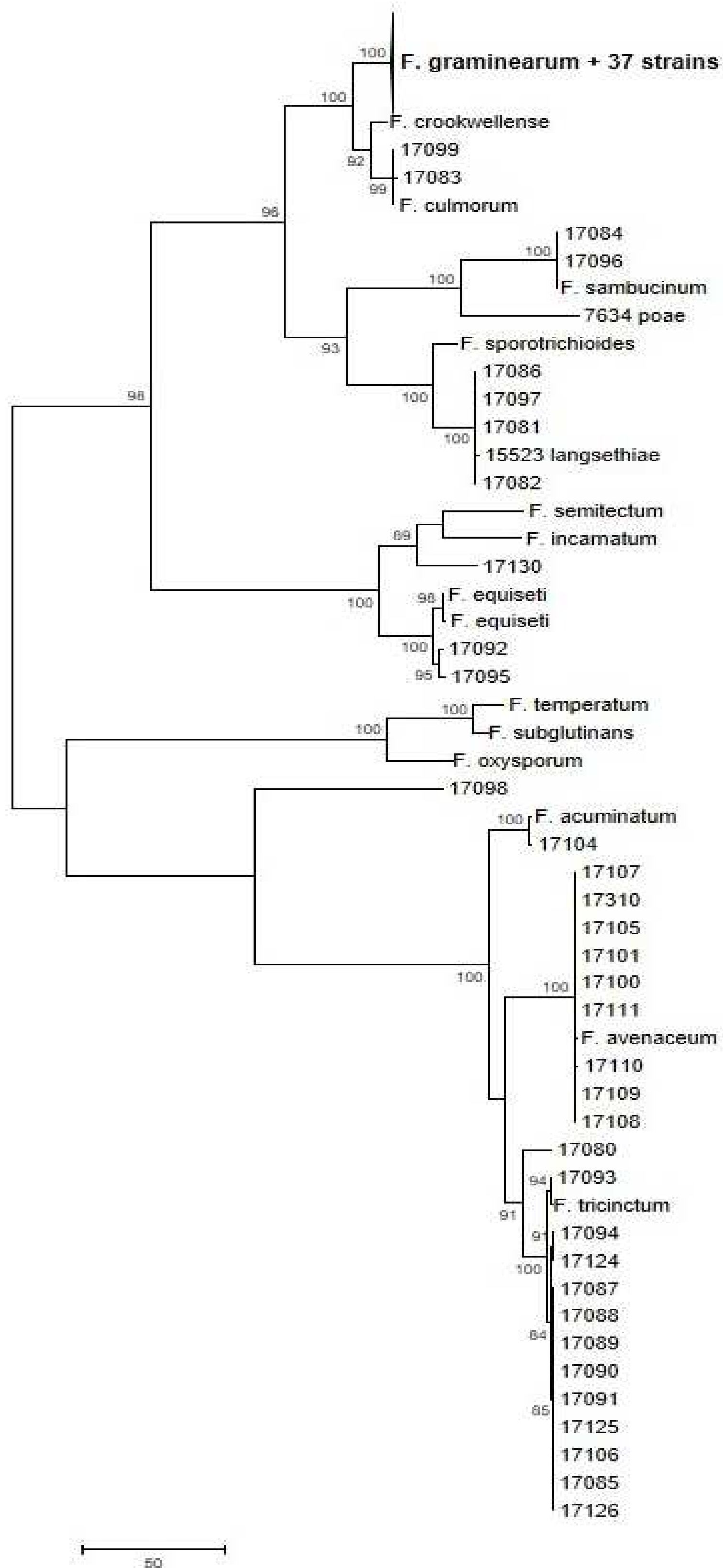


**Figure S3.1** Phylogenetic tree generated from concatenated nucleotide sequences of *β-TUB, CaM and TEF-1α* fragments from 72 *Fusarium* strains of studied population and 17 reference strains, using the Maximum Parsimony clustering method. For references strains, the identification code is shown.

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# CHAPTER 4

# SCREENING OF CHEMICAL AND MOLECULAR CHEMOTYPE INCLUDING THE NOVEL TYPE A TRICHOTHECENE MYCOTOXIN NX-2, IN A *FUSARIUM GRAMINEARUM SENSU STRICTO* POPULATION ISOLATED WORLDWIDE FROM CEREALS

## 4.1 ABSTRACT

Fusarium Head Blight (FHB) is an important disease of small grain cereals worldwide, causing a significant reduction in grain yields and representing a global concern for human health due to mycotoxins (MTX) contamination of kernels at harvest.
*Fusarium graminearum* is reported as the main agent of the disease. This species has been re-classified as *F. graminearum* species complex (FGSC), whose members were described based on phylogenetic analyses.
Among the most important *Fusarium* MTX that can be accumulated in the cereals, there are trichothecenes, terpens compounds characterized by a 12, 13-epoxytrichothec-9-ene ring system. They are potent inibithors of proteic synthesis, and divided in type A and B according with the absence/presence of a carbonyl function group at C-8 of the ring. Each species of FGSC is capable of producing B-trichothecenes in planta. In particular, deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives (3-ADON, 15-ADON and 4-ANIV) are the most common MTX produced by species of the complex.
Recently, a novel type A trichothecene termed NX-2 has been detected from *F. graminearum sensu stricto (s. s.)* cultures isolated in North America. NX-2 is similar to 3-ADON, lacking the carbonyl group in C-8. Genetic analysis revealed a different TRI1 allele in those isolates, responsible for the different ability to hydroxylate C-8.
In this study, we used polymorphisms occurring in 2 alleles of TRI1 gene to develop specific PCR-based assays, for the detection of NX-2 genotype, and therefore chemotype.
A total of 92 strains of *F. graminearum* isolated from samples of cereals, mainly wheat, collected in Austria, Germany, China and different geographical areas of Italy, were amplified by using PCR specific assay here developed in order to investigate their potential capability of producing this novel type A trichothecene, and to assess their molecular and possible chemical chemotype.

## 4.2 Introduction

FHB is a disease of wheat, barley and other cereal crops worldwide (McMullen et al., 2012) that can significantly reduce grain yield and quality. FHB agents can produce MTX, mostly trichotohecenes, that pose a serious threat to food safety and animal health (Desjardins and Proctor, 2007).

FHB of small grain cereals is associated with up to 17 *Fusarium* species, but only a few of them are important worldwide in terms of diffusion and economic impact (Ferrigo et al., 2016).

Worldwide, the predominant pathogen in the FHB complex is *F. graminearum*, which was shown to be a species complex (FGSC). Sixteen phylogenetically distinct species have been identified within the FGSC (O'Donnell et al. 2000; O'Donnell et al. 2004; O'Donnell et al. 2008; Sarver et al. 2011), and each of them is able to produce type B trichothecenes: DON, NIV and their acetylated derivatives 3-ADON, 15-ADON and 4-ANIV. Within each FGSC species, different chemotypes can co-exist and often they refers to in geographic distribution. The DON chemotype define the capability to produce DON and acetyl-DONs and the NIV chemotype to produce NIV and 4-ANIV (Ward et al., 2002).

Another group of *Fusarium* trichothecenes is the Type A which is produced by soil fungi and plant pathogens *F. langsethiae* (Imathiu et al., 2013), *F. sporotrichioides* and *F. poae* (Foroud and Eudes, 2009). This group includes T-2 toxin and HT-2 toxin, which are among the most toxigenic members of this MTX family.

Type A trichothecenes are characterized by a hydroxyl group, or no substituent (at all) at C-8, whereas type B trichothecenes carry a keto group at the same position (McCormick et al., 2011).

Recently, a novel type A trichothecene termed NX-2 has been detected from *F. graminearum* cultures isolated in North America (Varga et al., 2015). NX-2 is similar to 3-ADON, but lacks the carbonyl group in C-8 (Fig. 4.1). Genetic analysis revealed a different TRI1 allele in the isolates analyzed, which was verified to be responsible for the difference in hydroxylation at C-8.

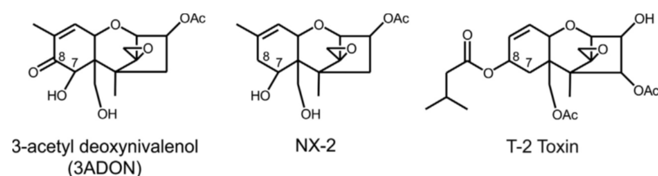


**Figure 4.1.** Chemical structures of 3-acetyl deoxynivalenol, a 7-hydroxy, 8-keto, type B trichothecene; NX-2, a 7-hydroxy type A trichothecene; and T-2 toxin, a 8-acyl type A trichothecene (Kelly et al., 2016).

The genetic polymorphism in the trichothecene biosynthetic genes (TRI) cluster (Alexander et al., 2009; Lee et al., 2002) is reported reported as responsible for diversity among type B trichothecenes. DNA sequence polymorphisms in TRI13 have been used to separate strains/species with the 3-ADON and 15-ADON as well as the NIV chemotypes (Wang et al., 2008).

The aim of our work was to analyze, with chemical and genetic approaches, chemotypes of the *F. graminearum* isolates included in the study and to investigate their possible ability to produce the novel trichothecene toxin NX-2. The toxicity of these compounds lead to understand the importance of this study for food safety.

## 4.3 Materials and methods

### 4.3.1 Phylogenetic analyses

A total of 92 strains, previously assigned to FGSC by sequence analysis (see chapter 2 and 3 of this thesis), isolated from cereals samples collected in Austria, Germany, China and Italy, were used in this study to investigate phylogenetical relationships among isolates, further characterized within the FGSC.

Phylogenetic analyses of ß-TUB and TEF-1α gene sequences of selected *F. graminearum* strains and 17 reference strains of the FGSC, listed in Table 4.1 (O'Donnell et al., 2000, 2004, 2008; Ward et al., 2002; Starkey et al., 2007; Yli-Mattila et al., 2009; Sarver et al, 2011),were performed with MEGA 5.2 version software (Tamura et al., 2011), by using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods.

**Table 4.1** List of the 17 reference strains of the FGSC and *F. oxysporum* used as the outgroup strain considered in the phylogenetic analyses.

| NUMBER CODE | SPECIES | NUMBER CODE | SPECIES |
|---|---|---|---|
| NRRL 2903 | *F. austroamericanum* | NRRL 31238 | *F. brasilicum* |
| NRRL 5883 | *F. graminearum s. s.* | NRRL 34461 | *F. sp.* |
| NRRL 6101 | *F. asiaticum* | NRRL 37605 | *F. vorosii* |
| NRRL 25797 | *F. mesoamericanum* | NRRL 38380 | *F. gerlachii* |
| NRRL 26754 | *F. acaciae-mearnsii* | NRRL 45665 | *F. ussurianum* |
| NRRL 26916 | *F. bothii* | NRRL 46710 | *F. aethiopicum* |
| NRRL 28439 | *F. graminearum s. s.* | NRRL 54197 | *F. louisianense* |
| NRRL 28721 | *F. meridionale* | NRRL 54220 | *F. nepalense* |
| NRRL 29306 | *F. cortaderiae* | NRRL 7618 | *F. oxysporum* |

### 4.3.2 Molecular identification of trichothecenes chemotype

In order to identify the trichothecene genotype of the 92 *F. graminearum* strains, two different PCR assays, targeting different genes of the (TRI cluster) trichothecene biosynthetic pathway, were applied.

The first assay uses the primers pair designed in this study on the basis of DNA polymorphisms found in the TRI1 gene (Table 4.2), in order to investigate the potential capability of producing a novel trichothecene type A, NX-2. Primers were designed with the aid of Primer Express Software version 2.0 (Applications-Based Primer Design Software) (see supplementary Fig. S4.1).

Amplifications were performed in 15 µl volume reactions and were set up with 1×PCR buffer, 200 µM dNTP's, 300 nM each primer, 0.375 U of Taq DNA Polymerase and 25 ng of genomic DNA, according to the following PCR conditions: 95 °C-2 min; 35 cycles: 95 °C-30 s, 60 °C-30 s, 72 °C-50 s; 72 °C-7 min.

Three *F. graminearum* strains from North America (NRRL 66036, NRRL 66037 and NRRL 66038), reported in literature as producers of the new metabolite NX-2 (Varga et

al., 2015), were used as references to investigate the reliability of the 215F and 420R primers (Table 4.2) for the identification of the NX-2 chemotype among our set of isolates.

**Table 4.2** The primer sequences and the size of the PCR fragment used to identify the novel chemotype NX-2.

| PRIMER NAME | SEQUENCE (5′ TO 3′) | EXPECTED AMPLICON SIZE (bp) |
|---|---|---|
| ***F. graminearum* 02-264NX-2. 215F** | **AGCACCCAACAACACCTGTG** | **206 bp** |
| ***F. graminearum* 02-264NX-2. 420R** | **AAAACCGGGAATTTCAGTGTGTA** | |

The second assay uses a primer pair designed by Wang et al. (2008) in conserved regions of TRI13, upstream and downstream a polymorphic region of the gene, where different deletions were reported as related to different B chemotyes (NIV, 3-ADON, 15-ADON) (Fig. 4.2).

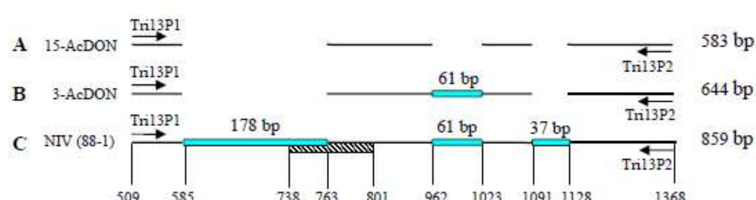


**Figure 4.2** Diagrammatic presentations of Tri13 genes are showing the gene structures of 3-ADON-, 15-ADON- and NIV-chemotype strains, and indicating the positions of primers designed for this study and the positions of nucleotides in the amplicon amplified by the primers in a NIV-chemotype (Wang et al., 2008).

Each reaction was carried out in 25 µL, containing, 25 ng of genomic DNA, 1× PCR buffer, 200 nM dNTPs, 0.375 U of Taq DNA polymerase, and 300 nM of each primer, at the following PCR conditions: 10 min at 94°C; 30 cycles of 1 min at 94°C, 40 s at 58°C and 40 s at 72°C; 10 min at 72°C.

PCR products were loaded on 1.5% agarose gel. The expected PCR products were 859 bp, 644 bp and 583 bp to distinguish NIV, 3-ADON and 15-ADON chemotypes, respectively.

### 4.3.3 Chemical analysis

All Chinese and Italian *F. graminearum* strains isolated were tested for the effective capability to produce DON, 3-ADON, 15-ADON and NIV by chemical analysis.

Strains were grown firstly on PDA medium, then, after 4 days pieces of cultures were transferred to 30 gr of autoclaved rice previously kept to 45% of moisture for one night and sterilized at 121°C for 15 minutes. Cultures were incubated at 26°C without light for 3 weeks, then dried at 55 °C for 36 h and ground to a fine powder and used for chemical analyses. Control rice was treated in the same way, except fungal inoculation.

Samples were purified with Mycosep 227 method (Weingaertner et al., 1997) and quantified by HPLC-PDA method (Quarta et al., 2006). Limit of detection for DON, 3 ADON and 15-ADON was 0.03 mg / g and for NIV was 0.02 mg / g.

Possible production of type B trichothecenes was also evaluated for 3 *F. graminearum* representative strains, already well characterized for their chemotype, on synthetic media: PDA (potato dextrose agar), WA (Wickerham agar) and YGA (Yeast Glucose Agar), in triplicate for 7, 14 and 21 days. Plugs (1 gr) of each fungal culture were purified with immunoaffinity columns and analysed with method reported in Pascale et al. (2014), based on the determination of the toxin using UPLC with DAD detector.

## 4.4 Results

### 4.4.1 Phylogenetic analyses

Two phylogenetic trees with ML (Figure 4.3) and MP (dendrogram shown in supplementary material, Fig. S4.2) methods were obtained from analysis of combined ß-TUB and TEF-1α sequences of 92 *F. graminearum* strains and 17 reference strains of FGSC.

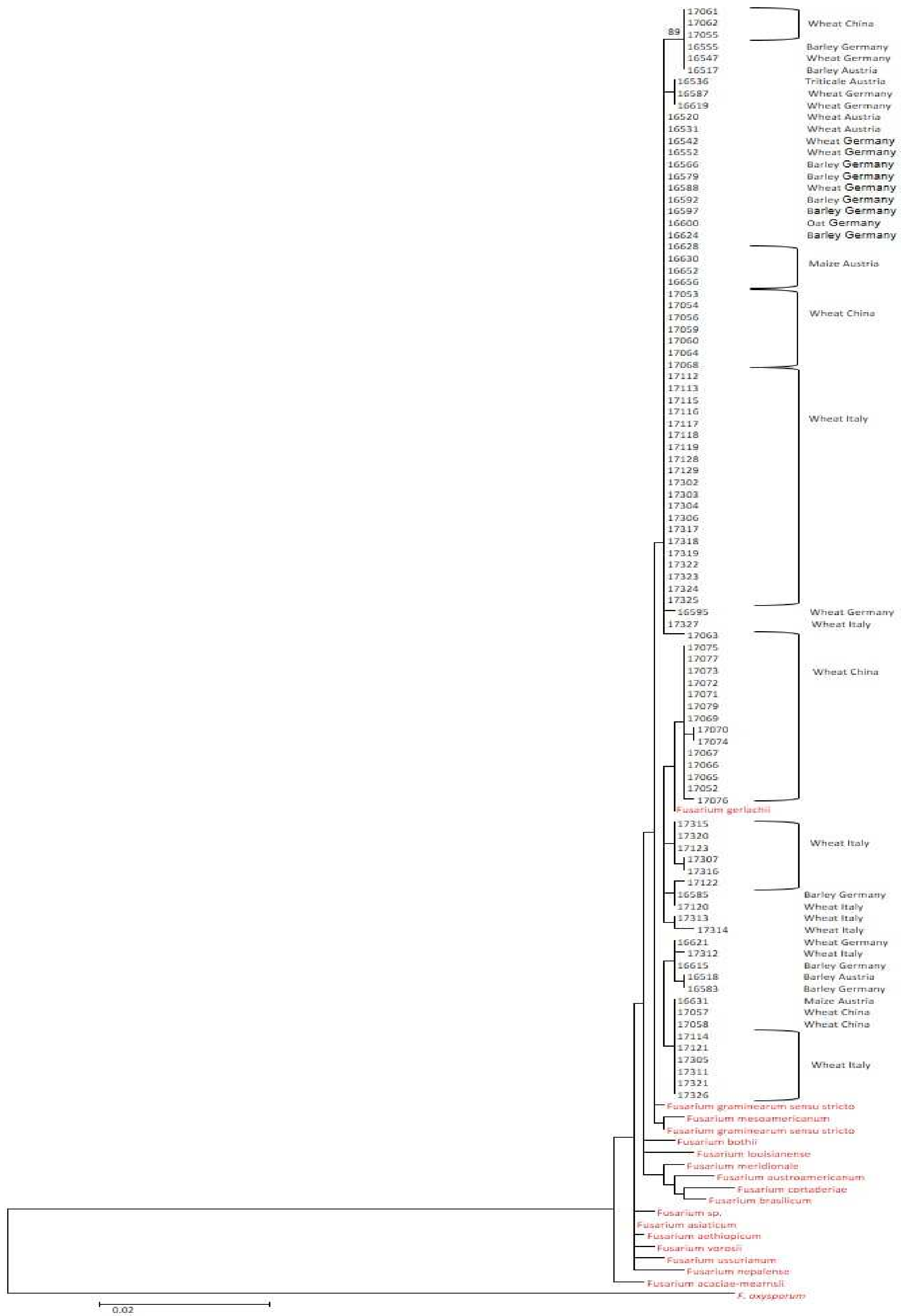


**Figure 4.3** Phylogenetic tree generated from concatenated nucleotide sequences of *ß-TUB and TEF-1α* fragments from 92 *Fusarium graminearum* strains of studied population, 17 reference strains of FGSC and *F. oxysporum* reference strain as outgroup. For references strains, the identification code NRRL is shown. Tree was constructed by using Maximum Likelihood clustering method with MEGA 5.2 version software.

Almost all the isolates (78 out of 92) resulted in a clade with NRRL 5883 and 28439, references strain *F. graminearum s. s.* (Fig. 4.3), and a clade of 14 strains isolated from wheat collected in China clustered closely related to NRRL 38380 and were assigned to *F. gerlachii*, a species discovered in the last decade in USA on wheat and shown to be a high producer of NIV.

### 4.4.2 Chemotype identification

The set of 92 strains of *F. graminearum* was analysed by P□R to identify their molecular chemotype.

The first experiment, based on specific primers (Supplementary Fig. S4.3) for NX-2 chemotype identification, revealed that the whole population analyzed don't have the allele of this gene for the production of new MTX (representative strains shown in Fig. 4.4).

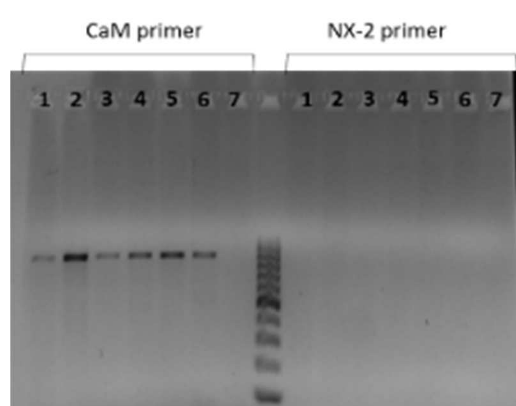


**Figure 4.4** Some of representative strains amplified with primers for the TRI1 gene allele specific for NX-2 chemotype and also for an housekeeping gene (CaM):
**1.** ITEM 17071 **2.** ITEM 17073 **3.** ITEM 17075 **4.** ITEM 17076
**5.** ITEM 17077 **6.** ITEM 17078 **7.** NTC

The second experiment, based on the detection of polymorphisms of the Tri13 gene, revealed different size amplicons for all the 92 strains tested.

All the Italian strains resulted in 15-ADON genotype; Chinese strains in 15-ADON and 3-ADON genotypes at the same extent; 6 of the 9 strains isolated in Austria were 15-ADON genotype and 10 of the 15 strains isolated in Germany were 3-ADON genotype.

### 4.4.3 Chemical results

Production of type B trichothecenes of *F. graminearum*strains is shown in Table 4.3.

**Table 4.3** List of *F. graminearum* isolates and their molecular identification, molecular chemotypes and trichothecenes production.

| N. | ITEM | Origin | Molecular identification | DNA-based chemotype | Trichothecenes production (µg/g) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | NIV | DON | 3-ADON | 15-ADON |
| **1** | 17079 | China | *F. gerlachii* | 3-ADON | 149 | 16 | n.r. | n.r. |
| **2** | 17052 | China | *F. gerlachii* | 3-ADON | n.r. | 332 | 3.9 | 0.8 |
| **3** | 17065 | China | *F. gerlachii* | 3-ADON | 5 | 153 | n.r. | n.r. |
| **4** | 17066 | China | *F. gerlachii* | 3-ADON | 49 | 16 | 79.1 | 16.5 |
| **5** | 17067 | China | *F. gerlachii* | 3-ADON | 34 | 577 | 42.4 | 6.6 |
| **6** | 17069 | China | *F. gerlachii* | 3-ADON | n.r. | 25 | 743.4 | 27.4 |
| **7** | 17070 | China | *F. gerlachii* | 3-ADON | 41 | 17 | 536.9 | 15.3 |
| **8** | 17071 | China | *F. gerlachii* | 3-ADON | n.r. | 177 | 1.5 | 0.2 |
| **9** | 17072 | China | *F. gerlachii* | 3-ADON | n.r. | n.r. | n.r. | n.r. |
| **10** | 17073 | China | *F. gerlachii* | 3-ADON | n.r. | n.r. | n.r. | n.r. |
| **11** | 17074 | China | *F. gerlachii* | 3-ADON | 6 | 245 | 19.2 | 1.1 |
| **12** | 17075 | China | *F. gerlachii* | 3-ADON | 77 | 114 | 259.5 | 24.3 |
| **13** | 17076 | China | *F. gerlachii* | 3-ADON | 129 | 21 | 472.4 | 38.2 |
| **14** | 17077 | China | *F. gerlachii* | 3-ADON | n.r. | 56 | n.r. | n.r. |
| **15** | 17053 | China | *F. graminearum s. s.* | 15-ADON | 253 | 33 | 0.6 | 71.5 |
| **16** | 17054 | China | *F. graminearum s. s.* | 15-ADON | n.r. | 169 | 16.3 | 81.4 |
| **17** | 17055 | China | *F. graminearum s. s.* | 15-ADON | 49 | 14 | n.r. | 34.9 |
| **18** | 17056 | China | *F. graminearum s. s.* | 15-ADON | 69 | 1 | n.r. | 21.3 |
| **19** | 17057 | China | *F. graminearum s. s.* | 15-ADON | 61 | 23 | n.r. | 62.1 |
| **20** | 17058 | China | *F. graminearum s. s.* | 15-ADON | 92 | 32 | 0.9 | 49.0 |
| **21** | 17059 | China | *F. graminearum s. s.* | 15-ADON | 12 | 573 | n.r. | 22.5 |
| **22** | 17060 | China | *F. graminearum s. s.* | 15-ADON | 31 | 22 | 1.0 | 33.6 |
| **23** | 17061 | China | *F. graminearum s. s.* | 15-ADON | 22 | 16 | n.r. | 72.0 |
| **24** | 17062 | China | *F. graminearum s. s.* | 15-ADON | 1577 | 12 | 0.9 | n.r. |
| **25** | 17063 | China | *F. graminearum s. s.* | 15-ADON | 32 | 17 | n.r. | 40.5 |
| **26** | 17064 | China | *F. graminearum s. s.* | 15-ADON | 19 | 470 | n.r. | 10.3 |
| **27** | 17068 | China | *F. graminearum s. s.* | 15-ADON | 75 | n.r. | n.r. | n.r. |
| **28** | 17078 | China | *F. graminearum s. s.* | 15-ADON | n.r. | n.r. | n.r. | n.r. |
| **29** | 17112 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 15 | n.r. | n.r. |
| **30** | 17113 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 15 | n.r. | 33.3 |
| **31** | 17114 | Italy | *F. graminearum s. s.* | 15-ADON | 84 | 289 | 0.3 | 12.9 |
| **32** | 17115 | Italy | *F. graminearum s. s.* | 15-ADON | 112 | 68 | 7.6 | 18.1 |
| **33** | 17116 | Italy | *F. graminearum s. s.* | 15-ADON | 98 | 11 | n.r. | 83.0 |
| **34** | 17117 | Italy | *F. graminearum s. s.* | 15-ADON | 203 | 36 | n.r. | 20.8 |
| **35** | 17118 | Italy | *F. graminearum s. s.* | 15-ADON | 47 | 228 | n.r. | 37.4 |
| **36** | 17119 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 40 | n.r. | 2.6 |
| **37** | 17120 | Italy | *F. graminearum s. s.* | 15-ADON | 216 | 177 | n.r. | n.r.r |
| **38** | 17121 | Italy | *F. graminearum s. s.* | 15-ADON | 71 | 300 | n.r. | 2.9 |
| **39** | 17122 | Italy | *F. graminearum s. s.* | 15-ADON | 14 | 10 | n.r. | 32.9 |
| **40** | 17123 | Italy | *F. graminearum s. s.* | 15-ADON | 15 | 480 | n.r. | 37.9 |
| **41** | 17127 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 14 | n.r. | n.r. |
| **42** | 17128 | Italy | *F. graminearum s. s.* | 15-ADON | 75 | 29 | n.r. | 82.6 |
| **43** | 17129 | Italy | *F. graminearum s. s.* | 15-ADON | 24 | 7 | n.r. | 5.1 |
| **44** | 17302 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 17 | 3.5 | 8.0 |
| **45** | 17303 | Italy | *F. graminearum s. s.* | 15-ADON | 4 | 12 | n.r. | 24.1 |
| **46** | 17304 | Italy | *F. graminearum s. s.* | 15-ADON | 42 | 7 | n.r. | 27.0 |
| **47** | 17305 | Italy | *F. graminearum s. s.* | 15-ADON | 14 | 42 | 2.2 | 6.6 |
| **48** | 17306 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 476 | n.r. | 55.4 |
| **49** | 17307 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 15 | n.r. | n.r. |
| **50** | 17308 | Italy | *F. graminearum s. s.* | 15-ADON | 20 | 18 | n.r. | 47.8 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **51** | 17309 | Italy | *F. graminearum s. s.* | 15-ADON | 22 | 398 | 0.4 | 2.8 |
| **52** | 17311 | Italy | *F. graminearum s. s.* | 15-ADON | 1229 | 24 | 1.1 | n.r. |
| **53** | 17312 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 15 | n.r. | 52.3 |
| **54** | 17313 | Italy | *F. graminearum s. s.* | 15-ADON | 30 | 12 | 5.1 | 1.5 |
| **55** | 17314 | Italy | *F. graminearum s. s.* | 15-ADON | 10 | 6 | n.r. | 31.2 |
| **56** | 17315 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 13 | 1.4 | 5.3 |
| **57** | 17316 | Italy | *F. graminearum s. s.* | 15-ADON | 2 | 487 | n.r. | 0.5 |
| **58** | 17317 | Italy | *F. graminearum s. s.* | 15-ADON | 7 | 467 | 0.7 | 2.9 |
| **59** | 17318 | Italy | *F. graminearum s. s.* | 15-ADON | 5 | 56 | 4.9 | 23.2 |
| **60** | 17319 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 299 | n.r. | 38.2 |
| **61** | 17320 | Italy | *F. graminearum s. s.* | 15-ADON | 275 | 404 | 2.8 | 0.8 |
| **62** | 17321 | Italy | *F. graminearum s. s.* | 15-ADON | 14 | 152 | n.r. | 21.3 |
| **63** | 17322 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 174 | n.r. | n.r. |
| **64** | 17323 | Italy | *F. graminearum s. s.* | 15-ADON | n.r. | 11 | 0.5 | 0.5 |
| **65** | 17324 | Italy | *F. graminearum s. s.* | 15-ADON | 14312 | 4 | n.r. | n.r. |
| **66** | 17325 | Italy | *F. graminearum s. s.* | 15-ADON | 8 | 238 | 3.3 | 20.5 |
| **67** | 17326 | Italy | *F. graminearum s. s.* | 15-ADON | 71 | 44 | 1.5 | 34.4 |
| **68** | 17327 | Italy | *F. graminearum s. s.* | 15-ADON | 84 | 83 | 8.2 | 62.2 |
| | Blank | | | | n.r. | n.r. | n.r. | n.r. |

Results of chemical analysis confirmed the highest production of 15-ADON by the main part of the strains. A lot of tested strains co-produced DON and NIV compounds in different quantities.

In some samples, toxin production was not detected by chemical analyses, although a molecular chemotype was assigned to them.

Chemical analysis of the strains grown on the three different synthetic media revealed no production of MTX by strains of *F. graminearum,* known to be type B trichothecenes producer on rice (results are shown in Tables S4.1a, b).

## 4.5 Discussion

In the present study, we characterized phylogenetic diversity of a population of *F. graminearum* worldwide and evaluated this species composition over 5 different cereals, wheat, barley, maize, oat and triticale.

DNA sequences based identification and phylogenetical analysis of BT and TEF-1α genes showed that 14 strains isolated from wheat collected in China were closely related to reference strain of *F. gerlachii*.

All the other strains of the population isolated in Austria, Germany, China and Italy, was shown to be *F. graminearum s. s.* revealing no correlation between clusters and geographical origin.

Related to the 5 cereals considered in the study, isolates from wheat were scattered within the dendrogram in different small clusters due showing low intraspecific variability of the strains, but with very low bootstrap values. So phylogenetic analysis evidenced, in this set of strains, a lack of relationship between strains and host plant for the isolates analyzed.

*F. graminearum s. s.* is nowadays the most frequently isolated species worldwide and can belong to different chemotypes: 3-ADON (producing DON and 3-ADON), 15-ADON (producing DON and 15-ADON) and NIV (producing NIV and 4-ANIV) (Pasquali et al., 2014; Ward et al., 2002).

In this study, conventional PCR tests, one from literature and one developed in this study, have been used to determine the trichothecene genotype of the individual isolates in order to study the distribution of these in different geographical areas, and chemical analyses have been used to detect the real capability to produce trichothecenes.

The data obtained on the Italian *F. graminearum* population analyzed in the present study, showing the *F. graminearum s. s.* as the exclusive species identified, confirmed the last decade previous reports from Europe that have shown *F. graminearum s. s.* as the dominant FGSC pathogen of cereals elsewhere in Europe (Yli-Mattila et al., 2008). This level of genetic homogeneity among the FGSC strains isolated from cereals could facilitate a better management of the disease, simplifying the development of breeding strategies for FHB resistance. However, among the Chinese FGSC strains here analyzed, 14 strains isolated from wheat were identified as *F. gerlachii*; to date, this is the first report on the occurrence of this species in China.

In 2007 Starkey (Starkey et al, 2007) discovered this new species on wheat exhibiting head blight symptoms, isolated in the upper Midwest of the U.S. It was morphologically similar to *F. graminearum* including colony characters on PDA, but with slightly different conidial features from other species within the FGSC, and was described as a single chemotype NIV.

All 14 species identified in this study as *F. gerlachii* showed molecular chemoype 3-ADON and chemical analyses detected production of both NIV and DON chemotypes.

It could be interest to verify the true extent of *F. gerlachii* occurrence on Chinese cereals since this species has been shown to be a NIV producer, the most toxic chemotype toward humans and animals of the FGSC (Desjardins 2006; Ward et al., 2002). Those data suggest an increase vigilance of plant control and plant quarantine programmes for reducing the risk that, through international trade of agricultural commodities, FGSC strains with novel toxigenic potential could be spread globally.

Molecular analyses on Italian strains *F. graminearum s.s* confirmed the 15-ADON to be the only chemotype observed.

These data partially agree with previous reports from Italy (Prodi *et al.,* 2009, 2011; Somma et al, 2014), that collected strains only from durum wheat in restricted areas of Northern and Central Italy, showing 15-ADON as the most dominant chemotype.

It is important to underline that the 3-ADON chemotype strains were reported to be more aggressive toward wheat plants and are known to have a better fitness than the other chemotypes (Zhang et al., 2010), although not all reports agree with this assumption. Therefore, the absence of the 3-ADON chemotype shown in our study is appreciable information for the Italian situation.

However, since the studies of Prodi *et al.* (2009, 2011) detected at level of 3-ADON chemotype occurrence higher than our report, a wider investigation on the whole Italy should be carried out, including not only wheat, but also other cereals.

Chemical results didn't confirm the distinctions of DON genotype versus NIV genotype in all the 40 italian strains tested for the chemical capability to produce trichothecenes because most of them showed to be both NIV and DON producer. However, among the group of 40 strains molecularly identified as 15-ADON trichothecene genotype, although all confirmed to be DON producers, only 33 strains were also 15-ADON producers. The fact that in *F. graminearum s. s.*, strains genotypically characterized do not express phenotypically the same chemotype has been often reported (Desjardins, 2008) and is a main reason for which the evaluation of the possible risk related to *F. graminearum s. s.* contamination of wheat cannot be based only on the trichothecene genotype detection. Indeed, the chemical validation of molecular genotypes is often missed in many reports and can be the reason for misleading data that not always reflect the true situation of the trichothecene contamination profile (Desjardins, 2008). As an example, previous chemical analyses of cereal grains from China have consistently reported DON as the major occurring trichothecene, with NIV trichothecene genotype usually present at lower levels than DON (Desjardins, 2008), as is demonstrated also in this study.

Therefore a thorough validation of the trichothecene genotypes by chemical analyses remains an important tool for reporting the actual frequency of the different chemotypes within a target population.

In conclusion, our work provided a detailed report on worldwide *F. graminearum* population from cereals, mainly wheat.

This study confirmed the prevalence of *F. graminearum s. s.* species in Italy, characterized by high level of genetic homogeneity among the strains, and the

predominance of 15-ADON chemotype on strains. Unique to this study is the first detection of *F. gerlachii* among Chinese strains studied.

It is important that genotype profiles must be validated by performing chemical analyses in order to reflect the effective toxin production capability of each species of the FGSC (Desjardins, 2008).

However, the knowledge of trichothecene genotypes occurring in each country is still too limited and wider maps of trichothecene genotypes contaminating cereals need to be designed worldwide and therefore also at whole European level to better evaluate the strategies for facing the eventual import of new species/strains with new toxigenic potential from outside Europe.

## 4.6 Supplementary material

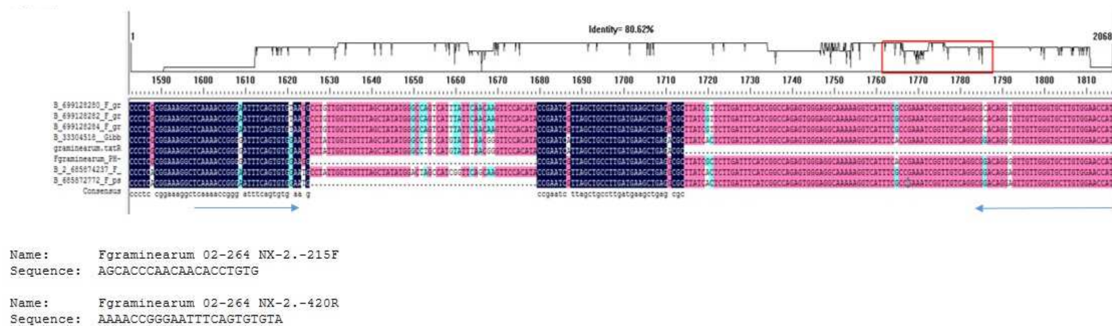


**Figure S4.1.** Primer pairs designed for the specific detection of TRI1 allele for *F. graminearum* in order to investigate the potential capability of producing a novel trichothecene type A, NX-2.

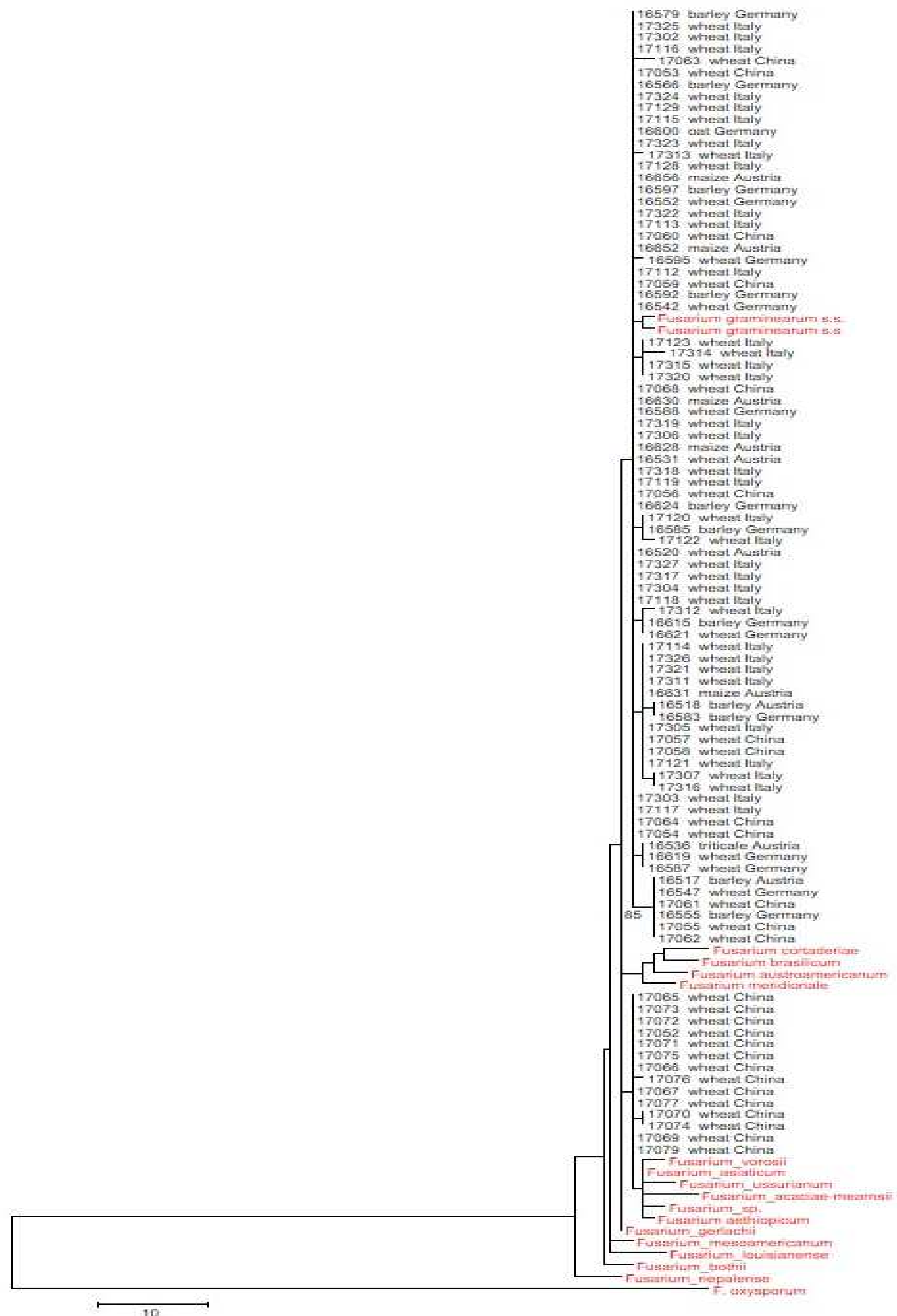


**Figure S4.2** Phylogenetic tree generated from concatenated nucleotide sequences of *ß-TUB and TEF-1α* fragments from 92 *Fusarium graminearum* strains of studied population, 17 reference strains of FGSC and *F. oxysporum* reference strain as outgroup. For references strains, it shows the identification code NRRL. Tree was constructed by using Maximum Parsimony clustering method with MEGA 5.2Version software.

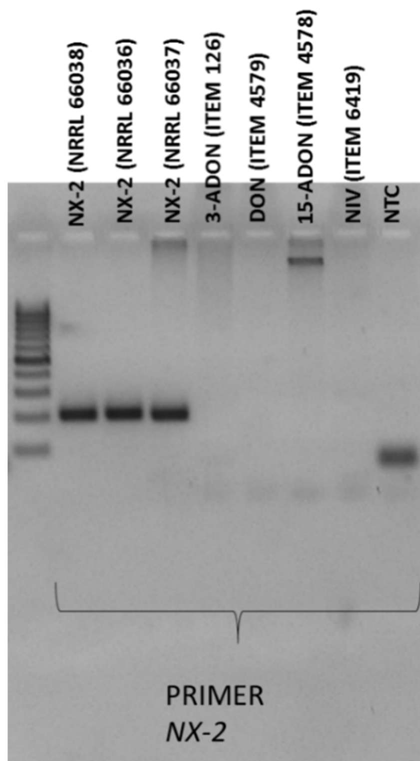


**Figure S4.3. Polymerase chain reaction of reference strains for NX-2 chemotype analysis**
Designed primers were tested obtaining amplifications with 3 NX-2 producing strains positive (NRRL 66038, 66036, 66037) and 4 strains (3-ADON, DON, 15-ADON and NIV) known to be producers.

**Table S4.1a.** Trichothecenes production (µg/g) on rice of three *F. graminearum* strains.

| | DON | 3-ADON | 15-ADON | NIV |
|---|---|---|---|---|
| **ITEM 126** | 2371 | 1433 | 50 | n.r. |
| **ITEM 6352** | 2909 | n.r. | 45 | n.r. |
| **ITEM 6415** | n.r. | n.r. | n.r. | 72 |

**Table S4.1b.** Trichothecenes production (µg/g) on three different agar substrates of three *F. graminearum* strains after 7, 14 and 21 days.

| | | DON | 3-ADON | 15-ADON | NIV | |
|---|---|---|---|---|---|---|
| **PDA** | 126 | n.r. | 3 | n.r. | n.r. | **7 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | 5 | 0.5 | n.r. | n.r. | **14 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | 6 | n.r. | n.r. | n.r. | **21 days** |
| | 6352 | n.r. | n.r. | 0.05 | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| **WA** | 126 | n.r. | n.r. | n.r. | n.r. | **7 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | n.r. | n.r. | n.r. | n.r. | **14 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | n.r. | n.r. | n.r. | n.r. | **21 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| **YGA** | 126 | n.r. | n.r. | n.r. | n.r. | **7 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | n.r. | n.r. | n.r. | n.r. | **14 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |
| | 126 | 5 | n.r. | n.r. | n.r. | **21 days** |
| | 6352 | n.r. | n.r. | n.r. | n.r. | |
| | 6415 | n.r. | n.r. | n.r. | n.r. | |

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# CHAPTER 5

# ANALYSIS OF PATHOGENIC PATHWAY OF *FUSARIUM GRAMINEARUM* AND *FUSARIUM CULMORUM* ON INOCULATED DURUM WHEAT PLANTS

## 5.1 ABSTRACT

Contamination of wheat by *Fusarium* species is one of the major sources of mycotoxins (MTX) accumulation in food and feed. Quantification of biomass of *Fusarium* species is essential to understand the interaction between individual species and plants in *Fusarium* disease development.

In this study, two different trials were performed in climatic chambers on durum wheat plants artificially inoculated with *F. graminearum* and *F. culmorum* spore suspensions, at different plant growth stages. The plants were sampled at different growth stages and cut in different parts: in total, 64 wheat samples inoculated with *F. graminearum* and 64 samples with *F. culmorum* were tested.

Two different quantitative real-time PCR assays were carried out to detect *F. graminearum* and *F. culmorum* DNA in wheat samples. A quantitative PCR assay based on the TEF-1α gene, by using SYBR Green chemistry, was used to detect *F. culmorum*; on the other hand, a TaqMan assay was used to detect *F. graminearum*, with primers and probe built on a DNA fragment species specific for *F. graminearum.*

In addition, chemical analyses were carried out on a set of wheat samples, in order to quantity deoxynivalenol (DON), a MTX produced by *F. graminearum* and *F. culmorum.*

The presence of *F. graminearum* was detected in ears and kernels of plants inoculated at flowering, a key stage for the successful contamination by *Fusarium.* On the other hand, the presence of *F. culmorum* was detected only in samples inoculated and sampled at the same timing, without spreading after inoculation.

DON was detected also in samples with low amount ù'of the fungi, or their absence given its ability to move into the vascular system. DON was never detected in kernels.

## 5.2 Introduction

The *Fusarium* genus has a global distribution and many of its species are phytopathogenic fungi infecting a wide range of crop plants including cereals such as maize, wheat, oat and barley. *Fusarium* contamination is a major agricultural problem as quality and yield can be reduced. However, the most important aspect is that many *Fusarium* species produce MTX responsible for serious diseases in humans and farm animals.

Fusarium Head Blight (FHB) is one of the worrisome diseases in small-grain cereals, and is caused by several *Fusarium s*pecies. The *Fusarium* species predominantly found in association with FHB in small-grain cereals all over Europe are *F. graminearum (syn. Gibberella zeae*) and *F. culmorum* (Xu and Nicholson, 2009).

In particular, the main toxins produced by *F. graminearum* and other closely related species, including *F. culmorum*, are the type B trichothecenes deoxynivalenon (DON) and nivalenol (NIV) (Ward et al., 2008). Throughout Europe, DON is the most frequently detected trichothecene toxin in wheat (Nielsen et al., 2011). Indeed, the DON contamination in wheat is regulated by the European Commission with maximum levels fixed at 1.75 µg/g for dried matter for unprocessed durum wheat and 1.25 µg/g for soft wheat (EC 1126/2007).

MTX are very stable metabolites and once the grain is contaminated by MTX, its decontamination is very difficult. Fungus-infested plants can be difficult to identify because symptoms are often not visible, even if heavily contaminated by MTX. On the other hand, field products that appear to be heavily contaminated by fungus do not necessarily contain MTX, even if the fungus has been identified usually produces MTX.

The ability of *F. graminearum* to migrate from infected seeds to the ears of the wheat heads was reported by Poels et al. (2006). However, Moretti et al. (2014) showed accumulation in the kernels although the absence of *F. graminearum* in the upper parts,

both heads and kernels, of wheat plants previously inoculated at sowing. The authors suggested that a sort of barrier at head level for the fungus and not for the MTX inibhited the colonization of heads by the *F. graminearum* occurring in the seed.

Poor knowledge of the ability of *F. graminearum,* inoculated at other stages of the plant, to migrate on the whole plant and to contribute to the DON contamination of kernels is available.

Clement and Parry (1998) demonstrated the ability of *F. culmorum* to move far from the point of inoculation, and Covarelli in 2012 demonstrated that *F. culmorum* can extensively colonize stem tissues but does not reach the head by the time of plant maturity, altough DON was detected in tissues beyond those colonized by the fungus, translocating to the head. However, further analyses could expand the knowledge about the role of this fungus in FHB disease.

Molecular tools allow to detect the presence of fungal species in plant tissues with high accuracy even in lack of symptoms. In particular, several real-time PCR assays have been developed for the quantification of individual *Fusarium* species in infected grain or plant tissues (for a review see Niessen, 2007).

The aim of this study was to analyse the pathogenic pathway of *F. graminearum* and *F. culmorum* on durum wheat plants artificially inoculated in different parts and at different plant growth stages, monitoring the presence of these two species by real-time PCR.

Moreover, chemical analyses of DON content were carried out on same samples to deeply investigate fungal and MTX contamination.

## 5.3 Materials and methods

### 5.3.1 Wheat samples

Saragolla, a cultivar of durum wheat susceptible to *Fusarium* infection, was used in two trials prepared in a climatic chamber to study *F. graminearum* and *F. culmorum* pathways in wheat plants. The plants were grown in a climatic chamber with controlled light, temperature and humidity conditions. They were artificially inoculated on different parts (leaves, ears and kernels) and at different phenological plant phases (soil, seeds, tillering, booting and flowering) with fungal spore suspensions of *F. graminearum sensu stricto* ITEM 126 and *F. culmorum* ITEM 11007.

Sampling was carried out at different plant growth stages: tillering, booting, milky ripening, waxy ripening, vitreus maturity and harvest. The plants, cut in different portions, for a total of 64 wheat samples inoculated with *F. graminearum*, 64 inoculated with *F. culmorum* and 12 samples from not inoculated wheat plants used as control, were then stored at -20°C until analyses.

### 5.3.2 Quantitative real-time PCR assays

The wheat samples were lyophilized and homogenized in a blender.

The genomic DNA extraction was performed by using Wizard® Magnetic DNA Purification System kit for Food (Promega), starting from 100 mg of plant material and following the manufacturer's instructions. The accurate estimation of DNA quantity was obtained with spectrophotometer “NanoDrop ND-1000” (Thermo Fisher Scientific) and the purity was evaluated by the ratio A260/A280 (wavelength to which absorb nucleic acids and both nucleic acids and proteins, respectively), considering "pure" the DNA sample with the ratio around 1.8.

To detect *F. graminearum* DNA amount, a TaqMan assay with primers and probe built on a specie specific DNA fragment was conducted according to Waalwijk et al. (2004). Primers and probe sequence were listed in Table 5.1.

**Table 5.1**. Primers and probe sequences used in this study.

| SPECIES | PRIMER/PROBES | SEQUENCE (5'-3') | REFERENCES |
|---|---|---|---|
| *F. graminearum* | *graminearum* MGB-F | GGCGCTTCTCGTGAACACA | Waalwijk et al., 2004 |
| | *graminearum* MGB-R | TGGCTAAACAGCACGAATGC | |
| | *graminearum* MGB probe | AGATATGTCTCTTCAAGTCT | |
| *F. culmorum* | FculC561 fwd | CACCGTCATTGGTATGTTGTCACT | Nicolaisen et al., 2009 |
| | FculC614 rev | CGGGAGCGTCTGATAGTCG | |

Each reaction were performed in 15 μL, containing 100 ng of extracted total genomic DNA of wheat samples, 1X iTaqUniversal probes supermix (BIO-RAD), 200 nM of probe and 300 nM of both forward and reverse primers.

TaqMan reactions were performed in a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems), and run in the Viia$^{TM}$7 Real Time PCR System (Applied Biosystems). Thermal cycling conditions consisted of a single cycle of 2 min at 50 °C to degrade uracil containing DNA and 2 min at 95 °C to inactivate uracil-N-glycosidase, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 sec.

In each experiment a 1:10 dilution series of genomic DNA obtained from pure culture of the corresponding pathogen (*F. graminearum* strain ITEM 126), a negative control (water as template) and a positive control (a wheat sample contaminated by a known quantity of fungal DNA) were included. The standard curve obtained from 6 dilutions of fungal genomic DNA, from 10 ng to 0.1 pg, was used to quantify, by comparison, the *F. graminearum* DNA in each wheat sample, tested in triplicate. DNA amount was expressed as pg of DNA / mg of wheat plant tissue.

To detect *F. culmorum*, SYBR Green real-time PCR assays, based on the TEF-1α gene, were performed by using primers listed in Table 5.1, according to Nicolaisen et al. (2009). Real-time PCR was carried out in a total of 12.5 μl consisting of 1X SYBR Green PCR Master Mix (Applied Biosystems), 250 nM of each primer, 0.5 μg/μl bovine serum albumin (BSA) and 100 ng of template DNA. Each wheat sample was tested in triplicate. PCR was performed by using the following cycling protocol: 2 min at 50 °C; 2 min at 95 °C; 40 cycles of 95 °C for 1 sec and 62 °C for 20 sec. A final dissociation stage (15 sec at 95 °C, 1 min at 60 °C and 15 sec of a gradual increase of temperature to 95 °C) was added to perform the melting curve analysis. In each experiment, run in the Viia™7 Real Time PCR System (Applied Biosystems), a 1:10 dilution series of genomic DNA (6 dilutions, from 10 ng to 0.1 pg) obtained from pure cultures of the corresponding pathogen (*F. culmorum* strain ITEM 11007), a positive control and a negative control were included. The *F. culmorum* DNA amount in the wheat samples, calculated by comparison with the standard curve, was expressed as pg of fungal DNA / mg of wheat plant tissue.

### 5.3.3 Chemical analyses

A subset of 48 wheat samples, selected based on PCR assay results, were analyzed at ISPA-CNR Bari for DON occurrence.

The analysis of DON was carried out using the method based on the purification of the extracts realized with immunoaffinity columns and on the determination of the toxin realized by UPLC with DAD detector, according to Pascale et al., 2014. The limit of detection of the toxin for this analysis is 0.03 mg/g.

## 5.4 Results and Discussion

The quantities of *Fusarium* species detected on leaves, ears and kernels, expressed as pg of fungal DNA / mg of plant material, are shown for each inoculum stage and sampling times, in Figure 5.1 for *F. graminearum* and in Figure 5.2 for *F. culmorum.*

In general, *F. graminearum* and *F. culmorum* were detected in few samples, in range varying from 0.01 to 82 pg of fungal DNA/mg of plant tissue for *F. graminearum*, and from 0.04 to 342 pg/mg for *F. culmorum.*

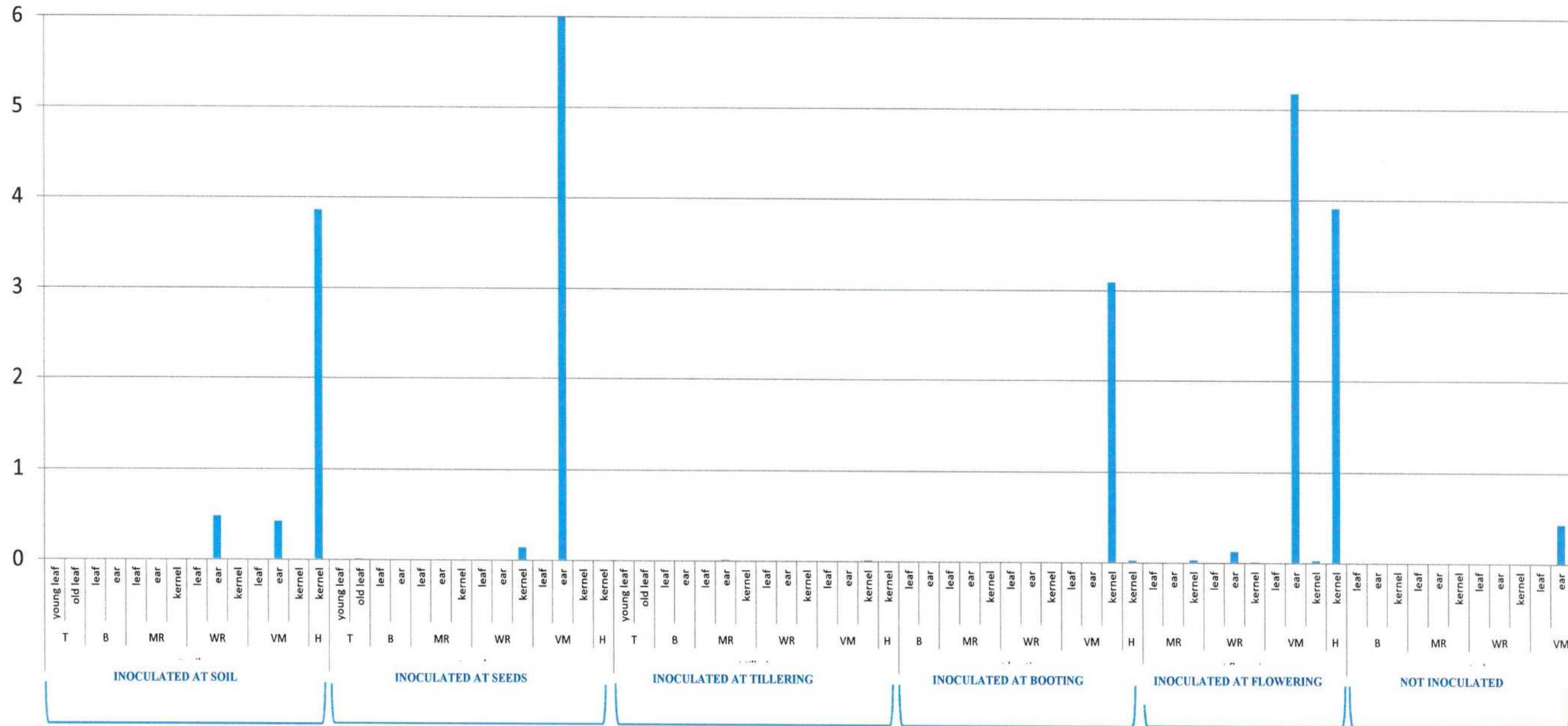


**Figure 5.1** DNA amount of *F. graminearum* (Fg) in 64 inoculated and 12 not inoculated wheat field samples as detected by TaqMan realtime PCR assay. Sampling times are indicated as follows:**T** (tillering), **B** (Booting), **MR** (Milky ripening), **WR** (Waxy ripening), **WM** (Vitreus maturity), **H** (Harvest). Each part of plant (leaf, ear and kernel) was inoculated with spore suspension at soil, at seeds, at tillering and at booting.

As shown in Figure 5.1, *F. graminearum* DNA was detected only in ears and kernels. The results showed that plants grown in the proximity of inoculated soil, the fungus could be detected in ears sampled at waxy and vitreous ripening and in kernels at harvest. According with these data, *F. graminearum* that infected the plants from the soil could colonize the ears.

Also in plants grown from inoculated seeds, a very high level *F. graminearum* was detected in ears, although only when they were sampled at vitreous maturity; in samples inoculated at booting, only kernels sampled at vitreous maturity showed *F. graminearum* contamination.

The data shown above that report a possible migration of *F. graminearum* from the stems to the ears of wheat plants should be carefully evaluated, since also a possible cross contamination, occurred in the climatic chamber, should be considered as explanation of such levels of ear contamination. Indeed, the lack of the fungus contamination in plants inoculated at same stages (soil and sowing) and sampled at previous stages, led to believe that cross contamination played a major role in such contamination levels. This hypothesisis supported by the evidence that a positive sample was detected also in ears at vitreous maturity among the controls not inoculated with *F. graminearum*, confirming the idea that cross contamination in the climatic chamber occurred.

In plants inoculated at flowering, *F. graminearum* was detected in ears sampled at waxy ripening, in ears and kernels sampled at vitreous ripening and in kernels at harvest. These data confirm the key role of fungal attack at flowering for the final kernel contamination.

Analysing the results of *F. culmorum* contamination, shown in Figure 5.2, in plants grown in the proximity of inoculated soil, the fungus was detected in leaves sampled at tillering and in ears sampled at booting and at waxy ripening. The latter result, considering also the very high level of *F. culmorum* DNA amount, could be due to a contamination in the

climatic chamber, hypothesis supported also by the contaminated ears sampled at milky ripening from plants of the not inoculated control. In plants grown from inoculated seeds, ears and kernels at vitreous maturity showed *F. culmorum* contamination. In plants inoculated at tillering, *F. culmorum* was detected on leaves, while plants inoculated at booting *F. culmorum* was detected only on boot, as expected. No contamination was detected in the subsequent stages, as well as *F. culmorum* was absent inoculating at flowering.

Thus, the presence of *F. culmorum* was detected only in plants inoculated and sampled at the same timing. This could mean that the fungus could not find the optimal conditions for spreading in the plants.

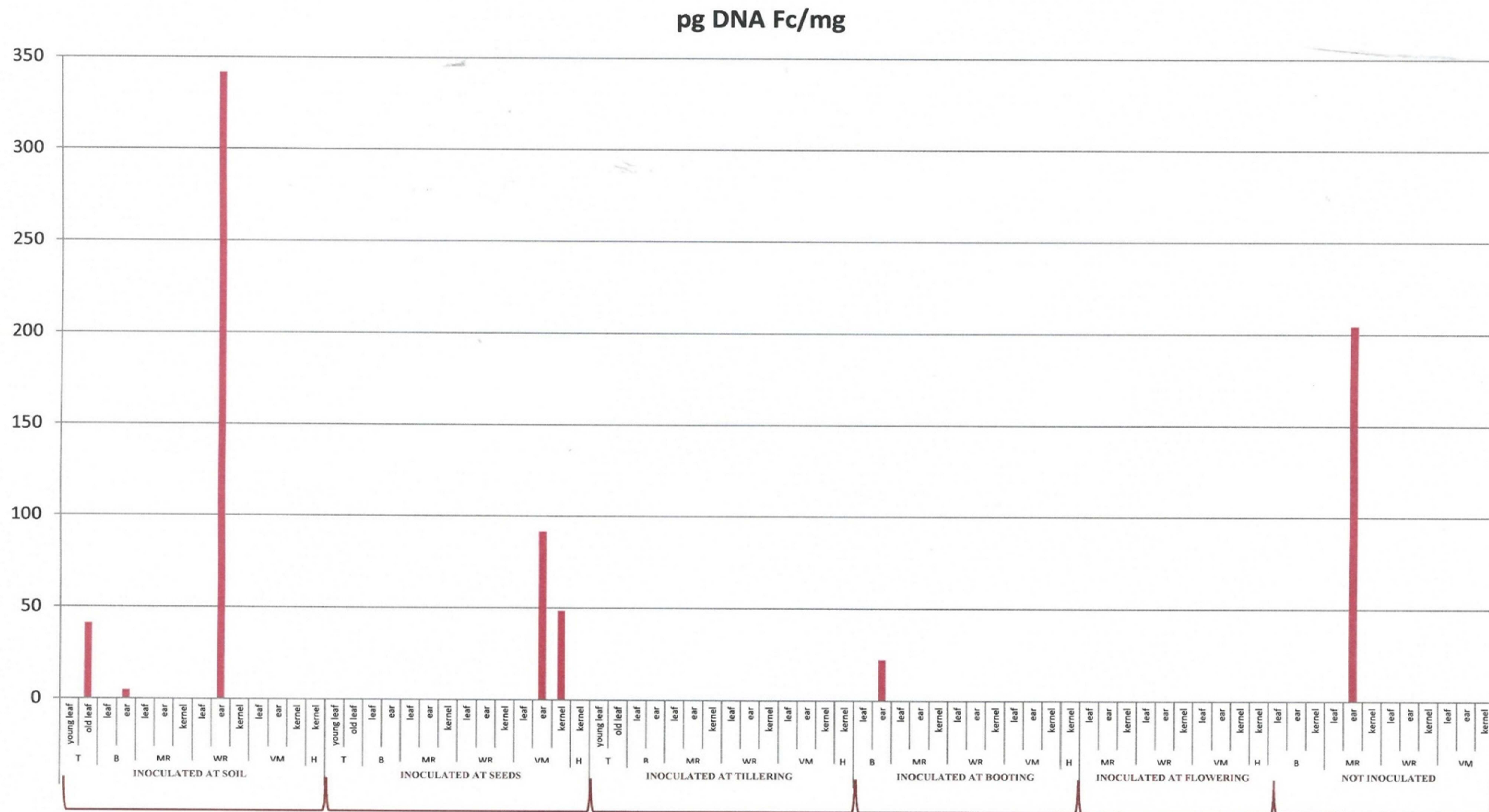


**Figure 5.2** DNA amount of *F. culmorum* (Fc) in 64 inoculated and 12 not inoculated wheat field samples as detected by the SYBR Green real time PCR assay. Sampling times are indicated as follows:**T** (tillering), **B** (Booting), **MR** (Milky ripening), **WR** (Waxy ripening), **WM** (Vitreus maturity), **H** (Harvest).
Each part of plant (leaf, ear and kerne) were inoculated at soil, at seeds, at tillering and at booting.

Selected wheat samples (48 samples) were also chemically analysed to detect the DON accumulation in the plants. The results, expressed in µg/g, are shown in Table 5.2.

**Table 5.2** Concentration of DON toxin and quantification of fungal biomass detected in 48 wheat samples, inoculated with *F. graminearum* and *F. culmorum*.

| *Species inoculated* | **INOCULUM STAGES** | **SAMPLING TIMES** | **PART OF PLANT** | **DON (µg/g)** | **pg of fungal DNA/mg of plant tissue** |
|---|---|---|---|---|---|
| ***F. graminearum*** | at soil | waxy ripening | ear | 29.0 | 0.48 |
| | at soil | vitreus maturity | ear | 11.4 | 0.42 |
| | at soil | harvest | kernel | n.r. | 3.86 |
| | at seeds | tillering | old leaf | 9.1 | 0.01 |
| | at seeds | waxy ripening | kernel | n.r. | 0.14 |
| | at seeds | vitreus maturity | ear | 9.3 | 82.06 |
| | tillering | milky ripening | ear | n.r. | 0.01 |
| | tillering | vitreus maturity | kernel | n.r. | 0.02 |
| | booting | vitreus maturity | kernel | 0.2 | 3.09 |
| | booting | harvest | kernel | n.r. | 0.03 |
| | flowering | milky ripening | kernel | n.r. | 0.03 |
| | flowering | waxy ripening | ear | 8.1 | 0.13 |
| | flowering | waxy ripening | kernel | n.r. | 0.01 |
| | flowering | vitreus maturity | ear | 3.6 | 5.18 |
| | flowering | vitreus maturity | kernel | n.r. | 0.03 |
| | flowering | harvest | kernel | 0.2 | 3.91 |
| | control | vitreus maturity | ear | n.r. | 0.44 |
| ***F. culmorum*** | at soil | tillering | old leaf | 18.9 | 41.13 |
| | at soil | booting | leaf | n.r. | 0.04 |
| | at soil | booting | ear | n.r. | 4.8 |
| | at soil | milky ripening | leaf | n.r. | 0 |
| | at soil | milky ripening | kernel | n.r. | 0 |
| | at soil | waxy ripening | ear | 1.0 | 341.91 |
| | at soil | waxy ripening | kernel | n.r. | 0 |
| | at soil | vitreus maturity | kernel | n.r. | 0 |
| | at seeds | booting | ear | n.r. | 0 |
| | at seeds | milky ripening | ear | n.r. | 0 |
| | at seeds | waxy ripening | ear | 1.6 | 0 |
| | at seeds | vitreus maturity | ear | 1.4 | 91.68 |
| | at seeds | vitreus maturity | kernel | n.r. | 48.54 |
| | tillering | tillering | young leaf | n.r. | 0.12 |
| | tillering | tillering | old leaf | n.r. | 0.4 |
| | tillering | booting | ear | n.r. | 0 |
| | tillering | milky ripening | leaf | 0.7 | 0 |
| | tillering | milky ripening | ear | n.r. | 0 |
| | tillering | waxy ripening | ear | 8.6 | 0 |
| | tillering | vitreus maturity | leaf | 16 | 0 |
| | tillering | vitreus maturity | ear | 8.8 | 0 |
| | tillering | vitreus maturity | kernel | n.r. | 0 |
| | tillering | harvest | kernel | n.r. | 0 |
| | booting | booting | ear | n.r. | 22.38 |
| | booting | milky ripening | kernel | n.r. | 0 |
| | booting | harvest | kernel | n.r. | 0 |
| | flowering | milky ripening | ear | n.r. | 0 |
| | flowering | vitreus maturity | ear | n.r. | 0 |
| | flowering | vitreus maturity | kernel | n.r. | 0 |
| | flowering | harvest | kernel | n.r. | 0 |
| | control | milky ripening | ear | n.r. | 204.64 |

Only 16 wheat samples of 48 (30%) resulted contaminated by DON, which amount ranged from 0.2 to 29 µg/g.

Comparing molecular and chemical analyses, part of plants contaminated by *F. graminearum* and *F. culmorum* resulted also contaminated by DON, as shown in Table 5.2. In some samples, mostly ears, the level of DON was relevant, despite the fungus was detected at very low level or was absent. This finding support the hypothesis that DON moves through the vessels in the plant to the ears, also in absence of fungal infection in the same part of plant. Unlike, no DON contamination was detected in kernels. These results are in accordance with the findings reported in Moretti et al (2014) for *F. graminearum* and in Covarelli et al (2012) for *F. culmorum*, about the inability of *F. graminearum* and *F. culmorum* to migrate from the stems to ears or kernels. On the contrary, the ability of DON to move into the vascular system of plants and contaminate also ears and kernels lacking the fungal contamination is confirmed.

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# CHAPTER 6

## FUSARIC ACID CONTRIBUTES TO VIRULENCE OF *FUSARIUM OXYSPORUM* ON PLANT AND MAMMALIAN HOSTS

Vahid Rahjoo1,†, Michael Sulyok2, Veronica Ghionna1,‡, Cristina Lopez-Diaz1, Adela Martin-Vicente3, Javier Capilla3, Antonio Di Pietro1,* and Manuel S. Lopez-Berges1,§,**

1Departamento de Genetica, Facultad de Ciencias, Universidad de Cordoba. Campus de Excelencia Agroalimentario (ceiA3), E-14071 Cordoba, Spain.
2Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenz Str. 20, 3430 Tulln, Austria.
3Mycology Unit, Medical School, Universitat Rovira i Virgili, IISPV, 43204 Reus, Spain.
*Corresponding author at: ge2dipia@uco.es

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## 6.1 ABSTRACT

Fusaric acid (FA) is amongst the oldest identified secondary metabolites produced by *Fusarium* species, known for a long time to display strong phytotoxicity and moderate toxicity to animal cells; however, the cellular targets of FA and its function in fungal pathogenicity remain unknown. Here, we investigated the role of FA in *Fusarium oxysporum*, a soil-borne cross-kingdom pathogen that causes vascular wilt on more than 100 plant species and opportunistic infections in humans. Targeted deletion of fub1, encoding a predicted orthologue of the polyketide synthase involved in FA biosynthesis in *F. verticillioides* and *F. fujikuroi*, abolished the production of FA and its derivatives in *F. oxysporum.* We further showed that the expression of fub1 was positively controlled by the master regulator of secondary metabolism LaeA and the alkaline pH regulator PacC through the modulation of chromatin accessibility at the fub1 locus. FA exhibited strong phytotoxicity on tomato plants, which was rescued by the exogenous supply of copper, iron or zinc, suggesting a possible function of FA as a chelating agent of these metal ions. Importantly, the severity of vascular wilt symptoms on tomato plants and the mortality of immunosuppressed mice were significantly reduced in fub1Δ mutants and fully restored in the complemented strains. Collectively, these results provide newinsights into the regulation and mode of action of FA, as well as on the function of this phytotoxin during the infection process of *F. oxysporum.*

## 6.2 Introduction

Fungi produce major plant diseases and destroy or contaminate each year a significant part of the global agricultural production, making them by far the most damaging class of plant pathogens (Fisher et al., 2012, Strange and Scott, 2005). Moreover, opportunistic fungal pathogens of humans can provoke life-threatening systemic infections, particularly on immunocompromised patients (Fridkin, 2005). The soil-inhabiting fungus *Fusarium oxysporum* has been ranked in the Top 10 fungal pathogens in molecular plant pathology based on scientific/economic importance and causes vascular wilt disease in more than 100 different crops (Armstrong and Armstrong, 1981, Dean et al., 2012). In addition, *F. oxysporum* isolates can cause opportunistic infections in humans ranging from superficial or locally invasive to disseminated, depending on the immune status of the host (Nucci, Anaissie, 2007). *F. oxysporum* f. sp. *lycopersici* FGSC 9935 is a fully sequenced isolate (Ma *et al.*, 2010) able to kill both tomato plants and immunodepressed mice (Ortoneda *et*

*al.*, 2004). Therefore, this isolate represents an excellent model for studying the genetic basis of cross-kingdom pathogenicity in fungi. Many fungi produce secondary metabolites that are toxic to plants or animals (Berthiller *et al.*, 2013). Fusaric acid (FA), a picolinic acid derivative originally isolated from *Fusarium heterosporium* (Yabuta *et al.*, 1937), was the first fungal phytotoxin isolated from infected host plants (Gaumann, 1957) and is known for its high phytotoxicity (Niehaus *et al.*, 2014, Stipanovic *et al.*, 2011). FA also exhibits toxicity towards animals including notochord malformation in zebrafish (Yin *et al.*, 2015) or neurotoxicity in mammals (Porter *et al.*, 1995), and towards bacteria (Bacon *et al.*, 2006, Ruiz *et al.*, 2015). Although several studies on the mode of action of FA have been conducted, the cellular basis for its toxicity remains poorly understood. Suggested mechanisms include the modification of cell membrane potential, inhibition of ATP synthesis, chelation of metal ions or electrolyte leakage (D'Alton and Etherton, 1984, Pavlovkin, 1998, Ruiz et al., 2015, Marre *et al.*, 1993). Recently, chromatin condensation, cytochrome *c* release, DNA fragmentation and hydrogen peroxide accumulation were reported in FA treated plant cell cultures suggesting a possible involvement of programmed cell death in FA toxicity (Jiao *et al.*, 2013, Samadi and Shahsavan Behboodi, 2006).

The polyketide synthase (PKS) Fub1 was recently identified as the first enzyme of the FA biosynthetic pathway in *F. verticillioides* (Brown *et al.*, 2012). The *fub1* gene is part of the FA gene cluster, and its inactivation is sufficient to completely block FA production (Brown et al., 2012, Niehaus et al., 2014). In the present work we studied the role of Fub1 in *F. oxysporum*. We found that *fub1* is essential for production of FA and its derivatives in this fungus, and that its transcription is regulated by LaeA, a master regulator of secondary metabolism. We further demonstrate that loss of Fub1 and FA in *F. oxysporum* leads to reduced virulence in tomato plants and immunodepressed mice.

Finally, we show that phytotoxicity of FA can be reduced by supplying copper or zinc to the plants. Our results establish a functional role for FA in fungal virulence on plants and mammals.

## 6.3 Experimental Procedures

### 6.3.1 Fungal isolates and culture conditions

*Fusarium oxysporum* f. sp *lycopersici* race 2 wild-type isolate 4287 (FGSC 9935) was used in all experiments. Fungal strains were stored as microconidial suspensions at - 80 °C with 30% glycerol. For extraction of genomic DNA and microconidia production, cultures were grown in PDB at 28 °C (Di Pietro and Roncero, 1998). For analysis of gene expression and relative chromatin accessibility, freshly obtained microconidia were germinated for 14-16 h in PDB or GMM. Germlings were harvested by filtration, washed three times in sterile water and transferred to fresh PDB, CDL or GMM with or without 50 mM CuSO4, FeSO4 or ZnSO4 for the indicated time periods. pH 5 and pH 7 buffered conditions were achieved using 100 mM 4- Morpholineethanesulfonic (MES), when indicated. For determination of colony growth, 2 x 104 microconidia were spotted onto PDA, CDA or minimal medium agar (MMA) or GMM with or without FA (0–0.75 mg/mL), with or without 200 mM BCS or 4 mM TPEN, and with or without CuSO4, FeSO4 or ZnSO4 (0.05–5 mM). Plates were incubated at 28 8C for the indicated time periods.. All experiments included two replicates and were performed at least three times with similar results.

### 6.3.2 Fungal strains

PCR reactions were routinely performed with VELOCITY$^{TM}$ DNA Polymerase (Bioline, London, UK) using a MJ Mini$^{TM}$ Personal Thermal Cycler (Bio-Rad, Madrid, Spain) (see Table S6.1, Supporting Information, for a complete list of primer sequences used in the

study). All fungal transformations and purification of the transformants by monoconidial isolation were performed as described (Di Pietro and Roncero, 1998). The cassette for targeted replacement of the entire coding region of the *F. oxysporum fub1* gene with the hygromycin B-resistance marker (Punt *et al*., 1987) was assembled by a fusion PCR method (Szewczyk *et al*., 2006). DNA fragments flanking the *fub1* coding region were amplified from genomic DNA of *F. oxysporum* wild-type with primers *fub1*-F1 + *fub1*-R1 and *fub1*-F2 + *fub1*-R2, respectively, while the hygromycin B-resistance marker, under control of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator, was amplified from pAN7-1 plasmid (Punt et al., 1987) with primers *fub1-hph*-F + *fub1-hph*-R. The three DNA fragments were then PCR fused with primers *fub1*-F1n + *fub1*-R2n. The obtained *fub1*Δ allele was used to transform protoplasts of the *F. oxysporum* wild-type strain to hygromycin B-resistance (Fig. S6.1B). Transformants showing homologous insertion of the construct were genotyped by PCR of gDNA with primers *fub1*-F1 + *fub1*-R2 (not shown) and by Southern blot analysis (Fig. S6.1C). To generate a construct for complementation of the *fub1*Δ strain, a 9645 bp fragment spanning from 1077 bp upstream of the wild-type *F. oxysporum fub1* translation initiation codon to 1092 bp downstream of the translation termination codon was amplified by PCR with primers *fub1*-F1 + *fub1*-R2. The amplified fragment was used to co-transform protoplasts of the fub1D strain with the phleomycin B resistance gene under the control of the *A. nidulans gpdA* promoter and trpC terminator, amplified from the pAN8-1 plasmid (Mattern et al., 1988) with the primers gpdA215b1trpC28b (Fig. S6.2A). Several phleomycin-resistant cotransformants were analysed for the presence of a functional fub1 allele by PCR with the gene-specific primers fub1-F31fub1-R3 (Fig. S6.2B). Among the different complemented strains, we selected one in which the production of FA and derivatives (Fig. 6.1) and fub1 transcript levels returned to wild-type values (Fig. S6.2C, D).

### 6.3.3 Nucleic acid manipulations and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA and gDNA were extracted from *F. oxysporum* mycelia following previously reported protocols (Chomczynski and Sacchi, 1987, Raeder and Broda, 1985). The quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) respectively. Routine nucleic acid manipulations were performed according to standard protocols (Sambrook and Russell, 2001). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990). RT-qPCR was performed as described (Lopez-Berges *et al.*, 2010, Lopez-Berges *et al.*, 2012) using FastStart Essential DNA Green Master (Roche) in a CFX Connect Real-Time System (Bio-Rad). Gene-specific primers (see Table S6.1) were designed to flank an intron, if possible. Transcript levels were calculated by comparative ΔCt and normalized to *act1*.

### 6.3.4 Analysis of chromatin structure

Mycelia of *F. oxysporum* strains grown under the indicated conditions were harvested by filtration, lyophilized and ground to a fine powder in a Mini-BeadBeater 8 (BioSpe Products, Bartlesville, OK, USA). Nuclease digestion was performed as described (Basheer *et al.*, 2009, Gonzalez and Scazzocchio, 1997, Lopez-Berges et al., 2013). Briefly, 20 mg of lyophilized mycelium was suspended in 1 ml of micrococcal nuclease (MNase) buffer (250 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM CaCl2, 3 mM MgCl2), and 300 ml of the suspension were treated for 5 min with 3 U of MNase (Sigma, Madrid, Spain) at 37 °C. The reaction was terminated by adding stop buffer (2% SDS, 40 mM EDTA). DNA was obtained by phenol/chloroform extraction, precipitated, washed with 70% ethanol and dissolved in water (see Fig. S6.3). Quantitative real-time PCR was

performed as described above using promoter- and gene-specific primers (see Table S6.1). Chromatin accessibility was expressed by comparative ΔCt as the ratio between amplification levels from untreated gDNA relative to those obtained from MNase digested gDNA. Values were represented relative to those of the wild-type strain.

### 6.3.5 Mycotoxin quantification

The quantification of FA and derivatives was performed as described previously (Lopez-Berges et al., 2013). Samples were obtained from fungal colonies grown for 3 days at 28 8C on PDA or CDA, and from mycelia and supernatant of the wild-type strain germinated in PDB for 16 h, and then transferred for 3 h to fresh PDB or CDL buffered to pH 5 or pH 7. Samples were homogenized in acetonitrile–water–glacial acetic acid (79 : 20 : 1, v/v/v) with a Homogenizer Workcenter T10 basic (IKAVR , Wilmington, NC, USA) for 1 min at a rate of 4 mL solvent per gram of sample. The mix was re-homogenized after 2 min of repose, filtered, centrifuged for 10 min at 12 000 g and the supernatant was lyophilized. Dry crude extracts were reconstituted in the solvent, and mycotoxin detection and quantification were performed with a QTrap 5000 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series UPLC System (Agilent, Waldbronn, Germany), as described previously (Malachova et al., 2014). Supernatant samples were lyophilized directly and then reconstituted in the solvent for quantification.

### 6.3.6 Determination of FA toxicity on tomato plants

Three-week-old seedlings of tomato plants (cultivar Monika) were individually root immersed in inoculum tubes containing pH 6 sterile water with different FA concentrations or 200 mM BPS, 200 mM BCS or 4 mM TPEN, and placed in a glasshouse for the indicated time periods time periods. Copper, iron and zinc foliar spraying was performed, when indicated, 2 h before the root immersion. Briefly, plant roots were

carefully covered with cling film and leaves spayed two times with 0.025% $CuSO4$, $FeSO4$ or $ZnSO4$ in 0.1% tween 20 solutions. When the leaves were completely dry, the roots were washed 3 times in sterile water before the immersion in the indicated solutions. Symptoms were monitored daily and scored 3 and 6 days after the FA or chelator treatment.

**6.3.7 Plant infection assays**

Tomato root inoculation assays were performed as described (Di Pietro and Roncero, 1998), using 2-week-old tomato seedlings (cultivar Monika). The severity of disease symptoms and plant survival was recorded daily for 30 days. Ten plants were used for each treatment. Virulence experiments were performed at least three times with similar results. Plant survival was calculated by the Kaplan-Meier method and compared among groups using the log-rank test. Data were analysed with the software GraphPadPrism 4. The quantification of fungal biomass in planta was performed as described previously (Pareja-Jaime *et al.*, 2010) using total gDNA extracted from tomato roots orstems infected with *F. oxysporum* strains at 3 or 7 dpi. Relative amounts of fungalgDNA were calculated by comparative ΔCt of the *Fusarium act1* gene normalized to the tomato *EFα1* gene (see Table S6.1).

**6.3.8 Animal infection assays**

Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, No. 123; http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm). Experimental conditions were approved by the Animal Welfare Committee of the Faculty of Medicine, Universitat Rovira i Virgili. Infection assays with immunodepressed mice were performed as described (Ortoneda et al., 2004). Briefly, groups of 10 Oncins France (OF) 1 male mice (Charles River, Criffa

S.A.) were immunosuppressed with an intraperitoneal 200 mg/kg dose of cyclophosphamide (Laboratorios Funk S.A, Barcelona, Spain) 2 days before the inoculation, and then every 5 days, and infected by injecting 0.2 ml of an inoculum of 107 conidia into a lateral vein of the tail. Survival was recorded daily for the indicated time periods. Infection experiments with each individual strain were performed at least three times. Survival was estimated by the Kaplan-Meier method and compared among groups using the log-rank test. To determine fungal tissue burden, randomly chosen surviving mice inoculated with $10^7$ conidia were sacrificed at 5 dpi. Kidneys, livers and lungs were aseptically removed, weighed, and homogenized in sterile saline, and 10-fold serial dilutions were spread onto PDA. The plates were incubated at 28 °C, the colonies were counted after 3 days, and the number of colony forming units (CFU) per gram of organ was calculated. Fungal colony counts were converted to $\log_{10}$ and compared using the analysis of variance test. Data were analyzed with the software GraphPad Prism 4.

**Accession numbers**

Sequence data can be found in the GenBank/EMBL database or in the *Fusarium* Comparative Genome database under the following accession numbers: Fub1, FOXG_15248; Act1, FOXG_01569; EFα1, NC_015443; pAN7-1 (PgpdA-hygr-TtrpC), Z32698; pAN8-1 (PgpdA-phleor-TtrpC), Z32751.

## 6.4 Results

### 6.4.1 Inactivation of the PKS Fub1 abolishes FA production in *F. oxysporum*

A BLASTP search in the *Fusarium* Comparative Database (Broad Institute), using Fub1 from *Fusarium fujikuroi* (FFUJ_02105) as a bait, identified a single predicted Fub1 orthologue (FOXG_15248) displaying 89% overall identity with the query protein. Manual inspection of the *F. oxysporum* fub1 locus identified all other members of the

FA gene cluster previously described in *F. verticillioides* (Brown et al., 2012) and *F. fujikuroi* (Niehaus et al., 2014) (Fig. S6.1A, see Supporting Information). Interestingly, two additional putative genes were present between fub3 and fub4 in different F. oxysporum isolates, including the reference strain FOL 4287 (Fig. S6.1A) (Brown et al., 2015). Both genes are neighbours in other *Fusarium* species, but not located in the FA gene cluster. For example, in *F. fujikuroi*, the orthologues of these two genes, FFUJ_11046 and FFUJ_11047, are located on chromosome 10, whereas the FA gene cluster is located on chromosome 3 (Fig. S6.1A). It is currently unknown whether the insertion of these two additional genes has any effect on the FA gene cluster. Recently, additional components of the cluster, including two Zn(II) 2Cys6 transcription factors, have been identified in different *Fusarium* species (Brown et al., 2015; Studt et al., 2016). To study the role of Fub1 in FA production by *F. oxysporum*, we replaced the entire FOXG_15248 coding sequence with the hygromycin B resistance gene (hphr), generating several fub1D strains (Fig. S6.1B and S6.1C). To determine whether FOXG_15248 was responsible for FA production in *F. oxysporum*, extracts from cultures of the different strains grown on potato dextrose agar (PDA) or Czapek- Dox agar (CDA) were analysed by high-performance liquid chromatography/electrospray ionization-tandem mass spectrometry (HPLC/ESI-MS/MS). This approach allows the reliable and sensitive quantification of several hundred fungal analytes, including almost all mycotoxins for which standards are commercially available (Malachova et al., 2014). FA and its derivative fusarinolic acid (FnA) were detected in wild-type extracts, but not in those of the fub1Δ mutant (Fig. 6.1A). Interestingly, the total amount of mycotoxin (FA1FnA) was approximately 2.5 times higher in CDA than in PDA cultures, with an FA : FnA ratio of 29.5 : 1 in the former and 1 : 2.75 in the latter (Fig. 6.1A). These data are consistent with those reported in *F. fujikuroi* (Niehaus et al.,

2014). In addition to FA and FnA, beauvericin (Bea) and bikaverin (Bik) were detected in all samples. Interestingly, both compounds were more abundant in the fub1D cultures compared with the wild-type, especially in CDA (Fig. 6.1B). Reintroduction of the intact fub1 allele into fub1D, yielding the complemented fub1DC strain (Fig. S6.2, see Supporting Information), fully restored the wild-type FA levels (Fig. 6.1). Thus, Fub1 is responsible for the production of FA and its derivatives in *F. oxysporum*. Next, we tested the potential toxicity of FA on *F. oxysporum*. When the wild-type strain or fub1Δ were cultured on PDA supplemented with 0.25 or 0.5 mg/mL FA, both showed a significant and comparable reduction in radial growth, whereas no growth was detectable at 0.75 mg/mL FA (Fig. S6.3, see Supporting Information).

**6.4.2 Effect of pH and nutrients on fub1 transcript levels and FA production**

In *F. fujikuroi*, fub1 transcription is positively regulated by the pH response factor PacC at pH 8, but not at pH 4 (Niehaus *et al.*, 2014). We noted that CDA has an initial pH of 6.860.2, whereas PDA has an initial pH of about 5.660.2. Moreover, the pH in CDA, in which NaNO3 is the sole nitrogen source, tended to increase during fungal growth (data not shown).

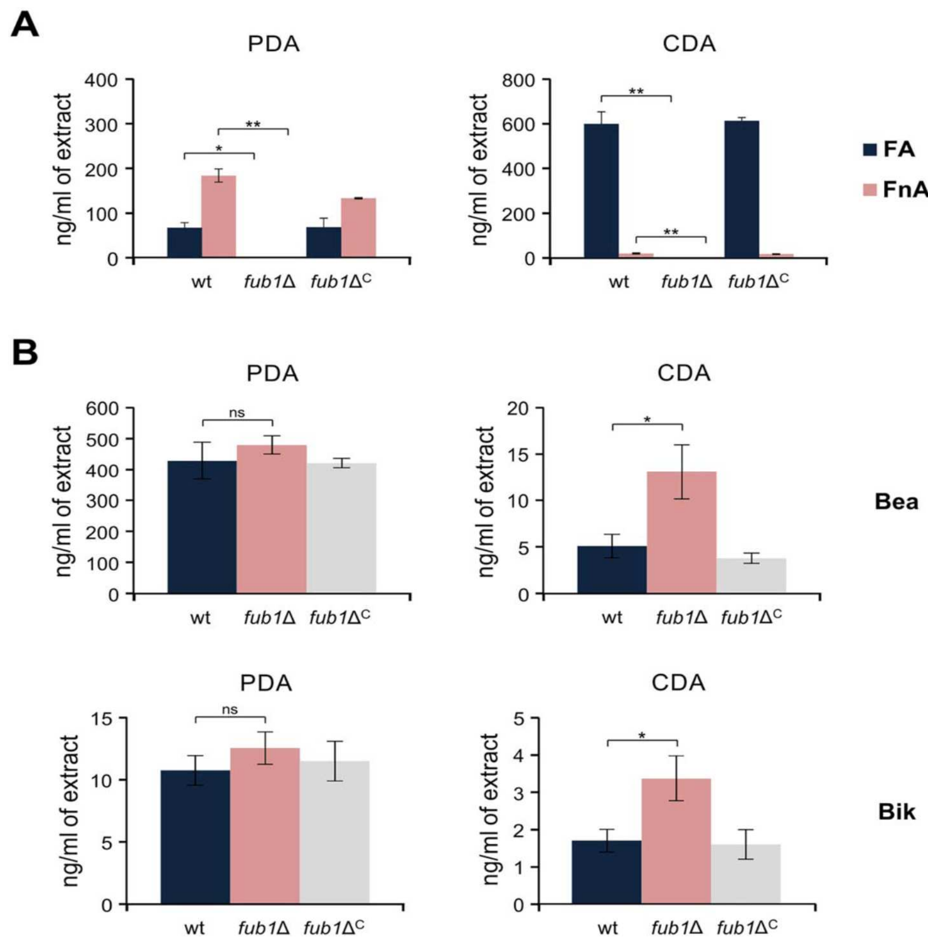



**Figure 6.1** Fub1 is required for the production of fusaric acid (FA) and its derivatives in *Fusarium oxysporum*. (A) The amounts of FA and fusarinolic acid (FnA) in cultures of the indicated strains, grown for 3 days on potato dextrose agar (PDA) or Czapek-Dox agar (CDA), were quantified by liquid chromatography/tandem mass spectrometry and expressed as nanograms per millilitre of extract. (B) The quantification of beauvericin (Bea) and bikaverin (Bik) was performed as in (A). wt, wildtype strain. Bars represent standard errors from two independent fungal cultures. *P<0.05; **P<0.001; ns, not significant.

To discriminate between the effects of medium composition and pH on FA biosynthesis, we germinated conidia of the wild-type strain in potato dextrose broth (PDB) and transferred the germlings to Czapek- Dox liquid (CDL) or fresh PDB buffered to either pH 5 or pH 7 (see Experimental procedures for details). Unexpectedly, in both media, fub1 transcription and FA production were much higher under moderate acidic conditions, although, as expected, CDL induced more FA (Fig. 6.2A, B). The effect of pH on FA production was stronger than that of the medium composition, as reflected by the finding that FA production was higher in PDB at pH 5 than in CDL at pH 7 (Fig. 6.2A, B). Our data indicate that both pH and nutrients are important factors in the regulation of FA biosynthesis, and that this regulation may

differ between *F. oxysporum* and *F. fujikuroi*. We next tested the role of PacC in fub1 regulation using a pacC loss-of-function mutant (Caracuel et al., 2003). When germlings of the different strains were grown in glutamine minimal medium (GMM) buffered to either pH 5 or pH 7, fub1 transcript levels were 10 times lower in pacCD relative to the wild-type at both pH values (Fig. 6.2C). Thus, PacC functions as a positive regulator of fub1 within this pH range.

### 6.4.3 Chromatin structure at the fub1 locus is controlled by the global regulator of secondary metabolism LaeA and the pH response factor PacC

Although the fub1D mutants did not show a detectable growth defect on PDA or CDA, we noted that their growth in the presence of hygromycin B was markedly reduced (Figs 6.3A and S6.4, see Supporting Information). Interestingly, the complemented fub1$\Delta^C$ strains showed a similar growth defect on hygromycin, whereas the transformants carrying an ectopic insertion of the knockout construct (Ect) (Fig. S6.1C) did not (Figs 6.3A and S6.4). We hypothesized that this phenotype could be caused by a chromatin regulatory effect on transcription of the hph hygromycin resistance gene inserted at the fub1 locus. In line with this hypothesis, we found that hph transcript levels were between 30 and 50 times higher in Ect than in fub1Δ and fub1$\Delta^C$ (Fig. 6.3B). LaeA is a global regulator of secondary metabolite gene clusters in different fungi (Bok and Keller, 2004; Butchko et al., 2012; Lopez-Berges et al., 2013; Wiemann et al., 2010), and has been reported previously to regulate FA production in *Fusarium* (Lopez-Berges et al., 2013; Niehaus et al., 2014).

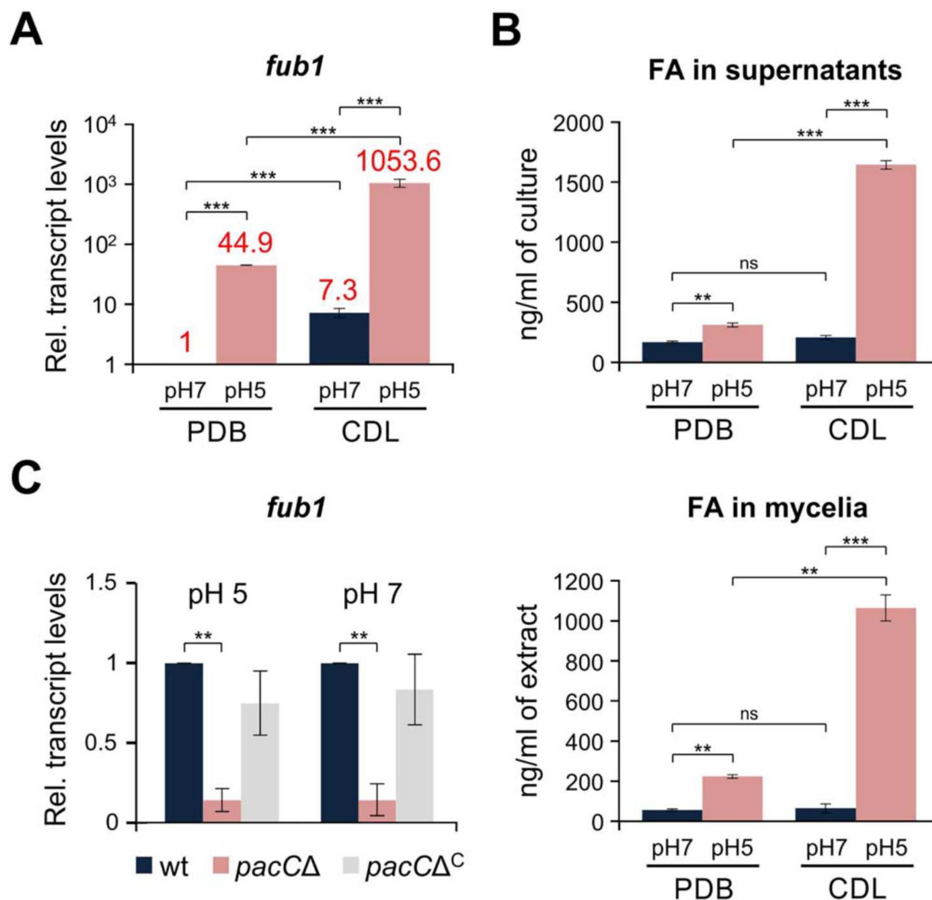



**Figure 6.2** pH and medium composition regulate fub1 transcript levels and fusaric acid (FA) production. (A) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed in the wild-type strain germinated for 16 h in potato dextrose broth (PDB) and then transferred for 3 h to fresh PDB or Czapek-Dox liquid (CDL) buffered at the indicated pH with 100 mM 2-(N-morpholino) ethanesulfonic acid (MES). Transcript levels of fub1 are expressed relative to those in PDB at pH 7 (see numbers above the columns for exact data). Bars represent standard errors from two independent biological experiments with three technical replicates each. (B) Quantification of FA in culture supernatants (top panel) and mycelia (bottom panel) of the wild-type strain grown as in (A) performed by liquid chromatography/tandem mass spectrometry. Bars represent standard errors from two independent fungal cultures. (C) Quantitative real-time RT-PCR was performed in the indicated strains germinated for 16 h in glutamine minimal medium (GMM) and then transferred for 3 h to fresh GMM buffered at the indicated pH with 100 mM MES. Transcript levels of fub1 are expressed relative to those of the wild-type at both pH values. Bars represent standard errors from two independent biological experiments with three technical replicates each. **P<0.001; ***P<0.0001; ns, not significant; wt, wild-type.

Transcript levels of fub1 in the wild-type and the laeA$\Delta^C$ strains were between 300 and 500 times higher than in the laeAΔ mutant (Fig. 6.3C). We next examined the role of LaeA in chromatin remodelling and transcriptional regulation at the *F. oxysporum* fub1 locus, using real-time quantitative polymerase chain reaction (PCR) with promoter- and gene-specific primers (Fig. 6.4A) on genomic DNA (gDNA) obtained from mycelia treated with micrococcal nuclease (MNase) (Fig. S6.5, see Supporting Information).

Relative chromatin accessibility, calculated as the ratio of amplification from untreated versus MNase-treated mycelia, was about six times higher in wild-type and laeAΔ$^{C}$ compared with laeAΔ (Fig. 6.4B). Moreover, relative chromatin accessibility was significantly lower at pH 7 in comparison with pH 5, and in a pacCD strain at both pH values compared with the wild-type (Fig. 6.4C, D), in line with the previous finding that fub1 transcript levels are lower at pH 7 and in pacCΔ (Fig. 6.2A, C). We conclude that chromatin accessibility and transcription at the fub1 locus, as well as the production of FA and its derivatives, are positively regulated by LaeA, moderate acidic pH and PacC.

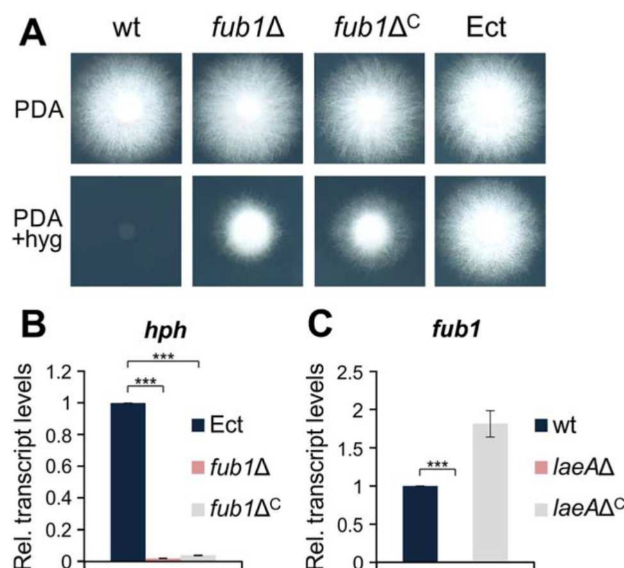



**Figure 6.3** Transcript levels of fub1 are controlled by LaeA. (A) Colonies of the indicated strains grown on potato dextrose agar (PDA) with or without 50 mg/mL hygromycin B for 3 days at 28 _C. (B, C) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed in the indicated strains germinated for 16 h in potato dextrose broth (PDB) and then transferred to fresh PDB for 1 h. Transcript levels of the hph (B) and fub1 (C)genes are expressed relative to those of the ectopic transformant and the wild-type strain, respectively. wt, wild-type strain; Ect, ectopic transformant. Bars represent standard errors from two independent biological experiments with three technical replicates each. ***$P<0.0001$.

### 6.4.4 Fub1 and FA are not required for the growth of *F. oxysporum* under copper-, iron- or zinc-limiting conditions

The ability of FA to chelate metal ions, such as iron or copper, has been known for a long time (Lakshminarayanan and Subramanian, 1955; Malini, 1966; Pan et al., 2010; Tamari and Kaji, 1952). In a recent study, FA has been shown to chelate different metal ions, including Fe21, Fe31, Cu21, Mn21 and Zn21 (Ruiz et al., 2015; Yin et al., 2015). We

thus asked whether the production of FA is required for the growth of F. oxysporum under metallimiting conditions. The depletion of copper, iron or zinc was achieved by the addition of the specific chelators bathocuproinedisulfonic acid disodium salt (BCS), bathophenanthrolinedisulfonic acid disodium salt hydrate (BPS) and N,N,N0,N0-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), respectively (Fig. 6.5A). Unexpectedly, although copper is an essential micronutrient in most living organisms (Vulpe and Packman, 1995), we observed no detectable growth defect in *F. oxysporum* grown under copper limitation (Fig. 6.5A), even in the presence of BCS concentrations up to 1 mM (data not shown). A similar result has been reported previously in Aspergillus fumigatus (Park et al., 2014). By contrast, depletion of iron and zinc resulted in severe growth defects, as expected. In any case, inactivation of Fub1 had no additional effect on growth (Fig. 6.5A). Next, we asked whether Fub1 was required for fungal growth at toxic concentrations of copper, iron or zinc. When the wild-type and fub1D strains were grown on GMM with up to 5 mM of the different metal ions, no significant differences were observed between strains (Fig. 6.5B). These results demonstrate that FA is not essential for growth under limiting or toxic concentrations of copper, iron or zinc. However, we noted that transcript levels of fub1 were significantly reduced in the presence of these three metal ions (Fig. 6.5C).

**6.4.5 FA toxicity in tomato plants is reversed by the exogenous addition of copper, iron and zinc**

The production and phytotoxic properties of FA have been studied over the past 75 years (Bacon et al., 1996; Dong et al., 2014; Gaumann, 1957, 1958; Yabuta et al., 1937), and a number of mechanisms for FA toxicity have been suggested, most related to modifications in the plant cell membrane (D'Alton and Etherton, 1984). To test FA toxicity, roots of 3-week-old tomato plants were immersed in sterile water with or without

FA. Plants maintained in the presence of 0.5–1 mM FA exhibited a progressive depigmentation of the stem, most probably a result of anthocyanin degradation, followed by a general loss of turgor and, finally, wilting of the entire plant (Fig. 6.6A). Importantly, the external addition of copper, iron or zinc, either to the FA solution or by foliar spraying, a process by which leaves can take up ions through the stomata and distribute them throughout the plant (Eddings and Brown, 1967; Neumann and Prinz, 1975), rendered plants more resistant to FA and significantly increased stem strength and pigmentation (Fig. 6.6B–D). Furthermore, the phytotoxic effect of FA was partially recapitulated by immersion of the roots in a solution containing the membrane-permeable chelator TPEN (Fig. 6.7), but not the membrane-impermeable chelators BPS or BCS (data not shown). Collectively, these results suggest that the phytotoxicity of FA is mediated by chelation of metal ions inside the plant.

### 6.4.6 FA is a virulence factor of *F. oxysporum* on tomato plants and immunodepressed mice

We noted that the expression of fub1 in *F. oxysporum* was markedly up-regulated during the early stages of plant infection (Fig. 6.8A) and therefore tested the role of FA production in virulence. Tomato plants whose roots were inoculated with conidia of the *F. oxysporum* wild-type or fub1$\Delta^{C}$ strains showed progressive wilt symptoms and usually died before day 25 post-inoculation (dpi) (Fig. 6.8B). In contrast, plants inoculated with the fub1Δ mutant displayed a significantly reduced mortality rate (Fig. 6.8B) and most survived the assay, developing only mild disease symptoms.

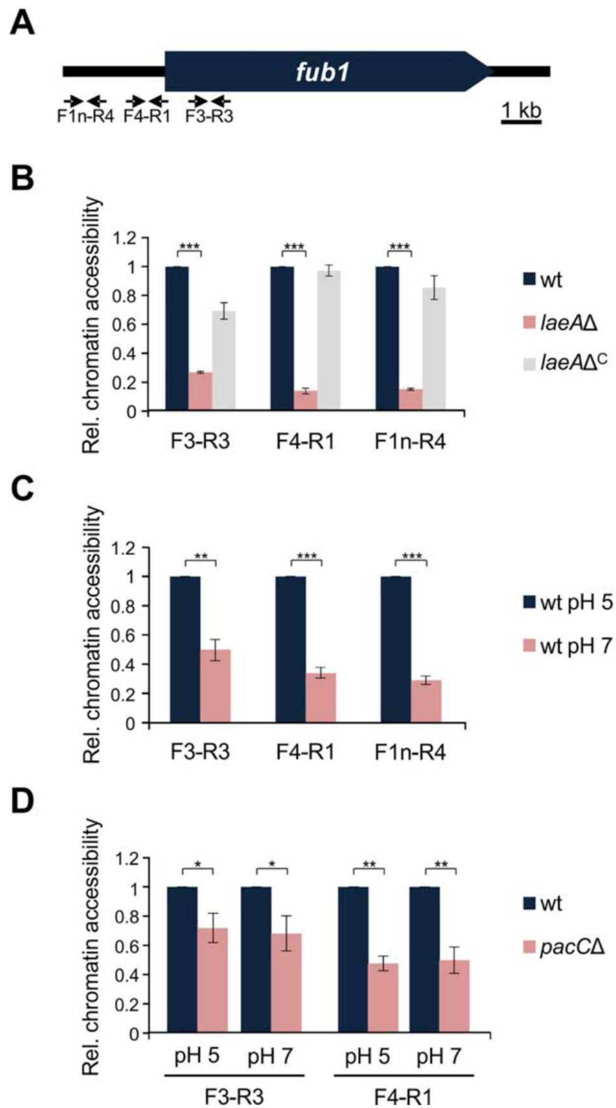



**Figure 6.4** LaeA, pH and PacC regulate chromatin modifications at the fub1 locus. (A) Physical map of the promoter region of the fub1 gene located in the fusaric acid (FA) gene cluster. Primers used for chromatin analysis are indicated. (B–D) Real-time quantitative polymerase chain reaction (PCR) performed on genomic DNA (gDNA) of the indicated strains grown as in Fig. 3B, C. Relative chromatin accessibility was calculated as the ratio of amplification levels obtained with gDNA from untreated mycelia versus gDNA from micrococcal nuclease (MNase)-treated mycelia, and represented relative to that of the wild-type (B), the wild-type at pH 5 (C) or the wild-type at different pH values (D), with each of the indicated primer pairs (see Table S1). wt, wild-type strain. Bars represent standard errors from two independent biological experiments with three technical replicates each. *P<0.05; **P<0.001; ***P<0.0001.

Moreover, the amount of fungal biomass in roots and stems was markedly reduced in fub1D in comparison with the wild-type and complemented strains (Fig. 6.8C). Thus, FA is required for full virulence of F. oxysporum in tomato plants. As the tomato pathogenic *F. oxysporum* strain can also infect and kill immunosuppressed mice (Ortoneda et al., 2004), we tested the role of FA production during infection of a mammalian host.

Inoculation with $10^7$ conidia of the wild-type or fub1DC strain resulted in the killing of all animals before 15 dpi (Fig. 6.9A), and transcripts of fub1 were detected inside the host (Fig. S6.6, see Supporting Information).

However, animals inoculated with two independent fub1D mutants showed significantly delayed mortality (Fig. 6.9A). In contrast with plant infection, the fungal burden in kidney, liver and lung of surviving mice did not differ significantly between the strains (Fig. 6.9B). These results suggest that FA contributes to the virulence of *F. oxysporum* in mammals, but is not required for dissemination in the host.

## 6.5 Discussion

FA was discovered almost 80 years ago (Yabuta et al., 1937) and was the first fungal toxin whose production was detected *in planta* (Gaumann, 1957). Its strong phytotoxicity (Niehaus et al., 2014, Stipanovic et al., 2011), moderate toxicity in animals (Yin et al., 2015, Porter et al., 1995) and bacteria (Bacon et al., 2006, Ruiz et al., 2015), and its pharmacological properties (Wang and Ng, 1999, Song and Yee, 2001) make the study of FA biosynthesis and regulation of high interest.

Moreover, FA inhibits the growth of fungi, including its producer *Fusarium.* However, FA producing strains use a variety of strategies, such as active export or enzymatic modification, to protect themselves from the toxin (Crutcher et al., 2015; Studt et al., 2016). Since the recent discovery of the FA biosynthetic gene cluster, different components of the cluster have been characterized (Brown et al., 2012, 2015; Niehaus et al., 2014; Studt et al., 2016). Inactivation of Fub1, the PKS acting in the first step of the FA biosynthetic pathway, completely abolishes the production of FA and its derivatives in different *Fusarium* species (Brown et al., 2012; Niehaus et al., 2014) (this work).

Here, we used targeted deletion of fub1 to demonstrate, for the first time, a role of FA in

the virulence of the crosskingdom pathogen *F. oxysporum* on plant and mammalian hosts.

### 6.5.1 Chromatin-mediated regulation of FA production

The regulation of FA production has been studied for close to 80 years. Initially it was proposed that FA is mainly produced under alkaline conditions (Yabuta *et al.*, 1939), whereas later studies suggested that nitrogen sufficiency and slightly acidic media are optimal for FA production (Pitel and Vining, 1970). Here we compared two different media, potato dextrose (PD) and czapek dox (CD), both in solid and liquid versions. Although PD is a richer and more complex medium, we found that production of FA was higher in CD, a medium that has been known for a long time to promote FA production (Loffler and Mouris, 1992). By contrast, Bik and Bea were preferentially produced in PD. The exact reason for this difference is currently unknown. We hypothesized that pH could act as a key regulatory factor, and observed significantly higher *fub1* expression and FA production at pH 5 compared to pH 7. Our results are in contrast to those reported in *F. fujikuroi* showing a higher expression of *fub1* at pH 8 in comparison to pH 4, requiring the alkaline pH regulator PacC (Niehaus et al., 2014). We also confirmed that PacC is required for full expression of fub1 at both pH 5 and pH 7. The seemingly contradictory results between *F. oxysporum* and *F. fujikuroi* could be explained by the different experimental conditions used in the two studies: 2-(Nmorpholino) ethanesulfonic acid (MES)-buffered versus unbuffered media, respectively (Niehaus et al., 2014).

It is known that pH in an unbuffered culture can change rapidly during fungal growth. However, the optimum pH for fub1 expression and FA production in *F. oxysporum* could be around pH 5 or higher, a range in which PacC is still active. In line with this hypothesis, pacC transcript levels were similar at pH 5 and pH 7, but almost undetectable at pH 4 (Caracuel et al., 2003). The global regulator of secondary metabolism LaeA (Bok and

Keller, 2004; Butchko et al., 2012; Lopez- Berges et al., 2013; Wiemann et al., 2010) has been shown previously to regulate FA production in *Fusarium* (Lopez-Berges et al., 2013; Niehaus et al., 2014).

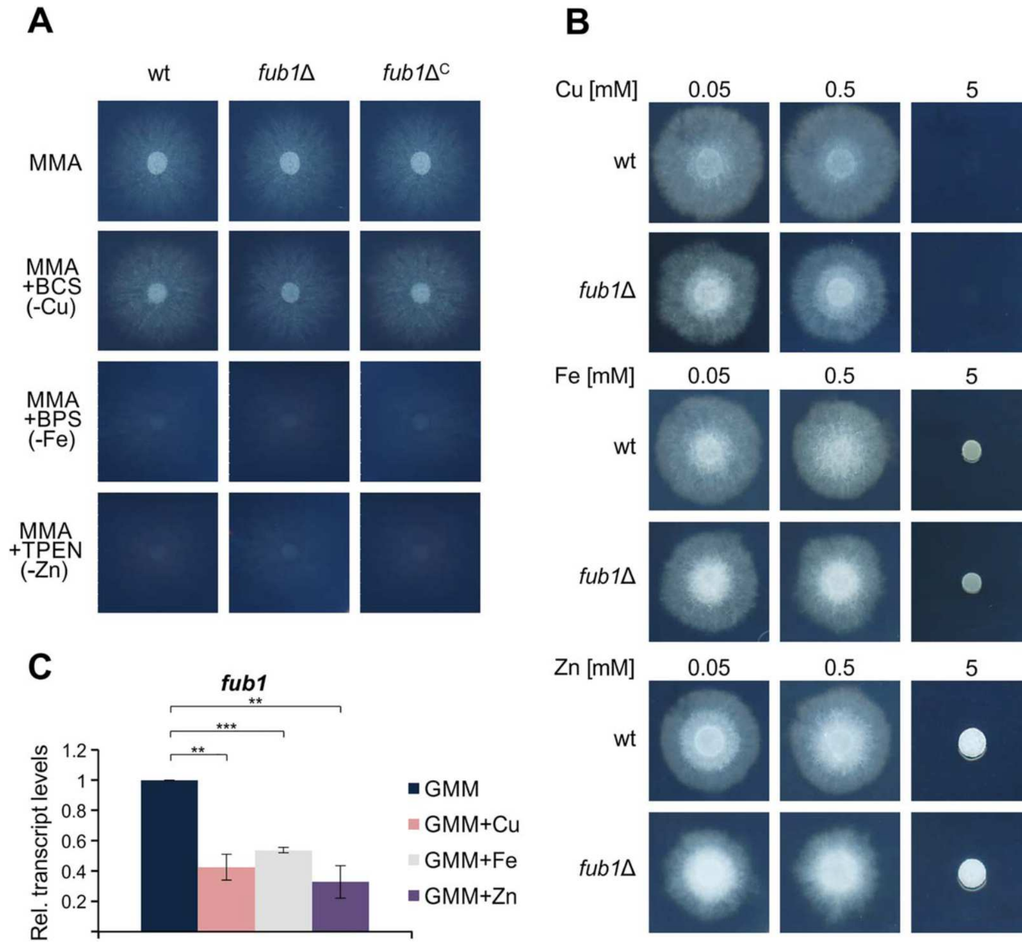



**Figure 6.5** Expression of fub1 is repressed by copper, iron and zinc. (A) Growth of the indicated strains on minimal medium agar (MMA) with or without the indicated metal chelators. Plates were cultured for 3 days at 28 _C. (B) Growth of the indicated strains on solid glutamine minimal medium (GMM) supplemented with the indicated concentrations of copper, iron or zinc. Plates were cultured for 3 days at 28 _C. (C) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed in the wild-type strain germinated for 16 h in GMM without copper, iron or zinc and then supplemented, or not, with 50 mM CuSO4, FeSO4 or ZnSO4 for 2 h. Transcript levels of fub1 are expressed relative to those in GMM. wt, wild-type strain; BCS, bathocuproinedisulfonic acid disodium salt; BPS, bathophenanthrolinedisulfonic acid disodium salt hydrate; TPEN, N,N,N0,N0-tetrakis(2-pyridylmethyl)ethylenediamine. Bars represent standard errors from two independent biological experiments with three technical replicates each. **P<0.001; ***P<0.0001.

LaeA contains a conserved *S*adenosylmethionine-(SAM-) binding site essential for its function, contributes to histone H3 lysine 9 trimethylation (Reyes-Dominguez *et al.*, 2010) and links transcriptional and epigenetic control of gene expression (Sarikaya-Bayram *et al.*, 2014).

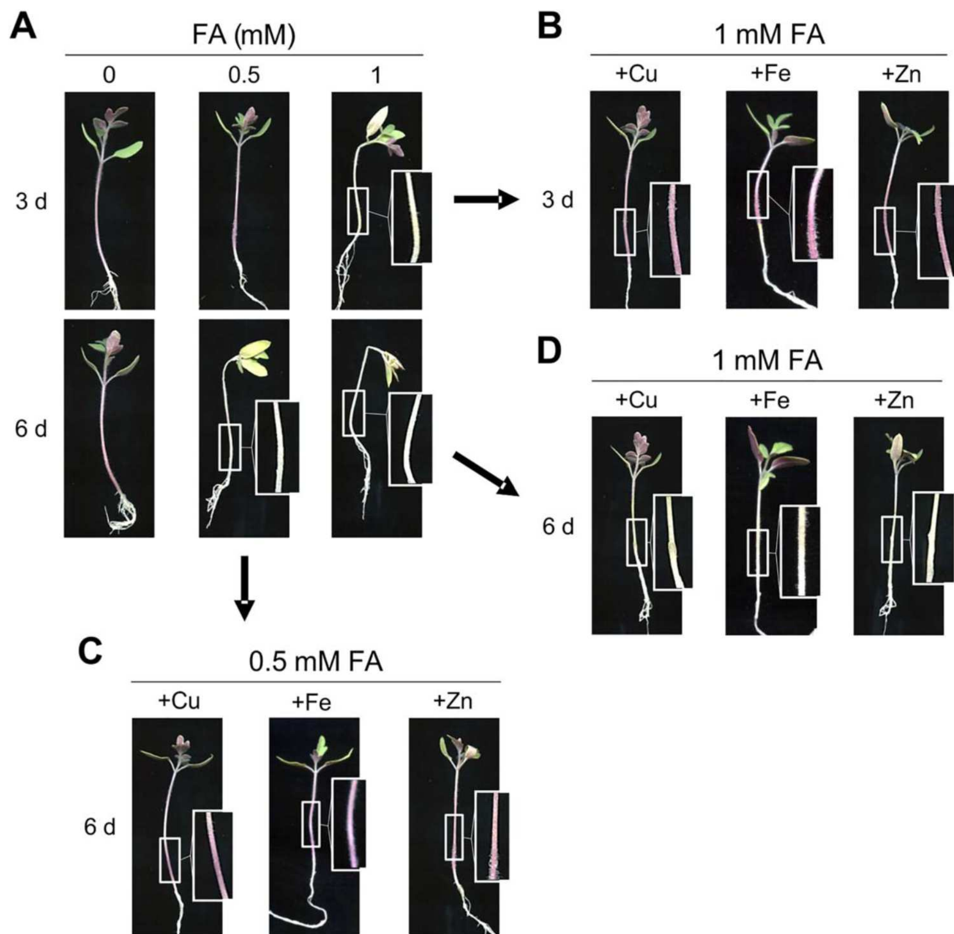



**Figure 6.6** Phytotoxic effect of fusaric acid (FA) on tomato plants is remediated by exogenous copper, iron or zinc. (A) Roots of 3-week-old seedlings of tomato plants (cultivar Monika) were immersed in sterile water with the indicated concentrations of FA for 3 and 6 days. (B–D) Leaves of plants were sprayed with 0.025% CuSO4, FeSO4 or ZnSO4 solutions before immersing roots in FA solution. Boxed areas are shown at double magnification.

In line with previous reports suggesting a positive role of LaeA in FA biosynthesis (Niehaus et al., 2014, Lopez-Berges et al., 2013), we show here that inactivation of LaeA leads to a significant decrease in chromatin accessibility at the FA gene cluster. These findings, together with the reduced expression of the *hph* gene when inserted at the place of *fub1*, suggest a major regulatory function of LaeA in remodeling chromatin structure at the *F. oxysporum* FA locus. In addition, we showed that moderate acidic pH and PacC contribute to an increase in chromatin accessibility at the fub1 locus. Although our data suggest that this contribution requires LaeA, this remains to be confirmed experimentally. The fact that inactivation of LaeA has, by far, the strongest effect on the expression of the FA gene cluster and FA production suggests that other stimuli, such as nutrients or pH,

may converge on this master regulator of secondary metabolism to regulate expression of the gene cluster.

FA has long been known for its ability to chelate metal ions (Tamari and Kaji, 1952, Malini, 1966, Lakshminarayanan and Subramanian, 1955, Pan et al., 2010). However, the regulation of FA biosynthesis by metals has not been studied so far. Here we show that transcript levels of *fub1* are negatively regulated by copper or zinc. Similarly, transcript levels of *sidC*, a LaeA-regulated gene functioning in the biosynthesis of the siderophore ferricrocin, are also downregulated in the presence of iron (Perrin *et al.*, 2007, Lopez-Berges et al., 2013, Eisendle *et al.*, 2004). Althought this suggests that FA might function in metal uptake, we found that *fub1* was not essential for growth of *F. oxysporum* during copper, iron or zinc limiting conditions, most likely because more specific and efficient uptake mechanisms are present in filamentous fungi such as high affinity copper and zinc transporters (Vicente ranqueira *et al.*, 2005, Park et al., 2014), and siderophore-assisted iron uptake (Schrettl and Haas, 2011). Alternatively, metalchelatingFA might be used by *Fusarium* to inhibit microbial competitors in the soil. Indeed, FA is exported in *F. fujikuroi* and *F. oxysporum* f. sp. *vasinfectum* via the Major Facilitator Superfamily (MFS) transporters Fub11 and FubT, respectively (Crutcher et al., 2015, Studt et al., 2016). However, we found that a lack of FA production was not detrimental during fungal growth in toxic copper, iron or zinc conditions.

**Figure 6.7** The membrane-permeable zinc chelator N,N,N0,N0-tetrakis(2- pyridylmethyl)ethylenediamine (TPEN) causes similar phytotoxicity symptoms in tomato plants to fusaric acid. Leaves of tomato plants were pretreated, or not, with a 0.025% ZnSO4 solution and roots were immersed in sterile water containing 4 mM of the zinc chelator TPEN for 3 and 6 days. Boxed areas are shown at double magnification.

### 6.5.2 Mechanism of FA phytotoxicity and role in virulence

Early studies established the phytotoxic activity of FA and its role in the induction of wilt symptoms in plants (Yabuta et al., 1937, Gaumann, 1957, Gaumann, 1958). Our study confirmed that tomato seedlings develop typical wilt symptoms when their roots are exposed to FA. The fact that wilting was observed in cotyledons and lower leaves suggests that FA is transported and distributed throughout the entire plant. Similar wilt symptoms were reported in water melon seedlings (Hong-Sheng *et al.*, 2008). The precise mechanism of phytotoxicity of FA remains unknown. A number of studies suggested that could be related to its ability to chelate different metal ions (Tamari and Kaji, 1952, Lakshminarayanan and Subramanian, 1955, Gaumann, 1958, Ruiz et al., 2015). Here we show that addition of copper or zinc to FA treated plants significantly reduces wilting. Importantly, inhibition of FA toxicity was also functional when the metal ions and FA were applied to different parts of the plant (leaves and roots, respectively) indicating that

the chelating mechanism occurs inside the plant. Although additional mechanisms of FA toxicity cannot be ruled out, our results clearly link FA phytotoxicity with metal chelation.

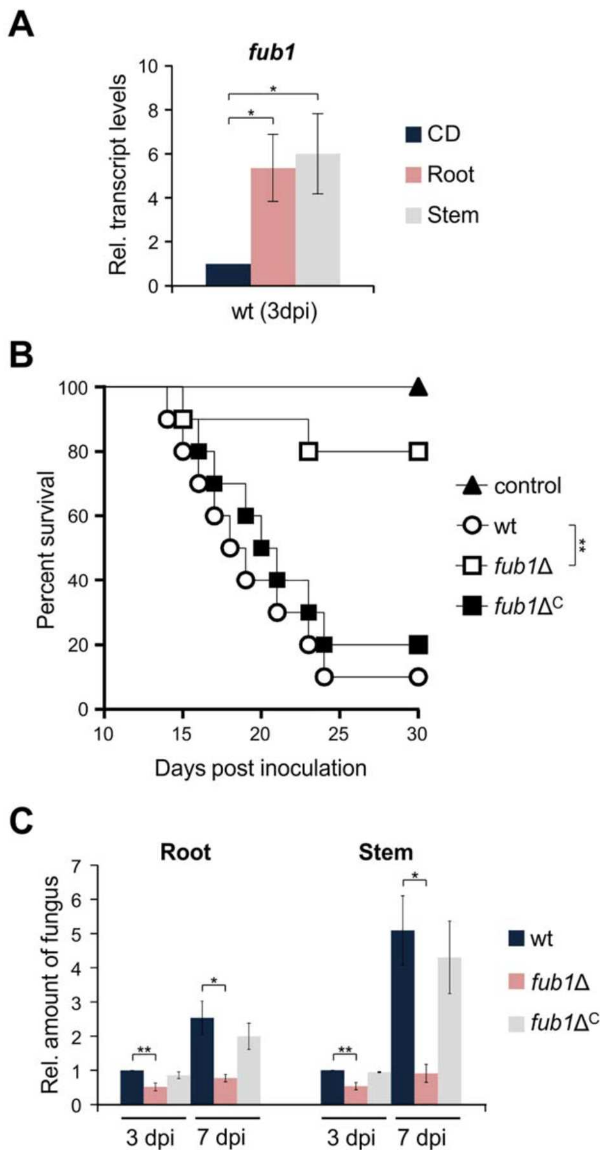



**Figure 6.8** Fusaric acid (FA) production is required for full virulence of *Fusarium oxysporum* on tomato plants. (A) Quantitative real-time reverse transcriptionpolymerase chain reaction (RT-PCR) was performed in the wild-type strain germinated for 16 h in potato dextrose broth (PDB) and then transferred to Czapek-Dox liquid (CDL) for 3 h or for inoculated tomato roots and stems at 3 days post-inoculation (dpi). Transcript levels of fub1 are expressed relative to those in CDL. (B) Groups of 10 tomato plants (cultivar Monika) were inoculated by dipping roots into a suspension of 5 3 106 freshly obtained microconidia/mL of the indicated fungal strains. Percentage survival was plotted for 30 days. Data shown are from one representative experiment. Experiments were performed three times with similar results. (C) Quantitative real-time PCR was used to measure the relative amount of fungal DNA in total genomic DNA extracted from tomato roots and stems at 3 and 7 dpi with the indicated strains. Amplification levels are expressed relative to those of plants infected with the wild-type strain. wt, wild-type strain. Bars represent standard deviations from two independent biological experiments with three technical replicates each.*P<0.05; **P<0.001.

FA has been one of the first fungal toxins for which a functional role in virulence has been proposed (Gaumann, 1957, Gaumann, 1958) and several studies have provided circumstantial evidence linking FA production to plant pathogenicity (Venter and Steyn, 1998, Gapillout *et al.*, 1996, Dong et al., 2014). However, so far no formal proof for such a role has been provided.

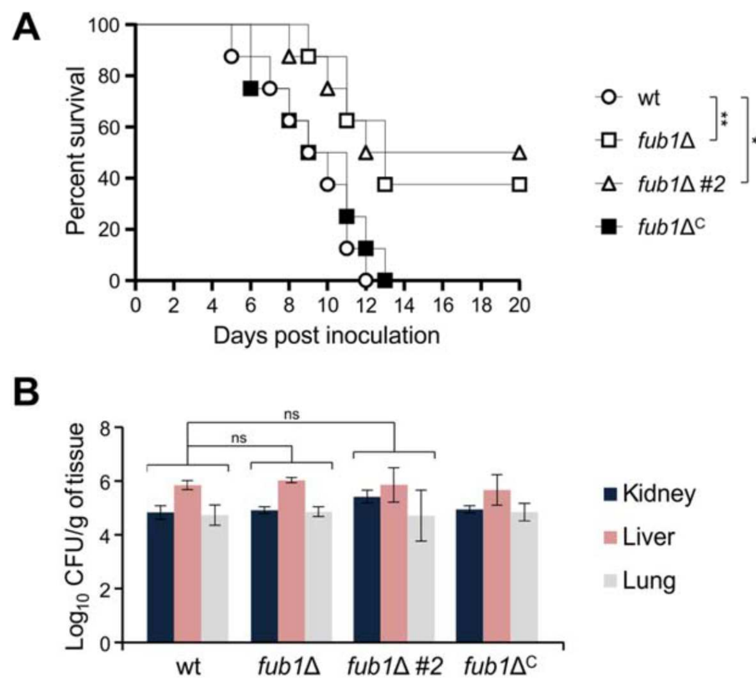



**Figure 6.9** Fusaric acid (FA) is a virulence factor in mice. (A) Groups of 10 immunosuppressed Oncins France 1 male mice were inoculated with 107 microconidia of the indicated strains by lateral tail vein injection. Percentage survival was plotted for 20 days. Data shown are from one representative experiment. Experiments were performed three times with similar results. (B) Four randomly chosen surviving mice inoculated with 107 microconidia of the indicated strains were sacrificed at 5 days post-inoculation (dpi) and homogenates obtained from the indicated organs were quantitatively cultured on potato dextrose agar (PDA). wt, wild-type strain. *P<0.05; **P<0.001; ns, not significant.

Here we demonstrate that mutants lacking *fub1,* which are unable to produce FA or its derivatives, are significantly reduced in their capacity to cause mortality in tomato plants. Interestingly, these mutants also caused less mortality in immunodepressed mice, showing for the first time the relevance of FA production during fungal infection of mammals. Previously, the MTX beauvericin was also shown to contribute to infection of *F. oxysporum* in plants and mice. This suggests that the production of secondary metabolites, many of which are regulated by the Velvet complex and LaeA, could play a

role in the capacity of *F. oxysporum* to attack both plant and animal hosts. In line with this idea, mutants lacking VeA or LaeA are significantly attenuated in virulence on tomato plants and mice (Lopez-Berges et al. 2013), as are Velvet complex mutants in other human and plant pathogenic fungi (Bok et al., 2005, Webster and Sil, 2008, Laskowski-Peak et al., 2012, Jiang et al., 2011, Lee et al., 2012, Merhej et al., 2012, Wiemann et al., 2010, Myung et al., 2009, Lopez- Berges et al., 2013). Additional studies, including investigations on combinatory/synergic effects of co-occurrence MTX, are required to fullyunderstand the role of SM production in cross-kingdom pathogenicity of *F. oxysporum*.

## 6.6 Supplementary material

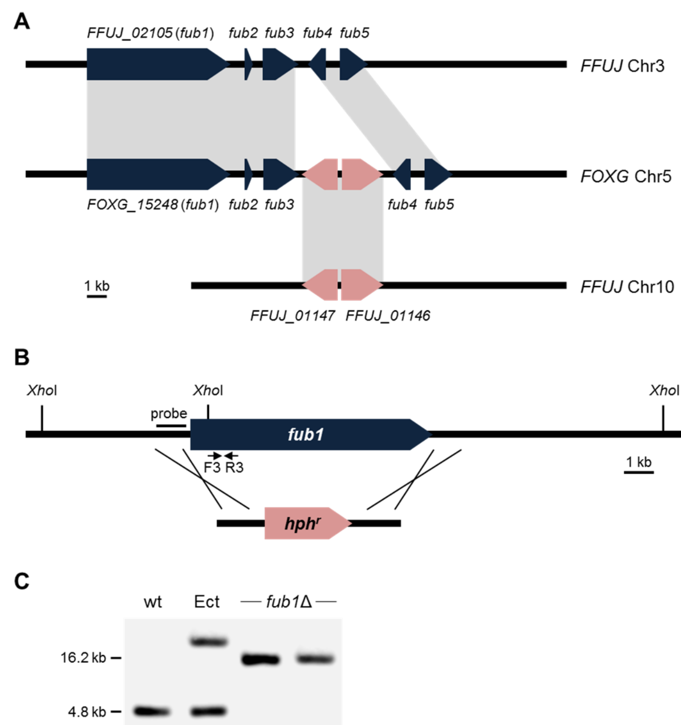



**Figure S6.1 Identification of the *F. oxysporum* FA gene cluster and *fub1* knockout strategy**

(**A**) Conserved synteny between *F. fujikuroi* and *F. oxysporum* FA gene clusters. Note that the two genes inserted between *fub3* and *fub4* in *F. oxysporum* are present in *F. fujikuroi* in another chromosome. (**B**) *F. oxysporum fub1* locus and targeted gene disruption construct. (**C**) Southern blot analysis. gDNA of the indicated strains was treated with *Xho*I, separated on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the DNA probe indicated in (**B**). wt, wild-type.

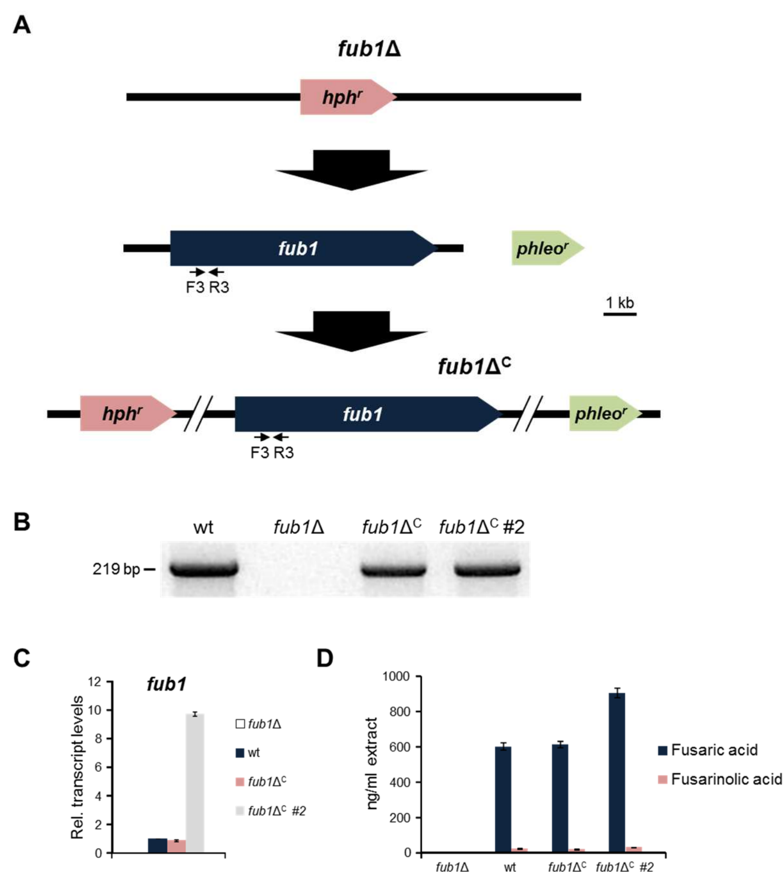



**Figure S6.2 Generation and selection of the *fub1*Δ complemented strain (*fub1*Δ$^{C}$)**

**(A)** Strategy of *fub1*Δ complementation by co-transformation with a *fub1* wild-type allele and the phleomycin resistance marker. Relative position of the PCR primers used for genotyping are indicated. *phleo*$^{r}$, phleomycin resistance gene. **(B)** PCR amplification of gDNA of the indicated strains using primers F3 and R3. The complemented strains, *fub1*Δ$^{C}$ and *fub1*Δ$^{C}$ #2, produce a banding pattern consistent with the integration of an intact *fub1* allele. wt, wild-type strain. **(C)** Quantitative real-time RT-PCR performed in the indicated strains germinated for 16 h in potato dextrose broth (PDB) and then transferred for 3 h to czapec-dox liquid (CDL). Transcript levels of *fub1* are expressed relative to those in the wild-type strain. Bars represent standard errors from two independent biological experiments with three technical replicates each. **(D)** Fusaric acid (FA) and fusarinolic acid (FnA) in cultures of the indicated strains grown for 3 d on czapek-dox agar (CDA) was quantified by liquid chromatography/tandem mass spectrometry and expressed in ng/ml of extract.

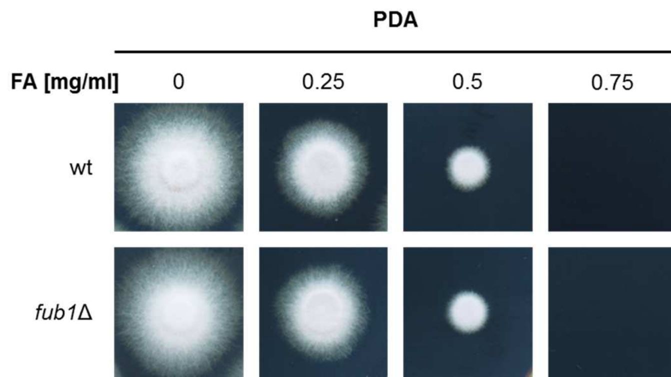



**Figure S6.3 Mycelial growth on potato dextrose agar (PDA) with or without FA**

Growth of the indicated strains cultured for 3 d at 28ºC. wt, wild-type.

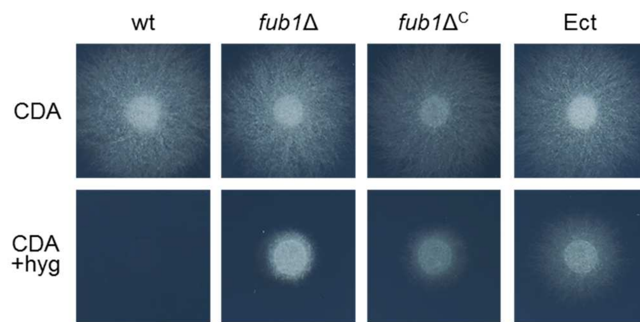



**Figure S6.4 Mycelial growth in czapek-dox agar (CDA) with or without hygromycin B**

Growth of the indicated strains cultured for 3 d at 28ºC. wt, wild-type.

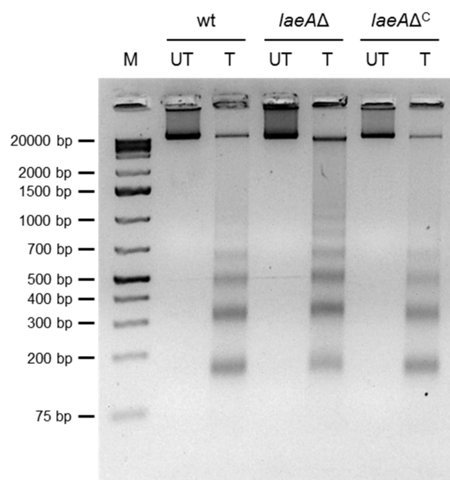
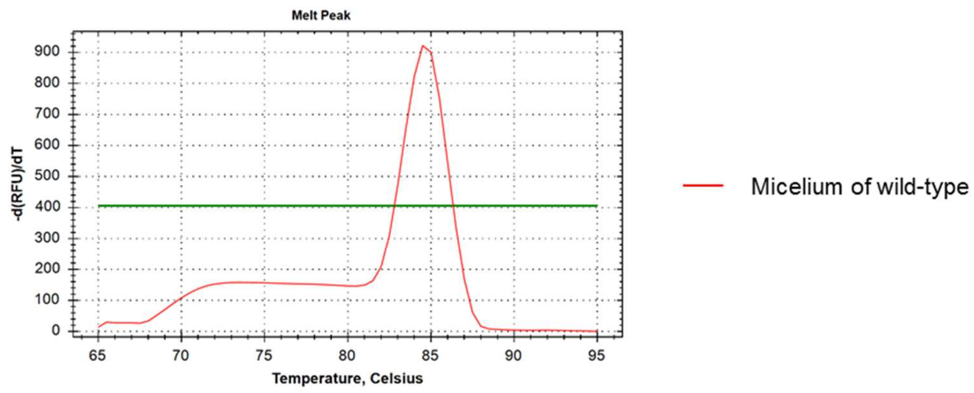



**Figure S6.5 Nucleosomal repeat length in *F. oxysporum***

Genomic DNA was extracted from lyophilized mycelium of the indicated strains, treated with MNase for 5 minutes at 37ºC (T), separated in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. DNA extracted from untreated mycelium was loaded as a control (UT). M, DNA marker. wt, wild-type.

**A**




**B**

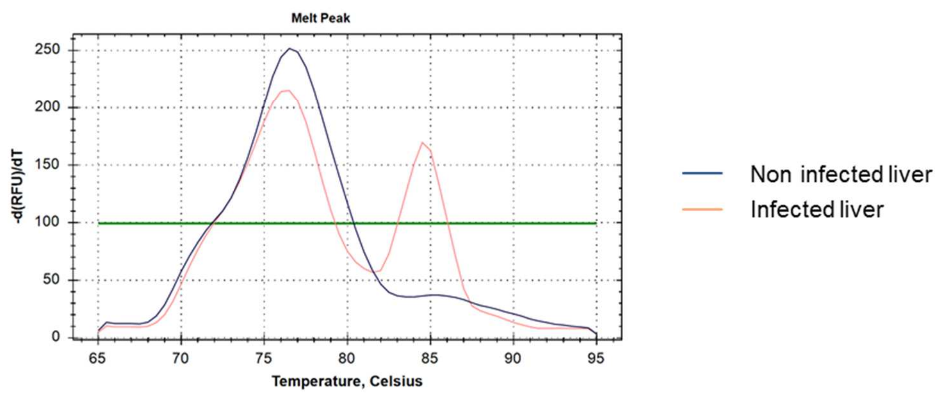



**Figure S6.6 *F. oxysporum fub1* is expressed during infection of mice**

**(A, B)** Melt curves in quantitative real-time RT-PCR experiments of the indicated samples. Note the detection of a non-specific amplicon in mice samples which makes quantification impossible.

**Table S6.1** Primers used in this study

| Name | Sequence (5'→3') | Use |
|---|---|---|
| fub1-F1 | GCTACCCATATAACACAACGG | knockout/complementation |
| fub1-F1n | AATACTCTCCTCTAAGCAGCC | knockout/qPCR |
| fub1-R1 | CTTGCTTTGGATTTGACTCTG | knockout/qPCR |
| fub1-F2 | TCTATGTTTTATTTCACTCAGGG | knockout |
| fub1-R2n | AAATCCCAAACCCCCTCAATC | knockout |
| fub1-R2 | GCGTTCCTCTTCACTAAATTAT | knockout/complementation |
| fub1-hph-F | TTCTTCAGAGTCAAATCCAAAGCAAGCGAGACCTAA TACAGCCCCTA | knockout |
| fub1-hph-R | ATGCCCTGAGTGAAATAAAACATAGACCTGTGCATT CTGGGTAAACG | Knockout |
| gpdA-15b | CGAGACCTAATACAGCCCCTA | knockout/complementation |
| tripter-8b | CCTGTGCATTCTGGGTAAACG | knockout/complementation |
| act-q7 | ATGTCACCACCTTCAACTCCA | qRT-PCR/qPCR |
| act-q8 | CTCTCGTCGTACTCCTGCTT | qRT-PCR/qPCR |
| fub1-F3 | GGGTTTGAGTTGTGCTGAGG | qRT-PCR/qPCR |
| fub1-R3 | AGAACGCTCATTATACTTGCTG | qRT-PCR/qPCR |
| hyg-G | CGTTGCAAGACCTGCCTGAA | qRT-PCR |
| hyg-12 | TTCGGGGCAGTCCTCGGC | qRT-PCR |
| EFα1-1 | TACTGGTGGTTTTGAAGCTGG | qPCR |
| EFα1-2 | AACTTCCTTCACGATTTCATCA | qPCR |
| fub1-F4 | GCAGTGGGTCATTCTCTCGG | qPCR |
| fub1-R4 | TTTACACATTACATACCGATTCA | qPCR |

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