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Fusarium species responsible for mycotoxin production in wheat crop: involvement in food safety

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Fusarium species responsible for mycotoxin production in wheat crop: involvement in food safety

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I would like to dedicate this thesis to my family especially

to my father, Hassan Alkadri and mother Ibtehal Alkadri, whom I didn't see for the past few years while I was busy in carrying out this scientific work. Their support, positive encouragement, constructive criticism and prayers still ring deep in my ears. No words could well thank you for everything you have done for me over the years, of all icons you are the most precious.

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To my Damascene poem Shaam and Abed, my small family, I have been justly questioned by you, whether there would ever be a completion date for this project?, now that I have come this far I would like to thank You for being with me all along and in every step, I want to write different words for you, to invent a language for you alone to fit the size of my love.

I dedicate this thesis to my beloved grandmothers and all my relatives, your words of support enlightened my way .
In a word, I know I always have an amazing family to count on when times are rough.
I also dedicate my thesis to the souls of my grandfathers, Ahmed Alkadri and Muhammad Munier Alkadri, which I am very proud to be their granddaughter.
Special dedication is to my beloved father in law Yasein Aljahmany.

I would like to dedicate this dissertation to the souls of my professors who died during my stay here, in Italy pursuing my studies, Prof. Jamal Hasnah, Prof. Ghassan Khayyat, Prof. Abdou Qasem and Prof. Ahmad Younes, God bless their souls and may they rest in peace and may almighty give their families all strength they need to overcome this great loss.

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Last but not least, and in view of the tragic events that are engulfing those days my beloved Syria, I wish to start the dedication to her, my wounded beloved homeland I want you as always strong and like the old Syrian mythological phoenix rising from its own ashes, you will overcome all challenges and revive stronger and more determined. I am your daughter, back after three years of hard and continuous efforts. My research is devoted to you and through you to all human beings. I wish that you accept this, as a sign of my love and affection for you My Syria, My Damascus.

Abbreviations

Ac-DON	acetyldeoxynivalenol
AF	aflatoxins
ANOVA	analysis of variance
AP_{index}	Petri-dish aggressiveness index
AUDPC standard	area under disease progress curve standard
AUHPC	area under healthy progress
BEA	beauvericin
bp	base pair
CE	collision energy
CEP	collision cell entrance potential
CID	collision-induced dissociation
CLA	carnation leaf piece agar
Clr	coleoptile length reduction
CTAB	hexadecyl-trimethyl-ammonium bromide
cv	cultivar
CXP	collision cell exit potential
DAI	days after inoculation
DI	disease incidence
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DP	declustering potential
DS	disease severity
ELISA	enzyme-linked immunosorbent assay
ENN	cyclohesadepsipeptide enniatin
FB	fumonisin B
EP	entrance potential
FDK	Fusarium damaged kernels
FHB	Fusarium head blight
FUS-X	fusarenon-X

GFC	*Gibberella fujikuroi* complex
Gr	germination rate reduction
HPLC-MS/MS	high-performance liquid chromatography combined with tandem mass spectrometry
HW	hectolitric weight
IDA	information dependent acquisition
KW	kernel weight
LOD	limits of detection
LOQ	limits of quantification
MSPD	matrix solid-phase dispersion
NIV	nivalenol
OTA	ochratoxins
PCR	polymerase chain reaction
PDA	potato dextrose agar
PPM	part per million
QqQ	triple quadrupole
QTL	quantitative trait loci
TDI	Tolerable Daily Intake
TRI	trichodiene synthase
ZEN	zearalenone

Key words

wheat, FHB, *Fusarium,* PCR, chemotype, mycotoxins, HPLC-MS/MS, aggressiveness, Petri-dish test, inoculation, cultivars, resistance.

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SUMMARY

Wheat is considered one of the main and strategic crops in Mediterranean countries. Syria is one of the few self-sufficient countries in wheat production (3.6 million tons in 2010) and an important exporter. In Italy durum wheat is considered a key crop and covers almost 50% of cereal cultivated areas.

Fusarium Head Blight (FHB), a worldwide cereal disease that harms the crops in different manners, causes significant yield reduction, inferior grain quality, and is responsible for the mycotoxins accumulation. *Fusarium graminearum* and *F. culmorum* are the prevalent causal agents. To our knowledge, there are no published records on the presence of FHB disease in Syria, while in Italy it has been present, especially in northern and central regions, since 1995. Cultivar resistance is one of the most promising and effective managing strategies against FHB but unfortunately, no resistant cultivars to FHB exist.

This research covered different aspects such as: identification, either morphologically or molecularly, of fungal population isolated from Italian and Syrian wheat kernels cultivated in distinct regions with particular attention to *Fusarium* species (CHAPTER I); investigation of the capability of different *Fusarium* species to produce mycotoxins after inoculation on different media (CHAPTER II); detection of mycotoxins in different Syrian and Italian wheat kernel samples (CHAPTER III); study the aggressiveness of *F. culmorum* isolates using different assays and validation of a new Petri-dish test (CHAPTER IV and V); study the behavior of different Syrian wheat cultivars grown under different conditions (field and growth chamber) toward FHB agents (CHAPTER VI).

CHAPTER I of this research concerns the collection of 48 Syrian and 46 Italian wheat kernel samples from six Syrian and seven Italian regions, respectively, in the years 2009 and 2010 and their analysis for *Fusarium* presence. *Fusarium* strains were morphologically and molecularly identified. *F. graminearum* and *F. culmorum* strains were chemotyped by multiplex PCR assays.

The mycological analysis of Syrian samples revealed the presence of different fungal genera as *Alternaria* (53%), *Cladosporium* (15%), *Penicillium* (12%), *Rhizopus* (6%), *Aspergillus* (5%), *Fusarium* (4%), *Epicocum* (2%) and also other fungi but in low percentages. *Fusarium* spp. were present in 62.5% of Syrian samples with a relative frequency of 4% in *Fusarium* infected

samples. 163 *Fusarium* strains were collected and identified to species level. The most frequent species were: *F. tricinctum* (30.1%), *F. culmorum* (17.8%), *F. graminearum* (12.9%), *F. equiseti* (14.1%), *F. verticillioides* (10.4%) and *F. proliferatum* (8%) while to a lesser extent *F. oxysporum* (3%), *F. semitectum* (1.8%), and *F. pseudograminearum* (1.8%). The 3Acetyl-Deoxynivalenol (3Ac-DON) and nivalenol (NIV) chemotypes were found in *F. culmorum* whilst all *F. graminearum* strains belonged to NIV chemotype. In Italian samples, *Fusarium* spp. were present at 67.4%. Among 93 *Fusarium* strains, *F. graminearum* (74.2%), *F. poae* (17.2%) and *F. culmorum* (8.6%) were identified morphologically and molecularly. 15Ac-DON was the prevalent chemotype in *F. graminearum*, while only 3Ac-DON chemotype was detected in *F. culmorum*.

CHAPTER II regards mycotoxin production by 60 Syrian *Fusarium* strains, belonging to nine species, grown on wheat medium screened by HPLC-MS/MS in the laboratory of Toxicology, Department of Preventive Medicine, Faculty of Pharmacy, Valencia University (Spain). The results showed that all *F. culmorum*, *F. graminearum* and *F. pseudograminearum* strains were zearalenone producers. The production of fumonisin exclusively by all *F. proliferatum* and *F. verticilliodes* strains, the scattered presence of DON, and the absence of emerging mycotoxins (except one strain) were the prominent characters of the analysed strains. The quantification of mycotoxin production from 28 different *Fusarium* strains, belonging to four species associated to FHB, was done on rice medium by HPLC-MS/MS in the laboratory of Molecular Phytopathology and Mycotoxin Research, Göttingen University (Germany) and had shown accordance with the chemotypes of the strains, i.e. potential ability to produce mycotoxins. ZEN was found to be the predominant mycotoxin in all the tested strains (except for one *F. equiseti*). The most important species, *F. graminearum* and *F. culmorum*, were characterized by the presence of NIV/FUS-X and DON or its derivatives, respectively. Moreover, all *F. culmorum* strains belonged to NIV chemotype were capable to produce both NIV and DON.

In CHAPTER III the estimation of mycotoxin contents on different Syrian and Italian wheat kernel samples, collected from different geographical areas from both countries, has been determined by HPLC-MS/MS. The results illustrated that 60% of Syrian samples were contaminated with mycotoxins: 27.5% with *Fusarium* mycotoxins, mainly DON, Fumonisin, ZEN in addition to emerging mycotoxins i.e. beauvericin, enniatins, and 55% with aflatoxins and ochratoxin. Italian seed samples were contaminated for 80.43% with *Fusarium* mycotoxins.

However, the levels of mycotoxins were under the European allowable limits (CE No 1881/2006 as amended by CE No178/2010) with the exception of one sample that had ZEN level higher than the allowable ones.

In CHAPTER IV, it has been studied the aggressiveness of different Syrian and Italian *F. culmorum* strains on a FHB susceptible cultivar, Simeto, using three different assays (floret inoculation in the growth chamber, ear inoculation in the field and Petri dish test). The aggressiveness levels of the isolates were further investigated for the relationship among aggressiveness parameters assessed in the three assays. Significant differences in aggressiveness levels within *F. culmorum* population and good correlations among the parameters of the three assays were found.

CHAPTER V is about the Petri dish test on two different wheat cultivars, Simeto and Duilio. The assessment was based on germination reduction, coleoptiles length reduction and area under disease progress curve standard. The data were well correlated with the *in vivo* data and allowed to validate a new parameter named Aggressiveness Petri dish Index (AP index). This Petri dish test is a reliable and fast method which could be used to screen the aggressiveness of *F. culmorum* strains before artificial inoculation in field trials.

In CHAPTER VI, the behavior of ten Syrian and one Italian (control) durum wheat cultivars towards FHB agents, under controlled and field conditions, was evaluated after artificial infection by Syrian and Italian *F. culmorum* strains. Syrian cultivars showed variable reactions to FHB agents in both trials. The cultivars Sham9, Sham5 and ACSAD1315 showed high susceptibility whilst the cultivars Jory and ACSAD1333 were the most tolerant to FHB agents. Moreover, Jory, the most tolerant cultivar had the lowest level of DON accumulation and could be a promising cultivar for breeding purposes. In conclusion, this is the first study on mycological and toxicological characterization of Syrian *Fusarium* species associated to FHB. The results should be taken in consideration in order to prevent, or at least reduce, the risks of any upcoming FHB epidemic, which might be caused by the changes in agricultural practices, imposed a cause of drought. Furthermore, these results should aid in the establishing of "Syrian allowed limits", for *Fusarium* mycotoxins (conventional and emerging) in cereal food and feed.

GENERAL INTRODUCTION

Wheat

Wheat (*Triticum* spp.) originates from south-west Asia, and some of the earliest remains of the crop were found in Syria, Jordan, and Turkey dated back to 10,000 years (Heun *et al*., 1997). It is the first food crop in all parts of the world where bread is the staple food for more than three-quarters of the world (Dib and Soussi, 2004). The importance of wheat has been mainly attributed to its ability to be ground into flour, semolina, and etc. that form the basic ingredients of bread and other bakery products and pastas, e.g. macaroni, spaghetti (Chandrika and Shahidi, 2006; Gallo *et al*., 2008). There are three main factors playing an important role in the success of wheat crop: (1) adaptation of its cultivation to a wide range of environments, (2) ease grain storage and transportation, and (3) production of a limitless variety of healthy and appealing food (Morris *et al*., 2011). FAO's estimation for global wheat production in 2010 stands at 653 million tonnes (FAO, 2010), while FAO's first forecast for world wheat production in 2011 stands at 676 million tonnes. In the first rank comes the EU as the main wheat producer with an expected production of 142 million tonnes in 2011 (FAO, 2011). Syria produces both durum (*T. durum* L.) and soft (common) (*T. aestivum* L.) wheat over the winter season; durum wheat in rainfed areas (60%) whilst soft wheat is cultivated mainly in irrigated areas (40%) (Sadiddin and Atiya, 2009). Depending on the rainfall, rainfed yields are highly unstable with a production average ranging from less than 0.5 tons per ha in drought years to over 1.7 tons per ha in years of good rainfall. Yields are more stable in an irrigated area with national average ranging from 3.0 to 4.4 tons per ha (www.pecad.fas.usda.gov). Syrian agricultural policy resulted in an increase in cultivation of wheat in the irrigated land from 229,000 ha in 1988 to 800,000 ha in 2003 and 1.9 million ha in 2005 with annual production between 4 and 5 million tonnes (NAPC, 2009) and reached 3.6 million tonnes in 2010 (FAO, 2011).

Wheat and its two main products, i.e. bread, and bulgur are familiar commodities in Syria (Haydar *et al*., 1990) with an average consumption of bread of 12.9 Kg per capita per month (FAO, 2003). In Italy, both types of wheat are cultivated, and in 2010 the annual production reached 2.9 million tons for soft wheat and 3.6 million tons for durum wheat (Istat, 2011). In particular, in the province of Bologna, Emilia-Romagna region in Northern Italy, the area under wheat cultivation increased from 8,200 ha in 1999 to 14,100 ha in 2007 (ISTAT data, http://agri.istat.it). Durum wheat is commonly used in Italy for pasta and semolina manufacturing

with a productivity that reaches 3,194,152 tons and 5,850,000 tons, respectively (FranceAgriMer, 2011).

Fusarium spp.

The teleomorph of the majority of *Fusarium* species belongs to the phylum *Ascomycota*, class *Ascomycetes*, order *Hypocreales*, genus *Gibberella*, and only a small number of *Fusarium* species, have telomorph in *Hemanectria* and *Albonectria* genera (Leslie and Summerell, 2006). *Fusarium* genus is common in nature, with pathogen and saprophyte species (Liddell, 1991). A high number of species is responsible for plant disease in several crops as well as cereals and at the same time can be pathogenic for human and animals. *Fusarium* spp. can be isolated from different plant organs, plant debris and soil (Summerell *et al.*, 2003). Leslie and Summerell (2006) reported that at least 80% of all cultivated plants are associated with one disease caused by a *Fusarium* species and reviewed all *Fusarium* species associated with plant diseases and especially cereals. *Fusarium* infection can occur at all the plant developmental stages, from seed germination to matured vegetative tissues, depending on *Fusarium* species involved and the host plant. Various *Fusarium* species can coexist in the same plant causing diseases with a complex etiology and able to produce secondary metabolites, mycotoxins (Logrieco *et al*., 2007). Early and precise identification of *Fusarium* spp. in every stage of infection is essential in predicting the potential toxicological risk to which the plants are exposed other than preventing these metabolites to be formed, since most of *Fusarium* species have specific mycotoxin profiles.

The ambiguous identification of mycotoxigenic *Fusarium* species is still the most critical issue. In fact, the genus *Fusarium* is characterized by the presence of a large number of species, now stands over 80, which are continuously changing, due to the various systems of taxonomy (Leslie and Summerell, 2006). These continuous changes are causing controversies among the researchers (Asan, 2011).

This genus is provided by few morphological features useful for distinguishing the different species based on traditional methods, but with some experience and using the morphological characteristics - colony features, macroconidia, microconidia, chlamydospores, and other microscopic features (Dongyou, 2009) - we can discriminate the most important pathogenic and toxigenic *Fusarium* species (Dongyou, 2009). Macroconidia of *Fusarium* species are sickle

shaped, with multi septa and resemble a banana or a canoe (Figure 1a, 1b), microconidia are one or two celled and develop from phialides. Chlamydospore with thick walls can be found in some species (Leslie and Summerell, 2006). The occurrence of asexual spores, the distinctive banana shaped macroconidia, is considered the main trait needed for the species to be placed in the genus *Fusarium* (Moretti, 2009). The septated macroconidia are produced in the aerial mycelium and particularly on mono and polyphialides, but often in specialized structures called sporodochia on short monophialides (Hawksworth *et al*., 1983) (Figure 1d). The term monophialide is refereed to a conidiation cell with a unique pore from which the endoconidia are released, while a polyphialide can have multiple openings. The other trait which is used is microconidia, which can vary in shape and size and are produced on the aerial mycelium, either in clumps or chains, both on monophialides and polyphialides (Figure 1c, 1e). Finally, chlamydospores, the resistance structures, with thick high lipid content walls, can be formed on the middle or the apex of the hyphae (Figure 2) (Sen and Asan, 2009). Depending on the different shapes and absence or presence of the above-mentioned structures in addition to the characteristics of the micro- and macro-conidiogenous cells, researchers can distinguish the different *Fusarium* species. The different shapes of macroconidia remain the most important features. All taxonomists suggest, as the correct steps to reach the goal of characterization and identification of the species, the use of strain cultures obtained from single spore isolation, grown on suitable media under optimal condition (Dongyou, 2009).

On the other hand, the increasing utility of DNA-based methods which are believed to be a revolution in *Fusarium* taxonomy prove the occurrence of underestimation of the true diversity in the genus *Fusarium* (O'Donnell, 2000). The molecular phylogenetic analysis recently proposed has been applied by many researchers in order to examine the taxonomy of the genus *Fusarium*, however many phylogenetic relationships remain unclear due to the presence of only few comprehensive phylogenetic analysis performed for this genus (Watanabe *et al.*, 2011). Analysis such as DNA sequencing and species-specific PCR assays must also be conducted.

In *Fusarium,* the translation elongation factor 1-α (TEF) gene, which encodes an essential part of the protein translation machinery, has become the marker of choice as a single-locus identification tool (Geiser *et al.*, 2004). This is due to the appearance of constant occurrence of TEF gene as single-copy in *Fusarium* with high level of sequence polymorphism among closely

related species even when compared with the intron-rich portions of protein-coding genes such as calmodulin, β-tubulin and histone H3 (Rahjoo *et al*., 2008).

Geiser *et al.* (2004) created the first generation of FUSARIUM- ID v.1.0, a publicly available database started with 441, but currently containing 5560, sequences representing a phylogenetically diverse selection of TEF sequences from the genus and placed it on a local BLAST server, which can be accessed at http://isolate.fusariumdb.org/index.php. Some species-specific PCR primers have been developed but in most cases they need to be more widely tested especially for analysis of strains from various crops and /or geographic locations (Rahjoo *et al*., 2008). Some researchers have used species-specific PCR assay to identify some *Fusarium* species as *F. culmorum* (Nicholson *et al*., 1998), *F. graminearum* (Waalwijk *et al*., 2003), *F. poae* (Parry and Nicholson, 1996) and *F. pseudograminearum* (Aoki and O'Donnell, 1999).

Figure 1. Spore morphology in the identification of Fusarium species.

A: Macroconidia, B: Macroconidial apical and basal cells, C: Microconidia, D: Phialides, E: Microconidial chains (Leslie and Summerell, 2006)

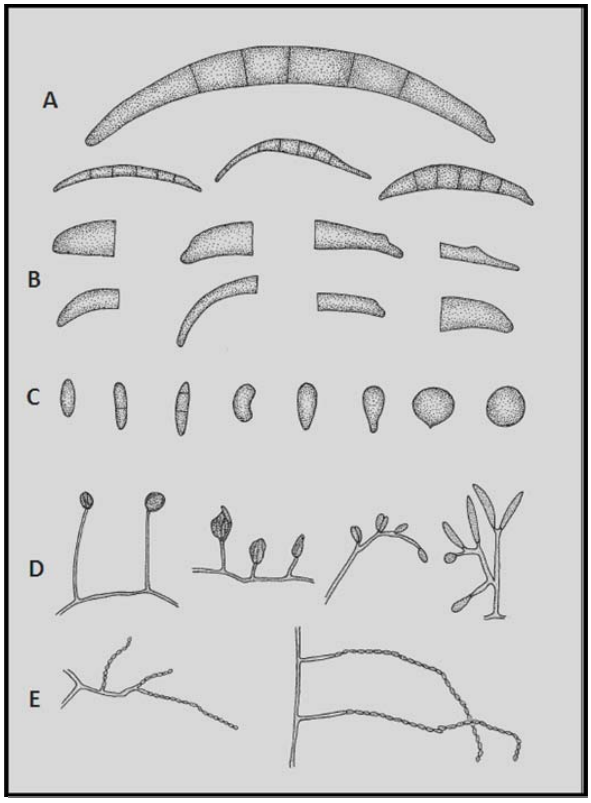
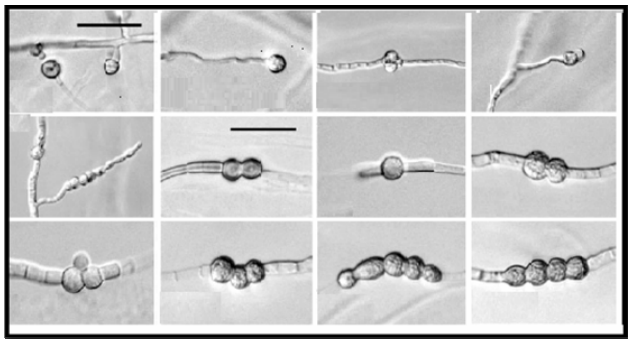


Figure 2. Chlamydospores of Fusarium species (Leslie and Summerell, 2006)



Fusarium head blight in wheat

The seed infection by *Fusarium* pathogens is a great risk for wheat cultivation. *Fusarium* species are widespread pathogenic fungi, which can cause *Fusarium* Head Blight (FHB) and *Fusarium* crown rot in wheat (Goswami and Kistler, 2004). *Fusarium* crown rot is known to occur in Syria (El-Khalifeh *et al*., 2006) while FHB or scab, which is a more dangerous and important disease, has not been reported in Syria yet. FHB epidemic incidences can arise suddenly: their appearance depends on environmental conditions such as rainfall occurring during flowering in the presence of susceptible hosts and aggressive isolates of the pathogen (Xu and Nicholson, 2009). Recent epidemic outbreaks of FHB were seen in South America, Asia, and Europe (Parry *et al*., 1995; McMullen *et al*., 1997). In Canada, the yield loss caused by FHB reached up to 70 % (Bai and Shaner, 1994), while in Europe the losses were estimated between 10 and 30 % (Bottalico and Perrone, 2002; Logrieco *et al*., 2002a). Severe epidemics in USA, in the years 1991-1997, caused a total loss of $2.6 billion and subsequently induced mycotoxin contamination of wheat and barley (Windels, 2000). In Italy FHB was first reported at the beginning of the 20th century, and it has been permanently present since 1995, especially in the north-central regions, with variable incidence and severity related to the year, area and cultivar (Pancaldi *et al*., 1996; Rossi *et al*., 2006). The aetiology of FHB is complex because of the involvement of several species of *Fusarium* and *Microdochium nivale* (Fr.) Samuels & I.C. Hallett. *Fusarium* species most frequently associated in Italy are *F. graminearum* Schwabe (teleomorph *Gibberella zeae* [Schwein.] Petch), *F. culmorum* (W.G. Sm.) Sacc., *F. avenaceum* (Fr.) Sacc. (teleomorph *Gibberella avenacea* R.J. Cook) and *F. poae* (Peck) Wollenw. (Pancaldi *et al*., 2010).

Epidemiology

Understanding the life cycle of *Fusarium* pathogens (Figure 3) is fundamental to understand the relation with seedling blight and head blight on small grains, including wheat. Plant debris is considered as the primary source of the inoculum which might be ascospores, hyphal fragments, macroconidia or chlamydospores (Goswami and Kistler, 2004). Boshoff (1996) reported that the saprophytic survival of the pathogen in residues could transfer inoculum from one season to another in addition to the capability of some *Fusarium* species to survive for long time in soil (Shaner, 2003). Cereal seeds sowed into *Fusarium* infested soil may result in the infection of plants and development of seedling rot. *Fusarium*- infected grains, resulting from the development of FHB, if used as seed, can provide an important source of inoculum for the development of seedling blight that will complete the disease cycle (Dill-Macky, 2003). Since hyphal fragments are believed to be an important source of inoculum for root infection, the air born inocula are also important in infecting the ears of the plants later in the growing season. Ear infection can happen either by rain-splashed conidiospores (asexual) transferred from the stem base or upward from the soil surface to the leaves by direct wind-dispersed ascospores (sexual) (Trail *et al.*, 2005). High humidity, is needed for ascospore release, during anthesis to produce FHB (Trail *et al*., 2002). Nelson *et al.* (1981) mentioned that the intensity of the infection is reduced when ascospore release does not correspond with anthesis. Disease signs are characterized by bleached spikeltes of the head and if the conditions are highly favorable to FHB, pink-red mycelium and conidia develop on the spikelets and infection spreads throughout the entire head (Figure 4). Infected kernels finally become shriveled and chalky white in appearance (von der Ohe, 2010).

Figure 3. Disease cycle of the causal agents of FHB in wheat

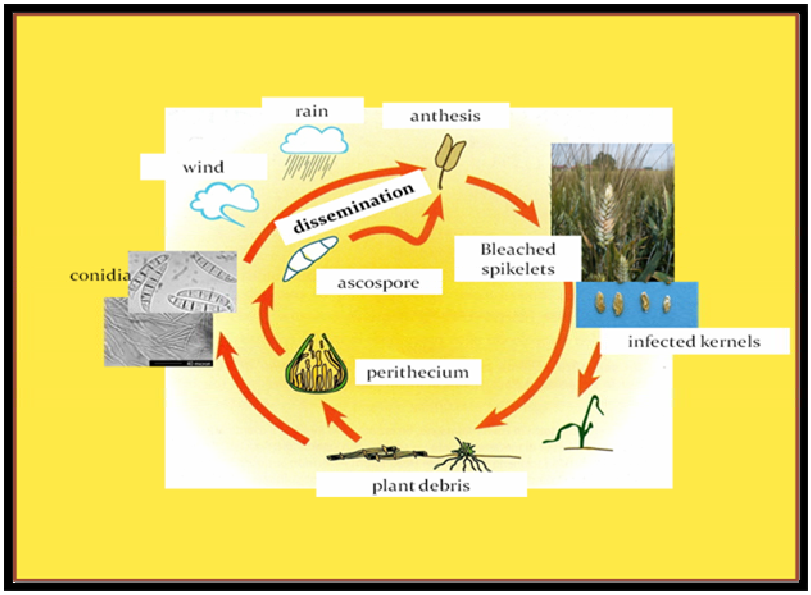


Figure 4. FHB symptoms: a) pink-red mycelium on the spikelets, b) bleached spike

a) *b)*

Conditions for Infection and Colonization

Field monitoring is the base for most studies, which considered the relationship between environmental factors and FHB. The conditions for all FHB causative agents in general are

similar: wet or moist conditions during flowering (De Wolf *et al*., 2003). Nonetheless, there are some differences in the temperatures required for germination and infection among and within the *Fusarium* spp. *In vitro* studies showed that isolates from different origins have optimal temperatures in accordance with their country of origins (De Wolf *et al*., 2003).

The optimum temperature for *F. avenaceum* and *F. graminearum* are approximately between 28 and 29 °C on detached spikes, and spikes inoculated with *F. culmorum* have a lower infection incidence with the corresponding optimum temperatures of 18 and 26.5°C (Rossi *et al*., 2001). The relationships between the prevalence and abundance of FHB species with the environmental variables are not similar: *F. graminearum* is more frequent with warmer/humid conditions; *F. poae* is associated with relatively drier and warmer conditions, and *F. avenaceum* and *F. culmorum* are both associated with cooler/wet/humid conditions (Xu and Nicholson, 2009).

Fusarium head blight and food quality

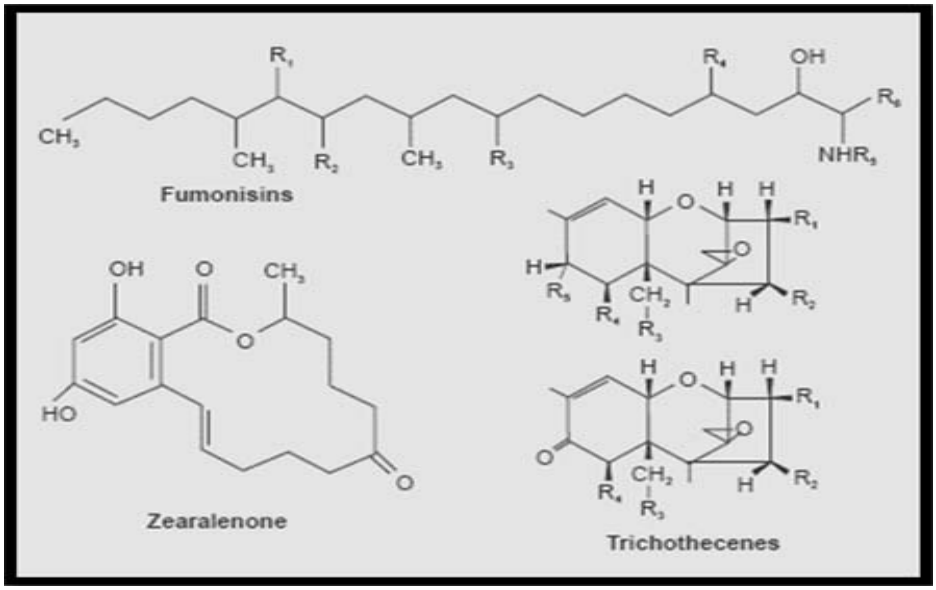
Dexter *et al*. (1997) mentioned that the effects of FHB on wheat processing quality have not received much attention. On the other hand, artificial inoculation of wheat heads under field conditions with *F. culmorum* and examination of FDK under scanning electron microscopy (SEM) has revealed structural alteration caused by the infection and colonisation of kernels by this pathogen. Moreover, in contrast to the healthy kernels in which the endosperm cells were filled with tightly packed large and small starch granules, surrounded by the protein matrix, the principal changes in the structure of infected kernels characterized by partial or complete lack of cell walls, visible symptoms of the amylolytic degradation of starch granules and lack of the protein matrix surrounding the starch granules. These symptoms are likely a sign of the activity of hydrolytic enzymes produced by the fungi growing in the infected kernels (Jackowiak *et al*., 2005). Similar images of the endosperm under scanning electron microscopy were observed on kernels of barley severely infected by *F. graminearum* (Schwarz , 2003). The structure and the quantity of gluten protein are strongly related to the quality and technological properties of wheat flour (Wang *et al*., 2005). Inferior backing quality caused by the degradation of wheat protein, i.e. gluten and glutanin as a result of the activity of proteolytic enzymes. This interprets the weaker dough with a lower loaf volume during manufacturing (Jackowiak *et al*., 2005).

Mycotoxins

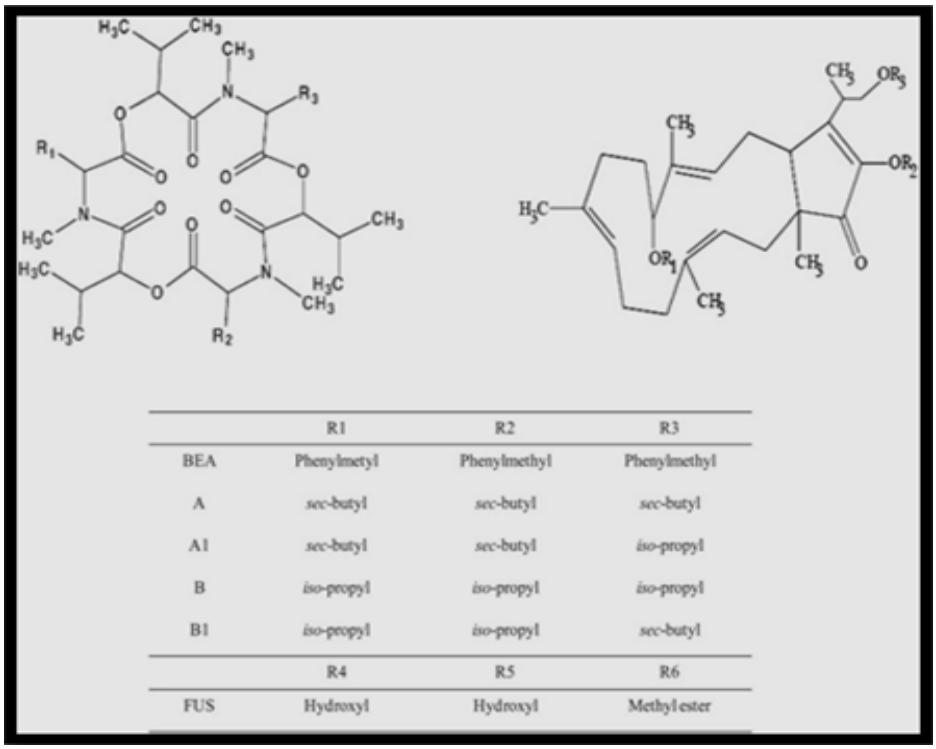
Infected grains can be contaminated considerably with secondary metabolites (mycotoxins) that are recognized as health hazard for both human and animals (Mankeviciene *et al*., 2007). Many authors have indicated the importance of mycotoxins in the mechanism of infection since they are involved in the inhibition of the host resistance reactions (Jansen *et al*., 2005; Maier *et al*., 2006). Mycotoxin presence depends on several factors, such as fungal strain, climatic and geographical conditions, cultivation techniques, susceptibility level of host plants and crop protection, especially during storage (Pancaldi *et al*., 2010). Rotation intervals among host crops, land preparation, use of fertilizers, irrigation, and weed control have been listed as influencing factors (Parry *et al*., 1995). Major mycotoxigenic fungi involved in the human food chain belong to the filamentous genera *Fusarium*, *Aspergillus* and *Penicillium* (Jestoi, 2008). *Fusarium* species, are an example of mycotoxigenic fungi, able to produce several major mycotoxins, such as deoxynivalenol (DON), acetylated-DON (ac-DON), nivalenol (NIV), HT-2 and T-2 toxins and zearalenone (ZEN) (Figure 5). In addition, cyclohesadepsipeptide enniatin (ENN) and beauvericin (BEA) production has also been reported (Torp and Langseth, 1999; Uhlig *et al*., 2006) (Figure 5). Poisoning with *Fusarium* mycotoxins causes acute and chronic symptoms such as nausea, internal organs damage, cancer, and infertility (Rocha *et al*., 2005; Minervini *et al*., 2004; Nielsen *et al*., 2009). DON mycotoxin, which is a member of trichothecene B group, is known to inhibit protein synthesis of eukaryotic cells and possesses neurotoxic and immunosuppressive activity (Snijders, 1994; Benett and Klich, 2003). While low concentration of DON in feed can reduce the food consumption of the animals and affect their appetite, higher doses induce vomiting (Benett and Klich, 2003). Consequently, mycotoxin is considered a major concern for food safety. Gareis *et al*. (1989) reported that DON contamination in European countries was found in more than 90% of samples harvested in years with FHB epidemics; Lepschy (1992) instead mentioned that even without any epidemics the levels of DON reached 0.1 PPM in German wheat samples. Due to food-safety concerns, approximately 100 countries have regulated the maximum levels of mycotoxins in food or feedstuffs by the end of 2003 (van Egmond *et al*., 2007). In the EU, the limits in grains and food products allowed a maximum DON content in unprocessed bread wheat of 1.25 mg kg-1, in bread and bakeries of 0.5 mg kg-1, and 0.2 mg kg-1 of baby food (Anonymous, 2005). No regulations are known in Syria for *Fusarium* mycotoxins allowable limits in food and feeds.

Figure 5. Chemical structures of mycotoxins produced by Fusarium species a) conventional, b) emerging (Sebastia et al., 2012)

a)



b)



	R1	R2	R3
BEA	Phenylmetyl	Phenylmethyl	Phenylmethyl
A	*sec*-butyl	*sec*-butyl	*sec*-butyl
A1	*sec*-butyl	*sec*-butyl	*iso*-propyl
B	*iso*-propyl	*iso*-propyl	*iso*-propyl
B1	*iso*-propyl	*iso*-propyl	*sec*-butyl
	R4	R5	R6
FUS	Hydroxyl	Hydroxyl	Methyl ester

Control

Several authors have reported different control measures for FHB (Parry *et al*., 1995; Pancaldi *et al*., 2010), that include the use of cultural control techniques, genetic resistance and the use of chemical or biological antagonists. The cultural control techniques comprehend the use of suitable crop rotations, appropriate use of fertilizers, irrigation and weed control. Lemmens *et al*. (2004) suggested that growing resistant cultivars, genetically developed, could be one of the solutions to solve the problem related to mycotoxin accumulation in wheat. Application of fungicides, e.g. Tebuconazol and Prothioconazol, as chemical control against FHB, is still difficult since, they depend on the environment and the genotype as well as on the fungicide applications that need to be very close to each other. The best time should be at flowering, but it is limited because is affected by the precipitation time (Von der Ohe, 2010). The nature of the limited time in which the heads are susceptible to FHB infection (only during anthesis, and for a short period after) makes this disease a potential target for the biological control. There are numerous reports of bacterial or fungal antagonists to *Fusarium* species, but the results in the field are inconsistent or even a complete failure (Xu and Nicholson, 2009). Concerning the biological antagonists, the goal seems far away to be reached (Parry *et al*., 1995). The agronomical practises are known to affect FHB. Application of fertilizers from 0 to 80 kg/ha increased FHB severity and DON grain contamination, and it can be explained by the fact that fertilizers increase the plant density and alter the microclimate (Lemmens *et al*., 2004). Tillage and stubble management show efficacy in influencing FHB, especially when the previous crops cultivated are maize or wheat. The tendency of maintaining crop residues in order to resist the erosion of the soil, illustrate the effect of this procedure on the progress of FHB as it serves as a nutrient source for fungal inocula on the soil surface (Dill-Macky, 2008). The intensity of FHB infection is greatly affected by the type of tillage since the severity of the infection is in general the lowest with the deep tillage, and reaches the highest level without tillage. The destruction of the residues prevents the source for the inocula (Paul *et al*., 2004; Bateman *et al*., 2007). The tillage and the type of the previous cultivated crop, affect the severity of the disease, but without significant alteration of the species composition. For the same reason, burning the stubble significantly reduce the survival of *F. graminearum* (Dill-Macky, 2008). Maize-wheat rotation is the most conducive for *F. graminearum*-induced FHB, but other crops may also influence the FHB population. FHB presence was 25% and 50% less where the previous cultivated crop was

soybean instead of wheat or maize (Dill-Macky, 2008). In order to achieve the goal of the individual management strategies for efficient reduction of disease symptoms and mycotoxin accumulation, it should be taken into consideration the composition of FHB pathogens. Thus, it is crucial to understand the FHB pathogen community structure for practical disease management (Xu and Nicholson, 2009).

FHB resistance

In plants, disease resistance is manifested by limited symptoms, which reflect inability of the pathogen to grow or multiply and spread, and often takes the form of a hypersensitive reaction (HR), in which the pathogen remains restricted to the site of infection as necrotic lesions (Van Loon, 1997). Mesterhazy (2002) described resistance of wheat to FHB as a complex phenomenon and reported different types of resistance (i) resistance to initial infection (Schroeder and Christensen, 1963); (ii) resistance to spreading (Schroeder and Christensen, 1963); (iii) resistance to kernel infection (Mesterhazy, 1995; Mesterhazy *et al*., 1999); (iv) tolerance to infection (Mesterhazy, 1995; Mesterhazy *et al*., 1999) and (v) resistance to DON accumulation (Miller *et al*., 1985). Most authors conclude that no wheat cultivar is immune, most are susceptible, but a few are moderately resistant (Parry *et al*, 1995). *Triticum durum* L. is more susceptible in comparison with *Triticum aestivum* L. to infection with pathogens of the genus *Fusarium*, therefore, its grains are more exposed to higher mycotoxin concentrations (Stack *et al*., 2002). Moreover, Buerstmayer *et al*. (2009) report that 46 different quantitative trait loci (QTLs) were identified for FHB resistance in common wheat, while only four QTLs were detected in durum wheat. Mesterhazy (1999) found very similar resistance reactions against *F. graminearum* and *F. culmorum,* and this held true for FHB, FDK, yield loss and the degree of DON contamination.The resistance to FHB is quantitatively inherited in all cereal species with a highly significant genetic variation among breeding materials (Snijders, 1990; Miedaner, 1997). Buerstmayr *et al.* (2000) reported that the cultivation of genetically resistant cultivars is the most cost-effective method to control the disease and it is well documented the presence of genetic variations against FHB among wheat and its relatives. Wheat cultivars with high and stable yield and good quality other than resistance against diseases, including FHB, is a great challenge (Buerstmayer *et al*., 2009). A quantitative trait of wheat resistant to FHB agents is induced by

several genes, and the environmental factors also play an important role (Bai *et al*., 2000). Thus, QTL approach has extensively been applied using molecular markers, nevertheless, it has not been found yet durum wheat sources with effective FHB resistant (Chen *et al*., 2007). The slow evaluation of resistance to FHB in cereal breeding is considered a problem since it is necessary to avoid escapees while evaluating resistance in whole plants over the years and in different environments (Browne and Cooke, 2004). Wheat breeding towards FHB resistance has become one of the major practices for wheat breeders. Toth *et al.* (2008) on FHB resistance breeding, reported that one pathogenic isolate of *Fusarium* species is sufficient, however it is also important to use an aggressive isolate because the low aggressive isolate may not allow to distinguish the different levels of resistance of wheat lines and cultivars.

Pathogenicity and aggressiveness are two important characteristics of *Fusarium* spp. (Von der Ohe *et al.*, 2010). Although the two words have different meaning, sometimes they have been confused. Pathogenicity reflects the measurement of the ability of a fungus to cause the disease, qualitatively, whereas, aggressiveness is a quantitative measurement of the rate at which level, the disease is reached with more aggressive pathogens (Shaner *et al.*, 1992). Aggressiveness assessment is fundamental (Wu *et al.*, 2005) to understand the interaction between host-pathogen in FHB-wheat system. Precise and accurate aggressiveness assays to quantify the levels of aggressiveness of *Fusarium* are needed.

OBJECTIVES

The overall objective of this research was to study the involvement in food safety of *Fusarium* species, responsible for mycotoxin production in wheat crop. The specific objectives were:

1. (a) determine the diversity of fungal species in wheat kernels (durum and soft wheat) from the different provinces of Syria;
   (b) identify *Fusarium* species isolated from Syrian and Italian samples;
   (c) determine the genotype and chemotype of selected *Fusarium* strains using molecular techniques;
2. (a) screen the mycotoxin production of several *Fusarium* spp. previously isolated from Syrian wheat kernels and identification;
   (b) quantify the mycotoxin production of *Fusarium* spp. associated with FHB and compare their chemotypes with mycotoxin production;
3. carry out a survey to obtain information on the incidence and levels of mycotoxins in Syrian and Italian wheat for human and animal consumption;
4. (a) compare aggressiveness of Syrian *F. culmorum* isolates with Italian ones using three different aggressiveness assays (ear inoculations in field, floret inoculations in growth chamber and Petri-dish test);
   (b) investigate the relationships among aggressiveness indices from different assays and their relation to FDK, kernel weight (KW) reduction and DON production;
   (c) compare aggressiveness of two different chemotypes of *F. culmorum* isolates using four disease parameters obtained with three aggressiveness assays;
   (d) evaluate FDK and KW reduction induced by two different chemotypes;
5. (a) evaluate the feasibility of the modified Petri-dish test using Area Under Healthy tissue Progress Curve (AUHPC) or Standardized Area Under Disease Progress Curve ($AUDPC_{standard}$) to determine the different levels of aggressiveness among Syrian and Italian *F. culmorum* strains;
   (b) validate this method by finding significant correlation among these results and the data from floret inoculation techniques under controlled conditions (growth chamber) and ear inoculations in field conditions;

(c) discover the relationships among the three parameters obtained by Petri-dish test ($AUDPC_{standard}$, germination rate reduction and coleoptile length reduction) and their relations to floret inoculation in both growth chamber and field;

(d) prove the stability and repeatability of this method among different durum wheat cultivars;

6. (a) investigate varietal differences in resistance to FHB infection and spread, kernel infection and mycotoxin accumulation comparing different Syrian durum wheat cultivars with Italian susceptible ones inoculated with different Syrian and Italian *F. culmorum* strains under controlled and field conditions;

(b) evaluate the relations among FHB-resistant types in different Syrian cultivars.

CHAPTER I

Mycoflora isolated from Syrian wheat kernels and characterization of *Fusarium* species in two Mediterranean countries, Syria and Italy

ABSTRACT

Wheat is one of the main crops in Mediterranean countries, and its cultivation has an important role in Syrian and Italian economy. *Fusarium* Head Blight (FHB) is considered an important disease in the Mediterranean basin and worldwide. In Syrian wheat, FHB and the accumulation of *Fusarium* mycotoxins have not been reported so far. We performed a mycological analysis of 48 Syrian durum and common, and 46 Italian durum wheat kernel samples collected from several wheat cultivation areas in Syria and Italy with different environmental conditions in years 2009 and 2010. Fungal genera were identified morphologically, and confirmation carried out molecularly for *Fusarium* isolates by species-specific PCR. For Syrian samples; most frequent fungal genera were *Alternaria* and *Cladosporium*. The relative frequency of *Fusarium* spp. in *Fusarium* infected samples was 4% while the percentage was 3% in all analyzed samples. The main *Fusarium* species associated with Fusarium head blight were *F. culmorum* 17.8%, *F. graminearum* 12.9%, *F. equiseti* 14.1%, and for the first time in Syrian wheat kernels, *F. tricinctum* 30.1%, *F. proliferatum* 8%, *F. semitectum* 1.8%, *F. pseudograminearum* 1.8% and *F. oxysporum* 3% were identified while the percentage of *F. verticillioides* was 10.4%. Chemotypes of Syrian *F. culmorum* and *F. graminearum* strains (3- and 15-acetyldeoxynivalenol, nivalenol) were determined by multiplex PCR. Syrian *F. equiseti* strains were checked for their potential ability to produce tricothecenes, and the results showed that 60% of *F. culmorum* strains were 3AcDON while 40% were NIV, all *F. graminearum* strains have NIV chemotype and 80.83% of *F. equiseti* strains have trichothecenes gene.

On the other hand, in the Italian samples the percentage of *Fusarium* infected samples was 67.4% Among 93 *Fusarium* strains were obtained; 69, 16 and eight strains were identified morphologically and molecularly to be *F. graminearum*, *F. poae and F . culmorum*, respectively. The chemotyping of these strains revealed that all *F. culmorum* were 3Ac-DON while 15Ac-DON was predominant in *F. graminearum* strains.

INTRODUCTION

Durum wheat constitutes the largest part of the staple food in the southern Mediterranean countries (El-Khalifeh *et al*., 2009). In Syria, wheat cultivation covers 83% of the cultivated area and has a central role in the diet (www.pecad.fas.usda.gov). There are five distinct agro- climatic zones in Syria based on rainfall (FAO, 2003) (Figure 1.1a). Wheat is present in all these areas. Rainfed wheat is concentrated in high rainfall zones 1 and 2 while irrigation is necessary in zones 3, 4 and 5.The largest zones are 5, 1 and 2, the wheat occupies roughly 53%, 42%, and 40% of the total cultivated land, respectively (NAPC, 2009).

Syria produces both durum and common wheat over the winter season. Common wheat is cultivated mainly in irrigated areas, durum wheat in rainfed areas. Depending on the rainfall, rainfed wheat yields are highly unstable with an average ranging from less than 0.5 tons per ha in a drought year to over 1.7 tons per ha in a year with good rainfall. Yields are more stable in the irrigated area with a national average ranging from 3.0 to 4.4 tons per ha (www.pecad.fas.usda.gov). Syrian agricultural policy brought to an increase in cultivation of wheat in irrigated land from 229,000 ha in 1988 to 800,000 ha in 2003 and to 1.9 million ha in 2005 (NAPC, 2009). In Italy, both types of wheat are cultivated with an annual production that reaches 2.9 million tons for common wheat and 3.6 million tons for durum wheat (Istat, 2011). In particular, in the province of Bologna, Emilia-Romagna region in Northern Italy, the area under wheat cultivation increased from 8,200 ha in 1999 to 14,100 ha in 2007 (ISTAT data 2011, http://agri.istat.it).

Seed infection by *Fusarium* pathogens is a great risk for wheat cultivation. *Fusarium* species are widespread pathogenic fungi, which can cause *Fusarium* Head Blight (FHB) and *Fusarium* crown rot in wheat (Goswami and Kistler, 2004). *Fusarium* crown rot is known to occur in Syria (El-Khalifeh *et al*., 2006) while FHB or scab, which is a more dangerous and important disease, has not been reported in Syria yet. In contrast, FHB was first reported in Italy at the beginning of the 20th century and has been permanently present in Italy since 1995, especially in the north-central regions, with variable incidence and severity related to the year, area and cultivar (Pancaldi *et al*., 1996, Rossi *et al*., 2006).

The aetiology of FHB is complex due to the involvement of several species of *Fusarium* and *Microdochium nivale* (Fr.) Samuels & I.C. Hallett. The *Fusarium* species most frequently associated are *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* [Schwein.] Petch),

F. culmorum (W.G. Sm.) Sacc., *F. avenaceum* (Fr.) Sacc. (teleomorph *Gibberella avenacea* R.J. Cook), and *F. poae* (Peck) Wollenw. Pancaldi and Torricelli ,(1999) in the study, performed during three years of observations (1994–1996) of FHB in durum wheat throughout Italy, have found that *F. culmorum* and *F. graminearum*, associated with *F. avenaceum*, were predominant in all three years, while *F. verticillioides*, *F. tricinctum* and *F. cerealis* only in 1994. *F. poae* was isolated with high frequency in 1996.

The distribution of *Fusarium* species in wheat and their prevalence over other fungi is affected by climatic conditions (temperature, humidity, etc.), agricultural practices (soil tillage, crop rotation, nitrogen fertilizers, pesticide treatment, etc.) and cultivar susceptibility (Parry *et al*., 1995; Saremi *et al.*, 1999; Doohan *et al*., 2003). FHB causes yield losses from 30 to 70% (Parry *et al*., 1995; McMullen *et al*., 1997). In addition to yield loss, colonization in wheat of *Fusarium* species can cause contamination of grain with mycotoxins, toxic fungal secondary metabolites, recognized as health hazard for both human and farm animals (Dexter *et al*., 1997).

Toxigenic *Fusarium* species produce a number of mycotoxins such as trichothecenes A and B, zearalenone, moniliformin, depsipeptides and fusaric acid. Most attention in the analysis of FHB-afflicting wheat grain has been so far devoted to deoxynivalenol (DON), acetylated forms of DON (3Ac-DON and 15Ac-DON), nivalenol (NIV), fusarenon X (Fus X), and zearalenone (ZEN). The knowledge of the occurrence of *Fusarium* species in different growing areas help to predict mycotoxin content in harvested grain (Desjardin, 2006; Pancaldi *et al.*, 2010). Poisoning with *Fusarium* mycotoxins causes acute and chronic symptoms such as nausea, internal organs damage, cancer, and infertility (Rocha *et al*., 2005; Nielsen *et al.*, 2009). The maximum limits allowed for mycotoxin levels in food for the protection of the consumer have been established, and for DON and ZEN in food Europe (EU-regulation1881/2006) have already defined the allowance limits.

DON in the recent years has been found in FHB-infected durum and common wheat kernels in several wheat-growing areas of Emilia- Romagna and other Italian regions (Lops *et al*., 1998; Pascale *et al*., 2002; Rossi *et al*., 2006).

In addition to *Fusarium* spp., other fungi infect wheat grain in the field, and cause quality loss due to undesirable colour and odour, e.g. *Alternaria* spp., *Cladosporium* spp., *Epicoccum* spp., *Rhizopus* spp. (Zillinsky, 1983). Some of these species are known to produce mycotoxins (Li *et al*., 2001).

The aims of the present research were (1) to determine the diversity of fungal species in wheat kernels (durum and common wheat) from different provinces of Syria, (2) to identify *Fusarium* species isolated from Syrian and Italian samples, (3) to determine the genotype and chemotype of selected *Fusarium* species strains using molecular techniques.

MATERIALS AND METHODS

Fungal isolation

During 2009 and 2010, 48 grain samples of durum and common wheat were collected from six Syrian regions distributed in five ago-climatic zones (Figure 1.1a) and 46 of durum wheat were collected from seven Italian regions (Figure 1.1b). For Syrian samples, *Fusarium* presence was studied in addition to fungal population. 400 kernels, selected randomly from each sample, were disinfected in a sodium hypochlorite solution with 2% of available chlorine for two minutes, rinsed with sterile water, dried on sterile filter paper, placed in Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with neomycin and streptomycin sulphate (100 mg/l and 200 mg/l, respectively) and incubated at 22°C in the darkness for seven days.

Micromorphology of fungal isolates was examined by light microscopy (Watanabe, 2002). All *Fusarium* isolates were sub-cultured on water agar (2% of Bacto agar, Difco) using single spore technique (Leslie and Summerell, 2006). Pure cultures of *Fusarium* spp. were grown at 22°C (12 h photoperiod) for 10 days on Carnation Leaf piece Agar (CLA) to produce macroconidia of uniform size and form, and on PDA for the morphology of the colony (Nelson *et al*., 1983; Leslie and Summerell, 2006). Strains of *Gibberella fujikuroi* complex (GFC) were characterized following method previously described by Nirenberg and O'Donnell (1998) and Marasas *et al.* (2001). The relative frequency of each genus was calculated as a percentage of the total number of fungal colonies.

DNA extraction

DNA was extracted from mycelium that was harvested from 7- day-old single- spore cultures grown on PDA, using CTAB (hexadecyl-trimethyl-ammonium bromide) method (Prodi *et al.,* 2011a).

Fungal mycelium, 100-200 mg was placed in 10x15 cm 500 gauge polyethylene bag,

pre frozen in liquid nitrogen and then grinded using a small hand roller until the mycelium tissue formed smooth paste. One-two ml (10 vols) of grinding buffer (2% CTAB, 100 mM Tris-HCL, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1.0 % Na sulphate, 2.0 % PVP-40, and 1 µl of proteinase K) were added and mixed thoroughly using a roller. The ground sap was poured into 2.0 ml microfuge tube and incubated in water bath at 65°C for 10-15 min to denature the proteins. The sample was centrifuged for 10 min at 11,000 rpm (room temperature). The supernatant (Clarified sap) was transferred to microfuge tube, added with an equal volume of chloroform: isoamyl alcohol (I.A.A.) (24:1) and mixed to emulsion by inverting the tube. The sample was centrifuged again for 10 min at room temperature. The aqueous upper phase was carefully transferred to a new Eppendorf tube. DNA was precipitated with 0.5 volumes of 5 M NaCl pH 8.0 and an equal volume of ice cold iso-propanol. The DNA extract was thoroughly mixed and incubated at - 20°C overnight. The precipitate was centrifuged at 11,000 rpm for 15 min, the supernatant removed, the DNA washed carefully with cold ethanol (70%) and again centrifuged for 3-4 min at 11,000 rpm. DNA was dried and redissolved in 50 µl sterile distilled water. Finally, the extracts were stored at –20°C.

Molecular identification of *Fusarium strains* by PCR amplification with specific primers

To confirm morphological identification, *F. graminearum, F. culmorum*, *F. equiseti*, *F. pseudograminearum, F. poae, F. proliferatum* and *F. verticillioides* strains were identified using species-specific primers Fg16F/Fg16R and Fc01F/Fc01R (Nicholson *et al.*, 1998), FEF1/FER1 (Mishra *et al.*, 2003), Fp1-1/Fp1-2 (Aoki and O'Donnell, 1999), Fp82 F/R (Parry and Nicholson,1996), PRO1/ PRO2 (Mule *et al*., 2004), and VER1/ VER2 (Mule *et al*., 2004), respectively. Amplification was done in a T3 thermocycler (Biometra, Göttingen, Germany) under the conditions described in the protocols.

Chemotype identification

F. graminearum and *F. culmorum* strains were characterized by multiplex PCR assays to differentiate their chemotypes regarding trichothecene synthesis. For *F. culmorum*, primers amplifying parts of *Tri3* and *Tri7* genes were used to identify 3Ac-DON, 15Ac-DON and NIV

chemotypes (Quarta *et al.*, 2005). The primer sets *Tri3*F971/*Tri3*R1679 and *Tri3*F1325/*Tri3*R1679 identified 15Ac-DON and 3Ac-DON chemotypes, respectively, while the primer set *Tri7*F340/*Tri7*R965 identified the NIV chemotype.

F. graminearum chemotypes were identified using a multiplex version of another chemotype-specific test (Starkey *et al.,* 2007) based on data published by Ward *et al.* (2002). The primers, designed in the region of the *Tri*12 gene encoding an efflux pump for trichothecenes, differentiate among three chemotypes for B trichothecene. One primer for each pair is common to all chemotypes (12CON) while the other is specific for 15Ac-DON chemotype (12-15F), 3Ac-DON chemotype (12-3F) and NIV chemotype (12NF) (Ward *et al.*, 2002). Primers Tox5/1 and Tox5/2, derived from the DNA-sequence of the trichodiene synthase gene (*Tri5*), were used to test the ability of *F. equiseti* strains to produce trichothecenes (Niessen and Vogel, 1998). Amplification products were resolved on 1.5% agarose gels stained with ethydium bromide (0.4 µg ml-1) and visualized under UV light, alongside a 100 bp DNA ladder (Promega, USA) (Figure 1.6).

RESULTS

Fungal colonization of Syrian wheat kernels

In the surveys carried out in 2009 and 2010 on durum and common grain wheat collected from the different provinces in Syria, 17 different genera of fungi were identified in surface-sterilized kernels. *Alternaria* spp. and *Cladosporium* spp. were the most frequent, with an average isolation frequency of 53% and 15%, respectively (Figure 1.2). Storage fungi like *Penicillium* and *Aspergillus* were isolated at 12% and 5%, respectively. The percentage of non-infected kernels ranged from 1.5% to 96% per sample.

High kernel infection with *Alternaria* and *Cladosporium* was detected in the samples collected from all six provinces investigated. *Fusarium* spp. was present in 62.5% of all samples (Table 1.1) with a frequency of 4% of isolated fungi (Figure 1.2). *Fusarium* species were mostly isolated from samples collected in Daraa and Damascus rural areas with frequencies of 7.6% and 4.7%, respectively (Figure 1.3).

Morphological and molecular identification of Syrian *Fusarium* species

163 *Fusarium* isolates were identified to species level. PCR was used to verify the morphological identification of *F. culmorum, F. equiseti*, *F. graminearum, F. proliferatum, F. verticillioides* and *F. pseudograminearum.* Genomic DNA of isolates putatively identified as *F. culmorum* (11 isolates)*, F. graminearum* (6 isolates)*, F. equiseti* (9 isolates), *F. proliferatum* (4 isolates), *F. verticillioides* (5 isolates) and two isolates of *F. pseudograminearum,* was used in this analysis. The products of DNA amplification with species-specific primers (described in MATERIALS AND METHODS) were 570 bp for *F. culmorum*, 400 bp for *F. equiseti*, 450 bp for *F. graminearum,* 585bp for *F. proliferatum,* around 578 bp for *F. verticillioides* and around 523 bp for *F. pseudograminearum*. These sizes correspond to published values for species-specific PCR products, confirming the morphological identification.

Morphological and molecular data (Figure 1.4, 1.5) revealed that the major *Fusarium* species were *F. tricinctum*, *F. culmorum, F. graminearum, F. equiseti, F. verticillioides and F. proliferatum* with relative frequencies of 30.1, 17.8, 12.9, 14.1, 10.4 and 8 %, respectively. *F. semitectum*, *F. pseudograminearum*, and *F. oxysporum* were present in low frequencies of 1.8, 1.8, and 3%, respectively.

Table 1.1 shows the distribution of *Fusarium* species in wheat samples from different Syrian provinces.

F. culmorum was not detected in samples from Aleppo and Idlib provinces, while it was isolated from all other provinces. Some *Fusarium* species were limited to specific regions, such as *F. graminearum* to Damascus and Deir Ezzor, *F. equiseti* to Daara and Damascus, and finally, *F. pseudograminearum* to Deir Ezzor. The presence of *F. tricinctum* was predominant in all provinces.

Morphological and molecular identification of Italian *Fusarium* species

The frequency of *Fusarium* infected samples was 67.4% (Table 1.2). Among 93 *Fusarium* strains, 69, 16, and eight strains were identified morphologically to be *F. graminearum*, *F. poae and F . culmorum* respectively. These results were confirmed by molecular identification (see above, additionally *F. poae*- 220 bp).

Chemotype identification

Qualitative chemotyping results of Syrian and Italian *F. graminearum*, *F. culmorum* and *F. equiseti* strains are shown in Figure 1.6.

Syrian strains

In six strains (60%) of *F. culmorum,* using the primers directed to *Tri3* (Tri3F1325/Tri3R1679), an amplification product of about 350 bp was obtained as expected for 3Ac-DON chemotypes (Quarta *et al.*, 2005). Five strains (40%), amplified by the Tri7F340/Tri7R965 primers (*Tri7* gene), generated a 625 bp fragment expected for NIV producers. The 700-bp fragment specific for 15Ac-DON chemotype was not found in any of the tested strains. All six *F. graminearum* strains tested using primers for *Tri*12 produced amplicons of 840 bp, as expected for NIV chemotype (Starkey *et al.,* 2007). None of the strains produced amplicons of 670 or 410 bp expected for 15Ac-DON and 3Ac-DON chemotypes, respectively (Figure 1.7a and c).

Nine *F. equiseti* isolates were tested for the presence of trichodiene synthase gene involved in trichothecene synthesis (Niessen and Vogel, 1998). The presence of a 658-bp amplification product in seven strains could indicate a potential production of trichothecene.

Italian strains

All *F. culmorum* strains were 3Ac-DON chemotype while NIV and 15Ac-DON chemotype were not detected. 87.2 % of *F. graminearum* amplified a 670 bp product as expected for 15Ac-DON, 23.19% amplified by 12NF primer and gave a 840 bp fragment expected for NIV, whereas 4.35% have an amplification product of 410 bp as expected for 3Ac-DON (Figure 1.7b and d).

DISCUSSION

Fungal diseases are the primary constraint for wheat production in Syria (El Khalifeh *et al.*, 2009). In the 48 Syrian wheat grain tested samples, *Alternaria* and *Cladosporium* were the most dominant fungal genera (71%). These fungi are known to cause grey or black discoloration of heads and seeds resulting in sooty moulds, black points or smudge (Zillinsky, 1983). Furthermore, some *Alternaria* spp. produce mycotoxins such as alternariol which contaminate food products (Bottalico and Logrieco, 1998; Li *et al*., 2001). Storage fungi *Penicillium* spp. and

Aspergillus spp. of which some species produce aflatoxins and ochratoxins on stored grains (Muthomi and Mutitu, 2003) were isolated in the frequency of 12% and 5%, respectively.

The fungal population found in this survey was consistent with what reported by Pinson-Gadais *et al*. (2007) that found that eight different fungal genera isolated and identified from durum wheat samples, were similar to the genera isolated in our work. FHB has not been reported in Syria so far; but *Fusarium* populations have been recently isolated from wheat grown in Syria (El-Khalifeh *et al*., 2009; Arabi and Jawhar, 2010). Our results revealed that 62% of the analysed wheat samples from six provinces in Syria were contaminated with *Fusarium* spp. The frequency of *Fusarium* species in *Fusarium* contaminated samples was around 4%, and the low prevalence of *Fusarium* species doesn't reflect true field situation, since severely infected and shrivelled kernels, which are very light in weight, are expelled during combine harvesting (Bai and Shaner, 1994).

Wheat samples from Daraa, and Damascus rural regions were the most contaminated. *F. tricinctum*, *F. equiseti* and *F. culmorum* were identified in both regions while *F. graminearum* only in the latter one. These results are in line with the report of El-Khalifeh *et al*. (2009) who found *F. equiseti* spread in Daraa province.

The Morphological and molecular identification of Italian *Fusarium* species illustrated that the prevalent strains belonged to *F. graminearum*, *F. culmorum* and *F. poae.* Our results agreed with previous reports indicating that among several species of *Fusarium* responsible for FHB, the prevailing ones in Italy were *F. graminearum, F. culmorum* and *F. poae* (Prodi *et al*., 2009; Shah *et al*., 2005; Pancaldi *et al*., 2010). In Italy and Europe, several authors have mentioned that *F. poae* is becoming more frequent with FHB complex (Pasquini *et al.,* 2006; Infantino *et al*., 2005; Xu *et al.,* 2005). This increase of *F. poae* was in the years where *F. graminearum* was less frequent (Parry *et al*., 1995; Pancaldi *et al*., 2010). The widespread presence of FHB agents in Syria is a worry because legal limits for mycotoxin content in food commodities are not established yet, whereas in Italy and other European countries, these limits already exist (EU regulations No. 856/2005) (Prodi *et al*., 2009). Genotype characterization could be a useful tool to map a population and identify population changes in the field (Karugia *et al*., 2009; Pasquali *et al*., 2010) as well as to predict the contamination with different trichothecenes, especially in regions lacking of such studies, as Syria. Interestingly, the majority of Syrian *F. equiseti* strains (7 out of 9 tested) possessed a gene encoding trichodiene synthase, which is necessary for

trichothecene production, in contrast to Canadian isolates, which rarely produce trichothecenes (Demeke *et al*., 2005). There are not too many reports on *F.equiseti* chemotypes, since its presence is still sporadic (Bottalico and Perrone, 2002; Pancaldi *et al*., 2010).

PCR assay indicated that 6 out of 11 *F. culmorum* isolates belonged to 3Ac-DON chemotype and 5 were potential NIV producers. These are the first data on *F. culmorum* chemotypes in Syria. The Syrian situation is similar to the Italian (Prodi *et al*., 2011b) and the European, in particular, in England, 3Ac-DON is more frequent than NIV chemotype (Jennings *et al*., 2004) and in Luxemburg, these two chemotypes are present for 53.2% and 46.8%, respectively (Pasquali *et al.,* 2010). Almost all *F. graminearum* isolates belonged to NIV chemotype result comparable with what reported in another area of Middle–East, Iran, by Haratian *et al*., (2008) who affirmed that the majority (46/57) of *F. graminearum* isolated by cereals grown in Iran were NIV chemotype. Furthermore, the result is in accordance with what reported by Lee *et al*. (2009) for both Southern and Eastern Korea. In the Netherlands (Waalwijk *et al*., 2003), England and Wales (Jennings *et al*., 2004), Italy (Prodi *et al.,* 2009), and the USA (Gale *et al.*, 2007) it has been shown the dominance of 15Ac-DON chemotype,.

The trend toward higher irrigation rates after the drought waves which hit Syria recently might have increased the risk of FHB. Temperatures higher than 18C° accompanied by high humidity and the increasing role of maize in crop rotation are suitable factors for FHB incidence (Dill-Macky and Jones, 2000; Parry *et al*. 1995).

Syrian agricultural policy should be aware of the presence of the different FHB causal agents and their ability in producing mycotoxins, then it is necessary to control their presence in food and feed and to develop a national legislation. This is the first report on the chemotypes and genotypes in *Fusarium* complex isolated from Syrian wheat kernels. The present study gives a contribute on the occurrence of fungal population as well as *Fusarium* species on wheat kernels in the post-harvest stages.

Regarding the chemotypes of the Italian *Fusarium* strains, the study pointed out that most of *F. graminearum* strains belonged to 15Ac-DON chemotype, data comparable with those reported by Prodi *et al*. (2009) who found that this chemotype was predominant (87.2 %) over 3Ac-DON and NIV. Our results are also in agreement with Gale *et al*. (2007) in USA, Jennings *et al*. (2004) in England as well as with Yli-Mattila *et al*. (2008) in southern Russia, 3Ac-DON chemotype was predominated in western Russia and Finland (Yli-Mattila *et al*., 2008).

All the italian isolates of *F. culmorum*, belonged to 3Ac-DON chemotype: the same result was also reported by Yoruk and Albayrak (2012).

The chemotype composition of the different *Fusarium* species is different in the two countries, Syria and Italy.

FIGURES AND TABLES

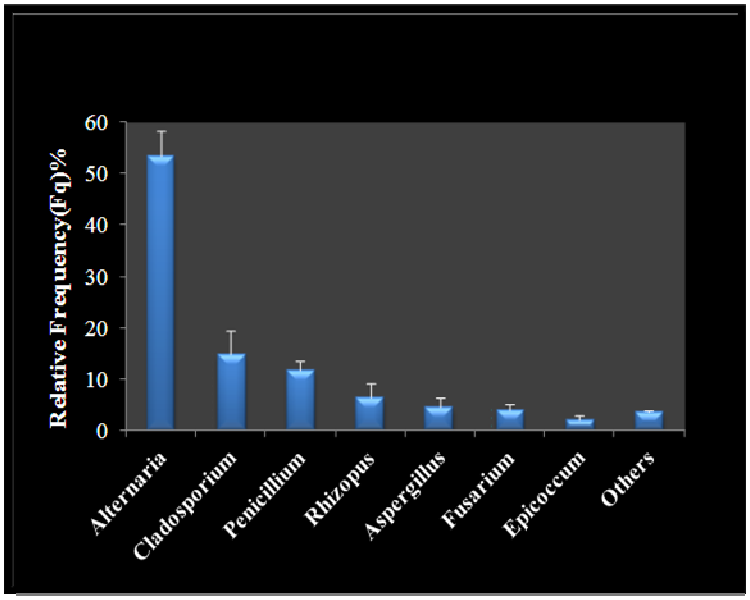
Figure 1.1. a) Agro- climatic zones in Syria based on rainfall (in mm) (FAO, 2003) and wheat samples collected provinces (▲) b) Italian samples collection provinces

a)

b)

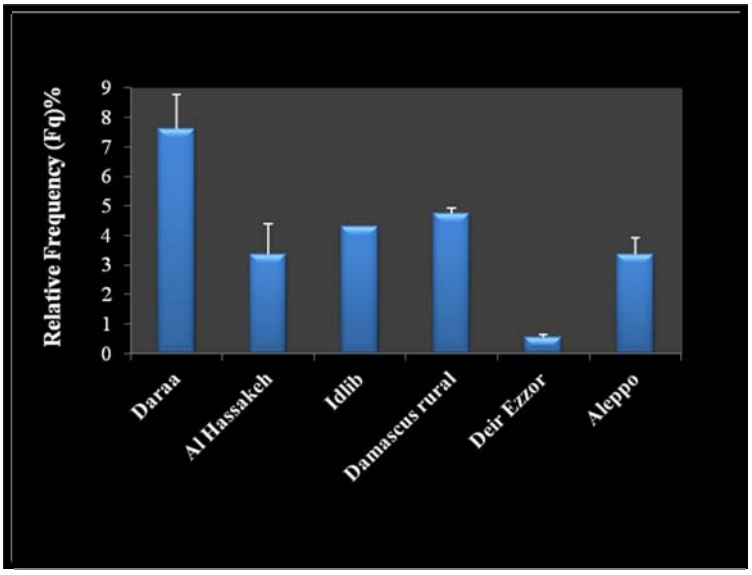
Figure 1.2. Average frequency of the major genera of fungi contaminating wheat kernels in Syria

**Others = species of Absidia, Chaetomium, Cylindrocarpon, Helminthosporium, Nigrospora, Phoma, Sclerotina, Septoria, Stemphylium.*



Error bars represent the standard error.

Figure 1.3. Frequency (%) of Fusarium isolates from wheat kernels samples from different provinces in Syria



Error bars represent the standard error.

Figure 1.4. Colonies of different mycotoxigenic Fusarium species on Potato Dextros Agar (PDA)

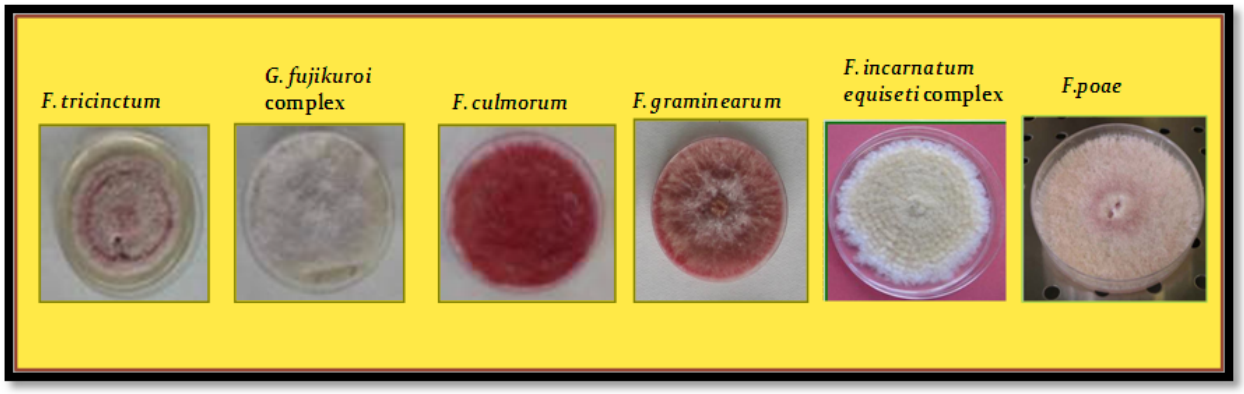


Figure 1.5. PCR assay: identification of Fusarium species by specific-species primers

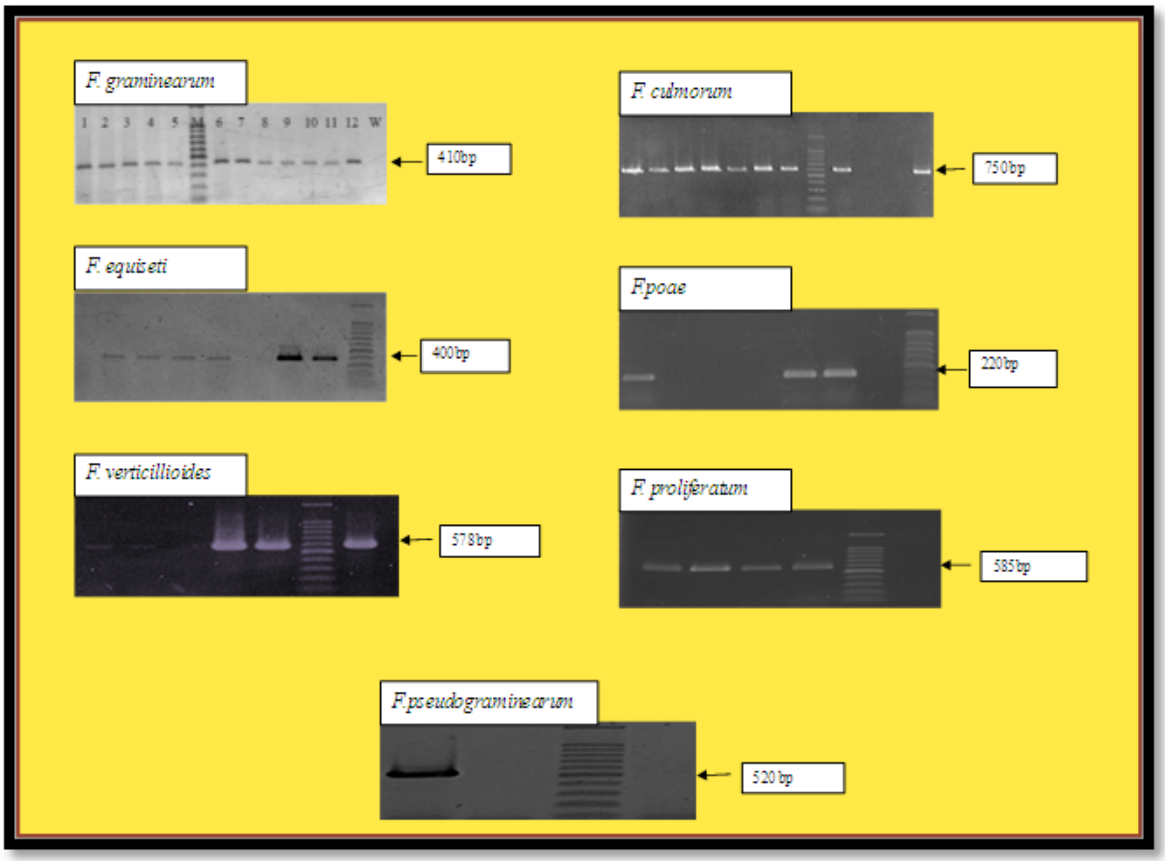


Figure 1.6. Results of chemotyping assay for F. graminearum, F. culmorum and F. equiseti

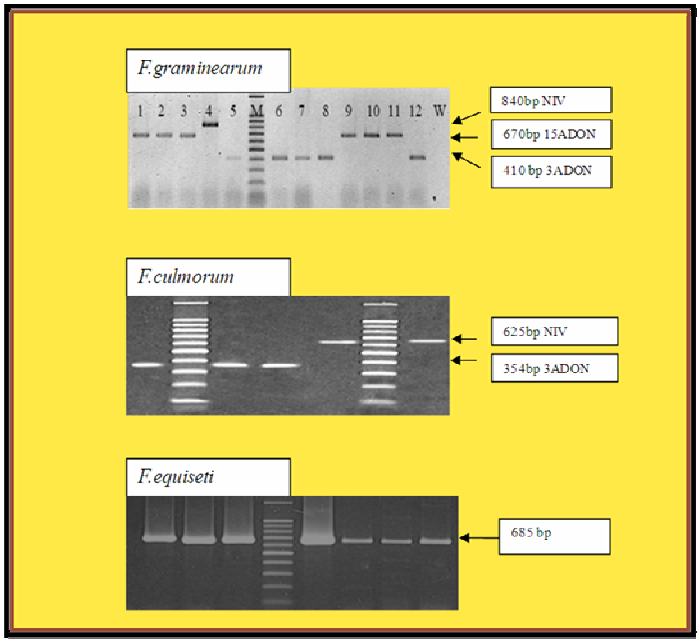


Figure 1.7. Percentage of Syrian and Italian F. graminearum and F. culmorum chemotypes

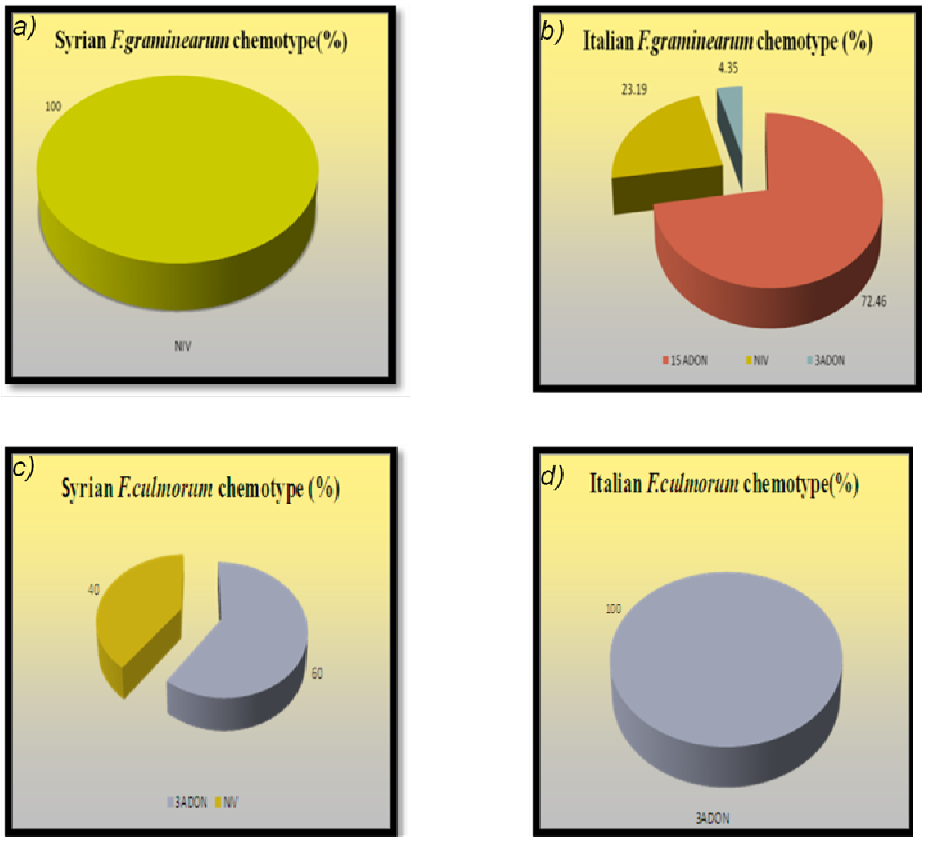


Table 1.1. Occurrence of *Fusarium* species in wheat grains from different provinces in Syria

Provinces	Number of samples	Number of *Fusarium* – infected samples	*Fusarium* species found
Daraa	6	5	*F. tricinctum*, *F. equiseti*, *F. culmorum*
ALHassakeh	7	6	*F. tricinctum*, *F. oxysporum*, *F. culmorum*, *F. semitectum, F. verticillioides, F. proliferatum*
Aleppo	4	4	*F. tricinctum*, *F. verticillioides, F. proliferatum*
Idlib	1	1	*F. tricinctum*, *F. verticillioides, F. proliferatum*
Deir Ezzor	11	7	*F. culmorum*, *F. tricinctum*, *F. graminearum*, *F. pseudograminearum*, *F. verticillioides, F. proliferatum*
Damascus rural	19	7	*F. tricinctum*, *F. culmorum*, *F. equiseti*, *F. graminearum, F. verticillioides, F. proliferatum*
Total (%)	100%	62.5%	

Table 1.2. Occurrence of *Fusarium* species in wheat grain from different provinces in Italy

Provinces	Number of samples	Number of *Fusarium* – infected samples	*Fusarium* species found
Emilia Romagna	12	12	*F. graminearum, F. poae*
Toscana	5	3	*F. culmorum*
Marche	12	9	*F. graminearum, F. poae, F. culmorum*
Umbria	7	4	*F. graminearum, F. poae, F. culmorum*
Lazio	3	1	*F. graminearum*
Sicilia	4	1	*F. graminearum, F. poae*
Basilicata	3	1	*F. culmorum*
Total (%)	100%	67.4%	

CHAPTER II

Screening and quantification of mycotoxin produced by Syrian *Fusarium* species

ABSTRACT

In Syria, wheat is considered as the main strategic crop with annual production ranged between 4 and 5 million tonnes during the last years and with consumption that reaches 12.5 Kg per capita per month. Infection with *Fusarium* spp. causes mycotoxin accumulation in the seeds; both major and minor mycotoxins. HPLC tandem mass spectrometry (HPLC-MS/MS) was used for screening mycotoxin's production from sixty different Syrian *Fusarium* strains, previously isolated and identified, cultured on wheat. The results revealed that zearalenone was detected in all the analysed samples of *F. culmorum* (11), *F. graminearum* (9) and *F. pseudograminearum* (2). Deoxynivalenol (DON) was not predominantly present, fumonisin B_1, B_2, B_3 (FB_1, FB_2, FB3) were present exclusively in all the analysed strains of *F. proliferatum* and *F. verticilliodes,* five and eight respectively. One strain of *F. tricinctum* was capable to produce several types of emerging mycotoxins; beauvericin (BEA), enniatin B, B_1 (ENB, ENB_1), enniatin A, A_1 (ENA, ENA_1). The main *Fusarium* Head Blight (FHB) causal agents were cultured on rice for a quantification of their mycotoxins. All the analysed strains - except one of *F. equiseti* -were ZEN producers with levels ranged from <0.1 to >100 ppm. Six *F. graminearum* strains were nivalenol (NIV) / fusarenon-X (Fus) producers. Six out of eleven strains of *F. culmorum* which belonged to 3Ac-DON chemotype produced DON and its derivatives i.e. 3Ac-DON and 15Ac-DON while the rest which had NIV chemotype produced both NIV/FUS and DON. *F. equiseti* strains have produced mainly ZEN while produced the other mycotoxins sporadically. *F. pseudograminearum* strain (F.1030) produced high amount of DON while the strain (F.1029) produced the highst level of ZEN.

It is worthy to say that this study – according to our knowledge - is the first which carried out for screening and quantification of mycotoxins produced by Syrian *Fusarium* species.

INTRODUCTION

Wheat (*Triticum* spp.) is the first food crop in the part of the world where bread is the staple food for more than three-quarters of its inhabitants (Dib and Soussi, 2004). Wheat and barley constitute almost two-thirds of the whole world production of small grain cereals and almost 80%

of the European small-grain production (Bottalico and Perrone, 2002). In Syria, wheat is considered the main strategic crop with an annual production varies from 4 to 5 million tons in recent years (NAPC, 2009) and in 2010 attained only 3.6 million tons (FAO, 2011). Sadiddin and Atiya (2009) reported that wheat production in Syria is divided roughly between 60% durum and 40% soft wheat. Wheat and its two main products, bread and bulgur, are basic foods in Syria (Haydar *et al*., 1990) with an average monthly consumption of bread per capita of 12.9 kg (FAO, 2003). Apart from its importance to human, wheat is equally important for livestock, since cereals and related products constitute a major source of energy and protein.

The colonization of cereal grains by fungi is a significant risk of contamination with mycotoxins, secondary metabolites of these fungi (Placinta *et al.*, 1999). Several *Fusarium* species are globally widespread pathogens on wheat that can cause root rot, seedling blight and *Fusarium* head blight (FHB), resulting in severe reductions in quality as well as crop yield, which may reach 75% (Wilcoxson *et al.*, 1988; Brennan *et al.*, 2005; Ward *et al.*, 2002). In addition to their pathogenicity, several *Fusarium* strains are capable of producing mycotoxins, which can be formed in pre-harvest infected plants or in stored grains (Bottalico, 1998). The major *Fusarium* mycotoxins are deoxynivalenol (DON), acetylated-DON (Ac-DON), nivalenol (NIV), HT-2 and T-2 toxins, fumonisin (FB_1, FB_2, FB_3) and zearalenone (ZEN) (Table 2.1). *Fusarium* species are also responsible for the production of another group of bioactive compounds called emerging or ‘‘minor” mycotoxins which have been recently reviewed and described (Jestoi, 2008; Mahnine *et al.*, 2011; Torp and Langseth 1999; Uhlig *et al*., 2006). There are only few information on the occurrence of these mycotoxins (Meca *et al*., 2010). *Fusarium* mycotoxins are responsible of acute and chronic symptoms such as nausea, internal organ damages, cancer and infertility (Rocha *et al.*, 2005; Minervini *et al*., 2004; Nielsen *et al.*, 2009).

Mycotoxins are considered as unavoidable; more than 25% of the world grain production is contaminated by mycotoxins. In particular, *Fusarium* mycotoxins (so called field mycotoxins) contaminate up to 100% of the grains (Surai *et al.*, 2008). For the importance of this topic, screening for the presence of *Fusarium* mycotoxins should be continuously carried out to avoid their hazardous risk. High-performance liquid chromatography combined with tandem mass spectrometry HPLC-MS/MS has become the most emerging analytical tool for multi determination, identification and characterization of mycotoxins and their metabolites depending

Table 2.1. Different *Fusarium* spp with their related mycotoxins (Logrieco *et al*., 2002a)

***Fusarium* species**	**mycotoxins**
F. heterosporum	ZEN, ZOH
F. acuminatum	T2, MON, HT2, DAS, MAS, NEO, BEA
F. anthophilum	BEA
F. avenaceum	MON, BEA
F. cerealis	NIV, FUS, ZEN, ZOH
F. chlamydosporum	MON
F. culmorum	DON, ZEN, NIV, FUS, ZOH, Ac-DON
F. equiseti	ZEN, ZOH, MAS, DAS, NIV, DAcNIV, FUS, FUC, BEA
F. graminearum	DON, ZEN, NIV, FUS, Ac-DON, DAcDON, DAcNIV
F. oxysporum	MON, BEA
F. nygamai	BEA, FB1, FB2
F. poae	DAS, NIV, FUS, MAS, T2, HT2, NEO, BEA
F. proliferatum	FB1, BEA, MON, FUP, FB2,
F. sambucinum	DAS, T2, NEO, ZEN, MAS, BEA
F. semitectum	ZEN, BEA
F. sporotrichioides	T2, HT2, NEO, MAS, DAS
F. subglutinans	BEA, MON, FUP
F. tricinctum	MON, BEA
F. verticillioides	FB1, FB2, FB3

Ac-DON – Mono-acetyldeoxynivalenols (3Ac-DON, 15Ac-DON); AcNIV – Monoacetylnivalenol (15-AcNIV); BEA – Beauvericin; DiAcDON – Diacetyldeoxynivalenol (3,15Ac-DON);DAcNIV – Diacetylnivalenol (4,15-AcNIV); DAS – Diacetoxyscirpenol; DON – Deoxynivalenol (Vomitoxin); FB1 – Fumonisin B1; FB2 – Fumonisin B2; FB3 – Fumonisin B3; FUP – Fusaproliferin; FUS –Fusarenone-X (4-Acetyl-NIV); FUC – Fusarochromanone; HT2 – HT-2 toxin; MAS – Monoacetoxyscirpenol; MON – Moniliformin; NEO – Neosolaniol; NIV – Nivalenol; T2 – T-2 toxin; ZEN – Zearalenone; ZOH – zearalenols (α and β isomers).

on their molecular mass, collision-induced dissociation (CID) and fragmentation behavior (Berthiller *et al.*, 2005a) but not on their chemical characteristics (Berthiller *et al.*, 2007). Additional advantages are offered by low detection limits (LOD), that allow to cover a wide range of analytes by their polarities and to give structural information, a requirement of minimal sample treatment. To our knowledge, there are no previous reports on mycotoxins produced by Syrian *Fusarium* isolates. Since wheat represents the major staple food for the people in Syria, it is important to assess the mycotoxin production capacity of *Fusarium* isolates and to determine the types and amounts of mycotoxins produced, to evaluate the risk that might be posed by contaminated food or feed.

The objectives of this study are 1) to screen the mycotoxin production of several *Fusarium* spp. from Syrian wheat kernels previously isolated and identified (Chapter I), 2) to quantify the mycotoxin production of *Fusarium* spp. associated withFHB and to compare their chemotypes with their mycotoxin production in culture.

MATERIALS AND METHODS

Fusarium strains

Fusarium strains examined for their mycotoxins production in this study were isolated during previous investigations of the mycological analysis of Syrian wheat kernels and identified morphologically and molecularly (Chapter I).

60 strains of different *Fusarium* species were used to screen their mycotoxin production while 28 strains of *Fusarium* spp. associated with *Fusarium* head blight were used to quantify their mycotoxin production.

The *Fusarium* species and strains used in the present study are listed in Table 2.2.

Screening of mycotoxin production

This part of the study was carried out in the laboratory of Toxicology, Department of Preventive Medicine, Faculty of Pharmacy, Valencia University (Spain), directed by Prof. Jordi Manes.

Buffered peptone water media were prepared, placed in the tubes (15 ml for each one), autoclaved at 121°C for 20 min then inoculated with a piece of mycelium of *Fusarium* single spore cultures and incubated at 27 °C for 4 days on a rotary shaker operating at 150 rpm. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^6 conidia per ml in each pre-inoculum tube (Kelly *et al*., 2006). Solid medium of wheat was utilized in this study. Briefly, 50 g of solid wheat was weighted in glass fruit jars, and then autoclaved for 20 min at 120 °C. The substrate was inoculated with conidia (concentration 10^6) and maintained at 27 °C for 4 weeks then the cultures were dried at 60 °C for 24 hours and finely ground. Non-inoculated control samples were inoculated with water and treated in the same manner.

50 g of inoculated wheat and the control were prepared using an Oster® food processor (Professional Series Blender model BPST02-B00) by mixing thoroughly. Representative portions of 1 g (wheat flour) were weighed and placed into a glass mortar (50 ml) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 15 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formate by applying a slight vacuum. Consequently, the extract was transferred to a 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hopkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol/water (50/50, v/v) and filtered through a 13 mm/0.22 μm nylon filter purchased from Membrane solutions (Texas, USA) (Figure 2.1).

For the preparation of fortified samples, 1 g of wheat flour "blank" sample (it was corroborated before the analysis that no analytes were present) was spiked with 0.2 ml of working mixture of the mycotoxins at the appropriate concentration. Then, spiked samples were left to stand 3 h at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between the mycotoxins and sample. Ten replicates were prepared for each spiking level. LC–tandem MS analyses were carried out in a system consisting of an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with a Turbo-V™ source (ESI) interface. The QTRAP® analyzer combines a fully functional triple-quadrupole and ion trap mass spectrometer within on the same instrument. An extra confirmation tool, Information Dependent Acquisition (IDA), was carried out only for samples that contain the

selected mycotoxins since the inclusion of this IDA experiment provides an unequivocal identification of mycotoxins in the matrix (Rubert *et al.,* 2011).

Separation of analytes (Figure 2.2, 2.3) was performed using a Gemini C_{18} (Phenomenex, 150 mm×2 mm, 3µm of particle size) analytical column preceded by a guard column with the same packing material. The flow rate was set to 0.250 ml min^{-1} and the oven temperature was 35C, being eluent A water (mobile phase A) slightly acidified with 0.1% of formic acid with 5mM ammonium formate, and B (mobile phase B) methanol with 5 mM ammonium formate. The elution gradient started with 10% of eluent B, increasing to 70% in 1.5 min and kept as isocratic during 1.5 min. After this step, B was increased to 80% in 5 min. The last step was to increase 100% B in 10 min. During the further 8 min the column was re-equilibrated to the initial conditions. The volume to injection was of 20 µl.

The analyses were performed using Turbo-V™ source in positive mode. The operation conditions for the analysis in positive ionization mode were the followings: Ion spray voltage 5500 V, curtain gas 15 (arbitrary units), GS1 and GS2, 50 and 60 psi, respectively, probe temperature (TEM) 500 ºC. Nitrogen served as nebulizer and collision gas. SRM experiments were carried out to obtain the maximum sensitivity for the detection of target molecules. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) were performed by flow injection analysis for each compound; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively for all analytes. The QTRAP® instrument was operated in SRM mode and with a resolution set to unit resolution for Q1 and Q3. For HPLC–MS/MS analysis, scheduled SRM (sSRM) was used with 60 s of SRM detection window and 1.5 s of target scan time. Analyst® version 1.5.2 software (AB Sciex) was used to control all components of the system and also for data collection and analysis.

Quantification of mycotoxin production

In this part of the study, the samples were prepared, and the mycotoxin levels were quantified in the laboratory of Molecular Phytopathology and Mycotoxin Research, Göttingen University (Germany), directed by Prof. Peter Karlovsky.

Rice media were prepared by autoclaving 50 g of polished rice with 70 ml of distilled water. After, then they were separately inoculated with agar plugs overgrown with each *Fusarium* strain and incubation incubated at 24°C for 4 weeks. Rice cultures were extracted with acetonitrile/water mixture and defatted as previously described (Adejumo *et al*., 2007) except that the residue after removal of extraction solvent was dissolved in 1 ml methanol/water (1:1, v/v). The analytes were separated on a polar-modified reverse-phase HPLC column (Polaris C18-Ether, 100 x 2 mm, 3 µm particle size; Agilent, Darmstadt, Germany) kept at 40°C with a methanol-water gradient elution (10% to 98% in 7 min followed by washing and equilibration steps) at a flow rate of 0.2 ml/min. Mass spectrometry detection in a multiple reaction monitoring mode was performed after electrospray ionization in a negative mode as described by Klotzel *et al*. (2006) except for DON (adduct 355.0 > 295.0 and 355.0 > 265.0), 3Ac-DON (337.0 > 246.8 and 337.0 > 217.0), 15Ac-DON (337.0 > 150.0 and 337.0 > 277.1), and nivalenol (adduct 371.0 > 311.0 and 371.0 > 281.0). Regarding the differentiation between 3Ac-DON and 15Ac-DON, all four mass transitions were detectable for both derivatives but the difference in the relative intensity of the signals allowed chemotype determination. Triple quadrupole 1200L (Varian, Darmstadt, Germany) was used as a detector. Limits of detection were 100 µg/kg for DON, 300 µg/kg for NIV, 20 µg/kg for ZEN, 250 µg/kg for 3Ac-DON und 15Ac-DON, and 200 µg/kg Fus X.

Table 2.2. Number of strains of each *Fusarium* species cultured on buffered peptone water medium

***Fusarium* species**	**Number of the strains**
F. culmorum	11
F. graminearum	9
F. equiseti	9
F. verticilliodes	8
F. proliferatum	5
F. semitectum	2
F. tricinctum	3
F. incarnatum equiseti complex	11
F. pseudograminearum	2

RESULTS

Screening of mycotoxin production

Table 2.3 shows the results of the screening of the main and minor mycotoxins. ZEN was detected in all the strains of *F. culmorum* (11), *F. graminearum* (9) and *F. pseudograminearum* (2) in addition to one strain of *F. semitectum* and *F. equiseti*. DON was not the predominant mycotoxins in the tested species, as the largest percentage was reached by *F. culmorum* strains (4 out of 11). Emerging mycotoxins, i.e. BEA, ENB, ENB_1, ENA, ENA_1 were produced by only one strain of *F. tricinctum*. Fumonisin B_1, B_2, B_3 (FB_1, FB_2, FB_3) were present exclusively in all the analysed strains of *F. proliferatum* and *F. verticilliodes.*

Quantification of the mycotoxins

The production of mycotoxins by the main *Fusarium* species, associated with FHB, was quantified and in Table 2.4, these results were reported.

All eleven *F. culmorum* strains were able to produce DON and its derivatives i.e. 3Ac-DON and 15Ac-DON. The quantification of DON produced by *F. culmorum* strains ranged from 5 to > 100 ppm, while the five strains which belonged to NIV chemotype tended to produce both NIV/FUS X as well as DON. All the tested *F. graminearum* strains (6) were NIV/FUS X producers. The *F. pseudograminearum* strains produced DON, 3Ac-DON and 15Ac-DON. Among nine strains of *F. equiseti*, two produced DON whilst three produced NIV.

Almost all the analysed strains, except one of *F. equiseti*, were ZEN producers with levels ranged from <0.1 to >100 ppm, and the highest production was reached by *F. pseudograminearum* (F1029).

DISCUSSION

Wheat is exposed to the contamination of fungal by-products, mycotoxins, which are considered a serious health problem for human and Syria showed pay great attention to mycotoxins being wheat consumption rate per capita very high. Screening revealed the dominant production of ZEN in all *F. graminearum, F. culmorum* and *F. pseudograminearum* strains. This result is in accordance Bottalico and Perrone , (2002) who reported that *F. graminearum* and *F. culmorum*

are the main producers of ZEN. Also, it is in agreement with Blaney and Dodman, (2002) who reported in their study in Queensland that all *F. pseudograminearum* and most *F. graminearum* strains produced ZEN on culture. Furthermore, the results were similar to Hussein *et al.* (1991) who found that the two tested *F. culmorum* strains were ZEN producers. ZEN contamination is commonly associated with DON (Bottalico and Perrone, 2002) but this co-existence in our results did not often exist, and it might be interpreted as the difference between production *in vitro* and *in vivo*.

Emerging mycotoxins are well known to be produced by different *Fusarium* spp. (Monti *et al*., 2000; Meca *et al.*, 2010). The capability of *F. tricinctum* to produce different types of emerging mycotoxins was reported by Jestoi (2008).

All the strains of *F. verticilliodes* (8) and *F. proliferatum* (5) were capable of producing fumonisins (FB_1, FB_2, FB_3). Our findings are in accordance with other authors (Farnochi *et al*., 2005; Hartl *et al*., 1999; Aoyama and Ishikuro., 2007) that recognize *F. proliferatum* and *F. verticilliodes* (before known as *F. moniliforme*) as the main producers of fumonisins among the other *Fusarium* spp.. The ability of *F.equiseti* to produce DAS was previously reported by Langseth *et al*. (1999).

The quantification of different *Fusarium* spp. associated with FHB revealed correspondence between the chemotypes of *Fusarium* spp. and the production of mycotoxins on rice medium. The production of small amounts of 15Ac-DON is common in strains belonging to 3Ac-DON chemotype due to acetylation of 15-hydroxyl of DON by the product of *Tri3* (Tokai *et al*., 2008). Moreover, recent results showed that trichothecene 15-acetyltransferase (product of gene Tri8) is active in all three chemotypes (Alexander *et al.*, 2011). Similar to our results, ten *F. culmorum* strains belonging to 3Ac-DON chemotype from France produced large amounts of DON (Bakan *et al.*, 2002). Co-production of DON and NIV is rare, and it has been reported only for a few European isolates of *F. culmorum* (Nielsen and Thrane, 2001). The ability of the NIV strains to produce DON or its derivatives besides NIV/FUS-X was also noticed in the study performed by Hestbjerg *et al*. (2002). In our work, all *F. culmorum* and *F. graminearum* strains belonging to NIV chemotype also produced Fus X, which is not surprising because Fus X is 4-acetyl-NIV and NIV producers contain active trichothecene-4-acetylase, product of gene Tri7 (Lee *et al*., 2002). In a previous work of Bakan *et al*. (2001) found only 12 to 35 strains of *F. culmorum* coproducers of these mycotoxins.

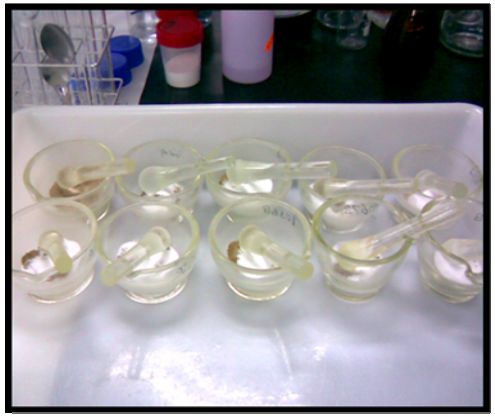
ZEN in detectable levels was found in almost all the tested strains from different species; *F. culmorum, F. graminearum, F. pseudograminearum* and *F. equiseti* (except one strain). Furthermore, four of seven *F. equiseti* strains possessed trichothecene gene produced tricothecene B. NIV production by *F. equiseti* was mentioned by several authores (Langseth *et al*., 1999 ; Nicholson *et al*., 2003), while the ability of some strains to produce DON and its derivatives is interesting, and need further investigation. There are possible explanations for the lack of detection of trichothecene production: (i) production in amounts below the detection level for the applied method (ii) mutations in the biosynthetic pathway, and (iii) down regulation of the biosynthetic pathway by unknown mechanisms (Hestbjerg *et al*., 2002).

Analysis of two strains of *F. pseudograminearum* showed an ability to produce DON and its acetylated more than NIV which is in accordance with O'Donnell *et al*. (2000) and Monds *et al*. (2005), who found that *F. pseudograminearum* isolates produce the mycotoxin 3Ac-DON rather than NIV. The diversity of mycotoxins produced gives us a picture about the capability of the tested strains to produce different mycotoxins.

This study, is the first study conducted on mycotoxin production by Syrian *Fusarium* species, and confirmed previous chemotyping of different *Fusarium* strains isolated from Syrian wheat kernels. The preliminary map, which can be drawn on the occurrence and prediction of the types of *Fusarium* mycotoxins, could be important for future work in Syria, which needs further investigation.

FIGURES AND TABLES

Figure 2.1. Different stages of mycotoxin extraction and detection by HPLCMS/MS

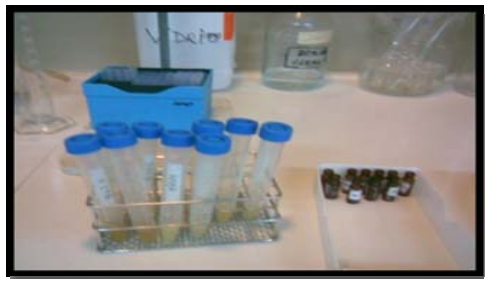



Table 2.3. Occurrence of mycotoxins in 9 *Fusarium* species, isolated from wheat kernels collected from different Syrian areas, and cultured on wheat media

***Fusarium* species**	**N.o**	**Number of the strains producing mycotoxins**										
		DON	**FB_1**	**FB_2**	**FB_3**	**DAS**	**ZEN**	**BEA**	**ENA**	**ENA_1**	**ENB_1**	**ENB**
F. culmorum	11	4	-	-	-	-	11	-	-	-	-	-
F. graminearum	9		-	-	-	-	9	-	-	-	-	-
F. pseudograminearum	2	2	-	-	-	-	2	-	-	-	-	-
F. semitectum	2	-	-	-	-	-	1	-	-	-	-	-
F. tricinctum	3	-	-	-	-	-	-	1	1	1	1	1
F. incarnatum equiseti complex	11	1	-	-	-	-	-	-	-	-	-	-
F. proliferatum	5	-	5	5	5	-	-	-	-	-	-	-
F. verticilliodes	8		8	8	8	-	-	-	-	-	-	-
F. equiseti	9	2	-	-	-	3	1	-	-	-	-	-

Table 2.4. Strains, chemotypes of different *Fusarium* species and quantification (mg/kg) of the mycotoxins producted in rice media

***Fusarium* species**	**Sample Strain**	**Chemotype**	**mycotoxins**					
			DON	**NIV**	**ZEN**	**Fus X**	**3Ac-DON**	**15Ac-DON**
F. culmorum	960	3Ac-DON	>100	-	17	-	42	6
	961	3Ac-DON	>100	-	8	-	9	2
	962	3Ac-DON	>100	-	18	-	10	2
	963	NIV	5	>100	5	30	-	-
	964	NIV	>100	>100	9	54	10	2
	965	NIV	9	>100	0.1	53	-	-
	966	3Ac-DON	>100	-	2	-	48	6
	967	NIV	10	>100	13	>100	0.4	-
	968	3Ac-DON	>100	-	50	-	54	7
	969	3Ac-DON	>100	-	33	-	37	4
	970	NIV	8	>100	1	52	0.2	-
F. graminearum	1012	NIV	-	2	7	2	-	-
	1014	NIV	-	1	6	1	-	-
	1016	NIV	-	2	4	2	-	-
	1017	NIV	-	3	6	3	-	-
	1018	NIV	-	2	4	2	-	-
	1022	NIV	-	3	3	2	-	-
F. pseudo-graminearum	1029		3	-	>100	-	10	1
	1030		>100	1	1	-	65	8
F. equiseti	982	*Tri5* gene	>100	-	8	-	15	3
	983	-	-	-	< 0.1	-	-	-
	984	*Tri5* gene	-	19	13	-	-	-
	985	*Tri5* gene	-	-	>10	-	-	-
	987	*Tri5* gene	23	4	8	1	25	0.3
	988	-	0.3	-	2	-	-	-
	990	*Tri5* gene	-	-	-	-	-	-
	991	*Tri5* gene	-	1	< 0.1	1	-	-
	992	*Tri5* gene	-	-	< 0.1	-	-	-

Figure 2.2. Chromatograms of spiked wheat flour under the optimum chromatographic conditions

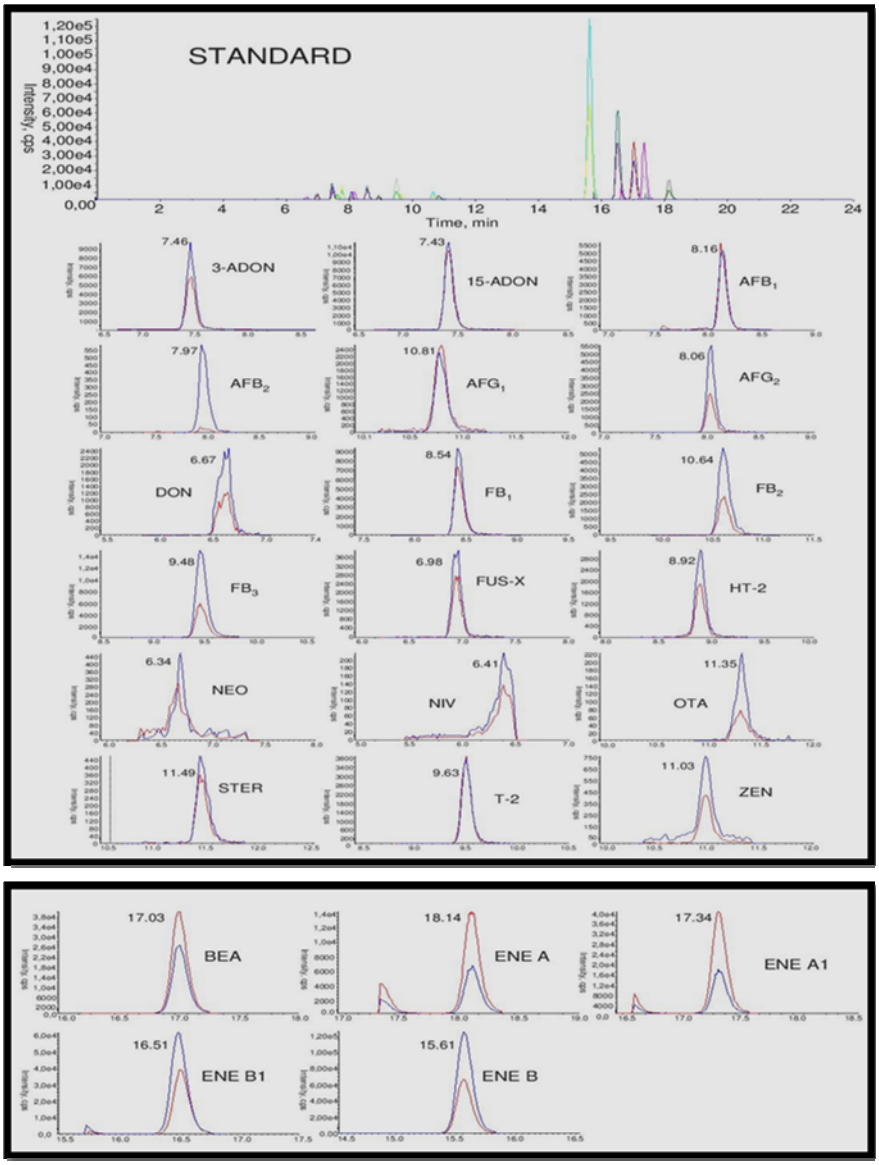
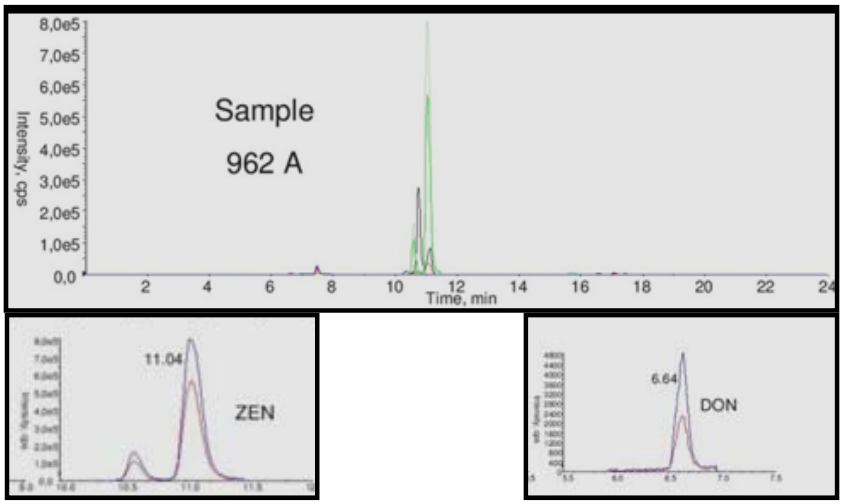
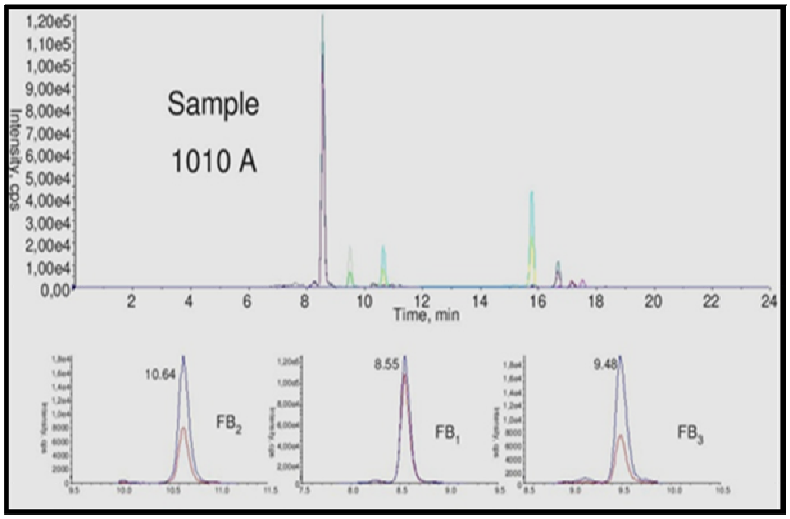


Figure 2.3. Chromatograms of mycotoxins prodyced by a) F. culmorum strain b) F. verticilliodes strain

a)



b)



CHAPTER III

Detection of mycotoxins in Syrian and Italian wheat kernels

ABSTRACT

This article describes the validation of an analytical method for the detection of 23 mycotoxins in wheat flour. The analytical method is based on the simultaneous extraction of selected mycotoxins by matrix solid-phase dispersion (MSPD) followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using a hybrid triple quadrupole-linear ion trap mass spectrometer (QTrap®). Information Dependent Acquisition (IDA), an extra confirmation tool for samples that contain the selected mycotoxins, was used. The results of the analysis of 40 Syrian and 46 Italian wheat flour samples were not identical. Whilst the Syrian samples were contaminated mainly with ochratoxins and aflatoxins, which are produced by storage fungi, these toxins were absent in the Italian samples. Moreover, the Syrian samples were contaminated with deoxynivalenol (DON) but not with its acetylated forms (15Ac-DON and 3Ac-DON), while the Italian samples were contaminated mainly with DON and 15Ac-DON. The emerging mycotoxins were predominant in the Italian samples versus the Syrians.

Among the analyzed samples, only one sample had zearalenone level above the European allowable limits (100 ppb). The climatic differences between Syria and Italy, both from Mediterranean basin, play a key role in the type of mycotoxins detected.

INTRODUCTION

Wheat (*Triticum* spp.) is the first food crop in the world where bread is the staple food for more than three-quarters (Dib and Soussi, 2004). The importance of wheat has been mainly attributed to its ability to be ground into flour and semolina, etc. that form the basic ingredients for bread and other bakery products and pastas (e.g. macaroni and spaghetti) (Chandrika and Shahidi, 2006). Furthermore, wheat and its products, bread and pasta, are basic foods, especially in the Mediterranean diet (Gallo *et al*., 2008). There are three main factors that play an important role in the success of wheat crop: (1) adaptation to a wide range of environments, (2) ease storage of its grains and transportation, and (3) limitless variety of healthy and appealing food to which its grains could be processed (Morris *et al*., 2011). FAO's estimation for global wheat production in 2010 stand to 653 million tonnes (FAO, 2010), while FAO's first forecast for world wheat

production in 2011 stands to 676 million tonnes. In the first rank, the European Union (EU) appears as the main wheat producer with an estimated production of 142 million tonnes in 2011 (FAO, 2011). In Syria, wheat is considered the main strategic crop with an annual production that ranged from 4 and 5 million tonnes, within the last years (NAPC, 2009) and reached 3.6 million tonnes in 2010 (FAO, 2011). Both types of wheat, durum and soft, are cultivated in Syria for roughly 60% and 40% respectively (Sadiddin and Atiya, 2009). Wheat and its two main products, i.e. bread, and bulgur are common food stuffs in Syria (Haydar *et al.,* 1990) with an average consumption of bread per capita per month of 12.9 Kg (FAO, 2003). On the other hand, durum wheat is commonly used in Italy due to its role in manufacturing pasta and semolina with a productivity that reaches 3,194,152 tons and 5,850,000 tons, respectively (www.franceagrimer.fr).

Cereals and their derivatives could be contaminated by fungi, which may occur during harvesting, handling, transportation and storage (Jestoi *et al.,* 2008). Pre and post-harvest infection by fungi, of wheat kernels in addition to quantity losses, can contaminate the grains with secondary metabolites (mycotoxins) that are recognized as health hazard for both human and animals (Mankeviciene *et al*., 2007). Mycotoxin presence depends on several factors, such as fungal strain, climatic and geographical conditions, cultivation technique, susceptibility level of host plants and crop protection, particularly during storage (Pancaldi *et al*., 2010). Rotation intervals between host crops, land preparation, use of fertilizers, irrigation, and weed control have also been listed as influencing factors (Parry *et al*., 1995). Major mycotoxigenic fungi involved in the human food chain belong to the filamentous genera *Fusarium*, *Aspergillus* and *Penicillium* (Jestoi, 2008).

Fusarium species are mycotoxigenic fungi which produce several major mycotoxins, such as deoxynivalenol (DON), acetylated-DON (ac-DON), nivalenol (NIV), HT-2 and T-2 toxins and zearalenone (ZEN). In addition, cyclohesadepsipeptide enniatin (ENN) and beauvericin (BEA) production has also been widely reported (Torp and Langseth, 1999; Uhlig *et al*., 2006). Some fungal species are able to produce multiple mycotoxins. *F. poae* has been reported to produce both type A trichothecenes, such as T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), and neosolaniol (NEO), and type B trichothecenes, nivalenol (NIV) and fusarenon-X (Fus) (Desjardins, 2006). *Aspergillus* spp. are capable to produce a group of mycotoxins called aflatoxins (B_1, B_2, G_1, G_2) (Sangare-Tigori *et al*., 2006). Poisoning with *Fusarium* mycotoxins

causes acute and chronic symptoms such as nausea, internal organ damages, cancer, and infertility (Rocha *et al*., 2005; Minervini *et al*., 2004; Nielsen *et al*. 2009). Liquid chromatography–mass spectrometry (LC–MS/MS) is an useful method for rapid-simultaneous detection and quantification of many mycotoxins and their metabolites (Vendl *et al.,* 2009). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the technique for multi-mycotoxin analysis because of its versatility, specificity and selectivity. Until recently, triple quadrupole (QqQ) LC-MS/MS equipment has been the most widely employed equipment for quantitative mycotoxin analysis (Ren *et al*., 2007; D'Arco *et al*., 2008; Beltran *et al*., 2009). Although the excellent sensitivity, selectivity and efficiency of QqQ, the qualitative information need to support the structural elucidation of the compounds that is still not available (Hernandez *et al*., 2005). This liability could be overcome with the hybrid mass spectrometer QTrap®, which is appropriate for both quantification and confirmation of mycotoxins (Martinez *et al.*, 2007; Gros *et al.*, 2009). In previous research (Rubert *et al*. 2010; Rubert *et al.*, 2011) the matrix solid-phase dispersion MSPD extraction procedures for the legislated mycotoxins have been developed and reported. As a follow-up to these studies, the objective of this work has been the development of a fast, selective and sensitive mycotoxin analytical method based on MSPD extraction followed by LC-MS/MS using a 3200 QTRAP® instrument applied to mycotoxins in wheat flour. To our knowledge, an MSPD method (followed by QTRAP® mass spectrometry) is scarcely used as a routine analytical technique for mycotoxin in the field, and for the analysis of these natural contaminants in wheat flour. Several surveys were conducted on the levels of mycotoxins in wheat all over the world such as USA, Canada, Serbia, Italy, Jordan (Jelinek *et al*., 1989; Roscoe *et al*., 2008; Skrbic *et al*., 2012; Gallo *et al*., 2008; Salem and Ahmad, 2010)
while, to our knowledge, in 1990 only one survey on aflatoxins in different syrian's food products, wheat included, was conducted in the city of Lattakia (Haydar *et al*., 1990). An estimate of the risk can support the decision to make strategic and tactical the control of the disease, for post-harvest management of kernels.
For these purposes, the survey described in this paper was designed to obtain the first information on the incidence and levels of mycotoxins in Syrian and Italian wheat for human and animal consumption.

MATERIALS AND METHODS

Occurrence of mycotoxins in Italian and Syrian wheat flour

The mycotoxin analyses were performed in the laboratory of Toxicology, Department of Preventive Medicine, Faculty of Pharmacy, Valencia University (Spain), directed by Prof. Jordi Manes.

Chemical and reagents

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). The dispersant used for MSPD was octadecyl silica (C_{18}) (50 μm), bonded silica from Analisis Vinicos S.L. (Tomelloso, Spain).

Deionized water (>18 MΩ cm^{-1} resistivity) was purified using the Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade formic acid (purity > 98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

The standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), sterigmatocystin (STER), α-zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FUS-X), neosolaniol (NEO), diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxins, aflatoxin M_1 (AFM_1) and deepoxy-deoxynivalenol (DOM-1) stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmBH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa).

The stock solutions of aflatoxins (AFs) and OTA at a concentration of 500 μg mL^{-1} were prepared in acetonitrile and stock solutions of STER, ZOL, ZEN, NIV, DON, 3-ADON, 15-ADON, FUS-X, NEO, FB_1, FB_2 and BEA were also prepared at a concentration of 500 μg mL^{-1} but in methanol. Stock solutions of FB_3, DAS, T-2 and HT-2 at a concentration of 100 μg mL^{-1} were prepared in acetonitrile. The internal standards (ISs) were AFM_1 (for AFs) at 0.05 μg mL^{-1}

and DOM-1 (for trichothecenes) at 0.150 μg mL^{-1}. Both of these solutions were prepared by dilution of individual stock solutions in methanol.

All solutions were kept in secure conditions at -20ºC.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol:water (50:50) (V/V).

Sampling

A total of 86 wheat grain samples, collected during 2009 and 2010 seasons, were analysed for the presence of mycotoxins. 40 of durum and soft wheat were collected from different areas of Syria including Deir Ezzor (11), Damascus rural (19), Daraa (3) and Al Hassakeh (7). 46 of durum wheat were collected from different Italian areas belonging to Emilia-Romagna (12), Toscana (5), Marche (12), Umbria (7), Lazio (3), Basilicata (3) and Sicilia (4). Figure 3.1 shows the regions in Italy and Syria where the wheat samples were collected.

According to EU guidelines (EU, 2006), three incremental samples of at least 1 kg were collected to obtain an aggregate sample of 3 kg total weight. After homogenization, samples were packed in a plastic bag and kept at -20 °C in a dark and dry place until analysis. Just before analysis, a subsample of 100 g was mixed thoroughly using an Oster® food processor (Professional Series Blender model BPST02-B00) to obtain wheat flour.

Extraction

Portions of 1 g of wheat ground sample were placed into a glass mortar (50 mL) and gently blended with 1 g of C_{18} for 5 min using a pestle to obtain a homogeneous mixture. This mixture was introduced into a glass column and eluted with 15 mL of a mixture of acetonitrile: methanol (50: 50) (v/v) and 1 mM ammonium formate by applying a slight vacuum. The extract was then transferred to a 25 mL conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 mL with a mixture of methanol: water (50: 50) (v/v) and filtered using a 13 mm/0.22 μm nylon filter purchased from Membrane Solutions (Texas, USA) before the injection of the prepared samples into the LC–MS/MS system.

For fortified samples (a sample enriched with a known amount of the analyte to be detected) (EU, 2002), 1 g of "blank" sample (sample in which it was corroborated that no analyte was present)

was spiked with 0.2 mL of a working mixture of the mycotoxins at the appropriate concentration e. Spiked samples were then left to stand for 3 hours at room temperature before the extraction to allow the solvent to evaporate and to establish equilibration between the spiked mycotoxins and wheat flour samples. Ten replicates were prepared at each spiking level.

Liquid chromatography - mass spectrometry analysis

LC-tandem MS analyses were conducted on a system consisting of a Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with a turbo ionspray electrospray ionisation (ESI) interface. The QTRAP® analyser combines a fully functional triple quadrupole and an ion trap mass spectrometer within the same instrument. Separation of analytes was performed using a Gemini-NX (Phenomenex, 150 mm × 4.6 mm, 5 μm of particle size) LC-column preceded by a guard column utilising the same packing material. The flow rate was set to 0.8 mL min^{-1}, and the oven temperature was 40º C, with eluent A water (mobile phase A) slightly acidified with 0.1% formic acid and 5 mM ammonium formate and eluent B (mobile phase B) methanol with 5 mM ammonium formate. The elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. During the subsequent 6 min, the column was cleaned and readjusted to the initial conditions and equilibrated for 7 min. The volume of the injections was 20 μL.

The analyses were performed using the Turbo V® ionspray in positive ionization mode (ESI+). The operating conditions for the analysis were the following: ion spray voltage, 5500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 55 and 65 psi, respectively; probe temperature (TEM), 500º C. Nitrogen served as the nebuliser and collision gas. SRM experiments were performed to obtain the maximum sensitivity for the detection of target molecules. The optimisation of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow injection analysis for each compound; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. The MS was operated in selected reaction monitoring (SRM) mode and with the resolution set to unit resolution for Q1 and Q3. For increased sensitivity and selectivity, MS/MS data acquisition was also performed in the SRM mode. For LC–MS/MS analysis, scheduled SRM (sSRM) was used with a 45 s SRM detection window and 2 s of target scan time. Scheduled SRM is defined

as SRM with the amount of time for detection that surrounds the retention time for each transition. Analyst® version 1.5.1 software (Applied Biosystems/AB Sciex) was used to control all components of the system and also for data collection and analysis.

In addition, to obtain additional confirmation, especially when trace concentration levels were required, IDA experiments were performed with SRM as the survey scan and the EPI mode and MS^3 mode were operated.

Validation of the method

Validation of the method was performed according to a previous study (Rubert *et al*., 2012). Quantification of each compound was performed using two SRM transitions and monitoring the SRM ratio. Enhanced product ion (EPI) scan (as an extra information tool) was used for confirmation of the positive mycotoxin findings. Matrix effect (ME) for each analyte is defined as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A) and was calculated for wheat. The ratio (B/A x 100) is defined as the absolute matrix effect (ME %). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%.

Matrix-matched calibration was used for reliable quantitative determinations. The linearity in the response was calculated using matrix-matched curves prepared by spiking one "blank" wheat sample and analysing it in triplicate at six concentration levels within the analytical range: from the limit of quantification (LOQ) to 100 times this LOQ.

All results were calculated comparing the area obtained for a blank extract spiked before the extraction (fortified samples) to the results obtained from a blank extract spiked after the extraction (matrix-matched sample). This experiment was repeated ten times within a day for an intra-day precision test and additionally performed once each day, for five consecutive days, for the inter-day test.

The recovery experiments were conducted by spiking the blank wheat sample in ten replicates at two concentration levels (LOQ and 10 times LOQ). In this way, intra-day and inter-day parameters of the method were determined at LOQ and 10 times LOQ concentration levels by repeating the analysis of the wheat samples in ten replicates on the same day and for five non-consecutive days.

Limits of detection (LODs) and limits of quantification (LOQs) were defined as the concentration at which the signal-to-noise (S/N) obtained was close to 3 and 10, respectively. These limits were calculated by Analyst version 1.5.1 software (Applied Biosystems/AB Sciex) and both parameters were determined by the analysis of decreasing concentrations of the spiked wheat sample.

Estimation of daily intake

For estimating the toxin uptake by the consumers, a mean concentration of each found mycotoxin was calculated, considering: only positive samples, both positives and negative samples and the high concentration level found. These mean contamination values of analytes respect food consumption data were divided by the body weight (standard body mass of 60 kg) to calculate the dietary daily intake per person.

RESULTS

Validation of the method

In the present work, a previous developed MSPD method (Rubert, *et al*., 2011) was further applied for extraction and determination multi-mycotoxin in different flours using LC–MS/MS. However, in order to validate the developed procedure for this matrix, parameters as recoveries, repeatability and reproducibility, as well as limits of detection (LODs) and limits of quantification (LOQs), were evaluated.

The LODs and LOQs were based on the minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Calculated values for wheat flour to guarantee quantification, matrix effects in wheat flour were deeply studied. For the matrix effects evaluation, six concentration between LOQ and 100 times LOQ levels were analyzed in methanol/ water (50/50, v/v) and in matrix-matched (spiked after blank) samples, and the slopes of the calibration curves were compared by the formula (slope matrix-matched wheat flour /slope standard in solvent × 100). In all cases, the calibration curves showed high linearity.

Matrix matched standards calibration was required to correct the matrix effect problems in order to obtain reliable quantification of these mycotoxins in wheat flour. Matrix-matched calibration does not increase the time of analysis since extraction procedure, and chromatographic analysis are fast methods. Recoveries and repeatability of the developed analytical method, for each analyzed compound, were carried out by injection of the spiked samples at two concentration levels (LOQ concentration level and 100 times LOQ concentration level), ten consecutive times within the day (intraday precision), and for five consecutive days (inter-day precision). For all compounds, the mean recoveries in wheat flour were satisfactory. These results were in accord with the performance criteria of the EU (EU, 2002).

Occurrence of mycotoxins in Italian and Syrian wheat flour

In this study, "positive sample" was considered when the concentration detected was upper than LOQ level. Moreover, the confirmation of these "positive samples" was carried out, according to the European Commission (EU, 2002): the q/Q ratios were evaluated from reference standards in solvent and compared to those experimentally obtained from spiked samples.

With this criterion, a total of 37 Italian samples and 24 Syrian were confirmed as positive samples. The specific mycotoxin in the positive samples was identified by searching in the appropriate retention time window (defined as the retention time ± three standard deviations of the retention time of a blank sample spiked at LOQ for each mycotoxin), and the confirmation was conducted by comparison of the signal intensity ratios of the two transitions (quantification and qualification) to the two transitions obtained using fortified blank samples.

Out of 46 Italian wheat samples, 80% were contaminated with at least one mycotoxin. In fact, 27% of positive samples were contaminated with more than one mycotoxin and 38% of them were contaminated with three or more mycotoxins.

DON was the mycotoxin with high incidence (59%) (Table 3.1) and in 24% of samples this mycotoxin was detected with its precursors 3-ADON and/or 15-ADON. Only in five samples, 3-ADON (2 samples) and 15-ADON (4 samples) were detected without the presence of DON.

Low correlation between DON and other type B trichothecenes such as NIV and FUS-X has been found. For example, NIV was detected in three samples and only in one sample accompanied with DON. FUS-X was found in three samples; two with the presence of DON.

Type A trichothecenes, T-2 and HT-2, were detected in 26% of samples; in 18% of samples were detected simultaneously, and the positive samples for these mycotoxins were detected in Marche region. The second mycotoxin with high incidence (35%) was ZEN, and its metabolite α-ZOL was present in three samples.

In spite of the high frequency of type A and B trichothecenes and ZEN, only in one sample from Marche, the level of ZEN exceeded the MLs extrablished by the EU (Table 3.4).

Co-occurrence of mycotoxins in Syrian wheat grains is presented in Table 3.2. Out of 40 Syrian samples, 24 samples (60%) were contaminated with at least one mycotoxin. Aflatoxins exhibited the highest incidence compared to the other examined mycotoxins; 14 out of positive samples (58%) were contaminated with AFB_2. AFG_2 was present in 2 wheat grain samples, and in one sample was with AFG_1. No sample contaminated with AFB_1 was detected in this study. STER was present only in four samples. The second most abundant mycotoxin was OTA. 12 samples (50%) out 24 samples were contaminated with OTA. In 6 samples, OTA was the only mycotoxin and in the other 6 samples, there was a co-occurrence of OTA and AFs. The incidence and levels of *Fusarium* toxins in Syrian samples were lower then Italian ones. Only in 11 out of 24 positive samples, *Fusarium* toxins were detected; ZEN was detected in 10 samples (42%) and its metabolite ZOL was detected in 3 samples. DON was found in 6 samples (25%) and Fumonisins (FB_1 and FB_2) were found only in 4 samples.

Results about the occurrence and contamination level of emerging mycotoxins are summarized in Table 3.3. The incidence and mean concentrations of emerging mycotoxins found in Italian wheat grain samples were higher than those found in wheat samples from Syria. Moreover, the occurrence of mycotoxins was different; while ENB was the most common mycotoxin in positive Italian samples (49%), BEA was the emerging mycotoxin with high incidence (21%) in positive Syrian samples. According to Italian results, co-occurrence of the 5 mycotoxins was verified only in one sample. In 4 samples, the four enniatins were present simultaneously. ENB and ENB_1 were detected simultaneously in 8 samples and ENB was detected together with ENA in 4 samples. Six samples were contaminated with only one mycotoxin; BEA was detected alone in one sample.

Regarding to Syrian samples, the simultaneous incidence of BEA and ENA and ENA1 was confirmed in 3 samples. ENB was detected in one sample while ENB_1 was absent in all the analyzed samples.

Estimation of daily intake

The results of estimating dietary intake was compared with temporary TDI (tTDI) of the respective mycotoxins, to evaluate the possible health risk associated with the intake (Table 3.5)

DISCUSSION

There are few studies regarding the presence of different groups of mycotoxins simultaneously in wheat grain samples; the present study evaluates for the first time a broad spectrum of mycotoxins in wheat grains from Italy and Syria. In this regard, the level of contamination with various mycotoxins including aflatoxins, fumonisins, type A and B trichothecenes, ZEN, ZOL, OTA, STER and emerging mycotoxins were assed. Moreover, an estimation of consumer risk was calculated.

In the field of mycotoxin analysis, several methods using the hybrid triple quadrupole-linear ion trap mass spectrometer had been described in literature (Berthiller *et al*., 2005b). In the current study, good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied. One of the main aims of this method was to detect as many mycotoxins as possible in a single run, but the problem encountered in the quantitative LC-MS/MS analysis was the existence of the matrix effects. Although sampling plans and performance features, that control requirements for methods in mycotoxin analysis have been regulated (FAO, 2004; EU, 2006), there is still a need for a specific performance criterion to overcome these matrix effects for mycotoxin analysis. Other contaminants such as pesticides or veterinary antibiotics have specific guidelines (Document SANCO, 2000; SANCO, 2003; SANCO, 2009) that recommend matrix-matched calibration as the optimal option to eliminate these interferences and obtain accurate results. In this work focused on mycotoxins, different techniques applied in other fields (pesticide and antibiotic analysis) have been evaluated to meet the established performance requirements in mycotoxin analysis (EU, 2002; EU, 2006). The validation of the method should be conducted in accordance with the performance criteria of the analytical method selected (EU, 2002). External matrix-matched calibration and internal standard calibration were therefore compared to evaluate matrix effects in wheat flour. A great number of approaches to evaluate and compensate for the matrix effects have been tested. However, the only way to ensure high accuracy in the results is

the use of isotopically labelled internal standards. Ideally, each analyte should be corrected by its own isotope-labelled molecule. Achieving this ideal situation is problematic in a multi residue method because of the commercial unavailability of several compounds and the economic restrictions on the acquisition of a large number of the ispotope-labelled compounds. The evaluation of different systems able to compensate for matrix effects is particularly important (Sforza *et al.*, 2006; Rubert *et al*., 2011).

On the light of our results obtained after mycotoxins analysis in the investigated sample, it is important to mention the fact that origin variations were noted on mycotoxins occurrence. Whearas in Italian samples only *Fusarium* mycotoxins, particularly type A and B trichothecenes, ZEN and ZOL were detected, *Aspergillus* and *Penicillium* mycotoxins such as aflatoxins and OTA had high incidence in Syrian samples (Figure 3.2).

The relation between the region and the presence of fungal isolates is well-known and based on various factors such as climatic factors, agronomic practices and competition with other species (Parry *et al.,* 1995; Saremi *et al.*, 1999; Doohan *et al*., 2003; Pancaldi *et al*., 2010). The changes in these factors might strongly alter the mycoflora composition from season to season (Visconti and Pascale, 2010). This relation is important in order to establish control strategies for fungal disease and the content of mycotoxins in food and animal feed.

Almost all the Italian infected samples were contaminated with the type B trichothecenes toxin DON, with the co-occurrence of its acylated form, 15Ac-DON in nearly half of the samples, and few with 3Ac-DON and NIV chemotypes. Our results are in agreement with the survey presented by Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001) which showed that DON was the most abundant trichothecene in Italian cereals. Furthermore, 12 EU member States indicated that DON was the most frequently detected mycotoxin in wheat samples (EU, 2003). Trichothecenes A, T-2 and HT-2, were also simultaneously detected with tricothecenes B, but to a lesser extent. On the other hand, it can be noticed that the infected Syrian samples were mainly contaminated with ochratoxins and aflatoxins, which are produced by storage fungi i.e. *Aspergillus* spp. and *Penicillium* spp. (Reyneri, 2006), isolated previously from the same samples (Chapter I). On contrary to our findings, Haydar *et al*. (1990) estimated aflatoxin contamination in sixty-three samples of nineteen food commodities of Syrian origin - no levels were reported-

and found that AFB_2 was present only in one sample whilst AFG_1 and AFG_2 were not detected in any commodity.

The absence of AFB_1 could be explained by two hypotheses: firstly, it is possible that it was presented at concentration levels lower than LOD, secondly, it is known that AFB_1 is rapidly decomposed to AFB_2; a much less toxic form (Carvajal and Arroyo, 1997) and AFB_2 degrades more slowly. STER was detected in few samples, and this mycotoxin is supposed to be the precursor of aflatoxins (Wilkinson *et al*., 2004). So, its low incidence compared with high incidence of AFs could be explained by the transformation of this toxin to AFs owing to long periods of storage.

These results indicate that more attention should be paid to post-harvest conditions to minimize the content of these toxins (Frenich *et al*., 2009). In addition, the presence of *Fusarium* mycotoxins (conventional and emerging) was also detected indicating pre-harvesting or post–harvesting infection however, this detection was not concomitant with isolation of *Fusarium* spp. in most of the samples (Chapter I). The absence of *Fusarium* spp. could be due either to removal of the external infected coat during the wheat harvest or the application of fungicides during the storage. It should be taken into account the increasing use of irrigation in Syria, due to persistent drought in recent years, influenced *Fusarium* infection, particularly FHB, even if the level of inoculum was low (Chapter I). It is worth to note that all *Fusarium* mycotoxins belonged to the type B trichothecenes, particularly DON, with complete absence of its acetylated forms; 3Ac-DON and 15Ac-DON. As *Fusarium* spp. and *Alternaria* spp. are responsible for emerging mycotoxin production (Meca *et al*, 2010), the low percentage of contaminated samples with these mycotoxins in Syrian samples versus the Italian ones is either due to the low percentage of *Fusarium* infection or the presence of non mycotoxigenic *Alternaria* strains.

Climatic differences between Syria and Italy are significant, although both belong to the Mediterranean basin. Syria has an arid and dry climate, very hot in the Summer and cold in Winter, with an average maximum inland Summer temperatures between 33° and 40°C. Italian climate is mainly temperate, it slightly varies according to the areas, the northern Italian regions have warm humid Summer with occasional rains, the southern part is hot and dry. The climatic conditions and the agricultural practices are crucial for FHB appearance and its severity in field

conditions (Xu and Nicholson, 2009). Referring to the above mentioned factors, this might explain the higher incidence of *Fusarium* mycotoxins in Italian samples versus the Syrian.
The research suggests that the most frequent isolated fungi can produce different amounts of two or more mycotoxins. The extend of the risk of grain co-contaminations with several mycotoxins depends on the level and kind of mycotoxins, human age, health status, feature or organ assessed and mycotoxin interactions, which can be additive, synergistic or antagonistic (Speijers and Speijers, 2004). Therefore, it is difficult, right now, to define safe levels of mycotoxins.

Aflatoxins, and in particular aflatoxin B_1, are considered to be genotoxic and carcinogenic and there is evidence that they can cause liver cancer in humans (Farombi *et al*.,2005). In accordance with expert scientific panels, it is not possible to identify an intake without risk (Yu *et al*., 1997). Therefore, the limits set for certain foodstuffs for direct human consumption are those considered to be as low as reasonably achievable (ALARA). In general, concentrations of mycotoxins analysed in our survey were below the tolerable levels established by EU. ZEN concentration was higher than the EU allowable limits in only one Italian wheat sample.

In recent years the consumption of wheat around the world has increased as well in grain as its derivates. Because of the simultaneous occurrence of different mycotoxins in the analyzed samples, there should be a continuous monitoring of wheat. More studies, attempting to understand the dynamics involved in mycotoxin production in grains, need to be carried out with the aim of reducing the presence of these mycotoxins.

FIGURES AND TABLES

Table 3.1. Summary of mycotoxin frequencies and levels found in Italian wheat samples according to the regions (n=3) (ng ml^{-1}). [a] Mean value of positive samples, [b] Mean value of total samples

Regions		DON	3Ac-DON	15Ac-DON	NIV	T-2	HT-2	FUS-X	ZEN	ZOL
EMILIA ROMAGNA (n=12)	Frequency	6/12	0/12	2/12	0/12	1/12	0/12	1/12	3/12	1/12
	Mean Value[a]	68.1	-	34.5	-	9	-	6	38	8
	Mean Value[b]	34	-	5.75	-	0.75	-	0.5	9.5	0.67
	Range	19-180	-	13-56	-	9	-	6	11-62	8
TOSCANA (n=5)	Frequency	5/5	0/5	2/5	0/5	0/5	0/5	0/5	2/5	0/5
	Mean Value[a]	82.2	-	12.5	-	-	-	-	18	-
	Mean Value[b]	82.2	-	5	-	-	-	-	7.2	-
	Range	53-120	-	12-13	-	-	-	-	17-19	-
MARCHE (n=12)	Frequency	5/12	3/12	6/12	3/12	7/12	7/12	1/12	5/12	1/12
	Mean Value[a]	248	25	41.8	183	12	15	14	88	5
	Mean Value[b]	103.3	6.25	21	46	7	9	1.1	37	0.4
	Range	62-551	11-33	14-101	67-290	2-51	5-32	14	8-231	5
UMBRIA (n=7)	Frequency	5/7	1/7	4/7	0/7	1/7	2/7	1/7	4/7	1/7
	Mean Value[a]	41	4	45	-	11	8.5	5	15	4
	Mean Value[b]	29	0.6	26	-	2	2	0.7	8	0.6
	Range	14-93	4	19-94	-	0-11	2-15	5	7-26	4
LAZIO (n=3)	Frequency	3/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3
	Mean Value[a]	818	-	-	-	-	-	-	46	-
	Mean Value[b]	818	-	-	-	-	-	-	15	-
	Range	13-1230	-	-	-	-	-	-	46	-
SICILIA (n=4)	Frequency	3/4	0/4	1/4	0/4	1/4	1/4	0/4	1/4	0/4
	Mean Value[a]	31	-	105	-	2	5	-	7	-
	Mean Value[b]	23	-	26	-	0.5	1.2	-	2	-
	Range	24-37	-	105	-	2	5	-	7	-
TOTAL (n=46)	Frequency	27/46	4/46	15/46	3/46	10/46	10/46	3/46	16/46	3/46
	Mean Value[a]	178	20	42	183	11	13	8	44	6
	Mean Value[b]	105	2	14	12	2.3	2.8	0.5	15	0.4
	Range	13-1230	4-33	12-105	67-290	2-51	2-32	5-14	7-231	4-8

Table 3.2. Summary of mycotoxin frequencies and levels found in Syrian wheat samples according to the regions (n=3) (ng ml^{-1}).

Regions		DON	AFB_2	AFG_1	AFG_2	STER	OTA	FB_1	FB_2	ZEN	ZOL
DEIR EZZOR (n=11)	Frequency	2/11	3/11	0/11	1/11	3/11	1/11	2/11	2/11	3/11	3/11
	Mean Value[a]	19	0.5	-	0.6	1.5	0.51	5	12	9	3
	Mean Value[b]	3.5	0.1	-	0.05	0.4	0.05	0.9	2	2.3	0.9
	Range	15-23	0.5-0.6	-	0.6	1.4-1.6	0.51	5	12	7-10	3-4
DAMASCUS RURAL (n=19)	Frequency	2/19	5/19	1/19	1/19	0/19	6/19	0/19	0/19	0/19	0/19
	Mean Value[a]	13.5	0.6	0.6	0.6	-	0.6	-	-	-	-
	Mean Value[b]	1.42	0.2	0.03	0.03	-	0.2	-	-	-	-
	Range	9-18	0.5-0.9	0.6	0.6	-	0.5-0.7	-	-	-	-
DARAA (n=3)	Frequency	0/3	1/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3
	Mean Value[a]	-	0.8	-	-	-	0.52	-	-	-	-
	Mean Value[b]	-	0.3	-	-	-	0.2	-	-	-	-
	Range	-	0.8	-	-	-	0.52	-	-	-	-
AL HASSAKEH (n=7)	Frequency	2/7	5/7	0/7	0/7	1/7	4/7	2/7	2/7	3/7	0/7
	Mean Value[a]	15.5	0.6	-	-	1.2	0.5	5.5	12	7	-
	Mean Value[b]	4	0.4	-	-	0.2	0.3	1.6	3	3	-
	Range	15-16	0.6-0.7	-	-	1.2	0.4-0.7	5-6	12	4-9	-
TOTAL (n=40)	Frequency	6/40	14/40	1/40	2/40	4/40	12/40	4/40	4/40	6/40	3/40
	Mean Value[a]	16	0.6	0.6	0.6	1.4	0.6	5	12	6	3
	Mean Value[b]	2.4	0.2	0.02	0.03	0.1	0.2	0.5	1.2	1	0.3
	Range	9-23	0.5-0.9	0.6	0.6	1.2-1.6	0.4-0.7	5-6	12	4-10	3-4

Table 3.3. Summary of "minor" mycotoxins levels found in Italian and Syrian wheat grain samples (n=3) (ng ml^{-1})

Origin	Parameter	BEA	ENA	ENA_1	ENB	ENB_1
	Frequency	6/46	6/46	11/46	18/46	11/46
ITALY	Mean Value[a]	2.7	8.5	14.7	27.4	28.8
(n=46)	Mean Value[b]	0.4	1.1	3.5	11	7
	Range	1.8-5.1	3.1-18.1	4.5-40.4	3.1-87.2	1.5-69.8
	Frequency	5/40	4/40	4/40	1/40	0/40
SYRIA	Mean Value[a]	1.60	1.7	1	0.9	-
(n=40)	Mean Value[b]	0.2	0.2	0.1	0.02	-
	Range	1.5-1.7	1.5-2.2	0.6-2.1	0.9	-

Table 3.4. Supplementary. Maximum levels for certain mycotoxins in cereal foodstuffs established by European Commission (EU, 2006)

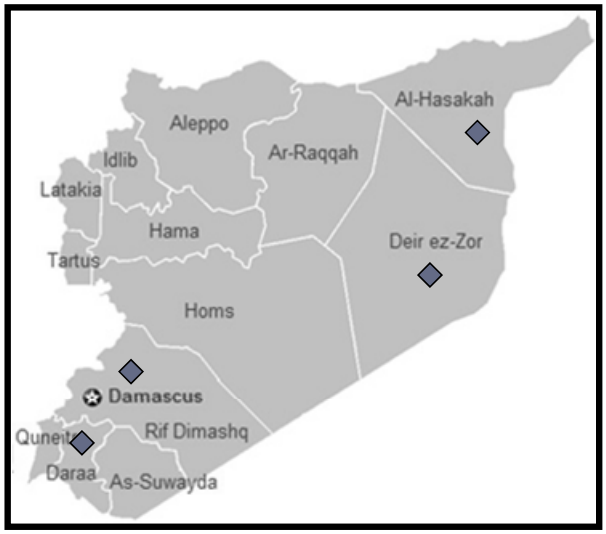
Mycotoxin	Level (µg kg^{-1})	Food stuff
AFB_1	2	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.7, 2.1.10 and 2.1.12
AFB_2		
AFG_1	4	
AFG_2		
OTA	5	Unprocessed cereals
DON	1750	Unprocessed durum wheat and oats
ZEN	100	Unprocessed cereals other than maize

Table 3.5. Estimation of daily intake, TDI- Tolerable Daily Intake, [1]-Mean concentration level of positive samples, [2]-Mean concentration level of total samples, [3]-High found concentration

	TDI (μg/kg bw/day)	Italian Samples Daily wheat Intake 0.4 kg/day			Syrian Samples Daily wheat Intake 0.42 kg/day		
		EDI[1]	EDI[2]	High EDI[3]	EDI[1]	EDI[2]	High EDI[3]
DON	1	1.2	0.7	8	0.1	0.01	0.2
3Ac-DON		0.1	0.01	0.2			
15Ac-DON		0.3	0.1	0.7			
NIV	0.7	1.2	0.1	1.9			
FUS-X		0.1	0.01	0.3			
T-2	0.06	0.1	0.01	0.2			
HT-2		0.05	3×10^{-3}	0.1			
ZEN	0.2	0.3	0.1	1.5	0.04	7×10^{-3}	0.07
ZOL		0.04	2×10^{-3}	0.05	0.02	2×10^{-3}	0.03
AFB_1		-	-	-			
AFB_2		-	-	-	4×10^{-3}	1×10^{-3}	6×10^{-3}
AFG_1		-	-	-	4×10^{-3}	1×10^{-4}	4×10^{-3}
AFG_2		-	-	-	4×10^{-3}	2×10^{-4}	4×10^{-3}
OTA	0.02	-	-	-	4×10^{-3}	1×10^{-3}	5×10^{-3}
STER		-	-	-	0.01	7×10^{-4}	0.01
FB_1	2	-	-	-	0.03	3×10^{-4}	0.04
FB_2		-	-	-	0.08	8×10^{-3}	0.08
BEA		0.02	3×10^{-3}	0.03	$9x10^{-3}$	$1x10^{-3}$	0.01
ENA		0.06	7×10^{-3}	0.1	0.01	$1x10^{-3}$	0.01
ENA_1		0.1	0.02	0.2	$6x10^{-3}$	$6x10^{-4}$	0.01
ENB		0.2	0.07	0.6	$5x10^{-3}$	$1x10^{-4}$	$5x10^{-3}$
ENB_1		0.2	0.05	0.5	-	-	-

Figure 3.1. Locations (◆) in Syria (a) and Italy (b) where wheat samples were collected for mycotoxins analysis.

a)



b)

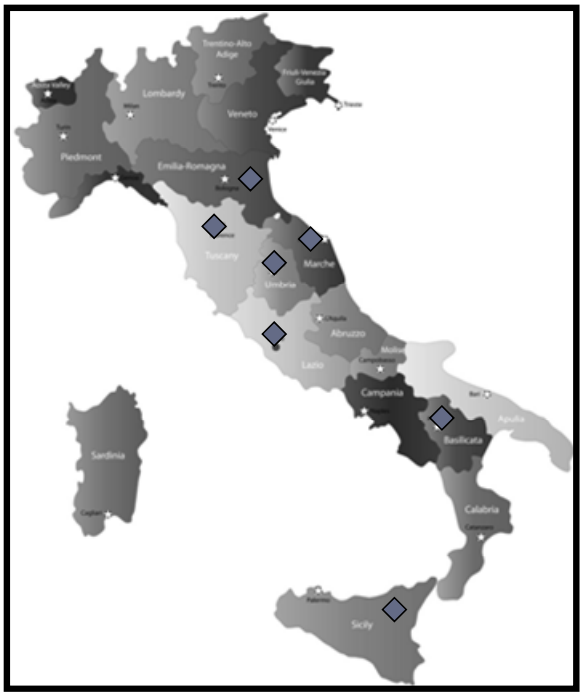
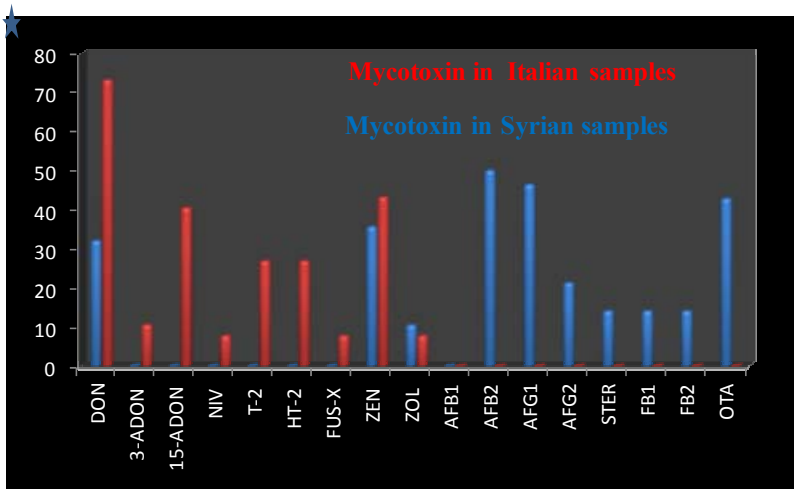


Figure 3.2. Percentage of kernel samples contaminated with mycotoxins



CHAPTER IV

Comparision of the aggressiveness between Syrian and Italian *Fusarium culmorum* strains

ABSTRACT

In this work eight Syrian and three Italian strains of *Fusarium culmorum*, previously characterized in chemotypes (3Ac-DON and NIV) (Chapter I), were studied for aggressiveness and Deoxynivalenol production. The aggressiveness was compared using different assays, ear inoculation in the field, floret inoculation in growth chamber and a new Petri-dish assay recently set up for *F. graminearum*. DON levels in 3Ac-DON chemotype strains were carried out by ELISA technique.

All *F. culmorum* strains were pathogenic, and a wide range of aggressiveness was observed. The aggressiveness levels of the strains were further investigated for the relationship among the following parameters: Fusarium damage kernel (FDK), kernel weight (KW) reduction and the chemotypes of the isolates.

The relationships among the aggressiveness levels found in different assays, and their relation to FDK and KW reduction have been confirmed by the results in this study. The highest correlation coefficient was found between the data of the seedling test in the Petri-dish (AUDPCstandard) and the data of the floret inoculation in the growth chamber (mean and terminal disease severity; r = 0.891 and 0.932, respectively) and ear inoculation in the field (r = 0.829). There was no significant difference in aggressiveness among the two different chemotypes as determined by three aggressiveness parameters from ear and floret inoculation assays. Moreover, there are also no significant differences in FDK and KW reduction between the two chemotypes. Different *F. culmorum* chemotypes showed no difference in aggressiveness and caused the similar damage level to wheat kernels. Highly significant correlations of AUDPCstandard from the Petri-dish test with both floret and ear inoculations revealed the potential of using Petri-dish test to screen for highly aggressive *F. culmorum* isolates for breeding purposes.

INTRODUCTION

Fusarium culmorum (W. G. Sm.) Sacc. is one of the most common pathogen for wheat (*Triticum* L.) causing Fusarium head blight (FHB), a worldwide devastating disease (Parry *et al*., 1995; McMullen *et al.*, 1997; Gilbert and Tekauz, 2000; Miedaner *et al.*, 2008).

This disease leads to high yield and grain quality losses, as well as accumulation of mycotoxins that is a hazardous health risk for animals and poses a safety concern in human food (Windels, 2000; Cumagun and Miedaner, 2004; Placinta *et al.*, 1999).

The presence of *Fusarium* species on wheat grain greatly affects the quality of harvested wheat leading to reduce seed germination and vigour, as well as baking qualities of flour by destroying starch granules, storage proteins and cell walls (Jackowiak *et al.*, 2005). The losses due to FHB in wheat in Europe often estimated from 10 to 30 % (Bottalico and Perrone, 2002; Logrieco *et al.,* 2002a), while the wheat yield losses in different regions in the USA ranged from 20-50% in 1993 with subsequent mycotoxin contamination (Windels, 2000).

Some *Fusarium* species are producers of trichothecene mycotoxins, of which the main are Deoxynivalenol (DON) and Nivalenol (NIV) (Cumagun and Miedaner, 2004) involved in the inhibition of host resistance reactions and in the increase of pathogen colonization (Jansen *et al.,* 2005; Maier *et al.*, 2006). The effect of mycotoxins on the quality of cereal grains has led to a great interest in FHB.

FHB epidemics in nature could strike suddenly, but its appearance could be inconsistent within the years, since it requires high humidity and rainfall during flowering in presence of susceptible host and aggressive strains of the pathogen.

The symptoms of FHB are premature ripening of the spikelets and peduncle tissue, which turn brown or tan (Osborne and Stein, 2007). The rating of FHB symptoms, as the amount of the disease caused in a race non-specific resistance, defines the aggressiveness of the pathogen, that differs from the pathogenicity, and their ability in causing the disease (Vanderplank, 1968). Pathogenicity is a qualitative measurement while aggressiveness is a quantitative measurement of the rate at which the disease level is reached with more aggressive pathogens (Shaner *et al.*, 1992). Aggressiveness is an important aspect essential for understanding the interaction between host-pathogen in FHB-wheat system (Wu *et al*., 2005). The mean FHB disease rating is usually measured in order to evaluate the aggressiveness of the isolates collected from different continents (Voss *et al.*, 2008), but other parameters such as symptom development, host colonization, type and amount of mycotoxin production should be considered (Mesterhazy, 1984; Miedaner and Schilling, 1996; Miedaner *et al.*, 2000; Desjardins *et al.,* 2004; Toth *et al.,* 2004; Akinsanmi *et al.,* 2006). The aggressiveness of *Gibberella zeae* or *F. culmorum* has a

quantitative-genetic basis besides other possible components (Miedaner *et al*., 1996; Miedaner *et al.*, 2000).

Several assays such as ear inoculations in field condition (Mesterhazy, 2002; Toth *et al*., 2008; Von der Ohe *et al*., 2010), floret inoculations under controlled conditions (growth chamber or green house) (Xue *et al*., 2004; Alvarez *et al*., 2010), coleoptile inoculations (Wu *et al.*, 2005), Petri-dish methods (Mesterhazy, 1978; Mesterhazy 1984; Koutnik and Lemmens, 2007; Purahong, 2010), etc. have been conducted to determine the aggressiveness levels. Whether different assays give the same result or not on the aggressiveness levels are still to be investigated. Wu *et al.* (2005) mentioned the existence of few studies about this last point.

It is a matter of debate the correlation between the proportion of scabbed wheat spikelets growing in greenhouse and field. Hall *et al.* (2001) found a very low correlation between the data obtained in these two different environments, whereas Bai *et al.* (2001) reported a significant association. Although the existence of this contrast, the high correlation of aggressiveness should minimize the costs in carrying out the aggressiveness test for pathologists, biotechnologists and plant breeders.

In addition to the aggressiveness assays, Fusarium damage kernel (FDK) and yield loss were two parameters, which have shown a high significant correlation between each other as well as with other indices of aggressiveness in field conditions (Mesterhazy, 2002; Toth *et al*., 2008). Buerstmayr *et al*. (1999) studied the aggressiveness of *F. culmorum* isolates in wheat kernels in Austria using ear inoculations in the field and reported that there was a high correlation between visual FHB symptoms, Area Under Disease Progress Curve (AUDPC) and FDK.

Mesterhazy (1997) and Lemmens *et al*. (1997) also reported that the percentage of FDK was highly correlated with trichothecene content. Other authors (Cumagun and Miedaner, 2004; Goswami and Kistler, 2005; Nicholson, 2009) found high correlations between aggressiveness and DON production in artificial infections. Miedaner and Reinbrecht (2001) compared different aggressiveness levels with different chemotypes of *F. culmorum*.

The aims of the present study were:

1) to compare aggressiveness of Syrian and Italian *F. culmorum* isolates by using different aggressiveness assays: ear inoculations in field, floret inoculations in growth chamber and Petri-dish test,

2) to investigate the relationships among aggressiveness indices from different assays and their relation to FDK, kernel weight (KW) reduction and DON production,

3) to compare aggressiveness of two different chemotypes of *F. culmorum* isolates using four disease parameters obtained with three aggressiveness assays,

4) to evaluate FDK and KW reduction induced by two different chemotypes.

MATERIALS AND METHODS

Aggressiveness and pathogenicity of *F. culmorum* strains

Fungal isolates

Several *F. culmorum* colonies were isolated from durum wheat kernels of different cultivars grown in localities in Syria and Italy in 2009. They were morphologically and molecularly identified and subsequently characterized for chemotypes (see Chapter I).

Eleven *F. culmorum* strains (8 Syrian and 3 Italian) were used for this study. The Italian strains were all 3Ac-DON chemotype while the Syrian strains belonged to NIV and 3Ac-DON chemotype (Tab. 4.1).

V8 broth – growth medium

One litre of V8 tomato juice (Campbell foods, Belgium) was mixed with 5 g of calcium carbonate. The mixture was then centrifuged at 4000 rpm for 20 min. The supernatant was collected in a new flask and diluted with distilled water 1:4 (v: v) and finally autoclaved at 121 °C for 15 min (Singleton *et al.*, 1992).

Macroconidia production

Each *F. culmorum* strain was cultured on PDA plate for 7 days and then pounced using a sterile cork borer, 5 mm diameter. Flasks of 300 ml, containing 150 ml autoclaved V8 broth were inoculated by two mycelium plugs and shaken on a thermo-controlled horizontal type shaker (Thermo scientific, USA) 120 rpm at 25 °C under incident sun light for two weeks (Figure 4.1).
The mixture of macroconidia, mycelium and V8 broth was filtered by a syringe filled with double layers of autoclaved cheesecloth to obtain only a macroconidia suspension, that was stored in a refrigerator at 4°C until the beginning of each experiment. The suspension of each strain was

adjusted by a haemocytometer (Thoma cell) to the final concentrations of $2x10^5$, $1x10^4$ and $1x10^6$ conidia/ml just one day before the assays - ear inoculations in the field, growth chamber floret inoculations and Petri-dish test, respectively. Double layers of autoclaved cheesecloth were used to eliminate the mycelia from germinating conidia in some isolates.

Ear inoculations in the field

Susceptible wheat, cv Simeto, was sown in autumn at the University of Bologna research field located in Cadriano, Emilia Romagna, Northern-Central Italy.

This field was subdivided into small subplots (1 m x 2.2 m), one subplot was representing one replication, and three replications were used for each of 11 *F. culmorum* strains and control treatment. At 30% anthesis, 60 ml of conidial suspension at concentration $2x10^5$ conidia/ml or sterile distilled water (control) was sprayed on each subplot by a hand sprayer.

Five groups of ten spikes per subplot were chosen and marked with plastic labels for disease assessment.The disease evaluations were measured as disease severity (DS) and disease incidence (DI) at 13 and 22 days after inoculation (DAI). For DS, the scale rating of Purahong *et al.* (2012) was used. This scale represents eight levels of percentage area infected on individual ears: 0% (no infection), 2%, 5%, 10%, 25%, 50%, 75% and 90% (90% or more) (Figure 4.2). DI was measured as the number of ears that are visibly diseased in relation to the total number assessed (50spikes/replicate). Mean FHB Index was calculated as the product of DI and DS divided by 100 (Von de Ohe, 2010), and it was used as the aggressiveness index of the fungal strains in the field condition (Figure 4.3).

To determine Fusarium damage kernel (FDK) and kernel weight (KW) reduction, 100 seeds from each replicate were automatically counted for the evaluations. The scabby "tombstone" kernel infection (Figure 4.4) was estimated visually and recorded as FDK (%) (Mesterhazy *et al*., 1999). Koch's postulate was fulfilled by the reisolation of *F. culmorum* from infected kernels. The kernel weight per each plot was recorded, and KW reduction (%) was calculated in respect to the control.

Floret inoculations in growth chamber

Seeds of the susceptible wheat cv Simeto were sown in seed trays (160 plants/tray) filled with autoclaved potting mix medium and subsequently placed in a growth chamber (25/19 °C day/night temperature, 14/10 hr light/dark cycle). Each seedling was individually transplanted into a new pot (8 cm in diameter and 15 cm in height) containing autoclaved potting mix medium and placed in a growth chamber with the conditions previously described. One week later, approximately 3 g of commercial fertilizer was applied to all plants. The plants were watered three times a week until harvest, to avoid water stress condition.

After two months, the first spike was observed and within one month the plant spikes were in the anthesis stage and ready for fungal artificial inoculation. 20 µl (10 µl/floret) of each macroconidia suspension of 11 *F. culmorum* strains (Tab.4.1) at a concentration of $1x10^4$ conidia/ml (sterile distilled water for control treatment) were injected into two florets at the middle of each spike (without wounding) and covered with polyethylene bags for 48 h, to ensure constant high humidity. Each *F. culmorum* suspension was injected to ten spikes from different pots, accounting for ten replications (Figure 4.5).

Disease evaluations were carried out at 7, 14 and 21 DAI. DS was determined as previously described in disease evaluation in the field. Mean and terminal (the 3rd disease severity evaluation, 21 DAI) severity were used as the aggressiveness index of the fungal strains in the growth chamber experiment (Figure 4.5).

The experiment was duplicated as described above with three replications. Koch's postulate was fulfilled by the re-isolation of *F. culmorum* from infected kernels.

Petri-dish test

Six *F. culmorum* strains (3 Syrian and 3 Italian) were examined by Petri-dish test (Tab.4.1). Wheat seeds of two susceptible Italian cultivars (Duilio and Simeto) were surface disinfected in 2% sodium hypochlorite solution for 8 min and then rinsed with sterile distilled water six times.

Sterile double layer filter papers were put in glass Petri-dishes (15 cm in diameter). Ten ml of macroconidia suspension at concentration of $1x10^6$ conidia/ml for each *F. culmorum* strain (sterile distilled water for control treatment) was poured on the filter paper. Twenty-five healthy seeds, disinfected as above described, were put on one side of the Petri-dish, and the dish was

inclined to soak the seeds in the fungal inoculum. Each seed was then placed on the filter with the embryo directed upward. An environment of high relative humidity and low air flow were secured, by placing the Petri dishes of the same treatment in a clean polyethylene bag, supplemented by a piece of wet cotton, and then incubated in an incubator at 22 ° C in the dark. Four replicates for each strain, and cultivar were set up (Figure 4.6).

The germinated seeds were counted 48h later and this value was considered at 100% germination, in addition to daily post inoculation, from 3 to 6 days; the healthy looking coleoptiles were counted. Seedlings infected with *Fusarium* were distinguished by the presence of brown spots on the coleoptiles and/or seeds completely covered by mycelium. Percentage of healthy coleoptiles was plotted as a function of time (from day 2 to 6; the value at day 2 is 100%), and the area under the curve was calculated (formula 1) (Koutnik and Lemmens, 2007). This value defines the area under the healthy progress curve (AUHPC) for each strain and ranges from 50 (most aggressive) to 400 (not aggressive). AUHPC can be transformed to AUDPCstandard (formula 2) (Koutnik and Lemmens, 2007): the value ranges from 0 (not aggressive strain) to 1 (most aggressive). The aggressiveness of the strain was calculated as the mean values of two wheat cultivars.

The experiment was repeated twice, and Koch's postulate was fulfilled by the reisolation of *F. culmorum* from either small or large brown spots on the coleoptiles.

$$\text{AUHPC} = \frac{B1 + 2B2 + 2B3 + 2B4 + B5}{2} \qquad (1)$$

$$\text{AUDPCstand} = \frac{400 - \overline{AUHPC}}{350} \qquad (2)$$

Where (1) AUHPC = area under healthy progress curve, B1 – B5 = percentage of healthy coleoptile at 1-5 evaluation (B1 always = 100%);

(2) AUDPC stand = area under disease progress curve standard.

Mycotoxin analysis

Wheat grains obtained from spikes artificially inoculated with the eight strains belonged to 3ADON chemotype, in addition to the control (non inoculated spikes) were ground and DON

level was estimated for each sample (27 (9×3)). 20 g from each sample was suspended in 100 ml double distilled water in 200 ml flasks. Sample suspensions were placed in a rotary shaker (200 rpm) for 5 min, 2 ml of the suspension was transferred into Eppendorf tubes and centrifuged (14000 rpm) for 5 min. One ml of the supernatant served as a stock solution for dilution preparations. Dilutions were prepared from the stock solution to optimize ELISA analysis. DON content of ground wheat grain samples was analyzed using AgraQuant DON (Romer Labs, Austria) (Figure 4.7).

Statistical analysis

Data analysis was performed using SPSS (SPSS Inc. Chicago, IL, v17, 1993–2007). The correlation coefficients among indices of aggressiveness from each method and between replicate experiments were determined using the Pearson product-moment correlation at a significant level of 5%. ANOVA incorporating the Games-Howell post hoc test at the 5% level of significance was used to differentiate aggressiveness of different *F. culmorum* strains.

RESULTS

Pathogenicity of *F. culmorum* strains

All the eleven *F. culmorum* strains tested with ear inoculations in the field and floret inoculations in the growth chamber were pathogenic, causing typical FHB symptoms. The bleached spikelets were observed on the first evaluation at 7 and 13 DAI in growth chamber and field experiments, respectively. There were no bleached spikelets in the control treatment (Tab. 4.2).

All the six *F. culmorum* strains tested with the Petri-dish test caused also brown spots on the coleoptiles and/or the mycelium completely covered the seeds of two susceptible wheat cultivars (Simeto and Duilio). No symptoms of *Fusarium* infection were observed in the control treatment (Tab.4.2).

Aggressiveness of *F. culmorum* strains

Significant differences in aggressiveness levels among *F. culmorum* strains were detected with all four aggressiveness parameters obtained with three assays (Tab. 4.2).

One *F. culmorum* strain (F11) was highly aggressive in all parameters examined. This proved that the highly aggressive isolates were stable with different aggressiveness assays. Two *F. culmorum* strains (F961, F960) were weakly aggressive in all parameters examined and ensured that these artificial inoculation methods work successfully.

For mean FHB index, the parameter from ear inoculations in the field, only one strain (F11) differed significantly from six strains (Tab. 4.2).

The floret inoculations in the growth chamber yielded two parameters for aggressiveness (mean and terminal severity) (Tab. 4.2). The mean severity of the different strains did not reach a significant level by ANOVA, while the terminal severity reached the significant level. Three strains (F960, F961, F966) were medium aggressive, six (F962, F963, F964, F965, F35, F24) medium-high aggressive and two (F11, F968) highly aggressive.

For the last parameter $AUDPC_{standard}$ (Petri-dish test), all the six *F. culmorum* tested were significantly different from the control, and all isolates were highly aggressive (Tab. 4.2).

The two highly aggressive isolates (F11, F35) caused highest FDK and kernel weight (KW) reduction, with values ranging from 49.66 – 47.57% and 21,82 – 25,36% respectively (Tab.4. 2).

Eight strains caused FDK values significantly different from the control, and all the eleven isolates of *F. culmorum* tested were significantly different from the control for KW reductions.

Mycotoxin production

The highest aggressive strain (F11) produced the highest amount of DON, and there was significant difference among the amounts produced by the isolates with values ranging from 0,24-6,12 ppm (Tab. 4.2) the level of DON produced by strains number 11 and 968 was over of the detection limit of ELISA kit (5 ppm).

Relation among the different aggressiveness parameters

Mean FHB index yielded higher positive correlation coefficients with FDK and KW reduction than the mean and terminal severity. $AUDPC_{standard}$ showed a high correlation coefficient with FDK but not with KW reduction (Tab. 4.3).

The best correlation coefficients were between the data of terminal severity in the growth chamber and $AUDPC_{standard}$ ($r = 0{,}932$, $P < 0.01$) and between the mean severity and $AUDPC_{standard}$ ($r = 0.891$, $P < 0.05$). The data in growth chamber and in the field yielded significant correlation coefficients ranging from 0.853 (mean FHB index and terminal severity) and 0.840 (mean FHB index and mean severity) ($P < 0.01$). The correlation between $AUDPC_{standard}$ and mean FHB index in the field was also significant ($r = 0.829$, $P < 0.05$) and comparable to the correlation of mean FHB index, mean and terminal DS.

All aggressiveness parameters showed a highly significant correlation with FDK. Field mean FHB index had the highest correlation with both FDK ($r = 0.877$ $P < 0.01$) and KW reduction ($r = 0.869$, $P < 0.01$) (Tab. 4.3). The remaining correlations between FDK and severity (both mean and terminal), FDK and $AUDPC_{standard}$, KW reduction and severity (both mean and terminal) were high, ranging between $r = 0.872{,}0.856$ (mean and terminal severity with FDK, $P < 0.01$) and 0.629 ,0.666 (mean and terminal severity with KW reduction, $P < 0.05$) (Tab. 4.3).

High correlations were found among DON production and all other parameters (mean FHB index, mean severity, FDK, terminal severity, $AUDPC_{standard}$) with values 0.909, 0.862, 0.844 ($P < 0.01$) 0.819, 0.845($P < 0.05$) respectively, but there was no correlation between DON production and KW reduction .

Aggressiveness of different chemotypes

There was no significant difference in aggressiveness between the two *F. culmorum* chemotypes (3Ac-DON and NIV) as determined by three parameters from the two assays in the growth chamber and in the field (Tab. 4.4). In addition, there were also no significant differences in FDK and KW reduction between the two different chemotypes (Tab. 4.4). In the field, mean FHB index and severity of 3Ac-DON were slightly higher than NIV. For FDK and KW reduction, NIV had slightly higher values than 3Ac-DON (Tab. 4.4). For $AUDPC_{standard}$ and DON production,

we could not differentiate between the two chemotypes because only DON producer isolates were used.

DISCUSSION

This study investigated two very important aspects of *F. culmorum*: pathogenicity and aggressiveness. All isolates tested with three different aggressiveness assays fulfilled the requirement for pathogenicity (ability to cause disease), thus they are pathogenic. The difference of aggressiveness within *F. culmorum* strains had been reported by Miedaner and Reinbrecht, (2001), and the same behavior after ear inoculations in the field and floret inoculations in the green house has been detected in the present study. Although the weather conditions were not ideal for the development of FHB in the year 2011 (year in which field experiment was carried out), the symptoms appearance allowed to distinguish the aggressiveness of the different *F .culmorum* isolates as well as to properly measure the different parameters of the disease severity.

Highly significant correlations of $AUDPC_{standard}$, with mean and terminal severity of floret inoculations in the growth chamber were found. These data are in agreement with those reported by Cumagun and Miedaner (2003), that compared how temperature and humidity affect disease severity in uncontrolled field conditions and in the greenhouse.

In addition, there was stability among the aggressiveness of the different *F. culmorum* strains in all the aggressiveness parameters. The aggressive strains had higher FDK losses and lower kernel weight than the less aggressive isolates. However, this difference did not reach the significant level. In this study, we found that all the aggressiveness indices, including mean FHB index, mean and terminal severity and $AUDPC_{standard}$, had a high significant correlation with FDK. This good correspondence among the previous mentioned parameters was reported by other authors (Buerstmayr *et al.*,1999; Mentewab *et al*. 2000; Lemmens *et al*. 2004). Toth *et al*. (2008) reported highly significant correlations (more than 0.90, $P< 0.001$) between aggressiveness index (disease severity) and FDK in a study of resistance of common wheat to isolates of *F. graminearum* species complex and *F. culmorum*. In contrast, other authors denied such a correlation among the same parameters (Alvarez *et al*., 2009). Therefore, our results indicate that

there are correlations of different aggressiveness parameters, FDK and KW reduction and are stable in the different floret inoculations in aggressiveness assays.

The comparison between aggressiveness of both chemotypes led us to conclude that different *F. culmorum* chemotypes have similar aggressiveness, and they can cause a similar damage level to wheat kernels. Furthermore trail with more strains is necessary to confirm this result.

In contrast to our results, Miedaner and Reinbrecht (2001) studied trichothecene content of rye and wheat genotypes inoculated with DON and NIV producers and found that the NIV producer was significantly less aggressive than the DON producer. This phenomenon could be ascribed to a lower phytotoxicity of NIV in cereals and unfavorable conditions for the NIV producer.

Our results confirm role of DON accumulation in the level of aggressiveness. In other words, aggressiveness is closely related to DON levels, whenever the level of DON increased there was an increase of aggressiveness and vice versa. Similar results were obtained for *F. culmorum* isolates by other authors (Gang *et al*., 1998; Hestbjergh *et al*., 2002; Bai *et al*., 2001).

These data are in accordance with the results of Mesterhazy (2002) and Atanassov *et al.* (1994) that suggested that DON and related trichothecenes have a role as a virulence factor in disease development. Other researchers supported this conclusion (Muthomi *et al*. 2000) and reported a close correlation between aggressiveness and DON production in *F. culmorum* isolates (Scherm *et al*., 2011).

Snijders (1994) mentioned that DON is a strong protein inhibitor, and this may cause inhibition of enzymatic activity in susceptible hosts, leading to a rapid increase of FHB. Alexander *et al*. (1997) did not consider trichothecenes necessary for pathogenicity, but they increase disease development. Thus, strategically, the disease resistant cultivars should not be associated with toxin-producing *Fusarium* species. There were no differences between Syrian and Italian *F. culmorum* strains regarding to the different parameters of the three assays, however they originated from two distinct geographical regions. This result might be interpreted as reported by Srobarova *et al.* (2008) that there are none or very low differences among *F. culmorum* isolates. Gargoliu *et al*. (2003) and Vanco *et al*. (2007) explained that the low level of genetic differentiation among populations of *F. culmorum* is usually observed in out crossing fungal species rather than in predominant asexually propagated populations.

These results revealed the possibility of using the Petri-dish test to screen for highly aggressive *F. culmorum* isolates for breeding purposes. These findings are comparable with those previously performed by Purahong *et al.* (2012) on *F. graminearum*.

FIGURES AND TABLES

Figure 4.1. F. culmorum strains grown on V8

Table 4.1. Chemotypes of *F. culmorum* tested with three different aggressive assays (11 strains were tested with floret inoculations in field and growth chamber and 6 with Petri-dish test)

No. of Strain	Location	Chemotype	Field	Growth chamber	Petri-dish test	DON production
960	ALHassakeh- Syria	3Ac-DON	yes	yes	yes	yes
961	Daraa- Syria	3Ac-DON	yes	yes	yes	yes
962	ALHassakeh- Syria	3Ac-DON	yes	yes	no	yes
963	Daraa- Syria	NIV	yes	yes	no	no
964	Daraa- Syria	NIV	yes	yes	no	no
965	Damascus rural-Syria	NIV	yes	yes	no	no
966	ALHassakeh- Syria	3Ac-DON	yes	yes	yes	yes
968	ALHassakeh- Syria	3Ac-DON	yes	yes	no	yes
11	Italy	3Ac-DON	yes	yes	yes	yes
24	Italy	3Ac-DON	yes	yes	yes	yes
35	Italy	3Ac-DON	yes	yes	yes	yes

Figure 4.2. FHB symptoms evaluated with rating scale of Purahong et al. (2012), the numbers represent the percentage area infected on individual ears: 0% (no infection), 2%, 5%, 10%, 25%, 50%, 75% and 90% (infection area is 90% or more)

Figure 4.3. Evaluations of DI and DS in plots inoculated with F. culmorum strains (a) F11 and (b) F968

a) b)

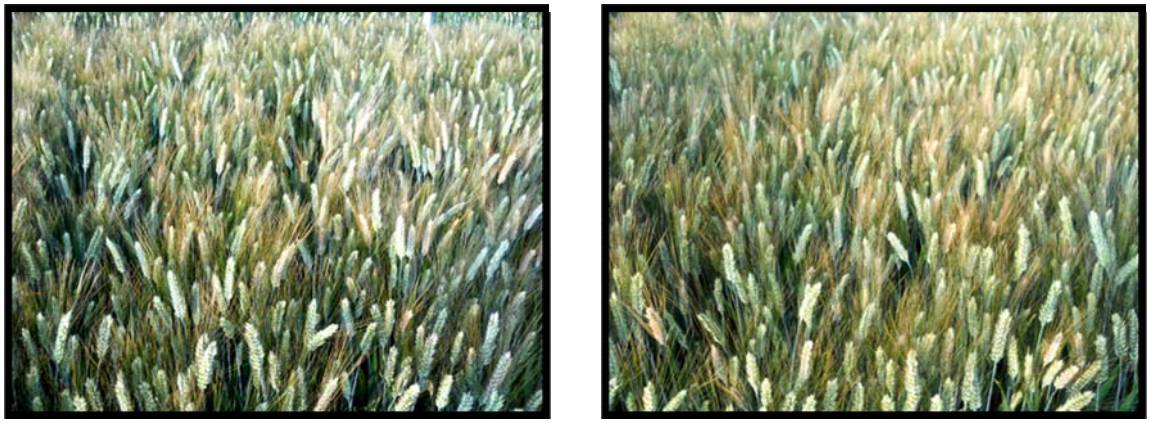


Figure 4.4. Fusarium damage kernels (FDK) (left) compared with healthy kernels (right)

Figure 4.5. Steps of floret inoculation and estimation of DS in growth chamber

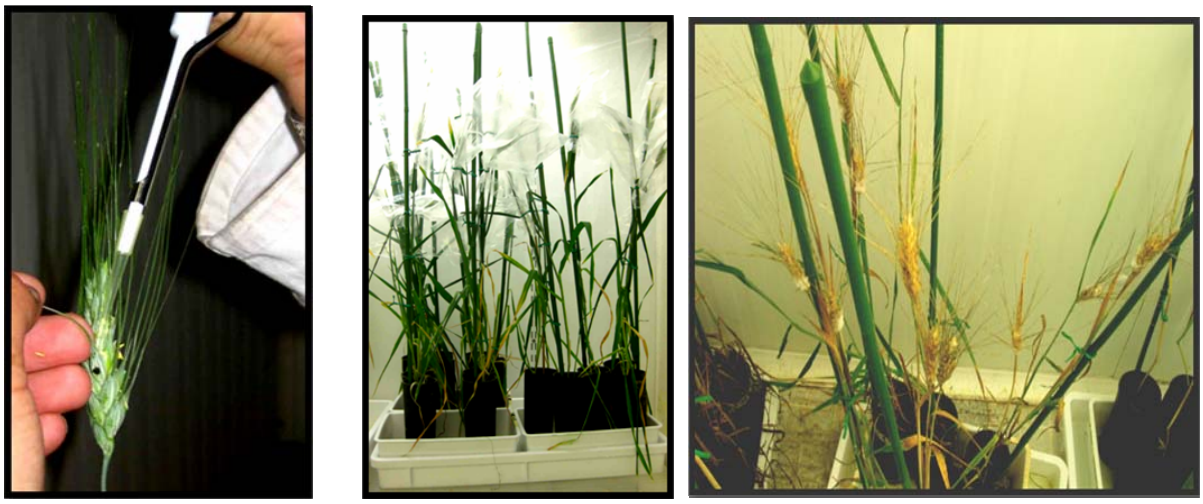


Figure 4.6. Steps of Petri-dish test

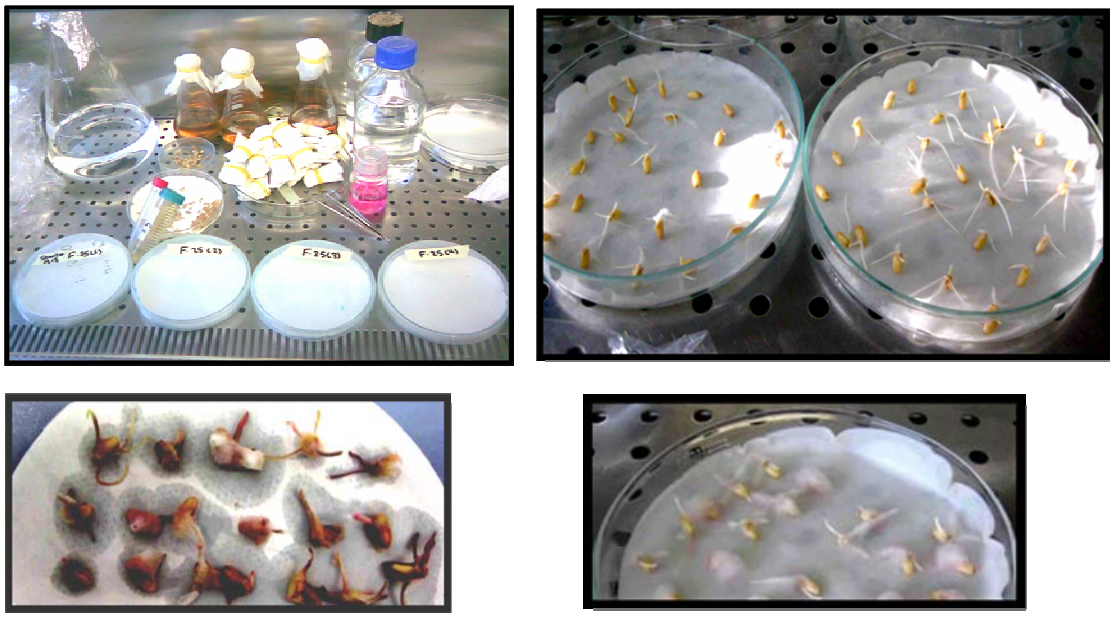


Figure 4.7. Detection of DON content in post harvest wheat kernels using ELISA technique

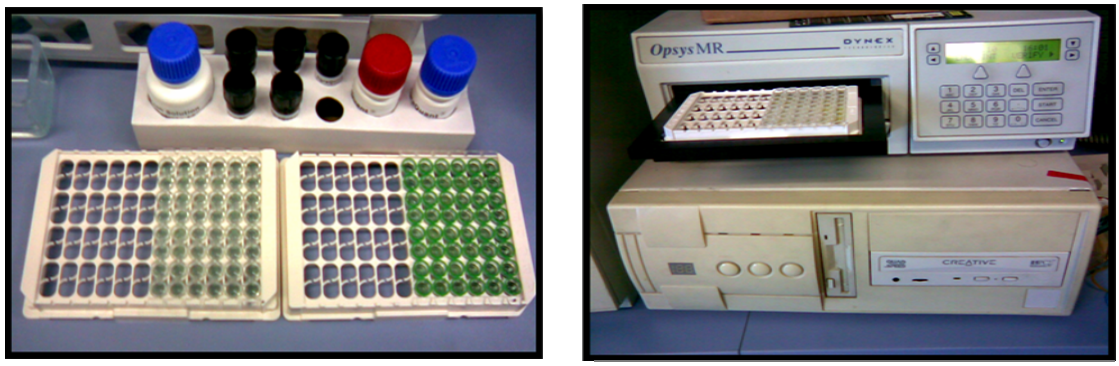


Table 4.2. Parameters used to estabilish aggressiveness levels in field experiment, growth chamber experiment and Petri-dish test: mean FHB index (0-90%), mean and terminal DS (0 – 90%), FDK (0 – 100%), KW reduction, DON production and AUDPCstandard averaged across 3, 8 and 4 replicates in field, growth chamber and Petri-dish test experiment, respectively

Strain	Field experiment				Growth chamber experiment		Petri-dish test
	Mean FHB index	FDK (%)	KW reduction (%)	DON production	Mean DS	Terminal DS	AUDPCstandard
control	0.196[a]	5.8[a]	0[a]	0.11[a]	0[a]	0[a]	0[a]
960	0.69[a]	29.77[ab]	16.08[b]	0.35[a]	31.4[b]	50.5[b]	0.91[bc]
961	0.97[a]	30a[b]	14.7 [b]	0.24[a]	33.7[b]	55.5[b]	0.94[cd]
962	2.88[abc]	41.48[b]	18.57[b]	1.78[a]	39.4[b]	67.5[bc]	nd
963	2.71[abc]	40[b]	16.07[b]	nd	38.7[b]	59.3[bc]	nd
964	1.47[ab]	39.78[b]	14.8[b]	nd	35.7[b]	61.4[bc]	nd
965	2.19[ab]	40.66[b]	18.67[b]	nd	33.7[b]	59.2[bc]	nd
966	0.69[a]	29.77[ab]	16.08[b]	0.35[a]	31.4[b]	50.5[b]	0.94[cd]
968	6.34[abc]	44.66[b]	20.54[b]	5.64[bc]	39.7[b]	68.6b[c]	nd
11	9.11[c]	49.66[b]	21.82[b]	6.12[c]	44.6[b]	82.5[c]	0.99[e]
24	1.15[a]	33.33[ab]	18.21[b]	0.94[a]	35.1[b]	61.5[bc]	0.96[de]
35	7.80[bc]	47.57[b]	25.36[b]	2.96[ab]	40.2[b]	67[bc]	0.97[de]

Means followed by the same letter in column within each *F. culmorum* strain are not significantly different by the least significant difference test (LSD); nd =not determined.

Table 4.3. Correlation analysis of different aggressiveness parameters obtained by three different assays (Field floret inoculations, growth chamber floret inoculations and Petri-dish test) and their relation to FDK and KW reduction (number of the strains used in growth chamber and field floret inoculations = 11, number of the strains analysed for their DON production = 8, number of the strains used in Petri-dish test= 6)

Parameters	Correlation analysis					
	Field mean FHB index	**Growth chamber mean DS**	**Growth chamber Terminal DS**	**AUDPC standard**	**FDK**	**KW reduction**
Growth chamber Mean DS	0.840**					
Growth chamber Terminal DS	0.853**	0.922**				
AUDPCstandard	0.829*	0.891*	0.932**			
FDK	0.877**	0.872**	0.856**	0.835*		
KW reduction	0.869**	0.629*	0.666*	ns	0.758*	
DON production	0.909**	0.819*	0.862**	0.845*	0.844**	ns

ns = no significant correlation

* and ** = Significant at P< 0.05 and P< 0.01, respectively.

Table 4.4. Comparison of mean FHB index (0-90%) from field experiment, mean and terminal DS (0 – 90%) from growth chamber experiment, FDK (field) (0– 100%) and KW reduction averaged among different strains within the same chemotype

Parameters	3Ac-DON	NIV	Significant
Mean FHB index (Field)	3.76	2.12	no
Mean DS (Growth chamber)	37.4	34.1	no
Terminal DS (Growth chamber)	62.8	60.6	no
FDK (Field)	39.45	40.37	no
KW reduction	14.38	14.58	no

*ANOVA Test was used to determine significant differences among different chemotypes

Table 4.5. Mean of disease severity of 11 isolates in wheat cultivar 'Simeto' carried out in growth chamber at 7, 14 and 21 DAI (1st, 2nd, 3rd evaluation, respectively)

Strain	Disease Severity			
	1st Evaluation	2nd Evaluation	3rd Evaluation	Mean
control	0.0	0.0	0.0	0.0
960	8.3	35.5	50.5	31.4
961	16.4	29.2	55.5	33.7
962	4.8	45.8	67.5	39.4
963	11.0	45.7	59.3	38.7
964	11.0	34.5	61.5	35.7
965	7.0	35.0	59.2	33.7
968	6.3	44.3	68.6	39.7
11	6.2	45.0	82.5	44.6
24	9.5	34.2	61.5	35.1
35	17.7	36.0	67.0	40.2

CHAPTER V

Validation of a modified Petri-dish test to quantify aggressiveness levels of *Fusarium culmorum* in durum wheat

ABSTRACT

Fusarium culmorum is a worldwide distributed agent causing Fusarium head blight (FHB) (scab) on wheat. Aggressiveness is a fundamental concept in order to understand the host-pathogen interaction in the FHB-wheat system. In this article, we modified and validated the Petri-dish test originally described by Mesterhazy (1978) to quantify the aggressiveness of 23 *F. culmorum* strains using different susceptible durum wheat cultivars for FHB. The results correlated to a high and significant degree with those obtained by using adult plants in the growth chamber and in the field (r= 0.891 and 0.829 ($P<0.05$), respectively). The Petri-dish test has repeatability with highly significant correlation coefficients in different wheat cultivars (r=0.991 for Dulio and 0.978 for Simeto ($P<0.01$) as well as stability with (r=0.992 ($P<0.01$). In this study, we also demonstrated that germination rate reduction and coleoptile length reduction are parameters involved with aggressiveness of *F. culmorum*. Petri-dish aggressiveness index is a new parameter for aggressiveness, which resulted from averaging three disease parameters from the modified Petri-dish method. The results obtained reveal that this modified Petri-dish test is rapid, reliable and stable with different durum wheat cultivars, and yields highly significant correlation coefficients with floret and ear inoculations, thus it is suitable to be used for quantification of aggressiveness of *F. culmorum*.

INTRODUCTION

Fusarium spp. are recognized as pathogens for many plant species. 19 species of *Fusarium* are associated with Fusarium Head Bligh (FHB), a disease of a wide range of host cereal crops as wheat (*Triticum* L.), barley (*Hordeum* L.), maize (*Zea mays* L.) and other grains, and among these *Fusarium culmorum* (WG Smith) Sacc. and *F. graminearum* Schwabe [teleomorph, *Gibberella zeae* (Schwabe) Petch] are the most important FHB causal agents (Gilbert and Tekauz, 2000; Tekauz *et al.,* 2000; Liddell, 2003; Goswami and Kistler, 2004). *F. culmorum* can colonize and cause disease in several plant parts, i.e. roots, stems and spikes (Parry *et al*., 1995). This low specificity makes its aggressiveness and spread in plant tissues variable and greatly influenced by environmental conditions.

FHB leads to considerable losses in grain quality and yield, which may reach 75% (Wilcoxson *et al.*, 1988). Moreover, the infected grains tend to show reduced germination and cause seedling blight, poor stand as well as reduced grain mass, test weight, baking and seed quality (Wiersma *et al.*, 1996; Bai and Shaner, 1996). The production of several mycotoxins by these fungi as trichothecenes, which include deoxynivalenol (DON), 15-acetyldeoxynivalenol (15Ac-DON), 3-acetyldeoxynivalenol, (3Ac-DON) and NIV, is considered a major concern to human and animal health (Yazar and Omurtag, 2008).

Pathogenicity and aggressiveness are two important characteristics of *Fusarium* spp. (Von der Ohe *et al.*, 2010). Although these two words have different meaning, sometimes they have been not properly used. Pathogenicity reflects the ability of the fungus to cause qualitatively the disease. Aggressiveness is a quantitative measurement of the rate at which the level the disease is reached; with aggressive pathogens (Shaner *et al.*, 1992). In order to understand the interaction between host-pathogen in FHB-wheat system, aggressiveness assessment is fundamental (Purahong *et al.*, 2012).

To reach this purpose several alternative methods have been developed including floral tissues, stems and seedling inoculation (Wu *et al.,* 2005; Bai *et al*., 2001; Mesterhazy, 1995; Snijders, 1990; Hare *et al*., 1999). Since for several decades, the floret inoculation assay has been used as a classical method, being considered reliable and precise, to assess the aggressiveness of fungal pathogen isolates, it is costly, time consuming, laborious, and greatly influenced by growth stages, environmental conditions and the inoculum, which can be applied quantitatively (Yang, 1994; Wu *et al.*, 2005). The other alternative inoculation methods (i.e. stem and seedling inoculation), even if are less time consuming and affect less the environment, have some limitations and/or disadvantages. Purahong *et al*. (2012) mentioned that the lack of data, that will allow to compare the aggressiveness of *F. graminearum* among the alternative methods and the single floret inoculation technique in controlled conditions and/or in the field, is a major problem.

The use of wheat coleoptile in *in vitro* method gave a good correlation with floret inoculation in the field and many other advantages over the other inoculation methods (Wu *et al*.,2005). These authors also reported that the success of this method depends on the cultivar since the correlation factor extensively varied within the cultivars. Mesterhazy (1978) was the first who set up this method with the aim to screen the resistance of the cultivars to FHB; the cultivars more resistant show higher healthy wheat seedling. Further modifications of this method were done by Brennan

et al. (2003) and Koutnik and Lemmens (2007). Purahong *et al.* (2012) set up a similar method that can quantify the aggressiveness of *F. graminearum* strains and fulfill all the requirements. According to our knowledge, this study on *F. culmorum* and the previous study carried out by our research group on *F. graminearum* (Purahong *et al*., 2012) are the first scientific works. It is also possible to assess, with this method, two additional parameters related to the aggressiveness of *F. culmorum:* germination rate and coleoptile length reduction. Browne and Cooke (2005) observed that *F. culmorum* induced a reduction of the germination rate of wheat seeds, in fact when kernels inoculated with *F. culmorum* in Petri-dish test are infected, their germination rate is likely reduced when compared with the non-inoculated ones. Infection by *F. culmorum* cause reduction of the coleoptile length and it has been related to the aggressiveness of the isolates; isolate posses more aggressiveness when the reduction of the coleoptile length is higher (Brennan *et al.*, 2003). To date, we are in need of an *in vitro* aggressiveness method, simple and rapid, for *F. culmorum* in wheat, to show the high correlation with floret inoculation in different cultivars.

The objectives of this research were: 1) to evaluate the feasibility of the modified Petri-dish test using AUHPC or $AUDPC_{standard}$ to determine the different levels of aggressiveness among Syrian and Italian *F. culmorum* strains; 2) to validate this method by finding significant correlation among these results and the data from floret inoculation techniques under controlled conditions (growth chamber) and ear inoculations in the field; 3) to discover the relationships among the three parameters obtained by Petri-dish test ($AUDPC_{standard}$, germination rate reduction and coleoptile length reduction) and their relations to floret inoculation in both growth chamber and field; 4) to prove the stability and repeatability of this method among different durum wheat cultivars.

MATERIALS AND METHODS

Fungal strains and plant materials

Twenty *F. culmorum* strains, were isolated previously from durum wheat kernels with visible FHB symptoms from different cultivars and localities in Italy during the years 2007- 2009 and three Syrian strains, from wheat kernels grown in AlHassakeh, Daraa provinces (Syria) in 2009, were characterized for mycotoxin chemotypes in chapter 1 and used for further studies (Table 5.1).

Macroconidia production

Each *F. culmorum* strain was cultured on PDA plate for seven days and then pounced using a sterile cork borer, 5 mm diameter. Two mycelium plugs were inoculated into 300 ml flask containing 150 ml autoclaved V8 broth, prepared as describes in Chapter IV (Singleton *et al.*, 1992), and shaken on a refrigerated rotary incubator (Thermo-Fisher MaxQ 4000) at 120 rpm and 25°C under direct sun light for two weeks.

The mixture of macro conidia, mycelium and V8 media was filtered by a sterile syringe filled with double layers of autoclaved cheesecloth. The spore concentrations were measured with a haemocytometer and stored at 4°C. Macroconidia of each strain were adjusted to the final concentrations of $1x10^6$ conidia/ml. The mycelia from germinating conidia were excluded using double layers of autoclaved cheesecloth.

A modified Petri-dish test

Wheat seeds of a susceptible cultivar Simeto, were surface sterilized in 2% sodium hypochlorite for 8 min and then rinsed with sterile distilled water six times. Each of 10 ml of *F. culmorum* macroconidia suspension at a concentration of $1x10^6$ conidia /ml (or sterile distilled water in the control treatment) was inoculated into Petri-dish (15 cm in diameter) with sterile double- layer filter papers (Perfect 2, Cordenons).

Sterile forceps was used to eliminate air bubbles under the filter paper. Twenty-five seeds, apparently healthy, were put on one side of the Petri-dish, which were rotated in a way to make the seeds to submerge under the fungal inoculum, and then each seed was placed on a filter paper with the embryo turned upward. To ensure the high relative humidity and low air movement, the Petri-dishes, from the same treatment, were put in a clean polyethylene bag supplemented with moist cotton and incubated in an incubator at 22 °C in the dark. Four replicates for each strain were set up. The germinated seeds were counted two days after inoculation (DAI), and this value was set at 100% germination.

The healthy looking coleoptiles were counted every single day from day 3 to 6 post inoculation. *Fusarium* infected seedlings were identified by brown spots on the coleoptiles and/ or mycelium covered the seeds completely (Figure 5.1). Percentage of healthy coleoptiles was plotted as a function of time (from day 2 to 6; the value at day 2 is 100%), and the area under the curve was

calculated (formula 1). The value of this area from each isolate is the area under the healthy progress curve (AUHPC) and ranges from 50 (very aggressive) to 400 (not aggressive). AUDPC can be transformed to AUDPC$_{standard}$ (formula 2): the value ranges from 0 (not aggressive) to 1 (very aggressive). If aggressiveness is low, the observation period can be extended over six days. The mean values of four replicates are taken as a measure for aggressiveness of the strain. The experiment was repeated once. Koch's postulate was fulfilled by the reisolation of *F. culmorum* from either small or large brown spots on the coleoptiles.

Germination rate reduction (Gr, formula 3) and coleoptile length reduction (Clr, formula 4) were determined by comparison with the non inoculated control at 6 DAI. The coleoptile length of six germinated seedling, which were considered as representative of each replicate, was measured for Clr.

Petri-dish aggressiveness index (formula 5) combined three parameters that could link with aggressiveness of fungal isolates.

$$AUHPC = \frac{B1+2B2+2B3+2B4+B5}{2} \quad (1)$$

$$AUDPCstand = \frac{400-\overline{AUHPC}}{350} \quad (2)$$

$$Gr = \frac{NGc-NGt}{NGc} \quad (3)$$

$$Clr = \frac{\overline{Clc}-\overline{Clt}}{\overline{Clc}} \quad (4)$$

$$Apindex = \frac{\overline{AUDPCstandard}+\overline{Gr}+\overline{Clr}}{3} \quad (5)$$

(1) AUHPC = area under healthy progress curve, B1 – B5 = percent of healthy coleoptile at 1-5 evaluation (B1 always = 100%);

(2) $AUDPC_{standard}$ = area under disease progress curve standard,
(3) Gr = germination rate reduction, NGc and NGt = number of germination seed in control and in treatment (*F. culmorum*),
(4) Clr = coleoptile length reduction, Clc and Clt = coleoptile length in control and in treatment (*F. culmorum*), respectively
AP_{index} = Petri-dish aggressiveness index.

Validation of the Petri-dish test

$AUDPC_{standard}$, FHB index and disease severity (DS) were used as an aggressiveness index in Petri dish test, ear inoculations in the field and floret inoculations in the growth chamber, respectively.

Among the strains, that have been tested by Petri-dish test, six strains (3 Italian and 3 Syrian) were also tested in growth chamber and in the field on the susceptible Italian wheat cv, Simeto. In the growth chamber, at wheat anthesis 20 µl (10 µl/floret) of each conidial suspension at a concentration of 10^4 conidia/ml or sterile distilled water (control) were injected into 2 florets at the middle of each spike (without wounding) (ten spikes from different pots were treated with each strain, accounting for ten replications). In the field, wheat ears at 30% anthesis, 60 ml of conidial suspension at concentration $2x10^5$ conidia/ml or sterile distilled water (control) was sprayed on each plot (1.0m x2.2 m) by a hand sprayer (3 plots were used for each fungal strains). More details about ear inoculations in the field and floret inoculations in the growth chamber were shown in chapter IV. The aggressiveness indices from different methods were correlated and compared.

Repeatability and Stability of Petri-dish test in different wheat cultivars

To verify the repeatability and stability of Petri-dish test among different host cultivars, another durum wheat cultivar "Duilio", susceptible, was inoculated with the same 23 *F. culmorum* strains, as previously described in Petri-dish test. Each strain was repeated four times, and the whole experiment was repeated. The repeatability was accessed using correlation analyses between $AUDPC_{standard}$ of the two experiments for each cultivar. The stability between different wheat cultivars was determined by correlation analyses between $AUDPC_{standard}$ of the two wheat

cultivars in two independent experiments. Details of the strains and raw data were used to calculate the correlations that validated the repeatability and the stability between different durum wheat cultivars (Table 5.1, Table 5.2 and Table 5.3).

Statistical analysis

Data analysis was performed using SPSS (SPSS Inc. Chicago, IL, v17, 1993 – 2007). The correlation coefficients among aggressive indices from each method and between replicate experiments were determined using the Pearson product-moment correlation at a significant level of 5%. ANOVA with LSD were used to differentiate the strains.

RESULTS

Modified Petri-dish test

The number of germinated seeds decreased in all *F. culmorum* treatments compared with the control, while in four strains (F820, F24, F93 and F722) the germination reduction was significant ($P< 0.05$). Clear symptoms of Fusarium infected seedlings have been observed since the first evaluation (3 DAI). Twenty-three strains showed a substantial variation in aggressiveness as defined by AUHPC ranging from 52.35 – 108.99 ($AUDPC_{standard}$ ranging from 0.83 - 0.99) (Table 5.2). There was no contamination in the control treatment (water), and AUHPC was equal to 400 ($AUDPC_{standard} = 0.00$) (Table 5.2).

Twenty-three strains also showed a large variation in aggressiveness as defined by germination rate reduction and coleoptile length reduction (Table 5.2). These reductions ranged from 25–45% and 51–92% for germination rate and coleoptile length, respectively (Table 5.2).

Three parameters obtained from Petri-dish test ($AUDPC_{standard}$, germination rate reduction and coleoptile reduction rate) were significantly correlated with each other.

The data of $AUDPC_{standard}$ and coleoptile length reduction were obtained from the same organ of the plant (coleoptile) and were highly significantly correlated ($r = 0.910$, $P< 0.01$) (Table 5.3). On the other hand, the correlations among the parameters from different plant organs were lower (germination rate reduction with $AUDPC_{standard}$ ($r = 0.824$; $P< 0.01$) and with coleoptile reduction ($r = 0.867$; $P< 0.01$) (Table 5.3).

Germination rate reduction was not correlated with floret inoculations in growth chamber and ear inoculations in the field. Data on coleoptile reduction showed higher correlations than those for germination rate reduction ($r = 0.951$; $P< 0.01$) in floret inoculation in the growth chamber, there was no correlation in the case of ear inoculation in the field (Table 5.3).

Comparative analysis of aggressiveness of *F. culmorum* strains using $AUDPC_{standard}$ (modified Petri-dish test), disease severity (floret inoculations in the growth chamber) and FHB index (ear inoculations in the field) showed highly significant correlations both between $AUDPC_{standard}$ and disease severity as well as $AUDPC_{standard}$ and FHB index, with correlation coefficients of 0.891 and 0.829 ($P<0.05$), respectively (Figure 5.2). The repeatability of Petri-dish test was confirmed by the high significant correlation of $AUDPC_{standard}$ of the two wheat cultivars inoculated. The correlation coefficient was 0.991 for Duilio and 0.978 for Simeto ($P<0.01$). The stability between different host cultivars was demonstrated with the high significant correlation coefficients, 0.992 ($P<0.01$), between data of $AUDPC_{standard}$ from different wheat cultivar.

The new parameter, Petri-dish aggressive index (APindex), calculated from the mean value of $AUDPC_{standard}$, germination rate reduction and coleoptile reduction, yields a satisfactory correlation with floret inoculation in growth chamber and field condition with correlation coefficients of 0.886 ($P< 0.05$) and 0.858 ($P<0.05$), respectively (Table 5.3).

DISCUSSION

This study was carried out to validate the Petri-dish test and to quantifythe aggressiveness levels of *F. culmorum*. Taking in consideration, that this test was validated by Purahong *et al*. (2012) for *F. graminearum*.

The values obtained from the three different parameters i.e. AUDPCstandard, germination rate reduction and coleoptile reduction were used to calculate the Petri-dish aggressiveness index. The modified Petri-dish test succeeded in differentiating the aggressiveness levels for twenty- three *F. culmorum* strains and to group them according to those levels. The results from the modified Petri-dish test were highly correlated with the data obtained by *in vivo* inoculations: the strains showed higher aggressiveness levels in Petri-dish test and floret inoculation in the growth chamber than in the ear inoculation in the field. This result could be interpreted by the great fluctuation of environmental factors and cross contamination in the field as well as the

inoculation methods. Among the environmental factors, temperature and moisture are considered crucial in influencing the disease severity and incidence in the field. Wu *et al*. (2005) reported that warm humid weather can stimulate the spread of FHB. Under field conditions, there are possibile cross contaminations by different fungal genera, species and strains, and among them *F. culmorum*, which can compete for nutrition (Xu *et al*., 2007; Xu and Nicholson, 2009). The possibility for the co-existence of several *Fusarium* species increases with the presence of arthropod vectors, wind and rain splash (Parry *et al*., 1995). In our field experiment the control plots developed much less disease symptoms in comparison with the inoculated ones. Inoculation methods as well as the target organ for the infection have a key role in determining the aggressiveness levels of the pathogen, *F. culmorum*. Wu *et al*. (2005) referred the quick germination and invasion of the *Fusarium* conidia in the coleoptile tissues. after head removal. Furthermore, Almgren *et al*. (1999) stated that the difference of the disease development rate between roots and leaves may be due to the presence of resistance genes which were not expressed in the roots. The way to inoculate the pathogens, spray and double point inoculations in the field and in controlled conditions, respectively, could explain the difference of FHB rate between field and growth chamber as *F. culmorum* inoculum in floret inoculations was placed directly into the mature ovary while in the field it has first to overcome the spike (Type I resistance; initial infection) by the external hyphal growth and then enter in the susceptible sites (Bushnell *et al*., 2003). The precise determination of wheat anthesis is a favourable condition for disease development in growth chamber rather than in the field. In durum wheat, there are no high FHB resistant cultivars (Jauhar *et al*., 2009), hence it should be sufficient to prove the repeatability and stability of Petri dish test by using different durum wheat cultivars with different *F. culmorum* strains. The repeatability and stability of Petri-dish method are considered one of the main advantages. Germination rate reduction and coleoptile length reduction compared with control can also be used in the assessment of *F. culmorum* aggressiveness and they could be obtained with the Petri-dish test. Aggressiveness levels reached by coleoptile length reduction have high correlation with floret inoculation in growth chamber, which is in accord with what stated by Purahong *et al*. (2012) for *F. graminearum*.

On the other hand, our data revealed no correlation between germination rate with floret and ear inoculation, and this is in contrast to the finding of Purahong *et al.* (2012) that found moderate to

weak correlation with the same parameters in a study on *F. graminearum*. Moreover, regarding the absence of correlation between germination rate and FHB rating in field experiments, our results disagreed with those reported by Browne (2007) who found weak negative relationship between germination rate reduction of wheat seeds induced by *Microdochium majus* and FHB rating obtained by spray inoculation of *F. graminearum* in the field. The difference was probably due to the number of the strains which was larger than ours or the difference among the pathogens used. In addition, our results were incompatible with those of Wu *et al.* (2005) regarding the coleoptile parameter, that found a moderate to high correlation between disease severity in the field and the length of the lesions on the cleoptile caused by *F. graminearum*. The good correlation of the new parameter "Petri-dish aggressiveness index" with floret and ear inoculation both in controlled and field conditions, is very close to the correlation obtained with $AUDPC_{standard}$. Petri-dish test has several advantages such as low costs, labour and time and at the same time can be performed all over the year and almost with no limitations in working with many isolates and/or replications. This test could be very usefull for both ear and floret inoculations, for screening and differentiation of different *F. culmorum* isolates for breeding purposes and for checking the aggressiveness levels of fungal inocula before inoculation in the field. Moreover, Petri-dish test is accounted to be a solution to facilitate the progress in studying FHB especially in the countries where it is forbidden to spray gene modified *Fusarium* strains in the field. The required period of 6 days to get results using this test, compared to the 2.5 months for the *in vivo* experiments shows how advantageous is this method. It is also important for the scientists or the breeders involved with FHB, since with this method they can quantify the aggressiveness levels of *F. culmorum*.

FIGURES AND TABLES

Figure 5.1. Symptoms on seedlings of durum wheat cv "Simeto" inoculated with F. culmorum: browning on the seedlings and mycelium covering the kernels

Table 5.1. *F. culmorum* strains included in this experiment

Strains	Location	Chemotype
9	Grosseto	3Ac-DON
10	Baricella (BO)	3Ac-DON
11	Grosseto	3Ac-DON
24	Grosseto	3Ac-DON
25	Grosseto	3Ac-DON
27	Grosseto	3Ac-DON
28	Grosseto	3Ac-DON
33	Grosseto	3Ac-DON
35	Grosseto	3Ac-DON
45	Grosseto	3Ac-DON
53	Savarna (RA)	NIV
93	Asciano (SI)	3Ac-DON
104	Braccagni (GR)	3Ac-DON
106	Pisa	3Ac-DON
385	ITEM	3Ac-DON
595	Grosseto	NIV
593	Grosseto	3Ac-DON
597	Grosseto	3Ac-DON
722	Grosseto	3Ac-DON
820	Grosseto	3Ac-DON
960	Daraa	3Ac-DON
961	Daraa	3Ac-DON
966	AlHassakeh	3Ac-DON

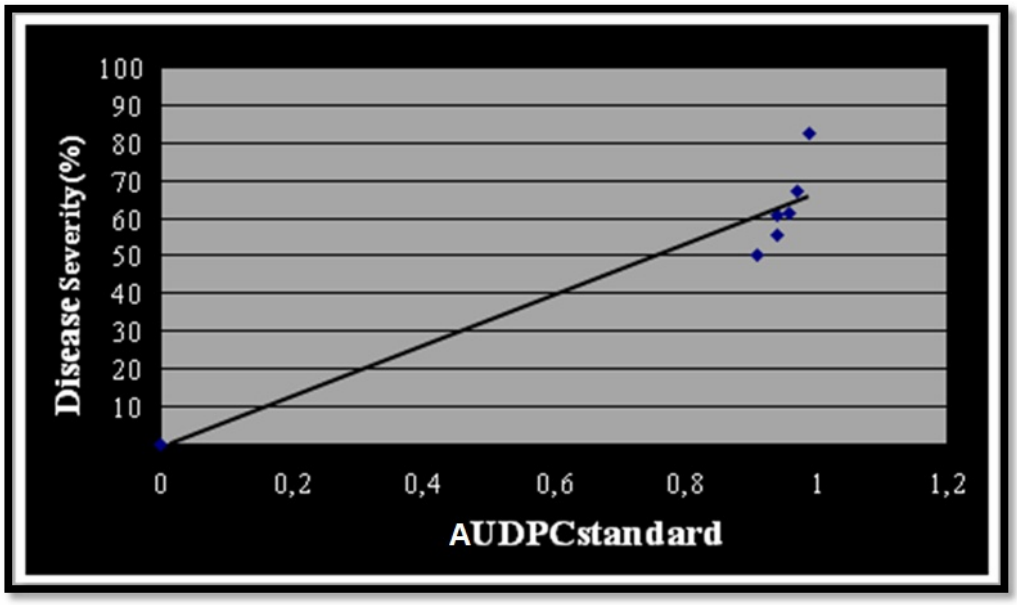
Table 5.2. Different parameters of *F. culmorum* strains: AUHPC = area under healthy progress curve, Clr = coleoptile length reduction, Gr = germination rate reduction, AUDPCstand = area under disease progress curve standard, APindex = Petri-dish aggressiveness index

Strain No	AUHPC	Coleoptile length	Clr	Gr	AUDPCstand	APindex
control	399,14	8,13	0^{a}	0^{a}	0^{a}	0^{a}
385	108,99	3,88	$0,51^{b}$	$0,25^{b}$	$0,83^{b}$	$0,53^{b}$
104	60,49	3,04	$0,60^{bc}$	$0,26^{b}$	$0,97^{cd}$	$0,61^{bc}$
595	79,13	2,89	$0,63^{bc}$	$0,30^{b}$	$0,92^{cd}$	$0,61^{bc}$
820	82,51	2,51	$0,69^{bc}$	$0,35^{b}$	$0,91^{c}$	$0,65^{c}$
106	76,96	1,74	$0,77^{bc}$	$0,346^{b}$	$0,92^{cd}$	$0,68^{c}$
597	68,08	1,57	$0,80^{bc}$	$0,30^{b}$	$0,95^{cd}$	$0,68^{c}$
960	74,94	1,64	$0,79^{bc}$	$0,36^{b}$	$0,93^{cd}$	$0,69^{c}$
593	58,20	1,07	$0,87^{c}$	$0,31^{b}$	$0,98^{cd}$	$0,72^{c}$
27	57,36	1,06	$0,86^{bc}$	$0,33^{b}$	$0,98^{cd}$	$0,72^{c}$
53	55,00	1,03	$0,87^{c}$	$0,30^{b}$	$0,99^{cd}$	$0,72^{c}$
25	58,71	1,23	$0,84^{c}$	$0,35^{b}$	$0,98^{cd}$	$0,72^{c}$
966	58,80	0,92	$0,88^{c}$	$0,32^{b}$	$0,97^{cd}$	$0,73^{c}$
24	59,83	1,25	$0,85^{c}$	$0,37^{b}$	$0,97^{cd}$	$0,73^{c}$
28	67,63	1,35	$0,83^{c}$	$0,40^{b}$	$0,95^{cd}$	$0,73^{c}$
9	66,43	1,66	$0,79^{bc}$	$0,45^{b}$	$0,95^{cd}$	$0,73^{c}$
722	58,73	0,91	$0,89^{c}$	$0,35^{b}$	$0,98^{cd}$	$0,74^{c}$
93	65,15	1,15	$0,85^{c}$	$0,40^{b}$	$0,96^{cd}$	$0,74^{c}$
961	57,44	1,18	$0,85^{c}$	$0,39^{b}$	$0,98^{cd}$	$0,74^{c}$
10	61,05	0,93	$0,88^{c}$	$0,39^{b}$	$0,97^{cd}$	$0,75^{c}$
45	55,92	0,84	$0,89^{c}$	$0,37^{b}$	$0,98^{cd}$	$0,75^{c}$
33	70,03	1,17	$0,85^{c}$	$0,45^{b}$	$0,94^{cd}$	$0,75^{c}$
35	64,03	0,84	$0,89^{c}$	$0,42^{b}$	$0,96^{cd}$	$0,76^{c}$
11	52,35	0,64	$0,92^{c}$	$0,39^{b}$	$0,99^{d}$	$0,77^{c}$

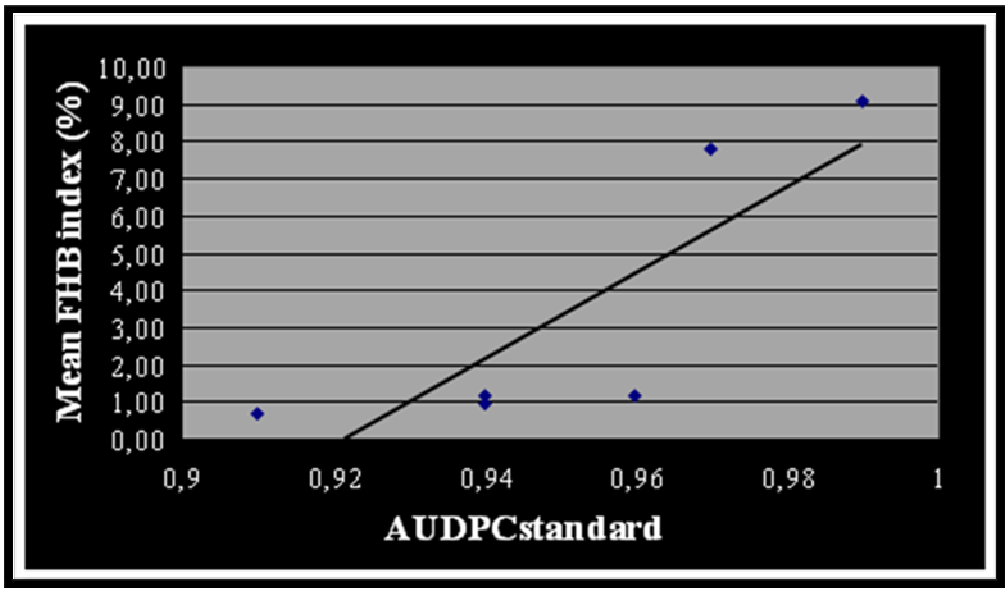
The different letters indicate the significant differences among the strains based on ANOVA Test, Games-Howell post hoc test at the 5% level of significance.

Figure 5.2. *Correlation between AUDPC$_{standard}$ and disease severity in floret inoculations in growth chamber (a) and FHB index in ear inoculation in the field (b)*

a)



b)



The correlation was determined by Pearson correlation coefficient AUDPC

r = 0.891, P< 0.05 (a) and r = 0.829, P< 0.05 (b).

Table 5.3. Correlation analysis among data of aggressiveness parameters obtained by three different methods; Petri-dish test, floret inoculations in growth chamber and field floret inoculations

AUDPCstand=AUDPCstandard; Gr= germination rate reduction; Clr = coleoptile length reduction; AP index = Petri-dish aggressiveness index; DSf = field disease severity and DSc =disease severity in growth chamber

Aggressiveness parameters	**Correlation analysis**				
	AUDPCstand	Gr	Clr	AP index	DSf
Gr	0.824**				
Clr	0.910**	0.867**			
DSf	0.829*	-	-	0.861*	
DSc	0.891*	-	0.951**	0.852*	0.835**

* and ** = Significant at P< 0.05 and P< 0.01, respectively.

CHAPTER VI

Behavior of Syrian durum wheat cultivars in field and growth chamber tests

ABSTRACT

Wheat (*Triticum durum* L.) is a strategic crop worldwide and considered as a susceptible plant species for Fusarium head blight (FHB); the destructive disease for all cereals. FHB reduces yield, grain quality and causes accumulation of deoxynivalenol (DON) in grains. Cultivar resistance is one of the most promising and effective management strategies for FHB. 11 wheat cultivars (ten Syrian and one Italian, as susceptible cv) were infected artificially with different Syrian and Italian strains of *F. culmorum* under growth chamber and field conditions in order to 1) study the behavior of wheat cultivar toward FHB and its relationship to different parameters: *Fusarium* head blight (FHB) index, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON) concentration, Hectolitric weight (HW), Disease severity and Disease development 2) ranking wheat cultivars with different types of resistance to FHB and DON accumulation. Different cultivars have shown different behavior toward FHB disease. The Syrian cultivars Jory and ACSAD1315 were the most tolerant in growth chamber and field, while the Syrian cultivars Sham 9 was the most susceptible under both conditions. DON accumulation and FDK (%) among the cultivars were different. However, the differences were not significant. Disease development showed significant difference between the cultivars under both conditions. The hectolitric weight parameter was affected by FHB also. DON accumulation was not correlated with any other parameters under both conditions.

There were correlations among the following parameters: FHB index correlated significantly at $P<0.01$ with FDK, HW, disease severity and disease development in growth chamber and field. FDK correlated significantly with HW, disease development in the field, disease severity ($p<0.01$) and disease development in the growth chamber ($p<0.05$). Moreover, disease development in the field showed significant correlation with disease development in the growth chamber as well as disease severity and HW ($P<0.05$). Based on the different parameters, we could group the tested cultivars according to their resistance to FHB.

INTRODUCTION

Resistance as defined by Argos (1988) is the ability of an organism to exclude or overcome, completely or partially, the effect of a pathogen or other damaging factor. In plant, disease

resistance is manifested by limited symptoms, reflecting the inability of the pathogen to grow or multiply and spread. Often it takes place the hypersensitive reaction (HR), in which the pathogen remains restricted to the site of infection as necrotic lesion (Van Loon, 1997). Mesterhazy (2002) described resistance of wheat (*Triticum* L.) to Fusarium Head Blight (FHB) as a complex phenomenon, and he reported different forms, types or components of physiological resistance: (i) resistance to initial infection (Schroeder and Christensen, 1963); (ii) resistance to spreading (Schroeder and Christensen, 1963); (iii) resistance to kernel infection (Mesterhazy, 1995; Mesterhazy *et al.*, 1999); (iv) tolerance to infection (Mesterhazy, 1995; Mesterhazy *et al.*, 1999) and (v) resistance to DON accumulation (Mesterhazy *et al.*, 1999).

FHB or scab, that is caused by 17 *Fusarium* species, mainly *F. culmorum* and *F. graminearum,* attracted attention in the recent years as a commonly worldwide distributed disease in wheat growing regions causing great yield losses (Brennan *et al.*, 2005), bleached and shrunken kernels, decreased seed and baking quality due to destruction of starch, proteins and cell walls of infected kernels (Lemmens *et al*., 1993; Chelkowski *et al*., 1998) beside the most serious health risk, accumulation of mycotoxins, that have harmful effects on human and animal (Windels, 2000; Cumagun and Miedaner, 2004). The most important mycotoxins associated with FHB in wheat are the trichothecenes; deoxynivalenol (DON) and nivalenol (NIV) (Cumagun and Miedaner, 2004), that are involved in the inhibition of host resistance reactions (Jansen *et al.,* 2005; Maier *et al.*, 2006). The majority of authors assert that no wheat cultivars are immune, most are susceptible, but a few are moderately resistant (Parry *et al*., 1995 ; Cai *et al*., 2005). *Triticum durum* L. is more susceptible to FHB than common wheat (*T. aestivum* L.); therefore, its grains are more exposed to higher mycotoxin concentrations (Stack *et al*., 2002). Although *F. graminearum* and *F. culmorum* are two distinct pathogens, Mesterhazy (1995) found that wheat cultivars have very similar resistance reactions against them and this can be true for FHB, Fusarium damaged kernels (FDK), yield loss and the degree of deoxynivalenol (DON) contamination.

Wheat (durum and common) is one of the most important crops in Syria, both for local consumption and as an export commodity (NAPC, 2006). The cultivation area is divided according to agro-meteorological conditions into five sub-regions, from I to V.

There are no reports about the existence of FHB in Syria. However, *Fusarium* species were frequently isolated and identified (EL-Khalifah *et al.,* 2009). In Syrian wheat kernel samples, the

main pathogens isolated were *F. culmorum* and *F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] (Chapter I). Besides different control practices, as fungicides, biological control, agronomical aspects e.g nitrogen application, crop rotation, and tillage/stubble (Xu and Nicholson, 2009), breeding for resistance has taken a high priority worldwide, and seems to offer the most promising tool (Browne and Cooke, 2005). The resistance to FHB is quantitatively inherited in all cereal species with a highly significant genetic variation among breeding materials (Snijders, 1990; Miedaner, 1997). A cultivar may be resistant to initial infection, hyphal spread and/or to mycotoxins (Lemmens *et al.*, 1993). Moreover, Buerstmayr *et al.* (2000) reported that the cultivation of genetically resistant cultivars is the most cost-effective method to control the disease. High priority has taken to FHB resistance in cereals worldwide however, the resistance evaluation has been slow due to the necessity to avoid escapees by evaluating the whole plant resistance in different environmental conditions and over several years (Browne and Cooke, 2004).

The objectives of the present experiment were, first, to investigate varietal differences in resistance to FHB infection, FHB spread, kernel infection and mycotoxin accumulation comparing different Syrian durum wheat cultivars with an Italian susceptible one inoculated with different Syrian and Italian *F. culmorum* strains under control and field conditions and second, to evaluate the relations among FHB-resistant types in the different Syrian cultivars.

MATERIALS AND METHODS

Plant materials and fungal strains

Ten Syrian durum wheat cultivars, used frequently in different areas in Syria, were obtained from Arab Center for the Study of Arid Zones and Dry Lands (ACSAD)-Syria. One susceptible Italian cultivar (Simeto) was used as reference. All the 11 cultivars were then used for experimental trials under field and control (growth chamber) conditions.

Six *F. culmorum* strains, isolated from durum wheat kernels (3 from Syria and 3 from Italy), aggressive and belonged to 3Ac-DON chemotype, were used as artificial inoculum.

Macroconidia production

Each *F. culmorum* strain was cultured on PDA plate for 7 days and then two mycelium plugs were inoculated into a flask of 300 ml, containing 150 ml autoclaved V8 broth (Singleton *et al.*, 1992), shaken on a refrigerated horizontal type shaker at 140 rpm, 25 °C under incident sun light for two weeks. The mixture of macroconidia, mycelium and V8 medium was filtered through a sterile syringe filled with double layers of autoclaved cheesecloth. The conidial concentrations were measured with a haemocytometer and stored at 4°C. Macroconidia concentration of each strain was adjusted to $1x10^4$ conidia/ml for floret inoculations in the growth chamber and to $2x10^5$ conidia/ml for field trials. Two mixtures were prepared; the first was consisted of the three Syrian *F. culmorum* strains (F960, F961, F966) and the second was of the three Italian ones (F11, F24, F35) previously characterized for aggressiveness in (Chapter IV).

Floret inoculations in growth chamber

Seeds of ten Syrian durum wheat cultivars and one susceptible Italian cultivar (Simeto) were embedded in seed trays (160 plants/tray) filled with autoclaved potting mix medium and placed in a growth chamber (25/19 °C day/night temperature, 14/10 hr light/dark cycle). Each seedling was transplanted individually into a new pot (diameter 8 cm and 15 cm height) containing autoclaved potting mix medium and placed in a growth chamber with the conditions described previously. One week after transplantation approximately 3 g of commercial fertilizer was applied to all plants. The plants were watered three times a week until harvest to avoid water stress condition. At wheat anthesis (GS = 63-65; Zadoks *et al*., 1974), 20 µl (10 µl/floret) of each conidial suspension at a concentration of $1x10^4$ conidia/ml were injected into two florets at the middle of each spike (without wounding) and covered with polyethylene bags for 48 h to ensure constant high humidity. Eight spikes from different pots were treated with each isolate, accounting for eight replications.

Disease evaluations were carried out at 7, 14 and 21 days after inoculation (DAI). For disease severity (DS), the scale of Purahong *et al.* (2012) was used. This scale represented the percentage area infected on individual ears: 0% (no infection), 2%, 5%, 10%, 25%, 50%, 75% and 90% (infection area is 90% or more) (Figure 4.2). Mean and terminal severity (the 3rd DS evaluation, 21 DAI) were used to compare the resistance of the different cultivars in the growth chamber

experiment. Koch's postulate was fulfilled by the re-isolation of *F. culmorum* from infected kernels.

Ear inoculations in the field

The same ten Syrian durum wheat cultivars and the Italian susceptible one (Simeto) were planted in the research field of University of Bologna located in Cadriano (Emilia Romagna, Northern-Central Italy) in autumn. For each cultivar, this field was subdivided into micro-plots of double rows (1m length, 15 cm from each other row and 20 cm between two successive micro- plots). 200 seeds were sown in each micro-plot (100 for row). The total field contained 44 micro-plots; 22 inoculated with the Syrian strains mixture (11 cultivars repeated twice) and the same design was followed for the same cultivars but inoculated with Italian strains mixture (Figure 6.1). At 30% anthesis, 60 ml of conidial suspension at concentration $2x10^5$ conidia/ml were sprayed on each micro-plot by using hand sprayer. Natural rain on this day ensured high humidity, so no addition irrigation was applied.

Five groups of 10 spikes per micro-plot were chosen and marked with plastic labels for disease assessment. The disease evaluations were measured as disease severity (DS) and disease incidence (DI) at 13and 19 days after inoculation (DAI). DS was determined as described previously in the growth chamber. DI was measured as the number of ears that are visibly diseased relative to the total number assessed (50 spikes/replicate). Mean FHB Index was calculated as the product of DI and DS divided by 100 (Von de Ohe, 2010).

To determine FDK, one hundred seeds were counted manually from each replicate post- harvest for further evaluations (Mesterhazy *et al*., 1999). The percentage of scabby "tombstone" infected kernels (Figure 4.4) was estimated visually and recorded as % Fusarium damage kernel (FDK) (Mesterhazy *et al*., 1999). The Hectolitric weight was measured post- harvest also. Koch's postulate was fulfilled by the re-isolation of *F. culmorum* from infected kernels.

DON analysis

Wheat grains were ground, and DON was extracted by weighing out 20 g of each sample. This weight was put into flasks of 150 ml containing 100 ml double distilled water. Sample suspensions were placed in a rotary shaker (200 rpm) for 3 min, 2 ml of the suspension was

transferred into Eppendorf tubes and centrifuged (14000 rpm) for 5 min. One ml of the supernatant served as a stock solution for dilution preparations. Dilutions were prepared from the stock solution to optimize ELISA analysis. The ground wheat grain samples were analyzed by AgraQuant DON (Romer Labs, Austria) Kit, an enzyme immunoassay for the quantitative analysis of DON in cereals. Two replicates for each cultivar inoculated with each mixture were analyzed, and DON content was calculated using a microtiter plate spectrometer, and a software package distributed by the manufacturer. Five standard solutions in water (0,0.25, 1, 2 and 5 mg⁄kg) provided with the immunoassay kit were used in each plate as standards.

RESULTS

Floret inoculations in growth chamber

Disease severity (DS) was the parameter evaluated in the growth chamber trial due to the limited number of spikes. Jory and ACSAD1333 were the most resistant cultivars based on the terminal disease assessment at 21 DAI (28.6%, 35% respectively) whereas the most susceptible cultivars were Sham9 and ACSAD1315 (76.4, 73.6% respectively) (Table 6.1). Typical FHB symptoms were observed in the inoculated spikelets whilst in control, no bleached spikelets were present.
To study the development of disease, we plotted the means of each estimation (7, 14 and 21 DAI) of each cultivar to calculate the slope. The results ranged between 17.8 and 37.1% indicating that Jory has type II resistance while the most susceptible cultivar for the spread of disease was Sham9. The difference was significant only between the lowest and the highest values (Table 6.1 and Figure 6.2).

Ear inoculations in the field and DON analysis

The values of mean FHB index ranged between 18.6 and 38.7%, the most resistance cultivars were Jory and ACSAD1333, which showed significant difference with the most susceptible cultivar Sham9. Whereas the values of the first estimation were between 0 and 2% for all the cultivars, the second estimation showed a wider range of differences in both types of inoculum (Syrian and Italian) (Table 6.2).

The values of FDK and DON levels were variables. However, there were no significant differences between the different cultivars. FDK ranged between 30 and 46% whilst DON levels ranged between 0.33 and 2.1 ppm.

Hectolitric weight values showed diversity among the cultivars which reached significant levels; the highest value was for Sham3 (96.1 Kg/m3) while Sham5 had the lowest one (90.01 Kg/m3) (Table 6.2).

Development of the disease was also heterogeneous among the cultivars using ANOVA comparisons between the mean of the estimations: less variability in Jory (17.8%), the highest value was for Sham9 (37.1%) (Figure 6.3). The cultivars inoculated either with Syrian or Italian inoculum had the same behavior.

Comparison between the results of growth chamber and field trials

Cultivar rankings generally fluctuated from trial to trial and from parameter to parameter within the same trial. In the growth chamber trial, Jory and ACSAD1333 were the most resistant to FHB while Sham9 and ACSAD1315 were the most susceptible (Table 6.1). For the disease development Jory and ACSAD1333 had the least values while Sham 5 and Doma1 had the highest. In the field, Jory and ACSAD1333 confirmed to be the most tolerant to FHB whilst Sham9, ACSAD1315 and Sham5 were the most susceptible (Table 6.1). Horani and ACSAD1333 showed less FDK in contrast to Sham5 and Sham9 that had the highest values. DON accumulation was lower in Jory and Sham9 than in Bohoth, Horany and Simeto. The grains of Sham3 gave the best yielding , while Sham5 gave the worst (highest and the lowest hectolitric weight) (Table 6.1). Jory and Horani showed the less development of the disease whilst the disease developed sharply in Sham9 and Sham5. The correlations among the different parameters in the growth chamber and the field are reported in Table 6.3.

Mean FHB index showed high positive correlation with FDK (%) and negative correlation with hectoliteric weight ($r = 0.912$ and $- 0.772$, $P < 0.01$, respectively). Furthermore, high correlation was found between DS and FHB index ($r = 0.843$, $P < 0.01$). Disease development in the field correlated significantly with disease development in the growth chamber ($r = 0.782$, $p<0.01$). DON accumulation did not show a correlation with any other parameters.

DISCUSSION

Few studies were carried out in Syria to evaluate the FHB resistance of the durum wheat cultivars. This study provides information about FHB infection in durum wheat cultivars from different regions in Syria , newly released cultivars are also included in this study. Cultivars differed in their behaviour to FHB in both trials; growth chamber and field. Durum wheat cultivars evaluated in this study also differed in DON accumulation. However, differences among cultivars were not significant. DON accumulation level was not correlated to other parameters, and this implies that cultivars with resistance to FHB do not necessarily show low DON accumulation. This is in agreement with Wisniewska *et al*. (2004) and Chrpova *et al*. (2007) who reported that some cultivars expressed high resistance to the accumulation of DON but FHB symptoms were clearly developed. Moreover, Bai *et al.* (2001) reported that severe visual symptoms may not always be associated with high DON levels. On the contrary, Lemmens *et al*. (1997) and Perkowski and Chełkowski (1993) observed a significant correlation between resistance to FHB and DON accumulation in seeds after natural infection. The contradiction between the different finding could be interpreted as the regulation of DON accumulation is rather complicated and depends on ecological conditions in addition to host and fungal genotype (Mesterhazy *et al*.,1999). Moreover, the toxin resistance and disease resistance are two different phenomena. The behaviour of the cultivars in growth chamber and the field was similar as the most resistant and the most susceptible cultivars kept their scores under both conditions. Furthermore, disease severity in the growth chamber was higher than in the field. This might be interpreted by the fact that in the growth chamber, the humidity and temperature were adjusted to be ideal for disease development in contrast to uncontrolled conditions in the field, although in 2011, the year in which field trial was carried out, the climatic conditions were not favourable to disease incidence and spreading. Type I–resistance to initial infection by the fungus is strongly influenced by environmental conditions and type II–spread of the pathogen within the spike is regarded as a stable measure of cultivar resistance (Wisniewska *et al.,* 2004). It is necessary to combine type I and type II resistance to get FHB resistant wheat plants.

Wheat cultivars also varied in FDK, although these differences were not significant. The highly correlation between FDK and FHB index is in accordance with Mesterhazy (2002) and Wegulo *et al*. (2011). Since hectolitric weight is considered a quality parameter influenced by FHB, then it could be used to determine the tolerance of wheat cultivars. According to our results, there were a

significant difference within hectolitric weights among the cultivars and a correlation between this parameter and FHB index. Ramirez-Marchand *et al*. (2003) reported the correlation between FHB and hectolitric weight. The results from this study indicate that among Syrian cultivars, there is tolerance to FHB and DON accumulation. “Jory” has a better behavior than “Simeto”, the most cultivated cultivar in southern Italy where the climatic conditions is quite similar to some Syrian areas. However, the majorityof grown cultivars are susceptible. These results can be used to take decisions regarding the choice of cultivars to plant in order to reduce losses due to FHB and DON accumulation. Evaluation of a wider range of durum wheat cultivars grown in the region for resistance to FHB and DON accumulation will provide more choices and increased benefits to producers and to the food processing industries. In spite that FHB data in this study are only from one year, we are confident that the precautions taken into account to obtain reliable results could be efficient enough, as noticed by the consistency of the results obtained from both field and growth chamber trials. Repeating the experiments is recommended to evade escapee of cultivars.

FIGURES AND TABLES

Figure 6.1. Field experiment for studying the behavior of Syrian wheat cultivar

Table 6.1. Values of different parameters for evaluating different types of resistance in 11 Syrian durum wheat cultivars tested under field and growth chamber conditions: FHB index, Fusarium-damaged kernels (FDK), DON level, Hectolitric weight (HW), Disease severity (DS) and Disease development (growth chamber and field)

Cultivars	Field					Growth chamber	
	Mean FHB index Type I	FDK Type III	DON (ppm) Type V	HW Type IV	Disease development Type II	DS Growth chamber	Disease development
Simeto	22,2^{ab}	34^{a}	1,88^{a}	94,09^{bc}	19,94^{ab}	56,8^{bc}	21,97^{bc}
ACSAD 1315	32,6^{ab}	45^{a}	1,67^{a}	91,96^{ab}	30,17^{ab}	73,6^{c}	30,41^{c}
ACSAD 1333	20,6^{a}	32^{a}	1,18^{a}	92,87^{abc}	20^{ab}	35^{ab}	14,4^{ab}
Bohoth1	28,8^{ab}	45^{a}	2,1^{a}	93,44^{abc}	28,4^{ab}	65,8^{c}	28,74^{c}
Douma1	29,7^{ab}	41^{a}	1,41^{a}	93,58^{abc}	28,16^{ab}	70,3^{c}	30,7^{c}
Horani	21,7^{ab}	30^{a}	1,81^{a}	95,26^{bc}	19,78^{ab}	50,8^{bc}	23,75^{bc}
Jory	18,6^{a}	33^{a}	0,33^{a}	94,3^{bc}	17,8^{a}	28,6^{a}	10,87^{a}
Sham1	23,8^{ab}	39^{a}	0,75^{a}	94,73^{bc}	23,5^{ab}	58,9b^{c}	25,73^{c}
Sham3	22,2^{ab}	33^{a}	0,86^{a}	96,1^{c}	21,4^{ab}	63,8^{c}	26,58^{c}
Sham5	33,7^{ab}	46^{a}	1,09^{a}	90,01^{a}	32,3^{ab}	70,9^{c}	30,83^{c}
Sham 9	38,7^{b}	46^{a}	0,63^{a}	91,28^{ab}	37,1^{b}	76,4^{c}	30,02^{c}

* Means with the same letter within a column are not significantly different at P = 0.05 according to ANOVA test

Table 6.2. Values of FHB index in the field, 1st and 2nd evaluations, due to two inocula (Italian and Syrian strains) of *F. culmorum* for 11 durum wheat cultivars (10 Syrian and 1 Italian)

Cultivars	Syrian mix		Italian mix	
	FHB index (1st evaluation)	FHB index (2nd evaluation)	FHB index (1st evaluation)	FHB index (2nd evaluation)
Simeto	1,10	10,84	3,48	33,62
ACSAD1315	1,03	38,84	3,86	26,39
ACSAD1333	0,61	21,48	0,54	19,78
Bohoth1	0,35	23,38	0,54	34,33
Douma1	0,15	26,91	3,00	32,58
Horani	1,78	15,99	2,16	27,52
Jory	1,17	15,83	0,41	21,41
Sham1	0,36	28,14	0,11	19,42
Sham3	1,25	24,22	0,38	20,23
Sham5	1,71	34,79	1,04	32,64
Sham9	0,64	37,85	2,56	39,53

Table 6.3. Correlations among different parameters: FHB index, Fusarium-damaged kernels (FDK), DON concentration, Hectolitric weight, Disease development and Disease severity (DS) in 11 durum wheat cultivars evaluated in the field and growth chamber for resistance to FHB

	FHB index	FDK	DON (ppm)	Hectolitric weight	Disease development in growth chamber	DS
FDK%	0.912**					
DON(ppm)	-	-				
HW	0.772**	-0,744**	-		-	
Disease development in growth chamber	0,793**	0,734*	-			
Disease development in field	0,994**	0,929**	-	-0,766**	0,782**	0,828**
DS	0.843**	0.773**	-	-	0,978**	

* and ** = Significant at P< 0.05 and P< 0.01, respectively

Figure 6.2. Disease development in growth chamber for 11 durum wheat cultivars after 7 (blue), 14 (red) and 21 (green) DAI

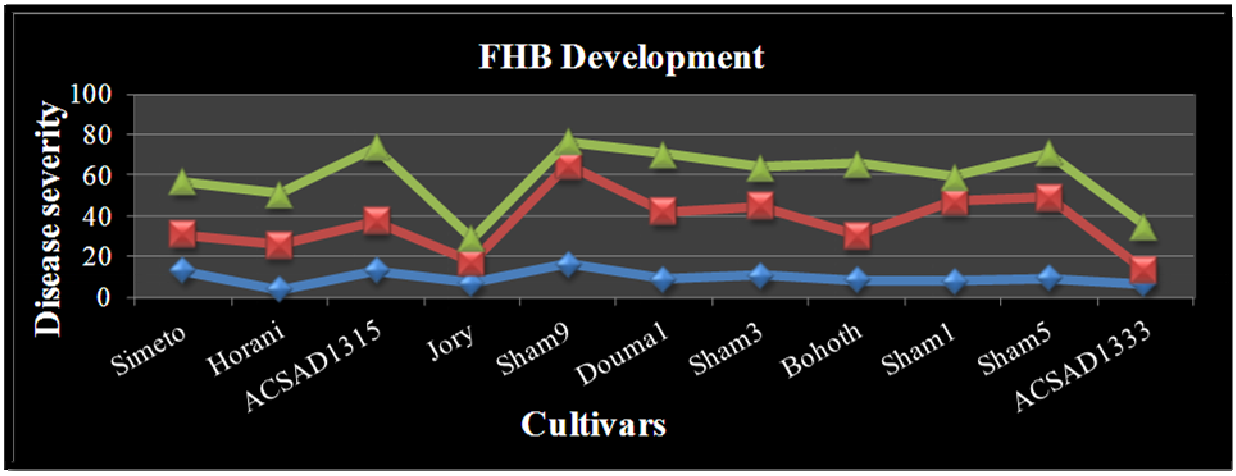
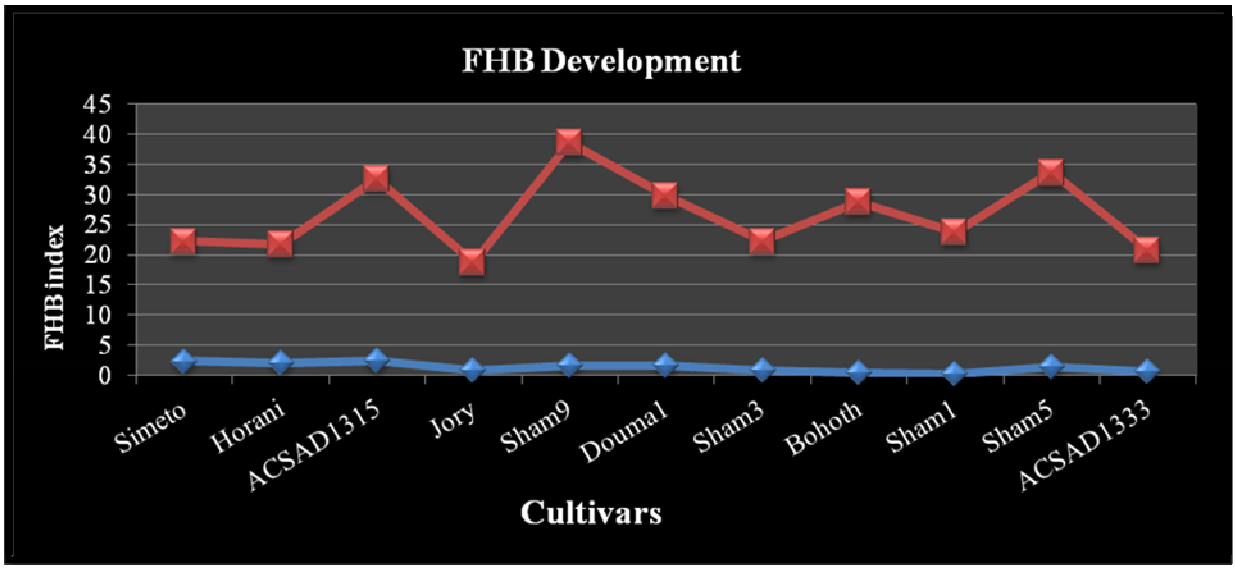


Figure 6.3. Disease development in the field for 11 durum wheat cultivars after 14 (blue) and 21 (red) DAI



GENERAL DISCUSSION

In this work, it was established, for the first time, the possibility of the occurrence of a serious FHB outbreak in Syria. We have isolated and identified, undoubtedly, FHB's causal agents in Syrian wheat samples, using different biological and molecular approaches. These *F. culmorum* strains were able to cause the disease on different Syrian and Italian cultivars under field and growth chamber conditions and to produce different typeof mycotoxins.

The morphological and molecular identification of the main *Fusarium* species associated with FHB in Syrian wheat kernel samples, collected from different regions, revealed the presence of *F. culmorum, F. graminearum, F. equiseti* and *F. pseudograminearum* while in the Italian samples, *F. graminearum*, *F. poae* and *F. culmorum*. The distribution and the prevalence of different *Fusarium* species are largely variable over the continents and regions due to environmental conditions (Logrieco *et al*., 2002b; Xu and Nicholson, 2009). The diversity between Syrian and Italian *Fusarium* species confirms the importance of the geography and the worldwide distribution, in fact differences in the distribution of alternative hosts, soil type, cultivar, cropping practice or temperature may all play a role in FHB causal agents (Jennings *et al*., 2004*;* Toth *et al*., 2005).

It is not possible to conduct a comparative study for Syrian samples because of the absence of studies related to FHB causal agents in Syria. The presence of these species in Italian samples was found by several researchers in Italy (Prodi *et al*., 2009; Shah *et al*., 2005; Pancaldi *et al*., 2010); *F. pseudograminearum* has always been associated with crown rot disease in wheat in Italy (Balmas, 1994), Turkey (Bentley *et al*., 2006) and Iran (Saremi *et al*., 2007), but only recently it has been found responsible of the major outbreaks of FHB in wheat in Australia (Akinsanmi *et al*., 2006). Miedaner *et al*. (2008) reported that *F. pseudograminearum* seemed to be restricted only to Australia as a FHB pathogen, but Kammoun *et al*. (2009) found in Tunisia this species representing 9% of the fungal complex associated with FHB in durum wheat. The association of *F. pseudograminearum* to FHB in new areas might be due to climate changes and a new source of disease and mycotoxin risks.

Pasquali *et al*. (2010) affirmed that genetic chemotyping on a large scale is a valid approach to predict mycotoxin contamination in wheat, this aspect is also confirmed in this present work. The chemotyping showed diversity among *F. graminearum* and *F. culmorum* in Syria and Italy. In the tested Syrian *F. graminearum* strains, there was a prevalence of NIV chemotype. This finding is

in accordance with Haratian *et al*. (2008) who reported that the majority of *F*. *graminearum* isolated from cereals grown in Iran, another area of Middle–East, was NIV chemotype. This result is not in accord with the data obtained in Europe, where the prevalent chemotype was 15Ac-DON. In Italy, Prodi *et al*. (2009) found 15Ac-DON predominant over 3Ac-DON and NIV, as well as in the Netherlands (Waalwijk *et al*., 2003), England and Wales (Jennings *et al*., 2004), USA (Gale *et al*. 2007) and southern Russia (Yli-Mattila *et al*., 2008), whilst 3Ac-DON chemotype was predominant in western Russia and Finland (Yli-Mattila *et al*., 2008) .

Syrian isolates of *F*. *culmorum* belonged to 3Ac-DON and NIV chemotypes; while the Italian strains belonged exclusively to 3Ac-DON. Most probably the limited number of Italian *F*. *culmorum* strains analysed in our study might be the cause of non appearance of NIV chemotype. Several studies detected 3Ac-DON and NIV chemotypes of *F*. *culmorum* in several European countries, like Germany (Muthomi *et al*., 2000), Italy (Prodi *et al*., 2011b), Norway (Langseth *et al*., 1999) and France (Bakan *et al*., 2001).

Interestingly, the majority of Syrian *F*. *equiseti* strains possessed a gene encoding trichodiene synthase, which is necessary for trichothecene production, gene not frequently present in Canadian isolates (Demeke *et al*., 2005). The studies about *F*. *equiseti* chemotypes are few since the presence of this species on wheat is sporadic (Bottalico and Perrone, 2002; Pancaldi *et al*., 2010).

The screening of mycotoxin production by Syrian *Fusarium* strains on wheat media revealed the tendency of these isolates to produce conventional mycotoxins rather than emerging ones.

ZEN production characterized all the tested strains of *F. graminearum, F. pseudograminearum* and *F. culmorum*. The same results were obtained by Bakan *et al.* (2002) on French *F. culmorum* tested strains. Furthermore, Al Mugrabi *et al*. (2011) found that all 59 German and French *F. graminearum* tested strains produced high amounts of ZEN. Blaney and Dodman, (2002) reported in their study in Queensland that all *F. pseudograminearum* and most *F. graminearum* strains produced ZEN on culture. In Europe, DON and ZEN were the most frequently encountered *Fusarium* mycotoxins in FHB induced by *F. graminearum* and *F. culmorum* (Bottalico and Perrone, 2002).

The quantification of ZEN reflected its presence in almost all the strains of the different *Fusarium* species associated to FHB (CHAPTER I).

The *in vitro* production of trichothecene B corresponded to the chemotypes previously identified. In fact, *F. culmorum* and *F. graminearum,* belonging to NIV chemotype, produced NIV and/or its acetylated form FUS-X while the remaining of *F. culmorum* strains, belonging to 3Ac-DON chemotype, produced 3Ac-DON, 15Ac-DON and DON. Tokai *et al*. (2008) reported that the production of small amounts of 15Ac-DON was common in strains belonging to 3Ac-DON chemotype due to acetylation of 15-hydroxyl of DON by the product of *Tri3*. Bakan *et al.* (2002), in the study carried out in France, reported that *F. culmorum* strains, belonging to 3Ac-DON chemotype, were able to produce DON. Syrian *F. culmorum* strains, belonging to NIV chemotype, produced different amounts of DON and/or its acetylated forms, as also reported by Al Mugrabi *et al*. (2011). This characteristic could be accounted as an enzymatic conversion step of NIV to DON or as a bi-product of biosynthesis (Al Mugrabi *et al*., 2011). The quantification is extremely important to acquire a definite picture of mycotoxin expression in the different *Fusarium* strains since the co-presence of the different chemotypes cannot be detected with molecular techniques and allows to define whether the plant products are adequate for human and animal consumption (Al Mugrabi *et al*., 2011).

The survey on mycotoxin content in Syrian and Italian wheat kernels has confirmed the presence of mycoflora on tested samples (CHAPTER I). *Fusarium* species were more frequent in the Italian samples, that were characterized by the contamination with both emerging and conventional *Fusarium* mycotoxins. DON and 15Ac-DON were the prevalent trichothecene B toxins with a limited level of NIV. This finding affirms the general concept that DON is the most common trichothecene found in cereal grains (Xu and Nicholson, 2009). In our study, the co-occurrence of ZEN and DON is in accordance with Chrpova *et al*. (2007) who reported that high DON content could be an indicator of high ZEN level. In Syrian samples the storage fungi, i.e. *Aspergillus* spp. and *Penicillium* spp., were isolated in higher percentage and the main mycotoxins detected were aflatoxins and ochratoxin (Reyneri, 2006). The absence of *Fusarium* species in some of the analysed samples (CHAPTER I) with the detection of *Fusarium* mycotoxins could be interpreted as an effect of post-harvest fungicide use in Syria.

The aggressiveness of several Syrian *F. culmorum* strains, belonging to both chemotypes, was assessed and the role of DON accumulation in pathogenesis was also evaluated, in order to estimate the risk of *Fusarium* strains in a possible future occurrence of FHB outbreak in Syria, especially in the areas where the agronomical practices are changing (introduction of irrigation).

The knowledge of aggressiveness of fungal strains is fundamental in breeding assays for testing the pathogen resistance of a host/genotype. In this work we observed no significant differences in aggressiveness between Syrian and Italian *F. culmorum* strains, and the absence of this diversity can be explained by the low level of genetic differentiation among *F. culmorum* populations (Miedaner *et al*., 2008). The data obtained from different assays indicated that DON production is proportional to disease development, and enabled us to classify the different Syrian strains of *F. culmorum* according to their aggressiveness.

The aggressiveness of the strains had the highest values in Petri dish test and the lowest in the field trial. These data could be explained by the different artificial inoculation methods performed in this experimental work (CHAPTER IV). Optimal humidity and temperature, suitable environmental conditions, for disease development (Parry *et al*., 1995; Birzele *et al*., 2002; De Wolf *et al.*, 2003) are difficult to be ensured under fluctuated and uncontrolled field environment versus the stable and controlled ones in the growth chamber and the Petri-dish test. Therefore, this method with its remarkable stability of results, reliability, rapidity and low-cost could be considered a plausible test for breeding purposes. This method has been already validated for *F. graminearum* by Purahong *et al*. (2012) and it will be possible to validate the modified Petri dish test for *F. culmorum* too.

The control of FHB, in years of a high pressure of natural inocula, is not risolutive with only good agronomic and chemical managements (Xu and Nicholson, 2009; Pancaldi *et al*., 2010). The adoption of resistant cultivars is the best method for FHB control (Parry *et al*., 1995; Bai and Shaner, 2004), but it is difficult to obtain FHB resistant cultivar, due to polygenic features. In our study, the evaluation of FHB resistance was based on different parameters, DS, FDK, HW and DON level. The positive correlation between visual disease symptoms and DON accumulation has been a debate in previous studies where the symptoms and DON contents could (Mesterhazy, 2002) or could not (Edwards *et al*., 2001) be related to each other. Xu *et al*. (2007) hypothesized that this inconsistency might be due to a variable competition and toxin-producing capability between and within pathogen species in field samples. Furthermore, other researchers suggested that in some cases resistance to FHB and DON accumulation may not depend from each other (Arseniuk *et al*., 1999). Our data show that the most tolerant cultivar ,"Jory", had the lowest DON level while the most susceptible, "Sham9", had low DON content. "Jory", with constant behavior under field and growth chamber conditions as well as the ability to accumulate a low

amount of mycotoxin, could be promising, but further research should be carried out to investigate these cultivars more thoroughly over several years and under different environments

In conclusion, our current study has proved the presence of FHB causative agents with high capability to infect and accumulate mycotoxins in different Syrian cultivars under different conditions. We draw the attention over the risk of FHB and its hazardous mycotoxins in Syria, especially with the increasing trend to irrigation and the changes of agronomical practices. These two factors are considered as key players in enhancing the occurrence of this disease. In view of the great risk that poses the FHB ,we would like to point out the importance and the need to establish a national Syrian allowable limits for *Fusarium* mycotoxins in food and feed, particularly for baby foods, who are the most vulnerable and the most susceptible.

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