

Molecular Detection of *Fusarium* spp. Producing mycotoxins on wheat samples from Syria

**Thesis Submitted to Damascus University in Partial Fulfillment of
M.Sc. Degree in Biotechnology**

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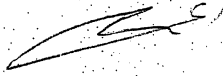
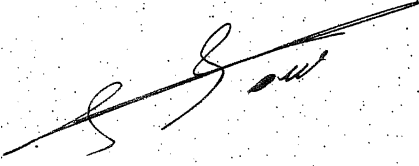
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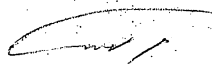
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Declaration

I hereby declare that research work described in this thesis entitled "**Molecular Detection of *Fusarium* spp. Producing mycotoxins on wheat samples from Syria**" has not been, and is not actually submitted to obtain any other university degree, and that all experiments and results indicated are of my own realization under the direction of my scientific supervisors. Sources of any other data, methods or results have been clearly mentioned in the text and in the reference list.

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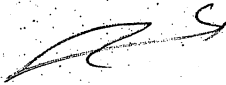
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Abstract

Fusarium head blight (FHB) is a common and worldwide disease of wheat which has a negative impact on grain yield and quality, FHB often implies production of mycotoxins (trichotecenes, fumonisins, zearalenone) that pose a threat to human and animal health.

Several *Fusarium* species were detected in wheat samples from Syria in the 2006 and 2007. Morphological and molecular identification using *Fusarium* species-specific PCR of single spore cultures was performed. The morphological classification for the isolates suggested the presence of at least nine *Fusarium* spp., which are by order of predominance:

F.avenaceum, *F.culmorum*, *F.crookwellense*, *F.graminearum*,
F.equiseti,

F.sporotrichoides, *F.acumonatum*, *F.oxesporum*, *F.triticeum* .

Whereas the molecular detection was done for the dominant species:
F.avenaceum, *F.culmorum*, *F.crookwellense*, *F.grameniarum*.

These four species were also tested for their ability to produce Deoxynivalenol and their aggressiveness in the field, comparing with German isolates, the field experiment took place in two locations (Hohenheim and Eckartsweire) and two different cultivars (Taifun and Amaretto) were used to be inoculated by the isolates .

The most aggressive species were *F.culmorum* (Ger.), *F.culmorum* (Syr.), and *F.graminearum* (Ger.) respectively, while *F.avenaceum* (Syr.), *F.graminearum* (Syr.), and *F.cereales* (Syr.) were less aggressive than the other species. Also the Variation in aggressiveness between isolates from different gene pools was rather high and significant.

On the other hand, the interaction between isolates and the two locations showed a significant difference, the results showed also a significant difference between the two cultivars.

The ability of the Syrian *F. graminearum* and *F. culmorum* isolates to produce trichothecenes was determined using chemotype –specific PCR markers, 15-acetyledoxynivalenol was more frequent in our isolates than 3-acetyledoxynivalenol . Syrian *F. graminearum* and *F. culmorum* production of deoxynivalenol in the field experiment was measured by ELISA assay . Isolates of *F. culmorum* produce high levels of deoxynivalenol (up to 53 ppm) whereas *F. graminearum* produce a low levels of deoxynivalenol (0 to less than 4.72 ppm).

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List of Abbreviations:

Abridgement	Meaning
Ab	Antibody
Ag	Antigen
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EWE	Eckartsweire
FHB	<i>Fusarium</i> head blight
HOH	Hohenheim
HPLC	High Performance Liquid Chromatography
LSD	Least significant difference
NIV	Nivalenol
PCR	Polymerase chain Reaction
PDA	Potato Dextrose Agar
ppm	Part Per Million
SNA	Synthetic nutrient agar
UV	Ultra Violet
ZEA	Zearalenon

1. Introduction

1.1. Importance of wheat in Syria:

Wheat is considered one of the major crops all over the world; it has an increasing demand for its importance as a foodstuff.

Wheat production in Syria occupies an important part in agricultural production, which has been noticeably increased through introduction of a high productive and environmentally adapted varieties by many local, Arab or international institutions such as GCSAR, ACSAD and ICARDA. Wheat production in Syria was highly decreased in the last few years (Table 1) , due to the unfavorable environmental conditions, which encouraged the development of plant diseases that decrease the quantity and the quality of wheat yield.

Table .1. Development of wheat production in Syria between the years 2000 and 2007.

*(Source: Ministry of Agriculture and Agrarian Reform, 2008).

Production /Tons/*	2000	2001	2002	2003	2004	2005	2006	2007
Irrigated	2396573	2744203	3275222	3421429	3392660	3471110	3567671	3130010
Non-Irrigated	707089	2000420	1500220	1491564	1144799	1197636	1363854	911090
Total	3105489	4744623	4775442	4912993	4537459	4668746	4931525	4041100

The alteration in the environmental conditions, especially the late rainfall accompanied with high temperature in spring that associated with wheat flowering, formed suitable conditions for development of many pathogenic and saprophytic fungi such as, *Alternaria* spp., and *Fusarium* spp.

The first suspicion of with FHB infection (on a harmful scale) was in 2002 in wheat production area in Syria and showed atypical symptoms of FHB (just white empty heads). No attempt was undertaken to determine the causative species, nor was there any attempt to investigate mycotoxin production . In 2003 a small scale survey In Al-Gahb took place and some species were identified (*F.graminearum*, *F.culmorum*, and *F.avenaceum*) (Talas, unpublished data) but also without any estimation of mycotoxins. The ocurrent study aimed to test the aggressiveness including DON producing (the most important secrete mycotoxin by *Fusarium* spp.) of some *Fusarium* species isolated from FHB infected wheat samples from Syria, and this study is a separate part of an embracement wider project with other objective and more isolates (Talas, personal communication). All the study was performed in the state plant-breeding institute at Hohenheim University – Germany.

1.2. *Fusarium* head blight:

Fusarium head blight (FHB) is considered a devastating fungal disease of wheat and other small-grain cereals all over the world (Windels, 2000) and is found in both temperate and semi tropical regions (Eeuwijk, 1995).

The first detailed description of this disease was made in England at the end of the 19th century; it was considered a major threat to wheat and barley during the early years of the twentieth century (CPC, 2003).

FHB has been recorded in many countries in the world:

- In America: Canada (1994), USA (1997).
- In Europe: Italy (1980), Germany (1984), France (1995).
- In Africa: Egypt (1989).
- In Asia: it was recorded in Iran (1989), Turkey (1993), Japan (1994), Saudi Arabia, (1995). (CPC, 2003).

The host species of this pathogen include a wide range of important crops such as wheat, barley, maize, rice, oats and other small grain cereal crops (Goswami *et al.*, 2004).

Fusarium head blight affects the developing heads of small grains directly, and causes losses that exceed 45 % in some years with a severe infection of FHB. *Fusarium* head blight also negatively affects the grain quality, often resulting in a mycotoxin contamination.

A number of reports had highlighted the effect of a severe FHB epidemic. Schaafsma *et al.*, (1998) reported that 85 % of the winter wheat crop of southern Ontario, Canada was destroyed by this disease in 1996, resulting in losses of more than \$100 million.

In the United States, FHB has reached epidemic levels in several years during the last decade, causing yield losses and price discounts resulting from reduced seed quality (Windels, 2000). Direct and secondary economic losses due to FHB for all crops in the Northern Great Plains and Central United States were estimated to be \$2.7 billion from 1998 to 2000 alone (Nganje *et al.*, 2002).

In European cereal cultivation areas, a reduction of 10 to 30% in crop yield has been calculated (Bottalico, 1998). Losses as high as 45% reported on several infected crops in China, Approximately 6.7 to 7.0 million hectares in 20 provinces in China, representing 25% of the total wheat acreage, are affected by FHB (Wang, 1996; Chen *et al.*, 1997). Also in Japan about 4 -7% of the total wheat production area, is affected by FHB in an average year, more than 40% of the crop was damaged and yields were reduced by 20% (Li *et al.*, 2000).

1.2.1. Life cycle of Fusarium head blight:

Primary inoculum for this disease comes from infected plant debris, on which the fungus over winters as a saprophytic mycelium. In spring, warm moist weather conditions enhance the development and maturation of conidia and perithecia that produce ascospores concurrently with the flowering of cereal crops (the complete phase of this fungus reported just in North America) (Markell and Franci, 2003).

The sticky ascospores are forcibly discharged from mature perithecia (Trail *et al.*, 2002) formed on the surface of crop debris (e.g. corn stubble) and dispersed by wind, rain or insects to host plants (Parry *et al.*, 1995; Sutton, 1982). Figure (1):

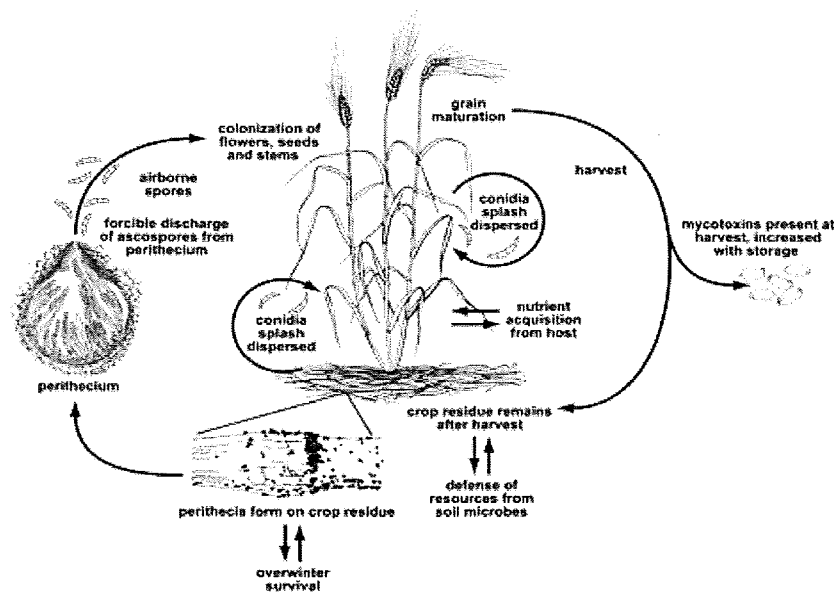


Figure 1: Life cycle of *F. graminearum* (sexual phase, *G. zeae*)

A comprehensive review of the FHB infection process was published recently (Bushnell *et al.*, 2003) which can be briefly summarized as: the deposition of spores on or inside spike tissue initiates the infection process; the anthers in wheat heads are the most susceptible to infection during the flowering stage (Sutton, 1982). The fungus initially does not penetrate directly through the epidermis. Rather, hyphae develop on the exterior surfaces of florets and glumes, allowing the fungus to grow toward stomata and other susceptible sites within the inflorescence (Bushnell *et al.*, 2003).

Hyphae may also form peculiar lobed structures between cuticle and epidermal cell wall on the surface of inoculated glumes (Pritsch *et al.*, 2000). Such subcuticular growth on the glume, lemma and palea is thought to serve as a mechanism for fungal spread and, in the case of adaxial floret surfaces, probably leads to direct penetration of epidermal cells (Bushnell *et al.*, 2003).

Other avenues for direct entry include stomata and underlying parenchyma, partially or fully exposed anthers, openings between the lemma and palea of the spikelet /floret during dehiscence (Bushnell *et al.*, 2003) and through the base of the wheat glumes where the epidermis and parenchyma are thin walled. Once inside the floret, the anthers, stigmas and lodicules are most easily colonized. The principal mode of fungal spread in wheat from floret to floret inside a spikelet and from spikelet to spikelet is through the vascular bundles in the rachis and rachilla (Ribichich *et al.*, 2000).

Several changes in the vascular bundles have been noted that may cause the xylem and phloem tissues in the infected rachis to become at least partially dysfunctional leading to premature death of the spikelet. Under wet conditions mycelia can spread over the exterior surfaces of the glume, lemma and palea in both wheat and barley (Bushnell *et al.*, 2003). The fungus appears to have a brief biotrophic relationship with its host before switching to the necrotrophic phase. This necrotrophic stage is associated with an increase in vigour of colonization by the fungus and eventually plant death leads to thorough colonization of the host substrate.

1.2.2. Symptoms:

Early infection reduces both size and number of kernels, and a few or no kernels develop if heads were inoculated at flowering, whereas progressively larger kernels develop in head inoculated at successively later times after flowering (Edwards, 2004).

Infected spikelets quickly lose chlorophyll and become pale in color, later they turn pink or peach colored, especially at the base and the edges of the glumes, if humidity continues to be high, diseased spikelets are invaded by saprophytic fungi and turn dark or black shortly before harvest (Goswami *et al.*, 2004). The uninfected part of the head above the point of infection often dries and dies, apparently because water and food supply are cut off (Edwards, 2004).

FHB of small-grain cereals is not a single-species problem like powdery mildew (*Erysiphe graminis*), leaf blotch of barley (*Rhynchosporium secalis*) or leaf blotch of wheat (*Septoria tritici*) but can be caused by up to seventeen different organisms. It is therefore often considered to be part of a complex of cereal diseases caused by several *Fusarium* species (Parry *et al.*, 1995).

Species of the genus *Fusarium* do not only cause severe losses of grain yield and quality reduction but *Fusarium* spp. can also produce secondary metabolites, which can accumulate in the plant and grains in the field. Many of these fungal metabolites are toxic to man and animals (D'Mello *et al.*, 1999).

1.3. Mycotoxins:

Mycotoxins are toxic secondary metabolites produced by toxigenic molds, and they are classified as secondary metabolites, since their biosynthesis is not required for fungal growth and reproduction (Desjardins, 2006).

Almost all toxigenic molds produce spores and are widely distributed in the environment. They can contaminate and produce mycotoxins in human food or animal feed during production, transport, processing and storage. Consumption of mycotoxins by humans or animals causes adverse health effects such as liver and kidney damage, cancer, and death.

Some mycotoxins are not destroyed during physical processing such as milling, baking, malting or freezing so mycotoxin-contaminated crops

may result in significant economic and commercial losses. For example most of the *Fusarium* mycotoxins are relatively resistant to heat. Zearalenone was not destroyed in corn, even after 44 h, at 150 °C (Bennett and Klich, 2003). Major mycotoxins; which include aflatoxins, trichothecenes, and fumonisins that are produced by various *Aspergillus* spp, and *Fusarium* species.

1.3.1. *Fusarium* mycotoxins :

The main *Fusarium* species responsible of mycotoxins production are:

F.graminearum, *F.culmorum*, *F.avenaceum*, *F.poa* (Marasas, 1991). The produced mycotoxins include the fumonisins, the trichothecenes, (including deoxynivalenol DON), and zearalenone (ZEA) (Marasas *et al.*, 1984). Some *Fusarium* strains are capable of producing mycotoxins, which can accumulate either pre, or post harvest or in stored grains (Botallico, 1998).

Biosynthesis of the mycotoxins depends on many factors, especially the strain of the pathogen, the substrate, the period of colonization of the substrate, temperature, and moisture (Sinha, 1996). The two primary factors, which influence the growth and mycotoxin production of fungi, are temperature and moisture (Ramirez *et al.*, 2003).

Species differ substantially in their optimal environmental conditions (optimal moisture level or water activity w.a) for mycotoxin production. In addition to the optimal water activity (wa), each fungus has an optimal temperature for mycotoxin production, which may differ from the optimal temperature for growth. For example, diseases caused by *Fusarium* in cereals are associated with different species under various conditions (Magan *et al.*, 1984).

The risk for mycotoxins contamination is related to the timing of the humidity determines the risk for mycotoxin contamination. Rainfall at the time of crop's anthers sensitivities encourage FHB infection and subsequent mycotoxin contamination (Sutton, 1982; Langseth and Elen, 1997; Inch and Gilbert 2003).

The concentrations of a trichothecene mycotoxin including deoxynivalenol (DON), in Norwegian oats were affected by the annual variations of weather. The highest amounts detected were after warm and dry springs with heavy rainfalls at the time of the mid flowering. On contrary, the cold summers with moderate rainfalls led to a lower level of DON contamination (Langseth and Elen, 1997).

In Finland, cold and rainy summers have led to the presence of lower levels of DON than those occurring in warmer and partly dryer growing seasons (Yli-Mattila *et al.*, 2005).

1.3.2. Trichothecenes :

The trichothecenes are a group of mycotoxins that are produced by a variety of different *Fusarium* species; Trichothecenes known to be produced by at least 24 different species of *Fusarium* (Marasas *et al.*, 1984).

The most important four species (from the range of produced mycotoxin point of view) are *F.equiseti*, *F.graminearum*, *F. moniliforme* and *F.sporotrichioides* . The trichothecenes are the most chemically diverse of all mycotoxins. They are all tricyclicsesquiterpenes with a basic 12,13-epoxy-trichothec 9-ene ring system Figure (2). They can be designated into four subclasses: **Type A** having a functional group other than a ketone at position C-8. **Type B** having a ketone at position C-8. **Type C** having a second epoxy group at C-7, 8 or C-9, 10 and **Type D** containing

a macrocyclic ring between C-4 and C-5 (Bottalico, 1998; Visconti *et al*, 1999).

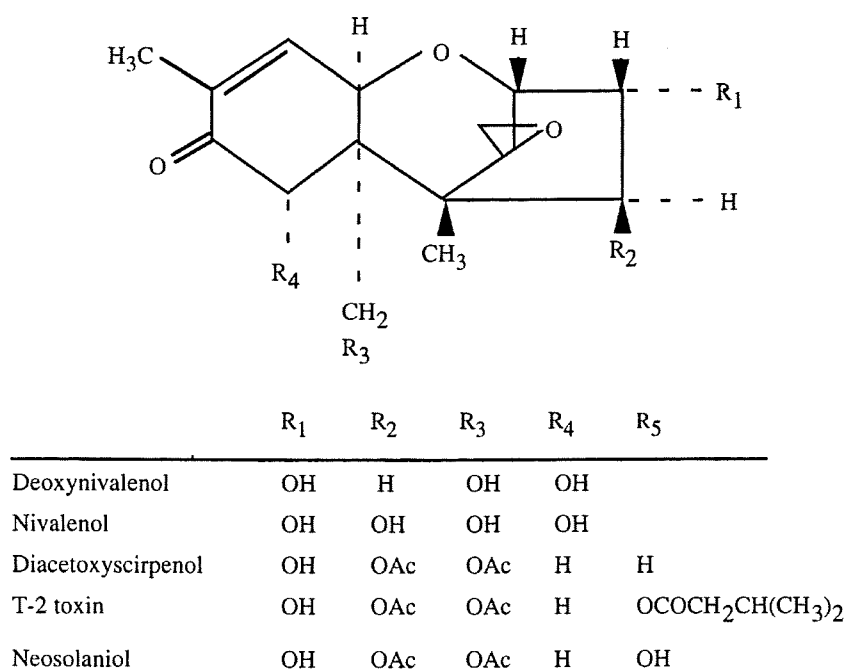


Figure .2. Chemical structure of some trichothecenes.

1.3.2.1. Deoxynivalenol:

Deoxynivalenol and its metabolites, 15-acetyldeoxynivalenol and 3-acetyldeoxynivalenol are members of a group of sesquiterpenoids known as the trichothecenes. This secondary metabolite (Figure 2) is characterized by the ketone moiety at the C8 position, which distinguishes Group B from Group A trichothecenes. Deoxynivalenol is heat-stable, survives processing (milling), and does occur in food products and feeds prepared from contaminated corn and wheat.

The most common producer of DON are *Fusarium graminearum* and *F. culmorum* (Marasas *et al.*, 1984), in addition to zearalenone, these *Fusarium* species can produce nivalenol, diacetoxyscirpenol, and other

trichothecenes (Bennett *et al.*, 1987). Deoxynivalenol is also the most important trichothecene because of its high incidence in cereals, including maize, wheat, barley, oats, etc., but it is not one of the most acutely toxic of this group of mycotoxins (Rotter *et al.*, 1996). At the cellular level the main toxic effect is inhibition of protein synthesis via binding to ribosomes. In animals, the main overt effect at low dietary concentrations (> 2 mg/g in feed for pigs) appears to be a reduction in food consumption (anorexia) and weight gain, while higher doses (> 20 mg/g) induce feed refusal, diarrhea and vomiting. Deoxynivalenol is known to alter brain neurochemicals, and the serotonergic system appears to play a role in mediation of the feeding behaviour and emetic response. Low doses of toxins in animals fed are able to recover from initial weight losses, while higher doses induce more long-term changes in feeding behaviour. Swine is more sensitive than other livestock species to the presence of deoxynivalenol in their feed. (Prelusky, *et al.*, 1994, Rotter, *et al.*, 1996).

1.3.4. T-2 toxin:

T-2 toxin is the most toxic of the *Fusarium* trichothecenes, although it is less widely distributed than deoxynivalenol. In pigs clinical signs of T-2 toxicosis include emesis, posterior paresis, lethargy, and frequent defecation. At natural levels of contamination in the diet T-2 toxin causes reduced feed intake and animal performance. At high concentrations in the diet, it produces diarrhea, emesis and feed refusal. T-2 toxicosis in poultry causes oral lesions, reduced feed consumption and growth rate in young animals and reduced egg production in laying hens (Beardall and Miller, 1994).

The impacts on poultry production become important at dietary concentrations above 2 mg/kg. In ruminants T-2 toxicosis results in a wide range of responses, such as feed refusal, leukopenia, depression, diarrhoea, coagulopathy, enteritis, and posterior ataxia. Reduction of humoral immunity is a common effect for pigs, poultry and ruminants exposed to low concentrations of T-2 toxin in the diet.

This increases susceptibility to other diseases and the effects of poor management practices. Most of the clinical signs caused by the ingestion of T-2 toxin are also observed with diacetoxyscirpenol and, at slightly lower extent, with HT-2 toxin (Prelusky *et al.*, 1994).

1.3.3. Zearalenone:

Zearalenone, a non-steroidal estrogenic mycotoxin, and its major metabolic products (a-zearalenol and b-zearalenol) are depicted in Alphazearalenol (Figure 3), the more estrogenic metabolite, is formed in humans and swine in greater amounts than in rats. This mycotoxin is a resorcylic acid lactone, originally isolated and crystallized from laboratory cultures of *F.graminearum* (Anthony, 1992).

Zearalenone is insoluble in water and heat-stable, and it persists in both human foods and animal feeds prepared from contaminated grains. This toxin is the only known phytoestrogen produced by fungi and is unique in that one of its derivatives, zeranol (a-zearaleanol), is useful commercially as a growth promoter in cattle. *Fusarium graminearum* (*Gibberella zeae*) and *F.culmorum* are the major zearalenone-producing species and are distributed worldwide (Marasas *et al.*, 1984).

F. crookwellense isolates from different geographical areas were compared for their ability to produce mycotoxins on corn in laboratory cultures and it showed the ability to produce nivalenol, 4-acetylnivalenol and zeralenone (Vesonder *et al.*, 1991; Sugiura *et al.*, 1994).

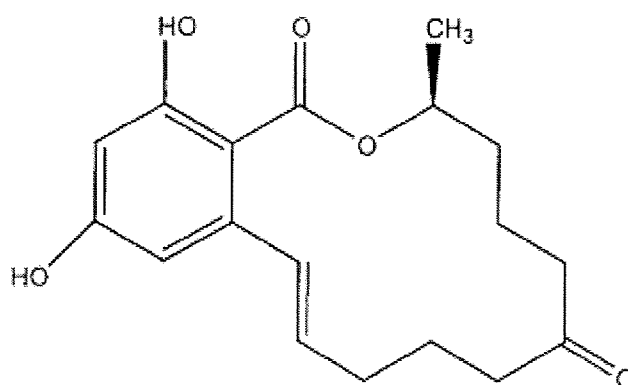


Figure .3. Chemical structure of Zearalenone.

1.3.4. Fumonisin:

Fumonisin is a group of mycotoxins mainly produced in maize grown under warm and dry conditions by *F. moniliforme* and *F. proliferatum*. Fumonisin B1 (FB1), B2 (FB2) and B3 (FB3) (Figure 4), are the major compounds present in maize fungal cultures and have been shown to occur naturally at biologically significant levels in maize and a variety of maize-based human foodstuffs and animal feeds in several countries throughout the world (Visconti *et al*, 1996), Fumonisin (particularly FB1 and in some cases FB2) has been shown to cause fatal toxicoses in animals (Marasas, 1995; Caramelli *et al.*, 1993).

The ingestion of fumonisin-contaminated maize has been associated with spontaneous outbreaks of equine leukoencephalomalacia, a neurological syndrome characterized by focal, often extensive,

liquefactive necrosis of the white matter of the cerebrum, and with acute pulmonary edema in pigs, a fulminant swelling of the lungs and thoracic cavity (Marasas, 1995).

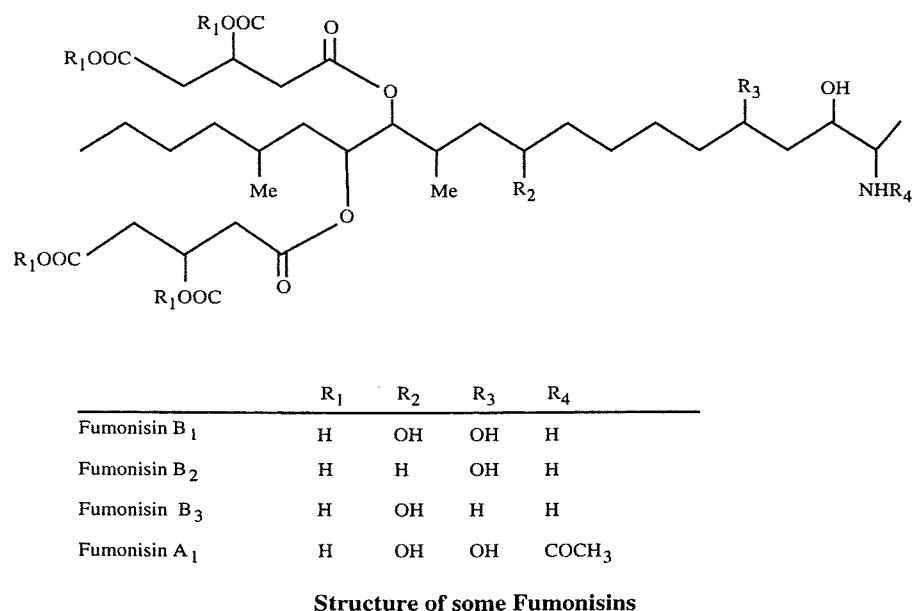


Figure .4. Chemical structure of some Fumonisins.

1.3.6. Toxic effects of *Fusarium* mycotoxins:

In a number of animal species, mycotoxins are considered to be toxic because of their effect on the synthesis of lipids present in nerve cells. However, this impact on mammals varies depending on the species, giving rise to symptoms such as a loss of appetite or energy, damage to the nervous system. (Ueno, 1983).

In human, a link is suspected between a high level of consumption of wheat contaminated by mycotoxins in certain regions of the world and the development of cancers of the esophagus. These toxins also have certain influence on the nerve cells and the Immune system since it amount of DON case a significant decrease of IgA, IgM and IgG in the animal test (Goyart *et al.*, 2006).

1.3.7. Legal limits for DON:

The U. S. Food and Drug Administration, has established advisory levels for DON-contaminated grain and grain-based products as follows:

- 1 ppm DON on finished wheat products, e.g. flour, bran, and germ, that may potentially be consumed by humans. The FDA has not established an advisory level for wheat intended for milling because the manufacturing practices and technologies available to millers can substantially reduce DON levels in the finished wheat products from those found in raw wheat. Because of significant variability in manufacturing processes, an advisory level for raw wheat is not practical.
- 10 ppm DON on grains and grain by-products destined for ruminating beef and feedlot cattle older than 4 months and for chickens with the added recommendation that these ingredients not exceed 50% of the diet of cattle or chickens.
- 5 ppm DON on grains and grain by-products destined for swine with the added recommendation that these ingredients not exceed 20% of their diet.
- 5 ppm DON on grains and grain by-products destined for all other animals, with the added recommendation that these ingredients not exceed 40% of their diet.

1.3.8. Trichothecene biosynthesis and evolution:

Much of the biochemical characterization of the trichothecene toxin biosynthetic pathway has been conducted in the fungus *Fusarium sporotrichioides*. Comparative studies have shown very similar genes

operative in *F. graminearum* except where differences would be expected based on acetylation patterns of the trichothecenes (Kistler *et al.*, 2004; Lee *et al.*, 2002) or oxygenation at positions C-4 and C-8 (Lee *et al.*, 2002; McCormick *et al.*, 2004; Meek *et al.*, 2003).

Three strain-specific profiles of trichothecene metabolites, called 'chemotypes' (Anderson *et al.*, 1989), have been observed for individuals of the *F. graminearum* species complex and related species (Ward *et al.*, 2002). Strains produce predominantly either DON or its C-4 oxygenated derivative, nivalenol; DON producers also make acetyl ester derivatives of DON at the three-, and 15-position oxygen. Thus, three trichothecene chemotypes are observed; NIV (nivalenol producers), 3ADON (DON producers that also make predominantly 3-acetyldeoxynivalenol) and 15ADON (DON producers that also make predominantly 15-acetyldeoxynivalenol). The 'Tri cluster' (Figure 5) contains a central core of genes involved in the biosynthesis of the basic trichothecene structure (Kimura *et al.*, 2003).

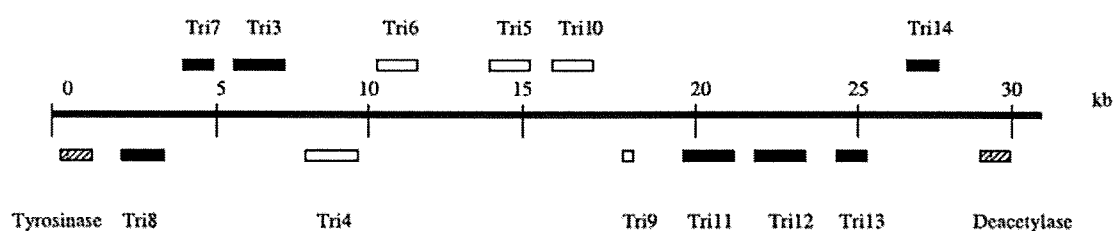


Figure .5. Diagram of the trichothecene biosynthetic (Tri) gene cluster.

1.3.9. Analytical methods of *Fusarium* mycotoxins:

The chemical diversity of mycotoxins and their varying concentration in a wide range of agricultural commodities, foods and biological samples poses a great challenge to analytical chemists. The different chemical and physicochemical properties of the mycotoxins require specific extraction, cleanup, separation and detection methods. There were two groups of methods used to analyze *Fusarium* mycotoxins, chromatographic methods and immunologic methods.

The chromatographic analytical method for mycotoxins in feed and foodstuff generally require toxin extraction from the matrix with an adequate extraction solvent, a clean-up step intended to eliminate interference from the extract, and finally, detection of the toxin by suitable analytical instrument technologies.

1.3.9.1. High performance liquid chromatography (HPLC):

High performance liquid chromatography with diode array detection (HPLC-DAD) is an efficient tool for chemical fingerprinting of fungal isolates and it is the most widely used laboratory methods to analyze for fumonisins and DON with detection by ultraviolet (UV) absorbance or fluorescence (Sydenham *et al.*, 1996; Trucksess *et al.*, 1996).

DON is extracted from grain samples with acetonitrile /water (84/16) and the sample extract is cleaned up by passing the extract through a clean-up column, concentrated under nitrogen, and an aliquot is analyzed by HPLC. Quantitation is based on UV absorbance at 220 nm (Bennett and Richard, 1994). This method gives high sensitivity results in short analytical time but it is more expensive than other methods such as ELISA.

1.3.9.2. Thin layer chromatography:

Thin layer chromatography (TLC), is a good methods for identifying and quantifying deoxynivalenol (DON), fumonisin, and zearalenone in grain samples. Thin layer chromatography (TLC) is a widely used method of screening for mycotoxins because it is simple, fast and has a low cost (Soares, 1992).

1.3.9.3. Gas–chromatography:

Gas–chromatography (GC) works similarly to other chromatographic methods, and is used for detecting a broad spectrum of *Fusarium* metabolites that have often been restricted to cover one or few biosynthetic families, e.g. trichothecenes. Comparing with the other method, (GC) is much more laborious and not easy to set up an integrated part of characterization and identification of *Fusarium* isolates (Betina, 1993).

1.3.9.4. Enzyme Linked Immuno Sorbent Assay (ELISA):

ELISA principle depends on the immunological assays. ELISA tests are based on serological identification of *Fusarium* mycotoxins in grain samples. These tests are popular because they are relatively inexpensive, easy to use, and their results can be obtained in a just few minutes, the development of ELISA to detect trichothecene mycotoxins in food and grain samples has been proposed and now is available for different mycotoxin from different companies (Pestka, 1995)

2. Objectives:

Deep studies are required about *Fusarium* head blight in Syria to establish a good comprehension of the local situation of this disease. Therefore the objectives of this study were:

- 1- isolate *Fusarium* head blight pathogens from infected wheat samples in Syria.
- 2- identify isolated *Fusarium*. spp by morphological and molecular methods
- 3- classify Syrian isolates of *Fusarium* for their ability to produce mycotoxins and their aggressiveness in the field.

3. Materials and methods:

3.1. Sampling:

Sampling was done on five wheat production areas in Syria, which we suspected to be infected representing favorable conditions for FHB infection (Figure 6).

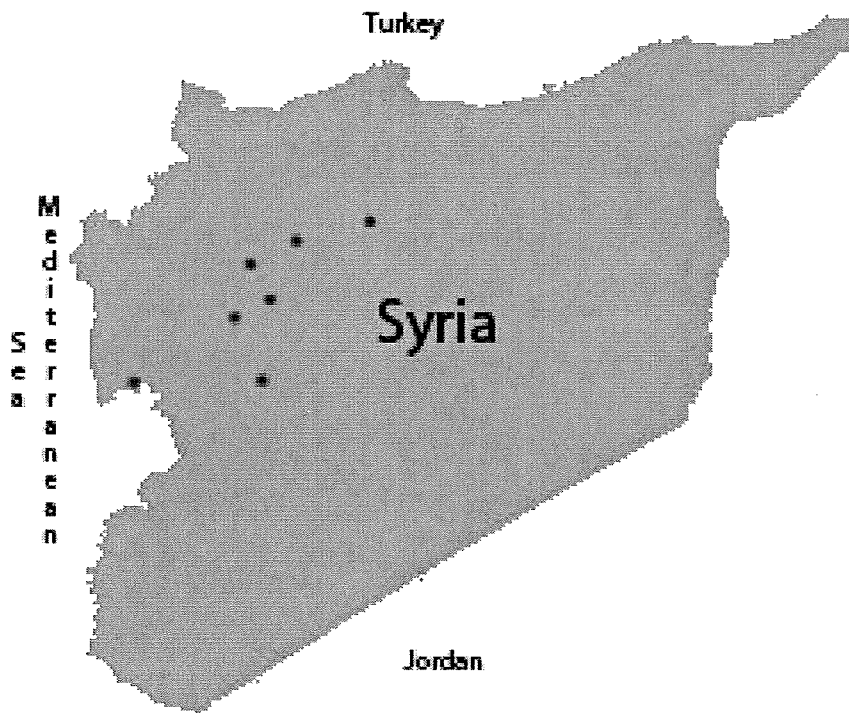


Figure .6. Shape shows the distribution of sampling areas in Syria.

These areas were visited in the years 2006 and 2007 during the flowering stage of wheat (April), Collection of samples was performed according to the primary symptoms and the information from the farmers in these fields (Talas, personal communication) (Table 2).

Table .2. Show the number and year of the samples collection.

Nr.	Area	Nr. of sampled fields	Year
1	Al-Ghaab	6	2006, 2007
2	Hama	4	2007
3	Aleppo	4	2007
4	Idleb	4	2007
5	Homs	5	2006, 2007

From each zone four to six fields were chosen to represent the area. Three to five samples of 15 heads have been collected from each field depending on the area of the field (from each field we took samples from the front of the field, from the middle, and from the end).

Samples were dried for one week at room temperature, then kept refrigerated at -20 °C until use.

3.2. Isolation and morphological identification:

Spikelets were disinfected externally using 2% Na-hypochlorite and 0.8% Streptomycin to prevent the growth of external pathogens, then Spikelets were washed by sterilized water.

Thereafter spikelets were dried on clean tissue and placed onto sterilized potato dextrose agar (PDA), 5 to 6 spikelets from each head was taken and placed in one Petri dish.

These samples were incubated for one week on 25 °C to enhance the growth of *Fusarium* spp on PDA. The next step was to carry *Fusarium* isolates from infected spikelet and re-inoculated them into a low nutrient medium (Synthetic nutrient agar SNA).

One single spore transferred onto a new SNA plate, we considered this plate as a source for two other plates, one was placed on PDA plate to conserve our isolate to continue this study, and the second one was placed on small SNA plate and but under UV light for 4 days to encourage macroconidia spores formation which is important for the morphological classification of the isolates using light microscopy.

The identification was done mainly depending on macroconidial characters (shape, size, number of septa and shape of the apical and basal cells). An impression should be developed on the overall spores neglecting the rare or unusual shapes or sizes. Other character kept in consideration is the existence or nonexistence of the microconidia besides to its shape. Also, for the microconidia, the difference between monophialides and polyphialides has been checked in a microscope water mount.

3.3. Molecular identification:

3.3.1. Nucleic acid extraction:

Each isolate was placed on PDA plate and incubated for 7 days to obtain the fresh mycelium. A suitable quantity of fresh mycelium (150 mg) was harvested and transferred into sterilized 1,5 ml Eppendorf (microcentrifuge) tube containing 300 μ L of TES extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS) and acid-washed, sterilized sea sand or 0.5-mm glass beads, after the contents were homogenized using hand-held disposable homogenizer, an additional 200 μ l TES was added containing 50 μ l Protinase K, the mixture was incubated at 65 °C for 30 min. One-half volume of 7.5 M ammonium acetate was added and incubated on ice for 10 min, then we centrifuge for 15 min at maximum speed.

The supernatant was mixed with one volume ice-cold isopropanol and incubated at -20 °C for 1-2 hour. After centrifuging the mixture for 10 min at max speed the pellet was washed for two times with 70% cold Ethanol then air-dried on clean sterile paper by turning the tubes upside-down for 10-15 min. The extracted DNA was eluted from the pellet with 1X TE buffer (10mM Tris-HCl pH 8, and 1mM EDTA).

3.3.2. Polymerase Chain Reaction (PCR):

PCR test with species-specific primers was performed to certify the initial taxonomy according to the following table (Demeke *et al.*, 2005) (Table 3):

Table 3: Species specific primers used in molecular identification.

<i>Fusarium</i> spp.	Primer name	Primer sequence 5'to3'	Size
<i>F.graminearum</i>	Fg 16 F	CTCCGGATATGTTGCGTCAA	400-500 bp Variable
	Fg 16 R	GGTAGGTATCCGACATGGCAA	
<i>F.culmorum</i>	Fc01 F	ATGGTGAACCTCGTCGTGGC	570 bp
	Fc01 R	CCCTTCTTACGCCAATCTCG	
<i>F.avenaceum</i>	Fa F	GCTAATTCTTAACCTACTAGGGGCC	220 bp
	Fa R	CTGTAATAGGTTATTTACATGGGCG	
<i>F.crockwellense</i>	CroA F	CTCAGTGTCCACCGGTTGCGTAG	842 bp
	CroA R	CTCAGTGTCCCAATCAAATAGTCC	

PCR for single species detection was performed in 0.2 ml tubes with 25µl of the reaction mixture containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2 mM of each dNTPs, 0.4 µm of each oligonucleotide primer, and 0.75 units of Taq DNA polymerase. DNA amplification performed using an initial denaturation temperature of 95° C for 3 min, and then 38 cycles of 95 °C for 30 sec, 62 °C for 20 sec and 72 °C for 45 sec. followed by a final extension at 72 °C for 5 min, PCR was carried out in a Biorad thermocycler.

Amplified products were separated by electrophoresis in 1.5% wt/vol agarose gels stained with 0.2 µg/ml ethidium bromides. The final products visualized on Transilluminator U.V. supplier.

3.3.3. Determination of *Fusarium* spp. chemotype:

Primers (Tri303F/Tri303R, Tri315F/Tri315R) were used to determine DON chemotypes (Waalwijk *et al.*, 2003) (Table 4).

Amplification reactions were carried out in 20 µL volumes containing 5–20 ng fungal DNA. The reaction mixture consisted of 80 mM each of dATP, dCTP, dGTP and dTTP, 0.2 mM of each primer, and 1 unit Taq DNA polymerase in 1x PCR buffer with 1.5 mM MgCl₂. A negative control, containing all reagents but no DNA, was used in every set of reactions. PCR was carried out in a Biorad thermocycler. The annealing temperature was (57°C) for 30 cycles. The cycling conditions consisted of denaturation (94°C) for 30 s, annealing for 30 s and extension (72°C) for 60 s followed by a final extension of (72°C) for 5 min. Then the samples were cooled at (4°C) until recovery. PCR products were separated by electrophoresis through 1.5% agarose gels. Gels were stained with 0.2 µg/ml ethidium bromide and photographed under UV light.

Table .4. The sequences of chemotypes *Fusarium* mycotoxins primers.

Name of the primers	Sequence (5'-3')	Size (bp)	Reference
Tri303F/Tri303R	GATGGCCGCAAGTGGAGCCGGACTGCCCTATTG	583	Jennings <i>et al.</i> (2004)
Tri315F/Tri315R	CTCGCTGAAGTTGGACGTAA GTCTATGCTCTCAACGGACAAC	863	Jennings <i>et al.</i> (2004)

3.4. Field trail:

Four species *F.avenaceum*, *F.culmorum*, *F.graminearum*, *F.crookwellense*, were chosen to be tested for their aggressiveness in the field conditions.

Selection of 12 isolates from each *F. graminearum* (Fg), *F.culmorum* (Fc), *F.crookwellense* (Fckw), and *F.avenaceum* (Fa) were prepared to be inoculated on spring wheat in the field conditions. For that purpose these isolates were inoculated into a liquid media containing: KH_2PO_4 (0,2%), KNO_3 (0,2%), KCl (0,1%), Mgso_4 (0,1%), Microicons (0,1%), with concentration 0,02g/100mg, and saccharose (0,2%) to enhance the isolates to produce more macroconidial spores.

We used one SNA plate of each isolate as an initial start to inoculate the Liquid media separately. The inoculated media were put on a rotary shaker until the maximum amount of spores produced, as a last step we adjusted the concentration of spores in each isolate to 2×10^5 spore/ml by means of Hemacytometer. The adjustment was made by the addition of water, and then the inoculums were kept in - 80 °C deep freezer till the time of field inoculation.

The experiment was planned following the split plots design with three replications by two growing areas: in Hohenheim and Eckartsweire (Badenwürttemberg – Germany). Two different spring wheat varieties were used in this experiment, (Taifun) which has a medium resistance towards FHB and (Amaretto) which has a relatively higher resistance towards FHB disease.

Each variety grown on two rows with one meter distance separating each block from the other by two rows of Triticale (Triticale hexaploide Lart.) that provide a good fence to the experiment's blocks due to its height.

The inoculation were performed by hand for 216 block in each area using air pressure 3 Bar {12 Syrian isolates of *Fg*, *Fc*, *Fckw*, *Fa*, and *F.graminearum* (FgHOH) from Germany, and *F.culmorum* (FcHOH) from Germany}.

The inoculation date took place when 50% of wheat flowering for each of (Taifun) and (Amaretto) wheals there is about one week difference between the two.

Readings of isolate's aggressiveness was performed in 15 days following the inoculation and for five times with 2 days intervals taking into consideration the percentage of infected heads in each block and the percentage of infected spikelets within the head itself.

3.5. Statistical analysis:

The results of aggressiveness test were obtained from three replications in each growing area (HOH, and EWE), the average of five readings (with interval of 2 days between each other) were analyzed using Plabstat program (Utz, 2005).

3.6. Analysis of Deoxynivalenol by ELISA:

Wheat samples were harvested from the field experiment, from Hohenheim and Eckartsweire for ELISA test.

3.6.1. Samples preparation for ELISA:

All the samples were grounded until it became fine powder then we kept the ground samples frozen until usage (-20 °C). 5 g of each sample was taken and put in 200 ml flasks and 100 ml of sterilized H₂O was added. We Shacked the flasks while filling in the water, then we put the flask on shaker for 5 minutes, 1000 µl of each sample was taken and kept into Eppendorf tube, these tubes were centrifuged for 5 minutes.

Supernatant was transferred into other signed Eppendorf-tube and it was kept frozen until usage for the ELISA-test.

3.6.2. ELISA test:

Commercial kits from R-biopharm company were used to perform ELISA test; each kit contains one Microtiter plate with 48 wells coated with anti-deoxynivalenol antibodies. The Deoxynivalenol standards include four concentrations 0 ppm (zero standard), 0.222 ppm, 0.666 ppm, 2 ppm, 6 ppm, the steps for ELISA test were as followed:

- 50 µl of standards or prepared sample were pipeted in the wells.
- 50 µl of enzyme conjugate was added to each well.
- 50 µl of anti-deoxnivalenol antibody solution was added to each well, mixed it gently and incubated for 5 min at room temperature.
- The mixture was dumped out of the wells and tapped it into a clean filter towel (three times) to remove all remaining liquid from the wells.

- The wells were washed three times with the washing buffer (tween 20), the washing step repeated two more times.
- 100 µl of substrate / chromogen was added to each well, we mix it gently by shaking the plate manually and incubated for 3 min at room temperature in the dark.
- 100 µl of stop solution was added to each well, we mix it manually and measure the absorbance at 450 nm.

ELISA test has been performed by Elisa reader (Tecan –sunrise).

4. Results and discussion:

4.1. Morphological identification:

About 200 *Fusarium* isolates were obtained from the wheat samples that we collected from different areas in Syria (cooperation with Talas) (Table 2).

Isolation was made from seeds with suspected symptoms of FHB. Different *Fusarium* species from the wheat samples were morphologically identified. Morphological identification was done based on standard morphological traits, such as conidium characteristics and micro-conidiophore morphology, shape and septation of macroconidia, along with cultural characteristics on PDA, such as pigmentation, colony morphology, density of aerial and medial mycelium and spore shape and type (Nelson, 1983; Seifert, 1996).

The morphological classification of the isolates suggested the presence of at least nine *Fusarium* species. (Figure 7):

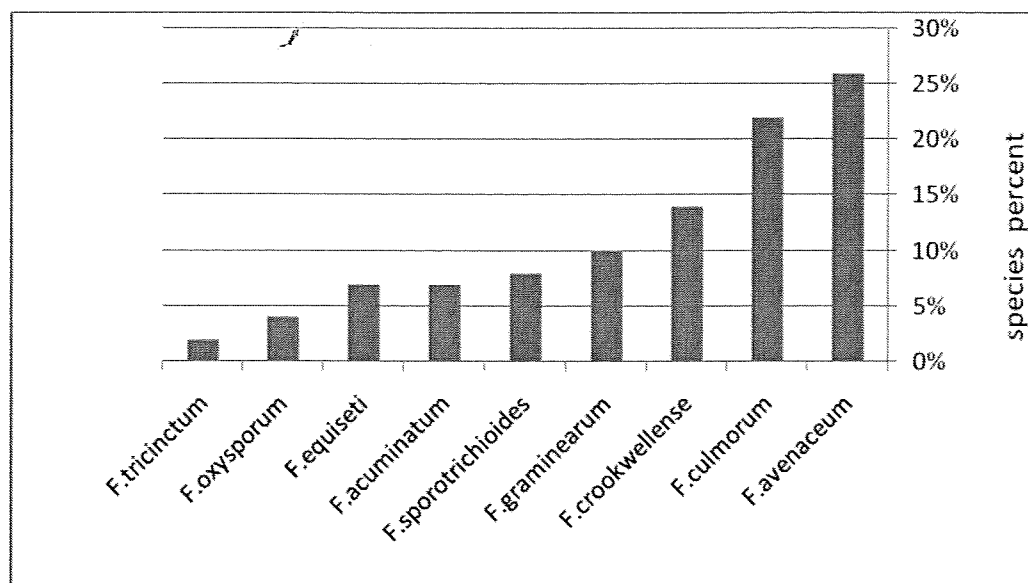


Figure 7: Percentage of *Fusarium* species within the 2006 and 2007 collection.

The presence of *Fusarium* species is mainly influenced by weather conditions like temperature and humidity (Brennan *et al.*, 2005).

In the two seasons (2006 and 2007) the warm and humid weather conditions during (April and May) encouraged the development of the species, *F.avenaceum*, *F.culmorum*, *F.crookwellense*, *F.grameniarum* and *F.equiseti*.

The same species within similar environmental conditions have been reported in south Italy (Bottalico and Perrone, 2002) and considered as the major cause of FHB (Stack, 1999).

Our results partially agree with previous studies in Syria about FHB on wheat. In 2005 Alazem, had collected wheat samples from Alghab area in Syria. *Fusarium* species from these samples were identified by morphological methods. Dominant species resulted by that study were: *F. moniliforme*, *F. culmorum*, *F. proliferatum* and *F. equiseti* (Alazem, 2007).

Whereas Kubrusli (1997) found that the dominant species were: *F.culmorum*, *F.nivale*, *F.grameniarum* and *F.equiseti*.

In North America, the three species of fungus most often responsible for the disease are *Fusarium avenaceum*, *F.graminearum*, and *F.culmorum* (Symons *et al.*, 2002). Also the *Fusarium* species predominantly found associated with Fusarium head blight (FHB) in wheat and other cereals all over Europe are: *F.graminearum*, *F.avenaceum* and *F.culmorum*.

Among the less frequently encountered species are several others, which are less pathogenic or more opportunistic, but also toxigenic, those include *F.cerealis*, *F.equiseti*, *F.sporotrichioides*, *F.tricinatum* (Wakulinski and Chekowski 1993; Bai and Shaner 1994; Parry *et al.*, 1995; Arseniuk *et al.*, 1999; Bottalico and Perrone, 2002).

We used in our molecular study 124 on *Fusarium* isolates representing the predominant four *Fusarium* spp., and have the most economic importance (Table 5).

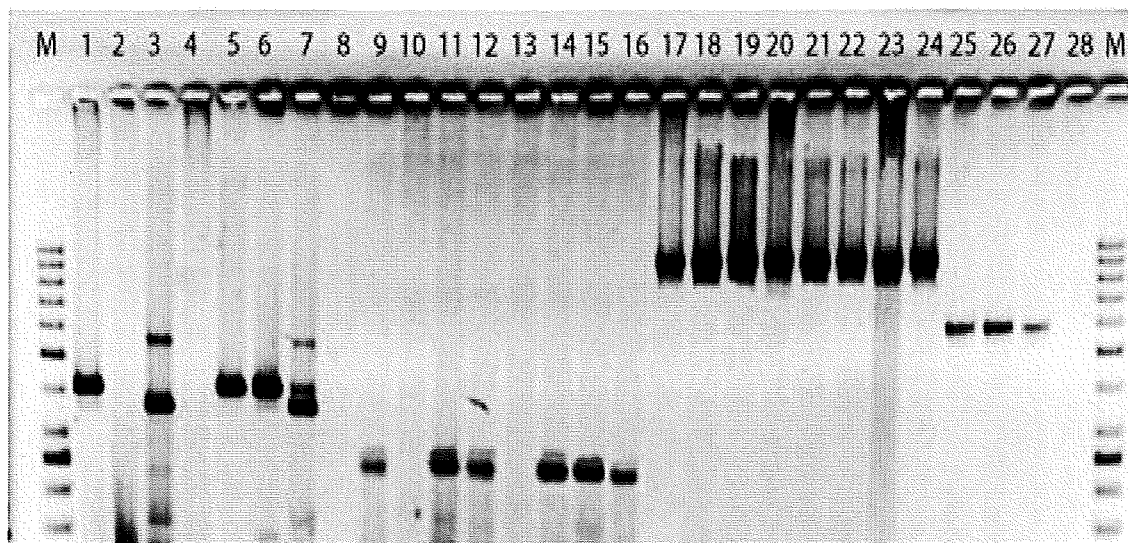
Table .5. Distribution of the 124 Syrian isolates within the predominant *Fusarium* spp.

<i>Fusarium</i> spp	<i>F.avenaceum</i>	<i>F.culmorum</i>	<i>F.graminearum</i>	<i>F.crookwellense</i> .
Nr. of isolates	43	35	26	20

1.1. Species -specific Polymerase Chain Reaction (PCR):

PCR test using species-specific primers (Demeke *et al.*, 2005), was performed on the four main *Fusarium* species classified as *F.avenaceum*, *F.culmorum*, *F. graminearum*, *F.crookwellense*.

Isolates of *F.crookwellense* yielded 842-bp DNA fragment using the primer set (CroAF/R). The primer (Fg16F/R) has amplified a different fragments all of it rang between 400-500 bp (exactly as mentioned by Demeke *et al*, 2003 and Nicholson *et al*,1998) , 20 isolate from our initial *F.graminearum* group (morphologically identified totaling 26 isolates). This pair of primers according to Demeke *et al.*, (2005) amplified the products from *F.graminearum* lineage 1, 2, 6 and 7. Lineage 7 was recovered only in North America where no amplification can be obtained from the lineage 3, 4, and 5. In our results no amplification was obtained from the other 6 isolates, those isolates need further study using other *F.graminearum* specific primers to conferm their identification or to put it under other species close to graminearum in morphology. *F.colmorum* species-specific PCR using the primer set (Fc01 F/R) amplified the expected 570-bp DNA fragment in 30 isolate from the 35 inicial *F.colmorum* group (Figure 8).



*Samples: 1 to 8 *F. graminearum*; 9 to 16 *F. avenaceum*; 17 to 24 *F. crookwellense* and from 25 to 28 *F. culmorum*.

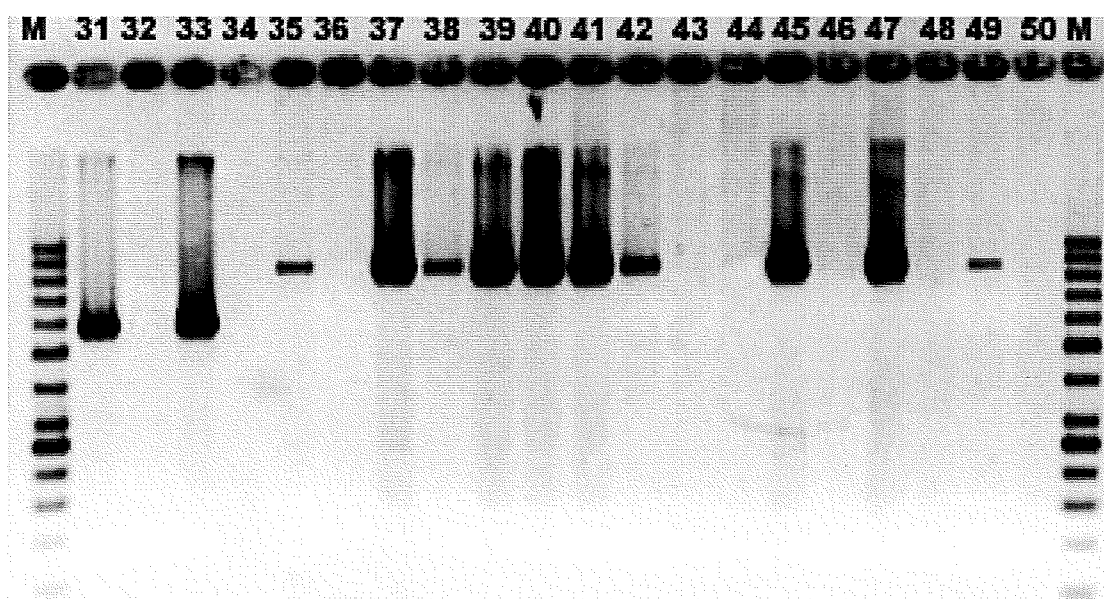
Figure. 8. Species-specific PCR products* of four *Fusarium* species (*F. culmorum*, *F. graminearum*, *F. avenaceum* and *F. crookwellense*) in wheat.

F. avenaceum species-specific primers (FaF/R) amplified the expected 220-bp DNA fragment in 30 isolates, the other 13 isolates considered within the same species may belong to *F. acuminatum*, because of the high similarity between these two species.

However, there are other primers reported to be specific to *F. avenaceum* that may detect these isolates (Doohan *et al.*, 1998).

1.2. PCR assay for *Fusarium* spp. mycotoxins chemotype:

The potential ability of twelve Syrian isolates of each of *F.graminearum* and *F.culmorum* to produce trichothecenes was evaluated using chemotype-specific PCR markers (Table 4).



*Samples 31to 33 :3-acetyledioxynivalenol; 34 water control; 35to 50 15-acetyledioxynivalenol.

Figure .9. PCR products* of *Fusarium* spp. mycotoxins chemotyping.

In our results, 15-acetyledoxynivalenol was more frequently produced by examined isolates compared to 3-acetyledoxynivalenol (Figure 9), whereas three isolates (Fc7, Fg 9 and Fg10) have none of these two chemotypes (Table 6) the thing suggesting it to be NIV producer because *F. culmorum* and *F. graminearum* are able to produce NIV, 3Ac-DON, and 15Ac-DON (Chandler *et al.*, 2003; Quarta *et al.*, 2005).

Table .6. Mycotoxin chemotypes of two *Fusarium* spp.

FC isolates	3-DON	15-DON	FG isolate	3-DON	15-DON
FC1	-	+	Fg1	-	+
FC2	-	+	Fg2	-	+
FC3	+	-	Fg3	-	+
FC4	-	+	Fg4	+	-
FC5	-	+	Fg5	-	+
FC6	-	+	Fg6	-	+
FC7	-	-	Fg7	-	+
FC8	-	+	Fg8	+	-
FC9	+	-	Fg9	-	-
FC10	+	-	Fg10	-	-
Fc11	-	+	Fg11	-	+
Fc12	-	+	Fg12	-	+

Gale *et al.* (2003) found that, North American's strains of *F.graminearum* predominantly produced 15ADON as their major acetylated form of DON. A small numbers of isolates from North Dakota and Minnesota obtained in 1999 and 2000 produced 3ADON as their major acetyl derivative (Gale *et al.*, 2003).

Results of this study agree with the studies mentioned in the Report of the EU Commission, the most common trichothecene from group B is 15ADON, followed by nivalenol, and 3-acetyldesoxynivalenol (Schothorst, 2003).

Whereas in another study in China, a large number of *Fusarium graminearum* isolates representing all regions in China with a known history of Fusarium head blight, were assayed using PCR to ascertain their trichothecene mycotoxin chemotypes. As a results they found that 3-AcDON was the predominant chemotype in their isolates (Jing-Bo *et al.*, 2007).

4.4. Field trial:

The results of aggressiveness test were obtained from a field trial planted in two sites (Hohenheim and Eckartsweire) with three replications in each growing area, and the average of five readings (with intervals of 2 days) has been analyzed using Plabstat program (Utz, 2005) (Table 7):

Table .7. Means percentage and rang of head blight aggressiveness rating for each group of isolates in Hohenheim (HOH) and Eckartsweier (EWE).

*Fusarium group	HOH		EWE	
	Mean%	Range %	Mean%	Range %
FC Ger	32.29	4 – 61.67	12.47	4.40 – 41
FG Ger	26.90	4.33 – 51.67	11.56	5.20 – 29.60
FC Syr	18.41	4.33 – 58.33	9.21	2.80 – 59.60
FA Syr	15.62	4.33 – 31.67	9.20	1 – 34.60
FcKW Syr	11.62	3 – 27.67	8.65	3.20 – 27.40
FG Syr	10.30	2.67 – 33.33	7.91	1.20 – 19.60

*FG Syr: *F. graminearum* (Syria), FC Syr: *F.culmorum* (Syria), FG Ger: *F.graminearum* (Germany), FC Ger: *F.culmorum* (Germany), FCKW Syr *F.crookwellense* (Syria), FA Syr: *F.avenaceum*.

We notice that the most aggressive groups according to the average for each Fusarium group were *F.culmorum* (Ger.), *F.graminearum* (Ger.) and *F.culmorum* (Syr.) respectively (Figure 10). While *F.avenaceum* (Syr.), *F. graminearum* (Syr.), and *F.crookwellense* (Syr.) showed obviously less aggressiveness than the other species. Putting in consideration the few number of single-spore randomly selected Syrian

isolates and selectively chosen of German isolates, this is the first pathogenicity comparison between such groups, this

kind of work could be continued to obtain more comprehensive information.

The other explanation of this difference is that some Syrian isolates were not fully adapted to the environmental factors existing in the experimental field in Germany. Aggressiveness results of *F.culmorum* (Syr.) in the German field conditions is comparable with results of Jasenka *et al.*, (2008) when they made artificial infection of ten winter wheat cultivars with *F. culmorum* and showed high severity levels of Fusarium head blight and negative effect on yield, as well as high DON accumulation. These results are also comparable with the results of Mesterhazy (1995). Variation in aggressiveness in the first location (HOH) between isolates from different gene pools was rather high and significant at level 5% (variance component was 100,02) with a high repeatability (99.68). This indicates that the observed variance in aggressiveness is related to genetic variation between the isolates and not because of the environmental factors prevalent at the experiment sites. Figure 10 shows that the aggressiveness of six groups (average of 12 isolates rating) in Hohenheim, the variation in this location evidently higher than in Eckartsweire.

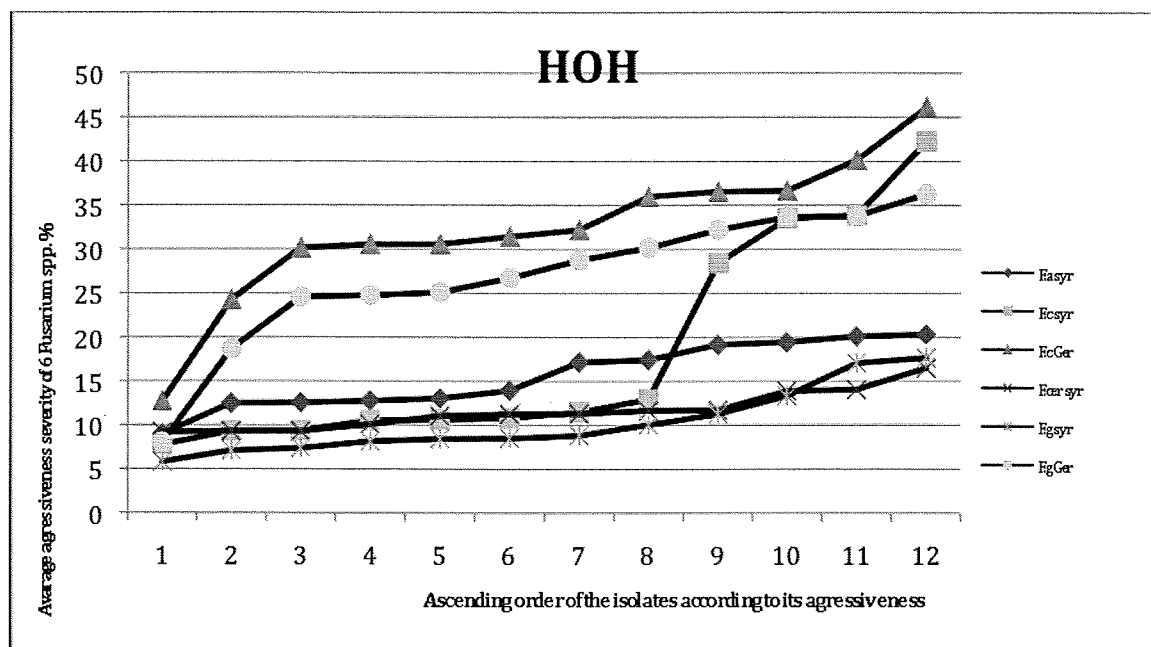


Figure .10. Comparison between *Fusarium* spp. populations from Germany and Syria in HOH.

On the other hand, variation between the isolates in the second location (EWE) was not so wide as in HOH with a significant variance component (8,09) and high repeatability (97,81). In spite of this, the species kept the same order of aggressiveness severity (as a small population or group) (Figure 11).

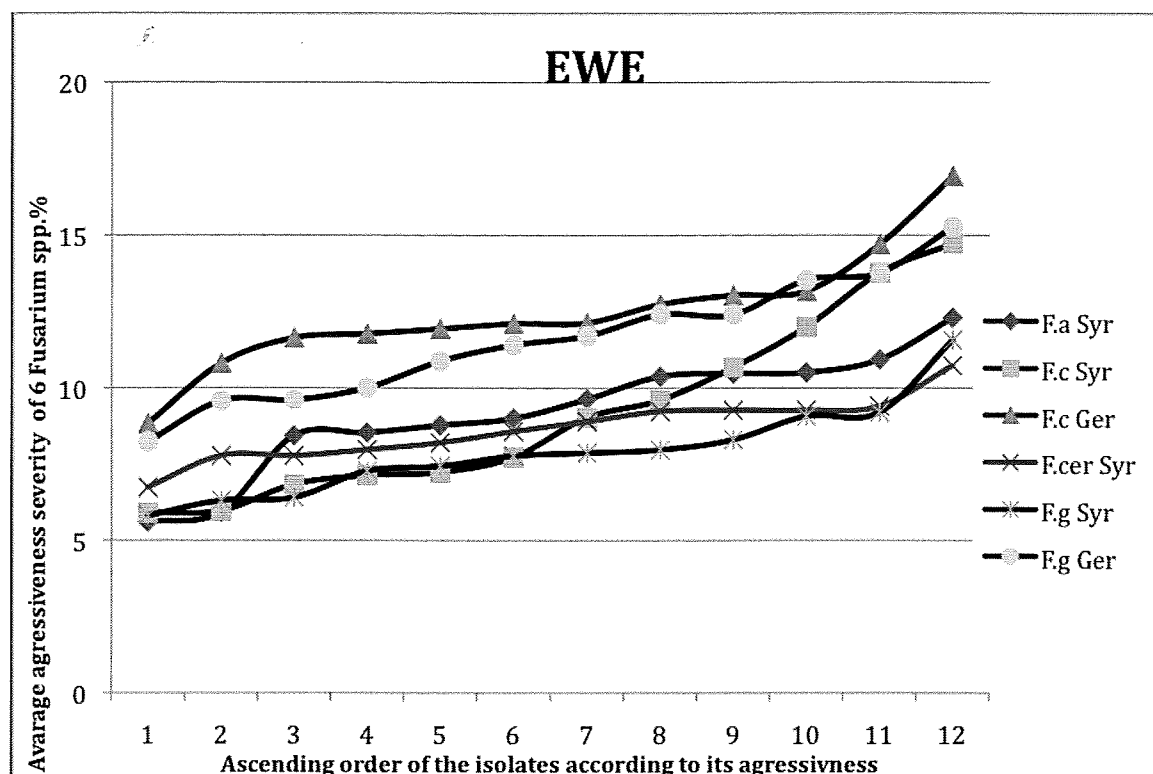


Figure .11. Comparison between *Fusarium* spp. populations from Germany and Syria in EWE.

The interaction between isolates and locations showed a significant variation between HOH and EWE, this was due to the delay of wheat sowing in EWE (because of late rainfall) this did not permit an optimal differentiation between the isolates (Table8):

Table 8: statistical analysis with plabstat over the two locations.

	Source	Var.cp	F	s.e	LSD5	Rep./Her.
HOH	Isolates	100.025	15.10**	5.8	7.46	99.68**
	Var./Isol.	8.09	1.68**	3.45	9.64	
EWE	Isolates	8.095	1.80**	2.12	6	97.81**
	Var./Isol.	1.38	0.79	2.57	7.26	
HOH/EWE	Isolates	28.98	16.33**	1.38	3.85	97.76**
	Var./Isol.	2.22	1.80**	1.67	4.67	
	Isol./Loc.	13.32	4.52**	1.94	5.44	

The results showed a significant difference between the two varieties (Taifun and Amaretto) and this was as expected since the beginning, because Taifun has a moderate resistance towards FHB while Amaretto has a relatively higher resistance towards FHB disease .

4.5. Analysis of Deoxynivalenol by ELISA:

In the present study the production of Deoxynivalenol by the *F. graminearum* and *F. culmorum* isolates, was investigated using ELISA assay.

The DON content was high in the inoculated two varieties Taifun and Amaretto, inoculated with Syrian *F.culmorum* in both sites Hohenheim and Eckartsweire , whereas the inoculated two varieties with Syrian

F.grameniarum had a low DON content. In the first location (HOH) the highest DON content was obtained from the two *F.culmorum* (Syr.) isolates FC5 (53 ppm) and FC3 (40 ppm), whereas the highest DON content between *F. graminearum* (Syr) was obtained from FG 2 (4.7ppm) (Table 9):

Table .9. Mean values of DON concentration produced by some Syrian Fusarium isolates taken from inoculated wheat in Hohenheim as assayed by DON –specific ELISA.

FC isolates	DON concentration (ppm)	FG isolate	DON concentration (ppm)
Fc(Syr) 1	1.2	Fg(Syr)1	0
Fc(Syr) 2	22.4	Fg(Syr)2	4.7
Fc(Syr) 3	40.1	Fg(Syr)3	0.1
Fc(Syr) 4	14.8	Fg(Syr)4	1.5
Fc(Syr) 5	53.9	Fg(Syr)5	0.1
Fc(Syr) 6	20.4	Fg(Syr)6	1.1
Fc(Syr) 7	0	Fg(Syr)7	0.1
Fc(Syr) 8	0.8	Fg(Syr)8	0.2
Fc(Syr) 9	0.9	Fg(Syr)9	0
Fc(Syr) 10	0.1	Fg(Syr)10	0
Fc (syr) 11	8.7	Fg(Syr) 11	0.1
Fc(Syr) 12	0.1	Fg(Syr) 12	0.1

The range of DON content from the German *F. culomrum* (Ger) was 12.4 to 44 ppm, and for German *F.graminearum* (Ger) the range was 4.3 to 85 ppm (Table 9).

Table .10. Mean values of DON concentration produced by some German Fusarium isolates taken from inoculated wheat in Hohenheim as assayed by DON –specific ELISA.

FC isolates	DON concentration (ppm)	FG isolates	DON concentration (ppm)
Fc(Ger) 1	27.2	Fg(Ger) 1	16.5
Fc(Ger) 2	18.9	Fg(Ger) 2	26.5
Fc(Ger) 3	44.22	Fg(Ger) 3	40.8
Fc(Ger) 4	30.1	Fg(Ger) 4	42.9
Fc(Ger) 5	19.1	Fg(Ger) 5	13.2
Fc(Ger) 6	12.4	Fg(Ger) 6	12.4
Fc(Ger) 7	43.4	Fg(Ger) 7	4.3
Fc(Ger) 8	12.9	Fg(Ger) 8	14.5
Fc(Ger) 9	25.5	Fg(Ger) 9	5.5
Fc(Ger) 10	26.8	Fg(Ger)10	16.2
Fc(Ger) 11	33.7	Fg(Ger) 11	25.8
Fc(Ger) 12	15.7	Fg(Ger) 12	38.4

In Eckartsweire, the second location the results were a little bit different because of the changes of the environmental conditions in the second location, the highest DON content obtained from Syrian *F.culmorum* was also from FC3 (39 ppm), whereas the highest DON from Syrian *F. graminearum* was from FG 6 (1.57ppm) (Table 11).

Table .11. Mean values of DON concentration produced by some Syrian Fusarium isolates taken from inoculated wheat in Eckartsweire. as assayed by DON –specific ELISA

FC isolates	DON concentration (ppm)	FG isolate	DON concentration (ppm)
Fc(Syr) 1	1.28	Fg(Syr)1	0
Fc(Syr) 2	19.82	Fg(Syr)2	1.127
Fc(Syr) 3	39.813	Fg(Syr)3	0.378
Fc(Syr) 4	1.152	Fg(Syr)4	1.188
Fc(Syr) 5	33.204	Fg(Syr)5	1.291
Fc(Syr) 6	3.159	Fg(Syr)6	1.579
Fc(Syr) 7	0.1	Fg(Syr)7	1.109
Fc(Syr) 8	0.42	Fg(Syr)8	0.1
Fc(Syr) 9	0.55	Fg(Syr)9	0
Fc(Syr) 10	1.367	Fg(Syr)10	0
Fc (syr) 11	0.8	Fg(Syr) 11	0.1
Fc(Syr) 12	2.7	Fg(Syr) 12	0.1

The range for German *F. culmorum* (Ger) was from 0.1 to 35 ppm, and the range for *F. graminearum* (Ger) was 2.9 to 43 ppm (Table 12).

Table 12: Mean values of DON concentration produced by some German Fusarium isolates taken from inoculated wheat in Eckartsweire as assayed by DON –specific ELISA

FC isolates	DON concentration (ppm)	FG isolates	DON concentration (ppm)
Fc(Ger) 1	0.1	Fg(Ger) 1	8.4
Fc(Ger) 2	16.6	Fg(Ger) 2	2.9
Fc(Ger) 3	33.0	Fg(Ger) 3	18.3
Fc(Ger) 4	14.1	Fg(Ger) 4	43.5
Fc(Ger) 5	13.8	Fg(Ger) 5	22.3
Fc(Ger) 6	10.7	Fg(Ger) 6	3.9
Fc(Ger) 7	0.4	Fg(Ger) 7	11.4
Fc(Ger) 8	7.2	Fg(Ger) 8	6.2
Fc(Ger) 9	5.6	Fg(Ger) 9	30.5
Fc(Ger) 10	35	Fg(Ger)10	20.3
Fc(Ger) 11	6.6	Fg(Ger) 11	18.9
Fc(Ger) 12	27.9	Fg(Ger) 12	22.9

The results of *F.culmorum* from Syria are agree with other studies which showed that DON content in wheat kernels usually is highly correlated with *F.culmorum* (Viscont *et al.*, 1996; Parry *et al.*, 1995).

The mean value of DON concentrations recorded internationally in positive *Fusarium* samples ranged from 0.03 to 1.78 $\mu\text{g/g}$ in wheat grain under field conditions in Italy (Visconti *et al.*, 2004), In Netherland DON values gave an average of 0.9 mg Kg^{-1} and 2 mg Kg^{-1} with uncleaned wheat (Snijders, 1992).

In a previous work in the same area (Stuttgart) DON contents ranged between 20– 163 mg Kg^{-1} in winter rye and from 43 – 67 mg Kg^{-1} in winter wheat (Miedaner *et al.*, 2003). In another study Gang *et al.*, (1998) inoculated winter rye with 42 isolates of *Fusarium culmorum*,

the mean values of DON contents ranged from 0.5 to 64.6 mg/kg in grains from field trials and from 0.3 to 376.3mg/kg in grains incubated *in vitro*.

On the other hand, *F.graminearum* from Syria did not that high aggressiveness or DON production comparing with its parallels from Germany, this could be related to the some missing blocks in the field trail or by chance because the tested isolates are just a single spores isolates dose not give a full image about the Syrian *F.graminearum* gene pool, further studies are now taking place in the state plant breeding institute at Uni-Hohenheim to get a better comprehension of the occurring FHB status in Syria examining more isolates with different environments.

5. Recommendations:

- Further observation of FHB development on wheat in the main wheat production areas in Syria, especially in the favorable seasons for FHB development.
- Extension of fusariotoxins detection studies other than DON and on other grain crops and their products in Syria.
- Continue detection of *Fusarium* mycotoxins in local and imported grain crops.
- Suggestion and adaption of Syrian standards for the main *Fusarium* mycotoxins in small grains and their commercial products.
- Installation of automatic forecasting system to predict favorable conditions for FHB and fusariotoxin development.

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أكثر من ذلك إذا اعتبرنا هذه العزلات حسب المنشأ كوحدة وراثية نلاحظ أن مجتمع *F. culmorum* من ألمانيا كان الأشد وطأة على كلا الصنفين من القمح يليه *F. culmorum* من سوريا ، ثم *F. graminearum* من ألمانيا بينما أظهرت أنواع *F. avenaceum*, *F. graminearum* *crookwellense*, شراسة مرضية اقل بشكل ملحوظ من الانواع السابقة واضعين بعين الاعتبار ان هذه العزلات هي عزلات مأخوذه من بوغات وحيدة مختارة بشكل انتقائي للعزلات الالمانية وبشكل عشوائي للعزلات السورية. اي انها ليست مدروسة سابقا او اي لا يوجد اي عمل سابق عليها في سوريا.

أما بالنسبة للموقع الثاني Eckartsweire لم يكن التباين في الشراسة بين العزلات على نفس القدر مثل الموقع الأول على الرغم من أن التباين كان معنويا أيضا.

ولكن طيف التباين كان اضيق من طيف التباين في الموقع الأول علما أن الأنواع الأربعة احتفظت بنفس الترتيب بالنسبة للشدة المرضية كما في الموقع الأول.

لوحظ أيضا وجود تفاعل معنوي بين العزلات المستخدمة ومنطقتي الزراعة كما وجد أيضا تفاعل معنوي بين العزلات وصنفي القمح المختارين.

٣- الكشف عن السموم الفطرية:

أظهرت نتائج الكشف عن النمط الكيميائي للسموم الفطرية ترددا عاليا ل 15-acetyledoxynivalenol أكثر من 3-acetyledoxynivalenol في العزلات السورية.

تم الكشف عن تركيز سم Deoxynivalenol في عينات القمح المأخوذة من التجربة الحقلية والملقحة بعزلات من نوعي *F. graminearum*، *F. culmorum* حيث كان أعلى تركيز للسم في الموقع الأول Hohenheim من بين عزلات *F. culmorum* ٣٥ جزء في المليون وبين عزلات *F. graminearum* كان أعلى تركيز ٤,٧٢ جزء في المليون

في الموقع الثاني Eckartsweire كان أعلى تركيز لعزلات *F. culmorum* ٣٩ جزء في المليون، أما بين عزلات *F. graminearum* فكان أعلى تركيز ١,٥٧ جزء في المليون.

تم حصاد التجربة الحقلية بعد وصول السنابل الى مرحلة النضج الكامل من أجل تحليل السموم الفطرية الناجمة عن هذه العزلات.

٤- الكشف عن السموم الفطرية: تم استخدام تقانة تفاعل بوليميراز التسلسلي

PCR للكشف عن النمط الكيميائي للسموم الفطرية المفترزة من قبل نوعي *F. culmorum*, *graminearum*

أما بالنسبة لتحليل السموم الفطرية فقد تم استخدام تقانة المقايسة المناعية المرتبطة بالانزيم ELISA للكشف عن تركيز سم الديوكسي نيفالينول (DON) في عينات القمح التي حصدت من التجربة الحقلية .

النتائج:

١- توصيف عزلات الجنس *Fusarium* من عينات القمح السوري :

تم عزل حوالي ٢٠٠ عزلة من الجنس الفطري *Fusarium* تنتمي إلى الأنواع التالية:

F.avenaceum(26%), *F.culmorum*(22%), *F.crookwellense*(14%),
F.graminearum(10%), *F.equiseti*(8%), *F.sporotrichioides*(7%),
F.acuminatum(7%), *F.oxysporum*(4%), *F.tricinctum* (2%)

وقد تم تصنيف جميع هذه العزلات تصنيفا مورفولوجيا ،أما بالنسبة للتصنيف بالطرق الجزيئية فقد تم الاهتمام بالانواع الاربعة التالية :

F. graminearum، *F. crookwellense* ،*F. culmorum*،*F. avenaceum*

٢- التجربة الحقلية:

التباين في الشدة المرضية:

بالنسبة للموقع الاول Hohenheim تباين الشراسة بين العزلات (من انواع مختلفة) كان كبيرا ومعنويا على مستوى ثقة ٠,٠٥% مع تكرارية عالية مما يؤكد أن التباين في الشراسة المرضية يعود إلى اختلاف وراثي بين العزلات نفسها وليس نتيجة للظروف البيئية المتعلقة بمكان التجربة .

مواد وطرائق البحث:

١- جمع العينات: جمعت العينات من خمس مناطق منتجة للقمح في سورية خلال موسمي ٢٠٠٦ و ٢٠٠٧ اشتبه بوجود الإصابة فيها بسبب الظروف الجوية التي سادت خلال هذين الموسمين والتي تشجع تطور هذا المرض

٢- عزل وتعريف الأنواع الفطرية التابعة للجنس *Fusarium*: تم عزل حوالي ٢٠٠ عزلة مختلفة من أنواع الجنس الفطري المذكور من خلال زرع حبوب القمح على وسط أغار البطاطا و الدكستروز بعد تعقيمها سطحيا وتحضيرها في ظروف مناسبة لنمو الفطر ومن ثم نقل العزلات الفطرية بعد زراعة بوغة وحيدة من كل عزلة الى وسط تصنيفي. و قدمت تصنيفها مورفولوجيا بالاعتماد على مفتاح (Nelson 1983) ، كما تم تصنيف العزلات بالطرائق الجزيئية باستخدام تقانة PCR من خلال استخدام مرئسات متخصصة للكشف عن هذه الأنواع الفطرية حيث تم الاهتمام بأربعة أنواع رئيسية لها تأثيرها الاقتصادي و مفرزة للسموم الفطرية (بشكل أساسي سم الديوكسي نيفالينول DON).

٣- التجربة الحقلية: تم زراعة التجربة الحقلية باستخدام تصميم القطع المنشقة بثلاث مكررات في موقعين في جنوب ألمانيا (Hohenheim و Eckartsweire) لدراسة القدرة الامراضية لهذه الأنواع الأربعة على صنفين ألمانيين هما (Taifun) و (Amaretto) احدهما متوسط التحمل لمرض لفحة السنابل والثاني أكثر تحملا.

أجريت العدوى الاصطناعية لهذين الصنفين باستخدام معلق بوغي محضر من العزلات السورية للأنواع الأربعة وتمت العدوى في منتصف فترة إزهار القمح كما تمت أيضا العدوى الاصطناعية باستخدام معلق بوغي من عزلات المانية معروفة من حيث قدرتها الامراضية وانتاجها للسموم الفطرية كشاهد ايجابي في التجربة . تم اخذ القراءات الحقلية بمعدل خمس قراءات بفاصل زمني ٢-٣ يوم وقدرت شدة الشراسة المرضية لكل عزلة على الصنفين النباتيين المستخدمين بتقدير نسبة السنابل والسنبيلات المصابة في الحقل. تم تحليل نتائج التجربة الحقلية بواسطة البرنامج الإحصائي Plabstat .

الكشف الجزيئي عن أنواع من الجنس *Fusarium* المفرزة للسموم الفطرية في عينات قمح من سورية

يعتبر القمح من المحاصيل الإستراتيجية في الجمهورية العربية السورية و يزرع في مناطق عديدة فيها وقد أدى الاهتمام المتزايد بهذا المحصول الاقتصادي إلى زيادة المساحات المزروعة وإنتاج العديد من الأصناف الجديدة المتحملة للظروف البيئية المحلية في العديد من المؤسسات البحثية العلمية مثل الهيئة العامة للبحوث الزراعية والمركز الدولي لدراسات المناطق الجافة (إيكاردا)، إلا أن انخفاضاً ملحوظاً بدأ يطرأ على إنتاج القمح في السنوات القليلة الماضية بسبب قلة الأمطار الهائلة بالإضافة إلى موسمية هطولها وخصوصاً في شهري آذار ونيسان اللذان يترافقان مع فترة إزهار القمح وارتفاع الحرارة والرطوبة النسبية في الجو، هذه الظروف البيئية أدت إلى ظهور بعض الأمراض الفطرية على القمح مثل مرض الطرف الأسود والذي تسببه عدة أنواع فطرية وبخاصة من الجنس *Alternaria* . كما أدت أيضاً لظهور مرض لفحة سنابل القمح الذي يسببه بشكل أساسي أنواع من الجنس *Fusarium* وهو يؤدي إلى انخفاض الغلة بشكل ملحوظ في محصول القمح كما تتجلى خطورته بقدرة بعض أنواع هذا الجنس على إفراز السموم الفطرية في الحقل واستمرار إفرازها حتى في ظروف التخزين مما يجعل القمح غير صالحاً للاستخدام البشري أو حتى لتغذية الحيوانات في حال تجاوز السموم نسباً معينة.

ونظراً لقلة الدراسات المتعلقة بهذا المسبب المرضي في سورية فإن دراستنا الحالية تعنى بالكشف عن بعض أهم أنواع *Fusarium* المفرزة للسموم الفطرية في عينات من القمح السوري.

تصريح

أصرح بأن البحث الموصوف في الأطروحة تحت عنوان " الكشف الجزيئي عن أنواع من الجنس *Fusarium* المفززة للسموم الفطرية في عينات قمح من سورية " لم يسبق أن قدم للحصول على أي درجة جامعية أخرى و لا هو مقدم حالياً لذلك و أن كافة الأعمال و النتائج المذكورة هي محصلة جهودي الشخصية و بتوجيه من المشرفين العلميين . و أن أي معلومات أو طرائق أو نتائج أخرى ذكرت في الأطروحة قد نسبت إلى مصادرها و مؤلفيها بوضوح في النص و في قائمة المراجع.

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رسالة معدة الى جامعة دمشق لاستكمال مستلزمات الحصول على درجة الماجستير في
التقانات الحيوية

إعداد المهندسة الزراعية

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