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Muhammad ABID

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Ecological role of mycotoxins produced by *Fusarium graminearum*: consequences of the presence of deoxynivalenol (DON) in crop residues on the soil microflora and soil fauna

Directeur de thèse

Christian STEINBERG

Jury :

Corinne LEYVAL	Directeur de Recherche, CNRS, Nancy	**Rapporteur**
Thierry MATEILLE	Directeur de Recherche, IRD, Montpellier	**Rapporteur**
Sylvain JEANDROZ	Professeur, Université de Bourgogne, Dijon	**Examinateur**
Florence FORGET-RICHARD	Directeur de Recherche, INRA, Bordeaux	**Examinateur**
Pierre MANGIN	Ingénieur d'Etude, INRA, UE Dijon	**Examinateur**
Christian STEINBERG	Directeur de Recherche, INRA, Dijon	**Directeur de thèse**

Dedicated To

My beloved Father and Mother

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Muhammad Abid
Dijon, France

ABSTRACT

Fusarium graminearum is a plant pathogenic fungus, causing devastating disease "Fusarium head blight" (FHB) in cereals including wheat and maize. It also contaminates the grains with mycotoxins including deoxynivalenol (DON) which are toxic to human and animals. This disease has resulted in the serious losses in grain yield and quality. We established through a first bibliographic review that during off season fungus survives saprophytically on the crop residues (ecological habitat) and serves as primary inoculum for the next season crop. However, we noticed also that the literature was poor about the role mycotoxins could play in the establishment of *F. graminearum* in such a habitat. The main aim of this thesis was therefore to test whether the presence of mycotoxins in the crop residues gives an advantage to *F. graminearum* to survive and develop a primary inoculum in the presence of the whole soil biota including fungi, bacteria, protozoa, nematodes and earthworms. We studied the impact of DON on the soil communities in the field as well as in microcosms, in wheat as well as in maize residues under tillage and no-tillage conditions. The disease development and the yield were noted in the field experiment. Some DON resistant active fungal decomposers and nitrogen fixing bacteria were picked and the dynamics of *F. graminearum* was observed by accelerating decomposition of crop residue in their presence, in the presence or absence of DON.

During this study, the dynamic and survival of *F. graminearum* and total fungal and bacterial communities were examined by using quantitative real time polymerase chain reaction (qPCR) as well as by plate counting. At the same time, the structures of microbial communities were determined by using terminal restriction fragment length polymorphism analysis (T-RFLP). The DON resistance of isolated fungal decomposers and nitrogen fixers was tested by using minimal inhibitory concentration test (MIC). Nematodes and earthworms were quantified through binocular observations. The fate of DON was determined by quantifying the mycotoxin by high performance liquid chromatography (HPLC).

The results suggested that DON in crop residues showed an impact on the biotic components of the soil but the impact depended on the communities and on the location of the residues (on surface or incorporated in the soil). The molecular biomass shows that the fungal and bacterial densities were significantly affected by the presence of DON. The presence of DON played significant role on the structure of bacterial and protozoan community while the nematodes and fungal communities remained unaffected. MIC results showed that the susceptibility of some competitive fungal strains towards DON was dependent on the dose of mycotoxin. The earthworms (*Lumbricus terrestris*) were not affected by the presence of mycotoxin. The degradation of DON in the residues was dependent on the time, the location of residues and the soil biota.

The quantification of *F. graminearum* suggested that the presence of DON gave no advantage in the survival and development of primary inoculum during the decomposition of crop residues in the soil. We conclude that fungal decomposers can be selected on their enzymatic potential towards organic matter more than on the DON resistance to increase the degradation of the straw left at the surface and limit the subsequent development of *F. graminearum.*

RÉSUMÉ

Fusarium graminearum est un champignon pathogène des plantes, responsable de la fusariose de l'épi (plus connue sous le nom de Fusarium Head Blight : FHB) sur céréales, notamment sur le blé et le maïs. En interaction avec la plante, le champignon produit des mycotoxines, parmi lesquellse le déoxynivalénol (DON), dont la finalité pour le champignon producteur est méconnue mais qui sont toxiques pour les humains et les animaux. Ainsi la qualité des grains contribue fortement aux pertes de rendement observées et les résidus contaminés restent au champ. Une première revue bibliographique (Leplat et al 2012) a mis en évidence l'importance des résidus de culture (habitat écologique) pour la survie saprophyte du champignon, pour sa reproduction sexuée et pour l'établissement de l'inoculum primaire susceptible d'infecter la prochaine culture. Une seconde revue bibliographique a souligné les lacunes en ce qui concerne le rôle que les mycotoxines pourraient jouer dans la survie de *F. graminearum* dans un cet habitat.

L'objectif principal de cette thèse était donc de vérifier si la présence de mycotoxines dans les résidus de récolte donne un avantage compétitif à *F. graminearum* vis-à-vis des composantes biotiques du sol et des résidus et notamment les champignons, les bactéries, les protozoaires, les nématodes et les vers de terre. L'impact du DON sur ces différentes communautés a été évalué dans des résidus de maïs et de blé, au champ et en microcosmes, en condition de labour et de travail superficiel du sol. Le développement de la maladie et ses conséquences sur le rendement ont été observés dans l'expérience de terrain à l'Unité Expérimentale de l'INRA de Dijon.

Au cours de cette étude, la survie et les dynamiques de développement de la souche modèle d'étude *F. graminearum* MIAE00376 et des communautés fongiques et bactériennes ont été mesurées en utilisant la réaction de polymérisation en chaîne en temps réel (Q-PCR) ainsi que par comptage sur boîtes. Dans le même temps, l'évolution des structures des communautés microbiennes a été déterminée par analyse du polymorphisme de longueur des fragments de restriction terminaux (T-RFLP). Les nématodes et les vers de terre ont été quantifiés par extraction et observations à l'œil ou a la loupe binoculaire. Le DON introduit dans le sol et les résidus a été extrait et quantifié au cours du temps par chromatographie liquide haute performance (CLHP). Des dynamiques de population de la souche MIAE00376 associée à différents microorganismes isolés de paille en décomposition et sélectionnés pour leur résistance au DON, à des bactéries fixatrice d'azote et à des *Fusarium* sp. appartenant au complexe fongique du FHB ont été mesurées en microcosmes de paille en présence ou non de DON

Les résultats suggèrent que le DON dans les résidus de culture a une incidence sur les composantes biotiques du sol, mais l'impact dépend des communautés et de la localisation des résidus (en surface ou incorporés dans le sol). La biomasse moléculaire montre que les densités bactériennes et fongiques ont été significativement affectées par la présence de DON. La présence de DON a joué un rôle significatif sur la structure des communautés bactériennes et protozoaires, plus faible sur les communautés fongiques et nul sur les nématodes voire positif sur les vers de terre.

Il est conclu que le DON est rapidement inaccessible en profondeur et un peu moins rapidement en surface (immobilisation ou dégradation), qu'il ne confère pas d'avantage compétitif au champignon producteur et que la gestion de l'habitat privilégié que constituent les résidus de culture pour *F. graminearum* peut être envisagée par le travail du sol en favorisant la décomposition rapide des résidus, par le labour ou l'utilisation d'organismes décomposeurs indigènes ou introduits.

List of Abbreviations

ANOVA	Analysis of variance
CFU	Colony Forming Unit
d.w.	Dry weight
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
LOD	Limit of detection
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FHB	Fusarium head blight
HPLC	High Performance Liquid Chromatography
INRA	Institut National de la Recherche Agronomique
ITS	Internal Transcribed Spacer
LOD	Limit of detection
LOQ	Limit of quantification
MIC	Minimal inhibitory concentration
OD	Optical density
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PVPP	Polyvinyl polypyrrolidone
Q-PCR	Real-time quantitative PCR
rDNA	Ribosomal DNA
SDS	Sodium dodecyl sulfate
T-RFLP	Terminal restriction fragment length polymorphism
TRFs	Terminal restriction fragments
WHC	Water holding capacity

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General Introduction

General Introduction

Studies of plant pathogenic fungi generally focus on infection processes, disease development and other concerns in plant–microorganism interactions, but the saprotrophic period of these pathogens' life cycle is not so well–known. Most soil fungi are decomposers or saprotrophs that feed on decaying organic material. Actually they play a key role in the decomposition of organic polymers that takes place in the soil. Fungi are considered as the primary decomposers in forests, where litter contains high concentrations of complex polymers. Fungi have a unique role in the degradation of plant–derived woody substrates containing lignocellulose, *i.e.* cellulose complexed with lignin (Finlay, 2007; Sinsabaugh, 2005). They also play an important role in arable soils by breaking down and recycling plant residues, primarily cellulose and hemicellulose (Stromberg, 2005). Among them, some plant pathogenic fungi take place and their role should be considered. Indeed, plant pathogenic fungi are categorised as either biotrophs or necrotrophs, and as either obligate pathogens or facultative saprotrophs. For example, the disease cycle of the deleterious fungus *Fusarium graminearum* (Fig. 1), the anamorph stage of *Gibberella zeae* (Schwein.) Petch, is well–studied (Trail, 2009). In a previous review, (Goswami and Kistler, 2004) provided an update on the pathogenesis, genetics, evolution and genomics of *F. graminearum* but the ecological requirements of its saprotrophic stage are less well–understood.

Fusarium head blight, root rot and foot rot (crown rot) are diseases that cause significant yield loss in several crops worldwide such as wheat (Fig. 2), maize , oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and rice (Parry et al., 2007; Pereyra and Dill-Macky, 2008; Trail et al., 2003). Yield losses caused by Fusarium head blight in Northern and Central America from 1998 to 2002 were evaluated to 2.7 billions of dollars (Nganje et al., 2002). Several species are involved in the fungal complex that causes these diseases. Many of them also produce mycotoxins, such as deoxynivalenol, commonly known as DON, and its acetylated forms 3–acetyl–4–deoxynivalenol and 15–acetyl–4–deoxynivalenol, nivalenol and zearalenone, these mycotoxins being commonly known as 3-ADON, 15-ADON, NIV and ZEA respectively (Desjardins and Proctor, 2007). These mycotoxins are of major concern because of their effect on human and animal health and because they persist during storage and are heat resistant ((JEFCA), 2001). The threshold of these mycotoxins in foodstuffs is regulated in Europe since 2007 (CE n°1881/2006 and n°1116/2007).

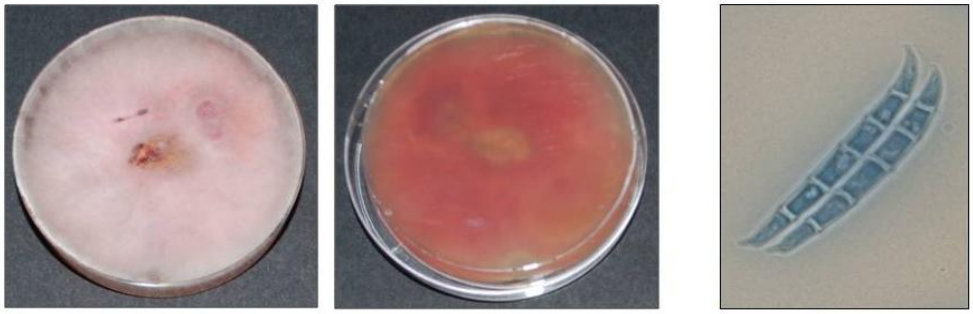

Fig. 1: Macroscopic and microscopic pictures of *Fusarium graminearum*, the causal agent of Fusarium head blight (photograph: courtesy of J. Leplat). Macroscopic pictures were taken after growth on potato dextrose agar. The undersurface shows the typical carmine red color of *F. graminearum* species. The microscopic picture shows macroconidia with the typical spindle shape which gives its name to the *Fusarium* genus. The cylindrical shape of the macroconidia, i.e., dorsal and ventral surfaces parallel, and the foot shape of the basal cell are typical of *F. graminearum* species.

Fig. 2: Wheat ear infested by *Fusarium graminearum* (Photograph: J. Leplat).

Among the species involved in the complex causing Fusarium disease on wheat, *F. graminearum* predominates in many parts of the world (Bottalico, 1998; Bottalico and Perrone, 2002; Parry et al., 2007).

Like the other *Fusarium* species in the complex, *F. graminearum* survives saprophitically on crop residues in the absence of its hosts (Sutton, 1982). Fusarium head blight severity and deoxynivalenol contamination significantly increase with the density of residues left from the preceding crop (Blandino et al., 2010). Moreover, surface residues provide a substrate for active growth of *F. graminearum* for a longer period of time than buried residues (Pereyra et al., 2004). Burying *F. graminearum*–infested crop residues deeper in the soil can efficiently reduce *F. graminearum* populations; however, the pathogen may survive for several years. During the decomposition process, the chemical composition and the availability of the plant material changes as some resources are used up while others are made available for saprotrophic growth. For *F. graminearum* to survive over time, it has to be able to use available resources and to compete with the different organisms that are invading the material, each of them being specific for each of the decomposition stages. To develop control strategies of *F. graminearum* primary inoculum, a better understanding of the complex interactions that determine its ability to grow and compete for crop residues is needed.

A review was performed in collaboration with Johann Leplat who worked on the saproptrophic survival of *F. graminearum* (Leplat 2012; Leplat et al 2012). The major conclusions were that

1) temperature, water, light and O_2 availabilities are key conditions for *F. graminearum* growth and the development of its sexual reproduction structures on crop residues, although the fungus can resist for a long time under extreme conditions,

2) *F. graminearum* survival is enhanced by important quantities of available crop residues and by rich residues, while sexual reproduction structures appear on poor residues,

3) *F. graminearum* seems to be a poor competitor over time for residues decomposition. The survival of this fungus could be controlled by the enhancement of the decomposition processes by other organisms. In addition, the development of *F. graminearum* on crop residues could be limited thanks to antagonistic fungi or thanks to soil animals growing at the expense of *F. graminearum*–infested residues, and

4) agricultural practices are key factors for the control of *F. graminearum* survival. A suitable crop rotation and an inversion tillage can limit the risk of Fusarium head blight development.

Most of the factors regulating the survival of *F. graminearum* interact and the combat the fungus must be taken to address these factors is illustrated in the schematic Fig. 3 (Leplat et al 2012). However, one point was not really considered in that review which focused mainly on the pathogenic fungus and less on the other soil inhabitants. Indeed, *F. graminearum* can produce huge amount of different mycotoxins *in planta*. At harvest, part of them is exported with the grains, what causes a major problem for human and animal health through the consumption of these contaminated grains. However part of them which is contained in small contaminated grains, spines, glumes, lemmas and pieces of straw returns back to the soil as crop residues.

The questions are:

- ⇨ What could be the impact of these mycotoxins on soil biotic components and what is their fate?
- ⇨ Does *F. graminearum* get a competitive advantage of this mycotoxins during its saprophytic phase?

In the present PhD work, we tried to answer these questions in the frame of a national project supported by the National Agency for Research (ANR; www.agence-nationale-recherche.fr) and coordinated by Dr. Florence FORGET (INRA-UPR 1264 MycSA Bordeaux). The project DON&Co (ANR 2010 CESA 01204) officially started the 1st March 2011 but the kick off meeting was held in November 2010. The title of this project is: "Production of mycotoxins in wheat: from the diversity of the *Fusarium* community to the toxicological impact of mycotoxins". In addition to the group of F. FORGET, the consortium includes six other partners (Arvalis-Institut du Végétal; INRA-UMR 1290 BIOGER-Grignon; INRA-UR 66 pharmacologie-toxicologie-Toulouse; ENVT-Toulouse et INRA-AgroSup-Université de Bourgogne-UMR 1347 Agroecologie, Dijon).

The project DON&Co aims at understanding how the composition of the *Fusarium* and *Microdochium* complexes impact the levels and type of trichothecenes accumulated in the grain and consequently its toxicity?

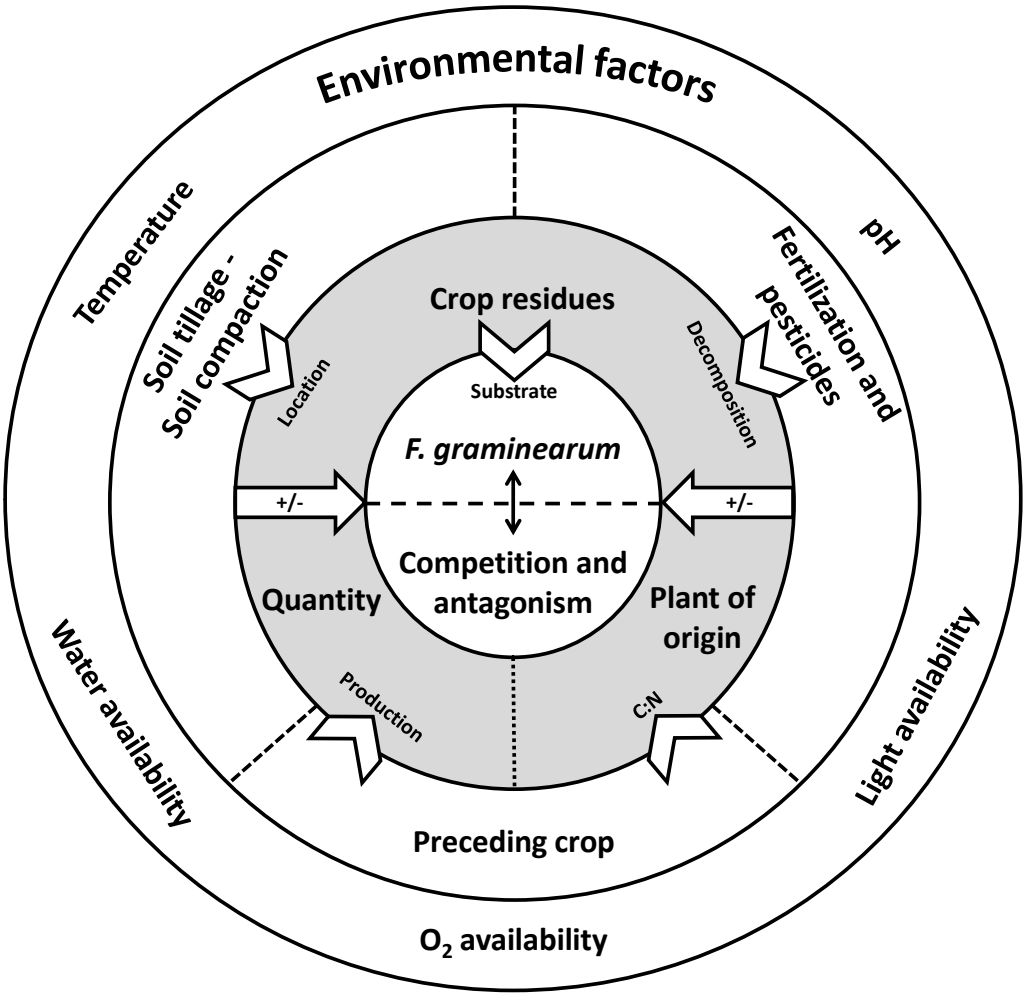

Fig. 3: Saprotrophic survival of *Fusarium graminearum*. Crop residues are the main habitat of *F. graminearum*. On the one hand, they provide spatial and trophic resources the fungus has to exploit in interaction with the rest of the microflora and the soil fauna. On the other hand, they buffer the impact of environmental factors, including agricultural practices (Leplat et al 2012).

This projects includes four main tasks:

- Diversity of *Fusarium* and *Microdochium* in relation with the types and levels of mycotoxins in the grains.
- An analysis of the mechanisms of production and regulation of toxins by different chemotypes of *F. graminearum* and *F. culmorum* to understand the biological significance of toxin production in planta.
- An analysis of the impact of agronomic factors (waste management, rotation, tillage system, fungicide) on the balance between species or between populations (chemotypes) within the *Fusarium* and *Microdochium* complexes. A deeper focus concerns the type of interactions between toxigenic *Fusarium* and crop residues.
- Toxicity to the consumer related to multicontaminations of cereals by DON and its acetylated derivatives.

A flow diagram illustrates the joints and complementarities between the various tasks of the project (Fig. 4). My PhD work is part of task 3.

I should mention that the Conseil Regional de Bourgogne contributed to the financial support of field experiments to promote the regional research for innovation in the frame of Le Plan d'Actions Régional pour l'Innovation (PARI)-Agrale 7: Impacts des pratiques agricoles sur le fonctionnement géochimique des sols et la qualité de l'environnement.

The present document will give first a general overview of what are mycotoxins and will focus more on *Fusarium* mycotoxins. Following this literature review, we limited our research using one strain of *F. graminearum* and one mycotoxin, the deoxynivalenol, as study models. A chapter is dedicated to the materials and methods used to address the above mentioned questions. Then we will explain how we dealt successively with a field approach and a microcosm approach using both classical microbiology, molecular biology and biochemistry tools. A general discussion will consider all the findings for each of the approaches to provide the answers on the one hand the impact of deoxynivalenol on bacteria, fungi, protozoa, nematodes and earthworms present in soil and residues and secondly to define the putative competitive advantage the mycotoxinogenic fungus gets from this molecule released into its surrounding environment.

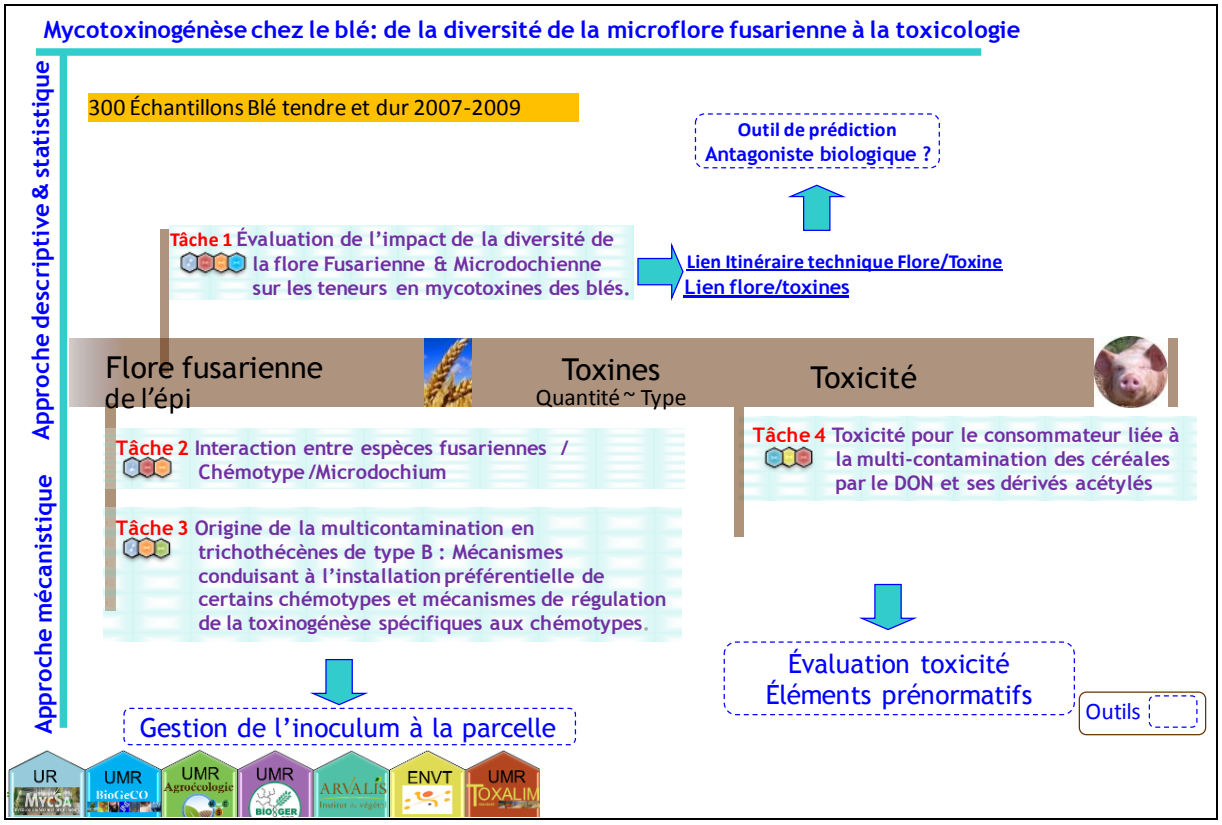

Fig. 4: Joints and complementarities between the various tasks of the project DON&Co supported by the ANR.

Chapter 1: Ecological role of mycotoxins

Ecological role of mycotoxins

1- Introduction:

The chemical machinery of a fungus produces different kind of metabolites and enzymes, which are necessary to perform the basic metabolic processes and the production of energy. Apart from these basic needed compounds, some low molecular weight organic compounds are also produced which are known as secondary metabolites. These chemical compounds are not needed for the basic requirements of the producer (Fox and Howlett, 2008). The fungal secondary metabolites may include the pharmaceutically beneficial compounds as penicillin (Demain, 1999), plant growth regulators as gibberellins (Candau et al., 1992) and as well as the harmful compounds (for human and animals) known as "Mycotoxins". These are the poisons produced by a moderate number of filamentous fungi also known as molds (Bhatnagar et al., 2002; Yu and Keller, 2005). Mycotoxins are dangerous to the vertebrates and reported to produce many severe diseases (known as Mycotoxicosis) in human and animals (Pestka, 2010; Richard, 2007). These chemicals may be acute to chronic poisons depending on their types, even in very low quantity to the high dose (Bryden, 2007; Döll and Dänicke, 2011).

The significance of mycotoxins was realized in 1961, with the discovery of first mycotoxin known as aflatoxin. In 1960, the brutal disease of young turkey birds known as Turkey X disease killed 100,000 turkeys on the consumption of mold contaminated feed. This was the first time that the proper cause of this disease by mold contaminated food was recognized (Desjardins and Hohn, 1997). However, they have always been dangerous to human and domestic animals in the past. The history of diseases caused by the consumption of these mycotoxins producing molds contaminated food is very old. One of the famous examples is Ergotism. Its epidemics called "St. Anthony's Fire", led to the death of thousands of humans in Europe in the Middle Ages (Dotz, 1980). Ergotism is caused by utilization of cereal grains (usually rye), infected with toxin producing fungi *Claviceps purpurea,* which produces toxins known as Ergot alcaloids. Alimentary toxic aleukia (ATA) is another notorious example which caused the death of more than 100,000 people in USSR from 1942 to 1948 (Pitt, 2000). Later investigations showed that ATA was caused by eating *Fusarium* spp. infected grains which were contaminated by T-2 mycotoxin.

The discovery of mycotoxins opened a new era of importance in the health of Human and animals and since then hundreds of mycotoxins have been discovered. These mycotoxigenic (toxin producing) fungi are present all around the world and causing the great loss to the world's agriculture (Goswami and Kistler, 2004). The disease caused by these molds, reduces the seed vigor, crop yield, grain quality, make them poisonous to human use, which leads to the great economic loss for a farmer as well as to a country (Windels, 2000; Wu, 2007). These mycotoxins are produced in the cereals, fruits as well as in vegetables, in the field, during transportation, storage and processing (Drusch and Ragab, 2003; Goswami and Kistler, 2004; Moss, 2008). The problem of mycotoxins fluctuates from year to year by the changes in the environmental conditions favorable for the production of mycotoxins and the development of the producing fungi (Logrieco et al., 2002). The world scientists, decided to limit the mycotoxin in food stuff consuming for the human and animal, in order to avoid the adverse effects. European Union (EU) has set the limits for most commonly found Agro-economical important mycotoxins in different crops including cereals as wheat, maize, oats (The commission of the European communities, 2007).The text (CE 1126/2007) published by these EU commission is provided in Annexe at the end of thesis.

The cereal diseases caused by the toxin producing fungi got the world's attention as the grains contaminated with mycotoxin reduce the quality as well as the production (Placinta et al., 1999). About 25% of the world's food is contaminated by mycotoxin annually. This phenomenon causes a great loss every year to the cereal producers and exporters. These fungal toxins are constant threat to the economic losses and the regular danger to the health of human beings and animals. Once they are produced in the food, it is difficult to eliminate them during cooking and industrial processing as they are highly stable to heat (Bullerman and Bianchini, 2007). The vertebrates including animals and human being are greatly affected by the presence of mycotoxins in the food. The disease symptoms caused by mycotoxins is known as "Mycotoxicosis". The investigations show that mycotoxins may cause very serious effects on the eukaryotic cells. They may inhibit the protein synthesis, DNA and RNA synthesis, disturb the mitochondrial function, affect the normal cell division (Bin-Umer et al., 2011; De Walle et al., 2010; Kouadio et al., 2007) and may lead to severe diseases in human and animals. T-2 toxins are found to induce maternal and fetal toxicity in pregnant mice and rats (Doi et al., 2008). Mycotoxins may cause acute and chronic poisoning to vertebrates. The adverse effects of mycotoxins on human and animal health are highly important topic of research since decades. They may produce disturbance in the immunity system, liver damage,

diarrhea, vomiting, cancer and many other sever diseases (Fokunang et al., 2006; Peraica et al., 1999; Placinta et al., 1999; Reddy et al., 2010; Ross et al., 1992; Shephard, 2008; Wild and Gong, 2010). The mycotoxins are not only present in the food and feed stuff, they are even reported in the indoor air and indoor environment damp including homes and buildings, and can cause the health risk by inhalation (Hendry and Cole, 1993; Jarvis and Miller, 2005; Robbins et al., 2000).

In this review, we will investigate the ecological role of mycotoxins in the environment shared by micro to macro world and prokaryotes to eukaryotes on the basis of the research conducted up to date.

2- Major Mycotoxins:

More than 300 fungal secondary metabolites are declared as mycotoxins due to their harmful effects on vertebrates. Most of these mycotoxins are produced by the fungal species belonging to three genera i.e. *Fusarium, Aspergillus* and *Penicillium.* All the species related to these three genera are not toxigenic. *Fusarium* is known as the field fungi due to the production of mycotoxins in the field while *Aspergillus* and *Penicillium* are called storage fungi as generally these two genera are known for the production of toxins postharvest or during the food storage. The list of some frequently found important mycotoxins and their major producers are given in the Table 1.

No doubt all the mycotoxins have their importance but the mycotoxins produced on the cereal are considered more important and extensive data is available on them. These mycotoxins are produced during the growth of crop while standing in the field, so very difficult to avoid them from food chain. Among the cereal mycotoxins, trichothecenes, zearalenone and fumonisins are considered most important cereal mycotoxins from animals and human health point because of both their toxicity and their frequency of presence in the cereals (González-Osnaya and Farrés, 2011; Luo et al., 1990; Omurtag, 2008; Thuvander et al., 1999). European Union has established the limits for some extensively found mycotoxins including deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUM) (The commission of the European communities, 2007).

Trichothecenes are a large group of agriculturally important mycotoxins containing more than 150 members which are mainly produced by different *Fusarium* species. Trichothecenes are accumulated in the kernels of *Fusarium spp* infected spikelets of many cereal crops including wheat, maize, barley, rye and oat etc and rendering them inapt for the consumption of human

and animals (Nielsen et al., 2009). Trichothecenes are simple to complex mycotoxins and divided in four types (A, B, C and D) based on the substitution pattern of EPT (12,13-epoxytrichothec-9-ene) (reviewed by McCormick et al., (2011)). Type A trichothecenes (TCTA) and Type B trichothecenes (TCTB) are the most frequently found important trichothecenes produced by different *Fusarium* spp (Table 1). Most of them are related to Fusarium head blight (FHB) disease (Foroud and Eudes, 2009). Type A trichothecenes are considered more toxic than Type B trichothecenes as T-2 is considered about 10 times more toxic than DON (Thuvander et al., 1999). These mycotoxins are reported in all the regions of the world due to the producing fungal adaptation to the different environments in USA, Asia and Europe (Ward et al., 2008; Yli-Mattila, 2011; Yli-Mattila et al., 2011). Different countries including USA, Canada and European Union made the legislations against certain frequently founds mycotoxins of this group. The most frequently found Type A trichothecenes includes T-2 toxin (T-2), HT-2 toxin (HT-2) and 4,15-diacetoxyscirpenol (4,15-DAS) (Mateo et al., 2002). The most frequently found major Type B trichothecenes includes nivalenol (NIV), deoxynivalenol (DON), 3-O-acetyl DON (3 ADON) and 15- O-acetyl DON (15 ADON) (Fig. 1).

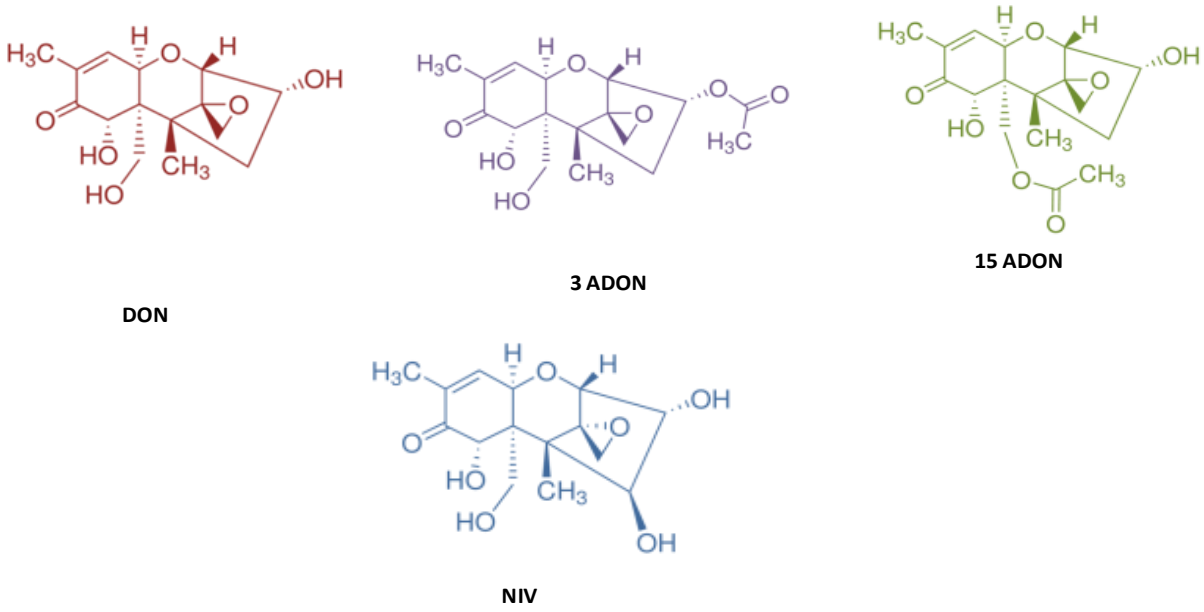

Fig. 1: Molecular structures of Trichothecene-B produced by *F. graminearum* and *F. culmorum*

The most commonly reported mycotoxin in the cereal crop is DON and considered one of the most important member of trichothecenes (Foroud and Eudes, 2009). DON is produced by *Fusarium graminearum* and *F. culmorum* on several cereal crops under wide range of environments (Doohan et al., 2003). These fungi are associated to the production of the notorious FHB disease of cereal. FHB has appeared in many outbreaks and caused high losses in different ages all around the world. DON interrupts the protein synthesis, reduces nutritional efficiency, reduces immunity system, causes diarrhea and vomiting etc (Fokunang et al., 2006; Placinta et al., 1999; Sobrova et al., 2010; Wild and Gong, 2010). In the Fusarium-infected cereals DON is also followed by very important type B trichothecenes i.e. NIV and 3ADON and 15 ADON but they occur at lower levels than DON. European Union (EU) has made the legislations against the consumption of DON in our cereal food products. The maximum limit for DON in unprocessed cereals other than durum wheat, oats and corn at 1250 µg / Kg (1250 ppb) and for maize at 1750 µg / Kg (1750 ppb) for human consumption. Higher doses are accepted for animal feeding (The commission of the European communities, 2007).

In trichothecene type A T-2 and HT-2 toxins are also of the main mycotoxins. They are very stable compounds and produced by *F. poae*, *F. sporotrichoides*, and *F. acuminatum*. T-2 toxin, inhibits both protein synthesis and mitochondrial translation (Bin-Umer et al., 2011) and produces many disease symptoms in animals as alimentary toxic aleukia (ATA), weight loss, decreases in leukocyte count and blood cell and can seriously damage the liver and stomach (Weber et al., 2010). The combined temporary tolerable daily intake (TDI) for T-2 and HT-2 is established at 0.06 µg / Kg body weight (Scientific Committee on Food on Fusarium-toxins, 2002).

Zearalenone (ZEA) is also one of the most commonly found mycotoxins produced by *Fusarium* species (Table 1) and co-occurs with trichothecenes in cereals (González-Osnaya and Farrés, 2011). It is mainly produced by *F. graminearum*, *F. culmorum* and *F. langsethiae.* It is a non-steroidal estrogenic fungal toxin towards animals and is associated to the grains infected by FHB in cereal crops. EU has made the legislations also for ZEA due to its frequent presence in the food and threat to the animals. The limit for ZEA is 100 µg/Kg (100ppb) in unprocessed cereals other than corn and is 200 µg / Kg in unprocessed maize to (200 ppb) (The commission of the European communities, 2007).

Fumonisins is a group of mycotoxins mainly divided in four types (i.e. A, B, C and P) on the basis of their chemical structure. Fumonisins are found all around the world (Marasas, 1996)

and are produced by many Fusarium species including *Fusarium verticillioides*, *F. moniliforme and F. subglutinans* in Europe (Marasas, 2001; Visconti and Doko, 1994). Fumonisins produce the mycotoxicoses in animals through the interruption of sphingolipid metabolism by hindering ceramide synthetase (Marasas et al., 2004).These mycotoxins are reported on many cereals including wheat, maize, barley and sorghum (Visconti and Doko, 1994) but more often in maize. Its limit in Europe is fixed at 2000 ppb for unprocessed maize and at 200 ppb for the processed maize-based foods and baby foods for infants and young children (The commission of the European communities, 2007).

Mycotoxin	Major fungi	Reference
Deoxynivalenol (DON)	*Fusarium graminearum, F. culmorum*	(Audenaert et al., 2009; Gang et al., 1998; Manka et al., 1985; Mirocha et al., 1989; Mirocha et al., 1994)
3- O-acetyl deoxynivalenol (3ADON)	*F. graminearum, F. culmorum*	(Audenaert et al., 2009; Manka et al., 1985; Mirocha et al., 1989; Mirocha et al., 1994)
15- O-acetyl deoxynivalenol (15ADON)	*F. graminearum, F. culmorum*	(Audenaert et al., 2009; Mirocha et al., 1989)
Nivalenol (NIV)	*F. graminearum, F. culmorum, F.poae*	(Gang et al., 1998; Mirocha et al., 1994; Thrane et al., 2004)
Zearalenone (ZEA)	*F. graminearum, F. culmorum, F. sporotrichioides,*	(Greenhalgh et al., 1983; Manka et al., 1985)
Fumonisins (FB1, FB2,F B3)	*F. proliferatum, F. verticillioides*	(Marasas, 2001; Ross et al., 1992; Visconti and Doko, 1994)
T-2 toxins (T-2)	*F. sporotrichioides, F. poae, F. acuminatum, F. langsethiae*	(Rabie et al., 1986; Thrane et al., 2004)
HT-2 toxins (HT-2)	*F. sporotrichioides, F. poae, F. langsethiae*	(Thrane et al., 2004)
Diacetoxyscirpenol (DAS)	*F. sporotrichioides, F. poae, F. langsethiae*	(Thrane et al., 2004)
Moniliformin (MON)	*F. subglutinans, F. acuminatum, F. concolor, F. equiseti, F. semitectum, F. acuminatum, F. concolor, F. oxysporum*	(Irzykowska et al., 2012; Lew et al., 1996; Marasas et al., 1986; Rabie et al., 1982)
Enniatin (EN)	*F. langsethiae, F. tricinctum*	(Meca et al., 2010; Thrane et al., 2004)
Beauvericin (BEA)	*F. sporotrichioides, F. poae*	(Thrane et al., 2004)
Aflatoxin (B1, B2, G1, G2)	*Aspergillus. flavus, A. parasiticus, A. nomius*	(Elshafie et al., 2002; Olsen et al., 2008; Yu et al., 1995)
Ochratoxin A	*A. ochraceus, A. carbonarius*	(Kapetanakou et al., 2009)
Patulin	*Penicillium expansum, P.patulum*	(Abrunhosa et al., 2001; Aziz and Moussa, 2002; Northolt et al., 1978)

Table 1: Some commonly found mycotoxins and major producer fungi.

3- Molecular basis of Trichothecenes:

The biosynthesis of mycotoxins is a complex process and mainly depends on the genetics of fungi. It can be affected by various factors including the environmental conditions and the nature of the substrate and the external (biotic or abiotic) stress (Paterson and Lima, 2011). The amount of mycotoxin produced cannot be estimated by the growth of fungi in the kernels as all the environmental conditions must be taken into account (Shim et al., 2003).

The fungal genetics have a fundamental importance in the mycotoxin type and the amount of mycotoxin production on the same substrate (Vogelgsang et al., 2008). There can be different chemotypes within the same species producing specific mycotoxins as well as the different species can produce the same chemicals (Reynoso et al., 2011; Yli-Mattila and Gagkaeva, 2010). The geographical area determines the chemical phenotype of mycotoxin producing fungi. Various trichothecene chemotypes are reported to be associated with FHB in the different parts of the world: 3ADON chemotype of *F. graminearum* population is dominant in the major wheat growing area in the North America and is rapidly replacing 15ADON chemotype (Ward et al., 2008). In Europe 15ADON is reported as the more dominating chemotype (Jennings et al., 2004; Prodi et al., 2009; Yli-Mattila, 2010). 3ADON and 15-ADON are more commonly found together in Japan (Suga et al., 2008). The biosynthesis of trichothecene in *Fusarium* is controlled by 15 Tri genes located at three different loci: a 12-gene core Tri cluster locus, a two gene TRI1-TRI16 locus and a single gene TRI101 locus (Alexander et al., 2009; Merhej et al., 2011). These genes play their roles at different steps of the pathway of trichothecene biosynthesis which starts from farnesyl pyrophosphate (FPP) and results in the formation of trichothecene through a series of reactions (Fig. 2). Calonectrine protein is formed from FPP through a cyclization by the functioning of Tri5, Tri4, Tri101, Tri11 and Tri3 (Alexander et al., 2011; Alexander et al., 2009; Brown et al., 2002; Lee et al., 2002). These steps are common in the both T-2 toxin producing *F. sporotrichioides* and Trichothecene B (TCTB) producing *F. graminearum*. The involevement of Tri13 and Tri7 leads to the formation of 3,4,15-Triacetoxyscirpenol. FsTri1, FsTri16 and Tri8 lead to the formation of T-2 toxin in *F. sporotrichioides*. In *F. graminaerum* FgTri1 genes makes the way to the formation of TCTB either from calonectrine or from 3,4,15-triacetoxyscirpenol (Brown et al., 2001, 2002; Lee et al., 2002). Tri6 and Tri10 genes are regarded as regulators genes for the other genes and their deletion in *F. graminearum* is reported to reduce the pathogenicity in plants (Seong et al., 2009).The functioning of Tri7 and

Tri13 genes determine the Chemotype NIV while these are not active in DON producing strains (Brown et al., 2002; Lee et al., 2002). Tri8 gene is found to behave differently in 3-ADON and 15ADON chemotypes in *F. graminearum*. In 3ADON chemotypes Tri8 gene deacetyl 3,15-Acetyl DON at 15 carbon atom to form 3ADON and in 15ADON chemotype it deacetyl 3,15-Acetyl DON at 3 carbon atom to convert it into 15ADON (Alexander et al., 2011).

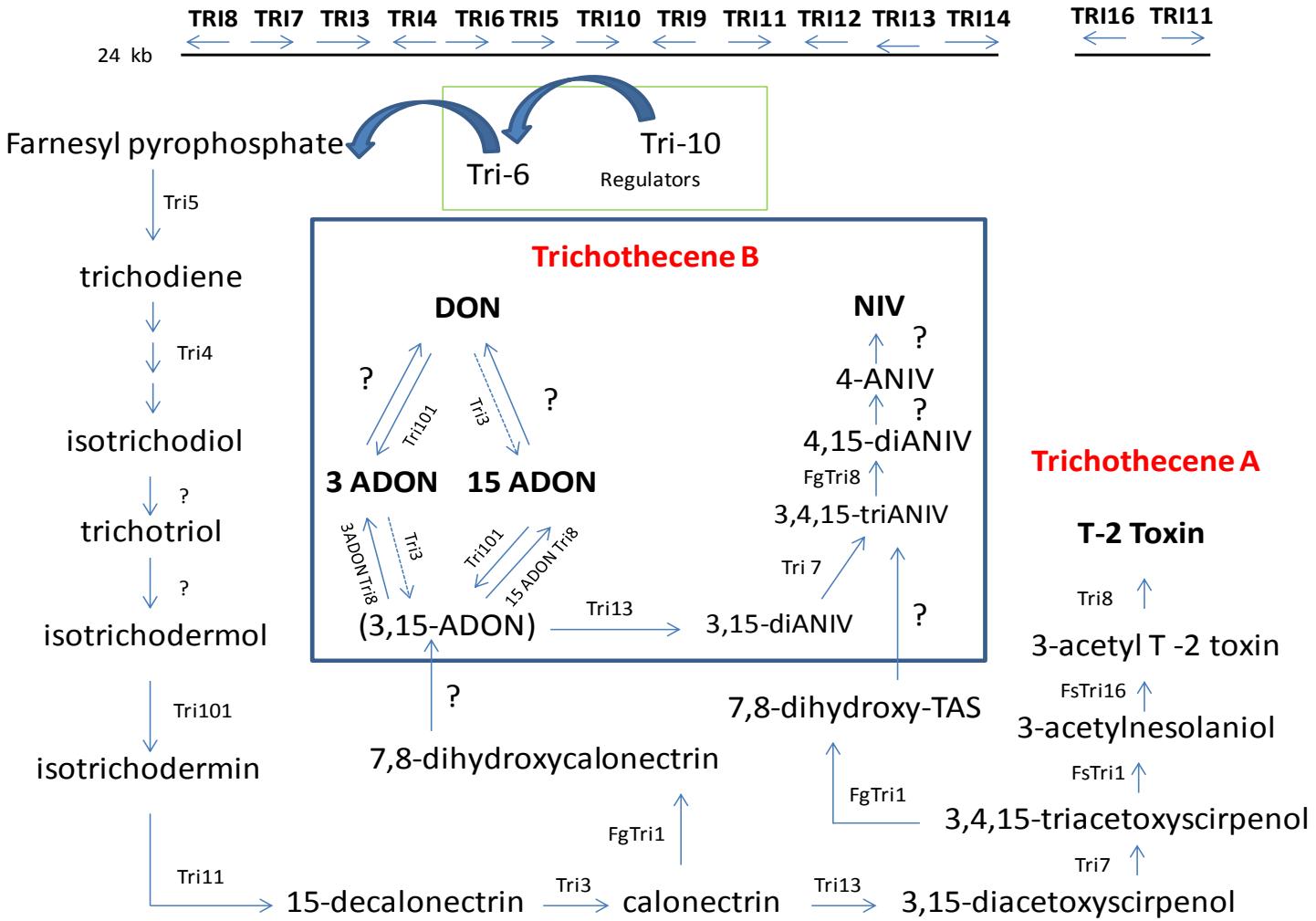

Fig. 2: A proposed biosynthetic pathway of trichothecene B and T-2 toxin in *F. graminearum* and *F. sporotrichioides* and the genes involved at different steps (adapted from Alexander et al 2009 and Alexander 2011). The question mark (?) indicates unidentified genes.

4-Factors affecting the production of Trichothecene B:

Generally, fungicides are considered to control the growth of pathogen in order to reduce the disease, ultimately the mycotoxins but by the time they have increased the fungal adaptation to them (Becher et al., 2010). In practical, fungicides are found in complex relationship with the disease development and the mycotoxin production (Magan et al., 2002). The use of some fungicides acts as a stress for fungus and enhance the production of mycotoxins (Mesterhazy et al., 1999; Moss, 2008). Prothioconazole fungicide can induce H_2O_2 in the plant cells which triggers the DON production (Audenaert et al., 2011). The biological components of the environments affect the production of mycotoxins as the presence of other fungi in the neighborhood can change the production of mycotoxins (Cooney et al., 2001; Xu et al., 2007). Apart from the genetics of the fungi, the genetic variability of host plants has also key importance in the disease production and the mycotoxin production (Mesterhazy et al., 1999) . The environmental factors including temperature and the water activity have also a great contribution in the production of mycotoxins by a fungal strain. In the literature, the studies are based on the production of mycotoxins in the in-vitro conditions. The temperature, humidity as well as the pH may increase or decrease the fungal growth and development as well as the production of mycotoxins (Garcia et al., 2011; Kokkonen et al., 2010; Lasram et al., 2010; Mateo et al., 2002; Medina and Magan, 2011; Mylona and Magan, 2011; Pose et al., 2010; Price et al., 2005; Ramirez et al., 2006; Schmidt-Heydt et al., 2008; Spadaro et al., 2010). The filamentous fungi may have different optimal temperature and humidity for the toxin production than for the optimal conditions required for its mycelia growth. (Ramirez et al., 2006) studied that *F. graminearum* showed highest growth at 25°C on water activity (aw) 0.995 while DON production was maximum at degrees 30°C on the same water activity. The optimal water activity (aw) for the growth of fungus was from 0.900 to 0.995 but for mycotoxin fabrication was from 0.950 to 0.995. The acidic pH usually favours the mycotoxin production (Keller et al., 1997; Spadaro et al., 2010). The acidic pH in the de-germed maize kernel favours the production FB1 mycotoxins than germed tissue in which the pH becomes basic (Shim et al., 2003). In an in vitro study (Merhej et al., 2010) demonstrated that Tri genes are stimulated as the pH goes from neutral to acidic and stimulates the production of trichothecene B (TCTB).

The increase in the resistance in the plants may decrease the production of the disease which ultimately can be helpful in the reduction of the accumulation of mycotoxins. The

composition of the substrate is the major factor in the mycotoxin production (Bouras and Strelkov, 2010; Kheiralla et al., 1992; Kokkonen et al., 2005; Mateo et al., 2002; Vogelgsang et al., 2008).The phase of the kernel development can have an effect on the production of mycotoxins. The embryonic stage of grains (presence of amylopectin) induces Fumonisin B1 (FB1) production by *F. verticillioides* and it is not produced in its absence (Bluhm and Woloshuk, 2005). Some plant molecules are also reported to activate the production of mycotoxins as polyamines trigger TRI genes expression and to increase the trichothecenes biosynthesis in *F. graminearum* (Gardiner et al., 2009). Sugar (sucrose, 1-kestose and nystose) activates Tri4 and Tri5 expressions and increases DON and 3ADON production in *F. graminearum* (Jiao et al., 2008).

5- Ecological role of mycotoxins:

5.1- Mycotoxins in the multitrophic interaction in soil:

It is foremost important to know the place of the mycotoxins in the environment to understand their possible role. Filamentous fungi are reported all around the world in the wide range of environment and produce disease in cereals as well as in fruits and vegetables in the field on the standing crops, after harvest and during storage (Olsen et al., 2008; Pietri et al., 2004; Placinta et al., 1999; Reddy et al., 2010). The disease incidence and the mycotoxin production varies from year to year and region to region.

Once the mycotoxins are produced they can get opportunity to flow in the environment. The production of mycotoxins in the field (as cereals) is difficult to control due to the open environmental conditions. Mycotoxins from the plants (i.e. grains, spike, spikelets, leaves and stem) enter to the environment shared by plants, vertebrates, micro and macro fauna and flora and even the producer themselves. One part of mycotoxins remains in the crop residue in the fields and the other goes to storage, milling and processing. The flow in the environment depends on the stability to adverse environmental conditions as temperature, pH, and solubility. Most of the mycotoxins are usually found resistant to the high temperature and adverse environmental conditions (Bullerman and Bianchini, 2007; Lauren and Smith, 2001) and are more or less soluble in the water. These properties make them to flow easily in the environment.

The crop residues in the field contaminated with mycotoxins are potentially dangerous to the environment. The rain water may solubilize the mycotoxins and take them to the field soil and drainage water (Fig. 3). A very few research is done in this potential environmental issue. Zearalenone (ZEA) is reported in the field soil and drainage water from the wheat contaminated with *F. graminearum* (Hartmann et al., 2008). The water samples collected animal feed processing mills and animal farms have also confirmed the presence of mycotoxins in the water which may be a threat to the environment (Aragon et al., 2011; Gajêcka et al., 2011; Gromadzka et al., 2009). The samples collected from various rivers of Switzerland confirmed the presence of deoxynivalenol and zearalenone (Bucheli et al., 2008). DON and ZEA were also recovered from the streams of United States (Wettstein et al., 2010). This may be dangerous to the water life of the river when in high quantity but this is still a question to be addressed.

The fungal toxins are found to be not only dangerous to the animals who consume them but they could also be transferred to humans by animal milk (Signorini et al., 2012) which goes back to the environment and make them risk for human health. Even a significant amount of DON is reported in the eggs of hens which were fed on the DON contaminated cereal feed (Sypecka et al., 2004) which is the indirect exposure of humans to the mycotoxins. Mycotoxins are also recovered from animal feces and human urine (Warth et al., 2012).

During the last few decades, of most part of research on mycotoxin focused on their effects on human and animals. Unfortunately, the role of mycotoxins in the life of fungi and their need for the producer fungi is not fully elicited and is still a mystery. By definition, mycotoxins are secondary metabolites and are not needed for the normal growth, development, energy production, the metabolic activity and the reproduction of their producers (Fox and Howlett, 2008; Karlovsky, 2008). As they are not needed for the primary functions of a fungi, so their role in the life of fungi becomes a big question.

Fungi may contest against other organisms in their ecology in diverse ways including growing faster than competitors, sporulation under stress conditions, hindrance for competitors, colonization of diverse hosts and substrates. We will try to summarize how these mycotoxins can help the fungi in life under the light of some basic but important questions and research taken place up to date.

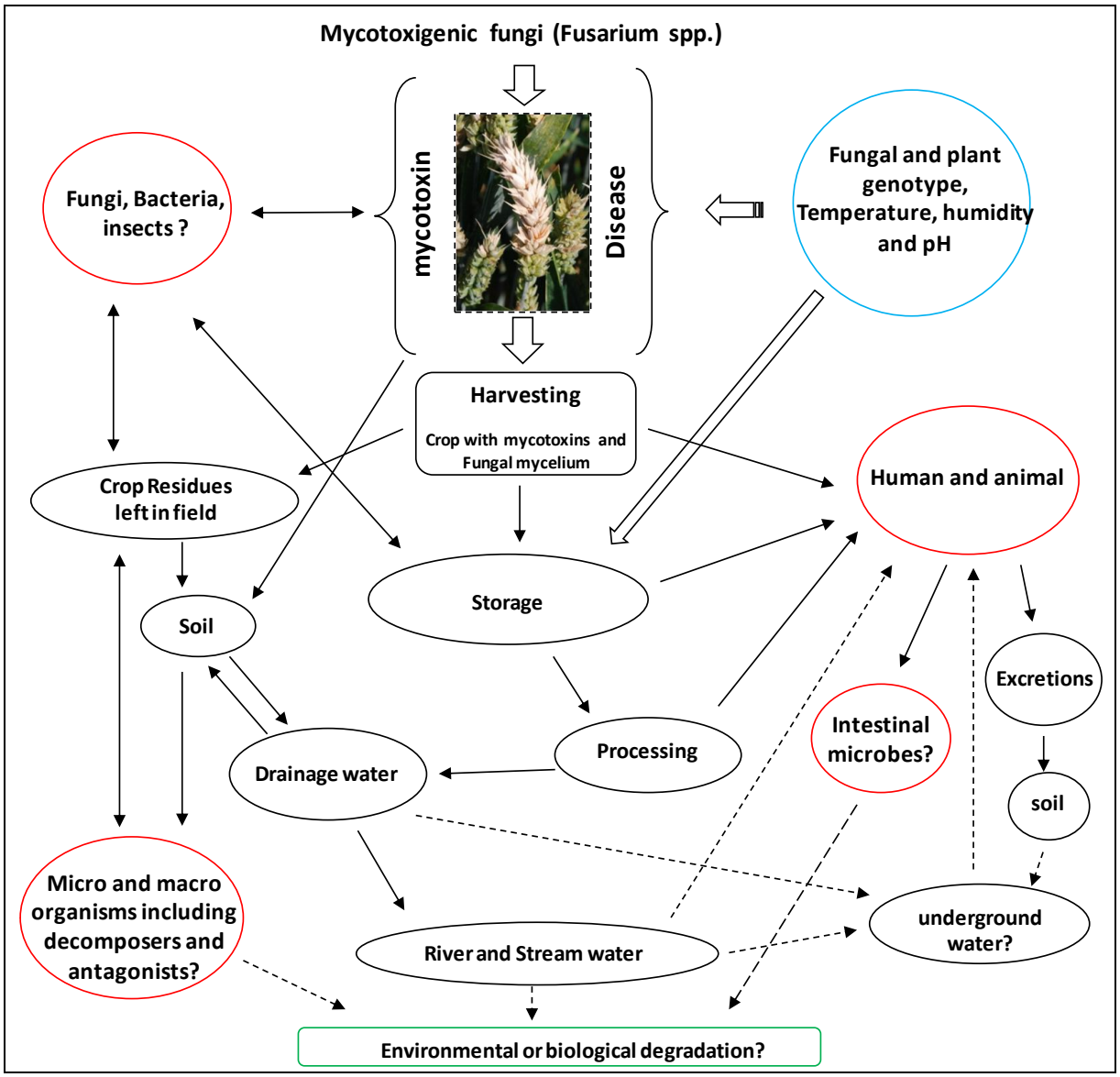

Fig. 3: Possible flow of mycotoxins in the environment (black coloured circles), its interaction with other organisms (red coloured circles) .The conditions which can favour mycotoxin production (blue coloured circle). Single end arrow represents the flow of mycotoxin or mycotoxin+producer fungi while two end arrow shows the competition between mycotoxigenic fungus and the other organisms. The question marks and the dotted arrows indicate the need for further research in these places.

5.2- Do mycotoxins help in disease production or fungal development and colonization on plants?

In general, most of the mycotoxin producing fungi are plant pathogens. So, the general perception is that mycotoxin helps in the production of disease. The role of mycotoxin in the primary infection is investigated extensively. All the reports shows that mycotoxins are not involved in the primary fungal infection on the plants for the disease production. The mycotoxin non producing mutants showed the same disease production as the mycotoxin producing wild type fungi (Bai et al., 2002). On the other hand, mycotoxin producing pathogens are found more virulent than their mutagens. In some studies NIV and DON are reported to play a significant role in the virulence of pathogen fungus and help in colonization in the plant tissue. In *Fusarium* spp. trichothecenes producing genes are activated and the mycotoxin production is increased during the colonization of stem and grains in cereals. (Bai et al., 2002; Desjardins et al., 1996; Hallen-Adams et al., 2011; Harris et al., 1999; Mesterhazy et al., 1999; Mudge et al., 2006). Wang et al., (2006) described that disrupted DON production in *F. graminearum* decreases the aggressiveness on wheat, barley and triticale seedling root infection. Mycotoxin producing fungi are found more aggressive than the mycotoxin non producing fungi. *F. culmorum* isolates producing DON were found more aggressive than the isolates producing NIV in wheat crop (Muthomi et al., 2002). This indicates that chemotype of a fungus also has a significant importance in the aggressiveness of a pathogen during disease development process. Previously it was also reported that hydrogen peroxide (H_2O_2) which may induce the programmed cells death is induced in the plant cells in response to the release of DON by fungus (Desmond et al., 2008; Gechev and Hille, 2005). The fungal toxins activate the cell death in the host cells which may help colonization the fungus (Desmond et al., 2008).

This discussion concludes that the mycotoxins are not involved in the production of disease but increase the aggressiveness of pathogen which helps the fungus rapid colonization. This rapid colonization may give an advantage to mycotoxin producer fungus over the other competitors during plant infection. Thus the fungus may occupy large part of plant material before it becomes ecological habitats in the soil which possibly help the fungus during its subsequent saprophytic survival.

5.3 - Do mycotoxins help in fungal survival under environmental stress?

This is one of the most important question comes in our mind about the role of mycotoxins in the fungal life which was completely ignored previously. The production of mycotoxins is affected by many environmental and biological stresses as we described earlier. Various environmental stresses increase the production of mycotoxins. This gives us a clue about the role of mycotoxins in the fungal life under stress. This hypothesis can be supported by the research argument which indicates that the sporulation and secondary metabolites are genetically connected and controlled by the common factors. For example the asexual spore production and the mycotoxins i.e. sterigmatocystin and aflatoxin production in *Aspergillus* spp. are related to the same genetic basis (Hicks et al., 1997; Kale et al., 1994; Kale et al., 2003). A direct relationship was observed in the conidia production of *Claviceps purpurea and* toxic alkaloids on agar plates (Pažoutová et al., 1977). It has also been investigated that the genes (FCC1) involved in the signal transduction are the same for regulating fumonisin biosynthesis as well as fungal conidia formation in *F. verticillioides* (Shim and Woloshuk, 2001).

The correlation between mycotoxins production and sporulation points out hypothesis that mycotoxins might be involved in the survival of fungus under adverse conditions. The production of secondary metabolites including many *Fusarium* and *Penicillium* mycotoxins and the sporulation are regulated by the same signaling G-proteins signalling pathways (reviewed by Brodhagen and Keller, (2006)). Zearalenone takes part directly or indirectly in formation of perithecia in *Gibberella zeae* (teleomorph of *F. graminearum*) (Wolf and Mirocha, 1973, 1977). The stress may stimulate the production of zearalenone (Kim et al., 2005) and can serve as a fungal tool for survival in the adverse conditions. On the other side, this perithecia formation was found to be highly dependent on mycotoxin dose. A high dose of zearalanone may inhibit the spore formation of the producer fungi (Mirocha and Swanson, 1983). This may help in the increasing fungal inoculum for infection and help in survival. Further investigations are needed in this regards in order to suggest the better control of fungi producing environmental threat.

5.4- Do mycotoxins help in competition with other organisms?

The above studies give us an indication to establish hypotheses about the role of mycotoxins in its ecology. It is usually believed that secondary metabolites are produced for the self defense against the other organisms (Kempken and Rohlfs, 2010) but this hypothesis is also still ignored in the case of mycotoxins. The mycotoxin producing fungi and their toxins interact with other micro and macro organisms (including fungi, bacteria, nematodes, protozoa, earthworms and arthropods) in their ecology as shown in Fig. 3. They come in contact whether on the plant during disease development, during storage or during the saprophytic survival in crop residues. The nature of interaction between them is still a question.

A general prospective is that mycotoxin may be one of the tools which can be used by the producer during the competition for food and survival against other fungi. Losada et al., (2009) reported that the competition with other fungi augmented the antifungal secondary metabolites in *Aspergillus* spp. In another study, (Ramakrishna et al., 1996) found that during a competition between T-2 toxin producing fungi *F. sporotrichioides* and two other fungi i.e. *A. flavus* and *Penicillium verrucosum* the T-2 toxin significantly increased in barley grains. The growth of *F. sporotrichioides* was negatively affected during this competition but it stimulated the production of T-2 mycotoxin. What is not clear in this study is to know if the two other competitors were negatively affected or not by the T-2 toxin and this toxin allowed *F. sporotrichioides* to survive. Therefore the role of mycotoxin is not so clear. *F. graminearum* mycotoxin production was 1500 % increased in the presence of *F. culmorum* and *F. poae* (Xu et al., 2007). They also found that the mycotoxin production is changed during the competition among two chemotypes. Cooney et al., (2001) found the production of DON by *F. graminearum* was affected negatively or positively in the presence of other *Fusarium* species. Recently, mycotoxin production and the growth of *Alternaria tenuissima* isolates was studied in relation to *F. graminearum* and *F. culmorum*. The growth of *Alternaria* and the Alternaria toxins (AOH, AME and ALT) were depressed and the DON and ZEA production was increased (Müller et al., 2012).

The impact of these mycotoxins on the other fungi and the mechanism of action on other fungi still need to be explored. Lutz et al., 2003 tested the impact of DON against a famous fungal antagonist *Trichoderma atroviride*. They reported that chitinase gene expression was repressed by the effect of DON. In another study, the impact of trichothecenes on yeast cells

was observed by McLaughlin et al., (2009) and later by Bin-Umer et al., (2011). The results illustrated that these mycotoxins inhibit the mitochondrial membrane potential and reactive oxygen species, depending on the dose of mycotoxins. The higher dose of trichothecene can inhibit the mitochondrial translation.

Bacteria are the most important competitors of fungi in the food web. They compete by using the food resources or the production of chitinase enzymes. some mycotoxins including patulin and penicillic acid, produced by *Penicillium* spp, are reported as quorum-sensing inhibitor (QSI) compounds against *Pseudomonas aeruginosa* which may provide the fungus a protection from the chitinase producing bacteria (Rasmussen et al., 2005). The growth of rumen bacteria *Ruminococcus albus* and *Methanobrevibacter ruminantium* were reported to be inhibited in the presence of Fusaric acid but not effected in the presence of DON or in the combination of DON+Fusaric acid (May et al., 2000). More studies are needed in this context about the relation between fungal mycotoxins and their impact on the bacterial growth on the different food resources.

Nematodes are one of the active members of the soil web and are potential competitors of mycotoxin producing fungi in soil and the plant debris or crop residues. They decompose the organic matter including crop residues. A very little is deliberated on the role of mycotoxins on the nematodes. In an in vitro experiment some mycotoxins including T2-toxin, moniliforrnin, verrucarin A and cytochalasin B are reported to reduce the viability of nematodes significantly. They were even died by the presence of mycotoxins but the rate of mortality depends on the mycotoxin concentrations (Ciancio, 1995). Two trichothecene compounds i.e. 4,15-diacetylnivalenol and diacetoxyscirpenol were described to reduce the mobility in second-stage juveniles which were hatched in the presence of these mycotoxins (Nitao et al., 2001). In a liquid medium DON reduced the egg hatching and the growth in the *Caenorhabditis elegans* after their birth from eggs(Gowrinathan et al., 2011).

Insects are the primary decomposers of the crop residues in the soil. Mycotoxinogenic fungi have to compete with insects on plants during food storage and as well as in the crop residues decomposition. The mycotoxins are found to have the ability to control the insects by infecting at different stages of insect life cycle. They have insecticidal effects depending on the type and quantity of mycotoxins (Wright et al., 1982). Mycotoxins may give the fungi an advantage against the fungivor insects. Indeed the latter are reported to prefer the non mycotoxin producing fungi over the mycotoxin producing fungi (Rohlfs et al., 2007). Honey bees are also found to be negatively affected by the presence of aflatoxin B1 (AB1) and

ochratoxin A (OTA), which depends on the concentration and the time of exposure to the mycotoxins (Niu et al., 2011). NIV, DON and FB1 are found to be cytotoxic to the SF-9 insect cells and can change the normal progression of the cell cycle and induce an apoptotic process (Fornelli et al., 2004).

5.5-Are mycotoxins needed for the saprophytic survival?

When crop are harvested, whether they are contaminated or not with mycotoxins, the crop residues were generally left in the field. They can be left at the surface or buried in the soil depending on the tillage system used. All micro flora and micro meso fauna we discussed are more or less involved in the colonization exploitation and decomposition of such trophic and spatial resources. Among the colonizers the filamentous fungi including plant pathogenic ones and mycotoxigenic ones play an important role in the decomposition process. They can subsequently over winter and develop the primary inoculum for the next season. Therefore, the place of mycotoxins and mycotoxin producers is worth being investigated.

Soil is a huge pool of different kind of microorganisms depending on the type of soil. One gram of soil may contain 1 µg to about 200 µg of DNA containing virus, bacteria, fungi, protozoa and nematodes etc in different ratios depending on the soil (Trevors, 2010). When these residues become in contact with the soil they become the ecological habitats for the mycotoxin producing fungus. These residues and the mycotoxin producer fungi are attacked by other decomposers and antagonists. In that contest a review of literature was performed to address the behavior of mycotoxigenic fungus *Fusarium graminearum* during its saprophytic phase in the crop residues (Leplat et al., 2012). Despite of this competition for the food filamentous fungi are reported to survive for many years in these crop residues and remain a permanent threat.

However, the role of mycotoxins in the fungal defense, substrate colonization and development, and competition with other soil biota still remains a question. May be these mycotoxins are used as a chemical weapon for the fungus in the saprophytic survival in the crop residues during off season.

6-Detoxification of mycotoxins:

Mycotoxins are found highly resistant to the environment and not easily degraded even by high temperature. A lot of researches are going on in regards to the detoxification of mycotoxins on the plants by themselves and by the microorganisms.

Plants produce UDP-glucosyltransferase (UGT) enzyme which can detoxify DON and 15ADON but have no impact on nivalenol accumulation (Poppenberger et al., 2003). UGT enzyme is induced in the presence of DON and converts it into DON-3-O-glucoside (D3G) which leads to the resistance in the plants against DON (Poppenberger et al., 2003; Shin et al., 2012).

Microorganism can be an effective source of detoxification of mycotoxins (Wu et al., 2009). Usually it may depend on incubation time, temperature and pH and can be stimulated or lessened by the presence of different minerals (Tinyiro et al., 2011). The bacteria are found to be good candidates in this regards. Aflatoxin B1 is degraded by a gram positive bacteria *Rhodococcus erythropolis* (Alberts et al., 2006) and by gram negative bacteria *Stenotrophomonas maltophilia* (Guan et al., 2008). S. *maltophilia* activity increased with the increase in temperature (up to 37° C) and the increase of pH (up to 8). The minerals also played a significant role. Mg^{2+} and Cu^{2+} activated the degradation of Aflatoxin B1 and Zn^{2+} strongly inhibited it. This shows that the conditions are very important for the mycotoxin degradation. So it might not be necessary for each mycotoxin degrading bacteria to degrade the same mycotoxin at each place with varying conditions. The bacteria *Bacillus subtili* and *B. natto* strains (*B. subtilis* 168 and *B. natto* CICC 24640) which are usually found in the food stuff are important decomposers of zearalenone and therefore can be useful to reduce mycotoxins from the food (Tinyiro et al., 2011). The soil has many kind of degrading bacteria which are reported to degrade different mycotoxins. Sato et al., (2012) isolated 13 bacteria aerobic (including gram-negative and gram-positive) bacteria from different environmental samples (2 bacteria from wheat leaves and 11 from soil) having good ability to degrade DON by converting it into 3-epi-DON. Soil-borne and organic matter resident bacteria *Bacillus licheniformis* strain was found to reduced zearalenone up to 95.8% and 98% in Luria–Bertani (LB) medium and in corn meal medium after 36 hours of incubation at 37° C (Yi et al., 2011). Soil-borne bacteria *Nocardioides* sp strain WSN05-2 was found to use the DON as a sole source of carbon and convert the DON into 3-epi-DON as an intermediate product in its degradation (Ikunaga et al., 2011).

There are not many reports about participation of fungi in the mycotoxin degradation. Only some *Rhizopus* spp are found the good decomposer of ochratoxin A (OTA) (Varga et al., 2005). Earthworms are considered the important decomposers, of soil and especially in the agriculture point of view. Deoxynivalenol (DON) is reported to be degraded by earthworm species as *Lumbricus terrestris* and *Aporrectodea caliginosa* (Oldenburg et al., 2008; Schrader et al., 2009; Wolfarth et al., 2011).

So, although there are few examples of plants, microorganisms and earthworms which can potentially degraded mycotoxins in given controlled conditions, the fate of DON in the crop residues and in soil itself is not really known. Besides, the biological degradation illustrated in previous example and the leaching underlined in the section 5, mycotoxins could potentially accumulate through the adsorption mechanism on the organic matter in the soil but nothing is known on that point too.

7- Conclusion and research prospects:

Apart from their impact on human and animal health a very few is known on the mycotoxins in the environment. However they can be present in all the compartments of the environment and many biotic agents may interact with positive and negative consequences on their fate (Fig. 1). Owing to the complexity of the flow of mycotoxins along the trophic webs, one may question about the ecological role of the mycotoxins in the environment. In the frame of controlling the primary inoculum of *F. graminearum* responsible for the Fusarium head blight on the wheat, we decided to focus more specifically on the role of DON towards micro flora and micro meso fauna in relation to the fate of this pathogenic fungus in its habitat in the crop residues. Therefore, in the first part of the thesis, I will describe the materials and methods we used during the study. Then first study was conducted in the field which raised some questions which needed to be addressed more deeply. So, the second part was conducted in the microcosms in the controlled conditions to see in which way the mycotoxins affect biotic inhabitants of the soil.

Chapter 2: Materials and Methods

Materials and Methods

1 - *Fusarium graminearum* strain MIAE00376:

F graminearum strain MIAE00376 was chosen for all the microcosm and field experiments. This strain was taken from the fungal and bacterial collection "Microorganisms of Interest for Agriculture and Environment" (MIAE), UMR-Agroécologie, INRA Dijon, France, (http://www2.dijon.inra.fr/umrmse/spip.php?rubrique47).

2 - Production of conidial suspension:

Conidia were produced by the method adapted from (Hassan and Bullerman, 2009). Briefly, the wheat bran liquid medium was prepared by adding 1% wheat bran in the distilled water and was autoclaved at 120°C for 20 min. The small plugs of 7 days old cultures of *F. graminearum* strain MIAE00376 grown on PDA were added and placed at 25°C on a rotary shaker (150 rpm) for seven days. The cultures were filtered through sterile cheesecloth (approximately 50–µm mesh size) to remove the mycelial mat and the remaining pieces of bran and conidial concentration was assessed through Malassez counting chamber under the microscope. The concentration (6.67×10^2 conidia/ml) was then adjusted by dilution with sterile distilled water.

3 - Chemotyping of *Fusarium graminearum* strain MIAE00376:

Objective:

F. graminearum can potentially produce different mycotoxins including deoxynivalenol (DON), 3–acetyl–4–deoxynivalenol (3-ADON) and 15–acetyl–4–deoxynivalenol (15 ADON), nivalenol (Niv) and zearalenone (ZEA) according to the various factors that are not always known. The geographical distribution induces some populations to produce one set of mycotoxins and the other populations the other mycotoxins. Each strain should therefore be characterized for its chemotype pattern. Therefore, the objective of this assay was to determine the chemotype of *F. graminearum* strain MIAE00376 to use in all the experiments.

Procedure:

This assay was performed on the grains of three varieties of winter wheat (*Triticum aestivum*) of different levels of susceptibility to Fusarium head blight (Maxwell > Charger >Apache respectively, GEVES data) and wheat bran (unknown susceptibility). Their humidity was adjusted up to 45% and then they were disinfected by autoclaving at 120°C for 20 min on three successive days.

They were aseptically transferred into the small tubes of 50 ml in such a way that each tube contained 15 g of wheat grains or 6 g of wheat bran. *F. graminearum* MIAE00376 conidia were prepared with the method described above for the conidial suspension production. 2.5×10^3 conidia/g of grains or wheat bran were distributed in each of the tube. The samples were incubated at 25°C in an incubator. Three replicates were made for each modality for each sampling time. Some tubes containing each of the substrate were left without inoculation as controls.

Sampling was done at 0, 4, 10, 21 and 60 days of incubation and placed at -20°C until mycotoxin extraction and further analysis. At each sampling time, the grains were ground and homogenized to access the content of the grains. Humidity of each replicate was measured with infrared balance (Mettler Toledo LJ16 Infrared Moisture Analyzer). Then mycotoxins were extracted for each sample.

For the extraction of mycotoxins, 3.5 g of ground grains or wheat bran were taken in 50 ml tubes and 10 ml of extraction solvent acetonitrile ((ACN)/H20mQ (84/16, v/v)) were added. The tubes were mixed by shaking manually and incubated at 250 rpm at 25°C in the rotary shaker for 3 h. Tubes were centrifuged for 3 min at 4500 rpm. Then 5 ml of the supernatant from each tube were filtered by the purification/exclusion column P-5 (R-biopharm, France). 2 ml of that filtrate was transferred in 4 ml vials. The organic phase was evaporated to dryness.The dry extract was dissolved in 200 µl of MeOH/H2O (1/1, v/v) by vortexing for 1 min. The samples were stored at -20°C until further analysis.

The analyses were performed by high performance liquid chromatography (HPLC, Beckman System Gold) coupled with a diode array detector (DAD) and managed by the 32 Karat software (v. 5.0). TCTB and ZEA were separated along a column HICHROM Ultrasphere 5octyl (150 mm × 4.6 mm, particle size 5µm) non-thermostated. A HICHROM Pre-Column Ultrasphere Octyl Guard® (4.6 X 45 mm; particle size 5µm) was used as a guard-column. Chromatographic system was ultra pure water pH 2.6 (eluent A) and ACN (eluent B). The gradient started with 5% B. From 0 to 14 min, it linearly increased to 30% B. Over further 10 min, there was another linear increase to 90% B. This phase was kept up to 29 min. The

gradient was then linearly decreased to 5% B during 2 min. Finally the column was equilibrated for 14 min before next injection. The flow rate was set at 1ml/min. The injection volume was 20 µl. The chromatograms were recorded for λ = 230, λ = 280 and λ = 320 nm. λ = 230 nm was used for detection and quantification of DON. Retention times were 6.4, 8.7, 14.4, 14.8 and 23.6 for NIV, DON, 15ADON, 3ADON and ZEA respectively.

Results:

Only one mycotoxin i.e. DON was found to be produced during the experiment in all the grains whether they were resistant or susceptible. The presence of other mycotoxins was not detected. So, *F. graminearum* strain MIAE00376 was found as DON chemotype. This experiment didn't allow to determine it more precisely (15 or 3 ADON one). Owing to the fact that this strain was isolated in France so it is quite likely that this strain should be 15 ADON which is more dominating in Europe (Jennings et al., 2004).

DON is detected after 10 days of incubation in the grains of 2 wheat varieties *i.e.* Maxwell & Charger. Production of DON by MIAE00376 was detected on Apache, which is the less susceptible of the three tested varieties, after 21 days of incubation. This production was directly proportional to the susceptibility up to 21 days. After 60 days this trend remained for two varieties Apache and Charger while in the case of Maxwell the DON concentration remained almost the same as in the 21 days. DON was not detected in wheat bran till 60 days of experimentation (Fig. 1). Anyway, this strain was not a NIV chemotype.

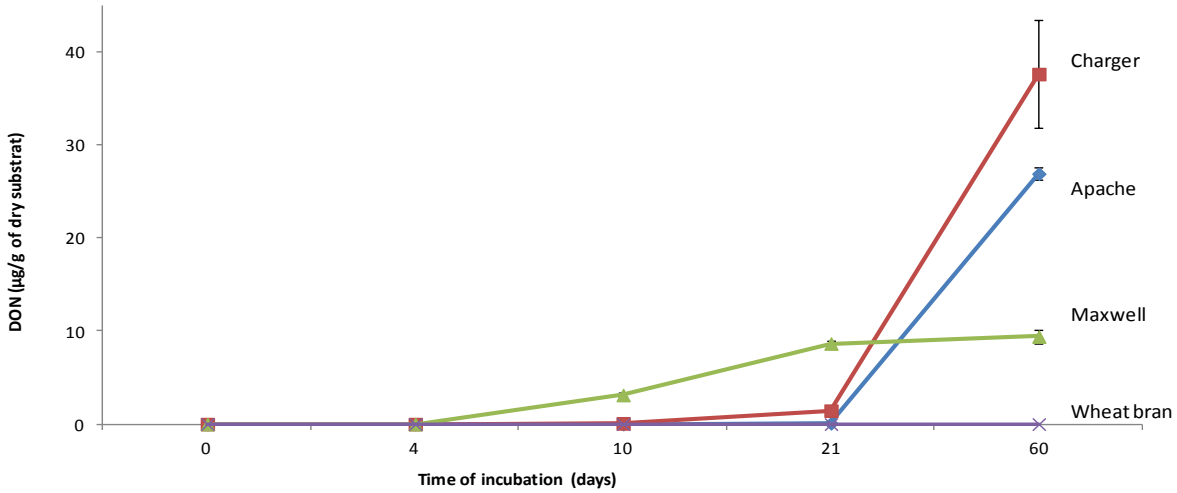

Fig. 1: Quantity of DON produced by *F. graminearum* strain MIAE00376 in the grains of three wheat varieties of different level of susceptibility to Fusarium Head Blight (Maxwell >

Charger >Apache respectively) on different time intervals up to 60 days and detected/quantified by HPLC-DAD.

4 - Soil

4.1- Field experiments

Field trials conducted during 2010-11 and 2011-12 campaigns were carried out on the field of Epoisses, experimental unit of INRA Dijon, 21120 Bretenière (5°05"E, 47°14"N). The estate is located in the plain, 15 km south of Dijon. Epoisses soil is a silty clay 54% silt, 38% clay, 8% sand, 2.3% organic matter, pH 8.

4.2- Microcosm experiments

The soil was collected from the meadow area near green house INRA, Dijon. The physico-chemical characteristics of this soil are close to the ones of the soil of the experimental unit of Epoisses where field experiments were conducted. This soil, instead of the one of Epoisses was sampled for practical reasons. This soil is also a silty-clay with 39.2% clay, 45.6% silt and 15.2% sand, 2.05% organic matter and pH 8. The soil C/N ratio was 9.85 and Cation Exchange Capacity was 21.6 cmol(+) kg^{-1} soil. It was sieved at 4 mm to remove gravels and the crop debris. A homogenous mixture of the soil was prepared by mixing manually. The humidity of soil was measured and adjusted to 17 % w/w i.e. 80% of its water holding capacity.

5 - Production of the field Inoculum:

5.1 - Maize inoculum:

Air dry maize stems were cut into 2-3 cm long pieces. Half of them were put in plastic boxes (65X30X17cm, Fig. 2a) and inoculated with plugs of 10 days old *F. graminearum* strain MIAE00376 grown on PDA culture. They were left for 3 weeks at room temperature. They were mixed manually in order to homogenize the inoculum once in a week (Fig. 2b). Half of the 2-3 cm pieces of maize stem were left non-inoculated.

a

Fig. 2a: fragments of maize stems used for inoculating field experimental plots

Fig. 2b: fragments of maize stems colonized by the inoculated *F. graminearum* strain MIAE00376 (characteristic pink color) 3 weeks post incubation.

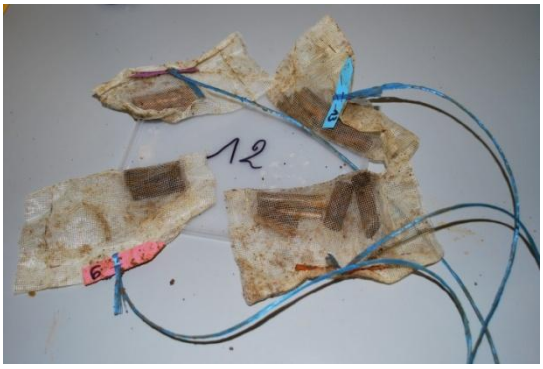

Fig. 2c : nylon bags containing the fragments of maize recovered after *in situ* incubation

5.2 - Nylon made bags:

Air dried maize stems were taken from the field area of Epoisses, and they were cut into small pieces of about 2-3 cm. They were disinfected by autoclaving twice at 120°C for 20 min. These stubbles were dried under laminar flow chamber in sterilized conditions for 2 days.

Half of them were inoculated with *F. graminearum* strain MIAE00376 by dipping them in the conidial suspension (10^6 conidia/ml) for two days and half were dipped in the sterile water to give the same water treatment and were kept uncontaminated. Both were placed at 25°C in the rotator shaker incubator at 125 rpm for 2 days in order to make the homogenous treatment for all the maize stubbles. These two types of maize stubbles were dried under laminar flow chamber in the sterile conditions separately. About 2.1 g (d.w.) (2 to 5 pieces) of these maize stubbles were filled in the nylon gauze bags with dimensions 15 × 10 cm (length × height) (Fig. 2c). Half of the bags were filled with inoculated stubbles and remaining half were filled with non-inoculated maize stubbles. Half of the bags contaminated with *F. graminearum* and half of the non-contaminated bags were contaminated with the solution of DON (Sigma Aldrich, CAS No. 51481-10-8) by injecting the required amount in equal amount in all the stubbles in a bag. Each DON contaminated bag contained 175µg DON / g (d.w.) of maize stubbles. In this way, four kinds of bags were prepared depending on the treatment on the maize stubbles. 1- Inoculated with *F. graminearum*, 2- Inoculated with *F. graminearum* and contaminated with DON, 3- Contaminated with DON, 4- With no treatment.

6 - Earthworms (*Lumbricus terrestris*) collection:

Lumbricus terrestris adult specimens were caught from the meadow situated near green house area of INRA Dijon, France. These earthworms were compelled to come on soil surface from the deep soil by using water and chemical expellant "Formalin" (Bouché, 1972).

Several trees were selected and one square meter soil area was marked around each of them. The grass in that marked area was cut up to the ground level. The marked areas were humidified by spraying about 100 l of tap water per square meter. Two days later, 20 l of 0.5 % formalin solution was sprayed per square meter of soil, which forced earthworms to come at the soil surface. These earthworms were caught and collected from the ground surface. This step was repeated twice for each marked area to catch maximum number of earthworms. The earthworms were washed immediately twice by tap water to remove the formalin from their skin. They were put in the soil in different containers and were placed at 10°C for 3 weeks.

At the time of launching the experiment, earthworms were put in the experimental soil at 17°C for 4 days. Biomass of earthworms was noted for each microcosm before putting them into the microcosms.

7 - Extraction and quantification of nematodes:

The extraction and the counting of nematodes were carried out using the elutriator described by Oostenbrink (1960). In order to extract the nematodes from each sample, 250 g moist soil or soil-straw mixture were put into a bucket containing 1 l water. The contents in the bucket were stirred to make the suspension and sieved at 1 mm sieve to remove the big fragments of straw. Then the sieved soil suspension containing nematodes was put in the elutriator using an upward flow of water (flow rate: 35 l/h) to accelerate the natural process of sedimentation of the coarse particles. After 15 min, the soil fine particles along with nematodes were collected.
When the elutriator was completely filled, the contents were poured into a bucket and sieved using four stacked sieves of 50 µm. The contents that didn't pass through the sieves were containing the nematodes, which were collected and transferred to a sieve containing a pre-moistened tissue paper. The sieve was placed in a large Petri dish containing water in an amount that could keep the permanent humidification of the sieve and migration of nematodes. The nematodes migrated below the sieve into the Petri dish.
After 48 h, the contents of the Petri dish were poured in a graduated cylinder to the volume up to 25 ml. Then 2 ml were spread on a plate grid specific for counting the individuals under a dissecting microscope. This counting was performed 2 times for each sample. For each sample, 2 ml of the extracted nematodes suspension was filled in the Eppendorf tubes and placed at -20°C for DNA extraction.

8 - Direct counts of bacteria and fungi (Colony Forming Units):

At each sampling time, total cultivable densities of fungi and bacteria were estimated by colony forming unit (CFU) on the appropriate media.
Briefly, 5 g of soil or soil-straw mixture were added to 45 ml of sterile water and shaken for 20 min in a three dimensional shaker at 700 oscillations/min. Tenfold dilutions were made for each sample from the mother suspension. Fungi were quantified on MEA (malt extract 10 g/l, agar 15 g/l) supplemented with citric acid (250 mg/l), and antibacterial antibiotics (chlortetracycline 50 mg/l and streptomycin sulphate 100 mg/l). Bacteria were quantified on YPGA (yeast 5 g/l, peptone 5 g/l, glucose 10 g/l, agar 15 g/l) supplied with cycloheximide (50 mg/l). Three repetitions for the bacteria and 5 repetitions for the fungi were performed.
Bacterial and fungal densities in all modalities on each sampling time were compared by analysis of variance (ANOVA) using XLSTAT-Pro version 7.1 (Addinsoft).

9 - Extraction of DNA:

Objective and principle:

The objective of this method was to extract total genomic DNA from 2 g of different substrates (i.e. soil, straw, soil-straw mixture and maize stubbles) or 2 ml of nematode suspension and to obtain non-degraded DNA. We needed DNA purified enough so that the impurities do not interfere with enzymatic reactions (PCR amplifications).

In this method, stored and frozen samples were thawed in extraction buffer. Lysis was mechanical (stirring in the presence of beads) and chemical denaturation of proteins and lipids by the action of the detergent sodium dodecyl sulfate (SDS) at high temperature followed by precipitation of complex protein/lipid-SDS denatured. The different components of the extraction buffer had the following roles: the high concentration of Ethylenediaminetetraacetic acid (EDTA) chelates the majority of divalent cations (Mg^2+) cofactor of many DNAses; the solution was buffered by Tris pH 8.0; the concentration of NaCl limited the possible partial denaturation of DNA at 70°C and released many DNA-protein interactions; SDS facilitated the lysis of membranes and protein denaturation. To separate the DNA from denatured proteins, deproteinization was to precipitate the proteins in the presence of a high salt concentration. This method had two advantages: the DNA-protein interactions were destroyed and the products were not toxic (unlike organic solvents, phenol and chloroform). The precipitates were separated by centrifugation at high speed. The clear supernatant contained the DNA. DNA was precipitated with isopropanol and washed with ethanol and two final purification steps were performed i.e. Polyvinylpyrrolidone (PVPP) and Geneclean, which were used to eliminate possible polluting elements (proteins, sugars, phenolic macromolecules, humic acids, etc). The PVPP polymer of high molecular weight made a complex with phenolic compounds and alkaloids. DNA bound to silica columns (Geneclean) in the presence of high concentrations of salts and was then eluted at low salt concentrations.

Extraction method:

The DNA was extracted from different substrates (i.e. soil, straw, soil-straw mixture and maize stubbles) by a procedure adapted from (Edel-Hermann et al., 2004). Two g of freeze dried and ground soil or soil-straw mixture or 0.5 g of freeze dried and ground straw or maize stubbleswere added to a 15 ml tube containing 4 g of 1.4 mm diameter silica beads, 5 g of 0.1 mm diameter ceramic beads and eight 2 mm diameter glass beads. Then 8 ml of lysis buffer

containing 100 mM Tris HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl and 2% (w/v) SDS were added to each sample. Samples were shaken for 90 s at 4 m/s in a Fast Prep-24 Instrument (MP Biomedicals, Illkirch, France) and incubated for 30 min at 70°C with mixing after 15 and 30 min. Samples were centrifuged at 7000 × g for 5 min at 15°C. The supernatants were recovered in 2 ml microcentrifuge tubes and incubated for 10 min on ice with 0.1 volume of 5 M potassium acetate. After centrifugation at 14000 × g for 10 min at 4°C, the nucleic acids in the collected supernatants were precipitated with one volume of ice-cold isopropanol for 15 min at -20°C. The precipitate was pelleted by centrifugation at 15000 × g for 20 min at 4°C, washed twice with 70% (v/v) ice-cold ethanol, air-dried and dissolved in 200 µl of 10 mM Tris HCl (pH 8.0). Out of 200 µl, only 100 µl of extracts of soil nucleic acids were used for the purification and 100 µl were freezed at -20°C for further use if needed.

These extracts of soil nucleic acids were purified twice by using a PVPP spin column to remove co-extracted humic acids. The two purification steps were miniaturized using Micro Bio-Spin columns (Bio-Rad, Marnes La Coquette, France) adapted to microcentrifuge tubes. For the preparation of PVPP columns, Micro Bio-Spin columns were filled with 93-95 mg of PVPP (Sigma-Aldrich, Saint Quentin Fallavier, France). The spin columns were washed twice by the addition of 400 µl of sterile water and centrifugation at 2000 × g for 4 min at 10°C. The columns were plugged at the bottom and 400 µl of sterile water were added. Before use, the PVPP spin columns were centrifuged at 2000 × g for 4 min at 10°C. 100 µl of crude DNA extract were loaded slowly onto the top center of the PVPP spin column and the purified extract was collected after 5 min of incubation of the column on ice, followed by 4 min of centrifugation at 1000 × g at 10°C. Then, the DNA extract was purified using a Gene clean kit (MP Biomedicals, Illkirch, France).

10 µl of the purified DNA extracts, were resolved by electrophoresis in a 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer, together with dilutions of calf thymus DNA (Bio-Rad). Gels were stained with ethidium bromide, photographed under a camera and the staining intensities were measured with Bio-1D^{++} software (Vilber-Lourmat, Marne-la-Vallée, France). The DNA concentrations in the DNA extracts were calculated using a standard curve of 25-150 ng of calf thymus DNA versus intensity.

DNA extractions from the nematodes-water suspensions were carried by using the same procedure as used for the soil with the following exceptions: 2 ml of nematodes-water suspension were used instead of 2 g of soil and 2 ml of 2 times more concentrated lysis buffer

were used for extraction of nucleic acids. The dry DNA was dissolved in 30 µl of 10 mM Tris HCl pH 8.0. For the purification it was not passed though the PVPP spin column but only purified by the Gene clean kit. The pure DNA obtained was quantified and stored at -20°C until it was further used for T-RFLP analysis.

10 - Fungal, bacterial, protozoan and nematode community structure analysis by terminal restriction fragment length polymorphism (T-RFLP):

Principle:

The analysis of ribosomal DNA (rDNA) by T-RFLP (terminal restriction fragment length polymophism) allows the characterization of communities by revealing length polymorphism of terminal restriction fragments.

For this purpose, PCR amplification of the rDNA was performed on the DNA template complex using fluorescently labeled primers specific for the community of fungi, bacteria, protozoa and nematodes. The amplified products were digested with restriction enzymes, and then labeled terminal restriction fragment were separated and analyzed by a sequencer.

Method:

The structural changes in the microbial and microfaunal communities during the straw decomposition were determined by using the DNA based technique T-RFLP.

The fungal communities were amplified by targeting the internal transcribed spacer (ITS) of the rDNA with the primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). Primer ITS1F was 5′end-labeled with the fluorescent dye D3 (Beckman Coulter, Fullerton, CA, USA). PCR amplifications were performed in a final volume of 25 µl by mixing 20 ng of sample DNA with 0.2 µM of each primer, 200 µM of each of dATP, dCTP, dGTP, and dTTP, 6 U of *Taq* DNA polymerase (MP Biomedicals, Illkirch, France), 0.16 ng/ml bovine serum albumin (BSA) and PCR reaction buffer containing 2 mM $MgCl_2$. DNA amplifications were performed in a Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation (30 s at 94° C), primer annealing (30 s at 55°C), and extension (1 min at 72°C) and a final extension of 10 min at 72° C.

To assess the bacterial community structure, the bacterial specific region targeted was 16S rDNA amplified by the primers 27F (AGAGTTTGATCCTGGCTCAG) (Edwards et al.,

1989) and 1392R (ACGGGCGGTGTGTACA) (Braker et al., 2001). Primer 27F was 5′end-labeled with the fluorescent dye D3 (Beckman Coulter). PCR amplifications were performed in a final volume of 25 µl containing 20 ng of sample DNA with 0.2 µM of each primer, 200 µM of dNTP, 6 U of *Taq* DNA polymerase, and PCR reaction buffer containing 1.5 mM $MgCl_2$. DNA amplifications were performed in a Mastercycler (Eppendorf) with an initial denaturation of 3 min at 94°C followed by 30 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 57°C), and extension (1 min at 72°C) and a final extension of 10 min at 72°C.

In the case of protozoan community structure, the amplification was done by the group-specific PCR primers Kin24SF (TAGGAAGACCGATAGCGAACAAGTAG) with 5′end labeled with the fluorescent dye D3 and Kin24SR (TTTCGGGTCCAAACAGGCACACT), which target 24S rDNA of the flagellate kinetoplastids (Rasmussen et al., 2001). PCR amplifications were performed in a final volume of 25 µl by mixing 20 ng of sample DNA with 0.2 µM of each primer, 400 µM each of dATP, dCTP, dGTP, and dTTP, 6 U of *Taq* DNA polymerase, 0.16 ng/ml of BSA, and PCR reaction buffer containing 3 mM $MgCl_2$. DNA amplifications were performed in a Mastercycler (Eppendorf) with an initial denaturation of 3 min at 94°C followed by 35 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 60°C) and extension (1 min at 72°C), and a final extension of 5 min at 72°C.

Nematode community structure was characterized using the specific primers NEMF1 (CGCAAATTACCCACTCTC) 5′end-labeled with the fluorescent dye D3 and S3 (AGTCAAATTAAGCCGCAG), which target the 18S gene of rDNA (Waite et al., 2003). PCR amplifications were performed in a final volume of 25 µl by mixing 20 ng of soil DNA with 0.3 µM of each primer, 250 µM of each of dATP, dCTP, dGTP, and dTTP, 6U of *Taq* DNA polymerase (MP Biomedicals), 16 ng/ml of BSA and PCR reaction buffer containing 2 mM $MgCl_2$. DNA amplifications were performed in a Mastercycler with an initial denaturation of 3min at 94°C followed by 30 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 53°C) and extension (1 min at 72°C), and a final extension of 10 min at 72°C.

PCR products were verified by electrophoresis in 2% agarose gels for fungi, protozoa and nematodes and 1% agarose gels for bacteria and stained with ethidium bromide for visualization under UV light. PCR products were purified by using a MinElute PCR purification kit (Qiagen, Courtaboeuf, France) according to the instructions of the

manufacturer, with two final elutions of the PCR products in 2 × 10 µl. The purified amplicons were quantified by comparison with known quantities of the molecular weight marker Smart Ladder (Eurogentec, Seraing, Belgium) in 2 % agarose gels for fungi, protozoa and nematode while 1 % agarose gels for bacteria. Then 120 ng of pure amplicons were digested with 5 U of restriction enzyme in a final volume of 100 µl. The restriction enzymes giving the more diversity were used for each community as *Hinf*1 was used for fungi, *Hae*III for bacteria, *Alu*I for protozoa and *Taq*I for nematode. The digestion reactions were incubated for 3h at 37°C, except for *Taq*I at 65°C.

The digested products were precipitated with 2 µl of 2.5 mg/ml glycogen (Beckman Coulter), 10 µl of 3 M sodium acetate (pH5.2), and 250 µl of ice-cold ethanol and were centrifuged for 15 min at 12000 × g at 4°C. The digested DNA were rinsed twice with 200 µl of ice-cold 70% ethanol and left at room temperature until the alcohol was evaporated. The DNA was re-suspended in 63 µl of sample loading solution (SLS; Beckman Coulter) and mixed with 30 µl of mixture containing 28.8 µl of SLS and 1.2 µl of size standard- 600 (Beckman Coulter). For each sample 3 times 30 µl were deposited into three wells of a 96 well plate and loaded on to a capillary electrophoresis sequencer CEQ™ 8000 (Beckman Coulter).

Analyses were run with the Frag 4-30 s method including a denaturation of 2 min at 90°C, an injection at 2000 V during 30 s, and a separation at 4,800V during 70 min. After electrophoresis, the length and the signal intensity of the fluorescently labeled terminal restriction fragments (TRF) were automatically calculated by comparison with the size standard using the CEQ 8000 Genetic Analysis System version 8.0.52. For each PCR product, the T-RFLP analyses were performed in triplicate. Mean values for the intensity of peaks found in at least two of the three analyses were considered for further statistical analyses of microbial community structure. The fragments between 60 to 640 bp corresponding to the size range of the standard were considered. The comparison of the TRF sizes between samples was automated by assigning them to discrete categories using the program Lis with an interval of 1.25 bp (Mougel et al., 2002). The communities characterized by the sizes of the TRF and their intensity measured by the height of the peaks were compared by principal component analysis (PCA) using the ADE-4 software (Thioulouse et al., 1997). This ordination method summarizes multivariate data to a few variables or dimensions and provides an arrangement of the communities in a two-dimensional diagram based on their scores on the two first dimensions. The significance of the resulting structures was checked using Monte-Carlo tests ($p < 0.05$) with 1000 random permutations of the data.

11- Quantification of bacteria, fungi and *F. graminearum* by real time polymerase chain reaction:

F. graminearum, overall fungal and bacterial densities were quantified in samples soil, straw, soil-straw mixture and maize stubbles by targeting a specific region of DNA using the molecular based technique real time polymerase chain reaction (real time Q-PCR). The quantification was based on the fluorescence intensity of SYBR Green dye, which binds to the double-stranded DNA. The real time Q-PCR reactions were carried out on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems TM, USA).

F. graminearum was quantified by using the specific pair of primers Fg16NF (ACA GAT GACAAGATTCAGGCACA)/Fg16NR (TTCTTTGACATCTGTTCAACCCA) (Nicholson et al., 1998) which amplify a 284 bp fragment of DNA. For the PCR, 1 µl of DNA was mixed with 0.25µM of each of the primers, 1 µg of T4 gene 32 protein (MP Biomedicals), 6.5 µl of Q-PCR SYBR Green ROX MIX (2X) (Thermo Fisher scientific Inc., USA) and DNAse-RNAse-free water to complete the final volume up to 13 µl. The real time Q-PCR conditions consisted of an initial step of 10 min at 95°C for enzyme activation and followed by 45 cycles of 15 s at 95°C (denaturation), 30 s at 64°C (annealing), 30 s at 72°C (elongation) and 30 s at 78°C (data acquisition). Then a melting curve analysis was performed as followed: 95°C for 30 s, 70°C for 30 s and then temperature was increased from 70 to 95°C at 2% (°C/min) ramp rate.

The fungal quantification was carried out by targeting a fungal specific 348 bp fragment of 18S rDNA using the primer set FR1 (AICCATTCAATCGGTAIT)/FF390 (CGATAACGAACGAGACCT) (Prévost-Bouré et al., 2011; Vainio and Hantula, 2000). For the PCR, 1 µl of DNA was mixed with 0.25 µM of each of the primers, 0.5 µg of T4 gene 32 protein, 6.5µl of QPCR SYBR Green ROX MIX (2X) and DNAse-RNAse-free water to complete the final volume up to13 µl. The conditions for the real-time Q-PCR were consisted of an initial step of 10 min at 95°C followed by 40 amplification cycles with 15 s at 95°C (denaturation), 30 s at 50°C (annealing), 60 s at 72°C (elongation and data acquisition). Then a melting curve analysis was followed with the cycling set as: 95°C for 15 s, 70°C for 15 s and then temperature was increased from 70 to 95°C at 2% (°C/min) ramp rate.

The bacterial densities were measured by targeting a bacterial specific 194 bp fragment of 16S rDNA using the pair of primers 341F (CCTACGGGAGGCAGCAG) and 534R, also

referred to as 515R, (ATTACCGCGGCTGCTGGCA) (López-Gutiérrez et al., 2004). For the PCR, 1 µl of DNA was mixed with 0.25 µM of each of the primers, 0.5 µg of T4 gene 32 protein, 7.5 µl of QPCR SYBR Green ROX MIX (2X) and DNAse-RNAse-free water to complete the final volume up to 13 µl. The real-time Q-PCR conditions consisted of an initial step of 10 min 95°C for enzyme activation, a second step corresponding to the 35 cycles PCR cycle with 15 s at 95°C (denaturation), 30 s at 60°C (annealing), 30 s at 72°C (elongation and data acquisition). Then a melting curve analysis followed the cycling set: 30 s at 95°C, 30 s at 72°C and then temperature was increased from 72°C to 95°C at 2% (°C/min) ramp rate.

Three technical replicates were performed for each biological sample for all the communities. For each community, a standard was prepared. For this purpose, the specific fragment of each community was amplified by using their specific primers. These fragments were cloned and then purified through Kit pGEM–T easy vector systems II (Promega, USA) by following the manufacturer's instructions. A standard curve was generated for each real time Q-PCR by using ten-fold dilution series of plasmid DNA corresponding to 10^9 to 10^2 copies of target DNA per PCR reaction. Two repeats of the standard curve were assessed during each replication. The curve was used to quantify the amount of target DNA in the different DNA samples. The threshold (CT) was significantly and linearly positively correlated to the logarithm of the theoretical quantity of each cloned specific DNA region of gene copies on the standard curve ($r^2 > 0.9871$for each target).

In microcosm experiment coefficient of correlation (r^2) values for *F. graminearum*, fungi and bacteria were 0.9981, 0.9871 and 0.9958 respectively and the efficiencies were 0.8373, 0.9629 and 0.9629 respectively. Final results were expressed as number of copies/g soil (dw).

In field experiment coefficient of correlation (r^2) values for *F. graminearum*, fungi and bacteria were 0.9945, 0.9882 and 0.9895 respectively and the efficiencies were 0.8027, 0.9073 and 0.9158 respectively. Final results were expressed as number of copies/g soil (dw).

12 - Preparation of deoxynivalenol (DON) solution:

Deoxynivalenol was obtained in powder form from Sigma Aldrich, France (CAS No. 51481-10-8). It was slightly soluble in water and highly soluble in ethanol (10 mg/ml). So a highly transparent solution was obtained by mixing 5 mg of DON powder in 3- 4 ml of pure ethanol.

The required concentrations were prepared by dissolving the transparent solution of DON in the sterile water.

13 - Extraction and quantification of DON from soil, straw, soil-straw mixture and maize residues:

A measured amount (about 2 g) of each sample were weighted and transferred to 50 ml tubes with 10 ml of sterilized ultrapure water in case of soil straw mixture while 20 ml in case of wheat straw and maize. In order to allow transfer of DON from matrix to water, samples were shaken at 230 rpm before centrifugation (10 min, 4 500 rpm). 10 ml (when soil-straw mixture) or 3 ml (when straw or maize stubbles) of supernatant were deposited on Immuno Affinity Column (IAC) DON-PREP® (P50 B, R-Biopharm AG, Darmstadt, Germany). Supernatant passed by gravity and DON was retained by IAC anti-DON contained on the gel of the column. Retained mixture was washed twice with 3 ml of sterilized ultrapure water using gravity property. Elution was done with 1.5 ml of 100% pure methanol (MeOH). Organic phase was evaporated to dryness and dissolved in 200 µl of MeOH/H2O ultrapure (1/1) by vortexing vigorously for 1 min. They were maintained at -20°C until they were analyzed by HPLC-DAD.

Detection and quantification of DON was carried out by a System Gold™ HPLC coupled with a 168 detector module, a dual-pump 128 programmable solvent module, a 7725i manual sample injector and 32 Karat™ software version 5.0, all purchased from Beckman-Coulter. An Ultrasphere Octyl Analytical® reverse-phase column (4.6 X 150 mm; particle size 5µm) was used as analytical column and a Pre-Column Ultrasphere Octyl Guard® (4.6 X 45 mm; particle size 5µm) as a guard-column. Chromatographic system was ultrapure H_2O pH 2.6 (eluent A) and ACN (eluent B). The gradient started with 5% B. From 0 to 14 min, it linearly increased to 30% B. Over further 2 min, there was another linear increase to 90% B. This phase was kept up to 18 min. The gradient was then linearly decreased to 5% B during 2 min. Finally the column was equilibrated for 4 min before next injection. The flow rate was 1 ml/min. The injection volume was 20 µl. The detection wavelength was 220 nm and the retention time was about 8.32 min.

Calibration curve was prepared by spiking matrix extract with standard solution of DON. Detection limit was determined as a concentration with a signal to noise ratio of 3:1. The quantification limit was at a signal to noise ratio of 10:1. This was done for each matrix type.

DON standard was purchased from N'Tox (St Jean d'Illac, France; http://www.ntox.fr/) in highest purity available. Organic solvents were of HPLC grade (VWR International).

14 - Minimum inhibitory concentration test:

Minimum inhibitory concentration (MIC) tests were performed to find the lowest concentration of DON required restraining the growth of different fungi and bacteria. Two types of methods were used in this regard i.e. radial growth measurement for the fungal strains and optical density measurement for fungi and bacteria.

14.1- Radial growth measurement:

This test was performed on potato dextrose agar PDA (39 g/l) Petri dishes having different concentrations (low to high) of DON. The radial growth of the fungi was measured for certain days according to the growth rate of fungi.

For this purpose, selected fungal strains were cultured on PDA Petri dishes at 25°C for 5-7 days. Specific amount of DON was mixed in the flasks containing liquid melting (60°C) PDA to make the different concentrations of DON as 0 µg/ml, 0.1 µg/ml, 0.3 µg/ml, 0.9 µg/ml and 2.7 µg/ml. 16 ml of PDA containing specific concentrations of DON were poured in the already marked Petri dishes having two perpendicular lines on their bottoms crossing each other in the middle of Petri dishes. 5 mm diameter pieces of 5-7 days old PDA cultures of selected fungi were placed in the center of the PDA Petri plates containing DON just at the intersection of the lines. Five replications were performed for each concentration for each fungal strain.

Radial growth of fungal strains were measured in mm on each of two lines regularly at 1, 2, 3, 4, 7, 8, 9 days or less when the fungal mycelium reached to the corresponding edge of the Petri plate on control media (0 µg/ml).

14.2- Optical density measurement:

14.2.1- Preparation of liquid minimal medium:

The liquid minimal medium (LMM) was prepared by using a protocol adapted from (Correl et al., 1987). Briefly, the media was prepared by adding to 1L of distilled water, 2 g of Na (NO_3), 1 g of KH_2PO_4, 2.5 g of $MgSO_4.7H_2O$, 0.5 g of KCL, 5 g of glucose and 2 ml of

already prepared oligo elements solution (Citric acid 50 g/l, $ZnSO_4.7H_2O$ 50 g/l, $FeSO_4.7H_2O$ 47.5 g/l, $Fe(NH_4)_2(SO_4)_2.6H_2O$ 10 g/l, $CuSO_4.5H_2O$ 2.5 g/l, $MnSO_4.H2O$ 0.5 g/l, H_3BO_3 0.5 g/l, $NaMoO_4.2H_2O$ 0.5 g/l). The media was sterilized by autoclaving at 120°C for 20 min.

14.2.2- Fungal growth measurement:

The selected fungal strains were grown on the PDA medium on the Petri plates for 5-7 days. For each strain a small square of PDA covered by mycelium was cut and put in 15 ml tubes containing 10 ml of LMM. The tubes were incubated at 25°C on a rotary shaker (150 rpm). After one week, the liquid cultures were filtered through sterile cheesecloth (approximately 50 µm mesh size) to remove the mycelium mats. The filtrates containing spores were centrifuged at 8000 × g for 20 minutes at 15°C. The supernatants were discarded and the spores were re-suspended in 1 ml of sterile water and counted by using a Malassez counting chamber.

Specific amounts of DON were mixed in LMM to make different final concentrations i.e. 0 µg/ml, 0.1 µg/ml, 0.3 µg/ml, 0.9 µg/ml 2.7 µg/ml, 8.1µg/ml and 10 µg/ml. Ninety-six wells microtiter plates were marked for the specific concentrations of DON and the selected fungal strains. The containing DON and the spores of selected fungal strains were filled in marked specific wells of microtiter plates. Each well was filled with a total amount of 270 µl of LMM containing DON and 10^6/ml spores of selected fungus. These microplates were incubated at 25°C and OD was measured at 590 nm wavelength at 0h, 22h, 27h, 43h, 51h, 67h, 70h, 75h, 91h and 137h.

14.2.3- Bacterial growth measurement:

In this assay, 12 ml hemolytic tubes were used instead of microtiter plates as these tubes could contain large amount of inoculum and media and were easy to place for constant shaking for bacteria homogenous growth. The different strains were cultured in LMM in 12 ml tubes for 3 days. The bacteria were counted by using a Malassez counting chamber. The specific quantity of DON was mixed in LMM to make the same final concentrations as above for fungi in 12 ml tubes. The bacterial suspensions were added to the media to make 10^3/ml bacteria to each tube having different concentrations of DON. Three replicates were prepared for each modality. Tubes were placed in an incubator rotary shaker at 25°C and 180 rpm. The OD was measured at 590 nm for each bacterial strain with different concentrations of DON at the same time as for fungi. At each sampling time 150 µl from each tube was put in a well of a microtiter plate and the OD was measured by spectrophotometer.

14.3- Data analysis:

From the data collected, the curves representing the change in OD or radial growth were plotted versus time. The growth of fungus and bacteria (OD values or radial growth) were calculated and compared to different concentrations of DON for all the strains. For this purpose, the growth (OD values) curve of strains over time for each replication was calculated by area under the growth progress curve (AUGPC) formula and then one way ANOVA was performed for each strain.

Chapter 3: Survival of *Fusarium graminearum* in soil and mycotoxin contaminated crop residues according to the tillage system

Survival of Fusarium graminearum in soil and mycotoxin contaminated crop residues according to the tillage system

1-Introduction:

Fusarium head blight (FHB) is one of the most dangerous disease of wheat, maize and other small grain cereals (Parry et al., 2007; Sutton, 1982). FHB causes considerable reductions in grain yield and quality, and its repeated epidemics result in massive losses worldwide (McMullen et al., 1997; Nganje et al., 2004; Windels, 2000). In addition, the grains are filled with a wide range of mycotoxins (Bottalico and Perrone, 2002; McMullen et al., 1997; Miller, 1995; Snijders, 1990) which make them hazardous to the humans and animals (Pestka, 2007, 2010). A complex of more than 17 Fusarium species is involved in causing this quite deleterious disease of the cereals worldwide (Bottalico, 1998; Dorn et al., 2011; Tan et al., 2012; Xu et al., 2008). *Fusarium graminearum* is the primary causal agent of FHB among the others fungal species of the complex and leading the disease worldwide (Bottalico, 1998; Bottalico and Perrone, 2002; Goswami and Kistler, 2004). FHB attacks on wheat have consequences on both the yield quantity and on the grains quality by leaving the grains small, light, degraded, wrinkled with degraded proteins, low baking quality and nutritive value (Champeil et al., 2004). Moreover, most of the FHB fungal complex produces various types of mycotoxins, some of which being quite deleterious for human and animal consumption (Bottalico and Perrone, 2002; Foroud and Eudes, 2009).

Studies of plant pathogenic fungi generally focus on infection processes, disease development and other concerns in plant–microorganism interactions, but the saprophytic period of these pathogen's life reveals the weakness that could be exploited to control the development of these fungi (Leplat et al., 2012; Parry et al., 2007). The most serious stage in *F. graminearum*'s life cycle is its survival in the field after the harvest of the host crop (Fig. 1). *F. graminearum* survives in the form of mycelium in non-decomposed crop residues and develop the primary inoculum for the next season crop (Parry et al., 2007). At the time of harvest, the mycotoxin contaminated crops are harvested and the residues are left in the field in the form of grains, glumes and lemmas, leaves, stem and roots. *F. graminearum* develops

fruiting body perithecia on the surface of infested residues and discharges the ascospores during the suitable environmental conditions. These ascospores serve as the primary inoculum during the development of head and initiate the disease on the plants (Parry et al., 2007; Trail, 2009; Yuen and Schoneweis, 2007).

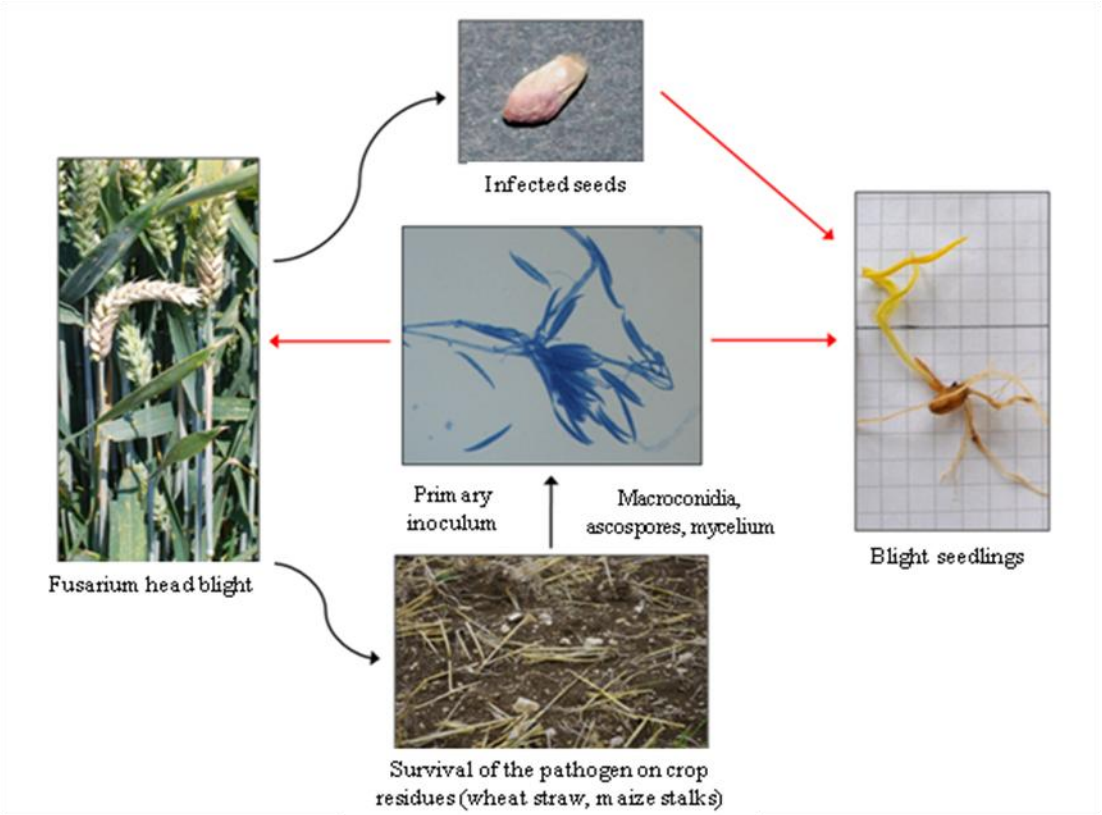

Fig. 1: Disease cycle of *Fusarium graminearum*. Black, sigmoid-like arrows indicate habitats provided by the crop and red arrows indicate infectious activity kept up by habitats (photograph: courtesy of J. Leplat). Crop residues allow the production of *F. graminearum* primary inoculum. The primary inoculum can provoke seedling blight as well as Fusarium head blight by splash dispersal. *F. graminearum*-infested wheat ears can cause the production of infected seeds which lead to seedling blight. (Leplat et al., 2012).

The management of these crop residues remains a big question. *F. graminearum* survival in the crop residues is the potential threat to the next crops, to FHB epidemics and to economic and health risk. Diverse strategies were generally proposed previously to eliminate the crop residues such as burning of crop residues or post harvest nitrogen applications but their use

produced questions for the soil quality, areal and ground-water pollution (Dormaar et al., 1979; Khan et al., 2007; Wood, 1991). The incorporation of the crop residues by the soil tillage was reported as an effective tool in the reduction of the *F. graminearum* inoculum and the disease incidence (Dill-Macky and Jones, 2000; Fernandez et al., 2008; Steinkellner and Langer, 2004). However, the world trend towards the tillage practices is changed and the world is heading toward the reduced or zero tillage. In the frame of a more sustainable agronomy, reduced tillage can reduce the soil erosion, conserve soil moisture, increase soil fertility and expected yield as well as it preserves biodiversity and ecosystem services (Cheatham et al., 2009; Coolman and Hoyt, 1993; Lestingi et al., 2010). These reduced tillage practices however may provide safe haven to the pathogen for their growth and development (Bockus and Shroyer, 1998). FHB is reported higher in the reduced tillage as compared to the tillage (Dill-Macky and Jones, 2000).

Therefore, the problem of crop residue as place of pathogen overwintering and a foundation of inoculum needs the solid approach that can address studies on the survival of the *F. graminearum*. The presence of mycotoxins in the crop residues cannot be ignored as they are produced on the crop during the development of disease and they are likely strongly involved in the mechanisms of interactions between the pathogenic fungi and the host plant leading to the accumulation of these deleterious compounds in the grains (Boutigny et al., 2008; Merhej et al., 2011; Ponts et al., 2009). Beyond the end products dedicated to human and animal consumption, the side products such as small and light diseased kernel, rachillas, glumes, lemmas and paleas on one side, stem, leaves and to a lesser extent roots on the other side can also be contaminated with the mycotoxins but these secondary products have received little attention as they go back to the soil at harvest. Most of them are water soluble and could be leached down to the ground water, they can also be adsorbed on the soil clay-humus complex or they can integrate the food webs with unknown so far deleterious impacts on the microflora and soil fauna providing thus a competitive advantage to the mycotoxigenic fungi. However, the ecological role of interference competition through toxin production is not well understood. In particular, it is unclear under what conditions the benefits of toxic killing outweigh the metabolic costs involved. A killer advantage could rely on local competitive interactions where the benefits of killing accrue to the toxin producer preferentially, but this notion has little empirical support (Matarese et al., 2012). In addition, the abundance of resources could modulate the benefits of toxin production. Indeed, this benefit should either be highest when resources are abundant and metabolic costs are relatively low or when resources are scarce and toxic killing is a 'last resort strategy' to obtain nutrients (Pintor et al.,

2010). Crop residues are supposed to provide an important trophic resource to the microflora and soil fauna, therefore the cost/benefit ratio mycotoxins production by *F. graminearum* remains really questionable

The most commonly associated mycotoxins with *F. graminearum* are deoxynivalenol (DON) and its acetylated forms 3-ADON and 15ADON (Foroud and Eudes, 2009). It is known that DON causes many disease symptoms in the animals and humans (Pestka, 2007, 2010) but the ecological role of this mycotoxin and its acetylated form towards the residue-borne microflora and the soil fauna remains obscure.

The objective of our study was to identify a putative ecological role of DON all through the saprophytic survival of *F. graminearum* during the decomposition of the crop residues in the field. We evaluated the impact of DON on the other crop residue colonizing small organisms including bacteria, fungi, protozoa and nematodes using fingerprinting methods. Because the tillage system determines the fate of crop residues, the structures of the microbial and microfauna communities were monitored both under mouldboard and shallow tillage systems.

2-Materials and Methods:

Field trials conducted during 2010-11 and 2011-12 campaigns were carried out on the field of Epoisses, experimental unit of INRA Dijon (Chapter 2, page 46)

Preliminary information:

For field experiments, two different but adjacent plots were used in 2010-11 and 2011-12 successively. For the first campaign (2010-11) the previous crop was wheat, for the second campaign (2011-12), the previous crop was maize. For both years, the wheat variety Charger, which is susceptible to Fusarium Head Blight (FHB), was cultivated in these plots according to various treatments (tillage system, inoculation with *F. graminearum* strain MIAE00376) and in both cases, the population dynamics of the strain in the crop residue was monitored by a molecular method and the developpement of FHB on the wheat crop was monitored by visual observations, regular subsamplings and image analyses, measurements of yield and DON contamination.

Campaign 2010-2011 did not provide any exploitable results. Briefly, the experimental set up was a classical split plot design including the following factors: (shallow *vs* mouldboard tillage) × (inoculated *vs* non inoculated crop residues) × 3 blocs. Inoculation was performed by dispersing quantified amounts of previously contaminated oats kernels at the surface of corresponding subplots (9 × 3 m). Unfortunately (from a scientific point of view) the climatic conditions were such that the *Fusarium* strain did not establish and that no FHB was recorded this year in Burgundy. Therefore the data acquired during this campaign were too few, they did not allow discriminating the treatments and so, they won't be neither presented nor commented in this manuscript. However an important conclusion drawn from this unsuccessful experimentation was that surface inoculation alone was risky and that it should be completed by a localized inoculation allowing to monitor the fate of the inoculum in the crop residues both left at the surface (shallow tillage) and buried (mouldboard tillage). For further field experimentations it was decided to use nylon gauze bags whose mesh (approximately 0.5 mm) allows exchanges between the content (inoculated or non inoculated crop residues) and the soil surrounding. In the present study, the subsequent experimentation was performed in 2011-12 in the plot previously cropped with maize. Maize was therefore the crop residue that was inoculated with the *Fusarium* strain MIAE00376 both at the surface and

using the nylon bags. Duplication over time of this field experiment could not be performed in the frame of the thesis.

2.1- Preparation of field:

During campaign 2010-2011, the plot was cropped with maize. On the 16th August 2011, stubbles were sampled and brought back to the lab to produce the inoculum. On the 16th September 2011, maize was harvested, the residues were ground and left at the surface up to the 25th September. At that date, part of plot was plowed (mouldboard tillage) and consequently the crop residues were buried. A cover-crop was used to break the surface of the soil (shallow tillage) of the second half of the plot and consequently, the crop residues remained at the surface or close to the surface of the soil. Field inoculation of *F. graminearum* was performed by dispersing 500 g of previously contaminated or non contaminated pieces of maize stubbles (Chapter 2, page 46) at the surface of corresponding subplots (9 × 3 m) on the 22nd September 2012, i.e. 3 days before any tillage procedure to allow for a good installation of the inoculum.

The experimental set up was a classical split plot design including the following factors: (shallow *vs* mouldboard tillage) × (inoculated *vs* non inoculated crop residues) × 3 blocks (Fig. 2). Wheat, variety Charger which is a susceptible variety towards FHB, was sown on the 24th October 2011. The density of sowing was 400 seeds/m^2. The subplots were separated by variety Maxwell which is also susceptible to FHB and higher in size to avoid any cross contamination between different treatments (Fig. 3).

In the following, mouldboard and shallow tillage will be considered as tillage and no tillage systems respectively.

In this way four kinds of subplots were constructed within each block:

1- Tillage × inoculated with *F. graminearum*

2- Tillage × non-inoculated

3- No tillage × inoculated with *F. graminearum*

4- No tillage × non-inoculated

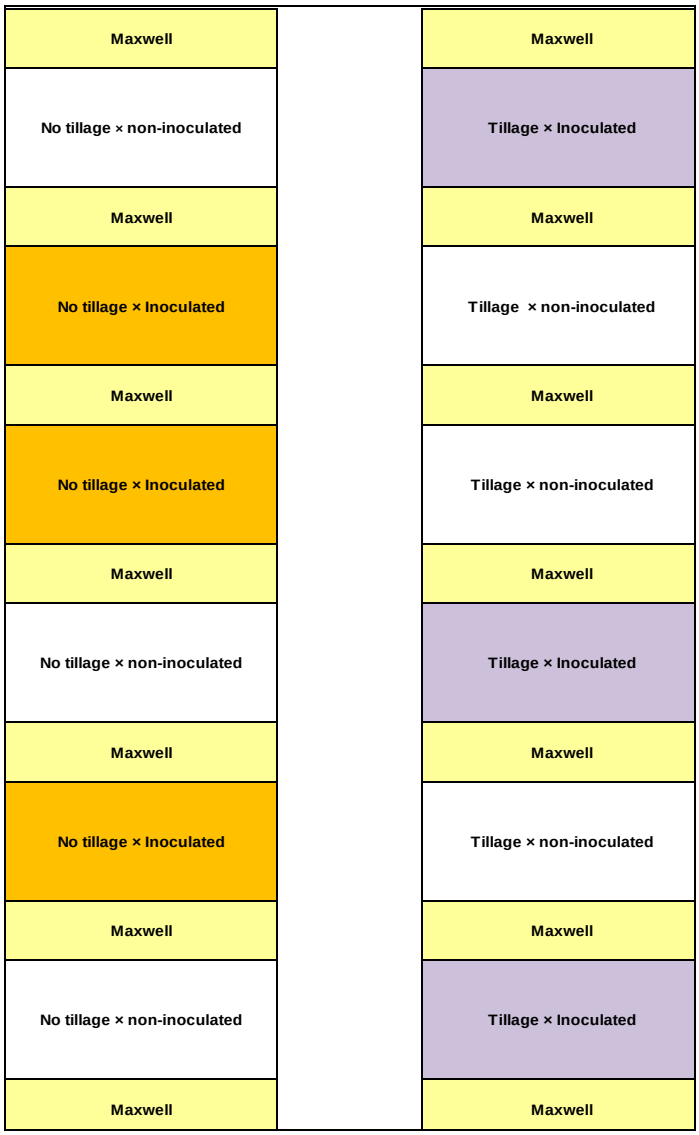

Fig. 2 : Experimental set up. For practical reason, the distribution of the subplots is not fully random. The left part of the plot is dedicated to shallow tillage while the right part was dedicated to mouldboard tillage.

Fig. 3 : Experimental plots set up to monitor the survival of *F. graminearum* strain MIAE in crop residues in relation to the FHB development, according to the tillage system.

a : subplots conducted under shallow tillage at the end of winter (March 2012)
b : subplots conducted under mouldboard tillage at the end of winter (March 2012)
c : tracking bags buried in subplots "a" (March 2012)
d : tracking bags buried in subplots "b" (March 2012)
e : Experimental subplots planted with the susceptible variety Charger are separated from each other by a cultivated subplot with a different variety (Maxwell) also susceptible to FHB and higher in size to avoid cross contamination between treatments (18 June 2012).
f : Symptoms of Fusarium Head Blight can be observed all through a no tilled subplot.(18 June 2012).
(photo a-d : courtesy of E. Gautheron; photo e-f : courtesy of C. Steinberg)

2.2- Preparation of nylon bags:

F. graminearum strain MIAE 0376 conidial suspension was produced (Chapter 2, page 43) in order to inoculate the maize stubbles and deoxynivalenol solution was also prepared (Chapter 2, page 56) in order to contaminate the maize stubbles. Then four kinds of bags were prepared depending on the treatment on the maize stubbles. 1- Inoculated with *F. graminearum*, 2- Inoculated with *F. graminearum* and contaminated with DON, 3- Contaminated with DON, 4- With no treatment.

2.3- Establishment of experiment:

A FHB susceptible variety of wheat (Charger) was sown in all the subplots on the 24 October 2011. The sowing density was 400 seeds / m^2. Two weeks (7 November 2011) after wheat sowing, all 4 kinds of bags were put in each of the 4 kinds of subplots in each block. They were placed on 1 cm deep in the subplots with no tillage and 10 cm deep in the subplots with tillage. For the last sampling time we used double number of bags to get enough material for the extraction of DNA and the measurement of DON in case if the decomposition process got too fast.

2.4 – Sampling, samples processing and analyses:

Small bags were sampled on 7 November, 13 December (week 5), 7 March (week 17) and 20 April (week 24). The maize stubbles were sorted out and filled in the separate vials. All the samples were freeze dried and ground to make a homogenous mixture. They were placed at -20°C.

From these samples DNA was extracted (chapter 2, page 50). From this DNA *F. graminearum*, fungal and bacterial densities were determined by using real time PCR (Chapter-2, page 55). The changes in the genetic structure in the fungal, bacterial, nematode and protozoan communities were determined by DNA based technique terminal restriction fragment length polymorphism (T-RFLP, Chapter 2, page 52).

From the homogenous mixture of ground maize stubbles, DON was extracted and quantified by using high performance liquid chromatography (HPLC, Chapter 2, page 57).

2.5-Disease development on the wheat crop:

Four blocks were used to monitor the wheat crop, among which were the 3 blocks used to monitor *F. graminearum* and the microflora in the nylon bags. The development of the culture was assessed by visual observations (counting seedlings or ears for example) and by objective quantifications (e.g. green surface) performed by image analysis from photographs (a template was prepared for that) or samples taken in the subplots and scanned before analysis. The image analysis software used was Mesurim Pro (http://pedagogie.ac-amiens.fr/svt/info/logiciels/Mesurim2/Index.htm;) thanks to the setting up performed by L. Falchetto (unpublished results) in the frame of J. Leplat's thesis (Leplat 2012)

The measured parameters were:

a. number of emerging seedlings after sowing, at the end of winter and of plants in the course of the season (counts)

b. cm^2 of green surface /m^2 after sowing and at the end of winter (image analysis)

c. number of tillers, ear height and green surface in early spring (counts and image analyses)

d. number or ears, and relative number of FHB contaminated ears and spikelets (counts and image analysis) (3 times in June)

e. yield and DON content.

ANOVA were performed on the sets of data to evaluate the role of the factors (inoculation and tillage system) and their interaction on the development of the disease and the resulting yield.

3- Results:

3.1- Process of decomposition:

The dry weight was calculated for each sampling time and the loss in the weight (%) of maize stubbles was noted in order to evaluate the state of decomposition of maize stubbles and microbial activity and therefore adjust the sub-sampling strategy. The weight of maize stubbles was reduced gradually and very fastly with the passage of time. We observed that 17% weight was lost after 5weeks, 51% weight loss after 17 weeks and 78% weight loss was observed after 24 weeks of setting up the experiment. We observed no effect of tillage on the loss of weight in the maize stubbles.

3.2- Structural changes in microbial and microfaunal communities colonizing the maize stubbles:

The genetic structures of fungal, bacterial, protozoan and nematodes communities were determined by using terminal restriction fragment length (T-RFLP) assay. The changes in the community structures of all these communities were determined after 0, 5, 17 and 24 weeks of the establishment of experiment. By this assay, the changes in the community structures were followed up to 24 weeks to observe the impact of DON on the community structures colonizing the maize stubbles in the field cultivated with wheat crop in the field with and without soil tillage. We also observed the impact of field inoculation as well as the bagged maize stubbles inoculation on the changes in the community structure to see how the presence of *F. graminearum* can change the colonization of other fungi, bacteria, protozoa and nematodes in the crop residues.

The TRFs (Terminal restriction fragments) occurring between 60 bp and 640 bp were taken into account. The mean numbers of TRFs per DNA sample were 90 for bacteria, 81 for protozoa, 37 for fungi and 83 for nematodes community. Principal component analyses (PCA) were performed by integrating the number and the intensity of different TRFs for each of the four communities. The result of the PCA was displayed as variations on a two dimensional diagram for each community. The significance was determined at each sampling time by Monte Carlo test with 95% level of significance to find the effect of different factors on the

changes in the structure of all these four communities (Table 1-4). For sake of clarity only some of the two dimensional diagrams are shown, results are summed up in tables to elaborate in short but comprehensively.

3.2.1-Fungal Community structures:

The T-RFLP results for the fungal communities are determined in Table 1. The results revealed that DON produced no impact on the structure of fungal community having colonized the maize stubbles at any sampling time. The fungal community structure was different in the maize stubbles inoculated with *F. graminearum* from the one in non-inoculated stubbles at each sampling time till the end of experiment. The colonizing fungal communities were significant affected by tillage system. This can be clearly observed in the Fig. 3 which shows the variation along two dimensions. One dimension (PC1) shows 41.04 % variation and the second dimension shows 15.62% variation. The fungal structure is more spread along PC1 which shows that fungal community structure was highly different in the stubbles contaminated with *F. graminearum* and the spread along PC2 shows that the tillage system was also the main factor which changed the community structure colonizing the crop residues.

Furthermore, the results showed the field inoculation produced no significant impact on fungal colonization. In addition, the passage of time (seasonal effect) produced a significant impact. The community profile changed from week 5 to 17 and also from week 17 to week 24.

3.2.2-Bacterial community structure:

The results showed that the bacterial community colonizing crop residues was not affected by DON at any sampling time (Table 2). The community structure was significantly affected by soil tillage and the maize stubbles inoculated by *F. graminearum*. The time also played a significant role in the changes in the bacterial community structure in the maize stubbles.

3.2.3-Protozoan community structure:

According to the results from T-RFLP analysis, the protozoan community was significantly affected by DON in the maize stubbles (Table 3). This impact of DON was observed throughout the experiment at each sampling time. This can clearly be seen in the two dimensional Fig. 4 of week 17 sampling time. The dimension 1 with 32.19% variation shows the clear impact of DON on the community structure. Furthermore, the soil tillage produced

also a significant impact on protozoan community colonization of maize stubbles. This impact was observed on week 5 but then on week 24 it was disappeared and then again appeared on week 24. The soil inoculation produced no impact on the protozoan communities as well. The maize stubbles inoculation with *F. graminearum* produced no influence for the first two sampling times but a significant impact was found after 24 weeks.

3.2.4- Nematodes community structure:

The nematodes community structure was not affected by the presence of DON in the maize stubbles (Table 4). The nematodes community which attacked the crop residues was only affected by the soil tillage and this was seen at each sampling time. The maize stubbles inoculation produced also an impact on the nematodes colonizing the crop residues. The field inoculated or not with *F. graminearum* produced no impact on the community colonizing the maize crop residues. The time factor played a significant role also on the nematodes communities. The community structure was significantly different between the time periods.

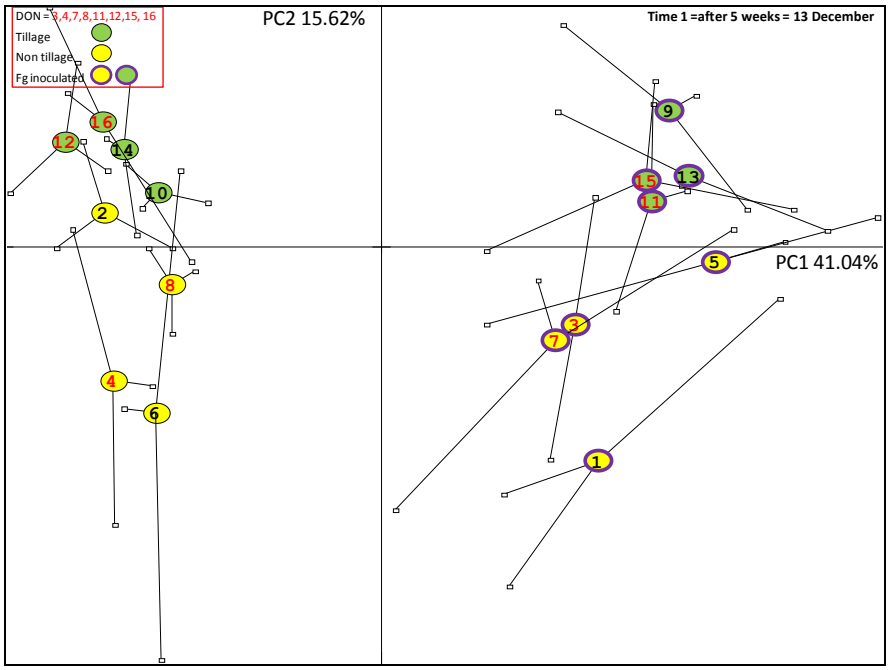

Fig. 3: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the fungal community structure. Each number represents the mean of 3 biological replicates for one treatment at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results. The red numbers represent the presence of DON.

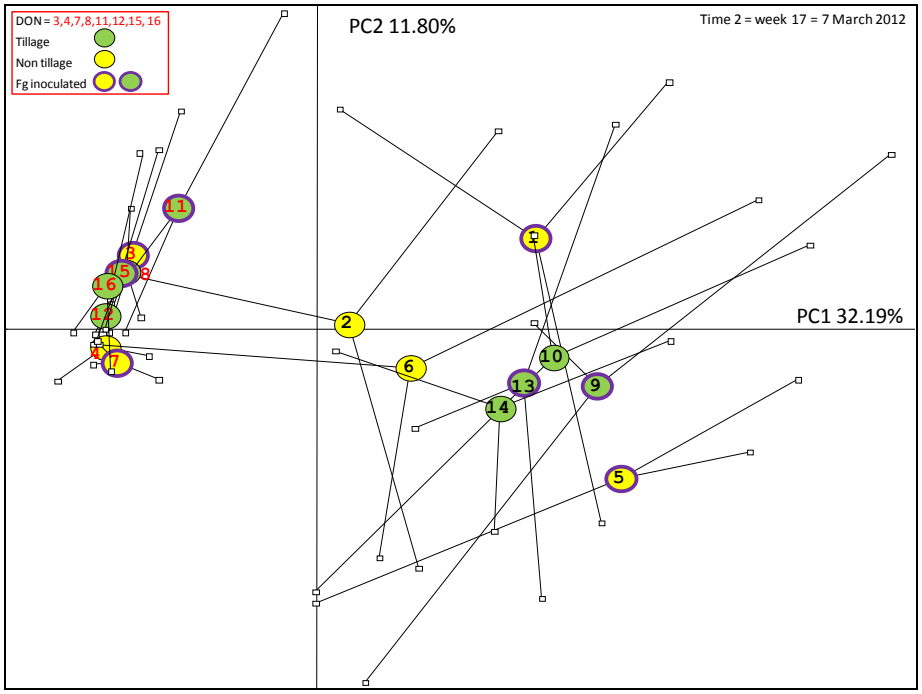

Fig. 4: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the protozoan community structure. Each number represents the mean of 3 biological replicate for one modality at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results. The red numbers represent the presence of DON.

Factors	Time – 1 (week 5) (7 November – 13 December)	Time – 2 (week 17) (13 December – 7 March)	Time - 3 (week 24) (7 March – 20 April)	Overall effect (7 November – 20 April)
Effect of Field inoculation	NS	NS	NS	NS
Effect of field tillage	S	S	S	S
Effect of DON	NS	NS	NS	NS
Effect of stubbles inoculation (in bags)	S	S	S	S
Effect of Blocks	NS	S	S	NS
Time		Time 1 Vs Time 2= S	Time 2 Vs Time 3= S	S

Table 1: The effect of different factors on fungal community structure colonizing maize stubbles in field on different time periods. The significance was checked by Monte Carlo test with 95% level of confidence (S= Significant; NS= non-significant)

Factors	Time – 1 (week 5) (7 November – 13 December)	Time – 2 (week 17) (13 December – 7 March)	Time- 3 (week 24) (7 March – 20 April)	Overall effect (7 November – 20 April)
Effect of Field inoculation	NS	NS	NS	NS
Effect of field tillage	S	S	S	S
Effect of DON	NS	NS	NS	NS
Effect of stubbles inoculation (in bags)	S	NS	S	S
Effect of Blocks	NS	NS	S	NS
Time		Time 1 Vs Time 2= S	Time 2 Vs Time 3= S	S

Table 2: The effect of different factors on bacterial community structure colonizing maize stubbles in field on different time periods. The significance was checked by Monte Carlo test with 95% level of confidence (S= Significant; NS= non-significant)

Factors	Time – 1 (week 5) (7 November – 13 December)	Time – 2 (week 17) (13 December – 7 March)	Time - 3 (week 24) (7 March – 20 April)	Overall effect (7 November – 20 April)
Effect of Field inoculation	NS	NS	NS	NS
Effect of field tillage	S	NS	S	S
Effect of DON	S	S	S	S
Effect of stubbles inoculation (in bags)	NS	NS	S	S
Effect of Blocks	NS	S	S	NS
Time		Time 1 Vs Time 2= S	Time 2 Vs Time 3= S	S

Table 3: The effect of different factors on protozoa community structure colonizing maize stubbles in field on different time periods
The significance was checked by Monte Carlo test with 95% level of confidence (S= Significant; NS= non-significant)

Factors	Time – 1 (week 5) (7 November – 13 December)	Time – 2 (week 17) (13 December – 7 March)	Time - 3 (week 24) (7 March – 20 April)	Overall effect (7 November – 20 April)
Effect of Field inoculation	NS	NS	NS	NS
Effect of field tillage	S	S	S	S
Effect of DON	NS	NS	NS	NS
Effect of stubbles inoculation (in bags)	NS	NS	S	NS
Effect of Blocks	NS	NS	S	S
Time		Time 1 Vs Time 2= S	Time 2 Vs Time 3= S	S

Table 4: The effect of different factors on nematodes community structure colonizing maize stubbles in field on different time periods

The significance was checked by Monte Carlo test with 95% level of confidence (S= Significant; NS= non-significant).

3.3- Fate of DON in maize crop residues in the field:

The DON was extracted and quantified in all treatments after 0, 5, 17 and 24 weeks of launching the experiment (Table 5 and 6). The quantification on the week 0 determined that DON present naturally in the maize stubbles was 51.83 (±28.79) µg/g (d.w.) (Table 5) and in the artificially contaminated maize stubbles it was 122.09 (±3.6) µg/g (d.w.) (Table 6). The quantity of DON in the maize stubbles was followed at each sampling time. We found that it reduced considerably with the passage of time with the degradation of crop residues. In the end of experiment (week 24) it remained 2.50 (± 1.3) µg/g (d.w.) in the naturally contaminated maize residues and 5.16 (± 3.3) µg/g (d.w.) in the artificially contaminated maize stubbles.

The results revealed that after passing 5 weeks of launching the experiment it was reduced almost 2 times in all types of maize stubbles whether naturally contaminated or artificially contaminated with DON. Furthermore, it was reduced about 5 times from week 5 to week 17 and more or less 2 times from week 17 to week 24. The overall analysis of results illustrate that the quantity of DON was reduced gradually and the tillage system produced no impact on the disappearance of mycotoxin. The rate of reduction of DON was about 2-5 µg/g (d.w.) of maize per week. The presence of *F. graminearum* in maize stubbles produced no impact on the quantity of DON. The results suggested that DON was not produced by *F. graminearum* during its saprophytic survival in the maize stubbles.

Field treatments	Maize stubbles treatments	Quantity of DON (µg/g)			
		Week 0 (7 Nov.)	Week 5 (13 Dec.)	Week 17 (7 Mach)	Week 24 (20 May)
Non tillage inoculated	Fg + DON	**122.09** ± 3.6	**56.61** ± 15.0	**07.01** ± 07.1	**01.96** ± 00.1
	0 Fg + DON	**122.09** ± 3.6	**49.72** ± 03.9	**09.71** ± 00.6	**03.99** ± 03.7
Non tillage non-inoculated	Fg + DON	**122.09** ± 3.6	**67.03** ± 11.6	**06.68** ± 02.6	**05.51** ± 05.5
	0 Fg + DON	**122.09** ± 3.6	**67.25** ± 13.7	**12.72** ± 03.4	**10.89** ± 02.7
Tillage inoculated	Fg + DON	**122.09** ± 3.6	**49.45** ± 09.5	**09.97** ± 02.5	**02.40** ± 00.4
	0 Fg + DON	**122.09** ± 3.6	**57.81** ± 13.2	**10.43** ± 01.8	**01.74** ± 01.4
Tillage non-inoculated	Fg + DON	**122.09** ± 3.6	**63.48** ± 01.1	**12.72** ± 00.2	**08.74** ± 05.6
	0 Fg+DON	**122.09** ± 3.6	**64.58** ± 01.7	**23.66** ± 01.0	**06.06** ± 05.1
Average concentration of DON		**122.09**	**59.40** ± 07.2	**11.60** ± 05.3	**05.16** ± 03.3

Table 5: The fate of DON in the artificially DON contaminated maize crop residues in the maize stubbles inoculated with *F. graminearum* (Fg) and non-inoculated (0Fg) in tillage and no tillage field system along the season of saprophytic survival of *F. graminearum*.

Field treatments	Maize stubbles treatments	Quantity of DON (µg/g)			
		Week 0 (7 Nov.)	Week 5 (13 Dec.)	Week 17 (7 March)	Week 24 (20 May)
Non tillage inoculated	Fg	**51.83** ± 28.8	**20.30** ± 12.44	**05.26** ± 01.94	**03.83** ± 0.2
	0 Fg	**51.83** ± 28. 8	**22.62** ± 06.83	**04.16** ± 02.29	**03.33** ± 02.3
Non tillage non inoculated	Fg	**51.83** ± 28. 8	**01.18** ± 01.67	**00.30** ± 00.42	**00.00** ± 00.0
	0 Fg	**51.83** ± 28. 8	**10.66** ± 12.59	**03.68** ± 03.75	**01.99** ± 01.8
Tillage inoculated	Fg	**51.83** ± 28.8	**44.48** ± 22.24	**08.32** ± 06.22	**02.78** ± 02.6
	0 Fg	**51.83** ± 28.8	**15.36** ± 0 5.26	**02.91** ± 02.69	**01.83** ± 01.9
Tillage non inoculated	Fg	**51.83** ± 28.8	**47.12** ± 13.94	**01.85** ± 01.85	**02.44** ± 2.24
	0 Fg	**51.83** ± 28.8	**14.26** ± 16.59	**03.89** ± 04.39	**03.77** ± 03.5
Average concentration of DON		**51.83**	**22.01** ± 16.054	**03.60** ± 02.49	**02.50** ± 1.26

Table 6: The fate of DON in the naturally DON contaminated maize crop residues in the maize stubbles inoculated with *F. graminearum* (Fg) and non-inoculated (0Fg) in tillage and no tillage field system along the season of saprophytic survival of *F. graminearum*.

3.4- Quantification of *F. graminearum*, fungi and bacteria:

The growth of *F. graminearum* and the total fungal and bacterial molecular biomass was quantified from the maize stubbles in all the treatments from 7 Nov to 20 April 2012 using real time polymerase chain reaction (Q-PCR).

The numbers of DNA copies for each week were calculated in terms of number of copies per gram (d.w.) of maize. Analyses Of Variance (ANOVA) were applied at week 5, week 17 and week 24 for *F. graminearum*, overall fungal and bacterial densities and the interaction was checked in relation to the different treatments including impact of DON, tillage system, field inoculation and maize stubbles inoculation.

Our results for the molecular biomass of *F. graminearum* (Fig 5, Table 7) indicate that the population of *F. graminearum* was not affect by the presence or the quantity of DON in the maize stubbles at any sampling time. The field tillage and the field inoculation also produced no impact on the population of *F. graminearum.* The field tillage produced a significant impact on the population of *F. graminearum* but it appeared only on week 17. Time produced a highly significant effect on the population of *F. graminearum* (Table 10).

In case of fungi as a whole, the molecular biomass was not affected by the quantity of DON on any sampling time (Table 8). The soil tillage and the field inoculation as well as the maize inoculation with *F. graminearum* produced no impact on the fungal biomass in the maize stubbles at any of the sampling times. The time produced a highly significant impact on the fungal biomass (Table 10).

In case of bacteria the molecular biomass remained unaffected by the quantity of DON in the maize stubbles at any sampling time (Table 9). The field tillage, field inoculation and the maize stubbles with *F. graminearum* produced no impact on the bacterial biomass. The biomass was only affected by time factor (Table 10).

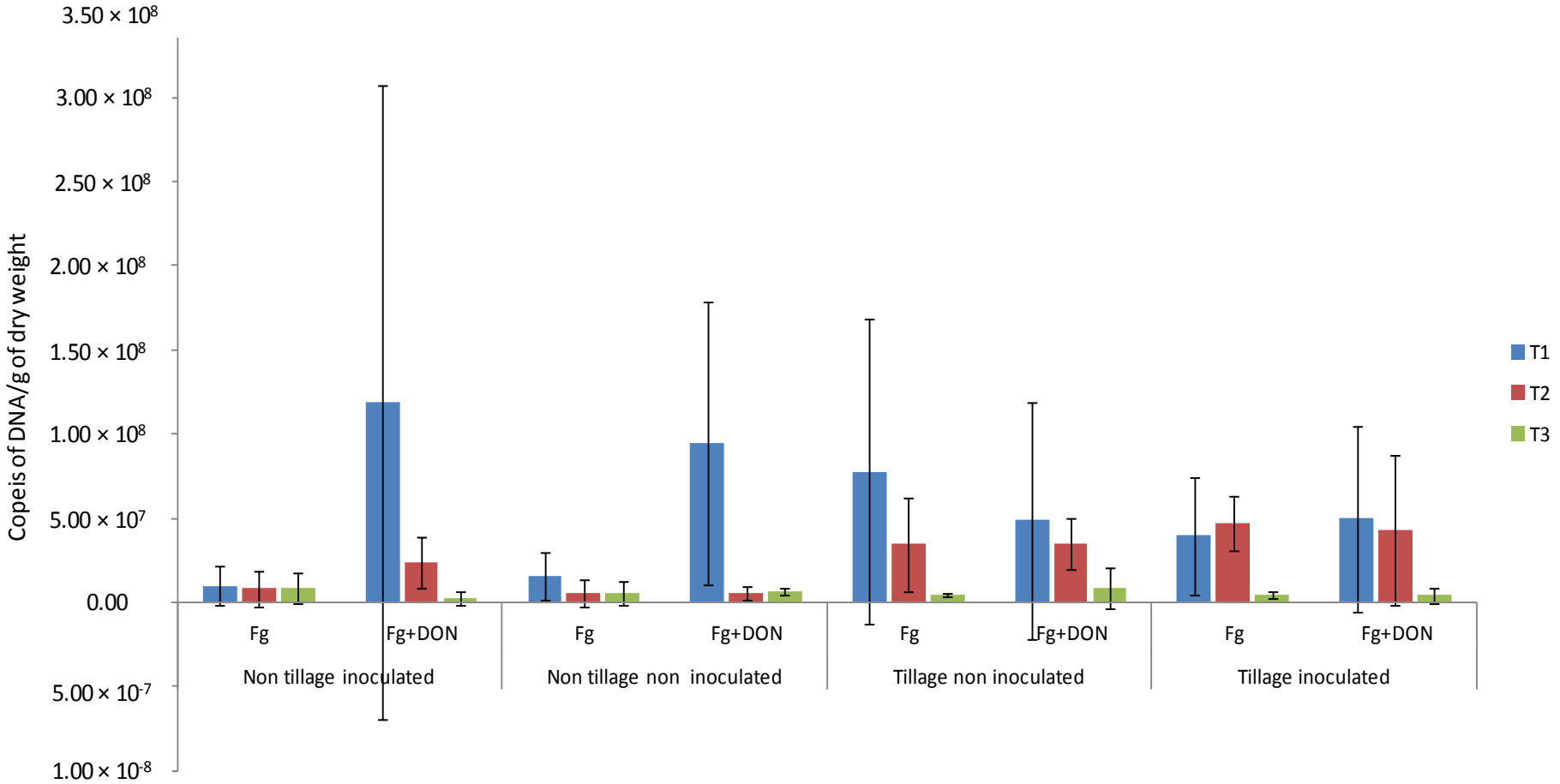

Fig. 5: The impact of DON on the biomass of *F. graminearum* in the maize stubbles put in the field tilled or not tilled and inoculated or not with *F. graminearum.* (T1= week 5, T2= week 17 and T3=week 24)

Treatments (Source of variance)	Time 1			Time 2			Time 3		
	df	MS	F		MS	F	df	MS	F
Effect of tillage	1	1.98×10^{14}	0.029	1	5.10×10^{15}	13.410*	1	7.57×10^{11}	0.022
Effect of field inoculation	1	1.33×10^{14}	0.019	1	6.49×10^{14}	1.115	1	1.26×10^{13}	0.364
Effect of DON	1	1.07×10^{16}	1.677	1	4.46×10^{13}	0.073	1	5.84×10^{11}	0.017

Table 7: Analysis of Variance (ANOVA) for Q-PCR (real time PCR) of *F. graminearum* for different treatments at three time periods.
df: degrees of freedom, MS : Mean Square, F : Fisher's F.* $p < 0.05$

Treatments (Source of variance)	Time 1			Time 2			Time 3		
	df	MS	F	df	MS	F	df	MS	F
Effect of tillage	1	3.68×10^{26}	1.071	1	1.24×10^{26}	2.035	1	1.56×10^{24}	0.192
Effect of field inoculation	1	7.48×10^{24}	0.021	1	1.82×10^{25}	0.287	1	2.32×10^{24}	0.285
Effect of maize stubbles inoculation	1	4.56×10^{26}	1.336	1	9.49×10^{23}	0.015	1	6.62×10^{24}	0.824
Effect of DON	1	6.51×10^{26}	1.931	1	1.23×10^{26}	2.020	1	2.34×10^{24}	0.288

Table 8: Analysis of Variance (ANOVA) for Q-PCR (real time PCR) of fungi for different treatments at three time periods.
df: degrees of freedom, MS : Mean Square, F : Fisher's F. * $p < 0.05$

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Treatments (Source of variance)	Time 1			Time 2			Time 3		
	df	MS	F	df	MS	F	df	MS	F
Effect of tillage	1	1.87×10^{22}	2.546	1	1.12×10^{22}	3.395	1	8.36×10^{21}	1.706
Effect of field inoculation	1	8.37×10^{21}	1.105	1	2.01×10^{20}	0.057	1	1.90×10^{21}	0.377
Effect of maize stubbles inoculation	1	6.40×10^{20}	0.083	1	1.53×10^{21}	0.439	1	7.28×10^{21}	1.480
Effect of DON	1	9.28×10^{21}	1.229	1	7.07×10^{20}	0.201	1	1.65×10^{21}	0.328

Table 9: Analysis of Variance (ANOVA) for Q-PCR (real time PCR) of bacteria for different treatments at three time periods
df: degrees of freedom, MS : Mean Square, F : Fisher's F. * $p < 0.05$

Treatments (Source of variance)	*F. graminearum*			Fungi			Bacteria		
	df	MS	F	df	MS	F	df	MS	F
Time	2	1.61×10^{16}	6.735*	2	1.73×10^{27}	12.515**	2	3.33×10^{22}	6.255*

Table 10: Analysis of Variance (ANOVA) for Q-PCR (real time PCR) of *F. graminearum*, fungi, bacteria and the impact of time.
df: degrees of freedom, MS : Mean Square, F : Fisher's F. **$p < 0.001$, * $p < 0.01$

3.5- Disease development on the wheat crop:

Campaign 2011-2012 was completely different from the previous one. While in 2010-2011, the dry climatic conditions prevented the wheat crop from FHB, in 2011-2012 wet weather and mild temperatures during the flowering period of wheat favored the epidemic development of Fusarium head blight. Therefore, the whole plot was affected what could explain why none of the various parameters measured along this campaign were significantly different between inoculated and non-inoculated subplots (Table 11).

However more significant differences were found when the comparison was performed between no tilled and tilled plots. The same number (374 and 378) of emerging seedlings were found out of the 400 seed initially sown/m^2 what makes about 94% of success for both sets of plots. However, the green surface was significantly higher in the no tilled plots than in the tilled one in autumn (22 Nov 2011). This can be explained by the fact that seeds were closer to the surface in no tilled plots than in tilled plots as a consequence of the tillage system. Therefore, the former seedlings could grow more rapidly. Conversely, at the end of winter (5 March) the trend was opposite because the seeds in the tilled plots were protected, deep in the soil, to face the cold winter period and thus were able to re-start their growth faster than the seedlings of the no tilled plot which suffered from the frost. Indeed, the percentage of seedlings that survived the winter period was significantly higher (91.7%) in the tilled plots than in the no tilled lots (79.9%). This handicap was more or less compensated as 1 month later (2nd April), the green surfaces were no more significantly different, thus indicating that globally, the coverage of the soil was the same in both tillage systems. Actually, the situation was different in the 2 systems as in the case of no tilled plots, the number of tillers was significantly higher (4.27/plant) that in tilled plots (3.44/plant) but the height of the seedlings at that stage (ears at 1 cm) was significantly higher in tilled plots (1,305) than in no tilled plots (0,895). Therefore, the growth scenario of the wheat variety Charger depends on the tillage system. At the time of flowering, the numbers of ears were similar (291/m^2 as a mean). After flowering, significantly different scenarii were also observed. Only the observations performed on the 12 and 19 June 2012 were taken into account, those performed on the 28th June were biased by the natural yellow coloration the ears getting matured. However, both the numbers of spikelets exhibiting FHB symptoms and the intensity of the symptoms, as assessed by image analyses, revealed that the wheat cultivated in the no tilled plots was significantly more affected by FHB than the wheat in the tilled plots. The consequences are

revealed by the significant difference in yield. The wheat grown in the tilled system produced 8473 kg grains/ha while the same variety of wheat grown in no tilled system produced 7666kg grains/ha i.e. 800 kg less. The significantly lower dry weight of 1000 grains in the no tilled system revealed that the grain were smaller in these plots. At the present day, the DON content of the different samples of grains issuing from the various subplots has not yet been quantified.

The statistical analysis performed on the whole data set at each sampling occasion indicate that the only block effect was observed at the end of winter and therefore it did not affect the whole kinetics of development of both the crop and the disease. In the same way, there was no significant interaction between the tillage system and the artificial inoculation of maize residues, probably because the natural infestation by indigenous *F. graminearum* and other fungi of the FHB complex obscured the artificial inoculation performed on half of the subplots. However, it is noticeable that the natural infestation was more severe in the no tilled subplots but it is difficult to draw general conclusion from this single experiment.

Variable	Date of notation	mean	tillage system			inoculation				Block					interaction			
				no tillage	tillage		no inocul	inocul		I	II	III	IV			no tillage	tillage	relative std dev
nb emerging seedlings	8-nov.	**376**	NS	374	378	NS	369	383	NS	400.7936	365.0793	357.1428	380.9523	NS	non ino	**365.0793**	**372.3545**	9.6%
															ino	**382.9365**	**383.5978**	11.6%
green surface /cm²	22-nov.	**229.8**	S	271.9	187.7	NS	231.525	228.0875	NS	235.8	218.3	230.8	234.2	NS	non ino	**282.9**	**180.12**	14.48%
															ino	**260.9**	**195.3**	11.94%
green surface /cm²	5-mars	**662.76**	HS	575.5125	750.025	NS	643.8375	681.7	S	655.9	690.7	695.6	608.875	NS	non ino	**517.9**	**769.775**	2.55%
															ino	**633.125**	**730.275**	12.18%
nb seedlings - end of winter	2-avr.	**332.01**	NS	315.4762	348.5449	NS	333.9947	330.0264	NS	341.2698	330.0264	332.672	324.074	NS	non ino	323.4127	344.5767	16.04%
															ino	307.5397	352.5132	11.88%
green surface /cm² - Ears at 1cm	2-avr.	**1901.625**	NS	1891.875	1911.375	NS	1864.75	1938.5	NS	1902.5	1891.75	1954.25	1858.00	S	non ino	1792,00	**1937.5**	1.65%
															ino	1991.75	**1885.25**	4.76%
NbTillers - Ears at 1cm	2-avr.	**3.855**	HS	4.27	3.44	NS	3.775	3.935	NS	3.85	3.71	4.15	3.71	NS	non ino	**4.02**	**3.53**	5.11%
															ino	**4.52**	**3.35**	15.9%
Height Ears at 1cm	2-avr.	**1.09975**	S	0.895	1.3045	NS	1.02	1.1795	NS	1.139	1.098	1.127	1.035	NS	non ino	**0.866**	**1.174**	17.41%
															ino	**0.924**	**1.435**	13.98%
% Survival (end of Winter)		**88.7**		79.9	91.7										non ino			
															ino			
nb ears		**291.84**	NS	287.5	296.18	NS	290.4	293.3	S	290.8	276,00	287.5	313,00	NS	non ino	**281.7**	**298.9**	2.08%
															ino	**293.2**	**293.4**	9.2%
% contaminated spickelets	12-juin	**7.8%**	S	8.9%	6.7%	NS	8.3%	7.4%	NS	7.3	7.7	8.3	7.9	NS	non ino	**9.7**	**6.9**	18,00%
															ino	**8.2**	**6.5**	19,00%
% contaminated surface	12-juin	**1.2%**	HS	1.5%	0.98%	NS	1.22%	1.28%	NS	1.08	1.08	1.45	1.38	NS	non ino	**1.31**	**1.13**	13.7%
															ino	**1.72**	**0.84**	25.2%
% contaminated spickelets	19-juin	**16.4%**	S	19.8%	13.0%	NS	15.5%	17.4%	NS	16.9	16.7	16.4	15.6	NS	non ino	**19.0**	**11.9**	19.7%
															ino	**20.6**	**14.1**	12.98%
% contaminated surface	19-juin	**7.5%**	S	9.4%	5.6%	NS	6.8%	8.2%	NS	7.5	7.6	7.6	7.3	NS	non ino	**8.7**	**4.9**	28,00%
															ino	**10.1**	**6.2**	24,00%
yield	23-juil.	**80.69**	HS	76.66	84.73	NS	80.02	81.37	NS	82.85	79.21	80.97	79.73	NS	non ino	**75.83**	**84.21**	2.32%
															ino	**77.49**	**85.24**	2.47%
dry weight	23-juil.	**71.59**	S	70.9	72.29	NS	71.41	71.77	NS	71.02	71.3	71.67	72.34	NS	non ino	**70.55**	**72.27**	1.21%
															ino	**71.25**	**72.3**	1.18%

Table 11 : monitoring of the wheat crop in *F. graminearum* inoculated and non inoculated plots under tillage and no tillage systems.

S: Significant (p=0.05); HS: Highly Significant (p=0.01); NS: Non Significant.

4-Discussion:

The soil has been too long considered an inert matrix in which farmers had to bring mineral fertilizers for growing plants. In recent years, the idea of a living soil is increasingly present in mind when managing crops. Even though the role of all biotic components of the soil is not really known, taking into account multitrophic interactions in the management of preceding crops, organic matter and tillage led to reflect upon the relationship between agricultural practices and ecology of soil-borne plant pathogens. The crop residues are subjected to the degradation by the soil microflora and fauna and the nutrients are liberated in the soil (Lupwayi et al., 2004).

In this experiment, the process of decomposition was measured in terms of the weight of maize stubbles in the nylon bags. We observed that once the maize residues were in the soil and attacked by the microbial communities the process of degradation started. We saw the soil communities worked very well and they degraded 51 % of the maize stubbles within 5 weeks post launching the experiment and then the process of degradation continued which was slower than before but at the end of 24 weeks we got only 22 % dry matter back from the soil. We found no clear difference between the maize stubbles placed on the soil surface (1 cm deep) and the one incorporated in the soil (10 cm deep) with soil tillage which may seem surprising as it is well known that the decomposition process is much more faster when crop residues are buried than when they remain at the surface of the soil. Anyway, the decomposition process was not affected by the quantity of DON in the maize stubbles which indicates the quantity of DON didn’t stop the decomposition process of the maize crop residues.

During this decomposition process it was very interesting to see the community profile associated with the process. We observed the community profile of fungi, bacteria, protozoa and nematodes which colonized the crop residues and may be participated in the process of decomposition. The first thing we observed that the quantity of DON in the maize stubbles played no impact on fungi, bacteria and nematodes but significantly affected the protozoan community structure whether the stubbles were on the soil surface or buried in the soil. This showed that the protozoa were highly sensitive towards the DON during their attack on the maize stubbles while the fungi, bacteria and the nematode populations were not much sensitive towards the DON quantity in the crop residues. Unfortunately, the role of protozoa in the decomposition process in arable crops is rather unknown therefore the impact of DON

on their community cannot really be interpreted in relation to any functional consequence for the fate of crop residues as for *F. graminearum* survival.

The impact of tillage practices on the community structures of these four soil-borne communities (fungi, bacteria, protozoa and nematodes) was also observed. We got very interesting information that almost all the communities were affected by the tillage practice. It showed that the soil micro- flora and fauna colonizing the maize stubbles was completely different when they are placed on the soil surface than the one which are present 10 cm deep in the soil. It was surprising that both played equal role in the decomposition process of the maize stubbles. This impact on the community structure of the different communities can be due to the soil aeration, water moisture and the carbon nitrogen ratios which in turn can have impact on the populations in the different soil communities (Doran, 1980; Spedding et al., 2004).

In addition, we examined the impact of *F. graminearum* which was already provided to the maize stubbles by inoculation, on the other communities attacking the crop residues. We found the fungal structure was completely different in the presence of *F. graminearum*. However, it was unclear whether the change was due to real changes in abundance ratios between populations initially presented before the introduction or if they are simply the *F. graminearum* TRF which are responsible for the observed changes. It was surprising to see that the presence of *F. graminearum* in the stubbles greatly affected the bacterial populations which were colonizing the crop residues which showed a relation between *F. graminearum* on the bacterial communities or it might be the nutrients used by the *F. graminearum* which discouraged the bacterial communities to colonize the crop residues. Moreover we saw that nematodes and protozoa community structure was also clearly affected throughout by the presence of *F. graminearum*. We may assume that *F. graminearum* was fighting or using some kind of mechanisms to conserve its food resources without using DON. It might also be proposed that *F. graminearum* rapidly colonized the maize stubbles to prevent populations of some other communities to colonize the maize crop residues.

Furthermore, our results indicated that the field inoculation by *F. graminearum* brought no change in the community structure of any of the four communities. Furthermore, we checked the impact of plots on the community profile of all the four communities and we observed that there was an effect of block on these communities. This effect was clear after 5 weeks on the fungal and bacterial community structure and significantly appeared after 17 weeks on the bacterial and nematodes community structure. This reveals the difficulty we faced in field experiments. Despite everything was done to ensure the best homogeneity in preparing the

plots and in distributing the nylon bags across the subplots, intrinsic heterogeneity was still present and caused variability among the samples. For practical reason and for the sake of feasibility, it was not possible to handle more than 3 biological replicates for which 3 technical replicates each were performed. Unfortunately, such variability created a limitation in the biological interpretation of the data. This was also true for the Q-PCR measurements. The molecular biomass of the *F. graminearum* was measured by Q-PCR to find out its survival in the presence of DON and what kind of benefit it could obtain from the quantity of DON present in the maize crop residues. Our results demonstrated that the quantity of DON gave no advantage to the *F. graminearum*. The population of the *F. graminearum* was reduced with the passage of the time or we can say with the decomposition of the maize stubbles. Though statistically the application of ANOVA on the data showed that there was no impact of DON but it is not so clear as we saw some higher densities of *F. graminearum* in terms of mean value but a big standard deviation was found among the biological replicates (plots). This standard deviation was observed throughout the experiment which restrains us to give a solid conclusion about the impact of DON on the growth of *F. graminearum* and the development of primary inoculum. Samples were carefully preserved at -20°C so we repeated the Q-PCR measurements on the whole set of samples. We found the same results so we can say it was the effect of block linked to the spatial heterogeneity of the plot which prevents to draw any conclusion about the effect of DON and tillage on *F. graminearum* population. This effect of the block can also be seen on the community profile of the overall fungal and bacterial communities. The results about the disease development showed that the primary inoculum was significantly higher in case of no tillage than soil tillage which shows that the population the *F. graminearum* might be higher in zero tillage than in tillage system. We also observed the impact of DON and the tillage system on the overall fungal and bacterial densities and we found no impact of DON and the tillage system on them. Same was the case with these two communities as with the population of *F. graminearum*. Even in the presence of the high variability among the plots (biological replicates), we could see that the decomposition of the crop residues produced a significant reduction in the fungal, bacterial densities as well as the *F. graminearum* population.

The quantification of DON in the maize crop residue at zero sampling time was a bit surprising as we found a certain big amount of DON in the maize stubbles (51 ± 28 µg/g of dry matter) although they were autoclaved two times at 120°C to make them disinfected. By the way we had 2.3 times more DON in the artificially contaminated maize stubbles to see the impact on the *F. graminearum* and the other soil communities. We monitored the fate of

DON by quantifying them in each sample at each sampling time. We also noticed that less than 100% of DON was recovered a few hours after it was introduced. The quantification of mycotoxins generally concerns plant material which is not contaminated with soil and adequate procedures are proposed to evaluate the quantity of DON in cereal grains. These procedure were not efficient to detect and quantify DON in soil-residues mixtures thus we had to set up a method to extract DON from the mixture and to recover it from the extractant. Ethanol was used as a solvent instead of acetonitrile and affinity column were used instead of classical columns. Preliminary assays performed in controlled conditions predicted a 100% recovering of DON introduced in complex mixture. Therefore, it is likely that part of the DON introduced into the soil-maize mixture in natural conditions was either rapidly adsorbed on this matrix or made unavailable by indigenous soil organisms. Anyway, the fate of DON could be monitored as it was possible to depict quantitative variations over time relatively to the initial quantification. We found that DON disappeared with the passage of time in all the samples. It was correlated to the passage of time and the degradation of the crop residues. It might be difficult to say that whether DON was degraded by the microbes or it was leached down or liberated in the soil due to the decomposition of the organic matter and presence of less material for its absorption. Our results also showed that DON was not increased in the crop residues during the completion with other soil biota. In the end of the experiment we found a very little amount of DON in the maize residues.

We monitored the development of the disease in the field to monitor the impact of the tillage and the inoculum we provided artificially to the field. It was not possible to correlate what was observed in the nylon bags with the development of the crop and the disease. Moreover, the climatic conditions were quite favorable to the epidemic spray of FHB what obscured the inoculated non–inoculated treatment of the natural maize crop residues. This experiment revealed, but this is not really new, that the tilled plot was less affected by FHB than the no tilled plot but it does not allow to ensure that tillage can prevent the crop from FHB disease. It is probably wiser to evaluate the tillage system on a longer duration.

The field experiment suggests that DON has an impact on soil microflora and soil fauna components. It is not known from the measurements we did if this impact should be considered as positive or negative towards the biotic components but it seems that this impact depends on the location of the crop residues and also, it seems that *F. graminearum* did not get any competitive advantage of the presence of DON. However a huge variability of the data acquired in this field experiments was a limitation to interpret and discuss the results and to provide a solid answer. This variability was partly attributed to the field heterogeneity and

to the climatic conditions so we suggest that such an approach should be conducted in controlled conditions using microcosms as experimental unit.

6-Conclusion:

The main conclusions of this field experiment are briefly explained in the figure-6. We conclude that most of the DON disappeared from the maize crop residues with the passage of time and left in very low amount after six months. The disappearance of DON was not dependent on the tillage system or stubbles inoculation. The fact that DON did not increase in the presence of F. graminearum in the maize residues could be explained either by the absence of production or by a concomitant production-disappearance (adsorption or degradation) process.

The quantification of *F. graminearum* by real time PCR showed that there were high variations among the blocks (biological replicates). Therefore, it was difficult to say anything about the impact of DON or tillage system. So the question about the advantage of the presence of DON to *F. graminearum* left unrevealed and need to be studied in controlled environment to eliminate environmental factors. Similarly, it was difficult to conclude about the impact of DON on the fungal and bacterial densities variability among the blocks. The measurement of disease and yield in the field showed that the field tillage significantly reduced disease development on the wheat crop and ultimately led to the high yield which showed the better survival of *F. graminearum* in the no tillage system as compared to the tillage system.

T-RFLP results showed that only protozoa community structure was affected by the presence of DON independent of the tillage system. The fungal, bacterial and nematodes communities remained unaffected by DON.

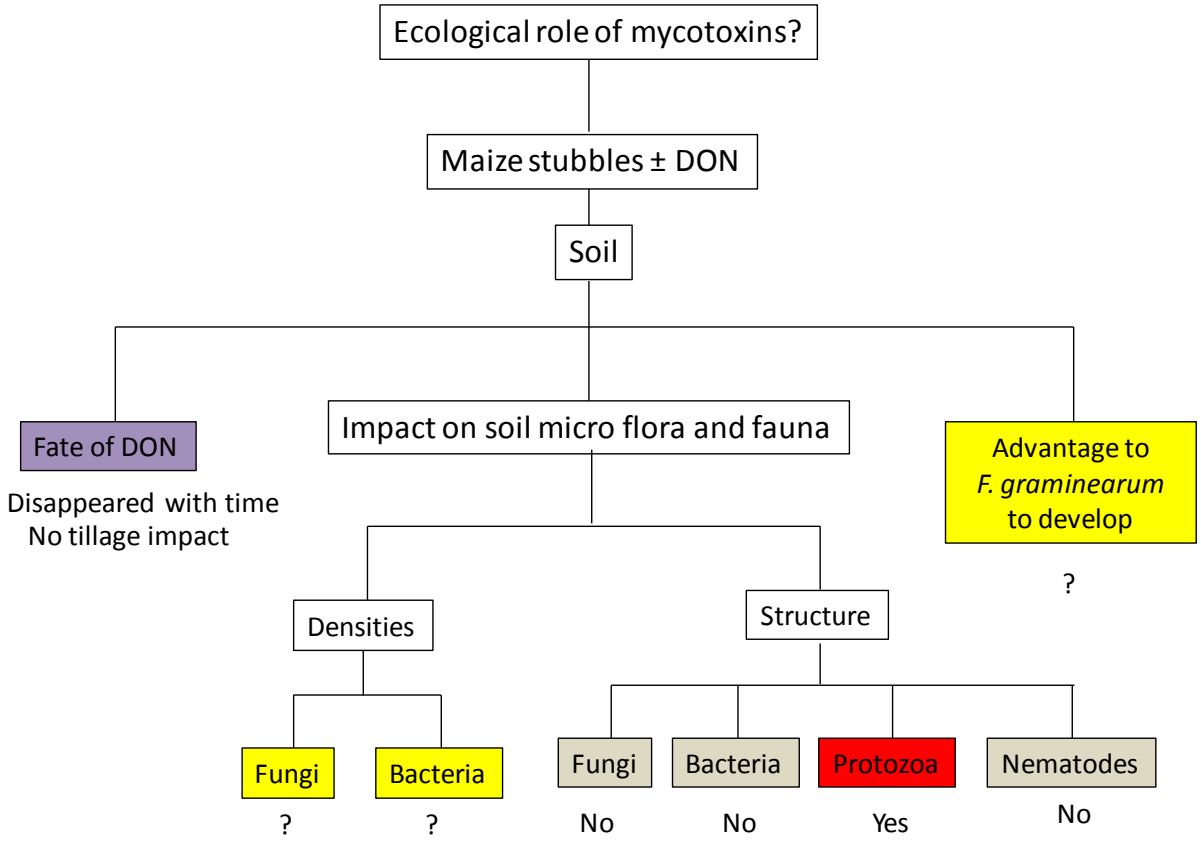


Fig. 6: Flow chart showing the main conclusion obtained during the different measurements in the maize stubbles nylon bags in the field experiment. The violet colour rectangle shows the disappearance of DON; yellow colour rectangles indicate that questions remained unanswered; red colour rectangle shows the impact of DON while the grey colour shows no impact of DON. Furthermore, Yes= DON had an impact; No= DON had no impact; ?= still remained questions.

Chapter 4: Fate of DON and its impact on the soil microflora and soil fauna communities

Fate of DON and its impact on the soil microflora and soil fauna communities

1. Introduction:

Mycotoxins are fungal secondary metabolites potentially harmful to humans and animals. Trichothecenes are one of the most important mycotoxins produced in the field by the genus *Fusarium* and are considered great threat to humans and animals (Rocha et al., 2005). They are produced in the field during the pathogen invasion on the crop. Their incidence in the cereal crops is a huge challenge for the agricultural industry (Bottalico and Perrone, 2002; Tanaka et al., 1988). Deoxynivalenol (DON) is one of the most important trichothecene metabolites and is found in cereals as wheat, maize, rye and barley. DON (also known as vomitoxin) is a type B trichothecene and is mainly produced by *F. graminearum.* It is one of the most commonly reported mycotoxins associated with the Fusarium head blight (FHB) diseased cereals (Bottalico, 1998; Foroud and Eudes, 2009; Paul et al., 2005). DON causes very destructive effects on mammals as immunity reduction, protein biosynthesis damage, food refusal, diarrhea and vomiting and many sever disease symptoms (Fokunang et al., 2006; Placinta et al., 1999; Wild and Gong, 2010). The European Union has set the threshold level for DON in the winter wheat at 1.250 mg/kg (CE N°1116/2007). Beyer et al., (2007) reported that 4.27% of Fusarium-damaged kernels can meet this limit of European Union.

FHB is the center of interest of the scientists worldwide and different approaches have been investigated to overcome the disease such as forecasting of disease, cultivation of resistant varieties, use of fungicides, cultural practices and biocontrol agents (Buerstmayr et al., 2009; Champeil et al., 2004; De Wolf et al., 2003; Mesterhazy et al., 1999; Prandini et al., 2009; Schisler et al., 2002). Despite all this experimental research, a durable solution to control FHB has not been found and is still a challenge for the agriculture in the world. The management of the disease is an ultimate tool for the reduction of the related mycotoxins, and the food losses, to make the food secure for increasing world population. The fungal toxins including DON are reported not obligatory for the production of disease on plants. The fungal mutants having no mycotoxin production ability are demonstrated to produce the disease as the wild type of *F. graminearum* fungus (Adams and Hart, 1989; Bai et al., 2002). However DON is reported to have an active role in the aggressiveness of the fungus (Mesterházy, 2002; Mudge et al., 2006) .

Crop residues are important components of arable soil functioning thanks to the nutrients they bring back to the soil. They are stimulating the high diversity of decomposer organisms in the process of litter decomposition and fuelling the multitrophic interactions among the various soil inhabitants with noticeable consequences for biogeochemical cycles. Potential mechanisms include fungi-driven nutrient transfer among litter species, inhibition or stimulation of microorganisms by specific litter compounds, and positive feedback of soil fauna due to greater habitat and food diversity (Hättenschwiler et al., 2005; Kreuzer et al., 2004; Rantalainen et al., 2004). How the species richness of decomposer fungi, bacteria and soil fauna including protozoa, or their relative frequencies of occurrence (i.e. community structure) influence the decomposition of organic matter in arable soil is poorly known compared to the one of forest litter (Buee et al., 2009). Using different sets of fungi isolated from plant litters, Deacon et al., (2006) showed that there was apparently a high degree of functional redundancy in assemblages of cultivable decomposer fungi what could buffer the impact of external event on the decomposition process. However, the authors pointed out that some less abundant taxa might have a key role in the degradation of complex or recalcitrant substrates. Moreover, community structures are not invariant during the process of decomposition over time. The number of trophic levels among soil fauna and soil microflora, species identity as well as keystone species may replace one another as their dynamic communities alter in space and time, each functional species adapting for occupation of the successive habitats resulting from the decomposition process (Frankland, 1998). In turn, the decomposition process may be depending on the multitrophic interactions. For instance, microbial succession with shifting enzymatic capabilities enhances decomposition, whereas antagonistic interactions among organisms that compete for similar resources or/and produce deleterious secondary metabolites slow litter decay (Deacon et al., 2006). Moreover, external factors such as the agricultural practices and more precisely the tillage system could affect both the consortium of decomposers and the decomposition process (Cookson et al., 1998). Therefore, one may question about how the phytopathogenic fungi during their saprotrophic phase can be included in this food web, and can survive the dynamics of successions. One example of a very strong and effective interaction is provided by the common earthworms (*Lumbricus terrestris* L) which remove sources of phytopathogenic fungi (*Venturia inaequalis*) in orchards thanks to their efficient grazing on leaf litter (Holb et al., 2006).

The diseased crop residues (grains, straw and stubbles), are colonized by the fungus and are the source of saprophytic survival during off seasons (Pereyra and Dill-Macky, 2008). The

modern age agriculture is moving towards reducing the soil tillage practices, in order to conserve the field soil (Bai and Shaner, 2004). However, this reduced tillage supports the survival of the fungus more than deep tillage (Pereyra and Dill-Macky, 2008). *F. graminearum* survives in these crop residues saprotrophically and serves as primary inoculum to the next crop (Parry et al., 2007; Trail, 2009). Under appropriate climatic conditions, the fungus receives the warm moist climatic condition which leads to the conidia formation. The ascospores are produced in the perithecia and make the fungal spread to the seasonal cereal crops as wheat (Trail, 2009).

Therefore we can wonder about the strategy used by *F. graminearum* to survive in the crop residues which appeared as a very coveted resource while this plant pathogenic fungus is not such a good saprophytic competitor (Leplat et al., 2012) unless the mycotoxins it produced *in planta* provide it with a significant competitive advantage towards the microbial and soil fauna communities. The presence of mycotoxins in the soil and their impact on the soil biota are still an enigma. There is no research performed on the fate of mycotoxin in the crop residues and the soil environment. It is still a question to understand the role of DON in the ecological habitat of microorganisms.

Owing this background the main question of this study was to understand the ecological role of mycotoxin during the survival of *F. graminearum* in the crop residues by monitoring the survival of other soil biota including fungi, bacteria, protozoa, nematodes and earthworms. The research was conducted in microcosms in non-disinfected soil containing straw as crop residue and DON as mycotoxin. All the conditions closed to the natural field environment were established in these microcosms.

2. Materials and methods:

A-DON chemotype *Fusarium graminearum* strain MIAE00376 (Chapter 2, page 44) was used in this experiment. The inoculum was produced according to the procedure described Chapter 2.

Lumbricus terrestris adult specimens were captured (Chapter 2, page 49) and were put in the soil in different containers and were placed at 10°C for 3 weeks. At the time of launching the experiment, earthworms were put in the experimental soil at 17°C for 4 days.

2.1- Soil and straw preparation:

The soil was collected from the meadow area near green house (described in chapter 2, page 46). The air dry straw was taken from the winter wheat (*Triticum aestivum*) originated from a field of Epoisses (Bretenières, France). The straw was cut into approximately 2-3 cm small pieces.

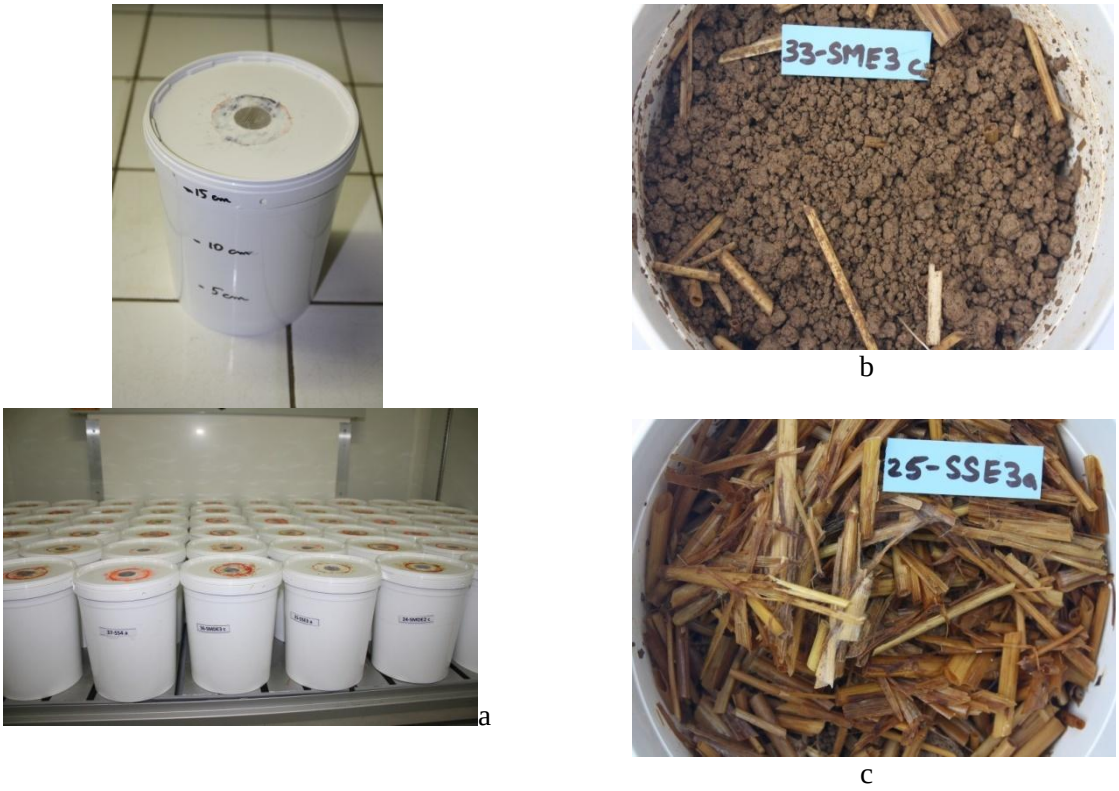
The water holding capacity of straw was found as 1 g of straw (d.w.) could hold 3.5 ml of water. The straw was made infected with *F. graminearum* by spraying and mixing with 1.5 ml containing conidial suspension (6.67×10^2 conidia/ml) per gram of straw (d.w.). Then the straw was divided into two halves, one half was made contaminated with DON by spraying and mixing with 1.5 ml DON solution (66.7 µg/ ml, DON solution preparation is described in Chapter 2, page 56) while the other half was moistened by spraying and mixing with distilled water to give the homogenous humidity to all straw. Finally, the straw contained 10^3 conidia/g of straw (d.w.) and the DON contaminated straw was containing DON at the rate of 100 µg/g of straw (d.w.).

2.2- Preparation of microcosms:

Small plastic pots of 11 cm × 15.5 cm × 6.5 cm in size (width × length × height) were used as experimental units (microcosms). In the lid of each pot, a hole was made for the breathing of earthworms and elimination of gases produced as a result of decomposition of straw. This hole was covered with plastic gaze to avoid the escape of earthworms.

Each microcosm was filled with 1kg of soil (dry weight, soil is described in chapter 2, page 48) and 10 g of *F. graminearum* infected straw (dry weight) either contaminated with DON or not. The straw was either incorporated in the soil or placed on the soil surface in the form of

layer. Four previously collected earthworms (chapter 2, page 49) of total biomass 13.67 (± 0.76) g were added to each required microcosm. In this way six types of microcosms were prepared representing six treatments: microcosms with or without DON, straw left at the surface or incorporated in the soil and the microcosms with or without earthworms. Three replicates (microcosms) were prepared for each treatment for each sampling time. These microcosms were placed in an incubator at 17°C (Fig. 1).



a b c

Fig. 1: Microcosms were incubating at 15°C in a climatic chamber (a). Straw was mixed with the soil in one half microcosms (b) while it was left at the surface of soil in the other half (c).

2.3- Sampling, samples processing and analyses:

Sampling was done on 0, 1, 2, 8 and 24 weeks after the establishment of experiment. At each sampling time, the earthworms were removed from the microcosms. The whole remaining contents (i.e. straw and soil) were mixed and were filled in the vials in a way that each vial had 30 g of soil-straw mixture. These vials were conserved at -20°C. Then all the samples were freeze-dried and ground to powder to make a homogenous mixture of straw and soil and conserved at -20°C. These samples were ready for the extraction of DNA. The humidity in the

microcosms was measured from time to time by weighing the microcosms and was adjusted by spraying water if needed.

At each sampling time all the earthworms were sorted out of the microcosms. They were washed twice by water to remove their outer soil and then dried on the tissue paper. They were counted and the total weights of all the earthworms were measured for each microcosm and were termed as their biomass.

Nematodes were extracted and quantified (Chapter 2, page page 49),

DON was extracted and quantified by HPLC (chapter 2, page 57).

DNA was extracted from soil and soil-straw mixture and nematodes suspension (Chapter 2, page 50). From the extracted DNA Fungal, bacterial, protozoan and nematode communities structure analysis by terminal restriction fragment length polymorphism (T-RFLP) (Chapter 2, page 52). The quantification of bacteria, fungi and *F. graminearum* was also determined by real time PCR (Chapter 2, page 55).

At each sampling time, total cultivable densities of fungi and bacteria were estimated by colony forming unit (CFU) on the appropriate media (Chapter 2, page 49)

3- Results:

3.1- Visual observation of decomposition process:

The process of straw disappearance was observed in the presence or absence of earthworms and in the presence or absence of DON up to six months. Visual observations of straw disappearance process showed that *Lumbricus terrestris* played an important role in the incorporation of straw in the soil (Fig. 2). Surprisingly, the straw contaminated with DON was more attractive for the earthworms.

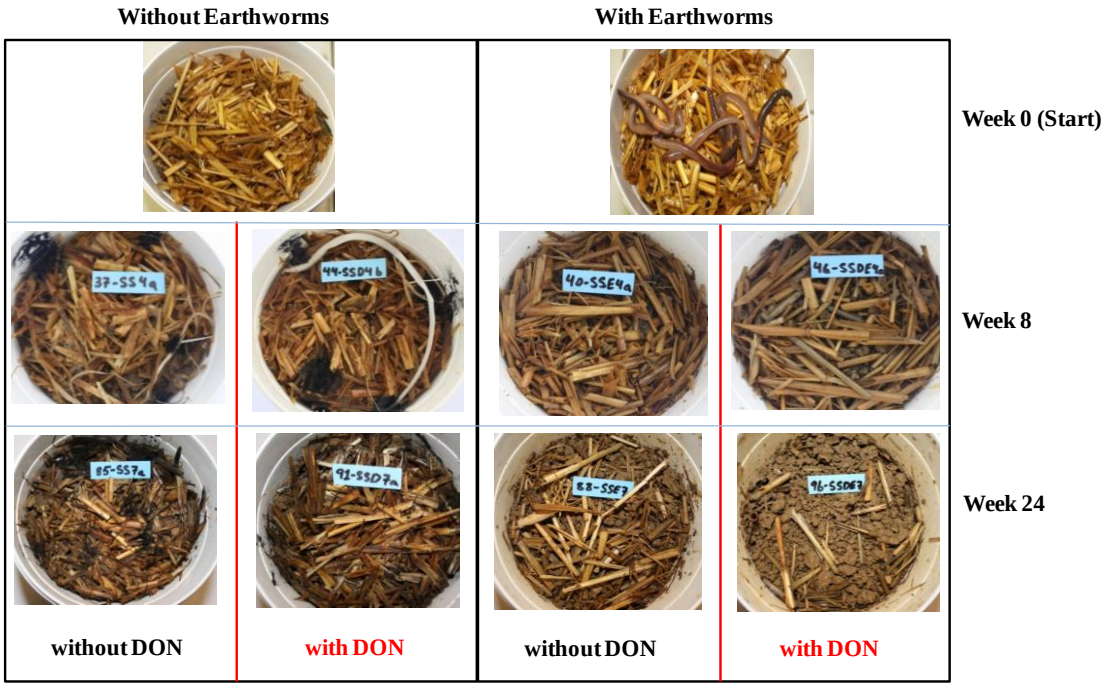


Fig. 2: The process of incorporation of straw placed on the soil surface in the presence or absence of earthworms and DON.

3.2- Fate of DON in wheat straw in soil:

The fate of DON during the incorporation of straw in the soil was followed up to six months (24 weeks) after the establishment of experiment. DON was extracted and quantified in each treatment in the presence of earthworms when the straw was left on the soil surface incorporated in the soil on 0, 1, 2, 8 and 24 weeks and in the absence of earthworms when the

soil was left at the surface on 0, 8 and 24 weeks whether the straw was contaminated or not with DON (Table -1).

Ten g straw inoculated with 10 µg DON/ g (d.w) were introduced into 1kg soil (d.w.) in order to provide each microcosm 1µg DON/g soil-straw mixture, which was close to the threshold limit recommended by European union for unprocessed wheat grains (1.250 µg of DON / g). The sample of representative homogenous DON contaminated soil-straw mixture at the same day of establishment of experiment (week 0) was considered as start point. The quantity of DON indicated that 0.831 µg of DON / g of soil-straw mix was present in the system at day zero or at start for all treatments.

Treatments	Quantity of DON (DON µg / g soil-straw mixture)				
	Week 0	**Week 1**	**Week 2**	**Week 8**	**Week 24**
SME	ND	Traces	0	0	Traces
SMDE	0.831	0.349 ± 0.03	0.033 ± 0.02	Traces	Traces
SSE	ND	0.105 ± 0.02	0.006 ± 0.01	0	0
SSDE	0.831	0.889 ± 0.02	0.885 ± 0.17	0.012 ± 0.00	0
SS	ND	ND	ND	0	0
SSD	0.831	ND	ND	0.400 ± 0.09	0

Table 1: Quantity of DON in the different treatments (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON) at the different time intervals during the incorporation of straw in the soil. ND = not determined; Traces = below the limit of quantification (LOQ = 0.0018 µg/g soil-straw mixture); 0 = below the limit of detection (LOD = 0.00034 µg/g soil straw mixture).

The percentage of recovery of the DON inoculated into the system was of 83% at day 0, suggesting that either part of the DON was actively adsorbed by the mineral and/or organic matrix made by the soil-straw mixture or that the extraction procedure was not 100% efficient. Anyway, relative to the initial value at day 0, DON disappeared very rapidly when the straw was incorporated in the soil as compared to left at the surface. Soon after the setting up of experiment (week 1), DON started disappearing in the treatments where the DON contaminated straw was mixed in the soil (SMDE) and remained about half (0.349 ± 0.034

µg/g) as compared to the starting point (0.831 µg/g). On the opposite, no disappearance of DON was found the first two weeks when the DON contaminated straw was placed at the surface of the soil in the presence of the earthworms. The disappearance of DON continued very rapid when DON contaminated straw was incorporated in the soil and the quantity of DON became significantly very low (0.033 ± 0.02 µg/g) after 2 weeks and soon went below the limit of quantification after 2 weeks. On the other hand, the disappearance of DON was very slow when the DON contaminated straw was left on the soil surface. DON started disappearing after 2 weeks and reached to a very low amount after 8 weeks (0.012 ± 0.00 µg/g) and completely disappeared up to week 24.

Surprisingly, a low quantity of DON was also found during the first week in the microcosms where the DON was not introduced and the straw was placed on the soil surface (0.105 ± 0.02 µg/g). It also disappeared and went below the limit of detection after first two weeks. Traces where found in the when the DON non-contaminated straw was mixed in the soil.

The comparison of quantity of DON on week 8 and 24 when the DON contaminated straw was placed on the soil surface in the presence or absence of earthworms showed that there is a link between the presence of earthworms and the reduction of DON from the system. On week 8, we found a significantly high amount of DON (0.400 ± 0.09 µg/g) as compared to the presence of DON (0.012 ± 0.00) in presence of earthworms in the microcosms. In all the treatments DON completely disappeared or went below the limit of quantification (LOQ = 0.0018 µg/g soil-straw mixture) or limit of detection (LOD = 0.00034 µg/g soil straw mixture) after 24 weeks.

3.3- Structural changes in the microbial and microfaunal communities:

The T-RFLP assay was performed for the four soil communities i. e. bacteria, fungi, protozoa and nematodes in all the treatments after 1, 2, 8 and 24 weeks of establishment of experiment. By this assay, the changes in these community structures was followed in the presence or absence of DON when the straw was incorporated in the soil in the presence of earthworms or left at the surface in the presence or absence of earthworms. The mean number of TRFs (Terminal restriction fragments) per soil sample was 115 for bacteria, 85 for protozoa, 69 for fungi and 98 for nematodes communities. PCA was performed by integrating the number and the intensity of different TRFs for each of the four communities. The result of the PCA was displayed as variations on a two dimensional diagram for each community.

The results showed that the presence of DON in the system had an impact on the microbial communities of the soil. The impact of DON was depending on the type of community and the location of the DON contaminated wheat straw i.e. whether it was incorporated or placed at the surface of the soil.

3.3.1- Bacterial community structure:

PCA results for the bacteria are explained in the two dimensional diagram (Fig. 3). One dimension (PC1) is showing 24.86 % variability and the second dimension (PC2) is showing 11.45 % variability. The bacterial community structure moved along the time frame in all the six treatments, which is more explained along the first dimension. The presence of DON contaminated straw produced a significant effect on the bacterial community structure immediately after the establishment of the experiment. The effect of DON contaminated straw was observed very clearly during the first two weeks whether the straw was placed at the surface or mixed in the soil. After the first two weeks the differences in the bacterial community structures started disappearing and at the eighth week, the differences disappeared between both the treatments contaminated or not with DON. This corresponds with the quantity of DON as it was disappeared or degraded up to the eight weeks in the presence of earthworms (Table -1).

The location of straw i.e. left at the surface or incorporated in the soil played a significant role on the community structure only during the first two weeks. The comparison among the treatments with and without DON when the straw was left at the surface of the soil on week 8 and week 24 showed that the presence of the earthworms played a significant role on the bacterial community structures changes. The effect of DON when the straw was left at the surface of the soil in the absence of earthworm was not clear throughout the experiment. On the opposite, the community structures after eight weeks was different in the presence or absence of earthworms when the straw was contaminated with DON while this effect was not found after 24 weeks.

3.3.2- Fungal community structure:

The two dimensional diagram for the fungal communities is explained in the one dimension (PC1) with 53.23 % and the second dimension (PC2) with 16.56 % variability (Fig. 4).

The genetic structure of the fungal community changed over time from week 1 to week 8 mainly when the straw has been left at the surface while it remained almost constant overtime

where the straw was incorporated in the soil. The same interaction of location-time can explain the variability revealed by PC 2.

The main point anyway was the fact that the fungal community structure as a whole was not directly affected by the presence of DON whether the straw was left at the surface or incorporated in the soil. On the same way it is difficult to illustrate the direct role of earthworms on the fungal community structure but an interaction DON-earthworms seem to affect the fungal community along PC1 when the straw was left at the surface.

3.3.3- Protozoa community structure:

In case of protozoa, the two dimensional diagram (Fig. 5) based on the PCA result is showing 31.57 % variability along one dimension (PC1) and 19.02 % on the second dimension (PC2). The community structure of the protozoa in the treatments in which the straw is placed at the surface showed more variability in the biological replicates. The presence of DON showed a clear impact on the community structure of protozoa throughout the experiment when the straw was left on the soil surface in the presence as well as in the absence of earthworms. DON did not impact the protozoan community structure when the straw was incorporated in the soil. The presence of earthworms produced no significant impact on the protozoan community structure when the community structures were compared between the treatments where the straw was left at the surface of the soil in presence or absence of earthworms on week 8 and week 24.

3.3.4- Nematode community structure:

The community structure of the nematode is explained in a two dimensional diagram, one dimension is explaining 27.74% variability and second 21.38% variability (Fig. 6). DON did not impact the nematode community. The changes in the nematode community structures were more explained by the time. The nematodes communities remained unchanged in the first two weeks but then they moved along the time factor and this movement with time continued till the end of six months. The placement of straw in the soil (incorporated or placed at the surface) and the earthworms did not affect the nematode community structure.

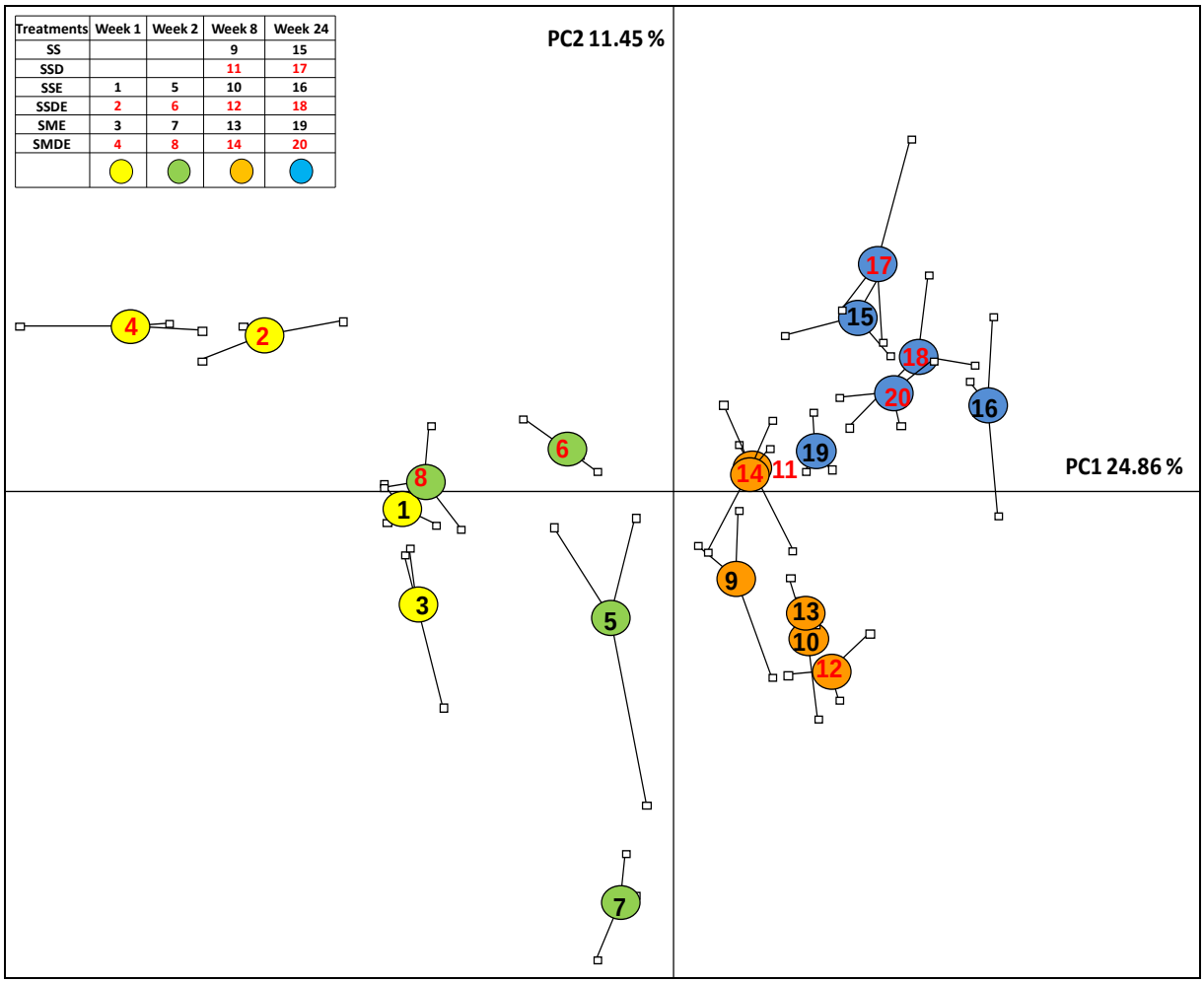


Fig. 3: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the bacterial community structure. Each number represents the mean of 3 biological replicate for one treatment at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results The red colour numbers represents the presence of DON. SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON.

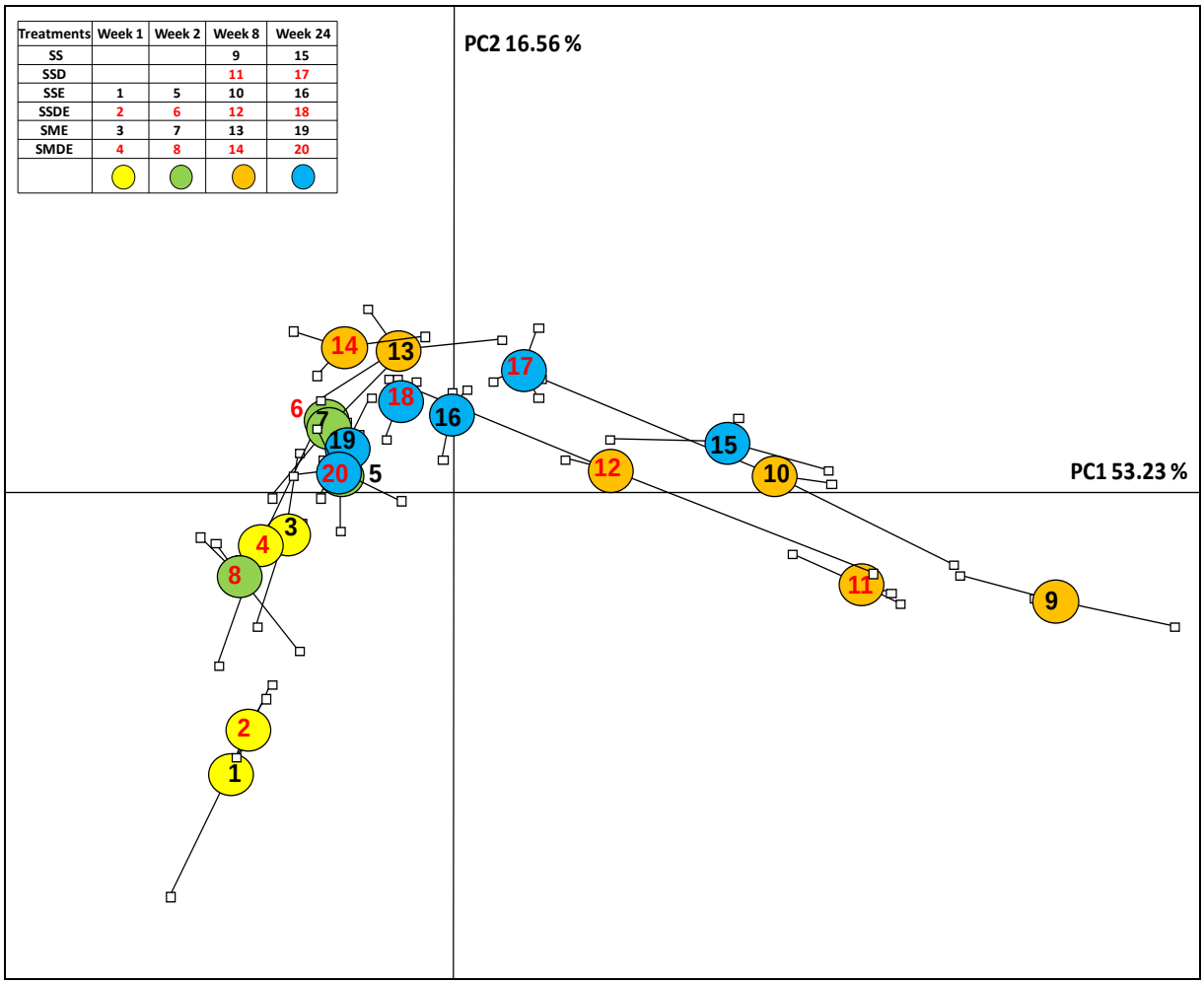


Fig. 4: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the fungal community structure. Each number represents the mean of 3 biological replicate for one treatment at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results The red colour numbers represents the presence of DON. SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON.

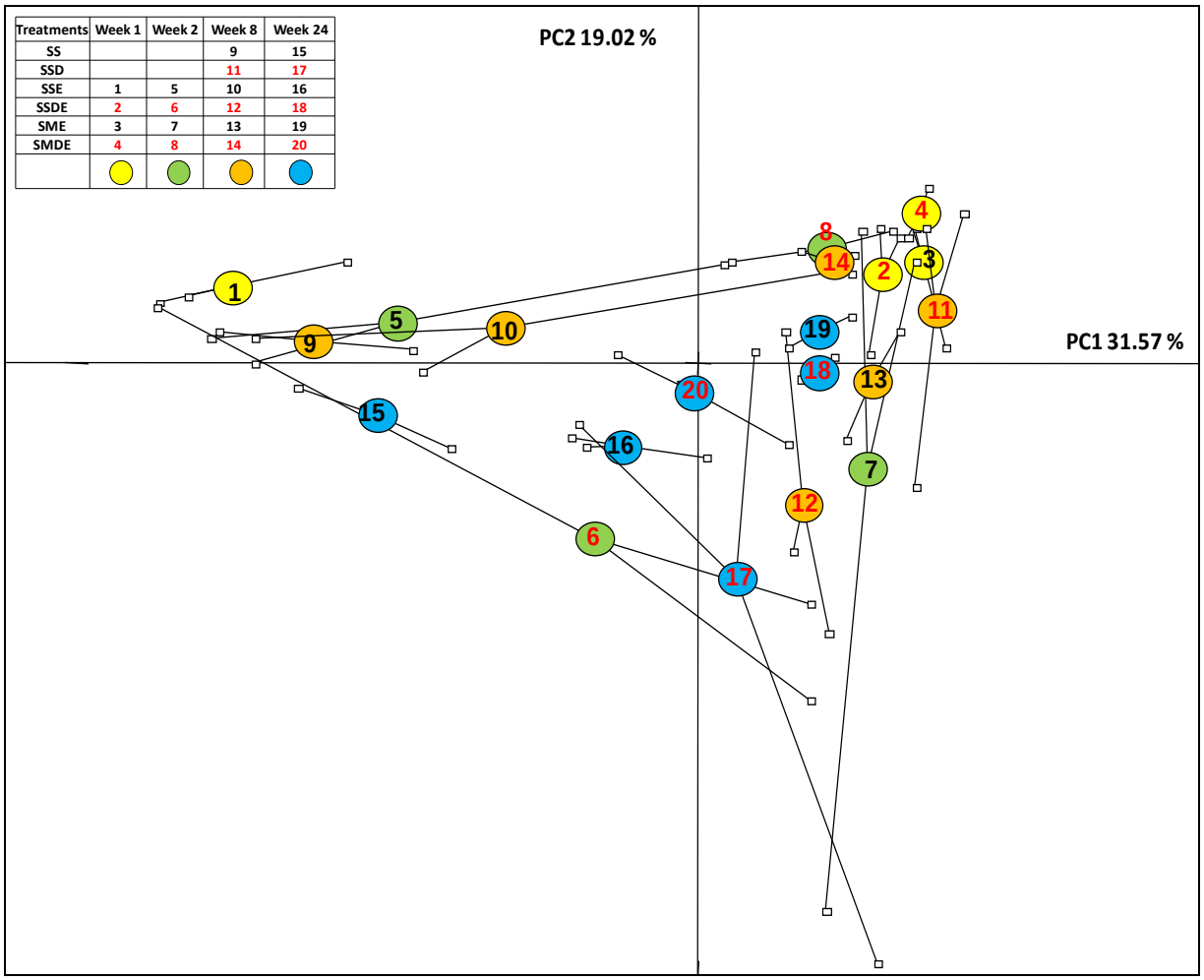


Fig. 5: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the protozoa community structure. Each number represents the mean of 3 biological replicate for one treatment at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results The red colour numbers represents the presence of DON. SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON.

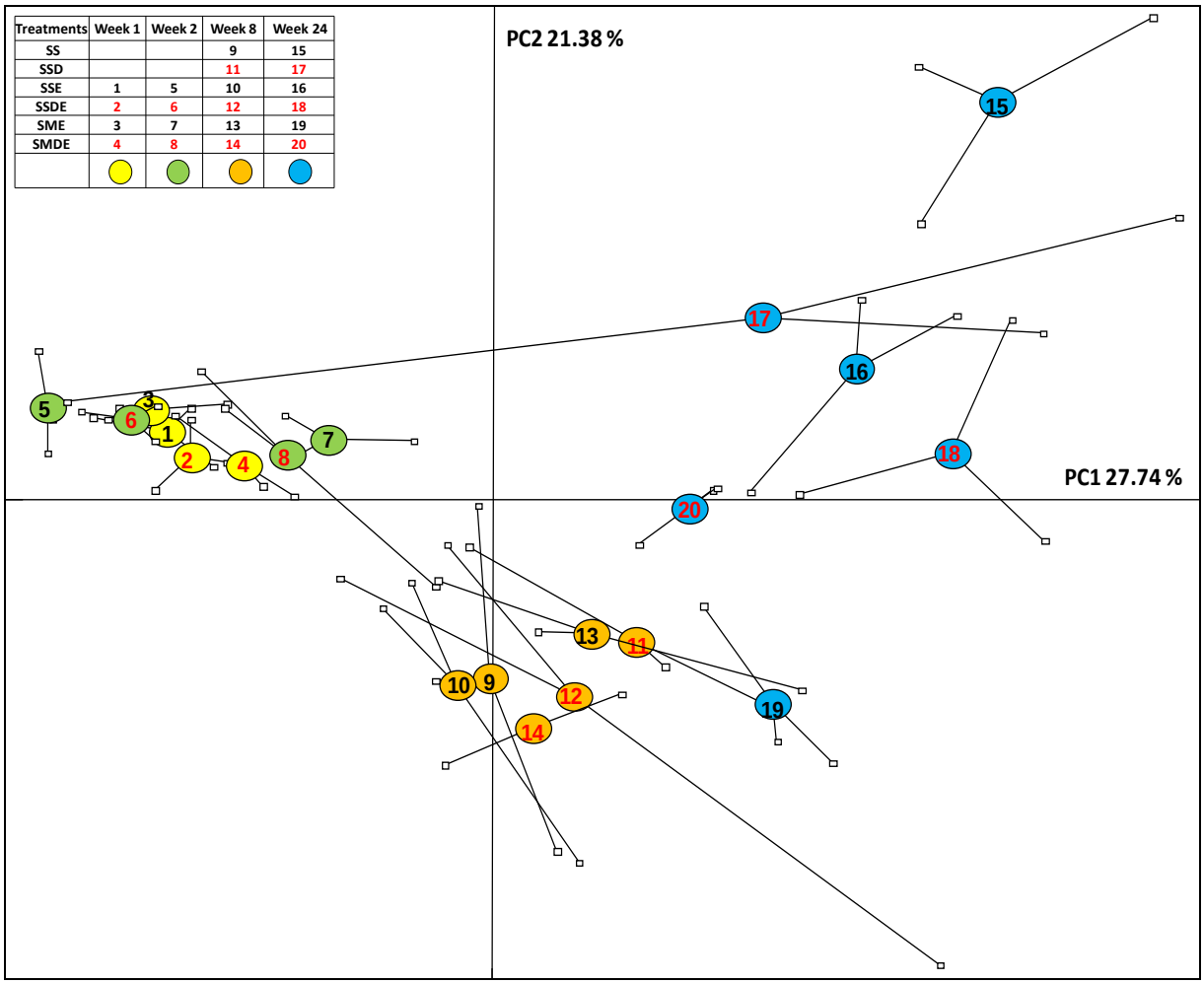


Fig. 6: Principal component analysis of T-RFLP data set in the two dimensional diagram from all the treatments for the nematodes community structure. Each number represents the mean of 3 biological replicate for one treatment at a specific time period. For each biological replicate, 3 technical replicates were performed and provided non significantly different results The red colour numbers represents the presence of DON. SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON.

3.4- Impact of DON on the microbial and faunal densities

3.4.1-Effect of DON on earthworms:

Each microcosm was provided with 4 adult earthworms of biomass 13.67 (±0.76) g at the time of setting up the experiment. This value of biomass was considered as starting point for all the treatments. At each sampling time, the total biomass of earthworms was measured (i.e. week 1, 2, 8 and 24). The average value of each treatment comprised three independent biological replicates (microcosms). The results illustrate that the presence of DON had no impact on the biomass of earthworms. The cumulative biomass of earthworms decreased remarkably after first two weeks and it continued along with the disappearance of straw until week 24 (Fig. 7). This fall in biomass was not related to the presence or absence of DON but seems to be more dependent on the exhaustion of the food. At the end of 24 weeks we found some new-borne earthworms in all the treatments, which indicates that even the reproduction of earthworms or the cocoon hatching was not affected by the presence of mycotoxin. These new-borne earthworms were counted and the biomass was measured. A significantly high number of new-borne earthworms were found when DON contaminated straw was left at the surface as compared to non-contaminated straw. On average 4 earthworms per microcosm (0.12 ± 0.04g) were found in the DON contaminated straw left at the surface, while only 1 earthworm (0.045 g) from the three biological replicates was found in the absence of DON contaminated straw. On the opposite no significant impact was observed when the straw was incorporated in the soil. On average 5 new-borne earthworms (0.174 ± 0.053g) per microcosm were observed in the treatment with DON contaminated straw and 4 new-borne earthworms (0.503 ± 0.52g) in the treatment without DON where the straw was incorporated in the soil.

We found that no earthworm was died till the 8 weeks while a very few mortality was observed on week 24, which was a bit higher in the presence of DON. The mortality of earthworms in the treatment when the straw left on the soil surface in the presence of DON was 41%, it was 33% without DON, it was 12% in the treatment when the straw was incorporated in the soil in the presence of DON and 0% in the absence of DON.

3.4.2- Effect of DON on nematodes:

The numbers of nematodes were counted and were compared in the presence or absence of DON when the straw was left at the surface or incorporated in the soil in the presence of earthworms after 1, 2, 8 and 24 weeks and in the absence of earthworms after 8 and 24 weeks. The number of nematodes ($2.4 \times 10^3 \pm 3.9 \times 10^2$) in the soil counted at the establishment of experiment was considered as starting point for all the treatments. The nematodes in the system increased soon after the establishment of the experiment (Fig. 8). The presence of DON had no influence on the nematode density during the first two weeks. Overall a negative trend was observed on the community of nematodes in the presence of DON in the week 8. This trend became significant on the week 24 where the straw was left at the surface of the soil with or without the presence of earthworms. On the other hand, earthworms were also found to suppress nematodes on the 24 week.

3.4.3 -Effect of DON on fungal and bacterial biomass:

The total fungal and bacterial molecular biomass was measured and expressed in the form of numbers of DNA copies/g of the soil-straw mixture at each sampling time (i.e. after 1, 2, 8 and 24 weeks). The soil-straw mixture just at the establishment of the experiment was taken as starting point for all the treatments.

The fungal and bacterial biomass increased after the establishment of microcosms and then started decreasing till the end of experiment (Figures-9 & 10). On the week-1 fungal densities were significantly higher where straw was incorporated in the soil. Further, the fungal as well as bacterial densities when the straw contaminated with DON was incorporated in the soil were significantly higher over the other treatments. On the week-2, their biomass was suddenly significantly suppressed where the DON contaminated straw was incorporated in the soil. This negative impact was observed even after 8 weeks. The fungal as well as bacterial density again increased on week 24 where DON contaminated straw was incorporated in the soil. On the other hand, DON contaminated straw left on the soil surface produced no impact on the fungal biomass in the presence of earthworms throughout the experiment. Fungal biomass was found higher in the absence of earthworms on week 24 while no impact was found on week-8. The bacterial biomass was not affected by the DON contaminated straw left at the surface in the presence or absence of earthworms.

3.4.4 - *Fusarium graminearum* biomass in relation with DON:

The densities of *F. graminearum* were observed in all the treatments in the presence or absence of DON. It was measured in the presence of earthworms (on week 0, 1, 2, 8 and 24) and in the absence of earthworms (on week 0, 8 and 24). The soil-straw mixture just at the establishment of the experiment was taken as starting point for all the treatments as for the fungi and bacteria (Fig. 11). The overall molecular biomass of *F. graminearum* increased just after the start of experiment especially in the treatments where the DON contaminated straw was incorporated in the soil. Later, no impact was observed till the week 8. DON was found to support *F. graminearum* again on week 24 when DON contaminated straw was incorporated in the soil as compared to the other treatments.

The DON contaminated straw produced no positive or negative impact on the *F. graminearum* density when the straw was left on the soil surface in the presence or absence of earthworms throughout the experiment.

3.4.5 - Colony Forming Unit (CFU):

The cultivable fungal and bacterial densities were also measured for each sampling date by colony forming unit (CFU) per gram of soil. There was no significant effect of mycotoxins on the fungal and bacterial cultivable communities whether the DON contaminated straw was incorporated or left at the surface of the soil.

The bacterial communities increased in the first week and then decreased along the time factor (Fig. 12), which is more or less same to the total molecular densities (DNA). The cultivable fungal communities on contrary to the total molecular densities increased after two weeks (Fig. 13).

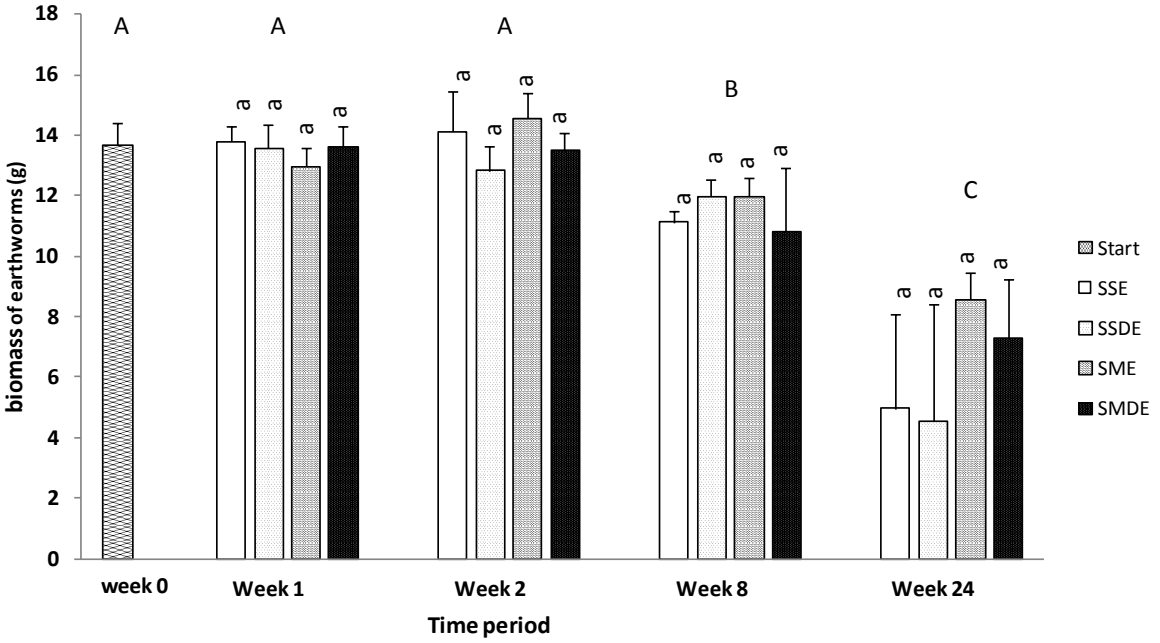


Fig 7: The impact of DON on biomass of earthworms with the passage of time in four treatments (SSE = Straw placed on Surface of soil + Earthworms, SSDE = SSE + DON, SME= Straw Mixed with the soil + Earthworms, SMDE = SME + DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference between the sampling times.

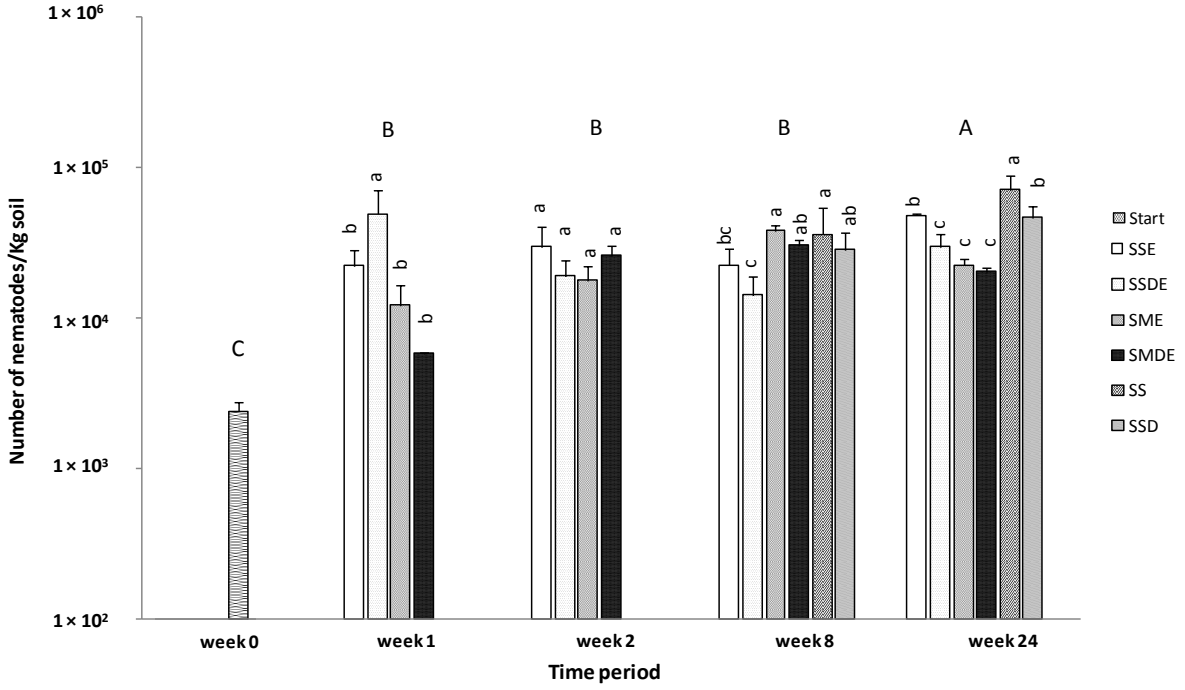


Fig. 8: The impact of DON on nematodes densities in microcosms, straw being incorporated or placed on the soil surface in the presence or absence of earthworms. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

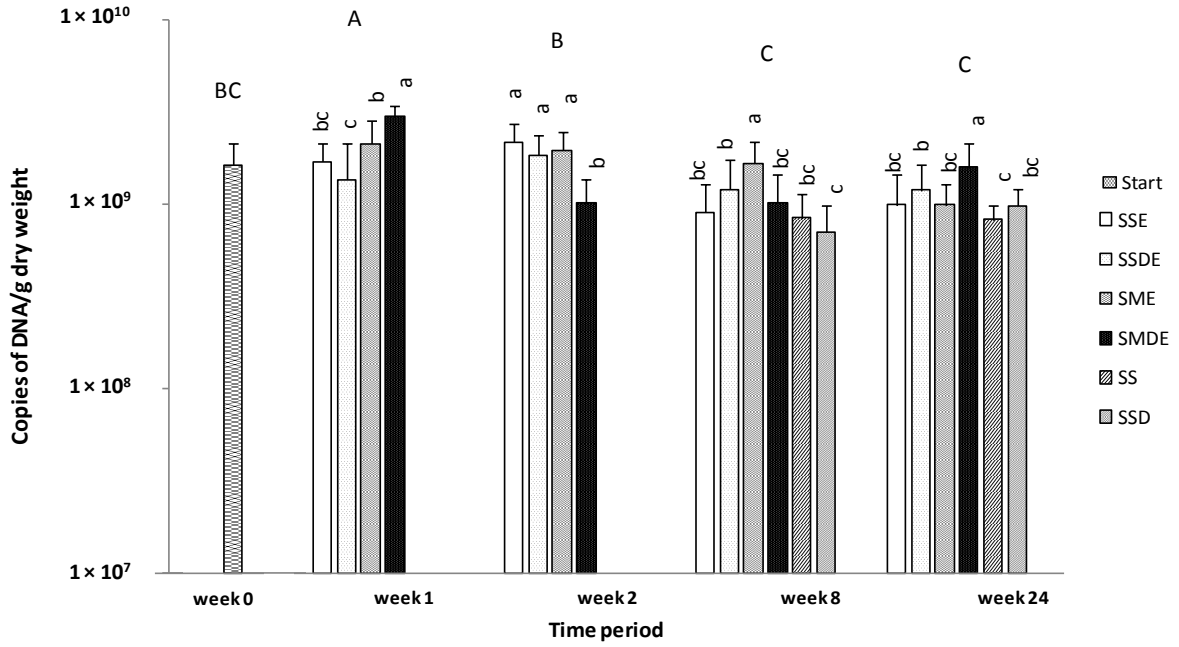


Fig. 9: The impact of DON on bacterial molecular densities in microcosms, straw being incorporated or placed on the soil surface in the presence or absence of earthworms. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

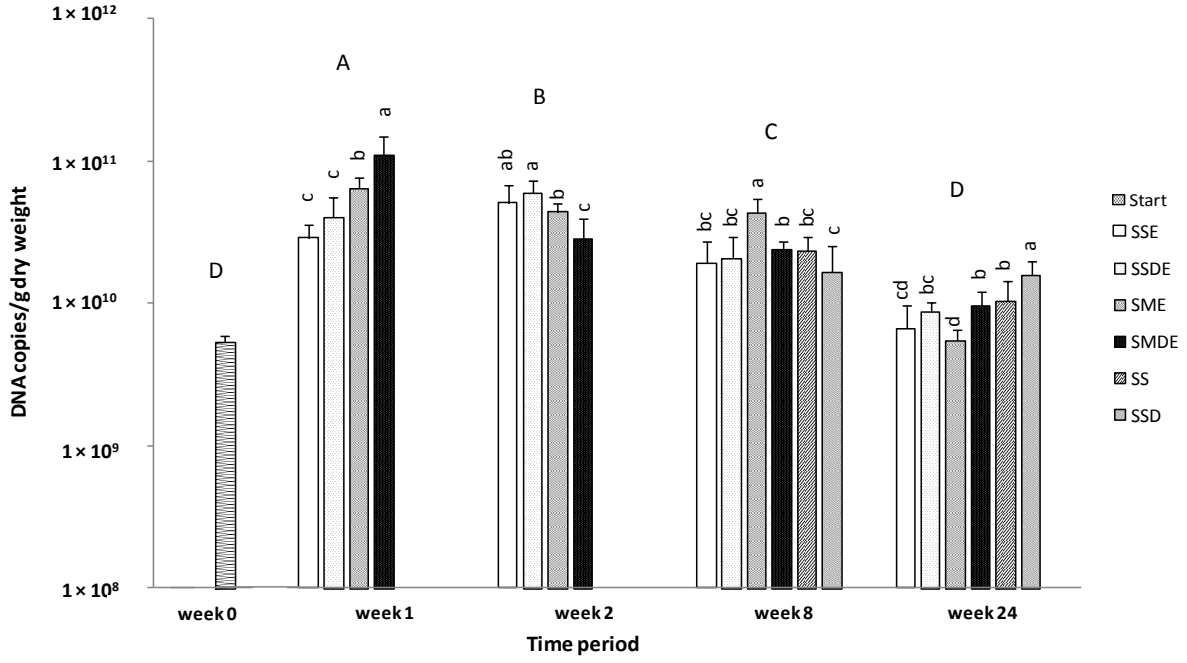


Fig. 10: The impact of DON on fungal molecular densities in microcosms, straw being incorporated or placed on the soil surface in the presence or absence of earthworms. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

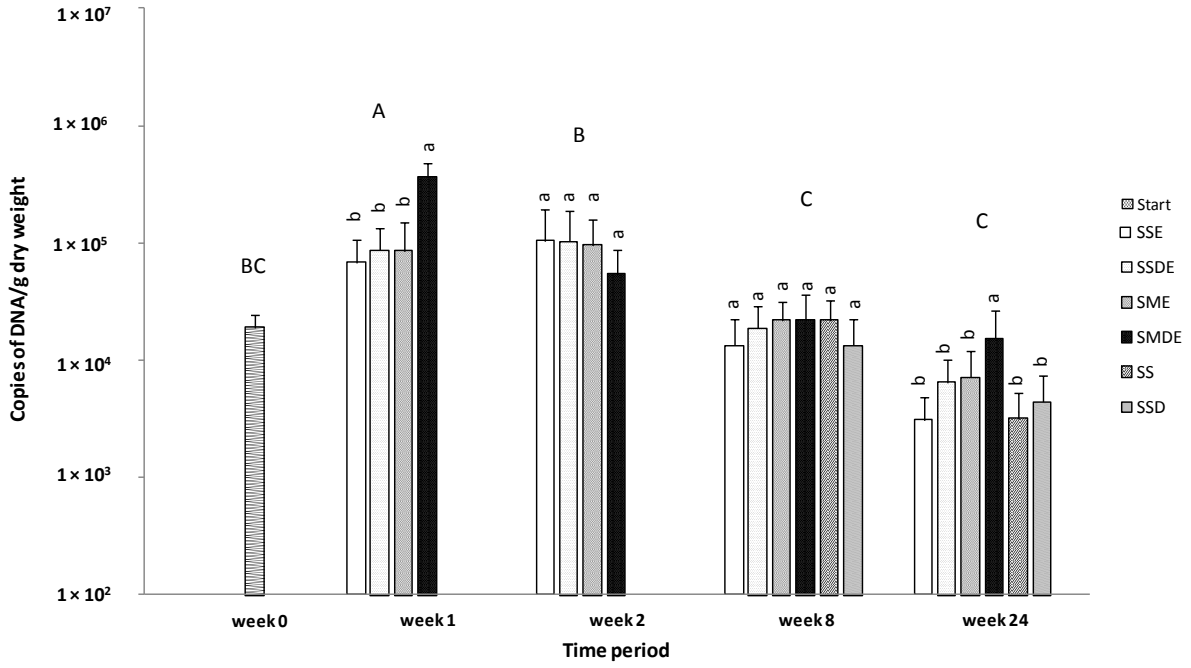


Fig. 11: Population dynamics of *F. graminearum* in wheat straw in the presence or absence of DON and earthworms, straw being placed on the soil or incorporated in the soil. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

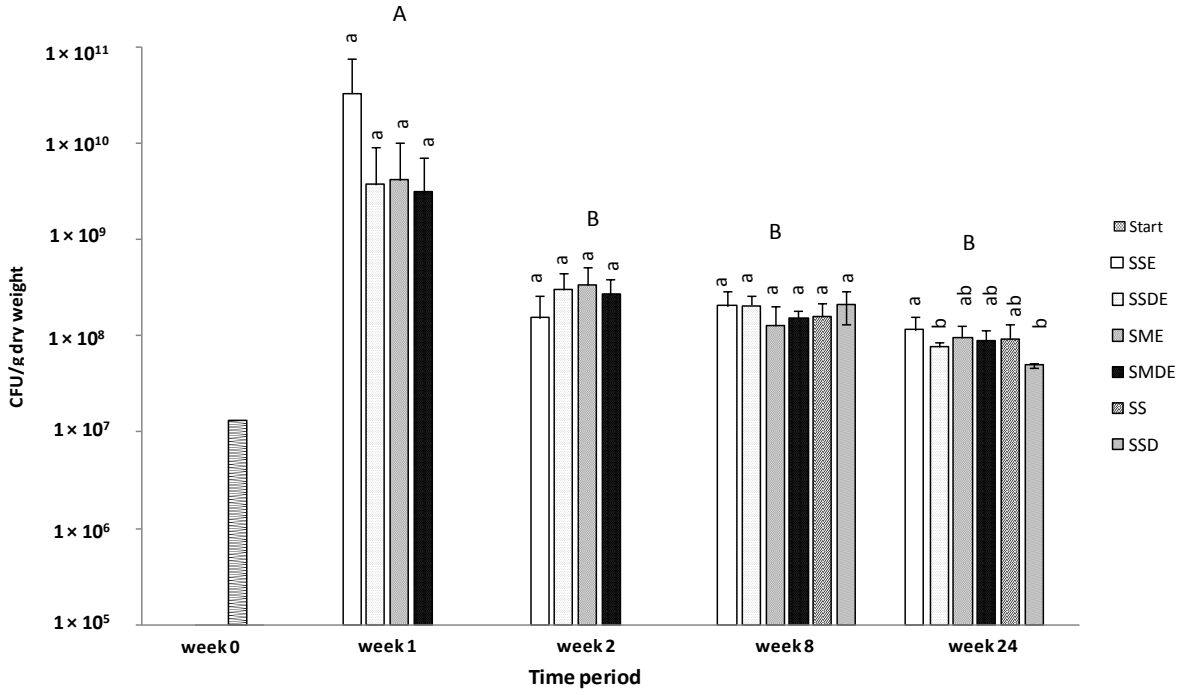


Fig. 12: The impact of DON cultivable bacterial colonies in microcosms, straw being incorporated or placed on the soil surface in the presence or absence of earthworms. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

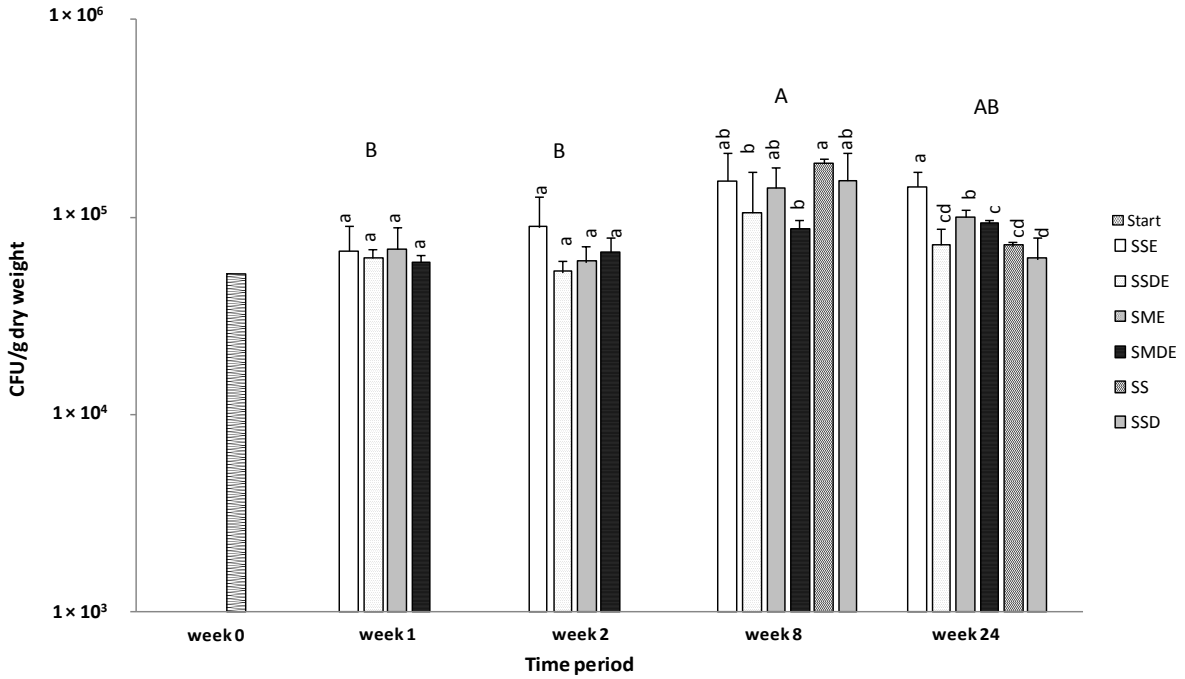


Fig. 13: The impact of DON cultivable fungal colonies in microcosms, straw being incorporated or placed on the soil surface in the presence or absence of earthworms. (SS=Straw at the Surface, SSD= SS+DON, SSE= SS+Earthworms, SSDE= SSD+Earthworms, SME= Straw Mixed with the soil+Earthworms, SMDE=SME+DON). A comparison was done among different treatments on all the sampling times by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05). Different small letters indicate significant difference between the treatments on the different sampling times. Different capital letters indicate the significant difference of cumulative total between the sampling times.

4- Discussions:

4.1-Fate of DON:

In this study, we followed the fate of DON in the winter wheat straw during the saprophytic survival of *F. graminearum* up to six months in the microcosms where the straw was left at the surface or incorporated in the soil in the presence of the whole soil biota.

It was observed that with the passage of time DON disappeared from all the microcosms after six months passage, whether the straw was left at the surface or incorporated in the soil. The rate of the disappearance of DON was considerably higher when the straw was incorporated in the soil as compared to when it was left at the surface thanks to greater exchange surface between the substrate and microorganisms. This may be because of the faster contact of the soil communities with DON when the straw was incorporated in the soil. The incorporation of crop residues is a great reservoir of microbial activity, they are decomposed faster as compared to the above surface (Lupwayi et al., 2004). In a recent in vitro study, Sato et al., (2012) reported some soil-borne bacteria can degrade DON efficiently. So the presence of similar functional bacteria and of the other soil communities could have made the DON unavailable in the system.

Furthermore, we also found that earthworms played a significant role in the degradation of DON. Our results revealed that even after passing 8 weeks, the concentration of DON in the absence of the earthworms was 40 times higher as compared to the presence of earthworms when the straw was placed at the surface of the soil. The earthworms increase the microbial activity by breaking down the big pieces of straw into small pieces and burrowing them, increasing even more the area of exchange between the contaminated straw and the soil microorganisms. This resulted in the faster disappearance of DON. This comparative assessment goes with the recent demonstration that earthworms have a specific preference for DON contaminated straw compared to non-contaminated straw (Oldenburg et al., 2008). Although this preference has not yet been explained, it is likely that the choice is related to the earthworm gut microflora, the later being able to degrade the DON and therefore to provide the earthworm with specific trophic resources (Schrader et al., 2009).

4.2-Impact of DON on the microbial and micro-faunal community structures:

We chased the changes in the community structures along six months to determine the impact of DON on the soil microbial and microfaunal communities when it is present in the crop residues. We observed that the community structure of bacteria and protozoa were significantly affected in the presence of DON while fungal and nematodes community showed respectively conditional and no reaction towards DON. From these results we cannot assume whether some populations of bacteria and protozoa were definitively favored or suppressed by the presence of the DON but at least it appeared that the ratio between populations within a community changed, what reveals some microorganisms are more susceptible to the toxin.

The bacterial community structures were significantly different in the presence of DON whether the straw was left at the surface or incorporated in the soil. This effect lasted only for the first two weeks. This corresponds to the presence of the high quantity of DON in all the treatments in the start of the experiment. After the passage of two weeks the quantity of DON was highly reduced in all the treatments and on the eighth week, there was no significant difference in the community structure of bacteria in DON contaminated and no contaminated treatments. In the mean while, our comprehensive analysis revealed that the bacterial community structure in the presence of DON was clearly different in the presence and absence of earthworms, which also goes to the difference in the quantity of DON in the both treatments. Surprisingly, the impact of DON was not clear in the absence of earthworms on the eighth week even we found a high amount of DON. The change in the bacterial structure can be positive in the sense that certain bacteria from the soil accumulated in order to degrade DON. Recently, some soil-borne bacteria were reported to be involved in the degradation of DON (Ikunaga et al., 2011; Sato et al., 2012). This impact of DON could also be negative and suppress certain populations of bacteria. The negative impact of DON on bacteria has never been studied or reported before.

In the case of fungi, overall the fungal genetic structure was different when the straw was left at the surface than when it was incorporated in the soil. The genetic structure was affected by the presence of DON only in the absence of earthworms. The presence of earthworms significantly affected the community structure of fungi all over the experiment in all the modalities. The presence of earthworms might have masked the effect of DON on the fungal

communities. Indeed, on the one hand certain fungal species are reported to be preferred over the other fungi as food by earthworms (Bonkowski et al., 2000). On the other hand, it might be because of the rapid degradation of DON in the presence of earthworms which suppressed the effect on the fungal communities.

On the protozoa the effect of DON was related to the location of the straw. The impact of DON is very clear throughout the experiment when the straw was placed on the soil in the presence as well as in the absence of the earthworms from the start till the end of six months. As DON was no more detectable at this stage so we can assume that the changes DON caused previously on the protozoan community were long lasting if not definitive. It would be worth to check for the possible erosion of the protozoan diversity. When the straw was incorporated in the soil the presence of DON showed less impact on the protozoa.

The nematodes communities showed no reaction against the presence of DON in all the treatments during the whole period of the experiment. The community structure was only changed with the passage of time which appeared after the passage of two weeks. The presence of earthworms had no impact on the nematodes communities.

4.3- Impact of DON on the soil microflora and faunal densities:

The impact of DON was observed on the biomass of earthworms and on the densities of nematodes, bacteria and fungi during the saprophytic survival of *F. graminearum* on the wheat straw during its decomposition in the soil.

Earthworms are well known bio-indicators of the soil health and have great importance in agriculture due to their ability to incorporate and degrade wheat crop residues and organic matter (Friberg et al., 2008; Friberg et al., 2005; Kreuzer et al., 2004; McLean et al., 2006). Our results are in agreement with these previous findings and they more specifically emphasize the role of *L. terrestris* in the degradation and incorporation of the straw in the soil. Despite the biomass of the earthworms reduced over time, probably because the food contents were exhausting, the positive and original point was that this total biomass was not affected by the DON contaminated straw. At the contrary, the DON contaminated straw was found more attractive for the earthworms which coincides with finding of 11 week study of (Oldenburg et al., 2008).

Furthermore, the presence of the new-borne earthworms in all the treatments containing DON contaminated straw showed that DON even had no negative effect on the reproduction of earthworms or the cocoon hatching. The presence of higher number of new-borne earthworms

when the DON contaminated straw was left at the surface of the soil showed that DON stimulated reproduction in the earthworms or increased the hatching of cocoons. We also counted the number of earthworms at each sampling time and compared to the initial number of earthworms put in the microcosms. We observed that the mortality of earthworms was appeared only in the end of six months. Overall more earthworms were died when the straw was left at the surface. We also found that mortality of earthworms was a bit higher in the presence of DON especially when the straw was incorporated in the soil. These mortalities produced no significant impact on the overall reduction in the biomass of earthworms which was not significantly different in all the treatments in the presence or absence of DON.

Nematodes are also a very important component of arable soil functioning and could too be considered indicators for evaluating the soil quality (Schloter et al., 2003). However, more than their role in decomposing organic matter and in controlling soil-borne fungal pathogens (Hasna et al., 2007) this is the effect of crop residues and the nature of the organic matter on the structure of nematodes communities which are taken into account (Berry et al., 2007). Indeed, the nematodes include both pathogenic and non-pathogenic taxa. Management practices including previous crops or organic amendments aim at influencing the structure of nematodes communities to increase the control of the pathogenic ones either through a direct effect of the organic matter or through a microbes mediated effect (Berry et al., 2007; Mateille et al., 2009). Unfortunately, little is known concerning the direct or microbial mediated interaction between nematodes and wheat straw. Our results showed that the number of nematodes increased with the passage of time during the decomposition of straw. This was contrary to the decomposition of straw and the biomass decrease of the earthworms. The impact of DON appeared very late in the experiment on the nematodes density. DON had no sudden poisoning effect on nematodes but the effect appeared later which may indicate that DON showed rather an acute progressive poisoning. There was no impact of DON on the first two weeks but after eight weeks a negative trend of DON was developed and it was found significant after 24 weeks. In a recent report deoxynivalenol is reported to reduce the developmental process and inhibit the nematodes (*Caenorhabditis elegans*) egg hatching in the liquid medium (Gowrinathan et al., 2011). The nematodes densities increased in the system with the decrease in the biomass of earthworms and on week 24 it is significantly clear that earthworms were higher in the absence of earthworms. It seems that earthworms and DON were playing also a role in the suppression of nematodes densities. Dominguez et al., (2003) previously reported that the nematode densities are in negative relation with biomass of earthworms during the decomposition of organic substrate. The effect of DON appeared on

the nematodes as well as on the earthworms late in the experiment. In case of nematodes it is significant while in case of earthworms it was not so high. This indicates that DON was not involved in the sudden death but a slow poisoning.

We also measured the molecular mass of bacteria and fungi to determine the impact of DON contaminated straw on them. We found that molecular biomass of bacteria and fungi were increased soon after the establishment of experiment and then started decreasing with the decomposition of the wheat straw. This same trend was observed on the plates in the case of bacteria and not confirmed in the case of fungi. The availability of the nutrients and the aeration in the start of the experiment seems to be involved in boosting their densities. The bacterial and fungal densities showed the reaction against the presence of DON only when the straw was incorporated in the soil and DON produced no impact when the straw was left at the surface. This might be because of the rapid contact of DON contaminated straw with bacterial and fungal communities. They showed positive response first just after the experimental setup and then DON significantly reduced their densities which were detectable up to 8 weeks till the detection of DON. The reaction of DON was same towards the bacterial and fungal densities. They showed the positive reaction in the start of the experiment but suddenly decreased significantly. This may show that the presence of DON served as an external stress and in response the fungi first produced more spores but later DON showed a negative reaction on growth of mycelium and spore production. The reaction of DON was limited only in the treatments where straw was incorporated in the soil. This might be again because of the rapid exposure of DON to the soil bacterial and fungal communities.

The growth and the development of *F. graminearum* were observed in the presence or the absence of DON. *F. graminearum* showed the same kind of behavior as the other fungal and bacterial communities. *F. graminearum* also showed the reaction towards the presence of DON only in the case where the straw was mixed in the soil. *F. graminearum* was found to get benefit of the presence of DON and the biomass was increased like overall fungal and bacterial communities. *F. graminearum* density was also decreased with the passage of time like the bacterial and fungal communities. Contrary to other fungal populations within the fungal communities, the presence of DON had no negative effect on the density *F. graminearum* as we compare its population with control in the experiment. The comparison among the treatments after 8 or 24 weeks showed also that the presence of earthworms had no significant impact in the reduction of *F. graminearum*.

The multiple interactions occurring in the system studied could have obscured a stronger impact of DON than the one revealed by the community structure fingerprintings or the community molecular biomass. At the contrary, the classic microbiological methods revealed some variability among the microorganisms in their response to the presence of DON but they are imprecise and cannot ensure that the observed differences must be attributed to DON, to biotic interactions or to technical limitations. Therefore, although molecular and classic microbiological assessments of the fate of bacteria and fungi in the crop residues in presence or absence of DON gave occasionally different results, they are complementary and they both revealed similar trends, the main ones being that the microbial communities, including the protozoa are affected at various extents by the presence of DON but that *F. graminearum* is not affected by the presence of DON it its surrounding.

5-Conclusions:

Finally, this study is original. It provides for the first time a wide overview and a comprehensive knowledge on the fate of DON in the crop residues in the soil and its impact on the soil microflora and fauna.

We conclude that DON didn't stay for a long time in the straw and disappeared completely in all the treatments during the six months. The location of straw played a significant role in the disappearance of DON. It disappeared more rapidly when the straw was incorporated in the soil as compared to when left at the surface of the soil. The presence of earthworms played a key role in the disappearance of DON which was highly slow in their absence. DON was not produced *in situ* or produced in very small quantity, which disappeared very rapidly during the saprophytic survival of *F. graminearum*.

DON significantly changed the community structure of bacteria which was dependent on the quantity of DON present in the microcosms. In case of protozoa the location of straw was more important and the affect of DON remained throughout the experiment. The effect on the fungal community structure was seen only in the absence of earthworms. The nematodes community structure remained unaffected by presence of DON.

The biomass of earthworms was not affected by their exposure to DON. The reproduction or cocoon hatching of earthworms was stimulated in the presence of DON when the straw was placed on the soil surface. Nematodes densities were negatively affected and their number were reduced but the impact appeared late in the experiment. The bacterial and the fungal densities were negatively affected only when the straw was incorporated in the soil. *F.*

graminearum got no advantage of the presence of mycotoxin in the straw whether it was left at the soil surface or incorporated in the soil but conversely, it was not negatively impacted by the presence of this mycotoxins in its surrounding.

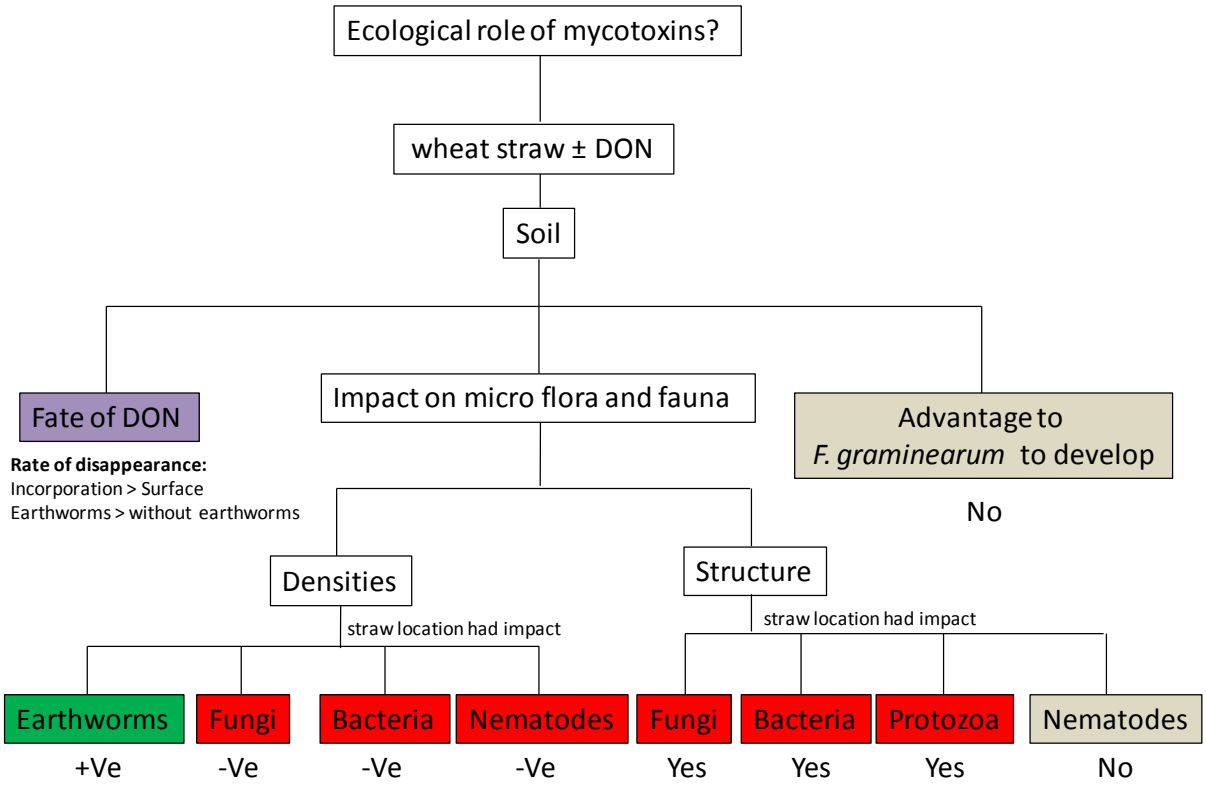


Fig. 14: Flow chart showing the main conclusion obtained during the different analyses in the microcosm experiment. The violet colour rectangle shows the disappearance of DON; the green colour rectangle shows the positive impact of DON; red colour rectangles show the impact of DON while the grey colour shows no impact of DON. Furthermore, Yes= DON had an impact; No= DON had no impact; +Ve = positive impact; -Ve = negative impact.

Chapter-5: Population dynamics of *Fusarium graminearum* in the presence of decomposers and deoxynivalenol

Population dynamics of *Fusarium graminearum* in the presence of decomposers and deoxynivalenol

1-Introduction:

Reduced tillage tends to be more practiced to preserve soil structure and reduce erosion, to maintain soil moisture, save energy and working time. Therefore, crop residues are going to be more and more frequently left on the soil surface rather than being incorporated into the soil. Consequently, many soil-borne plant pathogens survive in the previous year's crop residue, making diseases more problematic under reduced-tillage conditions (chapter 3). Indeed, reduced tillage can favor pathogens by protecting the pathogen's refuge in the residue from microbial degradation, by lowering soil temperature, or by increasing soil moisture (Bailey and Lazarovits, 2003; Bateman et al., 2007). Therefore additional controls are needed for pathogens. Besides breeding host plants for resistance towards bio-aggressors, chemical, biological and cultural controls can be used alone or in combination to limit damage from diseases (Bockus and Shroyer, 1998). In the case of soil- or residue- borne microorganisms, chemical control proved to be not really efficient to protect the plant from the pathogens (Bolton et al., 2010; Nel et al., 2007) while crop rotation schemes including intermediate crops such as Brassicaceae appeared more promising (Buhre et al., 2009; Paulitz et al., 2010). The use of biocontrol agents is a bit tricky because many parameters must be met to ensure an efficient bioprotection. Among these parameters, the proper timing of antagonist applications is probably the more difficult to adjust, unless the inoculum can establish in and be retained by the residues so that this inoculum can antagonize continuously the pathogen (Matarese et al., 2012). The problem is even more crucial with mycotoxigenic fungi such as *F. graminearum* and other *Fusarium* sp belonging to the Fusarium Head Blight (FHB) complex and whose mycotoxins may affect other residue-borne microorganisms or inoculated antagonists. Any competitive advantage conferred by mycotoxins would complicate efforts to control *F. graminearum* during its saprophytic growth on crop residues. For instance, deoxynivalenol (DON) produced by *F. graminearum* can repress chitinase gene expression in a biocontrol agent *Trichoderma atroviride* (Lutz et al., 2003). Conversely, the use of a nontoxigenic mutant of *F. graminearum* in controlled conditions revealed that DON did not provide any further advantage to the wild strain towards *T. atroviride* (Naef et al., 2006).

Most of the studies so far focused on the pathogenic phase of *F. graminearum* lifecycle and on the way to biocontrol the mycotoxins production but more rarely on the saprophytic phase of its lifecycle while this phase could be the weakness of the pathogenic fungus.

Indeed, on the one hand, the mycotoxigenic *Fusarium* sp. generally overwinter in crop residues and they remain potential threat to the coming susceptible crops, what is stated by most of the studies (Leplat et al., 2012; Parry et al., 2007). On the other hand most soil fungi are saprotrophs that feed on decaying organic materials and contribute at different extents to the decomposition process of plant–derived substrates. Therefore, they could limit the development of the future primary inoculum. Therefore, the direct role of mycotoxins towards decomposers and direct antagonists need to be assessed to understand the strategy used by *F. graminearum*, to survive for years in crop residues.

The main objective of this study was to find how strain MIE 00376 of *F. graminearum* could get profit of the presence of the DON it produces towards i) other fungal decomposers and ii) other DON non producing fungi of the FHB fungal complex. Assuming that nitrogen could be a limiting factor for the decomposition processes tested in subsequent experiments, some free fixing nitrogen bacteria were also included in the test. For this purpose the response to different doses of DON of different soil-borne fungi previously isolated from decaying wheat straw as well as the susceptibility of known strains of *Fusarium* sp. and *Microdochium nivale* issuing from the FHB fungal complex and of a set of rhizospheric bacteria was evaluated towards to choose DON resistant microorganisms. Then the population dynamics of *F. graminearum* was observed in presence of DON resistant microorganisms, alone or in combinations, in the presence or absence of DON in the microcosms.

2-Materials and Methods:

A set of 17 fungal strains and 4 bacterial strains was used in this study (Table 1). Four fungal strains are part of the FHB fungal complex and exhibit different abilities to produce or not various mycotoxins, 8 fungi were isolated from decaying straw at various stages of decomposition and are therefore associated to the decomposition process although their potential enzymatic activities were not tested for each of them, 2 strains are pathogenic fungi which could cause damping off and root rot on wheat but they were selected because of their saprophytic abilities, 3 strains are saprophytic fungi isolated from soil and previously characterized for their antagonistic activities towards some plant pathogenic fungi but not towards *F. graminearum*. Three bacterial strains were isolated from rhizospheric soil of cereals and were chosen for their ability to fix nitrogen freely while the 4th one was chosen as a common rhizospheric bacterium.

Among the 8 fungi isolated from decaying straw 4 were previously identified by sequencing the ITS and EF regions of rDNA. The sequences were then compared using blast and NCBI data library and were preserved to the MIAE collection. The other 4 fungi isolated from decaying straw in microcosm experiment (Chapter 4) were just identified phenotypically. All other fungal and bacterial strains have been previously identified and were preserved as so in the MIAE collection.

2.1- Minimal Inhibition Concentration test for screening:

Two types of methods were used to evaluate the minimal inhibition concentration needed to limit, or not, the microbial development. Radial growth of fungal colonies was measured on PDA medium in Petri dishes and optical density of fungal and bacterial growth was measured in liquid medium in microtiter plates as described in detail in the Chapter-2, , page 58.

Table 1: Strains and isolates of fungi and bacteria used and their respective Minimal Inhibitory Concentration (MIC) determined in this study

Fungal strains	Accession number[1]	Origin - Traits of interest	MIC[2]	Population dynamics[3]
Fusarium graminearum	MIAE00376	Maize stubbles - FHB complex-DON producer	> 10 µg/ml	X
F. culmorum	MIAE00288	Diseased plant - FHB complex-DON producer	< 2.7 µg/ml	X
F. poae	MIAE00300	Diseased plant - FHB complex-does not produce DON (but produces TCTA[4] and other TCTB[4])	< 0.3 µg/ml	X
Microdochium nivale	MIAE00318	Diseased plant - FHB complex-does not produce mycotoxin	< 0.9 µg/ml	X
T. koningiopsis	MIAE00725	Isolated from decaying straw	> 10 µg/ml	X
F. oxysporum	MIAE00726	-	> 10 µg/ml	X
F. oxysporum	MIAE00800	-	> 10 µg/ml	
Epicoccum nigrum	MIAE00727	-	> 10 µg/ml	
Gliocladium spp.	PC[5]	-	< 0.1 µg/ml	
Aspergillus spp.	PC	-	> 10 µg/ml	
A. niger	PC	-	> 10 µg/ml	
Penicillium spp.	PC	-	> 10 µg/ml	
Rhizoctonia solani	MIAE00078	Soil- responsible for root rot; good saprophytic abilities	< 2.7 µg/ml	
R. solani	MIAE00066	-	< 2.7 µg/ml	
F. oxysporum	MIAE00047	Fusarium wilt suppressive soil of Chateaurenard (France) – Antagonistic abilities toward pathogenic *F. oxysporum*	> 10 µg/ml	
Trichoderma gamssi	MIAE00029	Soil- antagonistic abilities towards pathogenic fungi	< 2.7 µg/ml	
T. velutinum	MIAE00044	-	< 0.9 µg/ml	
Bacterial strains				
Azospirillum brasilense	MIAE00334	Rhizospheric soil - free nitrogen fixing bacteria	> 10 µg/ml	
A. lipoferum	MIAE00337	-	> 10 µg/ml	
A. lipoferum	MIAE00338	-	> 10 µg/ml	X
Pseudomonas fluorescens	MIAE00587	Rhizospheric bacteria	< 2.7 µg/ml	

[1] accession number in collection MIAE (Micro-organisms on Agro-Environmental Interest - UMR-MSE, Dijon, France (http://www2.dijon.inra.fr/umrmse/spip.php?rubrique47).
[2] MIC : Minimal Inhibitory Concentration (µg DON/ml medium)
[3] strains which were selected for the population dynamic study are indicated with an X
[4] TCTA: trichothecenes A; TCTB: trichothecenes B,
[5] PC : personal collection. Strains were phenotypically identified. The molecular identification is being performed before the strains as a prerequisite to get an accession number from MIAE collection

2.2- Preparation of microcosms and analyses:

2.2.1-Inoculum production:

Three kinds of media were prepared:

1- Wheat bran liquid medium (WB): 2 % wheat bran in the distilled water

2- Luria betari (LB) media: 10 g/l Bacto trypton, 5 g /l yeast extract and 10 g/l NaCl, pH =7.0

3- Malt liquid media (ML): 10 g/l malt.

All of these media were autoclaved at 120°C for 20 min. Meanwhile, all the required fungal strains were cultured on the PDA and were placed at 25° C for seven days. Small plugs of 7 days old PDA cultures of different fungi were added to their appropriate media. *F. graminearum*, *F. poae* and *F. culmorum* were added WB medium, *M. nivale* and *T. koningiopsis* were added to ML medium and *F. oxysporum* and *A. lipoferum* were added to LB medium. They were placed at 25°C in the rotary shaker (150 rpm) for ten days for fungi and 2 days for bacteria. The fungal cultures were filtered through sterile cheesecloth (approximately 50 µm mesh size) to remove the mycelial mat. The number of bacteria and the conidial concentrations were assessed through Malassez chamber under the microscope. The concentrations were then adjusted by dilution with sterile distilled water. *M. nivale* didn't produce the spores, so the propagules were counted by colony forming unit (CFU) just before putting in the microcosms.

2.2.2-Straw treatments and microcosms preparation:

The air dry straw was taken from the winter wheat (*Triticum aestivum*) originated from a field of Epoisses (Bretennières, France). The straw was cut into approximately 2-3 cm small pieces and was sterilized by gamma radiations.

Small glass jars of dimensions 10 cm × 7 cm × 7cm (height × length × width) were used as microcosms. Two gram dry sterilized straw was filled in each of these microcosms. The equal amount of each fungi was added to the required microcosms in the form of spore suspensions (10^3 spores/g of straw (d.w.)) or in the form of propagules in case of *Microdochium nivale* (10^3 CFU/g of straw (d.w.)) or number of bacteria in case of bacteria (10^5 bacteria/g of straw (d.w.)). They were spread on the straw with micropipettes. DON solution (Chapter 2, page 56) at the rate of 10 µg/g straw (d.w) was added to each of the required microcosms. The final humidity in each microcosm was maintained as 80% which was equal to its water holding capacity. In the end half microcosms of each treatment were contaminated with DON solution

at the rate of 10 µg of DON/g of straw. All the microcosms were closed and were vigorously shaken by hand to mix all the contents of the microcosms. Half of the microcosms containing DON contaminated straw were prepared in extra to measure the quantity of DON in the start and the end of experiment. In this way 8 types of microcosm were prepared containing different combinations of different fungi which were further divided as with and without DON (table 2).

***F. graminearum* MIAE00376**	**FHB complex:** *F. poae* MIAE00300 *F. culmorum* MIAE00288 *M. nivale* MIAE00318	**Decomposers:** *F. oxysporum* MIAE00726 *T. koningiopsis* MIAE00725	**Decomposers + free N2 fixing bacteria:** *F. oxysporum* MIAE00726 *T. koningiopsis* MIAE00725 *A. lipoferum* MIAE00338	**DON**
X	X			X
X	X			
X		X		X
X		X		
X			X	X
X			X	
X				X
X				

Table 2: Experimental design to monitor the population dynamics of *F. graminearum* in presence of different combinations of different fungi with and without DON.

Three independent microcosms were prepared for each treatment. Some extra microcosms were prepared to measure the loss of humidity. All the microcosms were placed in an incubator at 25°C.

2.3 -Sampling and samples processing and analyses:

The sampling was done at 0, 14, 28 and 49 days. Three independent microcosms for each treatment were picked. The entire contents were shifted in the small vials and were freeze dried. They were ground to fine particles by sterilized pestles and mortars. These fine particles of straw were placed again at -20°C and were used for the extraction of DNA and

DON. The humidity was measured at each sampling time and was found 78-80% till the end of experiment.

The DNA from the straw was extracted and purified (chapter 2, page 50).

From the extracted DNA *F. graminearum* was quantified by using real time PCR (chapter 2, page 51).

DON was extracted from the straw in the start and at the end of experiment and it was quantified by using high performance liquid chromatography (HPLC, Chapter 2, page 57).

3-Results:

3.1-Minimal inhibition concentrations:

In the experiment we tested different soil-borne fungal and bacterial strains against different doses of DON from 0 µg/ml to 10 µg/ml on the Petri dish and OD measurement. The results showed that all the fungal species were not susceptible or resistant to DON (Table 1). The MIC values of DON for different fungal species showed that the susceptibility was dependent on the dose of DON. Some fungal strains were highly resistant even to the maximum dose we tested.

F. graminearum strain MIAE00376 chemotype DON was resistant to highest dose of DON we tested (10 µg/ml). This indicated that the producer of DON was resistant to the mycotoxin it produces. Among other fungal species all the FHB causing fungal species were susceptible to DON. The growth of *F. culmorum* was restricted at 2.7 µg/ml, *F. poae* was restricted at 0.3 µg/ml and *M. nivale* was inhibited at 0.9 µg/ml.

DON produced no impact on the growth of some antagonistic and decomposer fungal species including *F. oxysporum* strain MIAE00726, *F. oxysporum* strain MIAE00800, *T. koningiopsis* strain MIAE00725 and *E. nigrum* strain MIAE00727 were resistant to DON even to a very high amount 10 µg/ml. Some other universal antagonistic fungi of *Trichoderma* spp. i.e. *T. gamssi* strain MIAE00029 and *T. velutinum* strain MIAE00044 showed susceptibility towards DON. *T. gamssi* strain MIAE00029 was found susceptible at 2.7 µg/ml and *T. velutinum* susceptibility to DON was even lower as it was susceptible at 0.9 µg/ml. The 2 strains of *R. solani* were found to be both susceptible to 2.7 µg/ml of DON. Some fungi were isolated during decomposition of straw as putative active decomposer in the microcosm experiment (Chapter-4). Among them *Gliocladium spp.* was found highly sensitive towards DON that even the low amount 0.1 µg/ ml restricted the growth of fungus. On the other hand, other fungal species *Aspergillus niger* and *Penicillium spp.* were highly resistant to even the highest dose of DON we tested (10 µg/ml).

We also tested the susceptibility of some nitrogen fixing bacterial species (Table 1). Most of the bacterial species we tested were resistant towards DON even to the highest dose of DON used in the experiment (10 µg/ml). The fourth bacterial strain tested, *P. fluorescens* strain MIAE00587 was found sensitive to DON < 2.7 µg/ ml.

3.2-Population dynamics of *Fusarium graminearum*:

The population dynamics of *F. graminearum* in the presence of FHB-complex or the fungal decomposers with or without added nitrogen fixing bacteria and in the presence or absence of DON was monitored using qPCR in destructive independent microcosms including 2 g dw of straw inoculated with the various combinations of microorganisms (= treatments) according to the experimental set up.

The *F. graminearum* strain has developed similarly in all microcosms (Fig. 1). The number of DNA copies of strain MIAE00376 was 6.5×10^5 copies/g dw straw as an average just after the inoculation process. It increased within 2 weeks up to 1.2×10^9 copies/g of dw straw (average value) and then up to 1.43×10^9, (average value) 4 weeks post inoculation to reach 1.73×10^9 copies of DNA/g dw straw as an average value 7 weeks post inoculation. Data acquired on day 0 and those acquired on the other sampling dates were analyzed separately. Indeed, the inoculum value was supposed to be the same for all the microcosms and was not affected by any of the tested factors on day 0. However the densities of DNA copies of strain MIAEO376 appeared dissimilar among treatments just after the inoculation process at day 0 with a high relative standard deviation of 0.55. An ANOVA performed on the dataset at day 0 revealed that the dispersion of the densities of DNA was not related to the treatments what means that this dispersion was random and should not determine the subsequent putative effect of the tested factors on the population dynamics of the strain MIAE00376.

An ANOVA performed on the whole set of data acquired on week 2 , 4 and 7 post inoculation indicated that the density of DNA copies of the *Fusarium* strain significantly increased from week 2 to week 7 although this increase was not significant between week 2 and 4 and between week 4 and 7.

Important points were that in no case, neither the presence of DON nor the presence of fungi of the FHB complex had an effect of the population dynamics of strain MIAE00376. For cons, the presence of decomposers and the presence of decomposers with the nitrogen fixing bacteria significantly reduced the development of strain MIAE.

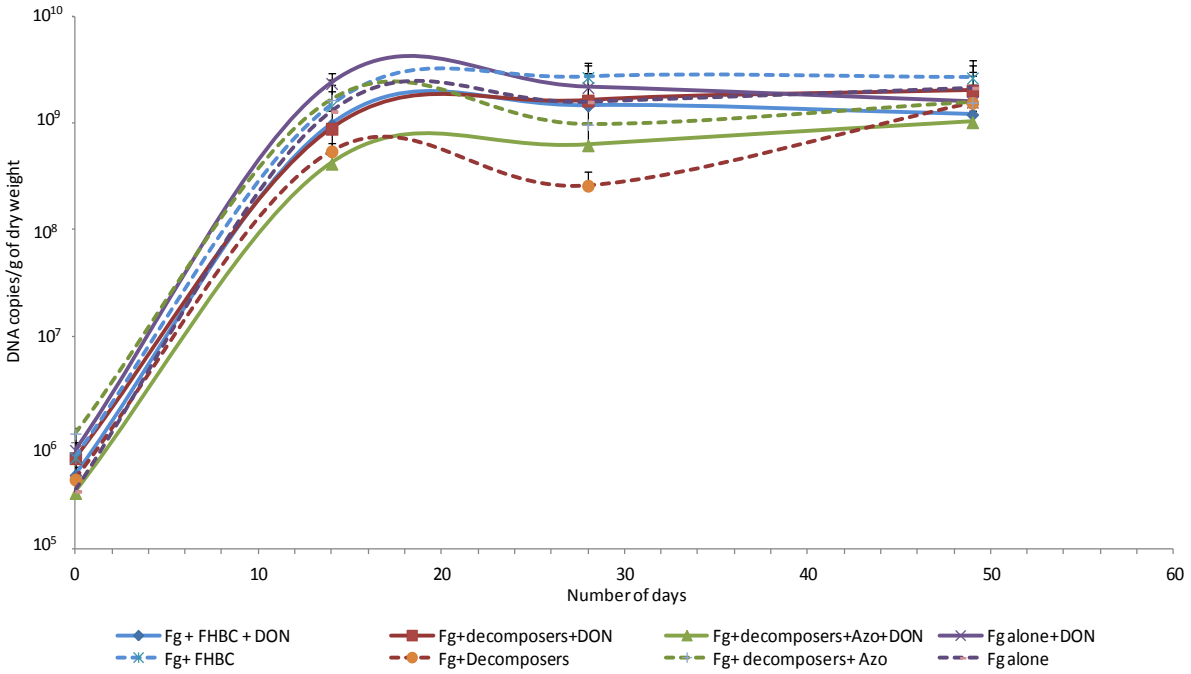


Fig. 1: Population dynamics of *F. graminearum* MIAE00376 (Fg) in straw with and without DON. The *Fusarium* strain was inoculated either alone or in combination with i) FHBC= fungi of the FHB complex (*F. poae* MIAE00300, *F. culmorum* MIAE00288 and *M. nivale* MIAE00318), ii) decomposers fungi isolated from decomposing straw (*F. oxysporum* MIAE00726 and *T. koningiopsis* MIAE00725), iii) decomposers + Azo = decomposers + a free N2 fixing bacteria (*A. lipoferum* MIAE00338). The population dynamics of *F. graminearum* was monitored using Q-PCR.

3.3- Concentration of DON:

DON was extracted and quantified in the microcosms at the initial stage (day of contamination) and at the end of the experiment (week 7) to evaluate the fate of the mycotoxins in the various treatments (Fig. 2). Theoretically, the concentration of DON introduced in the straw was 10 µg DON/g dw straw but average quantities found just after the contamination at day 0 varied from 3.8 to 4.4 µg/g in the 4 sets of microcosms in which *F. graminearum* strain was inoculated alone or in combination with other microorganisms. The relative standard deviations were really important, ranging from 0.36 to 0.78 and the concentrations were not significantly different among the 4 treatments. However, almost 60% of the inoculated DON was no more accessible for quantification soon after inoculation either

because the mycotoxin is adsorbed in the organic matrix, or because a technical error occurred. At week 7, the relative standard deviations were much more lower than at time 0, ranging from 0.1 to 0.26 but surprisingly, the concentration of DON remained significantly stable in the set of microcosms used to monitor the strain of *Fusarium* when it was in interactions with other inoculated microorganisms. At the contrary, the concentration was significantly lower in the set of microcosms where the strain MIAE00376 was alone, what is difficult to explain from a biological point of view. However, probably because of the high level of variability among the data acquired at week 0 and at a lesser extent at week 7, there was no significant difference between initial and final stage for the 4 treatments. In conclusion, only 40% of the mycotoxins introduced in the straw were quantifiable at day 0 but this proportion remained constant for the 7 weeks of incubation, i.e. the DON was not either degraded or used by the microorganisms present in the microcosms. Conversely, no DON was produced by *F. graminearum* both when the fungus was alone in the microcosms and when it was in interaction with the other fungal and bacterial species.

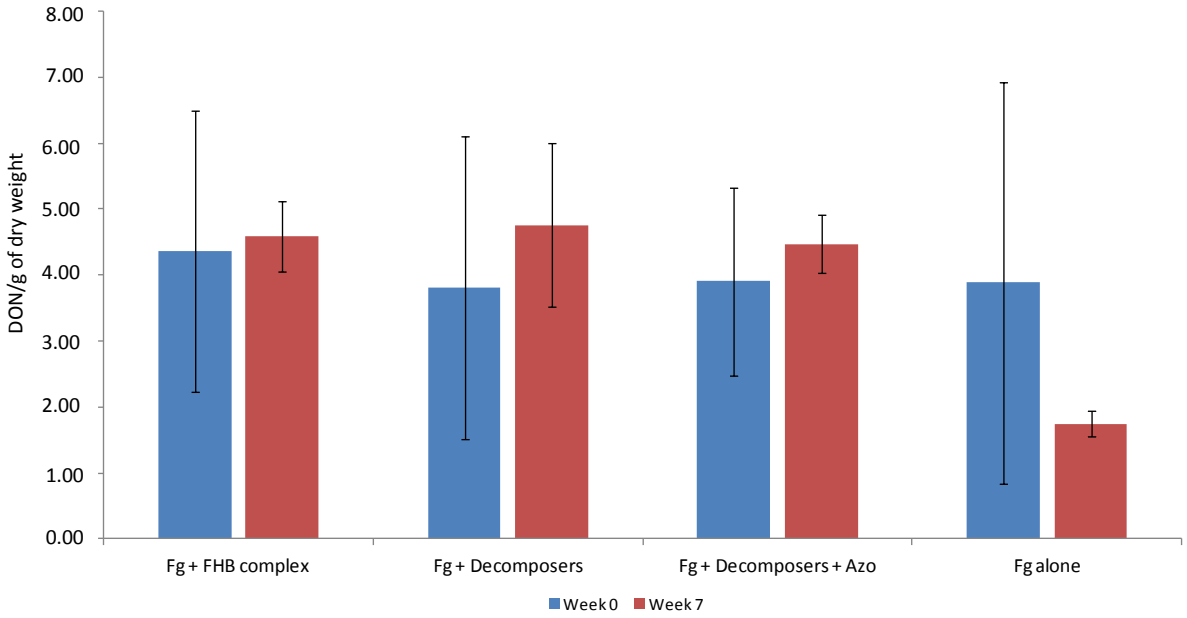


Fig. 2: Quantity of DON in the straw contaminated with DON inoculated with *Fusarium* strain MIAE00376 (Fg) was inoculated either alone or in combination with i) FHBC= fungi of the FHB complex (*F. poae* MIAE00300, *F. culmorum* MIAE00288 and *M. nivale* MIAE00318), ii) decomposers fungi isolated from decomposing straw (*F. oxysporum* MIAE00726 and *T. koningiopsis* MIAE00725), iii) decomposers + Azo = decomposers + a free N2 fixing bacteria (*A. lipoferum* MIAE00338).

4-Discussion:

Our results from MIC revealed that DON was not deleterious for its producer and the same results were confirmed from our results of the population dynamics of *F. graminearum* in microcosms. On the other hands it was not beneficial for its producer in the sense of increasing its population. Another mycotoxins, zearalenone (ZEA), produced by this fungus is reported to increase its sporulation what should promote greater dispersion and better development of the fungus, although this has not been tested directly in terms of population dynamics in the study cited (Wolf and Mirocha, 1973). In our study, at the contrary, we didn't directly test fungal sporulation nor physiologic or metabolisms changes but we can draw that DON is not used by the fungus to increase the population size during the saprophytic survival in the crop residues. These results are consistent with our field and microcosm experiment.

In addition, we observed that DON could be deleterious at various doses to other different fungal species. We tested the MIC of a limited number of strains from the FHB causing fungal species which we call FHB-complex but they were found susceptible at various extents according to the strains. These results lead to the hypothesis that DON produced by *F. graminearum* could eliminate members of the FHB-complex during the competition in the crop residues during saprophytic survival or on the plants during disease development and colonization. The competition among the FHB-complex and *F. graminearum* and the dominance of *F. graminearum* associated to an increase in the DON concentration during the fungal interaction was previously observed (Xu et al., 2007). Our results from the population dynamics and the quantification of DON negated this hypothesis. In our study, during any competition there was no significant increase in DON (Fig. 2) and the presence of artificially given dose of DON didn't give any advantage to *F. graminearum*. In order to get DON resistant decomposers for fast decomposition of crop residues to control the saprophytic survival of *F. graminearum*, strain isolated from decaying straw as well as others saprotrophic fungi and nitrogen fixing bacteria were also tested for their growth in presence of various doses of DON. MIC results revealed that among the other decomposers and antagonists we tested some of them were found susceptible to the different doses of DON. This result is consistent with the field and the microcosm studies previously conducted (chapter 3 and 4) which revealed that weak but significant modifications in the genetic structure of bacterial and fungal communities were detected when DON was present in the crop residues, according to the location of the latter. MIC results indicate that overall 47% of fungal species used in our test were susceptible towards DON depending on the concentration of DON. Deeper in the

details, the *F. oxysporum* strains which were also isolated from wheat straw or from soil were surprisingly highly resistant towards DON. Although *F. oxysporum* and *F. graminearum* are grouped in the same genus, they are quite different from a phytopathology point of view, with different specific host plants and different mode of actions towards these host plants and therefore, one may wonder why *F. oxysporum* could have built up mechanisms to resist to DON unless DON is not a mycotoxins produced to antagonize other fungi. However, the two species have a common phase in their life cycles, the saprophytic phase during which they can interact. *F. oxysporum* are generally reported to colonize the crop residues very fast and are more efficient as saprotrophs as compared to the FHB causing fungal pathogens (Pereyra et al., 2004).

Three different *Trichoderma* species which were previously isolated from wheat straw during decomposition and are known as crop residues decomposers and for their antagonistic ability as *T. gamsii* were tested (Anees et al., 2010; Matarese et al., 2012). Only one strain i.e. *Trichoderma koningiopsis* was found resistant to DON while the other two out of three *Trichoderma* spp were found sensitive towards the different doses of DON. Contrary to what was observed with *F. oxysporum* which competes with *F. graminearum* for the exploitation of common trophic resources, DON could be used by *F. graminearum* for defense against fungal species having antagonistic abilities. Indeed, DON can down regulate *T. atroviride* strain P1 chitinase genes (Lutz et al., 2003). In contrast to that a recently published study Matarese et al., (2012) suggested that *Trichoderma* spp. could be used as biocontrol agent and can even down regulate the production of DON and the growth of *F. graminearum* but it was substrate dependent.

Having in mind that nitrogen could be a limiting factor to decompose crop residues which are mainly carbon based, it was assume that this element could be input in the decomposition process via the activity of free nitrogen fixing bacteria, although this bacteria are more frequently found in the rhizosphere than in litters but they are generally associated to the rhizosphere of cereals, hence the idea of testing their susceptibility to DON (Sanguin et al., 2009; Venieraki et al., 2011). Only one out of four species were affected by DON. Overall the results from MIC values revealed that DON seems to be very selective in its targets and very selective towards different soil communities.

The MIC was an in vitro test which anyway allowed sorting out some DON resistant decomposers and nitrogen fixing bacteria which could help to destroy the crop residues which are considered the main cause of fungal primary inoculum. We used the decomposers which included *T. koningiopsis* strain MIAE00725 and *Fusarium oxysporum* strain MIAE00726.

Trichoderma spp. are also well known antagonists and as well as soil-borne fungi and are aggressive colonizer of maize and wheat crop residues and take an active part in the decomposition process (Broder and Wagner, 1988).

The test was scaled up small microcosms to include both i) crop residues, i.e. wheat straw, ii) interacting microorganisms i.e. decomposers in one case and fungi of the FHB complex in the other case, and iii) the monitoring over time of the impact of these microbial interactions on the *F. graminearum* strain on a specific substrate in presence or absence of DON.

Interesting results were obtained with this experimental set up and in a way, they confirmed the hypothesis we had about the possible control of *F. graminearum* by fastening the decomposition of their habitat i.e. the wheat crop residues but we need first to be cautious with the discussion of the results we got for various reasons. The first one is that only 40 % of the DON was accessible for quantification immediately after its inoculation in the crop residues. This could be due to an experimental mistake we did in preparing the solution. In previous experiments (chapter 3 and 4) less quantities of DON than the quantities added to maize stubbles or to wheat straw-soil mixtures were recovered but in that case, besides technical aspects, both biotic and abiotic factors were incriminated. In the present study, as experiments were conducted in sterile conditions, we can exclude that indigenous microorganisms degraded the mycotoxins. Therefore, the remaining alternative explanations could be linked to the difficulties faced to extract the DON from the straw, or to recover it from the solvent with the affinity columns used for that purposed despite previous tests were performed to specifically choose these affinity columns. At last, it is also quite possible that DON is rapidly physically adsorbed by the straw itself and could be released later, once the straw is decomposed.

The second reason we have to consider is that the MIC were evaluated with known increasing doses of DON from 0 up to 10µg/L incorporated in agar medium but we didn't check for the availability of the DON, therefore the same phenomena could have occurred as well. We have been anyway able to rank the strains for susceptibility to DON in the agar medium and to select the more resistant ones, what was, in some cases, confirmed by test performed in liquid medium. Therefore we are confident with the ranking and with the selection we did. However, it is now difficult to compare the doses used for the selection of resistant strain and the active doses of DON available in the microcosms. The active dose of DON in the microcosms was less than the one used for the MIC tests and this could explain why, no effect of DON was observed on the population dynamics of the *F. graminearum* strain MIAE 00376 when the strain was either alone or interacting with other microorganisms.

At last, the microcosms where a miniature system and the incubation lasted for only 7 weeks, therefore we noticed that the growth i.e. increase in the number of DNA copies of the *F. graminearum* strain was reduced in presence of a few strains representing decomposers but no significant decrease was observed.

Anyway, having in mind the above statements, one interesting point issuing from this experiment is that neither the presence of DON nor the presence of fungi of the FHB complex had an effect of the population dynamics of *F. graminearum* strain MIAE00376. So the mycotoxins produced by a member of this fungal complex do not provide any saprotrophic competitive advantage to the producer. This does not preclude the fact that the advantage can be found by the mycotoxins producer during the infectious phase of its lifecycle but our results do not help solving the question about the reasons why mycotoxins are produced.

The main and promising point that this experiment provided is that the presence of decomposers and the presence of decomposers with the nitrogen fixing bacteria significantly reduced the development of the *F. graminearum* strain MIAE. The decomposers can't be restricted to the only 2 or 3 fungal strains we used. The soil fauna and many other microorganisms may contribute to the process but we hereby underlined a weakness in the lifecycle of *F. graminearum* which should be exploited to improve the control of the pathogenic fungus during its saprophytic phase. This could be achieved either by stimulating indigenous decomposers, but this is not always easy to do unless a global and systemic way of managing the agricultural plots takes into account this parameter, or it can be achieved by means of inoculating a consortium of decomposers including enzymes-well equipped decomposers fungi and nitrogen fixing bacteria, onto the straw getting out of the combine harvester for cereals. Once again, the second alternative should be included in the frame of an overall reasoning of rotation, including intermediate crops and cropping practices.

Of course, this proposal cannot yet be applied and further experiments should be conducted to validate it but it shows that the management of the biotic diversity and biotic potential of the soil may help to suggest ecological ways to control pathogens.

5-Conclusion:

The main conclusion from this study is shown in the form of flow chart diagram (Fig. 3) From MIC tests we conclude that DON produced negative impact on different strains of fungi as well as bacteria and was dependent on the concentration of DON. From the microcosm study of population dynamics of *F. graminearum*, we conclude that DON gave no advantage to *F.*

graminearum when alone in the sterile straw or in the interaction with decomposers or decomposer+ N_2 fixing bacteria or FHB complex. *F. graminearum* growth was reduced by decomposers and decomposers + N_2 fixing bacteria. The quantification of DON revealed that DON was not increased or decreased in the sterile straw when *F. graminearum* was alone or in competition with other fungal and bacterial strains.

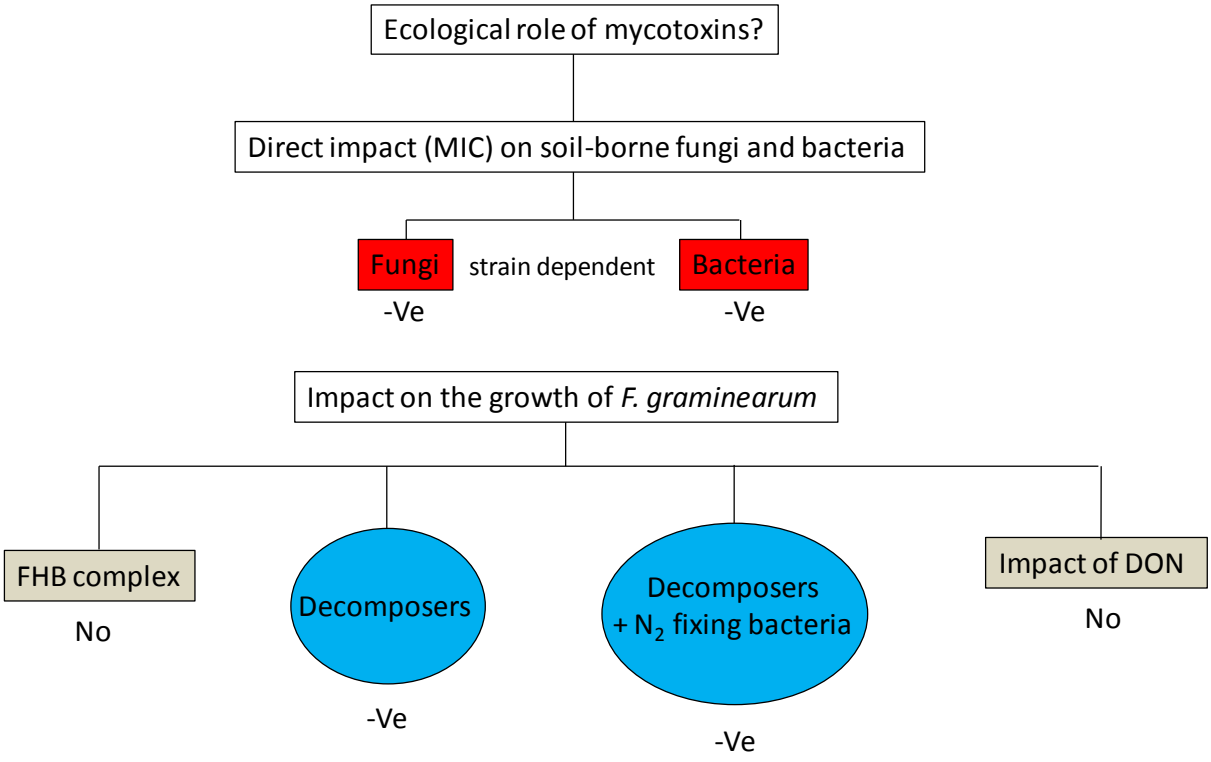


Fig 3: Flow chart showing the main conclusion obtained during MIC and microcosm experiment. The red colour rectangles show the impact of DON in the MIC assay. The grey colour rectangles show no impact of DON and FHB complex on the *F. graminearum* growth. The blue circles show the negative impact of decomposers on the *F. graminearum* growth. Furthermore, No= no impact; -Ve = negative impact.

General Discussion

General Discussion

Fusarium graminearum (teleomorph *Gibberella zeae*) is a major plant pathogenic fungus and is main responsible for Fusarium head blight (FHB) of cereals including wheat and maize causing enormous losses to the world (Nganje et al., 2004). *F. graminearum* is very cosmopolitan, occurring on all continents, being often associated with the cultivation of cereals but still in very different soil and climatic conditions. It is thus possible for this fungus to achieve the pathogenic and saprophytic phases of its life cycle, ensuring its sexual reproduction in developping in crop residues (Leplat et al., 2012; Parry et al., 2007). Moreover, *F. graminearum*, as other *Fusarium* sp. involved in the FHB complex, produces mycotoxins whose character deleterious to human and animal consumers has been shown and discussed in the literature review presented in Chapter 1. *F. graminearum* can produce various mycotoxins although deoxynivalenol (DON) appeared as the main one. (Fokunang et al., 2006; Pestka, 2010; Placinta et al., 1999; Sobrova et al., 2010; Wild and Gong, 2010). However, the literature review revealed that chemotypes could be different from one place to another at a very broad scale. By the way, most of the studies focused on the role of these mycotoxins in the fungus-host plant interactions but less is known about their role in the saprophyte phase of the *F. graminearum* life cycle, neither in the saprophytic phase of all the mycotoxigenic *Fusarium sp.* involved in the FHB complex.

It was therefore tempting to assess whether the mycotoxins produced by mycotoxigenic fungi could be used by these fungi as a strategic tool to enable them to adapt to different biotic conditions encountered on different continents and major regions where different soil, climatic and anthropogenic conditions determine a culture of cereals which the life-cycle of the fungus may be associated. Our goal was not to focus on the co-evolution between cereals and mycotoxigenic fungus, although this should be quite interesting. It was rather to identify a putative ecological interest for the fungus to produce mycotoxins whose energetic cost for the producer is important and can only be compensated by the acquisition of spatial or trophic resources scarce but allowing it to ensure its cycle.

It is in this context that our study is taking part using *F. graminearum*, DON producer and wheat pathogen as a biological model. One of the possible applied aim of this project was to be able to offer, taking into account the presence of mycotoxins, a way to control the development of the pathogen during its saprophytic stage which recently published literature review showed that it had weakness points (Leplat et al 2012).

In the first part of the thesis (**Chapter 1**), the review of literature showed the thirst of literature and research about the role of mycotoxins towards human health and subsequently towards economic cost for cereal growers. The frequency and the development of mycotoxins is great environmental challenge. The physico-chemical properties of DON shows that it is a highly stable molecule and even at high temperature (Kushiro, 2008; Trigo-Stockli, 2002). The aim of the production of mycotoxin production in the fungal life is not clear and needs further investigation as they are not required in the production of the disease (Mesterházy, 2002). Most of the studies showed that the most critical stage of the fungal life is its saprophytic survival in the crop residues where it can survive for several years (Leplat et al., 2012; Parry et al., 2007). The mechanism of survival of *F. graminearum* in these wheat residues is not fully understood. The current PhD study was conducted mainly to find the impact of mycotoxins on soil biotic components, the fate of mycotoxins in the crop residues and the importance of mycotoxins in the life of *F. graminearum* to gain a competitive advantage during its saprophytic phase.

In the **Chapter 3** we focused on the survival of *F. graminearum* in maize crop residues with or without DON in the field in the tillage and no tillage system and the role of deoxynivalenol (DON) in the saprophytic survival and development of primary inoculum of *F. graminearum* in the maize crop residues in the inoculated and non inoculated field in the tillage and no tillage system. The negative experience we got from previous experiments led us to combine surface and localized inoculations procedures. We used inoculated maize residues as a natural agronomical situation and we used some nylon gaze bags filled with maize residues inoculated or not with *F. graminearum* and contaminated or not with DON to observe the survival and the development of the *F. graminearum*. More exploitable data were acquired with the nylon bags where the biotic interactions were probably concentrated while they were probably diluted in dispersed crop residues. We observed the more disease in case of no tillage as compared to soil tillage where the crop residues were buried in the soil. We also measured the concentration of DON in the field experiment and found that with the passage of time DON started disappearing in the maize residues and remained in very low amount at the end of 24 weeks. The molecular quantification of *F. graminearum* by Q-PCR indicated that this fungus got no advantage of the DON in the maize stubbles during the development of primary inoculum and the inoculum decreased with the decomposition of the maize stubbles. The molecular biomass of the *F. graminearum* was not significantly higher in the no tillage as compared to the tillage system and this is in line with the previous studies (Dill-Macky and Jones, 2000). The molecular quantification represented that DON produced no impact on

overall fungal and bacterial densities in the maize crop residues. The community structure of fungi, bacteria, protozoa and nematodes were determined. DON produced significant impact on the protozoan community structure while the fungal, bacterial and nematodes community structure remained unaffected. Unfortunately too few data are available in the literature about the soil-borne protozoa so it was not really possible to propose an ecological interpretation of the changes we observed. This taxon appears to be forgotten by most soil microbial ecologists. It is a bit frustrating because they obviously play a role in the functioning of arable soil as they do in rhizospheric soils (Bonkowski, 2004; Bonkowski et al., 2009) and forest litter (Lenoir et al., 2007). The results demonstrated that the community structure of fungi, bacteria and nematodes were significantly different in tillage and no tillage systems. The soil inoculum produced no impact on any of the communities in terms of their molecular biomass as well as on the structure of the communities and the production of the disease. The fungal as well as the bacterial community structure was clearly changed by the *F. graminearum* presence in the crop residues. In the case of the fungal structure, it was unclear whether the change was due to real changes in abundance ratios between populations initially present before the introduction or if they are simply the *F. graminearum* TRF which are responsible for the observed changes. By cons, in the case of bacteria, there is indeed a change in the structure of their community which is induced by the presence of the fungus in inoculated residues. This shows some interaction between the *F. graminearum* and the microbial communities. This was also found in case of nematodes and protozoa but it appeared later in the experiment.

We moved from field experiment where external factors and investigated mechanisms were diluted by the scale of approach to microcosm experiment where controlled conditions were expected to emphasize the phenomena we suspected in field but were unable to clearly demonstrate. In the **Chapter 4**, we studied the fate of DON in the wheat crop residues in the soil in the presence of the whole soil biota including, fungi, bacteria, protozoa, nematodes and earthworms in the controlled conditions in the microcosms. The crop residues are the natural residence of pathogens as well as the residence of soil-borne eukaryotes and prokaryotes which are important members of the soil food web and take a great part in the liberation of nutrients by the decomposition of the organic matter (Clarholm, 1985; Saetre, 1998; Schaefer et al., 2009). Our results about the investigation of the fate of DON illustrated that DON disappeared from the wheat straw with the passage of time. These results were in the line with our field experiment conducted on the maize crop residues (**Chapter 3**). In addition, we found the disappearance of DON was considerably accelerated when straw was incorporated in the

soil rather placed at the surface the soil. Earthworms played also a significant role in the disappearance of DON. Our results from the quantification of DON in the crop residues in the field as well as in the microcosms illustrated that *F. graminearum* doesn't produce DON in the crop residues during the saprophytic survival in crop residues as they were never increased during its presence in these residues. We investigated the impact of DON on the community profile and the molecular biomass measurements of the fungi, bacteria, protozoa and nematodes illustrate that the presence of DON in the crop residues in the soil produces a significant impact on them depending on the community and the location of the straw. DON significantly changed the structure of fungal communities affecting the growth of some fungal species but not all. It may also reduce the quantity of overall fungal densities. The bacterial communities as well as densities were also affected by the presence of DON. Protozoan community structure again was affected by DON. The influence of DON was not clear on the nematodes as the structure of nematodes was not affected but DON seems to have an impact on the nematodes densities. DON doesn't cause acute poisoning but may be slow poisoning. Unexpectedly, the straw contaminated with DON was more attractive to the earthworms. Actually DON has a rather positive effect on earthworms. It was not clearly demonstrated in our experimental set but it is a global interpretation we are able to provide based on the combination of observations we did, the main ones being the fast incorporation of DON contaminated straw and the birth of new juveniles in the microcosms. A MIC looking like test, close to the one we used for fungi and bacteria should be set up and used to evaluate if indeed, DON is of real interest for earthworms. We evoked the fact that earthworms could host it in their gut a specific microflora which degradation activity of mycotoxins could provide by-products of interest for earthworms metabolism. This hypothesis needs to be tested.

The results from the molecular biomass measurement revealed that the presence of DON gave no advantage to *F. graminearum* for the growth and development of primary inoculum. Moreover, DON was found to produce no negative impact on *F. graminearum* growth. In all the results of **Chapter 3** and **Chapter 4** showed that *F. graminearum* biomass was significantly reduced with the decomposition of the crop residues. In the last **Chapter 5** we tested different doses of DON on the fungal and bacterial strains and the MIC test provided promising results that DON was deleterious towards the different fungal species including especially the FHB causing fungal strains and also antagonistic fungi. Overall, 46 % fungal species were found susceptible to DON. One out of four bacterial strains was also found susceptible to DON. The impact on the fungi and bacteria was dependent on the dose of

DON. These results were consistent with results of **Chapter 4** that revealed that DON can have differential impacts on fungal species. However, MIC showed the potential susceptibility of microbial strains to mycotoxins but the effect of DON fades when the tests are carried out in soil or crop residues. Indeed, the population dynamics of *F. graminearum* in the presence of decomposers and FHB producing fungi was determined up to 7 weeks. We observed the dynamics of *F. graminearum* in the straw in the presence of decomposers and a nitrogen fixing bacteria to control the fungus by increasing the decomposition their ecological habitat. Our results suggested that decomposers significantly reduced the inoculums of *F. graminearum* even in the presence of DON and the presence of DON gave no advantage to the *F. graminearum* population to increase the primary inoculum. The amount of DON was never increased or decreased during any competition with the decomposers or the FHB complex as well as alone in the microcosms during the seven weeks experiment. This gives evidence that DON was not used by *F. graminearum* against other organism.

Hence throughout the thesis we observed that *F. graminearum* gets no advantage of the presence of DON in the crop residues and in the soil environment. The presence of DON in the crop residues in the soil produces a significant impact on the soil inhibiting communities that may be positive or negative depending on the communities. The right selection of decomposers and rapid destruction of crop residues may be the right solution for the reduction of the disease.

Conclusions and Perspectives

General conclusions:

Our results illustrated that the presence of DON in the crop residues gives no benefit to *F. graminearum* to grow. We observed the development of *F. graminearum* in two kinds of crop residues including maize stubbles and wheat straw in the field as well as in microcosms in the soil. They were confirmed in straw alone without soil in competition with decomposers and FHB producing fungal complex as well as alone but all results revealed that the presence of DON gives no advantage in the development of *F. graminearum* primary inoculum.

We followed the fate of DON in the both kinds of crop residues including maize stubbles and wheat straw in the field and in the microcosms and our results revealed that DON disappears from the crop residues with the decomposition of the crop residues with the passage of time. The rate of disappearance of DON was dependent on the location of the crop residues and the soil communities. Earthworms were clearly associated with the disappearance of DON from the crop residues and the soil but the mechanisms and the possible attractive role of mycotoxins towards earthworm need to be further investigated. We found no evidence of the increase of DON in the crop residues during the saprophytic survival of *F. graminearum* in the crop residues.

Our results from community profiling and the molecular biomass measurement of the fungi, bacteria, protozoa and nematodes illustrate that the presence of DON in the crop residues in the soil produces the significant impact on them but different communities behaved differently against DON. DON significantly changed the structure of fungal communities in the absence of earthworms in the microcosm. The affect on the fungal communities was deleterious which was confirmed by the molecular density and minimal inhibitory concentration test. The location of the crop residues in the soil and the quantity of DON played a significant role on the growth of other fungal species. The bacterial communities as well as densities were also affected by the presence of DON. The minimal inhibitory test and the molecular density measurement in the microcosm experiment and the location showed a significant role of the DON impact on bacteria. Protozoan community structure was strongly affected by the DON presence in the crop residues. The influence of DON was not clear on the nematodes as the structure of their community was not affected but DON seems to have an impact on the nematodes densities which appeared later in the experiment in the microcosm. Our results about impact of DON on earthworms and their role in crop residues degradation demonstrate that 1) DON produced no negative impact on the earthworm biomass in the soil and 2) did not alter their important and already known role in the incorporation of

crop residues. In addition, the straw contaminated with DON was more attractive to the earthworms. The presence of DON also stimulated the reproduction or cocoon hatching in the earthworms depending on the location of the crop residues.

In the field experiment our results revealed that no tillage caused higher Fusarium head blight disease (FHB) and lower production than the deep tillage even in the season of high disease incidence. However, these results should not lead to the conclusion that tillage is the only solution to control *F. graminearum* and FHB. Less disease in tilled plot does not mean no disease at all. Mouldboard tillage provided an immediate positive result which is what the growers expect. However, this can also be seen as a way to postpone the resolution of the problem. In our experimentation all the results confirmed that *F. graminearum* biomass was significantly reduced with the decomposition of the crop residues. In the microcosm experiment based on wheat crop residues, we saw that some decomposers and a nitrogen fixing bacteria can reduce the growth the *F. graminearum* population during its saprophytic survival in these crop residues. These decomposers were selected on a very weak basis. For sure, this was not a clear and strong demonstration but this experiment underlined the possibility to use indigenous or/and inoculated selected consortia of microorganisms to control the pathogenic fungus during its saprophytic phase.

This was one of the applied objectives of this work and we can now provide a new track of investigation leading to a sustainable solution. Indeed, the tillage system is part of Integrated Pest Management (IPM) and its use should be thought in combination with rotation schemes, use of intermediate crops and management of the biodiversity within and around the plots.

However, the primary question of this work was about the ecological role of DON, used as a study model for mycotoxins, toward the soil microflora and soil fauna. Actually three issues are hidden behind this question (Fig-1).

The first one is "Does DON affect the biotic component of soil and crop residues?

and the answer is **Yes**!

The second one is Does DON provide a competitive advantage to the DON producer?

and the answer is **NO!**

F. graminearum gets no advantage of the presence of DON either towards bacteria and fungi, nor towards nematodes and protozoa, and or even towards fungi of the FHB complex. At the contrary, it seems that the fungus gets a handicap towards earthworms and the later should be considered in the IPM strategy.

The third one is "What are the conditions to make the benefits of mycotoxic secondary product outweigh the metabolic costs involved?

and the answer is **STILL PENDING!**

What means that this work opened track for more investigations in Ecology, both on the applied aspect and on the more conceptual aspect.

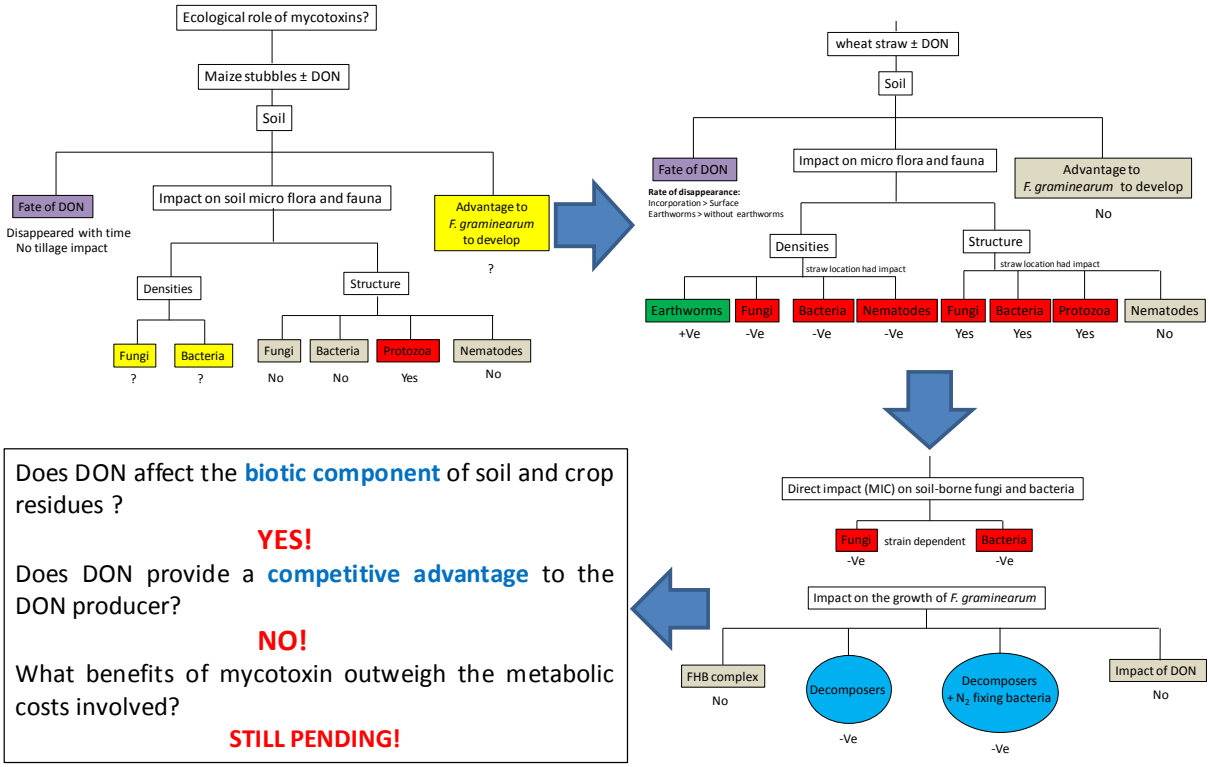


Fig-1: The flow chart showing the combination conclusions of three chapters (Chapter 3, 4, 5) to drive the main conclusion to respond the three main questions.

Future prospects:

This thesis work responded to the different questions and in the mean while opened new windows in terms of research perspectives. They can be ranked on short term and long term perspectives.

1 -Applied research could be the frame to propose a strategy to control *F. graminearum* using IPM strategies. In addition to the descriptive approach we used in the present work, the selection of efficient decomposers based on their enzymatic machinery and enzymatic potential could be used as a mean to investigate the mechanisms driving the assemblages of successive functional microbial groups (including bacteria, fungi and protozoa) during the decomposition process of wheat straw. The study could be later on extended to the FHB complex on one hand to explain the relationship between the various species composing this complex, in which way the external factors may determine its composition and the issue of competing strains during the saprophyte phase as well as at the time of interaction with a given host plant. On the other hand, the study could be also extended to various crop residues.

This proposal may include different topics which were raised during the present work:

-It might be interesting to find the competition among the different chemotypes in the presence or absence of different mycotoxins produced by them and the advantage for the one chemotype.

- DON was selective toward the different fungal strains. It can be important to see what kind of mechanism exists between the fungal strains and the mycotoxin produced by the fungus.

- The field experiment was performed for one year on a given plot (2010-11 with wheat as preceding crop, 2011-2012 with maize as preceding crop but the 2 plots were adjacent). Therefore the tillage system was shortly evaluated what could lead to biaised conclusion such as the shallow tillage promotes FHB disease. Actually, soil microflora and soil fauna established on the crop residues. Studying the succession of these organisms for a several years duration could be a way to evaluate the establishment of a specific functional community which might give rise to either a suppressive or a conducive system towards soil- and residues-borne plant pathogenic fungi.

- DON was attractive for the earthworms which were in turn quite efficient in incorporating the wheat straw, hence their role in an IPM strategy. Therefore, it might be interesting to check if DON has any analogy in their chemical structure with earthworm hormones or volatile organic compounds as already observed in the use of flower-insect

interactions. This investigation could be enlarged to other earthworms genera as well as to various mycotoxins. Another explanation for this DON attractively toward earthworms could be favoured in the gut of the earthworm. Indeed, we can assume that the gut microflora provides by-product of metabolic interest for the earthworm when the endophytic bacteria (if any) degrade DON in the earthworm gut. One point could be the role of this putative by-product on the reproduction of earthworms. Indeed, DON seemed to stimulate the reproduction or cocoon hatching of the earthworms so it might be interesting to see the long term effect of DON and other mycotoxins on the earthworms.

2 – Academic research. We still don't know the ins and outs of mycotoxin production by *F. graminearum* and other mycotoxigenic fungi. It seems that DON at least does not give advantage to the fungus during its sasprophytic phase. Many studies are presently dealing with the DON produced during the fungus-plant interaction but it is still unclear whether the mycotoxins are produced on purpose by the fungus to inhibit putative competitors, to weaken the host plant or as a fungal defense reaction towards the plant defense reactions.

From an ecological and theroretical point of view, a poisoning advantage relies on local competitive interactions where the benefits of poisoning accrue to the toxin producer preferentially. Therefore, it would be exciting to check if such theory could be applied to the mycotoxins producer *F. graminearum*.

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Annexes

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REVIEW ARTICLE

Survival of *Fusarium graminearum*, the causal agent of Fusarium head blight. A review

Johann Leplat · Hanna Friberg · Muhammad Abid · Christian Steinberg

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Abstract Wheat is one of the most cultivated crops worldwide. In 2010, 20 % of wheat and durum wheat were cultivated in Europe, 17 % in China and 9 % in Russia and in North America. Wheat yield can be highly decreased by several factors. In particular *Fusarium graminearum* Schwabe is a worldwide fungal pest impacting wheat production. *F. graminearum* is the causal agent of Fusarium head blight, root and stem-base rot of cereals. Losses caused by Fusarium head blight in Northern and Central America from 1998 to 2002 reached $2.7 billion. Moreover, *F. graminearum* produces mycotoxins which affect human and animal health. The threshold of these mycotoxins in foodstuffs is regulated in Europe since 2007. *F. graminearum* survives for several years saprotrophically in the soil, on dead organic matter, particularly on crop residues. *F. graminearum* adapts to a wide range of environmental variations, and produces extracellular enzymes allowing feeding on different crop residues. However, *F. graminearum* competes with other decomposers such as other *Fusarium* spp. belonging to the same complex of species. Actually, it is not known whether *F. graminearum* mycotoxins give *F. graminearum* a competitive advantage during the saprotrophic period. Anthropogenic factors including preceding crops, tillage system and weed management can alter the development of the soil biota, which in turn can change the saprotrophic development of *F. graminearum* and disease risk. We review the ecological requirements of *F. graminearum* saprotrophic persistence. The major conclusions are: (1) temperature, water, light and O_2 are key conditions for *F. graminearum* growth and the development of its sexual reproduction structures on crop residues, although the fungus can resist for a long time under extreme conditions. (2) *F. graminearum* survival is enhanced by high quantities of available crop residues and by rich residues, while sexual reproduction structures occur on poor residues. (3) *F. graminearum* is a poor competitor over time for residues decomposition. *F. graminearum* survival can be controlled by the enhancement of the decomposition processes by other organisms. In addition, the development of *F. graminearum* on crop residues can be limited by antagonistic fungi and soil animals growing at the expense of *F. graminearum*-infested residues. (4) Agricultural practices are key factors for the control of *F. graminearum* survival. A suitable crop rotation and an inversive tillage can limit the risk of Fusarium head blight development.

Keywords Crop residues · Ecological requirements · Habitat · Mycotoxins · Preceding crop · Saprotrophic development · Soil microbial ecology · Tillage · Wheat diseases

J. Leplat · M. Abid · C. Steinberg (✉)
INRA, UMR1347 Agroécologie,
Interactions Plante–Microorganismes (IPM),
21065 Dijon, France
e-mail: christian.steinberg@dijon.inra.fr

J. Leplat
e-mail: johann.leplat@dijon.inra.fr

M. Abid
e-mail: muhammad.abid@dijon.inra.fr

J. Leplat · M. Abid · C. Steinberg
Université de Bourgogne, UMR1347 Agroécologie,
Interactions Plante–Microorganismes (IPM),
21065 Dijon, France

J. Leplat · M. Abid · C. Steinberg
Agrosup, UMR1347 Agroécologie,
Interactions Plante–Microorganismes (IPM),
21065 Dijon, France

H. Friberg
Swedish University of Agricultural Sciences (SLU),
Department of Forest Mycology and Pathology,
Box 7026, 750 07 Uppsala, Sweden
e-mail: hanna.friberg@slu.se

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1 Introduction

Wheat (*Triticum aestivum* L. ssp. *aestivum*) is the second most cultivated crop in the world after maize (*Zea mays* L.). In 2010, 653 million tons of wheat and durum wheat were produced in the world, of which 140.7 million tons were produced in Europe (FAO 2011). Moreover, the wheat is one of the most traded crops worldwide, with 125.9 million tons traded in 2010 (AGPB 2012). The cultural practices trends due to economical and environmental reasons, i.e., reduction of soil tillage and pesticides use, raise the issue of re-emerging wheat diseases, such as fungal diseases (McMullen et al. 1997; Millennium Ecosystem Assessment 2005).

Studies of plant pathogenic fungi generally focus on infection processes, disease development and other concerns in plant–microorganism interactions, but the saprotrophic period of these pathogens' life cycle is not well known. Most soil fungi are decomposers or saprotrophs that feed on decaying organic material. In fact, they play a key role in the decomposition of organic polymers that takes place in the soil. Fungi are considered primary decomposers in forests, where litter contains high concentrations of complex polymers. Fungi have a unique role in the degradation of plant-derived woody substrates containing lignocellulose, i.e., cellulose complexed with lignin (Finlay 2007; Sinsabaugh 2005). They also play an important role in arable soils by breaking down and recycling plant residues, primarily cellulose and hemicellulose (Stromberg 2005). Among them, some plant pathogenic fungi take place and their role should be considered. Indeed, plant pathogenic fungi are categorised as either biotrophs or necrotrophs, and as either obligate pathogens or facultative saprotrophs. For example, the disease cycle of the deleterious fungus *Fusarium graminearum* (Fig. 1), the anamorph stage of *Gibberella zeae* (Schwein.) Petch is well studied (Trail 2009). In a previous review, Goswami and Kistler (2004) provided an update on the pathogenesis, genetics, evolution and genomics of *F. graminearum* but the ecological requirements of its saprotrophic stage are less well understood.

Fusarium head blight, root rot and foot rot (crown rot) are diseases that cause significant yield loss in several crops worldwide such as wheat (Fig. 2), maize, oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and rice (Oryza sativa L.) (Parry et al. 1995; Pereyra and Dill-Macky 2008; Trail et al. 2003). Yield losses caused by Fusarium head blight in Northern and Central America from 1998 to 2002 were evaluated to reach $2.7 billion (Nganje et al. 2002). Several species are involved in the fungal complex that causes these diseases. Many of them also produce mycotoxins, such as deoxynivalenol (commonly known as DON) and its acetylated forms 3-acetyl-4-deoxynivalenol (3-ADON) and 15-acetyl-4-deoxynivalenol (15-ADON), nivalenol (NIV) and zearalenone (ZEA) (Desjardins and Proctor 2007). These mycotoxins are of major concern because of their effect on human and animal health and because they persist during storage and are heat-resistant (JEFCA 2001). The threshold of these mycotoxins in foodstuffs is regulated in Europe since 2007 (CE N°1881/2006). Among the species involved in the complex causing Fusarium disease on wheat, *F. graminearum* predominates in many parts of the world (Bottalico 1998; Bottalico and Perrone 2002; Parry et al. 1995).

Like other *Fusarium* species in the complex, *F. graminearum* survives saprotrophically on crop residues in the absence of its hosts (Sutton 1982). Fusarium head blight severity and deoxynivalenol contamination significantly increase with the density of residues left from the preceding crop (Blandino et al. 2010). Moreover, surface residues provide a substrate for active growth of *F. graminearum* for a longer period of time than buried residues (Pereyra et al. 2004). Burying *F. graminearum*-infested crop residues deeper in the soil can efficiently reduce *F. graminearum* populations; however, the pathogen may survive for several years. During the decomposition process, the chemical composition and the availability of the plant material changes as some resources are used up while others are made available for saprotrophic growth. To survive over time, *F. graminearum* has to be able to use available resources and to compete with the different organisms that are invading the material, each of them being specific for each of the decomposition

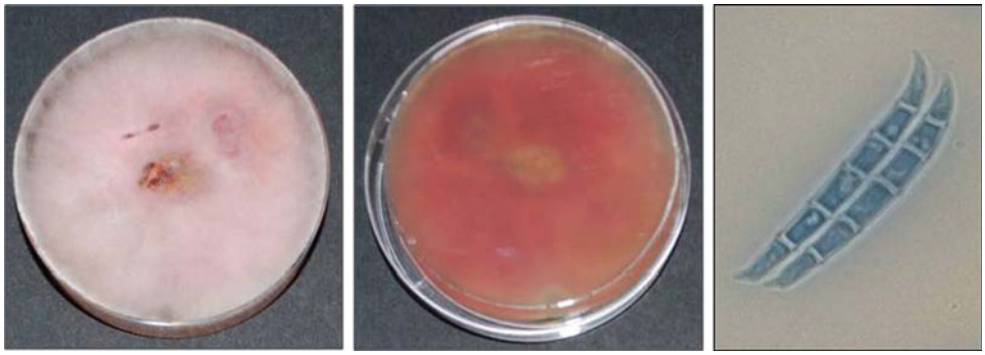

Fig. 1 Macroscopic and microscopic pictures of *Fusarium graminearum*, the causal agent of Fusarium head blight (photograph: courtesy of J. Leplat). Macroscopic pictures were taken after growth on potato dextrose agar. The undersurface shows the typical carmine red color of *F. graminearum* species. The microscopic picture shows macroconidia with the typical spindle shape which gives its name to the *Fusarium* genus. The cylindrical shape of the macroconidia, i.e., dorsal and ventral surfaces parallel, and the foot shape of the basal cell are typical of *F. graminearum* species

stages. To develop control strategies of *F. graminearum* primary inoculum, a better understanding of the complex interactions that determine its ability to grow and compete for crop residues is needed.

This review focuses on the saprotrophic phase of the life cycle of *F. graminearum*. Discussion includes several topics: (1) how environmental factors affect its saprotrophic survival; (2) in which ways crop residues provide a habitat for it and impact on its survival depending on their quantity and on plant species they come from; (3) whether crop residue colonization by it is a matter of competition and antagonism; and (4) what is the incidence of agricultural practices on its survival.

Fig. 2 Wheat ear infested by *Fusarium graminearum* (photograph: courtesy of J. Leplat). The ear shriveling suggests a low grains filling inferring yield losses. The orange spikelet is due to *F. graminearum* growth suggesting mycotoxins production

2 *Fusarium graminearum*

2.1 Fusarium diseases on wheat

On wheat, *Fusarium* fungi cause several distinct diseases (Colbach et al. 1996; Kohl et al. 2007). First, seedling diseases, which cause damping-off, seedling blight, and foot rot. In Europe and North America, these symptoms are mainly due to *Microdochium nivale* (Fr.) Samuels & I.C. Hallet, but *F. culmorum* (Wm.G. Sm.) Sacc., *F. graminearum* and *F. pseudograminearum* O'Donnell & T. Aoki are also frequently associated depending on the geographical conditions and climatic conditions (Bateman 1993; Smiley et al. 2005). Second, Fusarium head blight, which is the mature plant disease caused by a complex of species. The *Fusarium* species predominantly found in Europe are *F. graminearum*, *F. avenaceum* (Fr.) Sacc. and *F. culmorum* (Bottalico 1998; Bottalico and Perrone 2002; Nielsen et al. 2011a, b). A survey conducted in France between 2000 and 2002 showed that, in addition to *F. graminearum*, *F. avenaceum* and *F. poae* (Peck) Wollenw. were also found regularly, whereas *M. nivale* and *F. culmorum* were less frequent than previously recorded. Other species, such as *F. tricinctum* (Corda) Sacc., *F. sambucinum* Fuckel, *F. equiseti* (Corda) Sacc., *F. acuminatum* Ellis & Everh. and *F. sporotrichioide*s Sherb., were found in lower quantities (Ioos et al. 2004). The composition, the development and the structure of the *Fusarium* community depend on a combination of factors, among which climate plays a major role (Muller et al. 2010). *F. graminearum*, together with several other encountered species, can produce toxigenic compounds (Bottalico 1998). Like the other *Fusarium* species associated with Fusarium head blight, *F. graminearum* overwinters in soil and on infested crop residues (Fernandez et al. 2008; Pereyra and Dill-Macky 2008; Sutton 1982). The mycelium on crop residues allows the production of both macroconidia

(asexual spores) and ascospores (sexual spores produced in perithecia), which constitute the primary inoculum that causes primary infection of wheat heads (Parry et al. 1995; Shaner 2003; Yuen and Schoneweis 2007; Fig. 3).

F. graminearum is one of the predominant species involved in Fusarium diseases. *F. graminearum* overwinters on crop residues which provide the primary inoculum for Fusarium head blight development. Therefore, a better knowledge of *F. graminearum* survival on crop residues is important to control this disease development.

2.2 Saprotrophic growth

Cell-wall degrading enzymes produced by plant pathogenic fungi are considered important during the pathogenic part of the life cycle and may also be relevant during the saprotrophic part (Belien et al. 2006; Van den Brink and de Vries 2011). In the case of *F. graminearum*, scanning electron micrographs and immuno-labelling showed that the fungus penetrates and invades its hosts by secreting cell-wall-degrading enzymes (Kikot et al. 2009). The plant cell-wall components cellulose, xylan, and pectin are damaged when they are in direct contact with the pathogen growing inter- and intracellularly in the tissues of wheat spikelets (Wanjiru et al. 2002). Kikot et al. (2010) examined *F. graminearum* isolates for their production of different extracellular enzymes with activities of potential biotechnological interest: pectinases (polygalacturonase and polymethylgalacturonase), cellulase (carboxymethylcellulase) and hemicellulase (xylanase). Although enzymatic activities varied among the different isolates, polygalacturonase activity was evidenced early (after 2 days' incubation in the presence of oat bran) and was the highest for all isolates. Only some of the isolates showed a high level of polymethylgalacturonase activity; carboxymethylcellulase and endoxylanase activities were particularly high at late stages, i.e., after 4 and 7 days' incubation, respectively, and their maximum values were lower than pectinase values (Kikot et al. 2010). The production of these enzymes

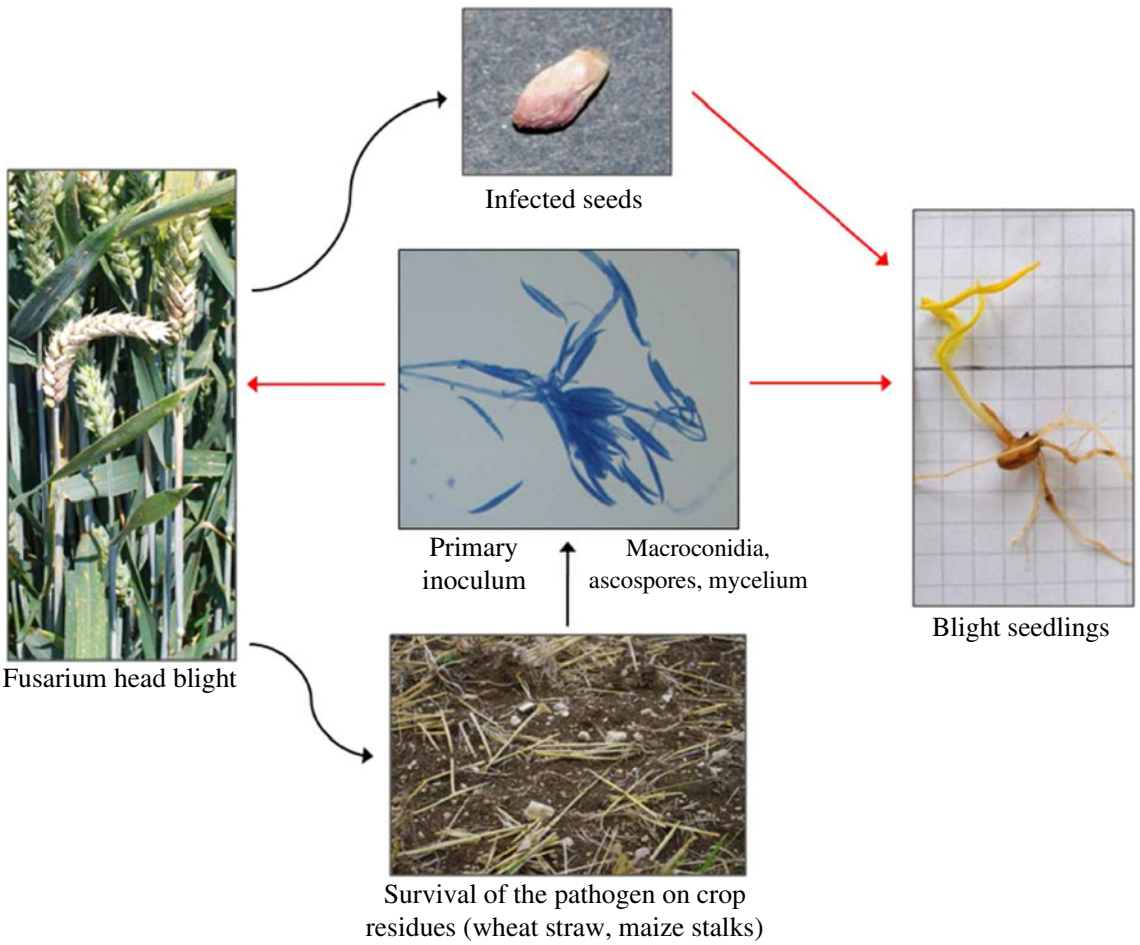

Fig. 3 Disease cycle of *Fusarium graminearum*. Black, sigmoid-like arrows indicate habitats provided by the crop and red arrows indicate infectious activity kept up by habitats (photograph: courtesy of J. Leplat). Crop residues allow the production of *F. graminearum* primary inoculum. The primary inoculum can provoke seedling blight as well as Fusarium head blight by splash dispersal. *F. graminearum*-infested wheat ears can cause the production of infected seeds which lead to seedling blight

requires inducers that are likely to be present in the substrate and regulated by various mitogen-activated proteins (MAP) kinases, some of which have already been identified (Jenczmionka and Schafer 2005). Besides being factors involved during infection, these polysaccharide-degrading enzymes are also important for the colonisation of crop residues.

The chemical composition of crop biomass differs from one plant species to another and from one plant part to another. This may influence the decomposition of the crop residues by microbial colonisers, and thereby the saprotrophic survival of pathogens such as *F. graminearum* (Khonga and Sutton 1988; Nicolardot et al. 2007). There are also consequences on soil organic C inputs, which have been discussed elsewhere (Johnson et al. 2007). A comparative screening of *F. graminearum* exoproteome on culture media containing glucose or hop (*Humulus lupulus* L.) showed that the number of enzymes secreted by the fungus was higher in the presence of plant material (Phalip et al. 2005). Eighty-four proteins were identified on medium containing hops, whereas only 23 were identified on medium containing glucose. Among them, 11 degraded cellulose, 19 degraded pectin and 25 degraded hemicellulose. Two amylases and two chitinases were also identified. Obviously, *F. graminearum* has the enzymatic ability to degrade compounds of the primary cell wall. Moreover, 30 xylanase-related genes were transcribed in the presence of different carbon sources, i.e., hop cell wall, xylan, xylose or carboxymethylcellulose, with different expression patterns for a specific enzyme, which suggests that *F. graminearum* can also adapt to a range of variations in its environment (Hatsch et al. 2006).

Briefly, *F. graminearum* can overwinter on crop residues thanks to its enzymatic ability to degrade and use these residues as nutrients.

2.3 Environmental factors controlling saprotrophic survival

Temperature, water activity and other physico-chemical factors can influence the different aspects of residues colonisation by *F. graminearum*. Its growth and the germination of conidia and ascospores are favoured by warm, humid conditions. Ramirez et al. (2006) found that the mycelial growth of two strains of *F. graminearum* reached an optimum at 25°C at water activities ranging between 0.950 and 0.995, and that no growth was observed below 5°C. Both strains were able to grow in drier conditions, at a minimum water activity of 0.900. By contrast, when the water activity was maintained at high levels, overall microbial activity was stimulated, resulting in a rapid decrease in the quantity of *F. graminearum* on wheat and maize residues buried in the soil (Burgess and Griffin 1968). Soil characteristics such as soil compaction affect water availability. *M. nivale* caused more foot rot in a non-compacted soil than in a compacted soil, probably due to reduced water availability leading to poor fungal development and mobility (Colbach et al. 1996; Toyota et al. 1996).

Inch and Gilbert (2003a) studied the maturation of *F. graminearum* sexual structures on damaged kernels of wheat at three different temperatures. Even if the fungus survives at −10°C, perithecia are only formed between 2°C and 20°C and ascospores only appear at 20°C. The optimal temperature range for the maturation of perithecia is between 15.0°C and 28.5°C, whereas the optimum for the production of ascospores is between 25°C and 28°C at high water activity (Dufault et al. 2006; Sutton 1982; Tschanz et al. 1976).

The optimal temperature for the production of compounds such as mycotoxins is different. For two strains of *F. graminearum*, mycotoxin production was highest at a temperature of 30°C at a water activity of 0.995 (Ramirez et al. 2006). Deoxynivalenol was only produced at water activities between 0.950 and 0.995 even though growth was possible between 0.900 and 0.995, which indicates that mycotoxin production requires more specific conditions than growth.

Although *F. graminearum* can survive on residues buried 20 to 25 cm deep for more than 4 years, it can only develop on plant debris in the upper centimetres of the soil (Champeil et al. 2004). In addition to favourable temperature and water availability, its development depends on soil aeration (Cassini 1970). Furthermore, some stages of its life cycle require light. For example, perithecia initiation and ascospore production are light-dependent (Gilbert and Tekauz 2000; Sutton 1982; Tschanz et al. 1976). A *F. graminearum* survival test on damaged kernels left on the soil surface or buried in the first layer of the soil at 5- and 10-cm depths for 24 months showed that perithecia were produced at all depths but ascospores were only formed at the soil surface (Inch and Gilbert 2003a).

Soil characteristics such as pH could also have an effect *on F. graminearum* survival. A negative correlation between soil pH values ranging between 4.4 and 6.4, and the amount of crown rot on wheat has been found, but it was not clear whether this is because *F. graminearum* has a better saprotrophic capacity in acidic soils or because it is more aggressive under these conditions (Smiley et al. 1996). Although mycelial growth and conidial germination were limited under acidic and alkaline conditions, *F. graminearum* could grow on media whose pH values range between 4 and 10 (Thompson et al. 1993). Macroconidia germination on solid media reached almost 100 % after 18 h at pH values ranging between 3 and 7 (Beyer et al. 2004). The time needed for freshly discharged ascospores to germinate and the rate of ascospore germination were both affected by the pH. The time required for 50 % of the viable ascospores to germinate was shortest at pH 3.5, and ascospore germination was highest at pH 3.7. Changing the pH from 3.7 to 2.5 and from 3.7 to 6.5 decreased the germination of ascospores by 66 % and 56 %, respectively (Beyer and Verreet 2005). This

might explain the better saprotrophic capacity of the fungus in soil at pH 4.4 than in soil at pH 6.4.

Although *F. graminearum* can survive when exposed to unfavourable environmental conditions, its growth and the development of its sexual reproduction structures require sufficient temperature, water, light and O_2 availabilities.

3 Effect of crop residues as *F. graminearum* growth substrates in the soil

3.1 Effect of crop residues quantities

There is a general relationship between the yield of a given crop and the amount of residues left on the ground after harvesting. In most cases, the residues/yield ratio is between 1 and 2 (Kumar et al. 2003; Scarlat et al. 2011). The amount of residue may vary from 2 to 9 tons ha^{-1}, depending on the type of crop: in rape (*Brassica napus* L.), barley and wheat leaves, values were 2.0, 2.5 and 3.5 tons residues ha^{-1}, respectively, whereas in alfalfa (*Medicago sativa* L.) and maize leaves they were 8.5 and 9.0 tons residues ha^{-1}, respectively (Morel 1996; Vilain 1989). Part of the residues is exported for further transformation (e.g., animal bedding, animal feed and biofuel production; Berndes et al. 2003); however, a large part of them is left in and on the soil (Malhi et al. 2011). The residues are either ploughed down (inversion tillage) or left at the surface when conservation tillage is being practiced, as in zero-tillage or other types of non-inversion tillage. Non-inversion tillage may increase wheat grain infection by *F. graminearum* as compared to inversion tillage whereby residues are buried in the soil. The effects vary to a great extent with climatic conditions and preceding crop type (Blandino et al. 2010; Fernandez et al. 2008). In conservation tillage, more than 30 % of the soil surface is covered by crop residues (Bockus and Shroyer 1998). Steinkellner and Langer (2004) found up to 9.10^3 colony–forming units of *F. graminearum* and of *F. culmorum* g^{-1} of soil when non-inversion tillage was used, whereas ten times as few colony-forming units were found after 20-cm deep inversion tillage. *F. graminearum* and *F. culmorum* survival is favoured by high quantities of available residues (Bateman et al. 1998). For example, maize production results in large amounts of residues which promote the production of inoculum (Champeil et al. 2004). Comparing four different densities of maize residues left on the soil surface showed that disease severity and deoxynivalenol occurrence in wheat grains both increased with residue quantity (Blandino et al. 2010; Maiorano et al. 2008).

To sum up, *F. graminearum* survival is enhanced by important quantities of available crop residues, which depends on the production capacity of the preceding crop and on the crop residues management.

3.2 Effect of plant species

Not only can climatic conditions and residue quantities influence *F. graminearum* development, but the nature of crop residues can also affect its biology. For example, wheat and durum wheat produce similar amounts of residues, but wheat infection is more severe after durum wheat than after wheat (Champeil et al. 2004). The production of reproductive structures also varies with the plant species. Pereyra and Dill-Macky (2008) found the induction of a higher ascospore production on wheat and barley than on maize or on some selected weed species (i.e., *Digitaria sanguinalis* (L.) Scop., *Setaria* spp., *Lolium multiflorum* Lam. and *Cynodon dactylon* (L.) Pers.), while no ascospore production was found on sunflower (*Helianthus annuus* L.) residues. Similarly, inoculum production varies over time, and depends on the plant part. *F. graminearum* survival on maize stems and ears on the one hand and on wheat stems, spikelets and grains on the other hand, was compared over 3 years. The length of macroconidia and perithecia production varied according to the type of residue. For example, perithecia were produced on all types of residues during the first year, while only wheat spiklets and grains allowed the perithecia production during the third year. (Khonga and Sutton 1988). Similarly, the amount of *Fusarium* was found to decrease faster on wheat internodes than on stem bases, and faster on nodes than on internodes (Kohl et al. 2007; Pereyra et al. 2004). Finally, *F. graminearum* ascospore production was higher on kernels than on nodes and floral bracts (Pereyra and Dill-Macky 2005). These observations can be partly explained by the chemical composition of the residues and particularly by their C/N ratio: the C/N ratio varies over time depending on the decomposition stage of the residues, and influences growth and the production of macroconidia and sexual structures. For example, the C/N value of wheat straw is high (134), whereas the C/N value of wheat leaves is ten times as low (13.4; Nicolardot et al. 2001). Macroconidia are produced on residues that are in the early stage of decomposition whereas perithecia production occurs later, when decomposition is much more advanced and growth conditions are less favourable. Rich residues with a low C/N ratio (such as maize stems, maize kernels, wheat spikelets and lowly infested wheat grains) provide a favourable habitat, allowing longer saprotrophic development before perithecia production, as compared to poor residues with a high C/N ratio, such as wheat stems and severely infested wheat grains (Khonga and Sutton 1988).

Briefly, *F. graminearum* survival depends on the C/N ratio of the residues, and consequently on the plant species, on the plant part and on the degradation rate of the residues. *F. graminearum* survival is enhanced by rich residues, with a low C/N ratio. *F. graminearum*

sexual reproductive structures appear on residues with high C/N, when growth conditions are less favourable to fungal development.

4 Competition and antagonism

4.1 Organisms' succession during residue decomposition

Organisms of different species and from different trophic groups are involved at different stages of the decomposition process (Frankland 1998; Thirup et al. 2001). The ability of a specific species to grow or survive on the material depends on its ability to use the nutrients available at a particular decomposition stage and on its ability to compete for them with other organisms that are colonising the material concomitantly. Among these groups, microarthropods, nematodes, protozoa, bacteria and fungi have important predator–prey interactions that may determine community assemblages during the early decomposition of crop residues depending on their nature (Ponge 2005). The fungal community seems to be the least affected by such interactions (Georgieva et al. 2005a), and the increase in fungal biomass that occurs later during succession is more correlated with the decomposition of different crop residues (Georgieva et al. 2005b). Therefore controlling the decomposition process could represent a way of controlling primary inoculum quantities of soil- and residue-borne plant pathogenic fungi such as *F. graminearum*. The succession of fungal populations and the decomposition process are also affected by external factors such as climatic conditions and agronomic practices, which may determine the competitive exclusion among the complex of *Fusarium* species (Doohan et al. 2003; Fernandez et al. 2008). Crop residues present in the soil or at the soil surface are degraded by a wide range of organisms that use the material for their growth. Larger soil organisms such as microarthropods and earthworms fragment the plant material, thereby making it more available for microbial degradation. In general, weak and aggressive pathogens dominate during the initial stages of degradation. Later, the material is more and more colonised by fungi that are specialised for saprotrophic growth (Frankland 1998; Kjöller and Struwe 2002). Fresh residue-colonising microorganisms are copiotrophs, which can be considered as r-strategists using easily available carbon sources and maximising their intrinsic growth rate when resources are abundant (Pianka 1970). The microorganisms that colonise residues at later stages of decomposition are oligotrophs, which can be considered as K-strategists. Compared with r-strategists, K-strategists have a slower growth rate, a better ability to degrade recalcitrant organic substances and better survival rates when resources are limited (Bastian et al. 2009).

To sum up, the decomposition of the crop residues is a complex process involving the whole soil biota. *F. graminearum* survival can be controlled by driving the balance of this process.

4.2 Interactions with soil microorganisms

By applying the r- and K-strategy concept to the comparison of the different microorganisms involved in crop residues decomposition, we could consider *F. graminearum* as an r-strategist during its saprotrophic phase because it can grow rapidly when fresh matter is available. This can be linked to the enzymes it excretes in the presence of plant material. The many cellulases, hemicellulases and pectinases that *F. graminearum* produces are important early in the decomposition process (Phalip et al. 2005). The relative abundance of *F. graminearum* can be decreased rapidly by keeping the humidity of wheat residues at a high level, which favours the activity of other microbes and can hence hamper *F. graminearum* growth (Burgess and Griffin 1968). Indeed, *F. graminearum* seems to be a poor competitor over time, particularly compared with other *Fusarium* species. For instance, the amount of *F. graminearum* found on wheat residues rapidly decreases whereas those of *F. solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. poae* and *F. sporotrichioides* increase. All these species are known to have better saprotrophic capacity in crop residues or in soil than *F. graminearum* (Pereyra and Dill-Macky 2008). Nevertheless, *F. graminearum* can survive on residues for more than 24 months (Pereyra et al. 2004). *Fusarium poae* and *F. sporotrichioides*, also involved in Fusarium head blight, are less aggressive than *F. graminearum* on plants, but they have a better saprotrophic capacity and can outcompete other organisms on residues (Fernandez et al. 2008). Few studies have been carried out about the interactions that occur among the different pathogens on residues in the field, but experiments in controlled conditions emphasise how specific these interactions are (Simpson et al. 2004; Velluti et al. 2000). *F. culmorum* growth and *M. nivale* var. *majus* growth were both limited when the strains were grown together in the same liquid medium, compared with their respective growth rates when they were inoculated alone (Simpson et al. 2004). On wheat seedlings, *F. culmorum* thoroughly inhibited *M. nivale* var. *nivale* and var. *majus* growth; however, when *M. nivale* var. *majus* was established before *F. culmorum*, it co-suppressed *F. culmorum* growth. Similarly, the presence of *F. graminearum* reduced *F. moniliforme* and *F. proliferatum* (T. Matsushima) Nirenberg growth on sterile maize grains, whereas its own growth was not affected by the other two species (Velluti et al. 2000).

Moreover, intraspecific diversity of phenotypic traits within *F. graminearum* species leads to interactions between *F. graminearum* populations for crop residues colonisation.

This can be revealed through different levels of aggressiveness or different chemotypes among any given set of *F. graminearum* strains interacting with host plants. This can also be revealed through the intrinsic competitive skills used by different *F. graminearum* populations to rapidly colonise and efficiently exploit crop residues such as maize stalks (Miedaner et al. 2004; Naef and Defago 2006).

The role of deoxynivalenol — and of mycotoxins in general — in the multitrophic interactions *F. graminearum* faces during its saprotrophic growth, is poorly understood. While the deleterious impact on animals, including humans, is rather well studied (Nielsen et al. 2011a, b; Pestka 2010; Sampietro et al. 2010), the impact it might have on soil organisms has less been considered (Abid et al. 2011). The different studies results are conflicting. In some cases, mycotoxins have been shown to have antimicrobial activities against *Trichoderma atroviride* P. Karst. (Lutz et al. 2003), and a possible regulatory role in bacterial–fungal interactions has been proposed (Duffy and Defago 1997), but in other case, the comparison of toxigenic and non-toxigenic *F. graminearum* strains showed that there was no evidence that deoxynivalenol played a role in the defence against *T. atroviride* (Naef et al. 2006). Moreover, the potential impact of mycotoxins on the microflora can be reduced by bacteria able to degrade these mycotoxins (Awad et al. 2010; Fuchs et al. 2002). Therefore the putative competitive advantage mycotoxins might confer to *F. graminearum* versus other antagonistic colonisers remains to be demonstrated.

F. graminearum seems to be a poor competitor over time for crop residues colonisation, even among *Fusarium* species. The enhancement of the residues decomposition processes by other organisms could be efficient to limit *F. graminearum* survival.

4.3 *Fusarium* species displacement on residues

It is possible to limit *F. graminearum* survival and growth on residues by adding microorganisms that can outcompete it. Already known biocontrol agents like *T. atroviride* and *T. harzianum* Rifai (Naef et al. 2006), or *Clonostachys rosea* (Link) Schroers, Samuels, Seifert and W. Gams (Gromadzka et al. 2009) are among the possible candidates. It is also possible to screen species isolated from residues (Luongo et al. 2005; Singh et al. 2009). The latter approach increases the likelihood that the microorganisms are adapted to the environment of the studied residues. Magan and Lynch (1986) tested ten fungal isolates for their capacity to colonise straw residues at different temperatures and at different water potentials. Among them, only *Penicillium* spp. and *F. culmorum* were able to grow at a low water potential, confirming the role played by water availability in competitive interactions. *T. harzianum*, *F. equiseti* and *F. nygamai* L.W. Burgess & Trimboli, which were all isolated from wheat stubble, have been shown to reduce *F. pseudograminearum* growth on culture medium (Singh et al. 2009). The displacement of *F. pseudograminearum* by these fungal isolates on barley straw was tested at different temperatures (5–35°C) and at different water potentials (−0.3 to −5.0 MPa). *T. harzianum* was the most efficient antagonist but displaced *F. pseudograminearum* very poorly at low temperatures and low water potentials. *F. equiseti* and *F. nygamai* gave moderate displacement. *F. equiseti* was the most efficient at low temperatures and low water potentials, showing that field conditions need to be taken into account when looking for an efficient antagonistic agent. *C. rosea* isolates were found to suppress sporulation of *F. graminearum* and *F. culmorum* on wheat straw and to suppress sporulation of *F. graminearum*, *F. culmorum*, *F. proliferatum* and *F. verticillioides* (Sacc.) Nirenberg on maize stalks under controlled conditions. When tested under field conditions, however, their efficiency was limited (Luongo et al. 2005).

With regard to sexual reproduction, a *Microsphaeropsis* sp. isolate significantly reduced ascospore production by *F. graminearum* on wheat and maize residues under controlled conditions (Bujold et al. 2001). *Microsphaeropsis* sp. suppressed ascospore production when inoculated on wheat residues 2 weeks before the pathogen was inoculated, concomitantly, or 4 weeks later. Furthermore, it still suppressed ascospore production when inoculated on maize residues 6 weeks after the pathogen. However, *Microsphaeropsis* sp. significantly reduced the production of perithecia on crop residues under field conditions at only a few sampling dates. This highlights once again that although the antagonist can be found efficient under laboratory conditions, field application may not be so easy.

Along with their effect on pathogen growth and sporulation, competitors for residues can also decrease mycotoxin production. When *T. atroviride* and *F. graminearum* were inoculated together on autoclaved maize leaves, deoxynivalenol production by *F. graminearum* was 36 % lower per biomass unit of the pathogenic fungus than when *F. graminearum* was inoculated alone (Naef et al. 2006).

To sum up, the use of specific outcompeting microorganisms could be an efficient option to limit *F. graminearum* survival on crop residues. Several fungal species with an interesting effect under laboratory conditions were identified. However, the field efficiency of these fungal strains is still limited, highlighting the difficulties to transpose laboratory experiments to field application.

4.4 Interactions with the soil fauna

The soil animals influence pathogenic fungi and other soil microbes directly by feeding on them and by dispersing them. They also have an indirect influence: they alter the

physical environment by fragmenting organic matter, burrowing through the soil, mixing it and by depositing faeces (Brown 1995; Coleman and Crossley 1996; Swift et al. 1979). The soil fauna is often divided up into groups, which is useful for understanding how animals move within the soil and influence soil structure and other abiotic and biotic conditions (Coleman and Crossley 1996; Lavelle and Spain 2001; Swift et al. 1979). The smaller soil animals grazing on mycelia with selective feeding habits have an important influence on saprotrophically growing fungi. The larger soil animals, which are less selective in their feeding habits, are important consumers of plant residues and soil. Their main influence can probably be seen through their effects on the fragmentation of organic material and on soil mixing (Friberg et al. 2005).

Anecic earthworms, such as *Lumbricus terrestris* L. and *Aporrectodea longa* Ude, which take fresh litter from the soil surface and pull it down into the soil through their burrows, can reduce the quantities of fungal pathogens such as *Fusarium* spp. (Moody et al. 1996). The fact that infested wheat straw with high levels of deoxynivalenol was incorporated faster than straw with low levels of deoxynivalenol shows that deoxynivalenol is not repellent for *L. terrestris* and possibly attractive (Oldenburg et al. 2008). Earthworms also grew better on infested straw than on non-infested straw, either because the decomposition by *F. graminearum* made compounds in the straw more easily available or because the fungal biomass itself was a source of nutrients for them. Since, fungal biomass and deoxynivalenol degradation increased in the presence of earthworms, it is possible that earthworms take part in deoxynivalenol degradation, maybe through the activity of their associated gut microorganisms (Schrader et al. 2009).

It seems that soil animals able to feed on *F. graminearum*-infested crop residues could participate in *F. graminearum* survival control.

5 Importance of agricultural practices for the disease development

5.1 Preceding crop

The preceding crop is an important factor determining the risk for Fusarium diseases on wheat (Blandino et al. 2010; Klem et al. 2007; Muller et al. 2010). If the preceding crop is a good host of the pathogen, the disease risk increases due to the inoculum initially present on the crop. The severity of Fusarium head blight caused by *F. graminearum* on wheat and the amount of mycotoxins produced have been found higher after maize than after soya, and continuous wheat cropping implied a higher risk of crown rot development caused by *F. graminearum* than a wheat–pea or wheat–fallow rotation (Dill-Macky and Jones 2000; Smiley et al. 1996; Teich and Hamilton 1985). Variations also exist among host plants: the disease is more severe after maize or durum wheat than after wheat or barley due to the quantities of residues produced and to their chemical composition. Schaafsma et al. (2005) found the number of viable propagules trapped at anthesis in wheat fields planted on maize or wheat stubble higher than the number of viable propagules in wheat fields following non-host crops, and the highest number of propagules was found after maize.

The composition of the fungal community causing Fusarium head blight changes depending on the preceding crop. In a survey performed in New Zealand on the community composition of *Fusarium* fungi isolated from harvested wheat grains in relation to the preceding crop, *F. graminearum* was recovered, along with *F. avenaceum*, *F. poae* and *F. culmorum* (Cromey et al. 2002). In each case, *F. graminearum* was the most common species. *F. graminearum* quantities were highest after maize, whereas *F. avenaceum* and *F. poae* quantities were highest after other crops. Where crown rot and Fusarium head blight were caused by *F. graminearum* and *F. culmorum*, the stem-bases were more frequently colonised by *F. culmorum* than by *F. graminearum*, but the situation was reversed on ears. And in this case, after maize, the stem-base disease was generally reduced whereas head blight increased (Bateman et al. 2007).

Most studies about the effects of different preceding crops on the development of *Fusarium* fungi were carried out on crops that are classically included in major rotation systems, such as wheat, maize or rape (Dill-Macky and Jones 2000; Klem et al. 2007; Muller et al. 2010). It could be worth assessing the role of specific, less frequently studied intermediate crops such as Indian mustard (*Brassica juncea* (L.) Czern & Coss), which has been shown to suppress *F. graminearum* growth (Kirkegaard et al. 1996; Sarwar et al. 1998).

Briefly, preceding crop is an important factor in the development of Fusarium head blight on wheat. Using a host of the disease as preceding crop carries a risk. Indeed, *F. graminearum* could settle on crop residues, in particular in case of maize which produces high quantities of residues. More care must be paid to the crop rotation scheme to limit the risk of Fusarium head blight development.

5.2 Soil tillage

Inversion tillage generally decreases the risk of Fusarium head blight compared with non-inversion tillage (Dill-Macky and Jones 2000; Fernandez et al. 2008; Steinkellner and Langer 2004). Several factors can explain why tillage can affect plant disease. Infection caused by splash dispersal

during rainfalls or overhead irrigation is increased in the presence of large amounts of infested crop residues at the soil surface (Bateman 2005; Osborne and Stein 2007; Sutton 1982). Bateman (2005) found that the presence of plant material infested by *F. culmorum* on the soil surface 3–4 weeks before anthesis was necessary to cause infection on ears. As for the preceding crop, tillage can have different effects on the different diseases caused by the same pathogens spectrum. In a study comparing disease on stem-bases and on ears, infection on stem-bases, mainly caused by *F. culmorum*, was decreased by non-inversion tillage, whereas infection on ears, mainly caused by *F. graminearum*, was increased (Bateman et al. 2007). However, a test using plating method to determine whether the quantity of *F. culmorum* in a soil sampled to a 10-cm depth was affected by soil tillage showed that the number of propagules per gram of soil was higher with non-inversion tillage than with inversion tillage (Bateman et al. 1998). As already mentioned, the fungus can survive in soil for a extend periods (more than 2 years) despite low O_2 availability and unfavourable abiotic conditions, but it cannot grow or produce ascospores (Champeil et al. 2004; Khonga and Sutton 1988).

Tillage has an effect on the decomposition rate of residues. Decomposition of buried residues is faster and more complete than decomposition of surface residues. Pereyra et al. (2004) found that for wheat residues that were buried for 24 months, only 2 % of dry matter remained, whereas 25 % remained when the residues were left at the soil surface. In a comparative study between barley straw and red clover foliage (*Trifolium pratense* L.), the limited contact between the soil matrix and the residues affected decomposition dynamics, particularly in the case of straw residues, which are rich in cellulose and hemicelluloses (Henriksen and Breland 2002). This slow decomposition may be due to insufficient colonisation and growth of holocellulose-degrading microorganisms. The influence of soil tillage on the composition of microbial communities was confirmed by the analysis of bacterial and fungal communities during the decomposition process. The genetic structure of communities on residues is specific to residue location within the soil (soil surface or incorporated in soil; Nicolardot et al. 2007). Comparison of buried and non-buried residues showed that the quantity of *F. graminearum* inoculum was twice as high on non-buried residues as on buried residues. Indeed, a slower decomposition at the soil surface allows a longer-lasting availability of nutrients that are essential for *F. graminearum* development (Pereyra et al. 2004). A positive effect of reduced tillage is that the soil fauna is better preserved and can play a more active role in the decomposition and mineralization of crop residues, thus limiting habitats for saprotrophically surviving plant pathogenic fungi.

Different tillage practices affect the presence of weeds. Weeds can be a source of inoculum: in particular, *F. graminearum* inoculum has been found on *Festuca*, *D. sanguinalis*, *Setaria* spp., *L. multiflorum* and *C. dactylon*. When *F. graminearum* was the most common isolated species, other fungi belonging to the complex such as *F. avenaceum*, *F. sambucinum* and *F. poae* were also found (Pereyra and Dill-Macky 2008). Over the years, the composition of fungi causing Fusarium head blight founds on weeds changes. In a study by Inch and Gilbert (2003b), *F. graminearum* was found on 11 out of 34 grass species investigated. *F. sporotrichioides*, *F. equiseti*, *F. avenaceum*, *F. culmorum* and *F. poae* were isolated too, but only *F. graminearum* and *F. sporotrichioides* were isolated in June. In July, *F. equiseti* and *F. culmorum* were isolated in addition to *F. graminearum* and *F. sporotrichioides*, while in August, all species were isolated. Although weed removal has been an important part in the control of different fungal plant diseases for many decades (Mantle et al. 1977; Miedaner et al. 2011), it is clear that the reduction soil tillage and sustainable pesticide use are clearly going to bring about with them a new focus on the role of weeds in Fusarium head blight mediation (Landschoot et al. 2011; Postic et al. 2012).

Shortly, soil tillage is an important factor in the development of Fusarium head blight on wheat. Inversion tillage reduces the risk of disease development in comparison to non-inversion tillage. Inversion tillage (1) hides the primary inoculum infecting wheat ears and prevents splash dispersal, (2) enhances the residues decomposition process and thus limits *F. graminearum* survival, and (3) allows controlling weed species which could be a source of *F. graminearum* inoculum.

5.3 Fertilization and pesticides

The fungi in the complex causing Fusarium head blight are able to use all forms of nitrogen; however, the effects of fertilizers are mainly indirect and mediated via increased plant vigour (Huber and Watson 1974). Different forms of nitrogen have different effects on the survival of pathogenic *Fusarium* spp. On one hand, in some cases, the number of propagules of *F. culmorum* in the soil and of *F. graminearum* on residues increases with inorganic nitrogen fertilization and with the application of calcium ammonium nitrate, respectively (Bateman and Coskun 1995; Lemmens et al. 2004; Yi et al. 2002). On the other hand, urea can hinder the reproduction of fungi, inhibit the formation of chlamydospores and also prevent ascospore maturation (Teich 1989).

The herbicides used can influence inoculum levels. As weeds can be inoculum source, applying herbicides could reduce the risks of disease break-outs; however, little information is available about the direct effect of herbicides on *F. graminearum*, or about the way its saprotrophic ability could

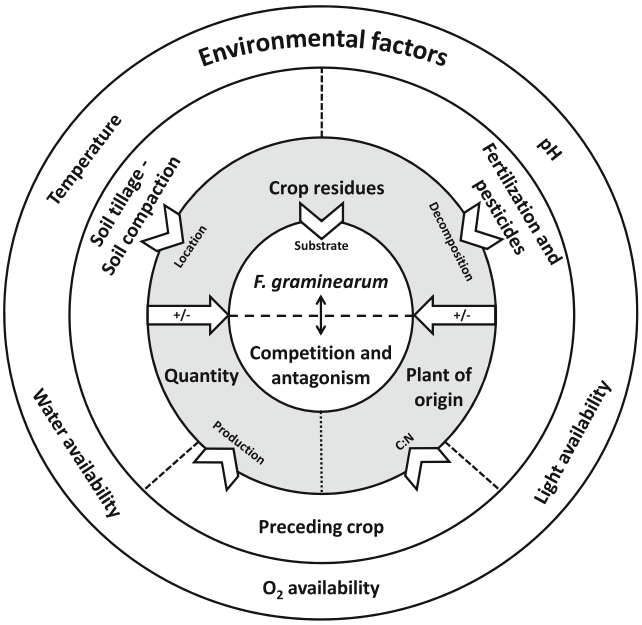

Fig. 4 Saprotrophic survival of *Fusarium graminearum*. Crop residues are the main habitat of *F. graminearum*. On the one hand, they provide spatial and trophic resources the fungus has to exploit in interaction with the rest of the microflora and the soil fauna. On the other hand, they buffer the impact of environmental factors, including agricultural practices

be affected by their presence. Some glyphosate-based herbicides can stimulate *F. avenaceum* and *F. graminearum* growth, under *in vitro* conditions as well as on crop residues in the field, which leads to an increase in wheat stem-base and ear infections (Fernandez et al. 2009; Hanson and Fernandez 2003). Weed control with glyphosate seems to provide a source of energy for *Fusarium* leading to a proliferation of populations. The numbers of other fungal species decrease when glyphosate is used, suggesting important changes in the structure of fungal communities that should in turn affect their functioning.

Data from European surveys have recently shown that organically produced cereal grains have an equal or a lower level of *Fusarium*-generated mycotoxins than conventionally produced cereals (Bernhoft et al. 2010; Birzele et al. 2002; Edwards 2009; Meister 2009). There are currently no clear explanations for that fact, but one factor may be differences in the microflora associated with the different cropping systems.

To summarise, little is known about the effect of fertilizers and pesticides on *F. graminearum* saprotrophic survival. Some glyphosate-based herbicides may stimulate *F. graminearum* survival by changing the structure of fungal communities and providing a source of energy.

6 Conclusion

An important part of the life cycle of *F. graminearum*, the main causal agent of Fusarium head blight, takes place outside the plant. The fungus produces an array of enzymes which allow it to use crop residues as a trophic and spatial resource for its saprotrophic development.

Figure 4 shows the proposed model exposing the role of the various factors that affect *F. graminearum* survival on crop residues. Depending on environmental factors, it is able to survive on crop residues, grow, and produce conidia and sexual structures which provide the primary inoculum causing disease on wheat heads. The development of *F. graminearum* is favoured by the presence of large amounts of residues and by nutrient rich residues, with a low C/N ratio. Since *F. graminearum* appears to be a poor competitor over time compared to other organisms that colonise crop residues, strategies based on competition for the growth substrate could be an efficient way to control the production of primary inoculum. Some fungal species have been found to suppress sporulation and ascospore production by *F. graminearum* under controlled conditions in that way, but their efficiency still remains to be confirmed under field conditions. In addition, strategies that favour residue decomposition, via the activity of the soil's microflora and fauna, may reduce *F. graminearum* survival.

Since *F. graminearum* overwinters on crop residues, agricultural practices including crop rotation and residue management play a large part in Fusarium head blight management. Primary inoculum production can be limited by using a non-host plant as a preceding crop. Inversion tillage buries the primary inoculum and thus prevents inoculum being splashed up to wheat heads. In addition, inversion tillage favours the decomposition of crop residues in comparison with non-inversion tillage. Finally, inversion tillage makes it possible to control the source of *F. graminearum* inoculum provided by weed species.

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RÈGLEMENT (CE) Nº 1126/2007 DE LA COMMISSION

du 28 septembre 2007

modifiant le règlement (CE) nº 1881/2006 portant fixation de teneurs maximales pour certains contaminants dans les denrées alimentaires en ce qui concerne les toxines du *Fusarium* dans le maïs et les produits à base de maïs

(Texte présentant de l'intérêt pour l'EEE)

LA COMMISSION DES COMMUNAUTÉS EUROPÉENNES,

vu le traité instituant la Communauté européenne,

vu le règlement (CEE) nº 315/93 du Conseil du 8 février 1993 portant établissement des procédures communautaires relatives aux contaminants dans les denrées alimentaires [1], et notamment son article 2, paragraphe 3,

considérant ce qui suit:

(1) Le règlement (CE) nº 1881/2006 de la Commission du 19 décembre 2006 portant fixation de teneurs maximales pour certains contaminants dans les denrées alimentaires [2] fixe les teneurs maximales applicables aux toxines de *Fusarium* dans certaines denrées alimentaires.

(2) Les teneurs maximales doivent être fixées de façon stricte à un niveau pouvant raisonnablement être atteint grâce au respect des bonnes pratiques agricoles et de fabrication, compte tenu du risque lié à la consommation des aliments.

(3) Les conditions climatiques durant la croissance, en particulier à la floraison, influent considérablement sur la teneur en toxines du *Fusarium*. Toutefois, de bonnes pratiques agricoles réduisant les facteurs de risque au minimum peuvent, dans une certaine mesure, empêcher la contamination par les champignons *Fusarium*. La recommandation 2006/583/CE de la Commission du 17 août 2006 sur la prévention et la réduction des toxines du *Fusarium* dans les céréales et produits céréaliers [3], y compris le maïs et les produits à base de maïs, énonce les principes généraux de prévention et de réduction de la contamination des céréales par les toxines du *Fusarium* (zéaralénone, fumonisines and trichothécènes), dont l'application doit être assurée par des codes d'usages nationaux.

(4) Des teneurs maximales ont été fixées en 2005 pour les toxines du *Fusarium* dans les céréales et les produits céréaliers, y compris le maïs et les produits à base de maïs. En ce qui concerne le maïs, tous les facteurs contribuant à la formation des toxines du *Fusarium*, en particulier de la zéaralénone et des fumonisines B_1 et B_2, n'étaient pas encore connus avec précision. Par conséquent il était prévu que les teneurs maximales concernant le maïs et les produits à base de maïs s'appliqueraient seulement à partir du 1er juillet 2007 dans le cas du déoxynivalénol et de la zéaralénone et à partir du 1er octobre 2007 dans le cas des fumonisines B_1 et B_2, à condition que d'autres teneurs maximales fondées sur de nouvelles informations sur leur présence et leur formation n'aient pas été établies entre-temps. Ce délai a permis aux exploitants du secteur alimentaire actifs dans la filière céréalière d'effectuer des études sur les sources de formation de ces mycotoxines et sur le type de mesures de gestion à prendre pour prévenir leur présence autant qu'il est raisonnablement possible de le faire.

(5) Il est nécessaire, à la lumière des nouvelles informations recueillies depuis 2005, de modifier les teneurs maximales concernant le maïs et les produits à base de maïs ainsi que la date d'application de ces teneurs.

(6) Des informations obtenues récemment attestent que les teneurs relevées dans le maïs de la récolte 2005 et 2006 sont supérieures à celles relevées dans celui de la récolte 2003 et 2004 pour ce qui concerne principalement la zéaralénone et les fumonisines et, dans une moindre mesure, le déoxynivalénol. Cette évolution est liée aux conditions météorologiques. Les teneurs en zéaralénone et en fumonisines prévues pour le maïs ne peuvent dès lors pas être respectées dans certaines conditions météorologiques, même si l'on applique des mesures de prévention de manière optimale. Il est par conséquent nécessaire, pour éviter une désorganisation du marché, de modifier les teneurs maximales tout en maintenant un niveau élevé de protection de la santé publique en veillant à ce que l'exposition de la population reste nettement inférieure à la valeur maximale recommandée établie aux fins de la protection de la santé.

(7) Il convient également, pour garantir une application correcte et aisée de ces teneurs maximales, que celles-ci s'appliquent à tout le maïs récolté au cours d'une saison et à tous les produits fabriqués à partir de ce maïs, c'est pourquoi la date d'application doit correspondre au début de la campagne de commercialisation de la prochaine récolte. Étant donné que la récolte du maïs en Europe débute d'ordinaire à la mi-septembre et s'achève à la fin octobre, il convient de rendre ces teneurs applicables à partir du 1er octobre 2007.

[1] JO L 37 du 13.2.1993, p. 1. Règlement modifié par le règlement (CE) nº 1882/2003 du Parlement européen et du Conseil (JO L 284 du 31.10.2003, p. 1).
[2] JO L 364 du 20.12.2006, p. 5.
[3] JO L 234 du 29.8.2006, p. 35.

(8) Eu égard à ce qui précède, le présent règlement doit s'appliquer à partir du 1er juillet 2007.

(9) Il convient en outre d'apporter un certain nombre de modifications techniques mineures.

(10) Il convient de prévoir que la teneur maximale ne s'applique pas au maïs brut destiné à être transformé par mouture humide (production d'amidon), car il est scientifiquement démontré que les toxines du *Fusarium* ne sont pas détectées ou ne sont détectées qu'en très faible quantité dans l'amidon de maïs quelles que soient les teneurs en toxines du *Fusarium* du maïs brut. Néanmoins, la protection de la santé publique et animale commande que les exploitants du secteur alimentaire actifs dans le domaine de la mouture humide contrôlent de manière intensive les sous-produits issus du processus de mouture humide destinés à l'alimentation des animaux afin de vérifier qu'ils respectent les teneurs maximales recommandées citées dans la recommandation 2006/576/CE de la Commission du 17 août 2006 concernant la présence de déoxynivalénol, de zéaralénone, d'ochratoxine A, des toxines T-2 et HT-2 et de fumonisines dans les produits destinés à l'alimentation animale ([1]).

(11) À partir d'un même lot de maïs brut, le processus de mouture à sec débouche sur des fractions de mouture dont la taille des particules est différente. Il est scientifiquement démontré que les fractions de mouture à particules plus fines présentent une teneur en toxines du *Fusarium* plus élevée. Les fractions de mouture de maïs sont classées selon différentes positions de la nomenclature combinée en fonction de la taille des particules. Cette classification repose sur le taux de passage dans un tamis d'une ouverture de mailles de 500 microns. Il convient de fixer des teneurs maximales différentes pour les fractions de mouture inférieures et supérieures à 500 microns afin de tenir compte du niveau de contamination des différentes fractions.

(12) Les mesures prévues par le présent règlement sont conformes à l'avis du comité permanent de la chaîne alimentaire et de la santé animale,

A ARRÊTÉ LE PRÉSENT RÈGLEMENT:

Article premier

Le règlement (CE) nº 1881/2006 est modifié comme suit:

1) À l'article 11, le point b) est remplacé par le texte suivant:

«b) 1er octobre 2007, pour ce qui est des teneurs maximales en déoxynivalénol et zéaralénone fixées aux points 2.4.3, 2.4.8, 2.4.9, 2.5.2, 2.5.4, 2.5.6, 2.5.8, 2.5.9 et 2.5.10 de l'annexe;»

2) La section 2 de l'annexe est modifiée comme suit:

a) Les dispositions relatives au déoxynivalénol (2.4), à la zéaralénone (2.5) et aux fumonisines (2.6) sont remplacées par celles de l'annexe du présent règlement.

b) Le texte de la note 20 de bas de page est remplacé par le texte suivant: «La teneur maximale est applicable à partir du 1er octobre 2007.»

c) La note 21 de bas de page est supprimée.

Article 2

Le présent règlement entre en vigueur le jour suivant celui de sa publication au *Journal officiel de l'Union européenne*.

Il s'applique à partir du 1er juillet 2007.

Le présent règlement est obligatoire dans tous ses éléments et directement applicable dans tout État membre.

Fait à Bruxelles, le 28 septembre 2007.

Par la Commission
Markos KYPRIANOU
Membre de la Commission

([1]) JO L 229 du 23.8.2006, p. 7.

ANNEXE

«2.4	**Déoxynivalénol** ([17])	
2.4.1	Céréales brutes ([18]) ([19]) autres que le blé dur, l'avoine et le maïs	1 250
2.4.2	Blé dur et avoine bruts ([18]) ([19])	1 750
2.4.3	Maïs brut ([18]) à l'exception du maïs brut destiné à être transformé par mouture humide (*)	1 750 ([20])
2.4.4	Céréales destinées à la consommation humaine directe, farine de céréales, son et germe en tant que produit fini commercialisé pour la consommation humaine directe, à l'exception des denrées alimentaires figurant aux points 2.4.7, 2.4.8 et 2.4.9	750
2.4.5	Pâtes (sèches) ([22])	750
2.4.6	Pain (y compris les petits produits de boulangerie), pâtisseries, biscuits, collations aux céréales et céréales pour petit-déjeuner	500
2.4.7	Préparations à base de céréales et aliments pour bébés destinés aux nourrissons et enfants en bas âge ([3]) ([7])	200
2.4.8	Fractions de mouture de maïs dont la taille des particules est > 500 microns auxquelles s'applique le code NC 1103 13 ou 1103 20 40 et autres produits de mouture de maïs dont la taille des particules est > 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	750 ([20])
2.4.9	Fractions de mouture de maïs dont la taille des particules est ≤ 500 microns auxquelles s'applique le code NC 1102 20 et autres produits de mouture de maïs dont la taille des particules est ≤ 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	1 250 ([20])
2.5	**Zéaralénone** ([17])	
2.5.1	Céréales brutes ([18]) ([19]) autres que le maïs	100
2.5.2	Maïs brut ([18]) à l'exception du maïs brut destiné à être transformé par mouture humide (*)	350 ([20])
2.5.3	Céréales destinées à la consommation humaine directe, farine de céréales, son et germe en tant que produit fini commercialisé pour la consommation humaine directe, à l'exception des denrées alimentaires figurant aux points 2.5.6, 2.5.7, 2.5.8, 2.5.9 et 2.5.10	75
2.5.4	Huile de maïs raffinée	400 ([20])
2.5.5	Pain (y compris les petits produits de boulangerie), pâtisseries, biscuits, collations aux céréales et céréales pour petit-déjeuner, à l'exclusion des collations au maïs et des céréales pour petit-déjeuner à base de maïs	50
2.5.6	Maïs destiné à la consommation humaine directe, collations à base de maïs et céréales pour petit-déjeuner à base de maïs	100 ([20])
2.5.7	Préparations à base de céréales (à l'exception des préparations à base de maïs) et aliments pour bébés destinés aux nourrissons et enfants en bas âge ([3]) ([7])	20
2.5.8	Préparations à base de maïs destinées aux nourrissons et enfants en bas âge ([3]) ([7])	20 ([20])

2.5.9	Fractions de mouture de maïs dont la taille des particules est > 500 microns auxquelles s'applique le code NC 1103 13 ou 1103 20 40 et autres produits de mouture de maïs dont la taille des particules est > 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	200 ([20])
2.5.10	Fractions de mouture de maïs dont la taille des particules est ≤ 500 microns auxquelles s'applique le code NC 1102 20 et autres produits de mouture de maïs dont la taille des particules est ≤ 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	300 ([20])
2.6	**Fumonisines**	Somme B_1 + B_2
2.6.1	Maïs brut ([18]) à l'exception du maïs brut destiné à être transformé par mouture humide (*)	4 000 ([23])
2.6.2	Maïs destiné à la consommation humaine directe, aliments à base de maïs destinés à la consommation humaine directe, à l'exception des aliments figurant aux points 2.6.3 et 2.6.4	1 000 ([23])
2.6.3	Céréales pour petit-déjeuner à base de maïs et collations à base de maïs	800 ([23])
2.6.4	Préparations à base de maïs et aliments pour bébés destinés aux nourrissons et enfants en bas âge ([3]) ([7])	200 ([23])
2.6.5	Fractions de mouture de maïs dont la taille des particules est > 500 microns auxquelles s'applique le code NC 1103 13 ou 1103 20 40 et autres produits de mouture de maïs dont la taille des particules est > 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	1 400 ([23])
2.6.6	Fractions de mouture de maïs dont la taille des particules est ≤ 500 microns auxquelles s'applique le code NC 1102 20 et autres produits de mouture de maïs dont la taille des particules est ≤ 500 microns non destinés à la consommation humaine directe auxquels s'applique le code NC 1904 10 10	2 000 ([23])

(*) L'exception s'applique uniquement au maïs dont l'étiquetage ou la destination, par exemple, font clairement apparaître qu'il est destiné à être utilisé dans un processus de mouture humide (production d'amidon).»