Integrated Master in Bioengineering

Fusarium graminearum and *F. culmorum* isolates: pathogen-distribution and DON- production in *Triticum aestivum* influenced by TRI-inhibitors

Master's Dissertation

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Developed in

BASF
We create chemistry

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Abstract

Due to a population growth and a higher demand on cereals and food, protection of crops, in particular cereal crops, is essential to avoid and decrease great losses caused by pests. Fusarium head blight (FHB) is a very common worldwide disease that affects several cereal crops. *Fusarium graminearum* and *Fusarium culmorum* are fungal pathogens that cause FHB in wheat. These species produce several mycotoxins, namely DON and zearalenone, which are toxic to plants and animals. To control FHB in the cereals, it is therefore crucial to have a good understanding and research of the pathogens and affected host. It is necessary to raise questions related with the distribution of the pathogens in the plant, the affected anatomy, as well as mycotoxin production and accumulation in the plant system.

Therefore, and to help clarify these questions, the first part of this study focused on the distribution of *F. graminearum* and *F. culmorum* in the wheat plant – *Triticum aestivum* – as well as DON production by these two pathogens in the plant system with time. In the second part of this study, natural tri-inhibitors (magnolol, carvacrol, ferulic acid and flavone), previously reported as effective in *in vitro* trials were tested in greenhouse experiments (*in vivo*). This was performed to evaluate the feasibility of their use to control Fusarium head blight (FHB).

It was concluded that DON concentration increases in the plant tissues with time, mainly in the ears and not so severely in the leaves. Surprisingly, this *F. culmorum* strain appeared to be the most aggressive, as it produced higher amounts of mycotoxin in the ears and F-leaves when compared with the *F. graminearum* strain. Furthermore, a statistically relevant linear relationship (R^2 > 0.90; p-value < α= 0.05) was found that correlates fungal growth and DON production in the infected ears of the wheat plant, meaning that an increase in the amount of fungi in the ears will most likely lead to an increase of mycotoxin in the respective tissues. Moreover, it was supposed that the mycotoxin was translocated within the plant system, as a considerable amount of DON was detected in the leaves of the plant, even when almost no fungi was present in the same samples. None of the tested natural tri-inhibitors was able, under the selected conditions, to decrease neither DON or the amount of fungi *in planta* trials, though they were reported as highly efficient decreasing DON mycotoxin *in vitro* trials. Prothiconazole, a standard used fungicide, was the only effective compound decreasing DON mycotoxin as well as fungi in the plants. Thus, more studies are required to consider proper formulations – tri-inhibitors or mixtures with fungicides – so that they can be absorbed not only into the wheat plants, but also disturb fungi growth and mycotoxin production as, for instance, prothioconazole does.

Keywords: Crop protection, fungicides, trichothecene inhibitors, DON mycotoxin, *Triticum aestivum*, *F. graminearum*, *F. culmorum*

Declaração

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Assinar e datar

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List of abbreviations and symbols

ADON	Acetyldeoxynivalenol
ATP	Adenosine triphosphate
BCAs	Biocontrol agents
Ct	Threshold cycle
CYTB	Cytochrome *b*
DMI	Demethylation inhibitors
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
Dpi	Days post inoculation
E	Amplification efficiency
EPPO	European Plant Protection Organization
F-1 leaf	First leaf below *flag* leaf
FHB	Fusarium head blight
F-leaf	*Flag* leaf
FUSACU	*Fusarium culmorum*
GIBBZE	*Fusarium graminearum*
NIV	Nivalenol
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
QoIs	Quinol oxidation inhibitors
qPCR	Quantitative PCR
STC	Solvent treated control
Tm	Melting temperature
TRI – genes	Genes responsible for Trichothecenes biosynthesis
UTC	Untreated control

1 Introduction

1.1 Framework and Project Presentation

BASF Crop Protection division focuses essentially on five areas: seed solutions, crops, soil management, animal farming and agricultural films. “Farming, the biggest job on Earth”, clearly presents the company passion and concern about the topic. Therefore, its positioning focuses on the current demands on food supply and farmers needs on new technologies and solutions. BASF Crop Protection is keen on developing sustainable solutions in farming with the goal of providing safe and affordable food for all.

Therefore, in the agricultural research center in Limburgerhof new active substances, seed treatments, biological crop protection solutions and formulation technologies are developed in the fields, greenhouses and laboratories where around 1700 employees work. Relative to fungicides, the company knows that fungal diseases cause crop losses and contaminate food with mycotoxins that can get into the animal food chain being a threat to human and animal health. As a result, this project focused on the interaction between two fungal pathogens – *Fusarium graminearum* and *Fusarium culmorum* – and the wheat plant – *Triticum aestivum*, with the ultimate goal of developing a new solution to decrease the level of DON mycotoxin in the wheat.

1.2 Project Planning

The concept was developed a few months before starting the internship. In February 2017, upon start, the work plan was designed. This initial plan was followed throughout the study. Based on the results obtained, some modifications were introduced whenever necessary. These changes were mainly performed to correct mistakes and obtain more accurate results.

1.3 Monitoring Meetings

In BASF, monitoring meetings took place every two or four weeks with Dr. Egon Haden, mostly when trials were finished to discuss results and progress. Initial meetings were scheduled as well as with other colleagues, either to discuss the planning or ask for judgement on the best techniques and methods to be followed.

In FEUP, two monitoring meetings were held with Dr. Olga Nunes (in April and in May) and emails were exchanged every week, to inform on the work performed, give notice on the progress made and ask for advice and judgement on the techniques and methods employed.

1.4 Employed Technologies

The technologies employed in this work were already established in the greenhouse and laboratory. Greenhouse technologies included spraying of the compounds and inoculation of fungi using specific equipment and protections. Laboratory technologies included DON analysis by the strip test from Charm Sciences, DNA extraction using DNA extraction kit by Macherey-Nagel and qPCR using Rotor Gene Q machine by QIAGEN.

1.5 Company Presentation

BASF (Badische Anilin- & Sodafabrik) is the largest chemical company in the world (Fig. 1). It was created in Germany, by Friedrich Engelhorn (1821-1902) that in 1861 began producing fuchsin (magenta), a red dye, and aniline, the raw material obtained from coal tar. On April 6 1865, Engelhorn founded a stock corporation in Mannheim under the name Badische Anilin- & Sodafabrik (BASF 2017).

Throughout history, BASF was pioneer in the production of several chemical compounds on industrial scale. One of the first achievements was the synthesis of the first natural dye in 1869: Alizarin, a red dye derived from the root of the madder plant, mainly used to dye cotton. Other new dyes such as eosin, true red and auramine followed (BASF 2017). This was performed by Heinrich Caro in collaboration with Berlin-based professors Carl Graebe and Carl Liebermann. In 1876, Heinrich Caro succeeded in synthesizing a pure blue dye for cotton – methylene blue, giving Germany's first patent for a coal tar dye for methylene blue. Methylene blue played an increasingly important role not just in the textile industry, but in medicine, too, being used by Robert Koch to stain the tubercle bacillus in his research on tuberculosis (BASF 2017).

BASF started conducting intensive research under the direction of Carl Bosch (1874 – 1940) for the synthesis of ammonia from nitrogen and hydrogen, as proposed by Fritz Haber (BASF 2017). Carl Bosch wanted fertilizers to be tested as thoroughly as dyes and customers should be given proper instructions for their use. This conducted to trials to determine the effect of fertilizers on soil and on plants. In 1914 the Agricultural Research Station in Limburgerhof was opened to investigate fertilizers and plant physiology (BASF 2017). This paves the way for BASF's worldwide activities in the field of agricultural chemistry.

"Chemistry for the Future is only possible if we have clear goals, and if we affirm and are willing to help shape the changes underway around us. [...] By moving into promising markets, BASF is adjusting to the challenges of growth and changing demand. [...] By means of responsible action, we have a duty to prove that chemistry and nature are not incompatible but instead form a whole. This is especially true for environmental protection, an area in which we are developing innovative solutions on our own initiative and on the basis of our worldwide expertise." – Hans Albers, Chairman of the BASF Board of Executive Directors from 1983 to 1990 on BASF 125th anniversary

Always concerned about the environment and its protection, BASF has conducted a series of experiments to develop the biodegradable plastics Ecoflex and Ecovio. With these plastics, BASF is one of the world's leading providers in the field of biodegradable and biobased plastics (BASF 2017).

BASF follows four strategic principles to seize the opportunities:

- ✓ Add value as one company
- ✓ Innovate to make their customers more successful
- ✓ Drive sustainable solutions
- ✓ Form the best team

The company has built since the beginning a comprehensive social policy tradition. Occupational safety and healthcare play a crucial role in its values.

Fig. 1 - BASF Headquarters Ludwigshafen am Rhein, Germany.

1.6 Dissertation Structure

This work is structured in seven main parts: the introduction, context and state of art, aim of the work, workflow, materials and methods, results and at last the discussion. In the first two parts, an introduction is made to the company and the current problems to be solved, as well as the already existent scientific background. Next, the aim of the work is presented, as well as the workflow, so that the reader can understand better the practical steps performed to achieve the results and conclusions. At last the discussion is presented, and the results are compared with previous studies to take the final conclusions and future improvements.

2 Context and State of Art

2.1 Population growth and sustainable agriculture

With population growth, ancient agricultural practices became not sufficient to feed every person in the planet. With time, the small-scale agriculture, practised by local farmers in small areas evolved to a large-scale agriculture, practised in very large fields. In fact, the world demographic explosion was only possible due to the increase of productivity of world agriculture, related with the development of motorized, mechanized and chemicalized agriculture in the developed countries.

Even though agriculture capacity has grown, there are several countries with famine and undernourishment. FAO (Food and Agriculture Organization of the United Nations) alerts that current population growth is demanding more livestock products, particularly in developing countries. The livestock sector is the world's largest user of agricultural land and feed crops and it also plays a major role in climate change, land and water management and biodiversity. This demand is projected to increase by 70% to feed a population that is expected to reach 9,6 billion by 2050 (Mazoyer and Roudart 2006). However, data from the Worldwatch Institute shows that the growing needs of humanity are approaching the nature exploitable limits (Mazoyer and Roudart 2006).

Because global population depends largely on agriculture to survive, maintenance of large crop fields is necessary. Pest control is one of the measures to maintain high productivity. Pathogenic organisms, hosts and environment relationships have been studied to implement measures to avoid crop loss. One of the measures to avoid crop losses was the development and introduction of pesticides in the market.

2.2 Affected crops and losses

Crop losses are usually related with both abiotic and biotic factors. Abiotic factors include irradiation, water supply, temperature and nutrient presence, while biotic factors are related with three major groups of pests: weeds (monocots, dicots and also parasitic weeds), animal pests (insects, birds, nematodes, rodents, and others), and pathogens (fungi, bacteria and also viruses). Global loss potential due to pests is approximately of 50% in wheat and more than 80% in cotton production (Oerke 2006). Generally, some of the most important affected crops produced worldwide are wheat, rice, maize, cotton, potatoes and soybeans (Oerke 2006).

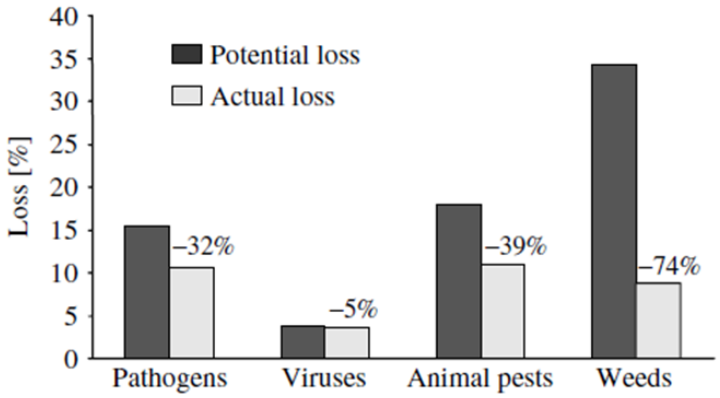

Fig. 2 - Average efficacy in pest control worldwide relative to pathogens, viruses, animal pests and also weeds. Reduction rates are calculated from estimates of production losses in barley, cottonseed, maize, oilseed rape, potatoes, rice, soybean, cotton, sugar beet, tomatoes and wheat, in 2001–03 (Oerke 2006).

Figure 2 shows the actual and potential loss in crops due to pests such as pathogens, viruses, animal pests and weeds. According to Oerke (2006), weeds represent the highest potential loss but also the highest efficacy of control (74%) when compared to other pests. This is because weeds may be controlled manually, by mechanical weeding or even by synthetic herbicides. Animal pests and pathogens show a considerable potential loss, and a good efficacy of control (39% and 32%, respectively) due to the availability of pesticides (Oerke 2006). Viruses present the lowest efficacy of control (5%) being most of the times controlled by insecticides, preventing the development of the virus vectors. Nevertheless, the control of pests that have a low potential loss does not justify an economic effort to increase efficacy in control. This means that efficacy in pest control usually increases with the loss potential (Oerke 2006).

Crop protection measures have been preventing crop losses worldwide (Fig. 3). Potential losses are higher in Africa because of high temperatures and common rainfall periods, which favour the development of pests. On the other hand, in moderate climate conditions present in Northwest Europe and North America pesticides are more available and training of farmers is an important issue to consider. Therefore, losses have been reduced considerably (Oerke 2006).

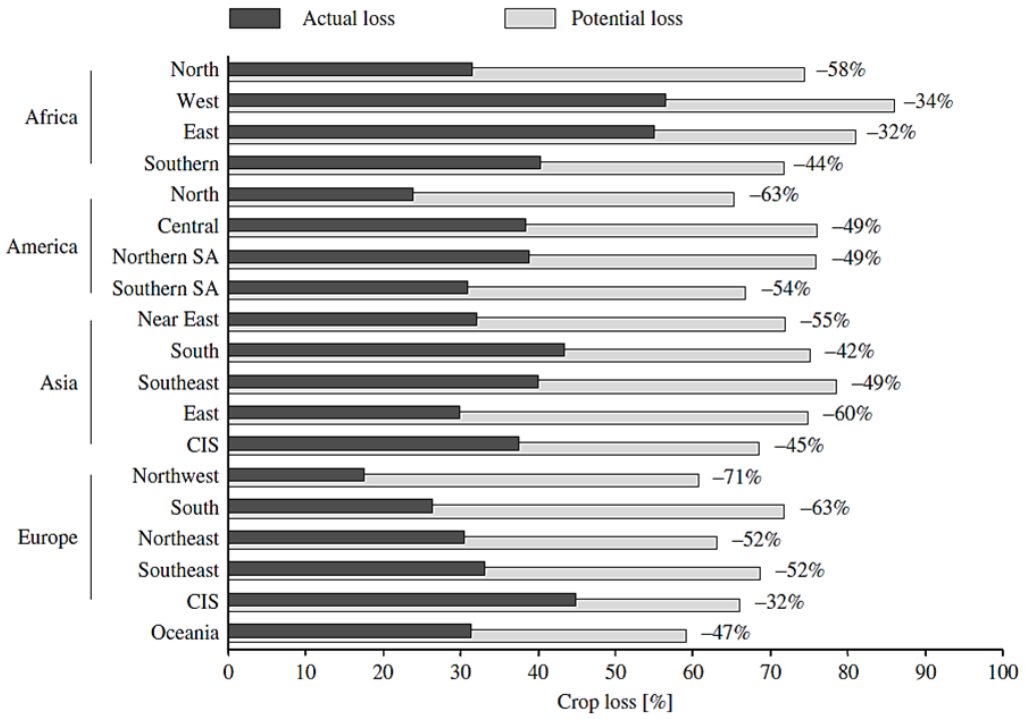

Fig. 3 - Regional differences in the efficacy of actual crop protection practices in 2001–03. Figures indicate reduction of total pest losses as calculated from regional loss potentials and actual loss rates (data based on estimates of monetary production losses in barley, cottonseed, maize, oilseed rape, potatoes, rice, soybean, cotton, sugar beet, tomatoes and wheat, by region) (Oerke 2006).

2.3 Fungi as pathogens

Koch determined that an organism is the source of an infection if (1) it can be isolated from the infected host, (2) be cultured in the laboratory, (3) be the cause of the same disease when inoculated into another host, (4) and be re-isolated from that host (Doohan 2005). According to these postulates there is a wide range of fungi classified as plant pathogens or phytopathogens.

Plant- pathogenic fungi can be mainly classified as biotrophs, which only grow when in contact with their host plants (e.g. fungi that cause rust), or non-obligate pathogens, which grow on dead organic matter as well as on living hosts (Doohan 2005). These last can be further distinguished as facultative saprophytes or facultative pathogens, differing on the time they spend most of their life cycle (on dead organic matter or as parasites in a living host) (Doohan 2005).

Some disease symptoms on plants caused by fungal parasitism include (a) localized on host leaves (spots), (b) disintegration of roots, (c) fruits and others (rots), (d) browning of leaves and branches (blight), (e) retarded development (decline), (f) fungal colonization of root or stem vascular tissue (wilts), and others. There is available data about diseases caused by different fungi in different hosts, and the symptoms occurring in each case (Doohan 2005). Therefore, it is possible to design a disease triangle, including the pathogen, the host and also the environment. These three factors are crucial in the disease severity of the plant. This means that it is important taking a further analysis of the different factors influencing the development of a disease in the plant (Doohan 2005).

Pathogens: The aggressiveness of the pathogens influence disease development in the plant, causing a faster or slower decadence of the host. Infective propagules, such as spores must be also present and the amount of inoculum influences disease development as well. The pathogen must be capable of competing with other organisms in order to survive and grow in the host plant (Doohan 2005).

The host: In order to have disease development, the host plant should be at a life cycle stage susceptible to infection. Furthermore, if a plant is resistant to a pathogen, it does not mean that it can be resistant to others. There are also plants that are disease tolerant, being capable to grow even when infected with some parasites (Doohan 2005). The genetics of each species (host and pathogen) also plays an important role.

Environmental conditions: As previously said, environment affects disease because of conditions such as temperature, wind, sunlight, moisture, and others (Doohan 2005). This is because pathogens also have optimum environmental growth conditions.

Disease cycle: This cycle is relative to the initiation of disease (infection) and to the dispersal of the pathogen to a new host plant (Fig. 4).

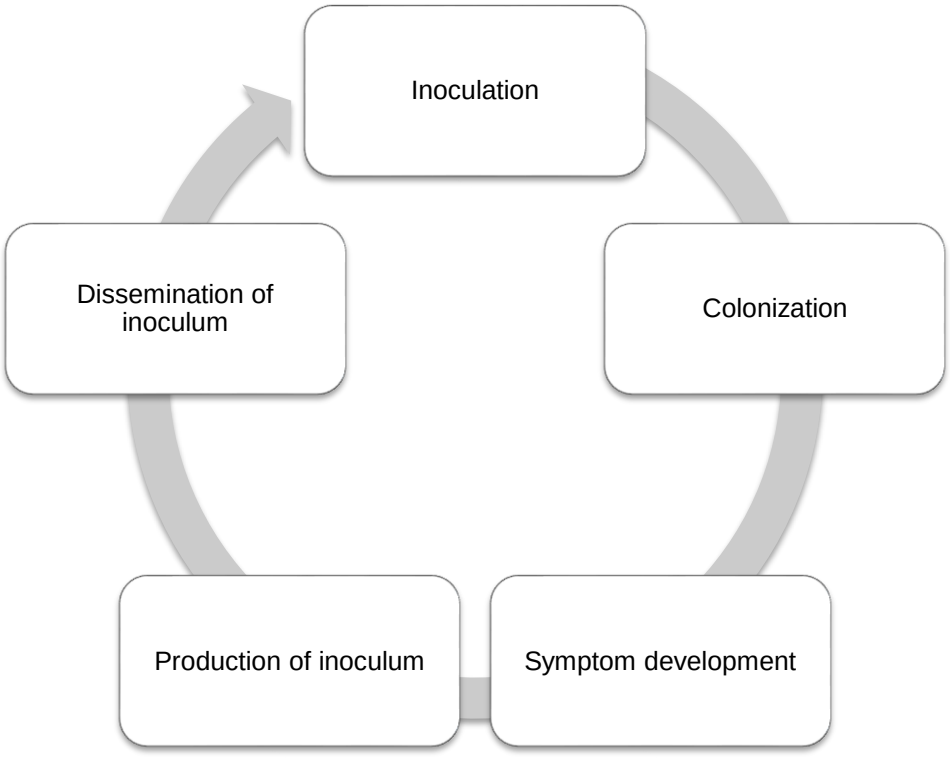

Fig. 4 - Generalized fungal disease cycle (Doohan 2005).

In this cycle, the inoculum is disseminated causing the infection of the plant tissue. The production (sexual or asexual spores, mycelium) and dissemination (wind, water, insect) of the inoculum depends on the type of pathogen. Penetration is usually through wounds or pores in the plant. Nevertheless, some fungi are capable of producing penetration structures called *appressoria*

(Doohan 2005). The proteins secreted by the pathogen may interact with the plant at several levels: (1) release of enzymes that breakdown host macromolecules to provide nutrition for the pathogen, (2) toxin proteins that disrupt cellular function of the host and lead to cell death and (3) immune modulator proteins that alert the host to pathogen attack to prevent colonization (Lowe et al. 2015).

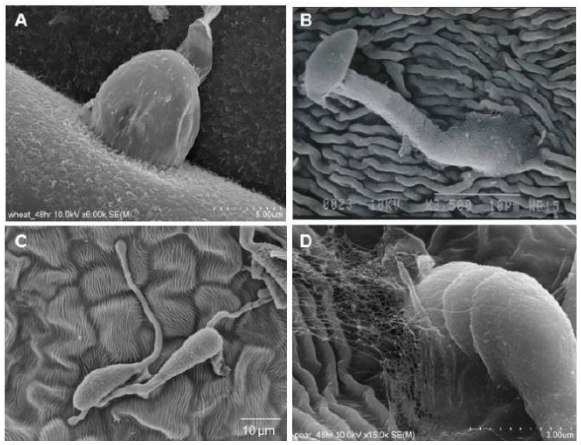

Fig. 5 - Scanning electron microscope image of *appressoria* of phytopathogenic fungi on the host plant surface (Ikeda et al. 2012).

After penetrating its host, the fungus starts growing and utilizing plant resources - key nutrients such as carbon – as a source of cellular energy – and nitrogen for synthesis of proteins and nucleic acids - leading to disease symptoms in the plant. There is usually a latent period until disease manifestation.

Mechanisms of parasitism: These include mechanical means (adherence to host tissue and penetration, development of *appressoria* (Fig. 5) as well as metabolite mediated means. These last ones may include production of enzymes that degrade cellular and intercellular constituents of plants, and production of polysaccharides, toxins and growth regulators (Doohan 2005). Focusing on toxins, they vary from low molecular weight metabolites to proteins. These can be host or non - host specific toxins, the first being specifically required for pathogenicity and affecting just one type of host, and the second having a wider range of action. Especially mycotoxins can also affect human and animal health (Doohan 2005).

Disease control: Diseases may be controlled through cultural practises such as (1) plant rotation in cropping systems, which help prevent inoculum formation, and (2) good sanitary practices preventing dissemination of inoculum (Doohan 2005). There is also fungicide control or even the use of biocontrol agents (BCAs) that affect directly or indirectly fungal diseases in plants, for example, when a certain microbial population inhibits fungal growth. BCAs can act directly through secretion of antifungal compounds, indirectly through physical exclusion of pathogen from the host as well as inducing host defence through several mechanisms (Doohan 2005).

2.4 *Triticum aestivum*: The structure and development of the wheat plant

The wheat (*Triticum*) plant belongs to the cereals group as well as barley (*Hordeum*), oat (*Avena*), rye (*Secale*) and the man-made hybrid triticale (*Triticosecale*). Currently, there are 30 species of wheat which can be divided according to the number of chromosomes present in the vegetative wheat plant cells: diploid (14 chromosomes), tetraploid (28 chromosomes) and hexaploid (42 chromosomes) (Perry and Belford 2000). This cereal is widely consumed in Europe, North of Africa, Asia and Oceania (Fig. 6).

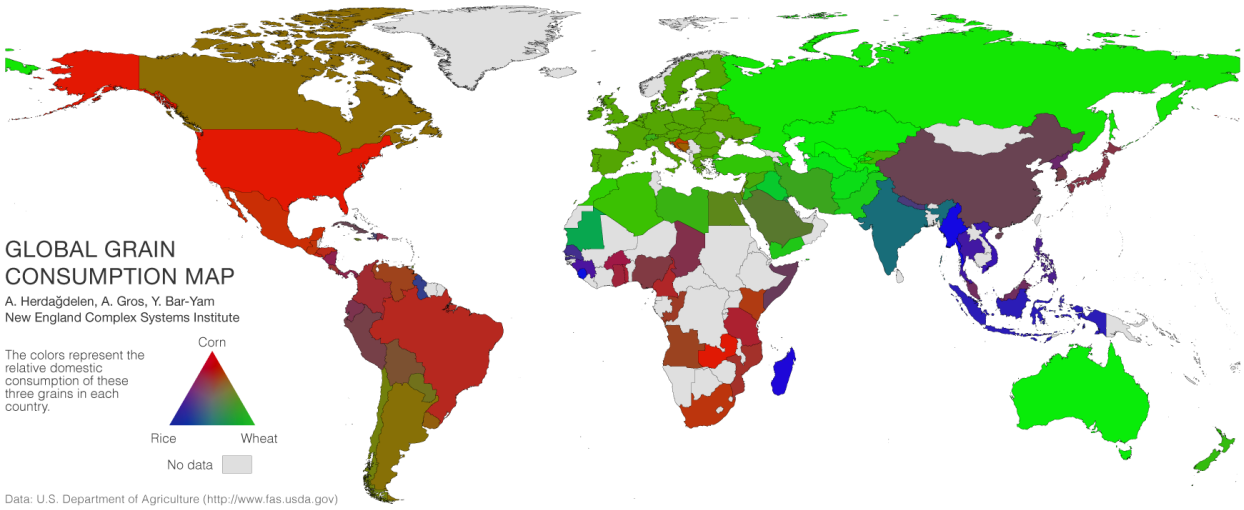

Fig. 6 – Global Grain Consumption Map (Herdağdelen and Bar-Yam 2011).

The ear. It is constituted by spikelets arranged on opposite sides of the central rachis, being attached to them by the rachilla at each node (Fig. 7). At the base of the spikelets there are the empty glumes, which enclose individual flowers called florets in grasses and only two or four florets form grains in every spikelet leading to a development of 30 to 50 grains in each ear. The flower produces one grain that develops in the *lemma* which is enclosed in the *palea* (Perry and Belford 2000).

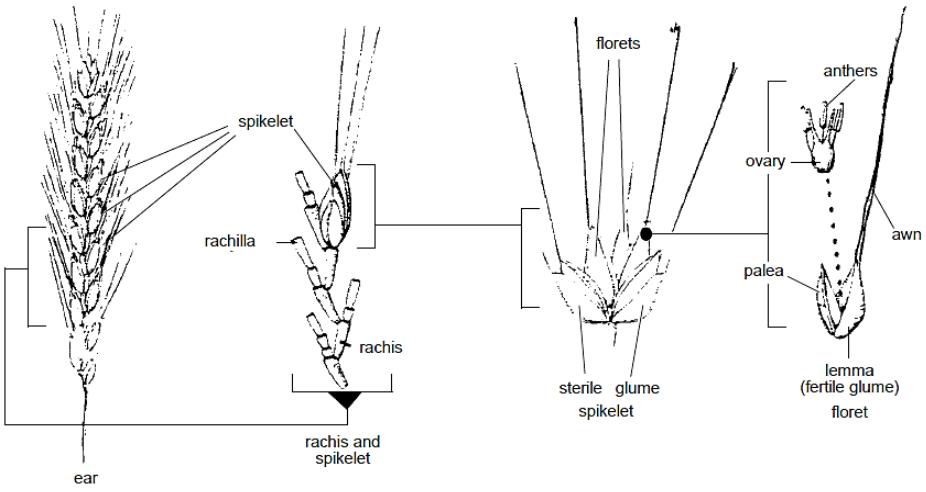

Fig. 7 - Structures of the wheat ear, spikelet and floret (Perry and Belford 2000).

The grain (also called kernel): This is the unit of reproduction in cereals, as well as the economic product (Fig. 8). It is between 3 and 8 mm long, and it is dry. In wheat, the pericarp is thin and is fused with the seed coat, while in other plants such as berries, the pericarp may be fleshy. The main constituents of the grains are the bran coat, the embryo, and the endosperm. The endosperm is about 83% of the grain while the embryo present on the germ is approximately 3%. The bran coat is an important source of enzymes and other nutrients for germination. The endosperm is the source of energy of the germinating seed, containing carbohydrates and proteins, being milled to produce flour. Nevertheless, before using the grains to produce flour it is first necessary to remove the hulls, which are a thin dried layer constituted by the glumes, lemmas and paleas that cover the grains (Perry and Belford 2000).

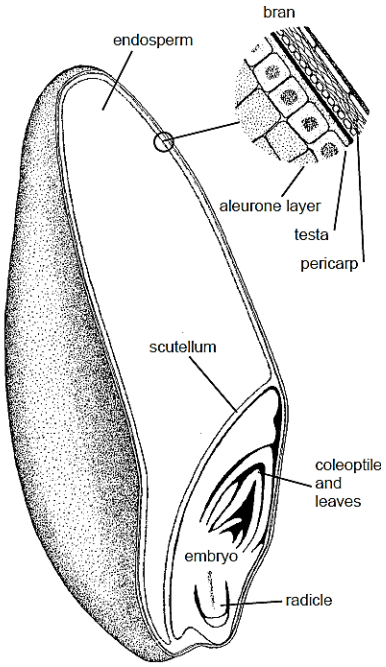

Fig. 8 - The structure of the wheat grain (Perry and Belford 2000).

The stem: The stem is made of several nodes and is wrapped in the sheaths of the surrounding leaves. The stem is essential to keep the cereal shoot erect and reducing lodging. In the stem also occurs the exchange of nutrients with the vascular channels. The tissue between two adjacent nodes is called internode. When the stem grows it also becomes a source of carbohydrates and nutrients for later grain filling (Perry and Belford 2000).

The leaf: As previously referred to, about three leaves are present around the shoot apex of the embryo at germination. After gemination more leaves are produced on different sides of the apex. The final leaf that develops before the ear is called the *flag leaf* (F-leaf). The leaf after the *flag* leaf is called first leaf (F-1 leaf). The wheat leaf is long and narrow with a *basal sheath* encircling the steam of the plant and contributing to the stem strength and also a leaf blade which is the primary photosynthetic tissue of the plant (Perry and Belford 2000).

Germination and emergence are considered to be very important stages in the life cycle of the wheat plant due to the fact that it is mainly in these stages that wheat is more vulnerable to pests and also environmental hazards (Tennant, Siddique, and Perry 2000). Environmental factors such as moisture, suitable temperatures and adequate oxygen are important for germination of non dorment seeds. The minimum water content of seed germination is said to be about 25 to 40% and the optimum temperatures between 22ºC and 25ºC (Tennant, Siddique, and Perry 2000).

2.5 Fusarium graminearum and *Fusarium culmorum*

The genus *Fusarium*, belonging to division Ascomycota, currently contains over 20 species. The identification of its species is difficult due to great variations not only in morphological but also in non-morphological characteristics (e.g. virulence) (Wagacha and Muthomi 2007). Therefore, separation of *Fusarium* species is based on primary characteristics – shape of macroconidia, presence or absence of microconidia, and others – secondary characteristics – presence or absence of chlamydospores and their configuration and position, as well as presence or absence of sclerotia. Other characteristics such as colony morphology, pigmentation, and growth rate can be helpful as well (Wagacha and Muthomi 2007).

Fusarium graminearum (anamorph) (also named by *Gibberella zeae* (teleomorph)) and *Fusarium culmorum* are two very important species worldwide because of their interaction with small grain cereals and also corn, occurring in a wide range of soils and environmental conditions (Turkington et al. 2014). They share very common characteristics as well as some differences. Whereas *F. graminearum* usually grows better in warmer, humid areas of the world, *F. culmorum* prefers colder areas such as north, central, west Europe and Canada (Wagacha and Muthomi 2007). *F. culmorum* has been concretely found as predominant species in Rhineland region in Western Germany and is reported as capable of causing severe disease and loss of grains (Wagacha and Muthomi 2007).

F. graminearum presents a “banana shaped” macroconidia, which contain several *septae* and produce ascospores, promoting its dispersal. In contrast, *F. culmorum* does not produce ascospores, producing instead macroconidia being the principal mode of dispersal. These conidia are dispersed by the wind or rain splashes to the wheat, infecting it during a short period. The success of infection depends on factors such as temperature, humidity, nitrogen fertilization and mainly availability of humidity (Wagacha and Muthomi 2007). They both use glucose as carbon source, undergoing aerobic respiration and grow rapidly on potato dextrose agar, creating a dense aerial mycelium that is usually white but it can also be yellow to tan.

2.6 Mycotoxin production

Fungi produce a large number of metabolites that are not essential for their survival, but might provide an ecological advantage in the respective environment. These secondary metabolites include growth regulators, pigments, useful compounds such as penicillin as well as mycotoxins (e.g., aflatoxins, trichothecenes, fumonisins) (McCormick et al. 2011). These mycotoxins may be a problem when accumulated in crops, mainly in those fed to animals, because they enter the food chain causing diseases both in humans and animals. In fact, trichothecenes are one of the major classes of mycotoxins, having a considerable economic impact on crops every year (McCormick et al. 2011). Trichothecenes are small and amphipathic molecules being capable of moving passively across cell membranes, being absorbed in the gastrointestinal system and proliferating through the tissues. These molecules are reported as inhibitors of eukaryotic protein synthesis, and ingestion of these toxins may cause feed refusal, vomiting, immunological problems, and others. They are also reported as phytotoxic, inhibiting root elongation, chlorosis and acting as a virulence factor in wheat head scab (McCormick et al. 2011).

Trichothecenes are a group of over 200 toxins and have in their structure a common tricyclic skeleton with an epoxide function, since they derive from the isoprenoid intermediate farnesyl pyrophosphate via a series of reactions such as cyclization, isomeration, oxygenation and acetylation reactions (Alexander et al. 2011). These molecules are currently classified in four groups (Types A, B, C and D). Types A, B and C can be distinguished by the substitution at the C-8 position. For instance, Type A trichothecenes have a ester function at C-8 (e.g., T-2 toxin), while Type B have a keto function at C-8 (e.g., nivalenol, deoxynivalenol and trichothecin) (McCormick et al. 2011).

As previously mentioned, the biosynthesis of trichothecenes in *Fusarium* starts with the cyclization of farnesyl pyrophosphate to form trichodiene (Fig. 9). The cyclase trichodiene synthase catalyzes this reaction and is encoded by *TRI5* gene. Trichodiene suffers a series of oxygenations which are catalysed by cytochrome P450 monooxygenase encoded by the *TRI4* gene, giving isotrichotriol. This molecule is then converted to isotrichodermin by an acetyltransferase encoded by gene *TRI101*. In Type B producing strains, TRI1 controls the addition of hydroxyl groups at C-7 and C-8 positions. The presence of C-7 hydroxyl group in *Fusarium* is correlated with the transformation of C-8 hydroxyl group to a keto function. The removal of the C-3 or the C-15 acetyl group by an esterase encoded by gene *TRI8*, is the final step in *Fusarium* Type B trichothecene biosynthesis. The differential activity of this esterase is defined by the DNA sequence of the organism, which makes it produce either 3-ADON or 15-ADON (McCormick et al. 2011).

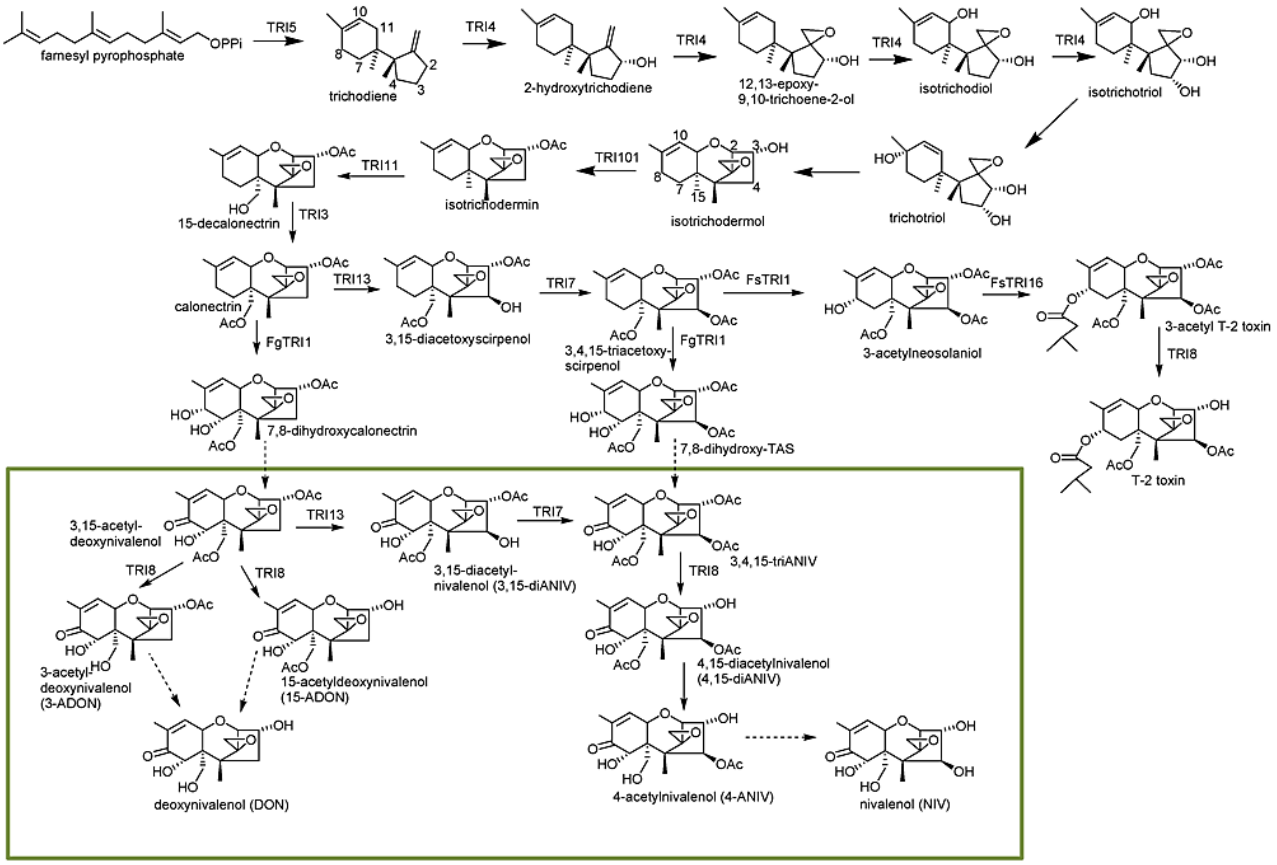

Fig. 9 – Proposed trichothecene biosynthetic pathway in *Fusarium*. The genes responsible for an enzymatic step are identified in the respective arrows. In dashed arrows the gene has not been appointed. The green box is identifying Type B trichothecenes (McCormick et al. 2011).

Deoxynivalenol (DON) belongs to Type B trichothecenes, and is produced by several *Fusarium spp*. Fungi belonging to the *F. graminearum* species complex (including also *F. culmorum*) present three major trichothecene production phenotypes: 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and NIV (nivalenol) (Alexander et al. 2011). DON acts as a virulence factor, being essential for the development of disease symptoms after the initial infection and considered the most economically important mycotoxin (Audenaert et al. 2014).

Due to its toxicity, the European Commission set legislative limits for the DON in cereal grains and cereal based products for human consumption. The maximum value for DON in unprocessed wheat is 1250 µg/kg, corresponding to 1.250 ppm (Edwardsa and Godleyb 2010). It withstands high temperatures, being documented as stable within the interval from 170ºC to 350ºC. This rises the problem of being present in food when wheat crops are not properly treated (Sobrova et al. 2010).

DON properties are summarized in table 1.

Table 1 - Physico-chemical properties of deoxynivalenol (DON).

Property	Information
Name	Deoxynivalenol (DON), vomitoxin
IUPAC Name	12,13-Epoxy-3-α,7-α,15-trihydroxy-9-trichothecen-8-one
Molecular formula	$H_{15}H_{20}O_6$
Molar mass	296.32 g/mol
Physical state	colourless fine needles
Melting point (ºC)	151–153 °C
Solubility (mg/L)	5.5×10^4 (25 °C)
Soluble in:	polar organic solvents (e.g., aqueous methanol, ethanol, chloroform, acetonitrile, and ethyl acetate) and water

2.7 Interaction with *Triticum aestivum:* Fusarium head blight

F. graminearum and *F. culmorum* produce several mycotoxins, as aforementioned. These mycotoxins, namely DON, seem to be involved on the development of Fusarium head blight in the wheat plant, due to its toxicity (Fig. 10). There is evidence suggesting that DON production during the infection is a strategy developed by the fungi to hijack the plant's defence system. The penetration of the fungi in the plant usually occurs after an initial superficial growth of the pathogen. After penetrating the host tissue, the fungi grows biotrophically into the intercellular spaces; the production of the mycotoxin (DON) is not considered important in this phase. Afterwards, the pathogen starts to be more invasive in its growth, inducing necrosis and cellular death in the host. This is named as the necrotrophic infection phase, when the production of DON occurs and is necessary for the growth of the fungi in the rachis of the wheat. Previous studies have shown that *tri5* knockout mutants are less virulent, as they do not produce trichodiene, implying that DON is essential for ear colonization (Audenaert et al. 2014).

The infection develops in the flowering stage of the wheat plant and is caused by ascospores and macroconidia produced by the fungi. Biotic and abiotic agents favour its dispersal, as well as certain weather conditions. Table 2 summarizes important disease related parameters.

Table 2 – Fusarium head blight development in the wheat plant caused by *F. graminearum* and *F. culmorum*. Propagules, weather conditions and symptoms are summarized (Bushnell, Hazen, and Pritsch 2003).

Disease	**Fusarium head blight**
Host	*Triticum aestivum*
Pathogens	*Fusarium graminearum* and *Fusarium culmorum*
Propagules	Macroconidia, ascospores, hyphal fragments and chlamydospores
Dispersal agents	Abiotic (wind, water, soil) and biotic (animals such as insects)
Favourable conditions for infection	Long moisture periods on early disease stages; continuous wetness at 25ºC
Affected stage of the plant life cycle	Flowering: The first visible symptoms of infection usually appear on the first florets to flower
Symptoms	First browning and pinkish tint given by the fungus; On advanced stages, production of macroconidia giving a pink colour to margins and surface of florets and glumes; Floret discoloration spreads apically and basally in the head of infected spikelets, and the entire apical portion of the head above an infected spikelet turns the colour of mature heads

Fig. 10 – Fusarium head blight development on the ears of the wheat plant (left). Infected and healthy kernels (right) (The American Phytopathological Society 2017).

The primary interest in the research of FHB is based on the toxicity of these mycotoxins, namely DON, to plants, animals and humans (Audenaert et al. 2014).

2.8 The need of fungicides: classification, advantages and disadvantages

The first use of pesticides, namely insecticides and fungicides (sulphur compounds and botanicals), is recorded by the Sumerians and the Chinese between 2500-1500 BC (Oerke 2006). Inorganic chemicals such as copper, sulphur and inorganic mercury were then adopted, being considered the first generation of fungicides (Oerke 2006). The second generation included organic chemicals as surface protectants while the third generation of fungicides (benzimidazoles, phenyllamides, azoles, morpholines and others) penetrate the plant tissue and control infections leading to plant recovery (Oerke 2006). Currently, fungicides may be classified for example as systemic, mobile, curative and protectant (Fig. 11) (Oliver and Hewitt 2014).

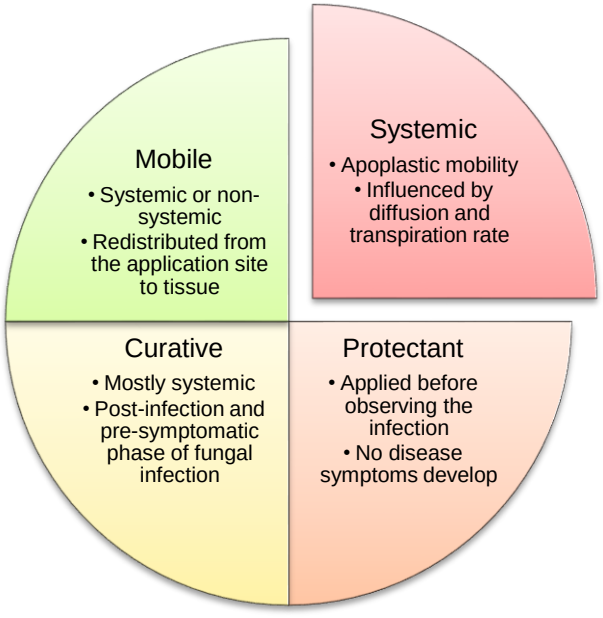

Fig. 11 – Fungicides classification.

Fungicides have different modes of action: they may inhibit nucleic acid synthesis, mitosis and cell division, respiration, among others. Benzimidazoles are compounds that inhibit mitosis and cell division acting on β-tubulin assembly. These molecules were the key for systemic fungicides development in the 1960s, including benomyl, carbendazim, thiophanate-methyl, fuberidazole and thiabendazole (Fig. 12) (Oliver and Hewitt 2014).

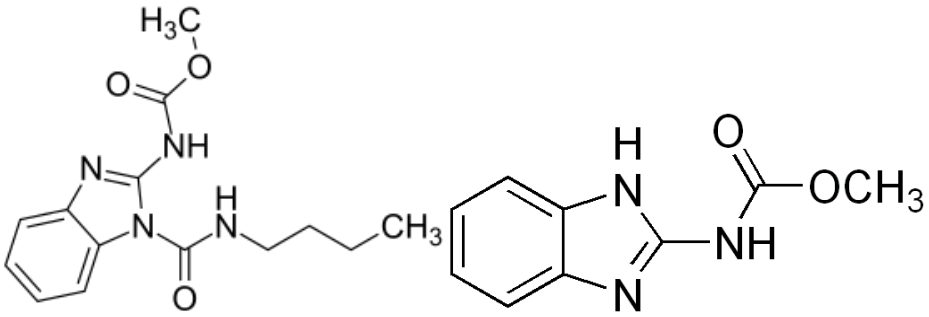

Fig. 12 - Benomyl (in the left) and carbendazim (in the right).

The group of fungicides, mainly known as QoIs also called strobilurins, are active in the inhibition of electron transfer in complex III in the mitochondrial electron transport chain (Fig. 13). Most commonly, they are known to inhibit the mitochondrial respiration by binding to the Qo site in cytochrome *b*. Cytochrome *b* is a protein which is present in the mitochondria of eukaryotic cells, namely in the subunit of cytochrome *b*c1 enzyme complex (complex III). In this inhibition, electron transfer is blocked between cytochrome *b* and cytochrome c_1 which results in an energy deficiency in the fungal cells, reducing the synthesis of ATP (Fernández-Ortuño et al. 2008).

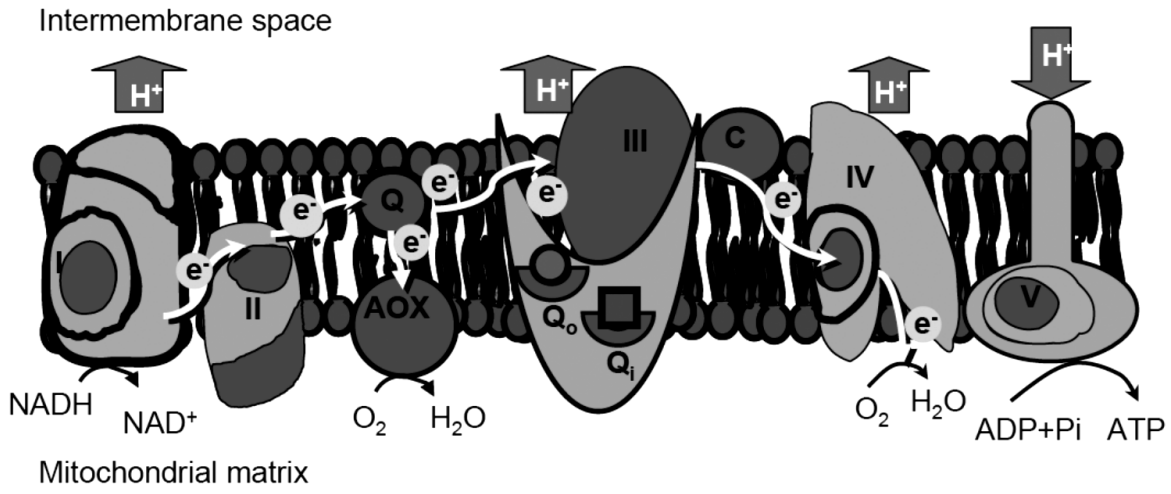

Fig. 13 – Representation of the mitochondrial electron transport system (complexes I, II, III, IV, V). Qo and Qi binding sites of the cytochrome *b*c1 enzyme complex (complex III) are delineated by a circle and a square representing Qo- and Qi-inhibitor molecules, respectively (Fernández-Ortuño et al. 2008).

These compounds have slow acting systemic properties, being able to provide long term disease control. Distribution within the crop involves continuous mechanism of absorption from

leaves into the plant and also by movement via vapour phase and reabsorption into cuticular waxes. These compounds are active against Ascomycota, Basidiomycota and Oomycota (Oliver and Hewitt 2014).

QoIs have been essential to crop protection since their introduction in the market, making over 50% of the sales together with demethylation inhibitors (DMI) (Oliver and Hewitt 2014). They have been used in different crops, such as cereals, grapevine, turf grass, vegetables and ornamentals. Nevertheless, an increasing concern relative to resistance to QoI fungicides has been growing. The resistance to these fungicides arises in first place with mutations in the mitochondrial cytochrome *b* gene (CYTB) (Fernández-Ortuño et al. 2008). Two important amino acid substitutions need to be considered: the first at position 143 from glycine to alanine (G143A) and the second from phenylalanine to leucine at position 129 (F129L). These substitutions have been detected in cytochrome *b* of some phytopathgenic fungi and oomycetes that are resistant to QoIs (Fernández-Ortuño et al. 2008). Usually, the nucleotide substitution resulting in alanine at position 143 is lethal since it will lead to a deficient cytochrome *b* (Grasso et al. 2006). However, some species survive even with this mutation (G143A), becoming resistant to QoI fungicides. Therefore, some techniques such as allele-specific PCR and quantitative PCR (qPCR) have been used to detect and quantify this G143A.

Several fungicides have been tested to reduce FHB and DON in harvested wheat. The most important that are currently used are the azoles. Within this group, tebuconazole, metconazole and prothioconazole (Fig. 14) were effective reducing DON content (Audenaert et al. 2014).

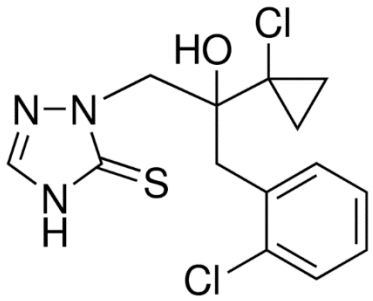

Fig. 14 – Prothioconazole molecule.

Regarding *in vitro* tests, strong inhibitory activities were also demonstrated by some natural compounds – phenolic and polyphenolic – against trichothecene producing strains such as *F. graminearum* and *F. culmorum*. Several compounds were tested at different concentrations, being after evaluated the vegetative growth and 3-ADON production by *F. culmorum* strain ISPaVe MCf21 (Pani et al. 2014). Carvacrol (Fig. 15), a phenylpropanoid, showed almost complete 3-acetyl-4-deoxynivalenol inhibition and no significant fungal growth inhibition at 0.25 mM. Ferulic acid and magnolol (Fig. 16), both hydroxylated biphenyls, demonstrated capacity to reduce 3-ADON production. Nevertheless, magnolol retained the capacity of inhibiting DON production by the fungus even at the lowest concentration and showed fungicidal activity even at 0.1 mM (Pani et al. 2014).

Other compounds such as flavone (Fig. 15), showed a very potent inhibition of *TRI4*: at 100 µM, flavone inhibited 68% the hydroxylation of trichodiene early in the biosynthesis of trichothecenes (Takahashi-Ando et al. 2008). Furanocoumarins, namely bergapten and xanthotoxin also shown to be efficient TRI4 inhibitors (Takahashi-Ando et al. 2008).

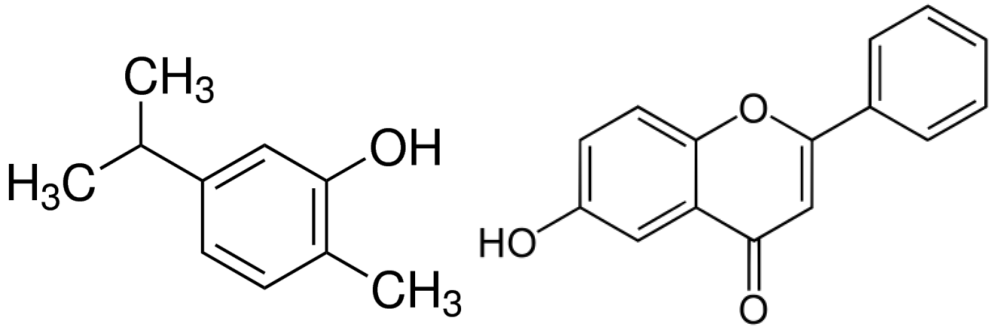

Fig. 15 – Carvacrol (left) and Flavone (right).

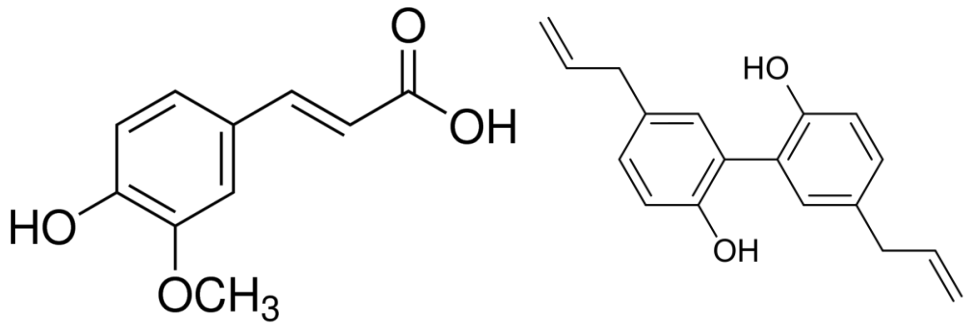

Fig. 16 - Ferulic acid (left) and Magnolol (right).

Fungicide use has great advantages in crop protection and is extensively used nowadays, but it also has disadvantages. Examples of these disadvantages are increased population risk exposure to these compounds, resistance development and environmental related issues. In fact, fungicides must be treated or eliminated in such way that they are not prejudicial to ecosystems and living organisms. Therefore, some attributes to be targeted in new fungicidal products are safety (to users, environment and consumers of the treated product), performance (broader disease control spectrum, extended control period, anti-resistance activity and improved crop safety), use (easy to use formulations, safe application) and cost (cheaper fungicides, lower use rates, fewer treatments, lower cost per treatment) (Oliver and Hewitt 2014). Alternatives to fungicides might be biological pest control (such as interfering with insects' reproduction), cultivation practises, among others. However, the main constraints to the implementation of biological pest control are said to be related with the its small market that faces not only high levels of costs evaluating environmental risks but also the large amount of documents required for the registration of the respective products (Bale, Lenteren, and Bigler 2007). Therefore, the procedure is costly and time consuming. Furthermore, the regulation of these agents must be performed without introducing negative trade-offs, meaning that the associated costs need to be set to a minimum level, without compromising the environment and the

human health (Bale, Lenteren, and Bigler 2007). Nevertheless, in the future, pest management is believed to depend strongly on biological control as it is the most sustainable and environmentally safest system (Bale, Lenteren, and Bigler 2007).

3 Aim of this work

This study focused on the distribution of *Fusarium graminearum* and *Fusarium culmorum* in the wheat as well as DON production in the plant with time. Specifically, it aimed at evaluating not only the plant mass loss but also the growth of both pathogens and DON production in the ears, F-leaf and F-1 leaf, occurring after inoculation of the fungi in the flowering stage of the wheat.

Another major objective was to evaluate the feasibility of using natural tri-inhibitors to control Fusarium head blight (FHB). Specifically, it aimed at evaluating the growth of both types of fungi and DON production in the wheat after the treatment of the plants with magnolol, carvacrol, ferulic acid and flavone.

4 Workflow

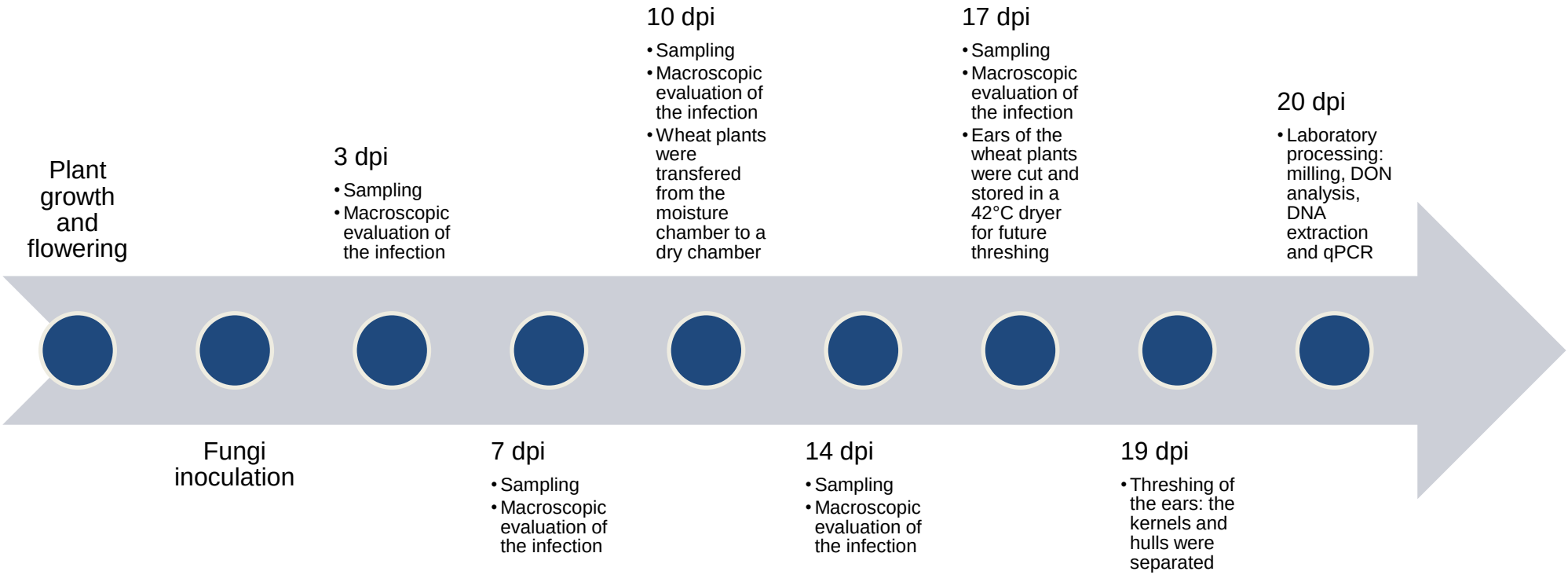

Fig. 17 - Trials A1 and A2. Study the distribution of *Fusarium graminearum* and *Fusarium culmorum* in the wheat as well as DON production in the plant with time. Workflow and check points.

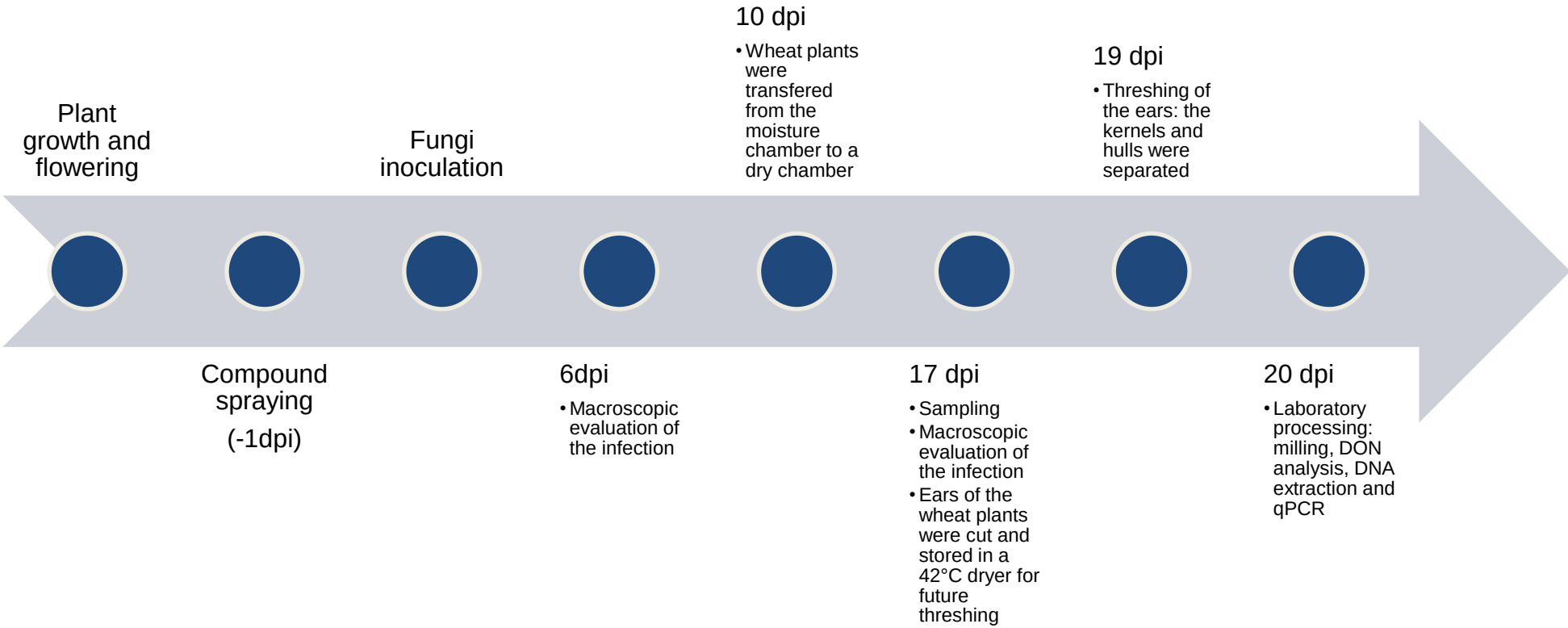

Fig. 18 – Trial A3. Test natural tri-inhibitors *in vivo*, namely magnolol, carvacrol, ferulic acid and flavone, reported as effective in *in vitro* trials, to evaluate the feasibility of their use to control Fusarium head blight (FHB). Workflow and check points.

5 Materials and Methods

This work consisted of 3 trials: Trial A1, Trial A2 and Trial A3. The first two trials (A1 and A2) focused on the study of the distribution of *F. graminearum* and *F. culmorum* in the wheat as well as DON production in the plant with time. Trial A3 was designed to test natural tri-inhibitors *in vivo*, namely magnolol, carvacrol, ferulic acid and flavone, reported as effective in *in vitro* trials, to evaluate the feasibility of their use to control Fusarium head blight (FHB). The workflow is presented in Figure 17 and Figure 18, respectively.

5.1 Trials A1 and A2: Distribution of *F. graminearum* and *F. culmorum* in the wheat plant. Fungi growth and DON production with time

5.1.1 Plant growth and flowering

The wheat variety (the USU – Perigee) used in this study was developed in the Utah State University (United States of America) with the support of Prof. Bruce Bugbee from the Department of Plants, Soils and Climate (Utah State University 2017) for space experiments. The big advantage of this variety is the shorter life cycle and the dwarf habit. The seed heads emerge faster and the plant is only 40 cm tall, being half the height of common wheat (Utah State University 2017).

The USU – Perigee plants were grown in pots of approximately 913 cm^3 capacity (~ 1 liter) containing the universal substrate – Spezial – Substrat – from Flora Gard, 100% natural, fertilizer-free and providing a good moisture absorption without soaking and mold formation. The total number of pots used in Trials A1 and A2 was respectively 53 and 51 with an average number of 10 wheat plants (10 to 12 seeds) per pot. The wheat plants were grown in a greenhouse, at 22ºC and 40% to 60% relative humidity until the flowering stage, taking approximately 7 weeks. The luminosity in the chambers was always above 6 K Lux: From beginning September to end of April the light period was from 4 am-6 pm; From April to end of August the light period was from 4 am-sunset. When the luminosity was below 6 K Lux a CHD AGRO 400-watt lamp from DH-Licht was used. The fertilizer Kamasol® brilliant blue from COMPO GmbH & Co KG was added separately. It contained 8% total nitrogen (N) (1.4% nitrate nitrogen, 2.8% ammonium nitrogen and 3.8% carbamide nitrogen), 8% water-soluble phosphate (P2O5), 6% water-soluble potassium oxide (K_2O), as well as low percentage of water soluble boron (B), copper (Cu), iron (Fe), Manganese (Mn), Molybdenum (Mo) and zinc (Zn). The wheat plants were watered every day and specifically at Mondays and Wednesdays, water with fertilizer Kamasol® brilliant blue (1 liter kamasol mixed with 2 liters water) was used. The wheat transferred to dry chambers (after day 10) was not watered.

5.1.2 Pathogen Inoculation

Pathogen inoculation was immediately scheduled after noticing flowering in the wheat plants. The plants of 25 pots were inoculated with a spore suspension of *F. culmorum* (EPPO code FUSACU), previously isolated from the North of Germany (Kiel), whereas those of other 25 pots were inoculated with *F. graminearum* (EPPO code GIBBZE), previously isolated from France (Marchélepot).

To prepare the spore suspension, the fungal strains were grown in PDA (Potato Dextrose Agar) from BD Difco™ for 10 to 15 days, at 22°C and 66% rH (relative humidity) in a UV climate incubator (Rubarth Apparate GmbH), with alternate light (12 hours day and 12 hours night). After the incubation period, the mycelia in the plate was mixed with water containing 1% TWEEN 80™ (Polysorbate 80 from Merck KGaA), which is a nonionic surfactant that acts lowering the surface tension between the spore solution and the surface of the plant material, allowing a better interaction between the fungi and the plant. To separate the mycelia from the spores, the suspension was filtered through a gauze that was placed on the top of a 50 ml Falcon tube from Sarstedt. The number of spores per volume of suspension was determined by microscopy. For this, 10 µl of each spore suspension was pipetted into a C-Chip™ disposable haemocytometer, with two individual Neubauer Improved (NI) counting chambers. The number of spores was counted with an Olympus CX31 microscope. The hemocytometer was placed on the microscope under the 10x objective to facilitate the localization of the grid. The volume of the spore suspension to be mixed with 300 ml deionized water (with 1% TWEEN 80™ from Merck KGaA) was calculated according to the number of spores per millilitre, to reach a final number of 10^4 spores/ml.

The flowered wheat plants were sprayed with the spore suspension using the SATAminijet 3000 BHVLP 0.8SR spray gun. The plants of 3 pots (Trial 1) and 1 pot (Trial 2) were not sprayed and were directly taken from the greenhouse for further analysis in the laboratory, being used as control. This was important to check absence of previous fungi infection in the tested wheat.

After the spraying step, the pots were placed in cars and covered not only to facilitate transportation but also to avoid disturbances in the environment (see Appendix, Plant growth and flowering, compound spraying and fungi inoculation). The pots were placed randomly in the cars to have independent samples. The cars were transported to a high moisture chamber, with an average temperature of 20°C and relative humidity between 80 and 90%. This chamber provides a good environment to maintain the wheat plants and favours fungi growth, as both water and nutrients are abundant and constantly available. Ten days after the inoculation, the pots (plants) were transferred from the high moisture chamber into a dry chamber, with an average temperature of 22°C and humidity of 40 to 50%. They stayed in this chamber until day 17 after inoculation, without being watered and therefore lacking optimal conditions, aiming to induce the natural ripening process. Therefore, this change in the chambers was performed to avoid not only plant rotting, but also to stop the optimal growth conditions of the fungi.

5.1.3 Sampling and Storage

The plants were analysed before the inoculation (control) as well as 3, 7, 10, 14 and 17 days after the inoculation (dpi). At each sampling day, 5 pots infected with *F. graminearum* and 5 infected with *F. culmorum* were collected from the greenhouse. All the wheat plants from the respective pots were cut in three anatomic sections: the ears, *flag* (F) -leaf and F-1 leaf (see Appendix, Sampling and Storage). On day 17 sampling was carried out as previously described, with the exception that all the ears were stored in a 42°C drier from Binder for two days, to further separate the kernels and the hulls upon threshing (see Appendix, Threshing and Milling). This was not performed in the previous sampling days because the respective samples (plants) did not provide sufficiently ripened kernels to do so. All the plants of the 5 pots were considered for mass measurements, but only those from 3 pots were used for DON analysis, DNA extraction and qPCR analysis. Hence, the anatomic sections of the plants from 2 pots were stored in bags for fall-back measurements at -20°C freezer from Kirsch or -80°C freezer from New Brunswick Scientific. The diagrams in section Workflow allow a better understanding of this process.

5.1.4 Mass loss and macroscopic evaluation

At each sampling day, the wet mass of 10 ears, 10 F-leaves and 10 F-1 leaves of each collected pot was measured with a Kern EG precision balance from Waagen-Kissling, aiming to observe plant mass loss as infection aggravated. A macroscopic evaluation of the infection was also performed on each sampling day, based on observation of the amount of mycelia present in the ears of the wheat plants. The results were given in average percentage of infected ear-surface per pot.

5.1.5 Threshing and Milling

To further extract the total DNA and quantify DON, it was necessary to homogenise the samples from each anatomic section of the wheat. Therefore, for each sampling day, the plant material from a total of 6 pots (3 for *F. graminearum* and 3 for *F. culmorum*) was homogenised separately, avoiding mixing of material from the different pathogens. Thus, a total of 30 ears, 30 F-leaves and 30 F-1 leaves were milled separately for each infection case. After threshing the ears cut on day 17, the kernels and hulls were separated and milled using Retsch Mixer Mill MM 400, while the fresh ears and leaves (from 3 to 14 dpi) were milled with Retsch Grindomix GM200, due to its higher capacity (see Appendix, Threshing and Milling). The homogenised material was placed in 50 ml Falcon tubes (Sarstedt) and stored in a -80°C freezer (New Brunswick Scientific).

5.1.6 DON Analysis

The lateral flow test or strip test (also called immunochromatographic test), has been applied in mycotoxin testing – related with food safety measures (Zheng, Richard, and Binder 2006). This test strip contains a simple and a conjugate pad, a membrane, an absorbent pad as well as an adhesive backing (Fig. 19). The sample is added onto the sample pad and the mycotoxin present binds to the anti-mycotoxin antibody gold particle complex present in the conjugate pad. After, they

migrate together along the membrane, which contains a test zone and a control zone, where the mycotoxin-protein conjugate and a 2nd antibody are dried, respectively. The control zone is always visible as the 2nd antibody always captures the anti-2nd antibody gold particle complex, validating the test. The visible line that appears in the strip is relative to the concentration of gold particle complexes. These strips are very convenient, as they are easy to use, very rapid and stable in different climates, and are suitable to use in the field (Zheng, Richard, and Binder 2006).

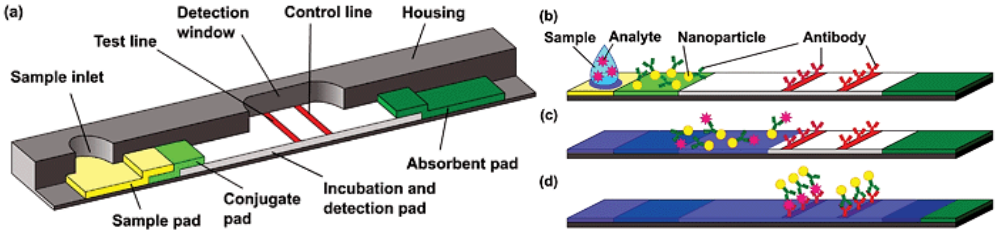

Fig. 19 – Structure and operation of the immunochromatographic test (Posthuma-Trumpie, Korf, and Amerongen 2008).

The previously homogenised material was defrosted and the mass of 1 g of each sample (ears and leaves) was measured using Kern EG 4200-2NM (Waagen-Kissling) and mixed with deionized water, using Variomag Poly Komet from Thermo Electron Corporation. An approximate volume of 2 ml of the homogenized mixed sample was pipetted into a 2 ml Eppendorf tube and centrifuged (VWR Ministar silverline) for approximately 1 minute. A volume of 50 µl of the supernatant was diluted in 450 µl deionized water and mixed, being transferred 50 µl of this mixture into 1000 µl of dilution buffer from Charm Sciences. A volume of 300 µl was pipetted into a ROSA Lateral Flow Mycotoxin Strip, which was incubated for 2 minutes at 46°C in a ROSA incubator from Charm Sciences (see Appendix, DON Analysis). The DON concentration was measured with the Charm EZ-M system reader (see Appendix, DON analysis), which was regularly calibrated.

5.1.7 DNA Extraction

DNA extraction was performed with a Macherey-Nagel kit (NucleoSpin® Plant II) to extract the total DNA from the plant material. From the previously homogenised and defrosted material, a small amount of each sample (approximately 0.5 ml) was first placed in a 2 ml Eppendorf tube and then left overnight in the -80°C freezer (New Brunswick Scientific). After freezing, the samples were homogenised using steel beads: 2 beads (diameter: 7 mm) that were placed together with the deep-frozen material and mixed for about 30 seconds. The homogenised material was transferred to a new tube and 800 µl of Buffer PL1 was added to perform cell lysis, as well as 20 µl of RNase A solution. The mixture was vortexed thoroughly and the suspension was incubated for 50 min at 65°C. The next step was filtration/clarification of crude lysate with a NucleoSpin® Filter that was placed into a new Collection Tube (2 ml). The lysate was loaded onto the column and was centrifuged for 2 min at 11,000 x g. The clear flow-through was collected and the NucleoSpin® Filter was discarded. The DNA binding conditions were adjusted with 900 µl of Buffer PC that was added and mixed thoroughly by pipetting up and down. DNA binding was performed with a NucleoSpin® Plant II

Column that was placed into a new Collection Tube (2 ml) and 700 µl of the sample was loaded, being centrifuged for 1 min at 11,000 x g. The flowthrough was discarded and the silica membrane was washed first with 400 µl Buffer PW1 followed by centrifugation for 1 min at 11,000 x g. The second wash was performed with 700 µl of Buffer PW2 followed by centrifugation for 1 min at 11,000 x g. The third and last wash was performed with 200 µl of Buffer PW2 followed by centrifugation for 2 min at 11,000 x g to remove wash buffer and dry the silica membrane completely. The last step was DNA elution: the NucleoSpin® Plant II Column was placed into a new 1.5 ml Eppendorf tube and 50 µl of Buffer PE (65 °C) was pipetted onto the membrane. The NucleoSpin® Plant II Column was incubated for 5 min at 65 °C and the sample was centrifuged for 1 min at 11,000 x g to elute the DNA. The DNA concentration as well as the quality of the extraction (260/280 and 260/230 ratios) were determined with NanoDrop 2000 UV-Vis Spectrophotometer from Thermofisher Scientific.

5.1.8 Quantitative PCR (qPCR)

Quantitative PCR (qPCR), allows a precise quantification of specific nucleic acids in a complex mixture, even if there is a very low concentration of the starting material. The amplification of the target sequence in qPCR is accomplished by using fluorescent technology. In this method, DNA produced in each amplification cycle is measured. It is known that qPCR overcomes some difficulties in PCR, increasing speed due to the reduced cycle number or even by introducing a higher sensitivity of the fluorescent dyes. Nevertheless, despite the advantages of qPCR, it has some drawbacks, such as requiring sophisticated equipment and more cost driven expenses (Fraga, Meulia, and Fenster 2008). Using qPCR, it is possible to obtain two values within exponential phase: the cycle threshold (Ct) from the threshold line – when reaction reaches a fluorescent intensity above background – and then the amount of DNA from the comparison of Ct values of unknown concentration samples. The cycle threshold depends on the amount of template present at the start of the reaction. Therefore, if at the start of the reaction there is a small amount of template, then the cycle threshold will be higher because more amplification cycles are required to rise the fluorescent signal above background. On the other hand, if at the start of the reaction there is a high amount of compound, the opposite occurs (Bio-Rad Laboratories 2006) . To have an optimized qPCR assay it is important to plot a standard linear curve (R^2>0.980 or r>|-0.990|), a high amplification efficiency (90-105%) and also consistency across replicate reactions. To generate a standard curve it is necessary to run serial dilutions of a given template with known concentrations (nanograms of genomic DNA or copies of plasmid DNA) (Bio-Rad Laboratories 2006). SYBR Green I dye is a fluorescent dye that binds to the minor groove of double stranded DNA. This bound DNA dye produces a stronger fluorescent signal when excited, when compared to unbound dye (Fig. 20). In ideal conditions, SYBR Green assay produces a similar pattern when compared with TaqMan probe-based assay (Life Technologies Corporation 2012).

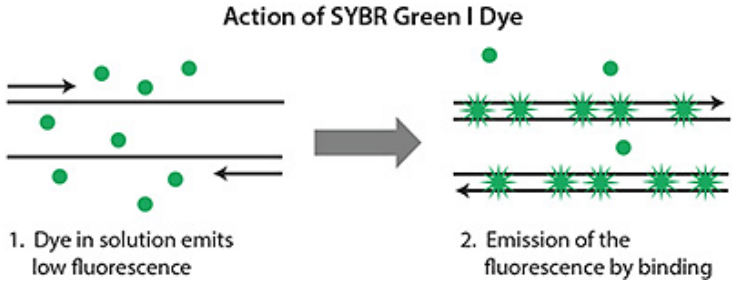

Fig. 20 - Action of SYBR Green I dye (SIGMA-ALDRICH Co. 2017).

When using SYBR Green I, it is important to check the specificity of the qPCR assay by analysing the reaction products using the melt curve function, and by running the products in agarose gel. An optimized SYBR Green I qPCR reaction should have a single peak in the melt curve, which corresponds to a single band on the agarose gel (Bio-Rad Laboratories 2006). The linear regression line is represented by the following expression:

$$y = mx + b \Leftrightarrow C_T = m \times \log quantity + b$$

Therefore, to determine the quantity of the DNA in the sample:

$$Quantity = 10^{\frac{(C_T - b)}{m}}$$

In this study, qPCR was used to quantify the relative amount of fungi in the wheat plant, hence the target sequence belonged to the gene encoding cytochrome *b* in *F. graminearum* and *F. culmorum* cells (see Appendix, Quantitative PCR(qPCR)). In order to obtain a sufficient DNA concentration to plot the standard curves, the biomass from three and two Petri plates from *F. graminearum* and *F. culmorum* was used, respectively. The DNA was extracted according to the previously described section (DNA extraction). A five-fold dilution series was performed for each sample (see Appendix, Quantitative PCR(qPCR)). A final volume of 22.5 µl from Takyon™ Rox SYBR® MasterMix dTTP Blue (Eurogentec) and 2.5 µl of DNA template were pipetted to a total reaction volume of 25 µl. The primers KES 978 (forward) (5'-TACCTTATGGACAAATGTCATTATGAG-3') and KES 1009 (reverse) (5'-TGCGCCGTACATTAAAAATAAAGTA-3'), targeting the sequence belonging to cytochrome *b* gene from *F. graminearum* and *F. culmorum*, were previously designed and optimized by BASF (see Appendix, Quantitative PCR (qPCR)). Rotor-Gene Q MDx by QIAGEN was used to perform the qPCR. The cycling conditions were 95°C for 5 minutes (hold), 95°C for 10 seconds followed by 60°C for 45 seconds (40 cycles) and a melt step with a ramp from 60°C to 95°C rising 1°C in each step. Having the Ct values and the concentration of the dilutions series, two standard curves were plotted, one for *F. graminearum* and other for *F. culmorum* (see Appendix Quantitative PCR(qPCR)). The melting curves relative to this qPCR as well as respective electrophorese results are displayed in the appendix (see Appendix, Quantitative PCR(qPCR)). The same primers, volumes and conditions mentioned above were maintained for all qPCR reactions in this study. The results are presented in relative amount of fungi (RAF), which is given by the following expression:

$$RAF(\%) = \frac{Fungal\ DNA\ concentration\ (\frac{ng}{\mu l})}{Concentration\ of\ the\ DNA\ template\ (\frac{ng}{\mu l})} \times 100$$

5.1.9 Statistical analysis

The statistical analysis was performed with R software version 3.3.3 and Microsoft Excel 2016. The one-way ANOVA was performed to take conclusions on the influence of both pathogens and different treatments in DON concentration as well as in the relative amount of fungi (RAF) in the different anatomical sections of the wheat (ears, F-leaves and F-1 leaves). This test was also applied for the different days of analysis.

The one-way analysis of variance (ANOVA) is commonly used to conclude about statistically significant differences between the means of two or more independent groups (Lund Research Ltd 2013). As a result, in an ANOVA it is common that null hypothesis affirms that the group means are equal and the alternative hypothesis affirms that there are at least two groups that present statistically significant differences between each other. The alternative hypothesis is accepted in case the p-value obtained is lower than the alpha value, which is the significance level. In this study, the output of ANOVA was obtained with function `anova ()` that returned the analysis of variance table for the fitted model. An alpha value of 0.05 was assumed for all tests, meaning that there was a 5% risk of accepting a difference between the group means (accepting the alternative hypothesis), when there was actually no difference and the null hypothesis is valid.

There are some assumptions that the one-way ANOVA requires: (1) there is a normal distribution of the dependent variable in each group compared in the test, (2) in each group the population variances are equal, meaning that there is homogeneity of variances and (3) independence of observations meaning that each sample is randomly selected and independent (Lund Research Ltd 2013). Therefore, for all the ANOVA tests performed in this work, these assumptions were tested in R software with the function `gvlma ()` that allows to check parameters such as skewness of the data, meaning the asymmetry of the data distribution as well as heteroscedasticity (absence of homoscedasticity), meaning that the variances between groups are different. Normal Q-Q plots are commonly used to check for normal distribution. As the title indicates, this plot sets the quantiles (also referred to as percentiles) against each other. It is possible to check a normal distribution by analysing the proximity of the data points to a straight line. The shape obtained in these type of plots is useful to highlight data anomalies such as asymmetry, heavy tails or outliers (Galili and Pearson 2011).

When the p-value is less than the significance level (in this study 0.05), post hoc tests may be performed to conclude which specific groups differed (Mendiburu 2017). The Tukey post hoc test was performed with the function `HSD.test ()` in the R software package `agricolae`, allowing multiple comparisons of treatments by means of Tukey. The function `TukeyHSD ()` might as well

be used, performing all pairwise comparisons for the groups and adjusting the p-value. This function gives a more detailed output when compared with `HSD.test ()`. The package `agricolae` was developed specially for agricultural research, and includes several procedures of experimental data analysis, such as the Tukey post hoc test referred to above, as well as non-parametric comparisons such as the Kruskal Wallis, Friedman, Durbin, and others commonly applied in genetics, procedures in biodiversity and descriptive statistics (Mendiburu 2017).

In case the ANOVA assumptions are not met, removing outliers that disturb the model might be a possible approach to address this issue (Prabhakaran 2017). Other two options are recommended: (1) transformation of the data so that it becomes normally distributed or (2) perform non-parametric tests such as Kruskal-Wallis test which does not require assumption of normality. In case homogeneity of the samples is violated (1) Welch or (2) Brown and Forsythe test might be performed (Lund Research Ltd 2013). The `agricolae` package includes a `kruskal ()` function that performs a non-parametric multiple comparison with Kruskal Wallis test. This function was used in this study to take conclusions when the ANOVA assumptions were not met (Mendiburu 2017).

A linear regression model describes the relationship between two variables (y and x), and can be expressed by the following equation:

$$y = ax + b$$

Two linear regressions were performed with the R software, to understand the relationship between DON production and the relative amount of fungi in the ears of the wheat plant. The function `lm ()` was used to fit a linear model to the data, and `summary ()` to produce the result summary of the model fitting function. This last function allows to obtain important parameters such as the p-values and the adjusted R-squares. The generic function `plot ()` allowed to obtain the normal Q-Q plots as well in ANOVA tests (Mendiburu 2017). In this study, the linear regression is described as the following:

$$DON\ concentration = a \times RAF\ (\%) + b$$

5.2 Trial A3: Test natural tri-inhibitors to access the feasibility of their use to control Fusarium head blight (FHB): Fungi growth and DON production with treatment

This trial differed from the previous two in (1) the spraying of the compounds, which happened one day before inoculating the fungi and (2) the sampling days, which in this case was only on day 17, meaning that all the tested pots remained in the greenhouse for 17 days after fungi inoculation. Furthermore, it differed as well in (3) the macroscopic evaluation, which was performed six days after inoculation as well as on the 17th day. In this trial a total of 50 pots were used (25 for *F. graminearum* and 25 for *F. culmorum)*. These 25 pots were further divided so that 4 could be used

for each treatment. Four of the remaining pots were used as solvent treated controls (STC), and one as untreated control (UTC). All the other technical procedures in this trial (plant growth and flowering, pathogen inoculation, threshing, milling and DON analysis, DNA extraction, quantitative PCR and Statistical analysis) were carried out as previously described for Trials A1 and A2.

5.2.1 Spraying the compounds

Compound spraying was scheduled immediately after noticing flowering of the wheat plants (approximately after 7 weeks, as described above). The plants were sprayed with prothioconazole (PTZ, synthesized by BASF), the standard fungicide, and with four different natural tri-inhibitors: ferulic acid (FA, from Sigma Aldrich), magnolol (MAG, from Sigma Aldrich), carvacrol (CAR, from HWI ANALYTIK GMBH) and flavone (FLA, synthesized by BASF). Additionally, solvent treated controls (STC) and untreated controls (UTC) were added. All the natural tri-inhibitors were sprayed at 400 ppm, while PTZ was sprayed at 125 ppm. Upon preparation, FA, MAG, FLA and PTZ mass was measured with a Mettler Toledo XS204 analytic balance and then mixed with 3 ml Acetone with 1% WETTOL™ (Calcium dodecylbenzenesulfonate from BASF) and 47 ml H_2O. CAR has a melting point of approximately 4ºC, and therefore was already in a liquid state. Therefore, carvacrol was first mixed with acetone (with 1% WETTOL™) and further diluted in the water. WETTOL™ (from BASF) was used as an emulsifier, as it is commonly present in pesticide formulations, reducing the surface tension and creating a stabilizing effect on the emulsion, allowing the droplets to remain dispersed and stable. The solvent treated controls (STC) were only sprayed with Acetone (with 1% WETTOL™) and H_2O. This was performed to observe the interaction of the solvent system with the pathogen/host relationship, since it may enhance or diminish functionalities influencing the performance. The untreated controls (UTC) were only sprayed with the fungi spore suspension, allowing to take conclusions about the efficacy of the tested compounds. All the pots were sprayed with special equipment, thereafter being placed in covered cars and taken to a high moisture chamber with an average temperature of 20°C and relative humidity between 80 and 90% (see Appendix, Plant growth and flowering, compound spraying and fungi inoculation). The compounds were sprayed onto the wheat plants one day before the inoculation of the fungi. This was important to simulate the field scenario where fungicides are mainly sprayed as a preventive measure against diseases and seldom as a curative measure when the infection is already present. This time lapse was given to simulate a protective treatment, in which the fungicides are applied as a protective measure, before infection of the wheat plants occur.

6 Results

6.1 Trials A1 and A2: Distribution of *Fusarium graminearum* and *Fusarium culmorum* in the wheat plant: Growth analysis and DON production with time

Data obtained in trials A1 and A2 was compared and presented the same pattern for DON concentration and relative amount of fungi (RAF %) in the wheat plant with time. Nevertheless, trial A1 required some small corrections and, consequently, A2 was performed to improve accuracy of the results. Therefore, the results presented in this section for DON analysis and qPCR are only relative to trial A2.

6.1.1 Pathogen growth: detection and macro-evaluation

The infection aggravated progressively with time. Both fungal species grew as expected. Three days after the inoculation small mycelia was already visible in the ears (Table 3 and Appendix: Pathogen Growth, Trials A1 and A2: Detection and evaluation). Even though the wheat plants were already inside a high moisture chamber, they were watered every day and the nutrients necessary for their maintenance were also present. Therefore, the fungi had all the necessary resources for their growth, progressively consuming the carbohydrates present in the ears of the plant. On day 7, the mycelia was already in higher amounts in the ears of the plants when compared to day 3. *F. graminearum* always covered a higher area of the plant than *F. culmorum*, being supposed as the most aggressive species. The loss of mass in the infected plants was evident throughout the process when compared to the controls (see Appendix: Pathogen Growth, Mass loss in the wheat plant with time). Necrosis in other plant tissues – such as the *flag* leaves - started to be visible after the 7th day of infection. By this time, 80% to 90% infection (average percentage of infected ear-surface per pot) was accounted for both cases. On day 10, the infection was already 100% and the leaves started to become yellow, showing necrosis signs. After day 10 the plants were taken to a dry chamber where they stood until day 17. By the end of the process – on day 17 – the wheat plants were dry, yellow and fully consumed by the fungi (see Appendix: Pathogen Growth, Trials A1 and A2: Detection and evaluation).

Table 3 - A macroscopic evaluation of the infection was performed on each sampling day, based on observation of the amount of mycelia of *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) present in the ears of the wheat plants. The results are given as the average percentage of infected ear surface per pot. The affected anatomy and symptoms were also registered for each pathogen. BS = Black Spots; YT = Yellow Tips; YL = Yellow Leaves; WM = White Mycelia; PM = Pink Mycelia.

Days Post Inoculation	**% Infection**		**Affected anatomy**						**Symptoms**					
	GIBBZE	FUSACU	GIBBZE			FUSACU			GIBBZE			FUSACU		
	Ears		Ears	F-leaf	F1-leaf	Ears	F-leaf	F1-leaf	Ears	F-leaf	F1-leaf	Ears	F-leaf	F1-leaf
0	0	0	-	-	-	-	-	-	-	-	-	-	-	-
3	2	2	X	-	-	X	-	-	WM	-	-	WM	-	-
7	100	90	X	X	-	X	X	-	WM	BS	YT	WM;PM	BS	YT
10	100	100	X	X	X	X	X	X	WM;PM	BS;YT;WM	BS;YT	WM;PM	BS;YT;WM	BS;YT
14	100	100	X	X	X	X	X	X	WM	BS;YL;WM	BS;YT	WM;PM	BS;YL;WM	BS;YT
17	100	100	X	X	X	X	X	X	WM	BS;YL;WM	BS;YT	WM;PM	BS;YL;WM	BS;YT

The average wet mass of 10 ears, 10 F-leaves and 10 F-1 leaves was plotted against time (Figure 21). The average mass values and standard deviations for each infection case are presented in appendix (see Appendix: Pathogen Growth, Mass loss in the wheat plant with time).

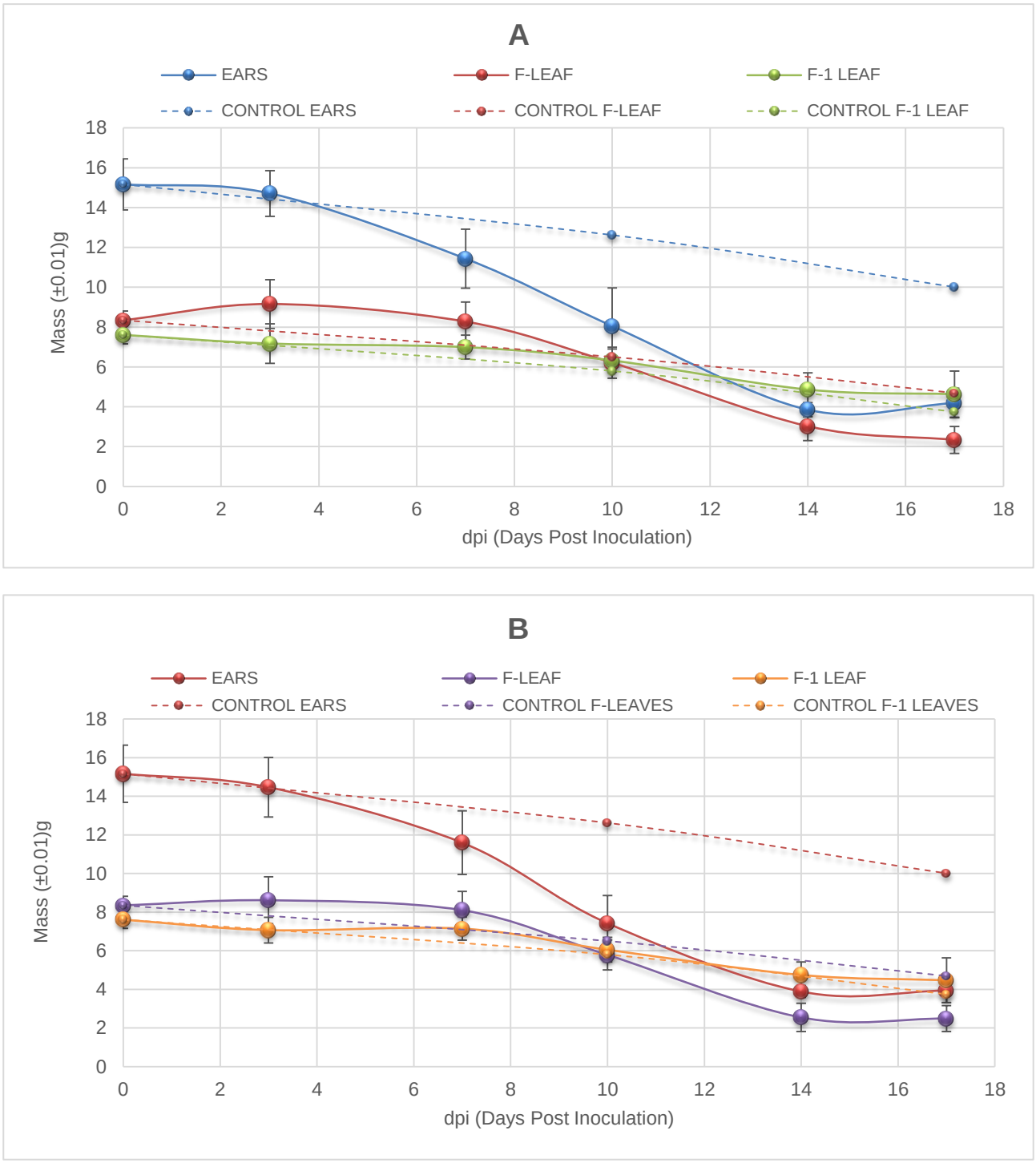

Fig. 21 – *F. graminearum* (GIBBZE) infection (A) and *F. culmorum* (FUSACU) infection (B). Average wet mass and standard deviation of 10 ears, 10 F-leaves and 10 F-1 leaves (one pot) in each sampling day. Error bars represent the Standard Deviation (0 dpi n=4; 3, 7, 10, 14 and 17 dpi n=10). The control lines are relative to non-inoculated plants that were exposed to the same conditions as the inoculated wheat. Data collected from trials A1 and A2.

Regardless the type of infection, the mass of the wheat plants decreased over time. The mass loss followed the same pattern for both types of infection, with the ears of the wheat suffering the highest loss of mass compared to the F-leaves and F-1 leaves (Figure 21). Overall, a critical loss of mass was observed in both infection types after day 7 in the leaves and after day 3 in the ears. After day 14 the mass in the ears, F-leaves and F-1 leaves remained constant, meaning that in general the mass of the wheat plant after this day would not fluctuate much. In the control lines it is possible to see that the highest decrease in mass is after day 10, when the healthy plants are transferred from the moisture chamber to the dry chamber. The mass of these plants did not change much in the leaves when compared to the infected wheat. A picture relative to mass loss in the plant is also displayed in appendix (see Appendix: Pathogen Growth, Mass loss in the wheat plant with time).

6.1.2 DON analysis

DON concentration increased progressively over time (0 dpi to 17 dpi) in both infection types, with *F. culmorum* apparently producing a higher amount of mycotoxin than *F. graminearum,* regardless the anatomic section (Fig. 22). The mycotoxin concentration was higher in the ears where the carbohydrates are present in higher amount, being the nutrient source for the fungi. Furthermore, the inoculation of the pathogens was focused on the ears of the wheat plant and not the leaves. Therefore, it was expected that fungi started growing in the ears, moving progressively to the leaves. A higher increase in DON production occurred from day 14 to day 17, in both infection types, which might have been caused by the stress conditions to which the plant was submitted (lack of water and dry air). This might have induced the fungi to produce more mycotoxin as a pressure measure, as it perceived that the host was starting to lack carbohydrates.

DON concentration only seems relevant in the F-leaves after day 7. In fact, the *flag* leaf was already affected on day 7, showing necrotic signals manifested by darkened spots, due to the DON toxicity in the tissues. The increase in DON in the leaves between days 14 and 17 was not so remarkable when compared to the ears. In this period, the higher DON concentrations in the F-leaves infected with *F. culmorum* than in those infected with *F. graminearum* are clear. The lowest DON concentration was measured in the F-1 leaf, as expected, only appearing relevant after day 10 (Fig. 22).

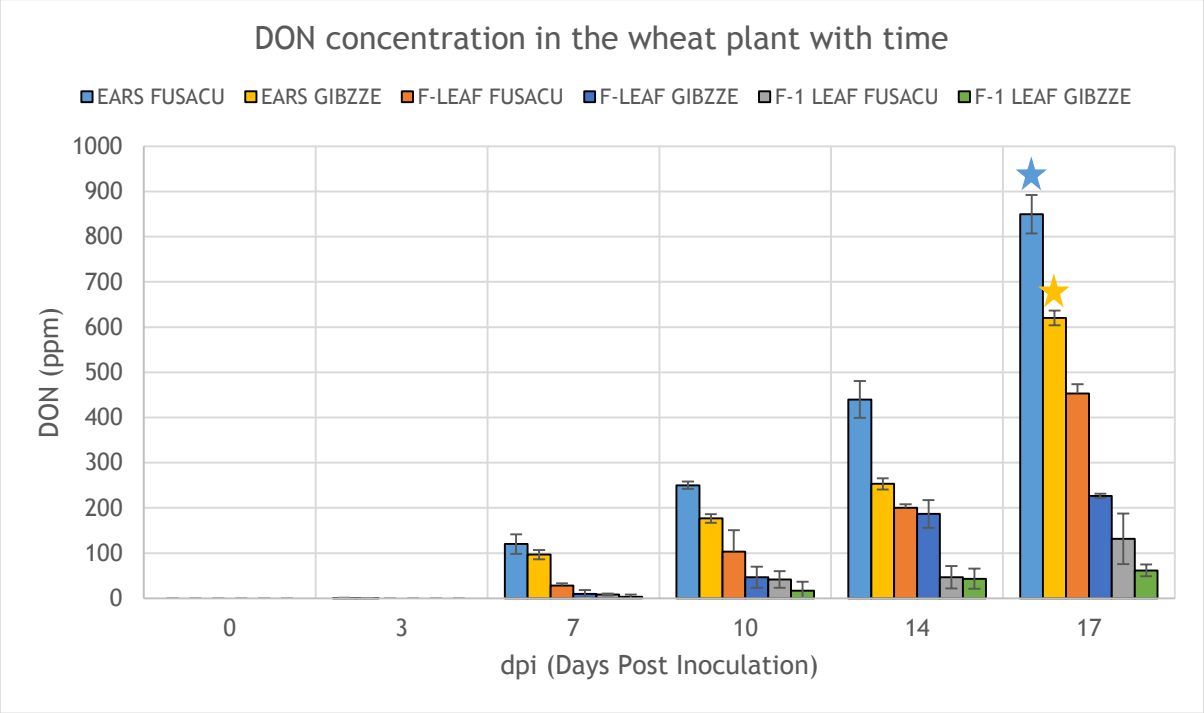

Fig. 22 – DON concentration in the ears, F-leaf and F-1 leaf of the wheat plant produced by *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) over time (days post inoculation). The error bars represent the Standard Deviation (0 dpi n=1; 3, 7, 10, 14 and 17 dpi n=3). The stars indicate the total DON concentration in the ears (sum of DON concentration in the kernels and hulls). The DON values determined in each sampling day and trial are presented in appendix (see Appendix: DON Analysis).

A one-way analysis of variance (ANOVA) was performed to search for significant differences in DON concentration produced by two different pathogens in the ears and leaves of the wheat. The null hypothesis was that DON concentration in each anatomic section was not significantly different between species. The alternative hypothesis was the opposite. To decide whether the null hypothesis is valid or not, p-value was compared with the significance level (α=0.05).

Table 4 – Results from one-way ANOVA obtained with R software version 3.3.3. Only significant results are displayed in the table. Full table is displayed in appendix (see Appendix: Statistical analysis, One-way ANOVA tables). α=0.05. '***' p≤0.001 '**' p≤ 0.01.

Days Post Inoculation	Anatomical section	p-value	Significance level	Assumptions
10	EARS	0.001	***	Acceptable
14	EARS	0.003	**	
17	EARS	0.002	**	
	F-LEAF	0.000	***	

On days 3 and 7, DON concentration in the wheat plants infected by both fungi was not significant, since the p-values were higher than 0.05 significance value (Table 4). Therefore, the null hypothesis was accepted. On days 10, 14 and 17, DON concentration in the ears of the wheat plants infected with *F. culmorum* was different from that of plants infected with *F. graminearum*, since the p-values of 0.001, 0.003 and 0.002 were lower than 0.05 significance value (Table 4). Therefore, the null hypothesis was rejected. The null hypothesis was also rejected in day 17 for the F-leafs. Therefore, it was possible to conclude that DON concentration was significantly higher in wheat plant infected *F. culmorum*, mainly in the ears.

6.1.3 Quantitative PCR (qPCR)

The qPCR allowed to determine the relative amount of fungi (RAF) in each of the anatomic sections of the wheat plant over time. Figure 23 shows the relationship between DON concentration and the relative amount of fungal DNA in the ears of the wheat plant, with time (dpi).

As expected, DON concentration and the relative amount of fungi increased with time (days post inoculation) (Fig. 23). As previously shown, the mycotoxin DON was also detected in the leaves of the wheat plant. Nevertheless, the concentration of cytochrome *b* in these anatomical sections of the plant was very low (< 1 ng/ µL), therefore the RAF values were close to 0%. The relative amount of fungi started to be considerable after day 10 for both infection types. Furthermore, these results showed that *F. culmorum* was not only the strongest DON producer, but seemed as well to be present in higher amounts in the ears of the plant when compared with *F. graminearum*.

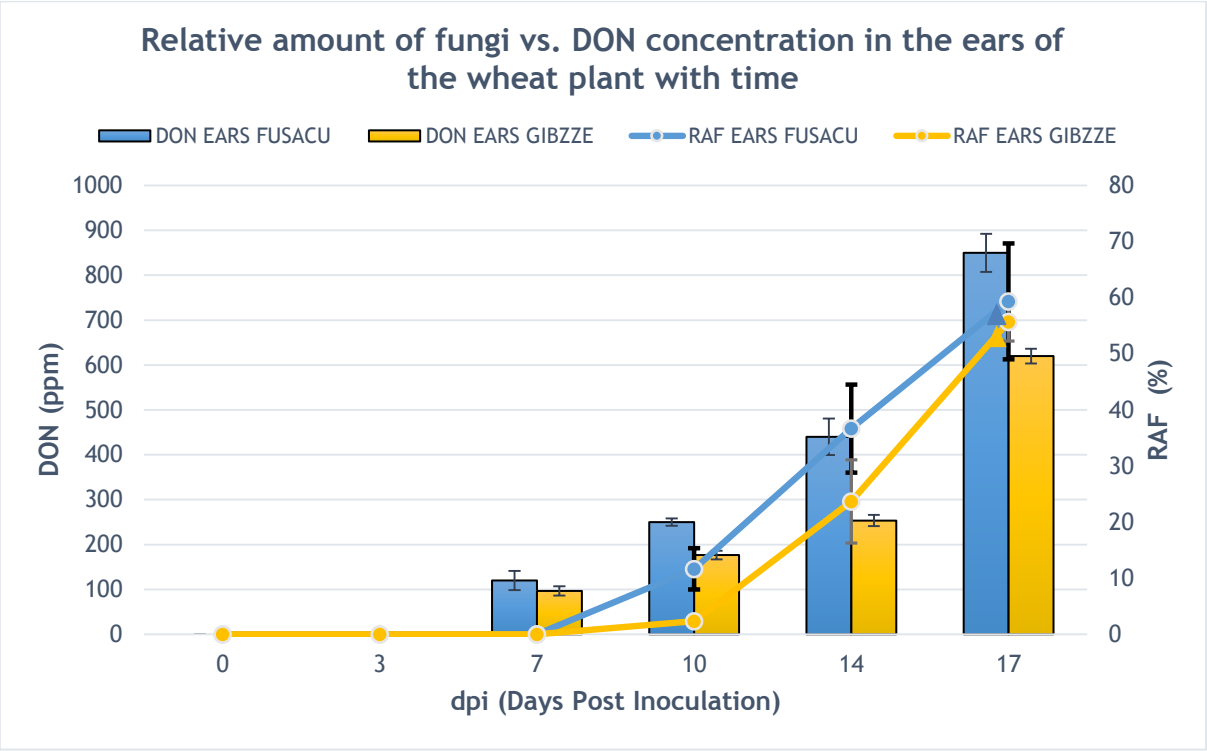

Fig. 23 – Plot of DON concentration (ppm, bars) and relative amount of *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) (RAF, line) (%) in the ears of the wheat plant over time. Error bars represent the standard deviation (0 dpi n=1; 3, 7, 10, 14 and 17 dpi n=3) of the average DON concentrations and the average RAF values, respectively. The triangles on the side of the two 17dpi markers are relative to the overall RAF value in the ears (sum of RAF values in the kernels and hulls). RAF values for each dpi and trial are presented in appendix (see Appendix, Quantitative PCR (qPCR)).

Linear regressions were performed to understand the relationship between DON production and the relative amount of fungi in the ears of the wheat plant (Fig. 24). Significant relationships between DON production in the ears of the wheat plant and the relative amount of each type of fungi (RAF) were found, since the p-value was much lower than the considered alpha value (0.05) and the assumptions of the linear regression were acceptable (Table 5). This means that an increase in the relative amount of fungi was related with an increase of DON concentration in the ears of the wheat plant.

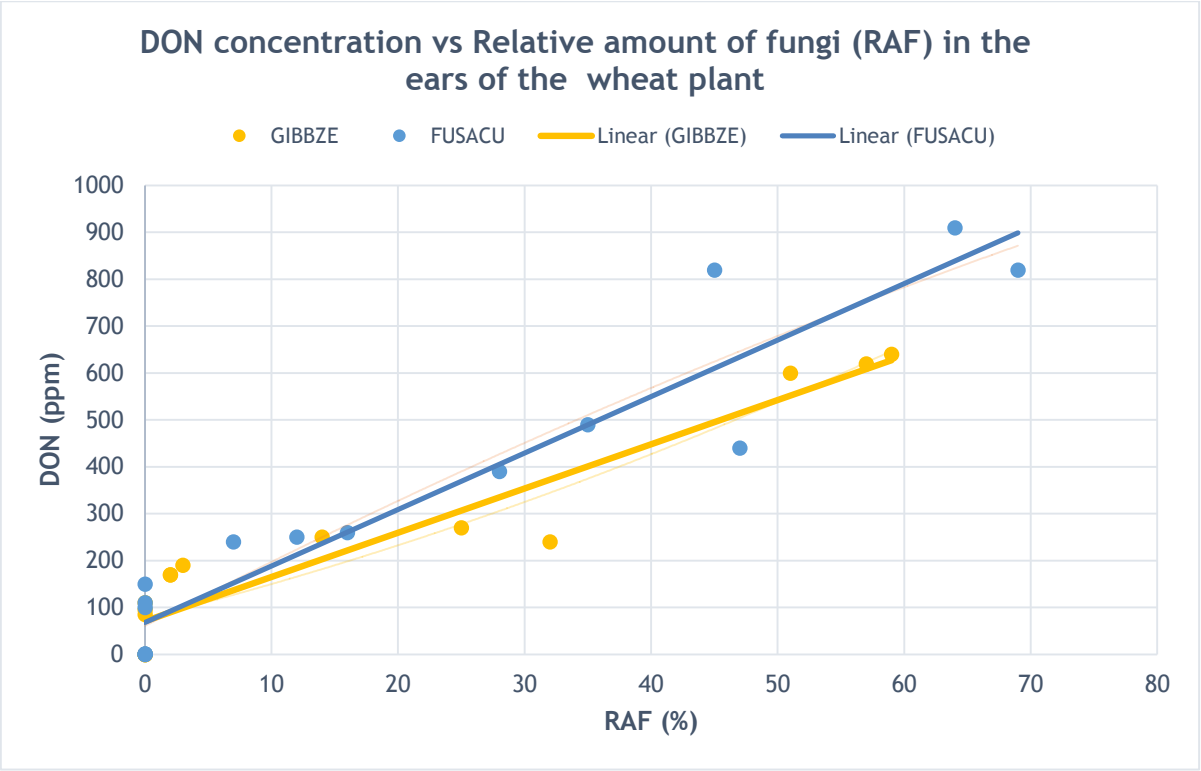

Fig. 24 – Relationship between DON production by *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) and relative amount of fungi (%), in the ears of the wheat plant.

Table 5 – Linear regression parameters obtained with R software version 3.3.3. α=0.05. '***' p≤0.001.

Linear regression parameters	a	b	Multiple R-squared	p-value	Significance level	Assumptions
FUSACU	12.043	68.296	0.9096	0.000	***	Acceptable
GIBZZE	9.4333	70.5838	0.9078	0.000	***	Acceptable

6.2 Trial A3: Test natural tri-inhibitors to access the feasibility of their use to control Fusarium head blight (FHB): Growth analysis and DON production with treatment

6.2.1 Pathogen growth: detection and macro-evaluation

With exception of prothioconazole treated plants, the infection aggravated progressively with time in the plants treated with tri-inhibitors as well as the untreated controls (UTC) and solvent treated controls (STC). Six days after inoculation some mycelia was already visible in the ears of the plants treated with the tri-inhibitors as well as in the controls (UTC and STC) (see Appendix: Pathogen Growth: Detection and evaluation, Trial A3: Detection and Evaluation). On day 17, it was clear that none of the tested tri-inhibitors inhibited the growth of both fungi, as mycelia fully covered the ears and partially the leaves of the wheat. Necrosis was visible as in the first trials, mainly in the leaves and 100% infection was accounted in all plants inoculated with *F. culmorum* and *F. graminearum* treated with the natural tri-inhibitors, as well as in both controls (STC and UTC). Only the plants sprayed with prothioconazole looked healthy, with almost no mycelium present. Furthermore, flavone seemed to be phytotoxic for the wheat, as the leaves became faster yellow and dry when compared to the other plants. Therefore, even if this compound could inhibit DON, it could not be used in further studies, since it may be toxic for the wheat plant (see Appendix: Pathogen Growth: Detection and evaluation, Trial A3: Detection and Evaluation).

6.2.2 DON analysis

DON was quantified in the kernels, hulls, F-leaves and F-1 leaves for the different treatments and infections (*F. culmorum* and *F. graminearum*) on the 17th day post inoculation, when the plants were collected from the greenhouse (Fig. 25).

The DON concentration in the different anatomic sections of the wheat plants followed the pattern described in Trials A1 and A2. The concentration of the mycotoxin was higher in the hulls of the wheat plant and showed similar concentration values in the kernels and F-leaves, being close to the limit of detection (0 ppm) in the F-1 leaves. Higher DON concentrations were determined in plants infected with *F. culmorum* than with *F. graminearum*. The natural tri-inhibitors magnolol (MAG), carvacrol (CAR) and ferulic acid (FA), as well as flavone (FLA) did not show any effect controlling DON in both infection types. In contrast, prothioconazole (PTZ) inhibited DON production by the fungi and acted as a fungicide, hampering their development in the plant. In this case, the mycotoxin concentration in the plant was much lower when compared to the tested natural compounds.

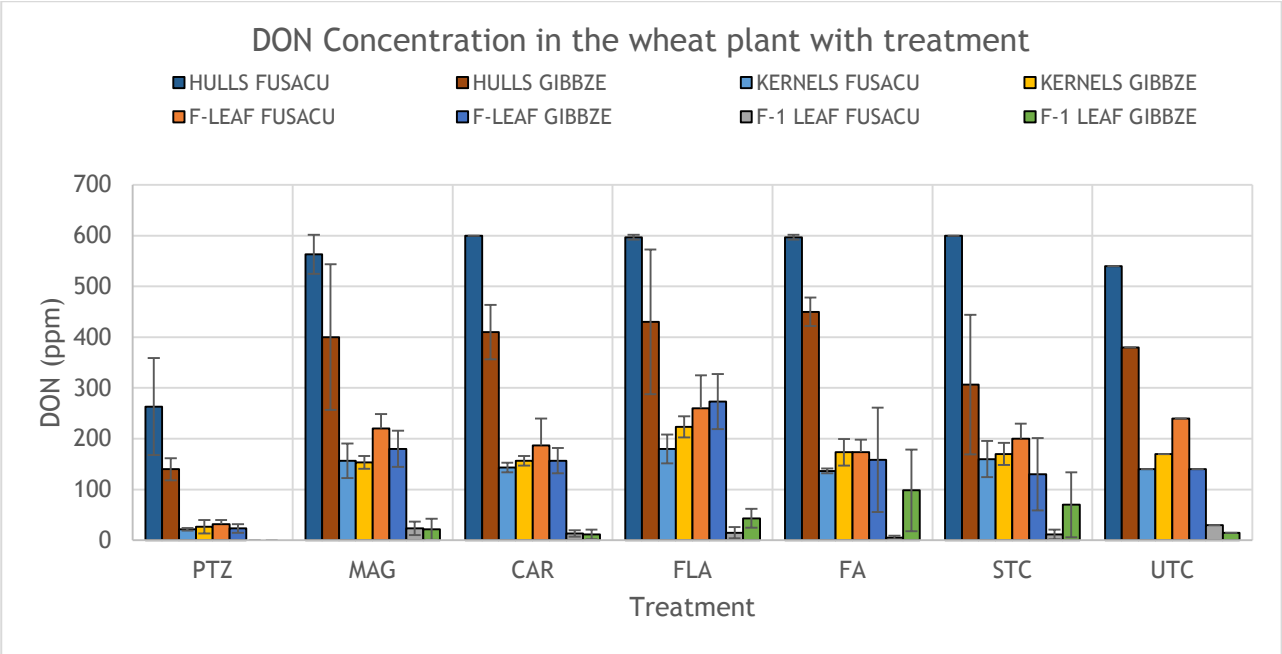

Fig. 25 – DON concentration produced by *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) in the wheat plant on day 17, after different treatments: prothioconazole (PTZ), magnolol (MAG), carvacrol (CAR), flavone (FLA) and ferulic acid (FA). DON concentration is as well plotted for two controls: solvent treated control (STC) and untreated control (UTC). In each diagram the error indicator represents the standard deviation (UTC n=1; PTZ, MAG, CAR, FLA, FA and STC n=3) of the average DON concentrations in the kernels, hulls, F-leaf and F-1 leaf. The DON values of each trial are presented in appendix (see Appendix: DON Analysis).

A one-way analysis of variance (ANOVA) was performed to search for statistically significant differences on DON concentration in the different anatomical sections of the plants subjected to the various treatments (Table 6). The null hypothesis was that different treatments did not have significant effects on DON concentration in the different anatomic sections of the wheat plant. The alternative hypothesis was the opposite. To decide whether the null hypothesis was valid or not, p-value was compared with the significance level (α=0.05).

Table 6 – The one-way ANOVA was performed with R software version 3.3.3. Only significant results are displayed in the table. Full table is displayed in appendix (see Appendix: Statistical analysis, One-way ANOVA tables). α=0.05. '***' p≤0.001 '**' p≤ 0.01.

	Anatomical section	F value	p-value	Significance level	Assumptions
treatment	HULLS	4.18	0.003	**	Not satisfied
	KERNELS	26.26	0.000	***	Acceptable
	F-LEAF	8.93	0.000	***	Not satisfied

The analysis of variance (Table 6) allows to conclude that there was a statistically significant effect of different treatments in DON concentration in the kernels of the wheat plant since the p-value was lower than 0.05 significance value and the ANOVA assumptions were acceptable. As a result, the Tukey test (post hoc test) was performed to evaluate which treatment groups differed.

Table 7 - Post hoc Tukey test performed with `TukeyHSD()` function in R software version 3.3.3. Only significant results are displayed in the table. α=0.05. '***' p≤0.001 '*' p≤ 0.05.

Anatomical section	Treatment	Adjusted p-value	Significance level
Kernels	FLA-CAR	0.029	*
	PTZ-CAR	0.000	***
	PTZ-FA	0.000	***
	PTZ-FLA	0.000	***
	PTZ-MAG	0.000	***
	STC-PTZ	0.000	***
	UTC-PTZ	0.000	***

The statistical parameters (Table 7) permitted to conclude that, as expected, the wheat treated with PTZ presented statistically significant differences compared to the wheat treated with the natural tri-inhibitors as well as with the controls. There was also a significant difference between the kernels treated with flavone and carvacrol. However, DON concentrations for both of these treatments were still significantly higher than with prothioconazole.

When performing the one-way ANOVA for the hulls and F-leaves, the ANOVA assumptions were not satisfied. The normal Q-Q plots are presented in appendix (see Appendix, Statistical analysis, Normal Q-Q Plots), for the cases in which the ANOVA assumptions were not met. By doing a basic analysis of such plots, it is easy to understand the presence of outliers in all of them, as well as the presence of asymmetry. All the normal Q-Q plots relative to the cases in which the assumptions were not met, were compared with the normal Q-Q plot relative to the linear regression of DON production by *F. culmorum* against relative amount of fungi in the wheat plant. In this case the assumptions were satisfied and in the Q-Q plot the presence of outliers and asymmetry was not highlighted meaning that there was a normal distribution of the data points. Consequently, the cases in which a violation of the ANOVA assumptions happened, the non-parametric test of Kruskal Wallis included in the package `agricolae` was performed.

Table 8 - The Kruskal Wallis test was performed to evaluate the difference between the treatment groups relative to DON production in the hulls and F-leaves of the wheat plant. Results obtained with `kruskal ()` function in R software version 3.3.3. Only significant results are displayed in the table. α=0.05. '***' p≤0.001 '**' p≤ 0.01 '*' p≤ 0.05.

Anatomical section	Treatment	p-value	Significance level
Hulls	CAR-PTZ	0.003	**
	FA-PTZ	0.003	**
	FLA-PTZ	0.002	**
	MAG-PTZ	0.01	**
	PTZ-STC	0.006	**
F-leaves	CAR-FLA	0.014	*
	CAR-PTZ	0.011	*
	FA-FLA	0.022	*
	FA-PTZ	0.007	**
	FLA-PTZ	0.000	***
	FLA-STC	0.017	*
	MAG-PTZ	0.001	***
	PTZ-STC	0.009	**
	PTZ-UTC	0.022	*

The Kruskal-Wallis analysis presented in table 8 allowed to conclude that in the hulls there was a significant difference between the plants treated with PTZ and the plants treated with the tri-inhibitors, as expected, as the p-values were lower than the alpha-value of 0.05. Similar conclusions were retrieved for the F-leaves. Furthermore, in this case it was possible to observe a significant difference between the plants treated with flavone and plants treated with carvacrol, ferulic acid and the solvent treated controls. In fact, by analysing the plot presented in figure 25, DON concentration in the leaves of the wheat plants treated with flavone was higher when compared with the concentration of the mycotoxin in the leaves of plants treated with ferulic acid, carvacrol and the STC.

6.2.3 Quantitative PCR (qPCR)

The relative amount of fungi was calculated for the kernels and hulls of the wheat plants exposed to the different treatments and infections (*F. culmorum* and *F. graminearum*) on the 17th day post inoculation, when the plants were collected from the greenhouse (Figure 26).

In the kernels and hulls of the plants treated with magnolol (MAG), ferulic acid (FA) and mainly with carvacrol (CAR), flavone (FLA) and even the solvent treated controls (STC), *F. graminearum* seems to be in higher amount than *F. culmorum* (Figure 26). In contrast, *F. culmorum* seems to be more present than *F. graminearum* in the hulls of the untreated control.

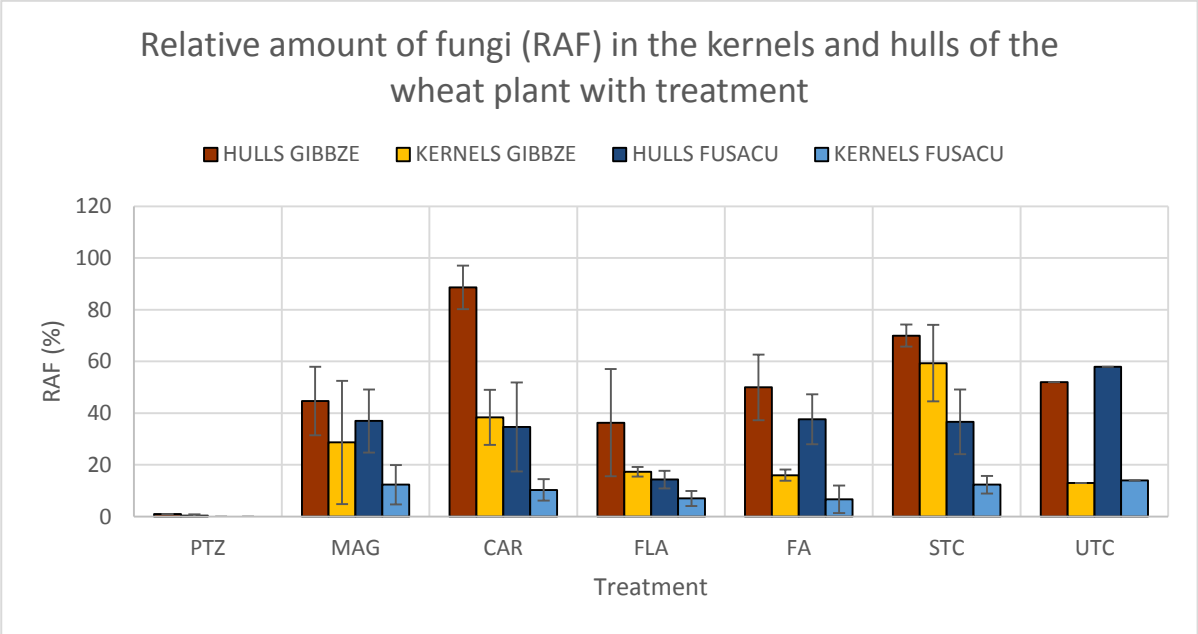

Fig. 26 – Relative amount of *F. graminearum* (GIBBZE) and *F. culmorum* (FUSACU) on day 17 in the kernels and hulls of the wheat plants after different treatments (prothioconazole (PTZ), magnolol (MAG), carvacrol (CAR), flavone (FLA) and ferulic acid (FA)). RAF is plotted as well for two controls: solvent treated control (STC) and untreated control (UTC). In each diagram the error indicator represents the standard deviation (UTC n=1; PTZ, MAG, CAR, FLA, FA and STC n=3) of the average of relative amount of fungi in the kernels and hulls of the wheat plant. RAF values for each dpi and trial are presented in appendix (see Appendix, Quantitative PCR (qPCR)).

A one-way analysis of variance (ANOVA) was performed to search for statistically significant effect of the treatments on the RAF values for the different anatomical sections. The null hypothesis was that different treatments did not show significant effects on RAF values in the HULLS and KERNELS of the wheat plant. The alternative hypothesis was the opposite. To decide whether the null hypothesis is valid or not, p-value was compared with the significance level (α=0.05).

Table 9 – The one-way ANOVA was performed with R software version 3.3.3. α=0.05. '***' p≤0.001 '*' p≤ 0.05.

	Anatomical section	F value	p-value	Significance level	Assumptions
treatment	HULLS	6.57	0.000	***	Acceptable
	KERNELS	2.86	0.025	*	Not satisfied

It was observed a statistically significant effect caused by the treatments on the RAF values in the hulls, since the p-value was lower than the significance value of 0.05 and the ANOVA assumptions were acceptable (Table 9). As a result, the Tukey test (post hoc test) was performed to evaluate which treatment groups differed.

Table 10 - Post hoc Tukey test was performed with `TukeyHSD ()` function in R software version 3.3.3. Only significant results are displayed in the table. α=0.05. '***' p≤0.001 '**' p≤0.01 '*' p≤ 0.05.

Anatomical section	Treatment	Adjusted p-value	Significance level
Hulls	PTZ-CAR	0.000	***
	PTZ-FA	0.009	**
	PTZ-MAG	0.018	*
	STC-PTZ	0.001	***
	UTC-PTZ	0.026	*

Significant differences between the RAF values in the hulls of plants treated with PTZ and of the plants treated with the tri-inhibitors or in both controls were observed (Table 10). Therefore, as expected, PTZ had a standing performance when compared to the tri-inhibitors reducing the fungi in the wheat plant.

However, when performing the one-way ANOVA for the kernels, the assumptions were not satisfied. Again, the normal Q-Q plot showed the presence of outliers and asymmetry in the data (see Appendix, Statistical analysis, Normal Q-Q plots). Consequently, a non-parametric test of Kruskal Wallis included in the package `agricolae` was performed (Table 11) to evaluate the difference between the treatment groups relative to RAF values in the kernels of the wheat plant.

Table 11 - The Kruskal Wallis test was performed with `kruskal ()` function in R software version 3.3.3. Only significant results are displayed in the table. α=0.05. '***' p≤0.001 '**' p≤ 0.01 '*' p≤ 0.05.

Anatomical section	Treatment	p-value	Significance level
Kernels	CAR-PTZ	0.000	***
	FA-PTZ	0.012	*
	FA-STC	0.049	*
	FLA-PTZ	0.005	**
	MAG-PTZ	0.002	**
	PTZ-STC	0.000	***
	PTZ-UTC	0.031	*

Again, as expected, and as previously observed for DON measurements, RAF values in the kernels of the plants treated with PTZ were lower than those of the plants treated with the tri-inhibitors or in the controls, meaning that prothioconazole efficiently inhibited fungi growth and DON production, while the tri-inhibitors did not.

7 Discussion

Fusarium graminearum and *Fusarium culmorum* are pathogenic fungi for several cereals, such as wheat and barley. These pathogens produce type B trichothecenes, namely nivalenol (NIV) and deoxinivalenol (DON). Trichothecenes are hazard mycotoxins for animals (including humans) and plants. They cause several negative effects such as weight loss, nausea, vomiting and convulsions. Therefore, these compounds should be avoided in cereal grains. As DON is phytotoxic for the plants, it leads to the development of Fusarium Head Blight (FHB), which is detected in the cereals by the manifestation of symptoms as floret discoloration in the head of infected spikelets. To control FHB in the cereals, it is crucial to understand the interactions between the pathogens and affected host. Knowledge on the distribution of the pathogens in the plant, the affected anatomy, as well as mycotoxin production and accumulation in the plant system is, thus, important to develop new methods of disease control.

The first part of this study aimed at shading some light on the distribution of *F. graminearum* and *F. culmorum* in the wheat plant as well as DON production by these two pathogens in the plant system with time.

Wegulo (2012) states that during the growing season in the field, FHB symptoms are premature bleaching of the wheat that starts with one or more spikelets on a spike and may continue until the entire spike is whitened. Bleached spikelets usually show chalky white or pink kernels known as *Fusarium*-damaged kernels (FDK). Nevertheless, kernels that appear to be healthy may be infected, and already containing DON. In fact, previous experiments showed that DON was already detected 36 hours after inoculation of wheat spikelets with *F. culmorum*. The same author (Wegulo, 2012) asserts that trichothecenes such as DON have multiple effects on eukaryotic cells such as inhibition of protein synthesis. In plants, DON and 3-ADON are considered to be phytotoxic, causing not only the symptoms above stated as well as growth inhibition of plant material as shown by Bruins et al. (1993) and Shimada and Otani (1990) in studies with seedlings, coleoptile segments, anther-derived callus, and anther-derived embryos. Furthermore, since DON is water soluble, it can easily be translocated in the plant system leading to other physiological effects. Kang and Buchenauer (1999) have also found DON and 3-ADON in mycelium free wheat plant tissues that were actually distant from *F. culmorum* inoculated spikelets. Therefore, they concluded that toxins might be translocated upwards via xylem vessels and phloem sieve tubes as well as downwards via phloem sieve tubes.

The results herein obtained corroborate these observations, since infection aggravated with time after inoculation of the pathogens, as expected. Macroscopic evaluation of the mycelia, allowed to conclude that fungi starts growing firstly in the ears, and then in the leaves, with progressive symptoms of disease such as full ear coverage by the mycelia, bleaching of the spikelets and necrosis of the tissues. The ears of the wheat plants were of great importance in this process, since

they were the focus of pathogen inoculation, being constituted by the spikelets, the rachis and the grains (or kernels). According to Perry and Belford (2000), the grains contain the endosperm, which is the source of energy of the germinating seed, being formed by carbohydrates and proteins. Both fungi use glucose as carbon source, undergoing aerobic respiration, and therefore their main source of nutrients in the plants are the grains. Consequently, the highest decrease of mass was observed in the ears of the wheat plant, with lower variations in the leaves caused as well by a progressive loss of water. These fungal species have many similarities and therefore, the pattern observed in the loss of mass was almost similar between both infection types. Thus, the FHB symptoms and disease development patterns caused by these two pathogens in the wheat are similar to the literature above stated.

Previous studies by Audenaert et al. (2014) indicate that DON production is a fungi mechanism to disturb the defence system of plant and assure colonization that leads to symptom development. According to Hope et al. (2005) both fungal species show optimum DON production at 25°C, with *F. culmorum* producing more DON and *F. graminearum* growing faster and producing DON over a wider range of water activity. In this study, DON analysis allowed to conclude that both species produce more DON in the ears of the wheat plant, when compared with the *flag* leaf and first leaf. These results were expected since the focus of the inoculation was this anatomical section of the plant. Nevertheless, it was concluded that *F. culmorum* strain isolated from north of Germany significantly produces more DON in the ears and F-leaves when compared with *F. graminearum* strain isolated from France. DON concentration in the ears infected with *F. culmorum* was above 850 ppm, compared with 600 ppm measured in the ears infected with *F. graminearum*. Since the maximum value for DON content in unprocessed wheat is 1.25 ppm and for animal feed is of 0.90 ppm (Edwardsa and Godleyb 2010), it is to conclude that in future greenhouse studies moisture chamber timing of 10 days might be reduced to 7 days, as in this case DON concentration for both infection cases was already 100 ppm. With trial time reduction, an optimization of the process can be implemented and laboratory analysis will be performed faster.

Wegulo (2012), Sneller et al. (2012) and Demeke et al. (2010), have shown a positive correlation between the amount of DON produced by *F. graminearum* and the fungal biomass. Demeke et al. (2010) measured DON and quantified *F. graminearum* DNA in grain samples, with gas chromatography and real-time polymerase chain reaction, respectively. A linear regression of DON on fungal DNA was plotted, revealing a strong relationship between the two values with R^2 higher than 0.90. Sanoubar, Bauer, and Seigner (2015), performed a Real-Time PCR (RT-PCR) analysis and confirmed that *F. graminearum* was in higher amounts – DNA range from 0.04 to 4945 $\mu g\ kg^{-1}$– in the infected grains of the wheat plant, compared with *F. culmorum* – 0.04 to 39.22 $\mu g\ kg^{-1}$. Both species were detected in wheat ears of several cultivars randomly collected from farmers in different areas of Bavaria, in the south of Germany. The TaqMan Real-time PCR analysis showed that *F. graminearum* was an efficient DON producer with a high positive correlation (R^2=0.7) between

DON and fungi DNA content, being therefore considered the most aggressive in FHB development. In contrast, a weak correlation was found between DON and *F. culmorum* DNA (R^2=0.03), suggesting this species as weaker DON producer when compared to the previous one. Nevertheless, previous studies indicate that *F. culmorum* and *F. graminearum* are the most pathogenic *Fusarium* species infecting cereal ears, despite the fact that in Sanoubar, Bauer and Seigner (2015) study these species were considered to have a low agressiveness all over Bavaria, as DON content was relatively low (10-2990 µg kg^{-1}). Yli-Mattila et al. (2008) also states that in previous studies (1) DON concentration correlated with the total DNA (R^2=0.83) of DON producing species *F. culmorum* and *F. graminearum* in the Netherlands wheat and (2) the correlation between DON and DNA levels was very good (R^2=0.90) in samples with high DON concentration. However, in Finnish wheat and barley it was found a better correlation between DON and DNA levels of trichothecene producing species *F. culmorum*, while *F. graminearum* was found as less predominant, presenting lower R^2 values for correlation (Yli-Mattila et al., 2008). Yli-Mattila et al. (2008) showed that SYBR Green was as well previously used in quantitative PCR to estimate the amount of *Fusarium spp.* in wheat. Finally, also Nicolaisen et al. (2009) affirms that in wheat samples from Danish fields it was found a good correlation between the occurrence of *Fusarium* species determined by RT-PCR and the toxin content, with *F. culmorum* and *F. graminearum* both present in most samples.

In this study, a linear correlation was found between DON production and relative amount of fungi (RAF%), with a R^2 higher than 0.90 and a p-value lower than the alpha significance value of 0.05, for both pathogens. Therefore, there was a statistically relevant relationship between fungal growth and DON production in the tissues of the wheat plant, being expected that the factors that lead to pathogen growth in the plant also promote DON production in the tissues, corroborating the previous studies. Additionally, the relative amount of fungi (RAF) in the samples did not vary much between the two species because of overlapping in standard deviation. In fact, RAF values were only significantly different in day 10, with *F. culmorum* being in higher amount in the ear samples. RAF values increased progressively with time for both infection cases due to fungi growth in the wheat plant. Even so, it was interesting to observe that RAF values of 0% on day 7 did not imply null DON content in the plant, as in fact the mycotoxin was already detected in the tissues 7 days post inoculation, suggesting its translocation in the plant vessels as previously reffered to. Consequently, it was possible to conclude that as RAF values did not vary much between species, and as *F. culmorum* was still able to produce more DON compared with *F. graminearum*, the first was considered the most aggressive species in this study.

The second part of this work allowed to test natural tri-inhibitors, namely magnolol, carvacrol, ferulic acid and flavone, reported as effective in *in vitro* trials. This was performed to evaluate the feasibility of their use to control Fusarium head blight (FHB).

According to Pani et al. (2014), these compounds are of great interest in agrochemical research, namely in the development of selective and environmentaly friendly mycotoxin inhibitors. Specific and strong inhibitory activities were therefore demonstrated by phenolic and polyphenolic natural compounds against *F. graminearum* and *F. culmorum* trichothecene producing strains *in vitro* (Pani et al., 2014). The interest in these compounds has been growing due to its application as antimicrobials and fungicidal agents. They are usually plant metabolites, that may be present in food preservatives, spices, being generally recognized as safe (GRAS). Pani et al. (2014) tested several compounds *in vitro* at different concentrations to evaluate the vegetative growth and 3-ADON production by *F. culmorum* strain ISPaVe MCf21 ($1x10^4$ conidia/ml incubated at 25ºC). After 14 days of incubation both media and mycelia were analysed using thin-layer chromatography (TLC) and liquid chromatography−mass spectrometry (LC−MS). Almost complete DON inhibition and no significant fungal growth was observed when carvacrol was added at 1.0 mM in the liquid medium, being considered a good type B trichothecene inhibitor (Pani et al., 2014). In the same study, magnolol displayed a remarkable fungicide activity when tested at 1.5 mM, retaining the capacity of inhibiting DON production even at the lowest concentrations. In a different study, Pani et al. (2016) showed that ferulic acid demonstrated high affinity with the catalic domain of the TRI5 protein, being considered a good inhibitor in trichothecenes byosynthesis. According to Kim et al. (2006) and Zhou, Su, and Yu (2004) phenolic acids are usually present in a free soluble form, however most of them (including ferulic acid) are insoluble and bound to cell wall polysacharides by ester linkages. Matern and Kneusel (1988) and Nicholson and Hammerschmidt (1992) report that phenolic acids accumulate rapidly at the infection site upon pathogen infection, hence it was supposed that during *Fusarium* progression to the kernels, it most likely encounters ferulic acid. Takahashi-Ando et al. (2008) showed that unsubstituted flavone was the most potent inhibitor of TRI4 and at 100 µM flavone inhibited 68% of the 2α-hydroxylation activity related with trichothecene biosynthesis using recombinant *S. cerevisiae* expressing *Tri4* (in liquid media at 28ºC). However, in the same study but using *F. graminearum* in liquid media (25ºC), 100 µM of flavone failed to inhibit trichothecene production at longer incubation periods (5 days) although it successfully inhibited TRI4 at this concentration (Takahashi-Ando et al. 2008). Nevertheless, at higher concentrations (200 µM) flavone was efficient in decreasing the amount of trichothecenes at day 10, without signififcant changes in dry weight of fungal biomass (Takahashi-Ando et al. 2008). However, according to Einhellig (2003) some flavonoids such as flavone were reported to be potent inhibitors of energy metabolism, blocking mitochondrial and chloroplast functions. Specifically, according to Peer and Murphy (2007), flavones have shown to interfere with ATP formation in the plant mitochondria and might interfere as well with plant hormones such as auxins and its action.

In this study, tests *in planta* were performed with magnolol, carvacrol, ferulic acid and flavone. The results showed that none of these natural tri-inhibitors was successful inhibiting DON biosynthesis by the fungi in the wheat plants, in the selected experimental conditions. DON concentration was higher in the ears of the wheat plant, specifically in the hulls followed by the

kernels. The mycotoxin concentration in the ears was even higher in plants treated with tri-inhibitors when compared with the untreated control (UTC). More to say, flavone seemed to be phytotoxic for the plants, due to the premature yellowing of the leaves. Interestingly, the relative amount of *F. graminearum* in the hulls and kernels of the plants was higher for some treatments when compared with the relative amount of *F. culmorum*. These was mainly observed for plants treated with carvacrol, flavone and the solvent treatment control, as the RAF standard deviations in these cases almost do not overlap between species. The untreated control apparently shows that *F. culmorum* is more present in the hulls compared with *F. graminearum*. Nevertheless, more untreated controls should have been analysed to take statistically significant conclusions between species. As expected, prothioconazole inhibited fungi growth, since it is a commonly used fungicide in agriculture to control FHB and as a result the qPCR did not detect almost any DNA from fungi in the hulls and kernels of the analysed samples treated with PTZ (RAF=0%). Moreover, DON concentration in the same plants was not that high when compared with DON content in tri-inhibitors treatments or even the controls. Thus, it is to conclude that a small amount of fungi produces a considerable amount of DON, and that there might be considered mycotoxin circulation in the plant system from the ears to the leaves, as previously stated in the literature. Furthermore, although *F. graminearum* was in higher amount in the plant tissues compared with *F. culmorum*, this last produced a higher amount of DON in the ears of the plant. Therefore, plants treated with tri-inhibitors might even have stimulated DON production by *F. culmorum*, which appears to be the most aggressive species in this study. Finally, this study has shown that flavone should be excluded in future studies as it may be considered as potentially phytotoxic for the wheat.

7.1 Limitations and Future Work

This study was performed with two strains of pathogens isolated from specific places – *F. graminearum* from France and *F. culmorum* from north of Germany. As previously explained, there is an interaction between the pathogens and the host that depends on several factors, including the genetics and biological characteristics of the species interacting. Consequently, the results presented in this study are restricted to the species used, despite the fact that both pathogen strains are reported as being severely aggressive. For a more accurate conclusion on the results obtained, different strains should be used and field trials should be performed. By doing this, a larger amount of data on DON measurements and relative amount of fungi would be gathered to take further conclusions. Future work should focus on the ears of the wheat plants, because the grains are the business core of wheat producers, and because it was concluded that the leaves are not primarily affected by the fungi, most probably due to their low content in carbohydrates. Therefore, fungicide development should have the ears of the wheat as a target to avoid and decrease DON and fungi growth.

Even though the natural tri-inhibitors were not successful avoiding DON production by the fungi for the selected conditions, further research should be performed. The research should be conducted to understand a possible way of establishing a direct contact between these compounds and the pathogens, allowing them to cross the plant barriers instead of staying exclusively at the surface of the plant material. A curative treatment instead of protective should as well be considered.

In the present study, it was concluded that DON concentration in the 7^{th} day post inoculation was already sufficiently high for further analysis in the laboratory. Hence, future trials can be carried out in shorter periods, which is an advantage for the company and for the development of new solutions.

8 References

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9 Appendix

9.1 Plant growth and flowering, compound spraying and fungi inoculation

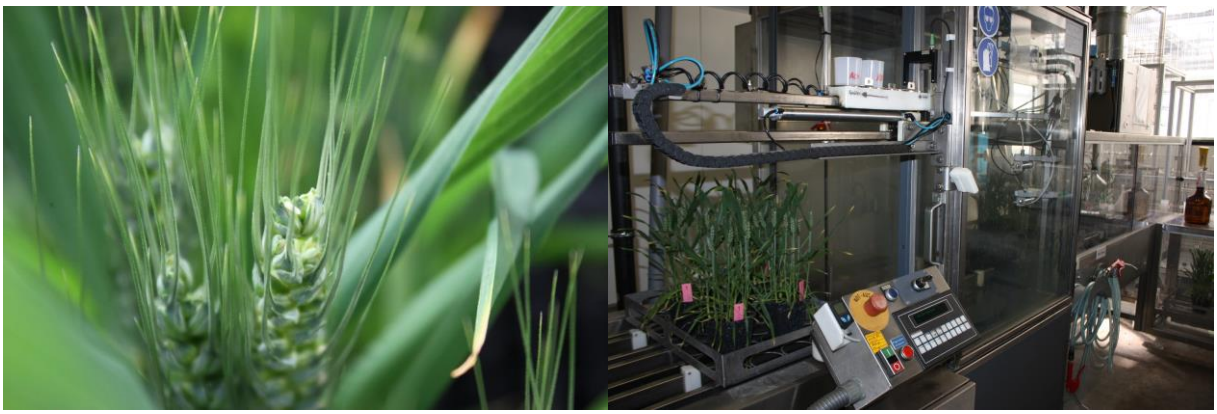

Fig. 27 – Wheat plant on the flowering stage (left) and spraying of the compounds (right).

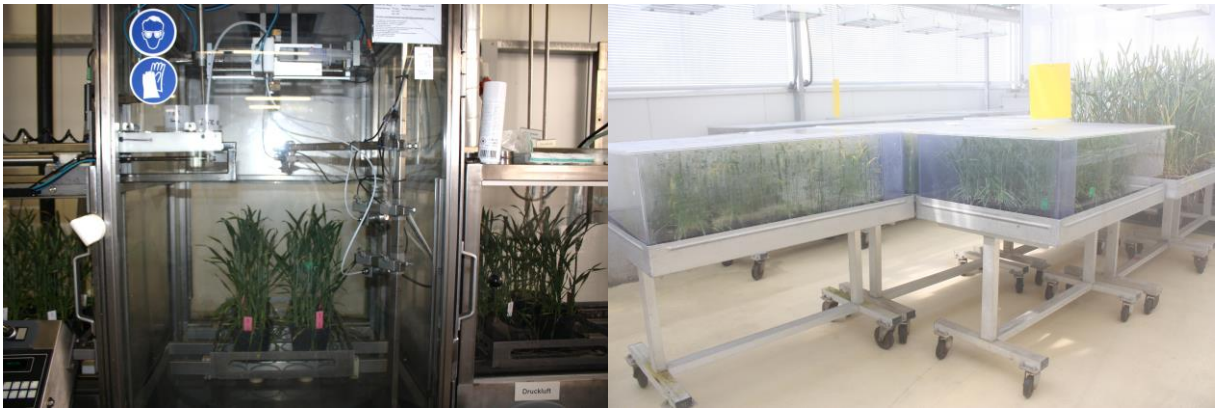

Fig. 28 – Spraying of the compounds inside the cabine (left) followed by storage of the plants in boxes that stay in the high moisture chamber (right).

Fig. 29 – The next day the spore suspension was prepared from *F. culmorum* and *F. graminearum* cultures (left) to be sprayed in the previously treated plants (right).

Fig. 30 – The plants are sprayed with the pathogens using a spray gun (left) and after stored in boxes that were kept in the high moisture chamber for 10 days (right).

9.2 Pathogen growth

9.2.1 Trials A1 and A2: Detection and evaluation

Fig. 31 – 3 days after inoculation (3 dpi). *F. culmorum* infected ear (left) and *F. graminearum* infected ear (right).

Fig. 32 – 7 days after inoculation (7 dpi). *F. culmorum* infected ear (left) and *F. graminearum* infected ear (right).

Fig. 33 – 10 days after inoculation (10 dpi). *F. culmorum* infected ear (left) and *F. graminearum* infected ear (right).

Fig. 34 – 14 days after inoculation (14 dpi). *F. culmorum* infected ear (left) and *F. graminearum* infected ear (right).

Fig. 35 – 17 days after inoculation (17 dpi). *F. culmorum* infected ear (left) and *F. graminearum* infected ear (right).

9.2.2 Trial A3: Detection and evaluation

Fig. 36 – 6 days post inoculation (6 dpi), untreated controls (UTC): *F. culmorum* infection (left) and *F. graminearum* infection (right).

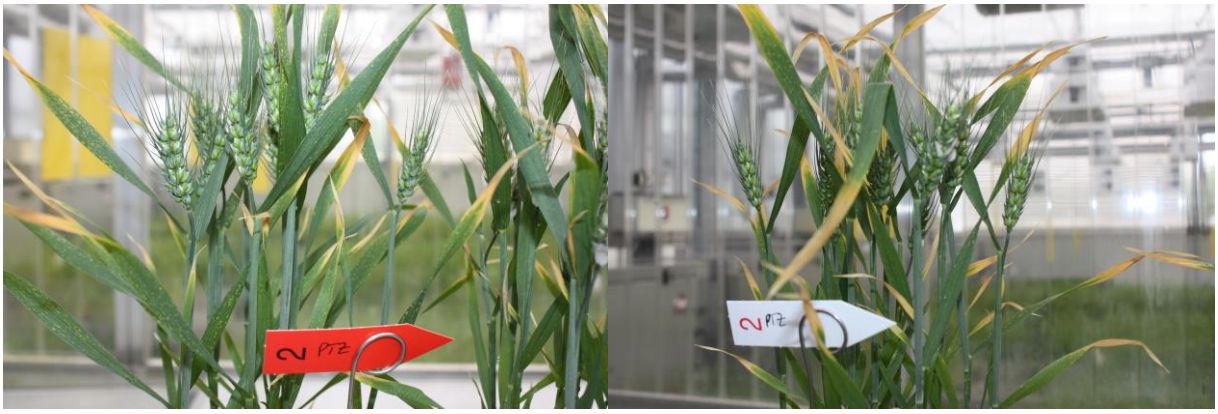

Fig. 37 – 6 days post inoculation (6 dpi), prothioconazole (PTZ) treated plants: *F. culmorum* infection (left) and *F. graminearum* infection (right).

Fig. 38 – 6 days post inoculation (6 dpi) infection with *F. culmorum*: Plants treated with ferulic acid (FA) (left) and plants treated with magnolol (MAG) (right).

Fig. 39 – 6 days post inoculation (6 dpi) infection with *F. graminearum*: Plants treated with magnolol (MAG) (left) and plants treated with carvacrol (CAR) (right).

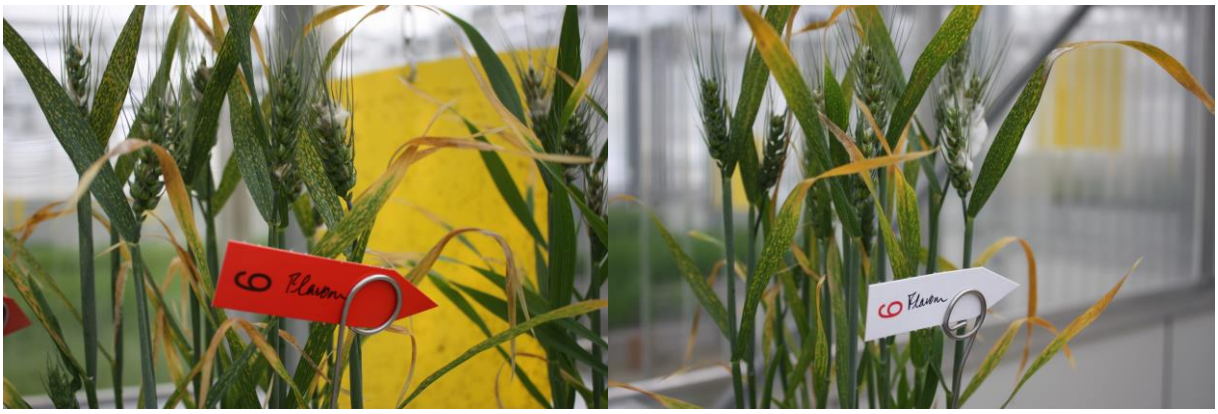

Fig. 40 – 6 days post inoculation (6 dpi), plants treated with flavone (FLA): *F. culmorum* infection (left) and *F. graminearum* infection (right).

Fig. 41 – Phytotoxicity caused by flavone in the plant.

9.2.3 Mass loss in the wheat plant with time

Fig. 42 – 7 days after inoculation (7 dpi): loss of water in the plant.

Table 12 – *F. graminearum* infection. Average wet mass and standard deviation for 10 ears, 10 F-leaves and 10 F-1 leaves (one pot), for each dpi. Collected data from Trials A1 and A2.

$(\overline{m} \pm stdev)g$

dpi	EARS	stdev	F-LEAF	stdev	F-1 LEAF	stdev
0	15.17	1.28	8.35	0.47	7.61	0.45
3	14.71	1.14	9.16	1.22	7.17	0.99
7	11.44	1.48	8.28	0.98	6.99	0.60
10	8.05	1.91	6.21	0.77	6.32	0.58
14	3.85	0.36	3.03	0.73	4.86	0.83
17	4.19	0.74	2.34	0.67	4.63	1.16

Table 13 - *F. culmorum* infection. Average wet mass and standard deviation for 10 ears, 10 F-leaves and 10 F-1 leaves (one pot), for each dpi. Collected data from Trials A1 and A2.

$(\bar{m} \pm stdev)g$

dpi	EARS	stdev	F-LEAF	stdev	F-1 LEAF	stdev
0	15.17	1.48	8.35	0.47	7.61	0.45
3	14.47	1.55	8.61	1.22	7.07	0.67
7	11.59	1.65	8.10	0.98	7.14	0.60
10	7.42	1.44	5.78	0.77	6.05	0.64
14	3.89	0.30	2.54	0.73	4.74	0.68
17	3.94	0.64	2.49	0.67	4.47	1.16

9.3 Sampling and Storage

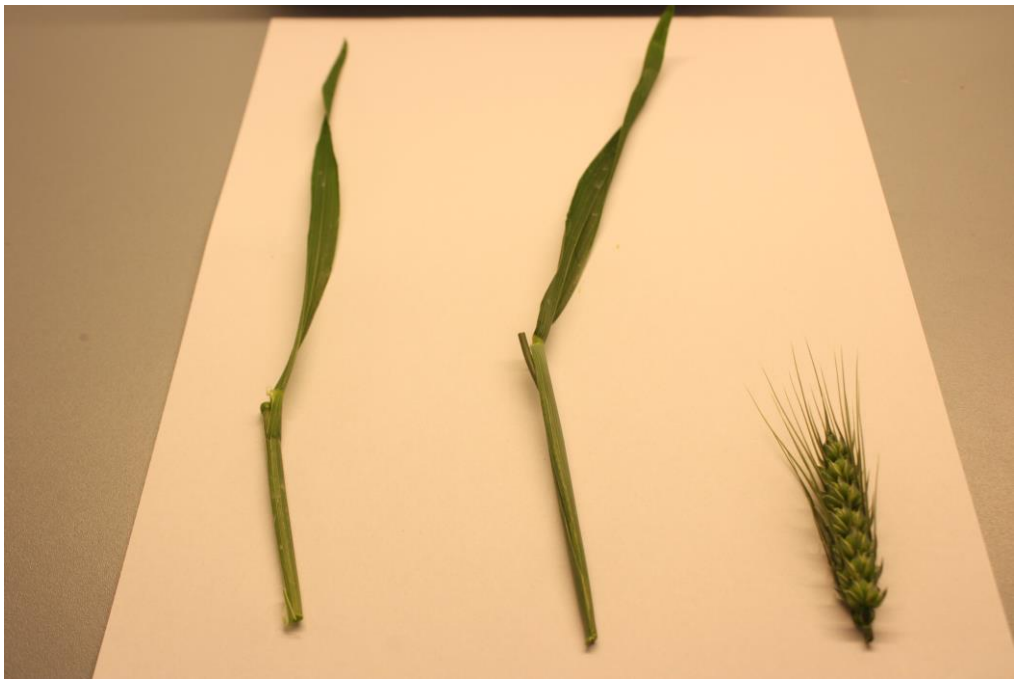

Fig. 43 – Cut of the plants upon sampling. F-1 leaf, F-leaf and ear of the wheat plant (from left to right, respectively).

9.4 Threshing and Milling

Fig. 44 – Threshing machine used after drying of the ears. Allowed separation of the kernels and hulls.

Fig. 45 – Plant material obtained after threshing. Infected kernels (left) and infected hulls (right).

Fig. 46 – Retsch Grindomix GM200 (left) to mill higher amounts of plant material (10 ears, 10 leaves or 10 F-1 leaves) and Retsch MM400 (right) to mill smaller amounts of plant material, typically kernels and hulls that fit properly in the respectively two containers of the machine.

9.5 DON Analysis

Fig. 47 – Prepared strips with 300 µl of sample (left). ROSA incubator (center) to put the strips for 2 minutes at 46°C and Charm EZ-M system to read DON concentration in the strips.

Table 14 – DON concentration in the ears of the wheat plant for different dpi. Results for trials A1 and A2 are displayed. In trial A1 DON measurements were not performed in the 14 dpi.

DON (ppm)

Trial	A1		A2	
dpi	GIBBZE	FUSACU	GIBBZE	FUSACU
0	0	0	-	-
0	0	0	-	-
0	0	0	0	0
3	0	0	0.5	0.5
3	0	5	0	1
3	0	5	0	1
7	180	120	110	110
7	230	200	85	150
7	160	230	95	100
10	190	400	170	250
10	250	370	190	240
10	140	260	170	260
14	-	-	250	390
14	-	-	240	440
14	-	-	270	490
17	850	810	600	820
17	510	990	640	910
17	830	870	620	820

Table 15 - DON concentration in the F-leaves of the wheat plant for different dpi. Results for trials A1 and A2 are displayed. In trial A1 DON measurements were not performed in the 14 dpi.

	DON (ppm)			
Trial	A1		A2	
dpi	GIBBZE	FUSACU	GIBBZE	FUSACU
0	0	0	-	-
0	0	0	-	-
0	0	0	0	0
3	5	0	0	0
3	0	0	0	0
3	0	0	0	0
7	15	15	10	25
7	0	25	20	35
7	5	0	0	25
10	95	150	15	70
10	150	190	55	70
10	80	170	70	170
14	-	-	170	200
14	-	-	230	190
14	-	-	160	210
17	600	550	220	480
17	530	580	230	430
17	450	340	230	450

Table 16 - DON concentration in the F-1 leaves of the wheat plant for different dpi. Results for trials A1 and A2 are displayed. In trial A1 DON measurements were not performed in the 14 dpi.

	DON (ppm)			
Trial	A1		A2	
dpi	GIBBZE	FUSACU	GIBBZE	FUSACU
0	0	0	-	-
0	0	0	-	-
0	0	0	0	0
3	0	0	0	0
3	10	0	0	0
3	0	0	0	0
7	10	5	10	10
7	0	5	0	5
7	0	0	0	10
10	30	70	5	40
10	45	20	0	20
10	0	50	45	65
14	-	-	70	20
14	-	-	45	40
14	-	-	15	80
17	100	190	55	85
17	120	200	80	210
17	60	80	50	100

Table 17 – Trial A3: DON concentration in the kernels and hulls of the wheat plant for different treatments.

TRIAL	*A3*			
	KERNELS		HULLS	
treatment	GIBBZE	FUSACU	GIBBZE	FUSACU
PTZ	15	20	160	240
PTZ	20	25	110	390
PTZ	45	20	150	160
MAG	150	190	530	580
MAG	170	110	470	600
MAG	140	170	200	510
CAR	150	150	350	600
CAR	150	150	400	600
CAR	170	130	480	600
FLA	200	140	550	600
FLA	220	200	230	600
FLA	250	200	510	590
FA	150	140	470	590
FA	160	130	410	600
FA	210	140	470	600
STC	160	110	230	600
STC	150	180	190	600
STC	200	190	500	600
UTC	170	140	380	540

Table 18 - Trial A3: DON concentration in the F-leaves and F-1 leaves of the wheat plant for different treatments.

TRIAL	*A3*			
	F-LEAF		F-1 LEAF	
treatment	GIBBZE	FUSACU	GIBBZE	FUSACU
PTZ	15	20	0	0
PTZ	35	35	0	0
PTZ	20	40	0	0
MAG	150	240	0	35
MAG	160	180	50	5
MAG	230	240	15	30
CAR	190	190	25	5
CAR	130	250	5	20
CAR	150	120	5	15
FLA	230	350	30	20
FLA	240	200	30	0
FLA	350	230	70	25
FA	210	200	60	0
FA	15	180	25	10
FA	250	140	210	5
STC	170	160	15	5
STC	30	210	35	25
STC	190	230	160	5
UTC	140	240	15	30

9.6 Quantitative PCR (qPCR)

The qPCR was performed to obtain the Ct values for each case and at last plot the curves. The Ct values should not vary much between fungi as the gene amplified was the same in both cases, using the same primers. The standard curves were plotted with the following data:

Table 19 – Prepared dilutions (50 ng/µl to 0.8 ng/µl), respective logarithm and Ct values obtained in the qPCR for *F. culmorum* (FUSACU) DNA and *F. graminearum* (GIBBZE) DNA.

Concentration (ng/µl)	*log concentration ng/µl*	*Ct FUSACU*	*Ct GIBBZE*
50,0	1,70	7,89	6,6
25,0	1,40	8,23	7,67
12,5	1,10	8,94	8,79
6,3	0,80	9,73	9,21
3,1	0,50	10,82	10,63
1,6	0,19	11,81	11,73
0,8	-0,11	12,65	11,6

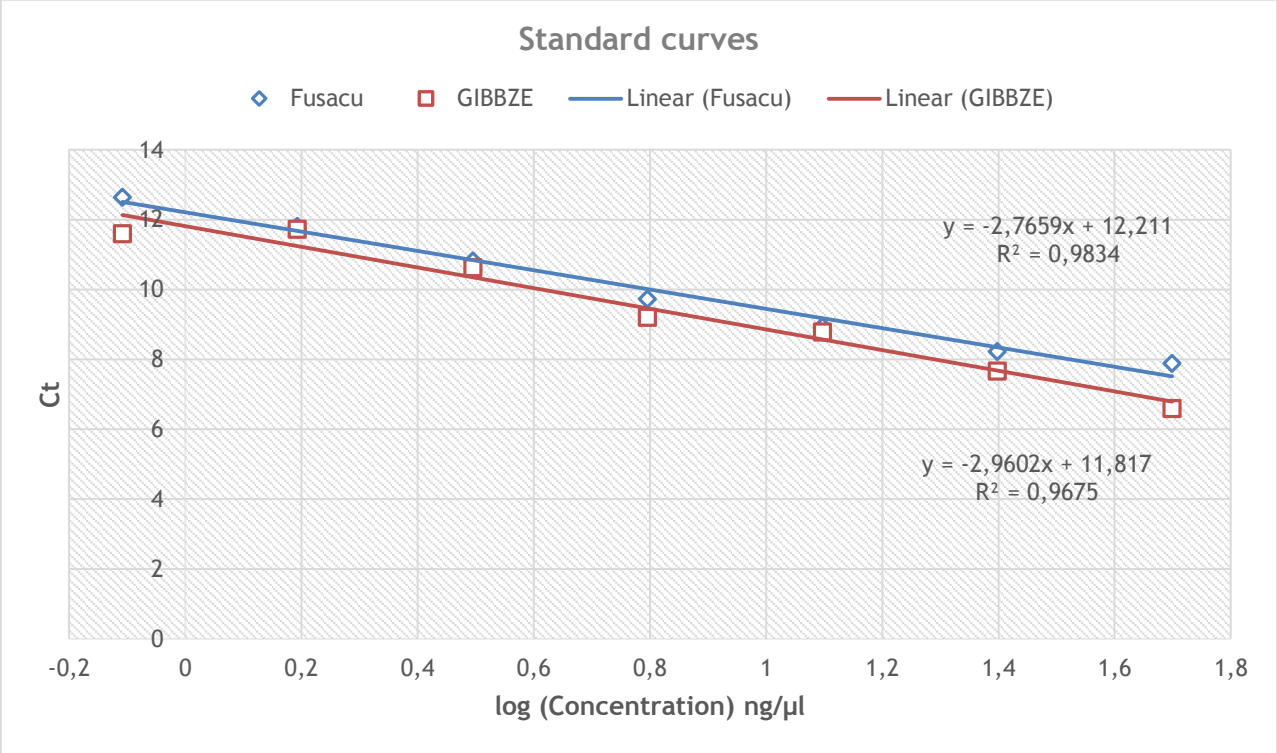

Fig. 48 – Standard curves obtained upon plotting of the Ct values against the logarithm of the concentrations for FUSACU and GIBBZE DNA.

A linear regression was performed which allowed to obtain the linear equations to calculate the fungi cytochrome b concentrations in the following experiments. The melting curve for this qPCR is displayed below:

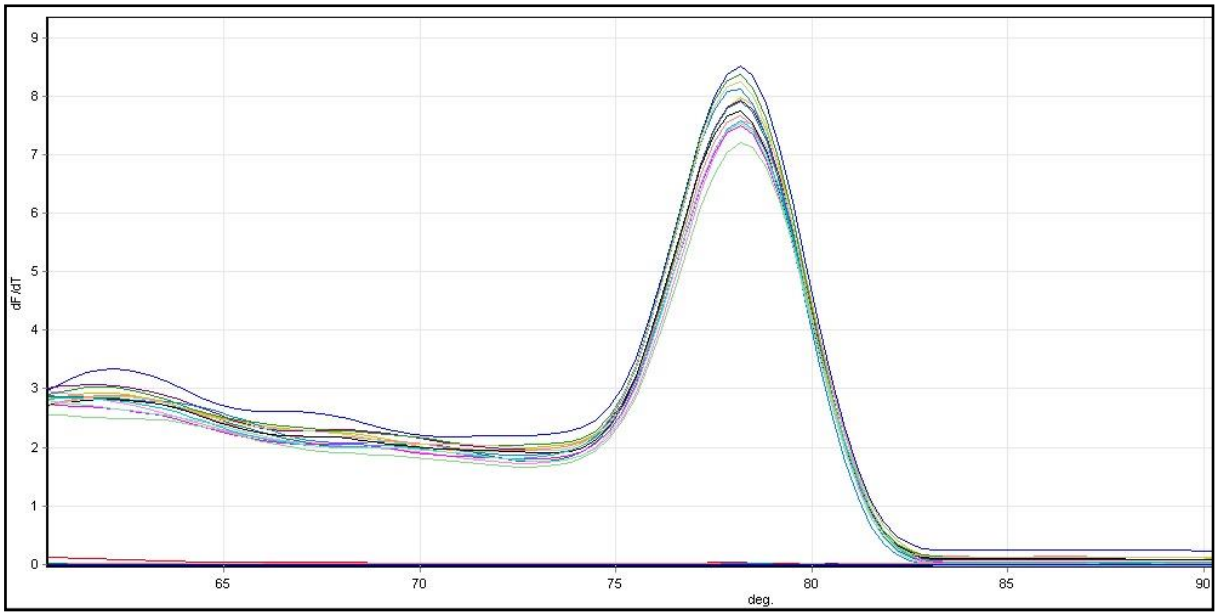

Fig. 49 – Melting curves obtained in the qPCR with FUSACU and GIBBZE DNA for the standard curves.

The plot of dF/dT translates the change of fluorescence in the reaction which acompanies the melting of the double-stranded PCR products. Therefore, this plot displays the change of fluorescence as distinct peaks. As a result, when there is a maximum peak, this means that the melting temperature (T_m) of a specific product occured. This allows to conclude if the primers are specific – only one peak – or not – more than one peak - , and also reveal the presence of primer dimers. Primer dimers have a vey small size and therefore, melt at lower temperatures when compared with the desired product.

This melting curve has indeed only one peak, for a melting temperature close to 80°C, meaning that the primers are specific. The differences in peak height are due to the initial concentration of the DNA: the most diluted (0.8 ng/ µl) DNA has a lower peak when compared to the least diluted one (50 ng/ µl), as the most concentrated DNA will result in a more intensive fluorescence signal.

As previously said, it is also important to run the qPCR products in an agarose gel to check for a single band. The amplification of the cytochrome b gene results in a product of 152bp.

5’-
TACCTTATGGACAAATGTCATTATGAGGTGCTACAGTTATTACTAATTTAATTAGTGCTGTTCCA
TGAATTGGACAAGACATAGTTGAGTCAACAAACACTATTATTAATTTATGTTTAGGTATTATTAC
TTTATTTTTAATGTACGGCGCA-3’

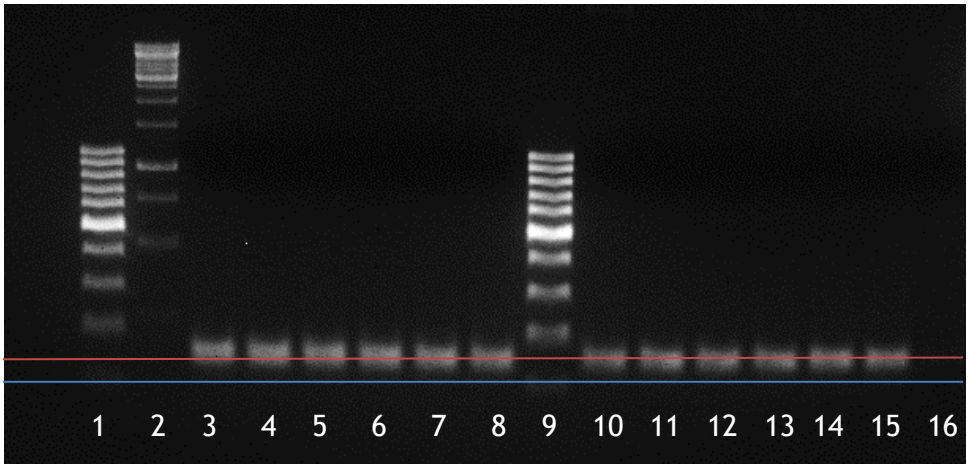

Fig. 50 – Electrophoresis results. Expected DNA is 152 bp (red line). The lower DNA marker is 100 bp (blue line). 1 and 9 – GeneRuler™ 100 bp DNA Ladder; 2 - GeneRuler™ 1 kb DNA Ladder; 4 to 9 – *F. graminearum* DNA; 11 to 15 – *F. culmorum* DNA; 16 – Negative control

The blue line is relative to the 100bp band while the red line stands at approximately 150bp, where the qPCR products are. These products result from the qPCR performed to obtain the standard curves, therefore the template DNA are the prepared dilutions from *F. graminearum* and *F. culmorum* cultures. After analysing the melting curve and the electrophoresis result, it is possible to conclude that the qPCR assay is specific and optimized.

Table 20 – Trial A2: Relative amount of fungi (RAF) in the ears of the wheat plant in different dpi.

	Relative amount of fungi (RAF) (%)	
dpi	GIBBZE	FUSACU
0	0	0
3	0	0
3	0	0
3	0	0
7	0	0
7	0	0
7	0	0
10	2	12
10	3	7
10	2	16
14	14	28
14	32	47
14	25	35
17	51	45
17	59	64
17	57	69

Table 21 - Trial A3: Relative amount of fungi (RAF) in the kernels and hulls of the wheat plant for different treatments.

Relative amount of fungi (RAF) (%)

	KERNELS		HULLS	
Treatment	GIBBZE	FUSACU	GIBBZE	FUSACU
PTZ	0	0	1	0
PTZ	0	0	1	0
PTZ	1	0	1	0
MAG	16	23	48	54
MAG	62	6	27	26
MAG	8	8	59	31
CAR	52	6	80	36
CAR	37	16	100	55
CAR	26	9	86	13
FLA	20	3	64	19
FLA	16	10	31	11
FLA	16	8	14	13
FA	14	0	34	45
FA	15	13	51	24
FA	19	7	65	44
STC	76	17	73	47
STC	40	11	73	44
STC	62	9	64	19
UTC	13	14	52	58

9.7 Statistical analysis

9.7.1 One-way ANOVA tables

Table 22 - The one-way ANOVA was performed to search for a statistically significant effect of different fungal species - *F. graminearum* and *F. culmorum* - on DON production for each time point (dpi) and anatomical of the wheat plant. Null hypothesis: different fungal species do not have a statistically significant effect on DON production in the EARS (F-LEAF / F-1 LEAF). Alternative hypothesis: different fungal species have a statistically significant effect on DON production in the EARS (F-LEAF / F-1 LEAF). Results obtained with R software version 3.3.3. α=0.05. '***' p≤0.001 '**' p≤ 0.01 '*' p≤ 0.05 '.' p≤ 0.1

Dpi (Days Post inoculation)	Anatomical section	**F value**	**p-value**	**Significance level**	**Assumptions of linear regression**
3	EARS	8.00	0.047	*	Acceptable
	F-LEAF	-	-	n.s.	
	F-1 LEAF	-	-	n.s.	
7	EARS	1.90	0.240	n.s.	
	F-LEAF	7.56	0.051	.	
	F-1 LEAF	1.80	0.251	n.s.	
10	EARS	69.14	0.001	***	
	F-LEAF	2.33	0.202	n.s.	
	F-1 LEAF	1.68	0.265	n.s.	
14	EARS	38.24	0.003	**	
	F-LEAF	0.35	0.587	n.s.	
	F-1 LEAF	0.020	0.895	n.s.	
17	EARS	51.19	0.002	**	
	F-LEAF	231.20	0.000	***	
	F-1 LEAF	2.99	0.159	n.s.	

Table 23 – Analysis of variance (ANOVA) performed to evaluate the effect of the treatments on DON production in the HULLS (KERNELS/F-LEAF/F-1 LEAF) of the wheat plant. Results obtained with R software. Null hypothesis: Different treatments do not show significant effects on DON production in the HULLS (KERNELS/F-LEAF/F-1 LEAF) of the wheat plant. Alternative hypothesis: Different treatments have a statistically significant effect on DON production in the HULLS (KERNELS/F-LEAF/F-1 LEAF). Results obtained with R software version 3.3.3. α=0.05. '***' p≤0.001

	Anatomical section	F value	p-value	Significance level	Assumptions of linear regression
treatment	HULLS	4.18	0.003	**	Not satisfied
	KERNELS	26.26	7.239e-11	***	Acceptable
	F-LEAF	8.93	1.089e-05	***	Not satisfied
	F-1 LEAF	0.99	0.448	n.s.	Not satisfied

9.7.2 Normal Q-Q plots

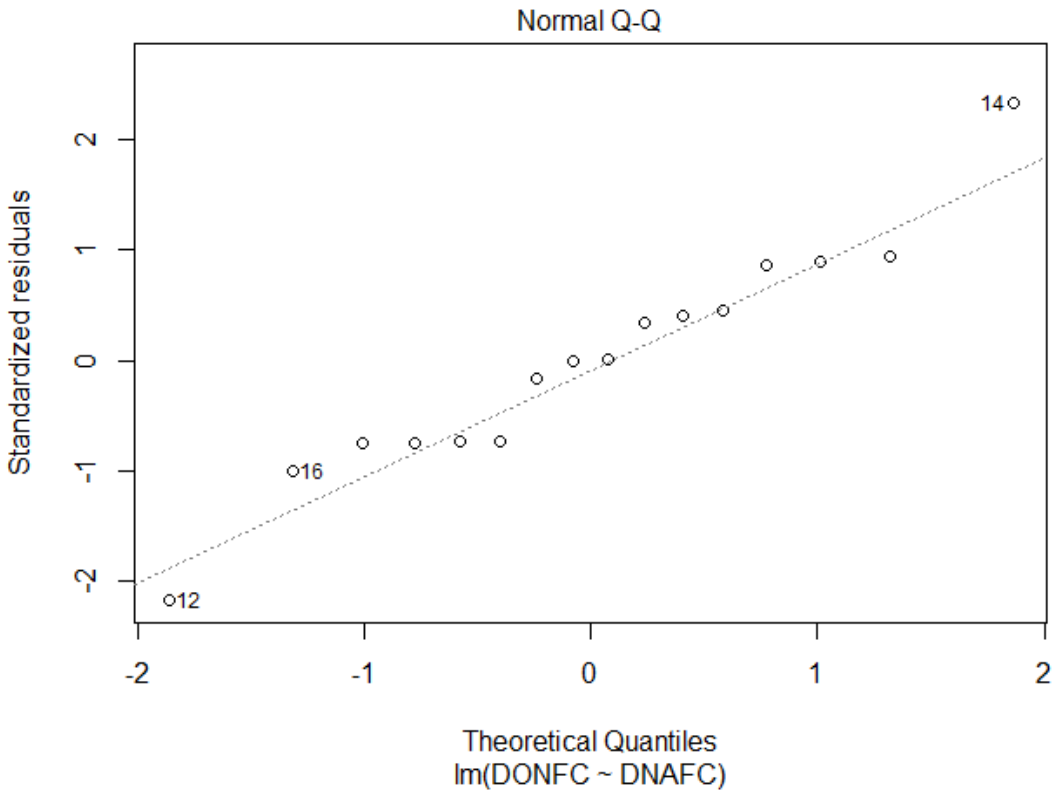

Fig. 51 – Normal Q-Q plot obtained with R software version 3.3.3, relative to linear regression of DON production by *F. culmorum* against relative amount of fungi (RAF) in the ears of the wheat plant.

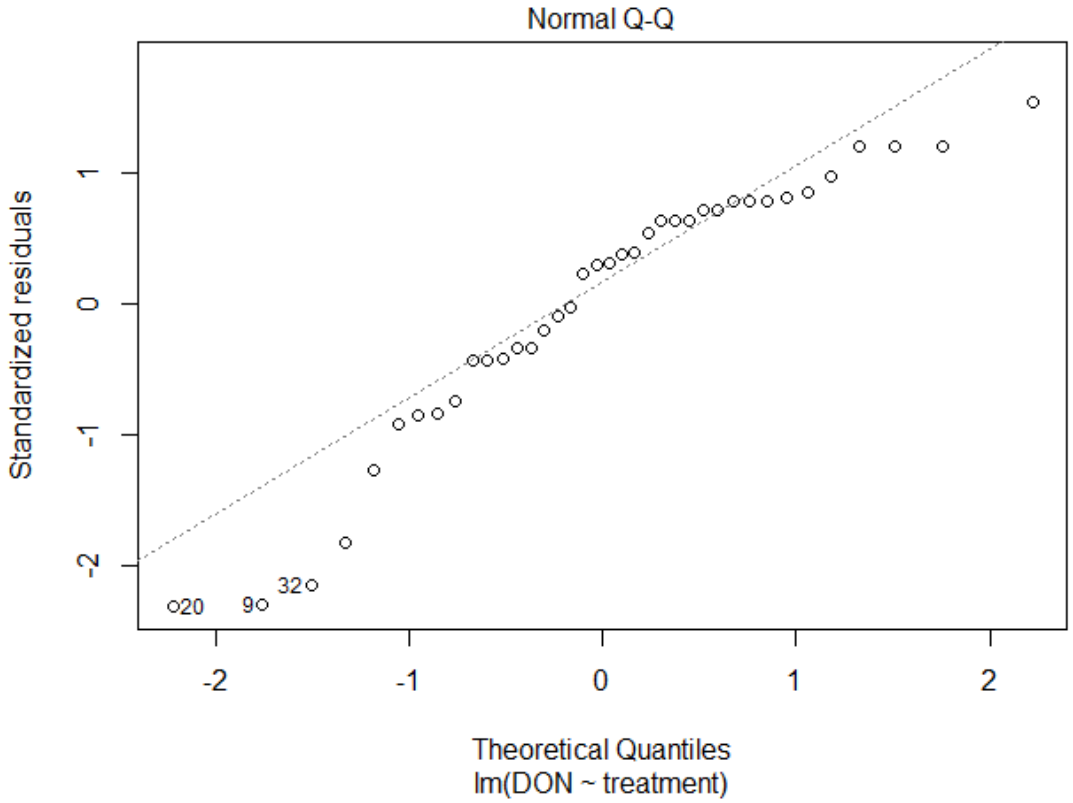

Fig. 52 – Normal Q-Q plot obtained with R software version 3.3.3, relative to the effect of different treatments on DON production in the hulls of the wheat plant.

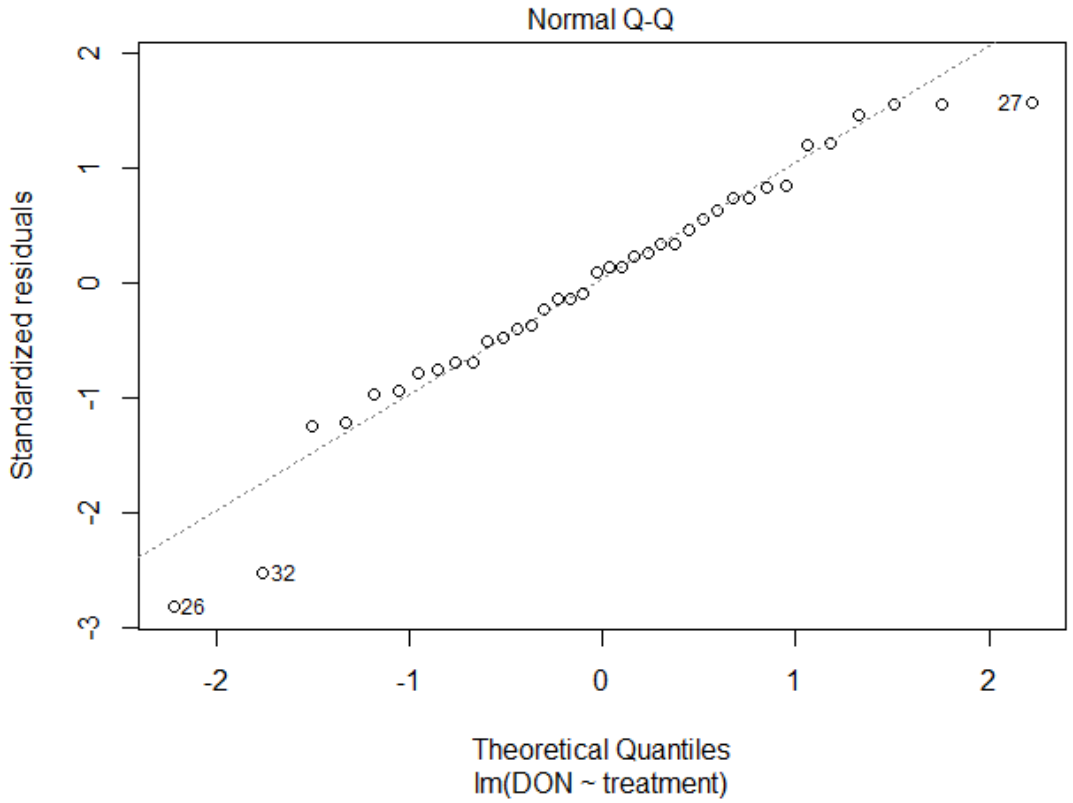

Fig. 53 – Normal Q-Q plot obtained with R software version 3.3.3, relative to the effect of different treatments on DON production in the F-leaves of the wheat plant.

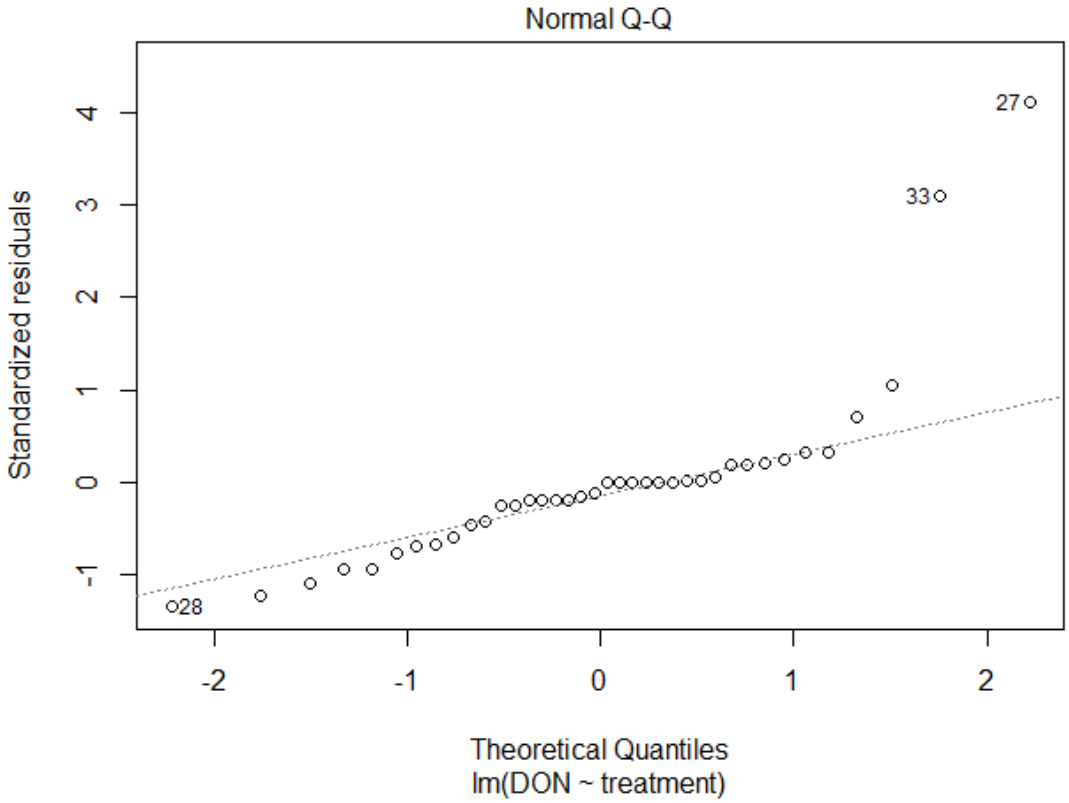

Fig. 54 – Normal Q-Q plot obtained with R software version 3.3.3, relative to the effect of different treatments on DON production in the F-1 leaves of the wheat plant.

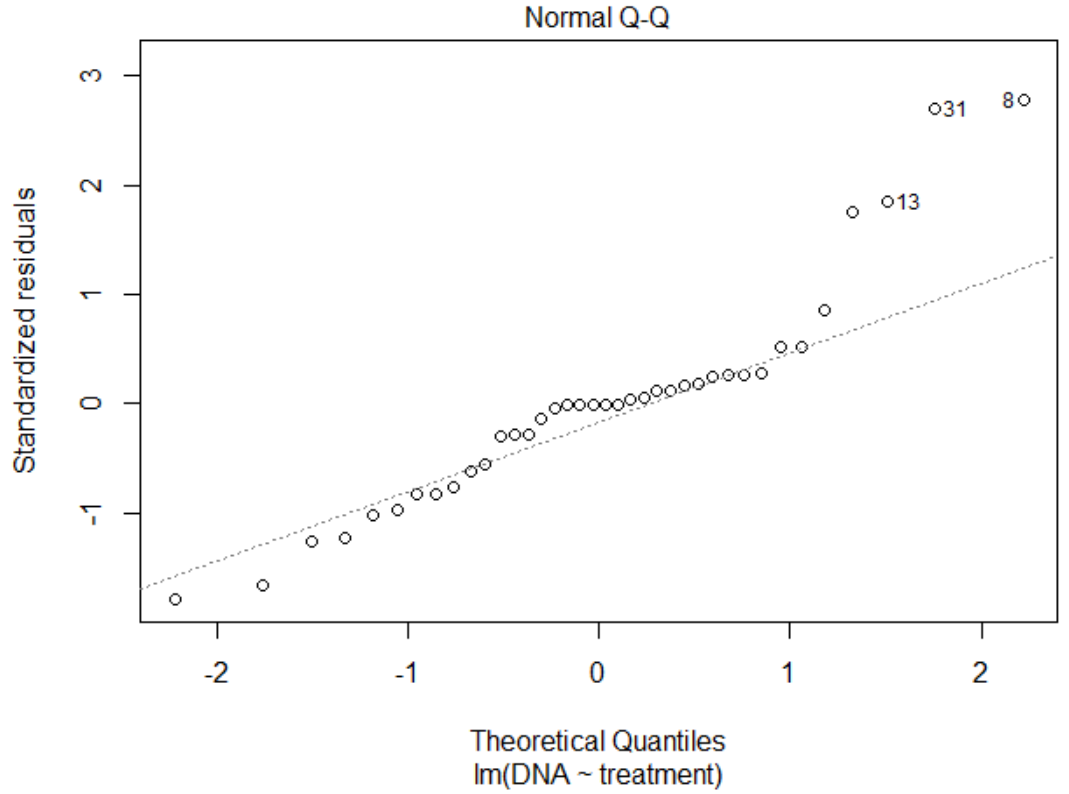

Fig. 55 -Normal Q-Q plot obtained with R software version 3.3.3, relative to the effect of different treatments on RAF values in the kernels of the wheat plant.

9.7.3 R script written in R software version 3.3.3.

```
#TRIALS A1 and A2: Study the distribution of Fusarium culmorum and Fusarium
#culmorum in the wheat as well as DON production in the plant with time.
#
#
#
#
#Perform analysis of variance to DON production in the EARS (F-leaf /F-1 leaf)
#of the wheat plant for each dpi.
#
#Null hypothesis: DON concentration in each anatomic section was not
#significantly different between species in the EARS (F-LEAF / F-1 LEAF).
#
#Alternative hypothesis: DON concentration in each anatomic section was
#significantly different between species in the EARS (F-LEAF / F-1 LEAF).
#
#
#alpha=0.05
#
#libraries

library(tidyr)

#Makes data easier to work with: it's easy to munge (with #dplyr), visualise
#(with ggplot2 or ggvis) and model (with R's hundreds of #modelling packages).
#The two most important properties of tidy data are: #Each column is a
#variable. Each row is an observation.

library(gvlma)

#The gvlma() function from gvlma offers a way to check the #important
#assumptions on a given linear model.

library(agricolae)

# Offers a broad functionality in the design of #experiments, especially for
#experiments in agriculture and improvements of #plants, which can also be used
#for other purposes.
```

```
#DATA FROM TRIAL A2


##EARS
DON_vs_species_EARS <- DON_vs_species_EARS[complete.cases(DON_vs_species_EARS),
]
test1 <- gather(DON_vs_species_EARS, key=species,value=DON,-dpi)
test1$dpi <- as.factor(test1$dpi)

anova(lm(DON~species,data=test1[test1$dpi %in% c(3),]))
gvlma(lm(DON~species,data=test1[test1$dpi %in% c(3),]))


anova(lm(DON~species,data=test1[test1$dpi %in% c(7),]))
gvlma(lm(DON~species,data=test1[test1$dpi %in% c(7),]))


anova(lm(DON~species,data=test1[test1$dpi %in% c(10),]))
gvlma(lm(DON~species,data=test1[test1$dpi %in% c(10),]))


anova(lm(DON~species,data=test1[test1$dpi %in% c(14),]))
gvlma(lm(DON~species,data=test1[test1$dpi %in% c(14),]))


anova(lm(DON~species,data=test1[test1$dpi %in% c(17),]))
gvlma(lm(DON~species,data=test1[test1$dpi %in% c(17),]))


##F-LEAF
DON_vs_species_F_LEAF <- DON_vs_species_F_LEAF[complete.cases(DON_vs_species_F_
LEAF),]
test2 <- gather(DON_vs_species_F_LEAF, key=species,value=DON,-dpi)
test2$dpi <- as.factor(test2$dpi)

anova(lm(DON~species,data=test2[test2$dpi %in% c(3),]))
gvlma(lm(DON~species,data=test2[test2$dpi %in% c(3),]))

anova(lm(DON~species,data=test2[test2$dpi %in% c(7),]))
gvlma(lm(DON~species,data=test1[test2$dpi %in% c(7),]))

anova(lm(DON~species,data=test2[test2$dpi %in% c(10),]))
gvlma(lm(DON~species,data=test2[test2$dpi %in% c(10),]))

anova(lm(DON~species,data=test2[test2$dpi %in% c(14),]))
gvlma(lm(DON~species,data=test2[test2$dpi %in% c(14),]))

anova(lm(DON~species,data=test2[test2$dpi %in% c(17),]))
gvlma(lm(DON~species,data=test2[test2$dpi %in% c(17),]))
```

```
##F-1 LEAF
DON_vs_species_F_1_LEAF <- DON_vs_species_F_1_LEAF[complete.cases(DON_vs_specie
s_F_1_LEAF),]
test3 <- gather(DON_vs_species_F_1_LEAF, key=species,value=DON,-dpi)
test3$dpi <- as.factor(test3$dpi)
anova(lm(DON~species,data=test3[test3$dpi %in% c(3),]))
gvlma(lm(DON~species,data=test3[test3$dpi %in% c(3),]))

anova(lm(DON~species,data=test3[test3$dpi %in% c(7),]))
gvlma(lm(DON~species,data=test3[test3$dpi %in% c(7),]))

anova(lm(DON~species,data=test3[test3$dpi %in% c(10),]))
gvlma(lm(DON~species,data=test3[test3$dpi %in% c(10),]))

anova(lm(DON~species,data=test3[test3$dpi %in% c(14),]))
gvlma(lm(DON~species,data=test3[test3$dpi %in% c(14),]))

anova(lm(DON~species,data=test3[test3$dpi %in% c(17),]))
gvlma(lm(DON~species,data=test3[test3$dpi %in% c(17),]))


#Plot DON (ppm) in function of relative amount of fungi (RAF) (%)
#in the EARS of the wheat plant for F. culmorum and F. graminearum infection

linearreg1 = lm(DONFC~DNAFC, data=DNA_vs_DON_FC)
summary(linearreg1)
gvlma(lm(DONFC~DNAFC,data=DNA_vs_DON_FC))
par(mfrow=c(2,2))
plot(lm(DONFC~DNAFC, data=DNA_vs_DON_FC))

linearreg2 = lm(DONFG~DNAFG, data=DNA_vs_DON_FG)
summary(linearreg2)
gvlma(lm(DONFG~DNAFG,data=DNA_vs_DON_FG))
par(mfrow=c(2,2))
plot(lm(DONFG~DNAFG, data=DNA_vs_DON_FG))
```

```
#TRIAL A3: Test natural tri-inhibitors, namely magnolol, carvacrol, ferulic
#acid and flavone, reported as effective in in vitro trials, to evaluate the
#feasibility of their use to control Fusarium head blight (FHB).
#
#
#
#
#Perform analysis of variance to DON production and RAF values in the
#HULLS(KERNELS/F-LEAF/F-1 LEAF) of the wheat plant for each treatment.

#Null hypothesis: Different treatments did not have significant effects on DON
#concentration and RAF values in the different anatomic sections of the wheat
#plant (HULLS, KERNELS, F-LEAF, F-1 LEAF).
#
#Alternative hypothesis: Different treatments have a statistically significant
#effect on DON concentration and RAF values in the
#HULLS (KERNELS/F-#LEAF/F-1 LE#AF).

##HULLS
anova(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_HULLS))
gvlma(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_HULLS))
par(mfrow=c(2,2))
plot(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_HULLS))

#Non parametric Kruskal test is performed
kruskal(DON_vs_SPECIES_vs_treatment_HULLS$DON,DON_vs_SPECIES_vs_treatment_HULLS
$treatment,alpha = 0.05,p.adj="none", group=FALSE, main = NULL,console=TRUE)

anova(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_HULLS))
gvlma(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_HULLS))
par(mfrow=c(2,2))
plot(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_HULLS))

#Alternative hypothesis accepted (p value lower than 0.05)
#Run post hoc test

test8<-aov(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_HULLS)
df<-df.residual(test8)
MSerror<-deviance(test8)/df
HSD.test(test8,'treatment',df,MSerror, group=TRUE,console=TRUE, main="RAF value
s in the hulls with different treatments")
TukeyHSD(test8,'treatment', conf.level = 0.95)
```

```
##KERNELS
anova(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_KERNELS))
gvlma(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_KERNELS))
par(mfrow=c(2,2))
plot(lm(DON~treatment,data=DON_vs_SPECIES_vs_treatment_KERNELS))

#Alternative hypothesis accepted (p value lower than 0.05)
#Run post hoc test

test9<-aov(DON~treatment,data=DON_vs_SPECIES_vs_treatment_KERNELS)
df<-df.residual(test9)
MSerror<-deviance(test9)/df
HSD.test(test9,'treatment',df,MSerror, group=TRUE,console=TRUE, main="DON conce
ntration in the kernels with different treatments")
TukeyHSD(test9,'treatment', conf.level = 0.95)

anova(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_KERNELS))
gvlma(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_KERNELS))
par(mfrow=c(2,2))
plot(lm(DNA~treatment,data=DNA_vs_SPECIES_vs_treatment_KERNELS))

# Non parametric Kruskal test is performed
kruskal(DNA_vs_SPECIES_vs_treatment_KERNELS$DNA,DNA_vs_SPECIES_vs_treatment_KER
NELS$treatment,alpha = 0.05,p.adj="none", group=FALSE, main = NULL,console=TRUE
)


##F-LEAF (ANOVA for RAF values not tested as RAF=0%)
anova(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_LEAF))
gvlma(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_LEAF))
par(mfrow=c(2,2))
plot(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_LEAF))

# Non parametric Kruskal test is performed
kruskal(DON_vs_species_vs_treatment_F_LEAF$DON,DON_vs_species_vs_treatment_F_LE
AF$treatment,alpha = 0.05,p.adj="none", group=FALSE, main = NULL,console=TRUE)


##F-1 LEAF (ANOVA for RAF values not tested as RAF=0%)
anova(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_1_LEAF))
gvlma(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_1_LEAF))
par(mfrow=c(2,2))
plot(lm(DON~treatment,data=DON_vs_species_vs_treatment_F_1_LEAF))

# Non parametric Kruskal test is performed
kruskal(DON_vs_species_vs_treatment_F_1_LEAF$DON,DON_vs_species_vs_treatment_F_
1_LEAF$treatment,alpha = 0.05,p.adj="BH", group=FALSE, main = NULL,console=TRUE
)
```

```
####Bibliography

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#of-variance-anova/.
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#tistical-guides/one-way-anova-statistical-guide-3.php.
# Prabhakaran, Selva. 2017. "Assumptions of Linear Regression." http://r-statis
#tics.co/Assumptions-of-Linear-Regression.html.
# Venables, W. N., D. M. Smith, and R Core Team. 2017. An Introduction to R.
# Yau, Chi. 2017. "R Tutorial." http://www.r-tutor.com/.


#Save the data
save.image(file="ANOVA_MT_AR.RData")
```