UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Department of Agro-forestry Ecosystems

Master's Degree in Plant Health in Sustainable Cropping Systems

"Zearalenone production by Fusarium graminearum and its effect on fungal communities in maize fields".

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TITULO: Producción de zearalenona por *Fusarium graminearum* y su efecto en comunidades de hongos presentes en campos de maíz

RESUMEN: (50-500 palabras)

La zearalenona es una de las micotoxinas más comunes que se encuentran en en el maíz, el trigo y la cebada, entre otros cultivos importantes. Siendo uno de sus principales productores *F. graminearum.* El objetivo de este trabajo es determinar si la zearalenona producida por *F. graminearum* cambia la composición de las comunidades de hongos presentes en los residuos de maíz. Esto fue evaluado usando una cepa silvestre de *F. graminearum* y un mutante de *F. graminearum* cuya capacidad para producir zearalenona ha sido inhabilitada. Los hongos fueron inoculados en frascos con muestras de maíz molidas, obtenidas de un campo de Alemania y harina compuesta de trigo, maíz y cebada como sustrato. Después de 21 días a 18 °C, se agregaron 40 mL de agua a cada frasco. Esta suspensión fue inoculada en 300 cajas Petri con medio malta maiz (BMM) y la cantidad de zearalenona presente en los frascos fue medida mediante cromatografía líquida de alta eficacia. Tras 24 horas, las unidades formadoras de colonia presentes en cada caja Petri fueron contadas y se realizó un análisis estadístico para detectar si hubo una diferencia significativa entre los distintos tratamientos. Los resultados no mostraron diferencias significativas entre los tratamientos y solo dos especies de hongos fueron aisladas, *Trichoderma viride* y *Mucor* sp. Pruebas de antagonismo demostraron que los hongos eran capaces de competir entre ellos. Debido a la existencia de *F. graminearum* en las muestras de maíz, es posible que esta población con un número reducido de especies fuera la consecuencia de una presión de selección que ocurrió antes de llegar al laboratorio.

Palabras clave: ***"Fusarium graminearum", "zearalenona", "micotoxinas"***

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Title: *The effect of zearalenone production by Fusarium graminearum and its effect on fungal communities in maize fields*

Summary: (50-500 words)

Zearalenone is one of the most common mycotoxins found in corn, wheat, and barley among other relevant crops. *F. graminearum* being one if the main producers. The objective of this work is to determine if the zearalenone produced by *F. graminearum* changes the composition of the fungal communities present in corn residues. This was evaluated using a wild-type strain of *F. graminearum* and a mutant whose ability to produce zearalenone has been impaired. The fungi were inoculated in flasks with flour composed of wheat, corn and barley as substrate and the corn residues, which were obtained from a field in Germany. After incubation for 21 days at 18 °C, 40 mL of water was added to each flask. This suspension was inoculated in 300 Petri dishes with (Biomalt maize extract medium) BMM medium and the amount of zearalenone present in the flasks was measured by high performance liquid chromatography. After 24 hours, the colony-forming units present in each Petri dish were counted and a statistical analysis was performed to detect if there was a significant difference between the different treatments. Results showed no statistical differences among treatments and only two fungi species were isolated, *Trichoderma viride and Mucor sp.* Antagonism tests showed that the fungi are all able to compete with one another. Because of the pre-existence of *F. graminearum* in the corn residues this was likely the consequence of a previous selection pressure on the local fungal population.

Key words: ***"Fusarium graminearum", "zearalenone", "mycotoxins"***

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1. Introduction

1.1 The phytopathogenic fungus *Fusarium graminearum*

The fungus *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* Petch) is the most common causal agent of Fusarium head blight disease and has been registered in all continents except Antarctica (Backhouse, 2014). A visual representation (Fig. 1), created by Backhouse (2014), using BIOCLIM species distribution modelling package and 346 georeferenced reports from around the world, shows the reported areas where the *F. graminearum* develops in wheat as black dots as well as areas which are suitable for its development as shaded area. Illustrating the widespread distribution of the disease.

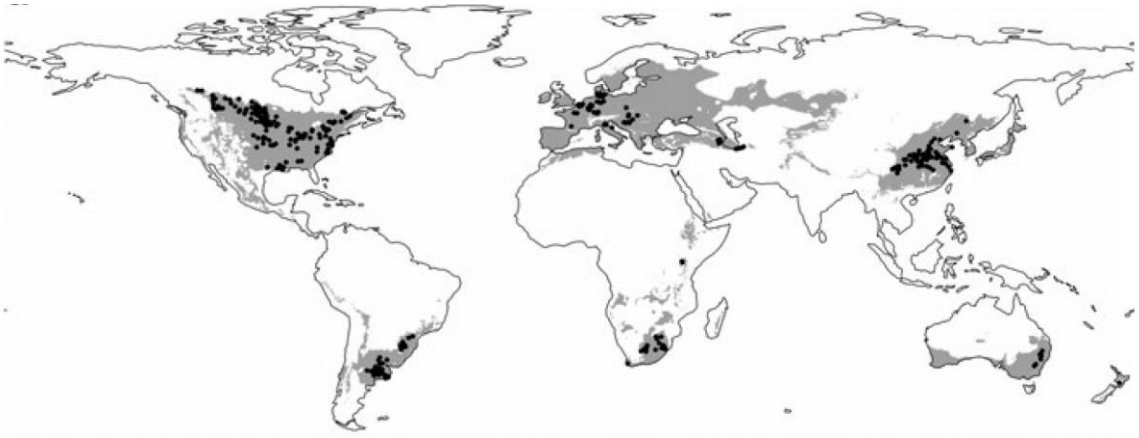

Figure 1. Recorded distribution and predicted distribution based on climate conditions during the month of wheat anthesis of Fusarium graminearum (Backhouse, 2014)

Fusarium spp*.* belongs to the kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Nectriaceae (Agrios, 2005). The genus *Fusarium* is divided into 7 clades, Watanabe *et al*., 2011*,* grouped the clades V, VI and VII to form the *Gibberella* clade due to their common teleomorph. The clade VII is subdivided as well into 4 sections: *Eupionnotes, Gibbosum, Discolor* and *Sporotrichiella*. *Fusarium graminearum* is member of the discolor section (Fig. 2).

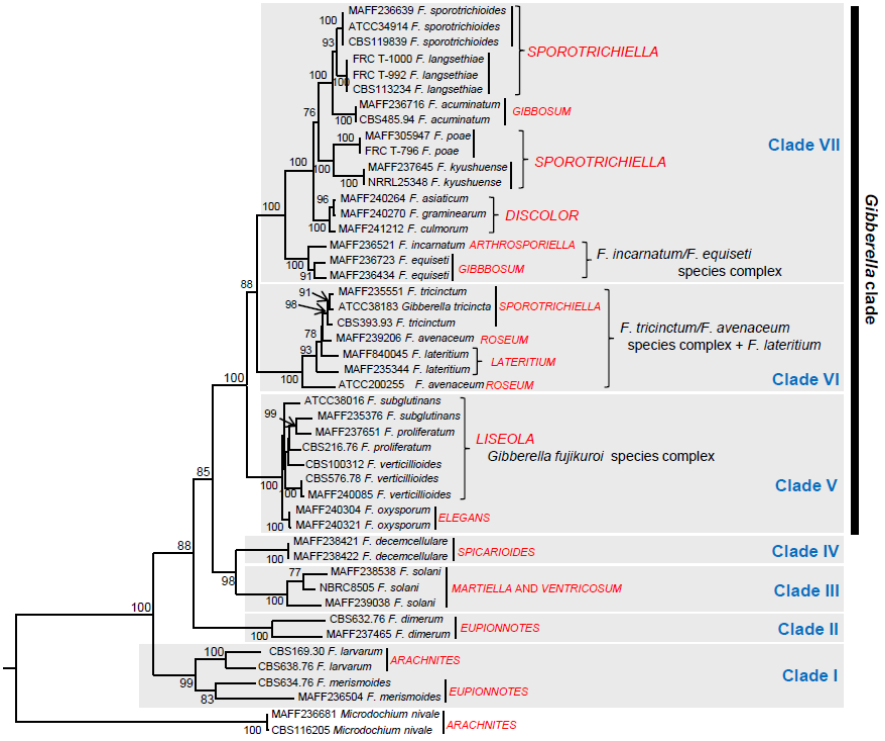

Figure 2. Maximum likelihood tree of the Fusarium genus and related genera inferred from the combined sequences of the rDNA cluster and the EF-α, and β-tub genes (Watanabe et al., 2011)

Fusarium spp. attack annual and perennial plants. The most economically relevant agricultural crops susceptible to being attack by *F. graminearum* are wheat, maize, and barley (Leslie & Summerell, 2006). Asymptomatic host plants have been reported (Goswami & Kistler, 2004) for *Gibberella zeae,* among them tendril-bearing plants and several monocotyledon weeds (Goswami & Kistler, 2004). *F. graminearum* can also survive on host debris, having a longer survivability in breakdown resistant tissues (Miller & Trenholm, 1994). These residues can be the inoculum sources for reinfection of crops via ascospores, macroconidia, chlamydospores and hyphal fragments.

The sexual stage structures or fruiting bodies are called perithecia. They are bottle shaped ascocarps, (Agrios, 2005), developing at a temperature range between 16°C to 28.5°C, optimally at 28.5°C (Wolf *et al.* 1972). Once perithecia are matured, and atmospheric moisture is below dehydration, they discharge ascospores (Tschanz *et al.* 1976). Ascospores are 3-septated, hyaline spores, which form perithecia on plant tissue and in host debris. They are produced at a temperature range from 11°C to 23°C; the optimum is at 16.6°C (Tschanz *et al.* 1976). They are released mainly at night (Ayers, 1975, as cited in Sutton, 1982), and commonly distributed by wind, (Atanastoff *et al.,* as cited in Sutton, 1982).

Macroconidia are usually 3-7 septate spores, which transform into chlamydospores when added to soil or when inoculated into maize ears. The temperature for macroconidia production ranges from 16 to 36°C, with an optimal 32°C (Sutton, 1982). In maize fields, *F. graminearum* requires moderate rain at silk emergence with periods of warm temperatures (Miller & Trenholm, 1994). In spring the warm and moist weather conditions cause the maturation of the conidia and perithecia which in turn produce ascospores that are dispersed by the wind, rain, or insects to other host plants. Infection occurs when spores land on a wheat, maize, or barley heads. When colonizing the corn silk, the initial symptoms appear at the apex of the ear, then the white mycelium turns pink and red, and spreads from the kernels to the entire ear (Goswami & Kistler, 2004). However, the symptoms are only visible once the crop approaches maturity, when the husks loosen and expose the kernels (Sutton, 1982).

Fusarium species that attack maize can also be divided by their infection symptoms into red-ear rot or red fusariosis and pink ear rot or pink fusariosis. The most common red-ear rot causing agents are *Fusarium graminearum, F. culmorum, F. cerealis* and *F. avenaceum*. While the most common pink-ear rot species are the anamorphs of *F. verticillioides, F. proliferatum* and *F. subglutinans* (Xu *et al*., 2003). Maize red ear rot is caused by members of the *Discolor* section, while maize pink ear rot is caused by members of the *Liseola* section. Pink fusariosis are common in drier and warmer climates while red fusariosis predominantly occur in areas with high precipitation and low temperatures during summer (Logrieco *et al.,* 2002).

1.2 Fusarium mycotoxins

In addition to the damage that *F. graminearum* causes to the crop, this fungus has the ability to produce toxic secondary metabolites called mycotoxins which represent a risk to both animal and human health. Mycotoxins are a group of metabolites that are naturally produced by fungi. They are toxic to higher vertebrates in low concentrations which may also have antimicrobial and phytotoxic properties (Bennett, 1987). Some authors estimate that up to a quarter of the world's food crops are affected yearly by mycotoxins (Logrieco *et al.* 2002). These types of infections cause losses in the US due to the rejection of toxin infected grains of up to 45.8 million dollars every year, and up to 320,000 dollars due to animal life losses (Wu, 2007).

Eight different mycotoxins have been reported to be produced by *Fusarium graminearum*. They can be divided into two groups: Trichothecenes which are subdivided into type A and type B, and Zearalenones. Among the most relevant are zearalenone, deoxynivalenol, diacetoxyscirpenol and nivalenol (Nesic *et al*., 2014). Deoxynivalenol (DON) and Nivalenol (NIV) are both members of the type B trichothecene group. Whose mechanism of action affects the DNA template in animals (Kiessling, 1986). DON also known as vomitoxin due to its effect on pigs. It is often found at levels which can cause adverse effects in human and animal health. Studies have shown that chronical exposure to high doses of the toxin affect the DNA, causing apoptosis in cells, inducing shock-like death in mice. When chronically exposed to low doses, monogastric animals suffer weight loss (Pestka, 2010). NIV is another type B mycotoxin, it induces apoptosis on the cellular level as well immunotoxicity and haematotoxicity in mice (EFSA, 2013). It has been proven to have a higher toxicity than deoxynivalenol (Minervini *et al*., 2003). However, it poses a lower concern for public health due to its low occurrence in food (EFSA, 2013). Zearalenones specifically, are non-aketide, non-steroidal mycotoxins, (Desjardins & Proctor, 2007), which are a type of polyketide, produced by enzymes present in fungi, called polyketide synthases (PKSs) (Gaffoor *et al*., 2005). These mycotoxins have also been demonstrated to have antifungal properties that help *Fusarium graminearum* in its competitive inter and intra-competitive interactions (Utermark & Petr, 2007), and associated with estrogenic syndromes (Nesic *et al*., 2014).

Zearalenone and deoxynivalenol in products are regulated under the European Commission recommendation (2006/576/EC), limiting the amount in mycotoxin present depending on the source product from 0.5 to 3 ppm in case of zearalenone and from 0.9 to 12 ppm in case of Deoxynivalenol (European Comission, 2006).

In maize, infestation with Fusarium is favored when the plant has suffered insect damage, particularly on maize ears that had been attacked by the European corn borer (*Ostrinia nubilalis*). However, control of insects has little effect on the concentrations of zearalenone (ZEA), deoxynivalenol (DON) or Nivalenol (NIV) (Xu *et al.,* 2003), reducing only the incidence of *Fusarium* species from the *Liseola* section, (Logrieco *et al.,* 2002). The best way to control the disease incidence and severity is through crop rotation and controlling insect pests that may facilitate the infection whereas for its mycotoxins, host-plant resistance mechanisms are the most effective methods. Currently there are completely resistant varieties such as Mp317, as well as partially resistant lines like OH43 and HP301.

A positive correlation has been reported between visual symptoms and mycotoxin accumulation, resistant plants showing lower concentrations of zearalenone in their grains (Quesada-Ocampo *et al.*, 2016). Schollenberger *et al.* (2005), reported the occurrence of low-level concentrations of α-ZEA and β-ZEA in vegetables, grains, oilseeds, nuts, and wheat-based and corn-based products. Showing the widespread distribution of this toxin in our food production systems.

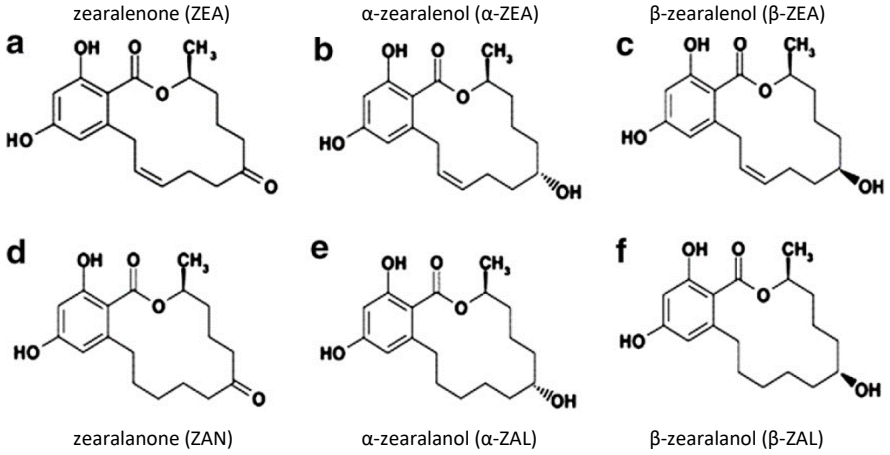

Figure 3. Chemical structures of ZEA and its derivatives *(Zinedine et al., 2006)*

The fungitoxic effect of zearalenone derivatives can be ranked in increasing order as β-zearalenone, α-zearalenone and zearalenone (Utermark & Petr, 2007). These derivatives differ only in the position of the hydroxyl group (Matthies *et al.,* 2001). Different authors have proven that zearalenone and deoxynivalenol can be reduced to alpha and beta zearalenone by microorganisms (Matsuura & Yoshizawa, 1985; Scott *et al*., 1992; Ji *et al*., 2016). However, this process does not change their oestrogenic properties and therefore it is not considered detoxification. In humans, zearalenone is rapidly absorbed by oral exposure. Studies have found it accumulated in reproductive and adipose tissue, and the testes (Ueno *et al.,* 1977). Zearalenone presents a potential danger for animal and human health when it is absorbed in high amounts or when chronically exposed to it (Zinedine *et al.,* 2006). As such, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), (2000) established a provisional maximum tolerable daily intake for zearalenone of 0.5 micrograms per kilogram of body weight.

1.3 Fungal soil interactions

The ecological metabolite hypothesis states that secondary metabolites modulate interactions between organisms, being most fungal secondary metabolites also capable of this effect (Chatterjee *et al.*, 2016). Several organisms commonly found with *F. graminearum* have the capacity to suppress, detoxify or reduce its mycotoxins, zearalenone has also been found to have antifungal and antibiotic properties (Hestbjerg *et al.,* 2002). These adaptations hint to its importance in the fungal community ecology.

Table 1. Processes described for binding, detoxification, or degradation of ZEA (Zinedine et al., 2007)

Process	Observation	References
Biological process		
Mannan-oligosaccharides derived from the cell wall of *S. cerevisiae* 1026	Binding capacity of about 80% of ZEA	Devegowda et al. (1996)
Mixed bacterial culture	Total degradation of ZEA. No ZEA or ZEA-like products were detected	Megharaj et al. (1997)
Ruminants protozoa	90–100% of ZEA metab to α-ZEA and to a lesser degree to β-ZEA	Kiessling et al. (1984)
Lactonohydrolase from *Clonostachys rosea* IFO 7063	Conversion of ZEA to less toxic compounds	Takahashi-Ando et al. (2002)
L. rhamnosus GG and *L. rhamnosus* LC705	Binding of ZEA	El-Nezami et al. (2002)
Lactic acid bacteria	Reduction of 68–75% in 4 days of fermentation in maize	Mokoena et al. (2005)
Trichosporon mycotoxinivorans	Degradation of ZEA to non-toxic metabolites	Molnar et al. (2004)
Gliocladium roseum	Metabolization of 80–90% ZEA to two isomeric compounds that are less toxic than ZEA	El-Sharkawy and Abul-Hajj (1988)
Physical process		
Montmorillonite	Adsorption of 108 mg/g	Lemke et al. (1998)
Montmorillonite	Adsorption of 0.19 mg/g	Ramos et al. (1996)
Bentonite	Adsorption of 0.11 mg/g	Ramos et al. (1996)
Sepiolite	Adsorption of 0.07 mg/g	Ramos et al. (1996)
Mg trisilicate	Adsorption of 0.02 mg/g	Ramos et al. (1996)
Cholestyramine	Adsorption of >0.3 mg/g	Ramos et al. (1996)
Crospovidone	Adsorption of 0.3 mg/g	Ramos et al. (1996)
Cross-linked polyvinylpyrrolidone	Adsorption of 0.5–2.1 mg/g	Alegakis et al. (1999)
Activated carbon	Binding 100% ZEA (pH 3 and 7.3)	Bueno et al. (2005)
Extrusion Cooking	Reduction of 83% of ZEA	Ryu et al. (1999)
Chemical process		
Ozone (O_3)	Total degradation of ZEA	McKenzie et al. (1997)
H_2O_2 (10%) at 80 °C for 16 h	83.9% of degradation of ZEA	Abd Alla (1997)

1.4 Objective.

The current project will study the effect of zearalenone in populations on plant residues from a field. Comparing the colony forming units (CFU) which grow when exposed to a *F. graminearum* wild population with zearalenone production and a genetically modified *F. graminearum* whose capacity for zearalenone production has been impaired.

2. Research Methodology

2.1 Sampling and cultivation

To conduct our research, we used maize residues which were collected from several maize fields in Schleswig-Holstein, close to Kiel, Germany. Our research was divided into two experiments; the first experiment was done to determine the concentration of spores to be used considering the zearalenone production and the second final experiment was done using the concentration obtained in the first experiment to evaluate the effect of zearalenone in the fungal populations.

Forty grams of maize residues were grinded to an average diameter of 4 mm and placed with 30 mL of water in a plastic bag to create a humid chamber. The straw was left to incubate for 6 days at 21°C to incentivize development of the fungi present in them. Two strains of the fungus *F. graminearum* were used, a wild type, and a mutant with a disruption in the PKS4 gene by an *Agrobacterium tumefaciens*-mediated transformation (Lysøe *et al.*, 2006).

Substituting the PSK4 central part with a hybB resistance gene, rendering the fungi unable to produce zearalenone, as proven by (Lysøe *et al.*, 2006).

2.2 Inoculum obtention. Specific nutrient-deprived agar (Table 2) was used to obtain spores. The media was inoculated with three pieces of agar plugs (0.5mm in diameter) of PDA overgrown with *F. graminearum* and placed in an incubator shaker INOVA® 44 at 130 RPM and 25°C for 7 days. Following incubation, the media was filtered using autoclaved cotton strips and the resulting liquid was centrifugated in a ROTINA 420® centrifuge, at 4754 RCF for 15 minutes. The sedimented spores were re-suspended in 1 mL of sterile tap water and their concentration per milliliter was quantified using a Thoma-hemocytometer. The samples that were not to be used in less than 5 days were placed in storage at -80°C. For this, a 1:1 ratio mixture with 30% glycerol was done and the resulting mixture was placed 30 minutes at room temperature prior to its placement in the -80°C fridge.

Table 2. Specific Nutrient-deprived Agar medium (SNA)

Compound	Amount
3Potassium nitrate (KNO_3)	1.0 grams
Monopotassium phosphate (KH_2PO_4)	1.0 grams
Magnesium sulfate heptahydrate ($MgSO_4 \times 7\ H_2O$)	0.5 grams
Potassium Chloride (KCl)	0.5 grams
Glucose	0.2 grams
Sucrose	0.2 grams
Tap water	1 liter

2.3 First experiment.

2.3.1 Determination of spore concentration. To determine which concentration of *F. graminearum* spores was adequate for the inoculation of corn residues and to avoid effects due to excess or lack of inoculum, a pre-experiment was carried out. The substrate consisted of 6 grams of flour composed of corn, barley and wheat in a 1: 1: 1 ratio, 6 mL of sterilized water suspension and 5 grams of the residues (4 mm), which were placed in Erlenmeyer flasks of 100 mL. Five concentrations of *F. graminearum* were used: 250, 1250, 6250, 31250 and 156 250 spores per flask. As a control, jars containing only flour in one case and flour with corn residues in the other. Five repetitions per treatment were used. The samples were incubated for 14 days at 18 ° C in the dark.

2.3.2 Zearalenone quantification. Each flask had 80 mL of 84% acetonitrile (ACN) + 1% Acetic acid (CH_3COOH) added. They were then placed in the shaker at 130 RPM and 25°C for 13 hours to extract the zearalenone content. The next day, 1 mL from the treated samples was placed into Eppendorf tubes for evaporation of liquids on a RVC 2-25 CDplus Compact benchtop midi concentrator at 35°C for 24 hours. After the evaporation, dried extracts were stored at -20°C.

Prior to High-performance liquid chromatography (HPLC) analysis, the samples were defatted by adding 800 microliters of a mixture of 70 % ultra-pure water mixture, obtained from an Arium® pro UV Ultrapure Water System and 30 % pure ethanol. To this mixture, 800 µL of 99.5 % cyclohexane were added. Dried extracts pellets were redissolved by subjecting the samples to a 3 second treatment in a BANDELIN® ultrasonic bath.

Subsequently, samples were shaken for five minutes in a VORTEX-GENIE 2® shaker and centrifuged at 19.204 RCF for 10 minutes in an EPPENDORF 5424 centrifuge. The supernatant was removed and 500 µL of the lower phase were placed into a new 2 mL Eppendorf tube. 500 µL of ultra-pure water was added to this new Eppendorf tube. The samples were centrifugated again at 20.238 RCF for 10 minutes. To remove any remnants of cyclo-hexane, the samples were placed on the RVC 2-25 CDplus Compact benchtop midi concentrator at 38°C for 15 minutes. With the cyclo-hexane removed, the samples were centrifuged at 19.204 RCF for 10 minutes. From the upper phase, 500 µl were taken and placed to HPLC vials for chromatographic analysis. To avoid contamination, the samples were filtered with HPCL filters Th. geyer® using a 2 mL syringe which was cleaned between samples with an 99.5 % Acetonitrile for LC-MS solvent solution. An Agilent 1290 Infinity II LC HPLC System was used for the chromatographical analysis. With this experiment the amount of zearalenone present in each the samples was determined.

2.4 Second experiment

2.4.1 CFU (colony forming units) quantification. *F. graminearum* wildtype, *F. graminearumΔZEA* and a control were used. The control consisted of 6 g of flour mixture and 6 g of maize chaff humidified with 6 mL of sterile tap water. The first treatment consisted of 6 g of flour mixture, 6 g of maize chaff and 30.000 spores of H16 *F. graminearum* wildtype inoculated in 6 mL of sterile tap water. The second treatment was created with 6 g of flour mixture, 6 g of maize chaff and H18 *F. graminearumΔZEA* spores inoculated in the same way as the first treatment. In total eighteen 100 mL Erlenmeyer flasks (six per treatment) were prepared. The flasks were sealed with a cotton plug to enable exchange of gases. Following the inoculation, all flasks were randomly placed in a closed carton box to avoid effects based on temperature and light gradients. The box was stored at 18°C for three weeks, to favor fungal growth and zearalenone production (Fig. 4, 5).

Figure 5 Flasks first (A) and last day of incubation (B).

Figure 4 Example of samples of each treatment (left) wildtype, (center) ZEA, (right) Control

2.4.2 Plating. After three weeks of incubation, 40 mL of sterile tap water were added to each of the eighteen flasks. They were then placed in the shaker at 25°C and 130 RPM for 2 hours to release the spores.

2.4.2.1 Estimation of number of dilutions to be done. A first test with four serial dilutions was conducted to determine the number of dilutions that would allow a counting of the CFU. From each flask one milliliter of the liquid was taken, from this, a 100 µL aliquot, was taken and placed into 900 µL of sterile tap water. This process was repeated four times to obtain concentrations of $1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-4}$ less than the spores per milliliter found in the initial dilution. The second, third and fourth dilutions were inoculated into petri dishes with Biomalt maize extract medium (Table 3), using a dose of 100 µL per dish.

Table 3. Biomalt maize extract medium (BMM) maize agar medium

Compound	Amount
Tap water	1 liter
Polenta ® corn flour	25 grams
Malt-Extract	8 grams
Potassium hydroxide (KOH) 10 M	Until pH is between 6.4-6.6
Agar-Agar Kobe I ROTH®	20 grams
Streptomycin	0.05 mg/mL
Leave overnight the tap water with polenta at 60°C and filter the maize through a tea-towel	

Two repetitions per concentration were done, summing a total of six petri dishes per flask. After leaving the plates to incubate for 72 hours at 18°C, the colony forming units were counted to determine which dilution would be used for the final experiment.

2.4.3 CFU counting. Once the ideal concentration was found, 16 petri dishes were used per treatment, summing a total of 300 plates. The plates had 100 µL of the selected dilution and were left for 24 hours at 18 °C and stored for 12 hours at 4°C until visual examination. They were observed under a ZEISS® Stemi 305 stereoscopic microscope at an 8x magnification and the number of colonies per petri-dish were quantified. For the statistical analysis, RStudio Desktop 1.3.1093 was used, a linear model was created with the lm function. The data normality was tested with a Shapiro-Wilk normality test. An analysis of variance test and a least square means post-hoc test using the emmeans R package were conducted to see if there were any statistically significant differences between treatments.

2.4.4 Zearalenone quantification. Using HPLC, the zearalenone present in the remaining straw was estimated and a statistical analysis was done.

2.4.5 Species identification. The resulting colonies were identified by morphological and molecular techniques, using PCR (Polymerase chain reaction) and sequencing for the latter. For the morphology, the fungi were transferred to Potato Dextrose Agar (PDA) plates (Table 4) and incubated for 72 hours. The mycelia was then observed under a microscope. For the molecular identification, the Primers ITS1 (TCCGTAGGTGAACCTGGGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used, (White *et al.*, 1990), amplyfing the fragments with the table 5 reagents, running a gel for verification of successful amplification and using isopropanol for purification of the DNA. The sequencing was done by sending the amplification of the ITS1 region to MACROGEN®.

Table 4. PDA medium

Compound	Amount
Organically grown potatoes with peel	200 grams
Glucose	20 grams
Tap water	1 liter

Table 5. PCR master mix

Mastermix	µL
Water	74.5
10x Buffer	10
$MgCl_2$	1
dNTP	4
Forward (EF1)	3
Reverse (EF2)	2
Taq	0.5
Sample (1:100)	1
Total volume	96
Volume per reaction	24

2.4.6 Antagonism tests. The identified fungi were then placed at 18°C in PDA plates to study possible antagonist interactions between them. One set of plates was inoculated with all the fungi at the same time, while the other was left for 24 hours with the isolated fungi, prior to inoculation of the *F. graminearum*. For the dual cultures, the plugs of 0.4 mm were placed at an approximated distance of 5 cm between them, whereas on the 3 interactions ones, the plugs were placed at an approximated distance of 4 cm between them. They were left for 72 hours and visually assesed for possible antagosnistic interactions.

3. Results and findings

3.1 First experiment.

3.1.1 Determination of spore concentration. The HPLC Zearalenone quantification from the first experiment, showed that the concentration of spores used for inoculation in the pre-experiment did not significantly affected its final zearalenone concentration. Zearalenone was present in all analyzed samples in concentrations ranging from 0.010 to 0.185 mg/kg. (Table 6). The test also showed that the maize straw was contaminated with *F. graminearum* since the control also had small amounts of zearalenone. Given the information condensed in table 6, we decided to use 20.000 spores per flask, which was close to the intermediate level concentration used in the previous experiment.

Table 6. First experiment HPLC zearalenone concentration results

Sample	c [mg/kg]	Sample	c [mg/kg]
Flour 1/5	<LOD	**1250 3/5**	0.021
Flour 2/5	<LOD	**1250 4/5**	0.028
Flour 3/5	<LOD	**1250 5/5**	0.101
Flour 4/5	<LOD	**6250 1/5**	0.010
Flour 5/5	<LOD	**6250 2/5**	0.054
Flour+straw 1/5	0.016	**6250 3/5**	0.047
Flour+straw 2/5	0.038	**6250 4/5**	0.037
Flour+straw 3/5	0.029	**6250 5/5**	0.185
Flour+straw 4/5	0.023	**31250 1/5**	0.030
Flour+straw 5/5	0.031	**31250 2/5**	0.069
250 1/5	0.026	**31250 3/5**	0.066
250 2/5	0.032	**31250 4/5**	0.056
250 3/5	0.061	**31250 5/5**	0.022
250 4/5	0.051	**156250 1/5**	0.018
250 5/5	0.049	**156250 2/5**	0.020
1250 1/5	0.044	**156250 3/5**	0.026
1250 2/5	0.043	**156250 4/5**	0.022
		156250 5/5	0.048

3.2 Second experiment

3.2.1 Plating and CFU quantification. After three weeks most flasks showed a big growth of white fluffy mycelium and green colored structures (Fig. 6).

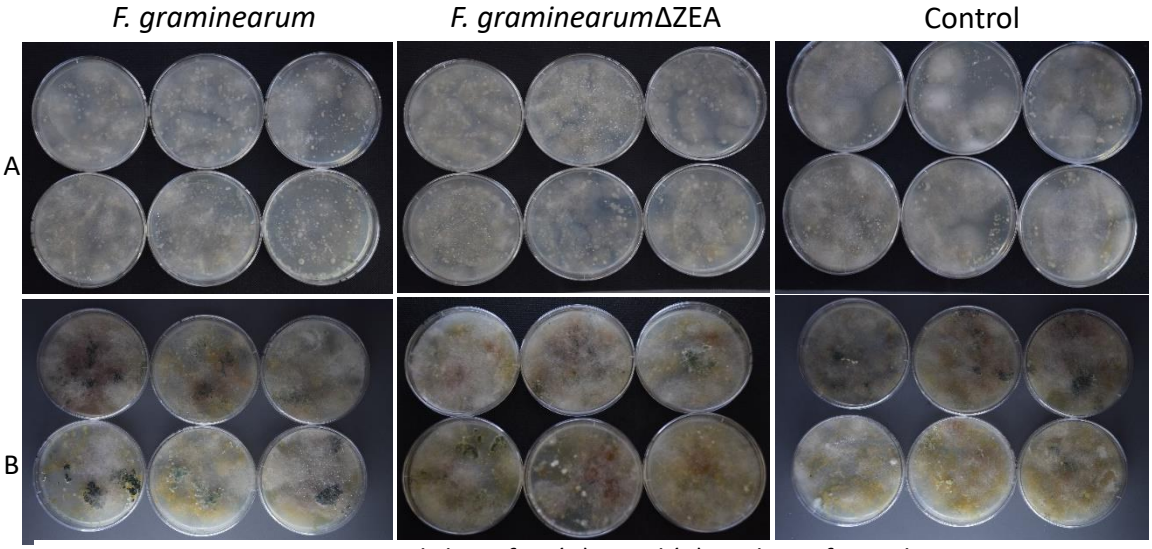

Figure 6. BMM dishes after (A) 3 and (B) 14 days of inoculation

This hinted to the presence of *F. graminearum* and *Trichoderma* sp. respectively. Even though this structure could also be produced by other fungi, *Trichoderma* sp. was later confirmed in the test described in section 3.2.2.

Estimation of number of dilutions to be done. In the initial plating experiment, we determined that the best concentration for the spores was the fourth dilution equivalent to 1 x10^{-4} of the original concentration, since it was the only one in which it was possible to count the colony forming units.

3.2.2 CFU counting. The plates inoculated with the determined dilution showed no morphological diversity based on the visual examination of the colony forming units. We observed two different morphotypes in total. One was predominant on almost all the plates from all the treatments. While the other was only rarely found either individually or in small clusters of colonies in some plates. For the identification of the predominant fungi, mycelia were transferred into PDA plates and incubated for 72 hours. By colony form, mycelial structure, and spores we determined that it was *Mucor* sp. (Fig. 8). For the second one a PCR amplification was done and sent to a laboratory for sequencing.

The resulting sequence was compared with the NCBI data base using their Basic Local Alignment Search Tool (BLAST) and was concluded that it was *Trichoderma viride* (Fig. 7).

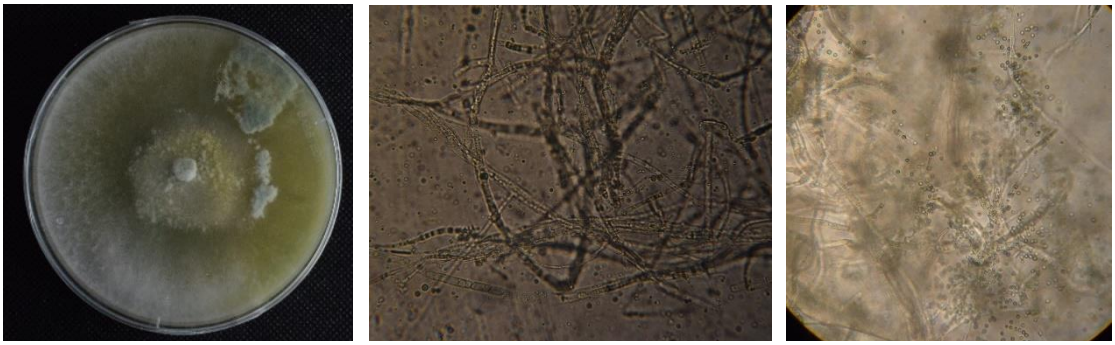

Figure 7. Trichoderma viride

Figure 8. Mucor sp.

From each flask, the average value of CFU per petri dishes was calculated and tested for normality using the Shapiro-Wilk normality test. It was concluded that the data had a parametric distribution (p - value = 0.9613). Based on this, an analysis of variance was performed, which showed that there were no statistically significant differences neither by temperature gradient nor by fungi (p - value = 0.224 and 0.455 respectively). To better visualize the results, a least square means post-hoc test grouped by treatment was conducted (Table 7, Figure 9). The results (Figure 10) showed that the flasks inoculated with the *F. graminearum* wildtype had less variation, while the control and the treatment with *F. graminearum*ΔZEA had outliers that greatly exceeded the values of *F. graminearum* treatment. However, none of this was not statistically significant. However, when observed by flask a great variability within the treatments is visible (Fig. 11).

Table 7. CFU LSD Least Square Means post-hoc test by fungi

Fungi	emmean	SE	df	lower.CL	upper.CL	group
F_gram_WT	21	5.34	13	9.45	32.5	1
Control	29.4	5.34	13	17.84	40.9	1
F_gram_ZEA	29.8	5.34	13	18.22	41.3	1

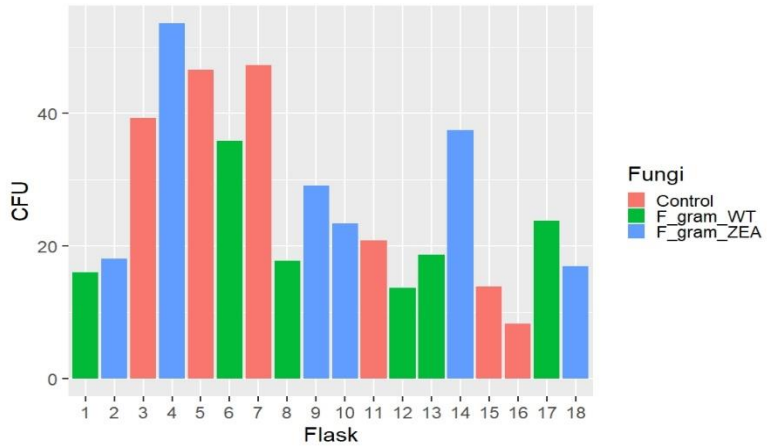

Figure 9. CFU grouped by flask

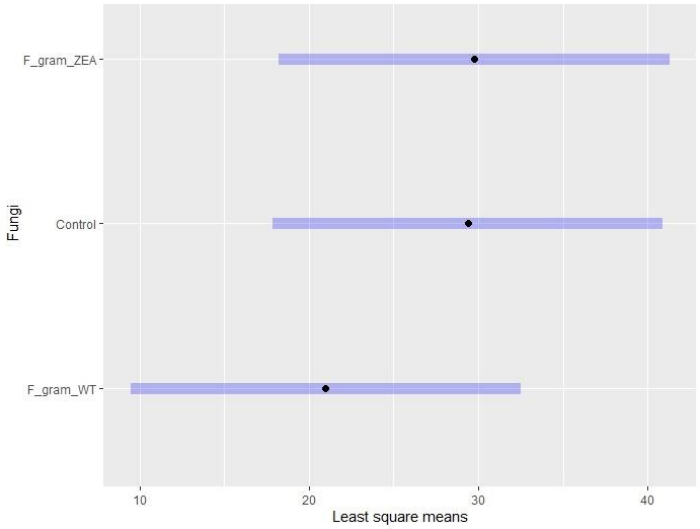

Figure 10. CFU Least square means POST-HOC test grouped by fungi

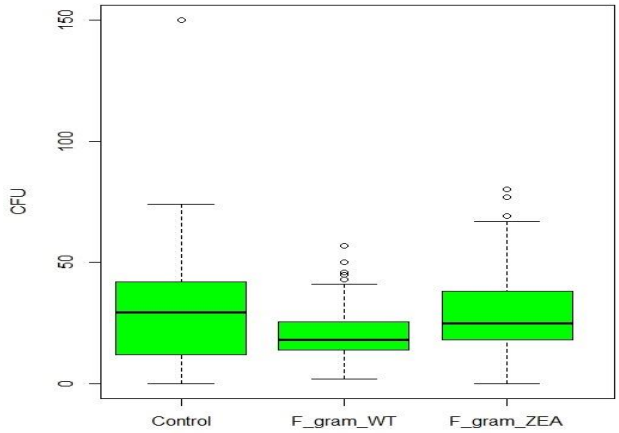

Figure 11. Average number of Colony forming units (CFU) grouped by treatment.

F_gram_WT= *F. graminearum wildtype, F_gram_ZEA=F. graminearumΔZEA*

3.3 Antagonism tests. In the dual culture dishes, in the *F. graminearum*ΔZEA and *F. graminearum, Trichoderma viride* outcompeted them having mycelial growth over both. In the *F. graminearum*ΔZEA with mucor, the first one had a less area, but the *Mucor sp.* plug was contaminated with *Trichoderma viride* which grew on top of both fungi. In the triple culture plates, both *F. graminearum*ΔZEA and *F. graminearum* were outcompeted by the *Trichoderma* which grew on top of *Mucor sp.*, (Fig. 12).

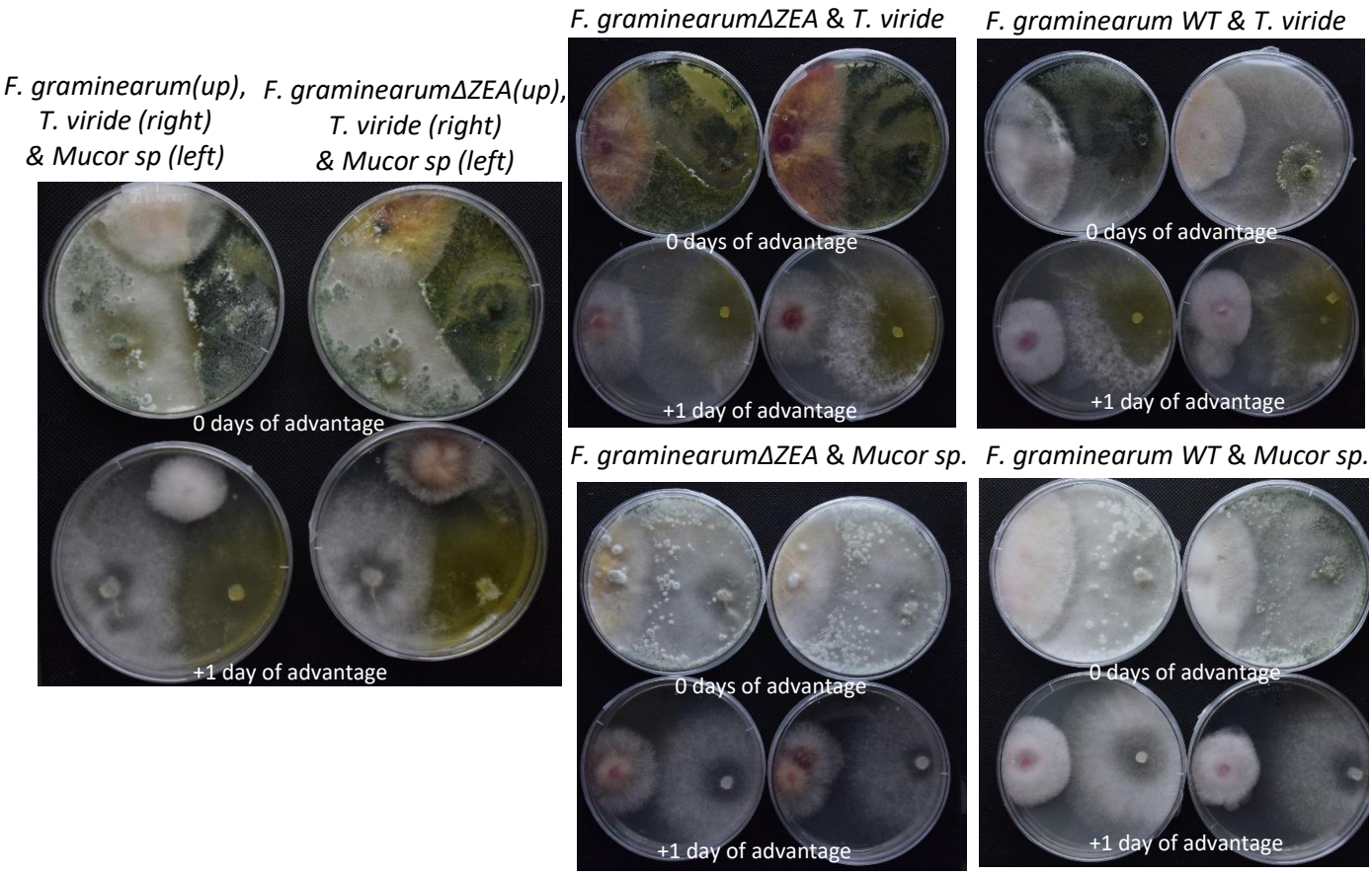

Figure 12. Antagonism tests results

3.4 Zearalenone quantification. The HPLC analysis showed that the zearalenone content was much higher compared to the previous experiment (Table 6). Ranging from 1.16 to 3.64 mg/kg. A linear model was created using R lm function and the values were tested for normality using the Shapiro-Wilk normality test. It was concluded that the data had a parametric distribution (p - value = 0.6682). Based on this, an analysis of variance test (Table 7) and a Least square means POST-HOC test (Table 8) were performed. The results showed that there were no statistically significant differences in the zearalenone content when grouped by treatment (p - value = 0.855).

Table 8. Second Experiment HPLC zearalenone concentration results

		***trans*-Zearalenone**	***cis*-Zearalenone**	***Total***
Sample	**Fungi**	**c [mg/kg]**	**c [mg/kg]**	**c [mg/kg]**
1	F_gram_WT	2.21	0.60	2.8
2	F_gram_ZEA	0.10	2.89	3
3	Control	0.48	0.68	1.16
4	F_gram_ZEA	0.46	1.44	1.91
5	Control	0.52	1.21	1.73
6	F_gram_WT	0.72	1.48	2.19
7	Control	1.57	1.48	3.05
8	F_gram_WT	0.51	1.56	2.07
9	F_gram_ZEA	0.65	3.00	3.64
10	F_gram_ZEA	0.28	2.17	2.45
11	Control	0.30	2.44	2.74
12	F_gram_WT	0.52	1.38	1.9
13	F_gram_WT	0.29	2.52	2.81
14	F_gram_ZEA	0.07	2.06	2.13
15	Control	1.14	2.06	3.2
16	Control	0.59	1.50	2.09
17	F_gram_WT	0.61	2.11	2.71
18	F_gram_ZEA	0.72	1.38	2.09
LOD		0.006	0.006	
LOQ		0.020	0.020	

Table 9. Analysis of variance of zearalenone content grouped by Treatment

	DF	Sum	Mean Sq	F. value	Pr(>F)
Fungi	2	0.132	0.0658	0.158	0.855
Residuals	15	6.253	0.4169		

Table 10. Least square means POST-HOC test grouped by Treatment

Fungi	emmean	SE	df	lower.CL	upper.CL	group
Control	2.33	0.264	15	1.77	2.89	1
F. graminearum WT	2.41	0.264	15	1.85	2.98	1
*F. graminearumΔ*ZEA	2.54	0.264	15	1.97	3.1	1

When grouped by fungi, the zearalenone amount in the treatments do not show many differences besides some outliers (Fig. 13). When observed by flask no clear tendency within flasks of the same treatment is visible (Fig. 14).

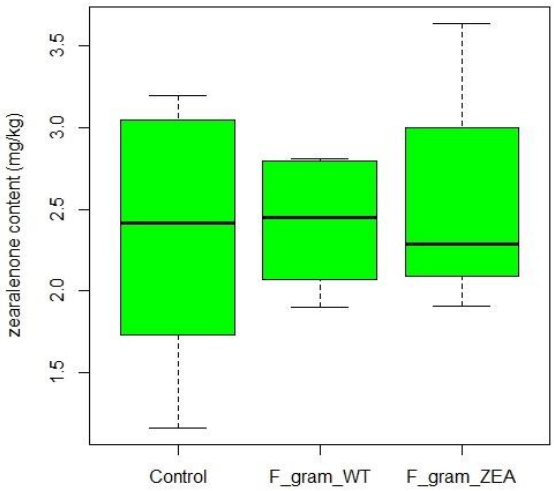

Figure 13. Zearalenone amounts (mg/kg) grouped by treatment

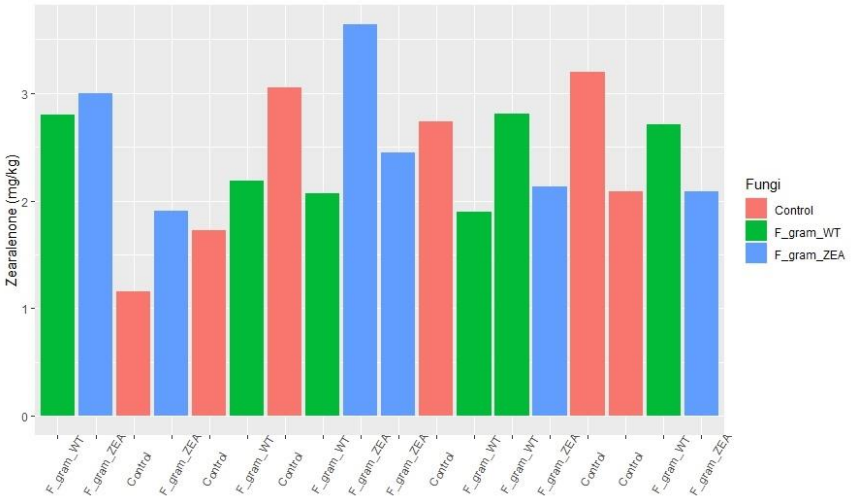

Figure 14. Zearalenone content grouped by Flask

4. Discussion

In the first experiment zearalenone was present in all the analyzed samples in concentrations from 0.010 to 0.18 mg/kg (Table 6), having no correlation with the number of spores inoculated. The experiment also demonstrated that the maize straw was contaminated with *F. graminearum* since the control presented small amounts of zearalenone. We therefore decided to use 20.000 spores per flask, as we described in section 3.1.1. Confirming our results, we found that Etcheverry *et al.*, (1998) also reported this independence between spore concentration and mycotoxin production. Reporting that *F. graminearum* produced similar amounts of zearalenone and deoxynivalenol with no difference among the different spore concentrations used.

In our final experiment, the concentration of zearalenone was higher than any of the concentrations used in the previous experiment, having values from 0.60 to 3.64 mg/kg. The difference could be explained as a consequence of the time the straw was humidified prior to each experiment. In the first experiment, 10 grams of straw with 15 mL of sterile tap water were placed in a plastic bag at 4°C for 14 days while in the final experiment the straw was exposed to humidity for 24 hours. The initial theory was that the spores of *F. graminearum* would out-compete the existing fungi populations due to their sheer numbers in the highest concentrations and the exposure to humidity would also eliminate the possibility of losing the *F. graminearum* spores due to a lack of humidity.

None of the treatments showed any statistical difference in their colony forming units (CFU), and only 2 fungi species grew in the petri dishes, none of them being *F. graminearum.* The effect of adding the spores of *F. graminearum*, seem to have been non-significant however a reduction trend in the number of CFU where *F. graminearum* was inoculated was observable in the statistical analysis. (Kosawang *et al.,* 2014) proved the importance of zearalenone in inter-competitive interactions by showing that *F. graminearum* mutants were unable to compete against *Clonostachys rosea* regardless if the latter had the ability produce zearalenone lactonase or not. The capacity of competing organisms to suppress partially or completely the production of a metabolite hints of it being an important factor in fungal interactions as theorized by (Hestbjerg *et al.,* 2002; Cooney *et al.*, 2001). They proved that *F. subglutinans* could act as an antagonist and reduce the deoxynivalenol production of *F. graminearum*. *F. subglutinans* co-occurs with *F. graminearum* but their ratios vary with location and season. Intuitively we would expect that the mutant and the wildtype had different results in the fungal populations and a different amount of CFU present in them.

Other experiments have shown that the season when the samples are taken is a factor that must be considered when conducting experiments with fungal communities are carried out (Cooney *et al.*, 2001). A combination of organisms with detoxification capabilities might be the possible causes for the lack of *F. graminearum* in the petri dishes. *Clonostachys rosea*, *Rhizopus* and *Aspergillus* are also widely distributed facultative saprophytes, whose isolates have also been reported to have zearalenone detoxification capabilities (Zhang *et al*., 2008; Ji *et al.*, 2016). Though no colony of them was found, it is possible that a fungus may not appear in the final plating but still caused alterations in the fungal population composition. Competition pressure in the initial establishment phase would alter the population composition. Metabolites have been proven to have a function as inhibitors of competing organisms and the competitiveness of each fungi depends on the fungi present in the environment. The advantages provided by a fungus secondary metabolites depend in whether the present fungi are susceptible to their secondary metabolites and in the microorganism's resistance to metabolites produced by the other microorganisms, (Chatterjee *et al.* 2016). It could be that the lack of differences was caused by a large inoculum of non-susceptible fungal species. Another explanation for these results can be theorized when revising the work of (Marin *et al.* 2004). He divided competitions between organisms into resource capture and resource combat situations. The fungi with rapid growth and spore generation are better suited in resource capture situations while those with volatiles and other secondary metabolites have an edge when it is a resource combat situation. Since *F. graminearum* spores and the straw were added to flour, the situation was predominantly a resource capture scenario. Studies of fungal populations on decomposing straw conducted by (Bowen & Harper, 1989) showed that pathogens which survive in infected straw can predominate in the substrate in the early stages of decay. This could mean that *F. graminearum* was not able to stablish itself and produce zearalenone in sufficient amounts to compete with the present fungi. Tests of zearalenone presence in infected maize straw show concentrations of up to 40 mg/kg which are significantly higher than the amounts found in our samples. Based on our observations we can theorize that the initial colonization of *F. graminearum* was able to produce measurable amounts of zearalenone and that later the population suffered from a gradual decrease as weeks passed. This matched the observations reported by (Sadasivan, 1939) in which *Fusarium spp.* populations are the primary colonizers of debris, since they usually attack living tissue, the report also mentions that the populations are gradually reduced.

The usage of straw obtained from the field adds a layer of uncertainty in the results since all the organisms present in the samples are not known nor the size of their inoculum. In the first experiment, the flasks presented the characteristical red pigmentation of *Fusarium spp*. This hints that subsequent experiments with the same inoculum source will give different results due to the heterogeneity of the fungi distribution within the straw. It is also possible that the *Mucor spp.* found in most plates had the ability to detoxify zearalenone since this ability has been reported for *Mucor bainieri* (Ji *et al.*, 2016). Colonies of *Trichoderma viride* which have also been proven to outgrow *F. graminearum* (Schöneberg *et al.*, 2015), were also found. Initially they were obscured by *Mucor spp.* since their colonies were initially similar, only varying in their developing speed since *Mucor* outgrew *Trichoderma viride.* But as time passed, green colored mycelia appeared in most plates, (84% of the plates presented this growth). It has been proven at the genus level that *Trichoderma spp.* isolates have been capable of detoxifying Deoxynivalenol through glycosylation (Ye *et al*., 2016).

Fields with alterations due to chemical crop protection products might show different results since they alter the enzyme production of fungi and bacterial populations, either stimulating or inhibiting their production, (Franco da Silva *et. al*, 2017). Weather factors have been proven to affect the microbial activity, decreasing their activity when in freezing conditions, which in turn increases the time needed for suppressive soils to reduce disease severity from soil diseases (Cook, 2014). Studies comparing fields with similar crop history but different management and/or weather might be a way to better grasp the effect of *F. graminearum* zearalenone on the fungal populations since there might be greater variability in the populations present in the samples.

In a previous study conducted by Sadasivan (1939), it was described how the composition of fungal populations on decomposing wheat straw, changes as time progresses and the straw further decays (Figure 15).

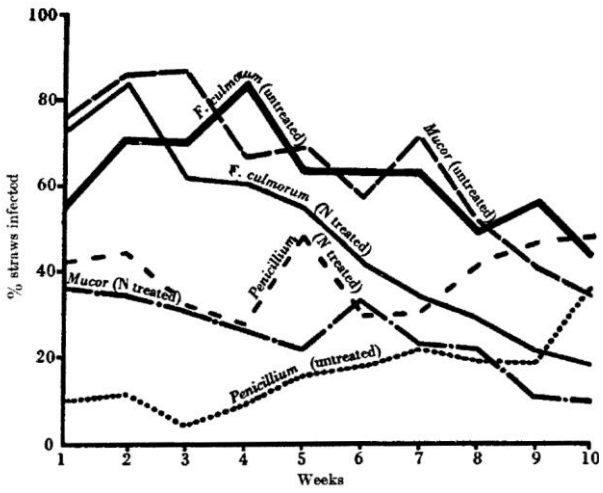

Figure 15. Sequence of fungal development on untreated and nitrogen-treated straws buried in the soils (Sadasivan, 1939)

In our experiment we have a limited amount of substrate, which is being consumed, therefore a case could be made that these two situations are comparable, since both environments gradually lose more nutrients, changing the organisms that are better suited to thrive in them. This would explain why there are significant amounts of zearalenone but no colony of *F. graminearum* was found during the plating step. Since *F. graminearum* was the only controlled organism and a very limited number or organisms developed, the high amounts of zearalenone compared to the previous experiment might be the cause of the reduced number of species since all the samples had *F. graminearum* due to the straw used as a substrate. Antagonist tests with all the possible combinations of *F. graminearum, F. graminearumΔZEA, Mucor spp. and Trichoderma viride* showed no adaptation that would allow them to suppress *F. graminearum* and its mutant. But literature review and the fact that they were found in a soil with natural *F. graminearum* populations, does suggest that they could have mechanisms to resist *F. graminearum* mycotoxins. A similar experiment with more samples from different fields or with sub samples from different parts of the field would better reflect the effect of *F. graminearum* on populations which will highly likely vary between the samples. If the samples are taken from multiple fields, the crop history and production system should also be considered. Since the levels of incidence of *F. graminearum* increase under no-tillage or chisel plow and previous crops can increase of decrease the incidence of the disease (Dill-Macky & Jones, 2000).

Another experiment design that would ensure that *F. graminearum* is present and can compete with the existing fungi is using mycelia as inoculum source. Weighting the media in which the plate and media prior to inoculation of the fungi and subtracting its volume to obtain the milligrams of mycelia that are being inoculated into the flasks, would give a reproducible way of inoculation. Several of these sections could be apply per flask to maximize its distribution in the samples, while still controlling for the total amount of mycelia inoculated to each flask. Another way for evaluating the effect of zearalenone in fungal populations, is the use of artificially synthesized zearalenone, applying it in multiple concentrations to maize straw collected from the fields which have not been used for *F. graminearum* hosts and evaluate the differences among the samples in the fungal composition and CFU (Brito *et al.,* 2015) conducted experiments of fungistasis of *F. graminearum* on different systems considering the bacterial profile of the soil and found that antibiosis and siderophore productions were the main cause for fungistatic effects. Bacterial identification would be needed to be considered in further experiments, since an unknown fungal population developed in our plates which may have an effect on the fungal populations.

5. Conclusion

The initial theory was that with enough spores, *F. graminearum* would be able to compete with the fungi naturally present in the straw. The first experiment confirmed that *F. graminearum* was able to produce zearalenone. However, the second experiment showed that even with zearalenone production, the fungi may disappear either due to a possible depletion of nutrients or change in their ratio. Or due to intense competition from organisms adapted to withstand its mycotoxins. In this final experiment, even though only two fungi were identified with sufficient certainty, the results also showed a large variability in the CFU between the flasks. This could be the consequence of an unaccounted factor, the effect of an organism which was not considered in the screening process or a lack of significant response due to the pre-existence of *F. graminearum* in the studied straw which may have altered the population composition from the field. Studies with more material from more environments without *F. graminearum* history would give a better estimation of the effect that zearalenone has in the fungal populations. Further experiments should be conducted to determine the reason why *F. graminearum* was not found in the samples using the previously mentioned designs.

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