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HOW BIOLOGICAL AND CHEMICAL FUNGICIDES IMPACT THE MAIZE MICROBIOME, *Fusarium verticillioides* POPULATIONS AND FUMONISINS CONTENT

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Prof. Dr. Flávio Henrique Vasconcelos de Medeiros
Orientador

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APROVADA em 10 de Dezembro de 2018

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“To all who believed and to believe with love in the heart

Extend”

“Á todos que acreditaram e acreditam com amor no coração

Dedico”

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À vida por sua beleza, plenitude e ensinamentos.

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RESUMO

As doenças de plantas vão além de danos econômicos e danos fisiológicos. Alguns patógenos como *Fusarium verticillioides* podem produzir metabólitos secundários, que são tóxicos aos seres humanos e animais, conhecidos por micotoxinas. A produção destas toxinas, no caso de *F. verticillioides*, mais conhecidas como fumonisinas são um problema socioeconômico. Assim, alternativas que reduzam a produção de fumonisinas são necessidades emergências, principalmente dentro da cultura do milho, onde esta toxina normalmente está mais presente. Assim, os objetivos deste trabalho foram (1) avaliar a dinâmica do microbioma da filosfera de milho sob a combinação do tratamento foliar com fungicida (azoxistrobina+ciproconazol) e agente de controle biológico (*Bacillus subtilis*) (2) avaliar novas técnicas de manejo integrado, usando a compatibilidade a fungicida com agente de controle biológico contra *F. verticillioides* e (3) avaliar os custos econômicos em dois sistemas de produção, um com apenas a aplicação de fungicidas e outros com a combinação de fungicidas e o agente de controle biológico na redução de perdas por fumonisinas. Ensaios com plantas de milho foram realizados em duas áreas diferentes onde foram coletados parte da espiga em dois tempos para análise de populações de bactérias (16S) e fungos (ITS) por sequenciamento de nova geração (NGS). Ainda foram analisadas a quantidade de ciclos presentes do patógeno (qPCR) e o teor de fumonisinas (LC-MS / MS) sobre plantas tratadas e inoculados com *F. verticillioides*. Na segunda parte foi avaliada a sensibilidade de 20 isolados de *F. verticillioides* a 10 princípios ativos de fungicidas (azoxistrobina, piraclostobina, captana, tiabendazol, fluatriafol, carbendazim, propiconazol, tetraconazol, tebuconazol e ciproconazol) em diferentes concentrações (0; 0,1; 1; 10 e 100 ppm) além da compatibilidade de bactérias (30 isolados) e fungos (30 isolados) antagonistas do filoplano de milho a ciproconazol e azoxistrobina. E por ultimo, o custo de produção em dois sistemas produtivos, sistema convencional (duas aplicações de fungicida) e sistema proposto (uma aplicação de fungicida combinada com agente de controle biológico), analisando a produtividade do sistema (ton/ha), a qualidade nutricional entre os dois sistemas (NIR) e as perdas por fumonisinas (LC-MS / MS) nos sistemas produtivos. As análises estatísticas foram realizadas através do teste de Tukey ($p<=0.05$) e a analise do microbioma da filosfera de milho por procedimentos de bioinformática. Foram observadas mudanças nas comunidades de bactérias e fungos entre os tratamentos. Houve maior número de copias de DNA de *F. verticillioides* e maior teor de fumonisinas no tratamento com duas aplicações de fungicida. Em relação à sensibilidade de isolados de *F. verticillioides* a fungicidas, tebuconazol e tetraconazol foram os princípios ativos com maior sensibilidade entre a população. Houve também isolados antagonistas a *F. verticillioides* compatíveis com ciproconazol e azoxistro bina. Em relação ao custo de produção/produtividade foi observado melhor relação no tratamento com duas aplicações de fungicidas, porém o teor de fumonisina acumulado foi maior, na relação de 8:1 (sistema convencional/sistema proposto). É possível concluir a importância do manejo integrado dentro do patossistema milho-*F. verticillioides*-fumonisinas como ferramenta aliada na redução de fumonisinas. Devendo ser trabalhado neste sistema os melhores momentos de aplicação dos agentes de controle biológico para maiores ganhos em produtividade.

Palavras-chave: Podridão rosada da espiga, controle químico, controle biológico, manejo integrado, custo de produção, fumonisinas.

ABSTRACT

Plant diseases go beyond economic damage and physiological damage. Some pathogens such as *Fusarium verticillioides* can produce secondary metabolites, which are toxic to humans and animals, known as mycotoxins. The productions of these toxins, in the case of *F. verticillioides*, better known as fumonisins are a socioeconomic problem. Thus, alternatives that reduce the production of fumonisins are emergencial needs, mainly within the maize crops, where this toxin is usually present. The objectives of this work were: (1) to evaluate the dynamics of the maize phyllosphere microbiome under the combination of foliar treatment with fungicide (azoxystrobin+cyproconazole) and biological control agent (*Bacillus subtilis*) (2) to evaluate new integrated management techniques , using the fungicide compatibility with biological control agent against *F. verticillioides* and (3) to evaluate the economic costs in two production systems, one with only the application of fungicides and others with the combination of fungicides and the biological control agent in the reduction of fumonisin losses. Tests with maize plants were carried out in two different areas as part of the ears was collected in two times for analysis of bacterial populations (16S) and fungi (ITS) by next-generation sequencing (NGS). The amounts of pathogen present cycles (qPCR) and fumonisin content (LC-MS / MS) on plants treated and inoculated with *F. verticillioides* was also analyzed. In the second part the sensitivity of 20 isolates of *F. verticillioides* to 10 active fungicides (azoxystrobin, pyraclostobin, captan, thiabendazole, fluatriafol, carbendazim, propiconazole, tetraconazole, tebuconazole and cyproconazole) was evaluated in different concentrations (0; 0,1; 1; 10 e 100 ppm) besides that bacteria (30 strains) and fungi (30 strains) antagonists of the maize phylloplane to cyproconazole and azoxystrobin. Finally, the production cost in two production systems, conventional system (two fungicide applications) and proposed system (fungicide application combined with biological control agent), analyzing the system productivity (ton/ha), nutritional quality between the two systems (NIR) and fumonisin losses (LC-MS/MS) in the production systems. Statistical analyzes was performed using the Tukey test (p<=0.05) and analysis of the maize phyllosphere microbiome by bioinformatics procedures. Changes were observed in the communities of bacteria and fungi between the treatments. There were more copies of *F. verticillioides* DNA and higher fumonisin content in the treatment with two fungicide applications. Regarding the sensitivity of *F. verticillioides* strains against fungicides, tebuconazole and tetraconazole were the most active ingredients among the population. There have also been isolated antagonists *to F. verticillioides* compatible with cyproconazole and azoxistorbin. In relation to the cost/yield, a better relation was observed in the treatment with two fungicide applications, but the accumulated fumonisin content was higher in the ratio of 8:1 (conventional system/proposed system). It is possible to conclude the importance of integrated management within the maize-*F. verticillioides*-fumonisins pathosystem as an allied tool in the reduction of fumonisins. The best moments of application of the biological control agents for greater gains in yield must be worked on in this system.

Key-words: Fusarium ear rot, chemical control, biological control, integrated management, cost of production, fumonisins.

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INTRODUCTION

1 LITERATURE REVIEW

1.1 SCENERY OF BRAZILIAN MAIZE PRODUCTION

Maize (*Zea mays* L.) is one of the most important cereals cultivated in the worldwide. Due to the multiplicity of forms for use, both for food as well as industrial and technological purposes, this cereal is more present in mankind than is imagined (RANUM; PEÑA-ROSAS; GARCIA-CASAL, 2014; SARAVANAKUMAR et al., 2017). Brazil in the 2017/2018 harvest ranked third among the world's largest producers, losing only to China and the United States of America. Thus, 81.3 million tons were produced in Brazilian's land, while the largest USA producer was 371 million tons and China was second with 215.9 million tons (CONAB, 2018a, USDA 2018).

The Brazilian crop is highlighted by the production in two harvests. The first crop or “summer crop” (planting between August to November) and the second crop or “winter crop” (planting between January and April), which is also known as "little crop" (JAMES, 2015). The variation between in the two crops depends on the regions, due to the dependence of the edaphoclimatic conditions that can advance or delay the planting in these regions. The most important requirements in the regions to start planting are the availability of water, one of the abiotic stress targets limiting maize plants (HEINEMANN et al., 2008, ALLEN;VALDES, 2016; DE OLIVEIRA et al., 2017) and the degree-days relationship that is linked to photoassimilation and plant growth (LOBELL et al., 2013). Hence, the last Brazilian maize crop produced 26.8 million tons in the first harvest, due to a 12% reduction in the area sown. In the second crop, there was also a reduction in the cultivated area combined with the strong water stress that resulted in losses of productivity, resulting in a production of 54.5 million tons (CONAB, 2018b).

The projection for the 2018/2019 crop is that 16,823.2 thousand hectares of maize being planted. A promising scenario is expected in this plantation for different positions, such as: (i) an export alternative to the Chinese market; (ii) improvements in exchange rates; (iii) higher application of maize to ethanol production; and (iv) increased consumption of the intern market for the formulation of protein concentrate in animal feed (CONAB, 2018a). In addition to the market projections, before the final product is guaranteed, various measures must be taken in the production processes. The final quality of the maize for export is

obtained before the projection of the future market and should have both pre-harvest and post-harvest quality, both for external than internal consumption (MAGAN; ALDRED 2007; KAMALA et al., 2016; AGEGNEHU et al., 2016).

Maize hybrids can be affected by several factors such as soil type, water availability and nutrition (CHAUHAN; SOLOMON; RODRIGUEZ, 2013; LOBELL et al., 2013), as well as interference from weeds, insects and diseases (REID et al., 1999; PARSONS; MUNKVOLD, 2010; BLACUTT et al., 2018) that are not always resolved with the use of breeding hybrids for tolerance or resistance against them (BERGVINSON; GARCIA-LARA, 2004).

1.2 MAIZE DISEASES

Maize diseases in the late 1990s were not as important as they are today. The changes in production systems were a decisive factor in expanding the dissemination of diseases, creating a barrier to achieving high productivity. Therefore, systems that related to increasing productivity are also responsible for the increased incidence and severity of diseases. Some factors, such as the expansion of the agricultural frontier, the expansion of planting seasons (first and second crop), the adoption of a no-tillage system without crop rotation, increased use of irrigation systems and susceptible materials have been working synergistically in favor of diseases in maize (COTA; COSTA; SILVA, 2015).

The maize diseases can cause quantitative and qualitative losses. These damages beginning from the time of sowing and can go until to harvest time, and often also progress in post-harvest (MITCHELL et al., 2016; ACHARYA et al., 2017). Diseases in maize can be divided into: (1) leaves diseases; (2) stalk and root rot diseases and (3) ear rot diseases. The most important leaves diseases present in Brazil are, *Pantoea ananatis* or “maize white spot” (CASELA; FERREIRA; PINTO, 2006), *Puccinia sorghi* or “maize common rust”, *Puccinia polysora* or “maize southern rust” (RAMIREZ-CABRAL; KUMAR; SHABANI, 2017), *Physopella zeae* or “maize tropical rust” (LIMA et al., 2006), *Cercospora zeae-maydis* and *Cercospora sorghi* or “maize gray leaf spot” (NEGA et al., 2016), *Exserohilum turcicum* or “northern maize leaf bligh” (PATAKY, 1992), *Stenocarpella macrospora* or “diplodia leaf streak” (PANISON et al., 2016), *Phaeosphaeria maydis* or “phaeosphaeria leaf spot” (ADAM et al., 2017), *Colletotrichum graminicola* or “maize anthracnose leaf blight” (NICOLI et al., 2016) and *Spiroplasma kunkelii* or “maize bushy stunt phytoplasma” (MENESES et al., 2016). The major diseases that cause stem and root rot are *Pythium* ssp. or “Phytium stalk

Rot" (MATTHIESEN et al., 2016), *Fusarium verticillioides* or "Fusarium stalk rot" (KIM et al., 2018), *Fusarium graminearum* or "Gibberella stalk rot" (MUELLER et al., 2016), *Colletotrichum graminicola* or "Anthracnose stalk rot" (MATIELLO et al., 2012), *Stenocarpella maydis* or "Diplodia stalk rot" (ZACCARON; WOLOSHUK; BLUHM, Burton, 2017) and *Macrophomina phaseolina* or "Charcoal rot" (KAISER; DAS, 1988). Maize ear rot may be associated with *Stenocarpella maydis* or "Diplodia ear rot" (LUNA; WISE, 2015), *Fusarium verticillioides* "Fusarium ear rot" (BLACUTT et al., 2018), *Fusarium gramineraum* or "Gibberella ear rot" (KEBEBE et al., 2015). Other Fusarium species of that have been described in association with *F. verticillioides* and *F. graminearum*, which are usually the most predominant species (ZHOU et al., 2018). Also in association with maize ear rot is the presence of fungi that use water activity to colonize maize grains such as *Aspergillus flavus* and *Aspergillus parasiticus* or "Aspergillus ear rot" (BHATNAGAR-MATHUR et al., 2015) and *Penicillium* spp or "Penicillium ear rot" (OGARA et al., 2017). Figure 1 shows the dynamics of diseases in the maize plant.

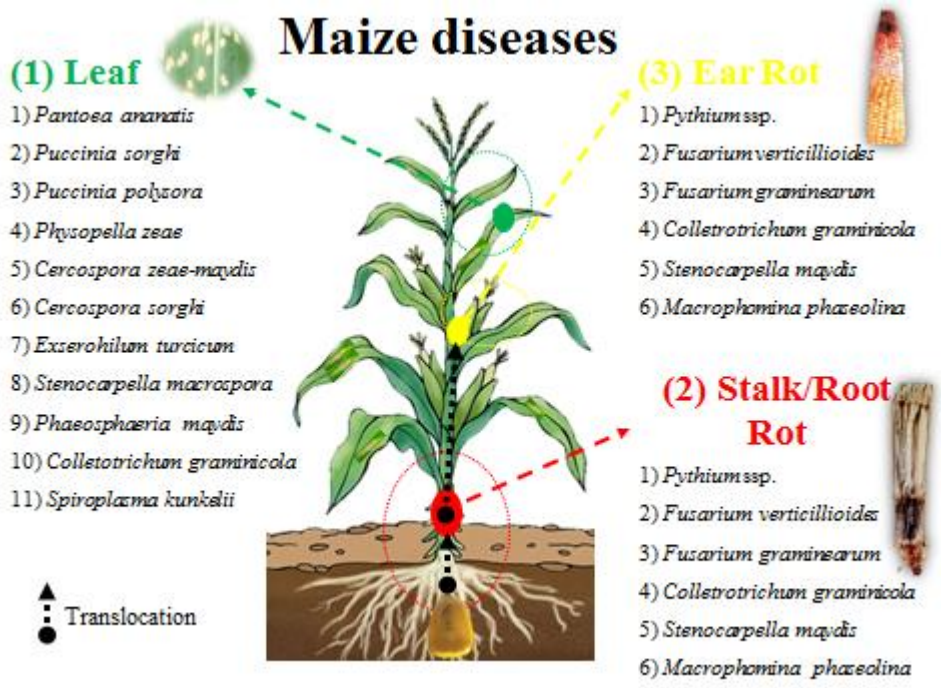

Figure 1. Major diseases of maize crop and its possible translocation between maize stalk/root rot and maize ear rot.

1.3 FUSARIUM EAR ROT (*FUSARIUM VERTICILLIOIDES*)

Fusarium verticillioides (Saccardo) (previously known as *Fusarium moniliforme* Sheldon) it is one of a most important pathogen in maize. Its presence in maize ears causes the symptom known by Fusarium Ear Rot (FER). This pathogen has the ability to colonize maize roots, stems and ears endophytically and causes diseases such as FER and Fusarium Stalk Rot, because it has the capacity to systematic translocation in the plant (SCHOEMAN et al., 2018). Besides a pathogen of maize *F. verticillioides* already gone associated with teosinte, millet, sorghum and tallgrass (DESJARDINS; PLATTNER; GORDON, 2000, HIRATA et al 2001, LESLIE et al 2004, O'DONNELL et al., 2007). However *F. verticillioides* has more affinity to infect maize plants wherever it is planting (SHEPHARD et al., 1996; SCHOEMAN et al., 2018). The fungus *F. verticillioides* can infect maize plants in several ways. The most observed form is the dispersion of macroconidia and microconidia of the fungus through the wind, this form of infection is usually responsible for causing the greatest infection of the grains (MUNKVOLD; HELLMICH; SHOWERS, 1997). The pathogen can also systemically colonize any plant through seed infection. This pathway usually occurs due to contaminated seeds (with *F. verticillioides*) or by survival in cultural remains from the no-tillage or monoculture systems of *F. verticillioides* reproductive structures (BLACUTT et al., 2018). Thus, the pathogen is able to live endophytically in the plant and can later colonize the stalks and ears, where the damage occurs (OREN et al., 2003; BLACUTT et al., 2018).

The characteristic symptoms of FER in maize ears, following these characteristics, in relation to the form, can have scattered or in groups, in relation to the color, can be white, pink or salmon-colored. The infected ears may turn tan or brown with um standard known by "starbust" (light-colored streaks radiating from the top of ears where silks were attached). Nonetheless, there is no uniformity in symptoms in the same field because the symptoms vary widely and range from asymptomatic infection to severe rotting of all plant parts, how would the visual symptoms (MORALES et al., 2018). In the same field is very common that have plants with disease and asymptomatic even under conditions of the genetically uniform host (OREN et al., 2003; MURILLO-WILLIAMS; MUNKVOLD, 2008; PARSONS; MUNKVOLD, 2012).

In addition to the pathogenic characteristic with the presence of symptoms and asymptomatic of *F. verticillioides* in maize plants (NGUYEN et al., 2016), this fungus has the capacity to produce toxins, known as mycotoxins (NELSON et al., 1993; DESJARDINS; PLATTNER; GORDON, 2000), which is the most common type is fumonisin (PASCALE et

al., 1997, DESJARDINS et al., 1998). Accrue in three possible interactions between the production of toxins and the symptomatology in this maize-FER system can be observed, as: (i) presence of symptoms and high levels of fumonisins; (ii) the presence of grain symptoms and low levels of fumonisins and (iii) the production of toxins in visually asymptomatic grains (MUNKVOLD; DESJARDINS, 1997; DESJARDINS et al., 1998; AFOLABI et al., 2007). In Figure 2 shows the interaction dynamics of *F. verticilliodes* for pathogenicity and how may occur the production of fumonisins in this interaction.

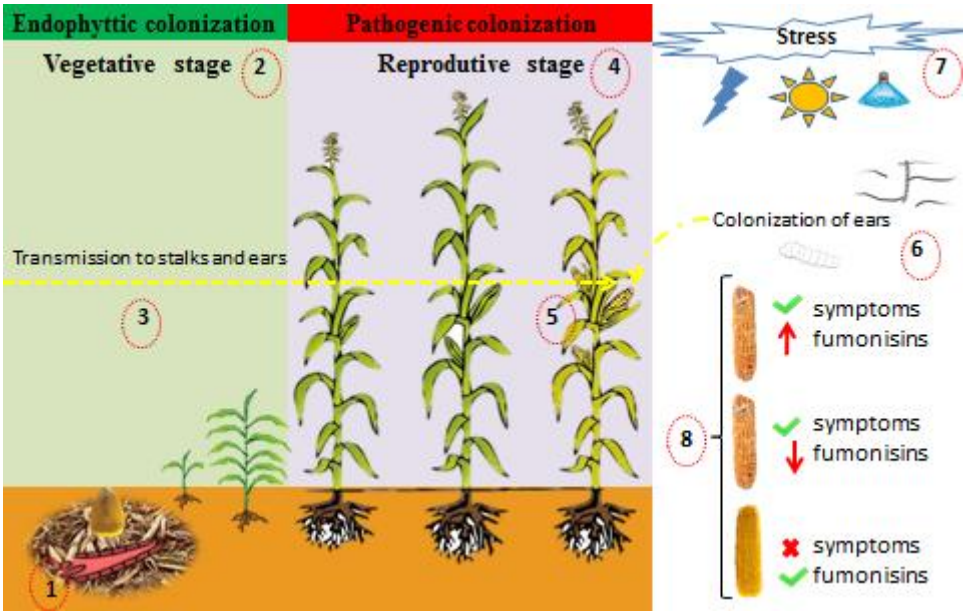

Figure 2. Dynamics of colonization and production of fumonisins by *Fusarium verticillioides* in maize, where (1) source of inoculum of the pathogen that survived in stubble crops, colonize the maize seeds; (2) in the vegetative stage this colonization still does not cause damage to the plants, at that moment the pathogen lives endophytically within the plant; (3) in the change of the vegetative stage to the reproductive stage occurs the transmission to the stalks and posterior to ears; (4) in the reproductive stage it is possible to verify the pathogenic form of F. verticillioides, causing stalk and ear rot and mycelial growth can be visualized (sometimes); (5) at the end of the reproductive cycle it is possible to verify that there was transmission of *F.verticillioides* of the seeds for ears, often not being possible visual analysis; (6) the other form of transmission occurs through the wind that carries the microconidia and macroconidia that will be deposited in the stigma-style, where with the presence of water and also caterpillar and larvae can able penetrate inside the ears; (7) after the possible means of colonization and the presence of *F. verticillioides* in the ears, stress conditions are determinant for the production of fumonisins, such as rainfall, droughts and use of chemical fungicides and, (8) the three possible interactions symptoms of *F. verticillioides* and the production of fumonisins, (1) presence of symptoms in the grains and high levels of fumonisins; (2) the presence of symptoms in the grains and low levels of fumonisins and (3) toxin production in asymptomatic grains.

A number of factors may contribute to the production of fumonisins in maize. The increased production of this secondary metabolite normally occurs in response to stress conditions (MILLER, 2001; ETCHEVERRY et al., 2002) as environmental conditions and water availability (NAGY; CABULEA; HAS, 1997; MAGAN et al., 1997). Conditions such

as drought and insect attack (PARSONS; MUNKVOLD, 2010) are reported as factors that increase the production of fumonisins in the grains. Miller (2001) suggests that there are five possible factors that interfere in the production of fumonisins such as, (i) temperature; (ii) drought stress, (iii) insect damage, (iv) other fungal diseases and (v) type of maize genotype. Several authors working with modeling suggest that the fumonisins-FER interaction is favored by dry or droughty conditions, in addition to the need for temperatures above 28 ° C to initiate the development and growth of FER (PASCALE et al., 1997; MAIORANO et al., 2009). Typically fumonisins have a higher expression between the ranges of temperatures that are ideal for production than maize plants for growth and development, so under stress conditions for plant normally the higher production of these metabolites is triggering off (MURILLO-WILLIAMS; MUNKVOLD, 2008). Another condition that has been reported with the increase of fumonisin levels is the use of chemical fungicides (MARÍN et al., 2013; CAO et al., 2014). Before the harvest, the same factors described as important, such as climate (temperature, humidity, and precipitation), exposure to insect pests, the presence of other pathogens, besides planting dates, maize genotype and cropping system can also influence the increased production of fumonisins in postharvest. Besides, postharvest conditions may influence fungus growth and a higher accumulation of fumonisins due to worse conditions to storage, normally with high humidity and the presence of insects, especially weevils (MARÍN et al., 2004, MILLER, 2001, DÍAZ-GÓMEZ et al., 2016).

1.4 FUMONISINS

Fumonisins are products of the secondary metabolism of some species of Fusarium, such as *F. fujikuroi*, *F. globosum*, *F. nygamai*, *F. proliferatum* and *F. verticillioides*, species present in Fusarium Fujikuroi Species Complex (FFSC) and also in other complex. In higher levels and more frequent are, *F. verticillioides*, *F. proliferatum* and *F. nygamai* (PROCTOR et al., 2004; AOKI; O'DONNELL; GEISER et al., 2014). Most isolates of *F. verticillioides* have the ability to produce fumonisins (DESJARDINS et al., 1995; RHEEDER; MARASAS; VISMER, 2002). Fumonisin production was discovered in 1975 when an isolate of *F. verticillioides* (MRC 826) from maize in the Transkei region of the Eastern Cape Province (South Africa) was described in an area of high incidence of esophageal cancer in humans (MARASAS, 1984).

Fumonisin B1 (FB1) and fumonisin B2 (FB2) are the most found and abundant fumonisins in maize (LI et al., 2015). The synthesis of fumonisins can inhibit ceramide

synthetase enzyme that is involved in the biosynthesis of sphingolipids (WANG et al., 1991). Those conditions disturbed the animal and human metabolism induces toxic responses (OLIVEIRA et al., 2015). The consumption of maize contaminated with fumonisins in animal feed is related of damage like the cause of eucoencephalomalacia (ELEM) and hepatosis in horses, pulmonary edema in swine, nephrosis, hepatosis in sheep, and have carcinogenic and hepatotoxic effects in rats (MARASAS et al., 2004). In humans diet the intake of fumonisins are associated with defects of the neural tube (GELINEAU-VAN WAES et al., 2005) and carcinogenic effects (MÜLLER; DEKANT; MALLY, 2012) and more the susceptibility to HIV (WU et al., 2011).

In the year 2001, several countries presented information on the presence of fumonisins in maize consumed in each country. The presence of 60% of fumonisins in the tested products was detected (JOINT FAO/WHO, 2001). With the support of this data, a report was made on the exposure of the European Union population to mycotoxins. Regarding the fumonisins between samples of maize without processing, 67% gave positive for FB1 and 51% gave positive for FB2 (BAKKER et al, 2009). Hence, the major problem behind fumonisins beyond human and animal health is the regulation of permitted levels, and the presence of these metabolites in food is considered a socioeconomic problem (VAN EGMOND; JONKER, 2004).

Lesgilation for present levels of fumonisins in food has been elaborated by countries. For example, in Brazil, this regulation applies to companies that import, produce, distribute and market certain categories of beverages, foods and raw materials. The “Resolution of board of directors - rdc no. 07, february 18, 2011” provides for Brazilian levels the following categories, (1) of immediate application; (2) of application in January 2012; (3) of application in January 2014; (4) of application in January 2016 where the maximum tolerated limits (LMT) are listed in Table 1 (ANVISA, 2011). While in the European Union these levels are divided between food for human consumption and animal consumption, see Table 2 (LERDA, 2011). In the United States of America, foodstuffs for human consumption and animal consumption also have different levels of fumonisins allowed. In addition, in the United States, the presence of FB3 has already been added, see Table 3 (updated by MAZUMDER; SASMAL, 2001).

Table 1. Brazilian legislation to control fumonisins levels marketed in maize.

Commodity	Maximum permitted levels FB1+FB2 (ppb)
Popcorn [1]	2000
Maize-based foods for infant feeding (early childhood and infants) [1]	200
Maize flour, maize cream, maizemeal, flakes, canjica, canjiquinha[2]	2500
Maize starch and other maize products[2]	2000
Maize in grain for further processing[3]	5000
Maize flour, maize cream, maizemeal, flakes, canjica, canjiquinha[4]	1500
Starch of maize and other maize products[4]	1000

[1,2,3,4] check above

Table 2. European Union legislation to control fumonisins levels marketed in maize.

Commodity/ In food	Maximum permitted levels FB1+FB2 (ppb)
Unprocessed maize not intended for wet milling	4000
Maize and maize based foods intended for direct human consumption	1000
Maize based breakfast cereals and maize based snacks	800
Processed maize based foods and baby foods for infants and young children	200
Commodity/ In Feed	**Maximum permitted levels FB1+FB2 (ppb)**
Maize and maize based products	60
Complementary and complete feeding stuffs for pigs, horses, rabbits and pet animals	5
Complementary and complete feeding stuffs for fish	10
Complementary and complete feeding stuffs for poultry, calves (< 4 months), lambs and kids	20
Complementary and complete feeding stuffs for adult ruminants (>4 months) and mink	50

Table 3. USA legislation to control fumonisins levels marketed in maize.

Commodity/ In food	Maximum permitted levels FB1+FB2+FB3 (ppb)
Degermed dry milled corn products (e.g. flaking grits, corn meal, corn flour with fat content of	2000
Cleaned corn intended for popcorn	3000
Whole of partially degermed dry milled corn products (e.g. flaking grits, corn meal, corn flour with fat content of <2.25%, dry weight basis); dry milled corn bran; cleaned corn intended for mass production	4000
Commodity/ In feed	Maximum permitted levels FB1+FB2+FB3 (ppb)
Corn and corn by-products intended for equids and rabbits	5
Corn and corn by-products intended for swine and catfish	20
Corn and corn by-products intended for breeding ruminants, breeding poultry and breeding mink (includes lactating dairy cattle and hens laying eggs for human consumption)	30
Ruminants > 3 months old being raised for slaughter and mink being raised for pelt production	60
Poultry being raised for slaughter	100
All other species or classes of livestock and pet animals	10

1.5 INTEGRATED MANAGEMENT AGAINST FUSARIUM EAR ROT (*FUSARIUM VERTICILLIOIDES*)

Within the context presented, the integrated management of *F. verticilliodes* in maize is an emergency situation and of extreme importance for food security. Thus, it is necessary to use integrated management practices, which aim not only to control the pathogen but also to reduce the levels of fumonisins. Several types of initiatives have been taken around the world to develop management strategies that can reduce losses and damages caused by this pathogen, whether in the field, storage or industrial processing, have been adopted (LESLIE; LOGRIECO, 2014).

Initially, the management of diseases in maize was mainly carried out through the use of resistant cultivars, associated with cultural measures (LANZA et al., 2016, DA COSTA et al., 2018). The control strategies of *F. verticilliodes* must occur before harvesting, and are integrated measures, such as crop rotation, tillage, adherence to optimal planting date and plant densities, and management of irrigation and fertilization (MUNKVOLD, 2003).

Although genetic resistance is one of the main strategies for disease management in maize, chemical control is now one of the most widely used measures by farmers (DA COSTA et al., 2012, LANZA et al., 2016).

The use of fungicides in maize is recent, mainly due to the high cost of application initially. It was widely discussed whether fungicide application would have an economic return and whether yield would actually pay the cost of the application (WARD; LAING; NOWELL, 1997). In just over 30 years, the first cases of fungicide use in maize for *Cercospora* zeae-maydis (maize gray leaf spot) were reported. And from this milestone, also cases of resistance to the active principle used benzimidazole began to appear in the United States (CARTER; STROMBERG, 1980, SMITH, 1998).

The success of chemical control of diseases in maize is already well known, especially of foliar diseases (ESKER et al., 2018). Munkvold et al. (2001) were successfully in controlling maize gray leaf spot with only one application of propiconazole in the non-susceptible hybrid. Brandão et at. (2002) with three applications of azoxystrobin were able to control maize common rust (*Puccinia sorghi*) regardless of the level of resistance of the hybrid. Reddy et al. (2013) were able to reduce the *in vitro* mycelial growth of *Exserohilum turcicum* with the combination of metriram + pyraclostrobin. However, when dealing with Fusarium ear rot, the results with fungicide use are not as efficient than in aerial part. Juliatti et al. (2007) obtained better results against *F. verticillioides* only with the combined application of pyraclostrobin + epoxiconazole in two applications. For Duarte et al. (2009) the best treatment for the reduction of *F. verticillioides* was with a combined application of azoxystrobin + cyproconazole. Lanza et al. (2016) in none combination of fungicide (picoxystrobin + cyproconazole, pyraclostrobin + epoxiconazole, trifloxystrobin + tebuconazole, azoxystrobin + cyproconazole and methyl thiophanate) in one or more applications has succeeded in reducing the incidence of *F. verticillioides* and the fumonisin content. The application of fungicides, especially of the strobilurins and triazoles group (separately or in combination) does not always reduce the incidence of ear rot grains (MAZZONI et al., 2011), and other mixtures such as fludioxonil + metalaxyl-M, can also induce the increase in the production of fumonisins type FB1 (FALCÃO et al., 2011).

The fungicides of the quinone-outside inhibitor fungicides (QoI) group can act on the maize plant by retarding senescence and thus increase the drying time in the field until it can be harvested for storage. This factor may result in a high moisture harvest and consequently be a predisposing factor to the increase of fumonisins by growers who cannot dry their grains due to the costs (NASON; FARRAR; BARTLETT, 2007; WISE; MUELLER, 2011; COSTA

et al., 2018). For Santiago et al. (2015) planting and harvesting dates can also affect grain quality, and harvest delay greatly exposes grain to abiotic stress, a fact that helps increase the production of fumonisins.

The low success of fungicides against *F. verticillioides* besides the residual problems in grains and seeds and the high purchase cost, open the opportunity for another management technique (CHANDRA NAYAKA et al., 2008). The biological control is a proposal within the integrated management for the reduction of ear rot grains and the fumonisins content produced by *F. verticillioides* (CAVAGLIERI et al., 2005; PEREIRA; NESCI; ETCHEVERRY, 2009; MEDINA et al., 2017). Biological control agents (BCAs) act in a friendly way to the plant, in addition to having no toxic residual effect. BCAs action is a nonchemical measure that has been reported in several cases to be effective as a chemical control (DIK; ELAD, 1999; ELAD et al., 1993). These BCAs may be naturally present or inserted in an agroecosystem and have a range of action on plant pathogens (GUETSKY et al., 2002). The proposal to use BCAs within the integrated management of plant diseases is to avoid diseases with high severity levels, reducing quantitative and qualitative damages (HAJEK; EILENBERG, 2018). Use of biological control agents with antagonistic effects on crop pathogens could represent a promising alternative (WHIPPS, 1997; BLOEMBERG; LUGTENBERG, 2001) because is a complementary action with chemical control, and can be applied in combination or alternation to control diseases below the threshold of economic damage (MEDEIROS et al., 2012).

For Pereira et al., (2010) it is necessary to observe the phenological stage of the plant to spray the BCA. The timing of application is fundamental to affect the colonization of *F. verticillioides* and even the production of fumonisin, due to the antagonistic effects being based on niches. In the mentioned work the presence of the antagonist in the seeds guarantees a longer period of contact with the plant and a greater chance of to exercise the antagonism, being able to act through different mechanisms of biological control throughout the plant growth. Thus, the best known biological control mechanisms are parasitism, mycoparasitism, competition, antibiosis, resistance induction and growth promotion (DE ALMEIDA HALFELD-VIEIRA et al., 2015).

Usually the competition between pathogens and non-pathogens (BCAs) by resource and nutrient is one of the most important mechanisms to reduce the severity of diseases, mainly soil-related pathogens, such as the genus Fusarium (PAL; GARDENER, 2006). In the present study, Chandra Nayaka et al. (2008) worked with a maize rhizosphere isolate of *Pseudomonas fluorescens* (Trev.) applied in the treatment of *F. verticillioides*. The results

showed that in different hybrids the seed and leaf treatment improved the growth parameters and reduced the incidence of *F. verticillioides* and the level of fumonisins to the maximum in comparison with the other treatments. Martínez-Alvarez et al. (2016) worked with a shelf life of a *Bacillus cereus* isolate against *F. verticillioides* and obtained promising results, reducing the severity of the disease and still obtaining storage time response of a powder formulation of the test antagonist. In addition to the cited example, we have several interactions with other agents of biological control against *F. verticiilioides*. Table 4 shows a summary, such as the moment of application, the triggering mechanism and the control result in maize of plants.

Table 4. Examples of BCAs, such as the time of application, the mechanisms involved and the results against *F. verticillioides*.

BCA	Time			Mechanisms						Results				Reference
	Seed	Vegetative phase	Reprodutive phase	Parasitism	Mycoparasitism	Competition	Antibiosis	Resistance induction	Growth promotion	Reduction ear rot grains	Reduction of incidence	Reduction of fumonisins levels	Increase yield	
Pseudomonas fluorescens	x	x				x	x				x	x		Chandra Nayaka et al., 2008
Bacillus cereus	x					x	x			x	x			Martínez-Alvarez et al., 2016

Continue (Table 4.) Examples of BCAs, such as the time of application, the mechanisms involved and the results against *F. verticillioides*.

BCA	Time		Mechanisms						Results			Reference
	Seed	Vegetative phase	Reprodutive phase	Parasitism	Mycoparasitism	Competition	Antibiosis	Resistance induction	Growth promotion	Reduction ear rot grains	Reduction of incidence	Reduction of fumonisins levels	Increase yield	
B. subtilis	x					x	x				x		x	Cavaglieri et al., 2005
Clonostachys rosea			x		x	x							x	Samsudin et al., 2017
B. amyloliquefaciens, Enterobacter hormaechei	x		x			x	x			x	x		x	Pereira et al., 2010
B. megaterium, B. cereus	x						x		x	x	x		x	Figueroa-López et al., 2016
Pediococcus pentosaceus			x				x				x		x	Dalie et al., 2010

1.6 CONCLUSION

For the control of *F. verticillioides* several factors should be considered, mainly decision making in an integrated way within the maize-*F.verticillioides* pathosystem. There are a number of BCAs that can assist with chemical fungicides to reduce fumonisins, but locality, type of hybrid and especially environmental conditions should, if possible, be estimated for the management techniques to be efficient. Thus, reducing the stress conditions in the maize production system, it is possible to reduce the levels of fumonisins obtaining food security for human and animal consumption.

1.7 OBJECTIVES

1.7.1 General objectives

Evaluation of the adoption of the biological control in the integrated management of Fusarium ear rots (*Fusarium verticillioides*) in maize.

1.7.2 Specific objectives

A) **1° CHAPTER:** "FUMINISIN AND MICROBIOME CHANGES ON FUSARIUM EAR ROT BIOCONTROL AND FUNGICIDES APPLICATIONS"

To evaluate the effect of the combined application of biological control agent-BCA (*Bacillus subtilis*) with fungicide (azoxystrobin + ciproconazole) on the following variables like, (i) parameters of yield of maize grains; (ii) incidence of fungi in grains; (iii) fumonisins content (FB1+FB2); (iv) number of copies present of *F. verticillioides* and (v) maize ear microbiome analyzes for understanding the importance of use de BCA tandem chemical fungicide.

B) **2° CHAPTER:** "RESPONSE-DOSE AND BIOCONTROL AGENTS COMPATIBILITY WITH FUNGICIDE IN THE INTEGRATED MANAGEMENT OF *FUSARIUM VERTICILLIOIDES"*

To evaluate the sensitivity of 20 isolates of *F. verticilliodes* to 10 fungicides (azoxystrobin, pyraclostobin, captan, thiabendazole, fluatriafol, carbendazim, propiconazole, tetraconazole,

tebuconazole, and cyproconazole) in different concentrations (0; 0,1 ;1; 10 and 100 ppm). To evaluate the compatibility of 30 antagonists strains (fungi and bacteria) to azoxystrobin and cyproconazole in different concentrations (0; 0,1; 1; 10 and 100 ppm). The evaluated parameters wiil be, (i) to evaluate the sensitivity of *F. verticillioides* strains to the main fungicides used in maize; (iii) To compare between 'old population' and 'new populations' the sensitivity of some fungicides to *F. verticillioides*; (iii) to evaluate the compatibility of fungicide with antagonistic fungi strains of *F. verticillioides* and (iv) to evaluate the compatibility of fungicides with antagonistic bacteria strains of *F. verticillioides*.

C) 3° CHAPTER: "**A LOOK BEYOND THE COST OF PRODUCTION IN THE REDUCTION OF FUMONISINS CONTENT"**

To evaluate two production systems: (1) Conventional system with two fungicide applications and (2) Proposed system, a fungicide application and a BCA application based on Bacillus subtilis BIOUFLA2. Within each system, we have evaluated, (i) the production cost, (ii) nutritional quality, and (iii) losses by fumonisins

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2 CHAPTER 1: Fuminisin and microbiome changes on Fusarium ear rot with biocontrol and fungicides applications

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Fusarim verticillioides (FV) known to cause the symptom of Fusarium ear rot (FER) is responsible for qualitatively and quantitatively affecting maize. In context of management of diseases in maize has been using overly fungicides of chemical origin. This fact, together with plant breeding, has created dependence on many hybrids to obtain high productive ceilings and leaf sanity only with the use of chemical fungicides. However, when it comes to grain sanity and mycotoxins content, the use of chemical fungicides alone does not guarantee the same. Thus, the objective of this work was to understand the impact of chemical and biological fungicides on the dynamics of FV, the mycotoxins content of the fumonisins type and the microbiome of bacteria and fungi. Three treatments were used in the phenological stages V9 and R1: T1 (control, two water applications), T2 (fungicide azosxystrobin + cyproconazole, V9 and Bacillus subtilis in R1) and T3 (fungicide in both moments). All treatments were inoculated with the same FV isolate ten days after the stigma-style exposure at the 105 conidia / mL concentration. Therefore, total productivity, Blotter test, total fumonisin content (type B1 and B2), number of copies of *F. verticillioides* (qPCR, quantitative Polymerase Chain Reaction) and sequencing were evaluated of fungi and total bacteria (NGS) for the analysis of the non-cultivable microbiome. It was observed that two applications of fungicides (T3) did not guarantee high yields of grains, besides being the treatment that presented a higher production of fumonisins differing statistically from the others ($p \leq 0.05$). In relation to qPCR the T3 treatment (twice fungicide) was also the one that had the highest average number of DNA copies at the moments observed differing from the others ($p \leq 0.05$). In relation to the microbioma, the combination between the chemical and biological fungicide was the one that recruited the largest number of Operational Taxonomic Units (OTUs) of fungi and bacteria beneficial to maize grains. Thus, this combination of chemical and biological fungicide is the treatment that most reduced levels of FV as well as total fumonisin levels, a fact that can be explained behind the analysis of the microbiome. In conclusion, the combination of the two products was the one that most preserved the naturally occurring biological control agents responsible for acting in the microbial recruitment, thus guaranteeing the greater protection of the grains and disfavoring the production of fumonisins.

Keywords: Integrated management of diseases, *Fusarium verticillioides*, fumonisins content, bacterial and fungi interactions.

2.1 INTRODUCTION

The production of maize (*Zea mays*) is essential for human and animal nutrition on a global scale and is also of inscreasing importance in the production of biofuels (Saravanakumar et al., 2017). However, the maize crop diseases compromise both grain yield and quality and the control relies mostly on the use of chemical fungicides. In this context, *Fusarium* ear rot (FER) caused by the *Fusarium verticillioides* (FV) in tropical or temperate weather conditions have been very relevant causing not only reduction in yield but also in grain quality (Blacutt et al., 2018). The quality is compromised by the fungal colonization of grains causing rotting but mainly by their contamination by mycotoxins which is a recurrent problem reported by the animal feed industry (Torres et al., 2014; Oliveira et al., 2017). The mycotoxins are substances that are produced by secondary metabolism of some fungi, inluding Fusarium species (Bennett and Klick, 2003).

In association with maize, there are different Fusarium species besides FV, like species that belong to FGSC (*Fusarium graminearum* species complex), *F. proliferatum, F. oxysporum, F. fujikuroi, F. equiseti, F. culmorum, F incarnatum, F. kyushuense,* and *F. solani*. Usually FV is within the most frequent species, as frequency above 40%. This species together with *F. proliferatum* can produce fumonisin-like mycotoxins (Zhou et al., 2018). Fumonisins type B (FB) are the most important produced by FV. The most abudants are FB1, FB2 and FB3, but FB1-type fumonisins are found at higher levels (70 to 80%), FB2 (15 to 25%) and FB3 (3 to 8%) as quantified *in vitro* (Rheeder et al., 2002). Moreover, in the interaction between maize-FV we have three possible results, such as (1) presence of symptoms in grains plus high levels of fumonisins; (2) presence of symptoms in grains plus low levels of fumonisins and (3) the production of toxins in visibly asymptomatic grains (Afolabi et al., 2007).

Climatic conditions during maize plant growth and insect damage are determinant factors for initial infection with FV. Once the plants are infected, the levels of fumonisins are regulated by stress abiotic factors, and the predominance of these factors will regulate whether this occurrence results in low or high levels of fumonisins. These factors act by activating the secondary metabolism of the fungus and can be linked to temperature conditions, water potential and use of fungicides (Marín et al., 2013; Cao et al., 2014). Furthermore, the fungicide applications also results (1) elimination of competing species by the same niche as Fusarium ssp. (2) affects the balance between the Fusarium species that

colonize the maize grain, thus predominating less sensitive species and later potential fumonisin producers, and (3) increase the activity of enzymes that act in the biosynthesis of fumonisin-producing genes (Edwards & Godley 2010).

All factors that allow the colonization, incidence and subsequent production of fumonisins by FV must be controlled, as these secondary metabolites have toxic effects in humans and animals (Medina et al., 2017). Epidemiological studies have been associated the consumption of fumonisins with the increased risk of esophageal cancer and more the susceptibility to HIV in humans. Also, defects have been detected in the neural tube, a structure that gives rise to the brain and the spinal cord, have also been associated with this fumonisins consumption. In animal feed, high incidences of fumonisins have been responsible for causing equine eucoencephalomalacia (ELEM) and porcine pulmonary edema, as well as several other adverse effects on animal health, such as weight reduction and immunotoxic relationship development (Wu et al., 2011).

In an attempt to reduce the risks of contamination by fumonisins, due to the inefficiency of management techniques in field conditions, mainly of chemical control with fungicides. In addition to the low quality of modern hybrids, biological control agents (BCAs) has been a promising alternative within FER integrated management. BCAs are also an efficient promise in the reduction of toxic products to the environment since they act in a more friendly way within the agroecosystem. The action mechanism of BCAs can act to reduce the content of fumonisins are per direct antagonism, competitive exclusion by niche occupation, production of secondary metabolites, hyperparasitism, and production of volatile organic compounds (Medina-Córdova et al., 2016; Medina et al., 2017). Bacillus species are promising for use with BCAs because produce antimicrobial compounds like lipopeptide antibiotics (Yánez-Mendizábal et al., 2012). Besides, has the ability to form endospores, an structure that to can endure adverse environmental conditions because has the mechanical strength, greater resistance to external factors such as desiccation, solar radiation and high temperatures (Martínez-Álvarez et al., 2016). Some authors have already proved the efficiency of isolates of *B. subtilis* protecting maize roots and acting to reduce colonization by FV (Cavaglieri et al., 2005). In addition to also reducing the levels of fumonisins type FB1 and FB2 by *B. amyloliquefaciens* in seed treatment (Pereira et al., 2009). In order to characterize bacterial and fungi networks and to reveal the particularly important taxa of phyllosphere microbiome in ears maize inside two different treatments (1) applying fungicide alone, and (2) applying fungicide tandem with *B. subtilis*. Both treatments in trials of field under pressure of artificial inoculum of FV. We evaluated (i) parameters of yield of maize

grains (ii) incidence of fungi in grains (iii) fumonisins content (FB1+FB2), (iv) number of copies present of FV, and (v) maize ear microbiome analyzes for understand the importance of use de BCAs tandem chemical fungicide.

2.2 MATERIALS AND METHODS

Experiment description

The 2-areas (area 1-A1 and area 2-A2) field experiments were conducted with four replicates from November 2016 to May 2017 at Muquem Farm in Universidade Federal de Lavras, Minas Gerais, Brasil. Where the differences between areas are location like A1 (-21°204'242"S, -44°980'322"W) and A2 (-21°11'26.1"S, 44°58'42,2"W). The A1 has been described with low attitude and normal level ground. While A2 has make up higher attitude and uneven terrain. In both areas (A1 and A2), the experiment was carried out on the no-tillage system as the only practice of soil revolving was the recommended fertilizing planting for maize, with the formulation 08-28-16 (NPK) with 500 kg per hectare. During fertilization, the soil was a scratched to receive 0,60 meter between the lines. Thus, the hybrid DKB PRO 2 (Dekalb Brazil) was seeded obtaining a final density after the germination of 70.000 plants per hectare. Each block was formed by four lines with maize plants at the proposed density and spacing. The length of each block was 5 meter, thus composing a total area of 9 m^2 (5 x 1,8 m) per block. The useful plot was composed of one by an area of 1,8 m^2 (3 x 0,6 m) aiming to remove all drift and border effects, thus composing only the two central lines (width) and with a one-meter retreat between the beginning and end of the plot (length). Cover fertilization was performed with 20-00-20 (NPK) with 450 kg per hectare in installment (first in V3 and second in V6). The control of weeds was done in a post-emergency, combining the systemic herbicides atrasine (2.000 g a.i. per hectare) and tembotriona (100 g a.i. per hectare) applying in two moments. The other cultural treatises were composed of the treatments of interest, with three treatments, applied in the phenological stages V9 and R1, composed of: T1 (control, two times water sprayed), T2 (fungicide azosxystrobin + cyproconazole sprayed in V9 and *Bacillus subtilis* sprayed in R1) and T3 (fungicide sprayed at both times).

Applying the treatments and inoculation-FV

As BCA was used a *B. subtilis* strain (BIOUFLA2) which was previously preserved in a peptone-glycerol medium under freezing. BIOUFLA2 was reactivated in YPD (yeast extract-

peptone-dextrose agar medium) and then the typical colonies were transferred to YPD liquid medium for 72 hours under 150 RPM shaking at 27°C. The extract obtained was diluted with water distilled to obtain a concentration of 108 CFU/mL. The fungicide used was a mixture of azoxystrobin + cyproconazole (Priori Xtra®, Syngenta Crop Protection). The application was performed with 300 mL/ha of the formulated ingredient. Before application to the leaves, in both treatments and periods of application, 0.5% of the mineral oil was added in the composition. The final volume applied for both BIOUFLA 2 and fungicide in the two periods of applyed was adjusted to the flow rate of 200 L/ha, and it was applied with the aid of a CO_2 compressor cylinder by sprinkling in a cone nozzle.

The FV strain of F425 origin (Collection of Embrapa Milho e Sorgo, Sete Lagoas-MG) described by Lanza et al., (2014) was reactivated in BDA (potato-agar-dextrose) and maintained in BOD for 7 days at 27 ° C. Petri plates with FV has grown were washed with distilled water and a suspension of 10^5 conidia/ mL was adjusted with the aid of Neubauer Camêra under optical microscope. The maize plants with 10 days after the stigma-style emission were inoculated with 5 mL of this strain per ear (Mendes et al., 2012).

Sample collection

The collections for the analysis of fungal and bacterial microbiome and the amount of FV DNA copies were performed in two periods. The first period was 7 days after inoculation with FV and the second period at harvest, ie when the water quantity of the grains reached 13% moisture (Figure 1). With the second collection also, the yield, weight of 1000 grains, number of rot grains, the weight of ear rot grains and incidence of fungi and fumonisin content (FB1 + FB2) were also analyzed. All these variables were analyzed from the grains harvested within the useful plot.

For analysis of the fungi and bacterial microbiome and qPCR, the will developed ears were sampled in three plants within the useful plot of each plot. A portion (20 mm^2 of diameter) in center of ear maize was used as the template and each maize plant was collected at two opposite points until it reached the ear. The plants that were once sampled were not considered for the following collections and the samplings were randomized. The collected samples were conditioned in ice until put in the freezer and later macerated with liquid nitrogen and freeze-dried for subsequent extraction of the total DNA.

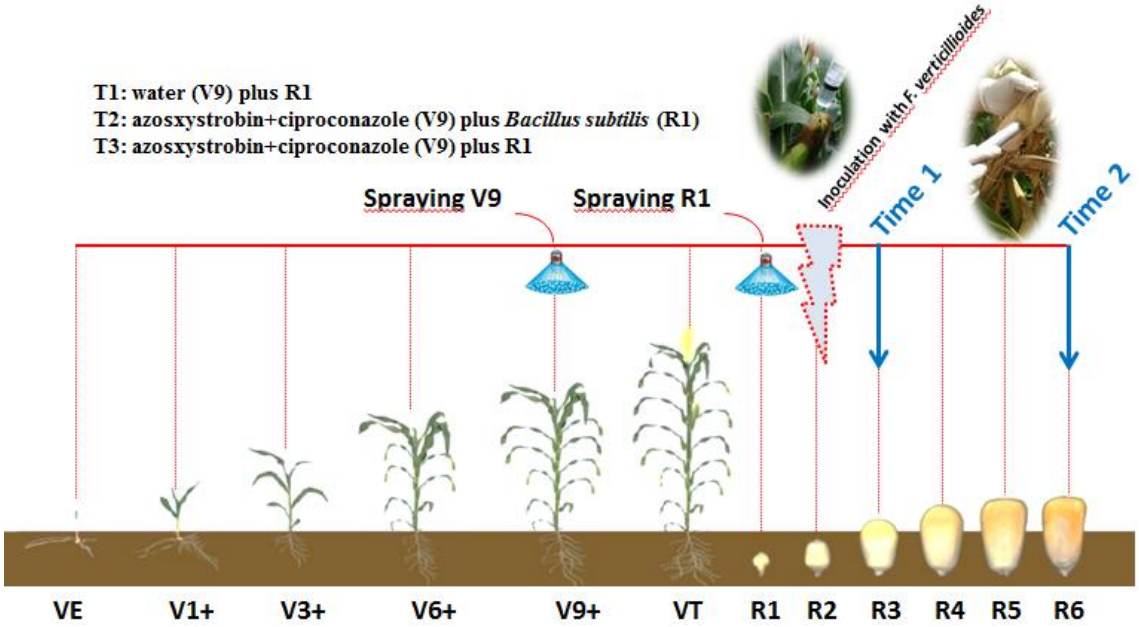

Figure 1. Phenological stages of maize plants showing the spraying (at V9 and R1) and samples collection (time 1 and time 2) at the treatments (T1, T2 and T3).

Productivity parameters

As grain yield parameters were analyzed, final yield was measured in ton/hectare (ton/ha) by collecting all the ears of the useful plot. The yield in kg was transformed to the redemption in one hectare (10.000 m^2). Besides, the weight of 1.000 grains was calibrated with manual counting and weighing (8 replications per variant) and adjusted for the basis of 13% moisture. With the same data used for 1000 grains weight was calculated also the number of ear rot grains (number) and the weight of ear rot grains (g).

Incidence of fungi on the grains

The incidence of fungi Fusarium ssp. and Stenocarpella ssp. on the grains harvested over was evaluated by Blotter test with freezing (BTF) as proposed in Manual of Sanitary Seed Analysis (Brasil, 2009). In total, 200 grains of each treatment were used. These were distributed over Petri dishes using 25 grains per plate. Before receiving the beans, each plate was lined with three layers of filter paper, moistened with sterile distilled water (incorporated with 0.2% agar). The grains were evenly distributed over each plate in order to avoid contact between them. The plates were incubated at 20 ° C and 12 hours photoperiod for 24 hours. Subsequently, they were frozen for 24 hours at -20 ° C, aiming to inhibit germination, killing the embryo by freezing. After this period they have incubated again at 20 ° C and 24 hours of photoperiod for 14 days. At the end of the exposure period, the percentage incidence of fungi of interest was measured using a stereoscope.

Total fumonisin content (FB1 + FB2)

For the detection of fumonisins content type FB1 and FB2 has been used the multi-mycotoxins technique used by Liquid chromatography-tandem mass spectrometry (LC-MS / MS) proposed by Sulyok et al., 2006. For the beginning, where twenty milliliters of acetonitrile/water/acetic acid (79: 20: 1, v / v / v) were used as the extraction solvent in 5 grams of maize grain samples from each plot. The methodology used for the extraction follows the same steps of Oliveira et al., 2016. Thus, the samples that have fumonisins content (FB1 and FB2) were quantified by analytes with a QTrap 5500 LC-MS / MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source and to 1290 Series HPLC System (Agilent, Waldbronn, Germany). In chromatographic process was separation by carried out at undertaken at 25 ˚C on a Gemini C18-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 x 140 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA) as also used by Oliveira et al. (2016). The fumonisins FB1 and FB2 were detected separately and the final total by sampling was summed up obtaining the total fumonisins content (FB1 + FB2) per plot.

Quantification of the fumonisin gene in total community DNA

In order to quantify fumonisin gene fragments in total DNA extracts, we applied the primer pair Verpro-F and VERTI-R as described by Waalwijk et al. (2008). The qPCR-based approach required a specific standard that was prepared by amplification of the Verpro-F/VERTI-R fragment from total DNA extracts and subsequent Sanger sequencing of the resulting fragment. The fragment's identity was confirmed by BLAST searches within the NCBInt (www.ncbi.nlm.nih.gov) collection. It was ligated into the pGEM®-T Easy Vector (Promega, Germany) and further processed as described by Bragina and colleagues (2013). Amplification-grade DNase I (Sigma-Aldrich, St. Louis, USA) treated total DNA extract were used to determine inhibitory effects of co-extracted substances. Based on this experiment, the total community DNA was diluted to 1:10 and target genes was amplified using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, USA). Two independent runs, with three replicates for each sample, were performed on the Rotor Gene 6000 (Corbett Research, Mortlake, Australia), using the following cycling protocol: 95 °C 5 min; 35 cycles of 95 °C for 25 s, 58 °C for 30 s, 72°C for 30 s and after the last cycle, as a final melt of double-stranded DNA, the temperature was ramped up to 95°C with steps of 1°C per minute. The

specificity of the amplicons was confirmed with both, melting-curve analysis and gel-electrophoresis of the qPCR products, respectively.

Statistical analyzes

In the field conditions the experiments were conducted in a randomized block design with four replicates per treatment. The statistical differences between the data of yield, weight of 1000 grains, number of ear rot grains, weight of ear rot grains and incidence of fungi and fumonisin content (FB1 + FB2) were calculated by analysis of variance (ANOVA) and values were compared by the Tukey test. Both evaluations were conducted at the significance level of 5% ($p \leq 0.05$). Analysis of the total microbiome of fungi and bacteria were reported as described below.

Total community DNA extraction

All samples were the freeze-dried and sent to a laboratory for further processing (Institute of Environmental Biotechnology, Graz University of Technology; Austria). Samples were manually ground with a sterile mortar and pestle in order to homogenize them. Subsequently, 0.5 g of each sample were weighted in and transferred to the next processing steps, while the remaining material was stored as a backup. The extraction of total community DNA was performed with the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol. After the procedure, the samples were analyzed with NanoDrop (Thermo Fisher Scientific, USA) to confirm the extraction efficiency. The DNA extracts were stored at -20 °C before further processing.

Amplification and barcoding of 16S rRNA gene fragments and the ITS region

The hypervariable V4 region of the 16S rRNA gene was amplified according to the protocol described by Caporaso et al., (2011) using the region specific primer set 515f/806r that included sample-specific barcodes. In order to avoid amplification of plant-derived mitochondrial and chloroplast DNA during amplification of bacterial 16S rRNA fragments, PCR reaction mixtures were supplemented with specific peptide nucleic acid (PNA) PCR clamps (0.75 µM of each PNA in the final reaction in a ratio of (pPNA:mPNA 1:1) as described by Lundberg et al., (2013). The PCR reaction mixture (30 µl) contained 1 × Taq&Go (MP Biomedicals, Illkirch, France), 0.2 mM of each primer, 1.5 µM PNA mix and 1 µl template DNA (96°C for 5 min, 30 cycles of 96°C for 1 min, 78°C for 5 s PNA annealing, 54°C for 1 min primer annealing, 74°C for 1 min, and final elongation at 74°C for 10 min).

PCR amplifications targeting the fungal ITS region were conducted using ITS1F (CTT GGT CAT TTA GAG GAA GTA A) and ITS2 (GCT GCG TTC TTC ATC GAT GC) primers carrying sample-specific barcode sequences (Schoch et al., 2012). DNA was amplified in PCR reactions (30 µl each) containing 0.9 µl MgCl (25 mM), 6 µl Taq&Go, 1.5 µl of 5 µM for each primer, 19.1 µl PCR water, and 1 µl of the DNA template with the following cycling conditions: 95°C, 5 min; 30 cycles of 95°C, 30 s; 58°C, 35 s; 72°C 40 s; and elongation at 72°C, 10 s).

For each sample and target, the PCR reactions were conducted in triplicates followed by pooling prior to purification of the amplicons with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Equimolar aliquots of all samples were pooled and sent for 300 bp paired-end Illumina HiSeq sequencing (GATC Biotech, Germany).

Bioinformatic processing

The sequences obtained from 16S rRNA gene and ITS amplicon samples were processed using scripts with default parameters provided by the open source software package QIIME 1.9.1 (http://qiime.sourceforge.net). Read pairs were joined (fastq-join; minimum overlap of 50 bases and maximum mismatch density of 0.25) followed by sorting according to primer sequences and sample-specific barcodes. Resulting reads were quality (Phred score ≥ 20) and length (290-300 bp) filtered. Chimeric sequences from16S rRNA gene amplicon samples were discarded after de novo detection using USEARCH 6.1 (Edgar et al., 2011). UCLUST algorithm using default parameters was applied to cluster remaining reads to operational taxonomic units (OTUs) at 97% similarity (Edgar 2010) followed by taxonomic assignment of representative sequences by RDP naïve Bayesian rRNA classifier (Wang et al., 2007) based on the reference database Greengenes release gg_13_8 (De Santis et al., 2006). Prior to further analysis, all OTUs assigned to plant plastids (chloroplasts and mitochondria) were discarded from datasets. Chimeric sequences from fungal ITS amplicon samples were identified with USEARCH 6.1 (Edgar 2011) based on reference sequences (sh_qiime_release_10.10.2017; UNITE Community, 2017). Subsequently, all chimeric sequences were removed from the data set. Reads were clustered to operational taxonomic units (OTUs) by applying reference-based UCLUST algorithm and taxonomically assigned using the blast algorithm and the UNITE reference data set as indicated above.

The beta diversity was assessed by computing of the Bray-Curtis distance metric within the QIIME pipeline. For the visualization, two-dimensional principal cooordinate analysis (PCoA) plots were generated for both datasets. For the network analysis, core OTUs

(threshold: 60%) were extracted for each treatment from both datasets. The integrated ‘make_otu_network.py‘ script was used to generate network files that were processed with Cytoscape v.3.6.1 (Shannon et al., 2003). Networks were rendered within this software environment with emphasis on the taxonomy of identified OTUs and their abundances.

2.3 RESULTS

Relation of productivity parameters

In relation to yield, when analyzed together, it is possible to observe that the treatment T3 (fungicide sprayed at both times) in area 1 (A1) is the one with the highest production values, with 19.68 tons / ha. And if we look between areas, this same treatment in area 2 (A2) is also the most productive among the others, producing 13.5 ton/ha. While T2 (fungicide azosxystrobin + cyproconazole sprayed in V9 and Bacillus subtilis sprayed in R1) produces A1 at 16 ton/ha and at A2 at 6.40 ton/ha showing no statistical differences with the control treatment (Figure 2A). Regarding the weight of 1000 grains, no treatment had any relevance within this variable, both between the areas and between treatments (Figure 2B). When analyzing the relationship between the weight of ear rot grains and the number of ear rot grains in the treatments and areas it is possible to observe that not always a larger number of burned grains result in greater or lesser weight grains or also the inverse relation with less number of burned grains can be observed (Figure 3).

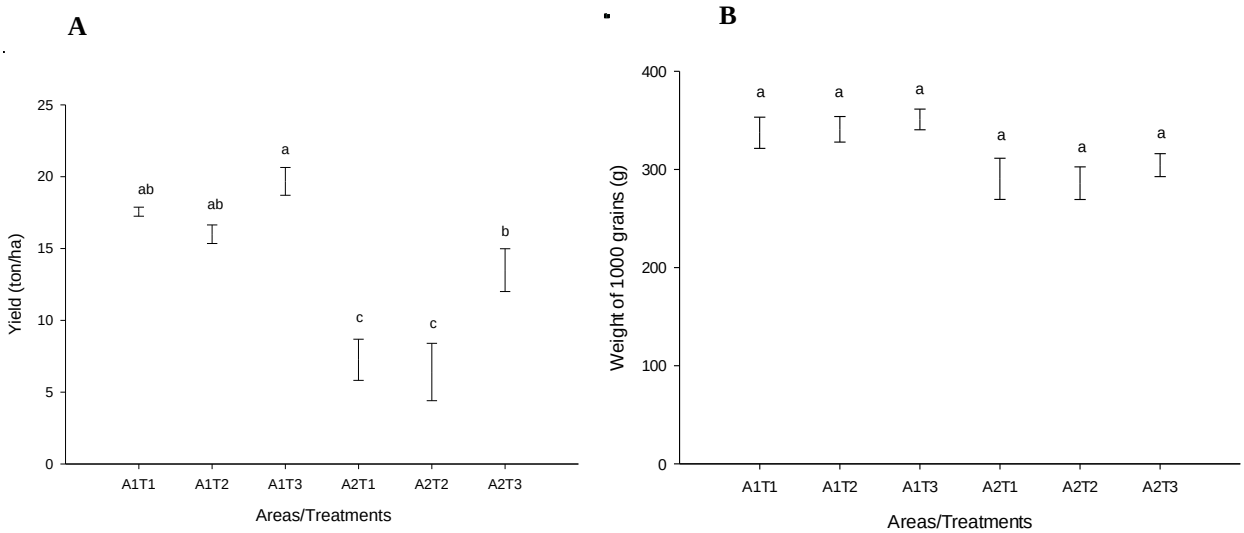

Figure 2. (A) Maize yield in ton/ha and (B) Weight of a thousand grains of maize in two locations (A1 and A2) into the treatments (T1, T2 and T3). Where T1: water (V9) + R1; T2: azosxystrobin+ciproconazole (V9) + *Bacillus subtilis* (R1) and T3: azosxystrobin+ciproconazole (V9) +R1. *Averages followed by the same letter don't statistically different by the Tukey test (p <= 0.05).

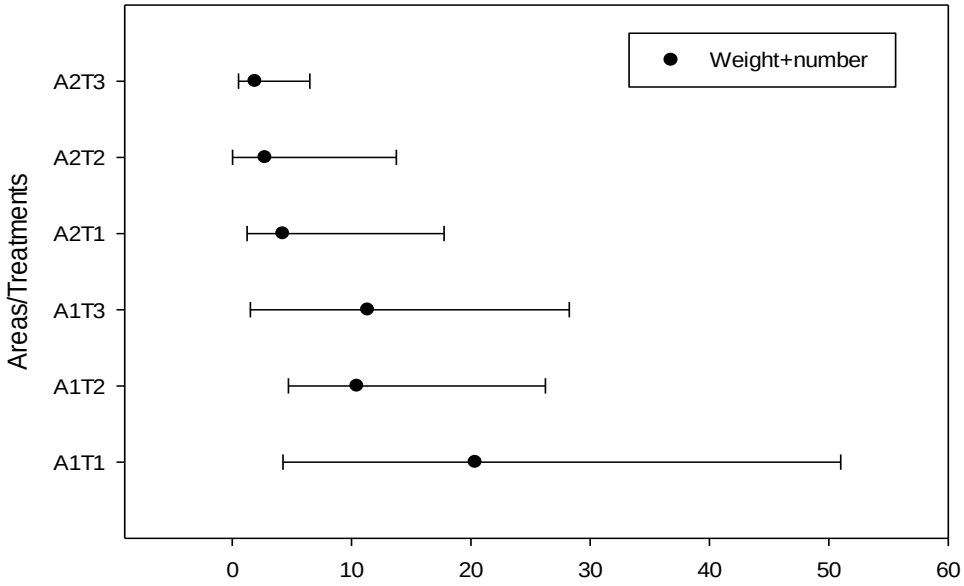

Figure 3. Relation between Fusarium ear rot: weight of ear grains versus number of weight in ear rot grains in two locations (A1 and A2) and three treatments, T1: water (V9) + R1; T2: azosxystrobin+ciproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin+ciproconazole (V9) +R1.

Qualitative analyses of maize grains

In the survey of fungi associated to grains from the different areas and treatments through blotter test, the fungi were *Fusarium* ssp. and *Stenocarpella* ssp and the ones found in all treatments and therefore they were considered for statistical purposes and no difference between areas or treatments was found (Figura 4).

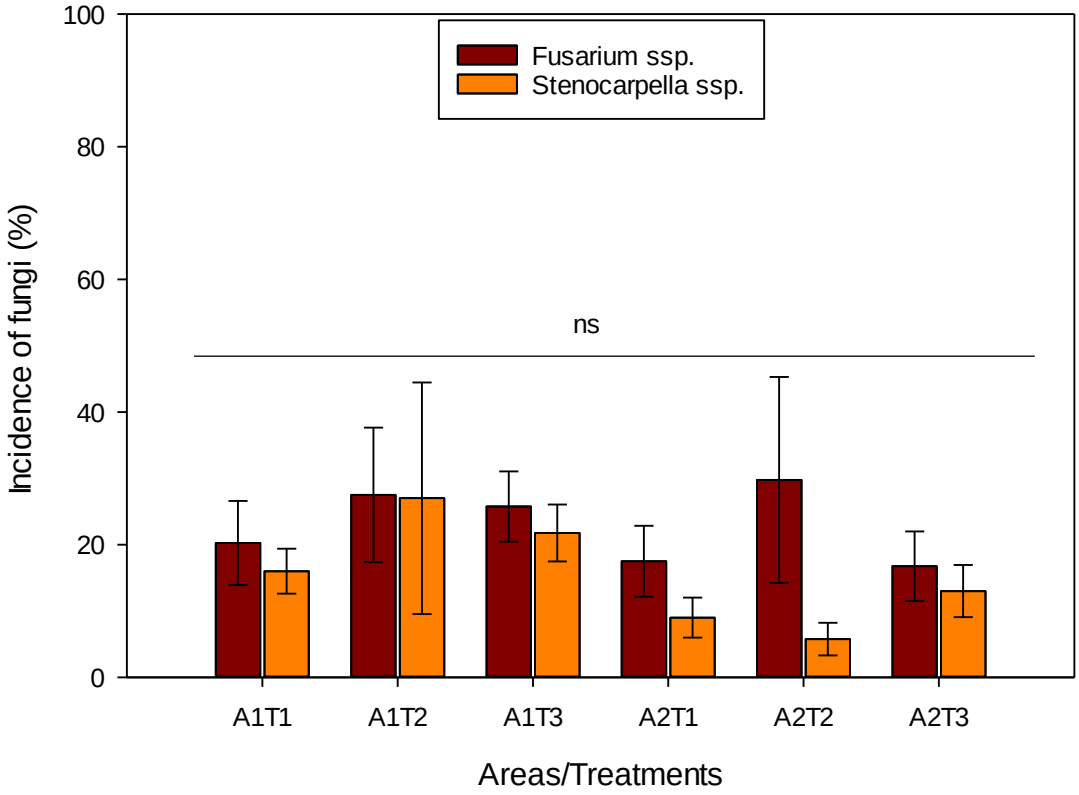

Figure 4. Incidence of *Fusarium* spp. and *Stenocarpella* spp. on maize grains under treatments (T1, T2 and T3) in two locations (A1 and A2). * **ns** shows that there was no significant difference between treatments.

Total fumonisins content

In the quantification of total fumonisin content (FB1 + FB2) in both areas with the described method, the T3 treatment with two fungicide sprays was the one with the highest levels (3.21 ppm) differing from the other treatments as the most important to promote the production of the mycotoxin. However the treatment of the sequential application of the fungicide with *B. subtilis* (0.41 ppm) resulted in similar level (0.91 ppm) of the mycotoxin accumulation for the untreated control plants (Figure 5).

qPCR of the fumonisin gene

In relation to fumonisin gene predictor quantification, it was possible show that at the time of the first grain sampling (as described in "sample collection" item), the DNA levels was higher in T1 (control) and T3 (both applied with fungicide) treatments for the two areas. While T2 (fungicide plus *B. subtilis*) was the one that resulted in lower DNA concentration. This same pattern was repeated in the second sampling (Figure 6).

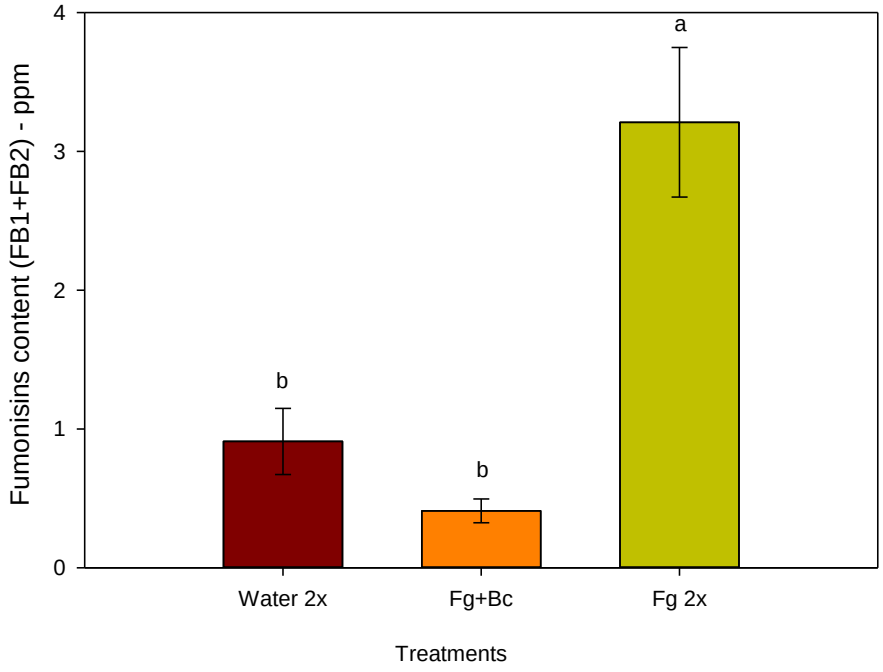

Figure 5. Fumonisin content in ppm (type FB1+FB2) in maize grains according to different treatments (Water 2x, Fg+Bc and Fg 2x). Where **Water 2x**: water (V9) + R1; **Fg+Bc**: azoxystrobin+ciproconazole (V9) + *B. subtilis* (R1) and **Fg 2x**: azoxystrobin+ciproconazole (V9) +R1. *Averages followed by the same letter don't statistically different by the Tukey test (p <= 0.05).

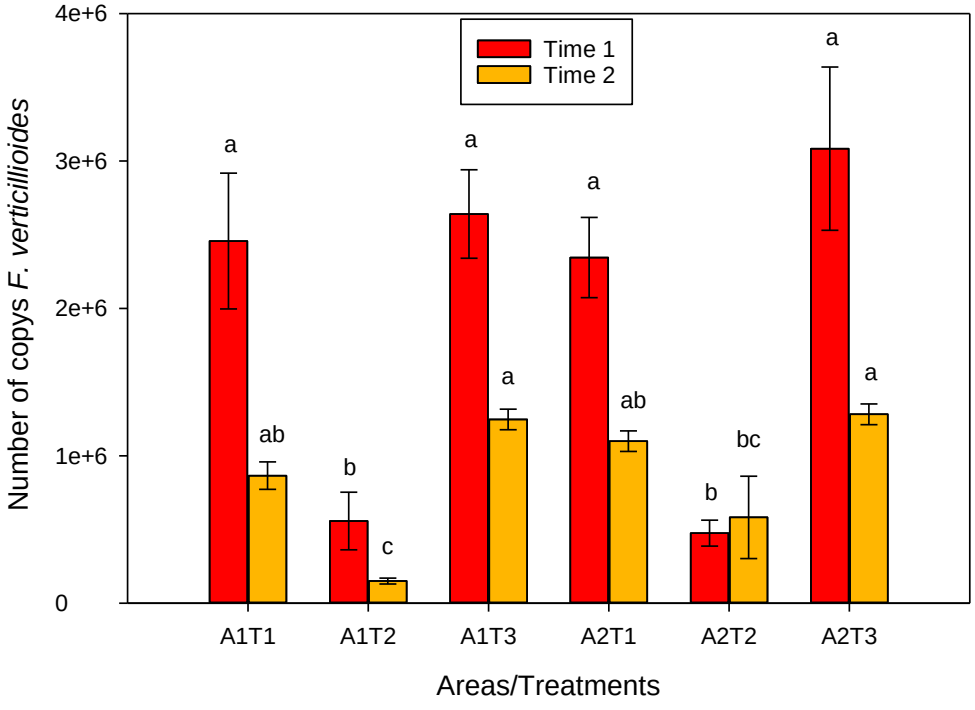

Figure 6. The number of copies of fumonisin gene in total DNA into two locations (A1 and A2) into the treatments (T1, T2 and T3). Where T1: water (V9) + R1; T2: azoxystrobin+ciproconazole (V9) + *B. subtilis* (R1) and T3: azoxystrobin+ciproconazole (V9) +R1. Also, the time for collection of samples in two times (time 1 and time 2). *Averages followed by the same letter don't statistically different by the Tukey test (p <= 0.05).

Bacterial community structure

Following quality filtering and removal of chimeric and plastid sequences, the 16S rRNA gene amplicon data set including 48 samples comprised a total read count of 7,275,437 reads with 10,637 to 509,978 sequences per sample (median 104,789; mean 151,572). UCLUST-based OTU picking at 97% similarity resulted in 23,844 OTUs. The maize plants were predominantly colonized by *Proteobacteria* (72.2% of the total reads) when the bacterial community was assessed at phylum level. Other abundant phyla were identified as *Bacteroidetes* (15.6%), *Firmicutes* (5.4%), and *Actinobacteria* (4.7%). At class level *Gammaproteobacteria* (40.4%), *Betaproteobacteria* (16.4%), *Alphaproteobacteria* (14.9%), *Flavobacteriia* (7.3%), and *Sphingobacteriia* (5.1%) were the most common lineages. When the community was assessed at order level (Figure 7), *Enterobacteriales* (24.4 %) were the most common lineage followed by *Burkholderiales* (15.9%), *Pseudomonadales* (9.3%), *Rhizobiales* (8.3%), *Flavobacteriales* (7.3%) and *Sphingobacteriales* (5.1%). The main bacterial families were assigned to *Enterobacteriaceae* (24.4%), *Oxalobacteraceae* (6%), *Pseudomonaceae* (5.2%), *Sphingobacteriaceae* (5.1%), *Burkholderiaceae* (5%), and *Xanthomonadaceae* (4.9%). At genus level two unassigned OTUs that belong to the *Enterobacteriaceae* family were predominant with 10.1% and 6.3% of the total reads respectively. Other abundant genera were identified as *Pseudomonas* (5.2%), *Burkholderia* (4.6%), *Erwinia* (4.2%), *Sphingobacterium* (4.1%), *Ralstonia* (4.1%), and *Acinetobacter* (4%).

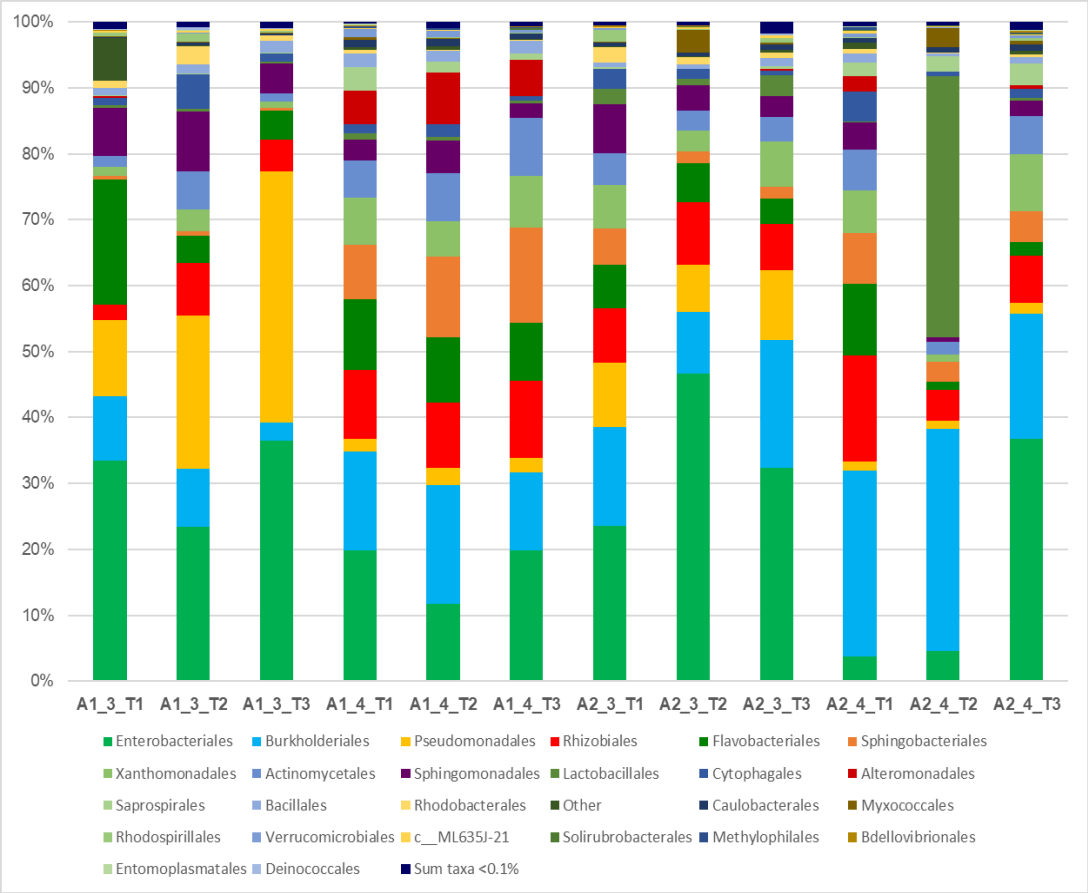

Figure 7. Taxonomic distribution of bacterial OTUs in treatments/times. Where: A1_3_T1, A1_3_T2 and A1_3_T3 are T1, T2 and T3 treatments collected at A1 and time 1; A1_4_T1, A1_4_T2 and A1_4_T3 T3 are the treatments T1, T2 and T3 collected at A1 and time 2; A2_3_T1, A2_3_T2 and A2_3_T3 are the treatments T1, T2 and T3 collected in A2 and time 1 and; A2_4_T1, A2_4_T2 and A2_4_T3 are the treatments T1, T2 and T3 collected in A2 and time 2. Where T1: water (V9) + R1; T2: azosxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin + cyproconazole (V9) + R1.

Treatment-induced changes in the bacterial community

General changes in the bacteriome were assessed with beta diversity analyses based on the Bray-Curtis distance metric. When the results were visualized in PCoA plots, no clear clustering of the samples was observed (Figure 8). Treatment-independent variations in the community composition were observed across all samples. In addition to the general assessment of differences in the bacterial communities, treatment-specific occurrence of OTUs was visualized in a network (Figure 9). This analysis led to the identification of various signature taxa for each treatment. In total eight unique OTUs were observed with the fungicide treatment in two times (T3), while the combined treatment, fungicide plus *B. subtills* (T2) had no unique OTUs at all. However, three distinct OTUs assigned to *Buchnera*, *Cupriavidus*, and *Leuconostoc* were shared only between the fungicide-treated plants and those subjected to the combined treatment. The OTUs that were solely found following the

the fungicide treatment, were assigned to the genera *Cellvibrio*, *Devosia*, *Flavobacterium*, *Leadbetterella*, *Pediococcus*, *Rarobacter*, *and Sphingobacterio*. One OTU remained unidentified at genus level. The treatment control (T1) had five unique OTUs, three remained unidentified at genus level, while one OTU was assigned to the genus *Burkholderia* and the other to *Pedobacter*.

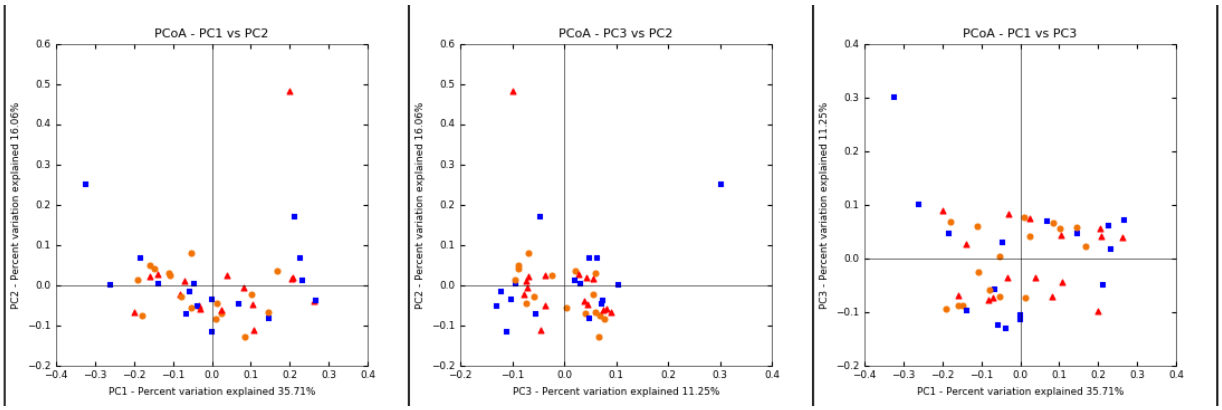

Figure 8. Beta diversity metrics of bacterial 16S rRNA genes among the phyllosphere microbiome of three treatments. Beta diversity community clustering was observed using Bray-Curtis distance metric. Each point corresponds to number sampling reported within each treatment, as T1 (red triangles), T2 (blue squares) and, T3 (orange circles) in three possibles PCoA plots. The percentage of variation can be observed by the plotted principal coordinates is indicated on the axes. Like in first and second PCoA variation rate in16,06% and last PCoA with 11,25%. Where T1: water (V9) + R1; T2: azosxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin + cyproconazole (V9) + R1.

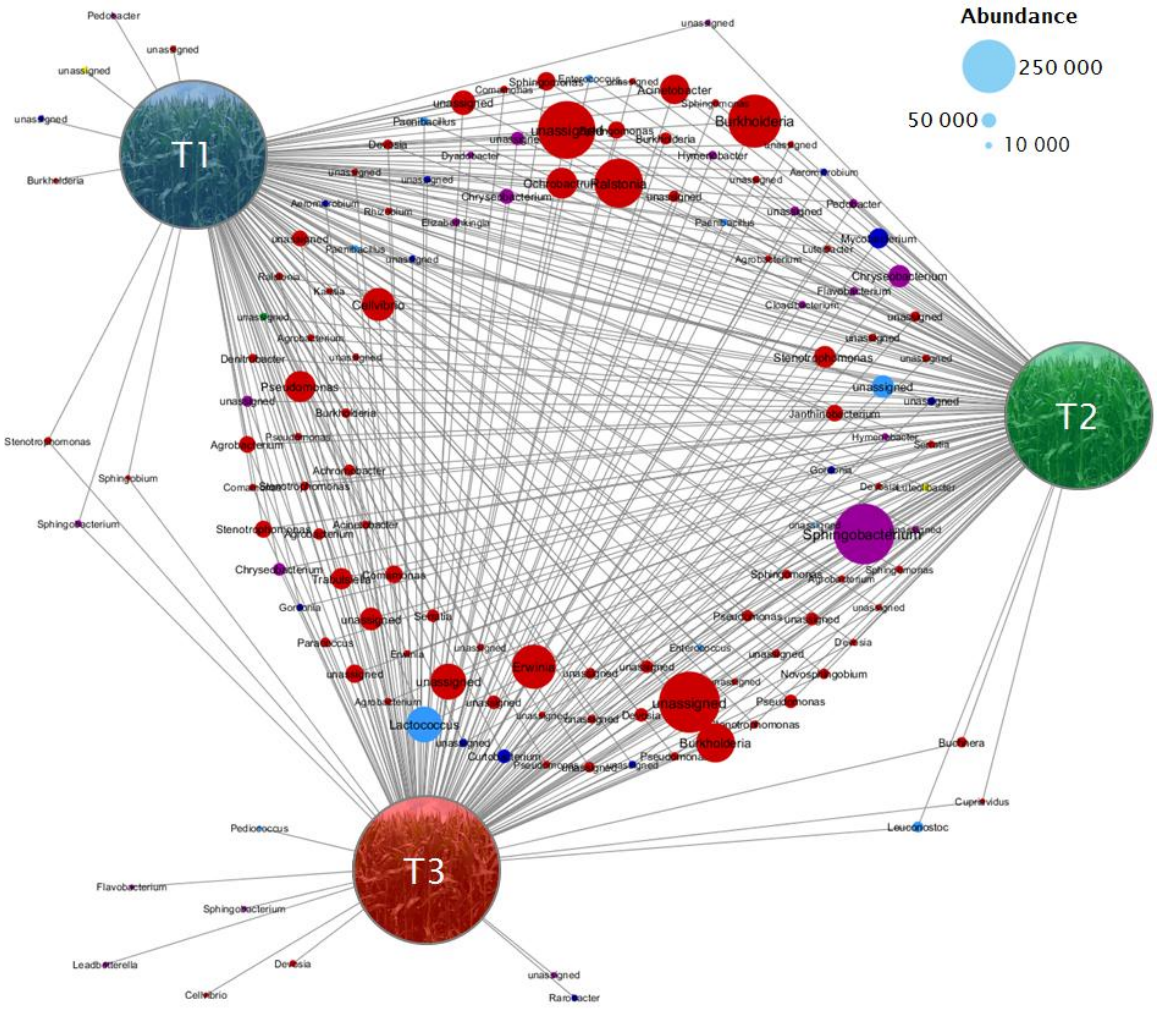

Figure 9. Co-occurrence network analyses of bacterial 16S rRNA genes processed by Cytoscape v.3.6 that show the taxonomy of identified OTUs and their abundances in three treatments, T1, T2 and, T3. Where T1: water (V9) + R1; T2: azoxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azoxystrobin + cyproconazole (V9) + R1.

Fungal community structure

Following quality filtering and removal of chimeric sequences, fungal ITS amplicon data set including 48 samples comprised a total read count of 7,093,161 reads with 39,485 to 309,719 sequences per sample (median 142,337; mean 147,774). UCLUST-based OTU picking at 97% similarity resulted in 4,212 OTUs. Taxonomic assignments in the fungal amplicon library showed that *Ascomycota* (96.3% of all reads) were the predominant lineage across all samples at phylum level. *Basidiomycota* (2.4%) were less abundant and 1.2% of the analyzed reads remained unassigned at phylum level. Within *Ascomycota* the fungal classes S*ordariomycetes* (44.9%), *Dothideomycetes* (37.8%), *Saccharomycetes* (5.7%), and *Eurotiomycetes* (4.7%). At order level, *Hypocreales* (34.5%), *Capnodiales* (30.2%), *Diaporthales* (7.4%), *Pleosporales* (6.4%), *Saccharomycetales* (5.7%), and *Eurotiales* (4.4%) were the most abundant lineages. The complete fungal community composition at order level

is visualized in Figure 10. Various highly abundant taxonomic groups with a total abundance between 7.4% and 29.4% were not assignable at family level. Assigned lineages included Nectriaceae (22.4%), Trichocomaceae (4.4%), Pleosporaceae (4.3%), and *Debaryomycetaceae* (3.1%).

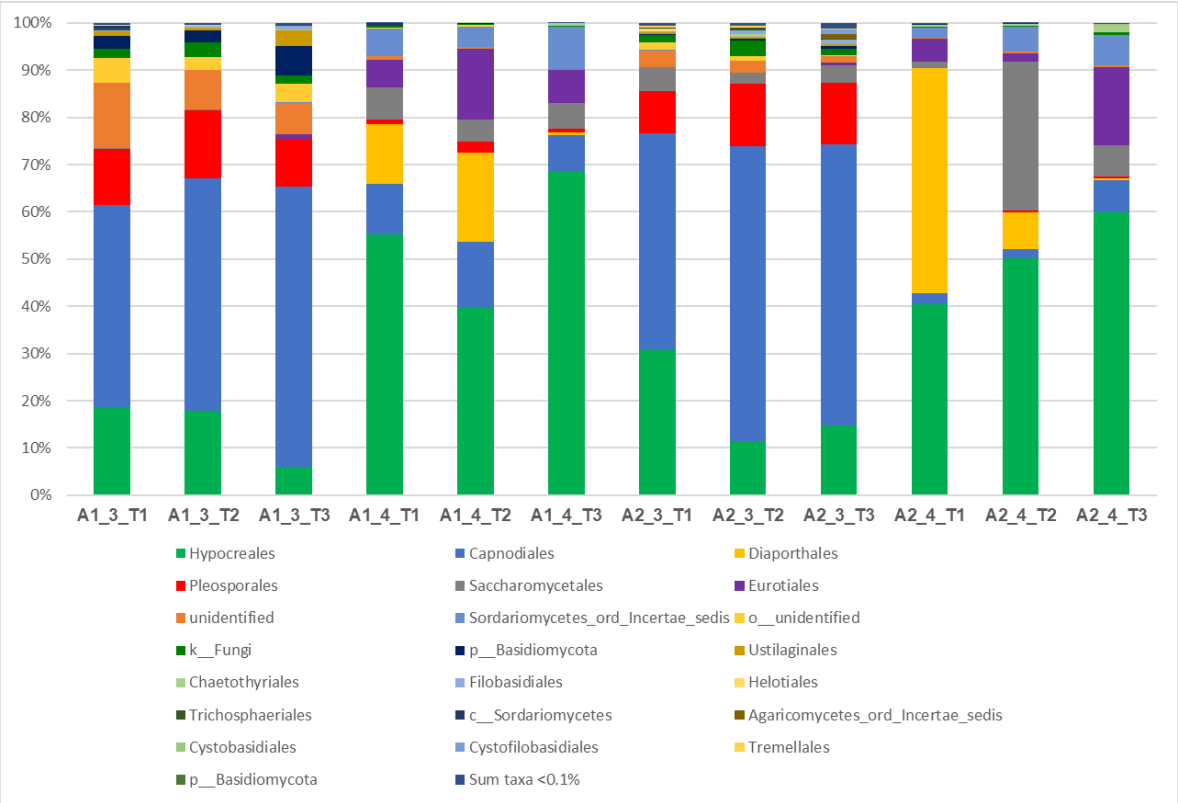

Figure 10. Taxonomic distribution of fungi by ITS region that show OTUs in treatments/times. Where: A1_3_T1, A1_3_T2 and A1_3_T3 are T1, T2 and T3 treatments collected at A1 and time 1; A1_4_T1, A1_4_T2 and A1_4_T3 T3 are the treatments T1, T2 and T3 collected at A1 and time 2; A2_3_T1, A2_3_T2 and A2_3_T3 are the treatments T1, T2 and T3 collected in A2 and time 1 and; A2_4_T1, A2_4_T2 and A2_4_T3 are the treatments T1, T2 and T3 collected in A2 and time 2. Where T1: water (V9) + R1; T2: azosxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin + cyproconazole (V9) + R1.

Response of the fungal community to different treatments

In order to assess effects of the treatments on the fungal communities in maize plants, beta diversity analyses based on the Bray-Curtis distance metric were implemented in the bioinformatic workflow. Visualizations with PCoA plots showed highly dispersed samples with no clear clustering (Figure 11), similar to the observations in the bacterial community. A network analysis was implemented to identify treatment-specific OTUs at family level due to the resolution of the ITS marker (Figure 12). Most fungal OTUs were found with the combined treatment of fungicide plus *B. subtilis* (T2). This treatment had 22 unique OTUs, and was followed by the fungicide two times treatment (T3; 19 unique OTUs), while the control group (T1) had only 10 unique OTUs. In the control treatment and the combined

treatment, the introduced OTUs were primarily assigned to members of the families Dothideomycetes and Sordariomycetes. Plants subjected to the combined treatment had one member of Tremellomycetes and Microbotryomycetes respectively. The fungicide sprayed in two times resulted in a more diverse composition of unique OTUs assigned to Cystobasidiomycetes, Dothideomycetes, Eurotiomycetes, Mortierellomycotina, Saccharomycetes, Sordariomycetes, and Ustilaginomycotina at a family level.

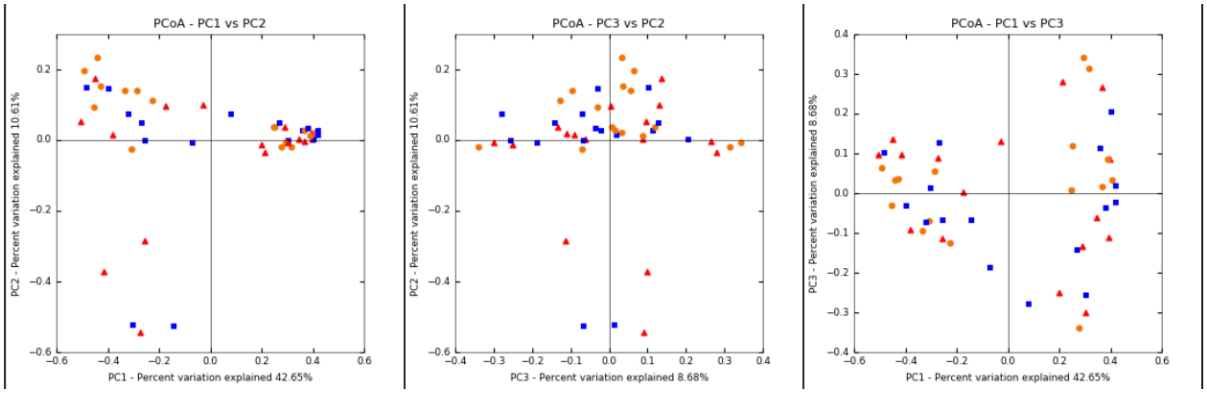

Figure 11. Beta diversity metrics of bacterial ITS genes among the phyllosphere microbiome of three treatments. Beta diversity community clustering was observed using Bray-Curtis distance metric. Each point corresponds to number sampling reported within each treatment, as T1 (red triangles), T2 (blue squares) and, T3 (orange circles) in three possibles PCoA plots. The percentage of variation can be observed by the plotted principal coordinates is indicated on the axes. Like in first and second PCoA variation rate in10,61% and last PCoA with 8,68%. Where T1: water (V9) + R1; T2: azosxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin + cyproconazole (V9) + R1.

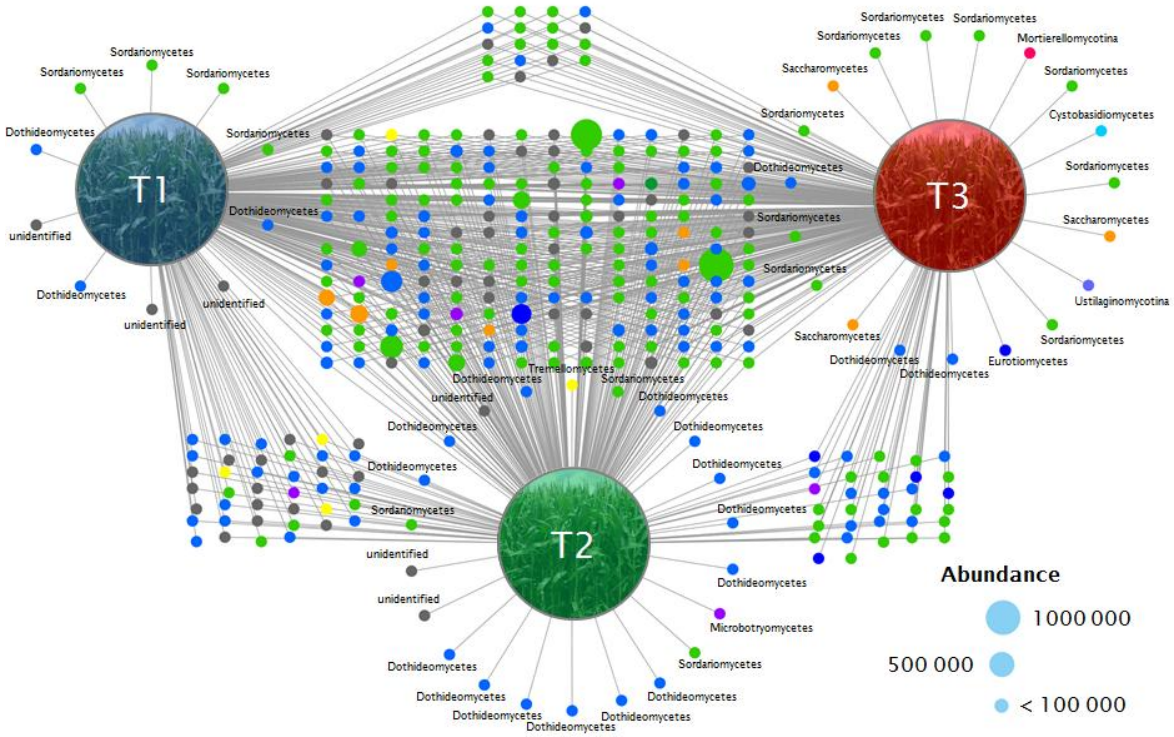

Figure 12. Co-occurrence network analyses of fungi ITS genes processed by Cytoscape v.3.6 that show the taxonomy of identified OTUs and their abundances in three treatments, T1, T2 and, T3. Where T1: water (V9) + R1; T2: azosxystrobin + cyproconazole (V9) + *B. subtilis* (R1) and T3: azosxystrobin + cyproconazole (V9) + R1.

2.4 DISCUSSION

The highest productivity observed in the system maize-FV-treatments-areas was no doubt with two foliar applications of the fungicide mixture (T3, azoxystrobin plus cyproconazole). The application of fungicide in modern hybrids is a dependency created by the plant breeding process, requiring at least one application to guarantee better yields (Shah et al., 2010). The use of fungicides is also an alliance to ensure better protection against foliar pathogens, a fact that is directly correlated with high levels of productivity, due to some fungicides mainly in the QoI group and DMI act by also increasing the photosynthetic rate (Blandino et al., 2012).

The weight of 1000 grains was not an efficient parameter to measure productivity when it comes to the use of the same maize hybrid. This variable can be controlled by the genetics of the material, thus not showing, as in this case, the influence or not of the application of foliar products (Lopes et al., 2007). It is also important to note that a greater or lesser number of ear rot grains in a treatment, do not guarantee greater or lesser weight. However, the levels of FV severity in the grains and also other damage factors, such as insects (Parsons and Munkvold

2010) and even mechanical damages in the harvest, are also responsible for not balancing this relationship (Tefera et al, 2011).

The presence of mycotoxins, in general, is linked to stress factors such as changes in the environment linked to biotic factors (like the microbiome neighboring) or abiotic factors (heat, pH, light and more). All these variables are linked to start of the secondary metabolism of many fungi and their initial biosynthesis is often associated with oxidative stress using reactive oxygen species (ROS) as pathways (Ponts et al., 2015). That reason, explains the value higher of total fumonisins (3.21 ppm) when there are two applications of fungicide (in T3 treatment). This value may be connected to a possible FV resistance against the active ingredients of the used fungicides (Hayes et al., 2014; Miguel et al., 2015) or an activation of biosynthetic pathways responsive to fumonisin production (Desjardins and Proctor 2007; Marín et al., 2013). Noteworthy the replacement of one fungicide application by *B. subtilis* is friendlier, reducing the total fumonisin production (0.41 ppm). Thus, we can infer that the second application is the most responsive to stress, maybe due to phenology coinciding with the critical period of incidence and colonization of the pathogen in the grains (Blacutt et al., 2018). This value is further reinforced by the presence of the artificial inoculation (mentioned in the item "applying the treatments and inoculation-FV").

Even though an isolate described as a fumonisin producer (Lanza et al., 2014) has been inoculated. It's possible to verify efficiency in the reduction of the dynamics of this gene in the treatment with the second application of *B. subtillis* in these evaluations. Proving the effectiveness of BIOUFLA2 (*B. subtilis*) strain and suggesting that to can been involved in colonization of the grains, competing for the same niche as FV, thus protecting maize grains and reducing colon colonization by FV (Cavaglieri et al., 2005; Pereira et al., 2007).

The next-generation sequencing (NGS) of fungi and bacterial communities that living in the leaves made it possible to understand the dynamics of the temporal and spatial variation, thus structuring and enhancing the interaction of microorganisms and their influence with the increase or reduction of disease severity (Rastogi et al., 2013). The phylloplane is a hostile environment for microorganisms due to the limited availability of nutrients and extreme environmental conditions such as variations in temperature and humidity, osmotic stress, ultraviolet radiation and nutrient limitation (Bulgarelli et al., 2013). These factors working together, often do not allow understanding the foliar interactions in communities because many times change very fast or are deleted in time.

The structure of the taxonomic distribution of the bacterial and fungal communities (Figure 7, Figure 10) represented by the OTUs is clearly affected by the treatments, by the

time of sampling and within the two analyzed areas. The order Enterobacteria is the largest representative of gram-negative pathogenic bacteria of maize, such as *Erwinia chrysanthemi* pv. *zeae* and *Pantoea ananatis*. The highest incidence of these bacteria is in T3 treatment (two fungicide applications) mostly when compared to treatment, time and area. It has been suggested that fungicide use may reduce diversity and temporarily alter the structure of the bacterial community. Thus, as already reported by Adetutu et al., (2008), the presence of azoxystrobin is present in the soil and acts interfering in the beneficiary communities. In addition, to acting increasing the number of pathogenic bacteria also (Xiuguo et al., 2009).

The β-diversity data presented by PCoA for fungi and bacteria (Figure 8; Figure 11) suggest that there are no clear clusters for this analysis, which means that the community displacements are induced by the treatments and were quite unspecific, is possible to observe a non-homegeneous distribution in the composition of each treatment. Thus, there was no variation in the identities of species among sites and treatments or a direct link between biodiversity at local scales (Anderson et al., 2011)

2.5 CONCLUSION

This work reports *Bacillus subtilis* strain (BIOUFLA2) as a potential biocontrol agent against *Fusarium verticillioides* a causal agent of maize ear rot. In addition, this work suggested that fungicide tandem with BIOUFLA2 treatments can influence the dynamics of microbial communities, in decreased of total fumonisins content and in the recruitment of friendly microorganism. As well, this treatment have a potential for insert of management integrated of plant disease, is a promising alternative. Therefore, further microbiome studies are necessary with new approach until going to a conclusion about relative species that occur so much bacterial and fungi.

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3 CHAPTER 2: Response-dose and biocontrol agents compatibility with fungicide in the integrated management of *Fusarium verticillioides*

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ABSTRACT

The use of more efficient practices in the management of plant diseases is necessary not only for better control of pathogens but also for less pollution and environmental contamination. Control of the *Fusarium verticillioides* fungus is a challenge because it is a pathogen normally present in all the maize producing areas of the world besides producing mycotoxins. Thus, the objective of this work was to evaluate the sensitivity of 20 isolates of *F. verticilliodes* to 10 fungicides (azoxystrobin, pyraclostobin, captan, thiabendazole, fluatriafol, carbendazim, propiconazole, tetraconazole, tebuconazole, and cyproconazole) in different concentrations (0; 0,1 ;1; 10 and 100 ppm). To evaluate the compatibility of 30 antagonists strains (fungi and bacteria) to azoxystrobin and cyproconazole in different concentrations (0; 0,1; 1; 10 and 100 ppm). Fungicides that showed the best control of mycelial growth was tetraconazole and tebuconazole againt population *F.verticillioides* at all concentrations. In the compatibility of the biological control agents, there was extreme, but there are within each group compatible isolates. Thus it is possible to integrate two worlds in the search for the control of plant diseases.

Keywords: sensibility of fungicides, micelial growth, biological control agents, compatibility.

3.1 INTRODUCTION

Plant disease management has long been carried out only with cultural practices such as fertility management, irrigation, and some physical and biological soil conservation practices (Gruhn and Yudelman, 2000; Winding and Rutgers, 2005). The doubling of the global demand for food gave rise to the 'Green Revolution' and thus doubled agricultural production in the last 50 years with the use of this advent. These tools have primarily resulted in higher productivity, due to increased use of inputs such as fertilizers and pesticides, and also the use of improved varieties and different technologies (Tilman et al., 2002, Carvalho, 2006).

These new production systems are very dependent on inputs, which increases production costs. In addition, with the intensive use of soil, they also present problems (McLaughlin & 1995). As for example, cereal production systems, which include two or three crops per year, have become progressively susceptible to diseases and pests because of the insufficient diversity in crop rotation (Nillor, 1996; Tilman et al., 2002). Thus, the production systems depend even more on the use of fungicides, insecticides, and herbicides to continue gaining high yields (Andert et al., 2016).

In the world maize production, many fungicides have already become inefficient for the control of leaves diseases, stalk rot and ear rot (Juliatti et al., 2007; Lanza et al., 2016). Intensive use and/or erroneous of some active principles use often makes pathogens few sensitivity or resistant to the presence these fungicides (Bartlett et al., 2002; Brent and Hollomon, 1995; Ronchi et al., 1997; Ward et al., 1997). Some fungicides, besides being ineffective in disease control, may be a stressing agent of mycotoxin-producing fungi, such as *Fusarium verticillioides* (D'mello et al., 1998; Falcão et al., 2011).

F. verticillioides is one of the most important pathogens in the maize crop, responsible for large quantitative and qualitative losses in the grains (Jurgenson et al., 2002; Bömke et al., 2008; Presello et al., 2008). This pathogen is mainly responsible for “pink ear rot” (Fusarium ear rot) as well as fumonisins, mainly type B1, B2, and B3 (Munkvold and Desjardins, 1997; Seo et al., 2001). Fumonisins have toxic effects on animals and humans when consuming high levels of content that are present in grains (Ross et al., 1992; Desjardins, 2006).

Nevertheless, the inefficiency of some fungicides within the integrated management of maize diseases, they are still necessary because some active principles are used in the basis of maize plant breeding (Wegulo et al., 1997). Many hybrids become so, mainly dependent for high yields just with the use of fungicides and yet effective against some leaf diseases (Ward

et al., 1996; Luna and Wise, 2015). An additional alternative to chemical control, biological control has shown promising results within the *F. verticillioides*-maize-fumonisins pathosystem, reducing the severity of Fusarium ear rot and fumonisin content (Cavaglieri et al., 2005; Luongo et al., 2005; Figueroa-López et al., 2016). Thus, the objective of this work was, (i) to evaluate the sensitivity of *F. verticillioides* strains to the main fungicides used in maize; (ii) To compare between 'old population' and 'new populations' the sensitivity of some fungicides to *F. verticillioides*; (iii) to evaluate the compatibility of fungicide with antagonistic fungi strains of *F. verticillioides* and (iv) to evaluate the compatibility of fungicides with antagonistic bacteria strains of *F. verticillioides*.

3.2 MATERIAL AND METHODS

Obtaining strains

Sensitivity of populations of *F. verticillioides* to fungicides

Twenty isolates of *F. verticillioides* (F1 - F20) from different maize producing regions of Brazil were used (Figure 1). The origin of the isolates is from Lanza et al. (2014), Collection Embrapa Environment and “Coleção Micologica de Lavras”.

Comparison between the sensitivity of new and old populations of *F. verticillioides* to fungicides

Eight isolates of *F. verticillioides* were used, four (F1, F3, F8 and F15) of the ‘new group’ (Figure 1) and four (source of “Coleção Micologica de Lavras (CML)”; CML 323, CML 766, CML 767 and CML 894) of the ‘old group’.

Compatibility of fungicides of fungi and bacteria isolates antagonistic to *F. verticillioides*

Strains of fungi (30 isolates) and bacteria (30 isolates) were used, 10 with high capacity, 10 with medium capacity and 10 with low capacity against antagonism with *F. verticillioides*. The strains originate from the maize phylloplane and were obtained from the combination of different leaf treatments (chemical and/or biological), previously classified by Pherez Perrony (2018) with an activity antagonistic against *F. verticillioides*.

Locality of origin of the strains

F1	Luís Eduardo Magalhães-BA	**F11**	Piracicaba-SP
F2	Belém-PA	**F12**	Rio Verde-GO
F3	Rio Verde-G0	**F13**	Boa Vista-RR
F4	Campos Novos-SC	**F14**	Sete Lagoas-MG
F5	Jaguariúna-SP	**F15**	Sinop-MT
F6	Londrina-PR	**F16**	Valença-RJ
F7	Manaus-AM	**F17**	Castro-PR
F8	Manduri-SP	**F18**	Campinas-SP
F9	Passo Fundo-RS	**F19**	Lavras-MG
F10	Perdizes-MG	**F20**	Picos-PI

Figure 1. Origin of the Brazilian isolates of *F. verticillioides* used in the test of relative mycelial growth with fungicides.

In vitro tests*:* preparation

The strains of *F. verticillioides* and antagonistic fungi were reactivated in BDA (Potato-Dextrose-Agar) synthetic medium and incubated for seven days in BOD (27 ° C/12 hours photoperiod). Petri plates of 90 mm received 10 mL of BDA medium at different

concentrations of the fungicides. In the culture middle of each plate was placed a 5 mm mycelial disk of each strain. The antagonistic bacteria were incubated in liquid NA (Agar-Nutrient) medium for multiplication under agitation of 200 RPM for two days.

Sensitivity of populations of *F. verticillioides* to fungicides

The inhibition of mycelial growth of the 20 isolates of *F. verticillioides* was done with 10 fungicides (azoxystrobin, pyraclostobin, captan, thiabendazole, fluatriafol, carbendazim, propiconazole, tetraconazole, tebuconazole and cyproconazole) in different concentrations (0; 0, 1; 1; 10 and 100 ppm) of the active principle alone and in three replicates. The control treatment (0 ppm) was the standard for the evaluation of the other concentrations, and when it grew throughout the plate, the relative mycelial growth of the other concentrations was evaluated. Means of vertical and horizontal mycelial growth were obtained in three replicates. The mean values of the relative growth data were transformed into boxplot (growth/concentration variation) and ANOVA (fungicide control efficiency/concentration) were also compared by Tukey's test ($p <= 0.05$)

Comparison between the sensitivity of new and old populations of *F. verticillioides* to fungicides

The evaluation of inhibition of mycelial growth of the eight ("new group" and "old group") isolated from *F. verticillioides* was done with three fungicides (azoxystrobin, tebuconazole and cyproconazole) in different concentrations (0, 0.1, 1, 10 and 100 ppm) of the active principle alone and in five replicates. The control treatment (0 ppm) was the standard for the evaluation of the other concentrations, and when it grew throughout the plate, the relative mycelial growth of the other concentrations was evaluated. Means of vertical and horizontal mycelial growth were obtained in three replicates. The averages of the relative growth data were submitted to ANOVA and compared by the Tukey test ($p <= 0.05$).

Compatibility of fungicides of fungi isolates with antagonistic action against *F. verticillioides*

The 30 strains of fungus antagonists was tested with two fungicides (azoxystrobin and cyproconazole) at concentrations of 0; 0.1; 1; 10 and 100 ppm during mycelial growth of five days. On the fifth day, the horizontal and vertical mycelial growth of the colonies was measured. With the control treatment (0 ppm) the percentage of mycelial growth of the other concentrations was obtained from the ratio [% growth / concentration = (100 * concentration

value to be calculated) / (control growth)]. Growth rate data means were transformed into boxplot between each level of the antagonists.

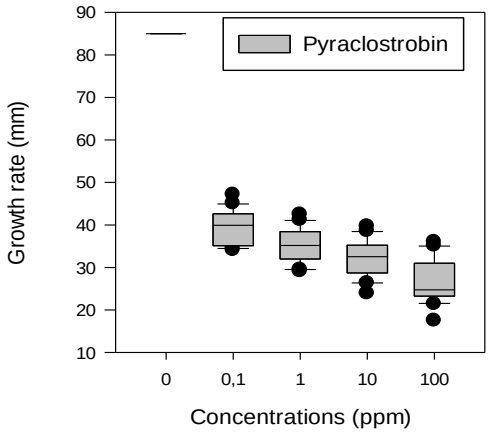
Compatibility with fungicides of bacteria isolates antagonistic to *F. verticillioides*

The 30 strains of antagonistic bacteria was tested with two fungicides (azoxystrobin and cyproconazole) at concentrations of 0; 0.1; 1; 10 and 100 ppm during the exposure time of 24 hours and 48 hours on Elisa plates. Thus, they were deposited in three replicates / bacteria/concentration in the following concentrations of fungicides 0; 0.1; 1; 10 and 100 ppm. With 100 µl of the fungicide concentration (at different concentrations) and 100 µl of the concentration of each bacterium (adjusted for 10^8 CFU) was received in each well of the plate. In the plates with 200 µl of final volume were readings with mass spectrometer at the wavelength of 490 nm, spectral value relative to bacterial mitochondrial activity. With the control treatment (0 ppm) the relative mitochondrial activity percentage was obtained by the relation [relative activity/concentration = (100 * value of the concentration to be calculated) / (mitochondrial activity of the control)]. The means of relative mycelial activity data were transformed into boxplot at both reading times (24h and 48h) between each level of the antagonists.

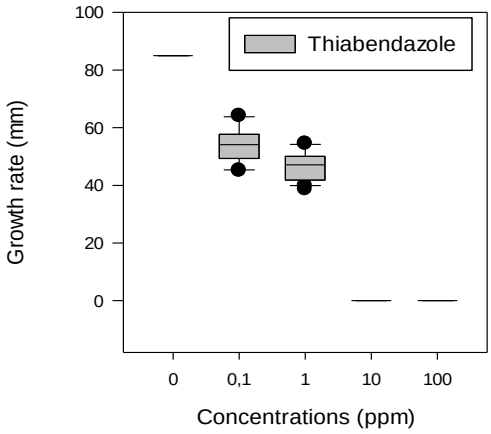
3.3 RESULTS

In the results of mycelial growth sensitivity of *F. verticillioides* to fungicides, the maximum growth value was 85 mm (Figure 2), value usually, relative to control (0 ppm). Piraclostrobin presented a dose-response effect, that is, with increasing concentration of the active principle there was an increase in the control of mycelial growth, but at the highest dose (100 ppm) there was still growth in all isolates. Thiabendazole at concentrations of 0.1 and 1 ppm reduced mycelial growth proportionally and at the dose of 10 ppm was able to inhibit the full growth of all isolates. Captan presented a similar behavior to the piraclostorin, but with lower variation among outliers. Fluatriafol also had a dose-response effect between the concentrations, reaching some isolate that at the highest concentration (100 ppm) completely sensitive. Carbendazim at the lowest concentrations (0.1, 1 and 10 ppm) is practically non-differentiated between the dosages, showing high insensitivity, but at the highest concentration it abruptly reduces mycelial growth in almost all of the group, and with outlier representative growing as the control at that concentration. Propiconazole has high control effectiveness and at the lowest concentration (0.1 ppm) it reduces the mycelial growth in relation to the control by half (0 ppm). Most isolates do not grow at the highest concentration, but outlier with low growth. Tetraconazole has high control efficiency, at the lowest concentration (0.1 ppm) it already reduces growth by more than half, in the concentration of 10 ppm it has low growth and in 100 ppm no isolate grows. Azoxystrobin has low efficiency at all concentrations and at the highest concentration inhibits just under half of the growth over the control (0 ppm). Cyproconazole shows greater reductions in mycelial growth at the concentration of 10 ppm, at the highest concentration (100 ppm), almost all of them isolated still have mycelial growth, but it is possible to observe an outlier zone. Tebuconazole is apparently the most efficient fungicide among all. At the lowest concentration (0.1 ppm) it reduces by more than half the growth in relation to the control, in 1 ppm it has low growth and at 10 ppm no isolate can develop mycelial growth (Figure 2).

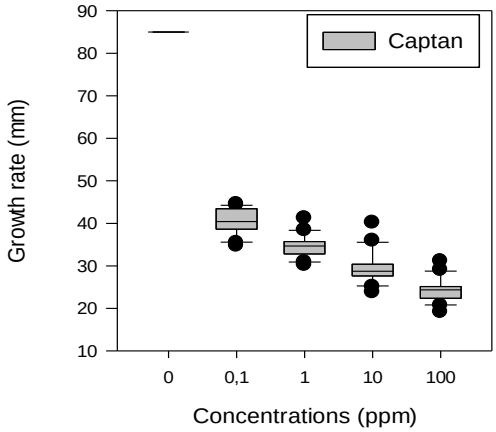
A


Pyraclostrobin
Growth rate (mm)
90
80
70
60
50
40
30
20
10
0
0,1
1
10
100
Concentrations (ppm)

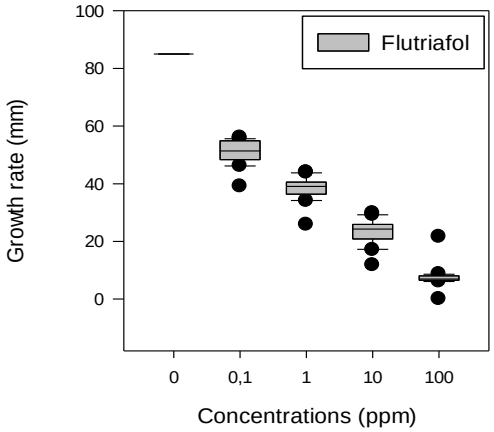
B


Thiabendazole
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

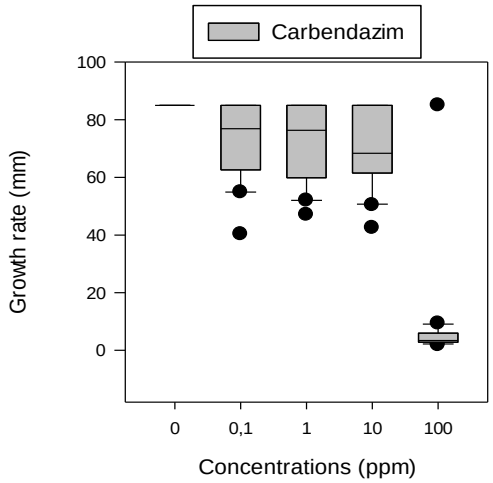
C


Captan
Growth rate (mm)
90
80
70
60
50
40
30
20
10
0
0,1
1
10
100
Concentrations (ppm)

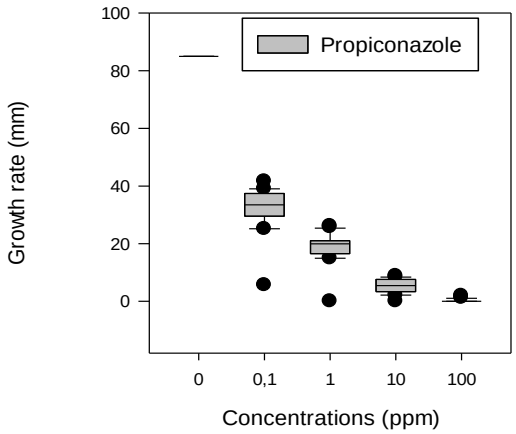
D


Flutriafol
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

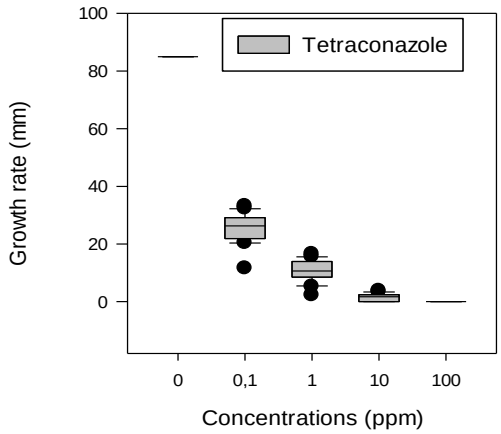
E


Carbendazim
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

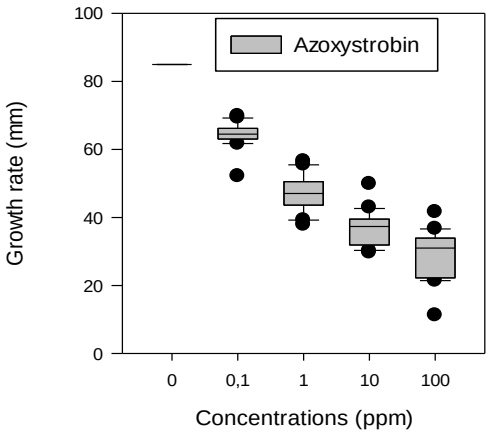
F


Propiconazole
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

G


Tetraconazole
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

H


Azoxystrobin
Growth rate (mm)
100
80
60
40
20
0
0
0,1
1
10
100
Concentrations (ppm)

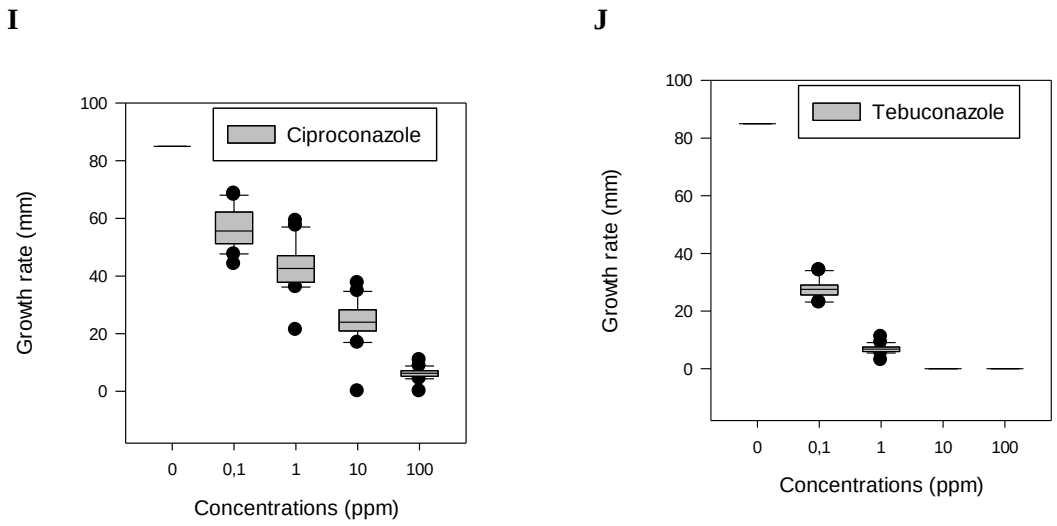


Figure 2. Micelial growth variation at concentrations of 0; 0.1, 1.0, 10 and 100 ppm of the fungicides (A) pyraclostobin, (B) thiabendazole, (C) captan, (D) fluatriafol, (E) carbendazim, (F) propiconazole, (G) tetraconazole, (H) azoxystrobin, (I) cyproconazole and (J) tebuconazole among *F. verticillioides* strains.

In relation to the effective control of the mycelial growth of the *F. verticillioides* population, the best fungicides tested were tetraconazole and tebuconazole (Table 1) for all concentrations, being better even at low concentrations (0.1 ppm) in relation to the other fungicides. Tetraconazole at the highest concentration (100 ppm) eliminates any growth of *F. verticillioides*. While tebuconazole at 10 ppm is also can able of doing it. Thiabendazole at low concentrations (0.1 and 1) does not have much effectiveness, but at the concentration of 10 ppm it limits any growth and in any isolate. The fungicides pyraclostrobin, captan, azoxystrobin and carbendazim are the least interesting in the use of *F. verticillioides*. Of medium behavior we have propiconazol, fluatriafol and ciproconazol which we could say are fungicides that reduce the severity of the disease, in this case mycelial growth.

Table 1. Efficacy of fungicides in the control of *F. verticillioides* in different concentrations and possible resistant strains.

Fungicide	0,1 ppm	1 ppm	10 ppm	100 ppm	Resistant*
Tetraconazole	25,59 a	10,77 a	1,56 a	0 a	-
Tebuconazole	27,74 a	6,92 a	0 a	0 a	-
Propiconazole	32,18 a	18,72 b	5,3 a	0,15 a	F17;F19
Piraclostrobin	39,18 b	35,31 c	29,55 cd	26,71 c	All
Captan	40,49 b	34,57 c	29,55 bc	24,21 c	All
Fluatriafol	50,92 c	38,32 cd	23,64 b	7,57 b	All, less F6
Thiabendazole	53,97 c	46,68 e	0 a	0 a	-
Ciproconazole	56,43 c	43,01 de	23,96 b	6,19 ab	All, less F6
Azoxystrobin	64,42 d	47,22 e	36,54 c	28,54 c	All
Carbendazim	72,44 e	72,34 e	70,94 e	8,11 b	All

***Growth above 100 ppm.

When we compare the new population, that is, the population that suffered more selection pressure with current use of fungicides. And the old populations, population less exposed to fungicide use. Due to the name, not so many fungicides are used in the maize crop (Table 2). We can observe that for azoxystrobin, that only the concentration of 10 pmm presents difference between the populations, being the new population considered more sensitive, with smaller mycelial growth. For cyproconazole there are no differences between populations. Tebuconazole at the concentration of 1 ppm has higher sensitivity of the old populations and at the concentration of 0.1 ppm this is invested for the new population.

Table 2. Mycelial growth in comparison between the sensitivity of new population and old populations of *F. verticillioides* to fungicides

Fungicide	Concentrations	New group		Old group	
		Mean (mm)	Range (mm)	Mean (mm)	Range (mm)
Azoxystrobin	100	66,52 **a**	53 - 76	65,45 **a**	58 - 73
	10	82,83 **b**	75 - 85	85 **a**	85 - 85
	1	85 **a**	85 - 85	85 **a**	85 - 85
	0,1	85 **a**	85 - 85	85 **a**	85 - 85
	0	85 **a**	85 - 85	85 **a**	85 - 85
Cyproconazole	100	6,55 **a**	5 – 9	7,41 **a**	0 - 10
	10	23,48 **a**	0 - 33	23,24 **a**	18 - 27
	1	48,96 **a**	32 - 58	48,96 **a**	23 - 66
	0,1	70,36 **a**	63 - 76	70,69 **a**	63 - 76
	0	85 **a**	85 - 85	85 **a**	85 - 85
Tebuconazole	100	0 **a**	0-0	0 **a**	0-0
	10	0 **a**	0-0	0 **a**	0-0
	1	22,63 **a**	18 - 26	20,92 **b**	17 - 23
	0,1	37,92 **b**	26 - 45	42,58 **a**	26 - 50
	0	85 **a**	85 - 85	85 **a**	85 - 85

The compatibility of the fungal isolates of *F. verticillioides* antagonists to azoxystrobin shows a number of combinations. At level 1 (best antagonist) the concentration effect clearly affects the average growth of biocontrol agents (Figure 3). This effect is reducing along the other two levels, being in level 3 (worse antagonist) where this effect is smaller. Even at level 1, up to the 10 ppm concentration, outliers that grow even at the control (0 ppm) are isolated, but at the highest concentration (100 ppm) the highest outlier growth is almost 60%. Level 2 (mean antagonist capacity) showed a better relationship, because in the highest concentration (100 ppm) there are outliers type isolates that almost reach maximum growth as control (0 ppm).

Level 3 (worst antagonists) presented a more constant relationship. Because of the low potential as a biological control agent and as high survival efficacy in the presence of azoxystrobin, it may be more likely to be equivalent to pathogens than promising biological control agents in integrated management.

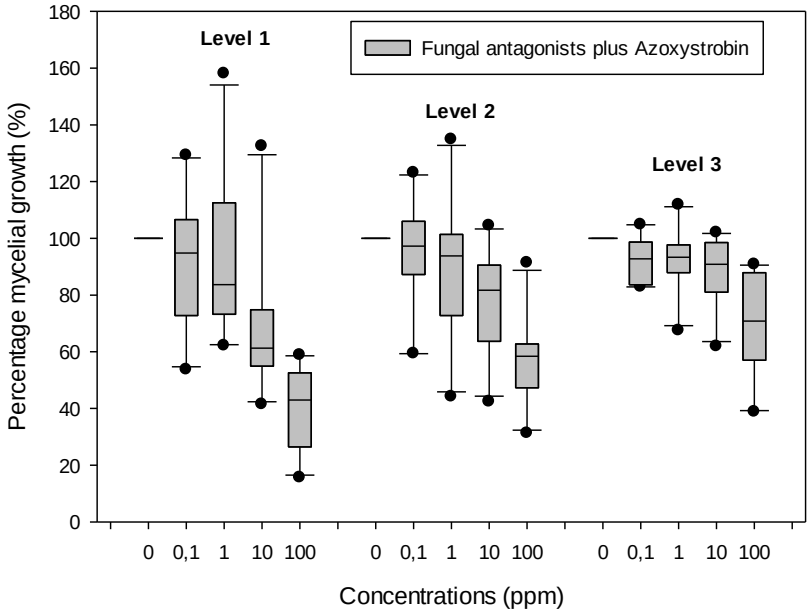


Figure 3. Percentage of mycelial growth of good (level 1), medium (level 2) and bad (level 3) fungus antagonists in azoxystrobin compatibility at different concentrations (0, 0.1, 1, 10 and 100 ppm). Black dots outside the distribution axes present the outliers.

In contrast to the isolates of fungal antagonists of *F. verticillioides* to ciproconazole (Figure 4), it is possible to observe in a contrary way that the isolates considered worse antagonist (level 3) have the growth compromised in the different concentrations without any outlier, showing in fact a control, not compatibility. At level 2 (mean antagonistic activity) this effect is reduced and at the highest concentration (100 ppm) some isolate achieves a growth relative to 50% of the control (0 ppm). At level 1 (best antagonists) a similar behavior is observed at level 2 with still isolated outliers at all concentrations. Still with growth above the control, less in the greater concentration (100 ppm).

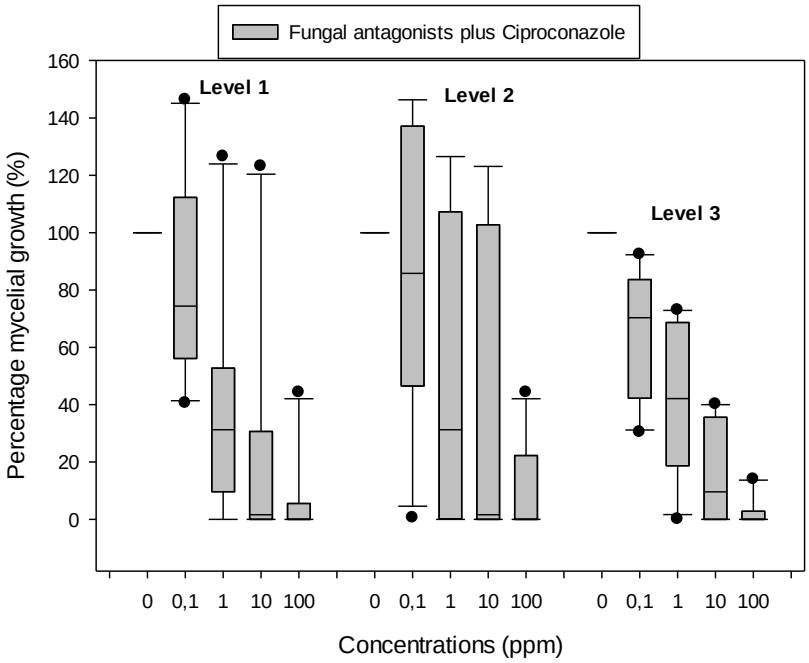


Figure 4. Percentage of mycelial growth of good (level 1), medium (level 2) and bad (level 3) fungus antagonists in cyproconazole compatibility at different concentrations (0, 0.1, 1, 10 and 100 ppm). Black dots outside the distribution axes present the outliers.

The mean activity of the antagonistic bacteria at 24 hours exposure is little affected in the presence of azoxystrobin (Figure 5A). Level 2 (medium antagonist) is the one that has the least effect, since the general average is above in all the concentrations. However, there are outliers at all levels, both for higher activity and lower, showing that some isolates may have some sensitivity and others may be stimulated in the presence of azoxystrobin. With longer exposure time of azoxystrobin to bacteria, for 48 hours (Figure 6), this effect is reduced in relation to 24 hours (Figure 5B). All levels and concentrations have averages of activity in the minimum equal to the control (0 ppm). At level 3 some outliers show that perhaps some bacteria, even in low concentration have not survived, a fact that may be a characteristic of the isolate not of the presence of the fungicide.

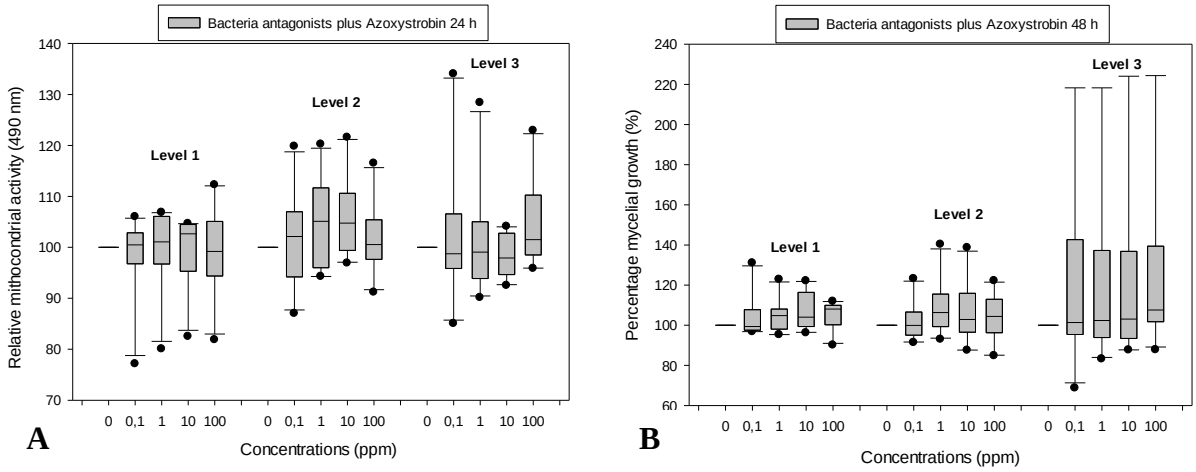


Figure 5. (A) Relative mitochondrial activity of good (level 1), medium (level 2) and bad (level 3) bacterial antagonists in cyproconazole compatibility at different concentrations (0, 0.1, 10, and 100 ppm) for 24 hours exposure and (B) Relative mitochondrial activity of good (level 1), medium (level 2) and bad (level 3) bacterial antagonists in cyproconazole compatibility at different concentrations (0, 0.1, 10, and 100 ppm) for 48 hours exposure. Black dots outside the distribution axes present the outliers.

The activity of cyproconazole-antagonistic bacteria in 24 hours of medium exposure is slightly altered (Figure 7A). At all concentrations and levels there are outliers with both high and low activity. However, this activity during 48 hours of exposure (Figure 8) is reduced, mainly at level 1, where the highest concentration (100 ppm) has a high activity reduction. Level 2 has few changes, while level 3 undergoes a random change, reducing in some isolated outliers sharply its activity. Also appearing to increase activity in some outlier isolates as at the 100 ppm concentration, which is almost at 160% and before (Figure 7B) was a little over 120%.

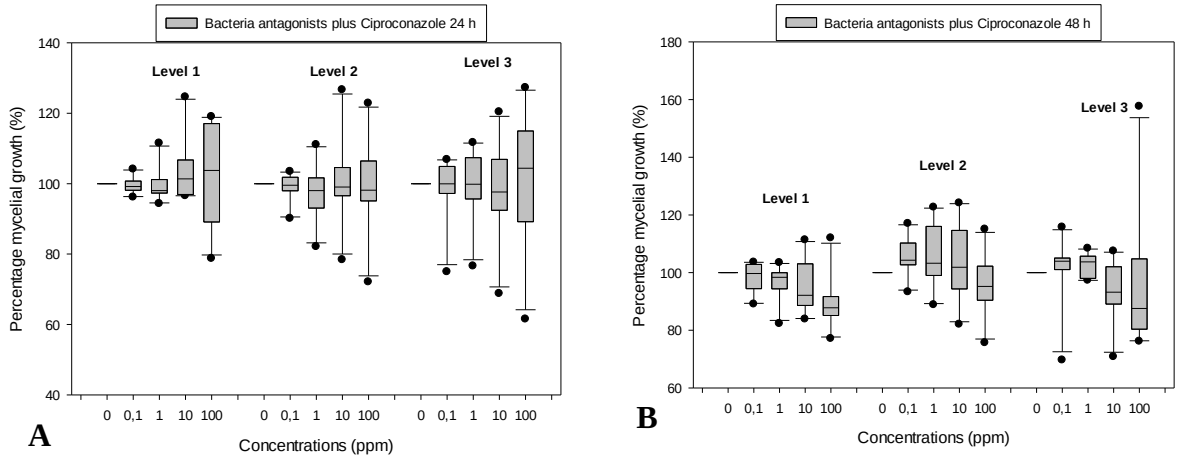


Figure 6. (A) Relative mitochondrial activity of good (level 1), medium (level 2) and bad (level 3) bacterial antagonists in cyproconazole compatibility at different concentrations (0, 0.1, 10, 100 and 100 ppm) for 24 hours exposure. (B) Relative mitochondrial activity of good (level 1), medium (level 2) and bad (level 3) bacterial antagonists in cyproconazole compatibility at different concentrations (0, 0.1, 10, and 100 ppm) for 48 hours exposure. Black dots outside the distribution axes present the outliers.

3.4 DISCUSSION

The insensitivity of *F. verticillioides* populations to fungicides, mainly from the group of strobilurins (QoI group) was the most observed in this work. In particular the active principle of azoxystrobin and not so much pyraclostrobin. This type of study on resistance and/or insensitivity has been increasing studying in different species of Fusarium, very little still with isolates of *F. verticillioides*. A model within this species is *F. graminearum* in wheat (Boyacioglu et al., 1992, Mesterházy 2002, Ramirez et al 2004, Haidukowski et al 2005, Chen et al., 2009, Al-Hatmi et al., 2014). The molecular mechanism behind the emergence of fungicide resistance among Fusarium species is still poorly known, due to the complexity of species and interactions within Fusarium ssp. Triazoles (inhibitors of ergosterol biosynthesis) have been highlighting concerns, as they are still more efficient products in the control. And prolonged use them in agriculture has raised concerns about the development of resistance in filamentous fungi like Fusarium ssp. Some studies have shown that some of the isolates from *F. graminearum* to tebuconazole (Spolti et al., 2012, Nicolli et al., 2018), which for *F. verticillioides* were also one of the most effective molecules together with tetraconazole.

Product compatibility within integrated disease management is a necessary feature, aiming not only to control disease, but also to manage the resistance of both chemical fungicides and biological fungicides (Omar et al., 2006). Several systems have shown compatibility in the integration of products (Fratel et al., 2005; McLean et al., 2001; Malathi et al., 2002). Thus, it is also necessary to adjust the doses used in fields, sometimes reducing the concentration so that the fungicides do not compromise the integrity of the biological control agents. In addition to evaluating the potential of some isolates also to act as pathogens, a fact that seems to happen at level 3 of the uncompetitive biological control agents (Figure 3, Figure 4) that may have been isolated endofically (Blacutt et al., 2018) interaction in the presence of fungicides. Behavior can be compared with that of *F. verticillioides* strains (Figure 2) in the presence of azoxystrobin and cyproconazole. The presence of outlier's isolates in the compatibility between the antagonists with fungicides shows that there is not a same relation for the entire group of antagonistic activity (level 1, level 2 and level 3), so it is interesting to study the behavior of each isolate separately applied in plants sprayed with fungicides and reisolating in various concentrations sprayed fungicides.

3.5 CONCLUSION

This work reports that there is some inefficiency on the most part of chemical fungicides in the control of *F. verticillioides*. And that this gap may be complemented by the use of biological agents compatible mainly in the use of azoxystrobin and cyproconazole. It was possible to observe that the sensitivity of the populations varied with the active principle and the dose of each product. When comparing populations between "old" and "new", there is a difference only in the active principle of tebuconazole. The antagonistic bacteria presented greater compatibility than the antagonistic fungi to the active principles of azoxystrobin and cyproconazole.

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4 CHAPTER 3: A look beyond the cost of production in the reduction of fumonisins content

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ABSTRACT

The systems of production in modern agriculture come every day presenting bigger problem of efficiency besides high costs. In the management of diseases of plants, the still more used form is the chemical control. However, some systems with maize-*Fusarium verticillioides*-fumonisins this form of handling has been incipient. Fumonisins are fungal toxins produced for *F. verticillioides* that contaminate many foods worldwide, such as maize. Thus, the objective of this work was to evaluate the insertion of a new control model, "Proposed System-PS" (replacing a chemical fungicide application with biofungicide application), compared to the "Conventional System-CS", that are most apply in all part of the country, with two fungicide applications. Within this two systems were analyzed the cost of production, the nutritional indices, the total fumonisins content and the effective losses. The production cost was obtained with market values of maize production inputs. Nutritional analysis was performed by the NIR technique. Fumonisins were quantified by the LC-MS / MS technique. The effective losses by fumonisins in the country were measured within each system starting from the value of total area of maize produced in the country. Thus, it was possible to conclude that CS is more effective in productivity and lower cost of production, with PS being more effective in the better nutritional composition and reduction of total fumonisins levels, with lower losses. It is possible to infer that within a human and animal health problem, not only production and low cost of production are efficient, but should be inserted a system that presents better quality, such as PS.

Key-words: *Zea mays* L; fungicide, *Bacillus subtilis*, losses.

4.1 INTRODUCTION

Brazil has the third largest area planted of maize (*Zea mays* L.) in the world, behind only the United States of America and China (CONAB 2018). However, we still do not have high production standards, mainly due to maize diseases (Acharya et al., 2017) insect attack (Lange et al., 2014) and environmental conditions (Mickelbart et al., 2015) unleashed by abiotic stress. At present, approximately 41% of the world's maize is planted in areas outside areas of high productivity, a fact that provides low yield, mainly due to interannual climatic variability (Ray et al., 2015; Tigchelaar et al., 2018). Thus, was harvesting 81.3 million tons were produced in Brazilian's land, while the largest USA producer was 371 million tons and China with 215.9 million tons in last crop 2017/2018 (CONAB, 2018a ; USDA 2018).

The economic importance of maize relies mostly on its use for animal feed but it is also intended for human consumption as an important source of energy and in the biofuels industry to make either biodiesel or ethanol. However, the major component of the use of this cereal is for the elaboration of animal food, representing about 70% of its total destination (Paes, 2006; Knothe et al., 2006). In the context of consumption, maize is a basic macro-ingredient in monogastric feed poultry, swine, and pets. The poultry industry accounts for 50% of the total maize demand (Pinazza, 2007).

The current scenario of grain production, demonstrate limitations in the production network of this cereal. These oscillatory characteristics compromise the potential of the sector as well as the activities that rely on the cereal production. Such risk factors that compromise the maize production chain encompasses: (1) factors governing yield intrinsic to the plant growth conditions; (2) factors governing grain quality loss due to grain contamination by fungi and specially by their toxic products, namely mycotoxins; (3) grower’s adoption of the most suitable crop management practices for the given region; (4) obscurity in the formation of prices, both realted internal and external markets; (5) breach of contracts; (6) precarious infrastructure for grain storage and, (7) logistical problems. All these factors may act together, discouraging maize production, reducing the preference for products of Brazilian origin and consequently impacting exports (Rasmussen et al., 2010; Caldarelli and Bacchi, 2012).

The maize feedstock is the main component in the manufacture of food. These should ensure good quality so that the formulations are within the nutritionally

stipulated standards. The non-standard feedstocks generate errors in the nutritional and mineral supply that lead to damages in the carcass losses, besides economic damage in the activity (Corte-Real et al., 2014). Many of these nutritional variations that compromise the quality of maize-based feeds come from the field, such as edaphoclimatic factors, hybrid technology, crop dealings, harvesting time, and storage conditions (Stringhini et al., 2000; Anfossi et al., 2010). Thus, the feedstock may suffer high or low depreciation depending on the field variables. In addition, all these factors act together on the physiological quality of maize grains, regulating the water activity inside the grains and even favoring ear rot (Freire et al., 2007). In summary, the poor physiological quality of the grains results in loss of performance of the animals, in the case of the poultry, this can be the result of the contamination by mycotoxins, mainly those of the fumonisin type. These fumonisins are secondary metabolites produced mainly by the fungus *Fusarium verticillioides* (Blacutt et al., 2018) which are responsible for producing mycotoxicosis in poultry, such as chemical and radiomimetic injury; cardiac toxicity; and skeletal, digestive, and reproductive disorders (Hoerr et al., 2017).

In economic terms, the cost components are grouped, according to their function in the productive process, in the categories of variable costs, fixed costs, operational cost, and total cost. Fixed costs are those that do not change in value in case of increase or decrease of production. They are therefore independent of the level of activity, also known as the cost of structure (CONAB, 2010). While, that of operational cost that is the cost of all resources that require the monetary disbursement by part of the productive activity for its recomposition, including the depreciation; and its purpose in the analysis is the decision option in cases where the financial returns are lower than the other alternatives, represented by the opportunity cost (Reis, 2007).

Thus, within the productive system, the management practices adopted are of great importance. Becoming another aggravating factor in the production of mycotoxins causing further the physiological depreciation of the maize grains. The biological control is disease management tool to be inserted within the maize production management system towards the reduction of mycotoxins (Chulze et al., 2014; Saravanakumar et al., 2017), whenever chemical control does not achieve satisfatory control (Falcão et al., 2011; Miguel et al., 2015) or even as a partner of the fungicide, to be used preventively to prevent the emergence of fungicide-insensitive pathogens in maize. Based on this, the objective of this work was to evaluate the

insertion of the biological control as a replacement of the chemical fungicide, thus evaluating two production systems: (1) Conventional system with two fungicide applications and (2) Proposed system, a fungicide application and a bio-product application based on *Bacillus subtilis* BIOUFLA2. Within each system, we have evaluated, (i) the production cost, (ii) nutritional quality, and (iii) losses by fumonisins.

4.2 MATERIALS AND METHODS

Description of maize grain production system

All the experiments were conducted in two different areas at the Universidade Federal de Lavras in crop 2016/2017. The values used (yield, production, nutritional quality and fumonisins content) refer to the averages obtained between the areas. In this context, two production systems of maize grains were analyzed. The first system, known as the conventional system is the most widely adopted system in most of Brazil and consider the exclusive adoption of fungicide as sole plant protection strategy, based on two fungicide applications. The second system or proposed system also relied on the fungicide application for the first plant spray with a replacement of the second application by the biological control product based on *Bacillus subtilis* BIOUFLA2. All the other agronomic practices for the grain production are described in table 1.

Table 1. Agronomic practices adopted in our trials for maize production, based on Borém et al. (2018) and IMEA (2017).

Sequence	Production inputs	Application time (phenological stage)	Quantity used	Product name
1	Plant Fertilizer	Before planting	500 kg/ha	Formulated fertilizer (08-28-16)
2	Seeds of Mayze Hybrid	Planting	70.000	DKB390 PRO2
3	Herbicide	V2	2L/ha	Atrasine

4	Herbicide	V2	200 mL/ha	Tembotriona
5	Cover Fertilizer	V2	225 kg/ha	Formulated fertilizer (20-00-20)
6	Cover Fertilizer	V4	225 kg/ha	Formulated fertilizer (20-00-20)
7	Herbicide	V5	2 L/ha	Atrasine
8	Herbicide	V5	200 mL/ha	Tembotriona
9	Inseticide	V8	125 mL/ha	Lambda-cyhalothrin+ chlorantraniliprole 1
10	Inseticide	V8	750 mL/ha	Imidacloprid
11	Fungicide	V9	300 mL/ha	azosxystrobin + cyproconazole
12	**CS** Fungicide	R1	300 mL/ha	azosxystrobin + cyproconazole
12	**PS** Biological control agent	R1	1L/ha	*Bacillus subtillis*

Production cost

Within each of the production systems was calculated the production cost. Total Variable Operating Cost was calculated by summing all inputs used in grain production, adding to it the cost of machine operations (Matsunaga et al., 1976). The yield value in tons/hectare, obtained within each system was adjusted by taking the percentage of rotten grains observed, resulting in the corrected yield (ton/ha). This value was multiplied by 60 kg (weight of maize bag) to have the productivity output (bags/ha). The average cost of the activity was obtained by dividing the Total Variable Operating Cost/productivity (bags/ha). Thus, the amount spent to produce a bag of maize within each system was calculated. The value of the inputs used for

the calculations was taken from the IMEA (2017) database based on the 2017/2018 harvest, as well as some used from local resellers.

Nutritional quality of maize grains

The nutritional values of maize grains in the proposed treatments (CS and PS) were analyzed through the Near Infrared Spectroscopy (NIR) technique. This is a near-infrared method that works in the 12,800 to 4,000 cm-1 range of samples. It is a rapid method of nutritional and non-destructive analysis of the samples. Infrared spectroscopy is used in this case for the quantitative analysis of organic components such as minerals, fibers and organic matter in maize grains (Gislum et al., 2004). The analysis of variance between the treatments composing with three replicates for each production system was carried out. The results were compared by the Tukey's test ($p <= 0.05$).

Quantification of total fumonisins and damages caused

For the detection of fumonisins content type FB1 and FB2, we used the multi-mycotoxins technique through Liquid chromatography-tandem mass spectrometry (LC-MS / MS). The methodology used for the extraction of the mycotoxin followed Oliveira et al. (2016). The detected values of each type of fumonisin were obtained individually and at the end summed FB1 plus FB2. The Brazilian legislation does not consider fumonisins content alone (BRASIL, 2011), thus the value of total fumonisins (FB1 FB2) was calculated for each sample. Using data from the total area of maize produced in Brazil in the two crops (CONAB, 2018), the values obtained of productivity, with more each yield systems and total fumonisins content were calculated, (i) the general estimate of losses by fumonisin (ii) losses relative in area (per hectare) by fumonisins, (iii) the relative percentage of losses by fumonisins and (iv) the losses ratio between CS/PS in systems by fumonisins. The cost of a mycotoxin in particular to a particular commodity group is calculated by assessing how much of the commodity should be discarded or discounted due to contamination. So, measuring the cumulative benefit of a particular intervention requires estimating how much mycotoxin levels are reduced as a result of the intervention and how much more of the commodity can thus be sold. The difference between the total market value of the commodity with and without the intervention is an estimated cost-effective problem (IARC, 2012).

4.3 RESULTS AND DISCUSSION

The total operating cost of the activity (ToCA) from the demonstrated values can be calculated, thus being denominated, [ToCa = (Total Variable Operating Cost - TVoC/ Productivity). Thus, we have the total operating cost of PS equal to 17.78 R$ and in CS equal to 11.90 R$. Thus, adopting a system for a value of $ 5.88 plus or minus does not represent profitability in an activity. The expensive price of Total Variable Operating Cost in the two systems is mainly due to the production inputs, such as fertilizers and the hybrid technology used (Pavinato et al., 2008, Galvão et al., 2014). This high value of production is caused by the high evolution of the maize production system, a fact common in modern agriculture, dependent on many agronomic practices adopted in the production systems. In regard to the price of the biological fungicide, it presents a slightly higher value than the chemical fungicide, due to the higher application rate than some systemic fungicides (such as azoxystrobin + ciproconazole). Thus, biofungicides, such as the one based on *B. subtillis*, act with microbial disrupters of pathogen cell membranes and induced resistance in plants (McGrath, 2004). While fungicides can be used to ensure better protection against foliar pathogens, a fact that is directly correlated with high levels of productivity, like QoI group (estrubirulins) and DMI (triazoles) act by also increasing the photosynthetic rate (Blandino et al., 2012). These conditions and the high dependence of the modern hybrids may have guaranteed higher productivities to CS than PS.

Table 2. Costing expenses in a no-tillage system "CONVENTIONAL SYSTEM (CS)" for maize production, based on Borém et al. (2015) and IMEA (2017).

DESCRIPTION UNITY	UNIT (SYMBOL)	AMOUNT	UNITARY VALUE (R$*)	R$*/ha
PRODUCTION INPUTS	-	-	-	**2.666,00**
Plant Fertilizer	kg	500	1,62	810
Herbicide 1	L	2	28	56
Herbicide 2	L	0,2	700	140
Maize Hybrid	bag	1	700	700

Cover Fertilizer	kg	450	1,54	693
Insecticide 1	L	0,125	300	37,5
Inseticide 2	L	0,750	200	150
Fungicide 1	L	0,6	132,5	79,5
MACHINE OPERATIONS				**562,14**
Planting			140	140
Herbicide application (V2)			21,43	21,43
Herbicide application (V4)			21,43	21,43
Cover fertilizer			15	15
Inseticide application	**h/M**	**1**	21,43	21,43
Fungicide application (V9)			21,43	21,43
Fungicide application (R1)			21,43	21,43
Harvest			300	300
Total Variable Operating Cost (R$)				**3.228,14**
Yield (ton/ha)				**16,6**
Ear rot grains (%)				**2**
Corrected yield (toneladas/hectare)				**16,27**
Productivity (bag/hectare)				**271,17**
Average cost (R$/bag)				**11,90**

*Real/R$ purchase price and payment in local currency **Quotation of the day (08/24/2018) for US Dollar 0,24 US$ EUR 0,21 € (for a convention in this financial currency multiply each of the values by the quotation described in US$ or €)

Table 3. Costing expenses in a no-tillage system "PROPOSED SYSTEM (PS)" for maize production, based on Borém et al. (2015) and IMEA (2017).

DESCRIPTION UNITY	UNIT (SYMBOL)	AMOUNT	UNITARY VALUE (R$*)	R$*/ha
PRODUCTION INPUTS	-	-	-	**2.686,25**
Plant Fertilizer	kg	500	1,62	810,00
Herbicide 1	L	2	28	56,00
Herbicide 2	L	0,2	700	140,00
Maize Hybrid	bag	1	700	700,00
Cover Fertilizer	kg	450	1,54	693,00
Inseticide 1	L	0,125	300	37,50
Inseticide 2	L	0,75	200	150,00
Fungicide 1	L	0,3	132,5	39,75
Bacillus subtilis	L	1	60	60,00
MACHINE OPERATIONS				**562,14**
Planting	h/M	1	140	140
Herbicide application (V2)			21,43	21,43
Herbicide application (V4)			21,43	21,43
Cover fertilizer			15	15
Inseticide application			21,43	21,43
Fungicide application (V9)			21,43	21,43
Bacillus subitilis (R1)			21,43	21,43
Hasvest			300	300
Total Variable Operating Cost (R$)			**3.248,39**	

Yield (ton/ha)	**11,2**
Ear rot grains (%)	**2,1**
Corrected yield (toneladas/hectare)	**10,96**
Productivity (bag/hectare)	**182,67**
Average cost (R$/bag)	**17,78**

*Real/R$ purchase price and payment in local currency **Quotation of the day (08/24/2018) for US Dollar 0,24 US$ EUR 0,21 € (for a convention in this financial currency multiply each of the values by the quotation described in US$ or €)

In relation to the nutritional analysis of maize grains, when statistically compared, there was no significance among the evaluated parameters ($p <= 0.05$) (Table 4).

Table 4. Nutritional comparison between systems CS and PS and relation rate between increased in number.

Nutritional variables	Conventional system (CS)	Proposed system (PS)	Better increase
Description (%DM)	%	%	-
Water content (%)	10.30	10.95	-
Dry matter (%)	89.69	89.05	-
%DM	%DM	%DM	-
Crude protein	9.65^{ns}	10.52^{ns}	-
Soluble protein %SB	46.22 ^{ns}	47.14 ^{ns}	-
Acid detergent insoluble N (ADIN)	0.26 ^{ns}	0.27 ^{ns}	-
Neutral Detergent Insoluble Protein (NDICP)	0.51 ^{ns}	0.51 ^{ns}	-
Neutral Detergent Fiber (NDF)	13.89 ^{ns}	12.88 ^{ns}	-
Organic Matter Insoluble in Neutral Detergent (OMIND)	13.21 ^{ns}	12.42 ^{ns}	-
Acid Detergent Fiber (ADF)	4.38 ^{ns}	4.41 ^{ns}	-
Lignin	0.56 ^{ns}	0.58 ^{ns}	-
Lignin % NDF	4.13 ^{ns}	4.48 ^{ns}	-
Starch	68,41 ^{ns}	69,27 ^{ns}	-

Lipids (EE)	3,57 [ns]	3,86 [ns]	-
Ashes	2,02 [ns]	2,01 [ns]	-
Calcium	0,02 [a]	0,02 [a]	-
Phosphor	0,45 [ns]	0,42 [ns]	-
Potassium	0,56 [ns]	0,54 [ns]	-
Magnesium	0,16 [ns]	0,15 [ns]	-
Sulfur	0,09 [ns]	0,10 [ns]	-

Among the final destination of grains, the feed for broiler chickens and laying hens are the ones that use this source the most. Thus, challenges related to animal nutrition are major pillars to increase poultry production in general. The obtaining of grains with values of digestibility and availability of nutrients can reduce the cost of production in this sector (Corte-Real et al., 2014). Thus, it is of great importance to investigate whether the raw materials are of good quality, guaranteeing the productive performance as well as the economic result of the activity. Essentially, due to the nutritional level be the main responsible for performance in the poultry industry (Fawcett and Webster, 1999).

Regarding the total fumonisin content present between the two production systems, a very discrepant value is observed between them. The CS with the mean fumonisins detected between 3210 μg/kg (ppb) or 3.21 ppm and the PS with 410 μg/kg (ppb) or 0.41 ppm (Table 5). In relation nutritional feed of animal, some losses at the end of the productive period of poultry have already been related to the use of poor quality maize, linked to the incidence of insects and fungi, which caused metabolic problems, often causing condemnation of the carcasses in the slaughterhouses (Stringhini et al. 2000).

In relation to maize products for human consumption, the levels detected in CS are above the allowable level for some foods, as observed in Table 5. This is because Brazilian legislation is more flexible in relation to more permitted levels than for example in USA legislation. In Brazilian conditions, that food with higher contamination (up to 5000 μg/kg) are foods that need to be pre-processed in order to reduce these levels (ANVISA, 2017). Under USA law, the largest allowable amount is 4 ppm or 4000 μg/kg (ppb). However within some groups as listed Brazilian legislation allows lower levels than the USA.

Table 5. Comparison of the total fumonisin allowed levels in Brazil and the United States with the values of each production system.

TOTAL FUMONISINS CONTENT OBTAINED (FB1+FB2)		
Conventional system (CS)	3210 μg/kg (ppb) / 3,21 ppm	
Proposed system (PS)	410 μg/kg (ppb) / 0,41 ppm	
MAXIMUM TOLERED LIMITS* (MTL) FOR FUMONISIN IN BRASIL		
Type food	Maximum Total Fumonisins μg/kg (ppb)	System accepted
Maize in grain for further processing	5000	CS/PS
Corn flour, corn cream, cornmeal, flakes, canjica, canjiquinha	1500	PS
Starch of maize and other maize products	1000	PS
FDA GUIDELINES FOR FUMONISINS IN HUMAN FOOD**		
Type food	Maximum Total Fumonisins (ppm)	System accepted
Degermed dry-milled corn products	2	PS
Whole dry-milled corn products	4	CS/PS
Dry-milled corn bran	4	CS/PS
Cleaned corn for masa production	4	CS/PS
Cleaned corn for popcorn	2	PS

*ANVISA (2017). **FDA (2014)

The physical, biochemical and bromatological qualities of maize may be related to the presence of mycotoxins, this type of impurity can alter the nutritional composition of maize, affecting the quality of the feed and causing loss of performance due to the lower bioavailability of some nutrients, due to the increase in fumonisins and your high incidence is considered being considered an anti-nutritional factor (Mazzuco et al., 2002; Moore et al., 2008; Pereira et al., 2008).

Losses occurring in the proposed cropping systems (CS and PS) per incidence of fumonisins can be extrapolated within the productive area (Table 6). It is possible to

observe that in the CS with the corrected yield of 16.27 ton/ha and mean total fumonisins of 3210 μg / kg, the prevalence within one hectare is 5.22 kg of maize grain contaminated with fumonisins. While in the PS of fumonisins of 410 μg / kg this value represents 0.44 kg of fumonisins contaminated with total fumonisins (FB1 + FB2). When comparing this estimate with the total area cultivated in the last harvest (2017/2018) of 16.7 million hectares, the CS accounts for 87.7 million of tons in losses, while the PS accounts for 7, 34 million of tons throughout the Brazilian area cultivated. The loss in cultivated area is 5.33 million hectares in the CSand 0.66 million hectares in the PS. Thus, this represents a percentage of 31.92% in the CS in relation to the total cultivated area and 3.95% in the PS. The established loss ratio between the systems is 8/1 (CS / PS).

Table 6. Estimation of fumonisin losses in Brazilian production within the production systems CS and PS

System	**Corrected yield (ton/ha)**	**Fumonisins content (FB1+FB2)- μg/kg**	**Fumonisins losses (kg/ha)**
Conventional system (CS)	16,27 ton/ha	3210 μg/kg	5,22 kg/ha
Proposed system (PS)	10,96 ton/ha	410 μg/kg	0,44 kg/ha
LOSSES IN TOTAL PLANTED AREA			
Brazilian crop area 2017/2018 (summer and winter crop) 16,7 Mha			
System/Losses	**General estimate of losses/ano**	**Lost relative in area (ha)**	**Percentage value**	**Relation**
CS – 5,22 kg/ha	87,17 Mton	5,33 Mha	31,92%	8,08
PS – 0,44 kg/ha	7,34 Mton	0,66 Mha	3,95%	1

In addition to the yield and productive damage, it is possible to investigate other types of damages with the presence of fumonisins, such as, export loss with the proposed formula "i, j, k = Pi * Wi, j * ri, j, k, "where i is the crop (eg maize, groundnuts); j is the country; k is the international standard mycotoxin (e.g.for fumonisin or aflatoxin); Pi is the world price for food crop per unit weight; Wi, j is the total export amount (in

metric tons) of crop i from country j; and ri, j, k is the fraction of export volume of crop i from country j rejected at international mycotoxin standard k. Damage to human health can also be measured, considering the economic damage of mycotoxins in health and its socioeconomic cost. Thus, it is possible to calculate the cost of the disease (COI), which is more appropriate in developed countries because a large part of the estimate is the cost of health care. The second is disability-adjusted life years (DALYs), which is appropriate for both developed and developing countries. A third metric, quality-adjusted life years (QALYs), is most commonly used to estimate the relative effectiveness of different public health interventions to improve overall quality of life. Both possible damages were proposed by IARC 2012 (International Agency for Research on Cancer) in the publication "Economics of mycotoxins: Evaluating costs to society and cost-effectiveness of interventions"

If both the market and the economic impacts of mycotoxins on health can be estimated, the cost-effectiveness of different interventions to reduce the risk of mycotoxins can then be assessed. As for example the insertion of the biological control agents (like *Bacillus subtilis*) aiming at the reduction of the levels in the productive system, as observed in this study. However, more observations at different levels and calculations of effective losses should be performed, aiming at the adoption of a system of crops that are sometimes less productive but with lower rates of recurrent mycotoxins.

Thus the occurrence of mycotoxins is an almost inevitable fact, due to the multiple actions that can generate its occurrence and make occurring high levels. The decision-making known as "door to inside" in field is unpredictable so that such damages can be reduced in the final product since this damage is irreversible.

4.4 CONCLUSIONS

The present study has shown that maize grain management system when replacing a chemical fungicide application with a biofungicide, the productivity levels were lower and the cost of production higher than in the conventional system (with two fungicide applications), but when the nutritional value and the fumonisin content in the grains are observed, these values are better in the proposed system (with the application of biocontrol agent). In addition, losses to the conventional system represent 8 times more than the proposed system (with biofungicide). Showing that,

the quality dynamics of the productive system should be taken into account no merely than productivity and cost in decision making.

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