ALINE VIEIRA DE BARROS

STENOCARPELLA STALK AND SEED ROT OF MAIZE AND THE ROLE OF MATING-TYPE GENES IN AGGRESSIVENESS OF *Fusarium graminearum* TO WHEAT

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração Fitopatologia, para obtenção do título de Doutora.

Dr. Eduardo Alves
Orientador

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2020

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APROVADA em 17 de Abril de 2020.

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LAVRAS - MG
2020

Aos meus amados pais, Sebastião e Rosani,

Aos meus irmãos e melhores amigos, Lívia e Davi

Ao meu companheiro de vida, Franklin

Dedico

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RESUMO GERAL

O fungo *S. maydis* (Berkeley) Sutton [Sin. *Diplodia maydis* (Berkeley) Saccardo] é um importante patógeno necrotrófico que causa tanto podridão de colmo quanto a podridão de espiga no milho. Similarmente, *F. graminearum* também é um patógeno importante do milho, causando a podridão de espiga orelha e de colmo de Gibberella (GER e GSR), mas também é um patógeno importante do trigo causando a doença conhecida como giberela (FHB). Essas doenças causam perdas econômicas significativas, não apenas devido à redução no rendimento de grãos, mas também podem reduzir o valor dos grãos colhidos devido à contaminação com micotoxinas. A presente tese investigou esses dois importantes patógenos, *Stenocarpella maydis* e *Fusarium graminearum* sendo estruturada em uma série de três estudos. No primeiro estudo, o objetivo principal foi estudar o processo infeccioso por *S. maydis* em colmos de milho utilizando diferentes técnicas de microscopia. Plantas de dois híbridos de milho, P30F53 e DKB 390 foram cultivadas em casa de vegetação e inoculadas com uma suspensão de conídios do patógeno na concentração de 6 x 10^4 conídios / mL na fase de crescimento V6. Após a inoculação, amostras de colmo foram coletadas às 0, 12, 18, 24, 36, 48, 72 e 96 horas após a inoculação e aos 14 e 21 dias após a inoculação, preparadas seguindo metodologia padrão e, em seguida, visualizadas em microscópio laser confocal e microscópio eletrônico de varredura. As imagens geradas permitiram visualizar a colonização dos caules pelo fungo, o que contribui para futuros estudos de interação com patógenos. O segundo estudo teve como objetivo avaliar os efeitos dos óleos essenciais extraídos de *Cinnamomum zeylanicum*, *Copaifera langsdorffii*, *Origanum vulgare*, *Cymbopogon martinii* e *Melaleuca alternifolia* nas doses de 0,25, 0,50, 0,75 e 1,00% no crescimento micelial de *S. maydis in vitro*. Além disso, os melhores óleos e concentrações foram utilizados no tratamento de sementes de milho para determinar seus possíveis efeitos na saúde e fisiologia das sementes. Verificou-se que os óleos de *C. zeylanicum* e *O. vulgare* foram capazes de inibir o crescimento micelial fúngico em concentrações superiores a 0,5% em níveis semelhantes ao tratamento padrão com fungicida. O óleo de *O. vulgare* apresentou melhor desempenho na redução da incidência de *S. maydis* nas sementes inoculadas em comparação com *C. zeylanicum* e no tratamento com fungicida. O óleo de *C. zeylanicum* a 0,75% apresentou taxas de germinação de sementes de 92,4% superiores às do controle não tratado e do controle tratado com fungicida. Em contraste, o óleo de *O. vulgare* nas duas concentrações testadas (0,25 e 0,50%) apresentou efeitos na fisiologia das sementes de milho. Por fim, o terceiro estudo desta dissertação teve como objetivo investigar o papel dos genes de 'mating-type' na patogenicidade de *F. graminearum* em trigo. Para tal, foram utilizados mutantes knockout (KOs), cada um dos MAT1, MAT1-1-1 e MAT1-2-1 no isolado padrão de *F. graminearum* (PH-1). Como esperado, todos os KOs perderam sua capacidade de produzir ascósporos. No entanto, quando o MAT1-1-1 KO foi pareado com o MAT1-2-1 KO, formaram peritécios férteis. A maioria dos mutantes (mas não todos) dos genes de especificidade individual resultou em reduções na agressividade e, na maioria dos casos, na produção de DON. Não houve redução consistente da agressividade nos mutantes da MAT. Em resumo, os três trabalhos desta tese fornecem novos conhecimentos para entender o ciclo de vida e também manejar esses importantes patógenos no Brasil e no mundo.

Palavras-chave: *Zea mays*, *Triticum aestivum*, processo infeccioso, óleos essenciais, giberela do trigo, micotoxina.

GENERAL ABSTRACT

The fungus *Stenocarpella maydis* (Berkeley) Sutton [Sin. *Diplodia maydis* (Berkeley) Saccardo] is an important necrotrophic pathogen causing both stalk and ear rot on maize. Similarly, *F. graminearum* is also an important maize pathogen causing both Gibberella ear and stalk rot (GER and GSR), but it is also an important pathogen of wheat causing Fusarium head blight (FHB). These diseases cause significant economic losses not only due to reduction in grain yield, but also can reduce crop value due to contamination of grain with mycotoxins. The present dissertation investigated these two important pathogens, *Stenocarpella maydis* and *Fusarium graminearum* and has been structured in a series of three studies. In the first study, the main objective was to study the infection process of *S. maydis* in maize stalks using different microscopy techniques. Plants of two maize hybrids, 30F53 and DKB 390 were grown in a greenhouse and inoculated with conidial suspension of the pathogen in the concentration of 6 x 10^4 conidia/mL at the V6 growth stage. After inoculation, samples were collected from stalk samples at 0, 12, 18, 24, 36, 48, 72 and 96 hours after inoculation and at 14 and 21 days after inoculation, prepared following standard and then, visualized both in confocal laser microscope and scanning electron microscope. The generated images allow us to visualize the colonization of the stalks by the fungus, which contributes to future pathogen interaction studies. The second study aimed to evaluate the effects of essential oils extracted from *Cinnamomum zeylanicum*, *Copaifera langsdorffii*, *Origanum vulgare*, *Cymbopogon martinii* and *Melaleuca alternifolia* at the rates 0.25, 0.50, 0.75 and 1.00% on *S. maydis* mycelial growth *in vitro*. Additionally, the best oils and concentrations were used to treat maize seeds to determine their possible effects on seed health and physiology. It was found that the oils from *C. zeylanicum* and *O. vulgare* were able to inhibit fungal mycelial growth at concentrations higher than 0.5% at similar levels of the standard treatment with fungicide. *Origanum vulgare* oil performed better in reducing the *S. maydis* incidence in inoculated seeds compared to *C. zeylanicum* and the fungicide treatment. The oil from *C. zeylanicum* at 0.75% showed seed germination rates of 92.4% which were higher than that from the non-treated and fungicide-treated control. In contrast, *O. vulgare* oil at both tested concentrations (0.25 e 0.50%) had physiological effects on maize seeds. Lastly, the third study of this dissertation aimed to investigate role of mating-type genes in pathogenicity of *F. graminearum* to wheat. For such, independent knockout mutants (KOs) each of MAT1, MAT1-1-1, and MAT1-2-1 in the PH-1 strain of *F. graminearum* were used. As expected, all the KOs lost their ability to produce ascospores. However, when MAT1-1-1 KO were paired with the MAT1-2-1 KO, formed fertile perithecia. Most (but not all) KOs strains of the individual specificity genes resulted in reductions in aggressiveness and, in most cases in DON production. There was no consistent reduction in aggressiveness on MAT mutants. In summary, the three works of this disseration provided new knowledge to understand the life cycle and also manage such important pathogens in Brazil and worldwide.

Keywords: *Zea mays*, *Triticum aestivum*, infection process, essential oil, Fusarium head blight, mycotoxin.

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GENERAL INTRODUCTION

The present dissertation has been structured in three studies of two important pathogens, *Stenocarpella maydis* and *Fusarium graminearum*. The fungus *S. maydis* (Berkeley) Sutton [Sin. *Diplodia maydis* (Berkeley) Saccardo] is an important necrotrophic pathogen causing both stalk and ear rot on maize. *Fusarium graminearum* is also an important maize pathogen causing both Gibberella ear and stalk rot (GER and GSR), but it is also an important pathogen of wheat causing Fusarium head blight (FHB). These diseases cause significant economic losses not only due to reduction in grain yield, but also can reduce crop value due to contamination of grain with mycotoxins.

In the first two studies it was investigated the infection process of *S. maydis* on maize stalks of two commercial hybrids and the use of essential oils as an alternative tool for control this pathogen on maize seeds. In a third study, which was conducted during my Sandwich period at the University of Kentucky, USA, it was investigated the role of mating-type genes, MAT1-1-1 and MAT1-2-1, in aggressiveness of *F. graminearum* to wheat in order to test whether the MAT proteins can individually regulate genes that can negatively impact aggressiveness and toxigenicity. Taken together, these works provide new knowlodge to understand and manage such important pathogens in Brazil and worldwide.

CHAPTER 1: LITERATURE REVIEW

Stenocarpella maydis

Importance of maize

Brazil is the third largest world producer of maize (*Zea mays* L.), behind only the United States and China (CONAB, 2016). Maize occupies a significant position in the national context, in terms of production value, cultivated area and volume produced, especially in the south, southeast and central-west regions of Brazil (FANCELLI; DOURADO NETO, 2004). Regarding its use, maize is an input for the production of several products. Around 70% of the world production and 80% of the maize produced in Brazil is destined for the pork and poultry production chain (EMBRAPA, 2015). It is also widely used for the food industry, mainly due to the quantity and nature of the reserves accumulated in the grains (KUNTZ, 2005).

The varied climatic conditions in Brazil allow the planting of two annual maize harvest seasons, differentiating it from the main producers in the world. Summer or first crop planting takes place during the rainy season, which varies between the end of August, in the South region, until the months of October / November, in the Southeast and Midwest. The second crop harvest is planted between the months of February and April.

By choosing the right maize hybrids it allows to obtain greater yield, however, abiotic and biotic factors, such as water, light, nutrients, pests and diseases, may limit the genetic potential of the cultivars (CRUZ et al., 2010). The appropriate planting timing and location help to escape from conditions that may favor certain pathogens or pests (WHITE, 1999; FANCELI; DOURADO NETO 2003; SANGOI et al., 2000).

Stalk rot

The main issues with stalk rot in maize can occur before tasseling, where the losses are due to the premature death of the plants, leading to a reduced plant population, compromised grain filling due to less absorption of water and nutrients, and also leading to premature plant lodge (CASELA; FERREIRA; PINTO, 2006). When the problem occurs after the physiological maturation of the grains, the losses

are related to the lodge of the plants, which may difficult the mechanical harvest and leads to the contact of the ears with the soil, which favors the rotting of the grains by saprophytes organisms (CASELA; FERREIRA; PINTO, 2006).

Stalk rot can start through root infections progressing to the lower internodes and then to the upper internodes, or directly due to stalk injuries caused by insects or mechanical damage during crop management and by contaminated seeds. Some factors during the grain filling phase tend to favor stalk rots. Among these factors, we can highlight the leaf spots that compromise the photosynthetic apparatus, insects injury both in leaf and in the stalk favoring the entry of pathogens, the soil moisture in excess or lack, the nutritional imbalance between K and N, the high plant density and excessive rainfall after flowering (CIMMYT, 2012).

Most stalk rot does not occur with a pattern of distribution, but randomly in the area where is still possible to find healthy plants alongside infected plants. The microorganisms responsible for stalk rot, mostly fungi, manage to survive in crop debris and through resistance structures in the soil, thus no-till cropping system, which is a recommended soil conservation technique and as a strategy to manage soil-borne diseases, can favor the increase of the inoculum amount in the soil, being possible to find a higher incidence of stalk rot in crops under this cropping system (CIMMYT, 2012).

The most important pathogens causing stalk rots are fungi of the genera *Stenocarpella* spp., *Colletotrichum* spp., *Fusarium* spp., *Macrophomina* spp., *Pythium* spp. Bacteria of the genera *Pseudomonas* spp., e *Erwinia* spp. have also been reported causing stalk rots (CIMMYT, 2012).

The fungus *Stenocarpella maydis*

The fungus *Stenocarpella maydis* (Berkeley) Sutton [Syn. *Diplodia maydis* (Berkeley) Saccardo] is a necrotrophic pathogen, responsible for causing both stalk and ear rot in maize. (CASA et al., 2006). The fungus produces subepidermal, globular or elongated pycnidia, with dark brown to black color, thick walls, diameter of 150-300 µm and a protruding papillate ostiole. Conidiophores are usually absent. They present enteroblastic, phialidic, cylindrical conidiogenous cells, formed in the internal cells of the pycnidium wall. Conidia are olive-brown to brown, cylindrical,

spindle-shaped, straight to slightly curved, measuring 15-34 x 5-8 µm, bicellular and commonly with 1 septum (0-2) (SUTTON; WATERSTON, 1966; SUTTON, 1980).

The fungus can be considered cosmopolitan because it is found in Europe, the Americas, Asia, and Africa (SUTTON; WATERSTON, 1966). Its economic importance is associated with the damage caused by stalk and ear rot that are frequently found in the maize production area (REIS; CASA; BRESOLIN, 2004; PEREIRA et al. 2005).

The mycelial development of the fungus is fast, the mycelium has a cottony aspect, in 5-6 days it is able to fully colonize a 90 mm diameter Petri dish. The colonies are white in PSA culture (Potato Sucrose Agar). Pycnidia formation occurs on the grains in seed pathology test or in solid culture media (PSA, cornmeal, and oatmeal agar) and in pieces of maize leaf superficially embedded in these media (LATTERELL; ROSSI, 1983; CASA; REIS; ZAMBOLIM, 1998).

S. maydis infects maize plants (SUTTON; WATERSTON, 1964; WHITE, 1999) and has also been reported in bamboo (*Arundinaria* sp.) (SUTTON; WATERSTON, 1966; SUTTON, 1980). The dispersion of *S. maydis* over long distances is possible mainly through infected seeds. Over short distances, rains associated with winds can facilitate the removal and dissemination of conidia from their pycnidia (SHURTLEFF, 1992). Temperatures between 28 and 30 °C (EDDINS, 1930; WHITE, 1999) with relative humidity above 50% (LATTERELL; ROSSI, 1983) are ideal conditions for conidia release and germination. This fungus is also found surviving as mycelium in seeds (MCGEE, 1988; CASA et al., 1998a, RHEEDER, et al., 1990) and forming survival structures, such as pycnidia, in crop debris (SMITH; WHITE, 1988; SHURTLLEF, 1992).

Stenocarpella stalk rot

Maize stalk rot is caused by two species of *Stenocarpella*:*Stenocarpella maydis* (syn. *Diplodia maydis*); and *Stenocarpella macrospora* (syn. *Diplodia macrospora*), which also causes ear rot and macrospora leaf spot (WHITE, 1999). Stalk rot is common in most maize growing regions worldwide, mainly in areas of no-tillage system under monoculture. (REIS; CASA; BRESOLIN, 2004; CASA; REIS; ZAMBOLIM, 2006; FLETT; WEHNER, 1991). Infection of ears by species of *Stenocarpella* can result in the production of mycotoxins which are toxic to humans

and animals (MARASAS et al., 1984; ODRIOZOLA et al., 2005; MASANGO et al., 2015).

Infected plants may die suddenly, with the green color of the internodes disappearing and becoming brown, spongy and easily crushed. The pith disintegrates and changes to a brown color. Dark sub-epidermal pycnidia can be seen grouped close to the nodes at the bottom of the stalk, and white fungal growth may also be present on the surface. The pathogen can also affect the upper internodes and cause ear rot. The abundant production of dark colored pycnidia in infected internodes at the end of the crop season allows the distinction of Stenocarpella stalk rot from other stalk rots (CASA; REIS; ZAMBOLIM, 2006).

This disease is more severe in regions with altitude above 700m, moderate temperatures and, mainly, humid environment. The predisposition to this disease is mainly increased by water stress before flowering followed by a rainy season, with high humidity 2 to 3 weeks after pollination (CHAMBERS, 1988; BENSCH, 1995) and temperature in the range of 28 to 30 °C (WHITE, 1999) under the no-till system (FLETT; WEHNER, 1991). Other very important factors that also cause stress, increasing the predisposition to the disease are plant population above the recommended, fertilization of planting below the recommended and lack of soil pH correction (WHITE, 1999).

The intensity of stalk and ear rot (ULLSTRUP, 1964; FLETT; WEHNER, 1991) and leaf spot (MORA; MORENO, 1984) is the result of the amount of infected crop debris present on the soil surface. In the crop debris, mainly maize stubble, the fungus *S. maydis* survives saprophytically producing pycnidia and releasing conidia in cirri which are the main source of primary inoculum (FLETT; WEHNER; SMITH, 1992). The viability period of *S. maydis* is longer in the straw kept on the soil surface than when it is buried (FLETT; WEHNER; SMITH, 1992).

The use of resistant varieties is the most practical and economical way to manage Stenocarpella rots, mainly in areas where the no-tillage system is adopted (WHITE, 1999; REIS; CASA; BRESOLIN, 2004). Use of healthy seeds, balanced soil fertility, management of infected crop debris after harvest, crop rotation, and lower plant density are the main strategies for disease management (CASA; ZAMBOLIM; REIS, 1998; DENTI; REIS, 2001; TRENTO; IRGANG; REIS, 2002).

The infectious process

The disease process is the compatible reaction between a pathogenic fungus and its susceptible host, under favorable environmental conditions. The onset of the infectious process begins with the deposition of the pathogen spore onto susceptible tissue, followed by its germination, formation and growth of the germ tube and the development of structures responsible for penetration (WYNN, 1981). The events between germination and penetration comprise the pre-penetration phase. This phase deserves great attention, since its understanding can elucidate how the plants are infected, and then, it can help to the definition of strategies that prevent the penetration of the pathogen and consequently improve the management of the disease (BRUNELLI et al., 2005).

Plant pathogens have developed different strategies and mechanisms to infect and colonize host tissues (DEAN, 1997; VAN KAN, 2006). The strategy used by necrotrophic fungal pathogens to infect plants usually begins with the adhesion of conidia to the leaf surface. This stage is followed by germination, penetration and sporulation (NICHOLSON et al., 1988; PRINS et al., 2000; LALUK; MENGISTE, 2010). Necrotrophic pathogens can also penetrate their hosts through wounds, natural openings, and directly through the surface by secreting lytic enzymes and non-selective toxins to the host tissue which is responsible for the dissolution of cell walls and the complete disintegration of cells (KOLATTUKUDY, 1985; HAVE et al., 2001; CABANNE; DONÉCHE, 2002; VAN KAN, 2006).

Fusarium graminearum

Importance of Fusarium Head Blight disease of wheat and small grains

Fusarium head blight (FHB) is the most important disease on wheat and small grains worldwide (MCMULLEN et al., 2012). *Fusarium graminearum* Schwabe [teleomorph. *Gibberella zeae* (Schwein.) Petch] is the most prevalent causal agent of FHB and of Gibberella stalk and ear rot on maize. The same diseases can also be caused by several other species within the *Fusarium graminearum* species complex (FGSC) (DEL PONTE et al., 2015; MCMULLEN et al., 2012). FHB is a fungal disease of major economic concern on wheat not only due to yield losses but also because

FGSC members are known to produce several mycotoxins, such as trichothecenes, which reduces grain quality (GOSWAMI; KISTLER, 2004; MCMULLEN et al., 2012). Deoxynivalenol (DON) is the most significant trichothecene and its maximum limits have been regulated in several countries (VAN EGMOND et al., 2007). Nivalenol (NIV) is another trichothecene produced by some FGSC members in wheat (DEL PONTE et al. 2012).

Fusarium Head Blight Disease Cycle

The fungus overwinters on crop debris as sexual fruiting bodies structures called perithecia. The ascospores produced in those structures comprise the primary inoculum for FHB (GOSWAMI; KISTLER, 2004). The ascospores are ejected from the mature perithecia (TRAIL et al., 2002). Ascospores are carried by wind or splashed by rain droplets onto flowering wheat heads (SUTTON, 1982; REIS, 1990; SCHMALE et al., 2005), while macroconidia are mainly dispersed via water splashes (PARRY; JENKINSON; MCLEOD, 1995). Infection of wheat heads by *F. graminearum* appears to occur via the glumes, following penetration of stomates and colonization of substomatal spaces (PRITSCH et al., 2000). The hyphae proceed into the rachis, and from there into the stems via the vascular tissues and pith (GUENTHER; TRAIL, 2005). External symptoms of FHB begin at the point of infection in the form of water soaking and brown discoloration and progress up and down along the rachis. Bleaching and distortion of awns as disease progresses can be seen before senescence are often associated with DON production by the fungus (PARRY; JENKINSON; MCLEOD, 1995).

Sexuality in *F. graminearum*

The sexual behavior of *F. graminearum* is not only related to its ability to overwinter and to start disease epidemics but also to produce genotypic variability via genetic recombination (BOWDEN; LESLIE, 1999; DESJARDINS et al., 2004; VOSS et al., 2010). *Fusarium graminearum* is homothallic ascomycete that is able to out-cross with other strains of *F. graminearum*, and also with other species within the FGSC, although there is variation in fertility among those species (BOWDEN; LESLIE, 1999). Factors that may affect the ability to out-cross among species/strains are poorly understood.

The proteins encoded by a single MAT locus control the sexual reproduction in filamentous ascomycetes. The MAT locus of *F. graminearum* comprise of two mating types, aka. idiomorphs, MAT1-1 and MAT1-2, situated in close proximity within the single MAT1 locus (YUN et al., 2000). The MAT1 locus spans about 7 kb on chromosome 2. The MAT1-1 idiomorph is comprised of three genes, MAT1-1-1, MAT1-1-2, and MAT1-1-3, while the MAT1-2 idiomorph contains only one gene, MAT1-2-1 (Figure 1). Both idiomorphs, which encode DNA binding proteins, are necessary for homothallism. When either idiomorph was deleted, the resulting mutant became obligately heterothallic (LEE et al., 2003).

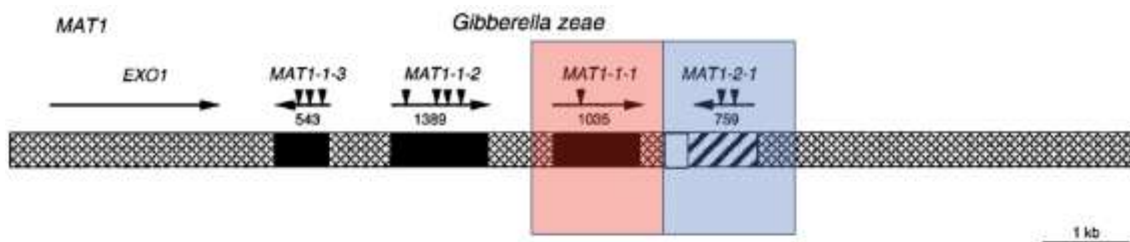

Figure 1. MAT1 locus of *Fusarium graminearum* (syn. *Gibberella. zeae*). The figure was adapted from Yun et al. (2000).

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CHAPTER 2 - MICROSCOPIC STUDY OF THE *Stenocarpella maydis* INFECTION PROCESS ON MAIZE STALKS

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ABSTRACT

Maize has a significant position in the national context, regarding the value of production, acreage and volume produced, besides its importance in human and animal feeding. Among the factors which limit maize production are the diseases, such as stalk rot caused by *Stenocarpella maydis*, which interferes in the perfect development of the crop and hence affecting its production and sanitary quality of the grains. The objective of this work was to study the infection process of *S. maydis* in maize stalks using different microscopy techniques. The experiment was conducted in a greenhouse where corn plants of cultivars P30F53 and DKB 390 were seeded in 10L pots. Plants were inoculated with conidial suspension of the pathogen in the concentration of 5 x 10^5 conidia/mL at the V6 growth stage. After inoculation, samples were collected from stalk samples at 0, 12, 18, 24, 36, 48, 72 and 96 hours after inoculation and at 14 and 21 days after inoculation. The samples were prepared

following protocols described elsewhere and then, the samples were visualized both in confocal laser microscope and scanning electron microscope. The generated images allow us to visualize the colonization of the stalks by the fungus, which contributes to future pathogen interaction studies.

Keywords: confocal laser scanning microscopy, Diplodia stalk rot, scanning electron microscopy*, Zea mays.*

INTRODUCTION

Brazil is ranked among the largest producers of maize (*Zea maydis* L.) worldwide (FAOSTAT, 2012). *Stenocarpella maydis* (syn. *Diplodia maydis*) is an important pathogen of maize, causing diseases of economic and food safety concern, i.e. Diplodia stalk rot and Diplodia ear rot (Pinto et al., 1997; Reis et al., 2004).

The fungus can infect all aerial organs of maize plants, including stalk, ears and leaves (Casa et al., 2006; Bermudez-Cardona et al., 2016). Symptoms of Diplodia stalk rot are a straw-brown discoloration of the lower nodes and internal disintegration of the pith. Signs in the form of embedded small black dots (pycnidia) appear around the lower nodes. (Casa et al., 2006; Casela et al., 2006). In general, stalk rot is most severe under wet conditions and temperatures ranging between 28 and 30 °C, mainly when rain occurs (Casa et al., 2006). *S. maydis* saprophytically survives in maize debris as both mycelia and pycnidia, which is the main source of inoculum in non-tillage areas. (Casa et al., 2006; Flett et al., 1992).

The main strategies to manage Diplodia stalk rot are sowing heathy seeds (Casa et al., 1998), genetic control (White, 1999; Reis et al., 2004), optimizing plant

populations (Denti and Reis, 2001; Trento et al., 2002), maintaining balanced soil fertility (White, 1999), and crop rotation (Flett and Mclaren, 2001).

The disease process occurs during a compatible reaction between the pathogenic fungus and a susceptible host, under favorable environmental conditions. The infection process starts with spore deposition on a susceptible tissue, followed by germination, penetration, colonization, and sporulation (Wynn, 1981). The events occurring before and directly after penetration have been investigated using scanning electron microscopy (Freitas et al., 2017), including for *S. macrospora* infecting maize leaves (Bermúdez-Cardona et al., 2016).

Considering the importance of Diplodia stalk rot, there is almost no information regarding the infection process of *S. maydis* on maize stalks. The goal of this study is to investigate and elucidate the events occurring during the infection process of maize stalks by *S. maydis*.

MATERIALS AND METHODS

Maize growing

Seeds of maize hybrids P30F53 (Leptra - Dupont®/Pionner®) and DKB 390 (PRO3 - Dekalb®), which are moderately susceptible and moderately resistant to *S. maydis*, respectively, were sown in plastic pots containing 5 kg of Rohrbacher® substrate mixed with soil (1:1). A total of five seeds were sown per pot, and each pot was thinned to three plants eight days after seedling emergence. Plants were kept in a greenhouse (temperature 26 ± 2 °C during the day and 14 ± 4 °C at night and relative humidity 72 ± 5%). Plants were watered daily and fertilized as needed.

Inoculum production and inoculation of maize plants

Plants were inoculated with a monosporic isolate of *S. maydis* obtained from the Mycological Collection of Lavras (CML). The *S. maydis* isolate was grown in Petri dishes containing oatmeal-agar medium (30g of oatmeal, 20g of agar per liter of medium) and was incubated for 35 days at 25 ± 2 °C and a light-dark cycle of 12-12h, respectively. Stalks of maize plants at V5 growth stage (around 30 days after emergence) were inoculated by injecting two milliliters of a conidia suspension of *S. maydis* (6×10^4 conidia mL^{-1}) into the first internode above the uppermost aerial root using an obtuse needle (Bensch et al., 1992). A mock inoculation treatment (sterile distilled water) was included as a negative control.

Sample preparation for scanning electron microscopy

Stalk fragments (around 5 mm^2) were collected at 6, 12, 18, 24, 36 h and 48 HAI (hours after inoculation) and at 14 and 21 DAI (days after inoculation). The fragments were placed into glass vials, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and stored at 4 °C for 24 hours. The samples were then washed twice with the same buffer, with each wash lasting 10 min. The fragments were then dehydrated in acetone series (25%, 50%, 75%, 90% and 100%). After dehydration, the fragments were subjected to a critical point dryer using the unit Critical Point Dryer BAL-TEC CPD 030 (Balzers®). The samples were coated with colloidal gold using a Sputtering BAL-TEC 050 (Balzers®). Images were acquired using a scanning electron microscopy Zeiss LEO Evo 40 XVP and a Smart SEM Zeiss software.

Samples preparation for confocal laser scanning microscopy

Samples collected and fixed as described before were clarified using KOH 10% for 48 h. Firstly, clarified specimens were treated with the fluorochrome Wheat Germ Agglutinin (WGA) AlexaFluor 488® Conjugate (Alexa488-WGA) (Thermo Fisher Scientific, Basingstoke, Hampshire, UK, CAT-W11261) 10 µg.mL^{-1} work solution in 0.1 M PBS (Phosphate buffer solution) pH 7.2 for 30 min under vacuum, to mark the fungus (Ha et al., 2016). After, the fluorochrome Calcofluor White (Fluorescent brightener 28, Sigma-Aldrich®, St. Louis, USA, CAS-4404-43-7) for 30 min were used to mark plant cell walls at 0.01 % (w/v) work solution in 0.1 M PBS pH 7.2 (Anderson et al., 2010). Other plant dye used was Pontamine Fast Scarlet S4B (Sigma-Aldrich®, St. Louis, USA, S479896) at 0.01% (w/v) work solution in 0.1 M PBS pH 7.2, for 30 min (Anderson et al., 2010; Liesche et al., 2013). Fluorescence images were acquired using a confocal laser microscope LSM780 Zeiss Observer Z.1 and Zen 2012 software. To observed fungus stained with Alexa488-WGA the follow conditions were used: excitation with Argon 488 ηm laser line and emission filter with 500 to 550 ηm. Plant tissues stained with CalcoFluor: excitation with Diode 405 ηm laser line and emission filter with 420 to 480 ηm range. For plant dying with S4B: HeNe 514 ηm laser excitation, and emission filter from 568 to 594 ηm.

RESULTS

Symptoms of Stenocarpella stalk rot on maize

Symptoms of Stenocarpella stalk rot started at 7 DAI for both maize hybrids, when also observed foliar wilt (Figure 1B) was compared to mock inoculated plants (Figure 1A). Necrotic brown lesions were also detected at the inoculation point which became darker as the disease progressed (Figure 1C). Brown discoloration of the

lower nodes and the internal disintegration of the pith were observed (Figure 1D). The abundant presence of pycnidia associated to stalk tissue where detected at 21 DAI (Figure 1D-E).

Fungal germination and penetration in maize stalks

The germination and penetration stage of fungal development were not observed during the times 6, 12, 18, 24, 36 and 48 HAI.

Observation of fungal colonization and sporulation on maize stalks using electron scanning microscopy

At 21 DAI, a dense and abundant presence of mycelia along with pycnidia were detected on stalk surface of both maize hybrids, P30F53 (Figure 2D) and DKB 390 (Figure 2G). Longitudinal sections from stalks of both hybrids, P30F53 (Figure 2E) and DKB 390 (Figure 2H), showed intense intracellular colonization. Fungal hyphae colonized phloem and parenchyma cells and xylem vessels in both hybrids (Figure 2F and 2I). Pycnidia at different stages of development were first detected at stalk surface at 21 DAI (Figure 2D and 2G).

Fungal colonization and sporulation on maize stalks using confocal scanning microscopy

The fungal colonization of stalk cells and pycnidia development were detected after staining of the samples with Alexa488-WGA (Figure 3D and F). Similarly, the colonization of vascular tissue by *S. maydis* was detected after staining of the samples with CalcoFluor (Figure 3E).

The presence of autofluorescence of plant tissues were detected in stalk samples, where the green color was stained by Alexa Fluor 488-WGA (lignin and cellulose stain) and CalcoFluor (cellulose stain) (Figure 3A, 3B, 3C). No fungal structures were observed in mock inoculated samples.

Fungal colonization of stalk cells by *S. maydis* (Figure 3I) were observed after staining samples with S4B (Figure 3G) and Alexa 488-WGA (Figure 3H) for plant and fungal tissues, respectively. Both stains can be used for studies that aim to elucidate the colonization of maize stalks by *S. maydis*.

DISCUSSION

The present study provides a better understanding about microscopic details of *S. maydis* colonization and reproduction in maize stalks. Microscopic studies of this pathosystem are lacking. Studies focusing on the infection process of *S. macrospora* on maize leaves have been reported elsewhere (Bermudez-Cardona et al., 2016; Brunelli et al. 2005). Those studies have shown that at 24 HAI, conidia started to germinate on leaves through both conidia cells both without any specific pattern across the leaves. Germination tube could develop or not to reach stomata and appressoria could be present or not. Those authors also noticed that the fungus can dissolve the epicuticular wax around the conidia and germ tubes on the leaf surface, which indicates fungal enzymes activity. Those results lead the authors to conclude that *S. macrospora* can directly penetrate maize leaves by the enzymatic activity but also through natural openings, mainly stomata. Unfortunately, our samplings failed to find the germination and penetration phase of the disease cycle. One possible explanation might be due to the inoculation procedure used in the present study and also due to the difficulty to work and find fungal structures on

maize vascular tissues. The conidial germination could be delayed under greenhouse conditions, especially lower relative humidity. Competition with other microorganisms and temperature fluctuation could be antagonistic to the optimal conditions to conidia germination (Bensch and Van Staden 1992).

The lack of differences observed on reproduction and colonization of *S. maydis* between the maize hybrids used in this study may suggest that the differential resistance between them is not related to the structural surface of their stalks. Inoculation method may have prevented us to see any difference between the two hybrids, once their resistance response could be expressed in different steps of disease cycle, such as germination or penetration.

Similarly to what was described for *S. macrospora* on maize leaves (Bermudez-Cardona et al., 2016), after fungal penetration, two distinct colonization patterns were observed. During the early stages of fungal infection, hyphae were less abundant in epidermal and also subepidermal cells. The integrity of cells wall was changed which may be due to the production of non-host selective toxins during the infection process (Bermudez-Cardona et al., 2016).

Fungal hyphae were noticed colonizing the epidermal and parenchyma cells, and fully colonizing the vascular system. The degree of cell wall degradation in maize tissue presented by this study suggests that enzymes are involved in *S. maydis* infection process of maize stalks. The occurrence of intracellular and intercellular growth events of *S. maydis* in maize stalk tissues observed in this study provides other evidence of enzyme activity during fungal colonization. In fact, the production of of cellulases by *S. maydis* cellulases by *S. maydis* has been already reported (Bemiller et al., 1969; Hernández-Domínguez et al., 2020). After the generalized cell degradation, fungal hyphae emerged throughout the necrotic tissue and the

reproduction phase started, which was characterized by the development of pycnidia. This behavior was similar to the observed in *S. macrospora* infecting maize leaves (Bermudez-Cardona et al., 2016).

Confocal scanning images allowed us to detect the fungus in stalks. The formation of fungal structures, i.e. pycnidia, and the colonization of vascular tissue was possible using Alexa488-WGA. The staining plant tissue using Calcofluor allowed us to differentiate fungal hyphae from vascular elements. This combination of stains was effective and could be used in tissue with autofluorescence such as maize stalks.

Taking into account the importance of stalk rot to maize production worldwide and the lack of information about *S. maydis* infection process, our results provide relevant knowledge to better understand this pathosystem.

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FIGURES

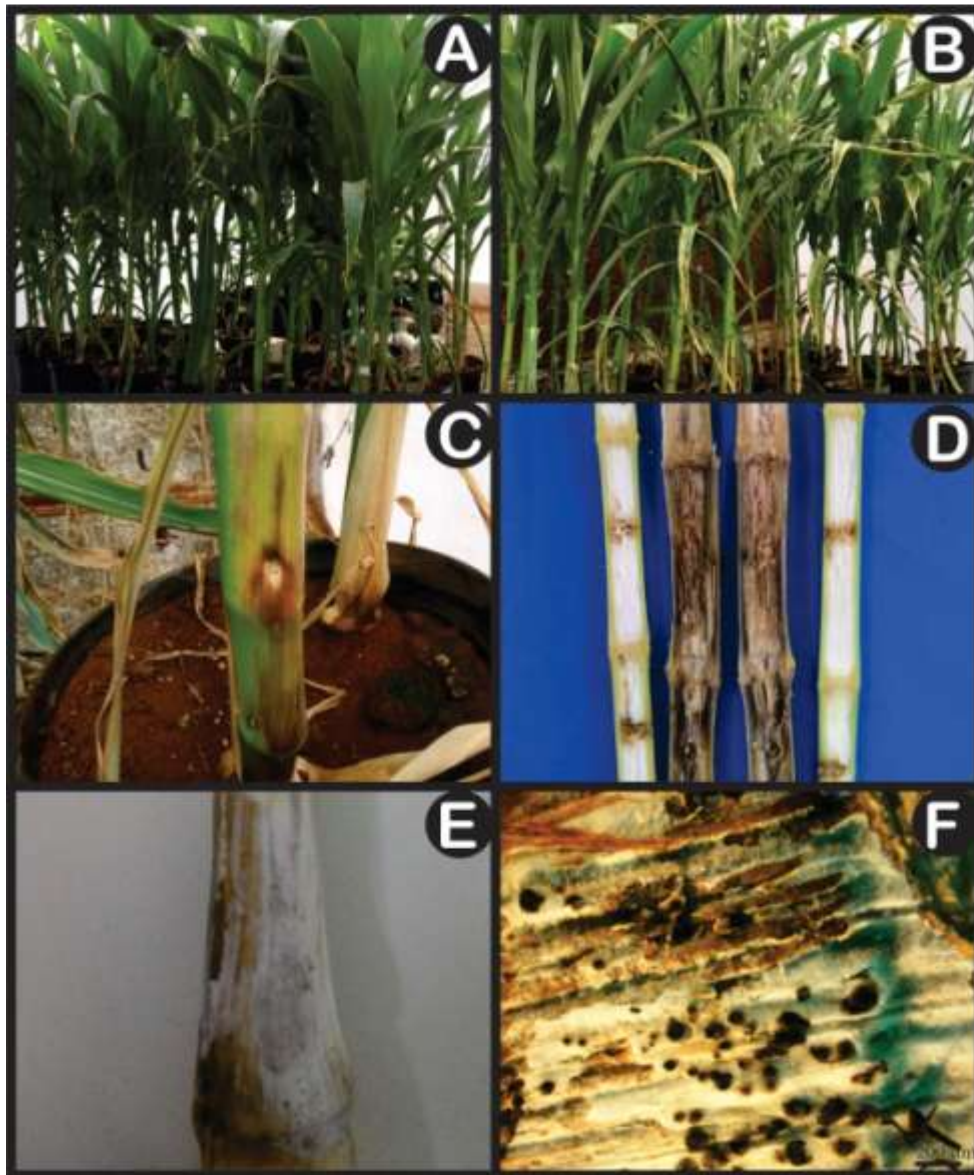

Figure 1. Photographs of symptoms of Stenocarpella stalk rot on maize. **A:** Mock treatment: plants inoculated with sterile water; **B:** Plants inoculated with a spore suspension of *Stenocarpella maydis* showing foliar wilt; **C:** Necrotic lesion on inoculated stalk; **D:** Longitudinal section of inoculated maize stalks (two central stalks) with pycnidia (dark structures) and mock treatment (left and right stalks); **E:** Stalks samples at 21 DAI showing abundant mycelia (white) and pycnidia (dark structures); **F:** Stereomicrograph of mycelia and pycnidia on stalk surface under dissecting microscope.

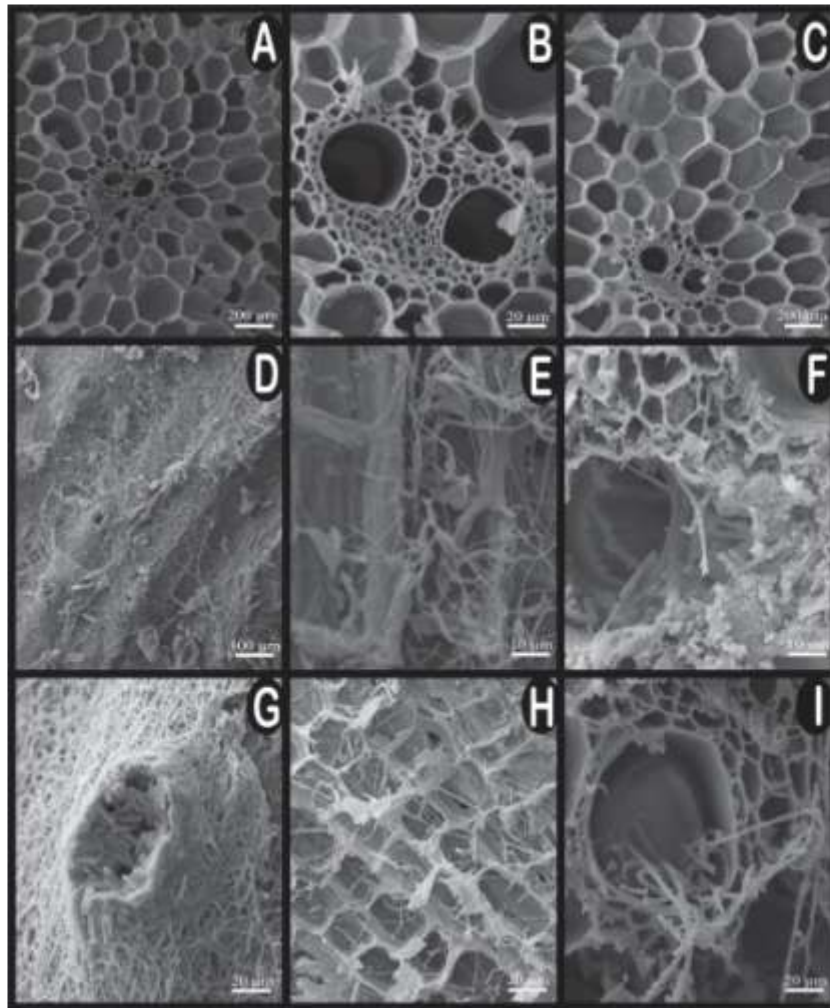

Figure 2. Scanning electron micrographs of maize stalks. **A:** Transversal section of hybrid DKB 390 stalks mock inoculated (plants inoculated with sterile water); **B:** Transversal section of mock inoculated stalks of hybrid P30F53; **C:** Transversal section of mock inoculated stalks of hybrid P30F53; **D:** Stalk surface of hybrid P30F53 showing dense mycelial and pycnidia; **E:** Longitudinal section of hybrid P30F53 showing fungal hyphae colonizing intracellularly plant tissue; **F:** Fungal colonization of vascular tissue of hybrid P30F53; **G:** Stalk surface of hybrid DKB 390 showing mycelia and pycnidia; **H:** Longitudinal section of hybrid DKB390, showing cell colonization; **I:** Vascular colonization of stalk tissue of hybrid DKB 390 by *S. maydis* hyphae.

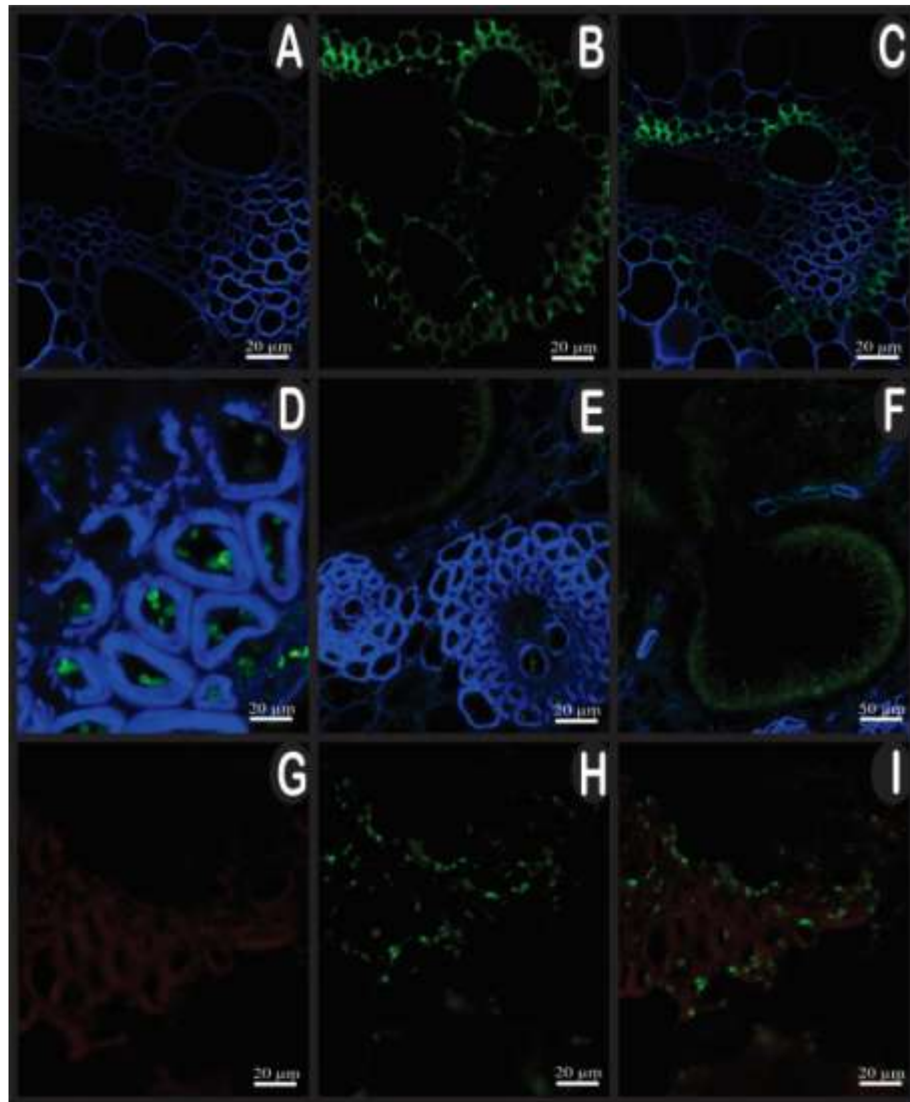

Figure 3. Scanning laser Confocal micrographs of maize stalks. **A:** Mock treatment – CalcoFluor staining plant tissue; **B:** Mock – autofluorescence of plant tissue; **C:** Mock - Calcofluor staining plant and vascular tissue, presence of autofluorescence of plant tissue and absence of *S. maydis*; **D:** Plant cells stained with CalcoFluor colonized by *S. maydis* stained with Alexa488-WGA at 21 DAI; **E:** Plant and vascular tissue of maize stained with CalcoFluor and fungal colonization of vascular tissue stained with Alexa488-WGA at 21 DAI; **F:** *S. maydis* pycnidia stained with Alexa488-WGA; **G:** Plant cells stained with S4B; **H:** *S. maydis* hyphae stained com Alexa488-WGA; **I:** Plant cells stained with S4B being colonized by *S. maydis* stained with Alexa488-WGA at 21 DAI.

CHAPTER 3: EFFECT OF ESSENTIAL OILS ON STENOCARPELLA ROT IN MAIZE SEEDS

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ABSTRACT

*Stenocarpella may*dis is an important maize pathogen causing both stalk and ear rots which is responsible for severe crop losses worldwide. Seed treatment is one of the most important strategies of the integrated management of this pathogen in the field. The objective of this study was to evaluate the effects of essential oils extracted from *Cinnamomum zeylanicum*, *Copaifera langsdorffii*, *Origanum vulgare*, *Cymbopogon martinii* and *Melaleuca alternifolia* at the rates 0.25, 0.50, 0.75 and 1.00% on *S. maydis* mycelial growth *in vitro*. Additionally, the best oils and concentrations were used to treat maize seeds to determine their possible effects on seed health and physiology. The oils from *C. zeylanicum* and *O. vulgare* were able to inhibit fungal mycelial growth at concentrations higher than 0.5% at similar levels of the standard treatment with fungicide. *O. vulgare* oil performed better in reducing the *S. maydis* incidence in inoculated seeds compared to *C. zeylanicum* and the fungicide treatment. The oil from *C. zeylanicum* at 0.75% showed seed germination rates of

92.4% which were higher than that from the non-treated and fungicide-treated control. In contrast, *O. vulgare* oil at both tested concentrations had physiological effects on maize seeds. Our findings suggest that the oils from *O. vulgare* and *C. zeylanicum* can be used as potential alternatives for maize seed treatment to control of *S. maydis*.

Keywords: *Origanum vulgare*; *Cinnamomum zeylanicum*; seed treatment.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide (FAOSTAT, 2012) not only for food but also fuel production as ethanol which is of great importance for the economy of several countries (CONAB, 2020). Maize yield can be affected by several factors such as water availability, soil fertility, plant density, cropping system, genetic potential of the hybrid and management of weeds, pests and diseases (Pinto et al., 1997; Reis et al., 2004).

Infected maize seeds are important sources of inoculum where the pathogens can cause seed and root rot, damping-off (Pinto, 1993). Infected seeds are is one of the main ways for the spread of *S. maydis*, which can introduce the fungus into new areas at higher distances (DAI et al., 1987).

Seed treatment contributes to the maintenance of plant population and reduces the spread of several pathogens (Fancelli; Dourado 2000). Failures in emergence are directly reflected in the final plant density and, consequently, in grain yield. Seed treatment is usually conducted using synthetic fungicides with different active ingredients; however, new active ingredients are constantly studied, including

natural composts, such as plant extracts and essential oils (Hillen et al., 2012; Christian; Goggi, 2008). Essential oils are obtained through different processes from vegetable raw material, and are compositions of substances with different chemical functions, such as aldehydes, alcohols, phenols, ketones, etc. (Sell, 2010). The antifungal potential of some essential oils has been widely evaluated with some promising results (Pauli; Schilcher, 2010). Thus, it is pertinent to conclude that essential oils can be used in sanitary seed treatments, however the effect of these oils on germination and seedling development must also be investigated (Christian; Goggi, 2008). Studies to test the effect of essential oils can indicate them as possible alternative products for old molecules that have been intensively used for the last decades (Duke et al., 2010).

The objective of this study was to evaluate the effects of essential oils extracted from *Cinnamomum zeylanicum*, *Copaifera langsdorffii*, *Origanum vulgare*, *Cymbopogon martinii* and *Melaleuca alternifolia* on *S. maydis* and to determine their possible effects on seed physiology and *S. may*dis control on seeds.

MATERIALS AND METHODS

All experiments were conducted at the Electron Microscopy and Ultrastructural Analysis Lab, of the Department of Plant Pathology, Universidade Federal de Lavras (UFLA). A single-spored *S. maydis* isolate obtained from Mycological Collection of Lavras (CML) was used in all experiments.

Effect of essential oils *in vitro*

The effect of essential oils on mycelial growth was tested in solid PDA (potato dextrose agar) media. A solution of sterile deionized water with 0.7% Tween 20 was

prepared for each of the essential oils: *Cinnamomum zeylanicum*, *Copaifera langsdorffii*, *Origanum vulgare*, *Cymbopogon martinii* and *Melaleuca alternifolia*. An aliquot of 1 mL of each solution was added in molten PDA (around 40 °C) to obtain the final concentrations of 0.25, 0.50, 0.75 e 1.00% and poured into 9-cm diameter Petri dishes. Water (plus 0.7% Tween 20) and Maxim XL® (Metalaxil + Fludioxonil) fungicide (1%) treatments were added as negative and positive controls, respectively. Plates of each treatment (replicates) were inoculated centrally with a 5 mm mycelial plug taken from the edge of a 5-day-old colony of *S. maydis* grown on PDA Plates were sealed with parafilm and incubated at 25 °C with a 12h photoperiod for seven days. The colony diameter was assessed every 24 h until the fungus growing on the unamended treatment reached the edges the plates (approximately 7 days) using a digital caliper. The experiment was conducted in a completely randomized design with four replicates. The entire assay was repeated once.

Effect of essential oils in maize seeds

Only the oil concentrations able to inhibit *S. maydis* mycelial growth at levels comparable to the fungicide treatment were used for the seed treatment tests. In total, seven treatments were included in the next tests. Non-treated and non-inoculated seeds (NT_NI); inoculated but non-treated (I_NT); inoculated and fungicide treated (I_FUNG); inoculated and treated with 0.25% *O. vulgare* oil (I_0.25OREG); inoculated and treated with 0.5% *O. vulgare* oil (I_0.50OREG); inoculated and treated with 0.50% *C. zeylanicum* oil (I_0.50CAN); inoculated and treated with 0.75% *C. zeylanicum* oil (I_0.75CAN). Seeds were first disinfested with 1.0% sodium hypochlorite solution for 2 min. For the treatment with each oil, around 850g of seeds of maize hybrid DKB390, moderately resistant to *S. maydis*, were

immersed for 10min in a 1% Tween 20 solution with the adjusted concentration of each oil. Fungicide treatment was performed by spraying a solution at the recommended concentration onto 850g of seeds until run-off. Negative control was treated with a 1% Tween 20 solution. Seed were dried on sterilized filter paper at room temperature.

Seeds were inoculated with a conidial suspension of *S. maydis* (6×10^4 conidia mL^{-1}) obtained from a 35-days old colony of *S. maydis* grown in Petri dishes containing oatmeal-agar medium (30g of oatmeal, 20g of agar per liter of medium) and were incubated at 25 ± 2 °C and a light-dark cycle of 12-12h, respectively.

In order to assess the effect of the treatments on the physiological quality, seed vigor and health were assessed through the percentage of germination, accelerated aging, emergency speed index and seed health.

Seeds were submitted to standard germination test in paper. For each treatment, 200 seeds were evenly distributed in eight replicates of 25 seeds each and incubated in germination chambers at 25 ± 3 °C, with 12 hours of photoperiod. Germination was assessed at the fourth and seventh day.

For the accelerated aging test, the seeds were placed on a mesh tray attached to a plastic box, containing 40 mL of distilled water at the bottom. The boxes were placed in an appropriate chamber for accelerated aging, for 72 hours, at 42 °C (Krzyzanowski et al., 1999). Eight replicates containing 25 seeds were used for each treatment. After this period, the seeds were submitted to the germination test, as described above.

In order to assess the seed health, the standard health blotter test was used. For such, eight plates (replicates) with 25 seeds per plate were used for each treatment as described previously (Machado 2000). After 14 days, seeds examined

under a stereomicroscope for the determination of *S. maydis* incidence as a proportion of infected seeds.

For the standard emergence test, seeds were sowed in plastic trays containing autoclaved sand as substrate with one seed per cell. Substrate of each treatment was watered with 600 ml of water and trays were placed 10 cm apart. Trays were transferred to a greenhouse (25°C ± 5 °C). The numbers of emerged seedlings were counted daily for 14 days. The emergence seed index (ESI) was calculated using the formula suggested by MAGUIRE (1962). Next, the initial and final plant populations (stands) were counted at 10 and 28 days after sowing.

Data analysis

Data were subjected to analysis of variance (ANOVA) by F-test and means were compared by Scott-Knott test (P < 0.05). All analyses were performed using R software (R Core Team 2019).

RESULTS

Essential oil from *C. langsdorffii*, *C. martinii* and *Melaleuca alternifolia* at all tested concentrations performed poorly in the inhibition of *S. maydis* mycelial growth (Figure 1). In contrast, oil extracts obtained from *C. zeylanicum* and *O. vulgare* at concentrations higher than 0.25% showed mycelial growth inhibition similar to the fungicide treatment (Figure 1).

S. maydis was found in non-inoculated (NT_NI) seeds in these experiments in an incidence of around 18% (Figure 2). The positive control (I_NT) showed significantly higher incidence compared to all oils (Figure 2). Seeds treated with *C. zeylanicum* oil either at 0.5 or 0.75% rate showed *S. maydis* incidence levels not

significantly different from the non-inoculated treatment (Figure 2). Additionally, seeds treated with *O. vulgare* provided the lowest *S. maydis* incidence.

Interestingly, seeds treated with *O. vulgare* significantly reduced the emergence rate in comparison to the other treatment groups (Table 1). In contrast, oil extracts from *C. zeylanicum* at 0.75% showed seed germination rates of 92.4% which were significantly higher than that from the non-treated and fungicide-treated controls (Table 1). Fungicide treatment significantly differed from the other treatments on the accelerated aging test ($P < 0.05$, Table 1). There were no significant effects of the treatment of maize seeds with any of the oils in the percentage of emergency at 14 days and in emergency speed index ($P < 0.05$, Table 1)

DISCUSSION

In the present study, we investigated the potential of essential oils extracted from some plant species to be used as alternatives for the control of *S. maydis*. We found that two oils (*O. vulgare* and *C. zeylanicum*) inhibited mycelial growth at levels similar to the standard fungicide labeled and used as seed treatment. In fact, previous work had shown that oil from *C. zeylanicum* inhibited fungal development at concentrations higher than 0.025% (Teixeira et al. 2013). Although, we showed both oils were only effective at concentrations higher than 0.5% our results corroborate their results. Previous study has also confirmed the activity of *C. zeylanicum* essential oil against *A. alternata* not only *in vitro* but also in the commercial orchards attributing its activity to trans-cinnamaldehyde (Perina et al., 2019).

Oil extracts from *O. vulgare* not only reduced *S. maydis* incidence in inoculated plants but also significantly the incidence in treated seeds had lower *S. maydis* then the non-inoculated treatment. It may be evidence that this oil not only

has effect on fungus growing at seed surface but also in reducing the fungal development during seed infection and colonization. In contrast, the treatment with *O. vulgare* oil had direct effect on seed quality, especially reducing germination. Similarly, other studies have described the negative effect on seed quality in other crops although not the effect on fungal development (Morais et al., 2008; Paudel et al., 2008). The negative effect of *O. vulgare* oil on seed germination might be related to the fast water absorption during seed and its composition which might toxic to maize seeds. Although there was no significant difference among the treatments on emergence, there was a high within treatment variation that prevented us to detect any difference between them.

Seeds treated with fungicide were the only treatment that was not affected in the accelerated aging test. It is possible that the fungicide protects the seed from the effects of the test and the seed vigor is less affected than non-treated seeds or seeds treated with essential oils.

Our findings suggest that the oils from *O. vulgare* and *C. zeylanicum* can be used as potential alternatives for maize seed treatment to control of *S. maydis*. However, the toxic effects of *O. vulgare* oil to seed physiology and the mechanisms underlying the fungicide/fungistatic effect of *C. zeylanicum* need to be further investigated. Thus, the use of these oils may contribute new alternative strategies on *S. maydis* management.

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FIGURES

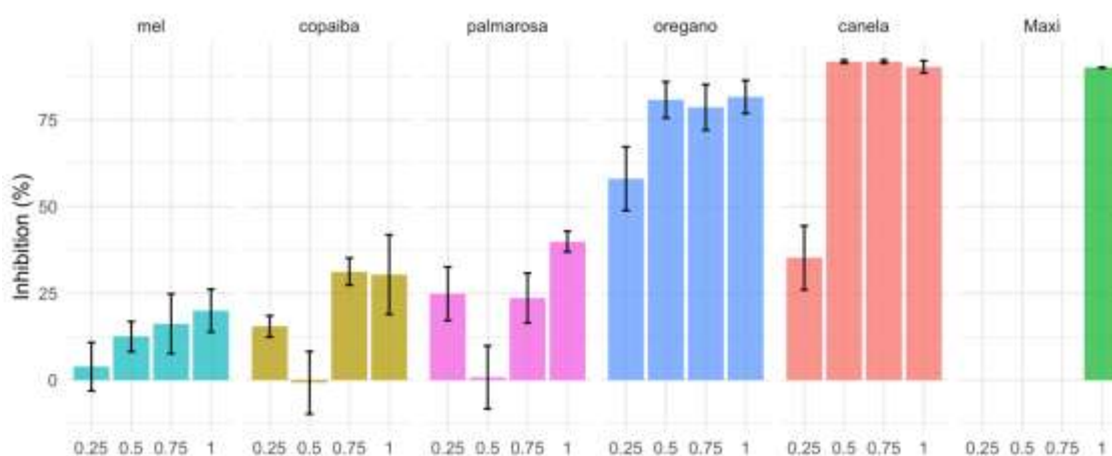

Figure 1. Percentage of inhibition of *Stenocarpella maydis* radial mycelial growth relative to the unamended check under *in vitro* conditions by each of the essential oils: *Cinnamomum zeylanicum* (canela), *Copaifera langsdorffii* (copaiba), *Origanum vulgare* (oregano), *Cymbopogon martinii* (palmarosa) and *Melaleuca alternifolia* (mel). The fungicide Metalaxil + Fludioxonil (Maxi) was included positive controls.

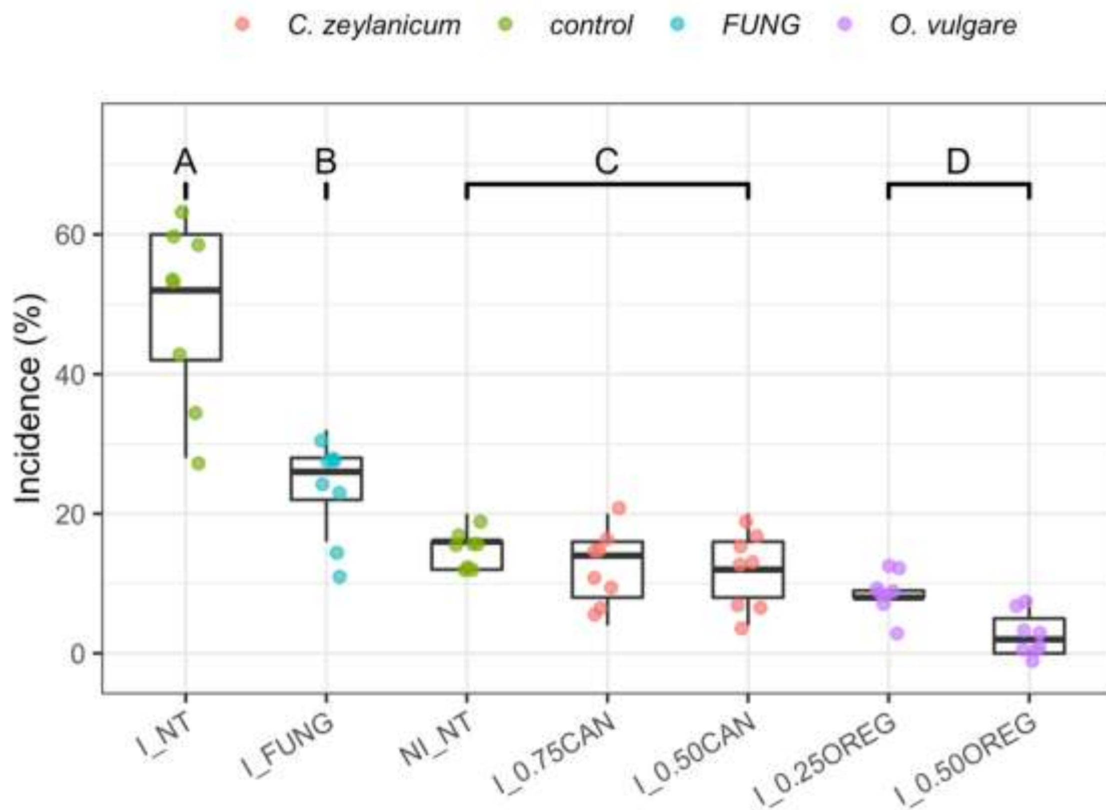

Figure 2. Incidence of *Stenocarpella maydis* (percentage of diseased seeds) in samples of maize seeds treated with essential oils of *Origanum vulgare* (OREG) and *Cinnamomum zeylanicum* (CAN) at 0.25 and 0.5% and 0.5 and 0.75% rates, respectively. Non-treated treatments, either inoculated (I_NT) or non-inoculated (NT_NI) were included as positive control. Non-inoculated and fungicide treated (I_FUNG) treatment were includeed as negative control. Treatment followed by the same letter are not significantly different from each other based on Scott-Knott test ($P > 0.05$).

TABLE

Table 1. Germination (percentage of normal, abnormal and dead seeds), accelerated aging (percentage of normal, normal but infected, abnormal and dead seeds) and emergency speed index of maize samples treated with *O Origanum vulgare* (OREG, 0.25% and 0.50%) and *Cinnamomum zeylanicum* (CAN, 0.50% and 0.75%) essential oils compared with the controls (non-inoculated, inoculated and fungicide treated seeds).

Treatments [a]	G (%)[b]				AA (%)[b]					
	Normal	Abnormal	Dead	Diseased.	Normal	Infected	Abnormal	Dead	E (%)[c]	ESI (%)
Mock	73.00 b	7.50 ns	15.50 b	4.00 d	46.08 b	1.47 d	20.55 a	31.89 b	81.00 ns	87.49 ns
Inoculated	46.42 c	9.06	15.08 b	29.18 b	34.38 b	32.57 a	6.65 b	26.40 b	79.00	90.42
Fungicide	65.38 b	8.51	15.00 b	11.03 c	71.23 a	0.00 d	5.61 b	23.15 b	82.00	93.38
0.25% OREG	27.58 d	9.38	17.4 b	45.61 a	32.50 b	24.00 b	13.00 b	30.50 b	71.50	74.62
0.50% OREG	36.44 c	15.23	31.51 a	16.22 c	35.95 b	13.78 c	25.89 a	24.39 b	71.00	83.31
0.50% CAN	23.26 d	12.74	34.58 a	29.42 b	45.43 b	6.59 d	16.72 a	31.26 b	53.00	54.79
0.75% CAN	92.06 a	1.52	0.48 c	0.00 d	34.00 b	3.50 d	12.50 b	50.00 a	41.00	46.46
Average										
CV	16.23	49.75	26.45	38.21	25.28	48.77	46.32	30.88	20.47	23.70

[a] Means followed by same letter did not differ from each other by Scott-Knott at 95% of significance.

[b] G = percentage of germination; AA = accelerated aging test; ESI = percentage of emergency at 14 days; ESI = emergency speed index.

[c] Means were compared using non-parametric test Kruskal-Wallis at 95% of significance.

CHAPTER 4: THE ROLE OF *Fusarium graminearum* MATING-TYPE GENES IN AGGRESSIVENESS TO SPRING WHEAT HEADS AND CORN STALKS

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ABSTRACT

Fusarium graminearum, the primary cause of Fusarium Head Blight (FHB) of wheat and barley in North America and worldwide, is a homothallic ascomycete capable of outcrossing. Ascospores produced in early spring from perithecia on infested crop debris serve as primary inoculum for the establishment of epidemics. The sexual behavior of this fungus is regulated by the mating type locus MAT1, which is comprised of two linked idiomorphs, (MAT1-1 and MAT1-2). It is assumed that the products of these two genes interact to form a heterodimer that regulates homothallic sexual development. We used several independent knockout mutants (KOs) of MAT1, MAT1-1-1, and MAT1-2-1 in the PH-1 strain of *F. graminearum.* As expected, all the KOs lost their ability to produce ascospores, while the MAT1-1-1 KO, when paired with the MAT1-2-1 KO, formed fertile perithecia. Most (but not all) of the KO strains were reduced in aggressiveness and in DON production when compared to the wild type in both spring wheat heads and maize stalks. However, the reduction was not consistent, and a few of the KO strains were not significantly different from the WT. These results suggest that the *F. graminearum* MAT1-1-1 and MAT1-2-1

specificity proteins individually might regulate genes that negatively impact the aggressiveness and toxigenicity of the heterothallic strains.

Keywords: *Triticum aestivum***,** Fusarium head blight, deoxynivalenol, mycotoxin**.**

INTRODUCTION

Fusarium graminearum, the primary cause of Fusarium Head Blight (FHB) of wheat and barley, and of Gibberella ear and stalk rot of maize, in North America is a homothallic ascomycete (Kazan etal., 2012; Mcmullen et al., 2012; Goswani and Kistler, 2004). Ascospores produced in early spring from perithecia on infested crop debris serve as the initial inoculum for the establishment of epidemics in small grains (Mcmullen et al., 2012; Goswani and Kistler, 2004). Production and dissemination of ascospores are potential targets for disease management and, as such, have been studied and described in detail (Trail et al., 2005; Maldonado-Ramirez et al., 2005; Schmale et al., 2012).

The mating-type locus of *F. graminearum* (MAT1) is similar to other fungi, i.e. *N. crassa* except that both idiomorphs (MAT1-1 and MAT1-2) are linked within the same locus in all strains (Yun et al., 2000). It is assumed that the products of two specificity genes (MAT1-1-1 and MAT1-2-1) interact to form heterodimers that regulate homothallic sexual development in *F. graminearum*, and that non-dimerized specificity proteins are generally absent from the thallus and play minimal roles outside of the ascogenous hyphae (Lee et al., 2003; Yun et al., 2000; Zheng et al. 2013).

Deletion of the entire MAT1 locus of *F. graminearum* (including both idiomorphs) reportedly had no effect on aggressiveness to heads of spring wheat (cv. Wheaton)that were point-inoculated with macroconidia in the greenhouse (Desjardins

et al., 2004). This suggests that genes regulated by the MAT heterodimer have no direct role in this process. As expected, the MAT1 knockout (KO) lost its ability to produce ascospores. A field study demonstrated that the KO was incapable of inducing a disease epidemic from colonized crop debris, presumably because only ascospores can be ejected high enough into the air to reach the wheat flowers (Desjardins et al., 2004).

The published MAT1 and MAT1-2-1 KO mutants were no longer available, so a former graduate student in the Vaillancourt lab used a split-marker and whole casseste replacement protocols to generate KO mutants (KOs) of MAT1, MAT1-1-1, and MAT1-2-1 in the PH-1 strain of *F. graminearum* (Figure 1) (Bec, 2011). These KOs were confirmed by Southern hybridization and evaluated for pathogenicity to susceptible point-inoculated winter wheat (Pioneer 2555) in the greenhouse. Consistent with the previous report (Desjardins et al., 2004), the MAT1 KOs were unaffected in aggressiveness to wheat heads. In contrast, KOs of the individual specificity genes, especially of MAT1-1-1, resulted in significant reductions in aggressiveness and, in most cases, in DON production. These results are unlikely to be due to changes in the expression of genes regulated by the MAT heterodimer, since the complete absence of the heterodimer in the MAT1 KOs has no effect. These conclusions were based on a single greenhouse experiment on soft red winter wheat (SRWW) with only two independent KOs of each type. It was necessary to confirm these results in a larger independent experiment.

The main objective of this work was to investigate the effect of the MAT gene deletions on aggressiveness and mycotoxin production of *F. graminearum* on a susceptible spring wheat variety, Wheaton, and in stalks of a susceptible maize hybrid, Golden Jubilee, with a larger number of independent KO strains in each case.

MATERIALS AND METHODS

Fungal Growth

Additional KO strains were recovered from silica gel stocks prepared by the former graduate student for analysis, and their genotypes and phenotypes were confirmed. All fungal strains were grown on mungbean agar [40 g mungbeans and 10 g Bacto® Agar per L (Bai and Shaner 1996)] at 23 °C for 7 days under constant light (Sylvania F032/741/ECO). Mutant strains were single-spored and stored on silica gel at -20 °C or -80 °C (Tuite, 1969, after Perkins, 1962). Strains were never subcultured more than once.

DNA extraction

To isolate fungal DNA, five mL of YEPD media (20 g dextrose, 10 g Bacto® peptone, 10 g yeast extract per L) in 150 mL flasks was inoculated with an 8-mm plug of an actively growing culture and incubated for 4-5 days at 25 °C with agitation (250 rpm). The mycelial mats were harvested by filtration, frozen at -80°C for one hour, and lyophilized in a freeze-drier. Lyophilized tissue was pulverized with sterile plastic pestle in 15 ml plastic tubes. The DNA was extracted using CTAB method. The DNA pellet was dried for 10 minutes in a transfer hood, then eluted in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) + 10 µl RNaseA at room temperature.

Species-specific PCR marker

The Fg16 primer pair that amplifies a *F. graminearum* complex-specific sequence (Nicholson et al., 1998) was used to confirm species identity for each

isolate. PCR using specific primer sets that amplifies MAT1-1-1 and MAT1-2-1 sequences were used to confirm the KOs in the putative mutant strains (Figure 2).

Sexual fertility

The sexual fertility was confirmed based on the ability of each isolate to produce perithecia and ascospores on carrot agar following a standard protocol with some adaptations (Cavinder et al. 2012). Carrot agar plates (6.0-cm-diameter) were inoculated by placing a 5-µLdrop of a spore suspension (1×10^4 macroconidia mL^{-1}) of each isolate in the center. Plates were incubated in a growth room at 23°C under constant luminosity until the mycelium reached the edge of the plate (around 4 days). Aerial mycelia were gently removed using a toothpick and then 1 mL of 2.5% Tween 80 in water was distributed with a sterile plastic micro-pestle. Plates were returned to the light. At 21 days after induction, perithecia and ascospore production were assessed under a stereomicroscope. Each plate was considered as one replicate and placed on different benches in the growth room (blocks, three plates per isolate). The entire assay was performed once.

Crossing procedure

The heterothallic sexual behavior of the mutant strains was confirmed by crossing them in various combinations. MAT KO strains were crossed by using the mycelial plug method described by Bowden and Leslie (1999). Carrot agar plates were inoculated with 10 µL of 10^4 macronidia suspension of each pairing strain was placed on opposite halves of a 60 mm Petri plate. After four days of incubation at 23° C, perithecial production was induced by applying 1000 µL of 2.5% Tween 60 to the surface of each plate, and gently rubbing the aerial mycelium with a sterile glass rod

to flatten it. Following induction, the plates were incubated at 23° C with constant fluorescent light. At 21 days after induction, perithecia and ascospore production were assessed under a stereomicroscope

Aggressiveness on wheat heads.

Seeds of spring wheat variety Wheaton, which is highly susceptible to FHB, were sown in cone containers filled with a mixture of ProMix BX and topsoil (3:1). Wheat plants were grown in greenhouse with a 14 h photoperiod and temperatures ranging from 25 to 28 °C. Seedlings were fertilized weekly with a solution of Peters 20-20-20 fertilizer (Scotts-Sierra Horticultural Product Co. Marysville, OH) and kept in the greenhouse until flowering. A spore suspension was prepared by growing each isolate on mung bean agar (MBA) for 7-14 days under constant lights. The macroconidia suspensions were filtered through two layers of cheesecloth. The concentration of each macroconidial suspension was then quantified using a hemocytometer adjusted to 1×10^4 macroconidia/mL. The wheat heads were inoculated with a 10-μL drop of the spore suspension (1×10^4 macroconidia/mL) placed inside each lateral floweret of the central spikelet at early- to mid-anthesis. Each head was individually covered with a plastic bag for 24 h and kept in a contained growth chamber set for 25 °C and a 14 h photoperiod until harvest (plant maturity). FHB severity was assessed at four, seven- and 10-days post-inoculation, as the number of FHB symptomatic spikelets per inoculated spike. Twenty replicates (individual head) were used per isolate. The experiment was performed three times. The experiments for the MAT1-1-1 or MAT1-2-1 mutant were performed once and using ten replicates.

Trichothecene production *in planta*.

Harvested wheat heads from the aggressiveness assay were dried at room temperature and kept in a cold room (4 °C) until analysis. Mycotoxin production by each of the 45 isolates was determined by bulking the samples from each experiment and considered as a replicate. First, the entire wheat heads were ground to obtain at least a 5-g sample of each replicate. The ground samples were sent to the Virginia Tech Deoxynivalenol (DON) testing Lab. The amount of DON and its acetylated forms (15ADON and 3ADON), NIV and ZON were quantified using a gas chromatography–mass spectrometry method as described previously (Fuentes et al. 2005; Mirocha et al. 1998).

FHB pathogenicity assay in maize stalks:

Three seeds of the susceptible sweet corn hybrid Golden Jubilee were planted in 25-cm pot filled with a mixture three parts of Pro-Mix BX (Premiere Horticulture Ltd., Riviere du Loup, PQ, Canada) and one part topsoil. One week after seedling emergence, plants were thinned out to retain two plants were per pot. Seedlings were fertilized weekly with a solution of Peters 20-20-20 fertilizer (Scotts-Sierra Horticultural Product Co. Marysville, OH) and kept in the greenhouse until maturity. At silking stage, the second internode above soil line were inoculated with a 20-µL drop of a $1x10^6$ macroconidial suspension, or sterile water as a control, into a 5mm downward diagonal wound. The wound was sealed with Parafilm to create a moist chamber. The inoculated internodes were harvested two weeks after inoculation. Disease were assessed by slitting open the corn stalks longitudinally and measuring the length of tissue discoloration. The experiment was performed twice.

Data analysis

Data were subjected to analysis of variance (ANOVA) by F-test and means were compared by Scott-Knott test ($P < 0.05$). All analyses were performed using R software (R Core Team 2019).

RESULTS

Knockouts confirmation

PCR with specific primers for each MAT gene were mostly consistent with Southern blot results reported in (Bec, 2011). However in a few cases, the results differed from expectations, which may have been due to false negatives or false positives, or to mis-labeling of the strains in the collection (Table 1). Strains that gave variant results were not used for further experiments.

Deletion of any of the three MAT loci resulted in mutant strains unable to reproduce by homothallic "selfing" behaving as obligate heterothallic strains (Figure 9; Table 1). Most, but not all, of the deleted mutant strains were capable of producing small perithecial initials containing no ascospores (Figure 3; Table 1). When crushed, the initials produced by mutant deletion strains contained no asci or ascospores (Figure 3). A cross of mat1-1-1 x mat1-2-1 resulted in normal sized fertile perithecia containing asci with eight ascospores (Figure 3) varying accordingly the crossing strains combination (Table 2). Strains with phenotypes that are inconsistent with expectations were not used for further experiments.

Aggressiveness on wheat heads and DON production *in planta*

Only mutants that gave consistent results by all three methods (Southern blot, PCR and sexual fertility on carrot agar) were used for pathogenicity assays. All mutant

strains were able to produce FHB symptoms on wheat heads (Figure 4). Most (but not all) strains that were less aggressive on SRWW (Bec, 2011) were also less aggressive on Wheaton (Figure 5). When additional strains were tested, there was no consistent reduction in aggressiveness of MAT mutants on Wheaton spring wheat (Figure 5). Overall, reductions in aggressiveness were correlated with reductions in DON production *in planta* (Figure 6).

To further investigate the effect of gene deletion, an additional 17 independent confirmed MAT1-1-1 mutants were tested in a single experiment. Individual mutant strains were consistent between experiments on Wheaton. However, only six (35%) were statistically reduced in aggressiveness (Figure 7). For MAT1-2-1, the PH1 WT strain did not behave as expected being less aggressive compared to the same strain in all the previous assays. Hence, it prevented us to conclude about the effect of MAT1-2-1 deletion when testing several independent mutants (Figure 8). However, individual mutant strains were consistent between experiments (Figure 5 and 7).

Aggressiveness on maize stalks

In comparison with the wild type, none of the mutants had statistical changes in aggressiveness in infection assays with maize stalks (Figure 9). However, it was noticed that individual MAT1-1-1 and MAT1-2-1 mutants varies in aggressiveness (Figure 9).

DISCUSSION

The current study focused on exploring the potential role of MAT genes in pathogenicity-related phenotypes of *F. graminearum*. Previous studies have shown a

direct role of MAT genes in pathogenicity among other fungi where mating-type genes are known to act directly as virulence factors (Zhan et al., 2007ab). Our results showed a surprisingly large amount of variation among individual MAT gene knockout transformants of *F. graminearum.* We confirmed that MAT KO mutants were not able to self, nor could they mate with either of the idiomorph KO strains. In contrast, the MAT1-1-1 and MAT1-2-1 KO strains were fertile but obligately heterothallic. It was found that when out-crossing mat1-1-1 x mat1-2-1 normal sized fertile perithecia containing asci with eight ascospores where produced. Interestingly, different combinations of strains resulted in different degrees of fertility. A previous study has reported that mat1-1-1 mutant displayed male-specific defects in mating although it was normal in female fertility whereas the mat1-2-1 deletion mutant displayed a female specific defect in the production of normal perithecia and ascospores in out-crosses with the resulting perithecia varying in size and fertility (Zheng et al., 2013).

In *F. graminearum,* the deletion the entire MAT1 locus of the Gz3639 strain was found that the mutants were infertile and lost their ability to out-cross with fertile strains (Desjardins et al., 2004). As expected, the majority of the PH-1 MAT KO mutants used in the present work were self-sterile, but capable of producing perithecial initials. This could be evidence that the MAT genes do not control the ability of *F. graminearum* to initiate development related to fertility, but only to produce asci and ascospores.

Previous study shown that although the MAT locus gene deletion mutants were unaffected in aggressiveness towards wheat heads, was noticed that the mat1-1-1 and mat1-2-1 mutants were reduced in aggressiveness in maize stalks (Zheng et al., 2013). The mat1-1-1 and mat1-2-1 mutants may be defective in adaptation to or

colonization of corn stalks, which are substrates for *F. graminearum* to produce perithecia in the field (Zheng et al., 2013). However, there was no consistent evidence that the MAT genes directly regulate aggressiveness to point-inoculated spring wheat and maize stalks. Our results may suggest that the effect of MAT genes deletion on aggressiveness in either wheat heads or maize stalks may be a strain-specific phenotype. However, the results of the pathogenicity assays in maize stalks need to be confirmed using more independent mutants. Once, in order to conclude about the effects of mating-type genes in *F. graminearum* it is important to further characterize multiple strains to better understand the underlying mechanisms of this large strain-specific variation.

We found that DON production was highly correlated with FHB intensity. This means that reductions in aggressiveness resulted in reductions in DON production in infected wheat heads. This is evidence that the deletion of the MAT genes had not direct effect on DON production *in planta*. DON is the best-known and most well-researched mycotoxin of *F. graminearum.* In fact, DON has been confirmed as an aggressiveness factor that facilitates fungus spread within wheat heads and maize ears in several studies (Bai et al. 2002; Desjardins et al. 1996; Harris et al. 1999; Maier et al. 2006).

More work is needed to confirm the results of the present chapter and to follow up on these findings including microscopic investigation of the infection process of knockout strains versus the wild type. In summary, there was no consistent evidence that the MAT genes directly regulate aggressiveness to point-inoculated spring or winter wheat and maize stalks due to the surprisingly large amount of variation among individual MAT gene knockout transformants of *Fusarium graminearum.* Unlinked random mutations may be responsible for the reduced aggressiveness of

some mutants and it is important to continue to evaluate other phenotypes of the confirmed KO mutants. The strains can be useful for genetic studies in the future to analysis of the role of MAT genes individually and together in mating and in regulation of genes that may be important for pathogenicity or aggressiveness.

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FIGURES

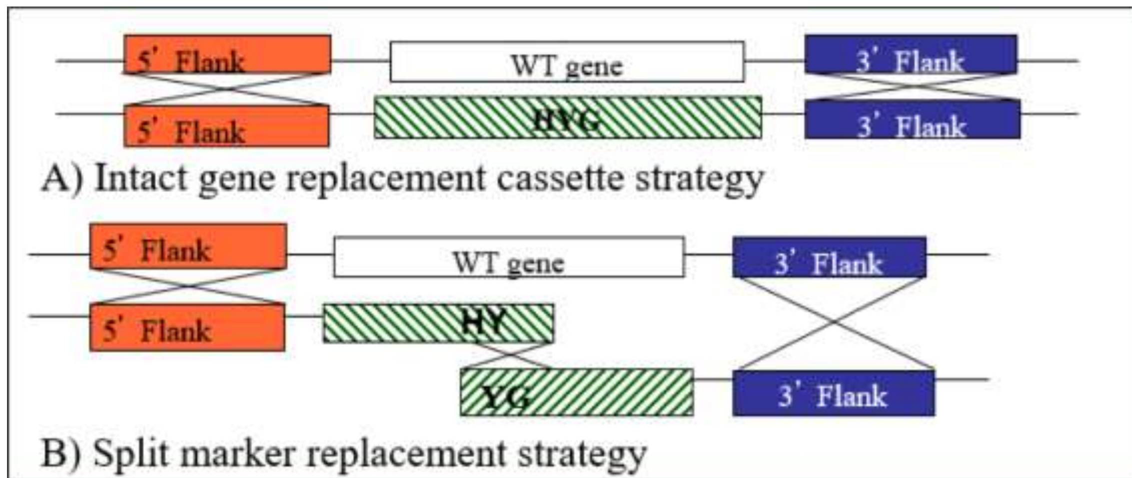

Figure 1. Gene replacement strategies used to generate MAT mutant strains. Adapted from Sladana Bec dissertation (2011).

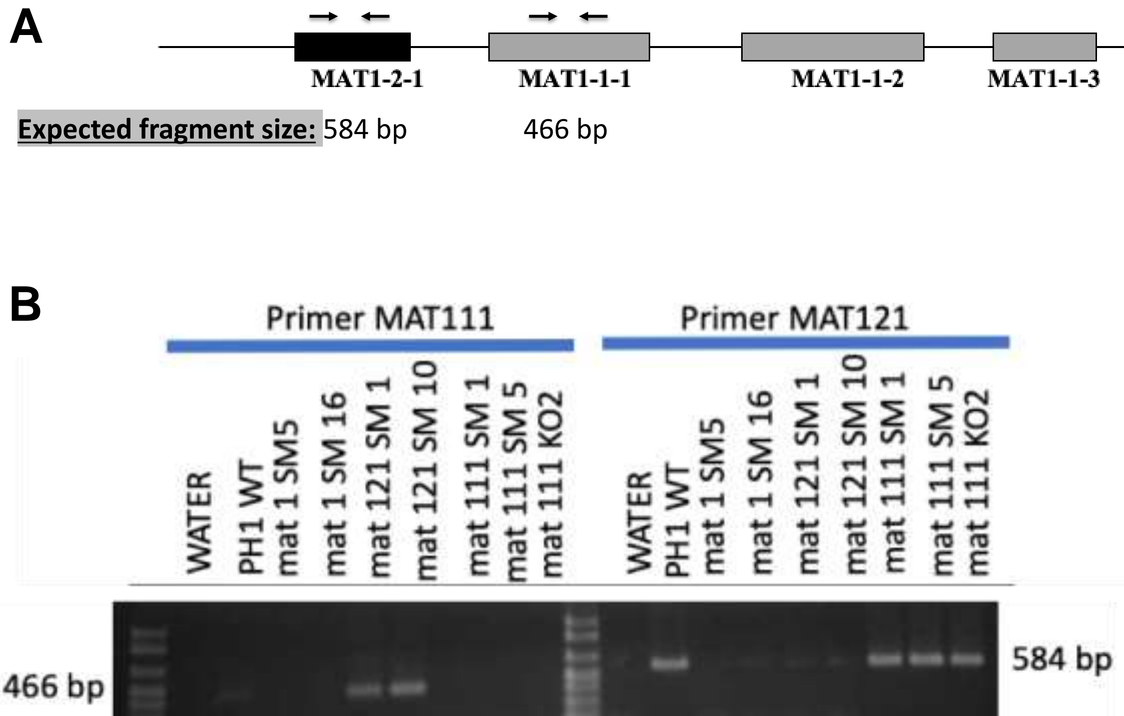

Figure 2: A) Region amplified by the primers MAT1-1-1 (Forward: AGTCCGAATGAAGCCCCAATACC; Reverse: CAGAACTTGCAGGTGCTGGGAGT), and MAT1-1-1 (Forward: TCTTCCACCCCCTGTGTCTACCA
; Reverse: TGCGAATGTCAGGATGCTCCA). **B**) Example of the assay for MAT deletion using PCR with specific primer sets. Arrows indicates the position where the primers sits.

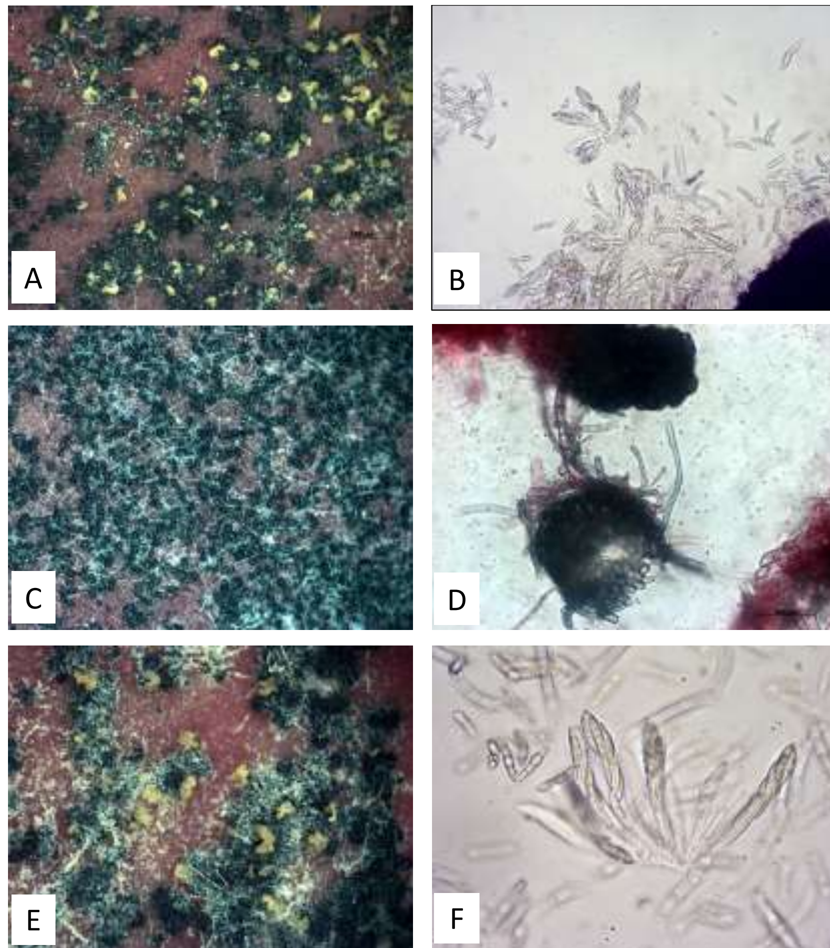

Figure 3. Phenotype on carrot agar at 23 ℃ incubated for 10 days post induction of wild-tyoe strain PH-1 (A-B), MAT mutant (C-D) and a cross between MAT1-1-1, and MAT1-2-1 (E-F) mutants.

Figure 4. Photographs of heads of the effect of the MAT genes deletion on the development of external FHB symptoms on the susceptible spring wheat cv. Wheaton. The images show FHB symptoms 7 days post-inoculation.

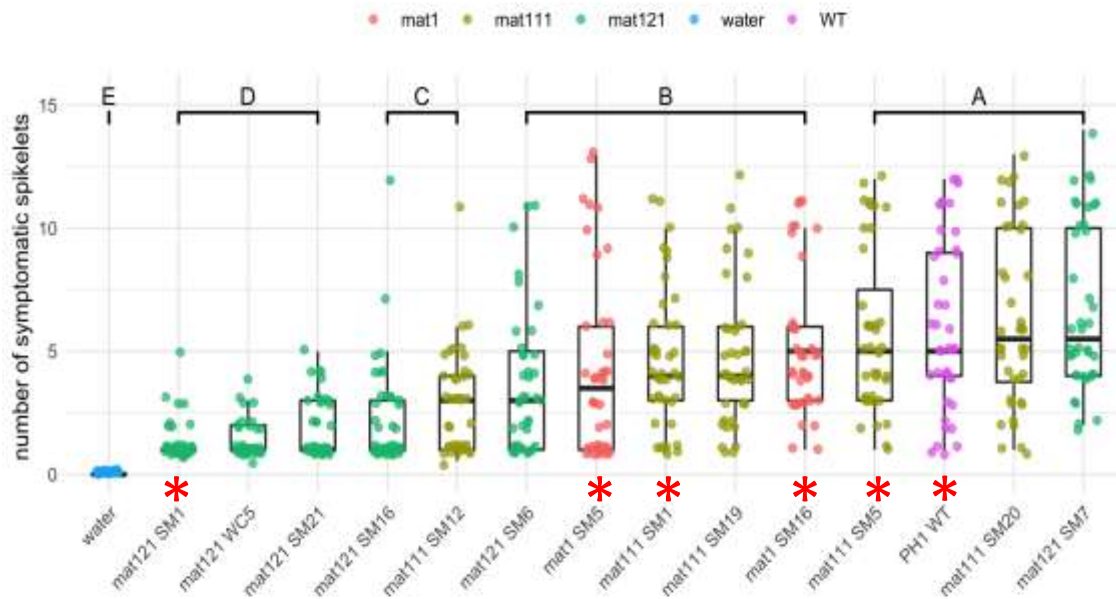

Figure 5: Effect of MAT locus deletions on aggressiveness to Wheaton spring wheat. [Statistically significant at α = 0.05]. Data are combined from two trials (*n* = 20) on cultivar Wheaton at 7 dpi. Strains behaved similarly in both trials. Asterisks indicate strains used for pathogenicity tests on Pioneer 2555 SRWW. Letters indicate Scott-Knott groups that are statistically different (α = 0.05).

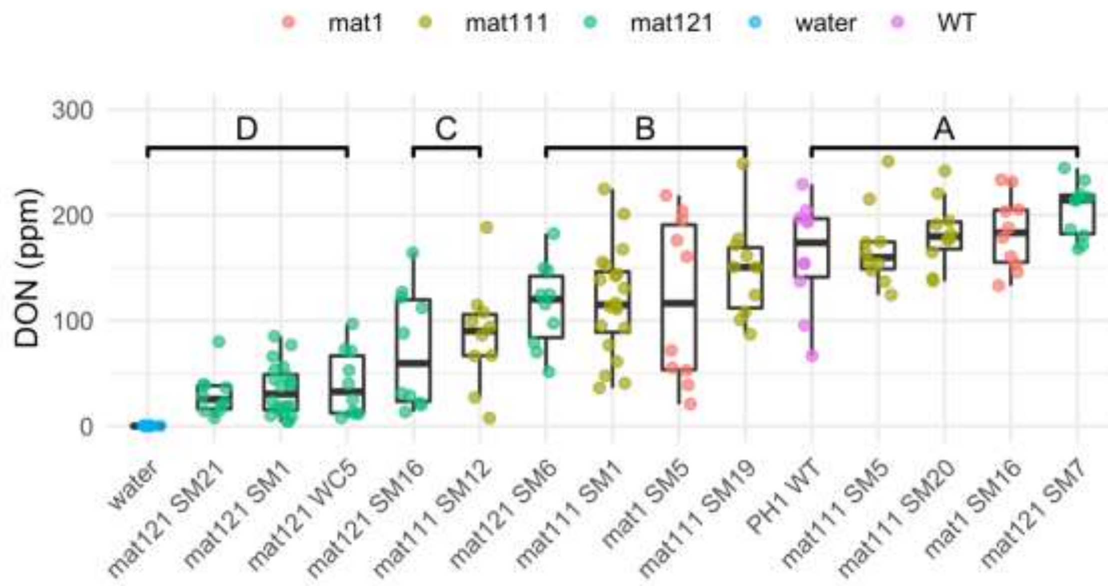

Figure 6: Effect of MAT locus deletions on the level of DON production on Wheaton spring wheat. Data are combined from two trials (*n* = 20) on cultivar Wheaton at 7 dpi. Strains behaved similarly in both trials. Each data point represents DON levels of four heads ground and pooled together. Letters indicate Scott-Knott groups that are statistically different (α = 0.05).

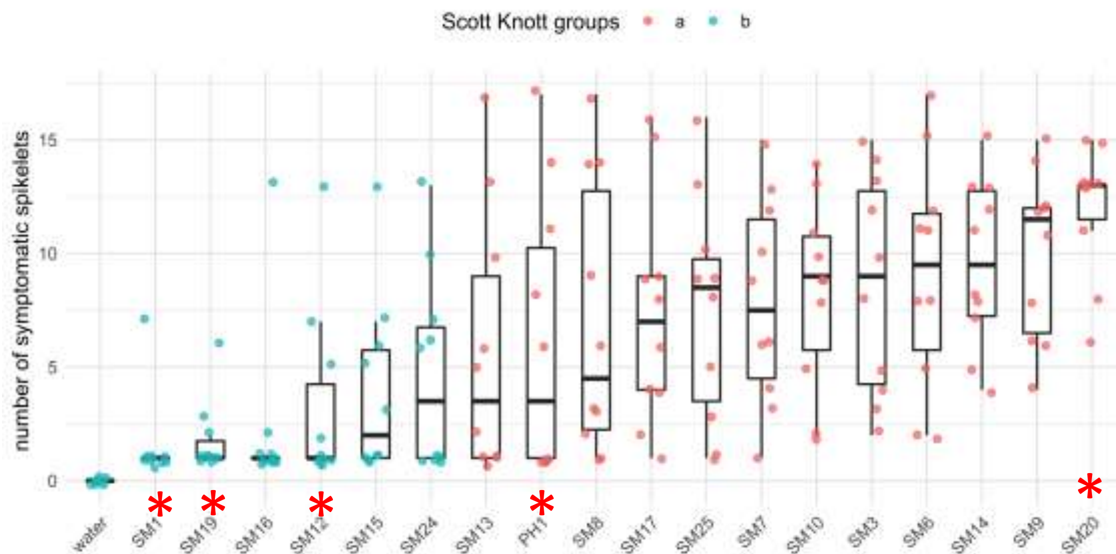

Figure 7: Effect of MAT1-1-1 deletion of multiple strains on aggressiveness on spring wheat cultivar Wheaton at 10dpi (*n* = 20). This experiment was done once. [Statistically significant at α = 0.05]. Red and blue dots indicate groups that are significantly different. Asterisks indicate strains that were also used in the previous experiments on Wheaton.

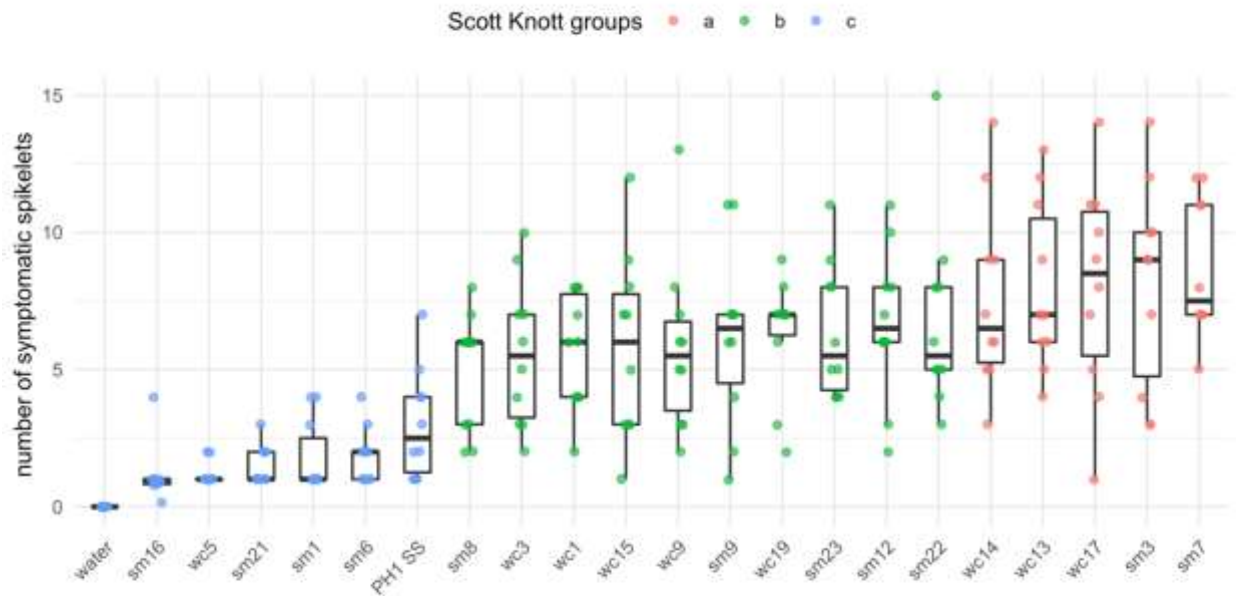

Figure 8: Effect of MAT1-2-1 deletion of multiple strains on aggressiveness on spring wheat cultivar Wheaton at 10dpi (n = 20). This experiment was done once. Asterisks indicate strains that were also used in the previous experiments on Wheaton. Other strains were "clean" knockouts. Colored dots indicate Scott-Knott groups that are statistically different (α = 0.05).

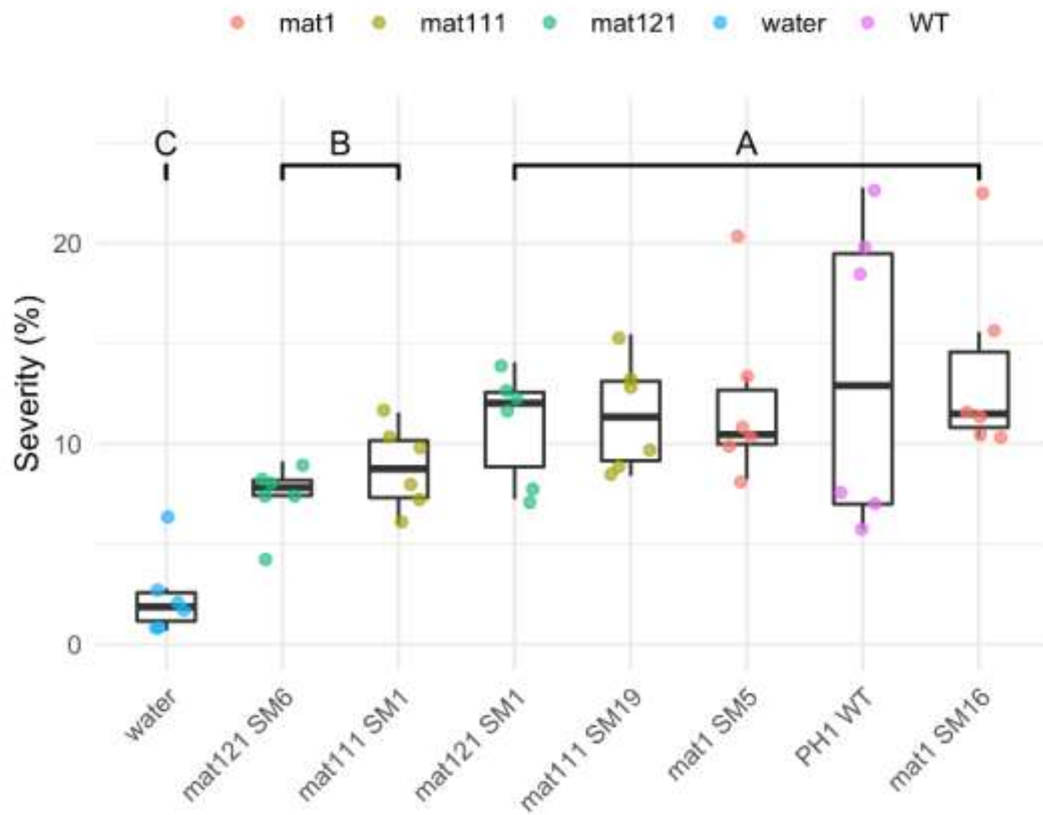

Figure 9. Effect of MAT locus deletions on aggressiveness on maize stalks cultivar Golden Jubilee, at 14 dpi (*n* = 16). This experiment was done twice. Strains behaved similarly in both trials. Letters indicate Scott-Knott groups that are statistically different (α = 0.05).

TABLES

Table 1. Summary of knockout mutant confirmation using specific PCR primers and on carrot agar.

	Mutation method	Number of strains	Infertile on carrot agar	PCR confirmation
MAT 1	SM	-	-	-
	WC	25	20	0
MAT 1-1-1	SM	25	16	17
	WC	-	-	-
MAT 1-2-1	SM	24	10	14
	WC	25	9	11

Table 2. Pairwise crosses of MAT1-1-1 and MAT1-2-1 mutants on carrot agar.

	MAT1-1-1 mutants[a]						
MAT1-2-1 mutants	**SM1**	**SM1-J**	**SM5-J**	**SM12**	**SM19**	**SM20**	**KO2**
WC5	prot.	+	prot.	prot.	prot.	prot.	prot.
SM1	+	prot.	+++	+++	+	prot.	+++
SM6	+	+	+	+	+	prot.	++
SM7	+++	+++	+++	+++	+	+++	++
SM16	+	mycelia	mycelia	mycelia	mycelia	+	prot.
SM21	+++	+++	+++	+++	+++	+++	+++

[a] prot. = protoperithecia, empty, with no ascospores; mycelia = presence of mycelia only; +, ++, +++ = classes of perithecia and ascospores production.

CURRÍCULO DA AUTORA

Aline Vieira de Barros, filha de Sebastião Eurestes de Barros e Rosani Fernandes Vieira de Barros, nasceu em 22 de abril de 1991, em Senador Firmino, Estado de Minas Gerais. Em 2009, ingressou no curso de Agronomia da Universidade Federal de Viçosa (UFV) onde lhe foi conferido o título de Engenheiro Agrônomo, no ano de 2014. Nesta instituição, foi bolsista de Iniciação Científica em Fitopatologia sob orientação do Prof. Dr. Laércio Zambolim. Em março de 2014, iniciou o curso de Mestrado em Fitopatologia na Universidade Federal Viçosa sob orientação do Prof. Dr. Fabrício Ávila Rodrigues, defendendo dissertação com o título em fevereiro de 2016. Em abril de 2016 começou o doutorado em Fitopatologia na Universidade Federal de Lavras sob orientação do professor Eduardo Alves tendo oportunidade de treinamento internacional com duração de um ano na University of Kentucky sob orientação da Prof. Dra. Lisa Vaillancourt.