AMANDA FLAUSINO DE FARIA

UNDERSTANDING PITFALLS AND PROPOSING STRATEGIES TO OPTIMIZE THE BIOLOGICAL CONTROL OF WHITE MOLD (*SCLEROTINIA SCLEROTIORUM*) IN SOYBEAN

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ENTENDENDO ARMADILHAS E PROPONDO ESTRATÉGIAS PARA OTIMIZAR O CONTROLE BIOLÓGICO DO MOFO BRANCO (*SCLEROTINIA SCLEROTIORUM*)

Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Graduate Program in Agronomy/, for the degree of Doctor in Phytopathology.

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ENTENDENDO ARMADILHAS E PROPONDO ESTRATÉGIAS PARA OTIMIZAR O CONTROLE BIOLÓGICO DO MOFO BRANCO (*SCLEROTINIA SCLEROTIORUM*)

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

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À DEUS primeiramente,

À minha família,

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ABSTRACT

We evaluated during seven crop seasons; 74 uniform field trials conducted throughout Brazil to assess the performance of biological control products on the reduction of the carpogenic *Sclerotinia sclerotiorum* sclerotium germination. The mean difference (MD) by three products classes (*Trichoderma*, *Bacillus* and Mixed) was summarized (59 trials, k = 340). Separate network meta-analysis to determine the influence of three classes (low, moderate, and high) of average monthly temperature (AMT) and average monthly rainfall (AMR) on biocontrol performance. All three product classes significantly reduced carpogenic germination, with fitted $\overline{MD}$ values of -10.6270 for *Bacillus*, -8.5928 for *Trichoderma*, and -7.0177 for Mixed. For (AMT) and (AMR), only interactions of genus and low or moderate AMT and AMR were statistically significant from zero ($p < 0.1$), indicating that high AMT and AMR affected biocontrol of carpogenic germination. The second manuscript, we evaluated carpogenic germination, colonization and crop yield, on soybean and succeeding crops (Wheat and Maize). Here, we found sclerotia germination rates from 31.0% to 61.6%, (P = 0.583) in soybean crop. Colonization by BCAs from 4.7% to 19.4% (P = 0.174). Also, biological and chemical application disease incidence (P = 0.520), range from 73.7% to 87.2% and yield production from 2001 to 2803kg/ha. For succeeding crops, carpogenic germination interaction (crop x treatments) effect was observed (P=0.328). However, the treatments reduced carpogenic germination (P=0.011), control was 65.7% and treatments until 47.0%. For Crop factor (p=0.022), maize germination rate from 51.71% to 63.85% and wheat crop from 39.24% to 62,65%. Evaluated colonization, treatments (P=0.157), crop (p<0.001) and interaction (crop x treatment) (p=0.048). For colonization in Maize range from 2.23% to 10.15 %. For Wheat, treatments range from 16.6% to 39.8%. Wheat yield production (p=0.177) and Maize yield (p=0.593). The last part of our study, we investigated that effect of dressing-seed by *Trichoderma* sp. to biological agent can growth endophytically and promote tissue development on wheat plants. Additionality, viability of *Trichoderma* persists on tissues after Glyphosate applied and their ability to colonize sclerotia of *Sclerotinia sclerotiorum*. At the 7^{th} day after planting shoot (P = 0.318) however root (P = 0.010) increased 28%, and at 20^{th} day after planting, for aerial parts (P=0.049). Plants treats with *Trichoderma* sp. increased shoot length by 10,7%. Additionally, root length (P = 0.008) increased by 18,7%. *T. asperellum* levels detected on roots at 20 days after planting up to 1×10^{4}. Followed by roots at 7 days after planting up to 1×10^{3}. For shoots at 7 and 20 days, up to 1×10^{2}. After Glyphosate, the presence of *T. asperellum* decreased to 1×10^{2} root, and to 1×10^{1} in the bottom leaf. Carpogenic germination (P = 0.419) that control (31.2%) and *Trichoderma* (26.7 %). Thus, its stability and improved efficacy can be improved considering the dominant whether (rainfall and temperature) and cover crop (wheat). In turn, wheat may serve as a crop to selective buildup the population of the antagonist.

Keywords: *Sclerotinia sclerotiorum*. Cultural and biologic practices. Network meta-analysis. Endophyte.

RESUMO

Foram avaliados durante sete safras; 74 ensaios de campo uniformes realizados em todo o Brasil para avaliar o desempenho de produtos de controle biológico na redução da germinação carpogênica de *Sclerotinia sclerotiorum*. A diferença média (DM) por três classes de produtos. A diferença média (DM) por três classes de produtos (*Trichoderma*, *Bacillus* e Mixed) foi resumida (em 59 ensaios, k = 340). Meta-análise de rede separada para determinar a influência de três classes (baixa, moderada e alta) da temperatura média mensal (TMA) e precipitação média mensal (TMA) no desempenho dos agentes de biocontrole. Todas as três classes de produtos reduziram significativamente a germinação carpogênica, com valores ajustados de $\overline{MD}$ -10,6270 para *Bacillus*, -8,5928 para *Trichoderma* e -7,0177 para Mixed. Para (AMT) e (AMR), apenas as interações de gênero e AMT baixa ou moderada e AMR foram estatisticamente significativas a partir de zero ($p < 0,1$), indicando que altos TMA e TMA, afetaram o biocontrole da germinação carpogênica. No segundo artigo, avaliou-se a germinação carpogênica, colonização e produtividade em soja e culturas subsequentes (trigo e milho). Neste trabalho, foram encontradas taxas de germinação de escleródios de 31,0% a 61,6%, (P = 0,583) na cultura da soja. A colonização por BCAs variou de 4,7% para 19,4% (P = 0,174). Além disso, a incidência da doença com aplicação biológica e química (P = 0,520) variou de 73,7% a 87,2% e a produtividade de 2001 a 2803kg/ha. Para as culturas subsequentes, observou-se efeito da germinação carpogênica na interação (cultura x tratamento) (P=0,328). Entretanto, os tratamentos reduziram a germinação carpogênica (P=0,011), o controle foi de 65,7% e os tratamentos até 47,0%. Para o fator Safra (p=0,022), a taxa de germinação do milho de 51,71% a 63,85% e a do trigo de 39,24% a 62,65%. Foram avaliados colonização, tratamentos (p=0,157), cultura (p<0,001) e interação (cultura x tratamento) (p=0,048). Para a colonização em milho variam de 2,23% a 10,15%. Para o trigo, os tratamentos variam de 16,6% a 39,8%. Produtividade de trigo (p=0,177) e milho (p=0,593). Na última parte de nosso estudo, investigamos o efeito de tratamento de sementes por *Trichoderma* sp no desenvolvimento endofitico do bioagente e desenvolvimento dos tecidos das plantas de trigo. Também, a viabilidade de *Trichoderma* persistir nos tecidos após a aplicação de Glyphosate e sua capacidade de colonizar escleródios de *Sclerotinia sclerotiorum*. Aos 7 dias após o plantio a parte aérea (P = 0,318), aumentou em 28% o desenvolvimento da raiz (P = 0,010) e aos 20 dias após o plantio, para a parte aérea (P=0,049). Os tratamentos com *Trichoderma* sp aumentaram o comprimento da parte aérea em 10,7%. Adicionalmente, o comprimento da raiz (P = 0,008) aumentou 18,7%. Níveis de T. *asperellum* detectados nas raízes aos 20 dias após o plantio até 1×10^{4}. Seguido de raízes aos 7 dias após o plantio até 1 x 10 3. Aos 7 e 20 dias, a detecção de até 1×10^{2}. Após aplicação de Glifosato, a presença de *T. asperellum* diminuiu para 1×10^{2} raízes e para 1×10^{1} na folha inferior. Germinação carpogênica (P = 0,419) que controle (31,2%) e *Trichoderma* (26,7 %). Assim, sua estabilidade e eficácia podem ser melhoradas considerando o domínio (precipitação e temperatura) e a cultura de cobertura (trigo). Por sua vez, o trigo pode servir como cultura para o acúmulo seletivo da população do antagonista.

Palavras-chave: *Sclerotinia sclerotiorum*. manejo cultural e biológico. meta-analises multivariada. Endofíticos.

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SUMMARY

1 CHAPTER 1: LITERATURE REVIEW

1.1 Background

Brazil was the most important producer of soybean in worldwide (CONAB, 2021). Among the diseases that can compromise crop productivity, white mold was able to reduce up to 70%. The pathogen has high genetic variability and can infect a wide range of hosts (MERRIMAN et al., 1976; BOLAND; HALL, 1994). The survival phase of the *Sclerotinia sclerotiorum* cycle is production of sclerotia, resistance structures that remain viable in the field for many years (ABAWI et al., 1979; BRUSTOLIN et al., 2016).

Disease management in Brazil was based on the integration of strategies. Cultural management and biological control are tools that can contribute to reduce the initial source of *Sclerotinia sclerotiorum*. The occurrence of the pathogen into production fields and its spread has been related to contaminated seeds. Samples of soybean seeds can contain either sclerotia presence or mycelium on internal teguments. Certificated and treated soybean seeds are necessary to regulate source of *Sclerotinia sclerotiorum* new introductions (HENNEBERG et al., 2012; JUHÁSZ et al., 2013; JULLIATI et al., 2015).

Proper row space and cultivars can reduce favorable conditions to disease development. Humidity and luminosity are able to modify the microclimate in aerial part and result in reduce the development of white mold disease. Crop rotation with non-host species like grasses, are important to reduce the carpogenic germination of sclerotia, and it was provided ground cover in the off-season (PENNYPACKER et al., 1999; SOUZA JACCOUD-FILHO et al., 2016).

Maize, wheat and grasses were effective to alter the dynamics of apothecia production. High C:N ratio, soil moisture maintenance, and physical barrier was important to reduction of disease incidence under field conditions (GRAU, 1988; CIVARDI et al., 2009; MEYER et al., 2014).

Biological control-based products play an essential role on the disease management system. In Brazil, around of 33 products are registered for the management of *S. sclerotiorum*. The active ingredients were based either *Trichoderma* and *Bacillus* genera or combine both (BETTIOL et al., 2009; AGROFIT, 2021). Bioproducts formulations were compound of the active ingredients, adjuvants and inert molecules, which improve dispersion and condition of establishment microorganism (VERMA et al., 2007).

Trichoderma spp. present larger number of mechanisms of action against *Sclerotinia sclerotiorum*. Parasitism, antibiosis and competition for nutrients make the genus a competitive potential against the pathogen (INBAR et al., 1996). Also, *Bacillus* spp. has effectives mechanisms of action such as antibiosis and emission of volatile molecules against the pathogen (CAWOY et al., 2011). Furthermore, both genera have secondary mechanisms of action that trigger plant resistance induction or growth promotion (TSAVKELOVA et al., 2006; MACHADO et al., 2012; 2015). Biocontrol based-products was evaluated under field conditions by network trials in many states of Brazil (MEYER et al., 2014).

Considering the effectiveness of both strategies on disease management, the use of grasses and biocontrol agents were a great potential to favor the permanence of biologic agents on system and contribute to *Sclerotinia sclerotiorum* management. In France, Wheat crop combined to application of *Coniotridium minitans* was reduced *Sclerotinia sclerotiorum* carpogenic germination (PENAUD et al., 2003).

In Brazil, the use of *Trichoderma* spp. in *Brachiaria* grass, was resulted in reduction of sclerotia carpogenic germination. Also, a decrease in the incidence of disease levels and grass growth promotion was observed (GÖRGEN et al., 2008). A novel trend of biological disease management was shown, in which a screening of endophytes from *brachiari*a grass was antibiosis and competition against *Sclerotnia sclerotiorum* (GAMA et al., 2020).

Those isolates that demonstrated antagonistic potential against pathogen were evaluated by *in vitro* and *in vivo* assays. The volatile molecules production was reduced sclerotia carpogenic germination, and the antagonism effect on mycelial growth of pathogen was demonstrated *in vitro*. Endophytes was exposed to glyphosate doses, and it was able to recolonize sclerotia after this exposition (ALVES et al., 2021).

It can be a novel tool to *Sclerotinia sclerotiorum* management on field conditions. Thus, consider the soybean production system as a whole, enhancing the conditions of bioagents establishment and permanence, may reflect in greater control of White mold disease.

1.2 Sclerotinia sclerotiorum

1.2.1 Pathogen description and history

White mold disease or white stem rot was caused by the etiologic agent *Sclerotinia sclerotiorum* (Lib.) of Bary. The pathogen was described on kingdom *Fungi*, phylum *Ascomycota*, class *Discomycetes*, order *Helotiales*, family *Sclerotiniaceae*, genus *Sclerotinia*. Hyaline and septate hyphae contain many branches, it was morphological features of the pathogen. After grown hyphae and mycelium formation, the colony has a white cottony appearance. At *Helotiales* order, *Sclerotinia sclerotiorum* forms resistance structures (sclerotia). Apothecium (fruiting body) was formed from sclerotia, and it was able to produce many infective ascospores (KOHN, 1979; BOLTON et al., 2006).

More than 400 species of plants was susceptible to its infection. Dicotyledonous subclass that pretense Herbaceous species, are the most common host of pathogen. Although it was reports on monocotyledons subclass (BOLAND; HALL, 1994). Weeds, forage plants and interest economic crops such as soybeans, are part of its host range (PHILLIPS et al., 1992; OLIVEIRA et al., 2015). While the pathogen cannot infect corn and small grasses (ROUSSEU et al., 2007). Furthermore, *S. sclerotirum* has high genetic variability among isolates, which makes its management was a big challenge (GARCIA et al., 2012; MACHADO et al., 2017).

The first studies of *Sclerotinia s.* as a causal agent of disease were in 1873 (PURDY et al., 1979). The first Brazilian report of the pathogen was in São Paulo state, that *S. sclerotiorum* infect potato plants in 1921 year (SACCÁ et al., 1937). In 1976, the presence of *S. sclerotiorum* on soybean production fields was reported in Paraná state (MENTEN et al., 1995). Also, the epidemic reduce in soybean productivity was reported by Ferreira et al. (1981) in some state. Currently, the main grain producing regions were cultivated with the presence of the pathogen. It was corresponding about 10 million hectares (JULIATTI, 2010; MEYER, 2019).

1.2.2 Epidemiology

Sclerotinia sclerotiorum was classified by host-pathogen relationships as monocyclic. It completes live-cycle and forms resistance structures, that will be a source in the subsequent soybean season. The presence of initial source added to favorable

environmental conditions and plant susceptibility, causes the development of the disease (BOLAND et al., 1988; WILLBUR et al., 2019). Survival phase

Sclerotia was a resistance structure formed by melanin deposition in a cluster of hyphae rich in β-1,3-glucans and proteins (LE TOURNEAU, 1979; VÁZQUEZ-GARCIDUEÑAS et al., 1998). Amount the formation process has three distinct stages: initiation, growth and maturation of the structure. At Maturation stage, that color varies from light to dark tones, due to the deposition of the melanin layer. Melanin confers resistance to structure (LE TOURNEAU et al., 1979).

It has an irregular shape and variable diameter according to the culture, that was produced (BOLTON et al., 2006). The viable sclerotia in the soil in that absence of host species can occur for consecutive years. This fact makes difficult management of pathogen in field conditions (MERRIMAN et al., 1976; BRUSTOLIN et al., 2016).

1.2.3 Dissemination

Soybean seeds was the main form of dissemination of *Sclerotinia sclerotiorum*. Dissemination occurs via the presence of sclerotia either seed lots or the presence of pathogen mycelium inside the internal membranes. Contaminated agricultural implements, rainwater or irrigation are known of spreading the pathogen (PELTIER et al., 2012; WALKER et al., 2020).

1.2.4 Infection and tissue colonization

Carpogenic germination was the main source of inoculum for the development of the disease (BOLTON et al., 2006). It is influenced by environmental factors such as the incidence of light, temperature and soil moisture (ABAWI et al., 1979; TEO et al., 1985). These factors on carpogenic germination varies according to the geographic origin of *S. sclerotiorum* isolates. If it was origins from temperate or subtropical regions, require varies conditions to germinate (HUANG et al., 1991; GODOY et al., 2017).

In Brazil, the combination of temperatures between 15 and 25 C^{o}, soil moisture close to field capacity, and the shading of the aerial part of the soybean, were conditions in that favor the production of apothecia (JACCOUD FILHO et al., 2017; MEYER et al., 2019). The number of apothecia produced can varies according to the size of the sclerotia,

and according to Dillard et al. (1995), a larger diameter can produce a greater number of apothecia.

In a period of ten to fourteen days, an apothecium can release millions of ascospores, which were disseminated via rain or wind to length distances (STEADMAN, 1979). Ascospores are the main source of pathogen infection and when released between flower formation (R1) and pods (R5), that can cause infection in soybean crops (ABAWI et al., 1979; BOLAND et al., 1988; DANIELSON et al., 2004).

The host infection process begins, when the ascospores were use mucilage to fix the flower structure, that was a source of substrate for its germination. Ascospore germination occurs at mild temperatures and high relative humidity conditions. After germination, it is able to hypha development and appressorium formation (SUTTON et al., 1983). Whereas, by penetrating on tissue, the process of cell colonization begins. The action of metabolites such as oxalic acid and lytic enzymes will be result in host tissue cell death (BOLTON et al., 2006).

Oxalic acid (OA) was the main virulence factor of *S. sclerotiorum* Maxwell; Lumsden (1970), that shown the defective mutants to OA do not cause disease symptoms (GODOY et al., 1990). Many studies explore the role of oxalic acid in host metabolism (BOLTON et al., 2006). Nonetheless, one of the most important functions that OA performs was the reduction of pH.

It can change range pH to 4-5, in which optimum value for the action of enzymes that degrade the cell wall (MAGRO et al., 1984; KIM et al., 2008; WILLIAMS et al. 2011; MCCAGHEY et al., 2019). Lytic enzymes such as pectinases, β-1,3-glucanases, glycosidases, cellulases, xylanases and cutinases were secreted by *Sclerotinia sclerotiorum.* Those enzymes can act different components of the plant cell wall, in which cell maceration result in tissue death (ANNIS et al., 1997; WHEELER, 1975; RIOU et al. 1991).

1.2.5 Reproduction

The pathogen has a sexual and asexual reproduction system. The asexual phase began at sclerotia germinate to form a mycelium. Hyphae growth will not result in conidia production. The mycelium can stay under the straw and under specific conditions, generate the infection of plants by direct contact, although this type of infection was not common (AMSELEM et al., 2011; HUZAR-NOVAKOWISKI et al., 2017).

Mycelial growth on Plant tissues, was regulated by many factors such as pH, nutrient availability and O2. These factors can influence on the resistance structure formation process. At low availability conditions, the pathogen was able to begin the hyphae fusion process in which to form new resistance structures (FORD et al., 1995; GLASS et al., 2004). The carpogenic germination as known the sexual phase of *Sclerotinia sclerotiorum*. It was defined when sclerotia form the apothecia. The ascocarps will be produce the infectives spores (ascospores) (LE TOURNEAU et al., 1979).

1.2.6 Symptoms

The symptoms start from the R3 reproductive stage of soybean (GRAU et al., 1994). That can occur in different plant organs such as stem, armpits, leaves and pods. The lesion was watery appearance and light brownish color. According to severity levels of disease will be evolves in size and color shade. Subsequently, the mycelial growth of the pathogen takes place in the host structures, that shown the mold, the most characteristic symptom of the disease. With mycelial growth, hyphae can fuse and give rise to new sclerotia in the colonized organs. Resistance structures return to the crop soil either at harvest or disseminated associated with seeds (GRAU et al., 1994; HEGEDUS et al., 2005).

The development of epidemics under field conditions is mainly related to inoculum density. For this reason, white mold disease management practices should preferably aim to reduce the initial source of *Sclerotinia sclerotiorum* (MEYER et al., 2016).

1.3 Cultural management

Estimates for the 19/20 harvest confirmed that Brazil was the world's largest producer of soybean. That number of 135 million tons was produced in an area of 38.2 million hectares (CONAB, 2021). Among the diseases that affect crop yield, White Mold causes significant crop yield losses. The presence of the etiological agent *S. sclerotiorum* is estimated in 27% of the areas to grain production. The management in Brazil is based on the integration of practices, including cultural and biological (BETTIOL et al., 2009, MEYER et al., 2014; JULIATTI et al., 2015).

Cultural practices can modify soil biotic and abiotic factors, and important tool to diseases caused by soil pathogens (KATAN et al., 1996). The cultural measures in the management of *Sclerotinia sclerotiorum* can contribute (i) to the prevention of the entry of the inoculum source in the field (ii) reduction favorable conditions to the development of the disease (iii) reduction of the initial source (HENNEBERG et al., 2012; JACCOUD-FILHO et al., 2016; GÖRGEN et al., 2008).

One of the main ways that spread pathogen was the presence of sclerotia in seed lots (YANG et al., 1998). The tolerance level according to the Ministry of Agriculture of Livestock and Supply, *Sclerotinia s.* on commercial soybean seed lots is “zero” (MAPA, 2009). Nonetheless, seeds can be infected by mycelium on internal tegument (YANG et al., 1998). The development of diseased seedlings can result in new sclerotia, that will be deposited in the field (JUHÁSZ et al., 2013). Thus, the use of certified and treated seeds can contribute to reduce the risk of the pathogen introduction and dissemination (HENNEBERG et al., 2012; TANCIC et al., 2013).

Row space and number of plants per row can contribute to the disease management (PENNYPACKER et al., 1999). The proper space presents the lowest White Mold severity (JACCOUD-FILHO et al., 2016). There are greater penetration of light, air and reduced soil moisture, in which influence the sclerotia germination (ADAMS, 1975; MCDONALD et al., 2013). Adequate plant populations can reduce the favorable microclimate for disease development. It is either smaller leaf area, increased light intensity or aeration on aerial part (HEIFFIG et al., 2006; JACCOUD-FILHO et al., 2016).

Although there were no cultivars with complete resistance to *S. sclerotiorum*, the adoption of suitable cultivars can contribute to disease management, both by plant architecture and by cycle length. The adoption of architecture cultivars that favors air circulation and light penetration. It can reduce favorable conditions to development of the disease (MEYER et al., 2014). Plants that early cycle can flower in a shorter time, and do not enter on window of pathogen infection. Nonetheless, late cycles plants can develop more leaves in vegetative growth. This makes the conditions for the occurrence of the disease more favorable (BOLAND; HALL, 1986; GRAU, 1988).

No-till system can contribute to inoculum reduction. The no-tillage system is the practice of crop rotation, in which direct seeding on straw crop without soil removal, allowing the cycling of nutrients (TRIPLETT et al., 2008). The most common crops used on soybean rotational system cultivation, grasses are indicated that both non-host of the

pathogen and providing soil cover for a longer period (HECKLER et al., 2012; TRIPLETT et al., 2008).

Brachiaria ruziziensis at different densities reduced the number of germinated sclerotia. Thus, number of apothecia produced and reducing germination up to 33.99% and 64.7 apothecia units/m2, respectively (CIVARDI et al., 2009). Plant cover can form a physical barrier to carpogenic germination. It is changing microclimatic conditions, inducing apothecia germination and reducing the viability of the sclerotia bank to soybean cultivation (GÖRGEN et al., 2008; CIVARDI et al., 2009).

Crop rotation and planting system reduced the initial source. The number of apothecia germinated in no-tillage was 55% lower than compared to conventional planting. The best results obtained in no-tillage system were soybean-corn-soybean and soybean-corn-wheat succession, that reduced apothecia formation at 50% and 75% lower than monoculture in conventional system (GARZA et al., 2002).

Soybean/winter cereal succession decreased the White Mold incidence (FELLER et al., 2021). Cover yield compared to the control without vegetation was reduced the disease incidence at 77.7%. Thus *in vitro* assays were reduced apothecia germination by the add 4 mm of straw under the sclerotia samples. Only the control (with the absence of coverage) was present germination of sclerotia. Nonetheless, treatments of black oat straw, sitting and triticale did not shown germination.

In a crop-livestock integration system, the effect Maize and *Brachiaria* grass merged or isolate, had an effect on reducing number of sclerotia and apothecia germination. The germination of sclerotia was demonstrated with crop residues, in which the strow preserve soil moisture. Thus, reduction of sclerotia size was observed, that can reflect a reduction in the incidence of white mold, although it was not evaluated in this study (GÖRGEN et al., 2010).

Other recommended management practices were the control of areas irrigated by pivot to maintain soil moisture, elimination of spontaneous species that may be a source of inoculum to *S. sclerotiorum*, and elimination of remaining plants from soybean cultivation (NAPOLEÃO et al., 2007; GARZA et al., 2002).

1.4 Biological control

The use of pesticides to control diseases, pests and spontaneous plants was directly related to environmental problems. That’s include the resistance of organisms, water and

soil contamination, biological imbalance, alteration in the cycling of nutrients and organic matter and also the elimination of beneficial organisms in the environment (ALMEIDA et al., 2017; SILVÉRIO et al., 2012). According to Pomella et al. (2009), biological products can reduce the use of chemical fungicides, that make the production system more sustainable.

The increase of biologic products-based were the lower cost in relation to chemical agents, sustainability, growth in industrial scale production, regulatory pressure for "safe and natural" products, limitation of chemical registrations, in addition to the implementation of the registration regulations by biological targets (DIAS, 2016; BETTIOL et al., 2009). Biological management is based on application of products-based on *Trichoderma* spp. and *Bacillus* spp. (BETTIOL et al., 2009). Nowadays, around 33 products are registered against *S. sclerotiorum*, in with formulations based on *Trichoderma* spp., *Bacillus* spp. or combined of both (AGROFIT, 2020).

The biological products application was in the vegetative stages (V2 and V4), before the closing of the crop lines, to attach the sclerotia present in the soil (MEYER et al., 2014; JULIATTI et al., 2015). The application conditions were recommended on periods with lower incidence of light and mild temperatures. It is important to ensure adequate conditions for the establishment and action of bioagents (POMELLA et al., 2009). The UV incidence is one of the most limiting factors of the *Trichoderma* genus (SAMETZ-BARON et al., 1997). Products-based of *Trichoderma* spp. have three types of active ingredients: mycelium, conidia and chlamydospores, in which conidia is the most used especially for its stability to the processing steps (VERMA et al., 2007). In general, formulates are compound to active ingredient, inert ingredients and adjuvants. That were act protection against UV rays, moisture retention, protection against desiccation and aid on product dispersion (HYNES et al., 2006).

Although different species and isolates of *Trichoderma* spp. can present different mechanisms of action, the antagonism to *S. sclerotiorum* was mainly know by parasitism (INBAR et al., 1996; GERALDINE et al., 2013). Parasitism consists of targeted recognition and growth, contact and penetrate of hyphae in the prey, haustorium development and production of lytic enzymes, that cause death of host tissue (HARMAN et al., 2004).

Many species of *Trichoderma* spp. have lytic enzymes such as a protease, β-glucosidase and chitinase, in which act in the cell wall degradation of phytopathogens (DELGADO et al., 2000). The melanin and internal components such as β-1,3-glucans

and proteins of sclerotia was a good target to enzymes action (LE TOURNEAU, 1979; VÁZQUEZ-GARCIDUEÑAS et al., 1998). Antibiosis is the production of secondary metabolites, that toxify antagonists, and it have an effect against soil pathogens (MARQUES et al., 2018). Thus, *Trichoderma* species colonize the plant's root system that act the production of phytohormones and gibberellins. Its hormones can promote plant growth. The induction of resistance by genera was common related (MACHADO et al., 2012; 2015).

Bacillus based products contains endospores and/or metabolites. Formulations carry inert components that ensure stability and help disperse the product (SCHISLER et al., 2004). Endospores were spores able to survive on scarcity nutritional conditions, different temperature ranges, pH and osmolarity. These formulations can provide to products a longer shelf life. Also, it is enhancing the effect in applications by stability at application time (BACKMAN et al., 1997; JACOBSEN et al., 2004).

Beyond the endospore production fact, different mechanisms of action such as parasitism, antibiosis and competition were attributed to the genus *Bacillus*. It makes an efficient biocontrol agent (BETTIOL et al., 2009). Competition for nutrients and space were effectiveness mechanism of action. Those bacteria need the lower nutritional demand than filamentous fungi. This fact describes *Bacillus* an efficient agent against phytopathogens (CAWOY et al., 2011).

Antibiosis is the production of metabolites such as lipopeptides, which were toxic to phytopathogens. *Bacillus* subtilis produced about 60 types of antibiotics, including iturine, which has a broad spectrum against phytopathogens (OHNO et al., 1995alme). *Bacillus amyloliquefaciens* strains can produce compounds such as iturin, surfactin and fengycin, that shown *in vitro* assays inhibition to *S. sclerotiorum* mycelial growth (ALVAREZ et al., 2012). Thus, the action of *Bacillus pumilus* cellular filtrate was inhibited the pathogen *in vitro* and controlling the disease at greenhouse.

Bacillus sp. parasitism is the presence of enzymes such as chitinase, xylanase, pectinase, among others, which were able to degrade the cell wall of different phytopathogens (ROHBAN et al., 2009). The other secondaries effects on plants were described, in which *Bacillus*-mediated growth promotion and trigged resistance in plants (TSAVKELOVA et al., 2006).

1.5 Joining forces against sclerotia

Both non-host species and biological control agents (BCAs) application were essential an integrated management against the *Sclerotinia sclerotiorum*. Nonetheless, the effect of combine two practices is still not common explored. In France, two applications of *Coniothyrium minitans* combined with wheat crop more reduce the incidence of White mold than the crop of interest. The treatments of Wheat that were applied *Coniothyrium minitens*, showed to 51% increase colonization and to 15% reduction in the incidence of *S. sclerotiorum* (PENAUD et al., 2003).

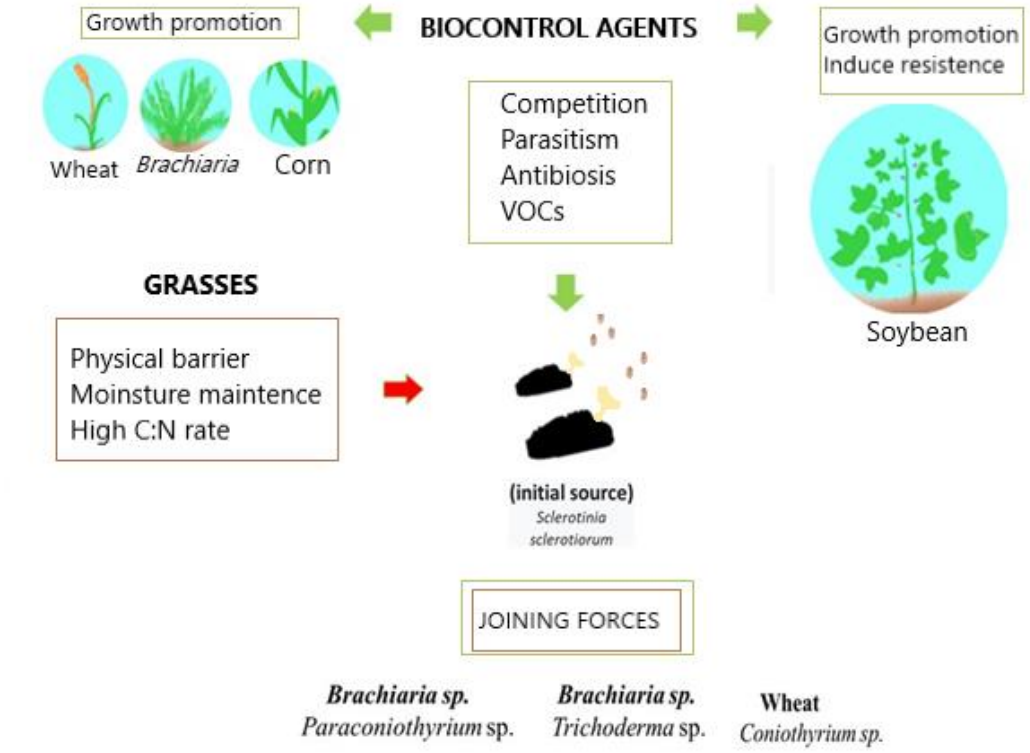
In Brazil, the use of *Brachiária* grass combine with *Trichoderma* spp. application was increased the reduction of carpogenic germination under field conditions (GÖRGEN et al., 2008). The reduction of apothecia germination occurred both by the physical action of the grass under the resistance structures and by the action of the biological control agent. Under different application rates of BCAs, all treatments showed higher colonization rates when combined with *brachiaria* grass. At doses of 0.5 to 1 l/ha, *Trichoderma* isolate shown highest colonization rates. There was effective on decreased disease incidence and increase crop productivity.

Nowadays, a screening of endophytic fungus on *B. ruziziensis*, *B. decumbens*, *B. humidicola* and *B. brizantha* shown the antagonistic potential against *S. sclerotiorum*. The genus *Paraconiothyrium* spp. was the most abundant endophyte. Of these, four isolates present growth inhibition of the pathogen up to 60% *in vitro* conditions (GAMA et al., 2020).

The potential of *Paraconiothyrium* spp. mechanism of action to reduction of the mycelial growth of *S. sclerotiorum* was explored. The volatile molecules produced reduced carpogenic germination of *S. sclerotiorum* sclerotia. In addition, grass growth promotion when inoculated with endophytic has been reported. Exploring the potential use of these microorganisms within the production system, in vivo tests showed that *Paraconiothyrium* isolates were able to remain active even after the application of glyphosate, suggesting a possibility of using a culture system (ALVES et al., 2021).

Thus, The Trojan, association of bioagents and non-host species, and bioproducts application refinement under field conditions can contribute of the management of the monocyclic pathogen. It needs to explorer the potential for the establishment and permanence of these bioagents on a whole soybean production system.

Figure 1. Biologic control agents to improve cultural and biologic strategies on management of *Sclerotinia sclerotiorum* soybean production system in Brazil.



Source: By the author, 2023.

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2 CHAPTER 2: FIRST MANUSCRIPT

Seven years of white mold biocontrol product's performance efficacy on Sclerotinia sclerotiorum carpogenic germination in Brazil: A *meta*-analysis

Abstract

Biocontrol is a plausible strategy to be considered on the management of white mold but the efficacy is not always the same. Therefore, the identification of the sources of such variable performance fosters a fine-tune product recommendation to achieve the highest performance. Based on seven soybean crop seasons, 59 uniform field trials were conducted throughout Brazil to assess the performance of *Sclerotinia sclerotiorum* sclerotia parasitism under different temperature and rainfall regimes. Hence, we carried out a meta-analysis to evaluate the effect of three treatment classes (*Trichoderma*-, *Bacillus*-, and a Mixed combination of both organisms) on the reduction of *S. sclerotiorum* sclerotia carpogenic germination (mean difference; *MD*) according to a multilevel network model (59 trials, k = 340). Throughout trials included in the meta-analysis, biocontrol reduced carpogenic germination ca. 70% of times, with fitted MD values of -10.6 for *Bacillus*, -8.6 for *Trichoderma*, and -7.0 for Mixed *Bacillus* and *Trichoderma* groups. Separate network models were then fitted to determine the influence of average monthly temperature (AMT) and average monthly rainfall (AMR) under three classes (low, moderate, and high) for each variable on the carpogenic germination. Overall, interactions of treatments and low or moderate AMT and AMR were significant (p < 0.1). Temperatures above 27ºC and precipitation higher than 250mm have not contributed to the reduction in carpogenic germination regardless of the considered active ingredient. Hence, biocontrol product´s reduce carpogenic germination in ca. 9% and dominant weather conditions. These relationships were important factors involved in sclerotia colonization and therefore, high temperatures and rainfall should not be indicated for the product's application for best BCAs performance.

Keywords: Sclerotinia sclerotiorum, network meta-analysis, carpogenic germination, weather conditions

1 INTRODUCTION

Soybean is the most important commodities in Brazil, with 38.9 million hectares sown in 2021 (Conab, 2021). It is estimated that *Sclerotinia sclerotiorum* (Lib.) Bary, the causal agent of white mold is present in 27% of sown area with soybean in Brazil (Embrapa, 2020; Meyer, 2020). It is a monocyclic pathogen that survives from one crop season to the other by producing sclerotia, a structure that is also the source for disease development. The amount of sclerotia is a determining factor for the epidemiology of the disease, and it increases with each soybean crop season. Also, sclerotia were still viable on the field after up to ten years (Abawi and Grogan, 1979; Brustolin et al., 2016; Lehner et al., 2017a). Under high humidity and low temperatures, they germinate carpogenically producing apothecia (Adams and Ayers, 1979; Grau and Hartman, 1999), causing a more severe epidemic.

Disease management is based on the integration of cultural, chemical, and biological management practices (Juliatti et al., 2015). Proper row spacing and plant density can reduce the favorable conditions to disease development (De Souza Jaccoud-Filho et al., 2016), while cultivating non-host species such as wheat can reduce the inoculum, physically inhibit apothecium germination, and improve the conditions for biological control agents (BCAs) (Görgen et al., 2009; Feller et al., 2021). Chemical fungicides are sprayed when plants reach the reproductive growth stage to protect the flowers during the critical period for ascospore infection (Meyer et al., 2014), but the widespread use of some compounds can select resistant pathogen population (Zhou et al., 2014; Lehner et al., 2015; Lehner et al., 2017b).

A new trend for white mold control has been the use of BCAs (Meyer et al., 2019; Medeiros et al., 2018). In tropical conditions, most products were based on *Trichoderma* spp. and *Bacillus* spp. (Cawoy et al., 2011; Juliatti et al., 2019). *Trichoderma* spp. penetrate the sclerotia via haustoria and secrete lytic enzymes that cause cell death (Vázquez-Garcidueñas et al., 1998; Geraldine et al. 2013). *Bacillus* spp. have been shown to control several soil pathogens (Hou et al., 2006; Zhang et al., 2010). They also produce endospores, which give them a larger shelf life (Angelo et al., 2010). *Bacillus* spp. Produce several antimicrobial metabolites in addition to antibiotics such as surfactins and iturin (Arguelles et al., 2009).

While these organisms show promising results in controlled conditions, field efficiency is variable (Zhang and Xue, 2010; Zeng et al., 2012). The effect of environmental

conditions on some of the biological processes of microorganisms has already been established. For example, temperature and water availability influence the dynamics of *Trichoderma* spp. spore germination, mycelial growth, mechanisms of action, and metabolite production (Magan, 1988; Kredics et al., 2000; Kredics et al., 2003). The same has been shown to affect *Bacillus* spp. growth and colony formation (Satapute et al., 2012; Mezanges et al., 2012; Ke et al, 2015).

Since 2013, uniform field trials (UFTs) have been carried out in Brazil to assess the effectiveness of biological control products in the colonization of *S. sclerotiorum* sclerotia (Meyer et al., 2014; Meyer et al., 2016), with variable results. Meta-analysis is a statistical analysis that allows researchers to combine the results of multiple independent studies to derive conclusions about a research question (Sutton and Higgins 2008) and establish statistical significance with studies that have conflicting results. It also allows researchers to evaluate the influence of different predictor variables in product performance.

Thus, the goal was to perform a multivariate meta-analysis to assess the overall effect of different treatment classes of biocontrol agents on the carpogenic germination of sclerotia of *S. sclerotiorum*, as well as to identify possible environmental factors that affect biocontrol of carpogenic germination.

2 MATERIALS AND METHODS

2.1 Database and soybean field trials

It was used the database consisted of raw data and reports from 74 UFT conducted during seven soybean crop seasons (from 2012/13 to 2016/17 and 2018/19 to 2019/20) as part of a nationwide white mold biological control trial network (Meyer et al., 2020). The soybean variety that cultived were different regarding of place and the choice of according of edaphoclimatic conditions of each region of the assay (Supplementary Table 1). It were used sclerotia produced in the laboratory at Embrapa Soja from a *S. sclerotiorum* strain obtained in an infested field in Campos Novos (SC, Brazil) and provided to carry out all field trials. A total of 30 sclerotia (within average length ranged from 3.24-8.17 mm to a width of 2.16-3-31 mm) were placed in a screened nylon enveloped (10x10cm), on Styrofoam tray and covered through the remains of previous crops. Four replicates were adopted for each field trial. The data used in the summarization was the average of

carpogenic germination for the four replicated of each experiment. Colonization essays were performed following the methodology of Meyer et al., (2019). In short, polystyrene trays containing sclerotia were deposited in the center of each experimental plot and covered with straw from the previous cultivated crop (either corn or wheat). Biocontrol products were applied twice to the plots following the manufacturer's instructions when soybean plants reached the V2 and V4 phelogical stages (two and four fully expanded trifoliate leaves, respectively). Twenty days after the second application, the sclerotia samples were removed from the field and sent to a laboratory, where they were placed in transparent plastic boxes containing 200g of 3x sterilized sand and kept at 17± 2 ºC and 90% field capacity under a 12:12 L/D photoperiod until carpogenic germination evaluations were stable, which occurred between 20-30 days of incubation. After carpogenic germination, sclerotia were quantified for the number of germinated and BCA-colonized sclerotia one. Sclerotia were considered colonized whenever they were covered with *Trichderma* mycelial matt or slimy *Bacillus* biofilm on the melanized surface of sclerotia was considered as such.

2.2 Studies and treatment selection criteria

Out of 74-reported UFT, eight were missing information regarding carpogenic germination, therefore have been excluded from the analysis. Seven additional UFT were excluded since had mean germination (near or exactly) 0% on the check treatment, assuming the sclerotia sent to those UFTs were non-viable. Fifty-nine UFT conducted in thirteen research institutions across seven Brazilian states (Universidade Federal de Lavras, Lavras, MG, Brazil; Embrapa Soja, Londrina, PR, Brazil; Universidade de Rio Verde, Rio Verde, GO, Brazil; Agro Carregal Pesquisa e Proteção de Plantas Eireli, Rio Verde, GO, Brazil; Assist Consultoria e Experimentação Agronômica, Campo Verde, MT, Brazil; Círculo Verde Assessoria Agronômica & Pesquisa, Luís Eduardo Magalhães, BA, Brazil; Centro Tecnológico de Pesquisa Agropecuária (CTPA), Goiânia, GO, Brazil; Estação Experimental Agrícola Campos Gerais (EEACG), Papagaios Novos, PR, Brazil; Fundação Chapadão, Chapadão do Sul, MS, Brazil; RB Consultoria, Passo Fundo, RS, Brazil; Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil; Universidade Federal de Jataí, Jataí, GO, Brazil; Universidade Federal de Uberlândia, Uberlândia, MG, Brazil) Figure 1A). From the remaining studies, only treatments that had formulations with live microorganisms as active ingredients were included (14 out of 17 tested

products). The included products were grouped based on the formulated biocontrol treatment (Supplementary Table 1), resulting in the following classes: Bacillus (4 formulations; 59 trials), Trichoderma (8 formulations; 59 trials), and Mixed (Bacillus + Trichoderma; 2 formulations; 9 trials) (Supplementary Table 1). For all treatments, the total volume sprayed was 150 L/ha using a CO_2 compressor cylinder at 3PSI and coupled to a 4-cone nozzle bar. The central plot was considering of four the central lines with 5 m of size and 0.5m distance between rows.

Temperature and rainfall were adopted as moderators (Fall et al., 2018). Information regarding both variables was obtained from each scientist carrying out the experiment. One data was generated per field trial. The average monthly temperature and rainfall were determined based on the whole soybean season. When necessary, the tools Graphreader (www.graphreader.com) (Tolmeijer et al., 2020) and GetData DigitizeIt® (version 2.26) (Perez et al., 2021) were used to access the trials data.

2.3 Quantitative synthesis of biocontrol agents' effect across trials

Data from each of the 59 UFT constituted an independent study in the meta-analysis. Mean germination from biocontrol and check treatments were collected from each study and used to find the effect size, estimated as the mean difference (MD), by subtracting the mean germination of check treatment at the specific study from the mean germination of the biocontrol product using the escalc function of the metafor package (Viechtbauer, 2010) of R.

2.4 Meta-analytic models

A multilevel network model was fitted using the rma.mv function of the metaphor package (Viechtbauer, 2010). The random components were considered the treatment and trial, with correlated random effects for the different treatments within trials. The amount of heterogeneity (2 and ρ) was estimated using the restricted maximum-likelihood estimator (Viechtbauer, 2005) and evaluated based on the significance of the Cochran's Q test. The within-study variance (V) was estimated from the coefficient of variation (CV) of an analysis of variance of the effects of treatment by first estimating the standard deviation (*SD*). Studies were weighted in inverse proportion to their sampling variances

(within-study variances). Wald-type tests and 95% CIs were obtained using an assumption of normality.

2.5 Effect of temperature and rainfall on carpogenic germination

Average monthly temperature (AMT) and average monthly rainfall (AMR) were included as moderator variables to account for at least part of the heterogeneity in the true effects (Borenstein et al., 2009). A separate multilevel network model was fitted for each moderator variable, with three categorical classes each. For average monthly temperature, classes were UFTs were grouped in low (AMT≤23 °C), moderate (23 °C<AMT ≤27 °C), and high (AMT>27 °C). For average monthly rainfall, classes were low (AMR<150 mm), moderate (150 mm≤AMR ≤200 mm), and high (AMR>250 mm). Parameters classes were calculated according to data amplitude.

3 RESULTS

3.1 Distribution of BCA and germination across trials

Trichoderma was the most frequently used treatment (8 formulations across 235 entries; 69.12% of all BCA entries), followed by Bacillus (4 formulations across 85 entries; 25%) and Mixed (2 formulations across 20 entries; 5.88%) (Figure 2B). Germination in check entries ranged from 0.83 to 100% (median: 49.99%), while germination in BCA-treated entries ranged from 0 to 100% (median: 37.74%), 0 to 99.17% (median: 29.17%), and 1.33 to 87.38% (median: 51.11%) for Bacillus, Trichoderma, and Mixed, respectively (Figure 2C). Mean difference values for Bacillus varied from -52.840 to 25.800, with 77.65% being negative (i.e., reduced germination), while for Trichoderma varied from -55.340 to 52.500 (71.91% negative) and for Mixed from -42.660 to 19.430 (75% negative) (Figure 3).

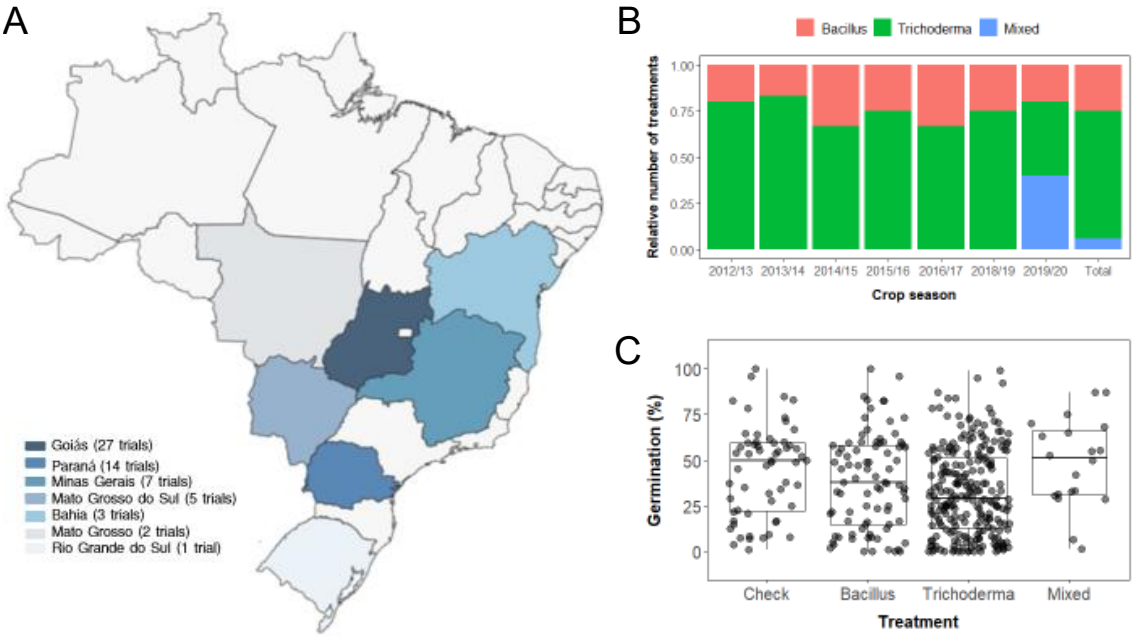

Figure 2. a) State distribution and number of trials of *Sclerotinia sclerotiorum* biocontrol throughout Brazil. b) Proportion of products by biocontrol treatment class used at each crop season. c) Carpogenic germination distribution of sclerotia of *Sclerotinia sclerotiorum* under different treatments. Check = water treatment; Mixed = *Bacillus* + *Trichoderma*.

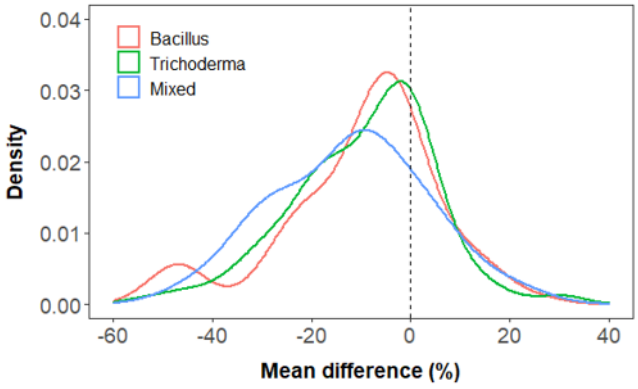

Figure 3. Distribution of mean difference values of the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* between biocontrol treatment classes and check (water) treatment. Mixed = *Bacillus* + *Trichoderma*. All values to the left of the dashed line indicate biocontrol treatment reduced carpogenic germination compared to check treatment.

3.2 Effect of biocontrol genera on carpogenic germination

A total of k = 340 entries was included in the carpogenic germination meta-analysis. BCA treatment as a whole affected carpogenic germination (< 0.0001). The overall mean difference (MD) was -10.6270 (95% CI: -13.5782 to -7.6757) for *Bacillus*, -8.5928 (95% CI: -11.1973 to -5.9883) for *Trichoderma*, and -7.0177 (95% CI: -12.3172 to -1.7182), for Mixed; all values were different from zero with probability levels of < 0.0001, < 0.0001, and 0.0094, respectively (Table 1). All pairwise MD comparisons but one (*Bacillus* and *Trichoderma*) had *p* values > 0.1 (Figure 4A). According to Cochran's test of heterogeneity, the true outcomes appear to be heterogeneous (QE = 3985.4536, *p* < 0.0001).

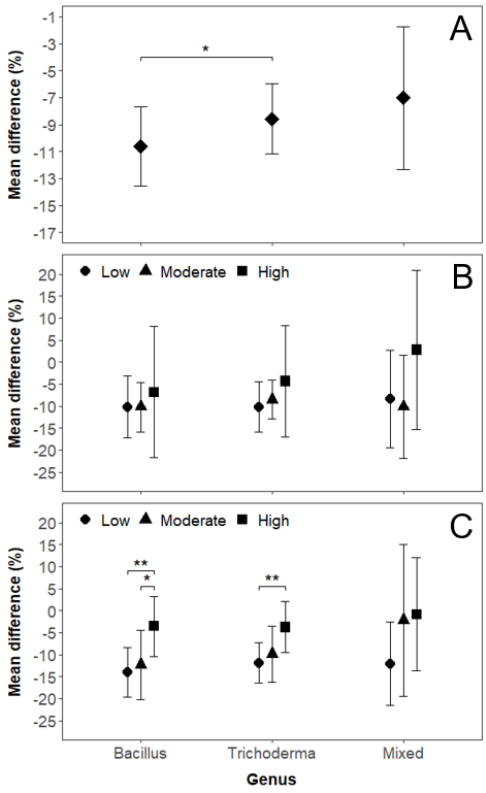

Figure 4. Mean difference (effect size) and 95% confidence interval for the effect of A) biocontrol treatment class and the interaction of treatments and three classes of average monthly temperature (AMT; B) and average monthly rainfall (AMR; C) on the carpogenic germination of sclerotia of Sclerotinia sclerotiorum. Mixed = Bacillus + Trichoderma. AMT = Low: AMT≤23 °C; Moderate: 23 °C<AMT ≤27 °C; High: AMT>27 °C. AMR = Low: AMR<150 mm; Moderate: 150 mm≤AMR ≤200 mm; High: AMR>250 mm. Pairwise comparisons linked by brackets are statistically significant with p = 0.05 (**) and p = 0.10 (*).

3.3 Moderator analysis

To account for some of the residual heterogeneity found in the first germination meta-analysis, two separate meta-analyses were performed considering the interaction between biocontrol agent and average monthly temperature (k = 182) or average monthly rainfall (k = 199). Based on Wald-type chi-square tests, both interactions affected carpogenic germination ($p = 0.0003$ and $p < 0.0001$ for AMT and AMR, respectively). Both models reduced the amount of residual heterogeneity, but Cochran's Q test was still significant ($QE = 1049.9133$, $p < 0.0001$; and $QE = 1121.8287$, $p < 0.0001$ for AMT and AMR, respectively).

For AMT, only MD from interactions with low and moderate AMT were statistically significant from zero ($p < 0.1$) on all treatment classes except Mixed:Low ($MD = -8.3712$; $p = 0.1367$) (Table 1). By treatment classes, all pairwise comparisons had p-values > 0.1 (Figure 5B). When AMT was high, Bacillus had the lowest MD (-6.8557), but it was not statistically different from check ($p = 0.3682$) (Table 2).

Similarly to AMT, only MD from interactions with low and moderate AMR were statistically significant from zero ($p < 0.1$), but results varied in that Mixed:Moderate (and not Mixed:Low) did not differ from check ($MD = -2.2034$; $p = 0.8013$) (Table 3). No treatment class performed well when rainfall levels were high: probability levels were 0.3082, 0.2026, and 0.8955 for Bacillus, Trichoderma, and Mixed, respectively. For Bacillus, pairwise comparisons between low and high, and moderate and high AMR had p values of 0.0202 and 0.0979, respectively. Regarding Trichoderma, the pairwise comparison between low and moderate had a p-value of 0.0315 (Figure 5C).

Table 1. Mean difference (effect size) and corresponding statistics for the effect of biocontrol genera on the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum.*

Genus	k[a]	Effect Size[b]				
		$\overline{MD}$	$se(\overline{MD})$	CI_{LB}	CI_{UB}	p value
Bacillus	85	-10.6270	1.5058	-13.5782	-7.6757	< 0.0001
Trichoderma	235	-8.5928	1.3289	-11.1973	-5.9883	< 0.0001
Mixed	20	-7.0177	2.7039	-12.3172	-1.7182	0.0094

[a] Total number of entries used in each analysis; [b] $\overline{MD}$ = mean difference of germination, calculated by subtracting mean germination of check treatment from mean germination of biocontrol product; $se(\overline{MD})$ = standard error of $\overline{MD}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\overline{MD}$; p value = probability value (significance level).

Table 2. Mean difference (effect size) and corresponding statistics for the effect of the interaction of biocontrol genera and average monthly temperature on the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum.*

Genus	AMT[a]	K[b]	Effect Size[c]				
			$\overline{MD}$	$se(\overline{MD})$	CI_{LB}	CI_{UB}	*p* value
Bacillus	Low	12	-10.2163	3.5983	-17.2688	-3.1639	0.0045
	Moderate	29	-10.2034	2.8766	-15.8414	-4.5654	0.0004
	High	3	-6.8557	7.6193	-21.7892	8.0779	0.3682
Trichoderma	Low	37	-10.1974	2.9122	-15.9053	-4.4896	0.0005
	Moderate	83	-8.5499	2.2536	-12.9667	-4.1330	0.0001
	High	6	-4.3518	6.4914	-17.0747	8.3711	0.5026
Mixed	Low	4	-8.3712	5.6251	-19.3962	2.6538	0.1367
	Moderate	6	-10.1605	5.9909	-21.9024	1.5814	0.0899
	High	2	2.7783	9.2532	-15.3576	20.9142	0.7640

[a] Average monthly temperature during trials. Low: AMT $\leq$ 23 °C; Moderate: 23 °C $<$ AMT $\leq$ 27 °C; High: AMT $>$ 27 °C; [b] Total number of entries used in each analysis; [c] $\overline{MD}$ = mean difference of germination, calculated by subtracting mean germination of check treatment from mean germination of biocontrol product; $se(\overline{MD})$ = standard error of $\overline{MD}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\overline{MD}$; *p* value = probability value (significance level).

Table 3. Mean difference (effect size) and corresponding statistics for the effect of the interaction of biocontrol genera and average monthly rainfall on the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum.*

Genus	AMR[a]	K[b]	Effect Size[c]				
			$\overline{MD}$	$se(\overline{MD})$	CI_{LB}	CI_{UB}	*p* value
Bacillus	Low	23	-14.0025	2.8558	-19.5998	-8.4052	< 0.0001
	Moderate	12	-12.3375	4.0146	-20.2059	-4.4691	0.0021
	High	13	-3.5452	3.4792	-10.3643	3.2740	0.3082
Trichoderma	Low	64	-11.9008	2.3453	-16.4976	-7.3040	< 0.0001
	Moderate	37	-9.9034	3.2896	-16.3509	-3.4558	0.0026
	High	38	-3.7736	2.9619	-9.5788	2.0316	0.2026
Mixed	Low	8	-12.0627	4.8142	-21.4982	-2.6271	0.0122
	Moderate	2	-2.2034	8.7574	-19.3677	14.9609	0.8013
	High	2	-0.8607	6.5553	-13.7089	11.9874	0.8955

[a] Average monthly rainfall during trials. Low: AMR $<$ 150 mm; Moderate: 150 mm $\leq$ AMR $\leq$ 200 mm; High: AMR $>$ 250 mm; [b] Total number of entries used in each analysis; [c] $\overline{MD}$ = mean difference of germination, calculated by subtracting mean germination of check treatment from mean germination of biocontrol product; $se(\overline{MD})$ = standard error of $\overline{MD}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\overline{MD}$; *p* value = probability value (significance level).

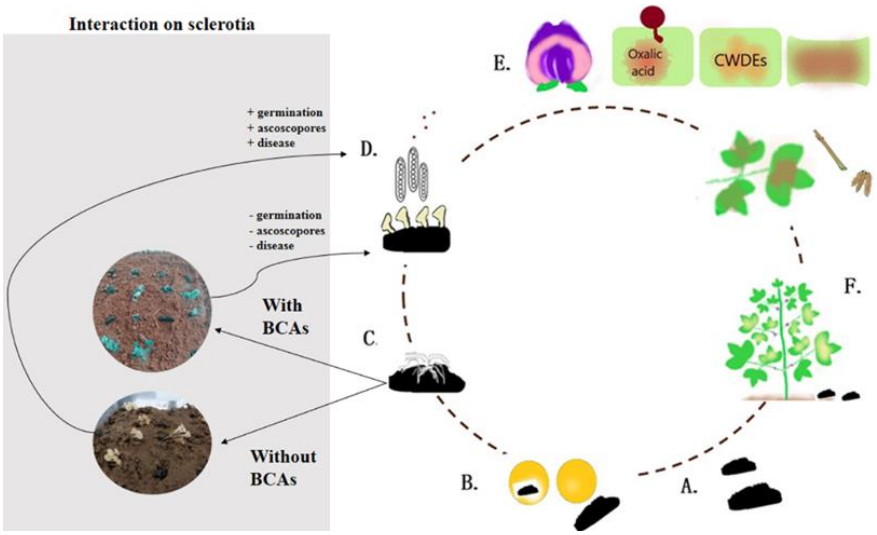

Figure 5. *Sclerotinia sclerotiorum* disease cycle on soybean (*Glycine max L.*). (A) Survive structure. (B) Seeds infect by sclerotia mycelial or selerotia presence on seed lots. (C) Asexual phase: Mycelial germination. (D) Sexual phase: sclerotia germination to produce apothecia and disperse ascospores into the air. (E) Ascospores colonize flowers, developed appressorium and secret Oxalic acid that support action of Cell well degraded enzymes (CWDES). Brown lesions the initial symptoms, can be disseminate into the stem, leaves and pods. (F) Selerotia from plant tissue can turn to the soil during harvest or disseminated by seeds. In the left or grey side arerelated the possible interaction on sclerotia, when spray BCAs and without BCAs sprays.

4 DISCUSSIONS

White mold (*Sclerotinia sclerotiorum*) can cause major yield losses in soybean, and its management is complicated given the pathogen's wide host range and ability to form resistance structures (Boland and Hall 1994; Abawi and Grogan, 1979). White mold control is achieved by integrating several practices, including biological control, a sustainable tool that acts by reducing the initial inoculum of *S. sclerotiorum* (Juliatti et al., 2015). Analyzing carpogenic germination due to BCA colonization in uniform field trials carried out in seven crop seasons in Brazil, we found that sclerotia sprayed with biocontrol products germinated significantly less than untreated sclerotia (Figure 4). Throughout trials, average monthly rainfall and average monthly temperature, affected the efficacy of the genera in reducing carpogenic germination.

Biological control of *S. sclerotiorum* is largely used in Brazil, with the first commercial product, based on *Trichoderma harzianum*, being registered in 2007 (MAPA, 2007). The number of biological products registered in Brazil has increased due to the lower developmental cost compared to chemical products and new legislation that made the registration process for biocontrol products based on biological targets (Guimarães et

al., 2019; Bettiol et al., 2012), allowing their use in several crops (unlike chemical products, which require crop-specific efficacy trials). Nowadays, there are 33 biocontrol products registered against *S. sclerotiorum*. All of the tested products were formulations of *Bacillus* and *Trichoderma* (Figure 1B). Both genera have desirable traits for the biocontrol industry: they multiply fast, have a long shelf-life, and are known to have multiple modes of action (Puyam, 2014; Jacobsen et al., 2004). All products registered for *S. sclerotiorum* control are based on *Trichoderma* and *Bacillus* (MAPA, 2021), whereas outside of Brazil, *Coniothyrium minitans* is the most widely available and tested BCA for white mold control (Peltier et al., 2012).

A recent trend has been the use of mixtures of isolates, species and/or genera: according to AGROFIT (2020), there are six mixed products for *S. sclerotiorum* control, with the first being registered in 2018. In our meta-analysis, there were two formulations combining *Trichoderma* and *Bacillus* in the 2019/20 crop season. The combination of two or more biocontrol agents can improve the management of disease possibly by positive synergism and reduced risk of variability (Guetsky et al., 2001). Indeed, Guetsky et al., (2002) found that the combination of *Trichoderma*, and *Bacillus* was more efficient to control *Botrytis cinerea* in strawberry leaves. However, each combination should be thoroughly analyzed as there is evidence there are more antagonistic interactions than synergistic interactions among BCAs (Xu et al., 2011). While mixed formulations only accounted for about 7% of all total entries (Supplementary Table S1), we expect to see more of those being tested in the next uniform field trials and a rationale for the combination of strains to be the higher plasticity in temperature and rainfall for sclerotia parasitism.

All treatment classes reduced germination compared to check treatment, with an average germination 10.63, 8.59, and 7.02% lower than untreated plots for products based on *Bacillus*, *Trichoderma*, and *Bacillus* + *Trichoderma* (Mixed), respectively. The amount of germinated sclerotia is a critical factor in white mold epidemics, given that a single sclerotium can produce several apothecia (Bolton et al., 2006), with each apothecium releasing up to 7.6×10^5 ascospores over 20 days (Clarkson et al. 2003). Indeed, there is a significant correlation between the number of apothecia (and indirectly, the number of viable sclerotia) and white mold incidence in soybean (Boland and Hall, 1988).

The current chemical control strategy is to apply fungicides when plants reach the flowering stage, when plants are the most vulnerable (Meyer et al., 2018). At this moment,

most sclerotia have already germinated and produced apothecia, under favorable conditions (Fall et al., 2018), so fungicide applications are mostly deployed to protect against infection and reduce ascospore germination, plant infection and tissue colonization rate. On the other hand, BCAs parasite sclerotia, thus decreasing the primary inoculum (Meyer et al., 2019); by implementing both practices, we affect two of the parameters that affect disease epidemics: the initial inoculum and the rate of disease progress (Campbell, 1998).

Environmental conditions can alter the dynamics of BCA interactions (Kredics et al., 2004). The effect of temperature on *Trichoderma* sp. varies from species and strains, affecting conidia germination, mycelium growth, antibiosis, and parasitism (Guigón-lópez et al. 2010). There is evidence that sclerotia parasitism by *Trichoderma* spp. is optimal around 17 ºC and 30 º C (Trutmann and Keane, 1990; Domingues et al., 2016), temperatures that span all three AMT classes in the current study. The optimal temperature for *Bacillus* spp. growth and metabolite production is also strain-dependent, varying from 15 to 37 ºC for *Bacillus subtilis*, for example, but most isolates prefer higher temperatures (Jiménez-Delgadillo et al. 2018; Sidorova et al. 2020). We found there was a significant effect of the interaction of AMT with biocontrol genera on carpogenic germination, but reduction varied between temperature classes inside each genus. Here, no BCA genus significantly reduced germination in high temperatures, even though both genera have been shown to grow in temperatures in this AMT class. Most trials included in this meta-analysis were carried out in regions with low and moderate AMT, resulting in low statistical power in high AMT, but we cannot discard the existence of other correlated variables that affected biocontrol performance. Mixed formulations had better results in moderate temperatures; moderate temperatures may benefit both species, resulting in better synergistic potential.

Water availability is one of the most important factors that affect both *Trichoderma* and *Bacillus*, with restrictive water conditions drastically affecting populational growth and interaction with other microorganisms (Magan, 1988; Luard and Griffin, 1981; West et al., 1985; Leuschner and Lillford, 1999; Kredics et al., 2004). The interaction between genus classes and high AMR did not affect carpogenic germination. The excessive rainfall might have washed away the products, especially if precipitation occurred just right after product application when populations were not yet established. It has been shown that different isolates react differently to a gradient of matric potential. Jones et al. (2015) found that depending on the isolate, not only drier soils but almost-

saturated soils compromised *Trichoderma* ability to affect sclerotia viability of *S. sclerotiorum* in controlled conditions. Also, it is not known how different matric potentials or fluid flows affect *Trichoderma* conidia adhesion and substrate colonization.

Environmental conditions deeply affect bacterial biofilm dynamics, with communities exposed to desiccation having a higher biofilm formation ability than communities exposed to saline or non-stressful conditions (Bogino et al., 2013). Although *B. subtilis* biofilm has been shown to have non-wetting characteristics (Arnaouteli et al., 2016), it is possible that *Bacillus* attachment to sclerotia substrate was compromised by high water availability, with bacteria being more likely to disperse actively (which depends on cell motility or extracellular polymeric substances degradation) and/or passively (due to physical factors such as liquid flow conditions) (Toyofuku et al., 2016).

5 CONCLUSIONS

As BCAs contributes to *Sclerotinia sclerotiorum* management through inoculum reduction from sclerotia parasitism and 70% of the evaluated products and fields resulted in reduction of inoculum viability. The combined analyses of several uniform field trials revealed that reduced carpogenic germination ca. 9% was mostly impaired by high levels of average monthly temperature (above 27^{o}C) and average monthly rainfall (250mm). Therefore, cooler temperatures and drier environmental conditions should be considered for maximum sclerotia parasitism.

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3 CHAPTER 3: SECOND MANUSCRIPT

Can Wheat and Maize on soybean crop rotation system with biological agents' application contribute to decrease initial source inoculum of *Sclerotinia sclerotiorum*?

Abstract

White Mold (*Sclerotinia sclerotiorum*) *reduces* soybean yield under favorable conditions. The range of hosts and the presence of resistance structure (sclerotia) turn its management a challenge. Sclerotia is the main source of inoculum and the quantity of this structure in the field affects disease incidence and causes yield losses. Biological and cultural practices reduce apothecia germination. Thus, the goal was evaluating in field production with high inoculum pressure of soybean crop, carpogenic germination, colonization by BCAs, incidence of withe mold and yield. Also, the ability of colonization of sclerotia by biocontrol agents in soybean of crops that commonly succeed it such as maize and wheat. Investigate biological contribution to decrease carpogenic germination compare the crop contribution to its performance. Carpogenic germination rates in soybean as cover crop ranged from 30.9 to 61.6 % with no significant contribution of the treatments (P = 0.583). A similar effect (P = 0.174) was found from 4.7% to 19.4% for colonization. Also, biological and chemical application disease incidence (P = 0.520), ranges from 73.750% to 87.18%, and yield production from 2001 kg ha^{-1} to 2803 kg ha^{-1}. For carpogenic germination, considering cover crops maize and wheat we found effects by treatments (P = 0.011), control by 65.71% and treatments up to 46.98%. Effect of factor crop (p=0.022), maize germination by 59.65% and wheat by 50.337%. No effects were found at interaction (crop x treatments), (P=0.328). Evaluated colonization, we found effect by treatments, (P=0.157), crop (p<0.001), and interaction (crop x treatment) (p=0.048). Colonization in maize trials range from 2.23 % to 10.15 %. Hight colonization was observed by *Trichoderma asperellum* A, follow by *Trichoderma harzianum*, *Trichoderma asperellum* B, and *Bacillus a. + Trichoderma h.* by 10.15 %, 9.947%, 2.738% and 2.232%, respectively. Wheat colonization rates from 16.65% to 39.82%. *Trichoderma asperellum B*, followed by *Trichoderma harzianum*, *Trichoderma asperellum* A, *Bacillus a. + Trichoderma h.* by 39.82%, 24.43%, 20.96% and 16.65%, respectively. Wheat yield (p=0.177) and Maize yield (p=0.593). Thus, these results suggest that biological applications in wheat crop have more possibility to help contribute to decrease *sclerotinia sclerotiorum* initial inoculum.

Keywords: Crop rotation, Wheat, *Sclerotinia sclerotiorum*, BCAs colonization

1 INTRODUCTION

White Mold disease, caused by *Sclerotinia sclerotiorum* can reduce soybean crop yield by up to 70% (MERRIMAN et al., 1976). An important factor for the epidemiology of the disease is the presence of a sclerotium that can remain in the fields of production even in the absence of a host, for subsequent years. (ABAWI et al., 1979; BRUSTOLIN et al., 2016). It forms mycelium or apothecium. The apothecium produces thousands of ascospores that can infect soybeans under favorable conditions occur disease development. (ABAWI et al., 1979) According the number of sclerotia in the soil disease development and incidence can reach high levels and affect the soybean yield (LEHNER et al., 2017).

Management of White Mold disease is based on breeding, chemical, cultural and biological practices. However, there is no resistant cultivar to *Sclerotinia sclerotiorum.* Chemical fungicide applications were targeted to protect the soybean flower against pathogen infection (JULIATTI et al., 2015 MEYER et al., 2020). Thus, cultural practices and biocontrol agents' application aim to reduce sclerotia viability in the soil and can contribute to the reduction of the initial inoculum source for the occurrence of the disease. (JULIATTI et al., 2015)

Soybean has an important commercial role in the Brazilian economy and the grower rely on a succession crop to increase its revenue, which is mostly maize or wheat. Actually, such succession with a monocot, can also benefit soybean planted afterwards by improving soil microbial activity, cycling nutrients and improving overall soil health. (RESTOVICH et al., 2012; SONG et al., 2022) Additionality, cultural practices such as plant population, plant architecture and life cycle, and succession crop contribute to the reduction of favorable conditions to *Sclerotinia sclerotiorum* survival, parasitism and disease (HENNEBERG et al., 2012; JACCOUD-FILHO et al., 2016).

Bacillus- and *Trichoderma*-based products were the main ingredients of BCAs use in Brazil to control the disease. The key of using those organisms as active ingredients rely on their peculiar mechanism of action against the pathogen (BETIOL et al., 2009). *Trichoderma* sp. can inhibit pathogens by competition, antibiosis and mycoparasitism and *Bacillus* can produce metabolites such as antibiotics and volatile compounds with deleterious effects against phytopathogens (ABDULLAH et al., 2018, KISHAN et al., 2017, MASSAWE et al., 2018). Additionally, both of the classes of biological control

agents can trigger induced resistance and act as biostimulant, promoting nutrient use efficiency, hormone homeostasis and overall crop growth.

Also, use of biological control agents combine with non-host crops can provide best niche for microorganisms and were able to decrease soil-borne pathogens. (KTHIRI et al., 2020; ASAD et al., 2014). For example, combining *Brachiaria* grass with *Trichoderma* sp. resulted in a physical barrier to apothecium release of the pathogen and offered environmentally buffered conditions to *S. sclerotiorum* sclerotia parasitism. Furthermore, it can reduce the sclerotia bank in the soil by sustaining moisture and lower temperature in the winter, which stimulate carpogenic germination in the absence of the plant host (GÖRGEN et al., 2008).

Thus, we aimed at evaluating the incidence of white mold in the field under high inoculum pressure on soybean crop. Also, the sclerotia of *Sclerotinia sclerotiorum* colonization by microorganisms after two foliar applications at early vegetive stage (V2 and V4) in soybean and both successive crops maize and wheat and their ability to decrease carpogenic germination rates.

2 MATERIAL AND METHODS

2.1 Sclerotinia sclerotiorum inocullum production

Sclerotinia sclerotiorum isolate used in all experiments was from a naturally infected soybean plant in Luminarias, MG soybean field production. Sclerotia was surface disinfested by 70% ethanol (60 s) followed by 5% sodium hypochlorite (60 s) and rinsed in sterile distilled water tree times (SANABRIA-VELAZQUEZ et al., 2019). To produce the sclerotia used in the experiments, the sclerotia stored at 4ºC was allowed to grow on PDA Potato Dextrose Agar: Agar (15g/L), Dextrose (20 g/L) and Potato extract (4 g/L). After 7 days incubation at 20 ±2 C^{o} and 12 H L:D photoperiod, mycelial plugs of the pure colony (5mm diameter) were transfered to with a new plate containing the same medium and allowed to grow for 15 days at 20 ±2 C^{o} and 12 H L:D photoperiod (ZANCAN el al., 2012).

2.2 Soybean crop field trial

The assay in 2020/2021 season was field production of Fazenda Santa Maria in the municipality of Conceição do Rio Verde/MG (21º53'26” S / 45º06'37” W) at 898

meters of altitude, from October 22th, 2020 to February 23th, 2021. The field had a history of high incidence (above 60%) of the white mold disease recorded before soybean planting and pre testing carpogenic germinated rates. The cultivar was Lança 58i60 RSF IPRO (Brasmax), sown with 45 cm between rows and 13.5 seeds per linear meter resulting in a population of 300,000 plants per hectare. Pre-planting fertilization was applied at 200 kg ha^{-1} rate of potassium chloride and 200 kg ha^{-1} of monoammonium phosphate (MAP). Seed treatment with the fungicide / insecticide methyl tiophanate, pyrachlostroibin and fipronil (Standak Top, BASF) in 200 mL per 100 kg of seeds. Seed Inoculation were 8 doses of *Bradyrhizobium* japonicum and 2 doses of *Azospirillum brasilense*.

At 15 days before soybean sowing the herbicides Glyphosate (Crucial), imethylammonium (2,4-dichlorophenoxy) acetate (2,4-D dimetilamina) Aminol 806 and Ethyl 2-(4-chloro-6-methoxypyrimidin-2-ylcarbamoylsulfamoyl) benzoate (Classic) were applied at 3.0 L ha^{-1}, 1.5 L ha^{-1} and 80 g ha^{-1}, respectively. Additionality, Cypermethrin (Nortox) in the dose of 200 mL ha^{-1}, in addition to the application of 2.0 kg ha^{-1} of boric acid via desiccation syrup. At vegetative stage 3, the herbicides Glyphosate (Crucial, Sumitomo) and haloxyfop-R-methyl (Verdict, FMC) were applied at 3.0 L ha^{-1}, 0.5 L ha^{-1} following the crops technical guidelines.

In the center of each experimental plot, nylon bags with 30 sclerotia units were placed in tray. The biocontrol treatments were applied twice, the first application in vegetative stage V2, followed by a second one at V4. Volume of 150 L ha^{-1} and the sprays were with CO_2 pressurized sprayer coupled to a PET bottle under at 2 atm pressure (202.65 kPa), (Table 4). White mold disease incidence was accessed at R5.1, R5.4 and R6, in 40 plants in the two central lines of each experimental plot. At the R8 reproductive stage measured productivity (kg ha^{-1}). Grain yield by weight of grains and considered in kg and converted to kg ha^{-1}. Grain moisture adjusted to 13% wet basis.

2.3 Maize crop field trial

The assay was in the Fazenda Represa, Municipality of São Joao Del Rey at coordinates -21.319610 latitude, -44.405584 longitude, and 993 m altitude. The cultivar MG30A37 sowed in March 2, 2022, with a spacing of 0.4 m between rows and a stand of 12 plants per linear meter. Pre-plating fertilization with MAP 13-33-30 at the recommendation of 150 kg per hectare. Top dressing fertilization was performed 24-00-

15 with a 300kg per hectare rate. At 16 days before maize sowing the herbicides Mesotrione + Atrazine (Calaris, Syngenta) and Glyphosate at 1.5 L ha^{-1} and 3.0 L ha^{-1}, respectively. The same herbicides were applied at 30 days and 45 days post emergence.

The duplicate trial was in a randomized block design, with five treatments and four replicates per treatment. The plots were composed of six lines of 5 meters length. In the center of each experimental plot, nylon bags with 30 unit of sclerotia were placed in tray. Two foliar applications were performed. The first application performed at the phenological stage V2 /V3, on March 17, 2021.

The second application, performed on April 1st, in the phenological stage V5/V6. Applications were performed similar to the above mentioned (Table 5). Sclerotia samples were collected twenty days after the second application. Subsequently, they were reassigned to the laboratory for sclerotium viability tests. Grain yield by weight of grains and considered in kg and converted to kg ha^{-1}. Grain moisture adjusted to 13% wet basis.

2.4 Wheat crop field trial

The assay was in the Fazenda Represa, Municipality of São Joao Del Rey at coordinates -21.260296 latitude, -44.324330 longitude, and 993 m altitude. The cultivar BRS264 sowed on April 10, 2022, with a spacing of 0.17 m between rows and a stand of 60 plants per linear meter. Pre-plating fertilization with MAP 11-52-00 at the recommendation of 150 kg per hectare. Top dressing fertilization was performed 24-00-15 with a 400kg per hectare rate. For weed control, the products clodinafop-propargil (Topik, Syngenta Crop Science) and Metsulfuron-methyl (Ally, FMC) were applied at 0.25 L ha^{-1} and 6g ha^{-1}, respectively.

The duplicate trial was in a randomized block design, with five treatments and four replicates per treatment. The plots were composed of eight lines of 5 meters in length. In the center of each experimental plot, nylon bags with 30 sclerotia samples were placed in the tray. Two foliar applications were performed. Time of application defined before the first top-dressing fertilization of Wheat. The first application performed at 12 days after planting. on April 17, 2021. The second application, performed May 5th, 23 days after planting. Applications were performed similar to the above mentioned (Table 6) Sclerotia samples were collected twenty days after the second application. Subsequently, they were reassigned to the laboratory for sclerotium viability tests. Grain yield by weight

of grains and considered in kg and converted to kg ha^{-1}, moisture adjusted to 13% wet basis.

Tabela 4. Biological Control product-based applied on soybean crop.

Treatment Product-based	**Active ingredient**	**Dose L-Kg/ha**	**1th apl.**	**2th apl.**
Control	-			
Trichoderma harzianum A	$1x(10)^{10}$	0,1	V2	V4
Trichoderma asperellum	$1x(10)^{9}$	0,1	V2	V4
B. amyloliquefaciens +*Trichoderma h.*	$1x(10)^{7}$	0,3	V2	V4
Trichoderma harzianum B	$1x(10)^{10}$	0,1	V2	V4
Trichoderma h. + *Trichoderma a.*	$1x(10)^{10}$	0,1	V2	V4
Fluazinan		3	R2	R2 +10

[a] (V2) = two fully expanded trifoliate leaves.
[b] (V4) = four fully expanded trifoliate leaves.
[c] (R2) = reproductive stage that start flower formation.

Tabela 5. Biological Control product-based applied on maize crop.

Treatment Product-based	**Active ingredient**	**Dose L-Kg/ha**	**1th apl.**	**2th apl.**
Control	-			
Trichoderma harzianum A	$1x(10)^{10}$	0,1	V2/V3	V5/V6
Trichoderma asperellum	$1x(10)^{9}$	0,1	V2/V3	V5/V6
B. amyloliquefaciens +Trichoderma h	$1x(10)^{7}$	0,3	V2/V3	V5/V6
Trichoderma harzianum B	$1x(10)^{10}$	0,1	V2/V3	V5/V6

[a] (V2/V3) = vegetative stage 2th and 3th leaf stage.
[b] (V5/V6) = vegetative stage 5th and 6th leaf stage.

Tabela 6. Biological Control product-based applied on wheat crop.

Treatment Product-based	**Active ingredient**	**Dose L-Kg/ha**	**1th apl.**	**2th apl.**
Control	-			
Trichoderma harzianum A	$1x(10)^{10}$	0,1	12 (DAP)	23 (DAP)
Trichoderma asperellum	$1x(10)^{9}$	0,1	12 (DAP)	23 (DAP)
B. amyloliquefaciens +Trichoderma harzianum	$1x(10)^{7}$	0,3	12 (DAP	23 (DAP)
Trichoderma harzianum B	$1x(10)^{10}$	0,1	12 (DAP	23 (DAP)

[a] (DAP) = days after planting.

2.5 Viability of Sclerotinia sclerotiorum

The samples from all experiments remained in the field 20 days after the last application before they were taken to the lab. In laboratory, the sclerotia were laid on top of plastic boxes containing 200g of soil autoclaved three times and moistened to 90% of the field capacity (cc). The boxes were stored at 17Cº, under 12hL/D for 40 days. Carpogenic germination and sclerotia colonization evaluations were performed every 15 days (MEYER et al., 2019)

2.6 Statistical Analysis

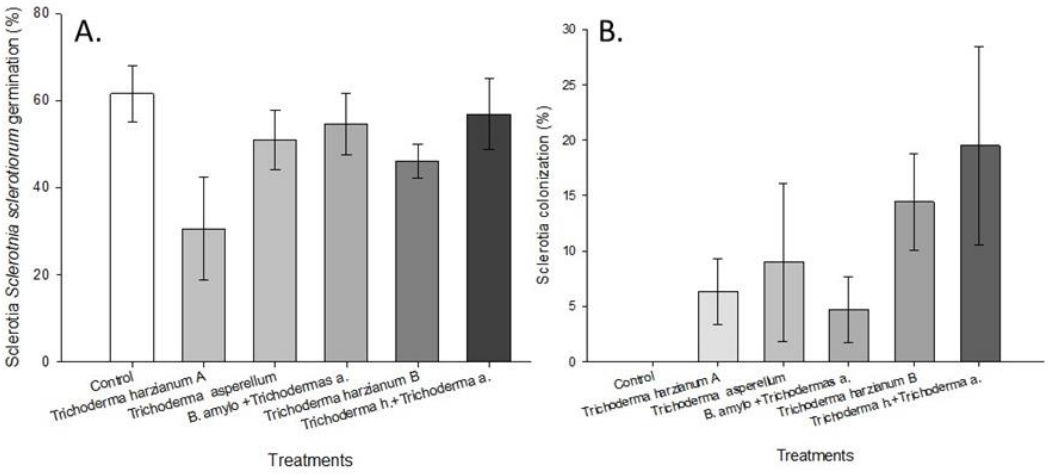
Soybean crop data submitted at Shapiro-Wilk test, Brown-Forsythe and Analysis of variance. Followed by mean comparison Tukey's Test for significant effects. The design of analyzed colonization and carpogenic germination of maize and wheat crops followed a double factorial (crop and treatment) and their interaction. Data submitted at Shapiro-Wilk test, Brown-Forsythe and Analysis of variance. Followed by mean comparison by Tukey's Test for significant effects ($p<0.05$).

3 RESULTS

3.1 Soybean crop Sclerotia germination and colonization

For soybean as the cover crop, there was no significant effect of the treatments ($P = 0.583$). The carpogenic germination ranged from 30.9 to 61. 6 %. The lowest reduced average germination by *Trichoderma harzianum* A, 31.00%, and *Trichoderma harzianum* B, 15.27%. (Figure 6A) Sclerotia colonization ($P = 0.174$) ranged from 4.7% to 19.4%, with *Trichoderma h.+Trichoderma a. + Bacillus a., Trichoderma harzianum* B and *B. amyloliquefaciens +Trichoderma harzianum*, colonization rates by 19.4%., 14.4% and 9.00% (Figure 6B).

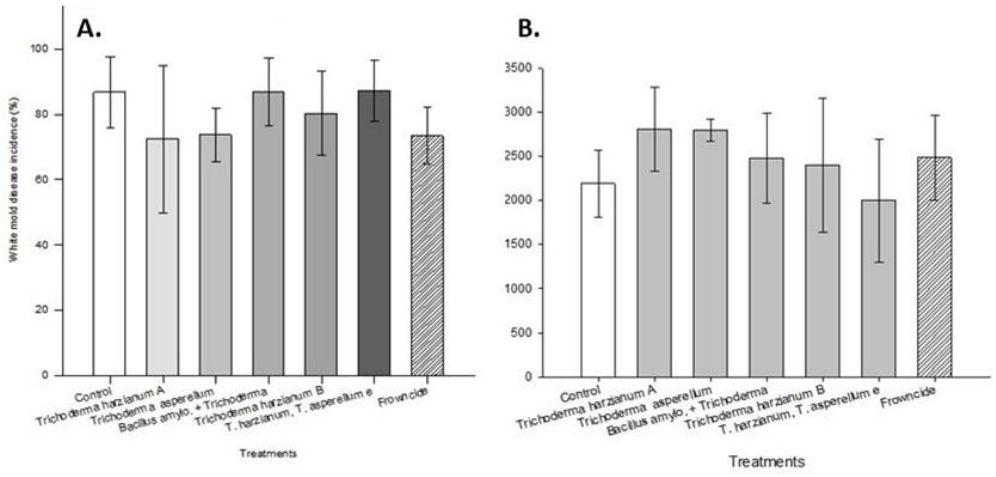
Figure 6. Soybean field trials with biological control product application. A) Sclerotia carpogenic germination. B) Sclerotia colonization.



3.2 White Mold disease incidence and soybean yield

White mold incidence was not influenced by the treatments (P = 0.520) and ranged from 73.7% to 87.2%. *Trichoderma harzianum* and *Trichoderma asperellum* decrease incidence by 12.7 % and 11% compared to the control, a result similar to the exclusive use of the two foliar sprays of the chemical fungicide 13.5%, (Figure 7A). Regarding yield, there was also no significant effect (P=0.503), ranging from 2001 to 2803.5 kg/ha. *Trichoderma harzianum*, *Trichoderma asperellum* and *Bacillus amilolyquefaciens* + *Trichoderma harzianum*, increased grains by 385 kg/ha. (Figure 7B).

Figure 7. Soybean field trials with biological control product application. A) White mold disease incidence. B) Soybean yield.



3.3 Sclerotia carpogenic germination of successive non host crops

There was no significant (P = 0.328) effect of the interaction (crop x treatments) and the factors were analyzed separately. In regard to the factor Crop (p=0.022), maize resulted in higher carpogenic germination 59.6% compared to wheat 50.3% (Figure 8). For treatment (P=0.011), the germination control was the highest 65.7%, followed by *Bacillus amyloliquefaciens* + *Trichoderma harzianum*, *Trichoderma asperellum* A, *Trichoderma asperellum* B and *Trichoderma harzianum* by 61.8%, 51.5 %, 48.9, 47.0 %. *Trichoderma asperellum* A, *Trichoderma asperellum B* and *Trichoderma harzianum* differed significantly from the Control, (Figure 9).

Figure 8. Carpogenic germination of *Sclerotinia sclerotiorum* sclerotia in wheat and maize field trials.

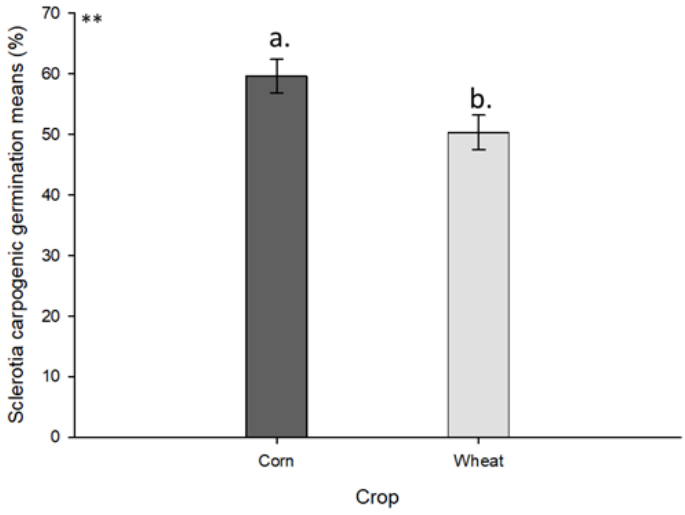
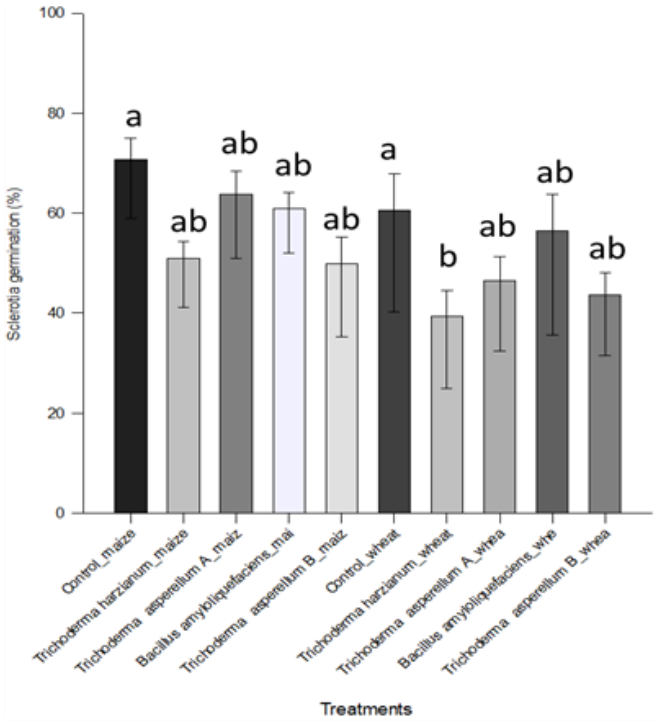


Figure 9. Effect of treatments on Carpogenic germination of *Sclerotinia sclerotiorum* sclerotia in wheat and maize field trials.

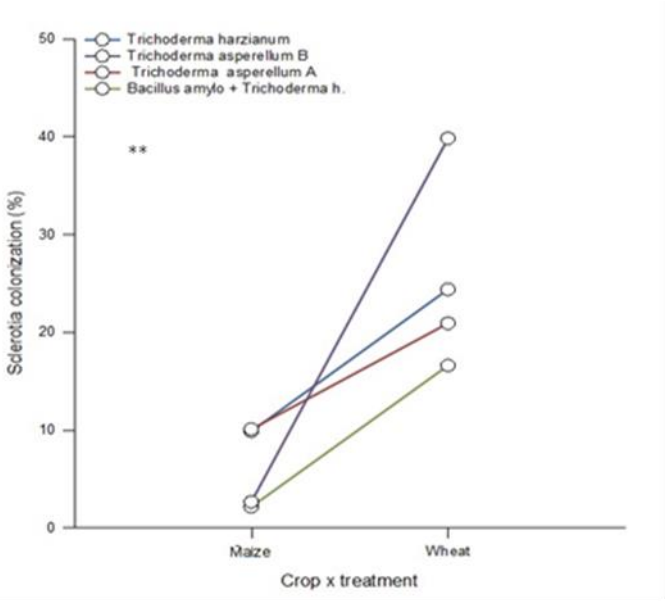


3.4 Sclerotia colonization by biological control agents

Interaction (crop x treatment) (p=0.048), colonization in maize trials range from 2.2 % to 10.2 %. For treatments, the effect was not significant (P=0.157), while for crop it was (p<0.001). In maize as cover crop, the sclerotia colonization averaged 6.3% and in wheat 25.5%, (Figure 10). The highest colonization was observed by *Trichoderma asperellum* A, follow by *Trichoderma harzianum*, *Trichoderma asperellum* B, and *Bacillus amyloliquefaciens* + *Trichoderma harzianum* by 10.2 %, 9.9%, 2.7% and 2.2%, respectively. The sclerotia colonization when wheat was the cover crop ranged from 16.6 to 39.8%. *Trichoderma asperellum B,* followed by *Trichoderma harzianum*, *Trichoderma asperellum* A, and *Bacillus amyloliquefaciens* + *Trichoderma harzianum* by 39.8%, 24.4%, 21,0% and 16.6%. respectively, (Figure 11).

Figure 10. Sclerotia colonization by biological control agents in wheat and maize field trials.

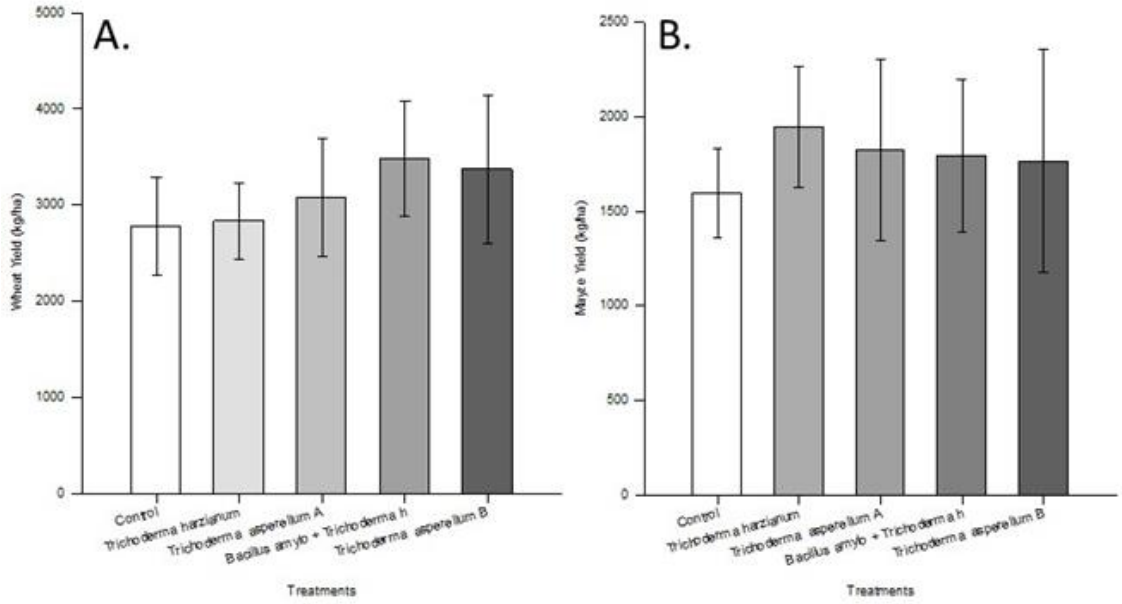
Figure 11. Carpogenic germination of *Sclerotinia sclerotiorum* sclerotia in wheat and maize field trials.



3.5 Maize and Wheat yield

Regarding wheat yield, there was no significant effect (p=0.177), treatments *Trichoderma asperellum B* and *Bacillus amyloliquefaciens* + *Trichoderma harzianum* responding positively with yield production by 700 and 300 kg. Maize yield (p = 0.203) the treatment *Trichoderma harzianum* present 400 kg.

Figure 12. Yield of wheat and maize crops on field trials with biological control-based products.



4 DISCUSSION

Soybean crop rotation systems with maize and wheat can benefit soybeans by providing nutrients and improving the physical and biological quality of the system (SILVA et al., 2022). White mold is one of the main diseases of soybean and, it causes a reduction in productivity of up to 70% (PELTIER et al., 2012). It was demonstrated that had a correlation of quantity of initial inoculum and incidence disease in field productions (LEHNER et al., 2017). Sclerotia remain in the field for years even in the absence of the soybean crop (BOLAND et al., 1988), although this may not be true for tropical agriculture, where favorable conditions for the pathogen germination is encountered throughout the year. Thus, non-host crops and biological cultural practices can reduce the source of infection of this pathogen (JULIATTI et al., 2015).

Regarding sclerotium germination when soybean was the cover crop, we found from 31.0% to 62% (Figure 6A), and colonization ranging from 4.7% to 19.4%, (Figure 6B). Carpogenic germination depends on the interaction of temperature, humidity soil and light and therefore the variation in germination relies on the species and geographical origin of pathogen isolate (BOLAND et al., 1988; PELTIER et al., 2012) and this within the range frequently reported for soybean (Meyer, 2021). Furthermore, biocontrol interaction requires favorable conditions and time for full establishment of the parasitic relationship. Indeed, the main mechanism of action involved in colonization is

mycoparasitism, which require growth of the antagonistic fungus and production of hydraulic enzymes, followed by pathogen sclerotium cell wall degradation (STEYAERT et al., 2003).

The disease incidence ranged from 73.7% to 87.2%, (Figure 8). A historical inoculum pressure implies in a reservoir of sclerotia in the field that may have contributed to the development of the disease. According to Lehner et al. (2017), of the concentration of viable sclerotia has direct relationship with the disease incidence under favorable environmental conditions. Additionality, in the 2020 and 2021 seasons, the effect of accumulated rain up to 300 mm contribute to favorable environment to infection and disease development, while these conditions were not favorable to BCA performance (ABAWI et al., 1971; FARIA et al., 2022)

Considering maize and wheat corps, treatments affected carpogenic germination (P=0.011). The products based on *Trichoderma asperellum* A, *Trichoderma asperellum B* and *Trichoderma harzianum* differed significantly to the Control considering either crop. According Görgen et al. (2009) the use of grass and *Trichoderma harzianum* application affect ability to the colonization of the biological control agent. The straw and canopy provide physical barrier to apothecia formation and buffers the niche to favor *Trichoderma harzianum* to establish and colonize sclerotia structures. Furthermore, there is evidence that the adoption of a biocontrol agent may modulate the native microbiome and recruits other organisms with antagonistic potential (SUI et al., 2022).

Furthermore, other factors may affect the establishment of antagonistic microbial communities. Along with the crop, there is a different weed management system technically recommended and the factors were not isolated from the effect of the plant itself and the change in crop management practice may result in variable biocontrol efficacy (ILLESCAS et al., 2020)

On the other hand, the tolerance of antagonistic community to pesticides assures stable efficacy (WIDENFALK et al., 2008). We have determined that glyphosate affect viability of *Trichoderma* spp, when exposure to the herbicide is 3h or more inhibition *Trichoderma* sp. was 100% (Supplementary data – Figure 13). Additionality, glyphosate herbicide on growth of *Trichoderma* sp. depends on dose and genera of biological control agent (RAMANAGOUDA et al., 2021). Exploring the system and analyze maize herbicides, viability in vitro tests shows that doses of Atrazine affect number of spores and mycelial growth of *Trichoderma atroviride* (SANTORO et al., 2014). Atrazine

reduced by 40.1% the number of spores produced compere to control. On colony growth, the effect is strong and decreased by 83.8% (SANTORO et al., 2014).

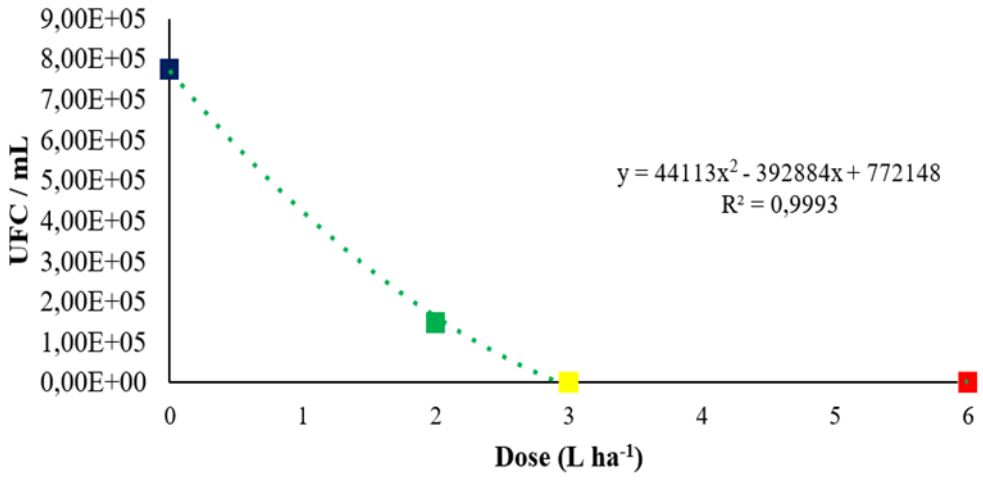
Thus, the role of this microbials combined with non-host as cover crop following soybean crop. In this case, considering the viability to biological agents to colonize sclerotia in wheat crop, can contribute to decrease sclerotia of *Sclerotinia sclerotiorum.* Thus, possible contribution to disease management in fields that have high inoculum pressure and incidence of the disease.

5 CONCLUSIONS

The parasitism of *Sclerotinia sclerotiorum* sclerotia was variable according to the considered crop. While the most important host is soybean, the application of the biocontrol products aiming at parasiting sclerotia at that crop was not the one that resulted in the highest parasitism and colonization of sclerotia. Actually, wheat was the most important plant to be considered as a succession of soybean when there is a high pressure of the disease. With a decrease from 65.7% to 47.0% sclerotia viability. Thus, for higher performance of biocontrol application in the management of white mold, a system-wide approach should be considered in the management of the diseases tackling the inoculum potential.

Supplementary data

Figure 13. Colony forming unit (CFU mL^{-1}) in the volume of 150 L ha^{-1}.



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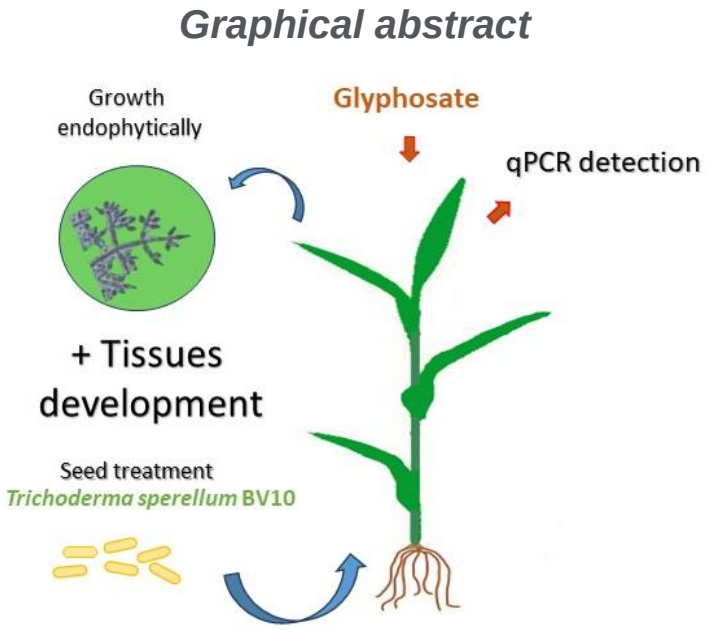
4 CHAPTER 4: THIRD MANUSCRIPT

How *Trichoderma asperellum* BV10 endophytic affect Wheat tissues development and persist after Glyphosate application?



Abstract

Sclerotinia sclerotiorum is a plant pathogen that infects and causes White Mold disease in a range of crop plants worldwide. The presence of sclerotia is an important factor for the epidemics of white mold. Biological and cultural practices were important tools to manage this disease, focusing on the reduction of sclerotia viability. We investigated whether treating seed with *Trichoderma asperellum* BV10 could result in the biocontrol population buildup endophytically on wheat plants. We evaluated the effect of the treated seeds on root length and aerial parts at 7 and 20 days after planting. Additionally, we analyzed quantified Trichoderma on plant tissues before and after Glyphosate treatment of wheat plants and its ability to colonize sclerotia of Sclerotinia sclerotiorum. At 7^{th} days after planting aerial part (P = 0.318) however roots increased by 28% (P = 0.010), additionality at 20^{th} days after planting, for aerial parts (P=0.049) increased plant height by 10.7%, and root length (P = 0.008) by 18, 7%. *Trichoderma asperellum* BV10 mycelium was detected trough the parenchyma root cells and into leaf tissues at the basis of leaf hair and epidermis at 20 days after planting. The *T. asperellum* levels detected on roots at 20 days after planting was up to 1 x 10^4 cfug. Followed by roots at 7 days after planting up to 1 x 10^3 cfu/g. For shoots at 7 and 20 days, up to 1 x 10^3 cfu/g were detected After wheat plants were killed with glyphosate, the presence of

T. asperellum decreased to 1 x 10 ^{3} in the roots, and in the bottom leaf to 1 x 10 ^{2} cfu/g Regarding carpogenic germination, we found germination rates for the water control (31.2%) and Trichoderma (26.7%) were similar. Thus, *T. asperellum* BV10 was able to colonize wheat plants endophytically, build up its population in roots and leaves but the glyphosate application reduced its population and hampered its potential to parasite sclerotia of *S. sclerotiorum*.

Keywords *Trichoderma asperellum. E*ndophytically. Wheat. Sc*lerotinia sclerotiorum.*

1 INTRODUCTION

Sclerotinia sclerotiorum is a plant pathogen that infects and causes white mold in a range of crop plants worldwide, such as common bean and soybean (BOLAND et al., 1994; BOTELHO et al. 2013; WILLBUR et al., 2019). Soybean is the one of most important crops consumed and exported in Brazil, in 2021 season 38.5 million hectares were planted and the production was 135.4 million tons of grains (CATTELAN; DALL'AGNOL, 2018; CONAB, 2021). White mold disease as able to reduced soybean yield losses of up to 70% (PURDY et al., 1979).

The presence of sclerotia is an important factor for the epidemics of white mold (ABAWI et al., 1979). Sclerotia germinates to form mycelium or apothecia (eruptive germination). Mycelium development infects the plants by direct penetration root or leaf tissue (JAMAUX, 1995). During eruptive germination, ascospores were formed from apothecia that provide other ways of pathogen infection (ABAWI et al., 1979).

Management of the disease is based on integrated disease management (SMOLIŃSKA et al., 2018; WILLBUR et al., 2019). However, the resistance to white mold is not yet available, chemical fungicide sprays at the reproductive stages of soybean protect the flowers from ascospores infection but does not have the same efficacy in the canopy and the bottom part of the plant which is the first reached by the ascospore is not as protected from the foliar application as the bottom part. (WILLBUR et al., 2019; MEYER et al., 2014). Thus, other disease management practices should be integrated such as biological and cultural practices particularly to reduce sclerotia viability (GERALDINE et al., 2013; VENTUROSO et al., 2013).

Indeed, one of the biological control strategies for the disease management is based on the mycoparasitism of sclerotia to reduce the release of ascospores by apothecia (MUKHERJEE et al., 2022). To evaluate the best approach of combining cover crops with biological control, Görgen et al. (2009) introduced *Trichoderma harzianum* by

spraying in *Brachiaria ruziziensis* on soybean field crops. *B. ruziziensis* decrease white mold disease incidence and its performance was improved by *Trichoderma harzianum*, measured as sclerotia colonization (GÖRGEN et al., 2009).

Trichoderma species was reported as sclerotium colonizer and the producer of protease, β-glucosidase, and chitinase stands out as lytic enzymes, and were related to the degradation of the fungal cell wall of (MUKHERJEE et al., 2022; HASSAN, 2014). Soybean crops planted in succcession with non-host plants such as maize and Wheat can reduce inoculum pressure and incidence of disease levels on field conditions (GARZA et al., 2002). Thus, the grasses reduced ascospore release with a physical barrier to apothecium formation and/or can provide favorable conditions to stimulate sclerotium germination without the host (BRUSTOLIN et al., 2016). *Trichoderma* sp. can develop mutualistic relationship with plants such as tolerance to drought stress and promote plant growth (PEDRERO-MÉNDE et al., 2021) Some can organisms can grow endophytically and not only buildup its population in the crop system but also persist after glyphosate spray and parasite sclerotia of *Sclerotinia sclerotiorum* in the so-called trojan horse mode of action (ALVES et al., 2021CHI).

Considering wheat-*Trichoderma* interactions already reported, exploring the *Trichoderma* endophytically growth on wheat would benefit not only the treated crop but the one that succeed it, which is predominantly soybean and could reduce the viability of *Sclerotinia sclerotium* inoculum.

Thus, we aimed at evaluating *Trichoderma asperellum* growth endophytically on wheat plants by light microscopy and qPCR and investigate the development of roots and leaves length at 7 and 20 days after planting. In addition, investigate the ability of *T. asperellum* to persist and parasite sclerotia of *Sclerotinia sclerotiorum* after Glyphosate application.

2 MATERIAL AND METHODS

2.1 *Trichoderma asperellum*, strain and inoculum.

The spore culture of *Trichoderma asperellum BV10* BV10, ($10x10^9$ ufc/g), was provided by Vittia Fertilizantes e Biológicos S.A (São Joaquim da Barra, São Paulo, Brazil). *S. sclerotiorum* was provided by Texas A&M AgriLife Research Center (Lubbock, Texas, United states). Sclerotia superficial disinfested by 70% ethanol (60 s)

followed by 5% sodium hypochlorite (60 s) and rinse in sterile distilled water tree times. (SANABRIA-VELAZQUEZ et al., 2019).

2.2 Wheat seed inoculation and developed length tissue

Four seeds of wheat-treated and untreated were sown in each pot. Seed treatment was carried out at 5ml of 10^7 spores rate of *Trichoderma asperellum BV10*, per kg of seed, 12 hours before planting (XUE et al., 2017; SUN et al., 2019). Two controls were used as negative control, non-inoculated seeds with autoclaved soil and non-inoculated seeds non-autoclaved soil. The soil was collected and autoclaved twice for 30 min at 121 C^o and 1,5 atm pressure with interval of 24h between autocleavages (BENNETT et al., 2003). The assays were performed with four plants per plot and five replicates per treatment. The plants were kept in a greenhouse for 34 days at 23°C ± 2, under a 12:12 L/D photoperiod. To check developed growth, the plant height (cm) and root length (cm), were determined at 7 and 20 days after planting (ALVES et al., 2021).

2.3 Tracking the population of *Trichoderma asperellum*, over time during plant colonization

To track the dynamics of the biocontrol in the plant through light microscopy and qPCR as proposed by Gerin et al. (2018) for *T. asperellum*, Initially, the DNA of the pure colony of the fungus has been used to validate the primers. At 7, 20 and 34 days after planting, samples have been processed for the biocontrol population determination in each plant tissue. Approximately 10 cm length of leaf tissues was split in the middle and analyzed on leaf up (5cm) and leaf down (5cm) tissue. Roots samples were the 10 cm bottom part of the pivoting root. At 30 DAPS, the plants were finalized by Glyphosate spray 2% (v/v) (ALVES et al., 2021) and a last plant tissue sampling was made 4 days after Glyphosate spray.

2.4 Light microscopy analyses

Leaf and roots samplings were evaluated with Trypan Blue (0.01%) according Nanjundappa et al. (2021). The tissues collected at 20 days after planting. Approximated 10 cm length of leaf tissues was split in middle and analyzed on leaf up (5cm) and leaf down (5cm) tissue. Roots samples was considering 10 cm of principal root. All tissues

were cut in 0,5 length and transferred to a solution with acetic acid and ethanol (1:3 v/v), incubated for 12h. The following treatment was with solution of acetic acid: ethanol: glycerol (1:5:5) v/v/v for five hours. Thus, all tissues were transferred to solution with Trypan Blue (0.01%) for 8 h. The fragments were stored on a glycerol (60%) until the microscopy analysis. Ten fragments per plant were evaluated on Olympus BX41 microscopy (NANJUNDAPPA et al., 2021).

2.5 DNA extraction and qPCR

The genomic DNA of *Trichoderma asperellum BV10*, was extracted from a suspension of pure conidia 1 x 10^{7} CFU/ml. Standard curves were 1:10 dilution factor, created and accepted when the slopes were upper −3.44 (95% efficiency) and the value of the correlation coefficient (R^2) was > 0.96. The amplification percentage efficiency was calculated as $(10^{(-1/\text{slope})}-1)\times 100$ (PERINI et al., 2011). Quantity and quality of DNA were assessed using ABI 7500 FAST, and amplifications experimental setup and data analysis. *Trichoderma asperellum* BV10, DNA on leaf and root extraction was used with the DNeasy Plant Pro Kit (Qiagen USA, Valencia, CA) protocol. For the bulk of DNA, 0,06 mg of tissue from 4 plants were used at each sampling. PCR mixes were done according to (GERIN et al., 2018). In short, adding 6.25 µL of Sso AdvancedTM Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, United States), 250 nM of primers developed to *Trichoderma asperellum BV10* Ta_rpb2fw and Ta_rpb2_rev3, 150 nM of Tarpb2_probe, 1 (single qPCR) µL of DNA template, and 12.5 µL of ultrapure water. Thus, the condition of thermal cycling was 95◦C for 2 min, 40 cycles at 95◦C for 5 s, and 64.5◦C for 30 s (GERIN et al., 2018).

2.6 Glyphosate application and sclerotia viability

To check the viability of *Trichoderma asperellum BV10*, colonized sclerotia, plants were finalized by glyphosate (Zapp Pro®) at 1.5 L / ha and 2%v/v. Four days after plants were finished, sclerotia of *Sclerotinia sclerotiorum* were placed on soil for 10 days. Two negative controls were untreated seeds on autoclaved soil and non-autoclaved soil. 10 days after sclerotia were laid on the soil, the sclerotia samples were removed from pots for a germination test (ALVES et al., 2021). The viability was set by placing in transparent plastic boxes containing 60g with sterile sand, watered to 90% of field

capacity, and kept at 20±3 C° for 60 days. Thus, the percentage of number of sclerotia colonized by *Trichoderma asperellum BV10*, and the number of germinated sclerotia mycelium and apothecia, were recorded (ALVES et al., 2021).

2.7 Statistics

For in vivo and viability sclerotia tests, the data were checked for normality thought Shapiro-Wilk's test, Equal Variance by Brown-Forsythe and comparation of means with treat and untreated samples by Student's t-test.

3 RESULTS

3.1 Development of leaf and roots

At 7th days after planting aerial part (P = 0.318), (Figure 14A). However, roots at 7th days after planting (P = 0.010), increased by 28% (Figure 14B). Additionally, increases aerial part and root lengths were regarding at 20 days after planting. For aerial parts (P=0.049), plants treated with *T. asperellum* increased length by 10.7%. root length (P = 0.008) increased by 18. 7% (Figure 15B).

Figure 14. Length of wheat tissues at 7 days after planting. A) Aerial part. B) Roots.

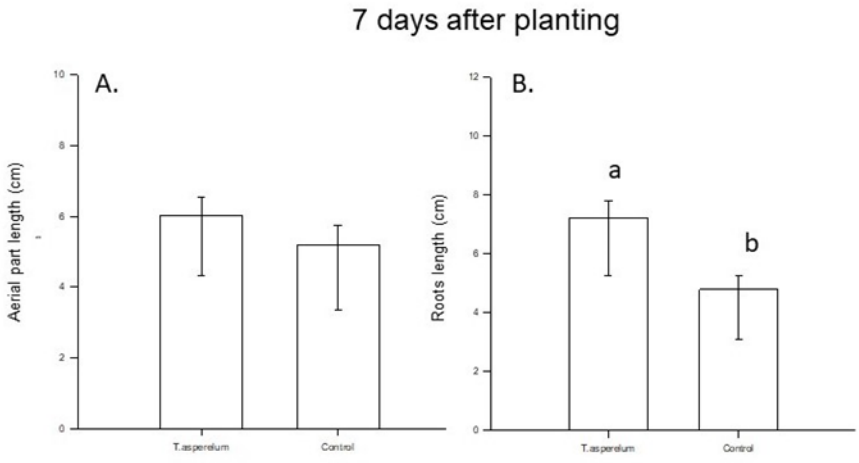
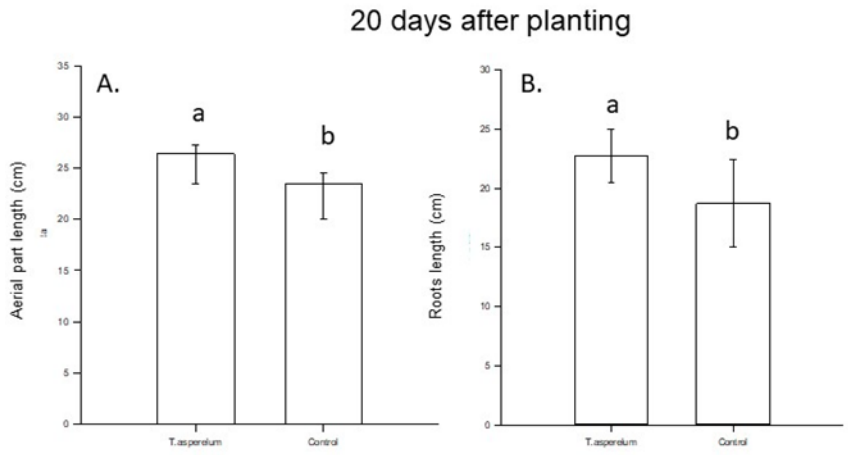


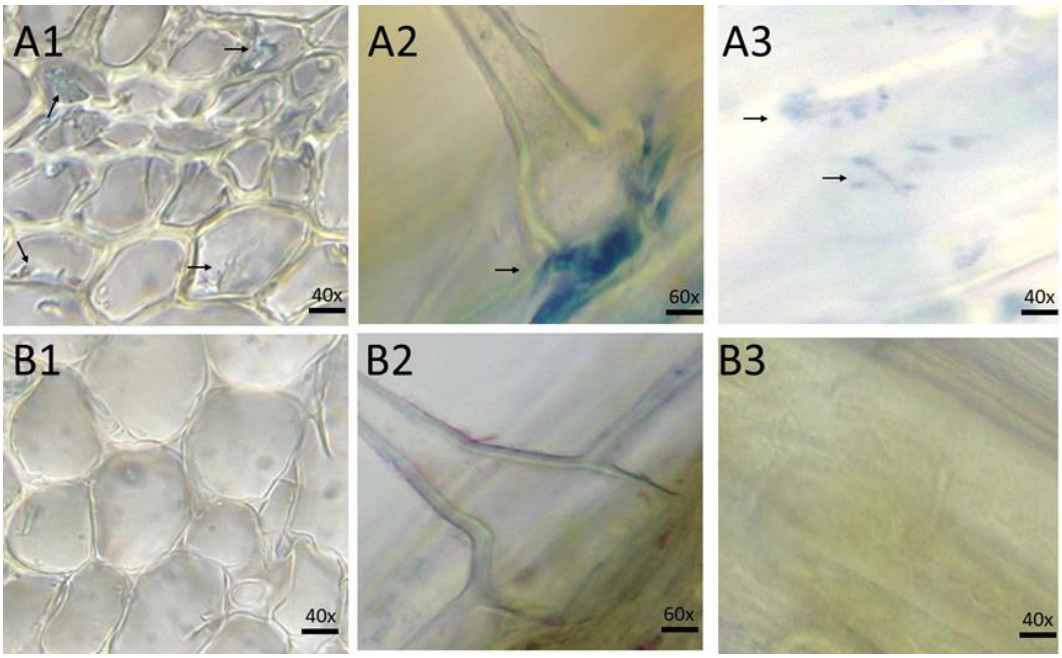
Figure 15. Length of wheat tissues at 20 days after planting. A) Aerial part. B) Roots.



3.2 Light microscopy analyses

The presence endophytically grown *Trichoderma* was determined by light microscopy at 20 days after planting. The transversal section showed *Trichoderma* hyphae growing through the parenchyma roots cells (Figure 16A1). On leaf tissues the presence of *Trichoderma* mycelium was detected on basis of leaf hair and into epidermis (Figure 16A2 and 16A3).

Figure 16. Microscopy light slides of tissues of wheat plants. A1) parenchyma roots cells with treat *Trichoderma* asperellum BV10 B1) parenchyma roots cells Control. A2) Leaf hair with treat *T. asperellum* B2) Leaf hair Control. A3) Leaf epidermis with treat *T. asperellum* B3) Leaf epidermis Control.



3.3 Tracking the population of Trichoderma asperellum BV10, over time during plant colonization and after Glyphosate application

The level of *T. asperellum* in plant tissue increased over time and reduced after glyphosate application. The distribution of the endophytic on tissues was higher in the roots, followed by bottom leaf and was not detected on the top leaves. The highest level of *Trichoderma* on roots at 20 days after planting 1×10^{4} cfu/g. Followed by roots at 7 days after planting up to 1×10^{3} cfu/g For aerial parts at 7 and 20 days, up to 1×10^{2} cfu/g (Figure 17). After wheat plants were treated with glyphosate, the presence of *T. asperellum* decreased in 1×10^{2} root, and bottom leaf part to 1×10^{1} (Figure 18).

Figure 17. Detecting endophytically presence of *Trichoderma* sp. by qPCR at 7 and 20 days after planting.

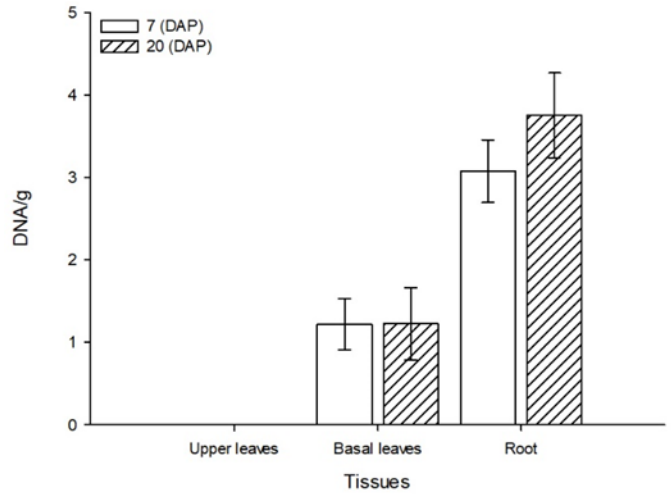
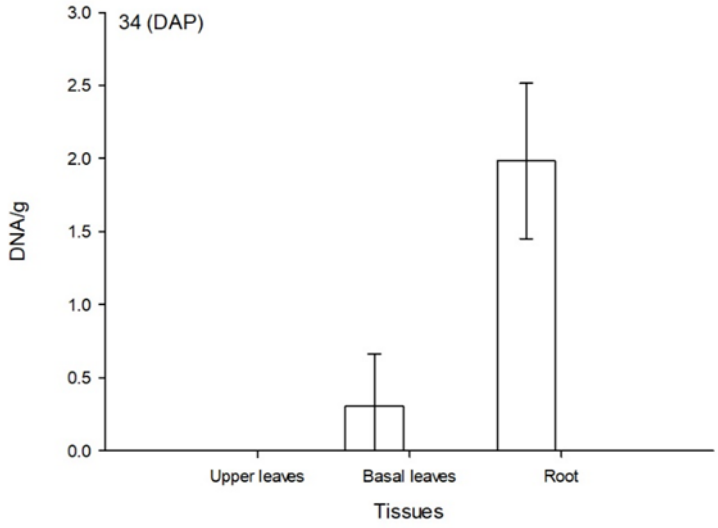


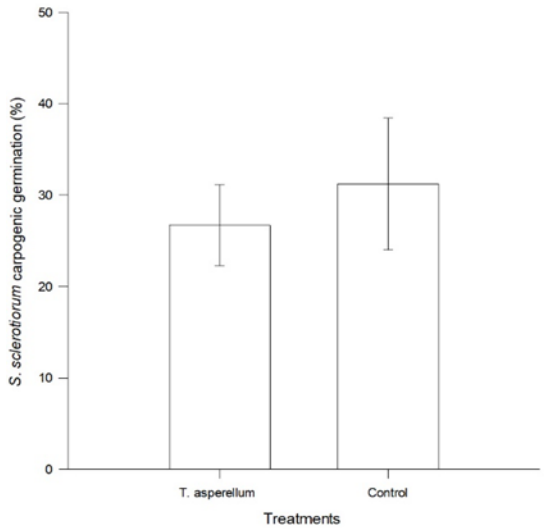
Figure 18. Detecting endophytically presence of *Trichoderma* sp. by qPCR at 34 days after planting, after Glyphosate application.



3.4 Sclerotia viability

For sclerotia viability (P = 0.419). Control carpogenic germination by 31.250 %. and *Trichodema asperellum* BV10, by 26.7 % 31.250 %. No colonization was observed in sclerotia.

Figure 19. *Sclerotinia sclerotiorum* sclerotia germination in soil with seed wheat treat with *Trichoderma asperellum* BV10 and control.



4 DISCUSSIONS

Analyzing the development of shoots and roots at 7th day there was no difference between the development of plants treated with *Trichoderma* and the negative control in aerial part (Figure 14A), but increase by 28% in roots, (Figure 14B). At 20th day, we verified significantly the development of the aerial part 10,7% and root in 18,7%, respectively (Figure 15A and 15B)**.**

According to Matar et al., 2022, endophytic isolates of *Trichoderma guizhouense* and *T. afroharzianum* promoted root growth in 37.6% and 37.7%, respectively. The aerial parts increase in length by 5.2% with both fungi. The seed treatment of wheat plants with *Glomus mossae* and *Trichoderma atroviride* MUCL 45632, increased the germination, reaching 88.7%. In addition, shoot and root dry biomass of seedlings were higher by 10.0%, 28.6%, 23.1% and 64.2% (COLLA et al., 2015).

Trichoderma promotes growth on roots and shoot length on wheat plants. *T. harzianum* IBLF006 WP, *T. harzianum* IBLF006 SC, *T. harzianum* ESALQ1306 *and T. asperellum* URM 5911 increased length root 0,50, 1,29, 4, 82 and 4,99 cm. Regarding shoot length, the isolates increased up 1,5 to 6,25 cm (OLIVEIRA et al., 2018) but its endophytic nature was not demonstrated.

Actually, such growth promotion may be related to the direct production of phytohormones by the fungus. such as auxin and indole-3-acetic acid (IAA) (NIETO-JACOBO et al., 2017; MATAR et al., 2022) Additionally, *Trichoderma* sp. excrete

elicitors like peptides, proteins and low-molecular-weight compounds that can modulate the signaling pathway of wheat plants, and trigger abiotic stress tolerance (ILLESCAS et al., 2021).

Our study showed *Trichoderma asperellum* BV10 could be retrieved endophytically from roots and leaves, on roots the concentration was higher than the shoots and actually, on the shoots it was only detected at the basal leave (Figure 16A). The endophytic colonization may not necessarily follow the plant growth and the colonization of the upper leaf could have occurred at a later sampling time point since we found higher detection of *Trichoderma asperellum* BV10, at 20 days after seedling (Figure 16A).

Furthermore, according to Al-Khawaldeh et al. (2020), and Błaszczyk et al. (2021), the roots have more endophytic microorganisms than other wheat plant tissues, and its mobility into other tissues may not necessarily occur. The abundance of endophytic microorganisms was variable according the growth stage of wheat and it can differ according to wheat plants genotype (COMBY et al., 2016; RANA et al., 2020).

At 4 days after Glyphosate application the rate of *Trichoderma asperellum* BV10, decrease up to 1×10^{3} into root tissues and less than 1×10^{2} on leaves (Figure 16B). The effect of the interaction microorganism-glyphosate can differ by species and isolate of *Trichoderma* sp. and the dose of the Glyphosate herbicide (RAMANAGOUDA et al., 2021). For example, Glyphosate tolerance ability of T. *asperellum, T. afroharzianum, T. harzianum,* isolates ranged from 11 to 48 mm of mycelial growth at 0.35 dose and by 6–39 mm at 0.4 per cent (RAMANAGOUDA et al., 2021)

Regarding carpogenic germination, we found germination rates for the control and *Trichoderm*a were 31.2 %, and 26.7 % respectively (Figure 17). Carpogenic germination rates was variable according the isolate and geographical origin of the pathogen (HUANG et al., 1991; GODOY et al., 2017). Additionality, some isolates need preconditioner to apothecia forming and others do not need (DILLARD et al., 1995)

Our study demonstrated no colonization of *Trichoderma* sp. on sclerotia. The units forming colony number can influence *Trichoderma* sp. colony growth. Colony growth is important factor for *Trichoderma* sp. can confront the antagonist and realize mechanism of action like competition and Mycoparasites (KÜÇÜK et al., 2003). Mycoparasites was a complex interaction of hype development, recognize the antagonist, penetrate the tissue and produce and secrete degraded cell wall enzymes (GERALDINE et a., 2013).

Thus, the explore tool of *Trichoderma* sp. endophyte wheat plants able to promote tissues developed, we can investigate how the interaction works on the field conditions. Additionality consider a system without wheat glyphosate finalized, explore your benefits to wheat treat plants and natural microorganisms' interaction in effect on sclerotia in soil fields production systems.

5 CONCLUSIONS

The *Trichoderma asperellum* BV10, was endophytic of wheat plants and regarding length increased by 10,7% of aerial part and 18,7% of roots. The high detection of the biological control agent was found on root tissues at 20 days after planting up to 10×10^4 and, at 7 days after planting up to 10×10^3. For aerial parts at 7 and 20 days up to 10×10^2. The detection intensity growth up from 7 to 20 days after planting. Glyphosate application decreased *Trichoderma* sp. detection at 34 days, in 1×10^3 root, and down leaf part to 1×10^2. Top dressing seed wheat no have effect on carpogenic germination we found germination by 26.7 % *Trichoderma asperellum* BV10 and control by 31.2%. No colonization by *Trichoderma asprellum* BV10 on *Sclerotinia sclerotiorum* sclerotia was detected.

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Conflict of interest

The authors declare that they have no conflict of interest.

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