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Conidia, enzymes and metabolites of Purpureocillium lilacinum Coad 4015 for the control of nematodes and insect pests: laboratory and field studies

Amanda Cristiane Queiroz Motta
Doctor Scientiae

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AMANDA CRISTIANE QUEIROZ MOTTA

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ABSTRACT

MOTTA, Amanda Cristiane Queiroz, D.Sc., Universidade Federal de Viçosa, August, 2025. **Conidia, enzymes and metabolites of Purpureocillium lilacinum Coad 4015 for the control of nematodes and insect pests: laboratory and field studies**. Adviser: Simon Luke Elliot. Co-adviser: Éverton Kort Kamp Fernandes.

Insect pests and plant-parasitic nematodes cause significant yield losses in agriculture, and the use of mycoparasitic fungi for their control has been increasing. However, a limited range of fungi has proven activity against these organisms, reinforcing the need to evaluate new strains and species with potential for biological control. This thesis aimed to characterize and evaluate the potential of a new strain of *Purpureocillium lilacinum* (COAD 4015) as a multi-target biological control agent, focusing on the management of nematodes and insect pests of agricultural importance. In Chapter 2, the strain was tested for the control of root-knot nematodes of the genus *Meloidogyne* in tomato crops. The application of conidia at different concentrations significantly reduced the number of eggs and juveniles in the soil and roots, in addition to promoting plant growth. The absence of fungal growth at 36°C indicated low risk to human health, reinforcing its safety for agricultural use. In Chapter 3, the efficacy of *P. lilacinum* was evaluated for the field control of the corn leafhopper (*Dalbulus maidis*). Two trials were conducted: one under commercial field conditions and another in a controlled experimental setting. The application of the fungus with an oil-based adjuvant improved conidial adhesion to the insect's cuticle, overcoming natural barriers such as brochosomes, and resulted in population reduction of the pest, as well as a significant increase in maize grain yield. In Chapter 4, the pathogenicity of conidia, enzymes, and metabolites was investigated against *D. maidis*, *Tenebrio molito*r, and four lepidopteran pest species. The results indicated low overall pathogenicity, with moderate effects observed only in *Rachiplusia nu* (enzymes) and marginal response in Spodoptera frugiperda. Notably, the addition of *P. lilacinum* to the chemical insecticide acephate (active ingredient of the commercial product Acefato®) resulted in a 50% reduction in *D. maidis* mortality compared to acephate alone, suggesting a possible negative interaction between treatments. Overall, the findings position *P. lilacinum* COAD 4015 as a promising candidate for integrated pest and nematode management, with potential for safe and effective formulations, provided its compatibility with chemical products is carefully evaluated.

Keywords: *Meloidogyne*; *Dalbulus maidis; Spodoptera frugiperda*;

Rachiplusia nu; *Chrysodeixis includens*; *Helicoverpa armigera*

RESUMO

MOTTA, Amanda Cristiane Queiroz, D.Sc., Universidade Federal de Viçosa, agosto de 2025. **Conídios, enzimas e metabólitos de Purpureocillium lilacinum Coad 4015 para o controle de nematoides e insetos-praga: estudos de laboratório e de campo**. Orientador: Simon Luke Elliot. Coorientador: Éverton Kort Kamp Fernandes.

Insetos pragas e nematoides fitoparasitas causam perdas significativas de produtividade na agricultura, e o controle com fungos micoparasitas vem aumentando. Entretanto, poucos fungos apresentam ação comprovada contra esses organismos, o que reforça a necessidade de avaliar novas cepas e espécies com potencial de controle biológico. Esta tese teve como objetivo caracterizar e avaliar o potencial de uma nova cepa de *Purpureocillium lilacinum* (COAD 4015) como agente de controle biológico multi-alvo, com foco no manejo de nematoides e insetos-praga de importância agrícola. No Capítulo 2, o isolado foi testado para o controle de nematoides do gênero *Meloidogyne* na cultura do tomate. A aplicação de conídios em diferentes concentrações reduziu significativamente a quantidade de ovos e juvenis no solo e nas raízes, além de promover o crescimento das plantas. A ausência de crescimento fúngico a 36°C indicou baixo risco à saúde humana, reforçando sua segurança para uso agrícola. No Capítulo 3, avaliou-se a eficácia de *P. lilacinum* no controle da cigarrinha-do-milho (*Dalbulus maidis*) em campo. Dois ensaios foram realizados: um sob condições comerciais e outro experimental. A aplicação do fungo com adjuvante à base de óleo melhorou a adesão dos conídios à cutícula do inseto, superando barreiras naturais como os brocossomos, e resultou em redução populacional da praga, além de aumento significativo na produtividade do milho. No Capítulo 4, investigou-se a patogenicidade de conídios, enzimas e metabólitos contra *D. maidis*, *Tenebrio molitor* e quatro espécies de lepidópteros-praga. Os resultados indicaram baixa patogenicidade geral, com efeito moderado apenas em *Rachiplusia nu* (enzimas) e resposta marginal em *Spodoptera frugiperda*. Notavelmente, a adição de *P. lilacinum* ao inseticida químico acefato resultou em redução de 50% na mortalidade de *D. maidis* em comparação ao inseticida isolado, indicando possível interferência negativa entre os tratamentos. Em conjunto, os resultados posicionam *P. lilacinum* COAD 4015 como um candidato promissor para o manejo integrado de pragas e nematoides, com potencial para formulações seguras e eficazes, desde que avaliada cuidadosamente sua compatibilidade com produtos químicos.

Palavras-chave: *Meloidogyne*; *Dalbulus maidis; Spodoptera frugiperda*; *Rachiplusia nu*; *Chrysodeixis includens*; *Helicoverpa armigera*

SUMARY

CHAPTER 1

1. General Introduction

Since the Green Revolution (1960-2000), there has been a significant increase in the use of fertilizers, new seed varieties, implements, agricultural machinery, and agrochemicals (pesticides, fungicides, and herbicides) to meet the demand for high yields (Pingali, 2012; Rana and Gupta, 2021). Among these technologies, one that has been increasingly used is chemical molecules (Shafiq et al., 2023). The improper and indiscriminate use of these chemical molecules can cause a series of health and environmental problems through direct or indirect contamination of air, soil, water, and the ecosystem in general, as well as affecting non-target organisms, such as pollinators (Singh et al., 2024).

Due to the harmful effects that chemical molecules can cause, there has been an increasing concern among the population for healthy and pesticide-free food (Sharma et al., 2021; Yi et al., 2020). Another crucial market regulator in the use of agrochemicals is the European Union (EU). The EU has strict rules on controlling the use of agrochemicals during agricultural crop cycles (Regulation (EC) No 1107, 2009). Non-compliance with the rules can compromise a large part of food exports to the EU, negatively affecting the economies of the world's largest food-exporting countries, including Brazil (FAO, 2023).

In Brazil, factors such as the tropical climate and uninterrupted cropping (green bridges), favor the development and attack of pest insects, which consequently generate a great need for control methods. Currently, chemical insecticides are still the most used control method in the country, which raises an alert due to the aforementioned problems, in addition to the main mode of action of these molecules occurring in the insects' nervous system, which is similar to that of humans (Osman, 2011).

Currently, one of the tools that has been widely adopted in Integrated Pest Management (IPM) is the use of biopesticides. Biopesticides are pest control products whose active ingredients are compounds or agents of natural origin, derived from animals, plants, microorganisms, such as bacteria, virus and fungi, and are formulated to control agricultural pests and pathogens (Kumar et al., 2021). They can be an effective substitute for synthetic chemical products due to their characteristic bioreactivity, reliability, and high efficacy dependency (Archana et al., 2022).

Among biopesticides, the most widely used biological control agents are entomopathogenic fungi. Fungi capable of infecting and killing insects were the first microorganisms to be used in biological pest control (Sisay and Legese, 2020) and are still widely studied and commercialized today (Karthi et al., 2024). These fungi comprise a diverse group of over 100 genera with approximately 750 species, reported in different insects (Sisay and Legese, 2020) and categorized into six phyla: Oomycetes, Chytridiomycota, Microsporidia, Entomophthoromycota, Basidiomycota, and the most common, Ascomycota (Litwin et al., 2020).

Ascomycetes are fungi that have septate hyphae that differentiate into structures for asexual reproduction such as conidiogenous, conidiophores or phialides, and conidia. The main route of entry for entomopathogenic fungi into the host is through contact of the conidia with the cuticle of the insect, without the need for ingestion. There are three steps that the entomopathogenic fungus follows to infect the insect: adhesion and germination, differentiation of the germ tube into an appressorium (specialized structure that generates mechanical pressure) and enzymatic activity to breach the host's cuticle, penetration into the hemocoel, and development of the fungus, which usually results in the death of the insect (Téllez-Jurado et al., 2009).

Upon initiating germination on the insect's cuticle, cascades of recognition reactions and enzymatic activation are simultaneously triggered in the host and parasite fungus (Samson et al., 1988). Subsequently, penetration occurs, and after successful infection, the entomopathogen grows in the hemocoel, rapidly depleting nutrients in the hemolymph and resulting in the death of the host. In addition, the fungus can invade and destroy other tissues or release toxic substances that interfere not only with the normal development and metamorphosis of the host but also with the immunological defense mechanisms necessary to combat invasive microorganisms, including fungi (Boucias and Pendland, 1998). The complex mode of action of entomopathogenic fungi prevents the evolution of populations of resistant pests (Islam et al., 2021).

The fungal bioinseticides currently available on the market are based on a few genera, specifically the anamorphic phase of hyphomycetous ascomycetes in the order Hypocreales, which includes key families such as Clavicipitaceae, Cordycipitaceae, Hypocreaceae, and Ophiocordycipitaceae (Wijayawardene et al., 2022). Within the Cordycipitaceae, important genera to control arthropods include *Beauveria*, *Akanthomyces*, *Lecanicillium* (formerly

Verticillium), and *Cordyceps* (formerly *Paecilomyces* or *Isaria*). In Clavicipitaceae, the genus *Metarhizium* stands out (Faria and Wraight, 2007; Quesada-Moraga et al., 2024).

Few entomopathogenic fungal species have been described in the literature, and even fewer are currently available for commercial use as biological control agents against insect pests. In addition to their role in Integrated Pest Management (IPM), certain species within the same fungal order have also been successfully employed in the control of plant-parasitic nematodes. Given that nematodes possess a chitin-rich outer layer—structurally like the insect cuticle—this raises the hypothesis that fungi used to control nematodes may also exhibit entomopathogenic potential.

Among these fungi, *Purpureocillium lilacinum* (Ophiocordycipitaceae) stands out as one of the most widely used in the biological control of nematodes. In this context, the present study aims to evaluate the entomopathogenic potential of a newly isolated strain of *P. lilacinum*, by testing its ability to infect and affect different species of insect pests. This General Introduction provides an overview of the historical development of modern agriculture, the growing challenges related to chemical pest control, and the increasing demand for environmentally friendly alternatives. In the following sections, we present the scientific foundations of this study, along with an overview of the experimental approaches and the specific objectives of the research.

1.1. *Purpureocillium lilacinum*

The fungus currently known as *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) was originally described as *Penicillium lilacinum* by Thom in 1910. It was later reclassified as *Paecilomyces lilacinus* by Samson in 1974, maintaining Thom as the original author of the species. In 1983, Dunn described a closely related taxon under the name *Paecilomyces nostocoides*. Subsequently, based on phylogenetic analyses, *P. lilacinus* was transferred to the newly established genus *Purpureocillium* by Luangsa-ard, Houbraken, Hywel-Jones, and Samson in 2011, becoming *Purpureocillium lilacinum* (Luangsa-ard et al., 2011).

Purpureocillium lilacinum is a cosmopolitan saprophytic fungus commonly isolated from soil, decaying plant material, insects, air and nematodes (Luangsa-ard et al., 2011). Its ability to parasitize nematode eggs has been recognized since at least 1979 (Jatala et al., 1979).

Several studies have demonstrated the ability of *P. lilacinum* to control plant-parasitic nematodes and their eggs, regardless of the crop in which it is established (Isaac et al., 2024; Khan and Tanaka, 2023; Rajendran et al., 2024). However, most published studies emphasize its high efficacy against nematodes of the genus *Meloidogyne*. Its potential as a biological control agent is recognized by the European Union (EU), where it is included in the official pesticide database as an approved biological nematicide. In Brazil, there are nine registered products based on *P. lilacinum* for the control of nematodes: Biostat WP, Gratto Nema, GNC008, Bio Gall, Agripon Nemacolt, Now Cover, Best Equilíbrio Ultra, BV0518, and Offensive (MAPA, 2024). In Switzerland, *P. lilacinum* is the only biological nematicide authorized for the control of *Meloidogyne* spp., with applications recommended pre-planting, at planting, and during vegetative development (Dahlin et al., 2019).

The infection of nematode eggs by *P. lilacinum* appears to rely primarily on physical contact to recognize nematode eggs (Xie et al., 2016). Infection begins with the growth of fungal hyphae over the gelatinous matrix that envelops the egg mass on the root surface (da Silva Amaral, 2021). This mucilaginous substance is rich in chitin and serves as the initial substrate for fungal adhesion and development. The fungus then forms penetration structures—such as appressoria and penetration pegs—that exert mechanical pressure and secrete hydrolytic enzymes. These enzymes degrade the eggshell layers, including the vitelline (protein), lipid, and inner chitin layers, facilitating fungal entry (da Silva Amaral, 2021). Once inside, the fungus colonizes the egg, sporulates, and produces mycotoxins, which disrupt embryo development and increase eggshell permeability (da Silva Amaral, 2021; Xie et al., 2016). In sedentary juveniles and adult females, *P. lilacinum* infects the nematode by direct contact with the cuticle; the fungus adhesion and penetration are facilitated by the formation of appressoria and the secretion of enzymes such as serine proteases and chitinases (da Silva Amaral, 2021).

The phylogenomic tree constructed from the genome assembly of *P. lilacinum* confirmed its evolutionary relationship with other fungi in the order Hypocreales, such as *Metarhizium* spp., *Pochonia chlamydosporia*, *Cordyceps militaris*, *Trichoderma reesei*, and *Fusarium* spp. (Prasad et al., 2015). Similar to these species, *P. lilacinum* possesses an expanded set of genes associated with nematode infection, including those encoding G protein-coupled receptors (GPCRs), proteases, and carbohydrate-degrading enzymes such as glycoside hydrolases and carbohydrate esterases, which are involved in the degradation of the nematode and nematode eggshell (Prasad et al., 2015).

The secondary metabolites (chemical compounds that are not essential for the growth and development of the organism) produced by *P. lilacinum* are also well described and contribute to its pathogenicity against nematodes (Chen and Hu, 2021; Prasad et al., 2015; Wang et al., 2016). This species is known to produce toxins such as leucinostatins (also known as paecilotoxins), paecilomide, and acremoxanthones, which can cause mortality and inhibit nematode reproduction (Khan and Tanaka, 2023; Prasad et al., 2015).

Some strains of *P. lilacinum* have also shown potential in controlling various phytophagous arthropods. Among the affected species are members of the orders Hemiptera (*Bemisia tabaci*, *Trialeurodes vaporariorum*, *Aphis gossypii*, *Myzus persicae*), Lepidoptera (*Spodoptera frugiperda*), Thysanoptera (*Frankliniella occidentalis*) and Tetranychidae (Acari) (*Tetranychus urticae*) (Fiedler and Sosnowska, 2007; Liu et al., 2022; Nguyen Thi et al., 2023).

Another beneficial effect of *P. lilacinum* is its ability to promote plant growth through phosphorus solubilization, phytohormone and secondary metabolite production, and organic matter decomposition (Rigobelo et al., 2024). It also possesses genes for cellulose and xylan degradation, suggesting its ability to colonize living plant roots and supporting its frequent occurrence in the rhizosphere, which may reflect a coevolutionary relationship not only with plants but also with nematodes (Xie et al., 2016).

Despite its broad potential for nematode and insect control and plant growth promotion, it is important to note that not all *P. lilacinum* isolates are risk-free. Some biocontrol strains show high genetic similarity to clinical isolates associated with human infections, particularly in immunocompromised individuals (Chen and Hu, 2021; Luangsa-ard et al., 2011). In this context, biosafety assessments—such as evaluating fungal growth at the human body temperature (37 °C)—are necessary to ensure that field-applied strains do not pose a threat to human health (Luangsa-ard et al., 2011; Yeo et al., 2003). Therefore, new *P. lilacinum* isolates should be carefully screened for pathogenic potential, enabling the development of safe and effective tools for integrated pest and nematode management, as well as plant growth promotion.

1.2. Plant parasitic nematodes: *Meloidogyne* spp.

All cultivated plants host one or more species of nematodes, which can be classified based on their feeding habits. These organisms may act as: (i) ectoparasites, feeding externally

on aerial parts or roots; (ii) sedentary semi-endoparasites, with part of their body inserted into the root and part remaining outside; or (iii) endoparasites, which fully penetrate the roots and feed internally. Endoparasites, in turn, can be further classified as migratory or sedentary (da Silva Amaral, 2021; Perry and Moens, 2011).

Nematodes of the genus *Meloidogyne*, commonly known as root-knot nematodes, are obligate endoparasites that spend most of their life cycle inside the roots of their host plants. They are extremely polyphagous and highly specialized biotrophic plant parasites with a worldwide distribution (Truong et al., 2021; Wyss and Grundler, 1992). The infective stage is the second-stage juvenile (J2), which hatches in the soil and penetrates the roots near the meristematic region. Once inside, it migrates intercellularly until reaching the vascular tissue, where it induces the formation of giant cells, which are highly metabolic structures that serve as a continuous nutrient source. This process is facilitated by the secretion of effector proteins that modulate the plant's cellular functions. The complete life cycle can last between 3 to 8 weeks, depending on the nematode species and environmental conditions, and includes four juvenile stages and the adult phase, in which sedentary females lay eggs in a gelatinous matrix external to the root (Truong et al., 2021).

This damage to the root system reduces the availability of water and nutrients to the plant, and as an indirect consequence of infection, the aboveground parts of the plant exhibit reduced growth, leaf chlorosis, poor yield, and wilting (Díaz-Manzano et al., 2023; Escobar et al., 2015). Moreover, the mechanical wounds caused by J2 during root penetration serve as entry points for other plant pathogens, facilitating secondary infections by fungi, bacteria, and viruses (da Silva Amaral, 2021).

Integrated nematode management primarily includes chemical control, cultural practices, and biological control. For the effective application of these methods, accurate taxonomic identification of nematodes is extremely important. Morphological identification at the genus level is less complex than at the species level. In the genus *Meloidogyne*, species-level identification is traditionally based on the analysis of the female's perineal pattern; however, this approach does not always allow for the separation of morphologically similar species, such as *M. incognita* and *M. inornata* (da Silva Amaral, 2021). To minimize this type of problem, molecular analyses have been suggested.

1.3. Economic importance of tomato and maize in Brazil

Tomato (*Solanum lycopersicum*) originates from the Andean region of South America and is the second most important vegetable crop worldwide, after potato (FAO, 2001; Peralta et al., 2008). In Latin America, Brazil is the second largest tomato producer, reaching nearly 4 million tons per year (FAO, 2018). Tomato production can be intended either for industry (processing tomato) or for fresh consumption (staked or trellised tomato). Regardless of the production system, tomatoes are highly susceptible and frequently affected by various diseases, insect pests, and phytophagous nematodes (Fornazier et al., 2010; Panno et al., 2021; Singh et al. 2007).

Among the different nematode genera that can infect tomato crops, *Meloidogyne* is considered the most aggressive (Carvalho, 2017). The susceptibility of tomato is so great that tomato plants are often used as hosts for nematode multiplication in experimental studies (Banora and Almaghrabi, 2019; Truong et al., 2021).

Among the crops of great economic importance in Brazil, we can highlight *Zea mays* L. (Poaceae). In the 2022/23 crop season, the estimated planting area for corn in Brazil was 77.1 million hectares, which represents a growth of 3.4% or 2.54 million hectares compared to the area of the 2021/22 crop season (Companhia Nacional de Abastecimento - Conab, 2023). According to Conab, a total production of 125.1 million tons of corn is expected, representing a 10.9% increase compared to the previous crop season. The cultivation of this economically important grain for Brazil can be affected by two extremely damaging insect pests, *Dalbulus maidis* (Hemiptera: Cicadellidae) and *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae).

1.4. Insects used in this study

Tenebrio molitor (Coleoptera: Tenebrionidae), commonly known as the yellow mealworm, is widely used as a model organism in studies of insect pathology, fungal isolation from soil, and pathogenicity testing (Costantin et al., 2025; Kim et al., 2018). It is an insect that is easy to rear and can be maintained in large numbers in the laboratory at low cost and with minimal maintenance.

Lepidopteran species of the family Noctuidae are highly voracious agricultural pests that are difficult to control, largely due to their ability to detoxify toxins through an efficient immune system (Berenbaum et al., 2021). In addition, they exhibit high dispersal capacity and

rapid reproduction within short time intervals. Below, the species addressed in this study are described individually.

Spodoptera frugiperda (J.E. Smith) (Noctuidae: Noctuinae), popularly known as the fall armyworm, is one of the most harmful pest species in Brazil due to its polyphagous habits and high biotic potential (Boregas et al., 2013; Cruz, 1995; Waquil et al., 2008). *Spodoptera frugiperda* has a wide geographical distribution (Boregas et al., 2013); between 2018 and 2021, it invaded 47 African countries, 18 Asian countries, and Australia, where it seriously threatens agricultural production (Wan et al., 2021). Fall armyworm represents a serious problem in countries where it is endemic and is one of the main pests of maize crops, attacking both the leaves and ears, and reducing yield by 34% to 52% (Valicente, 2015). The life cycle of *S. frugiperda* is completed in approximately 30 days under laboratory conditions, and the number of eggs per oviposition ranges from 100 to 200 per female, with a total of 1,500 to 2,000 eggs laid by a single female (Valicente, 2015).

Another group of Noctuidae of great economic importance consists of moths known as semiloopers or measuring worms, which include *Rachiplusia nu* Guenée and *Chrysodeixis includens* (Walker), both in the subfamily Plusiinae. *Rachiplusia nu* is a polyphagous species distributed in South America, especially in Argentina, southern Brazil, Paraguay, Uruguay, and Bolivia (Jakubowicz et al., 2019). *Chrysodeixis includens*, on the other hand, is widely distributed throughout the Western Hemisphere and is among the most destructive soybean pests in the southern United States (Debnath et al., 2024).The life cycle of *R. nu* is completed in approximately 25 days, with adult females living an average of 7 days and laying between 900 and 1,100 eggs during their lifetime (Alves et al., 2024). In *C. includens*, the life cycle can also be completed in less than a month; its females live between 13.5 and 14.8 days and lay up to 600 eggs, preferring the underside of leaves and mainly during the night (Debnath et al., 2024).

Helicoverpa armigera (Noctuidae: Heliothinae) is a pest widespread in both the Old and New Worlds and was first reported in Brazil in 2013 (Czepak et al., 2013; Santos, 2016). The life cycle of *H. armigera* is completed on average between 4 to 6 weeks, with adult females living up to 15 days and laying between 1,000 and 1,500 eggs during their lifetime (Gomes, 2016; Queiroz Santos, 2016; Czepack et al., 2013).

Dalbulus maidis (Hemiptera: Cicadellidae) was first reported in Brazil in 1938 and, until recently, was considered a secondary pest of maize (Oliveira and Frizzas, 2022). Since 2015,

however, *D. maidis* has been recognized as the primary pest of maize in Brazil due to its ability to cause economic losses both through direct feeding and by acting as a vector of the maize rayado fino virus (MRFV) and the mollicutes associated with corn stunt spiroplasma (CSS) and maize bushy stunt phytoplasma (BSP) (Nault, 1990, 1980). Maize is currently the only plant species in Brazil on which *D. maidis* is known to complete its life cycle (from egg to adult), and this insect is the only confirmed vector of the causal agents of maize stunting diseases in South America (Oliveira et al., 2020). Nevertheless, during the off-season, *D. maidis* can survive on alternative host plants, particularly those in the Poaceae family, when it does not migrate between maize fields or persist on volunteer maize plants (Oliveira et al., 2020). There are reports in the literature indicating that *D. maidis* is capable of flying distances of up to 20 km (Oliveira et al., 2013). The average generation time of *D. maidis* is approximately 25 to 30 days under field conditions (Todd et al., 1991). Females lay between 400 and 600 eggs throughout their lifetime, primarily inserting them into plant tissues on the basal half of young leaves (Oliveira, 2000; Waquil et al., 1999). At a temperature of 26 °C, adult survival averages 7 to 8 weeks, although some individuals may live up to 15 weeks (Oliveira, 2000; Waquil et al., 1999).

1.5. Application Technology

The application technology of phytosanitary products refers to the correct placement of the active ingredient on the intended target, in the required amount, in an economical manner, and with minimal contamination of non-target areas (Matuo et al., 2001). Proper pesticide application involves producing a spray with droplets that are large enough to avoid losses through evaporation and drift, but small enough to ensure good target coverage, resulting in effective control (Matthews et al., 2014).

The efficiency of pesticide application technology depends on several interrelated factors, such as weather conditions, soil characteristics, host type, nature of the pathogen, selection of the active ingredient and carrier, as well as the technical training of the operator and proper adjustment of the equipment (Contiero et al., 2018). Understanding and integrating these elements are essential to ensure an effective, safe, and environmentally responsible application (Contiero et al., 2018).

One of the tools that can support phytosanitary applications is the use of adjuvants. Adjuvants are not pesticides, and they are generally classified into two major groups according

to their function in the spray mixture: (i) modifiers of the physicochemical properties of the spray solution, also known as surfactants, and (ii) additives, which influence the absorption or performance of the active ingredient (Vargas et al., 2006). Surfactants include spreaders, humectants, detergents, dispersants, and stickers, substances that reduce surface tension and improve spray coverage and retention on target surfaces (Vargas & Roman, 2006). Additives such as mineral or vegetable oils, urea, and ammonium sulfate act mainly by enhancing the penetration of active ingredients through natural barriers like plant or insect cuticles (Vargas et al., 2006)

The low effectiveness of mycopesticides in controlling *D. maidis* may be associated with the presence of a natural barrier on its cuticle: brochosomes, protein-lipid structures that cover the insect's body and confer hydrophobic properties, making it difficult for fungal conidia to adhere. In addition, *D. maidis* exhibits an active self-cleaning behavior that removes applied conidia, further reducing the infection potential (Lopes et al., 2024). In their study, even with direct application of conidial suspensions of *Beauveria*, *Metarhizium*, and *Cordyceps*, adult mortality remained below 35%. Beyond the physical barrier provided by brochosomes, the self-cleaning behavior of the adults also contributed to the removal of fungal propagules from the insect's body. The authors conclude that the efficacy of mycopesticides can be enhanced by using adjuvants with adhesive properties, which improve conidia retention on the cuticle of *D. maidis*, thereby increasing infection rates and improving biological control outcomes (Lopes et al., 2024).

1.6. Objectives

This study aims to characterize and evaluate the biological control potential of a newly identified strain of *Purpureocillium lilacinum* (COAD 4015) through a multi-target approach, starting with its morphological and molecular characterization and assessment of its efficacy against root-knot nematodes (*Meloidogyne* spp.) in tomato (*Solanum lycopersicum*). Additionally, the study aims to assess the effectiveness of *P. lilacinum* conidia in controlling the corn leafhopper *Dalbulus maidis* under field conditions, both as a standalone treatment and in combination with a chemical insecticide and/or an oil-based adjuvant to improve conidial adhesion and persistence. Finally, we evaluated the pathogenic potential of different fungal components, conidia, enzymatic extracts, and secondary metabolites, against *Tenebrio molitor*, a model organism, as well as against key lepidopteran pests of agricultural importance, namely

Spodoptera frugiperda, *Helicoverpa armigera*, *Rachiplusia nu*, and *Chrysodeixis includens*, in order to explore the broader entomopathogenic potential of the isolate for integrated pest management.

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CHAPTER 2
Dual Action of *Purpureocillium lilacinum* COAD 4015: Biocontrol of *Meloidogyne* spp. and Plant Growth Promotion in Tomato

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ABSTRACT

Purpureocillium lilacinum is globally distributed saprophytic fungus known for its nematophagous activity and ability to promote plant growth. This study reports, for the first time, the use of *P. lilacinum* COAD 4015, in promoting tomato plant growth and controlling *Meloidogyne* nematodes. Tomato seedlings were inoculated with 3,120 second-stage juveniles and three conidial concentrations (3×10^7, 3×10^8, and 3×10^9 conidia·mL^{-1}) of *P. lilacinum* in Carolina Soil® substrate and maintained in BOD chamber conditions. After 45 days, all concentrations completely suppressed egg production in both soil and root samples. However, for second-stage juveniles (J2), reductions varied by dose. In roots, the observed reductions were 84.5%, 78.4%, and 71.1%, and in soil, 88.2%, 98.5%, and 33.8%, corresponding to 3×10^7, 3×10^8, and 3×10^9 conidia. mL^{-1}, respectively. Although all treatments reduced J2 counts, the results did not follow a clear dose-dependent pattern, as increasing conidial concentrations did not correspond to greater suppression of J2. The highest concentration (3×10^9 conidia·mL^{-1}) led to the most notable increase in plant height, root length, shoot fresh weight, and leaf number. temperature tests showed no fungal growth at 36°C, suggesting low pathogenic potential to humans. In summary, *P. lilacinum* COAD 4015 represents a promising and safe alternative for the integrated management

of *Meloidogyne* spp. in tomato crops, combining biological control efficacy with plant growth promotion. Additionally, the inability to grow at temperatures close to those of the human body reinforces its low pathogenicity risk, an essential factor for its potential large-scale agricultural use. This study provides the first report on this new isolate of *P. lilacinum,* its potential for the development of bioinput targeting root-knot nematodes.

Keywords: Nematophagous fungi, Root-knot nematodes, Biological control, Biostimulant

1. Introduction

Purpureocillium lilacinum is reported in different ecosystems worldwide and a saprophytic fungus frequently isolated from soil, decaying vegetation, insects, nematodes, and found in the air (Luangsa-ard et al., 2011). Its main highlight in agricultural research stems from its dual role: acting both as a biological control agent and as a plant growth-promoting fungus. Recently, *P. lilacinum* isolates have shown promising nematophagous activity and plant-stimulatory effects, drawing attention to the diversity of strains with distinct biological traits (Isaac et al., 2024; Khan and Tanaka, 2023; Kiriga et al., 2018).

The nematicidal potential of *P. lilacinum* has been widely reported, especially against *Meloidogyne*. Nematodes of *Meloidogyne* belong to the order Tylenchida (tylenchid nematodes) and are extremely polyphagous and highly specialized biotrophic plant parasites with a worldwide distribution (Truong et al., 2021; Wyss and Grundler, 1992). These nematodes invade the root in a vermiform developmental stage and migrate toward the differentiating vascular cylinder, where they induce the formation of Multinucleated Giant Cells (MGCs) that act as nurse cells located within the galls (Escobar et al., 2015; Wyss and Grundler, 1992). This damage reduces the availability of water and nutrients to the plant, and as an indirect consequence of infection, the aboveground parts of the plant exhibit reduced growth, leaf chlorosis, poor yield, and wilting (Díaz-Manzano et al., 2023; Escobar et al., 2015).

Meloidogyne can reduce the productivity of various crops, including tomato (Bernard et al., 2017), with yield losses reaching up to 85% (Padilla-Hurtado et al., 2022). Due to its high susceptibility to nematode infection and the ease of symptom observation, the tomato plant is widely used as a model in studies involving *Meloidogyne* spp. (Liu et al., 2022; Williamson and Hussey, 1996).

The nematicidal action of *P. lilacinum* is multifaceted, involving the production of hydrolytic enzymes such as chitinases, proteases, and collagenases, which degrade the structural components of nematode eggshells (Prasad et al., 2015). Additionally, this fungus produces toxic secondary metabolites, most notably leucinostatins, that contribute to nematode suppression. Furthermore, it has been reported to induce systemic resistance in host plants, enhancing their defense responses. Together, these mechanisms reduce nematode hatching, viability, and reproduction across a range of agricultural crops (Isaac et al., 2024; Khan and Tanaka, 2023; Prasad et al., 2015).

Purpureocillium lilacinum has also been reported to act as a plant growth-promoting fungus. Strains of *P. lilacinum* demonstrated the ability to produce significant levels of indole-3-acetic acid (IAA), a key phytohormone involved in root development and cell elongation, as well as solubilizing insoluble phosphate sources, enhancing nitrogen accumulation in plant roots (Baron et al., 2020). These effects translated into measurable improvements in plant height and biomass in greenhouse trials, indicating that *P. lilacinum* can improve plant nutrition and development through multiple mechanisms (Khan and Tanaka, 2023; Rigobelo et al., 2024).

Considering the intraspecific variability among *P. lilacinum* isolates, recent efforts have focused on identifying and characterizing new strains with superior or complementary functionalities. In this context, this study aimed to investigate the efficacy of the *P. lilacinum* COAD 4015 isolate, previously characterized by morphological and molecular analyses, as a candidate of interest for integrated management strategies targeting both plant growth improvement and control of nematodes of *Meloidogyne* in tomato (*Solanum lycopersicum*).

2. Material and Methods

2.1. Fungal isolate and morphological characterization

The *Purpureocillium lilacinum* isolate was obtained from the working collection of the Laboratório de Patologia de Invertebrados (LPI) at the Universidade Federal de Goiás (UFG). A dried culture is preserved in silica gel, and a living culture is stored in glycerol at both the LPI-UFG (accession number IP 551) and the Otávio de Almeida Drummond Collection (COAD-UFV) (accession number COAD 4015), at the Universidade Federal de Viçosa (UFV), both in Brazil. The morphological and molecular analyses were conducted by the team of the Laboratório de Micologia (LabMicol) at the Instituto de Patologia Tropical e Saúde Pública (IPTSP), UFG, Brazil.

For macroscopic characterization, the isolate was cultured on malt extract agar (MEA), oatmeal agar (OA), and potato dextrose agar (PDA) at 25°C for 7 days in the dark. Colony characteristics such as colony diameters, color, texture, pigment production, and margin appearance were evaluated. The colour chart of Rayner (1970) was used to evaluate colony colour. The shape, color, and size of conidiophores and conidia were observed using a Leica DM2500 microscope, images were captured using a Leica DFC7000T, and processed using the Leica Application suite software at the Centro Multiusuário de Pesquisa de Bioinsumos e

Tecnologias em Saúde (CMBIOTECs, UFG, Goiânia, Brazil) with differential interference contrast (DIC) optics.

2.2. Molecular Analysis

Genomic DNA was extracted from 7-day-old fungal cultures grown on PDA, following the protocols described by Bezerra et al. (2017). The primers pairs ITS4/ITS5 (White et al.,1990), LR0R/LR5 (Vilgalys & Hester, 1990), EF1-728F/EF1-2218R (Rehner, 2001), and RPB1-Af/RPB1-Cr (Sung et al., 2007) were used to amplify the internal transcribed spacer (ITS) region of rDNA, the large subunit (LSU) rDNA, the translation elongation factor 1-alpha (tef1), and the largest subunit of RNA polymerase II (rpb1), respectively.

PCR products were purified and sequenced using the same primer pairs and following Perdomo et al. (2013) and Bezerra et al. (2017). The obtained sequences were compared with those available in the NCBI GenBank database using the BLASTn tool. A maximum likelihood (ML) phylogenetic analysis was performed using a concatenated dataset of the ITS, LSU, and tef1 genes, conducted in IQ-TREE v1.6.12 software (Nguyen et al., 2015) with 5,000 bootstrap replications under default parameters. The tree was rooted with *Cordyceps gunii* ARSEF 6828, and bootstrap support values ≥ 70% were considered statistically significant and are shown at the nodes.

2.3. Fungal inoculum production for bioassays

Purpureocillium lilacinum COAD 4015 isolate was cultivated in 23 mL of potato dextrose agar (PDA) supplemented with 1 $g.L^{-1}$ of yeast extract (PDAY) in Petri dishes (90 × 15 mm) under a 12-hour photoperiod, 25 ± 2 °C, and RH ± 60% for 15 days. Conidia were dislodged with a microbiological loop and suspended in 50 mL of Tween 80® (0.01% v/v). Serial dilutions of the aqueous conidial suspension were made and quantified in a Neubauer chamber, with the concentration standardized to 1×10^7 conidia mL^{-1}.

Parboiled rice was placed in a plastic bag (30 cm x 20 cm) and moistened with 30% of its volume with distilled water for 10 minutes. Subsequently, the rice was cooked for 20 minutes. After the cooked rice reached room temperature, 1 mL of conidial suspension (described above) at a concentration of $1x10^7$ conidia mL^{-1} (*P. lilacinum* + sterile distilled water 0.01% Tween

80®) was inoculated. The bags were then incubated at 26 ± 1 °C with a photoperiod of 12 hours of light and 12 hours of darkness for 15 to 20 days (I. de C. Lopes, 2016). After this period, the rice was washed over a sieve using sterile deionized with sterile deionized water containing Tween 80® suspension (0.01% v/v) to remove the conidia. The conidial concentration was determined by counting the spores using a Neubauer chamber.

To evaluate viability, conidial suspension was prepared at a concentration of 1×10^5 conidia mL^{-1} for ease of counting. The suspension was inoculated with a volume of 2 µL at three different points of the Petri dish (55 × 15 mm) containing PDA. The plate was incubated for 14 hours at (27 ± 2 °C and RH ± 60%). After this period, a drop of cotton blue lactophenol and a cover slip were placed over the inoculum, and conidial germination was observed under a phase-contrast microscope with a magnification of 400×. At least 100 conidia for each of the three points were evaluated, and the percentage of germination was calculated. Conidia were considered to have germinated when the germ tube was longer than the maximum diameter of the conidia. The bioassays conducted in this study used suspensions of fungal propagules with viability higher than 90%.

2.4. Effect of temperature on the growth of *Purpureocillium lilacinum* COAD 4015

The isolate COAD 4015 was cultured on PDA plates (as described in section 2.3) and incubated at 36 ± 2°C and 25 ± 2°C (control) for 15 days. The experiment was conducted with four replicates per temperature, with each replicate consisting of one PDA plate. For inoculation, 2 µL of a conidial suspension (1×10^7 conidia·mL^{-1}) were pipetted onto the center of each plate. After the incubation period, colony growth was evaluated by measuring the colony diameter, allowing assessment of the isolate's ability to grow under high-temperature conditions.

2.5. Inoculation of *Purpureocillium lilacinum* and *Meloidogyne* spp. in tomato plants

To inoculate nematodes and *P. lilacinum,* tomato seedlings (*S. lycopersicum* var. Carolina) were transplanted 15 days after sowing into 700 mL plastic pots filled with commercial Carolina Soil® substrate (Carolina Soil, Brazil).

Second-stage juveniles (J2) of *Meloidogyne* spp. used as inoculum were obtained from soil collected in naturally infested soybean fields (cultivar NEO 700 i2x) located in São Gabriel do Oeste, Mato Grosso do Sul, Brazil. Nematodes were extracted from 250 cm^3 of homogenized soil using the centrifugal flotation technique in sucrose solution (Jenkins, 1964). Briefly, soil samples were mixed with 4 L of tap water in a bucket to break up clods, allowed to settle for 20 s, and the suspension was poured through a 20-mesh sieve nested over a 400-mesh sieve (0.038 mm opening). The material retained on the 400-mesh sieve was collected into a beaker using a wash bottle with water and transferred to calibrated centrifuge tubes. Samples were centrifuged at 1,750 rpm for 5 min, the supernatant discarded, and the pellet resuspended in sucrose solution (450 g sugar + 750 mL water; density 1.15–1.18). The mixture was homogenized with a glass rod and centrifuged again for 1 min at 1,750 rpm. The supernatant was poured through a 500-mesh sieve, rinsed thoroughly with water to remove sucrose, and the recovered J2 were collected into Falcon tubes. The suspension was quantified under a light microscope using a Peters counting slide, adjusted to the desired concentration, and used immediately for inoculation. Inoculation with *Meloidogyne* spp. was performed using 3,120 J2 per plant.

Two holes, each 3 cm deep, were made in the soil to access the rhizosphere of each plant for treatment application. Into each hole, the suspension containing eggs and J2 was first applied, followed by 10 mL of the fungal suspension according to the respective treatment.

The experiment included three treatments consisting of fungal suspensions at concentrations of 3×10^7, 3×10^8, and 3×10^9 conidia·mL^{-1}. Each treatment, as well as the control (plants without fungal application), had six replicates. Plants were irrigated daily according to substrate moisture requirements and maintained in an incubator at 25 ± 2 °C with a 12-hour photoperiod.

Forty-five days after inoculation, evaluations were performed to assess the effects of different concentrations of *P. lilacinum* COAD 4015 on plant height, root length, number of branches, number of leaves, fresh shoot and root weight, and stem diameter. Plants were carefully removed from the pots, and their root systems were washed under running water to remove excess substrate. They were then air-dried at room temperature. Shoot and root lengths were measured using a measuring tape; fresh biomass was recorded using an analytical balance, and stem diameter was measured with a digital calliper.

Nematodes in the substrate were extracted from 100 cm³ samples using the centrifugal flotation technique in sucrose solution (Jenkins, 1964), following the same procedure described above for field soil samples. Nematodes in the roots were extracted following the method of Hussey and Barker (1973) as adapted by Bonetti and Ferraz (1981), with clarification according to Coolen and D'herde (1972). The entire root system from each pot was cut into ~0.5–1 cm segments and blended at maximum speed for 30 s in 0.10% sodium hypochlorite (NaOCl). The suspension was poured through a 20-mesh sieve nested over a 500-mesh sieve (0.026 mm opening), and the material retained on the 500-mesh sieve was thoroughly rinsed with running water and collected into Falcon tubes containing water. For clarification, the suspension was transferred to calibrated centrifuge tubes, 1 cm³ of kaolin was added, and samples were centrifuged at 1,750 rpm for 5 min. The supernatant was discarded, the pellet was resuspended in sucrose solution (Jenkins method), homogenized, and centrifuged again for 1 min at 1,750 rpm. The supernatant was poured through a 500-mesh sieve, rinsed thoroughly with water to remove excess sucrose, and the recovered nematodes were collected into Falcon tubes for evaluation. Counts included both eggs and second-stage juveniles (J2) recovered from substrate (100 cm³) and root samples (per gram).

2.6. Statistical analysis

To evaluate the effect of different concentrations of *P. lilacinum* COAD 4015 (independent variable) on plant height, root length, number of branches, number of leaves, and fresh masses of the shoot and root (dependent variables), Analysis of Variance (ANOVA) was used. To evaluate the effect of fungal concentration on the number of nematodes in the soil and roots, ANOVA was also applied. The assumptions of normality and homogeneity of variances for ANOVA were verified using the Shapiro–Wilk and Levene's tests, respectively, and the correction was performed with Bonferroni. To evaluate the number of eggs, the non-parametric Kruskal–Wallis test was used, followed by Dunn's post hoc test. All statistical analyses were conducted using R software, version 4.1.2.

3. Results

3.1. Fungal isolate and morphological characterization

The isolate *P.* lilacinum COAD 4015 exhibited morphological characteristics consistent with the species description (Luangsa-ard et al., 2011). After 7 days of incubation at 25°C in the dark, colonies grown on MEA, OA and PDA displayed moderate growth (29, 25 and 27 mm diameter, respectively), with an initial white mycelial surface that gradually developed rose to lilac color as sporulation progressed (Figure 1A–B). The colony texture was floccose, and the reverse side showed light yellow to pale purple colour.

Microscopic examination on PDA revealed conidiophores, which were mononematous, erect, and arose from submerged hyphae (Figure 1C–G). Phialides were cylindrical to slightly swollen at the base, often with a distinct neck, and occurred singly or in whorls of two to four per branch.

Conidia were produced in dry, divergent chains (Figure 1H–I), being ellipsoidal to cylindrical, hyaline, and smooth-walled, measuring approximately 2.5–4.0 µm in length, in agreement with previous descriptions of *P. lilacinum* by Luangsa-ard et al. (2011).

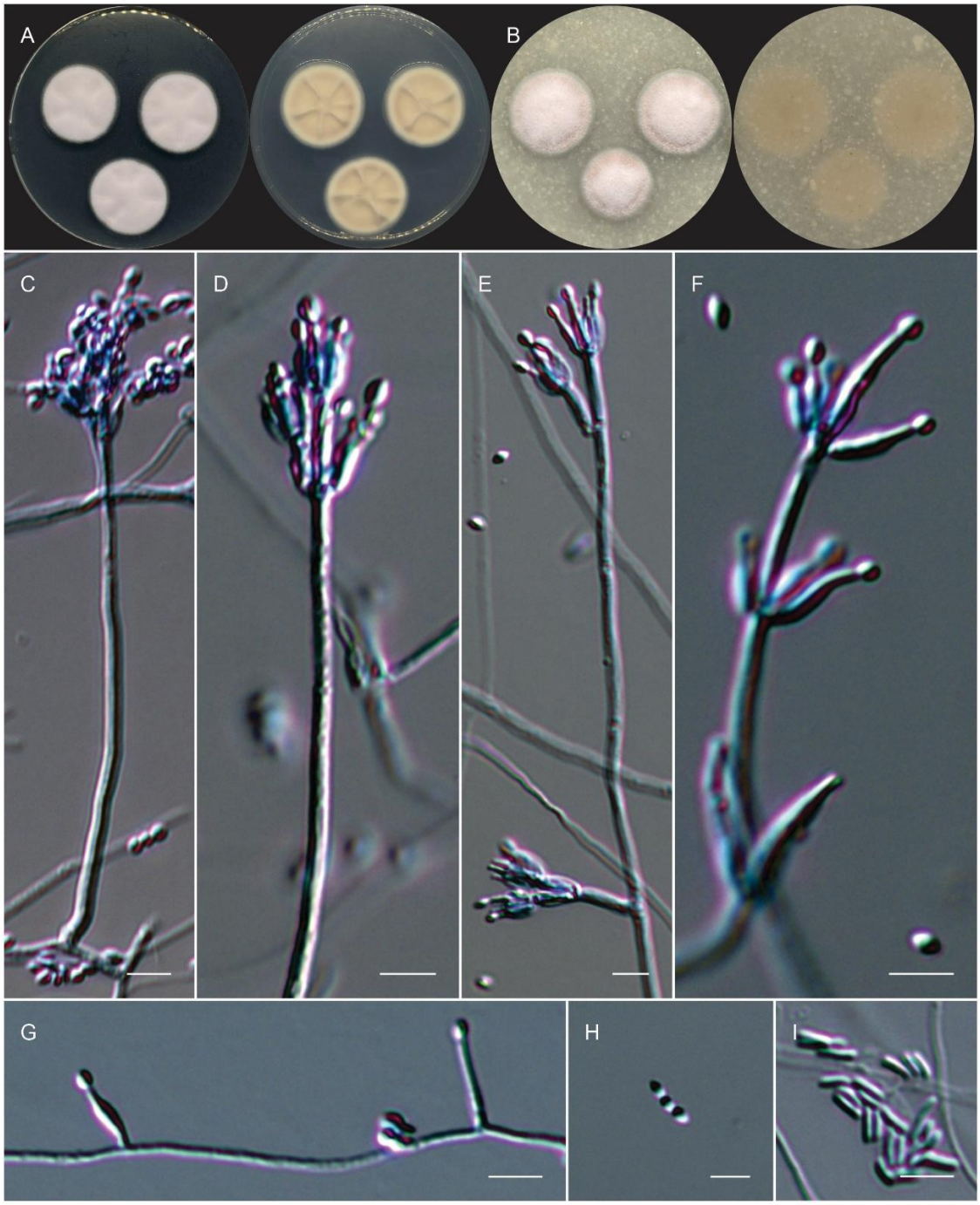

Figure 1. Morphology of *Purpureocillium lilacinum* COAD 4015. A. 7-day-old culture (observe and reverse) on malt extract agar (MEA). B. 7-day-old culture (observe and reverse) on oatmeal agar (OA). C-F. Conidiophores. G. Conidiophores acremonium-like. H. Chain of ellipsoidal conidia. I. Cylindrical conidia. Scale bar = 10 μm.

3.2. Molecular Analysis

BLASTn search using our ITS, LSU, *tef1*, and *rpb1* sequences showed 100% identity with reference sequences from the NCBI GenBank database (in all genes). Furthermore, ML analysis using a combined matrix (ITS, LSU, and *tef1*) showed that the isolate COAD 4015 clustered within other *P. lilacinum* sequences, together with the reference strains (CBS 284.36 and CBS 432.87), confirming its taxonomic identity (Figure 2). The generated sequences were deposited in GenBank under the following accession numbers: PV604125 (ITS), PV604126 (LSU), PV609772 (*tef1*), and PV609771 (*rpb1*) (Figure 2).

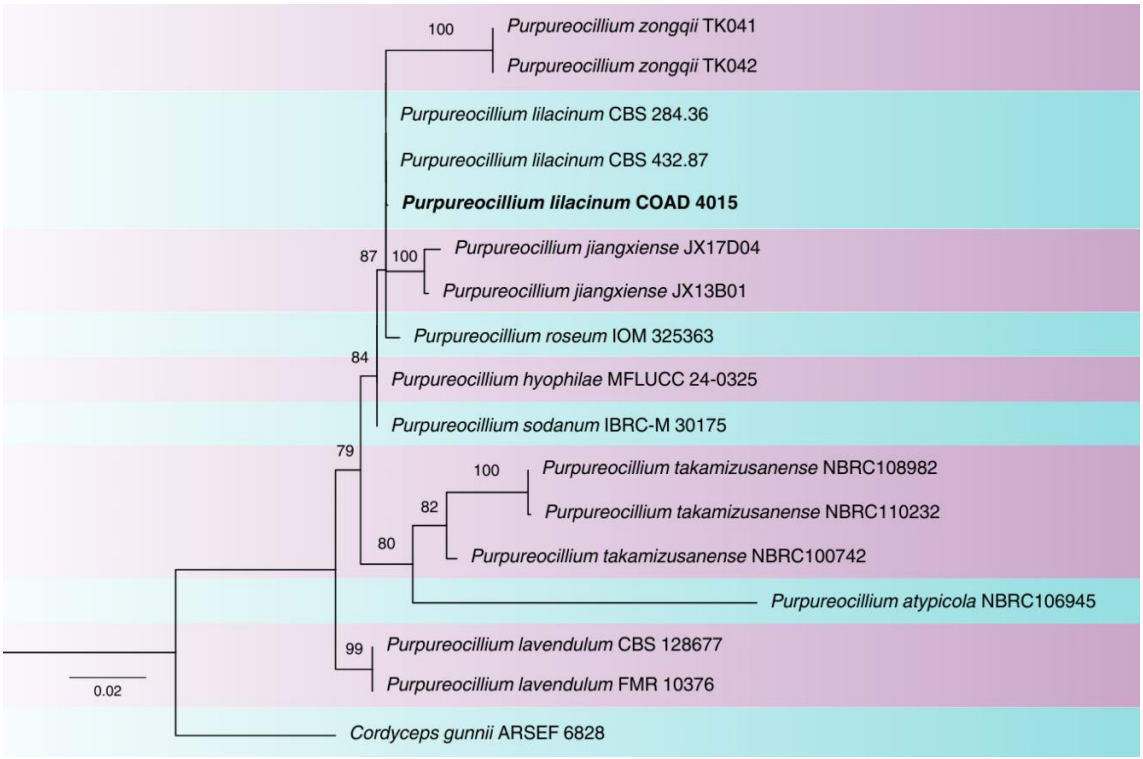

Figure 2. Maximum likelihood of *Purpureocillium* species based on a combined matrix of ITS, LSU, and tef1 genes conducted in IQ-TREE v. 1.6.12 software involving 5,000 replications and using the default parameters. Our isolate *Purpureocillium lilacinum* COAD 4015 is highlighted in bold. The IQ-TREE-BS values ≥ 70% are included near nodes. The tree was rooted with *Cordyceps gunii* ARSEF 6828.

3.3. Effect of temperature on the growth of *Purpureocillium lilacinum* COAD 4015

The growth of *P. lilacinum* COAD 4015 was strongly affected by temperature. At 25 °C, abundant mycelial growth was observed after 15 days of incubation, with a floccose texture and rose to lilac coloration on the upper surface, and a light yellow to pale purple coloration on the reverse. In contrast, at 36 °C, no fungal growth was observed on any of the PDA plates inoculated with *P. lilacinum* COAD 4015 during the same incubation period (Figure 3).

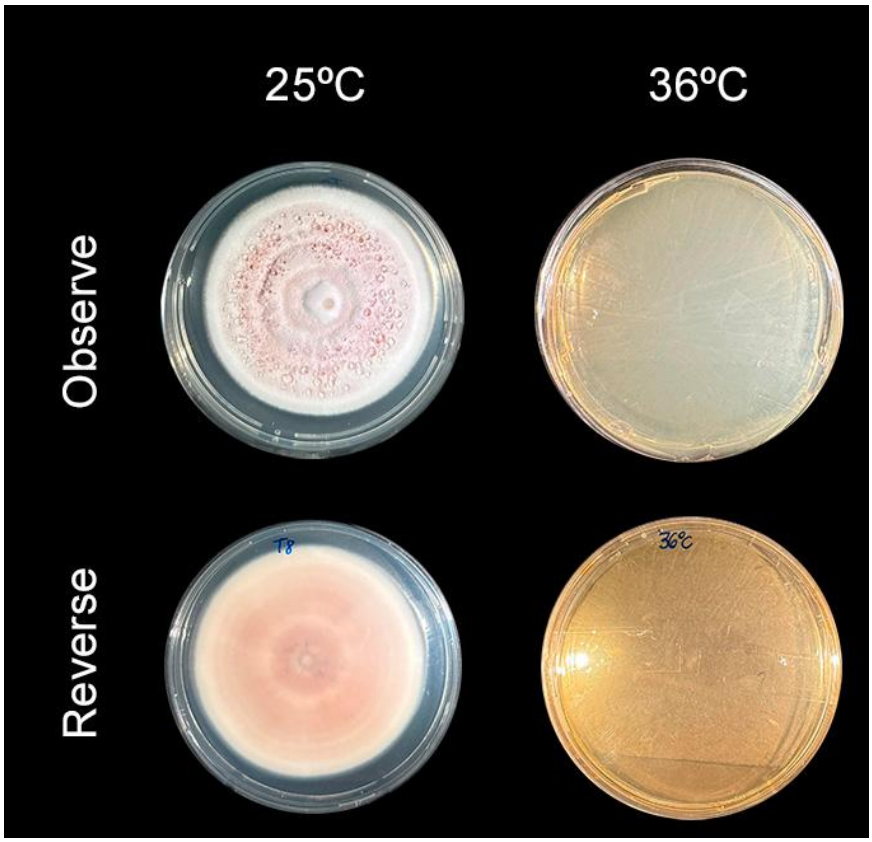

Figure 3. Growth of *Purpureocillium lilacinum* COAD 4015 on potato dextrose agar (PDA) after 15 days of incubation at 25 °C (left) and 36 °C (right). The top row shows the upper surface of the plates (observe), and the bottom row shows the reverse side (reverse).

3.4. Inoculation of *Purpureocillium lilacinum* and *Meloidogyne* spp. in tomato plants

All three concentrations (3×10^7, 3×10^8, and 3×10^9 conidia·mL^{-1}) of *P. lilacinum* completely suppressed the number of *Meloidogyne* spp. eggs in soil and tomato roots (Kruskal–Wallis = 22.429; df = 3; $p < 0.001$; Figure 4 A & C). Juvenile (J2) counts were highest in the

control, with means of 113 individuals in the soil and 162 in the roots. The 3×10^7 and 3×10^8 conidia.mL^{-1} treatments significantly reduced the number of juveniles in the soil compared to the control ($F_{(3,20)} = 5.235$, $p = 0.025$), reaching reductions of 88.2% and 98.5%, respectively. In contrast, the highest concentration (3×10^9 conidia.mL^{-1}) showed a reduction of 33.8%, but this was not statistically different from the control (Figure 4B). In roots, although mean J2 counts were also lower in all treated groups (with reductions of 84.5%, 78.4%, and 71.1% for 3×10^7, 3×10^8, and 3×10^9 conidia.mL^{-1}, respectively), no significant differences were observed among treatments ($F_{(3,20)} = 2.272$, $p = 0.139$; Figure 4D). Overall, the treatments reduced juvenile populations in both compartments, particularly in the soil, but no consistent dose–response pattern was detected.

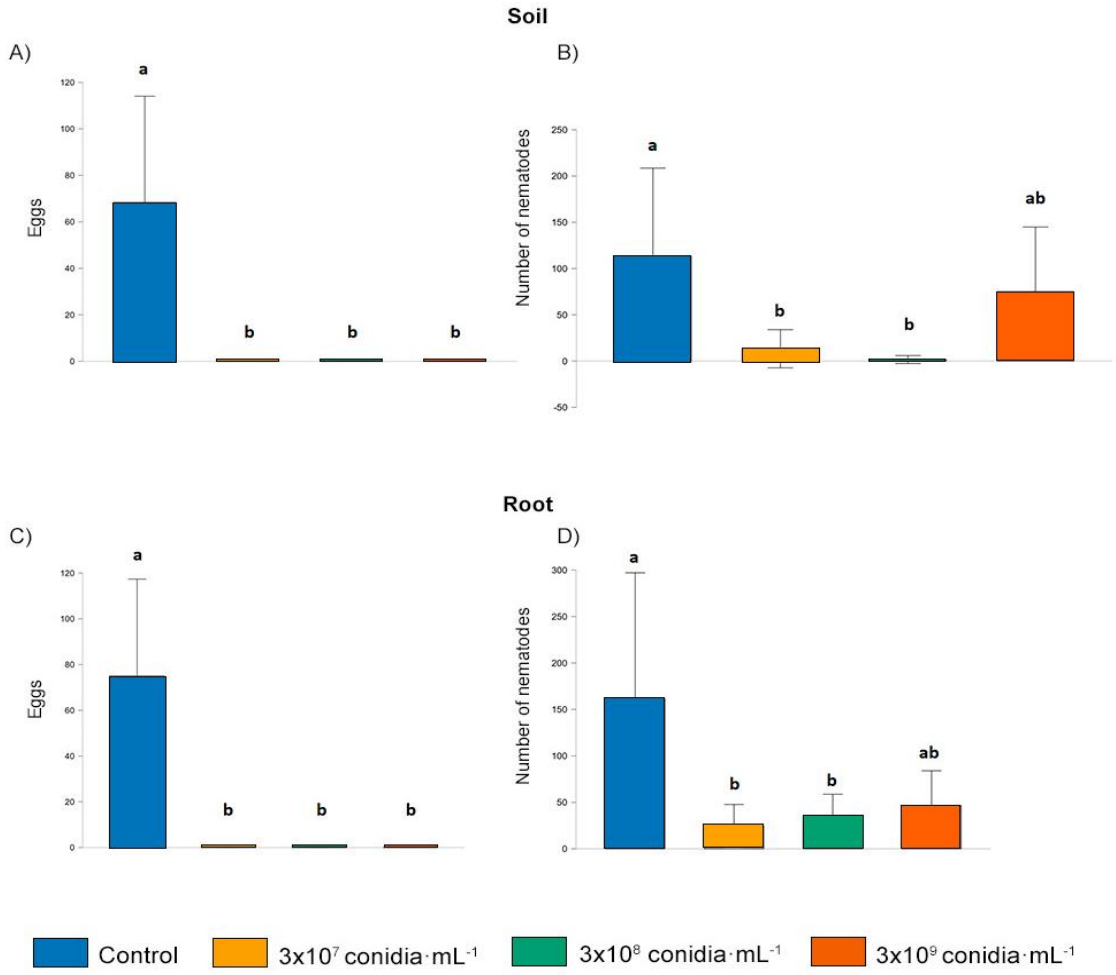

Figure 4. Mean numbers of eggs and nematodes in substrate (100 cm³) (A–B) and in roots (per gram) (C–D) of tomato plants, evaluated 45 days after inoculation with 3,120 J2 of *Meloidogyne* spp. and treatment with 10 mL of *Purpureocillium lilacinum COAD* 4015 at three concentrations (3×10^7, 3×10^8, and 3×10^9 conidia·mL^{-1}) and control (nematodes only). Bars

represent mean values ± 95% confidence intervals. Different letters indicate statistically significant differences among treatments, based on Kruskal–Wallis tests for egg counts (followed by Dunn's *post hoc* test) and ANOVA for nematode counts, with Bonferroni correction applied ($p < 0.05$).

Tomato plants grown in substrate treated with *P. lilacinum* showed visible improvements in shoot and root system growth compared to the untreated control after 45 days of the treatment (Figure 5).

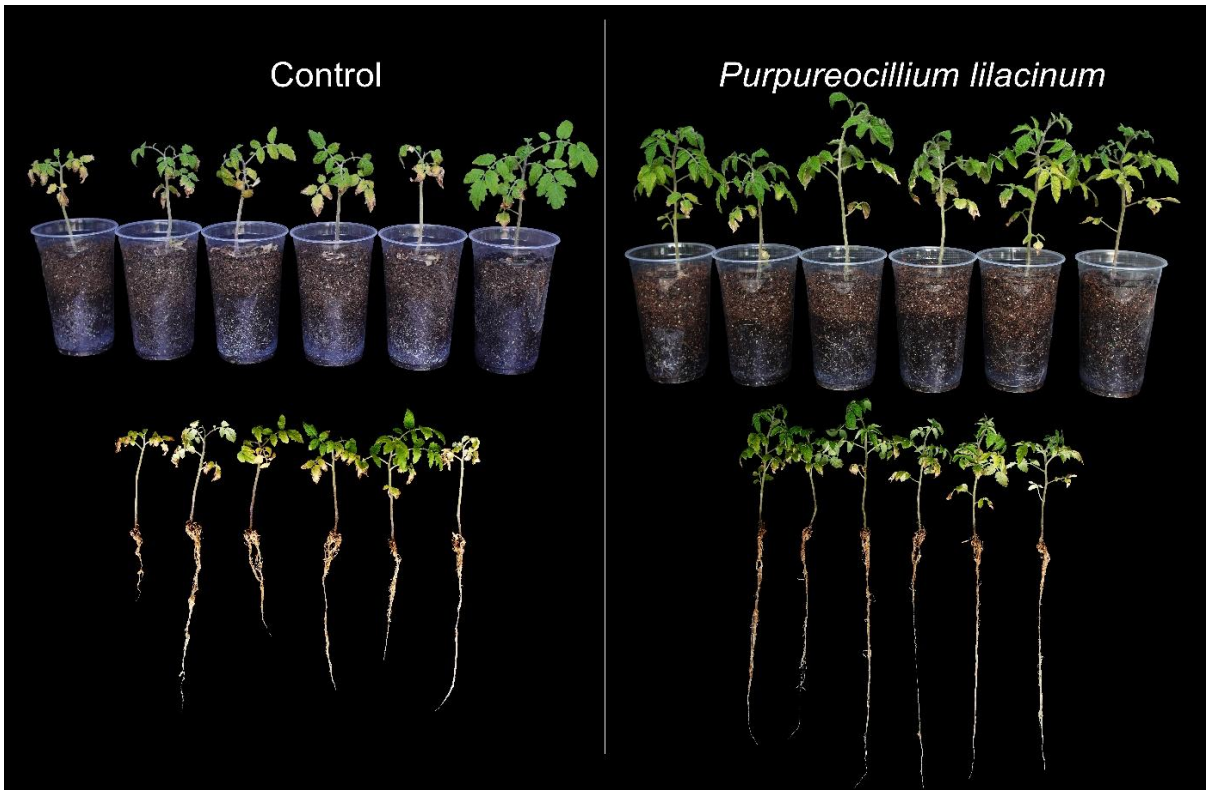

Figure 5. Tomato plants (*Solanum lycopersicum*) grown for 45 days at 25 ± 2 °C; 12:12 photoperiod after inoculation with *Meloidogyne* spp. and *Purpureocillium lilacinum* COAD 4015. On the left, control (only nematode); on the right, plants treated with *Purpureocillium lilacinum* COAD 4015 at a concentration of 3×10^9 conidia·mL^{-1}.

The highest concentration (3×10^9 conidia·mL^{-1}) of *P. lilacinum* resulted in taller plants compared to the intermediate concentration (3×10^7 conidia·mL^{-1}) and the control ($F_{(3,20)} = 11.196$, $p < 0.001$) but did not differ from the 3×10^8 conidia·mL^{-1} treatment (Figure 6A). A similar trend was observed for root length, where no difference was found between the 3×10^8

and 3×10^9 conidia·mL^{-1} treatments, both showing greater root length compared to the lowest concentration and the control ($F_{(3,20)} = 7.785$, $p < 0.001$; Figure 6B).

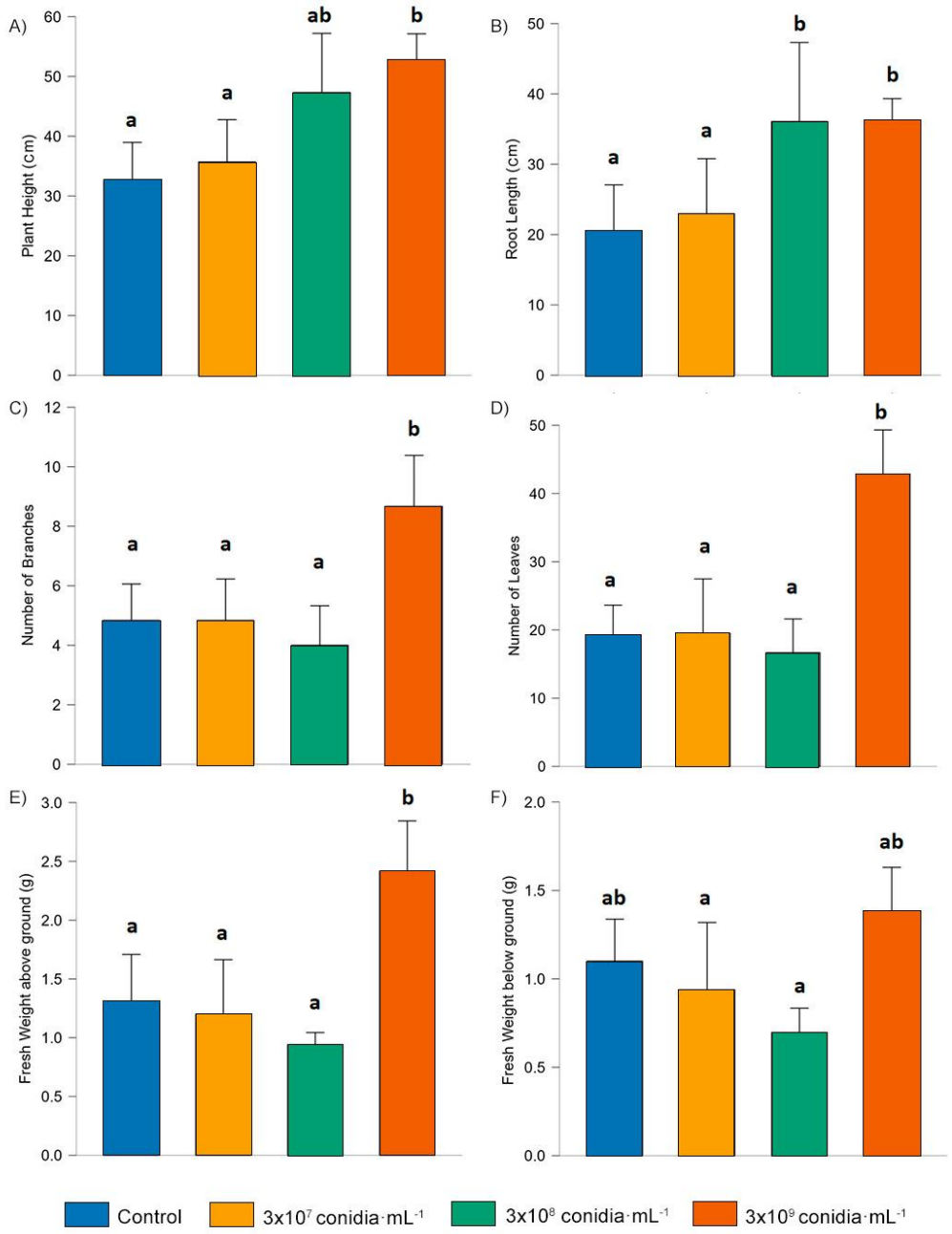

Figure 6: Growth parameters of tomato plants evaluated 45 days after treatment with *Purpureocillium lilacinum* COAD 4015 at three concentrations (3×10^7, 3×10^8, and 3×10^9 conidia·mL^{-1}) and control. The evaluated parameters were: (A) plant height, (B) root length, (C) number of branches, (D) number of leaves, (E) fresh weight above ground, and (F) fresh weight below ground. Bars represent mean values ± 95% confidence intervals. Different letters

indicate statistically significant differences among treatments, according to ANOVA with Bonferroni-adjusted post hoc tests ($p < 0.05$).

Tomato plants treated with the highest concentration of *P. lilacinum* (3×10^9 conidia·mL^{-1}) showed a greater number of branches ($F_{(3,20)} = 14.204$, $p < 0.001$), number of leaves ($F_{(3,20)} = 26.427$, $p < 0.001$), and shoot fresh weight ($F_{(3,20)} = 19.642$, $p < 0.001$) compared to the untreated control (Figure 6C, D, and E). Although fresh weight below ground was affected by the fungal treatment ($F_{(3,20)} = 7.754$, $p = 0.001$), no specific comparisons were significant, as indicated by the shared letters in Figure 6F.

4. Discussion

This study is the first report of a *Purpureocillium lilacinum* isolate (COAD 4015). The isolate did not show growth at temperature similar to those of the human body, suggesting it is not adapted to mammalian conditions, but shows effectiveness in controlling juveniles and eggs of *Meloidogyne* spp. and in promoting the growth of tomato plants.

The morphological characteristics observed for the isolate *P. lilacinum* COAD 4015, including colony appearance, conidiophore structure, and conidial morphology, were consistent with the descriptions available in the literature for the species (Luangsa-ard et al., 2011). However, given the morphological similarities between *Purpureocillium* species, especially those closely related within the Hypocreales, molecular tools were essential to confirm the taxonomic identity of the isolate (Lopes et al., 2023). The phylogenetic analysis based on the ITS, LSU, and *tef1* sequences placed the COAD 4015 within the *P. lilacinum* clustering together with reference strains (CBS 284.36 and CBS 432.87).

Purpureocillium lilacinum is a fungus commonly isolated from soil, decaying vegetation, insects, nematodes, and air, and has in some cases been associated with infections in humans and other vertebrates (Luangsa-ard et al., 2011). However, in our tests, the COAD 4015 fungal isolate was not able to grow at 36ºC demonstrating the absence of one important physiological feature to be treated as a possible opportunistic pathogen of humans. This result suggests that the isolate is not thermotolerant and, therefore, poses a low risk of pathogenicity to humans, reinforcing its safety for use as a biological control agent.

Purpureocillium lilacinum has been widely studied as a nematophagous fungus, with ability to infect eggs and juveniles of root-knot nematodes of the genus *Meloidogyne* spp. (Isaac et al., 2024; Kiriga et al., 2018), highlighting its potential for sustainable nematode management in agricultural systems.

In the present study, the number of eggs in both soil and roots was completely suppressed by all tested concentrations (3×10^7, 3×10^8, and 3×10^9 conidia. mL^{-1}), indicating 100% reduction compared to the untreated control. In contrast, Khan & Tanaka (2023) reported that *P. lilacinum* was able to penetrate hatched eggs of *Meloidogyne incognita* but achieved a maximum reduction of only 62.8% in egg masses in eggplant plants. The authors used a concentration of 1×10^8 CFU.mL^{-1}, which is numerically higher than the lowest concentration used in the present study (3×10^7 conidia. mL^{-1}), considering that only viable conidia were applied in this study, while Khan and Tanaka used a suspension of fresh mycelium. The differences between fungal strains and the type of propagule used may explain the greater efficacy observed in the present study for the control of *Meloidogyne* spp. eggs.

Although a reduction in the number of juveniles *Meloidogyne* spp. was observed in plants treated with *P. lilacinum* COAD 4015 in this study, no clear dose-dependent response was detected, as the reductions were not proportional to the fungal concentrations applied. It is possible that the highest concentration of *P. lilacinum* COAD 4015 led to antagonistic interactions among propagules or intraspecific competition for resources, which may have limited its nematicidal efficiency.

The reduction in the number of eggs and juveniles in both roots and soil supports the previously described nematicidal mechanisms for this genus, including the production of hydrolytic enzymes (such as chitinases and proteases), leucinostatins, and other secondary metabolites capable of degrading nematode eggshell structures and inhibiting hatching and development (Prasad et al., 2015).

Regarding plant growth promotion, a significant increase was observed in plant height, number of leaves, shoot fresh weight, and root length, especially at the highest concentration tested (3×10^9 conidia. mL^{-1}). Supporting our findings, Baron et al. (2020) evaluated different strains of *P. lilacinum* for their plant growth-promoting potential in maize (*Zea mays*), common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), even in the absence of pathogens. Their results showed significant increases in plant height and in both shoot and root biomass, particularly with strains LSM 65 and LSM 24.

These positive effects observed in plant development may be associated with the ability of *P. lilacinum* to produce significant levels of indole-3-acetic acid (IAA), a key phytohormone in root development and cell elongation, as well as to solubilize insoluble phosphate sources and enhance nitrogen accumulation in plant roots. As demonstrated by Baron et al. (2020), these characteristics contribute to increased root surface area, improved efficiency in water and nutrient uptake, and consequently greater plant biomass. Considering that Carolina Soil® was used in this study, a substrate with a balanced organic composition but limited nutrient availability, the beneficial effects observed indicate that the COAD 4015 isolate acted not only as a biocontrol agent against *Meloidogyne* spp., but also as an effective biostimulant under moderately nutrient-limited cultivation conditions.

Our results demonstrate that *P. lilacinum* COAD 4015 represents a promising and safe alternative for the integrated management of *Meloidogyne* spp. in tomato crops, combining biological control efficacy with plant growth promotion. Additionally, the inability to grow at temperatures close to those of the human body reinforces its low pathogenicity risk, an essential factor for its potential large-scale agricultural use. This study presents the first report on the COAD 4015 isolate's ability to promote tomato plant growth and control nematodes of the genus *Meloidogyne*, thereby expanding knowledge on the multifunctional potential of *P. lilacinum* COAD 4015 as a bioinput for sustainable agriculture.

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CHAPTER 3

Adjuvant enhanced field performance of *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) against *Dalbulus maidis* (Hemiptera: Cicadellidae)

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ABSTRACT

Self-cleaning behavior and the presence of brochosomes on the cuticle of *Dalbulus maidis* hinder the adhesion of entomopathogenic fungal conidia, making it important to test the use of adjuvants to enhance the use of fungi for its control. This study evaluated the efficacy of *Purpureocillium lilacinum* against *D. maidis* under field conditions, with emphasis on the use of an oil-based adjuvant (TA 35 Gold Bio). Two trials were conducted in commercial maize fields and one structured trial, comparing the treatments: *P. lilacinum* alone, *P. lilacinum* + adjuvant, adjuvant alone, chemical insecticide (Connect® – imidacloprid + beta-cyfluthrin), and untreated control. The use of the adjuvant increased droplet density by 65% compared to the fungus alone, favoring target coverage. Population reductions of *D. maidis* were observed as early as two days after application, with reinforced effects between seven and eight days, especially in the *P. lilacinum* +adjuvant treatment, which also achieved the highest grain yield (7,878.25 kg·ha^{-1}), statistically like the chemical insecticide (6,955.00 kg·ha^{-1}). Although no high field mortalities were recorded, all recovered insects showed signs of fungal infection. The pest's high dispersal capacity hindered statistical detection of mortality, highlighting the need for complementary studies under controlled conditions. The results indicate that the combination of *P. lilacinum* and adjuvant has potential for use in the integrated management of *D. maidis*, combining economic viability and sustainability.

Keywords: Entomopathogenic fungi, Maize leafhopper, Application technology.

1. Introduction

Adjuvants do not have biocidal action but are often used in pesticide spray solutions to improve their physicochemical properties, focusing on application quality and the phytosanitary efficacy of the treatment (Ferreira and Matuo, 2024). The enhancement of entomopathogenic fungi efficacy has been studied through the use of adjuvants, which protect these microorganisms against unfavorable environmental and climatic conditions, while also helping to improve spray coverage with the mycoinsecticide and increasing the chances of efficacy by promoting the adhesion of the fungus's propagative structures to the insect's body (Holka and Kowalska, 2023)

Insects exhibit self-cleaning behavior as a means of maintaining body integrity or as a defense strategy to prevent harmful stimuli (Yanagawa et al., 2014). In *Dalbulus maidis*, this self-cleaning behavior also occurs in adults, enabling the removal of conidia from their bodies using the spines on their hind tibiae, a morphological feature common among members of the Cicadellidae family (Grazia et al., 2024; Lopes et al., 2024). In addition, the presence of a natural barrier on their cuticle—the brochosomes, protein–lipid structures that cover the insect's body and confer hydrophobic properties—hinders the adhesion of fungal conidia (Lopes et al., 2024).

Dalbulus maidis (Hemiptera: Cicadellidae) has been considered the primary pest of maize in Brazil (Oliveira & Frizzas, 2022) due to its ability to cause economic damage through feeding and acting as a vector for the maize stripe virus (Maize rayado fino virus, MRFV) and the mollicutes associated with maize stunt spiroplasma (CSS) and maize bushy stunt phytoplasma (BSP) (Nault, 1990, 1980). These diseases can affect morphology, physiology, nutrition, and, most importantly, cause significant losses in maize grain production (da Cunha et al., 2023; Oliveira and Frizzas, 2022). Currently, to control *D. maidis* the main tools used is chemical control. However, many of the chemically active ingredients used for controlling this pest have been causing resistance in the field (Machado et al., 2024). Furthermore, it is important to highlight the problems that the continuous use of chemicals can cause to human health and the environment (Basso et al., 2021).

A widely used alternative in Integrated Pest Management (IPM) is the adoption of entomopathogenic fungi (EPF) for the control of *D. maidis*, especially fungi from the genera *Beauveria* and *Cordyceps* (Prado Ribeiro et al., 2023; Sperotto et al., 2025). Other fungi may also have potential for controlling this pest, such as *Purpureocillium lilacinum* (Hypocreales:

Ophiocordycipitaceae). This fungus is widely known for its pathogenicity to nematodes (Isaac et al., 2024; Khan and Tanaka, 2023) but it has also shown effectiveness in the control of sucking insect pests such as *Aphis gossypii*, *Tetranychus urticae*, and *Trialeurodes vaporariorum* (Fiedler and Sosnowska, 2007; Nguyen Thi et al., 2023). To date, no field studies have evaluated the efficacy of *P. lilacinum* against *D. maidis*.

To prevent the dislodgement of entomopathogenic fungal (EPF) conidia in the early stages of the invasion process in *D. maidis*, we tested an adjuvant applied together with the fungus *Purpureocillium lilacinum* in the field to enhance conidia adhesion to the insect body, as recommended by Lopes et al. (2024). Therefore, the objective of this study was to evaluate the efficacy of *P. lilacinum* in controlling *D. maidis* under field conditions, using an oil-based adjuvant. To this end, two field trials were conducted in commercial maize areas, applied side by side to assess the potential of *P. lilacinum* under real conditions; subsequently, another more structured trial was performed, also including the adjuvant, in order to improve control over variables and data accuracy.

2. Material and Methods

2.1. Fungal isolate and inoculum

The isolate of *Purpureocilium lilacinum* used in this study originated from the working collection of the Laboratório de Patologia de Invertebrados (LPI) at Universidade Federal de Goiás (UFG). It was identified molecularly and morphologically by the authors from Laboratório de Micologia (LabMicol) located at the Instituto de Patologia Tropical de Saúde Pública (IPTSP) of the Universidade Federal de Goiás (UFG), Goiânia, Brazil (Chapter 1). A dried culture is preserved in silica gel, and a living culture is stored in glycerol at both the LPI-UFG (accession number IP 551) and the Otávio de Almeida Drummond Collection (COAD-UFV) (accession number COAD 4015).

The fungus was cultivated in 23 mL of potato dextrose agar (PDA) supplemented with 1 $g.L^{-1}$ of yeast extract (PDAY) in Petri dishes (90 × 15 mm) under a 12-hour photoperiod at room temperature and humidity (27 ± 2 °C, RH ± 60%) for 15 days. Conidia were dislodged with a microbiological loop and suspended in 50 mL of Tween 80® (0.01% v/v). Serial dilutions of the aqueous conidial suspension were made to obtain the working concentration (1 × 10^7 conidia. mL^{-1}) with the aid of a Neubauer chamber.

Scaling up for field application was done on a rice substrate, following Lopes (2016). For this, parboiled rice was placed in a plastic bag (30 cm × 20 cm) and moistened with 30% by volume of distilled water. This was left for 10 minutes, and the rice was then cooked for 20 minutes. After the rice had cooled to room temperature, 1 mL of conidial suspension (1×10^7 conidia.mL^{-1} in Tween 80®, see above) was inoculated. The bags were then incubated at 26 ± 1 °C with a photoperiod of 12 L: 12 D for 15 to 20 days. After this period, the rice was washed with sterile distilled water and passed through a mesh sieve to remove the rice grains, allowing the collection of a fungal conidial suspension. The conidial concentration was determined using a Neubauer chamber and adjusted to 1×10^7 conidia. mL^{-1}. The suspension was applied in the field within 24 hours after preparation.

To evaluate the viability of each step (before and after inoculating in rice), a conidial suspension was prepared at 1×10^5 conidia mL^{-1} for ease of counting. The suspension was inoculated with a volume of 2 µL at three different points of a Petri dish (55 × 15 mm) containing PDA medium. The plate was incubated for 14 hours at room temperature and humidity (27 ± 2 °C, RH ± 60%). After this period, a drop of cotton blue lactophenol and a cover slip were placed over the inoculum, and conidial germination was observed under a phase-contrast microscope with a magnification of 400×. At least 100 conidia for each of the three points were evaluated, and percentage germination was calculated. Conidia were considered germinated when the germ tube was longer than the maximum diameter of the conidia. In all cases, germination was > 90% so the germination assay is not considered further in this paper.

2.2. Operational-scale field trial in two maize-growing regions

The first assay was conducted during the second harvest of 2024, in two distinct commercial maize-growing areas, with applications carried out in February and March, to evaluate the efficacy of the biological agent under real cultivation and management conditions.

The two areas were in the municipalities of Ouroana (18°03'58" S, 50°41'02" W) and Hidrolândia (16°52'05.0" S, 49°17'51.0" W), both in the state of Goiás, Brazil, where the maize hybrids Feroz Viptera 3 (SYN8A98 TLTG VIPTERA) and Ag 1051 were used, respectively. Each field had a total area of 5 hectares, which was divided into two equal parts: one half received a single application of the spray suspension containing *P. lilacinum*, while the other

half remained untreated as a control. The applications were performed using a drag sprayer with a 600-liter tank and a flow rate of 100 L·ha^{-1} (Figure 1). In Ouroana, the tank mix contained *P. lilacinum* alone, while in Hidrolândia, it was combined with orange oil. Applications were carried out in the morning, at 10:00 a.m. in Ouroana and 8:00 a.m. in Hidrolândia. The fungal suspension was prepared at a concentration of 1×10^7 conidia. mL^{-1}, with adjustments based on the spray volume typically used by each grower.

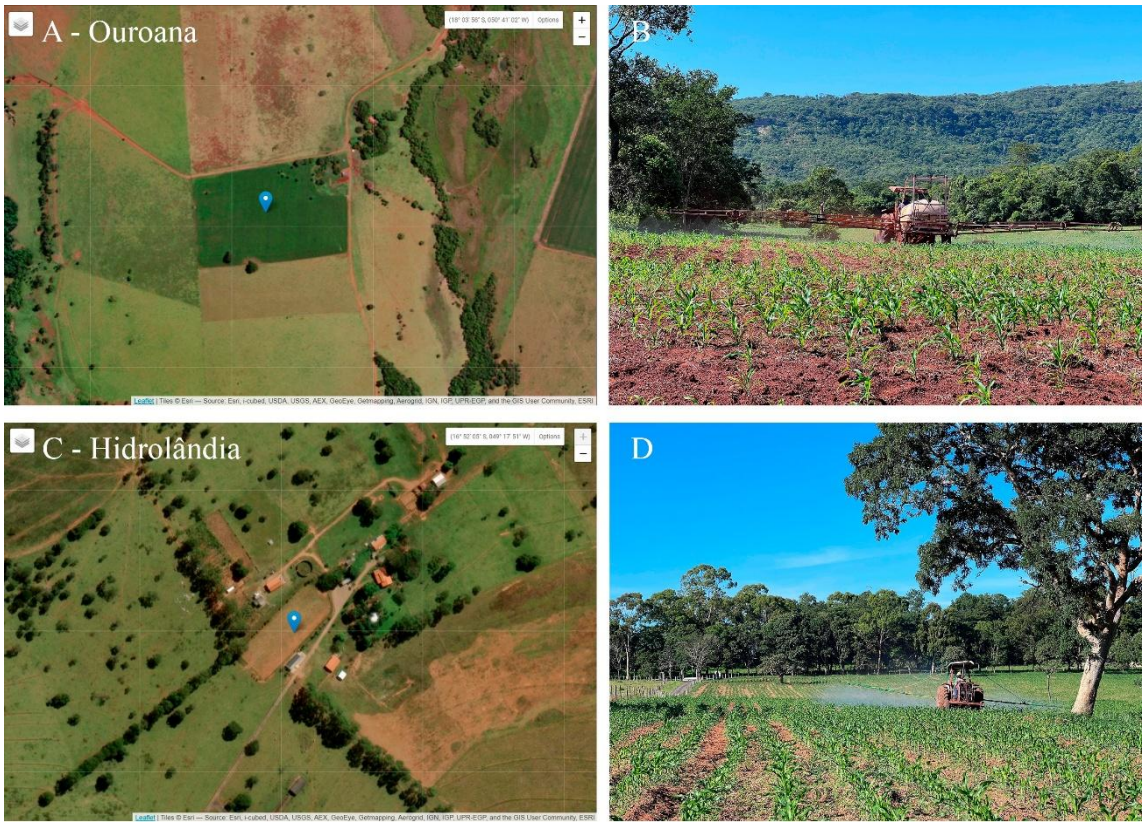

Figure 1. Experimental sites and application of treatments in commercial maize fields located in Goiás State, Brazil, where field mortality assays were carried out in 2024. Aerial views (A, C) and treatment applications (B, D) in the field trials conducted in 2024 in Ouroana (18°03'58" S, 50°41'02" W) (A–B) and Hidrolândia (16°52'05.0" S, 49°17'51.0" W).

The evaluations were made at intervals of 0 (prior to application), 2, 8, and 11 days after the application of *P. lilacinum.* For each evaluation time, 20 consecutive maize plants were selected from three random points within the area (with a minimum distance of 50 meters between them). Each plant was carefully inspected from top to bottom to assess the presence of live *D. maidis* individuals. Additionally, the number of dead insects adhering to the leaves and

exhibiting signs of fungal infection was recorded. These dead specimens were collected and transported to the laboratory, where they were placed in humid chambers (Petri dishes housed in plastic boxes containing distilled water) for subsequent fungal growth assessment.

2.3. Structured field trial for treatment evaluation and yield assessment

Given the difficulty in detecting significant differences under commercial field conditions, partly due to the migration behavior of the insect, a new trial was conducted under more structured and controlled field conditions to minimize external interference and improve data accuracy. Field mortality assays using *Purpureocillium lilacinum* against *Dalbulus maidis* were conducted in 2025 at the Universidade Federal dos Vales Jequitinhonha e Mucuri (UFVJM) - *Campus* Unaí, Minas Gerais, Brazil.

The maize used was hybrid BAYER 3510 RR2. At planting, fertilization was carried out with a formulated NPK (08-28-16) at a rate of 368 kg ha^{-1}. The experimental design followed a randomized complete block design (RCBD) in a factorial arrangement. Five blocks were established, each containing five plots (treatments), totaling 25 experimental units. Each plot measured 5 m × 5 m (25 m^2). The spacing between rows was 0.50 m, while the spacing between plants within each row was 0.30 m.

The treatments applied were: (1) *P. lilacinum* (1×10^7 conidia.mL^{-1}) applied with water only; (2) *P. lilacinum* (1×10^7 conidia.mL^{-1}) combined with an adjuvant (TA 35 Gold Bio, 150 mL ha^{-1}); (3) Chemical insecticide (imidacloprid + beta-cyfluthrin; Connect®, 750 mL ha^{-1}); (4) Water combined with adjuvant (TA 35 Gold Bio, 150 mL ha^{-1}) as a spray control and (5) Untreated control.

Three applications of each treatment were carried out at seven-day intervals, starting at the V5 growth stage, using a CO_2-pressurized backpack sprayer equipped with a hollow cone nozzle (MGA06, MagnoJet) and a flow rate of 150 L.ha^{-1}. The applications occurred at 0, 7, and 14 days after the beginning of the experiment. To assess spray coverage and droplet characteristics, water-sensitive papers (76 × 26 mm, Spraying Systems Co., Wheaton, USA) were placed horizontally on the soil surface, next to the base of the plants, with the sensitive side facing upward. Four papers were positioned per plot immediately before the first spraying. After application, the papers were collected, stored in envelopes, and later scanned in the laboratory using a flatbed scanner (600 dpi, 24-bit color). Droplet analysis was performed using

AccuStain 3.0 software (University of Illinois), providing estimates of volume median diameter (VMD), relative span (RS), percentage of coverage, and droplet density (droplets·cm^{-2}).

To evaluate treatment effects on insect mortality, field assessments were conducted at 3, 7, and 14 days after each application. However, due to the advanced development stage of the crop, the final evaluation (14 days after the third application) was not performed. At each assessment, 10 consecutive maize plants were sampled from the central rows of each plot. Plants were thoroughly inspected from top to bottom to record the number of live *D. maidis* individuals. Additionally, the number of dead insects adhered to the leaves and exhibiting visible signs of fungal infection was recorded.

The harvest of the experimental area was carried out at 120 days after planting in all plots of four blocks (block 5 was lost due to an environmental occurrence. The three central rows of each plot, each approximately 3 meters long, were selected for harvesting, totaling 9 linear meters per plot for yield assessment. Subsequently, the ears were separated from the remaining biomass (leaves and stems) of each plot and dried to reduce the grain moisture content. The material was stored in a naturally ventilated environment. A threshing machine powered by a combustion engine (Model SB, Tratores) was used to separate the grains from the cobs. The grains from each sample were weighed, and the estimated yield was calculated in tons per hectare (t. ha^{-1}) for each treatment, adjusting the grain moisture content to 13%.

2.4. Statistical analyses

Live insect counts (2024 and 2025) were analyzed using generalized linear models (GLMs) with a negative binomial distribution, with Time, Treatment, and the Time × Treatment interaction as fixed effects. The most appropriate error distribution was selected after testing Normal, Poisson, Quasi-Poisson, and Negative Binomial models, based on the envelope test.

Dead insect counts were analyzed separately for each locality and year. In the 2024 trials conducted in Ouroana, differences among treatments were assessed using the non-parametric Kruskal–Wallis test. In the 2024 trials conducted in Hidrolândia, a one-way ANOVA was applied at each evaluation time, and when significant, pairwise comparisons between treatments were performed using the non-parametric Mann–Whitney U test. In the 2025 trial, GLMs with a negative binomial distribution were applied, as described for the live insect counts.

Data on spray coverage, droplet density, and maize grain yield ($kg \cdot ha^{-1}$) were tested for normality of residuals (Shapiro–Wilk) and homogeneity of variances (Levene's test). When assumptions were met, data were analyzed by ANOVA, and significant differences between treatments were separated by Tukey's post hoc test at the 5% probability level. All analyses were performed in R (R Core Team, 2020) using the packages "glm", "MASS", "metan", "multcomp", and "Rmisc".

3. Results

3.1. Operational-scale field trial in two maize-growing regions

The population dynamics of *D. maidis* over time showed distinct patterns between Ouroana and Hidrolândia (Figure 2A–D). In Ouroana, the GLM with a negative binomial distribution indicated significant effects of treatment ($\chi^2 = 6.389$, df = 1, $p = 0.011$), time ($\chi^2 = 61.196$, df = 3, $p < 0.001$), and their interaction ($\chi^2 = 185.871$, df = 3, $p < 0.001$). The *P. lilacinum* treatment resulted in a lower number of live insects compared to the control on day 2 after application ($p < 0.001$) (Figure 2A). On day 8, the opposite was observed, with the *P. lilacinum* treatment showing significantly higher values than the control ($p < 0.001$). On days 0 and 11, no significant differences were observed between treatments ($p > 0.05$).

In Hidrolândia, the GLM with a Poisson distribution also indicated significant effects of treatment ($\chi^2 = 6.105$, df = 1, $p = 0.013$), time ($\chi^2 = 35.582$, df = 3, $p < 0.001$), and their interaction ($\chi^2 = 22.273$, df = 3, $p < 0.001$). The *P. lilacinum* treatment resulted in fewer live insects than the control on days 2 ($p < 0.001$) and 8 ($p = 0.036$) after application (Figure 2B). On days 0 and 11, no significant differences were found ($p > 0.05$).

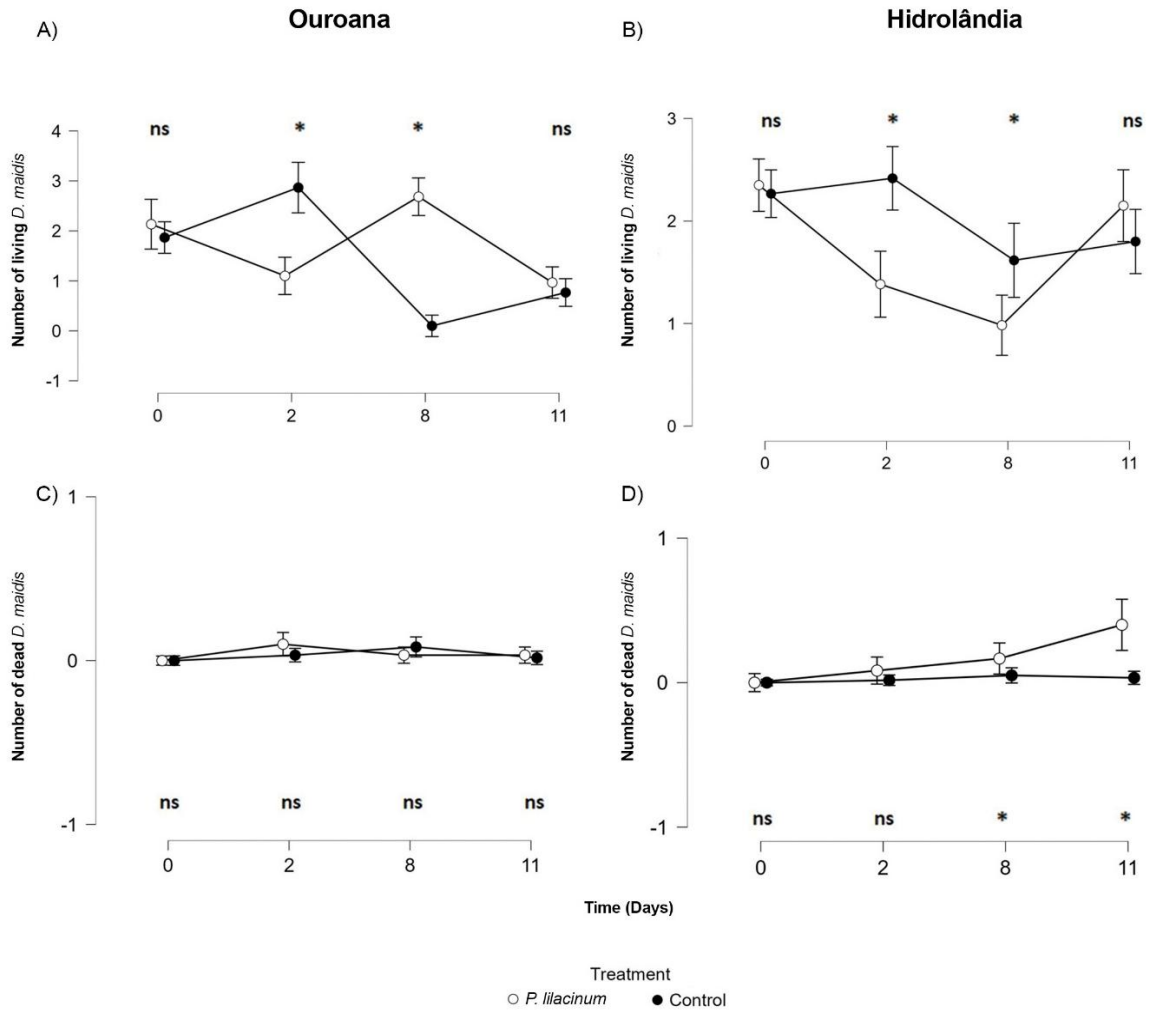

Figure 2. Mean numbers of *Dalbulus maidis* individuals (alive and dead) found on the leaves of 60 maize plants at 0 (pre-application), 2, 8, and 11 days after treatment with *Purpureocillium lilacinum* in field trials conducted in 2024 in Ouroana (A, C) and Hidrolândia (B, D), Goiás, Brazil. Graphs A and B show the number of live insects over time; the data were analyzed using generalized linear models (GLMs) followed by Tukey's post hoc test for pairwise comparisons ($p < 0.05$). Graph C was analyzed using the Kruskal–Wallis test, and Graph D using one-way ANOVA followed by the Mann–Whitney U test when significant. Asterisks indicate significant differences according to the respective tests mentioned above. NS = Not significant.

No significant difference was observed in the number of dead *D. maidis* individuals between treatments in Ouroana ($\chi^2 = 0.230$; df = 1; $p = 0.631$; Figure 2C). In Hidrolândia, there was a significant difference between treatments ($F = 18.534$; df = 1,478; $p < 0.001$), and

pairwise comparisons showed differences on days 8 ($p = 0.041$) and 11 ($p < 0.001$) after application, whereas on days 0 and 2 there were no differences ($p > 0.05$; Figure 2D).

Despite the lack of statistically significant differences in the number of dead *D. maidis* individuals between treatments, insect cadavers showing signs of sporulation were observed in both areas, treated and untreated (Figure 3). These visual findings suggest that the fungus *P. lilacinum* may have exerted some biological activity in the field. However, these effects were not captured quantitatively by the statistical models. These results suggest that the experimental design may not have been robust enough to detect potential differences between treatments in the field, indicating the need for adjustments in the experimental planning to enhance the analysis' sensitivity and allow for the detection of more subtle effects.

Figure 3. Presence of infected *Dalbulus maidis* in both treated and untreated field plots at all evaluation times following the application of *Purpureocillium lilacinum.*

3.2. Structured field trial for treatment evaluation and yield assessment

The results of the application technology assessment were based on four water-sensitive papers per plot, placed at the time of the first application in each of the four treatments.

Coverage did not differ significantly among treatments ($p = 0.390$), although there was a tendency for higher values in the *P. lilacinum* + adjuvant treatment (Table 1).

Table 1. Mean values of droplet coverage, density, and spectrum parameters for different treatments involving *Purpureocillium lilacinum*, adjuvant and chemical, applied using a hollow cone nozzle (MGA06, MagnoJet).

Treatments	Coverage (%)	CV	Droplet Density (cm^{-2})	CV	VMD (µm)	CV	$DV_{0.1}$ (µm)	CV	$DV_{0.9}$ (µm)	CV	SPAN
P. lilacinum	23.95	0.145	331.37a	0.188	400.80a	0.062	259.20b	0.074	550.40b	0.066	0.72b
***P. lilacinum* + adjuvant**	25.00	0.107	548.28b	0.050	361.60b	0.026	210.40a	0.033	537.20b	0.017	0.90a
Imidacloprid + beta-cyfluthrin	19.70	0.334	366.60ab	0.452	341.20b	0.058	238.20ab	0.091	475.20a	0.067	0.67b
Water + adjuvant	20.82	0.359	331.29a	0.325	356.00b	0.058	224.60a	0.045	511.00ab	0.084	0.80a
p	0.390^{ns}		<0.001*		<0.001*		<0.001*		0.011*		0.0131*

Means followed by the same letters do not differ according to the Tukey test at a 5% probability level. P: probability value; NS: not significant; CV: coefficient of variation; VMD: volume median diameter; $DV_{0.1}$: droplet diameter below which 10% of the spray volume is contained; $DV_{0.9}$: droplet diameter below which 90% of the spray volume is contained; SPAN: relative spray span index, calculated as ($DV_{0.9}$ – $DV_{0.1}$) / VMD.

Droplet density differed significantly among treatments ($p < 0.001$). The *P. lilacinum* + adjuvant treatment showed the highest density (548.28 drops·cm^{-2}), being superior to *P. lilacinum* alone (331.37) and water + adjuvant (331.29) (Tukey, $p < 0.05$). Imidacloprid + beta-cyfluthrin presented an intermediate value (366.60), not differing statistically from either the higher or the lower group. The combination of *P. lilacinum* + adjuvant increased droplet density by approximately 65% compared to *P. lilacinum* alone (Figure 4).

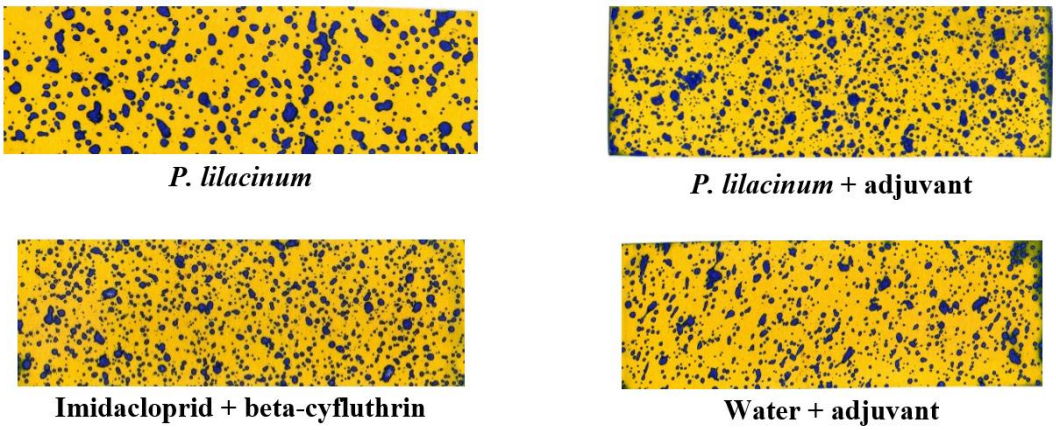

Figure 4. Droplet distribution on water-sensitive paper following the application of the treatments: *Purpureocillium lilacinum*, *P. lilacinum* + adjuvant, imidacloprid + beta-cyfluthrin and water + adjuvant, using a hollow cone nozzle (MGA06, MagnoJet).

The $DV_{0.1}$, which represents the droplet diameter below which 10% of the sprayed volume is contained (finer fraction of the application), showed the lowest values in the treatments with adjuvant (*P. lilacinum* + adjuvant: 210.40 µm; water + adjuvant: 224.60 µm), differing from *P. lilacinum* alone The chemical treatment (238.20 µm) did not differ statistically from the treatments with adjuvant nor from *P. lilacinum* alone ($p < 0.001$) (Table 1). This result indicates that the presence of the adjuvant increased the proportion of smaller droplets at the fine end of the spectrum. For $DV_{0.9}$, which corresponds to the droplet diameter below which 90% of the sprayed volume is contained (coarser droplet fraction), the highest values were obtained for the fungal treatments (*P. lilacinum*: 550.40 µm; *P. lilacinum* + adjuvant: 537.20 µm), with no difference compared to the water + adjuvant treatment (511 µm), but differing from the chemical treatment (475.20 µm) (Table 1).

Regarding the SPAN index, which represents the relative width of the droplet size spectrum, lower values indicate greater spray uniformity, whereas higher values indicate greater variability in droplet sizes. The imidacloprid + beta-cyfluthrin (0.67) and *P. lilacinum* alone

(0.72) treatments produced the most uniform sprays, with no statistical difference between them (Table 1). In contrast, *P. lilacinum* + adjuvant (0.90) and water + adjuvant (0.80) showed greater variability in droplet size, differing significantly from the more uniform treatments (p = 0.0131).

In 2025, the number of live *D. maidis* in the experimental structured was significantly affected by treatment ($\chi^2 = 26.16$; df = 4; $p < 0.001$), time ($\chi^2 = 390.84$; df = 6; $p < 0.001$), and their interaction ($\chi^2 = 61.92$; df = 24; $p < 0.001$) (Figure 5), indicating that the applied treatments, the evaluation time, and their combined effects significantly impacted the number of live insects.

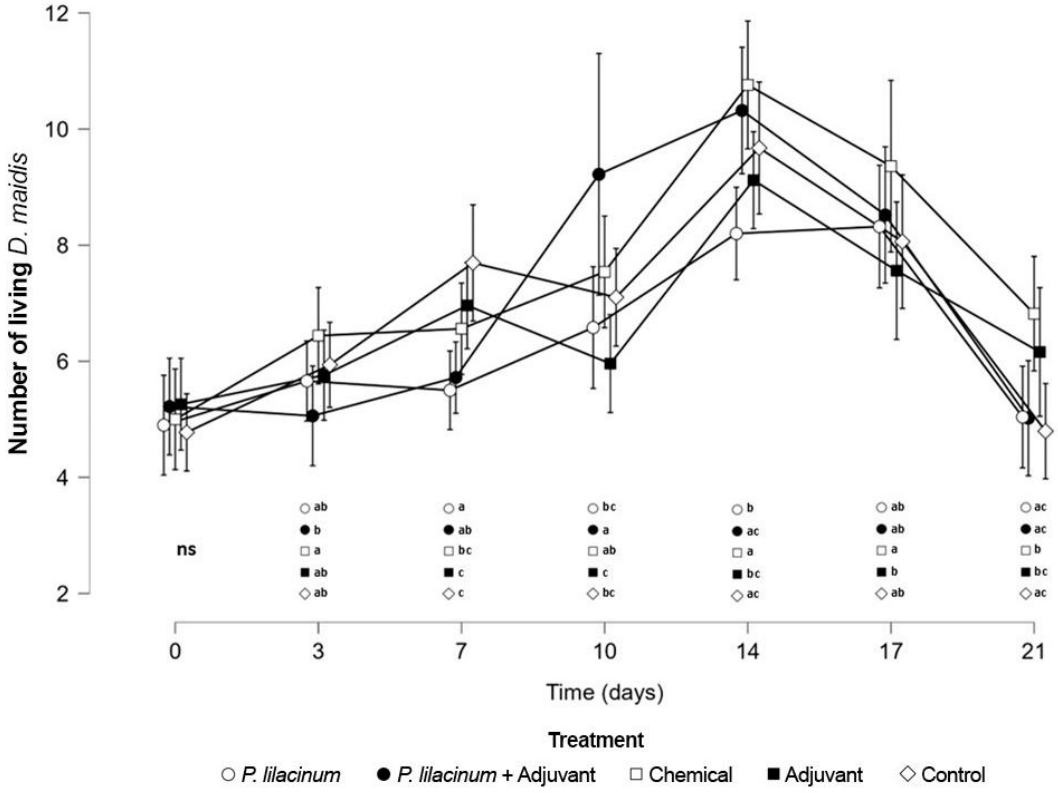

Figure 5: Number of live *Dalbulus maidis* recorded 3, 7, and 14 days after the first and second applications, and 3 and 7 days after the third application of *Purpureocillium lilacinum* (applications performed at days 0, 7, and 14). Treatments included *P. lilacinum*, *P. lilacinum*+ adjuvant, adjuvant alone, chemical control (Connect® – imidacloprid + beta-cyfluthrin), and untreated control. Data were analyzed using generalized linear models (GLMs) with a negative binomial distribution, and significant effects were separated by Tukey's post hoc test at the 5% probability level. Lowercase letters compare treatments within each evaluation time, and uppercase letters compare evaluation times within each treatment.

Three days after the first application, *P. lilacinum* + adjuvant showed the lowest mean number of live insects, differing significantly from the chemical treatment ($p = 0.005$); however, there was no difference between either of them and the other treatments and the control ($p > 0.005$).

On the seventh day after the first application, *P. lilacinum* alone showed the lowest mean number of insects, differing significantly from the chemical ($p = 0.031$), adjuvant ($p = 0.003$), and control ($p < 0.001$) treatments. The *P. lilacinum* + adjuvant treatment also showed reduced values compared with the adjuvant ($p = 0.013$) and the control ($p = 0.003$), but did not differ from *P. lilacinum* alone ($p > 0.001$) nor from the chemical treatment ($p > 0.001$).

Three days after the second application (day 10), the treatment that showed the lowest number of insects was the adjuvant, but there was no significant difference from the control ($p > 0.05$) and from *P. lilacinum* alone ($p > 0.05$). The fungus applied with adjuvant was the treatment that showed the highest number of live insects, with no difference from the chemical treatment ($p > 0.05$).

Seven days after the second application (day 14), the *P. lilacinum* alone treatment showed the lowest mean number of live *D. maidis*, differing significantly from the *P. lilacinum* +adjuvant ($p = 0.001$), chemical ($p < 0.001$), and control ($p = 0.027$) treatments, but with no difference from the adjuvant treatment ($p > 0.05$).

Three days after the third application (day 17), there was a difference only between the chemical and adjuvant treatments ($p = 0.027$), with the chemical treatment having the lowest mean number of insects. However, there were no differences among the other treatments ($p > 0.05$).

In the final evaluation, seven days after the third application (day 21), the *P. lilacinum* and *P. lilacinum* +adjuvant treatments again showed fewer live insects than the chemical treatment ($p = 0.04$ for both), but with no significant difference from the control ($p > 0.05$) and the adjuvant ($p > 0.05$).

Dead insects exhibiting signs of fungal infection were observed in all treatments over time. There was no significant interaction between treatment and time for the mean number of dead insects ($p = 0.3594$), except on day 17 (three days after the third application). On day 17,

the treatment containing only the chemical product showed the highest mean number of dead and sporulating insects, differing significantly from the adjuvant-only treatment, which recorded the lowest mean on that date. However, neither differed statistically from the other treatments (*P. lilacinum, P. lilacinum* + adjuvant, and the control), which showed intermediate values (Figure 6).

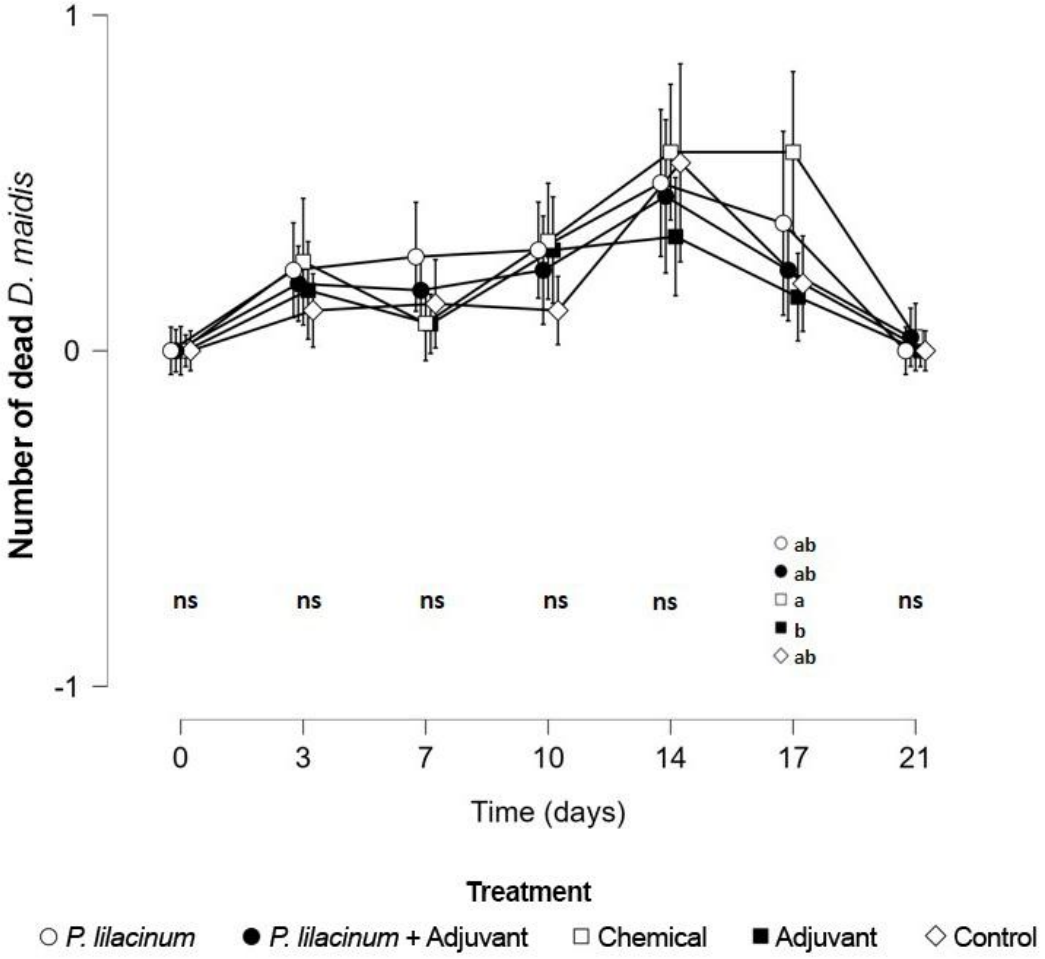

Figure 6. Average number of dead *Dalbulus maidis* found on the leaves of ten maize plants per treatment plot over 21 days of evaluation. Treatments included *P. lilacinum*, *P. lilacinum* + adjuvant, adjuvant alone, chemical (imidacloprid + beta-cyfluthrin), and untreated control. Data were analyzed using generalized linear models (GLMs) with a negative binomial distribution, and significant effects were separated by Tukey's post hoc test at the 5% probability level. Different lowercase letters indicate significant differences among treatments within each evaluation time. NS = indicates no significant difference. Error bars represent the standard error of the mean (SEM).

Maize grain yield showed significant differences among treatments (p = 0.0044), indicating that the management strategies influenced final production (Figure 7).

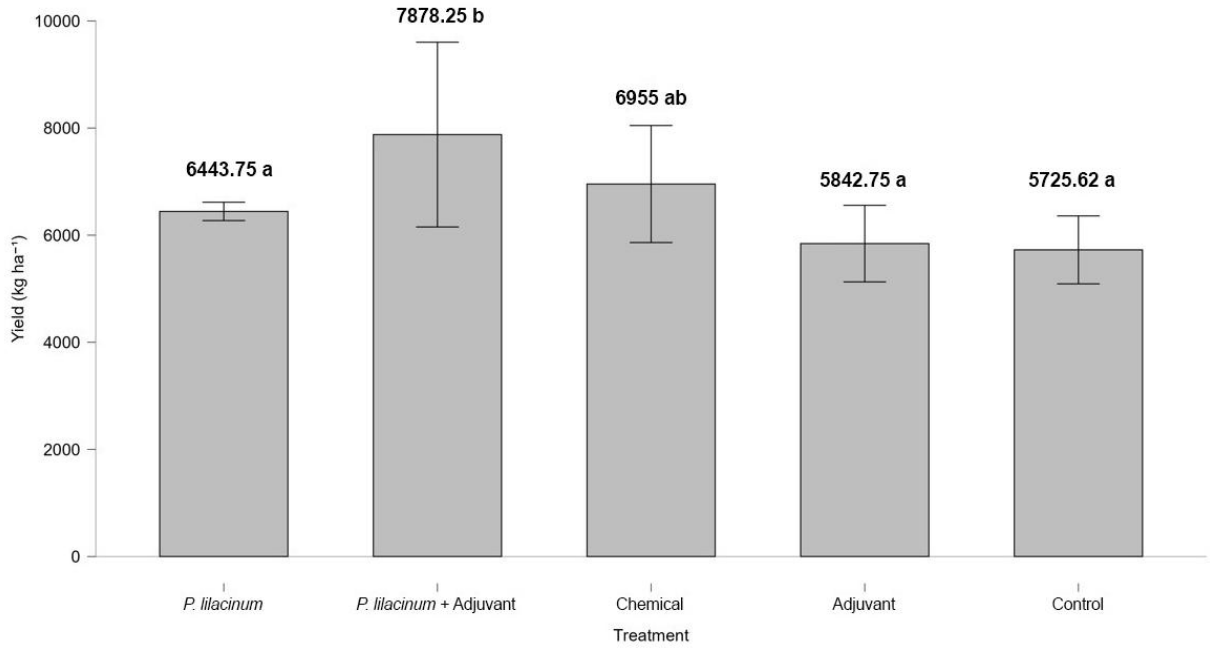

Figure 7. Maize grain yield (kg·ha⁻¹) under different treatments: *Purpureocillium lilacinum*, *P. lilacinum* + TA35 adjuvant, chemical insecticide (Connect® – imidacloprid + beta-cyfluthrin), adjuvant alone, and untreated control. Data were analyzed by ANOVA and significant differences were separated by Tukey's post hoc test at the 5% probability level. Means followed by the same letter do not differ significantly. Error bars represent the standard error of the mean (SEM).

Purpureocillium lilacinum combined with adjuvant and the chemical treatment resulted in the highest yields, reaching 7878.25 kg ha⁻¹ and 6955.00 kg ha⁻¹, respectively, both being significantly different from the other treatments.

4. Discussion

In this study, the addition of an adjuvant to mixtures with the fungus *Purpureocillium lilacinum* influenced the control of *Dalbulus maidis* in the field by altering droplet formation and density, thereby favoring deposition on the target. The fungus with adjuvant combination

promoted population reductions within the first days after application and resulted in the highest grain yield among treatments, although differences were not always statistically significant. These results reinforce the potential of using adjuvants to optimize the application of entomopathogenic fungi and enhance their efficacy in the integrated management of this pest.

Purpureocillium lilacinum (Ascomycota: Hypocreales) is primarily known as a pathogen of plant-parasitic nematodes (Dahlin et al., 2019; Khan and Tanaka, 2023). However, numerous studies have been conducted to evaluate its efficacy in controlling other microorganisms (Elsherbiny et al., 2021; Lan et al., 2017), disease-vectoring insects (Marti et al., 2006), as well as chewing (Bali et al., 2022; Goffré and Folgarait, 2015) and sucking insect pests (Fiedler and Sosnowska, 2007; Nguyen Thi et al., 2023). Nevertheless, to date, no studies have evaluated the effectiveness of *P. lilacinum* against *D. maidis* under field conditions.

Currently, around 100 genera of entomophagous fungi have been described (Sharma and Sharma, 2021), but only two, *Beauveria* and *Cordyceps*, are officially registered for the control of *D. maidis* in Brazil (Ministério da Agricultura, 2025). This highlights the need for further research on other genera and species of entomopathogenic fungi (EPF) for use against this pest.

Purpureocillium lilacinum has shown effectiveness in controlling sap-sucking insects in the field after seven days of application. Nguyen Thi et al. (2023) tested a *P. lilacinum* (PL1) isolate against *Bemisia tabaci* in cassava fields in Vietnam and reported a 77.46% reduction in nymph populations seven days after the first application. Similarly, Medeiros et al. (2018) showed that a *P. lilacinum isolate* found in *Aleurocanthus woglumi* nymphs on citrus in Maranhão, Brazil, caused up to 100% mortality in 2nd and 3rd-instar nymphs within seven days.

In the two commercial areas (Ouroana and Hidrolândia) where *P. lilacinum was* applied, a reduction in *D. maidis populations* was observed as early as the second day after application. A similar result was found in the structured experiment three days after the application of *P. lilacinum* with adjuvant. Maluta et al. (2023) reported changes in the feeding behavior of *D. maidis* 48 hours after applying *Cordyceps javanica*, including shorter stylet insertion times and reduced phloem ingestion. Such behavior may lead insects to abandon the host plant, potentially influencing the results observed in the present study.

In Hidrolândia, there was also a reduction in insect populations in the field on the eighth day, a result similar to that observed in the structured experiment on the seventh day after the first application of *P. lilacinum (*alone and with adjuvant) and seven days after the second application of *P. lilacinum.* From 14 days onwards, control consistency was lost. One factor

associated with this is insect migration. Field assessment of *D. maidis* populations presents considerable challenges, particularly due to their agile flight behavior and high dispersal capacity. Foresti et al. (2022) reported that *D. maidis exhibits* a mean spatial dependence of up to 463.5 meters and is classified as a commuting migrant, regularly traveling between separate crop areas. This high mobility, along with the ability to rapidly colonize crop edges from neighboring fields, can significantly affect the accuracy of field treatment evaluations, introducing statistical inconsistency. Therefore, to more precisely assess the efficacy of EPF against *D. maidis*, complementary experiments under controlled conditions are recommended to eliminate bias from natural insect dispersal.

In general, no significant difference in the number of dead insects was found over time among treatments in all fields treated with *P. lilacinum* (except in Hidrolândia on day 14 and in the structured experiment on day 17). However, all recovered insects showed signs of fungal infection. Only in some field-collected samples was it possible to re-isolate the applied *P. lilacinum* strain, as most were contaminated by other microorganisms.

In the structured field trial, an adjuvant was included to evaluate *P. lilacinum* performance against *D. maidis.* According to Lopes et al. (2024), *D. maidis* produces brochosomes, protein-lipid particles covering the body, that form a hydrophobic physical barrier repelling conidia applied with water alone. Additionally, this pest can self-clean using its third pair of spined legs, effectively removing fungal conidia. The authors emphasize that effective EPF application for *D. maidis* control requires adjuvants to improve conidial adhesion and increase insect exposure to the biocontrol agent.

Coverage in an application containing contact products for pest insect control is the most important parameter to be evaluated, as it ensures the distribution of the applied product over the target. Droplet density is the practical metric used to verify whether effective coverage has been achieved. For greater efficacy of contact product applications, a higher number of spray droplets per unit area (droplet density) will generally have a greater likelihood of reaching the critical threshold for pest control (Zhu et al., 2011). In the present study, the use of an adjuvant increased deposition: the adjuvant alone showed the highest coverage (25.00%) and density (548.28 droplets·cm^{-2}), and the combination *P. lilacinum* + adjuvant increased density by 65% compared to *P. lilacinum* alone. Still, these differences were not statistically significant among treatments, indicating only a positive trend and reinforcing the potential role of adjuvants in optimizing the application of entomopathogenic fungi.

The use of EPF in managing *D. maidis* goes beyond causing direct insect mortality. As previously mentioned, Maluta et al. (2023) demonstrated that, unlike chemical insecticides, EPF can reduce exploratory probing behavior, which contributes to decreasing the transmission of phloem-limited pathogens by *D. maidis*. This biological advantage is supported by the current study, in which the *P. lilacinum* with adjuvant treatment resulted in the highest grain yield (7878.25 kg ha^{-1}), although statistically similar to the chemical insecticide treatment (Imidacloprid + beta-cyfluthrin, 6955.00 kg ha^{-1}). These findings reinforce the practical and economic potential of biological control, positioning it as an efficient and sustainable strategy in *D. maidis* management.

To conclude, although no high mortality rates were observed with the use of *P. lilacinum,* the application of the fungus resulted in population reductions as early as two days after application, with reinforced effects observed between seven and eight days. The combination of *P. lilacinum* and adjuvant also presented the highest grain yield among treatments, confirming the economic viability of this approach. However, the assessment of dead insect counts in the field was limited by the pest's high dispersal capacity, which complicates monitoring and can bias statistical mortality analyses. Therefore, it is emphasized that field trials should be complemented with studies under controlled conditions to isolate the effect of the fungus and eliminate confounding factors related to *D. maidis* mobility.

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CHAPTER 4

Are *Purpureocillium lilacinum*, its enzymes and its metabolites active against insects?

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ABSTRACT

Understanding the pathogenic potential of *Purpureocillium lilacinum* against diverse insect pests is essential to explore its applicability beyond nematode control and guide the development of integrated pest management strategies. This study evaluated the efficacy of different forms of *Purpureocillium lilacinum*, conidia, enzymes, and metabolites, in the control of agriculturally important insect pests. The bioassays included the corn leafhopper (*Dalbulus maidis*), four lepidopteran species (*Spodoptera frugiperda*, *Rachiplusia nu*, *Chrysodeixis includens* and *Helicoverpa armigera*) and the model organism *Tenebrio molitor*. The conidia of *P. lilacinum* showed no significant pathogenicity when sprayed on *D. maidis*, even when combined with an insecticide (acephate) or an adjuvant at concentrations of 1×10^7 or 1×10^8 conidia mL^{-1}. On the other hand, when *P. lilacinum* was added to acephate, it reduced insect mortality by 50% compared to the application of the insecticide alone. Treatments with conidia (1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}), enzymes, and metabolites were not pathogenic to *T. molitor* after 10 seconds of immersion. Among the lepidopterans, only *R. nu* showed a significant reduction in survival (40%) following topical application of enzymes, while *S. frugiperda* exhibited marginal effects in response to conidia and metabolites. The results indicate low pathogenicity of the tested isolate but suggest that the combined application of conidia, enzymes, and metabolites may be promising and warrants further investigation, especially enzymes and metabolites through alternative infection routes such as ingestion.

Keywords: Biological control, *Dalbulus maidis*, *Tenebrio molitor,* Noctuidae.

1. Introduction

Biological control of insect pests using entomopathogenic fungi (EPF) or molecules derived from these organisms is increasingly used in agriculture due to its several advantages, such as specificity to target insects, less or no development of resistance and environmental safety (Islam et al., 2021). However, there are some limitations to the use of EPF, such as their susceptibility to environmental conditions (low humidity, high temperatures, and UV radiation) and the time required for the microorganism to cause insect death (Ferreira et al., 2024; Quesada-Moraga et al., 2024).

There are several steps that an entomopathogenic fungus follows to infect an insect, notably adhesion and germination of the spore on the insect's cuticle, penetration and replication into the hemocoel, which usually results in the death of the insect (Samson et al.,1988). The time for the entire process of infection to occur depends on the fungal species or isolate. On average, 14 days are required for the entire cycle, up to death and sporulation from the cadaver, but the death occurs between 4 and 10 days, depending on the type of fungus and number of infecting spores (Litwin et al., 2020; Sinha et al., 2016). To shorten this infection time and reduce crop damage caused by insect pests, studies have explored the use of enzymes and metabolites produced by the fungus as a complementary approach (Fan et al., 2007; Ferreira et al., 2024).

One of the components of the epicuticle (the outermost layer of the cuticle) is lipids, whereas the procuticle (the underlying layer of the cuticle) is mainly composed of protein (Islam et al., 2021). The levels of production of enzymes such as lipases, proteases and chitinases are determinant in the pathogenicity of EPF as they degrade the cuticle components (lipids, proteins, and chitin, respectively) facilitating the mechanical penetration of the fungus through these barriers (Samson et al.,1988).

On the other hand, secondary metabolites (chemical compounds that are not essential for the growth and development of the organism) produced by EPF can be toxic to insects (Vega et al., 2012; Vilcinskas et al., 1997). However, the role of these metabolites in pathogenesis is still unclear (Vega et al., 2012). Species in the order Hypocreales (Ascomycota), such as *Purpureocillium lilacinum*, are known for producing secondary metabolites that include toxins such as leucinostatins (paecilotoxin), paecilomide and acremoxanthones, which are able to cause mortality and inhibit reproduction in nematodes (Khan and Tanaka, 2023; Prasad et al., 2015). Although *P. lilacinum* is considered a promising entomopathogenic fungus for

controlling insect pests such as noctuids and hemipterans (Liu et al., 2022), its metabolites and enzymes have not yet been evaluated separately against these groups.

Dalbulus maidis (Hemiptera: Cicadellidae) is considered the primary pest of maize in Brazil (Oliveira and Frizzas, 2022) due to its ability to cause economic damage not only through feeding but also by acting as a vector for the maize stripe virus (Maize rayado fino virus, MRFV) and the mollicutes associated with maize stunt spiroplasma (CSS) and maize bushy stunt phytoplasma (BSP) (Nault, 1990, 1980). *Dalbulus maidis* can hinder infections by entomopathogenic fungi (EPF) during the early stages of the invasion process through grooming behavior and the presence of a protective brochosome coating, composed of proteins and lipids, on its cuticle surface (Lopes et al., 2024). Therefore, the use of an appropriate surfactant has been suggested to improve the adhesion of fungal conidia to the insect's body and enhance the effectiveness of biological control strategies.

Another group of insect pests that attack economically important crops such as maize, soybean, bean and cotton are noctuid lepidopterans. Species such as *Spodoptera frugiperda*, *Helicoverpa armigera*, *Rachiplusia nu* and *Chrysodeixis includens* stand out due to their wide geographical distribution, highly polyphagous behavior, and destructive potential (Barrionuevo et al., 2012; Cruz, 1995; Pomari-Fernandes et al., 2015; Specht et al., 2015). The management of these species has posed a major challenge to agriculture because of their remarkable adaptability and, in some cases, the development of resistance to conventional insecticides (Braga et al., 2024; Downes and Mahon, 2012; Eva Mamahit and Kolondam, 2023; Godoy et al., 2025).

This study aimed to evaluate the efficacy of *P. lilacinum*, as conidia, against the corn leafhopper *Dalbulus maidis*, both alone and in combination with a chemical insecticide commonly used for its control, in order to reduce the time required for pest suppression. In addition, an adjuvant was included in some treatments to assess whether its presence would influence the effectiveness of fungal control. We also tested the effects of fungal enzymes and secondary metabolites on *Tenebrio molitor*, commonly used as a model organism in pathogenicity assays. Furthermore, the same fungal components (conidia, enzymes, and metabolites) were evaluated against lepidopteran pests of agricultural importance: *Spodoptera frugiperda*, *Helicoverpa armigera*, *Rachiplusia nu* and *Chrysodeixis includens*.

2. Material and Methods

2.1. Fungal inoculum

The isolate of *Purpureocilium lilacinum* used in this study originated from the working collection of the Laboratório de Patologia de Invertebrados (LPI) at Universidade Federal de Goiás (UFG). It was identified molecularly and morphologically by the authors from Laboratório de Micologia (LabMicol) located at the Instituto de Patologia Tropical e Saúde Pública (IPTSP) of the Universidade Federal de Goiás (UFG), Goiânia, Brazil (Chapter 2). A dried culture is preserved in silica gel, and a living culture is stored in glycerol at both the LPI-UFG (accession number IP 551) and the Otávio de Almeida Drummond Collection (COAD-UFV) (accession number COAD 4015).

The fungus was cultivated in 23 mL of potato dextrose agar (PDA) supplemented with 1 $g.L^{-1}$ of yeast extract (PDAY) in Petri dishes (90 × 15 mm) under a 12-hour photoperiod at room temperature and humidity (27 ± 2 °C, RH ± 60%) for 15 days. Conidia were dislodged with a microbiological loop and suspended in 50 mL of Tween 80® (0.01% v/v). Serial dilutions of the aqueous conidial suspension were made to obtain the working concentrations (1×10^7, 1×10^8 and 1×10^9 conidia. mL^{-1}) with the aid of a Neubauer chamber.

To evaluate viability, a conidial suspension was prepared at 1×10^5 conidia mL^{-1} for ease of counting. The suspension was inoculated with a volume of 2 µL at three different points of a Petri dish (55 × 15 mm) containing PDA medium. The plate was incubated for 14 hours at room temperature and humidity (27 ± 2 °C, RH ± 60%). After this period, a drop of cotton blue lactophenol and a cover slip were placed over the inoculum, and conidial germination was observed under a phase-contrast microscope with a magnification of 400×. At least 100 conidia for each of the three points were evaluated, and percentage germination was calculated. Conidia were considered germinated when the germ tube was longer than the maximum diameter of the conidia. In all cases, germination was > 90% so the germination assay is not considered further in this paper.

2.2. Enzymes and Metabolites

Enzymes and metabolites of *P. lilacinum* were produced and evaluated for pathogenicity against *Tenebrio molitor* and noctuid species. For this, the fungal isolates (1 cm^2) were inoculated in 100 mL of Sabouraud Dextrose Broth (SDB). The culture was incubated on an

orbital shaker at 27 ± 1 °C and 170 rpm for 7 days. After this period, the liquid cultures were filtered through filter paper. The filtrate was then centrifuged at 10,000 rpm for 6 min at 4 °C, and the supernatants were stored at 4 ± 1 °C. For the metabolite treatment, the culture supernatant was autoclaved at 120 °C for 20 minutes to ensure the inactivation of conidia and enzymatic activity. Potato dextrose broth (PDB) medium was used as the control medium.

Proteolytic activity was measured using the caseinolytic method, according to Abidi et al. (2011), and was performed in triplicate. The volumes of the solutions used in this method were: 50 µL of sample and 200 µL of McIlvaine buffer (citrate-phosphate buffer) 100 mM, pH 8.0, containing 1% azocasein. The reaction mixture was incubated for 1 hour at 37 °C. The reaction was stopped by adding 600 µL of trichloroacetic acid (TCA) solution at 10%. Then, the samples were placed on ice for 15 minutes and subsequently centrifuged at 14,000 g for 10 minutes. The supernatant was collected, and 600 µL of it was neutralized with 700 µL of 1 N sodium hydroxide solution for absorbance determination in a microplate reader at 450 nm. One unit of protease activity was defined as the amount of enzyme required to increase absorbance at 450 nm by 0.01 after 60 minutes, under the assay conditions, as shown in the following formula:

$$U = \frac{\text{Absorbance of the reaction} - \text{Absorbance of the control}}{0.01}$$

Proteolytic activity was detected in the enzyme fractions of *P. lilacinum*. The first enzymatic extracts showed a mean absorbance of 0.6327, while the respective control presented a mean of 0.0115, resulting in 62.12 U/mL of protease. The second enzymatic extracts showed a mean absorbance of 0.5913, while the respective control presented a mean of 0.100, resulting in 49.13 U/mL of protease. These results indicate that enzymatic fractions retained measurable proteolytic activity, with higher enzymatic activity observed in the untreated enzyme extract.

2.3. Pathogenicity on *Dalbulus maidis*

The pathogenicity assays against *Dalbulus maidis* were conducted at Embrapa Arroz e Feijão, located in Santo Antônio de Goiás, Goiás, Brazil. The adult insects used in the experiments were obtained from a laboratory colony maintained on maize plants (BRS Caimbé) cultivated inside cages (1.75 × 1.10 m) covered with fine mesh, within a screened greenhouse.

Maize (BRS Caimbé) was sown in 1 L plastic pots filled with a mixture of soil and fertilizer (NPK 4:30:15), using one seed per pot. Seven days after planting (V0 stage), the base of each plant was isolated from the soil surface using a layer of black mesh fabric to prevent contact between the insects and the soil and to facilitate the observation and collection of dead individuals (Figure 1A). To ensure adequate irrigation and humidity, each pot was placed on a circular tray for plants, which was kept filled with water throughout the experimental period. Cylindrical cages (15 cm in diameter and 30 cm in height) were placed over each plant, positioned above the mesh, and covered with gauze to confine the insects (Figure 1B).

Dalbulus maidis adults of approximately five-days post-moult were collected from the rearing colony using a modified 50 mL Falcon tube (cut at the bottom and covered with gauze) to minimize handling stress (Figure 1C). For each plant, 15 insects were transferred 24 hours prior to treatment application. This pre-treatment period allowed insect acclimatization and enabled the exclusion of individuals that might have died due to handling injuries.

Seven treatments were evaluated: (1) *P. lilacinum* (1×10^7 conidia mL^{-1}); (2) *P. lilacinum* (1×10^8 conidia mL^{-1}); (3) *P. lilacinum* (1×10^7 conidia mL^{-1}) + Perito® (acephate, 1000 g ha^{-1}); (4) *P. lilacinum* (1×10^8 conidia mL^{-1}) + Perito® (acephate, 1000 g ha^{-1}); (5) Perito® (acephate, 1000 g ha^{-1}); (6) *P. lilacinum* (1×10^8 conidia mL^{-1}) + adjuvant (TA 35 Gold Bio®, 150 mL ha^{-1}); (7) adjuvant (TA 35 Gold Bio®, 150 mL ha^{-1}); and a control consisting of sterile distilled water containing 0.01% (v/v) Tween 80®. The experiment followed a randomized complete block design, each treatment included five replicates, with each replicate consisting of one maize plant infested with 15 *D. maidis* adults. Treatments were applied using an airbrush sprayer, delivering a total volume of 160 µL of each suspension per plant, including the control (Figure 1D and E). Mortality assessments were conducted at 1, 18, and 24 hours after treatment application, and subsequently every 48 hours up to 432 hours post-application. Dead insects were carefully collected (Figure 1F) and placed in a humid chamber to confirm mortality due to fungal infection, using a Leica stereomicroscope (Figure 1G). At the end of the experimental period, all plants were frozen, and the total number of surviving insects was recorded to confirm the actual insect density per replicate.

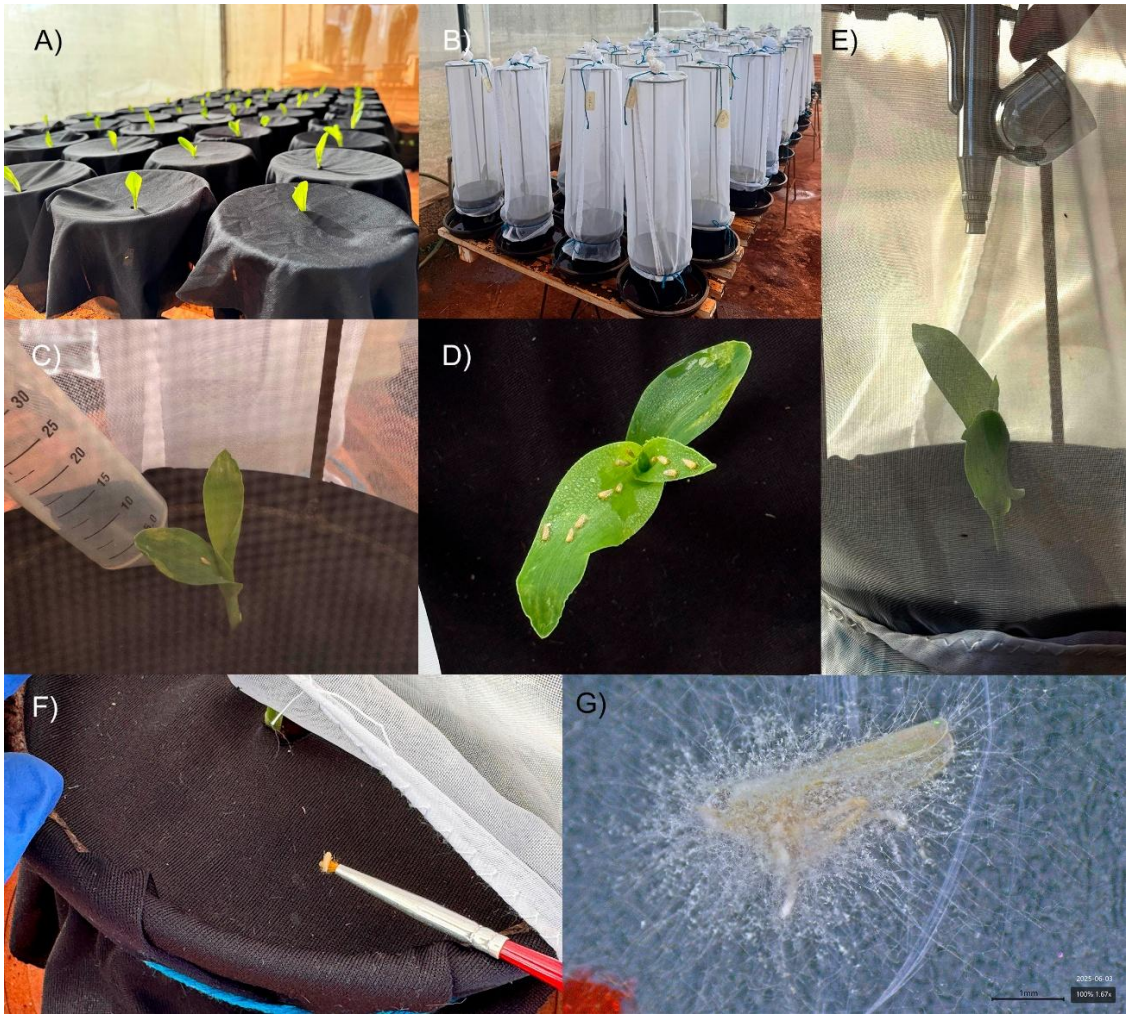

Figure 1. Experimental setup for the pathogenicity assay of *Purpureocillium lilacinum* against *Dalbulus maidis* under greenhouse conditions at Embrapa Arroz e Feijão. (A) Maize seedlings (BRS Caimbé) in pots with the base of the stem isolated using black mesh; (B) Cylindrical cages (15 cm × 30 cm) covered with gauze and placed over each plant; (C) Adult of *D. maidis* collected from the colony using modified 50 mL Falcon tubes; (D and E) Treatments and control were applied using an airbrush sprayer; (F) Collecting dead insects with soft brush; (G) Fungal infection confirmed using a stereomicroscope.

2.4. Pathogenicity on *Tenebrio molitor*

Pathogenicity assays of *P. lilacinum* against *T. molitor* from LPI were realized. Larvae of *T. molitor* were reared in opaque plastic boxes (38 × 28 × 12 cm) with ventilated lids and maintained at the insectary of the Institute of Tropical Pathology and Public Health (IPTSP) at room temperature (28 ± 3 °C), under a 12:12 h light:dark (L:D) photoperiod and 65 ± 10%

relative humidity. The diet was provided *ad libitum* and consisted of a mixture (1:1:1) of rabbit, quail and chicken feed (Supra®), all finely ground. Each container included a piece of egg carton (10 × 20 cm) as a shelter to allow emerging adults to hide and a piece of cabbage (10 × 10 cm) as a source of moisture. The substrate was replaced monthly, and the cabbage was renewed every two days.

Tenebrio molitor larvae of 100 to 140 mg were selected for the pathogenicity tests. The larvae were immersed for 30 seconds in 10 mL each of the fungal suspensions tested, which included three conidial concentrations (1.0×10^7, 1.0×10^8, and 1.0×10^9 conidia mL^{-1}), as well as in suspensions containing enzymes and metabolites (described in section 2.2). Control groups were immersed in sterile distilled water containing 0.01% (v/v) Tween 80®. Each treatment was conducted using five experimental units, Petri dishes (90 × 15 mm) with five larvae per unit, totaling 25 larvae per treatment. After treatment, the larvae were maintained at 27 ± 2 °C, 60% relative humidity, and a 12-hour photophase, and mortality was recorded daily until death or pupation and subsequent emergence. All assays were conducted in duplicate.

2.5. Pathogenicity to *Spodoptera frugiperda, Rachiplusia nu, Chrysodeixis includens* and *Helicoverpa armigera*

Pathogenicity assays were conducted with *P. lilacinum* against four lepidopteran species: *Spodoptera frugiperda*, *Rachiplusia nu*, *Chrysodeixis includens* and *Helicoverpa armigera*. Different fungal propagules were used: conidia, enzymes and metabolites. Neonate larvae (up to 24 hours after hatching) were used in all bioassays. Larvae were obtained from laboratory colonies maintained by AgBiTech.

For this assay, only the concentration of 1.0×10^7 conidia mL^{-1} was used, based on previous results with *T. molitor*. The treatments (conidia, enzymes, or metabolites) were applied topically to each larva of the different lepidopteran species, on sterile filter paper, with each larva receiving a 2 µL droplet directly on its dorsal surface. Control groups received sterile distilled water containing 0.01% (v/v) Tween 80®. After application, the larvae were carefully transferred to ELISA plates containing a modified Greene diet (without antibiotics) (Greene et al., 1976). Twenty-four neonate larvae were used per treatment for each caterpillar species. Mortality was recorded daily until death or pupation. All insects were maintained at 27 ± 2 °C, 60% relative humidity, and a 12-hour photophase. All assays were conducted completely randomized design in duplicate.

2.6. Statistical Analyses

To evaluate the effects of *P. lilacinum* treatments on insect survival, Kaplan–Meier survival curves were generated and compared using the log-rank test. Pairwise comparisons between treatment groups were performed with Bonferroni correction. The dependent variable was insect survival time (in days), and the independent variable was the treatment group (including conidial concentration, metabolites, enzymes, and combinations with adjuvant or insecticide). For *S. frugiperda*, *R. nu*, *C. includens* and *H. armigera*, the analysis included the 1×10^7 conidia mL^{-1} concentration, along with fungal metabolites and enzymes. For *T. molitor*, three conidial concentrations (1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}), metabolites, and enzymes were evaluated. For *D. maidis*, the analysis considered 1×10^7 and 1×10^8 conidia mL^{-1} concentrations, both alone and in combination with adjuvant and/or chemical insecticide, as well as the adjuvant and chemical alone. All statistical analyses were conducted in R (version 4.3.1) using the survival and survminer packages.

3. Results

3.1. Pathogenicity on *Dalbulus maidis*

Purpureocillium lilacinum alone or in combination with adjuvants did not significantly reduce *D. maidis* survival when compared to the control. Treatments with conidia at concentrations of 1×10^7 and 1×10^8 conidia mL^{-1} ($p = 0.121$ and $p = 0.886$, respectively), *P. lilacinum* (1×10^7 conidia mL^{-1}) + Perito® ($p = 0.608$), *P. lilacinum* (1×10^8 conidia mL^{-1}) + adjuvant ($p = 0.637$), and the adjuvant alone ($p = 0.328$) showed no significant pathogenicity and did not differ from the control (Figure 2).

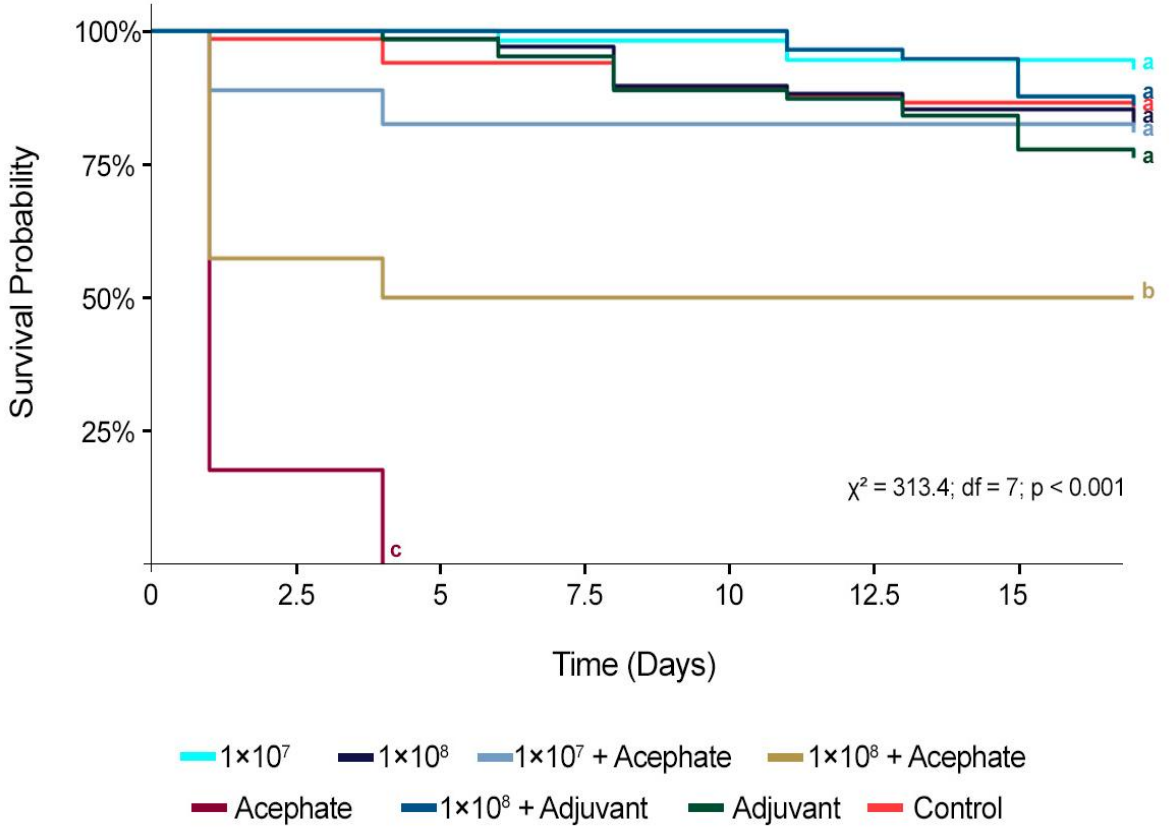

Figure 2. Survival curves of *Dalbulus maidis* adults (n ≈75) over an 18 day evaluation after application with: *P. lilacinum* (1 × 10^7 conidia mL^{-1}); *P. lilacinum* (1 × 10^8 conidia mL^{-1}); *P. lilacinum* (1 × 10^7 conidia mL^{-1}) + Perito® (acephate, 1000 g ha^{-1}); *P. lilacinum* (1 × 10^8 conidia mL^{-1}) + Perito® (acephate, 1000 g ha^{-1}); Perito® (acephate, 1000 g ha^{-1}); *P. lilacinum* (1 × 10^8 conidia mL^{-1}) + adjuvant (TA 35 Gold Bio®, 150 mL ha^{-1}); adjuvant (TA 35 Gold Bio®, 150 mL ha^{-1}); and a blank control. Different letters indicate statistically significant differences between treatments according to the log-rank test with Bonferroni correction ($p \leq 0.05$).

The most pronounced reduction in survival was caused by Perito® (acephate, 1000 g ha^{-1}) alone, reaching 100% mortality within 5 days after application. The combination of *P. lilacinum* at 1 × 10^8 conidia mL^{-1} with Perito® also significantly reduced survival, resulting in 50% mortality within the same period, and differed significantly from all other treatments (Figure 2).

3.2. Pathogenicity on *Tenebrio molitor*

Whether as conidia or its enzymes or metabolites, *P. lilacinum* had no pathogenic effects on *T. molitor* larvae. Survival curves showed similar patterns among treatments and the control group over the 14-day evaluation period ($\chi^2 = 7.38$; df = 5; p = 0.194) (Figure 3).

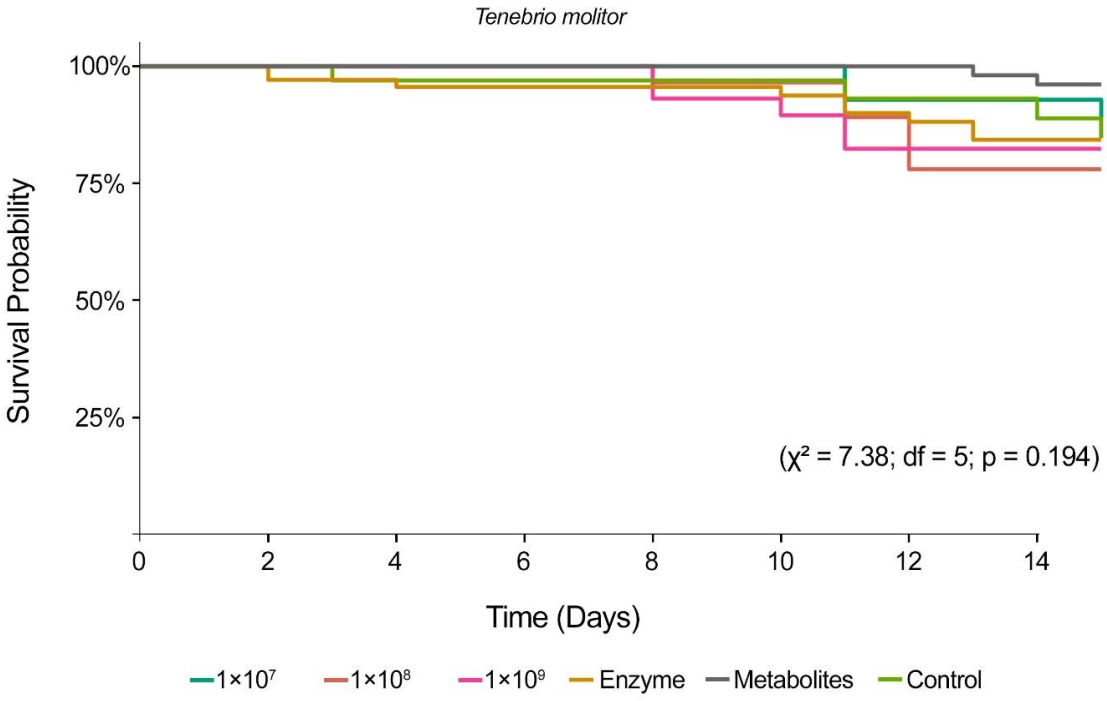

Figure 3: Survival curves of *Tenebrio molitor* larvae (n = 25) over 14 days after immersion for 10 seconds in 10 mL suspensions containing *Purpureocillium lilacinum* propagules (1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}), enzymes or metabolites.

3.3. Pathogenicity on *Spodoptera frugiperda, Rachiplusia nu, Chrysodeixis includens* and *Helicoverpa armigera*

Purpureocillium lilacinum had only marginal effects on the survival of *Spodoptera frugiperda* (Fig. 4A). Conidia (p = 0.042) and metabolite (p = 0.003) treatments presented significantly reduced survival compared to the control, with reductions of approximately 8% and 14%, respectively. However, no significant differences were observed among conidia (p = 0.860) and metabolites (p = 0.297) to enzyme treatments in this species (Figure 4A).

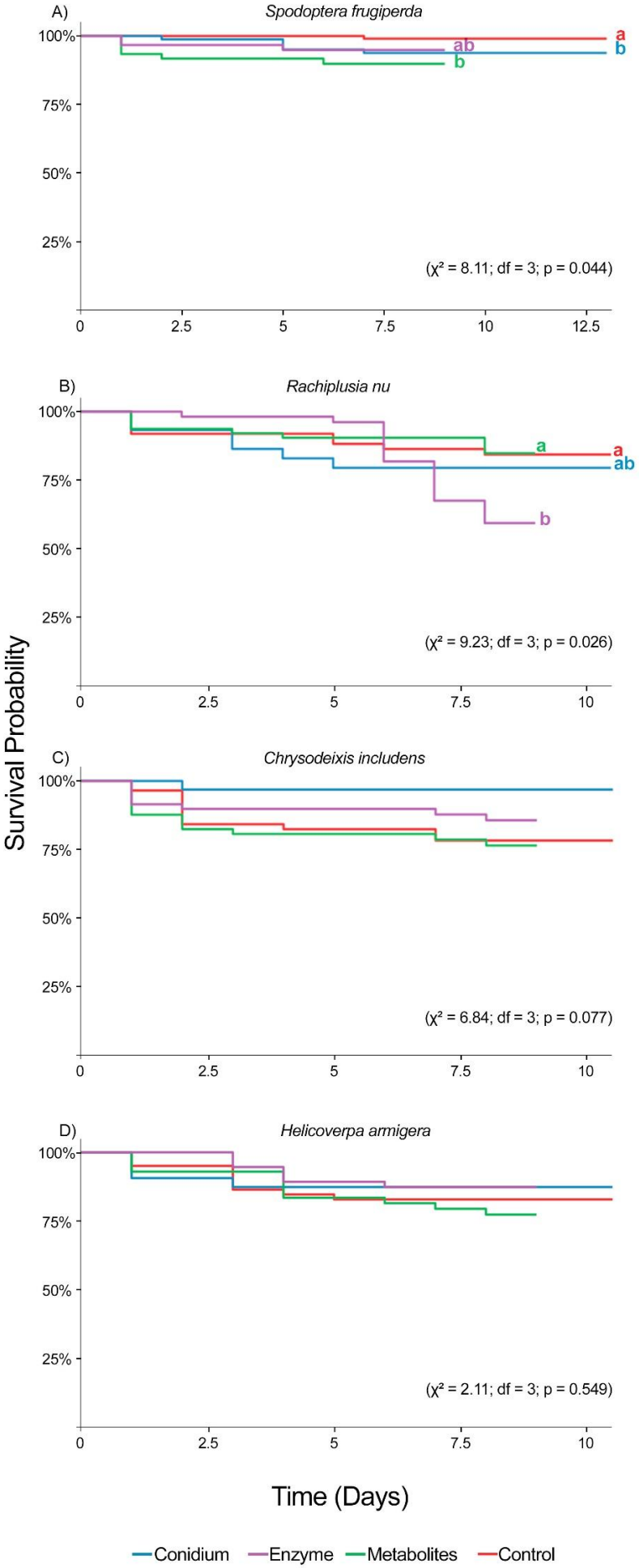
A)
Spodoptera frugiperda
100%
75%
50%
25%
a
ab
b
b
(χ² = 8.11; df = 3; p = 0.044)
0
2.5
5
7.5
10
12.5
B)
Rachiplusia nu
100%
75%
50%
25%
a
a
ab
b
(χ² = 9.23; df = 3; p = 0.026)
0
2.5
5
7.5
10
C)
Chrysodeixis includens
100%
75%
50%
25%
(χ² = 6.84; df = 3; p = 0.077)
0
2.5
5
7.5
10
D)
Helicoverpa armigera
100%
75%
50%
25%
(χ² = 2.11; df = 3; p = 0.549)
0
2.5
5
7.5
10
Survival Probability
Time (Days)
Conidium
Enzyme
Metabolites
Control

Figure 4: Survival curves of neonates of four lepidopteran species (n = 24 for each species): (A) *Spodoptera frugiperda*, (B) *Rachiplusia nu*, (C) *Chrysodeixis includens*, and (D) *Helicoverpa armigera*, over a 10-day evaluation period following topical application (2μL) of different forms of *Purpureocillium lilacinum* (conidia in 1×10^7 conidia.mL^{-1}, enzymes, and metabolites). Different letters indicate statistically significant differences between treatments according to the log-rank test with Bonferroni correction ($p \leq 0.05$).

Purpureocillium lilacinum conidia had no significant effect on the survival of *R. nu* ($p = 0.538$) (Figure 4B). The enzyme treatment resulted in a significantly lower survival rate compared to the control ($p = 0.018$), causing the highest mortality among all treatments and lepidopteran species tested, with an estimated reduction of approximately 33% in survival. However, no significant difference was detected between the enzyme and conidial treatments ($p = 0.196$).

Survival of *H. armigera* and *C. includens* was unaffected by *P. lilacinum*, whether as conidia, metabolites, or enzymes ($\chi^2 = 2.11$, df = 3, $p = 0.549$ and $\chi^2 = 6.84$, df = 3, $p = 0.077$, respectively; Figure 4C & D).

4. Discussion

Purpureocillium lilacinum, in the different forms tested in this study (conidia, metabolites and enzymes) was not pathogenic to the *D. maidis*, model organism *T. molitor* and noctuid species. However, conidia reduced the efficacy of chemical control (acephate) when co-applied in the corn leafhopper mortality assay.

Much has been discussed regarding the combination of chemical control with the application of entomopathogenic fungi for managing *D. maidis*. In this study, we observed that when *P. lilacinum* was combined with acephate, mortality was reduced by 50% compared to acephate application alone. The resistance of *Eurygaster integriceps* (Pentatomidae: Scutelleridae) was evaluated under treatment with a mixture of an organophosphate insecticide (acetylcholinesterase inhibitor) together with metabolites and conidia of *Beauveria bassiana* (Zibaee et al., 2009). The authors reported that fungal

infection and the application of its metabolites increased the activity of detoxifying enzymes in the insect, such as total esterases and glutathione S-transferase (GST). These enzymes help the insect neutralize or eliminate toxins, including pesticides, rendering them less susceptible to insecticide effects. The authors suggested that the combined use of entomopathogenic microorganisms should be planned carefully to avoid promoting cross-resistance.

The effect of the organophosphate insecticide malathion on wax moth (*Galleria mellonella*) larvae previously infected with *Metarhizium anisopliae* was evaluated. The larvae exhibited increased activity of glutathione S-transferases and total esterases in the hemolymph, along with greater tolerance to malathion, with an LD_{50} of 1.6 ± 0.5 mg/g compared to 1.1 ± 0.5 mg/g in non-infected insects, a statistically significant difference, suggesting that fungal infection can modulate the insect's response to chemical insecticides (Serebrov et al., 2006).

Populations of *D. maidis* from Brazil's main maize producing regions already exhibit reduced susceptibility to bifenthrin, acetamiprid, and imidacloprid (Machado et al., 2024). This reduced susceptibility may be related to the continuous use of chemical pesticides without rotation of active ingredients. On the other hand, the use of fungal biofungicides alongside these applications has been occurring in the field. Although studies on insect resistance to chemical insecticides applied in combination with or after treatment with entomopathogenic fungi and their metabolites have been reported for organophosphate groups (Serebrov et al., 2006; Zibaee et al., 2009), further testing is recommended to evaluate insect detoxification functions under EPF-chemical control integration, to preserve both the efficacy of biofungicides and chemical molecules and reduce the likelihood of resistance development.

To cause fungal infection, the EPF must adhere to the cuticle, germinate, and penetrate the integument (Téllez-Jurado et al., 2009). This initial step is crucial for the effectiveness of control. During penetration, the cuticle is degraded through mechanical pressure associated with various enzymes (Samson et al.,1988). The main enzymes described for *P. lilacinum* to degrade nematode eggshells are cuticle-degrading proteases and chitinases, which are also involved in the pathogenicity process of entomopathogenic fungi (Chen and Hu, 2021). Chitinases are essential enzymes that participate in insect molting by catalyzing the degradation of chitin, a major structural polysaccharide present

in both the cuticle and the peritrophic matrix (PM) of the midgut (Zhang et al., 2012). These enzymes hydrolyze the β-1,4-glycosidic linkages of chitin, facilitating the turnover of old cuticular and peritrophic structures during metamorphosis. Because the PM is a chitin-based, acellular barrier that protects the midgut epithelium and supports digestive compartmentalization, its integrity is critical for larval development and survival (Zhang et al., 2012; Zhu et al., 2016). Genomic sequencing of *P. lilacinum* and demonstrated that, in addition to producing carbohydrate-active enzymes (CAZymes), proteases, and pathogenesis-related genes, the fungus also produces secondary metabolites such as leucinostatins Wang et al. (2016). *P. lilacinum* can also synthesize paecilomide and acremoxanthones, which act as nematicidal compounds or reduce pest reproduction (Chen and Hu, 2021; Khan and Tanaka, 2023).

Application of conidia, metabolites, and enzymes to *T. molitor* was performed via immersion *Tenebrio molitor* can be infected by fungi through ingestion, and that fungal agents can reach the hemolymph and breach the gut barrier, leading to insect death. (Kim et al., 2018). Therefore, the immersion method employed in this study would allow both topical and ingestion-based action modes for *P. lilacinum*. However, we believe that *T. molitor* was not an appropriate model due to its thick, melanized cuticle, typical of Coleoptera. For this reason, a second test was conducted on Noctuidae species, this time using topical application on neonates.

Among the four noctuid species evaluated, only *Spodoptera frugiperda* and *Rachiplusia nu* showed significant reductions in survival after exposure to fungal treatments. Despite the statistically significant effect of conidia and metabolites on *S. frugiperda*, survival was only reduced by 10% and 15%, respectively, which can be considered low efficacy. However, topically applied enzymes on *R. nu* caused a 40% reduction in neonate survival.

The use of chitinases produced by *Penicillium ochrochloron* against *Helicoverpa armigera* was tested through topical application of the enzyme, which reduced pupation, increased larval mortality, and decreased adult emergence when isolated and concentrated via DEAE-cellulose ion exchange chromatography (Patil & Jadhav, 2015). For *Spodoptera littoralis*, oral application of crude protein extracts from *Metarhizium anisopliae* caused up to 100% mortality and induced severe damage to the larval midgut

epithelium, indicating that toxicity was mediated by heat- and protease-sensitive proteins (Quesada-Moraga et al., 2006).

It has been suggested that extracellular enzymes produced by entomopathogenic fungi, when combined with conidia, can reduce infection time in *S. frugiperda* larvae Ferreira et al (2024). Although the combination of enzymes and conidia of *P. lilacinum* was not tested in this study for the evaluated insect species, it is believed that this approach may be effective when applied to other isolates to shorten insect mortality time.

Purpureocillium lilacinum was not pathogenic when applied alone in the form of conidia, enzymes, or metabolites to *D. maidis*, *T. molitor*, *S. frugiperda*, *R. nu*, *C. includens* and *H. armigera*. However, we suggest that future tests should explore combined applications of these EPF forms, as previously reported in the literature. Additionally, ingestion-based mortality assays using enzymes appear promising and may offer an alternative strategy for insect pest management.

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FINAL CONSIDERATIONS

This work provides the first report on the *Purpureocillium lilacinum* isolate COAD 4015, highlighting its dual role as a biological control agent against root-knot nematodes (*Meloidogyne* spp.) and as a promoter of tomato (*Solanum lycopersicum*) plant growth. The isolate demonstrated complete suppression of egg production at all tested concentrations and significant reductions in the number of juveniles, confirming its nematicidal potential. Although no clear dose-dependent response was observed, suggesting possible intraspecific interactions at higher concentrations, the consistent reduction of nematode populations reinforces the efficacy of this fungus as a biocontrol agent.

In tomato plants, *P. lilacinum* COAD 4015 positively affected growth parameters, including plant height, number of leaves, shoot fresh weight, and root length, especially at the highest conidial concentration tested. These results indicate that this isolate can act as a biostimulant for *S. lycopersicum* under the conditions evaluated. Mechanisms such as the production of phytohormones (e.g., indole-3-acetic acid), phosphate solubilization, and improved nutrient uptake have been reported for other *P. lilacinum* strains, but specific assays are still required to confirm whether these processes are involved in the plant growth-promoting effects observed for the COAD 4015 isolate.

Incubation tests at 36 °C showed no fungal growth, indicating that the isolate is not able to grow at temperatures similar to those of the human body. This finding suggests low adaptation to mammalian conditions and that the isolate may be safe for agricultural use; however, further studies are required to determine its complete temperature tolerance profile and confirm its safety for large-scale applications.

Purpureocillium lilacinum COAD 4015 also demonstrated promising potential for use against the maize leafhopper *Dalbulus maidis* under field conditions. The incorporation of an oil-based adjuvant improved droplet formation and density, favoring the contact of fungal conidia with the target insect. The combination of *P. lilacinum* with the adjuvant resulted in early reductions of *D. maidis* populations and achieved the highest grain yield among treatments, like that obtained with the chemical insecticide tested. However, the pest's strong dispersal behavior likely limited the statistical detection of mortality, emphasizing the need for complementary experiments under

controlled environmental conditions to clarify the infection process and evaluate the persistence of fungal propagules on the insect cuticle.

The conidia, enzymes, and metabolites of *Purpureocillium lilacinum* COAD 4015 against *D. maidis*, four lepidopteran species (*Spodoptera frugiperda*, *Rachiplusia nu*, *Chrysodeixis includens*, and *Helicoverpa armigera*), and the model organism *Tenebrio molitor* revealed low pathogenicity under the tested conditions. Among the treatments, only the enzymatic fraction significantly reduced the survival of *R. nu* larvae (40%), suggesting that extracellular enzymes produced by *P. lilacinum* may play a role in insect pathogenesis.

Although the fungal conidia and metabolites did not induce significant mortality, the results indicate that the combined or sequential use of conidia, enzymes, and metabolites could enhance the pathogenic process, as synergistic effects have been reported for other entomopathogenic fungi. The co-application of *P. lilacinum* conidia with the chemical insecticide acephate reduced insect mortality by approximately 50% compared with the insecticide alone, suggesting a possible biochemical interaction that may involve the induction of detoxification enzymes in the insect host. This finding emphasizes the importance of evaluating biochemical compatibility before integrating *P. lilacinum* with chemical control strategies, to avoid potential cross-resistance or antagonistic effects.