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Selected isolates of endophytic *Trichoderma* decrease cutting by *Acromyrmex subterraneus subterraneus*

Pablo Fernandes Braga
Magister Scientiae

VIÇOSA - MINAS GERAIS
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Dissertation submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Simon Luke Elliot

Co-adviser: Thairine Mendes Pereira

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ABSTRACT

BRAGA, Pablo Fernandes, M.Sc., Universidade Federal de Viçosa, February, 2025. **Selected isolates of endophytic *Trichoderma* decrease cutting by *Acromyrmex subterraneus subterraneus***. Adviser: Simon Luke Elliot. Co-adviser: Thairine Mendes Pereira.

Endophytic fungi can colonize plant tissues, generally without causing harm to the host, and can cause a series of modifications in the host plants. These changes range from metabolic changes to morphological changes, leading to an optimization in the absorption of nutrients and resistance to stress, better development and protection against pests and diseases. The use of endophytic microorganisms has been gaining more and more prominence as bioinputs, whether to promote plant growth or protect plants against diseases and insect pests. Here, I initially present basic information on the topics related to my study, with emphasis mainly on endophytic fungi and their relationship with insects, especially leaf-cutting ants. I briefly provide an overview of the metabolic and morphological changes caused by endophytic fungi. Subsequently, I focus on the changes generated by fungi of the order Hypocreales and their influence on the plant-insect relationship. I then present the objects of study, leaf-cutting ants and the endophytic fungus Trichoderma. For this study, we initially used a screening process with 23 isolates of the endophytic fungus Trichoderma, looking for those with greater versatility in the use of different substrates, better growth in artificial medium, and greater spore production on rice substrates. Three isolates were selected in this screening. We investigated the methods of applying the fungus (foliar, root and both) with isolate TR71 and found that foliar application was the most effective, promoting greater plant growth and the fungus was recovered in all parts (leaf, stem and root). We investigated the colonization and growth promotion capacity of these three isolates in seedlings of a commercial eucalyptus hybrid (Eucalyptus urograndis). The tested isolates did not affect, in general, the development of the plant (number of leaves, height and roots). One isolate (TR71) did increase the number of root tips, however. The isolates were recovered from all inoculated plants, in all parts of the plants (leaf, stem and root), for the three isolates. Finally, we tested two endophytic isolates of Trichoderma for reduction of leaf cutting (leaf area removed by leaf-cutting ants). Both isolates reduced ant cutting on infected plants when offered with uninfected plants. We conclude that, while the tested isolates did not demonstrate efficacy in promoting plant growth, they did prove to be efficient as “bodyguards", protecting the plants from the leaf-cutting ants.

Trichoderma has demonstrated potential as a possible biological control agent for leaf-cutting ants, to reduce damage to eucalyptus.

Keywords: Endophytic Microorganisms, Endophytic Fungi, Plant-insect relationship, Leaf-cutting ants

RESUMO

BRAGA, Pablo Fernandes, M.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Isolados selecionados de *Trichoderma* endofítico diminuem o corte por *Acromyrmex subterraneus subterraneus***. Orientador: Simon Luke Elliot. Coorientadora: Thairine Mendes Pereira.

Os fungos endofíticos podem colonizar tecidos vegetais, geralmente sem causar danos ao hospedeiro, e podem causar uma série de modificações nas plantas hospedeiras. Essas alterações vão desde alterações metabólicas até alterações morfológicas, levando a uma otimização na absorção de nutrientes e resistência ao estresse, melhor desenvolvimento e proteção contra pragas e doenças. O uso de microrganismos endofíticos vem ganhando cada vez mais destaque como bioinsumos, seja para promover o crescimento vegetal ou proteger as plantas contra doenças e pragas de insetos. Aqui, inicialmente apresento informações básicas sobre os tópicos relacionados ao meu estudo, com ênfase principalmente nos fungos endofíticos e sua relação com insetos, especialmente formigas cortadeiras. Forneço brevemente uma visão geral das alterações metabólicas e morfológicas causadas pelos fungos endofíticos. Posteriormente, foco nas alterações geradas pelos fungos da ordem Hypocreales e sua influência na relação planta-inseto. Em seguida, apresento o objeto de estudo, formigas cortadeiras e o fungo endofítico Trichoderma. Para este estudo, inicialmente utilizamos um processo de triagem com 23 isolados do fungo endofítico Trichoderma, buscando aqueles com maior versatilidade no uso de diferentes substratos, melhor crescimento em meio artificial e maior produção de esporos em substratos de arroz. Três isolados foram selecionados nesta triagem. Investigamos os métodos de aplicação do fungo (foliar, radicular e simultâneo) com o isolado TR71 e verificamos que a aplicação foliar foi a mais eficaz, promovendo maior crescimento da planta e o fungo foi recuperado em todas as partes (folha, caule e raiz). Investigamos a capacidade de colonização e promoção de crescimento desses três isolados em mudas de um híbrido comercial de eucalipto (Eucalyptus urograndis). Os isolados testados não afetaram, de forma geral, o desenvolvimento da planta (número de folhas, altura e raízes). No entanto, um isolado (TR71) aumentou o número de pontas de raízes. Os isolados foram recuperados de todas as plantas inoculadas, em todas as partes das plantas (folha, caule e raiz), para os três isolados. Finalmente, testamos dois isolados endofíticos de Trichoderma para redução do corte de folhas (área foliar removida por formigas cortadeiras). Ambos os isolados reduziram o corte de formigas em plantas infectadas quando oferecidos com plantas não

infectadas. Concluímos que, embora os isolados testados não tenham demonstrado eficácia na promoção do crescimento das plantas, eles provaram ser eficientes como "guarda-costas", protegendo as plantas das formigas cortadeiras. Trichoderma demonstrou potencial como um possível agente de controle biológico para formigas cortadeiras, para reduzir danos ao eucalipto.

Palavras-chave: Microrganismos endofíticos, Fungos endofíticos, Relação planta-inseto, Formigas cortadeiras.

Summary

1 - General introduction

1.1 – What is an endophytic microorganism?

There is a great diversity of microorganisms that form endophytic associations with plants, being able to colonize and establish themselves inside plants without causing harm to the host (Caruso et al., 2022; Ji et al., 2022). These interactions can be beneficial, neutral or, in rare cases, harmful to the plant. In most cases, though, endophytic microorganisms provide benefits to the host (Schulz & Boyle, 2005). Among the main groups of microorganisms with endophytic potential, bacteria, fungi and viruses stand out, each with their own particularities and impacts on the health and growth of the plant.

Bacteria, mainly those known as PGPR (Plant Growth-Promoting Rhizobacteria), are well known due to their high efficiency in promoting root growth and colonization (Kumar et al., 2012; Shultana et al., 2022). They are effective in nitrogen fixation, in the degradation of toxic substances and in the production of plant hormones such as auxins, which stimulate root growth (Freire et al., 2020). They can also protect plants against pathogens, competing for nutrients or producing antimicrobial substances. Due to these characteristics, they are widely used for agricultural applications as biofertilizers and for disease control (Recep et al., 2009).

Endophytic viruses, on the other hand, are commonly thought of as phytopathogens. However, although diseases caused by viruses are widely documented, recent studies indicate that endophytic viruses can play beneficial roles, such as increasing the plant's resistance to other diseases or regulating the plant microbiome (Aghdam et al., 2023).

Endophytic fungi have a very ancient relationship with plants, with records of this interaction dating back around 400 million years ago (Krings et al., 2007). Endophytic fungi colonize plant tissues without causing apparent damage to the host, and can colonize all organs, from roots and stems to leaves, flowers and fruits (Rodriguez et al., 2009). These fungi can be divided into biotrophic and necrotrophic, the former being those that grow without causing damage to cells, while the latter are those that do cause damage to the cell (Eberl et al., 2019; Glazebrook, 2005). Endophytic fungi are naturally present

in all plants and play a fundamental role in them, helping with resistance to stress (heat, cold, pH, etc.) (Verma et al., 2022), and assimilation of nutrients (Behie et al., 2017; Chaudhary et al., 2022), in addition to providing other benefits such as protection against herbivory (Raman et al., 2012; Raman & Suryanarayanan, 2017; Tack et al., 2012).

The diversity of functions performed by endophytic fungi is vast and complex. They can assist not only in plant nutrition and protection but can also play a crucial role in the adaptation of plants to challenging environments (Freire et al., 2020; Khan et al., 2022; Verma et al., 2022). Another role that has been gaining increasing prominence is their use as biopesticides, helping to control diseases caused by phytopathogens, but also helping to control insect pests (Steyaert et al., 2003; Jaber & Ownley, 2018). For this, endophytic fungi can act directly on the target insect: the consumption of fungal structures present in plant tissues can lead to infection and death of the insect, since the fungus can colonize it after ingestion. Meanwhile, the production of compounds induced by the fungi can have a repellent action or alter the palatability of the plant (Dara et al., 2019; Aravinthraju et al., 2024). In the following text, emphasis will be placed exclusively on the role of fungi as plant endophytes and their influence on the host and on insect-plant interactions.

1.2 – Endophytic fungi

1.2.1 Infection process

The first step to be considered for infection is the ability of fungal spores to attach themselves to the surface of the plant, in addition to having the ability to overcome the physical and microbiological barriers present on the surface (Van Bael et al., 2017). To do this, fungi produce a series of structures and compounds to facilitate this process, such as adhesion hyphae and appressoria (Deising et al., 2000; Rodriguez et al., 2009; Shi et al., 2023). Effector compounds, responsible for assisting in the penetration process, include enzymes such as pectinases, cellulases and chemical compounds such as glycoproteins that play a fundamental role in the permanence and fixation of the fungus, degrading the cell wall and waxy layer (González-Teuber et al., 2021; Mengistu, 2020). The efficiency of adhesion is also a very important aspect to consider for successful infection (González-Teuber et al., 2021). There are several factors that can influence the

adhesion process, including abiotic factors such as temperature and humidity. There are also biotic factors, such as microbiota present in the leaf, which make it necessary to produce compounds that help in competition for space and nutrients(Stone et al., 2004; Mengistu, 2020).

After overcoming the first difficulties imposed on the fungus regarding its adhesion to the plants, they need to overcome the cell wall and cuticle (Schulz & Boyle, 2005). Therefore, fungi have a series of adaptations that help them overcome these physical barriers, such as their own structures and effector compounds (Caruso et al., 2022; Jha et al., 2023). One of these structures is the appressorium, a specialized structure for attaching fungal cells to the host and assisting in the invasion of plant tissues, in the case of plants. It exerts strong pressure on the cell wall, together with the release of effector compounds that will subsequently help penetrate and repair the tissue (Deising et al., 2000; Shi et al., 2023). The main penetration structure of fungi is the penetration peg, a structure produced from the appressorium, which exerts pressure on plant tissues to rupture the plant tissue and penetrate the plant (Deising et al., 2000; Shi et al., 2023). However, the pressure exerted by the appressorium alone is not sufficient for successful infection, and at this point the effector compounds act to facilitate this process (Ji et al., 2022; Li et al., 2024). These effectors are specific enzymes such as cutinases, cellulases, lignases and pectinases, which degrade the cuticle and cell wall components at the appressorium attachment site and subsequently facilitate the rupture of the plant tissue by the penetration peg (Li et al., 2024). Infections may also occur in areas of the plant that are already vulnerable, such as wounds or stomata, facilitating the success of the infection (Huang et al., 2018).

After penetration, the fungus faces further defense mechanisms that may hinder its colonization of the host (Glazebrook, 2005). However, fungi have biochemical adaptations that allow them to tolerate plant defenses. In addition, many microorganisms have the ability to modulate plant gene expression; one mechanism that is often observed is the regulation of the production of antimicrobial compounds or other plant defense mechanisms (Yuan et al., 2019; Batool et al., 2024). After overcoming the plants' defenses, the fungi begin to colonize the plant tissues, and they can disperse uniformly

throughout the intracellular spaces of the plants or in a localized manner in a plant organ (Mengistu, 2020; González-Teuber et al., 2021). Once the defense mechanisms are overcome, the fungus colonizes and establishes itself in the plant (Mengistu, 2020; Batool et al., 2024). In some cases, the fungus produces a structure during colonization called the haustorium, which develops inside the host cells (Spencer-Phillips, 1997). From within these cells, the haustorium allows the exchange of nutrients whereby the fungus absorbs carbohydrates from the plant host and the plant receives water and minerals from the fungi. (Spencer-Phillips, 1997). However, the dynamics of colonization present a great diversity of variations that will depend on the species of fungus or plant involved in this interaction(Mengistu, 2020; González-Teuber et al., 2021).

1.2.2 Metabolic exchanges

The fungus-plant interaction is dynamic, and endophytes can grow and adjust their metabolic activities to increase their gains from the interaction (Liao et al., 2022; Oliver & Ipcho, 2004). In the case of biotrophs, they may perform metabolic exchanges from which both partners benefit (Glazebrook, 2005). Biotrophs provide bioactive compounds to plants, in addition to increasing vegetative growth, as well as enhancing nutrient solubilization and water absorption, promoting healthier and better growth for the host (Liao et al., 2022). In return, fungi capture carbohydrates and nutrients from the inter- or intracellular environment of plants for their development (Liao et al., 2022). Meanwhile, necrotrophs produce structures or enzymes that lead to the rupture of host cells, leading to damage to plant tissue (Glazebrook, 2005). Through this cell lysis, necrotrophs absorb carbohydrates and other nutrients from plants, benefiting their growth (Liao et al., 2022) . However, metabolic exchanges are not limited to nutritional exchanges between the organisms involved but can also involve secondary metabolites (Zhi-Lin et al., 2007; Keshri et al., 2021; Jha et al., 2023). The secondary metabolites produced by fungi help, for example, to increase the plant's resistance to biotic stresses (such as pathogens and herbivores) and abiotic stresses (temperature, humidity etc.), in addition to promoting vegetative growth (Lee et al., 2021; Khan et al., 2022; Anand et al., 2023). Fungi of the genus *Trichoderma*, for example, are biotrophic and widely recognized to promote plant

growth; this is linked to the production of indole acetic acid (IAA) and other phytohormones that increase nutrient availability (Tseng et al., 2020).

1.2.3 Morphological changes

Endophytes altering metabolic and nutritional pathways in plants can also lead to differentiation in the plants' morphological patterns. Among these changes, increased root growth stands out, often observed in interactions with mycorrhizal fungi (Vannette & Rasmann, 2012). These fungi favor the formation of longer and more branched roots, which increases the plant's ability to explore the soil in search of water and nutrients. For example, in wheat crops (*Triticum aestivum*), inoculation with mycorrhizal fungi increased root volume by up to 30%, directly leading to greater tolerance to water stress (Beltrano et al., 2008). Other biometric patterns of plants can also be altered, leading to a greater number of branches, more abundant formation of shoots, and an increase in leaf area. *Trichoderma harzianum* infecting tomato seedlings (*Solanum lycopersicum*) endophytically increases leaf thickness and trichome density (Azarmi et al., 2011). These structures play a fundamental role in defense against herbivores. In addition, anatomical changes occur, resulting in greater efficiency in the transport of water and nutrients, so improving the performance of the plant in adverse conditions (Stone et al., 2004; Schulz & Boyle, 2005). Endophytic fungi can also increase the reproductive efficiency of plants, leading to an increase in the flowering rate (Rodriguez et al., 2009). The consequent increase in the number of flowers is linked to a greater number of fruits, in addition to helping with seed viability (Rodriguez et al., 2009).

1.3 – Hypocreales fungi as endophytes

Hypocreales is an order of fungi within the Ascomycota. This group of fungi is characterized by the formation of brightly colored fruiting bodies and spore-producing structures in the form of ascomata or perithecia. This group contains most of the fungi that are used as bioinputs, such as *Cordyceps* (= *Isaria*), *Trichoderma*, *Metarhizium* and *Beauveria*, among others (Mantzoukas & Eliopoulos, 2020). They include

entomopathogenic fungi, which can play a role in regulating insect populations naturally, and mycoparasites. These microorganisms are widely used as biological control agents in agriculture (Bamisile et al., 2021). However, these microorganisms are not only entomopathogenic (*Cordyceps, Beauveria, Metarhizium*) or mycoparasitic (*Trichoderma*). Many of them can also act as endophytes, establishing themselves within plant tissues without causing harm to their hosts (Ahsan et al., 2024; Moonjely et al., 2016). Among the genera in the group, the most relevant that act as endophytes are the genera *Metarhizium, Trichoderma, Beauveria* and *Cordyceps* (Mantzoukas & Eliopoulos, 2020). These fungi have been shown to have the ability to colonize a wide variety of plant tissues, even leading to changes in the production of antimicrobial compounds and in systemic plant resistance pathways (Aravinthraju et al., 2024; Dara, 2019; Geremew et al., 2024). In addition to metabolic changes, they also cause changes in cell wall thickening and vegetative growth (Russo et al., 2021). An example is the fungus *Metarhizium anisopliae*, which has been shown to be efficient in colonizing roots, thereby improving plant development, as well as helping to protect against insects in the soil, even capturing nitrogen from insects for the plant host (Behie & Bidochka, 2013; Behie & Bidochka, 2014).

The association of endophytic fungi with plants leads to direct and indirect effects on insects, influencing the dynamics of insect-plant interactions. (Rasool et al., 2021). An example is *Trichoderma*, which can be antagonists of the basidiomycete fungus that constitutes the garden of leaf-cutting ants (Nascimento et al., 2017; Rocha et al., 2017).

1.3.1 Changes caused by entomopathogenic fungi in plants

Entomopathogens are fungi with a widely recognized direct action on insects, playing a fundamental role in the biological control of pests. The objective of this section is to provide an introductory overview of these fungi, widely studied for their interaction with insects, particularly regarding their role as endophytes in plants. Through this analysis, we intend to highlight the most investigated fungi, elucidating their effects on insect-plant interactions, before addressing other fungi less explored in this context, but with high potential for biological control.

Colonization by fungi of the order Hypocreales, with a greater focus on entomopathogens, benefits plants in several ways, especially when these fungi establish themselves as endophytes, causing both nutritional and morphological changes. In addition to these changes, Hypocreales fungi play a significant role in modulating plant-insect interactions. They can induce the production of volatile organic compounds (VOCs) by plants, which help to inhibit herbivory. Compounds such as methyl jasmonate and 1-octen-3-ol are known for their insect repellent properties (Wasternack & Hause, 2013; Camele et al., 2023; Russo et al., 2023), while other VOCs attract natural enemies of these insects, contributing to the biological control of herbivore populations (Aravinthraju et al., 2024; Hartley & Gange, 2009). Furthermore, entomopathogenic endophytic fungi stimulate the production of non-volatile secondary metabolites in plants, such as alkaloids and terpenes, which have a toxic and repellent effect against herbivorous insects. Limonene, a terpene produced by some fungi, for example, has properties that cause disorientation and loss of appetite in insects (Galindo-Solís & Fernández, 2022). Fungi also promote the production of phenols, antioxidant compounds that hinder the digestion of plant tissues, reducing the feeding efficiency of herbivores and impairing their development (Singh et al., 2021).

Furthermore, these fungi act by regulating important hormonal factors in plants, such as jasmonic acid (JA), salicylic acid (SA), and ethylene, which play central roles in defense responses against insects. Jasmonic acid, for example, activates genes that produce toxic compounds and defense proteins against chewing insects, while salicylic acid helps strengthen the cell wall and increase resistance against pathogens and sucking insects (Wasternack & Hause, 2013; Zhang et al., 2020). Ethylene coordinates these responses and can make plants less palatable, helping to reduce herbivory (Alatalo et al., 2008; Lu et al., 2014). Therefore, the interaction between fungi of the order Hypocreales and plants has great potential to control herbivory and reduce insect damage (Eberl et al., 2019; Mantzoukas & Eliopoulos, 2020).

Entomopathogenic fungi also act directly on insects. Compounds such as destruxin (produced by *Metarhizium anisopliae*) (Wang et al., 2012) and beauvericin (produced by *Beauveria bassiana)* (Gupta et al., 1995),have toxic action against insects,

inhibiting their feeding and reproduction (Sree et al., 2008; Khoury et al., 2019). In addition, fungi can induce the production of compounds in plants that affect the palatability of leaves, making them bitter and less attractive to herbivores, in addition to altering the nutritional balance of leaves, reducing the attractiveness of plants (Hartley & Gange, 2009; Mantzoukas & Eliopoulos, 2020). They also stimulate the production of antidigestive compounds, such as proteases and chitinases, which make it difficult for herbivorous insects to digest plant tissue, leading to a reduction in attacks on plants over time (Fadiji & Babalola, 2020; Ji et al., 2022). In addition to changes in metabolism, entomopathogenic fungi can modify leaf morphology, making them less attractive to insects.

These modifications include an increase in the number of trichomes, making it difficult for insects to access the leaf blade, an increase in leaf thickness, making it difficult to pierce or color the leaves, and changes in leaf texture, mediated by changes in the deposition of cuticular waxes (Biere & Bennett, 2013; Qin et al., 2021). Fungal colonization by entomopathogens in an endophytic manner is not limited to indirect herbivory (through changes in plants), but can have direct effects on insects. When insects consume leaves infected with entomopathogenic fungi, they can be infected and killed by the fungi. In addition, these fungi can directly infect the larvae and nymphs of herbivorous insects when consumed by them (Geremew et al., 2024; Moonjely et al., 2016; Vinha et al., 2022). A classic example is the fungus *Beauveria bassiana*, which is known to infect and kill several insect species after ingestion (Klieber & Reineke, 2016; Qin et al., 2021). Volatile compounds, such as methyl jasmonate and salicylic acid, can make the plant repulsive to insects, reducing oviposition and interfering with the development of larvae when they consume contaminated tissues (Russo et al., 2021; Samal et al., 2023).

1.4 – Leaf-cutting ants

Leaf-cutting ants are insects belonging to the Attini tribe, which stand out for their cultivation of symbiotic fungi used as food. Among the genera that make up this tribe, the main ones that cut leaves for cultivating the fungus are *Acromyrmex* and *Atta*, which comprise the best-known and most studied species. They cultivate the fungus *Leucoagaricus gongylophorus* inside the nest in separate chambers, where it grows and

serves as food for the larvae and the queen, establishing an extremely intimate and obligatory relationship between the two organisms (Fisher et al., 1994; Bizarria et al., 2018). Although they are mainly known for their behavior of cutting and carrying leaves to the nest, they do not feed directly on this collected plant material. Instead, the leaves are used to grow the fungus. In natural ecosystems, these ants have great importance linked mainly to the cycling of organic matter, seed dispersal and dispersal of microorganisms (Folgarait, 1998; Montoya-Lerma et al., 2012) However, despite playing important ecological roles, leaf-cutting ants are also important agricultural and forestry pests. They attack a wide variety of crops, causing damage and economic losses, which is also observed in forestry, principally pine and eucalyptus.

1.4.1 Leaf-cutting ants as forestry pests

Leaf-cutting ants are widely distributed in tropical regions, where they represent one of the main agricultural pests (Montoya-Lerma et al., 2012, Elizalde et al., 2016; Sabattini & Bollazzi, 2023), attacking various crops such as sugar cane, citrus and pastures. They are also highly relevant as forest pests due to their behavior of cutting and transporting large quantities of leaves, which can result in substantial losses in forestry, especially in eucalyptus and pine (Elizalde et al., 2016). The impact of this can be very significant, with the loss of a large photosynthetic area of the plants. The high level of defoliation caused by the ants' cutting leads to a significant decrease in the attacked crops, resulting in the impairment of the plant's growth and development, especially in forestry (Crumière et al., 2021; Elizalde et al., 2016; Sabattini & Bollazzi, 2023).

In addition to direct damage, leaf-cutting ants can also increase the vulnerability of trees to other pests and diseases. Removing leaves exposes plants to stress, making them more susceptible to attacks by pathogens and other insect species that take advantage of the weakened plants (Zanetti et al., 2014). This cycle of impacts harms the overall health of the plantations and compromises their long-term sustainability.

1.4.2 Management and control of leaf-cutting ants

Managing leaf-cutting ants is considered a major challenge, given their complex behavioral patterns and the high level of inspection and control of all material deposited in the colony (Della Lucia et al., 2014; Montoya-Lerma et al., 2012). Traditionally, the most used management methods to control leaf-cutting ant attacks or to kill colonies are chemical control methods, such as pesticides and toxic baits (Montoya-Lerma et al., 2012; Zanetti et al., 2014; Poiani et al., 2023). Chemical management with pesticides has proven to be quite efficient in controlling these pests; however, the use of chemical control approaches brings with it a series of problems associated with the use of these techniques.

Pesticides contaminate water, soil and the environment, as well as posing risks to human health, in addition to the increasing resistance of insects to the compounds used over time (MacIntosh, 2010). This can generate major negative impacts on the environment and reduce the effectiveness of these interventions over time.(Underwood & Caputo, 1982, Burger, 2008; Kliucininkas & Velykiene, 2009; Molander et al., 2012). Additionally, the high-intensity and indiscriminate use of pesticides harms non-target organisms and can lead to losses to local biodiversity (Underwood & Caputo, 1982; Burger, 2008;). Therefore, it is imperative to seek more sustainable and long-term alternatives for the management of leaf-cutting ants.

In this context, biological control stands out as a promising strategy. Biological control involves using natural organisms, such as pathogens or predators, to reduce insect populations, without causing significant environmental damage (Headrick & Goeden, 2001). Widely known examples of biological control are the use of entomopathogenic fungi of the genera *Metarhizium* and *Beauveria*, (Espinosa et al., 2019, Elkhateeb et al., 2021; El-Maraghy et al., 2023), which are used for a wide variety of pests (Kim et al., 2013; Espinosa et al., 2019; Ramos et al., 2024). As for leaf-cutting ants, new research is increasingly being carried out aiming at the use of microorganisms or bioactive and natural extracts for their control. The development of new biological control methods is crucial to minimize the negative impacts of chemical treatments and ensure the sustainability of leaf-cutting ant management. Best practices for pest control contribute to

the balance between crop production and environmental preservation (Baron & Rigobelo, 2022).

1.5 – The endophytic fungus *Trichoderma*

Trichoderma is a fungus belonging to the Ascomycota group, a mycoparasite that can establish a symbiotic relationship with plants, establishing itself as endophytes (Rubini et al., 2005). As endophytes, these fungi contribute to plant growth, promoting greater nutrient absorption, increased resistance to stress, and stimulating the production of secondary compounds (Contreras-Cornejo et al., 2024; Hermosa et al., 2013; Zaidi et al., 2014). *Trichoderma* is a highly effective mycoparasite (Steyaert et al., 2003; Mukherjee et al., 2022), and is widely used in agriculture due to its ability to inhibit and control phytopathogens (Harman, 2006; Tseng et al., 2020). *Trichoderma asperellum,* for example, has demonstrated great efficacy in protecting plants, such as cocoa, against diseases (Rosmana et al., 2015). Furthermore, these fungi have great potential in biological pest control (Bailey et al., 2008).

The antagonistic relationship between this genus of fungus and that cultivated by leaf-cutting ants (Rocha et al., 2017), and its endophytic capacity, opens the door to studies of control strategies for these insects.

1.5.1 *Trichoderma* and plant resistance to pests

The use of endophytic fungi for pest control has gained prominence due to the multiple benefits that these organisms can provide, such as optimization of plant development, better nutrition and pest control (Bogas et al., 2024; Natsiopoulos et al., 2024; Rolón et al., 2024). Fungi such as *Trichoderma* can significantly alter insect-plant interactions through changes in the plant that protect it against herbivory (Gange et al., 2012; Natsiopoulos et al., 2024). Modulation of the production of secondary metabolites by fungi, such as tannins and other phenolic compounds, reduces the palatability and digestibility of plant material for insects (Gange et al., 2012). A practical example is observed in corn crops (*Zea mays*), where plants colonized by *Trichoderma atroviride*

present lower rates of damage from the caterpillar *Spodoptera frugiperda* (Contreras-Cornejo et al., 2018). Fungi induce plants to produce more phytoalexins, antimicrobial and antiherbivore compounds, increasing resistance (Agbessenou et al., 2022; Batool et al., 2022). They can also change the plant's nutritional profile, making it less attractive to insects and more difficult for pests to develop (Gange et al., 2012).

Fungi can influence the systemic acquired resistance (SAR) response that is important for this insect-plant interaction and can induce a more robust defense response in plants, increasing the production of defense-related proteins and hormones such as jasmonic acid (Agbessenou et al., 2022; Natsiopoulos et al., 2024), that are effective in combating attacks by herbivorous insects. In tomato crops (*Solanum lycopersicum*), for example, plants treated with *Trichoderma* showed greater resistance to attack by whitefly (*Bemisia tabaci*) (Jafarbeigi et al., 2020), resulting in lower incidence of the pest and higher productivity.

1.5.2 *Trichoderma* and leaf-cutting ants

Fungi such as *Trichoderma* have great potential to protect plants against attacks by leaf-cutting ants in plantations (Rocha et al., 2017; Batista et al., 2022; Bittleston et al.,2011). The natural diversity of endophytic fungi in plants is considerable (Arnold, 2000) and a great diversity of fungi is also observed in plant materials discarded by leaf-cutting ants (Coblentz & Van Bael, 2013; Rocha et al., 2014). Among the fungi recovered from these leaves are those with the potential to cause damage to the fungus garden cultivated by leaf-cutting ants. *Trichoderma* is frequently found on leaves rejected by colonies in different nests of leaf-cutting ants, but less so in leaves carried by them. This indicates that the ants initially do not recognize these fungi but may have quality control that leads to rejection and reduced cutting of plants with potential harmful organisms. This strategy can be used to mitigate damage (Rocha et al., 2014). This suggests that ants may recognize materials containing microorganisms that are potentially harmful to their gardens, and discard them (Coblentz & Van Bael, 2013; Rocha et al., 2017; Goes et al., 2020). Leaf-cutting ants tend to attack more plants that have fewer endophytic microorganisms on their leaves (Coblentz & Van Bael, 2013; Aponte Rolón et al., 2024).

Furthermore, in vegetation with many associated endophytes, there is a higher processing rate by ants, since they spend more time cleaning and checking the material. (Van Bael et al., 2012).

The interaction between the fungus *Trichoderma*, plants and the leaf-cutting ants constitutes a tripartite interaction, where the fungus can be harmful to the ants due to its antagonistic effect on the symbiotic fungus cultivated by the ants, but beneficial to the plants (Currie et al., 1999; Nascimento et al., 2017; Rocha et al., 2017). *Trichoderma* can colonize many plants, including eucalyptus, which is one of the main crops damaged by leaf-cutting ants. This fungus can also be found in anthills and in colony litter (Montoya et al., 2016). When ants cut and transport leaves to their nests, they also transport *Trichoderma* spores, which can colonize the plant material and negatively affect the health of the ants (Montoya et al., 2016; Poveda, 2021). The fungus can compete with *Leucoagaricus gongylophorus*, the symbiotic fungus cultivated by the ants, compromising the nutrition and development of the colony. The production of resistance hormones by plants, such as jasmonic acid and salicylic acid, can also act as an inhibitory factor for ants, reducing their consumption when these compounds are at high levels (Agbessenou et al., 2022; Natsiopoulos et al., 2024). The antagonistic relationship between *Trichoderma* and the fungus cultivated by leaf-cutting ants (Nascimento et al., 2017; Rocha et al., 2017), combined with their endophytic capacity, opens new possibilities for the development of biological control strategies for these insects.

1.6 - Dissertation Overview

Leaf-cutting ants adjust their leaf consumption based on several factors that include the presence and density of endophytic fungi (Coblentz & Van Bael, 2013). It has been shown that *Trichoderma* can colonize eucalyptus seedlings and potentially protect these from attack by leaf-cutting ants (Rocha et al., 2017). *Trichoderma* is also known to promote growth in other plants. In this study, we aimed to select isolates from endophytic *Trichoderma* that improve the growth of eucalyptus seedlings and also help to reduce leaf cutting by leaf-cutting ants.

To address this, we initially carried out a screening process with 23 fungal isolates of the genus *Trichoderma*, with the aim of selecting those that presented better performance in terms of growth, versatility in terms of the use of different substrates and endophytic potential. We selected three of these isolates, and these were evaluated in terms of their influence on plant development, looking for improvements in height, number of leaves and root development of seedlings of a commercial eucalyptus hybrid (*Eucalyptus urograndis*). We also evaluated colonization of all plant parts by fungi. Finally, we infected plants with two of these isolates and tested their influence on reducing the attack of leaf-cutting ants of the species *Acromyrmex subterraneus subterraneus*. We observed that the *Trichoderma* isolates used were not efficient in promoting eucalyptus growth. The only exception was with an isolate that presented a greater number of root tips. However, we observed that ants preferred to cut leaves from plants not colonized by the endophyte *Trichoderma*, highlighting the potential of this fungus to protect plants from leaf-cutting ants. However, the exact mechanisms of this interaction between *Trichoderma*, eucalyptus and leaf-cutting ants remain to be investigated, especially in the identification of the specific metabolites involved. Future studies should also explore the combination of different isolates to verify possible synergistic effects.

Chapter I

Selected isolates of endophytic *Trichoderma* decrease cutting by *Acromyrmex subterraneus subterraneus*

Selected isolates of endophytic *Trichoderma* decrease cutting by *Acromyrmex subterraneus subterraneus*

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Abstract

Endophytic fungi are microorganisms that can colonize the interior of plants and establish themselves without causing damage to the host plant. Fungi such as *Trichoderma* can promote a series of metabolic or morphological changes in plants, which can lead to improved development and protection against pests and pathogens. *Trichoderma* can also be antagonistic to the fungus *Leucoagaricus gongylophorus* that is cultivated by leaf-cutting ants and is the main food source for larvae. This study evaluated the potential of *Trichoderma* to establish itself as an endophyte and influence the growth of eucalyptus seedlings (*Eucalyptus urograndis*), in addition to its ability to reduce cutting by leaf-cutting ants. Twenty-three *Trichoderma* isolates were tested, selecting three (TR03, TR10 and TR71) with the best performance in growth, spore production and use of different carbohydrate sources. In the endophytism test, it was observed that the entire plant was colonized by the three tested isolates, demonstrating systemic colonization. In terms of growth promotion, there was no difference between the inoculated plants and the control regarding height and number of leaves, although TR71 presented a slightly lower number of leaves. Regarding root development, only the plants inoculated with TR71 presented more root tips, but the length and volume remained the same. The isolates did not promote seedling growth. To evaluate leaf cutting by leaf-cutting ants (*Acromyrmex subterraneus subterraneus*), in a choice experiment, seedlings inoculated with TR03 and TR10 and seedlings without fungus were exposed to nine ant colonies for seven days. The consumption of control plants was higher than that of those inoculated with the isolates. We conclude that *Trichoderma* isolates can establish themselves as endophytes, colonizing the entire plant, but do not have a great influence on the development of seedlings. However, despite this, they are important in modulating the cutting by leaf-cutting ants, proving to be promising isolates for reducing the damage in eucalyptus.

Keywords: Endophytic fungus, Hypocreales, Eucalyptus hybrid, Leaf-cutting ant, Growth promotion

Resumo

Fungos endofíticos são microrganismos que podem colonizar o interior das plantas e se estabelecer sem causar danos à planta hospedeira. Fungos como *Trichoderma* podem promover uma série de alterações metabólicas ou morfológicas nas plantas, o que pode levar a um melhor desenvolvimento e proteção contra pragas e patógenos. O *Trichoderma* também pode ser antagonista ao fungo *Leucoagaricus gongylophorus* que é cultivado por formigas cortadeiras e é a principal fonte de alimento para larvas. Este estudo avaliou o potencial de *Trichoderma* em se estabelecer como endófito e influenciar o crescimento de mudas de eucalipto (*Eucalyptus urograndis*), além de sua capacidade de reduzir o corte por formigas cortadeiras. Vinte e três isolados de *Trichoderma* foram testados, selecionando três (TR03, TR10 e TR71) com melhor desempenho em crescimento, produção de esporos e utilização de diferentes fontes de carboidratos. No teste de endofitismo, observou-se que toda a planta foi colonizada pelos três isolados testados, demonstrando colonização sistêmica. Em termos de promoção de crescimento, não houve diferença entre as plantas inoculadas e a testemunha quanto à altura e número de folhas, embora TR71 tenha apresentado um número de folhas ligeiramente menor. Em relação ao desenvolvimento radicular, apenas as plantas inoculadas com TR71 apresentaram mais pontas radiculares, mas o comprimento e o volume permaneceram os mesmos. Os isolados não promoveram o crescimento das mudas. Para avaliar o corte foliar por formigas cortadeiras (*Acromyrmex subterraneus subterraneus*), em um experimento de escolha, mudas inoculadas com TR03 e TR10 e mudas sem fungo foram expostas a nove colônias de formigas por sete dias. O consumo das plantas testemunha foi superior ao daquelas inoculadas com os isolados. Concluímos que isolados de *Trichoderma* podem se estabelecer como endófitos, colonizando toda a planta, mas não têm grande influência no desenvolvimento das plantas. No entanto, apesar disso, são importantes na modulação do corte de formigas cortadeiras, mostrando-se isolados promissores para redução dos danos em eucalipto.

Palavras-chave: Fungo endofítico, Hypocreales, Híbrido de eucalipto, Formiga cortadeira, Promoção de crescimento.

1 - Introduction

The interaction between fungi and plants is ancient in evolutionary history, with traces dating back to the Lower Devonian (±400Ma) (Krings et al., 2007). Endophytic fungi are fungi that live inside plants, colonizing their tissues and establishing symbiotic relationships with them (Schulz & Boyle, 2005). The relationships of endophytic fungi with plants are generally positive but can also be parasitic (Eberl et al., 2019). Studies on endophytes extend to a greater number of relationships between fungi and grasses, with fewer studies on woody trees, possibly because of the greater difficulty of working with these due to the greater resistance of the tissues (Hartley & Gange, 2009).

Endophytes bring with them a series of mechanisms that are beneficial to plants, acting directly and indirectly in the promotion of plant growth (Ahsan et al., 2024; Liu et al., 2022). Growth promotion occurs primarily through the production or induction of phytohormones by fungi (Nisa et al., 2015), such as gibberellins (Ahmad et al., 2010), and jasmonates (Wasternack & Hause, 2013). Fungi can act to increase nutrient absorption, helping to solubilize micro- and macromolecules, absorb water and nutrients from the soil, and in some cases as nitrogen fixers (Ahsan et al., 2024). Fungi can also cause morphophysiological changes in plants (Bamisile et al., 2021).

In addition to the factors related to the better development of plants by fungi, they also protect the plants (Espinosa et al., 2019; Mantzoukas & Eliopoulos, 2020). Endophytic fungi can produce toxic compounds and antifeedant compounds. These compounds decrease palatability and digestion, thus reducing insect attacks (Biere & Bennett, 2013; Eberl et al., 2019; Raman & Suryanarayanan, 2017). Endophytic fungi can cause impacts on the life cycle of insects, such as decreased oviposition and decreased survival of immature larvae, leading to problems in larval development and death (Russo et al., 2021; Tack et al., 2012; Zheng et al., 2023).

Among herbivorous insects, leaf-cutting ants stand out. There are two main genera: *Atta* and *Acromyrmex*, which play important ecological roles in natural ecosystems. This is mainly linked to the cycling of plant matter, seed dispersal, and the dispersal of microorganisms (Folgarait, 1998; Montoya-Lerma

et al., 2012). The fungus cultivated by the ants (*Leucoagaricus gongylophorus*) is the main food source for the larvae (Mueller et al., 2004). Leaf-cutting ants are also important agricultural pests, attacking a wide variety of crops. They are also important pests in terms of damage and economic losses in forestry, mainly in pine and eucalyptus (Hernández & Jaffé, 1995) Traditionally, the most used management methods for controlling leaf-cutting ants are chemicals. However, biological control is gaining relevance in this regard, with the use of bioactive compounds and entomopathogenic fungi (Montoya-Lerma et al., 2012; Zanetti et al., 2014).

Leaf-cutting ants have a system for inspecting the collected leaves to avoid substrates that could negatively affect the cultivated fungus from being deposited in the garden. Leaf-cutting ants cut leaves that have a lower incidence of endophytic fungi, due to the potential damage to the fungus cultivated by them (Coblentz & Van Bael, 2013; Van Bael et al., 2012). Even after the leaf is deposited in the garden, if there is any harmful microorganism, the colony may release different chemical signals that lead to the removal and disposal of the leaf (Goes et al., (2020).

Trichoderma (Ascomycota: Hypocreales) is a mycoparasite that is widely used in the biological control of plant pests (Bailey et al., 2008; Harman, 2000; Poveda et al., 2020; Tseng et al., 2020). This fungus is antagonistic to a wide diversity of microorganisms, with greater importance for those that cause diseases in plants. It acts directly on other fungi, causing the destruction of their cellular structures (Abdullah et al., 2008; Mirsam et al., 2021), in addition to producing antifungal and antibacterial compounds. *Trichoderma* can form a symbiotic relationship with plants as an endophyte (Rubini et al., 2005), and can promote plant growth and protection against herbivory. The antagonistic relationship between this genus of fungus and that cultivated by leaf-cutting ants, together with its endophytic capacity, opens the door to studies of control strategies for these insects (Rocha et al., 2017).

Due to the antagonism of *Trichoderma* to *L. gongylophorus*, the ants may reduce the cutting of plants colonized by it, to mitigate damage to the garden. Our initial objective was to establish a screening process for isolates, with a focus on endophytic potential. We also aimed to evaluate the increase in the growth of

eucalyptus seedlings, with the endophytic fungus *Trichoderma* and its effect on reducing leaf cutting by. We investigated (i) the selected isolates with a versatility in assimilation of plant-based carbohydrates, spore production and development in artificial media (ii) the ability of *Trichoderma* to establish itself as an endophyte in eucalyptus plants and its impact on plant development and (iii) the influence of the presence of *Trichoderma* in the plants on the level of herbivory of these plants by leaf-cutting ants.

2 - Materials and methods

2.1 - Molecular analysis of *Trichoderma* isolates

We performed molecular identification of the *Trichoderma* isolates used in the experiment. To obtain *Trichoderma* DNA, each of the 23 *Trichoderma* isolates was grown in PDA medium for 10 days and the FastDNA™ SPIN Kit for Plant and Animal Tissues was used to extract total DNA (MP Biomedicals, USA), following the protocol recommended by the manufacturer. Cell lysis was performed with the FastPrep® equipment (MP Biomedicals, USA), allowing the release of DNA without compromising its integrity. The extracted DNA was purified according to the kit instructions.

The amplification of the translation elongation 1-alpha (TEF) genomic region was performed by PCR. For amplification of the TEF gene, we used the primers 2218R and 983F, chosen for their specificity in identifying the *Trichoderma* genus. Each reaction had a final volume of 25 µL, containing 25 ng/µL of extracted DNA, 12.5 µL of Taq polymerase Kapa 2x ReadyMix (kk1006), 1 µL of each primer and 7.5 µL of ultrapure water.

The PCR reaction was carried out in an Eppendorf® thermocycler with the following steps: initial denaturation at 95°C for 2 min, followed by 10 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 1 min, 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min. The reaction was finished with an extension at 72°C for 5 minutes to ensure complete replication of the amplified fragments.

The amplification products were analyzed by 1% agarose gel electrophoresis to verify the presence and size of the fragments. After

confirmation, the samples were sent for purification and sequencing. After sequencing, forward and reverse sequences were joined into contigs and each sequence obtained was compared by BLAST to assess its similarity with the GenBank-NCBI database to identify the species used in this study.

2.2 - Isolate screening

The screening of 23 *Trichoderma* isolates was carried out to verify the ease and efficiency of their production in the laboratory. We investigated their growth in commonly used artificial media, spore production using rice as substrate and their ability to grow in different media, assimilating different carbohydrates.

2.2.1 - Growth in artificial media

The 23 *Trichoderma* isolates were cultivated in three solid media commonly used for fungal cultivation: PDA (potato dextrose agar), SDAY (saboraud yeast extract dextrose agar 25%) and malt extract 2%. A 5 mm (diameter) disk with the sporulated fungus was placed in the three media used, in 90 × 15 mm polypropylene Petri dishes. Three replicates were used for each isolate of each medium and incubated in complete darkness at 25 ºC for nine days.

The 23 isolates were also cultured in liquid versions of the same three media above. For this, a 2.5 mm (diameter) disk containing sporulated fungus was added to 50 mL Falcon tubes containing 20 mL of culture medium. Three replicates were used for each isolate evaluated from each medium and incubated in complete darkness at 25°C for nine days.

Individual photographs of each colony were taken every three days using a Nikon camera. In the case of the liquid media, *Trichoderma* colonies grew in flat disks floating in the medium, thus allowing photographs and diameter measurement. Individual photographs were taken from the top of the tubes. The images obtained each day were measured using ImageJ 1.48v software, with the diameter of each colony measured on the x and y axes. Mean colony diameters were subsequently calculated.

2.2.2 - Spore production on rice

For the rice substrate, 50 mL Falcon tubes containing 5 g of long-grain common rice and 4 mL of water were sterilized at 120 °C for 15 min and allowed to cool naturally. After this, three disks (2.5 mm) with the fungus, already sporulated in solid PDA medium, were placed in separate tubes, one for each of the 23 isolates, in triplicate. The tubes were stored in an incubator at 25 °C in complete darkness for 10 days.

In each tube, 20mL of 0.001% Tween 80® was added to the rice and homogenized. The suspension was filtered through sterile gauze to remove the rice grains and was subsequently diluted three times. Concentration of spores in each suspension was determined with a Neubauer chamber. Strains that presented at least one replicate with a concentration equal to or greater than $1x10^8$ conidia mL^{-1} were used in the next step.

2.2.3 - Phenotypic profile characterization

The 10 isolates that showed the highest spore production, equal to or greater than $1x10^8$ conidia mL^{-1}, were selected for the next stages of evaluation (TR71, TR10, TR29, TR32, TR57, TR03, TR13, TR31, TR23, TR04). These were cultivated in PDA medium for 10 days at 25ºC in complete darkness to obtain inoculum. For each isolate, a 96-well plate was prepared with 150 µL of culture medium (table 1) placed in triplicate into wells of a 96-well plate. After this period, 4 mL of 0.001% Tween 80® was poured on each of the plates with inoculum and spread using a Drigalski loop to suspend spores. A 2 mL aliquot of this suspension was transferred to a microtube to prepare the suspension to $1×10^6$ conidia mL^{-1}. Subsequently, 50 µL of spore suspension at $1x10^6$ conidia mL^{-1} were inoculated in all wells. The 96-well plates were evaluated daily for ten days and the growth of each strain in the different culture media was recorded, with the wells showing fungal growth being noted.

Table 1 –Carbohydrate sources used in culture media for phenotypic characterization of isolates of *Trichoderma*. Base culture media are shown in the first column. Supplemental carbohydrate sources are

then shown. For those that do not have added carbohydrate values, the pure base medium was used. To prepare the culture media used in the experiment, the reagents were calculated in a volume of 50 mL of each culture medium.

Culture medium base	Carbohydrate sources added			
	Sucrose	Glucose	Lignin	Cellulose
C-Synthetic Low-nutrient				
Saboraud dextrose yeast extract				
Saboraud dextrose yeast extract 1/4				
Malt				
Malt		5g		
Malt	5g			
Malt			0.020g	
Potato dextrose				
Potato dextrose	5g			
Grace				
Corn meal				
Corn meal		5g		
Corn meal	5g			
Water			0.020g	
Rice				
Rice		5g		
Rice	5g			
Yeast carbon				
Yeast carbon			0.020g	
Yeast carbon				5g
Yeast carbon		5g		
Yeast carbon	5g			
Yeast nitrogen				
Yeast nitrogen			0.020g	
Yeast nitrogen		5g		
Yeast nitrogen	5g			
Yeast nitrogen				5g

2.3 Comparison of *Trichoderma* fungus application methods

The method of application of the fungus to plants can influence its colonization. This experiment aims to determine the most efficient method of application to obtain better results, between applications by spraying, root application and both simultaneously.

2.3.1 Fungus application method

For rice substrate, a polypropylene bag containing 100 grams of long-grain common rice and 60 mL of water were sterilized for 15 minutes at 120ºC. After preparing the rice, five discs of 5 mm in diameter of TR71 isolate containing the sporulated fungus were added to each package and these were subsequently incubated for ten days in complete darkness at 25ºC. After this period, a spore suspension of each fungus was prepared. To prepare the suspension, all the rice with the sporulated fungus was placed in a blender with 500mL of 0.001% Tween 80® and processed to form the suspension. It was filtered through sterile gauze to remove excess rice grains. The number of spores was counted in a Neubauer chamber and suspensions were standardized to a concentration of $1x10^8$ conidia mL^{-1}.

To evaluate the efficiency of plant colonization and growth promotion using each method (root application, foliar application and simultaneous application – root and foliar together), eucalyptus seedlings were planted in 5-liter pots with commercial substrate (mecplant). The seedlings were divided into four treatments with 15 replicates each. For the first treatment, 30 mL of the suspension were applied as a soil drench close to the root of each seedling with a beaker. In the second treatment, the suspension was applied to the leaves by spraying until the point of runoff. In the third treatment, the spore suspension was applied to both roots and leaves as above. In the control, a blank 0.001% Tween 80® was applied to roots (30mL) and leaves (until the point of runoff) as above.

2.3.2 –Measurement of growth promotion and plant sampling for endophytic colonization

Plant height, number of leaves and diameter were recorded before the plants were inoculated. Height was measured from the first node to the apical meristem, using a measuring tape. The number of leaves on each plant was counted manually. The diameter of the plants was measured at the first node using a digital caliper. These measurements were repeated every three days after inoculation up to 30 days.

The leaves, stems and roots of the plants were sampled at the end of the experiment to evaluate the recovery of the applied isolate (TR71). Five leaves were randomly collected from each plant. Three fragments measuring approximately 5 x 5 mm were cut from each leaf with the aid of a scalpel blade. The stem was separated from the root at the first node and divided into the lower and upper regions. Using a scalpel, five fragments (±0.5cm) were removed from each region (lower and upper) of the stem. For roots, we removed five fragments (±0.5cm) of the pivotal root of each plant with a scalpel, totaling 30 fragments of each plant.

All fragments were surface sterilized by immersion in 70% alcohol for one minute, then in 1% sodium hypochlorite for one minute, with subsequent washing three times in sterile water. All sampled material was placed in PDA culture medium and incubated for 15 days in complete darkness at 25ºC and evaluated daily to verify whether *Trichoderma* grew from the fragments.

2.4 - Growth promotion and systemic infection

The experiment aimed to evaluate the ability of the isolates to infect and colonize eucalyptus seedlings, in addition to investigating the influence of this colonization on height development, crown formation and root growth. These are important parameters in the use of fungi as endophytes. Based on the results obtained in the previous section, the foliar application method was used due to the better colonization performance.

2.4.1 - Inoculation of *Trichoderma* in eucalyptus seedlings

For the rice substrate, the same methodology described in section 2.3 was used. After preparing the rice, five 5 mm discs of each of the three isolates (TR03, TR10, TR71) containing the sporulated fungus were added to each package and these were subsequently incubated for ten days in complete darkness at 25ºC. After this period, a spore suspension of each fungus was prepared, as previously described (section 2.3).

Sixty two-month-old hybrid eucalyptus seedlings, *Eucalyptus urograndis*, were placed in 5-liter pots containing commercial substrate (mecplant). Fifteen pots were separated for each of the four treatments. The spore suspension of each fungus was inoculated by spraying until runoff and water with Tween 80® at 0.001% was used as control.

The plants were placed outdoors, covered by a shade screen. The plants were distributed in a random block design, where the position of each pot and the plant used for each treatment in each block were defined by drawing lots. Each block consisted of 12 plants, four from each treatment, to ensure a balanced distribution between treatments. The plants remained in this location for 30 days and received daily irrigation with 250 ml of water until the end of the experiment.

2.4.2 – Growth promotion and plant sampling

For the growth promotion, the same methodology described in section 2.3.2 was used, except for the diameter measurement, which in this case was not used. After the final measurement, roots were removed and placed in 500mL bottle containing water and their development was analyzed using the WinRhizo 2013c software.

The leaves, stems and roots of the plants were sampled at the end of the experiment to evaluate the recovery of the applied isolates (TR03, TR10 and TR71), as described in section 2.3.2. Recognition of the strains was based on the morphology of the colony (Fig. S2).

2.5 - Effect of endophytic *Trichoderma* on attack by leaf-cutting ants

Here, we sought to test whether the presence of the endophytic fungus *Trichoderma* in eucalyptus seedlings reduces cutting by leaf-cutting ants in eucalyptus seedlings. For this, we used two previously selected isolates to conduct two choice experiments, in which inoculated and non-inoculated plants were offered to the ants simultaneously.

2.5.1 – Infected Plants, Ants and Experimental Arenas

To the first experiment 126 eucalyptus seedlings were planted in 3L pots using a commercial substrate (mecplant). Half of the plants were inoculated by spraying the leaves with a suspension of $1x10^8$ conidia mL^{-1} (prepared with water at 0.001% Tween 80) of the TR03 isolate, until runoff. The remaining plants were used as controls, in which blank Tween 80 was sprayed until the runoff. The plants were kept for 15 days outdoors, covered by a shade screen, to be offered to the ants during the experiment. The same procedure was performed to the second experiment with another 126 seedlings, for the experiment with the TR10 isolate.

For the experiment, 18 colonies of leaf-cutting ants (*Acromyrmex subterraneus subterraneus*) were used, with nine colonies used for each isolate tested (TR03 and TR10). The colonies were originally obtained from the Dendrology and Recanto das Cigarras at the Federal University of Viçosa and were maintained for approximately one year in the Laboratory of Insect-Microorganism Interactions. The ants were kept in plastic trays, with talcum powder on the edges to prevent escape, and fed daily with *Acalypha wilkesiana* leaves. In the experiment, each colony was placed individually in an arena 24 hours before the start, for acclimation. During this period, they were kept without food.

Experimental arenas for the preference test were made from two plastic trays, one of which was the tray containing the ant colony (32x21x7cm) and the other the foraging tray with the plants (60x40x11cm). The edge of each tray was coated with hydrated magnesium (pure talcum powder) to prevent the ants from

escaping. These two trays were connected by a 3/4 diameter plastic tube, the tube being inserted into the side of the foraging arena so that the plants were parallel to each other and at the same distance from the ants' entry point into the arena (Fig. 1).

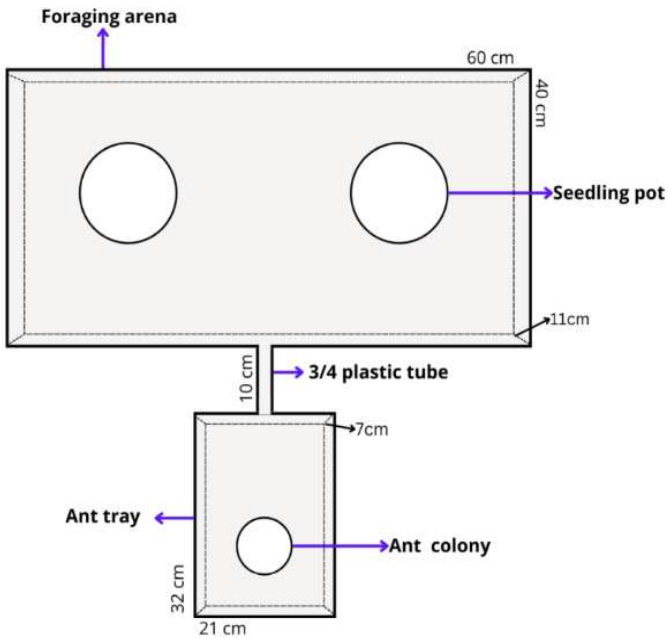

Figure 1 - Illustrative diagram of the experimental arena for preference testing that was used in the experiment.

2.5.2 - Choice experiment

The experiment was carried out twice, the first time using isolate TR03, and the second time using isolate TR10. In each case, nine plants inoculated with fungus and nine from the control group were randomly selected, one of each per colony. All the leaves of all 18 plants were photographed with a Nikon camera, with graph paper used as background for scaling. Subsequently, the seedlings were arranged in pairs (with fungus and without fungus) in each of nine arenas, alternating the side on which each treatment was arranged (right or left), in each arena. The ants were left to forage for 24 h, after which the plants were removed, and new plants were placed in the arenas. The plants were photographed again

after exposure to the ants to record the leaf-cuttings made by the ants. The experiment was terminated after 168 h (7 d) and each colony received seven plants of each treatment (one of each per day) throughout the experiment, totaling 14 plants per colony. At the end of the experiment, the leaf photographs were analyzed using ImageJ 1.48v software to measure the leaf area lost.

The arenas, as well as the plants, were photographed with a Nikon camera in three different periods (morning, afternoon and night). The number of ants recorded in each image was counted using ImageJ 1.48v software.

To confirm the presence of fungi in the seedlings used, all plants were sampled after being removed from the experimental arena. Sampling was carried out by removing five leaves randomly collected from each plant. These leaves were surface sterilized in 70% alcohol for one minute, then in 1% sodium hypochlorite for one minute and washed three times in sterile water. With the aid of a scalpel, three fragments were cut from each of the leaves measuring ±5x5 mm and these were placed on plates with PDA culture medium. The plates were incubated in incubators for 15 days at 25ºC in complete darkness. The plates were observed daily to confirm the recovery of the applied fungus through its growth from the plated fragments, thus confirming its presence. The fungus was identified by means of colony morphology and slide preparation.

2.6 - Statistical analyses

For the statistical analyses, the R software version 4.4.2 was used. To evaluate the effect of *Trichoderma* on the development of the seedlings, the height and number of leaves were evaluated after 30 days of experiment. For this, a mixed linear model was used, using the treatment as a fixed effect and the plant as a random effect. Due to the heterogeneity of the data, a generalized least squares model was used. Subsequently, the treatments were compared through planned comparisons between the groups.

To investigate whether *Trichoderma* can induce root development, we measured root total length, root volume and number of tips after 30 days. We considered these 3 root characteristics as response variables and presence of fungus as the predictor variable. Correlation among response variables was

assessed with *cor* function. The effect of fungi on development was assessed using a mixed linear model followed by ANOVA. Data heterogeneity was addressed using the lme package and generalized least squares (gls function) due to heterogeneous variance between treatments

To evaluate ant herbivory over time, a linear mixed model with the lmer package was used, followed by an ANOVA to evaluate the effect of the interaction between the predictor variables: day and treatment on leaf area consumed, considering the ant colony as a random effect. Data heterogeneity was addressed using the lme package and generalized least squares (gls function) due to heterogeneous variance between treatments. A planned comparison was then carried out between the different treatment levels, considering the interaction between day and treatment using the mulcomp package. The final model was adjusted excluding the day and focusing only on the treatment effect – Fungus and Control.

The number of ants throughout the days of the experiment was analyzed using a linear mixed model, followed by ANOVA, to examine the effect of the predictor variables day (first to seventh day of evaluation) and location (arena, control plant and plant infected). A planned comparison was performed to evaluate the effect of each day separately.

Data heterogeneity was adjusted using the lme and least squares (gls) package, due to the heterogeneity between the evaluated treatments.

3 - Results

3.1 Molecular identification of *Trichoderma* isolates

Molecular analyses allowed the identification of the *Trichoderma* species in nine isolates that had not previously been characterized (TR02, TR03, TR06, TR14, TR29, TR31, TR57, TR60, TR62 and TR66). However, isolates TR10,

TR11 and TR62 presented problems during the analysis, making it impossible to identify the species so far (table 2).

Table 2 Molecular identification of the *Trichoderma* isolates used during the experiment.

Isolate	Species	GenBank Accession Number	References
TR 01	*Trichoderma atroviride*	KT619061	Rocha *et al.* 2017
TR 05	*T. koningiopsis*	KT619057	Rocha *et al.* 2017
TR 07	*T. spirale*	KT619065	Rocha *et al.* 2017
TR 09	*T. atroviride*	KT619067	Rocha *et al.* 2017
TR 12	*T. atroviride*	KT619055	Rocha *et al.* 2017
TR 17	*T. spirale*	KT619064	Rocha *et al.* 2017
TR 22	*T. endophyticum*	KT619074	Rocha *et al.* 2017
TR 32	*T. inhamatum*	KT619076	Rocha *et al.* 2017
TR 33	*T. atroviride*	KT619073	Rocha *et al.* 2017
TR 35	*T. spirale*	KT619062	Rocha *et al.* 2017
TR 49	*T. spirale*	KT619069	Rocha *et al.* 2017
TR 58	*T. endophyticum*	KT619066	Rocha *et al.* 2017
TR 71	*T. koningiopsis*	KT619077	Rocha *et al.* 2017
TR 02	*T. merleae/ T. koningiposis*	In progress	This study
TR 03	*T. koningiopsis*	In progress	This study
TR 06	*T. merleae*	In progress	This study
TR 10	*Trichoderma sp.*	NA	This study
TR 11	*Trichoderma sp.*	NA	This study
TR 14	*T. koningiopsis*	In progress	This study
TR 29	*T. harzianum*	In progress	This study
TR 31	*T. pollinicola*	In progress	This study

TR 57	*T. koningiopsis*	In progress	This study
TR 60	*T. koningiopsis*	In progress	This study
TR 62	*Trichoderma sp.*	NA	This study
TR 66	*T. harzianum*	In progress	This study

3.2 - Isolate screening

The isolate screening consisted of evaluating the performance of the *Trichoderma* isolates in different aspects such as: growth in artificial media (solid and liquid), spore production on rice and phenotypic characterization, based on their growth on different substrates. At the end of the growth experiment on solid medium (table 3), we found that 21 isolates grew more than 8 cm on the plate in solid PDA, reaching their maximum size in 3 d, and only two isolates did not reach above this size. In malt extract, 21 isolates also reached sizes equal to or greater than 8 cm, with the exception of two isolates, a pattern that was repeated in the SDAY medium. However, in liquid medium (table 3), 21 isolates also grew to 2 cm and only TR01 and TR057 did not develop in potato-dextrose (PD). On the other hand, in malt and SDY liquid media only one isolate (TR49 and TR17, respectively) did not reach maximum size.

Table 3 Average growth diameter of isolates (average colony diameter on the x and y axes) in solid media (potato dextrose agar - PDA, saboraud dextrose agar yeast extract 25% - SDAY and malt extract 2%) and liquid media (potato dextrose - PD, saboraud dextrose yeast extract 25% - SDY and malt extract 2%) used in the experiment after nine days of incubation in complete darkness at 25ºC. In bold are highlighted those that did not reach above 8 cm in the solid medium and 2 cm in the liquid medium, having a lower development.

	Growth in culture medium (cm)					
	Solid			**Liquid**		
	BDA	**Malt**	**SDAY**	**BD**	**Malt**	**SDY**
TR03	8.83	8.76	8.77	2.31	2.1	2.29
TR04	9.01	8.74	8.61	2.2	2.2	2.06
TR10	8.29	8.28	**4.32**	2.09	2.05	2.15

TR13	9.12	8.99	9.1	2.2	2.23	2.49
TR23	8.81	8.86	8.5	2.29	2.12	2.27
TR29	8.9	8.98	8.79	2.12	2.17	2.15
TR31	9.12	**7.79**	8.82	2.15	2.19	2.35
TR32	9.07	8.87	8.77	2.77	2.4	2.21
TR57	8.91	8.67	8.73	**1.85**	2.11	2.48
TR71	**5.79**	8.3	8.15	2.03	2.27	2.15
TR01	**5.84**	**2.97**	**5.78**	**1.36**	2.15	2.16
TR02	8.78	8.67	8.56	2.26	2.17	2.29
TR06	8.82	8.59	8.69	2.1	2.12	2.14
TR09	8.76	8.62	9.07	2.17	2.17	2.19
TR11	8.79	8.61	8.57	2.08	2.11	2.21
TR12	8.95	8.75	8.69	2.23	2.29	2.17
TR14	9.01	8.68	9.16	2.38	2.2	2.38
TR17	9.09	8.62	8.91	2.37	2.08	**1.41**
TR22	9.05	8.48	8.89	2.1	2.16	2.22
TR33	8.97	8.81	8.59	2.34	2.18	2.21
TR49	8.92	8.87	8.78	2.31	**1.88**	2.16
TR60	9.02	8.57	8.69	2.22	2.17	2.24
TR66	9.11	8.68	8.7	2.32	2.19	2.27

In the spore production experiment, we evaluated spore production by all previously tested isolates (Table S1). Among these, after ten days of incubation at 25°C in complete darkness ten of them had spore production equal to or greater than $1x10^8$ conidia mL^{-1} in at least one of their replicates, with the better being isolates TR10, TR71 (table 4).

Table 4 Highest concentration of spores obtained in a replicate of each of the 23 *Trichoderma* isolates evaluated. After ten days of incubation in regular long-grain rice at 25ºC and in complete darkness. In bold, isolates that obtained at least one repetition with a concentration equal to or greater than $1x10^8$ mL^{-1} are highlighted.

Spore production	
Isolate	**Spore_concentration**
TR01	$4.75×10^7$

TR02	5.75×10^{7}
TR03	**1.75×10^{8}**
TR04	**3.62×10^{8}**
TR06	9×10^{7}
TR09	4×10^{7}
TR10	**5.03×10^{8}**
TR11	9.75×10^{7}
TR12	4.5×10^{7}
TR13	**1.6×10^{8}**
TR14	4.25×10^{7}
TR17	5.75×10^{7}
TR22	7.25×10^{7}
TR23	**1.42×10^{8}**
TR29	**8.05×10^{8}**
TR31	**1.75×10^{8}**
TR32	**3.03×10^{8}**
TR33	6.5×10^{7}
TR49	7.75×10^{7}
TR57	**1.78×10^{8}**
TR60	6.75×10^{7}
TR66	7.5×10^{7}
TR71	**7.02×10^{8}**

Of the ten isolates whose phenotypes were assessed, only three grew in at least 70% of the media used. This indicates that these isolates have a high capacity to develop in a greater diversity of nutritional conditions. Meanwhile, another three grew in 60% of the media, showing reasonable development on the plates, while four isolates grew at 50% or less of the media tested, showing poor performance (Fig. 2).

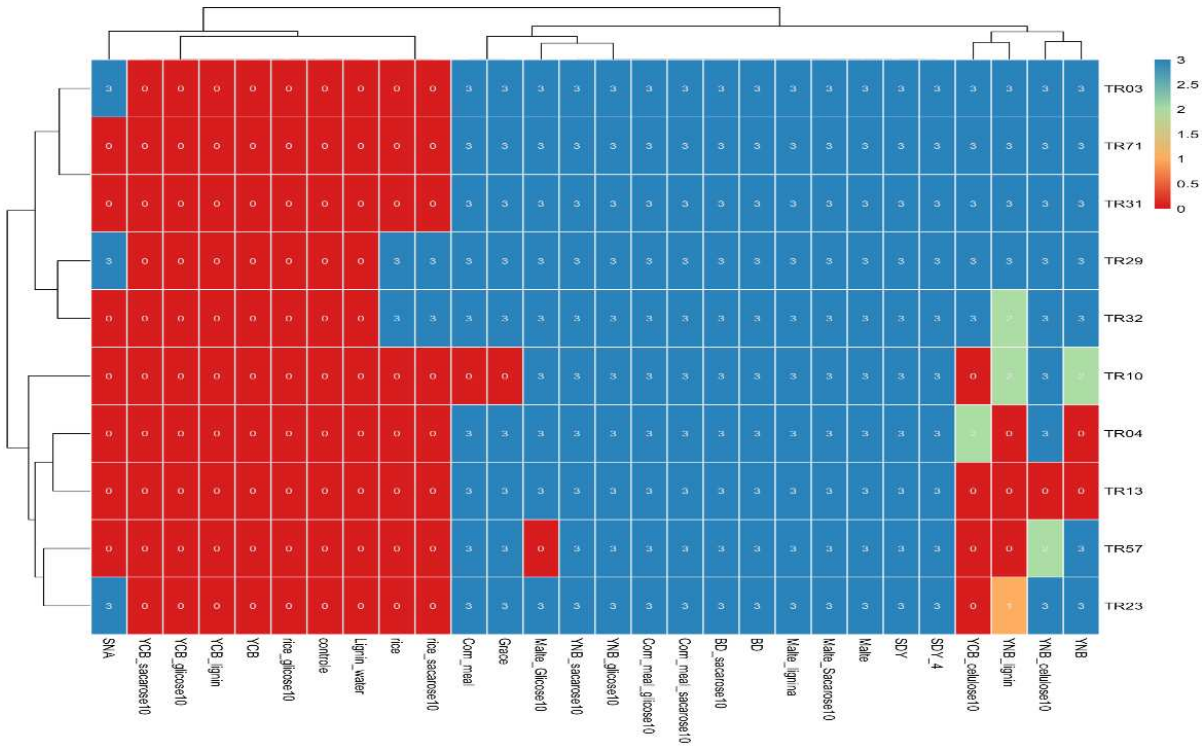

Fig.2 Growth of ten *Trichoderma* isolates (y-axis) in 96-well plate in 28 different liquid culture media (x-axis) with the addition of carbohydrates (sucrose, glucose, lignin and cellulose), to evaluate growth on different bases, as well as the assimilation of different carbohydrates. Those grown in two wells (green) or three (blue) are considered to have achieved effective growth in the medium. Those that showed growth in only one of the wells (orange) or none (red) are not considered successful.

Evaluating all the characteristics tested, the ones that presented the best performance were TR03, TR32, TR29. However, the last two (TR32 and TR29) did not grow in sufficient volume in rice later, which is an important characteristic for the selection of the isolate. Considering the good performance of the isolates TR71 and TR10, in addition to their great growth efficiency in rice, they were selected together with TR03 to evaluate their ability to grow endophytically.

3.3 Growth promotion and systemic infection

After defining the best way to apply the fungus to plants for the experiment (Fig. S1), the three selected isolates (TR03, TR10 and TR71) were applied to plants. After 30 days of evaluation, we found no difference in the number of leaves possessed by the plants by treatments (Fig. 3a; 38 to 51 leaves per plant). None of the isolates used showed a significant difference in height

from the control group (Fig. 3b). The plants in general had a height ranging from 22cm to 27cm on average.

The root development (Fig. S3) was evaluated in three aspects: length, volume and number of tips, we observed that the total root length dids not differ between treatments (Fig.3c; range 752cm to 810cm). There was also no difference in root volume (range 3.26m^3 to 3.86m^3) (Fig.3d). On the other hand, there was a significant difference in the number of tips in plants inoculated with TR71(Fig. 3e), indicating a greater number of branches ($p<0.005$).

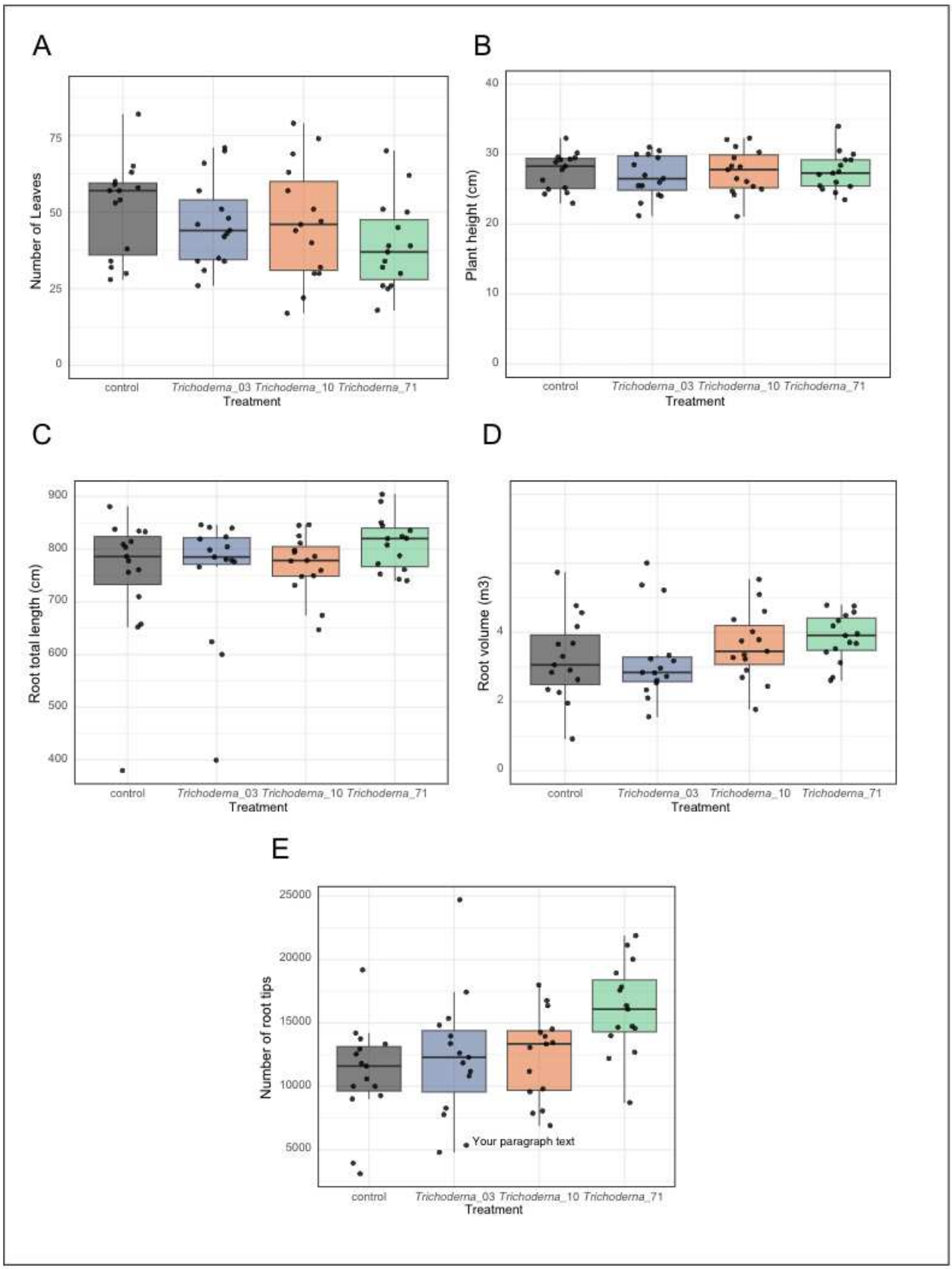

Fig. 3 Development of the aerial part of the eucalyptus seedlings, with the number of leaves (A) and the height of the seedlings (B), after 30 days of inoculum of the *Trichoderma* isolates. Root development after 30 days of application o in length (C), volume (D) and number of tips (E).

All plants were sampled (leaf, root and stem) after the experiment, and the applied isolate was recovered in all treatments, this being confirmed through colony morphology (Fig. S2) and microscopy (in 225 leaf fragments, 150 stem fragments and 75 roots fragments collected from each treatment) (Table 5). The isolates were recovered from all treatments, in all sampled plants, except in the control group. For leaf fragments, it was recovered in most of the sample in isolates TR03 and TR10, for this, both were used in the next step.

Table 5. Isolates recovered from plant samples. A total of 225 leaf fragments, 150 stem fragments and 75 root fragments were sampled. Isolates were recovered in all treatments (the number of recovered fragments is represented by "number of recovered fragments/numbers of sampled fragments").

	Regions sampled		
Isolates	**Leaves**	**Stem**	**Root**
Control	0/225	0/150	0/75
TR03	173/225	91/150	45/75
TR10	202/225	57/150	56/75
TR71	103/225	64/150	51/75

3.4 - Influence of endophytic *Trichoderma* on cutting by leaf-cutting ants

Evaluating the influence on the consumption of the *Trichoderma* isolate - TR03, a significant difference was also observed (Fig. 4a), with a greater consumption of uninfected plants compared to infected ones (p=1.792e-08). Total consumption was 0.28±0.45cm^2 from infected plants and 0.48±0.50 cm^2 from control plants. However, when considering the number of ants on the plants throughout the experiment (Fig. 4d) we observed that there was a significant difference between the number of ants exploring the control plant and the infected plants (F_{370}=116.5293, p<0.001). A greater number of ants were observed in the controls, although the values were initially the same. This indicates a decrease in visitation to infected plants after the first contact, with an average of 11.69±11.74 on plants with fungus and 29.10±20.52 on control plants.

To evaluate the preference for cutting by ants between plants infected and not infected with the fungus *Trichoderma* sp., the plants were exposed to the ants for 24 hours for seven consecutive days and the area consumed was evaluated. By the end of the study, the ants had shown preference for consuming uninfected plants, presenting a greater total consumption of uninfected plants than infected ones with isolate TR10 ($p<0.04$), with a consumed foliar area of $0.25\pm0.43cm^{2,}$ from infected plants and $0.31\pm0.0.45cm^2$ from uninfected (Fig. 4b). Meanwhile, when considering the number of ants on the plants throughout the experiment (Fig. 4d), we observed that there is a significant difference between the number of ants exploring the control plants, compared to those infected with fungus ($F_{559}= 140.013$, $p<0.001$). A higher number of ants was observed in the control, although the values were initially close. This indicates a lower visitation to the infected plants after the first contact, with an average of 23.31 ± 11.74 in the plants with fungus and 35.17 ± 13.77 in the control plants.

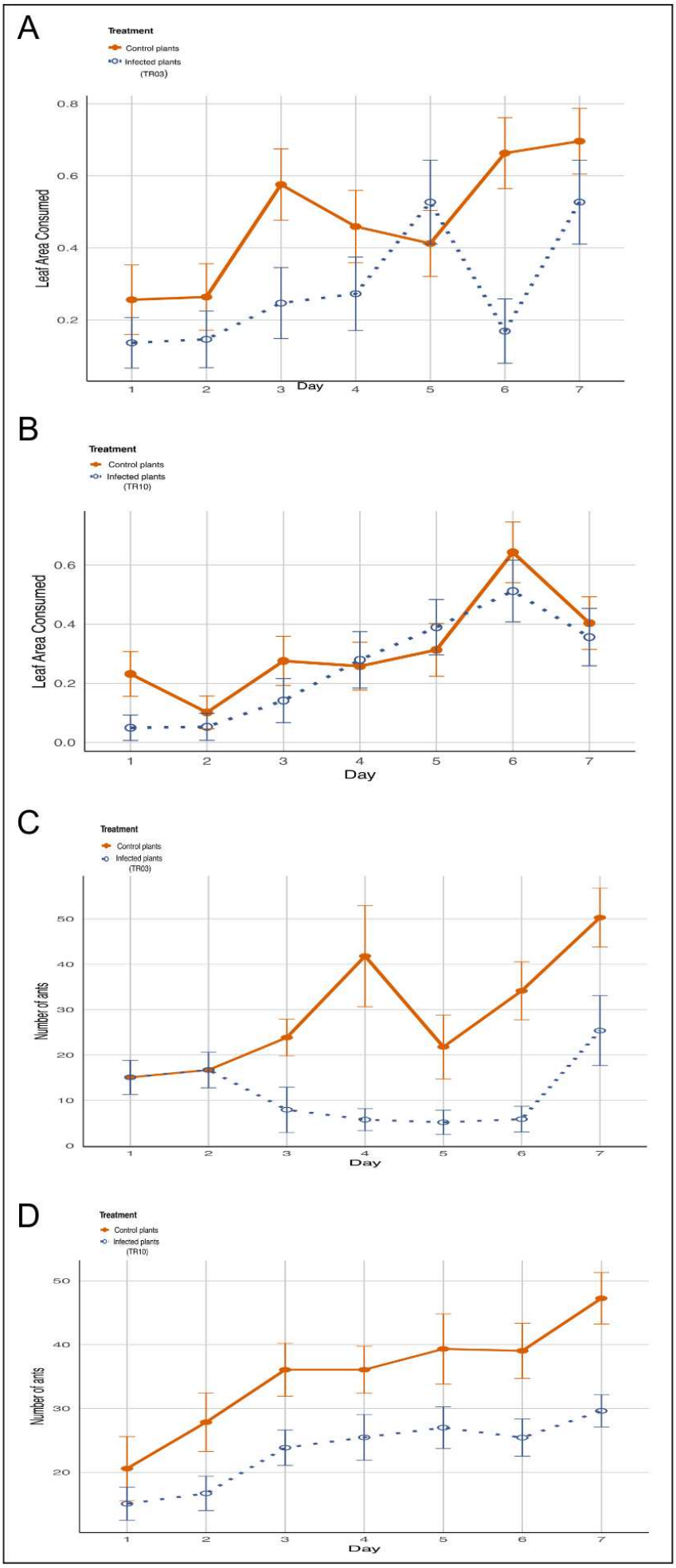
A
Treatment
Control plants
Infected plants (TR03)
Leaf Area Consumed
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
Day
B
Treatment
Control plants
Infected plants (TR10)
Leaf Area Consumed
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
Day
C
Treatment
Control plants
Infected plants (TR03)
Number of ants
50
40
30
20
10
0
1
2
3
4
5
6
7
Day
D
Treatment
Control plants
Infected plants (TR10)
Number of ants
50
40
30
20
1
2
3
4
5
6
7
Day

Fig.4 Leaf area consumed (cm^3) by ants over seven days of experimentation (A, B) with different treatments (plants inoculated with isolate TR03 (A) and inoculated with isolate TR10 (B)- Fungus, in blue and non-inoculated plants - Control, in orange). C (TR03) and D (TR10), number of ants (y-axis) in each area evaluated: control plants (orange) and infected plants (y-axis) during the seven days of the experiment (x-axis).

4 - Discussion

The use of different media allows the evaluation of the robustness of the isolates in the assimilation of various nutritional sources (Whipps, 1987).The PDA medium is rich in carbohydrates and starch, favoring isolates with a greater aptitude to assimilate simple carbon sources. In this medium, 21 isolates showed good growth, demonstrating their suitability for this type of substrate. The SDAY medium, rich in yeast nutrients and a source rich in nitrogen, also had 21 isolates developing well, attesting to a better capacity in the assimilation of this nutrient source. The medium with malt is a balanced source of sugars and proteins (Cvetkovic & Markov, 2002), being an intermediate medium between the two previous ones, also having 21 isolates with good growth in it.

The variation in growth among isolates indicates the metabolic diversity of these organisms, suggesting greater adaptability for biotechnological applications (Lee & Schmidt-Dannert, 2002). These results reinforce the importance of medium composition for the initial selection of isolates, ranging from simpler, intermediate and complex sources (such as PDA, Malt and SDAY) (Sharma & Pandey, 2010; Sknau & Meena, 2022; Taniwaki et al., 2001).

Spore production was evaluated in rice, an efficient substrate for both laboratory and industrial-scale fungal multiplication, in addition to simulating natural environmental conditions (Miranda-Hernández et al., 2016; Patiño-Medina et al., 2021). Of the 23 *Trichoderma* isolates analyzed, ten showed high spore production in at least one of the replicates evaluated. The ability to grow and produce many spores in rice indicates better starch assimilation and resource utilization, indicating great production potential with a biotechnological bias of the isolate, especially in the agricultural sector (Miranda-Hernández et al., 2016; Patiño-Medina et al., 2021).

The ten best-performing isolates were subjected to phenotypic characterization to evaluate their plasticity - that is, their ability to grow and utilize

different nutritional sources under varied conditions. (Oliveira et al., 2011; Yarrow, 1998). This analysis is essential to identify more versatile and adaptable strains, with greater potential for application in various agricultural systems (Gebremariam et al., 2021; Monteiro et al., 2024). In this study, culture media formulated substrates of plant origin or containing carbohydrates common in this type of environment were used, allowing the evaluation to be directed at the endophytic potential of the isolates.

Based on these results, the three isolates with the best performance in the most of parameters evaluated (TR03, TR10 and TR71) were selected for application in *Eucalyptus urograndis* seedlings. These isolates demonstrated robust growth, high spore production and great metabolic adaptability, so may be promising candidates for future applications in the agricultural sector.

Plants with foliar application stood out among the evaluated parameters (number of leaves and height), with the exception of diameter, which did not differ in any of the treatments. This result suggests that foliar application was more effective, promoting greater growth in height and number of leaves compared to the other treatments.

The difference in results can be explained by the dynamics of fungal infection in plants. The main route of entry of endophytic fungi occurs through lesions and stomata (Huang et al., 2018). Leaves, with greater stomatal density compared to roots (Hartung & Slovik, 1991; Votrubova et al., 1992; Fontana et al., 2017), offer more direct and effective contact for spore colonization. In contrast, root inoculum faces a greater physical barrier since roots are buried and have fewer stomata, making initial infection difficult (Hartung & Slovik, 1991; Votrubova et al., 1992; Fontana et al., 2017).

In some cases, simultaneous application showed lower rates than the others. This may be related to the high inoculum load inoculated simultaneously in different plant tissues, which may have led to an unfavorable physiological response, such as competition between fungi or induction of more intense defense mechanisms by the plant (Thakur et al., 2019). These results suggest that foliar application is the most promising approach for the inoculation of

endophytic fungi in *Eucalyptus*, maximizing the colonization potential and the beneficial effect on the plant.

After the application of fungal isolates, the eucalyptus seedlings did not show significant results regarding growth promotion. Although fungi of the *Trichoderma* genus are generally associated with growth promotion (Azarmi et al., 2011; Hermosa et al., 2013; Stewart & Hill, 2014), there may be different effects depending on the isolate used, as well as the plant tested (Bandeira et al., 2023). One of the factors that may have influenced the plant-microorganism interaction are the organisms involved themselves; perhaps the isolates used do not lead to growth promotion in *E. urograndis*. Mechanisms such as the action of the fungus on the plant may also be linked to the lack of significant difference presented in growth in general, such as the influence of the fungus on internal physiological parameters and processes of the plants, which are not directly reflected in growth (Vinale et al., 2008); Alam et al., 2021; Harman et al., 2021. These parameters may converge in a gain in height and canopy formation in the long term; however, since it is not a senescent plant, the experimental time used was not sufficient to capture these changes. However, although the absence of development in the parameters mentioned above (height and number of leaves) was observed, isolate TR71 presented a greater number of root tips. This characteristic may indicate a more specific and localized action of the isolate in question, indicating that it probably presents the production of some phytohormone linked to root development (Illescas et al., 2021).

Fungi of the genus *Trichoderma* are widely known for their potential as plant growth promoters (Nelson et al., 1995; Tseng et al., 2020), there is great metabolic plasticity within this group, which can result in different responses. Some isolates produce auxins and gibberellins, stimulating plant development, while others may not induce this response or even present neutral effects depending on the environmental conditions and the host (Vinale et al., 2008; Azarmi et al., 2011; Hermosa et al., 2013;). Different *Trichoderma* strains may present contrasting responses regarding root and shoot growth, reinforcing this functional variability (Bandeira et al., 2023). Although no significant growth promotion was observed, the preservation of morphological parameters can be interpreted as a positive result.

Considering that *E. urograndis* is a high-performance commercial hybrid, the absence of negative effects on growth reinforces the safety of using these isolates as bioinputs. Furthermore, the fact that they do not compromise plant development may be a favorable factor in agricultural contexts, especially in integrated management for controlling leaf-cutting ants, where the introduction of a biological agent should not negatively interfere with crop vigor.

In the ant consumption test, both isolates used showed a significant difference in consumption, with both cases consuming more control plants than infected plants. This indicates an inhibition of herbivory mediated by the presence of the fungus, which is in line with the results found by Batista et al., (2022) and demonstrates that ants can recognize the presence of the fungus (Rocha et al., 2014), Goes et al., 2020. This change in the feeding behavior of ants may be associated with the modification of the chemical profile of plants induced by the presence of *Trichoderma* (Gange et al., 2012; Natsiopoulos et al., 2024). Endophytic fungi can stimulate the production of antifeedant compounds, such as phenolics, alkaloids and other secondary metabolites, which make the plant less palatable to herbivores (Hartley & Gange, 2009; Gange et al., 2019; Mantzoukas & Eliopoulos, 2020; Natsiopoulos et al., 2024; Aravinthraju et al., 2024). Species of the genus *Epichloë*, for example, colonize grasses and produce alkaloids that are toxic to insects, reducing herbivory (Clay & Schardl, 2002).

Furthermore, the presence of *Trichoderma* as an endophyte may lead to a change in the volatiles emitted by eucalyptus seedlings that repel insects (Poveda, 2021; Agbessenou et al., 2022; Natsiopoulos et al., 2024;). In leaves carried by ants on the trail and discarded around the anthill, the presence of *Trichoderma* was observed with greater frequency in the discarded ones (Rocha et al., 2014). Our results corroborate this hypothesis, reinforcing the potential of *Trichoderma* as a "bodyguard" of plants against herbivory by leaf-cutting ants.

This effect can be attributed to both the induction of repellent compounds and the antagonistic action of *Trichoderma* against the ant symbiotic fungus, *Leucoagaricus gongylophorus* (Nascimento et al., 2017; Rocha et al., 2017), with production of lytic enzymes and antifungal metabolites that can negatively affect the colony, reinforcing the ants' aversion to colonized plants (Rodrigues et al., 2011).

5 – Final considerations and perspectives

The results presented by the three isolates with the best performance (TR03, TR10, TR71) suggest that the adaptability of fungi to different culture media, as well as their good performance in assimilating a wide variety of carbohydrates and high spore production, correlates with their performance as endophytes and lower consumption by leaf-cutting ants. *Trichoderma isolates* TR03 and TR10 have shown potential as a possible biological control agent for leaf-cutting ants, with the aim of reducing damage to *Eucalyptus*. However, the exact mechanisms of this interaction between *Trichoderma*, *Eucalyptus* and leaf-cutting ants still need to be investigated, especially in identifying the specific metabolites involved.

In addition, further investigations should explore the combination of different *Trichoderma* isolates to evaluate possible synergistic effects that could potentiate its action as a biological control agent. These advances could lead to the development of more effective and sustainable solutions for managing leaf-cutting ants, contributing significantly to the protection of eucalyptus plantations and other crops, with less environmental damage.

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Supplementary material

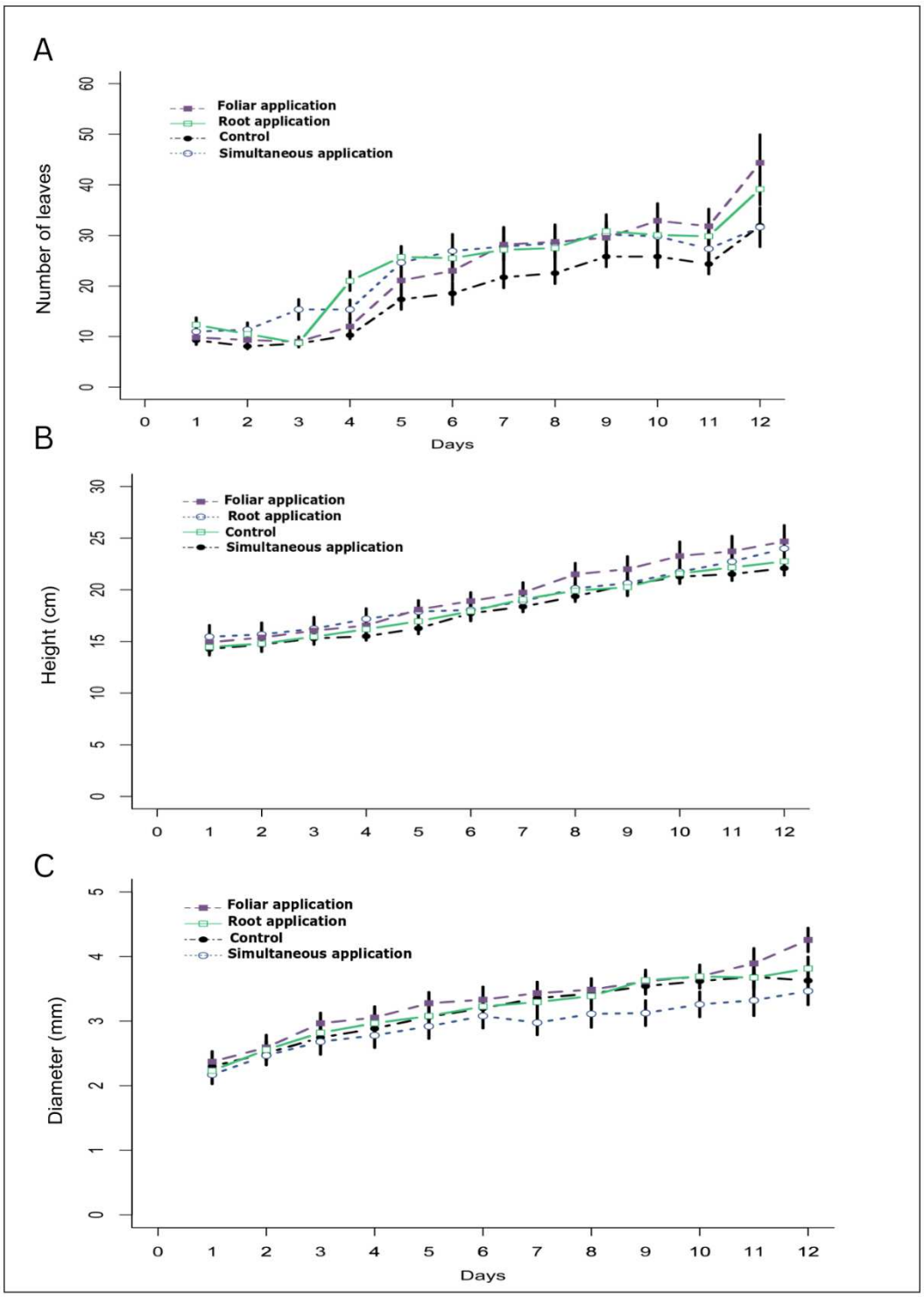

Fig S1 Development of eucalyptus seedlings using different *Trichoderma* fungus inoculum methods, using TR71 isolate. These are foliar applications (purple), root application (blue), simultaneous application (leaf and root joints - black) and control. Observing the number of leaves (A), plants inoculated with foliar application differed from other application methods (p=0.0004), having a greater number of leaves, and root

application had a difference in simultaneous application (p=0.03). Regarding the height of the plants (B), only the leaf treatment plants differed, being larger (average of 19.50±1.54cm, p<0.05). The diameter (C) showed no difference for any application method.

Table S1. Number of spores produced in rice after ten days by each of the 23 isolates in each of the replications used in the experiment. In bold, the repetitions that presented a spore concentration of at least $1x10^8$ ml-1 are highlighted. Isolates that had at least one repeat at this concentration are those selected for phenotypic characterization.

Isolate	Repetition	Spore_concentration
TR01	A	4.75×10^7
	B	0
	C	1×10^7
TR02	A	5×10^7
	B	5.75×10^7
	C	4.5×10^7
TR03	**A**	$\mathbf{1.75\times10^8}$
	B	7.0×10^7
	C	8×10^7
TR04	**A**	$\mathbf{3.62\times10^8}$
	B	6.25×10^7
	C	5.0×10^7
TR06	A	5.25×10^7
	B	3.5×10^7
	C	9×10^7
TR09	A	4×10^7
	B	2.5×10^7
	C	3.75×10^7
TR10	**A**	$\mathbf{1.15\times10^8}$
	B	$\mathbf{5.03\times10^8}$
	C	$\mathbf{4.48\times10^8}$
TR11	A	6.25×10^7
	B	4.5×10^6
	C	9.75×10^7
TR12	A	1×10^7
	B	1.25×10^7
	C	4.5×10^7
	A	$\mathbf{1.6\times10^8}$

	B	9.0×10^{7}
TR13	C	4.5×10^{7}
	A	2.25×10^{7}
TR14	B	3.75×10^{7}
	C	4.25×10^{7}
	A	5.75×10^{7}
TR17	B	1.25×10^{7}
	C	2×10^{7}
	A	7.25×10^{7}
TR22	B	5×10^{7}
	C	5.75×10^{7}
	A	$\mathbf{1.42\times10^{8}}$
TR23	B	5×10^{7}
	C	3×10^{7}
	A	$\mathbf{8.05\times10^{8}}$
TR29	B	3.75×10^{7}
	C	4.5×10^{7}
	A	2.25×10^{7}
TR31	**B**	$\mathbf{1.75\times10^{8}}$
	C	4.25×10^{7}
	A	$\mathbf{3.03\times10^{8}}$
TR32	B	5.75×10^{7}
	C	$\mathbf{1.32\times10^{8}}$
	A	6.5×10^{7}
TR33	B	5×10^{7}
	C	2.25×10^{7}
	A	5.75×10^{7}
TR49	B	6×10^{7}
	C	7.75×10^{7}
	A	$\mathbf{1.78\times10^{8}}$
TR57	B	8.75×10^{7}
	C	8.5×10^{7}
	A	5.5×10^{7}
TR60	B	6.75×10^{7}
	C	1.25×10^{7}
	A	2.5×10^{7}

TR66	B	3.25×10^{7}
	C	7.5×10^{7}
TR71	**A**	**6.4×10^{8}**
	B	**7.02×10^{8}**
	C	**5.72×10^{8}**

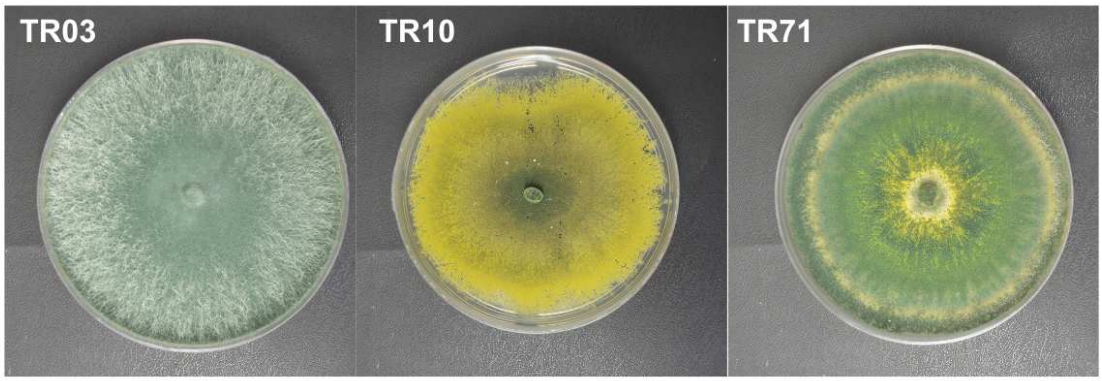

Fig S2 Different colony morphologies of the three *Trichoderma* isolates used in the endophytism experiment - TR03, TR10 and TR71. After nine days of incubation at 25ºC in complete darkness on potato dextrose agar (PDA).

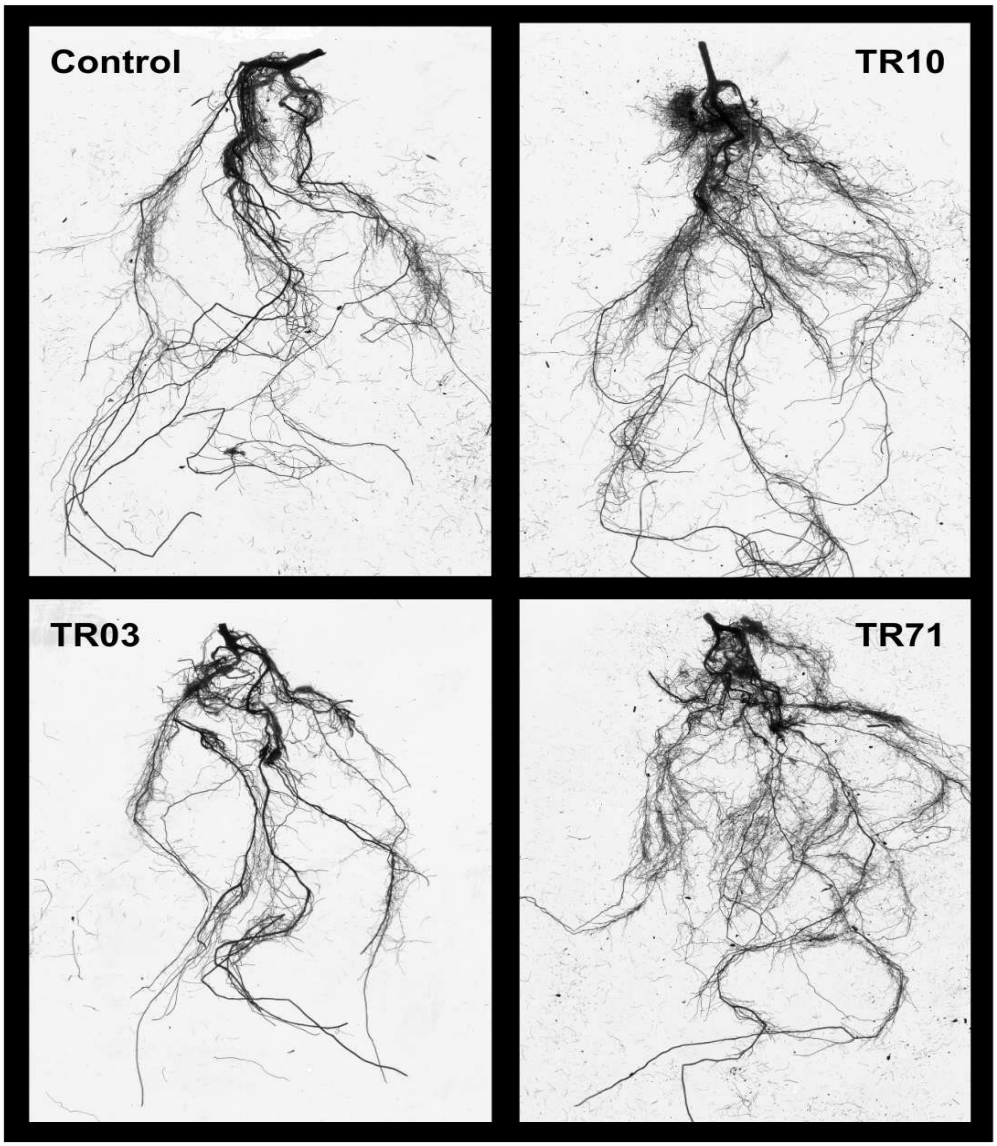

Fig. S3 Roots of the four treatments of the endophytism experiment - Control, and the *Trichoderma* isolates TR10, TR03 and TR71. At the end of 30 days of experiment. Image obtained using a scanner coupled to a computer with the WinRhizo 2013c software.