HANNA LORENA ALVARADO MORENO

ENDOPHYTIC *Trichoderma* spp. FOR THE CONTROL OF COMMON BEAN ANTHRACNOSE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Marisa Vieira de Queiroz

Coorientadores: Leandro Grassi de Freitas
Thalita Suelen Avelar Monteiro

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APROVADA: 30 de agosto de 2021.

Assentimento:

Hanna Lorena Alvarado Moreno
Autora

Marisa Vieira de Queiroz
Orientadora

A mi abuela Amelia Ramírez Pérez (*in memorian*), mujer futurista, cuyo deseo de aprender y comprender vive en mí, dedico.

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"Observa profundamente la naturaleza y entonces lo entenderás todo mucho mejor".

(Albert Einstein)

RESUMO

ALVARADO-MORENO, Hanna Lorena, D.Sc., Universidade Federal de Viçosa, agosto de 2021. ***Trichoderma* spp. endofíticos para o controle da antracnose do feijoeiro comum**. Orientadora: Marisa Vieira de Queiroz. Coorientadores: Leandro Grassi de Freitas e Thalita Suelen Avelar Monteiro.

Fungos do gênero *Trichoderma* podem proteger as plantas por mecanismos de ação como o micoparasitimo, antibiose, competição e/ou indução de resistência. Isolados provenientes de ambientes megadiversos como a floresta amazônica podem ter potencial de proteger plantas de interesse agronômico e fornecer informações que amplifiquem o conhecimento que hoje se tem sobre o gênero *Trichoderma*. Os objetivos do presente trabalho foram: selecionar isolados de *Trichoderma* spp. endofíticos provenientes da floresta amazônica com potencial como agentes de controle biológico (CB) da antracnose do feijoeiro comum (*Phaseolus vulgaris*), ocasionada pelo fitopatógeno *Colletotrichum lindemuthianum* e conhecer os mecanismos de ação pelos quais esses isolados selecionados conseguem proteger as plantas frente ao fitopatógeno. Inicialmente, foi feita a avaliação de 20 isolados de *Trichoderma* spp. em casa de vegetação e foram selecionados quatro isolados: *Trichoderma sp.* VIC44363 17F (T17F), *Trichoderma sp.* VIC44364 22F (T22F)*, Trichoderma koningiopsis* 24F (Tk24F) e *Trichoderma erinaceum* 610F (Te610F) que também promoveram o crescimento do feijoeiro comum. Os isolados T22F, Tk24F e Te610F foram estudados quanto ao mecanismo de ação, seja este o micoparasitismo, antibiose, competição e/ou indução de resistência. Para isso foi avaliado se o local de aplicação do antagonista, antes da aplicação do fitopatógeno, teria influência na eficiência do CB. Os isolados T22F*,* Tk24F e Te610F protegeram a planta quando foram aplicados à raiz e às folhas de forma preventiva, mas destacou-se o isolado Tk24F, pois protegeu a planta de forma sistêmica quando foi aplicado unicamente na raiz e de forma local quando foi aplicado na parte aérea; e da mesma forma o isolado Te610F destacou-se porque teve um efeito local, uma vez que a severidade da doença foi diminuída pela aplicação na parte aérea. Posteriormente com os experimentos *in vitro,* determinou-se que os isolados Tk24F e Te610F foram os antagonistas mais eficientes a *C. lindemuthianum* pelos mecanismos de micoparasitismo, antibiose e competição. Finalmente, o isolado T17F foi estudado mais detalhadamente devido a possibilidade de ele produzir micotoxinas que limitam sua utilização como agente de CB, pois pertence ao clado Brevicompactum, que é reconhecido por produzir micotoxinas do tipo tricotecenos. Foi feita a caracterização do isolado T17F mediante experimentos *in vitro* em que

foram avaliados os mecanismos de micoparasitismo, antibiose e competição e foi analisado o genoma deste isolado quanto a presença de *clusters* relacionados ao metabolismo secundário. Mediante testes *in vitro* foi determinado que a antibiose é o mecanismo utilizado pelo isolado T17F para antagonizar a *C. lindemuthianum* e outros fungos fitopatogênicos. Foi comparado o *cluster* de tricotecenos de *Trichoderma sp.* VIC44363 com os *cluster* de *T. brevicompactum* e *T. arundinaceum* e foi concluído que *Trichoderma sp.* VIC44363 tem potencial para produzir a micotoxina Tricodermina, mas pode não produzir Harzianum A, pois carece do gene *tri23*. *Trichoderma sp.* VIC44363 tem o potencial para produzir novos compostos, pois possui *clusters* diferentes dos encontrados em *T. brevicompactum. Trichoderma erinaceum* 610F e *T. koningiopsis* 24F são potenciais agentes de biocontrole de *C. lindemuthianum,* devido ao uso conjunto de mecanismos que inibem a infeção de *C. lindemuthianum*.

Palavras-chave: Endófitos. Transformação com RFP. Colonização. qPCR. Transcriptômica. Genômica. Brevicompactum. Viride.

ABSTRACT

ALVARADO-MORENO, Hanna Lorena, D.Sc., Universidade Federal de Viçosa, August, 2021. **Endophytic *Trichoderma* spp. for the control of common bean anthracnose**. Adviser: Marisa Vieira de Queiroz. Co-advisers: Leandro Grassi de Freitas and Thalita Suelen Avelar Monteiro.

Fungi of the *Trichoderma* genus can protect plants by mechanisms of action such as mycoparasitism, antibiosis, competition and/or systemic resistance induction. Isolates from megadiverse environments such as the Amazon rainforest may have the potential to protect plants of agronomic interest and provide information that amplify the current knowledge of the *Trichoderma* genus. The objective of the present work was to select *Trichoderma* spp. endophytes from the Amazon Forest with potential for use as biological control (BC) agents of common bean anthracnose caused by the phytopathogen *Colletotrichum lindemuthianum* and to know the mechanisms of action by which these selected isolates were able to protect the plants against the phytopathogen. First, 20 *Trichoderma* spp. isolates were evaluated in a greenhouse and four isolates were selected: *Trichoderma sp.* VIC44363 17F (T17F), *Trichoderma sp.* VIC44364 22F (T22F), *Trichoderma koningiopsis* 24F (Tk24F) and *Trichoderma erinaceum* 610F (Te610F) which also promoted the growth of common bean. The isolates T22F, Tk24F and Te610F were studied regarding the mechanism of action, whether it is mycoparasitism, antibiosis, competition and/or resistance induction. For this, it was evaluated whether the application site of the antagonist, before the application of the phytopathogen, would influence the BC efficiency. Isolates T22F, Tk24F and Te610F protected the plant when they were applied to the root and leaves in a preventive way, but the isolate Tk24F stood out because it protected the plant in a systemic way when it was applied only to the root and locally when it was applied in the aerial part; similarly, the isolate Te610F stood out because it had a local effect as the disease severity was reduced by application to the aerial part. Subsequently, with the *in vitro* experiments, it was determined that the isolates Tk24F and Te610F were the most efficient antagonists to *C. lindemuthianum* due to the mechanisms of mycoparasitism, antibiosis and competition. Finally, with the T17F isolate, a separate study was carried out due to the possibility of it producing mycotoxins that limit its use as a BC agent, as it belongs to the Brevicompactum clade, which is recognized for producing mycotoxins of the trichothecenes type. The characterization of the T17F isolate was carried out through *in vitro* experiments in which the mechanisms of mycoparasitism, antibiosis and competition were evaluated, and the genome was sequenced to perform a comparative analysis with genomes from other species

belonging to the same clade. By *in vitro* test it was determined that antibiosis is the mechanism used by the T17F isolate to antagonize *C. lindemuthianum* and other phytopathogenic fungi. By comparing trichothecenes cluster of the isolate *Trichoderma sp.* VIC44363 with the cluster of *T. brevicompactum* and *T. arundinaceum*, it was concluded that *Trichoderma sp.* VIC44363 can produce the mycotoxin trichodermin, as it lacks the *tri23* gene. *Trichoderma sp.* VIC44363 has the potential to produce new compounds, as it has a cluster that is different from those found in *T. brevicompactum*. *Trichoderma erinaceum* 610F and *T. koningiopsis* 24F are potential biocontrol agents for *C. lindemuthianum*, due to the joint use of mechanisms that inhibit the infection of *C. lindemuthianum*.

Keywords: Endophytes. Transformation with RFP. Colonization. qPCR. Transcriptomics. Genomics. Brevicompactum. Viride.

SUMÁRIO

INTRODUÇÃO

O feijoeiro comum (*Phaseolus vulgaris* L.) é uma cultura importante no Brasil pela sua relevância alimentar, econômica e social. Constitui a base da dieta alimentar do brasileiro e é a quarta cultura mais plantada no Brasil depois da soja, do milho e da cana-de-açúcar. O feijoeiro, que é plantado em quase todos os estados do Brasil, é principalmente cultivado por pequenos e médios agricultores cujas famílias dependem economicamente da sua produção. Porém, a produtividade é afetada pela pouca tecnificação das lavouras que são alvo de pragas e doenças. A antracnose do feijoeiro, ocasionada pelo fungo *Colletotrichum lindemuthianum* (Sacc. & Magnus), destaca-se entre as doenças mais limitantes da cultura devido à variabilidade genética que o fungo apresenta, que leva à quebra da resistência de cultivares selecionados em programas de melhoramento genético.

Diante disso, além da obtenção de novos cultivares, novas alternativas econômica e ambientalmente sustentáveis de controle de doenças precisam ser desenvolvidas para essa cultura. O controle biológico é uma dessas alternativas, pois são usados organismos que se associam a plantas de forma natural no campo, ocasionando menores impactos no ambiente do que os fungicidas químicos. Fungos do gênero *Trichoderma* se destacam entre os microrganismos usados como agentes de biocontrole por apresentarem plasticidade ecológica na interação com plantas.

O gênero *Trichoderma* tem uma longa história sobre seu potencial no controle biológico de fitopatógenos de mais de 80 anos de pesquisas. Um sem-número de estudos tem sido focados no potencial do gênero *Trichoderma* como agente de biocontrole desde que Weindling em 1932 relatou a sua capacidade de parasitar outros fungos. Tradicionalmente *Trichoderma* spp. têm sido descritos como fungos cosmopolitas do solo, possivelmente porque eram frequentemente isolados desse ambiente no mundo inteiro, associando-os ao fenômeno de solos supressivos. No entanto, atualmente sabe-se que *Trichoderma* spp. habitam muitos outros ecossistemas além do solo, especialmente madeira em decomposição e basidiocarpos, e que só algumas espécies como *T. harzianum* e *T. asperellum* são cosmopolitas. Também foi observado que no genoma de espécies de *Trichoderma* utilizadas no controle biológico de fitopatógenos há um amplo número de genes que codificam para enzimas hidrolíticas, e outras enzimas envolvidas na biossíntese de metabólitos secundários, transporte, catabolismo e biodegradação xenobiótica. Assim, *Trichoderma* spp. possuem a plasticidade genética para se adaptar a diferentes ambientes, por exemplo os tecidos

internos de plantas como endófitos e podem proteger as plantas contra patógenos por diferentes mecanismos como o micoparasitismo, antibiose, competição e indução de resistência. Isolados de *Trichoderma* spp. amplamente usados no biocontrole podem empregar mais de um desses mecanismos.

Grande parte da informação sobre a capacidade de atuação como controladores biológicos e os mecanismos de ação empregados por *Trichoderma* spp. no controle de fungos fitopatogênicos está baseada em estudos feitos com isolados de espécies oportunistas frequentemente isoladas do solo, como por exemplo *T. harzianum, T. virens* e *T. atroviride* que controlam fitopatógenos do solo. No clado Viride, a maior parte desses estudos tem sido feita com as espécies *T. viride* e *T. atroviride*. Todas as pesquisas realizadas com essas espécies têm sido muito importantes para construir o conhecimento que hoje se tem sobre o gênero *Trichoderma*. No entanto, nesse trabalho foram avaliados isolados de outras espécies menos estudadas de *Trichoderma* (*T. ararianum*, *T. endophyticum*, *T. endoviride*, *T. erinaceum*, *T. koningiopsis*, *T. lentiforme* e *T. ovalisporum*) e amplamente estudadas como *T. virens*, endófitos de seringueiras da floresta amazônica brasileira, quanto a capacidade de controlar o fitopatógeno foliar *C. lindemuthianum* em casa de vegetação. Posteriormente, os isolados selecionados foram estudados em condições controladas quanto aos mecanismos de ação empregados nesse controle, tais como o micoparasitismo, antibiose, competição e indução de resistência. Para determinar a capacidade desses isolados de micoparasitar a *C. lindemuthianum* e de colonizar as raízes e folhas do feijoeiro comum foram transformados geneticamente para expressar o gene da proteína fluorescente. Adicionalmente com o isolado de *T. koningiopsis* (Tk24F) foi utilizada uma abordagem transcriptômica para determinar a sua capacidade de ativar os genes associados ao sistema de defesa do feijoeiro comum. Finalmente, com o isolado de *Trichoderma sp.* VIC44363 (T17F) foi utilizada uma abordagem genômica para determinar seu potencial de produzir micotoxinas que impeçam a sua utilização como agente de controle biológico, mas também para determinar seu potencial de produzir novos compostos.

Capítulo 1

Mechanisms of action of endophytic *Trichoderma* isolates in the interaction with *Colletotrichum lindemuthianum*

Mechanisms of action of endophytic *Trichoderma* isolates in the interaction with *Colletotrichum lindemuthianum*

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Keywords: Plant-microbe interaction, mycoparasitism, colonization, genetic transformation, red fluorescence protein (RFP), *Trichoderma erinaceum*, *Trichoderma koningiopsis*.

ABSTRACT

Studies about mechanisms of action used by biological control agents provide important information in the selection and registration process of their products. There are a growing number of *Trichoderma* spp. described as endophytic, but there is not information on the relationship of some species with the host plant and on the mechanisms of action used in the antagonism of foliar phytopathogenic fungi. Therefore, 20 endophytic isolates of different *Trichoderm*a species from the Brazilian Amazon Forest were evaluated for the control of *Colletotrichum lindemuthianum*, the etiologic agent of anthracnose in common bean (*Phaseolus vulgaris* L.). Isolates *Trichoderma sp.* VIC44363 17F (T17F), *Trichoderma sp.* VIC44364 22F (T22F), *T. koningiopsis* 24F (Tk24F) and *T. erinaceum* 610F (Te610F) were selected for reducing the severity of symptoms in the greenhouse and promoting the growth of the aboveground part of the plant. Therefore, selected isolates were studied under controlled conditions regarding the use of direct or indirect mechanisms to protect common bean (*Phaseolus vulgaris* L.) against attack by *C. lindemuthianum*. Tests of antagonism in the greenhouse and *in vitro*, of colonization of common bean tissues and evaluation of the expression of genes related to the plant's defense mechanisms were carried out. It was determined that the isolates Tk24F and Te610F were the most efficient antagonists to *C.*

lindemuthianum due to the mechanisms of mycoparasitism, antibiosis and competition. It was proven that the isolate T22F can mycoparasitize and inhibit the growth of *C. lindemuthianum*, but less efficiently than the other studied isolates. *T. erinaceum* 610F and *T. koningiopsis* 24F are potential biocontrol agents against *C. lindemuthianum,* due to the joint use of mycoparasitism, antibiosis, and competition mechanism, thereby inhibition of the infection may be the prevalent mechanism of action to control *C. lindemuthianum* in this study.

INTRODUCTION

Trichoderma spp. can protect the plant against the attack of pathogens through several mechanisms of action. Knowing these mechanisms of action is advantageous in developing strategies for their use in the field, in determining possible risks to human health and the environment, and constitutes important information for the product registration process in which biological control agents are formulated (Kabaluk et al., 2010; Köhl et al., 2019). These mechanisms can be direct, such as mycoparasitism and antibiosis, or indirect, such as competition and the induction of systemic resistance in plants. However, it is often observed that prominent biological control agents can use more than one mechanism to antagonize phytopathogens (Köhl et al., 2019).

Mycoparasitism is the mechanism of action traditionally most studied in *Trichoderma*, generally used to refer to the destructive parasitism that ends in the death of the host/prey also qualified as necrotrophic (Karlsson et al., 2017; Sun et al., 2019) and mycotrophic (Druzhinina et al., 2011). However, necrotrophism will depend on the type of *Trichoderma* isolate, the host isolate and the state of the interaction (Karlsson et al., 2017; Druzhinina et al., 2011). In general, in mycoparasitism, morphological changes are observed as a positive tropism towards the prey (Deb and Das, 2020; Viterbo and Horwitz, 2010); coiling around hyphae, which has been associated with the presence of lectins (Barak et al., 1985) and involving heteromeric G-proteins and cyclic adenosine monophosphate (cAMP) (Omero et al., 1999; Rocha-Ramírez et al., 2002; Zeilinger et al., 2005). Lateral hyphae branches, finger-like clamps, papilla-like, appressoria and hook-like structures are also seen (Druzhinina et al., 2011; Monteiro et al., 2010; Tzean and Estey, 1992). In confrontation or paired culture experiments, growth over the host/prey fungus colony is also observed (Monteiro et al., 2010; You et al., 2016).

Trichoderma spp. can inhibit the growth and spore germination of phytopathogens by the antibiosis mechanism. *Trichoderma* species produce a wide variety of secondary metabolites (SM) with antimicrobial activity and involved in the induction of plant disease resistance. SM that stand out in *Trichoderma* spp. are siderophores, epipolythiodioxopiperazines (ETP), steroids, terpenoids, pyrones and peptaibols (Hermosa et al., 2014). Among the pyrones is 6-pentyl-alpha-pyrone (6-PP) associated with the coconut smell characteristic of some species of the Viride clade that has antimicrobial activity (Bisby, 1939). In addition, 6-PP can act as an auxin in the plant, which shows that SM can also be involved in resistance induction and plant growth promotion (Vinale et al., 2008).

Fungi of the *Trichoderma* genus can also protect plants by indirect mechanisms by inducing systemic plant resistance or by competing for space and nutrients with phytopathogenic microorganisms (Köhl et al., 2019). *Trichoderma* spp. can induce systemic resistance (ISR) to diseases by activating genes for resistance of the jasmonic acid and ethylene (JA/ET) signaling pathway in the plant, such as those that code for lyase-hydroperoxidase, peroxidases and ammonia lyase phenylanine. In addition, isolates of *Trichoderma* spp. produce nitrilases that participate in the biosynthesis pathway of ethylene or indole acetic acid, which are pheromones that promote plant root growth (Druzhinina et al., 2011). On the other hand, isolates from *Trichoderma* spp. can compete for space and nutrients secreted by plant roots due to their ability to grow quickly and in a wide variety of substrates. This is evidenced in its generalist genotype and ability to grow on different carbon sources, which facilitates association with plants (Baroncelli et al., 2016; Kubicek et al., 2011). The ability to use sucrose as a carbon source is associated with the symbiosis of *Trichoderma* species because the fungus can feed on sucrose present in the intercellular regions of plants, which can favor roots colonization and symbiosis establishment (Vargas et al., 2009).

Most of this information has been very well documented in the Viride clade with isolates of *Trichoderma viride* and *T. atroviride* species (Viterbo and Horwitz, 2010), however, isolated from less studied species such as *T. erinaceum* (Te) and T. *koningiopsis* (Tk), in this work showed to reduce the severity of anthracnose in bean plants in the greenhouse. There is little information about the mechanisms of action that isolates from these species can use in the antagonism of foliar phytopathogens such as *Colletotrichum lindemuthianum*. Therefore, the objective of the present

work was to characterize the isolates T22F, Tk24F and Te610F regarding their mechanisms of action in the control of *C. lindemuthianum* and the spectrum of action *in vitro* on other common bean phytopathogens. The ability to colonize roots and leaves in an alternative host plant was also evaluated.

RESULTS

Selection of *Trichoderma* spp. for the control of *C. lindemuthianum* in a greenhouse

Twenty isolates of different *Trichoderma* species were evaluated in two experiments, each with a control in which only the phytopathogen was applied (Pathogen Control) and a reference isolate that was evaluated in both experiments (610F). The coefficient of variation in the first experiment was 21.77% and in the second experiment 20.27%. In total, eight isolates showed differences in relation to pathogen control for the transformed variable of disease severity (Fig. 1). The isolates corresponded to four species: *Trichoderma* sp. VIC44364 (22F), *Trichoderma* sp. VIC44363 (14F and 17F), *T. erinaceum* (20F and 610F) and *T. koningiopsis* (24F and 265F). One isolate per species was selected for the following studies as they came from the same collection point. Isolates *Trichoderma* sp. VIC44363 17F (T17F), *Trichoderma* sp. VIC44364 22F (T22F), *T. koningiopsis* 24F (Tk24F) and *T. erinaceum* 610F (Te610F) decreased the disease severity in comparison with the pathogen control treatment (Fig. 1; Supplementary Figure 1).

Evaluation of the application site of selected isolates of *Trichoderma* spp., effective in the control of *C. lindemuthianum* in a greenhouse

In the selection experiments, conidia suspensions from the isolates were applied to the radicle of germinated seeds, before planting, and later to the root and shoot of the plants. Therefore, the effect of applying *Trichoderma* spp. only in the radicle and root and only in the aerial part compared to the three applications made in the experiment in which they were previously selected for decreasing the severity of the disease, to determine whether the effect in decreasing the severity of anthracnose was systemic (by the application in the radicle and root), local (by application to the aerial part) or the sum of the three applications (Table 1). In this case, three treatments were evaluated for each of the isolates and the control where only the pathogen was applied.

It was observed that in the experiments where both the root and the shoot had been treated, there were differences compared to the control treatment as previously observed in selection

experiments. However, the treatment in which the isolates T17F and T22F was applied only to the root or only in the aerial part, in the two repetitions of the experiment had no effect (Table 1). In contrast, isolate Tk24F reduced disease severity when applied to the root or aerial part, and isolate Te610F when applied to aerial part (Table 1).

Evaluation of selected isolates of *Trichoderma* spp. for growth promotion of common bean plants in a greenhouse

The four selected isolates induced a significant dry and fresh mass increase of the aboveground part of the plants (Table 2). The variables dry mass and fresh mass of the root did not show differences in the analysis of variance with the control where *Trichoderma* spp. isolates were not applied ($p=0.35$). In the second experiment, the same result as in the first experiment was observed.

Genetic transformation for fluorescent protein expression

For the analysis of mycoparasitism and colonization of common bean by *Trichoderma* spp. the transformation of the isolates T22F, Tk24F and Te610F with plasmids carrying genes encoding fluorescent proteins was performed. The isolate T17F was studied in a chapter apart since it belongs Brevicompactum clade. Protoplasts of isolates T22F, Tk24F and Te610F were transformed with 10 µg of plasmid DNA pPGW (RFP). Colonies of transformants were observed 3 days after addition of selection medium. 2 (0.2/µg of DNA), 22 (2.2 transformants/µg of DNA) and 15 (1.5/µg of DNA) transformants of the isolates T22F, Tk24F and Te610F, respectively, were obtained.

As plasmids randomly integrated into the genome of *Trichoderma* spp. and as the transformants are expressing a heterologous protein, they were evaluated in relation to different characteristics, such as radial growth rate (RGR), growth inhibition capacity against *C. lindemuthianum* (IM), mitotic stability (MS) and the efficacy in decreasing the severity of anthracnose in common bean (E) controlling wild isolates. Changes in RGR have been observed in some transformants that express RFP. Based on these results, transformants 22RFP-T1 (*Trichoderma* sp. VIC44364), 24RFP-T30 (*T. koningiopsis*) and 610RFP-T3 (*T. erinaceum*) were selected for mycoparasitism and common bean colonization experiments, as they showed no differences with wild isolates in RGR, IM, EM and E, by the t-student test ($p>0.05$) (Supplementary

Table S1). The presence of two plasmid copies in transformants 22RFP-T1 and 24RFP-T30, and a single copy in transformant 610RFP-T3 was determined (Supplementary figure S2).

Mycoparasitism of *C. lindemuthianum* by *Trichoderma* spp.

Trichoderma spp. were evaluated for the mycoparasitism capacity by the direct confrontation methodology to *C. lindemuthianum* by means of microscopical and macroscopical observations.

The same sequence of events was observed in the three *Trichoderma* spp. isolates evaluated in the process of formation of mycoparasitism structures (short lateral hyphal branches or finger-like clamps) (Fig 2). First, a parallel growth of hyphae of the three *Trichoderma* spp. evaluated to the hyphae of *C. lindemuthianum* before the formation of short lateral hyphal branches or clamp-like structures above the hyphae of the plant pathogen (Fig. 2A – 2B and 2E – 2F). Subsequently, an increase in the number of lateral hyphae or finger-like clamps was observed, which in some cases led to a decrease in the green fluorescence of the phytopathogen in the slides of isolates Tk24F and Te610F (Fig. 2C and 2G). The coiling of the hypha of *Trichoderma* spp. around the hyphae of the phytopathogen was observed only once on a slide of the Tk24F isolate (Fig. 2D and 2H).

Although mycoparasitism was observed in the three isolates evaluated, differences were observed in the amount and the time when short lateral hyphal branches or clamps-like structures were formed on a hypha segment of the phytopathogen (Figs. 3 and 4). In the slides observed 4 days after *Trichoderma* isolates inoculation in front of *C. lindemuthianum*, the T22F isolate grew concomitantly with the hyphae of the phytopathogen (Fig. 3A – 3D) and only in the observations made on the 8th day it was detected the formation of short lateral hyphal branches in some points of the slide (Fig. 4B – 4C). In the slides of isolates Tk24F and Te610F, the formation of short lateral hyphal branches or finger-like clamps was observed 4 days after inoculation (Fig. 3E – 3L) with a greater number of finger-like clamps per hypha segment of the isolate Te610F (Fig. 3I – 3L).

The Tk24F isolate seems to have an intermediate performance between the other two isolates, because in the observations made on the 4th day after inoculation, although the formation of finger-like clamps was observed, they occurred in lower quantity and density (number of finger-

like clamps per segment of hyphae) than those observed on Te610F slides (Fig. 3H and 3L). Mycotrophy was observed in isolates Tk24F and Te610F where the hyphal segments of the pathogen that were covered with the antagonist hyphae showed reduction or loss of green fluorescence (Fig. 3G and 3K).

The confrontation experiment showed the mycoparasitism capacity of the studied isolates, as they all grew over the phytopathogen colony (Fig. 4A, 4D and 4G). The lower mycoparasitism capacity of the T22F isolate was observed in the direct confrontation experiment, since there was not complete coverage of the pathogen colony, as occurred with the other two *Trichoderma* isolates (Fig. 4A).

Antibiosis of *C. lindemuthianum* by *Trichoderma* spp.

The inhibitory effect of *Trichoderma* spp. antimycotic compounds defunded throw cellophane membrane, on the colony growth and germination of conidia of the *C. lindemuthianum*, was evaluated. Isolate Te610F stood out in inhibiting the growth of the colony of *C. lindemuthianum* compared to isolates T22F and Tk24F. Isolate Te610F inhibited growth by 100% ±0.0% in both replicates of the experiment, followed by isolate Tk24F with 27.9% ±1.9% in the first experiment and 32.5% ±2.3% in the second experiment and to a lesser extent the isolate T22F with 8.7% ±0.8% and 8.2% ±7.7% inhibition.

The evaluation of germination inhibition of *C. lindemuthianum* conidia by compounds produced by *Trichoderma* spp. *in vitro* showed that all isolates secrete compounds with inhibiting capacity (Table 3). First, it was observed that the germination of *C. lindemuthianum* conidia was favored by the preparation of the culture medium in the liquid of an infusion carried out with parts of the plant, with around 90% of germination in the medium with seed infusion and 22%-35% of germination in PDA medium prepared with water (Table 3). On the other hand, isolate Te610F inhibited 100% of the germination of *C. lindemuthianum* conidia only when the medium contained the infusion of leaves or seeds, unlike the isolate Tk24F which inhibited germination indistinctly from the addition or not of infusion of plant parts to the medium. The isolate T22F, on the other hand, seems to have promoted the germination of the pathogen's conidia, as a higher percentage of germinated conidia was quantified in the treatment plates than in the control plates, and in addition, a greater length of the germ tubes was observed (Table 3). However, when the filtrate from the

suspension of conidia of the same isolate (T22F) was applied, before the application of the conidia of the phytopathogen, germination was effectively inhibited, contrary to what was observed when the filtrates of the other isolates were applied since they were better when applied in the suspension of conidia (Table 3).

Growth inhibition of *C. lindemuthianum* on leaf discs by *Trichoderma* spp. (mycoparasitism and/or antibiosis)

Inhibition of *C. lindemuthianum* infection in disinfested common bean leaf discs treated with *Trichoderma* spp. was evaluated. The number of leaf discs with signs of infection by the phytopathogen (incidence) decreases, but the treatment had no effect on reducing the size of lesions caused by the phytopathogenic fungus (severity) (Table 4). Isolate Te610F reduced the incidence of lesions both by applying conidia suspensions and suspension filtrate, unlike the isolates T22F and Tk24F, which only prevented the formation of lesions when conidia suspensions were applied (Table 4).

Colonization of common bean by *Trichoderma* spp. (competition potential)

On the first day of evaluation of root colonization by the 22RFP-T1 transformant (*Trichoderma sp.* VIC44364) just conidia were observed in the epidermis of the radicles. On the second day, growth of the fungus was observed in the root cap and epidermis of the three root zones studied (Fig. 5A – 5C). On the third day hyphae were observed growing in the exodermis of cuts made in the cell division zone and in the maturation zone (Fig. 5D – 5E) and on the fourth day fluorescence was observed in the cortex and in the zone of the conducting vessels (Fig. 5F) and hyphae growing on the leaf epidermis (Fig. 5G – 5H). Growth of the 22RFP-T1 transformant was not observed in the cuts made in the seed radicles that were transferred to the substrate in pots.

Conidia of the transformant 24RFP-T30 (*T. koningiopsis*) adhered to the epidermis of the radicles were observed on the first day of evaluation. On the second day of evaluation, growth of the fungus was observed in the cap and in the cell division zone up to the cortex (Fig. 6A – 6C), in the exoderm and in the cortex of the elongation and maturation zone (Fig. 6D – 6G). In observations made of germinated seeds transferred to the substrate in pots, growth of the fungus in the cortex of the maturation zone was observed four days after fungus inoculation (Fig. 6H). Likewise, two and four days after inoculation, the development of the fungus in the epidermis of leaf tissues in points

with small necrotic spots were observed (Fig. 6I – 6J). Some of the radicles of the germinated seeds kept in the cups were soft in the elongation and cell division zone on the fourth day.

On the first day of evaluation, hyphae of the 610RFP-T3 transformant (*T. erinaceum*) were observed growing in the root cap (Fig. 7A − 7B) and on the second day up to the cortex of the cell division zone (Fig. 7C – 7D). Growth was also observed in the epidermis of the elongation and maturation zone (Fig. 7E − 7H) and in the cortex and conductive vessels fourth days after fungus inoculation (Fig. 7I − 7L). Colonization of the epidermis of the radicles was also observed on the fourth day in germinated seeds transferred to the substrate (Fig. 7M) and in leaves inoculated with the fungus (Fig. 7N – 7P). In addition to the leaf epidermis, growth of the fungus was also observed in xylem cells on the fourth day of evaluation and small necrotic spots where the 610RFP-T3 transformant was growing (Fig. 7O and 7P).

Induction of resistance of common bean to *C. lindemuthianum* by *T. koningiopsis*

The induction of resistance genes in common bean was evaluated with the isolate Tk24F because, in the evaluation in a greenhouse, it was the only isolate that, when applied to the root, reduced the severity of anthracnose in common bean. The genes studied are involved in six processes of the common bean defense induction system (Mayo et al., 2016): (i) regulation of the balance between responses to necrotrophic and biotrophic pathogens: *WRKY33* (WRKY transcription factor); (ii) genes related to pathogenesis: *PR1* (pathogenesis related 1), *PR2* (β 1-3 endoglucanase), *PR3* (chitinase class I) and *PR4* (pathogenesis related 4); (iii) ethylene signaling pathway: *ERF1* (ethylene-responsive transcription factor 1), *ERF5* (ethylene-responsive transcription factor 5) and *CH5b* (endochitinase precursor); (iv) phytoalexin biosyntheses: *PAL1* (phenylamine and histidine ammonia-lyase); (v) osmotin biosynthesis; *OSM34* (osmotin-like protein); and (vi) Ca2+ signaling: *CNGC2* (cyclic nucleotide-gated ion channel 2) (Supplementary Table S2).

In general, higher expression levels of the evaluated genes related to defense mechanisms were observed in plants that were treated only with the phytopathogen *C. lindemuthianum* or together with the isolate Tk24F (Fig. 8). The same trend in the expression levels of the genes evaluated was observed with the two genes used as normalizers, *Act11* and *Ide* (Fig. 8; Supplementary Figure S3). Eight of the 11 genes evaluated showed greater expression in the

treatment with the phytopathogen, but due to the variances in the expression values of some of the genes evaluated not being statistically homogeneous ($p<0.05$), it was not possible to determine whether the difference in the expression of these genes was significant (Supplementary Table S3). However, when the isolate Tk24F was previously applied to the root, there was lower expression of the *PR1*, *PR2*, *PR3*, *PR4*, *ERF5*, *CHA5b* and *PAL1* genes, and higher expression of the *ERF1* gene. With the *WRKY33*, *PR1* and *ERF1* genes, it was possible to establish significant differences in expression (Supplementary Table S3). The *WRKY33* gene showed a significant increase in expression (1.760 times greater than in the control treatment) in plants treated with the isolate Tk24F in the root and with the phytopathogen. On the other hand, the *PR1* gene showed a significant increase in expression in plants treated with the isolate Tk24F in leaves and with the phytopathogen or treated only with the phytopathogen, which represented an increase of 9.790 and 6.480 times compared to the control treatment, respectively. As for the *ERF1* gene, there was an increase in expression in plants treated with the isolate Tk24F both in the root and in the leaves and with the phytopathogen or treated only with the phytopathogen, which represented increases of 3.732, 2.350 and 2.261 times compared to the control treatment, respectively. The only gene that had reduced expression was *CNGC2* in treatments where the phytopathogen was applied (Fig. 8), but without statistically significant differences by ANOVA analysis ($p>0.05$) (Supplementary Table S3). It was also the only gene where the exclusive application of the isolate Tk24F in the root had a different effect from the control treatment, showing less expression than the treatments where the phytopathogen was applied (Fig. 8).

Potential to control other common bean phytopathogens by *Trichoderma* spp. (mycoparasitism and antibiosis)

Isolate Te610F showed a greater potential to control *Fusarium oxysporum* f. sp. *phaseoli* and *Sclerotinia sclerotiorum* compared to the other isolates studied. Isolate Te610F completely covered the colony of *F. oxysporum* and *S. sclerotiorum* while the other isolates only partially covered the colonies of the phytopathogens (Supplementary Figure S4). Isolate Te610F also inhibited the growth of the two phytopathogens to a greater extent compared to T22F and Tk24F (Table 5).

DISCUSSION

For the first time it is reported that *Trichoderma* sp. VIC44363 (Brevicompactum clade), *Trichoderma* sp. VIC44364, *T. koningiopsis* and *T. erinaceum* (Viride clade) can control *C. lindemuthianum in vivo*. The species that have been reported to control *C. lindemuthianum in vivo* are *T. viride*, *T. asperellum*, *T. harzianum*, *T. strigosum* and *T. theobromicola* on cowpea and bean (Dania and Gbadamosi, 2019; Padder and Sharma, 2011; Pedro et al., 2012). For other *Colletotrichum* species, different species from those reported in this study were also described as biological control agents. For example, species such as *T. harzianum* (T-39), *T. hamatum* (T-105), *T. atroviride* (T-161) and *T. longibrachiatum* (T-166) (Freeman et al., 2004) are capable to control *Colletotrichum acutatum* that infect strawberries in the greenhouse, as well as T22 isolate of *T. harzianum* that is able to control *Colletotrichum graminicola* that causes disease in maize (Harman et al., 2004b).

Isolates *Trichoderma sp.* VIC44364 22F (T22F), *T. koningiopsis* 24F (Tk24F) and *T. erinaceum* 610F (Te610F), all of them belonging Viride clade, and *Trichoderma* sp. VIC44363 17F (T17F) belonging Brevicompactum clade, were selected since they decreased disease severity when applied before the pathogen, aiming at a preventive approach to the subsequent arrival of the pathogen. Applications were made to germinated seeds before planting and to the aerial part and roots 4 days before the application of the phytopathogen. In all experiments it was observe difference in the disease severity between selected isolates and the pathogen control treatments, although differences in the percentage was observe because experiments were established during the winter in different years. Therefore, the second step of the present work was to determine the influence of the application site of the inoculum of the selected isolates of *Trichoderma* spp. in decreasing the severity of the disease. It was observed that the Tk24F isolate had an effect on the disease severity when applied only to the root or only to the aerial part. On the other hand, isolate Te610F reduced symptoms when applied to the aerial part. However, the severity was not lower when the two applications were made with these two isolates. Isolates T17F and T22F reduced the severity just when the two applications were made in all the experiments performed (Table 1).

Given the above, it can be said that the Tk24F isolate had a systemic effect because the severity of a foliar phytopathogen was reduced even when applied only to the root, and local, because it also had an effect when applied only to the aerial part (Table 1). As for the isolate

Te610F, it can be said that the effect of the application was local because the disease severity was reduced by the application in the aerial part (Table 1). Finally, in relation to the isolate T22F, it can be said that it needs both applications to be able to protect the plant against the attack of *C. lindemuthianum*. The T17F isolate was studied in a chapter apart since it belongs Brevicompactum clade. Therefore, the third objective of the present study was to establish by which direct mechanism (mycoparasitism and/or antibiosis) and/or indirect (competition for the colonization of plant tissues) the three isolates belonging Viride clade could control *C. lindemuthianum*, and if the isolate Tk24F could induce systemic resistance in the plant, as it was the only isolate that had a systemic effect in protecting the plant by application to the radicle and root. To study mycoparasitism and colonization of common bean tissues, the three isolates were genetically transformed to express the red fluorescent protein.

For the transformation of the three isolates, the methodology that employs PEG-CaCl2 modified by Malmierca et al. (2012) was used. To our knowledge, there are no reports in the literature on the transformation of *Trichoderma sp.* VIC44364 and *T. erinaceum*. Transformants expressing the fluorescent protein gene have been useful for studies of plant tissue colonization (Rajasekaran et al., 2008), histopathological studies (Sarrocco et al., 2006) and quantitative studies of soil proliferation (Orr and Knudsen, 2004), which shows its importance in the study of interaction with plants and other microorganisms. These transformants together with wild isolates were then used to study the mechanisms of action possibly used by these fungi in the control of *C. lindemuthianum* in common bean.

Mycoparasitism in *in vitro* studies has been associated with the ability of fungi to grow on top of the colony of other fungi and the way in which the mycoparasite hyphae are placed on the host/prey hyphae (Monteiro et al., 2010). The three *Trichoderma* isolates studied fulfilled these two premises, but in microscopic observations the isolates Tk24F and Te610F stood out for their aggressiveness evidenced by the greater number of lateral hyphae or hairpins on the prey hyphae that eventually lost fluorescence due to possibly mycoparasite isolate degraded the *C. lindemuthianum* hyphae. The aggressiveness of the isolate Te610F was also evidenced in the direct confrontation test by the disappearance of the mycelium from the phytopathogen colony after 10 days of evaluation (data not shown). On the other hand, the T22F isolate was much less aggressive

to *C. lindemuthianum*, as it took longer to form lateral hyphae or staples and in smaller numbers, and no evidence of disintegration of host hyphae was observed until after 8 days of evaluation.

This correspondence between the aggressiveness with which the isolates grew on top of the phytopathogen in the confrontation test and a greater formation of hairpins and lateral hyphae growing and digesting the hyphae of the phytopathogen confirms that folding precedes the destruction of the prey hyphae (Benhamou and Chet, 1993), although these two phenotypes are not associated with the same regulatory cascade (Reithner et al. 2007). Reithner et al. (2007) observed that mutants Δ*tmk*1 (mitogen-activated protein kinase - MAPK) lost the ability to grow on top of the colony of *Botrytis cinerea* and *Rhizoctonia solani* although coiling continues to be observed in these mutants. However, dense coiling or formation of lateral hyphae and hook-like structures over the prey hyphae prior to hyphae disintegration is a characteristic phenomenon observed in efficient mycoparasite species such as *T. harzianum* T-203 (Benhamou and Chet, 1993) and *T. harzianum* ALL42 (Monteiro et al., 2010).

The mycoparasitism structures observed in this study were the short lateral hyphal branches and finger-like clamps that were formed after growth parallel to the host hyphae, and coiling was observed much less frequently. The fact that coiling was observed less frequently indicates that there must be a variation in the mycoparasitism strategy, which may be due to the species of *Trichoderma* or the host fungus used, as observed at the molecular level and in the analysis of the effectiveness of mycoparasitism in confrontation experiments in other studies (Atanasova et al., 2013; Monteiro et al., 2010; Moreno-Ruiz et al., 2021).

In mycoparasitism, *Trichoderma* uses several biochemical tools such as hydrolytic enzymes and secondary metabolites that enhance its effectiveness (Atanasova et al., 2013; Saravanakumar et al., 2016). Isolates Tk24F and Te610F present these tools, as they were able to secrete antifungal compounds in the culture medium (where they were previously cultivated on cellophane paper) and inhibited the growth of the pathogen on leaf discs. Secondary metabolites such as peptaibols and cell wall degrading enzymes such as chitinases, β-1,3-glucanase, glucosidase, acid phosphatase, mannosidase and proteinases act synergistically in the antagonism of phytopathogenic fungi (Lorito et al., 1996; Monteiro et al., 2010). The production of 6-pentyl-α-pyrone associated with the coconut smell characteristic of some species of the Viride clade, and

present in the Te610F isolate, may also explain the effectiveness in reducing the growth of the phytopathogenic fungus (Bisby, 1939; Reithner et al., 2007; Vinale et al., 2008).

The evaluation against different common bean pathogens served to have an indication of the spectrum of action of the antagonistic capacity of the evaluated isolates. It was observed that isolates Tk24F and Te610F could potentially have the same antagonistic effect with other phytopathogens, in the same way as that observed against *C. lindemuthianum in vitro*. This was also observed with the isolate *T. longibrachiatun* T6, which inhibited the growth of *C. lindemuthianum* and other phytopathogenic foliar, fruit and soil fungi in the work done by Zhang et al. (2018).

As in the work made by You et al. (2016), the experiment with leaf discs allowed us to observe the effect of applying the suspension of conidia and filtrates on the growth and sporulation of the phytopathogen, using the leaf as a substrate. In this experiment, the three isolates inhibited the growth of *C. lindemuthianum*, as previously observed in the experiments on plants, including the isolate T22F, which did not have an outstanding performance in the other *in vitro* tests. Likewise, isolates Tk24F and Te610F inhibited the germination of *C. lindemuthianum* conidia when the culture medium was prepared with plant infusion. This may be associated with the plant's induction in the fungus of the production of certain types of bioactive compounds, as reported by Mukherjee et al. (2012) for *T. virens* when they demonstrated that seven genes encoding putative NRPS and PK/NRPS cluster enzymes were up-regulated by contact with maize roots.

The induction of resistance in plants is associated with the activation of genes associated with the hormonal signaling pathway of salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET). The SA-mediated signaling pathway is associated with resistance to biotrophic and hemibiotrophic pathogens, whereas the JA/ET pathway is often related to resistance to necrotrophic pathogens (Penninckx et al., 1998) and resistance induced by fungi used in biological control such as *Trichoderma* (Shoresh et al., 2005; 2010). *Colletotrichum lindemuthianum* is a hemibiotrophic fungus that, in interaction with common bean, seems to modulate the biosynthesis and hormone signaling pathway. It was observed that the cytokinin and ethylene response genes were up-regulated and those for jasmonic acid, gibberellin and abscisic acid were down-regulated, indicating that these hormones may play a central role in this pathosystem (Oblessuc et al., 2012; Padder et al., 2016).

In the present study, the pathogenesis-related genes (*PR1*, *PR2*, *PR3* and *PR4*) were over-expressed when the phytopathogen was applied, but this over-expression was reduced when the Tk24F isolate was applied to the root before the application of the phytopathogen probably because there was less severity disease in those plants. The ethylene signaling pathway genes (*ERF1* and *ERF5*) were also overexpressed when the phytopathogen was applied, with a slight increase in *ERF1* gene expression when the *Trichoderma* isolate was previously applied to the root, with no significant difference. These results contrast with those found by Mayo et al. (2016) in which most of the genes that were evaluated were down-regulated in plants treated with *Trichoderma velutinum* and *R. solani* in the root or only with the phytopathogen. This was also observed in relation to the repression of the *ERF1* gene by the transcription factor *WRKY33*, as in the present study these two genes were overexpressed in plants treated with *T. koningiopsis* and *C. lindemuthianum* or overexpressed more slightly when only the phytopathogen was applied, with no significant difference. These results show a clear difference in the response of common bean to a necrotrophic fungus such as *R. solani* and an hemibiotrophic one such as *C. lindemuthianum*.

The only genes that were underexpressed by the application of *C. lindemuthianum* were the one involved in Ca^{2+} signaling (*CNGC2*) and the transcription factor WRKY33, last one significantly overexpressed when *Trichoderma* was previously applied to the root. The *WRKY33* gene codes for a transcription factor involved in the response to biotic and abiotic stress in plants that positively or negatively regulates genes associated with the AS and JA/ET signaling pathway of the defense system (Bakshi and Oelmüller, 2014). The upregulation of the *WRKY33* gene has been associated with the induction of JA-induced defense genes, such as defensins (PDF1.1 and PDF1.2) and for ethylene biosynthesis (Birkenbihl et al., 2012; Li et al., 2012). The overexpression of the *WRKY33* gene together with a lower overexpression of the gene related to the ethylene pathway and the genes related to pathogenesis, in plants preventively applied with *Trichoderma* and subsequently applied with *C. lindemuthianum*, show less activation of those genes may because plants had less disease severity as observed in the greenhouse experiments. This result is not evidence enough to prove induction of resistance as one more mechanism of the Tk24F isolate, in addition to the mycoparasitism and antibiosis reported above, possibly due to the time of evaluation used.

Colonization of roots and leaves of common bean was evidenced even in the internal parts of the root after 4 days of inoculation. Isolates Tk24F and Te610F were observed in the internal tissues of the root, including seedlings transferred to the substrate. This corresponds to what was observed in colonization studies such as that done by Bailey et al. (2008) with endophyte isolates of different *Trichoderma* species in *Theobroma cacao* roots. The isolates in the present study were also isolated as endophytes from rubber trees in the Amazon and, given the above, it is evident that they can also colonize the internal tissues of alternative hosts such as common bean, showing their competitive capacity and rhizo-competence. On the other hand, although root colonization has been associated with induction of plant resistance (Harman, 2011), as observed in this study with isolate Tk24F, isolates T22F and Te610F did not show evidence of inducing plant resistance in the pathosystem *C. lindemuthianum*-common bean in the greenhouse experiments, despite having the ability to colonize common bean roots as observed in the present study.

Trichoderma spp. isolates selected in this study for their effectiveness in controlling bean anthracnose also promoted plant shoot growth (Table 2). Isolates effective in controlling a disease can also be plant growth promoters (Macena et al., 2020; Pedro et al., 2012), especially species that associate with plant roots (Harman et al., 2004a) and by production of bioactive compounds (Vinale et al., 2008). In previous studies, isolates that promoted common bean growth were also effective in controlling *C. lindemuthianum* as mentioned above (Macena et al., 2020; Pedro et al., 2012). These isolates that belong to the species *T. harzianum*, *T. strigosum* and *T. theobromicola*, when inoculated in the substrate before planting, increased shoot dry mass by up to 30% (Pedro et al., 2012). Isolates of the species *T. harzianum*, *T. asperellum*, *T. virens* and *T. longibrachiatum* that were applied to sprouted bean seed radicles increased the length of shoots and plant roots by 6.8% (Hoyos-Carvajal et al., 2009). Likewise, it was observed that *T. asperellum* and *T. harzianum* increased the biomass of soybean shoots and roots and were effective in controlling *Sclerotinia sclerotiorum* (Macena et al., 2020).

In summary, the isolates that previously showed to be more efficient in controlling bean anthracnose by reducing disease severity with a single foliar application (Te610F and Tk24F) in the initial greenhouse experiment, in the *in vitro* tests stood out for being more aggressive as to the mechanism of mycoparasitism and antibiosis. Isolates Te610F and Tk24F were more aggressive mycoparasitizing *C. lindemuthianum*, since a greater number of lateral hyphae was observed over

the host hyphae before its disintegration; inhibition of germination of the phytopathogen conidia when applied in suspension; and colonization of bean leaves (Table 4). However, isolate Te610F also stood out in inhibiting the growth of the phytopathogen by producing enzymes and secondary metabolites diffused in the medium, which may explain its effectiveness in controlling *C. lindemuthianum* with only one foliar application before the application of the phytopathogen. However, with the Tk24F isolate it was no possible to determine whether it can stimulate the plant's defense system to favor a lower disease severity when preventively applied to germinated seeds and to the root, considering its ability to colonize the plant's roots. Finally, the T22F isolate, which required three applications to reducing disease symptoms in the greenhouse experiments, also had a lower performance in the experiments of the present study. However, T22F can mycoparasitize and inhibit the growth of *C. lindemuthianum* and the performance of several applications is enough to reduce the disease severity in the greenhouse (Table 6).

Our study shows that different species can have several mechanisms of action that, in synergy, can constitute different strategies to control the same phytopathogen. However, it also shows that to be more efficient in controlling the pathogen, which translates into needing less field applications, *in vitro* tests must present levels close to 100% in the evaluated variables. Conducting *in vitro* experiments that explore a single mechanism of action to select potential biological control agents ignores the importance of synergy between mechanisms of action and consequently runs the risk of discarding isolates that in the plant could show their true potential. An example of this are the most important commercialized strains for biological control such as *T. harzianum* T39, *T. virens* G-6 and *T. harzianum* T22, as their efficacy is associated with the presence of several mechanisms of action (Elad, 2003; Howell, 2006; Vinale et al., 2006). The test with leaf discs, for example, was useful to get this approximation to the result previously observed in the greenhouse.

In conclusion, *T. erinaceum* 610F and *T. koningiopsis* 24F are potential biocontrol agents against *C. lindemuthianum,* due to the joint use of mycoparasitism, antibiosis, and competition mechanism. Results *in vitro* and *in vivo* in leaf disc where conidia germination of *C. lindemuthianum* were inhibit by the preventive application of the *T. erinaceum* 610F lead us to think that the inhibition of the infection is the prevalent mechanism of action of this isolate. This work also shows the importance of having approaches with multiple selection criteria when a pre-selection of potential biological control agent *in vitro* is required, as already done by Bouaoud et

al. (2018) and You et al. (2016). This study constitutes only the baseline to explore these isolates in further studies of mechanisms of action with metatranscriptomics and metabolomics (Mhlongo et al., 2018), and in the same way, it serves as an important tool to delineate field experiments, regarding the form of application and formulation, aiming to take advantage of the mechanisms in which they stood out the most.

MATERIAL AND METHODS

Fungal isolates, growing conditions and common bean variety

Trichoderma spp. were originally collected and isolated from the inner tissues of leaves, stems, and roots of *Hevea guianensis* (Araújo et al., 2018) and *Hevea brasiliensis* (Araújo et al., 2020) in the Brazilian Amazon Forest (Supplementary Table S4) and taxonomically identified by Brito (2017). The phytopathogenic fungi used in this study were *C. lindemuthianum* pathotype 89 isolate A_2 2-3, *Fusarium oxysporum* f. sp. *phaseoli* and *Sclerotinia sclerotiorum*. All of them belong to the collection of microorganisms of the Laboratory of Molecular Genetics of Microorganisms/Bioagro, Universidade Federal de Viçosa and were preserved in 10% glycerol at 5 - 10 °C until they were reactivated for experiments in Potato Dextrose Agar (PDA) medium, brand Kasvi ®.

Trichoderma spp. isolates were incubated for 4 to 5 days at 28 ºC in PDA medium or rice to induce sporulation. The rice was previously moistened and sterilized at 121 °C for 15 min. The isolate of *C. lindemuthianum* was incubated for 10 days at 20 ºC in PDA and in YMA medium (2 g/L yeast extract, 10 g/L malt extract and 15 g/L agar) to induce sporulation. The *F. oxysporum* isolate was incubated for 8 days and the *S. sclerotiorum* isolate for 3 days at 25 ºC, in PDA medium. All fungi grew with a 12 h light/12 h dark photoperiod.

The common bean variety used in the experiments was *Pérola*.

Selection of *Trichoderma* spp. for the control of *C. lindemuthianum* in a greenhouse

Twenty isolates of different *Trichoderma* species were evaluated in a greenhouse for control of anthracnose in common bean (Supplementary Table S4). Seeds were disinfested with 0.5% sodium hypochlorite for 5 minutes, washed three times with sterile purified water (SPW) and excess water removed. The seeds were allowed to germinate in 16 x 32 cm aluminum trays, 32

seeds per tray, with moist filter paper and covered with plastic film, for three days at 25 °C in the dark. The filter paper was moistened every 24 hours. The germinated seeds were placed in 50 ml plastic cups with the radicle facing downwards and inoculated with 1.0 ml of a conidia suspension of the *Trichoderma* spp. adjusted to a concentration of 1 X 10^7 conidia/mL counted in the Neubauer chamber (Supplementary Figure S5). The cups were covered with plastic film and left to incubate overnight at 25 °C in the dark and after this period, the seeds were planted in 500 mL pots with commercial substrate Tropstrato® previously moistened to field capacity and fertilized with 0.5 g of compound fertilizer (4-14-8). When the plants reached stage V2 (completely expanded primary leaf) they were atomized in the aerial part (abaxial and adaxial part of the leaves) with 3 ml of a suspension of 1 X 10^7 conidia/ml and in the substrate with 5 ml. The plants were left for 24 hours in a fog chamber at 25 ± 2 °C with a relative humidity of 90% and a photoperiod of 12 h light/12 h dark. Then, the plants were returned to the greenhouse and after 4 days the application with the pathogen was made.

To prepare the pathogen conidia suspension, the YMC plates were washed with SPW and, to remove the mucilage, the conidia were washed by three successive centrifugations at 3400 rpm for 3 min, between which the supernatant was removed, and SPW was added again. The suspension was adjusted to a concentration of 1 X 10^6 conidia/mL and atomized in the aerial part of the plants (abaxial and adaxial part of the leaves) with 3 mL of the suspension. The plants were left for 24 hours in a fog chamber at 25 ± 2 °C with a relative humidity of 90% in the dark. Afterwards, the plants were returned to the greenhouse and kept there until symptoms of the disease were observed. The evaluation was made 7 days after application of the pathogen. For the evaluation, the two cotyledon leaves were cut and taken immediately to the laboratory to obtain the images of the lower surface of the leaf blade, which were later analyzed to determine the percentage of injured area (severity). Images were obtained by digitizing them in an Epson L365 printer/scanner and the injured area was quantified with the Quant V. 1.0 program (Vale et al., 2001).

The 20 isolates were evaluated in two experiments. In the first experiment, 11 treatments were evaluated; nine *Trichoderma* spp. isolates, a control in which the plants were treated only with water (healthy control), and another control in which the plants in addition to being treated with water, were treated with the pathogen (pathogen control). In the second experiment, 13 treatments were evaluated; the 11 missing isolates, a reference isolate that had been evaluated in

the first experiment and the two controls. A completely randomized experimental design and three replications per treatment were used. The experiments were carried out between the months of June and July 2019, when temperatures were favorable for the development of the disease (T max.=32 °C; T. min.= 9 °C).

The percentage of injured area (x) was evaluated was transformed into angular data by the formula arcsine$\sqrt{}$(x/100). In the statistical analysis, the assumptions of normality (Shapiro-Wilk test at 5% significance) and homogeneity of variance (Bartlett's test at 5% significance) were proven. Differences between treatments were established by analysis of variance with 5% of significance, and means were compared by Scott-Knott tests (α=0.05) with the statistical program R version 3.5.3 (2019-03-11) Copyright (C) 2019 with the platform x86_64-w64-mingw32/x64 (64-bit) and ExpDes.pt and Nortest packages.

Evaluation of the application site of selected isolates of *Trichoderma* spp., effective in the control of *C. lindemuthianum* in a greenhouse

Trichoderma spp. isolates selected for decreasing the severity of anthracnose in common bean when applied to the root and aerial part before application of *C. lindemuthianum* were evaluated. To do this, each isolates was evaluated in three treatments: a treatment in which *Trichoderma* isolate was applied to the radicle before planting, and the root when the plants reached stage V2 (fully expanded primary leaf) (R); a treatment in which the aerial part was applied when the plants reached the V2 (AP) stage; and a third treatment in which application was made to the root and aerial part (R+AP).

Seed treatment, inoculation of radicles and plants, conformation of experimental units, application with *C. lindemuthianum*, experimental conditions, disease severity evaluations and statistical analysis were made in the same way as in the selection experiment explained above.

A completely randomized experimental design was used, with 14 treatments and five repetitions per treatment. Nine treatments corresponded to the three selected isolates with the three forms of application: application to the radicle and root (R), application to the aerial part (PA), and application to the root and the aerial part (R+PA). The other two treatments corresponded to the healthy control where only water was applied (Healthy Control) and the control where the phytopathogen was applied (Control of the Pathogen). The experiment was carried out in the

greenhouse two times between the months of August and September 2019 and June 2020 when temperatures were favorable for the development of the disease (Tmax=38°C; Tmin=11°C).

Evaluation of selected isolates of *Trichoderma* spp. to promote common bean growth in a greenhouse

Trichoderma spp. isolates that were selected for decreasing the severity of anthracnose in common bean were also evaluated for growth promotion. *Trichoderma* spp. were cultivated in the same way as described above. However, inoculation was performed according to the methodology of Mayo et al. (2015), which consisted of submerging previously disinfested seeds in a suspension of 2 x 10^7 conidia/mL and leaving them to dry for 12 hours in the laminar flow cabin. Subsequently, two seeds were planted per 1 L pot with commercial substrate Tropstrato® without adding fertilizer and thinned after emergence to ensure one plant per experimental unit. The evaluation was carried out 25 days after planting and consisted of removing the plants, removing the substrate from the roots to immediately obtain the weight of the aerial part and root fresh mass. Afterwards the plants were left in a greenhouse at 60°C for 72 to 96 hours and weighed again to measure the dry mass of the aerial part and the root.

A completely randomized experimental design was used, with five treatments and 10 repetitions per treatment. The treatments consisted of four selected isolates and a control treatment whose seeds were treated with water. The experiment was carried out in a greenhouse two times between the months of August and September 2019 and June 2020 with a maximum temperature of 40 °C and a minimum of 11 °C.

In the statistical analysis of the fresh and dry mass weight, the assumptions of normality (Shapiro-Wilk test at 5% significance) and homogeneity of variance (Bartlett's test at 5% significance) were proven. Differences between treatments were established by analysis of variance with 5% of significance, and means were compared by Scott-Knott tests (α=0.05) with the statistical program R version 3.5.3 (2019-03-11) Copyright (C) 2019 with the platform x86_64-w64-mingw32/x64 (64-bit) and ExpDes.pt and Nortest packages.

Genetic transformation with the fluorescent protein gene

Trichoderma spp. have been genetically transformed to express the fluorescent proteins RFP. The plasmids used were pPGW that contains the cassette of the gene for resistance to

hygromycin B (*hph* - hygromycin B phosphotransferase from *E. coli*) and the gene that codes for the red fluorescent protein (RFP) (Mehrabi et al., 2015). The plasmid pPGW containing the *hph-rfp* cassette was kindly provided by the researcher from Embrapa Amazônia Ocidental Dr. Gilvan Ferreira da Silva. The construct has the *hph* gene under the control of the PtrpC promoter from *Aspergillus nidulans* and *rfp* (red flourescent protein) under the control of the *PtoxA*-5'UTR promoter from *Pyrenophora tritici-repentis*.

The protoplast transformation protocol described by McCormick et al. (2010) and modified by Malmierca et al., (2012) was used with some other modifications. The solution of lytic enzymes was prepared with 5 mg/mL of Lysing enzymes and 25 mg/mL of Driselase (Sigma, St. Louis, MO) and with the osmotic stabilizers NaCl 0.7 M for the isolates T22F and Tk24F and KCl 0 .6 M for the Te610F isolate. Transformants were selected by resistance to hygromycin B (Sigma, St. Louis, MO) at the minimum inhibitory concentration established, which was 150 µg/ml for isolates T22F and Tk24F and 90 µg/ml for isolate Te610F.

Colletotrichum lindemuthianum was genetically transformed to express the fluorescent protein GFP with plasmid pSM1 that also contains the cassette of the gene *hph* and the gene that codes for the green fluorescent protein (GFP) (Pöggeler et al., 2003). For this, the transformation protocol described by Soares et al. (2014) with some modifications was used. The enzyme solution was prepared with 30 mg of Lysing enzymes (Sigma, St. Louis, MO), 7 mg/ml of Bovine Serum Albumin (BSA) and with the osmotic stabilizer 10 mM phosphate buffer; 0.6 M KCl without added glucanex. The obtained protoplasts were washed with ST (1M Sorbitol, 100 mM Tris-HCl, pH 7.5) and STC (1M Sorbitol, 100 mM Tris-HCl pH 7.5, 50 mM CaCl2) by means of centrifugations at 3000 g at 4°C for 10 minutes. Then the protoplasts were resuspended in STC to a final concentration of 10^7 protoplasts/ml.

The fluorescence of the transformants was confirmed on the Olympus® BX53 fluorescence microscope equipped with the U-FBW (460/495 nm excitation filter) and U-FGW (530/550 nm excitation filter) filter cubes.

Transformants analysis

Monosporic purification of the transformants was carried out, which were then inoculated in PDA medium supplemented with hygromycin B at a concentration of half the MIC. From the

purified colonies of the transformants, the radial growth rate (RGR) was determined, mitotic stability, biological activity by cellophane membrane test and efficacy in the control of *C. lindemuthianum* in common bean in a greenhouse were analyzed.

To determine the RGR, 5 mm diameter discs were placed in PDA culture medium 1 cm away from the edge of the plate and measurements were taken of the colony radius every 24 hours until reaching the opposite edge of the plate. Daily growth in millimeters was calculated and the average of the repetitions was calculated. To determine mitotic stability, transformants were inoculated into rice in the same way as for the greenhouse experiments. Subsequently, a second inoculation was made in PDA medium without hygromycin B and the fluorescence was verified in an Olympus® BX53 microscope. The cellophane membrane test was performed as described by Mayo et al. (2012) and as explained below in the antibiosis experiments, with the phytopathogen *C. lindemuthianum*.

Next, with the transformants that showed no differences when compared to the wild-type isolate, the number of copies of the transformation cassette inserted into the genome was verified by Southern blot (Southern, 1975). The total DNA was extracted according to the methodology of Specht et al. (1982) modified by Cardoza et al. (2006, 2011), with some other modifications. The mycelium was washed with sterilized purified water (SPW) and filtered through filter paper conditioned in a Buchner funnel coupled to a vacuum filtration system. The extraction buffer used was 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 150 mM NaCl and 1% SDS. Total DNA from transformants and wild-type isolates was digested with the restriction enzyme *Hind*III (Promega), and the generated fragments were subjected to electrophoretic separation on a 0.8% agarose gel. The denaturation, neutralization and transfer of DNA to the membrane was done as described by Sambrook et al. (1989), and hybridization and detection were performed using the DIG High Prime DNA Labeling and Detection Starter kit (Roche, Applied Science), following the manufacturer's recommendations. The *hph* gene was used as a probe and the labeling was performed by PCR using the PCR DIG Probe Synthesis kit (Roche, Applied Science), following the manufacturer's recommendations. The *hph* gene was amplified with the primers HPH1 (5'-CAGCGAGCCTGACCTATTGC-3') and HPH2 (5'-GCCATCGGTCCAGACGGCCGCGC-3') in a thermocycler with an initial denaturation temperature of 95 °C for 2 min; 30 cycles of

denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 40 s; final extension at 72 °C for 7 min, which produced an amplicon of 667 bp.

The transformants with the lowest plasmid copy numbers were used in the experiment to control *C. lindemuthianum* in common bean in a greenhouse, as described above for the evaluation of the application site of *Trichoderma* spp. efficacy in the control of *C. lindemuthianum* in the greenhouse. The efficacy was calculated using the following formula: $\%E = [(C - T)/C] \times 100$ (C: percentage of the leaf area with necrosis in the control treatment that was only applied with the phytopathogen; T: percentage of the area with necrosis in leaves treated with *Trichoderma* spp. before application with the phytopathogen).

In all transformants analysis experiments the wild type isolate was used as a control. In the *in vitro* experiments, three replicates were performed, and in the *in vivo* experiment, five replicates were performed. In all tests, a completely randomized experimental design was used. The normality of the data was verified with the Shapiro-Wilk test of the northern package, the measures of central tendency and distribution were calculated, as well as the paired t-student test of the wild isolate and of each transformant, in the statistical program R (R Core Team, 2019). This was done to determine if there had been any alteration in the evaluated phenotypic characteristics due to plasmid integration.

Mycoparasitism

Direct confrontation for microscopic observations

Sterile cellophane paper circles were placed in petri dishes with 1.5% Water Agar medium and on the cellophane membrane the GFP-expressing *C. lindemuthianum* transformant and the isolates of *Trichoderma* spp. (22RFP, 24RFP or 610RFP) that express RFP. A modified methodology of El-Naggar et al. (2008) was used with modifications. At three points *C. lindemuthianum*-GFP was inoculated and 2 days later the transformants of *Trichoderma* spp. in three points at 1, 1.5 and 2 cm from the points previously inoculated with *C. lindemuthianum*-GFP. The plates were left to incubate at 25°C in the dark and 1 cm squares were cut out in the regions where the hyphae of the two fungi joined after 4 and 8 days of inoculation of the *Trichoderma* spp. transformants. The square of membrane was left on the agar until observations were made under

the fluorescence microscope to prevent dehydration, at which time a drop of water was placed on the slide to place the square of the membrane where the fungi were inoculated on top. Images were captured with the EVOS M5000 Imaging System (Thermo Scientific, Waltham, Massachusetts, USA) equipped with GFP (470/525 nm) and RFP (531/593 nm) light cubes in fluorescent, brightfield and color brightfield imaging modes and single color and multicolor imaging methods. The experiment was carried out in duplicate with three biological replicates.

Direct confrontation assay for macroscopic observations

Trichoderma spp. were evaluated for mycoparasitism capacity by the direct confrontation methodology to *C. lindemuthianum* according to the methodology of Mayo et al. (2015).

Discs of 5 mm in diameter from the edge of colonies of the active fungi growing in PDA plates, under the growth conditions of each fungus, were transferred to new plastic plates of ~90 mm in diameter with 20 mL of PDA medium. Discs were placed on opposite sides 1 cm away from the edge of the plate. Discs of *C. lindemuthianum* were placed on the culture medium and 4 days later the discs of the *Trichoderma* spp. were placed, due to the slow growth of the phytopathogen compared to other fungi. In the control plates, discs only of phytopathogenic fungi were placed. After 10 days of incubation, the photographic record was taken with a Nikon® CoolPix P510 camera and it was evaluated whether there was growth of *Trichoderma* spp. above the colonies of the phytopathogens. The experiment was carried out in duplicate with three biological replicates.

Antibiosis

Trichoderma spp. were evaluated for their ability to produce antimycotic compounds that inhibited the growth and germination of *C. lindemuthianum* conidia by the cellophane membrane methodology Mayo et al. (2015).

Discs of 5 mm in diameter from the edge of actively growing fungal colonies were transferred onto PDA plates. Discs from *Trichoderma* spp. were transferred to the surface of sterilized cellophane membranes deposited on PDA medium and after 48 hours of culture, the membranes were removed and phytopathogen discs were placed on PDA medium. In the control plates, the *Trichoderma* spp. isolates were not previously cultivated. After the time necessary for each phytopathogenic fungus to completely cover the medium in the control plates, measurements

were taken of the diameter of the colonies in the treatment plates to determine the percentage of pathogen growth inhibition by the compounds secreted by *Trichoderma* spp.

To evaluate the effect of the growth medium of *Trichoderma* spp. in the germination of conidia of *C. lindemuthianum*, aliquots of a suspension of fungal conidia or filtrate of this suspension were placed in place of discs. *Trichoderma* spp. were cultivated in rice to obtain conidia and *C. lindemuthainum* was cultivated in YMC medium as in the greenhouse experiment. The culture media used were PDA (Kasvi®) prepared with purified water (PDA), PDA prepared with *Pérola* bean leaf infusion (PDA-LB) and PDA prepared with *Pérola* bean seed infusion (PDA-SB). Leaf infusion was prepared with 1 g of dried leaves and seed infusion with 4 g of seeds in 100 mL of purified water and both were boiled for 10 min. Then, 5 µL aliquots of *Trichoderma* spp. at a concentration of 1×10^7 conidia/mL or the filtrate from the suspension of conidia at two equidistant points of a Petri dish with culture medium, where two cellophane membranes were deposited. After 24 h, the surface membrane was removed and 5 µL aliquots of *C. lindemuthianum* conidia were deposited at a concentration of 1×10^6 conidia/mL in the same points where the *Trichoderma* isolates were previously inoculated. In the plates where the filtrates were deposited, a single cellophane membrane was placed which was not removed to place the conidia of *C. lindemuthianum*. The conidia of *C. lindemuthianum* were washed three times as done in the greenhouse efficacy experiment. After 30 h, at least 100 conidia were counted between germinated and non-germinated to determine the percentage of inhibition of the pathogen's conidia germination under the optical microscope in the 40X objective.

The percentage of inhibition was calculated by the formula $\%IM = [(C - T)/C] \times 100$ (C: colony diameter or germination of the phytopathogen conidia on the control plate; T: colony diameter or germination of the phytopathogen conidia exposed to *Trichoderma* spp metabolites). A descriptive statistic of the percentage of inhibition of growth or germination of the pathogen was performed.

Growth inhibition of *C. lindemuthianum* on leaf discs

To assess the inhibitory effect of *Trichoderma* spp. on the growth of *C. lindemuthianum* on common bean leaf, the co-inoculation methodology on leaf discs of You et al. (2016) was used with modifications.

Cotyledonal leaves of common bean cultivar *Pérola* were disinfested in 70% alcohol for 30 sec, 1% sodium hypochlorite for 1 min and washed three times in SPW for 1 min. Disc with 13 mm diameter were cut from the disinfected leaves with a metal punch and placed inside a ~100 mm diameter glass plate with filter paper moistened with SPW, for a total of ten discs per plate. Discs were inoculated with 10 µL of a 1×10^7 conidia/mL suspension of *Trichoderma* spp. and with the filtrate of this suspension, which was obtained through filtration in a millipore membrane with pores of 0.45 µm. In the control treatment, 10 µl of SPW were applied to the leaf discs. After two days, the discs were inoculated with 10 µL of a suspension of 1×10^6 conidia/mL of *C. lindemuthianum*. The plates were left in incubation at 25ºC with 12 hours of photoperiod for 7 days and after this period the presence or absence of growth/sporulation of the phytopathogen was evaluated and the diameter of the lesion was measured. The response variables were number of discs with growth of the phytopathogen (NDPG) and lesion diameter (LD).

The assumptions of normality and homogeneity of variance were confirmed (Shapiro-Wilk and Bartlett test α=0.05), differences between treatments were established by analysis of variance (α=0.05) and means were compared by Scott-Knott test (α=0.05) with the statistical program R version 4.0.5 (2021-03-31) Copyright (C) 2021, the x86_64-w64-mingw32/x64 (64-bit) platform and the ExpDes.pt packages and north.

Colonization

Common bean radicles and leaves were inoculated with the transformants of the *Trichoderma* spp. that express RFP (22RFP, 24RFP or 610RFP).

Previously disinfested seeds were placed in aluminum trays with moistened filter paper. Disinfestation was carried out with 0.5% sodium hypochlorite for 5 min and to remove it, three washes with SPW were performed. Seeds were kept in the dark for 3 days at 25 °C and moistened each day with SPW. The germinated seeds were transferred to 50 ml cups with the radicle facing downwards and covered with cotton. The radicles were inoculated with 1.0 ml of a suspension of conidia of the RFP transformants adjusted to a concentration of 1 X 10^7 conidia/ml counted in the Neubauer chamber. The cups were covered with plastic wrap and left to incubate overnight at 25 °C in the dark. Twelve germinated seeds per treatment were kept in the cups at 25°C with a photoperiod of 12 hours light/ 12 hours dark until the day of evaluation. Evaluations were made of

three seeds per treatment every day for 4 days, during which time the seeds were moistened with 0.5 mL of SPW to avoid radicle dehydration. The evaluations consisted of carefully washing the radicles under running water and making four slices less than 1 mm thick every centimeter and making observations under the fluorescence microscope. Cuts were made from the tip of the radicle (Division Zone − DZ), 1 cm (Elongation zone − EZ) and 2 cm (beginning of the Maturation zone − MZ) from the tip of the radicle (Fig. 9).

Additionally, three seeds per treatment were transferred to pots with a substrate, maintained at a temperature of 25 – 28 °C with a 12h light/12h dark photoperiod. A single evaluation was made 4 days after inoculation in the same way as for seeds kept in cups.

Detached cotyledonary leaves from 10-day-old plants were disinfected and inoculated with 0.5 ml of a conidia suspension of the RFP transformants adjusted to a concentration of 1 X 10^7 conidia/ml. Disinfestation was carried out with 70% alcohol for 30 seconds, 0.5% sodium hypochlorite for one minute and three washes in SPW for one minute. The petiole of the leaves was covered with cotton moistened with SPW and placed in glass Petri dishes. The plates with the leaves were kept at 25 °C with a photoperiod of 12 h until the day of evaluation. The evaluations were carried out on the second and fourth day after inoculation and consisted of observation under a fluorescence microscope (Fig. 9) of longitudinal (Longitudinal Section − LS) and transverse (Cross Section − CS) sections of the leaf ribs.

In the evaluations of the two experiments, observations were made of the sections and the photographic recording of the images was made with the EVOS M5000 Imaging System (Thermo Scientific, Waltham, Massachusetts, USA) equipped with the RFP light cube (531/593 nm) in the fluorescent, brightfield and color brightfield imaging modes, and single color and multicolor imaging methods. The experiment was carried out once with three biological repetitions.

Resistance induction

Plants and experimental conditions

The isolate *T. koningiopsis* 24F was evaluated because it was the isolate that reduced the severity of anthracnose in common bean when applied to the root. Seeds of common bean cultivar *Pérola* were treated, inoculated, and planted as described above in the greenhouse efficacy

experiments. The treatments consisted of the application of only water on the root and leaves (Control-C); conidia of the isolate Tk24F (T-AP) in shoots; conidia of the isolate Tk24F and *C. lindemuthianum* (T-AP+A_2 2-3) in shoots; conidia of the isolate Tk24F (T-R) in the radicle; conidia of the isolate Tk24F in the radicle and of *C. lindemuthianum* (T-R+A2 2-3) in the aerial part; conidia of *C. lindemuthianum* (A_2 2-3) in the aerial part. Ten days after the application of the phytopathogen, when symptoms were observed in the treatment with *C. lindemuthianum*, the cotyledon leaves were sampled and immediately frozen in liquid N2, macerated, and stored at − 80 ^{o}C until RNA extraction.

RNA extraction and cDNA synthesis

RNA extraction was performed with the RNeasy Plant Mini kit from Qiagen® following the manufacturer's instructions. RNA integrity was assessed after 1% agarose gel electrophoresis and concentration and purity were estimated with NanoDrop (Thermo Scientific, Wilmington, DE, USA) considering the ideal A260/280 absorbance ratio in the range 1.8 up to 2.0. RNA was treated with RQ1 DNAse (Promega) and cDNA was synthesized with ImProm II Reverse Transcriptase System A3800 (Promega) according to the manufacturer's instructions.

qPCR conditions and analysis

For the qPCR reaction, the SYBR Green PCR Master Mix (Applied biosystems, Thermo Fisher Scientific, Woolston Warrington, UK) was used in a 10 µL reaction volume containing 5 µL of the mix, 0.5 µL of each primer at a concentration 0.5 µM and 1 µL cDNA diluted 1:4. The primers used were those selected by Mayo et al. (2016) for the analysis of the expression of genes related to the defense response of common bean plants (Supplementary Table S2). Each qPCR reaction was done in triplicate. The efficiency curve of each primer pair was determined using cDNA dilutions 1:4, 1:16, 1:64, 1:256 and 1:1024. The efficiency (E) of each pair of primers was calculated using the equation $E = (10^{-1/slope} - 1) \times 100$. Quantification was performed by a relative curve obtained for each gene from the mean Ct as a function of log10 cDNA concentration. The housekeeping genes used to normalize expression levels were the gene that codes for actin (*Act11*) and the one that codes for enzyme that degrades insulin (*Ide*). Differences in gene expression levels between the evaluated treatments were determined by means of ANOVA ($p<0.05$) and comparison of means was made using the Scott-Knott test using the statistical

program R version 4.0.5 (2021 -03-31) Copyright (C) 2021, the x86_64-w64-mingw32/x64 (64-bit) platform and the ExpDes.pt and Nortest packages.

Potential to control other common bean phytopathogens (mycoparasitism and antibiosis)

To verify the spectrum of action of the antagonistic capacity of the isolates of T22F, Tk24F and Te610F, mycoparasitism was evaluated by the direct confrontation methodology against the fungi *Fusarium oxysporum* f. sp. *phaseoli and Sclerotinia sclerotiorum* as done with *C. lindemuthianum* and described above. To test the antibiosis, the membrane growth methodology was used with the phytopathogens *F. oxysporum* f. sp. *phaseoli* and *S. sclerotiorum* as done with *C. lindemuthianum*.

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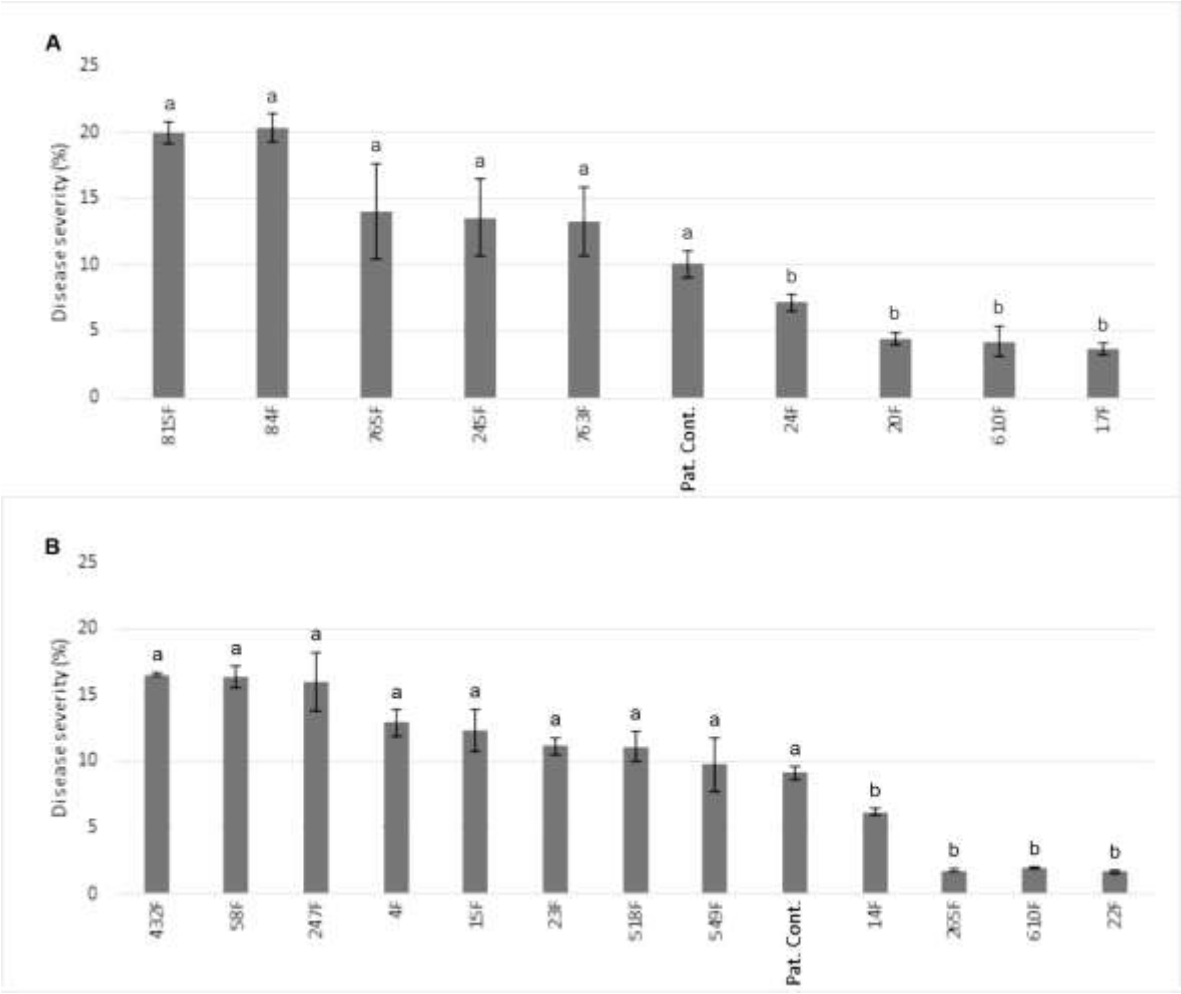


Fig. 1. Evaluation of *Trichoderma* spp. in the control of anthracnose in bean plants in a greenhouse (percentage of severity). A. Experiment 1. **B**. Experiment 2. Bars with the same letter do not show differences according to the Scott-Knott test (α=0.05). Pat. Cont.: Pathogen Control. Isolate 610F was evaluated in both experiments as a reference isolate.

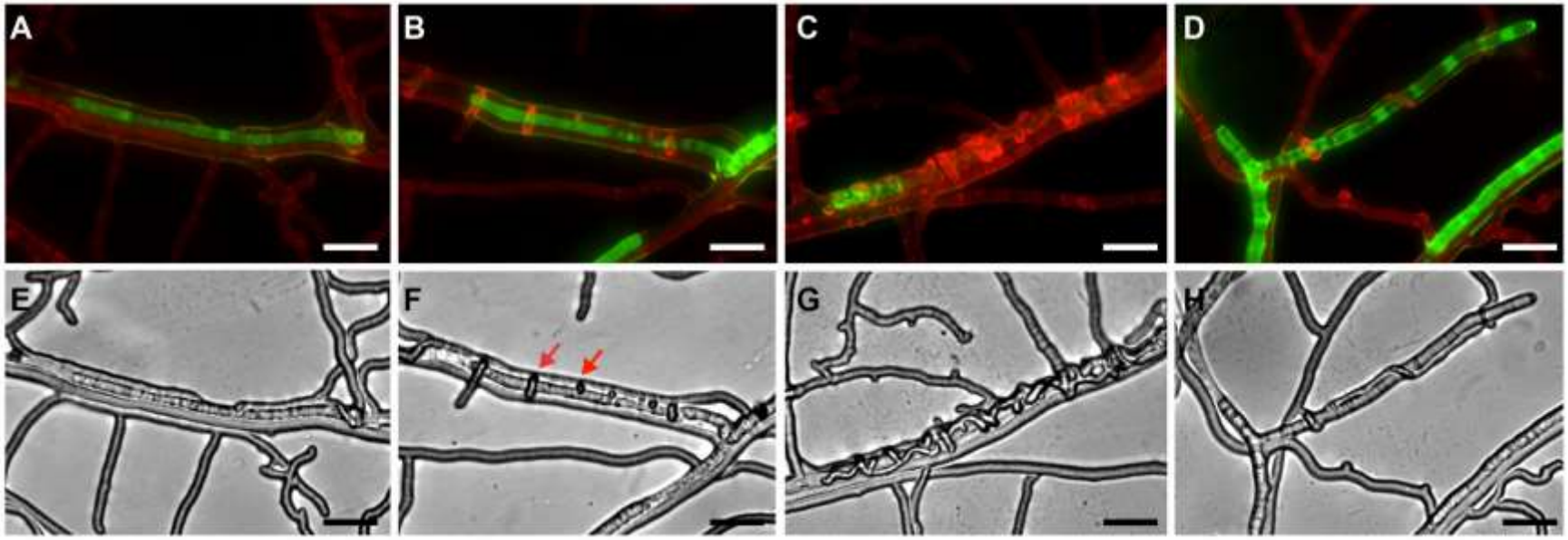


Fig. 2. Sequence of events observed in the formation of lateral hyphae or clamps and coiling in the process of mycoparasitism of *Colletotrichum lindemuthianum* hyphae by *Trichoderma* spp. A: Hyphae of *Trichoderma sp.* VIC44364 22F (red fluorescence) growing parallel to hyphae of *C. lindemuthianum* (green fluorescence). **B**: Formation of clamps (red arrows) of *Trichoderma sp.* VIC44364 22F on the hyphae of *C. lindemuthianum*. **C**: Successive clamps-like structure formation of *T. erinaceum* 610F and degradation of the *C. lindemuthianum* hypha observed by the absence of green fluorescence in part of the *C. lindemuthianum* hypha segment. **D**: *T. koningiosis* 24F hypha coiling over the *C. lindemuthianum* hypha. **E-H**: Brightfield observations of A-D images, respectively. Bar = 20 µm.

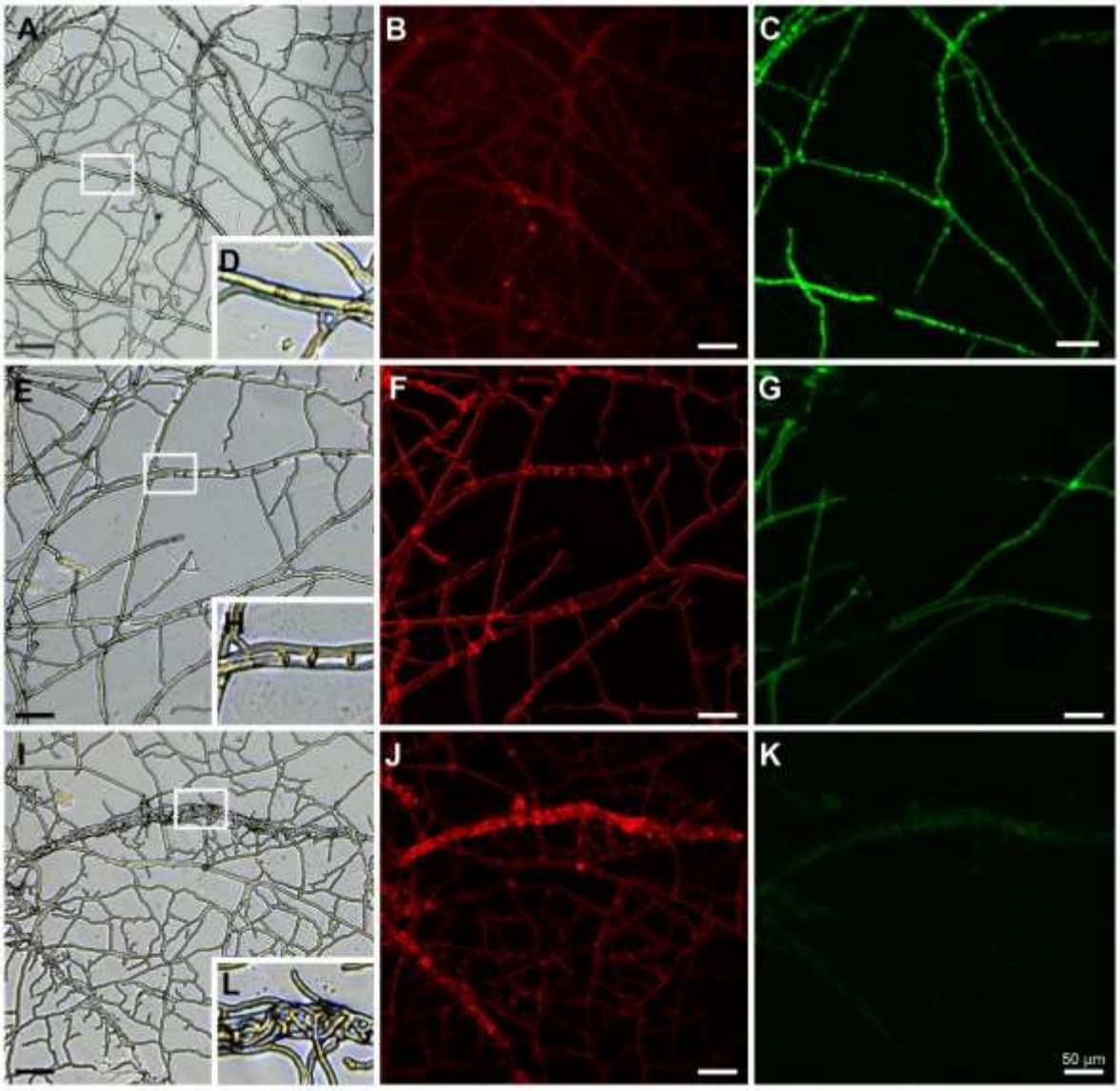


Fig. 3. Mycoparasitism of *Colletotrichum lindemuthianum* by *Trichoderma* spp. 4 days after inoculation. A – **D**: *Trichoderma* sp. VIC44364 22F (T22F). **E** – **H**: *T. koningiopsis* 24F (Tk24F). **I** – **L**: *T. erinaceum* 610F (Te610F). **A**, **D**, **E**, **H**, **I**, and **L**: observations in color brightfield mode. **D**, **H** and **L**: detail of the quantity of lateral hyphae or finger-like clamps formed on the hyphae of the phytopathogen. **B**, **F** and **J**: observations in single color fluorescence mode with the RFP light cube (*Trichoderma* spp. RFP transformants). **C**, **G** and **K**: observations in single color fluorescence mode with the GFP light cube (*Colletotrichum lindemuthianum*). Bar = 50 µm.

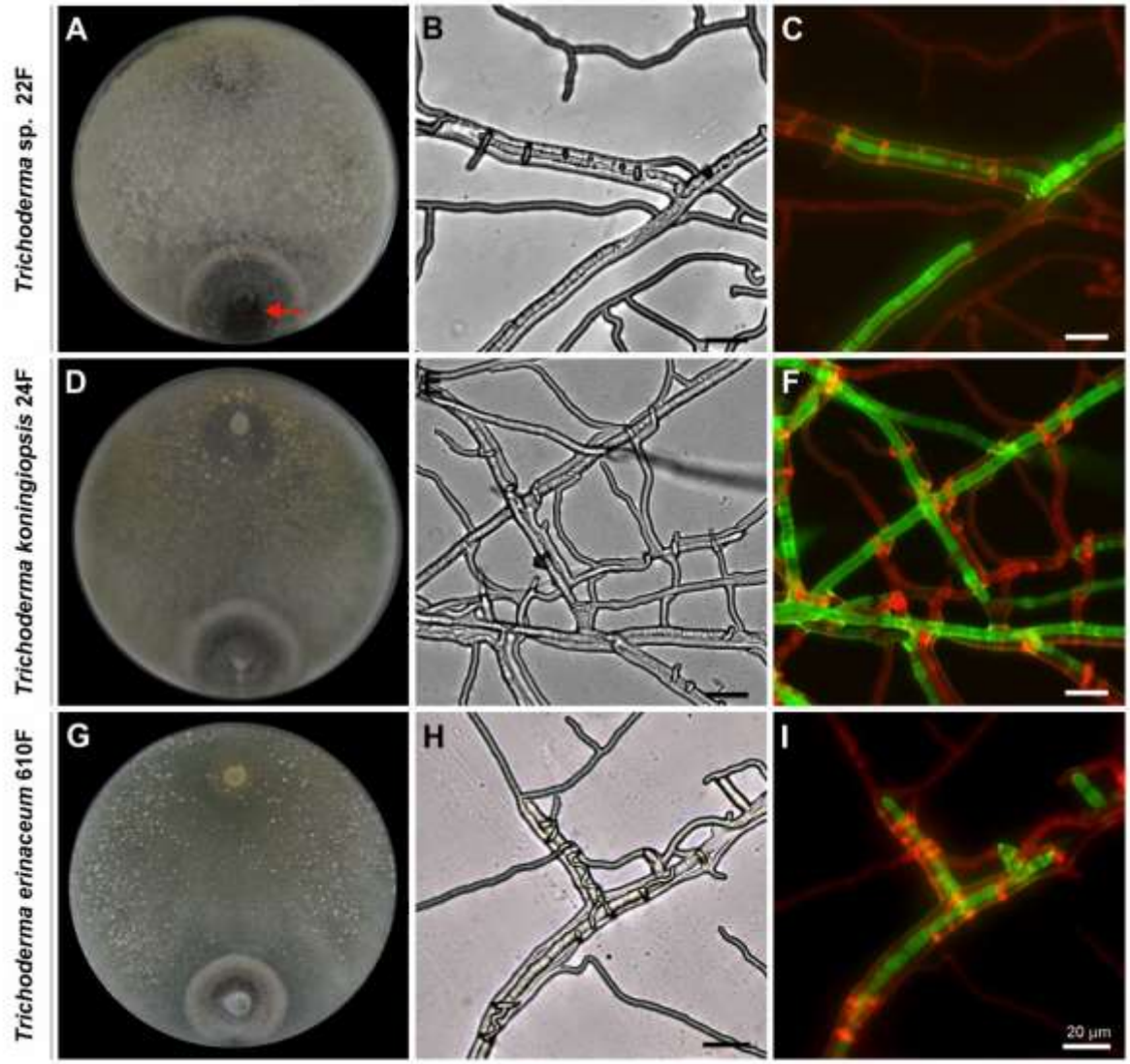


Fig. 4. *Trichoderma* spp. mycoparasitizing the fungus *Colletotrichum lindemuthianum*. The red arrow shows the part of the colony of *C. lindemuthianum* that was not covered by *Trichoderma sp.* VIC44364 22F. **A** – **C**: *Trichoderma sp.* VIC44364 22F (T22F). **D** – **F**: *T. koningiopsis* 24F (Tk24F). **G** - **I**: *T. erinaceum* 610F (Te610F). **A**, **D** and **G**: direct confrontation experiment after 10 days of incubation. **B**, **E** and **H**: observations in brightfield mode of images C, F and I, respectively, 8 days after inoculation. Bar = 20 µm.

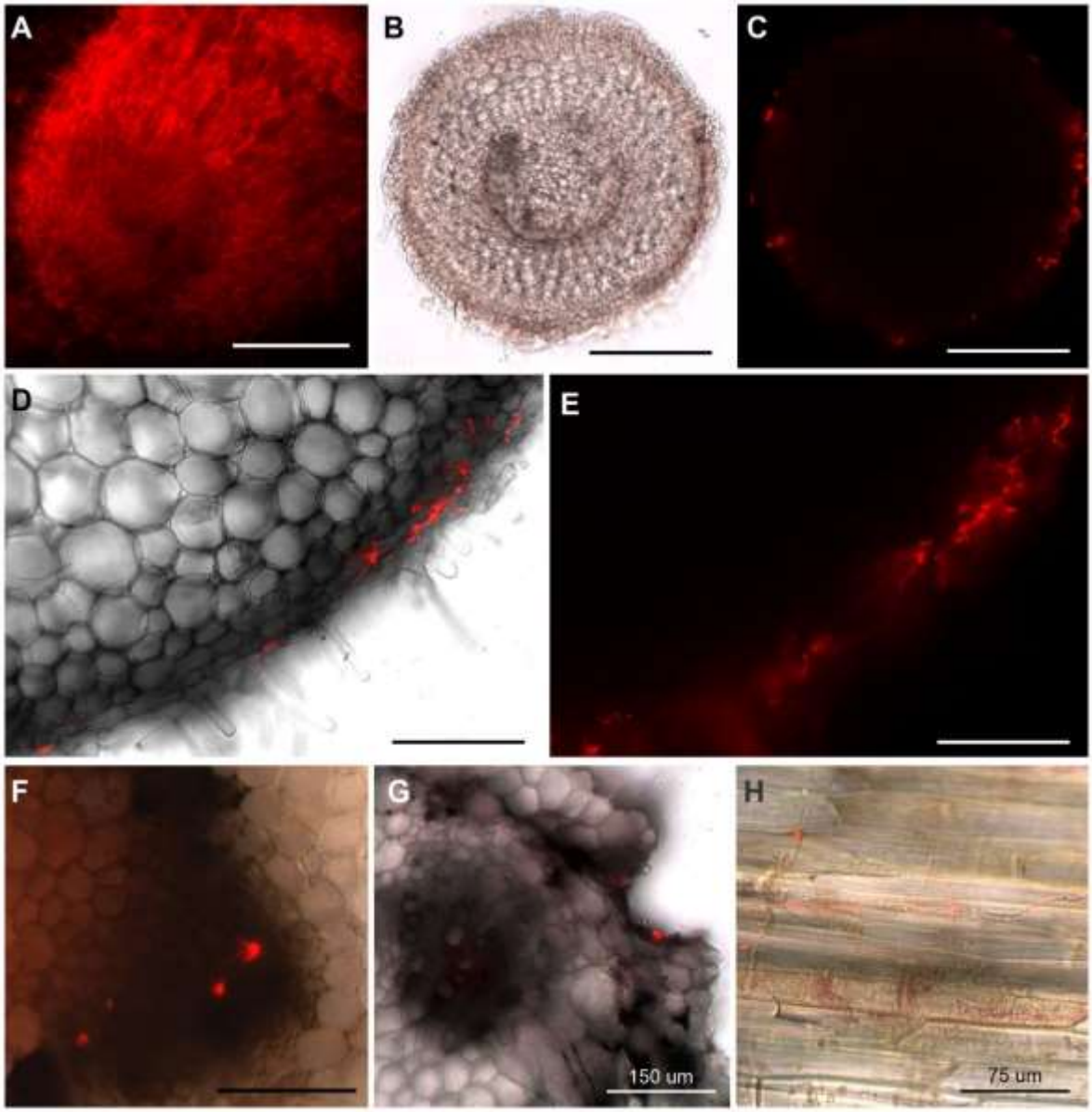


Fig. 5. Colonization of common bean root and leaf tissues by transformant 22RFP-T1 (*Trichoderma* sp. VIC44364). A – **C**: fungal mycelium colonizing the epidermis in the root cell division zone. **D** – **E**: fungal mycelium colonizing the exoderm in the maturation zone. **F**: fungal mycelium in conducting vessels in the elongation zone. **G** – **H**: fungal mycelium on leaf epidermis. **A**: observation of the root cap in fluorescence mode single color method. **C** and **E**: observations in fluorescence mode single color method of images B and D, respectively. **A** - **G**: bar = 150 µm. H: bar = 75 µm.

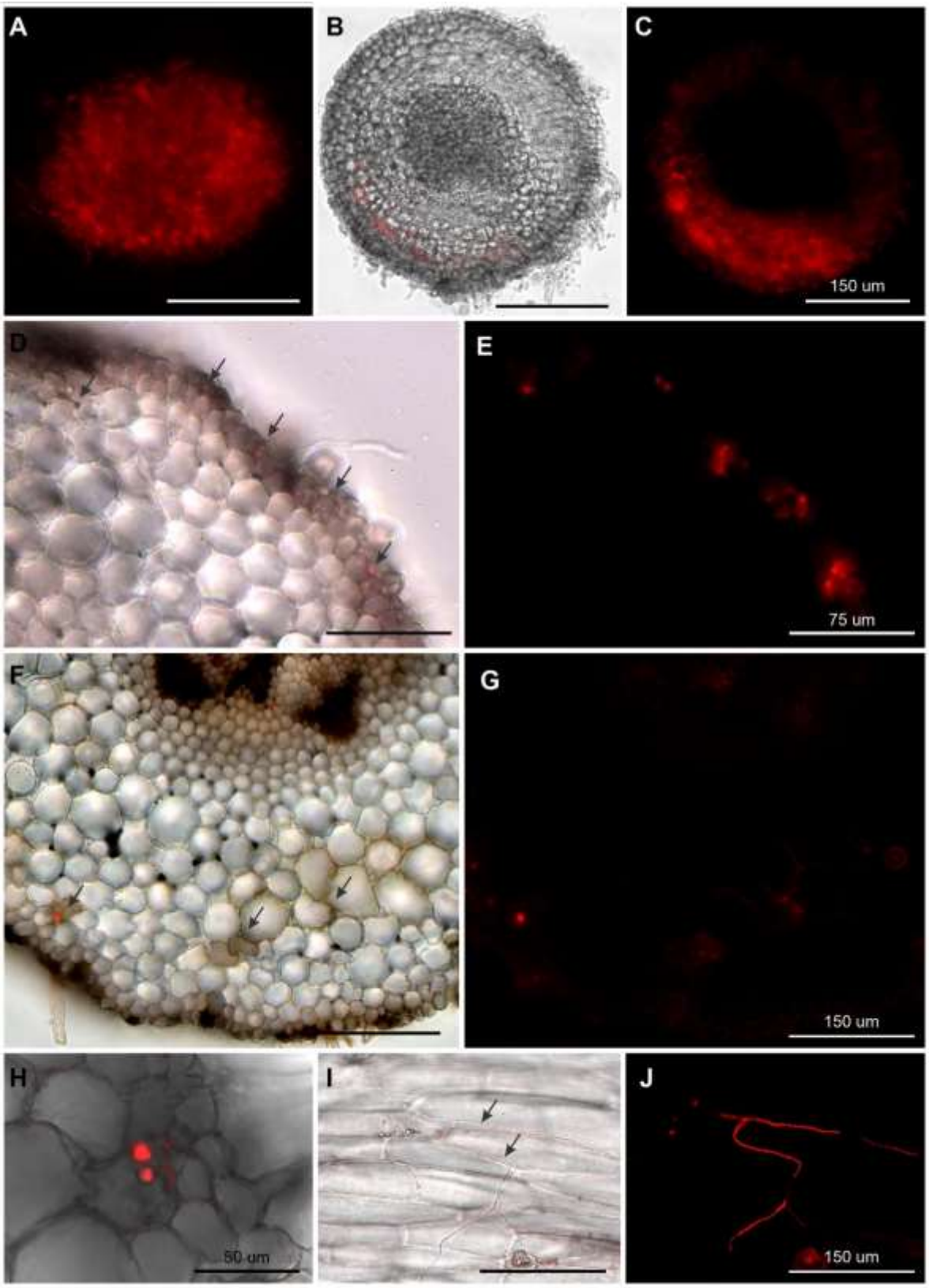


Fig. 6. Colonization of common bean root and leaf tissues by transformant 24RFP-T30 (*Trichoderma koningiopsis*). Arrows showing the places with fungus growth. **A** – **C**: fungal

mycelium colonizing the epidermis, exoderm and cortex in the cell division zone. **D** – **E**: fungal mycelium colonizing the exoderm in the elongation zone. **F** – **G**: fungal mycelium colonizing the cortex in the maturation zone. **H**: fungus mycelium colonizing the maturation zone of radicles of the germinated seeds that were transferred to substrate in pots. **I** – **J**: fungal mycelium colonizing the leaf epidermis. **A**: observation of the root cap in fluorescence mode single color method. **C**, **E**, **G** and **J**: observations in fluorescence mode single color method of images B, D, F and I, respectively. **A**, **B**, **C**, **F**, **G**, **I**, **J**: bar = 150 µm. **D**, **E**: bar = 75 µm. **H**. bar = 50 µm.

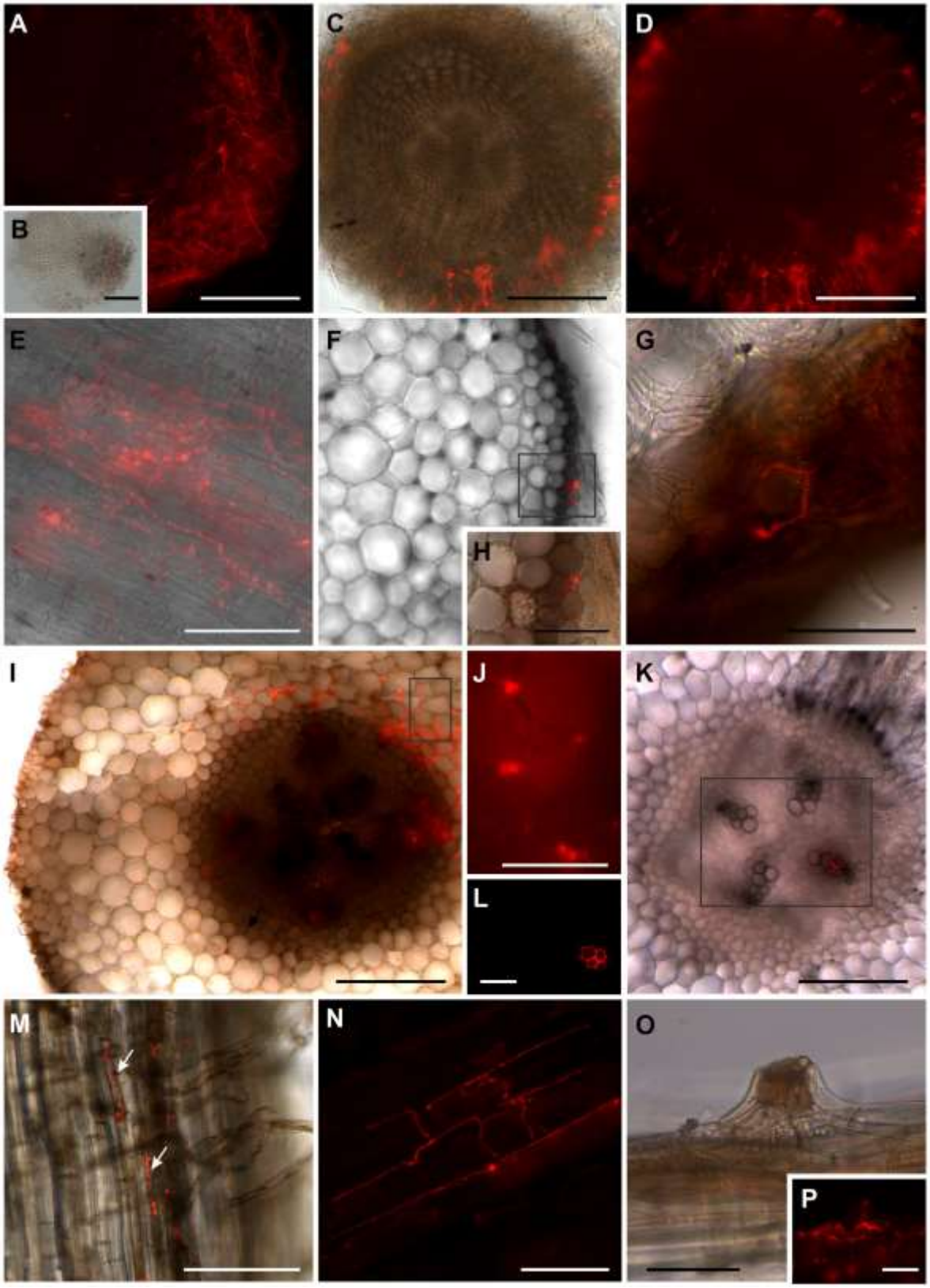


Fig. 7. Colonization of common bean root and leaf tissues by transformant 610RFP-T3 (*Trichoderma erinaceum*). A – D: fungal mycelium colonizing the epidermis, exoderm and

cortex. **E**, **G**: fungal mycelium colonizing the epidermis in the elongation zone. **F**, **H**: fungal mycelium colonizing the epidermis in the maturation zone. **I** – **L**: fungal mycelium colonizing the cortex and xylem of the elongation and maturation zone. **M**: fungal mycelium colonizing radicles of the germinated seeds that were transferred to substrate in pots. **N** – **P**: fungal mycelium colonizing the leaf epidermis. **A**: observation of the root cap in fluorescence mode single color method of the **B** image. **G**: hypha that appears to push through the root epidermis. **H**: detail of the **F** image in color brightfield mode multicolor color method. **J**, **L** and **P**: detail of the **I**, **K** and **O** images in fluorescence mode single color method, respectively. **A**, **B**, **C**, **D**, **F**, **I**, **M**: bar = 150 µm. **K**, **O**, **P**: bar = 75 µm. **H**, **G**, **J**, **L**, **N**: bar = 50 µm.

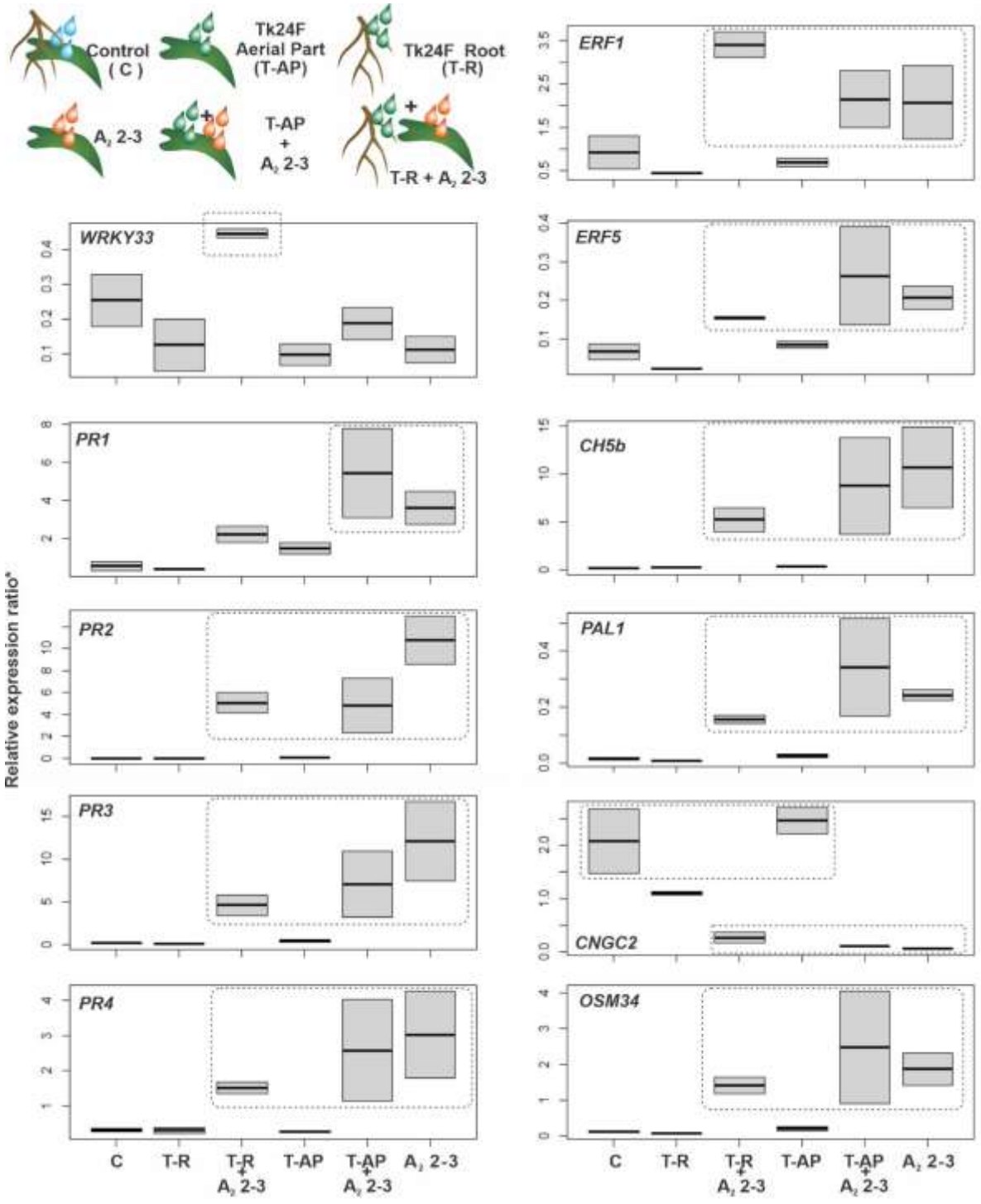

Control
(C)
Tk24F
Aerial Part
(T-AP)
Tk24F Root
(T-R)
A2 2-3
T-AP
+
A2 2-3
T-R + A2 2-3
Relative expression ratio*
WRKY33
PR1
PR2
PR3
PR4
ERF1
ERF5
CH5b
PAL1
CNGC2
OSM34
C
T-R
T-R + A2 2-3
T-AP
T-AP + A2 2-3
A2 2-3

Fig. 8. Effect of application of *Trichoderma koningiopsis* 24F (Tk24F) conidia on the leaf and root of common bean plants inoculated with *Colletotrichum lindemuthianum* on the expression of genes related to the plant defense system normalized with the *Act11* gene. C: Control treated with water in root and leaves. T-R: root treated with Tk24F. T-AP: aerial part treated with Tk24F. A_2 2-3: part of the area treated with *Colletotrichum lindemuthianum*.
*The relative expression ratio was calculated using the standard curve obtained for each gene.

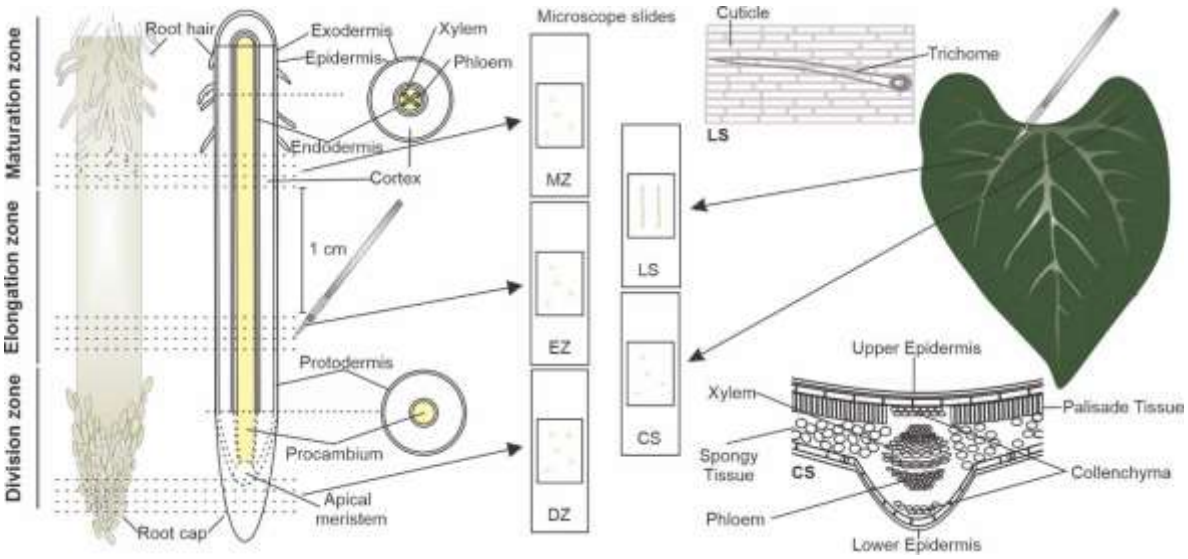
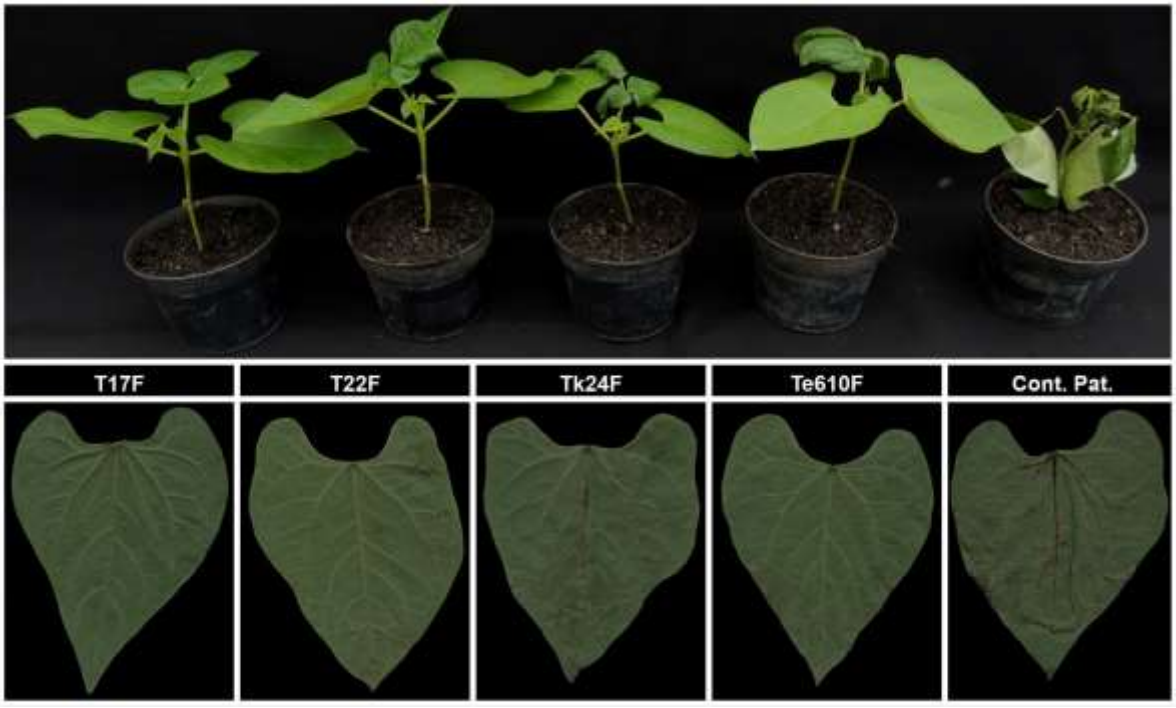


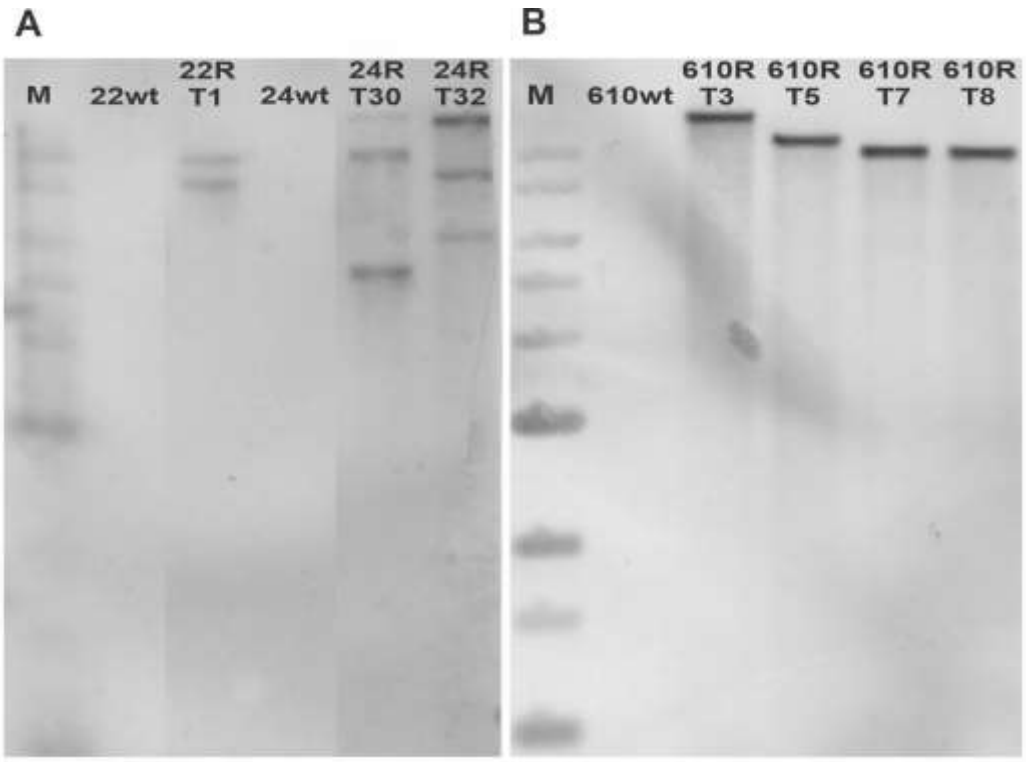
Fig. 9. Methodology for the study of colonization of common bean root and leaf tissues inoculated with *Trichoderma* spp. that express the red fluorescent protein.

On the left of the diagram are marked the root zones and the sections where the cuts were made for the observations under the fluorescence microscope; DZ: sections from the cell division zone, EZ: sections from the elongation zone, MZ: sections at the beginning of the maturation zone. On the right of the diagram are indicated the places on the leaf where the cross sections (CS) and the longitudinal sections (LS) of the ribs were made for observations under the fluorescence microscope.

Supplementary Figure 1. Common bean plants treated in the root and aerial part with *Trichoderma* spp. selected (isolates T17F, T22F, Tk24F and Te610F) and control where only the phytopathogen was applied (Cont. Pat.).



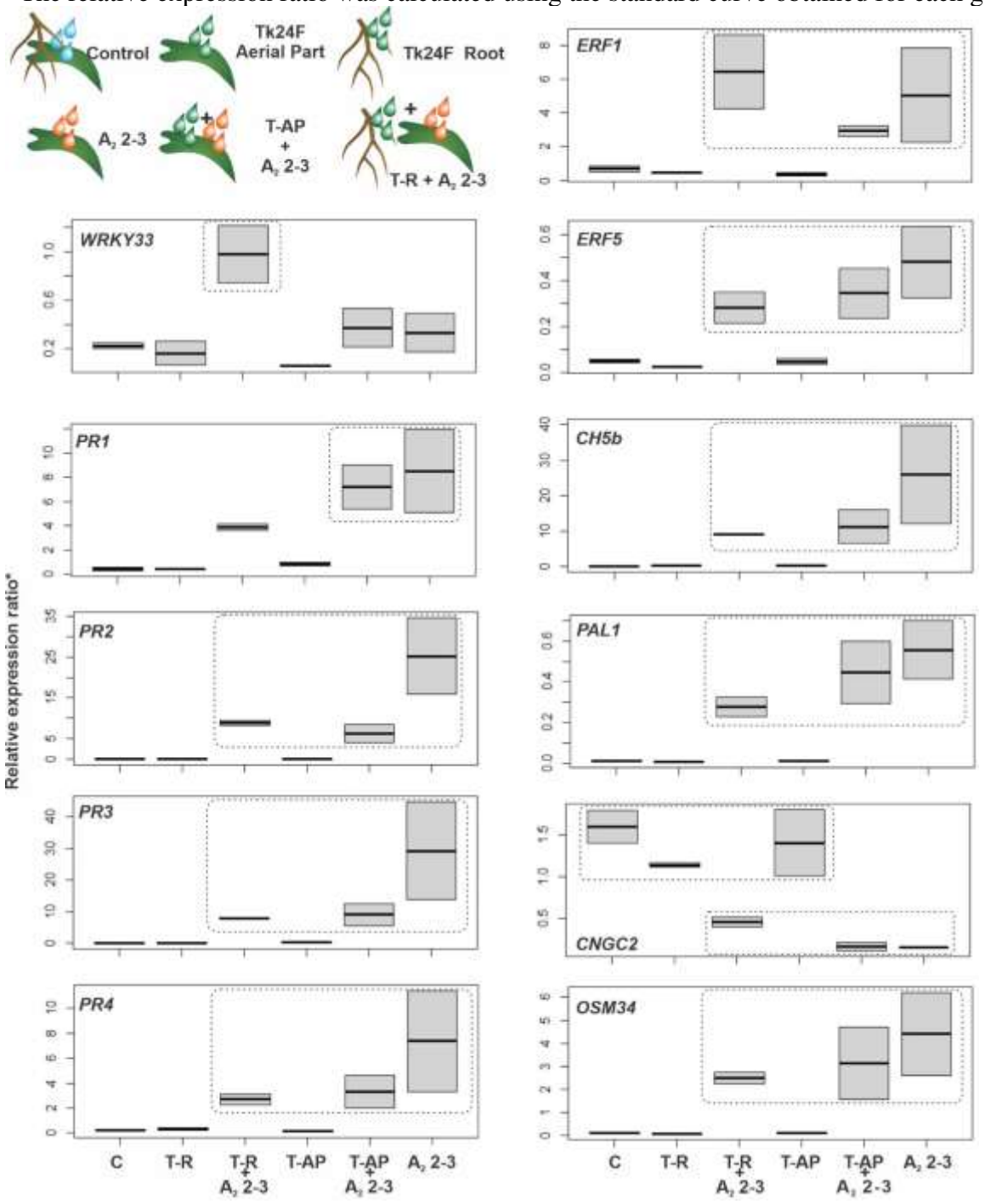
Supplementary Figure 2. Southern-blot analysis of *Trichoderma spp.* isolates transformants expressed RFP (R). DNA was digested with *Hind*III enzyme and hybridized with the *hph* gene as a probe.



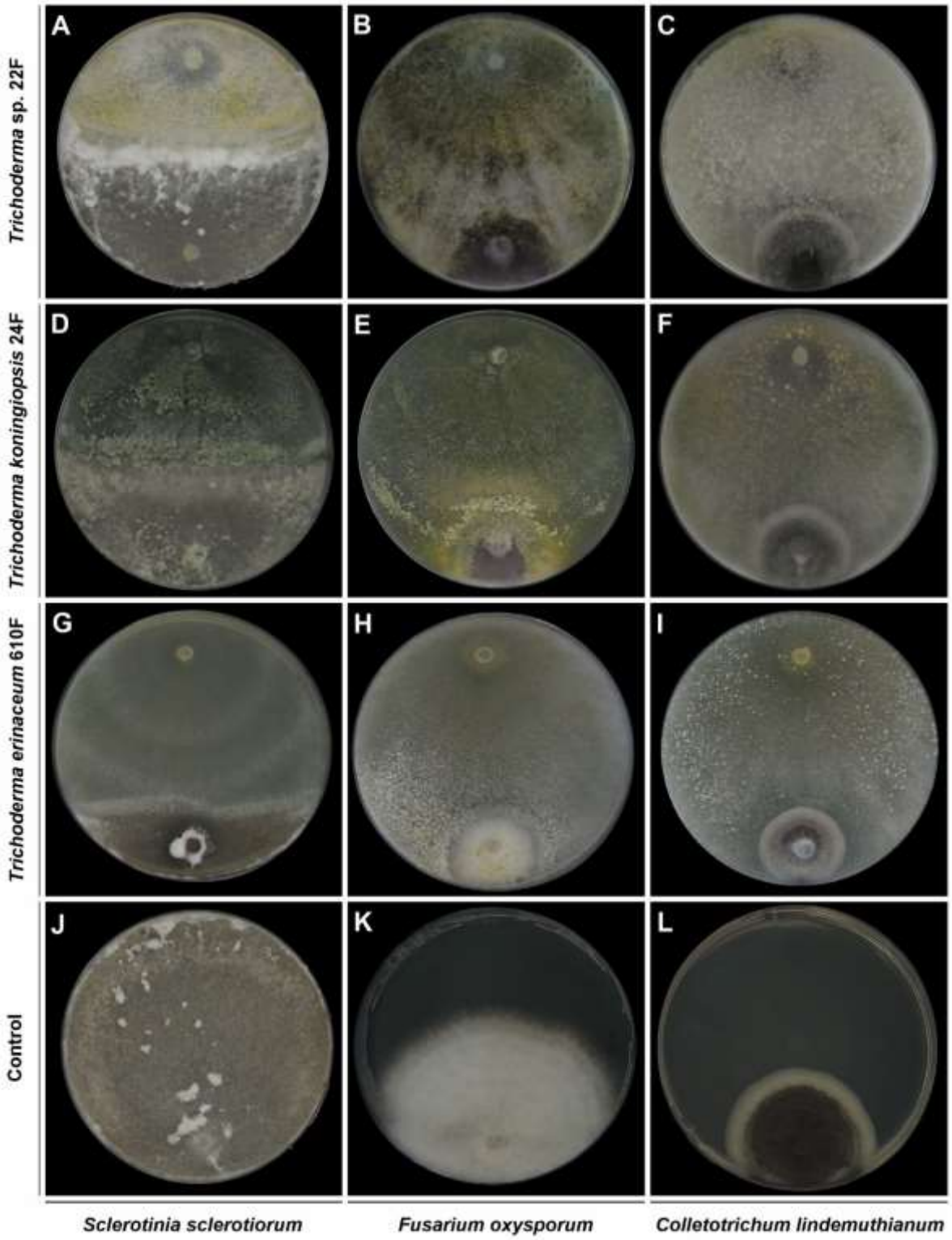
*R: RFP; wt: wild type.

Supplementary Figure 3. Effect of application of *Trichoderma koningiopsis* 24F (Tk24F) conidia on the leaf and root of common bean plants inoculated with *Colletotrichum lindemuthianum* on the expression of genes related to the plant defense system normalized with the *ide* gene. C: Control treated with water in root and leaves. T-R: root treated with Tk24F. T-AP: aerial part treated with Tk24F. A_2 2-3: part of the area treated with *Colletotrichum lindemuthianum*.

*The relative expression ratio was calculated using the standard curve obtained for each gene.



Supplementary Figure. 4. Mycoparasitism of *Trichoderma* spp. against common bean phytopathogens in the direct confrontation experiment.



Supplementary Figure 5. Germinated seeds of bean cultivar *Pérola*, used to inoculate the radicle with conidia suspensions of *Trichoderma* spp. A: Tray with moist filter paper for seed germination. B: Length of the radicle of the seeds used in the experiment. C and D: Cup with germinated seed and root in contact with the conidia suspension. Bar = 1 cm.

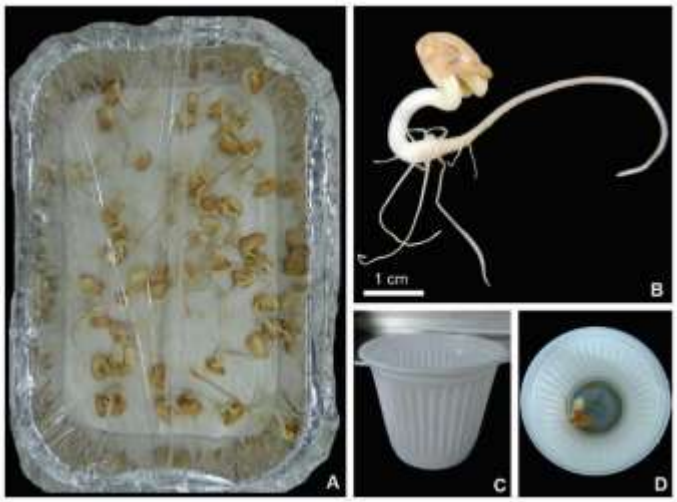


Table 1. Assessment of anthracnose severity after treatment of different common bean tissues with *Trichoderma* spp.

Treatment	Applicated plant part trial (Disease severity $\tilde{x}$ ± IC)[a]		
	Experiment 1 (CV: 29.65)		
	Root (R)	**Aerial part (AP)**	**R+ AP**
Trichoderma sp. VIC44363 17F	2.58 ± 0.32 **b**	2.01 ± 0.24 **b**	2.23 ± 0.58 **b**
Trichoderma sp. VIC44364 22F	3.61 ± 0.26 **a**	4.16 ± 0.47 **a**	1.32 ± 0.29 **b**
T. koningiopsis 24F	1.29 ± 0.17 **b**	2.21 ± 0.34 **b**	2.72 ± 0.29 **b**
T. erinaceum 610F	4.06 ± 0.50 **a**	2.56 ± 0.64 **b**	2.51 ± 0.56 **b**
Pat. Cont.		5.81 ± 1.04 **a**	
	Experiment 2 (CV: 37.11)		
Trichoderma sp. VIC44363 17F	4.83 ± 1.01 **a**	4.31 ± 0.39 **a**	3.08 ± 0.64 **b**
Trichoderma sp. VIC44364 22F	2.27 ± 0.46 **b**	2.92 ± 0.67 **b**	2.33 ± 0.41 **b**
T. koningiopsis 24F	2.44 ± 0.78 **b**	1.43 ± 0.45 **b**	1.41 ± 0.46 **b**
T. erinaceum 610F	6.25 ± 0.48 **a**	1.49 ± 0.19 **b**	2.68 ± 0.44 **b**
Pat. Cont.		5.14 ± 0.65 **a**	

[a] Percentage of lesion area. Means with confidence intervals (CI) followed by the same letter do not show differences according to the Scott-Knott test (α=0.5). Pat. Cont.: Control where only the pathogen was applied.

Table 2. Evaluation of bean growth promotion in a greenhouse by *Trichoderma* spp.

Treatments	Dry mass (x̃ ± IC g)*		Fresh mass (x̃ ± IC g)	
	Root (R)	**Aerial Part (AP)**	**Root (R)**	**Aerial Part (AP)**
	Experiment 1			
Trichoderma sp. VIC44363 17F	0.59 ±0.07	2.15 ±0.21 **a**	10.07 ±1.40	22.91 ±2.22 **a**
Trichoderma sp. VIC44364 22F	0.61 ±0.09	2.30 ±0.22 **a**	9.97 ±1.67	23.12 ±2.10 **a**
T. koningiopsis 24F	0.55 ±0.07	2.20 ±0.21 **a**	10.56 ±1.13	22.57 ±1.95 **a**
T. erinaceum 610F	0.57 ±0.07	2.30 ±0.22 **a**	11.38 ±1.54	23.10 ±1.88 **a**
Control	0.57 ±0.07	1.81 ±0.26 **b**	10.76 ±1.74	18.70 ±2.96 **b**
CV (%)	10.48	16.41	10.21	14.33
	Experiment 2			
Trichoderma sp. VIC44363 17F	0.24 ±0.05	0.55 ±0.05 **a**	4.01 ±0.99	5.55 ±0.78 **a**
Trichoderma sp. VIC44364 22F	0.25 ±0.06	0.55 ±0.06 **a**	4.59 ±0.92	5.60 ±0.64 **a**
T. koningiopsis 24F	0.24 ±0.05	0.52 ±0.08 **a**	4.20 ±0.74	5.29 ±0.67 **a**
T. erinaceum 610F	0.22 ±0.04	0.51 ±0.05 **a**	3.99 ±0.59	4.75 ±0.51 **b**
Control	0.22 ±0.06	0.45 ±0.03 **b**	3.89 ±0.62	4.37 ±0.27 **b**
CV (%)	23.82	11.53	19.08	11.79

*Weight in grams. Means with confidence intervals (CI) followed by the same letter do not show differences according to the Scott-Knott test (α=0.5). Data without letters showed no statistical differences by one-way ANOVA (p>0.05). Cont.: Control where only water was applied.

Table 3. Germination of *Colletotrichum lindemuthianum* conidia

		***Colletotrichum lindemuthianum* conidia germination (%)[a]**							
		T22F (*Trichoderma* sp.)		**Tk24F (*T. koningiopsis*)**		**Te610F (*T. erinaceum*)**		**Control**	
Treatments	**Media**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Conidia suspension	PDA[b]	44.8 ±4.3	95.7 ±4.1	0.0 ±0.0	8.7 ±0.2	31.2 ±19.2	77.5 ±17.3	22.3 ±3.9	34.7 ±7.2
	PDA-SB[c]	68.6 ±3.2	91.3 ±5.3	0.0 ±0.0	0.3 ±0.5	0.0 ±0.0	0.0 ±0.0	51.8 ±7.0	61.4 ±5.9
	PDA-LB[d]	99.7 ±0.6	77.0 ±0.4	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	97.5 ±4.3	87.6 ±5.5
Filtrates	PDA-LB	74.8 ±33.1	32.2 ±29.8	76.8 ±31.6	75.6 ±27.1	78.8 ±22.8	67.7±26.3	96.1±0.9	65.5 ±25.6

*1 and 2 represent repetition of the experiments.

[a] Data are the mean ± standard deviation of three independent biological repetitions.

[b] Potato Dextrose Agar medium.

[c] Potato Dextrose Agar medium prepared with infusion of *leaves* of common been.

[d] Potato Dextrose Agar medium prepared with infusion of *seeds* of common been.

Table 4. Inhibition of *Colletotrichum lindemuthianum* growth in leaf discs treated with suspensions of conidia or filtrates of these suspensions

Treatments	Replicates	NDPG[b] ($\bar{x}$ ±SD)		LD[c] ($\bar{x}$ ±SD)	
		CS[d]	F[e]	CS	F
***Trichoderma* sp. 22F**	**1**	4.3 ±3.0 **c**	9.3 ±0.6 **a**	9.9 ±2.6	9.6 ±0.7
	2	6.7 ±0.6 **b**	7.3 ±1.5 **a**	6.4 ±1.9	7.0 ±1.6
***T. koningiopsis* 24F**	**1**	6.7 ±1.5 **b**	9.0 ±1.0 **a**	7.4 ±3.3	9.8 ±1.5
	2	7.0 ±1.0 **b**	6.6 ±0.6 **a**	7.5 ±1.7	8.8 ±1.8
***T. erinaceum* 610F**	**1**	2.3 ±1.5 **c**	4.0 ±1.0 **b**	3.6 ±3.2	7.6 ±5.1
	2	5.3 ±1.5 **b**	4.7 ±1.1 **b**	6.4 ±3.5	8.6 ±1.9
Control	**1**	9.6±0.6 **a**		7.5 ±1.7	
	2	8.7 ±0.6 **a**		8.6 ±2.7	

[a] Data are the mean ± standard deviation of three independent biological repetitions. Different letters indicate statistical differences between treatments determined by the Skott-Knott mean comparison test ($p<0.05$). Data without letters showed no statistical differences by one-way ANOVA ($p>0.05$)

[b]NDPG: Number of leaf disc with phytopathogen growth

[c]LD: Lesion diameter in milimeters

[d]CS: Conidia suspension

[e]F: Filtrates

Table 5. Growth inhibition of common bean phytopathogens by *Trichoderma* spp.

Phytopathogens	Inhibition in growth (%)[a]					
	***Trichoderma* sp. 22F**		***T. koningiopsis* 24F**		***T. erinaceum* 610F**	
	1	**2**	**1**	**2**	**1**	**2**
***F. oxysporum*[b]**	24.5 ±0.5	14.4 ±1.1	53.7 ±3.6	59.1 ±1.3	43.3 ±3.4	100.0 ±0.0
***S. sclerotiorum*[c]**	5.9 ±0.0	5.9 ±8.4	25.4 ±0.0	28.7 ±6.0	100.0 ±0.0	100.0 ±0.0

*1 and 2 represent repetition of the experiments.

[a] Data are the mean ± standard deviation of three independent biological repetitions.

[b]*Fusarium oxysporum* f. sp. *phaseoli*

[c]*Sclerotinia sclerotiorum*

Table 6. Summary of the results of the experiments according to the evaluated mechanism of action.

Machanism of action	**Mycoparasitism**			**Antibiosis**				**Colonization**			**Induced sistemic resistant**
Experiment / **Isolate**	Confrontation	Fluorescece microscopy	Leaf-disc/ Suspension	Celofane	Germination		Leaf-disc/ Filtrates	Root Petri dish	Leave Petri dish	Root Pot	qPCR
					Suspension	Filtrates					
T22F	✓	✓	✓	✓	⊗	✓	⊗	✓	✓	⊗	
Tk24F	✓✓	✓✓	✓	✓✓	✓	⊗	⊗	✓	✓	✓	?
Te610F	✓✓	✓✓✓	✓	✓✓✓	✓	⊗	✓	✓	✓	✓	

The checkmark means that the evaluated mechanism was positively verified with the experiment in reference, more than one mark means that a better performance was observed compared to the other two isolates. The cross means that the evaluated mechanism was negatively verified with the reference experiment. The interrogation mark means that the evaluated mechanism was unconclusive with the reference experiment.

Supplementary Table 1: Comparison of phenotypic characteristics evaluated in transformants and wild type isolates of *Trichoderma* [a]

Isolate/Transformant	RGR (mm/d)[b]	MS[c]	IM (%)[d]	PCN[e]	E (%)[f]
T22F (*Trichoderma sp.*)	**17.7 ±1.0**	**Yes**	**20.3 ±2.7**	**0**	**85.1 ±18.7**
22RFP-T1	**17.8 ±0.7**	**Yes**	**19.5 ±2.4**	**2**	**88.5 ±13.9**
Tk24F (*T. koningiopsis*)	**18.1 ±0.9**	**Yes**	**25.18 ±3.0**	**0**	**84.4 ±9.6**
24RFP-T11	19.1 ±1.7*	Yes	32.6 ±5.9	NA	NA
24RFP-T14	21.7 ±0.6*	Yes	85.9 ±13.1*	NA	NA
24RFP-T20	18.8 ±0.7*	Yes	41.2 ±8.7	NA	NA
24RFP-T30	**20.9 ±0.9**	**Yes**	**29.6 ±5.5**	**2**	**74.5 ±15.4**
24RFP-T32	17.7 ±2.3	Yes	35.2 ±2.5	3	NA
24RFP-T34	13.7 ±0.1*	Yes	26.9 ±3.7	NA	NA
Te610F (*T. erinaceum*)	**18.4 ±0.5**	**Yes**	**100.0 ±0.0**	**0**	**58.2 ±23.7**
610RFP-T1	14.7 ±0.1*	Yes	100.0 ±0.0	NA	NA
610RFP-T3	**17.7 ±1.0**	**Yes**	100.0 ±0.0	**1**	**59.74 ±29.5**
610RFP-T4	18.9 ±0.3	Yes	100.0 ±0.0	NA	NA
610RFP-T5	17.1 ±0.4	Yes	100.0 ±0.0	1	43.1 ±45.1
610RFP-T6	15.3 ±0.0*	Yes	100.0 ±0.0	NA	NA
610RFP-T7	20.6 ±2.4	Yes	100.0 ±0.0	1	6.7 ±89.4
610RFP-T8	18.6 ±0.3	Yes	100.0 ±0.0	1	85.0 ±13.4
610RFP-T11	16.7 ±2.1	Yes	100.0 ±0.0	NA	NA
610RFP-T15	17.8 ±0.3	Yes	92.4 ±2.6*	NA	NA

In bold, the wild isolates and selected transformants

[a] Data are the mean ± standard deviation of three independent biological replicates, and asterisks indicate significant differences between the wild-type isolate and the transformant determined using Student's t-test (* $P < 0.05$; ** $P < 0.01$)

[b] Radial growth rate

[c] Mitotic stability

[d] Inhibition percentage

[e] Plasmid copy number

[f] Efficacy in controlling the phytopathogen

Supplementary Table 2: Primers used to amplify common bean genes for the qPRC study.

Primer name	Sequence (5' to 3')	Gene amplified	Reference	Efficiency[a]
IDE-F	GCAACCAACCTTTCATCAGC	Insulin degrading enzyme	Borges et al. 2012	103.98
IDE-R	AGAAATGCCTCAACCCTTTG			
Act11-F	TGCATACGTTGGTGATGAGG	Actin-11	Mayo et al. 2016	103.42
Act11-R	AGCCTTGGGGTTAAGAGGAG			
WRKY33-F	TTTCACAGGACAGGTTCCAGC	WRKY transcription factors	Mayo et al. 2016	97.78
WRKY33-R	CCTTTGACAGAAATGACTGAAGGA			
PR1-F	TGGTCCTAACGGAGGATCAC	Pathogenesis Related 1	Mayo et al. 2016	97.05
PR1-R	TGGCTTTTCCAGCTTTGAGT			
PR2-F	GTGAAGGACGCCGATAACAT	Beta 1-3 Endoglucanase	Mayo et al. 2016	103.45
PR2-R	ACTGAGTTTGGGGTCGATTG			
PR3-F	TGGAGTTGGTTATGGCAACAA	Chitinase classI	Mayo et al. 2016	100.29
PR3-R	ATTCTGATGGGATGGCAGTGT			
PR4-F	CGCAGTGAGTGCATATTGCT	Pathogenesis-related 4	Mayo et al. 2016	97.38
PR4-R	TGTTTGTCACCCTCAAGCAC			
ERF1-F	CGCTCTCAAGAGGAAACACTCC	Ethylene-Responsive Transcription Factor1	Mayo et al. 2016	96.24
ERF1-R	TGAATCAGAAGGAGGAGGGAAT			
ERF5-F	GGCTCCAAGTGGATTGAGAAC	Ethylene-Responsive Transcription Factor 5	Mayo et al. 2016	97.92
ERF5-R	TCAGAATCAGATAACTACAAAGCACAA			
CH5b-F	CAGCCAAAGGCTTCTACACC	Endochitinase precursor	Mayo et al. 2016	102.97
CH5b-R	TTGTTTCGTGAGACGTTTGC			
PAL1-F	TGAGAGAGGAGTTGGGCACT	Phenylalanine and histidine ammonia-lyase	Mayo et al. 2016	95.95
PAL1-R	TTCCACTCTCCAAGGCATTC			
OSM34-F	GAACGGAGGGTGTCACAAAATC	Osmotin-like protein	Mayo et al. 2016	95.71
OSM34-R	CGTAGTGGGTCCACAAGTTCCT			
CNGC2-F	ATTCAATTTGCTTGGAGACGTT	Cyclic nucleotide-gated ion channel 2	Mayo et al. 2016	96.78
CNGC2-R	ACAGTTTTATTGAAGGCCAGGA			
hGS-F	GTGGCTATATGGTGCGTACAAA	Homoglutathione synthetase	Mayo et al. 2016	100.22
hGS-R	GAAACAAGAATGCATCTCCTCA			

[a] In this work.

Supplementary Table 3: Relative expression levels of genes associated with resistance induction in common bean treated with *Trichoderma koningiopsis* 24F (T) and *Colletotrichum lindemuthianum* (A_2 2-3) in aerial part (AP) and roots (R) normalized with the gene *Act11*[a]

Gene	Control	T-R[b]	T-R+A_2 2-3	T-AP[c]	T-AP+A_2 2-3	A2 2-3[d]
WRKY33	0.254 ±0.106 **b**	0.124 ±0.106 **b**	0.447 ±0.019 **a**	0.096 ±0.045 **b**	0.187 ±0.066 **b**	0.110 ±0.054 **b**
PR1	0.556 ±0.338 **b**	0.376 ±0.026 **b**	2.237 ±0.608 **b**	1.489 ±0.449 **b**	5.448 ±3.318 **a**	3.606 ±1.221 **a**
PR2	0.005 ±0.004	0.017 ±0.001	5.044 ±1.330	0.051 ±0.031	4.800 ±3.503	10.768 ±3.091
PR3	0.178 ±0.057	0.149 ±0.096	4.616 ±1.748	0.407 ±0.209	7.027 ±5.447	12.056 ±6.578
PR4	0.318 ±0.077	0.313 ±0.112	1.511 ±0.238	0.277 ±0.028	2.579 ±2.037	3.031 ±1.741
ERF1	0.916 ±0.534 **b**	0.441 ±0,022 **b**	3.417 ±0.417 **a**	0.690 ±0.126 **b**	2.151 ±0.925 **a**	2.070 ±1.205 **a**
ERF5	0.067 ±0.029	0.024 ±0.000	0.154 ±0.004	0.085 ±0.012	0.264 ±0.181	0.207 ±0.043
CH5b	0.141 ±0.064	0.224 ±0.003	5.218 ±1.798	0.322 ±0.023	8.764 ±7.137	10.693 ±5.945
PAL1	0.016 ±0.007	0.009 ±5.511	0.155 ±0.021	0.025 ±0.008	0.342 ±0.247	0.242 ±0.027
OSM34	0.125 ±0.045	0.088 ±0.002	1.413 ±0.332	0.210 ±0.071	2.473 ±2.215	1.865 ±0.634
CNGC2	2.079 ±0.860	1.103 ±0.036	0.275 ±0.145	2.465 ±0.352	0.109 ±0.014	0.068 ±0.016

[a] Data are the mean ± standard deviation of three independent biological replicates. Different letters indicate statistical differences between treatments determined by the Skott-Knott mean comparison test ($p<0.05$). Data without letters showed no statistical differences by one-way ANOVA ($p>0.05$)

[b]T-R: Root application of isolate Tk24F

[c]T-AP: Aerial part application of isolate Tk24F

[d]A_2 2-3: Aerial part application of *Colletotrichum lindemuthianum* pathotype 89 isolate $A_2$2-3

Supplementary Table 4: *Trichoderma* spp. isolates evaluated for the control of anthracnose in common bean plants in a greenhouse.

N	Species	Isolate	Source
1	*Trichoderma* sp. VIC44364	22F20C_AC	Stem de *Hevea brasiliensis* − Acre, Brazil
2	*T. ararianum*	432F8C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
3	*Trichoderma* sp. VIC44363	14F5R_AM	Root de *Hevea brasiliensis* − Amazonas state of Brazil
4		15F5R_AM	Root de *Hevea brasiliensis* − Amazonas, Brazil
5		17F5R_AM	Root de *Hevea brasiliensis* − Amazonas, Brazil
6	*T. endophyticum*	23F18C_AC	Stem de *Hevea brasiliensis* − Acre, Brazil
7		549F18C_AC	Stem de *Hevea brasiliensis* − Acre, Brazil
8	*Trichoderma* sp. VIC44362	763F25F_AM	Leaf of *Hevea brasiliensis* − Amazonas, Brazil
9	*T. erinaceum*	20F5C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
10		610F5C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
11	*T. koningiopsis*	24F6C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
12		58F6C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
13		245F13C_AC	Stem de *Hevea brasiliensis* − Acre, Brazil
14		265F6C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
15		815F11R_AM	Root de *Hevea brasiliensis* − Amazonas, Brazil
16	*T. lentiforme*	247F12R_AC	Root de *Hevea brasiliensis* − Acre, Brazil
17		518F1C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
18		765F5C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil
19	*T. ovalisporum*	4F9R_AC	Root de *Hevea guianensis* − Acre, Brazil
20	*T. virens*	84F15C_AM	Stem de *Hevea brasiliensis* − Amazonas, Brazil

Capítulo 2

Characterization of secondary metabolism gene cluster of *Trichoderma sp.* VIC44363

Characterization of secondary metabolism gene cluster of *Trichoderma sp.* VIC44363

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Abstract

Background: Few representatives of the *Trichoderma* genus of the Brevicompactum clade have been studied in relation to their interaction with plants, as they produce mycotoxins that can be harmful to plants, animals, and humans. However, the study of the species *Trichoderma arundinaceum* has shown that trichothecenes such as Harzianum A (HA) have antimicrobial properties without being phytotoxic, which has allowed the study of their interaction with plants. *Trichoderma brasiliensis* (T17F), a new species/isolate from the Amazon rainforest has been shown to antagonize *Colletotrichum lindemuthianum* and promote plant growth, therefore the purpose of this work was to determine the mechanism responsible for the antagonism, the spectrum of action and through comparative genomics to propose hypotheses about the causes of the antimicrobial and beneficial effect for the plant.

Results: It was determined that antibiosis is the mechanism used by T17F isolate to antagonize *C. lindemuthianum* and other phytopathogenic fungi. The composition and organization of the trichothecenes cluster of the T17F isolate was compared with the cluster of *T. brevicompactum* and *T. arundinaceum* and it was concluded that *Trichoderma sp.* VIC44363 may not produce HA, as it lacks the *tri23* gene, but has the potential to produce new compounds, as it has a cluster different from those found in *T. brevicompactum* genome.

Conclusions: T17F isolate has the potential to produce trichodermin, but also other compounds that may be related to the antagonistic capacity and growth promotion of common bean, as it has clusters absent in *T. brevicompactum*.

Keys-words: Trichothecenes, antibiosis, comparative genomics, SM.

Background

In the universe of applications of fungi of the *Trichoderma* genus, biological control is one of the most studied fields due to the ability of these fungi to protect plants against phytopathogenic fungi and nematode (1, 2). Mycoparasitism is considered the ancestral characteristic of the genus *Trichoderma* (3), but it can also protect plants through mechanisms such as antibiosis, competition and systemic resistance induction, in which secondary metabolites (SM) are involved (4). Some taxonomic groups within the genus seem to have adopted strategies in which the production of SM can be essential, as they have a greater diversity of them, demonstrated by the large number of SM clusters present in their genomes and by the chemical characterization (5, 6, 7). This wide variety of SM and the potential to produce new compounds makes these taxonomic groups an interesting object of study to be explored in industry and biotechnology, as well as in agriculture to develop alternatives for the sustainable management of agricultural crops (8, 9).

The species of the genus *Trichoderma* of the clades Harzianum and Virens stand out as the taxonomic groups with the highest number of SM clusters compared to species of the clades Longibrachiatum and Section Trichoderma (10). The *Trichoderma* core genome proposed in the same study has six clusters of polyketide synthases (PKS), 10 of non-ribosomal peptide synthetases (NRPS) and three of terpene synthases (TS), which shows the importance that SMs should have in Trichoderma's ecology, as was also reported by Vicente et al. (7) in their studies of TS clusters. The species of the Brevicompactum clade, which were not included in Vicente's study, are also characterized for being important producers of SMs, especially trichothecenes such as trichodermin and Harzianum A (HA) (11, 12). Most species of the Brevicompactum clade produce HA, unlike *Trichoderma brevicompactum* and *Trichoderma rodmanii* which produce trichodermin (13). Trichothecenes are mycotoxins of the sesquiterpenes group that are produced by some species of phytopathogens, entomopathogens and *Trichoderma* (14). One of these genera is *Fusarium*, in which some species are producers of trichothecenes, for instance T2, nivalenol, deoxynivalenol, tricotecolone and tricothecin (14, 15, 16).

The study of trichothecenes in species of the *Fusarium* genus has received special attention because they are pathogenic factors of health importance in agricultural crops, as cereals, in which they represent a risk because they are toxic to animals and humans when

present in food (17, 18, 19). The possibility of producing trichothecenes means that *Trichoderma* species of the Brevicompactum clade are not considered for the biological control of phytopathogens, although they are effective *in vitro* inhibitors of the growth of other fungi (20, 17, 21, 22). However, in addition to antimicrobial activity, trichothecenes are also studied for the treatment of cancer (23), for the design of new bioactive compounds (24) and to understand its effect on interaction with plants (21, 25).

Trichoderma brevicompactum and *T. arundinaceum* are the most studied species of the Brevicompactum clade regarding antimicrobial activity, interaction with plants and biosynthesis pathway of trichothecenes. Trichodermin extracted from the endophytic isolate of *T. brevicompactum* 0248 showed outstanding antifungal activity against *Rhizoctonia solani* and *Botrytis cinerea* and to a lesser extent against *C. lindemuthianum* compared to the positive control Carbemdazim (22). Likewise, the extract or the purified trichodermin of other *T. brevicompactum* isolates such as ADL-9-2 (26) and IBT 40837 (17) inhibited the growth of bacteria or/and fungi. However, in the same study a negative effect on tomato growth and an increase in the severity of the damage caused by *Botrytis cinerea* on the plant was observed (17). On the contrary, the isolate *T. arundinaceum* IBT 40837, in addition to showing an antimicrobial effect on phytopathogenic yeasts and fungi, also elicit plant defense responses in tomato plants (21). The *T. brevicompactum* IBT 40841 and *T. arundinaceum* IBT 40837 isolates have their genomes sequenced and have been the reference isolates used in studies on the production pathway of tricodermin and HA, respectively (17, 27, 14).

Tri gene clusters and trichothecene production are not common and are discontinuously distributed among ascomycete fungi. Proctor et al. (14) compared the genes of trichothecene biosynthesis and determined how they are organized in the genome of 14 species from nine fungal genera. This work permitted to determine which genes are common to all studied fungi (*tri3*, *tri5* and *tri14*) and to propose the formation of a tri-ancestral cluster, which would be composed of 10 genes. Trichothecenes produced by fungi have more than 150 variations and are constituted by a 12,13-epoxytrichotec-9-ene (EPT) core structure, which differ in the patterns and types of oxygenations or acetylations of one or more positions of the EPT (14). Harzianum A, for example, is constituted by an EPT which on carbon eight has an acyl group derived from a polyketide synthase esterified to an oxygen on carbon 4. The genes (*tri*) and the respective biosynthetic proteins (TRI) of the HA production pathway in *T. arundinaceum* that consists of three phases have been described. In the initial phase of the HA synthesis pathway,

a terpene synthase (TRI5) acts, in the second phase a cytochrome P450 monooxygenase (CYP, TRI4) and finally another CYP, TRI22. The phase leading to the formation of octa-2,4,6-trienedioyl-CoA is mediated by the polyketide synthase TRI17 and CYP TRI23, the acetylation of trichodermol to form the 4-acetyl EPT (trichodermin) is mediated by the acyl transferase TRI3 and the transacylation that results in the substitution of 4-acetyl to form HA is mediated by TRI18 (14, 28, 29, 27, 30). In the trichodermin synthesis pathway, produced by *T. brevicompactum*, neither the last replacement phase nor the CYP TRI23-mediated octa-2,4,6-trienedioyl-CoA synthesis occurs, as it lacks the tri23 gene. In the main cluster there are other genes such as *tri6* which codes for a Cys2His2 Zn fingers (TRI6) protein with which regulates the expression of the other *tri* genes (29).

Few representatives of the Brevicompactum clade have been studied regarding their interaction with plants and its potential to produce new compounds. Studies with new representatives of the clade from environments such as the Amazon forest and associated with plants such as endophytes will allow us to advance in the knowledge of the role that trichothecene-producing fungi play in nature and in the survival of the fungi themselves (12). Given the recognized potential of *Trichoderma* species in the production of secondary metabolites, the genomic approach allows the identification of metabolites that would otherwise not be found, as they are not produced under laboratory conditions (5, 31). The genome mining of *Trichoderma* species also represents an opportunity to identify interesting genes in the development of transgenic plants tolerant to biotic and abiotic stresses (32).

The present work aimed to characterize secondary metabolism gene cluster of *Trichoderma sp.* VIC44363 T17F originating from the Amazon forest isolated as an endophyte from native rubber trees and which, when applied to bean plants, protected them from severe attack by *C. lindemuthianum* and promoted growth (chapter 1). It was then verified that the main mechanism used by T17F isolate to antagonize *C. lindemuthianum* was antibiosis and that the diffusion of antimicrobial compounds also inhibited other phytopathogenic fungi, demonstrating its broad spectrum of action. The genome of the T17F isolate was sequenced and analyzed regarding the potential to produce secondary metabolites (SM) and the presence and organization of the trichothecenes cluster, comparing it with the *T. brevicompactum* and *T. arundinaceum* clusters.

Results

Genetic transformation with the fluorescent protein gene

In order to evaluate the antagonism mechanisms and colonization capacity of common bean, the transformation of the *Trichoderma sp*. VIC44363 T17F isolate with a plasmid (pPGW) carrying a gene encoding red fluorescent protein (RFP) was performed. Seventeen transformants were obtained. The transformation efficiency was 1.7 transformants/µg of plasmid DNA.

No changes were observed in the evaluation of the phenotypic characteristics of the analyzed transformants. The transformation did not alter the radial growth rate, the antimicrobial activity in the cellophane membrane test, nor the efficacy in controlling *C. lindemuthianum* on bean plants (Supplementary Table 1). The transformant 17RFP-T2 was selected since it was determined a single copy of plasmid pPGW in its genome (Supplementary Figure 1).

Antagonistic capacity of T17F isolate in the common bean - *C. lindemuthianum* pathosystem

Mycoparasitism: T17F isolate did not cover the colony of *C. lindemuthianum* in the dual confrontation assays after 10 days of incubation and only some pustules were observed on the margin of the colonies of the phytopathogen (Fig. 1a). In microscopic analyses, a concomitant growth of the hyphae of the two fungi was observed more frequently, without showing a tropism towards the host hyphae by T17F isolate (Fig. 1 d-e). However, the folding structure of the hyphae of the T17F isolate on the hyphae of *C. lindemuthianum* was observed at some points on the slides after 7 days of incubation (Fig.1 f-g).

Antibiosis: The T17F isolate secreted compounds in the culture medium through the cellophane (enzymes and metabolites) and dialysis membranes (metabolites with molecular weight below 12 kDa) and these compounds inhibited the growth of *C. lindemuthianum* by 100% compared to the plates of the control that were inoculated with the phytopathogen without the previous growth of *Trichoderma* (Fig. 1b). Similarly, in the antibiogram test, there was inhibition of germination of *C. lindemuthianum* conidia and the formation of inhibition halos of 47.99 ± 2.59 mm and 50.31 ± 1.77 mm in the two replicates of the experiment (Fig. 1c).

Competition: No evidence of root and leaf colonization of common bean was found in observations made during 4 days after inoculation. In some sections only conidia were observed, but they never germinated and/or adhered to the superficial layers or internal layers or in the root cap (Supplementary Figure 2).

Mycoparasitism and antibiosis in leaf discs: The T17F isolate inhibited the growth of *C. lindemuthianum* when previously applied to disinfested fresh leaf discs and placed in Petri dishes with moist filter paper. The application of conidia suspensions and T17F isolate suspension filtrate decreased the number of discs with growth signs of the phytopathogen (NDPG) but had no effect on reducing the size of lesions caused by the phytopathogen (LD) (Table 1).

Antagonistic capacity of T17F isolate to other phytopathogenic fungi *in vitro*

Mycoparasitism and antibiosis: T17F isolate did not cover the colonies of Bc, Fo and Ss in the direct confrontation assays after 10 days of incubation, however, it was observed the formation of some pustules on the margin of the colonies of *Botrytis cinerea* (Supplementary Figure 3). On the other hand, in the growth tests on cellophane membrane and dialysis, and in the antibiogram tests, antimicrobial activity was observed against all studied phytopathogens, which together show the *Fusarium oxysporum* isolate as the least susceptible, with a smaller inhibition halo in the antibiogram test, followed by the *B. cinerea* isolate (Table 2; Supplementary Figure 3). The lower susceptibility of the *F. oxysporum* isolate was also observed in the membrane test with lower percentages of inhibition, especially in the dialysis membrane test with an inhibition percentage of up to 48.7% (Table 2).

Genomic analysis

Genome assembly: The genome was assembled in 582 scaffolds, with a total size of 38.5 Mb, the largest scaffold was 2.4 Mb, the N50 scaffold was 939 Kb and the L50 scaffold was 13.

Phylogenetic relationship of *Trichoderma* sp. VIC44363 with other species of the genus through the analysis of 27 housekeeping genes

The genome of *T. brevicompactum* IBT 40841 and *T. arundinaceum* IBT 40837 used in the present study were those previously reported by Proctor et al. (14). *Trichoderma sp.* VIC44363 clustered with *Trichoderma brevicompactum* with bootstrap values of 100 in the

phylogenetic trees of 18 of the 27 genes analyzed. In the *act1* sequence tree the bootstrap was 99, in *erg1, ku70* and *pgk1* was 98, in *lae1*, *tef1* and *tub2* was 97, in *tub1* was 95 and 93 in the *his3* tree. *Trichoderma sp.* VIC44363 also clustered with *Trichoderma turialbense* in the *his3*, *tef1* and *tsr1* sequence trees. The topologies of all phylogenetic trees were similar except for the *his3* sequence tree.

The concatenated alignment had 70,761 sites, of which 43,606 were conserved, 26,847 were variable, and 21,384 were informative for parsimony. Comparing the species *Trichoderma sp.* VIC44363 and *T. brevicompactum* only 434 sites were variable, a smaller difference than between other closely related species such as *Trichoderma reesei* and *Trichoderma parareesei* that differ in 1,333 sites. In the consensus phylogenetic tree, *Trichoderma sp.* VIC44363 grouped into the Brevicompactum clade along with *T. brevicompactum* and *T. turialbense* (Fig. 2).

Comparison of secondary metabolism cluster between *Trichoderma* sp. VIC44363 and *T. brevicompactum*

Sixty-three SM clusters were predicted, with one or more core genes encoding polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), PKS-NRPS hybrids, indole synthases (IS) and terpene synthases (TS). When compared with the predicted clusters for *T. brevicompactum*, there was a minimal difference regarding the number of clusters, with *Trichoderma sp.* VIC44363 having one more PKS cluster (Table 3). However, there are differences in the gene composition of the clusters due to additions and deletions of genes and only half of the clusters are conserved (Table 3).

Furthermore, the indole cluster and a NRPS cluster of *Trichoderma sp.* VIC44363 had no homologous clusters in *T. brevicompactum* (Table 3). The NRPS cluster has other biosynthetic genes in addition to the core gene TBRAS_501 which were predicted to encode a methyl transferase, two aldo/keto reductases, another NRPS and an alkanesulfonate monooxygenase (Table 4). Likewise, the single PKS cluster has other biosynthetic genes in addition to the TBRAS_2582 core gene that have been predicted to encode another PKS, a P450 monooxygenase, a hydrolase, and a transport-related gene (Table 4).

Comparison of the trichothecene biosynthesis cluster between *Trichoderma* sp. VIC44363, *T. brevicompactum* and *T. arundinaceum*

Most proteins of the *T. arundinaceum* HA biosynthesis pathway were identified among the predicted proteins in the genome analysis of *Trichoderma sp.* VIC44363 with the AUGUSTUS program. The homology was also verified by the percentage of identity and coverage determined with the NCBI Blastp tool. Most of the proteins encoded by major cluster genes (TRI4, TRI10, TRI22, TRI12, TRI14 and TRI6) have greater than 95% identity, just as TRI5 has 99% identity. The TRI17 and TRI18 proteins showed identities of 94.3% and 93.5%, respectively. The TRI3 protein had the lowest identity of 85.5% with the TRI3 of *T. arundinaceum* and 93.1% with the TRI3 of *T. brevicompactum* (Fig. 3). A protein homologous to TRI23 was not found. The *tri17* and *tri18* genes are arranged in a cluster along with *tri23* in the *T. arundinaceum* genome (Fig. 4). Therefore, the *tri* genes in *Trichoderma sp.* VIC44363 are the same and have the same organization as in *T. brevicompactum*, but there are differences in gene size and *Trichoderma sp.* VIC44363 lacks three binding sites of the TRI6 protein in the promoter regions of the *tri14*, *tri22* and *tri10* genes (Fig. 4).

Discussion

The species of the Brevicompactum clade have been a separate chapter in the well-known history of the genus *Trichoderma* in biological control as they are producers of trichothecenes mycotoxins, trichodermin and Harzianum A (HA). Although the isolates of species of this clade stand out for inhibiting the growth of a wide spectrum of phytopathogenic fungi *in vitro*, as observed in this study and in other previous studies (20, 17, 21, 22), the production of trichothecenes limits their use as biological control agent due to their risk to human and animal health (11, 33) and because trichothecenes such as trichodermin is phytotoxic (15, 17). However, in *in vivo* experiments carried out with *T. arundinaceum* IBT40837, HA producer, the phytotoxic effect was not observed, which has enabled the study of the trichothecene-plant interaction with this isolate (12). In addition, trichodermin has been studied for use in the design of new bioactive molecules (24) and, together with HA, for the treatment of tumor cells (23, 34). However, species of the Brevicompactum clade are producers of other bioactive metabolites such as peptabiotics and potential producers of new compounds (11, 35). Exploring new representatives of the Brevicompactum clade from megadiverse environments such as the Amazon rainforest opens up a world of possibilities, in addition to its

use in biological control, in the area of biotechnology and in understanding the effect of trichothecenes on the interaction of fungi with plants.

In comparison with other clades of the genus, in the Brevicompactum clade few representatives have been studied. *Trichoderma* sp. VIC44363 T17F is an isolate close to the species *T. brevicompactum* which was not toxic when applied to germinated seeds and leaves of common bean and which was also beneficial to the plant by decreasing the severity of anthracnose and promote its growth (chapter 1), contrary to what was previously reported with the *T. brevicompactum* isolate IBT40841 which was toxic to tomato and aggravated the lesions caused by *Botrytis cinerea* (17). Therefore, the T17F–plant–*C. lindemuthianum* interaction was studied, with transformants that expressed the red fluorescent protein, and in leaf discs, to establish the possible mechanism that T17F isolate used to antagonize *C. lindemuthianum* in bean plants and its spectrum of action with other phytopathogenic fungi was verified *in vitro*. Additionally, the genome was sequenced and compared with the genome of *T. brevicompactum* IBT40841 for SM clusters to generate hypotheses of the possible causes of the difference in the effect of the two species/isolates on the interaction with plants.

The T17F isolate was transformed with the red fluorescent protein without affecting the phenotypic characteristics. The transformation did not alter the radial growth, the production of compounds that inhibited the growth of *C. lindemuthianum* and the effectiveness in decreasing the symptoms of anthracnose in bean by the evaluated transformants. Transformant 17RFP-T2 was used to assess the colonization capacity of *P. vulgaris* root and leaf tissues.

The ability of T17F isolate to mycoparasitize *C. lindemuthianum* and the ability of the fluorescent transformant to colonize the root and leaf tissues of bean plants was evaluated. On the other hand, in leaf discs, the capacity of T17F isolate to produce compounds in the leaf and to diffuse compounds in the substrate that inhibited the growth of *C. lindemuthianum* was also evaluated. There was no evidence that mycoparasitism or competition are the prominent mechanisms in the control of *C. lindemuthianum*, as the T17F isolate after 7 days of incubation curled on the hypha of *C. lindemuthianum* only in some points of the slide, being observed more often a concomitant growth with hyphae of the phytopathogenic fungus, but also no colonization of plant tissues was observed in the evaluated inoculation condition. However, both the application of the suspension and the application of the fungus filtrate grown on rice substrate inhibited the growth of the phytopathogen and the appearance of lesions caused by *C.*

lindemuthianum in leaf discs. Metabolites may be the main responsible for the antimicrobial effect because the growth of *C. lindemuthianum* was inhibited by molecules less than 12 kDa transferred to the medium through the dialysis membrane by T17F isolate. The cellophane membrane is permeable to proteins larger than 90 kDa and the dialysis membrane only to molecules smaller than 12 kDa (36).

In *in vitro* experiments against other phytopathogenic fungi, a more prominent result was also observed in the antibiosis tests (membranes and antibiogram) than in the mycoparasitism test (confrontation). Contrary to what was observed with *C. lindemuthianum* and *B. cinerea*, which were completely inhibited by the metabolites diffused by T17F isolate through the dialysis membrane, *F. oxysporum* was more inhibited when, together with the metabolites, enzymes were transferred through the cellophane membrane. The results differ from those observed with the isolates *T. arundinaceum* Ta37 and *T. harzianum* T34 in the inhibition of *B. cinerea* growth because the inhibition was greater in the test with the cellophane membrane (21). On the other hand, the T17F isolate could not grow on the colony of phytopathogenic fungi in the confrontation test, contrary to what was observed with mycoparasitic fungi such as *T. harzianum* (37), *T. atroviride* T6 (38), *T. koningiopsis* (39) (chapter 1) and *T. erinaceum* Te610F (chapter 1) which in the confrontation experiment grew on the colonies of the phytopathogenic fungi they were confronted with.

The species of the Brevicompactum clade are characterized by producing several compounds with antimicrobial activity, among them, polypeptide antibiotics such as alamethacins (35, 40) and the trichothecenes tricodermin and HA (41, 11, 14). In agreement in the present study, it was observed that the production of metabolites was the main mechanism used by T17F isolate to antagonize *B. cinerea*, *C. lindemuthianum*, S. *sclerotiorum* and *F. oxysporum* isolates, as reported in previous works with other species of the Brevicompactum clade. Isolates of the species *T. brevicompactum* and *T. arundinaceum* have been reported as growth inhibitors of the phytopathogenic fungi *R. solani*, *B. cinerea* (22, 26), *Myrothecium roridum, Fusarium sporotrichioides* (42) *Cochliobolus miyabeanus*, *Fusarium oxysporum* f. sp. *cucumerium*, *Fusarium oxysporum* f. sp. *niveum* and *Phomopsis asparagi* (26) which was attributed to the production of trichodermin in *T. brevicompactum* and of HA in *T. arundinaceum*. However, against *C. lindemuthianum* Shentu et al. (22) observed that trichodermin had a slight inhibitory effect contrary to what was observed in the present work, in which the growth of *C. lindemuthianum* was completely inhibited and the least inhibited

fungus was *F. oxysporum*. Resistance to trichothecenes in *F. oxysporum* has been related to the expression of the *tri12b* gene, which is activated in the presence of trichothecenes (43). The *tri12b* gene codes for a membrane transporter protein important in the detoxification and virulence of trichothecene-producing *Fusarium* species (44).

The phylogenetic study of *Trichodema sp.* VIC44363 through the analysis of 27 housekeeping genes confirmed that *T. brevicompactum* and *T. turrialbense* are the closest species to *Trichodema* sp. VIC44363 and that the species *T. arundinaceum* and *T. protudens* constitute the sister group. Therefore, the comparison of the SM clusters with the predicted clusters in the genome of *T. brevicompactum* was made.

In the genome of *Trichodema* sp. VIC44363, the same types of SM clusters found in the genome of *T. brevicompactum* were found. The total number of clusters in *Trichodema* sp. VIC44363 was 63 and in *T. brevicompactum* 62, higher than the number found by Kubicek et al. (10) in the genomes of species of the Harzianum, Virens, Longibrachiatum and Section Trichoderma clades. In the same study, *T. harzianum* stood out for having the highest number with 59 MS cluster. However, the number of PKS, NRPS and TS clusters was close to those described in the genome of *T. harzianum*, however *T. brevicompactum* and *Trichodema* sp. VIC44363 have IS clusters absent in the genomes of the species of the clades studied by Kubicek et al. (10).

Although the same number and type of SM clusters were predicted in *T. brevicompactum* and *Trichodema* sp. VIC44363 genomes, only half of the clusters were conserved and three were exclusive to *Trichodema* sp. VIC44363. Among the unique clusters in *Trichodema* sp. VIC44363 is the IS cluster possibly involved in alkaloid metabolism. Alkaloids are considered phytoalexins with broad-spectrum antimicrobial activity (45). The other two exclusive *Trichodema* sp. VIC44363 clusters are of the NRPS and PKS type. The NRPSs produce a wide variety of compounds that can be formed by different amino acids than those that constitute proteins such as peptaibols that have antimicrobial and anticancer activity. Likewise, PKs are antibiotics or toxins with diverse structures that are important in interacting with plants (46).

It was determined that *Trichodema* sp. VIC44363 has almost all genes associated with the trichothecene production pathway. However, the gene encoding a protein homologous to TRI23 was not found. The TRI23 protein is a cytochrome monooxygenase P450 responsible

for the conversion of octatrienoate to octatrienedioate present in HA-producing species, but absent in tricodermin-producing species (13). *Trichoderma brevicompactum* also lacks the *tri23* gene and the *tri17* and *tri18* genes are in different places than those found in the genome of *T. arundinaceum,* where these genes are organized in a cluster along with *tri23* (Fig. 4). Therefore, the *tri* genes in *Trichodema* sp. VIC44363 are the same and have the same organization as in *T. brevicompactum*, but there are differences in gene size and *Trichodema* sp. VIC44363 lacks three TRI6 protein binding sites in the promoter regions of the *tri14* genes, *tri22* and *tri10* (Fig. 4). Therefore, *Trichodema* sp. VIC44363 has the potential to produce trichodermin, but presents differences in the nucleotide sequence.

Given the above, it will be important to determine with gas chromatography coupled to a mass spectrophotometer (GC-MS) the presence of trichodermin in the extract of metabolites produced by *Trichodema* sp. VIC44363 in the potato medium, which induces the production of the compound. But it would also be interesting to determine whether trichodermin is produced when rice is used as a substrate for the cultivation of the fungus and in the interaction with the plant, as the characteristic phytotoxic effect of trichodermin on leaf discs and common bean plants was not observed (chapter 1). Given the possibility that other compounds are responsible for the antagonism given the wide range of predicted and exclusive SM clusters in T17F isolate, it would also be interesting to know if they are involved in the antagonism exerted against *C. lindemuthianum* and the other studied phytopathogens.

Conclusions

The study of SM-producing fungi as diverse as fungi of the *Trichoderma* genus opens countless possibilities for uses and applications due to their biotechnological potential. Even compounds traditionally considered harmful due to their chemical nature may have prospects for use in medicine and the understanding of their ecological role can help in the development of strategies for their management. An example of this are the trichothecene-producing fungi belonging to the Brevicompactum clade that produce other compounds of interest, as they have several clusters above the average, which is even higher than that found in clade species as important in biological control as the Harzianum clade and the Virens clade.

The T17F isolate of the *Trichodema* sp. VIC44363 species, which is an endophyte fungus originating from rubber trees native to the Amazon rainforest, showed an antagonistic effect against *C. lindemuthianum* and other phytopathogenic fungi without being phytotoxic

and promoting the growth of common bean. In the present study, it was shown that this antagonism is associated with the production of SM and by genome analysis arises that this effect may be associated with the production of trichothecenes such as tricodermin, as it has all the genes responsible for the production, but not with the production of HA, as it lacks the *tri23* gene. However, other compounds may be related to the antagonistic capacity and growth promotion of common bean, as although *Trichodema* sp. VIC44363 is so closely related to *T. brevicompactum*, most clusters present gene deletions and additions, as well as clusters are found that could potentially produce other compounds different from those produced by *T. brevicompactum.*

Methods

Fungal isolates, culture media, growing conditions, and plant material

Trichoderma sp. VIC44363 COAD 2324 (=T17F) (Octávio de Almeida Drummond Collection - COAD) is the type isolate of the species proposed as new within the Brevicompactum clade by Brito (47), which was previously shown to be effective in the control of *Colletotrichum lindemuthianum* race 89 isolate A2 2-3 in common bean, without causing phytotoxicity (Chapter 1). The phytopathogenic fungi of common bean (*Phaseolus vulgaris*) were also used in the present study: *Colletotrichum lindemuthianum* race 89 isolated A2 2-3 (transformed with plasmid pPGW), *Fusarium oxysporum* f. sp. *phaseoli* UFV01 (Fop) and *Sclerotinia sclerotiorum* (Ss); and from the vine (*Vitis vinifera*) pathogen *Botrytis cinerea* (Bc). All fungi belong to the collection of microorganisms at the Laboratory of Molecular Genetics of Microorganisms at the Universidade Federal de Viçosa.

All fungi were preserved in 10% glycerol at 5 - 10 °C until they were reactivated for the experiments in Potato Dextrose Agar Difco® (PDA). To obtain *C. lindemuthianum* conidia, the YMA culture medium (0.2% yeast extract, 1.0% malt extract and 1.5% agar) was used, and for the fungi of other genera, the PDA medium. To obtain the broth that was filtered for the antibiogram test, the T17F isolate was grown in CM medium (5 g/L of malt extract, 5 g/L of yeast extract and 5 g/L of glucose) to obtain the pre-inoculum which later served to inoculate potato broth (PDB) as previously described (28). The T17F isolate was incubated at 28 °C and *C. lindemuthianum* at 22 °C, however the other fungi were incubated at 25 °C, all with 12:12 photoperiod.

Phaseolus vulgaris cultivar *Pérola* was used for the fungus-plant interaction experiments.

Genetic transformation with the red fluorescent protein gene

T17F isolate was genetically transformed with plasmid pPGW that contains the gene for resistance to hygromycin B (*hph*) and the gene that codes for the red fluorescent protein (RFP) (48) (chapter 1). The protoplast transformation protocol described by McCormick et al. (49) and modifed by Malmierca et al. (21), with some other modifications described in chapter 1. The osmotic stabilizer used to prepare the enzyme solution was KCl 0.6 M and the transformants were selected by resistance to hygromycin B (Sigma, St. Louis, MO) at the established minimum inhibitory concentration which was 100 µg/mL. The fluorescence of the transformants was confirmed on an Olympus® BX53 microscope equipped with a U-FGW filter cube (excitation filter 530/550 nm).

The transformants were purified by obtaining monosporic cultures from which the radial growth rate (RGR), mitotic stability, biological activity by the cellophane membrane test and the efficacy experiment in the control of *C. lindemuthianum* in common bean were determined in a greenhouse as described in chapter 2. The number of copies of plasmids inserted into the genome was also verified by Southern blot hybridization (50) and the total DNA was extracted according to the methodology of Specht et al. (51) modified by Cardoza et al. (28, 52) as described in chapter 1. The transformants with the lowest copy number were evaluated for their efficacy in controlling *C. lindemuthianum* in common bean under greenhouse conditions as described in chapter 1. The efficacy (E) was calculated as $\%E = [(C - T)/C] \times 100$ (C: percentage of the leaf area with necrosis in the control treatment where only the phytopathogen was applied; T: percentage of the area with necrosis in leaves treated with *Trichoderma* spp. before the application of the phytopathogen).

In the *in vitro* experiments, three replicates were performed, and in the efficacy experiment, five replicates were performed, using a completely randomized design. Normality was verified and the paired t-student test of the wild isolate and each transformant was carried out in the statistical program R (53). In all experiments the transformants were evaluated together with the wild type isolate for analysis comparison.

Antagonistic capacity of T17F isolate to *C. lindemuthianum*

Mycoparasitism

Direct confrontation experiment: The ability of T17F isolate to grow on the *C. lindemuthianum* colony was determined using the direct confrontation method described by Malmierca et al. (21) after 10 days of incubation.

Microscopic observations: *Colletotrichum lindemuthianum* and T17F isolate were inoculated on a cellophane membrane placed in 1.5% water agar. *Colletotrichum lindemuthianum* was inoculated 2 days before inoculation with T17F isolate and observations were made in the contact regions between the hyphae of the two fungi after 4 and 8 days of inoculation with T17F isolate (54) (chapter 1). Representative images were recorded with the EVOS M5000 Imaging System (Thermo Scientific, Waltham, Massachusetts, USA) in brightfield imaging mode. The experiment was carried out in duplicate with three biological replicates.

Antibiosis

Membrane growth experiment: T17F isolate was evaluated for its ability to produce antimicrobial compounds and inhibit the growth of *C. lindemuthianum* by cellophane membrane and dialysis methodology (Sigma, Darmstadt, Germany; cutoff size, 12 kDa) in PDA medium described by Malmierca et al. (21). The percentage of inhibition was calculated by the formula $\%I = [(C - T)/C] \times 100$ (C: phytopathogen colony diameter on the control plate; T: phytopathogen colony diameter exposed to T17F isolate metabolites) after the pathogen colony cover medium surface. Finally, a descriptive statistic of the variable percentage of pathogen growth inhibition was performed (Chapter 1).

Antibiogram: The antifungal activity of the filtrate of the T17F isolate was evaluated according to the methodology of the antibiogram (55). To obtain the filtrate, the potato broth in which the T17F isolate was grown was passed through a syringe with gauze and filter paper and finally filtered through a millipore membrane with a 0.45 µm pore size. 50 µL aliquots of the cell-free filtrate were transferred to the wells of the plates with conidia of the pathogens, incubated for 5 hours at 4 °C and transferred to incubators with the growth conditions described for each phytopathogenic fungus for 48 hours. After this period, the photographic record was made, and the diameter of the inhibition halo was measured.

Growth inhibition of *C. lindemuthianum* on *P. vulgaris* leaf discs

The co-inoculation methodology on leaf discs by You et al. (39) with the modifications described in chapter 1 was used. The number of discs with growth of the pathogen (NDPG) and the lesion diameter (LD) in each of the 10 leaf discs were calculated and the average of the 10 discs in each plate was calculated after 7 days of incubation. The assumptions were confirmed and the differences between treatments established by the analysis of variance (α=0.05) and the means were compared by the Scott-Knott test (α=0.05) with the statistical program R version 4.0.5 (2021- 03-31) Copyright (C) 2021, the platform x86_64-w64-mingw32/x64 (64-bit) and the packages ExpDes.pt and Nortest.

Root and leaf colonization of *P. vulgaris*

The transformant 17RFP-T2 was used to evaluate the colonization of radicles and leaves of common bean cultivar *Pérola* as reported in chapter 1. In the radicles the evaluations were made daily for 4 days of germinated seeds kept in cups and on the fourth day of germinated seeds that were transferred to pots with substrate. The radicle evaluations consisted of making four slices less than 1 mm thick every centimeter and making observations under the fluorescence microscope. Cuts were made from the tip of the radicle (Division Zone − DZ), 1 cm (Elongation zone − EZ) and 2 cm (beginning of the Maturation zone − MZ) from the tip of the radicle. The evaluations of the leaves were made on the second and fourth days after inoculation and consisted of making longitudinal (Longitudinal Section − LS) and transversal (Cross Section − CS) cuts of the leaf ribs to be observed under a fluorescence microscope (Chapter 1). The evaluations of the two experiments consisted of making observations of the sections and photographic recording of representative images with the EVOS M5000 Imaging System (Thermo Scientific, Waltham, Massachusetts, USA) equipped with the RFP light cube (531/593 nm) in the imaging modes fluorescent, brightfield and color brightfield, and single-color and multicolor imaging methods. The experiment was done once with three biological replicates.

Antagonistic capacity of T17F isolate against other phytopathogenic fungi in vitro

To verify the spectrum of action of the antagonistic capacity of the T17F isolate, mycoparasitism was evaluated by the direct confrontation methodology against the fungi *B. cinerea*, *F. oxysporum* f. sp. *phaseoli* and *S. sclerotiorum* in the same way as made with *C. lindemuthianum* described above. To test antibiosis, the membrane growth methodology of

Malmierca et al. (21). was used with the phytopathogens *B. cinerea*, *F. oxysporum* f. sp. *phaseoli* and *S. sclerotiorum* and antibiogram with the conidia producing phytopathogens *B. cinerea* and *F. oxysporum* f. sp. *phaseoli* as done with *C. lindemuthianum*, as conidia are needed for the development of the methodology.

Genome analysis

Genome sequencing

The genome of *T. brevicompactum* IBT 40841 and *T. arundinaceum* IBT 40837 used in the present study are available in the National Center for Biotechnology Information (NCBI) database with the accession number PXNZ00000000.1 and PXOA00000000.1, respectably. The genome of *Trichodema* sp. VIC44363 COAD 2324 was generated in the present study. For DNA extraction, the fungus was grown in PDB (Kasvi®) under agitation at 200 rpm and 28 °C for 2 days. Total DNA was extracted according to the methodology of Specht et al. (51) precipitated in 1/10 of 3M sodium acetate pH 7 and 3 volumes of ice cold ethanol and dried in a rotary evaporator. The genome was sequenced with Illumina NovaSeq, and the construction library used was the Truseq DNA Nano library.

Phylogenetic analysis

The conserved regions (exons) of 27 housekeeping genes were analyzed (Supplementary Table 2). For this, the gene sequences without introns were manually obtained with the help of the SnapGene program. Later, the aligned was made in the MEGA software with the MUSCLE program. The phylogenetic trees were built using the maximum likelihood method with 1000 bootstraps in the IQtree program from the alignments of each gene separately and the alignment concatenated with the SequenceMAtrix 1.8 program.

Secondary Metabolism Clusters

The Augustus prediction and annotation tool was used (56), Blast2GO (57) and anti-SMASH software version 5.0.0 (58), to predict and annotate clusters of genes for SM biosynthesis present in the genome of *Trichodema* sp. VIC44363 COAD 2324 and to search for trichothecene biosynthesis genes. Additionally, the NCBI Blastp tool was used.

Supplementary material

Additional file 1: Supplementary Table 1. Comparison of phenotypic characteristics of transformants and wild type T17F

Additional file 2: Supplementary Figure 1. Molecular characterization of transformants. Southern-blot analysis of T17F transformants expressed RFP. DNA was digested with *Hind*III enzyme and hybridized with the *hph* gene as a probe.

Additional file 3: Supplemental Figure 2. Colonization of common bean rootlets with the transformant 17RFP-T2, 4 days after inoculation. Observations in colored brightfield mode (**a**, **b**) and in fluorescence mode with the RFP light cube (531/593 nm) (**c**, **d**).

Additional file 4: Supplemental Figure 3. Antagonism of T17F isolate to phytopathogenic fungi. T17F isolate antagonizing to: (**a**, **b**, **c**) *B. cinerea*. (**d**, **e**, **f**) *F. oxysporum* f. sp. *phaseoli* (**g**, **h**) *S. sclerotiorum*. Experiments of: (**a**, **d**, **g**) Direct confrontation. (**b**, **e**, **h**) Cellophane membrane. (**c**,**f**) Antibiogram.

Additional file 5: Supplementary Table 2. Housekeeping genes used for phylogenetic analyses.

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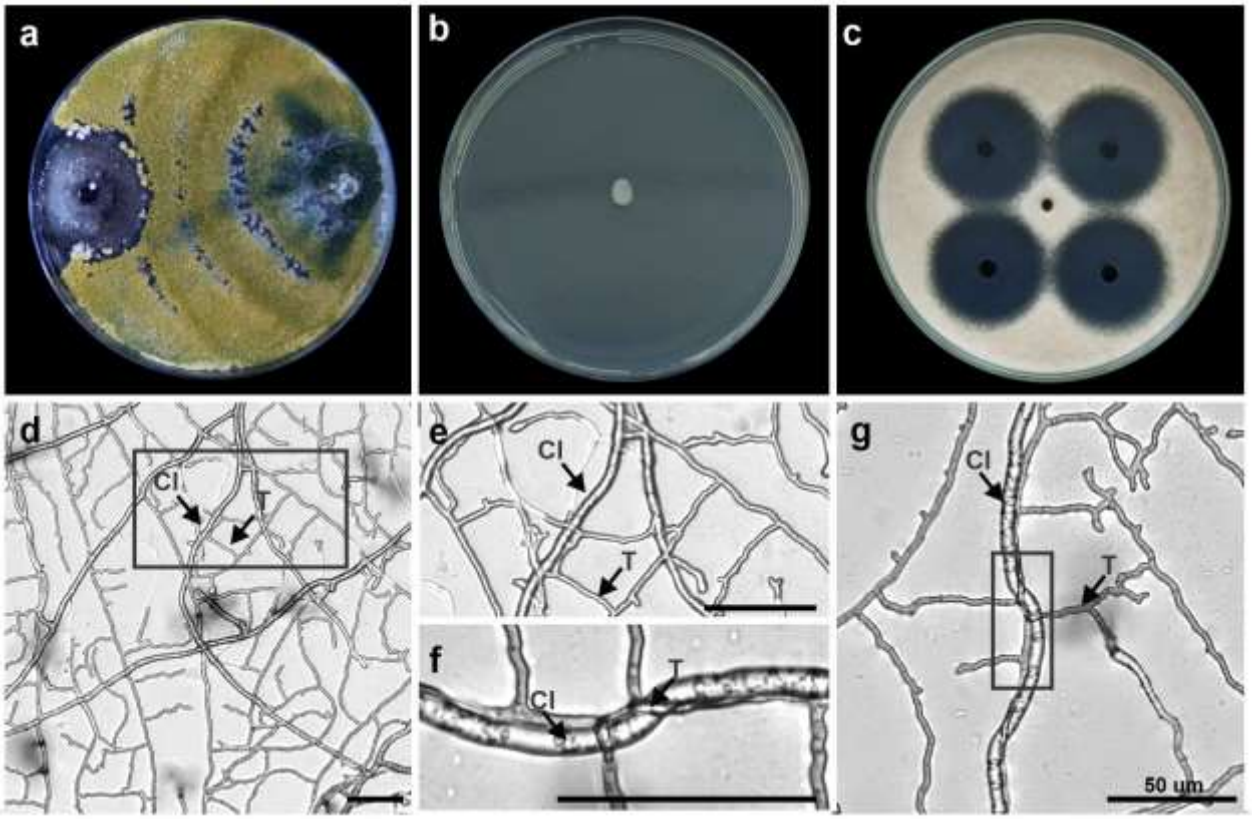


Fig. 1 Antagonistic capacity of the isolate T17F to *Colletotrichum lindemuthianum*. **a** Direct confrontation experiment between T17F and *C. lindemuthianum*. **b** Inhibition of *C. lindemuthianum* by enzymes and metabolites secreted into the culture medium by T17F, after growth on cellophane paper. **c** Inhibition of *C. lindemuthianum* by purified metabolites produced by T17F. **d-g** Mycoparasitism of T17F on *C. lindemuthianum*, 8 days after inoculation. Observations in brightfield mode. Cl: *C. lindemuthianum*. T: T17F. **d-e** Detail of the hyphae of the two fungi growing concomitantly without evidence of mycoparasitism. **f-g** Detail of structure related to mycoparasitism. Bar = 50 µm

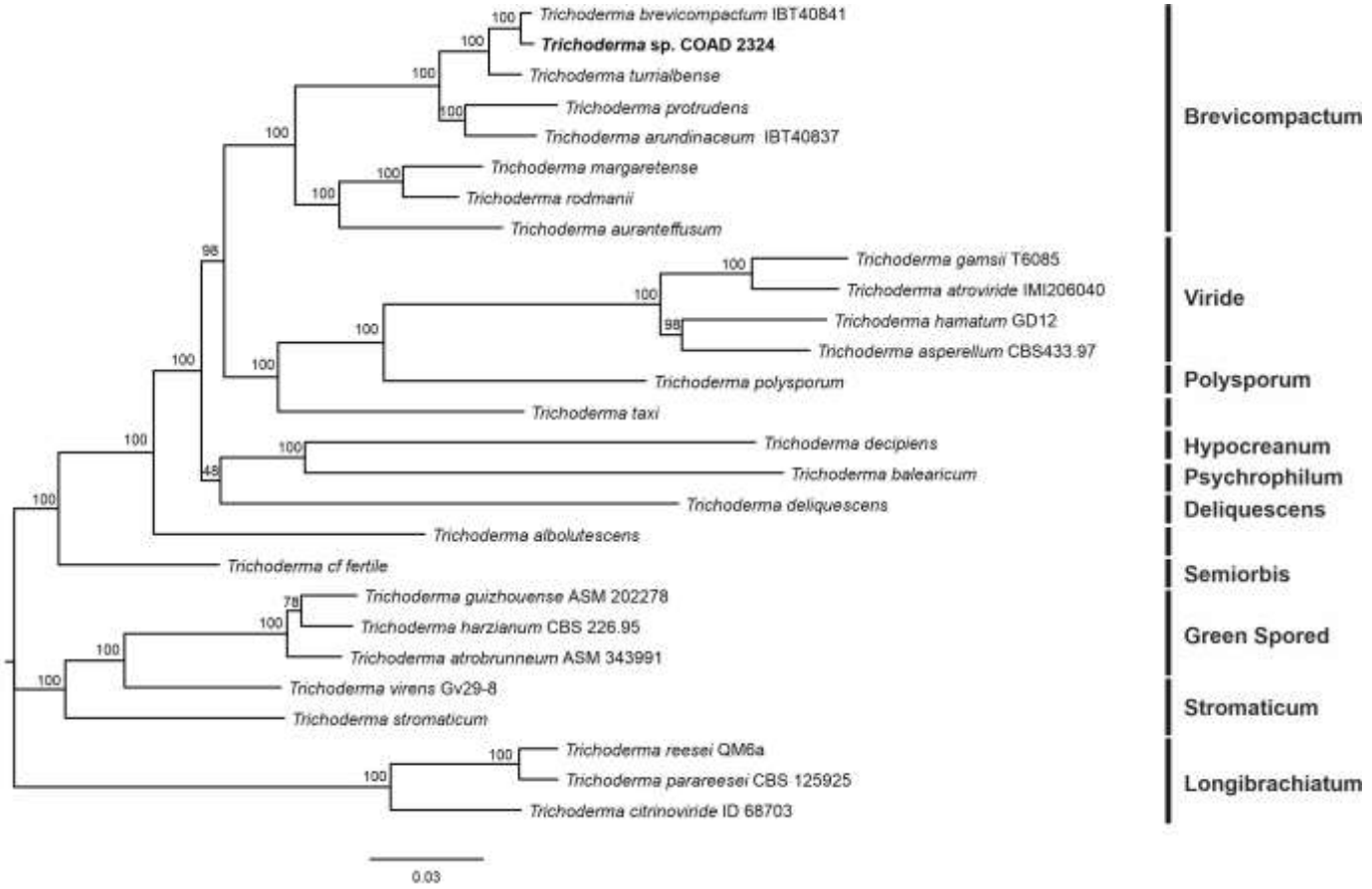


Fig. 2 Maximum likelihood phylogenetic tree for 27 housekeeping concatenated genes of *Trichoderma* species with genome sequenced and *Trichoderma* sp. COAD 2324. The numbers present in each node represent the bootstrap with 1000 pseudoreplicates. On the right side are the names of the complexes.

Fig. 3 Sequence alignment of the TRI3 protein from *Trichoderma sp.* VIC44363 (Tbras) and *Trichoderma brevicompactum* (Tbrev). Highlighted the identity regions.

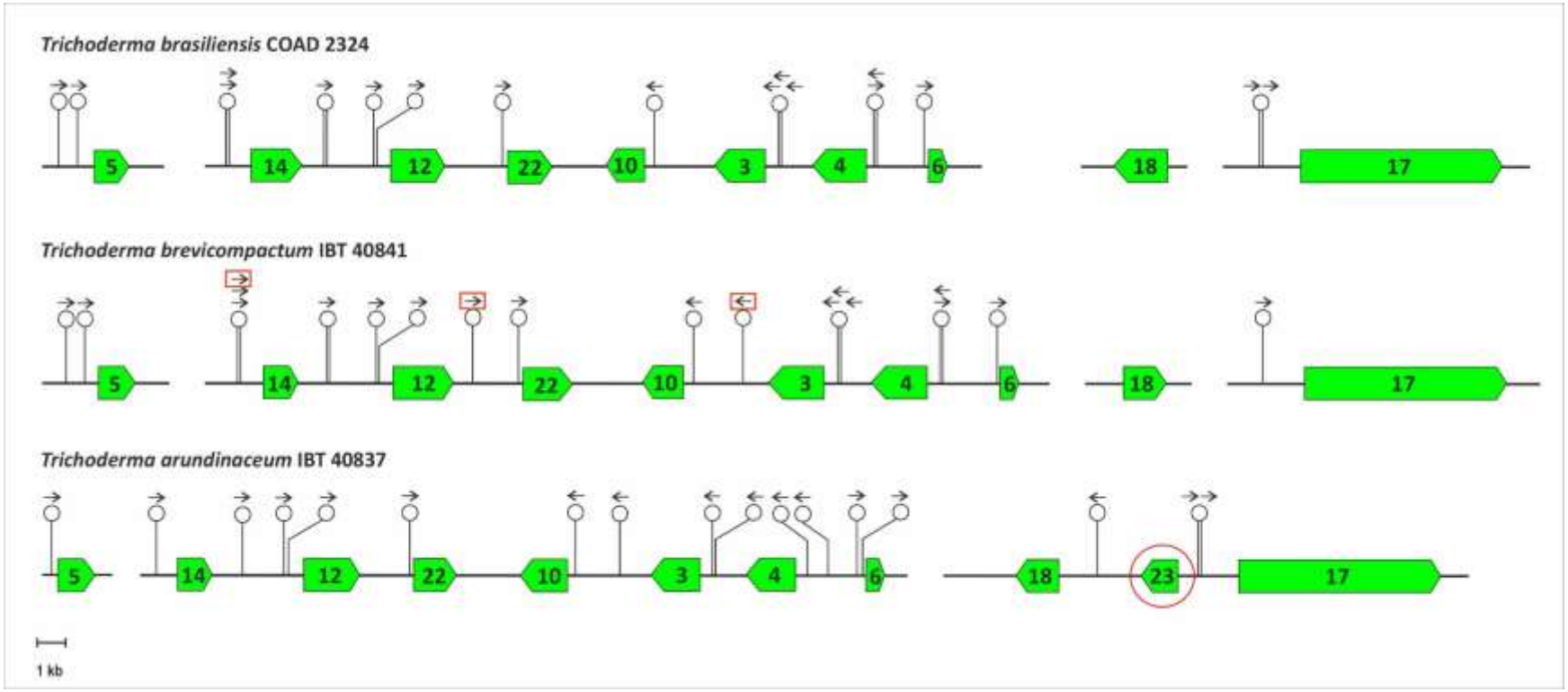
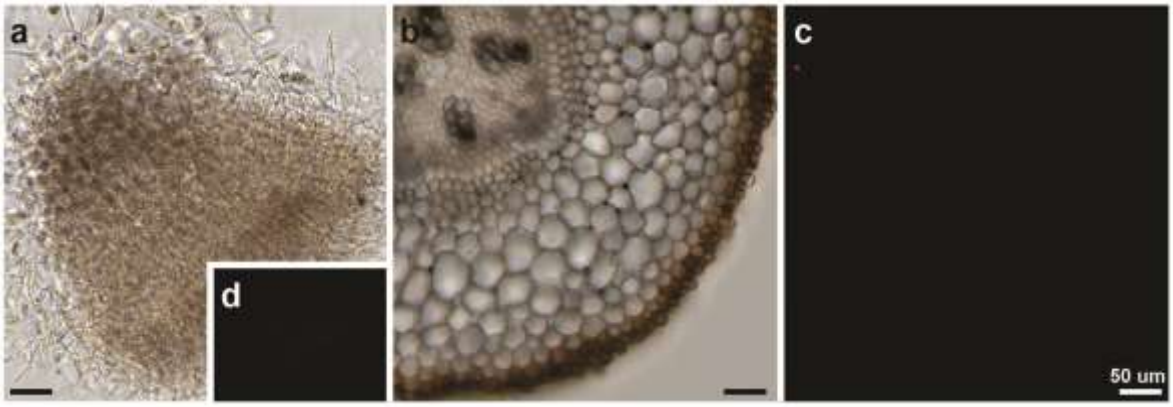


Fig. 4 Comparison of the content and cluster organization of *tri* genes in *Trichodema sp. VIC44363* with species producing trichothecenes (*T. brevicompactum* [producer of tricodermin] and *T. arundinaceum* [producer of Harzianum A]) and position of putative linkage motifs of TRI6 according to the binding sequences (TNAGGCC) described by Hohn et al. (33). represented by circles in the promoter region. Green arrows with numbers inside represent the homologous *tri* genes previously described and the number inside the arrows indicate the *tri* gene designation (e.g. 5 indicates *tri5*). Arrows above the circles indicate the tape where the motif was found. Elements absent in *Trichodema sp. VIC44363* are highlighted in red. Scale = 1kb.

Supplementary figure 1. Molecular characterization of transformants. Southern-blot analysis of T17F transformants expressed RFP. DNA was digested with *Hind*III enzyme and hybridized with the *hph* gene as a probe.

Supplementary figure 2. Colonization of common bean rootlets with the transformant 17RFP-T2, 4 days after inoculation. Observations in colored brightfield mode (**a**, **b**) and in fluorescence mode with the RFP light cube (531/593 nm) (**c**, **d**).



Supplementary figure 3. Antagonism of T17F isolate to phytopathogenic fungi. T17F isolate antagonizing to: (**a**, **b**, **c**) *B. cinerea*. (**d**, **e**, **f**) *F. oxysporum* f. sp. *phaseoli* (**g**, **h**) *S. sclerotiorum*. Experiments of: (**a**, **d**, **g**) Direct confrontation (10 days of incubation). (**b**, **e**, **h**) Cellophane membrane (3 to 8 days of incubation). (**c**,**f**) Antibiogram (3 day of incubation).

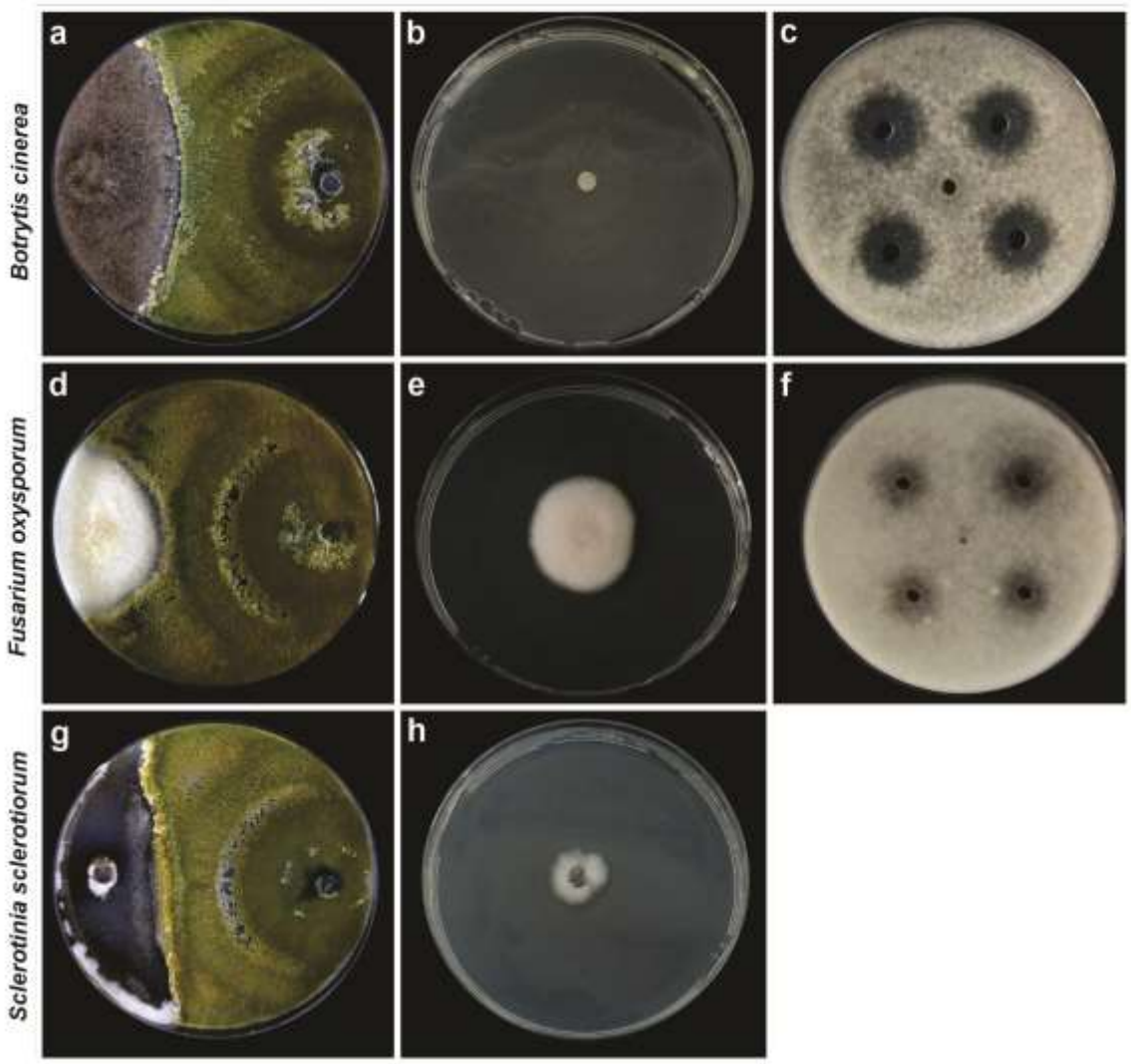


Table 1 Inhibition of *Colletotrichum lindemuthianum* growth on leaf discs that have been treated with suspensions of T17F isolate conidia or filtered from these suspensions after 7 days of incubation

		NDPG ($\bar{x}$ ±SD)		LD ($\bar{x}$ ±SD)	
Treatment	Replicates	**SS**	**F**	**SS**	**F**
T17F	**1**	5.0 ±2.0*	4.0 ±2.6*	5.5 ±4.0	4.6 ±2.1
	2	4.7 ±2.5*	6.7 ±1.1*	7.3 ±1.4	8.3 ±1.7
Control*	**1**	9.6 ±0.6		6.9 ±4.0	
	2	8.7 ±0.6		8.6 ±2.7	

NDPG Number of leaf discs with pathogen growth, *LD* Lesion diameter, *SS*: Spore's suspension, *F*: Filtrates

Asterisks indicate significant differences between control and T17F treatment determined using the Student's t-test ($P < 0.05$)

Table 2 Inhibition percentage (IM) of three phytopathogenic fungi by enzymes/metabolites produced by T17F isolate in growth tests on cellophane and dialysis membranes (12 kDa cutoff) and evaluation of the inhibition halo (mean ± standard deviation)

Pathogen	Cellophane (IM [%])		Dialysis (IM [%])		Antibiogram (Halo [mm])	
	1	2	1	2	1	2
B. cinerea	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	15,5 ± 0,1	21,9 ± 0,1
F. oxysporum	70.7 ±0.5	70.1 ±0.4	19.2 ±2.9	48.70 ±1.1	12,3 ± 0,7	11,7 ± 1,8
S. sclerotiorum	94.9 ±2.5	92.6 ±0.8	NT	NT	NT	NT

IM Percentage of Inhibition in growth on cellophane or dialysis membrane, *Halo* of inhibition in growth in milimeters, *NT* no tested in the experiment indicated

Table 3 Comparison between *T. brevicompactum* and *Trichodema* sp. VIC44363 of the secondary metabolism gene cluster, detected in the AntiSMASH analysis

	General Comparison			Specific comparison in TBRAS				
Cluster type	**TBREV***	**TBRAS**	**Conserved Clusters**	**Less conserved**	**With gene additions (A)**	**With gene deletions (D)**	**A and D**	**Exclusive**
Indole	1	1	0/1					1
NRPS	21 (-3)	18	12/18	3	1		1	1
NRPS_Indole	1	1	1/1					
NRPS_PKS	4 (+2)	6	3/6		2	1		
NRPS_PKS_Hyb	7	7	4/7	1	2			
NRPS_Terpene	1	1	0/1		1			
PKS	22 (-3)	20	8/20	3	2	5	1	1
PKS_Terpene	1	1	0/1		1			
Terpene	8	8	8/8					
Total	66 (62)	63	36/63	7	10	6	2	3

*In parentheses cluster number considered as part of another cluster as in *Trichodema* sp. VIC44363, since they were in contiguous contigs. TBREV: *T. brevicompactum,* TBRAS: *Trichodema* sp. VIC44363

Table 4 Predicted open reading sequences (ORFs) in the genomic region of the single clusters in *Trichodema* sp. VIC44363

Sequence name (Gene)	**Protein (aa)**	**Predited function Highest identity**	**Blastp score**	**sim mean Blast2Go**
TBRAS_6940	577	key lime pathogenicity	$5.6e^{-274}$	65.58
TBRAS_6941	345	DNA replication complex GINS protein PSF3	$2.3e^{-89}$	95.93
TBRAS_6942	357	Ankyrin repeat-containing domain protein	$4.3e^{-59}$	58.43
TBRAS_6943*	**369**	**dimethylallyl transferase**	**$1.6e^{-152}$**	**64.99**
TBRAS_6944	1100	hypothetical protein TRIVIDRAFT_50219	0.0	74.44
TBRAS_6945	66	predicted protein	$3.0e^{-22}$	67.95
TBRAS_6946	472	sterigmatocystin biosynthesis regulatory	$3.2e^{-219}$	84.3
TBRAS_494	338	CFEM protein	$3.4e^{-37}$	67.59
TBRAS_495	877	CFEM protein	$3.2e^{-148}$	79.28
TBRAS_496	745	hypothetical protein TRIVIDRAFT_204382	0.0	74.35
*TBRAS_497**	283	Methyltransferase type 11	$3.4e^{-107}$	81.13
*TBRAS_498**	119	Aldo/keto reductase	$7.5e^{-32}$	75.09
TBRAS_499	169	---NA---	0.0	0
*TBRAS_500**	542	nonribosomal peptide synthetase	$2.3e^{-75}$	51.94
TBRAS_501*	**1180**	**non-ribosomal peptide synthetase**	**0.0**	**95.64**
TBRAS_502	1636	NACHT and WD domain-containing protein	0.0	87.14
TBRAS_503	349	hypothetical protein TARUN_2637	$3.3e^{-197}$	89.4
*TBRAS_504**	263	aldo-keto reductase	$3.8e^{-113}$	84.71
TBRAS_505	134	---NA---	0.0	0
TBRAS_506	345	carbohydrate esterase family 16 protein	$1.3e^{-193}$	95.23
TBRAS_507	673	hypothetical protein M431DRAFT_14402	0.0	95.54
*TBRAS_508**	477	alkanesulfonate monooxygenase	$3.6e^{-279}$	97.09
TBRAS_2579	817	HET domain protein	0.0	56.74
TBRAS_2576	210	isochorismatase family domain-containing	$9.9e^{-96}$	95.85
TBRAS_2577	756	Zn2Cys6 transcriptional regulator	$1.2e^{-295}$	79.67
TBRAS_2578	567	hypothetical protein TARUN_1511	$4.2e^{-306}$	89.44
TBRAS_2580	258	metal dependent phosphohydrolase	$1.0e^{-134}$	86.76
*TBRAS_2581**	124	putative polyketide synthase	$3.2e^{-49}$	82.16

TBRAS_2582	**2229**	**putative polyketide synthase**	**0.0**	**76.94**
*TBRAS_2583**	414	p450 monooxygenase	$1.5e^{-188}$	78.24
TBRAS_2584	195	carbonic anhydrase	$7.6e^{-90}$	87.64
TBRAS_2585	302	CipA oxidoreductase	$1.0e^{-141}$	89.46
*TBRAS_2586**	270	carbon-nitrogen hydrolase	$1.5e^{-117}$	71.82
TBRAS_2587	277	monooxygenase	$9.0e^{-105}$	46.9
TBRAS_2588	284	hypothetical protein TARUN_7623	$4.0e^{-132}$	76.75
*TBRAS_2589**	495	high-affinity nicotinic acid transporter	$1.0e^{-271}$	87.3

*Core biosynthetic genes in bold, additional biosynthetic and transporter-related genes in italic.

Supplementary table 1. Comparison of phenotypic characteristics of transformants and wild type T17F

Isolate/Transformant	RGR (mm/d)	MS	IM (%)	GCN	Efficacy (%)
T17F-WT	**17.3 ±0.4**	**Yes**	**100.0 ±0.0**	**0**	**59.6 ±18.4**
17RFP-T2	**16.9 ±0.0**	**Yes**	100.0 ±0.0	**1**	**64.4 ± 13.2**
17RFP-T3	17.5 ±0.0	Yes	100.0 ±0.0	2	NT

In bold wild type isolates and transformants selected. Data are the mean ± standard deviation of three independent biological replicates. Radial Growth Rate (RGR), Mitotic Stability (MS), Percentage of Inhibition (IM), Gene Copy Number (GCN), Phytopathogen control Efficacy (Efficacy), No tested (NT)

Supplementary table 2. Housekeeping genes used for phylogenetic analyses.

Gene	No. Introns	Predicted protein product
act1	1	Actin
cpr1	2	Cytochrome P450 reductase
dpa1	4	DNA Polymerase Alpha Subunit
dpd1	2	DNA Polymerase Delta Subunit
dpe1	2	DNA Polymerase Epsilon Subunit
erg1	0	Ergosterol Monooxygenase Subunit
fas1	2	Fatty Acid Synthase Alpha Subunit
fas2	1	Fatty Acid Synthase Beta Subunit
hgr1	1	Hydroxy Methyl Glutaryl Co-A Reductase
his3	1	Imidazoleglycerol-Phosphate Dehydratase
ku70	2	ATP-dependent DNA helicase II
lae1	5	Global Regulatory Gene/Chromatin Methyl Transferase
lcb1	3	Sphinganine Palmitoyl Transferase Subunit 1
lcb2	0	Sphinganine Palmitoyl Transferase Subunit 2
mcm7	4	DNA replication Licensing Factor
pgk1	2	Phosphoglycerate Kinase
rpb1	2	RNA polymerase Largest Subunit
rpb2	2	RNA polymerase 2nd Largest Subunit
sph1	1	S-protein homologue 1
sph2	3	Sphingosine kinase
tef1	3	Translation Elongation Factor 1-alpha
top1	5	Topoisomerase
tps1	1	Trehalose Phosphate Synthase
tsr1	1	Ribosomal Biogenesis Protein
tub1	3	Tubulin Alpha Subunit
tub2	4	Tubulin Beta Subunit
ubt1	3	Ubiquitin Thiolesterase

CONCLUSÕES

Os isolados *Trichodema* sp. VIC44363 17F (T17F), *Trichodema* sp. VIC44364 22F (T22F)*, T. koningiopsis* 24F (Tk24F) e *T. erinaceum* 610F (Te610F) reduzem a severidade dos sintomas da antracnose do feijoeiro comum em casa de vegetação e promovem o crescimento da parte aérea da planta.

O fitopatógeno foliar *C. lindemuthianum*, nas condições de casa de vegetação do presente estudo, pode ser controlado mediante aplicações preventivas, isto é, aplicações às sementes germinadas, raízes e folhas de feijoeiro.

Os isolados T17F e T22F só diminuem a severidade da antracnose do feijoeiro quando aplicados as sementes germinadas, as raízes e as folhas.

Os isolados Tk24F e Te610F controlam *C. lindemuthianum* quando aplicados unicamente na parte aérea do feijoeiro possivelmente porque o antagonizam pelos mecanismos de micoparasitismo, antibiose e competição

O isolado T22F possui a capacidade de micoparasitar e inibir o crescimento de *C. lindemuthianum,* porém de forma menos eficiente do que os outros isolados selecionados.

Os isolados T22F, Tk24F e Te610F também têm o potencial de micoparasitar e inibir o crescimento *in vitro* de outros fungos fitopatogênicos do feijoeiro comum como *F. oxysporum* f. sp. *phaseoli* e *S. sclerotiourum,* mostrando seu amplo espectro de ação.

A inibição da infeção pode ser o mecanismo predominante do isolado Te610F para antagonizar a *C. lindemuthianum.*

Antibiose é o mecanismo utilizado pelo isolado T17F para antagonizar a *C. lindemuthianum* pela difusão de compostos ao meio, que também inibiram o crescimento de *Fusarium oxysporum* f. sp. *phaseoli* e *S. sclerotiourum*.

O isolado T17F tem o potencial de produzir a micotoxina tricodermina, aparentemente não produz Harzianum A, mas também tem o potencial de produzir novos compostos, pois possui cluster diferentes aos de *T. brevicompactum.*