UNIVERSIDADE FEDERAL DE VIÇOSA

Evaluation of antagonistic fungi of *Hemileia vastatrix* as biocontrol agents for coffee leaf rust and asian soybean rust (*Phakopsora pachyrhizi*)

Caio Mattos Pereira
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2025

CAIO MATTOS PEREIRA

Evaluation of antagonistic fungi of *Hemileia vastatrix* as biocontrol agents for coffee leaf rust and asian soybean rust (*Phakopsora pachyrhizi*)

Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Robert Weingart Barreto

VIÇOSA - MINAS GERAIS
2025

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
P436e
2025

Pereira, Caio Mattos, 1994-
Evaluation of antagonistic fungi of *Hemileia vastatrix* as biocontrol agents for coffee leaf rust and asian soybean rust (*Phakopsora pachyrhizi*) / Caio Mattos Pereira. – Viçosa, MG, 2025.
1 tese eletrônica (164 f.): il. (algumas color.).

Texto em inglês.
Orientador: Robert Weingart Barreto.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Fitopatologia, 2025.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2025.611
Modo de acesso: World Wide Web.

1. Café - Doenças e pragas - Controle biológico. 2. *Hemileia vastatrix* - Controle biológico. 3. Ferrugem asiática - Controle biológico. I. Barreto, Robert Weingart, 1955-. II. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-Graduação em Fitopatologia. III. Título.

CDD 22. ed. 633.73996

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

CAIO MATTOS PEREIRA

Evaluation of antagonistic fungi of *Hemileia vastatrix* as biocontrol agents for coffee leaf rust and asian soybean rust (*Phakopsora pachyrhizi*)

Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: July 30, 2025.

Assent:

Caio Mattos Pereira
Author

Robert Weingart Barreto
Adviser

ACKNOWLEDGMENTS

First and foremost, I thank God, because without Him, nothing would be possible.

To my parents, Amilton and Lusmar, whom I strive to emulate every day, for their unwavering love, advice, and unconditional support.

To my grandfather, Levindo, for his affection and example of strength.

To Ana Carolina de Almeida, for all her love, understanding, companionship, help, and patience. She made the journey and its challenges lighter.

To my advisor, Prof. Robert Weingart Barreto, for welcoming me into his lab as an undergraduate student, and for his guidance, teachings, trust, advice, and continued encouragement ever since.

To Drs. Adans A. Colmán, Maria del Carmen H. Rodríguez, Sara S. Sarmiento, and Miraine K. Ndacnou, for their early work with the antagonistic fungi assessed in this thesis.

To the interns who were directly involved in the biocontrol experiments: Eduardo A. Furtado, Emanuelle B. Rozaes, Sara R. Foletto, and Sofia S. S. Silva. Your help was essential to achieving these results.

To the current and former members of the Laboratório de Micologia/ Clínica de Doenças de Plantas, for their friendship, contributions, knowledge exchange, advice, and companionship over the years. A special thanks to Mr. Adão Célio da Silva and Marcela I. da Silva for their invaluable help on many occasions.

To the institution, the Universidade Federal de Viçosa, for its excellence in education and the opportunities provided.

To the professors of the Departamento de Fitopatologia, for the opportunities, conversations, and knowledge shared during my doctoral training.

To the staff of the Departamento de Fitopatologia, for their attention, availability, and services provided. In particular, to Mr. Mário L. Ferreira, for all his assistance with the experiments conducted at the UEPE Fitopatologia – Viveiro de Café.

This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).

To everyone who supported me and contributed in any way to help me reach this point and complete another chapter of my formation, thank you.

ABSTRACT

PEREIRA, Caio Mattos, D.Sc., Universidade Federal de Viçosa, July, 2025. **Evaluation of antagonistic fungi of *Hemileia vastatrix* as biocontrol agents for coffee leaf rust and asian soybean rust (*Phakopsora pachyrhizi*)**. Adviser: Robert Weingart Barreto.

Coffee leaf rust (CLR, *Hemileia vastatrix*) is the most important disease affecting coffee cultivation and threatens the livelihood of approximately 100 million people worldwide. Recommended and formerly effective measures for disease management have shown limitations. Therefore, innovative and sustainable strategies for CLR management are urgently needed. Since 2015, a search for fungal antagonists of *H. vastatrix* has been carried out in Africa (Cameroon, Ethiopia) and South America (Brazil and Paraguay), and some isolates belonging to *Aspergillus*, *Calonectria*, *Clonostachys*, *Cordyceps*, *Fusarium*, and *Trichoderma* stood out by reducing disease severity in *in vitro* assays and in greenhouse trials. The objective of this thesis was to assess these most promising isolates under field-related conditions for CLR control and to investigate whether their antagonistic activity could also extend to other agronomically important rusts. Experiments included: (I) trials in potted coffee plants maintained outdoors under shade and inoculated with *H. vastatrix*; (II) trials in experimental coffee fields; (III) *in vitro* assays testing the isolates against other rust pathogens; and (IV) trials with soybean plants grown in pots and inoculated with *Phakopsora pachyrhizi*. Coffee plants grown in pots and treated with fungal antagonists exhibited reductions in the area under the disease progress curve for incidence ($AUDPC_{INC}$) – up to 47% – and severity ($AUDPC_{SEV}$) – up to 80%. In field experiments, a reduction of up to 43% in $AUDPC_{SEV}$ was observed. In *in vitro* assays, urediniospores of *Austropuccinia psidii*, *Puccinia recondita*, *P. pachyrhizi*, and *Uromyces appendiculatus* had significant reductions in germination rates, with inhibition reaching up to 51%. On soybean plants, fungal antagonists reduced Asian soybean rust $AUDPC_{SEV}$ by up to 52%. These results indicate that *Clonostachys* spp., *Cordyceps cateniannulata*, and *Fusarium coffeibaccae* hold potential for CLR management under field conditions and may also be effective against other diseases caused by Pucciniales, such as Asian soybean rust. These findings reinforce the potential of fungal antagonists as promising tools for the sustainable management of plant diseases.

Keywords: field experiment; severity; endophytes; mycoparasites; Pucciniales

RESUMO

PEREIRA, Caio Mattos, D.Sc., Universidade Federal de Viçosa, julho de 2025. **Avaliação de fungos antagonistas de *Hemileia vastatrix* como agentes para o biocontrole da ferrugem do cafeeiro e ferrugem asiática da soja (*Phakopsora pachyrhizi*)**. Orientador: Robert Weingart Barreto.

A ferrugem do cafeeiro (FC, *Hemileia vastatrix*) é a doença mais importante da cafeicultura e ameaça o sustento de cerca de 100 milhões de pessoas em todo o mundo. As medidas recomendadas, e até então eficazes, para o manejo da doença têm apresentado limitações. Assim, formas inovadoras e sustentáveis de manejo da FC tornam-se necessárias. Desde 2015, uma busca por fungos antagonistas de *H. vastatrix* foi conduzida na África (Camarões, Etiópia) e na América do Sul (Brasil e Paraguai), e isolados de *Aspergillus*, *Calonectria*, *Clonostachys*, *Cordyceps*, *Fusarium* e *Trichoderma* se destacaram por reduzir a severidade da doença em testes *in vitro* e em casa de vegetação. O objetivo desta tese foi avaliar esses isolados mais promissores em condições de campo para o controle da FC e investigar se sua atividade antagônica também poderia se estender a outras ferrugens de importância agronômica. Os experimentos incluíram: (I) testes com plantas de café mantidas em vasos ao ar livre sob sombra e inoculadas com *H. vastatrix*; (II) testes em cafezais experimentais; (III) testes *in vitro* testando os isolados contra outros agentes causais de ferrugens; e (IV) testes com plantas de soja e inoculadas com *Phakopsora pachyrhizi*. Plantas de café cultivadas em vasos e tratadas com os antagonistas fúngicos exibiram reduções na área abaixo da curva de progresso da doença para incidência ($AACPD_{INC}$) – até 47% – e severidade ($AACPD_{SEV}$) – até 80%. Em experimentos de campo, redução de até 43% na $AACPD_{SEV}$ foi observada. Em ensaios *in vitro*, urediniósporos de *Austropuccinia psidii*, *Puccinia recondita*, *P. pachyrhizi* e *Uromyces appendiculatus* tiveram reduções significativas nas taxas de germinação, com inibição atingindo até 51%. Em plantas de soja, os antagonistas fúngicos reduziram a $AACPD_{SEV}$ da ferrugem asiática da soja em até 52%. Os resultados indicam que *Clonostachys* spp., *Cordyceps cateniannulata* e *Fusarium coffeibaccae* possuem potencial para o manejo da FC em condições de campo e também para o manejo de outras doenças causadas por Pucciniales, como a ferrugem asiática da soja. Tais evidências reforçam o potencial de fungos antagonistas como ferramentas promissoras para o manejo sustentável de doenças de plantas.

Palavras-chave: ensaio de campo; severidade; endofíticos; micoparasitas; Pucciniales

SUMMARY

General Introduction

1. Introduction

More than 3 billion cups of coffee are consumed around the world every day, making coffee the second most popular beverage (after bottled water) and a placing it among the main agricultural commodities traded globally (GUERRA-GUIMARÃES *et al.*, 2023; MUÑOZ-PAJARES *et al.*, 2023; SILVA *et al.*, 2022). Coffee belongs to the genus *Coffea* (Rubiaceae), which includes more than 100 species. However, only two species, *Coffea arabica* and *Coffea canephora*, are economically relevant, accounting for approximately 60% and 40% of global coffee production, respectively (MUÑOZ-PAJARES *et al.*, 2023; SILVA *et al.*, 2022). Coffee supports a global industry worth US$ 200 billion annually (BERTRAND *et al.*, 2023; DAVIS *et al.*, 2020) and also provides income for more than 120 million people, mainly in developing countries (MAGHULY *et al.*, 2020; RHINEY *et al.*, 2021).

However, global coffee production is at risk due to the plant vulnerability to a variety of plant pests and diseases, exacerbated by climate change, which is projected to negatively impact many of the current coffee growing areas by reducing coffee yields and increasing coffee exposure and susceptibility to pests and diseases (KOUTOULEAS *et al.*, 2022; MAGHULY *et al.*, 2020; MAGRACH; GHAZOUL, 2015).

Diseases are considered to be the main limiting factor in coffee production, and nearly 26% of the world's annual coffee production is lost to diseases. Among the fungal diseases, coffee leaf rust (CLR – *Hemileia vastatrix*), coffee berry disease (CBD – *Colletotrichum kahawae*), and coffee wilt disease (CWD – *Fusarium xylarioides*) are of major concern (KOUTOULEAS *et al.*, 2023; MAGHULY *et al.*, 2020). Nevertheless, only CLR has escaped from its original range in Africa.

CLR has been studied by plant pathologists for more than 150 years (KOUTOULEAS *et al.*, 2024). In addition to *H. vastatrix*, *H. coffeicola* (both belonging to the family Zaghouaniaceae), is also capable of attacking cultivated *Coffea*, but is still mostly neglected by researchers and (questionably) regarded as having limited economic importance, mostly because it is still restricted to the African continent (RITSCHEL, 2005). Losses attributed to *Hemileia vastatrix* are estimated to be around US$ 1-2 billion (KAHN, 2019; MCCOOK, 2006).

The first (anecdotal) report of CLR dates back to 1861, when the disease was observed in East Africa, near Lake Victoria (GUERRA-GUIMARÃES *et al.*, 2023; TALHINHAS *et al.*,

2017). In 1869, the causal agent, *H. vastatrix* (BERKELEY; BROOME, 1869), was formally described, and the first registered epidemic of CLR broke out in Ceylon (now Sri Lanka), leading to the destruction of coffee plantations in that former British colony, with disastrous economic and social consequences (GUERRA-GUIMARÃES *et al.*, 2023; MCCOOK; VANDERMEER, 2015).

By 1950, *H. vastatrix* had spread to coffee-producing countries in Africa and Asia, and arrived in the Americas in 1970 (SILVA *et al.*, 2022). Initially, the pathogen was observed in Brazil, and from there it reached all coffee-growing regions of Latin America by the mid-1980s, (MCCOOK; VANDERMEER, 2015). Recent reports indicate the presence of the pathogen in Hawaii, USA –the last significant coffee-growing region which was free of CLR (KEITH *et al.*, 2022) – as well as in Florida, USA (URBINA; AIME, 2023). Thus, the disease is now present in all coffee-producing regions.

In the last decades, a series of CLR epidemics, known as “The Big Rust” (BAKER, 2014) occurred in Americas, negatively impacting coffee production on the continent. The rust outbreaks began in 2008 and caused significant losses in Colombia, until 2011, reducing the country's coffee production by 31% (AVELINO *et al.*, 2015). In the following years, between 2012 and 2013, the “Big Rust” event expanded to Central America and Mexico, and between 2014 and 2015, the impacts of the epidemics were also registered in Ecuador and Peru (MCCOOK; VANDERMEER, 2015). Losses varied between affected countries, but it estimated that between 2012 and 2014, coffee production in Central America was reduced by 17% (MCCOOK; VANDERMEER, 2015).

Given the heavy dependence on this agricultural cash-crop, farmers and workers involved in coffee industry in Latin American countries saw their main source of income severely hit by *H. vastatrix*. The “Big Rust” economic and social consequences were severe (GUERRA-GUIMARÃES *et al.*, 2023), and contributed to the mass caravan migrations through Mexico towards the United States (WARD *et al.*, 2017).

There are recognized limitations in the main management measures currently available for CLF. The use of resistant cultivars, considered the most effective and durable practice (TALHINHAS *et al.*, 2017), has in certain occasions failed, due to the emergence of pathogen races capable of suppressing the resistance genes commonly used (MUÑOZ-PAJARES *et al.*, 2023). Most currently resistant *C. arabica* cultivars are derived from ‘Híbrido de Timor’ (HDT) and its progeny. However, over the last years, resistance of some of these cultivars has been

supplanted by races of *H. vastatrix* in some countries (MUÑOZ-PAJARES *et al.*, 2023; SERA *et al.*, 2022; SILVA *et al.*, 2022). A recent example is the cultivar "Lempira", an HDT derivative particularly important in Honduras, whose resistance was reported to have been overcome in 2017 (MORALES; GRAJEDA, 2021; ZAMBOLIM; CAIXETA, 2019).

Coffee cultivation at high altitudes (above 1,000 m) had been considered an effective practice for escaping CLR (BELACHEW *et al.*, 2020); however, with climate change (particularly the increase in global temperatures), the disease has become more severe in highland platations (AVELINO *et al.*, 2015; GUERRA-GUIMARÃES *et al.*, 2023).

The use of fungicides is broadly adopted for managing CLR in more industrialized farms. However, this measure faces increasing limitations and objections due to the high cost, the environmental impact, the risk of the emergence of *H. vastatrix* populations resistant to systemic fungicides, and the increasing demand for coffee beans free of pesticide residues (LE *et al.*, 2023; MUÑOZ-PAJARES *et al.*, 2023).

The humanitarian problem posed by the "Big Rust" and the difficulty of managing the disease properly are promoting the search and implementation of more sustainable crop protection strategies. These include advanced approaches, such as the use of RNA interference (RNAi) using spray-induced gene silencing (SIGS) and nanoparticles (SALAZAR-NAVARRO *et al.*, 2024), and more traditional, but poorly investigated methods of management of CLR, particularly the biological control (KOUTOULEAS *et al.*, 2023).

The search for biological control agents has revealed promising strains of some bacterial genera, such as *Achromobacter, Bacillus*, *Brevibacillus*, *Luteibacter, Pseudomonas*, *Paenibacillus,* and *Rhodococcus* (CRUZ *et al.*, 2024; MAFFIA *et al.*, 2009; OGATA-GUTIÉRREZ *et al.*, 2024; SILVA *et al.*, 2012). In connection, there are now commercial products, in Brazil, registered for CLR management. Examples are: a *B. velezensis*-based product (Bio-Imune®, Vittia) and a product based on the combination of *B. pumilus*, *B. subtilis* and *B. velezensis* (Bombardeiro®, Biotrop). Serenade® (Bayer), also based on *B. velezensis,* is registered in Mexico for CLR control.

In contrast, there are few studies evaluating the potential of fungi for the management of CLR. Thus, much remains to be explored, including the possibility of performing the classical biological control (EILENBERG *et al.*, 2001; SCOTT, 1995) with antagonists of *H. vastatrix* obtained from the native regions of *H. vastratix* in Africa. Also, little has been

investigated on the diversity and biocontrol potential of fungal antagonists of *H. vastatrix* present in the Americas which might be used for mycofungicide development.

Prior to the publications of our research group, *H. vastatrix* was already known to be a host of different groups of fungicolous fungi, some of which were recognised as mycoparasites. According to (MAFFIA *et al.*, 2009), *Akanthomyces lecanii* (syn. *Lecanicillium lecanii*) would be the fungal species most commonly associated with the pathogen's pustule. However, this was elucidated by COLMÁN (2018), as several other genera also fit into the white forming-colony fungi complex on CLR pustules, including *Acremonium*, *Ijuhya*, *Lecanicillium*, *Ovicillium*, *Pleurodesmospora*, *Sarocladium,* and *Simplicillium*, among others.

Few of the earlier studies have attempted to precisely identify the mycoparasites of *H. vastatrix*. The pioneering example was that of Carrión and Rico-Gray's publication (2002). A survey of fungi growing on uredia of *H. vastatrix* in Mexico, yielded specimens of five fungal species. These were identified as *Acremonium byssoides*, *Calcarisporium arbuscula*, *C. ovalisporum*, *Sporothrix guttuliformis*, and *Fusarium pallidoroseum*. Nevertheless, no molecular data was included in this paper and these identifications, based on morphology alone, are now regarded as dubious.

More recently, renewed interest in potential fungal antagonists of *H. vastatrix* resulted in novel surveys and publications. In samples collected from Mexico and Puerto Rico, 15 putative mycoparasitic fungi were identified on leaf discs containing CLR lesions. Based on single-molecule DNA sequencing, fungi belonging to the family Cordycipitaceae (*Lecanicillium* sp. and *Simplicillium* spp.) and the order Tremellales (*Bullera* sp. and *Fellomyces* spp.) were the most frequently detected (JAMES *et al.*, 2016). Until now, based on internal transcribed spacer region (ITS) sequences, *Akanthomyces*, *Bulleribasidium*, *Ceramothyrium*, *Cladosporium*, *Didymella*, *Fusarium*, *Hannaella*, *Meira*, *Neoceratosperma*, *Trichothecium* have been identified in association with CLR lesions in Ecuador (CRUZ *et al.*, 2024). In Hawaii (USA), *Akanthomyces*, *Cladosporium*, *Clonostachys, Fusarium*, and *Simplicillium* have also been reported (LUIZ *et al.*, 2024).

In 2015, with funding from World Coffee Research (WCR), a project was initiated to investigate fungal antagonists of *H. vastatrix*. More than 1,500 fungal strains were obtained from collections in South America (Brazil and Paraguay) and in Africa (Cameroon and Ethiopia). These strains were arbitrarily divided into two categories according to the way they

were obtained: mycoparasitic strains (found growing on CLR pustules) or endophytic strains (isolated from leaves, berries or stems of coffee).

A selected group of isolates obtained during the surveys in Africa and South America as potential biocontrol agents for CLR had their identity clarified and biocontrol potential preliminarily evaluated, namely: *Calonectria hemileiae* (CROUS *et al.*, 2018; SALCEDO-SARMIENTO *et al.*, 2021), a cladosporioid fungus *Digitopodium tectonae* (COLMÁN *et al.*, 2021), sixteen species of *Trichoderma* (RODRÍGUEZ *et al.*, 2021), *Cryptococcus depauperatus* (GUTERRES *et al.*, 2021), *Clonostachys farinosa*, *C. rhizophaga* and *C. rosea* (KAPEUA-NDACNOU *et al.*, 2023a), *Aspergillus flavus* (KAPEUA-NDACNOU *et al.*, 2023b), *Cordyceps catennianullata* (PEREIRA *et al.*, 2024a), and twelve species of *Cladosporium* (PEREIRA *et al.*, 2024b). In addition, other groups of fungi – *Alternaria* spp. (SALCEDO-SARMIENTO, 2018), *Lecanicillium*-like spp. (COLMÁN, 2018), and *Fusarium* spp. (NÓBREGA, 2021), were studied in theses and dissertation, and results have been submitted or are being prepared for publication.

During the development of these studies, results from preliminary screenings and tests, involving the evaluation of inhibition of *H. vastatrix* urediniospores germination on microscope slides or the protection of coffee plants against CLR under greenhouse conditions, have pushed the screening towards a selection of what has been treated as “elite isolates”. This group of ten isolates is still regarded as too large to be included in the next stage of evaluation. An important step needed to refine and reduce the list of potential candidates and selecting the most effective isolates for the biocontrol of *H. vastatrix* is that of field trials. Therefore, the general objectives of this thesis are:

1) To evaluate selected fungal isolates in experiments under field conditions, involving potted coffee plants maintained under shade;
2) To evaluate the most promising isolates in two experimental coffee fields.

One complementary hypothesis, stemming from the studies of fungal antagonists of CLR, which was also contemplated in this thesis, was that fungal antagonists of *H. vastatrix* might have a non-specific effect on rust fungi in general, and might be of use for controlling other agriculturally important Pucciniales. Two additional objectives were then added:

3) To verify whether fungal antagonists of CLR can inhibit the germination of other Pucciniales *in vitro*;

4) To preliminarily assess the biocontrol potential of selected isolates for reducing soybean rust on potted soybean plants in the field.

2. References

AVELINO, J.; CRISTANCHO, M.; GEORGIOU, S.; IMBACH, P.; AGUILAR, L.; BORNEMANN, G.; LÄDERACH, P.; ANZUETO, F.; HRUSKA, A. J.; MORALES, C. The coffee rust crises in Colombia and Central America (2008–2013): impacts, plausible causes and proposed solutions. **Food Security**, [*s. l.*], v. 7, n. 2, p. 303–321, 2015.

BAKER, P. The 'Big Rust': an update on the coffee leaf rust situation. **Coffee Cocoa Int**, [*s. l.*], v. 40, n. 6, p. 37–39, 2014.

BELACHEW, K.; SENBETA, G. A.; GAREDEW, W.; BARRETO, R. W.; DEL PONTE, E. M. Altitude is the main driver of coffee leaf rust epidemics: a large-scale survey in Ethiopia. **Tropical Plant Pathology**, [*s. l.*], v. 45, n. 5, p. 511–521, 2020.

BERKELEY, M. J.; BROOME, C. E. *Hemileia vastatrix*. **Gard. Chron**, [*s. l.*], v. 6, p. 1157, 1869.

BERTRAND, B.; DAVIS, A. P.; MARAVAL, I.; FORESTIER, N.; MIEULET, D. Potential beverage quality of three wild coffee species (*Coffea brevipes*, *C. congensis* and *C. stenophylla*) and consideration of their agronomic use. **Journal of the Science of Food and Agriculture**, [*s. l.*], v. 103, n. 7, p. 3602–3612, 2023.

CARRIÓN, G.; RICO-GRAY, V. Mycoparasites on the coffee rust in Mexico. **Fungal Diversity**, [*s. l.*], v. 11, p. 49–60, 2002.

COLMÁN, A. A. **Diversidade oculta de micoparasitas formadores de colônias brancas sobre *Hemileia vastatrix* (ferrugem do cafeeiro)**. 2018. Tese - Universidade Federal de Viçosa, Viçosa 2018.

COLMÁN, A. A.; EVANS, H. C.; SALCEDO-SARMIENTO, S. S.; BRAUN, U.; BELACHEW-BEKELE, K.; BARRETO, R. W. A fungus-eat-fungus world: *Digitopodium*, with particular reference to mycoparasites of the coffee leaf rust, *Hemileia vastatrix*. **IMA Fungus**, [*s. l.*], v. 12, n. 1, p. 1, 2021. https://doi.org/10.1186/s43008-020-00052-w.

CROUS, P. W.; LUANGSA-ARD, J. J.; WINGFIELD, M. J.; CARNEGIE, A. J.; HERNÁNDEZ-RESTREPO, M.; LOMBARD, L.; ROUX, J.; BARRETO, R. W.; BASEIA, I. G.; CANO-LIRA, J. F. Fungal Planet description sheets: 785–867. **Persoonia-Molecular Phylogeny and Evolution of Fungi**, [*s. l.*], v. 41, n. 1, p. 238–417, 2018.

CRUZ, D.; JARAMILLO-RIOFRÍO, A.; HERRERA, P.; AGUINSACA, R.; CHAMBA, M. Fungal Diversity Detected by ITS-5.8S from *Coffea arabica* Leaves Infected by Rust (*Hemileia vastatrix*) in Southern Ecuador. **Diversity**, [*s. l.*], v. 16, n. 10, p. 633, 2024.

DAVIS, A. P.; GARGIULO, R.; FAY, M. F.; SARMU, D.; HAGGAR, J. Lost and Found: *Coffea stenophylla* and *C. affinis*, the Forgotten Coffee Crop Species of West Africa. **Frontiers in Plant Science**, [*s. l.*], v. 11, 2020.

EILENBERG, J.; HAJEK, A.; LOMER, C. Suggestions for unifying the terminology in biological control. **BioControl**, [*s. l.*], v. 46, n. 4, p. 387–400, 2001.

GUERRA-GUIMARÃES, L.; DINIZ, I.; AZINHEIRA, H. G.; LOUREIRO, A.; PEREIRA, A. P.; TAVARES, S.; BATISTA, D.; VÁRZEA, V.; SILVA, M. do C. L. da. Coffee Leaf Rust Resistance: An Overview. *In*: MUTATION BREEDING IN COFFEE WITH SPECIAL REFERENCE TO LEAF RUST. Berlin, Heidelberg: Springer Berlin Heidelberg, 2023. p. 19–38.

GUTERRES, D. C.; NDACNOU, M. K.; SAAVEDRA-TOBAR, L. M.; SALCEDO-SARMIENTO, S.; COLMÁN, A. A.; EVANS, H. C.; BARRETO, R. W. *Cryptococcus*

depauperatus, a close relative of the human-pathogen *C. neoformans*, associated with coffee leaf rust (*Hemileia vastatrix*) in Cameroon. **Brazilian Journal of Microbiology**, [*s. l.*], v. 52, n. 4, p. 2205–2214, 2021. https://doi.org/10.1007/s42770-021-00592-2.

JAMES, T. Y.; MARINO, J. A.; PERFECTO, I.; VANDERMEER, J. Identification of Putative Coffee Rust Mycoparasites via Single-Molecule DNA Sequencing of Infected Pustules. **Applied and Environmental Microbiology**, [*s. l.*], v. 82, n. 2, p. 631–639, 2016.

KAHN, L. H. Quantitative framework for coffee leaf rust (*Hemileia vastatrix*), production and futures. **International Journal of Agricultural Extension**, [*s. l.*], v. 7, n. 1, p. 77–87, 2019.

KAPEUA-NDACNOU, M.; DE ABREU, L. M.; DE MACEDO, D. M.; DA NÓBREGA, T. F.; PEREIRA, C. M.; EVANS, H. C.; BARRETO, R. W. Assessing the Biocontrol Potential of *Clonostachys* Species Isolated as Endophytes from Coffea Species and as Mycoparasites of *Hemileia* Rusts of Coffee in Africa. **Journal of Fungi**, [*s. l.*], v. 9, n. 2, 2023a. https://www.mdpi.com/2309-608X/9/2/248.

KAPEUA-NDACNOU, M.; NÓBREGA, T. F.; BATISTA, L. R.; EVANS, H. C.; ABREU, L. M.; BEGOUDÉ, D. A. B.; REIS, T. A.; BARRETO, R. W. *Aspergillus flavus* from coffee in Cameroon: a non-aflatoxigenic endophytic isolate antagonistic to coffee leaf rust (*Hemileia vastatrix*). **Journal of Applied Microbiology**, [*s. l.*], v. 134, n. 5, p. lxad076, 2023b. https://doi.org/10.1093/jambio/lxad076.

KEITH, L. M.; SUGIYAMA, L. S.; BRILL, E.; ADAMS, B. L.; FUKADA, M.; HOFFMAN, K. M.; OCENAR, J.; KAWABATA, A.; KONG, A. T.; MCKEMY, J. M.; OLMEDO-VELARDE, A.; MELZER, M. J. First Report of Coffee Leaf Rust Caused by *Hemileia vastatrix* on Coffee (*Coffea arabica*) in Hawaii. **Plant Disease**, [*s. l.*], v. 106, n. 2, p. 761, 2022.

KOUTOULEAS, A.; COLLINGE, D. B.; BOA, E. The coffee leaf rust pandemic: An ever-present danger to coffee production. **Plant Pathology**, [*s. l.*], v. 73, n. 3, p. 522–534, 2024.

KOUTOULEAS, A.; COLLINGE, D. B.; RÆBILD, A. Alternative plant protection strategies for tomorrow's coffee. **Plant Pathology**, [*s. l.*], v. 72, n. 3, p. 409–429, 2023.

KOUTOULEAS, A.; SARZYNSKI, T.; BORDEAUX, M.; BOSSELMANN, A. S.; CAMPA, C.; ETIENNE, H.; TURREIRA-GARCÍA, N.; RIGAL, C.; VAAST, P.; RAMALHO, J. C.; MARRACCINI, P.; RÆBILD, A. Shaded-Coffee: A Nature-Based Strategy for Coffee Production Under Climate Change? A Review. **Frontiers in Sustainable Food Systems**, [*s. l.*], v. 6, 2022.

LE, C. T. M.; ISHII, H.; OKANE, I.; YAMAOKA, Y. Sequence analysis of *cytochrome b* gene in Vietnamese isolates of *Hemileia vastatrix*, the causal agent of coffee leaf rust, in relation to potential QoI fungicide resistance. **Tropical Plant Pathology**, [*s. l.*], 2023.

LUIZ, B. C.; SUGIYAMA, L. S.; BRILL, E.; KEITH, L. M. Survey of potential fungal antagonists of Coffee Leaf Rust (*Hemileia vastatrix*) on Coffea arabica in Hawai'i, USA. **Brazilian Journal of Microbiology**, [*s. l.*], v. 55, n. 3, p. 2839–2844, 2024.

MAFFIA, L. A.; HADDAD, F.; MIZUBUTI, E. S. G. Controle biológico da ferrugem do cafeeiro. *In*: BETTIOL, W; MORANDI, M. A. B. (org.). **Biocontrole de doenças de plantas: uso e perspectivas**. 1. ed. Jaguariúna: Embrapa Meio Ambiente, 2009.

MAGHULY, F.; JANKOWICZ-CIESLAK, J.; BADO, S. Improving coffee species for pathogen resistance. **CABI Reviews**, [*s. l.*], 2020.

MAGRACH, A.; GHAZOUL, J. Climate and Pest-Driven Geographic Shifts in Global Coffee Production: Implications for Forest Cover, Biodiversity and Carbon Storage. **PLOS ONE**, [*s. l.*], v. 10, n. 7, p. e0133071, 2015.

MCCOOK, S. Global rust belt: *Hemileia vastatrix* and the ecological integration of world coffee production since 1850. **Journal of Global History**, [*s. l.*], v. 1, n. 2, p. 177–195, 2006.

MCCOOK, S.; VANDERMEER, J. The Big Rust and the Red Queen: Long-Term Perspectives on Coffee Rust Research. **Phytopathology**, [*s. l.*], v. 105, n. 9, p. 1164–1173, 2015.

MORALES, Y.; GRAJEDA, R. Virulence genes of new population of coffee rust (*Hemileia vastatrix*) affecting coffee variety'Lempira', in Honduras; resistant and susceptible varieties. *In*: MUTATION BREEDING, GENETIC DIVERSITY AND CROP ADAPTATION TO CLIMATE CHANGE. [*S. l.*]: CABI Wallingford UK, 2021. p. 338–343.

MUÑOZ-PAJARES, A. J.; VÁRZEA, V.; SILVA, M. do C. The story of coffee: legend and truth. **Trends in Plant Science**, [*s. l.*], v. 28, n. 5, p. 501–504, 2023.

NÓBREGA, T. F. ***Fusarium* spp. antagonists to *Hemileia vastatrix* from Brazil, Paraguay and Africa: Taxonomy and biocontrol potential**. 2021. Tese - Universidade Federal de Viçosa, Viçosa 2021.

OGATA-GUTIÉRREZ, K.; CHUMPITAZ-SEGOVIA, C.; LIRIO-PAREDES, J.; ZÚÑIGA-DÁVILA, D. Antifungal Activity of Phyllospheric Bacteria Isolated from *Coffea arabica* against *Hemileia vastatrix*. **Microorganisms**, [*s. l.*], v. 12, n. 3, p. 582, 2024.

PEREIRA, C. M.; BAUTZ, K. R.; RODRÍGUEZ, M. del C. H.; SAAVEDRA-TOBAR, L. M.; KAPEUA-NDACNOU, M.; BELACHEW-BEKELE, K.; ELLIOT, S. L.; EVANS, H. C.;

BARRETO, R. W. *Cordyceps cateniannulata*: An endophyte of coffee, a parasite of coffee leaf rust and a pathogen of coffee pests. **Fungal Biology**, [*s. l.*], v. 128, n. 5, p. 1917–1932, 2024a.

PEREIRA, C. M.; SARMIENTO, S. S.; COLMÁN, A. A.; BELACHEW-BEKELE, K.; EVANS, H. C.; BARRETO, R. W. Mycodiversity in a micro-habitat: twelve *Cladosporium* species, including four new taxa, isolated from uredinia of coffee leaf rust, *Hemileia vastatrix*. **Fungal Systematics and Evolution**, [*s. l.*], v. 14, n. 1, p. 9–33, 2024b.

RHINEY, K.; GUIDO, Z.; KNUDSON, C.; AVELINO, J.; BACON, C. M.; LECLERC, G.; AIME, M. C.; BEBBER, D. P. Epidemics and the future of coffee production. **Proceedings of the National Academy of Sciences**, [*s. l.*], v. 118, n. 27, 2021.

RITSCHEL, A. Monograph of the genus *Hemileia* (Uredinales). [*s. l.*], 2005.

RODRÍGUEZ, M. del C. H.; EVANS, H. C.; DE ABREU, L. M.; DE MACEDO, D. M.; NDACNOU, M. K.; BEKELE, K. B.; BARRETO, R. W. New species and records of *Trichoderma* isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa. **Scientific Reports**, [*s. l.*], v. 11, n. 1, p. 5671, 2021. https://doi.org/10.1038/s41598-021-84111-1.

SALAZAR-NAVARRO, A.; RUIZ-VALDIVIEZO, V.; JOYA-DÁVILA, J.; GONZALEZ-MENDOZA, D. Coffee Leaf Rust (*Hemileia vastatrix*) Disease in Coffee Plants and Perspectives by the Disease Control. **Phyton**, [*s. l.*], v. 93, n. 5, p. 923–949, 2024.

SALCEDO-SARMIENTO, S. **New mycoparasitic fungi with potential for the biocontrol of *Hemileia vastatrix* (coffee leaf rust)**. 2018. Tese - Universidade Federal de Viçosa, [*s. l.*], 2018.

SALCEDO-SARMIENTO, S.; AUCIQUE-PÉREZ, C. E.; SILVEIRA, P. R.; COLMÁN, A. A.; SILVA, A. L.; CORRÊA MANSUR, P. S.; RODRIGUES, F. Á.; EVANS, H. C.; BARRETO, R. W. Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant. **iScience**, [*s. l.*], v. 24, n. 4, p. 102352, 2021. https://www.sciencedirect.com/science/article/pii/S2589004221003205.

SCOTT, J. K. 7 Classical biological control of plant pathogens. *In*: Advances in Plant Pathology, 1995. p. 131–146.

SERA, G. H.; DE CARVALHO, C. H. S.; DE REZENDE ABRAHÃO, J. C.; POZZA, E. A.; MATIELLO, J. B.; DE ALMEIDA, S. R.; BARTELEGA, L.; DOS SANTOS BOTELHO, D. M. Coffee Leaf Rust in Brazil: Historical Events, Current Situation, and Control Measures. **Agronomy**, [*s. l.*], v. 12, n. 2, p. 496, 2022.

SILVA, M. do C.; GUERRA-GUIMARÃES, L.; DINIZ, I.; LOUREIRO, A.; AZINHEIRA, H.; PEREIRA, A. P.; TAVARES, S.; BATISTA, D.; VÁRZEA, V. An Overview of the Mechanisms Involved in Coffee-*Hemileia vastatrix* Interactions: Plant and Pathogen Perspectives. **Agronomy**, [*s. l.*], v. 12, n. 2, p. 326, 2022.

SILVA, H. S. A.; TOZZI, J. P. L.; TERRASAN, C. R. F.; BETTIOL, W. Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust. **Biological Control**, [*s. l.*], v. 63, n. 1, p. 62–67, 2012.

TALHINHAS, P.; BATISTA, D.; DINIZ, I.; VIEIRA, A.; SILVA, D. N.; LOUREIRO, A.; TAVARES, S.; PEREIRA, A. P.; AZINHEIRA, H. G.; GUERRA-GUIMARÃES, L.; VÁRZEA, V.; SILVA, M. do C. The coffee leaf rust pathogen *Hemileia vastatrix*: one and a half centuries around the tropics. **Molecular Plant Pathology**, [*s. l.*], v. 18, n. 8, p. 1039–1051, 2017.

URBINA, H.; AIME, M. C. The first USA continental record of coffee leaf rust (*Hemileia vastatrix*) on coffee (*Coffea arabica*) in southwest Florida, USA. **Plant Disease**, [*s. l.*], 2023.

WARD, R.; GONTHIER, D.; NICHOLLS, C. Ecological resilience to coffee rust: Varietal adaptations of coffee farmers in Copán, Honduras. **Agroecology and Sustainable Food Systems**, [*s. l.*], p. 1–18, 2017.

ZAMBOLIM, L.; CAIXETA, E. T. An overview of physiological specialization of coffee leaf rust: New designation of pathotypes. **International Journal of Research in Agronomy**, [*s. l.*], v. 2, n. 1, p. 28–41, 2019.

Chapter 1

Biocontrol potential of selected fungal antagonists of *Hemileia vastatrix* (coffee leaf rust): *in planta* evaluations

Prepared according to the format of the Pest Management Science

Biocontrol potential of selected fungal antagonists of *Hemileia vastatrix* (coffee leaf rust): *in planta* evaluations

Caio Mattos Pereira[1], Miraine Kapeua-Ndacnou[1], Ana Carolina de Almeida[1], Eduardo Côrtes de Araujo Furtado[1], Emanuelle Barboza Rozaes[1], Sofia Sampaio Sumam Silva[1], Harry Charles Evans[2], Robert Weingart Barreto[1]

[1]Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570–900, Brazil.

[2]CAB International, Bakeham Lane, Egham TW20 9TY, UK

*Corresponding author: rbarreto@ufv.br

Abstract

BACKGROUND: Coffee leaf rust (CLR), caused by *Hemileia vastatrix*, is the most devastating disease affecting *Coffea* spp. worldwide. Current management strategies are becoming increasingly limited due to the suppression of resistance genes, the risk of fungicide resistance in pathogen populations, and climate change. Therefore, alternative approaches, including biological control, are necessary. This study aimed to evaluate the potential of fungal antagonists for managing CLR under two distinct conditions: potted trials in shaded environments and field experiments in Brazil. Previously, out of 1,500 fungal isolates initially obtained from coffee tissues and *H. vastatrix* uredinia, ten were selected based on their efficacy in *in vitro* and greenhouse assays.

RESULTS: In potted plant assays conducted over two consecutive seasons, *Fusarium coffeibaccae* and a *Clonostachys*-based consortium significantly reduced CLR severity (by up to 79.8%) and incidence (by up to 47.4%) compared to the untreated control. Most fungal antagonists outperformed a commercial *Bacillus velezensis*-based biofungicide. Endophytic colonization was confirmed for most antagonists, particularly *Trichoderma harzianum* and *Aspergillus flavus*. In field trials, *Clonostachys rosea*, *F. coffeibaccae*, and *T. virens* achieved the greatest reductions in CLR severity (up to 39.1%), although all were less effective than copper fungicide, which reduced severity by 73.4%.

CONCLUSION: Several fungal isolates demonstrated promising biocontrol potential against CLR in both potted plant assays and field conditions. Although none matched the efficacy of copper fungicide in field trials, some isolates provided disease suppression comparable to that of a commercial biofungicide, justifying further development and formulation studies. These findings may contribute to the development of future sustainable CLR management strategies under diverse environmental conditions.

Keywords: *Coffea arabica*; *Aspergillus*; *Calonectria*; *Clonostachys*; *Cordyceps*; *Fusarium*; *Trichoderma*; disease management

1. Introduction

Coffee leaf rust (CLR), caused by *Hemileia vastatrix* (Zaghouaniaceae), is the most destructive fungal diseases, affecting coffee plantations. It may reduce coffee production by up to 50% and is a permanent threat to a multibillion-dollar global industry, as well as the source of income for more than 100 million people, mainly in developing countries.[1–4]

In recent decades, particularly between 2008 and 2014, a severe wave of CLR outbreaks occurred in Latin America, the so-called "Big Rust".[5] These outbreaks were driven primarily by socio-economic conditions, crop management decisions, and climatic factors. In some countries, they reduced coffee production by up to 70%.[6] The impacts on coffee production, harmed farms profitability in northern Latin American countries and the livelihoods and food security of smallholder coffee producers, laborers and their families.[7,8] *Hemileia vastatrix* has been described as one of the main contributing factors to the mass migration of peasants from Central America through Mexico towards the United States following the "Big Rust".[9,10]

The most broadly adopted measures for CLR management, such as the use of resistant cultivars, fungicide spraying, or the cultivation of coffee plants in pathogen-free highland areas, have faced increasing limitations due to several factors: the emergence of *H. vastatrix* races that have overcome host resistance genes; the costs associated with fungicide application, the risk of selecting resistant pathogen populations and increasing market aversion to pesticide residues in coffee; and modified climatic patterns that have influenced the occurrence of CLR even in areas that were mostly free of the disease.[4,7,11,12]

Thus, alternative, innovative, and sustainable plant protection strategies have become necessary,[13] including the use of biological control agents. Although bacteria have been more extensively studied for their potential to reduce CLR,[14–17] antagonistic fungi have received much less attention.[18] Research on fungi has mainly focused on the mycodiversity associated with the pathogen's uredinia.[19–21] One exception is that of a study conducted by Sánchez-Lucio,[22] who evaluated the potential of *Trichoderma* and *Lecanicillium* isolates to reduce CLR under field conditions.

In 2015, a search for antagonistic fungi of *H. vastatrix* was initiated, covering selected areas in Africa (Cameroon and Ethiopia) and South America (Brazil and Paraguay). After three years of surveys a large and diverse assemblage of fungal isolates (more than 1,500 fungal isolates) was obtained either from healthy tissues of *Coffea* spp. or from

hyperparasitized uredinia of *H. vastatrix*. Part of this diversity has been described in previous publications.[18,23–30]

Approximately 600 isolates have been preliminarily evaluated for their biocontrol potential against *H. vastatrix* under controlled conditions. Isolates belonging to the genera *Aspergillus*, *Calonectria*, *Clonostachys*, *Cordyceps*, *Fusarium*, and *Trichoderma* significantly reduced disease levels during *in vitro* tests and, subsequently, in greenhouse trials. [18,27–32] Based on these preliminary results, the present study aimed to further evaluate the most promising isolates under field conditions, including test on potted coffee plants maintained in shaded areas and in experimental coffee fields. This next step of screening aimed at further reducing the list of antagonist candidates for use in biological control based on their actual ability to reduce CLR incidence and severity under less controlled environmental conditions. Only fungal species that are a recognized part of Brazilian mycodiversity were included in this study.

2. Material and methods

2.1. Origin and selection of isolates

The isolates (Table 1) were obtained from collections conducted between 2015 and 2017 in Cameroon, Ethiopia, Kenya, and Brazil. These isolates were selected based on promising and consistent results from *in vitro* and in greenhouse screenings conducted prior to this study.[18,27–29,31,32] These isolates were also selected because they were obtained in Brazil or because they have already been documented as occurring in Brazil.

Briefly, these fungi were isolated following the methodology described by Rodríguez *et al*.[30] and were arbitrarily classified as endophytes when obtained from fragments of healthy leaves, stems, and fruits, which were subjected to thorough surface sterilization, or as mycoparasites when obtained from abnormal or mycoparasitized uredinia of *H. vastatrix*. The selected antagonists are kept at −85 ºC in the "Coleção Octávio Almeida Drummond" (COAD) culture collection at the Universidade Federal de Viçosa (UFV).

2.2. Mass production of antagonistic fungi and preparation of inoculum

Prior to mass production, selected fungi had to be recovered from COAD. Small samples of fragments of fungal colonies were extracted from the cryovials, transferred to the center of plates containing potato-dextrose agar (PDA) and maintained in an incubator at 25 ± 2 ºC, under a 12-hour photoperiod for seven days. The resulting colonies were checked for purity and typical characteristics of each fungal genus, then transferred to fresh PDA plates and maintained under the same conditions as described above. These plates were used as seeds for increased inoculum production.

For bulk inoculum production, baking trays (30 × 20 cm) filled with parboiled rice (for every 100 g of parboiled rice, 1 g of $CaCO_3$, and 60 mL of deionized water were added) were fit into polypropylene bags (60 × 40 cm) and autoclaved for 40 minutes at 1.0 atm and 121 °C. The wrapped trays with rice were then removed from the autoclave and left at room temperature for at least 12 hours. The rice on each of the trays was then inoculated individually, under aseptic conditions in a laminar flow cabinet. Each bag received ten mycelial plugs (1 × 1 cm) from the margin of the sporulating cultures. The inoculated material was placed in a growth room at 25 ± 2 ºC, under a 12-hour photoperiod (light provided by a fluorescent lamp, 25 W – 127 V, and a black light lamp, 28 W – 127 V, positioned 35 cm above the trays) for seven days.

The rice was stirred daily, and the material was gently pressed through the plastic bag to avoid the formation of large aggregates and to ensure homogeneous colonization. Subsequently, when the rice was fully colonized, baking trays were removed from polypropylene bags and then transferred to white paper lunch bags (7.5 kg), and maintained under the same conditions, as described above, for five days. Thereafter, the colonized rice was stored in new 60 × 40 cm polypropylene bags in a fridge at 4 ± 2 ºC until use, for up to a maximum of 30 days.

The concentration of each batch was estimated in terms of conidia per gram of colonized rice. For this purpose, 5 g of rice colonized by each CLR fungal antagonist isolate (at a moisture content of 15 ± 2%) was sampled and transferred to a plastic beaker containing 50 mL of a 0.05% (v/v) Tween 20 solution prepared with tap water. The material was ground in a rotary homogenizer (MA 102, Marconi, Piracicaba, Brazil) until the rice was visually completely crushed. The resulting suspension was filtered through a domestic sieve and then through voile fabric. Conidial concentration in the filtered suspension was estimated with a Neubauer chamber, and this value was used to calculate the number of conidia per gram of colonized rice.

Immediately before application, the inoculum preparation for experiments was performed as follows. For each antagonist isolate, the amount of colonized rice required to obtain a suspension containing 5×10^8 conidia/ mL – corresponding to an average of 1×10^8 colony-forming units per gram (CFU/g) of colonized rice – was weighed. The colonized rice was transferred to plastic beakers containing a solution of Tween 20 (0.05% v/v) in tap water, and the mixture was ground and filtered as described above. The resulting suspension was checked for purity and abundance of sporulation in a microscope. Finally, adjuvants such as moisturizing, adhesive and/or against UV-protective agents were added to fungal suspensions, namely: 1% (v/v) glycerol, 1% (v/v) sunflower oil and 2% (m/v) skim milk powder.[33–37]

2.3. Urediniospores production of *Hemileia vastatrix*

Urediniospores of the pathogen were produced according to the method described in Salcedo-Sarmiento *et al*.[18]. Briefly, the abaxial side of leaves of six-month-old healthy *Coffea arabica* (cv. “Caturra”) plants were sprayed until runoff with a urediniospores suspension of *H. vastatrix* race II (kindly provided by the Laboratório de Biotecnologia do Cafeeiro – BioCafé, UFV) supplemented with Tween 20 (0.05% v/v), and adjusted to a concentration of 5×10^5 urediniospores/ mL. Subsequently, the inoculated plants were kept in a humid chamber, in the dark, at 22 ºC for 48 hours, and then transferred to benches in a room maintained at a constant

temperature of 22 °C, under a 12-hour photoperiod. After 30 to 45 days, when sporulation was intense, urediniospore were collected from the leaf surface using gelatin capsules and transferred to 1.5 mL microtubes for immediate use. After each inoculation, urediniospores viability was assessed,[18] and germination rates of at least 70% were consistently achieved.

2.4. Selected antagonists *vs Hemileia vastatrix*: potted coffee plants

2.4.1. Design and implementation of the experiment

Coffee plants (*C. arabica* cv. "Catuaí-vermelho" – IAC 144), were grown from seed. These were sown in a sandbox at the Unidade de Ensino, Pesquisa e Extensão em Fitopatologia – UEPE Viveiro de Café – of the UFV, located in Viçosa municipality, Minas Gerais state, Brazil. After emergence, when the cotyledons had expanded, the seedlings were transferred to plastic bags (12 × 4.5 cm) and kept in the shaded nursery. When the coffee plants reached three to five pairs of fully expanded leaves, they were transplanted into 2-litre pots and transferred to a shaded area, where the experiments were conducted (20° 45′ 11″ S; 42° 52′ 16″ W). One week after transplanting, the coffee plants were fertilized with Basacote® Plus 9M 16-8-12(+2) (Compo Expert, Germany) and watered whenever necessary.

Fungal antagonists were applied as conidial suspensions adjusted to 5×10^8 conidia/ mL (corresponding to an average of 1×10^8 CFU/g of colonized rice). The suspension was sprayed onto the aerial part of the plants, covering both sides of the leaves until run-off, using a manual hand-pump sprayer adapted to a plastic bottle containing the suspension. A *Bacillus*-based treatment (Bio-Imune®; *Bacillus velezensis* BV02; 3×10^9 CFU/mL; Vittia Group, São Joaquim da Barra, SP, Brazil) was sprayed at the same intervals as for fungi and using the doses recommended by the manufacturer.

Each treatment (Table 2), except for T15 – Control, was sprayed six times at 10-day intervals. Three applications were performed prior to inoculation with *H. vastatrix*, and three were conducted after pathogen inoculation.

Hemileia vastatrix was inoculated 72 hours after the third antagonist spraying. A suspension of urediniospores (5×10^5 urediniospores/ mL) supplemented with Tween 20 (0.05% v/v) was sprayed on the abaxial surface of the leaves of all of the plants. In order to favor urediniospore germination and penetration of the pathogen, inoculation was carried out in the late afternoon and the plants were covered with black plastic bags (105 L), which were removed 48 hours after inoculation.

The experiment was conducted in a randomized block design with 15 treatments and 20 plants per treatment, divided into four blocks, and replicated three times over distinct periods: the first year (Y1 - pilot study) from November 2022 to February 2023; the second year (Y2) from November 2023 to February 2024; and the third year (Y3) from November 2024 to February 2025.

2.4.2. Assessments

2.4.3. Assessment of coffee leaf rust and plant growth parameters

Disease intensity began to be assessed 45 days after inoculation (DAI) with *H. vastatrix*. Incidence and severity were recorded four times at 15-day intervals. Incidence was assessed by calculating the percentage of leaves showing rust symptoms per plant. Disease severity was assessed as follows: each coffee plant was considered into three sections (base, middle, and apex), and a pair of fully expanded leaves was marked in each of those sections. These leaves were then assessed visually using the diagrammatic scale proposed by Figueiredo *et al.*[38]

After the last disease intensity assessment, height of each plant was measured with a tape measure, considering the length of the orthotropic (main) stem from its base to the apical bud. The aerial part (branches and leaves) of the five plants within each block were collected, pooled by organ, placed in paper bags and kept in a laboratory drying oven at 70 ºC for 72 hours, until reaching constant weight. Subsequently, the dry biomass of branches and leaves was weighed separately on an analytical scale.

2.4.4. Evaluation of endophytic colonization by fungal antagonists

In addition, selected fragments of stems and leaves were collected aiming to verify the possible endophytic colonization of fungal antagonists. An “indirect method”, as described by Kapeua-Ndacnou *et al*.,[28] with modifications, was adopted as follows. Marked leaves from each third, the youngest pair of leaves, and stem fragments were sampled from each plant to form a composite sample per tissue for each treatment. The specimens were washed in running water and dried at room temperature. Leaves were cut into discs of approximately 5 mm in diam. Stem fragments had their bark removed and were cut into small pieces of approximately 1 cm in length.

The samples were superficially disinfested under aseptic conditions, by sequential immersion in alcohol 70% (1 min), 2% sodium hypochlorite (3 min) and two successive dips in sterile water (2 min each) and then transferred to sterile filter paper for drying of excess water. In sequence, the fragments were deposited on plates containing PDA supplemented with penicillin (10 mg/ L) and Triton X-100 (15 mL/ L). Three plates were used per plant sample, with nine fragments placed on each plate. Plates were then left under the conditions as described above. After 10 days the percentage of positive emergence of the inoculated antagonist was recorded. Confirmation of fungus identity was made based on colony morphology and microscopic examination.

2.5. Selected antagonists *vs Hemileia vastatrix*: field trial

2.5.1. Experimental fields

Based on previous assays, selected antagonists were screened under field conditions. The most promising isolates were brought for further evaluation of biocontrol potential in coffee field. Two experiments were established in close but separate coffee fields, established earlier for use in CLR biocontrol experiments. These were located at the UEPE Viveiro de Café, UFV, in Viçosa, Minas Gerais, Brazil. The experimental fields (EF) had areas of approximately 0.15 ha (EF1: - 20° 44′ 39″ S; 42° 51′ 00″ W) and 0.3 ha (EF2: 20° 44′ 36″ S; 42° 50′ 54″ W), and included 4-year-old *C. arabica* cv. Catuaí-vermelho (IAC 144) plants spaced by 2.5 m between rows and 0.5 m between plants.

In each area, the experiment was arranged in a randomized block design, with 12 treatments (Table 3) and four replications (blocks). Each experimental unit contained seven plants. Blocks were separated by an untreated coffee row. Experimental units were separated by an untreated plant.[15] The EF1 area was watered with a drip irrigation system, whereas the EF2 area was rainfed. Before the experiment, no disease management practices had been applied in either area; other cultural practices were conducted as needed, following specific recommendations.[39]

2.5.2. Experimental conditions

The most promising fungi were cultivated, as described above for inoculum production and conidial suspension were prepared as previously described. Conidial suspensions were

calibrated at 5×10^7 conidia/ mL, corresponding to an average of 1×10^7 CFU/g of colonized rice. In addition to the biofungicide based on *B. velezensis*, a copper oxychloride-based fungicide (Recop®; copper oxychloride 840 g/kg; metallic copper 50%; Albaugh Agro Brasil Ltda., São Paulo, SP, Brazil) was included for comparison, following the manufacturer's recommendations.

Sprayings began in November 2024, and were carried out at 21-day intervals until April 2025. Treatments (Table 3) were applied using a 20-litre battery-powered backpack sprayer (SS-20B, Brudden, Pompeia, Brazil), thoroughly covering the entire plant, with particular attention paid to spraying the abaxial surface of the leaves in the middle third of the plants. Applications were preferably performed in the late afternoon, avoiding spraying if rainfall was forecast within four hours after the time of application. In this experiment, coffee plants were naturally infected with *H. vastatrix* by existing inoculum already present in the plots and by inoculum from coffee plantations maintained nearby.

2.5.3. Assessments

Disease assessments were performed at 30- to 35-day intervals from November 2024 to June 2025. Five central plants in each experimental unit were evaluated excluding the first and last plant in each sequence in the individual row out of the evaluation scheme. Leaves were sampled in the 3rd or 4th node (starting from the apex of branch) of four opposite plagiotropic branches located in the middle third of the coffee plants,[40,41] resulting in, at least, 40 leaves per experimental unit. The leaves were collected, temporarily placed in paper bags labelled with each experimental field, block and treatment, and brought to the laboratory for evaluation of CLR incidence and severity.

In each experimental unit, the total number of collected leaves and symptomatic leaves (*i.e.*, those with at least one sporulated pustule) were quantified, and the incidence was determined. Symptomatic leaves were then scanned (Scanjet G4050, HP, Palo Alto, USA) and the resulting images were processed with the *pliman* package (v. 2.1.0)[42] in order to estimate the severity of CLR. In each experimental unit, average severity was calculated by dividing the sum of the estimated severity values by the total number of leaves sampled within that unit.

2.6. Data analysis

All data processing, analyses, and graphical work were conducted with R version 4.3.3.[43] Graphics were obtained via the *ggplot2* package.[44]

2.6.1. Pot experiment

In order to evaluate the effect of the applied treatments, data from the second and third years were combined, and disease scores were summarized by calculating the area under the disease progress curve (AUDPC) using the "AUDPC ()" function from the *epifitter* package[45] for incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$) between 45 and 90 DAI. In addition, severity scores obtained from the last assessments (final severity) of those years were combined and used to estimate the control efficacy of each treatment.

Generalized linear mixed models (GLMMs) were fitted using the "glmmTMB ()" function from the *glmmTMB* package,[46] with a Gaussian distribution and log link function for $AUDPC_{INC}$, and a Gamma distribution with log link function for $AUDPC_{SEV}$. Treatment was included as a fixed factor, whereas year, block, plant, and leaf were treated as random factors. Model selection was based on the lowest Akaike Information Criterion (AIC) value. Residual diagnostics were performed to assess the homogeneity of variances, normality of errors and random effects, and residual uniformity with the "simulate_residuals" function (DHARMa *package*[47]) and the "check_model ()" function (*performance* package[48]). Whenever significant differences among treatments were detected, via the "Anova ()" function (*car* package[49]), marginal means were estimated with the "emmeans ()" function (*emmeans* package[50]), and post-hoc comparisons were performed using the Tukey's test ($p < 0.05$) with the "cld ()" function from the *multcomp* package.[51]

A GLMM was also fitted to final severity with Gamma distribution and log link function. Treatment included as a fixed factor, while trial, block, plant, and leaf were treated as random factors. Model selection and assumptions checking, and means comparison were performed as described above. Based on the estimated means, the percentage of control efficacy (C) was calculated by comparing each treatment with either the untreated control (T15 - Control) or the formulation-based treatment (T14), according the following formula:

$$C(\%) = 1 - \frac{\text{Treatment}}{\text{Control}} \times 100$$

The plant height and aerial dry mass were also modelled with GLMMs, considering a Gamma distribution with an identity link function for height, and a Gamma distribution with a log link function for dry mass. All subsequent data processing followed the procedures described above.

Additionally, individual analyses were conducted for each year (Supplementary Table 2-4). When necessary, values were log-transformed [log (X + 1)] to meet assumptions of residual normality and homogeneity of variance among treatments. These assumptions were statistically and visually assessed using the "check_normality ()" and "check_heteroscedasticity ()" functions (*performance* package[48]), and the "simulateResiduals ()" function from (*DHARMa* package[47]). Data were fitted to a linear model, subjected to analysis of variance (ANOVA), and treatment means were back-transformed and estimated. Whenever significant differences among treatments were detected, post-hoc comparisons were performed as described above.

2.6.2. Field experiments

Data from the two experimental fields were combined, and diseases scores were summarized as $AUDPC_{INC}$ and $AUDPC_{SEV}$ as described above. GLMMs were fitted to both response variables using a Gamma distribution with a log link function. Treatment was included as a fixed factor, whereas field and block were specified as random effects. Model selection, assumptions checking, and means comparison were performed following the procedures described above. In addition, analysis per experimental field were conducted following the same procedures described above (Supplementary Table 5).

3. Results

3.1. Selected antagonists vs *Hemileia vastatrix*: potted coffee plants

3.1.1. Disease progress

Three preventive applications of antagonists onto coffee plants, followed by inoculation with *H. vastatrix* and then by three subsequent applications of antagonists, resulted in distinct rust levels, *i.e.,* different disease progress curves for CLR incidence and severity (Fig. 1a, b). From the beginning of the assessments (45 DAI), plants treated with the fungal antagonists exhibited lower incidence and severity values compared to those treated with the *Bacillus*-based product (T13) or left untreated (control, T15). This trend persisted at subsequent evaluations at 60, 75, and 90 DAI, particularly for severity.

In the combined analysis of the two assays (Y2 and Y3), the main factor – treatment – significantly affected the variables $AUDPC_{INC}$ and $AUDPC_{SEV}$ ($p < 0.01$). The analysis of the treatment effects on $AUDPC_{INC}$ revealed significant differences among treatments (Table 4). Fungal antagonists led to reductions in $AUDPC_{INC}$ values ranging from 10.7% to 47.4% relative to control. For this variable, the treatment T10 (M442 – *F. coffeibaccae*) showed the lowest mean $AUDPC_{INC}$ (70.4) and was statistically inferior to all other treatments, forming a distinct group (group A). The treatment T11 (*Clonostachys* mix) followed, with a mean of 89.2 and was also significantly different from most of the treatments, which had intermediate $AUDPC_{INC}$ values and did not differ significantly among themselves (groups ABC to CDE). On the other hand, the *Bacillus* treatment (T13) and the untreated control (T15) had the highest $AUDPC_{INC}$ values (131.0 and 133.8, respectively) and were grouped together (group E). This indicated a lack of efficacy in CLR control for the *Bacillus*-based product under our experimental conditions.

Significant differences between treatments were also observed for $AUDPC_{SEV}$ (Table 4), which was strongly and positively correlated with $AUDPC_{INC}$ ($r = 0.82$, $p < 0.01$). Values ranged from 2.43 to 12.05, and fungal antagonists led to reductions of up to 79.8% compared to the untreated control. Similarly to results found for CLR incidence, treatment with *F. coffeibaccae* (T10 – M442) showed the lowest mean (2.43) and was statistically different from all other treatments (group A). Treatments T11 (*Clonostachys* mix) and T09 (M121 – *C. hemileiae*) also performed well (group AB), but with slightly higher means than T10 (2.87 and 2.91, respectively). Most of the fungal treatments also formed intermediate groups (ranging from ABC to BC), resulting in moderate reduction in $AUDPC_{SEV}$. The

Bacillus-based product (T13) and the untreated control (T15) had the highest $AUDPC_{SEV}$ values and formed a distinct group (group D).

Final severity (Table 5) showed a strong positive and significant correlation with $AUDPC_{INC}$ ($r = 0.79$, $p < 0.01$), and a very strong positive and significant correlation with $AUDPC_{SEV}$ ($r = 0.96$, $p < 0.01$), with a similar trend observed across variables. Final severity values ranged from 1.48% to 5.85%. Treatment T11 (*Clonostachys* mix) had the lowest final severity and was statistically different from all other treatments (group A). This treatment also resulted in a control efficacy of 73%. Most of the fungal treatments and the formulation-based treatment formed an intermediate group (groups AB to BC), with control efficacies ranging from 46.11% to 68.17% when compared with the untreated control (T15) (Table 5). Treatments more effective than the formulation showed control efficacies ranging from 2.63% to 44.36% when compared with the formulation-based treatment (T14) (Table 5). Plants treated with the *B. velezensis* commercial product (T13) showed the highest severity score of 5.85% at the last assessment and were, under the trial conditions, ineffective for controlling CLR, as it grouped with the untreated control (T15) (groups CD and D) and exhibited 5.78% more disease than the control (final severity = 5.53%), suggesting that the product, instead of controlling the disease may even worsen its impact.

3.1.2. Plant growth parameters

In contrast to disease-related parameters, higher values of plant height and aerial dry mass (Table 4) were observed in plants treated with the *B. velezensis* product (T13) (29.6 cm and 4.82 g) and in the untreated control (T15) (29.5 cm and 4.62 g). Among the fungal treatments, plants mean height ranged from 22.2 cm to 26.7 cm, whereas mean aerial dry mass varied between 2.67 g and 4.23 g. Plant height and aerial dry mass were very strongly and positively correlated ($r = 0.86$, $p < 0.01$). However, plant height showed a null to weak negative correlation with disease parameters, whereas aerial dry mass exhibited no significant correlation. Interpretation of those results is, at this stage, challenging but deserves more careful complementary investigations, particularly for practical consequences for coffee growth in the field and coffee productivity.

3.1.3. Endophytic colonization by fungal antagonists

Following the final assessment, plant samples were collected and processed to attempt reisolation of the applied fungal antagonists. The frequency of recovery varied among treatments (Table 6), but *T. harzianum* (T06 – M290) and *A. flavus* (T08 – E486) were the most frequently reisolated fungi, showing consistent rates across both experiments, particularly from leaves in the lower and middle thirds of the plant. *Clonostachys rosea*, *C. rhizophaga*, *C. cateniannulata*, *F. coffeibaccae*, *T. virens*, and *T. theobromicola* were all recovered from coffee tissues, showing the ability of establishment (even if erratic) as endophytes. Variable levels of recovery were found for those species. It depended on the plant tissue sampled as well as by differences between experimental years (i.e., Y2 and Y3). *Calonectria hemileiae* was not recovered in any attempt in either year. Among the combination treatments, the *Clonostachys*-based treatment (T11) showed variable recovery, whereas the *Trichoderma*-based treatment (T12) consistently yielded *Trichoderma* colonies in both experimental trials.

3.2. Selected antagonists *vs Hemileia vastatrix*: field trial

At the beginning of the field assessments, CLR incidence was low across all treatments (Fig. 3a). However, a few peaks in severity were observed (Fig. 3b), which may be associated with residual symptoms from CLR epidemics that occurred during the season prior to the experiment. In subsequent assessments, from December 2024 to February 2025, the incidence and severity curves followed similar trends. From March onward, the mean values of incidence and severity remained low in leaves treated with the copper fungicide (T09), whereas disease progression was observed in all other treatments.

After estimating the AUDPC for incidence (Table 7), it was observed that the fungicide-based treatment (T09) had the lowest mean $AUDPC_{INC}$, differing significantly from all other treatments (group A) and reducing the accumulated area by 66.2% as compared with the control (T12). Plots that received the *A. flavus* isolate (T06), the solution used in antagonist suspension alone (T11), and the *C. hemileiae* isolate (T07) had the highest means of $AUDPC_{INC}$ and also differed significantly from the other treatments (group B), which were statistically similar (group AB) to both the control (T12) and the commercial product based on *B. velezensis* (T10). Plants treated with the bacterial product had a reduction of the accumulated incidence area by 13.9%, whereas the best-performing

fungal antagonists were *C. cateniannulata*, *T. virens*, and *F. coffeibaccae*, which reduced $AUDPC_{INC}$ by 17.3%, 12.2%, and 10.3%, respectively.

Regarding the AUDPC for severity (Table 7), it was strongly and positively correlated with $AUDPC_{INC}$ ($r = 0.86$, $p < 0.01$). The fungicide (T09) produced the best results, leading to the lowest $AUDPC_{SEV}$ and significatively differing from all other treatments (group A), and reducing $AUDPC_{SEV}$ by 73.4% as compared to the control (T12). On the other hand, the highest $AUDPC_{SEV}$ was recorded for plots that received the *Aspergillus*-based treatment (T06), which was statistically similar (group B) to the *Calonectria* treatment (T07), the solution used in antagonist suspension alone (T11), and the control (T12). All other treatments were similar, having an intermediate effect. *Clonostachys rosea* (T01), *F. coffeibaccae* (T08), and *T. virens* (T03) reduced $AUDPC_{SEV}$ by 39.1%, 32.8%, and 32%, respectively, compared to the control. The *Bacillus*-based product (T10) reduced values by 21.1%, whereas *C. cateniannulata* (T05) led to reduction in disease severity of 15.5%, as compared with the control.

Final severity (Table 8) had a weak positive but significant correlation with $AUDPC_{INC}$ ($r = 0.31$, $p < 0.01$) and $AUDPC_{SEV}$ ($r = 0.31$, $p < 0.01$). Mean final severity values ranged from 0.07% to 0.28%. The chemical fungicide-based treatment (T09) had the lowest final severity and was statistically different from all other treatments (group A). This treatment also resulted in a control efficacy of 49.4%. Plants treated with *A. flavus* (T06) had the highest severity (group B), being ineffective for reducing CLR severity. Other treatments formed an intermediate group (AB), including the control (T12), the formulation based-treatment (T11) and the *B. velezensis* commercial product (T10), with a control efficacy of 2.9%. Among fungal antagonists, *Clonostachys rosea* (T01), *F. coffeibaccae* (T08), *T. harzianum* (T04), *T. virens* (T03), and *C. rhizophaga* (T02), exhibited control efficacy higher than the biofungicide product, with values ranging between 29.2% and 9.1%. *Cordyceps cateniannulata* (T05) and *C. hemileiae* (T07) did not reduced final severity, and had negative values of control efficacy as compared to control.

4. Discussion

The most promising fungal isolates, selected from an original assemblage of *ca.* 1500, obtained during the surveys conducted between 2015-2017, were assessed for their effect on reducing the incidence and severity of CLR in coffee plants cultivated in pots and maintained in a shaded area. Additionally, the most promising isolates were also tested in two experimental coffee fields. This study intended to integrate the most promising fungal antagonists under the same experimental conditions. The initial selection of isolates was based on results from *in vitro* and greenhouse screenings conducted by our research group, which provided valuable insights to reduce the list from over 1,500 isolates to 600, and then to 10.

Calonectria hemileiae was selected based on its proven ability to mycoparasitize *Hemileia vastatrix* uredinia, inhibiting urediniospore germination by up to 80% and reducing CLR severity by 70–90% under greenhouse conditions.[18] A non-aflatoxigenic *A. flavus* isolate was included due to its significant suppression of CLR severity when applied to leaves and soil.[28] *Cordyceps cateniannulata*, originally isolated as a coffee endophyte, demonstrated the ability to parasitize *H. vastatrix* uredinia and coffee insect pests; in addition, both its conidial suspension and culture filtrate inhibited urediniospore germination by an average of 38%.[29] The *F. coffeibaccae* isolate stood out among 93 *Fusarium* mycoparasitic isolates due to its ability to inhibit urediniospore germination by 74%, completely suppress symptoms in leaf disc assays, and reduce CLR severity by up to 66% under greenhouse conditions.[32] *Trichoderma* isolates, initially assessed through leaf disc and urediniospore germination assays,[31] also performed well, with the selected isolates achieving the greatest reductions in CLR severity among the 31 evaluated isolates under greenhouse conditions.[52] Similarly, selected isolates of *Clonostachys* spp. consistently inhibited urediniospore germination (94–100%) and reduced CLR severity in greenhouse trials.[27]

Taken together, these findings supported the inclusion of these isolates in further testing under more realistic conditions. However, some isolates exhibited variable efficacy depending on the experimental conditions. *Fusarium coffeibaccae* had a good and consistent biocontrol performance under the conditions in which the tests were conducted. Other fungal isolates, particularly *C. rhizophaga*, *C. rosea*, and *C. cateniannulata*, also provided good results, but their biocontrol effects varied between experiments and also in pots *vs.* field experiments.

Although some antagonist isolates were effective in reducing CLR intensity under our experimental conditions, the potential effects of a broader diversity of antagonists of *H. vastatrix* remain to be tested. We were able to assess only a small fraction of isolates from our collection, and other organisms may perform even better under different environmental conditions. Another factor that guided the selection of antagonists was the occurrence of each species in Brazil. Only species obtained in Brazil, or having already been documented as occurring in Brazil, were evaluated in our work. *Lecanicillium hemileae* is a case in point of a mycoparasite that, seemingly broadly occurs in Africa but is absent in Brazil,[53] which was not tested during our study because it would represent a classical introduction of a fungal species which is not known to occur in Brazil.

Fusarium coffeibaccae was found (Nóbrega, unpub. results) to have a cosmopolitan distribution in association with coffee. In a comprehensive study of the *Fusarium lateritium* species complex, Costa *et al.*[54] analyzed isolates of *F. coffeibaccae* from Brazil, China, Ethiopia, Guinea, Kenya, Malawi, New Caledonia, New Guinea, Papua New Guinea, and Zimbabwe, obtained from different tissues of *C. arabica* and *C. canephora* plants, including bark, fruits, leaf surface, stem, and vascular bundle. In addition, previous observations within the scope of this project identified *F. coffeibaccae* as a mycoparasite of *H. vastatrix* in Brazil and African countries,[32] suggesting a close relationship between the antagonistic fungus and the pathogen's uredinia as well.

Fusarium coffeibaccae produces a red pigment on PDA,[54] and the isolate selected for evaluation in our study also produced the same pigment. This pigment may be a source of secondary metabolites with potential to reduce CLR. Nóbrega[32] evaluated the potential of *Fusarium* filtrates in *in vitro* assays and observed high inhibition rates of pathogen germination, indicating anti-CLR activity by isolates of this species, but the effects of filtrates or organic extracts and the identity of the produced metabolites still remain to be further evaluated.

Cordyceps cateniannulata applications resulted in intermediate control levels of CLR. However, the results obtained under *in vitro* conditions, which demonstrated its mycoparasitic and antibiosis potential against *H. vastatrix*, as well as high mortality rates of two major coffee pests – the coffee berry borer (*Hypothenemus hampei*) and the leaf miner (*Leucoptera coffeella*)[29] – justified the continuation of the studies on this fungus in future stages of this study. In case the recognized multitrophic activity of *C. cateniannulata*

is translated into benefits for control of three, among the worst phytosanitary problems for coffee, this species cannot be neglected in future biocontrol investigations.

Clonostachys rosea and *C. rhizophaga* provided moderate to high levels of CLR reduction when applied alone or in combination (in the pot experiment). Fungi of this genus are extensively studied for their potential as biocontrol agents against many diseases,[55] with *C. rosea* being among the most broadly and intensively studied fungus biocontrol species.[56] Such studies have already resulted in commercial biofungicides, some of which are available in Brazil, Canada, China, the European Union, Russia, and the USA.[56] In Brazil there is one product (Kamoi Pro/BioRoof®) based on *C. rosea*, registered for use against *Botrytis cinerea. Clonostachys rhizophaga* has received less attention; however, there are a few examples of studies indicating its potential for reducing the severity of potato early blight caused by *Alternaria grandis*[57] and root rot in cocoa caused by *Rosellinia* spp. [58] Although there are records of *C. rosea* and *C. rhizophaga* as causal agents of crop diseases – for example, *C. rosea* causing root rot in soybean in the United States,[59] and rot in faba bean in Iran;[60] and *C. rhizophaga* causing chickpea wilt in Syria,[61] or root rot in lentil and pea in France[62] – it is known that the isolates evaluated in our study were capable of colonizing coffee plants as endophytes without inducing disease symptoms, demonstrating that in the case of *C. arabica*, these species, or the isolates under study, are not coffee pathogens.[27]

In pot experiments, it was observed that, compared to the control, our fungal antagonists led to a reduction of coffee growth, reducing plant height by up to 25% and shoot dry mass by up to 42%. Conversely, applications of the commercial product based on *B. velezensis* led to an increase in plant height of 0.3% and enhanced shoot dry mass accumulation by 4.3%. In the literature, many bacteria and fungi are reported to establish endophytic associations with coffee plants, and in several instances enhancing coffee plant growth.[63] However, there are cases in which these microorganisms vary in their effects: while some strains benefit coffee plants, others do not promote plant growth.[16] Furthermore, genera better known for bringing benefits to host plants may provoke harm to some plants. For example, *Trichoderma virens* is well-known as a biocontrol fungus; however, specific strains of this species which produce the secondary metabolite gliotoxin, can negatively impact some plants. In tomato seedlings, for instance, a decrease in total biomass accumulation was observed due to the phytotoxicity of this metabolite.[64] Strains of *Clonostachys rosea* and *Cordyceps militaris* can also produce secondary metabolites that

are phytotoxic to plants. Dihydrovertinolide, produced by *C. rosea,* inhibited shoot and root growth of lettuce by 23% and 65%, respectively, compared to untreated controls,[65] whereas cordycepin, isolated from *C. militaris*, inhibited the germination and growth of radish.[66]

These studies suggest that purported biocontrol fungi may be, in fact, harmful to some plants depending on the species involved, fungal strain, concentration and type of secondary metabolites, host sensibility, and plant age. The substantial levels of reduction in biomass observed for many associations that were evaluated demands an explanation. It might be conjectured that such a large suppression of growth might not be observed on mature and productive coffee plants or that coffee yield would not be harmed, but these are matters that would require careful evaluations. It is also possible that induced systemic resistance is at play in our CLR antagonist applications. This growth reduction-impact may be a "price" paid for the establishment and maintenance of fungi in coffee tissues or an energetic investment by the plant in activating its defense mechanisms, as the activation and expression of defense pathways demands the reallocation of nutrient resources, leading to a reduction in growth, which is described as a “growth-defense trade-off”.[67] It is well documented in the literature, for crops such as rice, tobacco, lettuce and beans that induced systemic resistance (by acibenzolar-S-methyl applications, for example) can lead to reduction in height, biomass and leaf area, depending on plant age, frequency of application and dose utilized.[68,69] Such objectionable effects are considered as mostly temporary and losses in productivity may be averted through correct applications, as for commercial acibenzolar-S-methyl products such as Bion®. Our results emphasize, nevertheless, that special caution must be taken during further evaluations of our (or any) CLR biocontrol candidates. There must be no room for a situation where “the cure is worse than the disease”.

Despite the negative effects observed on coffee growth in the pot experiments, it would be premature to assume a reduction in coffee yield. Due to the time it takes for coffee plants to reach production, and the absence of yield evaluations for our field study, we were unable to obtain this data. However, considering the defoliation caused by CLR, it is reasonable to hypothesize that plants with higher CLR intensity would experience a greater impact on yield than those with less CLR, and maybe effect of fungal antagonists in CLR control would compensate the observed growth reduction. Although we could not determine how our antagonists reduce CLR intensity in the field, in addition to inducing plant resistance, mycoparasitism and the production of cell wall-degrading enzymes and

antibiotic compounds are established modes of action of biocontrol agents[70,71] that should be further examined in this context.

Studies on endophytes associated with coffee plants began to emerge in the late 1990s, initially focusing on bacterial strains. Later, in 2005, research expanded to fungal endophytes.[63] Since then, most studies have focused on fungal communities' composition in coffee tissues from different coffee-growing regions,[27,28,30,72–78] rather than functional traits such as growth promotion. To date, only one study has reported growth-promoting effects of fungal endophytes (*Trichoderma* sp. strains) in coffee,[79] while a few others have demonstrated positive effects of *Bacillus* species,[17,80] accordingly to our findings using the commercial product based on *B. velezensis*.

The success of endophyte-plant associations can be influenced by several factors, including fungal species or strain, inoculum concentration, timing of application, host species, developmental stage, physiological status, tissue type, environmental conditions, and even the sampling and isolation techniques employed.[81,82] Despite inconsistencies in our attempts to reisolate fungal endophytes from treated coffee plants, *T. harzianum* (T06 – M290) and *A. flavus* (T08 – E486) were the most frequently recovered isolates in the potted plant assays. In a previous greenhouse study, the same *Aspergillus* isolate also exhibited inconsistent recovery,[28] which has been attributed to the limited duration of the evaluation, tissue sampled, and methodology utilized. In contrast, *T. harzianum* appears to be capable of establishing long-term association with different hosts. In our potted experiments, this species was reisolated from coffee tissues up to two months after the last antagonists' application. Similar persistence has been reported in other crops, with *T. harzianum* recovered from sweet orange tissues after eight months[83] and from tomato plants after four months.[84]

In contrast to the results observed in the potted experiments, where plants treated with the *Bacillus*-based commercial product had a higher $AUDPC_{SEV}$ than the control group, in the field experiments this treatment reduced $AUDPC_{SEV}$ by 33.3% compared to the control, indicating that, depending on the environmental conditions and inoculum pressure, results may vary substantially. While some fungal antagonists performed similarly to the bacterial commercial product, such as *F. coffeibaccae*, which reduced $AUDPC_{SEV}$ by 32.6%, others performed better. For example, *C. rosea* and *C. rhizophaga* reduced severity values by approximately 42%. However, treatments based on fungal antagonists did not result in statistically significant differences compared to the control, as $AUDPC_{INC}$ and

$AUDPC_{SEV}$ values did not differ significantly, and the chemical fungicide was the most effective treatment. Even though, these results indicate that some antagonists have the potential to be used for CLR management, particularly with regard to disease severity and especially in zero-pesticide systems of coffee production.

Studies assessing the biocontrol potential of microorganisms under field conditions are still scarce in the literature. Some articles have focused on antagonistic bacteria, such as those conducted in Brazil[15] and Peru.[85] *Akanthomyces lecanii* (syn. *Lecanicillium lecanii*) was reported to delay incubation and latent period of CLR in greenhouse and field conditions,[86] to contribute indirectly at controlling *H. vastatrix* in a coffee agroecosystem,[87] and more recently, there were claims of *Trichoderma* and *Akanthomyces* isolates being effective in reducing the disease in an experimental coffee field.[22] These publications were based on studies conducted in Colombia, and, the later in Mexico, and in both cases, unfortunately, there is no information on the correct identity of the fungal isolates at play. The work by Sánchez-Lucio *et al* [22] was inadequate and left the identification of the isolates only at the generic level whereas the works of Vélez and Rosillo[86] and Jackson *et al*.[87] identified the fungus as *L. lecanii* – a common mistake which is only now being clarified by Colmán *et al.*[53]. These publications provide, nevertheless, indications that antagonistic fungi can indeed contribute to controlling CLR.

Considering the benefits that microorganisms may offer to the coffee plantations, the use of "microbial consortia", *i.e*., application of mixtures of microbial species/strains, may become a promising approach.[88] During our pot experiments, an attempt was made to test a combination of a small assemblage of *Clonostachys* isolates and, in separate, an assemblage of selected *Trichoderma* strains of distinct species. This yielded good results for reducing CLR incidence and severity. An approach that could be explored, besides mixtures of biocontrol candidates with phylogenetical affinities, would be combining fungal antagonists obtained from different ecological niches, namely: mycoparasites obtained from *H. vastatrix* pustules with endophyte “bodyguards” obtained from coffee tissues. Some synergistic interaction might result from such combinations. Also, combining *B. velezensis*, with its growth-promoting properties, with fungal antagonists might also represent an interesting option for future investigation.

As our experiments were conducted under field conditions, we opted to use an incipient formulation designed to protect the fungal antagonists from environmental adversities, and, at least in the experiment with potted plants, the formulation-based

treatment reduced disease-related parameters. Beyond its protective properties, each component of the formulation is known to exert direct or indirect effects against phytopathogens and plant diseases.

Sunflower oil has been reported as effective against powdery mildews, such as *Erysiphe neolycopersici*, *Podosphaera pannosa*, and *P. xanthii*,[89–91] and has also inhibited urediniospore germination of rust fungi such as *Uromyces appendiculatus* and *Puccinia recondita*, achieving up to 54% and 83% control, respectively, at 1% (v/v).[92] Similarly, aqueous skim milk solutions have reduced powdery mildew severity in cucurbits.[93–95] Glycerol has shown both direct antifungal activity[96] and indirect effects via induction of plant resistance,[97] including in coffee plants.[98]

When urediniospores were exposed to the formulation used in our experiments, no significant inhibition of germination was observed (Fig. S2). Similarly, fungal antagonists cultured in the presence of the formulation exhibited no mycelial growth inhibition, and some isolates even showed enhanced growth (Fig. S3, Fig. S4). Therefore, the disease suppression observed in formulation-based treatments in certain assays may be attributed, at least in part, to the indirect effects of these components. Nonetheless, a potential direct effect against *H. vastatrix* cannot be discarded, and more detailed experiments are warranted to explore these possibilities.

The results presented herein represent significant steps towards the selection and practical evaluation of the vast and diverse range of fungi collected as potential biocontrol agents of CLR. This long process, which followed the field surveys, included the elucidation of the taxonomy of selected groups of taxa, *in vitro* and greenhouse assays and now has reached the stage of more focused *in planta* studies. At each stage, details such as arbitrary choices that were made and adopted for experiments such as coffee cultivar, plant age concentration of inocula, frequency and mode of application, choice of isolates, and timing of rust inoculation, may have played a critical role in the results that were obtained for CLR control as well as plant development. Environmental conditions have certainly influenced our results. In two instances, entire pot experiments were lost because the disease did not establish or because the colder temperatures were favorable for severe outbreak of “Phoma leaf spot”.

We attempted to utilize a scheme of spraying intended to favour the establishment of the fungal antagonist population, regardless of whether this would be viable in crop

situations. It is inevitable that a considerable component of uncertainty is involved in this form of prospective study and we acknowledge that further studies are necessary to clarify the mode of action of each biocontrol candidate, confirm biocontrol potential of our antagonists which had a good performance as well as to provide additional opportunities for those candidates which showed inconsistent results.

It is hoped that future studies will address such issues as well as investigating the potential of microbial consortia, optimization of inoculum yield, evaluation of varying antagonist inoculum concentrations, spraying intervals, and formulations containing various adjuvants which may provide protective effects against adverse environmental conditions. Effects and identity of secondary metabolites produced by fungi such as *Calonectria hemileiae*, *F. coffeibaccae* and others, should be also explored. Practical matters such as economic feasibility of production and use biofungicide products based on our fungi and efficacies under field conditions should also be considered.

The wealth and diversity of fungal antagonists of *H. vastatrix* is still barely known to science and it is to be expected that an expansion of the fungal surveys, particularly in Africa, would unveil far larger list of antagonists. Perhaps it would even reveal a “holy grain of biocontrol” – a classical biocontrol agent that would be capable, alone, to bring the populations of *H. vastatrix* in the Americas down to a lower status. Nevertheless, it is possible that, by finding the right protocol, the fungi that are already available in our “toolbox” may become valuable options for CLR management.

5. References

1 Bertrand B, Davis AP, Maraval I, Forestier N, and Mieulet D, Potential beverage quality of three wild coffee species (*Coffea brevipes*, *C. congensis* and *C. stenophylla*) and consideration of their agronomic use, *J Sci Food Agric* **103**:3602–3612 (2023).

2 Davis AP, Gargiulo R, Fay MF, Sarmu D, and Haggar J, Lost and Found: *Coffea stenophylla* and *C. affinis*, the Forgotten Coffee Crop Species of West Africa, *Front Plant Sci* **11** (2020).

3 Maghuly F, Jankowicz-Cieslak J, and Bado S, Improving coffee species for pathogen resistance, *CABI Reviews* (2020).

4 Rhiney K, Guido Z, Knudson C, Avelino J, Bacon CM, Leclerc G, *et al.*, Epidemics and the future of coffee production, *Proceedings of the National Academy of Sciences* **118** (2021).

5 Baker P, The "Big Rust": an update on the coffee leaf rust situation, *Coffee Cocoa Int* **40**:37–39 (2014).

6 Dupre SI, Harvey CA, and Holland MB, The impact of coffee leaf rust on migration by smallholder coffee farmers in Guatemala, *World Dev* **156**:105918 (2022).

7 Avelino J, Cristancho M, Georgiou S, Imbach P, Aguilar L, Bornemann G, *et al.*, The coffee rust crises in Colombia and Central America (2008–2013): impacts, plausible causes and proposed solutions, *Food Secur* **7**:303–321 (2015).

8 McCook S and Vandermeer J, The Big Rust and the Red Queen: Long-Term Perspectives on Coffee Rust Research, *Phytopathology* **105**:1164–1173 (2015).

9 Koutouleas A, Collinge DB, and Boa E, The coffee leaf rust pandemic: An ever-present danger to coffee production, *Plant Pathol* **73**:522–534 (2024).

10 Ward R, Gonthier D, and Nicholls C, Ecological resilience to coffee rust: Varietal adaptations of coffee farmers in Copán, Honduras, *Agroecology and Sustainable Food Systems*:1–18 (2017).

11 Guerra-Guimarães L, Diniz I, Azinheira HG, Loureiro A, Pereira AP, Tavares S, *et al.*, Coffee Leaf Rust Resistance: An Overview, Mutation Breeding in Coffee with Special Reference to Leaf Rust, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 19–38 (2023).

12 Zambolim L, Current status and management of coffee leaf rust in Brazil, *Trop Plant Pathol* **41**:1–8 (2016).

13 Koutouleas A, Collinge DB, and Ræbild A, Alternative plant protection strategies for tomorrow's coffee, *Plant Pathol* **72**:409–429 (2023).

14 Cruz IG la, Martínez-Bolaños M, Chávez-Ramírez B, and Estrada-de los Santos P, Biocontrol of *Hemileia vastatrix*, the Causal Agent of Coffee Leaf Rust, by *Paenibacillus* sp. NMA1017, *Plant Dis* **108**:3163–3169 (2024).

15 Haddad F, Maffia LA, Mizubuti ESG, and Teixeira H, Biological control of coffee rust by antagonistic bacteria under field conditions in Brazil, *Biological Control* **49**:114–119 (2009).

16 Silva HSA, Tozzi JPL, Terrasan CRF, and Bettiol W, Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust, *Biological Control* **63**:62–67 (2012).

17 de Souza MPP, Cipriano MAP, Braghini MT, de Sousa LP, Mondego JMC, Patrício FRA, *et al.*, Beneficial bacteria improve seedling growth, nutrition and promote biological control of coffee diseases, *J Appl Microbiol* (2025).

18 Salcedo-Sarmiento S, Aucique-Pérez CE, Silveira PR, Colmán AA, Silva AL, Corrêa Mansur PS, *et al.*, Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant, *iScience* **24**:102352 (2021).

19 Carrión G and Rico-Gray V, Mycoparasites on the coffee rust in Mexico, *Fungal Divers* **11**:49–60 (2002).

20 Cruz D, Jaramillo-Riofrío A, Herrera P, Aguinsaca R, and Chamba M, Fungal Diversity Detected by ITS-5.8S from *Coffea arabica* Leaves Infected by Rust (*Hemileia vastatrix*) in Southern Ecuador, *Diversity (Basel)* **16**:633 (2024).

21 Luiz BC, Sugiyama LS, Brill E, and Keith LM, Survey of potential fungal antagonists of Coffee Leaf Rust (*Hemileia vastatrix*) on *Coffea arabica* in Hawaii, USA, *Brazilian Journal of Microbiology* **55**:2839–2844 (2024).

22 Sánchez-Lucio R, González-Gaona E, Padilla-Ramírez JS, Gallegos-Robles MA, González-Salas U, Gómez-Jaimes R, *et al.*, Effectiveness of *Trichoderma* spp. and *Lecanicillium* spp. strains in the control rust (*Hemileia vastatrix* Berkeley & Broome) in coffee cultivation in Nayarit, Mexico., *Biotecnia* **26**:136–143 (2024).

23 Colmán AA, Evans HC, Salcedo-Sarmiento SS, Braun U, Belachew-Bekele K, and Barreto RW, A fungus-eat-fungus world: *Digitopodium*, with particular reference to mycoparasites of the coffee leaf rust, *Hemileia vastatrix*, *IMA Fungus* **12**:1 (2021).

24 Pereira CM, Sarmiento SS, Colmán AA, Belachew-Bekele K, Evans HC, and Barreto RW, Mycodiversity in a micro-habitat: twelve *Cladosporium* species, including four new taxa, isolated from uredinia of coffee leaf rust, *Hemileia vastatrix*, *Fungal Syst Evol* **14**:9–33 (2024).

25 Crous PW, Luangsa-Ard JJ, Wingfield MJ, Carnegie AJ, Hernández-Restrepo M, Lombard L, *et al.*, Fungal Planet description sheets: 785–867, *Persoonia-Molecular Phylogeny and Evolution of Fungi* **41**:238–417, Naturalis Biodiversity Center (2018).

26 Guterres DC, Ndacnou MK, Saavedra-Tobar LM, Salcedo-Sarmiento S, Colmán AA, Evans HC, *et al.*, *Cryptococcus depauperatus*, a close relative of the human-pathogen *C. neoformans*, associated with coffee leaf rust (*Hemileia vastatrix*) in Cameroon, *Brazilian Journal of Microbiology* **52**:2205–2214 (2021).

27 Kapeua-Ndacnou M, de Abreu LM, de Macedo DM, da Nóbrega TF, Pereira CM, Evans HC, *et al.*, Assessing the Biocontrol Potential of *Clonostachys* Species Isolated as Endophytes from Coffea Species and as Mycoparasites of *Hemileia* Rusts of Coffee in Africa, *Journal of Fungi* **9** (2023).

28 Kapeua-Ndacnou M, Nóbrega TF, Batista LR, Evans HC, Abreu LM, Begoudé DAB, *et al.*, *Aspergillus flavus* from coffee in Cameroon: a non-aflatoxigenic endophytic isolate antagonistic to coffee leaf rust (*Hemileia vastatrix*), *J Appl Microbiol* **134**: lxad076 (2023).

29 Pereira CM, Bautz KR, Rodríguez M del CH, Saavedra-Tobar LM, Kapeua-Ndacnou M, Belachew-Bekele K, *et al.*, *Cordyceps cateniannulata*: An endophyte of coffee, a parasite of coffee leaf rust and a pathogen of coffee pests, *Fungal Biol* **128**:1917–1932 (2024).

30 Rodríguez M del CH, Evans HC, de Abreu LM, de Macedo DM, Ndacnou MK, Bekele KB, *et al.*, New species and records of *Trichoderma* isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa, *Sci Rep* **11**:5671 (2021).

31 Rodríguez M del CH, *Trichoderma* spp. associated with coffee in Africa: taxonomy and potential uses for the biological control of coffee leaf rust (*Hemileia vastatrix*) and promotion of plant growth, Universidade Federal de Viçosa. Thesis (2018).

32 Nóbrega TF, *Fusarium* spp. antagonists to *Hemileia vastatrix* from Brazil, Paraguay and Africa: Taxonomy and biocontrol potential, Universidade Federal de Viçosa. Thesis (2021).

33 Posadas JB, Maricel AL, Mini Jorge I, and Lecuona Roberto E, Natural tolerance to UV-B and assessment of photoprotectants in conidia of six native isolates of *Beauveria bassiana* (Bals-Criv) Vuillemin, *World Appl Sci J* **20**:1024–1030, Citeseer (2012).

34 Hemalatha S, Ramaraju K, and Jeyarani S, Influence of ultraviolet light protectants on the persistence of the entomopathogenic fungus *Beauveria bassiana*, *Journal of Biopesticides* **10**:130–134, Crop Protection Research Centre (2017).

35 Devi K, Joshi N, and Sangha KS, Oils as UV protectants of *Beauveria bassiana* conidia and bioefficacy against *Lipaphis erysimi* (Kalt), *Indian Journal of Entomology* **80**:761 (2018).

36 Grewal GK and Joshi N, Evaluation of adjuvants on growth and virulence of *Metarhizium rileyi* against *Spodoptera litura* (F.), *Indian Journal of Entomology*:1–4 (2022).

37 Aguirre K, Perez LM, and Montealegre JR, *In vitro* evaluation of drying supports and adhesive polymers as adjuvants for biocontrol of *Diplodia seriata* by *Trichoderma harzianum* and *Clonostachys rosea*, *Phytopathol Mediterr* **60**:227–237 (2023).

38 Figueiredo YF, Oliveira JM, Almeida KA, de Fátima Pereira P, Pedroso LA, de Resende Faria Guimarães M, *et al.*, Coffee leaf rust assessment: comparison and validation of diagrammatic scales for *Coffea arabica*, *Eur J Plant Pathol* **164**:411–427 (2022).

39 Sakiyama N, Martinez HEP, Tomaz MA, and Borém A, Café arábica do plantio a colheita, *Viçosa: Editora UFV*:316 (2015).

40 Belan LL, Belan LL, Satiro LS, Moraes WB, de Jesus Junior WC, and Moraes WB, A comparison of sampling methods to accurately estimate the incidence of leaf rust on conilon coffee, *Australasian Plant Pathology* **50**:729–737 (2021).

41 Capucho AS, Zambolim L, Lopes UN, and Milagres NS, Chemical control of coffee leaf rust in *Coffea canephora* cv. conilon, *Australasian Plant Pathology* **42**:667–673 (2013).

42 Olivoto T, Lights, camera, pliman! An R package for plant image analysis, *Methods Ecol Evol* **13**:789–798 (2022).

43 R Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria (2024).

44 Wickham H, Data Analysis, pp. 189–201 (2016).

45 Alves KS and Del Ponte EM, Analysis and simulation of plant disease progress curves in R: introducing the epifitter package, *Phytopathology Research* **3**:22 (2021).

46 McGillycuddy M, Warton DI, Popovic G, and Bolker BM, Parsimoniously Fitting Large Multivariate Random Effects in glmmTMB, *J Stat Softw* **112** (2025).

47 Hartig F, DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models, *CRAN: Contributed Packages*, The R Foundation (2016).

48 Lüdecke D, Ben-Shachar M, Patil I, Waggoner P, and Makowski D, performance: An R Package for Assessment, Comparison and Testing of Statistical Models, *J Open Source Softw* **6**:3139 (2021).

49 Fox J and Weisberg S, An R companion to applied regression, Sage publications (2018).

50 Lenth R V., emmeans: Estimated Marginal Means, aka Least-Squares Means, *CRAN: Contributed Packages* (2017).

51 Hothorn T, Bretz F, and Westfall P, Simultaneous Inference in General Parametric Models, *Biometrical Journal* **50**:346–363 (2008).

52 Kapeua-Ndacnou M, Isolates of *Aspergillus*, *Clonostachys* and *Trichoderma* from Africa as potential biocontrol agents against coffee leaf rust, Universidade Federal de Viçosa. Thesis (2022).

53 Cólman AA, Araújo JPM, Evans HC, Mansur PSC, Salcedo-Sarmiento S, Silva AL, *et al.*, Hidden diversity behind the *Lecanicillium*-like white colony-forming mycoparasites on *Hemileia vastatrix* (coffee leaf rust), *Persoonia*.

54 Costa MM, Sandoval-Denis M, Moreira GM, Kandemir H, Kermode A, Buddie AG, *et al.*, Known from trees and the tropics: new insights into the *Fusarium lateritium* species complex, *Stud Mycol* **109**:403–450 (2024).

55 Reyes-Estebanez M and Mendoza-de Gives P, The Genus *Clonostachys* (Bionectria) as a Potential Tool Against Agricultural Pest and Other Biotechnological Applications: A Review, *Microbiol Res (Pavia)* **16**:86 (2025).

56 Jensen DF, Dubey M, Jensen B, and Karlsson M, *Clonostachys rosea* to control plant diseases, Burleigh Dodds Science Publishing (2022).

57 da Silva HAO, Teixeira WD, Borges Á V., Silva Junior AL, Alves KS, Rodrigues Junior OM, *et al.*, Biocontrol of potato early blight and suppression of *Alternaria grandis* sporulation by *Clonostachys* spp., *Plant Pathol* **70**:1677–1685 (2021).

58 García RAM, Martijn ten Hoopen G, Kass DCJ, Sánchez Garita VA, and Krauss U, Evaluation of mycoparasites as biocontrol agents of *Rosellinia* root rot in cocoa, *Biological Control* **27**:210–227 (2003).

59 Bienapfl JC, Floyd CM, Percich JA, and Malvick DK, First Report of *Clonostachys rosea* Causing Root Rot of Soybean in the United States, *Plant Dis* **96**:1700–1700 (2012).

60 Afshari N and Hemmati R, First report of the occurrence and pathogenicity of *Clonostachys rosea* on faba bean, *Australasian Plant Pathology* **46**:231–234 (2017).

61 Abang MM, Kabbabeh S, Ahmed S, Murad S, Chilvers MI, Peever TL, *et al.*, First Report of Chickpea Wilt Caused by *Clonostachys rhizophaga* in Syria, *Plant Dis* **93**:666–666 (2009).

62 Gautheron E, Tayeh N, Gautheron N, André A, Gibert S, Steinberg C, *et al.*, First Report of *Clonostachys rhizophaga* Causing Root Rot on Lentil in France and Cross-Pathogenicity on Pea, *Plant Dis* **109**:505 (2025).

63 Asad S, Priyashantha AKH, Tibpromma S, Luo Y, Zhang J, Fan Z, *et al.*, Coffee-Associated Endophytes: Plant Growth Promotion and Crop Protection, *Biology (Basel)* **12**:911 (2023).

64 Zaid R, Koren R, Kligun E, Gupta R, Leibman-Markus M, Mukherjee PK, *et al.*, Gliotoxin, an Immunosuppressive Fungal Metabolite, Primes Plant Immunity: Evidence from *Trichoderma virens* -Tomato Interaction, *mBio* **13** (2022).

65 Supratman U, Suzuki T, Nakamura T, Yokoyama Y, Harneti D, Maharani R, *et al.*, New metabolites produced by endophyte *Clonostachys rosea* B5 − 2, *Nat Prod Res* **35**:1525–1531 (2021).

66 Quy TN, Xuan TD, Andriana Y, Tran H-D, Khanh TD, and Teschke R, Cordycepin Isolated from *Cordyceps militaris*: Its Newly Discovered Herbicidal Property and Potential Plant-Based Novel Alternative to Glyphosate, *Molecules* **24**:2901 (2019).

67 Panda S, Kazachkova Y, and Aharoni A, Catch-22 in specialized metabolism: balancing defense and growth, *J Exp Bot* **72**:6027–6041 (2021).

68 Walters D, Walsh D, Newton A, and Lyon G, Induced Resistance for Plant Disease Control: Maximizing the Efficacy of Resistance Elicitors, *Phytopathology* **95**:1368–1373 (2005).

69 Chamnongpol S, Willekens H, Moeder W, Langebartels C, Sandermann H, Van Montagu M, *et al.*, Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco, *Proceedings of the National Academy of Sciences* **95**:5818–5823 (1998).

70 Pal KK and Gardener BM, Biological Control of Plant Pathogens, *The Plant Health Instructor* (2006).

71 Köhl J, Kolnaar R, and Ravensberg WJ, Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy, *Front Plant Sci* **10** (2019).

72 Santamaría J and Bayman P, Fungal Epiphytes and Endophytes of Coffee Leaves (*Coffea arabica*), *Microb Ecol* **50**:1–8 (2005).

73 Vega FE, Simpkins A, Aime MC, Posada F, Peterson SW, Rehner SA, *et al.*, Fungal endophyte diversity in coffee plants from Colombia, Hawaii, Mexico and Puerto Rico, *Fungal Ecol* **3**:122–138 (2010).

74 Oliveira R, Souza R, Lima T, and Cavalcanti M, Endophytic fungal diversity in coffee leaves (*Coffea arabica*) cultivated using organic and conventional crop management systems, *Mycosphere* **5**:523–530 (2014).

75 Haddad F, Saraiva RM, Mizubuti ESG, Romeiro RS, and Maffia LA, Isolation and selection of *Hemileia vastatrix* antagonists, *Eur J Plant Pathol* **139**:763–772 (2014).

76 Oliveira MNV, Santos TMA, Vale HMM, Delvaux JC, Cordero AP, Ferreira AB, *et al.*, Endophytic microbial diversity in coffee cherries of *Coffea arabica* from southeastern Brazil, *Can J Microbiol* **59**:221–230 (2013).

77 Saucedo-García A, Anaya AL, Espinosa-García FJ, and González MC, Diversity and Communities of Foliar Endophytic Fungi from Different Agroecosystems of *Coffea arabica* L. in Two Regions of Veracruz, Mexico, *PLoS One* **9**: e98454 (2014).

78 Fulthorpe R, Martin AR, and Isaac ME, Root Endophytes of Coffee (*Coffea arabica*): Variation Across Climatic Gradients and Relationships with Functional Traits, *Phytobiomes J* **4**:27–39 (2020).

79 Mamani-Huayhua G, Leon-Ttacca B, Palao-Iturregui LA, and Borja-Loza YR, Biocontrol of coffee yellow rust (*Hemileia vastatrix* Berk. & Br.) with *Trichoderma* sp. endophyte strains, *Cultivos Tropicales* **42** (2021).

80 Asyiah IN, Mudakir I, Hoesain M, Pradana AP, Djunaidy A, and Sari RF, Consortium of endophytic bacteria and rhizobacteria effectively suppresses the population of *Pratylenchus coffeae* and promotes the growth of Robusta coffee, *Biodiversitas* **21** (2020).

81 Santos ML dos, Berlitz DL, Wiest SLF, Schünemann R, Knaak N, and Fiuza LM, Benefits Associated with the Interaction of Endophytic Bacteria and Plants, *Brazilian Archives of Biology and Technology* **61** (2018).

82 Zhao C, Onyino J, and Gao X, Current Advances in the Functional Diversity and Mechanisms Underlying Endophyte–Plant Interactions, *Microorganisms* **12**:779 (2024).

83 Nemec S, Datnoff LE, and Strandberg J, Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus, *Crop Protection* **15**:735–742 (1996).

84 Sivan A and Chet I, Integrated control of fusarium crown and root rot of tomato with *Trichoderma harzianum* in combination with methyl bromide or soil solarization, *Crop Protection* **12**:380–386 (1993).

85 Ogata-Gutiérrez K, Chumpitaz-Segovia C, Lirio-Paredes J, and Zúñiga-Dávila D, Antifungal Activity of Phyllospheric Bacteria Isolated from *Coffea arabica* against *Hemileia vastatrix*, *Microorganisms* **12**:582 (2024).

86 Vélez PE and Rosillo AG, Evaluación del antagonismo del hongo *Verticillium lecanii* sobre *Hemileia vastatrix* en condiciones de invernadero y de campo (1995).

87 Jackson D, Skillman J, and Vandermeer J, Indirect biological control of the coffee leaf rust, *Hemileia vastatrix*, by the entomogenous fungus *Lecanicillium lecanii* in a complex coffee agroecosystem, *Biological Control* **61**:89–97 (2012).

88 Nunes PSO, Lacerda-Junior G V., Mascarin GM, Guimarães RA, Medeiros FHV, Arthurs S, *et al.*, Microbial consortia of biological products: Do they have a future?, *Biological Control* **188**:105439 (2024).

89 Ko WH, Wang SY, Hsieh TF, and Ann PJ, Effects of Sunflower Oil on Tomato Powdery Mildew Caused by *Oidium neolycopersici*, *Journal of Phytopathology* **151**:144–148 (2003).

90 Wojdyla AT, Effect of vegetable and mineral oils on the development of *Sphaerotheca pannosa* var. *rosae*–the causal agent of powdery mildew of rose, *Bulg J Agric Sci* **21**:855–862 (2015).

91 Ma Y, Ke Y, Zhu H, Li B, Li B, Zhang F, *et al.*, Oleozon: A novel control strategy against powdery mildew in cucumber, *Journal of Phytopathology* **165**:841–847 (2017).

92 Arslan U, Efficacy of plant oils on the control of bean rust and wheat leaf rust, *Fresenius Environ Bull* **23**:2259–2265 (2014).

93 Ferrandino FJ and Smith VL, The effect of milk-based foliar sprays on yield components of field pumpkins with powdery mildew, *Crop Protection* **26**:657–663 (2007).

94 Keinath AP and DuBose VB, Controlling powdery mildew on cucurbit rootstock seedlings in the greenhouse with fungicides and biofungicides, *Crop Protection* **42**:338–344 (2012).

95 Medeiros FHV, Bettiol W, Souza RM, Alves E, Pinto ZV, and Iost R, Microorganisms, application timing and fractions as players of the milk-mediated powdery mildew management, *Crop Protection* **40**:8–15 (2012).

96 Kim YH, Jang SJ, Yun YB, Kim SS, and Kuk YI, Effect of ethanol and glycerol on suppression rates of various crop pathogens, *Research on Crops* **18**:164–169, Gaurav Society of Agricultural Research Information Centre (2017).

97 Novaes AP da S, Nascimento F dos S, Rocha A de J, Soares JM da S, Ramos AP de S, Junior LC de S, *et al.*, Glycerol as an Inducer of Disease Resistance in Plants: A Systematic Review, *Horticulturae* **10**:1368 (2024).

98 Gazolla LC de OF, Almeida DP, Castro ISL, Castro AG de, Arruda LV de S, Vieira NM, *et al.*, Regulação transcricional do metabolismo de glicerol-3-fosfato induz resistência à ferrugem do café, Embrapa Café (2019).

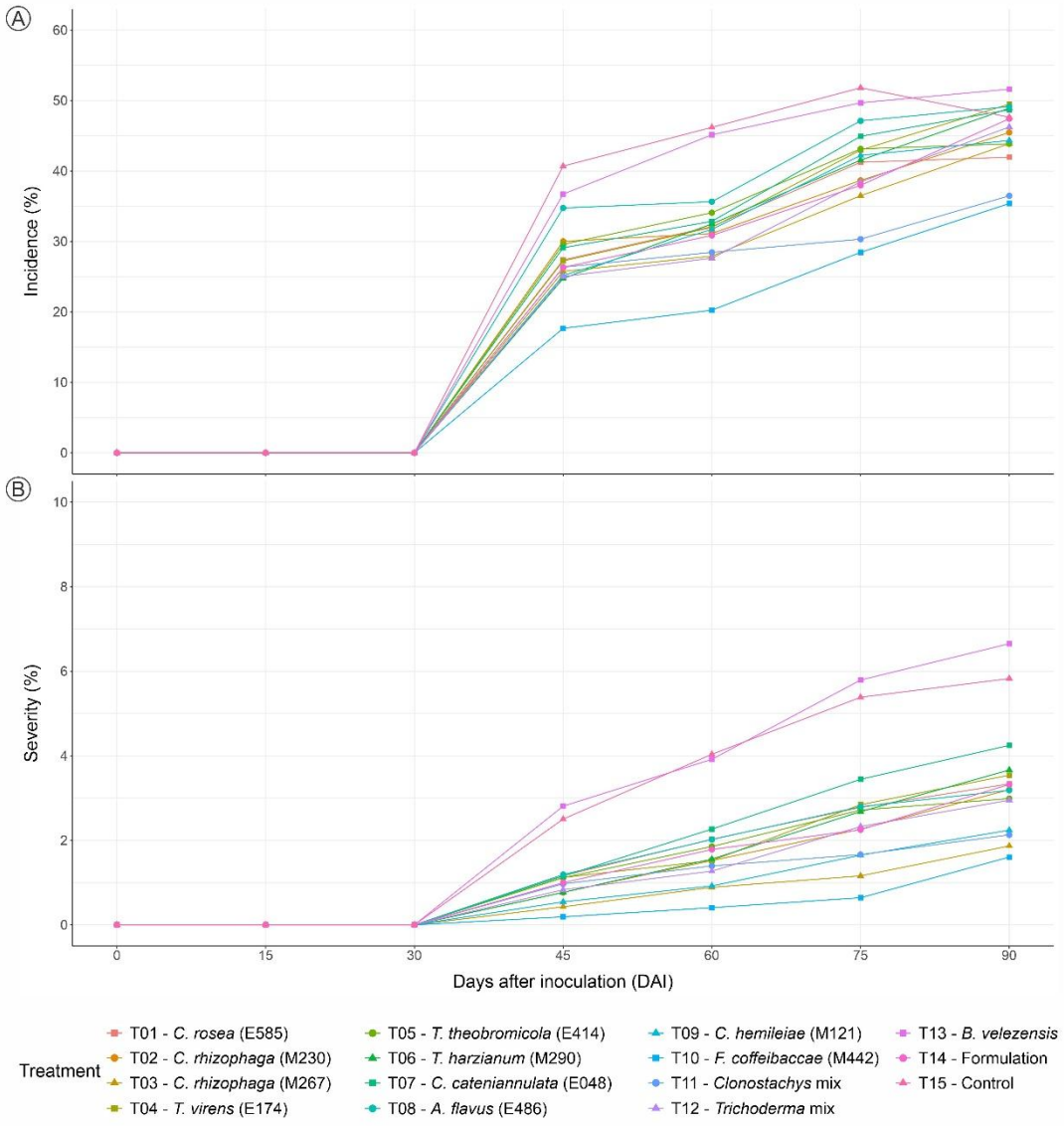

Figure 1. Combined analysis of the progression of coffee leaf rust in coffee plants (*Coffea arabica* cv. 'IAC 144') treated with fungal antagonists and inoculated with *Hemileia vastatrix* in two independent experiments (conducted from November 2023 to February 2024 and from November 2024 to February 2025). (A) Progress curve of incidence (%) and (B) severity (%). T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria hemileiae* (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of *Trichoderma* strains (E174 + E414 + M290); T13 = *Bacillus velezensis*; T14 = formulation; and T15 = untreated control.

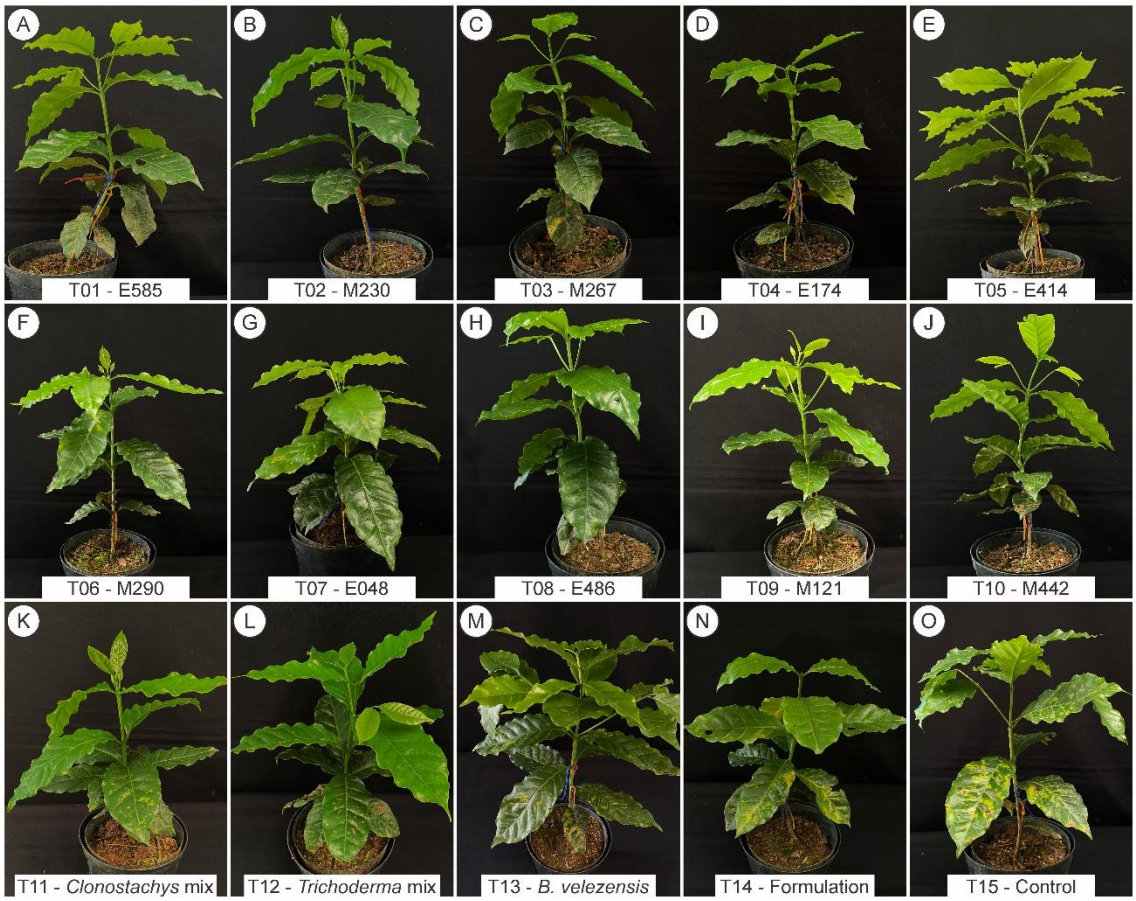

Figure 2. Coffee plants (*C. arabica* cv. 'IAC 144') 90 days after inoculation with *Hemileia vastatrix* and treated with: (A) *Clonostachys rosea* (E585); (B) *C. rhizophaga* (M230); (C) *C. rhizophaga* (M267); (D) *Trichoderma virens* (E174); (E) *T. theobromicola* (E414); (F) *T. harzianum* (M290); (G) *Cordyceps cateniannulata* (E048); (H) *Aspergillus flavus* (E486); (I) *Calonectria hemileiae* (M121); (J) *Fusarium coffeibaccae* (M442); (K) a mix of *Clonostachys* strains (E585 + M230 + M267); (L) a mix of *Trichoderma* strains (E174 + E414 + M290); (M) Commercial product based on *Bacillus velezensis*; (N) formulation; and (O) untreated control.

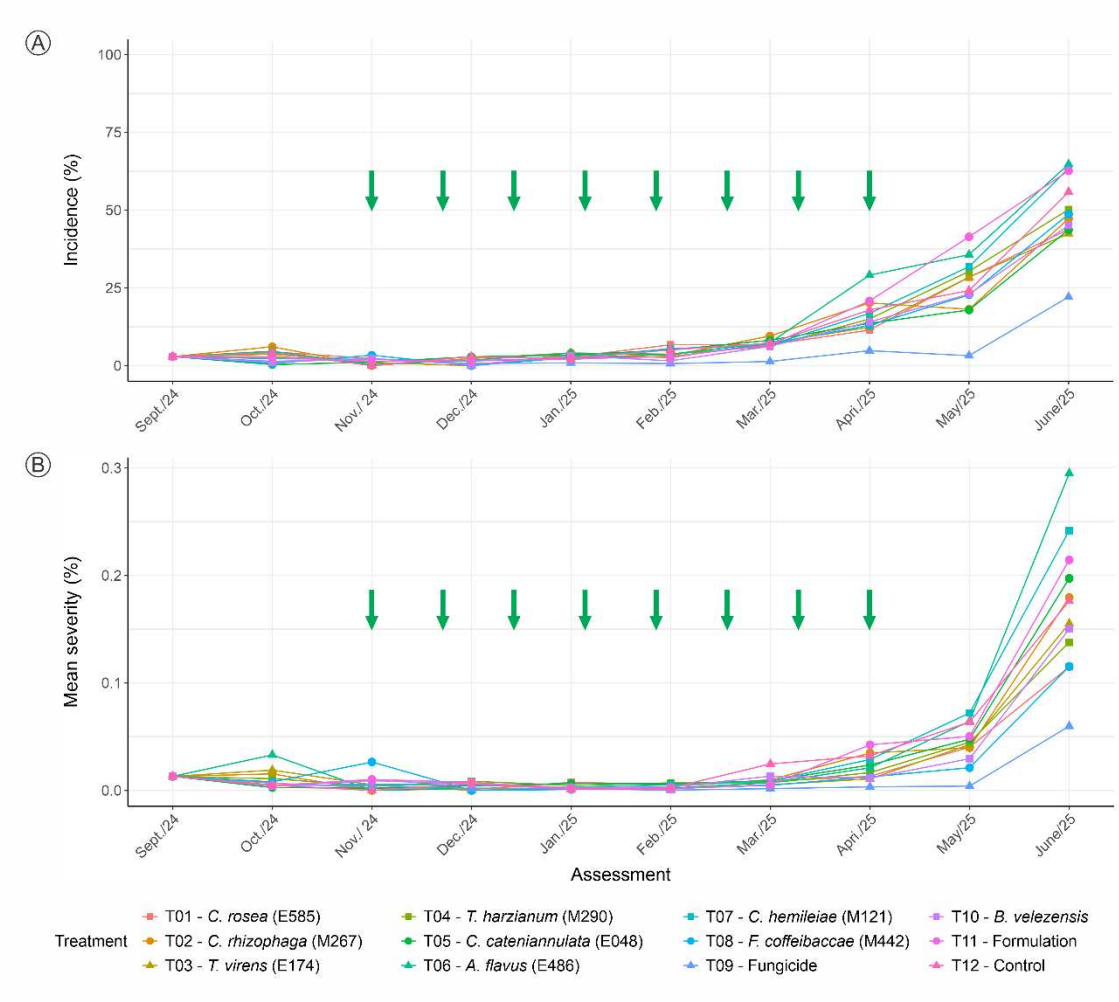

Figure 3. Combined analysis of coffee leaf rust progression in coffee plants (*Coffea arabica* cv. 'IAC 144') treated with fungal antagonists in two experimental fields. (A) Incidence (%) progress curve and (B) mean severity (%). Treatments: T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *Aspergillus flavus* (E486); T07 = *Calonectria hemileiae* (M121); T08 = *Fusarium coffeibaccae* (M442); T09 = Copper oxychloride fungicide; T10 = Commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control. Green arrows indicate the timing of treatments applications.

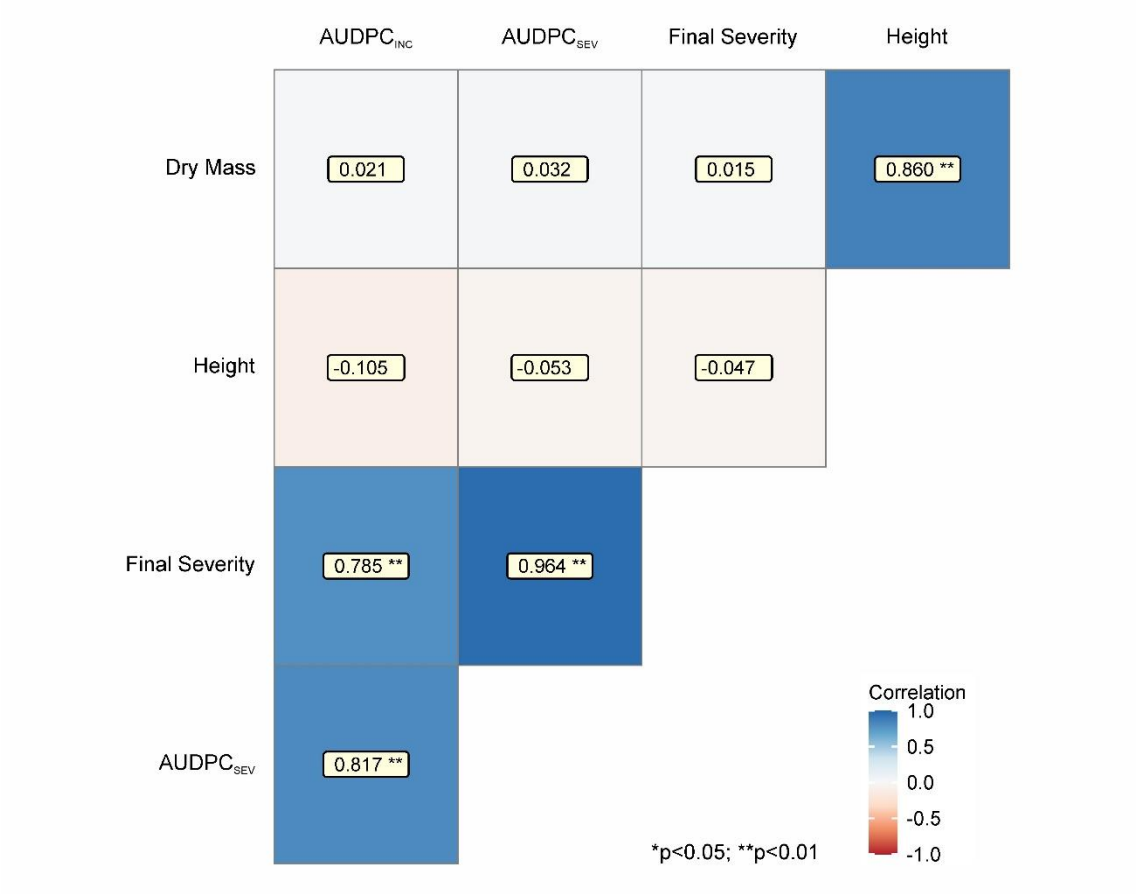

Figure S1. Spearman's correlation matrix among disease-related ($AUDPC_{INC}$, $AUDPC_{SEV}$ and Final Severity) and plant growth variables (Height and Shoot Dry Mass) evaluated in coffee plants treated with fungal antagonists in the potted experiments over two independent experiments (conducted from November 2023 to February 2024 and from November 2024 to February 2025). Correlation coefficients (r) are shown within each cell. Asterisks indicate statistically significant correlations ($p < 0.05$ = *, $p < 0.01$ = **). Color scale represents the strength and direction of correlation (blue = positive, red = negative).

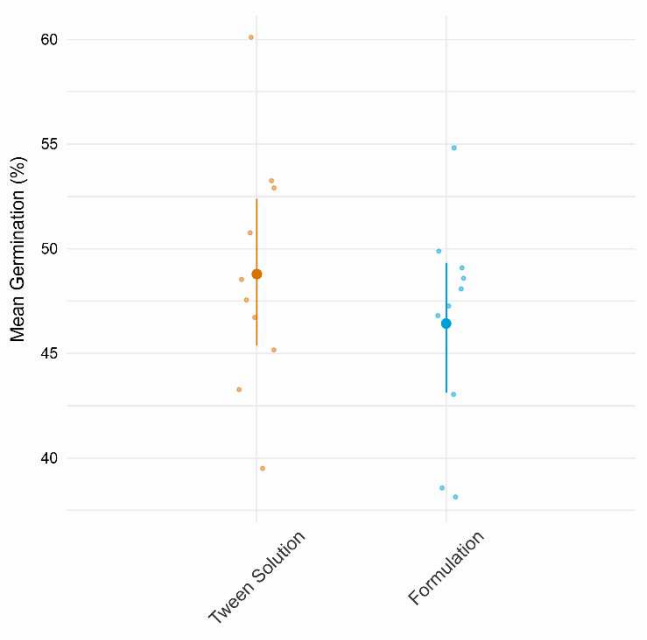

Figure S2. Mean urediniospore germination values (%) of *Hemileia vastatrix* with 95% confidence intervals (error bars) after exposure to a Tween solution (Tween 20, 0.05% v/v) and to the formulation used in the experiments, composed of Tween 20 (0.05% v/v) supplemented with sunflower oil (1% v/v), glycerol (1% v/v), and skim milk (2% m/v). Mean germination rates (Tween solution = 49.4%; formulation = 47.3%) were compared using a t-test, and no statistically significant difference was observed (p = 0. 469).

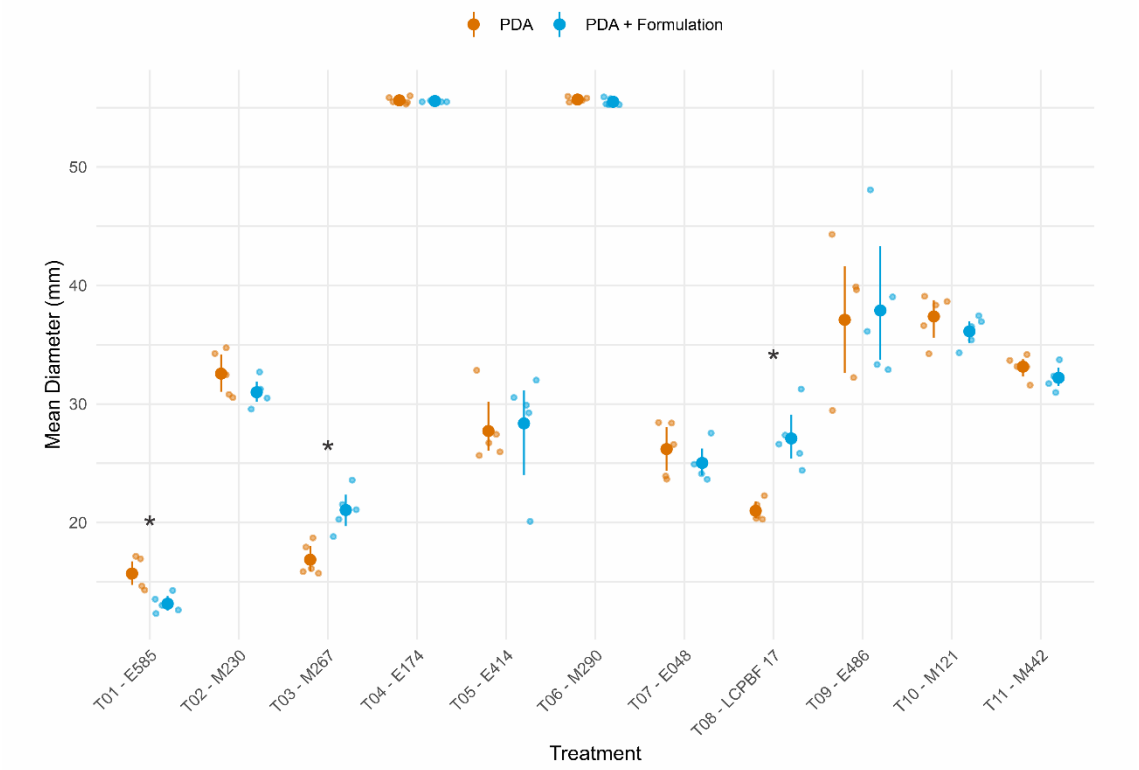

Figure S3. Mean mycelial growth values (mm) of fungal antagonist with 95% confidence intervals (error bars) after 5 days of incubation on PDA or PDA supplemented with the formulation used in the experiments (sunflower oil (1% v/v), glycerol (1% v/v), and skim milk (2% m/v). T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *C. cateniannulata* (LCBPF 17); T09 = *Aspergillus flavus* (E486); T10 = *Calonectria hemileiae* (M121); T11 = *Fusarium coffeibaccae* (M442). Mycelial growth was compared using a t-test; statistically significant differences ($p < 0.01$) are indicated with an asterisk (*).

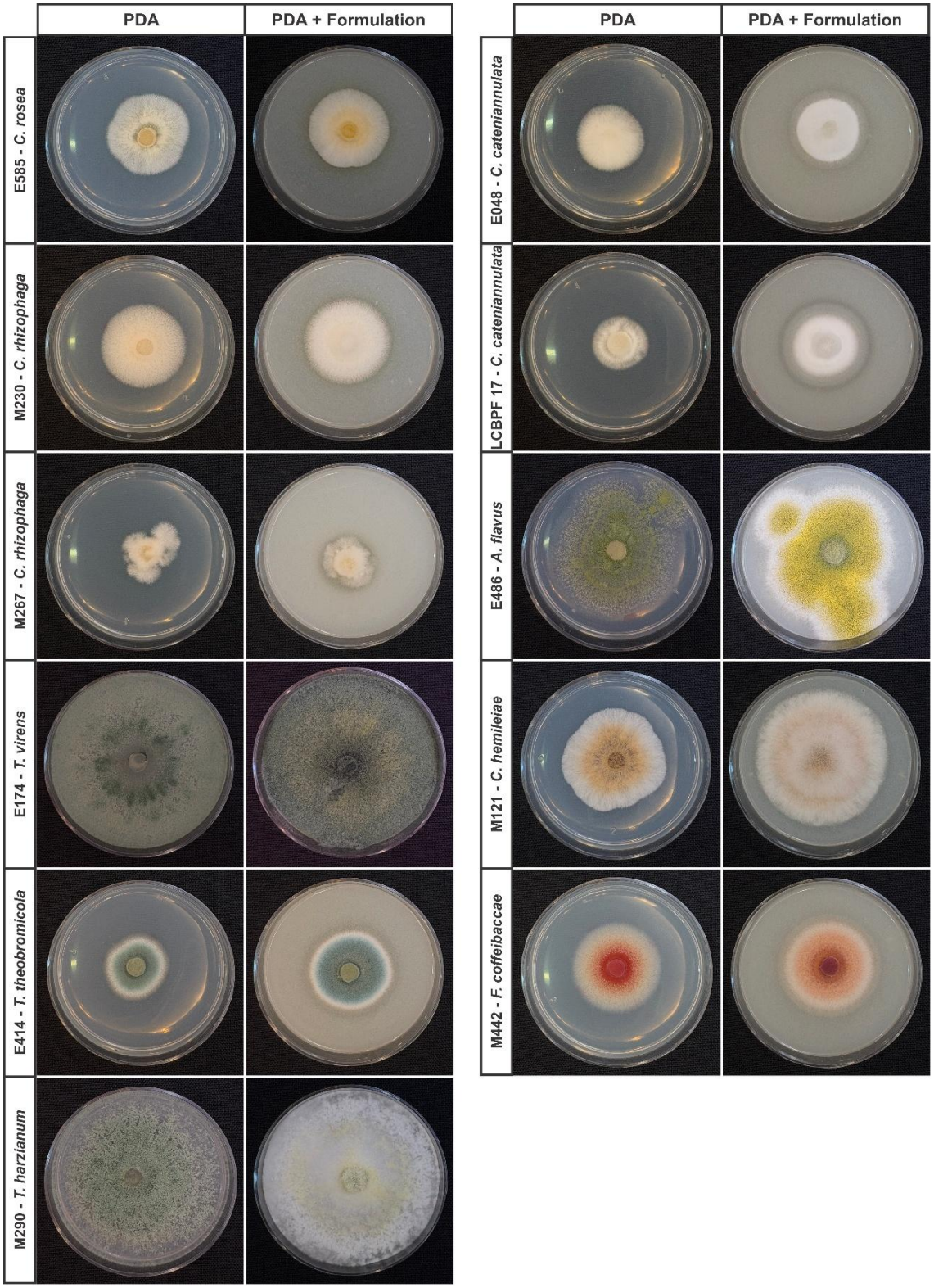

Figure S4. Mycelial growth of fungal antagonists on PDA and PDA supplemented with the formulation (sunflower oil 1% v/v, glycerol 1% v/v, skim milk 2% m/v) after 5 days.

Table 1. Description of the selected isolates assessed against *Hemileia vastatrix* in experiments *in planta* –field conditions.

Species	Strain code	Culture collection code	Origin	Host	Reference
Aspergillus flavus	E486	COAD 3307	Cameroon	Fruits of *C. canephora*	Kapeua-Ndacnou *et al.*[28]
Calonectria hemileiae	M121	COAD 2544	Brazil	Pustules of *H. vastatrix* on *C. arabica* leaves	Salcedo-Sarmiento *et al.*[18]
Clonostachys rosea	E585	COAD 2984	Ethiopia	Stem of *C. arabica*	Kapeua-Ndacnou *et al.*[27]
Clonostachys rhizophaga	M230	COAD 2980	Cameroon	Pustules of *H. vastatrix*/ *coffeicola* on *C. canephora* leaves	Kapeua-Ndacnou *et al.*[27]
Clonostachys rhizophaga	M267	COAD 2982	Cameroon	Pustules of *H. vastatrix*/ *coffeicola* on *C. canephora* leaves	Kapeua-Ndacnou *et al.*[27]
Cordyceps cateniannulata	E048	COAD 3349	Cameroon	Stem of *C. arabica*	Pereira *et al.*[29]
Fusarium coffeibaccae	M442	COAD 3041	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Nóbrega[32]
Trichoderma virens	E174	COAD 2400	Cameroon	Stem of *C. brevipes*	Rodríguez *et al.*[30]
Trichoderma theobromicola	E414	COAD 2406	Cameroon	Stem of *C. canephora*	Rodríguez *et al.*[30]
Trichoderma harzianum	M290	COAD 3672	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Tobar (Personal obs.)

Table 2. Treatments applied to the coffee plants in the pot experiments.

Treatment	Description[1, 2]
T01 – E585	Conidia suspension of *Clonostachys rosea*
T03 – M230	Conidia suspension of *Clonostachys rhizophaga*
T02 – M267	Conidia suspension of *Clonostachys rhizophaga*
T03 – E174	Conidia suspension of *Trichoderma virens*
T05 – E414	Conidia suspension of *Trichoderma theobromicola*
T06 – M290	Conidia suspension of *Trichoderma harzianum*
T07 – E48	Conidia suspension of *Cordyceps cateniannulata*
T08 – E486	Conidia suspension of *Aspergillus flavus*
T09 – M121	Conidia suspension of *Calonectria hemileiae*
T10 – M442	Conidia suspension of *Fusarium coffeibaccae*
T11 – *Clonostachys* Mix	A mix of conidia suspensions of E585, M230 e M267
T12 – *Trichoderma* Mix	A mix of conidia suspensions of E174, M414 e M290
T13 – *B. velezensis*	Commercial product based on *Bacillus velezensis*[3]
T14 – Formulation	Tween 20 solution (0,05 % v/v) + glycerol (1 % v/v) + sunflower oil (1% v/v) and skimmed milk powder (2% m/v)
T15 – Control	Just *H. vastatrix* applied[4]

[1]Conidial suspension calibrated at 5×10^8 conidia/mL (corresponding to an average of 1×10^8 CFU/g of colonized rice).

[2]Spray volume ranged from 15 to 50 mL per plant, depending on plant size. Treatments were applied six times at 10 day-intervals, except for T15, which remained untreated.

[3]The commercial product based on *Bacillus velezensis* was applied at a field rate of 6 L/ha. Considering a spray volume of 400 L/ha, the corresponding concentration was 15 mL of product per litre of spray solution (Bio-Imune®; *Bacillus velezensis* BV02; 3×10^9 CFU/mL; Vittia Group, São Joaquim da Barra, SP, Brazil).

[4]All plants were inoculated with *Hemileia vastatrix* 72 h after the third antagonists' spraying, with a suspension calibrated at 5×10^5 urediniospores/mL.

Table 3. Treatments applied to the coffee plants in the field experiments.

Treatment	Description[1, 2]
T01 – E585	Conidia suspension of *Clonostachys rosea*
T02 – M267	Conidia suspension of *Clonostachys rhizophaga*
T03 – E174	Conidia suspension of *Trichoderma virens*
T04 – M290	Conidia suspension of *Trichoderma harzianum*
T05 – E48	Conidia suspension of *Cordyceps cateniannulata*
T06 – E486	Conidia suspension of *Aspergillus flavus*
T07 – M121	Conidia suspension of *Calonectria hemileiae*
T08 – M442	Conidia suspension of *Fusarium coffeibaccae*
T09 – Fungicide	Copper oxychloride-based fungicide[3]
T10 – *B. velezensis*	Commercial product based on *Bacillus velezensis*[4]
T11 – Formulation	Tween 20 solution (0,05 % v/v) + glycerol (1 % v/v) + sunflower oil (1% v/v) and skimmed milk powder (2% m/v)
T12 – Control	Untreated plants

[1]Conidial suspension calibrated at 5×10^7 conidia/ mL (corresponding to an average of 1×10^7 CFU/g of colonized rice).

[2]Spray volume ranged from 0.3 L to 0.5 L per plant, depending on plant canopy. Treatments were applied every 21 days from November 2024 to April 2025, except for the T12, which remained untreated.

[3]The commercial fungicide based on copper oxychloride (Recop®; 840 g/kg; 50% metallic copper; Albaugh Agro Brasil Ltda., São Paulo, SP, Brazil) was applied at a rate of 3.5 kg/ha. Considering a spray volume of 500 L/ha, the concentration was 7 g of product per litre of spray solution.

[4]The commercial product based on *Bacillus velezensis* (Bio-Imune®; *Bacillus velezensis* BV02; 3×10^9 CFU/mL; Vittia Group, São Joaquim da Barra, SP, Brazil) was applied at a field rate of

6 L/ha. Considering a spray volume of 400 L/ha, the corresponding concentration was 15 mL of product per litre of spray solution.

Table 4. Area under disease progress curve for incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$) of coffee leaf rust, and height and shoot dry mass of coffee plants treated with antagonists in the combined analysis of the pot experiments conducted over two distinct periods.

Treatment[1]	$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]		Height (cm)		Shoot dry mass (g)	
	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]
T01 – E585	101.03 BC	[69.12; 147.67]	5.24 BC	[1.71; 16.02]	25.5 BC	[20.6; 30.5]	4.23 BC	[2.08; 8.58]
T03 – M230	104.1 BCD	[71.3; 151.98]	4.53 ABC	[1.48; 13.87]	25.5 BC	[20.5; 30.4]	3.05 AB	[1.5; 6.2]
T02 – M267	91.59 ABC	[62.43; 134.38]	3.45 ABC	[1.13; 10.55]	24.5 ABC	[19.5; 29.4]	2.67 A	[1.32; 5.42]
T03 – E174	107.24 BCDE	[73.51; 156.44]	4.7 BC	[1.53; 14.37]	26.7 CD	[21.7; 31.7]	3.4 ABC	[1.67; 6.9]
T05 – E414	110.63 BCDE	[75.92; 161.23]	4.87 BC	[1.59; 14.91]	25.5 BC	[20.5; 30.4]	2.94 AB	[1.45; 5.97]
T06 – M290	108.8 BCDE	[74.62; 158.62]	4.07 ABC	[1.33; 12.46]	25.6 BC	[20.6; 30.6]	2.99 AB	[1.47; 6.07]
T07 – E48	113.17 BCDE	[77.71; 164.82]	5.6 C	[1.83; 17.12]	25.1 ABC	[20.1; 30]	3.32 ABC	[1.63; 6.73]
T08 – E486	119.43 CDE	[82.09; 173.74]	5.32 BC	[1.74; 16.29]	23 AB	[18.1; 28]	2.68 A	[1.32; 5.43]
T09 – M121	107.92 BCDE	[74.01; 157.37]	2.91 AB	[0.95; 8.89]	25.4 BC	[20.4; 30.3]	3.57 ABC	[1.76; 7.25]
T10 – M442	70.36 A	[47.3; 104.65]	2.43 A	[0.79; 7.44]	24 ABC	[19.1; 28.9]	3.14 AB	[1.55; 6.38]
T11 – *Clonostachys* Mix	89.19 AB	[60.75; 130.95]	2.87 AB	[0.94; 8.77]	22.2 A	[17.3; 27.1]	3.14 AB	[1.55; 6.37]
T12 – *Trichoderma* Mix	98.94 ABC	[67.65; 144.69]	3.49 ABC	[1.14; 10.69]	26.4 CD	[21.4; 31.3]	4.04 BC	[1.99; 8.19]
T13 – *B. velezensis*	130.98 DE	[90.23; 190.15]	11.43 D	[3.73; 34.98]	29.6 D	[24.6; 34.7]	4.82 C	[2.38; 9.79]
T14 – Formulation	101.82 BC	[69.69; 148.76]	4.49 ABC	[1.47; 13.75]	24.5 ABC	[19.5; 29.4]	3.53 ABC	[1.74; 7.16]
T15 – Control	133.76 E	[92.19; 194.09]	12.05 D	[3.94; 36.88]	29.5 D	[24.5; 34.5]	4.62 C	[2.27; 9.37]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria*

hemileiae (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of *Trichoderma* strains (E174 + E414 + M290); T13 = Commercial product based on *Bacillus velezensis*; T14 = formulation; and T15 = untreated control.

[2]$AUDPC_{INC}$ = area under disease progress curve for incidence.

[3]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[4]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[5]CI = confidence interval, LL = lower limit; UP = upper limit.

Table 5. Combined analysis of final severity and control efficacy of the treatments applied to coffee plants and treated with *H. vastatrix* in potted plants in two distinct experiments.

Treatment[1]	Final Severity (%)		Control Efficacy (%)			
	Mean[2]	95% CI [LL; UL][3]	C[4]	95% CI [LL; UL][3]	C[5]	95% CI [LL; UL][3]
T01 – E585	2.73 AB	[1; 7.44]	50.63	[50.63; 50.74]	-2.63	[-2.9; -2.04]
T03 – M230	2.84 AB	[1.04; 7.73]	48.64	[48.71; 48.77]	-6.77	[-6.92; -6.12]
T02 – M267	1.97 AB	[0.72; 5.37]	64.38	[64.37; 64.53]	25.94	[25.73; 26.53]
T03 – E174	2.56 AB	[0.94; 6.98]	53.71	[53.68; 53.69]	3.76	[3.46; 4.08]
T05 – E414	2.40 AB	[0.88; 6.53]	56.60	[56.65; 56.67]	9.77	[9.68; 10.2]
T06 – M290	2.54 AB	[0.93; 6.91]	54.07	[54.15; 54.19]	4.51	[4.43; 5.1]
T07 – E48	2.98 BC	[1.09; 8.11]	46.11	[46.18; 46.31]	-12.03	[-12.17; -11.22]
T08 – E486	2.59 AB	[0.95; 7.05]	53.16	[53.20; 53.22]	2.63	[2.49; 3.06]
T09 – M121	1.76 AB	[0.65; 4.79]	68.17	[67.98; 68.21]	33.83	[33.67; 33.75]
T10 – M442	1.92 AB	[0.71; 5.25]	65.28	[65.02; 65.16]	27.82	[27.39; 27.55]
T11 – *Clonostachys* Mix	1.48 A	[0.54; 4.04]	73.24	[73.19; 73.40]	44.36	[44.12; 44.9]
T12 – *Trichoderma* Mix	1.98 AB	[0.73; 5.4]	64.20	[64.04; 64.17]	25.56	[25.31; 25.51]
T13 – *B. velezensis*	5.85 D	[2.15; 15.95]	-5.79	[-5.91; -5.84]	-119.92	[-120.61; -119.39]
T14 – Formulation	2.66 AB	[0.98; 7.23]	51.90	[51.72; 52.02]	–	–
T15 – Control	5.53 CD	[2.03; 15.07]	–	–	–	–

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria hemileiae* (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of

Trichoderma strains (E174 + E414 + M290); T13 = Commercial product based on *Bacillus velezensis*; T14 = formulation; and T15 = untreated control.

[2]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[3]CI = confidence interval, LL = lower limit; UP = upper limit.

[4]C = Control efficacy calculated relative to the untreated control (T15).

[5]C = Control efficacy calculated relative to the formulation treatment (T14).

Table 6. Recovery (%) of applied antagonists to coffee plants in the pot experiment during two experiments (2023/ 24 and 2024/25).

Treatment[1]	Year 2 (2023/ 2024)					Year 3 (2024/ 2025)				
	Leaf				Stem	Leaf				Stem
	Lower	Middle	Upper	Apical Bud		Lower	Middle	Upper	Apical Bud	
T01 – E585	14.8	7.4	0.0	0.0	0.0	14.8	3.7	0.0	0.0	0.0
T03 – M230	14.8	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T02 – M267	14.8	7.4	7.4	7.4	0.0	0.0	3.7	0.0	0.0	0.0
T03 – E174	18.5	0.0	18.5	18.5	0.0	3.7	7.4	18.5	3.7	7.4
T05 – E414	0.0	0.0	0.0	0.0	0.0	22.2	18.5	7.4	3.7	0.0
T06 – M290	88.9	85.2	51.9	3.7	0.0	70.4	59.3	29.6	18.5	11.1
T07 – E48	22.2	11.1	11.1	11.1	0.0	14.8	0.0	0.0	3.7	11.1
T08 – E486	29.6	22.2	3.7	0.0	14.8	100.0	100.0	96.3	100.0	7.4
T09 – M121	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T10 – M442	7.4	0.0	0.0	0.0	7.4	0.0	7.4	3.7	3.7	5.6
T11 – *Clonostachys* Mix	0.0	0.0	0.0	0.0	0.0	3.7	11.1	7.4	0.0	0.0
T12 – *Trichoderma* Mix	92.6	85.2	81.5	3.7	3.7	37.0	22.2	18.5	0.0	3.7

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria hemileiae* (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of *Trichoderma* strains (E174 + E414 + M290).

Table 7. Values of the area under the disease progress curve of incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$) combining the results from the experiment in two experimental coffee fields.

Treatment[1]	$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]	
	Mean[4]	95% CI [LL, UL][5]	Mean[4]	95% CI [LL, UL][5]
T01 – E585	26.9 A	[12.3; 58.8]	0.05 A	[0.021; 0.12]
T02 – M267	65.8 AB	[32.4; 133.5]	0.114 AB	[0.05; 0.26]
T03 – E174	68.5 AB	[33.9; 138.4]	0.126 AB	[0.056; 0.285]
T04 – M290	69.9 AB	[34.6; 141.2]	0.128 AB	[0.057; 0.289]
T05 – E048	71.4 AB	[35.5; 143.8]	0.147 AB	[0.066; 0.332]
T06 – E486	71.6 AB	[35.4; 144.5]	0.148 AB	[0.066; 0.332]
T07 – M121	72.1 AB	[35.7; 145.6]	0.159 AB	[0.071; 0.355]
T08 – M442	79.6 AB	[39.8; 159.2]	0.16 AB	[0.072; 0.356]
T09 – Fungicide	85.5 AB	[42.9; 170.2]	0.188 B	[0.085; 0.416]
T10 – *B. velezensis*	90.3 B	[45.5; 179.3]	0.22 B	[0.1; 0.482]
T11 – Formulation	108 B	[55; 212.1]	0.231 B	[0.105; 0.505]
T12 – Control	112.2 B	[57.3; 219.7]	0.248 B	[0.114; 0.54]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *Aspergillus flavus* (E486); T07 = *Calonectria hemileiae* (M121); T08 = *Fusarium coffeibaccae* (M442); T09 = Commercial based fungicide base on copper oxychloride; T10 = commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

[2]$AUDPC_{INC}$ = area under disease progress curve for incidence.

[3]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[4]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[5]CI = confidence interval, LL = lower limit; UP = upper limit.

Table 8. Combined analysis of final severity and control efficacy of the treatments applied to coffee plants in the assay in two experimental fields.

Treatment[1]	Final Severity (%)		Control Efficacy (%)			
	Mean[2]	95% CI [LL; UL][3]	C[4]	95% CI [LL; UL][3]	C[5]	95% CI [LL; UL][3]
T01 – E585	0.109 AB	[0.053; 0.223]	29.22	[29.20; 29.17]	49.37	[49.24; 49.54]
T02 – M267	0.14 AB	[0.068; 0.288]	9.09	[8.57; 9.46]	34.97	[34.84; 35.12]
T03 – E174	0.133 AB	[0.065; 0.273]	13.80	[13.33; 14.06]	38.34	[38.23; 38.41]
T04 – M290	0.13 AB	[0.064; 0.266]	15.61	[15.55; 15.50]	39.64	[39.45; 39.81]
T05 – E048	0.155 AB	[0.075; 0.317]	-0.06	[-0.63; 0.26]	28.42	[28.28; 28.53]
T06 – E486	0.284 B	[0.139; 0.579]	-83.41	[-83.80; -83.44]	-31.19	[-31.45; -30.99]
T07 – M121	0.238 AB	[0.116; 0.489]	-53.93	[-55.23; -52.82]	-10.1	[-10.63; -9.51]
T08 – M442	0.121 AB	[0.059; 0.249]	21.41	[20.95; 21.81]	43.79	[43.66; 43.97]
T09 – Fungicide	0.078 A	[0.038; 0.161]	49.41	[48.88; 49.80]	63.82	[63.57; 64.03]
T10 – *B. velezensis*	0.15 AB	[0.073; 0.308]	2.90	[2.22; 3.41]	30.54	[30.31; 30.79]
T11 – Formulation	0.216 AB	[0.106; 0.442]	-39.80	[-40.31; -39.55]	–	–
T12 – Control	0.155 AB	[0.076; 0.315]	–	–	–	–

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *Aspergillus flavus* (E486); T07 = *Calonectria hemileiae* (M121); T08 = *Fusarium coffeibaccae* (M442); T09 = Commercial based fungicide base on copper oxychloride; T10 = commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

[2]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[3]CI = confidence interval, LL = lower limit; UP = upper limit.

[4]C = Control efficacy calculated relative to the untreated control (T15).

[5]C = Control efficacy calculated relative to the formulation treatment (T14).

Supplementary Table 1. Area under disease progress curve for severity ($AUDPC_{SEV}$), final severity, and height of coffee plants treated with antagonists in the first year (pilot study) of the pot experiment.

Treatment[1]	$AUDPC_{SEV}$[2]		Final Severity (%)		Height (cm)	
	Mean[3]	95% CI [LL; UL][4]	Mean[3]	95% CI [LL; UL][4]	Mean [NS]	95% CI [LL; UL][4]
T01 - E585	4.94 BCD	[2.07; 11.01]	2.04 AB	[0.63; 5.25]	37.1	[33.7; 40.4]
T02 - M230	2.74 BC	[1.03; 6.35]	1.88 AB	[0.55; 4.88]	36.5	[33.1; 39.8]
T03 - M267	2.36 AB	[0.85; 5.56]	1.42 A	[0.35; 3.85]	37.1	[33.7; 40.5]
T04 - E174	2.83 BC	[1.07; 6.55]	1.98 AB	[0.6; 5.1]	35.6	[32.3; 39]
T05 - E414	2.42 AB	[0.88; 5.67]	0.96 A	[0.14; 2.8]	34.4	[31; 37.8]
T06 - M290	5.76 CD	[2.46; 12.74]	2.64 ABC	[0.89; 6.59]	31.8	[28.5; 35.2]
T07 - E048	0.93 A	[0.18; 2.54]	0.95 A	[0.14; 2.78]	37.3	[34; 40.7]
T08 - E486	4.73 BCD	[1.97; 10.57]	2.27 AB	[0.73; 5.75]	34.7	[31.3; 38]
T09 - M121	3.95 BC	[1.6; 8.92]	1.54 A	[0.4; 4.11]	34.3	[30.9; 37.7]
T10 - M442	2.47 ABC	[0.9; 5.8]	1.56 A	[0.41; 4.15]	33.5	[30.1; 36.8]
T11 - *B. velezensis*	9.3 DE	[4.13; 20.22]	4.69 BCD	[1.8; 11.21]	35.2	[31.7; 38.7]
T12 - Formulation	4.72 BCD	[1.97; 10.54]	2.28 AB	[0.73; 5.77]	36.3	[32.9; 39.6]
T13 - Water + Tween	18 E	[8.25; 38.62]	7.92 D	[3.23; 18.49]	33.8	[30.5; 37.2]
T14 - Control	9.38 DE	[4.17; 20.39]	6.34 CD	[2.53; 14.93]	35.3	[32; 38.7]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria*

hemileiae (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = Commercial product based on *Bacillus velezensis*; T12 = formulation; T13 = 0.05% (v/v) Tween 20 solution; and T14 = untreated control.

[2]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[3]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[4]CI = confidence interval, LL = lower limit; UP = upper limit.

[NS] Means do not significantly differ by Tukey's multiple range test at $P > 0.05$.

Supplementary Table 2. Area under disease progress curve for incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$), final severity, height and shoot dry mass of coffee plants treated with antagonists in the second year of the pot experiment.

Treatment[1]	$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]		Final Severity (%)		Height (cm)		Shoot dry mass (g)	
	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]
T01 – E585	98.4 BCD	[67; 129.8]	2.75 BC	[1.94; 3.91]	1.011 ABC	[0.54; 1.68]	29.4 BC	[27.4; 31.4]	5.8 B	[4.97; 6.62]
T03 – M230	80.2 ABC	[48.7; 111.6]	2.64 BC	[1.86; 3.74]	1.285 BC	[0.73; 2.08]	28.2 ABC	[26.2; 30.3]	4.87 AB	[4.04; 5.7]
T02 – M267	94.8 BCD	[63.4; 126.3]	2.71 BC	[1.91; 3.84]	1.123 BC	[0.62; 1.84]	29.4 BC	[27.4; 31.4]	4.97 AB	[4.14; 5.8]
T03 – E174	85.4 ABCD	[54; 116.8]	2.26 ABC	[1.59; 3.2]	0.771 ABC	[0.37; 1.33]	31.9 CD	[29.9; 33.9]	6.58 B	[5.75; 7.4]
T05 – E414	79.8 ABC	[48.3; 111.2]	2.58 BC	[1.82; 3.66]	0.887 ABC	[0.45; 1.5]	28.2 ABC	[26.2; 30.3]	4.66 AB	[3.83; 5.49]
T06 – M290	66.6 AB	[35.2; 98.1]	1.63 AB	[1.15; 2.31]	0.648 AB	[0.29; 1.16]	26.1 AB	[24.1; 28.2]	4.73 AB	[3.9; 5.55]
T07 – E48	82.2 ABC	[50.8; 113.6]	2.23 ABC	[1.57; 3.17]	0.724 AB	[0.34; 1.27]	27.6 AB	[25.6; 29.6]	5.08 AB	[4.26; 5.91]
T08 – E486	93.9 BCD	[62.5; 125.3]	2.9 C	[2.04; 4.13]	0.955 ABC	[0.5; 1.61]	26.4 AB	[24.4; 28.5]	4.51 AB	[3.68; 5.33]
T09 – M121	73.8 ABC	[42.4; 105.2]	1.61 AB	[1.13; 2.28]	0.58 AB	[0.24; 1.06]	28.4 ABC	[26.4; 30.4]	5.34 AB	[4.52; 6.17]
T10 – M442	69.1 AB	[37.7; 100.6]	2.2 ABC	[1.55; 3.12]	1.191 BC	[0.66; 1.94]	28.6 BC	[26.5; 30.6]	5.13 AB	[4.31; 5.96]
T11 – *Clonostachys* Mix	60.2 A	[28.8; 91.6]	1.32 A	[0.93; 1.87]	0.351 A	[0.08; 0.73]	24.6 A	[22.5; 26.6]	3.5 A	[2.68; 4.33]
T12 – *Trichoderma* Mix	68.3 AB	[36.9; 99.7]	1.6 AB	[1.13; 2.27]	0.504 AB	[0.19; 0.95]	29.3 BC	[27.3; 31.3]	6.27 B	[5.44; 7.1]
T13 – *B. velezensis*	102.3 CD	[70.8; 133.7]	5.85 D	[4.12; 8.32]	1.773 CD	[1.07; 2.79]	33.8 D	[31.8; 35.8]	10.02 C	[9.19; 10.84]
T14 – Formulation	76.4 ABC	[45; 107.8]	2.54 BC	[1.79; 3.61]	1.169 BC	[0.65; 1.91]	28.7 BC	[26.7; 30.7]	6.15 B	[5.32; 6.97]
T15 – Control	116.9 D	[85.5; 148.4]	7.36 D	[5.19; 10.44]	2.813 D	[1.78; 4.29]	34.5 D	[32.5; 36.6]	9.2 C	[8.37; 10.03]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria hemileiae* (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of *Trichoderma* strains (E174 + E414 + M290); T13 = Commercial product based on *Bacillus velezensis*; T14 = formulation; and T15 = untreated control.

[2]$AUDPC_{INC}$ = area under disease progress curve for incidence.

[3]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[4]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[5]CI = confidence interval, LL = lower limit; UP = upper limit.

Supplementary Table 3. Area under disease progress curve for incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$), final severity, height and shoot dry mass of coffee plants treated with antagonists in the third year of the pot experiment.

Treatment[1]	$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]		Final Severity (%)		Height (cm)		Shoot dry mass (g)	
	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]
T01 – E585	117.7 ABC	[97.1; 138]	12.22 CDE	[8.41; 17.75]	3.33 ABC	[2.09; 5.09]	21.4 ABC	[19.1; 23.9]	3 C	[2.57; 3.43]
T03 – M230	134.9 BCD	[114.4; 155]	9.75 BCD	[6.72; 14.15]	3.45 ABC	[2.18; 5.26]	22.2 ABC	[19.8; 24.7]	1.93 ABC	[1.5; 2.36]
T02 – M267	103.6 AB	[83.1; 124]	4.52 AB	[3.12; 6.56]	1.64 AB	[0.91; 2.67]	20 AB	[17.7; 22.3]	1.41 A	[0.98; 1.85]
T03 – E174	141.3 BCD	[120.7; 162]	11.96 CDE	[8.23; 17.37]	3.93 BC	[2.51; 5.94]	21.9 ABC	[19.6; 24.4]	1.71 AB	[1.28; 2.14]
T05 – E414	148.1 BCD	[127.6; 169]	11.45 CDE	[7.89; 16.62]	3.68 BC	[2.34; 5.59]	22 ABC	[19.6; 24.5]	1.84 AB	[1.41; 2.27]
T06 – M290	155.1 CD	[134.5; 176]	12.33 CDE	[8.51; 17.88]	5.45 C	[3.58; 8.11]	23.6 ABC	[21.1; 26.1]	1.89 AB	[1.45; 2.32]
T07 – E48	151.2 BCD	[130.6; 172]	15.83 CDE	[10.91; 22.96]	5.58 C	[3.67; 8.29]	22 ABC	[19.7; 24.5]	2.18 ABC	[1.74; 2.61]
T08 – E486	153.7 BCD	[133.2; 174]	11.74 CDE	[8.1; 17.01]	4.05 BC	[2.6; 6.11]	19.5 AB	[17.3; 21.8]	1.6 AB	[1.17; 2.03]
T09 – M121	143.6 BCD	[123; 164]	7.36 BC	[5.07; 10.68]	2.78 ABC	[1.71; 4.31]	21.7 ABC	[19.4; 24.2]	2.38 ABC	[1.95; 2.81]
T10 – M442	81.3 A	[60.8; 102]	2.57 A	[1.77; 3.73]	1.04 A	[0.49; 1.83]	19.8 AB	[17.5; 22.2]	1.93 ABC	[1.5; 2.36]
T11 – *Clonostachys* Mix	120.2 ABC	[99.6; 141]	8.73 BCD	[6.02; 12.65]	3.1 ABC	[1.93; 4.76]	19.2 A	[17; 21.5]	2.53 BC	[2.1; 2.96]
T12 – *Trichoderma* Mix	135.2 BCD	[114.6; 156]	10.15 BCDE	[7; 14.71]	3.74 BC	[2.38; 5.68]	23.1 ABC	[20.7; 25.7]	2.6 BC	[2.17; 3.03]
T13 – *B. velezensis*	175.7 D	[155.2; 196]	23.89 E	[16.46; 34.67]	7.44 C	[4.97; 10.94]	25.4 C	[22.9; 28.1]	2.15 ABC	[1.71; 2.58]
T14 – Formulation	135 BCD	[114.5; 156]	10.3 BCDE	[7.08; 14.98]	3.97 BC	[2.55; 6.01]	20.4 ABC	[18.1; 22.8]	2.01 ABC	[1.58; 2.45]
T15 – Control	167.4 CD	[146.9; 188]	19.97 DE	[13.75; 29.01]	6.08 C	[4.02; 9]	24.6 BC	[22.1; 27.2]	2.22 ABC	[1.79; 2.65]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *Aspergillus flavus* (E486); T09 = *Calonectria hemileiae* (M121); T10 = *Fusarium coffeibaccae* (M442); T11 = a mixture of *Clonostachys* strains (E585 + M230 + M267); T12 = a mixture of *Trichoderma* strains (E174 + E414 + M290); T13 = Commercial product based on *Bacillus velezensis*; T14 = formulation; and T15 = untreated control.

[2]$AUDPC_{INC}$ = area under disease progress curve for incidence.

[3]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[4]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[5]CI = confidence interval, LL = lower limit; UP = upper limit.

Supplementary Table 4. Values of the area under the disease progress curve of incidence ($AUDPC_{INC}$) and severity ($AUDPC_{SEV}$) of the assay in coffee two experimental fields.

Treatment	Experimental Field 1				Experimental Field 2			
	$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]		$AUDPC_{INC}$[2]		$AUDPC_{SEV}$[3]	
	Mean[4]	95% CI [LL; UL][5]	Mean[4]	95% CI [LL; UL][5]	Mean [NS]	95% CI [LL; UL][5]	Mean [NS]	95% CI [LL; UL][5]
T01 – E585	119 B	[74.6; 189.8]	0.229 B	[0.134; 0.389]	11.8	[2.32; 57]	0.047	[0.019; 0.117]
T02 – M267	118.5 B	[74.3; 189]	0.335 B	[0.197; 0.568]	10.9	[2.13; 52.7]	0.03	[0.011; 0.077]
T03 – E174	94 B	[58.9; 149.9]	0.239 B	[0.139; 0.409]	22.5	[4.56; 107.9]	0.057	[0.023; 0.141]
T04 – M290	100.2 B	[62.8; 159.8]	0.205 AB	[0.12; 0.351]	22.2	[4.51; 106.7]	0.128	[0.055; 0.298]
T05 – E048	104.4 B	[65.4; 166.5]	0.312 B	[0.183; 0.528]	13.9	[2.75; 66.9]	0.065	[0.027; 0.16]
T06 – E486	149 B	[93.4; 237.6]	0.386 B	[0.229; 0.65]	63.1	[13.08; 301.6]	0.167	[0.073; 0.381]
T07 – M121	121.5 B	[76.2; 193.8]	0.329 B	[0.194; 0.559]	29.6	[6.04; 141.6]	0.174	[0.076; 0.397]
T08 – M442	89.8 B	[56.3; 143.3]	0.171 AB	[0.098; 0.296]	44.4	[9.16; 212.3]	0.103	[0.043; 0.243]
T09 – Fungicide	19.7 A	[12.3; 31.4]	0.065 A	[0.036; 0.115]	11.1	[2.18; 53.8]	0.035	[0.014; 0.091]
T10 – *B. velezensis*	94.2 B	[59.1; 150.2]	0.172 AB	[0.1; 0.295]	39.6	[8.15; 189.5]	0.134	[0.058; 0.311]
T11 – Formulation	114.3 B	[71.6; 182.2]	0.248 B	[0.145; 0.422]	80	[16.63; 382.2]	0.196	[0.087; 0.444]
T12 – Control	145.8 B	[91.4; 232.5]	0.342 B	[0.202; 0.576]	24.4	[4.96; 116.9]	0.093	[0.039; 0.221]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *Aspergillus flavus* (E486); T07 = *Calonectria hemileiae* (M121); T08 = *Fusarium coffeibaccae* (M442); T09 =

Commercial based fungicide base on copper oxychloride; T10 = commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

[2]$AUDPC_{INC}$ = area under disease progress curve for incidence.

[3]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[4]Values followed by the same letter are not significantly different by Tukey's multiple range test at $P > 0.05$.

[5]CI = confidence interval, LL = lower limit; UP = upper limit.

[NS] Means do not significantly differ by Tukey's multiple range test at $p < 0.05$.

Chapter 2

Mycoparasites of *Hemileia vastatrix* (coffee leaf rust) may also be antagonists of other rust fungi

Prepared according to the format of the Brazilian Journal of Microbiology

Mycoparasites of *Hemileia vastatrix* (coffee leaf rust) may also be antagonists of other rust fungi

Caio Mattos Pereira[1], Emanuelle Barboza Rozaes[1], Sofia Sampaio Sumam Silva[1], Sara Rocha Foletto[1], Eduardo Côrtes de Araujo Furtado[1], Robert Weingart Barreto[1*]

[1]Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570–900, Brazil.

*Corresponding author: rbarreto@ufv.br

Abstract

Rust fungi (Pucciniales) are among the most devastating plant pathogens worldwide, posing serious threats to agriculture and natural ecosystems. In this study, we evaluated whether fungal isolates originally selected for their antagonistic activity against *Hemileia vastatrix* could also inhibit the germination of urediniospores from four other rust pathogens: *Austropuccinia psidii* (myrtle rust), *Phakopsora pachyrhizi* (Asian soybean rust), *Puccinia recondita* (wheat leaf rust), and *Uromyces appendiculatus* (bean rust). Urediniospores of each pathogen were exposed to conidial suspensions of eleven fungal isolates under *in vitro* conditions. Several antagonists significantly reduced spore germination, with the most consistent and effective inhibition observed for *Fusarium coffeibaccae* (M442) and two isolates of *Cordyceps cateniannulata* (E48 and LCBPF 17). These isolates achieved mean inhibition rates of up to 51%, depending on the isolate-pathogen combination. A combined analysis confirmed their broad-spectrum potential across all rust species tested. Although *in vitro* assays are conducted under simplified and controlled conditions, they are a valuable preliminary step for identifying promising biocontrol candidates; however, further validation under greenhouse and field conditions remains indispensable. Our results suggest that *H. vastatrix* antagonists may represent a valuable source of broad-spectrum biocontrol agents for managing multiple rust diseases in a sustainable manner.

Keywords: Fungal antagonists · Urediniospore germination inhibition · Biological control · Broad-spectrum · Pucciniales

1. Introduction

Pucciniales is the largest group of fungal plant pathogens, encompassing biotrophic basidiomycetes with diverse life cycle and host ranges – the rust fungi [1,2]. These fungi are both environmentally and economically important, being the cause of some of the most serious losses to agriculture, forestry, and ecosystems worldwide [3,4]. Members of *Pucciniales* cause diseases on numerous significant plants, such as wheat, maize, oat, soybean, bean, coffee, apple and even entire plant families, such as the Myrtaceae [5].

Rusts are among the most feared plant diseases, threatening global food security, national economies, and entire ecosystems. Examples of devastating epidemics caused by rust fungi include the wheat stem rust "race UG99" (*Puccinia graminis* f. sp. *tritici*), the myrtle rust (*Austropuccinia psidii*), and the coffee rust (*Hemileia vastatrix*) – the latter responsible for a devastating outbreak, starting in 1895 in Ceylon (now Sri Lanka), and again between 2008 and 2015 across Central and South Americas [1,4,5].

The most recent wave of coffee leaf rust (CLR) epidemics, termed as “The Big Rust” [6], had a severe impact on coffee production throughout northern South America and Central America, generating major economic, environmental, and social impact. Traditional CLR management strategies, such as the use of resistant cultivars, cultivation in rust-free areas (mainly above 1,000 m), and fungicides spraying are faced with increasing limitations. Thus, alternative approaches for coffee protection against CLR must be explored, including biological control [7].

In this context, a project launched in 2015 aimed at identifying potential biocontrol agents for CLR management. More than 1,500 fungal isolates were obtained from African countries (Cameroon and Ethiopia) and South America countries (Brazil and Paraguay). Following an initial *in vitro* screening, the collection was narrowed from approximately 600 potentially interesting isolates to 10 selected candidates, which were evaluated against *H. vastatrix* under greenhouse conditions. The most promising isolates were subsequently tested under field conditions (results published separately). Here we preliminarily tested the conjecture that the previously selected isolates in the CLR biocontrol studies might also have biocontrol potential against other rust species.

Wheat, soybean, and bean are major staple food crops worldwide [8]. These highly important crops are attacked by numerous fungal pathogens, including, among their worst pathogens the rust fungi *Puccinia recondita* (syn. *P. triticina* – wheat leaf rust), *Phakopsora*

pachyrhizi (Asian soybean rust), and *Uromyces appendiculatus* (bean rust), respectively. Each of these are capable of causing severe damage to their hosts under favorable conditions [9–12]. *Austropuccinia psidii* (myrtle rust) is a globally invasive neotropical rust of the Myrtaceae that came to international prominence due to the threat it poses to native ecosystems in Hawaii, Australia, New Zealand, and other regions [13], besides being the worst pathogen of guava and also important for many cultivated eucalypt species and cultivated hybrids [14]. Given the relevance of these pathogens, and based on some promising results previously obtained on the use of fungal antagonists against CLR [15–17], we conducted preliminary experiments towards assessing the potential of fungal antagonists of *H. vastatrix* at inhibiting the germination of urediniospores of *A. psidii*, *P. pachyrhizi*, *P. recondita*, and *U. appendiculatus* in *in vitro* assays.

2. Material and methods

2.1. Origin of isolates

The fungal antagonists (Table 1) used in the following assays were obtained as described by Rodríguez *et al.*[18]. In summary, these isolates were classified according to their source: endophytic fungi – obtained from healthy coffee tissues leaves, stems, and fruits – and mycoparasitic fungi – isolated from *H. vastatrix* uredinia showing fungal overgrowth or abnormal appearance. Only isolates of species known to occur in Brazil or directly obtained from Brazil were included in this study. All antagonists are kept at −85 ºC in the "Coleção Octávio Almeida Drummond" (COAD) culture collection at the Universidade Federal de Viçosa (UFV).

2.2. Inoculum preparation

Before inoculum preparation, selected fungal isolates were recovered from the culture collection. From cryovials, a small fungal sample of each isolate was collected and transferred to the center of plates containing potato dextrose-agar (PDA). Plates were left at 25 ± 2 ºC under a 12-hour photoperiod for seven days. Colonies were checked for purity and then transferred to fresh PDA plates, which were maintained under the same conditions. These plates were used as seeds for bulk inoculum production.

Bulk inoculum production was conducted as follows: portions of 100 g of parboiled rice, supplemented with 1 g of $CaCO_3$, and hydrated with 60 mL of deionized water, were placed in baking trays (30 × 20 cm), enclosed in polypropylene bags (60 × 40 cm), and autoclaved for 40 minutes at 1.0 atm and 121 ºC. The wrapped trays with rice were left to cool at room temperature for at least 12 hours. The rice on each tray was then seeded, under aseptic conditions in a laminar flow cabinet, with ten mycelial plugs (10 × 10 mm) taken from the margins of sporulating colonies. The inoculated material was then transferred to benches in a controlled-temperature room (25 ± 2 °C, 12-hour photoperiod) under fluorescent (25 W – 127 V) and black light (28 W – 127 V) lamps positioned 35 cm above the bench. The material was maintained under these conditions for seven days. During this period the rice was stirred daily, and the material was gently pressed through the polypropylene bag in order to loosen the aggregates and ensure homogeneous colonization. Once rice was fully colonized, the baking trays were removed from polypropylene bags and transferred to white paper lunch bags (7.5 kg), and left to dry under the same room conditions for an additional five days, until reaching a

moisture content of 15 ± 2%. Finally, the material was stored in new polypropylene bags at 4 ± 2 ºC until use, for up to a maximum of 30 days.

Estimation of the concentration of conidia per gram of colonized rice was performed as follows: 5 g of substrate containing each fungal isolate were weighed and transferred to a beaker containing 50 mL of 0.05% (v/v) Tween 20 solution in tap water. The mixture was homogenized using a rotary blender (MA 102, Marconi, Piracicaba, Brazil) until complete blending. The suspension was filtered sequentially through a household sieve and a layer of voile fabric. Conidial concentration in the resulting suspension were quantified with the help of a Neubauer counting chamber, and the total concentration per gram of substrate was calculated accordingly. At the time of the experiment setup, an amount of colonized rice was weighed to prepare a conidial suspension adjusted to 5×10^5 conidia/mL (corresponding to an average of 1×10^5 colony-forming units per gram of colonized rice) for each fungal antagonist. The colonized substrate was blended and filtered as described earlier. However, for the *in vitro* assays, suspensions were prepared using sterile 0.05% (v/v) Tween 20 solution in deionized water instead of tap water.

2.3. *In vitro* effect of fungal antagonists on the germination of selected rust fungi

In vitro tests were conducted to assess the ability of fungal antagonists to inhibit the germination of the following rust species: *Austropuccinia psidii* (myrtle rust), *Puccinia recondita* (wheat leaf rust), *Phakopsora pachyrhizi* (Asian soybean rust), and *Uromyces appendiculatus* (bean rust).

Urediniospore germination was evaluated by following a methodology adapted from Reis *et al.* [19] and Blum *et al.* [20]. Observation of rust germination was conducted on plates which contained special media which were prepared as follows: 5 g of fresh, healthy leaves from each rust host (myrtle rust: *Syzygium jambos*; wheat leaf rust: *Triticum aestivum*; soybean rust: *Glycine max*; and bean rust: *Phaseolus vulgaris*) were macerated in a domestic blender with 1,000 mL of deionized water. The resulting leaf juice was filtered through a household sieve, and 15 g of agar was added to each diluted leaf juice. This was then autoclaved for 20 minutes and poured into 90-mm plates. Germination of urediniospores of each rust species was evaluated on plates containing media based on leaves of their original host.

Urediniospores of each pathogen were obtained from different sources (Fig. 1) and harvesting periods. For *A. psidii*, urediniospores were collected in May 2025 from rose apple plants (*Syzygium jambos*), inoculated for use in ongoing experiments conducted in our lab by another group of researchers. In that case the rust spores were produced on plants kept in a growth room under controlled conditions (22 ± 2 °C, 12-hour photoperiod). Urediniospores of *P. recondita* were collected from plants belonging to an experimental wheat (*Triticum aestivum*) plot cultivated at the "Unidade de Ensino, Pesquisa e Extensão do Vale da Agronomia" by the wheat breeding program of UFV between August and October 2024. Urediniospores of *P. pachyrhizi* and *U. appendiculatus* were obtained from naturally infected soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) plants, respectively, maintained for teaching purposes in plots at the Infectarium of UFV (https://www.infectario.ufv.br/), between June and August 2024.

Diseased leaves were collected and brought to the laboratory. Uredinia were inspected for the possible presence of mycoparasites and only contamination-free samples were used. Leaf fragments bearing uredinia were then placed into a 500 mL laboratory media bottle containing 250 mL of a sterile 0.05% (v/v) Tween 20 solution in deionized water. Each bottle was shaken in order to release the spores [19], and the resulting suspension was adjusted to 5×10^5 urediniospores/mL with the help of a Neubauer chamber.

Fungal antagonists spores (treatments) and rust urediniospores were mixed at a 1:1 ratio in sterile 1.5 mL microtubes. Aliquots of 30 µL from each mixture (treatment-pathogen) were pipetted onto four spots per Petri plate, with three replicate plates per treatment. The control consisted of a 0.05% (v/v) Tween 20 solution mixed at a 1:1 ratio with each urediniospore suspension. Plates were left at 22 ºC for 6 hours (8 hours for *A. psidii*) in the dark. Germination was interrupted by adding a drop of lactofuchsin to each suspension and then covered with a coverslip.

Urediniospore germination was assessed under a light microscope by scanning the area under the coverslip and ranking as germinated or not germinated the first 100 urediniospores found during the scanning process with a careful procedure avoiding duplication of counting. A urediniospore was considered as germinated when its germ tube was longer than twice its diameter. The percentage of germination inhibition was then calculated. Each experiment was performed twice for each rust fungus/antagonist combination. Evaluations were conducted in a randomized block design, with each observer (three in total, corresponding to the blocks)

responsible for assessing the four predefined points on a plate for each treatment, thereby ensuring consistency throughout the analyses.

2.4. Statistical analysis

Germination data for each rust fungus was assessed individually. Initially, a linear model was applied, considering "treatment" and "experiment" as fixed factors. However, a significant interaction between "treatment" and "experiment" was observed, along with heteroscedasticity across experiments. Consequently, the experiments were combined in a single model using generalized linear mixed models (GLMMs) to account for experiment-level variability through a random effects structure. "Treatment" was modeled as a fixed factor, while "block" and "experiment" were included as random factors. Germination data, expressed as the number of germinated spores out of 100 observed, were modeled assuming a beta-binomial distribution with a logit link function.

Analyses were performed with the "glmmTMB ()" function (*glmmTMB* package [21]). Model diagnostics were performed using the "simulate_residuals ()" function (*DHARMa* package [22]) and the "check_model ()" function (*performance* package [23]) to assess variance homogeneity, normality of errors and random effects, and residual uniformity. Marginal means were estimated with the "emmeans ()" function (*emmeans* package [24]), and pairwise comparisons were performed using the "cld ()" function from the *multcomp* package [25], considering Tukey's test ($p < 0.05$).

The percentage of germination inhibition (PGI) was calculated based on the estimated mean germination of each treatment and the control using the following formula:

$$\text{PGI}\ (\%)\ =\ 1 - \frac{\text{Treatment}}{\text{Control}} \times 100$$

Where "Treatment" refers to the mean urediniospore germination obtained from the antagonists mixed with the urediniospore suspension, and "Control" refers to the mean germination of urediniospores treated with the 0.05% (v/v) Tween 20 solution.

A final combined analysis was conducted by pooling data from pathogens exposed to the treatments and from all assays performed. A GLMM was employed, considering "treatment" as a fixed factor, and "pathogen", "experiment", and "block" as random factors. All other procedures followed those previously described.

R software version 4.3.3. [26] was used to analyze the data and generate the graphs (with the *ggplot2* package [27].

3. Results

In the combined analysis of two experiments, *Austropuccinia psidii* urediniospore germination (Fig. 2, Table 2) was significantly inhibited by exposure to fungal antagonists ($p < 0.01$). Mean germination rates among antagonist treatments ranged from 58.3% to 67.6%, whereas the control (Tween 20) exhibited a significantly higher germination rate of 76.6%, differing significantly from all antagonist treatments. Inhibition percentages were calculated in relation to the control, and moderate reductions (12–14%) were observed for *A. flavus* (E486), *C. hemileiae* (M121), *F. coffeibaccae* (M442), and three *Trichoderma* isolates (E174, E414, M290), with germination means ranging from 65.6% to 67.6%. Intermediate inhibition levels (17–20%) were recorded for *C. cateniannulata* (E48) and *C. rhizophaga* (M230 and M267), with germination between 61.5% and 63.6%. The most pronounced reductions were achieved by *C. rosea* (E585) and *C. cateniannulata* (LCBPF 17), both showing the lowest germination rate (58.3%), representing approximately 24% inhibition relative to the control. Although statistical groupings overlapped, these two isolates appeared to be the most promising candidates for suppressing urediniospore germination, based on our results. In addition, *A. psidii* urediniospore that germinated after exposure to fungal antagonists developed abnormal germ tubes. Treatments based on *C. rosea* (E585), *C. rhizophaga* (M267), *T. harzianum* (M290), an isolate of *C. cateniannulata* (LCBPF 17), *A. flavus* (E486), *F. coffeibaccae* (M442), and *C. hemileiae* (M121) resulted in shorter germ tubes, while *T. virens* (E174) and another isolate of *C. cateniannulata* (E48) induced the formation of swollen germ tubes.

For *Phakopsora pachyrhizi* (Fig. 3, Table 3), urediniospore germination also differed significantly among treatments ($p < 0.01$), ranging from 27.3% to 42.2%. Conversely the control reached 56.0% urediniospore germination. The most effective treatment in terms of inhibition of urediniospore germination was *F. coffeibaccae* (M442), with germination reduced to 27.3% (51.3% inhibition), followed by *C. cateniannulata* (E48), with 31.1% germination (44.5% inhibition). Both differed significantly from the control. Most other treatments, including *A. flavus* (E486), *C. rosea* (E585), *C. rhizophaga* (M230, M267), *C. cateniannulata* (LCBPF 17), and *Trichoderma* isolates (E174, E414, M290), resulted in intermediate inhibition (25–36%). *Calonectria hemileiae* (M121) exhibited the highest germination among the treated groups (42.2%), with the lowest inhibition (24.6%). Although fungal antagonists did not induce the development of abnormal germ tubes in *P. pachyrhizi*, at least one isolate (*C. rhizophaga,* M267) appeared to degrade the germ tube after its formation (Fig. 4d).

In the case of *Puccinia recondita* (Fig. 4, Table 4), germination rates also varied significantly across treatments ($p < 0.01$), ranging from 35.1% to 49.0%, compared to 57.9% in the control. *Fusarium coffeibaccae* (M442) again demonstrated the strongest inhibitory effect, with germination at 35.1% (39.4% inhibition). This was followed by *C. cateniannulata* isolates (LCBPF 17 and E48), with germination values of 38.9% and 41.7%, respectively, corresponding to 33% and 28% inhibition. Other treatments, such as *A. flavus*, *C. hemileiae*, *Clonostachys* spp., and *Trichoderma* spp., showed intermediate inhibition effects (15–26%). While some isolates fell into overlapping statistical groups, M442 and E48 were consistently the most effective. Beyond that, it was observed that some germinated urediniospores of *P. recondita* exposed to *C. rosea* (E585), *C. rhizophaga* (M267), and *C. cateniannulata* (E48) exhibited bifurcation at the tips of their germ tubes.

Results for *Uromyces appendiculatus* (Fig. 5, Table 5), revealed significant differences ($p < 0.01$), with mean germination ranging from 44.5% to 64.7%, as compared to 77.8% for control. *Fusarium coffeibaccae* (M442) led to the greatest reduction (44.5% germination; 42.8% inhibition), followed by *C. cateniannulata* (E48) and *T. harzianum* (M290), both with inhibition above 33%. Moderate effects (22–29% inhibition) were seen for *A. flavus*, *C. hemileiae*, *C. rhizophaga* (M267 and M230), *C. cateniannulata* (LCBPF 17), and *T. theobromicola* (E414). The least effective treatments included *C. rosea* (E585) and *T. virens* (E174), with inhibition rates below 20%. *Uromyces appendiculatus* urediniospore that germinated after exposure to fungal antagonists also developed abnormal germ tubes. In this case, isolates of *C. rhizophaga* (M230 and M267) and *C. cateniannulata* (E48 and LCBPF 17) induced the formation of shorter germ tubes, and *T. virens* (E174), *F. coffeibaccae* (M442), and *C. hemileiae* (M121) led to swollen germ tubes.

In the global analysis (Table 6), which combined data from the four rust species, *F. coffeibaccae* (M442), and both isolates of *C. cateniannulata* (E48 and LCBPF 17) showed consistent performance across assays, with mean inhibition rates of 36.4%, 31.5%, and 27.8%, respectively, all calculated relative to the overall control mean. These were followed by intermediate-performing antagonists, including *Clonostachys* spp. (E585, M230, M267), *T. harzianum* (M290), and *A. flavus* (E486), which achieved inhibition rates ranging from 21.7–24.7%. The least effective antagonists were *C. hemileiae* (M121), *T. theobromicola* (E414), and *T. virens* (E174), with inhibition below 22%. Overall, these results had a consistent pattern of performance across rust pathogens, supporting the possibility of a broader biocontrol potential for the fungal antagonists of CLR.

4. Discussion

We have shown, in earlier studies, that *H. vastatrix* uredinia harbor a high diversity of fungal species, many of which exhibit antagonistic activity against CLR under various experimental conditions. In this study, a hypothesis of a broad effect across the Pucciniales of the *H. vastatrix* antagonists was preliminarily tested through *in vitro* assays. It provided evidence that this conjecture may be correct. It was found that the most promising fungal isolates selected for deployment against *H. vastatrix* also appear to be deleterious for *A. psidii, P. pachyrhizi, P. recondita,* and *U. appendiculatus*. It is too early to conclude that this represents true biocontrol potential, but it justifies the continuation of the study, particularly taking into account the major relevance of those rust species and of rust diseases as a whole.

Several successful biocontrol agents, such as strains of *Trichoderma harzianum* [28] and *Bacillus subtilis* [29], are known to have a broad spectrum of target pathogens, being effective against many plant pathogenic fungi and even bacteria, and nematodes too. However, to the best of our knowledge, *Bacillus velezensis* B157 remains the only antagonist originally selected for the biological control of coffee leaf rust and later evaluated against other phytopathogens. This bacterium is a prominent biocontrol agent first isolated from coffee leaves and assessed against *H. vastatrix* under various conditions, including *in vitro*, greenhouse, and field experiments [30,31]. More recently, it was tested against *Alternaria linariae*, the causal agent of tomato early blight, and was shown to reduce disease severity under greenhouse conditions [32].

Surveys for potential biological control agents targeting rust pathogens have been conducted over the years. For example, Chock *et al.* [33] isolated eleven fungal strains from *A. psidii* pustules on wild and naturalized Hawaiian Myrtaceae (*Syzygium jambos* and *Metrosideros polymorpha*) and evaluated isolates of *Bionectria ochroleuca* (syn.: *C. rosea)*, *Cladosporium cladosporioides*, and *Clonostachys rosea* for their effects on disease development. Although most results were not statistically significant, one *C. rosea* isolate reduced the average formation of *A. psidii* pustules. More recently, fungal genera such as *Cladosporium*, *Clonostachys*, *Fusarium*, *Sarocladium*, *Trichoderma*, and *Trichothecium* have been found in association with *A. psidii* in Brazil [34], and selected isolates are currently under evaluation *in vitro* and *in planta*. Some promising results are being yielded (unpub. results, N. M. P. Silva).

The biocontrol properties of *Simplicillium lanosoniveum* against *P. pachyrhizi* have been studied since 2007. These have demonstrated that *S. lanosoniveum* is a mycoparasite, but also inhibits urediniospore germination, and reduces both pustule formation and disease severity in soybean [35–37]. In addition, other microorganisms evaluated for soybean rust control include strains of *B. subtilis*, *B. pumilus*, and *B. licheniformis* [11,38], as well as *Metarhizium anisopliae* and *M. humberi* [39].

Biocontrol of bean rust has been investigated with the involvement of several organisms with distinct modes of action. An Italian research group reported that a *Cladosporium tenuissimum* isolate was capable of mycoparasitizing *U. appendiculatus* urediniospores [40], and that its secondary metabolites were able to inhibit urediniospore germination [41]. Evidence of systemic resistance induction was also observed when *T. harzianum* and *Pseudomonas aeruginosa* were applied to *Phaseolus vulgaris* seeds, activating plant defense responses and reducing rust severity under greenhouse conditions [42]. In addition, other bacteria have shown potential to control the disease through mechanisms such as mycoparasitism – *Pseudomonas putida* [43] – and antibiosis – *B. subtilis* [44].

Similarly, several microbial species have been tested for the control of wheat leaf rust, including *T. harzianum*, *B. subtilis*, *Saccharomyces cerevisiae* [45], *Akanthomyces lecanii* (syn. *Lecanicillium lecanii*), *Beauveria bassiana*, *C. cladosporioides*, *Cordyceps fumosorosea*, *M. anisopliae* [9], *A. attenuatus* (syn. *L. attenuatum*) [12], *B. megaterium* [46], *Penicillium brevicompactum*, *C. rosea*, *Simplicillium aogashimaense*, *Neoascochyta* sp., *Corniculantispora psalliotae* (syn.: *Lecanicillium psalliotae*) and *Epicoccum nigrum* [47], with promising results obtained in *in vitro*, greenhouse, and field trials.

In vitro tests have long been a valuable tool in preliminary research on the biocontrol potential of microbial antagonists [48], and are commonly used as an initial step in the screening process during the development of biological control products. This approach allows for the rapid, cost-effective, reliable, and reproducible testing of multiple strains against one or more pathogens. Moreover, it provides preliminary insights into the potential mechanisms of action, especially antibiosis and mycoparasitism [48,49].

However, some limitations must be considered. *In vitro* assays may produce artefacts or lead to the premature exclusion of promising candidates due to their lack of good performance under simplified and too artificial conditions. These assays do not

account for complex plant–microbe–pathogen interactions that occur in natural systems. Consequently, other modes of action involving plant responses (induced systemic resistance), interference with different steps of the phytopathogen life cycle (competition), or effects resulting from long-term exposure are not adequately considered in such *in vitro* tests [48–50]. Therefore, some microbial strains that perform poorly *in vitro* may still prove highly effective when used *in planta*. Despite these challenges, the sequential use of *in vitro* screening followed by *in planta* validation remains a practical and informative strategy for selecting promising biological control agents [51]. This is particularly true for situations such as faced during the evaluation of a large range of potential biocontrol candidates for CLR.

Despite variable inhibition rates across rust fungi, the fungal antagonists evaluated in this study were selected based on their consistent and promising performance against *H. vastatrix* and CLR under various conditions, including *in vitro* assays (Table S1). Under controlled conditions, *C. hemileiae*, for instance, inhibited urediniospore germination by 80–100%. In germinated urediniospores, the germ tubes were either overgrown by *C. hemileiae* mycelia or exhibited morphological abnormalities such as swelling and reduced length [15]. Similar distortions were observed when urediniospores of *H. vastatrix* were exposed to conidial suspensions of *Clonostachys* isolates, with inhibition rates ranging from 96% to 100%. Fungal filtrates were also evaluated and caused germ tube alterations; although slightly less effective, these treatments reduced germination by 60–79% [16]. When exposed to either conidial suspensions or fungal filtrates of *C. cateniannulata*, urediniospores of *H. vastatrix* developed short and swollen germ tubes, and germination was inhibited by an average of 38% [17]. In addition, isolates of *Fusarium* spp. and their culture filtrates [52], as well as *Trichoderma* spp. isolates [53], also exhibited significant inhibition of *H. vastatrix* urediniospore germination.

Overall, our results indicate that antagonist fungi of *H. vastatrix* are capable of reducing urediniospore germination of all four *Pucciniales* which were tested, thus suggesting broad-spectrum potential. The observed inhibitory effects, as well as the formation of shorter, swollen, or bifurcated germ tubes, point to the likely involvement of antibiosis as an important mechanism of action. However, it remains unclear whether these morphological abnormalities interfere with disease development. Further studies are needed to investigate this possibility and to elucidate additional modes of action, especially under more complex and dynamic conditions, which are essential for achieving effective and consistent disease control [48].

Fusarium coffeibaccae (M442) and *Cordyceps cateniannulata* (E48 and LCBPF 17) consistently ranked among the most effective antagonists and are candidates for performance validation in future greenhouse and field trials against the pathogens assessed here. Furthermore, considering the limitations of *in vitro* assays, even isolates that showed moderate or low efficacy under artificial conditions may still exhibit relevant biocontrol activity in *in planta* experiments. As previously observed for CLR, these antagonists are expected to significantly reduce disease development of other rusts, in which case they will represent much needed tools for sustainable, biologically based strategies for disease control across different crops.

5. References

1. Kaishian P, Layug CRK, Anderson M, Berg DR, Aime MC. Rust HUBB: DNA barcode-based identification of Pucciniales. *IMA Fungus*. 2024;15(1):3. doi:10.1186/s43008-023-00132-7

2. Tavares S, Ramos AP, Pires AS, et al. Genome size analyses of Pucciniales reveal the largest fungal genomes. *Front Plant Sci*. 2014;5. doi:10.3389/fpls.2014.00422

3. Aime MC, Bell CD, Wilson AW. Deconstructing the evolutionary complexity between rust fungi (Pucciniales) and their plant hosts. *Stud Mycol*. 2018;89:143-152. doi:10.1016/j.simyco.2018.02.002

4. Crous PW, Verkley GJM, Groenewald JZ, Houbraken J. *Fungal Biodiversity*. 2nd ed. (Crous PW, Verkley GJM, Groenewald JZ, Houbraken J, eds.). Westerdijk Fungal Biodiversity Institute; 2019. https://books.google.com.br/books?id=NOoJxQEACAAJ

5. Oliver RP. Diseases caused by fungi. In: *Agrios' Plant Pathology*. Elsevier; 2024:339-427.

6. Baker P. The 'Big Rust': an update on the coffee leaf rust situation. *Coffee Cocoa Int*. 2014;40(6):37-39.

7. Koutouleas A, Collinge DB, Ræbild A. Alternative plant protection strategies for tomorrow's coffee. *Plant Pathol*. 2023;72(3):409-429. doi:10.1111/ppa.13676

8. Simpson BB, Ogorzaly MC. *Economic Botany: Plants in Our World.* 3rd ed. McGraw-Hill Science; 2000.

9. Sheroze A, Rashid A, Shakir AS, Khan SM. Effect of biocontrol agents on leaf rust of wheat and influence of different temperature and humidity levels on their colony growth. Published online 2003.

10. Burmeister L, Hau B. Control of the bean rust fungus *Uromyces appendiculatus* by means of *Trichoderma harzianum*: leaf disc assays on the antibiotic effect of spore suspensions and culture filtrates. *BioControl*. 2009;54(4):575-585. doi:10.1007/s10526-008-9202-9

11. Dorighello DV, Bettiol W, Maia NB, de Campos Leite RMVB. Controlling Asian soybean rust (*Phakopsora pachyrhizi*) with *Bacillus* spp. and coffee oil. *Crop Protection*. 2015;67:59-65. doi:10.1016/j.cropro.2014.09.017

12. Nxumalo T. Development of a Biocontrol Agent, *Lecanicillium Attenuatum* (Zare & Gams), of Wheat Leaf Rust (*Puccinia Triticina* Eriks.). Published online 2018. Accessed July 10, 2025. https://core.ac.uk/download/pdf/286605317.pdf

13. Carnegie AJ, Pegg GS. Lessons from the Incursion of Myrtle Rust in Australia. *Annu Rev Phytopathol*. 2018;56(1):457-478. doi:10.1146/annurev-phyto-080516-035256

14. Roux J, Greyling I, Coutinho TA, Verleur M, Wingfeld MJ. The Myrtle rust pathogen, *Puccinia psidii*, discovered in Africa. *IMA Fungus*. 2013;4(1):155-159. doi:10.5598/imafungus.2013.04.01.14

15. Salcedo-Sarmiento S, Aucique-Pérez CE, Silveira PR, et al. Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant. *iScience*. 2021;24(4):102352. doi:https://doi.org/10.1016/j.isci.2021.102352

16. Kapeua-Ndacnou M, de Abreu LM, de Macedo DM, et al. Assessing the Biocontrol Potential of *Clonostachys* Species Isolated as Endophytes from Coffea Species and as Mycoparasites of *Hemileia* Rusts of Coffee in Africa. *Journal of Fungi*. 2023;9(2). doi:10.3390/jof9020248

17. Pereira CM, Bautz KR, Rodríguez M del CH, et al. *Cordyceps cateniannulata*: An endophyte of coffee, a parasite of coffee leaf rust and a pathogen of coffee pests. *Fungal Biol*. 2024;128(5):1917-1932. doi:10.1016/j.funbio.2024.05.004

18. Rodríguez M del CH, Evans HC, de Abreu LM, et al. New species and records of *Trichoderma* isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa. *Sci Rep*. 2021;11(1):5671. doi:10.1038/s41598-021-84111-1

19. Reis EM, Carlini R de C, Zanatta M. Optimal, lower and upper temperature thresholds for uredospore germination and germ tube growth of *Phakopsora pachyrhizi*. *Summa Phytopathol*. 2022;48(1):25-27. doi:10.1590/0100-5405/192967

20. Blum MMC, Reis EM, Vieira FT, Carlini R. In vitro effect of substrate, temperature and photoperiod on *Phakopsora pachyrhizi* urediniospore germination and germ tube growth. *Summa Phytopathol*. 2015;41(2):101-106. doi:10.1590/0100-5405/1999

21. McGillycuddy M, Warton DI, Popovic G, Bolker BM. Parsimoniously Fitting Large Multivariate Random Effects in glmmTMB. *J Stat Softw*. 2025;112(1). doi:10.18637/jss.v112.i01

22. Hartig F. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. *CRAN: Contributed Packages*. Published online 2016.

23. Lüdecke D, Ben-Shachar M, Patil I, Waggoner P, Makowski D. performance: An R Package for Assessment, Comparison and Testing of Statistical Models. *J Open Source Softw*. 2021;6(60):3139. doi:10.21105/joss.03139

24. Lenth R V. emmeans: Estimated Marginal Means, aka Least-Squares Means. *CRAN: Contributed Packages*. Published online October 20, 2017. doi:10.32614/CRAN.PACKAGE.EMMEANS

25. Hothorn T, Bretz F, Westfall P. Simultaneous Inference in General Parametric Models. *Biometrical Journal*. 2008;50(3):346-363. doi:10.1002/bimj.200810425

26. R Core Team. R: A Language and Environment for Statistical Computing. Published online 2024. Accessed May 10, 2025. https://www.R-project.org/

27. Wickham H. Data Analysis. 2016:189-201. doi:10.1007/978-3-319-24277-4_9

28. Fraceto LF, Maruyama CR, Guilger M, et al. *Trichoderma harzianum*-based novel formulations: potential applications for management of Next-Gen agricultural challenges. *Journal of Chemical Technology & Biotechnology*. 2018;93(8):2056-2063. doi:10.1002/jctb.5613

29. Valenzuela Ruiz V, Gándara-Ledezma A, Villarreal-Delgado MF, et al. Regulation, Biosynthesis, and Extraction of *Bacillus*-Derived Lipopeptides and Its Implications in Biological Control of Phytopathogens. *Stresses*. 2024;4(1):107-132. doi:10.3390/stresses4010007

30. Haddad F, Maffia LA, Mizubuti ESG, Teixeira H. Biological control of coffee rust by antagonistic bacteria under field conditions in Brazil. *Biological Control*. 2009;49(2):114-119. doi:10.1016/j.biocontrol.2009.02.004

31. Haddad F, Saraiva RM, Mizubuti ESG, Romeiro RS, Maffia LA. Isolation and selection of *Hemileia vastatrix* antagonists. *Eur J Plant Pathol*. 2014;139(4):763-772. doi:10.1007/s10658-014-0430-9

32. da Silva Junior AL, Borges Á V., da Silva HAO, et al. Lipopeptide-enriched extracts of *Bacillus velezensis* B157 for controlling tomato early blight. *Crop Protection*. 2023;172:106317. doi:10.1016/j.cropro.2023.106317

33. Chock MK, Hoyt BK, Amend AS. Mycobiome Transplant Increases Resistance to *Austropuccinia psidii* in an Endangered Hawaiian Plant. *Phytobiomes J*. 2021;5(3):326-334. doi:10.1094/PBIOMES-09-20-0065-R

34. da Silva NMP, Guterres DC, Borges LS, Barreto RW, Furtado GQ. Surveying potentially antagonistic fungi to myrtle rust (*Austropuccinia psidii*) in Brazil: fungicolous Cladosporium spp. *Brazilian Journal of Microbiology*. 2023;54(3):1899-1914. doi:10.1007/s42770-023-01047-6

35. Ward NA, Schneider RW, Aime MC. Colonization of soybean rust sori by *Simplicillium lanosoniveum*. *Fungal Ecol*. 2011;4(5):303-308. doi:10.1016/j.funeco.2011.03.008

36. Ward NA, Robertson CL, Chanda AK, Schneider RW. Effects of *Simplicillium lanosoniveum* on *Phakopsora pachyrhizi*, the Soybean Rust Pathogen, and Its Use as a Biological Control Agent. *Phytopathology*. 2012;102(8):749-760. doi:10.1094/PHYTO-01-11-0031

37. Gauthier NW, Maruthachalam K, Subbarao K V., et al. Mycoparasitism of *Phakopsora pachyrhizi*, the soybean rust pathogen, by *Simplicillium lanosoniveum*. *Biological Control*. 2014;76:87-94. doi:10.1016/j.biocontrol.2014.05.008

38. Dorighello DV, Forner C, de Campos Leite RMVB, Bettiol W. Management of Asian soybean rust with *Bacillus subtilis* in sequential and alternating fungicide applications. *Australasian Plant Pathology*. 2020;49(1):79-86. doi:10.1007/s13313-019-00677-5

39. Holz S, D'Alessandro CP, Maximo HJ, et al. The potential of using *Metarhizium anisopliae* and *Metarhizium humberi* to control the Asian soybean rust caused by *Phakopsora pachyrhizi*. *Biocontrol Sci Technol*. 2023;33(4):366-382. doi:10.1080/09583157.2023.2191299

40. Assante G, Maffi D, Saracchi M, Farina G, Moricca S, Ragazzi A. Histological studies on the mycoparasitism of *Cladosporium tenuissimum* on urediniospores of *Uromyces appendiculatus*. *Mycol Res*. 2004;108(2):170-182. doi:10.1017/S0953756203008852

41. Nasini G, Arnone A, Assante G, Bava A, Moricca S, Ragazzi A. Secondary mould metabolites of *Cladosporium tenuissimum*, a hyperparasite of rust fungi. *Phytochemistry*. 2004;65(14):2107-2111. doi:10.1016/j.phytochem.2004.03.013

42. Abeysinghe S. Systemic resistance induced by *Trichoderma harzianum* RU01 against *Uromyces appendiculatus* on *Phaseolus vulgaris*. *J Natl Sci Found*. 2009;37(3):203-207. doi:10.4038/JNSFSR.V37I3.1214

43. Abo-Elyousr KAM, Abdel-Rahim IR, Almasoudi NM, Alghamdi SA. Native Endophytic *Pseudomonas putida* as a Biocontrol Agent against Common Bean Rust Caused by *Uromyces appendiculatus*. *Journal of Fungi*. 2021;7(9):745. doi:10.3390/jof7090745

44. Bettiol W, Brandão MSB, Saito ML. Controle da ferrugem do feijoeiro com extratos e células formuladas de *Bacillus subtilis*. Published online 1992.

45. Eldoksch HA, Atteia MF, Abdel-Moity SMH. Management of brown leaf rust, *Puccinia recondita* of wheat using natural products and biocontrol agents. *Pak J Biol Sci*. 2001;4:550-553.

46. Mabrouk OI, Farouk AM, Draz IS, Zayto MA, Omar GE, najeeb khadija. Alternative Management of Wheat Leaf Rust Caused by *Puccinia triticina* Revealing Histological and Biochemical Defense Mechanisms. *Egypt J Phytopathol*. 2025;0(0):0-0. doi:10.21608/ejp.2025.415660

47. Wilson A, Cuddy WS, Park RF, et al. Investigating hyperparasites as potential biological control agents of rust pathogens on cereal crops. *Australasian Plant Pathology*. 2020;49(3):231-238. doi:10.1007/s13313-020-00695-8

48. Köhl J, Kolnaar R, Ravensberg WJ. Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. *Front Plant Sci*. 2019;10. doi:10.3389/fpls.2019.00845

49. Collinge DB, Jensen DF, Rabiey M, Sarrocco S, Shaw MW, Shaw RH. Biological control of plant diseases – What has been achieved and what is the direction? *Plant Pathol*. 2022;71(5):1024-1047. doi:10.1111/ppa.13555

50. Besset-Manzoni Y, Joly P, Brutel A, et al. Does *in vitro* selection of biocontrol agents guarantee success in planta? A study case of wheat protection against *Fusarium* seedling blight by soil bacteria. *PLoS One*. 2019;14(12):e0225655. doi:10.1371/journal.pone.0225655

51. Williams MEP, Knox OGG, Forsyth LM. Biological control agents: the importance for specific and targeted screening techniques to enable their effective and successful implementation. *Eur J Plant Pathol*. Published online April 24, 2025. doi:10.1007/s10658-025-03035-y

52. Nóbrega TF. *Fusarium Spp. Antagonists to Hemileia vastatrix from Brazil, Paraguay and Africa: Taxonomy and Biocontrol Potential*. Thesis. Universidade Federal de Viçosa; 2021.

53. Rodríguez M del CH. *Trichoderma spp. Associated with Coffee in Africa: Taxonomy and Potential Uses for the Biological Control of Coffee Leaf Rust (Hemileia vastatrix) and Promotion of Plant Growth*. Thesis. Universidade Federal de Viçosa; 2018.

54. Kapeua-Ndacnou M, Nóbrega TF, Batista LR, et al. *Aspergillus flavus* from coffee in Cameroon: a non-aflatoxigenic endophytic isolate antagonistic to coffee leaf rust (*Hemileia vastatrix*). *J Appl Microbiol*. 2023;134(5):lxad076. doi:10.1093/jambio/lxad076

55. Domingues MM, SANTOS PL, GÊA BCC, et al. *Cordyceps cateniannulata* and *Cordyceps javanica*: first report of pathogenicity to *Glycaspis brimblecombei* (Hemiptera: Aphalaridae). *An Acad Bras Cienc*. 2022;94(suppl 3). doi:10.1590/0001-3765202220211566

56. Kapeua-Ndacnou M. *Isolates of Aspergillus, Clonostachys and Trichoderma from Africa as Potential Biocontrol Agents against Coffee Leaf Rust*. Thesis. Universidade Federal de Viçosa; 2022. doi:10.47328/UFVBBT.2022.194

Figure 1. Sources of urediniospores used in the *in vitro* assays. (A–B) *Austropuccinia psidii* on rose apple (*Syzygium jambos*) grown and inoculated under controlled conditions. (C–D) *Phakopsora pachyrhizi* naturally infecting soybean (*Glycine max*) growing in plots at the Infectarium of the Universidade Federal de Viçosa (UFV). (E–F) *Puccinia recondita* naturally infecting a wheat (*Triticum aestivum*) stand in experimental plot at UFV. (G–H) *Uromyces appendiculatus* on common bean (*Phaseolus vulgaris*) in plots at the Infectarium.

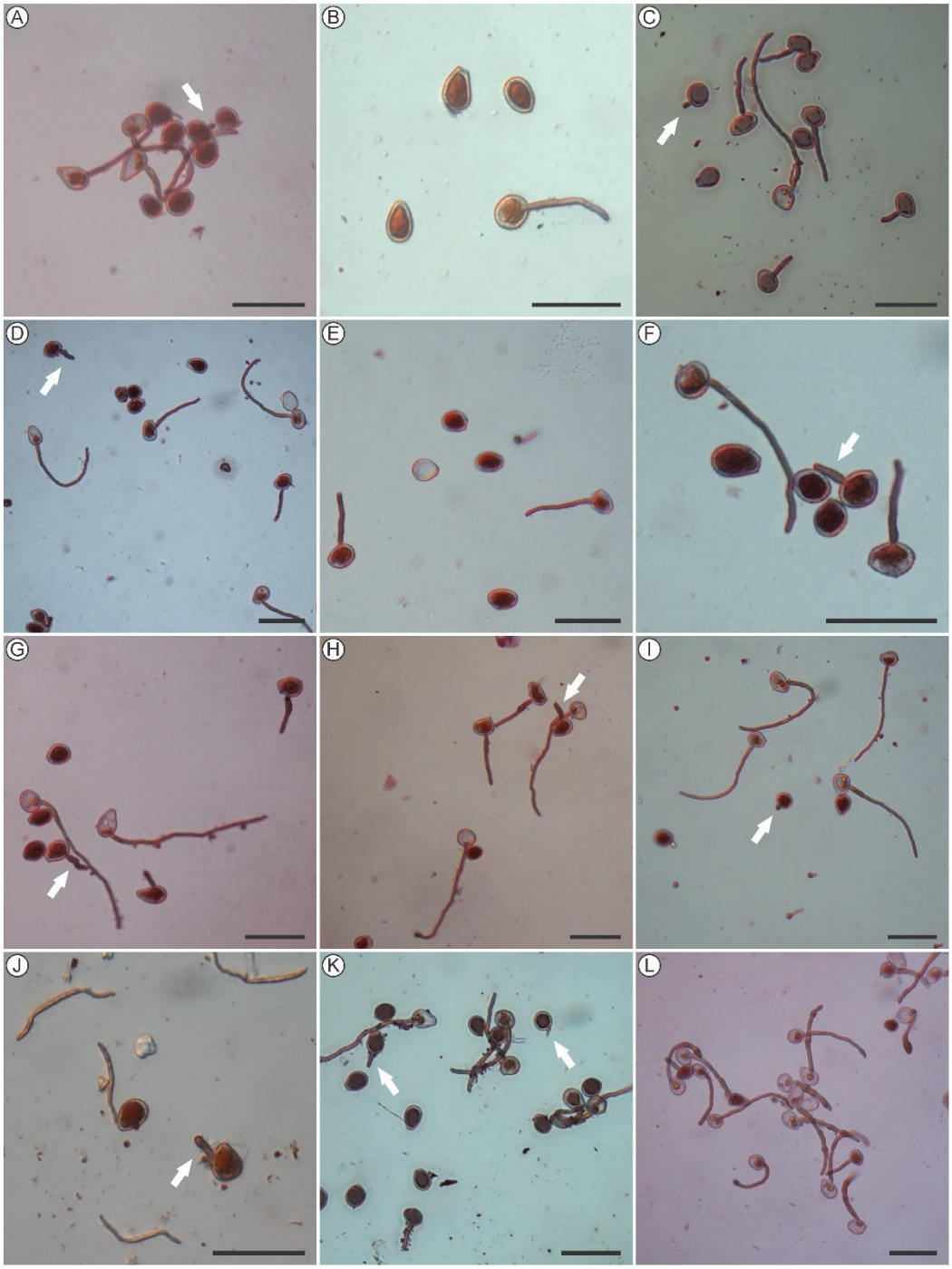

Figure 2. *Austropuccinia psidii* urediniospores after exposure to conidial suspensions of fungal antagonists: (A) *Clonostachys rosea* (E585), (B) *C. rhizophaga* (M230), (C) *C. rhizophaga* (M267), (D) *Trichoderma virens* (E174), (E) *T. theobromicola* (E414), (F) *T. harzianum* (M290), (G) *Cordyceps cateniannulata* (E048), (H) *C. cateniannulata* (LCBPF 17), (I) *Aspergillus flavus* (E486), (J) *Fusarium coffeibaccae* (M442), (K) *Calonectria hemileiae* (M121), and (L) 0.05% (v/v) Tween 20 solution (control). White arrows indicate abnormal germ tubes, including shortened or swollen structures. Scale bars: A-L 50 µm.

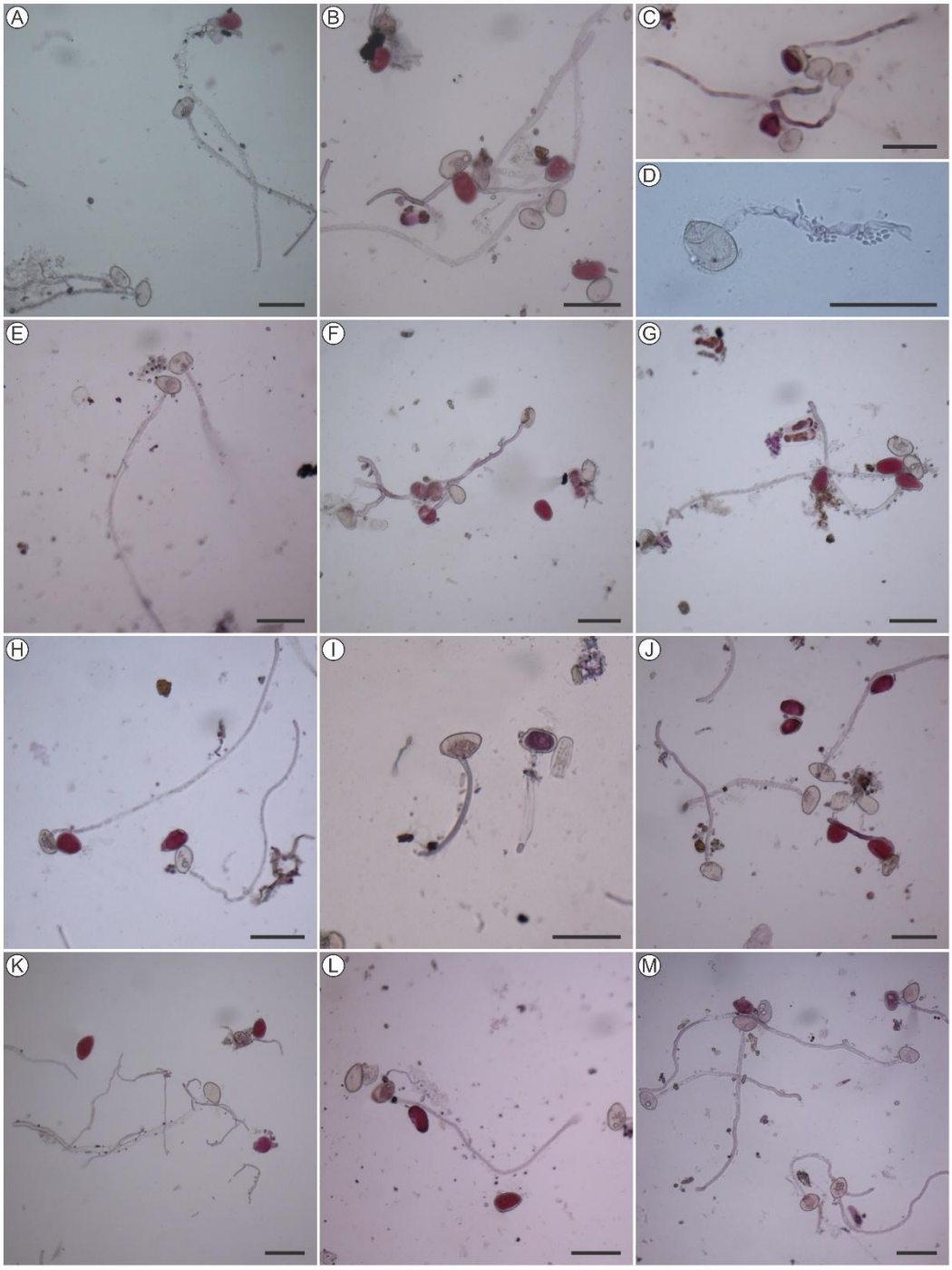

Figure 3. *Phakopsora pachyrhizi* urediniospores after exposure to conidial suspensions of fungal antagonists: (A) *Clonostachys rosea* (E585), (B) *C. rhizophaga* (M230), (C, D) *C. rhizophaga* (M267), (E) *Trichoderma virens* (E174), (F) *T. theobromicola* (E414), (G) *T. harzianum* (M290), (H) *Cordyceps cateniannulata* (E048), (I) *C. cateniannulata* (LCBPF 17), (J) *Aspergillus flavus* (E486), (K) *Fusarium coffeibaccae* (M442), (L) *Calonectria hemileiae* (M121), and (M) 0.05% (v/v) Tween 20 solution (control). Scale bars: A-M 50 μm.

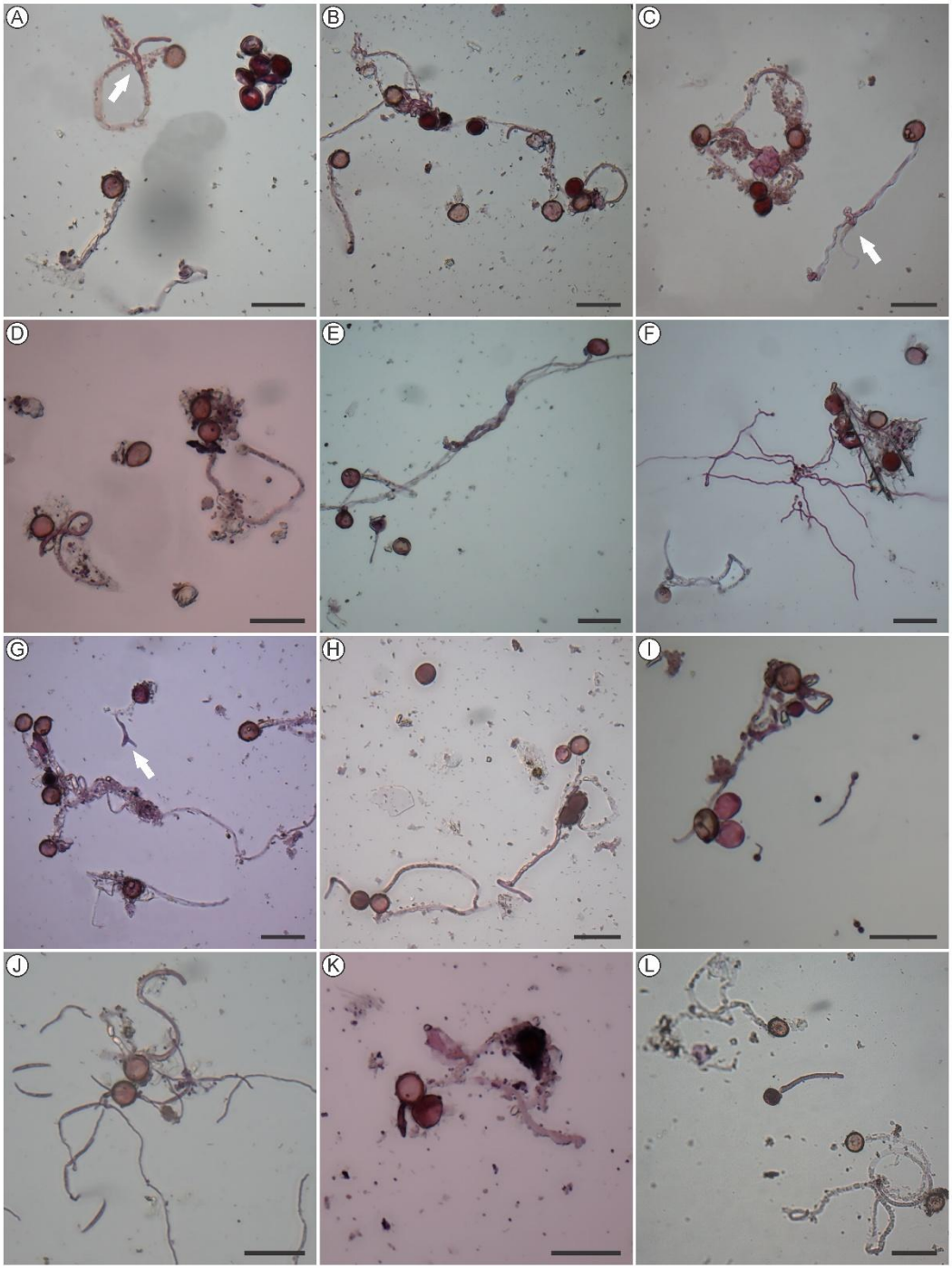

Figure 4. *Puccinia recondita* urediniospores after exposure to conidial suspensions of fungal antagonists: (A) *Clonostachys rosea* (E585), (B) *C. rhizophaga* (M230), (C) *C. rhizophaga* (M267), (D) *Trichoderma virens* (E174), (E) *T. theobromicola* (E414), (F) *T. harzianum* (M290), (G) *Cordyceps cateniannulata* (E048), (H) *C. cateniannulata* (LCBPF 17), (I) *Aspergillus flavus* (E486), (J) *Fusarium coffeibaccae* (M442), (K) *Calonectria hemileiae* (M121), and (L) 0.05% (v/v) Tween 20 solution (control). White arrows indicate bifurcated abnormal germ tubes. Scale bars: A-L 50 µm.

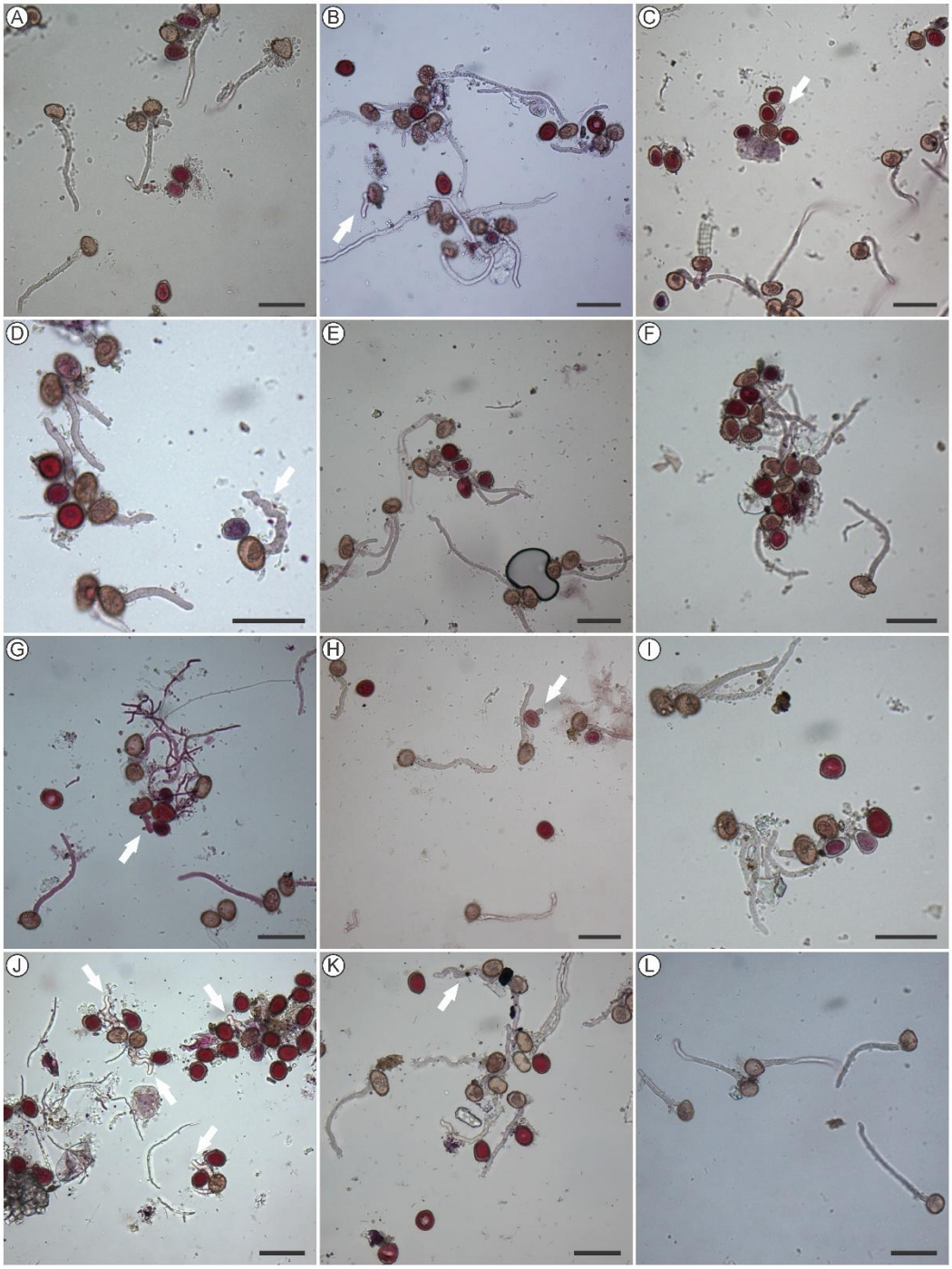

Figure 5. *Uromyces appendiculatus* urediniospores after exposure to conidial suspensions of fungal antagonists: (A) *Clonostachys rosea* (E585), (B) *C. rhizophaga* (M230), (C) *C. rhizophaga* (M267), (D) *Trichoderma virens* (E174), (E) *T. theobromicola* (E414), (F) *T. harzianum* (M290), (G) *Cordyceps cateniannulata* (E048), (H) *C. cateniannulata* (LCBPF 17), (I) *Aspergillus flavus* (E486), (J) *Fusarium coffeibaccae* (M442), (K) *Calonectria hemileiae* (M121), and (L) 0.05% (v/v) Tween 20 solution (control). White arrows indicate abnormal germ tubes, including shortened or swollen structures. Scale bars: A-L 50 µm.

Table 1. Description of the selected isolates assessed against rust fungi.

Species	Strain code	Culture collection code	Origin	Host/ substrate	Reference
Aspergillus flavus	E486	COAD 3307	Cameroon	Fruits of *C. canephora*	Kapeua-Ndacnou *et al.* [54]
Calonectria hemileiae	M121	COAD 2544	Brazil	Pustules of *H. vastatrix* on *C. arabica* leaves	Salcedo-Sarmiento *et al.* [15]
Clonostachys rosea	E585	COAD 2984	Ethiopia	Stem of *C. arabica*	Kapeua-Ndacnou *et al.* [16]
Clonostachys rhizophaga	M230	COAD 2980	Cameroon	Pustules of *H. vastatrix*/ *coffeicola* on *C. canephora* leaves	Kapeua-Ndacnou *et al.* [16]
Clonostachys rhizophaga	M267	COAD 2982	Cameroon	Pustules of *H. vastatrix*/ *coffeicola* on *C. canephora* leaves	Kapeua-Ndacnou *et al.* [16]
Cordyceps cateniannulata	E048	COAD 3349	Cameroon	Stem of *C. arabica*	Pereira *et al.* [17]
Cordyceps cateniannulata	LCBPF 17	COAD 3809	Brazil	From native soil, using *T. molitor* larvae as bait	Domingues *et al.* [55]
Fusarium coffeibaccae	M442	COAD 3041	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Nóbrega [52]
Trichoderma virens	E174	COAD 2400	Cameroon	Stem of *C. brevipes*	Rodrígues *et al.* [18]
Trichoderma theobromicola	E414	COAD 2406	Cameroon	Stem of *C. canephora*	Rodrígues *et al.* [18]
Trichoderma harzianum	M290	COAD 3672	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Tobar (Personal obs.)

Table 2. Mean urediniospore germination (%) and percentage of germination inhibition (%PGI) of *Austropuccinia psidii* after exposure to fungal antagonists under *in vitro* conditions.

Treatment[1]	Germination (%)		Inhibition (%)	
	Mean[2,3]	95% CI [LL; UL][4]	PGI[5]	95% CI [LL; UL][4]
T01 – E585	58.3 A	[42.6; 72.6]	23.89	[15.68; 32.59]
T03 – M230	61.4 AB	[45.7; 75.1]	19.84	[12.78; 27.69]
T02 – M267	63.6 AB	[48.0; 76.8]	16.97	[10.80; 24.05]
T03 – E174	67.6 B	[52.4; 79.8]	11.75	[7.32; 17.090]
T05 – E414	67.3 B	[52.1; 79.6]	12.14	[7.55; 17.56]
T06 – M290	67.0 B	[51.7; 79.3]	12.53	[7.90; 18.20]
T07 – E48	61.5 AB	[45.8; 75.1]	19.71	[12.78; 27.53]
T08 – LCBPF 17	58.3 A	[42.5; 72.6]	23.89	[15.68; 32.75]
T09 – E486	65.6 B	[50.2; 78.3]	14.36	[9.06; 20.57]
T10 – M442	66.4 B	[51.1; 78.9]	13.32	[8.36; 19.15]
T11 – M121	66.2 B	[50.8; 78.7]	13.58	[8.59; 19.62]
T12 – Control	76.6 C	[63.2; 86.1]	-	-

[1] T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *C. cateniannulata* (LCBPF 17); T09 = *Aspergillus flavus* (E486); T10 = *Fusarium coffeibaccae* (M442); T11 = *Calonectria hemileiae* (M121); T12 = 0.05% (v/v) Tween 20 solution.

[2] Values are means of two experiments.

[3] Different letters indicate statistically significant differences according to Tukey's test ($p < 0.05$).

[4] CI: confidence interval; LL: lower limit; UL: upper limit.

[5] PGI: percentage of germination inhibition, calculated relative to T12 – Control.

Table 3. Mean urediniospore germination (%) and percentage of germination inhibition (%PGI) of *Phakopsora pachyrhizi* after exposure to fungal antagonists under *in vitro* conditions.

Treatment[1]	Germination (%)		Inhibition (%)	
	Mean[2,3]	95% CI [LL; UL][4]	PGI[4]	95% CI [LL; UL][4]
T01 – E585	36.6 BC	[32.1; 41.4]	34.64	[31.90; 37.18]
T03 – M230	38.3 BC	[33.7; 43.1]	31.61	[29.11; 34.05]
T02 – M267	39.4 C	[34.8; 44.2]	29.64	[27.30; 31.90]
T03 – E174	38.2 BC	[33.6; 43.0]	31.79	[29.28; 34.25]
T05 – E414	38.8 C	[34.2; 43.6]	30.71	[28.29; 33.07]
T06 – M290	41.4 C	[36.7; 46.3]	26.07	[23.85; 28.18]
T07 – E48	31.1 AB	[26.9; 35.6]	44.46	[41.45; 47.36]
T08 – LCBPF 17	41.3 C	[36.6; 46.2]	26.25	[24.01; 28.38]
T09 – E486	39.3 C	[34.7; 44.1]	29.82	[27.47; 32.09]
T10 – M442	27.3 A	[23.4; 31.6]	51.25	[48.03; 54.21]
T11 – M121	42.2 C	[37.4; 47.0]	24.64	[22.70; 26.81]
T12 – Control	56.0 D	[51.1; 60.8]	-	-

[1] T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *C. cateniannulata* (LCBPF 17); T09 = *Aspergillus flavus* (E486); T10 = *Fusarium coffeibaccae* (M442); T11 = *Calonectria hemileiae* (M121); T12 = 0.05% (v/v) Tween 20 solution.

[2] Values are means of two experiments.

[3] Different letters indicate statistically significant differences according to Tukey's test ($p < 0.05$).

[4] CI: confidence interval; LL: lower limit; UL: upper limit.

[5] PGI: percentage of germination inhibition, calculated relative to T12 – Control.

Table 4. Mean urediniospore germination (%) and percentage of germination inhibition (%PGI) of *Puccinia recondita* after exposure to fungal antagonists under *in vitro* conditions.

Treatment[1]	Germination (%)		Inhibition (%)	
	Mean[2,3]	95% CI [LL; UL][4]	PGI[4]	95% CI [LL; UL][4]
T01 – E585	49.0 D	[43.3; 54.7]	15.37	[13.72; 17.05]
T03 – M230	45.9 CD	[40.3; 51.6]	20.73	[18.61; 22.80]
T02 – M267	47.3 CD	[41.7; 53.0]	18.31	[16.40; 20.11]
T03 – E174	42.9 BCD	[37.4; 48.6]	25.91	[23.34; 28.35]
T05 – E414	49.3 D	[43.6; 55.1]	14.85	[13.09; 16.48]
T06 – M290	44.6 BCD	[39.1; 50.4]	22.97	[20.50; 25.10]
T07 – E48	41.7 BC	[36.2; 47.4]	27.98	[25.24; 30.65]
T08 – LCBPF 17	38.9 AB	[33.5; 44.5]	32.82	[29.81; 35.82]
T09 – E486	45.5 CD	[39.9; 51.2]	21.42	[19.24; 23.56]
T10 – M442	35.1 A	[30.0; 40.6]	39.38	[35.96; 42.53]
T11 – M121	43.8 BCD	[38.2; 49.5]	24.35	[21.92; 26.82]
T12 – Control	57.9 E	[52.2; 63.4]	-	-

[1] T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *C. cateniannulata* (LCBPF 17); T09 = *Aspergillus flavus* (E486); T10 = *Fusarium coffeibaccae* (M442); T11 = *Calonectria hemileiae* (M121); T12 = 0.05% (v/v) Tween 20 solution.

[2] Values are means of two experiments.

[3] Different letters indicate statistically significant differences according to Tukey's test ($p < 0.05$).

[4] CI: confidence interval; LL: lower limit; UL: upper limit.

[5] PGI: percentage of germination inhibition, calculated relative to T12 – Control.

Table 5. Mean urediniospore germination (%) and percentage of germination inhibition (%PGI) of *Uromyces appendiculatus* after exposure to fungal antagonists under *in vitro* conditions.

Treatment[1]	Germination (%)		Inhibition (%)	
	Mean[2,3]	95% CI [LL; UL][4]	PGI[4]	95% CI [LL; UL][4]
T01 – E585	63.6 CD	[55.9; 70.6]	18.25	[15.04; 21.82]
T03 – M230	57.8 BCD	[49.9; 65.3]	25.71	[21.42; 30.21]
T02 – M267	55.3 BC	[47.4; 63.0]	28.92	[24.19; 33.71]
T03 – E174	64.7 D	[57.1; 71.7]	16.84	[13.72; 20.14]
T05 – E414	58.0 BCD	[50.1; 65.5]	25.45	[21.18; 29.93]
T06 – M290	52.0 AB	[44.1; 59.9]	33.16	[27.92; 38.32]
T07 – E48	51.5 AB	[43.6; 59.3]	33.80	[28.64; 39.02]
T08 – LCBPF 17	56.7 BCD	[48.7; 64.3]	27.12	[22.62; 31.89]
T09 – E486	60.8 BCD	[53.0; 68.1]	21.85	[18.05; 25.87]
T10 – M442	44.5 A	[36.8; 52.4]	42.80	[36.94; 48.53]
T11 – M121	60.9 BCD	[53.1; 68.2]	21.72	[17.93; 25.73]
T12 – Control	77.8 E	[71.5; 83.1]	-	-

[1] T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M230); T03 = *C. rhizophaga* (M267); T04 = *Trichoderma virens* (E174); T05 = *T. theobromicola* (E414); T06 = *T. harzianum* (M290); T07 = *Cordyceps cateniannulata* (E048); T08 = *C. cateniannulata* (LCBPF 17); T09 = *Aspergillus flavus* (E486); T10 = *Fusarium coffeibaccae* (M442); T11 = *Calonectria hemileiae* (M121); T12 = 0.05% (v/v) Tween 20 solution.

[2] Values are means of two experiments.

[3] Different letters indicate statistically significant differences according to Tukey's test ($p < 0.05$).

[4] CI: confidence interval; LL: lower limit; UL: upper limit.

[5] PGI: percentage of germination inhibition, calculated relative to T12 – Control.

Table 6. Summary of urediniospore germination (%) and inhibition (%PGI) across eight *in vitro* assays with four rust pathogens and fungal antagonists.

Treatment	Species	Germination (%)		Inhibition (%)	
		Mean[1, 2]	95% CI [LL; UL][3]	PGI[4]	95% CI [LL; UL][3]
T10 - M442	*Fusarium coffeibaccae*	43.0 A	[33.1; 53.4]	36.39	[30.01; 42.63]
T07 - E048	*Cordyceps cateniannulata*	46.3 AB	[36.2; 56.6]	31.51	[25.82; 37.26]
T08 - LCBPF 17	*Cordyceps cateniannulata*	48.8 BC	[38.5; 59.1]	27.81	[22.54; 33.28]
T02 - M230	*Clonostachys rhizophaga*	50.9 CD	[40.6; 61.2]	24.7	[19.79; 29.64]
T06 - M290	*Trichoderma harzianum*	51.3 CD	[40.9; 61.5]	24.11	[19.40; 29.12]
T03 - M267	*Clonostachys rhizophaga*	51.4 CD	[41.1; 61.6]	23.96	[19.27; 28.77]
T01 - E585	*Clonostachys rosea*	52.0 CD	[41.7; 62.2]	23.08	[18.48; 27.73]
T09 - E486	*Aspergillus flavus*	52.9 CD	[42.5; 63.0]	21.75	[17.43; 26.34]
T11 - M121	*Calonectria hemileiae*	53.3 D	[42.9; 63.4]	21.15	[16.91; 25.65]
T05 - E414	*Trichoderma theobromicola*	53.4 D	[43.0; 63.5]	21.01	[16.78; 25.48]
T04 - E174	*Trichoderma virens*	53.5 D	[43.1; 63.6]	20.86	[16.64; 25.30]
T12 - Control	–	67.6 E	[57.7; 76.3]	–	–

[1] Values are means from two independent experiments per pathogen (*Austropuccinia psidii*, *Phakopsora pachyrhizi*, *Puccinia recondita*, and *Uromyces appendiculatus)* pooled in a combined model.

[2] Different letters indicate statistically significant differences according to Tukey's test ($p < 0.05$).

[3] CI: confidence interval; LL: lower limit; UL: upper limit.

[4] PGI: percentage of germination inhibition, calculated relative to T12 – Control.

Table S1. Inhibition of *Hemileia vastatrix* urediniospore germination (%) by conidial suspensions of selected fungal antagonists in previous *in vitro* assays.

Species	Strain code	Culture collection code	Inhibition of germination (%)	Reference
Aspergillus flavus	E486	COAD 3307	95 – 100 (fungal filtrate)	Kapeua-Ndacnou *et al.* [54]
Calonectria hemileiae	M121	COAD 2544	80 – 100	Salcedo-Sarmiento *et al.* [15]
Clonostachys rosea	E585	COAD 2984	99	Kapeua-Ndacnou *et al.* [16]
Clonostachys rhizophaga	M230	COAD 2980	25	unpub. results, C.M. Pereira
Clonostachys rhizophaga	M267	COAD 2982	94	Kapeua-Ndacnou *et al.* [16]
Cordyceps cateniannulata	E048	COAD 3349	37	Pereira *et al.* [17]
Cordyceps cateniannulata	LCBPF 17	COAD 3809	20	unpub. results, C.M. Pereira
Fusarium coffeibaccae	M442	COAD 3041	74	Nóbrega [52]
Trichoderma virens	E174	COAD 2400	99.5	Kapeua-Ndacnou [56]
Trichoderma theobromicola	E414	COAD 2406	100	Kapeua-Ndacnou [56]
Trichoderma harzianum	M290	COAD 3672	20	unpub. results, C.M. Pereira

Chapter 3

Testing the possible biocontrol effect of fungal antagonists obtained from *Hemileia vastatrix* (coffee leaf rust) on a different rust fungus: nine selected isolates *vs Phakopsora pachyrhizi* (Asian soybean rust)

Prepared according to the format of the Crop Protection

Testing the possible biocontrol effect of fungal antagonists obtained from *Hemileia vastatrix* (coffee leaf rust) on a different rust fungus: nine selected isolates *vs Phakopsora pachyrhizi* (Asian soybean rust)

Caio Mattos Pereira[1], Ana Carolina de Almeida[1], Sofia Sampaio Sumam Silva[1], Sara Rocha Foletto[1], Robert Weingart Barreto[1*]

[1]Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570–900, Brazil.

*Corresponding author: rbarreto@ufv.br

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, poses a significant threat to global soybean production, and current management strategies relying on fungicides and resistant cultivars face limitations. This study tested nine fungal isolates – originally selected for biocontrol of coffee leaf rust (*Hemileia vastatrix*) – for their capacity to suppress ASR under semi-controlled, pot-based conditions. Soybean plants were sprayed weekly with conidial suspensions of *Aspergillus flavus, Calonectria hemileiae*, *Clonostachys rhizophaga*, *C. rosea*, *Cordyceps cateniannulata*, *Fusarium coffeibaccae*, *Trichoderma harzianum*, and *T. virens*, a Tween 20–adjuvant formulation alone, or a commercial *Bacillus velezensis* biofungicide. Seventy-two hours after the second application, plants were inoculated with *P. pachyrhizi*, and disease progress was monitored at 14, 21, 28 and 35 days after inoculation to calculate the area under disease progress curve and final severity for control-efficacy estimates; plant height, shoot and root dry mass, and endophytic colonization were also assessed. Treatments with *C. cateniannulata* and *C. rosea* achieved the highest control efficacies (62.4% and 62.3%, respectively), while other isolates yielded moderate suppression (7.4–35.6%), and the *B. velezensis* biofungicide failed to reduce disease. Most antagonists successfully colonized leaf tissues endophytically. These results demonstrate that fungal antagonists selected against *H. vastatrix* can also inhibit ASR, and identify *C. cateniannulata* and *C. rosea* as promising biocontrol agents warranting further investigation of their mechanisms of action, field performance, and integration into sustainable disease-management programs.

Keywords: Environmental conditions, *Cordyceps cateniannulata*, *Clonostachys rosea*, Endophytes, Mycoparasites, Antibiosis.

1. Introduction

Soybean (*Glycine max*) is the most important cultivated legume. It is cultivated worldwide and is now a fundamental source of protein, oil, and animal feed (Bai et al., 2003; Hossain et al., 2025). Abiotic and biotic stresses are major threats to yields. Impact of plant diseases – caused by numerous fungi, bacteria, viruses, and nematodes – result in yield losses which have been estimated, for example, by Savary et al. (2019), to be of 21.4% in reduction of the production. Among the diseases, rust, caused by *Phakopsora pachyrhizi*, is considered one of the most important diseases affecting soybeans (El-Hasan et al., 2022; Goellner et al., 2010; Hossain et al., 2024).

Phakopsora pachyrhizi causes the Asian soybean rust (ASR), which provokes rapid defoliation of susceptible soybean plants and is capable of provoking yield losses ranging from 10% to 100% (Chicowski et al., 2024; Yorinori et al., 2005). It is broadly regarded as a major challenge to the global soybean sector, particularly for growers in South America (Hossain et al., 2024). *Phakopsora pachyrhizi* was first recorded in 1902 in Japan, and various Asian countries have since reported its occurrence and severe yield (Hossain et al., 2024). ASR reached the Western Hemisphere in 1994, and was first reported on soybean in Hawaii (Killgore and Heu, 1994). It arrived in South America in 2001, in Paraguay (Rupe and Sconyers, 2008). It has been established in Brazil since 2002 (Freire et al., 2008), where it causes extremely high yield losses and raised production costs dramatically, forcing farmers to spend billions of dollars annually with fungicide applications (Chicowski et al., 2024).

In Brazil, the current ASR management strategy includes: avoiding soybean sowing and eliminating volunteer plants during the off-season; using early-maturing cultivars; planting at the beginning of the legally authorized period; using cultivars with resistance genes; monitoring for initial ASR symptoms and, most important, fungicide applications (Godoy et al., 2020). However, these management methods face important limitations, such as the lack of durable resistance in soybean cultivars, due to the complex gene-for-gene interactions with *P. pachyrhizi* (Chicowski et al., 2024) and the emergence of fungicide-resistant or less sensitive populations of *P. pachyrhizi*, resulting from the repeated use of systemic fungicides belonging to the same chemical groups – strobilurins and triazoles fungicides (Fontes et al., 2024).

Considering the substantial damage caused by ASR if inappropriately managed, and the limitations and costs of current control practices, there is a need for effective and more

sustainable alternatives. Biological control is a promising tool for managing ASR (Twizeyimana et al., 2023) and has gained increasing attention in recent years. In Brazil, four commercial biofungicides are currently registered for ASR management (MAPA, 2025). These products – and most published studies – have focused on species of *Bacillus* (Dorighello et al., 2020, 2015; dos Santos et al., 2025; Twizeyimana et al., 2023; Twizeyimana and Hartman, 2019), while fungal antagonists have received comparatively less attention. Fungal species such as *Corniculantispora psalliotae* (syn. *Verticillium psalliotae*) (Saksirirat and Hoppe, 1991), *Simplicillium lanosoniveum* (Gauthier et al., 2014; Ward et al., 2012, 2011), and *Trichothecium roseum* (Kumar and Jha, 2002) have been reported to affect *P. pachyrhizi* under various conditions, but research on these fungi has not progressed. More recently, promising results have been reported with *Trichoderma* spp. (dos Santos et al., 2025; El-Hasan et al., 2022) and *Metarhizium* spp. (Holz et al., 2023).

A study aimed at selecting fungal biocontrol agents to be deployed against *Hemileia vastatrix*, the etiological agent of coffee leaf rust (CLR), that started in 2015, obtained over 1,500 fungal strains (Pereira et al., 2024a). Only approximately 600 isolates were brought to a preliminary *in vitro* stage of evaluation of biocontrol potential. The list of isolates was significantly reduced and only the most promising isolates were identified and tested under greenhouse conditions (Colmán et al., 2021; Guterres et al., 2021; Kapeua-Ndacnou et al., 2023b, 2023a; Pereira et al., 2024b, 2024a; Rodríguez et al., 2021; Salcedo-Sarmiento et al., 2021) and in coffee fields (results published separately). This effort led to the selection of nine isolates belonging to the genera *Aspergillus*, *Calonectria*, *Clonostachys*, *Cordyceps*, *Fusarium*, and *Trichoderma*.

A hypothesis was raised that some of these fungal isolates, with biocontrol capability against CLR may also have antagonistic effects against other rust species. This was preliminary tested *in vitro* by pairing selected *H. vastatrix* antagonists with *Austropuccinia psidii* (myrtle rust), *Puccinia recondita* (syn. *P. triticina* – wheat leaf rust), *P. pachyrhizi* (ASR), and *Uromyces appendiculatus* (bean rust). Those preliminary *in vitro* results (published separately) indicated an antagonistic activity of the CLR potential biocontrol agents against other rust fungi. Here we conducted a study focused on the investigation of those antagonists' ability to antagonize *P. pachyrhizi in planta* in order to further explore the conjectured broader biocontrol effect of those isolates, and possible potential that any isolate may have in controlling ASR.

2. Material and methods

2.1. Fungal strains

Fungal isolates (Table 1) were obtained from collections conducted between 2015 and 2017 in Brazil, Cameroon, and Ethiopia. These isolates were originally obtained either as endophytes from healthy coffee tissues (leaves, stems, and fruits) or as mycoparasites associated with *H. vastatrix* uredinia (Rodríguez et al., 2021). Over 1,500 isolates were initially obtained, and based on promising and consistent results from *in vitro* and in greenhouse screenings conducted prior to this study (Kapeua-Ndacnou et al., 2023b, 2023a; Nóbrega, 2021; Pereira et al., 2024a; Rodríguez, 2018; Salcedo-Sarmiento et al., 2021), as well as their documented occurrence in Brazil, these isolates were selected for evaluation against soybean leaf rust. All fungi are preserved at −85 ºC in the "Coleção Octávio Almeida Drummond" (COAD), the culture collection of the Universidade Federal de Viçosa (UFV).

2.2. Inoculum production and preparation

Prior to mass multiplication, selected fungal isolates stored in the COAD were recovered and a small sample of each isolate was transferred to the center of plates containing potato dextrose-agar (PDA). Plates were kept at 25 ± 2 ºC with a 12-hour photoperiod for seven days, and were daily monitored to confirm purity and identity. After initial growth, the fungi were sub-cultured onto fresh PDA plates and incubated under the same conditions as above. These secondary cultures served as inoculum sources for inoculum production.

Inoculum production was carried out as described in Chapter 1. Similarly, the procedures for estimating the number of conidia per gram of colonized rice and for preparing conidial suspensions at the concentration of 5×10^8 conidia/mL – corresponding to an average of 1×10^8 colony-forming units per gram (CFU/g) of colonized rice) – for each antagonist isolate followed the same methodology previously detailed. Adjuvants such as glycerol (1% v/v), sunflower oil (1% v/v), and skim milk powder (2% m/v), which provide moisturizing, adhesive, and UV-protective properties (Aguirre et al., 2023; Devi et al., 2018; Grewal and Joshi, 2022; Hemalatha et al., 2017; Posadas et al., 2012), were added as a formulation to enhance fungal performance under field conditions, as previously described.

2.3. Control of soybean leaf rust under field conditions

2.3.1. Design and implementation

Three seeds of an ASR-susceptible soybean cultivar (IAC Foscarin 31) were sown in 2-L plastic pots filled with a 1:1 mixture of pasteurized soil and commercial greenhouse substrate (Tropstrato Hortaliça Plus, Vida Verde, Mogi Mirim, Brazil), composed of pine bark, coconut fiber, vermiculite, and carbonized rice husk, enriched with macro- and micronutrients. After emergence, seedlings were visually evaluated, and the most vigorous plant was retained in each pot. Pots were maintained on improvised benches under open-air conditions, exposed to natural, uncontrolled field conditions (20° 45′ 11″ S; 42° 52′ 16″ W).

Treatments sprayings began when plants reached the V1–V2 growth stage, characterized by the presence of one to two fully expanded trifoliolate leaves. Fungal antagonists suspended in a Tween 20 solution containing adjuvants, the Tween 20–adjuvant solution without fungal inoculum (hereafter referred to as the formulation), and a commercial *Bacillus velezensis*-based biofungicide (Bio-Imune®; *Bacillus velezensis* BV02; 3×10^9 CFU/mL; Vittia Group, São Joaquim da Barra, SP, Brazil), used as indicated by the manufacturer's label, were applied at seven-day intervals.

Treatments (fungal antagonists, the formulation, and the biofungicide) were sprayed onto the aerial part of the plants, covering both sides of the leaves until run-off, using a manual hand-pump sprayer adapted to a plastic bottle containing the suspension. A total of four weekly applications were performed, always in the late afternoon. The negative control consisted of plants inoculated only with *Phakopsora pachyrhizi*, without any antagonist treatment.

Seventy-two hours after the second application – *i.e.*, between the second and third sprays – and at the V4 growth stage, plants were inoculated with a suspension of *P. pachyrhizi* urediniospores. The inoculum was prepared from urediniospores obtained from naturally infected soybean plants maintained in plots for teaching purposes at the Infectarium of UFV (https://www.infectario.ufv.br/). Symptomatic leaves were collected and uredinia were examined for the possible presence of mycoparasites. Only contamination-free samples were placed in a 500 mL flask containing 250 mL of a 0.05% (v/v) Tween 20 solution in tap water. The bottle was shaken vigorously for at least 3 minutes in order to release the spores (Reis et al., 2022). The suspension was filtered through a household sieve and a layer of gauze, and then adjusted to 1×10^5 urediniospores/mL using a hemocytometer. The resulting suspension was immediately used for inoculation.

Soybean plants were inoculated with *P. pachyrhizi* in the late afternoon also using a manual hand-pump sprayer adapted to a plastic bottle. The urediniospore suspension was

applied to the abaxial leaf surface until run-off. Following inoculation, the plants were placed in a dark room at 25 ± 3 °C for 48 hours to favour pathogen infection. After this period, plants were returned back to raised benches, where they remained under ambient conditions until the end of the experiment.

The experiment was conducted between January 2025 and April 2025, and followed a completely randomized block design with 12 treatments (Table 2), four blocks, and included five pots per block.

2.4. Assessments

2.4.1. Disease Severity

Disease severity was evaluated four times at seven-day intervals, beginning 14 days after inoculation (DAI), when the first symptoms of soybean leaf rust appeared. Assessments were conducted on the third and fourth trifoliate leaves of each plant using the diagrammatic scale proposed by Franceschi *et al.* (2020).

In order to quantify the effect of treatments on ASR development, severity scores were summarized by calculating the area under the disease progress curve ($AUDPC_{SEV}$) between 14 and 35 DAI, using the "AUDPC ()" function in the *epifitter* R-package (Alves and Del Ponte, 2021), Additionally, final severity (*i.e.*, severity at the last assessment – 35 DAI) was used to estimate the control efficacy (C) of each treatment in relation to either the untreated control (T12 – Control) or the formulation-based treatment (T11), according to the formula:

$$C(\%) = 1 - \frac{\text{Treatment}}{\text{Control}} \times 100$$

2.4.2. Plant Growth

Following the final assessment of disease severity, additional evaluations were carried out to estimate the impact of treatments on plant development. Height of each plant was measured from the basal collar to the apical meristem using a tape measure. The aerial parts (stems and leaves) of the five plants within each treatment and block were cut, pooled, and placed in labeled paper bags. Root systems of every plant were carefully washed under running tap water to remove excess soil and substrate, allowed to air-dry on raised benches, and

similarly bagged. The material was dried in a forced-air oven at 70 °C for 72 hours until a constant weight was reached. The dry mass of each plant part was then determined using an analytical precision balance.

2.4.3. Endophytic Colonization

In order to verify the possibility of endophytic colonization by each fungal antagonist to which the soybean plants were exposed, we employed the "indirect isolation method", as described by Kapeua-Ndacnou et al. (2023b) and used in several other studies published by our research group. Minor adjustments to the methodology were included here. Samples were taken from each soybean plant as follows: for each antagonist-based treatment, leaflet samples were collected from three distinct portions of the plant: the third fully expanded trifoliate leaf (from the base), the fourth fully expanded trifoliate leaf (also from the base), and the terminal trifoliate. For each plant portion and treatment, a composite sample was formed, consisting of leaflets from multiple plants exposed to the same antagonist. Each composite sample was processed individually, and 5 mm-diameter fragments were excised from the leaflets using a cork borer.

Under aseptic conditions, leaf disc fragments were surface-disinfested by sequential immersion in 70% ethanol (1 min), 2% sodium hypochlorite (3 min), followed by two rinses in sterile deionized water (2 min each). The excess water of disinfected fragments was removed by placing each fragment on pieces of sterile filter paper for 2 min, and then fragments were transferred to plates containing PDA supplemented with penicillin (10 mg/L) and Triton X-100 (15 mL/L). For each composite sample, three plates were prepared, each containing nine leaf fragments. Plates were left under conditions previously described, and the presence of fungal colonies was recorded after 10 days. The emergence of colonies of the antagonists from disinfected fragments was verified by observation of colony morphology, further confirmed by examination of slides mounted with colony fragments under a light microscope (Olympus BX51, Tokyo, Japan).

2.4.4. Data analysis

Linear mixed models (LMMs) were fitted to all disease-related variables using the "lmer ()" function from the *lme4* package (Bates et al., 2015). "Treatment" was included as a fixed

effect, while “block” and “plant” were initially considered as random effects to account for variability within and between experimental units. However, when the initial model structure indicated singularity, suggesting that one of the random effects contributed negligible variance, the model was simplified by removing “plant” as a random term, ensuring parsimony and improving model stability. In addition to $AUDPC_{SEV}$ and final severity, plant height, total dry mass, shoot dry mass, and root dry mass were also analysed using this modelling approach.

To meet the assumptions of normality and homoscedasticity, disease-related variables were log-transformed using the function “log (x + 1)” prior to model fitting. Model assumptions regarding residual homogeneity, normality, and the distribution of random effects were evaluated using the “simulate_residuals ()” function from the *DHARMa* package (Hartig, 2016) and the “check_model ()” function from the *performance* package (Lüdecke et al., 2021). Analysis of variance were performed with the “Anova ()” function (*car* package, Fox and Weisberg, 2018) and marginal means were estimated with the “emmeans ()” function from the *emmeans* package (Lenth, 2017). Whenever significant differences were detected, post-hoc comparisons were performed using the Tukey’s test ($p < 0.05$) with the “cld ()” function from the *multcomp* package (Hothorn et al., 2008).

All data processing, statistical analyses, and visualizations were performed in R version 4.3.3 (R Core Team, 2024), with graphical outputs generated using the *ggplot2* package (Wickham, 2016).

3. Results

3.1. Disease severity and treatment efficacy

Severity assessment began at the onset of the first symptoms of ASR, 14 days after inoculation (DAI) with *P. pachyrhizi*. In the first (14 DAI) and second (21 DAI) assessments, all treatments showed similar mean severity values for the third and fourth trifoliate leaves (Fig. 1). This pattern remained unaltered for the third assessment (28 DAI), with the exception of the *Bacillus*-based treatment (T10), which exhibited higher mean severity as compared to all other treatments, including untreated control (T12). In the final assessment (35 DAI) (Fig. 2), more disease and higher severity means were again observed in plants treated with the *Bacillus* product (T10), whereas fungal antagonists produced distinct results. Plants treated with the *T. virens* (T03) and *F. coffeibaccae* (T09) isolates displayed severity levels similar to those of the untreated control (T12). Isolates of *C. cateniannulata* (T06), *C. rhizophaga* (T02), and *A. flavus* (T07) had severity values close to those observed in plants treated with the formulation (T11). Isolates of *C. hemileiae* (T08) and *T. harzianum* (T04) had comparable severity means, as did one of the isolates of *C. cateniannulata* (T05) and *C. rosea* (T01), which showed the lowest severity means in this assessment.

Successive severity assessments allowed the calculation of the $AUDPC_{SEV}$ for each treatment (Table 3), which was significantly affected by the treatments under non-controlled conditions ($p < 0.01$).

The lowest disease progress rate was observed in plants treated with *C. cateniannulata* (isolate E048, T05) and *C. rosea* (T01). Treatment with those candidate-antagonists led to significantly lower $AUDPC_{SEV}$ values (groups A and AB, respectively) than that of the commercial *B. velezensis*-based product (T10), which showed the highest $AUDPC_{SEV}$ (group D). No significant differences were observed between untreated plants (T12), plants treated with the formulation alone (T11), and those treated with *A. flavus* (T07), *F. coffeibaccae* (T09) or *T. virens* (T03), all of which had intermediate to high $AUDPC_{SEV}$ values (group C). The other treatments — *C. rhizophaga* (T02), *T. harzianum* (T04), *C. cateniannulata* (isolate LCBPF 17, T07), and *C. hemileiae* (T11) — yielded intermediate to low $AUDPC_{SEV}$ values (groups BC and ABC), but did not differ statistically from the untreated control.

Final severity means (Table 3) were strongly and positively correlated with $AUDPC_{SEV}$ ($r = 0.97$, $p < 0.01$). The lowest final severities were observed in soybean plants treated with *C. rosea* (T01) and *C. cateniannulata* (T05), which were statistically similar (group A), and

differed from the untreated plants (T12) (group BC). These treatments yielded the highest control efficacies, reducing disease levels by 62.46% and 62.37%, respectively, when compared with the untreated control (T12), and by 58.47% and 58.37%, respectively, when compared with the formulation-based treatment (T11).

In contrast, the commercial *B. velezensis*-based biofungicide (T10) had the highest final severity score and was statistically similar to the untreated control (group C), providing no ASR suppression under the conditions which this experiment was conducted. This treatment resulted in 86.43% more disease than the untreated control. All other treatments, including other fungal antagonists and the formulation-only treatment, yielded intermediate final severities (groups AB and B), with control efficacies ranging from 7.4% to 35.6%, when compared with the untreated control (T12).

3.2. Plant growth responses

Soybean plants treated with antagonists generally exhibited greater height than the untreated control (Table 4). Treatments such as *T. virens* (T03), *T. harzianum* (T04), *A. flavus* (T07). *F. coffeibaccae* (T09), and the commercial *B. velezensis*-based product (T10) formed a group (letters C and BC) that resulted in significantly taller plants than the untreated plants (T12, group A), resulting in height increases ranging from 19.95% to 31.14% compared to untreated plants (T12). The remaining treatments constituted an intermediary group (AB and ABC), not statistically different from the control, but still showing a numerical increase in plant height between 10.7% and 16.78%.

Conversely, total dry mass accumulation was negatively affected by several fungal treatments as compared to the control and showed no correlation with disease-related variables ($r < 0.3$). The highest total dry mass – comprising both shoot and root biomass – was recorded for untreated control plants (T12, group B). An intermediate group including *T. virens* (T03), *T. harzianum* (T04), *A. flavus* (T07), *F. coffeibaccae* (T09), the biofungicide (T10), and the formulation-only treatment (T11), showed values statistically similar to the control (group AB), although reductions ranged from 7.16% to 21.75%. The lowest total dry mass values (group A) were observed associated with treatments using *C. rosea* (T01), *C. rhizophaga* (T02), both isolates of *C. cateniannulata* (T05 and T06), and *C. hemileiae* (T08), resulting in reductions of up to 30.59% relative to the untreated plants.

Shoot dry mass (Table 4) showed a very strong positive correlation with total dry mass (r = 0.90, p < 0.01); however, no statistically significant differences were detected among treatments, including fungal antagonists, the biofungicide, and the controls (p = 0.1). Despite the lack of statistical significance, numerical reductions of up to 17% in shoot biomass were observed as compared to untreated plants.

Root dry mass (Table 4) had a moderate positive correlation with total dry mass (r = 0.66, p < 0.01) and a weak positive but significant correlation with shoot dry mass (r = 0.35, p < 0.05). The lowest root dry mass values were associated with treatments using *C. rosea* (T01), *C. rhizophaga* (T02), *T. virens* (T03), *T. harzianum* (T04), *C. cateniannulata* (T05 and T06), *C. hemileiae* (T08), and *F. coffeibaccae* (T09) (groups A, AB and ABC), with reductions ranging from 51.25% to 63.89% as compared to the untreated control (T12, group D). The remaining treatments, *B. velezensis*-based product (T10), *A. flavus* (T07), and the formulation (T11), showed intermediate root dry mass values and were statistically similar to the control (groups ABCD, BCD, and CD), although reductions of 21.48%, 30.94%, and 32.66%, respectively, were recorded.

3.3. Endophytic colonization by fungal antagonists

Most fungal antagonists applied to soybean plants were successfully reisolated from trifoliate leaf tissues (Table 5). Exceptions were plants that received a *C. cateniannulata* isolate (T06, LCBPF 17) or *T. virens* (T03). In general, the third trifoliate leaf was the most favorable for reisolation, with *F. coffeibaccae* (T09) showing the highest recovery rate (detected from 70.4% of the foliar fragments). Moderate recovery frequencies were also observed for *T. harzianum* (T04; 18.5%), *C. rhizophaga* (T02; 22.2%), and *C. rosea* (T01; 11.1%).

Fewer isolates were recovered from the fourth trifoliate leaf, with only four treatments yielding successful results: *A. flavus* (T07), *C. rhizophaga* (T02), *F. coffeibaccae* (T09), and *T. harzianum* (T04), with recovery rates of 7.4%, 11.1%, 22.2%, and 33.3%, respectively. From apical trifoliates, colonization was minimal. Only *T. harzianum* and *F. coffeibaccae* were reisolated, each detected in 3.7% of samples.

4. Discussion

The isolates evaluated in this study were selected based on previous research targeting *H. vastatrix* and the suppression of CLR under controlled conditions. From a larger collection, fungal antagonists were prioritized for their consistent performance under *in vitro* and greenhouse experiments. These included mycoparasitic fungi such as *Calonectria hemileiae* (Salcedo-Sarmiento et al., 2021) and *Fusarium coffeibaccae* (Nóbrega, 2021), which inhibited urediniospore germination and significantly reduced CLR severity, as well as isolates of *Clonostachys* spp. (Kapeua-Ndacnou et al., 2023a), *Trichoderma* spp. (Kapeua-Ndacnou, 2022; Rodríguez, 2018), and a non-aflatoxigenic *A. flavus* isolate (Kapeua-Ndacnou et al., 2023b), all of which showed promising disease suppression and high antagonistic activity in preliminary trials. One isolate of *C. cateniannulata*, was also included due to its ability to parasitize *H. vastatrix* uredinia and coffee pests, and inhibit urediniospore germination (Pereira et al., 2024a). However, as discussed in Chapter 1, some isolates exhibited reduced efficacy under potted trials in shaded environments or field experiments, highlighting the importance of evaluating biocontrol candidates beyond controlled environments.

Publications involving the evaluation of potential biocontrol candidates against fungal pathogens of plants normally focus on the antagonism of one agent against the target pathogen to which this was obtained in nature (Adams and Ayers, 1981; Budge and Whipps, 1991; Krauss et al., 2013; Moricca et al., 2001; Venkatesan et al., 2023). This approach has also characterized the work of our research group, with the exception of Pereira et al. (2024a), who tested *C. cateniannulata* against arthropods. All other cases involved the preliminarily evaluation of the potential of fungi obtained as endophytes of coffee plants or as mycoparasites of *H. vastatrix* as potential biocontrol agents of CLR. Here, a peripheral and innovative investigation explored the hypothesis that the so-called “elite isolates”, previously short-listed from the CLR-biocontrol studies and assessed against selected rust fungi (Chapter 2), might also possess biocontrol potential against ASR.

There are some well-known examples of broad-spectrum biocontrol fungi, including *Beauveria bassiana* (Mascarin and Jaronski, 2016), *Clonostachys* spp. (Reyes-Estebanez and Mendoza-de Gives, 2025), *Cordyceps* spp. (Shrestha et al., 2016), *Metarhizium* spp. (St. Leger and Wang, 2020), *Pochonia chlamydosporia* (Ciancio et al., 2016), *Purpureocillium lilacinum* (Rigobelo et al., 2024b), and *Trichoderma* spp. (Sood et al., 2020) for the control of insects, nematodes and suppression of fungal plant diseases. These are important, but nevertheless, limited examples, considering the broad biodiversity of mycoparasites and other fungal

antagonists of plant pathogenic fungi that exist in natural ecosystems. The question remains as to whether fungal antagonists are in general broad-range in terms of biocontrol effect or specialized. There is a general lack of examples of detailed investigations on the host-range of mycoparasites and it is unclear, at this stage, whether the observed effects obtained in this study are due to secondary metabolites produced by the isolates that were tested, by direct parasitism of the target rust or other form of interaction. Future pathogenicity tests, inclusive following Koch's postulates steps, as described by Salcedo-Sarmiento et al. (2021) and Pereira et al. (2024a), should be conducted that will help elucidating the pathological status towards *P. pachyrhizi* and also other rusts.

The significant inhibition of the germination of urediniospores of *A. psidii*, *P. pachyrhizi*, *P. recondita*, and *U. appendiculatus* (Chapter 2) justified the next-step presented here. Conidial suspensions of *Cordyceps cateniannulata* (E048, T06) and *C. rosea* (T01) were the most effective at reducing $AUDPC_{SEV}$ values of ASR by up to 50% and leading to final severity levels of up to 60% lower than control. These results were consistent to the high *in vitro* urediniospore-germination inhibition observed earlier for these two isolates (Chapter 2). These ranked among the top three isolates at reducing the germination of *P. pachyrhizi* isolates by 44% and 35%, respectively. In contrast, *F. coffeibaccae*, which appeared to be the most promising fungi among isolates during the *in vitro* test, had limited effect under field conditions when applied as a conidial suspension, with modest reductions in $AUDPC_{SEV}$ (5.66%) and final severity (17.1%).

This inconsistency may be attributed to differences in experimental conditions. While *in vitro* assays provide optimal and controlled environments that favour antagonist action, *in planta* and under open-environment conditions, numerous biotic and abiotic factors can interfere with the expression of antagonistic activity. These include interactions with the host plant, competition with resident phyllosphere microbiota, and environmental variables such as temperature and humidity fluctuations, wind, rain, and UV radiation, all of which may reduce the efficacy of foliar-applied antagonists (Boland, 1990; Collinge et al., 2022; Segarra et al., 2015; Tokas et al., 2023). Furthermore, the observed variability may be also associated with the activity of secondary metabolites produced by this antagonist. Supporting this hypothesis, parallel experiments indicated that *F. coffeibaccae* organic extracts applied at 5,000 µg/mL inhibited *P. pachyrhizi* urediniospore germination by 98% *in vitro*, and reduced $AUDPC_{SEV}$ and final severity by 57% and 59%, respectively, under potted trials (unpub. results, A.C. de Almeida).

The *B. velezensis*-based biofungicide had a poor performance under prevailing environmental conditions under which the experiment was conducted. These results are similar to those obtained in its evaluation as a treatment during CLR biocontrol experiments involving coffee plants maintained under shaded conditions (Chapter 1). This limited performance may be associated with factors such as inoculum concentration, application method, or post-inoculation conditions that favored pathogen germination and penetration. However, consistent with our findings, field trials conducted in Brazil during the 2020/21 and 2021/22 growing seasons indicated only a moderate efficacy of *Bacillus*-based biofungicides (Godoy et al., 2021, 2022). *Bacillus velezensis*-based biofungicides alone (2020/21) or a consortium of *B. subtilis*, *B. velezensis* and *B. pumilus* (2021/22) applications led to modest disease control efficacies – 11% and 14%, respectively. Nevertheless, when combined with systemic fungicides, control efficacy increased to 57% (2020/21) and 56% (2021/22), although no significant yield gains were observed compared to the chemical fungicide alone in those studies (Godoy et al., 2021, 2022). These findings highlight the need to carefully assess the cost-benefit of integrating biofungicides into disease management programs. However, their role in managing *P. pachyrhizi* resistance to site-specific fungicides provides a strong motivation for their use, not to mention their potential for promoting plant growth and suppressing other plant pathogens (Chinheya et al., 2017; dos Santos et al., 2024; Mosela et al., 2022; Zhou et al., 2024).

Results from the present work reinforces the experience-based knowledge that the efficacy of biocontrol agents varies depending on inoculum pressure, experimental conditions, environment factors, and the specific pathosystem. A valuable attribute of any biocontrol agent is its ability to be effective across multiple hosts and environmental scenarios. In this context, the effectiveness of *C. rosea* and *C. cateniannulata* in suppressing ASR indicates the potential value of isolates of those two species as broad-spectrum biocontrol candidates. Their efficacy should be further validated across different soybean cultivars and agroecological regions, and their activity against other plant pathogens should be investigated to fully assess their potential and reliability as biocontrol tools.

It is noteworthy that *Cordyceps* spp. are already recognized for their entomopathogenic potential, and commercial formulations based on *C. javanica* and *C. fumosorosea* are available and used for pest management. *Cordyceps cateniannulata* emerges as a particularly promising candidate due to its multitrophic activity. In addition to its efficacy against ASR, this species has demonstrated biocontrol activity against CLR (results published separately) and key coffee pests such as *Hypothenemus hampei* (coffee berry borer) and *Leucoptera coffeella* (coffee leaf

miner) (Pereira et al., 2024a). Further studies have also reported its effectiveness against other economically significant arthropod pests, including *Allantus luctifer* (Zhang et al., 2024), *Glycaspis brimblecombei* (Domingues et al., 2022), *Spodoptera frugiperda* (Zhou et al., 2020), *Stenoma impressella* (Montes-Bazurto et al., 2020), and the mite *Tetranychus urticae* (Guan et al., 2025).

To date, research and commercial biocontrol of ASR has mainly focused on *Bacillus* species (Dorighello et al., 2020, 2015; dos Santos et al., 2025, 2024; Twizeyimana et al., 2023; Twizeyimana and Hartman, 2019). However, the biocontrol effects of a few fungi have been studied since 1991. *Corniculantispora psalliotae* was reported as degrading *P. pachyrhizi* through the action of lytic enzymes and mycoparasitism (Saksirirat and Hoppe, 1991), and *Trichothecium roseum* as inhibiting germination of urediniospores of *P. pachyrhizi* (Kumar and Jha, 2002). *Simplicillium lanosoniveum* has also been tested against *P. pachyrhizi* both *in vitro*, in greenhouse, and in field experiments. Promising results were obtained, and its modes of action, including mycoparasitism and antibiosis, were documented (Gauthier et al., 2014; Ward et al., 2012, 2011). Unfortunately, no recent progresses have been reported on the continuation of those studies.

The rate of translation of extensive studies of potential biocontrol agents into practical applications as biocontrol products mass-produced by the industry is notoriously low. A case in point is that of *Clonostachys rosea.* Decades of intensive research led by many different groups of researchers worldwide, as recently listed in Nagaraj and Kolanthasamy (2025), dedicated to this seemingly biocontrol-champion species, have produced somewhat limited practical results. There are two *C. rosea*-based products patented in Canada – Vectorite® and Endofine® and, in Brazil, there is one product (Kamoi®, manufactured by Agrivalle) registered for use against gray mold (*Botrytis cinerea*). Despite being listed as registered in the official Brazilian website, AGROFIT (MAPA, 2025), this product does not appear in the product list at Agrivalle's website at this time. This overall situation is rather puzzling.

Other studies have also emphasized the biocontrol potential of certain fungi and their metabolites against ASR. For example, *Trichoderma* isolates and their secondary metabolite 6-pentyl-α-pyrone (6PAP) significantly reduced spore germination and rust severity (El-Hasan et al., 2022). *In vitro*, 6PAP, at 200 ppm completely inhibited urediniospore germination. In detached leaf assay, the metabolite suppressed diseased development by 98%, while conidial suspensions reduced rust disease by an average of 78%. On attached leaves, similar reductions were observed on treated tissues, while a lower yet notable decrease in disease was recorded on

adjacent and untreated leaves, with reductions of 47% on average associated with *Trichoderma* and 32% with 6PAP. Fungi better known as entomopathogens have also been tested against ASR. Similarly, culture filtrates of *M. anisopliae* and *M. humberi* inhibited *P. pachyrhizi* germination by up to 96% *in vitro*, while conidial suspensions had no effect (Holz et al., 2023). In greenhouse trials, conidial suspensions of *M. anisopliae* reduced rust symptoms by 51%, and culture filtrates of both species suppressed disease by an average of 86%. When applied to the substrate, only *M. anisopliae* reduced symptoms rust symptoms (by 40%) and was reisolated from the substrate and soybean roots.

These results suggest that, although unspecialized to *P. pachyrhizi*, under controlled conditions, both *Trichoderma* and *Metarhizium* species can reduce the development of ASR via direct (e.g., antibiosis) or indirect mechanisms (e.g., induced resistance). Considering the performance of our antagonists, it could be conjectured that their efficacy may similarly involve antibiosis or induced systemic resistance, and considering their original association to another rust fungus, perhaps even mycoparasitism. However, further studies are required to elucidate and confirm these modes of action.

Recent approaches in the biocontrol discipline have also explored the use of consortia of biological control agents (Nunes et al., 2024). In field trials conducted over two consecutive years (2021 and 2022), dos Santos et al. (2025) reported that bioproducts based on *B. velezensis* and *T. asperellum*, when applied alone or in combination, significantly reduced AUDPC values of three foliar soybean diseases: target spot (*Corynespora cassiicola*), brown spot (*Septoria glycines*), and ASR. The best results were achieved when *T. asperellum* was applied to crop debris, followed by a foliar application of *B. velezensis* and use of systemic fungicide, leading to reductions in disease severity and increases in yield. Considering this scheme of treatments, future studies could evaluate the potential of combining our fungal antagonists with *B. velezensis*-based products or chemical fungicides in the context of an integrated ASR management scheme.

Results observed in our investigation on the effect of our fungal isolates on soybean growth are mirrored by results obtained by authors who investigated other microbial systems such as for *Trichoderma* spp. (Battaglia et al., 2024; Bononi et al., 2020), *Purpureocillum lilacinum* (Rigobelo et al., 2024a), and a consortium involving *Beauveria bassiana*, *M. anisopliae*, *Pochonia chlamydosporia*, *P. lilacinum*, and *T. asperellum* (Farias et al., 2018). In our study, antagonist-based treatments slightly increased plant height but led to reductions in dry mass as compared to the untreated plants. Notably, root dry mass decreased by up to 62.17%

in plants treated with fungal antagonists, and by 21.48% for those treated with the *B. velezensis*-based product. These reductions may result from the potential phytotoxic effects of secondary metabolites produced by the antagonists. Some strains of the biocontrol fungi *C. militaris*, *C. rosea*, and *T. virens* are known to produce compounds such as cordycepin, dihydrovertinolide, and gliotoxin, respectively, which can inhibit plant development (Quy et al., 2019; Supratman et al., 2021; Zaid et al., 2022).

In addition, the observed reduction in biomass may reflect the metabolically costs associated with the establishment and maintenance of antagonists within soybean tissues, as well as the reallocation of metabolic resources toward the activation and expression of defense pathways (Chamnongpol et al., 1998; Walters et al., 2005). Another limiting factor involved in our experiment could be the lack of nodulating rhizobia, which were not inoculated on our plants and are well known to play a key role in nitrogen fixation, plant development, and yield (Nakei et al., 2022). It is important to acknowledge that we were conducting a preliminary study: plants were neither fertilized nor inoculated with *Bradyrhizobium* spp., nor grown to reproductive stages. Our primary objective was to explore the potential of the antagonists in a pathosystem distinct from their original target (*H. vastatrix* on coffee) and to observe possible reductions in disease levels. Given the promising results we have obtained, future studies should address the compatibility of our antagonists with *Bradyrhizobium* spp., the effects of rust and biological treatments on fertilized plants, and their impact on production.

The endophytic fungal communities of soybean plants have been extensively studied in countries such as Brazil, China, Korea, Argentina, and the USA. *Alternaria*, *Cladosporium* and *Fusarium* are among the most commonly isolated genera from healthy soybean roots, stems, leaves, and seeds, but isolates belonging to *Aspergillus*, *Calonectria*, *Clonostachys*, *Cordyceps*, and *Trichoderma* have also been obtained (Impullitti and Malvick, 2013; Kim et al., 2022; Pimentel et al., 2006; Russo et al., 2016; Stuart et al., 2018; Xiao et al., 2021; Yang et al., 2018, 2014). Some of these endophytic fungi have been evaluated for their potential to suppress seed-borne pathogen of soybean, such as *Cercospora* spp., *Diaporthe eres*, and *Septoria glycines* (Kim et al., 2022). Although endophytic fungi have already been obtained in China – the center of origin of soybean (Sedivy et al., 2017) – to the best of our knowledge, no study has investigated their potential to suppress *P. pachyrhizi*. Thus, based on the principles of classical biological control (Eilenberg et al., 2001; Scott, 1995) and the strategy of the long-lasting WCR-funded project aimed at CLR, searching for antagonistic fungi of *P. pachyrhizi* in its

center of origin, in East Asia (Hossain et al., 2024), remains a completely untapped opportunity that could yield co-evolved mycoparasites with high biological control potential against ASR.

During our attempts to reisolate the applied fungal antagonists from soybean tissues, a diversity of results has been obtained. Recovery rates varied dramatically from species to species and even between isolates of the same species and even for different isolation/ leaf position points. In general, higher reisolation rates were obtained from the third trifoliate, suggesting a decline in colonization or persistence toward the upper parts of treated plants. Only two species of antagonists were reisolated from apical leaves, *F. coffeibaccae* and *T. harzianum*, but both at low frequencies (3.7%). These results could be influenced by the sampling methodology, the tissue from which these fungi were reisolated, when plants were processed, or the inherent ability of each isolate to establish long-term associations with soybean tissues or to move along following plant growth. Other factors are reported to affect recovery rates, including plant age, environmental conditions, and soybean cultivars (Pimentel et al., 2006; Russo et al., 2016).

Still, *F. coffeibaccae* and *T. harzianum* may have a greater capacity to colonize soybean tissues. Although this was not directly correlated with rust control, the results expand the known ecological niches that some selected antagonists may occupy. Finding endophytic biocontrol isolates that might function as plant bodyguards remains one of the “holy grails” in the biocontrol discipline. It seems that there are no examples of practical applications of such bodyguards other than “endophyte-enhanced turfgrass” and a few other examples involving associations of *Epichloë* spp. with grasses. But this has been a topic of intense research along the last decades and the search for novel examples continues, since potential benefits include, besides protections against pests and diseases, others such as draught resistance and growth promotion (Murphy et al., 2018).

In conclusion, our results demonstrate that fungal antagonists obtained and selected to be deployed against *H. vastatrix*, when applied to soybean plants, can also reduce ASR severity. Results indicated that *C. rosea* and *C. cateniannulata* were the most effective and should be further investigated. Although larger and repeated field trials are needed to validate these findings, our experiments are of practical relevance, as they reflect environmental variability that likely influenced the outcomes, differently from the more artificial conditions of *in vitro* and greenhouse experiments. Future work should also investigate formulation development and compatibility (including component selection and concentrations), optimization of antagonist application (e.g., conidia concentrations and spraying intervals), evaluation of consortia of

isolates, and potential combinations with chemical fungicides. Such efforts will be essential to unlocking the full potential of these fungal antagonists and advancing their practical use in sustainable ASR control strategies.

5. References

Adams, P.B., Ayers, W.A., 1981. *Sporidesmium sclerotivorum:* Distribution and Function in Natural Biological Control of Sclerotial Fungi. Phytopathology 71, 90. https://doi.org/10.1094/Phyto-71-90

Aguirre, K., Perez, L.M., Montealegre, J.R., 2023. *In vitro* evaluation of drying supports and adhesive polymers as adjuvants for biocontrol of *Diplodia seriata* by *Trichoderma harzianum* and *Clonostachys rosea*. Phytopathol Mediterr 60, 227–237. https://doi.org/10.36253/phyto-14096

Alves, K.S., Del Ponte, E.M., 2021. Analysis and simulation of plant disease progress curves in R: introducing the epifitter package. Phytopathology Research 3, 22. https://doi.org/10.1186/s42483-021-00098-7

Bai, Y., Zhou, X., Smith, D.L., 2003. Enhanced Soybean Plant Growth Resulting from Coinoculation of *Bacillus* Strains with *Bradyrhizobium japonicum*. Crop Sci 43, 1774–1781. https://doi.org/10.2135/cropsci2003.1774

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H., Dai, B., Grothendieck, G., Green, P., Bolker, M. Ben, 2015. Package 'lme4.' convergence 12, 2.

Battaglia, M.E., Martinez, S.I., Covacevich, F., Consolo, V.F., 2024. *Trichoderma harzianum* enhances root biomass production and promotes lateral root growth of soybean and common bean under drought stress. Annals of Applied Biology 185, 36–48. https://doi.org/10.1111/aab.12909

Boland, G.J., 1990. Biological control of plant diseases with fungal antagonists: Challenges and opportunities. Canadian Journal of Plant Pathology 12, 295–299. https://doi.org/10.1080/07060669009501003

Bononi, L., Chiaramonte, J.B., Pansa, C.C., Moitinho, M.A., Melo, I.S., 2020. Phosphorus-solubilizing *Trichoderma* spp. from Amazon soils improve soybean plant growth. Sci Rep 10, 2858. https://doi.org/10.1038/s41598-020-59793-8

Budge, S.P., Whipps, J.M., 1991. Glasshouse trials of *Coniothyrium minitans* and *Trichoderma species* for the biological control of *Sclerotinia sclerotiorum* in celery and lettuce. Plant Pathol 40, 59–66. https://doi.org/10.1111/j.1365-3059.1991.tb02293.x

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Van Montagu, M., Inzé, D., Van Camp, W., 1998. Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. Proceedings of the National Academy of Sciences 95, 5818–5823. https://doi.org/10.1073/pnas.95.10.5818

Chicowski, A.S., Bredow, M., Utiyama, A.S., Marcelino-Guimarães, F.C., Whitham, S.A., 2024. Soybean-*Phakopsora pachyrhizi* interactions: towards the development of next-generation disease-resistant plants. Plant Biotechnol J 22, 296–315. https://doi.org/10.1111/pbi.14206

Chinheya, C.C., Yobo, K.S., Laing, M.D., 2017. Biological control of the rootknot nematode, *Meloidogyne javanica* (Chitwood) using *Bacillus* isolates, on soybean. Biological Control 109, 37–41. https://doi.org/10.1016/j.biocontrol.2017.03.009

Ciancio, A., Colagiero, M., Pentimone, I., Rosso, L.C., 2016. Formulation of *Pochonia chlamydosporia* for Plant and Nematode Management, in: Bioformulations: For Sustainable Agriculture. Springer India, New Delhi, pp. 177–197. https://doi.org/10.1007/978-81-322-2779-3_10

Collinge, D.B., Jensen, D.F., Rabiey, M., Sarrocco, S., Shaw, M.W., Shaw, R.H., 2022. Biological control of plant diseases – What has been achieved and what is the direction? Plant Pathol 71, 1024–1047. https://doi.org/10.1111/ppa.13555

Colmán, A.A., Evans, H.C., Salcedo-Sarmiento, S.S., Braun, U., Belachew-Bekele, K., Barreto, R.W., 2021. A fungus-eat-fungus world: *Digitopodium*, with particular reference to mycoparasites of the coffee leaf rust, *Hemileia vastatrix*. IMA Fungus 12, 1. https://doi.org/10.1186/s43008-020-00052-w

Devi, K., Joshi, N., Sangha, K.S., 2018. Oils as UV protectants of *Beauveria bassiana* conidia and bioefficacy against *Lipaphis erysimi* (Kalt). Indian Journal of Entomology 80, 761. https://doi.org/10.5958/0974-8172.2018.00189.X

Domingues, M.M., Santos, P.L., Gêa, B.C.C., Carvalho, V.R., Oliveira, F.N., Soliman, E.P., Pereira, F.F., Zanuncio, J.C., Wilcken, C.F., 2022. *Cordyceps cateniannulata* and *Cordyceps javanica*: first report of pathogenicity to *Glycaspis brimblecombei* (Hemiptera: Aphalaridae). An Acad Bras Cienc 94. https://doi.org/10.1590/0001-3765202220211566

Dorighello, D.V., Bettiol, W., Maia, N.B., de Campos Leite, R.M.V.B., 2015. Controlling Asian soybean rust (*Phakopsora pachyrhizi*) with *Bacillus* spp. and coffee oil. Crop Protection 67, 59–65. https://doi.org/10.1016/j.cropro.2014.09.017

Dorighello, D.V., Forner, C., de Campos Leite, R.M.V.B., Bettiol, W., 2020. Management of Asian soybean rust with *Bacillus subtilis* in sequential and alternating fungicide applications. Australasian Plant Pathology 49, 79–86. https://doi.org/10.1007/s13313-019-00677-5

dos Santos, D.K.Q., dos Santos, F.M., Roso, A.S., Camargo, D.P., de Souza Viera, L., Morandini, G.B., Santos, J.R.P., da Silva, J.C.P., 2025. Friends or foes: co-application of *Trichoderma*- and *Bacillus*-based products with chemicals to manage soybean foliar diseases. BioControl 70, 257–270. https://doi.org/10.1007/s10526-024-10298-7

dos Santos, F.M., de Souza Viera, L., Camargo, D.P., Queiroz dos Santos, D.K., Brião Muniz, M.F., Dressler da Costa, I.F., Pereira Santos, J.R., Pereira da Silva, J.C., 2024. *Bacillus*-based product protects soybean, improves grain yield and produces metabolites able to promote plant growth and antagonize pathogenic fungi. Journal of Phytopathology 172. https://doi.org/10.1111/jph.13353

Eilenberg, J., Hajek, A., Lomer, C., 2001. Suggestions for unifying the terminology in biological control. BioControl 46, 387–400. https://doi.org/10.1023/A:1014193329979

El-Hasan, A., Walker, F., Klaiber, I., Schöne, J., Pfannstiel, J., Voegele, R.T., 2022. New Approaches to Manage Asian Soybean Rust (*Phakopsora pachyrhizi*) Using *Trichoderma* spp. or Their Antifungal Secondary Metabolites. Metabolites 12, 507. https://doi.org/10.3390/metabo12060507

Farias, C.P., Carvalho, R.C. De, Resende, F.M.L., Azevedo, L.C.B., 2018. Consortium of five fungal isolates conditioning root growth and arbuscular mycorrhiza in soybean, corn, and sugarcane. An Acad Bras Cienc 90, 3649–3660. https://doi.org/10.1590/0001-3765201820180161

Fontes, B.A., Silva, L.C., Picanço, B.B.M., Barros, A.V., Leal, I.M.G., Quadros, L.P., Rodrigues, F.Á., 2024. Resistance in Soybean Against Infection by *Phakopsora pachyrhizi* Is Induced by a Phosphite of Nickel and Potassium. Plants 13, 3161. https://doi.org/10.3390/plants13223161

Fox, J., Weisberg, S., 2018. An R companion to applied regression. Sage publications.

Freire, M.C.M., Oliveira, L.O. de, Almeida, Á.M.R. de, Schuster, I., Moreira, M.A., Liebenberg, M.M., Mienie, C.M.S., 2008. Evolutionary history of *Phakopsora pachyrhizi* (the Asian soybean rust) in Brazil based on nucleotide sequences of the internal transcribed spacer region of the nuclear ribosomal DNA. Genet Mol Biol 31, 920–931. https://doi.org/10.1590/S1415-47572008005000026

Gauthier, N.W., Maruthachalam, K., Subbarao, K. V., Brown, M., Xiao, Y., Robertson, C.L., Schneider, R.W., 2014. Mycoparasitism of *Phakopsora pachyrhizi*, the soybean rust pathogen, by *Simplicillium lanosoniveum*. Biological Control 76, 87–94. https://doi.org/10.1016/j.biocontrol.2014.05.008

Godoy, C.V., Seixas, C.D.S., Meyer, M.C., Soares, R.M., 2020. Ferrugem-asiática da soja: bases para o manejo da doença e estratégias antirresistência.

Godoy, C.V., Utiamada, C.M., Meyer, M.C., Campos, H.D., Lopes, I. de O.N., Tomen, A., Muhl, A., Schipanski, C.A., Serciloto, C.M., Junior, E.R. de A., Moresco, E., Roy, J.M.T., Navarini, L., Belufi, L.M. de R., da Silva, L.H.C.P., Júnior, I.P.A., Fantin, L.H., Sato, L.N., Júnior, M.M.G., Senger, M., Müller, M.A., Debortoli, M.P., Martins, M.C., Carlin, V.J., 2021. Eficiência de fungicidas multissítios e produto biológico no controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2020/2021: resultados sumarizados dos experimentos cooperativos.

Godoy, C. V, Utiamada, C.M., Meyer, M.C., Campos, H.D., Lopes, I., Mochko, A.C.R., Farias, A. de, Muhl, A., Tomen, A., Dias, A.R., 2022. Eficiência de fungicidas para o controle das doenças de final de ciclo da soja, na safra 2021/2022: Resultados sumarizados dos ensaios cooperativos.

Goellner, K., Loehrer, M., Langenbach, C., Conrath, U., Koch, E., Schaffrath, U., 2010. *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Mol Plant Pathol 11, 169–177. https://doi.org/10.1111/j.1364-3703.2009.00589.x

Grewal, G.K., Joshi, N., 2022. Evaluation of adjuvants on growth and virulence of *Metarhizium rileyi* against *Spodoptera litura* (F.). Indian Journal of Entomology 1–4. https://doi.org/10.55446/IJE.2021.358

Guan, J., Liu, J., Gou, J., Xu, Z., Zhou, Y., Zou, X., 2025. Endophytic colonization via *Cordyceps cateniannulata* induces a growth-enhancing effect and increases the resistance of tomato plants against *Tetranychus urticae* (Koch). Crop Protection 193, 107182. https://doi.org/10.1016/j.cropro.2025.107182

Guterres, D.C., Ndacnou, M.K., Saavedra-Tobar, L.M., Salcedo-Sarmiento, S., Colmán, A.A., Evans, H.C., Barreto, R.W., 2021. *Cryptococcus depauperatus*, a close relative of the human-pathogen *C. neoformans*, associated with coffee leaf rust (*Hemileia vastatrix*) in Cameroon. Brazilian Journal of Microbiology 52, 2205–2214. https://doi.org/10.1007/s42770-021-00592-2

Hartig, F., 2016. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. CRAN: Contributed Packages.

Hemalatha, S., Ramaraju, K., Jeyarani, S., 2017. Influence of ultraviolet light protectants on the persistence of the entomopathogenic fungus *Beauveria bassiana*. Journal of Biopesticides 10, 130–134.

Holz, S., D'Alessandro, C.P., Maximo, H.J., Nascimento de Souza, P.H., Raruang, Y., Demétrio, C.G.B., Delalibera Júnior, I., Chen, Z.-Y., Pascholati, S.F., 2023. The potential of using *Metarhizium anisopliae* and *Metarhizium humberi* to control the Asian soybean rust caused by *Phakopsora pachyrhizi*. Biocontrol Sci Technol 33, 366–382. https://doi.org/10.1080/09583157.2023.2191299

Hossain, Md.M., Sultana, F., Mostafa, M., Adhikary, S., Yamanaka, N., 2025. Advancing soybean rust resistance: Strategies, mechanisms, and innovations in gene pyramiding. Physiol Mol Plant Pathol 139, 102770. https://doi.org/10.1016/j.pmpp.2025.102770

Hossain, Md.M., Sultana, F., Yesmin, L., Rubayet, Md.T., Abdullah, H.M., Siddique, S.S., Bhuiyan, Md.A.B., Yamanaka, N., 2024. Understanding *Phakopsora pachyrhizi* in soybean: comprehensive insights, threats, and interventions from the Asian perspective. Front Microbiol 14. https://doi.org/10.3389/fmicb.2023.1304205

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. Biometrical Journal 50, 346–363. https://doi.org/10.1002/bimj.200810425

Impullitti, A.E., Malvick, D.K., 2013. Fungal endophyte diversity in soybean. J Appl Microbiol 114, 1500–1506. https://doi.org/10.1111/jam.12164

Kapeua-Ndacnou, M., 2022. Isolates of *Aspergillus*, *Clonostachys* and *Trichoderma* from Africa as potential biocontrol agents against coffee leaf rust (Thesis). Universidade Federal de Viçosa, Viçosa - MG. https://doi.org/10.47328/UFVBBT.2022.194

Kapeua-Ndacnou, M., de Abreu, L.M., de Macedo, D.M., da Nóbrega, T.F., Pereira, C.M., Evans, H.C., Barreto, R.W., 2023a. Assessing the Biocontrol Potential of *Clonostachys* Species Isolated as Endophytes from Coffea Species and as Mycoparasites of *Hemileia Rusts* of Coffee in Africa. Journal of Fungi 9. https://doi.org/10.3390/jof9020248

Kapeua-Ndacnou, M., Nóbrega, T.F., Batista, L.R., Evans, H.C., Abreu, L.M., Begoudé, D.A.B., Reis, T.A., Barreto, R.W., 2023b. *Aspergillus flavus* from coffee in Cameroon: a non-

aflatoxigenic endophytic isolate antagonistic to coffee leaf rust (*Hemileia vastatrix*). J Appl Microbiol 134, lxad076. https://doi.org/10.1093/jambio/lxad076

Killgore, E., Heu, R., 1994. First Report of Soybean Rust in Hawaii. Plant Dis 78, 1216B. https://doi.org/10.1094/PD-78-1216B.

Kim, J., Roy, M., Ahn, S.-H., Shanmugam, G., Yang, J.S., Jung, H.W., Jeon, J., 2022. Culturable Endophytes Associated with Soybean Seeds and Their Potential for Suppressing Seed-Borne Pathogens. Plant Pathol J 38, 313–322. https://doi.org/10.5423/PPJ.OA.05.2022.0064

Krauss, U., ten Hoopen, M., Rees, R., Stirrup, T., Argyle, T., George, A., Arroyo, C., Corrales, E., Casanoves, F., 2013. Mycoparasitism by *Clonostachys byssicola* and *Clonostachys rosea* on *Trichoderma* spp. from cocoa (*Theobroma cacao*) and implication for the design of mixed biocontrol agents. Biological Control 67, 317–327. https://doi.org/10.1016/j.biocontrol.2013.09.011

Kumar, S., Jha, D.K., 2002. *Trichothecium roseum*: a potential agent for the biological control of soybean rust. Indian Phytopathol 55, 232–234.

Lenth, R. V., 2017. emmeans: Estimated Marginal Means, aka Least-Squares Means. CRAN: Contributed Packages. https://doi.org/10.32614/CRAN.PACKAGE.EMMEANS

Lüdecke, D., Ben-Shachar, M., Patil, I., Waggoner, P., Makowski, D., 2021. performance: An R Package for Assessment, Comparison and Testing of Statistical Models. J Open Source Softw 6, 3139. https://doi.org/10.21105/joss.03139

MAPA (Ministério da Agricultura e Pecuária), 2025. Agrofit: sistema de Agrotóxicos Fitossanitário. https://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons (accessed 7.19.25).

Mascarin, G.M., Jaronski, S.T., 2016. The production and uses of *Beauveria bassiana* as a microbial insecticide. World J Microbiol Biotechnol 32, 177. https://doi.org/10.1007/s11274-016-2131-3

Montes-Bazurto, L.G., Bustillo-Pardey, A.E., Medina-Cárdenas, H.C., 2020. *Cordyceps cateniannulata*, a novel entomopathogenic fungus to control *Stenoma impressella* Busck (Lepidoptera: Elachistidae) in Colombia. Journal of Applied Entomology 144, 788–796. https://doi.org/10.1111/jen.12818

Moricca, S., Ragazzi, A., Mitchelson, K.R., Assante, G., 2001. Antagonism of the Two-Needle Pine Stem Rust Fungi *Cronartium flaccidum* and *Peridermium pini* by *Cladosporium tenuissimum*

In Vitro and *In Planta*. Phytopathology 91, 457–468. https://doi.org/10.1094/PHYTO.2001.91.5.457

Mosela, M., Andrade, G., Massucato, L.R., de Araújo Almeida, S.R., Nogueira, A.F., de Lima Filho, R.B., Zeffa, D.M., Mian, S., Higashi, A.Y., Shimizu, G.D., Teixeira, G.M., Branco, K.S., Faria, M.V., Giacomin, R.M., Scapim, C.A., Gonçalves, L.S.A., 2022. *Bacillus velezensis* strain Ag75 as a new multifunctional agent for biocontrol, phosphate solubilization and growth promotion in maize and soybean crops. Sci Rep 12, 15284. https://doi.org/10.1038/s41598-022-19515-8

Murphy, B., Doohan, F., Hodkinson, T., 2018. From Concept to Commerce: Developing a Successful Fungal Endophyte Inoculant for Agricultural Crops. Journal of Fungi 4, 24. https://doi.org/10.3390/jof4010024

Nagaraj, G., Kolanthasamy, E., 2025. Unveiling the antimicrobial and biocontrol potential of the Ascomycete fungus, *Clonostachys rosea*: A review. The Microbe 6, 100226. https://doi.org/10.1016/j.microb.2024.100226

Nakei, M.D., Venkataramana, P.B., Ndakidemi, P.A., 2022. Soybean-Nodulating Rhizobia: Ecology, Characterization, Diversity, and Growth Promoting Functions. Front Sustain Food Syst 6. https://doi.org/10.3389/fsufs.2022.824444

Nóbrega, T.F., 2021. *Fusarium* spp. antagonists to *Hemileia vastatrix* from Brazil, Paraguay and Africa: Taxonomy and biocontrol potential (Thesis). Universidade Federal de Viçosa, Viçosa.

Nunes, P.S.O., Lacerda-Junior, G. V., Mascarin, G.M., Guimarães, R.A., Medeiros, F.H.V., Arthurs, S., Bettiol, W., 2024. Microbial consortia of biological products: Do they have a future? Biological Control 188, 105439. https://doi.org/10.1016/j.biocontrol.2024.105439

Pereira, C.M., Bautz, K.R., Rodríguez, M. del C.H., Saavedra-Tobar, L.M., Kapeua-Ndacnou, M., Belachew-Bekele, K., Elliot, S.L., Evans, H.C., Barreto, R.W., 2024a. *Cordyceps cateniannulata*: An endophyte of coffee, a parasite of coffee leaf rust and a pathogen of coffee pests. Fungal Biol 128, 1917–1932. https://doi.org/10.1016/j.funbio.2024.05.004

Pereira, C.M., Sarmiento, S.S., Colmán, A.A., Belachew-Bekele, K., Evans, H.C., Barreto, R.W., 2024b. Mycodiversity in a micro-habitat: twelve *Cladosporium* species, including four new taxa, isolated from uredinia of coffee leaf rust, *Hemileia vastatrix*. Fungal Syst Evol 14, 9–33. https://doi.org/10.3114/fuse.2024.14.02

Pimentel, I.C., Glienke-Blanco, C., Gabardo, J., Stuart, R.M., Azevedo, J.L., 2006. Identification and colonization of endophytic fungi from soybean (*Glycine max* (L.) Merril) under different environmental conditions. Brazilian Archives of Biology and Technology 49, 705–711. https://doi.org/10.1590/S1516-89132006000600003

Posadas, J.B., Maricel, A.L., Mini Jorge, I., Lecuona Roberto, E., 2012. Natural tolerance to UV-B and assessment of photoprotectants in conidia of six native isolates of *Beauveria bassiana* (Bals-Criv) Vuillemin. World Appl Sci J 20, 1024–1030.

Quy, T.N., Xuan, T.D., Andriana, Y., Tran, H.-D., Khanh, T.D., Teschke, R., 2019. Cordycepin Isolated from *Cordyceps militaris*: Its Newly Discovered Herbicidal Property and Potential Plant-Based Novel Alternative to Glyphosate. Molecules 24, 2901. https://doi.org/10.3390/molecules24162901

R Core Team, 2024. R: A Language and Environment for Statistical Computing.

Reis, E.M., Carlini, R. de C., Zanatta, M., 2022. Optimal, lower and upper temperature thresholds for uredospore germination and germ tube growth of *Phakopsora pachyrhizi*. Summa Phytopathol 48, 25–27. https://doi.org/10.1590/0100-5405/192967

Reyes-Estebanez, M., Mendoza-de Gives, P., 2025. The Genus *Clonostachys* (Bionectria) as a Potential Tool Against Agricultural Pest and Other Biotechnological Applications: A Review. Microbiol Res (Pavia) 16, 86. https://doi.org/10.3390/microbiolres16040086

Rigobelo, E.C., de Carvalho, L.A.L., Santos, C.H.B., Frezarin, E.T., Pinheiro, D.G., Nicodemo, D., Babalola, O.O., Desoignies, N., 2024a. Growth promotion and modulation of the soybean microbiome INTACTA RR PRO with the application of the fungi *Trichoderma harzianum* and *Purpureocillum lilacinum*. Sci Rep 14, 21004. https://doi.org/10.1038/s41598-024-71565-2

Rigobelo, E.C., Nicodemo, D., Babalola, O.O., Desoignies, N., 2024b. *Purpureocillium lilacinum* as an Agent of Nematode Control and Plant Growth-Promoting Fungi. Agronomy 14, 1225. https://doi.org/10.3390/agronomy14061225

Rodríguez, M. del C.H., 2018. *Trichoderma* spp. associated with coffee in Africa: taxonomy and potential uses for the biological control of coffee leaf rust (*Hemileia vastatrix*) and promotion of plant growth (Thesis). Universidade Federal de Viçosa, Viçosa.

Rodríguez, M. del C.H., Evans, H.C., de Abreu, L.M., de Macedo, D.M., Ndacnou, M.K., Bekele, K.B., Barreto, R.W., 2021. New species and records of *Trichoderma* isolated as mycoparasites

and endophytes from cultivated and wild coffee in Africa. Sci Rep 11, 5671. https://doi.org/10.1038/s41598-021-84111-1

Rupe, J., Sconyers, L., 2008. Soybean Rust. Plant Health Instructor. https://doi.org/10.1094/PHI-I-2008-0401-01

Russo, M.L., Pelizza, S.A., Cabello, M.N., Stenglein, S.A., Vianna, M.F., Scorsetti, A.C., 2016. Endophytic fungi from selected varieties of soybean (*Glycine max* L. Merr.) and corn (*Zea mays* L.) grown in an agricultural area of Argentina. Rev Argent Microbiol 48, 154–160. https://doi.org/10.1016/j.ram.2015.11.006

Saksirirat, W., Hoppe, H. -H., 1991. Secretion of Extracellular Enzymes by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm.) Viégas During Growth on Uredospores of the Soybean Rust Fungus (*Phakopsora pachyrhizi* Syd.) in Liquid Cultures. Journal of Phytopathology 131, 161–173. https://doi.org/10.1111/j.1439-0434.1991.tb04741.x

Salcedo-Sarmiento, S., Aucique-Pérez, C.E., Silveira, P.R., Colmán, A.A., Silva, A.L., Corrêa Mansur, P.S., Rodrigues, F.Á., Evans, H.C., Barreto, R.W., 2021. Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria mycoparasite* and the coffee plant. iScience 24, 102352. https://doi.org/https://doi.org/10.1016/j.isci.2021.102352

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N., Nelson, A., 2019. The global burden of pathogens and pests on major food crops. Nat Ecol Evol 3, 430–439. https://doi.org/10.1038/s41559-018-0793-y

Scott, J.K., 1995. 7 Classical biological control of plant pathogens. pp. 131–146. https://doi.org/10.1016/S0736-4539(06)80009-7

Sedivy, E.J., Wu, F., Hanzawa, Y., 2017. Soybean domestication: the origin, genetic architecture and molecular bases. New Phytologist 214, 539–553. https://doi.org/10.1111/nph.14418

Segarra, G., Puopolo, G., Giovannini, O., Pertot, I., 2015. Stepwise flow diagram for the development of formulations of non-spore-forming bacteria against foliar pathogens: The case of *Lysobacter capsici* AZ78. J Biotechnol 216, 56–64. https://doi.org/10.1016/j.jbiotec.2015.10.004

Shrestha, B., Tanaka, E., Hyun, M.W., Han, J.-G., Kim, C.S., Jo, J.W., Han, S.-K., Oh, J., Sung, G.-H., 2016. Coleopteran and Lepidopteran Hosts of the Entomopathogenic Genus *Cordyceps sensu lato*. J Mycol 2016, 1–14. https://doi.org/10.1155/2016/7648219

Sood, M., Kapoor, D., Kumar, V., Sheteiwy, M.S., Ramakrishnan, M., Landi, M., Araniti, F., Sharma, A., 2020. *Trichoderma*: The "Secrets" of a Multitalented Biocontrol Agent. Plants 9, 762. https://doi.org/10.3390/plants9060762

St. Leger, R.J., Wang, J.B., 2020. *Metarhizium*: jack of all trades, master of many. Open Biol 10. https://doi.org/10.1098/rsob.200307

Stuart, A.K. da C., Stuart, R.M., Pimentel, I.C., 2018. Effect of agrochemicals on endophytic fungi community associated with crops of organic and conventional soybean (*Glycine max* L. Merril). Agriculture and Natural Resources 52, 388–392. https://doi.org/10.1016/j.anres.2018.10.005

Supratman, U., Suzuki, T., Nakamura, T., Yokoyama, Y., Harneti, D., Maharani, R., Salam, S., Abdullah, F.F., Koseki, T., Shiono, Y., 2021. New metabolites produced by endophyte *Clonostachys rosea* B5 − 2. Nat Prod Res 35, 1525–1531. https://doi.org/10.1080/14786419.2019.1656629

Tokas, D., Singh, S., Yadav, R., Singh, A.N., 2023. Plant-microbe interactions: Role in sustainable agriculture and food security in a changing climate, in: Plant-Microbe Interaction - Recent Advances in Molecular and Biochemical Approaches. Elsevier, pp. 363–391. https://doi.org/10.1016/B978-0-323-91876-3.00008-7

Twizeyimana, M., Hammer, P.E., Gachango, E., Craig, K., Espejo, B., Biggs, M.B., Kremer, J., Ingham, D.J., 2023. Diverse environmental bacteria displaying activity against *Phakopsora pachyrhizi*, the cause of soybean rust. Front Plant Sci 14. https://doi.org/10.3389/fpls.2023.1080116

Twizeyimana, M., Hartman, G.L., 2019. Effect of Selected Biopesticides in Reducing Soybean Rust (*Phakopsora pachyrhizi*) Development. Plant Dis 103, 2460–2466. https://doi.org/10.1094/PDIS-02-19-0384-RE

Venkatesan, R.M., Muthusamy, K., Iruthayasamy, J., Prithiviraj, B., Kumaresan, P. V., Lakshmanan, P., Perianadar, I.V., 2023. First Report of *Clonostachys rosea* as a Mycoparasite on *Sclerotinia sclerotiorum* Causing Head Rot of Cabbage in India. Plants 12, 199. https://doi.org/10.3390/plants12010199

Walters, D., Walsh, D., Newton, A., Lyon, G., 2005. Induced Resistance for Plant Disease Control: Maximizing the Efficacy of Resistance Elicitors. Phytopathology 95, 1368–1373. https://doi.org/10.1094/PHYTO-95-1368

Ward, N.A., Robertson, C.L., Chanda, A.K., Schneider, R.W., 2012. Effects of *Simplicillium lanosoniveum* on *Phakopsora pachyrhizi*, the Soybean Rust Pathogen, and Its Use as a Biological Control Agent. Phytopathology 102, 749–760. https://doi.org/10.1094/PHYTO-01-11-0031

Ward, N.A., Schneider, R.W., Aime, M.C., 2011. Colonization of soybean rust sori by *Simplicillium lanosoniveum*. Fungal Ecol 4, 303–308. https://doi.org/10.1016/j.funeco.2011.03.008

Wickham, H., 2016. Data Analysis. pp. 189–201. https://doi.org/10.1007/978-3-319-24277-4_9

Xiao, J., Sun, J.-G., Pang, B., Zhou, X., Gong, Y., Jiang, L., Zhang, L., Ding, X., Yin, J., 2021. Isolation and screening of stress-resistant endophytic fungus strains from wild and cultivated soybeans in cold region of China. Appl Microbiol Biotechnol 105, 755–768. https://doi.org/10.1007/s00253-020-11048-2

Yang, H., Ye, W., Ma, J., Zeng, D., Rong, Z., Xu, M., Wang, Y., Zheng, X., 2018. Endophytic fungal communities associated with field-grown soybean roots and seeds in the Huang-Huai region of China. PeerJ 6, e4713. https://doi.org/10.7717/peerj.4713

Yang, Y., Yan, M., Hu, B., 2014. Endophytic Fungal Strains of Soybean for Lipid Production. Bioenergy Res 7, 353–361. https://doi.org/10.1007/s12155-013-9377-5

Yorinori, J.T., Paiva, W.M., Frederick, R.D., Costamilan, L.M., Bertagnolli, P.F., Hartman, G.E., Godoy, C. V., Nunes, J., 2005. Epidemics of Soybean Rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Dis 89, 675–677. https://doi.org/10.1094/PD-89-0675

Zaid, R., Koren, R., Kligun, E., Gupta, R., Leibman-Markus, M., Mukherjee, P.K., Kenerley, C.M., Bar, M., Horwitz, B.A., 2022. Gliotoxin, an Immunosuppressive Fungal Metabolite, Primes Plant Immunity: Evidence from *Trichoderma virens*-Tomato Interaction. mBio 13. https://doi.org/10.1128/mbio.00389-22

Zhang, X., Wu, Y., Peng, X., Liu, C., Yang, G., Chen, Q., Jin, D., 2024. *Cordyceps cateniannulata*, a new potential strain for controlling *Allantus luctifer* from China. PeerJ 12, e18345. https://doi.org/10.7717/peerj.18345

Zhou, Y., Chen, J., Feng, Y., Xiang, P., Li, J., Chen, L., Guo, Y., 2024. Biocontrol Potential of *Bacillus* strains against soybean cyst nematode (*Heterodera glycines*) and for promotion of soybean growth. BMC Microbiol 24, 371. https://doi.org/10.1186/s12866-024-03514-y

Zhou, Y.-M., Xie, W., Ye, J.-Q., Zhang, T., Li, D.-Y., Zhi, J.-R., Zou, X., 2020. New potential strains for controlling *Spodoptera frugiperda* in China: *Cordyceps cateniannulata* and *Metarhizium rileyi*. BioControl 65, 663–672. https://doi.org/10.1007/s10526-020-10035-w

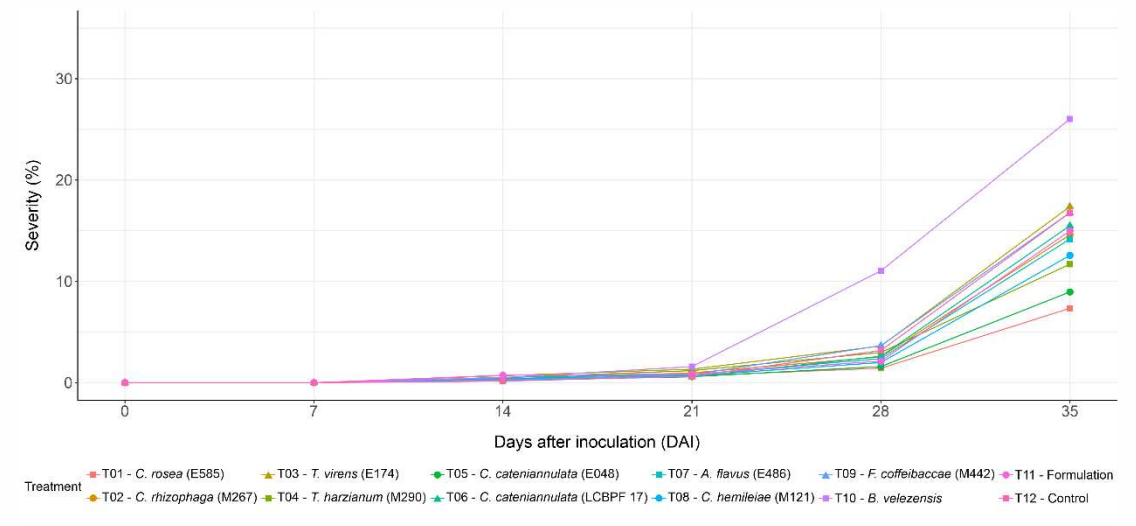

Figure 1. Progress curve of severity (%) of Asian soybean rust in soybean plants (*Glycine max* cv. 'IAC Foscarin 31') treated with antagonists and inoculated with *Phakopsora pachyrhizi*. T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *C. cateniannulata* (LCBPF 17); T07 = *Aspergillus flavus* (E486); T08 = *Calonectria hemileiae* (M121); T09 = *Fusarium coffeibaccae* (M442); T10 = Commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

Figure 2. Soybean plants (*Glycine max* cv. 'IAC Foscarin 31') 35 days after inoculation with *Phakopsora pachyrhizi* and treated with: (A) *Clonostachys rosea* (E585); (B) *C. rhizophaga* (M267); (C) = *Trichoderma virens* (E174); (D) *T. harzianum* (M290); (E) *Cordyceps cateniannulata* (E048); (F) *C. cateniannulata* (LCBPF 17); (G) *Aspergillus flavus* (E486); (H) *Calonectria hemileiae* (M121); (I) *Fusarium coffeibaccae* (M442); (J) Commercial product based on *Bacillus velezensis*; (K) formulation; and (L) untreated control.

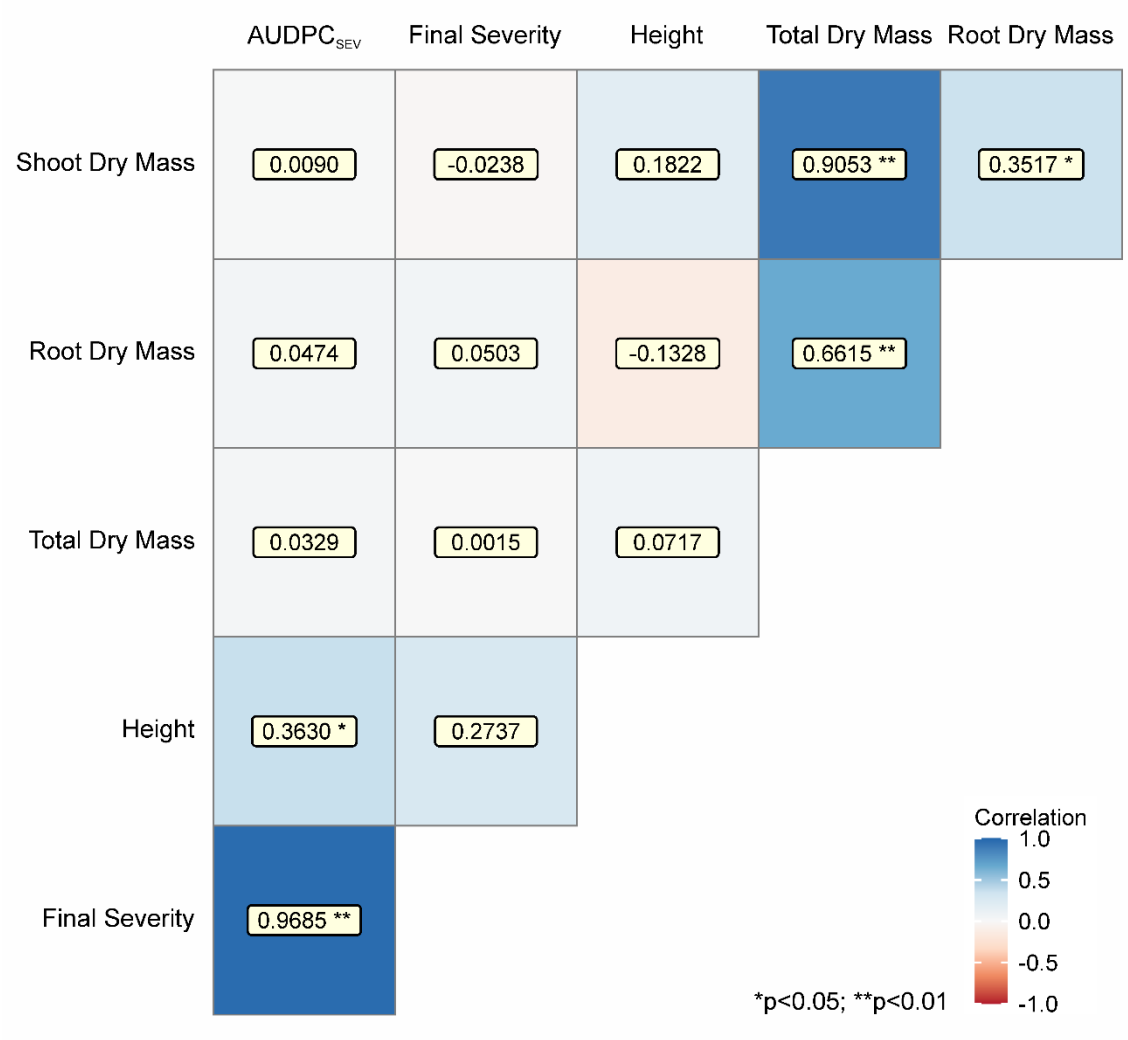

Figure S1. Spearman's correlation matrix among disease-related ($AUDPC_{SEV}$ and Final Severity) and plant growth variables (Height, Total Dry Mass, Shoot Dry Mass, and Root Dry Mass) evaluated in soybean plants treated with fungal antagonists. Correlation coefficients (r) are shown within each cell. Asterisks indicate statistically significant correlations ($p < 0.05$ = *, $p < 0.01$ = **). Color scale represents the strength and direction of correlation (blue = positive, red = negative).

Table 1. Description of the selected isolates assessed against *Phakopsora pachyrhizi* in soybean plants maintained in pots.

Species	Strain code	Culture collection code	Origin	Host	Reference
Aspergillus flavus	E486	COAD 3307	Cameroon	Fruits of *C. canephora*	Kapeua-Ndacnou *et al.* (2023b)
Calonectria hemileiae	M121	COAD 2544	Brazil	Pustules of *H. vastatrix* on *C. arabica* leaves	Salcedo-Sarmiento *et al.* (2021)
Clonostachys rosea	E585	COAD 2984	Ethiopia	Stem of *C. arabica*	Kapeua-Ndacnou *et al.* (2023a)
Clonostachys rhizophaga	M267	COAD 2982	Cameroon	Pustules of *H. vastatrix*/ *coffeicola* on *C. canephora* leaves	Kapeua-Ndacnou *et al.* (2023a)
Cordyceps cateniannulata	E048	COAD 3349	Cameroon	Stem of *C. arabica*	Pereira *et al.* (2024)
Cordyceps cateniannulata	LCBPF 17	COAD 3809	Brazil	From native soil, using *T. molitor* larvae as bait	Domingues *et al.* (2022)
Fusarium coffeibaccae	M442	COAD 3041	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Nóbrega (2021)
Trichoderma virens	E174	COAD 2400	Cameroon	Stem of *C. brevipes*	Rodríguez *et al.* (2021)
Trichoderma harzianum	M290	COAD 3672	Ethiopia	Pustules of *H. vastatrix* on *C. arabica* leaves	Tobar (Personal obs.)

Table 2. Treatments applied to the soybean plants in the pot experiments.

Treatment	Description[1,2]
T01 – E585	Conidia suspension of *Clonostachys rosea*
T02 – M267	Conidia suspension of *Clonostachys rhizophaga*
T03 – E174	Conidia suspension of *Trichoderma virens*
T04 – M290	Conidia suspension of *Trichoderma harzianum*
T05 – E48	Conidia suspension of *Cordyceps cateniannulata*
T06 – LCBPF 17	Conidia suspension of *Cordyceps cateniannulata*
T07 – E486	Conidia suspension of *Aspergillus flavus*
T08 – M121	Conidia suspension of *Calonectria hemileiae*
T09 – M442	Conidia suspension of *Fusarium coffeibaccae*
T10 – *B. velezensis*	Commercial product based on *Bacillus velezensis*[3]
T11 – Formulation	Tween 20 solution (0,05 % v/v) + glycerol (1 % v/v) + sunflower oil (1% v/v) and skimmed milk powder (2% m/v)
T12 – Control	Just *P. pachyrhizi* applied[4]

[1]Conidial suspension calibrated at 5×10^8 conidia/mL (corresponding to an average of 1×10^8 CFU/g of colonized rice).

[2]Spray volume ranged from 5 to 20 mL per plant, depending on plant size. Treatments were applied six times at 7 day-intervals, except for T12, which remained untreated.

[3]The commercial product based on *Bacillus velezensis* was applied at a field rate of 6 L/ha. Considering a spray volume of 400 L/ha, the corresponding concentration was 15 mL of product per litre of spray solution (Bio-Imune®; *Bacillus velezensis* BV02; 3×10^9 CFU/mL; Vittia Group, São Joaquim da Barra, SP, Brazil).

[4]All plants were inoculated with *Phakopsora pachyrhizi* 72 h after the second antagonists' spraying, with a suspension calibrated at 5×10^5 urediniospores/mL.

Table 3. Final severity of Asian soybean rust and control efficacy of the treatments applied to soybean plants and inoculated with *P. pachyrhizi* in potted experiment.

Treatment[1]	$AUDPC_{SEV}$[2]		Final Severity (%)		Control Efficacy (%)			
	Mean[3]	95% CI [LL; UL][4]	Mean[3]	95% CI [LL; UL][4]	C[5]	95% CI [LL; UL][4]	C[6]	95% CI [LL; UL][4]
T01 – E585	4.48 AB	[0.72; 16.4]	4.26 A	[0.32; 19.90]	62.46	[58.71; 84.73]	58.47	[54.56; 82.43]
T02 – M267	8.03 BC	[1.83; 27.7]	8.96 B	[1.50; 38.70]	21.05	[19.70; 28.57]	12.67	[11.64; 17.78]
T03 – E174	9.57 C	[2.32; 32.6]	10.51 B	[1.89; 44.80]	7.40	[7.05; 10.12]	-2.43	[-3.44; -2.28]
T04 – M290	7.85 BC	[1.78; 27.1]	8.93 B	[1.49; 38.50]	21.32	[20.12; 29.00]	12.96	[12.1; 18.28]
T05 – E048	4.21 A	[0.63; 15.6]	4.27 A	[0.32; 20.00]	62.37	[58.50; 84.59]	58.38	[54.33; 82.26]
T06 – LCBPF 17	7.21 ABC	[1.58; 25.1]	9.24 B	[1.57; 39.80]	18.59	[17.42; 25.29]	9.94	[9.13; 14.01]
T07 – E486	8.48 C	[1.98; 29.1]	10.01 B	[1.76; 42.80]	11.80	[11.20; 16.11]	2.43	[2.28; 3.44]
T08 – M121	6.61 ABC	[1.39; 23.2]	7.31 AB	[1.08; 32.10]	35.59	[33.40; 48.31]	28.75	[26.71; 40.5]
T09 – M442	8.67 C	[2.04; 29.7]	9.41 B	[1.61; 40.50]	17.09	[15.97; 23.25]	8.28	[7.53; 11.65]
T10 – *B. velezensis*	20.27 D	[5.69; 66.6]	21.16 C	[4.56; 87.20]	-86.43	[-117.07; -80.91]	-106.23	[-149.86; -99.08]
T11 – Formulation	8.63 C	[2.02; 29.6]	10.26 B	[1.82; 43.80]	9.60	[9.12; 13.12]	–	–
T12 – Control	9.19 C	[2.2; 31.4]	11.35 BC	[2.10; 48.20]	–	–	–	–

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *C. cateniannulata* (LCBPF 17); T07 = *Aspergillus flavus* (E486); T08 = *Calonectria hemileiae* (M121); T09 = *Fusarium coffeibaccae* (M442); T10 = Commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

[2]$AUDPC_{SEV}$ = area under disease progress curve for severity.

[3]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[4]CI = confidence interval, LL = lower limit; UP = upper limit.

[5]C = Control efficacy calculated relative to the untreated control (T12).

[6]C = Control efficacy calculated relative to the formulation treatment (T11).

Table 4. Height, shoot and root dry mass of soybean plants treated with fungal antagonists and inoculated with *Phakopsora pachyrhizi*.

Treatment[1]	Height (cm)		Total dry mass (g)[2]		Shoot dry mass (g)		Root dry mass (g)	
	Mean[3]	95% CI [LL; UL][4]	Mean[3]	95% CI [LL; UL][4]	Mean[NS]	95% CI [LL; UL][4]	Mean[3]	95% CI [LL; UL][4]
T01 – E585	48 ABC	[42.9; 53.5]	8.13 A	[6.98; 9.29]	6.43	[5.61; 7.25]	1.7 ABC	[1.27; 2.16]
T02 – M267	47.7 ABC	[42.6; 53.2]	8.37 A	[7.21; 9.52]	6.83	[6.02; 7.65]	1.52 AB	[1.11; 1.97]
T03 – E174	53.9 C	[48.5; 59.7]	9.04 AB	[7.89; 10.2]	7.53	[6.72; 8.35]	1.51 AB	[1.1; 1.95]
T04 – M290	50.3 BC	[45; 55.9]	8.96 AB	[7.81; 10.12]	7.57	[6.75; 8.39]	1.4 AB	[0.99; 1.83]
T05 – E048	48 ABC	[42.8; 53.4]	8.32 A	[7.16; 9.48]	7.01	[6.20; 7.83]	1.3 A	[0.91; 1.73]
T06 – LCBPF 17	45.5 AB	[40.5; 50.8]	8.36 A	[7.20; 9.52]	7.09	[6.27; 7.91]	1.26 A	[0.87; 1.69]
T07 – E486	49.3 BC	[44.1; 54.9]	9.80 AB	[8.65; 10.96]	7.37	[6.55; 8.19]	2.41 BCD	[1.93; 2.92]
T08 – M121	47.7 ABC	[42.5; 53.1]	7.85 A	[6.70; 9.01]	6.48	[5.66; 7.29]	1.38 AB	[0.98; 1.81]
T09 – M442	49.8 BC	[44.6; 55.4]	8.86 AB	[7.70; 10.01]	7.53	[6.71; 8.35]	1.32 A	[0.92; 1.74]
T10 – *B. velezensis*	49.9 BC	[44.6; 55.5]	10.50 AB	[9.35; 11.66]	7.75	[6.93; 8.57]	2.74 CD	[2.24; 3.28]
T11 – Formulation	46.7 ABC	[41.6; 52.1]	8.85 AB	[7.69; 10.00]	6.48	[5.66; 7.30]	2.35 ABCD	[1.87; 2.86]
T12 – Control	41.1 A	[36.3; 46.2]	11.31 B	[10.16; 12.47]	7.76	[6.94; 8.58]	3.49 D	[2.93; 4.07]

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *C. cateniannulata* (LCBPF 17); T07 = *Aspergillus flavus* (E486); T08 = *Calonectria hemileiae* (M121); T09 = *Fusarium coffeibaccae* (M442); T10 = Commercial product based on *Bacillus velezensis*; T11 = formulation; and T12 = untreated control.

[2]Total dry mass was obtained by adding shoot and root dry masses.

[3]Values followed by the same letter are not significantly different by Tukey's multiple range test at $p < 0.05$.

[4]CI = confidence interval, LL = lower limit; UP = upper limit.

[NS] Means do not significantly differ by Tukey's multiple range test at $p < 0.05$.

Table 5. Recovery (%) of applied antagonists to soybean plants in the pot experiment.

Treatment[1]	Trifoliate leaf		
	Third	**Fourth**	**Apical**
T01 – E585	11.1	0.0	0.0
T02 – M267	22.2	11.1	0.0
T03 – E174	0.0	0.0	0.0
T04 – M290	18.5	33.3	3.7
T05 – E048	3.7	0.0	0.0
T06 – LCBPF 17	0.0	0.0	0.0
T07 – E486	7.4	7.4	0.0
T08 – M121	11.1	0.0	0.0
T09 – M442	70.4	22.2	3.7

[1]T01 = *Clonostachys rosea* (E585); T02 = *C. rhizophaga* (M267); T03 = *Trichoderma virens* (E174); T04 = *T. harzianum* (M290); T05 = *Cordyceps cateniannulata* (E048); T06 = *C. cateniannulata* (LCBPF 17); T07 = *Aspergillus flavus* (E486); T08 = *Calonectria hemileiae* (M121); T09 = *Fusarium coffeibaccae* (M442).

General Conclusions

Starting from an assemblage of over 1,500 fungal isolates of possible fungal antagonists of *Hemileia vastatrix* (coffee leaf rust – CLR) obtained in African and South American countries, which led to a somewhat arbitrary selection of 600 isolates to be evaluated *in vitro*, and subsequently for further greenhouse *in planta* tests conducted by our research group, a short-list of 10 isolates was chosen as the most promising isolates.

Such isolates were: *Aspergillus flavus* (E486), *Calonectria hemileiae* (M121), *Clonostachys rhizophaga* (isolates M230 and M267), *C. rosea* (E585), *Cordyceps cateniannulata* (E48), *Fusarium coffeibaccae* (M442), *Trichoderma harzianum* (M290), *T. theobromicola* (E414), and *T. virens* (E174).

- Trials conducted both involving potted plants and also in experimental coffee fields indicated that *F. coffeibaccae* (M442) and *C. rhizophaga* (M267) consistently demonstrated biocontrol efficacy across varying environmental conditions.
- Results of potted coffee plant experiments have shown that consortia of *Clonostachys* or *Trichoderma* isolates were able to significantly reduce the area under the disease progress curve for severity (76% and 71%, respectively).
- Prospective investigation of a possible "broad antagonism" of the most promising isolates to other rusts has shown, first in *in vitro* assays, that *F. coffeibaccae* (M442) and *C. cateniannulata* (E48 and LCBPF 17) significantly inhibited urediniospore germination of *Austropuccinia psidii*, *Phakopsora pachyrhizi*, *Puccinia recondita*, and *Uromyces appendiculatus*, suggesting a partial confirmation of the conjecture. Other antagonist isolates had a variable influence on urediniospore germination.
- As a consequence of the results of the *in vitro* study, an experiment focused on *P. pachyrhizi* (Asian soybean rust) was conducted. The following isolates demonstrated a good biocontrol performance: *C. cateniannulata* (E48 and LCBPF 17), *C. rosea* (E585), and *C. hemileiae* (M121). Reductions of the area under the disease progress curve of up to 54% were achieved.
- Based on the combination of the results that were obtained, four fungal isolates stood out as the most promising antagonists: *F. coffeibaccae* (M442), *C. cateniannulata* (E48), *C. rosea* (E585), and *C. rhizophaga* (M267).
- Fungal treatments led to significant growth reductions in coffee and soybean plants. This needs to be clarified, particularly with respect to its impact on field productivity. This may result from metabolic costs of fungal establishment and maintenance, resources reallocation

toward the activation of plants defence responses, or an eventual phytotoxicity of secondary metabolites produced by these antagonists.

- Although *F. coffeibaccae* had previously been isolated from *Coffea* spp. tissues, it was not consistently recovered as an endophyte from treated coffee plants in this study. However, it was readily and frequently reisolated from soybean plants treated with M442 isolate. To the best of our knowledge, this is the first-time association of this species with soybean and suggests that *F. coffeibaccae* may colonize plant hosts from distinct botanical families.
- Contrarily to earlier results published by our group, *A. flavus* (E486) and *T. harzianum* (M290) did not effectively control rust both in coffee and in soybean but these exhibited strong endophytic colonization ability, as they were consistently reisolated from treated coffee plants.
- A continuation of this long-term study is justifiable both academically and in practical terms, for the benefit of the discipline of biological control of plant diseases. Many important questions still remain unanswered, but the potential of these isolates to become tools for the management of some of the most important plant diseases threatening crop plants deserves a close and continued evaluation.