MIRAINE KAPEUA NDACNOU

ISOLATES OF *Aspergillus*, *Clonostachys* AND *Trichoderma* FROM AFRICA AS POTENTIAL BIOCONTROL AGENTS AGAINST COFFEE LEAF RUST

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

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Assent:

Miraine Kapeua Ndacnou
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My deep gratitude to the God Almighty, for the graces granted during this academic journey, which has not always been obvious.

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"*All our dreams can come true if we have the courage to pursue them*."

(Walt Disney).

BIOGRAFIA

KAPEUA NDACNOU Miraine, filha de LIÉLÉ Béatrice Rose e TCHUEBON Daniel, nasceu na cidade de Douala, Littoral-Camarões, no dia 15 de agosto de 1983, onde cursou o ensinos fundamental e médio, concluindo em junho de 2001.

Em setembro de 2001 ingressou no curso de graduação em Química na University of Yaoundé I, Yaoundé-Camarões, graduando-se em maio de 2005. Em outubro de 2005 iniciou o curso de primeiro ano de Mestrado na mesma área e mesma universidade, concluindo em julho de 2006.

Em fevereiro de 2010 iniciou o curso de Mestrado em Química, opção Química Orgánica sempre na University of Yaoundé I, Yaoundé-Camarões, concentrado seus estudos em Fitoquímica e controle de doenças de plantas a partir de extratos/compostos derivados de plantas medicinais. Desenvolveu parte de seu mestrado na IRAD (Institute of Agricultural Research for Development), em Regional Biocontrol and Applied Microbiology Laboratory na mesma cidade, onde já trabalhava como estagíaria desde julho de 2009. Defendeu sua dissertação em novembro de 2011.

Em Dezembro de 2012 foi contratada em IRAD como assistente de pesquisa e afiliada ao Regional Biocontrol and Applied Microbiology Laboratory. E em julho de 2017, iniciou na UFV como bolsista da CAPES, os estudos no programa de Pós-Graduação em Fitopatologia, em nível de Doutorado; concentrando sua pesquisa nas áreas de micologia e controle biológico de doenças de plantas.

ABSTRACT

KAPEUA NDACNOU, Miraine, D.Sc., Universidade Federal de Viçosa, March, 2022. **Isolates of *Aspergillus*, *Clonostachys* and *Trichoderma* from Africa as potential biocontrol agents against coffee leaf rust.** Adviser: Robert Weingart Barreto.

Hemileia vastatrix – the coffee leaf rust (CLR) fungus – is native from Africa and an exotic invasive species in all other areas of the world where coffee is grown. It causes the worst disease of coffee, but in Africa, it is not regarded as the worst pathogen of the crop. Management of CLR has relied on escaping the disease through the highland plantation, using resistant coffee varieties and fungicide applications. There are limitations for each of these strategies and novel approaches for CRL management are necessary. It has been conjectured that natural enemies of *H. vastatrix* endemic to Africa might have been overlooked and that such antagonists might have the potential for CLR management. Surveys conducted since 2015 in Cameroon, Ethiopia and Kenya, have revealed numerous mycoparasitic fungi attacking CLR in the field as well as endophytic fungi growing inside healthy coffee plants (several of which were, or are being, described as new to science), which might play a bodyguard role, protecting the plants against the disease. Results of previous studies and of the present research seem to confirm this hypothesis. A series of taxonomic and polyphasic studies allowed to identify isolates of *Aspergillus* and *Clonostachys* as belonging to *Aspergillus flavus*, *Clonostachys byssicola*, *C. rhizophaga* and *C. rosea f. rosea*. All the selected endophytic isolates (confirmed here regardless of the genus, to grow as endophytes in coffee), including *Trichoderma* strains were demonstrated to inhibit the germination of *H. vastatrix in vitro*. The isolate of *A. flavus* was shown not to produce aflatoxin. Beforehand applications of a series of isolates, of *Clonostachys*, *Trichoderma* and also of *A. flavus*, on young *C. arabica* plants (a combination of soil and foliar applications) followed by inoculation with *H. vastatrix* led to the significant reductions in CLR severity. Two *Clonostachys rhizophaga* isolates (COAD 2981 and COAD 2982), and one *C. rosea* isolate (COAD 2984) yielded the best highly significant ($p < 0.001$) results for reduction of CLR severity among the *Clonostachys* isolates. *Trichoderma guizhouense* (COAD 2398), *T. virens* (COAD 2400) and *T. theobromicola* (COAD 2406) also produced highly significant ($p < 0.001$) levels of CLR severity reduction. This is the first study reporting anti-CLR biocontrol potential for African isolates of *A.*

flavus, *Clonostachys* and *Trichoderma*. And, they may pave the way towards preventive treatment of coffee plants with ‘bodyguard’ endophytes antagonistic to *H. vastatrix* as well as their use for control of CLR in the field. A continuation of this work is required to better assess this possibility.

Keywords: Classical biocontrol. Coffee rust. Endophytes. Phylogeny. Taxonomy.

RESUMO

KAPEUA NDACNOU, Miraine, D.Sc., Universidade Federal de Viçosa, março de 2022. **Isolates of *Aspergillus*, *Clonostachys* and *Trichoderma* from Africa as potential biocontrol agents against coffee leaf rust.** Orientador: Robert Weingart Barreto.

Hemileia vastatrix – o fungo da ferrugem do café (CLR) – é nativo da África e uma espécie exótica invasora em todas as outras áreas do mundo onde o café é cultivado. Ele causa a pior doença do café, mas na África não é considerado o pior patógeno da cultura. O manejo da CLR tem se baseado na fuga da doença através do plantio de terras altas, utilizando variedades de café resistentes e aplicações de fungicidas. Existem limitações para cada uma dessas estratégias e novas abordagens para o manejo da LCR são necessárias. Foi conjecturado que os inimigos naturais de *H. vastatrix* endêmicos da África podem ter sido negligenciados e que tais antagonistas podem ter potencial para o manejo da CLR. Pesquisas realizadas desde 2015 em Camarões, Etiópia e Quênia revelaram numerosos fungos micoparasitas atacando CLR no campo, bem como fungos endofíticos crescendo dentro de cafeeiros saudáveis (vários dos quais foram ou estão sendo descritos como novos para a ciência), o que pode desempenham um papel de guarda-costas, protegendo as plantas contra a doença. Resultados de estudos anteriores e da presente pesquisa parecem confirmar essa hipótese. Uma série de estudos taxonômicos e polifásicos permitiu identificar isolados de *Aspergillus* e *Clonostachys* como pertencentes a *Aspergillus flavus*, *Clonostachys byssicola*, *C. rhizophaga* e *C. rosea f. rosa*. Todos os isolados endofíticos selecionados (confirmados aqui independentemente do gênero, para crescer como endofíticos em café), incluindo cepas de Trichoderma, demonstraram inibir a germinação de *H. vastatrix in vitro*. O isolado de *A. flavus* mostrou não produzir aflatoxina. A aplicação prévia de uma série de isolados, de *Clonostachys*, *Trichoderma* e também de *A. flavus*, em plantas jovens de *C. arabica* (uma combinação de aplicações no solo e foliares) seguida de inoculação com *H. vastatrix* levou a reduções significativas na severidade da CLR. Dois isolados de *Clonostachys rhizophaga* (COAD 2981 e COAD 2982) e um isolado de *C. rosea* (COAD 2984) produziram os melhores resultados altamente significativos ($p < 0,001$) para redução da severidade de CLR entre os isolados de *Clonostachys*. *Trichoderma guizhouense* (COAD 2398), *T. virens* (COAD 2400) e *T. theobromicola* (COAD 2406)

também produziram níveis altamente significativos ($p < 0,001$) de redução da severidade da CLR. Este é o primeiro estudo relatando o potencial de biocontrole anti-CLR para isolados africanos de *A. flavus*, *Clonostachys* e *Trichoderma*. E podem abrir caminho para o tratamento preventivo de cafeeiros com endófitos 'guarda-costas' antagonistas a *H. vastatrix*, bem como seu uso para controle de CLR no campo. A continuação deste trabalho é necessária para melhor avaliar esta possibilidade.

Palavras-chave: Biocontrole clássico. Ferrugem do Cafeeiro. Endófitos. Filogenia. Taxonomia.

SUMMARY

GENERAL INTRODUCTION

Coffee (*Coffea arabica*) is one of the most important cultivated plants, and agricultural products and represents the most valuable agricultural commodity in the world (Monroy *et al*. 2019, USDA 2020). Up to 25 million of small producers depend on coffee for a living worldwide (FAO 2022), and its beverage is daily consumed by 30-40% of the population in the world (Hudyakova 2020).

Despite the immense economic, social and cultural relevance of coffee, the sustainability of its production is under threat by many factors, particularly for the smallholders in Africa, Asia and Central and South Americas, which combined are responsible for about 80% of the coffee global production. Among the most important threats, is its principal disease coffee leaf rust (CLR) caused by *Hemileia vastatrix*. Severe infections can cause a reduction of photosynthesis due to the early defoliation, with losses of the yield of 30–50% (Talhinhas *et al*. 2017) and annual costs worldwide estimated at a billion of dollars (Hein & Gatzweiler, 2006).

It had been reported that between 2008-2013, losses of up to 90% in coffee production occurred in the Central American countries (Honduras, Nicaragua, Panama, El Salvador and Guatemala) and Colombia, due to the increased attack by CLR (Avelino *et al*. 2015, Talhinhas *et al*. 2017). Various factors have been associated to the epidemic, including climate change and susceptibility of favorite varieties of *Coffea* (catuaí e caturra) (Avelino *et al*. 2015). The breakdown of resistance has also been observed in varieties that were previously resistant to CLR, such as the Lempira – a Timor hybrid derivate – particularly important in Honduras. Chemical control, the main CLR management strategy in Brazil, is too expensive for many poor family-level producers and its adoption compromises the reputation of quasi-organic of Central American coffees.

A major socio-economic crisis resulted from these CLR outbreaks particularly owing to the loss of the escape factor offered by cultivation in areas at altitudes above 1100 masl (Avelino *et al*. 2006). Avelino *et al*. (2015) mention that after an epidemic recorded in 2011, infestations of equal intensity were observed in regions up to 1400 masl. In these mountainous areas of Guatemala, El Salvador, Honduras, Costa Rica

and Mexico, smallholder families depend on harvesting high-quality “organic” coffee for survival. This crisis triggered migratory movements in the form of migrant caravans leaving El Salvador, Guatemala and Honduras crossing Mexico towards a better life in North America, as widely portrayed in the media.

The novel approach of surveying, identifying and evaluating fungi in Africa antagonistic to the CLR fungus in its center of origin was proposed at an emergency summit in Guatemala and the World Coffee Research funded the initiative based at the Departamento de Fitopatologia of the Universidade Federal de Viçosa – DFP/UFV (state of Minas Gerais, Brazil). Fungi have long been recognized as among the most interesting antagonists of *H. vastatrix,* either in the form of mycoparasites on CLR pustules or having endophytic lifestyle members of – and possibly functioning as ‘microbial bodyguards’ of the coffee plant, defending it against CLR. (Boosalis 1964, Barros *et al*. 1999). In the case of *H. vastatrix*, a considerable diversity of possible antagonist mycoparasites associated with the pustules of the rust has already been reported. For instance, *Acremonium byssoides, Bullera* sp., *Calcarisporum arbuscula, C. ovalisporum, Sporotrix guttuliformis, Colletotrichum gloeosporioides, Exophiala eucalyptorum, E. opportunistica, Fellomyces thailandicus, F. mexicanus, Fusarium pallidoroserum, F. decemcellulare, Lecanicillium lecani, L. fusisporum, Simplicillium lanosoniveum, S. cylindrosporum, S. lamellicola, S. minatense, Tilletiopsis pallescens* (Carrión & Rico-Gray 2002, James *et al*. 2016) among others. Nevertheless, most of the existing records of potential fungal antagonists of CLR are from the Neotropics, an exotic situation both for *H. vastatrix* and its coffee host. Nevertheless, even for the above-listed fungal species, very little has been performed in terms of evaluation of their biocontrol potential, until the work at DFP/UFV started.

According to Vandermeer *et al*. (2009), *Lecanicillium lecanii* would be the best known and the most commonly associated mycoparasite with *H. vastatrix*. Its white structures are easily visible on the pustules in the field. However, even for this species, supposedly quite common, and whose name has become a kind of all-purpose label and used for naming any of the fungi that form white colonies on pustules of *H. vastatrix*, as shown in a recent study by Colmán (2018). Even under questionable identity for the fungi involved, there are some publication presenting promising

biocontrol results (Alarcón & Carrión 1994, Meza & Leguizamón 1995, Gonzalez & Martinez 1998, Canjura-Saravia *et al*. 2002).

Since 2015, four Ph.D. theses and one MSc dissertation have been prepared based on the work on antagonistic fungi of CLR based at DFP/UFV. The sixth work in this series is presented here and was focused on fungi belonging to three genera : *Aspergillus*, *Clonostachys* and *Trichoderma*. All of them have a history of prior use in the biological control of fungal pathogens (Fravel 2005, Loguercio *et al*. 2009, Ten hoopen *et al*. 2010, Macedo *et al*. 2012, Krauss *et al*. 2013, Mbarga *et al*. 2014, Keyser *et al*. 2015, Tchameni *et al*. 2017, Pakora *et al*. 2018, López *et al*. 2019). The isolates of *Trichoderma* spp. used in this study have already been identified and characterized by Rodríguez *et al*. (2021). The objectives of this study were to characterize fungal isolates of the genera *Aspergillus* and *Clonostachys*, mycoparasites of coffee rust or obtained as endophytes in samples of *Coffea* spp. in the origin center of coffee; and to test their anti-CLR biocontrol potential together with selected isolates of *Trichoderma* spp.

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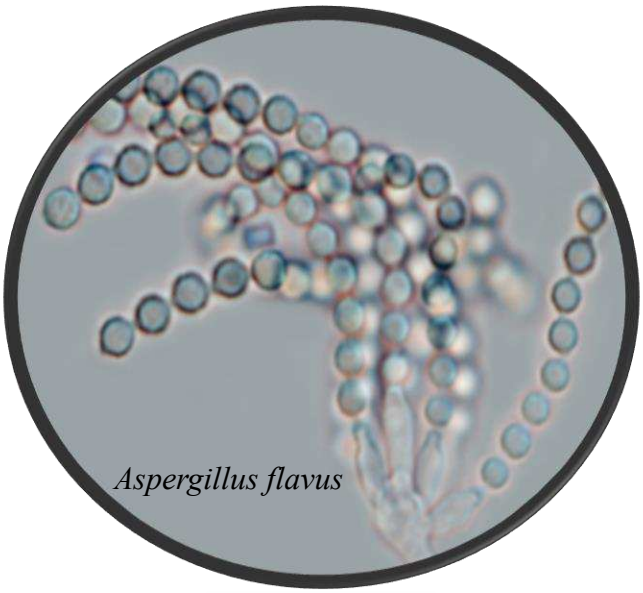
CHAPTER 1 (article in press in the Journal of Applied Microbiology**)**

***Aspergillus flavus*: a non-aflatoxigenic endophytic isolate from coffee in Cameroon antagonistic to coffee leaf rust (*Hemileia vastatrix*)**



RUNNING HEADLINE: *Aspergillus flavus* from coffee in Cameroon

Aspergillus flavus: a non-aflatoxigenic endophytic isolate from coffee in Cameroon antagonistic to coffee leaf rust (_Hemileia vastatrix_)

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Abstract

Aims

To confirm the identity and endophytic ability of an *Aspergillus* species isolated from coffee and to assess its aflatoxin status and biocontrol potential against coffee leaf rust (CLR).

Methods and Results

Evidence from both morphological and molecular analyses – including four regions (ITS, RPB2, β-tubulin and CAL) – identified the isolate (COAD 3307) as *Aspergillus flavus.* Inoculation of healthy *Coffea arabica* with COAD 3307 confirmed its establishment as an endophyte in leaves, stems and roots. Inoculation of *C. arabica* plants by applications of COAD 3307 to aerial parts and the soil, significantly (p > 0.0001) reduced CLR severity as compared to controls. Thin-layer chromatography (TLC) showed that COAD 3307 is not an aflatoxin-producing isolate.

Conclusions

COAD 3307 is an endophytic isolate of *A. flavus* – a species that has never been recorded as an endophyte of coffee in Cameroon and only once before from coffee

roots in Ethiopia. This non-aflatoxin-producing strain showed an anti-CLR effect and thus merits further evaluation as a biocontrol agent.

Significance and impact of the study
CLR is the most devastating disease of coffee and there is an urgent need for non-pesticide sustainable tools for its management. Non-aflatoxin-producing isolates of *A. flavus* have been safely and successfully used as biocontrol agents on other crops. Therefore, COAD 3307 isolated from coffee in Cameroon may become a useful tool for the management of CLR.

Keywords: Aflatoxins; Biological control; *Coffea*; Endophytes; Phylogenetics; Taxonomy.

Introduction

Coffee is one of the major agricultural commodities in the world (USDA 2020). Fungal diseases are among the most important limitations to production in all coffee-growing countries, and the most devastating disease worldwide is coffee leaf rust (CLR), caused by *Hemileia vastatrix* (Talhinhas *et al*. 2017). Management of CLR has relied on the use of resistant varieties or escape from CLR through highland plantations, as well as on fungicide applications (Avelino *et al*. 2006; Belan *et al*. 2015). Nevertheless, problems with resistance loss, climate change and economic or market limitations to chemical control have led CLR to gain additional importance since the early 2010s, particularly in northern South America and Central America (Avelino *et al*. 2015; Talhinhas *et al*. 2017; Belachew *et al*. 2020). This prompted World Coffee Research (WCR) to fund an initiative aimed at assessing the biocontrol potential of fungal antagonists of CLR in the center of origin of the genus *Coffea* in Africa (WCR 2019) where, in general, CLR is less important than other diseases. A plethora of fungi have been isolated, thus far, and the identities of these isolates are being progressively elucidated (Crous *et al*. 2018; Colmán *et al*. 2021; Guterres *et al*. 2021; Rodríguez *et al*. 2021). The extensive list includes both putative mycoparasites isolated from CLR pustules, and endophytic fungi isolated from the inner tissues of healthy coffee plants in the field (Rodríguez *et al*. 2021). Such endophytic fungi can colonise inside host-plants without causing any disease symptoms (Sudha *et al*. 2016) and, in addition,

some may protect their plant hosts against abiotic and biotic stress, the so-called "bodyguard-effect" (Rocha *et al*. 2017).

Several studies on the diversity of fungal endophytes associated with *Coffea* species have been published previously. Nevertheless, with the exception of Mulaw *et al*. (2013), who investigated the root endophytes of coffee in Ethiopia, these studies have been restricted to the Americas, a region of the world where coffee is an exotic cultivated species. Fulthorpe *et al*. (2020) detected a range of fungi belonging to 30 genera – including *Aspergillus* – via terminal restriction length polymorphism analysis of coffee roots from Costa Rica and Nicaragua. Bongiorno *et al*. (2016) isolated endophytic fungi from coffee leaves in an organic coffee plantation in Brazil and identified these as belonging to *Colletotrichum*, *Cercospora*, *Cladosporium*, *Mycosphaerella, Ophiognomonia*, *Schizophylum* and *Trichoderma*. Based on extensive surveys in the Neotropics, Vega *et al*. (2006; 2008; 2010) reported several species belong to the genus *Aspergillus*; including *A. niger, A. oryzae* and *A. tubingiensis*, associated with coffee leaves, stems and seeds. However, it was conjectured that a richer and possibly novel mycobiota associated with CLR pustules or healthy tissues of *Coffea* might be awaiting discovery in its African centre of origin. Sampling in wild and semi-wild conditions in Africa has since confirmed this assumption and novel fungi belonging to genera such as *Digitopodium* (Colmán *et al*. 2021), *Fusarium* (Nóbrega 2021) and *Trichoderma* (Rodríguez 2019; Rodríguez *et al*. 2021) have been discovered during the surveys, principally in Cameroon and Ethiopia. Species belonging to various other genera are currently under investigation. Additionally, an isolate of *Aspergillus*, obtained from the inner parts of healthy coffee berries in Cameroon, was also found and is discussed herein.

This study aimed at: (i) characterizing the isolate of *Aspergillu*s isolated during the coffee survey in Cameroon; (ii) evaluating its capacity to establish as an endophyte in coffee plants; (iii) determining the safety of this isolate in terms of mycotoxin production and; (iv) evaluating its potential as a biocontrol agent of CLR.

Materials and methods

Isolation

The fungal isolate used in this study was obtained from healthy berries of *Coffea canephora* collected in Cameroon (Buea, Southwest Region). For details of the survey strategy, and the isolation protocol for endophytes growing in healthy coffee tissues (stems, leaves and berries), see Rodríguez *et al*. (2021). Over 1500 isolates were obtained during the surveys in Africa but only one pertained to the genus *Aspergillus.* This isolate was maintained as mono-conidial colonies, grown on PDA at 25 ± 2°C for ten days under a 12-h daily light regime. These colonies were preserved in silica-gel at 4 °C, as well as in cryotubes with 10% glycerol at -80 °C (Dhingra and Sinclair, 1995), and deposited in the culture collection (Coleção Octávio de Almeida Drummond, COAD) of the Universidade Federal de Viçosa (UFV), under accession number COAD 3307.

DNA extraction, amplification and phylogeny

Genomic DNA was extracted from COAD 3307 grown in a potato-dextrose broth (PD) in flasks kept in the dark for two days. Biomass was removed and dried on sterile filter paper in a desiccator for two days and then transferred to a sterile plastic tube containing zirconium spheres and placed in a grinder (L-Beader-3, Loccus Biotecnologia, Brazil). After 20 seconds grinding, the resulting suspension was drained into a sterile plastic tube and used for DNA extraction. This was performed with the Wizard Genomic DNA Purification Kit following the manufacturer's protocol.

Target regions of the internal transcribed spacer (ITS), second-largest subunit of RNA polymerase (RPB2), β-tubulin (β-tubul) and calmodulin (CAL) were amplified using fungal specific primers ITS1F and ITS4 for ITS (White *et al*. 1990), 5F2 and 7CR for RPB2 (O'Donnell *et al*. 2008), T1 and T22 for β-tubulin (O'Donnell and Cigelnik 1997) and 228F and 2RD for CAL (Carbone and Kohn 1999; Groenewald *et al*. 2013). PCR products were analyzed on GelRed ™ (Biotium Inc., Hayward, CA, USA) and visualized under UV light to verify the size and purity of amplificons. The PCR products were sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The nucleotide sequences were edited with software SeqAssem ver. 07/2008 (Hepperle 2004).

The consensus sequences were compared with others deposited in the GenBank database using the MegaBLAST program. Sequences from GenBank were aligned using MUSCLE (Edgar 2004) and built in MEGA X 10.1 software (Kumar *et al*. 2018).

All of the ambiguously aligned regions within the dataset were excluded from the analyses. Gaps (insertions/deletions) were treated as missing data.

Bayesian inference (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each locus separately and then with the concatenated sequences. The alignments consisted of 10 parsimony-informative positions 10/799 bp for ITS, 60/995 bp for RPB2, 50/507 for β-tubulin and 65/534 bp for CAL. Before launching the BI, the best nucleotide substitution models were determined for each gene with MrMODELTEST 2.3 (Posada and Buckley 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The F81 + I model of evolution was used for ITS region, SYM + I was used for RPB2, K80 was used for β-tubulin and HKY + G was used for CAL. One concatenated tree with the four regions was generated with Sequence Matrix (Vaidya *et al*. 2011) and estimated on the CIPRES web portal using MrBayes on XSEDE 3.2.6 (Miller *et al*. 2011).

Additionally, a Maximum Likelihood (ML) tree was generated with the Nearest-Neighbor-Interchange (NNI) ML heuristic method and the Tamura-Nei substitution model as tree inference options, through the RaxML program, using the CIPRES web portal. The chain stabilities of the phylogenetic tree were assessed by using the bootstrap re-sampling strategy with 1000 bootstrap test replicates. The resulting tree topologies using the two methods (ML and BI) were then compared and the phylogram layout was edited with CorelDRAW Graphics Suite 2017. Sequences derived from this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) (Table 1).

Morphological characterization

COAD 3307 was cultured on both Czapeck Dox agar – CZ (sucrose 30 g; $NaNO_3$ 3 g; K_2HPO_4 1 g; $MgSO_4$ 0.001 g; KCl 0.5 g; $FeSO_4$ 0.01 g; bacteriological agar 15 g; distilled water 1L) and malt extract agar – MEA (malt extract powder 20 g; bacteriological agar 20 g; distilled water 1L). The protocols of Nyongesa *et al*. (2015) and Frisvad *et al*. (2019) were followed including the maintenance of the plates at 25 ± 2 °C in the dark for seven days.

After incubation, the culture morphology was observed following the description terminology of Crous *et al*. (2009); and the color terminology of Rayner (1970). Microscopic characters, including conidiophores, vesicles, phialides, conidial heads

and conidia, were described from slides mounted in 60% lactic acid. The slides were prepared by following the slide-culture methodology as described in the literature (Gonçalves and Alfenas 2016), with COAD 3307 colonies formed on blocks of CZ and MEA media placed over microscope slides, and incubated at 25 ± 2°C in the dark for three days. Observations and photography of fungal structures were made either with an Olympus SZX7 dissecting microscope or with an Olympus BX53 light microscope, adapted with differential interference contrast lighting and fitted with a digital image capture system (Olympus Q-Color 3™ camera).

A minimum of thirty representative structures constituted the biometric data. Photoplates and images structures were prepared in CorelDRAW Graphics Suite 2017.

Establishment of COAD 3307 as an endophyte in* Coffea arabica *and reduction of coffee leaf rust severity

Preparation of inoculum of fungi and test plants

Production of conidia – COAD 3307 was grown in potato dextrose-agar (PDA) plates, at 25 °C under a 12-hour light regime for five days. These colonies served for scaling up the production of inoculum in the next step of the solid-state fermentation process. Five polypropylene bags (12 x 25 cm) containing 50 g of rice, 0.22 g of $CaCO_3$ and 40 ml of distilled water per bag were autoclaved at 121 °C for 20 min. After removal from the autoclave, and left on a bench until reaching room temperature, the bags were seeded individually under aseptic conditions inside a laminar flow cabinet. Each bag received six culture plugs (5 mm diam) of COAD 3307 taken from actively growing colonies on PDA and aseptically transferred to each bag. The bags were then placed in a growth room at 22 °C under a 12-hour light regime (light provided by two white fluorescent day light bulbs, FLC, 25W−127V) and one near-UV lamp (SCT, 28W−127V) placed 35 cm from the bags, and incubated for seven days. Every two days, the bags were rolled and their contents were hand-squeezed to avoid the formation of large aggregates and to allow for good aeration in order to stimulate sporulation. Five non-inoculated rice bags were used for treating control plants.

Production of urediniospores of *H. vastatrix* – Abundant fresh viable urediniospores needed for use in inoculation experiments were obtained as described in Salcedo-

Sarmiento *et al*. (2021). Twelve young and healthy six-month-old coffee plants (cv. Caturra) were hand-spray inoculated with a urediniopore suspension of race II of *H. vastatrix* (1×10^5 spores mL^{-1}) containing 0.05% Tween 20, until runoff and placed in a dew chamber (conditions as described in Salcedo-Sarmiento *et al*., 2021). After 30-45 days abundant orange rust sporulation was present on the underside of leaves. Urediniospores were collected and preserved as described in Salcedo-Sarmiento *et al*., (2021), or collected directly from infected coffee leaves on the same day of the preparation of urediniospore suspensions for application. Only batches of urediniospores having at least 80% viability were used.

Preparation of test plants – Healthy, five, four-month-old, shade-house-grown coffee plants, *C. arabica* cv. Catuaí-vermelho (IAC 144), with 4-5 pairs of leaves were utilized. These were grown from seeds in the UFV nursery until transplantation for use in the assays.

Combined test of COAD 3307 establishment as an endophyte and of resulting protection against CLR

As COAD 3307 was the sole member of *Aspergillus* obtained as an endophyte during the surveys, and it was only obtained once from the inner part of coffee berries from a single locality, confirmation of the ability of this isolate to colonize other coffee organs as an endophyte was considered to be necessary. Two similar assays were then conducted to: a) verify the establishment of COAD 3307 as an endophyte in Arabica coffee after inoculation; b) verify if an anti-CLR “bodyguard-effect” resulted from COAD 3307 application. These tests were carried out from December 2019 - May 2020 (assay I), and June − November 2020 (assay II).

For the tests, inoculation of coffee plants with COAD 3307 involved a combination of soil application (rice colonized with COAD 3307) and foliar sprays (conidial suspensions). Five coffee plants were COAD 3307-treated whereas another five plants received no COAD 3307 inoculum (instead these received uncolonized rice and were sprayed with sterile distilled water (SDW) at 0.05% Tween 20 until run-off) and served as controls.

The two groups of young coffee plants were transferred from their original planting bags into larger (24×17×10 cm) polyethylene plastic bags, containing a mixture of pasteurized soil and either COAD 3307 colonized rice (50 g/plant) – with an estimated

10^9 conidia g^{-1} of rice (treated plants) – or uncolonized rice (untreated plants). After the transfer to the new bags, the test plants were left on a greenhouse bench and watered regularly. Thirty days after the transplantation of the plants, the aerial parts of the COAD 3307-treated plants were, spray-inoculated with a suspension of 10^7 conidia ml^{-1} of COAD 3307 until run-off and left on the greenhouse bench, controls were sprayed with SDW at 0.05% Tween 20 only. The conidia suspension was prepared by suspending the colonized rice in a solution of 0.05% Tween 20. This spray-inoculation procedure was repeated at one-month intervals three times.

Assay I endophytic colonization. Thirty days after transplantation of the coffee plants, and immediately prior to the plants included in the assay being sprayed with COAD 3307, conidial suspension or SDW (controls), one individual plant was arbitrarily selected from both the COAD 3307-treated plants and the plants control for isolation from leaves, stems or roots. These plants were destroyed during the process of attempting to isolate COAD 3307. The following protocol was followed: each of the plants chosen for endophyte isolation was thoroughly washed under a tap, to remove all debris, and treated according to a modified version of the protocol of Arnold *et al*. (2003). Each organ of the coffee plants – roots, stems and leaves – was dissected separately using a sterilized scalpel. The root and stem pieces were 2 cm long and leaf fragments consisted of 5 mm-diam leaf disks. Stem pieces had their bark removed before disinfection. Disinfection was performed through a sequence of dips in 70% ethanol (1 min), 2% sodium hypochlorite (3 min), 70% ethanol (1 min) followed by rinsing three times with SDW, and placed on sterilized filter paper under aseptic conditions to remove excess water. Ten pieces from each organ of a plant were then plated separately on a modified semi-selective medium, TME (Papavizas and Lumsden 1982). Three plates were used for each organ of the plant, then sealed with plastic film and placed in a growth chamber for two weeks at 25 °C under a daily 12-hour light regime (light conditions as above). TME consists of 200 ml V8 juice, 3 g $CaCO_3$, 15 g agar and 800 ml of distilled water. After autoclaving at 121 °C for 15 min and cooled (40-50 °C), the medium was aseptically supplemented with the antibiotics penicillin-G, nystatin and chloramphenicol, 10 ml L^{-1}; and, additionally Triton-x 2 ml L^{-1} was added to the medium.

The plates were observed every two days, in order to detect the presence or absence of COAD 3307 colonies. Whenever colonies similar to COAD 3307 appeared, the plates were further checked under a dissecting microscope (Olympus SZX7) and, if

needed, opened in a laminar flow and pieces of the colony were mounted in microscope slides and examined under a light microscope (Olympus BX-51). The final results of the observation for confirmation of recovery of COAD 3307 were ranked either as positive (+) – at least one colony of COAD 3307 obtained from a tissue sample, or negative (-) – not a single sample of that organ producing a COAD 3307 colony.

Assay II endophytic colonization. Procedures were mostly as described above. The main difference was that the attempt to isolate COAD 3307 was only carried out once, at the end of the five-month period. This was perfomed after the final assessment of CLR severity (as described below).

Antagonism of COAD 3307 to *Hemileia vastatrix* was tested on coffee plants, in parallel with the test of the ability of COAD 3307 to establish as an endophyte in roots, stems and leaves. The assays followed the steps described above but procedures for assays I and II differed. In order to assess the effect of COAD 3307, all plants (including the controls) were inoculated with *H. vastatrix*. Inoculation of the rust was through spray-inoculation with a suspension of urediniospore at 2×10^5 spores mL^{-1} until run-off. Inoculation with *H. vastatrix* occurred 72 h after the last spray-inoculation with COAD 3307 and inoculum was applied in the late evening to optimize conditions for urediniospore germination and host infection

Asssay I CLR severity. Inoculation of *H. vastatrix* was conducted, as described above, at the third month after the transplant of the coffee plants to larger bags and 72h after the third foliar application of a conidial suspension of COAD 3307. It involved all the remaining four plants (two COAD 3307-treated and two control plants).

Three weeks after inoculation with *H. vastatrix*, eight leaves were arbitrarily selected showing typical pale yellow spots (initial symptom of CLR) and identified with plastic tags. A total of sixteen leaves of COAD 3307-treated and sixteen control leaves, not treated with COAD 3307, were labeled. Each individual plant represented a repetition for CLR severity evaluation. The medium of CLR severities evaluated from the eight leaves per plant, was the CLR severity of the plant. One month after urediniospore application, the first CLR severity assessment of individual leaves was performed. CLR severity was assessed three times at two-week intervals. Each leaf was given a note following the standard protocol developed by Belan *et al*. (2020), which comprises seven levels of CLR severity; ranging from 0.0 to 50.9%, where 0.0% indicates a complete absence of sporulation and 50.9%, the maximum disease severity level.

Asssay II CLR severity. As previously mentioned, no recovery of COAD 3307 was attempted until the end of the assay. Therefore, all five COAD 3307-treated and five control plants were included in the CLR severtity evaluation. Evaluation of disease severity followed the same procedure described above. A total of 40 leaves of COAD 3307-treated plants were marked and evaluated and 40 leaves of control plants. Evaluation of disease severity for individually-marked leaves was performed three times as described for assay I and only the data collected from the third disease severity assessment were statistically analyzed in this study, due to the monocyclic character of the pathogen.

Inhibition of Hemileia vastatrix urediniospore germination by COAD 3307 filtrate

COAD 3307 was grown in 125 ml flasks containing 75 mL of potato dextrose broth (PD) amended with Penicillin G and seeded with five plugs of COAD 3307 taken from five day-old colonies on PDA plates. The flasks were placed in a shaker at 29 ± 1°C, 196 rpm, for one week. Subsequently, the suspension was filtered through sterile filter paper in a Büchner funnel. The filtrate was used in the test as described below.

A suspension of *H. vastatrix* urediniospores was prepared from a stock of urediniospores, collected and stored as previously described. Uredinispores were suspended in a 0.05% Tween 20 solution, and the concentration of urediniospores was calibrated to 2×10^5 spores mL^{-1} using a haemocytometer.

Two microscope slides were cleaned with 70% ethanol and placed inside polypropylene boxes (11 × 11 × 3.5 cm) which had also been cleaned with 70% ethanol. The boxes were lined with a layer of sterilized paper towels saturated with SDW. A 15 µL drop of *H. vastatrix* urediniospore suspension was transferred with a micropipette, to the center of each slide and a second 15 µL drop of filtrate of COAD 3307 was placed over one of the drops of urediniospore suspension inside the box and the two drops were gently mixed with the tip of the micropipette and the box was covered. Slides with only one drop of urediniospore suspension served as controls. The study involved four replicates. All four boxes were kept in the dark for six hours at 22 °C. After which, urediniospore germination was interrupted by adding a 15 µL drop of lactofuschin to each drop. and then observed under a light microscope (Olympus BX-51). The number of germinated *vs* non-germinated urediniospores was estimated by observing at least 100 spores on each slide. Urediniospores were considered to

have germinated when the germ tubes had a length equal or longer than the urediniospore diam (Capucho *et al*. 2009) Germination inhibition (% GI) was calculated following the equation: % GI = (1 – X / C) × 100, where C = germinated urediniospores on the control slide and X = germinated urediniospores exposed to the antagonist (Silva-Castro *et al*. 2018).

Aflatoxin analysis

Production of aflatoxin by COAD 3307 was preliminarily assessed as follows: COAD 3307 was grown in flasks containing yeast extract with supplements (YES) and incubated for seven days at 25 °C, after which aflatoxin B1, B2, G1 and G2 production was evaluated through the agar plug-technique, according to Filtenborg and Frisvad (1980). Aflatoxins were searched for through separation in thin-layer chromatography (TLC) plates (Merck; Silica Gel 60.25 mm, 20 x 20), using toluene–ethyl acetate–90% formic acid (50:40:10) as the mobile phase (Abbas 2021). The possible presence of aflatoxins was checked through examination under ultraviolet light at 366 nm in a chromatovisor (CAMAG, Willington, NC, USA). Presence of aflatoxin in COAD 3307 was verified by the retention factor (Rf) and the presence/absence of a fluorescent spot which should be similar to the standard for aflatoxins (Sigma).

Statistical analysis

The median values of the percent severity of coffee leaf rust were calculated. Data from each treatment were submitted to the test of normality using the Shapiro-Wilk, before proceeding with the Mann-Whitney U test. The distribution of the estimated CLR severities was represented in the form of boxplots. The medians were compared by the Mann-Whitney U-statistic using the JASP software, version 0.16.0 (Statistics Program, from University of Amsterdam, https://jasp-stats.org/, 2021).

Results

Phylogeny

The alignment of *Aspergillus* to construct the phylogenetic trees included, 49 strains (Table 1) representing many of the known *Aspergillus* spp. in section *Flavi* for which molecular information is available. It included COAD 3307 with *Aspergillus tamarii* as an outgroup. The combined matrix consisted of 2835 characters, including alignment gaps. A high degree of similarity in the topology of the single trees (not shown) and the concatened tree was observed. The phylogenetic analysis revealed that COAD 3307 clustered with *Aspergillus flavus* in a clade with bootstrap support value > 70 %, and a Bayesian posterior probability value > 0.95 (Figure 1).

Taxonomy

***Aspergillus flavus* Link,** Mag. Ges. Naturf. Freunde Berlin 3: 16, 1809
MycoBank: MB209842.
Material examined. CAMEROON: Southwest Region, Buea; isolated as an endophyte from berry of *Coffea canephora*, 22 November 2015, H.C. Evans, M.K. Ndacnou (Culture COAD 3307).
For a complete description see Samson *et al*. (2000).
Morphology of COAD 3307:
On CZ: Conidiophores cylindrical, (37-) 100−300 (-544) × 3−11 µm, roughened, hyaline. Vesicles globose to subglobose, 3−26 µm wide, hyaline. Conidial heads radiate, monoseriate on young colonies but biseriate on older colonies, 22−59 × 22−61 µm. white-yellow. Phialides ampuliform, borne directly on the vesicle, 5−11 × 2−4 µm. Conidia mostly globose, 3−5 µm diam, one-celled, hyaline, finely roughened.
On MEA: Conidiophores cylindrical, (61-) 100−300 (-500) × 3−10 µm, hyaline, roughened. Conidial heads radiate, monoseriate in young colonies but biseriate in older colonies, 21−54 × 18−74 µm, yellow-green. Vesicles globose to subglobose, 4−27 µm wide. Phialides ampuliform, borne directly on the vesicle, 5−10 × 2−6 µm. Conidia mostly globose, 2−5 µm diam, hyaline, finely roughened.
In culture: On CZ colonies fast-growing (69−71 mm diam after seven days in the dark). Colonies flat, margin fimbriate, aerial mycelium velvety, ranging from to pure yellow to straw-colored with a white border, sporulation abundant. On MEA fast-growing (59−65 mm diam after seven days). Colonies raised concave, margin undulate, aerial mycelium cottony, velvety centrally and citrine green, white in the outer rings, sporulation abundant. Globose sclerotia, black to gray, produced after 14 days – more

abundant on CZ than on MEA. A comparison of the morphology and growth features of COAD 3307 with published descriptions of *Aspergillus flavus* further confirmed it as belonging to this species (Klich and Pitt 1988; Nyongesa *et al*. 2015; Frisvad *et al*. 2019).

Establishment of COAD 3307 as an endophyte in Coffea arabica *and reduction of CLR severity*

The endophytic colonization of the tissues of coffee plants by COAD 3307 was confirmed, although the results were not entirely consistent (Table 2). Such an inconsistency may result from the limited duration of the evaluation (for a perennial plant species) and from the methodology utilized for this study. For example, in assay I, COAD 3307 was recovered from roots, stems and leaves at the second and fourth rounds of isolations. However, it was only recovered from stems and leaves in the first round of isolations and only from stem samples in the third round of isolations. In assay II, only leaves and roots yielded COAD 3307. In essay I COAD 3307 was isolated from stems in all rounds of isolations. Nevertheless, in essay II stems were the only organs from which COAD 3307 was not isolated. In assay I, indication was obtained of a fast establishment of COAD 3307 resulting from soil inoculation. Isolation from coffee leaves in plants that had only been inoculated (at that stage) with soil application of COAD 3307 resulted in pure colonies of COAD 3307. Nevertheless, strangely, no COAD 3307 were obtained from coffee roots in this first round of isolations. No *Aspergillus* colonies were obtained from any of the non-inoculated or control plants (Figure 3). These tests also generated evidence that COAD 3307 is not pathogenic to *C. arabica*, since no disease symptoms appeared on treated plants during the experimental period.

COAD 3307 applications produced significant reductions ($p > 0.0001$) in CLR severity as compared to untreated controls (Figure 4). The severity for COAD 3307-treated plants was Mdn = 0.15, as compared to Mdn = 12.1 for the controls, U=224.500, $p > 0.0001$in assay I; and disease severity for COAD 3307-treated plants was Mdn = 1.9, whereas, in assay II, the value for the controls was Mdn = 7.9, U=1225, $p > 0.0001$. Such results matched well with the level of infection of leaves by CLR, which was much higher in control plants than in plants treated with *A. flavus* COAD 3307 (Figure 5).

Inhibition of Hemileia vastatrix *urediniospore germination by COAD 3307 filtrate*

The percentage of urediniospore germination in the controls varied between 67–95% whereas, in the presence of the filtrate, germination was reduced to 0−5%.

Aflatoxin analysis

COAD 3307 is in the same clade with known aflatoxin-producing *A. flavus* isolates, but here COAD 3307 was shown to be a non-aflatoxin-producing isolate. This is of especial relevance to its potential use as a biocontrol agent since aflatoxins are well-known as carcinogens (group 1, IARC, International Agency for Research on Cancer).

Discussion

Fungi in the *Aspergillus flavus*-group, section *Flavi*, have been reported from soil and various foodstuffs worldwide (Klich and Pitt 1988; Ingenbleek *et al*. 2019); being especially common in nuts, oil seeds and cereals, as well as occurring in dried fruits (Samson *et al*. 2000). It is possible that, although still unreported, *A. flavus* also occurs as endophytes in the fruits of these crop plants, as found here in the case of COAD 3307 in coffee berries. *Aspergillus flavus* has been reported only once as a root endophyte of coffee, in Ethiopia (Mulaw *et al*. 2013). Its occurrence had also been previously reported on coffee in Brazil by Batista *et al*. (2003), Borges *et al.* (2009) and Ferreira *et al.* (2011), but all those publications refer to its presence on harvested/stored/processed coffee and not to its isolation as an endophyte. Here, the molecular analysis of COAD 3307 confirmed it as belonging to *Aspergillus flavus*, with a high posterior probability (> 0.95), and a bootstrap support superior at 70% (Figure 1). Nevertheless, based on the principal morphological characteristics used by Klich and Pitt (1988) to differentiate *A. flavus* from the other species, COAD 3307 clearly fits into *A. flavus* (Figure 2). Features such as its conidia having a diameter smaller than 6 µm; conidia being hyaline or subhyaline, instead of brown (after 7 days at 25 °C), and smooth to finely roughened; stipe length being below 600 µm and producing non-floccose colonies, places COAD 3307 within the *A. flavus* species concept. Additionally, COAD 3307 grew well on CZ and MEA and reached a minimum of 59 mm diam after 7 days when incubated at 25 °C in the dark. Another cultural feature of

relevance was the production of black to gray sclerotia under these conditions. These were more abundant on CZ than on MEA, as decribed in Frisvad *et al*. (2019). However, some disparities were noted. For example, the color of conidial masses on MEA, colony topography, color and texture, as well as the presence of exudate droptlets in COAD 3307 are not as commonly reported for *A. flavus*. On CZ, instead of having yellow-green conidial masses, conidial masses COAD 3307 produced yellowish-white conidial masses, and the texture was velvety instead of floccose, as described in Frisvad *et al*. (2019) for *A. flavus*. Moreover, exudate droplets, as described by Frisvad *et al*. (2019), were absent in COAD 3307 (Figure 2b). However, such cultural differences have been regarded as insufficient for species separation in *Aspergillus* by Klich and Pitt (1988).

Inoculation of young *Coffea arabica* plants with COAD 3307 resulted in its endophytic establishment, as demonstrated by its recovery from roots, stems and leaves. Although COAD 3307 was originally isolated from berries of *C. canephora* it was shown here to be capable of colonizing other tissues of a different host. Further complementary studies are needed to determine the duration and extent of the endophytic colonization by COAD 3307. Other species of *Aspergillus* have been shown to be capable of growing as endophytes, and also to confer benefits to their host plants. For example, Sun *et al*. (2018) demonstrated positive endophytic effects of *Aspergillus oryzae* on radish (*Raphanus sativus*); being found to promote plant growth, as well as to provide some protection against the insect pest, *Plutella xylostella*.

The *in vitro* presence of haloes around the growing colonies of COAD 3307 (Figure 3), suggests an ongoing accumulation of secondary metabolites. It can be posited that such metabolites are behind the strong inhibition (95− 100 %) of urediniospore germination observed when the rust spores were exposed to culture filtrates. Additionally, assays *in planta* have shown that treatment with COAD 3307 can significantly ($P > 0.0001$) reduce CLR severity on *C. arabica* (Figures 4 and 5).

Aflatoxins type B and/or G are produced by the majority of strains of *A. flavus* (Klich and Pitt 1988; Batista *et al*. 2003; Frisvad *et al*. 2019); whilst non-aflatoxin producing strains of *A. flavus* are considered to be rare (Klich and Pitt 1988). It is fortunate, therefore, that the sole endophytic isolate of *Aspergillus* obtained from *Coffea* during the surveys appears to be a member of the latter group of *A. flavus* strains. A rich profile of secondary metabolites has also been reported from *A. flavus*: kojic acid, diketopiperazine, flavimin, aspergillic acid, aflatrem, cyclopiazonic acid and 3-

nitropropionic acid (Assante *et al*. 1981; Wicklow and Shotwel 1983; Klich and Pitt 1988; Danmek *et al*. 2011; Frisvad *et al*. 2019). The analysis of aflatoxin production by COAD 3307 indicated the absence of this mycotoxin. It is acknowledged here that our results are preliminary and that a more detailed (HPLC-MS/MS) analysis is still needed – such as used in Wei *et al.* (2013) – in order to confirm the safety of COAD 3307 as a biocontrol agent and to exclude the risk of exposure to mycotoxins. The absence of such mycotoxins is known to be common in commercial strains of *A. oryzae* and *A. sojae*, used in the food industry or in biotechnological processes (Frisvad *et al*. 2019), but wild strains may also lack the ability to produce mycotoxins (Klich and Pitt 1988). Many isolates of *A. flavus* section *Flavi* are well known as noxious mycotoxin (aflatoxin) producers and may also be involved in aspergillosis, a life-threatening human disease (Houbraken *et al*. 2020). Nevertheless, *A. flavus* is a highly variable and multifaceted species. Isolates such as COAD 3307 may be more common in special niches, such as endophytes of plants than previously thought.

The high levels of inhibition of urediniospore germination observed from exposure to filtrate of COAD 3307 are similar to those obtained by Salcedo-Sarmiento *et al*. (2021) for the mycoparasite *Calonectria hemileiae* (Salcedo-Sarmiento *et al*. 2021) and merits further investigation on the metabolite(s) involved.

The use of fungicides remains the principal way to control coffee diseases (Belan *et al*. 2015), including CLR (Talhinhas *et al*. 2017). Nevertheless, its supplementation or replacement with other forms of management, such as biological control, is being considered by farmers, the coffee industry and consumers as necessary, especially as the demand for organic coffee increases. This is reflected in the recent launching of the ECOFFEE multi-stakeholder initiative (https://www.cirad.fr/en/press-area/press-releases/2021/ecoffee-r-d-to-reduce-pesticides-in-the-coffee-sector) aimed at reducing or even excluding the use of chemical pesticides in coffee production worldwide. The CLR biocontrol research activities initiated in 2015 by World Coffee Research (WCR 2019) could contribute to ECOFFEE reaching its ambitious goals. The COAD 3307 isolate of *A. flavus* may have a role to play, either alone or in combination with other fungal antagonists of *H. vastatrix*. With its anti-CLR biocontrol potential preliminarily confirmed *in planta* during this study, it may serve as a basis for viable biofungicides such as *Aspergillus flavus* AF36 and Afla-guad NRRL21882 which have been registered since 2003 and 2004 with the U.S. Environmental Protection Agency for use in cotton and peanut plantations, respectively (Fravel 2005), and repeatedly

validated as effective for the control of aflatoxin producing-strains of *A. flavus* in the maize field in the USA, including in a recent multi-year study (Weaver and Abbas 2019). However, further studies including field experiments are needed to confirm the results presented herein.

Conflicts of interest

We declare no conflict of interest.

Acknowledgments

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Authors' contributions

Conceived the study: HCE and RWB. Collected the fungi: MKN, HCE and RWB Designed the experiments: MKN, LRB, TFN and RWB. Performed the experiments:

MKN, LRB, TFN. Analysed the data: MKN, LRB, TFN. Writing original draft: LRB, MKN and RWB. Reviewed and edited the original draft: HCE, RWB and DABB.

Figures legends

Figure 1. Bayesian Inference (BI) tree constructed with the ITS, β-tubulin, CAL and RPB2 sequences of strains representatives of different taxa in *Aspergillus* section *Flavi*. The phylogenetic tree was rooted with *Aspergillus tamarii*. Bold branches represent bootstrap support values for ML greater than 70% and Bayesian posterior probabilities greater than 0.95. Branches supported in just one of the methods are given near nodes. The isolate obtained in this study is indicated in red. Branch lengths are proportional to distance. ^{T} Ex-type strain. The bar indicates the number of substitutions per site.

Figure 2. *Aspergillus flavus* COAD 3307. a-b Left to right, colonies on CZ and MEA after 7 days. c Sclerotia on MEA after 14 days. d-g Conidiophores and conidia. h-i Conidial heads. j monoseriate. k-l Conidia. Scale bar = 10 µm**.**

Figure 3. Isolations from coffee tissues inoculated (a–c) with *Aspergillus flavus* COAD 3307. Left to right: leaf, stem and root. Note COAD 3307 colonies emerging from a–c (arrowed).

Figure 4. Coffee leaf rust severity on plants sprayed with *Hemileia vastatrix* urediniospore suspensions alone (control) *vs* on plants previously treated with the isolate COAD 3307 of *Aspergillus flavus*. Box-plots showing results of assays I and II. Highly significant differences (*) found for both experiments as determined by Mann-Whitney U-statistic. (I) U = 224.5, $p > 0.0001$, medians resulting of the assessment of two plants/treatment with eight leaves/plant; (II) U = 1225, $p > 0.0001$, medians resulting of the assessment of five plants/treatment with eight leaves/plant.

Figure 5. Example images of the contrast in severity of coffee leaf rust between: (A) a *Coffea arabica* plant treated with the antagonist (COAD 3307 *A. flavus) vs* (B) an untreated (control) plant, 2 months after inoculation with a suspension of urediniospores of *Hemileia vastatrix*.

Table 1. Details on the combined dataset used in this study.

Species name	Strain number	GenBank accession numbers			
		ITS	BenA	CaM	Rpb2
Aspergillus aflatoxiformans	CBS 143679 = DTO 228-G2T = IBT 32085	MG662388	MG517706	MG518076	MG517897
	CBS 121.62 = IMI				
A. aflatoxiformans	093070 = NRRL A-11612 = IBT 3651 = IBT 3850 =	EF409240	MG517719	MG518089	MG517910
	DTO 010-H7 = DTO 223-C2 = DTO 228-H6				
A. aflatoxiformans	DTO 087-A2	MG662405	MG517652	MG517990	MG517840
A. aflatoxiformans	DTO 228-G1 = IBT 32079	MG662389	MG517705	MG518075	MG517896
A. aflatoxiformans	DTO 228-G3 = IBT 32086 = CBS 135587	MG662387	MG517707	MG518077	MG517898
A. arachidicola	CBS 117610^{T}	MF668184	EF203158	EF202049	MG517802
A. arachidicola	CBS 117611	–	MG517620	MG518006	MG517803
A. arachidicola	CBS 117615	–	MG517627	MG517999	MG517810
A. arachidicola	DTO 228-H9	MG662384	MG517721	MG518091	MG517912
A. austwickii	CBS 143677^{T}	MG662391	MG517702	MG518072	MG517893

A. austwickii	CBS 135406	MG662386	MG517712	MG518082	MG517903
A. austwickii	CBS 143678		MG517703	MG518073	MG517894
A. austwickii	DTO 228-F9 = IBT 32078	MG662390	MG517704	MG518074	MG517895
A. cerealis	CBS 143674^{T}	MG662394	MG517693	MG518063	MG517884
A. cerealis	CBS 143675		MG517694	MG518064	MG517885
A. cerealis	CBS 143676	MG662393	MG517695	MG518065	MG517886
A. flavus	Ex-type: CBS 569.65 = NRR L1957 = ATCC 16883 = IMI 124930 = QM 9947 = WB 1957	AF027863	EF661485	EF661508	EF661440
A. flavus	CBS 110.55	FJ491463	EF203135	MG518005	MG517821
A. flavus	CBS 117638	–	MG517619	MG518011	MG517801
A. flavus	CBS 117637	–	MG517618	MG518010	MG517800
A. flavus	CBS 485.65	EF661563	MG517643	MG518014	MG517828
A. flavus	CBS 501.65	EF661563	MG517642	MG518015	MG517827
A. flavus	CBS 542.69	EF661554	MG517641	MG518016	MG517826
A. flavus	CBS 574.65	JN185448	JN185446	JN185447	JN185449
A. flavus	**COAD 3307**	MZ452982	MZ467047	MZ467046	MZ467045
A. minisclerotigenes	CBS 117635^{T}	EF409239	EF203148	MG518009	MG517799
A. minisclerotigenes	CBS 117633	MG662408	EF203153	MG518007	MG517797

A. minisclerotigenes	CBS 117634	MG662402	MG517617	MG518008	MG517798
A. mottae	CBS 130016^{T}	JF412767	MG517687	MG518058	MG517878
A. mottae	MUM 10.233	–	HM803090	HM803013	HM802982
A. novoparasiticus	CBS 126849^{T}	MG662397	MG517684	MG518055	MG517875
A. novoparasiticus	CBS 126850	MH279415	MG517686	MG518057	MG517877
A. parasiticus	CBS 100926^{T}	AF027862	EF661481	EF661516	EF661449
A. parasiticus	CBS 104.22	–	MG517621	MG517994	MG517804
A. parasiticus	CBS 119.51	–	MG517622	MG518000	MG517805
A. parasiticus	CBS 138.52	–	MG517623	MG517997	MG517806
A. parasiticus	CBS 260.67	MG662400	EF203156	MG518013	MG517830
A. pipericola	CBS 143680^{T}	MG662385	MG517717	MG518087	MG517908
A. sergii	CBS 130017^{T}	JF412769	MG517688	MG518059	MG517879
A. sojae	CBS 100928^{T}	KJ175434	EF203168	EF202041	MG517831
A. sojae	CBS 133.52	EF661546	EF661482	EF661517	EF661450
A. sojae	DTO 173-C3	–	MG517658	MG518028	MG517846
A. subflavus	CBS 143683^{T}	MH279429	MG517773	MG518143	MG517964
A. subflavus	S843b	MH279449	MG517792	MG518164	MG517983

A. tamarii	CBS 104.13^{T}	AF004929	EF661474	EF661526	EU021629
A. transmontanensis	CBS 130015^{T}	JF412774	HM803101	HM803020	HM802980
A. transmontanensis	MUM 10.205	JF412771	HM803087	HM803021	HM802979
A. transmontanensis	MUM 10.211	JF412772	HM803102	HM803023	HM802968
A. transmontanensis	MUM 10.221	JF446612	HM803093	HM803028	HM802972

Table 2. Evidence of *Aspergillus flavus* (COAD 3307) endophytic colonization of *Coffea arabica* through isolation from different organs of inoculated *vs* non-inoculated plants*

Essay I

Month after first inoculation	COAD 3307 recovered (+)** or not (–)** from tissues					
	Inoculated plants			non-inoculated plants		
	leaf	stem	root	leaf	stem	root
1st*	+	+	–	–	–	–
2nd*	+	+	+	–	–	–
3rd*	–	+	–	–	–	–
5th*	+	+	+	–	–	–

Essay II

Month after first inoculation	leaf	stem	root	leaf	stem	root
5th*	+	–	+	–	–	–

* Inoculations in four rounds in both essays, combining soil application – COAD 3307 colonized rice (50 g/plant) – with an estimated 10^9 conidia g^{-1} of rice (first application) and three foliar sprays (conidial suspensions – 10^7 conidia ml^{-1} of COAD 3307 until runnoff) at 30 days-intervals after soil application. Control (non-inoculated plants) received 50g uncolonized rice per plant or sprayed with sterile distilled water (SDW) in parallel with the COAD 3307-treatement of the other group of plants.

** + = At least one fragment producing a colony of *A. flavus* ; − = No *A. flavus* colony obtained from any fragment in plates after 2−3 weeks.

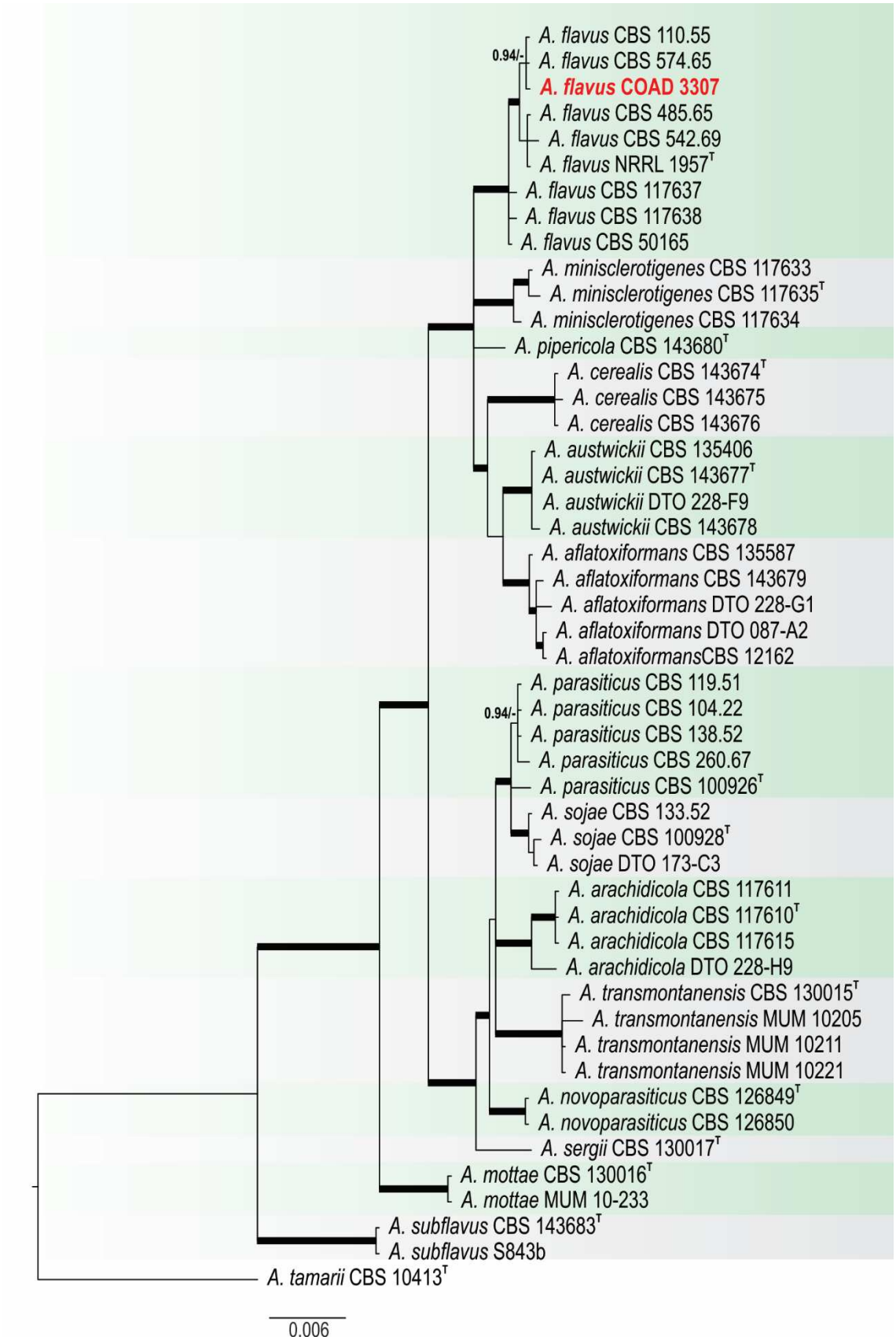
0.94/-
A. flavus CBS 110.55
A. flavus CBS 574.65
A. flavus COAD 3307
A. flavus CBS 485.65
A. flavus CBS 542.69
A. flavus NRRL 1957T
A. flavus CBS 117637
A. flavus CBS 117638
A. flavus CBS 50165
A. minisclerotigenes CBS 117633
A. minisclerotigenes CBS 117635T
A. minisclerotigenes CBS 117634
A. pipericola CBS 143680T
A. cerealis CBS 143674T
A. cerealis CBS 143675
A. cerealis CBS 143676
A. austwickii CBS 135406
A. austwickii CBS 143677T
A. austwickii DTO 228-F9
A. austwickii CBS 143678
A. aflatoxiformans CBS 135587
A. aflatoxiformans CBS 143679
A. aflatoxiformans DTO 228-G1
A. aflatoxiformans DTO 087-A2
A. aflatoxiformansCBS 12162
A. parasiticus CBS 119.51
0.94/-
A. parasiticus CBS 104.22
A. parasiticus CBS 138.52
A. parasiticus CBS 260.67
A. parasiticus CBS 100926T
A. sojae CBS 133.52
A. sojae CBS 100928T
A. sojae DTO 173-C3
A. arachidicola CBS 117611
A. arachidicola CBS 117610T
A. arachidicola CBS 117615
A. arachidicola DTO 228-H9
A. transmontanensis CBS 130015T
A. transmontanensis MUM 10205
A. transmontanensis MUM 10211
A. transmontanensis MUM 10221
A. novoparasiticus CBS 126849T
A. novoparasiticus CBS 126850
A. sergii CBS 130017T
A. mottae CBS 130016T
A. mottae MUM 10-233
A. subflavus CBS 143683T
A. subflavus S843b
A. tamarii CBS 10413T
0.006

Figure 1.

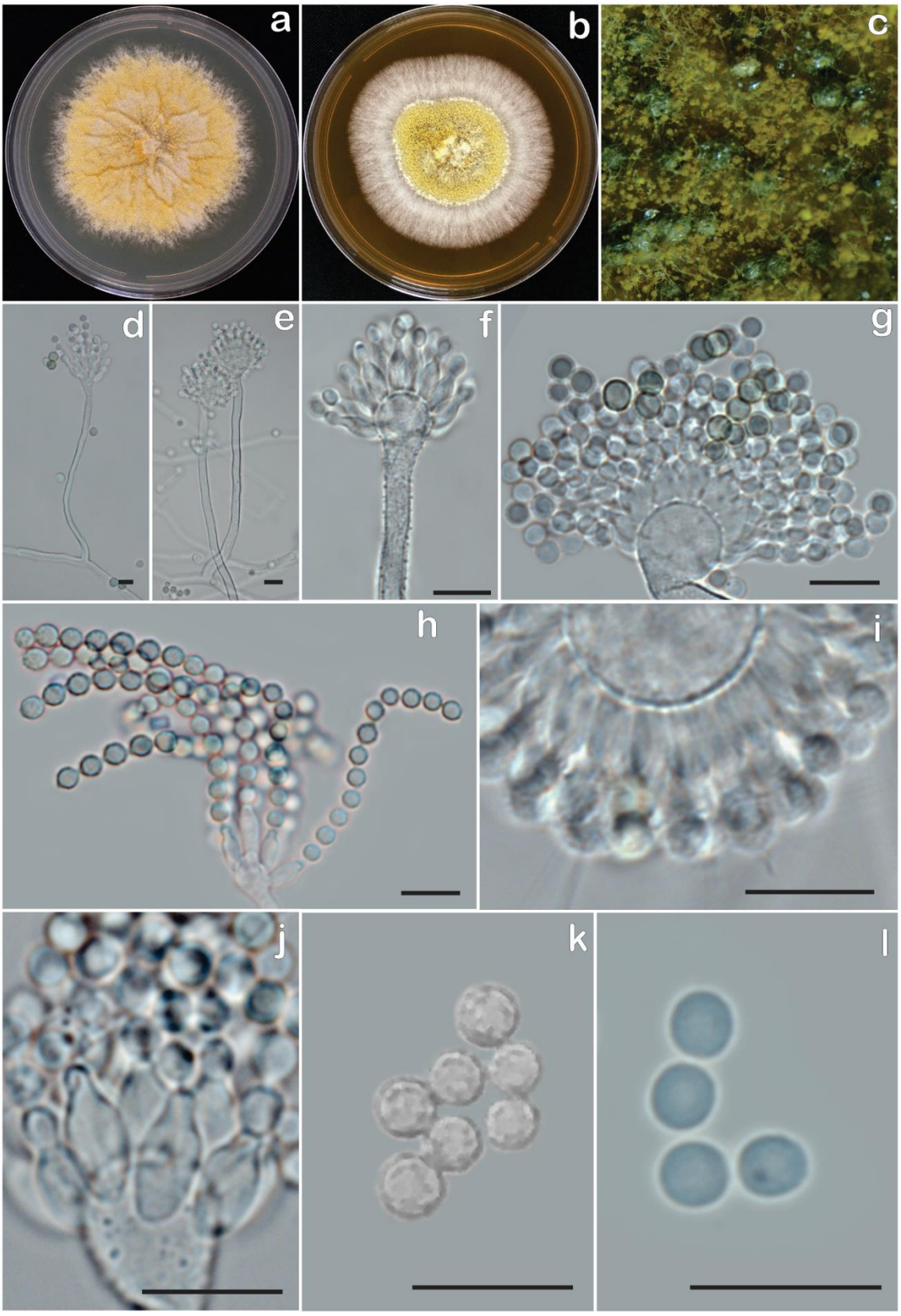

Figure 2.

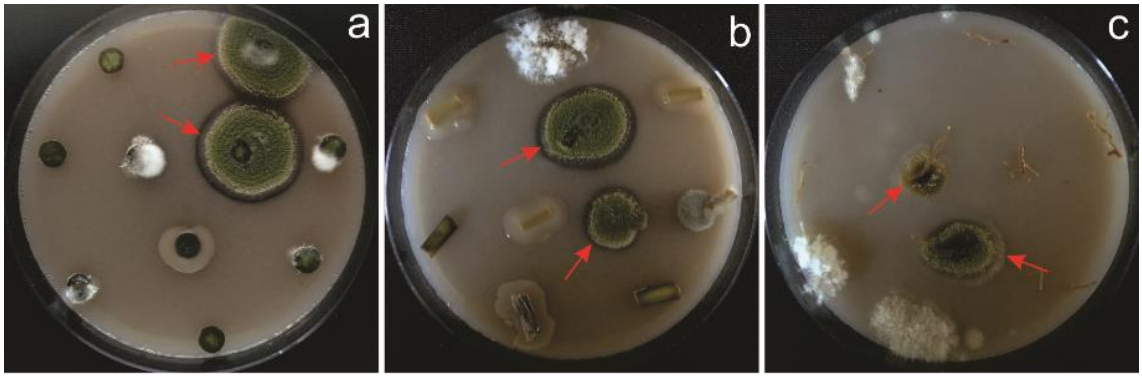

Figure 3.

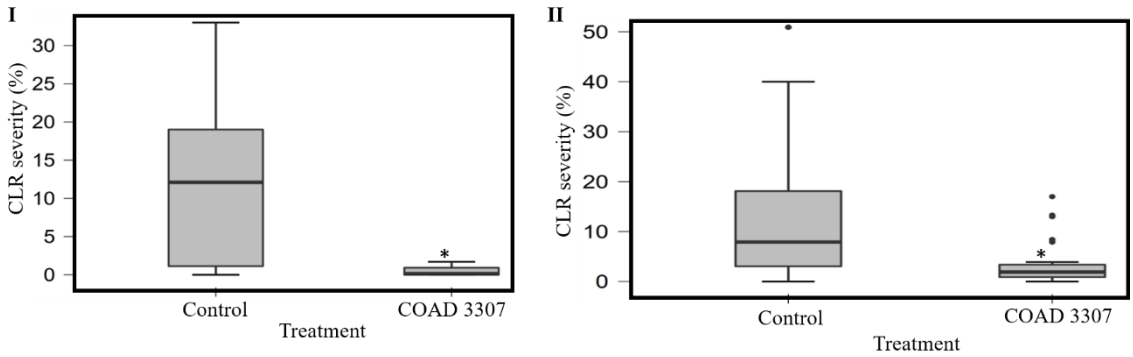

Figure 4.

Figure 5.

CHAPTER 2 (Article in the format of Brazilian Journal of Microbiology)

Clonostachys* species occurring as endophytes in *Coffea* species and mycoparasites on the rust fungus *Hemileia vastatrix

Clonostachys species occurring as endophytes in *Coffea* species and mycoparasites on the rust fungus *Hemileia vastatrix*

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Abstract

During surveys conducted in South America and Africa to identify fungal natural enemies of the coffee leaf rust, *Hemileia vastatrix*, over 1,500 strains were isolated either as endophytes from healthy tissues of *Coffea* species or as mycoparasites growing on *Hemileia* rust pustules. Based on morphological data, 28 isolates were provisionally assigned to the genus *Clonostachys*, a polyphasic study of their morphological, cultural and molecular characteristics – including *Tef1*(translation elongation factor 1 alpha), *RPB1*(largest subunit of RNA polymerase II), *TUB* (β-tubulin) and *ACL1* (ATP citrate lyase) regions – confirmed eight of these isolates as belonging to species of the genus *Clonostachys*, namely: *C. byssicola*, *C. rhizophaga* and *C. rosea f. rosea*. Data placing these fungi in each of the taxa are presented and discussed herein. These are the first records of *Clonostachys* associated with healthy coffee stem inner tissues and with coffee leaf rust.

Keywords: Bionectriaceae. Coffee leaf rust. Endophytic fungi. Phylogenetics. Mycoparasites.

Introduction

Coffee is ranked as the most economically valuable agriculture commodity [1]. Only two species of the genus *Coffea* are of commercial relevance: *C. arabica* and *C. canephora* (Rubiaceae). Although Africa plays a relatively small role in the world coffee production – Ethiopia, the largest coffee producer in the continent, being the fifth larger producer in the world but having only around 4% of the market share – the crop is of great social relevance, as well as worldwide, for the income and employment it generates. It is produced mostly by smallholder communities [2]. However, there are increasing threats to coffee production from pests, diseases and adverse climatic conditions [3, 4].

Amongst these limitations for coffee production is its most devastating disease, coffee leaf rust (CLR) caused by *Hemileia vastatrix*, a biotrophic fungus [5]. The recent report of CLR in the Hawaiian archipelago, means that it has now spread to every significant coffee-growing region in the world [6]. The search for a sustainable, non-chemical and effective form of management for CLR is a major challenge [7, 8]. Outbreaks of CLR in northern South America and Central America, starting in early 2010 [3], have caused economic and social distress in the region and appear to be connected to increasing temperature (climate change); leading to the failure of the strategy of escaping *H. vastatrix* by planting coffee in highland situations. Biological control of *H. vastatrix* has, thus far, received relatively little attention.

Although there are several studies on potential antagonists of CLR, such as endophytic fungi growing inside healthy coffee tissues [9, 10, 11, 12, 13], or mycoparasites growing on pustules of *H. vastatrix* [14, 15, 16, 17, 18], these have been focused predominantly in the Neotropics, where the rust and coffee are exotic species, and have yet to translate into practical advances in CLR management. Until recently, little attention had been given to the mycobiota associated with *Coffea* in Africa as a source of antagonists to pathogens attacking the crop. A notable exception is Mulaw et al. [19] a work dealing with *Trichoderma* spp. isolated as endophytes from roots of *C. arabica* in Ethiopia; and focusing on their antagonism to *Fusarium xylarioides*, the causal agent of tracheomycosis. Fungal antagonists of CLR in Africa remain largely unknown.

In 2015, surveys were initiated in Africa for potential fungal antagonists of *H. vastatrix*, and an unexpectedly high diversity of fungi was revealed [20-26]. Amongst

the more than 1,500 isolates, some were assigned provisionally to the genus *Clonostachys* (Ascomycota: Hypocreales: Bionectriaceae). Here, we report on the taxonomic studies to elucidate their identity.

Materials and Methods

Survey and isolation of purported antagonists of *H. vastatrix*

Surveys for fungal antagonists of *H. vastatrix* were concentrated in Africa; focusing on Cameroon and Ethiopia – representing regions within the centres of origin of *C. canephora* and *C. arabica*, respectively – and *Coffea* species growing in wild or semi-wild situations were targeted. Other *Hemileia* species, in addition to *H. vastatrix*, were also found on *Coffea* in these ecosystems. Details of the strategy and the isolation methodology employed to isolate endophytic fungi and purported mycoparasites are given in previous publications [20,22].

Morphology and cultural studies

Isolates were mounted in 60% lactic acid and observed under a light microscope (Olympus BX53), fitted with differential interference contrast light and a digital image capture system (Olympus Q-Color 3™ camera).

Additionally, a slide-culture method [28], was used for selected isolates. Small blocks of oatmeal agar (OA) were inoculated on their sides and a sterile coverslip was placed on top of the block. This was transferred to an incubator, adjusted to 25 ± 2°C under a (12h/12h) daily light regime (light provided by two white fluorescent day light bulbs, FLC, 25W−127V, placed 35 cm above the plates), for 5−6 days. Subsequently, the agar blocks were removed and the coverslips and slides, bearing the fungal cultures, were mounted (as described in [28]) for further examination.

Morphological data of relevance for species delimitation in *Clonostachys* and related taxa – shape of conidiophores, size of stipe and penicillus, shape and size of phialides and conidia, among others – were recorded for at least thirty representative structures. Photo images were prepared in CorelDraw Graphics Suite 2018.

Colony characters were recorded after 10 days of growth on potato dextrose agar (PDA), OA and 3 % malt extract agar (MEA) [37], at 25 ± 2°C under a 12 h/day

light regime. Colony morphology was described based on the standard terminology [29]. Colony color terminology followed Rayner [30]. Each isolate was replicated on three separate plates. Colony diameter was also measured after 10 days of incubation under the conditions described above.

Twenty-eight out of over 1,500 isolates obtained during the surveys were assigned provisionally to *Clonostachys*. These isolates were either stored on potato carrot-agar (PCA) slants at 4 °C for short-term use or, for long-term storage, kept at -80 °C in cryotubes with 10% glycerol as described in the literature [31]. Selected isolates (Table 1) were deposited in the culture collection of the Universidade Federal de Viçosa (UFV), Viçosa-MG, Brazil – Coleção Octavio de Almeida Drummond (COAD).

Preliminary molecular identification

The preliminary molecular identification included only an analysis of the sequences of the translation elongation factor 1 alpha (*tef1-α*) region of the rDNA gene [32,33]. This approach aimed at excluding from further study those isolates not belonging to the genus *Clonostachys*.

The isolates were grown on PDA and then seeded in small plates containing 5 mL of potato dextrose (PD) broth and incubated for 5 days at 25 °C under a 12/12 h daily light/dark regime. After that period, the mycelial material was removed from the plates and air dried on sterile filter paper at room temperature for 24 h. Dried mycelial material from each isolate was then transferred to sterile tubes for DNA extraction. Extraction was performed with the Wizard Genomic DNA Purification kit (Promega, Madison, EUA). Manufacturer's guidelines were strictly followed. The primer pair EF1-728F/EF2 was used for amplification and sequencing of *tef1-α* region. The following cycling conditions were used during PCR: Initial denaturation at 94.0 °C for 5 min; followed by 38 cycles of 94.0 °C for 30 s, 54.0 °C (annealing temperature) for 30 s and 72.0 °C for 30 s; with a final extension of 7 min at 72.0 °C. The amplicons were analyzed on GelRed ™ (Thermo Fisher Scientific) and visualized under UV light to verify the size and purity of amplificons. Then, they were purified with ExoSAP-IT™ (Thermo Fisher Scientific), and the PCR product was sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The first approach towards a molecular identification of the isolates was performed through BLASTn searches of the

sequences, in the NCBI (National Center for Biotechnology Information) nucleotide sequence database.

Those with high identity threshold corresponding to the genus *Clonostachys* (as indicated in Table 1) were selected for further evaluation.

DNA, Polymerase Chain Reaction (PCR) and sequencing of three additional specified gene regions

Isolates recognized as belonging to *Clonostachys*, by the combination of morphological and molecular (translation elongation factor 1 alpha) data, were further analyzed molecularly. Three additional gene regions were sequenced for those isolates, following the methodology described above, namely: *ACL1* (ATP citrate lyase), *RPB1* (largest subunit of RNA polymerase II), and *TUB* (β-tubulin). This was carried out using the primers pairs acl1-230up/acl1-1220low for *ACL1* [34], Fa/R8 for *RPB1* [35] and T1/T2 for *TUB* [36]. The PCR reactions were performed in a total volume of 12 µl as follow: 1 µl genomic DNA of concentration 30 ng μl^{-1}, 1 µl BSA, 0.5 µl DMSO, 0.5 µl Per each primer, 2.5 µl Water MilliQ and 6 µl Dream Taq. The cycling conditions for β-tubulin were according to the published description [37]. However, the protocol for *ALC1* was modified: initial denaturation was at 94.0 °C for 5 min; followed by 38 cycles of 94.0 °C for 30 s, 56.0 °C (annealing temperature) for 30 s and 72.0 °C for 30 s; with a final extension of 7 min at 72.0 °C. For *RPB1* initial denaturation was at 94.0 °C for 5 min; followed by 38 cycles of 94.0 °C for 30 s, 56.0-50.0 °C (annealing temperature) for 30 s and 72.0 °C for 30 s; with a final extension of 7 min at 72.0 °C. Amplicons were analysed on GelRed ™ (Thermo Fisher Scientific) and visualized under UV light to verify the size and purity of amplificons. Then, they were purified with ExoSAP-IT™ (Thermo Fisher Scientific) and the gene regions were sent for sequencing in Macrogen Inc., South Korea (http://www.macrogen.com).

Sequences-phylogenetic analyses

In order to assemble and edit the nucleotide sequences, the software DNA Dragon 1.7.3 – DNA Sequence Contig Assembler Software developed by SequentiX – Digital DNA Processing (https://www.sequentix.de/) was utilized [38]. BLASTn searches of Genbank were performed through the MegaBlast program, to verify the taxonomic and

locus identities of the consensus sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of reference of species with high percent of identity were selected for the alignment, according to the same gene region used for amplifying the gDNA of isolate. The algorithm Muscle, from the software Aliview, version 1.26 [39] was used to align sequences. Datasets from individual genes and the multilocus combination (*ACL1, RPB1, TEF1-α* and *TUB*) dataset were investigated using the programs RAxML-HPC on XSEDE 8.2.12 for the Maximum likelihood analysis, and MrBayes on XSEDE 3.2.7a for the Bayesian Inference analysis, via the CIPRES web portal [40]. Maximum likelihood (ML) analysis was performed with 1,000 bootstrap.

Bayesian Inference (BI) analyses were launched after defining the best nucleotide substitution models for each gene. The JModeltest 2.1.10 software [41] was applied and the different models were selected according to the Corrected Akaike Information Criterion (AICc). The likelihood settings from best-fit model of evolution TrN+G were used for *ACL*, TIM1ef+I for *RPB1*, TIM3ef+I for *TEF1-α* and HKY+G for *TUB*. Phylogenetic analyses were conducted with alignments of 44 parsimony-informative positions 44/821 bp for *ACL*, 46/1027 bp for *RPB1*, 46/563 bp for *TEF1-α* and 42/562 bp for *TUB*, following the standard configuration of two runs and four chains for each run. Two independent analyses were run for 20 x 10^6 generations and chains were sampled every 1000 generations for each dataset. 0.25 fraction of the initial trees were discarded as burn in before constructing trees consensus. The average standard deviation of split frequencies (ASDSF) was evaluated for the assessment of the convergence between independent runs, and automatically stopped when reached a determined value of ASDSF. A BI concatenated tree with the four gene regions was also constructed with MrBayes under the previous four best-fit models and following the same previous standard configuration via the CIPRES web portal [40]. *Clonostachys pseudochroleuca* CBS 192.94T was included as the outgroup. The tree topologies resulting from both BI and ML methods were visualized, compared and the phylogram edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and Inkscape.

Sequences of this study, partially deposited in Genbank (http://www.ncbi.nlm.nih.gov/genbank) and those from different genes (*ACL1, RPB1, TEF1-α* and *TUB*) retrieved from Genbank are listed in Table 1.

Results

Phylogeny

BLASTn searches applied to the sequences from the region *TEF1-α* of isolates preliminarily identified as *Clonostachys*-like, revealed that only eight among the 28 "suspected *Clonostachys*" isolates belonged to the purported genus (with a threshold of identity ≥ 96 %). Amongst these eight, three were isolated as endophytes from stems of *C. arabica*, four were isolated from colonies growing on rust uredinia on *C. canephora*, and one from a colony growing on rust. uredinia on *C. arabica* (Table 1). The BI and ML concatenated trees obtained by combining the available sequences, from all four loci (*ACL1, RPB1, TEF1-α* and *TUB*) of species of the genus *Clonostachys* had an equivalent topology. The BI tree is shown in Fig. 1. It revealed that the collection of *Clonostachys* isolates from coffee and *Hemileia* uredinia belong to: *Clonostachys byssicola*, *C. rhizophaga* and *C. rosea*. These are well known taxa which have already been described and discussed in detail in the literature [42, 37, 43].

Four of the isolates obtained as purported mycoparasites of *Hemileia* uredinia formed a clade with *C. rhizophaga*. Two endophytic isolates grouped within the *C. rosea* clade. The other two isolates – one an endophyte and the other a mycoparasite, formed a clade with *C. byssicola*. The topologies of single gene trees were not all equivalent (trees not shown) to those of the concatenated tree. In the *ACL1* and *RPB1* all the species strains were monophyletically grouped in both ML and BI analyses. The four isolates in the monophyletic group of *C. rhizophaga* were always divided into two groups, however in the concatened tree they are well-grouped and form a brother group with the strains already identified as *C. rhizophaga*. In the *TEF1* tree the strains already identified as *C. rhizophaga* appeared in two separate clades. The same was seen in the *TUB* tree for the *C. byssicola* strains, which also appeared in two separate clades in ML and BI analyses. However, all isolates of the three species formed monophyletic clades in the concatenated tree, with support values, ranging from 70 to 100% ML bootstrap support and BI posterior probabilities ≥ 0.98 (Fig. 1). The sequencing of the amplified *TUB* region within the gDNA of the four isolates of *C. rhizophaga* showed paralogous sequences, thereby being absent in Table 1.

Taxonomy

A comparison of the morphology of the isolates COAD 2983 and COAD 2986; COAD 2979, COAD 2980, COAD 2981 and COAD 2982; and COAD 2984 and COAD 2985, with published descriptions of *Clonostachys byssicola*, *C. rhizophaga* and *C. rosea* [42, 44], further confirmed their placement within the accepted boundaries of each of these species as shown in Fig. 1 (Table 2).

Clonostachys byssicola Schroers, Stud. Mycol. 46: 80 (2001) [42]. Sexual morph: *Bionectria byssicola* (Berk. & Broome) Schroers & Samuels, Z. Mykol. 63(2): 152 (1997).

Material examined. ETHIOPIA: Southern Nations, Nationalities and Peoples Region, Kaffa Zone, Bonga District, Komba Wild Forest Reserve, cloud forest, 2000 m; isolated as a mycoparasite on uredinia of *Hemileia cf. coffeicola* (COAD 2983) and as an endophyte in stems of wild *Coffea arabica* (COAD 2986), 25 Nov 2015, H.C. Evans and K. B. Bekele.

For a detailed description and illustrations, see Schroers [42].

Conidiophores dimorphic: Primary conidiophores verticillate, phialides divergent (2–4 whorls) 19−54 × 2−3 µm, stipes 10–63 µm and penicillus 19−55.5 µm long; secondary conidiophores adpressed, bi- to terverticillate, phialides adpressed (4−7 whorls) 10−32.5 × 1−2.5 µm.

Conidia septate, cylindrical, and/or slightly curved, 2.5−10 (–14) × 1.5−4 µm.

In culture (COAD 2983, on OA) (Fig. 3): low convex, edge entire, aerial mycelium felty or granular (due to sporulation), rosy buff, conidial mass white, or pale yellow; reverse rosy buff; sporulation abundant. Average colony diam after 10 days: 67.5 mm– on OA, 55 mm – on PDA and 64.5 mm – on MEA.

Notes – When compared with COAD 2983, some morphological details given in [42] for *C. byssicola* were different. Primary conidiophore phialides were shorter (12.4−48 × 1.4−2.8 µm), stipes and penicillus were longer (10−100 µm and 20−100 µm, respectively); secondary conidiophores had more verticillus (2–5), phialides were in smaller whorls (3−5) and were shorter (7.6−27.8 µm). Such morphological discrepancies between COAD 2983 and the data given in [42] were not regarded as significant for taxonomic recognition

Clonostachys rhizophaga Schroers, Stud. Mycol. 46: 85 (2001) [42].

Material examined. CAMEROON: Eastern Province, Somalomo, 700 m; isolated as mycoparasites of uredinia of *Hemileia vastatrix/coffeicola* on leaves of *Coffea canephora*, 22 Nov 2015, H.C. Evans (Culture COAD 2979, 2980, 2981 and 2982).

For a complete description and illustrations, see Tehon & Jacobs [45].

Conidiophores dimorphic: Primary conidiophores verticillate, phialides divergent (2–5 whorls) (9.5–) 11−35 × 1−3 µm, stipes 20−92 µm and penicillus 19−87 µm long; secondary conidiophores penicillate, mono- to quaterverticillate, phialides adpressed (3−6 whorls) (5−) 14.5−17(−28) × 1– 2.5(−7) µm.

Conidia: ellipsoidal/cylindrical, minutely curved to curved, with laterally displaced hilum; 3.5−9 (–11) × 2−5.5 (−7.5) µm.

In culture (COAD 2979, on OA) (Fig. 3): flat or effuse, with undulate/fimbriate edge, aerial mycelium felty to cottony, dense and finely granular, white to rosy buff, pigmenting the medium with yellow diffusate, conidial mass whitish; reverse whitish to buff; sporulation abundant. Average colony diam after 10 days: 66.5 mm − on OA, 46 mm − on PDA and 40 mm − on MEA.

Notes – When compared with COAD 2979, some morphological details given in [42] for *C. rhizophaga* differed: primary conidiophores penicillus were longer (30−100 µm) in his description. Also, the phialides in his secondary conidiophores were narrower (2.2−3.2 µm). Here, we interpret such discrepancies as representing a variation within the species, not meriting taxonomic recognition.

Clonostachys rosea (Link) Schroers, Samuels, Seifert & W. Gams, Mycologia 91(2): 369 (1999) [44] ***f. rosea***.

Material examined. ETHIOPIA: Southern Nations, Nationalities and Peoples Region, Kaffa Zone, Bonga District, Komba Wild Forest Reserve, cloud forest, 2000 m; isolated as endophyte from stems of *Coffea arabica*, 25 Nov 2015, H.C. Evans (Culture COAD 2984 and 2985).

For a complete description and illustrations, see Schroers [42].

Conidiophores dimorphic: Primary conidiophores verticillate, phialides divergent (2–5 whorls) 20.5−40.5 × 1.5−3 µm, stipes 20−122 µm and penicillus 25.5−73.5 µm long; − secondary conidiophores adpressed, bi- to quaterverticillate, phialides adpressed (5−7 whorls) 10−19 × 1−3 µm.

Conidia: cylindrical, less curved, distally broadly rounded, with one slightly flattened side, inconspicuous hilum; conidia size 4−10 × 1.5−3.5 µm.

In culture (COAD 2985, on OA) (Fig. 3): radially striate, with lobate edges, aerial mycelium felty in strands, granulose, white periphery to dense rosy buff towards the centre, conidial mass white; reverse whitish to rosy buff; sporulation abundant. Average diam in 10 days: 67 mm − on OA, 53 mm − on PDA and 64.5 mm − on MEA. *Notes* – A comparison of the morphology of *C. rosea* f. *rosea*, as described by Schroers [42] with that of COAD 2985 revealed some differences. In [42] the primary penicilli were longer (30−120µm long) as well as the stipes (25−200 µm). The discrepancies highlighted here are interpreted as normal variation within the species.

Discussion

Most species of *Clonostachys* are obscure and known only from their original descriptions, but *C. rosea* has been intensively studied as discussed in [46]. *Clonostachys* has been isolated mainly from soil and litter but they have also been isolated from bryophytes, wood, bark, black pepper, grapevine, insects, nematodes and wine [47, 46, 43]. Some *Clonostachys* species occur as mycoparasites, including on *Botrytis cinerea*, an important pathogen causing gray mold on numerous crops [48, 49, 50].

Clonostachys species have been reported as potential biological control agents of several plant pathogens. *Clonostachys byssicola* has been shown to inhibit the growth of *Phytophthora palmivora* on cocoa [51, 52], and reduce the incidence of banana crown rot [53]; and *C. cf. byssicola* has been reported as mycoparasitic on *Rosellinia* spp. causing black root rot of cocoa [54]. *Clonostachys rhizophaga* was found to reduce the severity of potato early blight caused by *Alternaria grandis* [55] and as a mycoparasite on the chickpea pathogen *Didymella rabiei* [56]. *Clonostachys rosea,* the most broadly studied species for biocontrol purposes, is a well-documented antagonist of *Botrytis cinerea* [48, 49, 50], *Pythium aphanidermatum* [57], *Phytophthora palmivora* [52], and *Fusarium graminearum* [58].

Research involving *C. rosea* has resulted in the development of several biofungicides, including Kamoi which has been registered for use against gray mold (*B. cinerea*) [59] in Brazil; whilst another strain of *C. rosea* is marketed for control of soil and seed-borne pathogens [60]. In a review [61], the authors expressed surprise

that few commercial biofungicides based on *C. rosea* existed, considering that this species has an enormous biocontrol potential.

Here, we report for the first time the occurrence of members of *Clonostachys* as purported mycoparasites of *Hemileia* rusts of coffee, namely: *C. rhizophaga* from parasitized uredinia of *H. vastatrix/coffeicola* on leaves of *C. canephora* in Cameroon and *C. byssicola* from pustules of *Hemileia* sp. on *C. arabica* from Ethiopia, Also, the only previous report of a member of *Clonostachys* occurring as an endophyte in coffee tissues appeared in [11] – *Clonostachys cf. rosea* having been isolated from a coffee leaf in Colombia. We have obtained both *C. byssicola* and *C. rosea f. rosea* from the inner healthy tissues of coffee stems of *C. arabica* in Ethiopia (Table 1).

Isolates of *C. byssicola* (COAD 2983, COAD 2986) and of *C. rosea* (COAD 2984 and 2985) of this study were monophyletic in all four genes studied. Nevertheless, inconsistencies were found for strains based on sequences already deposited in GenBank, in the phylogenetic trees of regions such as *TUB* for *C. byssicola* and *TEF1* for *C. rhizophaga*. Trees for these genes (not shown) generated polyphyletic topologies. Similar results (ambiguity for single gene analysis as compared to consistent monophyly for multilocus analysis of *Clonostachys*) have already been reported with *C. byssicola* in *TUB* [47, 37, 44] and *C. rhizophaga* in *TEF1* [37]. Although this occurred for single genes for some strains belonging to *C. byssicola* and *C. rhizophaga*, monophyly was evident in single-gene trees for *ACL1* and *RPB1*. More importantly, a monophyletic topology was found for the concatenate/multilocus study (Fig. 1).

Members of the subclade of *C. rhizophaga* clade formed by the four mycoparasite isolates of *H. vastatrix* showed contrasting *in vitro* appearances. All of these four isolates of *C. rhizophaga* were obtained from healthy coffee leaves from the same locality in Cameroon. COAD 2981, contrarily to the other three isolates, did not sporulate when cultivated on various media (MEA, OA, PDA), and sporulated poorly when cultivated on autoclaved rice. On OA and PDA, all four isolates released a yellow pigment into the medium which was intense and darker for COAD 2981 and COAD 2980 (Fig.1). It was noted that pigmentation produced by COAD 2981 when grown on autoclaved rice was intense and turned the substrate a dark yellow to orange. The isolates COAD 2979, COAD 2980 and COAD 2982, placed in the same clade, formed abundant secondary conidiophores on OA. The fact that one of the four isolates of *C. rhizophaga* involved in this study cannot sporulate as the others, reveals that the

species *rhizophaga* can suffer degeneration along the time. Secondary conidiophores were thought to be rare in *C. rhizophaga*, according to Schroers [42], who was of the opinion that this species represents an "evolutive degeneration" withing the genus. In his monograph on the genus [42], Schroers interpreted the abundant production of secondary conidiophores in a particular strain of *C. rhizophaga* (CBS 100004) as representing a feature of a "wild form" of *C. rhizophaga*. In that study, CBS 100004 appeared in the same clade with *C. rhizophaga* CBS 361.77^{T} and the latter is included in our study. From the views presented in [42], the production of abundant secondary conidiophores, as seen in our isolates, as well as the phylogenetic affinity of CBS 361.77^{T} with our isolates, indicates a "wilderness state" for our isolates. This may reflect their recent isolation from natural forest situations. One observation which could be of relevance for a better understanding of *C. rhizophaga* is the finding of spores which were morphologically similar to the ascospores of the sexual stage of *Clonostachys* in an OA culture of COAD 2980 (Fig. 2.g); although ascomata were not found and no sexual stage has been reported for this species [42]. Our study suggests that there is significant variability within the taxonomic boundaries of isolates of *C. rhizophaga.*

One of the first steps in any program on biological control is identifying the potential biocontrol agents, resulting from the surveys. Here, we found that the assemblage of *Clonostachys* isolates belong to three known species. All records of *Clonostachys* isolated from *Hemileia* pustules represent novel host-associations and two represent new records of *Clonostachys* growing endophytically in coffee. Although the presence of *C. byssicola*, *C. rhizophaga* and *C. rosea* on pustules of *Hemileia*, or inside healthy coffee tissues, is not sufficient to infer any potential for their use in biocontrol, the numerous examples in the literature of *Clonostachys* species being involved in or used for controlling plant diseases, justify the continuation of our studies to investigate the biocontrol potential of these isolates. Nevertheless, it will be necessary to take into account the existing documented cases of *Clonostachys* species (including isolates belonging to *C. rosea* and *C. rhizophaga*) as crop pathogens. For example: *C. rhizophaga* causing chickpea wilt in Syria [62] and infecting water chestnut in China [63]; *C. rosea* causing root rot of soybean in the USA [64]; and, root and foot rot of faba bean in Iran [65]. We have studied an isolate of *C. rosea* causing rot on pseudobulbs of an orchid and fulfilled Koch's postulates with this isolate, but failed to induce the disease on the same orchid with a *C. rosea* isolate used

as a biocontrol against gray mold (R. W. Barreto, unpub. obs.). The genetic background for the pathogenicity of such isolates deserves to be fully investigated since there seem to exist “slayers and saviors” within the “*Clonostachys* package”.

Studies are now underway to investigate the biocontrol potential against CLR and safety of the isolates of *C. byssicola*, *C. rhizophaga* and *C. rosea f. rosea* obtained during the surveys. It is hoped that some of these isolates will prove to be either effective co-evolved coffee “bodyguards” with good endophyte competence - as suggested by Muvea et al. [66] - or high impact mycoparasites amenable for use as a protectant or treatment against CLR.

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Author contribution All authors contributed to the study conception and design. Conceptualization, funding acquisition, supervision, and final writing were performed by R. W. Barreto. Material preparation, data collection, and morphological analysis were D. M. de Macedo and M. Kapeua-Ndacnou. Molecular data analysis and the first draft and final editing of the manuscript were performed by M. Kapeua Ndacnou and all authors critically revised the work. All authors read and approved the final manuscript.

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Data availability DNA sequence data used in the study are not yet available in public repository of GenBank.

Declarations

Ethics approval Not applicable.
Consent to participate Not applicable.
Consent for publication Not applicable.
Conflict of interest The authors declare no conflict of interests.

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Figures legends

Fig. 1 Bayesian Inference (BI) based on analysis of a combined dataset of *ACL1, RPB1, TEF1-α* and *TUB* sequences data. The concatenated tree is rooted with *Clonostachys pseudochroleuca* (CBS 192.94^{T}). Branches in bold represent Bayesian posterior probabilities 1.00 and bootstrap support for ML 100%, with indicated values at the nodes. The sequences generated in this study are in blue. Branch lengths are proportional to distance. *T ex-type strain. The bar indicates the number of substitutions per site.

Fig. 2 *Clonostachys* spp obtained during the survey for fungal antagonists of *Hemileia vastatrix.* Images taken from slide cultures on OA (5−6 days of growth at 25 °C in light/dark – 12/12 hs daily light/dark regime). (a−c) *C. byssicola* COAD 2983: a) secondary conidiophore; b) primary conidiophore; c) conidia. (d−g) *C. rhizophaga* COAD 2982 and COAD 2980: d) secondary conidiophore; e) primary conidiophore; f, g) conidia. (h−j) *C. rosea f. rosea* COAD 2984: h) secondary conidiophore; i) primary conidiophore; j) conidia. a−c COAD 2983; d−f COAD 2982; g COAD 2980; h−j COAD 2984. Scale bar = 10 µm**.**

Fig. 3 Colonies of *Clonostachys* species after 10 days at 25 °C 12/12 h daily light/dark regime. Left to right: PDA−OA−MEA media. A−C) *C. byssicola* COAD 2983; D−E) *C. rhizophaga* COAD 2979; F−G) *C. rosea f. rosea* COAD 2985.

Table 1

Details on the combined sequences data used in the phylogenetic analysis

Species	Strain n°	Host/Substrate	Locality/Country	GenBank accession n°			
				TEF1-α	*RPB1*	*ACL1*	*TUB*
Clonostachys byssicola	CML 0422	Soil from secondary forest	Benjamin Constant, AM	KX184964	KX184899	KX184833	KF871150
	CML1942	Soil from Amazon forest	Benjamin Constant, AM	KX184968	KX184903	KX184837	KF871148
	CML1943	Soil from Amazon forest	Benjamin Constant, AM	KX184965	KX184900	KX184834	KF871151
	CML2309	*Fragaria ananassa*	Bento Gonçalves, RS	KX184966	KX184901	KX184835	KF871149
	CML2311	Parasitizing colony of *Sordaria* sp.	Lavras, MG	KX184969	KX184904	KX184838	KF871152
	CML2402	Fruit of *Annona squamosa*	Januária, MG	KX184970	KX184905	KX184839	KX185030
	CML2404	Fruit of *Annona x atemoya*	Jaíba, MG	KX184971	KX184906	KX184840	KF871153
	CML 2510/CBS 364.78^{T}	Bark	Venezuela	KX184967	KX184902	KX184836	AF358153
	CML 2511/CBS 365.78	Wood	Venezuela	KX184972	KX184907	KX184841	AF358154
	CML2533	Bryophyte	Itumirim, MG	KX184973	KX184908	KX184842	KX185031
	CML2541	Litter	Itumirim, MG	KX184974	KX184909	KX184843	KX185032
	CML2552	*Piper nigrum*	Montes Claros, MG	KX184975	KX184910	KX184844	KX185033
	CML2654	Litter	Barroso, MG	KX184976	KX184911	KX184845	KX185034
	CML2665	Litter	Lavras, MG	KX184977	KX184912	KX184846	KX185035
	COAD 2983	***Hemileia sp., Coffea arabica***	**Arat Selisa Forest Reserve, Ethiopia**		**OM038397**	**OM038404**	**OM038391**

	COAD 2986	**Endophyte/stems, wild *Coffea arabica***	**Arat Selisa Forest Reserve, Ethiopia**		**OM038397**	**OM038401**	**OM038390**
C. chloroleuca	CML 2537	Bryophyte	Itumirim, MG	KX184989	KX184924	KX184858	KX185038
	CML 1941^{T}	Native soil from Cerrado	Montividiu, GO	KX184988	KX184923	KX184857	KF871172
	CML 1927	Soil under soybean field	Montividiu, GO	KX184987	KX184922	KX184856	KF871171
	CML 1922	Native soil from Cerrado	Montividiu, GO	KX184986	KX184921	KX184855	KF871170
	CML 1921	Native soil from Cerrado	Montividiu, GO	KX184985	KX184920	KX184854	KF871166
	CML 1920	Native soil from Cerrado	Montividiu, GO	KX184984	KX184919	KX184853	KX185037
	CML 1919	Native soil from Cerrado	Montividiu, GO	KX184983	KX184918	KX184852	KF871167
	CML 1918	Native soil from Cerrado	Montividiu, GO	KX184982	KX184917	KX184851	KX185036
	CML 1917	Native soil from Cerrado	Montividiu, GO	KX184981	KX184916	KX184850	KF871169
	CML 1916	Soil under cotton field	Montividiu, GO	KX184980	KX184915	KX184849	KF871174
	CML 1912	Native soil from Cerrado	Montividiu, GO	KX184979	KX184914	KX184848	KF871168
	CML 1213	Native soil from Cerrado	Montividiu, GO	KX184978	KX184913	KX184847	KF871173
C. rhizophaga	CML 2522	Soil	Lavras, MG	KX184994	KX184929	KX184863	KX185039
	CML2514 /CBS 361.77^{T}	Culture contaminant	Switzerland	KX184993	KX184928	KX184862	AF358158
	CML 2312	Parasitizing colony of *fusarium oxysporum*	Lavras, MG	KX184992	KX184927	KX184861	KF871157
	CML 1984	Native soil from Cerrado	Montividiu, GO	KX184991	KX184926	KX184860	KF871155
	CML 1210	Soil under soybean field	Montividiu, GO	KX184990	KX184925	KX184859	KF871156
	COAD 2979	***H. vastatrix/coffeicola, Coffea canephora***	**Somalomo Town, CMR**		**OM038395**		
	COAD 2980	***H. vastatrix/coffeicola, Coffea canephora***	**Somalomo Town, CMR**		**OM038394**	**OM038402**	

	COAD 2981	***H. vastatrix/coffeicola, Coffea canephora***	**Somalomo Town, CMR**		**OM038393**		
	COAD 2982	***H. vastatrix/coffeicola, Coffea canephora***	**Somalomo Town, CMR**		**OM038396**	**OM038403**	
C. pseudochroleuca	CML 2562/CBS 192.94^{T}	Bark	French Guiana	KX185016	KX184950	KX184885	AF358171
C. rosea f. catenulata	CML 2517/CBS 443.65	Soil	United States	KX184996	KX184931	KX184865	AF358166
	CML 2516/CBS 154.27^{T}	Soil	United States	KX184995	KX184930	KX184864	AF358160
C. rosea f. rosea	CML 2549	Litter	Itumirim, MG	KX185001	KX184935	KX184870	KX185040
	CML 2518/CBS 710.86^{T}	Soil, on Sclerotia of *Sclerotinia minor*	Netherlands	KX184999	KX184934	KX184868	AF358161
	CML 2310	*Fragaria ananassa*	Caxias do Sul, RS	KX184998	KX184933	KX184867	KF871146
	CML 0817	Endophyte, *Lychnophora pinaster*	Ingaí, MG	KX184997	KX184932	KX184866	KF871147
	COAD 2984	**Endophyte/stems, *Coffea arabica***	**Arat Selisa Forest Reserve, Ethiopia**		**OM038392**	**OM038399**	**OM038389**
	COAD 2985	**Endophyte/stems, *Coffea arabica***	**Arat Selisa Forest Reserve, Ethiopia**			**OM038400**	**OM038388**

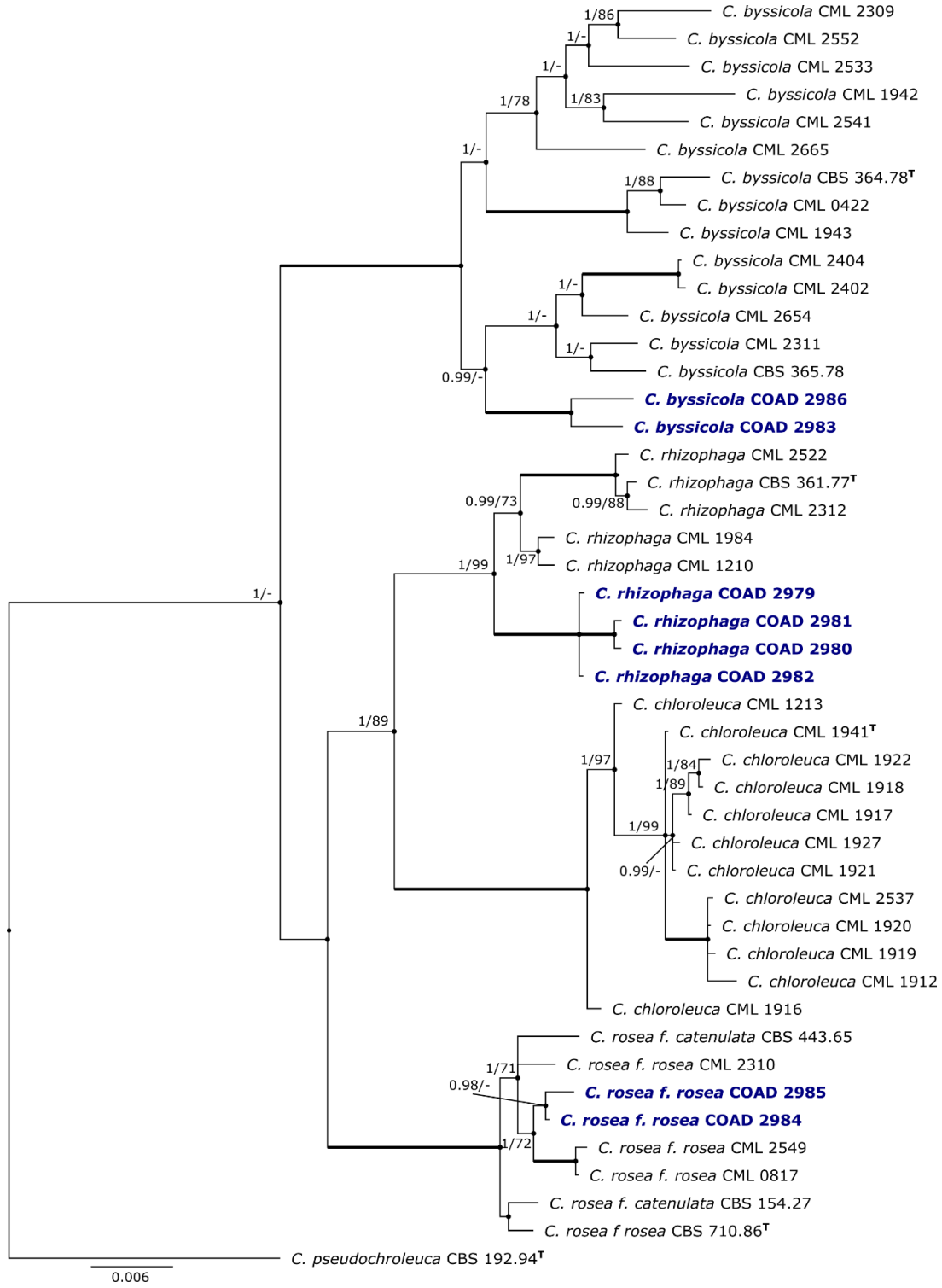
In bold the GenBank accession numbers of sequenced isolates of *Clonostachys* species during this study. ^{T} Ex-type strains.

Table 2

Morphology of *Clonostachys* isolates from coffee and *Hemileia vastatrix* compared to published descriptions of approximating *Clonostachys* species in the phylogenetic tree

Character	COAD 2983/2986	*C. byssicola**	COAD 2979/2980/ 2981/2982	*C. rhizophaga**	COAD 2984/2985	*C.rosea f. rosea**
Stipe length (µm)	10−63	10−100	20−92	10−100	20−122	25−200
Penicillus (µm)	19−55.5	20−100	19−87	30−100	25.5−73.5	30−120
Phialide shape	divergent	divergent	divergent	divergent	divergent	divergent
Phialides in whorls	2−4	2−4	2−5	2−5	2−5	2−5
Phialide size (µm)	19−54 × 2−3	12.4−48 × 1.4−2.8	(9.5)11−35 × 1−3	15.6−48.2 × 2.2−3.2	20.5−40.5 × 1.5−3	25−45 × 1.6−3
Secondary conidiophore	adpressed	adpressed to divergent	penicillate	penicillate	adpressed	adpressed or divergent
Phialide size (µm)	10−32.5 × 1−2.5	7.6−27.8 × 1.4−2.8	(5−)14.5−17(−28) × 1–2.5(−7)	5.8−25.2 × 2.2−3.2	10−19 × 1−3	8−18 × 2−3
Phialide shape	adpressed	± adpressed	adpressed	adpressed or divergent	adpressed	adpressed
Phialides in whorls	4−7	3−5	3−6	3−5	5−7	−
N° of verticillate	2−3	2−5	1−4	3−4	2−4	2−4
Conidia size (µm)	2.5−10(-14) × 1.5−4	3.2–10.8 × 1.8–4	3.5−9(11) × 2−5.5(−7.5)	4.8−9 × 2.4−4.2	4−10 × 1.5−3.5	5.6−10 × 2−3.6

**C. byssicola*, *C. rhizophaga* and *C. rosea* morphological data from Schroers [42].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C. byssicola CML 2533
C. byssicola CML 1942
C. byssicola CML 2541
C. byssicola CML 2665
C. byssicola CBS 364.78T
C. byssicola CML 0422
C. byssicola CML 1943
C. byssicola CML 2404
C. byssicola CML 2402
C. byssicola CML 2654
C. byssicola CML 2311
C. byssicola CBS 365.78
C. byssicola COAD 2986
C. byssicola COAD 2983
C. rhizophaga CML 2522
C. rhizophaga CBS 361.77T
C. rhizophaga CML 2312
C. rhizophaga CML 1984
C. rhizophaga CML 1210
C. rhizophaga COAD 2979
C. rhizophaga COAD 2981
C. rhizophaga COAD 2980
C. rhizophaga COAD 2982
C. chloroleuca CML 1213
C. chloroleuca CML 1941T
C. chloroleuca CML 1922
C. chloroleuca CML 1918
C. chloroleuca CML 1917
C. chloroleuca CML 1927
C. chloroleuca CML 1921
C. chloroleuca CML 2537
C. chloroleuca CML 1920
C. chloroleuca CML 1919
C. chloroleuca CML 1912
C. chloroleuca CML 1916
C. rosea f. catenulata CBS 443.65
C. rosea f. rosea CML 2310
C. rosea f. rosea COAD 2985
C. rosea f. rosea COAD 2984
C. rosea f. rosea CML 2549
C. rosea f. rosea CML 0817
C. rosea f. catenulata CBS 154.27
C. rosea f rosea CBS 710.86T
C. pseudochroleuca CBS 192.94T
0.006

Fig. 1

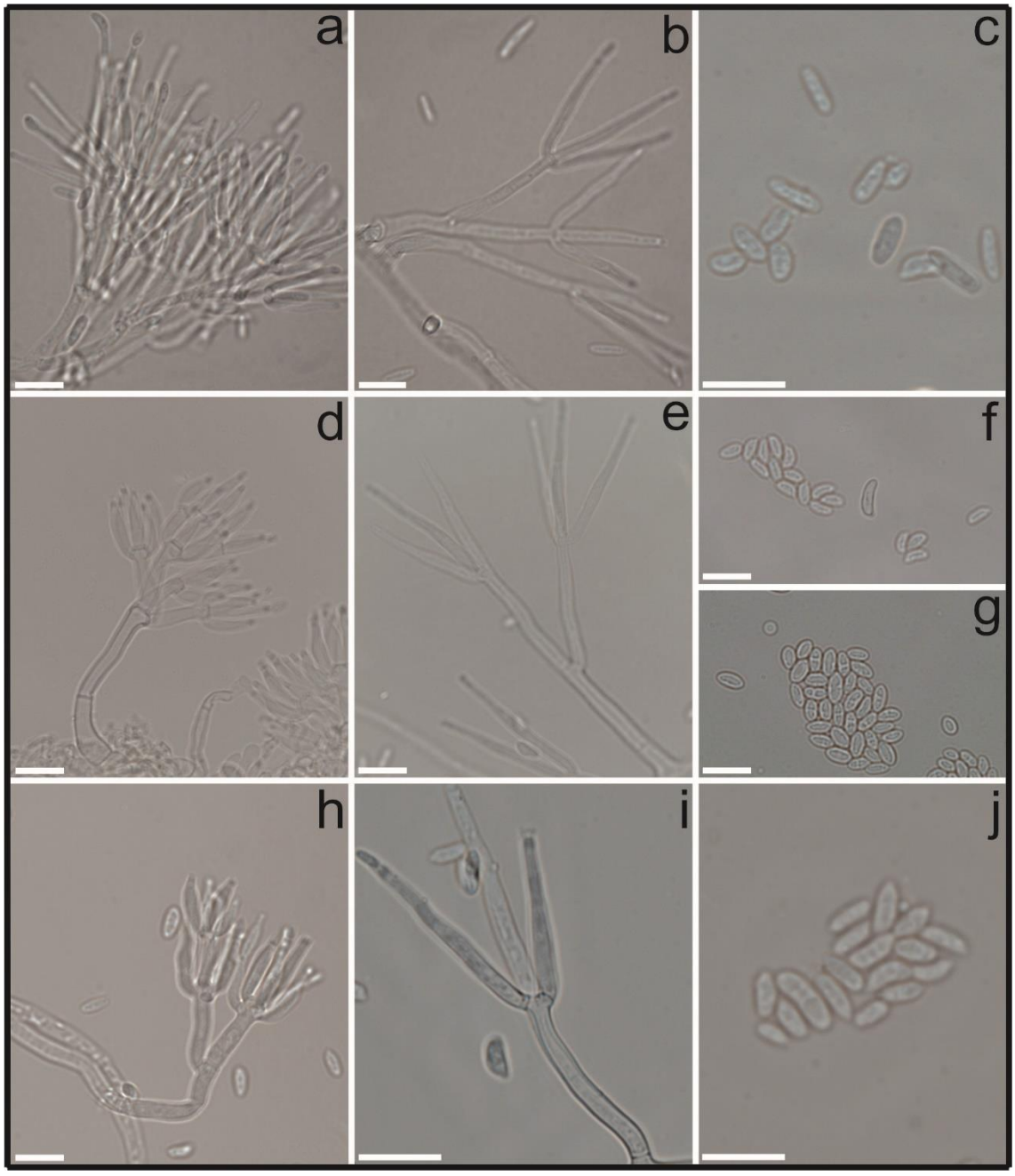


Fig. 2

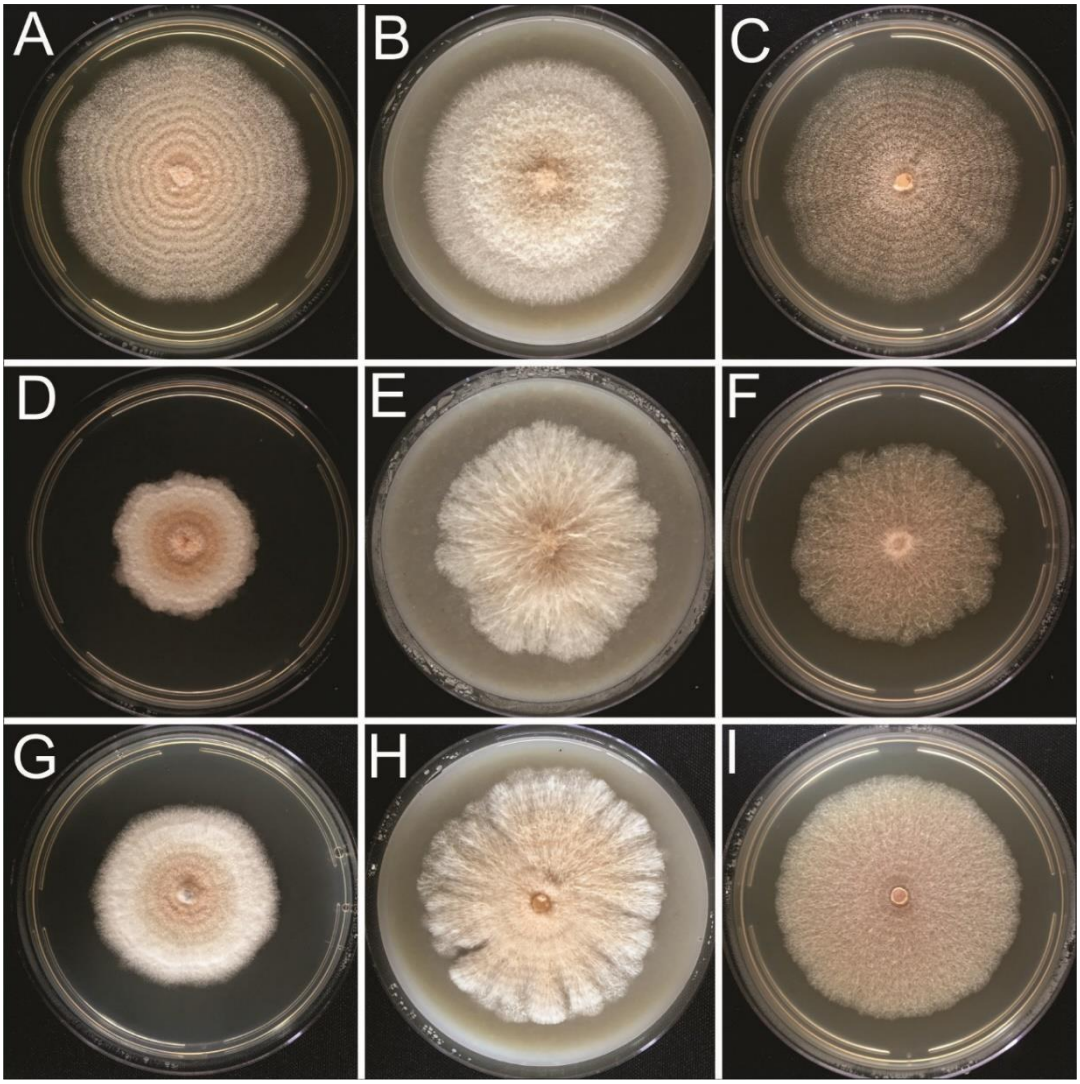
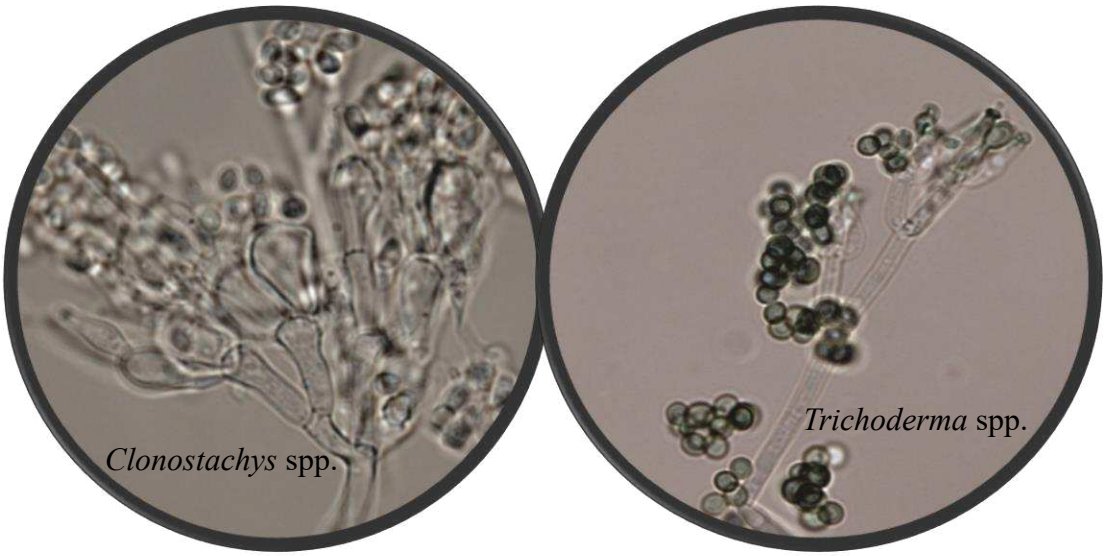


Fig. 3

CHAPTER 3 (Formatted in the journal Frontiers in Microbiology)

In planta **and** ***in vitro*** **antagonism of isolates of** ***Clonostachys*** **and** ***Trichoderma*** **to the coffee leaf rust fungus** ***Hemileia vastatrix***



In planta and *in vitro* antagonism of isolates of *Clonostachys* and *Trichoderma* to the coffee leaf rust fungus *Hemileia vastatrix*

Abstract

Escaping disease through highland cultivation, application of fungicides and the adoption of resistant have been the main control methods used against coffee leaf rust (CLR), the most devastating disease of *Coffea arabica*. Nevertheless, each of those strategies have been facing limitations and novel sustainable approaches are now necessary to mitigate losses to CLR. Biological control of *H. vastatrix* with co-evolved antagonists, namely fungal endophytes and mycoparasites, isolated from *Coffea* species in Africa have been investigated in recent years. Here we report the results of assays aimed at testing the potential of 31 *Trichoderma* endophyte isolates, three endophitic and five mycoparasitic isolates of *Clonostachys* to reduce CLR severity on Arabica coffee under greenhouse conditions. Foliar and soil applications were made of the potential antagonists 5 isolates of *Trichoderma* and 6 of *Clonostachys* isolates had a significant ($p < 0.001$, $z > 4$) effect at reducing CLR severity. Treatments involving the isolates COAD 2398 (*T. guizhouense*), COAD 2406 (*T. theobromicola*), COAD 2400 (*T. virens*), COAD 2981 and COAD 2982 (*C. rhizophaga*) and COAD 2984 (*C. rosea*) consistently produced high reduction in CLR severity as compared with other isolates. In parallel *in vitro* tests involving the suspension of conidia of each of the antagonists together with urediniospores of *H. vastatrix* also resulted in the highest levels of inhibition of urediniospore germination. All 39 isolates included in this study were also evaluated for their ability to establish as endophytes in *C. arabica* during the study and proved to be able to colonize roots, stems and leaves of treated plants. This work provides the first evidences that *Clonostachys* and *Trichoderma* isolates may be used as biological control agents against CLR. Nevertheless, further studies are needed to clarify the mechanisms behind the antagonism of those isolates to *H. vastatrix* and to further demonstrate their potential for field treatment against CLR in coffee plantations.

Keywords: Biological control, *Coffea arabica*, endophytes, mycoparasites, secondary metabolites, severity disease

1 Introduction

Hemileia vastatrix is a biotrophic fungus which caused the worst disease of Arabica coffee (*Coffea arabica*) coffee leaf rust (CLR). The yield losses from CLR can reach 30-90% depending on the circumstances (Avelino *et al*. 2015, Zambolim 2016). In Ethiopia where the coffee originates, as well as in other African coffee-producing countries (as in Kenya, origin of *H. vastatrix*), the disease exists but is not regarded as significant as a problem as others such as attack by coffee berry borer (Souza *et al*. 2011, Ayalew 2014).

Management of CLR has relied in strategies such as escaping the disease through highland cultivation, fungicide sprays and the use of resistant varieties (Souza *et al*. 2011, Belan *et al*. 2015). Nevertheless, the traditional strategies to mitigate losses by CLR have confronted with new or old limitations. One example is that of the increased attack of CLR in former escape highland areas in Northern South America and Central America (Avelino *et al*. 2006), possibly caused by increasing mean temperatures because of climate change (Avelino *et al.* 2015). Repeated events of resistance loss have been acknowledged by Zambolim (2016). Additionally, worries with possible contamination of coffee with pesticide residues and with the environmental impact of broad scale application of pesticides have raised the public opposition to the use of chemical pesticides (Ayalew 2014). A recent response to the trend towards reduction of reliance on intense use of pesticides in coffee is the multi-stakeholder project recently launched by CIRAD's (ECOFFEE R&D Initiative) which has the long-term ambitious goal of promoting a pesticide-free worldwide coffee production (https://www.cirad.fr/en/press-area/press-releases/2021/ecoffee-r-d-to-reduce-pesticides-in-the-coffee-sector).

There are several publications dealing with alternatives to pesticides for CLR control such as the use of chitosan oligomers (Silva-Castro *et al*. 2018), among many others. One potential, but so far, poorly exploited alternative for non-pesticide management of CLR is biological control. Several publications have produced promising preliminary results for biological control candidates such as *Bacillus subtilis* (Bettiol and Varzea 1992), *Bacillus thuringiensis* (Guzzo and Martins 1996), *Bacillus*

thuringiensis, *Pseudomonas putida* and *Fusarium* spp. (Haddad *et al*. 2009, 2013, 2014) and *Trichoderma* spp. (Chambe-Mamani *et al*. 2021) among others.

The epidemics of CLR that started in the early 2010s in northern South America and Central America (Avelino *et al*. 2015, Ramírez-Camejo *et al*. 2022) are in contrast with the less prominent status of *H. vastatrix* as a problem for coffee growers in Africa. It has been suggested that the activity of natural enemies of *H. vastatrix* in the center of origin of coffee and the CLR fungus in Africa might be behind this (Colmán *et al.* 2021, Rodrigues *et al.* 2021). The exploitation of such natural enemies through the classical biological control (CBC) approach (Evans *et al*. 2003, Mejía 2015) might contribute to mitigate the losses to CLR in coffee plantations outside Africa. The search for coevolved fungal natural enemies of *H. vastatrix* in Africa was initiated in 2015 in a project sponsored by the World Coffee Research (WCR 2019). Fungi belonging to *Trichoderma* and *Clonostachys* are amongst the most intensively studied for use as biocontrol tools to manage fungal diseases in agriculture (Sun *et al*. 2020). The surveys in Africa have yielded ninety-four isolates of *Trichoderma* and eight isolates of *Clonostachys* from *Coffea* sp. either as purported mycoparasites on CLR pustules or growing as endophytes in healthy coffee tissues (Rodriguez *et al*. 2021, Kapeua-Ndacnou *et al*. chapter 2). The identity of those isolates was elucidated and they were found to belong to 16 species of *Trichoderma* (including four which were described as new to science) and three species of *Clonostachys*.

Fungal endophytes and mycoparasites have been reported as rich sources of antimicrobial metabolites, such as enzymes capable of degrading chitin and β- glucan, plant growth promoting substances, or to boost of plant resistance against abiotic and biotic stresses (Sudha *et al*. 2016, Harman and Uphoff 2019, Sun *et al*. 2020). The common modes of action behind these biological properties of endophytes/mycoparasites have been discussed in several publications (Benítez *et al*. 2004, Bailey *et al*. 2008, Mejía *et al*. 2008, 2015).

The present study covers *in vitro* and greenhouse *in planta* evaluations of the antagonistic effect of selected *Trichoderma* and *Clonostachys* isolates collected during the surveys in Africa against *H. vastatrix.*

2 Materials and Methods

2.1 *Clonostachys* and *Trichoderma* isolates

Isolates included in this study were obtained as described in Rodríguez et al. (2021) – *Trichoderma* spp. and Kapeua-Ndacnou *et al*. (chapter 2) – *Clonostachys*. These isolates are kept in store at the culture collection of the Universidade Federal de Viçosa – Coleção Octávio de Almeida Drummond (COAD) in Viçosa, MG-Brazil. These are kept in store in cryotubes with 10% glycerol at -80 °C as well as in silica-gel at 4 °C (Dhingra and Sinclair, 1995). A total of 31 isolates of *Trichoderma* spp. (Table 1) used were isolated endophytically from healthy leaves, stems and berries of coffee trees in Africa (Cameroon, Ethiopia and Kenya), identified and characterized and eight isolates of *Clonostachys* spp. (Table 4) were arbitrarily selected for use in this study. The choice of the 31 *Trichoderma* isolates included in this work was based on the results, either of the *In vitro* assessment of inhibition of *H. vastatrix* urediniospores germination, or/and the capacity of reduction of CLR severity on leaf disc (e.g. COAD 2396) obtained in Rodríguez (2019).

2.2 Assay settings

All experiments were conducted inside a greenhouse belonging to the Departamento de Fitopatologia, Universidade Federal de Viçosa (State of Minas Gerais, Brazil). Control of the temperature in the greenhouse is partial and temperatures ranged between 25−32 °C during the assays.

Healthy, four-month-old, shade-house-grown coffee plants, *C. arabica* cv. Catuaí-vermelho (IAC 144), with 4 to 5 pairs of true leaves were utilized. These were grown from seeds in the UFV nursery until transplantation for usage in the assays. The substrate used for cultivation of test plants was a mixture of disinfected soil, prepared at the UFV Clínica de Doenças de Plantas, and rice colonized by the antagonist.

All plants used in the experiment were kept on benches in the greenhouse. In order to protect plants from the effect of direct rain splash from the above nozzles of the automatic irrigation system in the greenhouse, provisional tents were mounted with

bamboo frames and plastic sheets (Appendix). Individual plants kept under the tent were regularly watered individually.

In planta antagonism of selected *Trichoderma* isolates against *H. vastatrix* was tested between Nov 2019 and Apr 2020 (assay I), and Jun to Nov 2020 (assay II). These were arranged in a block design, with 32 blocks including a block for the control (plants untreated with antagonist). Each block included five repetitions, each of which were represented by one young coffee plant grown in plant nursery-type black polyethylene plastic bag containing 50 g of rice colonized by an isolate of *Trichoderma* spp., mixed with the pasteurized soil (sufficient quantity to fill a bag). Assay I included a total of 160 coffee plants and assay II included 60 coffee plants (reasons for differing numbers explained later).

In planta antagonism of selected *Clonostachys* isolates against *H. vastatrix* was tested between Dec 2019 and May 2020 (assay I), and between Jun and Nov 2020 (assay II). General conditions were as described above for *Trichoderma* Both assays I and II included 40 coffee plants. The block serving as control was as described for *Trichoderma*.

2.3 Inoculum preparation

Hemileia vastatrix – Abundant fresh viable urediniospores needed for use in inoculation essays were obtained as described in Salcedo-Sarmiento *et al*. (2021): Twelve young and healthy six-month-old coffee plants (cv. Caturra) were hand-spray inoculated with an urediniopore suspension of race II of *H. vastatrix* (1×10^5 spores mL^{-1}) containing 0.05% Tween 20, until runoff and placed in a dew chamber (conditions as described in Salcedo-Sarmiento *et al*., 2021). After 30-45 days abundant orange sporulation was present on the underside of leaves. Urediniospores were collected and preserved as described in Salcedo-Sarmiento *et al*., (2021) or collected directly from infected coffee leaves on the same day of the preparation of urediniospore suspension for application. Only batches of urediniospores having at least 80% of viability were used.

Trichoderma spp. isolates – Each isolate was grown in potato dextrose-agar (PDA) plates, in an incubator adjusted to 25 °C and a 12 h light/12 h daily regime for

five days. These colonies served for scaling-up the production of inoculum in the next step, when solid-state fermentation took place as follows. For each isolate five polypropylene plastic bags (12 x 25 cm) containing 50 g of parboiled rice, 0.22 g of $CaCO_3$ and 40 ml of distilled water each were prepared. These were autoclaved at 121 °C for 20 min and left in a lab bench until cooling to room temperature. Each bag was aseptically seeded in a laminar flow cabinet with six culture 5mm diam plugs taken from the margin of actively growing colonies on PDA. The bags were then placed in a growth room at 22 °C under a 12-hour light regime (light provided by two white fluorescent day light bulbs, FLC, 25W−127V) and one near-UV lamp (SCT, 28W−127V) placed at least 35 cm away from the bags. They remained under these conditions for seven days. Every two days, the bags were rolled and their contents were hand-squeezed to avoid the formation of large rice/mycelial aggregates and to allow for good aeration in order to stimulate sporulation. Five bags containing non-inoculated parboiled rice, but otherwise prepared as described above, were used for treating control plants.

Depending on the amount of sporulation produced by each isolate inside the bags, one to three grams of colonized rice were suspended in 0.05% Tween 20 solution and placed in 125 ml flasks on a shaker at 25°C and 130rpm for 20 min, for releasing the conidia from the substrate. The content of flasks was then filtered through cheesecloth and the concentrations of the conidial suspension was then adjusted with a haemocytometer to 10^8 conidia ml^{-1}, before use.

Clonostachys spp. isolates – All isolates were grown in oatmeal-agar (OA) plates at 25 °C under a 12-hour light regime, until the plates were fully colonized. Ten ml of sterile distilled water (SDW) were then added to each of the plates and the surface of the colonies were scrapped with a rubber spatula, in order to produce a concentrated suspension of mycelium and conidia. The content of the plates was aseptically transferred into 125 ml flasks containing 20 ml of 2% malt extract broth (MEB). Each flask was seeded with a single *Clonostachys* isolate. The flasks were left in a lab bench at room temperature for three days and were agitated manually every day. After this period 3 ml of the colonized liquid medium from each flask was aseptically transferred to each of five individuals (12 x 25 cm) polypropylene bags. These bags had been previously prepared as described above for *Trichoderma* and

the protocol described above for solid-state fermentation was also as described above for *Trichoderma*. However, the period of incubation of the *Clonostachys* isolates in the bags was longer and lasted 10 days.

In a second round of production of *Clonostachys* inoculum seeding of bags was performed as described for *Trichoderma* but the isolates being grown in OA plates before culture plugs were removed for seeding.

Depending on the intensity of sporulation observed for each *Clonostachys* isolate, six to ten grams of colonized rice were used for the preparation of the suspension in 0.05% Tween 20 and the procedure for producing the conidial suspension for inoculation was as described for *Trichoderma.* Concentrations of conidial suspension were adjusted with a haemocytometer to 10^6 – 10^8 conidia ml^{-1}, before spraying onto the aerial part of plants.

Inoculation procedures − 250 g of parboiled rice colonized by each isolate of *Trichoderma* spp (prepared as previously described) – with an estimated 10^9 – 10^{10} conidia g^{-1} of rice, using a haemocytometer, were thoroughly mixed separately with pasteurized soil (a volume adequate for partly filling five 24×17×10 cm coffee nursery bags). Then, this inoculum + substrate mixture was transferred to the nursery bags with one healthy young coffee plant transferred from planting bag where these were being grown individually until then. Controls consisted of plants treated as previously described but receiving a mixture of uncolonized rice. After the transfer to the larger bags (24×17×10 cm), all plants were left on the greenhouse bench. The same previous procedure was used for *Clonostachys* isolates with concentrations adjusted from 10^7 – 10^9 conidia g^{-1} of rice.

Sprayings of the antagonists on aerial parts of the coffee plants was conducted a month after the soil inoculation, with conidial suspensions of each isolate were made separately until runoff using a hand sprayer connected to an air compressor. The foliar sprayings were repeated three times, with one-month of interval.

Inoculation of the rust fungus was through spray-inoculation of urediniospores of *H. vastatrix* with a $2×10^5$ urediniospores mL^{-1} until runoff. Inoculation with *H. vastatrix*

occurred 72 h after the last spray-inoculation with the isolates of *Trichoderma* and *Clonostachys* (3 months after transplantation). Inoculation of the CLR fungus was conducted by the end of the evening in order to favor urediniospore germination and host infection.

2.4 Dual purpose essay: *in planta* antagonism of *Trichoderma* spp. an *Clonostachys* spp. against *Hemileia vastatrix* and evaluation of endophytic establishment in *Coffea arabica*

Most isolates of *Clonostachys* spp. and *Trichoderma* spp. were originally obtained as endophytes in healthy coffee tissues (leaves, stems and berries) in Africa. In order to further confirm their ability to grow as coffee endophytes, plants exposed to inoculation of each of the isolates during the *in planta* antagonism experiment were also used for preliminary evaluating whether tissues of roots, stems and leaves were colonized by these fungi endophytically. The isolates which were obtained from *H. vastatrix* uredinial pustules were also included in this study. Two similar (pseudo-repeat) assays were then conducted to verify: a) the establishment of different antagonist isolates as endophytes in Arabica coffee after inoculations; b) if an anti-CLR antagonistic "bodyguard-effect" (Gange *et al*. 2019) resulted from each isolate applications.

The overall conditions for the tests are described below:

Inoculation of young *C. arabica* cv. Catuaí-vermelho with each antagonist isolate, either *Clonostachys* or *Trichoderma* spp., involved a combination of soil application and conidial suspension spray of aerial parts (as described above). Thirty days after the transplantation of the plants, the aerial part of each treated plants group was separately spray-inoculated with a suspension of, each *Trichoderma* (10^8 conidia ml^{-1}) or *Clonostachys* (10^6 – 10^8 conidia ml^{-1}) isolate, (as described above).

Assay I endophytic colonization. 30 d after the transplantation of the coffee plants, one individual plant was arbitrarily selected among each isolates-treated plants and one from untreated control plants, and used in the attempt at obtaining evidence of endophytic colonization of *Trichoderma* and *Clonostachys* from leaves, stems or roots. This was repeated at 30 days intervals, prior to the round of spray of antagonists on aerial part of each plant group. The final round of endophyte isolation-attempt was

conducted after the final assessment of CLR severity (that is 5 months days after the coffee plants had been transplanted to larger bags). The plants that showed the lowest degree of CLR severity in each treatment were selected for the attempt at endophyte isolations.

At each round of endophyte isolation attempt, the following protocol was followed: each individual plant was thoroughly washed under the tap, to remove all debris and treated according to a slightly modified version of the protocol of Arnold *et al*. (2003). Each coffee plant-organ (roots, stems and leaves) was detached and with flame-sterilized tools. Root and stem fragments were cut in 2 cm long pieces and 5 mm diam leaf disks were taken from selected healthy leaves (At the bottom, middle and top of the young plant). Stem fragments had their bark removed before disinfection. Disinfection of fragments of each plant part was performed through a sequence of dips, namely: 70% ethanol (1 min), 2% sodium hypochlorite (3 min), 70% ethanol (1 min). This was followed by rinsing the fragments three times with SDW and placing the fragments on sterilized filter paper under aseptic conditions for removal of excess of water. Ten pieces of each organ of each plant were then plated separately on modified semi-selective medium (Papavizas and Lumsden, 1982). Three plates were used for each organ of the plant, then sealed with plastic film and placed in a growth chamber for two–three weeks at 25 °C under a daily 12-hour light regime (light conditions as above). Semi-selective medium consists of 200 ml V8 juice, 3 g $CaCO_3$, 15 g agar and 800 ml of distilled water. After autoclaving at 121 °C for 15 min and cooled (40-50 °C), the medium was aseptically supplemented with 10 ml l^{-1} of each following antibiotics: Penicillin-G, nystatin and chloramphenicol. This volume was taken from a stock solution of individual antibiotic prepared at 1g/100ml. Additionally, 2 ml l^{-1} of Triton-x was added to the medium.

The plates were observed every two days, in order to detect the presence or absence of typical *Trichoderma* or *Clonostachys* colonies. Whenever colonies similar to *Trichoderma* or *Clonostachys* appeared, the plates were further checked under a dissecting microscope (Olympus SZX7) and, if needed, opened in a laminar flow and fragments of the colony were mounted in microscope slides and examined under a light microscope (Olympus BX-51) in order to check the identity based on fungus morphology. The final results of the observation for confirmation of recovery of *Trichoderma* isolates was ranked either as positive for recovery of *Trichoderma* or

Clonostachys growing endophytically in tissues (+ = 1−10, ++ = 11−20 and +++ ≥ 21 fragments produced colony of antagonist isolate) or negative (-) – not a single sample of that organ producing a *Trichoderma* or *Clonostachys* colony.

For *Clonostachys* a different medium was used for isolations: OA medium (40 g oatmeal and 20 g agar per liter of medium) aseptically amended with 10 ml l^{-1} of each antibiotic – Penicillin-G and chloramphenicol.

Assay II endophytic colonization. Eleven "best-performing" *Trichoderma* isolates were selected based on results from assay I, both for their ability to establish as endophytes and to reduce CLR severity. These were tested once again to further confirm their anti-CLR potential. All eight isolates of *Clonostachys* were also included in essay II. Procedures were mostly as described above. The main difference between Assay I and II was, besides the reduction of the number of *Trichoderma* isolates included, the restriction of the attempts to confirm the endophytic colonization of *Trichoderma* or *Clonostachys* isolates to a single round, performed at the end of a five-month period, since transplanting. This followed the same isolation protocol and was performed after the final assessment of CLR severity (as described below).

Assay I *in planta* antagonism to *Hemileia vastatrix* (as reflected by the effect on CLR severity). Antagonism of different isolates to *H. vastatrix* was tested on coffee plants, in parallel with the test described above for evaluation of the ability of these same isolates to establish as an endophyte in coffee tissues. The assays followed the steps described above but procedures for assays I and II differed. In order to assess the effect of different isolates, all plants (including those in controls) were inoculated with *H. vastatrix* as described above and conducted as described above. CLR inoculation was conducted only once at the third month after the transplant of the coffee plants to larger bags and, 72h after the third foliar application of conidial suspensions of either *Trichoderma* or *Clonostachys* isolates. It involved all the remaining sixty-two plants for the 31 *Trichoderma* treatments, sixteen plants representing the eight *Clonostachys* treatments and two plants for the control treatment (two plants for each treatment). Other plants had been removed for the verification of endophyte colonization before that stage (as previously explained).

Three weeks after inoculation with *H. vastatrix*, eight leaves per plant were arbitrarily selected which showed typical pale yellow spots (initial symptom of CLR) and identified with plastic tags. A total of sixteen leaves of each *Trichoderma* or *Clonostachys* isolate-treated and sixteen control leaves, not treated with *Trichoderma* or *Clonostachys* isolates, were labeled. Each individual plant represented an experimental unit. The medium of CLR severities evaluated from the eight leaves per plant, was the CLR severity of the plant. One month after urediniospore application, the first CLR severity assessment of individual leaves was performed. CLR severity on individual leaves was assessed three times at two-week intervals. Each leaf was given a note following the standard protocol developed by Belan *et al*. (2020), which comprises seven levels of CLR severity; ranging from 0.0 to 50.9%, where 0.0% indicates a complete absence of sporulation and 50.9%, the maximum disease severity level.

Assay II *in planta* antagonism to *Hemileia vastatrix* (as reflected by effect on CLR severity). As previously mentioned, no attempt at recovering *Trichoderma* or *Clonostachys* from roots, stems or leaves was attempted in Assay II until the end and only eleven isolates were involved this time. Therefore, all five of each *Trichoderma* or *Clonostachys* isolate-treated coffee plants and five control plants were included in the CLR severity evaluation. Evaluation of disease severity followed the same procedure described above. A total of 40 leaves in both the control and each *Trichoderma* or *Clonostachys* isolate-treated plants were marked and evaluated. Evaluation of disease severity for individually-marked leaves was conducted three times as described for assay I. Nevertheless, only the data collected from the third disease severity assessment were statistically analyzed in this study, due to the monocyclic character of the disease: Throughout a growing season, in favorable conditions, the infection of the plant by the pathogen, is followed by colonization of internal plant tissues, sporulation and dispersal (Zambolim 2016).

2.5 Inhibition of *Hemileia vastatrix* urediniospore germination by endophytes isolates

Isolates of *Trichoderma* or *Clonostachys* spp. that showed the highest levels of reduction of CLR severity as compared to, both the control and other *Trichoderma*

or *Clonostachys* isolates (COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*) and COAD 2406 (*T. theobromicola* or COAD 2982 and COAD 2981 (*C. rhizophaga*) and COAD 2984 (*C. rosea*)) were included in one additional test conducted for checking their effect in terms of *in vitro* inhibition of germination of urediniospores of *H. vastatrix*. Suspension of spores and filtrate of each isolate were tested.

Trichoderma isolates were separately grown in 125 ml flasks containing 75 mL of potato dextrose broth (PD), and seeded with five plugs of the corresponding isolate taken from five day-old colonies on PDA plates. *Clonostachys* isolates were also separately grown in 125 ml flasks containing 75 mL of oatmeal broth, and seeded with five plugs of the corresponding isolate taken from five day-old colonies on OA plates. The flasks were placed in a shaker at 29 ± 1°C, 196 rpm, for one week. Subsequently, the supernatants were each filtered through sterile filter paper in a Büchner funnel. Each filtrate was used in the test as described below. A suspension of *H. vastatrix* urediniospore was prepared from a stock of urediniospores, collected and stored as previously described. Uredinispores were suspended in a 0.05% Tween 20 solution, and the concentration of urediniospore was calibrated to 1×10^5 spores ml^{-1} using a haemocytometer. Suspension of each of the 3 *Trichoderma* isolates and 3 *Clonostachys* isolates was prepared from colonized rice suspended in a 0.05% Tween 20 solution, and the conidial concentration was calibrated to 10^7 and $10^6 - 10^7$ conidia ml^{-1} with a haemocytometer, respectively.

Two microscope slides were cleaned with 70% ethanol and placed inside polypropylene boxes (11 × 11 × 3.5 cm) which had also been cleaned with 70% ethanol. The boxes were lined with a layer of sterilized paper towel saturated with SDW. One 15 µL drop of *H. vastatrix* urediniospore suspension was transferred with a micropipette, to the center of each slide and a second 15 µL drop of filtrate of a specific isolate was placed over one of the drops of urediniospore suspension on one of the slides inside the box, but not on the other, and the two drops (of antagonist filtrate and urediniospore suspension) were gently mixed with the tip of the micropipette and the box was covered. Slides with only one drop of urediniospore suspension served as controls. The study involved four replicates for each *Trichoderma* or *Clonostachys* isolate. All twenty-four boxes were kept in the dark for six hours at 22 °C, after which, urediniospore germination was interrupted by adding a 15 µL drop of lactofuschin to

each drop, and then observed under a light microscope (Olympus BX-51). The same previous protocol was used for the test with conidial suspensions of the same selected *Trichoderma* and *Clonostachys* isolates. The number of germinated *vs* non-germinated urediniospores was estimated by observing the first 100 urediniospores observed on each slide. Urediniospores were considered to have germinated when the germ tubes had a length equal or longer than the urediniospore diam (Capucho *et al*. 2009). Germination inhibition (% GI) was calculated following the equation:

$$\% \text{ GI} = (1 - X / C) \times 100$$

where C = germinated urediniospores on the control slide and X = germinated urediniospores exposed to the antagonist (Silva-Castro *et al*. 2018).

2.6 Statistical analysis

The median values of the percent severity of coffee leaf rust were calculated. Data from each treatment were submitted to the assumptions of normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests respectively, before proceeding with the Kruskal-Wallis test. The distribution of the estimated CLR severities was represented in the form of boxplots. The medians were compared by the Dunn's Post Hoc test (p = 0.05) using the JASP software, version 0.16.0 (Statistics Program, from University of Amsterdam, https://jasp-stats.org/, 2021).

3 Results

3.1 Dual purpose assay: *in planta* antagonism of *Trichoderma* spp. and *Clonostachys* spp. against *Hemileia vastatrix* and evaluation of endophytic establishment

3.1.1 Endophytic establishment of Trichoderma in Coffea arabica

The endophytic colonization of the coffee plants tissues by *Trichoderma* isolates was confirmed, but results were inconsistent (Tables 2 and 3). The methodology adopted for this study and the limited duration of the evaluation (for a perennial plant), may be behind such an inconsistency. For instance, consistent recovery was observed

for eight isolates among the 31 treatments (COAD 2398, COAD 2400, COAD 2406, COAD 2408, COAD 2412, COAD 2482, COAD 2483 and COAD 2503) selected for the circumstance in assay I, two months after the assessment of the CLR severity (Table 2). On the other hand, in assay II, only four among the 11 isolates (E133, COAD 2400, COAD 2406 and COAD 2412) were consistently recovered at the end of experimental period (Table 3). It was regarded as surprising that only three among the isolates (COAD 2400, COAD 2406 and COAD 2412) maintained this consistency in both assays at the end of the experimental period. However, in assay I all *Trichoderma* isolates treatments except COAD 2397, were demonstrated to be able to colonize at least one organ of coffee plants after thirty days of their exposure to the inoculum. A sharp contrast was observed in assay II with COAD 2396, where no fragment of roots, stems or leaves of the plants produced a colony of the isolate, five months after exposure to the inoculum.

3.1.2 Endophytic establishment of Clonostachys in Coffea arabica

The endophytic colonization of the coffee plants tissues by *Clonostachys* isolates was confirmed, but, as for *Trichoderma* with inconsistent results (Tables 5 and 6). As above-mentioned, the methodology adopted for this study and the limited duration of the evaluation (for a perennial plant), may be behind such an inconsistency. The consistency in the recovery was only observed with plants treated with COAD 2979 at each attempt in assay I, and with all treatments except COAD 2981, two months after the assessment of the CLR severity (Table 5). On the other hand, four of eight treatments (COAD 2980, COAD 2983, COAD 2985 and COAD 2986) yielded a consistent recovery of *Clonostachys* isolates at the end of experimental period in assay II (Table 6). The consistency observed with COAD 2979 in assay I during all the rounds of recovery was not repeated in assay II. Whereas, the consistency observed with COAD 2982 and COAD 2984 in assay I at the last round of recovery, was not repeated in assay II. However, in assay I, all *Clonostachys* isolates treatments were able to colonize at least one organ of the coffee plants after thirty days of their exposure to inoculum of the antagonist. No *Clonostachys* colonies were obtained from organs of any of the non-inoculated or control plants in both assays.

3.1.3 In planta antagonism of Trichoderma spp. *against coffee leaf rust severity*

A total of 31 isolates of *Trichoderma* species were screened *in planta*, i.e. 6 belonging to *T. pseudopyramidale* sp.nov., 5 to *T. botryosum* sp.nov., 4 to *T. theobromicola*, 3 to *T. koningiopsis*, 2 to *T. guizhouense*, 2 to *T. parareesei*, 2 to *T. breve*, 1 to *T. lentissimum* sp. nov., 1 to *T. hamatum*, 1 to *T. spirale*, 1 to *T. virens* and 1 to *T. atroviride*. Two remaining 2 isolates (E133 and E139) have not yet been fully identified (Table 1).

In assay I, the Kruskal-Wallis test revealed that the percentages of severity were significantly affected by all treatments at $p < 0.001$ (Figure 2). Pairwise comparisons showed that a group of four treatments (E133, COAD 2501, COAD 2503 and COAD 2410), a group of seven treatments (COAD 2400, COAD 2398, COAD 2406, COAD 2408, COAD 2412, COAD 2430 and COAD 2482) and that a group of four treatments (COAD 2396, COAD 2510, COAD 2514 and COAD 2429) reduced CLR severity percentages ($p < 0.01$, $p < 0.001$ and $p < 0.05$ respectively) as compared to the controls. There were no significant differences between a group of 14 treatments (COAD 2397, COAD 2399, E139, COAD 2402, COAD 2403, COAD 2537, COAD 2504, COAD 2417, COAD 2592, COAD 2509, COAD 2426, COAD 2515, COAD 2521, COAD 2483) and controls. Two isolates (COAD 2520 and COAD 2527) appeared to stimulate an increase in CLR severity when compared with the controls. Among the 15 isolates/treatments that produced a significant reduction in CLR severity as compared to the controls, isolates COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*), COAD 2406 (*T. theobromicola*) and COAD 2408 (*T. spirale*) were those that more significantly reduced CLR severity percentages ($p < 0.001$ and $z > 4$).

These four isolates of *Trichoderma* isolates were included in the second round of evaluation (assay II). The Kruskal-Wallis test revealed that the percentages of severity were also significantly affected by all treatments at $p < 0.001$ (Figure 3). Pairwise comparisons showed that one isolate (COAD 2412) produced results that did not differ from controls ($p = 0.186$). Whilst, seven isolates i.e. COAD 2396, COAD 2398, COAD 2400, COAD 2406, COAD 2410, COAD 2482 and COAD 2503 were able to significantly ($p < 0.001$) reduce CLR severity as compared to the controls. It was observed the seemingly absence of COAD 2396 in coffee tissues in the attempt to confirm the presence of endophytic growth (Table 3) coupled with the significant ($p <$

0.001) reduction of CLR associated with its inoculation as a potential antagonist. The highest level of CLR severity reduction was given by isolates COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*), COAD 2406 (*T. theobromicola*) and COAD 2410 (*T. koningiopsis*) at the value $z > 4$ and $p < 0.001$.

Thus, five *Trichoderma* isolates (COAD 2398, COAD 2400, COAD 2406, COAD 2408 and COAD 2410) can both grow endophytically in *C. arabica* organs (Tables 2 and 3), and significantly ($p < 0.001$ and $z > 4$) reduce CLR severity (Figures 2 and 3).

3.1.4 In planta antagonism of Clonostachys spp*. against coffee leaf rust*

Eight isolates of *Clonostachys* species were screened *in planta*, i.e. four belonging to *C. rhizophaga*, two to *C. rosea* and two to *C. byssicola* (Table 4).
In assay I, the Kruskal-Wallis test revealed that CLR severity was significantly affected by all treatments at $p < 0.001$ (Figure 6). Pairwise comparisons also showed that all treatments were significant ($p < 0.001$) in the reduction of CLR severity percentages when compared to the controls. Of the 08 treatments, treatments COAD 2981 and COAD 2982 (*C. rhizophaga*), and COAD 2984 (*C. rosea*) more significantly reduced CLR severity percentages ($p < 0.001$ and $z > 4$) than others.

In assay II, the Kruskal-Wallis test revealed that the percentages of severity were also significantly affected by all treatments at $p < 0.001$ (Figure 7). Pairwise comparisons showed that COAD 2983 and COAD 2985 were significantly different from the controls at $p < 0.05$ and $p = 0.01$, respectively. While, the others (COAD 2979, COAD 2980, COAD 2981, COAD 2982, COAD 2984 and COAD 2986) were significantly ($p < 0.001$) different from the controls in the reduction of CLR severity percentages. The highest levels of CLR reduction were produced by the four isolates (COAD 2979, COAD 2980, COAD 2981 and COAD 2982) of *C. rhizophaga*, COAD 2984 (*C. rosea*) and COAD 2986 (*C. byssicola*) at $p < 0.001$ and $z > 4$.

Thus, six (COAD 2979, COAD 2980, COAD 2981, COAD 2982, COAD 2984 and COAD 2986) isolates were capable both to endophytically colonize *C. arabica*

organs (Tables 5 and 6) and significantly ($p < 0.001$ and $z > 4$) reduce CLR severity (Figures 6 and 7).

3.2 Inhibition of *H. vastatrix* urediniospore germination

Isolates of *Trichoderma* and *Clonostachys* – Selected for their consistently in reducing CLR severity in both assays – were evaluated for their ability to inhibit *H. vastatrix* urediniospore germination.

The percentage of urediniospore germination in the controls varied between 76–92%, treatment. In the presence of the conidial suspension of the *Trichoderma* spp., germination decreased from 0−20% depending of the isolates. COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*) and COAD 2406 (*T. theobromicola*) inhibited *H. vastatrix* urediniospore germination to 80.7, 99.5 and 100%, respectively. Whereas, in the presence of the filtrate of the same *Trichoderma* spp. isolates, the percentage of urediniospore germination decreased, 10−25% depending of the isolates. COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*) and COAD 2406 (*T. theobromicola*) inhibited the *H. vastatrix* urediniospore germination to 90, 81.7 and 75.2%, respectively.

Results for exposure of *H. vastatrix* urediniospores to *Clonostachys* spp. suspension varied from 0−6% depending of the isolates. COAD 2982 (*C. rhizophaga*), COAD 2984 (*C. rosea*) and COAD 2981 (*C. rhizophaga*) inhibited the *H. vastatrix* urediniospore germination to 94, 99 and 100%, respectively. Exposure to *Clonostachys* spp filtrates of the same, the urediniospore germination varied from 21−40% depending of the isolates. COAD 2984 (*C. rosea*), COAD 2982 (*C. rhizophaga*) and COAD 2981 (*C. rhizophaga*) inhibited the *H. vastatrix* urediniospore germination to 60, 67 and 79%, respectively.

Similarly, to what has been portrayed by Salcedo-Sarmiento *et al.* (2021) we also observed distortions of urediniospores-germ tubes when *H. vastatrix* urediniospores were exposed to both conidial suspension and filtrate of *Clonostachys* or *Trichoderma*.

4 Discussion

At this stage of the study, the results showed that mycoparasites of *H. vastatrix* and endophytic fungi originally isolated from healthy leaves, stems and berries of *Coffea* species (*C. arabica*, *C. canephora*, *C. brevipes* and *C*. sp.) in Africa, can establish as endophytes of *C. arabica* somewhere else. Moreover, they can both inhibit *in-vitro* the germination of *H. vastatrix* urediniospores and reduce *in planta* the CLR severity. Such results are, potentially of great scientific and practical relevance for the management of the most devastating and economically important coffee disease (Zambolim 2016).

Inoculation of the young *C. arabica* with *Trichoderma* isolates resulted in their endophytic colonization, as demonstrated in general by their recovery from roots, stems and leaves at for one sample taken from treated plants. Among the 31 isolates screened, seven (COAD 2399, COAD 2403, COAD 2501, COAD 2504, COAD 2417, COAD 2515 and COAD 2521) have not been recovered from leaves during all the study. Similar with this finding, Rodríguez (2019) reported that COAD 2399 of *T. lentissimum* sp. nov. was not recovered from coffee leaves in all evaluations. Perhaps their colonization could be limited to stems and roots of coffee plants only. Curiously, those seven isolates except for COAD 2515 were originally isolated from stem.

All isolates of *Clonostachys* spp., either mycoparasitic or endophytic isolates were recovered as endophytes of *C. arabica* at each round (assays I and II). This demonstrates that *Clonostachys* isolates originally obtained as mycoparasites in CLR uredinia can also survive and grow as endophytes in coffee tissues. This was the case for COAD 2979, COAD 2980, COAD 2981, COAD 2982 (*C. rhizophaga*) and COAD 2983 (*C. byssicola*). Mejía *et al*. (2008) reported a similar finding for *C. rosea* on cocoa – a mycoparasite capable of establishing as an endophyte into cacao seedling.

There is published evidence that some mycoparasites can establish long-lasting endophytic associations with plants. For example, Nemec *et al*. (1996) provided evidence of endophytic growth of *Trichoderma harzianum* on sweet orange seedlings, even after eight months of inoculation. The authors also reported that *T. harzianum* proved to be also a good colonizer of tomato and pepper seedlings roots. It is difficult, based on our results, to explain the inconsistency in the isolation of the isolates of *Trichoderma* and *Clonostachys* from the coffee plants tissues. It can be conjectured

that this would be due to the limited period (five months) covered in the study since the plants were first exposed to *Clonostachys* and *Trichoderma* spp. isolates. Evans *et al*. (2003) also reported similar inconsistency three months after having inoculated pre-germinated cacao beans with some endophytic *Trichoderma* isolates. Bailey *et al*. (2008) have reported *Trichoderma* isolates as good colonizers of *Theobroma cacao* seedlings. Although no colony of COAD 2396 (*T. atroviride*) was recovered from plant organs in assay II, the reduction of disease severity referring to the isolate application was significant ($p < 0.001$) when compared to the controls. This observation lets to think that the reduction of the severity would be due to the effect of the protection of leaves surface by substances resulting of the COAD 2396 suspension spraying, during the infection phase by the pathogen. However, this deserves to be better addressed in future studies. It is a matter of debate whether the method used in this work (and by earlier workers) to assess endophytic colonization of a biocontrol fungus is adequate or biased against the recording the presence of low population endophytes. Perhaps endophytes present in coffee plant tissues at a lower density are impossible to be detected in such culture plating, and may be lost inside specific fragments of plant tissues because they would take longer to emerge or are overgrown by more opportunistic endophytic fungi and escape observation.

In summary, most of the different fungal isolates of *Trichoderma* spp. and *Clonostachys* spp. tested here, were capable of colonising coffee as endophytes, were (at least occasionally) reisolated, and didn't produce any evidence of disease symptoms in their coffee hosts. This indicates that the isolates are non-pathogenic endophytes while interacting with *C. arabica*. This is of special relevance, considering the occasional records of both *Clonostachys* spp. (Bienapfl *et al*. 2012, Yang *et al*. 2019) and *Trichoderma* spp. (Hatvani *et al*. 2007, Nicosia *et al*. 2015) as being the etiological agent of some crop diseases. Nevertheless, this comes as no surprise, since both *Trichoderma* spp and *Clonostachys* spp. are regarded as power-horses of the pathogen biocontrol industry.

To the best of our knowledge, this work provides the first examples of protection of coffee plants against CLR with *Clonostachys* spp. Previous studies on CLR biocontrol have concentrated in natural enemies collected in the Americas. Some relevant examples involved the use of bacteria (Bettiol & Varzea 1992, Shiomi *et al*.

2006, Haddad *et al*. 2013, Haddad *et al*. 2014) and fungi (Gómez *et al*. 2017, Salcedo-sarmiento *et al*. 2021). There are even two obscure records of evaluation of *Trichoderma* (Alomía-Lucero & Cosinga-Eslava 2021, Chambe-Mamani *et al*. 2021) against CLR.

Evidence was gathered that the prior inoculation of some *Trichoderma* and *Clonostachys* isolates can result in endophitic colonization and, in some cases, to mitigation of CLR severity. A correspondence was apparent between the repeated recovery of some isolates from leaves of plants and the good levels of reduction of CLR severity for such isolates. This seems to indicate the better endophytes are better "bodyguards" against CLR (Figure 4). The study undertaken by Rodríguez (2019) had shown that COAD 2396 (*T. atroviride*) and COAD 2410 (*T. koningiopsis*), both also included in this study, had no effect at reducing CLR severity *in planta*. Her result was not confirmed here. In fact, those two isolates had a good performance at reducing CLR severity ($p < 0.001$) as compared to controls (Figures 2 and 3). Relying solely on foliar spray-inoculation of those antagonist isolates (as chosen by Rodríguez, 2019) may explain the dissimilarity of the results in the present study and hers. In fact, *T. atroviride* is broadly recognized as amongst the best anti-pathogenic fungi biocontrol fungi (Schuster and Schmoll 2010).

In what concerns *Clonostachys* isolates, a significant ($p < 0.001$) reduction of CLR severity was obtained with each treatment at 60 days post-inoculation of plants with *H. vastatrix* as compared to the controls. The different isolates were repeatedly recovered from leaves of plants at the end of assays. As such, antagonistic activities at the foliar level may have directly interfered on the process of infection and colonization of *H. vastatrix* resulting in lower levels of disease severity (Figure 8).

It is likely that the benign penetration and establishment of endophytes inside the *C. arabica*, produces changes in the expression of some genes involved in its defense pathways against the fungal pathogen, such as hydrogen peroxide, peroxidases, ascorbate peroxidase, jasmonic acid components, pathogenesis related protein, chitinases, beta glucanases, endochitinases etc (Guzzo and Martins 1996, Howell *et al.* 2003, Nga *et al*. 2010, Mejía *et al*. 2014, Harman and Uphoff 2019, Salcedo-Sarmiento *et al*. 2021).

Trichoderma and *Clonostachys* species have already been reported as potential biocontrol agents against numerous plant pathogens. Some examples are: *Fusarium* root rot in tomato field controlled by *T. harzianum* (Nemec *et al*. 1996); *Trichoderma* species, *Clonostachys rosea* and *Clonostachys byssicola* strains showing evidence of control *in vitro* of *Crinipellis roreri* frosty pod rot (Evans *et al*. 2003); *Trichoderma* species isolates aggressively parasiting *Moniliophthora roreri* (the frosty pod rot fungus) in culture plates (Bailey *et al*. 2008); *Clonostachys rosea* reducing sporulation of *M. roreri* on cacao pods (Mejía *et al*. 2008); *T. aggressivum* f. *europaeum* proven to be a good antagonist in detached leaves and plant assays against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Mycosphaerella melonis* etc (Sánchez-Montesinos *et al*. 2021); *Clonostachys* spp. (e.g. *C. rhizophaga*), reducing the of severity of *Alternaria grandis* potato early blight (Da Silva *et al*. 2021), and many others.

During the *in vitro* tests where germination of urediniospores of *H. vastatrix* was challenged by *Trichoderma*, *Clonostachys*, no germination of conidia of either *T. guizhouense* COAD 2398, *T. virens* COAD 2400 or *T. theobromicola* COAD 2406 was observed. The same was observed for *C. rhizophaga* COAD 2981 and COAD 2982, and *C. rosea* COAD 2984. This was interpreted as unappropriated conditions of the antagonist isolates in this study. As such, any inhibitory effect observed for these isolates was likely to be caused by the metabolites present in the cultures and carried into the conidial suspension during the conidial harvest process. Saraiva *et al*. (2020) ascribed the high inhibition (94%) of *Botrytis cinerea* conidia mixed with *C. rosea* filtrate to the compounds produced by *C. rosea*. Inhibition percentages of over 94 % were obtained in this study for exposure of urediniospores to the conidial suspension of some the isolates of *Clonostachys* spp. Similar results have been reported by Salcedo-Sarmiento *et al*. (2021) when the rust spores were confronted to *Calonectria hemileiae* conidial suspension. The antagonistic effect of bacterial isolates (Shiomi *et al*. 2006, Haddad *et al*. 2013) on *H. vastatrix* was ascribed to the production of antifungal metabolites. Besides being fungal endophytes, *Clonostachys* spp. and *Trichoderma* spp. are known to produce metabolites and enzymes with a broad spectrum of biological activities such as antimicrobial, resistance inducer etc (Sudha *et al*. 2016, Saraiva *et al*. 2020). The best efficiency of *T. virens* strains as biocontrol agents has been related to overproducing gliovirin and gliotoxin antibiotics (Lumsden *et al*. 1992,

Benitez *et al*. 2004). *Clonostachys rosea* are known to be producer of cyclopeptides, piperazines, pyranones, sorbicillinoids, clonostach acids etc and *C. byssicola* a producer of piperazines (Han *et al*. 2020). In this case, it is needed to know if the effect of COAD 2400 (*T. virens*) and other *Trichoderma* isolates on urediniospore inhibition could also be related to the production of these antibiotics or other compounds. Further investigations along those lines also need to be conducted with COAD 2981 and COAD 2982 (*C. rhizophaga*), and COAD 2984 (*C. rosea*).

The antagonistic potential revealed by the endophytic *Trichoderma* and *Clonostachys* isolates applied on the coffee plants before *H. vastatrix* implies that, they might have proceeded via competition of space or nutrient, systemic resistance induction or antibiosis. Guzzo and Martins (1996) demonstrated that there was a correlation between the enzyme activities-increases and the local and systemic protection of coffee leaves treated with *Bacillus thuringiensis* (Thuricide HP). Salcedo-Sarmiento *et al*. (2021) also demonstrated the mycoparasitism and induction of coffee plants resistance to *H. vastatrix* by *C. hemileiae*. Similar results have been reported by other authors (Siddiquee *et al*. 2012, Qualhato *et al*. 2013) during the *in vitro* antagonistic tests between *Trichoderma* species and other phytopathogens. Therefore, further studies would be useful to allow a better understanding of the results reported herein.

It should noted that assays were conducted under greenhouse-controlled conditions. Artificial inoculation of test plants with the CLR fungus may have given it an unnatural advantage to infect the plants and establish high disease levels. It is expected that real-life field situations will both challenge *H. vastatrix* and the *Trichoderma* and *Clonostachys* antagonists, perhaps tipping the balance in favour of pathogen or biocontrol agent population and CLR disease progress. It is hoped that, as placed by Haddad *et al*. (2009) in the natural infection conditions, the preventive application of the antagonistic microorganisms could reduce the severity to a significant degree. A combination of antagonistic fungi also deserves being evaluated since a synergistic effect could be obtained.

Nemec *et al*. (1996) have mentioned that the capacity of a beneficial microorganism to survive in a foreign environment other than the original one, and to

successfully colonize plant root for the period when protection against pathogen is needed, is one of the most important selection criteria for a biological control agent. Based on this assumption we can postulate that COAD 2398 (*T. guizhouense*), COAD 2400 (*T. virens*), COAD 2406 (*T. theobromicola*), COAD 2410 (*T. koningiopsis*), COAD 2408 (*T. spirale*), COAD 2979, COAD 2980, COAD 2981 and COAD 2982 (*C. rhizophaga*), COAD 2984 (*C. rosea*) and COAD 2986 (*C. byssicola*) forms an assemblage of good candidates for further investigation as potential biocontrol agents to be deployed against CLR. However, the combination of results of the two assays indicate that the list could be further reduced to "potential elites" including only *T. guizhouense* COAD 2398, *T. virens* COAD 2400, *T. theobromicola* COAD 2406, *C. rhizophaga* COAD 2981 and COAD 2982, and *C. rosea* COAD 2984. Field experiments could be started with a list limited to those six isolates.

On the other hand, the association coffee plant-endophyte that lead to a protection of the coffee plants against pathogen as showed along this study, might result in the production of "improved *Coffea* biota". "Improved *Coffea* biota" following the idea of the enhanced plant holobiomes by Harman and Uphoff (2019). An ideal scenario would be that of having certified selected bodyguard endophyte-inoculated coffee plants ready to be taken to the field for plantation and having an advance protection against *Hemileia vastatrix.* The above list of isolates may prove useful in such a scheme, depending on their performance along further studies.

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6 Figures and Tables

Table 1. *Trichoderma* fungal endophytes screened in the present study, their origin, related organism and culture collection.

Isolate code	Substrate	Country	Organism	Culture collection
E02	Leaf, *Coffea* sp.	Kenya	*T. atroviride*	COAD 2396
E39	Stem, *Coffea* sp.	Kenya	*T. guizhouense*	COAD 2397
E40	Stem, *Coffea* sp.	Kenya	*T. guizhouense*	COAD 2398
E89	Stem, *Coffea cf. arabica*	Kenya	*T. lentissimum* sp. nov.	COAD 2399
E133	Leaf, 17-2 B L	Cameroon	*Trichoderma sp.*	COAD xxxx
E139	Leaf, 17-2 Di L	Cameroon	*Trichoderma sp.*	COAD xxxx
E174	Stem, *C. brevipes*	Cameroon	*T. virens*	COAD 2400
E243	Stem, *C. canephora*	Cameroon	*T. breve*	COAD 2402
E276	Stem, *Coffea arabica*	Cameroon	*T. botryosum* sp.nov.	COAD 2403
E414	Stem, *C. canephora*	Cameroon	*T. theobromicola*	COAD 2406
E420	Stem, *C. canephora*	Cameroon	*T. theobromicola*	COAD 2501
E425	Stem, *C. canephora*	Cameroon	*T. spirale*	COAD 2408
E449	Leaf, *C. canephora*	Cameroon	*T. koningiopsis*	COAD 2537
E453	Leaf, *C. canephora*	Cameroon	*T. koningiopsis*	COAD 2503
E456	Leaf, *C. canephora*	Cameroon	*T. koningiopsis*	COAD 2410
E507	Stem, *C. canephora*	Cameroon	*T. theobromicola*	COAD 2504
E508	Stem, *C. canephora*	Cameroon	*T. theobromicola*	COAD 2412
E586	Stem, *Coffea arabica*	Ethiopia	*T. hamatum*	COAD 2417
E619	Stem, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2592
E705	Leaf, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2509
E706	Leaf, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2510
E716	Leaf, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2514
E720	Leaf, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2426
E723	Leaf, *Coffea arabica*	Ethiopia	*T. pseudopyramidale* sp. nov.	COAD 2515
E737	Stem, *Coffea arabica*	Ethiopia	*T. botryosum* sp.nov.	COAD 2520
E741	Stem, *Coffea arabica*	Ethiopia	*T. botryosum* sp.nov.	COAD 2521
E765	Leaf, *Coffea arabica*	Ethiopia	*T. botryosum* sp.nov.	COAD 2527
E769	Berry, *Coffea arabica*	Ethiopia	*T. breve*	COAD 2429
E800	Leaf, *Coffea arabica*	Ethiopia	*T. botryosum* sp.nov.	COAD 2430
E840	Stem, *Coffea arabica*	Ethiopia	*T. parareesei*	COAD 2482
E841	Stem, *Coffea arabica*	Ethiopia	*T. parareesei*	COAD 2483

Table 2. Evidence of isolates of *Trichoderma* species endophytic colonization of *Coffea arabica* through isolation from different organs of inoculated *vs* (control) non-inoculated plants.

Assay I

	Month after first inoculation												
	1st			2nd			3rd			5th			
Isolate C.C	leaf	stem	root	leaf	stem	root	leaf	stem	root	leaf	stem	root	Isolate code
Control	−	−	−	−	−	−	−	−	−	−	−	−	Control
COAD 2396	+	+	+	−	+	+	−	+	+				**E02**
COAD 2397	−	−	−	+	−	+	+	+	+				E39
COAD 2398	−	+	+	+	+	+	−	+	+	+++	+++	++	**E40**
COAD 2399	−	+	+	−	+	+	−	+	+				E89
COAD xxxx	+	+	+	−	+	+	+	+	+				**E133**
COAD xxxx	+	+	+	−	+	+	+	+	+				E139
COAD 2400	−	−	+	−	+	+	−	−	+	+	+	+	**E174**
COAD 2402	+	+	+	+	+	+	−	+	++				E243
COAD 2403	−	+	+	−	+	+	−	+	++				E276
COAD 2406	+	+	+	−	+	+	−	−	+	+	+	+	**E414**
COAD 2501	−	+	+	−	+	−	−	+	+				**E420**
COAD 2408	−	−	+	−	−	+	+	+	+	+	+	+	**E425**
COAD 2537	+	+	+	−	−	+	+	+	+				E449
COAD 2503	−	−	+	−	+	+	+	+	+	+	+	+	**E453**
COAD 2410	−	+	+	−	+	−	−	−	+				**E456**
COAD 2504	−	+	+	−	−	+	−	+	+				E507
COAD 2412	−	+	+	−	+	+	−	+	−	+	++	+	**E508**
COAD 2417	−	+	+	−	−	+	−	+	−				E586
COAD 2592	−	+	+	−	+	+	+	+	+				E619
COAD 2509	+	+	+	+	+	+	−	+	+				E705
COAD 2510	−	+	+	−	+	+	+	+	++				**E706**
COAD 2514	−	+	+	+	+	+	−	+	+				**E716**
COAD 2426	−	+	+	+	+	+	+	+	+				E720
COAD 2515	−	+	−	−	+	+	−	+	+				E723
COAD 2520	−	−	+	−	+	+	+	+	+				E737
COAD 2521	−	−	+	−	−	+	−	−	+				E741
COAD 2527	−	+	+	−	+	+	+	+	+				E765
COAD 2429	−	−	+	+	+	+	+	+	+				**E769**
COAD 2430	−	+	+	−	+	+	−	−	+	+	++	+	**E800**
COAD 2482	−	+	+	+	+	+	−	++	+	++	++	++	**E840**
COAD 2483	−	+	+	−	+	+	+	+	++				E841

* Inoculations in four rounds in both assays, combining soil application – individual isolate colonized rice (50 g/plant) – with an estimated 10^9– 10^{10} conidia g^{-1} of rice (first application) and three foliar sprays

(conidial suspensions – 10^7– 10^9 conidia ml^{-1} of isolate until runnoff) at 30 days-intervals after soil application. Control (non-inoculated plants) received 50g uncolonized rice per plant or sprayed with sterile distilled water (SDW) in parallel with each *Trichoderma* isolate-treatment of the other groups of plants.

** + = 1−10, ++ = 11−20 and +++ ≥ 21, at least one piece producing a colony of *Trichoderma* isolate; − = No *Trichoderma* colony obtained from any in plates after 2−3 weeks.

*** In bold, isolates that reduced significantly ($p < 0.05$ at least) CLR severity, and C.C: Culture collection.

Table 3. Evidence of isolates of *Trichoderma* species endophytic colonization of *Coffea arabica* through isolation from different organs of inoculated *vs* (control) non-inoculated plants.

Assay II

	Month after first inoculation			
	5th			
Isolate C.C	leaf	stem	root	Isolate code
Control	−	−	−	Control
COAD 2396	−	−	−	**E02**
COAD 2398	++	++	−	**E40**
COAD xxxx	+++	++	+	E133
COAD 2400	+	+	+	**E174**
COAD 2406	++	++	+	**E414**
COAD 2408	+++	++	−	E425
COAD 2503	+	+	−	E453
COAD 2410	+	+	−	**E456**
COAD 2412	+	+	+	E508
COAD 2430	++	+	−	E800
COAD 2482	+++	++	−	E840

* Inoculations in four rounds in both assays, combining soil application – individual isolate colonized rice (50 g/plant) – with an estimated 10^9–10^{10} conidia g^{-1} of rice (first application) and three foliar sprays (conidial suspensions – 10^7– 10^9 conidia ml^{-1} of isolate until runnoff) at 30 days-intervals after soil application. Control (non-inoculated plants) received 50g uncolonized rice per plant or sprayed with sterile distilled water (SDW) in parallel with each *Trichoderma* isolate-treatement of the other groups of plants.

** + = 1−10, ++ = 11−20 and +++ ≥ 21, at least one piece producing a colony of *Trichoderma* isolate; − = No *Trichoderma* colony obtained from any in plates after 2−3 weeks.

*** In bold, isolates that reduced significantly ($p < 0.001$) CLR severity, and C.C: Culture collection.

Figure 1. Example images of recovery from coffee tissues inoculated (d–f) with *Trichoderma* isolate COAD 2398. Left to right: leaf, stem and root. Note COAD 2398 colonies emerging from d–f (green colour).

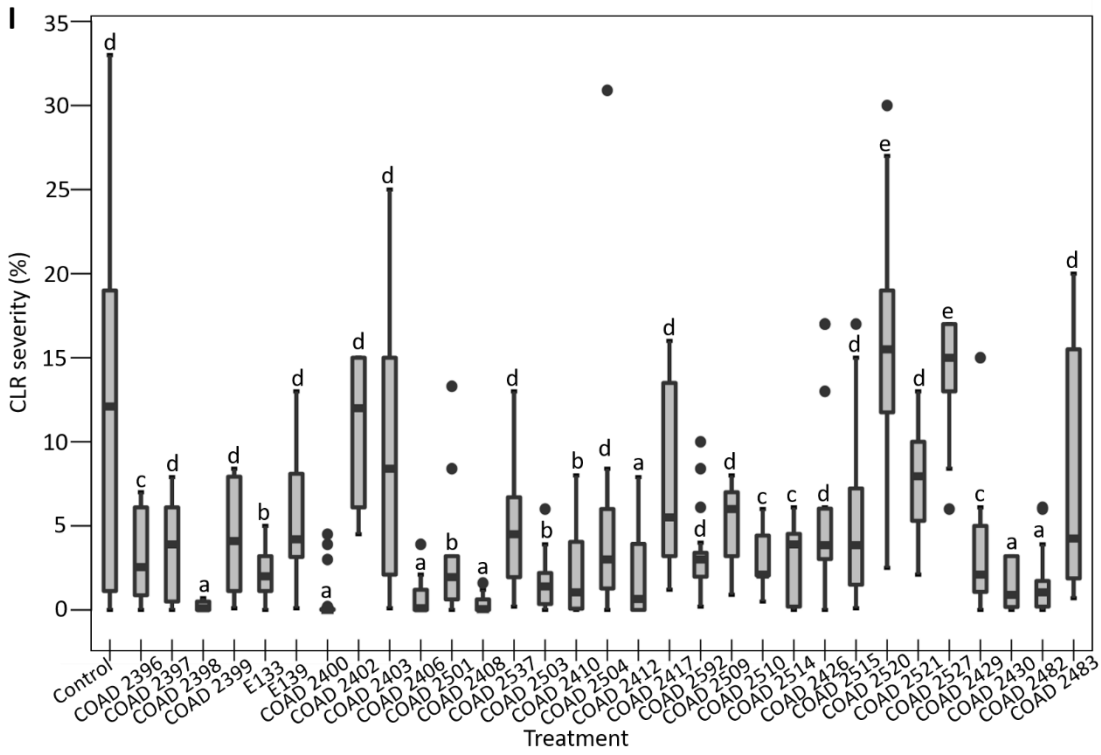


Figure 2. Effect of *Trichoderma* spp. isolates on the CLR severity at 60 days, after inoculation of *coffea arabica* plants with *H. vastatrix* 3 days after their last treatment with isolates. Each median value is the result of two replicates, with eight leaves evaluated per replicate. Medians followed by the same letter do not differ significantly (Dunn, $p < 0.001$). Leaves assessment was following a standard diagram ranged from 0.0 to 50.9%, the maximum severity level.

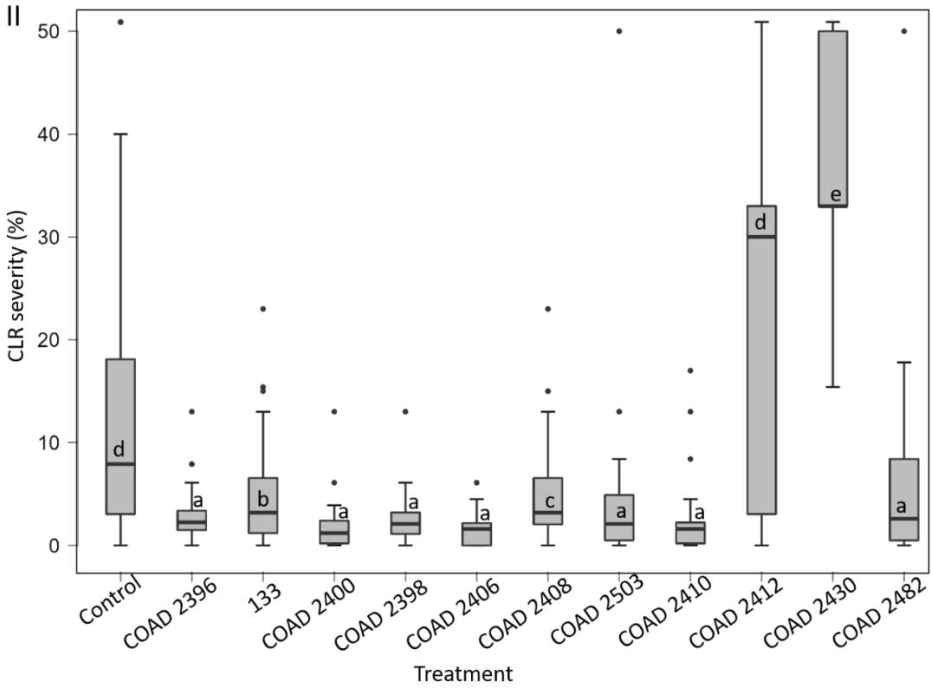


Figure 3. Effect of *Trichoderma* spp. isolates on the CLR severity at 60 days, after inoculation of *coffea arabica* plants with *H. vastatrix* 3 days post-treatment with the isolates. Each median value is the result of five replicates, with eight leaves evaluated per replicate. Medians followed by the same letter do not differ significantly (Dunn, $p < 0.001$). Leaves assessment was following a standard diagram ranged from 0.0 to 50.9%, the maximum severity level.

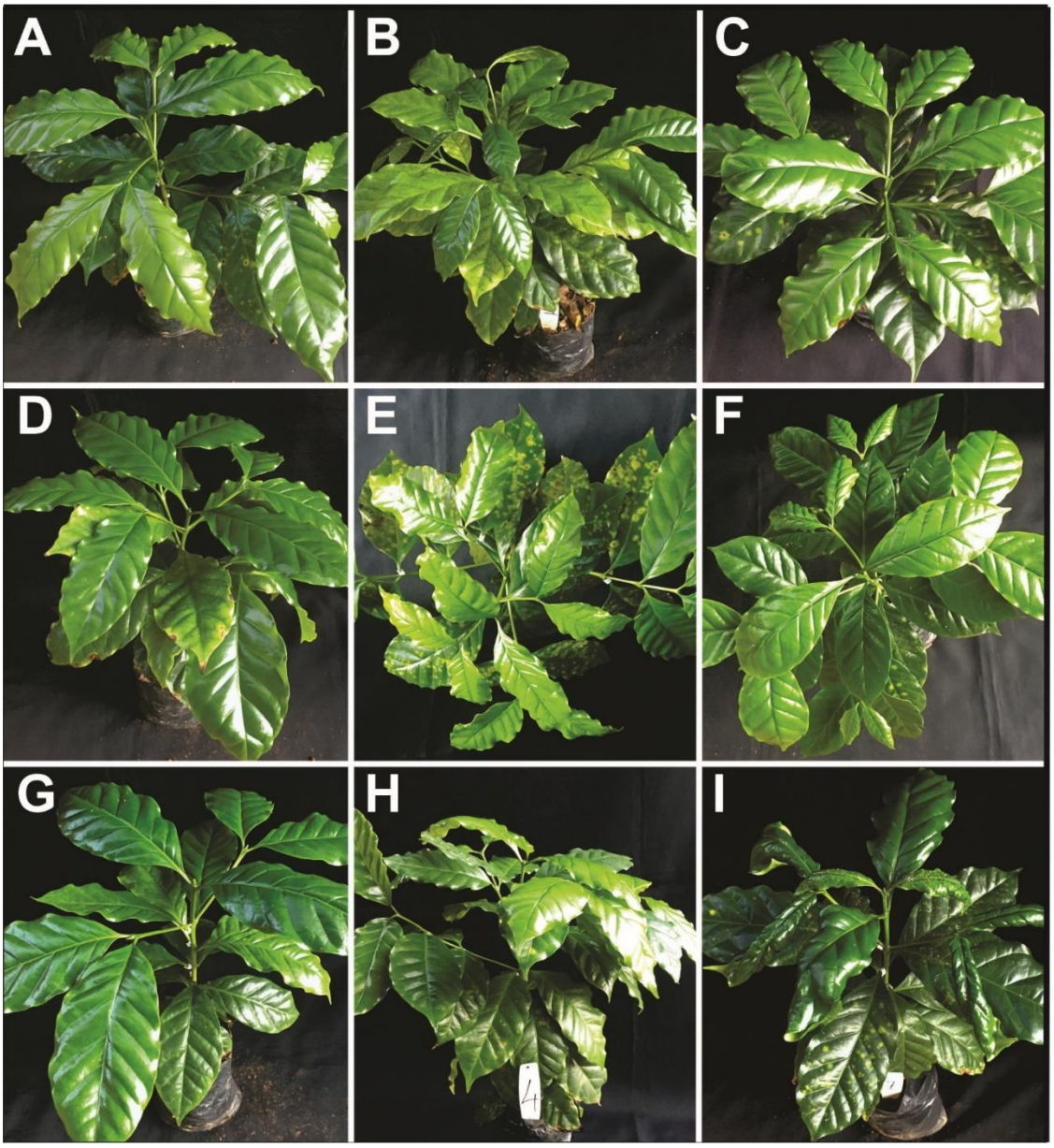


Figure 4. Evidence of effect of *Trichoderma* isolates on biocontrol of *H. vastatrix* after 60 days post-inoculation of *Coffea arabica* with pathogen. *C. arabica* grown in soil inoculated with non-colonized rice and leaves sprayed with SDW (E). *C. arabica* grown in soil inoculated with isolate-colonized rice and sprayed with isolate spore: (A) COAD 2400 *T. virens*, (B and I) COAD 2406 and COAD 2412 *T. theobromicola*, (C) COAD 2430 *T. botryosum* sp.nov., (D) COAD 2398 *T. guizhouense*, (F) COAD 2482 *T. parareesei*, (G) COAD 2408 *T. spirale* and (H) COAD 2503 *T. koningiopsis*.

Table 4. *Clonostachys* fungal endophytes and mycoparasites screened in the present study, their origin, related organism and culture collection.

Isolate code	Substrate	Country	Organism	Culture collection
M201	*H. vastatrix/coffeicola, Coffea canephora*	Cameroon	*Clonostachys rhizophaga*	COAD 2979
M230	*H. vastatrix/coffeicola, Coffea canephora*	Cameroon	*C. rhizophaga*	COAD 2980
M260	*H. vastatrix/coffeicola, Coffea canephora*	Cameroon	*C. rhizophaga*	COAD 2981
M267	*H. vastatrix/coffeicola, Coffea canephora*	Cameroon	*C. rhizophaga*	COAD 2982
M288	*Hemileia sp., Coffea arabica*	Ethiopia	*C. byssicola*	COAD 2983
E585	Stem, *Coffea arabica*	Ethiopia	*C. rosea*	COAD 2984
E588	Stem, *Coffea arabica*	Ethiopia	*C. rosea*	COAD 2985
E749	Stem, wild *Coffea arabica*	Ethiopia	*C. byssicola*	COAD 2986

Table 5. Evidence of isolates of *Clonostachys* species endophytic colonization of *Coffea arabica* through isolation from different organs of inoculated *vs* (control) non-inoculated plants.

Assay I

	Month after first inoculation												
	1st			2nd			3rd			5th			
Isolate C.C.	leaf	stem	root	leaf	stem	root	leaf	stem	root	leaf	stem	root	Isolate code
Control	−	−	−	−	−	−	−	−	−	−	−	−	Control
COAD 2979	+	+	+	+	+	+	+	+	+	+	**++**	+	M201
COAD 2980	+	+	−	+	+	+	+	**−**	+	+	**++**	+	M230
COAD 2981	**+**	**+**	**−**	**+**	**+**	**+**	**−**	**+**	**+**	**+**	**+**	**−**	**M260**
COAD 2982	**+**	**+**	**+**	**+**	**+**	**+**	**−**	**−**	**+**	**+**	**++**	**+**	**M267**
COAD 2983	+	+	+	+	+	−	**−**	+	+	+	+	+	M288
COAD 2984	**+**	**+**	**−**	**++**	**+**	**+**	**+**	**−**	**+**	**+**	**+**	**+**	**E585**
COAD 2985	+	+	−	+	+	+	**−**	**−**	+	**++**	**++**	+	E588
COAD 2986	+	−	+	+	+	+	**−**	+	+	+	+	+	E749

* Inoculations in four rounds in both assays, combining soil application – individual isolate colonized rice (50 g/plant) – with an estimated 10^7–10^9 conidia g^{-1} of rice (first application) and three foliar sprays (conidial suspensions – 10^6–10^8 conidia ml^{-1} of isolate until runnoff) at 30 days-intervals after soil

application. Control (non-inoculated plants) received 50g uncolonized rice per plant or sprayed with sterile distilled water (SDW) in parallel with each *Clonostachys* isolate-treatment of the other groups of plants.

** + = 1−10, ++ = 11−20 and +++ ≥ 21, at least one fragment producing a colony of *Clonostachys* isolate; − = No *Clonostachys* colony obtained from any in plates after 2−3 weeks.

*** In bold, isolates that reduced significantly ($p < 0.001$, $z > 4$) CLR severity, and C.C: Culture collection.

Table 6. Evidence of isolates of *Clonostachys* species endophytic colonization of *Coffea arabica* through isolation from different organs of inoculated *vs* (control) non-inoculated plants.

Assay II

	Month after first inoculation			
	5th			
Isolate C.C.	leaf	stem	root	Isolate code
Control	−	−	−	Control
COAD 2979	+	+	−	M201
COAD 2980	+	+	+	M230
COAD 2981	+	−	−	M260
COAD 2982	**+**	**+**	**−**	**M267**
COAD 2983	**++**	+	+	M288
COAD 2984	**+**	**−**	**+**	**E585**
COAD 2985	+	+	+	E588
COAD 2986	+	+	+	E749

* Inoculations in four rounds in both assays, combining soil application – individual isolate colonized rice (50 g/plant) – with an estimated 10^7−10^9 conidia g^{-1} of rice (first application) and three foliar sprays (conidial suspensions – 10^6−10^8 conidia ml^{-1} of isolate until runnoff) at 30 days-intervals after soil application. Control (non-inoculated plants) received 50g uncolonized rice per plant or sprayed with sterile distilled water (SDW) in parallel with each *Clonostachys* isolate-treatement of the other groups of plants.

** + = 1−10, ++ = 11−20 and +++ ≥ 21, at least one piece producing a colony of *Clonostachys* isolate; − = No *Clonostachys* colony obtained from any in plates after 2−3 weeks.

*** In bold, isolates that reduced significantly ($p < 0.001$, $z > 4$) CLR severity, and C.C: Culture collection.

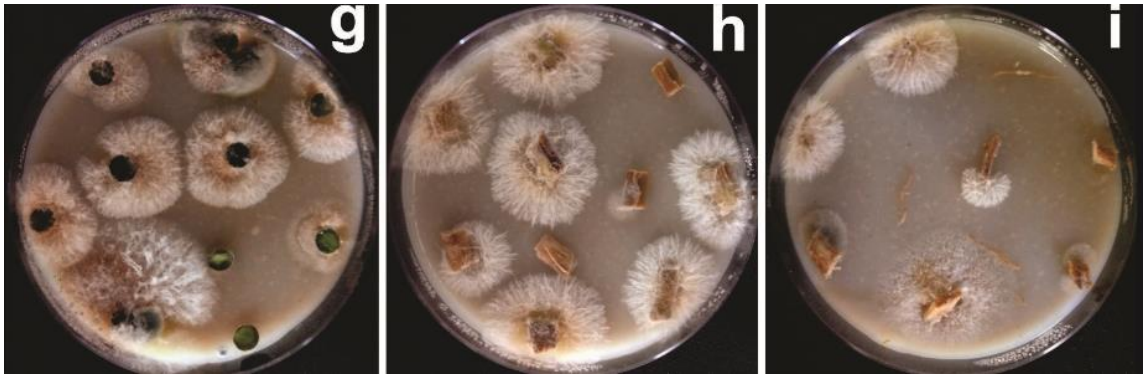


Figure 5. Example images of recovery from coffee tissues inoculated (g–i) with *Clonostachys* isolate COAD 2985 & COAD 2979. Left to right: leaf, stem and root.

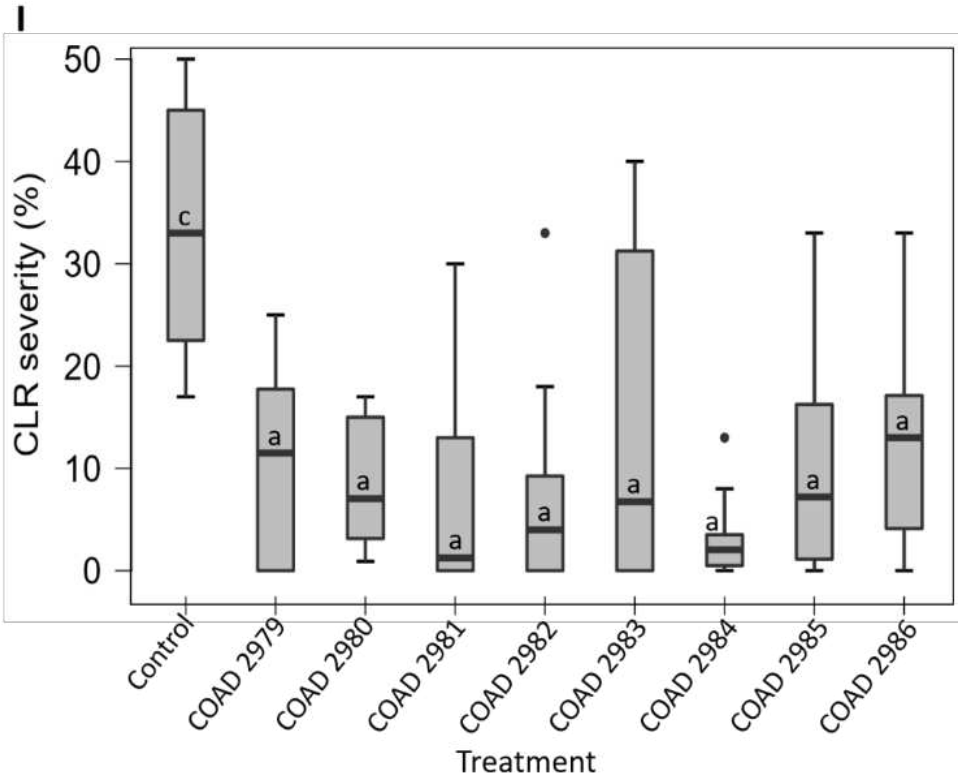


Figure 6. Effect of *Clonostachys* spp. isolates on the CLR severity 60 days after inoculation of *coffea arabica* plants with *H. vastatrix* 3 days post-treatment with the isolates. Each median value is the result of two replicates, with eight leaves evaluated per replicate. Medians followed by the same letter do not differ significantly (Dunn, $p < 0.001$). Leaves assessment was following a standard diagram ranged from 0.0 to 50.9%, the maximum severity level.

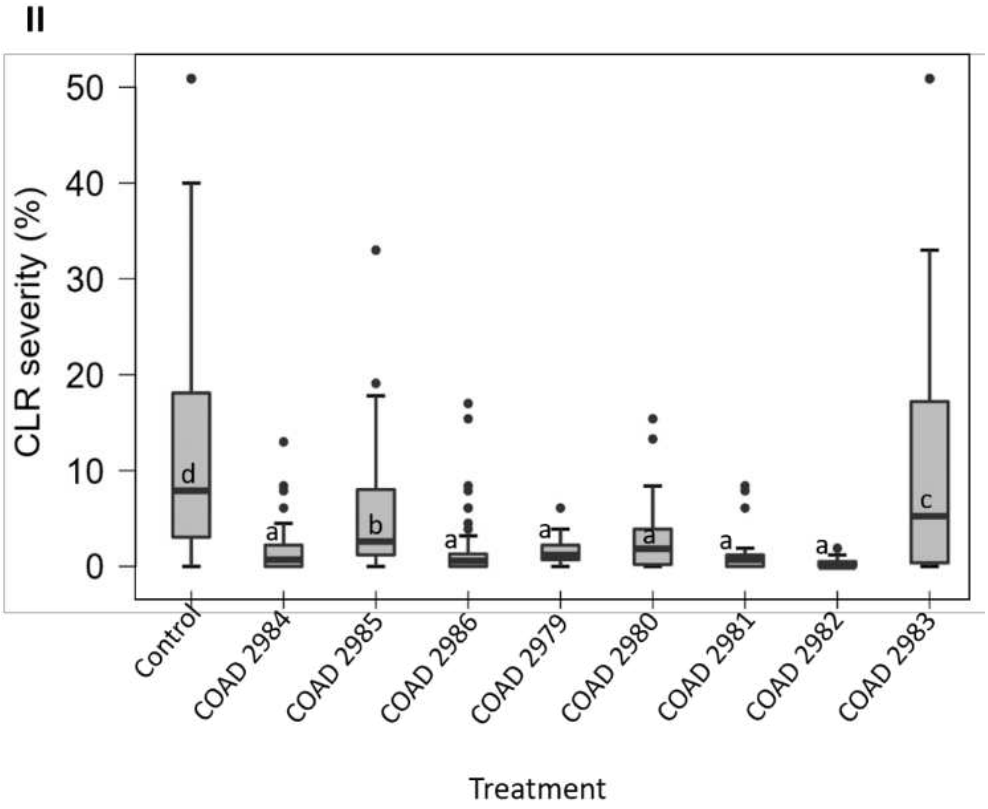


Figure 7. Effect of *Clonostachys* spp. isolates on the CLR severity 60 days after inoculation of *coffea arabica* plants with *H. vastatrix* 3 days post-treatment with the isolates. Each median value is the result of five replicates, with eight leaves evaluated per replicate. Medians followed by the same letter do not differ significantly (Dunn, $p < 0.001$). Leaves assessment was following a standard diagram ranged from 0.0 to 50.9%, the maximum severity level.

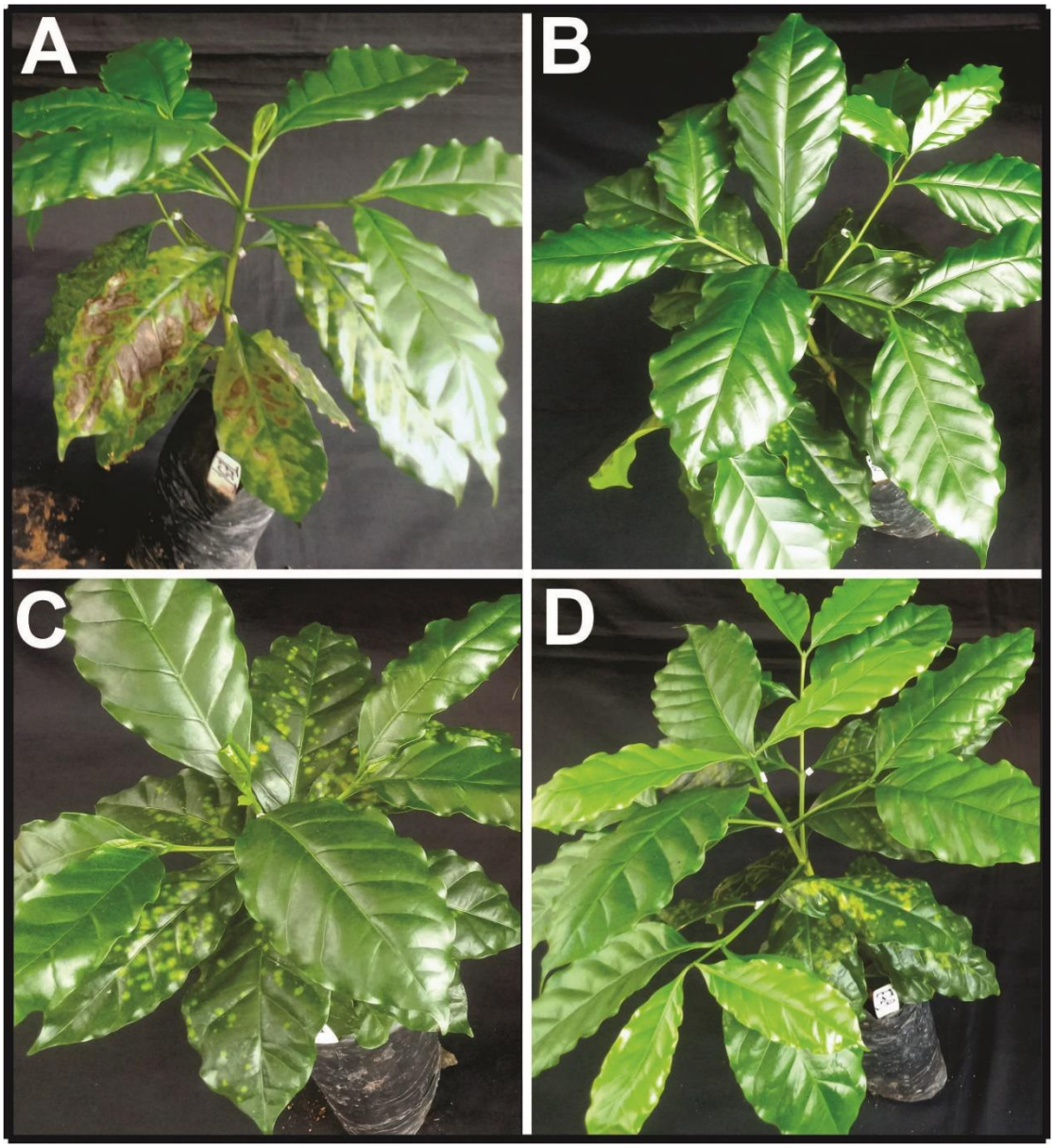
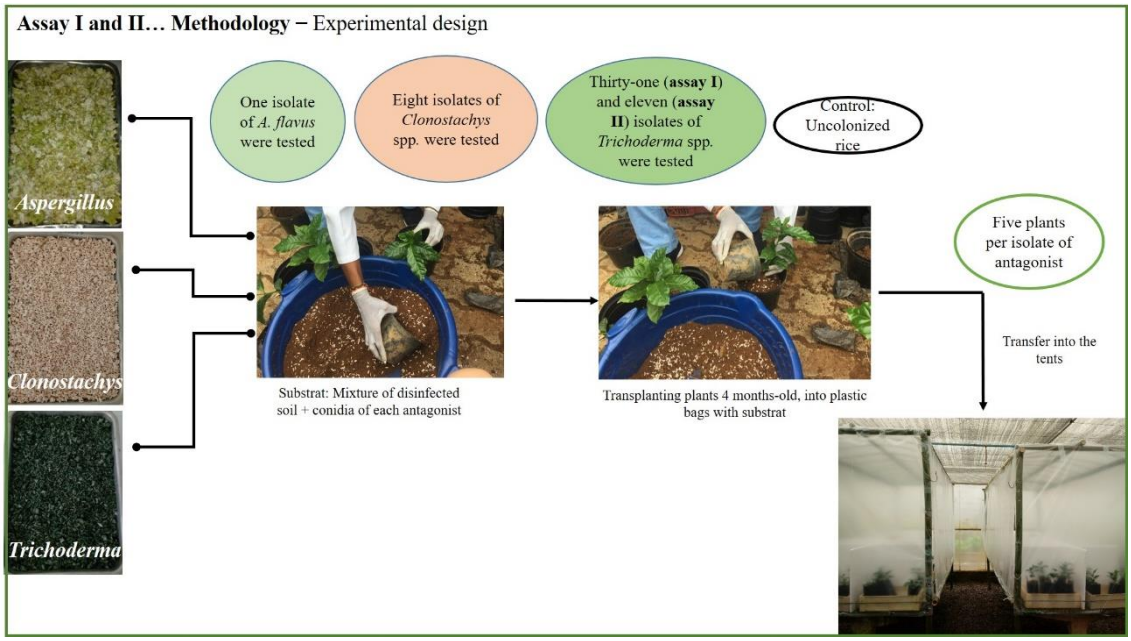
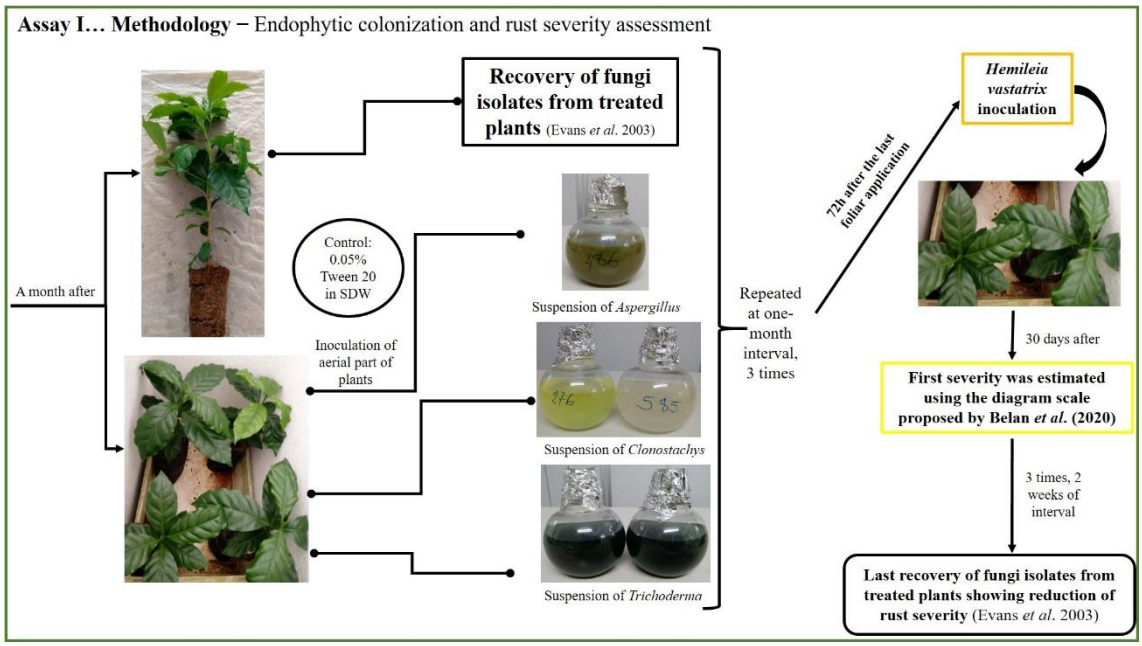
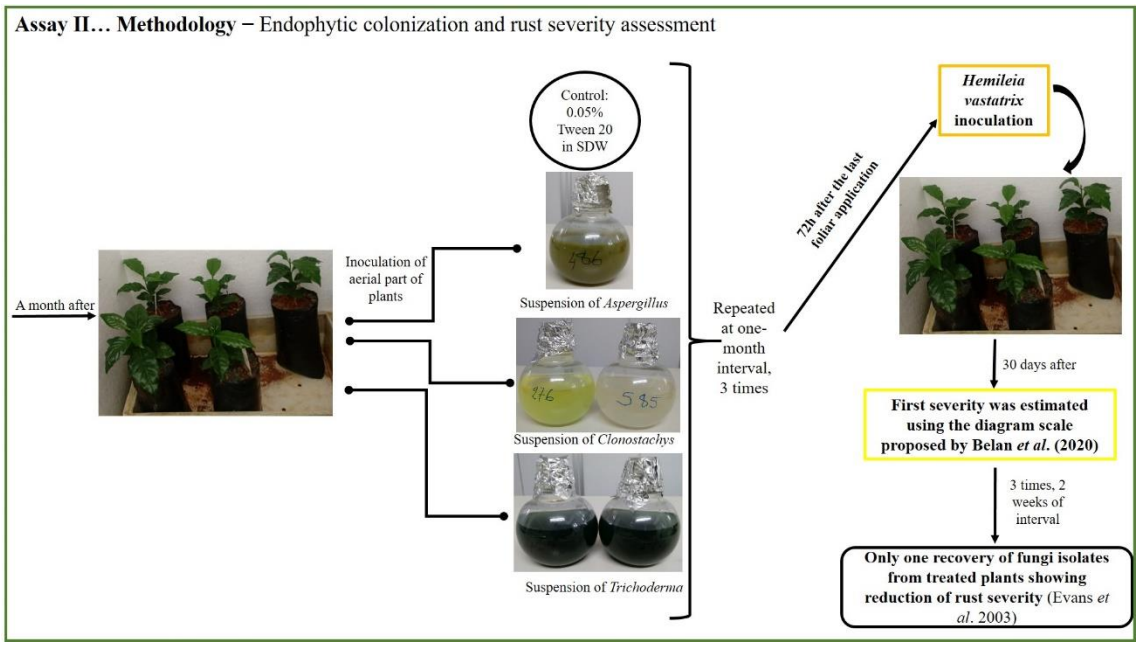


Figure 8. Evidence of effect of *Clonostachys* isolates on biocontrol of *H. vastatrix* after 60 days post-inoculation of *coffea arabica* with pathogen. Coffee plant grown in soil inoculated with non-colonized rice and leaves sprayed with SDW (A). Coffee plants grown in soil inoculated with isolate-colonized rice and sprayed with isolate spore: (B) COAD 2984 *C. rosea*, (C) COAD 2982 *C. rhizophaga*, (D) COAD 2986 *C. byssicola*.

APPENDIX. Simplified scheme of the methodology used in the dual purpose assay *in planta* with *A. flavus*, *Clonostachys* spp., and *Trichoderma* spp., potential antagonists to coffee leaf rust.






Assay II… Methodology − Endophytic colonization and rust severity assessment
A month after
Inoculation of aerial part of plants
Control: 0.05% Tween 20 in SDW
Suspension of Aspergillus
Suspension of Clonostachys
Suspension of Trichoderma
Repeated at one-month interval, 3 times
72h after the last foliar application
Hemileia vastatrix inoculation
30 days after
First severity was estimated using the diagram scale proposed by Belan et al. (2020)
3 times, 2 weeks of interval
Only one recovery of fungi isolates from treated plants showing reduction of rust severity (Evans et al. 2003)

GENERAL CONCLUSIONS

- COAD 3307 – an isolate of *Aspergillus* obtained as an endophyte of healthy coffee berries from *Coffea canephora*, in Africa was identified as *Aspergillus flavus*.
- Preliminar analysis of aflatoxin production by COAD 3307 indicated that it is a non-aflatoxigenic isolate.
- COAD 3307 was experimentally confirmed to be an endophyte in *Coffea arabica* and significantly ($p < 0.0001$) reduced CLR severity when compared with controls. It represents the first report of *Aspergillus flavus* with potential of biocontrol against *Hemileia vastatrix*.
- Eight isolates belonging to *Clonostachys*, also obtained in surveys in Africa, were identified as *Clonostachys byssicola*, *C. rhizophaga* and *C. rosea*. *Clonostachys byssicola* and *C. rhizophaga* are reported here, for the first time as endophytes in *Coffea* spp. and/or as purported mycoparasites on *Hemileia* pustules.
- All eight isolates *Clonostachys* were demonstrated to be capable of growing as endophytes in *Coffea arabica* and significantly ($p < 0.001$) reducing CLR severity when compared with the controls. This is first report of *Clonostachys* as potential biological control agents against *H. vastatrix*.
- *Clonostachys rhizophaga* COAD 2981 and COAD 2982, and *C. rosea* COAD 2984 yielded the highest levels of CLR severity reduction at $p < 0.001$, in the two assays.
- All thirty-one *Trichoderma* isolates investigated here were demonstrated to be capable of growing as endophytes in *Coffea arabica*, and fifteen among them significantly ($P < 0.5$ at least) reduced CLR severity when compared with controls. This is first report of these isolates belonging to nine different *Trichoderma* species with biological control potential against *H. vastatrix*.
- *Trichoderma guizhouense* COAD 2398, *T. virens* COAD 2400, *T. theobromicola* COAD 2406 showed highest CLR severity reduction at $p < 0.001$, in all assays when compared to other *Trichoderma* isolates.
- Biological control appears to offer a promising and sustainable method for managing the worst pathogen of coffee worldwide and deserves being intensively investigatedas a management option.