MARIA DEL CARMEN HERRERA RODRIGUEZ

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Trichoderma spp. ASSOCIATED WITH COFFEE IN AFRICA: TAXONOMY AND POTENTIAL USES FOR THE BIOLOGICAL CONTROL OF COFFEE LEAF RUST AND PLANT GROWTH PROMOTION

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

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(Coorientador)

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Robert Weingart Barreto
(Orientador)

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–*Uma das coisas mais maravilhosas da vida é que o aprendizado é contínuo*‖

(Clarice Lispector)

BIOGRAFIA

María del Carmen Herrera Rodríguez, filha de Margarita Rodríguez Madrid e Enrique Herrera Bandala, nasceu na cidade de Atzalan, Veracruz, México no dia 02 de Março de 1988. Realizou os estudos básicos na cidade de Plan de Arroyos, no mesmo estado.

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Em março de 2015, iniciou o programa de doutorado em Fitopatologia na Universidade Federal de Viçosa (UFV) baixo a supervisão do Prof. Robert W. Barreto.

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ABSTRACT

RODRÍGUEZ, María del Carmen Herrera, D.Sc., Universidade Federal de Viçosa, March, 2019**.** ***Trichoderma*** **spp. associated with coffee in Africa: taxonomy and potential uses for the biological control of coffee leaf rust and plant growth promotion**. Adviser: Robert Weingart Barreto. Co-advisers: Harold Charles Evans and Lucas Magalhães de Abreu.

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* is the most important disease in coffee. Difficulties in obtaining long lasting resistance, the failure of the strategy of escaping the disease by highland coffee cultivation and the increasing restrictions to the use of fungicides prompted the search for alternative forms of management. Biological control is an alternative of major strategical importance in such a scenario. Although there are publications dealing with biological control of *H. vastatrix*, none of these studies involved fungi of the genus *Trichoderma* or the classical approach of biological control involving antagonists to *H. vastatrix* from its native range in Africa. The present work concentrated in the study of endophytic and mycoparasite *Trichoderma* – collected in Africa and obtained from coffee plants in wild or semi-wild conditions. Taxonomy of 94 isolates which were obtained, screening for potential antagonists to *H. vastatrix* and a preliminary evaluation of beneficial physiological effects of one selected isolate were performed. Among the isolates of *Trichoderma* obtained from Ethiopia, Cameroon and Kenya 16 distinct species *Trichoderma* were recognized. Twelve were known to science, namely: *Trichoderma agressivum*, *T. andinense, T. atroviride, T. guizhouense, T. hamatum, T. koningiopsis, T. lentiforme, T. parareesei T. petersenii, T. spirale, T. theobromicola* and *T. virens.* Four were described as new taxa: *Trichoderma* sp. nov.1*, Trichoderma* sp. nov.2, *Trichoderma* sp. nov.3 and *Trichoderma* sp. nov.4. All sporulating isolates were tested for their ability to reduce the germination of *H. vastatrix* urediniospores *in vitro* and of reducing CLR severity on leaf disc and plants. Additionally, to *Trichoderma* spp. some other taxa obtained as endophytes of coffee in Africa were also included in the screening. The ability of *Trichoderma* spp. to colonize coffee plants as endophytes after inoculation under controlled conditions was also investigated. Seventeen isolates of *Trichoderma* inhibited the germination of *H. vastatrix* urenidospores. Isolates COAD 2396 (*T. atroviride*), E48 (*Cordyceps* sp.) and E486 (*Aspergillus* sp.) reduced the severity of the disease to less than 50% of the levels observed in the controls when applied at 72h, 24h before or simultaneously with *H. vastatrix* on coffee leaf discs. Over 60% reduction of disease severity was obtained when E48 (*Cordyceps* sp.) was applied

on coffee plants 72h before inoculation with *H. vastatrix*. All species of *Trichoderma* were able to colonize coffee stems and leaves as endophytes except for COAD 2399 – *Trichoderma* sp. nov1. Additionally, to those studies one selected isolate (COAD 2482 - *T. parareesei*) was tested for growth stimulation in a model-plant species (tomato). This involved four tomato cultivars: Cereja, Italiano, Maça and Santa Cruz. The results showed that the application of the COAD 2482 isolate produced a beneficial effect which was only statistically significant for the cultivar "Santa Cruz". For that cultivar the significant effect was on the growth and development of the root system.

RESUMO

RODRÍGUEZ, María del Carmen Herrera, D.Sc., Universidade Federal de Viçosa, março de 2019**. *Trichoderma* spp. associado ao café na África: taxonomia e usos potenciais para o controle biológico da ferrugem do cafeeiro e a promoção do crescimento das plantas.** Orientador: Robert Weingart Barreto. Coorientadores: Harold Charles Evans e Lucas Magalhães de Abreu.

A ferrugem do cafeeiro (CLR) causada pelo fungo *Hemileia vastatrix* é a doença mais importante no café. Dificuldades na obtenção de resistência duradoura, o fracasso da estratégia de fuga da doença pelo cultivo de café em terras altas e as crescentes restrições ao uso de fungicidas, motivaram a procura de formas alternativas para seu manejo. O controle biológico é uma alternativa de importância estratégica em tal cenário. Embora existam publicações que tratam do controle biológico de *H. vastatrix*, nenhum desses estudos envolveu fungos do gênero *Trichoderma* ou a abordagem de controle biológico clássico envolvendo antagonistas de *H. vastatrix* de sua região nativa na África. O presente trabalho concentrou-se no estudo de endofíticos e micoparasitas de *Trichoderma* - coletadas na África e obtidas de cafeeiros em condições selvagens ou semi-selvagens. Taxonomia de 94 isolados obtidos, triagem de potenciais antagonistas para H. vastatrix e uma avaliação preliminar dos efeitos fisiológicos benéficos de um isolado selecionado foram realizados. Entre os isolados de *Trichoderma* obtidos da Etiópia, Camarões e Quênia foram reconhecidas 16 espécies distintas de *Trichoderma*. Doze são conhecidas pela ciência, nomeadas: *Trichoderma agressivum, T. andinense, T. atroviride, T. guizhouense, T. hamatum, T. koningiopsis, T. lentiforme, T. parareesei T. petersenii, T. spirale, T. theobromicola e T. virens*. Quatro foram descritos como novos táxons: *Trichoderma* sp. nov.1, *Trichoderma* sp. nov.2, *Trichoderma* sp. nov.3 e *Trichoderma* sp. nov.4. Todos os isolados de boa esporulação foram testados quanto à sua capacidade de reduzir a germinação dos urediniósporos de *H. vastatrix in vitro* e na redução da severidade da CLR no disco folia e em plantas. Além de *Trichoderma* spp. alguns outros taxa obtidos como endófitos do café na África também foram incluídos na triagem. A capacidade de *Trichoderma* spp. Colonizar cafeeiros como endófitos após a inoculação sob condições controladas também foi investigado. Dezessete isolados de *Trichoderma* inibiram a germinação de urenidosporos de *H. vastatrix*. Os isolados COAD 2396 (*T. atroviride*), E48 (*Cordyceps* sp.) e E486 (*Aspergillus* sp.) Reduziram a severidade da doença para menos de 50% dos níveis observados nos controles quando aplicados em 72h, 24h antes ou simultaneamente com *H. vastatrix* em discos de folhas de café. Mais de 60% de redução na severidade da doença foi obtida quando E48

(*Cordyceps* sp.) foi aplicado em cafeeiro 72h antes da inoculação com *H. vastatrix*. Todas as espécies de *Trichoderma* foram capazes de colonizar caules e folhas de café como endofíticos, com exceção de COAD 2399 - *Trichoderma* sp. nov.1. Além desses estudos, um isolado selecionado (COAD 2482 - *T. parareesei*) foi testado para estimulação do crescimento em uma espécie de planta-modelo (tomate). Isso envolveu quatro cultivares de tomate: Cereja, Italiano, Maça e Santa Cruz. Os resultados mostraram que a aplicação do isolado COAD 2482 produziu um efeito benéfico que foi estatisticamente significativo apenas para a cultivar "Santa Cruz". Para essa cultivar, o efeito significativo foi no crescimento e desenvolvimento do sistema radicular.

GENERAL INTRODUCTION

Coffee is considered one of the top commodities in the world. Domestic consumption of coffee in the countries that produce and export it is very high and, now even in traditional tea-consuming countries, in South and East Asia, such as China, India, Indonesia, the Philippines, the Republic of Korea, Taiwan and Vietnam are becoming important coffee consumers (ICO 2018).

Brazil is the largest coffee producer of *C. arabica* and the second largest of *C. canephora* worldwide with a total production of 63,400 of thousand 60-kilogram bags (USDA 2018). However, Brazil, as well as the other coffee producing countries, faces great challenges for production as climate change, pests and diseases.

Coffee leaf rust (CLR) is the most important disease of coffee. It is caused by the fungus *Hemileia vastatrix* (Basidiomycota, Pucciniales) which was first described in 1869 (Eskes and Kushalappa 1989). This is a disease which mainly attacks young leaves. It starts as with yellow-orange lesions on the abaxial side of leaves which later coalesce with adjacent spots forming large chlorotic areas covered with powdery orange urediniospores and later leading to the formation of necrotic areas (Eskes and Kushalappa 1989). The damage of coffee rust results from a combination of reduction of photosynthetic area of leaves and heavy to complete defoliation of plants resulting in reduction of flowering and fruiting leading to reduction in coffee production of up to 35% (Eskes and Kushalappa 1989; Pereira et al. 2012; Talhinhas et al. 2017).

Since 2008, very destructive epidemics of CLR occurred in Central America, Mexico, Colombia, Peru and Ecuador; leading to major collapse in production (Avelino et al. 2015). Losses varied from country to country. In El Salvador, for instance production decreased by 54 % in 2013–14 as compared with 2012–13 (http://www.promecafe.org/). This generated a direct impact to smallholding coffee producers, in the economy and food security of entire countries due their high dependence on this commodity (Avelino et al. 2015). According to calculations by PROMECAFE, employment in Central America decreased by 16 % in 2012–13 and by 7 % in 2013–14 due to the coffee rust epidemic (Avelino et al. 2015).

The use of resistant coffee varieties is considered the best strategy for CLR management both in economic and environmental terms. Nevertheless, it is often not

sufficient for an adequate control level to be reached, not to mention the history of problems of resistance break down – such as in the recent case of the ―loss of resistance‖ of the cultivar Lempira in Honduras (Libert-amico and Paz-Pellat 2018; Prensa 2017; Ward, Gonthier, and Nicholls 2017). For adequate levels of control to be obtained the use of cupric and systemic fungicides such as triazoles and strobilurins is often necessary (Pereira et al. 2012; Talhinhas et al. 2017; Zambolim 2016). In view of the difficulty of obtaining durable resistance and increasing restrictions for fungicides and copper-based products (Carvalho, Cunha, and Chalfoun 2002), biological control has become an attractive option to be added to integrated management of CLR.

The use of endophytic and mycoparasitic fungi is an environmentally sustainable approach to the management of plant diseases (Steyaert et al. 2003). In the last decades endophytic fungi have been the focus of attention for their use in biological control because they can establish symbiotic relationships with their host and may provide protection against pathogens and herbivores (Allen et al. 2007; Fernandez-Conradi et al. 2018; Kuldau and Bacon 2008; Mejía et al. 2008; Saikkonen, Saari, and Helander 2010).

Endophytes usually live internally in plant tissues (leaves, stems, bark, petioles and reproductive structures) (Faeth and Fagan 2002) and can remain all or part of their life cycle within the host without causing any obvious damage to their plant hosts (Arnold 2007; Rodriguez et al. 2009). These microorganisms may have the capacity to protect their host by providing greater tolerance to drought, saline stress, protection against herbivores and resistance to diseases (Aly et al. 2011; Hartley and Gange 2009). The potential practical use of such ―endophytic bodyguards‖ is well illustrated by the commercialization of endophyte-enhanced grass seeds (Johnson et al. 2013; Lugtenberg, Caradus, and Johnson 2016).

Mycoparasitism is the direct attack of one fungus on another (Steyaert et al. 2003) the extent of damage that mycoparasitic fungi can cause to populations of their host-species can be very significant, as revealed by the threat represented to commercial mushroom cultivations by fungi such as *T. aggressivum* (Samuels et al. 2002)

Although some studies on the biological control of *H. vastatrix* have been published, inclusively involving surveys for antagonistic microbes (Haddad et al. 2009, 2014; Shiomi et al. 2006; Silva et al. 2012) none addressed the classical approach

focusing on natural enemies obtained from the center of origin of coffee and *H. vastatrix* in Africa. The classical biological control involves the importation of natural enemies of a pest or pathogen from the native range (Wapshere et al. 1989). This approach has resulted in examples of great success on the control of insect pests and weeds offering several advantages over other control methods (Scott 1995) but there are few examples of classical introductions against plant pathogens, and no examples of this approach being used against *H. vastatrix*.

Here, an opportunity for inaugurating classical biological control against the most important disease of one of the top tropical crops is first explored.

A survey was initiated in 2015, in cooperation, with partner scientists in organizations in Africa involving searches for fungal antagonists of CLR in Cameroon, Ethiopia and Kenya. A large diversity of fungal species, both growing as mycoparasites or as endophytes was obtained. Part of this diversity has been investigated by Colmán (Colmán 2018) and Salcedo (Salcedo 2018). A range of isolates, belonging in the genus *Trichoderma*, was also obtained growing as endophytes in coffee tissues and as mycoparasites on coffee rust. *Trichoderma* spp. may occur as saprophytes, as symbionts of plants and also as parasites of other fungi (Harman 2006; Harman et al. 2004). Several species are broadly used as biocontrol agents against plant pathogens (Ojha and Chatterjee 2011, Sharma et al. 2011). Parasitism of several phytopathogenic fungi and oomycetes by *Trichoderma* has been well documented such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Phytophthora capsici*, *Moniliophthora perniciosa*, among others (Mokhtari et al. 2018; Ojha and Chatterjee 2011; Qualhato et al. 2013; Souza et al. 2006). Additionally, some species of *Trichoderma* are also known to promote plant growth and tolerance to abiotic stresses (Harman et al. 2004; Hermosa et al. 2012).

Here, results of a research on species of *Trichoderma* found in association with *H. vastatrix* or its *Coffea* hosts is presented, including the elucidation of the taxonomy of the range of isolates obtained, a preliminary evaluation of their potential for biocontrol of CLR and a preliminary evaluation of plant growth-promoting ability of one selected isolate using tomato as a model-plant.

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Chapter 1

Coffee in Africa harbours a large diversity of *Trichoderma*

Abstract

A survey for *Trichoderma* occurring as endophytes and in association with coffee leaf rust pustules (*Hemileia vastatrix*) – CLR - was performed in Africa particulary in Kenya, Cameroon and Ethiopia. Ninety-four isolates were obtained in this study, 76 from healthy leaves, stems and berries of *Coffea* spp. and 18 directly from colonized rust pustules. The phylogenetic study for all isolates involved the combination of genes translation elongation factor-1α (*tef1*), *rpb2* and *cal* for 12 selected isolates. GCPSR criteria were used for the recognition of species; morphological and cultural characters was performed allowing for the identification of taxa. Results presented herein revealed a previously unrecorded wealth of *Trichoderma* species in *Coffea* spp. and in connection with coffee leaf rust. Sixteen species were identified during a survey. These included *Trichoderma agressivum, T. andinense, T. atroviride, T. guizhouense, T. hamatum, T. koningiopsis, T. lentiforme, T. parareesei, T. petersenii, T. spirale, T. theobromicola and T. virens*. Additionally, four novel *Trichoderma* taxa were found and are described in the present work. The true potential of such *Trichoderma* isolates for the coffee plants in terms of possible physiological benefits but, in particular as a tool to protect (fungal bodyguards) coffee plantations against its worst disease (CLR) will be investigated.

Keywords: *Coffeea* spp.; coffee rust; endophyte; fungal diversity; *Hemileia vastatrix*; molecular phylogeny; mycoparasite.

INTRODUCTION

Species of the of the ascomycete genus *Trichoderma* are widely distributed in different environments and have a variety of biological activities (Jiang et al. 2016). In the last two decades various studies have investigated the diversity and taxonomy of *Trichoderma* and numerous novel species have emerged using of DNA sequence data (du Plessis 2015). However, despite the various surveys aimed at covering the diversity of this genus, such studies concentrated mostly in Asia, Europe and America (Chaverri et al. 2011; Hoyos-carvajal, Orduz, and Bissett 2009; Jaklitsch 2011; Kubicek et al. 2003), Africa has been poorly covered in terms of assessment of diversity of *Trichoderma* until now, except from some studies covering a few specific regions or ecological niches (du Plessis 2015; du Plessis et al. 2018) . In the case of *Trichoderma* occurring as endophytes there is one study covering species of this genus obtained from coffee roots in Ethiopia (Mulaw et al. 2010).

Fungi belonging in the genus *Trichoderma* have a recognized relevance as decomposers (Cox et al. 2001; Druzhinina et al. 2006). For a long time, members of *Trichoderma* were considered to be soil saprotrophs of little practical relevance (Harman et al. 2004; Mukherjee et al. 2013). Currently it is widely accepted that such a generalization was mistaken. Many species of *Trichoderma* can develop as endophytes of plants, living in various tissues such as sapwood, leaves, stems and roots (Bailey and Melnick 2013; Evans et al. 2003; Harman et al. 2004). Such interaction between *Trichoderma* spp. and their host-plants is intimate and may be rather complex, involving many steps at each level from direct contact to internal colonization of tissues (Mukherjee et al. 2013). Endophytic *Trichoderma* may simply behave as innocuous commensals or can stimulate the plant defense system inducing plant host resistance against phytopathogens, promote tolerance to abiotic stresses, stimulate plant growth and contribute towards the solubilization of nutrients for the host plant‘s benefit (Hermosa et al. 2012; Lorito et al. 2010; Mastouri, Björkman, and Harman 2012; Shoresh, Harman, and Mastouri 2010).

Studies on the *Trichoderma* in perennial crop plants, particularly in their original wild to semi-wild situations, have revealed considerable diversity of species, including several novel taxa. Notable examples are cocoa (*Theobroma cacao*) and rubber (*Hevea brasiliensis*) (Chaverri et al. 2011; Samuels et al. 2006) in the Amazon(Chaverri et al.

2011; Hanada et al. 2008; Holmes et al. 2004; Samuels et al. 2000, 2006; Samuels and Ismaiel 2009).

Members of *Trichoderma* compete naturally in the wild with other groups of fungi to occupy niches and obtain nutrients and are capable of producing a range of secondary metabolites (Harman et al. 2004; Kubicek and Harman 1998). Another characteristic of several *Trichoderma* is their mycoparasitic ability which has led some to be considered as potential tools for the control of several phytopathogenic fungi. There are some practical example of applications of mycoparasitic *Trichoderma*, por example – that of *T. stromaticum*, a mycoparasite of *Moniliphthora perniciosa* - the causal agent of witches'broom of cacao - the most important disease of the crop in the Neotropics. It colonizes the necrotic broomed tissue of diseased plant and fruit bodies of the fungus decreasing inoculum production (Souza et al. 2006). A product based on *T. stromaticum* (Tricovab) has been produced and distributed to farmers in southern Bahia (Brazil) for many years (Bettiol et al. 2012).

Other *Trichoderma* species colonize and degrade resistance structures (sclerotia) of other phytopathogenic fungi (Druzhinina et al. 2011; Elad, Barak, and Chet 1984). and have been produced by the industry and used as commercial biofungicides (Whipps and Lumsden 2001). Although the known diversity of *Trichoderma* is already rather high (more of 200 species names) (Bissett et al. 2015), most research on mycoparasitism has been performed with only a few of these species, including *T. harzianum* sensu lato, *T. atroviride*, *T. virens*, *T. asperellum* and *T. asperelloides* (Druzhinina et al. 2011). The mycoparasitism of *Trichoderma* on rust fungi is not something extensively known or studied. Nevertheless, the existence of parasitism of rusts by species of *Trichoderma* is to be expected since several *Trichoderma* species have been described from fungi belonging to phylum basidiomycota (Samuels et al. 2002, 2000).

Trichoderma is recognized as one of the leading biocontrol fungi for control of plant diseases but its diversity is very large and its potential can be regarded as virtually untapped.

In this context we focused our research on surveying and describing *Trichoderma* spp. occurring as endophytes and mycoparasites in species of *Coffea* in Africa.

MATERIAL AND METHODS

Isolation of endophytes and mycoparasites

The fungal isolates were all obtained during survey collections to Africa namely:

20th May to 5th June 2015 – Kenya

15th Nov to 23rd Nov 2015 – Cameroon

23rd Nov to 29th Nov 2015 – Ethiopia

29th May to 5th Jun 2017 – Ethiopia

17th Jan to 29th Jan - Ethiopia

Surveys were performed in cooperation with African scientists from African research organizations, particularly of IRAD (Institut de Recherche Agricole pour le Developpement) – Cameroon - Jimma University and Ethiopian Institute of Agricultural Research – Ethiopia. *Ad hoc* collections were also performed by these scientists.

During those survey visits were directed to areas where wild species of *Coffea* occur (Cameroon and Kenya) and where *Coffea arabica* (Kenya and Ethiopia) and *Coffea canephora* (Cameroon – Congo Basin) occur in the wild or cultivated in semi-wild conditions. At each selected site, existing plants were examined for a period of 2 hours in search for pustules of *H. vastatrix* – with particular interest in collecting rust colonies bearing mycoparasites or appearing to be abnormal (unusal colours, lack of sporulation or others). These were dried in a plant press for later processing in the laboratory (identifications and isolations). Also, at each site, samples of at least three separate adult plants were collected consisting on healthy leaves, berries and 3 cm diam stem sections of each individual. Isolates were obtained from healthy leaves, stems and berries of *Coffea arabica, C. brevipes*, *C. canephora* and *C. eugenioides*. Protocol for isolations followed the procedure described by Evans et al. (2003) with modifications and were performed as described below:

- Stems had their bark thoroughly rubbed with cotton whool soaked in 70 % alcohol and, after the alcohol had evaporated, removed using a flamed blade. The exposed panel was further cleaned with a scalpel and the surface further pared with a smaller blade. Nine, triangular slivers of sapwood (*ca*. 8 × 5 mm) were excised with a scalpel from the panel and transferred individually with fine

forceps to three plastic Petri dishes containing selective media: potato dextrose agar, one-fifth strength (20% PDA), supplemented with 10mg/l penicillin-streptomycin solution. These were sealed immediately with electrical tape and stored in plastic sandwich boxes. During these procedures, all instruments were surface sterilized in 90% ethanol and flamed using a portable, alcohol burner. On arrival in the lab the plates were transferred to a 25 °C incubator and examined regularly over an 8-wk period. Hyphal tips or spores were excised or picked from colonies as they appeared on or around the wood samples and transferred to 5 cm diameter, plastic Petri dishes containing 20 % PDA or potato carrot agar (PCA) and incubated under black light at 25 °C to promote sporulation.

- Young mature healthy leaves (third from the branch tip) were thoroughly rubbed with cotton whool soaked in 70 % alcohol and, after the alcohol had evaporated, had three small (*ca*. 5 × 5 mm) square fragments excised from the centre (following and including the midrib) and were surface sterilized by 3 min immersion in 10% bleach, followed by immersion in sterile water in stoppered plastic tubes and thorough agitation and plated as described for stems. The following steps were as described for stems.
- Whenever available *Coffea* berries were also sampled and treated similarly as described above for leaf samples but after surface cleaning with acohol each fruit was skinned and inner parts were divided in three slices which were then surface sterilized before plating. Further steps followed the same procedure as described above.

For the mycoparasite isolates (isolates found forming colonies that emerged directly from pustules of *H. vastatrix*) were obtained by direct mycoparasite conidia from infected rust pustules onto PDA plates under a dissecting microscope upon arrival to the laboratory. A list of relevant samples is provided separately (Table 1 supplementary material).

DNA extraction, polymerase chain reaction (PCR) and sequencing

Strains were grown in 3 cm diam plates containing 5 mL of potato dextrose (PD) at 25 °C in the dark for 4-5 days. DNA was extracted from the mycelium grown on the surface of the broth. DNA was extracted with the Wizard Genomic DNA Purification kit (Promega, Madison, EUA) by following the manufacturer‘s instructions. The

fragments *rpb2* (primers fRPB27cR - RPB25F2) (Liu, Whelen, and Hall 1999) and *tef1* (primers EF2 - EF1728M) were amplified for all isolates and additionally *cal* (primers CAL228 -CAL737) (Carbone and Kohn 1999) was amplified for a subset of 12 isolates.

The polymerase chain reaction (PCR) amplifications were performed in a total reaction volume of 12.5 µl, including 0.25 µl of each primer, 1.25 µl of BSA, 6.25 of Taq polymerase [including dNTPs], 0.25 µl of genomic DNA [30ng/µl]; 0.25 µl DMSO and 4 µl of sterile ultrapure water. PCR conditions for *rpb2* were 95 ºC / 5 min., followed by 38 cycles at 95 ºC / 1 min., 58 ºC / 2 min., 72 ºC / 2 min. and 72 ºC/10 min. For *tef1*, conditions were 94 ºC / 2 min., followed by 9 cycles at 94 ºC / 35s, 66 ºC / 55s, and 35 cycles at 94 ºC / 35s, 56 ºC / 55s and 72 ºC / 1min 30s. Conditions for *cal* were 95 °C / 8 min., followed by 35 cycles at 95 °C / 15s, 55 °C / 20s, 72 °C / 1 min and extension at 72 °C / 5 min. PCR products were visualized by Gelred™ (Thermo Fisher Scientific) staining following electrophoresis of 4 µl of each product in 1 % agarose gel. The PCR products were sequenced by Macrogen Inc., South Korea (http://www.macrogen.com).

Phylogenetic analysis

Consensus sequences were assembled from forward and reverse sequencing chromatograms using SeqAssem (Hepperle 2004); *tef1*, *rpb2* and *cal* contigs of all strains were compared to homologous sequences deposited in NCBI-Gen-Bank. Sequences generated in the present study were deposited in the NCBI-GenBank database (Table 1 supplementary material) and sequences obtained in other studies were used in our phylogenetic analyses and were retrieved from the NCBI-GenBank database (Table 2 supplementary material). Sequence alignments were performed using MUSCLE implemented in MEGA 6 (Tamura et al. 2013). In total, the dataset comprised 204 partial *tef1* (sequences 540 pb); 195 partial *rpb2* sequences (905pb) and 25 partial *cal* sequences (443pb).

Two concatenated trees with *tef1* and *rpb2* sequences were created, one with taxa of the clade *harzianum* (more numerous) and one with the rest of the taxa (Fig. 1 and 2); a third concatenated analysis with partial sequences of three genes*, tef, rpb2* and *cal*, was constructed with a subgroup of sequences to clarify the phylogenetic relationships of some species within the clade *harzianum* (Fig. 3), such trees containing

134 taxa with 1539 characters, 72 taxa with 1608 characters and 25 taxa with 1927 characters, respectively. The concatenated alignment were generated in Sequence matrix v1.8 (Vaidya and Lohman 2009). Single-gene trees were also generated. Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian Inference (BI) were performed for the concatenated and single-gene trees. Prior to phylogenetic analyses, the most appropriate nucleotide substitution model for each locus was selected using MRMODELTEST v. 2 (Nylander 2004). Nucleotide substitution models in the two-gene concatenated trees were HKY+I+G and SYM+I+G (Fig.1), GTR+I+G and SYM+I+G (Fig. 2), for tef1 and rpb2, respectively. For the three-gene concatenated tree, the models were HKY+I, K80+I and K80+G (Fig. 3) for tef, rpb2 and cal, respectively. For all trees the BI and ML analysis were estimated in the CIPRES Science Gateway Platform using Mr. Bayes 3.2.6 and RaxML-HPC v.8, respectively (Miller, Pfeiffer, and Schwartz 2010; Stamatakis 2006) and MP in MEGA 6. Phylogenetic trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Corel Draw.

Phylogenetic species were recognized based on two main criteria proposed by (Dettman et al. 2003): Genealogical Concordance (the clade was present in the majority of the single-locus genealogies, as revealed by a majority-rule consensus tree) and Genealogical Non-discordance (the clade was well supported in the least one single-locus genealogy, as judged both by MP and BI and was not contradicted in any other single-locus genealogy at the same level of support).

Morphological characteristics

The results of the phylogenetic analysis of the assemblage of *Trichoderma* isolates guided the selection of isolates to be included in the morphological analysis and characterization of novel taxa. One or two isolates of each new taxon were examined. Procedures for morphological observation of *Trichoderma* spp. followed Samuels and Hebbar (2015). Macroscopic characteristics of colony (mycelium color, radial growth, presence/absence of concentric rings, ‒pustules‖ (=sporulation shrubs), pigmentation and presence/ absence of odor) were evaluated in PDA (Potato Dextrose Agar), CMD (Corn-meal Agar) and SNA (Synthetic Nutrient Deficient Agar) after 7 days of cultivation of isolates at 25°C under 12 h daily light regime (light provided by two white and one near-UV lamps placed 35 cm above the plates). Rates of growth were evaluated at 72 and 96 h on the three culture media at 25 and 30°C in the dark.

Observations of fungal structures were made on an Olympus BX 51 microscope and were based on slide cultures prepared with colonies of each isolate growing from PDA and CMD blocks, as described by Waller, Ritchie, and Holderness (1998). After 4-5 days of growth at 25°C under the same light regime described above the slides were mounted in 3% KOH for observation and illustrations. Descriptions included biometric data of phialides, conidia and chlamydospores. Measurements were taken from images generated with a digital camera Olympus Q-Color 3 by using the cellSens software.

RESULTS

Phylogenetic analyses and GCPRS

76 endophytic and 18 mycoparasitic *Trichoderma* isolates were obtained during this survey. The combined data set indicated that 94 *Trichoderma* strains grouped into sixteen monophyletic groups based on criteria of ML/MP $\geq$70% bootstrap and BI$\geq$0.9 posterior probability support (Fig. 1 and 2). The concatenated trees generated for BI, ML and MP analysis shared a similar topology, providing high support to the final trees. Phylogenetic trees and DNA sequence alignment data are available from TreeBase (study S24367).

Following Samuels and Hebbar (2015), it was identified five clades between endophytic and mycoparasitic *Trichoderma* isolates, namely clades: *Viridae, Virens, Stricpile, Longibrachiatum and Harzianum*. Five isolates were grouped into three known species belonging to the clade *harzianum* (*T. lentiforme, T. guizhouense* and *T. aggressivum*) and one isolate identified as *T. virens* [clade virens] and 3 as *T. spirale* [clade strictpile] (Fig. 1). Three isolates were grouped in *T. parareesei*, belonging to the Longibrachiatum clade and an additional isolate, obtained from Brazil (as mycoparasite on CLR pustules), also fell within this clade, and was identified as *T. andinense.* Twenty one isolates were grouped into 5 species of the viride clade: *T. koningiopsis, T. petersenii, T. theobromicola, T. hamatum and T. atroviride* (Fig. 2). Fifty - nine isolates grouped in three phylogenetic species belonging to the clade harzianum and one isolate belonging to the viride clade did not correspond to known species and were considered as new taxa, described in this work as *Trichoderma* sp. nov.1, *Trichoderma* sp. nov.2, *Trichoderma* sp. nov.3 and *Trichoderma* sp. nov.4 (Fig. 1 and 2). In order to clarify the phylogenetic relationship between *T.* sp. nov.4. and *T. pyramidale*, an analysis was performed with the addition of calmodulin sequences. The results of such analysis supported the distinction between *T. pyramidale* from the new species (Fig. 3).

The isolates identified in this study as *Trichoderma* sp.nov.4 were positioned as paraphyletic with *T. pyramidale* reference isolates, in the *tef* tree (Fig. 4) and in the *rpb2* tree (Fig. 5), the sequence of the only available reference isolate of *T. pyramidale* (S73) was distant from the *Trichoderma* sp. nov.4 clade, supporting the distinction of these taxa the phylogenetic relationships of these two groups were also evaluated with calmodulin sequences, which reinforced their distinction as two distinct taxa (Fig. 3).

The *tef* and *rpb2* trees were highly congruent (Fig. 4 and 5 complementary material) with the topology of the concatenated tree (Fig. 1), except for the isolates identified as *T. lentiforme* (indicated with numbers 1 and 2, Fig. 5), when evaluated in the *rpb2* tree. The reference isolates of this species were placed in two polyphyletic species and the isolates of this study attributed to *T. lentiforme* were positioned outside monophyletic groups (they remained as singletons) in this analysis.

Diversity and distribution

Although the collections of plant material were not systematic and there was no purpose of quantitativaly studying the frequency of colonization of plants by species of *Trichoderma* in this study, it was possible to observe indications of some patterns of occurrence of taxa originating in terms of region/locality, host *Coffea* species and organ. It was observed that *T. koningiopsis* was obtained only from leaves; *T. theobromicola, T. guizhouense* and *T. spirale* were only isolated from stems and *Trichoderma* sp. nov.2 only from berries. The other species were distributed in more than one plant organ. The predominant taxa in all organs was *Trichoderma* sp.nov.3

- The species predominantly isolated as mycoparasite, in Ethiopia and with one isolate in Cameroon, was *Trichoderma* sp. nov.4. The species *T. aggressivum, T. andinensis, T. parareesei* and *T. petersenii*, were isolated only once during the survey, respectively from Kenya, Brazil, and Ethiopia. In Cameroon, 24 isolates belonging to seven species were found, namely: Six species in stem samples (*Trichoderma* sp. nov.3, *T. koningiopsis, T. lentiforme, T. spirale, T. theobromicola* and *T. virens*), one in leaves (*T. koningiopsis*) and one species with one isolated only as mycoparasite (*Trichoderma* sp. nov.4) (Table 3).
- In Ethiopia 64 isolates, belonging to seven species were isolated, namely: 20 *Trichoderma* sp. nov.3 isolates from leaves, stem and fruits; 34 isolates of *Trichoderma* sp. nov.4, found in leaves, stems and as mycoparasites; three

isolates of *T. parareesei* from stem and as mycoparasite; three isolates of *T. hamatum* from stems and fruits; two isolates of *Trichoderma* sp. nov.2 from fruits; one isolate of *T. petersenii* growing as mycoparasite and one isolate of *T. lentiforme* from fruit (Table 3).

- In Kenya, four species were collected, namely: one isolate of *T. aggressivum* growing as mycoparasite*;* one isolate of *T. atroviride* from leaf; one isolate of *T. guizhouense* and one of *Trichoderma* sp. nov.1 from stems (Table 3).

When the diversity of *Trichoderma* from coffee in West Africa (Cameroon) is compared with that of East Africa (Kenya and Ethiopia), *Trichoderma lentiforme*, *Trichoderma* sp. nov.4 and *Trichoderma* sp. nov.3 are the only species which are present both in Ethiopia and Cameroon. The four species found in Kenya were absent from coffee samples in other countries. Although this suggests strong endemism and isolation of the *Trichoderma* mycobiota of coffee in Kenya, this need to be regarded with caution since sampling in Kenya was limited and this might have led to a chance artifact.

The only Brazilian isolate was identified as *T. andinense*. Nevertheless, it is inadequate to conclude that there is an impoverished *Trichoderma* mycobiota on coffee in Brazil since the isolation protocol described above for stems, leaves and berries was only utilized in Africa and this occurrence appeared in an *ad hoc* occasional isolation during a search for mycoparasites of CLR pustules in Brazil.

When comparing the number of taxa of *Trichoderma* present in *C. canephora* and *C. arabica*, we observed that both harbor five species and are distributed differently in their tissues. The only *Trichoderma* spp. found both in *C. arabica* and *C. canephora* were *T. lentiforme* and *Trichoderma* sp. nov.3.

In *C. arabica* the highest diversity of *Trichoderma* spp. was found in fruits (four species) whereas in *C. canephora* the highest diversity was found on stems (four species). Nevertheless, sampling was concentrated on *C. arabica* and conjectures based on samples collected on *C. canephora* may be inadequate.

Taxonomy

Four additions to the genus *Trichoderma* emerged from the phylogenetic study of the isolates obtained during this survey on *Coffea* spp. Morphological and cultural

information proved useful to confirm their separation from closely related known species of *Trichoderma* providing evidence towards their recognition as valid taxa as proposed below.

***Trichoderma* sp. nov.1** M. C. H. Rodríguez, H. Evans, R.W. Barreto (Fig. 9)

Typification: KENYA, Marsabit Lake Paradise, forest, alt 1340m, isolated as stem endophyte of *Coffea* sp. in 2015. H. C. Evans ex-type culture COAD 2399. Genbank: *TEF1*= MK044086; *RPB2*= MK044179.

Colonies on PDA: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 28-30 mm. At 30°C, colonies reach 7mm radius after 72h. At 25°C, mycelium mostly surface, grayish white aerial, olive green sporulation and beginning in the center of the colony with the formation of concentric rings. Absence of pigmentation in the media and no odor.

Colonies on CMD: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 29-31 mm. At 30°C, colonies reach 1mm radius after 72h. At 25°C, mycelium mostly hyaline, low and olive-green sporulation, no formation of concentric rings and no odor. At 35°C no growth occurs.

Colonies on SNA: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25 C = 30 mm. At 30°C, colonies reach 9mm radius after 72h. At 25°C, hyphae hyaline and smooth, low and olive-green sporulation; absence of concentric rings and no pigmentation. Formation of amorphous and cottony pustules, measuring 1-3.5 mm in diam.

Conidiophores pyramidal with phialides held in whorls; lageniform phialides, (4–) 5-9.7 (–10.7) × (1.9–) 2–3 (–3.2) µm mean (7.6 × 2.7 µm) length/width ratio , supporting cells (4.9–) 5.7–11.9 (–12.5) × (1.6–) 1.75–2.9 (–3) µm, mean (8.1× 2.1) length/width; Conidia globose to broadly ellipsoid, 2.2– 3.9 (–4.3) × (1.9–) 2–2.9 (–3.2) µm, mean (2.8×2.6 µm), green-colored, smooth; Chlamydospores globose to subglobose, abundant, intercalary and terminal, (5.4–) 6.9–12.3 (–13.5) × (3.6–) 4–7.1 (–10.2) µm, mean (9.4 × 6.6) (on CMD and PDA at 5th day) at 25°C.

Notes: *T.* sp. nov.1 was found phylogenetically to be close to *T. gamsii* (Jaklitsch et al. 2006) and *T. lieckfeldtiae* (Samuels and Ismaiel 2009). The new species is

morphologically similar to *T. gamsii* in branching pattern pyramidal type, lageniform phialides and the formation of chlamydospores; only presents slight differences in size in phialides and conidia being *Trichoderma.* sp. nov.1is smaller than *T. gamsii*. The most prominent differences are between *T.* sp. nov.1 and *T. lieckfeldtiae*, where *T. lieckfeldtiae* shows a fast growth in PDA and SNA at 25 °C, it has the branching patter pachybasium type, phialides are smaller *Trichoderma* sp. nov.1 and don‘t have formation of clamydospores.

***Trichoderma* sp. nov.2** M. C. H. Rodríguez, H. Evans, R.W. Barreto (Fig. 10)

Typification: ETHIOPIA: Kaffa Region, Bonga District Gedam village, alt 1550m. Isolated from berries of *Coffea arabica*. Kifle Belachew Bekele; ex-type: COAD 2415. GenBank: *TEF1*= MK044109; *RPB2*= MK044202.

Colonies on PDA: Optimum growth at 25 C. Colony radius after 72h when grown at 25°C = 51-53 mm. At 30°C, colonies reach 34mm radius after 72h. At 25°C, filling the plate after 4 days; mycelium mostly white aerial, low and green sporulation, no formation of concentric rings. Absence of pigmentation in the media and no odor. At 35°C no growth occurs.

Colonies on CMD: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 42-44 mm. At 30°C, colonies reach 19mm radius after 72h. At 25°C, mycelium mostly hyaline, low sporulation, green conidia, no presence of concentric rings and no odor. At 35°C no growth occurs.

Colonies on SNA: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C= 29-36 mm. At 30°C, colonies reach 12mm radius after 72h. At 25°C, hyphae hyaline and smooth, low sporulation; green conidia, of the formation of concentric rings, no pigmentation in the media.

Conidiophores pyramidal with verticillate paired lateral branches; phialides generally formed on terminal branches, held in divergent whorls of three to four, (5.2–) 5.3-12.2 (–13.2) × (1.7–) 2–2.8 (–3.48) µm, mean (7.4 × 2.5 µm) length/width ratio; Supporting cells (5.4–) 7.9–9.7 (–10.1) × 1.7–2 (–3) µm, mean (8.5×1.9 µm) Length/Width ratio; conidia ellipsoidal to ovoide, smooth , 2.2–3 (–3.2) × (1.9–) 2.3–3.1 (–3.4) µm, mean (2.8 × 2.8 µm) length/width , green, smooth. Chlamydospores

terminal and intercalary, globose, 3.3–5 (–6.6) × 3.0–4.6 (–5.3) µm, mean (4.4 × 3.7 µm) (abundantly formed in CMD after 4 days) Fig. 10.

Notes: Phylogenetic analyses placed *T. sp.* nov.2 close to *T. amazonicum* (Chaverri et al. 2003) and *T. pleuroticola* (Chaverri et al. 2011). The new species can be distinguished from its relatives by no growth at 35°C, presence of a slight odor coconut-like on PDA and conidia with blue-green color in PDA microculture. Morphologically, *T. sp. nov.2* presents a major difference with *T. amazonicum* which has a branching patchy pachybasium type, elliptical to subglobose conidia, minor phialides, ampuliforms and chlamydospore-like structures in clusters. Compared with *T. pleuroticola*, *T. sp. nov.2* has more shared morphological characteristics such as branching pattern pyramidal type, globose conidia and formation of chlamydospores; On the phialides, the new taxon has larger and lageniform phialides.

***Trichoderma* sp. nov.3** M. C. H. Rodríguez, H. Evans, R.W. Barreto (Fig. 11)

Typification: ETHIOPIA: Kaffa Region, Bonga District, Biosphere Reserve, Gela wild Coffee; alt. 1550 to 1900 masl. endophytic in *Coffea arabica* berrie; Kifle Belachew Bekele; ex-type: COAD 2422. GenBank: *TEF1*= MK044119; *RPB2*= MK044212.

Colonies on PDA: Optimum growth temperature at 30°C. Colony radius after 72h when grown at 25°C = 56–61 mm. At 30 and 35°C, colonies reach 65mm and 25mm radius after 72h, respectively. At 25°C, fills the plate after 4 days, cottony white aerial mycelium, green sporulation starting from the center of the plate, with the formation of concentric rings. Presence a sweet odor. No exudates or soluble pigments.

Colonies on CMD: Optimum growth temperature at 30°C. Colony radius after 72h when grown at 25°C= 55–58 mm. At 30 and 35°C, colonies reach 63mm and 43mm radius after 72h, respectively. At 25°C, mycelium mostly hyaline, low sporulation, no presence of concentric rings and no odor.

Colonies on SNA: Optimum growth temperature at 30°C. Colony radius after 72h when grown at 25 C =49–53 mm. At 30 and 35°C, colonies reach 61mm and 28mm radius after 72h, respectively. At 25°C, hyphae hyaline and smooth, green sporulation; presence concentric rings; no exudates or soluble pigments.

Conidiophores pyramidal bearing in whorls or pairs lateral or terminal phialides. Phialides lageniform to ampuliform, 4.0–8.0 (–8.6) × (1.9–) 2.3–3.1 × 4–8 (–8.6) µm (L/W), 1.2–2.4 µm in width at the base. Supporting cells 5.4–15.6 × 1.8–3.4 µm (L/W); Conidia globose to bradly ovoid, 1.4–3.3 × 1.6–2.8 µm (L/W) green smooth. Chlamydospores abundant globose to ellipsoidal, terminal and intercalar, 4.4–8.1 × 3.8–7.3 µm (L/W).

Notes: *Trichoderma* sp. nov.3 grouped phylogenetically close *to T. afarasin* and *T. endophyticum* (Chaverri et al. 2015) . The new species is morphologically similar its close relatives, the conidiophore pyramidal type, size of conidia and phialide very close, ampuliform. In PDA at 25°C the growth is similar, the growing in SNA is more slowy than PDA, T. afarasin and *T.* sp. nov.3 presents sometines a sweet odor. The main difference of *T. botryosa* and its relatives is the presence of chlamydospores.

***Trichoderma* sp. nov.4** M. C. H. Rodríguez, H. Evans, R.W. Barreto (Fig.12)

Typification: ETHIOPIA: Kaffa Region, Bonga District, Mankira-Grugutto - isolated from leaves and stems from *Coffeea arabica.* CAMEROON. Mycoparasite of coffee rust in Somalomo Town, Eastern Province, Cameroon. ex-type: COAD 2426 and COAD 2433. GenBank: *TEF1*= MK044131, MK044157; *RPB2*= MK044224, MK044250; *CAL*= MK084870, MK084869.

Colonies on PDA: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25°C = 43-45 mm. At 30 and 35°C, colonies reach 40mm and 22mm radius after 72h, respectively. The colony fills the plate after 4 days of incubation at 25 and 4 days in 30°C on PDA, CMD e SNA. At 25 C, colonies with white mycelium; presence of pigmentation yellow in the central reverser of plate.

Colonies on CMD: Optimum growth temperature at 25°C or 30°C. Colony radius after 72h when grown at 25 C = 43-44 mm. At 30 and 35°C, colonies reach 44mm and 25mm radius after 72h, respectively, mycelium mostly hyaline, no sporulation, no presence of concentric rings and odor.

Colonies on SNA: Optimum growth temperature at 25°C. Colony radius after 72h when grown at 25 C = 40-42 mm. At 30 and 35°C, colonies reach 33mm and 23mm radius after 72h, respectively at 25°C, hyphae hyaline and smooth, low sporulation; absence of concentric rings and pigmentation. Formation of amorphous and cottony

pustules, measuring 1-3.5 mm in diam. Formation of tufts or amorphous pustules, in de border white and cottony turning yellowish and in the center green after 5-6 days, eventually dark green.

Conidiophores are pyramidal to tree type, phialides ampulinform to lageriform formed usually in whorls (5–) 5.3–8.6 (–9.1) × (1.9–) 2.2–2.9 (–3.2) µm (mean 6.4 × 2.63 µm) length/width ratio, base (1.3–) 1.3–2.3 (–2.5) µm width. supporting cells (2.5–) 3.3–7.1 (–8.2) × 1.9–2.8 (–3.2) µm (mean 6 × 2.5 µm) length/wide; Conidia globose, subglobose or ovoid, green, smooth, (1.8–) 2.1–2.9 × (2.1–) 2.3–2.9 (–3.02) µm (mean 2.5 × 2.6 µm) length/ width. chlamydospores globose to subglobose (3.21–) 3.2–8.1 (–9.07) × (3.5–) 3.9–7.5 (–8.5) µm (mean 6.0 × 6.1 µm) length/width at 25°C on CMD e SNA.

Notes: *Trichoderma* sp. nov.4 grouped phylogenetically close to *T. pyramidale* (Chaverri et al. 2015). The two species share several characteristics in common such as pyramidal conidiophore, similar growth rate in PDA and SNA at 25 ° C and formation of amorphous pustules with white-yellow border; On the other hand, morphologically *T. pyramidale* presents phialides and conidia larger than *Trichoderma* sp. nov.4. The new taxa forms chlamydospores on CMD and presents a yellow pigmentation in the reverse and central part of the colony in PDA at 25 ° C.

DISCUSSION

Most of the species found in our work were possible to identify with high support using the combination of *tef1* and *rpb2*, however, for the isolates proposed here as *Trichoderma* sp. nov.4 it was necessary to include the calmodulin gene in the analysis in order to resolve if they belonged or not to *T. pyramidale. Trichoderma* sp. nov.4 is morphologically rather similar to *T. pyramidale* but *Trichoderma* sp. nov.4 has a faster growth rate in PDA A 30°C as compared with *T. pyramidale* and growths at 35°C, a feature which is absent in *T. pyramidale* (Chaverri et al 2015).

On the other hand, *Trichoderma* sp. nov.4 formed two monophyletic subclades, one clade containing endophytic isolates and another including isolates obtained directly from CLR pustules (mycoparasites). Since both subclades come from phylogenetically well supported clade by ML, MP and BI, we decided to keep them in a single species and consider them to represent an infraspecific grouping not deserving

taxonomic recognition at this stage. No significant morphological differences or differences in the growth rates for isolates belonging to these subclades were found.

There are some publications dealing with the diversity of endophytic fungi associated with coffee (Oliveira et al. 2014, 2013; Santamaría and Bayman 2005; Saucedo-García et al. 2014; Vega et al. 2010). Nevertheless, such studies were based in surveys restricted to the Americas and Hawaii, where coffee is an alien introduced species. The endophytic mycobiota found in such studies is dominated by genera such as C*olletotrichum, Fusarium, Penicillium, Pestalotia* and *Xylaria*. Such assemblage may consist mainly of opportunistic and occasional endophytes of little biological significance to their hosts. *Trichoderma* appeared infrequently in such studies. Conversely, samples from Africa in this study yielded a considerable number and variety of *Trichoderma*, including four new species. It is not possible to determine, at this stage, whether the new taxa described herein are geographically restricted to Africa or to coffee. Nevertheless, we find it significant that a far richer diversity of *Trichoderma* was found in coffee in Africa as compared with occurrences of endophytes belonging to this genus in coffee outside Africa. There are two publications reporting the occurrence of *Trichoderma* in association with coffee in Ethiopia (Mulaw et al. 2013, 2010). Such studies focused on strains isolated from the rhizosphere and root tissues of *C. arabica*. The authors reported as resulting from their investigation the occurrence of *T. harzianum* sensu lato, *T. hamatum*, *T. asperelloides*, *T. spirale*, *T. atroviride*, *T. koningiopsis*, *T. gamsii* and *T. longibrachiatum.* Only three such taxa found in those studies appeared in our isolations from stems, leaves and fruits, namely: *T. hamatum*, *T. spirale* and *T. koningiopsis.* Possibly, what Mulaw et al. (2013, 2010) identified as *T. harzianum* sensu lato in fact included some or the taxa found in our survey, but it is not possible to verify that possibility at this stage.

Trichoderma hamatum is known to be a cosmopolitan species which is commonly isolated from soil and had already been recorded as an endophyte of roots of *C. arabica* (Mulaw et al. 2013, 2010) whereas *T. spirale* and *T. koningiopsis* are also known to be cosmopolitan, although more commonly isolated from tropical habitats as was observed in our survey and in other studies (Druzhinina et al. 2005; Hoyos-carvajal et al. 2009; Kubicek et al. 2003; Samuels et al. 2006).

During our isolations of endophytes some *Trichoderma* species were obtained from more than one plant organ. That was the case for *T. koningiopsis* and *T. spirale*, which were isolated from leaves and stems of *C. canephora*. *T. hamatum*, which was obtained from stem and berries of *C. arabica, T. hamatum, T. koningiopsis* and *T. spirale* were already known to occur as endophytes in other plants (Samuels and Hebbar, 2015). Nevertheless, only *T. hamatum* had been previously reported as endophytic from *C. arabica* [obtained from roots by Mulaw et al. (2013). Other species (besides the four novel species) found here, for the first time as endophytes in coffee were: *T. guizhouense, T. lentiforme, T. theobromicola* and *T. atroviride.* Such species were known from other habitats such as tropical soils, decaying wood and bark, growing on other fungi, on mushroom compost, obtained from leaf-cutting ant colonies and as endophytes in *Theobroma cacao* and *T. gileri* (Samuels and Hebbar, 2015; Chaverri et al. 2015; Montoya et al 2016; Evans et al. 2003; Dodd et al. 2003). *Trichoderma guizhouense* has a worldwide distribution but had only been previously reported as an endophyte from cola trees in Africa (Chaverri et al. 2015). *Trichoderma lentiforme* and *T. theobromicola* were only known from South America (Samuels et al. 2006)(Chaverri et al. 2015;). These are all new geographical and host records for Africa but perhaps simply reflects the poor sampling of *Trichoderma*, also suggesting that many species of *Trichoderma* are in fact either cosmopolitan or pantropical.

Mycoparasitism - the ecological relationship where one fungus parasitizes another fungus (Karlsson et al. 2017) – has been reported for several species of *Trichoderma*. Examples are *T. atroviride, T. hamatum, T. longibrachiatum, T. reesei* and *T. virens.* Mycoparasitic *Trichoderma* spp. have a wide range of hosts, including true fungi such as *Botrytis cinerea, Rhizoctonia solani, Alternaria alternata* and *Fusarium* spp. and oomycetes such as *Pythium ultimum* (Druzhinina et al. 2011; Karlsson et al. 2017). However, the species found to be mycoparasites of *H. vastatrix* and reported here - *T. aggressivum, T. andinense, T. parareesei, T. petersenii* and *Trichoderma* sp. nov. 4 – are the first in the genus to be reported attacking the CLR fungus. Three among these species are both well-known mycoparasitic species and were found here growing as endophytes in coffee: *T. atroviride, T. hamatum* and *T. virens*. Among these *Trichoderma* sp. nov. 4 is a species deserving special attention as a potential biocontrol agent for CLR, since it was the mycoparasitic species for which the greatest number of isolates was obtained.

Mycoparasitic fungi associated with coffee rust have been studied in regions of the world where coffee is not a native species, such as in Mexico (Carrión and Rico-gray 2002). It is interesting to note that this survey and taxonomic study yielded six purported mycoparasites (*Acremonium byssoides, Calcarisporium ovalisporum, C. arbuscula, Fusarium pallidoroseum, Sporothrix guttuliformis* and *Verticillium lecanii* = *Lecanicillium lecanii*). A more recent publication reporting results of an investigation, involving the use of single-molecule DNA sequencing of fungal rRNA gene barcodes from pustules of *H. vastatrix* in México and Puerto Rico yielded fifteen fungi associated with coffee rust pustules as mycoparasites, but none of them belonged to *Trichoderma* (James et al. 2016).

Information on the ecology of the new *Trichoderma* spp. and their role in nature is limited because only few strains of each species have been isolated during the survey, the exception being *Trichoderma* sp.nov.3 and *Trichoderma* sp. nov.4, which seem to have a close connection to *H. vastatrix* and its *Coffea* hosts.

This study complements research conducted in some countries already explored in the African continent and despite focused on a relatively small number of samples obtained from few localities of three African countries (Cameroon, Ethiopia and Kenya) the significant diversity of *Trichoderma* and other fungal taxa, obtained during this brief survey. This indicates that a large mycobiota is associated with Coffee exists in the few situations where coffee still exists in the wild.

Here, in this step of naming some of the species of *Trichoderma* obtained from *Coffea* spp. and CLR. It may help, paving the way for more focused evaluations of the biocontrol potential of *Trichoderma* spp. against CLR as well as other potential applications.

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T. virens DIS 162
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Harzianum
virens
0.03

Figure 1.Bayesian phylogenetic tree of clades Harzianum, Stricpile and Virens (A). The tree was based on a concatenated *tef1* and *rpb2* sequence dataset. Bootstrap values (≥70%) of the ML and MP analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.

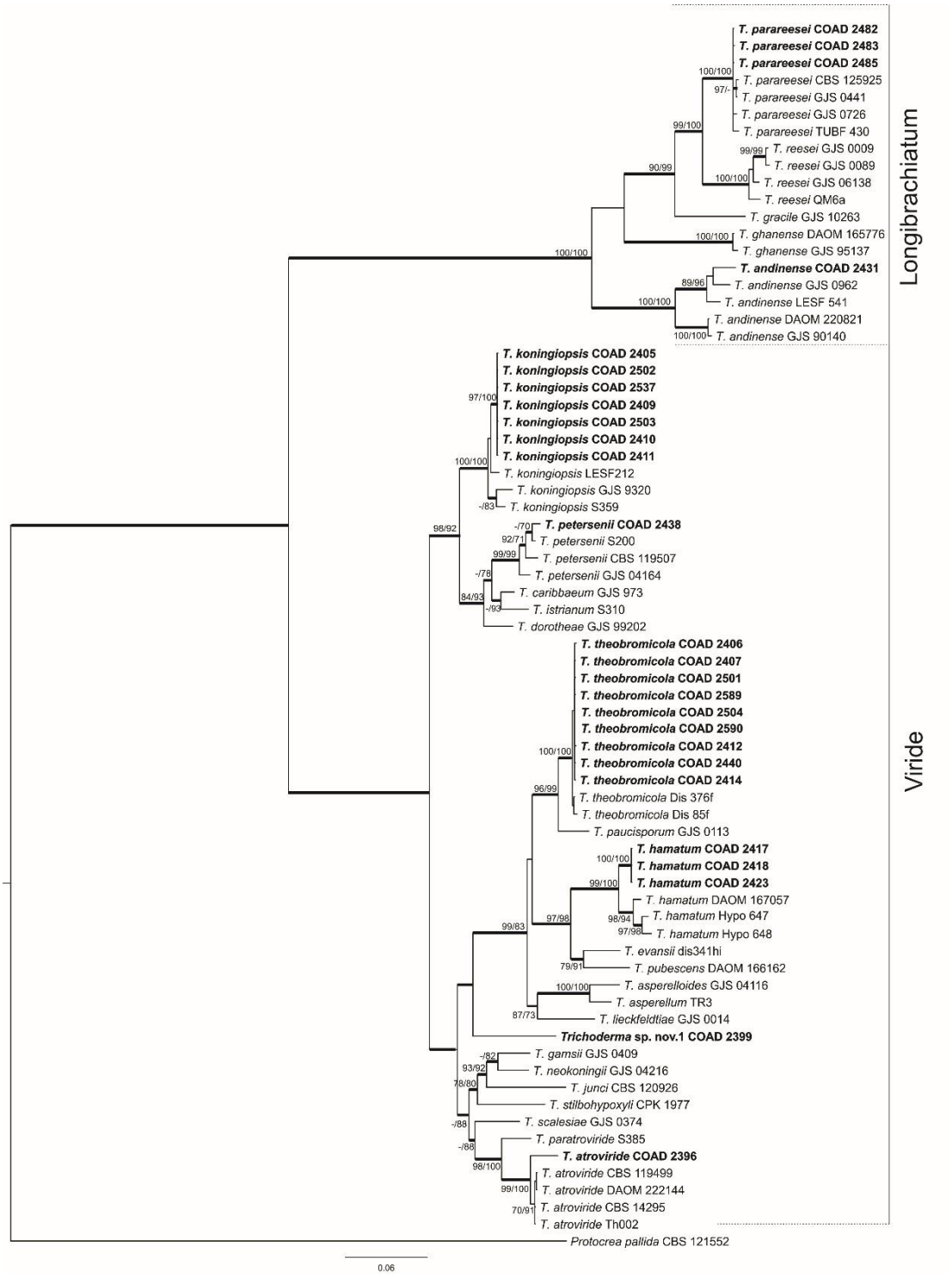


Figure 2. Bayesian phylogenetic tree of clades Longibrachiatum and Viride (B). The tree was based on a concatenated *tef1* and *rpb2* sequence dataset. Bootstrap values (≥70%) of the ML and MP analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.

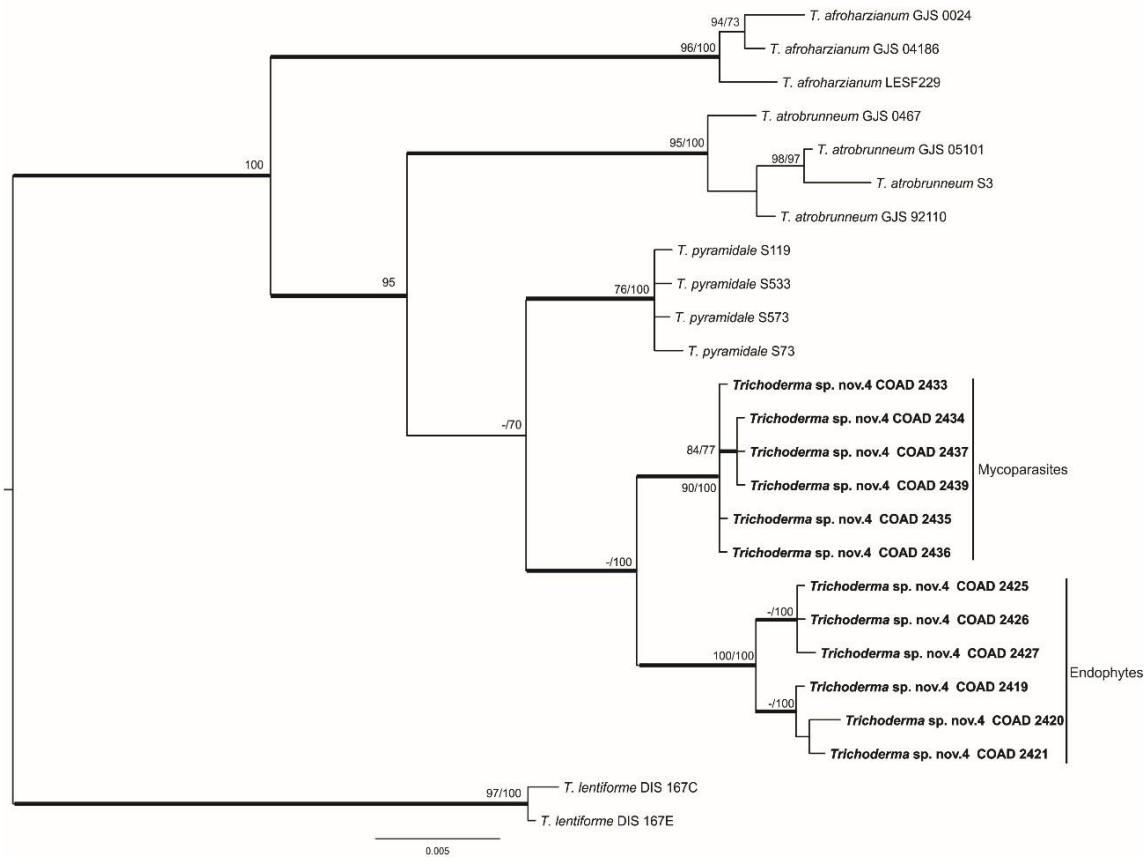
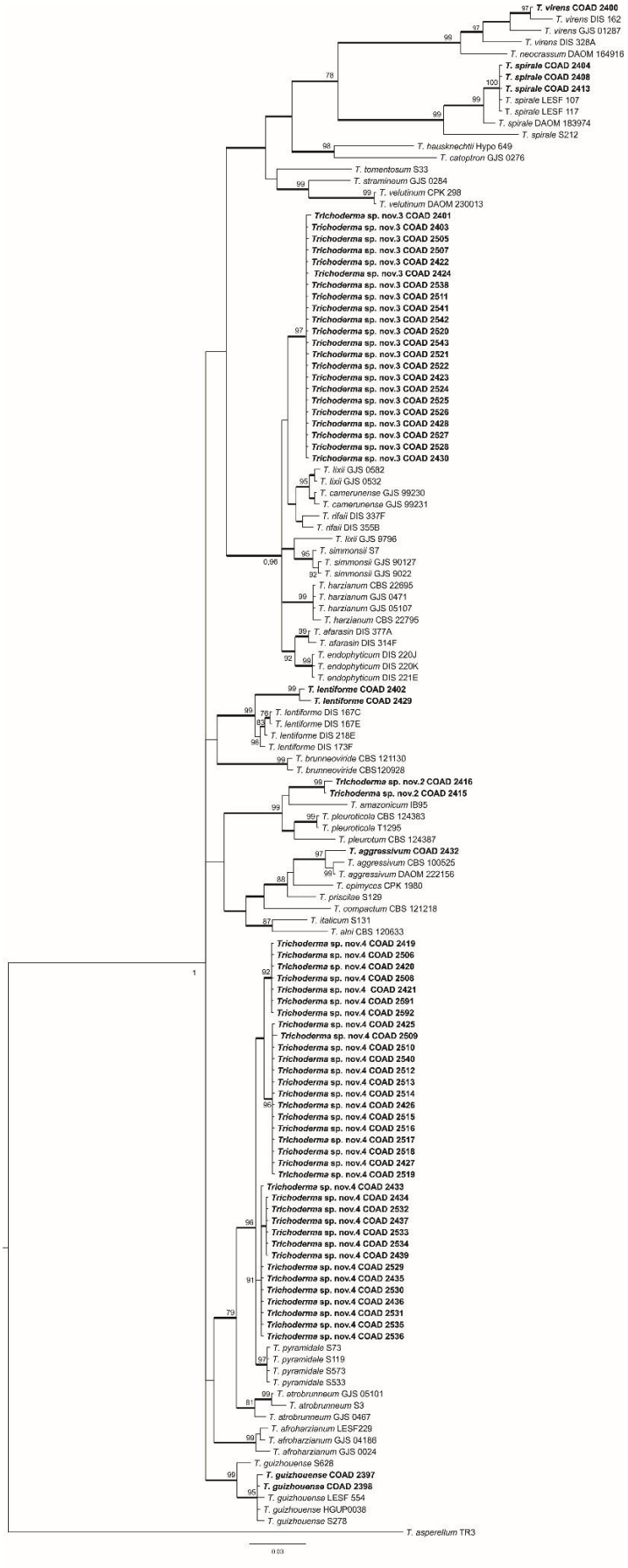


Figure 3.Bayesian phylogenetic tree (C). The tree was based on a concatenated *tef1*, *rpb2* and *cal* sequence dataset. Bootstrap values (≥70%) of the ML and MP analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.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T. spirale COAD 2408
T. spirale COAD 2413
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T. spirale LESF 117
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T. tomentosum S33
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T. velutinum CPK 298
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T. asperellum TR3
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Figure 4. Bayesian phylogenetic tree (D). The tree was based on *tef1* sequence dataset. Bootstrap values (≥70%) of the ML analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.

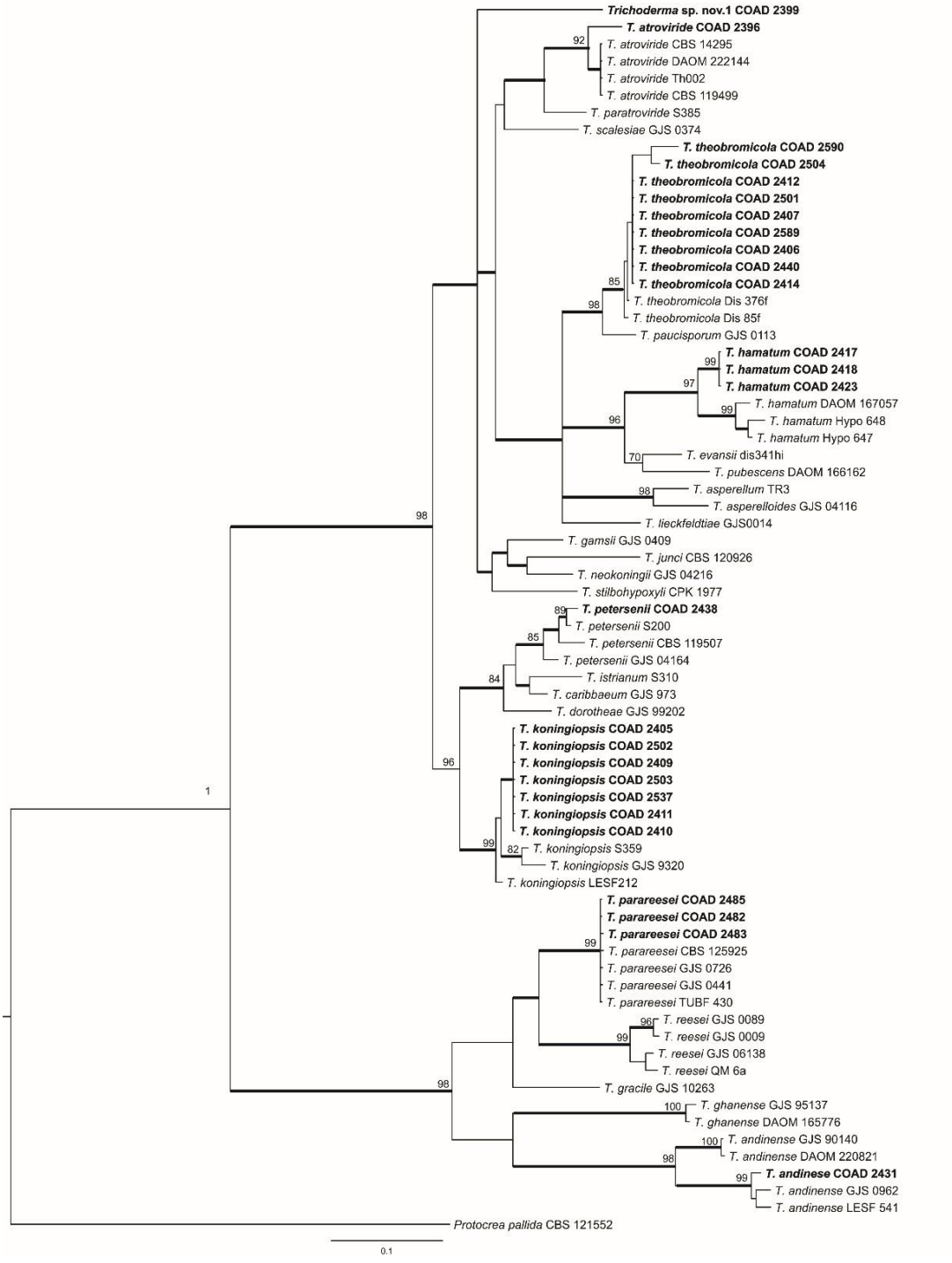
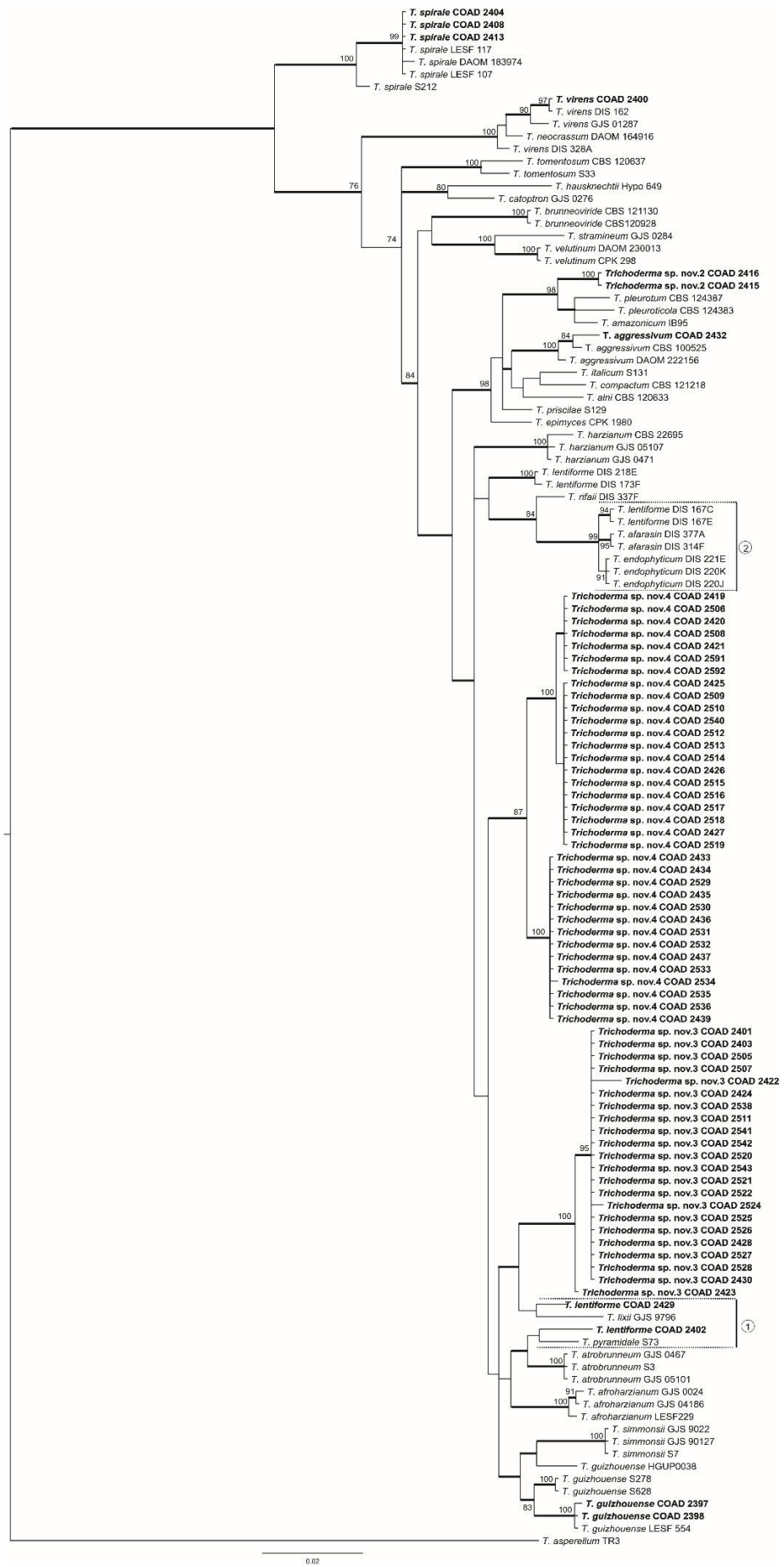


Figure 5. Bayesian phylogenetic tree (E). The tree was based on *tef1* sequence dataset. Bootstrap values (≥70%) of the ML analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.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T. catoptron GJS 0276
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T. italicum S131
T. compactum CBS 121218
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T. lentiforme DIS 173F
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T. asperellum TR3
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Figure 6. Bayesian phylogenetic tree (D). The tree was based on *rpb2* sequence dataset. Bootstrap values (≥70%) of the ML analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.

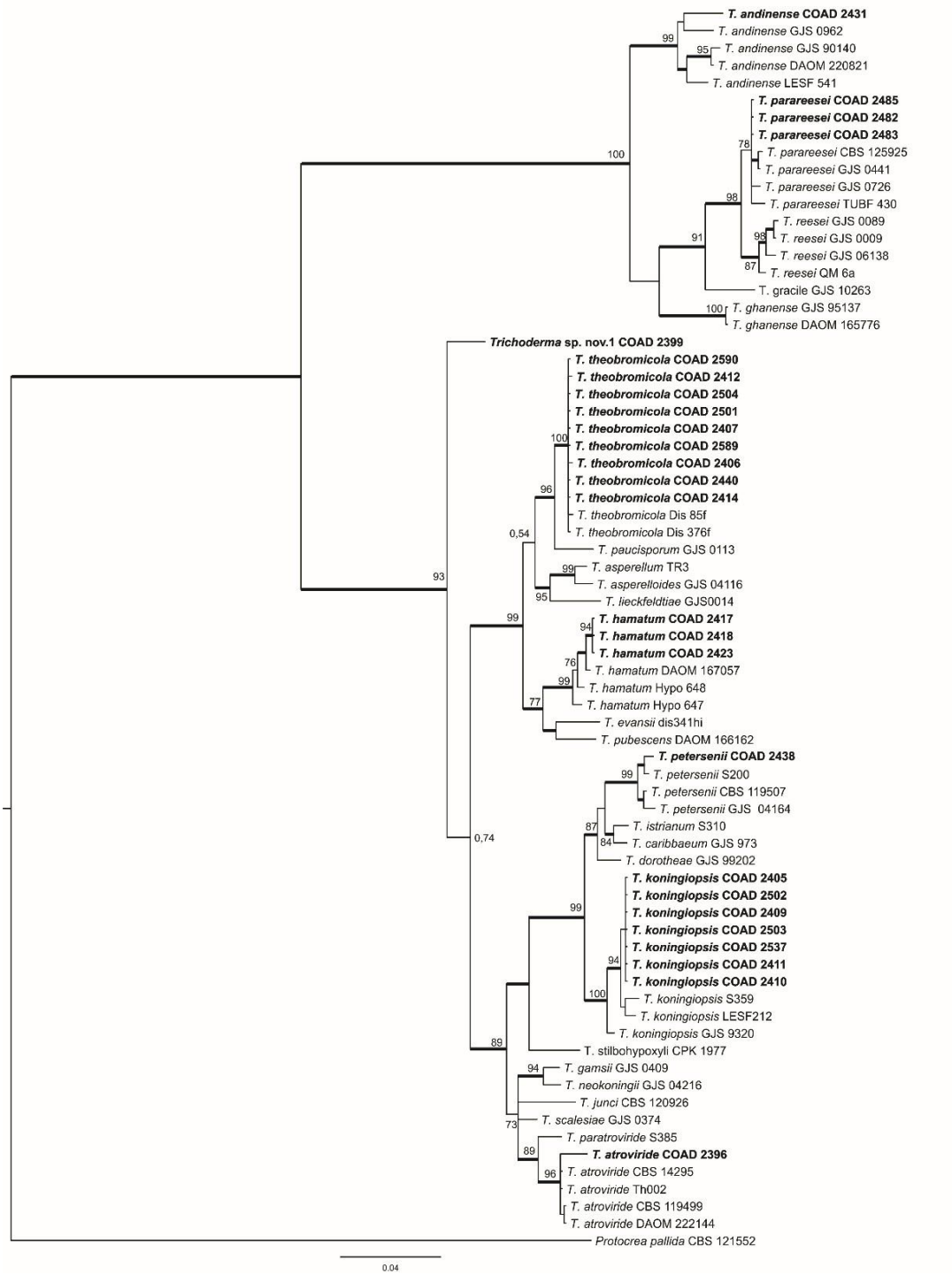

Figure 7. Bayesian phylogenetic tree (D). The tree was based on *rpb2* sequence dataset. Bootstrap values (≥70%) of the ML analyses, as well as posterior probability scores (≥0.9) from a Bayesian analysis of the same dataset, are indicated at well supported nodes together with thickened branches. In bold the isolates obtained in this study.

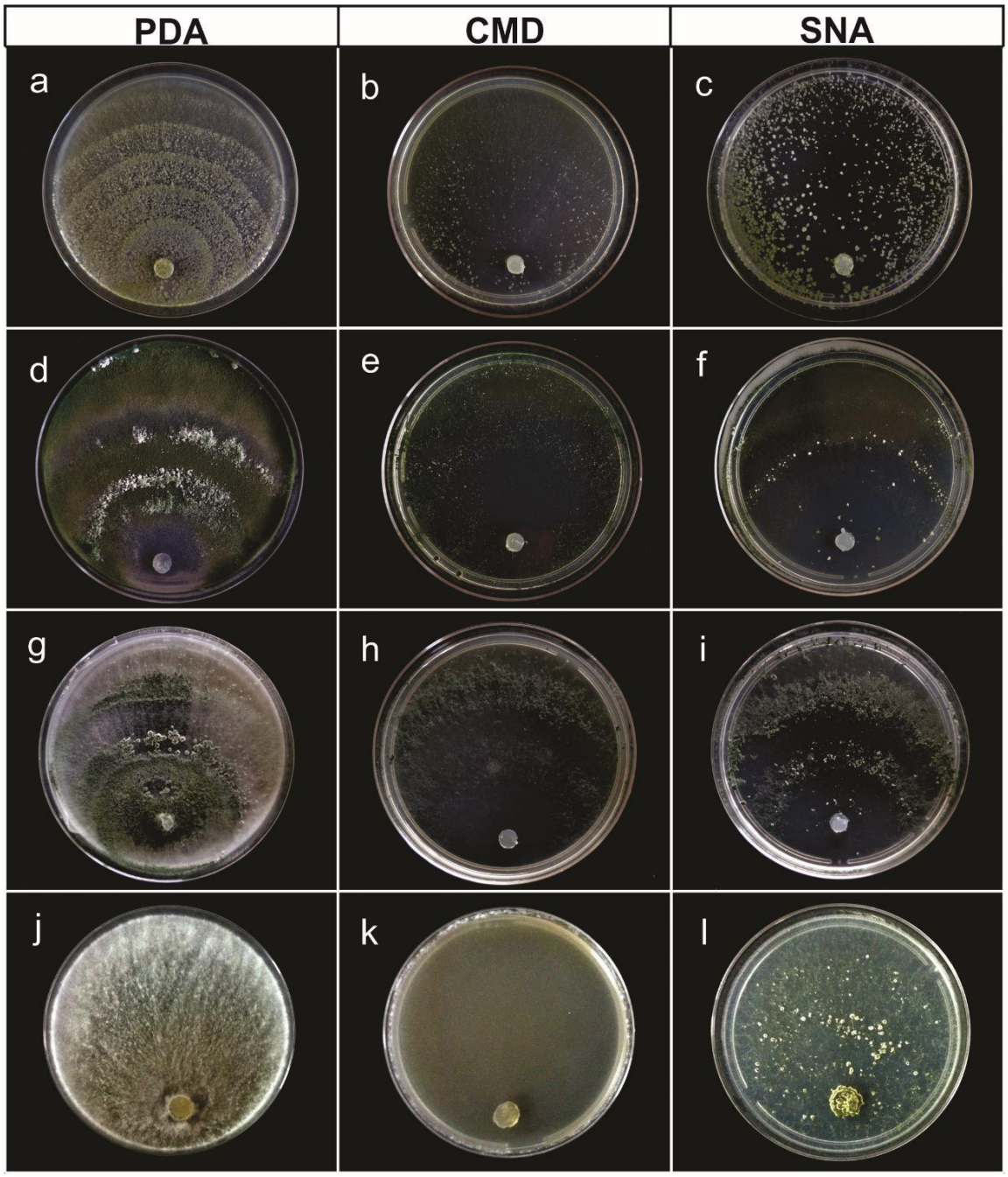

Figure 8. Colony photographs of the new *Trichoderma* species on PDA, CMD and SNA. All colonies incubated at 25°C under a 12h day/night lighting regime and photographed on day seven. (a – c) *Trichoderma* sp. nov.1; (d –f) *Trichoderma* sp. nov.2; (g –i) *Trichoderma* sp. nov.3; (j –l) *Trichoderma* sp. nov.4.

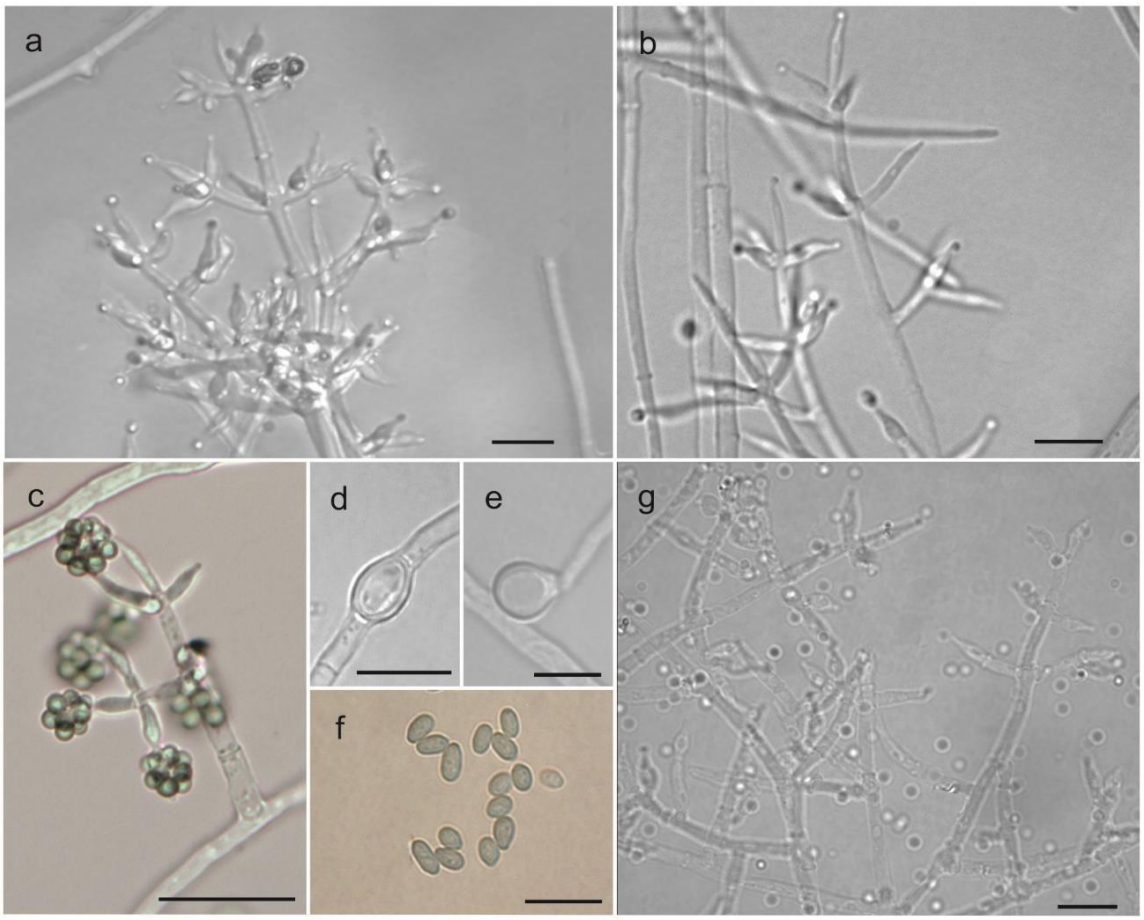

Figure 9. Morphological features characteristic of *Trichoderma* sp.nov.1 (COAD 2399). a, b, c, g conidiophores and phialides formed on SNA and CMD. d, e clamydospores on CMD. f conidia. Bars: a, b, d, e, f =10µm; c, g =20µm.

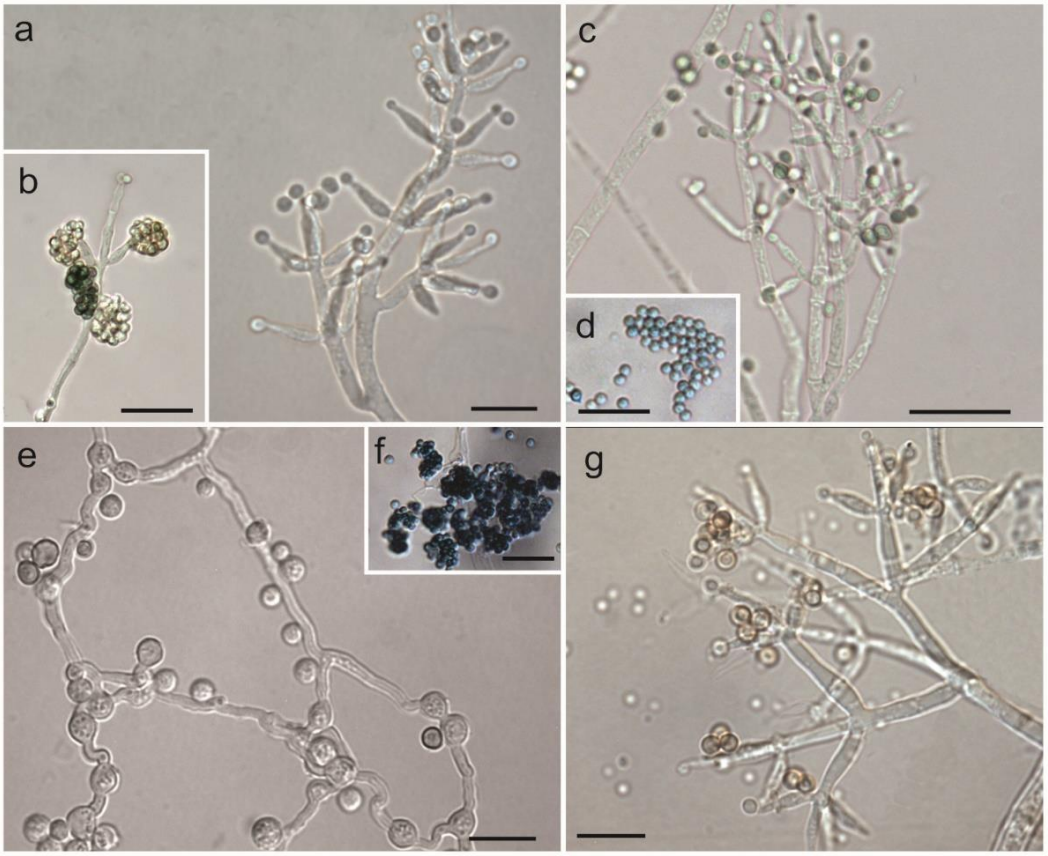

Figure 10. Morphological features characteristic of *Trichoderma* sp. nov.2 (COAD 2416). a, b, c, g conidiophores and phialides formed on SNA and CMD. e clamydospores on CMD. f, d blue conidia. Bars: a, e, g =10µm; b, c, d, f =20µm.

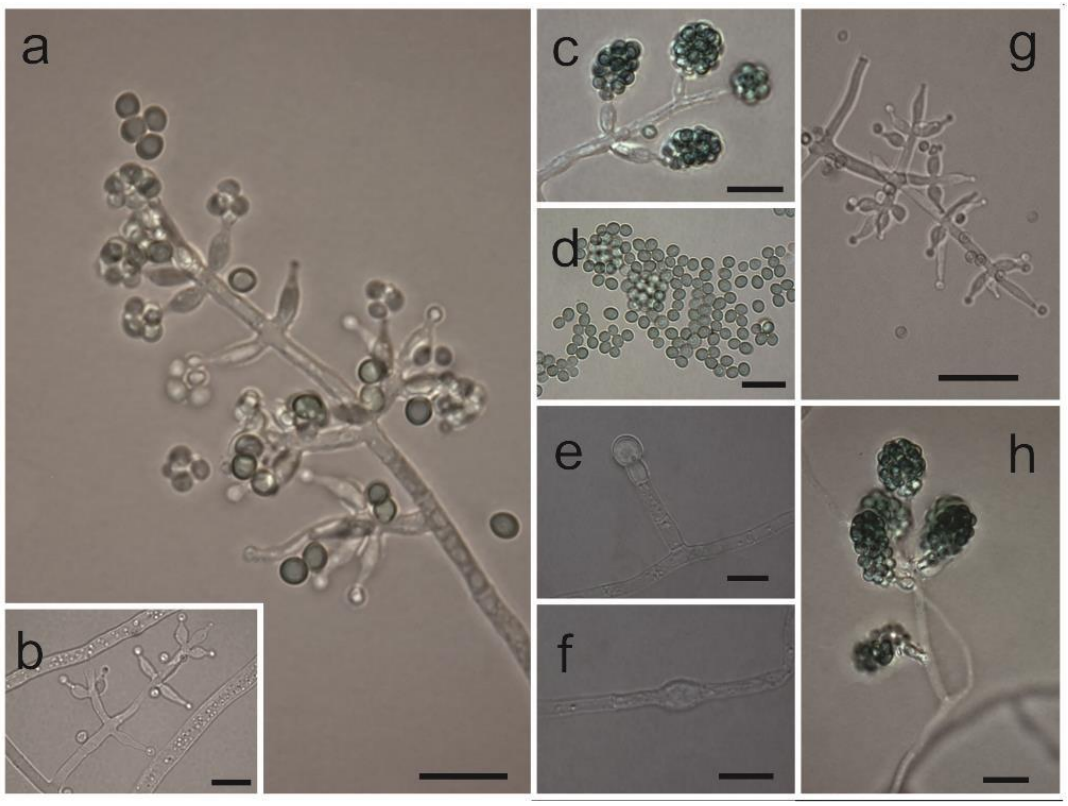

Figure 11. Morphological features characteristic of *Trichoderma* sp. nov.3 (COAD 2422). a, b, g, conidiophores and phialides formed on SNA. c, h conidia grouped in bunches. e, f clamydospores on CMD. d conidia. Bars: a, b, c, d, e, f, h =10µm; g =20µm.

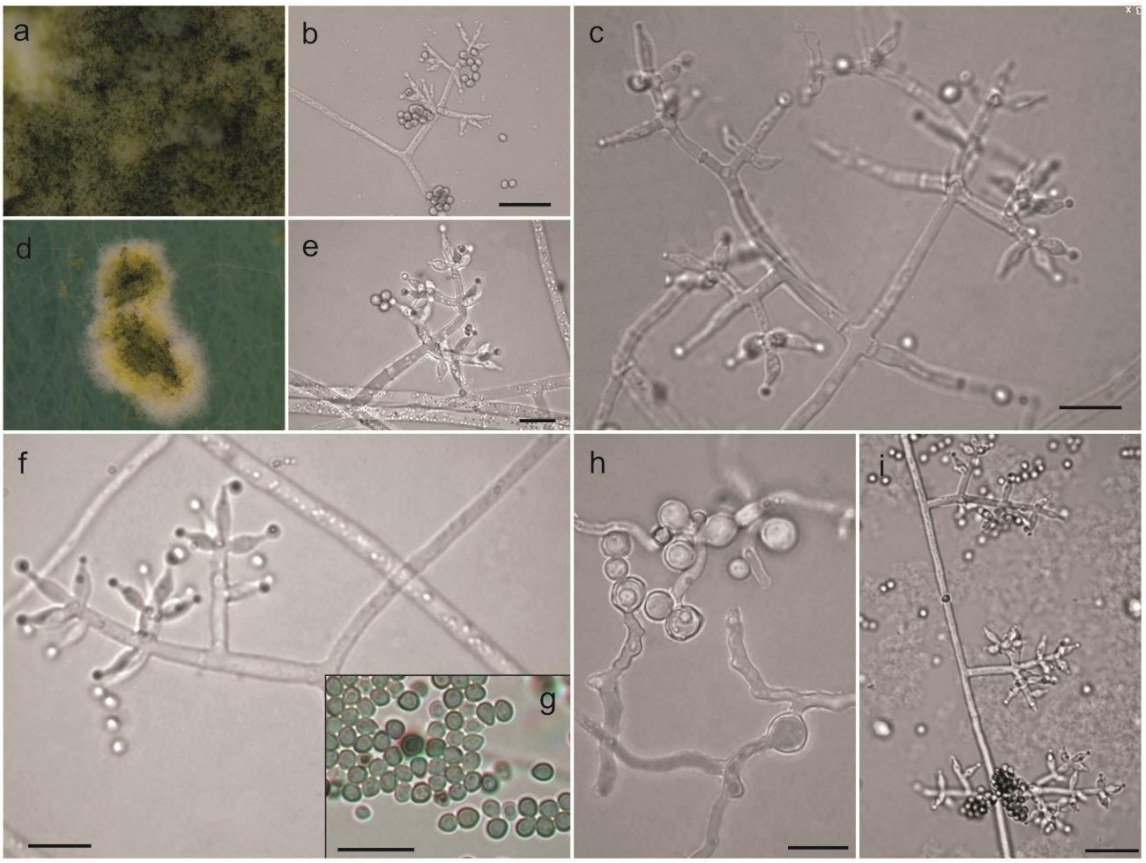

Figure 12.Morphological features characteristic of *Trichoderma* sp. nov.4 (COAD 2426). a, d stereo microscope images from SNA. b, c, e, f, i conidiophores and phialides formed on SNA. h clamydospores on CMD. g conidia. Bars: e, c, f, h =10µm; b, i =20µm.

SUPPLEMENTARY

Table 1.*Trichoderma* strains used in the phylogenetic analyses, with their corresponding geographic origin and host.

				Genbank accession numbers		
Taxon	**Isolate**	**Country**	**Substrate**	***tef***	***rpb2***	***cal***
Trichoderma parareesei	COAD 2485	Ethiopia	*Hemileia sp.* Mycoparasite	MK044082	MK044265	—
Trichoderma atroviride	COAD 2396	Kenya	leaf, *Coffea sp.* Endophyte	MK044083	MK044177	—
Trichoderma guizhouense	COAD 2397	Kenya	stem, *Coffea sp* Endophyte	MK044084	MK044176	—
Trichoderma guizhouense	COAD 2398	Kenya	stem, *Coffea sp.* Endophyte	MK044085	MK044178	—
Trichoderma sp. nov.1	COAD 2399	Kenya	stem, *Coffea sp*. Endophyte	MK044086	MK044179	—
Trichoderma virens	COAD 2400	Cameroon	stem, *Coffea brevipes* Endophyte	MK044087	MK044180	—
Trichoderma sp. nov.3	COAD 2401	Cameroon	stem, *Coffea canephora* Endophyte	MK044088	MK044181	—
Trichoderma lentiforme	COAD 2402	Cameroon	stem, *Coffea canephora* Endophyte	MK044089	MK044182	—
Trichoderma sp. nov.3	COAD 2403	Cameroon	stem, *Coffea arabica* Endophyte	MK044090	MK044183	—
Trichoderma spirale	COAD 2404	Cameroon	stem, *Coffea canephora* Endophyte	MK044091	MK044184	—
Trichoderma koningiopsis	COAD 2405	Cameroon	leaf, *Coffea canephora* Endophyte	MK044092	MK044185	—
Trichoderma theobromicola	COAD 2406	Cameroon	stem, *Coffea canephora* Endophyte	MK044093	MK044186	—
Trichoderma theobromicola	COAD 2407	Cameroon	stem, *Coffea canephora* Endophyte	MK044094	MK044187	—
Trichoderma theobromicola	COAD 2501	Cameroon	stem, *Coffea canephora* Endophyte	MK044095	MK044188	—
Trichoderma spirale	COAD 2408	Cameroon	stem, *Coffea canephora* Endophyte	MK044096	MK044189	—
Trichoderma koningiopsis	COAD 2502	Cameroon	leaf, *Coffea canephora* Endophyte	MK044097	MK044190	—
Trichoderma koningiopsis	COAD 2537	Cameroon	leaf, *Coffeea canephora* Endophyte	MK044098	MK044191	—
Trichoderma koningiopsis	COAD 2409	Cameroon	stem, *Coffea canephora* Endophyte	MK044099	MK044192	—
Trichoderma koningiopsis	COAD 2503	Cameroon	leaf, *Coffea canephora* Endophyte	MK044100	MK044193	—
Trichoderma koningiopsis	COAD 2410	Cameroon	leaf, *Coffea canephora* Endophyte	MK044101	MK044194	—
Trichoderma koningiopsis	COAD 2411	Cameroon	leaf, *Coffea canephora* Endophyte	MK044102	MK044195	—
Trichoderma theobromicola	COAD 2504	Cameroon	stem, *Coffea canephora* Endophyte	MK044103	MK044196	—

Trichoderma theobromicola	COAD 2412	Cameroon	stem, *Coffea canephora* Endophyte	MK044104	MK044197	—
Trichoderma spirale	COAD 2413	Cameroon	stem, *Coffea canephora* Endophyte	MK044105	MK044198	—
Trichoderma theobromicola	COAD 2440	Cameroon	stem, *Coffea canephora* Endophyte	MK044106	MK044199	—
Trichoderma theobromicola	COAD 2414	Cameroon	stem, *Coffea canephora* Endophyte	MK044107	MK044200	—
Trichoderma sp. nov.2	COAD 2416	Ethiopia	berry, *Coffea arabica* Endophyte	MK044108	MK044201	—
Trichoderma sp. nov.2	COAD 2415	Ethiopia	berry, *Coffea arabica* Endophyte	MK044109	MK044202	—
Trichoderma hamatum	COAD 2417	Ethiopia	stem, *Coffea arabica* Endophyte	MK044110	MK044203	—
Trichoderma hamatum	COAD 2418	Ethiopia	stem, *Coffea arabica* Endophyte	MK044111	MK044204	—
Trichoderma sp. nov.3	COAD 2505	Ethiopia	stem, *Coffea arabica* Endophyte	MK044112	MK044205	
Trichoderma sp.nov.4	COAD 2419	Ethiopia	stem, *Coffea arabica* Endophyte	MK044113	MK044206	MK084875
Trichoderma sp.nov.4	COAD 2506	Ethiopia	stem, *Coffea arabica* Endophyte	MK044114	MK044207	—
Trichoderma sp.nov.4	COAD 2420	Ethiopia	stem, *Coffea arabica* Endophyte	MK044115	MK044208	MK084874
Trichoderma sp. nov.3	COAD 2507	Ethiopia	berry, *Coffea arabica* Endophyte	MK044116	MK044209	—
Trichoderma sp.nov.4	COAD 2508	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044117	MK044210	—
Trichoderma sp.nov.4	COAD 2421	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044118	MK044211	MK084873
Trichoderma sp. nov.3	COAD 2422	Ethiopia	berry, *Coffea arabica* Endophyte	MK044119	MK044212	—
Trichoderma hamatum	COAD 2423	Ethiopia	berry, *Coffea arabica* Endophyte	MK044120	MK044213	—
Trichoderma sp. nov.3	COAD 2424	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044121	MK044214	—
Trichoderma sp. nov.3	COAD 2538	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044122	MK044215	—
Trichoderma sp.nov.4	COAD 2425	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044123	MK044216	MK084871
Trichoderma sp.nov.4	COAD 2509	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044124	MK044217	—
Trichoderma sp.nov.4	COAD 2510	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044125	MK044218	—
Trichoderma sp. nov.3	COAD 2511	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044126	MK044219	—
Trichoderma sp.nov.4	COAD 2540	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044127	MK044220	—
Trichoderma sp.nov.4	COAD 2512	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044128	MK044221	—
Trichoderma sp.nov.4	COAD 2513	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044129	MK044222	—
Trichoderma sp.nov.4	COAD 2514	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044130	MK044223	—
Trichoderma sp.nov.4	COAD 2426	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044131	MK044224	MK084870

Trichoderma sp.nov.4	COAD 2515	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044132	MK044225	—
Trichoderma sp.nov.4	COAD 2516	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044133	MK044226	—
Trichoderma sp.nov.4	COAD 2517	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044134	MK044227	—
Trichoderma sp.nov.4	COAD 2518	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044135	MK044228	—
Trichoderma sp.nov.4	COAD 2427	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044136	MK044229	MK084872
Trichoderma sp.nov.4	COAD 2519	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044137	MK044230	—
Trichoderma sp. nov.3	COAD 2541	Ethiopia	stem, *Coffea arabica* Endophyte	MK044138	MK044231	—
Trichoderma sp. nov.3	COAD 2542	Ethiopia	stem, *Coffea arabica* Endophyte	MK044139	MK044232	—
Trichoderma sp. nov.3	COAD 2520	Ethiopia	stem, *Coffea arabica* Endophyte	MK044140	MK044233	—
Trichoderma sp. nov.3	COAD 2543	Ethiopia	stem, *Coffea arabica* Endophyte	MK044141	MK044234	—
Trichoderma sp. nov.3	COAD 2521	Ethiopia	stem, *Coffea arabica* Endophyte	MK044142	MK044235	—
Trichoderma sp. nov.3	COAD 2522	Ethiopia	stem, *Coffea arabica* Endophyte	MK044143	MK044236	—
Trichoderma sp. nov.3	COAD 2423	Ethiopia	stem, *Coffea arabica* Endophyte	MK044144	MK044237	—
Trichoderma sp. nov.3	COAD 2524	Ethiopia	stem, *Coffea arabica* Endophyte	MK044145	MK044238	—
Trichoderma sp. nov.3	COAD 2525	Ethiopia	stem, *Coffea arabica* Endophyte	MK044146	MK044239	—
Trichoderma sp. nov.3	COAD 2526	Ethiopia	stem, *Coffea arabica* Endophyte	MK044147	MK044240	—
Trichoderma sp. nov.3	COAD 2428	Ethiopia	berry, *Coffea arabica* Endophyte	MK044148	MK044241	—
Trichoderma sp. nov.3	COAD 2527	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044149	MK044242	—
Trichoderma lentiforme	COAD 2429	Ethiopia	berry, *Coffea arabica* Endophyte	MK044150	MK044243	—
Trichoderma sp. nov.3	COAD 2528	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044151	MK044244	—
Trichoderma sp. nov.3	COAD 2430	Ethiopia	leaf, *Coffea arabica* Endophyte	MK044152	MK044245	—
Trichoderma parareesei	COAD 2482	Ethiopia	stem, *Coffea arabica*Endophyte	MK044153	MK044246	—
Trichoderma parareesei	COAD 2483	Ethiopia	stem, *Coffea arabica* Endophyte	MK044154	MK044247	—
Trichoderma andinense	COAD 2431	Brazil	*Hemileia vastatrix*, Mycoparasite	MK044155	MK044248	—
Trichoderma aggressivum	COAD 2432	Kenya	*Hemileia sp*. Mycoparasite	MK044156	MK044249	—
Trichoderma sp.nov.4	COAD 2433	Cameroon	*Hemileia sp*. Mycoparasite	MK044157	MK044250	MK084869
Trichoderma sp.nov.4	COAD 2434	Ethiopia	*Hemileia sp*. Mycoparasite	MK044158	MK044251	MK084868
Trichoderma sp.nov.4	COAD 2529	Ethiopia	*Hemileia sp.* Mycoparasite	MK044159	MK044252	—

Trichoderma sp.nov.4	COAD 2435	Ethiopia	*Hemileia sp*. Mycoparasite	MK044160	MK044253	MK084867
Trichoderma sp.nov.4	COAD 2530	Ethiopia	*Hemileia sp*. Mycoparasite	MK044161	MK044254	—
Trichoderma sp.nov.4	COAD 2436	Ethiopia	*Hemileia sp*. Mycoparasite	MK044162	MK044255	MK084865
Trichoderma sp.nov.4	COAD 2531	Ethiopia	*Hemileia sp*. Mycoparasite	MK044163	MK044256	—
Trichoderma sp.nov.4	COAD 2532	Ethiopia	*Hemileia sp*. Mycoparasite	MK044164	MK044257	—
Trichoderma sp.nov.4	COAD 2437	Ethiopia	*Hemileia sp*. Mycoparasite	MK044165	MK044258	MK084866
Trichoderma sp.nov.4	COAD 2533	Ethiopia	*Hemileia sp*. Mycoparasite	MK044166	MK044259	—
Trichoderma sp.nov.4	COAD 2534	Ethiopia	*Hemileia sp*. Mycoparasite	MK044167	MK044260	—
Trichoderma petersenii	COAD 2438	Ethiopia	*Hemileia sp*. Mycoparasite	MK044168	MK044261	—
Trichoderma sp.nov.4	COAD 2535	Ethiopia	*Hemileia sp*. Mycoparasite	MK044169	MK044262	—
Trichoderma sp.nov.4	COAD 2536	Ethiopia	*Hemileia sp*. Mycoparasite	MK044170	MK044263	—
Trichoderma sp.nov.4	COAD 2439	Ethiopia	*Hemileia sp*. Mycoparasite	MK044171	MK044264	MK084864
Trichoderma theobromicola	COAD 2589	Cameroon	Stem, *Coffea canephora* Endophyte	MK044172	MK044266	—
Trichoderma theobromicola	COAD 2590	Cameroon	Stem, *Coffea canephora* Endophyte	MK044173	MK044267	—
Trichoderma sp.nov.4	COAD 2591	Ethiopia	Stem, *Coffea arabica* L. Endophyte	MK044174	MK044268	—
Trichoderma sp.nov.4	COAD 2592	Ethiopia	Stem, *Coffea arabica* L. Endophyte	MK044175	MK044269	—

Table 2. *Trichoderma* from NCBI GenBank accessions used in the phylogenetic analyses

			GenBank accessions		
Taxon	**Strain**	**Country**	***tef1***	***rpb2***	***cal***
T. afarasin	Dis 377A	—	FJ463322	FJ442799	
T. afarasin	DIS 314F	—	FJ463400	FJ442778	—
T. afroharzianum	**CBS 124620 = G.J.S. 04-186**	**Peru**	**FJ463301**	**FJ442691**	**FJ442370**
T. afroharzianum	G.J.S. 00-24	México	AF443940	FJ442726	AF442880
T. afroharzianum	LESF229	Brazil	KT279013	KT278945	—
T. aggressivum	DAOM 222156	Canada, Ontario	AF348098	FJ442752	—
T. aggressivum	**CBS 100526 (T), CBS 100525; CBS 100525**	**Ireland; UK: England**	**AF348096**	**AF545541**	—
T. alni	Hypo 254 = CBS 120633 (T)	UK: England	EU498312	EU498349	—
T. amazonicum	IB 95	Peru	HM142377	HM142368	—
T. andinense	DAOM 220821	Venezuela	EU280042	KJ842208	—
T. andinense	GJS 09-62	Peru	JN133570	JN175533	—
T. andinense	LESF541	Brazil	KT279037	KT278979	—
T. andinense	**G.J.S. 90-140 = CBS 354.97 = ATCC 208857 (T)**	**Venezuela**	**AY956321**	**JN175531**	—
T. asperelloides	G.J.S. 04-116	Vietnam	GU248412	GU248411	—
T. asperellum	**CBS 433.97 = TR3 (T)**	**USA**	**AF456907**	**EU248617**	—
T. atrobrunneum	S3	Italy	KJ665376	KJ665241	—
T. atrobrunneum	CBS 130440= G.J.S. 04-67	Italy	FJ463360	FJ442724	FJ442329
T. atrobrunneum	GJS 05-101	—	FJ463392	FJ442745	FJ442331
T. atrobrunneum	CBS 548.92= G.J.S. 92-110	France	—	—	AF442883
T. atroviride	CBS 119499	Austria	FJ860611	FJ860518	—

T. atroviride	DAOM 222144	Canada	AF456889	FJ442754	—
T. atroviride	**CBS 142.95**	**Slovenia**	**AY376051**	**EU341801**	—
T. atroviride	Th002	Colombia	AB558906	AB558915	—
T. brunneoviride	**Hypo 170 = CBS 121130; Hypo 442 = CBS 120928**	**—**	**EU498316**	**EU498358**	—
T. camerunense	GJS 99-230	—	AF348107	—	—
T. camerunense	GJS 99-231	—	AF348108	—	—
T. caribbaeum	CBS 119093 = G.J.S. 97-3 (T)	Guadeloupe	KJ665443	KJ665246	—
T. catoptron	G.J.S. 02-76 = CBS 114232 (T)	Sri Lanka	AY737726 + AY391963	AY391900	—
T. compactum	CBS 121218	—	KF134798	KF134789	—
T. dorotheae	G.J.S. 99-202 (T)	New Zealand	DQ307536	EU248602	—
T. endophyticum	CBS 130733 =Dis 220j	Ecuador	FJ463330	FJ442690	—
T. endophyticum	Dis 220k	Ecuador	FJ463328	FJ442765	—
T. endophyticum	Dis 221e	Ecuador	FJ463316	FJ442775	—
T. epimyces	CPK 1980	—	EU498319	EU498359	—
T. evansii	DIS341hi = CBS 123079 (T)	Ecuador	EU883566	EU883558	—
T. gamsii	G.J.S. 04-09	USA	DQ307541	JN133561	—
T. ghanense	**G.J.S. 95-137 = IAM 13109 (T)**	**Ghana**	**AY937423**	**JN175559**	—
T. ghanense	DAOM 165776	USA	JN175610	JN175560	—
T. gracile	CBS 130714 = G.J.S. 10-263 (T)	Malaysia	JN175598	JN175547	—
T. guizhouense	S278	Croatia	KF134799	KF134791	—
T. guizhouense	LESF554	Brazil	KT279017	KT278952	—
T. guizhouense	S628	Greece	KJ665511	KJ665273	—
T. guizhouense	**HGUP 0038 = CBS 131803**	**China**	**JN215484**	**JQ901400**	—
T. hamatum	**DAOM 167057 (T)**	**Canada**	**EU279965**	**AF545548**	—

T. hamatum	Hypo 647	France	KJ665513	KJ665274	—
T. hamatum	Hypo 648 = CBS 132565	France	KJ665514	KJ665275	—
T. harzianum	GJS 04-71	—	FJ463396	FJ442779	—
T. harzianum	GJS 05-107	—	FJ463329	FJ442708	—
T. harzianum	CBS 227.95	U.K.	AF348100	—	—
T. harzianum	**CBS 226.95**	**U.K.**	**AF348101**	**AF545549**	—
T. hausknechtii	Hypo 649 = CBS 133493 (T)	France	KJ665515	KJ665276	—
T. istrianum	S310 = CBS 130539 (T)	Croatia	KJ665523	KJ665281	—
T. italicum	S131 = CBS 132567 (T)	Italy	KJ665525	KJ665282	—
T. junci	CBS 120926 = Hypo 399 (T)	Denmark	FJ860641	FJ860540	—
T. koningiopsis	S359	France	KJ665546	KJ665285	—
T. koningiopsis	LESF212	Austin - Texas, USA	KT278985	KT278914	—
T. koningiopsis	**G.J.S. 93-20 (T)**	**Cuba**	**DQ284966**	EU241506	—
T. lentiforme	Dis 173f	Brazil	FJ463347	FJ442787	—
T. lentiforme	Dis 167e	Brazil	FJ463333	FJ442764	FJ442366
T. lentiforme	Dis 167C	—	FJ463309	FJ442689	FJ442365
T. lentiforme	Dis 218e	Ecuador	FJ463310	FJ442793	—
T. lieckfeldtiae	G.J.S. 00-14 = CBS 123049 (T)	Colombia	EU856326	EU883562	—
T. lixii	C.P.K. 1724= G.J.S. 05-32	Cameroon	EF191328	—	—
T. lixii	C.P.K. 1720 =G.J.S. 05-82	Cameroon	EF191326	—	—
T. lixii	**G.J.S. 97-96 = CBS 110080 = C.P.K. 2784 (T epi)**	**Thailand**	**FJ716622**	**KJ665290**	—
T. neocrassum	**DAOM 164916 = CBS 336.93 = C.P.K. 63 (T ana)**	**Canada**	**EU280048**	**AF545542**	—
T. neokoningii	G.J.S. 04-216 = CBS 120070 (T)	Peru	KJ665620	KJ665318	—
T. parareesei	**CBS 125925, TUB F-1066**	**Mexico**	**GQ354353**	**HM182963**	—

T. parareesei	G.J.S. 07-26	Ghana	GQ354373	HM182966	—
T. parareesei	G.J.S. 04-41	Brazil	GQ354372	HM182964	—
T. parareesei	TUB F-430	Sri Lanka	GQ354351	HM182968	—
T. paratroviride	S385 = CBS 136489 (T)	Spain	KJ665627	KJ665321	—
T. paucisporum	G.J.S. 01-13 = CBS 118645 (T)	Ecuador	DQ109540	FJ150787	—
T. petersenii	S200	Portugal	KJ665636	KJ665327	—
T. petersenii	**CBS 119507 = Hypo 45**	**Austria**	**FJ860670**	**FJ860568**	—
T. petersenii	**G.J.S. 04-164**	**USA**	**DQ289004**	**FJ442783**	—
T. pleuroticola	**CBS 124383 (T)**	**Korea**	**HM142381**	**HM142371**	—
T. pleuroticola	T1295	—	EU279973	—	—
T. pleurotum	CBS 124387 (T)	Korea	HM142382	HM142372	—
T. priscilae	S129	Italy	KJ665689	KJ665332	—
T. pubescens	DAOM 166162 (T)	USA	AY750887	EU248613	—
T. pyramidale	S119	Italy	KJ665696	—	—
T. pyramidale	S573	Italy	KJ665698	—	—
T. pyramidale	**S73 = CBS 135574 (T)**	**Italy**	**KJ665699**	**KJ665334**	—
T. pyramidale	S533	Spain	KJ665697	—	—
T. reesei	G.J.S. 00-89	Brazil	JN175599	JN175548	—
T. reesei	G.J.S. 00-09	Mexico	JN175600	JN175549	—
T. reesei	G.J.S. 06-138	Cameroon	GQ354370	HM182972	—
T. reesei	**QM 6a**	**Solomon Islands**	**Z23012**	**HM182969**	—
T. rifaii	Dis 337F	—	FJ463321	FJ442720	—
T. rifaii	DIS 355B	—	FJ463324	—	—
T. scalesiae	G.J.S. 03-74 (T)	Ecuador	DQ841726	EU252007	—
T. simmonsii	S7	Italy	KJ665719	KJ665337	—
T. simmonsii	CBS 123765= G.J.S. 90-127	USA, North Carolina	AF443936	FJ442798	—

T. simmonsii	CBS 123799= IMI 393966= G.J.S. 90-22	USA, Wisconsin	AF443933	AY391925	—
T. spirale	S212	Spain	KJ665740	KJ665348	—
T. spirale	LESF117	Brazil	KT279024	KT278958	—
T. spirale	LESF107	Brazil	KT279022	KT278956	—
T. spirale	**DAOM 183974; DIS 311D**	**—**	**EU280049**	**FJ442694**	—
T. stilbohypoxyli	Hypo 256 = C.P.K. 1977	UK: England	FJ860702	FJ860592	—
T. stramineum	G.J.S. 02-84		AY737746	AY391945	—
T. theobromicola	**DIS 85f (T)**	**Peru**	**EU856321**	**FJ007374**	—
T. tomentosum	CBS 120637 = C.P.K. 2498	Austria	FJ860629	FJ860532	—
T. tomentosum	S33	—	KF134801	KF134793	—
T. velutinum	**DAOM 230013; C.P.K. 298**	**—**	**AY937415**	**KF134794**	—
T. virens	Dis 328A	Ecuador	FJ463363	FJ442738	—
T. virens	Dis 162	Costa Rica	FJ463367	FJ442696	—
T. virens	CBS 123790 = G.J.S. 01-287	Ivory Coast	AY750894	EU341804	—
Protocrea pallida	CBS 121552 = Hypo 376 CBS 299.78 (T)	Denmark	EU703900	EU703944	—

Table 3. Number of taxa collected in this survey per country-source

Species	Ethiopia				Cameroon				Kenya			
	Leaf	Stem	Berry	Mycoparasite	Leaf	Stem	Berry	Mycoparasite	Leaf	Stem	Berry	Mycoparasite
Trichoderma aggressivum												1
Trichoderma atroviride									1			
Trichoderma sp. nov.3	6	11	3			2						
Trichoderma sp. nov.2			2									
Trichoderma guizhouense										2		
Trichoderma hamatum		2	1									
Trichoderma koningiopsis					6	1						
Trichoderma lentiforme			1			1						
Trichoderma sp. nov.1										1		
Trichoderma parareesei		2		1								
Trichoderma petersenii				1								
Trichodema sp.nov.4	16	5		13				1				
Trichoderma spirale						3						
Trichoderma theobromicola						9						
Trichoderma virens						1						

Chapter 2

Screening endophytic fungi of *Coffea* spp. from Africa for potential antagonists of coffee leaf rust

Abstract

Coffee leaf rust (CLR) (*Hemileia vastatrix*) is the main coffee disease since the historical outbreaks in Sri Lanka in mid 19th century. It still represents a major challenge for coffee production, despite the management tools available such as the use resistant varieties, escaping the disease through highland plantation of coffee and spraying with contact or systemic fungicides. Although biological control has been investigated to some extent, this was mainly limited to the use of antagonists present outside the native range of *Coffea* and *H. vastatrix.* Classical biological control with natural enemies from Africa is a novel approach which has been investigated since 2015. Here a screening of selected endophytic fungi obtained from coffee tissues in Africa was conducted. Only sporulating fungi belonging to selected taxa (particularly members of *Trichoderma*) were investigated. One hundred and thirty isolates were tested against *H. vastatrix in vitro.* Four of these were brought to the next stage of *in planta* testing. Seventeen isolates inhibitted urenidiospore germination. E526, E816, COAD 2417, COAD 2535 and COAD 2439 reduced urediniospore germination in 80% or more, as compared with controls. Isolates COAD 2396, E48 and E486 reduced CLR severity above 50% on detached coffee leaf disc treated with conidial suspensions 72h, 24h before or simultaneously with *H. vastatrix.* Isolates E16, COAD 2403, E215, E400, COAD 2410, E462 and COAD 2417 showed a reduction of more than 25% in two time intervals (24 and 72h before the inoculation of *H. vastatrix*). Isolates COAD 2396, E48, E400 and COAD 2410 were brought forward and tested for their ability to reduce CLR severity in young coffee plants. Most of the isolates had no effect at reducing CLR severity in plants. Nevertheless, E48 produced a significant reduction of CLR severity (60% reduction as compared to control) when applied 72h before of pathogen inoculation. The ability of some of the isolates to establish endophytic colonies in coffee was tested. All the isolates tested were recovered from stems 60 days after inoculation Establishment of endophytic fungi on leaves was less effective. COAD 2396, COAD 2482, COAD 2502 and COAD 2592 were recovered from leaves with percentage of 1.6-35%. Although a modified and repeated broader scale experiment is regarded to be necessary, the present results of the screening indicate that some legitimate _bodyguard-

endophytic fungi‘ are present in the assemblage and may be useful to be deployed as classical biocontrol agents against CLR.

Keywords: Biological control; colonization; disease severity; *Hemileia vastatrix,* mycoparasites; *Trichoderma*.

Introduction

Coffee leaf rust (CLR), caused by *Hemileia vastatrix* is the main coffee disease since the historical outbreaks in Sri Lanka in mid 19th century and occurs in virtually all regions where arabica (*Coffea arabica*) and conilon (*Coffea canephora*) coffee are grown (Avelino et al. 2015; CJ 1990; Zambolim 2016).

During the period 2008-2013 a great epidemic of CLR occurred in Central America, Mexico, Colombia, Peru and Ecuador endangering a part of the economy of entire countries and the livelihood of their population which depended on this crop (Avelino et al. 2015). There was a great social crisis that contributed in part to the ongoing caravans of refugees who marched to the USA / Mexico border as has been widely covered by the media throughout 2018 (e.g. The Guardian, 2018). A combination of factors led to this ongoing crisis including agronomic, climatic and economic factors (Talhinas et al. 2017).

The use of copper fungicides as well as systemic fungicides such as triazoles and strobilurins and the use of resistant varieties are the most effective strategy for the management of the disease (Talhinhas et al. 2017; Zambolim 2016). However, although different varieties have been developed from resistant genetic stock, few have been shown to have a durable and broad resistant to coffee rust (Zambolim 2016). There are also increasing market and regulation restrictions for the use of fungicides (Carvalho et al. 2002). This scenario stimulates the search for alternative methods of control of CLR, one of which is biological control.

One of the most important and traditional approaches in biological control is classical biological control. This involves the importation of natural enemies of a pest or pathogen from the native range and release into the areas, countries or continents where – free from their co-evolved natural enemies – such species became a noxious invasive pest, weed or pathogen causing economic or ecological harm (Wapshere et al. 1989). This approach has resulted in examples of great success on the control of insect pests and weeds offering several advantages over other control methods (Scott 1995). Nevetheless there are few examples of classical biological control being used against plant pathogens.

Among the natural enemies of fungi there are mycovirus, bacteria, fungi and other groups of organisms (Xie and Jiang 2014), but possibly the most important

antagonists to fungi are other fungal species, namely mycoparasites (Barnett 1963) and endophytic fungi .The latter have been demonstrated to produce benefits for their host plants by protecting them against herbivores or pathogens, serving as ‒bodyguards‖ to the plant (Haddad et al. 2014a, 2009; Khare et al. 2018; Silva et al. 2012).

Biological control of *H. vastatrix* has been previously investigated. Such studies did not follow the classical approach but were concentrated on the utilization of antagonistic bacteria (Haddad et al. 2014a; Shiomi et al. 2006; Silva et al. 2012) and fungi (e.g. González and Martinez, 1998; Alarcón and Carrión, 1994) collected outside the native range of *H. vastatrix* or their coffee hosts (Africa). González and Martinez (1998) claimed to have demonstrated the effectiveness of *Verticillium lecanii* (= *Lecanicillium lecanii*) against *H. vastatrix* in field conditions. Unfortunately, it is impossible to confirm such claims since the authors did not deposit cultures of their fungus and even the identity of their fungus is unclear, as recently discussed by Colmán (2018). The same applies to the publication of Alarcón and Carrión (1994). The only published surveys of fungal natural enemies of CLR are those of Carrión and Rico-Grey (2002) and James et al. (2016). Although or relevance, their surveys were performed in the the New World, where both coffee and *H. vastatrix* are introduced species.

However, starting in 2015, surveys have been conducted in Africa for endophytic fungi growing inside *Coffea* spp. and mycoparasites of *H. vastatrix* (Colmán 2018; Salcedo 2018). Here, preliminary results of a screening of the endophytic fungi obtained from coffee tissues (mostly in wild and semi-wild situations) aimed at evaluating their potential for use as classical biocontrol agents to be deployed against CLR was conducted. The emphasis was on isolates of *Trichoderma* spp., a genus known to include several effective antagonists to plant pathogens and broadly used in biocontrol programs.

Material and methods

General information and procedures

Isolates

The fungal isolates were all obtained during expeditions to Africa, as decribed in chapter 1. namely:

Isolates tested in the *in vitro* and in planta tests, as described below, were withdrawn from the culture collection of the Universidade Federal de Viçosa (Coleção

Octávio de Almeida Drummond - COAD) where they are maintained in silica-gel at 4°C and also in 10% glycerol at -80°C (Dhingra and Sinclair 1995).

Coffee plants

Young coffee plants cv. Catuaí-Vermelho (IAC 144) were prepared by the coffee nursery of the Departamento de Fitopatologia of Universidade Federal de Viçosa and left in plastic pots containing 5 kg of substrate, namely: a 2.5:1:0.5 mixture of soil, manure and sand. The seedlings were kept in a greenhouse and used both as source of healthy leaves (for extraction of leaf discs - which were to be used as described below) or to be inoculated during assessment of *in planta* effect of antagonists on reducing CLR severity. These plants were used for the latter purpose when they were 6-7 months old.

Production of inoculum of *Hemileia vastatrix* and antagonistic fungi

Mass production of urediniospores of *H. vastatrix* followed the methodology described by Zambolim and Chaves (1974) with some modifications. A 10^5 spores/mL suspension of urediniospores (from stock originally identified as belonging to race II) was applied with a hand spray over the abaxial part of young and fully developed leaves of coffee plants cv. Caturra (a highly susceptible coffee cultivar, known to sustain abundant CLR sporulation). These plants were kept in the dark for 48 hours, with 100% humidity and at a temperature of 22°C. Later they were transferred to a growth chamber to allow for rust symptoms and sporulation to occur, for a period of 30-45 days at 22 ° C and under a 12-hour light regime (light provided by White lamps), mature uredinospores were collected with a paintbrush and placed inside microtubes. Next the microtubes were put, with their lids opened, inside a desiccator for 48-h and stored at -80°C for preservation longer than 90 days or at 5°C for 30 days.

Fungal endophyte isolates to be screened were grown in either potato dextrose-agar (PDA) or potato carrot-agar (PCA) plates (depending on how they performed in terms of sporulation on each medium in previous evaluations) and maintained at 25 °C during 10-15 days until spore harvest for suspension preparation.

Screening for potential antagonists to *Hemileia vastatrix*

After non-sporulating isolates and those seemingly appearing to belong to ‒low priority taxa‖, such as members of *Colletotrichum* and *Fusarium* were rejected, one

hundred and thirty isolates remained for evaluation of anti-CLR activity. A screening scheme was mounted including: a) an evaluation of inhibitory effect of each isolate on germination of urediniospores of *H. vastatrix* and b) assessment of potential to reduce CLR severity on detached coffee leaf disks. Sixty-nine isolates of other genera and 61 isolates preliminarily identified as belonging to *Trichoderma* were included in those tests. The urediniospores were brush-collected from pustules of leaves of coffee plants var. caturra; later were suspended in a 0.1% Tween 80 solution at a concentration calibrated to 10^5 urediniospores/mL with a Neubauer chamber. Plates containing sporulating endophyte colonies were flooded with 10mL of 0.1% Tween 80 solution and the surface of the colonies was scrapped with a paintbrush. Suspensions of each isolate were calibrated to 10^6 spores/mL using a Neubauer chamber (Haddad et al. 2014b; Shiomi et al. 2006; Silva et al. 2012).

Inhibition of uredioniospore germination

Glass microscope slides –fresh from the box‖ were disinfected with 70% alcohol and then placed at room temperature until the residue evaporated. Two slides were placed inside each of several 11 × 11 × 3,5 cm poliestirene boxes (gerbox). These were previously prepared by careful washing, drying and internally cleaned with 70% ethanol and then lined with sterilized and moist towel paper. A 15 µL aliquot of *H. vastatrix* urediniospore suspension was transferred to the center of each slide followed by the transfer of a 15 µL aliquot of the potential antagonist suspension. The two drops were mixed with the micropipette tip on each slide. After that procedure the boxes were covered with their lids and sealed with PVC film in order to avoid evaporation of the water. Boxes were left in a bench in a controlled temperature room at 22 ± 1ºC in the dark for six hours. Control consisted on equivalent apparatus treated identically but with slides supporting aliquots of *H. vastatrix* urediniospores as before but without antagonist addition. After the six hours period, germination was interrupted by adding a drop of lactophenol over each drop of urediniospore or urediniospore/antagonist suspension – either pure or mixed with possible antagonists (Fig.1a). The urenidiospores were considered to have germinated when the germ tube size was longer than the urediniospore diam. Germination inhibition was calculated by the following equation:

$$\text{Germination inhibition } \% = (c - x / c) \times 100$$

Where, c = germinated urediniospores in the control and x = germinated urediniospores exposed to possible antagonist (Silva-Castro et al. 2018)

Experiments were performed independently. Each experiment had its own set of controls. The experimental design was in a completely randomized design for each experiment. The data were log+1 transformed before using analysis of variance (ANOVA), and differences among isolates were analyzed using the Scott–Knott cluster test ($\rho < 0.05$ was considered significant), with the use of the statistics program R version 3.5.0. (The R Foundation for Statistical Computing, 2009).

Reduction of CLR severity on coffee-leaf discs

The strains were evaluated for their potential to reduce CLR severity on coffee leaf discs. This method followed the original procedure described by Eskes and Kushalappa (1989) but modified as described in Salcedo (2018). Polystyrene boxes (gerbox) were previously cleaned as mentioned above and then linned with a layer of 1.5 cm thick plastic foam, previously chlorine-sterilized and saturated with sterile water. Healthy leaves (2^{nd} and 3^{rd} pair) were collected from coffee plants (cv. ―Catuaí-Vermelho IAC 144) and were subjected to progressive disinfection by dipping into 70% alcohol for 1 min, sodium hypochlorite 2% for 1 min and rinsed with sterile water. Excess water was removed with sterile towel paper. Two cm diam leaf discs were removed from such leaves with a steel punch. Twelve discs were placed into the gerboxes, with abaxial surface facing up, over the layer of foam. A 25 µL aliquot of urediniospore suspension of *H. vastatrix* (10^5 urediniospores/mL suspended in 0.1% Tween 80), and a 25 µL aliquot of the potential antagonist suspension (10^6 spores/mL) were placed on each disc. The two drops were mixed and distributed with the micropipette tip on leaf disc. Controls received a 25 µL aliquot of the suspension of *H. vastatrix* and a 25µL aliquot of the Tween 80 solution (0.1%) (Fig. 1b). The suspensions of antagonists and of urediniospores of *H. vastatrix* were prepared as described above.

Three application times were tested for each antagonist against *H. vastatrix*, as given below (Fig. 1b):

- 0h- an aliquot of 25 µl of the antagonist suspension plus *H. vastatrix* uredinial suspension were deposited simultaneously on leaf disc
- 24h- an aliquot of 25 µl of the antagonist suspension placed on leaf disc 24 hs before depositing the urediniospore suspension of *H. vastatrix*

- 72h-*Idem* an aliquot of 25 µl of the antagonist suspension placed on leaf disc 72 hs before depositing the urediniospore suspension of *H. vastatrix*

Immediately after their preparation, the boxes containing the disc treatments were placed in the dark for 24h at 22 ± 1ºC. (Fig. 1b). Since the number of isolates selected for evaluation was large, these were evaluated in separate batches (independent experiments) and each batch had its control.

The severity of the disease was evaluated 30 days after inoculation, using a rating scale from 0 to 5 according to the percentage of leaf area with lesions (0 = 0%; 1 = 0.1% to 2.5%; 2 = 2.6% to 5%; 3 = 5.1% to15%; 4 = 15.1% to 25%, and 5>25%) (Silva et al. 2012).

The data were log+1 transformed before the analysis of variance (ANOVA), and differences among isolates were analyzed using the Scott–Knott cluster test ($\rho < 0.05$ was considered significant), with the use of the statistics program R version 3.5.0. (The R Foundation for Statistical Computing, 2009).

Reduction of CLR severity in planta

Four endophyte isolates for which results obtained in the screening described above, namely E48 (*Cordyceps* sp.), COAD 2396 (*T. atroviride*), E400 (*Pestalotiopsis* sp.), COAD 2410 (*T. koningiopsis*) were brought forward to further testing on live plants.

Six-month-old coffee plants (cv. ―Catuaí-Vermelho IAC 144) were used for this test. A conidial suspension of each antagonist and *H. vastatrix* were prepared as described above. Inoculation of *H. vastatrix* suspension and of antagonist conidial suspension were performed with a hand sprayer until runoff .

Similarly, to leaf disc test, three application times were tested for each antagonist against *H. vastatrix:*

- 0h- the antagonist suspension plus *H. vastatrix* uredinial suspension applied simultaneously on plants.
- 72h- the antagonist suspension was applyed on plants 72 hs in advance of spraying with urediniospore suspension of *H. vastatrix*

- 2wk - *Idem* the antagonist suspension was applied on plants two weeks in advance of spraying with urediniospore suspension of *H. vastatrix*

After inoculation with *H. vastatrix* uredinospore suspension, at every instance, the plants were immediately left afterwards in the dark for 48hs under 100% relative humidity at 22°C. After inoculation with the endophyte conidial suspension alone, plants were left in a dew chamber under a 12h light regime (light provided by white lamps) for 48h. Subsequently all the plants were transferred to growth chambers at 22 ± 2ºC (Shiomi et al. 2006). The plants were irrigated every third day until the evaluation.

Disease severity was quantified as follows: - Images of two leaves collected 45 days after inoculation from each repetition for each treatment and abaxial images of each leaf were obtained and digitalized with a scanner (HP G4050. Percentage of rusted leaf area was estimated with Quant software (Vale, Filho, and Liberato 2003).

Three independent experiments were carried out: one for E48, another for COAD 2396 and one for E400 and COAD 2410 together.

A random blocks experimental design was adopted for each experiment in a factorial scheme (two antagonist treatments – not inoculated or inoculated × 3 application times – 0h, 72hs or 2weeks before) with five replications (one plant = one experimental unit) per experiment. For the tthird experiment, a random blocks experimental design, in a factorial scheme (four antagonist treatments – not inoculated or inoculated × 2 application times – 0h or 72hs) was utilized.

For each experiment application of the systemic fungicide – tebuconazole at 200 g i. a. L-1 (Nativo®) was used as a positive control (F) applied four days after the pathogen.

The means were compared by Tukey test ($\alpha = 0.05$) using of the statistics program R version 3.5.0. (The R Foundation for Statistical Computing, 2009).

***Trichoderma* endophytic colonization of inoculated coffee plants**

A test was conducted involving 15 distinct endophytic species of *Trichoderma* obtained in the survey in order to verify whether it would be possible to restablish endophytic colonies on coffee plants starting with inoculum produced *in vitro.* Conida were obtained from 10 days old colonies formed on PCA (Himedia) incubated at 25°C under a 12 h light regime (light provided by white lamps). Conidial suspension was

prepared as described above and adjusted to 1×10^6 conidia/mL of a 0.1% (v/v) Tween 80 solution. Control plants were treated with 0.1% (v/v) Tween 80 solution.

Five plants were treated with conidial suspension of each species of *Trichoderma* by spraying the aerial part of each plant with a hand sprayer until runoff. Treated plants were left in a dew chamber (with plastic bags moistened internally) for 48 hs at 25-28°C after inoculation in greenhouse conditions.

In order to verify whether each fungal species had established on treated coffee plants samples were taken from each coffee plant at four different lengths of time after inoculation, namely: 15, 30, 45 and 60 days after inoculation. Twelve $5mm^2$ fragments of leaves of each plant/date/repetition were taken for isolations at each attempt of recovering endophytic colonies and to verify colonization of the stem, fragments approximately 5 mm long were taken of each plant/date/repetition only 60 days after inoculation. The protocol for surface disinfestation of fragments was that described by Arnold et al. (2000). Fragments were dipped for 1 min in ethanol 70%, transferred for 2 min to sodium hypochlorite 2% and then left for 1 min in sterilized water, before. Fragments were then briefly deposited on the surface of sterile filter paper to remove excess water and placed at equal distances on the surface of PCA medium on plates (six fragments per plate evenly spread). Plates were left in a growth chamber at 24 °C and the colonies that were growing in the culture medium were observed every day with stereoscopic microscope MOTIC SMZ-168 for detection the colonies of *Trichoderma* and to mark how many and which fragments were colonized. Whenever colonies suspected of being *Trichoderma* were observed a slide was made and visualized under the microscope (Olympus CX 31) to confirm that it was *Trichoderma* colony. The final evaluation was made ten days after attempted isolation and consisted on counting the total number of fragments which became colonized by *Trichoderma* spp.

Results and discussion

Inhibition of uredioniospore germination

A total of 13 experiments were performed. Of the 130 isolates tested, only those isolates that promoted inhibition of *H. vastatrix* were statistically analyzed as summarized in Table 1.

Only 17 isolates among the 130 which were tested produced a significant inhibition of germination of *H. vastatrix* urediniospores. Isolates E526, E816, COAD

2417, COAD 2535 and COAD 2439 produced an inhibition of over 80% of urenidospores germination as compared with controls. These were preliminarly identified as respectively belonging to *Mucor* sp.*, Pestalotiopsis* sp.*, T. hamatum* and *Trichoderma* sp. nov.4*,* respectively (Table 1). Deformations of urediniospore germ tubes were observed.

A study published by Shiomi et al. (2006) listed 23 endophytic isolates of bacteria obtained from leaves and branches of coffee which inhibited germination of *H. vastatrix* urediniospores significantly (40% reduction or more as compared with controls). Inhibition of spore germination by antagonistic species may be caused by antifungal secondary metabolites known to be produced by several fungal endophytes as documented in the literature (Gao et al. 2010; Gunatilaka 2006). Deleterious effect on *H. vastatrix* urediniospore germination have been reported for other organisms such as *Pseudomonas putida* and *Bacillus thuringiensis.* Supernatant, culture broth and bacterial cells of these bacteria were capable of promoting inhibition of up to 90% of the germination in essays conducted by Haddad et al. (2013).There are several mechanisms for endophytic microorganisms to inhibit plant pathogens such as antibiosis, competition for space or food or induced resistance (Hardoim et al. 2015). It is well known that *Trichoderma* species may produce numerous secondary metabolites (Mukherjee et al. 2013). *Trichoderma hamatum* produces substances such as viridol which are deleterious to fungal phytopathogens (Vinale et al. 2014). Similarly, secondary metabolism substances produced by *Pestalotiopsis foedans* were shown to have strong antifungal activities against *Botrytis cinerea* and *Phytophthora nicotianae* (Xu et al. 2016). Future investigations of E526, E816, COAD 2417, COAD 2535 and COAD 2439 may reveal them to produce useful substances to be deployed against CLR or other fungal pathogens of plants.

Reduction of CLR severity on coffee-leaf discs

Isolates E2 (*T. atroviride*), E48 (*Cordyceps* sp.) and E486 (*Aspergillus* sp.) promoted reduction of CLR severity levels of 50% or above for all time intervals evaluated and the Scott–Knott cluster test indicated that these fungi differed significantly from the control. Next, isolates E16 (unidentified), COAD 2403 (*Trichoderma* sp. nov3), E215 (unidentified), E400 (*Pestalotiopsis* sp.), COAD 2410 (*T. koningiopsis*), E462 (Phoma-like) and COAD 2417 (*T. hamatum*) showed a reduction in disease severity levels of over 25% for two of the application times (Table 2 and Fig. 2).

The best application time for reduction of severity of the disease was when the antagonist was applied 72 or 24h before the *H. vastatrix*. A similar effect of antagonism against *H. vastatrix* on leaf discs was observed with endophytic bacteria. *Bacillus lentimorbus, B. megaterium, Brevibacillus choshinensis, Salmonella enterica, Pectobacterium carotovorum, Microbacterium testaceum* and *Cedecea davisae* significantly reduced disease severity when applied 24 or 72h before *H. vastatrix* (Shiomi et al. 2006; Silva et al. 2012). In the study conducted by Silva (2012) they included 17 endophytic fungi in their test aimed at evaluating their antagonistic activity against *H. vastatrix*. Nevertheless, in contrast with our results, no inhibition was obtained with their isolates. A better effect obtained by applying the antagonists ahead of the pathogens was regarded by Beattie and Lindow (1995) as likely to be obtained because of an epiphytic colonization and occupation of sites of infection on the plant tissue occurring, in such circumstances before the arrival of the inoculum of the pathogen. In the case of antagonistic *Trichoderma* spp. besides establishing epiphytically these may also colonize the coffee tissue endophytically after foliar applications as demonstrated here (Fig. 3).

The isolates which had the best levels of CLR severity reduction on all intervals were provisionally identified as *T. atroviride* (COAD 2396), *Cordyceps* sp. (E48), and *Aspergillus* sp. (E486). Members of *Trichoderma* are well known biocontrol agents, inclusively broadly used commercially against a range of phytopathogens (Druzhinina et al. 2011). Despite the substantial amount of information published of application of members of this in biocontrol of diseases, there are few studies on the use of *Trichoderma* spp. against biotrophic pathogens such as rusts and none targetting *H. vastatrix*. However, in a study conducted with endophytic fungi to control *Melampsora x columbiana* it was discovered that *T. atroviride* has an antagonistic effect against this rust, diminishing its urenidial density and consequently affecting the development of the disease on leaf disc and on live plants (Busby et al. 2016; Raghavendra and Newcombe 2013). This is an encouraging example justifying a special interest to the study of the isolate of *T. atroviride* from coffee.

Suprisingly, the majority of the isolates which inhibited the germination of *H. vastatrix* did not produce a reduction in CLR severity in the leaf disc essays, and vice-versa. This may be indicating that different antagonism mechanisms are at play of action of the isolates that inhibit germination (antibiosis) is different from the

mechanism of action of the isolates that decreased the severity of the pathogen on the leaf disc. Endophytes than show antibiosis tended to be slower growing (Mejía et al. 2008) what can explain such phenomenon.

Only COAD 2417 (*T. hamatum*) and E546 (*Mucor* sp.) had both an effect at inhibiting urediniospore germination and at reducing CLR severity in coffee leaf discs (Table 1, 2); suggesting that these isolates are exceptional for combining more than one mechanism of action (possibly antibiosis and competition) against *H. vastatrix*.

Reduction of CLR severity in planta

COAD 2396 (*T. atroviride*), E48 (*Cordyceps* sp.), E400 (*Pestalotiopsis* sp.) and COAD 2410 (*T. koningiopsis*) were selected among the endophytic fungal isolates which decreased CLR severity in the leaf disc test and were also tested in plants. Most of the isolates procuced no significant reduction of CLR severity when compared with the control (Fig. 3, 4 and 5). Nevetheless, it was observed that there were differences between treatments with different intervals between the inoculation of the endophyte and inoculation with *H. vastatrix.* The best time of application ($p<0.05$) for reduction of the disease was 72h before the inoculation of *H. vastatrix* (Fig. 3 and 4). These results coincide with those obtained earlier in the context of this project with the mycoparasite *Calonectria hemileiae* (Salcedo et al. 2018) which effectively reduced the severity of CLR by more than 50% in an experiment following a similar procedure as described here Salcedo (2018).

Haddad et al. (2014b) also was observed that the number of *H. vastatrix* pustules and sporulation has a relationship with the time of application of the antagonists, indicating that, in their case, the antagonists should be applied near inoculation of the pathogen for better effectiveness.

A brief essay involving E48 alone resulted in a reduction of CLR severity of 64% as compared with the control, when the endophyte was applied with a 72h delay from inoculation with *H. vastatrix* and 26% when a delay of 2 weeks was attempted (Fig. 3) However, results of this essay were not significant, and a repetition of the experiment is required. Particularly since these results seem to suggest that E48 has biocontrol potential.

The contrasting results obtained in the *in vitro* screening on leaf discs and those obtained *in vivo* can be attributed to a higher level of environmental control in the *in vitro* tests as compared with the *in vivo* experiments. Legitimate endophytes may require longer than 72 hs to establish in coffee tissues and begin providing protection against CLR and problems with timing and ‒too artificial conditions‖ of leaf disc screening may have led to the rejection of good biocontrol agents. Considering those issues, it is logical, in the case of the endophyte, and particularly in the case of the several species of *Trichoderma* spp. to organize another round of experiments involving different methods of inoculation and repeated inoculations before challenging coffee plants with *H. vastatrix* and deciding on their true potential.

Colonization of *Trichoderma spp.*

The isolates of *Trichoderma* spp. tested were able to endophytically-colonize coffee leaves and stems when spray-inoculated. Some of the isolates appeared to be efficient endophytic colonizers of coffee leaves, for example *T. parareesei* (COAD 2482) was recovered from the leaves at a rate of 1.6-35% in the 4 evaluated intervals; followed by *T. atroviride* (COAD 2396), *T. koningiopsis* (COAD 2502), *Trichoderma* sp. nov.4 (COAD 2592) and *T. aggressivum* (COAD 2432) than were recovered (1.6-30%) in 3 of the evaluated intervals (Fig. 6). *Trichoderma* sp. nov.1 (COAD 2399) was not recovered from the leaves in any of the evaluations carried out. In the stem all of the isolates were recovered. The most frequently isolated were *T. parareesei* (COAD 2482), *T. lentiforme* (COAD 2429) and *Trichoderma* sp. nov.4 (COAD 2592) with a recovery percentage of 28.3, 18.3 and 11.6%, respectively (Fig. 7). No *Trichoderma* was recovered from the controls.

These results indicate that all *Trichoderma* spp. can establish themselves endophtically on their original coffee host, even with a simple and crude method of inoculation. As expected, some have preference for woody tissues whereas others are better at colonizing leaves, reflecting the observations made during the original surveys in Africa. Competition between epiphytic and endophytic communities were found in coffee leaves (Santamaría and Bayman 2005) would likely further limit the ability of some *Trichoderma* species to get or maintain colonization of coffee leaves. It is also known that some species of endophytic *Trichoderma* are also soil fungi. Spraying spore suspensions of such species in order to establish endophytic colonies may be a less effective way to deliver such inoculum. Exposure to different ultraviolet light,

dissecation and temperature fluctuations may influence colonization rates (Gomes et al. 2018).

An important observation which was made was that *T. atroviride* (COAD 2396), *T. parareesei* (COAD 2482) and *T. aggressivum* (COAD 2432) were recovered not only the tissues exposed to inoculums but were also recovered from newer tissues formed much later, indicating that, at least for those species there is an ability to systemically, and possibly permanently, colonize coffee plants (Fig.3).

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Table 1.Inhibition of germination of urediniospores of *Hemileia vastatrix* by selected endophytic and mycoparasite fungi.

	Isolate	Species	Inhibition (%)**
Experiment 1			
	E417	Unidentified	55.93b (±4.92)
	COAD 2516	*Trichoderma* sp. nov.4	59.25b (±4.66)
	COAD 2517	*Trichoderma* sp. nov.4	63.59b (±5.93)
	E526	*Pestalotiopsis sp.*	84.07a (±1.32)
Experiment 2			
	E122	Unidentified	43.91a (±11.68)
	E546	*Mucor sp.*	67.41a (±3.61)
Experiment 3			
	E789	*Pestalotiopsis sp.*	59.10a (±24.09)
	E22	*Pestalotiopsis sp.*	75.99a (±8.96)
	E27	*Pestalotiopsis sp.*	75.93a (±5.28)
	COAD 2418	*T. hamatum*	78.77a (±6.00)
	E816	Unidentified	98.79a (±1.20)
Experiment 4			
	COAD 2483	*T. parareesei*	36.44b (±2.7)
	COAD 2482	*T. parareesei*	51.00b (±10.28)
	COAD 2417	*T. hamatum*	89.7a (±3.08)
Experiment 5			
	COAD 2533	*Trichoderma* sp. nov.4	39.26b (±8.69)
	COAD 2535	*Trichoderma* sp. nov4.	87.32a (±9.76)
	COAD 2439	*Trichoderma* sp. nov.4	86.91a (±7.79)

* 30 µL drop containing suspension of urediniospores of *Hemileia vastatrix* and suspension of conidia of antagonist.

** Inhibition representing 100 spores observed on a slide under the microscope after 6 hs of incubation in the dark. Percentage give as = (c – x / c) × 100; where, c = germinated urediniospores in the control and x = germinated urediniospores exposed to antagonist

[1]Means followed by the same letter do not differ from each other (Scott–Knott ρ = 0.05). Value between parentheses indicates standard error (se). Note: only the isolates that had an inhibitory effect against *H. vastatrix* are shown in this table.

Table 2.Reduction of coffee leaf rust severity on coffee-leaf discs by endophytic and mycoparasitic fungi.

	Isolate	72h before	Isolate	24h before	Isolate	0h
Experiment 1						
	control	3.33a (±0.37)	control	2.25a (±0.50)	control	2.33b (±0.48)
	E120	0.58c (±0.22)	E120	1.16b (±0.47)	E120	2.16b (±0.27)
	E24	3.66a (±0.16)	E24	1.9a (±0.15)	E24	3.25a (±0.32)
	E25	2.29b (±0.26)	E25	1.0b (±0.23)	E25	1.87b (±0.20)
	E30	2.5b (±0.39)	E30	0.66b (±0.22)	E30	3.75a (±0.25)
	E5	0.83c (±0.34)	E5	1.0b (±0.32)	E5	0.75d (±0.35)
	E55	1.0c (±0.30)	E55	0.41c (±0.19)	E55	1.16c (±0.24)
	E68	3.41a (±0.28)	E68	0.66b (±0.28)	E68	1.75b (±0.48)
	COAD 2510	0.41c (±0.14)	COAD 2510	1.83a (±0.40)	COAD 2510	1.0c (±0.34)
	E110**	0.33c (±0.14)	**E110**	0.33c (±0.14)	E110	1.75b (±0.35)
	E113**	0.25c (±0.17)	**E113**	1.83a (±0.36)	E113	2.16b (±0.27)
	COAD 2396***	0.08c (±0.08)	**COAD 2396**	0.00c (±0.00)	**COAD 2396**	0.41d (±0.41)
Experiment 2						
	control	2.83a (±0.32)	control	3.16a (±0.24)	control	2.75a (±0.50)
	COAD 2401	0.83b (±0.32)	COAD 2401	2.58a (0.45)	COAD 2401	2.58a (0.45)
	COAD 2403**	1.16b (±0.34)	**COAD 2403**	1.66b (±0.51)	COAD 2403	3.91a (±0.45)
	COAD 2501**	1.66b (±0.37)	**COAD 2501**	0.66b (±0.35)	COAD 2501	2.16a (±0.32)
Experiment 3						
	control	2.91a (±0.35)	control	2.33b (±0.18)	control	2.75b (±0.25)
	E176	3.00a (±0.24)	E176	2.25b (±0.30)	E176	2.75b (±0.39)
	E215**	1.83b (±0.53)	**E215**	1.66c (±0.44)	E215	3.1b (±0.53)
	E322	2.25b (±0.27)	E322	2.75b (±0.30)	E322	3.83a (±0.27)
	E359	1.66b (±0.25)	E359	3.16a (±0.32)	E359	3.16b (±0.40)
	E431	3.33a (±0.22)	E431	4.08a (±0.22)	E431	4.00a (±0.17)
	E437	3.41a (±0.33)	E437	3.16 (±0.24)	E437	3.41b (±0.33)
	E440	2.81a (±0.20)	E440	3.02b (±0.33)	E440	2.66b (±0.39)
	E462**	1.91b (±0.48)	**E462**	0.75d (±0.21)	E462	2.51b (±0.22)
	E464	1.91b (±0.28)	**E464**	0.75d (±0.30)	E464	2.50b (±0.31)
	E465	2.58a (±0.25)	E465	3.91a (±0.14)	E465	3.66a (±0.18)
	E537	1.16b (±0.32)	E537	2.75b (±0.32)	E537	3.00a (±0.36)
	E578	3.58a (±0.19)	E578	2.75b (±0.42)	E578	4.33a (±0.18)
	COAD 2589	2.08b (±0.49)	COAD 2589	3.33a (±0.30)	COAD 2589	4.33a (±0.35)
	E583	2.91a (±0.28)	E583	2.25b (±0.27)	E583	2.41b (±0.19)
	E591	3.08a (±0.25)	E591	3.08b (±0.35)	E591	2.00c (±0.36)
	COAD 2420	3.66a (±0.28)	COAD 2420	3.5a (±0.31)	COAD 2420	3.66a (0.33)
	COAD 2524	3.33a (±0.25)	COAD 2524	3.41a (±0.33)	**COAD 2524**	1.83c (±0.38)
	COAD 2429	3.41a (±0.33)	COAD 2429	2.75b (±0.21)	COAD 2429	4.00a (0.25)
Experiment 4						
	control	4.16a (±0.16)	control	4.00a (±0.17)	control	2.41b (±0.31)
	E275	1.16c (±0.36)	**E275**	0.16d (±0.16)	E275	2.50b (±0.54)
	E321	3.83a (±0.16)	E321	3.33a (±0.14)	E321	3.91a (±0.08)

	E373	1.00c (±0.30)	E373	0.083c (±0.24)	E373	2.3b (±0.39)
	E437	1.00c (±0.17)	E437	1.25b (±0.27)	E437	3.5a (±0.19)
	E464	0.00d (±0.00)	E464	2.1b (±0.29)	E464	3.4a (0.25)
	E466	3.00b (±0.24)	E466	1.75b (±0.27)	E466	1.50b (±0.15)
	E477	1.33c (±0.43)	**E477**	0.00d (±0.00)	E477	2.58b (±0.35)
	E489	2.50b (±0.28)	E489	2.83a (±0.47)	E489	1.75b (±0.37)
	E581**	0.75c (±0.25)	**E581**	0.00d (±0.00)	E581	3.66a (±0.64)
	E883	4.00a (±0.36)	E883	3.33a (±0.25)	E883	3.00a (±0.17)
Experiment 5						
	control	2.41b (±0.43)	control	4.41a (±0.28)	control	2.08b (±0.33)
	E122	1.25b (±0.32)	E122	1.50b (±0.43)	E122	3.4a (0.31)
	E16	2.00b (±0.44)	**E16****	1.58b (±0.55)	**E16**	0.91d (±0.46)
	E365	3.66a (±0.25)	E365	1.58b (0.33)	E365	2.58b (0.45)
	E387	1.16b (±0.32)	E387	4.00a (0.22)	**E387**	0.25d (±0.13)
	E48***	0.25c (±0.13)	**E48**	0.25c (±0.13)	**E48**	1.16c (±0.27)
	COAD 2414	3.16a (±0.16)	COAD 2414	2.91a (±0.33)	COAD 2414	4.50a (±0.26)
	E70	2.00b (±0.40)	E70	4.00a (±0.21)	E70	4.75a (±0.17)
	COAD 2514	4.08a (±0.31)	COAD 2514	3.66a (±0.22)	COAD 2514	2.50b (±0.41)
	E88	2.00b (±0.31)	E88	3.50a (±0.39)	E88	3.58a (±0.22)
Experiment 6						
	control	2.58a (±0.35)	control	2.91a (±0.25)	control	3.16a (±0.27)
	E128	2.91a (0.35)	E128	2.16b (0.27)	E128	3.41a (±0.22)
	E22	3.66a (0.30)	E22	2.25b (0.25)	E22	2.83a (±0.16)
	E27	2.41a (0.19)	E27	2.66a (0.28)	E27	2.41a (±0.19)
	E486***	0.00d (±0.00)	**E486**	0.33c (±0.14)	**E486**	1.58b (±0.37)
	E569	0.66c (±0.22)	E569	0.75c (±0.27)	E569	2.58a (±0.19)
	E642	1.83b (±0.38)	**E642**	0.66c (±0.22)	E642	3.91a (±0.28)
	E789	3.08a (±0.14)	E789	1.00c (±0.21)	E789	2.58a (±0.22)
	E816	1.91b (±0.46)	E816	3.33a (±0.18)	E816	3.91a (±0.31)
	E9	2.25b (±0.32)	E9	0.75c (±0.21)	**E9**	1.25b (±0.30)
Experiment 7						
	control	1.66a (±0.30)	control	0.66a (±0.28)	control	3.83a (±0.29)
	E238	2.33a (±0.48)	E238	0.25a (±0.13)	E238	3.16a (±0.47)
	E269	1.16b (±0.29)	E269	0.00a (±0.00)	E269	3.91a (±0.25)
	E306	1.66a (±0.25)	E306	0.25a (±0.13)	E306	3.25a (±0.42)
	E338	2.33a (±0.37)	E338	0.33a (±0.14)	E338	3.00a (±0.49)
	E546	0.66b (±0.25)	E546	0.16a (±0.11)	E546	2.25a (±0.53)
	E566	2.25a (±0.37)	E566	0.08a (±0.08)	E566	3.08a (±0.46)
	E658	0.75b (±0.25)	E658	0.08a (±0.08)	E658	2.91a (±0.45)
	E698	0.91b (±0.22)	E698	0.00a (±0.00)	E698	4.16a (±0.40)
Experiment 8						
	control	4.08a (±0.25)	control	3.16a (±0.40)	control	3.08a (±0.48)
	COAD 2397	3.4a (±0.43)	COAD 2397	4.7a (±0.25)	COAD 2397	3.5a (±0.59)
	COAD 2398	0.91c (±0.22)	COAD 2398	3.5a (±0.48)	COAD 2398	1.75b (±0.27)
	COAD 2404	1.91b (±0.60)	COAD 2404	3.5a (±0.35)	COAD 2404	2.3b (±0.54)
	COAD 2424	3.00a (0.57)	COAD 2424	4.00a (±0.44)	COAD 2424	2.66a (±0.14)

	COAD 2422	1.91b (0.45)	COAD 2422	2.58b (±0.41)	COAD 2422	3.83a (±0.24)
	COAD 2412	2.41b (±0.62)	COAD 2412	2.91b (±0.58)	COAD 2412	1.33c (±0.35)
	COAD 2417**	0.33d (±0.25)	**COAD 2417**	1.25c (±0.49)	COAD 2417	4.08a (±0.28)
	COAD 2505	0.25d (±0.25)	COAD 2505	3.66a (±0.48)	COAD 2505	3.16a (±0.61)
	COAD 2425	2.75a (±0.37)	COAD 2425	2.75b (±0.61)	COAD 2425	1.83b (±0.34)
	COAD 2511	2.66a (±0.46)	**COAD 2511**	2.41b (±0.48)	COAD 2511	3.08a (±0.39)
	COAD 2515	3.16a (±0.32)	COAD 2515	3.66a (±0.44)	COAD 2515	2.75a (±0.41)
	COAD 2519	2.25b (±0.39)	COAD 2519	5.00a (±0.00)	**COAD 2519**	1.16c (±0.40)
	COAD 2520	2.33b (±0.58)	COAD 2520	3.58a (±0.41)	COAD 2520	2.25b (±0.52)
	COAD 2528	2.9a (±0.51)	COAD 2528	4.50a (±0.14)	COAD 2528	2.16b (±0.40)
	COAD 2430	2.4b (±0.45)	COAD 2430	4.50a (±0.22)	COAD 2430	3.08a (±0.35)
	COAD 2482	3.16a (±0.44)	COAD 2482	3.25a (±0.30)	COAD 2482	2.58a (±0.43)
	COAD 2483	2.25b (±0.05)	COAD 2483	5.00a (±0.00)	COAD 2483	2.7a (±0.25)
	COAD 2399	1.75b (±0.47)	COAD 2399	2.25b (±0.32)	**COAD 2399**	**0.58c (±0.33)**
Experiment 9						
	control	1.91a (±0.39)	control	1.50b (±0.54)	control	3.83a (±0.42)
	E367	2.58a (±0.43)	E367	0.75b (±0.27)	E367	0.83b (±0.32)
	COAD 2506	2.58a (±0.45)	COAD 2506	3.08a (±0.51)	COAD 2506	3.33a (±0.49)
	COAD 2423	1.91a (±0.35)	COAD 2423	2.16a (±0.53)	**COAD 2423**	0.66b (±0.28)
Experiment 10						
	control	3.08a (±0.41)	control	1.58b (±0.46)	control	4.00a (±0.46)
	COAD 2530	0.91c (±0.43)	COAD 2530	2.16a (±0.20)	COAD 2530	3.41a (±0.54)
	COAD 2531	0.41c (±0.41)	COAD 2531	3.16a (±0.44)	COAD 2531	5.00a (±0.00)
	COAD 2532	2.25b (±0.65)	**COAD 2532**	0.16c (±0.16)	COAD 2532	4.4a (±0.25)
	COAD 2534	3.5a (±0.41)	COAD 2534	2.8a (±0.36)	COAD 2534	4.5a (±0.22)
	COAD 2535	3.33a (±0.37)	COAD 2535	1.5b (±0.48)	COAD 2535	5.00a (±0.00)
	COAD 2536	4.25a (±0.25)	COAD 2536	1.08b (±0.39)	COAD 2536	5.00a (±0.00)
Experiment 11						
	control	4.08a (±0.25)	control	3.1a (±0.40)	control	3.08a (±0.48)
	COAD 2432	0.66b (±0.28)	COAD 2432	3.58a (±0.64)	COAD 2432	2.58a (±0.43)
	COAD 2433	2.83a (±0.36)	COAD 2433	1.83b (±0.44)	COAD 2433	1.16a (±0.32)
	COAD 2435	1.58b (±0.35)	COAD 2435	3.00a (±0.53)	COAD 2435	1.75a (±0.46)
	COAD 2436	1.91b (±0.49)	**COAD 2436**	1.58b (±0.43)	COAD 2436	2.75a (±0.52)
	COAD 2533	2.41a (±0.37)	COAD 2533	4.3a (±0.14)	COAD 2533	1.4a (±0.37)
	COAD 2438	1.75b (±0.44)	**COAD 2438**	1.58b (±0.39)	COAD 2438	3.08a (±0.48)
	COAD 2485	2.33a (±0.46)	COAD 2485	3.66a (±0.59)	COAD 2485	1.83a (±0.50)
Experiment 12						
	control	3.00a (±0.65)	control	3.58a (±0.19)	Control	3.08a (±0.35)
	COAD 2590	2.91c (±0.37)	COAD 2590	3.0a (±0.50)	COAD 2590	3.6a (0.54)
	COAD 2423	1.58b (±0.45)	COAD 2423	3.41a (±0.55)	COAD 2423	4.41a (±0.28)
	E417	2.16b (±0.47)	E417	2.50a (±0.51)	E417	3.75a (±0.39)
	E504	2.1b (±0.44)	**E504**	1.00b (±0.42)	E504	2.0a (±0.39)
	E526	1.8b (±0.40)	E526	1.5b (±0.41)	E526	3.9a (±0.55)
	COAD 2420	2.16b (±0.50)	COAD 2420	2.08b (±0.39)	COAD 2420	4.8a (±0.16)
	COAD 2507	1.33 (±0.35)	COAD 2507	2.0b (±0.36)	COAD 2507	4.0a (±0.39)

	COAD 2591	0.58d (±0.28)	COAD 2591	2.5a (±0.28)	COAD 2591	4.0a (±0.28)
	COAD 2426	1.66b (±0.35)	COAD 2426	1.75b (±0.44)	COAD 2426	3.16a (0.40)
	COAD 2402	3.75a (±0.17)	COAD 2402	3.16a (±0.61)	COAD 2402	2.8a (0.40)
	COAD 2541	0.16d (±0.16)	COAD 2541	2.5a (±0.46)	COAD 2541	3.5a (0.23)
	COAD 2540	3.25a (±0.42)	COAD 2540	1.66b (±0.39)	COAD 2540	3.66a (±0.30)
	COAD 2516	2.08b (±0.39)	COAD 2516	1.41b (±0.45)	COAD 2516	4.2a (±0.44)
	COAD 2517	3.25a (±0.25)	COAD 2517	2.41a (±0.51)	COAD 2517	3.83a (±0.40)
	COAD 2518	3.0a (±0.46)	COAD 2518	1.66b (±0.41)	COAD 2518	3.66a (±0.28)
	COAD 2427	2.16b (±0.45)	COAD 2427	2.75a (±0.46)	COAD 2427	4.00a (±0.21)
	COAD 2509	2.66a (±0.28)	COAD 2509	3.41a (±0.35)	COAD 2509	4.08a (±0.25)
Experiment 13						
	control	1.58b (±0.28)	control	1.66b (±0.43)	control	1.66c (±0.25)
	E133	0.33c (±0.33)	E133	0.9c (±0.45)	E133	2.1b (±0.45)
	E364	1.16b (±0.32)	E364	1.2d (±0.25)	E364	1.2e (±0.25)
	E400**	0.00c (±0.00)	E400	1.58b (±0.25)	**E400**	0.08e (±0.08)
	COAD 2405	1.22c (±033)	COAD 2405	1.00d (±0.00)	COAD 2405	1.66d (±0.33)
	COAD 2410**	0.00c (±0.00)	COAD 2410	1.50b (±0.28)	**COAD 2410**	0.16e (±0.11)
	COAD 2592	1.83a (±0.11)	COAD 2592	2.00d (±0.24)	COAD 2592	0.75d (±0.27)
	COAD 2421	0.33c (±0.25)	COAD 2421	1.2d (±0.22)	COAD 2421	3.66a (±0.33)
	COAD 2538	1.16c (±0.08)	COAD 2538	0.08d (±0.08)	COAD 2538	1.83b (±0.29)

Effect of endophytic fungi over severity of coffee rust on leaf discs cv. –Catuaí vermelho when applied at 72, 24h before, or simultaneously with of *H. vastatrix*

50 µL drop containing suspension of urediniospores of *Hemileia vastatrix* and suspension ofconidia of potential antagonist.

** Fungi that showed a significant reduction in the severity of the disease in 2 application times

*** Fungi that showed a significant reduction in the severity of the disease in 3 application times

[1]Means followed by the same letter do not differ from each other (Scott–Knott a = 0.05). Value between parentheses indicates standard error (se). Averages of the severity of the disease evaluated with the scale (0 = 0%; 1 = 0.1% to 2.5%; 2 = 2.6% to 5%; 3 = 5.1% to15%; 4 = 15.1% to 25%, and 5>25%) (Silva et al. 2012)

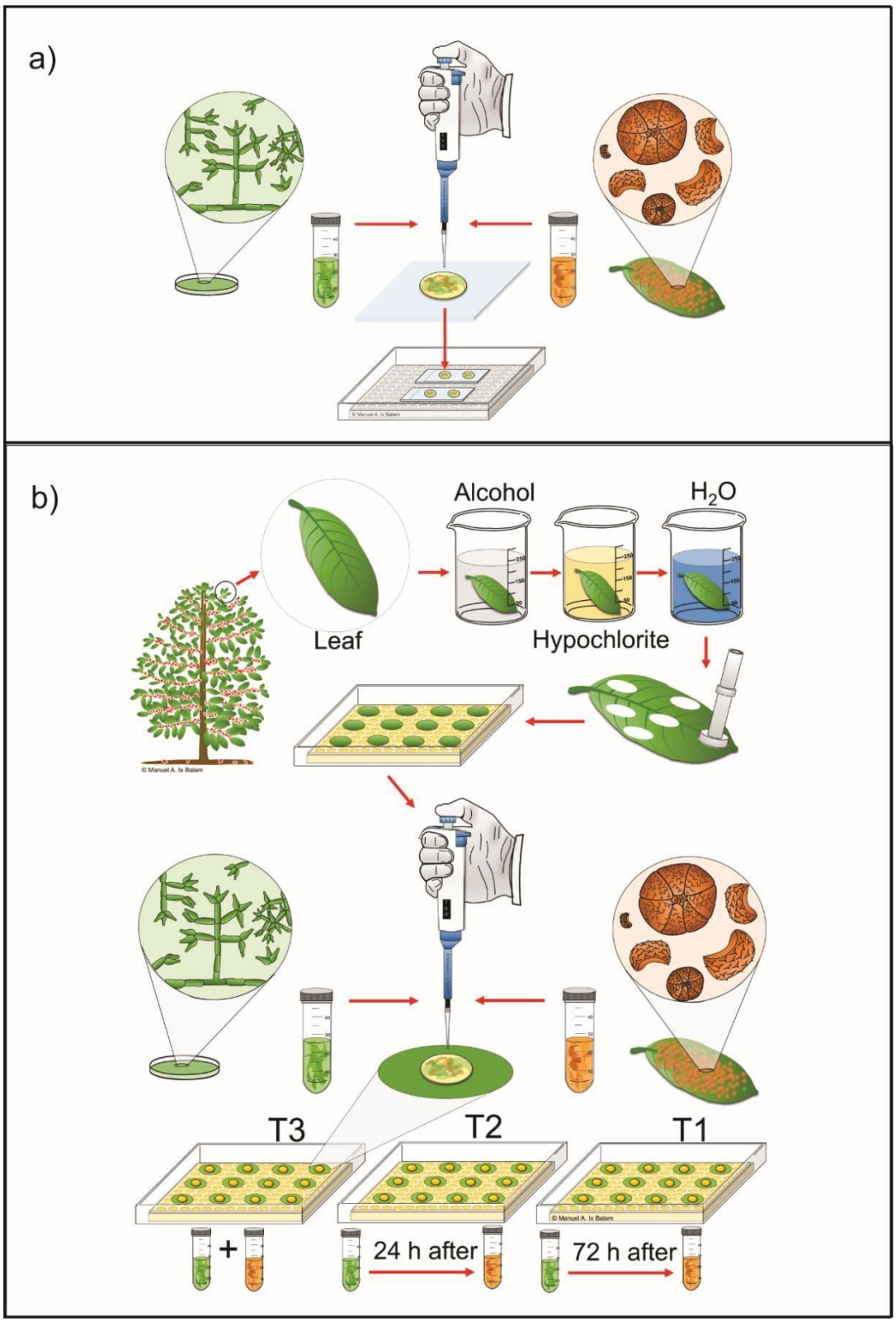

Fig. 1a) Steps followed in the experiments aimed at evaluating the reduction of germination of urediniospores of *Hemileia vastatrix* (orange) by endophytic fungi (green). b) Ibid experiments evaluating reduction of coffee leaf rust severity on coffee-leaf discs by endophytic fungi.

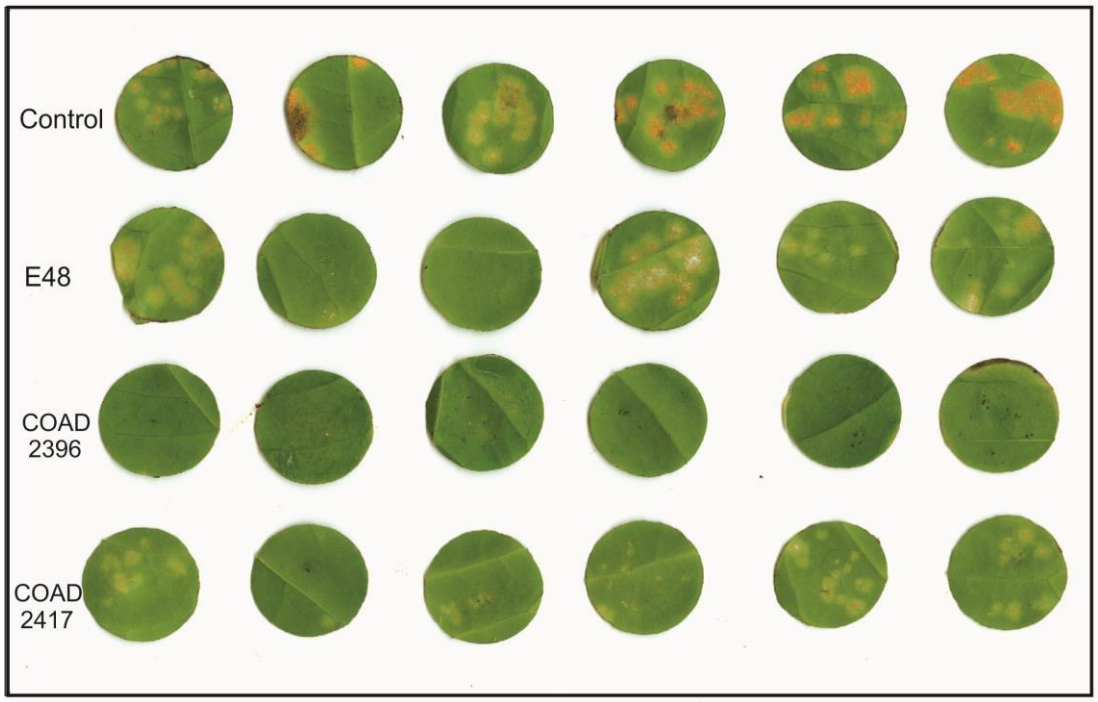

Fig. 2 Examples of images captured of coffee leaf discs inoculated with *Hemileia vastatrix* alone (control) as compared with treated with E48 (*Cordyceps* sp.), COAD 2396 (*T. atroviride*) and COAD 2417 (*T. hamatum*) 24 hours before deposition of urediniospores of *H. vastatrix.* Images captured 35 days after experiment began. Note significant reduction in disease on endophyte treated versus control.

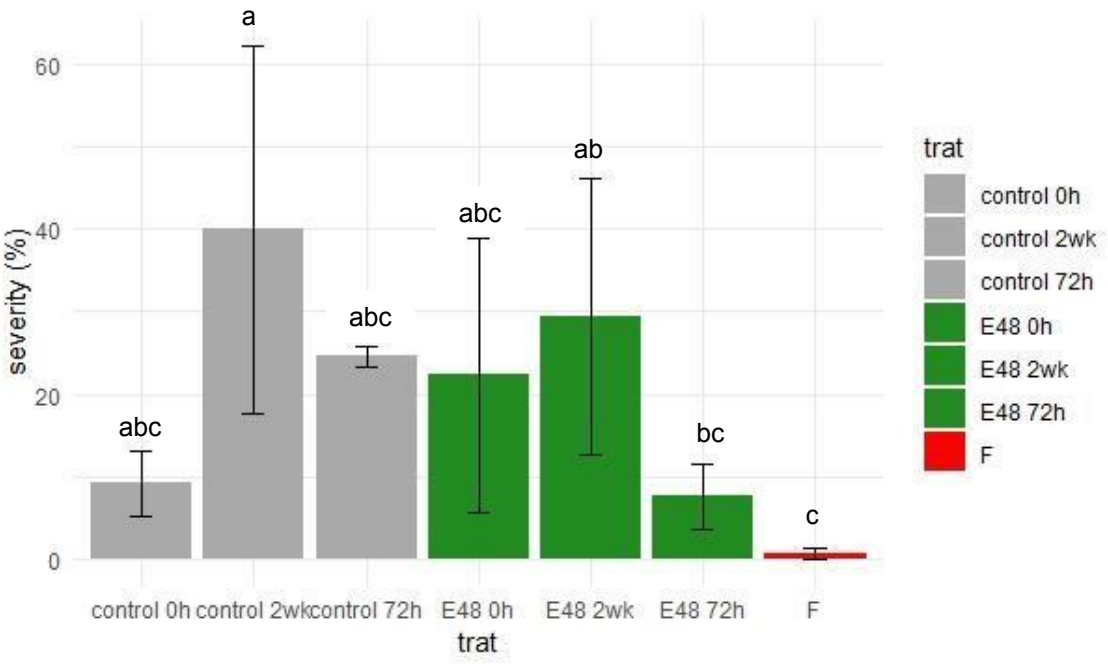

Fig. 3 Coffee leaf rust severity on plants inoculated with *Hemileia vastatrix* and treated or untreated with E48 (*Cordyceps* sp.). Endophyte applications 72 h, two weeks before or simultaneously with inoculation with *H. vastatrix*. F (Fungicide treatment). Means followed by the same letter do not differ from each other (Tukey $\alpha = 0.05$).

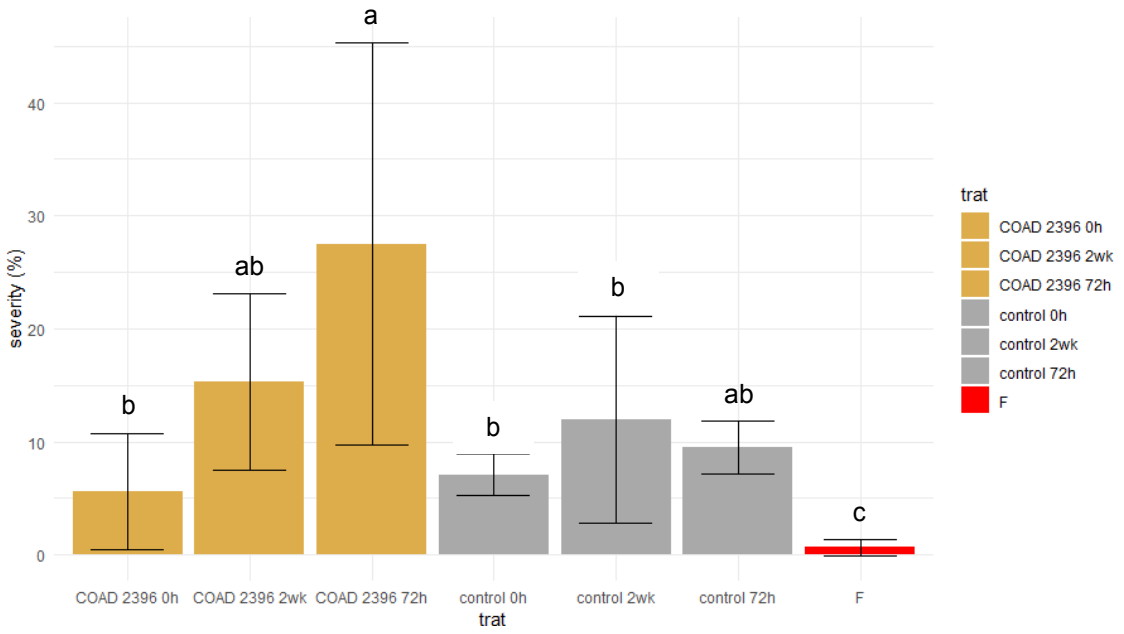

Fig. 4 Coffee leaf rust severity on plants inoculated with *H. vastatrix* and treated or untreated with COAD 2396 (*T. atroviride*). Endophyte applications 72 hs, two weeks before, or simultaneously with inoculation with *H. vastatrix*. F (Fungicide treatment). Means followed by the same letter do not differ from each other (Tukey α = 0.05).

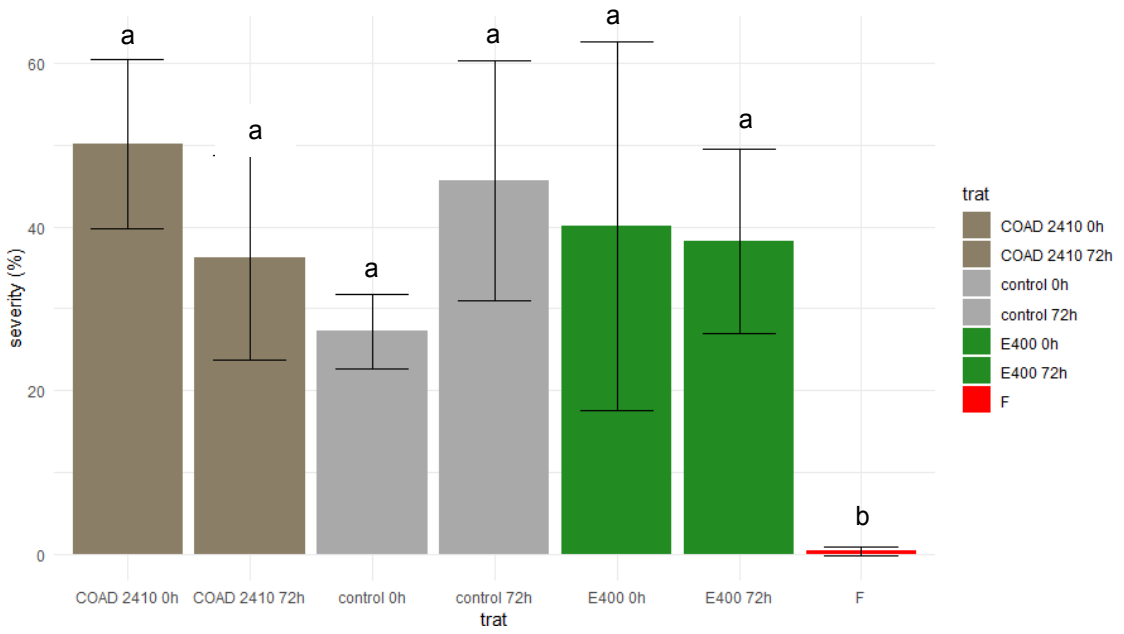

Fig. 5 Coffee leaf rust severity on plants inoculated with *H. vastatrix* and treated or untreated with E400 (*Pestalotiopsis* sp.) and COAD 2410 (*T. koningiopsis*). Endophyte applications 72 h, 24 h or simultaneously with *H. vastatrix*. F (Fungicide treatment). Means followed by the same letter do not differ from each other (Tukey α = 0.05).

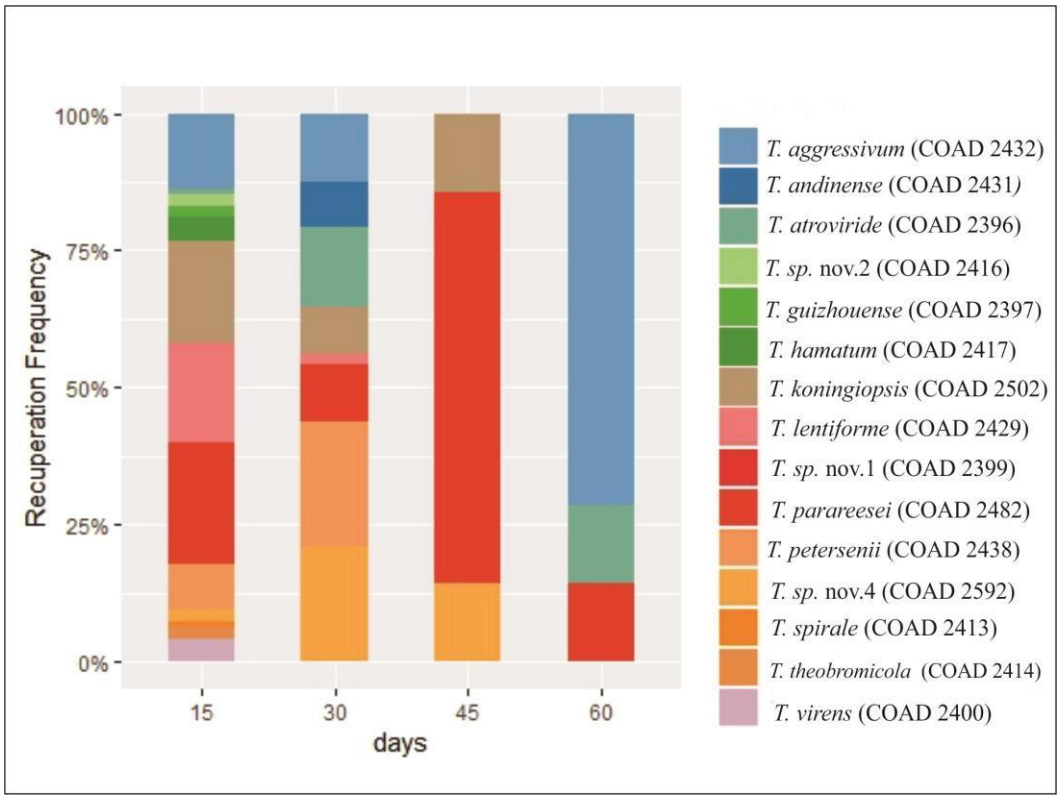

Fig. 6 Recovery percentage of *Trichoderma* spp. from leaves of inoculated coffee plants 15, 30, 45 and 60 days after inoculation. *Trichoderma* spp. absent from control plants.

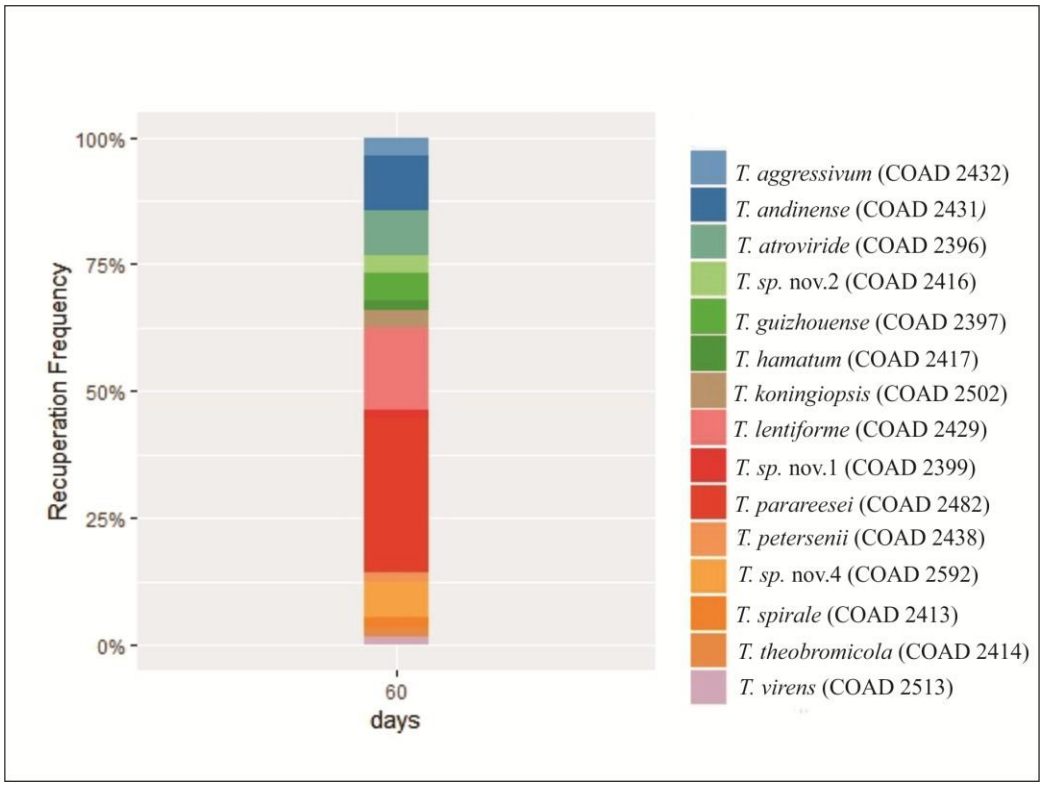

Fig. 7 Recovery percentage of *Trichoderma* spp. from stems of coffee plants 60 days after spray inoculation. Note tha all species of *Trichoderma* tested managed to establish on stems. *Trichoderma* spp. absent from control plants.

Chapter 3

Trichoderma parareesei grows endophytically and promotes root development of tomato

Abstract

Trichoderma spp. are among the most broadly studied fungi as antagonists of plant pathogens (biological control agents) or as plant growth-promoting fungi. During a search for fungi antagonistic to coffee leaf rust in Africa 16 species of *Trichoderma* were obtained. One of these was *Trichoderma parareesei,* which is a recently described species for which there is still relatively little information available in the literature. It was originally described from soil, but was obtained, during an ongoing survey for coffee rust fungal antagonists in Africa, growing as an endophyte in coffee and also as a mycoparasite in coffee rust pustules. This species is known to produce cellulases abundantly. Recent studies have indicated that this fungus can promote plant growth under stress conditions and stimulate disease resistance. Tomato is commonly used as a model-plant to evaluate plant growth promotion capacity of symbiotic microbes. Prior to testing *T. parareesei* on its original coffee host, tests were performed on selected commercial cultivars of tomato. Preliminary test demonstrated the ability of *T. parareesei* to grow endophytically in tomato and suggested that beneficial effects of the association to tomato might result. Two experiments were carried out in greenhouse conditions. These have equivalent designs but one involved plants cultivated in pasteurized soil and the other involved plants grown in non-pasteurized soil. In both experiments *T. parareesei* was incorporated to the soil, firstly one week after the germination of the tomato seeds and a second addition of *T. parareesei* inoculum was performed one month after transplanting. Five growth parameters were evaluated: plant height, stem diameter at the base, dry mass of aerial part, length of root system and dry mass of the root system. Results varied widely depending on the tomato cultivar utilized. Although an increase in several of the variables evaluated was noticed as a result of the application of *T. parareesei*, for most cultivars this was not significant ($\rho<0.05$). One clear exception was increase of length and root mass for the 'Santa Cruz' cultivar. This was observed both in pasteurized and non-pasteurized soil. In the former an increased length of root system and an increase of dry mass of the root of respectively 54.6 and 57.6% (pasteurized soil) and 33.3 and 225% (non-pasteurized soil) was obtained. Its significant beneficial effect for tomato roots of cultivar Santa

Cruz deserve further attention as well as other potential benefits which were not evaluated here such as protection against plant pathogens or abiotic stresses.

Keywords: *Coffea*, plant growth promotion; tomato cultivars.

Introduction

Trichoderma spp. are a common inhabitant of the soil which may saprophytic or mycoparasitic. The success of several species of *Trichoderma* as biological control agents is due to several factors such as rapid growth, efficiency in the use of nutrients, ability to modify the rhizosphere, strong antagonism to phytopathogens and efficiency in promoting growth in plants (Harman et al. 2004; Lorito et al. 2010; Tucci et al. 2011).

During a search for fungal antagonists of coffee leaf rust (CLR) caused *by Hemileia vastatrix* in the native range of coffee in Africa a large number of isolates of mycoparasitic and endophytic fungi was obtained. Special attention was given to members of *Trichoderma* spp. sixteen species, including four new taxa, were identified. One of these species was recognized as *T. parareesei*. This particular taxon caught our attention because some of the isolates belonging to that species, such as E840, besides inhibiting the germination of *H vastatrix* urediniospores, was fast-growing, sporulated abundantly and appeared to produce secondary metabolites which intensively pigmented the medium. Additionally, preliminary essays suggested that it might have plant growth promoting properties.

Trichoderma parareseei was recently described as a new species, originally obtained from soil of subtropical and tropical areas in South America, México and Africa (Atanasova et al. 2010) It was recognized as an efficient cellulase-producing species (Druzhinina et al. 2010). It is a sister-species of *Trichoderma reesei* another effective cellulose and hemicelluloses-degrading fungus, which is currently used in industrial processes (Li et al. 2017; Reese et al. 1950). One of the differences between these two species is that *T. parareesei* lacks a sexual morph and is considered to be ancestral to *T. reesei* (Atanasova et al. 2010). *Trichoderma parareesei* grows faster and is a more prolific conidial producer than its sister-species when grown in different carbon source media and is well adapted to a range of light conditions. In tests of confrontation against pathogens it showed a stronger potential for use in biocontrol than *T. reesei* (Alani and Albaayit 2018; Druzhinina et al. 2010; Rubio et al. 2014).

Although relatively little has been published about *T. parareesei* – particularly on its effect on plant physiology and as an antagonist of phytopathogens - there is one study (Rubio et al. 2014) showing that *T. parareesei* increases the development of

secondary roots of tomato induces systemic resistance against pathogens, and promotes plant growth in adverse conditions such as under saline stress.

Tomato (*Solanum lycopersicum*) has been used as a model plant for various studies related to plant growth and resistance to diseases (Chang et al. 2016). There are several practical reasons that have placed the tomato as a model plant such as ease of cultivation, fecundity, short generation time and in addition to its great economic value (Chang et al. 2016). There are several publications involving promotion of growth in tomato by *Trichoderma* spp. (Azarmi et al. 2011; Fontenelle et al. 2011; Li et al. 2015; Tucci et al. 2011).

A preliminary test, involving eight isolates (COAD 2397, COAD 2502, COAD 2429, COAD 2482, COAD 2592, COAD 2396, COAD 2414 and COAD 2536) of selected *Trichoderma* species obtained in African involving inoculations of tomato plants (cultivar "Santa Clara") has indicated that *T. parareesei* might have plant growth-promoting properties. This led to it being further evaluated in the essays described herein.

Material e methods

Fungus isolate and inoculum preparation

The isolate COAD 2482 originally isolated from coffee stem in Kaffa Region, Bonga District, Ethiopia was obtained from the culture collection of the Universidade Federal de Viçosa (Coleção Octávio Almeida Drummond – COAD). COAD 2482 was kept for regular use during experiments in potato carrot-agar (PCA) slant tube and was preserved in COAD deep-freezing (-80°C) in 10% glycerol and also in sílice gel until its use. Fungus inoculum was prepared as follows: isolate COAD 2482 was grown under a 12 h light regime in plates containing PCA at 25 °C during 7-10 days, after which abundant sporulation was present. Each plate was flooded with 10 mL of a solution of Tween 80 (0.1%) and the conidia and mycelium was rubbed with a paintbrush. The resulting suspension was calibrated to $1x10^6$ spores/ml with the help of a Neubauer chamber.

Seed germination

Fifty tomato seeds of the cultivars –Santa Cruz‖, –Italiano‖, –Maça‖ and –Cereja‖ were evenly spread over a layer of sterilized filter paper soaked s with sterile

water in individual plastic boxes. Each box was sealed with plastic film in order to maintain a high relative humidity and these were left 22°C during 7 days.

Plant growth promotion assays

After germination, twenty-two newly germinated tomato seedlings of each cultivar were submerged in a *T. parareesei* 1×10^6 conidia/mL in 0.1% Tween 80 solution conidial suspension for a period of 30 min. Twenty-two seedlings of each cultivar served as controls and were treated similarly but submerged in a 0.1% Tween 80 solution.. Subsequently each seedling was individually transplanted to 500 mL plastic pots containing a pasteurized substrate (Tropstrato HT Hortaliças®). The plants were wet every day with distilled water for one week and kept in the greenhouse After one month the plants were transplanted to 2L pots containing unpasteurized soil (experiment 1) and pasteurized soil (experiment 2). Pasterurization of the soil was performed by solarization on a Ghini type solariser (Embrapa 2017). At this stage a second inoculation with *T. parareseei* was carried out. At this instance 20 mL of 1×10^6 conidia/mL in 0.1% Tween 80 solution were added in the soil around the plant to each pots. Only 20mL of the Tween 80 (0.1%) suspension were added to controls. Two plants inoculated with E840 of each cultivar and two plants of each control of each cultivar were selected and used exclusively for the COAD 2482 recovery test in the final evaluation.

The evaluation was performed 60 days after the first inoculation in the following way: the height was measured with one meter of metal tape from the base of the plants to the last pair of leaves. The diameter of the stem was measured at the base using a digital pachymeter. The roots were cut and washed and subsequently measured with a ruler. For the dry mass, the aerial part and the roots were collected and placed inside paper bags and then in a stove at 65-70° C until obtaining constant mass.

Recovery of *Trichoderma parareesei* from tomato plants

For the recovery of the *T. parareesei* to confirm endophytic colonization of tomato plants after the application of COAD 2482 an indirect isolation protocol was followed. Root, leaf and stem samples were obtained from two plants previously selected of each tomato cultivar (as mentioned above) – inoculated and non-inoculated with *T. parareesei.* one day after the evaluation. 10 fragments of each plant were taken. These were sequentially dipped for 1 min in 70% ethanol, 2 min in sodium 2%

hypochlorite and for 1 min in sterilized water (Arnold et al. 2000).These were next rolled over sterile filter paper for removal of the water film and transferred to plates containing PCA and left in an incubator at 22°C and examined daily under a stereoscopic microscope (MOTIC SZM-168) for observation of possible emergence of fungal colonies. Whenever colonies suspected of being E840 were observed a slide was made and visualized under the microscope (Olympus CX 31) to confirm that it was *Trichoderma* colony.

Experimental design

Two independent experiments were carried out, one on pasteurized soil and the other on non-pasteurized soil. In both experiments the experimental design mentioned below was used.

Each experiment was organized as a two-way factorial in a completely randomized design (four cultivars – Santa Cruz, Italiano, Maça and Cereja two *Trichoderma* treatments – inoculated (Tr+) × non-inoculated (Tr-) with ten replications (one potted plant).

Data analysis

A generalized linear model with a Gaussian family was fitted to the data. The statistical analyses were conducted in the R statistical computing environment using the base *glm* functions for the Anova models. Least square means (lsmeans) were calculated and treatment means were compared based on *t-test* ($\rho = 0.05$).

Results

Recovery of *Trichoderma parareesei* from tomato

The fungus was recovered from the plants belonging to the four cultivars and that were inoculated with *T. parareesei*. From the cultivars "Santa Cruz", "Italiano" and "Cereja" the fungus was recovered from the root and of the hybrid "Maça" was recovery from the stem. The isolate was not recovered from any of the plants belonging to the controls (Fig. 2).

Plant growth promotion assays in greenhouse

For most variables which were evaluated there seemed to be a trend towards increased tomato growth resulting from the application of *T. parareesei* on the different cultivars either in pasteurized or non-pasteurized soil (Table 1). Nevertheless, this was

not significant and the interaction fungi application × cultivar was not significant ($\rho > 0.05$). Exceptions were for root length and dry mass in –Santa Cruz‖ cultivar – either on pasteurized or non-pasteurized soil (Table 1). For the other variables: height plant, stem diameter and aerial dry mass influence was restricted to the tomato variety involved.

Generally, each of the cultivars showed a different response to *T. parareesei*. "Santa Cruz" plants inoculated with COAD 2482 showed an increase for all variables when compared with non-inoculated plants on pasteurized and non-pasteurized soil (Table 1). The most significant effect observed for –Santa Cruz‖ was for root length and root biomass. Root length had an average increase of 12.4 and 5.3 cm as compared to controls respectively on pasteurized and unpasteurized soil. This represented 54.6 and 33.3% increases, respectively. For root biomass (dry mass of the root) the increase was of 0.15g (57.5% as compared with control) and 0.63g (225% as compared to control) respectively on pasteurized and non-pasteurized soil (Table 1).

The cultivar "Cereja" plants inoculated with COAD 2482 showed an increase of most of the variables evaluated when grown in pasteurized soil. The exception was root length which in average grew 7% less in length when compared with control. On non-pasteurized soil only two variables showed an increase in plants inoculated with COAD 2482, namely: stem diameter (3.9%) and root length (5.3%) (Table 1).

For the other two cultivars "Maça‖ and –Italiano" the results were very variable; for example, the cultivar "Maça" showed an increase of 5, 2.8 and 1.8% respectively for height, root dry mass and stem diameter when compared with control on pasteurized soil. However, in the experiment involving non-pasteurized soil a general decrease of growth parameters was observed in E840 treated plants (Table 1).

The –Italiano‖ cultivar plants inoculated with COAD 2482 showed an increase for all growth parameters when grown in no-pasteurized soil whereas the opposite happened in pasteurized soil (Table 1).

The results obtained from pasteurized soil show that the height and the stem diameter tended to increase (although not statistically significant) in cultivars –Santa Cruz‖, –Italiano‖ and –Cereja‖ when inoculated with *T. parareesei*. Similarly, on unpasteurized soil a tendency of improved stem diameter growth was noticed for –Santa

Cruz‖, –Italiano‖ and –Cereja‖ plants treatred with COAD 2482 – but, yet again, with no statistical significance supporting the observation cultivars (Table 1).

Discussion

It was observed that *T. parareesei* (COAD 2482) is able to colonize endofitically the stems and roots of the four cultivars included in the test (Fig.2). This suggests that despite not having an intense effect on growth promotion for most plant parts of most cultivars except cultivars "Santa Cruz", *Trichoderma parareesei* has good endophytic capacity, a feature not yet known for this species before this study. As it is generally expected that endophytic *Trichoderma* may promote host growth (Stewart and Hill 2014), It is reasonable to conjecture that the trends observed of growth stimulation for other cultivars, although not detected as significant if given a longer duration for future experiment may have reveale benefits for other cultivars other than –Santa.Cruz‖.

Host genotype had a clear influence in the growth reaction produced by *T. parareesei.* There was stimulation, no effect or inhibition of growth of different plant parts depending on the host variety. For most varieties tested cultivars COAD 2482 was not particularly effective at promoting plant growth. The clear exception being root growth stimulation for the –Santa Cruz‖ cultivar. Over 50% increase in length and dry mass of the root obtained in pasteurized soil (Table 1) is a significant result. A similar phenomenon was reported for tomato cultivar "Marmande" with the application of *T. parareesei* by (Rubio et al. 2014). These authors obtained increased *in vitro* growth of number and length of lateral roots for plants belonging to this cultivar when exposed to *T. parareesei.* This increase in root mass is generally attributed to the stimulation that *Trichoderma* exerts on the plant for the production of hormones such as auxins and gibberellins, where auxins have a more important role for the growth and development of the root (Blake et al. 2000; Stewart and Hill 2014).

Another consequence of the application of COAD 2482 observed in the hybrid "Santa Cruz" was the *ad hoc* observation of a faster flowering being stimulated with the application of *T. parareesei* as compared with controls for –Santa Cruz‖. It has already been reported that inoculation with *Trichoderma* species may have a positive effect on the flowering of some plants (Stewart and Hill 2014)- a favorable factor for crop production. Nevertheless, this is a novel and unexpected observation for *T. parareesei* × tomato. This deserves further investigation in future experiments to be conducted.

On pasteurized soil, the isolate COAD 2482 showed a negative effect on plant growth when compared with the control in "Italian" cultivar. The same happened with cultivar "Maça" on non-pasteurized soil. Reactions for cultivar "Cereja" were unequal with a mixture of positive and negative effects of association with the fungus. Such irregular results for growth promotion in tomato have already been documented for *T. harzianum* and *T. atroviride* as influenced by host genotype Tucci et al. (2011) found that *T. atroviride* increased plant growth in two tomato varieties and reduced it in three other varieties. Harman (2006) found that *T. harzianum* T22 promoted growth in some maize cultivars, had no effect in others and had a negative impact on plant growth of others. We have not investigated the mechanisms underlying these differential responses. However, in addition to the strong influence of the genotype, as mentioned above, there are other factors that can intervene in the effect of *T. parareesei* on growth promotion such as: the method of inoculantion and the inoculums concentration as documented for other *Trichoderma*-plant associations (Baker et al. 1984; Stewart and Hill 2014). Increasing concentrations of inoculum of *T. harzianum* were observed to have increasing effects on the stimulation of seed germination and at increase in length of stem and root of *Passiflora edulis* (Cubillos-hinojosa et al. 2009) also found that higher gains in dry weight and leaf area were obtained in radish and pepper plants treated with *T. harzianum* delivered in peat-bran as compared with conidial suspension (Kleifeld and Chet 1992). It is likely that more factors have an influence on the performance of endophytic *Trichoderma* as beneficial partners of cultivated plant hosts, making the task of understanding these fungus-plant relationships and translating them into practical applications particularly challenging.

Several properties have made species of *Trichoderma* choice for candidates to be used in biocontrol and as active ingredients in biocontrol products (biofungicides) and as plant growth promoting agents. These are discussed in various publications such as in Bailey and Melnick (2013) and Stewart and Hill (2014).*Trichoderma parareesei* is a fast-growing fungus which sporulates abundantly in culture and is easy to manipulate. These are all hightly desired features for a potential biocontrol agent.

Although the results obtained in this study might indicate that *T. parareesei* is not particularly effective at stimulating tomato growth cultivars it is important to highlight that the present results are only partial and may have hidden benefits that might have appeared only in a longer period of time. Additionally, there is the

possibility of *T. parareesei* (COAD 2482) providing other important benefits to its hosts which were not evaluated in the experiments described above such as: protection against environmental stresses such as drought or soil salinity and protection against pathogens. For the latter there are published results (Rubio et al. 2014) showing that *T. parareesei* promotes systemic resistance against *Botrytis* sp. in tomato and is highly antagonistic against *Pythium irregulare* the evaluation of *T. parareesei* (COAD 2482) in longer duration experiments in tomato, inclusively involving challenging COAD 2482-colonized plants with *Phytophthora infestans*, not to mention testing it on its original host *Coffea arabica* may reveal untapped possible uses for this as yet poorly known species and isolate.

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Table 1 Effect of inoculation of *Trichoderma parareesei* on growth parameters of different tomato cultivars.

Variable/factor	**Treatment**					
	Pasteurized soil			**Non-pasteurized soil**		
cultivar	Tr (-)	Tr (+)	Tr effect (%)	Tr (-)	Tr (+)	Tr effect (%)
Height (cm)						
Santa Cruz	62.9cd (±2.46)	66.0d (±1.49)	+4.9	58.7cd (±2.86)	59.3d (±1.91)	+1.0
Italiano	49.6a (±1.65)	47.1a (±2.52)	-5.1	41.7a (±3.11)	48.6ab (±2.92)	+16.5
Maça	53.5ab (±1.98)	56.2abc (±2.19)	+5.0	49.9b (±1.66)	46.1ab (±1.93)	-7.7
Cereja	55.5abc (±1.54)	59.1bcd (±2.51)	+6.4	51.0bc (±1.88)	50.4b (±2.66)	-1.2
Stem diameter (mm)						
Santa Cruz	4.9ab (±0.36)	5.4bc (±0.14)	+10.2	4.7abc (±0.23)	5.0bc (±0.09)	+6.3
Italiano	4.4a (±0.13)	4.3a (±0.16)	-2.3	4.1a (±0.21)	4.6ab (±0.10)	+12.1
Maça	5.4bc (±0.18)	5.5bc (±0.15)	+1.8	5.4c (±0.21)	4.9bc (±0.23)	-9.3
Cereja	5.5bc (±0.21)	5.9c (±0.18)	+7.2	5.1bc (±0.18)	5.3bc (±0.24)	+3.9
Root length (cm)						
Santa Cruz	22.7ab (±2.58)	35.1b (±4.78)	+54.6	15.9a (±1.55)	21.2a (±2.00)	+33.3
Italiano	23.3ab (±3.01)	18.5a (±2.65)	-20.7	17.0a (±2.02)	18.4a (±2.11)	+8.2
Maça	26.3ab (±2.56)	21.6ab (±2.05)	-17.9	19.5a (±2.17)	16.5a (±1.06)	-15.4
Cereja	34.5b (±2.27)	32.1b (±3.58)	-7.0	20.4a (±1.14)	21.5a (±2.08)	+5.3
Dry mass of the root (g)						
Santa Cruz	0.26a (±0.03)	0.41ab (±0.05)	+57.6	0.28a (±0.03)	0.91b (±0.12)	+225
Italiano	0.29a (±0.04)	0.25a (±0.05)	-13.8	0.32a (±0.10)	0.37a (±0.09)	+15.6
Maça	0.35ab (±0.04)	0.36ab (±0.04)	+2.8	0.44a (±0.07)	0.32a (±0.02)	-27.3
Cereja	0.44ab (±0.05)	0.526b (±0.06)	+18.1	0.39a (±0.06)	0.38a (±0.09)	-2.6
Dry mass of the aerial part (g)						
Santa Cruz	2.4ab (±0.26)	2.5ab (±0.23)	+4.1	2.8bc (±0.29)	3.2c (±0.28)	+14.2
Italiano	2.2ab	1.9a	-13.7	1.4a	1.7ab	+21.4

	(±0.24)	(±0.16)		(±0.21)	(±0.20)	
Maça	3.5abc (±0.29)	3.5bc (±0.40)	0.0	2.7bc (±0.30)	2.0ab (±0.17)	-26
Cereja	4.1c (±0.34)	4.8c (±0.65)	+17	2.3abc (±0.23)	2.3abc (±0.38)	0.0

*Lsmeans estimates for the effect in tomato hybrid and *T. parareseei* treatment on height stem diameter, root length, dry mass of root and dry mass of aerial part in the cultivars Santa Cruz, Italiano, Maça and Cereja grown in pasteurized and unpasteurized soil.

**Treatments with soil non inoculated (Tr-) or inoculated (Tr+) with *T. parareesei*.

[1] Lsmeans estimates for each variable followed by a same letter do not differ according to a pairwise comparison t-test at 5% probability

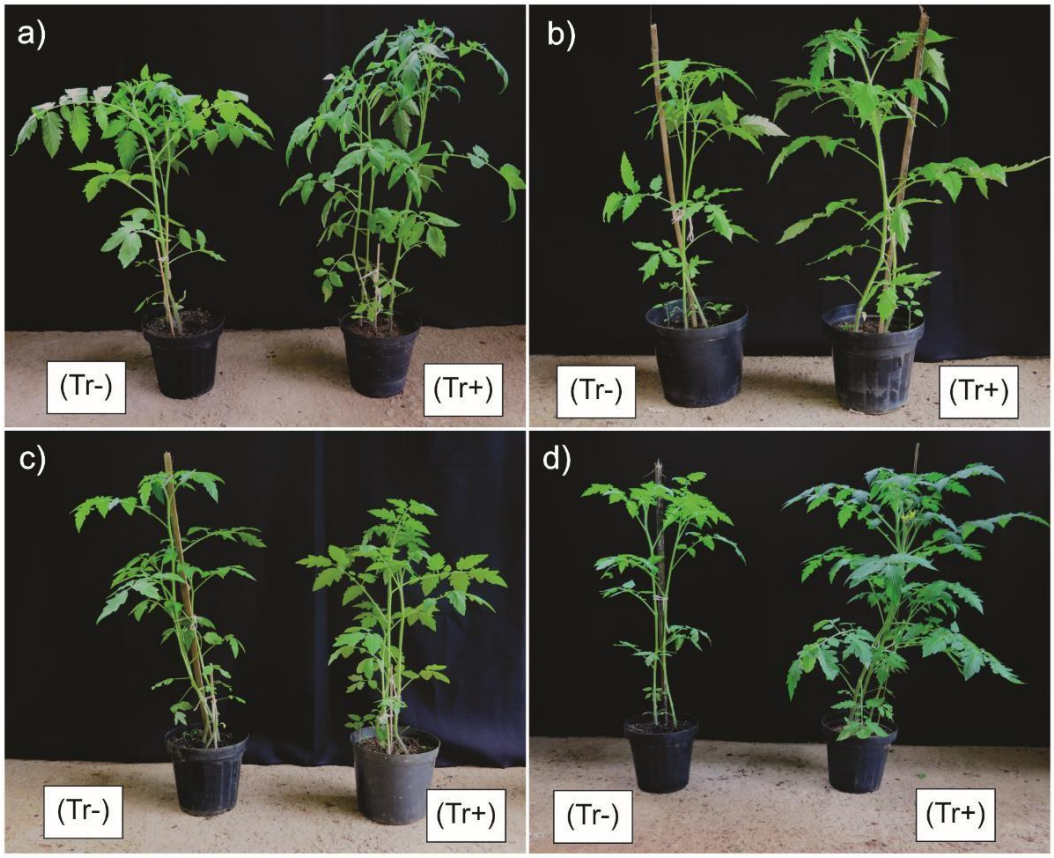

Fig 1. Effects of *T. parareesei* (COAD 2482) on the growth and development of different cultivarsf tomato cultivars. a) ‒Santa Cruz‖; b) ‒Italiano‖; c) ‒Maça‖ and d) ‒Cereja‖. (Tr-) No *T. parareesei*; (Tr+) treated with *T. parareesei*.

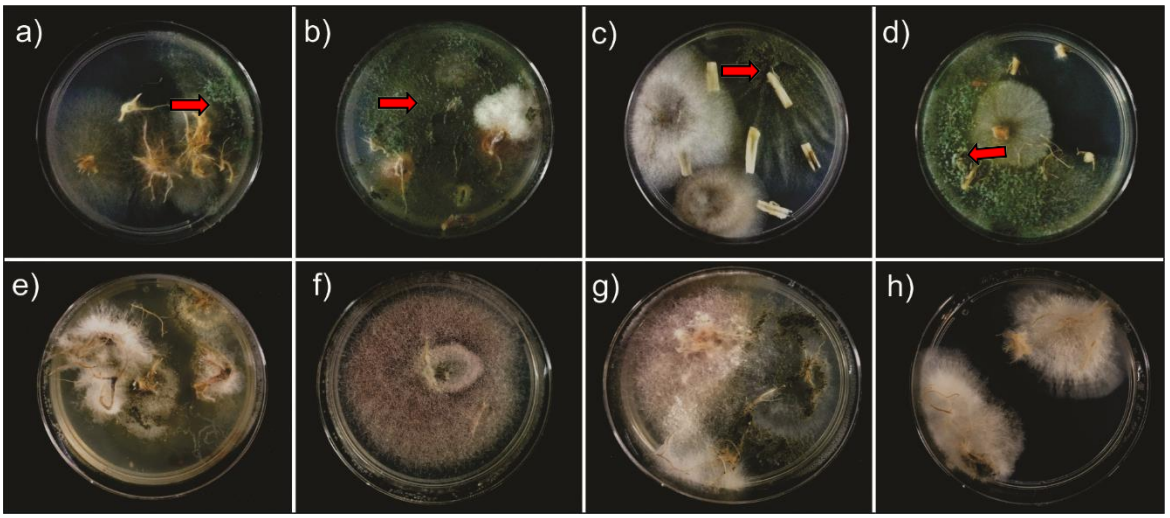

Fig 2. *Trichoderma parareesei* colonies emerging from roots and stems of the different tomato cultivars 2 months after the first inoculation. a-d: roots and stem of plants tomato treated with COAD 2482: cultivars "Santa Cruz", "Italiano", "Maça" and "Cereja". e-h: plants of controls: cultivars "Santa Cruz", "Italiano", "Maça" and "Cereja". Note presence of typical *Trichoderma* colonies (red arrowed) only emerging from treated with plants with the isolate COAD 2482 of each cultivar (a, b, c, d) and absent from untreated plants/controls (e, f, g, h). Colonies emerging from controls are other genera of fungi than can be endophytic or saproprophytes.

GENERAL CONCLUSIONS

- Sixteen species of *Trichoderma* were identified from collections made during the surveys in Africa, the majority representing novel fungus-host associations four of which representing taxa that are new to science.
- Eleven of the *Trichoderma* species were growing as endophytes in coffee, three were mycoparasites and two were obtained in both niches (as endophytes and mycoparasites).
- Isolates of endophytic fungi belonging to *Trichoderma*, *Pestalotiopsis Mucor*, *Aspergillus* and *Cordyceps* of endophytes and mycoparasites collected in Africa reduce coffee rust severity *in vitro.*
- The *in planta* effect of endophytic fungi on reducing CLR was lower when compared to results obtained for the *in vitro* tests for most isolated tested.
- Applications of the isolate E48 – which belongs *Cordyceps* sp. reduced CLR severity *in planta* by more than 50%.
- *Trichoderma parareesei* effectively promoted the growth or root system of tomato cv. Santa Cruz.