THAISA FERREIRA DA NÓBREGA

TAXONOMY OF *Fusarium* spp. ASSOCIATED WITH UREDINIA OF *Hemileia vastatrix* IN BRAZIL, CAMEROON, ETHIOPIA AND PARAGUAY AND THEIR BIOCONTROL POTENTIAL

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

Orientador: Robert Weingart Barreto

Coorientador: Lucas Magalhães Abreu

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Thaisa Ferreira da Nóbrega
Autora

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Orientador

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"Somewhere, something incredible is waiting to be known."

(Carl Sagan)

BIOGRAFIA

THAISA FERREIRA DA NÓBREGA, filha de Maria Ferreira da Silva Nóbrega e Arnaldo Modesto Menezes da Nóbrega, nasceu na cidade de Sobradinho, Bahia, Brasil, no dia 06 de março de 1991, onde cursou o ensino fundamental. Realizou o ensino médio na cidade de Petrolina, PE, Brasil, concluindo em dezembro de 2008.

Em 2010 ingressou no curso de graduação em Engenharia Agronômica na Universidade do Estado da Bahia, Juazeiro, Bahia, Brasil. Graduando-se em fevereiro de 2015.

Em abril de 2015 iniciou o curso de Mestrado em Agronomia/Horticultura Irrigada na Universidade do Estado da Bahia, Juazeiro, Bahia, Brasil, concentrando seus estudos nas áreas de Fitopatologia, Micologia e pós-colheita. Desenvolveu parte de seu mestrado na Embrapa Semiárido (Petrolina, PE), defendendo sua dissertação em março de 2017.

Ainda em março de 2017, iniciou no Programa de Pós- Graduação em Fitopatologia, em nível de Doutorado, na UFV, concentrando sua pesquisa nas áreas de Micologia (Taxonomia de fungos) e controle biológico de doenças de plantas.

RESUMO

NÓBREGA, Thaisa Ferreira, D.Sc., Universidade Federal de Viçosa, maio de 2021. **Taxonomia de *Fusarium* spp. associadas com uredínia de *Hemileia vastatrix* no Brasil, Camarões, Etiópia e Paraguai e seu potencial para o biocontrole.** Orientador: Robert Weingart Barreto. Coorientador: Lucas Magalhães de Abreu.

A ferrugem do cafeeiro (CLR) é a principal doença que atinge esta cultura, provocando grandes prejuízos em todas as regiões produtoras do mundo. Causada pelo fungo *Hemileia vastatrix*, a CLR é controlada principalmente pela aplicação de fungicidas químicos, pelo plantio de cultivares resistentes e pelo escape em cultivo em terras altas. No entanto, surtos iniciados no norte da América do Sul e na América Central desde o início de 2010, demonstraram a necessidade de novas abordagens de manejo. O controle biológico com fungos antagonistas é uma alternativa possível que não foi investigada intensivamente para CLR. Aqui relatamos os resultados de um estudo concentrado em isolados de *Fusarium* spp. coletados de pústulas CLR colonizadas no Brasil e Paraguai, porém mais focados, pela primeira vez, em isolados obtidos da região nativa de *H. vastatrix* e cultivadas com *Coffea* spp. na África (Camarões e Etiópia). A ideia de fundo do estudo era a de um possível uso de candidatos a biocontrole eficazes, seguros e confiáveis sob a abordagem clássica de controle biológico. Aqui, a taxonomia de isolados de *Fusarium* micoparasitas putativos obtidos durante as pesquisas foi elucidada e uma triagem preliminar de 69 isolados foi conduzida a fim de testar seu potencial contra CLR *in vitro* e *in planta*. O estudo taxonômico polifásico revelou sete espécies distintas, quatro das quais são descritas como novas para a ciência, a saber: *F. auranto-glutinosus* sp. nov., *F. cameroonicum* sp. nov., *F. hemileiae* sp. nov., *F. lateritium*, *F. pseudosolani* sp. nov., *F. perseae* e *Fusarium* sp. Mais de 80% dos isolados foram caracterizados como *F. hemileiae* sp. nov. pertencente ao complexo de espécies *Fusarium lateritium* (FLSC). Em testes *in vitro*, 17 isolados reduziram a severidade de CLR em mais de 80% em três regimes de tempo de aplicação. Três isolados de *F. hemileiae* sp. nov. (COAD 3011, COAD 3041 e COAD 3065) reduziram a severidade em 100% e foram selecionados para testes adicionais. Aplicações preventivas de suspensões de conídios dos três isolados selecionados em plantas foram eficientes na redução da severidade de CLR (até 89%) em casa de vegetação, atingindo taxas de controle superiores ao tratamento com fungicida de cobre. As aplicações preventivas de filtrados de cultura de *F. hemileiae* de dois isolados (COAD 3011 e COAD 3065) também reduziram a severidade de CLR, mas de forma menos eficaz do que a suspensão de conídios

(68% e 76%, respectivamente). Aplicações de suspensões de conídios e filtrados após a formação de pústulas em plantas não impediram ou reduziram o progresso de CLR. Não foram observadas colônias de *F. hemileiae* crescendo sobre pústulas em plantas sintomáticas pulverizadas com a suspensão de antagonista. No entanto, em paralelo, foi observado que a suspensão de esporos de COAD 3041 fez com que a maioria dos urediniósporos coletados de folhas pulverizadas com o antagonista perdesse a viabilidade ou produzisse tubos germinativos aberrantes. Presume-se que *F. hemileiae*, embora seja um antagonista eficaz e potente de *H. vastatrix* em algumas condições, pode não ser um micoparasita do fungo CLR. Pesquisas futuras podem confirmar que um dos isolados selecionados de *F. hemileiae* sp. nov. tem potencial para se tornar o ingrediente ativo de um novo biofungicida anti-CLR com benefícios para os cafeicultores, consumidores e meio ambiente.

Palavras-chave: Análise filogenética. Espécie nova. Ferrugem do cafeeiro. Fungo antagonista.

ABSTRACT

NÓBREGA, Thaisa Ferreira, D.Sc., Universidade Federal de Viçosa, May, 2021. **Taxonomy of *Fusarium* spp. associated with uredinia of *Hemileia vastatrix* in Brazil, Cameroon, Ethiopia and Paraguay and their biocontrol potential.** Adviser: Robert Weingart Barreto. Co-adviser: Lucas Magalhães de Abreu.

Coffee leaf rust (CLR) is the main disease that affects this crop, causing great damage in all producing regions of the world. Caused by the fungus *Hemileia vastatrix*, CLR is mainly controlled by the application of chemical fungicides, the planting of resistant cultivars and the escape through highland cultivation. Nevertheless, outbreaks have started in Northern South America and Central America since the early 2010s which have demonstrated the need for new management approaches. Biological control with antagonistic fungi is a possible alternative which hasn't been intensively investigated for CLR. The results on a study concentrated on isolates of *Fusarium* spp. collected from colonized CLR pustules in Brazil and Paraguay, but more focused, for the first time, on isolates obtained from the native region of *H. vastatrix* and cultivated *Coffea* spp. in Africa (Cameroon and Ethiopia) were reported here. The background idea of the study, was that of a possible use of effective, safe and reliable biocontrol candidates under the classical biological control approach. Here, the taxonomy of putative mycoparasitic *Fusarium* isolates obtained during the surveys was elucidated and a preliminary screening of 69 isolates was conducted in order to test their potential against CLR *in vitro* and *in planta*. The polyphasic taxonomic study revealed seven distinct species, four of which are described as new to science, namely: *F. auranto-glutinosus* sp. nov., *F. cameroonicum* sp. nov., *F. hemileiae* sp. nov., *F. lateritium*, *F. pseudosolani* sp. nov., *F. perseae*, and *Fusarium* sp. More than 80% of the isolates were characterized as *F. hemileiae* sp. nov. belonging the *Fusarium lateritium* species complex (FLSC). In *in vitro* tests, 17 isolates reduced the severity of CLR by more than 80% in three application time-regimes. Three isolates of *F. hemileiae* sp. nov. (COAD 3011, COAD 3041 and COAD 3065) reduced the severity by 100% and were selected for further testing. Preventive applications of conidial suspensions of the three selected isolates in plants were efficient in reducing CLR severity (up to 89%) in greenhouse, reaching control rates superior to the copper fungicide treatment. Preventive applications of *F. hemileiae* culture filtrates from two isolates (COAD 3011 and COAD 3065) also reduced CLR severity, but less effectively than conidial suspension (68 and 76%, respectively). Applications of conidial suspensions and filtrates after pustule formation in plants did not prevent or reduce CLR

progress. No *F. hemileiae* colonies were observed growing over pustules on symptomatic plants sprayed with the antagonist suspension. However, in parallel it was observed that the spore suspension of COAD 3041 made the most urediniospores collected from antagonist sprayed leaves to lose viability or to produce aberrant germ tubes. It is conjectured that *F. hemileiae*, although being an effective and potent antagonist to *H. vastatrix* under some conditions may not be a mycoparasite of the CLR fungus. Future research may confirm that one of the selected isolates of *F. hemileiae* sp. nov. has the potential to become the active ingredient for a novel anti-CLR biofungicide with benefits for coffee farmers, consumers and the environment.

Keywords: Phylogenetic analysis. New species. Coffee rust. Antagonistic fungus

SUMMARY

GENERAL INTRODUCTION

Coffee is one of the most valuable primary products traded in the world, surpassed only by petroleum (Caixeta, 2017). Coffee is produced in around 72 countries, and almost all nations consume it. In the last year (02/2020 – 02/2021) the world exports of *Coffea arabica* totalized 81.05 million bags (60 kg) and *Coffea canephora* exports amounted to 47.52 million bags. Four countries account for approximately 70% of the world's supply: Brazil, Vietnam, Colombia and Indonesia (ICO, 2021). Brazil is the world's largest producer and exporter of coffee, and is the second largest consumer of the product, currently having around 1.75 million hectares in production. In 2020, Brazil produced 63,077 thousand bags of grain, which was considered a record within the historical series. The states of Minas Gerais, Espírito Santo, São Paulo, Bahia and Rondônia contributed 97% of the national production in this harvest (CONAB, 2021).

Coffee leaf rust (CLR) is the worst disease affecting coffee worldwide and causes losses of one to two billion US dollars annually (McCook, 2006). Two species of *Hemileia* can cause CLR, *H. vastatrix* and *H. coffeicola*, but *H. coffeicola* is of limited economic importance and is geographically confined in Cameroon (Ritschel, 2005). CLR (*H. vastatrix*) symptoms and signs include large orange spore masses on the lower leaf surface, leading to premature leaf fall. The causal agent was first described based on samples collected in Sri-Lanka (formerly Ceylon) in 1869. The emergence of CLR destroyed the coffee industry of Sri-Lanka with devastating social and economic consequences (Morris, 1880). *Hemileia vastatrix* has spread to all coffee producing regions of the world since this first disastrous outbreak in Sri Lanka (Várzea and Marques, 2005).

In the years 2012 to 2014, CLR was responsible for losses ranging between 25 and 60% in the countries producing *C. arabica* in Central America (Nicaragua, El Salvador, Guatemala and Panama) and South America (Ecuador). More than 50% of the Central America coffee producing area was affected by the new outbreak of CLR, causing an estimated loss of US $ 500 million in the 2012/2013 harvest alone (Avelino et al., 2015; Zambolim and Brenes, 2018). This generated a direct impact to smallholding coffee producers, in the economy of entire countries and to the food security of the coffee-growing countries of Central America due their high dependence on this commodity (Avelino et al., 2015).

Currently, the main control strategy used for CLR (particularly in Brazil) are the application of contact copper-based and systemic fungicides such a triazoles and strobilurins

(Talhinhas et al., 2017). However, chemical control, is regarded as representing an environmental hazard and a potential threat to consumers health (Kanoun-Boulé et al., 2008; Loland and Singh, 2004), not to mention the financial cost aspect. Although highly desired, obtaining durable genetic resistance has been a challenge for breeders, particularly in the face of the emergence of new races of *H. vastatrix* (Brito et al., 2010). Moreover, the demand for environmentally benign and sustainable alternatives is on the rise for agriculture in general, including coffee. A recent example of a broad stakeholder's initiative to meet that demand is, what was called ECOFFEE (https://www.andifes.org.br/?p=88783). This is an ambitious programme launched by CIRAD aiming at, in the middle term, significantly reduce to even abolish the application of chemical pesticides in coffee worldwide. Before that, since 2014, the World Coffee Research has been funding research, based at the Universidade Federal de Viçosa (UFV), having the purpose of investigating fungi as biological control agents to be deployed against CLR, particularly under the classical biological control approach.

Classical biological control targets noxious invasive species. It involves the use of natural enemies collected the center of origin of the invasive species (a pathogen, arthropod pest or weed), which (after detailed study and evaluation – particularly safety) may be released in invaded. The biological control agent population may become established, build up and result in permanent pest population reduction and continuous and ever-increasing economic benefits (Cock et al. 2010). *Coffea arabica* originates from the highlands of Ethiopia (Kaffa and Ennarea), where it occurs spontaneously as an understory plant (Caixeta, 2017). CLR was first recorded by an English explorer in 1861 near Lake Victoria (East Africa) on wild *Coffea* species (Talhinhas et al., 2017) but its original distribution in Africa is unknown. The WCR funded-UFV conducted CLR biocontrol project focused on the search, description and evaluation of endophytic fungi from native coffee plants (potentially with an anti-CLR "bodyguard effect") and putative mycoparasites obtained from colonized *H. vastatrix* pustules collected in Africa. In parallel some collections were also made in Brazil and Paraguay. This generated a large and highly diverse collection of isolates, many of which are now being described as representing fungi which are new to science (Cólman, 2018; Crous et al., 2018; Colmán et al., 2021; Rodriguez et al., 2021). Among those fungal isolates several belonged to *Fusarium* spp. and were investigated during the course of this work.

Fungi having direct association with other fungi are known as fungicolous fungi (Barnett, 1963; Rudakov, 1978; Sun et al., 2019). Most of the fungicolous species are mycoparasites or hyperparasites (Barnett, 1963; Boosalis, 1964; Bartkowska, 2007; Baiswar et

al., 2014) and can be important in suppressing fungal diseases of plants and as potential biocontrol agents (Bartkowska, 2007; Baiswar et al., 2014; Videira et al., 2015). More than 50 commercial fungicolous fungi-based biological control products or based on their secondary metabolites have been successfully developed to control plant fungal pathogens (Karlsson et al. 2015; Sun et al., 2019).

The term "mycoparasite" specially refers to the fungi that have the ability to parasitize their fungal host (Barnett, 1963; Sun et al., 2019). In rust fungi (Pucciniales), mycoparasites are often found colonizing several stages of the life cycle (uredinium, telium and aecium) decreasing the viability or the number of spores that would serve as inoculum to infect new organs or new plants (Carrión & Rico-Gray, 2002). *Acremonium byssoides*, *Aphanocladium meliolae*, *Cladosporium hemileiae*, *Calcarisporium* spp., *Calonectria hemileiae*, *Digitopodium hemileiae*, *Lecanicillium* spp., *Paecilomyces lilacinus*, *Simplicillium* spp., *Sporothrix* spp., *Talaromyces wortmannii*, *Verticillium* spp. are names appearing in the literature reported as mycoparasites of *H. vastatrix* (Steyaert, 1930; Gams, 1971; Lim and Nik, 1983; Arriola et al., 1998; Carrión and Rico-Gray et al., 2002; Gómez-De La Cruz et al., 2017; Colmán et al., 2021; Salcedo-Sarmiento et al., 2021). Many of those names appearing in the older literature are either obsolete (eg. *Verticillium* – certainly representing fungi belonging to the assemblage of *Lecanicillium*-like fungi) or represent incomplete or doubtful taxonomic identifications. Most of these records are not supported by either material deposited in fungaria nor in culture collections and have no molecular data available in public databases which might be used for the confirmation of their identity.

Members of the genus *Fusarium* occupy a broad diversity of habitats and occupy a wide variety of ecological niches; occurring as saprophytes on a range of substrates and as parasites – including of other fungi, as well as of plants – but have also been commonly reported recently as non-pathogenic endophytes of numerous plants and also been prospected for novel medicinal compounds (Toghueo, 2019). A number of species of *Fusarium* have been reported as mycoparasites (Carter, 1971; Hoch et al., 1979; Upadhyay et al., 1981; Munkvold and Marois, 1993; Mathivanan and Murugesan, 1999; McMahan et al., 2001; Hoopen et al., 2003; Ghule et al., 2018; Lira et al., 2019; Sun et al., 2019; Torbati et al., 2019). However, there are few confirmed records of *Fusarium* as mycoparasites of rust fungi. One of these is *Fusarium pallidoroseum* reported as a mycoparasite of the CLR) fungus, *Hemileia vastatrix*, in Mexico (Carrión and Rico-Gray, 2002). This is also a record requiring confirmation.

Here, results of a research on species of *Fusarium* found in association with *H. vastatrix* is presented, including the elucidation of the taxonomy of the range of isolates obtained and, a preliminary evaluation of their potential for biocontrol of CLR.

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CHAPTER 1

New Species and Records of *Fusarium* Isolated as Putative Mycoparasites from *Hemileia* Leaf Rusts of Coffee in Africa and South America

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(Formatted according to the guidelines of the journal Frontiers in Microbiology)

Abstract

A broad survey of fungi over-growing and colonizing pustules of coffee leaf rusts, caused by species of the genus *Hemileia*, was conducted in Africa and South America. It revealed a large mycodiversity which is now being catalogued and described. Among the significant groups of fungi collected from this niche, 93 isolates belonged to the genus *Fusarium*. A polyphasic approach was adopted to identify such isolates; involving cultural and morphological features and multigene phylogenetic analyses. As a result, the isolates were delimited into four species complexes, representing four new and two known taxa. The vast majority of the isolates fell within the *F. lateritium* species complex. The most common taxon – comprising *ca.* 80% of the total number of isolates – is recognized as a new species, described herein as *Fusarium hemileiae* sp. nov.; being recorded from Brazil, Cameroon and Ethiopia. The *F. lateritium* complex remains an ill-defined group and awaits recollection and epityfication. The data presented here contribute to the future improvement of the phylogenetic resolution of this complex. Additionally, two new taxa were recognized in the *F. solani* complex: *Fusarium pseudosolani* sp. nov. and *Fusarium cameroonicum* sp. nov., both from Cameroon. One other isolate belonging to this complex from Ethiopia was identified as *F. perseae*. *Fusarium auranto-glutinosus* sp. nov. – recorded from Brazil, Ethiopia and Paraguay – is also described as a taxonomic novelty within the *F. incarnatum-equiseti* complex. A possible new species within the *F. graminearum* complex is also described but is not named here because of insufficient phylogenetic support based on a single isolate. The present study provides the taxonomic background for an ongoing evaluation of these putative mycoparasites as potential biocontrol agents against the main constraint to coffee production worldwide, coffee leaf rust caused by *Hemileia vastatrix*.

Keywords: biocontrol, *Fusarium* systematics, *Hemileia coffeicola, Hemileia vastatrix*, novel species, phylogenetics

1 Introduction

Members of the genus *Fusarium* occupy an immense diversity of habitats and a wide variety of ecological niches; occurring as saprophytes on a range of substrates and as parasites – including of other fungi, as well as of plants – but have also been reported recently as non-pathogenic endophytes (Toghueo, 2019). The genus includes some of the most important species of plant-pathogenic fungi, on some of the most important crops worldwide (Summerell, 2019). In addition, species of *Fusarium* may produce secondary metabolites (most notably trichothecenes and fumonisins) in food products, some of which have known deleterious effects on humans and other animals (Leslie and Summerell, 2006). Conversely, there are species of *Fusarium* which are antagonists of plant pathogens and are considered to be of interest for use in the biological control of plant diseases (Paulitz et al., 1987; Park et al., 1988; Lemanceau and Alabouvette, 1991; Mathivanan and Murugesan, 1999; Altomare et al., 2000; Nel et al., 2006; Ghule et al., 2018; Kamel et al., 2019); whilst *F. venenatum* is the cornerstone of the burgeoning mycoprotein industry for human consumption (Trinci, 1992; O'Donnell et al., 1998; Wiebe, 2004). Additionally, they have recently been bioprospected as sources of novel medicinal compounds (Toghueo, 2019).

The taxonomy of *Fusarium* remains controversial, with nomenclatural disputes among researchers making the naming of species confusing, especially for those uninitiated in the 'politics' of this fungal group (Sandoval-Denis et al., 2019; O'Donnell et al., 2020). Application of both biological and phylogenetic species concepts have helped to resolve some of the old problems for the genus (Taylor et al., 2000), but new problems have arisen along the way (Brown and Proctor, 2013). Currently, there are more than 300 phylogenetically distinct species of *Fusarium* – nearly half of them not having been formally described – distributed in 23 species complexes (Summerell, 2019). The terminology species complex based on morphological and phylogenetic data was formally used first used in the genus in *Gibberella fujikuroi* species complex (GFSC) (Nirenberg and O'Donnell, 1998). The species complex nomenclature is not formally recognized but usually indicates a well-defined monophyletic group of taxa that tend to share morphological characters. Ideally, the identification and characterization of new species of *Fusarium* should be based on a polyphasic approach and, therefore, morphological, biological and phylogenetic species concepts are employed to support the descriptions of the new *Fusarium* taxa proposed herein. Species that can be identified with at least two of these features (morphological, biological and phylogenetic species concepts) are considered to be

much more robust than those that are named based on a single source of taxonomic characters (Brown and Proctor, 2013).

Although the greatest attention given to *Fusarium* spp. is in connection with their role as causal agents of crop diseases, this is largely unrealistic. Most taxa at the specific and even infra-specific level within *Fusarium* are not plant pathogens and pose no threat to food security. A number of species of *Fusarium* have been reported as mycoparasites (Carter, 1971; Hoch et al., 1979; Upadhyay et al., 1981; Munkvold and Marois, 1993; Mathivanan and Murugesan, 1999; McMahan et al., 2001; Hoopen et al., 2003; Ghule et al., 2018; Lira et al., 2019; Sun et al., 2019; Torbati et al., 2019). However, there are few confirmed records of *Fusarium* as mycoparasites of rust fungi. One of these is *Fusarium pallidoroseum* (*=Fusarium semitectum*) reported as a mycoparasite of *Hemileia vastatrix*, causal agent of CLR, in Mexico (Carrión and Rico-Gray, 2002).

CLR is universally recognized as the worst disease of coffee and has been the cause of heavy crop and economic losses throughout history (McCook, 2006). Recent rust outbreaks have had a major impact in Central and South America with losses of up to 20% in annual production (Villarreyna et al., 2020). In Colombia, coffee production decreased by 31% during the years 2008 and 2011 due to the severity of disease (Avelino et al., 2015). Traditionally, the rust has been controlled by the use of resistant coffee cultivars, escape by cultivation at higher altitude and fungicide application (Zambolim, 2016). However, losses due to CLR are still significant and all of these strategies have been challenged by problems such as: breakdown of resistance by new physiological *races* of the rust (Haddad et al., 2009); climate change with higher global temperature favoring the attack of the fungus in highland situations (Zambolim, 2016); and because of increasing concern about pesticide residues, following the rise of the organic coffee market, together with their environmental impact, coupled with the high – and often prohibitive – cost of fungicide applications for smallholders in developing countries (Matthews, 2015). Thus, there is an obvious need for sustainable methods of CLR management (Jackson et al., 2012; James et al., 2016; Gómez-De La Cruz et al., 2018). Biological control using natural enemies of *H. vastatrix* still remains a poorly explored method of management of the disease.

To address this need, a project was initiated in 2015 to investigate the potential of fungi antagonistic to *H. vastatrix*. Surveys in Africa and South America had revealed that coffee in its African center of origin harbors a large diversity of endophytic species belonging to the

"flagship" biocontrol-genus *Trichoderma*. In sharp contrast, coffee in its exotic range in Brazil appears to be a 'void'; completely lacking in *Trichoderma* endophytes. Among the 16 species of *Trichoderma* isolated and described, four were new to science (Rodríguez et al., 2021). A practical evaluation of the potential of some of these taxa as 'bodyguards' of coffee against the attack of CLR is showing promising results (Unpubl. Data). Other mycological novelties have also emerged from a study of the mycoparasites of *H. vastatrix*; including *Calonectria hemieliae,* recently described from Brazil (Crous et al., 2018) and *Digitopodium* from Africa (Colmán et al., 2021). *Calonectria hemieliae* has also been evaluated in more detail and was found to be highly effective at protecting coffee against CLR and capable of significantly reducing disease severity (Salcedo-Sarmiento et al., 2021). We use the term mycoparasite with reference to fungi that have the ability to parasitize fungal hosts (Barnett, 1963; Sun et al., 2019).

Here, we investigated the taxonomy of a broad range of putative mycoparasitic *Fusarium* isolates associated with pustules of *H. vastatrix* and other species of *Hemileia* on wild, semi-wild and cultivated coffee collected in its center of origin in Africa, as well as in exotic plantations in South America.

2 Materials and Methods

2.1 Collection and Isolation

The fungal isolates investigated here were collected during field surveys between 2015 and 2018 in Africa (Cameroon, Ethiopia) and South America (Brazil, Paraguay). Local scientists were involved in the surveys wherever possible. The surveys in Africa were concentrated in areas where relatives of cultivated *Coffea* occur and where commercial species are found in wild or semi-wild conditions (Cameroon – *Coffea canephora*; Ethiopia – *C. arabica*). In South America, sampling was undertaken in coffee plantations and in unmanaged sites, often where abandoned plantations were overgrown by secondary forest or where coffee seeds had been brought into the forest by the wildlife; generating semi-wild, understory coffee populations. A detailed account of the collecting protocol and processing of the collections is given in Cólman et al. (2021) and Rodríguez et al. (2021).

Leaves of *Coffea* showing rust symptoms and appearing to be overgrown or colonized by whitish colony-forming fungi were selected for examination in the laboratory. The presence

of mycoparasites can be cryptic and difficult to confirm in the field and rusted material, even without evidence of mycoparasite colonization, was also collected and dried in a plant press for a more detailed examination in the laboratory, either in Ethiopia, the UK or Brazil.

Rust pustules showing evidence of colonization by other fungi were transferred with a scalpel to a drop of lacto-glycerol on a slide and mounted for preliminary observation and identification, using a stereoscopic microscope (Olympus SZX7). This study concentrated on moniliaceous fungi belonging to the complex classified as "white colony-forming fungi". This complex was found to include species of *Fusarium* together with unrelated moniliaceous ascomycete asexual morphs belonging to genera such as *Acremonium, Lecanicillium*, *Pleurodesmospora* and *Simplicillium* (Cólman, 2018). These taxa are being treated in a separate publication (in prep.).

Isolations were performed by direct transfer of spores collected from parasitized rust pustules onto potato dextrose agar (PDA) plates supplemented with 0.1 g/L streptomycin sulfate. Long-term preservation was performed on silica gel, as described by Dhingra and Sinclair (1985), and also at -80° C in cryogenic microtubes containing 10% glycerol solution. Representative cultures were deposited in the local, officially recognized, culture collection: Coleção Octávio de Almeida Drumond of the University Federal of Viçosa (COAD).

2.2 DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was isolated from fungal mycelium grown on PD (potato-dextrose broth) using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocols. Initially the translation elongation factor 1-alpha (EF-1α) was amplified for all of the *Fusarium* isolates with the primer combination EF-1 and EF-2 (O'Donnell et al., 1998). Based on these results, some isolates were selected as representing potentially separate taxonomic units for amplification of other gene regions, namely: RNA polymerase second largest subunit (RPB2), β-tubulin, calmodulin (CAL), histone H3 (HIS), 3-O-acetyltransferase (Tri101), phosphate permase (PHO). The polymerase chain reaction (PCR) amplifications were performed in a total reaction volume of 12.5 µl, including 0.5 µl of each primer, 2.5 µl of BSA, 6.25 of Taq polymerase (including dNTPs), 1 µl of genomic DNA (30ng/µl); 0.5 µl DMSO and 1.25 µl of sterile ultrapure water. PCR conditions and primers used are listed in **Table S1**.

The amplified products were visualized by GelredTM (Thermo Fisher Scientific) staining following electrophoresis of 4 µl of each product in 1% agarose gel. Subsequently, these were purified with ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientifc) according to the manufacturer's recommendations and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com).

2.3 Phylogenetic Analyses

The nucleotide sequences were edited with the SeqAssem software (Hepperle, 2004). All of the sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). A BLASTn search was performed to check for similarity with other sequences. The sequences obtained in this study were aligned with additional sequences retrieved from GenBank, by using the multiple sequence alignment program MUSCLE® (Edgar, 2004) implemented in the MEGA X 10.1 software (Kumar, 2018). The alignments were checked, and manual adjustments were made when necessary. The phylogenetic analyses included two different algorithms: Maximum Likelihood (ML) and Bayesian Inference (BI).

One independent analysis was conducted for each species complex (SC). Alignments of the *Fusarium lateritium* species complex (FLSC) consisted of 202 parsimony-informative positions/684 bp for EF-1α, 153/946 bp for RPB2 and 133/743 for β-tubulin. Alignments of the *Fusarium solani* species complex (FSSC) consisted of 117 parsimony-informative positions/709 bp for EF-1α and 149/864 bp for RPB2. Alignments of the *Fusarium incarnatum-equiseti* species complex (FIESC) consisted of 198 parsimony-informative positions/750 bp for EF-1α, 206/892 bp for RPB2 and 134/743 for CAL. Alignments of the *Fusarium graminearum* species complex (FGSC) consisted of 36 parsimony-informative positions/648 bp for EF-1α, 5/902 bp for RPB2, 28/1216 for β-tubulin, 21/449 bp for HIS, 34/1336 bp for TRI101 and 31/886 for PHO. Bayesian Inference analyses were conducted in the MrBayes v. 3.2.6 (Ronquist et al., 2012), on XSEDE platform available on CIPRES Science Gateway Portal (Miller et al., 2011). Bayesian Inference analyses were performed employing a Markov Chain Monte Carlo method. Before launching the BI, the best-fitted model of nucleotide substitution for the phylogenetic analysis was estimated using MrMODELTEST 2.3 (Posada and Buckley, 2004), namely GTR + G model for regions EF-1α and β-tubulin and SYM + G for RPB2 for FLSC analyzes; GTR + I + G model for EF-1α and SYM + I + G for RPB2 for FSSC analyzes; GTR + G + I model for regions EF-1α and RPB2 and SYM + G for CAL for FIESC analyzes;

and GTR + G model for regions EF-1α, K80 + I for RPB2, GTR for β-tubulin, GTR + I for regions HIS and PHO and HKY + I for FGSC analyzes. Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). Two independent analyses composed of four MCMC chains were run simultaneously, starting from random trees for 10,000,000 generations. The trees were sampled every 1,000th generation for a total of 20,000 trees. The first 5000 trees were discarded as the burn-in phase of each analysis. The posterior probabilities were determined from a majority-rule consensus tree generated from the remaining 15,000 trees.

ML analyses were conducted in the RAxML v. 8.2.9 (Stamatakis, 2014) platform available on CIPRES Science Gateway Portal (Miller et al., 2011). Default parameters were used and bootstrapping was carried out using the rapid bootstrapping algorithm with the automatic halt option (1,000 initial replicates). The trees were visualized in FigTree (Rambaut and Drummond, 2012). The resulting tree topologies using the two methods (ML and BI) were then compared and the phylogram layout was edited with Inkscape 1.0 (https://inkscape.org/pt-br/).

2.4 Morphological Studies

The results of the phylogenetic analyses of the assemblage of *Fusarium* isolates guided the selection of isolates to be included in the morphology studies. Selected representative isolates were described for their *in vitro* features following the standard procedures adopted for *Fusarium* spp. (Leslie and Summerell, 2006), based on colonies formed on oatmeal-agar (OA) and PDA after 14 days incubation at 22 °C under 12 h daily light regime (light provided by two white and one near-UV lamps placed 35 cm above the plates). Color nomenclature followed Rayner (1970). To estimate growth rate, three plates were used for each isolate and two perpendicular measurements of colony diameter were made during four days of incubation.

Micro-morphological characters of each taxon given below were based on observation of structures formed on carnation leaf-agar (CLA) (Leslie and Summerell, 2006) and synthetic nutrient-poor-agar (SNA) (Leslie and Summerell, 2006) after 10 to 14 days, under the same conditions described above for growth rate.

Slides were prepared from small colony fragments taken from plates using a sterile dissecting needle and were mounted in a drop of lacto-glycerol and examined under an Olympus BX 51. Fungal structures were based on a minimum of 30 measurements, the highest and lowest

values are given. Photographs were taken using an Olympus BX 53 microscope adapted with differential interference contrast lighting and a digital image capture system (Olympus Q-Color 3™). Inkscape 1.0 was used to edit the images and prepare the plates.

3 Results

3.1 Fungal Isolation

From a total of almost 700 isolates obtained during the surveys, 93 were found to belong to the genus *Fusarium*. Their origins are as follows: Ethiopia – 55 isolates; Brazil – 27 isolates; Cameroon – 10 isolates and Paraguay – one isolate, and these are listed in **Table 1**.

3.2 Phylogenetic Analyses

The first analysis based on EF-1α sequences was conducted in order to generate a preliminary discrimination/grouping of the 93 isolates and also inferring provisionally their phylogenetic affinity towards the different recognized species complexes (SC) of *Fusarium* (**Figure S1**). Seventy-seven isolates belonged to the *Fusarium lateritium* species complex (FLSC), namely: 50 from Ethiopia, 25 from Brazil and 2 from Cameroon. Only two of the Brazilian isolates grouped in clade III, all other 75 isolates belonged to clade I (Geiser et al., 2005). Nine isolates belonged to the *Fusarium solani* species complex (FSSC), six to the *Fusarium incarnatum-equiseti* species complex (FIESC), and one isolate to the *Fusarium graminearum* species complex (FGSC). Twenty-eight representative isolates were selected from that assemblage for amplification of additional regions.

A more inclusive analysis of the twelve isolates belonging to the FLSC was performed based on a three-locus dataset (RPB2, EF-1α and β-tubulin) and included 2373 bp (RPB2 945 pb, EF-1α 684 bp, and β-tubulin 742 bp) with 44 representative taxa reference sequences obtained from GenBank (**Table S2**). The ML and BI concatenated trees showed identical topologies, the ingroup taxa were resolved into six highly supported main clades (**Figure 1**).

Ten isolates from various geographic origins (Brazil, Ethiopia and Cameroon) clustered in a clade treated in the literature as clade I (Gaiser et al., 2005). This clade is fully supported (BS = 99%; PP = 1.0) and included only isolates from coffee. It clearly represents a distinct lineage within the FLSC. Clade II includes the isolate FRC L-405 (= NRRL 25485 = BBA 63887) obtained from citrus in New Zealand and described by Gerlach and Nirenberg (1982)

as a typical member of *F. stilboides*. The data analysis shows that clades I and II represent two phylogenetically distinct species. Therefore, a new species is proposed below to accommodate the isolates belonging to clade I.

Two isolates from Brazil (COAD 2937 and COAD 2944) formed a single clade with high support within clade III of FLSC (**Figure 1**), phylogenetically very close to the strain FRC L-55 (=NRRL 13622 from elm, Louisiana, USA) of *F. lateritium.* This clade is interpreted as representing *F. lateritium sensu stricto*.

The mycoparasitic isolates of *Fusarium* on *H. vastatrix* belonging to the FSSC grouped in three clades (**Figure 2**). The two-locus analysis was based on RPB2 and EF-1α sequences, included 1573 bp (RPB2 864 bp and EF-1α 709 bp) with 55 representative taxa reference sequences obtained from GenBank. Each gene was analyzed separately prior to multi locus analysis. The isolate COAD 2954 grouped with the type of *Fusarium perseae* (CPC 26829 = CBS 144142) with high support (BS = 93%; PP = 1.0). Two distinct and highly supported clades were found and are described here as two novel species, one of them represented by strains COAD 2949, COAD 2963, COAD 2964, COAD 3027 (PP = 1.0) and the other for COAD 2945, COAD 2947, COAD 2948, COAD 3029 (BS = 96%; PP = 1.0). The three clades are within the clade III of the FSSC (O'Donnell, 2000). With the exception of *F. perseae* COAD 2954 from Ethiopia, all others isolate obtained in this study and belonging to the FSSC were from Cameroon.

For the FIESC, three gene regions were used in the phylogenetic study: CAL, RPB2 and EF-1α. The analyses included 2385 bp (CAL 743 pb, RPB2 892 pb and EF-1α 750 bp) with 70 representative taxa sequences obtained from GenBank. The isolates COAD 2950, COAD 2965, COAD 2952, COAD 2935, COAD 2936, COAD 2942 formed a single highly supported clade (BS = 100%; PP = 1.0), within the "*equiseti* clade" (O'Donnell et al., 2009), in all analyzes and for all genes. This new lineage is described here as a new species (**Figure 3**) which includes isolates from Ethiopia, Brazil and Paraguay.

Only one isolate obtained in this study belonged to the FGSC (COAD 2951). Six gene regions were included in the phylogenetic analysis: HIS, PHO, RPB2, EF-1α, TRI101 and β-tubulin. The alignment included 5437 bp (HIS 449 pb, PHO 886 pb, RPB2 902 pb, EF-1α 648 bp, TRI101 1336 bp and β-tubulin 1216 pb) with 33 representative sequences obtained from GenBank. The placement of COAD 2951 in a separate clade and the high support in all terminal

branches of the phylogenetic tree suggests that this is a new species of *Fusarium* (**Figure 4**). However, we prefer here to wait until more isolates become available in order to justify the proposal of a new species.

3.3 Taxonomy

Based on the phylogenetic analyzes, the 93 isolates of *Fusarium* from *Hemileia* rusts were grouped into seven species belonging to four species complexes of *Fusarium*, namely: FLSC, FSSC, FIESC and FGSC. *Fusarium lateritium* and *F. perseae* are newly reported from coffee rust pustules and four new species are proposed and described below.

Fusarium auranto-glutinosus T.F. Nóbrega & R.W. Barreto sp. nov. **Figure 5.**

MycoBank: MB839066.

Etymology: Named for the orange color and mucilaginous aspect of the sporodochial mass *in vitro*.

Type: BRAZIL, Minas Gerais, Viçosa, Mata do Paraíso, isolated from pustule of *Hemileia vastatrix* on leaf of *Coffea arabica*, A.A. Cólman, 13 March 2015 (holotype VIC 47351, ex-type culture COAD 2935).

Mycelium branched, septate, hyaline, smooth, 2–5 µm diam. ***Aerial conidiophores*** branched or reduced to conidiogenous cells, sometimes of only one cell, bearing 1–2 phialides at the apex, 10–35 × 1–2 µm, hyaline, smooth. ***Aerial phialides*** mono or polyphialidic, similar to phialides on sporodochia or slightly longer, 5–18 × 1–2 µm. ***Aerial conidia*** like mesoconidia, fusoid, relatively straight to slightly curved, 7–21 × 2–4 µm; apical cell hook-shaped or conical, basal cell rounded, 1–3 septate, hyaline, thin-walled, smooth. ***Sporodochia*** abundantly produced on CLA and on the surface of SNA; orange, slimy. ***Conidiophores in the sporodochia*** branched, compact, usually with one or two phialides only, 20–35 × 2–4 µm, hyaline, smooth. ***Sporodochial phialides*** monophialidic, short, doliiform, 8–12 × 2–4 µm. ***Sporodochial conidia*** fusoid, relatively straight to slightly curved with parallel walls for most of the spore length, medium, 27–50 × 2–5 µm; apical cell hook-shaped, basal cell foot-shaped, 4–7-septate, hyaline, thin-walled, smooth. ***Chlamydospores*** abundant on PDA, globose or oval, intercalary or terminal, usually single but sometimes in chains, 6–14 × 5–12 µm, thin-walled, hyaline, smooth.

Culture characteristics*:* on PDA – flat, edge entire, aerial mycelium abundant, felty, flesh colored, reverse saffron, sporulation abundant; on OA – mostly immersed, edge entire, some effuse elevations of thin white aerial mycelium, reverse colorless, sparse sporulation, spiral hyphae present; fast growing on PDA and OA, 10.8 mm/day and 14 mm/day, respectively.

Notes: *Fusarium auranto-glutinosus* is represented by six isolates: three from Ethiopia; two from Brazil and one from Paraguay (**Table 1**; **Figure 3**).

Fusarium cameroonicum T.F. Nóbrega & R.W. Barreto sp. nov. **Figure 6.**

MycoBank: MB839067.
Etymology: Named after Cameroon, the country where it was collected.

Type: CAMEROON, Eastern Region, Somalomo, isolated from pustule of *Hemileia coffeicola*, on leaf of *Coffea canephora*, 22 November 2015, H.C. Evans (holotype VIC 47497, ex-type culture COAD 2947).

Mycelium branched, septate, hyaline, smooth, 1–5 µm diam. ***Aerial conidiophores*** often reduced to simple lateral phialides, but also long and branched conidiophores formed, 26–141 × 2–5 µm, hyaline, smooth. ***Aerial phialides*** monophialidic, long and cylindrical, 35–80 × 2–4 µm, dichotomously branched, thin-walled, smooth. ***Aerial conidia*** produced abundantly on PDA, OA and SNA; ovoid, obovoid, allantoid, reniform or ellipsoid, 7–16 × 2–6 µm, 0–1 septate, thin-walled, hyaline, smooth; forming small false heads at the tip of conidiogenous cells. ***Sporodochia*** sparsely produced on CLA; mucilaginous, cream to pale luteous**.** ***Conidiophores*** *and* ***phialides in sporodochia*** indistinct. ***Sporodochial conidia*** fusiform, slender and slightly curved or straight, tapering towards the basal part, medium-long, 51–67 × 4–6 µm; apical cell bent, basal cell foot-shaped, 5–6 septate, hyaline, thin-walled, smooth. ***Chlamydospores*** common on SNA; globose or ellipsoid, intercalary in the hyphae or formed terminally, 5–12 × 3–8 µm, hyaline, smooth or rough.

Culture characteristics*:* on PDA – flat, dentate edge, aerial mycelium dense, felty, buff to white surface and honey or white reverse, with concentric rings, forming brown or white mycelial cords, sporulation abundant; on OA – mostly immersed with some effuse elevations of thin white aerial mycelium often forming spiral hyphae, edge entire, with concentric rings, colorless reverse, sporulation abundant; relatively fast-growing, 5 mm/day on PDA and 7 mm/day on OA.

Notes: *Fusarium cameroonicum* was isolated on four occasions from the same locality in the Eastern Region of Cameroon, in a plantation of Robusta coffee (*Coffea canephora*), where the dominant rust species is the grey coffee rust, *Hemileia coffeicola*, known locally as 'rouille farineuse de caféier'.

Fusarium hemileiae T.F. Nóbrega & R.W. Barreto sp. nov. **Figure 7.**

MycoBank: MB839068.

Etymology: Referring to its direct and consistent association with the rust *Hemileia vastatrix*.

Type: BRAZIL, Rio de Janeiro, Duas Barras, Fazenda do Campo, isolated from pustule of *Hemileia vastatrix* on leaf of *Coffea arabica*, R.W. Barreto, 02 May 2015 (holotype VIC 47359, ex-type culture COAD 2943).

Mycelium branched, septate, hyaline, smooth, 1–4 µm diam. ***Aerial conidiophores*** as lateral branches, cylindrical, long, 50–86 × 2–4 µm. ***Aerial phialides*** monophialidic cylindrical, 14–37 × 2–4 µm, thin-walled, smooth. ***Aerial conidia*** falcate, 16–36 × 3–5 µm, with rounded ends, 1–3 septate, thin-walled, hyaline, smooth. ***Sporodochia*** rarely produced on CLA; mucilaginous, orange. ***Conidiophores*** *and* ***phialides in sporodochia*** indistinct**.** ***Sporodochial conidia*** thin, and inequilaterally fusoid, with the widest point above the center, either pointed or beaked at the apex, 30–57 × 3–5 µm; apical cell hook-shaped, basal cell foot-shaped, 3–7 septate, thin-walled, hyaline, smooth. ***Chlamydospores*** abundant on SNA; globose or oval, intercalary, usually forming aggregates, 9 – 21 × 8 – 15 µm, wall thin, hyaline, smooth.

Culture characteristics*:* on PDA – flat, fimbriate margins, aerial mycelium abundant and felty, coral with isabelline halo on the edge concentric rings present, dark vinaceous reverse with red diffusible pigments, sporulation abundant; on OA – mostly immersed with some effuse elevations of aerial mycelium, irradiated, edge entire, honey, reverse colorless, sporulation abundant, spiral hyphae present; relatively slow-growing, 7.3 mm/day on PDA and 6 mm/day on OA.

Notes: *Fusarium hemileiae* was by far the commonest species isolated during the surveys in both Ethiopia (50 isolates) and Brazil (23 isolates), with only two records from Cameroon (**Table 1**); representing over 80% of the total isolations. In addition, there were 11 matches in GenBank (**Figures 1** and **S1**). All were isolated from coffee (Arabica and Robusta) bark, fruits

and seeds, across the pantropics (Africa – Ethiopia, Guinea, Malawi, Zimbabwe; Australasia – New Caledonia, New Guinea; South America – Brazil) (Geiser et al., 2005). No information was provided by the authors on the pathological status of these isolates.

Fusarium hemileiae differs from *F. stilboides* (the closest species in the phylogenetic study) by having longer aerial phialides (up to 37 μm), shorter sporodochial macroconidia (up to 57 μm) and having fewer septa (up to 7 septa). Conversely, according to Gerlach and Nirenberg (1982), *F. stilboides* has phialides which are up to 20 μm in length and macroconidia that can be up to 160 μm long with up to 16 septa.

Fusarium lateritium Nees, in Esenbek *Syst. Pilze* (Würzburg): 31 (1817). **Figure 8**.

Mycelium branched, septate, hyaline, smooth, 2–5 μm diam. ***Aerial conidiophores*** usually reduced to conidiogenous cells or aggregated in sporodochia; dichotomously branched, compact, with doliiform phialides at apex, 29–42 × 2–4 μm, hyaline, smooth. ***Aerial phialides*** monophialidic, cylindrical, 3–16 × 1–2 μm, thin-walled, hyaline, smooth. ***Aerial conidia*** filiform, mostly straight to gently curved, 9–37 × 2–4 μm; apical cell hook-shaped or conical, basal cell rounded, 1–3 septate, thin-walled, hyaline, smooth. ***Sporodochia*** abundantly produced on CLA and SNA; mucilaginous, orange. ***Conidiophores in sporodochia*** branched, compact, usually with one or two phialides only, 22–59 × 2–4 μm, hyaline, smooth. ***Sporodochial phialides*** monophialidic, cylindrical, 7–16 × 2–4 μm, thin-walled, hyaline, smooth. ***Sporodochial conidia*** filiform, straight to slightly curved, walls parallel for most of the length, 40–62 × 2–5 μm; apical cell hook-shaped, basal cell foot-shaped, 4–6-septate, thin-walled, hyaline, smooth. ***Chlamydospores*** common on SNA; mostly single, globose or oval, terminal or intercalary, 11–26 × 7–20 μm, thin-walled, hyaline, smooth.

Culture characteristics*:* on PDA – low convex, margin fimbriate, aerial mycelium wooly, dense, white with flesh colored center, white reverse with salmon center and presence of concentric rings; sporulation abundant; on OA – mostly immersed with some effuse elevations of thin white aerial mycelium, irradiating, margin entire, reverse colorless, sporulation scarce; relatively slow-growing, 5.5 mm/day on PDA and 6 mm/day on OA.

Material examined: BRAZIL, Ceará, Ubajara, Pousada Gruta, isolated from pustule of *Hemileia vastatrix* on leaf of *Coffea arabica*, 13 September 2018, R.W. Barreto (COAD 2944).

Notes: *Fusarium lateritium* was found only in Brazil on two occasions (**Table 1**). It is a pantropical plant pathogen but has also been implicated in diseases of humans. Cambuim et al. (2007) identified it as an emergent pathogen of immunocompromised patients in Brazil, whilst Dey and Bhattacharya (2019) recently reported *F. lateritium* as a causal agent of respiratory allergies in India. There are also previous reports of this species as a mycoparasite. For example, Vajna (1987) found that in dual cultures, *F. lateritium* could penetrate and destroy the hyphae of a range of fungi, including *Botrytis cinerea*.

Fusarium perseae (Sand.-Den. & Guarnaccia) O'Donnell, Geiser & T. Aoki, in Aoki, Geiser, Kasson & O'Donnell, *Index Fungorum* 440: 3 (2020). **Figure 9.**

≡ Synonymy: *Neocosmospora perseae* Sand.-Den. & Guarnaccia, in Guarnaccia, Sandoval-Denis, Aiello, Polizzi & Crous, *Fungal Syst. Evol.* 1: 136 (2018).

Mycelium branched, septate, hyaline, smooth, 3–5 µm diam. ***Aerial conidiophores*** often reduced to simple lateral phialides or as long and branched conidiophores, 70–126 × 2–5 µm, hyaline, smooth. ***Aerial phialides*** monophialidic, long and cylindrical, 23–65 × 1–5 µm, dichotomously branched, thin-walled, hyaline, smooth. ***Aerial microconidia*** produced abundantly on PDA, OA and SNA; oval, allantoid or ellipsoid, 6–18 × 2–4 µm, 0–1 septate, thin-walled, hyaline, smooth; single or forming small false heads at the tip of conidiogenous cells. ***Sporodochia*** abundantly produced on CLA; mucilaginous, cream to pale luteous**.** ***Sporodochial conidiophores*** short, 21–30 × 3–3 µm, densely packed, irregularly branched with 1–3 terminal phialides. ***Sporodochial phialides*** monophialidic, doliiform, subulate to subcylindrical, 13–18 × 2–3 µm, thin-walled, smooth. ***Sporodochial conidia*** slender and slightly curved or straight, medium-long, 46–59 × 3–6 µm; apical cell bent, basal cell foot-shaped, 4–5 septate, hyaline, thin-walled, smooth. ***Chlamydospores*** common on SNA; globose or oval, intercalary or formed terminally, 5–13 × 5–8 µm, hyaline, smooth or rough-walled.

Culture characteristics*:* on PDA – flat, margins entire, aerial mycelium dense, felty, forming visible brown mycelial cords, buff with salmon reverse, forming concentric rings, sporulation abundant; on OA – mostly immersed with some effuse elevations of thin white aerial mycelium, edge entire, forming concentric rings, reverse colorless, sporulation abundant; fast-growing, 7.5 mm/day on PDA and 9.2 mm/day on OA.

Material examined: ETHIOPIA, Oromia Region, Jimma, isolated from pustule of *Hemileia vastatrix* on leaf of *Coffea arabica*, K.B. Bekele, November 2017 (COAD 2954).

Notes: This species has previously only been known as the causal agent of a trunk canker disease of avocado trees in Italy (Sandoval-Denis et al., 2018).

Fusarium pseudosolani T.F. Nóbrega & R.W. Barreto sp. nov. **Figure 10**.

MycoBank: MB839069.
Etymology: Named because of its morphological similarity to *F. solani sensu stricto*.

Type: CAMEROON, Somalomo, Eastern Region, isolated from pustule of *Hemileia coffeicola*, on leaf of *Coffea arabica*, H.C. Evans, 22 November 2015 (holotype VIC 47498, ex-type culture COAD 2949).

Mycelium*:* branched, septate, hyaline, smooth, 3–5 µm diam. ***Aerial conidiophores*** often reduced to simple lateral phialides or as long and branched conidiophores, 30–70 × 1–2 µm, hyaline, smooth. ***Aerial phialides*** monophialidic, cylindrical, 35–66 × 2–3 µm, dichotomously branched, thin-walled, hyaline, smooth. ***Aerial conidia*** produced abundantly on PDA, OA and SNA; oval, allantoid or ellipsoid, 4–16 × 2–5 µm, 0–1 septa, thin-walled, hyaline, smooth; single or forming small false heads at the tip of conidiogenous cells. ***Sporodochia*** sparsely produced on CLA; mucilaginous, cream to pale luteous***. Conidiophores*** and ***phialides in sporodochia*** indistinct. ***Sporodochial conidia*** fusiform, slender and slightly curved or straight, medium-long, 48–65 × 4–5 µm; apical cell bent, basal cell foot-shaped, 4–7 septate, thin-walled hyaline, smooth. ***Chlamydospores*** common on SNA; globose or oval, intercalary or terminal, 6–14 × 4–8 µm, hyaline, smooth or rough walled.

Culture characteristics*:* on PDA – flat, edge entire, aerial mycelium dense cottony with spiral hyphae arising from colonies, buff, reverse pale luteous fading to yellowish white from the center to margin, sporulation abundant; on OA – mostly immersed with some effuse elevations of thin white aerial mycelium and spiral hyphae arising from colonies, margin entire, reverse colorless with concentric rings; sporulation abundant; relatively fast-growing, 8 mm/day on PDA and 10.5 mm/day on OA.

Notes: *Fusarium pseudosolani* was isolated from the same locality as *F. cameroonicum* (see Notes above), and is represented by four isolates (**Table 1**).

***Fusarium* sp. 1. Figure 11.**

Mycelium**:* branched, septate, hyaline, smooth, 1–5 μm diam. ***Aerial conidiophores reduced to simple lateral phialides. ***Aerial phialides*** doliiform, short, 7–13 × 1–3 μm. ***Aerial conidia*** moderately curved to straight with the ventral surface straight and the dorsal side smoothly arched, medium, 23–38 × 2–4 μm; apical cell hook shaped, basal cell foot shaped, mostly 1–3-septate, hyaline, thick-walled; occasionally 0–1 septate, spindle-shaped, like mesoconidia, 15–23 × 2–4 μm. ***Sporodochia*** absent. ***Chlamydospores*** mostly formed within the macroconidia, rarely formed in the mycelium, usually in chains, globose, hyaline, wall finely roughened.

***Culture characteristics**:* on PDA – raised, margins fimbriate, cottony and dense (filling all the space up to the plate lid), vinaceous center and honey at the edges, reverse dark vinaceous, colored with diffusible pigments of the same color, no sporulation; on OA – entire edge, irradiated, aerial mycelium felty, salmon, aerial mycelium absent in the center of the plate, reverse coral with diffusible pigments of the same color, concentric rings present, sporulation absent; fast growing – 18.2 mm/day on PDA and 19.5 mm/day on OA.

Material examined: ETHIOPIA, Kaffa Region, Arat Selisa Forest Reserve, isolated from pustules of *Hemileia* cf. *coffeicola*, on leaf of wild *Coffea arabica*, 26 November 2017, H.C. Evans (COAD 2951).

Notes: This was isolated only once from a still unidentified *Hemileia* species, close to *H. coffeicola*, occurring on wild Arabica coffee in the understory of cloud forest (ca. 2000 m a.s.l.), and is provisionally assigned to the *Fusarium* sp., but surely a member of the *Fusarium graminearum* species complex (see **Figure 4).**

4 Discussion

The terminology and concepts applicable to the fungi growing on other fungi were discussed in Sun et al. (2019). Here, we report the frequent association of fungi belonging to the genus *Fusarium* with pustules of *Hemileia* leaf rusts of coffee, which we term putative mycoparasites until the relationship is confirmed experimentally. The isolation and identification of six species during this study probably represents a gross underestimation of the diversity of *Fusarium* associated with coffee rusts of the genus *Hemileia* since only a small part of their geographic range was covered during the surveys. Nevertheless, almost 100 *Fusarium* isolates were obtained from this niche. Analyses of the morphological and phylogenetic relationships among the isolates showed that these belong to four distinct species complexes. However, there was a notable predominance (over 80%) of isolates belonging to clade I of the FLSC. There is still limited information on this species complex and the phylogenetic relationships within this group remain poorly explored and several taxa still need to be fully characterized (Leslie and Summerell, 2006).

Even the limits between *F. stilboides* and *F. lateritium* are unclear, and most authors prefer to use the collective name "*Fusarium lateritium*" to designate these taxa (Geiser et al., 2005; Vitale et al., 2011; Cavalcanti et al., 2020). Geiser et al. (2005) were the first to study phylogenetic relationships within the FLSC and concluded that these two names can be divided into three main groups. The first group (I) consisted of coffee isolates originating from a broad geographical range in Africa, Asia, South America and Oceania. The second group (IIA and IIB) consisted of citrus isolates from New Zealand, the Philippines and New Caledonia; including isolate L-405 (NRRL 25485/BBA 63887) described morphologically as a member of *F. stilboides* by Gerlach and Nirenberg (1982). A third distinct group (III) was represented by coffee isolates from Papua-New Guinea; including the reference isolate L-55 of *F. lateritium*.

Unfortunately, there are no DNA sequences available for the type of *F. stilboides*. For *F. lateritium* var. *longum*, a neotype has been designated (CBS 633.76 / NRRL 25484/BBA 63665) by Gerlach and Nirenberg (1982). However, only Internal Transcribed Spaces and 5.8S gene (ITS) and the D1/D2 domain of the 26S Large Subunit (LSU) sequences are available for this isolate (Vu et al., 2019). These are of limited use for *Fusarium* phylogenies (O'Donnell and Cigelnik, 1997; O'Donnell et al., 2013). Uncertainty about the status of *F. lateritium* var. *longum* is compounded by Bilai (1955) who proposed that *F. lateritium* var. *longum* represents a synonym of *F. stilboides*.

Even in the absence of type cultures for resolving FLSC, we have assembled a significant number of isolates and generated numerous sequences for relevant genome regions for isolates in group I of FLSC. Hence, it became possible to propose that group I of FLSC, in fact represents a distinct and novel species. This is also in agreement with the views of Geiser et al. (2005) that group I can be separated from other *Fusarium* spp. on both molecular and morphological grounds.

The isolates grouping in clade I produce a red pigment on PDA and form concentric rings of aerial mycelium form long conidiophores and produce macroconidia of medium length with a curved apical cell and a blunt basal cell. This morphology is highly compatible with other taxa belonging to this clade (Geiser et al., 2005). The newly proposed species *F. hemileiae* appears to be associated only with *Coffea* and, as shown here, to be common in pustules of *H. vastatrix*. In addition to the isolates obtained during our survey, 11 reliable matches for *F. hemileiae* were found in GenBank. Moreover, these were all isolates obtained from coffee plants. Conversely *F. stilboides* (clade II) represents an unresolved group which is found associated with a range of different substrates.

Fusarium lateritium sensu stricto is considered here as represented by clade III. The strains COAD 2937 and COAD 2944 are morphologically identical to previous descriptions (Booth, 1971; Leslie and Summerell, 2006). This clade includes isolates from America and Africa also from coffee, as well as elm. The isolates in clade VI have been described as morphologically similar to *F. lateritium* (Vitale et al., 2011). However, our analysis revealed a high phylogenetic distance between these isolates and all others included in this complex. It is possible that in the future, with the inclusion of additional molecular data, this clade will be excluded from the FLSC.

Some isolates belonging to the FLSC have been linked with *Fusarium* wilt symptoms of coffee in Brazil (Pfenning and Silva, 1999; Belan et al., 2018) and with *Fusarium* bark disease of coffee in Zimbabwe (Gotora et al., 2014). Mycoparasitism of CLR is a previously unreported niche for fungi in this complex. A full elucidation of their taxonomy is of relevance, together with studies on details of their biology, in order to better assess their potential for use as a biocontrol agent against coffee leaf rust. Several studies have already demonstrated that isolates of *F. lateritium sensu lato* have antagonist activity against important plant pathogens such as *Sclerotinia sclerotiorum, Eutypa* spp. and *Fusarium culmorum* (Carter, 1971; Munkvold and Marois, 1993; McMahan et al., 2001; Kamel et al., 2019).

The FSS and FIES complexes include cryptic species or infraspecific taxa which are important plant pathogens; causing, in particular, root rots of a range of plant species and may produce secondary metabolites (mycotoxins) that can accumulate in agricultural products (O'Donnell et al., 2008; Villani et al., 2016). Morphological and molecular phylogenetic data independently showed that three species are associated with coffee rust pustules inside the FSSC. Strain COAD 2954 belongs to *F. perseae,* which was recently described as a new taxon causing trunk cankers on avocado in Italy (Sandoval-Denis et al., 2018). Here, we obtained it from an entirely disparate geographic region and occupying a completely different ecological niche. *Fusarium pseudosolani* and *F. cameroonicum* represent two distinct lineages within clade III (O'Donnell, 2000). These two new species have a similar morphology but *Fusarium pseudosolani* has smaller aerial mycelial conidiophores and macroconidia with more septa when compared to *F. cameroonicum.* Both species fall within the "*F. solani*-morphotype" concept. Nevertheless, these new species have significantly longer macroconidia, which are slender and with a bent apical cell, whereas other members of the FSSC have shorter macroconidia with rounded ends. They are also phylogenetically close to FSSC 38, isolated from coffee borer beetles in Benin and Uganda and with proven symbiotic-mutualist relationship with this important insect pest of coffee (Rojas et al., 1999; Morales-Ramos et al., 2000; O'Donnell et al., 2012).

In the last decade, there has been an intense discussion relating to the exclusion of the FSSC from the *Fusarium* genus and its inclusion in the genus *Neocosmospora.* This controversy is outside the scope of our study and here we have chosen to use the name *Fusarium solani*, as recommended in recent publications (Aoki et al., 2012; Geiser et al., 2013; O'Donnell et al., 2020).

In our phylogenetic study, *F. auranto-glutinosus*, emerged as a sister clade of the *F. equiseti* clade, and this was consistent for all three genes included in the analysis (data not shown). *Fusarium auranto-glutinosus* has fast-growing, salmon-colored colonies on PDA; producing macroconidia that are straight for most of their length, with a hook-shaped apical cell and a foot-shaped basal cell. Mesoconidia are also present; being 1-3 septate, relatively straight to slightly curved with a hook-shaped or conically-shaped apical cell and a rounded basal cell. In addition, smooth-walled, oval or globose chlamydospores are produced in abundance. This combination of features resembles members of the clade *incarnatum*, although the *F. auranto-glutinosus* clade has been placed closer to the *equiseti* clade.

Only one isolate, COAD 2951, among the 93 isolates belongs to the FGSC. This complex is within the *Fusarium sambucinum* species complex (FSamSC). To our knowledge, this is the first example of mycoparasitism in this group. *Fusarium* sp. 1 (COAD 2951) clustered in a clade (with weak support), together with six known *Fusarium* spp.: *F. acaciae-mearnsii, F. aethiopicum, F. asiatium, F. nepalense, F. ussurianum* and *F. vorosii*. Each of these species was strongly supported in the final branches. This suggests that *Fusarium* sp. 1 may be phylogenetically distinct, although it is morphologically indistinguishable from the other isolates in the FGSC and cryptic species are known to be common in this complex.

A rich and hidden diversity of species of the genus *Fusarium*, associated with pustules of *Hemileia* rusts of coffee, was uncovered during the surveys; indicating that the mycoparasitic habit may be a common and ecologically significant feature within the genus. Of particular interest is the number of isolates within the *F. lateritium* species complex – both in South America (Brazil) and Africa (Ethiopia) – suggesting an evolutionary trend towards mycoparasitism in this group. More intensive sampling will be required to test this hypothesis. The greater majority of these isolates belong to the new species *F. hemileiae*, with additional isolates found in GenBank from coffee plants in other continents (Asia, Oceania). The over-riding question is: did this species evolve on *Coffea arabica* in its Ethiopian center of origin and was then taken around the tropics with its host or is it a resident pantropical species that opportunistically adapted to the coffee/CLR niche? During the 19th century, *C. arabica* was moved from Africa – typically as live plants in Wardian cases – to form the basis of new plantations, especially in Asia (Ayres, 2005). Undoubtedly, this is how CLR reached Sri Lanka and India and caused such devastation and profound socio-economic changes (McCook, 2006). It has been posited that *H. vastatrix* reached Brazil – and thence spread throughout the Americas – as spores carried on the trade winds across the Atlantic Ocean (Bowden et al., 1971). The common occurrence of *F. hemileiae* in both Brazil and Ethiopia suggests that this purported mycoparasite may have arrived together with its rust host on imported planting material of *C. arabica*.

In sharp contrast, the two new species associated with *H. coffeicola – F. cameroonicum* and *F. pseudosolani* – were found only in Cameroon on Robusta coffee: whilst *F. hemileiae* was rarely recorded during the surveys in Cameroon. *Hemileia coffeicola* is still confined to Africa but the few studies on its biology have shown that this can be a damaging pathogen of the 'exotic' Arabica coffee in West Africa, with reports from São Tomé (Rodrigues, 1957), Togo

(Partiot et al., 1979) and Côte d'Ivoire (Lourd and Huguenin, 1982). Rodrigues (1957) found that at altitudes above 500 m, the rust caused heavy losses on *C. arabica* and that in screening tests all cultivars of Arabica were susceptible, even those showing resistance to *H. vastatrix*. Whether these *Fusarium* species are specific to this rust host is of interest and relevance should *H. coffeicola* ever 'break' out of Africa, especially since the available evidence suggests that this 'neglected' coffee rust poses a serious threat to *C. arabica* worldwide. There is evidence from our surveys that at least one other mycoparasite of *H. coffeicola*, *Paranectriella carrissiana*, which produces conspicuous peithecia on the rust pustules – and which is reported to be common on this rust in West Africa (Câmara and Luz, 1939) – has a high level of host specificity since it was found to be ubiquitous on and damaging to both *H. coffeicola* in Cameroon and *H.* cf. *coffeicola* in Ethiopia, but never occurred in association with *H. vastatrix* in either of these countries.

Some *Fusarium* isolates were obtained as endophytes from coffee tissues (leaf, fruit and stem) during the surveys, and these are being examined. However, *Trichoderma* was the genus more commonly isolated as an endophyte than as a mycoparasite (Rodríguez et al., 2021). During similar surveys in South America, to assess the mycoparasites and endophytes associated with frosty pod disease (*Moniliophthora roreri*) and its wild *Theobroma* host, only two *Fusarium* species were recorded as mycoparasites, whilst 10 species were isolated as endophytes from stems and pods (Evans et al., 2003). What is obvious, from such surveys of plants and their coevolved fungal pathogens in their centers of origin, is that there is a rich and untapped mycodiversity that offers novel alternatives for the management of major crop diseases such as CLR.

5 Conclusion

The present study has built the taxonomic framework in order to select named and characterized *Fusarium* isolates for the applied studies and to enable a more focused assessment of their potential as biocontrol agents of *Hemileia vastatrix*.

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7 Figures and Tables

Table 1 Origin of the *Fusarium* isolates obtained during the surveys and used in the phylogenetic analyses.

Species	Strain number COAD	Sampling site	GenBank accession number						
			EF-1α	RPB2	β-tubulin	CAL	H3	TRI101	PHO
***Fusarium auranto-glutinosus* sp. nov.**	2935	Viçosa, MG, Brazil	MT259992	MT259998	-	MT260004	-	-	-
Fusarium auranto-glutinosus sp. nov.	2936	Viçosa, MG, Brazil	MT259993	MT259999	-	MT260005	-	-	-
Fusarium auranto-glutinosus sp. nov.	2942	San Lorenzo, Paraguay	MT259994	MT260000	-	MT260006	-	-	-
Fusarium auranto-glutinosus sp. nov.	2950	Kaffa, Ethiopia	MT259996	MT260002	-	MT260007	-	-	-
Fusarium auranto-glutinosus sp. nov.	2965	Kaffa, Ethiopia	MT259995	MT260001	-	MT260008	-	-	-
Fusarium auranto-glutinosus sp. nov.	2952	Tepi, Ethiopia	MT259997	MT260003	-	MT260009	-	-	-
Fusarium cameroonicum sp. nov.	2945	Somalomo, Cameroon	MT259968	MT259985	-	-	-	-	-
***Fusarium cameroonicum* sp. nov.**	2947	Somalomo, Cameroon	MT259970	MT259987	-	-	-	-	-
Fusarium cameroonicum sp. nov.	2948	Somalomo, Cameroon	MT259971	MT259988	-	-	-	-	-
Fusarium cameroonicum sp. nov.	3029	Somalomo, Cameroon	MT625758	MW796582	-	-	-	-	-
Fusarium hemileiae sp. nov.	2938	Vitória, ES, Brazil	MT232888	MT259960	MT259975	-	-	-	-
Fusarium hemileiae sp. nov.	2939	Lambari, MG, Brazil	MT232890	MT259961	MT259976	-	-	-	-
***Fusarium hemileiae* sp. nov.**	2943	Duas Barras, RJ, Brazil	MT232891	MT259962	MT259977	-	-	-	-
Fusarium hemileiae sp. nov.	2940	Conceição da Barra de Minas, MG, Brazil	MT232892	MT259963	MT259978	-	-	-	-
Fusarium hemileiae sp. nov.	2941	Nepomuceno, MG, Brazil	MT232893	MT259964	MT259979	-	-	-	-
Fusarium hemileiae sp. nov.	2946	Somalomo, Cameroon	MT232894	-	MT259980	-	-	-	-

Fusarium hemileiae sp. nov.	2966	Bore, Ethiopia	MT232895	MT259965	MT259981	-	-	-	-
Fusarium hemileiae sp. nov.	2967	Bonga, Ehiopia	MT232896	MT259966	MT259982	-	-	-	-
Fusarium hemileiae sp. nov.	2968	Hawassa, Etiópia	MT232897	-	MT259983	-	-	-	-
Fusarium hemileiae sp. nov.	2953	Goba, Etiópia	MT232898	MT259967	MT259984	-	-	-	-
Fusarium hemileiae sp. nov.	3009	Viçosa, MG, Brazil	MT625693	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3010	Manhuaçú, MG, Brazil	MT625694	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3011	Florestal, MG, Brazil	MT625695	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3012	Florestal, MG, Brazil	MT625726	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3013	Três Corações, MG, Brazil	MT625727	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3014	Vargínia, MG, Brazil	MT625696	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3015	BR 265, Brazil	MT625697	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3016	Universidade Federal de Lavras, MG, Brazil	MT625698	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3017	Conceição da Barra de Minas, MG, Brazil	MT625699	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3018	Conceição da Barra de Minas, MG, Brazil	MT625728	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3019	Conceição da Barra de Minas, MG, Brazil	MT625700	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3020	Itutinga, MG, Brazil	MT625701	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3021	Itutinga, MG, Brazil	MT625702	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3022	Itutinga, MG, Brazil	MT625703	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3023	Rio de Janeiro, RJ, Brazil	MT625704	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3024	Araponga, MG, Brazil	MT625729	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3026	Carmo da Cachoeira, MG, Brazil	MT625706	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3025	Samambaia, MG, Brazil	MT625706	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3028	Somalomo, Cameroon	MT625707	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3030	Bonga, Ethiopia	MT625730	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3031	Bonga, Ethiopia	MT625708	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3032	Jimma, Ethiopia	MT625709	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3033	Bonga, Ethiopia	MT625710	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3034	Bonga, Ethiopia	MT625711	-	-	-	-	-	-

Fusarium hemileiae sp. nov.	3035	Bonga, Ethiopia	MT625712	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3036	Bonga, Ethiopia	MT625713	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3037	Bonga, Ethiopia	MT625714	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3038	Tepi, Ethiopia	MT625715	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3039	Boseka, Ethiopia	MT625731	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3040	Mizan-Aman, Ethiopia	MT625716	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3041	Gera, Ethiopia	MT625717	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3042	Gera, Ethiopia	MT625718	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3043	Metu, Ethiopia	MT625692	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3044	Metu, Ethiopia	MT625732	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3045	Metu, Ethiopia	MT625719	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3046	Metu, Ethiopia	MT625720	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3047	Metu, Ethiopia	MT625721	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3048	Tepi, Ethiopia	MT625733	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3050	Metu, Ethiopia	MT625722	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3051	Agaro, Ethiopia	MT625723	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3052	Agaro, Ethiopia	MT625724	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3053	Gimbi, Ethiopia	MT625725	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3054	Agaro, Ethiopia	MT625739	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3055	Bonga, Ethiopia	MT625740	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3056	Bonga, Ethiopia	MT625741	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3057	Agaro, Ethiopia	MT625742	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3058	Jimma, Ethiopia	MT625734	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3059	Bale Robe, Ethiopia	MT625743	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3060	Bonga, Ethiopia	MT625744	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3061	Aleta Wendo, Ethiopia	MT625745	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3062	Hawassa, Ethiopia	MT625746	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3063	Hawassa, Ethiopia	MT625747	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3064	Hawassa, Ethiopia	MT625748	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3065	Hawassa, Ethiopia	MT625735	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3066	Hawassa, Ethiopia	MT625749	-	-	-	-	-	-

Fusarium hemileiae sp. nov.	3067	Aleta Wendo, Ethiopia	MT625750	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3068	Aleta Wendo, Ethiopia	MT625751	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3069	Dilla, Ethiopia	MT625736	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3070	Jimma, Ethiopia	MT625752	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3071	Jimma, Ethiopia	MT625737	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3072	Hawassa, Ethiopia	MT625753	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3073	Goba, Ethiopia	MT625754	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3074	Dilla, Ethiopia	MT625755	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3075	Jimma, Ethiopia	MT625738	-	-	-	-	-	-
Fusarium hemileiae sp. nov.	3076	Dilla, Ethiopia	MT625756	-	-	-	-	-	-
Fusarium lateritium	2937	Viçosa, MG, Brazil	MT232883	MT232887	MT232885	-	-	-	-
Fusarium lateritium	2944	Ubajara, CE, Brazil	MT232884	MT232888	MT232886	-	-	-	-
Fusarium perseae	2954	Jimma, Ethiopia	MT259974	MT259991	-	-	-	-	-
Fusarium pseudosolani sp. nov.	2963	Somalomo, Cameroon	MT259969	MT259986	-	-	-	-	-
***Fusarium pseudosolani* sp. nov.**	2949	Somalomo, Cameroon	MT259972	MT259989	-	-	-	-	-
Fusarium pseudosolani sp. nov	2964	Somalomo, Cameroon	MT259973	MT259990	-	-	-	-	-
Fusarium pseudosolani sp. nov	3027	Somalomo, Cameroon	MT625757	MW796581	-	-	-	-	-
Fusarium sp. (FGSC)	2951	Bonga, Ethiopia	MT260010	MT260011	MT260012	-	MT260013	MT260014	MT260015

Note: Types of new species in bold.

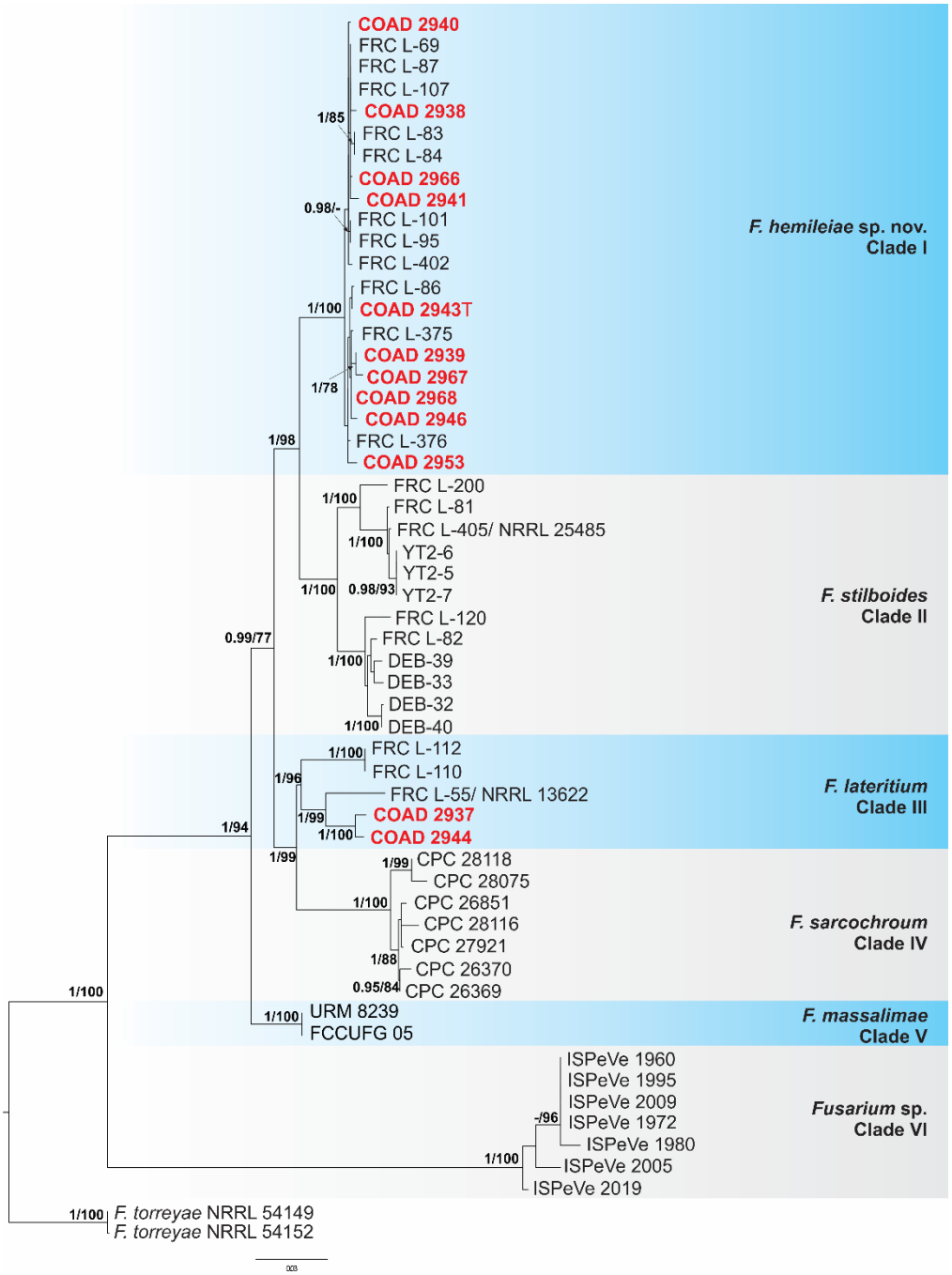

Figure 1 Maximum Likelihood (ML) phylogram obtained from the combined RPB2, EF-1α and β-tubulin sequences of members of the *Fusarium lateritium* species complex. Numbers on the nodes are Bayesian posterior probability/RAxML bootstrap values above 0.90/70%, respectively. Mycoparasitic strains on *Hemileia* in this study are shown in red. The tree is rooted with *Fusarium torreyae* (NRRL 54152 and NRRL 54149).

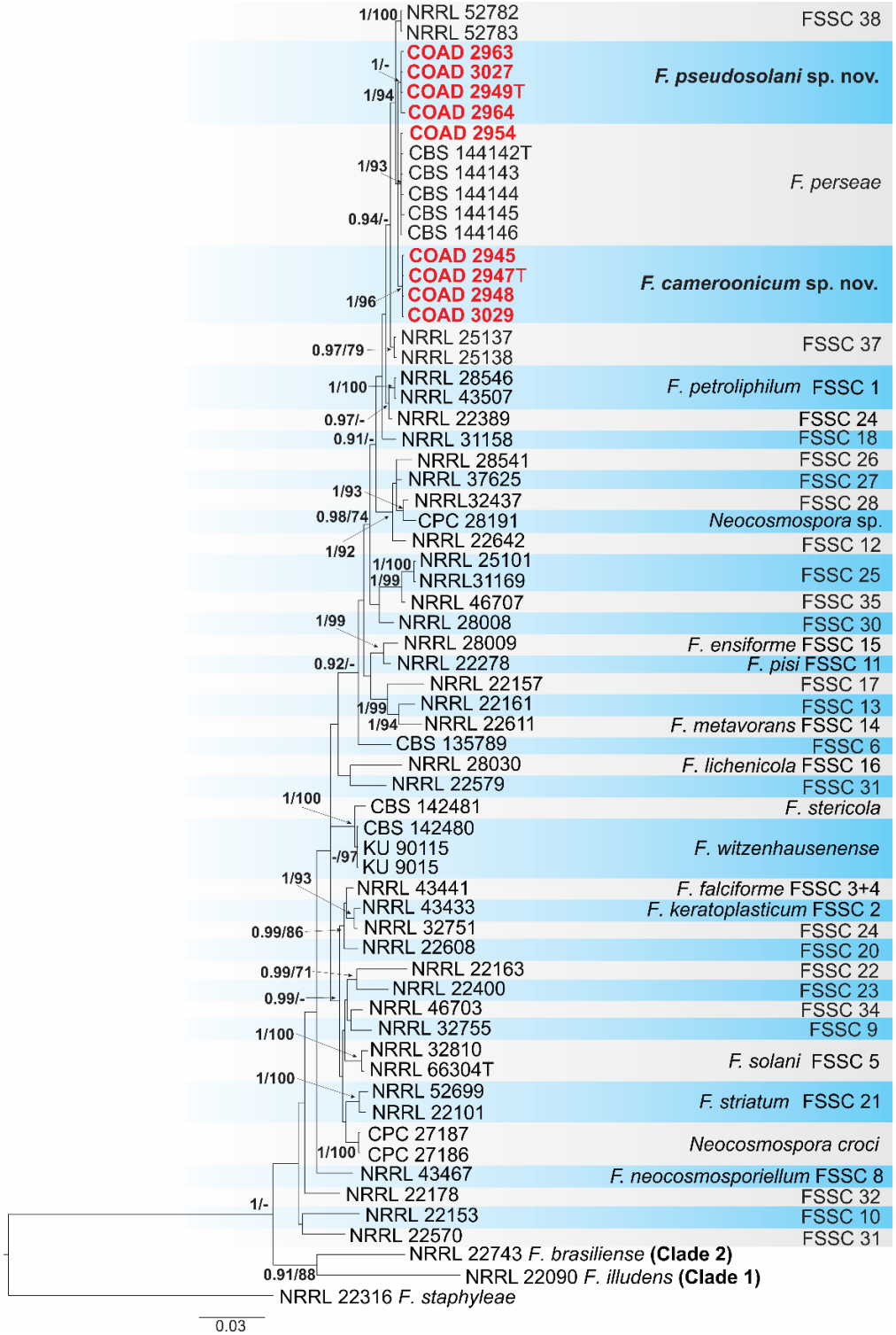

Figure 2 Bayesian Inference (BI) phylogram obtained from the combined RPB2 and EF-1α sequences of members of the *Fusarium solani* species complex (FSSC). Numbers on the nodes are Bayesian posterior probability/RAxML bootstrap values above 0.90/70%, respectively. Mycoparasitic strains on *Hemileia* in this study are shown in red. Numbers in parentheses indicate species within FSSC. Type material indicated with T. The tree is rooted with *Fusarium staphyleae* NRRL 22316.

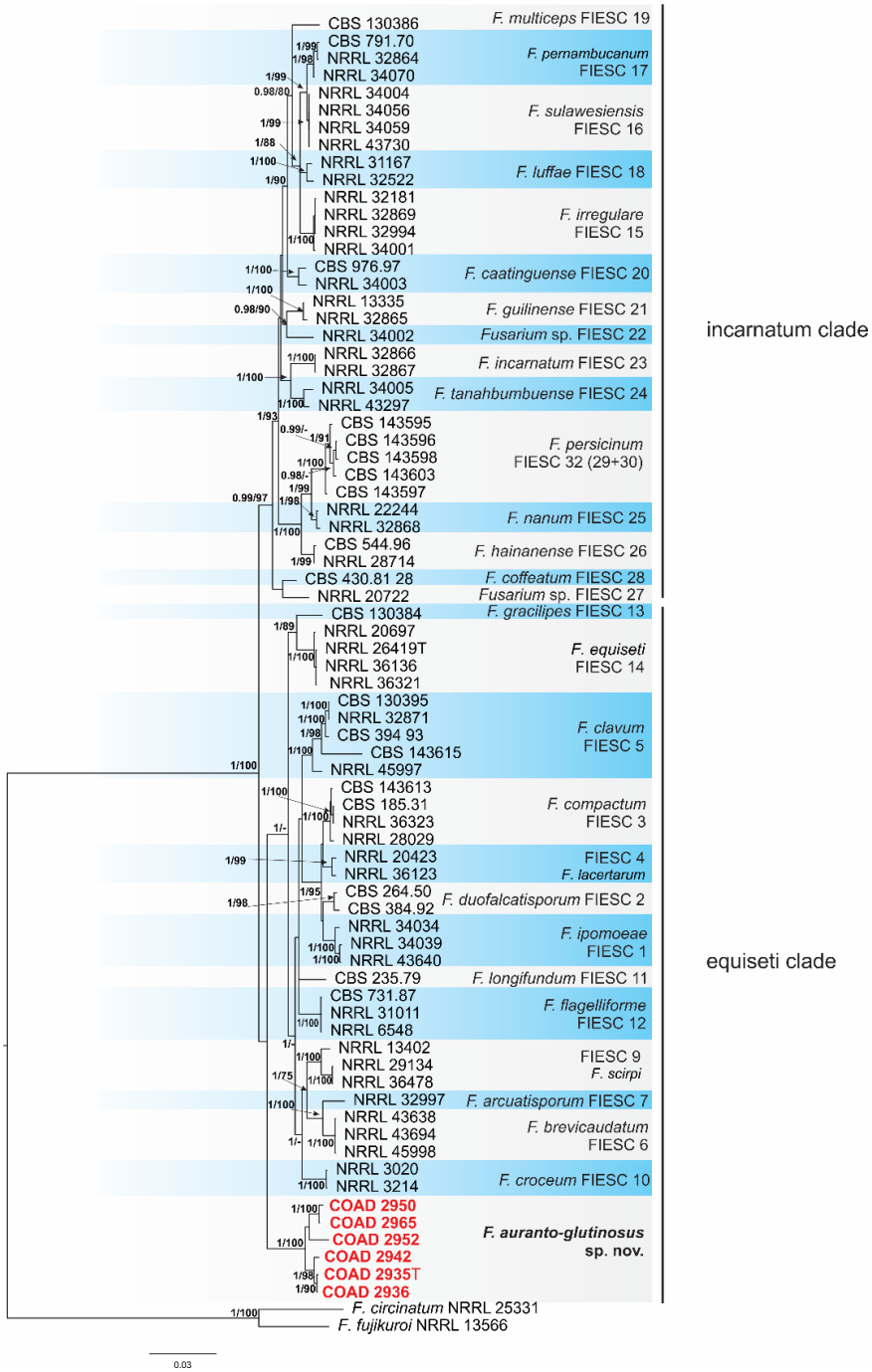

Figure 3 Bayesian Inference (BI) phylogram obtained from the combined CAL, RPB2 and EF-1α sequences of members of the *Fusarium incarnatum-esquiseti* species complex (FIESC). Numbers on the nodes are Bayesian posterior probability/RAxML bootstrap values above 0.90/70%, respectively. Mycoparasitic strains on *Hemileia* pustules included in this study are shown in red. Numbers in parentheses indicate species within FIESC. Type material indicated with T. The tree is rooted with *Fusarium circinatum* NRRL 25331 and *F. fujikuroi* NRRL 13566.

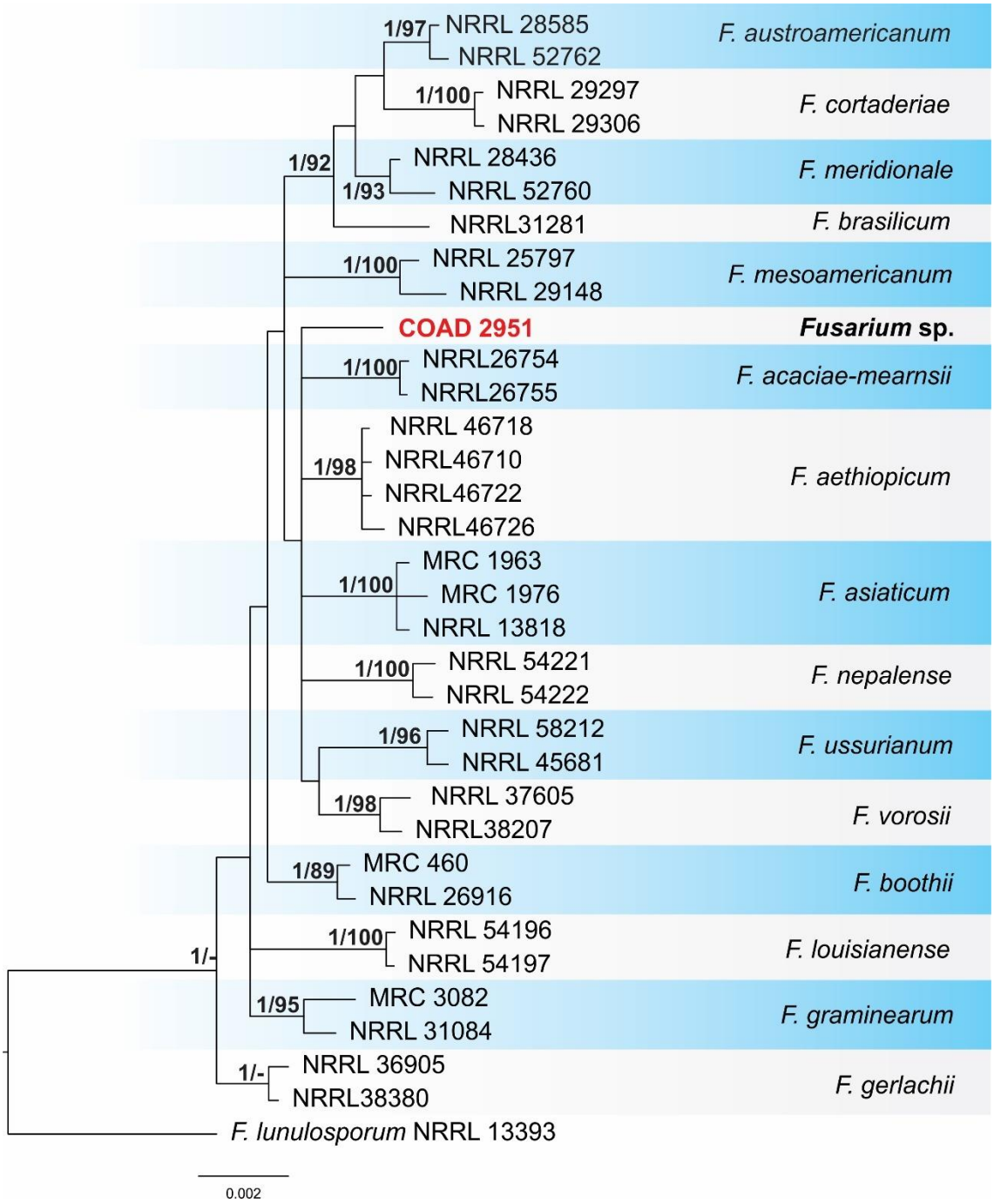

Figure 4 Bayesian Inference (BI) phylogram obtained from the combined HIS, PHO, RPB2, EF-1α, TRI101 and β-tubulin sequences of members of the *Fusarium graminearum* species complex. Numbers on the nodes are Bayesian posterior probability/RAxML bootstrap values above 0.90/70%, respectively. Mycoparasitic strains obtained from *Hemileia* pustules in this study are shown in red. The tree is rooted with *Fusarium lunulosporum* NRRL 13393.

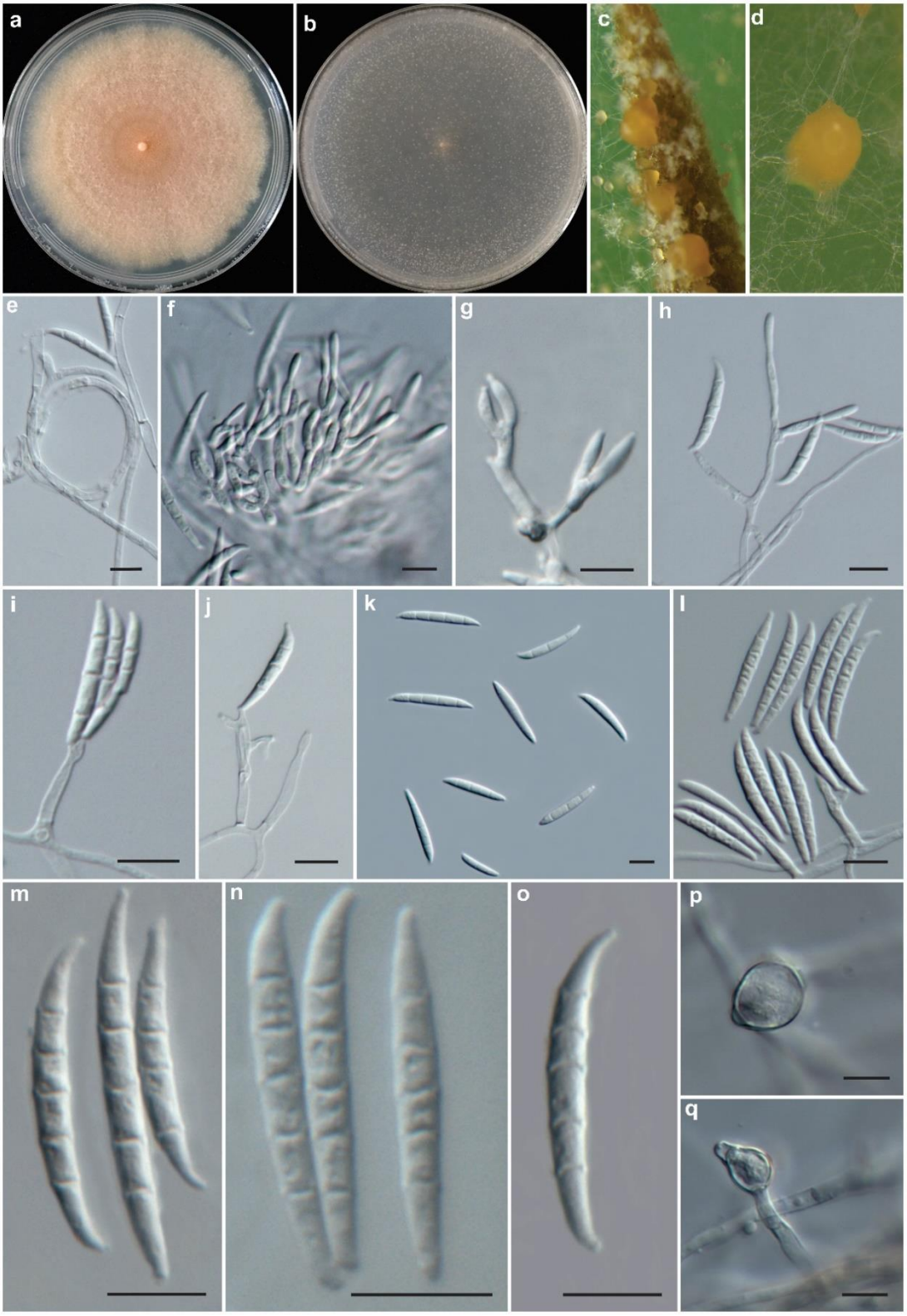

Figure 5 *Fusarium auranto-glutinosus* (COAD 2935). (**A)** Culture on PDA. **(B)** Culture on OA. **(C–D)** Sporodochia on carnation leaf-agar. **e** Spiral hyphae. **(F–G)** Sporodochial conidiophores and phialides. **(H–J)** Conidiophores and phialides formed on aerial mycelium. **(K)** Mesoconidia formed on aerial mycelium. **(L)** Macroconidia formed on aerial mycelium. **(M–O)** Sporodochial conidia. **(P)** Chlamydospores. Scale bars =10 μm.

Figure 6 *Fusarium cameroonicum* (COAD 2947). **(A)** Culture on PDA. **(B)** Culture on OA. **(C)** Sporodochia on carnation leaf-agar. **(D)** Spiral hyphae. **(E–G)** Conidiophores and phialides formed on aerial mycelium. **(H–I)** Microconidia formed on aerial mycelium. **(J–M)** Sporodochial conidia (same scale). **(N–O)** Chlamydospores. Scale bars =10 μm.

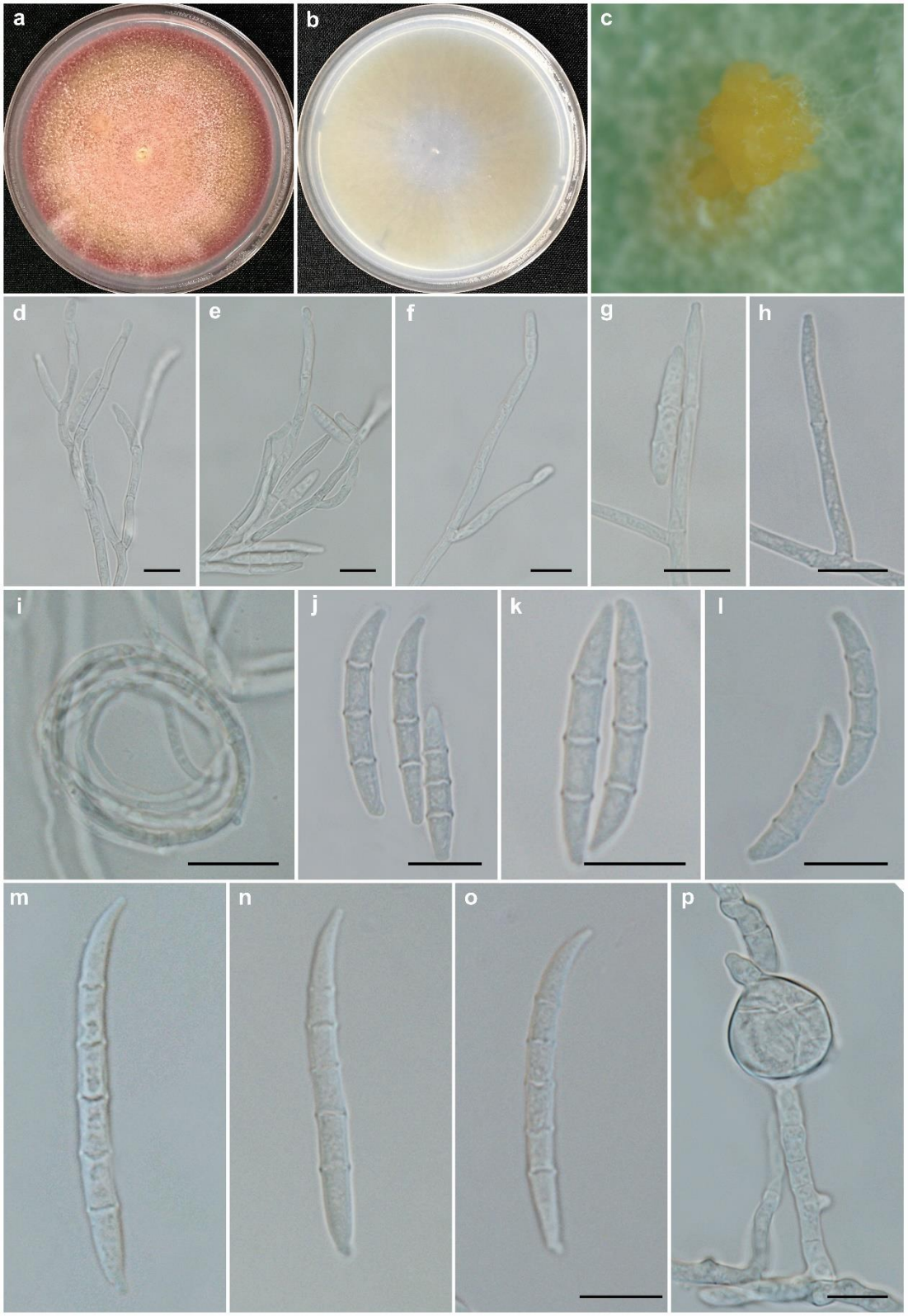

Figure 7 *Fusarium hemileiae* (COAD 2943). **(A)** Culture on PDA. (**B)** Culture on OA. **c** Sporodochia on carnation leaf-agar. **(D–H)** Conidiophores and phialides formed on aerial mycelium **(I)** Spiral hyphae. **(J–L)** Conidia on aerial mycelium. **(M–O)** Sporodochial conidia (same scale). **(P)** Chlamydospores. Scale bars =10 μm.

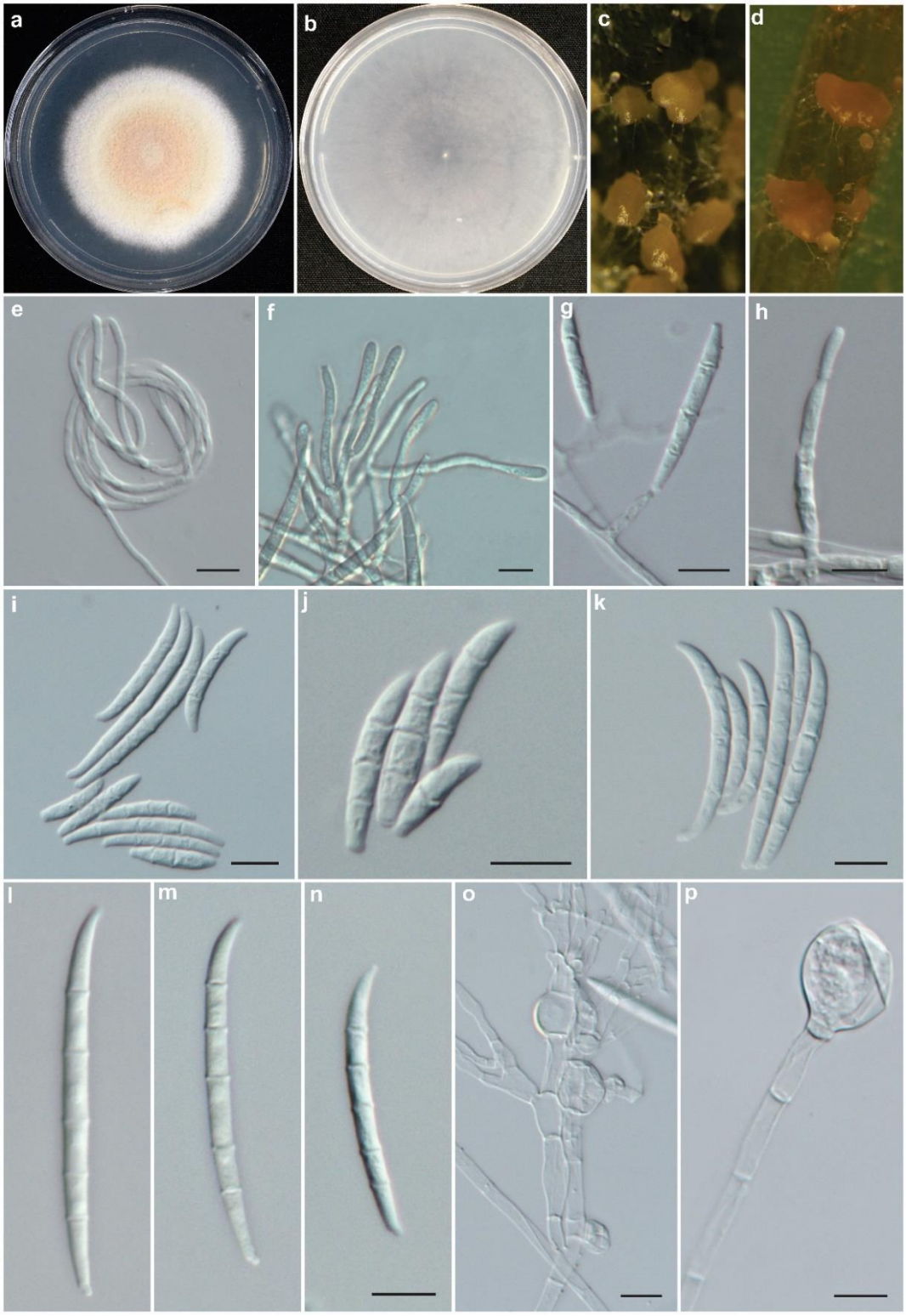

Figure 8 *Fusarium lateritium* (COAD 2944). **(A)** Culture on PDA. **(B)** Culture on OA. **(C–D)** Mucilagenous drops of conidia over sporodochia on carnation leaf-agar. **(E)** Spiral hypha. **(F)** Conidiophores and phialides formed on sporodochia. **(G–H)** Phialides formed on aerial mycelium. **(I–K)** Conidia formed on aerial mycelium. **(L–N)** Sporodochial conidia (same scale). **(O–P)** Chlamydospores. Scale bars =10 μm.

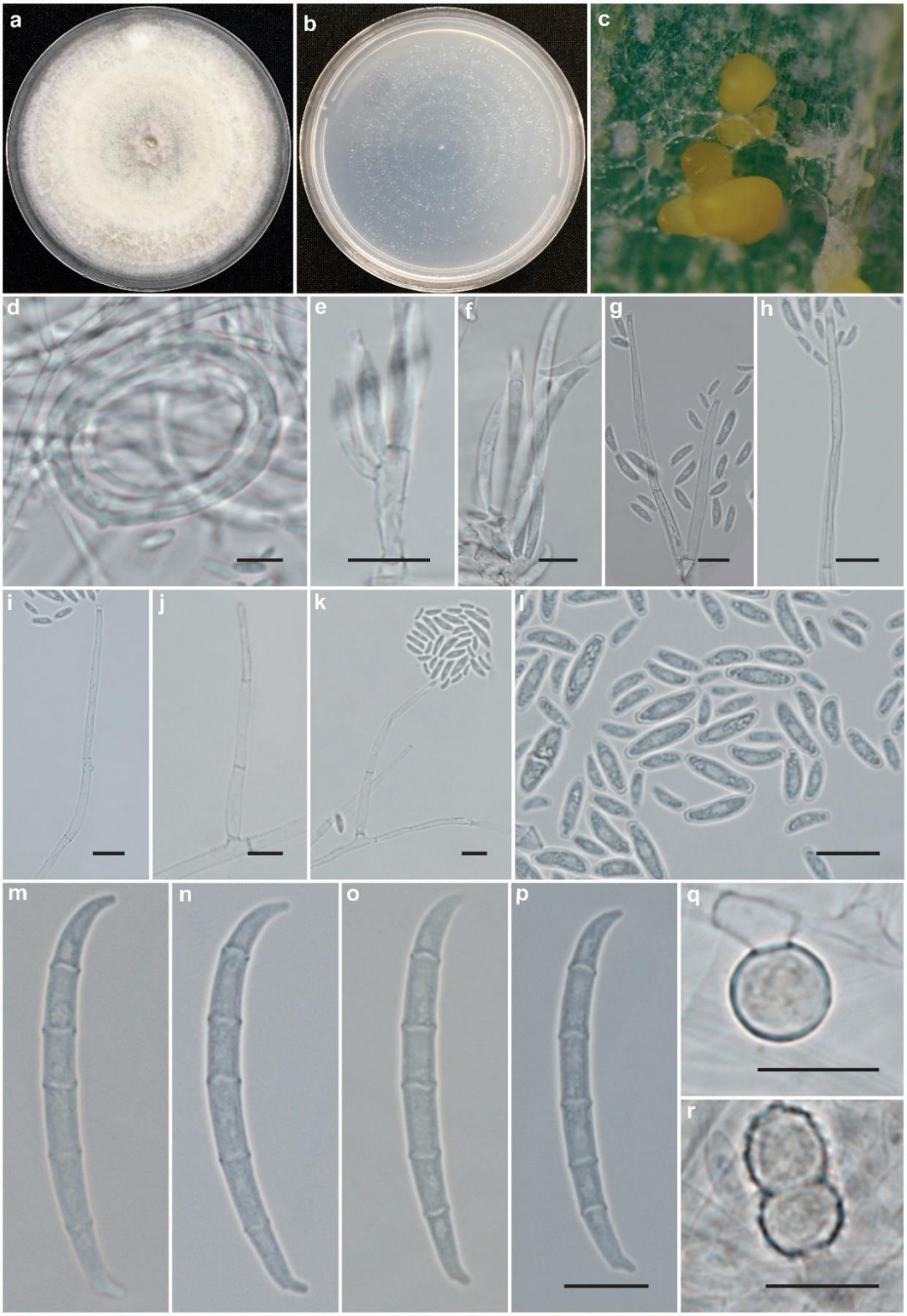

Figure 9 *Fusarium perseae* (COAD 2954). **(A)** Culture on PDA. **(B)** Culture on OA. **(C)** sporodochia on carnation leaf-agar. **(D)** Spiral hyphae. **(E**–**F)** Conidiophores and phialides formed on sporodochia. **(G**–**J)** Conidiophores and phialides formed on aerial mycelium. **(K)** False heads on aerial mycelium. **(L)** Microconidia formed on aerial mycelium. **(M**–**P)** Sporodochial conidia (same scale). **(Q**–**R)** Chlamydospores. Scale bars =10 μm.

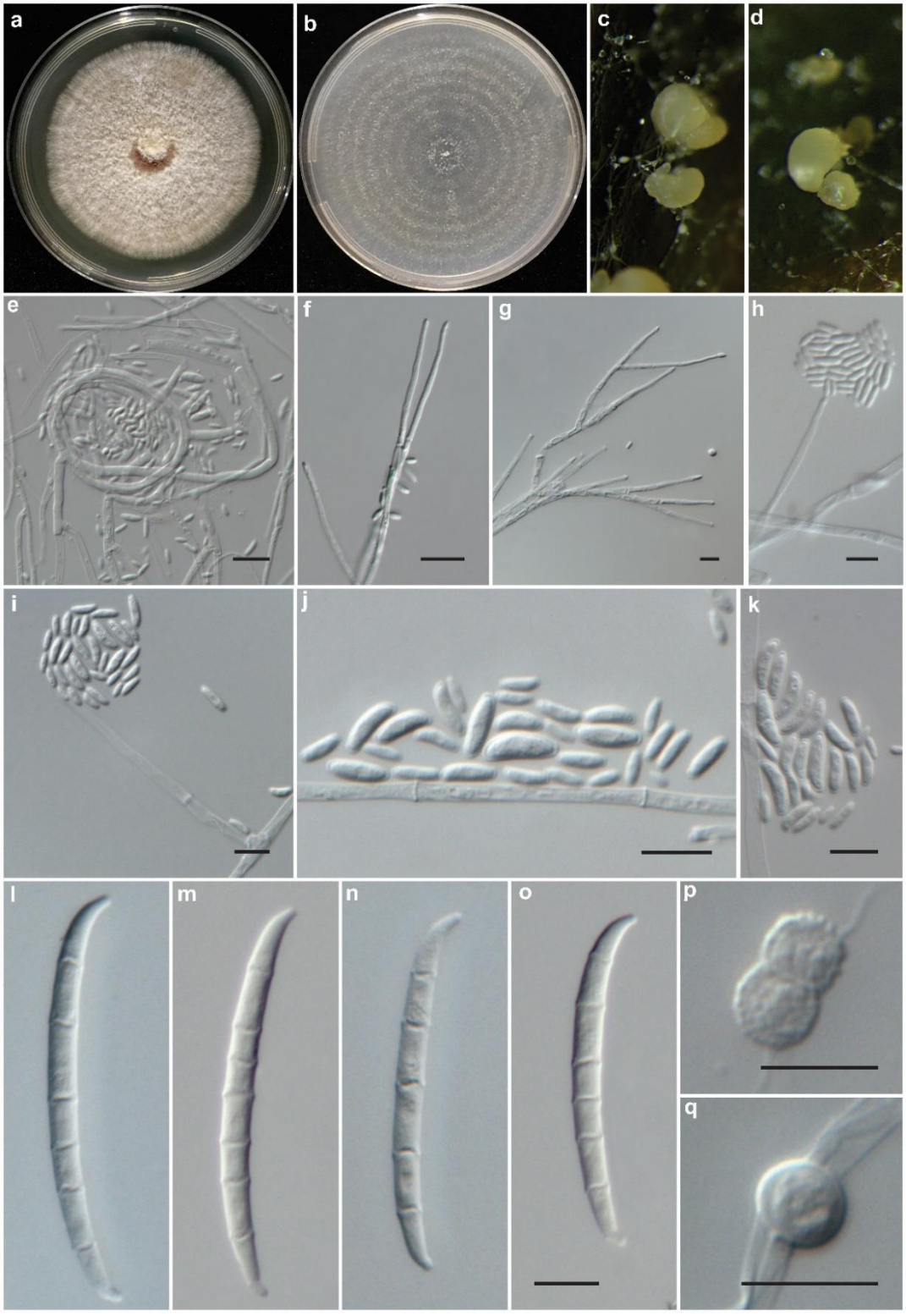

Figure 10 *Fusarium pseudosolani* (COAD 2949). **(A)** Culture on PDA. **(B)** Culture on OA. **(C–D)** Sporodochia on carnation leaf. **(E)** Spiral hyphae. **(F–G)** Conidiophores and phialides formed on aerial mycelium. **(H–I)** False heads on aerial mycelium. **(J–K)** Microconidia formed on aerial mycelium. **(L–O)** Sporodochial conidia (same scale). **(P–Q)** Chlamydospores. Scale bars =10 μm.

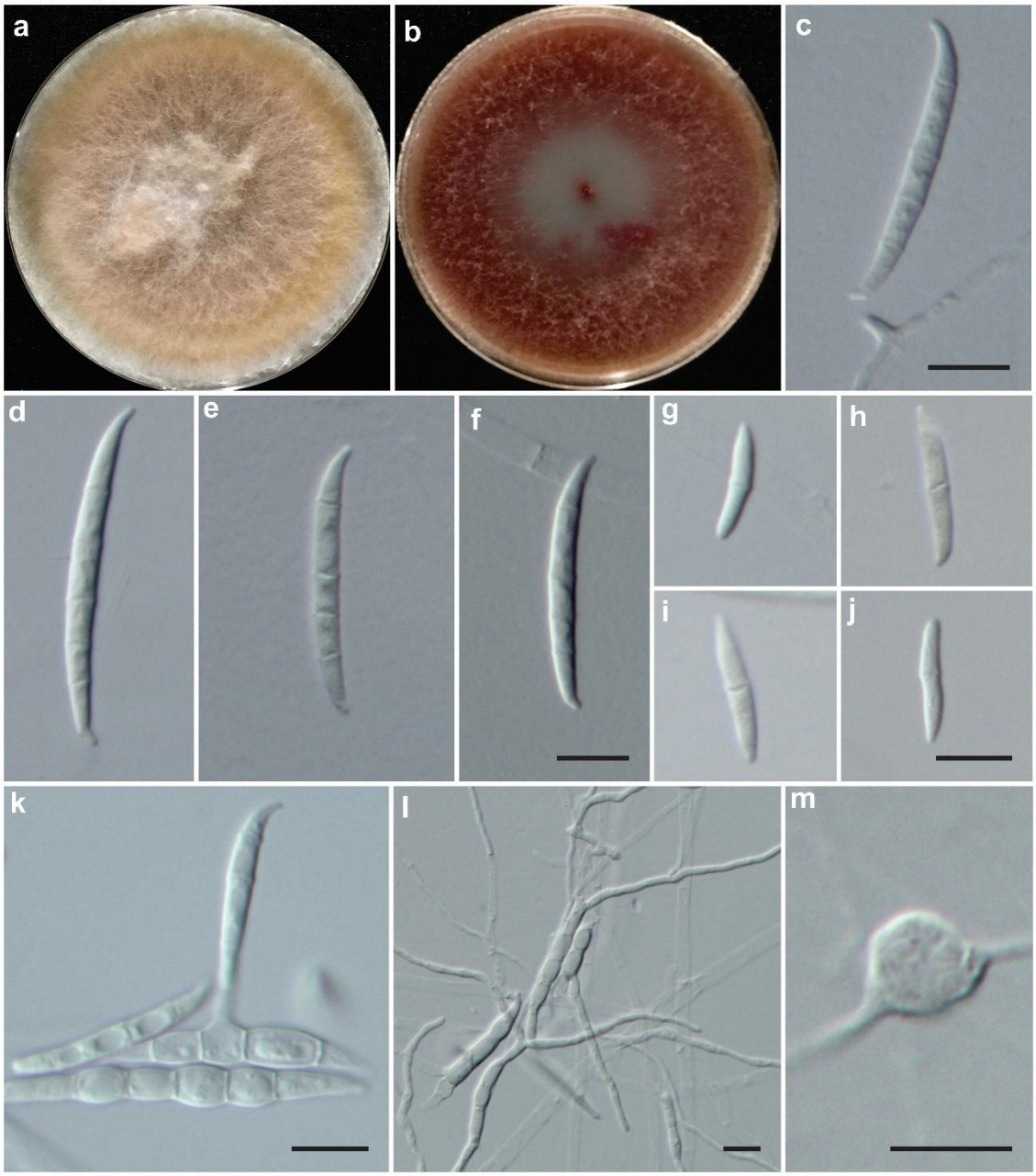

Figure 11 *Fusarium* sp. 1 (COAD 2951). **(A)** Culture on PDA. **(B)** Culture on OA. **(C)** Phialide formed on aerial mycelium. **(D–F)** Macroconidia formed on aerial mycelium (same scale). **(G–J)** Short conidia formed on aerial mycelium (same scale). **(K–L)** Chlamydospores formed within macroconidium. **(M)** Chlamydospore formed in hypha. Scale bars =10 μm.

8 Supplementary material

Table S1 Primers and PCR conditions.

Target gene	Primers	Primers sequence 5'→3'	Conditions for PCR	References
EF-1α	EF1	ATGGGTAAGGARGACAAGAC	94 ºC / 1 min., followed by 35 cycles at 94 ºC / 30s., 53 ºC / 45s., 72 ºC / 1 min. and 72 ºC/ 5 min.	(O'Donnell et al., 1998)
	EF2	GGARGTACCAGTSATCATGTT		
RPB2	5f2	GGGGWGAYCAGAAGAAGGC	94 ºC / 90s., followed by 39 cycles at 94 ºC / 30s, 55 ºC / 90s, 68 ºC / 2 min. and 68 ºC / 5 min.	(O'Donnell et al., 2008)
	7cr	CCCATRGCTTGYTTRCCCAT		
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	95 °C / 2 min., followed by 38 cycles at 94 °C / 35s, 52 °C / 55s, 72 °C / 2 min. and extension at 72 °C / 10 min.	(O'Donnell & Cigelnik, 1997)
	T22	TCTGGATGTTGTTGGGAATCC		
CAL	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	95 °C / 4 min., followed by 35 cycles at 95 °C / 30s, 59 °C / 30s, 72 °C / 45s and extension at 72 °C / 7 min.	(Carbone & Kohn, 1999; Groenewald et al., 2013)
	CAL-2rd	TGRTCNGCCTCDCGGATCATCTC		
HIS	H3-1a	ACTAAGCAGACCGCCCGCAGG	92 °C / 1 min., followed by 30 cycles at 92 °C / 1 min., 63 °C / 1 min., 72 °C / 1 min. and extension at 72 °C / 5 min.	(Glass & Donaldson, 1995)
	H3-1b	GCGGGCGAGCTGGATGTCCTT		
TRI101	TRI101F	CCATGGGTCGCRGGCCARGTSAA	95 °C / 2 min., followed by 30 cycles at 95 °C / 40s, 54 °C / 30s, 72 °C / 90s and extension at 72 °C / 7 min.	(Proctor et al., 2009)
	TRI101R	AACTCSCCRTCIGGYTTYTTNGGCAT		
PHO	PHO1	ATCTTCTGGCGTGTTATCATG	97 °C / 1 min., followed by 35 cycles at 96 °C / 30s, 50 °C / 1 min., 72 °C / 1 min. and extension at 72 °C / 10 min.	(Skovgaard & Nirenberg, 2001)
	PHO6	GATGTGGTTGTAAGCAAAGCCC		

Table S2. GenBank accession numbers of sequences derived from strains used in the phylogenetic analysis.

Species	Strain	EF-1α	RPB2	β-tubulin	CAL	H3	TRI101	PHO
Fusarium acaciae-mearnsii	NRRL 26754	AF212448.1	—	AF212742.1 AF212802.1 AF212765.1	—	AY452841.1	AF212595.1	AF212522.1 AF212485.1
Fusarium acaciae-mearnsii	NRRL 26755	AF212449.1	KM361658.1	AF212743.1 AF212803.1 AF212766.1	—	AY452842.1	AF212596.1	AF212523.1 AF212486.1
Fusarium aethiopicum	NRRL 46718	FJ240296.1	KM361670.1	FJ240286.2	—	FJ240242.1	FJ240340.1	FJ240329.2
Fusarium aethiopicum	NRRL 46726	FJ240298.1		FJ240288.2	—	FJ240244.1	FJ240342.1	FJ240331.2
Fusarium aethiopicum	NRRL 46722	FJ240297.1		FJ240287.2	—	FJ240243.1	FJ240341.1	FJ240330.2
Fusarium aethiopicum	NRRL 46710	FJ240295.1		FJ240285.2	—	FJ240241.1	FJ240339.1	FJ240328.2
Fusarium arcuatisporum (FIESC 7)	NRRL 32997	GQ505624	GQ505802	—	GQ505536	—	—	—
Fusarium asiaticum	MRC 1976	MH582250.1	MH582121.1		—	—	—	—
Fusarium asiaticum	MRC 1963	MH582249.1	MH582120.1		—	—	—	—
Fusarium asiaticum	NRRL 13818	AF212451.1	JX171573.1	AF212745.1 AF212805.1 AF212768.1	—	AY452821.1	AF212598.1	AF212525.1 AF212488.1
Fusarium austroamericanum	NRRL 52762	JF740836.1			—	—	—	—
Fusarium austroamericanum	NRRL 28585	AF212439.1	KM361661.1	AF212734.1 AF212793.1 AF212756.1	—	AY452825.1	AF212586.1	AF212513.1 AF212476.1
Fusarium boothii	NRRL 26916	GQ915503.1	KM361659.1	GQ915437.1	—	GQ915470.1	AF212591.1	AF212518.1

Fusarium boothii	MRC 460	MH582232.1	MH582211.1	—	—		—	—
Fusarium brasilicum	NRRL31281	AY452964.1		—	—	AY452861.1	—	—
Fusarium brasilicum	NRRL 31238		KM361663.1	—	—	—	—	—
Fusarium brasiliense	NRRL 22743	EF408407.1	EU329525.1	—	—	—	—	—
Fusarium brevicaudatum (FIESC 6)	NRRL 43638	GQ505665	GQ505843	—	GQ505576	—	—	—
Fusarium brevicaudatum (FIESC 6)	NRRL 43694	GQ505668	GQ505846	—	GQ505579	—	—	—
Fusarium brevicaudatum (FIESC 6)	NRRL 45998	GQ505673	GQ505851	—	GQ505584	—	—	—
Fusarium caatinguense (FIESC 20)	CBS 976.97, NRRL 36575	GQ505656	GQ505834	—	GQ505568	—	—	—
Fusarium caatinguense (FIESC 20)	NRRL 34003	GQ505627	GQ505805	—	GQ505539	—	—	—
Fusarium circinatum	CBS 405.97T, NRRL 25331	KM231943	HM068354	—	—	—	—	—
Fusarium clavum (FIESC 5)	CBS 394.93, NRRL 25795	GQ505597	GQ505775	—	GQ505509	—	—	—
Fusarium clavum (FIESC 5)	CBS 130395, NRRL 34032	GQ505635	GQ505813	—	GQ505547	—	—	—
Fusarium clavum (FIESC 5)	NRRL 32871	GQ505619	GQ505797	—	GQ505531	—	—	—
Fusarium clavum (FIESC 5)	NRRL 45997	GQ505672	GQ505850	—	GQ505583	—	—	—

Fusarium clavum (FIESC 5)	CBS 143615, CPC 30868, HoCo1	LT970777	LT970749	—	LT970730.1	—	—	—
Fusarium coffeatum (FIESC 28)	CBS 430.81, NRRL 28577	GQ505603	GQ505781	—	GQ505515	—	—	—
Fusarium compactum (FIESC 3)	CBS 185.31, NRRL 36318	GQ505646	GQ505824	—	GQ505558	—	—	—
Fusarium compactum (FIESC 3)	NRRL 28029	GQ505602	GQ505780	—	GQ505514	—	—	—
Fusarium compactum (FIESC 3)	CBS 186.31 = NRRL 36323	GQ505648	GQ505826	—	GQ505560	—	—	—
Fusarium compactum (FIESC 3)	CBS 143613, CPC 30866, FiPo2	LT970776	LT970748	—	LT970729.1	—	—	—
Fusarium cortaderiae	NRRL 29297	AY225885.1	KM361662.1	AH012625.2	—	AY452856.1	AY222642.1	AH012627.2
Fusarium croceum (FIESC 10)	NRRL 3020	GQ505586	GQ505764	—	GQ505498	—	—	—
Fusarium croceum (FIESC 10)	NRRL 3214	GQ505587	GQ505765	—	GQ505499	—	—	—
Fusarium duofalcatisporum (FIESC 2)	CBS 264.50, NRRL 36401	GQ505651	GQ505829	—	GQ505563	—	—	—
Fusarium duofalcatisporum (FIESC 2)	CBS 384.92, NRRL 36448	GQ505652	GQ505830	—	GQ505564	—	—	—
Fusarium ensiforme (FSSC 15)	NRRL 28009	DQ246869.1	EF470136.1	—	—	—	—	—

Fusarium equiseti (FIESC 14)	CBS 107.07, NRRL 36136	GQ505644	GQ505822	—	GQ505556.1	—	—	—
Fusarium equiseti (FIESC 14)	CBS 185.34, NRRL 36321	GQ505647	GQ505825	—	GQ505559.1	—	—	—
Fusarium equiseti (FIESC 14)	CBS 245.61, NRRL 20697	GQ505594.1	JX171595	—	GQ505506.1	—	—	—
Fusarium equiseti (FIESC 14)	CBS 307.94T, NRRL 26419	GQ505599	GQ505777	—	GQ505511.1	—	—	—
Fusarium falciforme (FSSC 3+4)	NRRL 43441	DQ790478.1	DQ790566.1	—		—	—	—
Fusarium flagelliforme (FIESC 12)	NRRL 6548	GQ505589	GQ505767	—	GQ505501	—	—	—
Fusarium flagelliforme (FIESC 12)	NRRL 31011	GQ505606	GQ505784	—	GQ505518	—	—	—
Fusarium fujikuroi	NRRL 13566	AF160279	JX171570	—	AF158332.1	—	—	—
Fusarium gerlachii	NRRL 36905	DQ459742.1	KM361664.1	DQ459640.1	—	DQ459725.1	DQ452409.1	DQ459708.1 DQ459691.1
Fusarium gerlachii	NRRL 3880	DQ459743.1	—	DQ459675.1 DQ459658.1 DQ459641.1	—	DQ459726.1	—	—
Fusarium gracillipes (FIESC 13)	CBS 130384, NRRL 43635	GQ505662	GQ505840	—	GQ505573	—	—	—
Fusarium graminearum	MRC 3082	MH582244.1	MH582207.1		—	—	—	—
Fusarium graminearum	NRRL 31084	HM744693.1	JX171644.1	HQ141668.1	—	AY452852.1	—	HQ154524.1

Fusarium guilinense (FIESC 21)	NRRL 13335	GQ505590	GQ505768	—	GQ505502	—	—	—
Fusarium guilinense (FIESC 21)	NRRL 32865	GQ505614	GQ505792	—	GQ505526	—	—	—
Fusarium hainanense (FIESC 26)	CBS 544.96, NRRL 26417	GQ505598	GQ505776	—	GQ505510	—	—	—
Fusarium hainanense (FIESC 26)	NRRL 28714	GQ505604	GQ505782	—	GQ505516	—	—	—
Fusarium hemileiae	FRC L-69	AY707155	—	AY707137	—	—	—	—
Fusarium hemileiae	FRC L-83	AY707158	—	AY707140	—	—	—	—
Fusarium hemileiae	FRC L-84	AY707159	—	AY707141	—	—	—	—
Fusarium hemileiae	FRC L-86	AY707160	—	AY707142	—	—	—	—
Fusarium hemileiae	FRC L-87	AY707161	—	AY707143	—	—	—	—
Fusarium hemileiae	FRC L-95	AY707162	—	AY707144	—	—	—	—
Fusarium hemileiae	FRC L-101	AY707163	—	AY707145	—	—	—	—
Fusarium hemileiae	FRC L-107	AY707164	—	AY707146	—	—	—	—
Fusarium hemileiae	FRC L-375	AY707169	—	AY707151	—	—	—	—
Fusarium hemileiae	FRC L-376	AY707170	—	AY707152	—	—	—	—
Fusarium hemileiae	FRC L-402	AY707171	—	AY707153	—	—	—	—
Fusarium illudens	NRRL 22090	AF178326.1	JX171601.1	—	—	—	—	—
Fusarium incarnatum (FIESC 23)	NRRL 32866	GQ505615	GQ505793	—	GQ505527	—	—	—
Fusarium incarnatum (FIESC 23)	NRRL 32867	GQ505616	GQ505794	—	GQ505528	—	—	—

Fusarium ipomoeae (FIESC 1)	NRRL 34034	GQ505636	GQ505814	—	GQ505548	—	—	—
Fusarium ipomoeae (FIESC 1)	NRRL 34039	GQ505639	GQ505817	—	GQ505551	—	—	—
Fusarium ipomoeae (FIESC 1)	NRRL 43640	GQ505667	GQ505845	—	GQ505578	—	—	—
Fusarium irregulare (FIESC 15)	NRRL 32181	GQ505610	GQ505788	—	GQ505522	—	—	—
Fusarium irregulare (FIESC 15)	NRRL 32869	GQ505618	GQ505796	—	GQ505530	—	—	—
Fusarium irregulare (FIESC 15)	NRRL 32994	GQ505621	GQ505799	—	GQ505533	—	—	—
Fusarium irregulare (FIESC 15)	NRRL 34001	GQ505625	GQ505803	—	GQ505537	—	—	—
Fusarium keratoplasticum (FSSC 2)	NRRL 43433	DQ790473.1	DQ790561.1	—	—	—	—	—
Fusarium lacertarum (FIESC 4)	CBS 102300, NRRL 36123	GQ505643	GQ505821	—	GQ505555.1	—	—	—
Fusarium lacertarum (FIESC 4)	CBS 130185T, NRRL 20423	GQ505593	JX171581	—	GQ505505.1	—	—	—
Fusarium lateritium	FRC L-110	AY707165	HM068358.1	AY707147	—	—	—	—
Fusarium lateritium	FRC L-112	AY707166	—	AY707148	—	—	—	—
Fusarium lateritium	FRC L-405, NRRL 25485	AY707172	—	AY707154	—	—	—	—

Fusarium lateritium	FRC L-55	AY707173	HM068350.1	—	—	—	—	—
Fusarium lichenicola (FSSC 16)	NRRL 28030	KR673968.1	EF470146.1	—	—	—	—	—
Fusarium longifundum (FIESC 11)	CBS 235.79, NRRL 36372	GQ505649	GQ505827	—	GQ505561	—	—	—
Fusarium louisianense	NRRL 54196	KM889632.1	—	KM889627.1	—	KM889637.1	KM889662.1	KM889647.1
Fusarium louisianense	NRRL 54197	KM889633.1	KM361667.1	KM889628.1	—	KM889638.1	KM889663.1	KM889648.1
Fusarium luffae (FIESC 18)	NRRL 31167	GQ505608	GQ505786	—	GQ505520	—	—	—
Fusarium luffae (FIESC 18)	NRRL 32522	GQ505612	GQ505790	—	GQ505524	—	—	—
Fusarium lunulosporum	NRRL 13393	AF212467.1	KM361655.1	AF212821.1	—	AY452835.1	AF212614.1	AF212541.1
Fusarium massalimae	URM 8239	MN939763	MN939767	MN939759				
Fusarium massalimae	FCCUFG 05	MN939764	MN939768	MN939760				
Fusarium meridionale	NRRL 28436	AF212435.1	KM361660.1	AF212730.1 AF212789.1 AF212752.1	—	AY452824.1	AF212582.1	AF212509.1 AF212472.1
Fusarium meridionale	NRRL 52760	JF740835.1	—	—	—	—	—	—
Fusarium mesoamericanum	NRRL 29148	AF212442.1	—	AF212736.1 AF212796.1 AF212759.1	—	AY452847.1	AF212589.1	AF212516.1 AF212479.1
Fusarium mesoamericanum	NRRL 25797	AF212441.1	KM361657.1	AF006364.1 AF212795.1 AF212758.1	—	AY452826.1	AF212588.1	AF212515.1 AF212478.1

Fusarium metavorans (FSSC 6)	CBS 135789	KU711773.1	KU604374.1	—	—	—	—	—
Fusarium multiceps (FIESC 19)	CBS 130386, NRRL 43639	GQ505666	GQ505844	—	GQ505577	—	—	—
Fusarium nanum (FIESC 25)	NRRL 22244	GQ505596	GQ505774	—	GQ505508	—	—	—
Fusarium nanum (FIESC 25)	NRRL 32868	GQ505617	GQ505795	—	GQ505529	—	—	—
Fusarium neocosmoporiellum (FSSC 8)	NRRL 43467	EF452940.1	EF469979.1	—	—	—	—	—
Fusarium nepalense	NRRL 54221	KM889630.1	—	KM889625.1	—	KM889635.1	KM889660.1	KM889645.1
Fusarium nepalense	NRRL 54222	KM889631.1	KM361668.1	KM889626.1	—	KM889636.1	KM889661.1	KM889646.1
Fusarium pernambucanum (FIESC 17)	CBS 791.70, NRRL 36548	GQ505655	GQ505833	—	GQ505567	—	—	—
Fusarium pernambucanum (FIESC 17)	NRRL 32864	GQ505613	GQ505791	—	GQ505525	—	—	—
Fusarium pernambucanum (FIESC 17)	NRRL 34070	GQ505642	GQ505820	—	GQ505554	—	—	—
Fusarium perseae	CBS 144142T, CPC 26829	LT991902	LT991909	—	—	—	—	—

Fusarium perseae	CBS 144143, CPC 26830	LT991903	LT991910	—	—	—	—	—
Fusarium perseae	CBS 144144, CPC 26831	LT991904	LT991911	—	—	—	—	—
Fusarium perseae	CBS 144145, CPC 26832	LT991905	LT991912	—	—	—	—	—
Fusarium perseae	CBS 144146, CPC 26833	LT991906	LT991913	—	—	—	—	—
Fusarium persicinum (FIESC 32)	CBS 143595, CPC 30847, MoPo1	LT970778	LT970750	—	LT970731.1	—	—	—
Fusarium persicinum (FIESC 32)	CBS 143596, CPC 30848, MoPo2	LT970779	LT970751	—	LT970732.1	—	—	—
Fusarium persicinum (FIESC 32)	CBS 143598, CPC 30850, MoSm17-1	LT970780	LT970752	—	LT970733.1	—	—	—
Fusarium persicinum (FIESC 32)	CBS 143597, CPC 30849, MoSm119	LT970784	LT970756	—	LT970737.1	—	—	—
Fusarium persicinum (FIESC 32)	CBS 143603, CPC 30855, MoSm29	LT970782	LT970754	—	LT970735.1	—	—	—
Fusarium petroliphilum (FSSC 1)	NRRL 28546	DQ246887.1	EU329544.1	—	—	—	—	—
Fusarium petroliphilum (FSSC 1)	NRRL 43507	DQ790490.1	DQ790578.1	—	—	—	—	—

Fusarium petroliphilum (FSSC 1)	NRRL 54988	KR673958.1	KR673995.1	—	—	—	—	—
Fusarium petroliphilum (FSSC 1)	NRRL 54995	KC808215.1	KC808356.1	—	—	—	—	—
Fusarium pisi (FSSC 11)	NRRL 22278	AF178337.1	EU329501.1	—	—	—	—	—
Fusarium sarcochroum	CPC 26369	LT746207	LT746320	—	—	—	—	—
Fusarium sarcochroum	CPC 26370	LT746208	LT746321	—	—	—	—	—
Fusarium sarcochroum	CPC 26851	LT746209	LT746322	—	—	—	—	—
Fusarium sarcochroum	CPC 27921	LT746210	LT746323	—	—	—	—	—
Fusarium sarcochroum	CPC 28075	LT746211	LT746324	—	—	—	—	—
Fusarium sarcochroum	CPC 28116	LT746212	LT746325	—	—	—	—	—
Fusarium sarcochroum	CPC 28118	LT746213	LT746326	—	—	—	—	—
Fusarium scirpi (FIESC 9)	CBS 447.84, NRRL 36478	GQ505654	GQ505832	—	GQ505566.1	—	—	—
Fusarium scirpi (FIESC 9)	CBS 448.84, NRRL 29134	GQ505605	GQ505783	—	GQ505517.1	—	—	—
Fusarium scirpi (FIESC 9)	NRRL 13402	GQ505592	JX171566	—	GQ505504.1	—	—	—
Fusarium solani (FSSC 18)	NRRL 32301	DQ246929.1	EU329567.1	—	—	—	—	—
Fusarium solani (FSSC 25)	NRRL 52783	JF740851.1	JF741177.1	—	—	—	—	—
Fusarium solani (FSSC 37)	NRRL 25137	JF740757.1	JF741084.1	—	—	—	—	—

Fusarium solani (FSSC 37)	NRRL 25138	JF740758.1	JF741085.1	—	—	—	—	—
Fusarium solani (FSSC 38)	NRRL 52781	JF740849.1	JF741175.1	—	—	—	—	—
Fusarium solani (FSSC 38)	NRRL 52782	JF740850.1	JF741176.1	—	—	—	—	—
Fusarium solani (FSSC 38)	NRRL 25101	JF740728.1		—	—	—	—	—
Fusarium solani (FSSC 5)	NRRL 32810	DQ247118.1	EU329624.1	—	—	—	—	—
Fusarium solani (FSSC 5)	NRRL 66304, FRC S-2364, CBS 140079, G.J.S. 09-1466	KT313611.1	KT313623.1	—	—	—	—	—
Fusarium sp.	ISPaVe2005	FN551000.1	—	FN554670.1	—	—	—	—
Fusarium sp.	ISPaVe2019	FN550980.1	—	FN554671.1	—	—	—	—
Fusarium sp.	ISPaVe 1980	FN550968.1	—	FN554648.1	—	—	—	—
Fusarium sp.	ISPaVe 1960	FN550954.1	—	FN554635.1	—	—	—	—
Fusarium sp.	ISPaVe 1972	FN550956.1	—	FN554640.1	—	—	—	—
Fusarium sp.	ISPaVe 1995	FN550998.1	—	FN554659.1	—	—	—	—
Fusarium sp.	ISPaVe 2009	FN550961.1	—	FN554666.1	—	—	—	—
Fusarium sp. (FIESC 22)	NRRL 34002	GQ505626	GQ505804	—	GQ505538	—	—	—
Fusarium sp. (FIESC 27)	NRRL 20722	GQ505595	GQ505773	—	GQ505507	—	—	—
Fusarium sp. (FSSC 12)	NRRL 22642	DQ246844.1	EU329522.1	—	—	—	—	—
Fusarium sp. (FSSC 13)	NRRL 22161	AF178330.1	EU329494.1	—	—	—	—	—
Fusarium sp. (FSSC 14)	NRRL 22611	DQ246841.1	EU329518.1	—	—	—	—	—

Fusarium sp. (FSSC 17)	NRRL 22157	AF178359.1	EU329493.1	—	—	—	—	—
Fusarium sp. (FSSC 18)	NRRL 31158	DQ246916.1	EU329559.1	—	—	—	—	—
Fusarium sp. (FSSC 20)	NRRL 22608	DQ246838.1	EU329517.1	—	—	—	—	—
Fusarium sp. (FSSC 22)	NRRL 22163	AF178328.1	EU329496.1	—	—	—	—	—
Fusarium sp. (FSSC 23)	NRRL 22400	AF178343.1	EU329509.1	—	—	—	—	—
Fusarium sp. (FSSC 24)	NRRL 32751	DQ247070.1	EU329611.1	—	—	—	—	—
Fusarium sp. (FSSC 24)	NRRL 22389	AF178340.1	EU329506.1	—	—	—	—	—
Fusarium sp. (FSSC 25)	NRRL 31169	DQ246923.1	KR673999.1	—	—	—	—	—
Fusarium sp. (FSSC 26)	NRRL 28541	DQ246882.1	EU329542.1	—	—	—	—	—
Fusarium sp. (FSSC 27)	NRRL 37625	FJ240353.1	EU329637.1	—	—	—	—	—
Fusarium sp. (FSSC 28)	NRRL 32437	DQ246979.1	EU329581.1	—	—	—	—	—
Fusarium sp. (FSSC 30)	NRRL 28008	DQ246868	EF470135.1	—	—	—	—	—
Fusarium sp. (FSSC 31)	NRRL 22579	AF178352.1	EU329515.1	—	—	—	—	—
Fusarium sp. (FSSC 31)	NRRL 22570	AF178360.1	EU329513.1	—	—	—	—	—
Fusarium sp. (FSSC 32)	NRRL 22178	AF178334.1	EU329498.1	—	—	—	—	—
Fusarium sp. (FSSC 34)	NRRL 46703	HM347126.1	EU329661.1	—	—	—	—	—
Fusarium sp. (FSSC 35)	NRRL 46707	HM347127.1	EU329665.1	—	—	—	—	—
Fusarium sp. (FSSC 7)	NRRL 32323	DQ246951.1	EU329576.1	—	—	—	—	—
Fusarium sp. (FSSC 7)	NRRL 32794	DQ247103.1	EU329622.1	—	—	—	—	—
Fusarium sp. (FSSC 9)	NRRL 32755	DQ247073.1	EU329613.1	—	—	—	—	—
Fusarium staphyleae	NRRL 22316	MH582426.1	EU329502.1	—	—	—	—	—
Fusarium stercicola	CBS 142481	KY556524	KY556552	—	—	—	—	—
Fusarium stilboides	FRC L-81	AY707156	—	AY707138	—	—	—	—
Fusarium stilboides	FRC L-82	AY707157	—	AY707139	—	—	—	—
Fusarium stilboides	FRC L-120	AY707167	—	AY707149	—	—	—	—

Fusarium stilboides	FRC L-200	AY707168	—	AY707150	—	—	—	—
Fusarium stilboides	YT2-5	MF521452.1	—	MF576377.1	—	—	—	—
Fusarium stilboides	YT2-6	MF521453.1	—	MF576378.1	—	—	—	—
Fusarium stilboides	YT2-7	MF521454.1	—	MF576379.1	—	—	—	—
Fusarium stilboides	DEB 40	KF918552.1	—	KJ001545.1	—	—	—	—
Fusarium stilboides	DEB32	KF918549.1	—	KJ001542.1	—	—	—	—
Fusarium stilboides	DEB 39	KF918551.1	—	KJ001544.1	—	—	—	—
Fusarium stilboides	DEB 33	KF918550.1	—	KJ001543.1	—	—	—	—
Fusarium striatum (FSSC 21)	NRRL 22101	AF178333.1	EU329490.1	—	—	—	—	—
Fusarium striatum (FSSC 21)	NRRL 52699	JF740782.1	JF741108.1	—	—	—	—	—
Fusarium sulawesiensis (FIESC 16)	NRRL 34004	GQ505628	GQ505806	—	GQ505540	—	—	—
Fusarium sulawesiensis (FIESC 16)	NRRL 34056	GQ505640	GQ505818	—	GQ505552	—	—	—
Fusarium sulawesiensis (FIESC 16)	NRRL 34059	GQ505641	GQ505819	—	GQ505553	—	—	—
Fusarium sulawesiensis (FIESC 16)	NRRL 43730	GQ505669	GQ505847	—	GQ505580	—	—	—
Fusarium tanahbumbuense (FIESC 24)	NRRL 34005	GQ505629	GQ505807	—	GQ505541	—	—	—

Fusarium tanahbumbuense (FIESC 24)	NRRL 43297	GQ505657	GQ505835	—	GQ505569	—	—	—
Fusarium torreyae	NRRL 54149	HM068337.1	HM068359.1	—	—	—	—	—
Fusarium torreyae	NRRL 54152	HM068340.1	HM068361.1	—	—	—	—	—
Fusarium ussurianum	NRRL 58212	MG989748.1		—	—	—	—	—
Fusarium ussurianum	NRRL 45681	FJ240301.1	KM361666.1	FJ240291.2	—	FJ240247.1	FJ240345.1	FJ240334.2
Fusarium vorosi	NRRL 38207	DQ459747.1	—	DQ459679.1 DQ459662.1 DQ459645.1	—	DQ459730.1	DQ452414.1	DQ459713.1 DQ459696.1
Fusarium vorosii	NRRL 37605	DQ459745.1	KM361665.1	DQ459643.1	—	DQ459728.1	DQ452412.1	—
Fusarium witzenhausenense	CBS 142480	KY556525	KY556553	—	—	—	—	—
Fusarium witzenhausenense	KU90.15	MG237868	MG237866	—	—	—	—	—
Fusarium witzenhausenense	KU90.1.15	MG237869	MG237867	—	—	—	—	—
Fusarium cortaderiae	NRRL 29306	AY225886.1	—	AH012626.2	—	AY452855.1	AY225882.1	AH012628.2
Neocosmopora croci	CPC 27187	LT746217.1	LT746329.1	—	—	—	—	—
Neocosmospora sp.	CPC 28191	LT746218.1	LT746331.1	—	—	—	—	—
Neocosmospora sp.	CPC 27186	LT746216	LT746329	—	—	—	—	—

[1] BBA Biologische Bundesanstalt für Land- Forstwirtschaft, Berlin-Dahlen, Germany; CBS: Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; FCCUFG: Fungal Culture Collection of the Universidade Federal de Goiás, Universidade Federal de Goiás, Goiânia, Goiás, Brazil; FRC Fusarium Research Center, University Park, PA, USA; ISPeVe Culture Collection of the Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; KU Internal Culture Collection of Ecological Plant Protection Department at University of Kassel, Witzenhausen, Germany; MRC Medical Research Council Fusarium Collection, Tygerberg, South Africa;

NRRL Agricultural Research Service Culture Collection, Illinois, USA; URM: University Recife Mycology culture collection Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil;

*No information about cultural collections. DEB: School of Biology, Universiti Sains, Malaysia; YT2: College of Agriculture, Shanxi Agricultural University, China

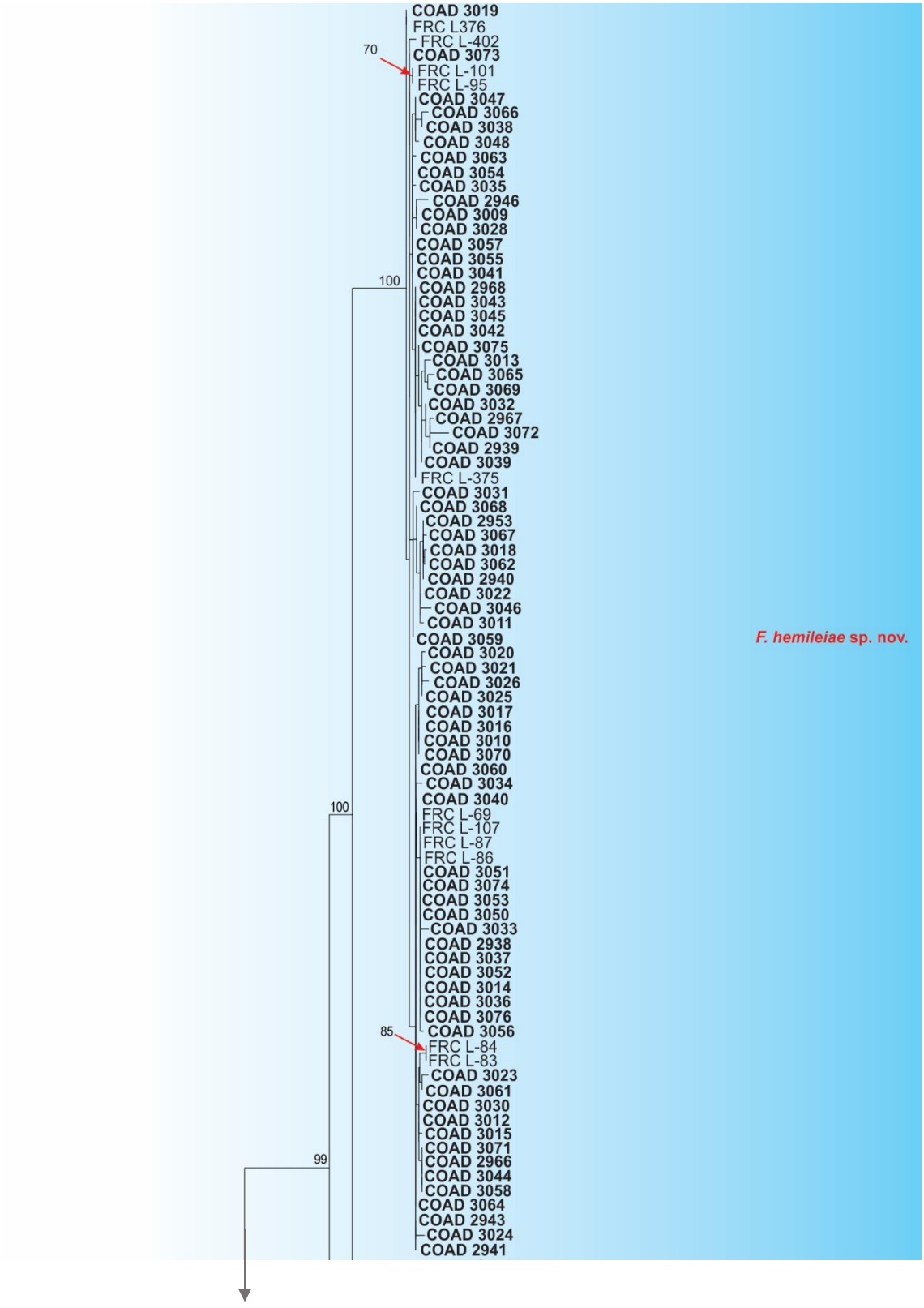

Figure S1 Maximum Likelihood (ML) phylogram obtained from the EF-1α sequences of members of the *Fusarium* species. Numbers on the nodes are bootstrap values above 70%. Mycoparasitic strains on *Hemileia* in this study are shown in bold. The tree is rooted with *F. xylarioides* (NRRL 25486).

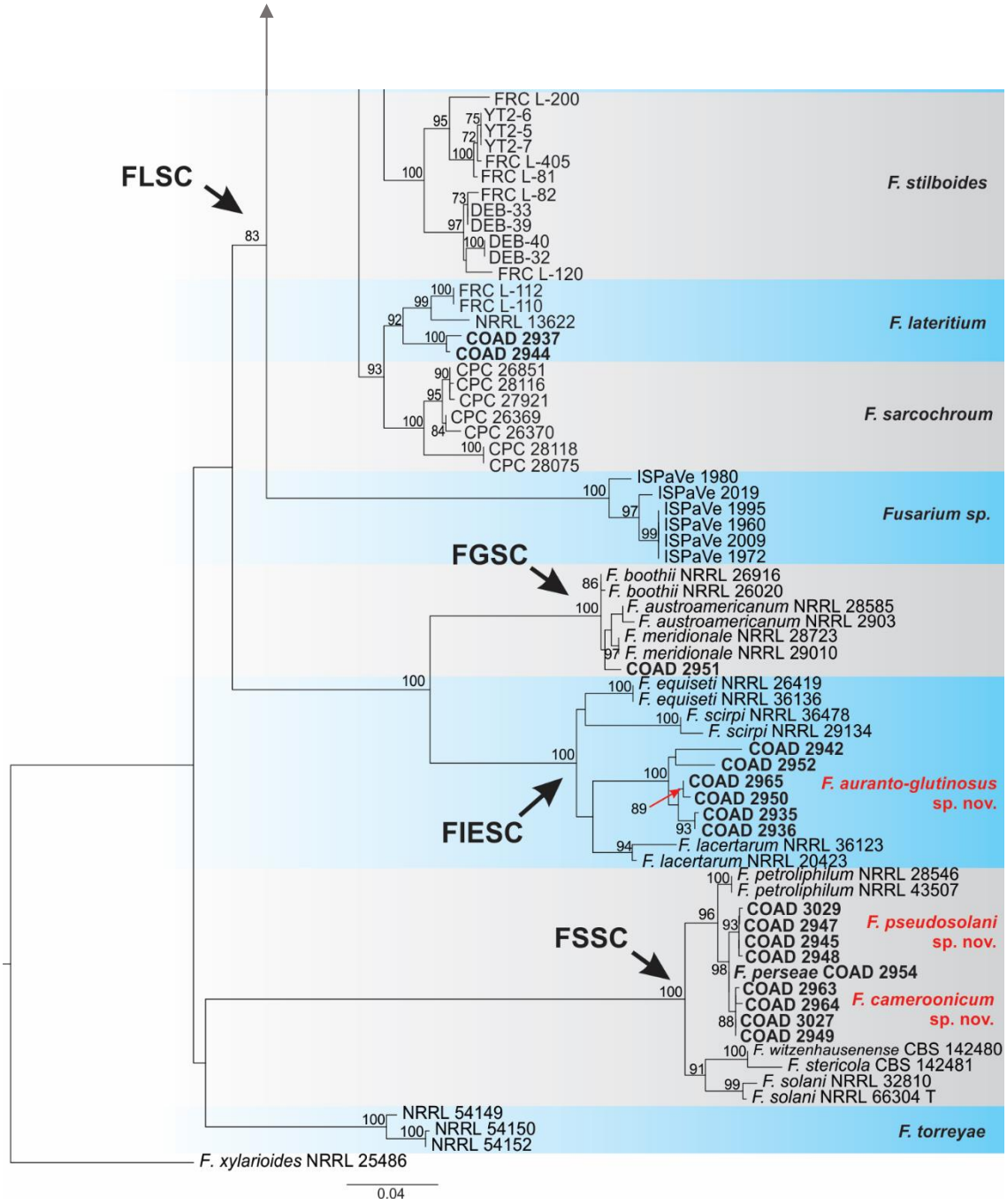

Figure S1 (Continued)

CHAPTER 2

Biological control of the coffee leaf rust fungus *Hemileia vastatrix* with antagonist species of *Fusarium*

Biological control of the coffee leaf rust fungus *Hemileia vastatrix* with antagonist species of *Fusarium*

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Abstract

Coffee leaf rust, caused by *Hemileia vastatrix*, is the main coffee disease worldwide. Recent outbreaks in Central America have highlighted the need to novel approaches to its management. Although biological control has been investigated as an alternative for its management, there are no significant examples of its application. Little attention has been given to antagonists of *H. vastatrix* occurring in its original range and that of coffee in Africa. A search for such antagonists was initiated in late 2014 (in South America and Africa) and 93 isolates of *Fusarium* spp. were obtained from abnormal rust pustules and treated as putative mycoparasites of *H. vastatrix*. These were examined in detail and found to belong to seven species, including four which were found to be new and described in a separate publication. Sixty-nine isolates of such *Fusarium* spp. were tested against *H. vastatrix in vitro* at three different application regimes and 17 of these reduced coffee leaf rust severity by over 80%. Three isolates of *F. hemileiae* (COAD 3011, COAD 3041 and COAD 3065) completely inhibited disease development and were further evaluated in additional experiments. In slide inhibition tests, combining *H. vastatrix* urenidiospores and conidia of *F. hemileiae* in suspensions led to a reduction in urediniospore germination by up to 97%. Spore suspensions of the three isolates of *F. hemileiae* applied prior to rust inoculation on coffee reduced disease severity by 85 to 89% as compared with controls sprayed with *H. vastatrix* uredinisopores alone. Liquid culture filtrates from *F. hemileiae* cultures were then used for suspending *H. vastatrix* urediniospores and observed. Although inhibition of urediniospore germination was significant such levels were lower than those obtained with *F. hemileiae* conidial suspensions. Nevertheless, for preventive applications *in planta*, the filtrates of COAD 3011 and COAD 3065 reduced the severity in 68% and 76%, respectively. Neither application of filtrates or conidial suspensions of *F. hemileiae* on coffee plants already producing sporulating rust lesions controlled the disease. However, urediniospores present in leaves treated with the conidial suspension of COAD 3041, were not viable. Preliminary results indicate that *F. hemileiae* is a promising biocontrol agent against *H. vastatrix*, particularly as a protection treatment.

Keywords: Biocontrol, disease severity, filtrates, mycoparasite, spore suspensions

1 Introduction

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* (Mikronegeriaceae) is considered the most important coffee disease (*Coffea arabica* and *C. canephora*) in the world. Severe infections cause decreased photosynthesis due to intense defoliation, with yield losses reaching 50% in prolonged drought conditions (Zambolim and Brenes, 2018). Annual losses worldwide are estimated at US$ 1 billion (Hein and Gatzweiler, 2006). Starting between the years of 2012–2014, major CLR epidemics hit Central and South America with losses ranging from 25 to 60% in countries like Nicaragua, San Salvador, Guatemala, Panama and Ecuador (Avelino et al., 2015, Talhinhas et al., 2017; Zambolim and Brenes, 2018). This crisis led to mass migration of people to Mexico and the USA with great economic and social impact (Ward et al., 2017). The main control strategies used against CLR have been escape through highland cultivation, use of resistant varieties and fungicide applications. Each of these strategies has limitations, as discussed in Talhinhas et al. (2017).

There is an obvious need for novel approaches to sustainably tackle the threat of CLR. One such strategy is biological control, a well-established approach for managing some fungal diseases on crops (Bettiol, 1996; Bettiol, 2011; Thambugala et al., 2020). In the case of CLR, the biological control has been getting attention in lest years with the availability of some commercial products with *Trichoderma* sp. and *Bacillus* spp. as an active ingredient (AGROFIT, 2021)

Since 2014 a programme aimed at surveying and evaluating potential fungal biocontrol agents of *H. vastatrix* collected in Africa and South America was conducted. It involved both endophytic fungi isolated from healthy tissues of *Coffea* spp. in Africa and putative mycoparasites of *H. vastatrix* collected in Cameroon, Ethiopia, Brazil and Paraguay. This study has revealed a great diversity of potential CLR antagonists which is now being documented (Crous et al., 2018; Colmán et al., 2021; Rodriguez et al., 2021;). More recently a manuscript recording/describing six species of *Fusarium* found during the surveys was submitted for publication (Chapter 1).

Members of *Fusarium* spp. occupy a broad diversity of ecological niches, playing very diverse roles ranging from plant and animal pathogens, to saprophytes in a variety of substrates, to plant endophytes and very commonly as saprophytes in soils (Torbati et al., 2019). Several *Fusarium* spp. have already been reported as mycoparasites, such as *F. solani* on *Mucor*

spinosus (Dubey and Dwivedi, 1986) and *F. udum* on *Aspergillus luchuensis* and *Syncephalastrum racemosum* (Upadhyay et al., 1981).

Recently, Ghule et al. (2018), identified four species of *Fusarium* (*F. delphinoides, F. brachygibbosum, F. pseudonygamai* and *Fusarium* sp. nov.) parasitizing *Plasmopara vitticola* in India, and demonstrated that these species had biocontrol potential against this pathogenic oomycete. Torbati et al. (2019) studying the diversity of fungi parasitizing species of mushrooms (Basidiomycota) in Iran, found nine species of *Fusarium* (*F. acuminatum, F. gamsii, F. iranicum, F. oxysporum, F. proliferatum, Fusarium* sp. FIESC 3, *Fusarium* sp. FIESC 5, *Fusarium* sp. FIESC 29 and *Fusarium* sp. FIESC 30), distributed in four different species complexes. *Fusarium concentricum* and *F. solani* have also been reported as mycoparasites of myrtle rust (Lira et al. 2019). Additionally, Sun et al. (2019) provided a list of fungicolous fungi which included seven *Fusarium* spp., namely: *F. bactridioides, F. dominicanum, F. graminearum, F. heterosporum, F. longipes, F. merismoides* and *F. oxysporum.*

The mechanisms involved in the association between *Fusarium* spp. and other fungi can go beyond mycoparasitism. This genus is widely known for its ability to produce mycotoxins that contaminate stored grains and cereals. Some of these are known to be a health threat to humans and other animals (Placinta et al., 1999). However, the secondary metabolites produced *by* Fusarium spp. include other non-toxigenic compounds, which are also biologically active, and may have practical application in microbial antagonism. It is believed that they may play important roles against competing microorganisms in the same environment (Nesic et al., 2014). Several studies have explored the potential of these secondary metabolites (SM) of *Fusarium* spp. to control pathogenic microorganisms and have yielded promising results. Some examples resulted from studies of the following species: *F. chlamydosporum* (Mathivanan and Murugesan, 1999), *F. lateritium* (Kamel et al., 2019), *F. proliferatum* (Li et al., 2014), *F. solani* (Tayung et al., 2011) and *F. semitectum* (Altomare et al., 2000). In addition, non-pathogenic strains of *F. oxysporum* have been studied as antagonists to pathogenic strains belonging to the same species (Paulitz et al., 1987; Park et al., 1988; Lemanceau et al., 1993; Nel et al., 2006). In the soil they were found to be able to compete for nutrients, affect spore germination, the production of chlamydospores, compete for infection sites in the roots or trigger defense reactions of the plant to induce systemic resistance (Fravel et al., 2003).

If available, the use of biological products was regarded by Várzea and Marques, (2005) to be highly desirable in CLR management of rust, because *H. vastatrix* has a high capacity to overcome cultivar resistance through the emergence of new races. On the other hand, the use of chemical fungicides often prompts the selection of resistant populations of phytopathogens leading to their inefficiency (Deising et al., 2008). Non-conventional control measures of CLR, which prove less harmful to the environment and to human health are increasingly appealing. Here, the diversity of *Fusarium* spp. reported in chapter 1 is exploited, including screening and evaluation of biocontrol potential towards practical applications in CLR control.

2 Material and Methods

2.1 Surveys

Fungal isolates were all obtained during expeditions to Cameroon, Ethiopia, Brazil and Paraguay as described in chapter 1.

Sixteen-nine isolates that showed easy growth and sporulation in culture were selected to be tested *in vitro*, as described below. These are listed in Tables 1 and 2. Each of these isolates was deposited in 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).

2.2 Production of inoculum of *Hemileia vastatrix*

Young coffee plants were inoculated with *H. vastatrix*. The abaxial surface of the expanded leaves of coffee seedlings cv. Caturra were sprayed with a urediniospore suspension calibrated, with a haemocytometer, to 10^5 urediniospores/mL (suspended in in a 0.3% Tween 80solution). The urediniospores utilized were from a *H. vastatrix* race previously identified as race II (Capucho, 2009). These plants were kept in the dark for 48 hours, with 100% humidity and at a temperature of 22°C. Posteriorly the seedlings were taken to a growth chamber at 22°C and under a 12-hour light regime (light provided by fluorescent daylight lamps). After 30-45 days, mature urediniospores were collected by carefully scraping the surface of the leaves with gelatin capsules and then transferred to 1.5 mL plastic microtubes. The microtubes were then left inside a desiccator (containing blue silica gel) for up to 30 days under 6 ± 2 ºC. Prior to use in experiments the urediniospores were checked for viability through spore counting germinated in a drop of 20 uL of urediniospore suspension (10^5 spore/mL) on a microscope

slide in a humid chamber and incubated at 22ºC in the dark for 6 h. Only urediniospores batches with a viability greater than 70% were used in the experiments.

2.3 Production of inoculum and culture filtrates of *Fusarium* spp.

2.3.1 Production of inoculum in plates

To prepare the *Fusarium* spp. conidial suspensions, each monosporic isolate was transferred to a Petri dish containing potato-dextrose-agar (PDA) culture medium and incubated at 25ºC under a 12h light photoperiod. After 15–20 days of incubation, an aliquot of 5 mL of SDW with 0.3% Tween 80 was poured over each colony and later scraped with the help of metal clamp. The suspensions obtained were calibrated, using a Neubauer Chamber, to a concentration of 10^6 conidia/mL. The conidial suspensions were used immediately.

2.3.2 Production of inoculum in bags

Fusarium hemileiae mass inoculum was produced as follows: 200 g portions of parboiled rice grains and 120 mL of tap water were placed into 35×40 cm each polypropylene bags and autoclaved one time at 121 ºC under pressure of 1kgf/cm^2 for 20 min. An aliquot of each conidial suspension was obtained by pouring 5 mL of SDW into a plate containing a 10 days-old colony of the individual isolate formed on PDA. Each aliquot was used to aseptically seed a bag of autoclaved rice. The bags were stored in a temperature-controlled room (22 ± 1 °C, 12 light hours – light provided by NUV BLB and daylight fluorescent lamps) and thoroughly shaken once every 24 h in order to allow for a homogeneous colonization. After 4 days, the bags were opened and kept under the same conditions for another 48 h to favor sporulation. Subsequently, the colonized rice was spread over thoroughly cleaned 20×30 cm aluminum trays and stored under the same conditions as for the bags for another 24 h. Then the colonized rice was placed in new plastic bags and stored in a cold chamber (4ºC) until use. On the days the treatments were applied, 150 mL of Tween 80 solution in SDW at 0.3% was added in each bag contending colonized rice and agitated to loosen the conidia of the grains. Then, the resulting suspension was passed through a sieve and used immediately. A small sample was used to calculate the concentration using a Neubauer chamber.

2.3.3 Production of filtrates

Filtrates from each isolate was obtained by seeding250 mL flasks with three mycelium discs obtained from the margin of actively growing colonies of the isolate in PDA plates. Each

Erlenmeyer flasks contained 100 mL of PD medium. The seeded flasks were kept on a shaker under constant rotation (130 rpm) and at room temperature for 13 days. After that arbitrary period the content of each flask was filtered three times through a two layer of sterile filter paper placed over a porcelain filter attached to a kitassato to which a vacuum pump was attached. The filtrates were placed in sterilized glass flask and covered and stored at 4 ºC until the day of use (up to 8 days).

2.4 Screening for potential antagonists of *Hemileia vastatrix* in coffee leaf discs

The selected isolates were evaluated for their potential to reduce CLR severity *in vitro* on coffee leaf discs. This method followed the original procedure described by Eskes and Kushalappa (1989) but modified as described in Salcedo-Sarmiento et al. (2021).

Transparent plastic boxes, previously disinfected with 70% ethanol, were lined with a sterile layer of foam saturated with sterile distilled water (SDW) and covered with a disinfested square plastic (PVC) grid. 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 1% for 2 min and rinsed with SDW. Excess water was removed with sterile absorbent paper. Two cm diameter discs were removed from the leaf with a cork punch and immediately used. Twelve discs were placed into each plastic box with abaxial surface facing up. The suspensions of the antagonists were prepared according with the item 2.3.1. A 25 µL aliquot of urediniospore suspension of *H. vastatrix* (10^5 urediniospores/mL suspended in SDW with 0.3% Tween 80), and a 25 µL aliquot of the potential antagonist suspension were placed on each disc. Controls received a 25 µL aliquot of the suspension of *H. vastatrix* and a 25µL aliquot of the SDW with 0.3% Tween 80.

Three application times were tested for each antagonist against *H. vastatrix*, as given below:

I. 0h- an aliquot of 25 µL of the antagonist suspension plus *H. vastatrix* uredinial suspension were deposited simultaneously on leaf disc;
II. 24h- an aliquot of 25 µL of the antagonist suspension placed on leaf disc 24 hours before depositing the urediniospore suspension of *H. vastatrix;*
III. 72h- an aliquot of 25 µL of the antagonist suspension placed on leaf disc 72 hours before depositing the urediniospore suspension of *H. vastatrix*;

The simplified outline of the methodology describe here are available in the Figure 1.

Immediately after the inoculation with *H. vastatrix*, the boxes containing the disc treatments were placed in the dark for 48h at 22 ± 1ºC. After this period, the boxes were maintained under a 12h photoperiod (light provided by daylight fluorescent lamps), at 22 ± 1ºC. 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 35 days after inoculation, using a rating scale from 1 to 5 according to the percentage of leaf area with lesions: 1 = 0%; 2 = 1–25%; 3 = 26–50%; 4 = 51–75%; and 5 > 75% of leaf area with lesions (chlorotic, rust pustules and/or necrotic) (Shiomi et al., 2006). Disease index (DI) was calculated according to the equation:

$$DI\ (\%) = [\sum(r \times a) / (R \times A)] \times 100$$

Where ***r*** is the rating value, ***a*** is the number of infected leaf disks with a rating of ***r***, ***R*** is the maximum rating value and ***A*** is the total number of leaf disks tested (Chaube and Singh, 1991).

2.5 Inhibition of urediniospore germination by *Fusarium hemileiae*

2.5.1 Filtrates in different culture media

Preliminary observations and literature information suggested that some *Fusarium* spp. isolates might produce anti-CLR metabolites. In order to verify this possibility, we first performed an essay to determine which would the best culture medium for anti-CLR metabolite production. The test involved three selected isolates of *F. hemileiae*, namely: COAD 3011, COAD 3041 and COAD 3065. Three different liquid media (broths) were tested: Potato-Dextrose (PD), Malt Extract (ME) (Tuite, 1969) and Rice Broth – broth of 30g of crushed rice in 100ml of water (RB). Controls consisted of non-colonized batches of each culture medium and an absolute control consisted of a 0.3% Tween 80 solution in SDW.

Filtrates of each isolate were prepared as follows: Three mycelium discs taken from the margin of actively growing colonies in plates containing PDA were aseptically added to each of the 100 mL flasks containing 50 mL of each culture medium described above. These were kept on a shaker under constant rotation (130 rpm) and at room temperature (±25ºC) for 13 days. Then they were filtered three times thorough two layers of filter paper placed over a

porcelain filter coupled to kitassato connected to a vacuum pump. The filtrates were placed in sterilized glass flask and covered and stored at 4 ºC until the day of use (up to 8 days).

Microscope slides were disinfected with 70% alcohol and then placed on top of a clean and bench surface at room temperature until dry. Three slides were placed inside each of several 11 × 11 × 3.5 cm polystyrene boxes. These were previously prepared by careful washing, drying and internally cleaning with 70% ethanol and then lined with sterilized and moist paper towel and covered with a disinfected square plastic (PVC) grid. Two 10 μL aliquot of a *H. vastatrix* urediniospores suspension (prepared as described in the item 2.4) were transferred to the center of each half side of each slide. Subsequently 10 μL drops of each treatment to be tested (suspensions were obtained with describe in the item 2.3), totaling 6 combined drops of suspension of *H. vastatrix* + Filtrate (repetitions) for each gerbox (treatment). The drops of urediniospores and conidia were mixed with a single micropipette tip (which was subsequently discarded). After that procedure the boxes were covered with their lids. Boxes were left in a bench in a controlled temperature room at 22 ± 1ºC in the dark for six hours. After the six hours period, germination of spores was interrupted by adding of a 10 μL lactofusin aliquot to each drop. The two points of each slide was then examined under the10× power of a light microscope (Olympus SZX7). The first 100 urediniospores viewed at each point were ranked as germinated (when the germ tube size was longer than the urediniospore diameter) or non-germinated (if otherwise).

The experimental design was in a completely randomized design for each experiment and the germination of urediniospores was evaluated using Kruskal-Wallis. Significant differences in their distribution were confirmed when p-value < 0.05. The analysis was made using program Rbio version 137 (Bhering, 2017).

2.5.2 Filtrates vs. Conidial suspensions

One additional test was performed to compare the levels of inhibition of urediniospore germination resulting from exposure to either filtrates or spore suspensions of three selected isolates, namely: COAD 3011, COAD 3041 and COAD 3065. Filtrates included in this essay were produced in the same manner as described in 2.5.1, but using only PD being used as growing medium. Spore suspensions were preparate as described in the item 2.3.1. Suspensions with a concentration above the desired were centrifuged in a microcentrifuge at 40,000 rpm for 5 min to precipitate most of the spores, then the supernatant was calibrated to the concentration

of 10^6 spores/mL using an hemocytometer. Dilutions were avoided so that there are no differences between the conditions used for each isolate, since aliquots of different volumes would be necessary according to the initial concentration of the suspensions. Furthermore, as the mechanisms involved in the control are still unknown, dilutions could reduce the amount of potentially bioactive SM present in the suspensions. Two control treatments were chosen for this experiment: i) exposure of urediniospores to pure PD (control for filtrate treatments) and ii) exposure of urenidiospores to Tween 80 solution in SDW at 0.3% (control for *Fusarium* spp. suspensions). Procedures used for assembling the experiment were as described in 2.5.1 involving the mixture of drops of either filtrates or conidial suspensions with urediniospore suspensions. Evaluation of urediniospore germination for each treatment were also as described in 2.5.1.

2.6 Effect of treatment of coffee plants with *Fusarium hemileiae* on coffee leaf rust severity

Group of 105 healthy one year-old coffee plants (cultivar Catuaí-Vermelho IAC 144) was used in the experiments below. These were grown in plastic bags (16 × 25 cm) containing a 2:5:1:0.5 mixture of soil, manure and sand. The plants were kept in a greenhouse throughout the experiment at 26 ± 2 ºC and a relative humidity of 85 ± 5% and watered daily.

Three *F. hemileiae* isolates (COAD 3011, COAD 3041 and COAD 3065), selected for having consistently yielded the best results for *in vitro* screening for CLR control on coffee leaf disks (as described above) were brought forward to further testing on live plants. Two independent experiments were conducted and followed the schemes given below.

Experiment 1. Two hand spray applications of each conidial suspensions or filtrates (as described above) of each isolate of *F. hemileiae* on separate plants were performed throughout the aerial part of the plant (covering both sides if leaves, about 14 mL for each plant) prior to inoculation with *H. vastatrix*. The concentration of the spore suspensions was quantified for each application (Table 3). The interval between the two spray-applications was of seven days. The inoculation with *H. vastatrix* occurred three days after the second application of treatments. One liter of urediniospore suspension (10^5 spore/mL) was used for the inoculation of all plants. Being inoculated with the rust, the plants were covered with black plastic for 48 hours to favor urediniospore germination. CLR severity was quantified 30 days after inoculation with *H. vastatrix*. The treatments consisted of: 1) Control: Tween 80 solution in SDW 0.3%; 2) Fungicide: applications of Copper Oxychloride (Recop® – Albaugh Agro Brasil Ltda) 0.5%

(1g/200 mL of water); 3) Conidial suspension of *F. hemileiae* COAD 3011; 4) Conidial suspension of *F. hemileiae* COAD 3041; 5) Conidial suspension of *F. hemileiae* COAD 3065; 6) Filtrate from COAD 3011; 7) Filtrate from *F. hemileiae* COAD 3041; and 8) Filtrate from COAD 3065.

Experiment 2. All plants included in the experiment were inoculated with *H. vastatrix* and then were covered with black plastic for 48 hours. Nine hundred mL of urediniospore suspension (10^5 spore/mL) was used for the inoculation of all plants. After three weeks rust pustules were observed on all plants. CLR severity was evaluated at this stage and the anti-CLR treatments were applied for the first time. Seven days later, a second application of the anti-CLR treatments occurred. CLR severity was evaluated again for all plants of all treatments seven days later. Concentration of the *F. hemileiae* conidial suspensions of each isolate were quantified at each round of application (Table 1). The difference between the initial and the final severity was calculated for each treatment. The treatments consisted of: 1) Control: Tween 80 solution in SDW 0.3%; 2) Conidial suspension of *F. hemileiae* COAD 3011; 3) Conidial suspension of *F. hemileiae* COAD 3041; 4) Conidial suspension of *F. hemileiae* COAD 3065; 5) Filtrate from COAD 3011; 6) Filtrate from COAD 3041; and 7) Filtrate from COAD 3065.

To verify whether the application of the treatments in experiment 2 had any effect on the viability of the urediniospores, at the time of the final evaluation, a little sample of the urediniospores was collected carefully scraping the surface of the leaves with gelatin capsules from each of the leaves under evaluation for each treatment (56 leaves for each treatment). These were mixed in one 1.5 mL plastic microtube and the germination was quantified by following a similar methodology as described in item 2.5.

The experiments were conducted in a completely randomized design with seven repetitions for each treatment, with the severity determined for eight leaves (counted from the apex to the base) of each plant. The mean severity of the 8 leaves evaluated constituted the severity value of each repetition (plant). Severity was estimated using the diagrammatic scale proposed by Belan et al. (2020). The data were $\sqrt{severity}$ transformed (so that they fit a normal distribution) before using analysis of variance (ANOVA) and the means were compared by Dunnett's test ($\alpha = 0.05$) using of the statistics program Rbio version 137 (Bhering, 2017).

3 Results

3.1 Isolates

Fusarium spp. were found to be common in association with CLR pustules, and were obtained from Brazil, Cameroon, Ethiopia and Paraguay. In chapter 1 their taxonomy elucidated and it was found that the isolates belonged to seven different species, four of which were identified as new to science. The second stage of the work (evaluation of biocontrol potential) progressed before the results of the study presented in chapter 1. Sixty-nine selected isolates were evaluated as CLR biocontrollers (Tables 2 and 3) contemplated the following species: fifty-five isolates of *Fusarium hemileiae*, four isolates of *Fusarium pseudosolani*, three isolates of *Fusarium cameroonicum*, three isolates of *Fusarium auranto-glutinosus*, two isolates of *Fusarium* sp., one isolate of *Fusarium lateritium* and one isolate of *Fusarium perseae.*

3.2 Screening for potential antagonists of *Hemileia vastatrix* in coffee leaf discs

Seventeen isolates of *F. hemileiae* promoted 80% or more reduction of CLR severity for all regimes (time intervals) evaluated as compared to their respective controls (Tables 1 and 2; Fig 2a-b). Three *F. hemileiae* isolates provided complete control of in all regimes. These were: COAD 3011, COAD 3041 and COAD 3065 (Fig 2). These isolates were selected for subsequent tests.

The isolates belonging to the *Fusarium solani* species complex (*F. cameroonicum, F. perseae* and *F. pseudosolani*) either had a poor performance at reducing CLR severity or even contributed to an increase in disease severity – particularly when applied 24h and 72h before *H. vastatrix* inoculation. One strain of *F. cameroonicum* (COAD 2963) was an exception and reduced CLR severity by 58, 72 and 63% when respectively applied: simultaneously, 24h and 72h prior to inoculation with the rust (Table 2 and 3).

Fusarium auranto-glutinosus (COAD 2935, COAD 3026 and COAD 2965) and *F. lateritium* (COAD 2937) provided good levels of disease reduction (more of 70%), only when applied 72h before *H. vastatrix*. In the other regimes, the isolates of those species had a low performance. In some instances, *F. auranto-glutinosus* applications even increased CLR severity (Table 2).

3.3 Inhibition of urediniospore germination by *Fusarium hemileiae*

3.3.1 Filtrates in different culture media

Fusarium hemileiae isolates (COAD 3011, COAD 3041 and COAD 3065) were included in the tests aimed at selecting the best culture medium to be used for obtaining active anti-CLR filtrates (Fig 3a). All isolates performed better at producing filtrates with high anti-CLR activity (p-value = 0.001417) when produced in PD medium. The resulting filtrates were biologically active and reduced the germination of urediniospores by more than 80% as compared with PD-only control. For ME, although there was a significant effect (p-value = 0.009192), this was negative. The control treatment only with ME inhibited germination more than the filtrates produced in this medium. Similarly, RB alone also inhibited the germination of urediniospores. A curious result was observed with the filtrate from COAD 3011 grown in RB. That filtrate stimulated the germination of urediniospores, yielding a germination level of urediniospores that reached 95%, resulting in a significant effect (p-value = 0.003297), with germination percentage higher than the control in RB only (32%) and similar to the control with SDW only (97%).

3.3.2 Filtrates vs. Conidial suspensions

The comparison that was performed between culture filtrates vs. conidial suspensions for their ability to inhibit the germination of urediniospores (involving COAD 3011, COAD 3065 and COAD 3041) (Fig 3b), resulted in conidial suspensions performing generally better than filtrates – a statistically significant effect (p-value = 0.0003809) was demonstrated. COAD 3011, COAD 3065 and COAD 3041 spore suspensions reduced urediniospore germination by 97%, 94% and 74%, respectively, as compared with control. In contrast, for the filtrates, the germination was not distinguishable among treatments (p-value = 0.1881). However, the isolates caused a reduction higher 80% with respect to control treatment when the means are compared.

It was observed that, besides the significant number of spores that did not form any germ tubes, numerous urediniospores produced germ tubes, when exposed to the *F. hemileiae* isolates or their filtrates which were abnormal – short and inflated – and probably non-functional (Fig. 3e-f). Nevertheless, abnormal urediniospore germ tubes were less common when they were exposed to filtrates. In that case inhibition occurred mostly by complete lack of germ tube formation.

3.4 Effect of treatment of coffee plants with *Fusarium hemileiae* on coffee leaf rust severity

Experiment 1. Spraying with filtrates and conidial suspensions *F. hemileiae* (COAD 3011, COAD 3065 and COAD 3041) reduced CLR severity were applied before of *H. vastatrix* inoculation (Fig 4 and 5). The filtrate of COAD 3041 produced no significant CLR severity reduction as compared to the control. On the other hand, the filtrates COAD 3011 and COAD 3065 a significant CLR severity reduction as compared with the control. Results for the filtrates from COAD 3011 and COAD 3065 were similar to that produced by the fungicide treatment, yielding similar indexes of reduction of CLR severity (68% for COAD 3011, 76% for COAD 3065 and 70% for fungicide treatment). The best control of CLR was obtained with the application of spore suspensions. Similar levels of reduction of disease severity were obtained for the three isolates (85% for COAD 3011, 86% for COAD 3041 and 89% for COAD 3065) (Fig 4 and 5).

Experiment 2. Post *H. vastatrix* sporulation applications were in general ineffective at reducing CLR progress, with no significant difference found between *F. hemileiae* applications and control. In one particular case (COAD 3011), the filtrate was found to have a negative effect in terms of control and contributed to greater progress of the CLR (Fig 6a). When the urediniospores from treated leaves were examined for germination, only COAD 3041 spore suspension treatment provoked a reduction in germination of urediniospores (15% of spore germinated) (Fig 6b). In addition, similarly to what was observed during the microscope slide inhibition tests, the germ tubes of urediniospores exposed to COAD 3041 were inflated and deformed (Fig 6c-d). Germination was normal and above 90% for all other treatments.

4 Discussion

The main control management strategies for CLR today are fungicide applications, the use of resistant cultivars and escaping the disease through highland cultivation. However, there are significant limitations for each of these approaches. Chemical control, in particular, may be costly, is broadly regarded as damaging to the environment and prone to the emergence of fungicide resistance, besides representing a health hazard (Kanoun-Boulé et al., 2008; Loland and Singh, 2004). Preventive treatments are usually performed with copper fungicides, while curative treatments are performed with systemic fungicides (e.g., strobilurins and triazoles) (Talhinhas et al., 2017). Although ecologically friendly and of low cost to the farmens, obtaining durable genetic resistance in the face of the emergence of new breeds of *H. vastatrix* remains challenging (Brito et al., 2010). Finally, besides controversial for its effects on CLR control, the reduction of shade coverage in coffee growing regions, sometimes promoted as

mitigating for losses to CLR, has been shown to have a strong and damaging effect on biodiversity (Perfecto et al., 2003). Therefore, it is evident that research should intensify in order to develop new, effective and sustainable solutions for the control of CLR.

Several isolates of *F. hemileiae* tested here showed biocontrol potential against CLR in leaf disc tests, 17 of these reduced the severity of CLR by over than 80% in the three application times. In order to reduce the number of isolates under investigation to a manageable level, only isolates that provided complete control of CLR were selected for inclusion in later studies. It is necessary to stress that several other isolates with excellent performance remain to be tested in future studies. The conidial suspensions of the three selected isolates of *F. hemileiae* were very efficient in inhibiting the germination of urediniospores in slide tests and most urediniospores were found either not germinating or to produce highly deformed germ tubes. Similar results were obtained by González and Martinez (1996) – for *Lecanicillium lecanii* and Salcedo-Sarmiento et al. (2021) – for *Calonectria hemileiae*.

The species *F. hemileiae* was by far the most common (80%) among the isolates of *Fusarium* spp. collected in our surveys as purported mycoparasites of *H. vastatrix*. It was also discovered that this is likely to be a cosmopolitan species, but always associated with coffee (and probably to *H. vastatrix*). According with showed in the chapter 1, other isolates this species found in GenBank, are reported in Africa, Asia, South America and Oceania (Geiser et al., 2005). Isolates of *F. hemileiae* appeared as the most effective at reducing CLR severity. Some isolates of other species were, at least under particular conditions, either innocuous to CLR or shown to even increase CLR severity, for an unknown reason, namely: *F. cameronicum*, *F. pseudosolani* and *F. perseae* (with few exceptions). Promising biocontrol results have already been reported in the literature for *F. lateritium*, which is phylogenetically close to *F. hemileiae*. This species provided excellent biocontrol effect against *Eutype* dieback in vines and apricots when spore suspensions were applied preventively to plants (Carter, 1983; Munkvold and Marois, 1993; McMahan et al., 2001). Isolates belonging to this species are also were studied as potential biological control agents for *Sclerotinia sclerotiorum* in lettuce (Sitepu and Wallace, 1984). However, in our study only a single isolate of *F. lateritium* (COAD 2937) was found and it provided no significant CLR control in leaf disc tests.

Fusarium spp. are well known for their ability to produce a diverse range of SM. Some are infamous as mycotoxins whereas others are beneficial and have been explored for their bioactive potential for agricultural applications (Li et al., 2020). In this context, we tested the

antifungal activity of filtrates from isolates of *F. hemileiae* against CLR. A positive effect was observed in preventive applications in plants for filtrates from isolates COAD 3011 and COAD 3065. Such filtrates had a similar effect at controlling CLR to the application of a copper fungicide. Other studies support the hypothesis that species of *Fusarium* can produce SM with antifungal properties. Altomare et al. (2000) isolated two chemical molecules from rice cultures of *Fusarium semitectum* which had considerable antifungal activity against several phytopathogenic and/or mycotoxigenic filamentous fungi in several biological assays, namely: *Alternaria alternata, Ascochyta rabiei, Aspergillus, flavus, Botrytis cinerea, Cladosporium cucumerinum, Phoma tracheiphila* and *Penicillium verrucosum. Fusarium lateritium* produced SM capable of controlling plant pathogens such as *Eupenicillium brefeldianum*, *Penicillium echinulatum* and *Alternaria phragmospora* (Kamel et al., 2019), in addition to producing compounds such as enniatin and lateropyron, which have antibiotic and antifungal activity (Bishop and Ilsley, 1978; Bushnell et al., 1984; Pieper et al., 1992). *Fusarium solani* was found to produce several compounds with antifungal action during *in vitro* experiments (Tayung et al., 2011). *Fusarium chlamydosporum* was found to produce SM capable of inhibit the germination of *Puccinia arachidis* urediniospores (Mathivanan and Murugesan, 1999). Although there were positive results in this study, the anti-CRL activity of the three filtrates was always lower than that observed for conidial. Nevertheless, in our opinion, this does not invalidate the continuation of the investigation towards the use of *F. hemileiae* filtrates, particularly at this early stage of the studies. It may be possible to optimize SM production of *F. hemileiae*, as the biosynthesis of fungal SM has long been known to be responsive to environmental conditions, including the source of carbon and nitrogen, temperature, light and pH (Keller et al., 2005). This remains to be explored.

Field and lab observations suggested that *Fusarium* spp. found growing directly over CLR pustules would represent legitimate mycoparasites. This interpretation being correct, one would expect that inoculum of *Fusarium* spp. (and other mycoparasites) would perform better is applied after the emergence of rust pustules in coffee. Therefore, the efficiency of *F. hemileiae* was evaluated through inoculation of conidial suspensions on coffee plants bearing CLR symptoms. Contrarily to what expected no beneficial effect in terms of CLR control was observed for either urediniospore suspension or filtrate applications in our test. However, in parallel it was observed that the spore suspension of COAD 3041 made the most urediniospores collected from antagonist sprayed leaves to lose viability or to produce aberrant germ tubes. A similar result was also observed by Vandermeer et al. (2009). These authors found that *L.*

lecanii was able to reduce the viability of the urediniospores, although the mycoparasite did not effectively control the CLR. It is important to evaluate the epidemiological implications of the results obtained here, since reducing the rate of disease progress in the management of polycyclic diseases, such as CLR, is an important approach that has been effectively explored with chemical fungicides. The reduction in the viability of urediniospores implies a reduction in the number of secondary cycles, and consequently, an increase in disease control. Another interesting fact is that the conidia of all the isolates germinated normally after seven days on the leaves, showing a good ability to survive under adverse conditions.

Mycoparasites are defined as fungi that have the ability to parasitize their fungal hosts (Barnett, 1963; Sun et al., 2019). It is known that some species of fungi can colonize uredinia, urediniospores and germ tubes of various rust fungi (Moricca et al., 2001). *Acremonium byssoides*, *Calcarisporium* spp., *C. hemileiae*, *Digitopodium hemileiae*, *Fusarium pallidoroseum*, *Lecanicillium* spp., *Simplicillium* spp., *Sporothrix* spp., *Talaromyces wortmannii*, have been reported as mycoparasites of *H. vastatrix* (Carrión and Rico-Gray et al., 2009; Gómez-De La Cruz et al., 2017; Colmán et al., 2021; Salcedo-Sarmiento et al., 2021). It has been shown that the hyphae of some of these species can penetrate the urediniospores of *H. vastatrix* (Vandermeer et al., 2009; Crous et al., 2018; Colmán et al. 2021). Although *F. hemileiae* was isolated from 'mycoparasitized' *H. vastatrix* pustules, the true mycoparasitic status still remains to be completely tested. Only in few occasions have Koch's postulates steps been fulfilled for mycoparasites and only recently this was experimentally conducted for a *H. vastatrix* mycoparasite – *C. hemileiae* (Salcedo-Sarmiento et al., 2021). For *F. hemileiae*, preliminary *in vitro* (data not shown) searches for mycelial growth and invasion of *F. hemileiae* in *H. vastatrix* urediniospores failed to detect direct parasitism. This needs to be revisited but the absence of the formation of colonies of *F. hemileiae* on CLR pustules in one test suggest that other biocontrol mechanism must be involved in reducing the observed severity and that, perhaps *F. hemileiae* is not a true mycoparasite. However, at this point in the research it is still difficult to decipher which mechanisms are involved in biocontrol.

The spore suspensions of the three isolates of *F. hemileiae* applied preventively to plants were efficient at controlling CLR, with severity reductions up to 89% in relation to the control treatment, and with a protective effect superior to that provided by a cupric fungicide. Analogous to this, Haddad et al. (2014) looking for antagonists of *H. vastatrix* isolated and tested three strains of *Fusarium* sp. obtained directly from leaves or by washing coffee plant

debris in organic crops in Manhumirim, MG, Brazil. Two isolates (F171 and F173) also showed some positive results in experiments on plants, reducing the frequency of infection and/or the number of urediniospores per leaf by 60-100% when applied, mainly, four days before or after inoculation of the pathogen. It is not unlikely that Haddad et al.'s fungus was *F. hemileiae* – the most ubiquous species in our survey.

Fusarium hemileiae is a newly described species that belongs to the *Fusarium lateritium* species complex (FLSC) (Chapter 1). This species appears to be commonly associated with *Coffea* and to be common in pustules of *H. vastatrix* but has never appeared in the coffee pathology literature (Chapter 1, Geiser et al., 2005). Never, during our experiments, a pathogenic effect of *F. hemileiae* sprays was observed on coffee test plants. Even after 2 months, all plants developed normally, without any symptoms other than those caused by CLR.

Our results strongly suggest that the isolates COAD 3011, COAD 3041 and COAD 3065 of *F. hemileiae* have good biocontrol potential against CLR. Interestingly, the best control was observed in plants sprayed with spore suspensions before inoculation with *H. vastatrix* on leaf discs and on coffee plants, suggesting that *F. hemileiae* may work better as a protector against *H. vastatrix*. In this context, field experiments will be conducted to assess whether the control levels obtained *in vitro* and, in the greenhouse, will persist under field condition. *Fusarium hemileiae* has several desirable characteristics for a biocontrol agent: i) it is easily mass-produced in simple and inexpensive substrates; ii) in preliminary tests (data not shown) the stored conidia remained viable for a long time (observed up to six months); iii) conidia survived well on coffee leaves without any special treatment (needs to be evaluated in the field). Therefore, *F. hemileiae* appears to have the potential to become a novel anti-CLR biofungicide in the future, for the benefit of the farmers, consumers and the environment.

5 References

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6 Tables and Figures

Table 1. Spore concentration/mL of the suspensions applied in the experiments 1 and 2 in plants.

	Experiment 1		**Experiment 2**	
Isolated	*First application*	*Second application*	*First application*	*Second application*
COAD 3011	2.75×10^4	5.2×10^4	3×10^6	1×10^7
COAD 3041	4.8×10^8	9×10^6	4×10^8	1×10^8
COAD 3065	1.4×10^8	8×10^6	4×10^8	5×10^7

Table 2. *Fusarium* spp. isolates tested in leaf discs in experiment 1, their origins and the respective severity values (disease index-DI) obtained after 35 days. The potential antagonists were applied 0, 24 and 72 hours before deposition of urediniospores of the *Hemileia vastatrix*.

Isolate COAD	Species	Sampling site	DI at different times of application of the *H. vastatrix*		
			0 h	24 h	72 h
3065	*F. hemileiae*	Hawassa, Ethiopia	0	0	0
3010	*F. hemileiae*	Manhuaçú, MG, Brazil	8	0	5
3047	*F. hemileiae*	Metu, Ethiopia	0	3	7
3050	*F. hemileiae*	Metu, Ethiopia	0	12	0
3057	*F. hemileiae*	Agaro, Ethiopia	10	0	8
2943	*F. hemileiae*	Duas Barras, RJ, Brazil	7	12	3
3022	*F. hemileiae*	Itutinga, MG, Brazil	3	0	12
3044	*F. hemileiae*	Metu, Ethiopia	13	0	17
3068	*F. hemileiae*	Aleta Wendo, Ethiopia	10	13	5
2968	*F. hemileiae*	Hawassa, Etiópia	13	12	17
3019	*F. hemileiae*	Conceição da Barra de Minas, MG, Brazil	8	7	22
3012	*F. hemileiae*	Florestal, MG, Brazil	7	10	40
3018	*F. hemileiae*	Conceição da Barra de Minas, MG, Brazil	27	15	12
3025	*F. hemileiae*	Samambaia, MG, Brazil	8	38	0
3310	*F. hemileiae*	Nepomuceno, MG, Brazil	2	28	13
2935	*F. auranto-glutinosus*	Viçosa, MG, Brazil	47	17	10
3067	*F. hemileiae*	Aleta Wendo, Ethiopia	28	8	18
2963	*F. cameroonicum*	Somalomo, Cameroon	30	8	17
2937	*F. lateritium*	Viçosa, MG, Brazil	20	27	3
2938	*F. hemileiae*	Vitória, ES, Brazil	0	23	25
3015	*F. hemileiae*	BR 265, Brazil	28	40	27
3309	*F. hemileiae*	Nova Friburgo, RJ, Brazil	8	38	38
3308	*Fusarium* sp.	São João del-Rei, MG, Brazil	60	22	5
3009	*F. hemileiae*	Viçosa, MG, Brazil	62	63	32
2939	*F. hemileiae*	Lambari, MG, Brazil	18	72	63
3014	*F. hemileiae*	Vargínia, MG, Brazil	65	67	33
3034	*F. hemileiae*	Bonga, Ethiopia	48	0	18
2967	*F. hemileiae*	Bonga, Ethiopia	18	0	22
3026	*F. hemileiae*	Carmo da Cachoeira, MG, Brazil	47	27	8
2942	*F. auranto-glutinosus*	San Lorenzo, Paraguay	33	27	13
3059	*F. hemileiae*	Bale Robe, Ethiopia	52	15	52
2965	*F. auranto-glutinosus*	Kaffa, Ethiopia	60	35	13
2947	*F. pseudosolani*	Somalomo, Cameroon	47	5	48
2946	*F. hemileiae*	Somalomo, Cameroon	90	43	60
3027	*F. cameroonicum*	Somalomo, Cameroon	63	47	50
2948	*F. pseudosolani*	Somalomo, Cameroon	58	53	42
2964	*F. cameroonicum*	Somalomo, Cameroon	63	70	42
3029	*F. pseudosolani*	Somalomo, Cameroon	58	73	62

3030	*F. hemileiae*	Bonga, Ethiopia	62	73	55
Control			72	29	47

Table 3. *Fusarium* spp. isolates tested in leaf discs in experiment 2, their origins and the respective severity values (disease index-DI) obtained after 35 days. The potential antagonists were applied 0, 24 and 72 hours before deposition of urediniospores of the *Hemileia vastatrix*.

Isolate COAD	Species	Sampling site	DI at different times of application of the antagonists		
			0 h	24 h	72 h
3011	*F. hemileiae*	Florestal, MG, Brazil	0	0	0
3041	*F. hemileiae*	Gera, Ethiopia	0	0	0
3061	*F. hemileiae*	Aleta Wendo, Ethiopia	3	0	0
3073	*F. hemileiae*	Goba, Ethiopia	5	0	2
3066	*F. hemileiae*	Hawassa, Ethiopia	7	0	5
3038	*F. hemileiae*	Tepi, Ethiopia	0	2	7
2946	*F. hemileiae*	Somalomo, Cameroon	13	0	5
3028	*F. hemileiae*	Somalomo, Cameroon	8	0	12
3036	*F. hemileiae*	Bonga, Ethiopia	7	7	0
3072	*F. hemileiae*	Hawassa, Ethiopia	12	0	7
3045	*F. hemileiae*	Metu, Ethiopia	10	3	5
3046	*F. hemileiae*	Metu, Ethiopia	3	8	5
3062	*F. hemileiae*	Hawassa, Ethiopia	0	0	23
3040	*F. hemileiae*	Mizan-Aman, Ethiopia	18	7	0
2953	*F. hemileiae*	Goba, Etiópia	0	18	5
3311	*F. hemileiae*	Jimma, Ethiopia	10	3	13
3043	*F. hemileiae*	Metu, Ethiopia	13	7	25
3056	*F. hemileiae*	Bonga, Ethiopia	8	2	23
3020	*F. hemileiae*	Itutinga, MG, Brazil	15	2	25
3060	*F. hemileiae*	Bonga, Ethiopia	5	47	10
3063	*F. hemileiae*	Hawassa, Ethiopia	0	42	30
3042	*F. hemileiae*	Gera, Ethiopia	3	38	45
3017	*F. hemileiae*	Conceição da Barra de Minas, MG, Brazil	20	8	17
2966	*F. hemileiae*	Bore, Ethiopia	20	10	17
3069	*F. hemileiae*	Dilla, Ethiopia	23	13	40
3076	*F. hemileiae*	Dilla, Ethiopia	17	38	50
2945	*F. pseudosolani*	Somalomo, Cameroon	50	60	52
3035	*F. hemileiae*	Bonga, Ethiopia	55	40	45
2954	*F. perseae*	Jimma, Ethiopia	27	65	92
3312	*Fusarium* sp.	Ethiopia	35	77	40
Control			72	93	61

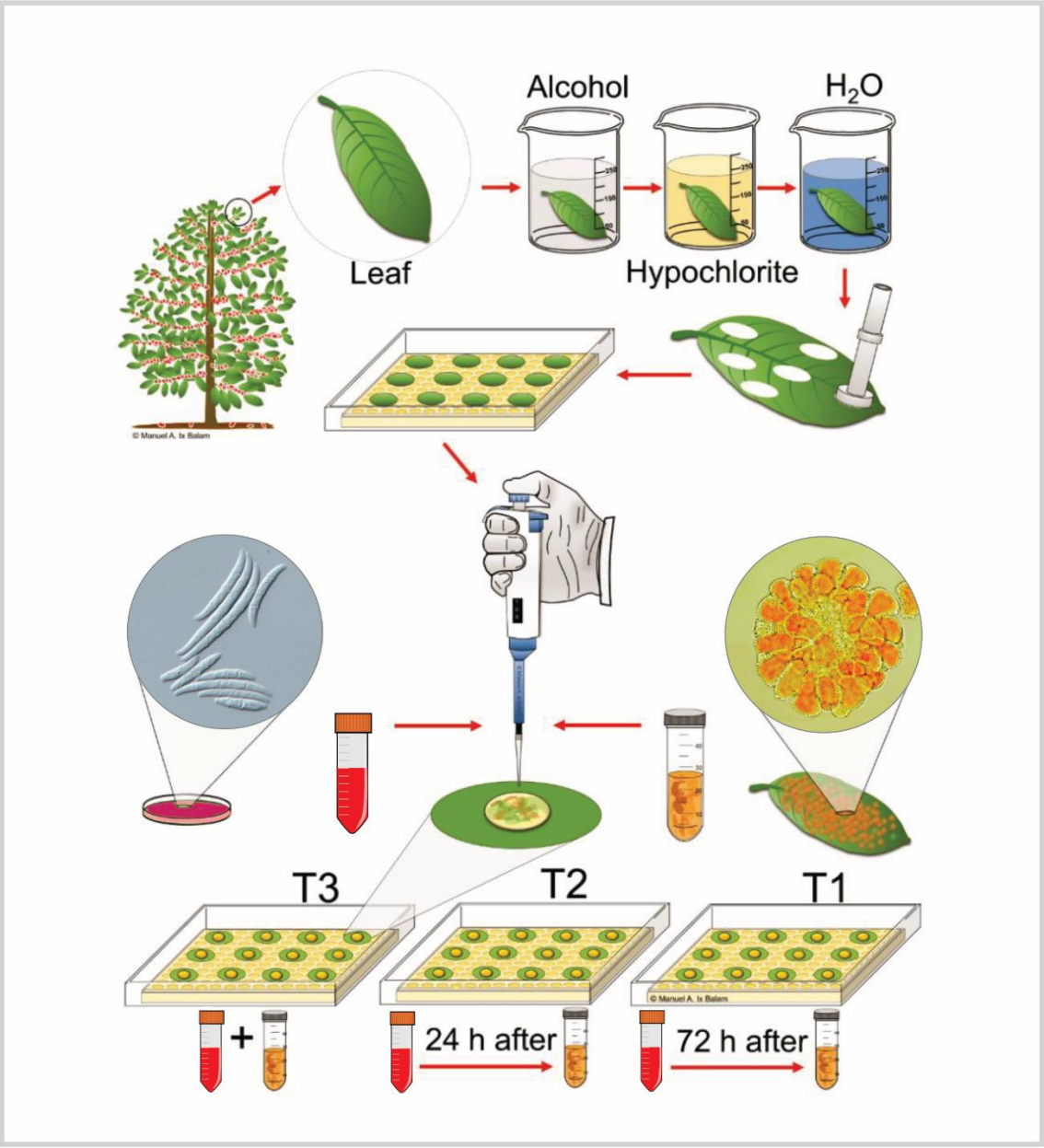

Fig. 1 Simplified outline of the methodology used in the screening of *Fusarium* spp. (red) potential antagonists to *Hemileia vastatrix* (orange) in leaf discs. Picture adapted of Rodríguez (2019).

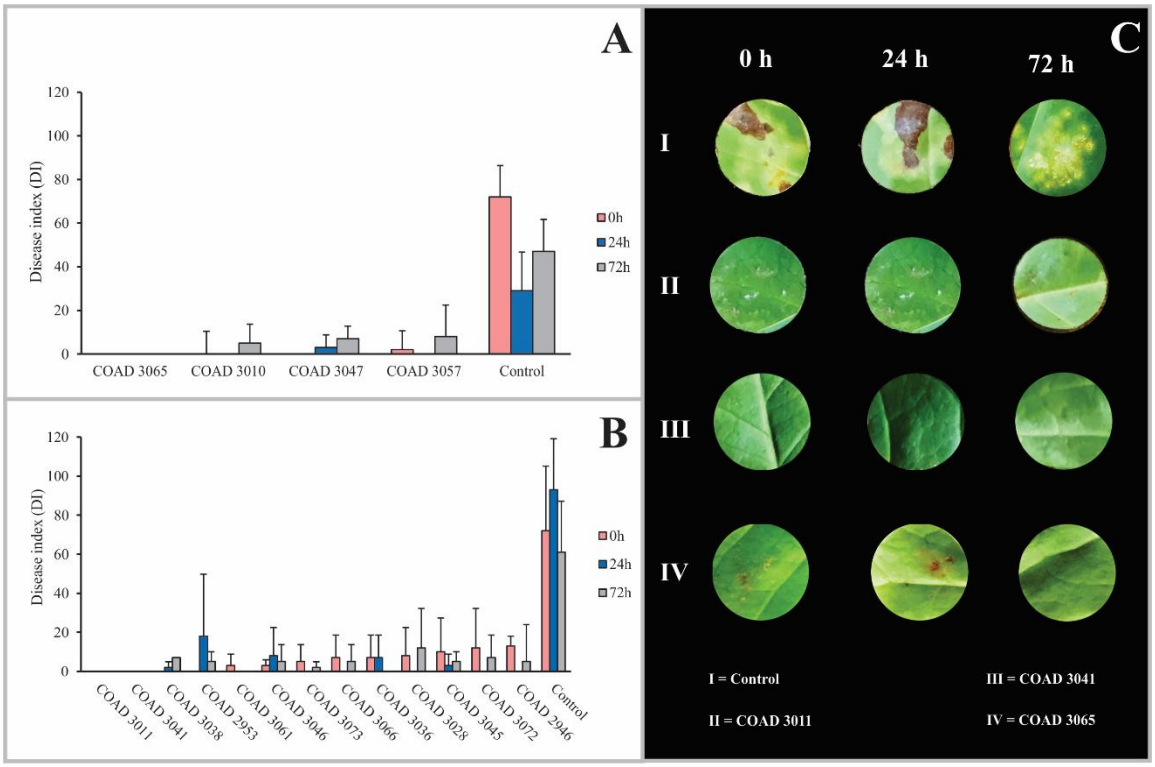

Fig 2 Severity evaluated through of the Disease Index (DI) in leaf discs in three different times. **(A)** Isolates that reduced the severity in more 80% in the all times of the experiment 1. **(B)** Isolates that reduced the severity in more 80% in the all times of the experiment 2. **(A-B)** Error bar = Standard Deviation **(C)** Pictures captured of coffee leaf discs inoculated with *Hemileia vastatrix* alone (control) as compared with treated with COAD 3011, COAD 3041 and COAD 3065 of the *Fusarium hemileiae*, 0, 24 and 72 hours before deposition of urediniospores of *H. vastatrix.* Images captured 35 days after experiment began.

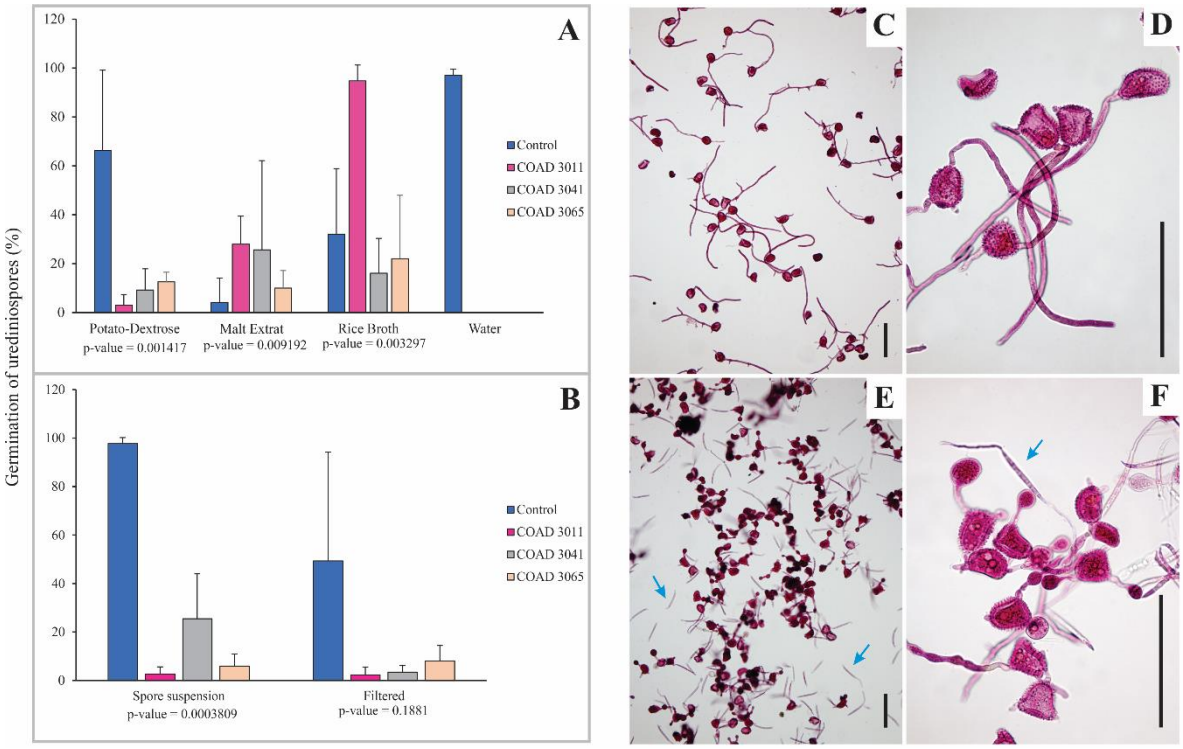

Fig. 3 Assays of inhibition of germination of the urediniospores of *Hemileia vastatrix*. **(A)** Test for filtrate formulation of the three isolates of *Fusarium hemileiae* in different cultures mediums. **(B)** Comparison between the bioactivity of filtrates and spore suspensions of the of the three isolates of *Fusarium hemileiae*. **(A-B)** Kruskal-Wallis significant differences in their distribution were confirmed when p-value < 0.05. Error bar = Standard Deviation. **(C-D)** Normal germination of rust urediniospores in control's treatments. **(E-F)** Urediniospores showing deformations in the germinative tubs in the spore suspensions treatments, blue arrows show conidia of *F. hemileiae*. **(C-F)** Scale bar = 100 µm.

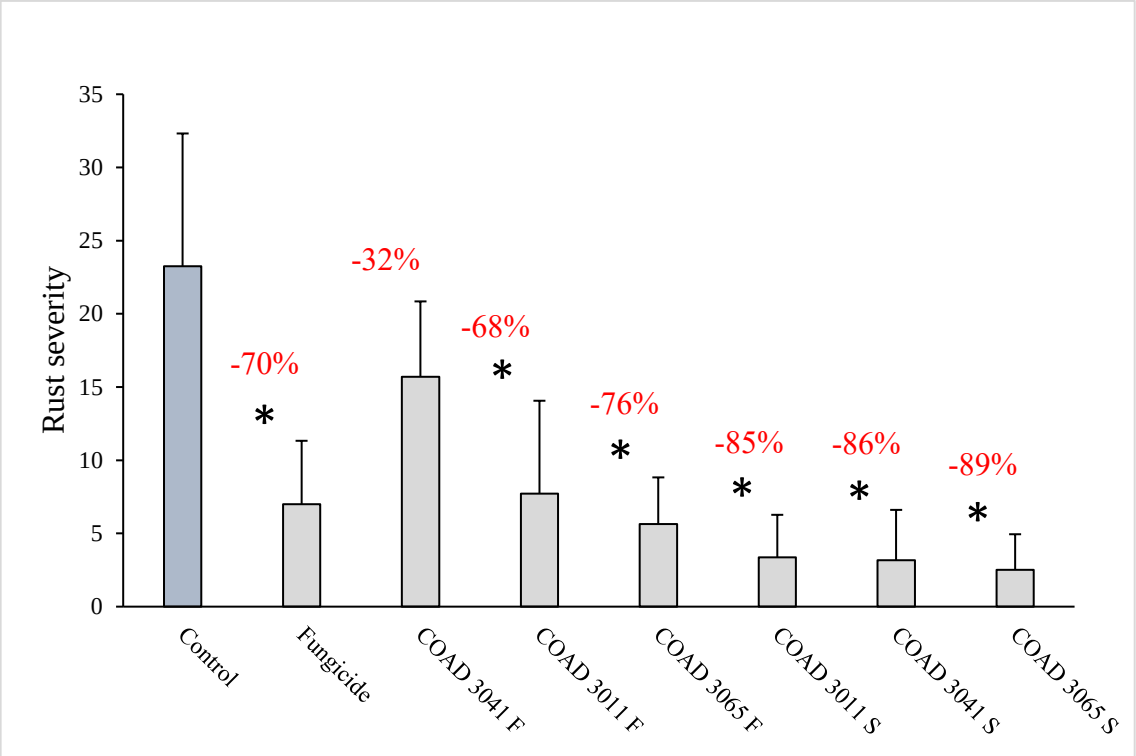

Fig. 4 Experiment 1 – Coffee leaf rust severity on plants inoculated with *H. vastatrix* and preventively treated or untreated (Control) with filtrates (F) and spore suspensions (S) of the three isolates of *Fusarium hemileiae* and Copper Oxychloride (Fungicide). In red: percentage of mean severity reduction in relation to the control. Treatments indicated with asterisk differ from control (Dunnett's $\alpha = 0.05$).

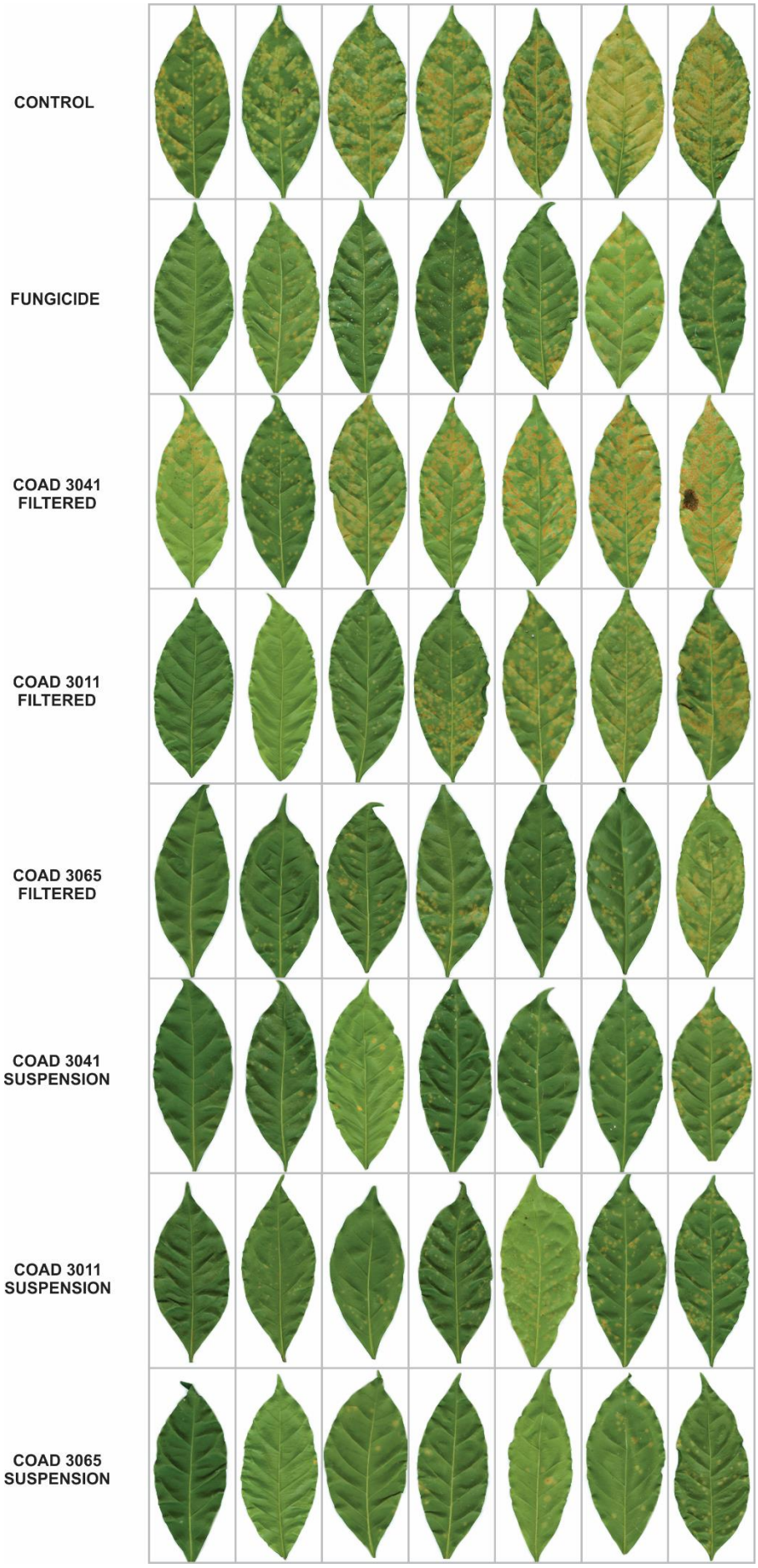

Fig. 5
Experiment 1 - Pictures captured of coffee leaf inoculated with *Hemileia vastatrix* alone (control) as compared with treated preventively with Copper Oxychloride (Fungicide), filtrates and spore suspensions of the three isolates of *Fusarium hemileiae.*

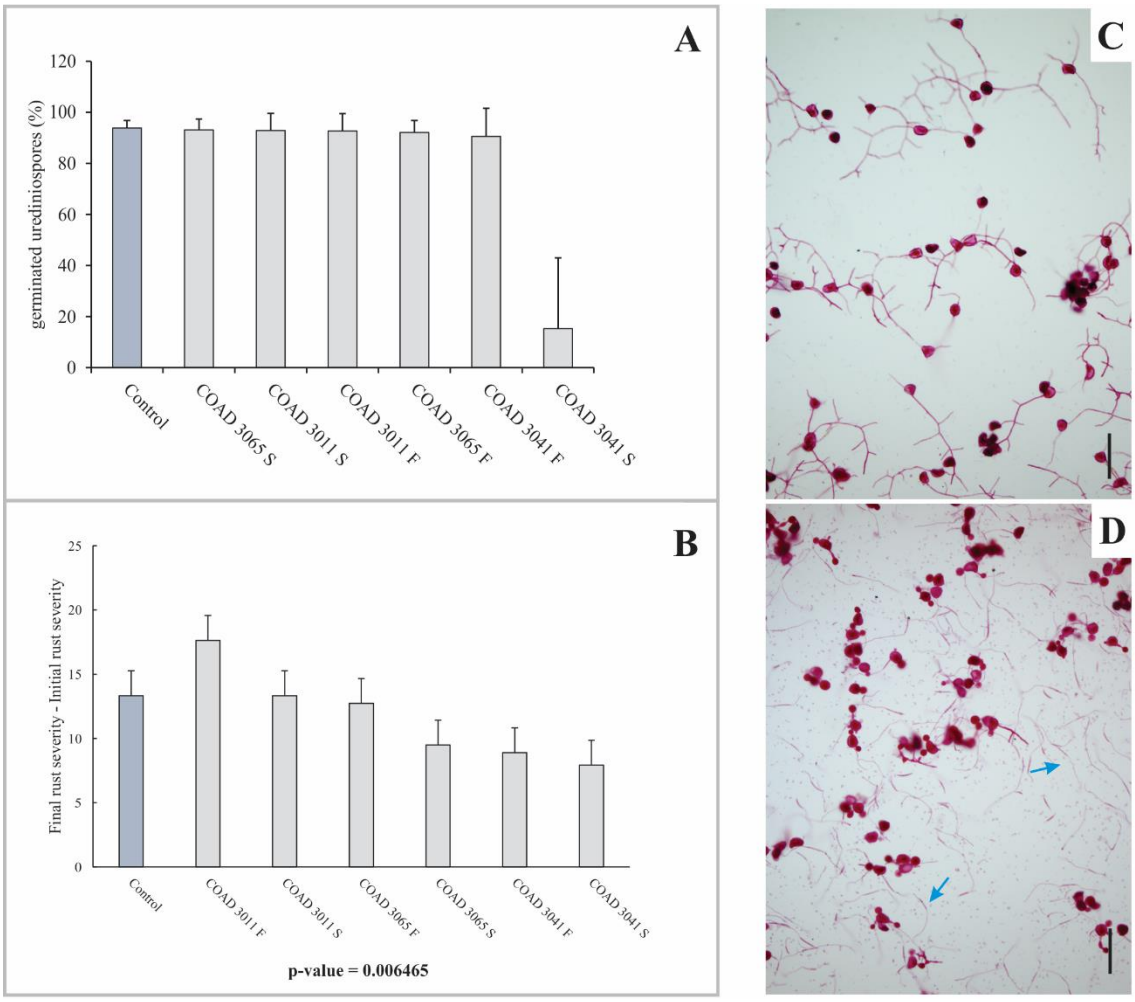

Fig. 6 Experiment 2 – **(A)** Progress of coffee leaf rust severity on plants inoculated with *H. vastatrix* untreated (Control) and treated with filtrates (F) and spore suspensions (S) of the three isolates of *Fusarium hemileiae* after rust pustules formation. Treatments indicated with asterisk differ from control (Dunnett's $\alpha = 0.05$). **(B)** Viability of urediniospores in the treated leaf. Kruskal-Wallis significant differences in their distribution were confirmed when p-value < 0.05. **(A-B)** Error bar = Standard Deviation. **(C)** Normal germination of rust urediniospores in control's treatments. **(D)** Urediniospores showing deformations in the germinative tubs in treatment with spore suspension COAD 3141, blue arrows show germinate conidia of *F. hemileiae*. **(B-C)** Scale bar = 100 µm.

GENERAL CONCLUSIONS

- Ninety-three putative mycoparasite *Fusarium* strains of *H. vastatrix* were isolated and characterized.
- The isolates were grouped in seven distinct species, four of which were found to be new to science.
- One new species and a known species have been grouped within the *Fusarium lateritium* species complex, namely*: F. hemileiae* sp. nov. and *F. lateritium.*
- Two new species and a known species have been grouped within the *Fusarium solani* species complex, namely: *F. cameroonicum* sp. nov., *F. pseudosolani* sp. nov. and *F. perseae.*
- One new species has been grouped within the *Fusarium incarnatum-equiseti* species complex, namely: *F. auranto-glutinosus* sp. nov.
- One isolate was grouped within the *Fusarium graminearum* species complex: *Fusarium* sp.
- The vast majority of the isolates belonged to the species *F. hemileiae* sp. nov.
- The isolates belonging to the *Fusarium solani* species complex (*F. cameroonicum, F. perseae* and *F. pseudosolani*) either had a poor performance at reducing CLR severity or even contributed to an increase in disease severity in leaf disc tests. Isolates of *Fusarium auranto-glutinosus* and *F. lateritium* provided good levels of disease reduction, only when applied 72h before *H. vastatrix* in leaf discs tests.
- Several isolates of *F. hemileiae* sp. nov. were effective in reducing coffee leaf rust severity *in vitro*.
- Sprays of conidial suspensions of three isolates of *F. hemileiae* sp. nov. applied prior to inoculation with *H. vastatrix* were effective in reducing coffee leaf rust severity *in planta*.
- Filtrates from cultures of two isolates of *F. hemileiae* sp. nov. had an anti-CLR protective effect comparable to copper fungicide applications *in planta*.
- Conidial suspensions of *F. hemileiae* sp. nov. performed better at inhibiting the germination of urediniospores and in reducing CLR severity then the sprays with *F. hemileiae* sp. nov. culture filtrates.