KEMINY RIBETT BAUTZ

Cordyceps cateniannulata: ITS ENDOPHYTIC CAPACITY AND TWO-IN-ONE BIOCONTROL AGENT FOR COFFEE LEAF RUST AND TWO COFFEE PESTS

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

Advisor: Simon Luke Elliot

Co-advisors: Madelaine Venzon
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Simon Luke Elliot
Advisor

Aos meus pais Lindaura e Laumiro,

minha base e os amores da minha vida.

Dedico

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ABSTRACT

BAUTZ, Keminy Ribett, D.Sc., Universidade Federal de Viçosa, May, 2023. ***Cordyceps cateniannulata*: its endophytic capacity and two-in-one biocontrol agent for coffee leaf rust and two coffee pests**. Adviser: Simon Luke Elliot. Co-advisers: Madelaine Venzon and Robert Weingart Barreto.

Coffee is one of the most important agricultural products in the global economy. Coffee leaf rust, caused by *Hemileia vastatrix*, is the worst of all coffee diseases, and coffee berry borer – *Hypothenemus hampei* and coffee leaf miner *Leucoptera coffeella* – are the two main insect pests of coffee. The management of this disease and these pests is mainly carried out by the application of chemicals products and, however, their management continues to challenge farmers and researchers. Biological control by entomopathogenic fungi is a strategic tool in the fight against diseases and pests that threaten coffee production. Typically, members of the fungal genus *Cordyceps* are best known as obligate parasites of arthropods. However, there is increasing evidence that *Cordyceps*, and related genera in the Hypocreales, can grow both as plant endophytes and as parasites of arthropods. *Cordyceps cateniannulata* was isolated from healthy coffee tissues in Cameroon during a survey for fungi antagonistic to *H. vastatrix*. Preliminary tests have shown that *C. cateniannulata* is a mycoparasite of *H. vastatrix*. Since the genus *Cordyceps* (Cordycipitaceae: Hypocreales) is well known as including species that are pathogens it was hypothesized that *C. cateniannulata* could be pathogenic to two of the manly pests of coffee. In the present study, we present results pertaining to the potential of *C. cateniannulata* as a two-in-one biocontrol agent for the management of coffee leaf rust and two coffee pests, in addition to its endophytic colonization of coffee. In chapter 1, we evaluated the systemic endophytic capacity of *C. cateniannulata* in coffee seedlings, effects of *C. cateniannulata* against uredospores of *H. vastatrix* and the pathogenicity of *C. cateniannulata* against insect pests of coffee and also the beetle *Tenebrio molitor* as a model. By recovering *C. cateniannulata* from inoculated coffee plants, via both soil drench and foliar applications, we confirmed its endophytic status. Further controlled inoculation studies showed that this strain of *C. cateniannulata* is also a mycoparasite of coffee leaf rust, as well as an entomopathogen; infecting and killing coffee berry borer and coffee leaf miner. In chapter 2, we evaluated the virulence of *C. cateniannulata* against *T. molitor* and *H. hampei* under laboratory conditions, a comparison of the virulences of *C. cateniannulata* and *Beauveria bassiana* against *H. hampei*

and a test of the application of *C. cateniannulata* to coffee branches against *H. hampei* under field cage-test conditions. *Cordyceps cateniannulata* caused a significant concentration-dependent increase in insect mortality. No difference was found between the virulence of *C. cateniannulata* and *B. bassiana* to *H. hampei* in this essay. Under this preliminary field cage test study, *C. cateniannulata* application resulted in a small but significant reduction in insect survival but it did not reduce the number of fruits bored by coffee berry borer. In chapter 3, we evaluated the potential of *C. cateniannulata* against coffee leaf miner in an *in planta* experiment and compared the potential of *C. cateniannulata, B. bassiana* and a mixture of both fungi in the same assay. In addition, we investigated the interaction between *C. cateniannulata* and *B. bassiana* in an *in vitro* assay. *Cordyceps cateniannulata* and *B. bassiana*, as well as the mixture, caused high mortality of coffee leaf miner pupae and no significant difference was found between the treatments. Furthermore, no synergistic effect was observed on pupal mortality after application of the mixture of both fungi; however, *C. cateniannulata* sporulated in more pupae than *B. bassiana*. In the *in vitro* experiment, *C. cateniannulata* alone and in coculture showed larger colony sizes than *B. bassiana* alone and in coculture. Moreover, there was no halo of inhibition for either fungus. Here, we provide a report on the potential of *C. cateniannulata* as novel entomopathogenic fungus to be deployed for the control of coffee leaf rust, coffee berry borer and coffee leaf miner.

Keywords: Biological control. Entomopathogenic fungi. Fungal endophytes. *Hemileia vastatrix*. *Hypothenemus hampei*. *Leucoptera coffeella*. Mycoparasites.

RESUMO

BAUTZ, Keminy Ribett, D.Sc., Universidade Federal de Viçosa, maio de 2023. ***Cordyceps cateniannulata*: sua capacidade endofítica e agente de biocontrole dois em um para a ferrugem e duas pragas do cafeeiro.** Orientador: Simon Luke Elliot. Coorientadores: Madelaine Venzon e Robert Weingart Barreto.

O café é um dos produtos agrícolas mais importantes na economia global. A ferrugem do cafeeiro, causada por *Hemileia vastatrix*, é a pior de todas as doenças do cafeeiro e sendo a broca-do-café – *Hypothenemus hampei* e o bicho-mineiro *Leucoptera coffeella* – os dois principais insetos-praga do café. O manejo dessa doença e das pragas do cafeeiro é feito principalmente pela aplicação de produtos químicos e, no entanto, seu manejo continua desafiando agricultores e pesquisadores. O controle biológico utilizando fungos entomopatogênicos é uma ferramenta estratégica no combate às doenças e pragas que ameaçam a cafeicultura. Tipicamente, os membros do gênero *Cordyceps* são mais conhecidos como parasitas obrigatórios de artrópodes. No entanto, há evidências crescentes de que *Cordyceps* e gêneros relacionados em Hypocreales podem crescer tanto como endófitos de plantas quanto como parasitas de artrópodes. *Cordyceps cateniannulata* foi isolado de tecidos saudáveis de café em Camarões durante uma pesquisa de fungos antagonistas da *H. vastatrix*. Testes preliminares mostraram que *C. cateniannulata* é um micoparasita da *H. vastatrix*. Visto que o gênero *Cordyceps* é bem conhecido por incluir espécies que são patógenos, levantou-se a hipótese de que *C. cateniannulata* poderia ser patogênico para duas das principais pragas do café. No presente estudo, apresentamos resultados referentes ao potencial de *C. cateniannulata* como um agente de biocontrole dois em um para o manejo da ferrugem do cafeeiro e duas pragas do cafeeiro, além de sua colonização endofítica no café. No capítulo 1, avaliamos a capacidade endofítica sistêmica de *C. cateniannulata* em mudas de café, os efeitos de *C. cateniannulata* contra uredósporos da *H. vastatrix* e a patogenicidade de *C. cateniannulata* contra insetos-praga do café e também o besouro *Tenebrio molitor* como agente modelo. Ao recuperar *C. cateniannulata* de cafeeiros inoculados, tanto por meio de irrigação no solo quanto por meio de aplicações foliares, confirmamos seu status endofítico. Outros estudos de inoculação controlada foram conduzidos para mostrar que está cepa de *C. cateniannulata* é também um micoparasita da ferrugem do café, bem como um entomopatógeno; infectando e matando a broca-do-café e o bicho-mineiro. No capítulo 2, avaliamos a virulência de *C. cateniannulata* contra *T. molitor* e *H. hampei* em condições de laboratório, comparamos as

virulências de *C. cateniannulata* e *Beauveria bassiana* contra *H. hampei* e testamos a aplicação de *C. cateniannulata* a ramos de café contra *H. hampei* em condições de campo-jaula. *Cordyceps cateniannulata* causou um aumento significativo dependente da concentração na mortalidade de insetos. Nenhuma diferença foi encontrada entre a virulência de *C. cateniannulata* e *B. bassiana* para *H. hampei*. Sob o estudo preliminar de teste de campo em gaiola, a aplicação de *C. cateniannulata* resultou em uma pequena, mas significativa redução na sobrevivência do inseto, mas não reduziu o número de frutos furados pela broca-do-café. No capítulo 3, avaliamos o potencial de *C. cateniannulata* contra bicho-mineiro em um experimento *in planta* e comparamos o potencial de *C. cateniannulata, B. bassiana* e uma mistura de ambos os fungos no mesmo ensaio. Além disso, investigamos também a interação entre *C. cateniannulata* e *B. bassiana* em um ensaio *in vitro*. *Cordyceps cateniannulata* e *B. bassiana*, assim como a mistura, causaram alta mortalidade das pupas do bicho-mineiro e nenhuma diferença significativa foi encontrada entre os tratamentos. Além disso, nenhum efeito sinérgico foi observado na mortalidade de pupas após a aplicação da mistura de ambos os fungos; no entanto, *C. cateniannulata* esporulou em mais pupas do que *B. bassiana*. No ensaio *in vitro*, a colônia de *C. cateniannulata* isoladamente e no cocultivo apresentaram tamanho maior do que *B. bassiana* isoladamente e no cocultivo. Além disso, não houve halo de inibição para nenhum dos fungos. Aqui, nós fornecemos um relatório sobre o potencial de *C. cateniannulata* como novo fungo entomopatogênico a ser implantado para o controle da ferrugem do café, broca do café e bicho-mineiro.

Palavras-chave: Controle biológico. Endofitismo. Fungos entomopatogênicos. *Hemileia vastatrix*. *Hypothenemus hampei*. *Leucoptera coffeella*. Micoparasita.

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CHAPTER I

Article

Inter-kingdom host-jumps 'extraordinaire': *Cordyceps cateniannulata*, an endophyte of coffee, a parasite of coffee leaf rust and a pathogen of coffee pests

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Summary

- *Cordyceps* is a genus of fungi pertaining to the Hypocreales, most species of which are obligate parasites of arthropods.
- Here, we report the occurrence of a *Cordyceps* species entering into a multi-trophic, multi-kingdom association. *Cordyceps cateniannulata*, isolated from stem tissues of *Coffea* in Cameroon, is shown to function as an endophyte, a parasite of coffee leaf rust and a pathogen of coffee pests.
- A detailed polyphasic taxonomic study of the isolate was conducted, including a multilocus phylogenetic analysis based on ITS, LSU and TEF sequences, to confirm its identity.
- An emended description of *Cordyceps cateniannulata* is provided herein. Previously, *C. cateniannulata* was known predominantly as an entomopathogen associated with and isolated from a range of insect hosts.
- By recovering *C. cateniannulata* from inoculated coffee plants, via both soil drench and foliar applications, we confirmed its endophytic status.

- Further controlled inoculation studies were conducted to show that this strain of *C. cateniannulata* is also a mycoparasite of coffee leaf rust (*Hemileia vastatrix*), as well as an entomopathogen; infecting and killing coffee berry borer (*Hypothenemus hampei*) and coffee leaf miner (*Leucoptera coffeella*).
- This is the first record of *C. cateniannulata* as an endophyte of coffee and as a mycoparasite, as well as a new record for Africa.
- This represents an extraordinary and unprecedented case of multi-kingdom host jumping; involving plant, fungal and animal hosts. The implications for biological control with a more integrated and sustainable approach to the management of coffee pests and diseases is discussed.

Key words: biological control, entomopathogenic fungi, fungal endophytes, *Hemileia vastatrix*, *Hypothenemus hampei*, *Leucoptera coffeella*, mycoparasites, taxonomy.

1. Introduction

Cordyceps is the largest genus within the Cordycipitaceae, encompassing more than 400 species, with a global distribution (Sung *et al.*, 2007). The genus includes species showing considerable variation in degrees of host-specialization (Luangsa-ard *et al.*, 2005). Typically, members of the genus *Cordyceps* are best known as obligate parasites of arthropods (Samson *et al.*, 1988). However, there is increasing evidence that *Cordyceps*, and related genera in the Hypocreales, can grow both as plant endophytes and as parasites of arthropods (Vega *et al.*, 2008; Jaber & Ownley, 2018; Branine *et al*., 2019; Gange *et al*., 2019; Nicoletti & Becchimanzi, 2020; Nishi *et al.,* 2021; Quesado-Moraga *et al.,* 2020, 2022). The possibility of exploiting this association to control crop pests has been considered – with the entomopathogens functioning as plant 'bodyguards' (Elliot *et al.*, 2000; Jaber & Ownley, 2018) – and this potential has been or is being investigated for various crops (Canassa *et al.*, 2019, 2020; Sun *et al.*, 2020); including coffee (Posada *et al.*, 2007).

During a project funded by World Coffee Research (WCF) to assess the potential of biological control as a disease-management strategy to improve coffee production in Central America, surveys were conducted in the African centre origin of *Coffea*, targeted primarily at sourcing fungal antagonists associated with coffee leaf rust (CLR), caused by *Hemileia*

vastatrix (Pucciniales: Zaghouaniaceae), which could act as 'bodyguards'. Over 1,500 potential antagonists were obtained; included endophytic fungi – isolated from healthy tissues of coffee plants – and mycoparasites – isolated from CLR pustules (Rodríguez, 2019; Colmán *et al*., 2021; Rodríguez *et al.*, 2021).

Among the isolates which showed promise during preliminary *in vitro* screening, one – initially classified as a *Lecanicillium*-like endophyte – showed high antagonistic activity against CLR but its taxonomic status remained uncertain. Here, we present the results of a study that led to the elucidation of its identity as a species of *Cordyceps* (Cordycipitaceae: Hypocreales) and the subsequent research towards determining its infection biology as both an insect pathogen of coffee pests and as a mycoparasite of CLR, as well as assessing its biocontrol potential.

2. Material and Methods

2.1. The *Cordyceps* isolate

The isolate under study was obtained as an endophyte from the healthy stem of *Coffea arabica* in Cameroon. It was one among many endophytic isolates obtained from both wild and cultivated species of the genus *Coffea* in Africa; following the procedures described in detail by Rodríguez *et al.* (2021), with stem samples being taken *in situ* from mature trees. This is a modified protocol first used for sampling endophytes directly in the field from wild *Theobroma* species (Evans *et al*., 2003)

The isolate was conserved by storage in silica-gel and in 10% glycerol maintained at -80 °C (Dhingra & Sinclair, 1995; Gonçalves *et al.*, 2016). For routine use, the fungus was transferred to tubes containing potato carrot agar (PCA) and kept at 5 °C. The isolate was deposited in the fungal culture collection of the Universidade Federal de Viçosa (UFV), "Coleção Octávio Almeida Drummond" (COAD), under the code COAD 3349. A dried, metabolically-inactive colony was deposited in the UFV fungarium under the accession number VIC 47513.

2.2. Morphological characterization

Fungal structures for description were obtained by taking material directly from actively-growing culture or by using the slide technique, as described by Mafia & Alfenas (2016). Slides were mounted in lactofuchsin or lactophenol, and examined under a light

microscope, model Olympus BX-51, equipped with an Olympus Q COLOR3 digital camera. Images were processed with Inkscape (ver. 1.2.1). Biometric data were taken from at least 30 representative structures.

For culture description, COAD 3349 was grown on potato dextrose agar (PDA) or oatmeal-agar (OA). The cultures were maintained in an incubator at 25 ± 2 °C, in the dark for 14 d. Cultural characteristics were described using the nomenclature of Crous *et al.* (2009) and the colour terminology followed that of Rayner (1970).

2.3. Molecular characterization

2.3.1. DNA extraction

COAD 3349 was transferred to Petri plates containing PDA and maintained in the same conditions as described above for 5 d. After this period, the mycelium was collected and transferred to sterile 1.5 ml centrifuge microtubes containing stainless-steel spheres. For mycelium maceration, microtubes were placed in a mechanical cell disruptor, model L Beader 3 (Loccus Biotecnologia, Cotia, SP, Brazil), adjusted to 4,000 rpm in two cycles of 10 s each. Total DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA), following the manufacturer's instructions.

2.3.2. PCR and sequencing

Genomic regions of relevance for the phylogenetic studies of the Cordycipitaceae (Kepler *et al.*, 2017) were amplified through polymerase chain reaction (PCR) with the following primers: ITS-5 and ITS-4 (White *et al.*, 1990), for the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA); LROR and LR5 (Vilgalys & Hester, 1990), for the partial 28S rDNA (LSU); and the pair 983F and 2218R (Rehner & Buckley, 2005), for the partial translation elongation factor-1α (TEF-1α) gene.

Each PCR reaction was performed using a total volume of 12.5 µl, containing: 6.25 µl of Dream-Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA); 4 µl of sterile ultrapure water; 1.25 µl of bovine serum albumin (BSA); 0.25 µl of dimethyl sulfoxide (DMSO); 0.25 µl of each primer in the pair; and 0.25 µl of fungal genomic DNA (30 ng µl-1). PCR reactions started with an initial denaturation of double-stranded DNA molecules, followed

by 35-40 cycles of denaturation, primer annealing and final extension. Temperature and time conditions followed the recommendations of each primer pair.

PCR products (4 µl of each reaction) were electrophoresed on a 1% agarose gel, stained with GelRed™ (Biotium Inc., Hayward, CA, USA), and visualized under UV light. The remaining product of each PCR reaction was treated with ExoSAP IT purification kit (Amersham Biosciences, Arlington Heights, IL, USA), according to the manufacturer's instructions. Amplified fragments were sequenced by Macrogen (Seoul, South Korea, http://www.macrogen.com).

2.3.3. Phylogenetic analyses

Consensus sequences were assembled using SeqAssem ver. 07/2008 software (Hepperle, 2022). Sequences generated from COAD 3349 were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Data used in the phylogenetic inferences were extracted from the following publications: Luangsa-ard *et al.* (2005), Tasanathai *et al.* (2016), Kepler *et al.* (2017), Mongkolsamrit *et al.* (2018) and Hu *et al.* (2021). Additional sequences were obtained from the GenBank database (Table 1). Sequences of two *Purpureocillium lilacinum* isolates (Luangsa-ard *et al.*, 2005) were used to root the tree. All sequences were aligned with the MUSCLE algorithm present in the MEGA X ver. 10.2.2 software (Kumar *et al.*, 2018) and the combined alignment was obtained through SequenceMatrix ver. 1.8 software (Vaidya *et al.*, 2011).

Phylogenetic analyses, based on individual genic sequences and combined sequences, were performed by maximum likelihood analysis (ML) and Bayesian inference (BI). For both methodologies, tools within the CIPRES Science Gateway portal (Miller *et al*., 2010) were used. Prior to the phylogenetic studies, the best-fit model of nucleotide substitution was determined for each gene/locus. For this purpose, jModelTest2 ver. 2.1.6 (Darriba *et al.*, 2012), also available within the CIPRES portal, was used. A GTR+I+G substitution model was used for all the genic regions under study.

Inferred tree by the ML method used RAxML-HPC ver. 8.2.12 (Stamatakis, 2014). The bootstrap resampling strategy was set to 10,000 replicates aiming to define the best phylogenetic tree. For BI analyses, MrBayes ver. 3.2.7a was used (Ronquist & Huelsenbeck, 2003), employing the Markov Chain Monte Carlo (MCMC) method. Two simultaneous and independent analyses were initiated, each with four MCMC chains. In each analysis, trees were

produced for 5,000,000 generations, with sampling every 500 generations, resulting in 10,000 sampled trees. The first 2,500 trees (25%) were discarded as the burning phase and the posterior probabilities values were determined using the 7,500 remaining trees, in each analysis. Resulting trees, from the BI and ML methods, were visualized in TreeGraph 2 ver. 2.15.0-887 (Stöver & Müller, 2010) and edited using CorelDRAW Graphics Suite 2019.

2.4. Demonstration of endophytism in coffee by *Cordyceps cateniannulata*

2.4.1. Inoculum preparation and viability assessment

COAD 3349 was grown on PDA and incubated at 25 ± 1 °C in the dark for 15 d. After this period, five mycelial discs (5 mm diam) from the margin of the sporulating culture were transferred to one polypropylene bag (15 × 25 cm) containing autoclaved rice (100 g of parboiled rice autoclaved together with 60 ml of distilled water for 15 min at 1.0 atm pressure at 121 °C), then sealed with an elastic band before being placed in an incubator at 25 ± 1 °C in the dark for 10 d. Every other day, the bags were removed from the incubator and their contents were shaken to allow for homogeneous colonization of the substrate by the fungus.

A conidial suspension was prepared as follows: 30 g of COAD 3349-colonized rice were removed from the bag and transferred to a Falcon® tube containing 50 ml of 0.01% Tween-80 (Difco™) in sterile distilled water (SDW). The suspension was stirred for three min in a vortex shaker and then filtered through sterile gauze. Conidial concentrations were determined using a haemocytometer and adjusted to 1×10^8 conidia ml^{-1}. Conidial viability was checked by transferring 100 µl of the suspension to 9-cm diam PDA + chloramphenicol (0.15 g l^{-1}) plates. The plates were kept in an incubator at 25 ± 1 °C for 24 h. After this period, a drop of lactofuchsin was placed on the conidial suspension and covered with a coverslip. The area was scanned under a light microscope (x 40) and 100 conidia were assessed. Those that had a germ tube equal to or greater than the diameter of the conidia were considered to have germinated. Only suspensions of COAD 3349 with a germination rate of 95% or above were used in the experiments.

2.4.2. Inoculation of coffee plants

Coffee arabica plants (var. “Caturra”) were grown from seed for one year, and ca. 70 cm in height, in the coffee nursery of the Departamento de Fitopatologia at the UFV. These plants were transferred to 3 L pots containing pasteurized soil, manure and sand (1.5:1:0.5) and

maintained in a greenhouse (Salcedo-Sarmiento *et al.* 2021). Each plant was fertilized with Basacote® Plus 9M 16-8-12(+2) and watered regularly (100 ml of water per plant every 2 d).

Inoculation of COAD 3349 was performed either by soil drenching (SD) or leaf brushing (LB). SD application involved 12 coffee plants and 30 ml of a 1×10^8 ml^{-1} conidial suspension (Franzin *et al.* 2022); the soil close to the base of each coffee plant was irrigated carefully with the suspension to avoid contact with the aerial part of each plant. LB also involved 12 coffee plants, and the conidial suspension (concentration as above) was applied to the abaxial side of selected leaves with a soft camel-hair brush, as follows: apical bud, first leaf pair, second leaf pair, and two older/mature leaves (adapted from Posada *et al.*, 2007; Rocha *et al.*, 2017). Twelve non-inoculated plants served as controls and received 30 ml of a sterile 0.01% Tween-80 solution as a soil drench (as described above). Similarly, 12 control plants were used for the LB applications with the same Tween solution. All plants were placed in a controlled temperature room at 26 ± 1 ºC, 75% ± 5% relative humidity and under a photoperiod of 12 h (light provided by white fluorescent lamps yielding a photon flux density of approximately 350 $\mu molm^{-2}$ s^{-1} at plant canopy height).

2.4.3. Evaluation of endophytic colonization of inoculated coffee plants

Isolations aimed at determining the advance of endophytic colonization by COAD 3349 were performed 3, 7, 15, and 40-d post inoculation (dpi). For each evaluation time, three separate plants were used per treatment. All SD and LB-inoculated coffee plants were moved from the growth chamber to the laboratory. Plants were uprooted and individual root systems were washed thoroughly under a running tap to remove substrate. Samples of roots, stems, and leaves were collected from each SD plant and surface-sterilized by immersion in 70% ethanol for 1 min, then in 5% sodium hypochlorite for 2 min, and again in 70% ethanol for 1 min and, finally, rinsed three times in SDW, then dried on sterile filter paper (Canassa *et al.*, 2019). Each LB-inoculated plant had three leaves arbitrarily chosen from two positions, medium height and lower branch, and the main root was sampled by dividing it in three parts, whilst the root tip portions were discarded (Posada *et al.,* 2007). Individual LB-treated plants and controls had seven leaf portions sampled: the entire apical bud, first pair of leaves, second pair of leaves, and two older leaves (methodology adapted from Rocha *et al.*, 2017). The stems and roots were sampled as described for SD plants.

Six pieces of each leaf were cut using a sterile 5 mm diam paper punch. For each plant, five samples of roots and five of stems were cut with sterile secateurs into pieces 3 to 5 mm

long. These were placed in 90 mm diam PDA plates, supplemented with 0.15 g l^{-1} chloramphenicol, and then incubated at 25 ± 1 °C for 15 d. The frequency of recovery of *C. cateniannulata*-like colonies from each type of sample was recorded, after confirmation of identity by light-microscope observation of morphological characteristics.

2.5. Proof of mycoparasitism

2.5.1. Fungal growth and inoculum preparation

COAD 3349 was transferred to plates containing PDA and incubated for 7 d at 25 ± 2 °C, under a photoperiod of 12 h. Then, with a flamed metal cork borer, five 1-cm diam discs were taken from the margin of the actively-growing colony and transferred to a flask containing 75 ml of potato dextrose (PD) broth. The flasks were kept on a rotary flask shaker for 7 d at 25 ± 2 °C. The mycelium was then shredded within the medium using a rotary homogenizer (model MA 102, Marconi, Piracicaba, SP, Brazil) for 1 min. The resulting suspension was passed through a domestic plastic sieve and the concentration of the suspension was calibrated with a haemocytometer. A concentration of 1×10^5 conidia ml^{-1} was used as the standard. Also, a 10 ml aliquot of this suspension was taken and filtered through a sterile syringe filter with a pore size of 0.22 µm. The conidial suspension and the fungal filtrate were used in the experiment described below.

2.5.2. Assessing the effect of *Cordyceps cateniannulata* COAD 3349 on germination of *Hemileia vastatrix* urediniospores

Fresh urediniospores were harvested from *Coffea arabica* plants bearing CLR pustules in the greenhouse, as described by Salcedo-Sarmiento *et al.* (2021), then suspended in a PD broth with 100 µl of Tween-20 and the suspension was calibrated to 1×10^5 urediniospores ml^{-1}.

Three plastic boxes (11 × 11 × 3.5 cm) were disinfected with 70% alcohol and lined with sterilized paper towels saturated with SDW. Each box received an internal acrylic frame (surface-cleaned with 70% ethanol). Two microscope slides, previously cleaned with 70% alcohol, were placed over each frame. A 15 µl drop of the urediniospore suspension was pipetted centrally onto each slide. Subsequently, a 15 µl drop of either a COAD 3349 conidial suspension or fungal filtrate was added on top of the uredinial suspension and the droplets were mixed with the tip of the micropipette. Another batch of microscope slides was used as the

control with each slide receiving a 15 µl drop of urediniospore suspension and a 15 µl drop of filtered PD broth. The boxes were then placed in the dark, at 22 ± 1ºC, for 6 h. After which, in order to halt the germination process, a 15 µl drop of lactofuchsin was added to each droplet and covered with a coverslip.

Under a light microscope, the germination of *H. vastatrix* urediniospores was evaluated by examination of the first 100 urediniospores seen during the scanning of each droplet. Urediniospores were rated as germinated when their germ tubes had a length equal or longer than the diameter of the urediniospore (Capucho *et al.*, 2009). For each treatment, six droplets were examined and the mean germination percentage was calculated. The experiment was replicated three times.

2.5.3. Assessing the ability of *Cordyceps cateniannulata* COAD 3349 to colonize *Hemileia vastatrix* uredinia

In order to verify whether COAD 3349 is able to parasitize and complete its cycle from spore to spore on *H. vastatrix* uredinia, three rusted plants, having at least ten fully expanded pairs of leaves, were selected from those maintained for inoculum multiplication. These plants were sprayed until runoff with a conidial suspension (1×10^8 conidia ml^{-1}). Subsequently, the plants were kept in a dew chamber, at 25 ± 2 ºC, for 5 d. The plants were then transferred to a greenhouse bench. Another three rusted plants were sprayed with a 0.01% Tween-20 solution to serve as controls.

After 21 d, diseased leaves were collected, observed under a dissecting microscope, and rust pustules were examined for the presence of COAD 3349. Pustules with fungal overgrowth were sampled by either mounting the mycelium in lactofuchsin and examining under a light microscope, or transferring the mycelium to PDA plates. These plates were maintained under the same conditions as described above and whenever *C. cateniannulata*-like colonies were formed, slides were prepared and observed under a light microscope to confirm the presence of COAD 3349.

In order to fully document the possible colonization of *H. vastatrix* pustules by COAD 3349, leaves bearing CLR pustules were collected from *C. cateniannulata*-treated plants, as well as from untreated CLR-bearing controls, and dried in a plant press. Leaf pieces with either COAD 3349-colonized CLR pustules or ‘healthy’ CLR pustules from controls were selected, mounted on stubs with double-sided adhesive tape and left overnight in a desiccator. These

specimens were gold-coated using a Balzer's FDU 010 sputter coater. A Carl-Zeiss Model LEO VP 1430 scanning electron microscope (SEM) was used, operating at 12 Kv with a working distance ranging from 10 to 30 mm, to observe the specimens and generate representative electromicrographs of the purported mycoparasitism as compared to 'healthy' CLR pustules.

2.6. Proof of entomopathogenicity

2.6.1. Insect sourcing and rearing

The mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae) was used in this study as a model insect (Souza *et al.*, 2015) in order to refine a methodology for inoculation of *C. cateniannulata* COAD 3349, and for familiarization with the host-pathogen interaction. Mealworms were obtained from a stock kept in the Laboratório de Interação Inseto-Microrganismo at the UFV and reared on wheat bran and carrots at 25 ± 2 °C, in 60 ± 10% RH with a 12 h photoperiod. Larvae of undetermined instar but of similar size and weighing between 100 and 140 g were used (Morales-Ramos *et al.*, 2010). Adult females of the coffee berry borer (CBB), *Hypothenemus hampei* (Coleoptera: Scolytidae), were collected from coffee plantations within the campus of the UFV. The CBB were removed from the fruit with the aid of a sterile knife and then placed in petri dishes until they were used in the experiments. Only CBBs with completely melanized teguments were used (Silva *et al.*, 2014). All CBB individuals were surface-sterilized by immersion in 5% sodium hypochlorite for 2 min, rinsed three times in SDW, then dipped in 70% ethanol for 1 min, followed by rinsing three times in SDW and drying on sterile tissue paper (method adapted from Bayman *et al.*, 2021). Pupae of the coffee leaf miner (CLM), *Leucoptera coffeella* (Lepidoptera: Lyonetiidae), were collected from infested leaves of coffee plants in a greenhouse at the Departamento de Fitopatologia of the UFV.

2.6.2. Demonstration of pathogenicity of *Cordyceps cateniannulata* COAD 3349

In order to verify whether COAD 3359 is able to parasitize and complete its cycle from spore to spore on mealworms, batches of healthy larvae were exposed to the fungus by pipetting 200 µl of conidial suspension – calibrated to 5×10^8 conidia ml^{-1} – onto sterile filter paper lining in a 60 mm diam Petri plate (adapted from Bayman *et al.*, 2021). Plates lined with sterile filter paper and moistened with 200 µl of sterile 0.01% Tween-80 solution, were used as controls. Twenty healthy larvae were then placed in each of 20 plates and then left in the dark at 25 ± 1

°C. After 48 h, the filter paper lining was removed and replaced with new sterile filter paper moistened with 200 µl of SDW. This was done to avoid repeated infections in plates that received COAD 3359. The insects were not fed during the experimental period. Larval mortality and plate humidity were checked daily for 10 d. Individuals were classified as dead when touching them with sterile tweezers evoked no movement and then observed daily for 10 days for any evidence of colonization by *C. cateniannulata*. This assay was conducted in a completely randomized design with 20 replicates for each treatment. Fungal structures were sampled with a fine pointed needle and transferred to PDA plates. These plates were kept at 25 ± 1 °C and whenever *C. cateniannulata*-like colonies were formed, slides were mounted and observed under a light microscope in order to confirm the presence of COAD 3349 through the morphological characteristics of this species as described in the item on the taxonomy.

The pathogenicity assay of COAD 3349 on adult female CBBs was similar to the assay described above for mealworm larvae, but inoculation involved pipetting a smaller volume of conidial suspension (100 µl of conidial suspension at 1.9×10^8 conidia ml^{-1} in a 49 mm diam Petri plate). Mortality and plate humidity were checked daily for 10 d, as above. The insects were not fed during the experimental period. Additionally, dead insects were placed on PDA supplemented with 0.15 g l^{-1} chloramphenicol, instead of leaving them in the plates as for the mealworm, because the small size of CBB made it difficult to observe fungal sporulation. These were incubated in the dark at 25 ± 1 °C and checked daily. This bioassay was conducted in a completely randomized design with 45 replicates for each treatment. The identity of the fungus growing on pupae and adults of CBB was confirmed as above.

The pathogenicity of COAD 3349 to CLM pupae was also assessed. Leaves naturally infested with CLM pupae were individually placed in plates (150 mm diam) lined with moist filter papers, and the number of pupae on each leaf was checked under a dissecting microscope and recorded (ranging from 3 to 8 pupae per leaf). Fourteen leaves bearing CLM pupae were individually exposed to a conidial suspension (1×10^8 conidia ml^{-1}). A hand-held atomizer was used to spray the leaf surfaces until runoff. Ten leaves received a sterile 0.01% Tween-80 solution as controls. All plates were placed in the dark in an incubator at 25 ± 1 °C for 9 d and the mortality of pupae and adults was recorded daily. Dead adults were placed in moist chambers to incubate, while dead pupae showing fungal growth were placed on PDA, supplemented with 0.15 g l^{-1} chloramphenicol, and incubated in the dark at 25 ± 1 °C. This bioassay was conducted in a completely randomized design with 14 replicates treated with the

fungus and 10 for the control. The identity of the fungus growing on pupae and adults of CLM was confirmed as above.

To document the process of colonization of COAD 3349 in CBB and CLM, three treated and another three untreated individuals of each insect were mounted on stubs and examined under a SEM, following the same procedure described above.

2.7. Data analysis

Survival data and median lethal times (LT_{50}) were tested by survival analysis with the Kaplan-Meier estimator. The general similarity among the curves was tested with log-rank tests while pairwise comparisons were performed by the Bonferroni method ($P < 0.05$). For the test of COAD 3349 against CLM, pupal death data were analysed through a Generalized Linear Model (GLM) with binomial distribution, and the data were compared using Chi-squared test (χ^2) ($P < 0.05$). For CLM adult deaths, the data were also analysed through a GLM with a binomial distribution, with quasi-binomial applied to correct over-dispersion, and the data were compared with F tests ($P < 0.05$). For the analysis of pupae and adult mortality, the weight function was used in the model to ponder the difference in the initial number of pupae and adults in each repetition. Data analyses were performed using R (version 4.2.2) statistical software package (R Development Core Team, 2022).

The data obtained for germination of *H. vastatrix* from the three experiments were analysed using Levene's test to verify whether data could be combined. The data were analysed by one-way analysis of variance (ANOVA) and the means were compared by Tukey's test ($P < 0.05$), using the Minitab software (version 19; Minitab Corporation).

3. Results

3.1. Phylogeny

The final alignment of *Cordyceps* sequences used for the phylogenetic trees assemblage included: 58 isolates of 30 species of *Cordyceps*; including previously described *C. cateniannulata* isolates (BUC2007, CBS 152.83 and TBRC 7258), as well as COAD 3349. Two sequences of *Purpureocillium lilacinum*, CBS 431.87 and CBS 284.36 (Luangsa-ard *et al*., 2005), were used as an outgroup to root the tree. The combined alignment with ITS, LSU and

TEF-1α included 2,273 characters (ITS: 549; LSU: 815; TEF-1α: 909). 1,588 were considered to be conserved (ITS: 348; LSU: 686; TEF-1α: 554), and 548 (ITS: 162; LSU: 83; TEF-1α: 303) were considered parsimoniously informative.

The concatenated phylogenetic analysis revealed that COAD 3349 grouped in a well-supported clade (96 % bootstrap support and a 1.0 posterior probability value) with the three described isolates of *C. cateniannulata* used in this study (Fig. 1).

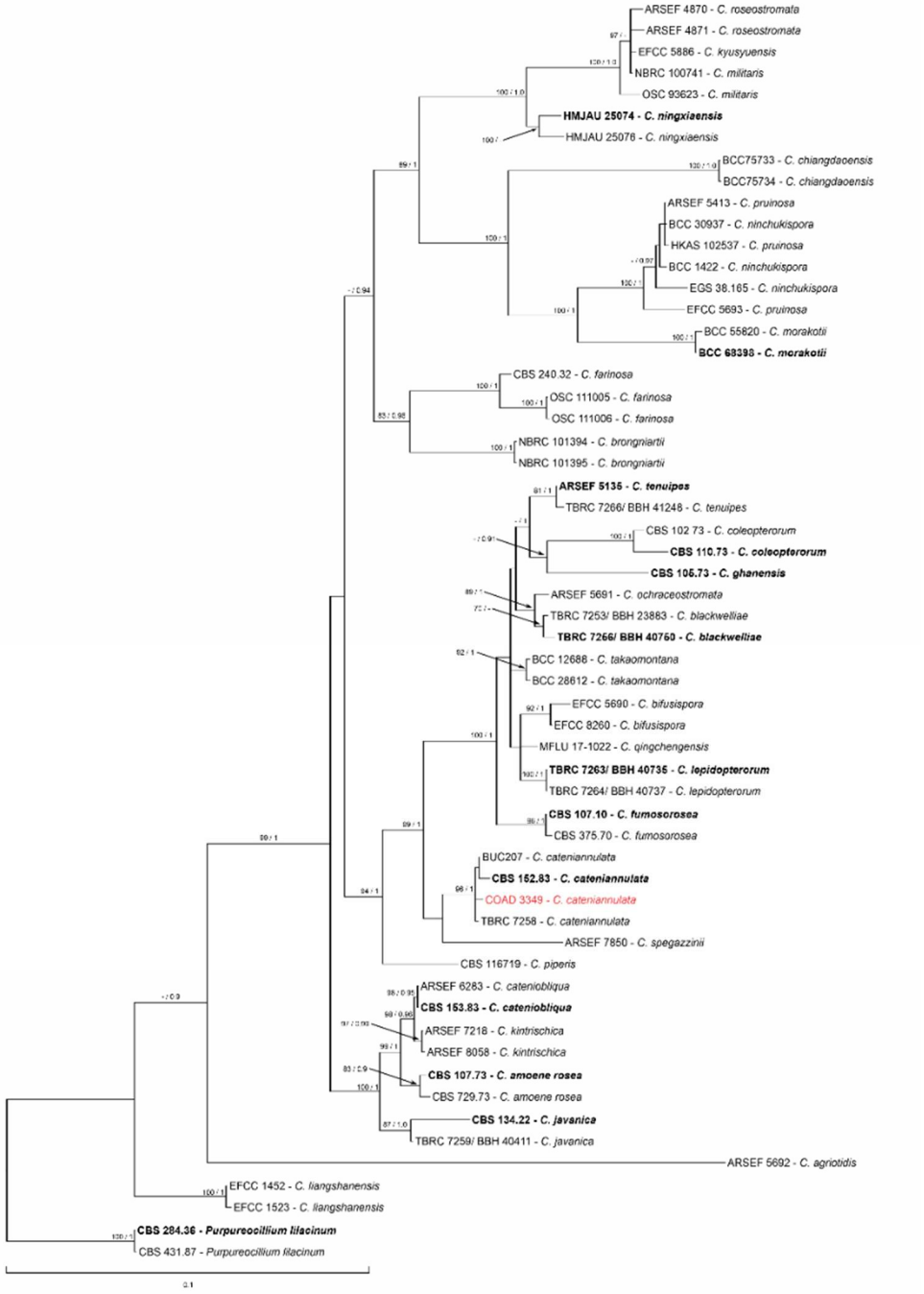

Figure 1. Phylogenetic analysis based on three combined loci (ITS, LSU, and TEF-1α) showing the relationship between the isolate COAD 3349 and strains of *Cordyceps cateniannulata*. Bootstrap support (Maximum likelihood) and/or Bayesian posterior probabilities values higher than 70% or 0.90 are presented above the nodes ("–" indicates lack of minimum support value). Ex-type cultures are indicated in bold and the isolate under study is highlighted in red.

3.2. Taxonomy

Cordyceps cateniannulata (Z.Q. Liang) Kepler, B. Shrestha & Spatafora – in Kepler *et al*., *IMA Fungus* **8**: 346, 2017. MycoBank: 820976. **emend. desc.**

≡ *Paecilomyces cateniannulatus* Z.Q. Liang – in Liang, *Acta Phytopathologica Sinica* **21**: 33, 1981.

= *Isaria cateniannulata* (Z.Q. Liang) Samson & Hywel-Jones – in Luangsa-ard *et al*., *Mycological Research* **109**: 588, 2005.

Material examined: CAMEROON: isolated from stem of *Coffea arabica*. 2017, M. K. Ndacnou (COAD 3349).

Emended description based on COAD 3349 (Fig. 2).

Cultural characteristics: slow-growing, 35–37 mm diam after 14 d at 25 °C in the dark: flat to low, convex, regular and circular with entire edges, white, felt-like to cottony aerial mycelium, with hyphal tufts in the colony centre (PDA), or with few tufts distributed radially (OA); sporulation abundant (PDA) or moderate (OA).

Description: sexual morph not seen. *Mycelium* branched, septate, 1–2 µm diam, hyaline, smooth. *Vegetative propagules* (*gemmae*) hockey-stick shaped, 10–60.5 × 1.5–3.5 µm, constricted at base 1–3 µm, 0 to 4 septate, hyaline, thin and smooth-walled. *Conidiophores* verticillate, straight to slightly sinuous, 6.5–81 × 1.5–2.5 µm, 0–7 septate, hyaline, smooth; *Conidiogenous cells* (*phialides*) ampulliform, 3–8 (–11.5) × 1–2.5 µm, hyaline, smooth. *Conidia* catenulate, in long chains; often coalescing in rings, 12–40 µm diam, ovoid or ellipsoid, 2–3.5 × 1–2 µm, hyaline and smooth.

Notes: *Cordyceps cateniannulata* was originally described by Liang (1981a, b) as *Paecilomyces cateniannulatus*; occurring as an entomopathogen on unidentified coleopteran adults and pupae of *Archips* (*Cacoecia*) *ingentanus* (Lepidoptera, Tortricidae) in China. Only the asexual morph of this species has been reported, thus far. Based on phylogenetic studies,

the fungus was later transferred to the genus *Isaria* by Luangsa-ard *et al.* (2005), as *I. cateniannulata*. Subsequently, according to present nomenclatural rules, it was recombined into *Cordyceps* by Kepler *et al.* (2017). The species was originally named by Liang (1981a), but with a limited description and lacking illustrations. Subsequently, a fuller, illustrated description was published (Liang, 1981b). Later, Shimazu (2001) re-described it from Japan, based on specimens isolated from a range of substrates – including soil and leaves, as well as dipteran, coleopteran, hymenopteran and lepidopteran hosts – but with no molecular data. Liang (1981b) reported that phialides have a globose inflated base with a distinct neck, 3–8 × 1.5–3 µm; producing hyaline, smooth, ovoid to ellipsoid conidia, 2–3.5 × 1–1.5 µm, in imbricate, irregular chains that "slime down to form a ring, ca. 30 µm diam". The isolate obtained from coffee in Africa, and described herein (COAD 3349), is morphologically similar to those described by Liang (1981a, b); although Shimazu (2001) reported phialides up to 12 µm in length and with slightly larger conidia, 2.0-4.5 x 1.5-2.5 µm. The vegetative structures interpreted here as gemmae (Fig. 2 F-H), are reported for the first time and may be a feature of isolate COAD 3349 since they were not included in the previous descriptions (Liang, 1981a b; Shimazu, 2001). The gemmae, or vegetative propagules, have a distinctly constricted base – suggesting ready detachment from the hyphae – and regularly germinate in situ; indicating that these function as an additional asexual propagule.

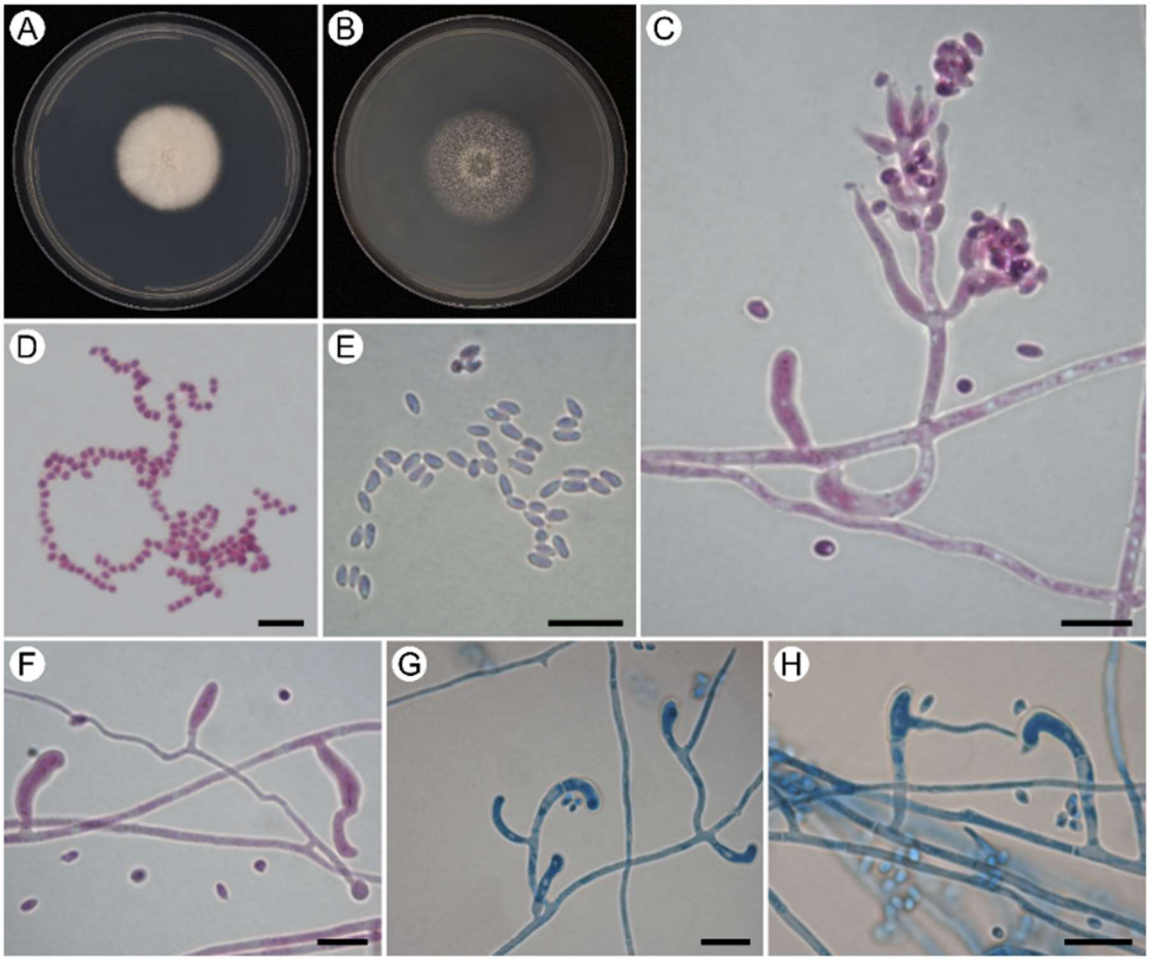

Figure 2. Morphological characteristics of *Cordyceps cateniannulata* COAD 3349. Fourteen days-old colonies on (A) PDA and (B) OA. (C) Conidiophore and conidiogenous cells. (D) Catenulate conidia formed in long chains. (E) Conidia. (F, G, H) vegetative propagules (gemae). Bars: 10 µm.

3.3. Endophytism of *Cordyceps cateniannulata* in coffee

Both inoculation methods (SD and LB) led to the colonization of coffee tissues by COAD 3349, although with different degrees of efficiency. SD-treated plants resulted in its isolation from leaves, stems and roots; whereas, in the LB inoculations, it was restricted to the leaves and failed to move systemically in other tissues. For the control plants, COAD 3349 was not isolated from the stems, roots or leaves. *Cordyceps cateniannulata* was distinguished readily from the background endophytes by its distinctive snow-white colonies (see Fig. 3).

For SD-inoculated plants, colonies were isolated from the stems of two of the three plants after 3 dpi (Fig. 3H). After 7 dpi, COAD 3349 was isolated from both the stems and roots of two plants (Fig. 3G). After 15 and 40 dpi, it was isolated only from the leaves of one plant (Fig. 3I) and was not found in the stems or roots.

For LB-inoculated plants, the fungus was recovered from all the apical buds and young leaves but only from one older leaf after 3 dpi (Fig. 3F). After 7 dpi, there was no recovery of COAD 3349 from any of the tissues sampled. After 15 dpi, COAD 3349 was isolated from young and old leaves of one plant. Finally, after 40 dpi, COAD 3349 was isolated only from the young leaves of one plant (Fig. 3F), but not from the stems and roots (Fig. 3D, E).

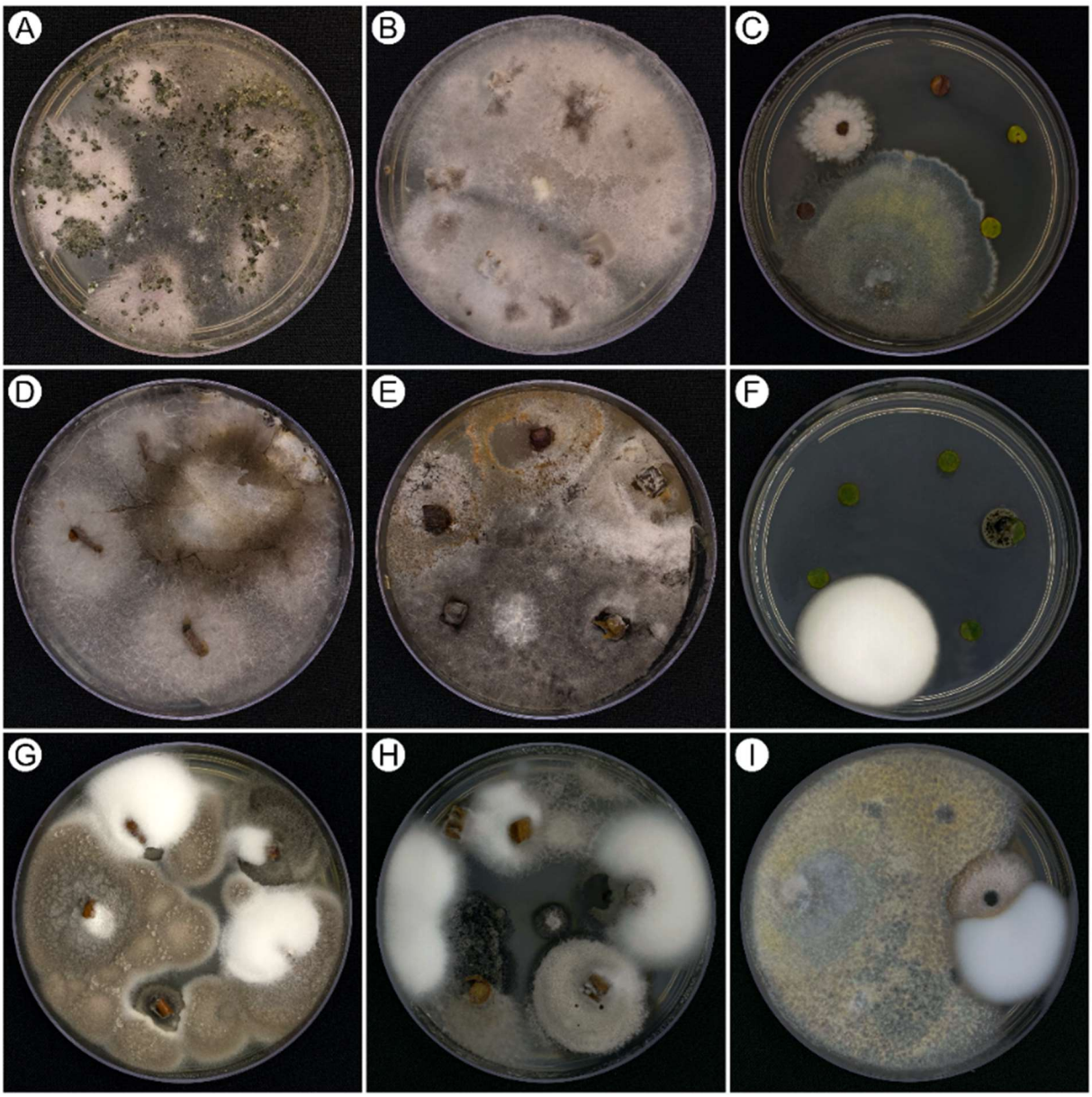

Figure 3. Demonstration of endophytism in coffee by *Cordyceps cateniannulata* COAD 3349 at three-, seven-, fifteen- and forty-days post-inoculation through soil drench and foliar application. Shown are: (A) Root fragments from a control plant. (B) Stem fragments from a control plant. (C) Leaf fragments from a control plant. Note that there is no presence of *C. cateniannulata* in these fragments. (D) Root fragments of a plant treated with foliar brushing of *C. cateniannulata*. (E) Stem fragments of a plant treated with foliar brushing of *C. cateniannulata*. (F) leaf fragments of a plant treated with foliar

brushing of *C. cateniannulata* (visible white mycelium only on the leaf). (G) Root fragments of a plant treated with soil drench of *C. cateniannulata* (visible white mycelium). (H) Stem fragments of a plant treated with soil drench of *C. cateniannulata* (white mycelium visible). (I) Leaf fragments of a plant treated with soil drench of *C. cateniannulata* (white mycelium visible in all three tissues).

3.4 Inhibition of *Hemileia vastatrix* and its mycoparasitism by *Cordyceps cateniannulata*

Rates of urediniospore germination were homogenous in the three experiments, allowing for combination of the data (Levene's test, P = 0.755; Table 1).

Germination of *H. vastatrix* urediniospores was reduced when exposed to a conidial suspension of COAD 3349 or to a culture filtrate when compared with the controls (Table 2; Fig. 4A, B, and D; Tukey test $P < 0.05$). Moreover, germ-tubes of most of the urediniospores that germinated after exposure to COAD 3349 were shorter and swollen when compared with the controls (Fig. 4C, E). It is not known, however, if their infectivity was affected as a result of these abnormalities.

The pustules of *H. vastatrix* on the control coffee plants (sprayed with Tween solution only) developed normally with no evidence of fungal mycelium, as observed under a stereomicroscope (Fig. 4F) and a SEM (Fig. 4H). Isolations from the uredinia did not yield any colonies of *C. cateniannulata*. Conversely, the pustules on plants sprayed with a conidial suspension of COAD 3349 were overgrown by white colonies of *C. cateniannulata* (Fig. 4G). Mycelium from these colonies was mounted under a light microscopic and a SEM, and the conidiophores and conidia were readily identified as typical of *C. cateniannulata*. Under the SEM, hyphae were observed colonizing the uredinia (Fig. 4I), leading to degradation and erosion of the pustules (Fig. 4J); as well as of the urediniospores (Fig. 4K), with evidence of spore penetration by *C. cateniannulata* hyphae (Fig. 4K).

Table 1. Effect of conidia or fungal filtrate of *Cordyceps cateniannulata* COAD 3349 on uredospores germination of *Hemileia vastatrix*.

Treatment	Uredospore germination (%)[1 2]

Conidia	44.44b (± 1.16)
Fungal Filtrate	43.28b (± 1.67)
Control	70.83a (± 1.82)
CV (%)	27.27

[1]Mean value (± standard error).
[2]Mean values followed by the same letter do not differ significantly according to Tukey's test ($P < 0.05$).

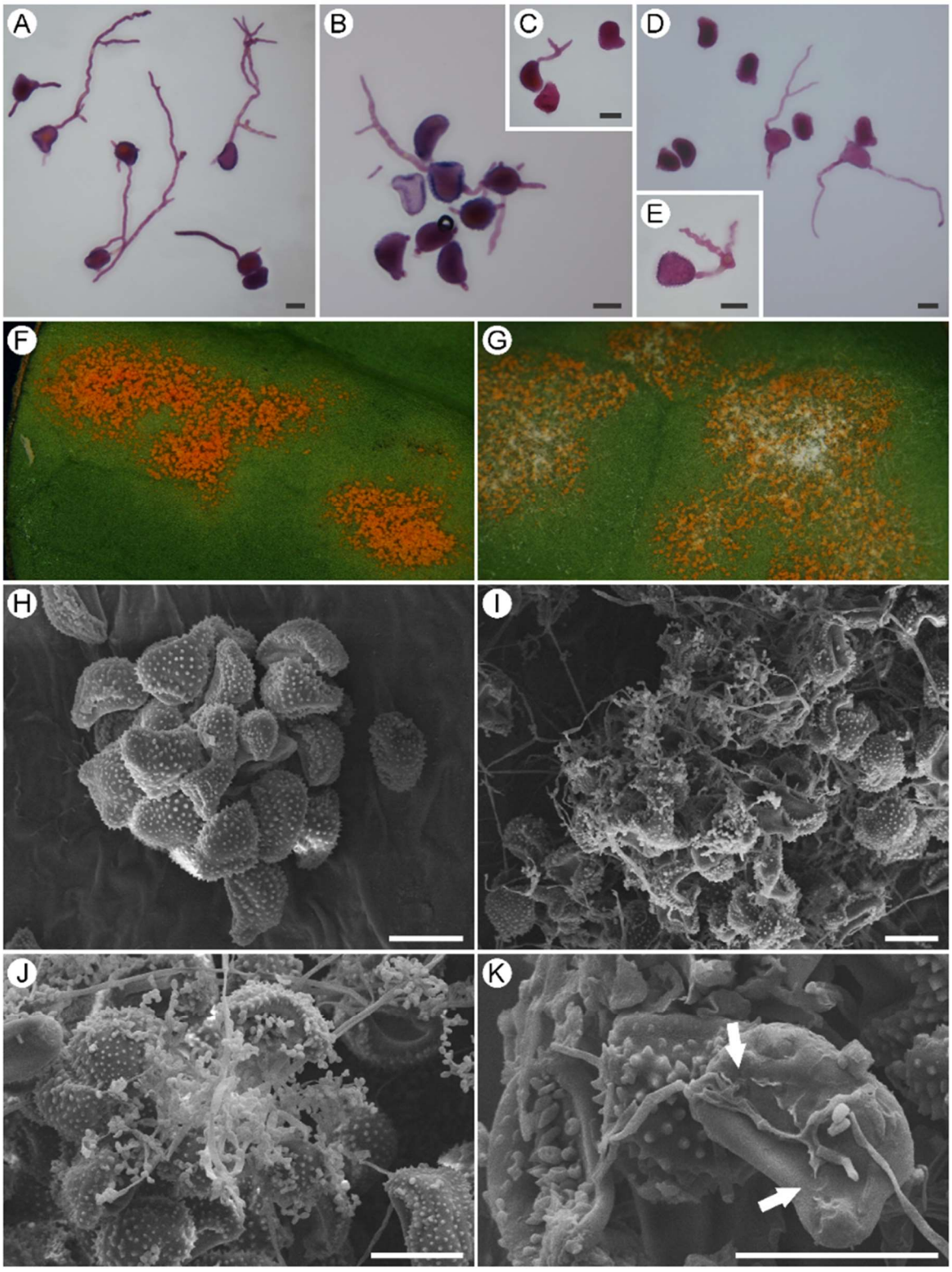

Figure 4. Evidence of the effects of *Cordyceps cateniannulata* COAD 3349 against *Hemileia vastatrix* in *in vitro* and *in planta* assays. Shown are: (A) Germinating urediniospores of *H. vastatrix* suspended in potato dextrose (PD) broth and incubated for 6h. (B) Urediniospores of *H. vastatrix* treated with COAD 3349 conidia suspension and incubated for 6h, (C) note morphological alterations in *H. vastatrix* germ tube. (D) Urediniospores of *H. vastatrix* treated with COAD 3349 fungal filtrate and incubated for 6h, also with (E) morphological alterations in the pathogen germ tube. (**F**) Coffee leaf bearing *H. vastatrix* uredinia and treated with tween solution. (G) Coffee leaf bearing *H. vastatrix* uredinia and

treated with COAD 3349 conidia suspension. Scanning electron microscopy micrographs of (H) a healthy urenidia of *H. vastatrix* and (I, J) uredinia of *H. vastatrix* covered by COAD 3349 mycelia. (K) Urediniospores of *H. vastatrix* overgrown by COAD 3349, arrows indicate possible sites of penetration or exit of COAD 3349 mycelium from the urediniospores. Bars: (A, B, C, D, E, H, I, J, K) = 20 μm.

3.5. *Cordyceps cateniannulata* as an insect pathogen

The coffee pests CBB and CLM, as well as mealworms, were all shown to be susceptible to COAD 3349 infection and their survival rates decreased under laboratory conditions (Figs. 5A, 5C, 6A), as detailed below.

Mealworm – COAD 3349 killed all mealworms after 4 d, with a LT_{50} after 3 d (CI95 % = 0.0528-0.426), compared with 100 % survival of the controls after 10 d (n = 20; log-rank test, $\chi^2 = 43.8$, df = 1, $P < 0.0001$; Fig. 5A), which completed their life cycle to adulthood (Fig. 5C). The sporulation rate on *C. cateniannulata*-killed individuals was 85 % (Fig. 5D-F) and zero in the controls.

Coffee berry borer – Adult female CBBs exposed to COAD 3349 were all killed after 10 d, with a LT_{50} after 5 d (CI 95 % = 0.0948-0.333). While there was some mortality in the controls (note that these were field-collected so age was not controlled), this did not reach 20 % and was significantly lower than that of infected insects (n = 45, log-rank test, $\chi^2 = 69.3$, df = 1, $P < 0.0001$; Fig. 5B). The rate of sporulation on *C. cateniannulata*-killed individuals was 98 % (Fig 5I). Under the SEM, it was possible to observe colonization of COAD 3349 on CBB cadavers (Fig. 5I-M). In contrast, the controls showed no fungal growth (Fig. 5G-H).

Coffee leaf miner – Mortality of CLM pupae exposed to COAD 3349 was higher as compared to controls (n = 38, $\chi^2_{[1, 22]} = 21.080$, $P < 0.0001$; Fig. 6A). Under the SEM, fungal hyphae were observed penetrating the pupae (Fig. 6J-M), with no fungal growth on the controls (Fig. 6D-E). Nine of the fungal-treated pupae reached maturity but, subsequently, the adults died and there was a significant difference between the control treatment ($F_{[1, 13]} = 45.068$, $P < 0.0001$; Fig. 6A). Under the SEM, fungal colonization of the adults was observed (Fig. 6N-Q). In contrast, control adults showed no fungal growth (Fig. 6F-G), and only three adults died as compared to nine exposed to the fungus. COAD 3349 colonized and sporulated on the larvae (Fig. 6H-I) whilst the controls remained free of infection (Fig. 6B-C). Typical *C. cateniannulata* colonies were isolated from all the inoculated insects.

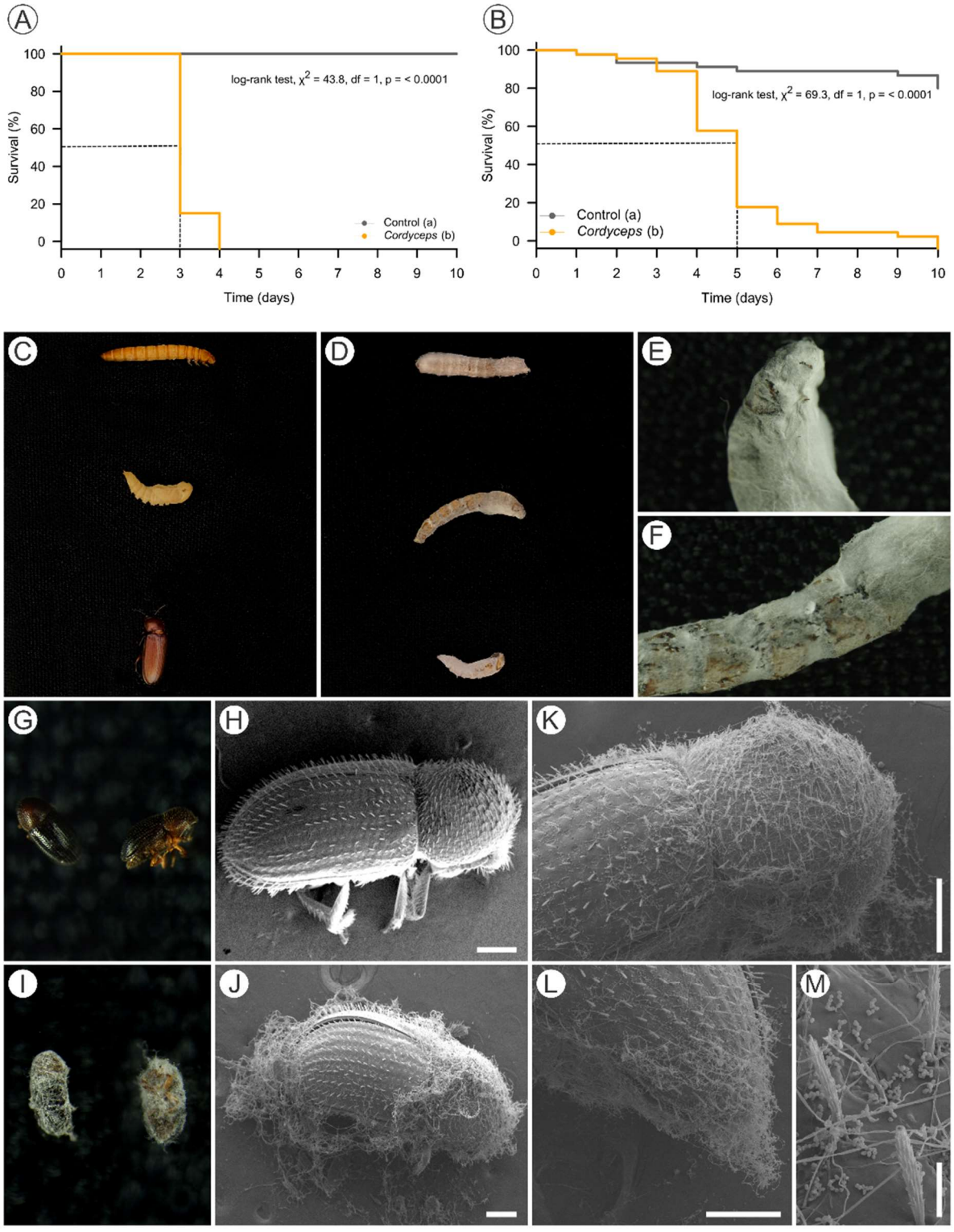

Figure 5. Larvae of *Tenebrio molitor* and adult females of *Hypothenemus hampei* exposed to *Cordyceps cateniannulata* COAD 3349 under laboratory conditions. (A) Survival curves for *Tenebrio molitor* larvae exposed to *C. cateniannulata* and blank control. (B) Survival curves for *H. hampei* adult females exposed to *C. cateniannulata* and blank control. (C) *Tenebrio molitor* larvae from control. Note that the individuals completed their life cycle to adulthood; (D) *Tenebrio molitor* larvae exposed to *C. cateniannulata.* Note that individuals are colonized by the fungus. Note the abundant colonization of *C. cateniannulata* (E) in the head and (F) body of *T. molitor.* (G) Female adults of *H. hampei* exposed to

blank control and (H) the same individual under SEM. (I) Female adults of *H. hampei* exposed to *C. cateniannulata.* (J) *C. cateniannulata* colonizing the body of the *H. hampei.* Note the fungus colonization (K) on the head, (L) the final part of the abdomen and (M) the presence of conidia and mycelium on the surface of the body of *H. hampei*. Different lower-case letters (A and B) indicate significant differences by the Bonferroni method ($p < 0.05$). Bars: (H, K, J, L) = 200 µm and (M) = 20 µm.

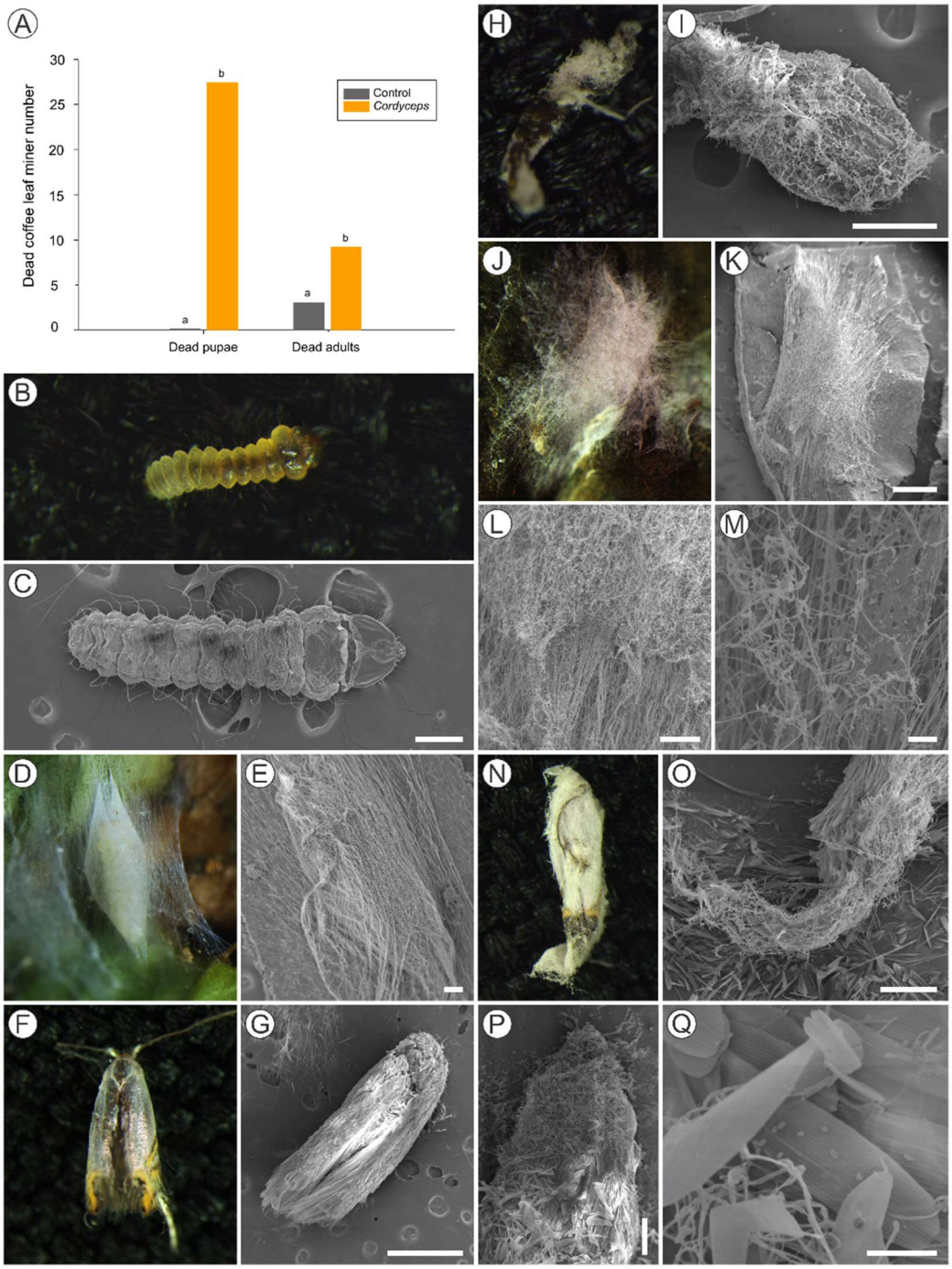

Figure 6. Larvae, pupae and adults of *Leucoptera coffeella* exposed to *Cordyceps cateniannulata* COAD 3349 under laboratory conditions. (A) Number of pupae and adults dead after exposure of *C. cateniannulata* and blank control. (B) Larva from control and (C) the same individual under SEM. (D)

Pupa from control and (E) the same individual under SEM. (F) Adult from control and (G) the same individual under SEM. Note that the larva, pupa and adult from control showed no colonization by *C. cateniannulata.* (H) *Cordyceps cateniannulata* colonizing the body of larva and (I) the hand. (J) *Cordyceps cateniannulata* colonizing the pupa. (K) Pupa colonized by the fungus seen in SEM. (L) *Cordyceps cateniannulata* colonizing among the silk threads and (M) presence of conidia and mycelium among the silk threads and in the pupa. (N) *Cordyceps cateniannulata* colonizing the body of adult and (M) the final part of the abdomen. (O) Presence of mycelium and (P) conidia on the wings of the adult. Different lower-case letters indicate significant differences by F tests ($p < 0.05$). Bars: (G, K) = 1 mm, (C, E, I, L, O, P) = 200 μm and (M, Q) = 20 μm.

4. Discussion

The results of this study have clarified the identity of the fungus isolated as an endophyte from coffee in Cameroon and, initially, considered to be a species of *Lecanicillium*. Using a polyphasic approach**,** this isolate has now been identified as a strain of *Cordyceps cateniannulata.* This strain (COAD 3349) produces distinctive vegetative propagules, or gemmae, not reported in previous descriptions of the fungus (Liang 1981a, b; Shimazu, 2001). Although this species has a wide geographic range – occurring in Brazil, China, Colombia and Japan (Liang, 1981a, b; Shimazu, 2001; Lazo, 2012; Montes-Bazurto *et al*. 2020; Zhou *et al*. 2020; Domingues, 2021) – there are no previous records of *C. cateniannulata* from Africa or from coffee. This additional record confirms that it has a pantropical distribution with an eclectic range of hosts.

Although members of the genus *Cordyceps* are best known as obligate parasites of arthropods, some species – such as *C. fumosorosea* and *C. ninchukispora* – have also been shown to have the ability to grow as endophytes in various crops under experimental conditions (Chang *et al*., 2017; Dash *et al.*, 2018; Sun *et al*., 2020; Doherty *et al*., 2021). Nevertheless, there are no confirmed instances where *Cordyceps* has been isolated from coffee plant tissues as an endophyte, as in the case reported here. Other members of the Hypocreales have been isolated as endophytes from the genus *Coffea,* such as *Beauveria bassiana* and *Clonostachys rosea* (Vega *et al*., 2008), and there are also reports of species of *Trichoderma* isolated as endophytes of coffee in Africa (Mulaw *et al*., 2013; Rodríguez *et al*., 2021). Here, the ability of *C. cateniannulata* to establish and grow as an endophyte in coffee was confirmed experimentally, when it was demonstrated that colonization can be initiated through the roots with the fungus becoming distributed systemically through the plant, in a similar fashion to *Metarhizium* colonization of coffee roots reported by Franzin *et al*. (2022).

Similar methods of endophyte inoculation (foliar spraying on the adaxial surface of the leaf, drench soil and stem injection) were reported by Posada *et al.* (2007) for four isolates of *B. bassiana* in coffee plants. However, they recorded a low recovery of isolates via foliar and soil inoculations, whilst the injection method was the most successful, and they concluded that the coffee leaf is a poor route for fungal colonization due to the presence of inhibitory cuticular components and few stomata on the adaxial compared to the abaxial surface. The better recovery of *C. cateniannulata* from leaves in our study is probably because the inoculum was applied exclusively to the lower leaf surface. Further inoculation protocols and longer periods of endophyte-recovery sampling are needed to ascertain the commercial feasibility of using *C. cateniannulata* as a 'bodyguard' to improve the management of coffee pests, such as the berry borer and the leaf miner, as well as CLR.

There have been a number of studies investigating the potential of *C. cateniannulata* as a biocontrol agent of a range of arthropods pests, predominantly in China. For example, against: the pine mite, *Cenopalpus lineola* (Acari: Tenuipalpidae) (FengBo *et al.*, 2011); the diamondback or cabbage moth, *Plutella xylostella* (Lepidoptera: Plutellidae) (Cuijuan *et al.*, 2014); the two-spotted mite, *Tetranychus urticae* (Acari: Tetranychidae) (Zhang *et al.*, 2016) and the fall armyworm of maize, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Zhou *et al*., 2020). And, more recently, such investigations have been initiated in South America; targeting the oil-palm pest, *Stenoma impressella* (Lepidoptera: Elachistidae) in Colombia (Montes-Bazurto *et al.*, 2020) and the psyllid pest of Eucalyptus, *Glycaspis brimblecombei* (Hemiptera: Aphalaridae) in Brazil (Domingues, 2021). Although the results have been promising, there are no reports that the fungus has been developed commercially and there was no indication that it was perceived in these studies as an endophyte.

Here, we report for the first time, the ability of *C. cateniannulata* to infect and kill adult beetles of *H. hampei* (CBB), as well as the three life stages of *L. coffeella* (CLM). Previous laboratory and field studies using entomopathogenic fungi to control CBB infestations have included *B. bassiana* (Haraprasad *et al.*, 2001; Posada & Vega, 2005; Hollingsworth *et al.*, 2020; Bayman *et al.*, 2021) and *Metarhizium anisopliae* (Pava-Ripoll *et al.*, 2008; De La Rosa *et al.*, 1995; 2000). For the CLM, there seems to be only a single study on the use of *M. anisopliae* against the egg and larval stages (Villacorta, 1983), when it was shown that the larvae were killed by the fungus either inside the mines or when they left the mine to pupate. In our study, the CLM larvae were only infected when they left the mines to pupate and became exposed to COAD 3349 inoculum. A similar scenario was reported for *B. bassiana* on

Leucoptera malifoliella (pear leaf blister moth) when late instar larvae were infected only when leaving the mines to pupate (Draganova & Tomov, 1998). These findings show the difficulty of controlling the *L. coffeella* in the larval stages and this needs further investigation to find out how the fungus can infect the larvae inside the mine.

Previous studies have indicated that some entomopathogenic fungi also have the ability to affect plant pathogens, either by: direct antagonism; functioning as mycoparasites; or, inducing systemic resistance in the host plant (Ownley *et al.,* 2010; Jaber, 2015, 2017; Jaber & Ownley, 2018; Dara, 2019; Canassa *et al*. 2020). For example, *Beauveria bassiana* – a ubiquitous pathogen of *H. hampei* (Rehner *et al*. 2006) – can also protect coffee fruits from anthracnose and fruit rots caused by different species of *Colletotrichum* (Serrato-Diaz *et al*. 2020). However, the mechanism of protection was not elucidated and no evidence of mycoparasitism was provided. In contrast, Jaber (2015) concluded that a significant reduction in disease caused by the grape downy mildew, *Plasmopara viticola*, was due to the presence of an endophytic strain of *B. bassiana*, previously inoculated in the leaves, which led to induced host resistance. Jaber (2018) also used a commercial biocontrol product based on *B. bassiana*, as well another based on *Metarhizium brunneum*, to assess both their endophytic ability and their potential antagonism to plant pathogens. Inoculation of wheat seeds showed that both fungi were able to colonise wheat tissues; promoting plant growth as well as reducing crown and root rot disease caused by *Fusarium culmorum*. Our results with *C. cateniannulata* show that spore suspensions and filtrates can inhibit or distort the germination process of *Hemileia vastatrix* – historically, the most serious constraint to coffee production (Berkeley & Broome, 1869; Ward, 1882; McCook, 2006; Talhinhas *et al.* 2017), especially in the Americas (Avelino *et al*., 2015) – as well as colonising and destroying the urediniospores; showing evidence of both parasitism and antagonism.

Nikoh & Fukatsu (2000) discussed the issue of host-jumping in entomoparasites, with particular focus on fungi of the genus *Cordyceps*, as mirrored by their evolutionary history and concluded that "remarkable host jumping events usually occurred anciently in the history of the endoparasitic taxa, the status before and after the host jumping cannot be surely assigned based on the characters of extant organisms". *Cordyceps cateniannulata* has now shown us that such jumps are not lost in the geological past and may be representative of the lifestyle of more fungi in the Hypocreales. For example, an unusual example of host-jumping is that of *Harposporium bredonense* (Evans & Whitehead, 2005): a hypocrealean fungus found under tree bark on cerambycid beetle larvae that produces two morphologically distinct synnemata; one of which

is phototrophic, forming typical *Hirsutella*-like phialides and conidia on emerging from bark crevices, whilst the other remains under the bark and produces *Harposporium*-like phialides and conidia. Morphologically, the latter pertains to a nematophagous genus in which the distinctive crescent-shaped conidia lodge in the gut and infect the nematode host after ingestion. It was posited that these conidia are deposited between the inner bark and the outer wood; subsequently, infecting free-living nematodes feeding on the detritus, whilst the *Hirsutella* conidia are aerially-dispersed to new beetle hosts. It remains to be determined if the two distinctive spore types in *C. cateniannulata* also have different host targets.

The evolution of plant and animal pathogens and the origin of the grass endophytes from insect pathogens in the Clavicipitaceae were documented by multigene phylogenetic analysis (Spatafora *et al.*, 2007; Sung *et al*., 2007). Introgression was detected in certain lineages of these hypocrealean fungi, and the main functions of the genes located in the introgressed regions are involved in host recognition, transcriptional regulation, stress response and cell growth regulation. These results indicate that contraction of gene families and introgression might be the main mechanisms that drive lifestyle differentiation and the evolution and host shift of hypocrealean fungi (Zhang *et al.,* 2018). As an order, the evolution of Hypocreales is characterized by a shift in nutritional mode from plant-based nutrition to animal and fungal-based nutrition (Spatafora *et al*., 2007), with a minimum of 5–8 independent and unidirectional interkingdom host jumps; including 3–5 to fungi, 1–2 to animals, and 1 to plants. Later, Kepler *et al.* (2012) concluded that co-occurrence in the same habitat allowed for these host shifts from animals to plants, and from plants to fungi.

The finding of such an extraordinary and expanded host range in *C. cateniannulata* poses many questions. How did it overcome the defences of such an ultra-diverse range of hosts? What are the features that allow it to colonize and obtain nutrients from plants, insects and fungi? What are the metabolites it produces in order to complete its cycle on its hosts? We know from the work of Behie *et al.* (2012, 2017) on insect-pathogenic endophytic fungi, that some species – especially, in the genus *Metarhizium* – are involved in tripartite nutrient transfer. Using radioactive isotopes, these authors showed that the fungus receives carbon from the plant partner and transfers nitrogen from its insect host to the plant roots. *Cordyceps cateniannulata* could be involved in a similar but even more complex four-way transfer. Branine *et al.* (2019) reviewed the biology of the so-called "endophytic insect-pathogenic fungi (EIPF)" in the context of the mutualistic associations with the plant host and their potential use in agriculture for controlling plant pests. They concluded that: "the study of EIPF systems has great potential

to elucidate fundamental questions on the ecology and evolution of multispecies interactions and to provide solutions to agricultural and medical problems. Subsequently, Quesada-Moraga *et al.* (2020, 2022) endorsed this potential of EIPF in agriculture; emphasizing, in particular, the importance of their endophytic lifestyle and rhizosphere competence, as well as the need to better understand their multi-trophic interactions.

Jaber & Ownley (2018) broadened the agricultural application of EIPF when they posed the question: "Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens?". After reviewing the evidence – mainly, relating to the hypocrealean genera *Beauveria* and *Lecanicillium*, as well as noting their ability to promote plant growth and to induce host resistance – they concluded that, although the results show promise, significant problems remain to be resolved before their true potential can be realised. In particular, they noted inconsistent plant colonisation, especially under field conditions, and the need to fully elucidate their modes of action. At present, these shortfalls also apply to *C. cateniannulata* – especially, as experiments are yet to be conducted to ascertain if the COAD 3349 strain can also stimulate plant growth and induce host resistance – but the potential is immense, and research should now be directed to resolving these issues: most notably, to help improve the management of coffee pests and diseases.

Coffee is one of the most important crop products in the global economy, representing a sector that trades in billions of dollars annually (Davis *et al.*, 2020; Salcedo-Sarmiento *et al.*, 2021). However, coffee production is subject to a range of debilitating pests and diseases with the major constraint worldwide being CLR (McCook, 2006; Avelino *et al*., 2015; Talhinhas *et al*., 2017). Nevertheless, the insect pests CBB and CLM can also cause significant losses (Green, 1984; De la Rosa *et al.*, 2000; Rehner *et al.*, 2006; Hollingsworth *et al.*, 2020; Bayman *et al.*, 2021). The limitations of current CLR management strategies imposed by climate change, breakdown of resistance in cultivated varieties and objections to, or the economic impracticability of chemical control, have stimulated interest in alternative and more innovative control methods (Avelino *et al.*, 2015; Zambolim, 2016). The question remains: can *C. cateniannulata* be integrated into any future pest-management strategy for coffee?

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CHAPTER II

Article

Cordyceps cateniannulata performance as a biocontrol agent of coffee berry borer (*Hypothenemus hampei*) under laboratory and field cage-test conditions

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Abstract

Coffee berry borer (CBB) – *Hypothenemus hampei* – is broadly recognized as one of the worst pests of coffee. There are numerous publications dealing with the use of two species of entomopathogenic fungi as biocontrollers of CBB: *Beauveria bassiana* and *Metarhizium anisopliae*. Here, we add a novel fungus to this list: *Cordyceps cateniannulata.* The pathogenicity of *C. cateniannulata* to CBB was recently demonstrated. Here we: (i) further assessed its virulence against *H. hampei*; (ii) compared the virulence of *C. cateniannulata* and *B. bassiana* against *H. hampei* under laboratory conditions; and (iii) assessed the effect of applications of *C. cateniannulata* on fruit-bearing coffee branches artificially infested with CBB under field cage-test (FCT) conditions. *Cordyceps cateniannulata* caused a significant concentration-dependent increase in insect mortality. No difference was found between the virulence of *C. cateniannulata* and *B. bassiana* to *H. hampei* in this essay. Under this preliminary FCT study, *C. cateniannulata* application resulted in a small but significant reduction in insect survival but it did not reduce the number of fruits bored by CBB. Under the conditions of this assay, and without the benefit of any adjuvant or special formulation, *C. cateniannulata* was not capable of protecting the fruits fast enough to avoid boring and establishment. Similar results were observed for *Beauveria,* where this fungus reduces CBB survival, but fruit protection is also low. As our results are still preliminary, but equivalent to

those reached with *Beauveria*, they seem to indicate *C. cateniannulata* also has potential as a biological agent against CBB.

Key words: Biological control; coffee pest; entomopathogenic fungus; virulence.

1. Introduction

Coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae) (CBB) is among the worst coffee pests worldwide (Aristizábal et al., 2016, 2017; Greco et al., 2018). The adult female builds a gallery inside the fruits for feeding and reproduction (Damon, 2000; Jaramillo et. al., 2009), causing qualitative and quantitative losses to production. Management of this insect is carried out mainly using chemical insecticides (Jaramillo et al., 2011). However, endosulfan – the insecticide most commonly used to control *H. hampei* – has been banned in many countries due to its high toxicity (Brun et al., 1989; Damon, 2000). There are several insecticidal chemicals that have replace endosulfan; however, there is still a need for new low-toxicity and environmentally friendly strategies for the management of *H. hampei*, including biological control. *Beauveria bassiana* has been used for the development of commercial bioinsecticides (Faria & Wraight, 2007) and some products based on this species are already registered for use in Brazil to control *H. hampei* (e.g., Bovemax EC®, Boveril®, Ballvéria®) (MAPA 2023).

The hypocrealean fungal entomopathogens *Beauveria bassiana, Cordyceps fumosorosea* (=*Isaria fumosorosea*) and *Metarhizium anisopliae* are used to control a range of insect pests globally (Faria & Wraight 2007). Two of these fungi have been studied as biocontrol agents for CBB: *B. bassiana* (Haraprasad et al., 2001; Posada & Vega, 2005; Hollingsworth et al., 2020; Bayman et al., 2021) and *M. anisopliae* (De La Rosa et al., 1995; 2000; Pava-Ripoll et al., 2008). Additionally, *C. fumosorosea* has been also reported infecting *H. hampei* in the field (Vega et. al., 1999).

Considering the significant losses caused by CBB to coffee production and that most attention has been given to a single entomopathogenic fungal species (*B. bassiana*) it is strategic to broaden the range of fungal tools to tackle this pest. *Cordyceps cateniannulata* was isolated from healthy coffee tissues in Cameroon during a survey for fungi antagonistic to coffee leaf rust – *Hemileia vastatrix* (Pucciniales), the worst coffee pathogen worldwide (McCook, 2006; Talhinhas et. al., 2017). Preliminary tests (published separately) have shown that *C. cateniannulata* is a mycoparasite of *H. vastatrix* (Pereira et. al., 2022 *chapter 1*.). As the genus

Cordyceps (Cordycipitaceae: Hypocreales) is well known as including species that are pathogens of arthropods (Zhang et. al., 2016; Ou et. al., 2019; Montes-Bazurto et. al., 2020; Zhou et al., 2020; Avery et. al., 2021; Domingues, 2021; Wang et. al., 2021) it was hypothesized that *C. cateniannulata* could be pathogenic to two of the principal pests of coffee: CBB (Baker, 1984) and coffee leaf miner *Leucoptera coffeella* (Green, 1984). This was shown to hold true in a recent study (Pereira et. al., *chapter 1*).

Cordyceps belongs to the family Clavicipitaceae, which comprises more than 400 species (Sung et al., 2007) with great diversity in terms of niche adaptation, which includes the parasitism of a wide range of insects (Luangsa-Ard et al., 2005). *Cordyceps cateniannulata* has been a potential biocontrol agent to be deployed against arthropods pests, for example: *Spodoptera frugiperda* in China (Zhou *et al*. 2020) and against *Glycaspis brimblecombei* in Brazil (Domingues, 2021), among others (Zhang *et al.* 2015; Ou *et al.* 2019; Montes-Bazurto *et al.* 2020), but so far there is no evidence of this species as a biocontrol agent for CBB and CLM.

Thus, in the present study, we provide a report on the potential of *C. cateniannulata* as novel entomopathogenic fungus to be deployed for the control of CBB. We first assessed the virulence of *C. cateniannulata* under a range of inoculum concentrations against *H. hampei* in the laboratory, in order to determine the lethal time of 50% of individuals exposed to *C. cateniannulata*. We then compared the virulence of *C. cateniannulata* and *B. bassiana* against *H. hampei*. Since *B. bassiana* has been broadly used for decades against CBB and is a well-established agent which became widely available as the active ingredient of several commercial mycoinsecticides (Faria & Wraight 2007; Monzón et. al., 2008; Gallardo et al., 2010; Mascarin & Jaronski 2016; Wraight et al., 2018), we the found that it would be worthy running a comparison between *B. bassiana* and *C. cateniannulata*. Finally, we assessed *C. cateniannulata* applications on fruit-bearing coffee branches exposed to *H. hampei* under filed cage-test conditions.

2. Material and Methods

2.1. Fungal isolates and preparation of conidial suspensions

Two entomopathogenic fungi, *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528, were used in this study. COAD 3349 and COAD 3528 were used for the laboratory

virulence test, while only COAD 3349 was used for the field cage-test. COAD 3349 was obtained from healthy *Coffee arabica* stems in Cameroon, Africa (Pereira et. al., *chapter 1*). COAD 3528 was obtained from coffee soil in Araponga, MG, Brazil. These isolates are kept in storage in silica gel and in 10% glycerol maintained at -80 °C both in the culture collection of UFV: Coleção Octavio de Almeida Drummond (COAD) of the Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil.

For use in experiments, the isolates in storage were inoculated on fungi were passaged though *Tenebrio molitor* larvae for reactivation then cultivated in dark at 25±1 °C in plates containing potato dextrose-agar (PDA) for 15 days to allow for growth and sporulation. Conidia of each isolate were harvested from the plates and the suspension was used for seeding bags containing autoclaved white rice (100 g of rice autoclaved with 60 ml of tap water for 15 minutes at 1.0 atm pressure and 120 °C). The bags were kept in the dark at 25±1 °C for 10 days. Every two days of interval the contents of the bags was agitated and squeezed to allow for homogenous colonization of the substrate by the fungus. After this period, colonized rice grains were suspended in 45 ml of sterile 0.01% Tween 80® solution. Subsequently, each suspension was sieved through two layers of sterile cheesecloth to remove rice grains and hyphal fragments and then vortexed for 30 seconds. Conidial concentrations were determined using a haemocytometer and adjusted to desired levels (below) in sterile 0.01% Tween 80® solution. The conidial viability of each isolate was checked by transferring 100 µl of the suspension onto plates (9 cm diam) containing PDA + chloramphenicol (0.15 g l^{-1}) and counting germinated conidia after 24 h at 25±1 °C. The first 100 conidia observed under the 40x objective of the microscope were ranked as germinated (germ tubes longer or of the same length of the conidial diam) or non-germinated (absence of germ tube or shorter than conidial diam).

2.2. Insect sourcing/rearing

Mealworm was used in this study as a model insect (Souza et. al., 2015) in order to refine a methodology for inoculation of *C. cateniannulata* and for training the observation of the host-pathogen interaction. This insect was obtained from a stock kept at the "Laboratório de Interação Inseto-Microrganismo" (UFV), Viçosa, MG, Brazil. This rearing is maintained with wheat bran and carrots at 25±2 °C, 60 ± 10% RH and 12 h photoperiod. Larvae of similar sizes and weighing between 100 and 140 grams were used in this study, as a substitute to precise age/instar determination (notoriously difficult for mealworm) (Morales-Ramos et al., 2010).

Adult female *H. hampei* were collected in the field in the campus of UFV. Insects with completely melanized teguments were used (Silva et al., 2014). For CBB it is also difficult to standardize the exact age of adults taken from the field. Before virulence testing, all insects were surface disinfected to remove microorganisms present externally in their bodies. In order to achieve that, the insects were immersed in 5% sodium hypochlorite for 2 min, rinsed three times in sterile distilled water, 70% ethanol for 1 min, rinsed again three times in sterile distilled water and then left over a layer of dry sterile filter paper for 15 min (adapted from Bayman et al., 2021). As for the FCT, CBB individuals were not disinfested prior to use.

2.3. Experiment I: Virulence of *Cordyceps cateniannulata* against *Tenebrio molitor* and *Hypothenemus hampei*

The pathogenicity of *C. cateniannulata* COAD 3349 to *T. molitor* and *H. hampei* (i.e. its capacity to infect these insects and cause disease) was demonstrated in a previous study (Pereira et. al., *chapter 1*). The virulence of this isolate to two insects species at different inoculum concentrations was evaluated herein as described next.

- *Tenebrio molitor.* 200 µl aliquots of COAD 3349 conidial suspensions were pipetted onto each of the filter paper layer lining 6 cm diam plates, as described by Bayman et al. (2021). Conidial suspensions consisted of 5×10^3, 5×10^4, 5×10^5, 5×10^6, and 5×10^7 conidia ml^{-1} in 0.01% Tween-80® solution and Plates on which the layer of filter paper received 200 µl of sterile 0.01% Tween-80® solution served as controls. Plates were left without their leads for 10 min in order to allow the even distribution of the suspensions and then 20 larvae of mealworm were placed individually inside each of 20 plates and then left in the dark at 25±1 °C. After 48 h, the layer of filter paper covering each plate was removed and new layers moistened with sterile water were placed lining each plate. This was done to avoid repeated infections in plates that received COAD 3359. Plates were examined daily and larval mortality was recorded for 14 days. The insect mortality was confirmed by touching it with tweezers and when it did not respond with any movement it was considered dead. Dead larvae were placed in a humid chamber under incubation conditions and observed for evidence of colonization by *C. cateniannulata*. This bioassay was conducted in a completely randomized design (CRD) with 20 replicates of each treatment.

- *Hypothenemus hampei.* Procedures were similar to this described for mealworm above but inoculation involved pipetting a smaller volume of conidial suspension (100 µl of conidial suspension per plate). Inoculum was calibrated to 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 in 0.01% Tween-80® solution and Plates on which the layer of filter paper received 100 µl of sterile 0.01% Tween-80® solution served as controls. Mortality was recorded daily for 10 days. The insect mortality was confirmed by touching it with tweezers and when it did not respond with any movement it was considered dead. Additionally, dead insects were placed on PDA supplemented with 0.15 g l^{-1} chloramphenicol instead of leaving them in the plates as for mealworm. This was because of the small size of CBB making it difficult to observe more subtle fungal sporulation. These were incubated in the dark at 25±1 °C. This bioassay was conducted in a CRD with 20 replicates of each treatment.

2.4. Experiment II: Comparison between virulence of *Cordyceps cateniannulata* and *Beauveria bassiana* to *Hypothenemus hampei*

Our isolate of *C. cateniannulata* COAD 3349 and an isolate of *B. bassiana* COAD 3528 available in our lab and originally obtained from coffee soil in Araponga, MG, Brazil were tested against *H. hampei*. Since *B. bassiana* is known to naturally infect *H. hampei*, whereas *C. cateniannulata* was never reported as found naturally infecting CBB, a comparison between the virulence of these two isolates was then conducted. Surface-disinfected insects (see description of procedure above) were exposed to these two isolates by pipetting 100 µl of conidial suspension of either COAD 3349 or COAD 3528. Inoculum concentration was calibrated at 1×10^3, 1×10^5 and 1×10^7 conidia ml^{-1} in 0.01% Tween-80® solution onto each of the filter paper layer lining plates (12×49 mm). The insects were exposed individually to the fungi as described above. This bioassay was conducted in a CRD with ca. 25 replicates of each treatment.

2.5. Experiment III: Field cage-test of *Cordyceps cateniannulata* against *Hypothenemus hampei*

The FCT experiment was carried out to determine whether the application of COAD 3349 on coffee branches had an impact on CBB and its feeding damage. This experiment was conducted in April-May 2022 (T_{max}: 29.5; T_{min}: 11.6; mean relative humidity: 81.78%; and

mean rainfall: 0.18 mm; Inmet 2022; Table 1). It consisted of applications of *C. cateniannulata* followed by the release of adult females 24 h after the last application

Table 1. Environmental variables of the place where the field cage-test was conducted. Maxima and minimum temperature, medium humidity, and rainfall are presented to according to the beginning until the end of the experiment.

Date	Max temp	Mim temp	Medium hum (%)	Medium Rainfall (mm)
25-Apr-22	27.7	18.3	80.8	0.0
26-Apr-22	28.4	15.8	83.2	0.0
27-Apr-22	28.2	16.5	78.1	0.0
28-Apr-22	28.3	14.2	79.5	0.2
29-Apr-22	29.4	13.8	81.3	0.2
30-Apr-22	28.3	16.5	78.7	0.0
1-May-22	27.8	14.8	79.2	0.2
2-May-22	28.6	14.4	81.2	0.0
3-May-22	28.5	14.1	76.3	0.0
4-May-22	29.5	15.5	73.0	0.0
5-May-22	24.0	18.8	93.5	0.0
6-May-22	25.2	16.3	86.4	2.4
7-May-22	23.7	17.5	87.0	0.0
8-May-22	25.2	16.1	82.5	0.0
9-May-22	24.8	13.9	80.0	0.2
10-May-22	22.4	11.6	85.7	0.2
11-May-22	26.6	11.7	81.1	0.2
12-May-22	27.3	13.7	82.1	0.0
13-May-22	24.7	17.7	85.2	0.0
14-May-22	26.7	16.0	80.8	0.0

The experiment was conducted on adult arabica coffee plants (*Coffea arabica,* variety "Catuaí") at the "Clínica de Doenças de Plantas" (UFV), Viçosa, MG, Brazil. Six plants were selected among a group of unmanaged *ca*. 16 years-old, 3m tall plants. Eight fruit-bearing branches were arbitrarily chosen for each plant, totalling 48 branches. Half of these were used as control with no application of COAD 3349 and half were treated with this isolate (Fig. 1A). As some variation of the exposure to sunlight between plants was noted, each plant had a control and a fungus-treated branch chosen. CBB-bored fruits were removed and, in addition more fruits were plucked in order to leave only 50 undamaged fruits on each branch, totalling 1,200 undamaged fruits per treatment. Subsequently, each branch was individually bagged with a gauze tube (cage) and its ends were tied to prevent the entry of other insects including *H. hampei*.

At the moment of application of *C. cateniannulata* on the branches, the cage was opened, and the fungal suspension was applied with a common hand sprayer (20 ml polyethylene terephthalate bottle with spray valve) until runoff. In order to avoid contamination of the control branches, these were covered with a plastic bag during the application of COAD 3349. Control-branches were treated with a spray until runoff of a sterile 0.01% Tween 80® solution. COAD 3349 applications were performed three times (at concentrations of 1×10^8, 1×10^8 and 1×10^8 conidia ml^{-1}) at four-day intervals. 960 adult females were selected from a sample collected in the field – insects with completely melanized teguments – were released within bags containing fungus-treated or untreated branches, 24 h after the last application of COAD 3349. Release was performed by placing the CLB individuals (20 per branch in 1.5 ml microtubes and tying the tube halfway along the bagged part of the branch), totalling 480 insects per treatment (Fig. 1B). These microtubes were then opened, for the insects to exit and start attacking the fruits, and the organza cages were closed (Fig. 1C).

Unexpected intense rainfall (2.4 mm) occurred on the day the insects were released. An additional, application of COAD 3349 suspension (1×10^8 conidia ml^{-1}) and of sterile Tween solution (on controls) was then undertaken six days after the release of the CBB individuals and 24 h after this application, another set of 20 insects was released within each organza cage. These procedures were carried out because a high mortality of CBBs (drowned inside the microtube) was observed and we suspect that the rain washed the conidia, as a suspension of conidia was used without the benefit of any adjuvant or a special formulation. However, on the day of the experiment evaluation, the number of CBBs drowned inside the microtube was counted to have the relation of the number of insects that were exposed to the experiment according to the new set of 20 insects that were released.

Insect mortality was assessed after 14 days. For this, all branches were removed with pruning shears and taken to the laboratory. The number of CBB-bored fruits and insects dead in the laboratory was counted. Fruits were opened individually with a knife, and dead insects, whenever found, were removed, placed in plates containing PDA supplemented with 0.15 g l^{-1} chloramphenicol and then incubated in the dark at 25±1 °C for 15 days, allowing for colonies to emerge from corpsed of *C. cateniannulata*-killed individuals. Corpses were not sterilized, so as to avoid a negative effect on fungal exteriorization and sporulation. Colonized and non-colonized individuals were repoted.

Additionally, in order to verify whether CBB individuals found alive inside the fruits might had come into contact with *C. cateniannulata*, two live adult females from each treatment

were arbitrarily chosen and placed each inside a separate 6 cm diam plates containing PDA supplemented with 0.15 g l^{-1} chloramphenicol on Petri dishes. The insects were allowed to wander over the surface of the medium for 15 days. These dishes were incubated in the dark at 25±1 °C for 15 days. The plates were observed daily, and the presence of *C. cateniannulata* colonies was recorded.

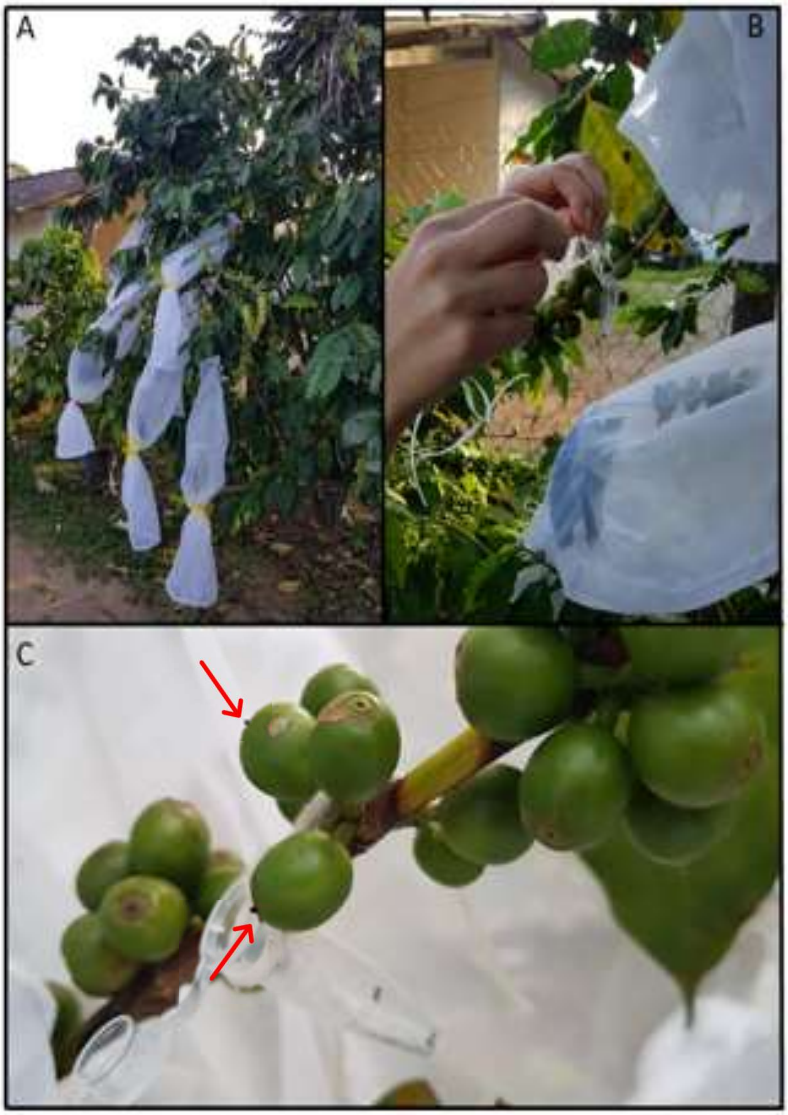

Figure 1. Field cage-test of treatment of coffee fruits with *Cordyceps cateniannulata* COAD 3349 and its effect on coffee berry borer (*Hypothenemus hampei*). (A) Branch covered with gauze tube (cage) with its ends tied to prevent the entry and exit of other insects including *H. hampei.* (B) Adult females of *H. hampei* released 24 h after the last application of *C. cateniannulata.* (C) Adult females (visible as back dots in the microtube and on the surface of fruits) starting the colonization process in the coffee fruit (red arrows).

2.6. Statistical analyses

All data analyses were performed using R (version 4.1.3) statistical software package (R Development Core Team, 2022).

Virulence tests in laboratory. Survival data were tested by survival analysis with the Kaplan-Meier estimator. The general similarity among the curves was tested with log-rank tests while pairwise comparisons were performed with the Bonferroni method ($p < 0.05$). Sporulation rate in dead insects was calculated through the percentage of individuals colonized by the COAD 3349 and COAD 3528, as shown below:

Percentage colonization = number of insects showing fungal growth / total number of dead insects × 100%.

Mortality of *H. hampei* in field cage-test. Mortality of *H. hampei* was analyzed through a Generalized Linear Model (GLM) with Binomial distribution and a Tools for General Maximum Likelihood Estimation (BBMLE) with the AICctab function was used to select the model based on the AICc criterion. The most suitable model based on the AICc criterion was the GLM with a Binomial distribution, and quasi-Binomial was applied to correct over-dispersion; the data were then compared with F tests ($p < 0.05$).

CBB-bored fruits in field cage-test. The number of CBB-bored fruits was also analyzed through a GLM with Poisson distribution and a Tools for General Maximum Likelihood Estimation (BBMLE) with the AICctab function was used to select the model based on the AICc criterion. The most suitable model based on the AICc criterion was the GLM with a Poisson distribution, and quasi-Poisson was applied to correct over-dispersion; the data were then compared with F tests ($p < 0.05$).

3. Results

3.1. Virulence of *Cordyceps cateniannulata* against *Tenebrio molitor* and *Hypothenemus hampei*

The survival of mealworm exposed to COAD 3349 differed among the treatments ($n = 20$, log-rank test, $\chi^2 = 73.5$, $df = 5$, $p = < 0.0001$; Fig. 3A). Individuals exposed to higher concentrations of the fungus (5×10^7, 5×10^6, 5×10^5 and 5×10^4 conidia ml^{-1}) exhibited higher mortality as compared to controls, whereas a low concentration (5×10^3 conidia ml^{-1}) caused low mortality of individuals and led to a level or mortality that did not differ from that obtained

for controls (Table 2). Groups exposed to concentrations of 5×10^7, 5×10^6, 5×10^4 and 5×10^4 conidia ml^{-1} reached 50% mortality on days 6.0, 7.0, 11.5, and 10.0 after inoculation, respectively (Table 2). In addition, we observed that all mealworm larvae presented an evident cuticular melanization after being exposed to COAD 3349 during the infection process (Fig. 2AB) whereas the mealworm individuals in control did not develop such cuticular melanization (Fig. 2CD).

Table 2. Mortality of *Tenebrio molitor* exposed to *Cordyceps cateniannulata* COAD 3349 at different conidial concentrations. Data shown are results of the Kaplan-Meier estimator and a posteriori pairwise comparisons of the Bonferroni method ($P < 0.05$).

Concentrations (conidia ml^{-1})	**P-value ($p < 0.05$) [1]**					**LT_{50} (d) [2]**	**Sporulation rate (%) [3]**
	Control	5×10^3	5×10^4	5×10^5	5×10^6		
5×10^3	0.1287	-	-	-	-	na	67
5×10^4	**< 0.0001**	0.1073	-	-	-	10.0	80
5×10^5	**0.0005**	0.7173	1.0000	-	-	11.5	83
5×10^6	**< 0.0001**	**0.0003**	0.4994	0.3195	-	7.0	89
5×10^7	**< 0.0001**	**< 0.0001**	**0.0016**	**0.0049**	1.0000	6.0	80

[1]p-values according to the Bonferroni method ($p < 0.05$).
[2]Lethal time of 50% of individuals killed after exposure to *C. cateniannulata* COAD 3349.
[3]Sporulation rate (percentage) of *C. cateniannulata* COAD 3349 in dead insects.
p-value in bold indicate significant differences by the Bonferroni method ($p < 0.05$).
* Read column table from left to right.

Figure 2. Appearance of mealworm (*Tenebrio molitor*) larvae exposed to *Cordyceps cateniannulata* COAD 3349 and control. (A) Mealworm larva three days after exposure to *C. cateniannulata.* Note cuticular melanization at ventral part of the body. (B) Mealworm larva four days after exposure to *C. cateniannulata.* Note general cuticular melanization. (C) Mealworm larva of control, three days after experiment started. Note no sign of cuticular melanization. (D) Mealworm pupa of control, four days after experiment started. Note absence of cuticular melanization.

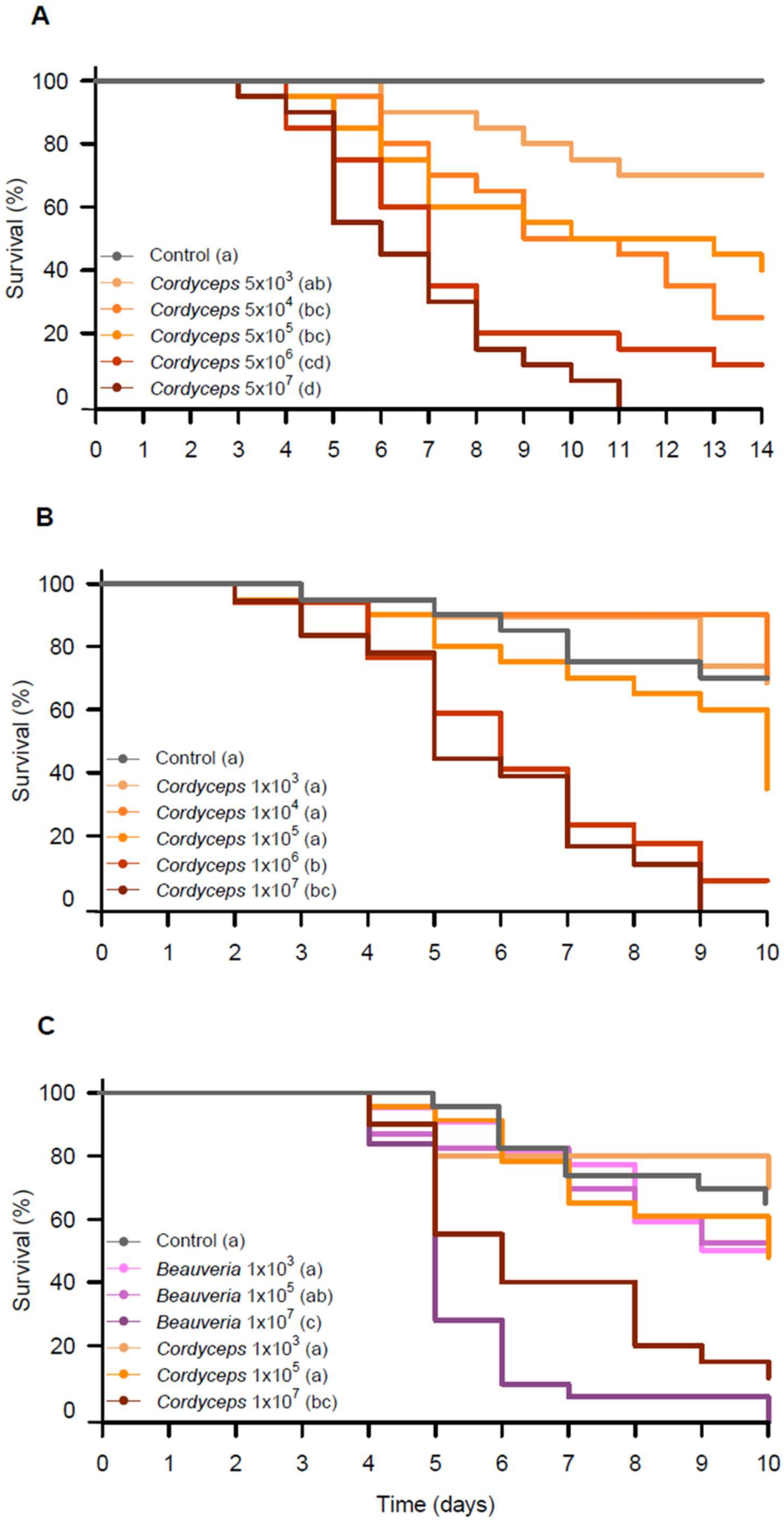
A
Survival (%)
Control (a)
Cordyceps 5x10^3 (ab)
Cordyceps 5x10^4 (bc)
Cordyceps 5x10^5 (bc)
Cordyceps 5x10^6 (cd)
Cordyceps 5x10^7 (d)
B
Survival (%)
Control (a)
Cordyceps 1x10^3 (a)
Cordyceps 1x10^4 (a)
Cordyceps 1x10^5 (a)
Cordyceps 1x10^6 (b)
Cordyceps 1x10^7 (bc)
C
Survival (%)
Control (a)
Beauveria 1x10^3 (a)
Beauveria 1x10^5 (ab)
Beauveria 1x10^7 (c)
Cordyceps 1x10^3 (a)
Cordyceps 1x10^5 (a)
Cordyceps 1x10^7 (bc)
Time (days)

Figure 3. Survival curves for *Tenebrio molitor* and *Hypothenemus hampei* exposed to increasing concentrations of *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528 and blank control. (A) *Tenebrio molitor* exposed to COAD 3349, (B) *H. hampei* exposed to COAD 3349 and (C) *H. hampei* exposed to COAD 3349 and COAD 3528. Different lower-case letters indicate significant differences by the Bonferroni method ($p < 0.05$).

The survival rate of adult female *H. hampei* exposed to COAD 3349 were significantly different between treatments (n = 20, log-rank test, $\chi^2 = 66.5$, df = 5, p = < 0.0001; Fig. 3B). Individuals exposed to the fungus at high concentrations (1×10^7 and 1×10^6 conidia ml^{-1}) exhibited a higher mortality as compared to controls, whereas at lower inoculum concentrations (1×10^5, 1×10^4 and 1×10^3 conidia ml^{-1}) a much lower mortality was observed and survival rates of COAD 3349 treated individuals did not differ statistically from controls (Table 3). Groups exposed to concentrations of 1×10^7, 1×10^6 and 1×10^5 conidia ml^{-1} reached 50% mortality at 5.0, 6.0 and 10.0 days after inoculation, respectively (Table 3). In addition, six individuals in control died during the experiment. Two among those six dead individuals showed sporulation of *B. bassiana* after incubation.

Table 3. Mortality of *Hypothenemus hampei* exposed to *Cordyceps cateniannulata* COAD 3349 at different conidial concentrations. Data shown are results of the Kaplan-Meier estimator and a posteriori pairwise comparisons of the Bonferroni method ($P < 0.05$).

Concentrations (conidia ml^{-1})	**P-values ($p < 0.05$) [1]**					**LT_{50} (d)[2]**	**Sporulation rate (%) [3]**
	Control	1×10^3	1×10^4	1×10^5	1×10^6		
1×10^3	1.0000	-	-	-	-	na	17
1×10^4	1.0000	1.0000	-	-	-	na	17
1×10^5	0.8653	0.7393	0.2945	-	-	10.0	54
1×10^6	**0.0005**	**0.0001**	**< 0.0001**	**0.0274**	-	6.0	69
1×10^7	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0011**	1.0000	5.0	78

[1]p-values according to the Bonferroni method ($p < 0.05$).
[2]Lethal time of 50% of individuals killed after exposure to *C. cateniannulata* COAD 3349.
[3]Sporulation rate (percentage) of *C. cateniannulata* COAD 3349 in dead insects.
p-value in bold indicate significant differences by the Bonferroni method ($p < 0.05$).
* Read column table from left to right.

3.2. Comparison between virulence of *Cordyceps cateniannulata* and *Beauveria bassiana* to *Hypothenemus hampei*

Mortality of adult female *H. hampei* exposed to COAD 3349 and COAD 3528 exhibited a significant statistic difference between treatments (n = 25; log-rank test, $\chi^2 = 71.7$, df = 6, p = < 0.0001; Fig. 3C). Individuals exposed to each of the fungi at concentrations of 1×10^7 conidia ml^{-1} had a higher mortality as compared to controls, whereas at concentrations of 1×10^5 and 1×10^3 conidia ml^{-1} mortality was low and did not differ from controls (Table 4). No significant difference in the virulence was found between COAD 3349 and COAD 3528 when compared directly in the same assay (at three concentrations of 1×10^3, 1×10^5 and 1×10^7 conidia ml^{-1}) (Table 4). Groups exposed to COAD 3349 at concentrations of 1×10^7 and 1×10^5 conidia ml^{-1} reached 50% mortality on days 6.0 and 10.0, respectively (Table 3). Groups exposed to COAD 3528 at concentrations of 1×10^7, 1×10^5 and 1×10^3 conidia ml^{-1} reached 50% mortality on days 5.0, 10.0 and 9.0, respectively (Table 3).

Table 4. Mortality of *Hypothenemus hampei* exposed to *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528 at different conidia concentrations. Data shown are results of the Kaplan-Meier estimator and a posteriori pairwise comparisons of the Bonferroni method (P < 0.05).

Concentrations (conidia ml^{-1})	**P-value (p < 0.05) [1]**						**LT_{50} (d) [2]**	**Sporulation rate (%) [3]**
	Control	COAD 3528 1×10^3	COAD 3528 1×10^5	COAD 3528 1×10^7	COAD 3349 1×10^3	COAD 3349 1×10^5		
COAD 3528 1×10^3	1.0000	-	-	-	-	-	9.0	27
COAD 3528 1×10^5	1.0000	1.0000	-	-	-	-	10.0	75
COAD 3528 1×10^7	**< 0.0001**	**< 0.0001**	**< 0.0001**	-	-	-	5.0	88
COAD 3349 1×10^3	1.0000	1.0000	1.0000	**0.0005**	-	-	na	0
COAD 3349 1×10^5	1.0000	1.0000	1.0000	**< 0.0001**	1.0000	-	10	42
COAD 3349 1×10^7	**0.0011**	**0.0348**	0.0935	0.2851	**0.0488**	**0.0409**	6.0	83

[1]p-values according to the Bonferroni method (p < 0.05).

[2]Lethal time of 50% of individuals killed after exposure to *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528.

[3]Sporulation rate (percentage) of *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528 in dead insects.

p-value in bold indicate significant differences by the Bonferroni method (p < 0.05).

* Read column table from left to right.

3.3. Field cage-test of *Cordyceps cateniannulata* against *Hypothenemus hampei*

Cordyceps cateniannulata COAD 3349 applied to coffee branches reduced the survival of adult female CBB as compared to controls ($F_{[1, 46]} = 18.551$, p = < 0.0001). The percentage of mortality of insects exposed to COAD 3349 was 13.0% whereas percentage mortality in

control was 4.5%. Additionally, COAD 3349 was recovered from 27 CBBs out of the 55 CBBs killed, whereas individuals from control did not develop COAD 3349. Sporulation of COAD 3349 was observed at CBB entry holes (Fig. 4A) and on many dead CBB inside the fruit (Fig. 4BC) and, interestingly, it appeared as if the mycelium of the fungus emerging from CBB cadavers grew towards the entrance hole of the fruits (Fig. 4D). Of the 24 live insects taken from cages treated with COAD 3349 and placed on PDA, only four developed colonies of *C. cateniannulata* only marginally. The number of bored fruits was higher in the branches exposed to the fungus than in the controls ($F_{[1, 46]} = 12.801$, $p = 0.0307$).

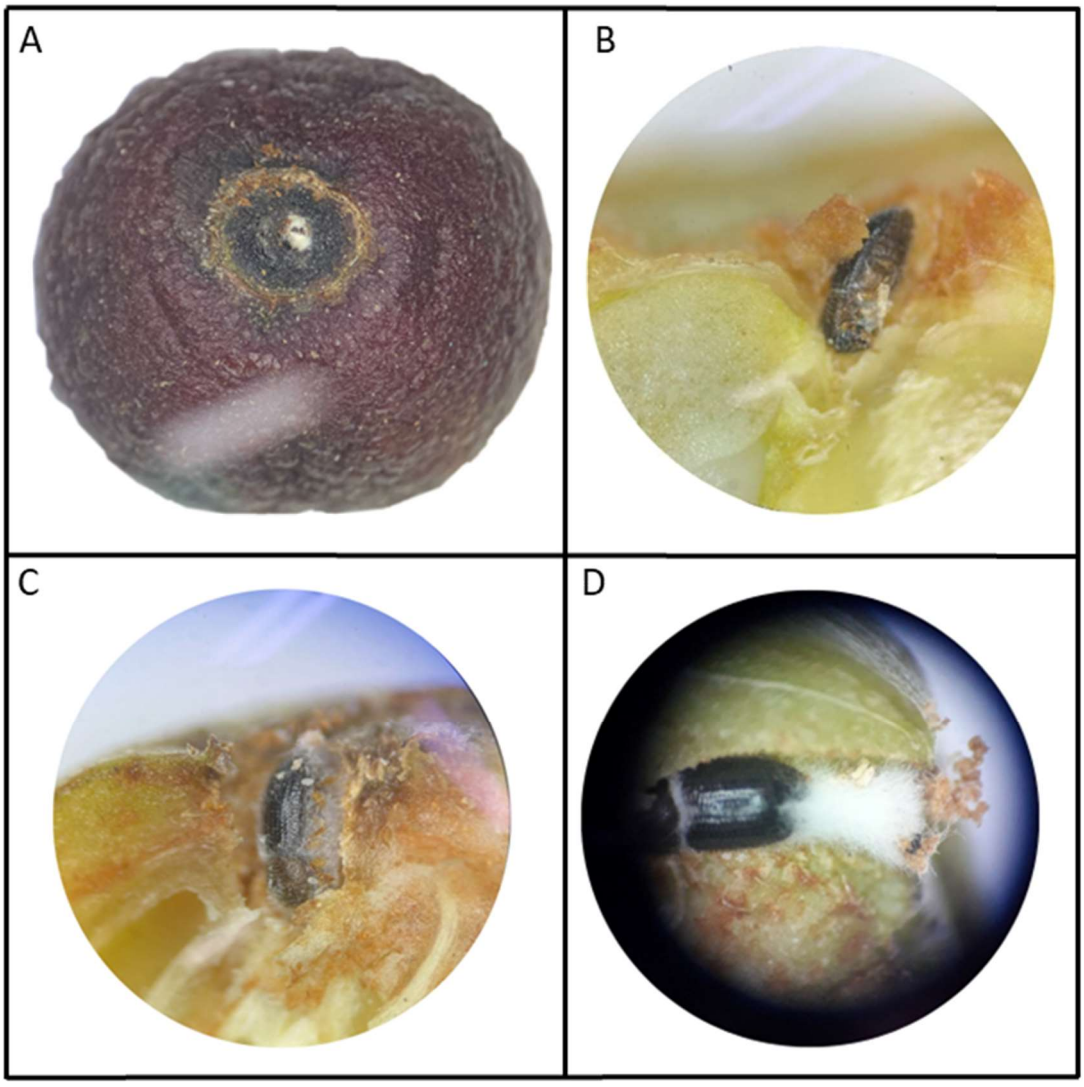

Figure 4. Interaction between *Cordyceps cateniannulata* COAD 3349 and adult female coffee berry borer (CBB) **(***Hypothenemus hampei*) individuals in Arabica coffee fruits. (A) Surface of coffee fruit showing an entrance hole and whitish sporulation of *C. cateniannulata*. (B) CBB individual killed inside the fruit. Note the presence of mycelium growing from ventral part of the body; (C) *C. cateniannulata* sporulating on a dead CBB individual inside fruit with mycelium emerging from the ventral part of the

body. (D) *C. cateniannulata* sporulating on a dead CBB individual. Note mycelium emerging from thorax and lower part of abdomen and growing towards the CBB entry hole.

4. Discussion

The pathogenicity of *C. cateniannulata* COAD 3349 to *H. hampei* and *Leucoptera coffeella* has been demonstrated previously, as has its systemic endophytic capacity in coffee plants (Pereira et. al., *chapter 1*). These findings stimulated us to evaluate the virulence of this fungus against *H. hampei* under laboratory and in a field cage-test. *Cordyceps cateniannulata* COAD 3349 was virulent to adult female *H. hampei* at the highest concentrations (1×10^7 and 1×10^6 conidia ml^{-1}), increasing CBB mortality, but not at lower concentrations (1×10^3, 1×10^4 and 1×10^5 conidia ml^{-1}). No significant difference was found in the virulence between *C. cateniannulata* and *B. bassiana* to *H. hampei* at three concentrations tested. Under field cage-test conditions, whereas COAD 3349 resulted in a small but significant reduction in insect survival, such reduction did not lead to a reduction in the number of fruits bored by these insects. In fact, the number of bored fruits significantly increased in COAD 3349-treated branches. Perhaps *C. cateniannulata* COAD 3349 does not protect plants quickly enough, but contributes to the reduction of the insect population.

Cordyceps cateniannulata COAD 3349 inoculations reduced *T. molitor* survival. Similar results were observed for the fungus *M. anisopliae* and *B. bassiana* against the same insect (Lestari & Rao 2017) and at different stages of insect life (Eski & Gezgin 2022). During fungal infection we observed that there was cuticular melanization throughout the body (Fig. 1AB). Such reaction in mealworm to infection by entomopathogenic fungi seems to be a novel observation, previously unreported in the literature. This melanization began to appear first at the ventral part of the larvae bodies (possibly at the points where the larvae come into contact with inoculum deposited on the filter paper) (Fig. 1A). Melanization then spreads throughout the rest of the body (Fig. 1b). It is known that melanization may be induced as a response to microbial invasion (Golkar et al., 1993; Ortiz-Urquiza & Keyhani, 2013; Vilcinskas, 2019). It may serve as a physical and toxic barrier against the advance of pathogenic microorganisms (Soderhall & Ajaxon, 1982; Stleger et. al., 1988; Hajek & Sleger, 1994). The accumulation of melanin at the penetration foci of the microorganism, may result in melanized dark spots around the fungal cells (Ortiz-Urquiza & Keyhani, 2013; Vilcinskas, 2019). However, such defence reaction may be insufficient to prevent the fungus from invading the host cuticle in compatible

host-pathogen interactions (Vilcinskas, 2019). This was the case for our mealworm *in vitro* test. Cuticular melanization induced as a response to *C. cateniannulata* COAD 3349 infection did not prevent disease development and insect death.

The survival of *H. hampei* exposed to *Cordyceps cateniannulata* COAD 3349 was also reduced. Similar results were observed for the fungus *M. anisopliae* against the same insect (Pava-Ripoll et al., 2008) and for the same fungal species against the mite *Tetranychus urticae* (Zhang et. al., 2016). These findings indicated that conidial concentration affects both mortality and lethal time, and that arthropods immune systems are able to respond and often repel fungal infection when inoculum pressure is low.

Beauveria bassiana was observed sporulating in a small portion of adult female *H. hampei* that were inoculated with *C. cateniannulata*. This is an expected result from the sourcing of insects used in the experiments from field areas where *B. bassiana* is known to occur naturally – and often seen sporulating at the entrance holes made by *H. hampei* (K.R.B. personal observation). The natural presence of *B. bassiana* infecting *H. hampei* has been broadly reported (Monzón et. al., 2008; Gallardo et al., 2010; Mascarin & Jaronski 2016; Wraight et al., 2018). *Beauveria bassiana* could already have been latently present in some of the CBB individuals. Note that in this test CBB were surface-sterilized prior to use. *Beauveria bassiana* is known to produce a variety of toxic metabolites (such as antimicrobial peptides) during the colonization process in a susceptible host (Quesada-Moraga & Vey 2003; Mascarin & Jaronski 2016). It is worth investigating if there is antibiosis at play between *B. bassiana* and *C. cateniannulata* (see chapter 3).

In our assay to comparing *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528 against *H. hampei*, we expected *B. bassiana* to perform better than *C. cateniannulata*, as *B. bassiana* isolates are found naturally infecting *H. hampei* (Monzón et. al., 2008; Gallardo et al., 2010; Mascarin & Jaronski 2016; Wraight et al., 2018). We found no difference, suggesting that *C. cateniannulata* and *B. bassiana* may have similar abilities to control *H. hampei*. In contrast, *B. bassiana* was more virulent to *H. hampei* than *M. anisopliae* under field conditions (De la Rosa et. al., 2000).

The field cage-test results are regarded here as only preliminary. These will need to be repeated and expanded to better elucidate the interaction and effect of application of *C. cateniannulata* on damage caused by *H. hampei* and its populations. COAD 3349 caused a limited, although statistically significant reduction in insect survival (13.0%). Application

strategies, formulation of inoculum with adequate adjuvants and other issues need to be addressed. The increase in the number of bored fruits in COAD 3349-treated branches was an unexpected outcome of the experiment. It requires an explanation. Perhaps the rapid process of fruit colonization *H. hampei* (boring of fruits observed only 50 min after CBB release in cages) (K.R.B. personal observation). In a field study, Paz and Leon (1972) observed that the penetration time of the drill in the fruit varies according to the stage of development of the fruit, for example in unripe fruits the time spent was 1 hour and for cherry fruits from 1 h 35 min to 2 h 35 min. This may have been accelerated even further by some form of recognition by CBB of the threat represented by the presence of inoculum of COAD 3349.

A temperature between 22 to 29° C and a relative humidity (RH) of 68 to 91% were reported to be ideal for the control of *T. urticae* with *C. cateniannulata* (referred to as *I. cateniannulata*) under field conditions (Zhang et. al., 2016). Conditions prevailing during our field-cage experiment were temperature ranging from 11.6 to 29.5° C and the mean RH from 81.7% throughout the experimental period (Table 1). It is known that the infectivity, development and survival of entomopathogenic fungi are greatly affected by temperature, RH, and solar radiation (Jaronski 2010). At the microclimatic level the situation may have been unfavourable for *C. cateniannulata* and favourable for CBB despite the seemingly overall adequate climatic conditions for the fungus. Impact to CBB population by inoculation with *C. cateniannulata* was minimal and 87.0% of exposed individuals were alive even 14 days after inoculation. Future field studies must be preceded by a closer investigation of the effect of environmental conditions over *C. cateniannulata.*

From the live *H. hampei* removed from the treatments and placed on agar, we observed that only four of these had contact with *C. cateniannulata*. At some point these insects came into contact with the fungus, but it did not impair the survival of the insects throughout the experimental period.

Most laboratory and field studies on biocontrol of CBB have focused on the use of the two best-known and most widely used entomopathogenic fungi (Lacey et. al., 2015): *B. bassiana* (Haraprasad et al., 2001; Posada & Vega, 2005; Hollingsworth et al., 2020; Bayman et al., 2021) and *M. anisopliae* (De La Rosa et al., 1995; 2000; Pava- Ripoll et al., 2008). There are numerous *B. bassiana*-based commercial products in Brazil (e.g., Bovemax EC®, Boveril®, Ballvéria®) (MAPA 2023) registered (and on-farm produced) being used for the biocontrol of CBB. Nevertheless, considering the extreme importance of CBB as a coffee pest, it is strategically important to expand the range of possibilities or even improve the effect of *B.*

bassiana products by adding new biocontrol agents to the list. In the present study, we preliminarily tested the potential of *C. cateniannulata* as an alternative for the control of *H. hampei*. This fungus caused high mortality of *H. hampei* under laboratory conditions but had a limited biocontrol effect in the field cage-test. However, further tests are required to better determine the potential of this newly reported CBB entomopathogen as a biocontrol option against this target.

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CHAPTER III

Article

Potential of the fungus *Cordyceps cateniannulata* as a biocontrol agent for the coffee leaf miner *Leucoptera coffeella* under greenhouse conditions

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Abstract

Coffee leaf miner (CLM) – *Leucoptera coffeella* – is broadly recognized as one of the worst pests of coffee. Considering the significant losses caused by *L. coffeella* in coffee production and the limited attention given to the use of fungal pathogens as tools to tackle this pest, we proposed a novel entomopathogenic fungi – *Cordyceps cateniannulata* to be deployed for CLM control. The pathogenicity of *C. cateniannulata* to CLM was recently demonstrated. Here, we evaluated the potential of *C. cateniannulata* against CLM in an *in planta* experiment and compared the potential of *C. cateniannulata, Beauveria bassiana* and a mixture of both fungi in the same assay. It includes (i) a test of the application of *C. cateniannulata* alone, *B. bassiana* alone and a mixture of both fungi to coffee plants against CLM and (ii) an *in vitro* assay to observe the interaction between *C. cateniannulata* and *B. bassiana* cultivated in coculture. *Cordyceps cateniannulata* and *B. bassiana*, as well as the mixture, caused high mortality of CLM pupae and no significant difference was found between the treatments. Furthermore, no synergistic effect was observed on pupal mortality after application of the mixture of both fungi; however, *C. cateniannulata* sporulated in more pupae than *B. bassiana*. In the *in vitro* experiment, *C. cateniannulata* alone and in coculture showed larger colony sizes than *B. bassiana* alone and in coculture. Considering the extreme importance of CLM as a coffee pest,

it is strategically important to expand the range of control possibilities for this insect, such as the use of the entomopathogenic fungus *C. cateniannulata*.

Keywords: biological control, coffee leaf miner, fungi entomopathogenic, mixture.

1. Introduction

Coffee leaf miner *Leucoptera coffeella* (Lepidoptera: Lyonetiidae) (CLM) is considered one of the most important coffee pests globally (Green, 1984). The larvae feed on the palisade parenchyma of the coffee leaves, creating mines and galleries, which reduces photosynthetic area, causes defoliation and consequently reduce fruit production (Reis et al. 2002; Parra & Reis 2013). Management of this coffee pest is mainly carried out using chemical insecticides; however, this has been documented as ineffective due to the insect's acquired resistance to most insecticides used, beyond costs and risks to humans and the environment (Guedes et al., 2016, 2017). Currently, the use of resistant plants with chemical and morphological characteristics that defend them against *L. coffeella* for implementation in integrated pest management programs has been reported (Souza 2022); however, there is still a need for new low-toxicity and environmentally friendly strategies for the management of *L. coffeella* (Dantas et. al., 2021), as well as biological control using fungi.

The hypocrealean fungal entomopathogens *Beauveria bassiana, Isaria fumosorosea* (= *Cordyceps fumosorosea*) and *Metarhizium anisopliae* are used to control a range of insect pests globally (Faria & Wraight 2007). For *L. coffeella*, there seems to be only a single study on the use of *M. anisopliae* against the egg and larval stages (Villacorta, 1983), but more recently, the pathogenicity of *Cordyceps cateniannulata* (= *Isaria cataniannulatus*) for larvae, pupae and adults of *L. coffeella* has been reported under laboratory conditions (Pereira et. al., *chapter 1*). Meanwhile, *B. bassiana* has been reported to be pathogenic for *Leucoptera malifoliella* (pear leaf moth) (Draganova & Tomov, 1998) and there seems to be a report of *B. bassiana* naturally infecting *L. coffeella* in the field (González & Velásquez 2007).

Considering the significant losses caused by *L. coffeella* in coffee production and the limited attention given to the use of fungal pathogens as tools to tackle this pest, we proposed an entomopathogenic fungi – *Cordyceps cateniannulata* – to be deployed for the control of CLM, as well as proposed for *Hypothenemus hampei* (Bautz et. al., *chapter 2*), also one of the

worst coffee pests worldwide (Baker, 1984). *Cordyceps cateniannulata* has great diversity in terms of niche adaptation, which includes the parasitism of a wide range of insects (Luangsa-Ard et. al, 2005) and has been a potential biocontrol agent to be deployed against arthropods pests, for example: *Spodoptera frugiperda* in China (Zhou et. al, 2020); *Glycaspis brimblecombei* in Brazil (Domingues, 2021); *Tetranychus urticae* in China (Zhang et. al, 2016) and *Stenoma impressella* in Colombia (Montes-Bazurto et. al, 2020).

Since *C. cateniannulata* is pathogenic for CLM under laboratory conditions (Pereira et. al., *chapter 1*), this stimulated us to evaluate the effects of this fungus against *L. coffeella* applied to coffee plants. In addition, we found that it would be worthy comparison between *C. cateniannulata* and *B. bassiana* for the control of this insect because *B. bassiana* is well established and widely used to control a variety of insect pests worldwide (Faria & Wraight 2007). We also tested the use of a mixture of both fungi to observe whether there is a synergistic effect between *C. cateniannulata* and *B. bassiana* on insect control. Furthermore, we evaluated the interaction of both fungi in an *in vitro* assay. It includes (i) a test of the application of *C. cateniannulata* alone, *B. bassiana* alone and a mixture of both fungi to coffee plants against CLM and (ii) an *in vitro* assay to observe the interaction between *C. cateniannulata* and *B. bassiana* cultivated in the same medium. Here, we provide a report on the potential of *C. cateniannulata* as novel entomopathogenic fungi to be deployed for the control of CLM.

2. Material and Methods

2.1. Fungal isolates, conidial suspensions and mixture of fungal

Two entomopathogenic fungi, our isolate of *C. cateniannulata* COAD 3349 and one isolate of *B. bassiana* COAD 3528, were used in this study. *Cordyceps cateniannulata* COAD 3349 was obtained from healthy *Coffee arabica* stems in Cameroon, Africa (Pereira et. al., *in chapter 1.*). COAD 3528 was obtained from coffee soil in Araponga, MG, Brazil. These isolates are kept in storage in silica gel and in 10% glycerol maintained at -80 °C both in the culture collection: Coleção Octavio de Almeida Drummond (COAD) of the Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil.

For use in experiments, the isolates in storage were passaged through *Tenebrio molitor* larvae for reactivation and then cultivated in plates containing potato dextrose-agar (PDA) in the dark at 25±1 °C for 15 days to allow for growth and sporulation. After this period, five

mycelium discs (5 mm diam) from the margin of the sporulating culture were transferred individually to polypropylene bags (15×25 cm) containing autoclaved rice (100 g of parboiled rice autoclaved together with 60 ml of distilled water for 15 min at 1.0 atm pressure at 121 °C), sealed with elastic bands and then placed in an incubator at 25±1 °C in the dark for 10 days. Every two days the bags were removed from the incubator and their contents were shaken to allow for a homogeneous colonization of the substrate by the fungi.

Conidial suspensions were prepared as follows: 40 g of both colonized rice were removed from their bags and transferred individually to a blender in 400 ml of Tween-80® solution (0.01%) which was switched on for 30 s. The suspensions were filtered through 10 and 120 mesh sieves (2 mm and 125 μm pore size, respectively) with agitation. Conidial concentrations were determined using a haemocytometer and adjusted to 1×10^8 conidia ml^{-1} in Tween 80® solution (0.01%) for both isolates. After that, a mixture of both isolates (COAD 3349 and COAD 3528) was prepared in a ratio of 1:1. For this, 200 ml of each isolate was transferred to a sterile beaker (500 ml) and mixed to maintain a homogenised mixture.

The conidial viability of each isolate was checked by transferring 100 μl of the suspension onto plates (9 cm diam) containing PDA + chloramphenicol (0.15 g l^{-1}) at 25±1 °C and after 24 h the count was performed of germinated conidia. The first 100 conidia observed under the 40× objective of the microscope were ranked as germinated (germ tubes longer or of the same length of the conidial diameter) or non-germinated (absence of germ tube or shorter than conidial diameter).

2.2. Insect sourcing, coffee plants and plant infestation

The rearing of the CLM was kept on coffee plants – *Coffea arabica,* variety "Catuaí" – in a greenhouse at the Departamento de Fitopatologia of UFV. Pupae of CLM were collected from infested leaves and taken to the laboratory of Laboratório de Interação Inseto-Microrganismo of UFV. In the laboratory, leaves containing the pupae were cut and placed individually in microtubes (2 ml) until adult emergence. Subsequently, adults were sexed and organized into couples for plant infestation.

Healthy *Coffea arabica* – variety "Catuaí" – 1 year old and ca. 70 cm were obtained from coffee nursery of the Departamento de Fitopatologia of the UFV. These plants were transferred to 3 L pots containing pasteurized soil, manure and sand (1.5:1:0.5) and maintained

in a greenhouse (Salcedo-Sarmiento *et al.* 2021). Each plant was fertilized with Basacote® Plus 9M 16-8-12(+2) and watered regularly (100 ml of water per plant every 2 d). The plants were carefully inspected prior to use in the experiment to ensure the absence of pests and diseases.

Thirty coffee plants were infested with two two-day-old CLM couples. Each plant was individually bagged with gauze and two couples were released on the plants and then the gauze was tied to prevent the insects from leaving (methodology adapted from Franzin et. al., 2022). Within of seven days, the couples mated and the females laid eggs on the surface of the leaves. After seven days, the adults were removed from the plants and was inspected the presence of eggs in each plant. The plants were kept in a greenhouse irrigated with sprinkler water at 9:00 am, 12:00 pm and 3:00 pm and then inspected daily to observe larval emergence and mine formation.

2.3. Effects of *Cordyceps cateniannulata* COAD 3349, *Beauveria bassiana* COAD 3528 and a mixture on coffee leaf miner

The greenhouse experiment was carried out to determine whether the application of COAD 3349, COAD 3528 and a mixture of COAD 3349:COAD 3528 on coffee plants had an impact on pupae and adults of CLM. To verify if there is a synergistic effect of the two fungi on the control of *L. coffeella*, we tested a mixture of COAD 3349 and COAD 3528 applied together. The treatments consisted of: (i) application of COAD 3349 alone (seven plants); (ii) application of COAD 3528 alone (seven plants); (iii) mixture of COAD 3349:COAD 3528 togheter (seven plants); and (iv) application of blank Tween-80® solution (0.01%) (nine plants). The applications of the fungi were performed three times (at concentrations of 1×10^8 conidia ml^{-1}) at four-day intervals, once in the presence of mines and twice in the presence of pupae. The total numbers and mortalities of pupae and adults were reported at the end of the experiment.

The plants received the first application of fungi at a concentration of 1×10^8 conidia ml^{-1} 12 days after removal of adults (the moment when mines were present) (Fig. 1A). The next applications were performed when pupae were presents. The application was carried out from larval stage as in the prelyminar test the COAD 3349 it did not show an ovicidal effect. For the applications, each plant was removed from the greenhouse individually for fungus application or control. At the moment of the applications, the gauze of each plant was opened, and the fungal suspension was applied with a common hand sprayer (500 ml polyethylene terephthalate

bottle with spray valve) until runoff – approximadly 100 ml per plant – mainly on the adaxial and abaxial part of the leaves (Fig. 1B). After applications, the gauzes were closed and the plants were taken back to the greenhouse (Fig. 1C). Subsequent applications were carried out in the same way as described above.

Plants were inspected daily to observe adult emergence. The gauze was carefully opened and the adults were collected using a microtube (2 ml) and taken to the laboratory. In the laboratory, the adults were kept inside a microtube closed with cotton to allow the passage of air. Dead adults were placed in moist chambers and then incubated in the dark at 25 ± 1 °C to be observed for the evidence of colonization by COAD 3349 and COAD 3528. The total numbers and mortalities of pupae and adults were verified at the end of the experiment. For this, all gauzes was opened and the leaves containing pupae were detached and taken to the laboratory. In the laboratory, each pupa was observed under a dissection microscope (Olympus SZ61) for evidence of mortality and COAD 3349 and COAD 3528 colonization.

Figure 1: *In planta* experiment of *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528 applied to coffee plants and their effects on pupae and adults of coffee leaf miner (*Leucoptera coffeella*). (A) Inoculation of COAD 3349 at concentration of 1 1×10^{8} conidia ml^{-1} on a whole coffee plant. (B) Coffee plants after application of COAD 3349 until runoff. Note the suspension aliquots on the leaf surfaces. (C) Plants covered with gauze tubes with their ends tied to prevent the entry and exit of insects including *L. coffeella*.

2.4. In vitro interaction between *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528

Due to the mixture of COAD 3349 and COAD 3528 that we carried out to observe whether there is a synergistic effect between the two fungi for the control of CLM in the previous experiment, we felt it would be worthwhile investigating whether there was antibiosis at play between both fungi in an *in vitro* assay. For this, each isolate was previously cultivated

in PDA and incubated at 25±1 °C in the dark for six days. After this period, a disc (5 mm diam) from the margin of each sporulating culture was plated 1.5 cm from the border of the Petri dishes (90×15 mm) containing 20 ml of PDA. COAD 3349 was plated on the opposite side of COAD 3528, maintaining a distance of 1.5 cm from the border of the Petri dish. For the control, a disc (5 mm diam) of PDA was plated on the opposite side of the COAD 3349 or COAD 3528. The combinations consisted of: (i) COAD 3349 *versus* PDA (n = 13); (ii) COAD 3528 *versus* PDA (n = 10); and (iii) COAD 3349 *versus* COAD 3528 (n = 10). The plates were incubated at 25 ±1 °C. The growth of fungi was evaluated every 12 h, for 25 days but we analyzed using the data from day 20. We measured the growth (diameter and width in cm) of COAD 3349 and COAD 3528 using a ruler and then averaged to obtain a measurement of each fungus.

2.5. Data Analysis

All data analyses were performed using R (version 4.1.3) statistical software package (R Development Core Team, 2022).

Pupal mortality was analyzed through a Generalized Linear Model (GLM) with binomial distribution and the data were then compared with the χ^2 test ($p < 0.05$). Paired comparisons between treatments were performed with Tukey tests ($p < 0.05$). The number of dead pupae sporulated with either COAD 3349 or COAD 3528 from the mixture treatment was analyzed with a GLM and the data were then compared using an F test ($p < 0.05$). The number of dead adults was analyzed through a GLM with a Poisson distribution and the data were then compared with the χ^2 test ($p < 0.05$). Paired comparisons between treatments were performed with Tukey test ($p < 0.05$).

The growth in centimeters of COAD 3349 colony in the presence of COAD 3528 colony or control (PDA) was analyzed through a GLM and then compared with the χ^2 test ($p < 0.05$). Paired comparisons between treatments were performed with Tukey tests ($p < 0.05$).

3. Results

3.1. Effects of *Cordyceps cateniannulata* COAD 3349, *Beauveria bassiana* COAD 3528 and a mixture on pupae and adults of coffee leaf miner

The mortality of pupae was significantly affected by exposure to COAD 3349, COAD 3528 or the mixture of both fungi (χ^2 = 48.75, df = 3, p = < 0.0001). Pupae exposed to each

fungus alone and the mixture had higher mortalities than the controls (p = < 0.0001; Tables 1 and 2; Fig. 1A-G). Meanwhile, mortality of pupae exposed to COAD 3349 did not differ from COAD 3528 (p = 0.0813; Tables 1 and 2). Likewise, mortality of individuals exposed to the mixture of both fungi did not differ from COAD 3349 applied alone (p = 0.8702; Tables 1 and 2) nor from COAD 3528 applied alone (p = 0.3084; Tables 1 and 2). Additionally, sporulation of COAD 3349 and COAD 3528 was observed from CLM pupae when treated with the mixture of both fungi; however, COAD 3349 sporulated in more pupae than COAD 3528 (χ^2 = 6.69, df = 1, p = 0.0237; Fig. 2G).

The number of dead adults was significantly affected by exposure to COAD 3349, COAD 3528 and the mixture of both fungi (χ^2 = 24.54, df = 3, p = < 0.0001). Adults emerged from pupae exposed to COAD 3528 had a higher mortalities than controls (p = < 0.0001; Tables 1 and 2), while dead adults that emerged from pupae exposed to COAD 3349 and the mixture did not differ from controls (p = 0.7129; p = 0.3968, respectively; Tables 1 and 2). Adults emerged from pupae exposed to COAD 3528 had a higher mortalities than those exposed to COAD 3349 (p = 0.0006; Tables 1 and 2) and the mixture (p = 0.0024; Tables 1 and 2). Meanwhile, dead adults that emerged from pupae exposed to COAD 3349 did not differ from the mixture (p = 0.9578; Tables 1 and 2).

Table 1. Mortality of pupae and adults of leaf coffee miner after three applications of *Cordyceps cateniannulata* COAD 3349*, Beauveria bassiana* COAD 3528, mixture of both fungi and control (0.001% Tween 80) *in planta* experiment. Data shown are results of Generalized Linear Model (GLM) and a posteriori Tukey pairwise comparisons (P < 0.05)

Treatments	p-values 5% [1]
Pupae	
COAD 3349 *versus* Control	**< 0.0001**
COAD 3528 *versus* Control	**< 0.0001**
Mixture *versus* Control	**< 0.0001**
COAD 3349 *versus* COAD 3528	0.0813
COAD 3349 *versus* Mixture	0.8702
COAD 3528 *versus* Mixture	0.3084
Adults	
COAD 3349 *versus* Control	0.7129
COAD 3528 *versus* Control	**< 0.0001**
Mixture *versus* Control	0.3968
COAD 3349 *versus* COAD 3528	**0.0006**
COAD 3349 *versus* Mixture	0.9578
COAD 3528 *versus* Mixture	**0.0024**

[1]p-values according to the Tukey test (P < 0.05).
p-values in bold indicate significant differences by the Tukey test (p < 0.05).

Table 2. Total number of pupae and adults of leaf coffee miner (*Leucoptera coffeella)* after exposure of *Cordyceps cateniannulata* COAD 3349*, Beauveria bassiana* COAD 3528, mixture of both fungi and control (0.001% Tween 80) *in planta* experiment

Treatments (n)[1]	Pupae		Adults	
	Total (n)[2]	Dead (n)[3]	Total (n)[2]	Dead (n)[3]
Control (9)	395	4	391	0
COAD 3349 (7)	393	389	4	4
COAD 3528 (7)	604	582	22	22
Mixture (7)	361	355	6	6

[1](n) Total number of plants/replication according to the treatment.
[2](n) Total number of pupae and adults according to all replication of each treatment.
[3] (n) Total number of pupae and adults dead to all replication of each treatment.

Figure 2. Pupae of coffee leaf miner (*Leucoptera coffeella)* exposed to *Cordyceps cateniannulata* COAD 3349, *Beauveria bassiana* COAD 3528, mixture of both fungi and control (0.001% Tween 80) *in planta* assay. (A) Pupae of *L. coffeella* from control. Note the empty pupae. (B) Pupae of *L. coffeella* from control. Note the pupae from control showed no colonization by COAD 3349 and COAD 3528. (C) Two pupae colonized by COAD 3349. (D) COAD 3349 colonizing among the pupal silk threads.

(E) Pupae exposed to COAD 3528. (F) COAD 3528 colonizing among the pupal silk threads. (G) Pupae exposed to the mixture of both fungi (COAD 3349: COAD 3528). Note on the left two pupae colonized by COAD 3349 and on the right a pupae colonized by COAD 3528.

3.2. In vitro interaction between *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528

The growth of COAD 3349 and COAD 3528 was significantly affected *in vitro* by the interaction of both fungi ($\chi^2 = 2.78$, df = 3, p = < 0.0001; Fig. 3A-F). COAD 3349 alone had a larger colony size than COAD 3528 alone (p= 0.0081; Table 3; Fig. 3ABDE) and than COAD 3528 from the co-culture (p= < 0.0001; Table 3; Fig. 3CF). COAD 3528 alone had a larger colony size when compared to COAD 3528 from the co-culture (p= < 0.0001; Table 3; Fig. 3BCEF). Meanwhile, the colony size of the COAD 3349 alone did not differ from COAD 3349 in the co-culture (p= 0.974; Table 3; Fig. 3ACDF). COAD 3528 alone had a smaller colony size than COAD 3349 from the co-culture (p= 0.0379; Table 3; Fig. 3BCEF). Finally, COAD 3349 from the co-culture had a larger colony size when compared to COAD 3528 from the co-culture (p= < 0.0001; Table 3; Fig. 1CF).

Table 3. *In vitro* interaction between *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528. Data shown are results of Generalized Linear Model (GLM) and a posteriori Tukey pairwise comparisons (P < 0.05)

Treatments	p-values 5% [1]
COAD 3349 alone *versus* COAD 3528 alone	**0.0081**
COAD 3349 alone *versus* COAD 3528 from co-culture	**< 0.0001**
COAD 3528 alone *versus* COAD 3528 from co-culture	**< 0.0001**
COAD 3349 alone *versus* COAD 3349 from co-culture	0.974
COAD 3528 alone *versus* COAD 3349 from co-culture	**0.0379**
COAD 3349 from co-culture *versus* COAD 3528 from co-culture	**< 0.0001**

[1]p-values according to the Tukey test (P < 0.05).
p-values in bold indicate significant differences by the Tukey test (p < 0.05).

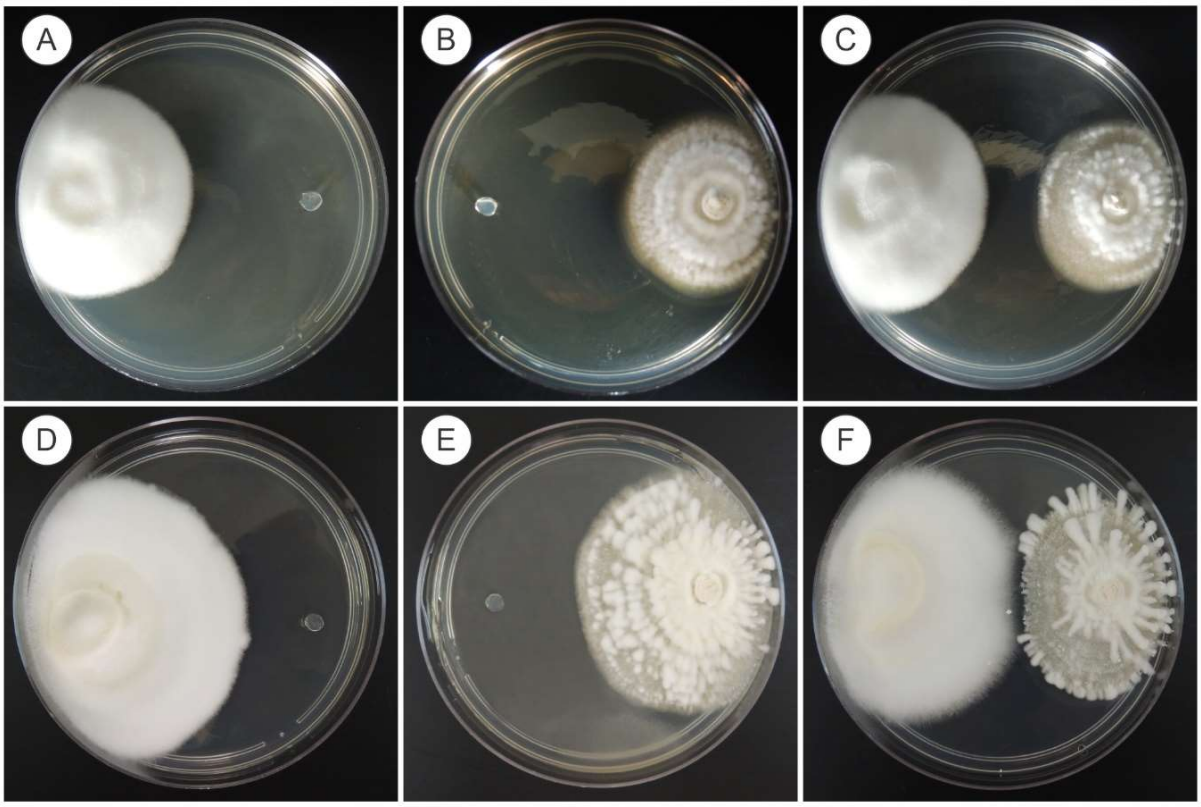

Figure 3. *In vitro* interaction between *Cordyceps cateniannulata* COAD 3349 and *Beauveria bassiana* COAD 3528. (A) COAD 3349 alone 20 days after inoculation. (B) COAD 3528 alone 20 days after inoculation. (C) COAD 3349 in confront with COAD 3528 20 days after inoculation. (D) COAD 3349 alone 25 days after inoculation. (E) COAD 3528 alone 25 days after inoculation. (F) COAD 3349 in confront with COAD 3528 25 days after inoculation. Note the absence of halo between the fungi.

4. Discussion

The pathogenicity of *C. cateniannulata* COAD 3349 to *L. coffeella* (CLM) has been demonstrated previously (Pereira et. al., *chapter 1*). This finding stimulated us to evaluate the effects of this fungus against *L. coffeella* applied to coffee *in planta* experiment. Furthermore, we felt it would be worthwhile to compare *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528 for control of this insect because *B. bassiana* is well established and widely used to control a variety of insect pests worldwide (Faria & Wraight 2007). *Cordyceps cateniannulata* COAD 3349 caused high mortality of CLM pupae with no significant difference with application of COAD 3528 as well as the mixture of both fungi. Furthermore, no synergistic effect was observed in pupal mortality after application of the mixture of both fungi; however, COAD 3349 sporulated in more pupae than COAD 3528 (Fig. 3G). In the *in vitro* experiment, COAD 3349 alone and the coculture showed a larger colony size when compared to COAD 3528 alone and the coculture (Fig 3A-F).

Here, we report for first time, the ability of *C. cateniannulata* COAD 3349 and *B. bassiana* COAD 3528 to infect and kill pupae and adults of *L. coffeella* CLM in an *in planta* experiment (Fig. 3A-G). Previous laboratory study reported the effect of *M. anisopliae* against egg and larvae of CLM (Villacorta, 1983), when it was shown that the larvae were killed by the fungus either inside the mines or when they left the mine to pupate. In our study, the first application of fungi was made when mines were present; probably the fungus did not infect the larvae inside the mines (Fig. 1AB), but rather when they left the mines to pupate. A similar scenario was reported for *B. bassiana* on *L. malifoliella* (pear leaf blister moth) when late instar larvae were infected only when leaving the mines to pupate (Draganova & Tomov, 1998). COAD 3349 and COAD 3528 probably do not protect the plants quickly enough for an immediate effect, but they contribute to the reduction of pupae and adults of CLM, potentially influencing population size the next generation of this coffee pest.

In our *in planta* experiment to compare COAD 3349 and COAD 3528 against CLM, we found no differences, suggesting that both isolates may have similar abilities to control CLM. Similar results were observed for the same fungus against adult females of *H. hampei* under laboratory conditions (Bautz et. al., *chapter 2*). Furthermore, no synergistic effect was observed on pupae mortality for the mixture of fungi when compared to COAD 3349 and COAD 3528 applied alone. However, COAD 3349 sporulated in more pupae than COAD 3528 (Fig. 3G). COAD 3349 excelled at colonizing and sporulating pupae, but it is unknown how COAD 3349 inhibited sporulation of COAD 3528. Costantin (2022) also reported no synergetic effect when applied a mixture of *B. bassiana* and *M. anisopliae* on *Tenebrio molitor* larvae*;* however, *B. bassiana* colonized more larvae than *M. anisopliae.* In contrast, a mixture of *B. bassiana* and *M. anisopliae* caused greater tick mortality than that achieved by the separate fungi (Maranga et. al., 2005).

Regarding the *in vitro* experiment, COAD 3349 showed a greater colony size than COAD 3528 when grown alone and in coculture (Fig. 3A-F). Meanwhile, COAD 3528 grew more when cultivated alone than in coculture, while COAD 3349 showed no difference in its growth in any of the cultivation forms. In addition, we did not observe the presence of an inhibition zone for both fungi when cultivated in coculture, but some unknown mechanism COAD 3349 used to inhibit COAD 3528 growth. Furthermore, it is possible to observe COAD 3349 colony touching the COAD 3528 colony 25 days after inoculation (Fig. 3F). Several toxic metabolites produced by *Beauvaria, Metarhizium* and *Pacilomyces* (= *Isaria* = *Cordyceps*) have been reported (Vey et. al., 2001) with antibiotic, fungicidal or insecticidal properties against

insect pests and diseases (Kershaw et. al., 1999, Vey et. al., 2001; Ross 2005). However, in our *in vitro* experiment, there seemed to be, at least to the unaided eye, no antifungal activity between COAD 3349 and COAD 3528. Meanwhile, *B. bassiana* showed moderate antifungal activity against the fungal phytopathogens *Aspergillus paraziticus, Fusarium moniliform* and *Fusarium oxysporum* (Sahab 2012).

The mixture of the two fungi did not result in a synergistic effect on pupal mortality, but COAD 3349 excelled in pupal colonization and sporulation. Furthermore, COAD 3349 inhibited COAD 3528 growth in the *in vitro* assay. Thus, further studies are needed to better determine the potential of both fungi together as a biocontrol option against this target.

Little attention has been given to the use of fungal pathogens as tools to combat CLM. Nevertheless, considering the extreme importance of CLM as a coffee pest, it is strategically important to expand the range of control possibilities for this insect. In the present study, we tested the potential of *C. cateniannulata* COAD 3349 to control *L. coffeella* CLM. This fungus, as well as *B. bassiana* COAD 3528, caused high mortality of CLM pupae and adults in an *in planta* experiment, but had a limited biocontrol effect on the larval stage. However, more testing is needed to find a way for the fungus to infect the larvae inside mines, since the larval stage that causes coffee damage. Here, *C. cateniannulata* COAD 3349 probably do not protect the plants quickly enough for an immediate effect, but it contribute to the reduction of pupae and adults of CLM, potentially influencing population size the next generation of this coffee pest.

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