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ORIGINAL ARTICLE

Polyphasic characterization and antimicrobial properties of *Induratia* species isolated from *Coffea arabica* in Brazil

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Abstract
Fungi belonging to the genus *Induratia* are endophytes that have received considerable attention because of the production of natural bioactive secondary metabolites, such as volatile compounds, with antimicrobial activity. In this study, we distinguished *I. coffeana* and *Induratia* sp. isolated from *Coffea arabica* in Brazil based on three-loci phylogeny, ITS, *RPB2*, and *TUB2*. The *Induratia* isolates showed high morphological plasticity and produced different volatile organic compounds, indicating that these compounds might not be correlated with their phylogenetic assignment. However, PLS-DA was able to discriminate *Induratia* isolates into three different clusters: one associated with *I. coffeana* and two with *Induratia* sp. *Induratia* isolates showed biofumigant activity against *Botrytis cinerea* with emphasis on *I. coffeana* (CML 4019), which inhibited the pathogenic fungus in postharvest strawberries. Nematicidal activity against *Meloidogyne incognita* was also observed in filtrates and volatile compounds produced by *Induratia* isolates. Moreover, we observed that *I. coffeana* (CML 4019) metabolites showed antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *E. faecium* and that extracts of seven other *Induratia* isolates reduced the pre-formed biofilm of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Keywords Endophytic fungi · *Muscodor* · Phylogenetic analysis · Volatile organic compounds · Biofumigation · Antibiofilm activity

Introduction

Among the diverse microbial communities that inhabit plants internally, endophytic fungi interact with host plants, increasing their productivity and resistance to biotic and abiotic stresses. These microorganisms can use different mechanisms during symbiotic interactions, including the synthesis of a large number of specific secondary metabolites that play many different physicochemical and biological roles, such as enzymes, antibiotics, toxins, soluble compounds, and volatile organic compounds (VOCs). Some of these compounds are used for the development of

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pharmaceuticals and agrochemicals, while others as mycotoxins tend to be more problematic because of their widespread occurrence as contaminants of food for humans and livestock, as well as mold-contaminated indoor environments (Hardoim et al. 2015; Lata et al. 2018; Hyde et al. 2019; Yadav 2019). Three complementary reviews cover a good part of literature about the breadth of fungal secondary metabolite diversity, with information of the biosynthesis of the most important fungus-derived metabolites including those produced from Xylariales, their biological activities, and recent developments that have contributed to human health and agriculture (Bills and Gloer 2016; Helaly et al. 2018; Becker and Stadler 2021).

The genus *Muscodor*, an important endophyte previously accommodated in the order Xylariales, has been reported as an efficient biocontrol agent for the synthesis of VOCs. *Muscodor albus*, strain cz620 isolated from *Cinnamomum zeylanicum*, showed production of VOCs with broad antimicrobial activity, and it was the first biological control agent commercially available and effective in controlling fungal decay in apples and peaches, green mold, and sour rot of stored lemon and damping-off disease of sugar beet by mycofumigation (Strobel et al. 2001; Worapong et al. 2001; Worapong et al. 2002; Mercier and Jiménez 2004; Mercier and Smilanick 2005; Strobel and Ezra 2005; Grimme et al. 2007; Hutchings et al. 2017).

Since the description of *M. albus* by Worapong et al. (2001), 26 *Muscodor* species from different hosts and ecological niches have been reported. However, the identification of most species was based on cultural morphology, VOC profiles, and molecular phylogenetic analyses using the ITS *rRNA* gene, which is highly questionable. Stadler et al. (2013) reviewed the nomenclatural changes for taxonomy of Xylariaceae after the introduction of the One Fungus-One Name (1F1N) concept and suggested the abandonment of some ill-defined anamorph genera, such as *Muscodor*. The authors presented confident arguments to do not integrate *Muscodor* into the Xylariaceae. More recently, Chen et al. (2019) concluded that the single ITS region cannot resolve *Muscodor* species and described a new species named *Muscodor yunnanensis* using four loci (ITS rRNA, 28S rRNA, *RPB2*, and *TUB1*) in the phylogenetic analyses. Certainly, incongruities and apparent mismatches between phenotypes and genotypes observed when ITS is used as the primary barcode for some families (Xylariaceae) and genera of fungi result from the number of copies intragenomic of *rDNA* genes and differences in nucleotide identity (Stadler et al. 2020). Thus, these authors propose that additional genomes should be checked for such ITS polymorphisms to reassess the validity of this non-coding part of the fungal DNA for molecular identification. Finally, Samarakoon et al. (2020) used ITS, LSU, *RPB2*, and *TUB2* DNA sequences of 89 isolates, transferred all *Muscodor* species to *Induratia*, and proposed the new family Induratiaceae. They also reported some probable structures of sexual states as apiospores and described the new species *I. ziziphi*, isolated from a dead branch of *Ziziphus* sp., and *I. thailandica* from a dead unknown host wood.

Until then, in addition to molecular identification, the *Muscodor* species discrimination included morphological characteristics and VOC profiles assessed by gas chromatography coupled with mass spectrometry. However, the quality of data obtained appears highly questionable, as few concise study comparatives included type and authentic isolates of Xylariales, which were originally used to erect the genus *Muscodor* (Stadler et al. 2013). Samarakoon et al. (2020) reported for the first time the production of conidiophores, and apiosporous ascospores to *Induratia* species, which are morphologically distinct from the Xylariales. In the same study, the authors reported that secondary metabolite profiles can be valuable for chemotaxonomic purposes, but the data require a high degree of standardization once secondary metabolite production is dependent on the culture medium and the growth phase and inclusion of a significant number of species.

In Brazil, *Induratia coffeana*, *Induratia yucatanensis*, and *Induratia vitigena* have been isolated from the stems and leaves of *Coffea arabica* (Hongsanan et al. 2015; Monteiro et al. 2017), and *Induratia braziliensis* has been isolated from the leaves of *Schinus terebinthifolius*, a Brazilian medicinal plant (Pena et al. 2019). These species produce VOCs with antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, *Phoma* sp., *Botrytis cinerea*, *Fusarium solani*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Pestalotia longisetula*, *Aspergillus ochraceus* (Monteiro et al. 2017), and *Penicillium digitatum* (Pena et al. 2019). Induratia species also reduced the symptoms of disease caused by three phytopathogens, *Colletotrichum lindemuthianum*, *Sclerotinia sclerotiorum*, and *Pseudocercospora griseola*, when re-inoculated in common bean seedlings (Mota et al. 2021). In addition, these fungi produce non-volatile secondary metabolites, including extracellular amylase, cellulase, lipase, pectinase, phytase, protease, endo β-1,4 glucanase, and exo β-1,4 glucanase, and molecules that modulate enzymes that act in human hemostasis (Bastos et al. 2020; Monteiro et al. 2020).

Here, we report the identification based on the phylogeny of three loci (ITS, *RPB2*, and *TUB2*), morphological characterization, and VOC analysis of 12 *Induratia* isolates obtained from coffee plants growing spontaneously in a secondary forest in Brazil. We also show the antifungal, antibacterial, and anti-nematode activities of VOCs and non-volatile metabolites produced by these endophytic fungi.

Material and methods

Fungal isolates

In this study, we evaluated twelve endophytic *Induratia* isolates, deposited in the Coleção Micológica de Lavras (CML) at the Departamento de Fitopatologia at the Universidade Federal de Lavras (UFLA), Brazil (Table 1). They were isolated from fresh and healthy leaves and stems of coffee plants (*Coffea arabica*) growing spontaneously in a secondary forest in Mata do Paraíso (20° 48′ 03.4″ S, 42° 51′ 42.6″ W), Zona da Mata region, Viçosa, Minas Gerais, Brazil.

DNA extraction, amplification, and sequencing

Fresh mycelia scraped from the margin of colonies grown on 2% malt extract broth (Himedia Laboratories, Mumbai, India) was used for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, São Paulo, Brazil) according to the manufacturer's protocol. The ITS was amplified using the primers ITS5 and ITS4 (White et al. 1990), and the following PCR conditions were used: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; and a final extension for 10 min at 72 °C. A portion of the second largest subunit of RNA polymerase II (*RPB2*) was amplified using the primers 5F2 and 7cR (Liu et al. 1999) with the following cycling conditions: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension for 10 min at 72 °C. *β-tubulin* (*TUB2*) was amplified using the primers T1 and T22 (O'Donnell and Cigelnik 1997) under the following cycling conditions: 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension for 10 min at 72 °C. PCR reactions were performed using the GoTaq® Colorless Master Mix kit (Promega, São Paulo, Brazil) in a MyCycler thermal cycler (Bio-Rad, Hercules, USA). The amplified fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). Bidirectional DNA sequences for each region were generated by Macrogen (Rockville, Maryland, USA), using the Sanger method using the same primers used for PCR amplification.

Phylogenetic analyses

Consensus sequences were obtained from a bidirectional DNA sequence using the SeqAssem ver. 07/2008 (Hepperle 2004). Sequences from the type and reference of *Induratia* species available in GenBank were added to the analyses (Table 1). Multiple sequence alignments were performed using Clustal W, as implemented in MEGA X (Kumar et al. 2018). Phylogenetic analyses of each gene separately were carried out to verify which of the gene regions would be more informative for the delimitation of *Induratia* species, as well as for the combined dataset (ITS-*RPB2*-*TUB2*). Analyses were performed with maximum parsimony (MP) and maximum likelihood (ML) using Mega X with 1000 bootstrap replications. The most suitable substitution model was determined based on the lowest Bayesian information criterion (BIC) using Mega X, and the parameters of the matrices used to generate phylogenies are shown in Online Resource 1. *Emarcea castanopsidicola* and *E. eucalyptigena* were used as outgroup taxa. The alignments were deposited in TreeBASE (www.treebase.org; study number: S28186). URL:http://purl.org/phylo/treebase/phylows/study/TB2:S28186?x-access- code = 19e96d3d50543e82ca8d617a8e8334a5&format = html.

Morphological characterization

Induratia isolates were incubated on potato dextrose agar (PDA) medium at 25 °C for 7 days to assess the differences in growth rates and colony morphologies. Then, mycelial discs (5 mm diameter) were excised from *Induratia* colonies and transferred to Petri dishes containing one of the following media: PDA, synthetic nutrient-poor agar (SNA), cornmeal agar (CMA), and water agar (WA) at 25 °C for 15 days. The isolates were also inoculated on the center of a Petri dish containing PDA and incubated in darkness at 15, 20, 25, and 30 °C. After 15 days of incubation, colony characteristics were observed, and their diameters were measured. The growth rate was determined by measuring the colony area in three replicates. The growth rates and colony morphology were also evaluated at 25 °C for 40 days in Petri dishes containing PDA, in addition to odor production and coil presence. The coils and hyphae were also measured. The characteristics of colonies were analyzed by visual inspection, and the morphology was microscopically analyzed using the *Induratia* isolates cultivated on PDA and SNA media. Light microscopy was performed with the Zeiss observer Z.1 Epifluorescence Microscope with Apotome System and Zeiss Axion Vision software at the Laboratório de Microscopia Eletrônica e Análise Ultraestrutural at the Universidade Federal de Lavras, Brazil. Images were processed using Corel Draw software.

For electron microscopy (SEM), discs of 10-day-old PDA and SNA cultures grown at 25 °C were fixed in Karnovsky solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.05 M sodium cacodylate buffer, $CaCl_2$ 0.001 M, pH 7.2) for 24 h. The samples were dehydrated in an ascending series of acetone solutions (25%, 50%, 75%, 90%, and 100% in triplicate) for 10 min at each step. The samples were brought to a critical point, dried, and coated with gold. The structures were observed using a LEO EVO 40 scanning

Table 1 *Induratia* isolates used in this study and GenBank accessions

Species	Isolates code[a]	GenBank accession number[b]			References
		ITS	*RPB2*	*TUB2*	
Induratia coffeana	CML 4009	MN658674	MN689579	MN689587	This study
Induratia coffeana	CML 4010	MN658675	MN689580	MN689588	This study
Induratia coffeana	CML 4011	MN658676	MN689581	MN689589	This study
Induratia coffeana	CML 4012	MN658677	MN689582	MN689590	This study
*Induratia coffeana**	CML 4014	MN658683	MN746440	MN746443	This study
*Induratia coffeana**	CML 4016	MN658684	MN746441	MN746444	This study
*Induratia coffeana**	CML 4017	MN658685	MN746442	MN746445	This study
Induratia coffeana	CML 4018	MN658678	MN689585	MN689593	This study
Induratia coffeana	CML 4019	MN658679	MN689586	MN689594	This study
Induratia coffeana	CML 4020	MN658680	MT822682	MT822682	This study
Induratia sp.	CML 4013	MN658681	MN689583	MN689591	This study
Induratia sp.	CML 4015	MN658682	MN689584	MN689592	This study
Induratia coffeana	N-L-7	MG309792	N/A	N/A	Mao et al. (2018)
Induratia coffeana	Y-L-43	MG309793	N/A	N/A	Mao et al. (2018)
Induratia coffeana	W-S-35	MG309794	N/A	N/A	Mao et al. (2018)
Induratia alba	9–6	HM034857	KC243321	HM034844	Zhang et al. (2010)
Induratia brasiliensis	LGMF 1256T	KY924494	MF510171	N/A	Pena et al. (2019)
Induratia camphorae	NFCCI 3236T	KC481681	N/A	N/A	Meshram et al. (2017)
Induratia cinnamomi	BCC 38842T	GQ848369	N/A	N/A	Suwannarach et al. (2010)
Induratia coffeana	COAD 1900	KP862879	KP862880	N/A	Hongsanan et al. (2015)
Induratia coffeana	COAD 1842T	KM514680	KP862881	N/A	Hongsanan et al. (2015)
Induratia crispans	MONT 2347T	EU195297	N/A	N/A	Mitchell et al. (2008)
Induratia darjeelingensis	NFCCI 3095T	JQ409997	N/A	N/A	Saxena et al. (2014)
Induratia equiseti	JCM 18233T	JX089322	N/A	N/A	Suwannarach et al. (2013)
Induratia fengyangensis	CGMCC 2862T	HM034856	HM034849	HM034843	Zhang et al. (2010)
Induratia ghoomensis	NFCCI 3234T	KF537625	N/A	N/A	Meshram et al. (2015)
Induratia heveae	RTM5–IV3T	KF850712	N/A	N/A	Siri-udom et al. (2016)
Induratia indica	NFCCI 3235T	KF537626	N/A	N/A	Meshram et al. (2015)
Induratia kashayum	NFCCI 2947T	KC481680	N/A	N/A	Meshram et al. (2013)
Induratia musae	JCM 18230T	JX089323	N/A	N/A	Suwannarach et al. (2013)
Induratia oryzae	JCM 18231T	JX089321	N/A	N/A	Suwannarach et al. (2013)
Induratia rosea	MONT 2098T	AH010859	N/A	N/A	Worapong et al. (2002)
Induratia strobelii	NFCCI 2907T	JQ409999	N/A	N/A	Meshram et al. (2014)
Induratia suthepensis	JCM 18232T	JN558830	N/A	N/A	Suwannarach et al. (2013)
Induratia suturae	MSUB 2380T	JF938595	N/A	N/A	Kudalkar et al. (2012)
Induratia thailandica	MFLUCC 17-2669T	MK762707	MK791283	MK776960	Samarakoon et al. (2020)
Induratia tigerensis	NFCCI 3172T	JQ409998	N/A	N/A	Saxena et al. (2015)
Induratia vitigena	MONT P-15T	AY100022	N/A	N/A	Daisy et al. (2002)
Induratia yucatanensis	MEXU 25511T	FJ917287	N/A	N/A	González et al. (2009)
Induratia yunnanensis	CGMCC 3.18908T	MG866046	MG866059	MG866066	Chen et al. (2019)
Induratia ziziphi	MFLUCC 17-2662T	MK762705	MK791281	MK776958	Samarakoon et al. (2020)
Emarcea castanopsidicola	CBS 117105T	AY603496	MK791285	MK776962	Duong et al. (2004) and Samarakoon et al. (2020)
Emarcea eucalyptigena	CBS 139908T	KR476733	MK791286	MK776963	Duong et al. (2004) and Samarakoon et al. (2020)

[a]*BCC* BIOTEC Culture Collection, Thailand, *BISH* Herbarium Pacificum (BISH), Bishop Museum, Hawaii, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, *CGMCC* China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, *CML* Coleção Micológica de Lavras, Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil, *COAD* Culture Collection of the Universidade Federal de Viçosa, Brazil, *CBS* CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, *JCM* Japan Collection of Microorganisms, Japan, *JDR* Herbarium of Jack D. Rogers, *LGMF* LabGeM Culture Collection, Federal University of Parana (UFPR), Curitiba, Brazil, *MEXU* Herbario Nacional, *MFLUCC* Mae Fah Luang University Culture Collection, Chiang Rai, Thailand, *MONT* Montana State University Herbarium, Plant Sciences and Plant Pathology, Montana State University, Bozeman, Montana, USA. Types of species are indicated by ^{T}

Table 1 (continued)

[b]Sequences generated in this study are indicated in bold and isolates for which sequences are unavailable are indicated by "N/A"

[*]In the ITS tree grouped with type isolate of *Induratia yucatanensis* (MEXU 25511)

electron microscope at the Laboratório de Microscopia Eletrônica e Análise Ultraestrutural at the Universidade Federal de Lavras, Brazil. The images were digitally generated and recorded at a working voltage of 20 kV and a working distance of 9 mm. Images were processed using Corel Draw software.

Characterization of VOCs

The VOC analyses of *Induratia* isolates were conducted at the Centro de Análises e Prospeção Química (CAPQ) of the Universidade Federal de Lavras, Brazil. The fungi were inoculated in duplicates in 20 mL SPME vials containing PDA medium, and after 7 days, the VOCs were extracted by headspace solid-phase microextraction (SPME) (Arthur and Pawliszyn 1990). The SPME extraction was performed as follows: divinylbenzene, carboxen, and polydimethylsiloxane (DVB/CAR/PDMS) fiber, extraction temperature of 55 °C at 250 rpm, extraction time of 35 min, and desorption time of 2 min in the GC injector. A GC-MS QP 2010 Ultra (Shimadzu, Japan) gas chromatograph coupled with a mass spectrometer equipped with an AOC-5000 (Shimadzu, Japan) automatic injector for liquids and gases and an HP-5 (5% phenyl-95% dimethylsiloxane) 30 m × 0.25 mm × 0.25 µm column was used to separate the VOCs. The injector, interface, and ion detector temperatures were 250 °C, 240 °C, and 200 °C, respectively. The injector was operated in splitless mode. The carrier gas was grade 5.0 He with a flow of 1.0 mL min^{-1}. The GC oven temperature was increased at a rate of 3 °C min^{-1} from 40 to 160 °C and then at 10 °C min^{-1} to 240 °C. The VOCs were identified by comparing the mass spectra obtained via the Automated Mass Spectral Deconvolution and Identification System (AMDIS) v. 2.6 software to those in the NIST library using the Mass Spectral Search Program v. 1.7 (NIST, Washington DC, USA) software (https://webbook.nist.gov/chemistry/). For comparison of the mass spectra, only spectra with a similarity greater than 80% were considered. To improve identification, experimental retention indices (RIExp) were obtained by injecting a homologous series of alkanes and comparing them with those reported in the literature (RI Lit) (Adams 2007).

Partial least squares: discriminant analysis

The total ion chromatograms of the VOCs were arranged in a matrix with 24 samples × 4922 chromatographic signals and then subjected to partial least squares discriminant analysis (PLS-DA) against the classes (*I. coffeana* and *Induratia* sp.) from the maximum likelihood phylogenetic tree based on concatenated ITS-*RPB2*-*TUB2* sequences. The proper number of latent variables (LV) was determined by leave-one-out cross-validation. All calculations and graphs were performed using the Chemoface software version 1.64.

Activity VOCs of *Induratia* isolates against *Botrytis cinerea*

The effect of VOCs produced by *Induratia* isolates on the growth of *B. cinerea* was determined using bipartite Petri dishes containing PDA medium. A mycelial disc of each endophytic strain (5 mm diameter) was cultivated for 7 days at 25 °C on one side of the plate. After this period, the pathogen was placed on the other side of the plate. The effect of volatile compounds was evaluated by measuring the average diameter of pathogen growth after 7 days of exposure to volatile compounds. The experiment was carried out in a randomized design with 13 treatments and three replicates each. The negative control contained only a pathogen plug. The fungistatic or fungicidal activity of the VOCs produced was evaluated by aseptically transferring conidia of *B. cinerea* from bipartide Petri dishes to new PDA medium.

Botrytis cinerea was cultivated on PDA medium for 7 days at 25 °C. A suspension of 1.0×10^5 conidia/mL was inoculated into the strawberries. Two inoculated strawberries were deposited in a polyethylene terephthalate box containing an open Petri dish with an *Induratia* strain previously cultivated on PDA medium for 7 days. The boxes were sealed with a plastic film and incubated at 25 °C. After 72 h, the effect of the volatile compounds produced was evaluated based on the presence or absence of pathogen growth on the inoculated strawberries. The negative control consisted of boxes with strawberries inoculated with *B. cinerea* without endophytic fungi. The experiment was carried out in a randomized design with 13 treatments, each with three replicates.

Activity of metabolites of *Induratia* isolates against pathogenic bacteria

The fungi were grown in plates containing PDA medium for 7 days at 25 °C. After growth, 5 discs approximately 5 mm in diameter from the fungal colonies were transferred to 1 L of PD medium and incubated at 25 °C in the dark at 125 rpm for 12 days. The supernatants were separated from the mycelia by vacuum filtration. The extraction was carried

out with the addition of ethyl acetate to the supernatants, in the proportion of 1:0.5 (supernatant/ethyl acetate), followed by removal of the solvent by rotary evaporation.

Extracts of *Induratia* isolates were tested against the following bacterial strains of clinical importance to determine their antibacterial activity: *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *E. faecium* ATCC 700221, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, and *Pseudomonas aeruginosa* ATCC 27853.

Each extract was diluted in 100% DMSO and filtered through a 0.22 µm filter to prepare a 100 × concentrated stock solution to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). This solution was diluted 1:100 in Cation Adjusted Mueller Hinton broth (CAMHb) (BBL Mueller–Hinton II broth; Becton, USA) to reach a final concentration of 1% DMSO according to CLSI (2015), followed by two-fold serial dilutions in 1% DMSO CAMHb to reach a range of 512 to 0.06 µg/mL in the 96 wells microplates. Bacteria colonies grown in CAMHb for 24 h were added to fresh media in tubes to adjust the suspension to 0.5 McFarland, followed by a 1:10 dilution and distribution of 5 µL pipetted in each microplate well. The tests were performed in duplicate. The results were observed after 24 h of incubation at 37 °C, and the MIC was considered the concentration at which microorganism growth inhibition could be visually observed. No extract was added to 1% DMSO CAMHb as a positive control for bacterial growth. As a negative control, only the broth was incubated to rule out any contamination.

After visual reading of the MIC, the MBC was determined using 100 µL of the following wells inoculated as a single drop on a CAMH-Agar plate: the well containing one dilution above the MIC, the well containing the MIC, and two wells containing concentrations below the MIC. The plate was incubated at 37 °C for 24 h prior to the visual observation. MBC had the lowest concentration, with no bacterial growth. The MBC/MIC ratio was calculated to classify the activity as bactericidal or bacteriostatic. Compounds with a rate less than or equal to four were considered bactericidal; rates above four indicated bacteriostatic activity (Pankey and Sabath 2004).

The biofilm eradication abilities of the extracts of *Induratia* species were evaluated as previously described by Qin et al. (2014) with some modifications. Briefly, the biofilm producers *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 8095 were cultured for 18 h in brain heart infusion broth (BHI; Kasvi, Curitiba, Brazil) supplemented with 0.75% (w/v) glucose. The bacterial suspension was adjusted to an OD600 of 1 and diluted to 1:40 in the same broth. Next, 200 µL of the bacterial dilution was added to the wells of a 96-well plate and incubated for 24 h at 37 °C. After bacterial adhesion, the plate was washed three times in PBS (pH 7.4), and the pre-formed biofilm was incubated with either fresh media (biofilm growth control) or with 512 µg/mL of the extracts, at 37 °C for 24 h. The wells were then washed with PBS before staining with crystal violet (0.2% w/v), and the extract activity was evaluated at 595 nm using a microplate reader (Polaris, Celer, Brazil). Twelve experimental replicates were performed for each extract, and the standard error of the mean was calculated. We considered biofilm reduction percentage any positive value obtained from the following formula: reduction (%) = $100 - (100 \times Abs_T/Abs_{GC})$, where Abs_{GC} is the mean of the absorbance at DO_{595} of the biofilm growth control and Abs_T is the mean of absorbance at DO_{595} of the biofilm under the fungal extract test. One-way ANOVA was used to compare the absorbance values of the extract treatments with the biofilm growth control ($p < 0.05$) was considered statistically significant.

Activity of metabolites and VOCs of *Induratia* isolates against *Meloidogyne incognita*

The effects of metabolites and VOCs of *Induratia* isolates on second-stage juveniles (J2) of *M. incognita* were evaluated. *Meloidogyne incognita* eggs were obtained from tomato roots according to the technique described by Bonetti and Ferraz (1981). To obtain J2 of *M. incognita*, a hatching chamber consisting of a 500-mesh sieve (mm) was placed in a glass funnel. Only J2 that hatched after 72 h was used in the experiments.

To prepare the filtrates, three discs (5 mm) collected from 7-day-old PDA *Induratia* colonies were inoculated in 50 mL potato dextrose (PD) medium at 25 °C and 160 rpm for 10 days. After centrifugation of the culture at 5000×*g* for 10 min, the supernatant was filtered through a 0.22 µm Durapore® membrane. The fungal filtrates were undiluted (100%) and diluted in sterile water (80%, 60%, and 40%). One hundred microliters of the filtrate was added to a 96-well microplate, and a 20 µL aliquot containing 25 individuals J2 was added to each well. Sterile water and the active ingredient of the nematicide Furadan (2,3-dihydro-2,2-dimethyl-7-benzofuranol, N-methyl-carbamate) at 200 µg/mL were used as controls. The analyses were performed by counting the percentage of immobile J2 after 24 h of incubation and after of 48 h incubation when 50 µL of NaOH (1 M) was added to each well to assess the mortality of J2 (Chen and Dickson 2000). J2 was considered dead if the mobility did not recover. Nematicidal activity was considered when all J2 of *M. incognita* remained immobile after 48 h.

To select the *Induratia* isolate producing VOCs toxic to J2 of *M. incognita*, discs of 5 mm were collected from 7-day-old PDA *Induratia* colonies, inoculated on one side of split Petri dishes containing media PDA or yeast extract supplemented (YES) incubated at 25 °C for 10 days. The J2 were

placed on the other side of the split Petri dish, sealed with plastic film, and incubated at 25 °C in the dark. After 72 h, the percentage of mobile J2 cells was determined. Culture medium without endophytic fungi was used as a control. The *Induratia* isolate producer of VOCs, which caused greater immobility in J2 of *M. incognita*, was selected for the next experiment.

The selected *Induratia* isolate was cultivated in the selected medium for 6, 9, and 12 days at 25 °C. The J2 of *M. incognita* was exposed to VOCs produced by the *Induratia* isolate for 72 h. The mobility percentage of J2 was then determined. The J2 exposure to VOCs was removed from the plate dish and inoculated into 30-day-old tomato seedlings in 75 cm^3 cell seeding trays containing substrate Plantmax® substrate. The trays were kept in a greenhouse, and the inoculated seedlings were sprayed manually whenever necessary. The number of eggs and galls per root system was quantified after 45 days, as described by Bonetti and Ferraz (1981). The control was a culture medium without *I. Coffeana* (CML 4011).

The assay was performed in five replicates for each sample. The treatments were subjected to analysis of variance using the R Statistics software, and the means were compared using the Scott and Knott test (1974), with $p < 0.05$. Data transformation (in the square root of $X+1$) was used to minimize the lack of normality of the biological data.

Results

Induratia species phylogeny

The ITS tree grouped the 12 isolates into three clades, two of which corresponded to the species *I. coffeana* ($n=7$ isolates) and *I. yucatanensis* ($n=3$). The third clade was not well resolved and contained the reference *Induratia equiseti*, *Induratia suturae*, *I. thailandica*, *I. vitigena*, and *I. ziziphi* together with *Induratia* sp. (CML 4013) and *Induratia* sp. (CML 4015) from this study (Fig. 1). In the analysis of the *RPB2* gene (Online Resource 2), *Induratia* sp. (CML 4013) and *Induratia* sp. (CML 4015) were phylogenetically related to a clade containing the types of *I. thailandica* and *I. ziziphi*. Considering the *TUB2* gene tree (Online Resource 3), the *Induratia* sp. (CML 4015) was phylogenetically more closely related to the types of *I. thailandica* and *I. ziziphi* than *Induratia* sp. (CML 4013). In the combined tree, *Induratia* sp. (CML 4013) was phylogenetically related to *Induratia* sp. (CML 4015) (Fig. 2).

The other isolates were clustered together with the *I. coffeana* reference isolate based on the *RPB2* gene tree. Using the *TUB2* gene and combined dataset trees, these isolates formed a strongly supported clade belonging to *I. coffeana* and showed polymorphism within the ITS, *RPB2*, and *TUB2* genes, indicating intraspecific variability.

Macromorphology and micromorphology

There was a slight difference in the isolate's mycelial growth in the different culture media at 25 °C with better growth in PDA medium where colony diameter ranged from 3.2 (*I. coffeana* CML 4020) to 5.2 cm (*Induratia* sp. CML 4015). When grown in PDA medium at different temperatures, some of the isolates grew better at 20 °C with colony diameters ranging from 3.4 to 7.2 cm. *Induratia coffeana* (CML 4009, CML 4012) did not grow at 30 °C, whereas *Induratia* sp. (CML 4013) had the largest colony diameter at the same temperature (Online Resource 4). Conidia and fruiting bodies were not observed in *Induratia* species.

The *I. coffeana* isolates showed high morphological plasticity. Colonies grew slowly on PDA medium, with a linear growth rate of 2.34 to 3.35 mm diameter/day at 25 °C with a 12 h photoperiod (Table 2). The colonies had weak moldy odors, different mycelial growth densities as floccose or cottony surfaces, and color colonies (surface and reverse) ranging from beige to white after 40 days (Fig. 3). The hyaline hyphae were branched, with 2.05 to 3.18 µm diameters, frequently intertwining and forming rope-like strands. Except for *I. coffeana* (CML 4017), all isolates formed coils with diameters ranging from 24.0 to 31.3 µm. Cauliflower structures were observed in some *Induratia* isolates.

The colony of *Induratia* sp. (CML 4013) grew on PDA medium, with rate of 3.31 mm diameter/day, reaching 68.9 mm diameter in 40 days at 25 °C with a 12-h photoperiod (Table 2). The colony was white on the surface with a soft beige reverse (Fig. 4). Forty-day-old colonies were soft beige in the center and white on the edge. Hyphal growth at the colony surface had a cotton-like pattern, concentric circles, and a weak moldy odor. They had rope-like hyaline hyphae with 1.99 µm diameter with coils and small compacted coil structures measuring 12.1 µm in diameter.

Induratia sp. (CML 4015) presented a slow-growth colony on PDA medium, with a daily average of 2.97 mm, reaching a 44.5 mm diameter in 15 days at 25 °C with a 12-h photoperiod (Table 2). The colony was white on the surface with a soft beige reverse (Fig. 4). Forty-day-old colonies were soft beige, with a floccose surface, 2.16 µm diameter hyaline hyphae; frequently intertwined and formed rope-like with fused coils, which measured an 18.5 µm diameter; and produced a weak moldy odor.

VOCs identification

We identified VOCs produced by all samples using SPME and GC-MS. We identified 51 VOCs consisting mainly of terpenes, alcohols, esters, and carboxylic acids, but four

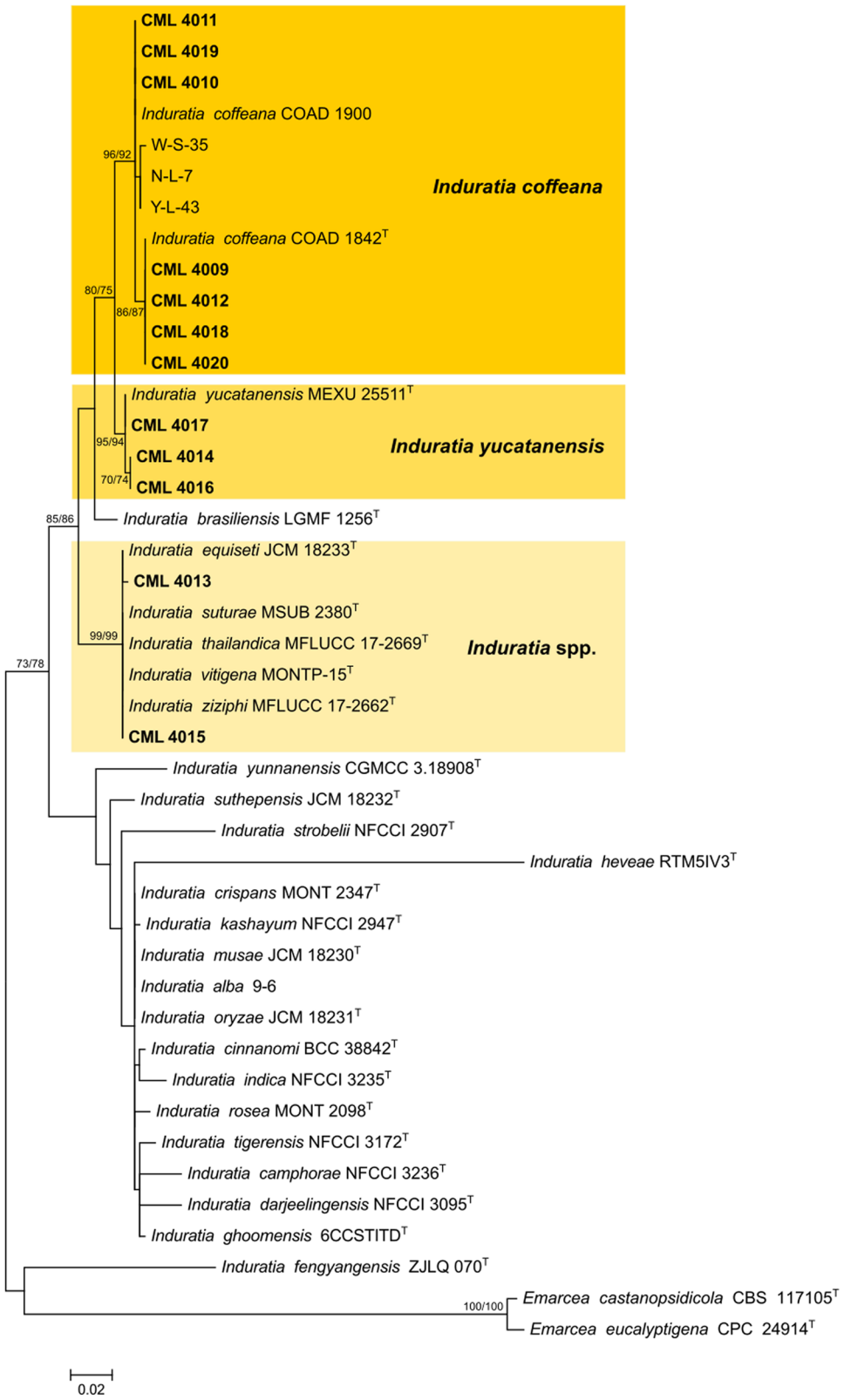


Fig. 1 Maximum likelihood phylogenetic tree based on ITS sequences showing relationships among *Induratia* species. Isolates from this study are highlighted in bold. Bootstrap values ≥ 70% (ML/MP) are shown at the internodes. The scale bar represents the expected number of nucleotide substitutions per site. The tree is rooted to *Emarcea castanopsidicola* and *E. eucalyptigena*. ^{T} = Type

peaks remained unidentified sesquiterpenes (Table 3). The mass spectra of these unidentified peaks revealed a fragmentation pattern of non-oxygenated sesquiterpenes, although the correct structure could not be assigned. The production of VOCs of the *Induratia* isolates were not identical. In samples of *I. coffeana* (CML 4017), only seven VOCs were

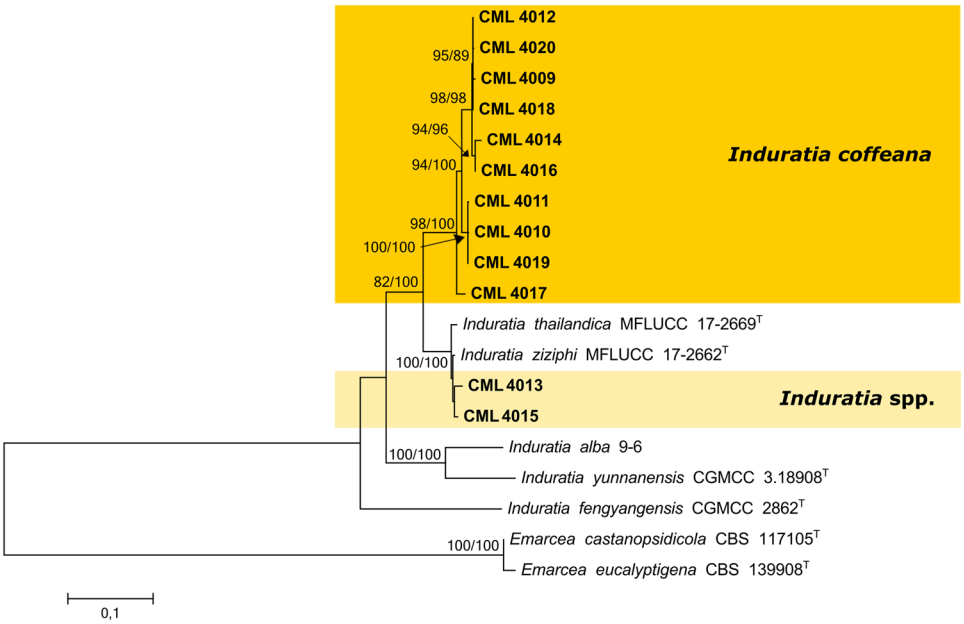


Fig. 2 Maximum likelihood phylogenetic tree based on concatenated ITS-*RPB2-TUB2* sequences showing relationships among *Induratia* species. Isolates from this study are highlighted in bold. Bootstrap values ≥ 70 (ML/MP) are shown at the internodes. The scale bar represents the expected number of nucleotide substitutions per site. The tree is rooted to *Emarcea castanopsidicola* and *E. eucalyptigena.* ^{T} = Type

detected, while in *Induratia* sp. (CML 4015) and *I. coffeana* (CML 4016), 29 were detected.

Partial least squares: discriminant analysis

PLS-DA was able to discriminate *Induratia* isolates into three different clusters. A cluster of *I. coffeana* and two clusters of *Induratia* sp., which were scattered in the scores graph, suggest that these two samples were neither *I. coffeana* nor of the same species (Fig. 5). The main compounds produced by *Induratia* isolates were as follows: (4) 1-butanol, 3-methyl- (5) 1-butanol, 2-methyl-, (12) methyl 2-methylpropanoate, (16) methyl 2-methylbutanoate, (38) β-guaiene (cis), (40) β-guaiene (trans), (42) Δ-cadinene, (45) unidentified sesquiterpene, (46) 2-methylpropanoic acid, and (47) 2-methylbutanoic acid. LV1 is classified based on the high production of (46, 47) 2-methylpropanoic and 2-methylbutanoic acids, in addition to their methyl esters, and 2-methyl butanol and 3-methyl butanol. Higher LV1 values indicate high production of carboxylic acids in samples, and lower values of LV1 denote samples with higher production of 2-methyl butanol and 3-methyl butanol (Fig. 5). The LV2 order follows the production of some sesquiterpenes, in which a high value of LV2 indicates high production of these compounds (38, 40, 42, and 45), mainly by *Induratia* sp. (CML 4015), whereas *Induratia* sp. (CML 4013) produced more carboxylic acids (compounds 46 and 47) (Fig. 5).

Activity of *Induratia* species VOC's in *B. cinerea*

The isolates *I. coffeana* (CML 4009, CML 4010, CML 4011, CML 4012, and CML 4019) completely inhibited *B. cinerea* growth compared to the control, while the other isolates partially inhibited the growth of the pathogen (Table 4). The transfer of pathogen conidia to new PDA media showed fungicidal activity against *B. cinerea* of *I. coffeana* isolates (CML 4010, CML 4011, CML 4019). When strawberries were inoculated with *B. cinerea*, the VOCs produced by *I. coffeana* (CML 4019) showed an effective biofumigation effect and caused smaller decay of the infected tissues at 25 °C for 3 days compared to the fruits of the control (Fig. 6).

Antibacterial activity of *Induratia* species extracts

The *I. coffeana* (CML 4019) extract showed antibacterial activity against *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *E. faecium* ATCC 700221 with MICs of 512 µg/mL. The extracts of other *Induratia* isolates did not inhibit the tested bacteria at 512 µg/mL. We were only able to determine the MBC of *E. faecium* ATCC 700221 (512 µg/mL), indicating bactericidal activity of *I. coffeana* (CML 4019) for this species. We could not identify the extract activity for the other bacteria because the MBC was higher than the highest concentration tested.

We assessed the ability of the *Induratia* isolates to reduce pre-formed biofilms of *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 8095 (Table 4), even with no evidence of antibacterial activity in the planktonic form at 512 µg/mL. Only the *I. coffeana* (CML 4009) extract eradicated the pre-formed biofilm of *S. aureus* ATCC 8095 (100% reduction). The extracts produced by *I. coffeana* (CML 4011, CML 4016, CML 4017) and *Induratia* sp. (CML 4013, CML 4015) also reduced the pre-formed biofilm of *S. aureus*

Table 2 Morphological characteristic of *Induratia* isolates on PDA medium at 25 °C

Induratia Isolates	Colony diameter (mm)			Mycelium pigment production	Hyphal growth at colony front	Mycelial growth	Hyphae (µm)	Coins (µm)
	15 days	Growth per day	40 days					
I. coffeana CML 4009	50.3	3.35	70.4	Soft beige in the center and white on the edge	Cottony-like pattern, concentric circles	Hyphae frequently intertwining and forming rope-like with coils appear as fused, and forming cauliflower structures	2.05	24.9
I. coffeana CML 4010	35.0	2.34	55.9	Beige (salmon)	Flocculose surface	Rope-like with coils and cauliflower structures, and swollen cell	3.14	31.3
I. coffeana CML 4011	40.5	2.70	69.9	Soft beige in the center and white on the edge	Cottony-like pattern	Hyphae frequently intertwining and forming rope-like with coils appear as fused	2.46	28.1
I. coffeana CML 4012	49.0	3.27	78.8	Soft beige in the center and white on the edge	Flocculated cottony-like pattern	Hyphae frequently intertwining and forming rope-like with coils appear as fused	2.24	26.3
I. coffeana CML 4014	42.0	2.80	68.9	Soft beige in the center and white on the edge	Flocculose surface, concentric circles	Hyphae frequently intertwining and forming rope-like with coils appear as fused, and forming cauliflower structures	3.13	27.9
I. coffeana CML 4016	40.5	2.70	75.4	Soft beige	Flocculose surface, concentric circles	Hyphae frequently intertwining and forming rope-like with coils appear as fused, and forming cauliflower structures	2.61	29.2
I. coffeana CML 4017	46.5	3.10	74.4	White	Smooth cottony-like pattern, concentric circles	Hyphae frequently intertwining and forming rope-like with coils appear as fused and forming cauliflower structures, and swollen cell	3.18	-
I. coffeana CML 4018	39.7	2.65	56.4	Soft beige in the center and white on the edge	Cottony-like pattern	Rope-like with coils and cauliflower structures	2.90	28.5
I. coffeana CML 4019	42.3	2.82	70.2	Beige (salmon)	Cottony-like pattern	Hyphae frequently intertwining and forming rope-like with coils appear as fused, and forming cauliflower structures	2.43	28.4
I. coffeana CML 4020	38.1	2.54	74.0	White	Flocculated cottony-like pattern, concentric circles	Hyphae frequently intertwining and forming rope-like with coils appear as fused, and forming cauliflower structures	3.02	24.0
Induratia sp. CML 4013	49.6	3.31	68.9	Soft beige in the center and white on the edge	Cottony-like pattern, concentric circles	Rope-like with coils and with small and compacts coils structures	1.99	12.1
Induratia sp. CML 4015	44.5	2.97	72.9	Soft beige	Flocculose surface	Hyphae frequently intertwining and forming rope-like with coils appear as fused	2.16	18.5

Data are expressed as mean ± standard deviation

ATCC 8095. The pre-formed biofilm of *S. epidermidis* ATCC 35984 was reduced by extracts of *I. coffeana* (CML 4009, CML 4011, CML 4012, CML 4017, CML 4020) and *Induratia* sp. (CML 4013, CML 4015) (Table 4).

Activity of metabolites and VOCs of *Induratia* species against *M. incognita*

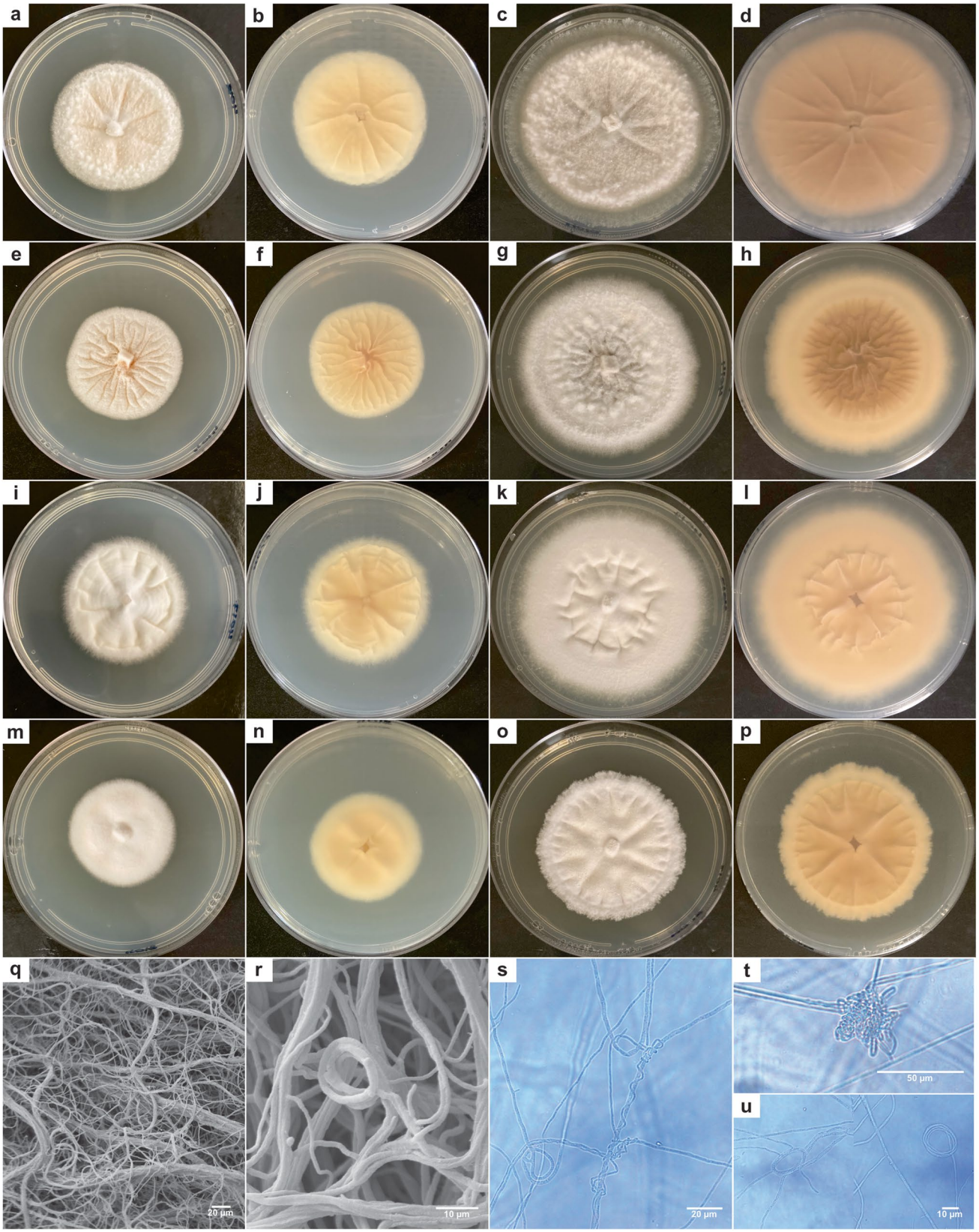
The metabolites produced by most *Induratia* isolates caused toxicity in J2 of *M. incognita*. Except for *I. coffeana* (CML 4012, CML 4014, CML 4020), nematicidal activity was observed in undiluted filtrates produced by other *Induratia* isolates, causing 100% J2 immobility after 24 h of exposure and 100% J2 mortality after adding NaOH at 48 h (Table 5). Although the percentage of immobility remained high for most of the filtrates produced by *Induratia* isolates, the percentage of mortality was reduced with the dilution of the filtrates.

The VOCs produced by *Induratia* isolates in YES medium were more toxic than those produced in PDA medium because of the highest reduction in the mobility of J2 of *M. incognita*. The VOCs produced by *I. coffeana* (CML 4011) showed the highest toxic effect on J2, with a motility of 28% (Table 5). When this isolate was cultivated in YES medium for 6, 9, and 12 days, there was no significant difference in the percentage of mobility of J2 after 72 h of exposure to VOCs, with a reduction of at least 84% in J2 mobility (Table 6). The J2 of *M. incognita* recovered from exposure to VOCs produced by *I. coffeana* (CML 4011) lost the ability to infect tomato roots after 45 days and did not form galls in these plants. A significant reduction in the number of eggs of *M. incognita* was also observed after exposure to VOCs from *I. coffeana* (CML 4011) cultivated in12 and 9 days. Exposure to VOCs produced after 6 days of fungal culture fully suppressed the development of nematodes without gall and egg formation in tomato plants *in vivo*.

Discussion

During our investigation of the endophytic fungi in coffee plants grown spontaneously in a secondary forest in Minas Gerais state, Brazil, we found 12 isolates belonging to the genus *Induratia* identified by molecular phylogeny based on the ITS, *RPB2*, and *TUB2* gene sequences. Although the ITS rDNA gene infers phylogenetic relationships to some Xylariales genera, it provides insufficient resolution for *Induratia* species (Stadler et al. 2013, 2020; Chen et al. 2019; Samarakoon et al. 2020). The inclusion of the *RPB2* and *TUB2* sequences provided informative characters, and the three-loci phylogeny consistently distinguished the *Induratia* species (Chen et al. 2019; Samarakoon et al. 2020). However, there are few *RPB2* and *TUB2* gene sequences available in GenBank. *Induratia* isolates (CML 4014, CML 4016, CML 4017) clustered with the *I. yucatanensis* reference isolate (González et al. 2009) in the ITS tree, as there is only a sequence of this region deposited in GenBank. In the studies by Monteiro et al. (2020), Bastos et al. (2020), and Mota et al. (2021), the same *Induratia* species isolated from coffee plants were identified using only ITS phylogeny and reported as *I. yucatanensis*. However, when the *RPB2* and *TUB2* sequences were used, the *Induratia* isolates (CML 4014, CML 4016, CML 4017) clustered with *I. coffeana.* This result can be reforced if we compare the morphologies of these three isolates with the isolate described by Gonzáles et al. (2009). *Induratia yucatanensis* showed a smaller colony diameter than the three *Induratia* isolates (CML 4014, CML 4016, and CML 4017). Furthermore, the authors reported a strong odor in colonies, and they did not describe the presence of cauliflower structures that were present in *Induratia* species (CML 4014, CML 4016, CML 4017). Two *Induratia* isolates were identified as *Induratia* sp. (CML 4013 and CML 4015). The alignment without gaps in the nucleotide sequences of the three loci revealed that the *Induratia* sp. (CML 4013) differs from *Induratia* sp. (CML 4015) in 7/429 nucleotides in the ITS sequence, 7/865 nucleotides in the *RPB2* gene, and 25/698 nucleotides in the *TUB2* gene. These two isolates were phylogenetically most closely related to *I. equiseti*, *I. suturae*, *I. vitigena*, *I. thailandica*, and *I. ziziphi* based on the ITS tree. However, the sequences of the *RPB2* and *TUB2* genes of *I. equiseti*, *I. suturae*, and *I. vitigena* are not available in GenBank, preventing species identification of *Induratia* sp. (CML 4013) and *Induratia* sp. (CML 4015), which could be putative new *Induratia* species. Moreover, we observed differences in cultural characteristics and the VOCs produced between these isolates and those species that were phylogenetically most closely related.

The morphological characteristics of *Induratia* species were based on colony and mycelial characteristics, as reproductive structures have never been observed in any culture medium (Strobel et al. 2001; Zhang et al. 2010; Chen et al. 2019; Mao et al. 2018). However, Samarakoon et al. (2020) found the sexual state of two specimens from Thailand, *I. ziziphi* and *I. thailandica*. In our study, the *Induratia* isolates showed colonies ranging from white to beige, smooth, or flocculated with branched hyphae forming coils and cauliflower structures when cultivated at 25 °C on PDA medium. *Induratia* sp. (CML 4013 and CML 4015) showed a morphology similar to that of *I. coffeana*, but with smaller coil structures. In addition, we used other culture media, including CMA, which is known to stimulate sporulation and production of the sexual state. However, none of the isolates produced sexual or asexual spores. The optimal growth temperature range for the tested *Induratia* isolates was 20–25 °C, with ten isolates grow at 30 °C.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◂**Fig. 3** Morphology of *Induratia coffeana*. *I. coffeana* (CML 4012) colony on PDA medium after 15 days, surface (**a**) and reverse (**b**) after 45 days surface (**c**) and reverse (**d**). *I. coffeana* (CML 4014) colony on PDA medium after 15 days, surface (**e**) and reverse (**f**) after 45 days surface (**g**) and reverse (**h**). *I. coffeana* (CML 4017) colony on PDA medium after 15 days, surface (**i**) and reverse (**j**) after 45 days surface (**k**) and reverse (**l**). *I. coffeana* (CML 4018) colony on PDA medium after 15 days, surface (**m**) and reverse (**n**) after 45 days surface (**o**) and reverse (**p**). *I. coffeana* (CML 4014) hyphae frequently intertwining and forming rope-like (**q**, **s**), coils (**r**, **u**), cauliflower structures (**t**)

The genus *Induratia* produces VOCs known for its lethal effects against a wide variety of pathogenic fungi (Strobel 2018; Kaddes et al. 2019). Analysis and comparison of these compounds have been described as important tools for chemotaxonomic characterization (Kudalkar et al. 2012; Suwannarach et al. 2013; Siri-udom et al. 2016; Saxena and Strobel 2020). However, as secondary metabolite profiles may have taxonomic significance, in addition to analyses using gas chromatography coupled with mass spectrometry (GC-MS), it is recommended to include methods such as high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy and a series of cultures of isolates in different culture media and the growth phase (Samarakoon et al. 2020). Stadler et al. (2014) showed that *Daldinia* spp. produce specific secondary metabolites at high concentrations that can be taxonomically significant. In this study, we found that *I. coffeana* and *Induratia* sp. produced different VOCs. This indicates that the VOCs might not be correlated with their phylogenetic assignment, at least among these *Induratia* isolates. However, PLS-DA was able to discriminate *Induratia* isolates into three different clusters: *I. coffeana* isolates, *Induratia* sp. (CML 4013), and *Induratia* sp. (CML 4015). Some VOCs, such as 2-methylpropanoic acid, have already been identified in other *Induratia* species that exhibit antifungal and antibacterial activities, while other compounds such as naphthalene and thujopsene produced by *Induratia* species with anti-insect and antifungal bioactivity were absent in our isolates (Daisy et al. 2002; Kudalkar et al. 2012; Suwannarach et al. 2013).

Induratia isolates obtained from Brazilian coffee plants have already been evaluated against phytopathogenic fungi. *Induratia coffeana* (CML 4019) inhibited the growth of three pathogenic fungi and reduced the symptoms of anthracnose, white mold, and angular leaf spot when re-inoculated in common bean plants (Mota et al. 2021). We also verified that the VOCs produced by *I. coffeana* (CML 4019) completely inhibited the growth of *B. cinerea* in PDA medium and strawberry fruits and reduced fruit decay at room temperature. This pathogen causes gray mold in strawberries, affecting fruit in the field, storage, transport, and market, leading to significant economic losses (Petrasch et al. 2019). Yalage Don et al. (2020) exposed *B. cinerea* to a mixture of VOCs (ethanol (ethyl alcohol), 2-methyl-1-propanol (1-propanol, 2-methyl-), 3-methyl-1-butanol (1-butanol, 3-methyl), and 2-phenyl ethanol (phenylethyl alcohol) produced by *Aureobasidium pullulans*. The authors verified the induced electrolyte loss and oxidative stress in this pathogen. The VOCs 3-methyl-1-butanol produced by the isolate *Candida intermedia* C410 has also been reported to inhibit mycelial growth and conidial germination of *B. cinerea* (Huang et al. 2011). All these VOCs were produced by *I. coffeana* (CML 4019), and future investigations could confirm whether the biofumigation was the result of one or more VOCs produced by this isolate. *I. coffeana* (CML 4019) is a promising candidate for biological control, mainly during the postharvest period.

Because bacterial pathogens require new antibiotics due to multidrug resistance worldwide, we decided to search for new compounds in extracts of *Induratia* isolates. We used four Gram-negative and four Gram-positive bacteria that belong to the ESKAPE group and are responsible for most healthcare-associated infections world, with multidrug resistance profiles (Santajit and Indrawattana 2016). The inhibition of any of the tested pathogens is significant because of the complications resulting from infections associated with them (Minarini et al. 2020). The *I. coffeana* (CML 4019) extract showed activity against the Gram-positive bacteria *S. aureus*, *E. faecalis*, and *E. faecium*. *Staphylococcus aureus* is an opportunistic pathogen that causes a variety of diseases in humans and other animals, and the treatment of these infections is a challenge due to the emergence of multidrug-resistant strains and their ability to escape the host's immune system attack (Rossi et al. 2014). Enterococci have emerged as the leading cause of antibiotic-resistant infections and are currently ranked among the most common nosocomial pathogens infecting the urinary tract, surgical sites, and the bloodstream, especially in immunocompromised hosts (Richards et al. 2000; Shepard and Gilmore 2002). Despite being commensal, *E. faecalis* has traits that turn it into an opportunistic pathogen (Shankar et al. 2002).

We also assessed the ability of the extracts to reduce preformed biofilms of *S. aureus* and *S. epidermidis*. The *I. coffeana* (CML 4009) extract eradicated the pre-formed biofilm of *S. aureus* ATCC 8095 (100% reduction) and reduced the biofilm of *S. epidermidis* ATCC 35984. Some extracts produced by *I. coffeana* and *Induratia* sp. also reduced the preformed biofilm of two bacteria. These extracts are sources of compounds able, for instance, to inhibit quorum sensing or the production of EPS and disperse the biofilm leading the bacterial cells to a more vulnerable phenotype. Compounds that reduce or disperse biofilms can be used in combination with antibiotics to treat infections (Algburi et al. 2017). Extracts with action in just one species suggest that these mechanisms are more specific, while extracts with action in

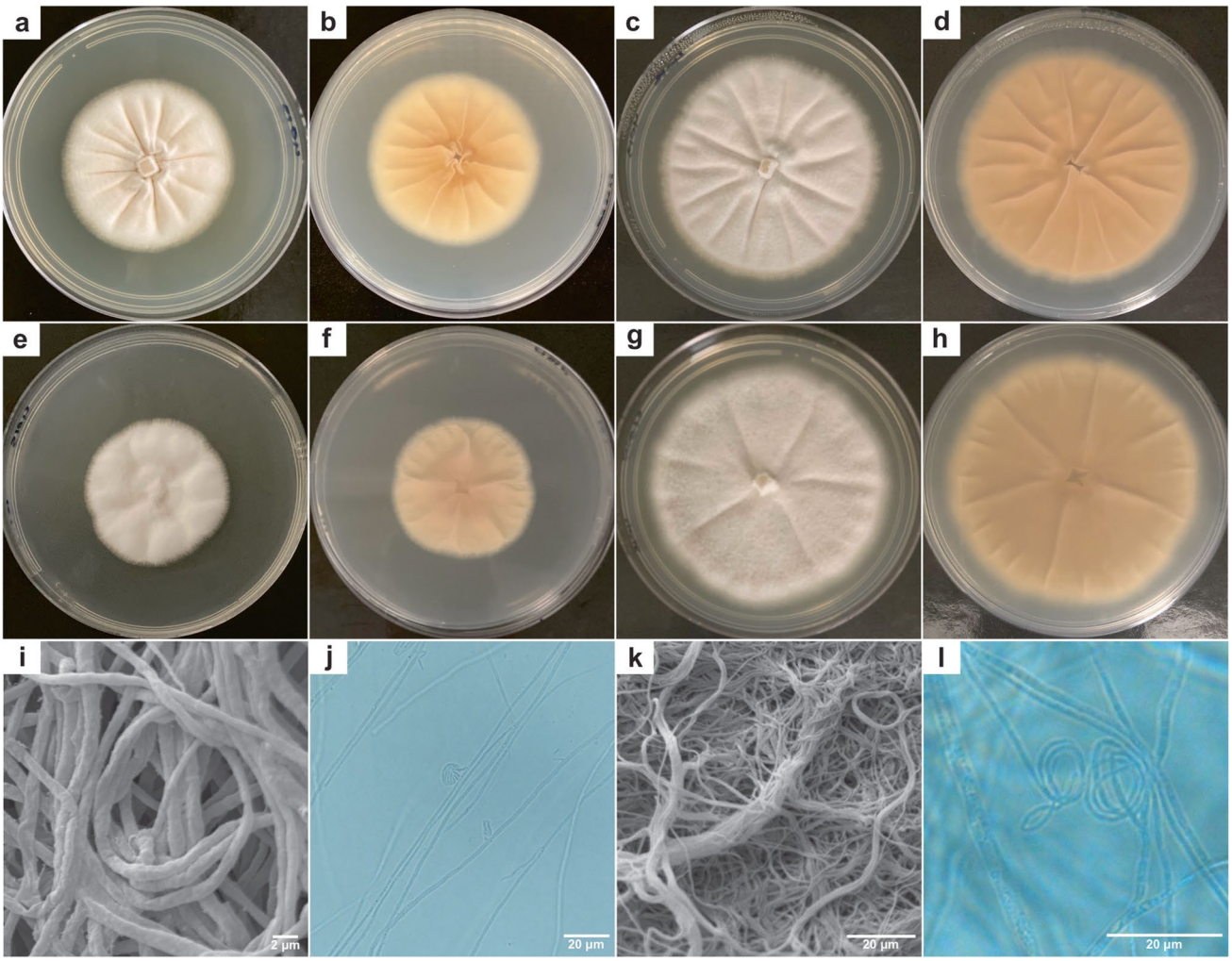

Fig. 4 Morphology of *Induratia* sp. (CML 4013). Colony on PDA medium after 15 days, surface (**a**) and reverse (**b**). Colony on PDA medium after 40 days growth, surface (**c**) and reverse (**d**). Rope-like with coils and with small and compacts coils structures (**i**), cauliflower structures (**j**). Morphology of *Induratia* sp. (CML 4015). Colony on PDA medium after 15 days growth, surface (**e**) and reverse (**f**). Colony on PDA medium after 40 days, surface (**g**) and reverse (**h**). Hyphae frequently intertwining (**k**), and coils appear as fused (**l**)

both likely have a broad mechanism, such as in targets present in different species. Although we have no information regarding the nature of the compounds, this study indicates the presence of antibiotic substances with the potential to be used in the medical field.

Meloidogyne incognita is among the most injurious phytonematodes, particularly tomato crops, causing high harvest losses in Brazil and worldwide (Machado 2014). An alternative control technique is microorganisms that have the capacity to parasitize eggs, juvenile forms, and sometimes even adult nematodes (Lopes et al. 2016). The VOCs produced by *Induratia* species caused mortality or immobility of J2 of *M. incognita*, and distinct compounds may have different modes of action on the nematode body. The compound 2-butanone produced by the deep-sea bacterium *Virgibacillus dokdonensis* MCCC 1A00493 exhibited repellent activity *in vitro* against *M. incognita* (Huang et al. 2020). *Induratia coffeana* (CML 4012) and *Induratia* sp. (CML 4013) also produced this compound, which likely caused J2 immobility. In previous studies, Grimme et al. (2007) reported that a mixture of the synthetic components of *I. albus* VOCs (1-butanol, 2-methyl, 1, butanol, 2-methyl acetate, propanoic acid, and 2-methyl propyl ester) protected tomatoes from the root-knot nematode *M. incognita*. *Induratia* isolates produced many of these VOCs, which were lethal to *M. incognita* juveniles after 24 h of exposure. In our study, *I. coffeana* (CML 4011) showed nematicidal activity against both VOCs and filtrate. VOCs produced by this isolate reduced infectivity and nematode reproduction in tomato plants, and studies are necessary to identify the molecule or a mixture that is toxic to *M. incognita*.

Table 3 Volatile organic compounds produced by *Induratia* isolates and extracted and identified by SPME–GC-MS

Compound number	Chemical class/compound	RI (exp.)[1]	RI (lit.)[2]	*Induratia* isolates											
				4009	4010	4011	4012	4014	4016	4017	4018	4019	4020	4013	4015
	Alcohols														
1	Ethyl alcohol	–	–	x	x	x	x	x	x		x	x	x	x	x
2	1-Propanol	–	558										x		
3	1-Propanol, 2-methyl-	626	622	x	x	x	x	x	x		x	x	x	x	x
4	1-Butanol, 3-methyl-	734	734	x	x	x	x	x	x		x	x	x	x	x
5	1-Butanol, 2-methyl-	735	738	x	x	x	x	x	x		x	x	x	x	x
6	1-Pentanol	765	768			x									
7	1-Hexanol	869	867			x			x						
8	2-Furanmethanol	872	866		x	x		x			x	x		x	
9	2-Nonanol	1102	1098		x				x						
10	Phenylethyl Alcohol	1113	1114	x	x	x	x	x	x		x	x	x	x	
	Esters														
11	Ethyl acetate	612	613					x	x				x		x
12	Methyl 2-methylpropanoate	681	685	x	x	x	x	x	x		x	x	x	x	x
13	Methyl 2,2-dimethylpropanoate	717	–						x			x			
14	Ethyl 2-methylpropanoate	752	762		x			x	x				x	x	x
15	2-Methylpropyl acetate	768	767		x	x		x	x			x	x	x	x
16	Methyl 2-methylbutanoate	771	771	x	x	x	x	x			x			x	x
17	Butyric acid, thio-, S-methyl ester	845	–	x	x	x						x			
18	3-Methylbutyl acetate	874	876		x	x		x	x			x	x	x	x
19	2-Methylbutyl acetate	876	880		x	x		x	x		x	x	x	x	x
20	3-Methyl-3-butenyl acetate	882	885												x
21	Ethyl-1-hexyl acetate	1147	1153		x	x		x	x		x			x	x
22	Phenylethyl acetate	1253	1256												x
	Terpenes														
23	α-Phellandrene	1006	1005			x					x	x			
24	α-Terpinene	1016	1014								x				
25	Cimene	1024	1023			x						x			
26	β-Phellandrene	1030	1025	x	x	x					x	x		x	
27	o-Guaicol	1085	1087						x						
28	Terpineol	1125	1130								x				
29	Cis-Piperitol	1197	1196								x				
30	Sesquiterpene[3]	1316	-												x
31	Longifolene (iso)	1387	1390						x				x	x	x
32	β-Elemene	1392	1390						x				x		x
33	β-Cedrene	1421	1420					x	x		x		x		

Table 3 (continued)

Compound number	Chemical class/compound	RI (exp.)[1]	RI (lit.)[2]	*Induratia* isolates											
				4009	4010	4011	4012	4014	4016	4017	4018	4019	4020	4013	4015
34	α-Bergamotene	1438	1436	x	x	x	x	x	x		x	x	x	x	
35	α-Guaiene	1441	1439		x		x	x	x	x	x		x	x	x
36	Sesquiterpene[3]	1487	–												x
37	Sesquiterpene[3]	1488	–	x	x	x	x		x		x	x	x	x	
38	β-Guaiene (cis)	1493	1492					x	x	x	x		x		x
39	α-Selinene	1499	1498						x						
40	β-Guaiene (trans)	1506	1502										x		x
41	α-Bulnesene	1506	1505		x		x	x	x	x	x		x	x	x
42	Δ-Cadinene	1523	1522												x
43	α-Calacorene	1548	1544												x
44	α-Epoxy-cedrene	1577	1574												x
45	Sesquiterpene[3]	1657	–	x	x	x	x	x	x	x	x	x	x	x	x
	Acids														
46	2-Methylpropanoic acid	810	793	x	x	x	x	x	x		x	x	x	x	x
47	2-Methylbutanoic acid	889	884	x	x	x	x	x			x	x		x	x
	Aldehydes														
48	Pentanal	698	697							x					
49	Pentanal, 2-methyl-	744	746							x					
50	Hexanal	801	800	x						x					x
	Ketones														
51	2-Butanone	601	597				x							x	
52	4-Heptanone	869	871		x		x		x				x		
53	2-Nonanone	1090	1091		x			x	x			x	x		
	Others														
54	2-Nonene, (E)-	905	903		x										
55	Benzene, methoxy-	915	917								x				

x compound identified in sample

[1]Experimental retention index

[2]Literature retention index

[3]Typical mass spectra of sesquiterpene

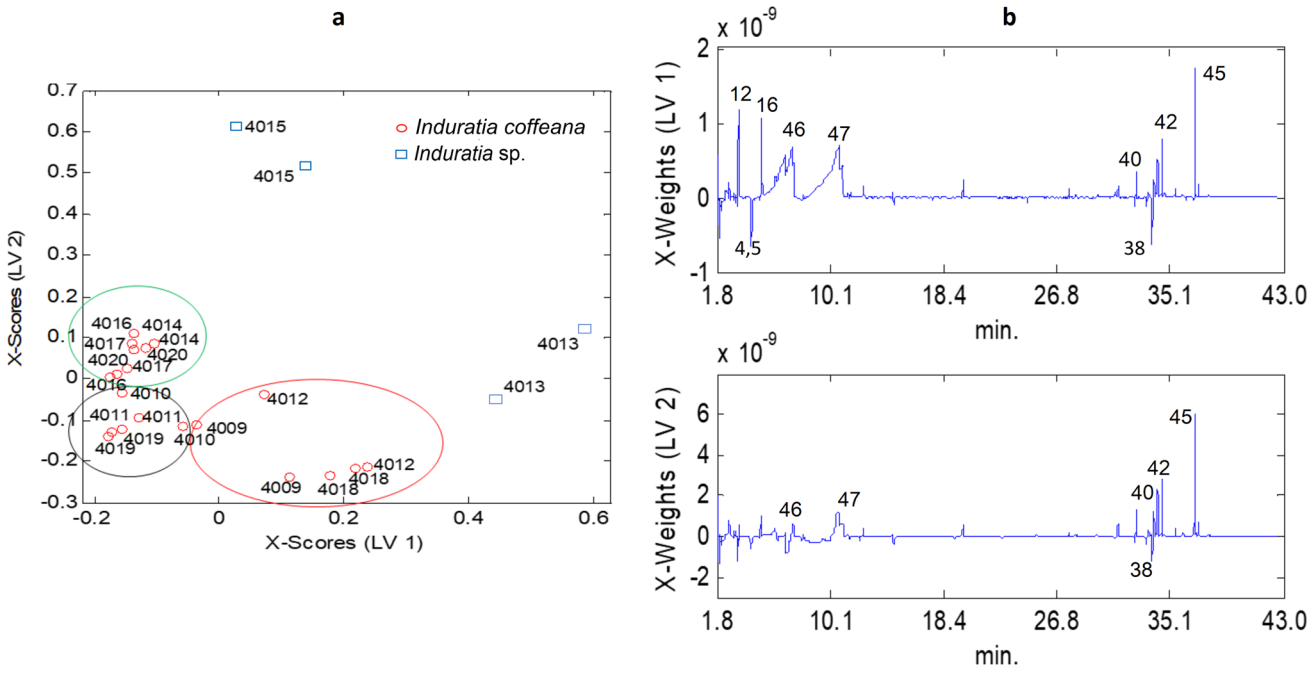

Fig. 5 PLS-DA scores (**a**) and weights (**b**) for the volatiles organic compounds from *Induratia coffeana* and *Induratia* sp.

Table 4 Effect of VOCs produced by *Induratia* isolates on the growth of *Botrytis cinerea* and of extracts (512 µg/mL) in biofilm reduction of *Staphylococcus epidermidis* and *Staphylococcus aureus*

Induratia isolates	Volatile compounds		Extracts	
	B. cinerea colony (cm)	Activity	*S. epidermidis* ATCC 35984 (%)	*S. aureus* ATCC 8095 (%)
I. coffeana (CML 4009)	0.00a	Fungistatic	34 ± 7*	100 ± 2*
I. coffeana (CML 4010)	0.00a	Fungicidal	N.R.O	N.R.O
I. coffeana (CML 4011)	0.00a	Fungicidal	51 ± 5*	20 ± 8**
I. coffeana (CML 4012)	0.00a	Fungistatic	59 ± 5*	N.R.O
I. coffeana (CML 4014)	1.33b	Fungistatic	N.R.O	N.R.O
I. coffeana (CML 4016)	1.50b	Fungistatic	N.R.O	43 ± 11**
I. coffeana (CML 4017)	2.16b	Fungistatic	21 ± 4**	46 ± 8*
I. coffeana (CML 4018)	1.50b	Fungistatic	N.R.O	N.R.O
I. coffeana (CML 4019)	0.00a	Fungicidal	N.R.O	N.R.O
I. coffeana (CML 4020)	1.50b	Fungistatic	20 ± 8**	N.R.O
Induratia sp. (CML 4013)	1.33b	Fungistatic	36 ± 9**	33 ± 9**
Induratia sp. (CML 4015)	2.00b	Fungistatic	9 ± 5**	55 ± 7*

VOCs-the means followed by the same letters do not differ by Scott-Knott's test at 5% probability

Extracts-N.R.O-no reduction observed; the results are means ± standard errors of the means of 12 replicates. *$p < 0.0001$, **$p < 0.05$

In conclusion, ten *I. coffeana* and two *Induratia* sp. associated with Brazilian coffee showed slight morphological differences and produced VOCs consisting mainly of terpenes, alcohols, esters, and carboxylic acids. These VOCs showed toxicity against *B. cinerea* and *M. incognita*, indicating the potential of the isolates as a biofumigant. In addition, it was observed that some *Induratia* isolates were able to disperse the pre-formed biofilm of *S. aureus* and *S. epidermidis*, indicating the production of promising compounds of clinical interest that, once identified, would be worth purifying and checking their activity and selectivity in pure state.

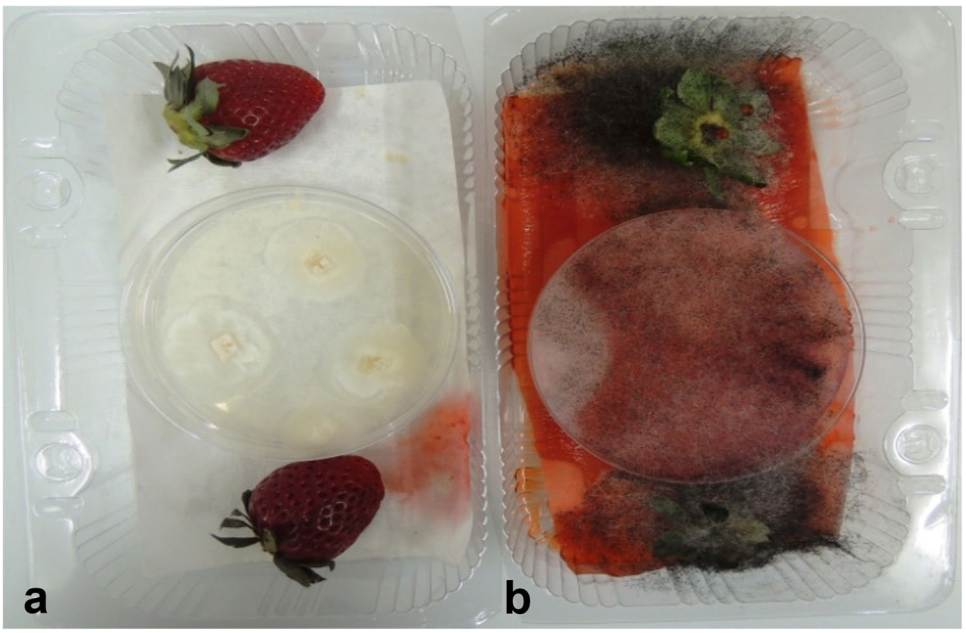

Fig. 6 Volatile compounds produced by *Induratia coffeana* at 25 °C after 3 days. Strawberries infected with *B. cinerea* with *I. coffeana* (CML 4019) in PDA medium (**a**). Strawberries infected with *B. cinerea* with PDA medium (**b**)

Table 5 Immobility and mortality percentage second-stage juveniles of *Meloidogyne incognita* exposure to filtrates of *Induratia* isolates and mobility percentage after 72-h exposure to volatile compounds of *Induratia* isolates in PDA and YES media

Induratia isolates	Filtrates concentrations immobility (24 h) and mortality (48 h)								Volatile compounds	
	100%		80%		60%		40%			
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	YES	PDA
I. coffeana CML 4009	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	4^{c}	100^{a}	12^{b}	84.6^{d}	95.2^{b}
I. coffeana CML 4010	100^{a}	100^{a}	100^{a}	100^{a}	96.8^{a}	89.82^{a}	99.2^{a}	14.92^{b}	48^{b}	84.5^{a}
I. coffeana CML 4011	100^{a}	100^{a}	89.6^{b}	96^{a}	90.4^{a}	94.9^{a}	99.2^{a}	81.72^{a}	28^{a}	97.2^{b}
I. coffeana CML 4012	80^{a}	80^{b}	99.2^{a}	100^{a}	99.2^{a}	71.17^{a}	99.2^{a}	12.12^{b}	44.7^{b}	86.5^{a}
I. coffeana CML 4014	100^{a}	42.93^{c}	100^{a}	4.8^{c}	80.8^{b}	0.8^{c}	45.6^{c}	0.8^{c}	68^{c}	98.2^{b}
I. coffeana CML 4016	100^{a}	100^{a}	100^{a}	96^{a}	100^{a}	9.74^{c}	95.2^{a}	0.8^{c}	77.5^{c}	96.6^{b}
I. coffeana CML 4017	100^{a}	100^{a}	100^{a}	55.25^{b}	80^{b}	10.48^{c}	99.2^{a}	2.47^{c}	65.2^{c}	79.7^{a}
I. coffeana CML 4018	100^{a}	99.2^{a}	100^{a}	96^{a}	99.2^{a}	78.92^{a}	85.6^{b}	71.58^{a}	45.5^{b}	98.7^{b}
I. coffeana CML 4019	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	5.6^{c}	100^{a}	3.2^{c}	48^{b}	79.2^{a}
I. coffeana CML 4020	100^{a}	83.19^{b}	100^{a}	48.7^{b}	58.4^{c}	19.37^{b}	0.8^{d}	19.33^{b}	68.7^{c}	80.7^{a}
Induratia sp. CML 4013	100^{a}	96^{a}	100^{a}	7.65^{c}	100^{a}	0.95^{c}	100^{a}	13.32^{b}	53.7^{b}	93.7^{b}
Induratia sp. CML 4015	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	28.65^{b}	100^{a}	0.8^{c}	79.5^{c}	96.5^{b}
Control									99.2^{e}	98.7^{b}
Furadan	100^{a}	100^{a}								
Water	23.67^{b}	9.46^{a}								

The means in the column followed by the same letters do not differ by Scott-Knott's test at 5% probability

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Author contributions PGC designed the project, supervised its execution, and wrote, reviewed, and edited the manuscript; OLP, MVdQ, and MCP contributed with collection of material, isolation, identification, and preservation of fungal isolates and reviewed the manuscript; UGdPL and EAG contributed to the sequencing of gene sequences and reviewed the manuscript; DGT contributed with morphological

Table 6 Mobility percentage of second-stage juveniles of *Meloidogyne incognita* exposure to 72 h of VOCs of *Induratia coffeana* (CML 4011) cultivated during 6, 9, and 12 days in YES medium and average values of number of galls and eggs in root of tomatoes

Variables	12 days		9 days		6 days	
	I. coffeana (CML 4011)	Control	*I. coffeana* (CML 4011)	Control	*I. coffeana* (CML 4011)	Control
Mobility (%)	15.4 ± 6.7^{a}	95 ± 3.7^{c}	13 ± 11.4^{a}	95 ± 3.5^{c}	14.9 ± 6.4^{a}	58 ± 8.8^{b}
Number of galls per root system	0 ± 0^{a}	30 ± 13.9^{c}	0 ± 0^{a}	14.4 ± 9.1^{b}	0 ± 0^{a}	$13{,}7 \pm 7.4^{b}$
Number of eggs	37.2 ± 21.7^{a}	403 ± 380^{b}	54.9 ± 34^{a}	201.3 ± 58.2^{a}	0 ± 0^{a}	623.7 ± 515^{b}

Data are expressed as mean ± standard deviation. The means in the line followed by the same letters do not differ by Scott-Knott's test at 5% probability

characterization and the assessment of anti-nematodes activity of *Induratia* isolates; SdSCG contributed with sequence analysis, preparation of phylogenetic trees, and overall analysis of the data and wrote, reviewed, and edited the manuscript; MPP and CAN contributed with characterization of VOCs and reviewed the manuscript; ILBCC, DKRB, BM, and ISeC contributed with the assessment of antibacterial activity of *Induratia* extracts in bacterial pathogens and reviewed the manuscript. All authors commented on the previous versions of the manuscript and approved the final version of the manuscript.

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Data availability The biological reference material was deposited and available in official collections and DNA sequences and alignments at GenBank and TreeBASE, respectively.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

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