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Richness of Nigrospora spp. (Apiosporaceae) in Manihot esculenta Cranz in Brazil and the description of three new species

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Abstract

Manihot esculenta Crantz, commonly known as cassava, is an important staple food in developing countries in Africa, Asia and Latin America. Despite its relevance, few studies in search of endophytic fungi have been carried out on this plant. *Nigrospora* spp. has a widespread range of hosts and can be found as saprobes, endophytes, and pathogens. *Nigrospora* has already been isolated from cassava plants in previous studies. However, its identification was made only at the genus level. In this study, healthy cassava stems were collected in the northeast region of Brazil from where specimens of this genus were obtained. The isolates were identified based on morphological features and phylogenetic analyses of internal transcriber space (ITS), part of the translation elongation factor 1 alpha (*TEF1-α*), and β-tubulin fragment (*TUB2*) genic regions. As a result, we report two new geographic occurrences of *N. pyriformis*, and *N. vesicularifera*, four new records *N. hainanensis*, *N. lacticolonia*, *N. pyriformis*, and *N. vesicularifera* associated with cassava, and the description of three new species named *N. endophytica*, *N. manihoticola* and *N. pernambucoensis*.

Introduction

Originally from South America, *Manihot esculenta* Cranz (Euphorbiaceae) is cultivated in more than 100 countries distributed throughout the tropical and subtropical zones (Carvalho 2006; FAO 2013, 2017). Commonly known as cassava, it is a perennial, shrubby plant that exhibits tuberous roots capable of storing large amounts of starch, which gives the plant a high energy value and importance for food security, especially in developing countries (Aguiar et al. 2013; FAO 2013).

Over the years, studies exploring the endophytic fungi community associated with cassava have identified specimens of *Nigrospora* in samples of healthy tissues taken from different varieties (Rivera et al. 1993; Ramírez-Camejo 2022). However, the elucidation of *Nigrospora* species, as well as their diversity in cassava, remain unknown because the identification of isolates was made only up to the genus level.

Nigrospora has a widespread range of hosts and can be found not only as endophytes, but also as saprobes, and human and plant pathogens (Ananya et al. 2014; Hao et al. 2020; Zhang et al. 2021). In addition, some species of the genus, as *N. oryzae* and *N. sphaerica*, presents biotechnological potential in the production of metabolites with antifungal activity against some plant pathogens (e.g., *Fusarium* spp. and *Colletotrichum musae*, Wu et al. 2018; Dawoud et al. 2021).

Nigrospora taxonomy was revised by Wang et al (2017) based on morphology and molecular data, and twelve new species were introduced in this genus. Since then, *Nigrospora* species have been identified and described based on morphological features and molecular analyses of internal transcriber space (ITS), translation elongation factor 1 alpha (*TEF1-α*), and β-tubulin (*TUB2*) genic regions. Some examples are *N. brasiliensis* and *N. falsivesicularis* that were obtained from *Nopalea cochenillifera* in Brazil and *Saccharum officinarum* in China, respectively (Crous et al. 2019; Raza et al. 2019). Using these technologies, we decided to investigate the identity of *Nigrospora* strains isolated from healthy stems collected from cassava plantations in the northeast region of Brazil.

Materials And Methods

Collection, isolation and storage

Healthy cassava stems were collected during surveys in cassava plantations in Pernambuco state, northeast region of Brazil, in 2019 and 2020. The samples were sent to the Laboratório de Micologia Ambiental, located in the Departamento de Micologia of the Universidade Federal de Pernambuco (Recife, Brazil) for processing.

The fungal endophytes were isolated using a methodology described by Sun et al. (2011) with minor modifications, as described below. Fifteen fragments (1 cm²) were cut per sample from the internal part of the cassava stems. Those fragments were surface sterilized with 70% ethanol for 1 min, 2% NaClO for 1 min, 70% ethanol for 30 s, rinsed in sterile distilled water, and then dried with sterilized tissue paper. The fifteen fragments were divided onto three Petri dishes containing Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol (0.05 g/L).

For purification, the isolates were transferred to 3% Water Agar (WA) medium. After three days of growth a hyphal tip was collected and transferred onto PDA and maintained at 25°C for seven days in the dark (Brito et al. 2020). After this period, those isolates were stored in tubes with sterile distilled water (Castellani 1967).

Dna Extraction, Pcr Amplification And Sequencing

Genomic DNA was extracted from 7-day-old colonies grown on PDA using the Wizard Genomic DNA Purification Kit according to the manufacturer's protocol. Mycelium was removed and placed in 2.0 mL microtubes containing four 3 mm diameter glass beads and 300 µL of Nuclei Lysis Solution. The tubes were then placed in the L-beader 6 (Loccus) equipment to macerate the mycelium and release the DNA from the cells.

PCR amplifications were performed in 12.5 µL of reaction media containing 1 µL of DNA sample, 0.5 µL of each forward and reverse primers, 4.25 µL of nuclease-free water and 6.25 µL of Go Taq Master Mix (Promega). The amplified and sequenced genic regions were the internal transcriber space (ITS) using the primer pair ITS1/ITS4 (White et al. 1990), part of the translation elongation factor 1 alpha (*TEF1-α*) using the primer pairs EF1-728F/EF1-986R, EF1/EF2 (Carbone & Kohn 1999; Jacobs et al. 2004), and β-tubulin fragment (*TUB2*) using the primer pair Bt-2a/Bt-2b (Glass and Donaldson 1995).

The PCR conditions for ITS and *TEF1-α* amplification followed Brito et al (2020). However, due to the non-amplification of some *Nigrospora* isolates, another prime pair was used (EF1-728F/EF1-986R). For these, the PCR conditions for *TEF1-α* were initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 30 s, and elongation at 72°C for 90 s, with a final extension at 72°C for 6 min. For the *TUB2* amplification, the conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 45 s, with a final extension at 72°C for 5 min. PCR final products were examined by electrophoresis in 1% agarose gels stained with GelRed™ in Tris-acetate EDTA (TAE 1x) buffer and visualized under UV light to verify amplification purity and size. The sequencing of the amplified PCR products was carried out at the Centro de Biociências at the Universidade Federal de Pernambuco (Recife, Brazil).

Phylogenetic Analyses

Nucleotide sequence editing was performed with MEGA v.7 (Kumar et al. 2015) and then compared with the GenBank data using the BLASTn tool. The new sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). A set of *Nigrospora* sequences and related genus (outgroup) obtained from GenBank were added to our new sequences (Table 1) and aligned using the online version of MAFFT v.7 (https://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013; Katoh et al. 2017). Alignments were checked and, when necessary, manual adjustments were made using MEGA v.7 (Kumar et al. 2015). The resulting alignment was deposited in TreeBASE (http://www.treebase.org/; submission ID 29813).

Table 1
Genbank access numbers of the isolates DNA sequences used in phylogenetic analysis. The specimens obtained in this study are highlighted in bold.

SPECIES	ISOLATES	HOST/SUBSTRATE	GENBANK ACESSION NUMBER[1]		
			ITS	TUB2	TEF1- α
Apiospora malaysiana	CBS 102053	*Macaranga hullettii*	KF144896	KF144988	KF145030
A. pseudoparenchymatica	LC7234*	Bamboo	KY494743	KY705211	KY705139
Nigrospora aurantiaca	CGMCC 3.18130*	*Nelumbo* sp.	KX986064	KY019465	KY019295
	LC7034	*Musa paradisiaca*	KX986093	KY019598	KY019394
N. bambusae	CGMCC 3.18327*	Bamboo	KY385307	KY385319	KY385313
	LC7245	Bamboo	KY385305	KY385321	KY385315
N. brasiliensis	CMM 1214*	*Nopalea cochenillifera*	KY569629	MK720816	MK753271
	CMM 1217	*Nopalea cochenillifera*	KY569630	MK720817	MK753272
N. camelliae-sinensis	CGMCC 3.18125*	*Camellia sinensis*	KX985986	KY019460	KY019293
N. chinensis	LC6851	Unknown	KX986049	KY019579	KY019450
	CGMCC 3.18127*	*Machilus breviflora*	KX986023	KY019462	KY019422
N. covidalis	CGMCC 3.20538*	*Lithocarpus sp.*	OK335209	OK431479	OK431485
	LC158337	*Lithocarpus sp.*	OK335210	OK431480	OK431486
N. endophytica	**ARM 687**	Manihot esculenta	**OM265226**	**OP572418**	**OP572415**
	URM8462 = ARM 973*	Manihot esculenta	**OM265233**	**OP572420**	**OP572416**
N. falsivesicularis	CGMCC 3.19678*	*Saccharum officinarum*	MN215778	MN329942	MN264017
	LC13553	*Saccharum officinarum*	MN215779	MN329943	MN264018
N. globospora	CGMCC 3.20539*	*Petasites hybridus*	OK335211	OK431481	OK431487
	LC15839	*Petasites hybridus*	OK335212	OK431482	OK431488

[1] ITS = internal transcribed spacers; *TUB2* = β-tubulin; *TEF1-α* = translation elongation factor 1-alpha;

[2] * = ex-type culture.

SPECIES	ISOLATES	HOST/SUBSTRATE	GENBANK ACESSION NUMBER[1]		
			ITS	TUB2	TEF1- α
N. gorlenkoana	CBS 480.73*	*Vitis vinifera*	KX986048	KY019456	KY019420
N. guangdongensis	CFCC:53917*	*Cunninghamia lanceolata*	MT017509	MT024495	MT024493
N. guilinensis	LC7301	*Nelumbo* sp.	KX986063	KY019608	KY019404
	CGMCC 3.18124*	*Camellia sinensis*	KX985983	KY019459	KY019292
N. hainanensis	CGMCC 3.18129*	*Musa paradisiaca*	KX986091	KY019464	KY019415
	ARM967	Manihot esculenta	**OM265228**	**OM793057**	**OM642834**
	ARM968	Manihot esculenta	**OM265229**	**OM793058**	**OM642835**
	ARM972	Manihot esculenta	**OM265232**	**OP572419**	**OM642837**
	ARM976	Manihot esculenta	**OM265236**	**OM793060**	**OP572417**
N. lacticolonia	CGMCC 3.18123*	*Camellia sinensis*	KX985978	KY019458	KY019291
	ARM 921	Manihot esculenta	**OM265227**	**OM642838**	**OM642833**
N. magnoliae	MFLUCC 19–0112*	*Magnolia candolli*	MW285092	MW438334	–
N. manihoticola	**URM8461 = ARM 645***	Manihot esculenta	**OM265224**	**OM869479**	**OM914791**
N. musae	CBS 319.34*	*Musa paradisiaca*	KX986076	KY019455	KY019419
	LC6385	*Camellia sinensis*	KX986042	KY019567	KY019371
N. oryzae	LC2724	*Symplocos zizyphoides*	KX985959	KY019486	KY019312
	LC4265	*Rhododendron sp.*	KX985994	KY019518	KY019335
N. osmanthi	CGMCC 3.18126*	*Osmanthus* sp.	KX986010	KY019461	KY019421
	LC4487	*Hedera nepalensis*	KX986017	KY019540	KY019438
N. pernambucoensis	**ARM651**	Manihot esculenta	**OM265225**	**OM869480**	**OM914792**
	URM8463 = ARM 974*	Manihot esculenta	**OM265234**	**OM869481**	**OM914793**
N.philosophiae-doctoris	CGMCC 3.20540*	*Disporum sessile*	OK335214	OK431484	OK431490

[1] ITS = internal transcribed spacers; *TUB2* = β-tubulin; *TEF1-α* = translation elongation factor 1-alpha;

[2] * = ex-type culture.

SPECIES	ISOLATES	HOST/SUBSTRATE	GENBANK ACESSION NUMBER[1]		
			ITS	TUB2	TEF1- α
	LC15838	*Disporum sessile*	OK335214	OK431484	OK431490
N. pyriformis	CGMCC 3.18122*	*Citrus sinensis*	KX985940	KY019457	KY019290
	ARM970	Manihot esculenta	**OM265231**	**OM642839**	**OM513904**
N. rubi	LC2698*	*Rubus* sp.	KX985948	KY019475	KY019302
N. saccharicola	LC12057	*Saccharum officinarum*	MN215789	MN329952	MN264028
	CGMCC 3.19362*	*Saccharum officinarum*	MN215788	MN329951	MN264027
N. sacchari-officinarum	CGMCC 3.19335*	*Saccharum officinarum*	MN215791	MN329954	MN264030
	LC13531	*Saccharum officinarum*	MN215792	MN329955	MN264031
N. singularis	CGMCC 3.19334*	*Saccharum officinarum*	MN215793	MN329956	MN264032
	LC12068	*Saccharum officinarum*	MN215794	MN329957	MN264033
N. sphaerica	LC2839	*Harpullia longipetala*	KX985964	KY019491	KY019317
	LC2840	*Harpullia longipetala*	KX985965	KY019492	KY019318
Nigrospora sp. 1	LC2725	*Symplocos zizyphoides*	KX985960	KY019487	KY019313
	LC4566	*Lithocarpus* sp.	KX986022	KY019545	KY019354

[1] ITS = internal transcribed spacers; *TUB2* = β-tubulin; *TEF1-α* = translation elongation factor 1-alpha;

[2] * = ex-type culture.

SPECIES	ISOLATES	HOST/SUBSTRATE	GENBANK ACESSION NUMBER[1]		
			ITS	TUB2	TEF1- α
Nigrospora sp. 2	LC6704	*Camellia sinensis*	KX986047	KY019571	KY019373
N. vesicularis	LC0322	*Unknown host plant*	KX985939	KY019467	KY019296
	CGMCC 3.18128*	*Musa paradisiaca*	KX986088	KY019463	KY019294
N. vesicularifera	CGMCC 3.19333*	*Saccharum officinarum*	MN215812	MN329975	MN264051
	ARM975	Manihot esculenta	**OM265235**	**OM642840**	**OM513905**
N. zimmermanii	CBS 290.62*	*Saccharum officinarum*	KY385309	KY385317	KY385311
	CBS 984.69	*Saccharum officinarum*	KY385310	KY385322	KY385316

[1] ITS = internal transcribed spacers; *TUB2* = β-tubulin; *TEF1-α* = translation elongation factor 1-alpha;

[2] * = ex-type culture.

Bayesian inference (BI) analysis with a concatenated matrix of the genic regions ITS, *TEF1- α* and *TUB2* was used for construct the phylogenetic tree. The analysis was performed using MrBayes v.3.2.7a (Ronquist and Huelsenbeck 2003) in the CIPRES Science Gateway (https://www.phylo.org/) (Miller et al. 2010). The nucleotide substitution model was calculated with MrMODELTEST 2.3 (Posada and Buckley 2004) for each gene region based on the Akaike Information Criterion (AIC). The SYM + I + G model of evolution was used for ITS, HKY + I + G was used for *TEF1- α*, and *TUB2*.

The BI analysis was run for 10,000,000 generations, with one tree sampled every 1.000 generations, resulting in 10,000 trees. The 2,500 trees with lowest probability values were discarded from the analysis and the probability values (Rannala and Yang 1996) were then determined from consensus tree constructed from the 7,500 remaining trees. FigTree software was used for the resulting phylogenetic tree visualization (Rambaut 2009). The tree was rooted to *Apiospora malaysiana* CBS 102053 and *A. pseudoparenchymatica* LC7234.

Morphology

The isolates were grown on WA maintained at 25°C, in the dark, for sporulation and observation of the shape and size of the microscopic structures using a light microscope. Colony descriptions were based on the growth of isolates on PDA (25°C, in the dark) for 12 days and assessed according to the color charts of Rayner (1970).

All strains obtained in this study are deposited in the personal culture collection ARM (Prof. Alexandre Reis Machado) held in Laboratório de Micologia Ambiental (Universidade Federal de Pernambuco, Recife, Brazil). Ex-type strains are deposited in the URM culture collection (*Micoteca URM Profa. Maria Auxiliadora de Queiroz Cavalcanti*), and the holotypes in the URM herbarium (*Herbário URM Pe. Camille Torrend*), both located at the Universidade Federal de Pernambuco (Recife, Brazil). New taxonomic descriptions and nomenclatures are deposited in MycoBank (www.mycobank.org).

Results

Phylogenetic analysis

Twelve *Nigrospora* isolates obtained as endophytes from *Manihot esculenta* were identified by phylogenetic analysis using sequences of ITS, *TEF1-α* and *TUB2* genic regions. Phylogenetic analyses were first performed for each genic region individually (data not shown), and then concatenated in the same matrix used to construct a consensus phylogenetic tree based on Bayesian inference. The concatenated alignment contained 1376 characters including gaps (491 ITS; 523 *TEF1-α*; 362 *TUB2*), 62 taxa. *Apiospora malaysiana* CBS 102053, and *A. pseudoparenchymatica* LC7234 were used as an outgroup.

According to the concatenated gene tree, seven isolates grouped with species already known - *N. hainanensis* (4), *N. lacticolonia* (1), *N. pyriformis* (1) and *N. vesicularifera* (1). This is the first time these species have been identified from *M. esculenta*. Five isolates did not cluster with any knows species of *Nigrospora*, and are new to the genus. They formed three new clades well supported with Bayesian posterior probability (BPP) value of 1 on the tree (Fig. 1). The novel species are described below as *N. endophytica*, *N. manihoticola* and *N. pernambucoensis*.

Taxonomy

Nigrospora endophytica A.C.Q. Brito & A.R. Machado, **sp. nov.** Figure 2

MycoBank number: MB845749

Type: BRAZIL, Pernambuco state, Condado municipality, in cassava field, 7° 35'18.959" S 35° 4' 59.022" W, alt. 118 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Jan 2020, A.C.Q. Brito, Holotype (URM94837), Ex-type culture (URM8462 = ARM973), Genbank accession numbers: OM265233 (ITS), OP572416 (*TEF1-α*), OP572420 (*TUB2*).

Etymology

Named in reference to the endophytic life style of this fungus.

Description: *Sexual morph* not observed. *Asexual morph*: *Hyphae* septate, smooth, branched, sometimes coiled, hyaline or pale brown to dark brown, 2.5–7.5 µm wide (av. 4.6 µm). *Conidiophores* rare, not different from vegetative hyphae, predominantly reduced to conidiogenous cells. *Conidiogenous cells* monoblastic,

determinate, solitary, smooth, pale brown or dark brown, globose, 6.2–10 µm diameter (av. 7.4 µm). *Conidia* acrogenous, solitary, simple, smooth, shiny, aseptate, pale brown to dark brown, globose or subglobose, 10–17.5 µm diam (av. 13.2 µm). *Culture characteristics* on PDA, rapid growth reaching 9 cm diameter in 7 days; colonies wooly, flat, edge entire; at first pale olivaceous grey in the center of the colonies with margin grayish white becoming completely olivaceous grey in 12 days (25°C, in the dark); reverse iron grey to grey olivaceous from center towards edge of the culture with smoke gray margin becoming entirely black in 12 days.

Habitat and known distribution

At the moment, *Nigrospora endophytica* is only found as an endophyte from internal tissues from the stem of *Manihot esculenta* Cranz. Its distribution is restricted to cassava fields in Condado and João Alfredo municipalities, Pernambuco state, northeast of Brazil.

Other material examined: BRAZIL, Pernambuco state, João Alfredo municipality, in cassava field, 7°49'40.6"S 35°33'13.5"W, approximately 87.2 Km of distance from ex-type location, also isolated as an endophyte from stem of *Manihot esculenta* Cranz, Oct 2019, S. S. Nascimento, (ARM687).

Notes: *Nigrospora endophytica* clustered in a well-supported clade, sister to *N. pernambucoensis* sp. nov (Fig. 1). In the phylogenetic analysis of genes individually, *N. endophytica* and *N. pernambucoensis* are closely related according to ITS genic region. Morphologically, *N. endophytica* showed very little spore production, taking much longer for conidia formation compared to *N. pernambucoensis* under the same culture conditions (WA culture medium, 25° C, in the dark). Conidiogenous cells and conidia occurring isolated and dispersed along the hypha in *N. endophytica* (Fig. 2), unlike in *N. pernambucoensis* where the hyphae develop clusters with formation of conidiogenous cells and conidia close to each other in a coiled manner (Fig. 4). Only globose conidiogenic cells were observed in *N. endophytica*, while *N. pernambucoensis* presented more than one shape of conidiogenic cells. Conidia size is smaller in *N. endophytica* (10–17.5 µm diam) than *N. pernambucoensis* (12.5–20 µm diam).

Nigrospora hainanensis M. Wang & L. Cai, Persoonia 39: 136 (2017).

Description of the species

Wang et al (2017).

Type

CHINA, Hainan Province, on leaf of *Musa paradisiaca*, 21 Sept 2015, F. J. Liu, Holotype (HMAS 247064), Ex-type culture (CGMCC3.18129 = LC7030).

Material examined

BRAZIL, Pernambuco state, Condado municipality, in cassava field, 7° 35' 18.959" S 35° 4' 59.022"W, alt. 118 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Jan 2020, A.C.Q. Brito, (ARM967, ARM968, ARM972, ARM976).

Notes

Nigrospora hainanensis has a known distribution mainly in Chinese provinces in different hosts, *Musa paradisiaca*, *Saccharum officinarum*, and *Oxalis corymbosa* (Wang et al. 2017; Raza et al. 2019; Zheng et al. 2021). Outside China, *N. hainanensis* was previously identified in Brazil, in *Napolea cochillifera*, as pathogen associated with cladode brown spot (CBS) (Conforto et al. 2019). In the present study, we identified five *N. hainanensis* strains as endophytes in a new host *Manihot esculenta* Cranz. In terms of geographical location, *N. hainanensis* isolates obtained by Conforto et al (2019) and in our study were identified in the same Brazilian state (Pernambuco). It is possible to observe that the species has no host specificity, and can be found in the environment both as a pathogen, and as an endophyte. So far, it appears that the known distribution of *N. hainanensis* is limited to China and Brazil.

Nigrospora lacticolonia M. Wang & L. Cai, Persoonia 39: 131 (2017).

Description of the species

Wang et al (2017).

Type

CHINA, Jiangxi Province, on *Camellia sinensis*, 24 Apr 2013, F. Liu, Holotype (HMAS 247070), Ex-type culture (CGMCC 3.18123 = LC3324).

Material examined

BRAZIL, Pernambuco state, Jucati municipality, in cassava field, 8° 47' 15.1" S 36° 25' 59.407" W, alt. 706, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Dec 2019, A. R. Machado, (ARM921).

Notes

The first occurrence of *Nigrospora lacticolonia*, in the Neotropical region, was on healthy leaves of *Guarea macrophylla*, collected in a Cocoa agroecosystem in Brazil (Santos et al. 2021). In our study, one strain of *N. lacticolonia* was identified on healthy stem of *Manihot esculenta* Cranz. Thus, this is the first record of *N. lacticolonia* in this host in the world, and this may also be a second report of this species in the Neotropical region. Previous records of *N. lacticolonia* have been reported in China on *Camellia sinensis, Musa paradisiaca*, and *Saccharum officinarum* (Wang et al. 2017; Raza et al. 2019), in Malaysia on *Hylocereus polyrhizus* (Kee et al. 2019), and in Oman on *Phoenix dactylifera* L. (Al-Nadabi et al. 2020). The lifestyle of *N. lacticolonia* can be endophytic (Santos et al. 2021) or pathogenic on plants (Raza et al. 2019; Kee et al. 2019; Al-Nadabi et al. 2020).

Nigrospora manihoticola A.C.Q. Brito & A.R. Machado, **sp. nov.** Figure 3

MycoBank number: MB845748

Type: BRAZIL, Pernambuco state, Chã Grande municipality, in cassava field, 8° 13'46.37" S 35° 27' 14.874"W, alt. 505 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, May 2019, A.C.Q. Brito, Holotype

(URM94836), Ex-type culture (URM8461 = ARM645), GenBank accession numbers: OM265224 (ITS), OM914791 (*TEF1-α*), OM869479 (*TUB2*).

Etymology

Named in reference to the host from which it was isolated *Manihot esculenta* Cranz.

Description: *Sexual morph* not observed. *Asexual morph*: *Hyphae* septate, smooth, branched, sometimes coiled, hyaline or pale brown to dark brown, 2.5–7.5 µm wide (av. 2.95 µm). *Conidiophores* rare, short, not different from vegetative hyphae, predominantly reduced to conidiogenous cells. *Conidiogenous cells* monoblastic, determinate, solitary, smooth, pale brown or dark brown, globose to subglobose to ovoid, 3.7–15 µm length × 3.7– 12.5 µm width (av. 6.7 × 6.8 µm). *Conidia* simple, smooth, solitary, acrogenous, aseptate, shiny, dark brown to black, globose to subglobose to ellipsoidal, 10–17.5 µm diam (av.12.41 µm). *Culture characteristics* on PDA, the colony exhibit rapid growth reaching 9 cm diameter in 7 days; edge entire with floccose appearance, initially white becoming grayish in 12 days (25°C, in the dark); reverse white with greenish black patches that spreads across the culture, turning the reverse fully black with age.

Habitat and known distribution

At the moment, *Nigrospora manihoticola* is only found as an endophytic from internal tissue from stem of *Manihot esculenta* Cranz. Its distribution is restricted to cassava field in Chã Grande municipality, Pernambuco state, Northeast of Brazil.

Notes

Nigrospora manihoticola clustered in a well-supported clade close to *N. hainanensis* in accordance with the genes individually and the concatenated gene tree (Fig. 1). Morphological differences between these two species can be seen in the hyphae, conidiogenous cells, and conidia. *Nigrospora manihoticola* exhibits slightly wider hyphae, and their coloration is predominantly pale brown and dark brown, and in *N. hainanensis* they are hyaline to pale brown. Coiled hyphae are present in *N. manihoticola* with the production of conidiogenous cells and conidia, but such feature is absent in *N. hainanensis*. The conidiogenous cells are hyaline with a globose or ampulliform shape in *N. hainanensis* (6.5–12.5 × 4.5–9.5 µm), while on *N. manihoticola* was observed pale brown or dark brown with globose to subglobose to ovoid shape (3.7–15 × 3.7– 12.5 µm). In addition to black conidia present in both species, *N. manihoticola* also displays dark brown conidia. Another morphological difference is the presence of setae that occurs on *N. hainanensis*, and was not observed on *N. manihoticola* (Wang et al. 2017). *N. hainanensis* was isolated from leaves of *Musa paradisiaca* in China (Wang et al. 2017), and has been associated with plant diseases in more than one host (Conforto et al. 2019; Raza et al. 2019; Zheng et al. 2021), while *N. manihoticola* was identified as endophytic lifestyle in internal tissues of the stem of *Manihot esculenta* Cranz in Brazil.

Nigrospora pernambucoensis A.C.Q. Brito & A.R. Machado, **sp. nov.** Figure 4

MycoBank number: MB845747

Type: BRAZIL, Pernambuco state, Condado municipality, in cassava field, 7° 35' 18.959" S 35° 4' 59.022" W, alt. 118 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Jan 2020, A.C.Q. Brito, Holotype

(URM94838), Ex-type culture (URM8463 **=** ARM974), GenBank accession numbers: OM265234 (ITS), OM914793 (*TEF1- α*), OM869481 (*TUB2*).

Etymology

Named in reference to the Brazilian state, Pernambuco, where the type was collected.

Description: *Sexual morph* not observed. *Asexual morph*: *Hyphae* septate, smooth, highly branched, sometimes coiled, hyaline to pale brown to dark brown, 2.5–6.2 µm wide (av. 3.7 µm). *Conidiophores* rare, short, not different from vegetative hyphae, predominantly reduced to conidiogenous cells. *Conidiogenous cells* monoblastic, determinate, discrete, solitary, smooth, pale brown to dark brown, globose to obpyriform 5– 22.5 µm length × 5–12.5 µm width (av. 8.5 × 6.8 µm). *Hyaline vesicles* around the septum delimiting the conidia and their conidiogenous cells. *Conidia* acrogenous, solitary, simple, smooth, shiny, aseptate, globose or ellipsoidal, pale or dark brown to black, 12.5–20 µm diam (av.14.9 µm). *Culture characteristics* on PDA, colonies wooly, edge entire, fast growing reaching 9 cm diameter in 7 days, initially pale olivaceous grey become olivaceous grey in 12 days (25°C, in the dark); reverse iron grey to grey olivaceous from center towards edge of the culture with olivaceous buff margin becoming entirely black in 12 days.

Habitat and known distribution

At the moment, *Nigrospora pernambucoensis* is only found as an endophytic from internal tissues from stem of *Manihot esculenta* Cranz. Its distribution is restricted to cassava fields in Condado and João Alfredo municipalities, in Pernambuco state, Northeast of Brazil.

Other material examined

BRAZIL, Pernambuco state, João Alfredo municipality, in cassava field 7°49'40.6"S 35°33'13.5"W, approximately 87.2 Km of distance from ex-type location, also isolated as an endophyte from stem of *Manihot esculenta* Cranz, Oct 2019, S. S. Nascimento, (ARM651).

Notes

Concatenated gene analysis clustered *Nigrospora pernambucoensis* in a well-supported clade, sister to *N. endophytica* sp. nov (Fig. 1). *Nigrospora pernambucoensis* has highly branched hyphae with a smaller diameter than *N. endophytica*. More than one shape of conidiogenous cells (globose and obpyriform) can be observed in *N. pernambucoensis*, while *N. endophytica* only globose shape were visualized (Fig. 4). Hyaline vesicles are present in *N. pernambucoensis* and absent in *N. endophytica.* Conidia can be black in color in addition to pale brown to dark brown, and are larger in *N. pernambucoensis* with 12.5–20 µm diam vs pale to dark brown conidia with 10–17.5 µm diam in *N. endophytica*.

Nigrospora pyriformis M. Wang & L. Cai, Persoonia 39: 136 (2017).

Description of the species

Wang et al (2017).

Type

CHINA, Jiangxi Province, on *Citrus reticulate*, 11 Mar 2012, X. M. Tan, Holotype (HMAS 247067), Ex-type culture (CGMCC3.18122 = LC2045).

Material examined

BRAZIL, Pernambuco state, Condado municipality, in cassava field, 7° 35' 18.959" S 35° 4'59.022"W, alt. 118 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Jan 2020, A.C.Q. Brito, (ARM970).

Notes

Nigrospora pyriformis has no host specificity, having already been isolated in *Camellia sinensis*, *Castanopsis* sp., *Chenopodium album*, *Citrus sinensis, Lindera aggregata, Musa paradisiaca*, *Rosa* sp., *Rubus reflexus*, and *Saccharum officinarum* (Wang et al. 2017; Raza et al. 2019; Chen et al. 2020). However, until the present study its distribution was restricted to China, and related to plant diseases, such as leaf spot (Chen et al. 2020). In our study, one strain of *N. pyriformis* was identified inhabit healthy stem of *Manihot esculenta* Cranz. It is a new host record for this species in the world. Besides that, this is the first occurrence of *N. pyriformis* outside of China.

Nigrospora vesicularifera M. Raza & L. Cai, Fungal Diversity 99: 96 (2019)

Description of the species

Raza et al (2019).

Type

CHINA, Guangdong Province, Zhanjiang City, Leizhou County, on *Saccharum officinarum*, Aug 2016, M. Raza and Y. Z. Diao, Holotype (HMAS 248077), Ex-holotype living culture (CGMCC 3.19333 = LC12052).

Material examined

BRAZIL, Pernambuco state, Condado municipality, in cassava field, 7° 35' 18.959" S 35° 4'59.022"W, alt. 118 m, isolated as an endophyte from stem of *Manihot esculenta* Cranz, Jan 2020, A.C.Q. Brito, (ARM975).

Notes

Nigrospora vesicularifera was introduced by Raza et al (2019) in a prospective study of pathogenic fungi associated with sugarcane diseases (*Saccharum officinarum*) in China. Since the description of the species no records of new hosts, new geographic occurrence or lifestyle (endophytic or pathogenic) quoting this species have been found. In the present study, *N. vesicularifera* was identified in a new host, *Manihot esculenta* Cranz, as endophytic in Brazil. This also represents a new geographic occurrence for this species outside Guangdong Province in China.

Discussion

To date only a few studies have been carried out to assess endophytic fungal communities in *Manihot esculenta* Cranz (cassava) (Rivera et al. 1993; Li et al. 2020; Hartanti et al. 2021; Ramírez-Camejo 2022). Among

these studies, Rivera et al (1993) were the first to mention *Nigrospora* as part of such communities in asymptomatic cassava stem tissues. Almost 30 years later, *Nigrospora* was cited again as part of cassava endophytic community, but this time on leaves in Panama (Ramírez-Camejo 2022).

In both studies, specimens of *Nigrospora* were identified only at the genus level. Therefore, in the present study, we decided to further investigate the *Nigrospora* strains obtained from our collections. As a result, seven species were identified as endophytes on cassava stem tissues in plantations in Pernambuco state, northeast region of Brazil. Of these, three were described here as new species (*N. endophytica*, *N. manihoticola, N. pernambucoensis*), four as new host record worldwide (*N. hainanensis*, *N. lacticolonia*, *N. pyriformis*, *N. vesicularifera*), and two species are also reported as a new geographic occurrences (*N. pyriformis*, *N. vesicularifera*).

Nigrospora (= *Khusia*) was introduced by Zimmerman (1902) for accommodate *N. panici* ex-type. Currently, the genus is placed in the family *Apiosporaceae* (*Amphisphaeriales*, *Sordariomycetes*) (Hyde et al. 2020). In this study, the use of morphology combined with phylogenetic data allowed us to more accurately identify our strains, following what was exposed by Wang et al (2017), because the existence of an overlap of morphological characteristics occurring within *Nigrospora*. In addition to this overlap of morphological characteristics, in our phylogenetic analysis of the ITS genic region, it was observed that this gene alone is not enough to separate all the species of the genus. This situation was also observed with our new strains. *Nigrospora endophytica* clustered in the same clade with *N. pernambucoensis* according to ITS genic region. However, phylogenic analyses performed in *TEF1-α* and *TUB2* separate these species into two different well-supported clades.

In terms of morphological characteristics, *N. manihoticola* can be differentiated from *N hainanensis* by having fertile coiled hyphae, with the production of conidiogenous cells and conidia, pigmented conidiogenous cells with a globose, subglobose to ovoid shape and absence of setae. Regarding morphology of *N. endophytica* and *N. pernambucoensis*, these species can be differentiated from each other based on conidiogenous cells and conidia that occur isolated and dispersed along the hypha in *N. endophytica*, unlike in *N. pernambucoensis* where the hyphae develop clusters with formation of conidiogenous cells and conidia close to each other in a coiled manner. Besides that, *N. pernambucoensis* has hyaline vesicles surrounding the conidia, and more than one shape of conidiogenic cells, features that are absent in *N. endophytica.*

Nigrospora hainanensis, *N. lacticolonia*, *N. pyriformis*, and *N. vesicularifera* are reported here for the first time as endophytes on cassava. All of these species have been described in China from different hosts, *Musa paradisiaca*, *Camellia sinensis*, *Citrus reticulata*, and *Saccharum officinarum*, respectively (Wang et al. 2017; Raza et al. 2019). Of the total number of strains obtained in our study, *N. hainanensis* was the most frequent, with four strains isolated. This species was associated with plant diseases, as cladode brown spot (CBS) on *Napolea cochillifera* in Brazil (Conforto et al. 2019), in the same Brazilian state from where our *Nigrospora* endophytes strains were obtained, and leaf spot on *Oxalis corymbosa* in China (Zheng et al. 2021). Although, *N. hainanensis* has been previously identified in Brazil, as far as we know this is the second report of the species in Brazil and also outside China, the country where the species was identified originally.

In the Neotropical region, *N. lacticolonia* has been identified as an endophyte on *Guarea macrophylla*, and cassava, so far (Santos et al. 2021, present study). However, the species was identified as pathogenic in other

countries, e.g. sugarcane diseases in China, reddish brown spot disease on dragon fruit in Malaysia, leaf spot diseases on date palm (Raza et al. 2019; Kee et al. 2019; Al-Nadabi et al. 2020). Among the *Nigrospora* spp identified in our study, *N. lacticolonia* is the one with the most widespread geographic distribution. On the other hand, *N. pyriformis*, and *N. vesicularifera* before the present study were geographically restricted to China. *Nigrospora pyriformis* has been shown to have no specific host (Wang et al. 2017; Chen et al. 2020). However, until the present study *N. vesicularifera* has only been identified once associated in a single host, *Saccharum officinarum* (Raza et al. 2019).

Declarations

Ethics approval and Consent to participate

Not applicable.

Consent for publication

All authors reviewed the manuscript and approved its submission to the Mycological Progress.

Availability of data and materials

Sequence data are deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), concatenated alignment is deposited in TreeBASE (http://www.treebase.org/). The ex-types and holotypes have been deposited in public collections (*Micoteca URM Profa. Maria Auxiliadora de Queiroz Cavalcanti,* and *Herbário URM Pe. Camille Torrend*, respectively) located at the Universidade Federal de Pernambuco (Recife, Brazil). New taxonomic descriptions and nomenclatures are deposited in MycoBank (www.mycobank.org).

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Author contributions

All authors contributed to the conception and design of the manuscript. ARM guided and supervised this research. CMSM revised the data. ACQB, JFM, AEAS, SSN, and ARM collected the samples used in this study. JFM did the molecular lab work. ACQB performed the morphological and phylogenetic analyses, done the illustrations, and wrote the first draft. All authors contributed to its development and completion. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figures

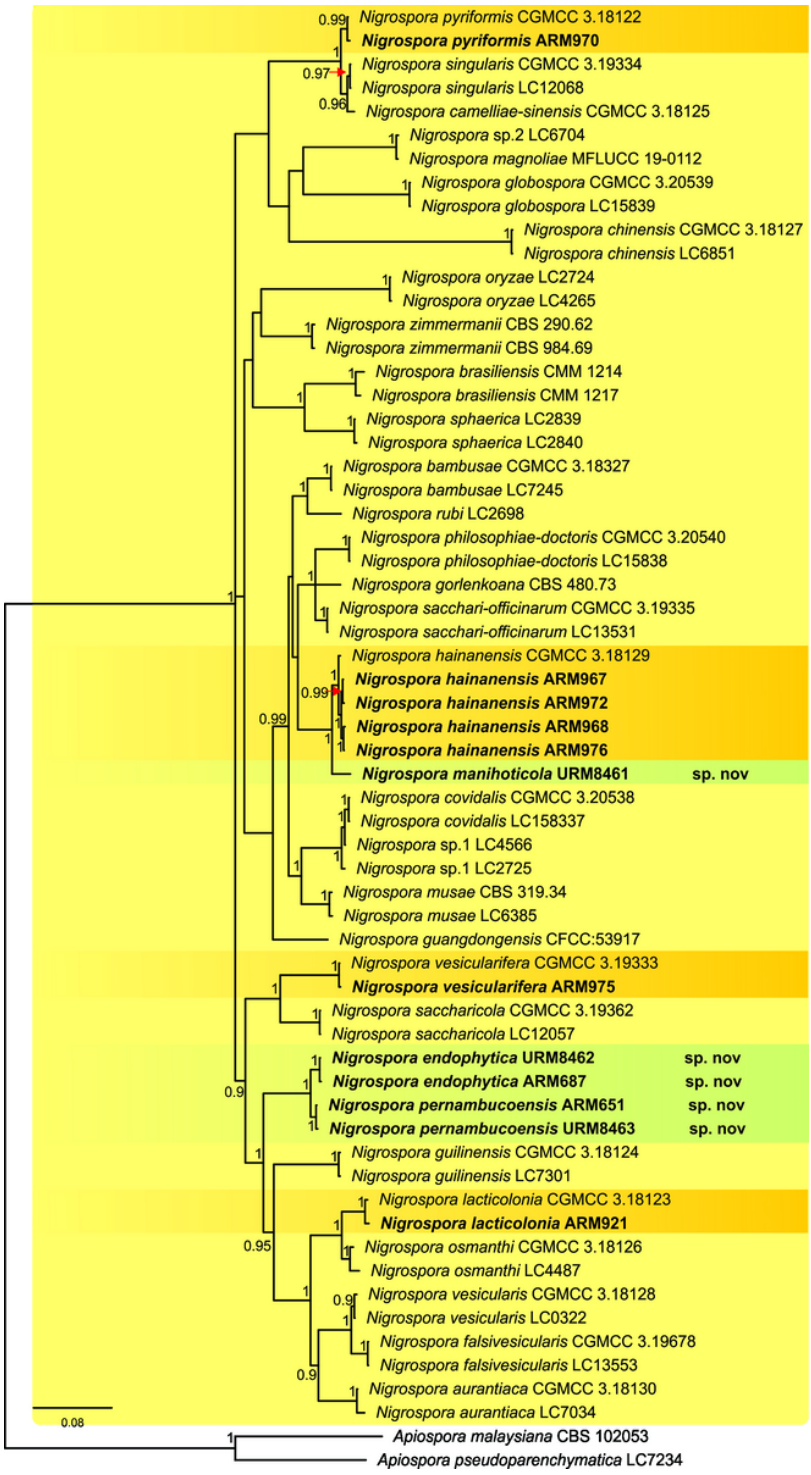

Figure 1

Bayesian inference tree obtained by using combined dataset of ITS, *TEF1-α* and *TUB2* gene regions. The isolates obtained in this study are highlighting in bold. The Bayesian posterior probabilities above 0.90 are indicated at the nodes. The tree was rooted to *Apiospora malaysiana* CBS 102053 and *A. pseudoparenchymatica* LC7234

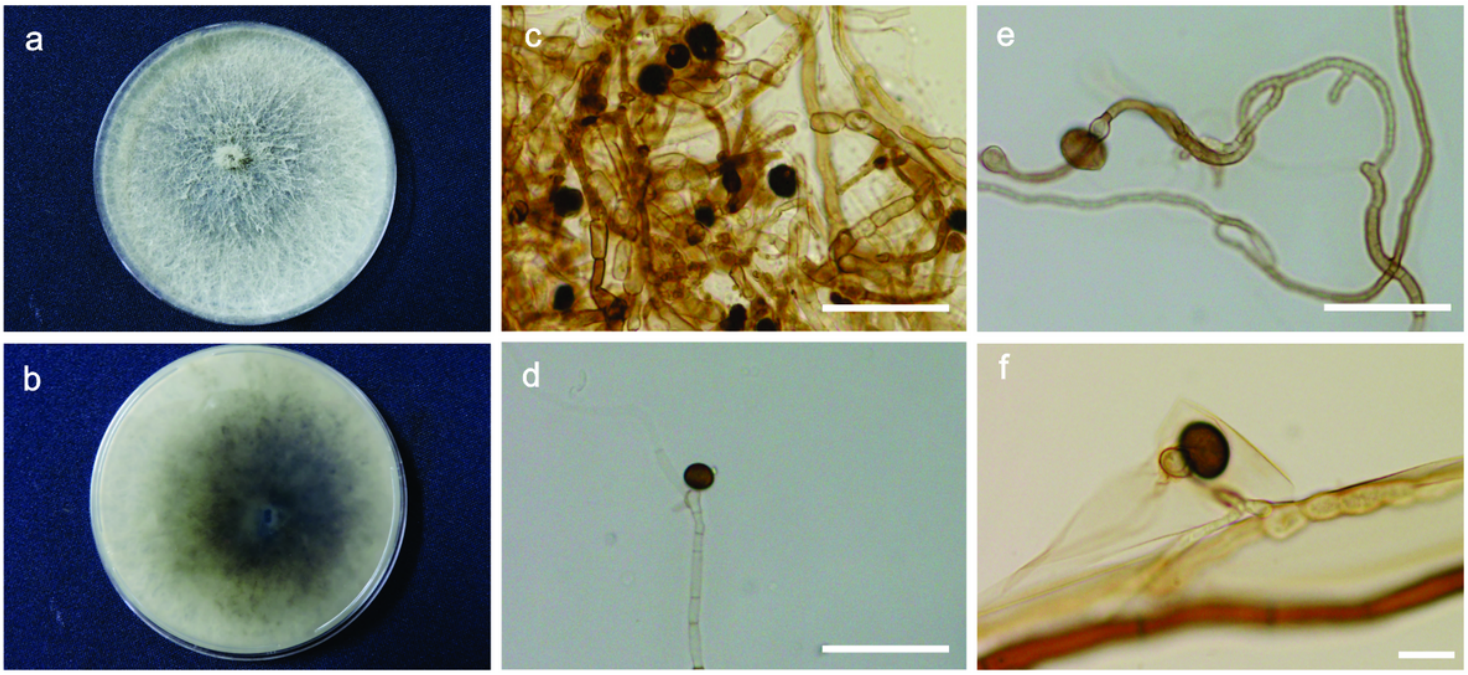

Figure 2

Nigrospora endophytica (URM8462). a, b Surface and reverse of colony on PDA after 7 days.**c** Pigmented hyphae and conidia on PDA. d – f Conidia and conidiogenous cells on WA. Scale bars: c – e = 50 µm; f= 10 µm

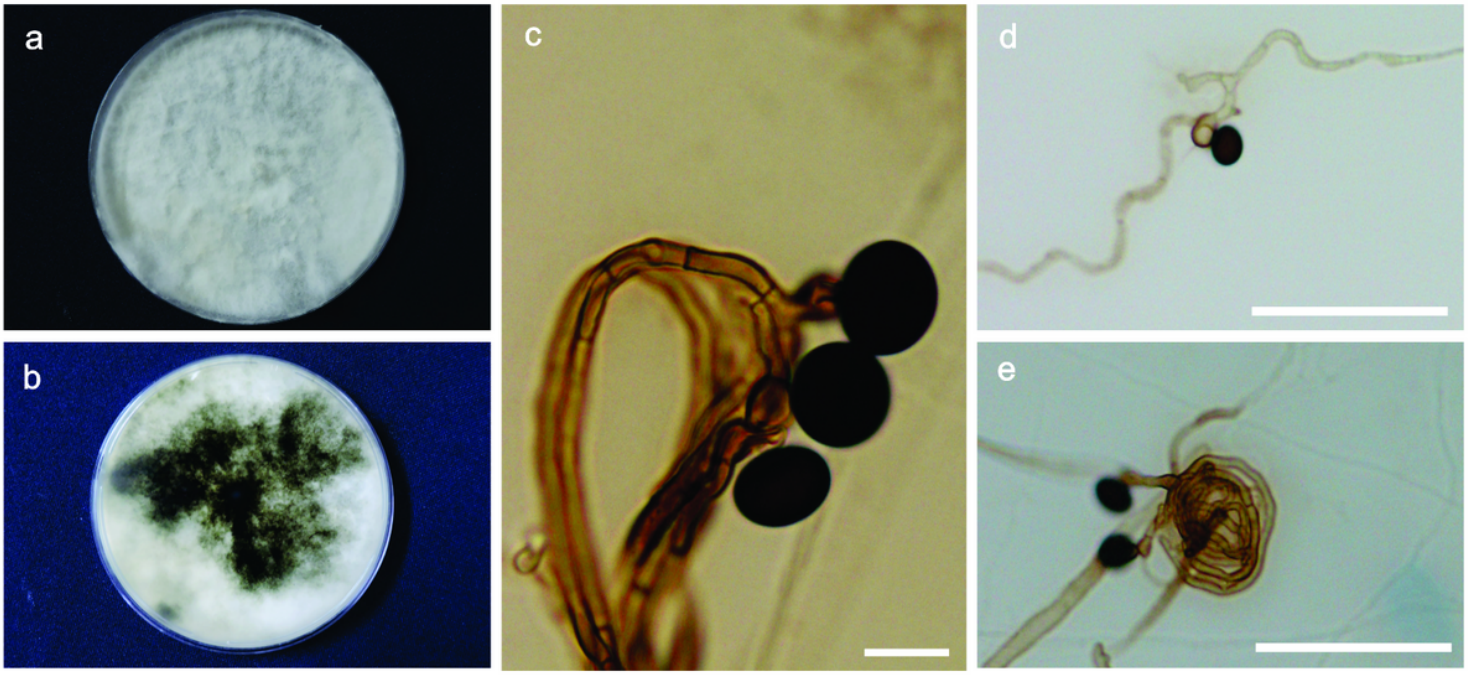

Figure 3

Nigrospora manihoticola (URM8461). a, b Surface and reverse of colony on PDA after 7 days. c Dark brown hyphae and conidiogenous cells with black conidia on WA. d Conidium and conidiogenic cell. e Coiled hyphae. Scale bars: c = 10 µm; d, e = 50 µm.

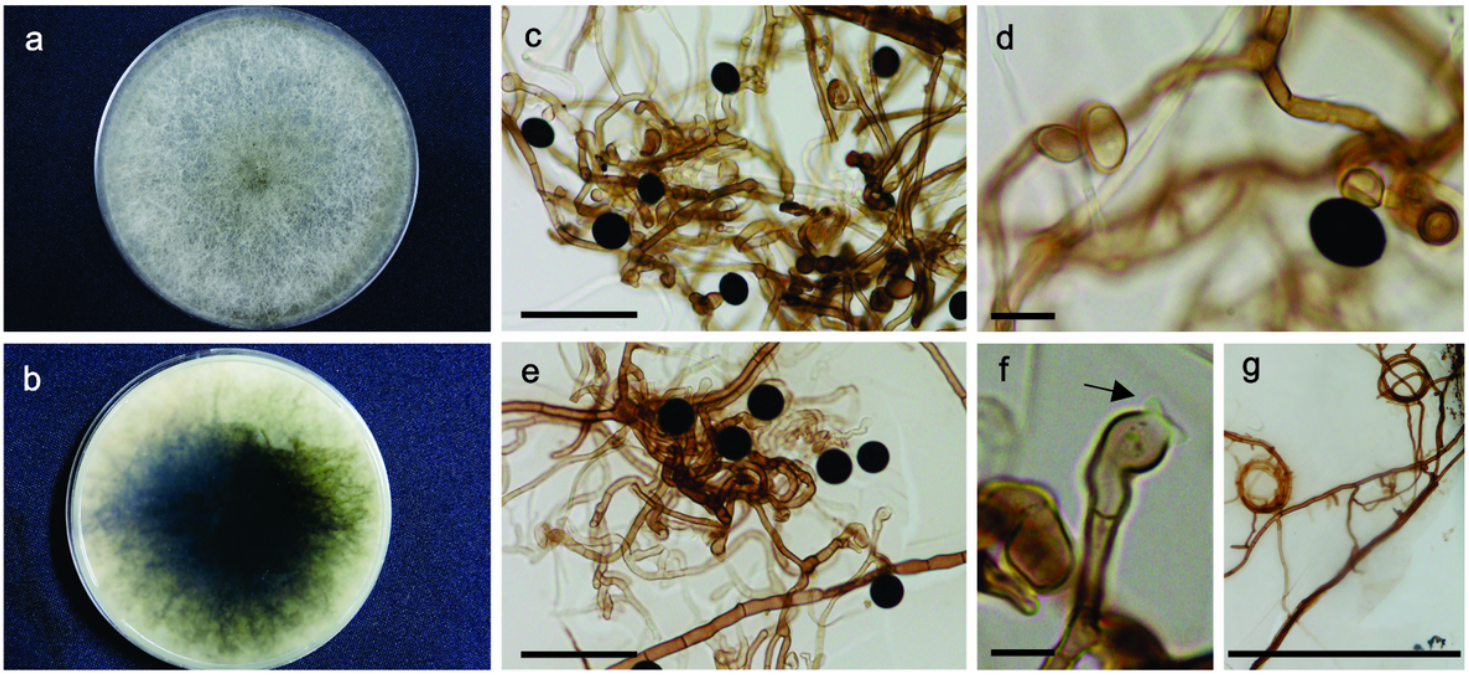

Figure 4

Nigrospora pernambucoensis (URM8463). a, b Surface and reverse of colony on PDA after 7 days. c Sporulation on PDA. d – g Sporulation on WA. d, e Conidiogenic cells and conidia. f Hyaline vesicles indicate by the black arrow. g Coiled hyphae. Scale bars: c, e = 50 µm; d ,f = 10 µm; g = 100 µm