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SHORT COMMUNICATION

New occurrences of Botryosphaeriaceae causing black root rot of cassava in Brazil

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ABSTRACT

Despite the occurrence of several diseases of cassava, the cassava black root rot (CBR) represents one of the main limiting factor for crop rentability in the world. However, the etiology of CBR is complex and it needs to be revised based on current molecular analysis. On this work, molecular and morphological studies allowed for the identification of three species of Botryosphaeriaceae causing black root rot disease of cassava in the states of Maranhão and Paraíba, Brazil, namely: *Lasiodiplodia euphorbicola*, *Lasiodiplodia pseudotheobromae* and *Neoscytalidium hyalinum*. This is the first report of these three fungal species as causal agents of CBR in the world.
Key words: *Lasiodiplodia* spp., *Manihot esculenta*, *Neoscytalidium hyalinum*, Botryosphaeriales, Dothideomycetes, soilborne fungi.

Cassava (*Manihot esculenta* Crantz) is an important food source. Its tuberous edible roots are high in calories and are a source of starch, the major form of carbohydrate consumed in the tropics for human and animal nutrition (Adeoti, 2010; CEPLAC, 2013). Furthermore, it tolerates adverse climatic and edaphic conditions and requires little care (Nweke et al., 2002). Due to these characteristics, cassava is an important activity for smallholders and it is widely cultivated in developing countries to reduce famine, providing a major source of nutrition for over 500 million people (FAO, 2013).

Currently, cassava productivity in Brazil, one of the probable centers of origin of cassava, is low. Cassava cultivation by family stallholders utilize low-level of technologies, including manivas (propagation materials) of poor physiological and phytosanitary quality (Silva et al., 2013). The high occurrence of disease transmitted by propagation material is one of the main factors that contribute to lower cassava productivity in Brazil (Cavalcante, 2001).

Some of these disease transmitted by propagative materials also occur in the postharvest phase. Among these is root rot, which is the main limiting factor for the production of cassava because it directly affects the marketable product. In Brazil numerous root rot fungi are listed as associated with cassava, namely: *Fusarium solani* (Mart.) Sacc., *Phytophthora capsici* Leonian, *P. drechsleri* Tucker, *P. nicotianae* var. *parasitica* (Dastur) G.M. Waterh., *P. richardiae* Buisman, *Scytalidium lignicola* Pesante, *Rhizoctonia solani* J.G. Kühn, *Rosellinia necatrix* Berl. ex Prill., and some Botryosphaeriaceae as *Diplodia manihotis* Sacc. and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Mendes and Urben, 2014). However, among these agents, only *Scytalidium lignicola* is considered to be the causal agent of cassava black root rot (CBR) (Laranjeira et al., 1994; Poltronieri et al., 1998; Muniz et al., 1999; Serra et al., 2009; Silva et al., 2013). It causes severe yield losses (Silva et al., 2013).

Nevertheless, the status of *Scytalidium lignicola* as the causal agent of CBR in Brazil needs to be revised based on molecular analysis. Recently, several species previously identified as *Scytalidium* were transferred to the genus *Neoscytalidium* (Seifert et al., 2011). *Neoscytalidium* is morphologically similar to *Scytalidium*, but under certain conditions this fungus forms synnanamorphs having pycnidia which contain *Fusicoccum*-like conidia. Moreover, these two genera belong to different orders of Ascomycota (Crous et al., 2006; Seifert et al., 2011; Phillips et al., 2013). Therefore, the identification of these fungi only by morphological characters can lead to errors. A morphological and molecular approach is required for a more accurate identification of the fungi associated with CBR (Hyde et al., 2010; Cai et al., 2011a; 2011b).

In 2011, six fungal isolates from cassava plants with symptoms of CBR collected in the states of Maranhão and Paraíba were initially identified as *Scytalidium* sp. and *Lasiodiplodia* sp. based upon morphological characteristics. Later, these isolates were provided by Embrapa Mandioca e Fruticultura to the Laboratório de Patologia de Sementes e de Pós-Colheita of the Universidade Federal de Viçosa for taxonomical and molecular studies. The aim of the present study was to identify these isolates based on morphological characters and molecular analysis and to verify their pathogenicity.

The isolates were grown on Petri dishes containing 2% water-agar (WA) overlaid with double-sterilized twigs of *Pinus* and incubated at 25°C with a photoperiod of 12 h to induce the formation of fruiting bodies and sporulation. The single-spore derived cultures were deposited in the Coleção de Culturas de Fungos Fitopatogênicos "Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco, Brazil. Sections of the fruiting bodies were prepared and mounted in lactophenol. Thirty measurements of conidia, paraphyses and conidiogenous cells were made with an Olympus CX31 light microscope. Images were obtained with an Olympus BX 51 light microscope fitted with a digital camera (Olympus EVOLT330).

Genomic DNA was extracted from colonies grown on PDA at 25°C for one week. Approximately 40 mg of mycelia were collected. Extraction was carried out through a process involving freezing the samples with liquid nitrogen and grinding them into a fine powder using a microcentrifuge tube pestle. The crushing was resumed after adding 100 µL of Nuclei Lysis Solution of the Wizard Genomic DNA Purification kit (Promega). Extraction continued as described by Pinho et al. (2012). PCR reagents, primers and conditions were as described by Machado et al. (2014). PCR products were directly sequenced at Macrogen (South Korea).

Nucleotide sequences were edited with the BioEdit software (Hall, 2012). All sequences were checked manually and positions with ambiguous nucleotides were clarified using sequences from both DNA strands. New sequences were deposited in GenBank (see Table 1 for accession numbers). Sequences of internal transcribed spacer regions 1 and 2 including the 5.8S rRNA gene (ITS), translation elongation factor 1-α (TEF1-α) and β-tubulin (βt) of additional species were retrieved from GenBank (Table 1). Consensus sequences were compared against GenBank's database using the MegaBLAST algorithm. The closest hit sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA v. 5 (Tamura et al., 2011). Alignments were checked visually, and manual adjustments were made when necessary. Ambiguously aligned sequences within the dataset were excluded from the analysis. The resulting alignment was deposited into TreeBASE (www.treebase.org) under accession number S15379. Phylogenetic analyses were conducted as described by Machado et al. (2014). The models of evolution selected according to the Akaike Information Criterion (AIC) were GTR+I for ITS, HKY+G for TEF and GTR+G for βt and the tree was rooted to *Spencermartinsia viticola* CBS117009.

Pathogenicity one representative isolate of each species was tested. Each selected isolate was grown in a Petri dish with PDA for 7 days at 25°C. Roots that were approximately 20 cm x 7 cm wide had their bark wounded superficially with a scalpel on the inoculation site. Six mm diam culture disks obtained from the margins of the growing culture were placed on the wounds. Wounded roots on which PDA plugs were deposited served as controls. Five roots were inoculated with each isolate and placed in plastic boxes that contained a portion of moistened cotton wool and were maintained in a moist chamber at approximately 25°C for two weeks.

Phylogenetic analysis (Figure 1) and morphological comparisons (Table 2) revealed three distinct species of Botryosphaeriaceae in association with CBR among the six fungal isolates: *Lasiodiplodia euphorbicola* A.R. Machado & O.L. Pereira (Figure 2H-K), *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous (Figure 2L-O) and *Neoscytalidium hyalinum* (C.K. Campb. & J.L. Mulder) A.J.L. Phillips, Groenewald & Crous (Figure 2C-G).

In recent years, morphological and molecular analyses have revealed a great diversity of species within plant pathogenic Botryosphaeriaceae (Begoude et al., 2010; Mehl et al., 2011; Ismail et al., 2012; Urbez-Torres et al., 2012; Marques et al., 2013a; 2013b; Machado et al., 2014). Despite the usefulness of morphological characters, molecular analysis became essential for recognizing taxa that are included in species complexes, such as *Lasiodiplodia* (Alves et al., 2008; Abdollahzadeh et al., 2010; Ismail et al., 2012; Urbez-Torres et al., 2012; Marques et al., 2013a). Molecular analysis can also distinguish taxa that show similar morphologies but are phylogenetically distant, such as the genera *Neocytalidium* and *Scytalidium*, which belong to Botryosphaeriaceae and Helotiaceae, respectively (Crous et al., 2006; Seifert et al., 2011; Phillips et al., 2013). Similarly, molecular analysis in this study revealed that two species of *Lasiodiplodia* are associated with CBR, and demonstrated that the *Scytalidium*-like fungus that causes this disease belongs to *Neoscytalidium*. Thus, it is possible that previous reports of fungi causing CBR in Brazil (Laranjeira et al., 1994; Poltronieri et al., 1998; Muniz et al., 1999; Serra et al., 2009; Silva et al., 2013) were misidentified as *Scytalidium lignicola*.

The species *Neoscytalidium hyalinum* (=*N. dimidiatum*) is a botryosphaeriaceous fungus that, under special conditions, forms *Scytalidium*-like and *Fusicoccum*-like synnanamorphs (Crous et al., 2006; Phillips et al., 2013). This is probably the main reason for the misidentification of this pathogen, which is often confused with the hyphomycete fungus *Scytalidium*. Therefore, it is clear that the identification of the etiologic agent of CBR requires a careful polyphasic approach.

Pathogenicity of the isolates representing the three species was confirmed two weeks after inoculation. All inoculated roots showed symptoms that were similar to those observed in the field, with the subsequent emergence of fungal structures occurring externally on the bark (Figure 2A). From the lesions, it was possible retrieve each of the inoculated fungi. Symptoms were not observed in control roots (Figure 2B).

Diseases caused by *Neoscytalidium hyalinum* tend to be more common in tropical countries (Phillips et al., 2013). In Brazil, this species was previously reported on *Jatropha curcas* L. and *Mangifera indica* L. (Machado

TABLE 1 - Genbank accession numbers of DNA sequences of Botryosphaeriaceae used in phylogenetic analysis. The specimens obtained in this study are highlighted in bold.

Species	Isolates	Host/Substrate	Genbank accession no.		
			ITS	EF1-α	βt
Neoscytalidium hyalinum	CBS 499.66	*Mangifera indica*	AY819727	EU144063	FM2111671
N. hyalinum	PD104	*Ficus carica*	GU251107	GU251239	GU251767
N. hyalinum	**CMM4022**	*Manihot esculenta*	KF369269	KF553902	KF720790
N. hyalinum	**CMM3895**	*M. esculenta*	KF369265	KF553898	KF720786
N. novaehollandiae	CBS122072	*Adansonia gibbosa*	EF585535	EF585581	-
N. novaehollandiae	CBS122610	*Acacia synchronicia*	EF585536	EF585578	-
Lasiodiplodia venezuelensis	WAC12539	*Acacia mangium*	DQ103547	DQ103568	-
L. venezuelensis	CMW13513	*Acacia mangium*	DQ103549	DQ103570	-
L. rubropurpurea	WAC12536	*Eucalyptus grandis*	DQ103554	DQ103572	-
L. gonubiensis	CBS115812	*Syzygium cordatum*	DQ458892	DQ458877	DQ458860
L. crassispora	CBS110492	Unknown	EF622086	EF622066	EU673134
L. crassispora	CMW22653	*Pterocarpus angolensis*	FJ888465	FJ888452	-
L. margaritacea	CBS122519	*Adansonia gibbosa*	EU144050	EU144065	-
L. pseudotheobromae	CBS116459	*Gmelina arborea*	EF622077	EF622057	EU673111
L. pseudotheobromae	CMM3887	*Jatropha curcas*	KF234559	KF226722	KF254943
L. pseudotheobromae	**CMM3894**	*M. esculenta*	KF369264	KJ452244	-
L. parva	CBS456.78	Cassava-field soil	EF622083	EF622063	-
L. parva	CBS495.78	Cassava-field soil	EF622085	EF622065	-
L. euphorbicola	CMM3651	*Jatropha curcas*	KF234553	KF226711	KF254937
L. euphorbicola	CMM3652	*Jatropha curcas*	KF234554	KF226715	KF254938
L. euphorbicola	CMM3609	*Jatropha curcas*	KF234543	KF226689	KF254926
L. euphorbicola	**CMM4018**	*Manihot esculenta*	KF369268	KF553901	KF720789
L. euphorbicola	**CMM3973**	*Manihot esculenta*	KF369267	KF553900	KF720788
L. euphorbicola	**CMM3897**	*Manihot esculenta*	KF369266	KF553899	KF720787
L. citricola	IRAN1521C	*Citrus* sp.	GU945353	GU945339	-
L. citricola	IRAN1522C	*Citrus* sp.	GU945354	GU945340	-
L. egyptiacae	CBS130992	*Mangifera indica*	JN814397	JN814424	-
L. egyptiacae	BOT-29	*Mangifera indica*	JN814401	JN814428	-
L. hormozganensis	IRAN1500C	*Olea* sp.	GU945355	GU945343	-
L. hormozganensis	IRAN1498C	*Mangifera indica*	GU945356	GU945344	-
L. subglobosa	CMM3872	*Jatropha curcas*	KF234558	KF226721	KF254942
L. subglobosa	CMM4046	*Jatropha curcas*	KF234560	KF226723	KF254944
L. macrospora	CMM3833	*Jatropha curcas*	KF234557	KF226718	KF254941
L. plurivora	STE-U5803	*Vitis vinifera*	EF445362	EF445395	-
L. gilanensis	IRAN1523C	Unknown	GU945351	GU945342	-
L. gilanensis	IRAN1501C	Unknown	GU945352	GU945341	-
L. iraniensis	IRAN1517C	*Citrus* sp.	GU945349	GU945337	-
L. iraniensis	IRAN1519C	*Mangifera indica*	GU945350	GU945338	-
L. brasiliense	CMM4015	*Mangifera indica*	JX464063	JX464049	-
L. brasiliense	CMM2186	*Carica papaya*	KC484812	KC481542	-
L. brasiliense	CMM2255	*Carica papaya*	KC484792	KC481523	-
L. brasiliense	CMM2313	*Carica papaya*	KC484793	KC481524	-
L. jatrophicola	CMM3610	*Jatropha curcas*	KF234544	KF226690	KF254927
L. mahajangana	CMW27801	*Terminalia catappa*	FJ900595	FJ900641	FJ900630
L. mahajangana	CMW27820	*Terminalia catappa*	FJ900597	FJ900643	FJ900632
L. theobromae	CMW28571	*Terminalia ivorensis*	GQ469924	GQ469897	-
Botryosphaeria rhodina	CBS164.96	Unknown	AY640255	AY640258	EU673110
B. rhodina	CBS124.13	Unknown	DQ458890	DQ458875	DQ458858
L. theobromae	CBS111530	Unknown	EF622074	EF622054	-
B. rhodina	CMW9074	*Pinus* sp.	AY236952	AY236901	AY236930
L. viticola	UCD2553AR	*Vitis vinifera*	HQ288227	HQ288269	HQ288306
L. viticola	UCD2604MO	*Vitis vinifera*	HQ288228	HQ288270	HQ288307
L. missouriana	UCD2193MO	*Vitis vinifera*	HQ288225	HQ288267	HQ288304
L. missouriana	UCD2199MO	*Vitis vinifera*	HQ288226	HQ288268	HQ288305
Spencermartinsia viticola	CBS117009	*Vitis vinifera*	AY905554	AY905559	EU673104

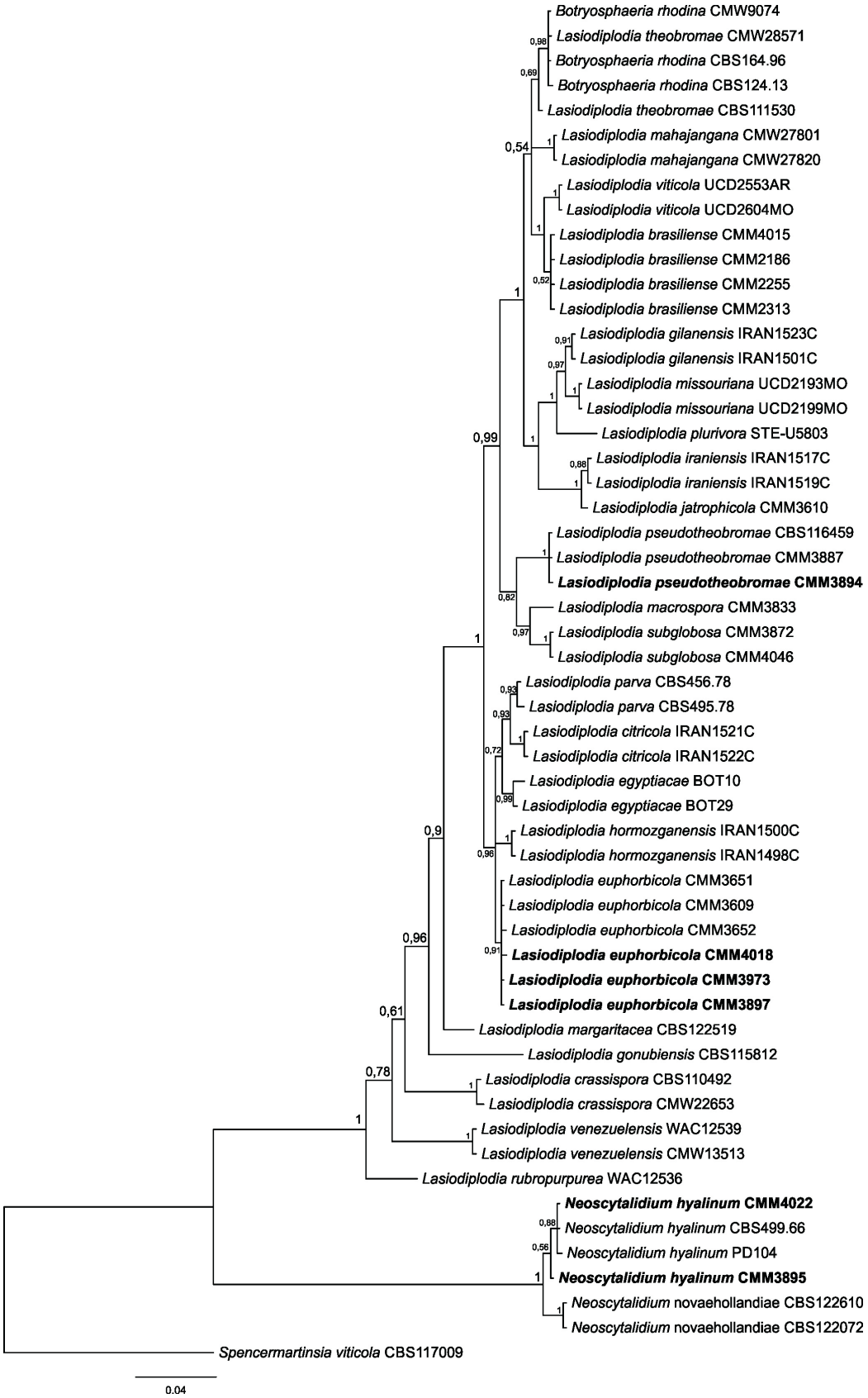

FIGURE 1 - Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS, TEF-1α and βt genes. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Spencermartinsia viticola* CBS117009. Isolates characterized in this study are highlighted in bold.

TABLE 2 - Biometric data of *Lasiodiplodia* spp. and *Neoscytalidium* spp. examined in this and in previous studies.

Species	Conidia (µm)	Paraphyses (µm)	Conidiogenous cells (µm)	Reference
L. theobromae	21–31 × 13–15.5	55 × 3–4	-	Alves et al., 2008
L. pseudotheobromae	23.5–32 ×14–18	58 × 3–4	-	Alves et al., 2008
	16–26 × 10–12	75 × 3–4	7–10 × 3–4	This study
L. euphorbicola	15–23 × 9–12	76 × 2–4	5–15 × 3–4	Machado et al., 2014
	17–24 × 10–12	40 × 2–3	5–12 × 2–3	This study
Species	**Conidia (µm)**	**Arthroconidia (µm)**	**Conidiogenous cells (µm)**	**Reference**
N. hyalinum	10–16(–21) × 3.5–6.5	4–16.5 × 8.5	6.5–14 × 2.5–4	Phillips et al., 2013
	8–12 × 4–5	4–12 × 2.5–8	6–10 × 1.5–2.5	Machado et al., 2014
	5–12 × 3–5	6–12 × 3–6	7–10 × 2–3	This study
N. novaehollandiae	10.5–12.5 × 4–5	5.5–7.5 × 3.5–4.5	7–10 × 2–3	Pavlic et al., 2008

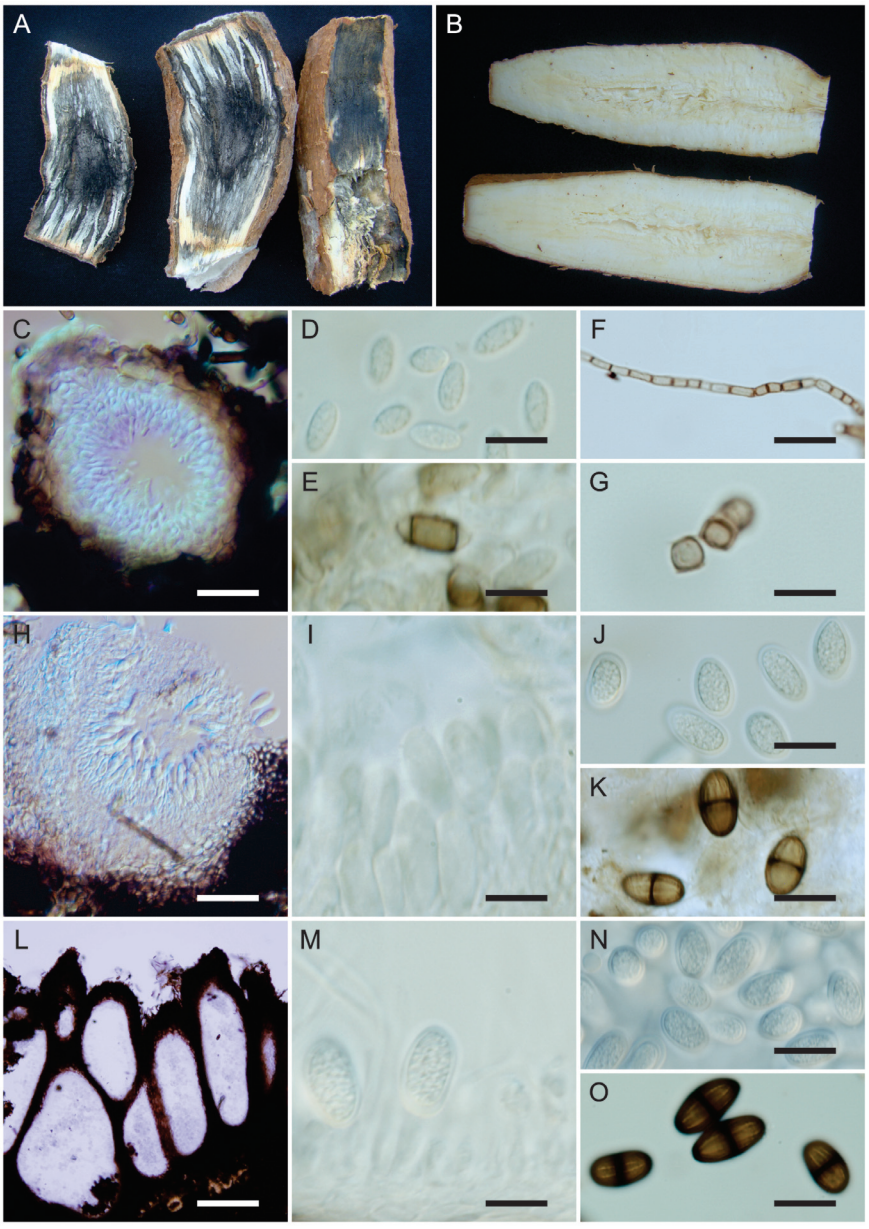

FIGURE 2 - Botryosphaeriaceae species causing black root rot of cassava. **A.** Symptoms of black root rot produced for Botryosphaeriaceae species in pathogenicity tests; **B.** Asymptomatic root used as control in pathogenicity tests; **C-G.** *Neoscytalidium hyalinum*. **C.** Conidiomata; **D, E.** Hyaline and septate mature conidia; **F, G.** Arthroconidia; **H-K.** *Lasiodiplodia euphorbicola*. **H.** Section of a conidiomata formed on *Pinus* twigs; **I.** Conidiogenous cells; **J, K.** Immature and mature pigmented conidia with longitudinal striations; **L-O.** *Lasiodiplodia pseudotheobromae*. **L.** Section of multilocular conidiomata formed on the host surface; **M.** Conidiogenous cells; **N, O**. Immature and mature pigmented conidia with longitudinal striations. Scale bars: C, H, L = 100 µm; I, J, K, M = 15 µm; N, O = 20 µm.

et al., 2012; 2014; Marques et al., 2013b). *Lasiodiplodia pseudotheobromae* has been described in *Carica papaya* L., *Jatropha curcas* and *Mangifera indica* (Marques et al., 2013a; Machado et al., 2014; Netto et al., 2014), whereas *L. euphorbicola* was reported only on *Jatropha curcas* and *Carica papaya* (Machado et al., 2014; Netto et al., 2014). This is the first report of the occurrence of *L. euphorbicola*, *L. pseudotheobromae* and *N. hyalinum* on cassava.

Since black rot is a major limiting factor for cassava production in Brazil, the correct identification of the associated pathogen(s) is essential for future studies of disease management and for the selection of resistant varieties, and provides new and relevant information for quarantine programs.

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