Lasiodiplodia hormozganensis causing basal stem rot on *Ricinus communis* in Brazil

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Abstract

Ricinus communis plants showing symptoms of root and stem rot were observed in the states of Bahia and Paraíba, Brazil. Based on the morphology and phylogenetic analyses of ITS and TEF-1 α combined, the causal agents of the observed symptoms were identified as *Lasiodiplodia hormozganensis* and *L. theobromae*, pathogenicity was confirmed by fulfilling Koch's postulates. To our knowledge, this is the first report from any part of the world of *L. hormozganensis* causing root and stem rot in *R. communis*.

Keywords *Botryosphaeriales* · Biofuel · Phylogeny · *Ricinus communis* · Stem rot

Castor (*Ricinus communis*) is a non-edible oilseed crop belonging to the family Euphorbiaceae. This crop is of great importance to the chemical industry as a raw material used in a variety of products such as lubricants, pharmaceuticals, cosmetics, paints, and plastics, since it is the only commercial source of a hydroxylated fatty acid (Severino et al. 2012). In Brazil, castor is commonly cultivated in the semiarid areas of the Northeast region, where few diseases have been reported (Severino et al. 2012). During a survey of castor diseases in the states of Bahia and Paraíba, castor plants were found showing symptoms of stem, collar, and root rot, eventually resulting in plant demise. Symptoms were usually observed on adult plants during capsule maturing stages, despite soil type, but usually more frequent under water-deficit stress. Symptomatic plants were collected in the field, and both roots and basal portion of the stem were

relocated to the Plant Pathology Laboratory of Embrapa Algodão for examination. Black pycnidia with two-celled spores, initially hyaline but turning brown on maturity, were observed suggesting that a *Lasiodiplodia* sp. was associated with the symptoms. Up to now, only *L. theobromae* had been reported causing stem rot and also dieback of castor plants in Brazil. The dieback is usually a consequence of injury caused during the harvesting of racemes in biannual varieties (Batista et al. 1996; Lima et al. 1997). On the other hand, basal stem and root rot of castor plants in Brazil are usually associated with *Macrophomina phaseolina* (Severino et al. 2012; Claudino and Soares 2014). Thus, the aim of the present work was to elucidate the etiology of the *Lasiodiplodia* sp. associated with basal stem and root rot of castor plants in Brazil.

Isolation of *Lasiodiplodia* sp. was secured by direct transfer of the spore mass to Petri dishes filled with Potato Dextrose Agar (PDA), as well as by transferring small tissue fragments obtained from transition areas between healthy and symptomatic tissues. These fragments were disinfected in 70% ethanol for 1 min followed by 1% sodium hypochlorite for 3 min and washed in sterile distilled water. Approximately 50 samples showing stem and root rot symptoms were collected during above-mentioned survey. From those, only six samples resulted in colonies with morphology compatible with *Lasiodiplodia* sp., while the majority were compatible

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with *M. phaseolina*. All six isolates obtained were deposited in the Coleção de Culturas de Microrganismos Fitopatogênicos of the Embrapa Algodão (CCMF-CNPA) with accession numbers CCMF-CNPA 0553, CCMF-CNPA 554, CCMF-CNPA 0555, CCMF-CNPA 0556, CCMF-CNPA 585, and CCMF-CNPA 0603.

The isolates of *Lasiodiplodia* sp. obtained were grown in Petri dishes with 2% Water Agar (WA - Agar Agar, type I Himedia®) and incubated at 25 °C for 12 days. Subsequently, the growing tips of hyphae of colonies were cut out and transferred to Petri dishes with PDA. The isolates were grown on Petri dishes containing 2% WA overlaid with triple-sterilized corn straw and incubated at 25 °C with a photoperiod of 12 h to induce the formation of pycnidia and spores. Observations, measurements and photographs were taken on an Olympus BX 53 microscope equipped with a digital camera Q-Color 5 Olympus.

All isolates had similar morphology: *conidiomata* stromatic, pycnidial, produced on corn straw on WA, superficial, dark, with an apical ostiole, stromatic wall composed of several layers of dark brown, thick-walled cells; *conidia* elipsooidal to cylindrical, one-celled and hyaline when immature, turning to dark brown, striate didymospores when mature (Fig. 1), typical of the genus *Lasiodiplodia*.

To extract genomic DNA, the isolates were grown on PDA at 25 °C for 1 week. Approximately 40 mg of mycelia were collected and placed in a 2 mL

microcentrifuge tube containing 600 µL of Nuclei Lysis Solution of the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.), 100 mg of Polyvinylpyrrolidone (PVP; Sigma-Aldrich Co.) and four steel beads. Next, the samples were mixed and crushed in the L-Beater 3 (Loccus Biotecnologia). After maceration, the extraction was continued as described by Pinho et al. (2012).

Target sequences of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS) and Translation Elongation Factor 1- α (TEF1- α) were amplified using primers ITS1 and ITS4 for ITS (White et al. 1990); EF1-728F (Carbone and Kohn 1999) and EF2R (Jacobs et al. 2004) or EF1-688F (Alves et al. 2008) and EF1-986R (Carbone and Kohn 1999) for partial TEF1- α . The PCR conditions and reagents were the same as those described by Machado et al. (2014).

Consensus sequences were compared against GenBank's database using their Mega BLAST program for a first identification. The ITS and TEF1- α sequences of additional species were retrieved from GenBank (Table 1) and aligned with sequences generated in this study using the multiple sequence alignment MUSCLE® program (Edgar 2004), an application of the MEGA v. 6 software program (Tamura et al. 2013). Alignments were checked, and manual adjustments were made where necessary.

Bayesian Inference (IB) analyses of the concatenated gene regions were performed as described by Machado

Fig. 1 Morphological characteristics of *L. hormozganensis* isolates, **a** colony of *L. hormozganensis* on PDA grown in the dark, **b** pycnidia with conidia exuded as a cirrus on corn straw, **c** Pycnidia releasing conidia immature hyaline, becoming pigmented with age, **d** conidia pigmented, septate and striate. Bars: c = 20 µm, d = 20 µm

Table 1 Isolate number, location, host/substrate and genbank accession numbers of DNA sequences of *Lasiodiplodia* spp. used in phylogenetic analyses

Species	Isolates	Location	Host/Substrate	Reference	Genbank accession number	
					ITS	TEF1- α
<i>Lasiodiplodia brasiliense</i>	CMM4015	Brazil	<i>Mangifera indica</i>	Netto et al. 2014	JX464063	JX464049
<i>L. brasiliense</i>	CMM2313	Brazil	<i>Carica papaya</i>	Netto et al. 2014	KC484793	KC481524
<i>L. caatinguensis</i>	IBL 40	Brazil	<i>Spondias purpurea</i>	Coutinho et al. 2016	KT154762	KT154755
<i>L. caatinguensis</i>	CMM 1325	Brazil	<i>Citrus sinensis</i>	Coutinho et al. 2016	KT154760	KT008006
<i>L. citricola</i>	IRAN1521C	Iran	<i>Citrus</i> sp.	Abdollahzadeh et al. 2010	GU945353	GU945339
<i>L. citricola</i>	IRAN1522C	Iran	<i>Citrus</i> sp.	Abdollahzadeh et al. 2010	GU945354	GU945340
<i>L. crassisporea</i>	CBS110492	Australia	Unknown	Burgess et al. 2006	EF622086	EF622066
<i>L. crassisporea</i>	CMW22653	Australia	<i>Pterocarpus angolensis</i>	Burgess et al. 2006	FJ888465	FJ888452
<i>L. egyptiaca</i>	CBS130992	Egypt	<i>Mangifera indica</i>	Ismail et al. 2012	JN814397	JN814424
<i>L. egyptiaca</i>	BOT-29	Egypt	<i>Mangifera indica</i>	Ismail et al. 2012	JN814401	JN814428
<i>L. euphorbiicola</i>	CMM3651	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234553	KF226711
<i>L. euphorbiicola</i>	CMM3652	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234554	KF226715
<i>L. euphorbiicola</i>	CMM3609	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234543	KF226689
<i>L. exigua</i>	CBS 137785	Tunisia	<i>Retama raetam</i>	Linaldeddu et al. 2014	KJ638317	KJ638336
<i>L. exigua</i>	BL 184	Tunisia	<i>Retama raetam</i>	Linaldeddu et al. 2014	KJ638318	KJ638337
<i>L. gilanensis</i>	IRAN1523C	Iran	Unknown	Abdollahzadeh et al. 2010	GU945351	GU945342
<i>L. gilanensis</i>	IRAN1501C	Iran	Unknown	Abdollahzadeh et al. 2010	GU945352	GU945341
<i>L. gonubiensis</i>	CBS115812	South Africa	<i>Syzygium cordatum</i>	Pavlic et al. 2004	DQ458892	DQ458877
<i>L. gravistriata</i>	CMM 4564	Brazil	<i>Anacardium humile</i>	Netto et al. 2016	KT250949	KT250950
<i>L. gravistriata</i>	CMM 4565	Brazil	<i>Anacardium humile</i>	Netto et al. 2016	KT250947	KT266812
<i>L. hormozganensis</i>	IRAN1500C	Iran	<i>Olea</i> sp.	Abdollahzadeh et al. 2010	GU945355	GU945343
<i>L. hormozganensis</i>	IRAN1498C	Iran	<i>Mangifera indica</i>	Abdollahzadeh et al. 2010	GU945356	GU945344
<i>L. hormozganensis</i>	CCMF-CNPA 0553	Brazil	<i>Ricinus communis</i>	This study	MG787090	MG806911
<i>L. hormozganensis</i>	CCMF-CNPA 0555	Brazil	<i>Ricinus communis</i>	This study	MG787091	MG806912
<i>L. hormozganensis</i>	CCMF-CNPA 0603	Brazil	<i>Ricinus communis</i>	This study	MG787092	MG806913
<i>L. hormozganensis</i>	CCMF-CNPA 0556	Brazil	<i>Ricinus communis</i>	This study	MG787093	MG806914
<i>L. iraniensis</i>	IRAN1517C	Iran	<i>Citrus</i> sp.	Abdollahzadeh et al. 2010	GU945349	GU945337
<i>L. iraniensis</i>	IRAN1519C	Iran	<i>Mangifera indica</i>	Abdollahzadeh et al. 2010	GU945350	GU945338
<i>L. jatrophiicola</i>	CMM3610	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234544	KF226690
<i>L. jatrophiicola</i>	CMM 4472	Brazil	<i>Anacardium occidentale</i>	Netto et al. 2014	KT325572	KT325585
<i>L. laeliocattleyae</i>	CBS 167.28	Italy	<i>laeliocattleyae</i>	Rodríguez-Gálvez et al. 2016	KU507462	KU507429
<i>L. laeliocattleyae</i>	LAREP1	Peru	<i>Mangifera indica</i>	Rodríguez-Gálvez et al. 2016	KU507484	KU507451
<i>L. margaritacea</i>	CBS122519	Australia	<i>Adansonia gibbosa</i>	Pavlic et al. 2008	EU144050	EU144065
<i>L. macrospora</i>	CMM3833	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234557	KF226718
<i>L. mahajangana</i>	CMW27801	Madagascar	<i>Terminalia catappa</i>	Begoude et al. 2010	FJ900595	FJ900641
<i>L. mahajangana</i>	CMW27820	Madagascar	<i>Terminalia catappa</i>	Begoude et al. 2010	FJ900597	FJ900643
<i>L. mediterranea</i>	CBS 137783	Italy	<i>Quercus ilex</i>	Linaldeddu et al. 2014	KJ638312	KJ638331
<i>L. mediterranea</i>	CBS 137784	Italy	<i>Vitis vinifera</i>	Linaldeddu et al. 2014	KJ638311	KJ638330
<i>L. missouriiana</i>	UCD2193MO	USA	<i>Vitis vinifera</i>	Úrbez-Torres et al. 2011	HQ288225	HQ288267
<i>L. missouriiana</i>	UCD2199MO	USA	<i>Vitis vinifera</i>	Úrbez-Torres et al. 2011	HQ288226	HQ288268
<i>L. parva</i>	CBS456.78	Colombia	Cassava-field soil	Alves et al. 2008	EF622083	EF622063
<i>L. parva</i>	CBS495.78	Colombia	Cassava-field soil	Alves et al. 2008	EF622085	EF622065
<i>L. plurivora</i>	STE-U5803	South Africa	<i>Vitis vinifera</i>	Damm et al. 2007	EF445362	EF445395
<i>L. pontae</i>	CMM1277	Brazil	<i>Spondias purpurea</i>	Coutinho et al. 2016	KT151794	KT151791
<i>L. pseudotheobromae</i>	CBS116459	Costa Rica	<i>Gmelina arborea</i>	Alves et al. 2008	EF622077	EF622057
<i>L. pseudotheobromae</i>	CMM3887	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234559	KF226722
<i>L. pyriformis</i>	CBS 121770	Namibia	<i>Acacia mellifera</i>	Slippers et al. 2014	EU101307	EU101352
<i>L. pyriformis</i>	CBS 121771	Namibia	<i>Acacia mellifera</i>	Slippers et al. 2014	EU101308	EU101353
<i>L. rubropurpurea</i>	WAC12536	Australia	<i>Eucalyptus grandis</i>	Burgess et al. 2006	DQ103554	DQ103572
<i>L. subglobosa</i>	CMM3872	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234558	KF226721
<i>L. subglobosa</i>	CMM4046	Brazil	<i>Jatropha curcas</i>	Machado et al. 2014	KF234560	KF226723
<i>L. thailandica</i>	CPC 22755	Thailand	<i>Phyllanthus acidus</i>	Trakunyingcharoen et al. 2014	KM006433	KM006464
<i>L. thailandica</i>	CPC22795	Thailand	<i>Mangifera indica</i>	Trakunyingcharoen et al. 2014	KJ193637	KJ193681
<i>L. theobromae</i>	CBS164.96	Papua New Guinea	Fruit along coral reef coast	Phillips et al. 2005	AY640255	AY640258
<i>L. theobromae</i>	CBS124.13	USA	Unknown	Alves et al. 2006	DQ458890	DQ458875
<i>L. theobromae</i>	CBS111530	Unknown	Unknown	Alves et al. 2008	EF622074	EF622054
<i>L. theobromae</i>	CCMF-CNPA 0554	Brazil	<i>Ricinus communis</i>	This study	MH485394	MH491477
<i>L. theobromae</i>	CCMF-CNPA 0585	Brazil	<i>Ricinus communis</i>	This study	MH485395	MH491478
<i>L. venezuelensis</i>	CMW13513	Venezuela	<i>Acacia mangium</i>	Burgess et al. 2006	DQ103549	DQ103570
<i>L. venezuelensis</i>	WAC12539	Venezuela	<i>Acacia mangium</i>	Burgess et al. 2006	DQ103547	DQ103568

Table 1 (continued)

Species	Isolates	Location	Host/Substrate	Reference	Genbank accession number	
					ITS	TEF1- α
<i>L. viticola</i>	UCD2553AR	USA	<i>Vitis vinifera</i>	Úrbez-Torres et al. 2011	HQ288227	HQ288269
<i>L. viticola</i>	UCD2604MO	USA	<i>Vitis vinifera</i>	Úrbez-Torres et al. 2011	HQ288228	HQ288270
<i>Diplodia mutila</i>	CBS 136015	Portugal	<i>Populus alba</i>	Alves et al. 2014	KJ361838	KJ361830

ITS internal transcribed spacer regions 1 and 2, including the 5.8S ribosomal rRNA gene, *TEF1- α* translation elongation factor 1- α

et al. (2014). The K80 + I evolution models for the ITS region were used in the analyses while HKY + I + G was selected for TEF1- α . The resulting trees were visualized in the Figtree v.1.3.1 program (Rambaut 2009) and later exported to graphic programs. Based on the results of combined phylogenetic analyses of ITS and TEF1- α gene regions, four isolates were identified as *Lasiodiplodia hormozganensis*, since they grouped with the type isolate of *L. hormozganensis* (IRAN1500C), and two isolates were identified as *L. theobromae*, the tree was rooted to *Diplodia mutila* (CBS 136015) (Fig. 2).

For the pathogenicity test (Fig. 3), castor plants of BRS Energia cultivar, approximately 3 months old grown in a greenhouse, were inoculated with isolates obtained during the course of this study, using five plants per isolate. For inoculation, the isolates were grown in petri dishes with PDA at 25 °C for 7 days. Healthy plants had their stalks wounded with a sterilized scalpel and a 6-mm-diameter PDA disk containing mycelium of each isolate was deposited on the wound. A portion of cotton moistened with autoclaved distilled water was placed on each disk and then covered with plastic film for the first 48 h after inoculation to maintain moisture. The control treatment consisted of PDA plugs, with no fungus grown, deposited on the injured stalks as described above. The plants were then kept in a greenhouse for 30 days. All inoculated plants showed a necrosis and constriction on inoculation area and a severe wilt symptom. The fungus was re-isolated from the all inoculated plants and its identity was confirmed as previously described thereby satisfying Koch's Postulates. No fungus was re-isolated from control plants.

As above-mentioned, *L. theobromae* was already known to be associated to stem rot of castor plants in Brazil. On the other hand, *L. hormozganensis* was described in Iran associated with *Olea* sp. and *Mangifera indica* (Abdollahzadeh et al. 2010). In Brazil, this species has been reported to cause dieback in mango and grape, and stem-end rot in mango and papaya in the

Fig. 2 Multilocus phylogenetic tree of *Lasiodiplodia* species inferred from Bayesian analysis based on the combined sequences of the ITS and TEF1- α . Bayesian posterior probabilities are indicated above the nodes and the tree was rooted to *Diplodia mutila* (CBS 136015). The bar represents the number of changes in the nucleotide sequence of each 100 bp. The species in this study are highlighted in bold

Fig. 3 Pathogenicity test on *R. communis*, **a** control, **b** plant inoculated with *Lasiodiplodia hormozganensis*

Northeast region of the country (Marques et al. 2013; Netto et al. 2014; Correia et al. 2015). Thus, the results of this work suggest this is the first report of *L. hormozganensis* causing basal stem and root rot on *R. communis*.

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