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Short Note

Fungal trunk pathogens associated with table grape decline in North-eastern Brazil

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Summary. During the last five years a decline of table grape plants has been noticed in nurseries, young plantations and vineyards of the Northeastern region of Brazil, where the management systems for grapevine production are adapted to the specific environmental conditions of a tropical viticulture. Samples of table grape plants showing decline symptoms were obtained from grapevine nurseries, young plantations and vineyards located in the São Francisco, Assú and Siriji Valleys in 2010, and were subjected to fungal isolation. Grapevine trunk pathogens were identified using morphological and molecular methods. Species recovered included *Botryosphaeria mamane*, *Campylocarpon fasciculare*, *C. pseudofasciculare*, *Lasiodiplodia crassipora*, *L. parva*, *L. pseudotheobromae*, *L. theobromae*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* and *Phaeomoniella chlamydospora*. They are all reported for the first time on grapevine in Brazil, with the exception of *L. theobromae*. Moreover, *Botryosphaeria mamane*, *Lasiodiplodia parva* and *L. pseudotheobromae* are reported for the first time on grapevine, and *C. fasciculare* is reported for the first time on the American continent.

Key words: Botryosphaeriaceae, *Vitis vinifera*.

Introduction

In 2011, 58,236 t of table grapes (*Vitis* spp.) were exported from Brazil, being the main fresh fruit export from this country and accounting for US$ 141 million (Agrianual, 2012). Most of these table grapes are produced in the Northeastern region, where 9,600 ha are cultivated in three different areas: the São Francisco Valley, located in the semi-arid region of Bahia and Pernambuco States; the Assú Valley, located in the semi-arid region of Rio Grande do Norte State; and the Siriji Valley, located in the humid region of Pernambuco State. The São Francisco Valley is the main table grape growing area in the region, accounting for 98% of the production. The Assú Valley is a new area of production of fine table grapes for export, which was implemented five years ago, while in the Siriji Valley table grapes have been grown for over 40 years with a production intended only for the local market (Araújo and Ramalho, 2009). North-eastern Brazil is a tropical region, thus the management systems for grapevine production are adapted to the specific environmental conditions of a tropical viticulture. In both the dry and wet tropics, the growth and cropping cycle of the vine can be manipulated to extend from 5 to 12 months by

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a combination of pruning, modifying vine water status and the use of chemical regulators. Thus, it is possible to achieve two and a half to three vegetative cycles per year (Camargo *et al*., 2008; Possingham, 2008).

During the last five years a decline of table grape plants has been noticed in nurseries, young plantations and vineyards of the Northeastern regions of Brazil. Symptoms included poor early growth, reduced vigour showing leaf yellowing, wilting and dieback, and different symptoms in wood such as V-shaped necroses when affected arms and trunks were cut in cross-section, and longitudinal brown and black streaking that appeared as necrotic black spots in cross-sections. These symptoms are similar to those described in other viticultural regions worldwide (Luque *et al*., 2009; Gramaje and Armengol, 2011), which have been associated with several decline diseases such as black dead arm, black foot, eutypiose, esca or Petri disease (Moller and Kasimatis, 1981; Mugnai *et al*., 1999; Halleen *et al*., 2006; Mostert *et al*., 2006; Úrbez-Torres, 2011). Nevertheless, grapevine decline and its associated pathogens are yet to be studied in tropical viticulture. Thus, the aim of this work was to determine the occurrence of fungal trunk pathogens in declining table grapes in Northeastern Brazil.

Figure 1. Table grape growing areas surveyed in Northeastern Brazil (the São Francisco, Assú and Siriji Valleys) in 2010.

Materials and methods

Sampling and isolation of fungi

Samples of table grape plants showing decline symptoms were obtained from grapevine nurseries, young plantations and vineyards located in the São Francisco, Assú and Siriji Valleys (Northeastern Brazil) in 2010 (Figure 1), and were subjected to fungal isolation. At least 5–7 plants were analyzed per sample.

Rootstocks, graft unions and scions were examined. Symptomatic wood fragments taken from the margin of dead and healthy tissue, and from internal necroses and brown-black vascular streaking were washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of necrotic, discolored or decayed tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, England) supplemented with 0.5 g L^{-1} of streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 25ºC in the dark for 14 to 21 days, and all colonies were transferred to 2% potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France). Thirty two representative isolates were selected for further analyses (Table 1). They were hyphal-tipped or single-spored with the serial dilution method, prior to morphological and molecular identification (Dhingra and Sinclair, 1995).

Fungal identification

Morphological identification

Species of the Botryosphaeriaceae were identified by colony and conidial morphology (Phillips, 2006). In order to enhance sporulation, cultures were placed with sterilized pine needles on 2% water agar (WA; Biokar-Diagnostics) at 25°C with a 12-h day (Philips TDL18W/33) (Slippers *et al*., 2004). Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology (cell wall, shape, color, and presence or absence of septa) from pycnidia was recorded. Species of *Campylocarpon* were identified by macroscopic characters such as colony texture, color, and the margin on PDA. Colonies grown on PDA were incubated for a further 20 days to determine the presence/absence of chlamydospores. Co-

Table 1. List of fungal trunk pathogens isolated from table grapes in Northeastern Brazil, with their geographical origin.

Species	Isolate	State/Town		Rootstock/scion[a]
Botryosphaeria mamane	BV1	Rio Grande do Norte	Assú	572/Red Globe
Campylocarpon fasciculare	BV2		Assú	572/Red Globe
	BV3		Assú	572/Red Globe
	BV4		Assú	572/Red Globe
	BV5		Assú	572/Red Globe
	BV6		Assú	572/Red Globe
Campylocarpon pseudofasciculare	BV7		Assú	572/Red Globe
Lasiodiplodia crassispora	BV8	Pernambuco	Petrolina	766/nd
Lasiodiplodia parva	BV9		Petrolina	572/nd
Lasiodiplodia pseudotheobromae	BV10		Machados	cv. Isabel
	BV11		Machados	cv. Isabel
	BV12		Machados	cv. Isabel
Lasiodiplodia theobromae	BV13		Machados	cv. Isabel
	BV14		Machados	cv. Isabel
	BV15		Petrolina	572/nd
	BV16		Petrolina	313/nd
	BV17		Petrolina	SO4/nd
	BV18		Machados	cv. Isabel
	BV19		Machados	cv. Isabel
	BV20		Petrolina	420 A/nd
	BV21	Rio Grande do Norte	Assú	572/Red Globe
	BV22		Assú	572/Red Globe
Neofusicoccum parvum	BV23	Pernambuco	Petrolina	313/nd
	BV24		Machados	cv. Isabel
Phaeoacremonium aleophilum	BV25		Petrolina	572/nd
	BV26		Petrolina	SO4/nd
	BV27		Petrolina	766/nd
Phaeoacremonium parasiticum	BV28		Machados	cv. Isabel
	BV29		Machados	cv. Isabel
Phaeomoniella chlamydospora	BV30		Petrolina	SO4/nd
	BV31		Machados	cv. Isabel
	BV32		Machados	cv. Isabel

[a] nd = not determined. Grapevines cv. Isabel are planted without rootstock.

nidia size was also measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Halleen *et al*., 2004). Morphological characters to distinguish species of *Phaeoacremonium* included conidiophore morphology, phialide type and shape, size of hyphal warts and colony characters and pigment production on MEA, PDA and oatmeal agar (OA, 60 g oatmeal; 12.5 g 16 agar; Difco, Osi, Maurepas, France) (Mostert *et al*., 2006). *Phaeomoniella chlamydospora* was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics on PDA and MEA (Crous and Gams, 2000).

DNA isolation and sequencing

Fungal mycelium and conidia from pure cultures grown on PDA for 2 to 3 weeks at 25ºC in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and stored at -20ºC.

Identification of Botryosphaeriaceae species was confirmed by analysis of elongation factor 1-α gen (EF) amplified using EF1-728F and EF1-986R primers (Carbone and Kohn, 1999). For *Campylocarpon* species identification, the internal transcribed spacer (ITS) region of DNA was amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns, 1993) and partial sequence of the first part of the β-tubulin gene, BT, were amplified using primers BT1a and BT1b (Petit and Gubler, 2005). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen Inc., Sequencing Center (Seoul, South Korea). *Phaeoacremonium* species were confirmed by sequence analysis of the β-tubulin gene using primer sets T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), and by comparison with the polyphasic, online identification system for *Phaeoacremonium* species recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx) developed by Mostert *et al*. (2006). *Pa. chlamydospora* was identified by PCR using primers Pch1-Pch2 (Tegli *et al*., 2000), and confirmed by sequencing the ITS region of DNA using the primers ITS1F and ITS4 (Gardes and Bruns, 1993).

Results and discussion

Based on their appearance in culture, the isolates obtained from the margin of dead and healthy tissue, and from internal necroses and brown-black vascular streaking of symptomatic wood fragments of trunks and branches could be assigned to three main fungal groups.

The first group was characterized by dark green or gray to dark gray fast-growing mycelium on PDA. With age, most of these cultures developed single or grouped, black, globose fruiting bodies (pycnidia) on the surface of pine needles on WA releasing either pigmented or hyaline conidia characteristic of Botryosphaeriaceae spp. (Van Niekerk *et al*., 2004; Phillips, 2006). BLASTn searches in GenBank showed that EF sequences of Botryosphaeriaceae isolates from Northeastern Brazil had 99 to 100% identity with isolates of *Botryosphaeria mamane* CMW 13416 (GU134938), *Lasiodiplodia crassipora* CMW 22653 (FJ888452), *L. parva* CMW 28309 (GQ469904), *L. pseudotheobromae* CMW 26702 (GQ471796), *L. theobromae* CMW 28311 (GQ469898) and *Neofusicoccum parvum* CMW 26718 (FJ900658).

The second group of isolates was characterized by pale to medium brown flat slow-growing cultures on MEA. Different types of phialides that were variable in size and shape were observed in the aerial mycelium, and either discrete or integrated in conidiophores. Sporulation was abundant and conidia were hyaline and aseptate. All morphological characters corresponded to the genus *Phaeoacremonium* (Mostert *et al*. 2006). BLASTn searches showed that BT sequences of these isolates had 99 and 100% identity with isolates of *Pm. aleophilum* CBS 631.94 (JQ691663) and *Pm. parasiticum* P46 (HQ605022), respectively.

The last fungal group was characterized by grey-olivaceous to olivaceous-black, slow-growing colonies with sparse aerial mycelium. They showed abundant straight, pigmented conidia and dark green-brown conidiophores with light green to hyaline conidiogeneus cells. These morphological characters corresponded to the genus *Phaeomoniella* (Crous and Gams, 2000). BLASTn searches showed that the ITS sequences of these isolates had 100% identity with isolates previously identified as *Phaeomoniella chlamydospora* Pach-302 (JQ822210).

Additionally, isolates obtained from the crown area showed colonies with white to off-white or slightly brownish cottony to felty aerial mycelium. Abundant curved macroconidia, up to 6-septate with

obtuse apical and basal cells, were observed. Microconidia were absent. These morphological characters corresponded to the genus *Campylocarpon* (Halleen *et al.*, 2004). In this case, BLASTn searches showed that the BT sequences of these isolates had 98 and 100% identity with isolates of *C. fasciculare* CBS 112611 (AY677225) and *C. pseudofasciculare* CBS 112679 (AY677214), respectively, and the ITS sequences of these isolates had 99% identity with isolates of *C. fasciculare* CBS 113559 (AY677303) and *C. pseudofasciculare* FI2034 (GU198190). Sequences and Blast results of representative isolates of each species derived in this study were lodged in GenBank (Table 2).

These results show the high diversity of fungal trunk pathogens found in table grapes in Northeastern Brazil. These include species of Botryosphaeriaceae (*Botryosphaeria mamane*, *Lasiodiplodia crassispora*, *L. parva*, *L. pseudotheobromae*, *L. theobromae* and *Neofusicoccum parvum*), species belonging to the genera *Campylocarpon* (*C. fasciculare* and *C. pseudofasciculare*) and *Phaeoacremonium* (*Pm. aleophilum* and *Pm. parasiticum*), and *Phaeomoniella chlamydospora*. These are all reported for the first time on grapevine in Brazil, with the exception of *L. theobromae* (Gava *et al.*, 2010), which was the only species present in all production areas surveyed. Moreover, *Botryosphaeria mamane*, *Lasiodiplodia parva* and *L. pseudotheobromae* are reported for the first time on grapevine, and *C. fasciculare* is reported for the first time in the American continent. These species could be distinguished

Table 2. List of fungal trunk pathogens isolated from table grapes in Northeastern Brazil, with their corresponding GenBank accession numbers and data of Blast results obtained from GenBank.

Species	Isolate	GenBank accessions[a]	Blast accessions[b]	Query length	Gaps[c]	Identities[d]	Maximum identity (%)
Elongation factor 1-α gen							
Botryosphaeria mamane	BV1	JX521846	GU134938	440	1/425	424/425	99
Lasiodiplodia crassispora	BV8	JX521847	FJ888452	458	0/443	443/443	100
Lasiodiplodia parva	BV9	JX521848	GQ469904	482	0/464	459/464	99
Lasiodiplodia pseudotheobromae	BV10	JX521849	GQ471796	480	0/462	462/462	100
	BV11	JX521850	GQ471796	485	0/462	462/462	100
	BV12	JX521851	GQ471796	478	0/457	457/457	100
Lasiodiplodia theobromae	BV13	JX521852	GQ469898	481	1/470	467/470	99
	BV14	JX521853	GQ469898	477	1/468	465/468	99
	BV15	JX521854	GQ469898	476	0/467	464/467	99
	BV16	JX521855	GQ469898	488	0/475	473/475	99
	BV17	JX521856	GQ469898	468	0/454	453/454	99
	BV18	JX521857	GQ469898	488	1/476	473/476	99
	BV19	JX521858	GQ469898	472	1/459	456/459	99
	BV20	JX521859	GQ469898	478	1/467	465/467	99
	BV21	JX521860	GQ469898	481	1/470	467/470	99
	BV22	JX521861	GQ469898	483	0/470	468/470	99
Neofusicoccum parvum	BV23	JX521862	FJ900658	457	0/440	435/440	99
	BV24	JX521863	FJ900658	447	0/431	427/431	99

(*Continued*)

Table 2. *Continues.*

Species	Isolate	GenBank accessions[a]	Blast accessions[b]	Query length	Gaps[c]	Identities[d]	Maximum identity (%)
β-tubulin gene							
Campylocarpon fasciculare	BV2	JX521835	AY677225	244	0/242	238/242	98
	BV3	JX521836	AY677225	212	0/212	208/212	98
	BV4	JX521837	AY677225	250	0/247	243/247	98
	BV5	JX521838	AY677225	227	0/227	223/227	98
	BV6	JX521839	AY677225	249	0/249	244/249	98
Campyl. pseudofasciculare	BV7	JX521840	AY677214	215	0/215	215/215	100
Phaeoacremonium aleophilum	BV25	JX521841	JQ691663	651	0/651	650/651	99
	BV26	JX521842	JQ691663	657	0/655	655/655	100
	BV27	JX521843	JQ691663	649	0/649	649/649	100
Phaeoacremonium parasiticum	BV28	JX521844	HQ605022	673	0/673	673/673	100
	BV29	JX521845	HQ605022	694	1/695	694/695	99
Internal Transcribed Spacer region of DNA							
Campylocarpon fasciculare	BV2	JX521864	AY677303	563	0/495	493/495	99
	BV3	JX521865	AY677303	563	0/495	493/495	99
	BV4	JX521866	AY677303	562	0/495	493/495	99
	BV5	JX521867	AY677303	558	0/495	493/495	99
	BV6	JX521868	AY677303	552	0/493	491/493	99
Campyl. pseudofasciculare	BV7	JX521869	GU198190	564	2/510	508/510	99
Phaeomoniella chlamydospora	BV30	JX521870	JQ822210	404	0/404	404/404	100
	BV31	JX521871	JQ822210	383	0/383	383/383	100
	BV32	JX521872	JQ822210	399	0/399	399/399	100

[a] Corresponding GenBank accession numbers of fungal trunk pathogens isolated from table grapes in Northeastern Brazil.
[b] GenBank accession numbers blasted with the isolates obtained in this study.
[c] Number of spaces introduced into the alignment to compensate for insertions and deletions in our sequence relative to blasted sequences.
[d] Number of nucleotides of our sequences/Number of nucleotides of blasted sequences.

based on their DNA sequence data and unique morphological characters.

The most frequently isolated Botryosphaeriaceae sp. was *L. theobromae*, which is an important pathogen of mango (*Mangifera indica* L.) and papaya (*Carica papaya* L.) crops in Northeastern Brazil (Costa *et al*., 2010; Pereira *et al*., 2012). *Neofusicoccum parvum* is also prevalent in this region causing tip dieback and stem-end rot symptoms on mango (Costa *et al*., 2010). Although further research is needed to explain the role that inoculum of Botryosphaeriaceae spp. from non-grapevine hosts surrounding vineyards has on the disease cycle, the wide host range of this family could potentially provide an important source of primary inoculum in table grapes, due to the numerous fruiting bodies produced on non-grapevine hosts (Úrbez-Torres, 2011).

Regarding the genus *Campylocarpon*, it was established in 2004 with two species *C. fasciculare* and *C. pseudofasciculare* associated with black-foot disease

of grapevine in South Africa (Halleen *et al*., 2004). Subsequently *C. pseudofasciculare* and *C. fasciculare* were reported in Uruguay (Abreo *et al*., 2010) and Spain (Alaniz *et al*., 2011), respectively.

Phaeomoniella chlamydospora and *Pm. aleophilum* are species commonly isolated from young vines showing a general decline (Mugnai *et al*., 1999). Numerous other species of the genus *Phaeoacremonium* have also been associated with grapevine decline in grape-growing regions throughout the world, although their importance is thought to be minor (Mostert *et al*., 2006; Essakhi *et al*., 2008; Gramaje *et al*., 2009). One of these species is *Pm. parasiticum*, which in the American continent had already been reported in Argentina, Chile and Peru (Mostert *et al*., 2006; Romero-Rivas *et al*. 2009). It is interesting to note that *Pm. parasiticum*, under its original name *Phialophora parasitica*, was the first species of *Phaeoacremonium* reported to cause phaeohyphomycosis in humans (Ajello *et al*., 1974). This species has also been reported in Brazil causing subcutaneous infections in humans (Guarro *et al*., 2003; Marques *et al*., 2006) and is a good example of the wide substrate range of *Phaeoacremonium* spp. (Mostert *et al*., 2006).

Fungal trunk pathogens are becoming an important problem to the table grape industry in Brazil. Our study improves the knowledge of the fungal species involved in table grape decline in this country and contributes to a better understanding of this complex syndrome under the specific conditions of a developing tropical viticulture.

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