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NEW OR UNUSUAL DISEASE REPORTS

First report of *Neofusiccoccum vitifusiforme* and presence of other Botryosphaeriaceae species associated with Botryosphaeria dieback of grapevine in Sicily (Italy)

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Summary. Since 2007, when a grapevine decline caused by *Lasiodiplodia theobromae* was reported for the first time in Sicily, vines showing similar declining symptoms have been also found in other grape-growing areas of western and central Sicily. We report the result of a study on fungi associated with grapevine decline in Sicily, with particular regard to those belonging to the *Botryosphaeriaceae*. Four species were found to be associated with declining vines, namely *Diplodia seriata, Lasiodiplodia* sp., *Neofusicoccum parvum* and *Neofusicoccum vitifusiforme*, the latter species reported for the first time on *Vitis vinifera* in Italy.

Key words: *Diplodia seriata*, *Lasiodiplodia* sp., *Neofusicoccum parvum*, *Vitis vinifera*, grapevine trunk disease.

Introduction

Species in the Botryosphaeriaceae Theiss & P. Syd. are cosmopolitan and have been reported as endophytes, parasites, and saprophytes on a broad range of both annual and perennial hosts (Barr, 1972; Punithalingam, 1980; von Arx, 1987; Smith *et al*., 1996; Burgess *et al*., 2005; Slippers and Wingfield, 2007) including grapevines (Chamberlain *et al*., 1964; Lehoczky, 1974, 1988; Hewitt, 1988; Leavitt, 1990, Úrbez-Torres, 2011). Investigations conducted during the last decade in different countries have shown that actually several species of Botryosphaeriaceae have a pathogenic role in *Vitis vinifera* causing trunk diseases (Phillips, 2002; van Niekerk *et al*., 2004, 2006; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2008; Úrbez-Torres and Gubler, 2009; Linaldeddu *et al*., 2010; Pitt *et al*., 2010), recently reported as Botryosphaeria dieback (Úrbez-Torres, 2011). Geographical distribution of some Botryosphaeriaceae species has been shown to be associated with climate. In particular, *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. is the prevalent species in warmer grape-growing areas of many countries such as Australia (Taylor *et al*., 2005; Pitt *et al*., 2010), California, Arizona, Mexico (Úrbez-Torres *et al*., 2006, 2008), Egypt (El-Goorani and El Meleigi, 1972), Spain (Aroca *et al*., 2008) and also in Italy (Burruano *et al*., 2008).

Among the 21 Botryosphaeriaceae reported to be associated with decline symptoms on grapevine (Úrbez-Torres, 2011), only five species have been to date recognized in Italy on *V. vinifera*: *Botryosphaeria dothidea* in Apulia (Carlucci *et al*., 2009), Marche (Romanazzi *et al*., 2009) and Central Italy (Spagnolo *et al*., 2011); *Diplodia seriata* in Molise (Cristinzio, 1978), Apulia (Pollastro *et al*., 2000) and Central Italy (Spagnolo *et al*., 2011); *L. theobromae* in Sicily (Burruano *et al*., 2008) and Apulia (Carlucci *et al*., 2009); *Neofusicoccum australe* in Sardinia (Linaldeddu *et al*., 2010) and *Neofusicoccum parvum* in Apulia (Carlucci *et al*., 2009) and Central Italy (Spagnolo *et al*., 2011). These

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species are associated with grapevine decline symptoms, as late sprout and or dead buds, sub-cortical brown streaking and wedge-shaped wood necrosis, and with "esca" symptoms. After the first report of Botryosphaeria dieback in a vineyard in West Sicily by Burruano *et al*. (2008), similar grapevine decline symptoms were also observed in other Sicilian grape-growing areas. Thus, the aim of the present study was to ascertain the occurrence and identity of the Botryosphaeriaceae species associated with Botryosphaeria dieback in Sicily.

Materials and methods

Over the four years (2008–2011) since the first report on grapevine decline in Marsala (Trapani), declining plants were gradually detected and some of these collected in different grape-growing areas in Sicily: 3 plants cultivar Insolia in Marsala, annually from 2008–2010, and in 2010–2011, 2 plants cv. Merlot in Salemi (Trapani), 2 cv. Grillo in Alcamo (Trapani), 2 cv. Alicante Bouchet in Montevago (Agrigento) and 2 cv. Insolia in Milena (Caltanisetta).

Sample collection and fungal isolation

In the summer of each year (July–September), declining grapevines were collected and sectioned in several portions both to detect and describe wood symptoms and for isolations. Symptomatic wood portions, after bark removal, were further cut into disks, about 2–3 cm in diameter, which were flame sterilized. Fragments at the margin between the healthy and affected tissue were excised aseptically and plated on 2% malt-extract agar (MEA, Oxoid, Milan, Italy). Petri dishes were kept at 25±1°C in the dark and examined daily for fungal development during two weeks. The isolation frequency (IF) of each fungal genus was calculated using the formula: IF = (Nif/Ntf)×100, where Nif is the number of colonies of a given fungus, and Ntf the total number of isolations attempted ×100 (Ragazzi *et al*., 2001).

The Botryosphaeriaceae isolates, which were selected on the basis of gross colony morphology, were transferred to PDA (Oxoid) and incubated until fungal spore production. Identification of fungal species was based on the morphology of monosporic colonies and conidial characters. Conidia (100) were observed and measured at 40× magnification with a light microscope equipped with an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd, Germany).

The optimum growth temperature of three isolates of each morphologically characterized species was determined by incubating the cultures in the dark at temperatures ranging from 5 to 40°C at 5°C intervals, with three replicates per temperature. For each colony two orthogonal diameters were measured after 2 and 3 days, and the colony diameter was expressed as mean radial growth in millimeters (Úrbez-Torres *et al*., 2008).

Molecular identification

Fungal genomic DNA of all isolates was extracted from monosporic cultures following a standard CTAB-based protocol (O'Donnell *et al*., 1998). Internal Transcribed Spacer (ITS) regions, ITS1 and ITS2, including the 5.8S gene, of the ribosomal DNA (rDNA) operon, were amplified with the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). Part of the translation elongation factor 1-alpha (EF1-α) gene was amplified with the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). The primers Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify a portion of the β-tubulin (BT) gene. The PCR reactions were performed following the PCR protocol described by Slippers *et al*. (2004). The ITS-RFLP technique was applied to identify groups among the collected isolates and to select representative isolates for sequencing. The PCR amplicons of the ITS regions were digested separately with *Cfo*I and *Hae*III restriction endonucleases (Slippers *et al*., 2007) following the manufacturer's instructions (Fermentas, Milan, Italy). The digestion reaction was incubated at 37°C overnight. The resulting restriction fragments were separated by electrophoresis on 2% (w/v) agarose gel and then molecular weights determined. Isolates for which identical RFLP patterns were obtained with both endonucleases were considered to belong to the same RFLP group. ITS, EF1-α and BT regions of a representative isolate of each RFLP group were sequenced in both directions using the same primers as for PCR reactions. Nucleotide sequences were compared to GenBank sequences through BLASTn searches.

Pathogenicity

Six-month-old shoots, 8–10 mm in diameter and 30 cm long, were collected from healthy, mature In-

solia grapevines, the leaves and tendrils removed, and the shoots surface sterilized with 70% ethanol. Four isolates of each species were used and inoculated onto three shoots. The shoots were first wounded, 10–15 cm from the apex, by removing the bark with a sterile scalpel. A 6-mm-diam. plug from of a 7-day-old colony on PDA was then placed on each wound and immediately covered with Parafilm. Control shoots were inoculated with non-colonised plugs of PDA. All inoculated shoots, were placed into a 3-cm-diameter tubes containing 200 mL of tap water, and covered with plastic bags to maintain humidity. After 21 days at 25°C with natural light, each shoot was evaluated for the length of vascular discoloration around the point of inoculation. Data were submitted to ANOVA and to the Student's *t*-test. Re-isolations from symptomatic tissues were carried out on PDA to fullfill Koch's postulates.

Results and discussion

Declining grapevines observed in the five vineyards of West and Central Sicily showed in field late sprouting and/or mortality bud, delayed growth, cankers and dieback. In particular, bud and canopy symptoms were more evident in spring, since as the season progressed the grapevines seemed to recover their vegetative growth. Longitudinal sections of symptomatic samples always showed, along the whole length or nearly so of the trunk, brown wood necrosis (Figure 1a) that was often wedge-shaped

Figure 1. Symptoms of *Botryosphaeria* dieback observed in the trunk of a grapevine: a) sub-cortical, dark longitudinal bands observed when bark has been removed and b) in cross section, often arc-shaped.

Table 1. Number of isolates and isolation frequency (IF) of the Botryosphaeriaceae species and other fungal taxa obtained from symptomatic wood of diseased grapevines in Sicily.

Fungal *taxa*	No. of isolates (IF) per vineyard					Total
	Alcamo	Marsala	Milena	Montevago	Salemi	
Diplodia seriata	47 (23.5)	7 (5.6)	153 (76.5)	4 (2.0)	89 (44.5)	300 (24.0)
Lasiodiplodia sp.	--	197 (43.8)	--	--	38 (18.9)	235 (18.8)
Neofusicoccum parvum	--	25 (1.6)	--	--	--	25 (2.0)
Neofusicoccum vitifusiforme	--	--	--	114 (57.0)	--	114 (9.1)
Total No. of Botryosphaeriaceae	47 (23.5)	229 (50.9)	153 (76.5)	118 (59.0)	127 (63.5)	670 (53.6)
Penicillium	18 (9.0)	69 (15.3)	59 (15.3)	32 (16.0)	15 (7.5)	193 (15.4)
Aspergillus	23 (11.5)	30 (6.7)	6 (3.5)	17 (8.5)	5 (2.5)	81 (6.4)
Alternaria	25 (12.5)	10 (2.2)	7 (3.5)	6 (3.0)	15 (7.5)	63 (5.0)
Cladosporium	5 (2.5)	7 (1.6)	8 (4.0)	8 (4.0)	4 (2.5)	32 (2.6)
Fomitiporia	--	--	23 (11.5)	--	--	23 (1.8)
Fusarium	6 (3.0)	--	--	--	--	6 (0.4)
Acremonium	4 (2.0)	--	--	--	--	4 (0.3)
Phoma	4 (2.0)	--	--	--	--	4 (0.3)
Rhizopus	--	3 (0.7)	--	--	--	3 (0.2)
Total No. of fungal isolates	132	348	256	181	166	1079
No. of wood fragments	200	450	200	200	200	1250

in cross section (Figure 1b). In addition, sub-cortical longitudinal brownish discolored bands were often detected (Figure 1c). Only in Milena and Salemi samples, white rot starting from pruning wounds spreading along the trunk was also observed.

From the symptomatic wood of all sampled grapevines, a total of 1079 fungal colonies were isolated (Table 1). Among these, Botryosphaeriaceae species predominated (670 isolates) with IF ranging between 23.5% (Alcamo samples) and 76.5% (Milena samples). Saprophytic and wood contaminant fungi belonging to *Penicillium*, *Aspergillus* and *Alternaria* were also present, while colonies of *Acremonium*, *Cladosporium*, *Fusarium*, *Phoma*, *Rhizopus* and *Fomitiporia mediterranea* were sporadically observed. Contrary to what is often reported by other authors (Pollastro *et al*., 2000; van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2006; Pitt *et al*., 2010), other causal agents of grapevine decline such as *Phaeomoniella chlamydospora, Phaeoacremonium* spp. and *Eutypa lata* were never isolated.

On the basis of colony and conidial morphology, and optimum growth temperature, Botryosphaeriaceae isolates were classified into four groups (Table 2), which were supported by the ITS-RFLP profiles (Figure 2). BLASTn searches of the ITS sequences of the four selected isolates showed a high homology with *D. seriata* (99%), *L. theobromae* (98%), *N. parvum* (99%) and *Neofusicoccum vitifusiforme* (99%) (Table 3). Sequences from EF1-α and β-tubulin gene regions were used to confirm identification that could not be clearly resolved with ITS sequence. The comparison of sequence data of both EF1-α and β-tubulin with those in GenBank confirmed the identification based on ITS sequences, with the exception of *L. theobromae*. Since a phylogenetic analysis is in progress in order to distinguish the two potential cryptic species in *L. theobromae* (Alves *et al*., 2008), we report this species as *Lasiodiplodia* sp.

Among these four species, *D. seriata* was associated with all samples from various grapevine growing

Table 2. Morphological features of the four groups of Botryosphaeriaceae isolated in this study.

No. group	No. isolates	Colony features	Mature conidia				Opt. growth T (°C)	Identity
			Colour and shape	Septa	Dimension (µm)[a]			
					Lenght	Width		
1	296	Dense grey-brown aerial mycelium	Brown, cylindrical to ellipsoid	no	(17–)20–22(-26.5)	(8.5–)10–11(–13)	30	*Diplodia seriata*
2	235	Abundant aerial mycelium that became dark green with age	Hyaline becoming dark brown, oblong with irregular longitudinal striations	1	(15–)19–22(–27)	(8.5–) 9–12-(16.5)	35	*Lasiodiplodia* sp.
3	25	Mycelium dull gray	Thin-walled, fusiform or ellipsoidal olivaceous or light brown	1–2	(12.5–)13–18(–23)	(5.0)6–7(–10)	30	*Neofusicoccum parvum*
4	114	Aerial mycelium that became grey whit age	Hyaline, fusoid to ellipsoid, widest in upper third, apex obtuse, base flattened and sub-truncate	no	(18–)20–21.4(–22)	(4.5–)5.0–5.6(–7)	30	*Neofusicoccum vitifusiforme*

[a] Minimum and maximum and dimensions in parentheses.

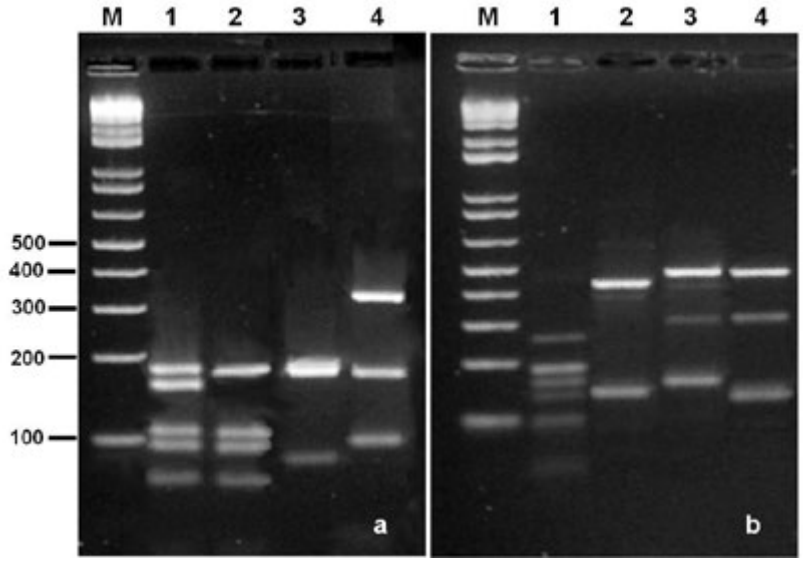

Figure 2. Restriction profiles with *Cfo*I (a) and *Hae*III (b) of the ITS PCR products of *N. parvum* (lane 1), *N. vitifusiforme* (lane 2), *D. seriata* (lane 3) and *Lasiodiplodia* sp. (lane 4). M, 1 Kb plus DNA ladder (Invitrogen).

Table 3. Molecular identification and GenBank accession numbers of the representative isolates of the *Botryosphaeriaceae* species isolated in this study.

Isolate	Molecular identification	DNA target[a]	GenBank accession No.	Blast match sequence		
				Reference accession No.[b]	Coverage (%)	Identity (%)
B4	*Lasiodiplodia* sp.	ITS	JN251118	*L. theobromae* AY640255	100	98
		EF1-α	--	--	--	--
		β -tubulin	--	--	--	--
B8	*Neofusicoccum vitifusiforme*	ITS	KC469638	*N. vitifusiforme* **AY343383**	87	99
		EF1-α	KC884948	*N. vitifusiforme* **AY343343**	99	99
		β -tubulin	KC884951	*N. vitifusiforme* HM176500	97	99
B19	*Neofusicoccum parvum*	ITS	JN251119	*N. parvum* **AY236943**	95	99
		EF1-α	KC884949	*N. parvum* **AY236888**	100	99
		β -tubulin	KC884952	*N. parvum* **AY236917**	96	99
B25	*Diplodia seriata*	ITS	JN251120	*D. seriata* **AY259094**	100	99
		EF1-α	KC884950	*D. seriata* **AY573220**	99	99
		β -tubulin	KC884953	*D. seriata* **DQ458856**	98	99

[a] ITS, internal transcribed spacer; EF1-α, elongation factor.
[b] Accession numbers of the ex-type or ex-epitype strains are in bold.

Figure 3. Discolouration on excised shoots of *V. vinifera* cv. Insolia 21 days after artificial inoculation with a) *D. seriata*, b) *Lasiodiplodia* sp., c) *N. parvum* and d) *N. vitifusiforme*. Arrows indicate the point of inoculation. The shoot in e) was wounded but was not inoculated with a fungus.

Table 4. Mean lesion length on excised grapevine green shoots cv. Insolia 21 days after artificial inoculation with Botryosphaeriaceae species.

Inoculum	Mean lesion length (cm) ±SE [a]	Re-isolation [b]
Diplodia seriata	4.9 ±2.0 b	100
Lasiodiplodia sp.	14.8 ±1.8 b	100
Neofusicoccum parvum	5.6 ±3.6 b	100
Neofusicoccum vitifusiforme	4.5 ±2.8 b	100
Control	1.1 ±0.1 a	0

[a] Values are means of three replicates per treatment. SE= standard error of the mean. Equal letters refers to not significantly different values (Student *t*-test; $P>0.05$).
[b] Reisolation percentage.

areas (Table 1). In particular, it was the only species isolated from the sub-cortical longitudinal brown bands and, exclusively in those of Alcamo (23.5%) and Milena (76.5%), also from the wood necrosis. This species was also obtained from wood necrosis of grapevines in Marsala, Montevago and Salemi, but not singularly and in different percentages (5.6, 2.5 and 44.5, respectively). *Lasiodiplodia* sp., on the contrary, was obtained both from Marsala and Salemi samples, with IF of 43.8% and 18.9%, respectively. *N. parvum* was only and sporadically isolated from Marsala grapevines (IF 1.6%), while *N. vitifusiforme*, from Montevago samples, with a high isolation percentage (57%).

With regard to the preliminary tests of pathogenicity, the inoculated fungi caused vascular discoloration, extending differently both upward and downward from the inoculation point, in the sampled shoots (Figure 3). However, the length of vascular discolorations was very variable both between the different species and within each species. The control shoots developed only a slight discolouration only around the wound site. ANOVA analysis showed significant differences in the extent of vascular discoloration between control and inoculated shoots, but no significant differences between Botryosphaeriaceae species (Table 4). The inoculated fungi were always reisolated from the inoculated canes and no botryosphaeriaceous fungi were reisolated from the control. These assays, even if with low number of replicates, allowed to ascertain the pathogenicity of the assayed Botryosphaeriaceae species on *V. vinifera.*

Our results show several Botryosphaeriaceae species associated with Botryosphaeria dieback in Sicily, as well as already observed in other countries (Úrbez-Torres *et al*., 2006; Candolfi-Arballo, *et al*., 2010; Pitt *et al*., 2010; Úrbez-Torres, 2011); this could be due to environmental conditions of every grape-growing area.

Regarding *N. vitifusiforme*, reported in *V. vinifera* for first the time in South Africa (van Niekerk *et al*., 2004), is now frequently associated with Botryosphaeria dieback in several grape-growing areas worldwide: Spain (Luque *et al*., 2009), New Mexico (Candolfi-Arballo, *et al*., 2010) and USA (Úrbez-Torres, 2011). The occurrence of *N. vitifusiforme* on *Olea europaea* in South Italy (Lazzizzera *et al*., 2008) shows the capability of this species to colonize different hosts, and may confirm the hypothesis of the role of secondary host as a inoculum source for grapevine trunk disease pathogens (Cloete *et al*., 2011). At present, *N. vitifusiforme* has been also recently reported as pathogen in fruit trees: *Prunus* spp. (Damm *et al*., 2007), *Malus* and *Pyrus* spp. (Cloete *et al*., 2011), *O. europaea* (Lazzizzera *et al*., 2008; Úrbez-Torres *et al*., 2012) and in blueberry (*Vaccinium corymbosum*, Kong *et al*., 2010), mostly associated with host wood necrosis. To date, this is the first report of *N. vitifusiforme* in *V. vinifera* in Italy.

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