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First report of *Neofusicoccum australe* associated with grapevine cordon dieback in Italy

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Summary. This is the first report of *Neofusicoccum australe* associated with grapevine dieback in Italy. Fungal isolates obtained from symptomatic tissues were identified on the basis of morphological and cultural characteristics as well as ITS sequence data. Pathogenicity was verified by inoculation of excised green grapevine shoots from cv. Cannonau under controlled laboratory conditions.

Key words: *Botryosphaeriaceae*, grapevine diseases, Sardinia.

Grapevine trunk diseases are a serious economic problem in Sardinian vineyards (Serra *et al*., 2008; Serra *et al*., 2010). These diseases are usually linked to infections by *Eutypa lata* and other fungal pathogens belonging to the genera *Fomitiporia*, *Phaeomoniella* and *Phaeoacremonium* (Mugnai *et al*., 1999, Mostert *et al*., 2006, Sosnowski *et al*., 2007). Moreover, several species of *Botryosphaeriaceae* have recently been recognized as important pathogens of grapevines worldwide (Phillips, 2002, Van Niekerk *et al*., 2006; Úrbez-Torres and Gubler, 2009, Pitt *et al*., 2010). Since only limited information about the occurrence and identity of the *Botryosphaeriaceae* associated with dieback and trunk diseases of grapevines in Sardinia is available, a field survey has been in progress since 2009.

Grapevines showing poor growth, defoliation, cankers and dieback (Figure 1a), were collected from six vineyards (local varieties Cannonau and Vermentino) in northern Sardinia. Common internal wood symptoms were wedge-shaped necrotic sectors and/or brown streaks below the bark running from cordon to rootstock, 2–3 cm wide and a few millimetres thick (Figure 1b, c). Fragments measuring approximately 5 mm^2 were aseptically cut from the margin of infected tissues and cultured in Petri dishes containing malt extract agar (MEA, Oxoid Ltd, Basingstoke, UK). After incubation at room temperature for 5 days, fungal colonies resembling *Botryosphaeriaceae* were sub-cultured onto both potato dextrose agar (PDA, Oxoid Ltd) and malt extract yeast agar (MEYA, 2% malt extract, 0.2% yeast extract, 1.5% agar) and incubated at room temperature until differentiation of the reproductive structures. To induce sporulation, the fungal isolates were sub-cultured on PDA with sterile pine needles. Microscope slides of conidia and ascospores were prepared in lactophenol for morphological examination. Spore dimensions were recorded using an Optika™ Vision Pro version 2.7 digital camera connected to a Leitz Diaplan (Ernest Leitz, Wetzlar, Germany) microscope. The colony appearance of cultures growing

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Figure 1. Grapevine disease symptoms associated with *Neofusicoccum australe*: cordon dieback (a); cordon wedge-shaped necrotic sector (b); trunk brown stripes under the bark (c).

on PDA at 25°C in the dark for 1 week was also recorded.

Fungal genomic DNA was extracted from 20 mg of mycelium following the CTAB method of Doyle and Doyle (1987). The entire Internal Transcribed Spacer region (ITS) of the rDNA, including the 5.8S gene, was amplified by polymerase chain reaction (PCR) using the primers ITS1 and ITS4 (White *et al*., 1990). The amplifications were performed using a Hybaid PCR Express and the following protocol: 95°C for 3 min initial denaturation; 35 cycles of 95°C for 15 s, 55°C for 20 s, 72°C for 1 min; and 72°C for 5 min final extension. The PCR products were purified using the Eurogold extraction kit (EuroClone S.p.A., Milano, Italy) following the manufacturer's instructions. Both strands were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv), aligned using ClustalW (www.ebi.ac.uk/clustalw/) and compared with sequences deposited in GenBank using the BLAST software (http://blast.ncbi.nlm.nih.gov).

A total of 113 Botryosphaeriaceous isolates were obtained from 15 of the plants sampled. Some isolates (44%) sporulated within one or two months. The conidial morphology and cultural fe-

atures of six isolates from one vineyard (cv. Cannonau) were in close agreement with the morphological description of both *Neofusicoccum australe* and *N. luteum* (Phillips *et al*., 2002, Slippers *et al*., 2004). On PDA at 25°C, these isolates developed a moderately dense mycelium, with a yellow pigment diffusing into the medium that became dark grey after 4 to 5 days. The conidia were hyaline, aseptate, fusiform with a truncate base and measured 18.7±2×6.8±0.9 μm, with a length/width ratio of 2.8±0.4 (mean±SD, n=100). Average conidial size was less than the 24.7×5.1 μm previously reported for *N. australe* by Slippers *et al*. (2004), but similar to the size reported for *N. australe* by Lazzizera *et al*. (2008). Conidial size was also similar to the size reported for *N. luteum* by Phillips *et al*. (2002). Only one isolate produced ascomata and conidiomata in the same Petri dish on PDA within 2 months. Ascospores were hyaline, aseptate, fusoid to ovoid, and measured 20.2±1.2×8.5±0.7 μm, with a length/width ratio of 2.4±0.2 (mean±SD, n=50).

The identity of this *Botryosphaeriaceae* species was defined by analysis of the ITS sequence of a representative isolate. BLAST searches in GenBank revealed that the sequence of this isolate differed by only 1 bp from the sequence of the ex-type isolate of *N. australe* (AY339262) and other isolates (e.g. EU856059, FJ157192). However, it had 100% similarity to sequences of other isolates identified as *N. australe* including isolates from olives in southern Italy (EF638770–EF638778) and pistachio in Spain (EU375516). Furthermore, the sequence of our isolate differed by three bp from the ex-type isolate (AY259091) and other isolates of *N. luteum*. Thus we consider that the isolate from Sardinian grapevines is *N. australe*.

The representative sequence of *N. australe* strain BOT48 obtained in this study was deposited in GenBank (accession number HQ011406). The strain BOT48 was stored in the culture collection of the Department of Plant Protection at the University of Sassari. Its pathogenicity was verified by inoculating it on five excised green grapevine shoots (30 cm in length) from cv. Cannonau. A mycelial plug (3 to 4 mm^2) taken from the margin of an actively growing colony on PDA was placed in a shallow wound (~3 mm) made by a scalpel on the middle of each shoot. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm®. The inoculated shoots were placed in a beaker containing 200 mL of sterile distilled water and maintained in an

Figure 2. Grapevine shoot showing dark-brown lesion 12 days after inoculation with *Neofusicoccum australe* (top). Asymptomatic control shoot (bottom).

enclosed transparent plastic bag at room temperature (18–30°C). Twelve days after inoculation, the shoots displayed dark-brown to black discoloration, measuring 7.3±3.5 cm (mean±SD) of the bark and vascular tissues (Figure 2). The pathogen was successfully reisolated from the margin of symptomatic tissues, thus fulfilling Koch's postulates. Five control shoots inoculated with sterile PDA plugs remained symptomless.

The plurivorous pathogen *N. australe* was originally thought to be native to the southern hemisphere (Slippers *et al*. 2004; Burgess *et al*., 2006). However, it has recently been reported on a variety of hosts, including grapevine, worldwide (Armengol *et al*., 2008, Amponsah *et al*., 2009, Espinoza *et al*., 2009). Until now, in Italy *N. australe* was only found associated with drupe rot of olives (Lazzizera *et al*., 2008). Interestingly, our isolates conform morphologically and phylogenetically more closely to the isolates of Lazzizera *et al*. (2008) than to typical isolates of *N. australe*. To our knowledge, this is the first report of *N. australe* on grapevine in Italy. Further investigations are currently underway to study the spread of this pathogen in Sardinian vineyards and its role in grapevine dieback.

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