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Analysis of genetic and virulence diversity of *Cylindrocarpon liriodendri* and *C. macrodidymum* associated with black foot disease of grapevine

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ABSTRACT

Inter-simple sequence repeat (ISSR) analysis was used to investigate the genetic diversity of 87 *Cylindrocarpon liriodendri* and *C. macrodidymum* isolates, the causal agents of black foot disease of grapevine. The four ISSR primers $(GT)_7$, $(CCA)_5$, $(CGA)_5$ and $(TCG)_5$, were able to provide reproducible and polymorphic DNA fingerprint patterns and detected relevant genetic diversity in *C. macrodidymum*. The cluster analysis of ISSR data showed 21 different genotypes that were grouped in seven ISSR groups, from which two corresponded to *C. liriodendri* (G1 and G2) and five to *C. macrodidymum* (G3–G7). Nineteen isolates selected from the seven ISSR groups were inoculated in grapevine seedlings obtained from cv. 'Tempranillo'. The pathogenicity tests detected virulence diversity in *C. macrodidymum*. The isolates belonging to ISSR groups G6 and G7 were significantly more virulent than the other *C. macrodidymum* and *C. liriodendri* isolates.

Introduction

Black foot disease caused by *Cylindrocarpon* spp. is a severe disease of grapevines, which affects mainly grapevine nurseries and young vineyards (Halleen *et al.* 2006a). This disease was first described in 1961 (Grasso & Magnano Di San Lio 1975) and, over the last decade, its incidence has increased significantly in different grapevine growing areas around the world (Rego *et al.* 2000; Halleen *et al.* 2004; Petit & Gubler 2005; Alaniz *et al.* 2007).

Vines affected by black foot disease show sunken necrotic root lesions with a reduction in root biomass. Removal of rootstock bark reveals black discolouration and necrosis of wood tissues, which develop from the base of the rootstock. Other symptoms include reduced vigour, shortened internodes, sparse foliage, and small leaves with interveinal chlorosis and necrosis, frequently leading to the death of the plants (Grasso 1984; Maluta & Larignon 1991; Scheck *et al.* 1998; Rego *et al.* 2000; Halleen *et al.* 2006a).

C. liriodendri and *C. macrodidymum* are the causal agents of black foot disease (Halleen *et al.* 2004, 2006b); however, two other *Cylindrocarpon* species were previously associated with this disease: *C. destructans* and *C. obtusisporium*.

C. destructans was first reported on grapevine in France in 1961 (Maluta & Larignon 1991) and then in other countries, such as Italy (Grasso, 1984), Portugal (Rego *et al.* 2000), Argentina (Gatica *et al.* 2001), Germany (Fisher & Kassemeyer 2003), New Zealand and South Africa (Halleen *et al.* 2004), Brazil

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(Garrido *et al.* 2004), and California (Petit & Gubler 2005). Recently, Halleen *et al.* (2006b) compared *C. destructans* isolates obtained from French, Portuguese, and South African diseased grapevines and other herbaceous and woody hosts with other species in the genus *Cylindrocarpon*. The results revealed that the grapevine isolates previously identified as *C. destructans* and *C. liriodendri* had identical morphological characters, ITS, and partial beta-tubulin (BT) gene sequences. *C. liriodendri* was first reported associated with root rot of tulip poplar (*Liriodendron tulipifera*) (MacDonald & Butler 1981). Consequently, all *C. destructans* isolates from grapevine included in this study, were re-identified as *C. liriodendri*. In California, Petit & Gubler (2007) reached the same conclusion in relation to *C. destructans* isolates from grapevine. Subsequently, *C. liriodendri* was also reported in Spain (Alaniz *et al.* 2007) and Australia (Whitelaw-Weckert *et al.* 2007).

C. obtusisporium was identified as the causal agent of black foot disease in Sicily (Grasso & Magnano Di San Lio 1974) and California (Scheck *et al.* 1998). Halleen *et al.* (2004), characterized morphologically and phylogenetically a collection of *Cylindrocarpon* isolates obtained from grapevine plants in South Africa, New Zealand, Australia, and France. Sequences of the LSU rDNA, ITS, and BT were used for phylogenetic inference. As a result these authors described the new species *C. macrodidymum*, which has subsequently been found in California (Petit & Gubler 2005), Chile (Auger *et al.* 2007), and Spain (Alaniz *et al.* 2007). Halleen *et al.* (2004) and Petit & Gubler (2005) indicated the possibility that all isolates from grapevine previously identified as *C. obtusisporium*, probably correspond to *C. macrodidymum*.

Isolates of *C. liriodendri* and *C. macrodidymum* have been characterized by means of phenotypical studies, ITS, BT, and LSU rDNA sequencing and pathogenicity tests (Halleen *et al.* 2004; Petit & Gubler 2005; Alaniz *et al.* 2007). Nevertheless, these studies did not investigate genetic and virulence diversity among isolates of both pathogens. A better understanding of genetic and virulence diversity among *C. liriodendri* and *C. macrodidymum* isolates would contribute to more efficient management of black foot disease of grapevine, in particular through the development of resistant rootstocks.

The inter-simple sequence repeat (ISSR) or random amplified microsatellites (RAMS) technique, originally described by Zietkiewicz *et al.* (1994) to analyse genetic diversity in plants and animals, has proven to be a powerful tool and has been shown to be applicable for fungi (Hantula *et al.* 1996). This technique combines the simplicity of the RAPD approach, the reliability of bands derived from known heritable domains of the genome, and the potential to differentiate populations or recently diverged species (Zhou *et al.* 2001). In the ISSR method, the DNA between the distal ends of two closely located microsatellites is amplified in multiple loci throughout the genome, using primers containing microsatellite sequences with or without degenerate anchors at the 5′ ends. Each amplified band corresponds to a unique DNA sequence delimited by two inverted microsatellites. This method produces polymorphic patterns between different individuals and was found useful in describing genetic diversity in several groups of fungi, such as: *Phytophthora cactorum* (Hantula *et al.* 1997), *Claviceps* spp. (Tooley *et al.* 2000), *Botryosphaeria* spp. (Zhou *et al.* 2001), *Phaeoisariopsis griseola* (Mahuku *et al.* 2002), *P. citrophthora* (Cohen *et al.* 2003), *Fusarium culmorum* (Mishra *et al.* 2003), *Rhizoctonia solani* (Elbakali *et al.* 2003), *Colletotrichum lindemuthianum* (Mahuku and Riascos 2004), *Gremmeniella* spp. and *Phomopsis* spp. (Borja *et al.* 2006).

The main objectives of this work were: (1) to test the utility of the ISSR method as a tool to study genetic diversity in *Cylindrocarpon* spp. from grapevines; (2) to analyse the intraspecific genetic variability of *C. liriodendri* and *C. macrodidymum* isolates using ISSR markers; and (3) to study the relationship between genetic and virulence profiles in both species.

Material and methods

Fungal isolates

Twenty-six *Cylindrocarpon liriodendri* and 56 *C. macrodidymum* Spanish isolates were included in this study. These isolates were recovered from roots and the basal ends of rootstocks from grapevines exhibiting symptoms of black foot disease in nurseries and young vineyards between 2001 and 2004. They were identified by means of phenotypical characters and BT phylogeny, and are representative of different locations and scion/rootstock combinations (Alaniz *et al.* 2007). Additionally, reference isolates of *C. liriodendri* (CBS 117640, CBS 117526, Cy13 Portugal and Cy15 Portugal, all from Portugal) and *C. macrodidymum* (CBS 112609, from Australia) were obtained from the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS), and from Portugal provided by C. Rego (Laboratório de Patologia Vegetal 'Veríssimo de Almeida', Lisboa, Portugal). The isolates were single-spored prior to use by means of the serial dilution method (Dhingra & Sinclair 1995) and stored in 15 % glycerol solution at −80 °C into 1.5 ml cryovials. Voucher specimens of the *Cylindrocarpon* isolates used in this research are stored in the fungal collection of the Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia. Additionally, reference *Cylindrocarpon* isolates are available in the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS), and in Portugal at the Laboratório de Patologia Vegetal 'Veríssimo de Almeida', Lisbon.

DNA extraction and ISSR amplifications

Fungal mycelium and conidia from pure cultures grown on potato–dextrose agar (PDA) for two weeks at 25 °C in the dark were scraped and mechanically disrupted by grinding them 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, Georgia) following the manufacturer's instructions. DNA was visualized under UV light on 0.7 % agarose gels stained with ethidium bromide and was stored at −20 °C.

A total of ten ISSR primers were evaluated by their capacity to produce polymorphic, scored and reproducible DNA fingerprint patterns in *Cylindrocarpon liriodendri* and *C. macrodidymum* isolates from grapevine. The primers included were four dinucleotide, and six trinucleotide repeats with or without 5′ anchors: 5′HBH$(AG)_7$A, 5′DBDA$(CA)_7$, 5′DVD$(CT)_7$C (Mahuku *et al.* 2002), 5′YHY$(GT)_7$G, 5′BDB$(ACA)_5$, 5′DHB$(CGA)_5$ (Hantula *et al.* 1996), 5′DDB$(CCA)_5$ (Hantula *et al.* 1997),