Phytopathol. Mediterr. (2001) 40, Supplement, S357–S361

Detection of Cylindrocarpon black-foot pathogens in grapevine by nested PCR

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Summary. Black-foot disease of grapevine has been associated with two closely related species of the genus *Cylindrocarpon*, *C. destructans* and *C. obtusisporum*. However, only *C. destructans* isolates could be obtained from young vines and diseased grapevine rootstock nurseries in Portugal. In the present study, an alternative to traditional methods of detection of *Cylindrocarpon* spp. fungi from infected grapevine is described. In 1996, Hamelin *et al.* designed species-specific primers (Dest1 and Dest4) for detection of *C. destructans* ITS variants from conifer seedlings. With these primers, a DNA fragment of 400 bp was produced by direct PCR using DNA extracted from cultures of *C. destructans* (60 isolates) obtained from grapevine plants. Whereas no fragments were detected when cultures of common wood grapevine fungi were analysed, an amplicon of the same size was obtained for isolates of *C. obtusisporum* revealing the failure of these primers to distinguish *Cylindrocarpon* species. The 400 bp fragment mentioned could also be produced by direct PCR after adding *C. destructans* to healthy grapevine tissue (cv. Periquita), followed by DNA extraction using frozen plant tissue in liquid nitrogen and polyvinylpyrrolidone (PVP) treatment. However, amplification failed to detect *C. destructans* in artificially inoculated grapevine plants (cv. Periquita). Consequently, a nested PCR was carried out by modifying the procedure described by Hamelin *et al.* The universal primer ITS4 and the fungus-specific primer ITS1F were used in a first-stage fungus-specific amplification, followed by a second-stage amplification with the primers Dest1 and Dest4 using the PCR products from stage one. This approach was found to be a simple and reliable method for collective detection of *Cylindrocarpon* spp. directly from infected grapevine tissues.

Key words: *Cylindrocarpon destructans*, *Vitis vinifera*, black-foot disease, grapevine, nested PCR.

Introduction

The involvement of fungi of the genera *Cylindrocarpon*, *Phaeomoniella* and *Phaeoacremonium* in the decline of grapevine has been reported worldwide. Whereas *Cylindrocarpon* species have been considered responsible for "black-foot disease of grapevine" (Grasso and Magnano di San Lio, 1975; Maluta and Larignon, 1991), *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. have been assigned to "young grapevine decline" or "Petri grapevine decline" (Mugnai *et al.*, 1999). Symptoms of black-foot disease and Petri grapevine decline[(1)] are similar and disease diagnosis relying only on the basis of the symptomatology observed is not possible.

Black-foot has been associated with infection by two closely related species of the genus *Cylindro-*

[(1)] At the general Assembly of the 2nd ICGTD meeting held in Lisbon 2001 it was unanimously decided that the disease will henceforth be called Petri disease.

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carpon, *C. destructans* (Zinssm.) Scholten and *C. obtusisporum* (Cooke & Harkness) Wollenw. Although in Portugal only *C. destructans* has been isolated from diseased grapevine rootstock nurseries and young vines, *C. obtusisporum* has been identified as the main causal agent of black-foot in other countries (Scheck *et al*., 1998). *C. destructans* is a ubiquitous soilborne pathogen which probably infects rootstock cuttings during the rooting process in grapevine nurseries (Rego *et al*., 2001a). This hypothesis regarding the infection route recently receive support from the fact that *C. destructans* was isolated unfrequently from canes of mother-plants of rootstocks (Rego *et al*., 2001). Since in Portugal grafting is traditionally performed directly in the vineyards (field grafting), the market for non-grafted rooted rootstocks is still important. In nurseries, approximately 300,000 rootstock cuttings are rooted within an 1-ha area. Within the nursery production areas, black-foot incidence has been evaluated by random sampling, followed by traditional methods of isolation and identification of micro-organisms present in symptomatic wood.

During the last years, molecular diagnostic methods for detection of plant pathogens directly in the soil or in plant tissue have been widely reported. Diagnostic methods have been developed for several host-pathogen combinations, including *Pm. aleophilum* and *Pa. chlamydospora* in grapevine tissue (Tegli *et al*., 2000) and these same fungi in grapevine tissue and soil (Eskalen *et al*., 2001). Furthermore, Hamelin *et al*. (1996) developed species-specific primers to detect *C. destructans* from infected conifer seedlings.

This paper describes a fast and reliable method for the detection of *Cylindrocarpon* black-foot pathogens isolated from infected grapevine tissues using nested PCR.

Materials and methods

Fungal isolates and cultural conditions

Twelve isolates of *C. destructans* from symptomatic rooted cuttings and young grapevines in Portuguese nurseries and vineyards were used for the present study. For validation of the method, another 47 isolates of *C. destructans*, also from grapevine, were included in this study. An isolate of *C. destructans*, Cy1 (IMI 357400, Rego, 1994), was used as reference strain. Four isolates of *C. obtusisporum* were kindly provided by W. Douglas Gubler (Davis, CA, USA). Isolates of *Fusicoccum* sp., *Botrytis cinerea*, *Pestalotiopsis menezesiana*, *Fusarium oxysporum*, *Phomopsis* sp., *Pa. chlamydospora* and *Truncatella angustata* commonly found in grapevine tissues were also studied. The isolates were maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 4°C and routinely subcultured on the same medium. For liquid cultures, 1.5 ml microcentrifuge tubes containing 500 µl of potato dextrose broth (PDB, Difco) were inoculated with some hyphal threads. The tubes were then incubated in a reciprocal shaker at 20°C in the dark for about 10 days.

Grapevine tissues

Pieces of healthy current-year canes of grapevine cv. Periquita were mixed with each of the above fungal cultures. Other experiments were carried out on wood tissues collected from potted grapevines (cv. Periquita) which had been artificially inoculated with different *C. destructans* isolates three months before.

DNA extraction

DNA of fungal cultures was extracted according to a modified protocol described by Cenis (1992). The mycelium was harvested by centrifugation (5 min at 13,000 rpm), washed with 500 µl of TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) and pelleted again. The TE was decanted and the mycelium was crushed with a conical grinder in 500 µl of extraction buffer (200 mM Tris-HCl, pH 8; 250 mM EDTA; 0.5% SDS, w:v). After that, 150 µl of 3M sodium acetate (pH 5.2) was added and tubes were placed at -20°C for about 20 min. Tubes were then centrifuged at 13,000 rpm for 10 min and each supernatant was transferred to a new tube, precipitated with the same volume of isopropanol, and centrifuged at 13,000 rpm for 10 min. Pellets were washed with 70% ethanol, air dried overnight, and resuspended in 50 µl of TE.

In another experiment, liquid fungal cultures (500 µl) were centrifuged and each pellet was added to pieces of healthy grapevine cane wood (250 mg), frozen in liquid nitrogen, and ground into fine powder with a mortar and pestle. The powder was transferred to microcentrifuge tubes (1.5 ml) followed by addition of 1 ml extraction buffer supple-

mented with 1% (w:v) polyvinylpyrrolidone (PVP, Sigma Chemical Company, St. Louis, MO, USA) and 300 µl of 3M sodium acetate (pH 5.2). The samples were further processed as described for the fungal cultures above.

Direct extraction of DNA from artificially inoculated grapevine plants was performed using pieces of wood (30 mg) collected from the basal end of the stems, which were processed as in the latter case.

PCR analyses

The specific primers Dest1 and Dest4 for *C. destructans* (Hamelin *et al*., 1996) were used for direct PCR amplification. Each PCR assay was performed as follows in a final volume of 25 µl: 50 mM KCl, 10 mM Tris-HCL (pH 9), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 100 µM of each dNTP (GIBCO-BRL, Life Technologies, Inc., Gaithersburg, MD, USA), 1 µM of each Dest1 and Dest4 primers, 1.25 U *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA) and 100 ng of DNA. Reactions were overlaid with mineral oil (Sigma). Amplification conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles each consisting of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, and a final extension at 72°C for 10 min.

For nested PCR, the universal primer ITS4 and the fungus-specific primer ITS1F were used in a first-stage fungus-specific amplification. The resulting PCR products were subsequently used in a second-stage amplification using the primers Dest1 and Dest4. The procedure was performed according to Hamelin *et al*. (1996) but using modified reaction mixtures as follows: 1.25 U *Taq* DNA polymerase (Promega), 100 ng of template DNA, no addition of gelatine.

All PCR reactions were repeated at least twice and the obtained products were separated by electrophoresis in 2% agarose gel in 0.5× TBE buffer (Sambrook *et al*., 1989). Gels were stained with ethidium bromide and photographed under UV light.

Results and discussion

Two species-specific primers (Dest1 and Dest4) that would overcome the genetic variability within isolates of *C. destructans* were designed by Hamelin *et al*. (1996) to detect the pathogen in the roots of conifer seedlings. Depending on the variant of *C. destructans* detected, these primers amplify a 400 or a 399 bp fragment.

In the present study, we determined how these primers directly amplified DNA extracted from a collection of *C. destructans* strains obtained from rootstock or grapevine varieties affected by black-foot disease. The expected amplicon of 400 bp was obtained with each of the 60 isolates tested (Fig. 1).

Another experiment focused on the specificity of these primers. No amplification resulted from PCR when the DNA of fungal cultures from other genera was used as template. However, an amplicon of 400 bp was obtained when the genomic DNA of *C. obtusisporum* isolates was amplified (Fig. 2).

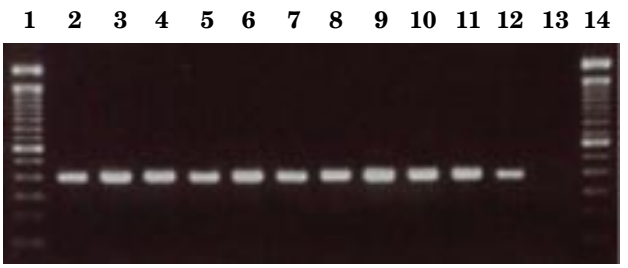

Fig. 1. Amplicons of direct polymerase chain reaction using the primer-pair Dest1 and Dest4 and DNA from different *Cylindrocarpon destructans* cultures. Lanes: 1 and 14, molecular weight markers (100 bp; GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA); 2–11, *C. destructans* isolates; 12, positive control (Cy1); 13, negative control.

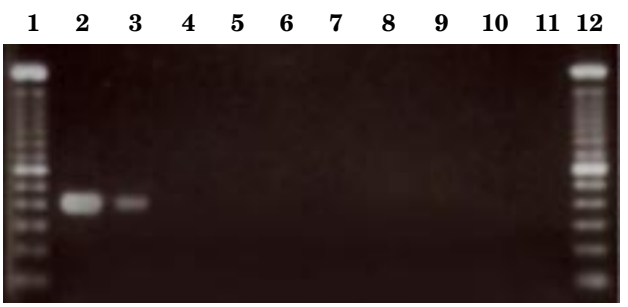

Fig. 2. Amplification products of direct polymerase chain reaction using the primer-pair Dest1 and Dest4 for different fungal cultures. Lanes: 1 and 12, molecular weight markers (100 bp; GIBCO-BRL, Life Technologies); 2, positive control (Cy1); 3–10, *Cylindrocarpon obtusisporum, Botrytis cinerea, Fusarium oxysporum, Fusicoccum* sp., *Phaeomoniella chlamydospora, Pestalotiopsis menezesiana, Phomopsis* sp. and *Truncatella angustata*; 11, negative control.

C. obtusisporum and *C. destructans* are closely related species and the variability within *C. destructans* has been reported (Samuels and Brayford, 1990). So, one aspect of future studies will be to evaluate of the taxonomic relationship between these two species of *Cylindrocarpon*. On the other hand, further research is required to determine common DNA sequences inside the ITS region of the two *Cylindrocarpon* species. However, in terms of practical application, the primers Dest1 and Dest4 appeared to be adequate for the collective detection of "grapevine black-foot pathogens".

Whereas *C. destructans* was detected by direct PCR assays using healthy grapevine tissue mixed with fungal cultures of *C. destructans* and primers Dest1/ Dest4, no amplification products were obtained using artificially inoculated grapevine potted plants (Fig. 3). However, the pathogen itself could be reisolated from those plants.

By modifying the procedure of Hamelin *et al.* (1996) for the conditions described, a nested PCR was developed for direct detection of *C. destructans* from grapevine tissues. Using the amplification products resulting from PCR with ITS1F and ITS4 primers as template, fragments of 400 bp were obtained in a second-round amplification using the primers Dest1 and Dest4 (Fig. 4). Although the nested-PCR was only carried out for grapevine infected with *C. destructans*, results obtained from direct PCR assays revealed the usefulness of this method for the collective detection of *Cylindrocarpon* spp. directly from infected grapevines tissues.

Further testing with nursery material will lead to improvements in this nested-PCR method, which offers new prospects for the certification of grapevine propagating material. The development of a related method for the detection of *Cylindrocarpon* spp. from the soil is also under study.

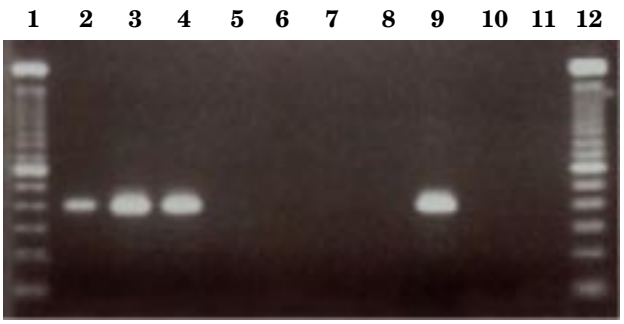

Fig. 3. Amplicons of direct polymerase chain reaction using the primer-pair Dest1 and Dest4 for *Cylindrocarpon destructans*, using DNA extracted from *C. destructans* isolates supplemented with healthy grapevine tissues, and from artificially infected grapevine plants. Lanes: 1 and 12, molecular weight markers (100 bp; GIBCO-BRL, Life Technologies); 2–4, extracts from healthy grapevines plus Cy7, Cy24 and Cy30 cultures respectively; 5–8 extracts from artificially infected grapevine plants with Cy2, Cy7, Cy30 and Cy68 isolates respectively; 9, positive control (Cy1); 10 and 11, negative controls (extracts from healthy grapevines and water respectively).

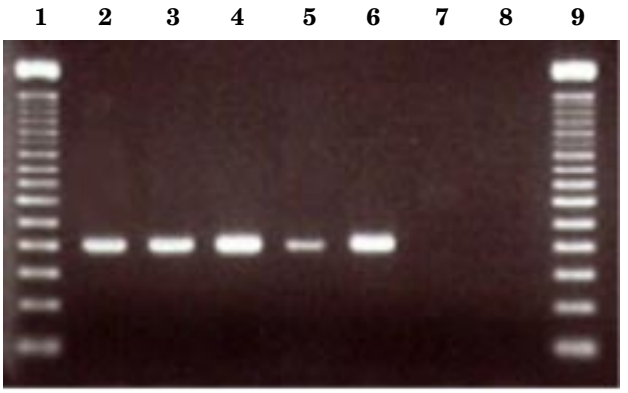

Fig. 4. Amplification products of a second-round PCR with primer-pair Dest1 and Dest4, using amplification products from ITS1F–ITS4 as a template. Lanes: 1 and 9, molecular weight markers (100 bp; GIBCO-BRL, Life Technologies); 2–5, extracts from artificially infected grapevine plants with Cy2, Cy7, Cy30 and Cy68 isolates respectively; 6, positive control (Cy1); 7 and 8, negative controls (extracts from healthy grapevines and water respectively).

Acknowledgements

The authors wish to thank Dr. Thomas Schulz for reviewing the manuscript.

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Accepted for publication: December 18, 2001