A PCR-based Technique for Detection *Cylindrocarpon destructans*, the Causal Agent of Grapevine Black Foot Disease

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Abstract

The symptoms of Grapevine black foot disease caused by *Cylindrocarpon destructans* including reduction of the plant vigour with small-sized trunks, reduction in root biomass, black discolouration and brown to dark streaks in wood mainly at the base of the rootstock. According to morphological and cultural characteristics of *Cylindrocarpon*, all isolates from infected roots of declined plants were identified as *C. destructans.* PCR technique was performed for accurate identification of *C. destructans* isolates. Ten isolates of the fungi were selected from different locations. Those isolates were subjected to species-specific PCR assay. Total genomic DNA was isolated from pure cultures of the isolates. The average DNA yields ranged between 1.5-6.7 µg/ml with a purity 1.6-1.8. The specific primers for *C. destructans* were used to amplify the ITS region of nuclear ribosomal DNA (rDNA) containing ITS1, ITS2 and the intervening 5.8 rRNA genes of *Cylindrocaropon.* TheITS sequence could successfully and appropriately confirmed that all isolates were correctly identified as *C. destructans*. This fungus obtained from this study was reported for the first time in Iraq.

Keywords: Black foot disease, Cylindrocarpon destructans, Grapevine, PCR

Introduction

Species of *Cylindrocarpon* Wollenw. are common soil inhabitants that often associated with roots of herbaceous woody plants. Grasso and Magnano di San Lio (1975) described black foot symptoms from nursery plants with black discoloration and gum inclusions in xylem vessels of affected rootstocks. Scheck *et al.* (1998) also described black foot symptoms as dark-brown to black streaking in the vascular tissue of young (2–5 year-old) grapevines.

There are two negative impacts of this disease on nursery seedling production. First, these fungi cause seedling mortality in nurseries up to 50% (Anderson *et al.* 1962; Anonymous, 1993). Second, infected seedlings have a lower survival rate after out planting to reforestation sites. An important factor compounding this problem is that in some cases the symptoms are not visible on infected seedlings but disease can develop after transplantation.

Detection of plant pathogens directly from infected tissues has been reported for several agricultural plant pathogens (Le´vesque *et al.* 1994 & O'Gorman *et al.* 1994). In addition, PCR has been used to detect soil pathogens directly from infested soil (Henson *et al*., 1993). This molecular detection approache is ideally suited for the study of root rot organisms because of the difficulty in isolating and identifying some of these fungi. Hamelin *et al*. (1996) designed species-specific primers (Dest1 and Dest4) to detect *C. destructans* from conifer seedlings. Using these primers in direct PCR assays on DNA extracted from *C. destructans* cultures isolated from grapevines in Portugal, obtained a DNA fragment of 400 bp; 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 product from stage one. This is a simple and reliable method for detection of *Cylindrocarpon* spp. directly from infected grapevines

(Nascimento *et al*., 2001).The objective of this study was develop an efficient and reliable detection method based on PCR for the identification and detection of *C. destructans* from infected seedlings.

Materials and Methods

Fungal isolation

Fungal isolation was done using two methods, i.e. isolation method from vine root and moist chamber isolation method.

Isolation from vine roots

Small pieces of vine roots collected from five locations in Duhok governorate (Bajelor, Badi, College nursery, Malta nursery and Nizarke) were surface sterilized respectively by placing in 70% ethanol for 30 s, 1% NaOCl for 1 min and again in 70% ethanol for 30 s and then dried by filter papers. Pieces of sterilized tissues were plated onto 2% potato dextrose agar (PDA) (Himedia Laboratories Pvt. Ltd. - India) containing 0.25 mg/ml chloramphenicol. Hyphae growing out from the tissue pieces were cut and subcultured onto fresh PDA plates, and incubated at 25±2 °C (Van Niekerk *et al*, 2004).

Moist chamber method

Cuttings were made from vine roots and placed in 90 mm petridishes containing sterilized moist filter paper. Plates were incubated at room temperature until fungal growth observed. Propagules (spores, mycelia) were transferred to Potato-dextrose-Agar (PDA) plates. Pure cultures of each isolate were obtained by excising a hyphal tip from colony margins and plating it onto fresh PDA.

Phenotypical characterization

All isolates were grown on PDA and MEA at 25°C in darkness or under NUV + fluorescent illumination with a 12-h photoperiod (Philips /36W) for 10 days until cultures sporulated. The colonies were further incubated for 20 days to determine the presence or absence of chlamydospores. The diameter of 20 chlamydospores per isolate was measured. Length and width of 40 conidia (microconidia and one-, two-, and three-septate macroconidia) were measured. Isolated fungi were identified based on the characters in culture and on natural substrates (Domsch *et al,* 1980; Watanabe, 2002; Petit & Gubler, 2005).

DNA extraction and PCR amplification of ITS region

Ten isolates (DC1-DC10) were selected to confirm the identification by a specific primer of the ITS region. Isolates were collected from five locations in Duhok governorate (Bajelor, Badi, College nursery, Malta nursery and Nizarke).

Genomic DNA was extracted according to a method reported by Borges *et al.* (1990). The specific primers of *C. destructans* ITS region (Dest1 5'-TTGTTGCCTCGGCGGTGCCTG-3', Dest4 5'-GGTTTAACGGCGTGGCCGCGCTGTT-3') were used to amplify the ITS region of nuclear ribosomal DNA (rDNA), containing ITS1, ITS2 and the intervening 5.8 rRNA gene (Hamelin *et al.,* 1996). The PCR reactions were carried out in a total volume of 25 µl, in thin-walled, 0.5 µl Eppendorf tubes.Master mix was prepared for 12 samples of each fungus (10 isolates plus 2 control) by mixing 30 µl of 10XPCR, 30 µl of dNTPs, 24 µl forward primer, 24 µl Reverse primer, 12 µl MgCL2, 4.8 µl of Taq polymerase enzyme and de-ionized distilled water was added to a final volume of 252µl. The solution mixed and spun for 10 second in a microcentrifuge. Then, the mixture was distributed in PCR tubes. All these steps were done on ice. Amplification was carried out in an automated thermal cycler (Delphy 1000, Oracle Biosystems, MJ Research Inc., Watertown, MA, USA) according to the following program: An initial denaturation at 94°C for 4 min, after which 30

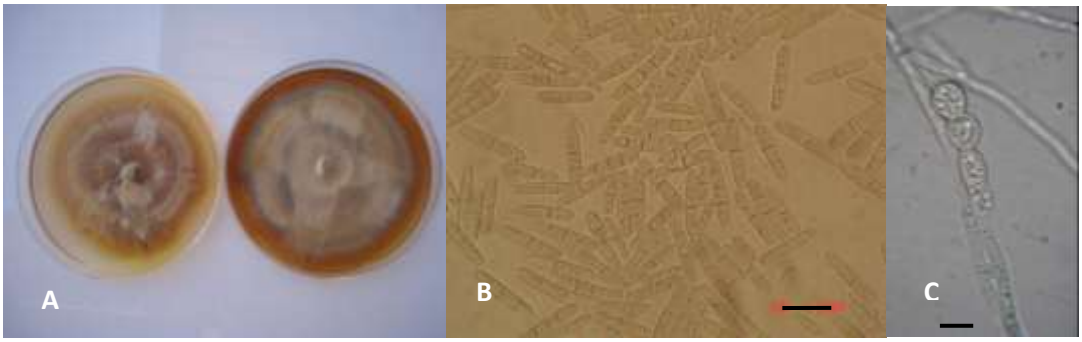
cycles of denaturation (1 min at 94°C), primer annealing (1 min at 58°C) and primer extension (1.5 min at 72°C) were performed (Alaniz *et al.,* 2007). A final extension was performed at 72°C for 10 min Amplification reactions were conducted at least twice, in two separate experiments. For each isolate, 5 µl of PCR products were mixed with 7µl loading buffer and then analyzed by electrophoresis in 2% (w:v) agarose gels with 1xTBE buffer visualized by UV fluorescence.

Results and Discussion

Phenotypical characterization of *C. destructans*

Cylindrocarpon destructans (Zinssm.) Scholten, Neth. JL. PL. Path. 70 (Suppl. 2) 9 (1964). Fig. (1) A – C. Telemorph: *Neonectria radicicola* (Gerlach & L. Nillsson) Mantiri and Samules Canada J. Bot. 79: 339 (2001).

Colonies on MEA reached a diameter of 78 mm on PDA and 80 mm on MEA after 20 days at 25°C. Colony surface slimy to felty; aerial mycelium typically sparse to felty, white to buff or a shade of brown. Colony reverse was orange to dark brown. Conidiogenous cells formed apically on densely, irregularly branching clusters of cells borne laterally on otherwise undifferentiated vegetative hyphae. Conidiophores 65 µm tall. Macroconidia cylindrical, mainly 4-celled, 18 – 45 (-47) × 4 – 8 µm. Microconidia, cylindrical, 1-celled, 8 – 11 (-12) × 3 – 4 µm. Chlamydospores yellowish brown, ovate to ellipsoidal, a few in a chain, 8 – 10 (-15) µm in diameter. Conidial dimensions were in concordance with the previous identification (Petit & Gubler, 2005).



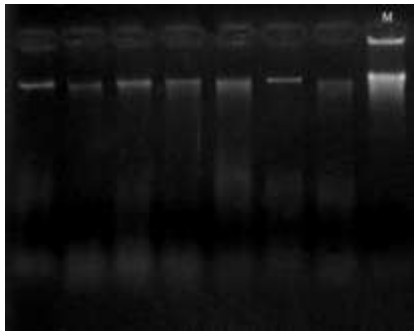
A) Twenty- day old colony on PDA-left, and MEA-right . B) Microconidia and macroconidia. Scale bar: 30 µm, C) Mycelia and Chlamydospores, Scale bar: 15 µm.

Figure 1. *Cylindrocarpon destructans*.

Molecular detection of *C. destructans*

Genomic DNA isolation and purification

Suitable yields of genomic DNA were obtained from repeated experiments with an average yield of 1.5-6.70 µg/ml and a purity of about (1.6-1.8) determined by spectrophotometer ratio A260/A280. The molecular weight of DNA samples was estimated using 1% agarose gel electrophoresis containing ƛ DNA sample as control (Fig. 2). Ratios above 2.0 correspond to RNA contamination, while ratios below 1.6 suggest protein contamination (Sinha *et al.*, 2001).

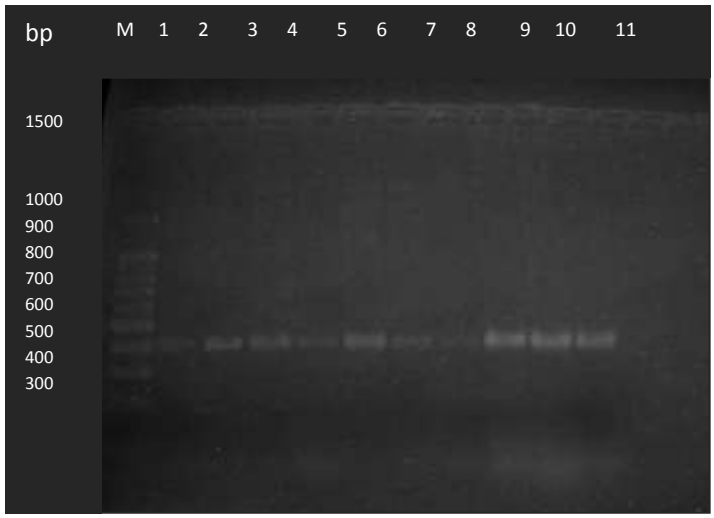


M: represents unrestricted λ DNA as a standard molecular weight marker. Lane1- 7: Whole Genomic DNA of some *C. destructans* isolates isolated from different locations of Duhok Governorate.

Figure 2. Agarose gel electrophoresis 1% at 70 volt for 45 minutes.

Species specific primers

All isolates of *C. destructans* collected from different locations of Duhok Governorate were amplified by two specific primers (Dest1, Dest4) which were designed by Hamelin *et al,* (1996). A PCR fragment of about 400-bp was obtained for all of them. The agarose gel electrophoresis of amplified products with this specific primer is shown in Figure (3). Other investigators have used the same primers to amplify the ITS region of *C. destructans* (Hamelin *et al.,* 1996; Alaniz *et al.,* 2007).



Lanes 1 (DC1)-10(DC10), *C. destructans* isolates. Lane 11, negative control of sterile distilled water; lane M, 1Kb Plus DNA Ladder.

Figure 3. Agarose gel of the PCR products using primer pairs Dest1 – Dest4.

This is the first molecular detection work on *C. destructans* in Iraq. In this study, the differences in the intensity of bands were not taken in consideration; despite they may reflect the differences in copy number of the priming sites in the individual genome. The band intensity may also be attributed to the difference of DNA concentration of individual isolates. This method did not require going through all classical methods and only in a few hours the results could be obtained. This approach is particularly well suited to soil organisms that are difficult to identify or isolate because of the presence of other aggressive species.

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-- back to Table of Content --