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Characterisation of *Cylindrocarpon destructans* isolates from grapevines in Portugal

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Summary. The isolates of *Cylindrocarpon destructans* used in the present study were selected from a collection of isolates from rootstock nurseries and grapevines showing symptoms of decline in Portugal during the last few years. These isolates were compared on the basis of gross cultural morphology, micromorphology and pathogenicity. The most variable parameter was colony colour. Analysis of micro- and macroconidial size of cultures on "Spezieller Nährstoffarmer Agar" plus 0.1% yeast extract medium (SNAY) revealed that all isolates except one, could be clustered in a single group. On carnation leaf agar (CLA) medium, however, all isolates clustered in a single group. All *C. destructans* isolates were pathogenic on rooted grapevine cuttings of cv. Periquita under greenhouse conditions. Internal black-foot symptoms were seen in inoculated plants and the pathogen was reisolated from 60 to 100% of these plants. All *C. destructans* isolates caused a significant (P=0.05) decrease in the height of inoculated plants, and the majority also caused a significant (P=0.05) reduction in the number of roots.

Key words: black-foot disease, fungal morphology, pathogenicity.

Introduction

Cylindrocarpon destructans (Zins.) Scholten is one of the fungi most frequently isolated from young Portuguese grapevines showing symptoms of decline (Rego, 1994; Oliveira *et al*., 1998; Rego *et al*., 2000). This fungus was first reported in France in 1961 as the causal agent of black-foot disease of grapevine and was later also considered to cause grapevine mortality in some new plantings in France (Maluta and Larignon, 1991) and in California (Scheck *et al*., 1998). *Cylindrocarpon obtusisporum* (Cooke & Harkness) Wollenw., a species closely related to *C. destructans*, has also been reported to produce black-foot symptoms on grapevine (Grasso and Magnano Di San Lio, 1974; Scheck *et al*., 1998).

According to Booth (1966), Samuels and Brayford (1990) and Brayford (1992), isolates of *C. destructans* from different hosts and geographic regions show great variations in their morphology and virulence. In some conditions, *C. destructans* is a relatively weak or opportunistic fungus that can cause disease in plants subjected to stress (Dumot *et al*., 1999).

In view of the high frequency of isolation of *C. destructans* from internally discoloured tissues of different grapevine materials (Rego *et al*., 2001), the aim of this work was to study the morphological and pathogenic characteristics of some isolates of *C. destructans* collected in Portugal

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to gain a better understanding of variation within this species.

Materials and methods

Colony characteristics and spore dimensions

Isolates of *C. destructans* used in this study were selected from a collection of isolates obtained from rootstock nurseries and vineyards in Portugal over the last seven years (Table 1). All isolates were derived from single macroconidia and maintained on slants of "Spezieller Nährstoffarmer Agar" plus 0.1% yeast extract (SNAY) at 5°C (Brayford, 1992). Identification was based on morphological characters according to Samuels and Brayford (1990). Colony diameters were measured in cultures grown on Petri dishes containing potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 20°C in darkness for 10 days. Perpendicular colony diameters were measured on three replicate plates. Some properties were also evaluated on carnation leaf agar (CLA) (Brayford, 1992). The colony characters, evaluated after 12 days on PDA were: colony texture, colour, zonation and the nature of the growing margin. Colony colour was compared with the colour designations of Saccardo (1891). Presence of chlamydospores was evaluated in PDA and production of sporodochia was recorded on CLA (Brayford, 1992). The length and width of 50 conidia from ten-day-old cultures on SNAY and CLA media were measured with a calibrated eyepiece micrometer at a magnification of ×630.

Pathogenicity tests

Rooted grapevine cuttings of the cv. Periquita were used for the pathogenicity experiments. Plants were uprooted and their roots washed. Ten-day-old fungal cultures grown in Czapek liquid medium were used as inoculum. For each fungal isolate, five plants were inoculated by dipping their roots for 30 min in a suspension of approximately 10^8 conidia per ml. Control plants were similarly treated but received sterile distilled water instead of inoculum. Rooted cuttings were then planted in 1 l plastic pots containing an autoclaved mixture of soil, peat and sand (2:1:1, v:v) and maintained in a greenhouse at 24±5°C day/18°C night with a 12 h day. Plants were watered weekly. After 3 months, plant height, number of internodes and the total number of roots were evaluated as described by Adalat *et al*. (2000). Reisolations were made as described by Rego *et al*. (2000) and the identity of *C. destructans* was confirmed on the basis of morphological characters (Samuels and Brayford, 1990). Data were subjected to analysis of variance (ANOVA) and means were compared with the Bonferroni test (P=0.05).

Table 1. Details of the *Cylindrocarpon destructans* isolates studied in this work.

Isolate	Year of collection	Origin (Region)	Rootstock/Cultivar
Cy1[a]	1992	Estremadura	99R/Seara Nova
Cy2	1992	Estremadura	99R/Seara Nova
Cy5	1992	Estremadura	99R/Boal Branco
Cy7	1992	Estremadura	99R/Perrum
Cy14	1995	Beira Litoral	99R
Cy16	1995	Beira Litoral	110R
Cy21	1996	Beira Alta	1103 P/Malvasia Fina
Cy24	1997	Estremadura	110R
Cy30	1998	Estremadura	99R
Cy32	1998	Estremadura	99R
Cy36	1998	Estremadura	110R
Cy68	1999	Estremadura	99R
Cy76	1999	Estremadura	99R

[a] IMI dried collection number 357400 (typical strain).

Results

Colony characteristics and conidium dimensions

Colony diameter on PDA varied from 39 mm to 53 mm after ten days of incubation at 20°C in darkness. Colony characteristics varied little between isolates (Table 2). Colony texture was cottony or felty with aerial mycelium in the centre or over the entire colony. The most strongly varying character was colony colour, which varied from white to cinnamon (Fig. 1). In PDA, zonation was absent, except in isolate Cy5. On the contrary, in CLA all the cultures formed concentric zones. The colony margin was even in both media. Chlamydospores were present in cultures grown on PDA and sporodochia were produced in both PDA and CLA (Fig. 2).

Conidiophores, unbranched or branched, arose abundantly from aerial mycelium and the agar surface. They produced terminal or lateral phialides (sensu Sutton, 1980) on short branches, or developed lateral branches ending in one or more phialides. Isolate Cy16 sometimes formed verticillate conidiophores. Conidiogenous cells were phialidic, hyaline, cylindrical or tapering towards the tip and straight, 22–45 μm long and 1.5–3.0 μm wide. Micro- and macroconidia were produced in culture on all media tested. Microconidia were copious, hyaline and cylindrical to ellipsoid with a typical protuberant flat abscission scar at the base except for isolate Cy16, which formed only sparse microconidia. Macroconidia formed in sporodochia were hyaline, mostly straight, with 3 septa, cylindrical or with a slightly curved apex and protruding basal abscission scar. Intercalary or terminal chlamydospores were hyaline, becoming golden brown in mature colonies, globose, 6–21 μm in diameter, thick-walled and smooth. Sporodochia were particularly abundant on the carnation leaf pieces of CLA (Fig. 2).

Table 2. Colony characters of *Cylindrocarpon destructans* isolates.

Isolate	Texture (PDA)[a]	Colour (PDA)	Zonation		Growing margin (PDA)	Chlamydospores (PDA)	Sporodochia (PDA-CLA)	Reverse of colony
			(PDA)	(CLA)[b]				
Cy1, Cy2	Cottony	White to cream-coloured	Absent	Concentric	Even	+	+	Rusty to cinnamon -coloured in the centre
Cy7, Cy14	Cottony with aerial mycelium	White to cream with cinnamon-coloured in the centre	Absent	Concentric	Even	+	+	Cinnamon -coloured
Cy5	Felty with aerial mycelium in the centre	White to cream with cinnamon-coloured in the centre	Concentric	Concentric	Even	+	+	Rusty to the cinnamon -coloured in centre
Cy16, Cy21, Cy24	Felty	White to cream with cinnamon-coloured in the centre	Absent	Concentric	Even	+	+	Cinnamon -coloured
Cy30, Cy32, Cy36, Cy68, Cy76	Cottony	Cream to cinnamon-colour	Absent	Concentric	Even	+	+	Cinnamon -coloured

[a] Potato dextrose agar.
[b] Carnation leaf agar.

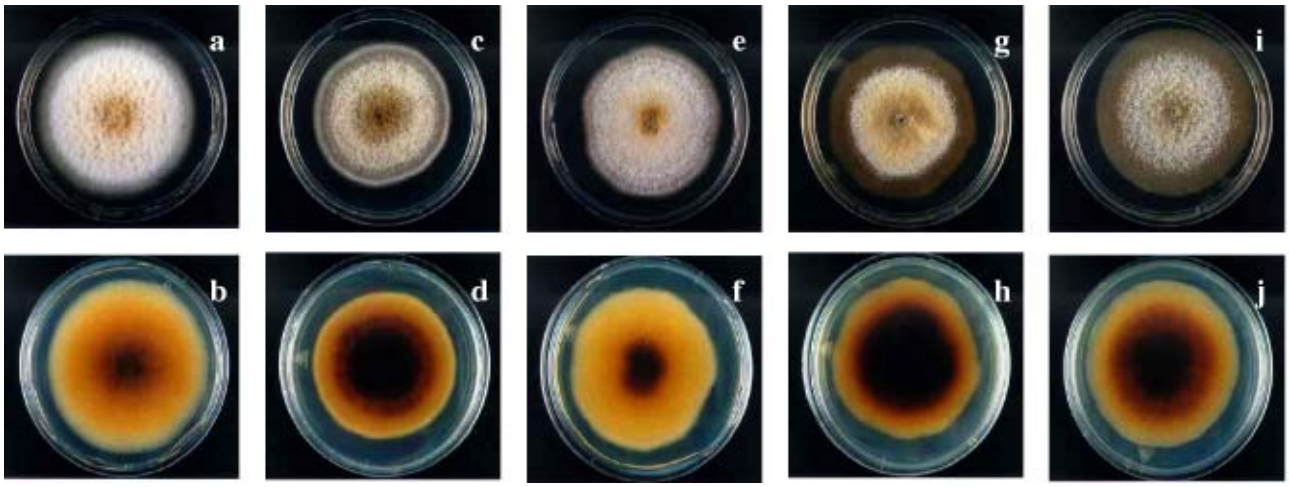

Fig. 1. Cultures of *Cylindrocarpon destructans* on PDA. Up: from top of colony; down: from bottom of colony. a and b, Cy2; c and d, Cy7; e and f, Cy5; g and h, Cy16; i and j, Cy68.

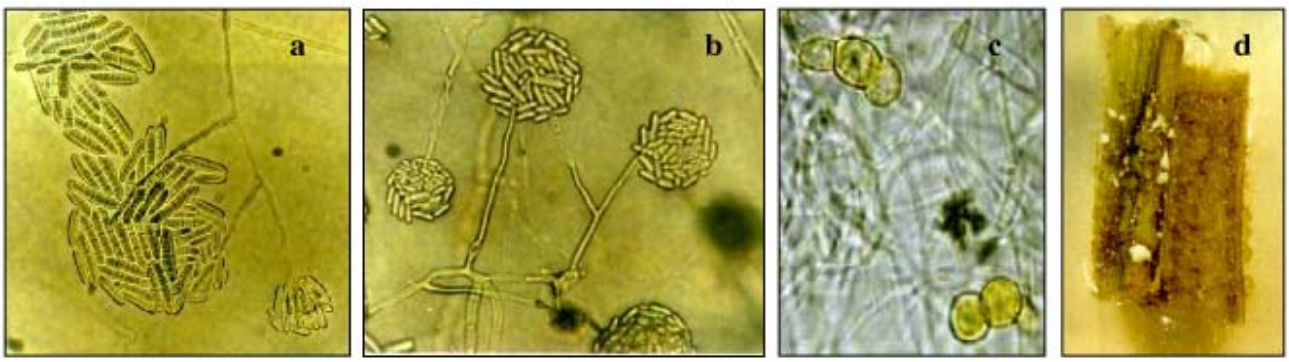

Fig. 2. *Cylindrocarpon destructans*: (a) macroconidia with 3 septa and microconidia (×400); (b) microconidia formed in phialides (×630); (c) chlamydospores (×630); (d) sporodochia.

Analysis of spore size on SNAY revealed that the majority of isolates studied clustered into one group, except for isolate Cy16, which had wider and longer conidia than the others. In CLA, spore dimensions were more homogeneous and all isolates could be grouped together (Table 3).

Pathogenicity tests on grapevine rooted cuttings of cv. Periquita

Inoculated rooted vine cuttings generally showed low growth with shortened internodes, sparse foliage and were stunted. The great majority of the rooted cuttings inoculated with *C. destructans* isolates exhibited typical black-foot symptoms. These included root lesions, vascular discolouration, and necrosis in the basal tissues of the plants. All symptoms developed within 3 months of inoculation (Table 4). The percentage of reisolation from inoculated plants ranged from 60 to 100% and the pathogen was never recovered from the controls (Table 4).

All isolates of *C. destructans* significantly (P=0.05) decreased the height of infected plants and most caused a significant (P=0.05) reduction in the number of roots (Fig. 3). Isolates Cy5, Cy16, Cy21, Cy30 and Cy68 were the most virulent as regards plant height and number of internodes. The stunting of inoculated plants was predominantly explained by the shorter internodes, since several isolates did not significantly decrease the number of internodes.

Table 3. Conidial size (μm) of *Cylindrocarpon destructans* isolates in "Spezieller Nährstoffarmer Agar" plus 0.1% yeast extract (SNAY) and carnation leaf agar (CLA).

Conidia		Medium[a]		
		SNAY		CLA
No. of septa	Size	Cy1, Cy2, Cy5, Cy7, Cy14, Cy21, Cy24, Cy30, Cy32, Cy36, Cy68, Cy76	Cy16	Cy1, Cy2, Cy5, Cy7, Cy14, Cy16, Cy21, Cy24, Cy30, Cy32, Cy36, Cy68, Cy76
0	Lenght	(4.5) 8.6 (13.5)	(13.5) 20.3 (43.5)	(4) 8.5 (12)
	Width	(2) 2.9 (4.5)	(4.5) 5.5 (7.5)	(1.5) 2.9 (4)
1	Lenght	(8) 16.4 (25.5)	(13.5) 29.1 (46.5)	(8) 14.3 (24)
	Width	(3) 4.3 (6)	(4.5) 5.7 (7.5)	(3) 3.6 (5)
2	Lenght	(15) 21.4 (28)	(33) 37.9 (43.5)	(15) 22.8 (29)
	Width	(4) 5 (6)	(6) 6.9 (8)	(3) 3.6 (6)
3	Lenght	(16.5) 28.4 (37.5)	(36) 42.1 (49.5)	(19) 27.2 (39)
	Width	(4) 5.7 (6)	(6) 7.1 (8)	(4.5) 5.7 (7)

[a] Averages were derived from 50 conidia observation for each isolate, (range -minimum and maximum- in parenthesis)

Table 4. Results of pathogenicity tests of *Cylindrocarpon destructans* isolates on rooted grapevine cuttings of cv. Periquita.

Isolate	Symptomatic plants	
	Infected plants[a] (%)	Reisolation (%)
Control	0 a	0 a
Cy1	n.d.	n.d.
Cy76	79.8 c	59.6 b
Cy2	69.6 b	60.4 b
Cy7	81.6 c	79.6 c
Cy14	94.6 e	79.6 c
Cy16	89.6 d	79.6 c
Cy5	79.8 c	79.8 c
Cy21	90.8 d	79.8 c
Cy24	90.4 d	80 c
Cy30	94.4 e	80.4 c
Cy36	99.6 f	99.6 d
Cy68	99.6 f	99.6 d
Cy32	91.8 d	100 d
LSD	2.12	2.12

n.d., Cy1 not determined; data in Rego (1994).
[a] Means in each column followed by the same letters are not different statistically (P=0.05).

Discussion

C. destructans has been frequently isolated from grapevine plants showing black-foot symptoms in Portugal (Rego, 1994; Oliveira *et al*., 1998). In other countries *C. obtusisporum* is reported to be the causal agent of this disease (Grasso and Magnano Di San Lio, 1974; Scheck *et al*., 1998). Both species are morphologically similar and the main distinguishing trait is the shape of the conidia. Macroconidia of *C. destructans* are typically straight but sometimes curved, cylindrical with an obtuse apex, while *C. obtusisporum* produces fusiform and gently curved conidia (Samuels and Brayford, 1990). However, strains of *C. destructans* from different provenances usually show great variation in morphology and it can be difficult to identify patterns of variation, especially when only the anamorph characters are considered. The teleomorph of *C. destructans* is considered to be *Nectria radicicola* Gerlach & Nilsson, but so far only the anamorph has been found on grapevine materials in Portugal. As regards *C. obtusisporum*, there is controversy about the correct name for the teleomorph, referred to by Booth (1966) as *Nectria tawa* Dingley (Samuels and Brayford, 1990).

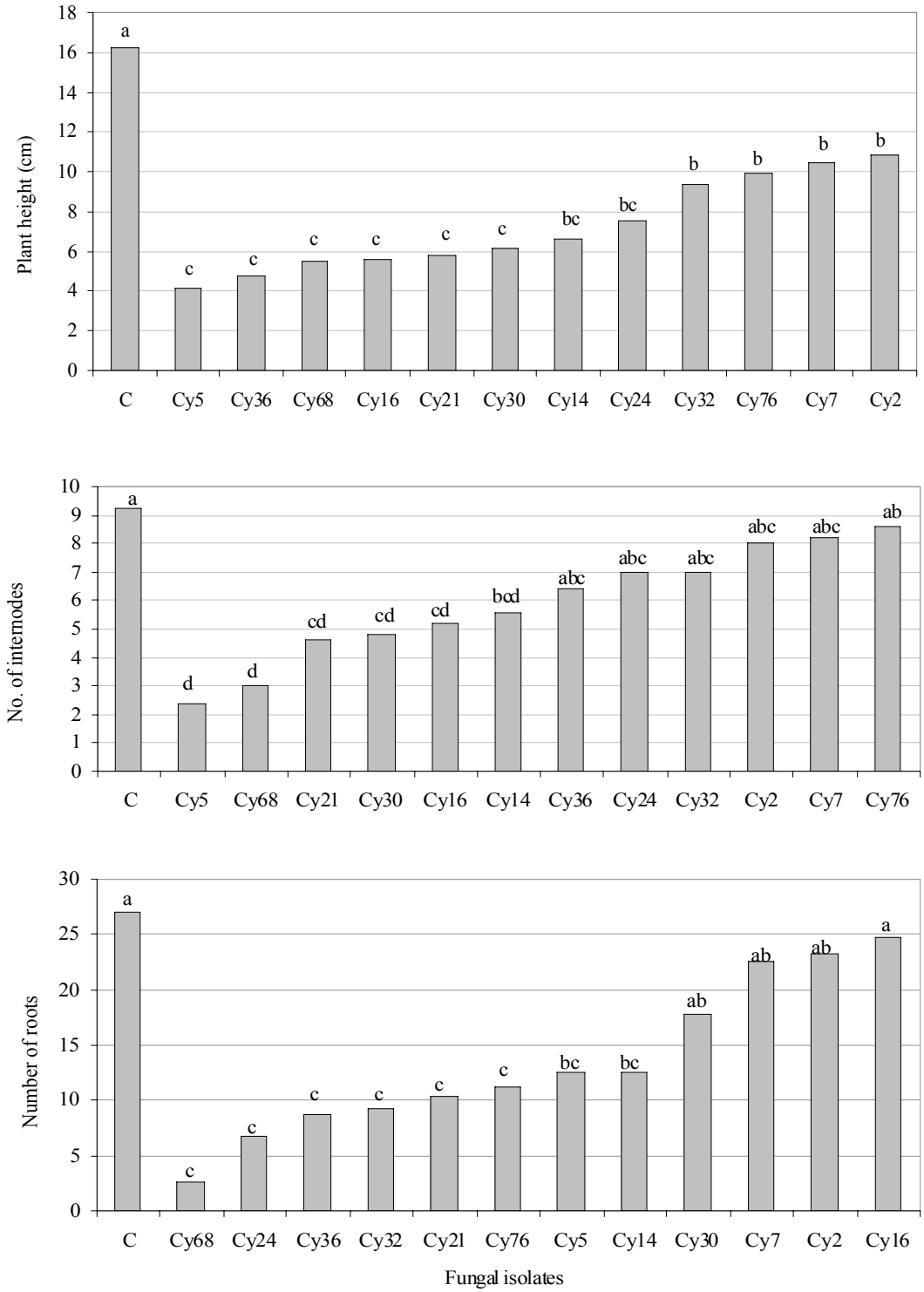

Fig. 3. Effect of *Cylindrocarpon destructans* isolates on plant height, number of internodes and number of roots in inoculated grapevine cuttings of cv. Periquita. C, control, Cy, *C. destructans* isolate.

In the present study, the morphological and pathogenic characteristics of 13 isolates previously identified as *C. destructans* were evaluated. All these isolates were obtained from grapevine plants showing black-foot symptoms and they showed the large variation of our present *C. destructans* collection. Although some isolates were stored for a number of years, there was no sign of cultural degeneration, probably because the cultures were maintained on a weak-nutrient agar (SNAY medium) as suggested by Brayford (1992). Some differences in colony characteristics were detected between isolates, most notably texture and colour. Nevertheless, these variations are acceptable and come within the current limits defined for *C. destructans*, which exhibits a large variation in colony characters (Booth, 1966; Samuels and Brayford, 1990). Conidia morphology and size of all isolates except Cy16 were within the limits defined for *C. destructans* (Samuels and Brayford, 1990). Isolate Cy16 differed from other isolates in the low number of conidia produced in culture and also in conidia size, which was slightly different from that normal for this species. This isolate also, sometimes formed verticillate conidiophores. These differences were detected in SNAY, which proved to be better than CLA for discriminating isolates on the basis of conidia size. We can conclude that Cy16 is not a typical strain of *C. destructans*. This isolate could also not be accommodated in *C. obtusisporum* on the basis of the shape of macroconidia.

Variations in the pathogenicity of isolates of *C. destructans* are reported and environmental factors and host stresses often affect disease development (Brayford, 1992). Saprophytic strains may also exist within this species (Booth, 1966). In this study, pathogenicity tests on various *C. destructans* isolates showed that all induced black discolouration at the basal end of inoculated plants and also significantly reduced plant height. However, isolates varied in their pathogenicity. According to the parameters analysed, isolates Cy2 and Cy7 were among the less virulent, but apparently this was not a consequence of the age of the isolates since the virulence of isolate Cy76, one of the most recent isolates, was similar to that of Cy2 and Cy7. *Cylindrocarpon* spp. are frequently part of a disease complex with other plant pathogens and comparison of artificial inoculations with natural infection processes should be made carefully (Brayford, 1992). The approach used in the present study only allows a relative comparison of the *C. destructans* isolates. Other pathogenicity-testing methods should be explored in future to simulate natural infections more closely.

Representative isolates of the present collection of *C. destructans* isolated from grapevine in Portugal were studied with regard to their morphological and pathological characters. In order to resolve many remaining questions, these studies are being extended by the combined use of morphological and molecular data on a wider *C. destructans* collection.

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