Variability within *Calonectria ovata* and its anamorph *Cylindrocladium ovatum* from Brazil

Pedro W. Crous[1], Acelino C. Alfenas[2] & Tatiana G. Junghans[2]

[1] Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa
[2] Departemento de Fitopatologia, Universidade Federal de Viçosa, 36.570 Viçosa, MG, Brazil

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Calonectria ovata is presently known only from the Amazonas province of Brazil, where it causes a severe leaf spot disease of various eucalypt species. Collections of *Ca. ovata* from two locations in the Amazon, namely Tucurui and Monte Dourado, revealed isolates from Tucurui to have much larger conidia than those from Monte Dourado. Twenty-four isolates from these sites were subsequently mated in all possible combinations, and also compared via isozyme analysis. Results from this study supported *C. ovatum* to be a homogeneous species. Although it was morphologically more variable than reported earlier, all isolates exhibited a biallelic heterothallic mating system, and were designated as either MAT 1-1 or MAT 1-2 based on their mating type. Furthermore, *C. ovatum* was found to be an important pathogen commonly associated with leaf spot and cutting rot symptoms of various eucalypt species grown in the Amazon.

Keywords: biallelic heterothallism, *Calonectria ovata*, Hypocreales, isozyme analysis, taxonomy.

Species of *Cylindrocladium* Morgan, which are known anamorphs of *Calonectria* De Not. (Rossman, 1979, 1983), are commonly associated with leaf spot, blight, cutting rot and various other economically significant diseases of eucalypts world-wide (Crous & al., 1991). *Cylindrocladium* spp. have hyaline, smooth, one- to multi-septate cylindrical conidia, and penicillate conidiophores with septate stipe extensions terminating in vesicles of characteristic shape. Species are primarily distinguished based on conidial size, septation and vesicle shape. During 1990, several isolates of a *Cylindrocladium* sp. were collected from the Amazonas province of Brazil that were characterized by having 1(–3)-septate conidia and ovoid vesicles. Based on their conidial morphology and ovoid vesicles, these collections could be distinguished from relatively similar species such as *C. floridanum* Sobers & C. P. Seym. (sphaeropedunculate vesicles), *C. candelabrum* Viégas (obpyriform vesicles) and *C. scoparium* Morgan (pyriform vesicles). The Brazilian species, which seemed to occur only in the Amazon, was subsequently described as *C. ovatum* El-Gholl & al. (1993). In a study characterizing *Cylindrocladium* species with 1-septate conidia and ovoid-like vesicles, Victor & al. (1997) employed RAPD banding patterns to distinguish *C. floridanum* (teleomorph: *Ca. kyotensis* Terash.) from mating types of *C. candelabrum* (teleomorph: *Ca. scoparia* Peerally), *C. scoparium* (teleomorph: *Ca. morganii* Crous & al.) and *C. ovatum*, and described the teleomorph of the latter as *Ca. ovata* Victor & Crous.

All known collections of *C. ovatum* were thus far obtained from Monte Dourado (Jari) in the Amazonas province of Brazil. During a recent survey of eucalypt diseases from the Amazon in Brazil, extensive collections were made of *Cylindrocladium* leaf spots and cutting rot from plantation trees and nursery cuttings, respectively. The species commonly encountered in plantations, which was also the dominant nursery pathogen, proved to be *C. ovatum*. A closer examination of these collections showed *C. ovatum* to be morphologically more variable than earlier acknowledged (El-Gholl & al., 1993; Crous & Wingfield, 1994). Collections from Tucurui were found to have larger conidia than those from Monte Dourado. Mating studies of *C. ovatum* done by Victor & al. (1997) induced perithecia with viable ascospores in only one mating, thus leaving some uncertainty as to the mating system involved. The aim of the present study, therefore, was to use isozyme analysis combined with mating studies to compare collections from Tecurui and Monte Dourado in an attempt to determine the acceptable morphological variation within, and mating system of *C. ovatum*.

Material and methods

Isolate collection and identification

Symptomatic eucalypt leaves and cuttings were placed in brown paper bags and transported to the laboratory within 48 h. Single conidial isolations were made on 2 % malt-extract agar (MEA) (Biolab), and incubated at 25 C. Colonies were subcultured onto divided plates containing MEA and carnation-leaf agar (CLA), and incubated at 25 C under near-ultraviolet light as explained in Crous & al. (1992). Identifications were based on the keys provided by Crous & Wingfield (1994). All measurements were made of fungal structures *in vitro* mounted in lactophenol. Thirty measurements of each structure were taken, and the 95 % confidence interval determined; extremes are given in parentheses. Twenty-six single conidial isolates were retained for the mating studies and isozyme analysis. Isolates 1–14 were collected from leaf spots on either *Eucalyptus tere-*

ticornis or *E. pellita* from Tucurui. Isolates 15–26 were collected from diseased cuttings and leaf spots of *E. grandis* × *saligna* trees from Monte Dourado. Representative isolates and mating types were deposited in the culture collections of the Departments of Plant Pathology at the Federal University of Viçosa, Brazil (UFV), and the University of Stellenbosch, South Africa (STE-U).

Mating studies

Twenty-six single-conidial isolates were paired in all possible combinations. Mating studies were conducted on CLA as explained in Crous & al. (1993). Plates were sealed in plastic bags, and incubated on the laboratory bench at 22 C. Ratings were done after 2 mo of incubation. Perithecia exuding masses of fertile yellow spores were accepted as positive, and the absence of perithecia or protoperithecia without visible exuding ascospore masses were regarded as an unsuccessful mating.

Isozyme analysis

The 26 isolates used in the mating study were also included in the isozyme study. A 5 mm diameter mycelial plug of each isolate was inoculated into separate 125 ml Erlenmeyer flasks containing 50 ml of semi-synthetic liquid medium (Alfenas, 1986) and incubated in the dark for 7 days. After incubation, the cultures were harvested under vacuum in a Buchner funnel containing a Whatman No. 1 filter paper. The mycelial cake was rinsed with distilled water and the excess moisture removed by squeezing the mycelium in filter paper. A sample of 200 mg of each culture was crushed in a frozen mortar and pestle, containing 1 ml ice-cold extraction buffer (0.34 M dibasic sodium phosphate, 0.2 M sucrose, 2.56 % PVP-40, 5.7 mM L-ascorbic acid, 5.8 mM DIECA, 2.6 mM sodium bisulphate, 2.5 mM sodium borate, 0.2 % -mercaptoethanol and 1 % polyethilenoglicol-6000; Alfenas & al., 1991). During homogenization, small quantities of Polyvinylpolypyrrolidone (PVPP) were added to the sample. The homogenate was adsorbed onto 12 × 5 mm chromatographic paper wicks Whatman No. 3 and stored in microcentrifuge tubes at −85 C until used for electrophoresis.

Electrophoresis was conducted on 13 % hydrolized starch gels containg 3 % sucrose, morpholine-citrate 0.04 M, pH 7.1, diluted 1:20 as gel buffer and morpholine-citrate 0.04 M, pH 6.1 in the electrode compartments. After electrophoresis the gels were stained for detection of enzyme activity (Alfenas & al., 1991). Cluster analysis and trees based on Euclidian distance among isolates was carried out using the Systat software (Wilkinson & al., 1992).

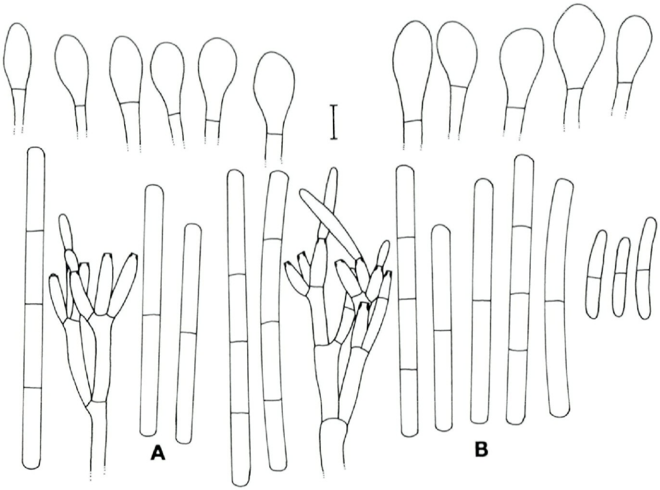


Fig. 1A, B. – Vesicles, conidiophores, macro- and microconidia of *Cylindrocladium ovatum* from Tucurui (A), and Monte Dourado (B). – Bar = 10 μm.

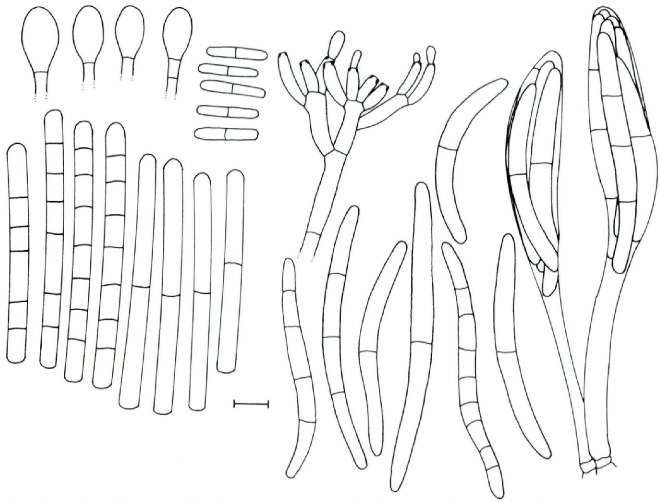


Fig. 2. – Vesicles, conidiophore, macro- and microconidia, asci and ascospores of *Calonectria ovata* and its anamorph *Cylindrocladium ovatum* after 2 mo on carnation leaf agar. – Bar = 10 μm.

Results

Isolate collection and identification

All isolates from Monte Dourado except isolate 19 and 25 fitted the description of the type strain of *C. ovatum* as characterized by Crous & Wingfield (1994). Conidia were primarily 1(–3)–septate, straight or curved, and (36–)50–65(–80) × 4–5(–6) µm. Not all isolates produced microconidia. Isolates 19 and 25 proved to be closer to *C. floridanum*, not *C. ovatum* as initially suspected. Conidia were (36–)45–55(–60) × 4–5 µm, and the sphaeropedunculate vesicles (10–) 12(–14) µm in diam. Conidia of *C. ovatum* from Tucurui were generally larger than those from Monte Dourado, straight or curved, 1(–3)–septate, (50–)65–80(–110) × 4–5(–6) µm. When mating studies were assessed after 2 mo, many of the conidia were observed to have developed additional septa, and were frequently up to 7-septate. In all collections, and in the older material examined after 2 months, vesicles remained typically ovate in shape, (8–)10(–14) µm diam. (Figs. 1, 2).

Mating studies

All isolates screened mated in the present study, except the *C. floridanum*-like isolates 19 and 25, which produced no perithecia in any of the matings. Isolates could be divided into two groups based on mating type, namely MAT 1–1 (5–7, 10, 15–17, 24, 26) and MAT 1–2 (1–4, 8, 9, 11–14, 18, 20–23), which is typical of a biallelic heterothallic mating system found in several ascomycetes (Yoder & al., 1986) (Fig. 3). Perithecia produced on CLA were orange, becoming red–brown with age, and turning red in 3 % KOH. Conidia were generally multiseptate after 2 mo on CLA, while the exuding ascospores were fusiform, 1–3(–7)-septate, (35–)55–70(–90) × (4–)5–6 µm (Figs. 4–11).

Isozyme analysis

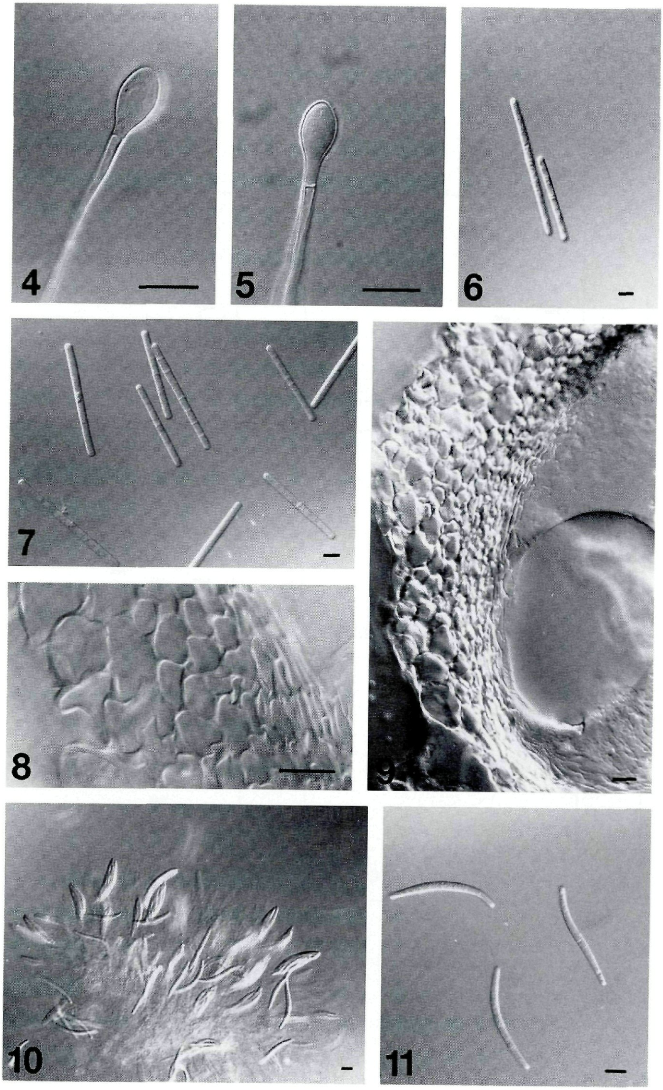
Among the 11 enzymes tested, eight [esterase (-EST), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), hexokinase (HK), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), glucose 6–phosphate dehydrogenase (6-PGDH) and sorbital dehydrogenase (SOD)] showed interpretable results. A total of 23 isoenzyme phenotypes, 31 polymorphic loci and one monomorphic locus were found (Fig. 12). All isolates were closely related and formed a tight cluster. Two distinct groups could be distinguished, group one consisting of the *C. floridanum*-like isolates 19 and 25, and group two representing the 24 *C. ovatum* isolates (Fig. 13). The *C. floridanum*-like isolates in group one were both from Monte Dourado,

Iso	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21	22	23	24	26
1	-																							
2	-	-																						
3	-	-	-																					
4	-	-	-	-																				
5	+	+	+	+	-																			
6	+	+	+	+	-	-																		
7	+	+	+	+	-	-	-																	
8	-	-	-	-	+	+	+	-																
9	-	-	-	-	+	+	+	-	-															
10	+	+	+	+	-	-	-	+	+	-														
11	-	-	-	-	+	+	+	-	-	+	-													
12	-	-	-	-	+	+	+	-	-	+	-	-												
13	-	-	-	-	+	+	+	-	-	+	-	-												
14	-	-	-	-	+	+	+	-	-	+	-	-	-	-										
15	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-									
16	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-								
17	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-	-							
18	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-						
20	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-					
21	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-				
22	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-			
23	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-		
24	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	
26	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-

Compatible matings = (+), incompatible matings = (–).
Isolates 5–7, 10, 15–17, 24 & 26 = MAT 1-1, and 1–4, 8, 9, 11–14, 18 & 20–23 = MAT 1-2.

Fig. 3. – Mating grid of *Calonectria ovata*. Isolates 1–14 are from Tecurui, and 15–26 from Monte Dourado.

Figs. 4–11. – *Calonectria ovata* and its anamorph *Cylindrocladium ovatum*. – 4, 5. Ovoid vesicles. – 6, 7. Variation in macroconidial size and septation. – 8, 9. Vertical section through a perithecium displaying its wall anatomy. – 10, 11. Asci and ascospores. – Bars = 10 µm.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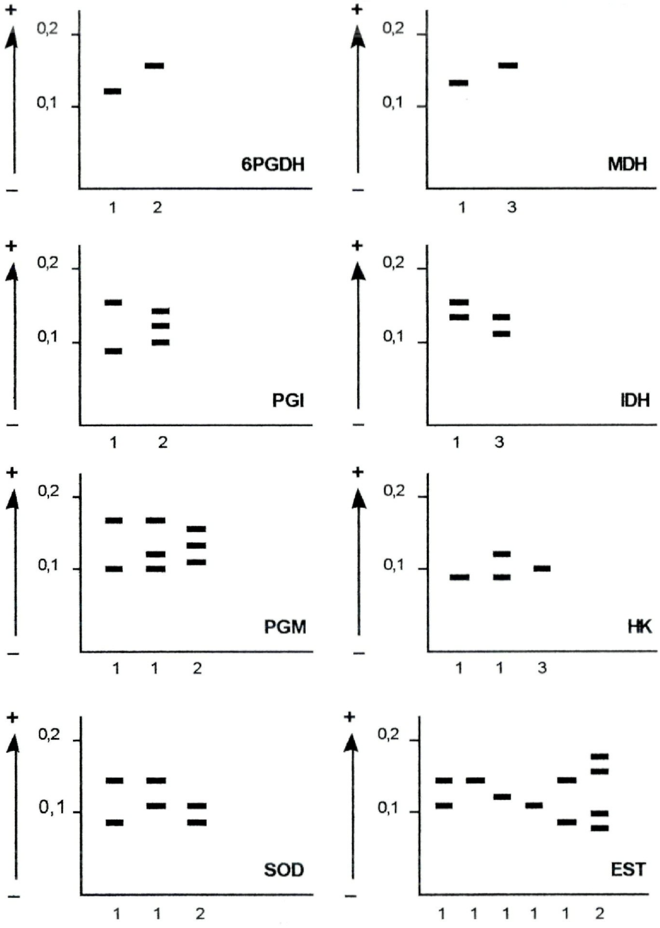

Fig. 12. – Isozyme phenotypes obtained with eight enzymes for *Cylindrocladium ovatum* isolates from Monte Dourado and Tucurui. – 1 = *C. ovatum*, 2 = *C. floridanum*, and 3 = phenotypes shared by isolates of *C. ovatum* and *C. floridanum*. – Enzymes used are the following: glucose 6-phosphate dehydrogenase (6-PGDH), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), isocitrate dehydrogenase (IDH), phosphoclucomutase (PGM), hexokinase (HK), sorbital dehydrogenase (SOD) and esterase (-EST).

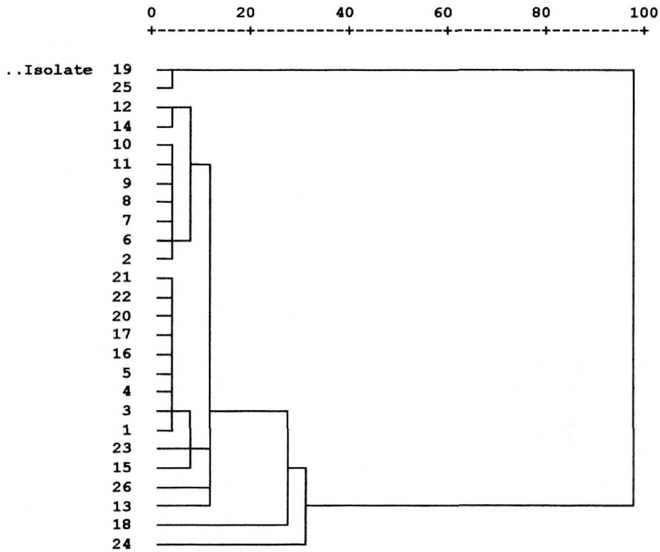

Fig. 13. – Dendrogram formed by single linkage cluster analysis, illustrating the similarities of 24 isolates of *Cylindrocladium ovatum*, and two isolates of *C. floridanum* (19 & 25), using isozymatic markers.

whereas the *C. ovatum* isolates in group two were collected in Monte Dourado and Tucurui.

Discussion

The present study was initiated to clarify the acceptable variation within *C. ovatum*, a species thus far only collected from the Amazonas province of Brazil. In the initial examination, isolates associated with leaf spots of *E. pellita* and *E. tereticornis* in Tucurui had much larger conidia than those from eucalypts in Monte Dourado, leading us to suspect that they may represent a new species. Little is known about the morphological variation and pathogenicity of *C. ovatum*. Although El-Gholl & al. (1993) noted conidia of *C. ovatum* to be straight, they were reported as either straight or prominently curved by Crous & Wingfield (1994). Curved conidia were once again observed in collections examined from Monte Dourado and Tucurui. Although conidia were reported to be primarily 1(–3)-septate, old cultures showed that conidia could in fact become up to 7-septate. No difference or distortion, however, was observed in the dimensions or shape of microconidia, vesicles or phialides (Fig. 2).

Mating type system

In mating nine isolates of *C. ovatum* at 15 and 25 C, respectively, only a few fertile perithecia were obtained in one combination at 25 C. This material was subsequently used to describe the teleomorph as *Ca. ovata* (Victor & al., 1997). In the present study, which was conducted at 22 C, nearly all matings produced perithecia with viable progeny. The numerous perithecia produced for *Ca. ovata*, as well as for other heterothallic *Calonectria* spp. (Crous & Schoch, unpublished), suggests that this is the temperature most suitable for inducing perithecia in heterothallic *Calonectria* spp. Based on the positive mating results derived here for the 24 isolates of *C. ovatum*, all isolates could either be classed as MAT1-1 or MAT1-2, which in turn mated with all isolates from the opposite mating type (Fig. 3). These findings also provided positive proof that *C. ovatum* is in fact a biallelic heterothallic species, as suspected by Victor & al. (1997). This mating system is present in many other ascomycetes (Dyer & al., 1996), and the use of mating type and molecular data to support the distinction of different anamorph species with similar sexual states has been employed in genera such as *Gibberella*, *Nectria*, *Ophiostoma* and *Tapesia* to name but a few (Hsieh & al., 1977; Brasier, 1991; Leslie, 1991; Robbertse & al., 1995; Dyer & al., 1996).

Morphological variation

Orange perithecia of *Ca. ovata*, which were also observed on infected cuttings collected *in vivo*, rendered ascospores which were straight, curved to sigmoid, 1–3(–7)-septate, (35–)55–70(–90) × (4–)5–6 μm, thus slightly larger than those reported by Victor & al. (1997) (35–70 × 4–6 μm) (Figs. 2, 10, 11). Although it was initially suspected that the slightly longer conidia of the Tucurui isolates (up to 110 μm) could be distinct from those of the Monte Dourado collections (up to 80 μm) (Fig. 1), isolates from both groups mated successfully with each other. Furthermore, at 30 % genetic distance most of the isolates of *C. ovatum* were grouped in two large clusters, which represented a mixture of isolates from both localities. Two isolates of *C. ovatum* from Monte Dourado, however, grouped separately. Based on the different isolates present in the two larger culsters, these findings prove that *C. ovatum* can have straight or curved conidia that can be up to 110 μm long, are generally 1(–3)-septate, but could become up to 7-septate in older cultures.

Although Ferreira (1989) listed *C. floridanum* as occurring in Brazil, little is in fact known about the relative importance of this pathogen in Brazil. *C. floridanum* is well-known as a root rot pathogen, (Sobers & Seymour, 1967; Kuhlman & al., 1980; Sharma & al., 1984; Boesewinkel, 1986; Crous & al., 1991; Juzwik & Testa, 1991), and we suspect that this is probably the first record of it causing cutting rot in eucalypt nurseries. Morphologically *C. floridanum* is very similar to *C. ovatum*, but is chiefly distinguished by having sphaeropedunculate vesicles, and smaller, 1-septate condia. The two isolates initially identified as *C. ovatum* in the present study (19 and 25) were later shown to be distinct both in mating studies, as well as in the isozyme analyses. A re-examination of these strains found them to have sphaeropedunculate vesicles (10–)12(–14) µm diam., and 1-septate condia (40–)50–55(–60) × 4–5 µm, thus placing them in the *C. floridanum* complex. Based on a study using RAPD and A+T-rich DNA markers, Victor & al. (1997) concluded that *C. floridanum* is heterogeneous, and consists of several groups, with group 1 being *C. floridanum sensu stricto*, found around Florida USA, in Europe and Japan, and group 2 found above the great lakes of North America, including Canada. Isolates from Hong Kong and Thailand were representative of yet other groups. Without employing additional molecular techniques, therefore, it is at present not possible to safely allocate the Brazilian isolates of *C. floridanum* to any of these groupings within the *C. floridanum* complex. Further studies would therefore be required to address these aspects.

Pathogenicity

El-Gholl & al. (1993) associated *C. ovatum* with a leaf spot disease of *E. urophylla*, and also proved its pathogenicity on *E. grandis*, *E. robusta*, *E. tereticornis* and *E. torelliana*. Blum & al. (1992) were, however, the first to prove that it was a pathogen of eucalypts (as *C. scoparium*, isolate Un-B 1026), causing damping off of several eucalypt species. The present study adds further information to its status as pathogen, and is the first record of *C. ovatum* being widely distributed throughout the eucalypt forests around Monte Dourado, and also being a serious cutting rot pathogen in nurseries. Considerable variation in resistance to leaf blight was observed in the field, suggesting that selection and clonal propagation of superior genotypes should be considered if this disease becomes a serious problem in the future.

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