

## Description and pathogenicity of *Cylindrocladium ovatum* sp.nov.<sup>1</sup>

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*Cylindrocladium ovatum* El-Gholl, Alfenas, Crous, et Schubert sp.nov., the cause of a leaf spot disease on *Eucalyptus urophylla* in Para, Brazil, is described and is shown to be a pathogen on *Eucalyptus grandis*, *Eucalyptus robusta*, *Eucalyptus tereticornis*, and *Eucalyptus torelliana*.

**Key words:** *Cylindrocladium ovatum*, *Eucalyptus urophylla*, leaf spot disease.

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Les auteurs décrivent le *Cylindrocladium ovatum* N. E. El-Gholl, A. C. Alfenas, P. W. Crous, et T. S. Schubert sp.nov., la cause de la tache noire foliaire sur l'*Eucalyptus urophylla* à Para, au Brésil, et ils démontrent sa pathogénicité sur les *Eucalyptus grandis*, *Eucalyptus robusta*, *Eucalyptus tereticornis*, et *Eucalyptus torelliana*.

**Mots clés :** *Cylindrocladium ovatum*, *Eucalyptus urophylla*, tache foliaire.

[Traduit par la rédaction]

### Introduction

*Cylindrocladium scoparium* and *Cylindrocladium spathulatum* have been reported as foliar pathogens of *Eucalyptus* spp. in Brazil. Following consistent isolations of a *Cylindrocladium* sp. from leaf spots on *Eucalyptus urophylla* S. T. Blake in Para, Brazil, a study was undertaken to identify the *Cylindrocladium* and to determine its pathogenicity on four of several species of *Eucalyptus* experimentally grown in Florida.

### Materials and methods

The fungus was isolated from pieces of excised leaf tissue surface sterilized with 0.3% sodium hypochlorite for 3 min and plated on acidified potato dextrose agar (APDA, pH 4.3). APDA was prepared from the broth of 200 g of freshly peeled, diced, and boiled Irish potatoes (*Solanum tuberosum* L.) supplemented with 20 g dextrose, 1 g KH<sub>2</sub>PO<sub>4</sub>, and 18 g Difco Bacto agar and made up to 1 L with deionized water. After cooling to 50°C, 1.4 mL of 50% lactic acid was added per litre of autoclaved medium to obtain a pH of 4.3. The *Cylindrocladium* from *E. urophylla* (UFV-89) was subcultured on carnation (*Dianthus caryophyllus* L.) leaf – water agar (CL–WA) and peanut (*Arachis hypogaea* L.) stem – water agar (PS–WA) to enhance sporulation (Snyder and Hansen 1947). CL–WA was prepared using dried, sterile carnation leaf segments (5 × 5 mm) on 1.8% water agar as described by Fisher et al. (1982). PS–WA was prepared by pouring cooled (50°C), autoclaved water agar (WA) over dried, propylene oxide fumigated (Hansen and Snyder 1947) pieces of woody peanut stem (4–6 cm long) floating on WA or partially submerged in solidified agar. Single conidium isolates were obtained by the method of Hansen and Smith (1932).

Five *Cylindrocladium* species with vesicle morphology resembling that of the *Cylindrocladium* under study from *E. urophylla* (UFV-89) were chosen: *C. ellipticum* Alfieri, C. P. Seymour & Sobers from *Mahonia bealei* Carr.; *C. scoparium* Morg. from *Eucalyptus cloeziana* F. v. Muell.; *C. floridanum* Sobers & C. P. Seymour from *Prunus persica* (L.) Batsch; *C. crotalariae* (C. A. Loos) D. K. Bell & Sobers; and *C. spathulatum* El-Gholl, Kimbrough, E. Barnard, Alfieri, & Schouties from *Eucalyptus viminalis* Labill. According to Peerally (1991), *C. ellipticum* has essentially the same morphological characters as *Cylindrocladium candelabrum* Viegas and is synonymous with *C. candelabrum*. Likewise, he considered *C. spathulatum* similar to *Cylindrocladium ilicicola* (Hawley) Boedijn & Reitsma and therefore reduced *C. spathulatum* to synonymy with *C. ilicicola*.

Vesicle morphology differences and microconidial production as well as isoenzyme and protein electrophoresis were used as distinguishing characteristics.

For isoenzyme electrophoresis, cultures were grown on potato dextrose agar at 26 ± 1°C in the dark for 4 days. Mycelial agar plugs were taken from the margins of actively growing colonies and transferred to a semisynthetic liquid medium containing 10 g sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.44 mg ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 0.48 mg FeCl<sub>3</sub>, and 0.31 mg MnSO<sub>4</sub> · H<sub>2</sub>O and made up to 1 L with distilled H<sub>2</sub>O. Four agar plugs were placed in each of three 125-mL Erlenmeyer flasks containing 50 mL of the liquid medium and were incubated at 26 ± 1°C in the dark for 7 days. The mycelium was then harvested by suction onto Whatman No. 1 filter paper and rinsed under vacuum with distilled water to remove any residual liquid medium.

For soluble protein extraction, the mycelial cake was placed in a centrifuge tube containing cold acetone in the presence of liquid nitrogen in a Polytron Homogenizer (Brinkmann Instruments, Westbury, N.Y.) for 3–4 min until completely homogenized. Grinding the mycelium in cold acetone was necessary to maximize resolution of

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sodium dodecylsulfate (SDS) proteins of the isolates under study. During grinding, the centrifuge tube containing the sample was kept in an ice bath. The extracts were maintained overnight at 10°C and were centrifuged at 40 000 × g for 30 min at 4°C. The supernatant was collected and stored at -20°C until used. For electrophoresis, protein extracts were mixed in equal amounts of sample buffer (25 mL glycerol, 0.06 M Tris-HCl solution, pH = 6.8, 2.5 mg bromophenol blue dye, and 25 mL distilled water), and 50 µL of each sample was applied on the gel. Discontinuous polyacrylamide gel electrophoresis (PAGE) for native and denatured proteins and isoelectric focusing was performed as described by Alfenas et al. (1984).

Tests were conducted to determine pathogenicity of mass isolate UFV-89 on 2-month-old seedlings of *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus robusta* Sm., *Eucalyptus tereticornis* Sm., and *Eucalyptus torelliana* F. v. Muell. experimentally grown in Florida. The plants were grown from seeds in Metro-Mix Growing Medium 300 (Grace Horticultural Products, W. R. Grace & Co., Cambridge, Mass.) in 15-cm diameter pots. Isolate UFV-89 was cultured on four CL-WA Petri dishes (60 × 15 mm) and on four PS-WA Petri dishes (100 × 15 mm). Cultures were grown at room temperature (25 ± 2°C) under laboratory conditions for 3 days, then were moved to an incubator and grown at 26 ± 1°C under continuous fluorescent lighting (GE F20 T12-CW) at an intensity of approximately 3000 lx for 5 additional days. Conidial suspensions from the eight dishes were combined to prepare the inoculum. The inoculum contained approximately 134 000 conidia/mL. Plants were sprayed with this suspension using 30 mL per plant. There were three replications for each species of *Eucalyptus* used, with an equal number of plants sprayed only with deionized water as controls. After inoculation, plants were individually enclosed in plastic bags (serving as moist chambers) for 3 days at room temperature, then were uncovered and placed on a greenhouse bench for a period of 8 weeks for observation. The temperature of the greenhouse fluctuated from 16 to 34°C; however, plants were mostly exposed to temperatures ranging from 21 to 27°C, and the relative humidity varied from 42 to 100%.

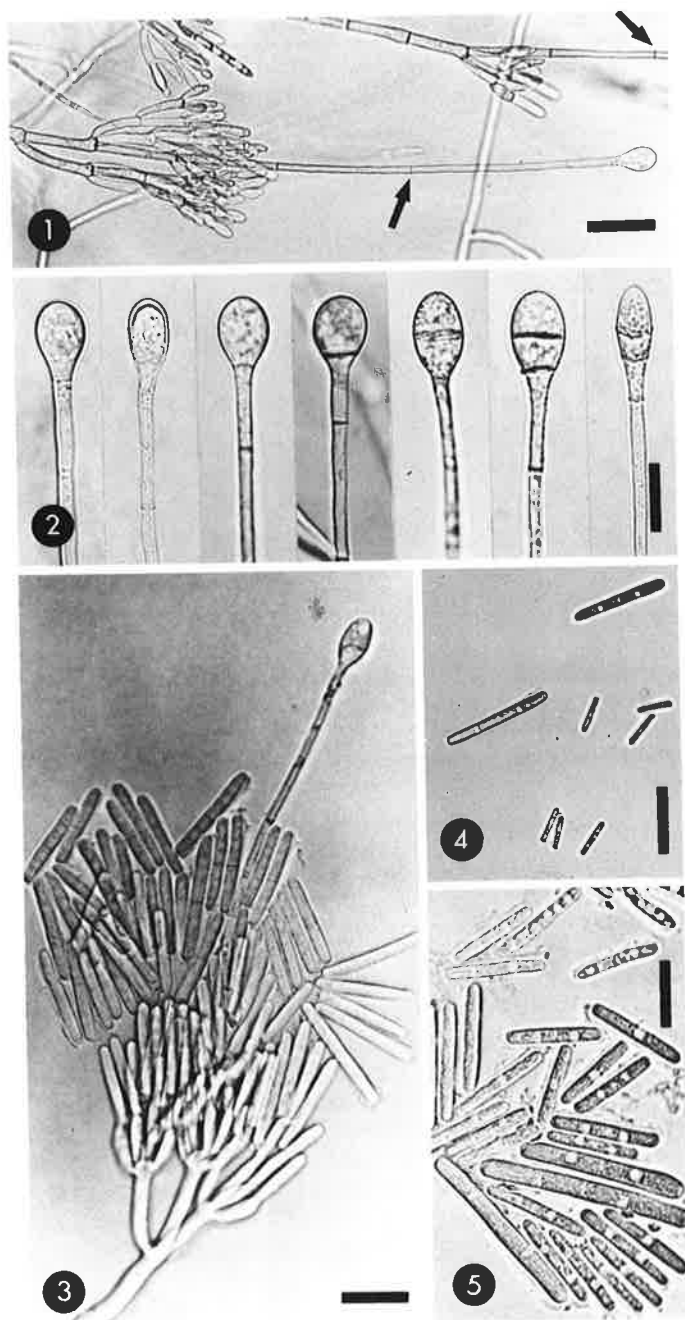
Cultures grown on CL-WA and PS-WA were incubated at room temperature under laboratory conditions and were observed over a 5-week period. Water-mounted conidiophores and conidia were photographed as soon as they were produced. Water-mounted vesicles were photographed when cultures were 1 week old. Conidiophore branches, phialides, and vesicles were measured within 5 min of preparation to avoid swelling of structures. Conidia produced on the peanut stem were mounted in cotton blue lactophenol for measurements. Microscopic examinations and measurements were made at ×400 or ×1000.

## Results and discussion

### Identification

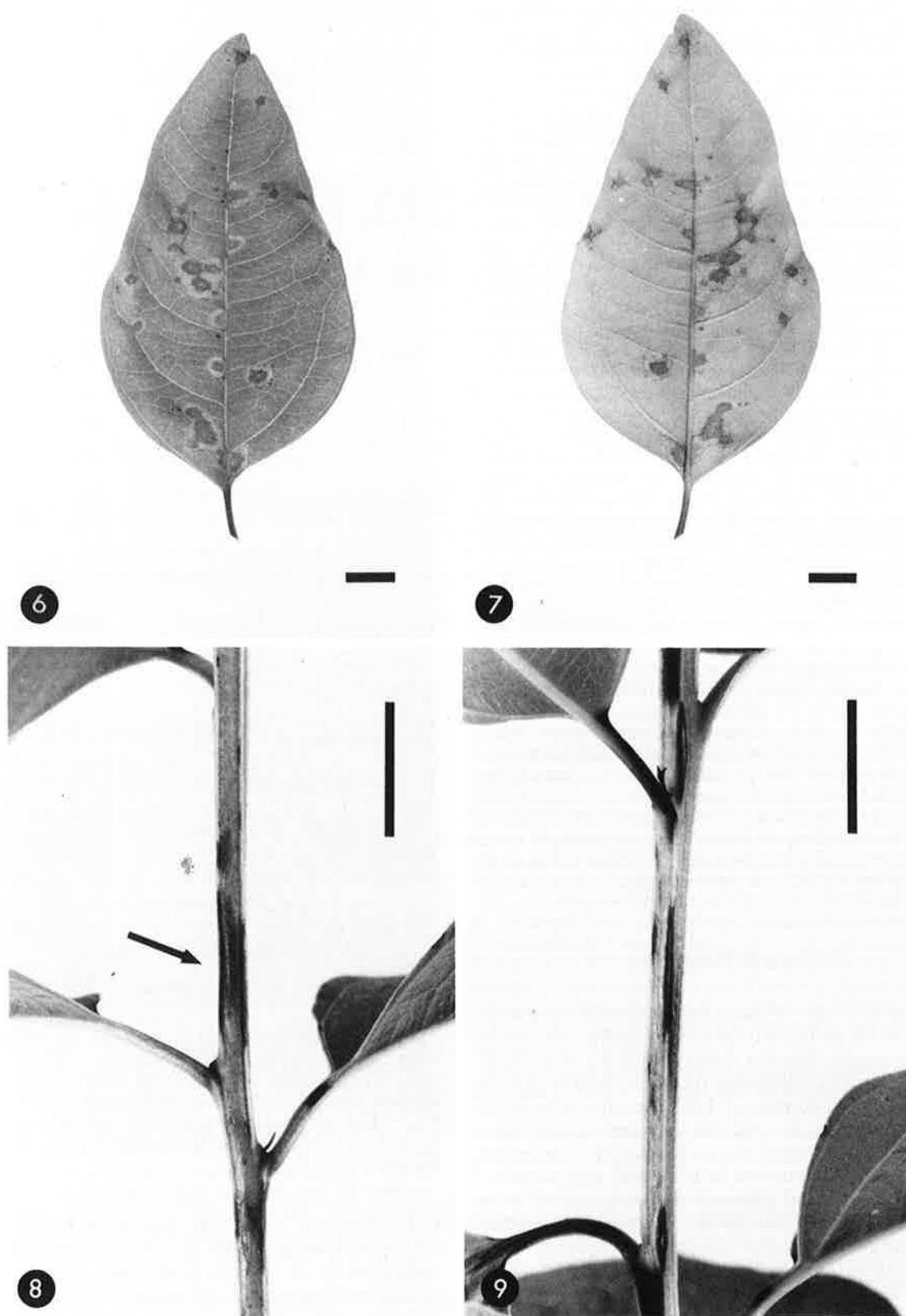
Conidiophores of the *Cylindrocladium* from *E. urophylla* (UFV-89) were borne laterally on a main central axis (stalk) (Fig. 1). The septate filament (extension of the main stalk) terminated in an oval to ellipsoidal (Peerally 1991) to globose or subglobose (Hawksworth et al. 1983; Peerally 1991) apical swelling (vesicle) (Fig. 2), with the dominant vesicle shape oval to ellipsoidal. Lateral filaments arose from some of the branches and terminated in oval to ellipsoidal microvesicles. As many as three lateral filaments per conidiophore were observed. Vesicles were nonseptate, 1-septate, or 2-septate (Fig. 2). In one instance, septate vesicles were produced within 7 days in relatively high numbers (48%) on CL-WA under conditions of this study. Forty percent were 1-septate, 8% were 2-septate, and all others were nonseptate.

The usefulness of vesicle morphology as a taxonomic criterion has been discussed by many authors. Bell and Sobers (1966) and Sobers and Seymour (1967) originally proposed vesicle morphology as a useful feature in their comparison of *C. crotalariae*,



FIGS. 1-5. *Cylindrocladium ovatum*. Fig. 1. Conidiophores with septate filaments (arrows). ×289. Scale bar = 35 µm. Fig. 2. Vesicles. ×578. Scale bar = 17.5 µm. Fig. 3. Microconidiophore bearing nonseptate and 1-septate microconidia and a vesicle. ×578. Scale bar = 17.5 µm. Fig. 4. Macroconidia and microconidia. ×289. Scale bar = 35 µm. Fig. 5. Macroconidia and microconidia. ×578. Scale bar = 17.5 µm.

*C. floridanum*, *C. scoparium*, and others. Hunter and Barnett (1978) and Rossman (1983) considered vesicle morphology too variable to be of any taxonomic value and stressed instead conidial characteristics as of primary taxonomic importance. Peerally (1991) and Uchida and Aragaki (1992), however, recognized that though vesicle morphology could be subject to some variation, it was not so variable as to disqualify it as a valuable character in distinguishing species of *Cylindrocladium*. This claim is substantiated by recognizable differences in soluble



FIGS. 6-9. Disease symptoms caused by *Cylandrocladium ovatum*. Fig. 6. Spots on the upper surface of an artificially inoculated *Eucalyptus robusta* leaf.  $\times 0.8$ . Fig. 7. Spots on the lower surface of the same leaf as in Fig. 6. Fig. 8. Stem girdling (arrow) on an artificially inoculated *Eucalyptus tereticornis* plant.  $\times 2.2$ . Fig. 9. Petiole necrosis and necrotic stem lesions on an artificially inoculated *E. tereticornis* plant.  $\times 2.2$ . Scale bars = 1 cm.

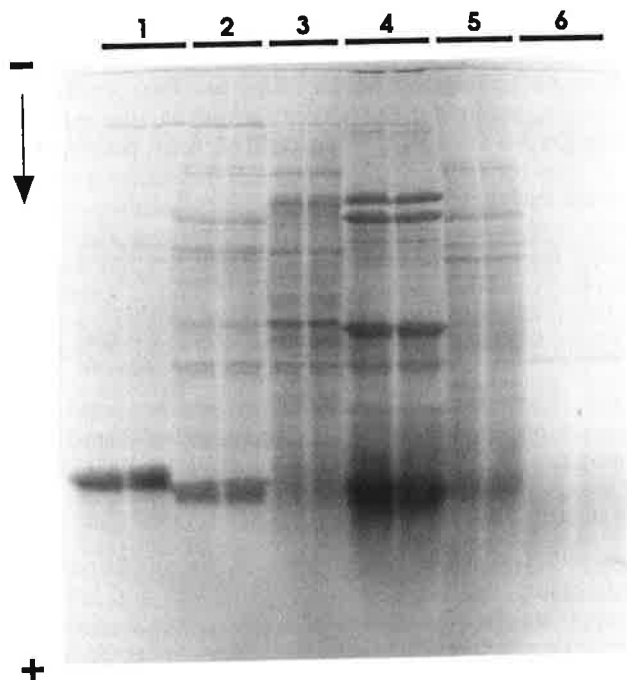


FIG. 10. Comparative banding patterns of *Cylindrocladium* species. From left to right every two lanes represent banding patterns of *C. ellipticum* (lane 1), *C. scoparium* (lane 2), *C. floridanum* (lane 3), *C. crotalariae* (lane 4), *C. spathulatum* (lane 5), and *C. ovatum* (lane 6). SDS proteins on 12–18% polyacrylamide gradient gel electrophoresis (PAGE).

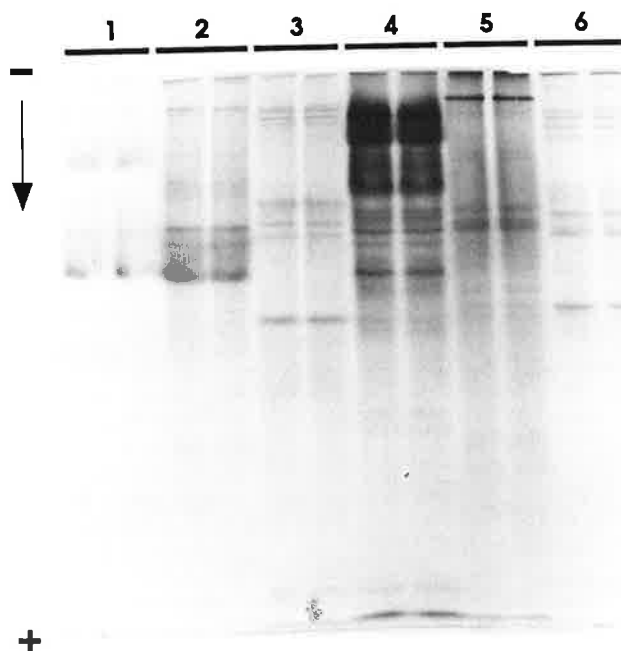


FIG. 11. Comparative banding patterns of *Cylindrocladium* species. From left to right every two lanes represent banding patterns of *C. ellipticum* (lane 1), *C. scoparium* (lane 2), *C. floridanum* (lane 3), *C. crotalariae* (lane 4), *C. spathulatum* (lane 5), and *C. ovatum* (lane 6). Non-SDS proteins on 10% PAGE.

protein and isoenzyme electrophoresis profiles of *Cylindrocladium* species that have superficially similar but distinct vesicle, conidium, and conidiophore morphology. We conclude that all features relating to conidium and conidiophore morphology, including shape and size of the vesicle, are useful and practical taxonomic characters when used judiciously with fresh isolates of *Cylindrocladium* species. Cultures from long-term storage often produce abnormal conidia and vesicles and should not be utilized in systematic and physiological studies.

Primary branches were nonseptate or 1-septate. Of 82 primary branches measured, 70 (85%) were nonseptate and 12 (15%) were 1-septate. Secondary branches were nonseptate (41 were measured). Tertiary branches were nonseptate (nine were measured). Phialides were hyaline, nonseptate, rarely 1-septate, and mostly doliiform (75 were measured). Although collarettes were noted on the microconidial phialides, they were not formed on the macroconidial phialides. On naturally infected *E. urophylla* leaves, the fungus produced straight or slightly curved 1-, 2- and 3-septate macroconidia (mostly 1-septate) that measured approximately  $69.5 \times 4.5 \mu\text{m}$ . In culture (APDA, CL-WA, or PS-WA), however, macroconidia were straight (not curved) and 1-septate. A total of 185 macroconidia produced on CL-WA and PS-WA were measured as well as 145 microconidia. This fungus (UFV-89) produced microconidia on CL-WA and PS-WA. In one case, microconidia were produced on PS-WA as early as 12 days from time of seeding. Microconidia were also observed on CL-WA, 19 days after seeding. Often, it took 3 weeks or longer for microconidia to form. Cultures that were obtained from single microconidia produced a mixture of macroconidia and microconidia (Figs. 4 and 5). Cultures that were obtained from single macroconidia also produced a mixture of both conidial types. Microconidia

were nonseptate or 1-septate (Fig. 3). The microconidiophore also had a septate filament (Fig. 3). The isolates of the five species of *Cylindrocladium* used for comparison were not observed to produce microconidia. No other species of *Cylindrocladium* has yet been characterized as having oval vesicles and a microconidial state as observed in UFV-89.

The *Cylindrocladium* from *E. urophylla* (UFV-89) was deposited in the American Type Culture Collection, Rockville, MD 20852, U.S.A., as ATCC 76225.

Isoenzyme and protein electrophoresis were used as a further tool in distinguishing the *Cylindrocladium* under study (UFV-89) from species of *Cylindrocladium* that show similarities in vesicle morphology, i.e., ellipsoidal to oval (Alfieri et al. 1970; Hawksworth et al. 1983; Peerally 1991; Snell and Dick 1971), spathulate (El-Gholl et al. 1986), or sphaeropedunculate (Bell and Sobers 1966; Snell and Dick 1971; Sobers and Seymour 1967) apical vesicles.

The *Cylindrocladium* from *E. urophylla* (UFV-89) displayed different SDS and non-SDS proteins and  $\alpha$ -isoesterases from those of the five isolates used for comparison. The banding patterns of isolate UFV-89 were different (Figs. 10–12).

Because of fundamental differences in characteristics mentioned above, the *Cylindrocladium* under study is different from all other reported species of *Cylindrocladium*. We propose here the following new species.

*Cylindrocladium ovatum* El-Gholl, Alfenas, Crous, et Schubert sp. nov.

Filamentum septatum, hyalinum, terminans in vesiculam ovoideam usque ad globosam aut subglobosam ( $12.4$ –) $25$ –( $47.5$ )  $\times$  ( $6$ –) $9.7$ –( $14.9$ )  $\mu\text{m}$  ((min.–)med. (–max.)). Vesiculae nonseptatae, 1-septatae, aut 2-septatae. Rami primi non septati, ( $16.8$ –) $27.8$ –( $39.6$ )  $\times$  ( $3.5$ –) $4.6$ –( $6.9$ )  $\mu\text{m}$  aut 1-septati ( $41.6$ –) $59.7$ –( $71.3$ )  $\times$  ( $4.3$ –) $4.8$ –( $5.5$ )  $\mu\text{m}$ ; rami

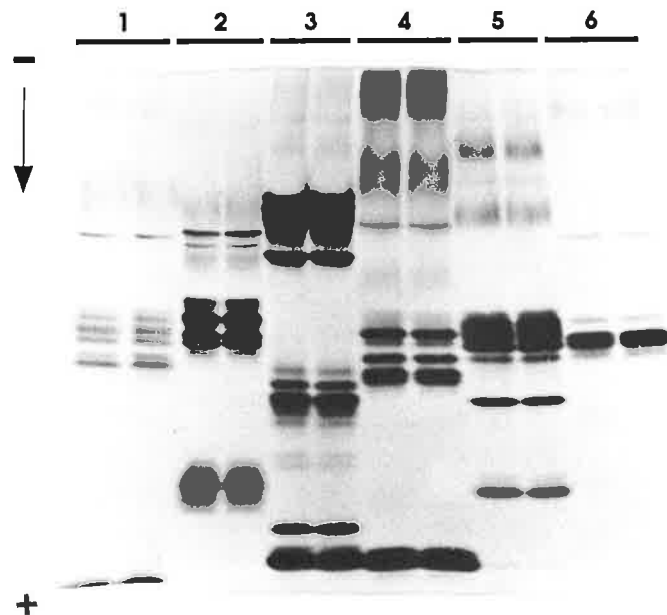


FIG. 12. Comparative banding patterns of *Cyindrocladium* species. From left to right every two lanes represent banding patterns of *C. ellipticum* (lane 1), *C. scoparium* (lane 2), *C. floridanum* (lane 3), *C. crotoniae* (lane 4), *C. spathulatum* (lane 5), and *C. ovatum* (lane 6).  $\alpha$ -Isoesterases on 10% PAGE.

secundi non septati, (10.9–)21(–28.7)  $\times$  (3.8–)4.3(–5.9)  $\mu\text{m}$ ; rami tertii non septati, (13.9–)17.6(–23.8)  $\times$  (3.8–)4.2(–5)  $\mu\text{m}$ . Phialides non septatae, raro 1-septatae, plerumque doliiformes, (8.9–)14.4(–28.7)  $\times$  (3–)4.5(5.5)  $\mu\text{m}$ . Macroconidia cylindrica, contenta granularia, (35.6–)56(–73.3)  $\times$  (3.5–)4.5(–5.8)  $\mu\text{m}$ , pro parte maxima 1-septata. Microconidia cylindrica, contenta granularia, non septata aut 1-septata, (12.9–)21(–31.9)  $\times$  (1.8–)2.5(–3.5)  $\mu\text{m}$ .

HABITAT: In foliis *Eucalypti urophyllae*.

LOCUS HUIUS HOLOTYPEI: Monte Dourado, Para, Brazil.

HOLOTYPE: FLAS F55638, March 1990.

TYPE CULTURAE: ATCC 76225, March 1990.

Filament septate, hyaline, terminating in an oval globose or subglobose vesicle (12.4–)25(–47.5)  $\times$  (6–)9.7(–14.9)  $\mu\text{m}$  ((min.–)med.(–max.)). Vesicles nonseptate, 1-septate, or 2-septate. Primary branches nonseptate, (16.8–)27.8(–39.6)  $\times$  (3.5–)4.6(–6.9)  $\mu\text{m}$  or 1-septate (41.6–)59.7(–71.3)  $\times$  (4.3–)4.8(–5.5)  $\mu\text{m}$ ; secondary branches nonseptate, (10.9–)21(–28.7)  $\times$  (3.8–)4.3(–5.9)  $\mu\text{m}$ ; tertiary branches nonseptate, (13.9–)17.6(–23.8)  $\times$  (3.8–)4.2(–5)  $\mu\text{m}$ . Phialides nonseptate, rarely 1-septate, mostly doliiform, (8.9–)14.4(–28.7)  $\times$  (3–)4.5(–5.5)  $\mu\text{m}$ . Macroconidia cylindrical, granular, (35.6–)56(–73.3)  $\times$  (3.5–)4.5(–5.8)  $\mu\text{m}$ , mostly 1-septate. Microconidia cylindrical, granular, nonseptate, or 1-septate, (12.9–)21(–31.9)  $\times$  (1.8–)2.5(–3.5)  $\mu\text{m}$ .

HABITAT: *Eucalyptus urophylla* leaves.

TYPE LOCALITY: Monte Dourado, Para, Brazil.

HOLOTYPE: FLAS F55638, March 1990.

TYPE CULTURE: ATCC 76225, March 1990.

#### Pathogenicity

Spots were observed on leaves of all inoculated plants within 3 days (Figs. 6 and 7). Midvein necrosis, petiole necrosis, and stem lesions (Figs. 8 and 9) were observed within 3 days only on *E. grandis*, *E. robusta*, and *E. tereticornis*. One *E. tereticornis* plant showed stem girdling (Fig. 8). However, stem girdling did not cause the plant to die even after 8 weeks

postinoculation. Leaf spots on *E. grandis*, *E. robusta*, and *E. tereticornis* were light to medium brown necrotic areas surrounded by light green border (Fig. 6). Two weeks after inoculation, the light green border surrounding the leaf spots became grayish to whitish. No midvein necrosis, petiole necrosis, or stem lesions were observed on *E. torelliana* seedlings. Although leaf spots were not initially surrounded by a border on this host, some developed an inconspicuous light green border 2 weeks after inoculation. Leaf blotches and leaf drop were observed on *E. grandis*, *E. robusta*, and *E. tereticornis* but not on *E. torelliana*. *Cyindrocladium ovatum* was reisolated from leaf spots, leaf blotches, necrotic petioles, necrotic main veins, and necrotic stem lesions.

Artificially infected *E. robusta* leaves were deposited at the University of Florida Herbarium, Department of Plant Pathology, Gainesville, FL 32611, U.S.A., as FLAS F55639.

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