Selective Medium for Quantitative Determination of Microslcerotia of *Cylindrocladium* species in Soil

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ABSTRACT

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A new selective medium was developed for monitoring the population density of microsclerotia of *Cylindrocladium* species in naturally and artificially infested soils. Colonies of *Cylindrocladium* on the new medium were small and surrounded by a distinctive dark halo that made colony recognition easy. Interference from undesired fungi was minimal. The recovery efficiency of the new medium ranged from 68 to 81.7%, depending on the number of microsclerotia added to soil.

Cylindrocladium attacks various economically important plant species in many areas of the world (1,5,11), including Brazil (2,3,7). Information regarding number of individual propagules or distribution of a pathogen in field soils is a prerequisite for predicting the severity of diseases caused by the pathogen. Selective media have been developed for isolation from soil and quantification of *C. scoparium* Morgan (12) and *C. crotalariae* (Loos) Bell and Sobers (6,8–10), but none of these media were satisfactory in preliminary tests for selective isolation and quantification of *Cylindrocladium* spp. from field soils in the Federal District of Brazil. Furthermore, the reported media have a major limitation in that colony recognition of *Cylindrocladium* on soil dilution plates is difficult and time-consuming. Our objective, therefore, was to develop a selective medium that would restrict growth of undesired soil fungi and also allow efficient recognition of *Cylindrocladium* colonies on soil dilution plates.

MATERIALS AND METHODS

Selective medium. The selective medium, designated castorbean agar media (CBA), had the following composition: 15 g of glucose, 0.5 g of yeast extract, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of oxgall, 2.4 mg of 2-(4-thiazolyl)-benzimidazol (thiabendazol), 112 mg of pentachloronitrobenzene, 200 mg of streptomycin sulfate, 200 mg of chloramphenicol, 20 g of agar, and 1,000 ml of castorbean (*Ricinus communis* L.) leaf extract. The castorbean leaf extract was prepared by comminuting in a blender (Walita HL 3229, Walita Eletro Domesticos Ltda, São-Paulo, 01.000 Brazil) 500 g of castorbean leaves with 1 L of a solution containing 8.7 g of sodium chloride and 1.7 g of ascorbic acid. The resultant slurry was passed through a cheesecloth and centrifuged for 20 min at 5,000 rpm, using a GSA rotor of a Sorvall RC-5 centrifuge (DuPont Company, Newtown, CT 06470). The extract thus obtained was mixed with the yeast extract, glucose, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and oxgall and the pH was adjusted to 6.5 with 5% KOH. After the agar was added, the medium was autoclaved at 121 C for 15 min. The fungicides and the antibiotics were added after the medium was cooled to 45 C.

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Collection and processing of soil samples. Soil samples were collected with a hand shovel from a depth of 0–20 cm from randomly determined locations in each of 170 fields in the Federal District of Brazil. The subsamples from each field were placed in polyethylene bags and mixed thoroughly by hand. All field samples were stored at room temperature (24 ± 2 C) until processed (no later than 6 days after collection). Before assay, a subsample was removed for moisture determination, and 20-g portions from each soil sample were separately processed using a method similar to that described by Krigsvold and Griffin (9). Each soil portion was separately blended with 100 ml of sterile water for 2 min at high speed. The blended soil suspension was then washed onto a 420-μm sieve that was placed over a 45-μm sieve. The soil on the sieve was washed thoroughly for 5 min with running tap water. Plant residues and soil particles collected on the two sieves were washed into a 500-ml beaker and treated with 0.25% NaOCl as described by Krigsvold and Griffin (9). The NaOCl was removed by washing the debris suspension on a 44-μm sieve. The debris on the sieve was then washed into a 100-ml beaker and the total volume adjusted to 75 ml. Ten milliliters of this suspension was pipetted into 100 ml of precooled (45 C) selective medium, which was then distributed into 10 petri plates (approximately 10 ml per plate). After 5 days of incubation at room temperature (24 ± 2 C), the *Cylindrocladium* colonies on the dilution plates were counted.

Efficiency of selective medium. To determine the efficiency of the new selective medium, microsclerotia of *C. clavatum* Hodges and May, *C. pteridis* Wof, *C. theae* Loos, *C. quinqueseptatum* Boedijn and Reitsma, *C. peruvianum* Batista, and *C. scoparium* were produced separately in 125-ml Erlenmeyer flasks containing 50 ml of castorbean leaf extract prepared as described. After 4–5 weeks of incubation on a laboratory bench at room temperature (24 ± 2 C), the contents of each flask (mycelium + microsclerotia + castorbean leaf extract) were blended in a blender for 10 min at high speed. The resultant slurry was washed under running tap water on a 80-μm sieve until all mycelial fragments were removed. The microsclerotia were then suspended in water and their number determined by direct plating and by microscopic counting as described by Phipps et al (10).

Air-dried nonsterile field soil samples free of *Cylindrocladium* propagules (confirmed by assaying the soil as described by Almeida et al [4]) were artificially infested separately with each *Cylindrocladium* sp. to be tested to give concentrations of 120, 50, 10, or 5 microsclerotia per gram of soil. Each soil sample was then mixed thoroughly by hand and immediately assayed as described for field soil. Four replicates per concentration per *Cylindrocladium* sp. were tested.

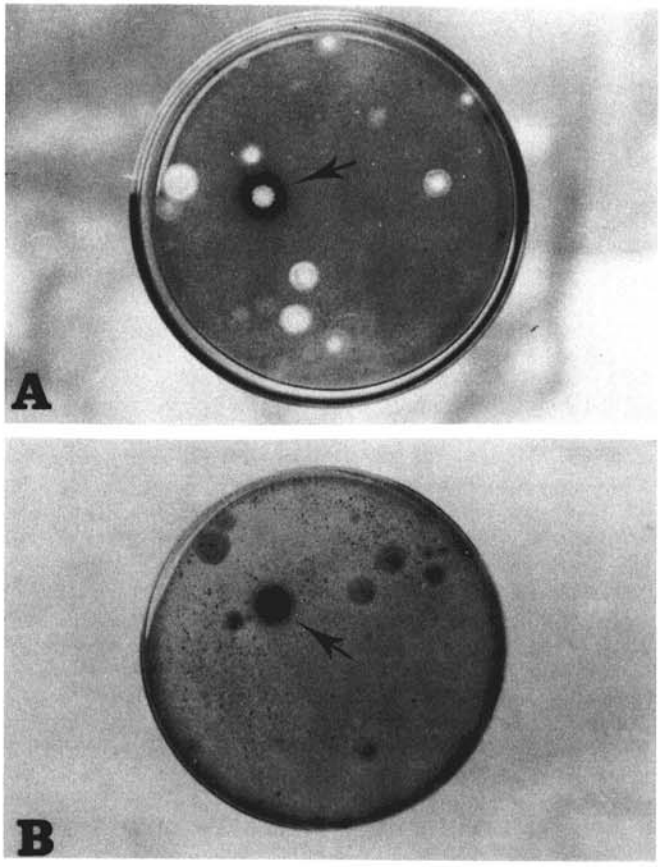

Fig. 1. Typical *Cylindrocladium* colonies on castorbean agar medium. **A,** Upper surface of assay plates. **B,** Bottom surface of assay plates. Arrows indicate *Cylindrocladium* colonies.

RESULTS AND DISCUSSION

Evaluation of selective medium. Colonies of *Cylindrocladium* on the selective medium were small, round, and surrounded by a distinctive dark halo (Fig. 1A). When the dilution plates were viewed from the bottom, *Cylindrocladium* colonies appeared as dark centers surrounded by a clear halo (Fig. 1B). These characteristics made *Cylindrocladium* colonies easily distinguishable from colonies of other fungi. Interference from other fungi was minimal, and none of the undesirable fungi appearing on the new medium developed colonies with a dark halo.

The formation of the dark halo around the *Cylindrocladium* colonies was induced by the castorbean leaf extract. In preliminary experiments, as the castorbean leaf extract concentration in the selective medium decreased, the density of the halo surrounding the *Cylindrocladium* colonies also decreased. Preliminary tests also showed that the dark halo developed only around *Cylindrocladium* colonies originating from microsclerotia. Colonies originating from mycelial fragments or conidia did not produce the dark halo.

When artificially infested soils were assayed, the recovery efficiency of the selective medium varied with the number of microsclerotia added to soil. At 120, 50, 10, and 5 microsclerotia per gram of soil, the mean recovery efficiency of the medium was 81.7, 80.0, 78.0, and 68.0%, respectively. The recovery efficiency of the medium did not differ with the six *Cylindrocladium* spp. tested. The new medium does not differentiate species of *Cylindrocladium*.

Comparisons with previously reported selective media. Comparisons of the CBA medium with previously reported selective media (6,8,9,12) were made by assaying artificially infested soils. The *Cylindrocladium* spp. tested, the microsclerotia concentrations added to soil, and the assaying procedure were the same as described for the efficiency test. The most important difference between CBA medium and those previously reported is that with the new medium, *Cylindrocladium* colonies were readily distinguishable on the dilution plates. Furthermore, identification of *Cylindrocladium* colonies is possible in a relatively short time compared with that of colonies on other media (5 days vs 8–10 days following incubation). Another advantage of the new medium is that colony recognition on soil dilution plates at low soil dilutions (1:1–1:7) is not hindered by crowding and overgrowth of undesired fungi, as occurs with other selective media (8,12).

Assay of naturally infested soils. The population densities of *Cylindrocladium* in 170 fields varied from not detectable to 86 microsclerotia per gram of oven-dried soil. Only 23% of the fields sampled contained detectable populations of *Cylindrocladium*. Isolates recovered from soil were identified as *C. clavatum*, *C. peruvianum*, *C. scoparium*, and *C. pteridis*. The most commonly isolated species was *C. clavatum*. The microsclerotia population of this particular species ranged from nine to 86 microsclerotia per gram of oven-dried soil.

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