Selective Medium for Isolating *Cochliobolus sativus* from Soil

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ABSTRACT

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A plate-count method using a selective medium has been devised for population studies of *Cochliobolus sativus* in field soils. The medium, quarter-strength potato-sucrose agar, contains antibiotics (streptomycin and neomycin) and fungicides (benomyl, captan, and dicloran). It restricts the development of undesirable fast-growing fungi, bacteria, and actinomycetes but permits growth of *C. sativus*. Its efficiency in determining *C. sativus* populations in field soils has been demonstrated using 1:100 soil dilutions in 0.1% water agar.

Additional key words: common root rot, inoculum density

Bipolaris sorokiniana (Sacc. in Sorok.) Shoem. (*Helminthosporium sativum* P. K. & B.; teleomorph *Cochliobolus sativus* (Ito & Kurib.) Drech. ex Dastur) is the principal pathogen involved in common root rot of small grains. It is present in soils mainly as conidia (4,6–9), but it also occurs as mycelium in plant debris (15). Because of their size, conidia can be extracted from soil by flotation utilizing surface tension phenomena and counted directly under the microscope (2,6,16,17). To determine whether the conidia are viable requires an additional test (6). Flotation and viability tests have been used extensively for Canadian soils (3–5,7–9), but without modification they are less efficient for soils of Rio Grande do Sul, Brazil.

The principle of a selective medium for a specific fungus is the selective exclusion of undesirable microorganisms that would otherwise interfere with the growth of that fungus on the isolation plate (22). The following, alone or in combination, may be used to achieve this selectivity: nutritional or osmotic modifications of agar media, additions of fungicides or bactericides, and exposure to ultraviolet radiation.

A dilution-plate method using a selective medium for *C. sativus* in soils would have the advantage of allowing populations of viable propagules to be determined in one operation. Earlier, Henry (15) in 1931 and Anwar (1) in 1949 tried unsuccessfully to quantify *C. sativus* conidia and mycelial fragments by plating soil dilutions. However, with modern fungicides and antibiotics it has been possible to formulate the selective medium described here and to reexamine the possibilities of using the dilution-plate method for enumerating *C. sativus* in soil.

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MATERIALS AND METHODS

Fungicides. Initially, broad-spectrum fungicides were tested, using Greenaway's technique (14), for their ability to inhibit the growth of undesirable fungi without being highly toxic to *C. sativus*. These included benzimidazole compounds such as benomyl, carbendazim, thiabendazole, and thiophanate-methyl. Later, pentachloronitrobenzene (PCNB), dicloran, pyrazophos, and captan were also tested.

Antibiotics. Streptomycin sulfate, chloramphenicol, polymyxin, neomycin, penicillin, griseofulvin, and vancomycin were tested for control of bacteria and actinomycetes. The most promising compounds, such as streptomycin and vancomycin, were then tested at different concentrations (0, 250, 500, and 1,000 μg/ml) against *C. sativus*.

Fungicides and antibiotics were added to cooled molten media after autoclaving.

Best results were obtained when plated media were allowed to dry in a dark cool place for 3–5 days before use because this decreased problems with bacteria and yeast contamination (18,20).

Control of fast-growing fungi. Although adding benzimidazoles to a medium suppressed many fungi, unfortunately they also increased the development of fast-growing fungi such as *Mucor* and *Rhizopus* spp. To eliminate these organisms, different media, carbon sources, water potentials, and ultraviolet radiation were tried. The media were potato-sucrose agar (PSA: sliced potatoes, 140 g; sucrose, 20 g; agar, 15 g), cornmeal agar, and peptone agar (23). Carbon sources (sucrose, lactose, maltose, galactose, glucose, mannitol, and fructose) were tested in water agar and potato agar. Variations of water potential were obtained by adding sodium chloride to PSA. Growth retardants such as sodium metabisulfite, ethanol, oxgall, sodium hypochlorite, and crystal violet were also tried in PSA. The plates were exposed to ultraviolet radiation after they had been inoculated with soil suspensions. The most promising results were obtained with a combination of PCNB, pyrazophos, neomycin, streptomycin, and benomyl in quarter-strength PSA (1/4 PSA). Combinations of the most promising medium (1/4 PSA), fungicides, and antibiotics were first tested using an aqueous suspension of conidia of *C. sativus*, *Rhizopus* spp., and *Mucor* spp. and then by using naturally infested soil. The best combination was compared with the flotation method (16) and used to determine propagule populations in several soils.

Soil dilution. Ten grams of air-dried, sieved soil were added to 100 ml of 0.1% water agar, which holds soil particles in suspension but does not gel (19). This soil suspension was shaken for 20 min before 10 ml was poured into 90 ml of 0.1% water agar and shaken for a further 5 min. A 1-ml aliquot was then pipetted into each plate of selective medium and spread uniformly over the surface by gently shaking and tilting the plate. Four plates for each soil sample were incubated at 24–26 C with a 12-hr photoperiod provided by 20W fluorescent tubes (General Electric) 50 cm above the plates. After 5 days, the colonies were identified and counted under a dissecting microscope.

RESULTS AND DISCUSSION

Benzimidazole compounds were found to decrease slightly the mycelial growth of *C. sativus* (ED_{50} >100 μg/ml). This confirms prior reports (10,12–14,21) of similar effects on dematiaceous fungi. Benomyl and carbendazim were both toxic to a large number of undesirable fungi without seriously affecting *C. sativus*, *Curvularia* spp., or *Alternaria* spp. Nevertheless, *Mucor* and *Rhizopus* spp. were not inhibited at any concentration tested. Many trials were conducted to inhibit the development of these fast-growing fungi. In tests of several naturally infested soils, the best combination based on qualitative criteria (no significant differences) was 1/4 PSA with streptomycin at 500 μg/ml, neomycin at 300 μg/ml, and benomyl at 25 μg/ml. This combination did not suppress two other unidentified fungi in some soils and it became necessary to add captan at 5 μg/ml and dicloran at 3 μg/ml. The final recipe contains 35 g of sliced potatoes, 5 g of sucrose, 15 g of agar, 5,000 μg of streptomycin, 3,000 μg of neomycin, 250 μg of benomyl, 50 μg of dicloran, and 30 μg of captan in 1 L. The presence of streptomycin and neomycin is sufficient to eliminate most bacteria and actinomycetes.

When compared with flotation, the dilution-plate method gave higher spore counts (Table 1). Possibly, Brazilian soils have different clay absorption charges as compared with those of Canadian soils and adsorb *C. sativus* conidia more firmly. The soil-dilution technique may also include other viable propagules such as mycelial fragments and infected plant debris, but these factors are not likely to explain all the differences between the two methods. Even in Canada, the flotation count is generally lower than the plate count. Ledingham and Chinn (16) found that the flotation count averaged 74% of the plate count (range of 59 to 96%). There is a report (11) stating that six to seven times more spores can be obtained using a modification of the flotation method. In practice, when soils contained more than 100 spores per gram, a 1:100 dilution could be used with fewer spores per gram, however, a 1:50 dilution was necessary (Table 2). This technique has been used successfully in studies of *C. sativus* population in soils of southern Brazil.

Table 1. Comparison of *Cochliobolus sativus* propagule numbers determined by flotation or by dilution plates with selective medium

Sampling dates[x]	Flotation count[y]	Plate count[z]	Relative recovery (plate/flotation)
17 March 1981	125	2,750	22.0
24 March 1981	125	2,880	23.0
31 March 1981	160	2,630	16.4
7 April 1981	207	2,500	12.0
Mean	154	2,690	18.4

[x] Air-dried, sieved soil, previously cultivated with barley. Stored in polyethylene bags in the laboratory.
[y] Method of Ledingham and Simmonds (16) but with 5% sodium pyrophosphate; counts made after 24 hr. Mean of four replicates.
[z] Soil dilution of 1:100 in 0.1% water agar. Mean of four replicates.

Table 2. Effect of soil dilution on estimates of *Cochliobolus sativus* propagules in soil using a selective medium

Soil dilution	Colonies/plate	Propagules per gram of soil	Average colony diameter (cm)
1:50	50.5	2,525 a[z]	0.49 a
1:100	28.0	2,800 a	0.65 a
1:200	14.0	2,750 a	0.87 b
1:400	7.0	2,800 a	0.88 b

[z] Values followed by the same letter are not statistically different at $P = 0.05$, Duncan's multiple range test. Measurements at 6 days with four replicates per treatment.

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