A Selective Medium for Assay of Colletotrichum coccodes in Soil

J. D. Farley

Assistant Professor, Ohio Agricultural Research and Development Center, Wooster 44691.

The author thanks Carol Jaberg for her assistance.

Published with the approval of the Director of Ohio Agricultural Research and Development Center, Wooster 44691 as Journal Article No. 31-72.

Accepted for publication 19 May 1972.

ABSTRACT

A selective medium was developed for the isolation and enumeration of *Colletotrichum coccodes* from soil. The medium consisted of the following ingredients/liter: 10 g polygalacturonic acid; 1.5 g KH_2PO_4; 4 g K_2HPO_4; 25 ml soil extract; 17 g Difco agar; and the following antimicrobial agents added after autoclaving: 0.1 g pentachloronitrobenzene; 0.1 g benomyl; 0.1 g streptomycin sulfate; 0.1 g tetracycline HCl, and 0.1 g chloramphenicol. The medium was used to recover *C. coccodes* from artificially and naturally infested soil and from overwintered tomato skins. Its selectivity is due to the selective inhibition of microorganisms by antimicrobial agents and the development of distinctive, brown-pigmented sclerotial colonies of *C. coccodes*.

Phytopathology 62:1288-1293.

Additional key words: tomato anthracnose, nystatin.

Anthracnose, caused by *Colletotrichum coccodes* (Wallr.) Hughes, is a major disease of canning tomatoes in the North Central and Northeastern sections of the United States. Illman et al. (7) and Chesters & Hornby (3) have suggested that the tomato anthracnose pathogen is closely related to *C. atramentarium*, a well-known soil organism which causes black dot of potatoes (14) and tomato root rot under greenhouse conditions (10). This suggestion is of more than taxonomical interest, since the knowledge acquired by *C. atramentarium* studies may be applied to tomato fruit anthracnose and, as Ludwig (9) suggested, "the philosophy to attack may be modified by the recognition of it as a well-known soil organism". The author has initiated research to study the behavior of *C. coccodes* in soil. A selective medium was developed which is the subject of this paper. An abstract on the selective medium has been published (4).

The development of agar media for the selective

isolation of specific fungi has generally been based on the selective exclusion of contaminating microorganisms and/or a selective enhancement of the fungus to be isolated (16). Preliminary observations indicated that sclerotia were a consistent and distinctive morphological feature of *C. coccodes* on agar, and that if their development were enhanced they would aid in selective detection of *C. coccodes* from other soil microorganisms. Such an approach had been utilized in the development of *Verticillium albo-atrum* selective media where identification of *V. albo-atrum* colonies was dependent on enhancement of microsclerotial formation (6, 11, 12). Preliminary tests indicated that the *Verticillium* selective medium developed by Menzies & Griebel (11) and modified by Green & Papavizas (6) showed promise as a starting point in developing a medium for recovery of *C. coccodes* from soil.

MATERIALS AND METHODS.—*Modification of the Verticillium medium.*—A *Verticillium* selective medium (11) containing the following ingredients/liter: 4.0 g K_2HPO_4, 1.5 g KH_2PO_4, 25 ml soil extract, 50 mg streptomycin, 50 mg chlortetracycline, 50 mg chloramphenicol, and 15 g agar, was modified by the addition of polygalacturonic acid (PGA) and antimicrobial agents. Soil extract was prepared (1) from Marengo silty clay loam. PGA was added to the *Verticillium* medium at concentrations of 0, 2, and 10 g/liter. Plates were seeded with three sclerotia of *C. coccodes*/plate, and sclerotial production was observed after 10 days' incubation at 24 C. The medium was adjusted to pH 5.0 with NaOH prior to autoclaving.

To test the effect of antimicrobial agents on linear growth of *C. coccodes*, fresh preparations of these agents (Table 1) were added to a control medium containing the following ingredients/liter: 4 g K_2HPO_4, 1.5 g KH_2PO_4, 25 ml soil extract, and 10 g PGA. Media containing these ingredients will be referred to as the basal medium in the text. All antimicrobial agents were prepared in aqueous suspensions or solutions and added after the medium had been autoclaved and cooled to 50 C. Pentachloronitrobenzene (PCNB) suspensions were prepared by dissolving 0.1 g analytical grade PCNB in 1 ml acetone and adding the acetone solution of PCNB to 100 ml of sterile distilled water to which 0.05 ml of a wetting agent, Triton X-100 (alkyl phenoxy polyethoxy ethanol, Rohm and Haas Co., Philadelphia, Pa.), had been previously added (5). Benomyl suspensions were prepared by addition of 70% wettable powder benomyl to sterile distilled water containing 0.05% Triton X-100. All other antimicrobial agents were added directly to distilled water. Media was delivered at a rate of 15 ml/plate and seeded with 5-10 sclerotia of four *C. coccodes* isolates. The average radial colony growth in mm was measured after 7 days' incubation at 24 C and expressed as percentage growth obtained on the control medium.

Soil and inoculum.—Four isolates of *C. coccodes* were used: C4 and C7 obtained by the author from ripe tomato fruit in Ohio and C9 and C17 from tomato fruit obtained from T. H. Barksdale, USDA, ARS, Beltsville, Md. Two nonsterilized Ohio soils were used, Wooster silt loam and Marengo silty clay loam.

C. coccodes sclerotia were collected from 1- to 2-month-old cultures grown on potato-dextrose agar (PDA) covered with uncoated cellophane. Sclerotia were aseptically scraped off the cellophane, ground in a Waring Blendor with water and fine mesh sand, and washed through a series of graded 60-, 100-, and

TABLE 1. Effect of antimicrobial agents on linear growth of four isolates of *Colletotrichum coccodes*

Antimicrobial agent	Active ingredient mg/liter	Linear growth of isolates (expressed as % of that obtained on basal medium[a])			
		C4	C7	C9	C17
Streptomycin sulfate	100	88	96	104	94
Tetracycline HCl	100	75	88	86	76
Chloramphenicol	100	96	101	90	102
Cycloheximide	2	117	141	113	130
Cycloheximide	10	72	82	94	89
Nystatin	100 units/ml	92	101	95	88
PCNB[b]	10	63	64	57	49
PCNB	100	45	48	56	48
PCNB	200	32	32	38	35
Benomyl	10	62	83	75	69
Benomyl	100	40	57	52	47
Thiabendazole[c]	10	67	95	80	74
Thiabendazole	100	15	26	32	17
Pimaricin	2	0	0	0	0

[a]Basal medium consisted of the following/liter: 10.0 g polygalacturonic acid; 4.0 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 17 g Difco agar; adjusted to pH 5.0 prior to autoclaving.

[b]Pentachloronitrobenzene.

[c]2-(4-Thiazolyl)benzimidazole.

120-mesh sieves. Sclerotia were collected on the 120-mesh sieve.

Recovery efficiency of the medium was tested by a seeding with diluted soil suspensions from soil artificially infested with *C. coccodes* sclerotia. Sclerotia were thoroughly mixed with air-dried soil at levels of 100, 1,000, or 10,000 sclerotia/g of oven-dry soil. Soil was moistened to 50% field capacity after infestation. Soil suspensions were prepared by adding sclerotial-infested soil to distilled water and mixing in an Omni-Mixer at ca. 4,000 rpm for 1 min. Final soil dilutions were placed in a beaker, and 1-ml amounts were removed from the beaker while the liquid was under continuous agitation by a magnetic stirrer. Each treatment consisted of three replications and each replication of 6-10 plates. All experiments were performed twice. Colonies were counted after 15-20 days incubation at 24 C.

RESULTS.—*Enhancement of sclerotia by PGA.*—Green & Papavizas (6) reported that addition of 2 g/liter of PGA to Menzies and Griebel's *Verticillium* medium (11) increased *Verticillium* microsclerotial production and thus enhanced distinction of *Verticillium* colonies from other fungi in soil-dilution plates. The effects of PGA on sclerotial development of *C. coccodes* in Menzies and Griebel's medium were studied. As PGA concentrations were increased, sclerotial production increased (Fig. 1), optimum concentration being 10 g/liter.

Attempts were made to recover *C. coccodes* from artificially infested soil with the *Verticillium* selective medium containing 10 g PGA/liter. Although *C. coccodes* was readily recovered from artificially infested soils at dilutions of 1:500 or 1:1,000, the number of fungal and bacterial contaminants at lower soil dilutions (1:50-1:100) prevented growth of recognizable colonies of *C. coccodes* (Fig. 2-A). Therefore, a search was made for antimicrobial agents which could be added to the medium to reduce contaminants.

Tolerance of C. coccodes to antimicrobial agents.—Table 1 reports data on the effect of antimicrobial agents on linear growth of *C. coccodes* colonies which arose from sclerotial-seeded plates. Of nine antimicrobial agents tested, pimaricin was the most inhibitory. Thiabendazole [2-(4-Thiazolyl)benzimidazole] and cycloheximide inhibited sclerotial formation, and thus were not tested further. *C. coccodes* was sufficiently tolerant to PCNB at 100 μg/ml, benomyl at 100 μg/ml, nystatin at 100 units/ml, and the three antibiotics tested at 100 μg/ml to warrant their incorporation in a selective medium. The effects of the antimicrobial agents were also tested on linear growth of colonies derived from conidia [from 4-day-old V-8 plates (2)] and from 3-mm discs from 4-day-old cultures on PDA. Results were similar to those in Table 1.

Synergism between PCNB and nystatin.—Recovery of *C. coccodes* from artificially infested soil with the basal medium containing the following antimicrobial agents/liter: 100 mg benomyl; 100 mg PCNB; 100 units/ml nystatin; 100 mg streptomycin; 100 mg tetracycline HCl; and 100 mg chloramphenicol was very low. An additive or synergistic toxic effect on *C. coccodes* between the antimicrobial agents was suspected. It was observed that a combination of nystatin and PCNB reduced linear growth of *C. coccodes* significantly and resulted in poor recovery from soil (Table 2). Nystatin was thus excluded from the medium.

TABLE 2. Effect of nystatin and pentachloronitrobenzene (PCNB) on linear growth and recovery of isolate C4 of *Colletotrichum coccodes* from sclerotial-infested soil

Treatment[a]	Linear growth (expressed as % of growth on control[b])	No. *C. coccodes* recovered[c]
Control	100	50
Control + PCNB	46	680
Control + PCNB + nystatin	27	15
Control + nystatin	102	23

[a]Concentration of PCNB and nystatin was 100 μg/ml and 100 units/ml, respectively.

[b]Control medium consisted of the following/liter: 10.0 g polygalacturonic acid; 4.0 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 100 mg streptomycin sulfate; 100 mg tetracycline HCl; 100 mg chloramphenicol; and 17.0 g Difco agar.

[c]Soil was infested with sclerotia (1,000/g oven-dry wt). Soil dilution was 1:100.

Effect of antimicrobial agents on isolation of C. coccodes from artificially infested soil.—The effects of PCNB, benomyl, and tetracycline in the basal medium supplemented with streptomycin and chloramphenicol on recovery of four isolates of *C. coccodes* from two soils infested with sclerotia (1,000/g oven-dry soil) were tested. Maximum recovery occurred when the basal medium was supplemented with PCNB, benomyl, and the three antibiotics (Table 3). Representative plates in Fig. 2 show the effects of PCNB, benomyl, and tetracycline in soil dilution plates seeded with 1:100 dilution of artificially infested Wooster silt loam. *C. coccodes* colonies are the black colonies. As illustrated, they are readily differentiated from other microorganisms.

PCNB and benomyl were very effective in reducing numbers of fungal contaminants. The efficiency of benomyl to reduce numbers of fungi in the *C. coccodes* selective medium was studied further. Soil suspensions from Marengo and Wooster soils were plated onto the basal medium containing the three antibiotics and varying concentrations of benomyl. Benomyl substantially reduced numbers of fungal colonies on soil dilution plates (Fig. 3). Numbers were reduced 90% in Marengo soil and 65% in Wooster soil by 10 μg/ml benomyl, and 100% and 90%, respectively, at 100 μg/ml.

The effects of tetracycline were significant. Its incorporation resulted in development of distinct,

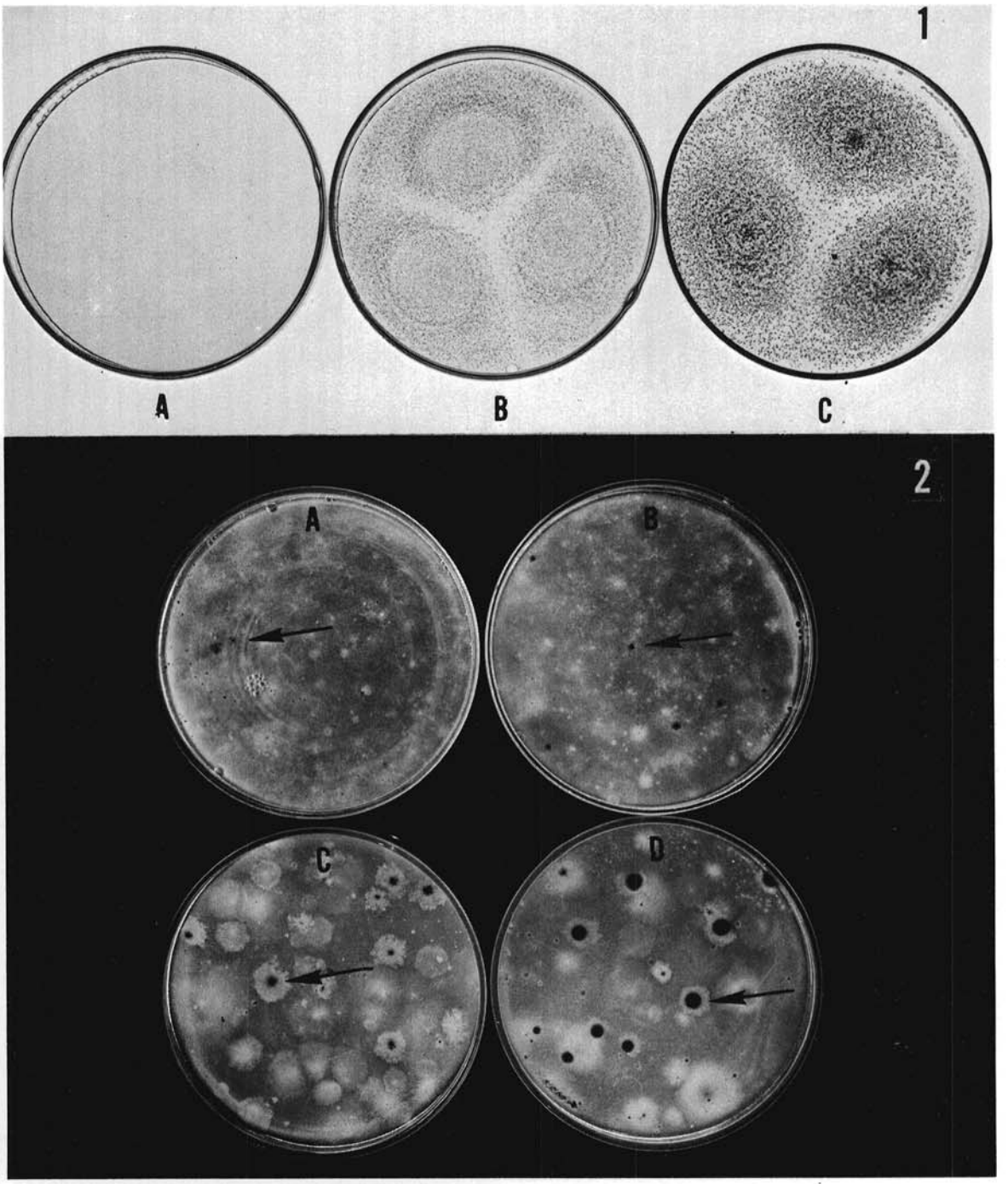

Fig. 1-2. 1) Effect of various concentrations of polygalacturonic acid (PGA) in the basal medium on sclerotial development of *Colletotrichum coccodes*. **A)** Basal medium; **B)** basal plus 2 g PGA/liter; **C)** basal plus 10 g PGA/liter. Plates were seeded with three sclerotia of isolate C4 and incubated 10 days at 24 C. **2)** Dilution plates from Marengo silty clay loam infested with 1,000 sclerotia/g of isolate C4 (soil dilution 1:100). **A)** Basal medium with 100 mg streptomycin sulfate and 100 mg chloramphenicol/liter; **B)** ingredients of A plus 100 mg pentachloronitrobenzene/liter; **C)** ingredients of B plus 100 mg benomyl/liter; **D)** ingredients of C plus 100 mg tetracycline HCl/liter. Arrows show *Colletotrichum coccodes* colonies. Plates were incubated 15 days at 24 C.

TABLE 3. Effect of antimicrobial agents on numbers of *Colletotrichum coccodes* recovered from two soils artificially infested with sclerotia of four isolates

Treatment	No. (100/g) recovered from two soils infested[a] with specified isolate							
	Marengo silty clay loam				Wooster silt loam			
	C4	C7	C9	C17	C4	C7	C9	C17
Control[b]	57	60	70	124	13	5	17	15
Control + PCNB[c]	58	37	58	108	67	95	8	73
Control + PCNB + benomyl	65	92	122	135	78	93	65	88
Control + PCNB + benomyl + tetracycline HCl	80	137	133	130	90	110	128	143

[a]Soils were infested with sclerotia (1,000/g oven-dry wt). Soil dilutions were 1:100.

[b]Control medium consisted of the following per liter: 10.0 g polygalacturonic acid; 4.0 g K_2HPO_4; 1.5 g KH_2PO_4; 25 ml soil extract; 100 mg streptomycin sulfate; 100 mg chloramphenicol; and 17 g Difco agar.

[c]Concentrations of pentachloronitrobenzene (PCNB), benomyl, and tetracycline HCl were 100 μg/ml.

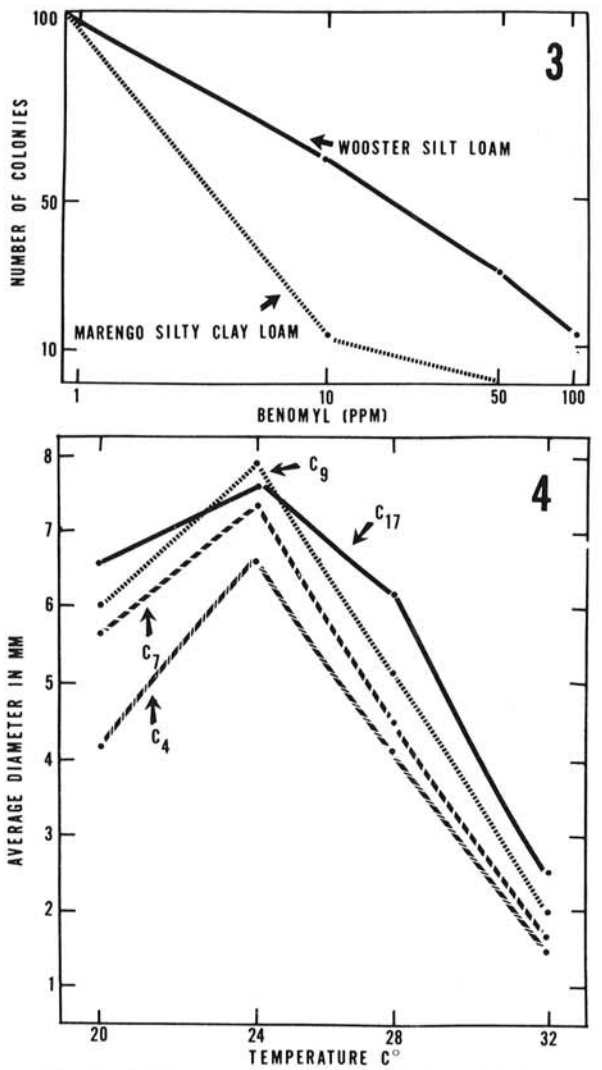

Fig. 3-4. 3) Number of fungal colonies from diluted soil suspensions plated on the basal medium amended with various concentrations of benomyl. For comparisons, numbers of colonies were adjusted on the basis of 100 colonies/plate without benomyl. **4)** Growth of four isolates of *Colletotrichum coccodes* on the selective medium after 7 days' incubation at various temperatures.

sclerotial *C. coccodes* colonies which contrasted sharply with background microflora (Fig. 2). Preliminary results indicated that the effects of tetracycline are indirect. Sclerotial enhancement only occurred on soil dilution plates (dilutions of 1:10 to 1:500), and did not occur in pure culture or on soil dilution plates having few contaminating organisms (i.e., dilutions above 1:500).

Incubation temperature with complete medium.—Results from previous experiments indicated that a medium with the following ingredients/liter was the most suitable for isolating *C. coccodes* from soil: 1.5 g KH_2PO_4; 4.0 g K_2HPO_4, 25 ml soil extract; 17 g Difco agar; 10 g PGA; 100 mg streptomycin sulfate; 100 mg tetracycline HCl; 100 mg chloramphenicol; 100 mg PCNB; and 100 mg benomyl. Tests were conducted to find the incubation temperature for maximum growth of *C. coccodes* in this medium. The medium was seeded with sclerotia (4-6/plate) of four isolates and incubated 7 days at temperatures of 20, 24, 28, and 32 C. Optimum growth of all four isolates occurred at 24 C (Fig. 4). Recovery of *C. coccodes* from artificially infested Marengo and Wooster soils also was best when soil dilution plates were incubated at 24 C.

Recovery with complete medium.—Sclerotia of *C. coccodes* isolates, C4, C7, C9, and C17 were added to nonsterilized Wooster and Marengo soils at concentrations of 100, 1,000, and 10,000 sclerotia/g of oven-dry soil. Assays made 1 hr and 14 days after the sclerotia and soil were mixed indicated that recovery was good (Table 4). Percent ranged from a low of 59% to a high of 146%.

Use of medium in the field.—Preliminary tests indicated that the medium described herein was useful in monitoring field populations as low as three propagules of *C. coccodes*/g of oven-dry soil. *C. coccodes* also was recovered from overwintered tomato skins from four Ohio tomato fields. Colonies typical of *C. coccodes*, developing on the medium seeded with dilutions from naturally infested soil or from tomato skins, were subcultured on potato-dextrose agar (PDA). PDA colonies were examined microscopically and found to be morphologically identical to *C. coccodes*. Uninjured tomatoes were atomized with conidia from the PDA

TABLE 4. Recovery of *Colletotrichum coccodes* from Wooster soil infested with sclerotia of four isolates

Sclerotia added/g of oven-dry soil	Recovery[a] after soil infestation with specified isolate							
	1 hr[b]				14 days[b]			
	C4	C7	C9	C17	C4	C7	C9	C17
100	93	117	63	102	134	119	85	161
1,000	1,420	977	595	977	988	960	493	1,215
10,000	11,730	10,540	9,180	9,180	14,620	11,900	8,300	11,900

[a]Dilution of 1:100 was used for recovery from soil infested with 100 and 1,000 sclerotia/g, and 1:1,000 for that with 10,000.

[b]Time after infestation.

colonies and incubated at high relative humidity. Ninety-100% of the colonies produced typical anthracnose tomato lesions, thus ensuring a correct identification of *C. coccodes* colonies on the selective medium.

DISCUSSION.—The *Verticillium* medium of Menzies & Griebel (11) was used as a starting point to develop the selective medium for recovery of *C. coccodes* from soil, and was amplified as needed to exclude contaminating organisms and enhance sclerotial formation of *C. coccodes*. PCNB commonly has been added to selective media to reduce numbers of fungi other than the target pathogen (8, 13, 15). In addition to this usefulness in the *C. coccodes* selective medium, PCNB amendment resulted in a compact-sclerotial colony which aided considerably in the identification of *C. coccodes* colonies from other fungi on soil dilution plates. The increase in recovery efficiency of *C. coccodes* when benomyl was added probably can be attributed to this significant reduction of fungal contaminants. Benomyl should be useful in other selective media.

All ingredients in the selective medium are defined chemically except agar and soil extract. Unfortunately, the use of these ingredients cannot be avoided without detriment to the medium's performance. Tests with the selective medium with and without soil extract indicated that it was essential for development of the type of *C. coccodes* colony desired.

Success of the medium is largely based on the development of a brown pigmented-sclerotial *C. coccodes* colony. The medium is not useful for isolating nonsclerotial fungi which may occasionally produce tomato anthracnose in the field, such as *Glomerella cingulata*. A survey of *Colletotrichum* spp. causing tomato fruit rot in Ohio (J. D. Farley, *unpublished data*) and by Illman et al. (7) in Canada and the north central and eastern tomato canning belt indicated that essentially all *Colletotrichum* spp. collected were sclerotial types. It is thus presumed that the selective medium described in this paper will be useful in the monitoring of field populations of *Colletotrichum* spp. in these areas.

LITERATURE CITED

1. ALLEN, O. N. 1957. Experiments in soil bacteriology [3rd ed.]. Burgess Publ. Co., Minneapolis, Minn. 117 p.
2. BARKSDALE, T. H. 1967. Light-induced in vitro sporulation of Colletotrichum coccodes causing tomato anthracnose. Phytopathology 57:1173-1175.
3. CHESTERS, C. G. C., & D. HORNBY. 1965. Studies on Colletotrichum coccodes. I. The taxonomic significance of variation in isolates from tomato roots. Trans. Brit. Mycol. Soc. 48: 573-581.
4. FARLEY, J. D. 1971. A selective medium for assay of Colletotrichum coccodes in soil. Phytopathology 61:891 (Abstr.).
5. FARLEY, J. D., & J. L. LOCKWOOD. 1968. The suppression of actinomycetes by PCNB in culture media used for enumerating soil bacteria. Phytopathology 58:714-715.
6. GREEN, R. J., JR., & G. C. PAPAVIZAS. 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of Verticillium albo-atrum in soil. Phytopathology 58:567-570.
7. ILLMAN, W. I., R. A. LUDWIG, & JOYCE FARMER. 1959. Anthracnose of canning tomatoes in Ontario. Can. J. Bot. 37:1237-1246.
8. KERR, A. 1963. The root rot-Fusarium wilt complex of peas. Australian J. Biol. Sci. 16:55-69.
9. LUDWIG, R. A. 1960. Host pathogen relationships with the tomato anthracnose disease. Proc. Campbell Plant Sci. Seminar. Camden, N.J. p. 37-56.
10. MAC NEILL, B. H. 1955. Colletotrichum root rot of greenhouse tomatoes. Plant Dis. Reptr. 39:45-46.
11. MENZIES, J. D., & G. E. GRIEBEL. 1967. Survival and saprophytic growth of Verticillium dahliae in uncropped soil. Phytopathology 57:703-709.
12. NADAKAVUKAREN, M. J., & C. E. HORNER. 1959. An alcohol agar medium selective for determining Verticillium microsclerotia in soil. Phytopathology 49:527-528.
13. NASH, SHIRLEY M., & W. C. SNYDER. 1962. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. Phytopathology 52:567-572.
14. SCHMIEDEKNECHT, M. 1956. Untersuchung des Parasitismus von Colletotrichum atramentarium (B. et Br.) Taub. an Kartoffelstanden (Solanum tuberosum L.). Phytopathol. Z. 26:1-30.
15. SINGH, R. S., & J. E. MITCHELL. 1961. A selective method for isolation and measuring the population of Pythium in soil. Phytopathology 51:440-444.
16. TSAO, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.