

Improved Selective Media for Estimating Populations of *Thielaviopsis basicola* in Soil on Dilution Plates

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ABSTRACT

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Modifications were made of Papavizas' VDYA-PCNB medium that increased its selectivity for *Thielaviopsis basicola*. Glucose was omitted, chloramphenicol and penicillin were substituted for streptomycin and chlortetracycline, and the concentrations of nystatin and PCNB were increased. These changes resulted in increased recovery of *T. basicola* from artificially or naturally infested soils, in reduced numbers of bacterial and fungal

contaminants, and made possible enumeration of *T. basicola* in soil amended with plant residues. The modified medium contained, per liter, 200 ml V-8 juice, 2 g yeast extract, 20 g agar, 1 g pentachloronitrobenzene, 1 g oxgall, 50 mg nystatin, 250 mg chloramphenicol, and 60 mg K penicillin G. The pH was adjusted to 5.2. Carrot root extract was suitable as V-8 juice as a base for the medium.

Additional key word: soybean.

The "most probable number" method (3) has been very useful in estimating populations of soil-borne pathogenic fungi, including *Thielaviopsis basicola*. However, despite careful standardization in procedures, wide variation among replications is not uncommon (9). Subsequently, two selective agar media, Papavizas' VDYA-PCNB (5) and Tsao's modified RB-M2 (8) were developed, and found suitable for enumeration and isolation of *T. basicola* from soil.

We found that VDYA-PCNB medium gave satisfactory results for some soils, but not for others. Moreover, it was not useful with soils having high microfloral populations such as existed in alfalfa-amended soil. The RB-M2 medium was less satisfactory than VDYA-PCNB. Since soil amendment with organic materials was being studied as a possible means of controlling *T. basicola* root rot in soybean (2), studies were undertaken to develop a medium with improved selectivity for estimating population levels of this fungus.

MATERIALS AND METHODS

The fungus.—*Thielaviopsis basicola* (Berk. & Br.) Ferr. isolates 157 and 170 were isolated previously from soybean fields in southwest Michigan. Endoconidia were obtained from 6-day-old cultures of the fungus on Czapek's agar supplemented with 5 g yeast extract per liter. Single-celled chlamydospores partially free of endoconidia were obtained from 4-week-old cultures by the method of Papavizas and Adams (6). The remaining endoconidia were removed by 6-10 centrifugations for 20

seconds each at 1,500 rpm (Chen and Lockwood, *unpublished*). The chlamydospore chains were broken by grinding the concentrated chlamydospore suspension in a glass tissue homogenizer. The ground suspension was filtered through four layers of cheesecloth to remove any residual hyphae. The concentrations of endoconidia or chlamydospores used in various tests were determined by hemacytometer counts.

Media.—Papavizas' VDYA-PCNB medium (5) and Tsao's modified RB-M2 medium (8), both previously described selective media for *T. basicola*, were used. We modified Papavizas' medium in various ways to improve its selectivity. Chloramphenicol and K penicillin G suppressed bacterial contaminants more effectively than streptomycin sulfate and chlortetracycline-HCl (present in the VDYA-PCNB medium), and did not decrease the number of *T. basicola* colonies recovered. Similarly, higher concentrations of PCNB and nystatin more effectively suppressed undesired fungi. Deletion of glucose also resulted in fewer bacterial and fungal contaminants.

From these preliminary studies, two modified media were selected for comparison with the original VDYA-PCNB medium. One of the media (TBM-V8) contained, per liter: 200 ml V-8 juice (Campbell Soup Co.) (as in the original medium), 20 g agar, 2 g yeast extract, 1 g pentachloronitrobenzene (PCNB, technical material or 75% wettable powder), 1 g oxgall, 50 mg nystatin, 250 mg chloramphenicol, and 60 mg K penicillin G. In the other medium (TBM-C), all constituents were the same except that 970 ml of an extract of carrot root prepared by autoclaving 200 g peeled carrot root slices in 1 liter of

TABLE 1. Comparison of three selective media for recovery of *Thielaviopsis basicola* from natural Conover loam soil infested with endoconidia of two isolates

| Medium | Propagules ($\times 10^3$)/g soil ^a | |
|------------------|--|-------------|
| | Isolate 157 | Isolate 170 |
| VDYA-PCNB | 39.3 | 37.4 |
| TBM-V8 | 42.5 | 45.4 |
| TBM-C | 48.0 | 46.5 |
| LSR ^b | 6.0 | 6.3 |

^aSoil dilution of 10^{-3} was used for recoveries; values are means of 10 petri plate replications. Inoculum density was approximately 50×10^3 endoconidia/g of oven-dry soil. Germination was 96% in carrot extract.

^bLeast significant range ($P = 0.05$) according to Tukey's 'w' procedure.

distilled water was substituted for the V-8 juice. Carrot extract supported 96-98% germination of endoconidia and chlamydozoospores of *T. basicola*. The pH of all three media was adjusted to 5.2. All antimicrobial agents were placed in a 250-ml Erlenmeyer flask containing 30 ml of distilled water and kept on a shaker for about 1 hour, then brought to 50 C in a water bath before being added to the autoclaved agar medium cooled to the same temperature.

Soil dilution plates.—Endoconidial or chlamydozoospore suspensions were mixed with natural or alfalfa-amended Conover loam soil (7) to give a known number of spores per gram of oven-dry soil. Alfalfa-amended soil was prepared by mixing dried ground alfalfa hay with Conover loam so that the alfalfa content was 1% (w/w). The amended soil was brought to about 50% moisture-holding capacity and sealed in a polyethylene bag for about two weeks before it was mixed with inoculum. Dilution series of the soils were made with distilled water. The first dilution of 10^{-1} (w/v) was shaken for 30 minutes before further dilution to 10^{-3} . One milliliter of the 10^{-3} dilution was mixed with 15 ml of molten (about 43 C) agar in 9-cm diameter petri plates. Five to 10 plates were used for each treatment. Counts of *T. basicola* colonies were made after 6 days of incubation at 24 C. To determine population levels of *T. basicola* in naturally-infested soils, dilutions of 10^{-1} or 10^{-2} usually were plated.

All experiments were repeated one or more times with similar results.

RESULTS

Experiments were first done to determine the effects of omitting each of the antimicrobial agents from the TBM media on recovery of *T. basicola* from soil infested with endoconidia, and on numbers of undesired fungi and bacteria. Absence of nystatin resulted in abundant growth of undesired fungi and a drastic reduction in the number of countable *T. basicola* colonies. Absence of PCNB gave similar, but less pronounced, results. Absence of chloramphenicol resulted in a large increase in bacterial colonies which obscured many *T. basicola* colonies. Deletion of penicillin also gave rise to more bacterial colonies. Absence of oxgall resulted in fewer *T. basicola* colonies and larger numbers of other fungi than

TABLE 2. Comparison of three selective media for recovery of *Thielaviopsis basicola* isolate 170 from alfalfa-amended Conover loam infested with endoconidia and chlamydozoospores

| Medium ^b | Propagules ($\times 10^3$)/g soil ^a | |
|---------------------|--|-------------------|
| | Endoconidia | Chlamydozoospores |
| VDYA-PCNB | 10.0 | 9.5 |
| TBM-V8 | 13.3 | 14.1 |
| TBM-C | 16.1 | 16.4 |
| LSR ^c | 2.8 | 3.6 |

^aSoil dilution of 10^{-3} was used in recoveries; values are means of 10 petri plate replications. Inoculum density was approximately 20×10^3 spores/g oven-dry soil. Germination was 96% in carrot extract.

^bSelective media: Papavizas' VDYA-PCNB (5), and two modified media (one contained V-8 vegetable juice and the other contained carrot juice).

^cLeast significant range ($P = 0.05$) according to Tukey's 'w' procedure.

were present in the control medium. *Thielaviopsis basicola* colonies were smaller in size and greater in number in the medium containing oxgall.

The VDYA-PCNB medium and the two modified media were then compared in experiments to recover *T. basicola* isolates 157 and 170 from natural Conover loam soil infested with endoconidia. As many or more *T. basicola* colonies were recovered with the modified media as with VDYA-PCNB (Table 1). Moreover, undesired fungal colonies ranged from 0-5 and bacteria 0-10 per plate with the modified media, whereas they ranged from 6-20 and 20 or more, respectively, with VDYA-PCNB (Fig. 1).

Recovery of *T. basicola* from alfalfa-amended Conover loam soil was compared using the same three media plus RB-M2. Endoconidia and chlamydozoospores of isolate 170 were used to infest the soil. No *T. basicola* colony was identified with the RB-M2 medium. These plates were covered with a dense growth of bacteria and other fungi within 3 to 4 days. Among the other three media greater numbers of *T. basicola* colonies were recovered with the TBM media than with VDYA-PCNB (Table 2). Undesired fungal and bacterial colonies ranged from 5-20 and 5-30, respectively, with the modified media, and usually occurred in uncountable numbers with VDYA-PCNB (Fig. 1).

The previous experiments were done with soils artificially infested with concentrations of *T. basicola* greater than 10^4 per gram which is greater than natural population levels. Therefore, experiments were done with the two modified media using a soil infested with 50 to 1,000 endoconidia of isolate 170 per gram. Soil dilutions of 10^{-1} to 10^{-3} were prepared. Colonies of *T. basicola* ranging in number from 40 to 100% of the soil population were counted on the media even at dilutions as low as 10^{-1} (Table 3).

Counts of *T. basicola* from naturally infested soils tended to be greater, and undesired bacteria and fungi fewer, on TBM-V8 or TBM-C than on VDYA-PCNB (Table 4, Fig. 2). Populations of *T. basicola* in the soil samples ranged from about 10 to 100 propagules per gram, except for soil sample 4 which had about 10^3

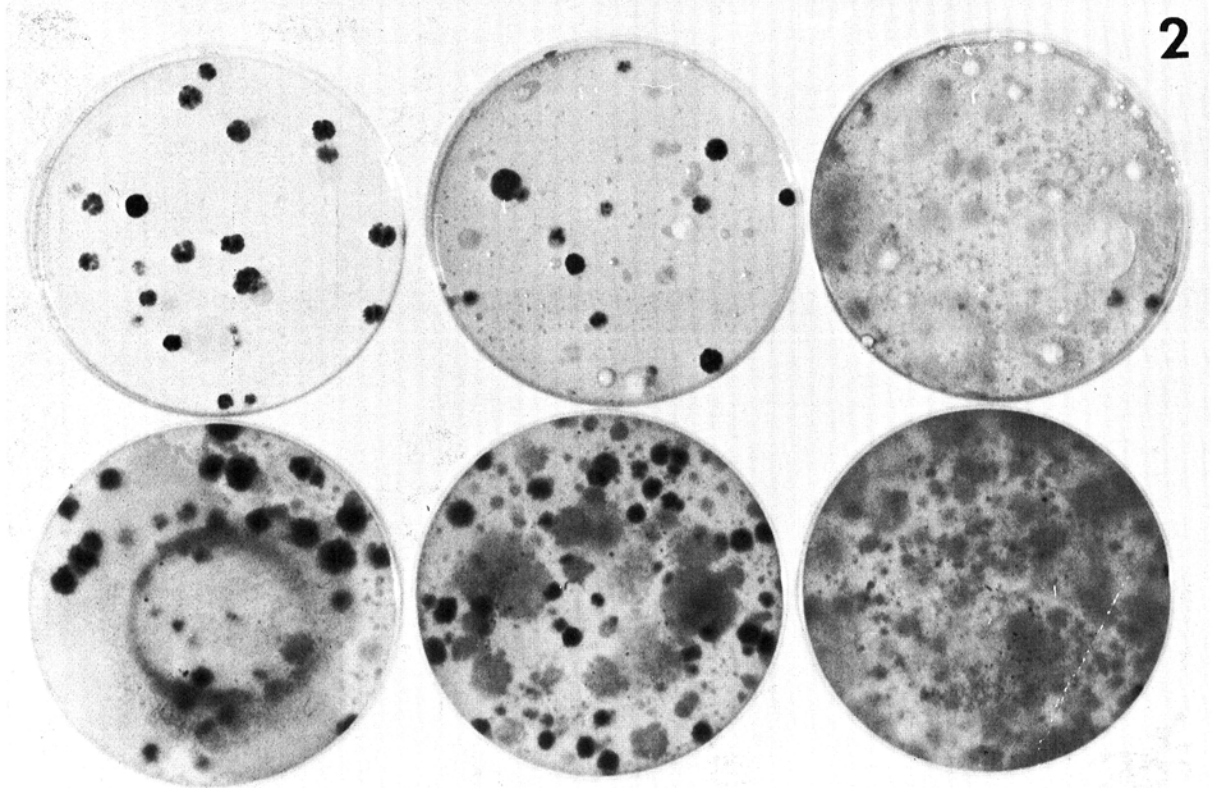
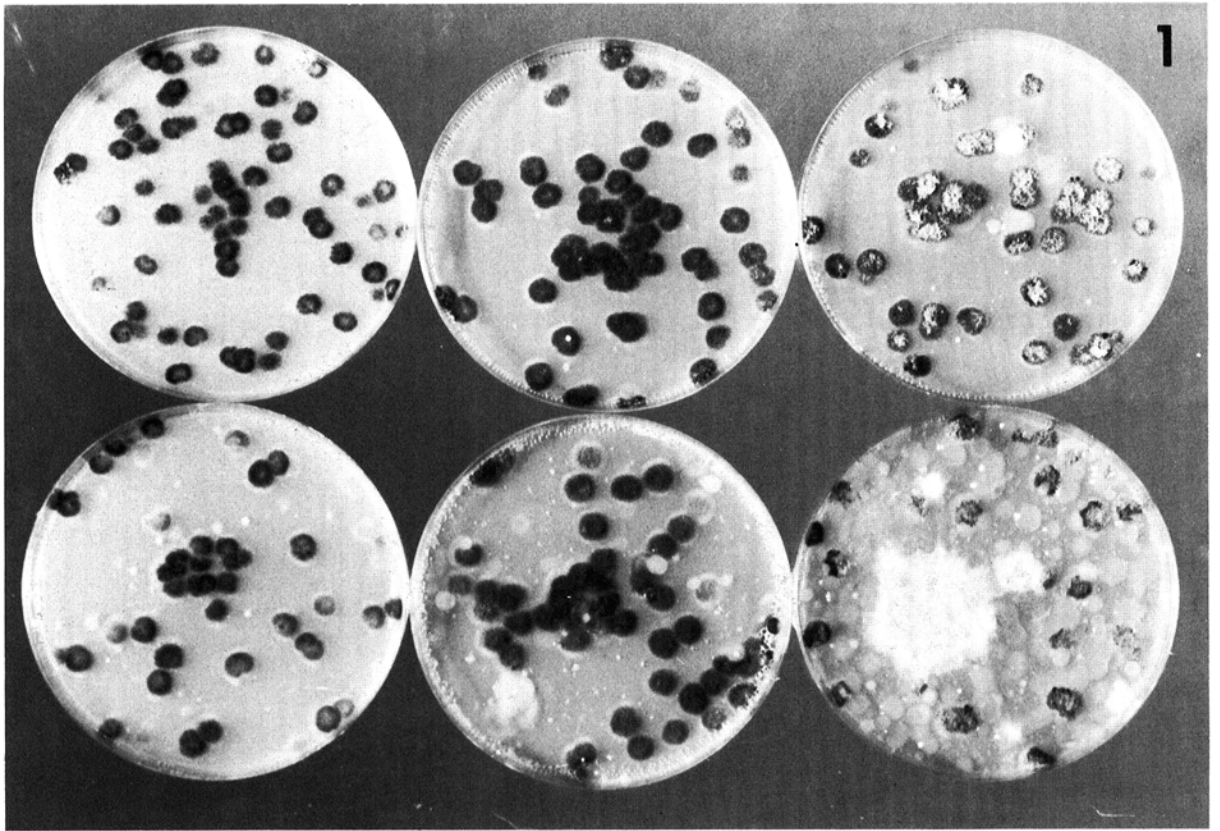


TABLE 3. Recovery of *Thielaviopsis basicola* isolate 170 from Conover loam soil infested with endoconidia, using two selective media

| Propagules applied (no./g soil) | Soil dilution plated | Propagules recovered ^a | |
|------------------------------------|-------------------------|-----------------------------------|-----------------------|
| | | TBM-V8 (no./g soil) | TBM-C (no./g soil) |
| 1,000 | 10 ⁻¹ | 550 | 426 |
| | 10 ⁻² | 700 | 620 |
| | 10 ⁻³ | 800 | 400 |
| 100 | 10 ⁻¹ | 70 | 80 |
| | 10 ⁻² | 40 | 100 |
| 50 | 10 ⁻¹ | 30 | 42 |

^aMean number based on five replicate petri plates. Germination of endoconidia was 97% in carrot extract. The TBM media were modifications prepared for these experiments; one was supplemented with vegetable juice (V-8) and the other with carrot juice (C).

propagules per gram. Several successive crops of soybeans had been grown in this soil in the greenhouse to increase the population of the pathogen. Of interest was the recovery of *T. basicola* with the two new media using a soil dilution of 10⁻¹. Although large numbers of bacteria and fungi other than *T. basicola* were present at this dilution, distinctive black colonies of the pathogen were easily counted from the bottoms of the petri plates (Fig. 2). Subsequent experiments, however, have shown that colonies of *T. basicola* cannot be counted at this dilution in all soils tested, however.

DISCUSSION

The improved selectivity of the modified media over that of VDYA-PCNB should enhance the usefulness of the dilution plate method for assessing populations of *T. basicola* in soil. The pathogen could be enumerated both when numbers of the pathogen were low, and in the presence of the large populations of bacteria and fungi that occurred in alfalfa-amended soil. A population level as low as 10 propagules per gram of soil could cause root rot of soybean in artificially infested soil (Maduewesi and Lockwood, unpublished).

Vegetable juice (V-8) or carrot root extract provided an excellent base for the media, probably through stimulation of rapid germination of the spores and by providing an adequate food source for mycelium development. Agar media that contained carrot root extract (4) or V-8 juice (5) supported faster and more complete germination of *T. basicola* spores than several other media. A proprietary carrot juice (1) and V-8 juice (6) also stimulated germination of *T. basicola* spores in soil.

The failure of the modified RB-M2 medium (8) in our

TABLE 4. Population levels of *Thielaviopsis basicola* in naturally-infested soils as estimated by the dilution plate method, using three selective media^a

| Soil sample | Dilution plated | Propagules/g soil ^b (no.) | | | |
|----------------|--------------------|--------------------------------------|--------|-----------|------------------|
| | | TBM-C | TBM-V8 | VDYA-PCNB | LSR ^c |
| 5 | 10 ⁻¹ | 2.5 | 10 | 0 | 17 |
| 8 | 10 ⁻¹ | 5.0 | 28 | 0 | 18 |
| 7 | 10 ⁻¹ | 15 | 28 | 13 | 16 |
| 2 | 10 ⁻¹ | 100 | 80 | 33 | 48 |
| 4 | 10 ⁻² | 1,475 | 1,250 | 675 | 640 |

^aSelective media: Papavizas' VDYA-PCNB (5), and two modified media, one supplemented with vegetable juice (V-8) and the other with carrot juice (C).

^bValues are averages of five petri plate replications.

^cLeast significant range ($P \leq 0.05$) by Tukey's 'w' procedure.

studies may have resulted from the presence of a high glucose concentration (10 g/liter) as the sole energy source. Glucose is less suitable than more complex energy sources for germination of *T. basicola* (4), and it promotes rapid development of numerous other microorganisms.

The combination of suitable carbon sources for *T. basicola*, which are relatively nonstimulatory to other microorganisms, and appropriate inhibitory agents at effective concentrations, provide the basis for the improved media described herein.

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Fig. 1-2. 1) Dilution plates from natural Conover loam (top row) and alfalfa-amended Conover loam (bottom row) infested with endoconidia (about 50×10^3 /g of soil) of *Thielaviopsis basicola* isolate 170. Left to right: TBM-C, TBM-V8, and VDYA-PCNB. A dilution of 10⁻³ was plated. Black colonies are those of *T. basicola*. 2) Dilution plates from a soil sample naturally infested with *Thielaviopsis basicola*. Upper row: soil dilution of 10⁻². Bottom row: soil dilution of 10⁻¹. Left to right: TBM-C, TBM-V8, and VDYA-PCNB. Black colonies are those of *T. basicola*. The plates in the bottom row were photographed from the underside. All plates were photographed at 6 days.

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