Ethanol-Potassium Nitrate Medium for Enumerating *Rhizoctonia solani*-like Fungi from Soil

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ABSTRACT

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A medium consisting of 0.2 g of potassium nitrate, 30 g of agar, 947 ml of distilled water, 53 ml of 95% ethyl alcohol, 0.38 ml of metalaxyl 2E, 0.02 ml of prochloraz 40EC, 100 mg of tobramycin, and 300 mg of streptomycin was developed for enumerating *Rhizoctonia solani*-like fungi from soil. Ethyl alcohol was mixed with the cooled medium at 50 C before antibiotics and fungicides were added. Selectivity decreased with age of the medium, thus freshly made media should be used. Most colony-forming units of *R. solani*-like fungi in field soils were present in organic matter and retained by a 65-mesh sieve when soil suspensions were screened. A few colony-forming units collected on a 200- or 270-mesh sieve were microsclerotia and resting hyphae free of organic matter.

Diseases are the limiting factor in carnation production in Hawaii, and *Rhizoctonia solani* Kühn and *Fusarium roseum* var. *graminearum* (Schwabe) Snyd. & Hans. are the most serious pathogens at Kula, Maui, Hawaii (7). Field studies in etiology and epidemiology of *R. solani* and related organisms can be facilitated with a simple and reliable assay procedure to estimate populations. This investigation was initiated to develop such a method.

Van Bruggen and Arneson (8) have recently summarized the methods for quantitative and qualitative determinations of *R. solani* in soil. To determine population densities, soil fractionation techniques and direct plating of soil by weight or in pellets have been used. The widely used medium developed by Ko and Hora (3), later modified by Gangopadhyay and Grover (2), becomes dark from gallic acid oxidation, making direct observation of colonies difficult. These media also tend to moderately suppress growth of organisms other than *R. solani*. We report here a medium selective for *R. solani* and binucleate *R. solani*-like (BRS) fungi that facilitates determining population densities of these fungi in soil. The phrase *Rhizoctonia solani*-like in this paper is used collectively to include *R. solani* (multinucleate) and BRS fungi.

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MATERIALS AND METHODS

R. solani isolates (AG2-1, AG2-2, and AG4) from soil and plant tissue and three isolates of BRS obtained from carnation fields at Kula, Maui, Hawaii, were used as testers for the medium. The effects of the various ingredients on radial growth of the test isolates were determined. The fungi were grown on 10% V-8 juice agar (100 ml of V-8 juice, 2 g of $CaCO_3$, 18 g of agar, and 900 ml of water), and 5-mm-diameter disks were placed in the center of the test medium. Plates were incubated at 28 C under continuous illumination, and radial growth was measured at 24-hr intervals.

The basal medium used was a modification of the GN medium of Ferriss and Mitchell (1) and consisted of 0.2 g of KNO_3, 30 g of agar, 0.09 g of metalaxyl (Ridomil 2E), 0.05 g of streptomycin sulfate (National Biochemicals Corporation), and 0.05 g of chloramphenicol (Sigma) per liter. The fungicide and antibiotics were added after the autoclaved medium had been cooled to 50 C.

Ethyl alcohol (95%) at concentrations of 0, 26.3, 52.6, 79, and 105.3 ml/L in the basal medium was tested as the carbon source (5). The ethanol was thoroughly mixed with the cooled medium before the antibiotics and fungicide were added.

Because the basal medium did not adequately suppress growth of soil fungi and bacteria, the fungicides fenarimol (Rubigan 12.5 EC), prochloraz (Boots 40EC, now available through Noram), and triadimefon (Bayleton 50WP) were tested for their effects on the growth of

the isolates at 1 ppm in the basal medium with 5% ethanol. The fungicides were also tested with field soil to observe their inhibitory effects on other groups of fungi. Prochloraz was subsequently tested at 2, 4, and 8 ppm. Streptomycin sulfate at 100, 200, and 300 ppm and chloramphenicol, tobramycin (Sigma), and nitrofurantoin (Sigma) at 50 and 100 ppm were also tested to suppress soil bacteria.

A modified soil-sieving method (4,9) was used to determine the population of *R. solani* and BRS fungi in soil from Maui carnation fields. Initially, 10-g soil samples in 100 ml of water were blended in a semimicro stainless-steel container for 30 sec and screened through a series of sieves decreasing from 65-, 200-, to 270-mesh (Tyler, 0.210-, 0.074-, and 0.053-mm pore size, respectively). This method reduced the soil organic matter to smaller sizes, inducing considerable variation in the number of colony-forming units present in the sample. To reduce this variation, soil samples in water were vigorously shaken for 1 min, then passed through a 200-mesh screen. The portion collected on the screen was backwashed into a beaker, and the new suspension was stirred and allowed to settle. After silt and other soil particles had settled, the liquid portion was decanted through a 200-mesh screen to collect the organic matter, which was washed into two or more petri plates containing the medium. The number of plates used depended on the amount of organic matter present in the sample. The soil residue in the beaker was also plated on four plates of the medium. The plates were kept open until all surface water had evaporated, then covered and incubated at room temperature (about 25 C) for 3 days. After the incubation period, the plates were gently washed with a soft camel's-hair brush under running tap water and incubated an additional 24 hr. Plates were washed again by the same procedure before examination and enumeration. Hyphal tips of each of the *R. solani*-like fungi enumerated were grown on water agar to obtain pure isolates. Pure colonies were grown on 2% V-8 juice agar (20 ml of V-8 juice, 0.4 g of $CaCO_3$, 18 g of agar, and 980 ml of water), and the nuclear number per cell of each isolate was determined after staining with safranin O (10). Anastomosis groupings were determined by standard procedures (6). Our medium was compared with water agar (3) and GN (1) media, using the above procedure and a soil sample from a Maui carnation farm with a long history of crown rot disease caused by *R. solani*.

RESULTS AND DISCUSSION

Growth of the test isolates decreased with increasing concentrations of ethyl alcohol and was totally inhibited by 10% ethyl alcohol for 7 days at 28 C. Alcohol at 5% allowed for restricted growth of all test isolates, making this the desired concentration.

All three fungicides inhibited growth of the BRS isolates equally, but only fenarimol inhibited the growth of the *R. solani* isolates. When inoculated with field soil, media with 1 ppm fenarimol and triadimefon allowed excessive growth of *Trichoderma* spp. and *Fusarium* spp., whereas prochloraz at 1 ppm restricted their growth. Prochloraz at 2, 4, and 8 ppm did not noticeably affect the growth of test isolates and, at the highest concentration, suppressed most soil fungi. Therefore, prochloraz at 8 ppm was chosen for the final medium.

Because three concentrations of streptomycin did not adversely affect growth of the test fungi, its level was established at 300 ppm. Tobramycin allowed better growth of the test isolates than chloramphenicol and nitrofurantoin. The use of 300 ppm streptomycin and 100 ppm tobramycin effectively inhibited

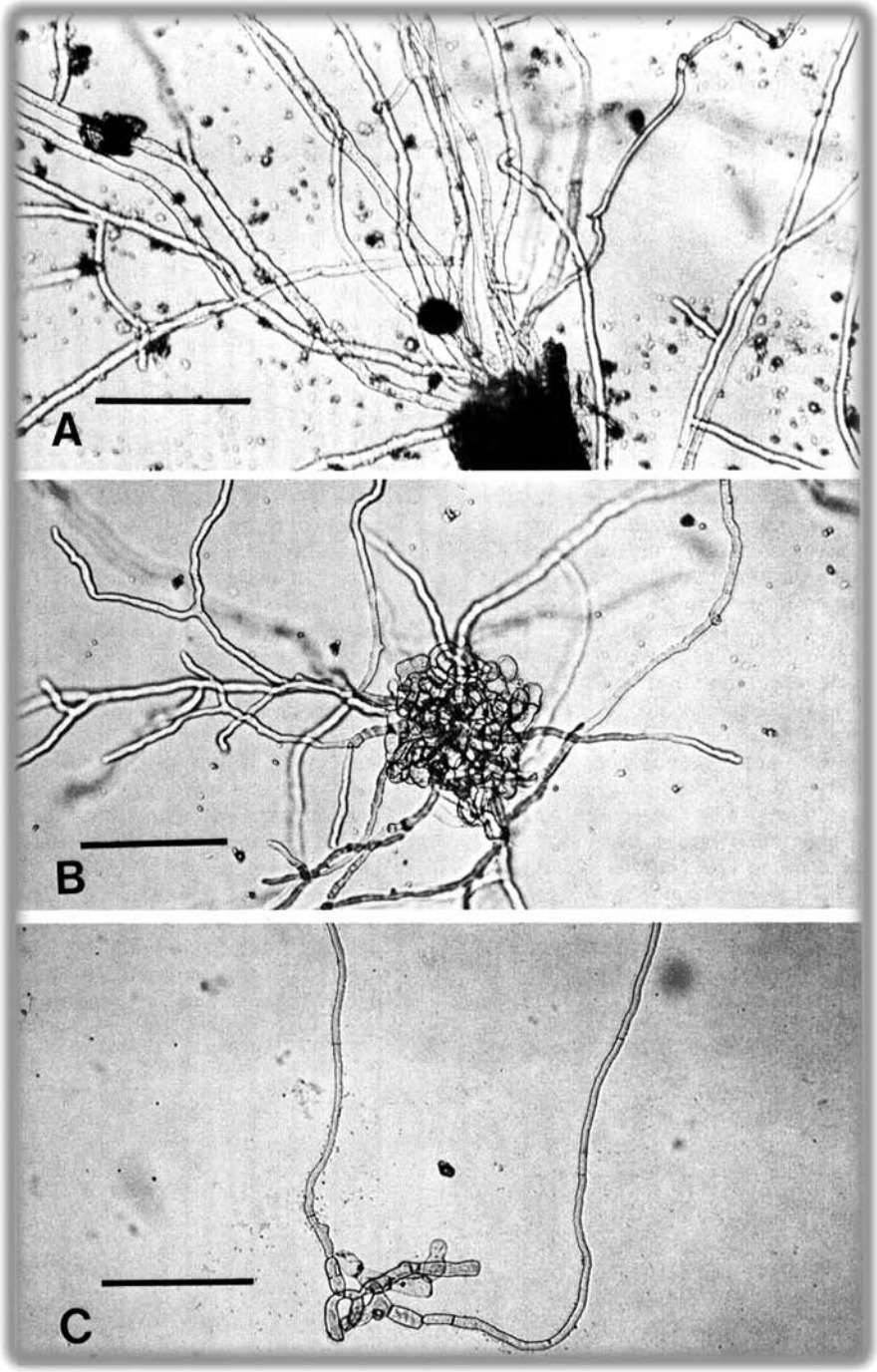

Fig. 1. Close-up of *Rhizoctonia solani*-like fungi colonies on ethanol-potassium nitrate medium showing colony origin. **(A)** Piece of organic matter derived from a decomposed root, origin of an *R. solani* colony in the isolation medium. This organic matter was commonly collected from unblended soil on a 65-mesh screen. Scale bar = 78 μm. **(B)** A sclerotium collected infrequently from unblended soil on a 200-mesh screen. Scale bar = 78 μm. **(C)** Resting hyphae collected from blended soil on a 270-mesh screen. Scale bar = 56 μm

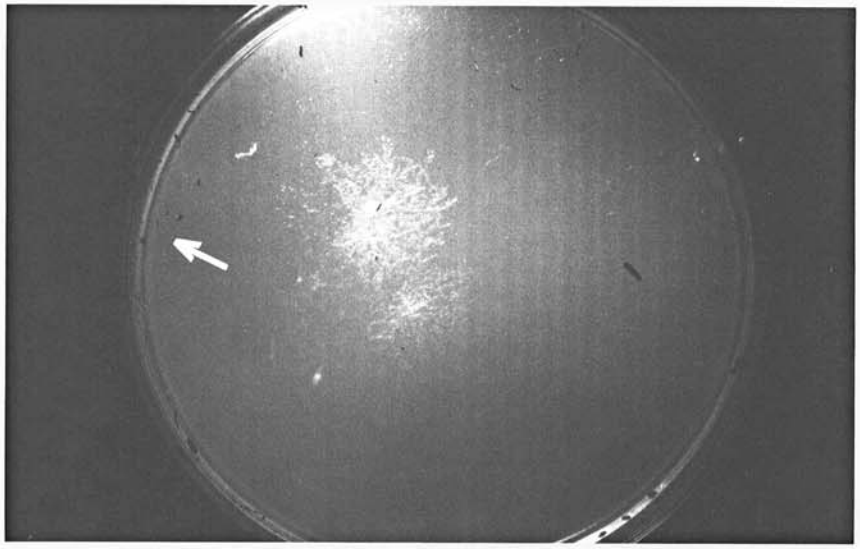

Fig. 2. *Rhizoctonia solani*-like fungi colonies on ethanol-potassium nitrate medium 4 days after plating soil organic matter from sample. A few pieces of organic matter (arrow) still remaining on the surface of the medium were not removed by the camel's-hair brush when washing the surface with tap water.

growth of soil bacteria.

The final medium consisted of the following: 0.2 g of potassium nitrate, 30 g of agar, 947 ml of distilled water, 5% ethyl alcohol (53 ml of 95% ethyl alcohol), 0.38 ml of metalaxyl 2E, 0.02 ml of prochloraz 40EC, 100 mg of tobramycin, and 300 mg of streptomycin. Ethyl alcohol was completely mixed with the cooled medium at 50 C before the antibiotics and fungicides were added. Selectivity of the medium decreased 15 days after preparation. Thus, freshly made media should be used.

R. solani and BRS fungi were readily isolated from field soil samples. In soils tested, most colony-forming units originated from sclerotia embedded in large pieces of organic matter collected on a 65-mesh screen (Fig. 1A), which conforms to other studies (9). Both *R. solani* and BRS fungi were recovered infrequently on 200-mesh screen as small microsclerotia averaging 70 μm (Fig. 1B). When soil samples were blended with tap water for 30 sec and screened through 200- and 270-mesh sieves, a few microsclerotia and pieces of resting hyphae (Fig. 1C) passed through the 200-mesh screen and were collected on 270-mesh screen. These gave rise to colonies of both fungi. Other sclerotia-producing basidiomycetous fungi were isolated on the medium, but they were readily distinguished from *R. solani* and BRS fungi by colony characteristics. The latter tended to have larger colonies with diffuse mycelial growth that were readily distinguished after washing the plates (Fig. 2). These other basidiomycetous fungi produced round sclerotia on 2% V-8 juice and had prominent clamp connections that were readily observed with a compound microscope.

A Kula carnation soil with high incidence of Rhizoctonia crown rot of carnation was plated in the medium of Ko and Hora (3), GN (1), water agar, and the ethanol-potassium nitrate medium. An average of 0.5, 0.5, 0.4, and 2.4 cfu of *R. solani*-like fungi per gram of soil were recovered on each medium, respectively. The growth of myriads of soil fungi was unrestricted on the first three media, totally obscuring colonies of *R. solani*-like fungi and rendering them unrecognizable 48 hr after soil plating, whereas on the present medium, colonies of *R. solani*-like fungi were discrete, relatively uninhibited, and readily visible (Fig. 2).

The use of soil sieves and the ethanol-potassium nitrate medium provided an excellent means for enumerating *R. solani*-like fungi from soil. When the nuclear condition of each individual colony of *R. solani*-like fungi isolated on this medium was determined by nuclear staining (10), the enumeration of *R. solani* and BRS fungi from soil was then possible.

ACKNOWLEDGMENT

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