Comparison of Media for Isolating *Rhizoctonia solani* from Soil

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ABSTRACT

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Three media used for isolating *Rhizoctonia solani* from soil were evaluated by comparing in vitro growth of *R. solani* AG-1 through AG-5, suppression of indigenous soilborne microflora, and efficiency of recovery of *R. solani* from soil. Growth of isolates of AG-1 was most rapid on water agar (WA), somewhat reduced on Ko and Hora's medium amended with 5 μl/L of prochloraz 40EC (KH_p), and greatly reduced on ethanol-potassium nitrate medium containing 2% ethanol (EPN_2). Isolates of AG-2-1, AG-2-2, AG-3, AG-4, and AG-5 grew equally well on WA and KH_p but grew very slowly on EPN_2. KH_p and EPN_2 were both highly effective in inhibiting soilborne aerobic fungi and bacteria. At least 97.9 and 98.8% of fungi detected in three soils using peptone-glucose agar (for quantifying total aerobic fungi) were inhibited on KH_p and EPN_2, respectively, after 7 days at 19–22 C; 99.9% of soilborne aerobic bacteria were inhibited on both media. At least 70% of aerobic bacteria failed to grow on WA after the same period, but all fungi detected in soil grew on WA. No difference was observed among media in estimates of populations of *R. solani* from three soils. Although efficiency of recovery of *R. solani* from soil was equal among media, it was much easier to locate and identify colonies of *R. solani* on the two selective media than on WA. Although materials for EPN_2 are costly, this medium would be useful for quantifying soilborne populations of *R. solani* when restricted growth of *R. solani* is desirable, such as when making standard plate counts of serially diluted organic matter extracted from soil. KH_p was a preferable medium when using a most-probable-number technique to quantify propagules in serially diluted organic matter, because of the low cost of materials and rapid growth of *R. solani* on the medium.

The study of diseases induced by soilborne plant pathogens is facilitated when data are available on population dynamics of these microorganisms in soil. Such information can provide important insight into the epidemiology of disease in the field, the growth and survival of pathogens in the absence of host plants, and mechanisms of disease resistance.

Varied techniques are available for estimating soil populations of *Rhizoctonia solani* Kühn, an important pathogen of numerous crops (3). Most of these techniques employ one of several culture media. Media commonly used include water agar, sometimes amended with compounds to inhibit bacterial growth (7,11,12,15,16,18,28); Ko and Hora's medium (6,8,9,26); tannic acid-benomyl medium (4,21); and ethanol-potassium nitrate medium (22), a recently developed medium selective for *R. solani* and related fungi.

In the present study, we evaluated in vitro growth of *R. solani*, efficiency of recovery of *R. solani* from soil, and suppression of native microbiota on water agar, Ko and Hora's medium, and ethanol-potassium nitrate medium. Because inconsistent results were obtained in our laboratory in numerous preliminary evaluations of tannic acid-benomyl medium, further research with this medium was not conducted.

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MATERIALS AND METHODS

Media. Water agar (WA) was prepared using 15 g of agar per liter of distilled water. The fungicide prochloraz 40EC was added at 50 C after autoclaving at 5 μl of formulated product per liter to all preparations of modified Ko and Hora's medium (KH_p) (6). Ethanol-potassium nitrate medium (EPN) was prepared with 5% ethanol (EPN_5) as originally described (22) or with 2% ethanol (EPN_2). Media were used within 48 hr of preparation.

Growth of *R. solani*. Isolates of *R. solani* consistently grew very slowly when sclerotia were placed onto EPN_5 in preliminary experiments. Therefore, the effect of ethanol concentration in EPN on mycelial growth of *R. solani* was studied by preparing the medium with ethanol concentrations of 0, 1, 2, and 5%. Sclerotia of an isolate of *R. solani* AG-2-2 recovered from a diseased sugar beet collected in Torrington, Wyoming, were produced as described by van Bruggen and Arneson (25). Individual sclerotia were placed into six petri dishes per treatment, and these were incubated at 27 ± 0.5 C in darkness. Colony radius was measured after 7 days.

Growth rates of 35 isolates representing *R. solani* AG-1, AG-2-1, AG-2-2, AG-3, AG-4, and AG-5 were examined on WA, KH_p, and EPN_2. Individual 5-mm plugs taken from the margin of actively growing cultures on potato-dextrose agar were transferred to petri dishes containing the media. Each isolate was transferred to two replicate petri dishes of each medium. Cultures were incubated at 27 C in darkness, and radial colony growth was measured daily for 5 days. Radial growth was averaged for each isolate-medium combination before data analysis. The experiment was repeated once.

The effect of the fungicide metalaxyl in KH_p on growth of *R. solani* was evaluated to determine if it could be added to the medium without adversely affecting growth of *R. solani*. KH_p was supplemented with 100 mg of metalaxyl (Ridomil 2E) (KH_{pm}) per liter of medium at 50 C after autoclaving. Sclerotia of *R. solani* AG-2-2 were placed onto KH_{pm} or unamended KH_p (one sclerotium per petri dish, six dishes per treatment), and cultures were incubated at 27 C in darkness. Colony radius was measured after 7 days.

Soils. A total of six soil samples were collected from four soil series. Three of the six soil samples were collected from agricultural fields with a history of Rhizoctonia root and crown rot of sugar beet, and three samples were collected from agricultural fields with no known history of the disease and which had not

been planted to sugar beet for at least 6 yr. The soil samples used were: 1) a Youngston clay loam (pH 7.8, 1.3% organic matter [OM]) naturally infested with *R. solani*, collected near Worland, Wyoming; 2) a Garland clay loam (pH 7.7, 1.4% OM) with no history of root rot, collected at the University of Wyoming Research and Extension Center (UW R&E Center) at Powell; 3) a Valentine sandy loam (pH 7.6, 1.3% OM) with no history of root rot, collected at the UW R&E Center at Torrington; 4) a Valentine sandy loam (pH 7.6, 1.3% OM) annually infested with an isolate of *R. solani* AG-2-2 and planted to sugar beet, collected at the UW R&E Center at Torrington; 5) a Wycolo sandy clay loam (pH 7.6, 1.9% OM) with no history of root rot, collected at the Laramie campus; and 6) a Wycolo sandy clay loam (pH 7.6, 1.9% OM) infested in 1984 with an isolate of *R. solani* AG-2-2 and planted to sugar beet biannually, collected at the Laramie campus. Soil samples were screened (6-mm openings) and stored moist in polyethylene bags at room temperature until use within 6 mo of collection.

Suppression of indigenous soilborne fungi and bacteria. Samples from soils having no history of Rhizoctonia root rot of sugar beet were passed through a No. 12 sieve (1.70-mm openings), after which 5-g aliquots were suspended in 50 ml of sterile 0.25% water agar and a 10-fold serial dilution was made in 0.25% water agar. Aliquots of 0.5 ml were spread onto 9-cm petri dishes containing solidified media (three replicates per dilution), and these were incubated at room temperature (19–22 C). Petri dishes were examined under a dissecting microscope (×15) after 7 days, and the total number of fungal and bacterial colonies were counted. Freshly sieved soil was weighed and dried at 105 C for 3 days to permit calculations of population densities on a dry-weight basis.

The media evaluated in the first test were KH_p; EPN_5; peptone-glucose agar (PGA), for quantifying total fungi (27); and a modified Henrici's medium (HM), for quantifying total bacteria (20). Subsequent tests included WA, KH_{pm}, and EPN_2. The experiment was performed using the Garland clay loam, the Valentine sandy loam, and the Wycolo sandy clay loam.

Quantitative recovery of *R. solani* from soil. Five replicate samples weighing 500 g from soils having a history of Rhizoctonia root rot were wet-sieved using a No. 12 sieve nested over a No. 60 sieve (250-μm openings). Material retained on the No. 60 sieve was suspended in tap water, stirred well, and allowed to settle for 15–20 sec. Floating organic matter was recovered on the No. 60 sieve. The soil was resuspended in tap water four more times, resulting in recovery of virtually all visible floating organic matter (26). Organic matter thus recovered was suspended in 50 ml of 0.25% WA, and a fourfold serial dilution in 0.25% WA was made. Six 0.2-ml aliquots from each dilution were transferred to petri dishes (three spots spaced 3.0–3.5 cm apart in each of two dishes) containing WA, KH_p, or EPN_2. These were incubated at 27 C in darkness for 3 days on WA and KH_p and for 7 days on EPN_2. The plates were incubated for 3 days because only 75.8% of colonies of *Rhizoctonia*-like fungi (RLF) detectable after 3 days on KH_p were detected after 1 day and only 90.9% after 2 days. After incubation, spots were examined individually at ×10–30 to locate colonies of RLF. Suspect colonies of RLF were examined at ×400 and ×1,000 for verification based on hyphal characteristics (5). Randomly selected colonies of RLF were hyphal-tipped and subcultured for characterization of nuclear condition, septal characteristics, and anastomosis grouping (5,10,23). The experiment was performed using samples infested with *R. solani* collected from the Valentine sandy loam, the Wycolo sandy clay loam, and the Youngston clay loam soils.

A most-probable-number table (MPN) was developed for estimating propagule density from the dilution series. The table was developed using a Hewlett Packard HP11-C calculator programmed to generate an MPN table using the algorithm of Alexander (1). Data on the number of spots at each dilution yielding actively growing colonies of RLF were compared with tabulated values to derive a maximum likelihood estimate of the number of colony-forming units per milliliter of diluent. These values were converted to colony-forming units per gram of dry soil using conversion factors based on drying of soil samples at 105 C for 3 days.

Data analysis. An analysis of variance was conducted for each experiment using the Statistical Analysis System (SAS Institute, Cary, NC). Where appropriate, data were transformed using the $\log_{10}$ transformation to meet the assumption of equality of variance (19). Linear contrasts were calculated, and regression analysis was used where appropriate (14,19). Fisher's protected least significant difference (LSD) statistic (19) was calculated when comparisons among all treatment means were desired.

RESULTS

Growth of *R. solani*. A significant ($P < 0.0001$) quadratic relationship was found between increasing ethanol concentration and decreasing growth of *R. solani* AG-2-2 (Fig. 1). Because the growth rate of *R. solani* on EPN medium containing 2% ethanol was about double that observed on EPN_5, EPN_2 was used in later experiments.

All isolates of *R. solani* AG-1 grew more rapidly on WA than on KH_p, and the mean growth rate of isolates of AG-1 was significantly ($P \leq 0.05$) greater on WA than on KH_p (Table 1). Isolates of AG-2-1 through AG-5 grew equally well on WA and KH_p, as differences between treatment means were nonsignificant. All five anastomosis groups tested grew significantly ($P \leq 0.05$) more slowly on EPN_2 than on either WA or KH_p.

Growth of *R. solani* AG-2-2 on KH_p was unaffected by the presence of metalaxyl at 100 mg/L in the medium. Mean colony radius after 7 days was 3.4 and 3.5 cm on KH_p and KH_{pm}, respectively, which was not significantly different ($P > 0.10$).

Suppression of indigenous soilborne fungi and bacteria. Populations of aerobic soilborne fungi on WA were not significantly different from those on PGA (Table 2), indicating that fungal growth was not restricted on WA. In all soils, fungal populations were significantly ($P \leq 0.05$) reduced on the selective media. Reductions in fungal populations on the selective media over those measured on PGA ranged from 97.9 to 99.9%. In two of three soils, EPN_5 was significantly more suppressive to indigenous fungi than KH_p. EPN_2 was significantly more suppressive than KH_p in one of two soils where it was tested. No significant reduction was observed in the number of fungal colonies when metalaxyl was added to KH_p.

Populations of aerobic soilborne bacteria were reduced significantly ($P \leq 0.05$) on all media tested (Table 2). Bacterial populations were reduced fivefold to 10-fold on water agar and were almost completely suppressed on the selective media. In two soils, small but statistically significant differences in suppression of bacteria were observed between the ethanol-based media (EPN_2 and EPN_5) and KH_p. Addition of metalaxyl to KH_p had no effect on suppression of soilborne bacteria.

Growth of soilborne actinomycetes was prolific on WA, although this was

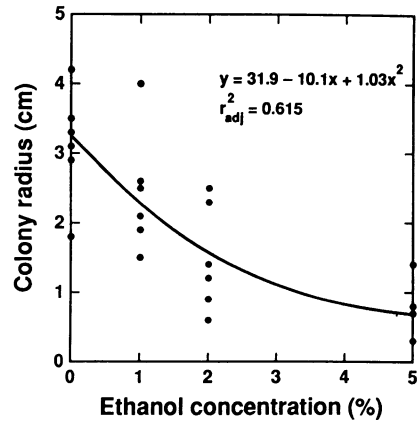

Fig. 1. Effect of ethanol concentration in ethanol-potassium nitrate medium on colony radius of *Rhizoctonia solani* AG-2-2 after 7 days.

not quantified. Growth of actinomycetes was observed to be highly restricted on EPN_2 and KH_p.

Quantitative recovery of *R. solani* from soil. All of 37 randomly selected isolates of RLF were identified as *R. solani* based on multinuclear condition, presence of dolipore septa, and anastomosis with known tester isolates of *R. solani*; 19 isolates were determined to be AG-2-2 and 18 to be AG-4. Because randomly selected isolates of RLF from the three soils were consistently identified as *R. solani*, all colonies of RLF were assumed to be *R. solani*.

Population densities of *R. solani* in the soils tested ranged from 2.6 cfu/100 g of dry soil in the Valentine sandy loam to 247 cfu/100 g of dry soil in the Youngston clay loam. Most colonies of *R. solani* originated from sclerotia and infested organic matter, and some originated from melanized hyphal fragments. Growth generally was greatest in colonies originating from sclerotia and pieces of infested debris larger than 1.0 mm. Relatively slow-growing colonies were often observed originating from smaller pieces of organic matter and from hyphal fragments.

In each of the soils tested, no significant difference ($P > 0.10$) was detected in estimates of mean population density of *R. solani* on the three media tested (*data not shown*). Inspection of petri dishes was relatively rapid using KH_p and EPN_2. Although not quantified, no difference was noted between the two selective media in inspection time. Inspection of petri dishes generally was slower using WA, on which growth of other microorganisms was abundant.

DISCUSSION

The selective media KH_p and EPN_2 provided excellent suppression of growth of most indigenous fungi and bacteria in several soils, whereas WA provided very little suppression of bacteria and no significant suppression of fungi. Although these observations were not unexpected, it was somewhat surprising to find that efficiency of recovery of *R. solani* from soil was statistically equal on WA and the two selective media. In initiating these experiments, we hypothesized that recovery of *R. solani* might be poorer on WA for at least two reasons. Conceivably, antibiosis from other microorganisms growing on the medium could inhibit slow-growing colonies originating from very small pieces of organic matter or from hyphal fragments. We also anticipated that slow-growing colonies could be difficult to locate on WA because of overgrowth from other fungi, particularly fast-growing zygomycetous fungi. However, efficiency of recovery of *R. solani* from soil using WA was not significantly different from that observed with KH_p and EPN_2, thus confirming the suitability of WA for estimating populations of *R. solani* in soil. It should be noted, however, that locating and quantifying colonies of *R. solani* was much more laborious on WA than on either KH_p or EPN_2 because of overgrowth of other fungi. Even after only 24 hr of incubation on WA, so many fungal colonies requiring microscopic inspection were present that use of this medium was somewhat time-consuming. Based on our experiences in conducting these experiments, we conclude that the convenience of quantifying colonies of *R. solani* on the two selective media was worth the extra time spent in media preparation.

As originally formulated (22), the EPN medium contained a final concentration of 5% ethanol. In our studies, this concentration was consistently found to be highly inhibitory to a representative isolate of *R. solani* when sclerotia were placed directly on the medium. In the original paper describing EPN_5 (22), organic matter extracted from soil was suspended in water and washed into petri dishes containing the medium, and the dishes were then left uncovered until all surface water had evaporated. In the process, some fraction of the ethanol probably was diluted and volatilized, leaving an unspecified concentration of ethanol in the medium. The use of EPN_5 in this way probably results in volatilization of a sufficient amount of ethanol from the medium to permit nearly normal growth of *R. solani*. Because many procedures for quantifying *R. solani* populations in soil rely on placement of soil, organic matter, or baits directly onto prepared media (2,4,6–8,11,13,16–18,28), we chose a final ethanol concentration of 2% in subsequent experiments with EPN, so as to retain some of the selective properties of the medium while permitting greater growth of *R. solani*.

Rapid growth of isolates of *R. solani* was observed on both KH_p and WA. The growth rates of isolates of *R. solani* AG-2-1 through AG-5 on KH_p were equal to

Table 1. Mean growth rates of isolates of *Rhizoctonia solani* AG-1 through AG-5 on culture media[y]

Medium	Radial growth rate (mm/day)					
	AG-1 (7 isolates)	AG-2-1 (6 isolates)	AG-2-2 (7 isolates)	AG-3 (5 isolates)	AG-4 (8 isolates)	AG-5 (2 isolates)
Water agar (WA)	22.9 a[z]	5.6 a	10.6 a	5.0 a	14.2 a	16.4 a
Ko and Hora's + prochloraz (KH_p)	12.8 b	6.3 a	9.9 a	5.7 a	11.2 a	13.7 a
Ethanol-potassium nitrate with 2% ethanol (EPN_2)	1.9 c	1.4 b	2.3 b	1.5 b	3.1 b	3.1 b

[y] Statistical analyses of all data were conducted on $\log_{10}$-transformed data.
[z] Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD statistic ($P = 0.05$).

Table 2. Populations of indigenous soilborne fungi and bacteria on several culture media[x]

Medium	Aerobic fungi (cfu/g dry soil)			Aerobic bacteria (cfu/g dry soil)		
	Garland clay loam	Wycolo sandy clay loam	Valentine sandy loam	Garland clay loam	Wycolo sandy clay loam	Valentine sandy loam
Peptone-glucose agar	193,000 a[y]	215,000 a	279,000 a	...	...	...
Henrici's medium	...	...	...	2.38×10^9 a	2.15×10^8 a	1.76×10^8 a
Water agar (WA)	ND[z]	192,000 a	272,000 a	ND	6.44×10^7 b	1.61×10^7 b
Ko and Hora's + prochloraz (KH_p)	4,100 b	537 b	3,540 b	499 b	207 c	0 c
Ko and Hora's + prochloraz and metalaxyl (KH_{pm})	ND	920 b	4,900 b	ND	153 c	0 c
Ethanol-potassium nitrate with 2% ethanol (EPN_2)	ND	123 c	1,088 b	ND	0 d	0 c
Ethanol-potassium nitrate with 5% ethanol (EPN_5)	2,288 b	16 d	272 c	8 c	0 d	0 c

[x] Statistical analyses of all data were conducted on $\log_{10}$-transformed data. Values are means of plate counts taken from 10-fold dilution series.
[y] Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD statistic ($P = 0.05$).
[z] ND = no data.

that observed on WA, and the growth rate of AG-1 on KH_p was quite rapid even though significantly slower than on WA. The rapid growth of *R. solani* on KH_p and WA permitted easy visualization of colonies of *R. solani* 3 days after placement of organic matter extracted from soil onto these media. In contrast, the growth rate of all five anastomosis groups tested was greatly reduced on EPN_2 despite the reduction in ethanol concentration from 5 to 2%. The slow growth of *R. solani* on EPN_2 required incubation of petri dishes for 7 days before examination.

Inhibition of growth of *R. solani* using EPN_2 could be advantageous under some circumstances. For example, plate counts of colonies of *R. solani* in a standard dilution series would be facilitated if colony growth were restricted using EPN_2, which would limit colony overgrowth. The same may also be true of using a pellet soil-sampler (8) that places soil pellets close to one another on the agar surface. With the MPN technique we used to quantify propagules of *R. solani* in a dilution series, aliquots of extracted organic matter can be well separated and only the presence or absence of viable propagules of *R. solani* in each aliquot needs to be determined. Colony overgrowth is therefore not a concern using an MPN technique. In this case, KH_p has an advantage over EPN_2 because the rapid growth rate of *R. solani* allows data to be collected after 3 days rather than 7.

A disadvantage we found using EPN_2 was the expense owing to the high cost of the antibacterial antibiotic tobramycin. Suppression of bacteria was excellent on KH_p, which contained the relatively inexpensive antibiotics chloramphenicol and streptomycin. It may be possible to substantially lower the cost of EPN_2 and still retain the advantages of the medium by substituting other antibiotics for tobramycin.

No detrimental effect on the growth of a representative isolate of *R. solani* was observed when the fungicide metalaxyl was added to KH_p. This was not surprising, in that the spectrum of activity of metalaxyl is limited principally to oomyceteous fungi (24). Nevertheless, this experiment was performed to rule out the possiblity of an interaction of metalaxyl with other components of KH_p. No improvement in suppression of indigenous fungi was found in two soils when metalaxyl was added to KH_p. This could be due either to low population densities of oomycetes in these soils or to suppression of oomycetes by other components of KH_p. In any case, the lack of an inhibitory effect of KH_{pm} on *R. solani* indicates that this modification of KH_p could be useful in soils with high populations of oomycetes.

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