Improved Medium for Isolation of *Trichoderma* spp. from Soil

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ABSTRACT

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A semiselective medium, previously developed for the isolation and enumeration of *Trichoderma* spp. from soils, was not effective with soils containing rapidly spreading Mucorales. The medium was improved and used effectively to recover *Trichoderma* spp. from soil by the addition of alkylaryl polyether alcohol at 2.0 ml/L alone or in combination with sodium propionate. The basal medium contained (per liter): V-8 juice, 200 ml; water, 800 ml; agar, 20 g; and glucose, 1 g. The improved medium, designated TME-SA, contained the following antimicrobial agents (μg/ml): neomycin sulfate, bacitracin, penicillin G, and chloroneb, 100; chlortetracycline hydrochloride, 25; nystatin, 20; and sodium propionate, 500. Alkylaryl polyether alcohol was added at 2.0 ml/L. For benomyl-tolerant biotypes of *Trichoderma* spp., the medium was supplemented with benomyl at 10 μg/ml and designated TME-ben10-SA. Both of the modified media allowed *Trichoderma* spp. to develop on the surface of the agar and effectively suppressed rapidly growing fungi such as *Rhizopus*.

A semiselective medium was described recently for the isolation and enumeration of *Trichoderma* spp. from soil (3). The new *Trichoderma* medium E (TME), with (TME-ben10) or without benomyl, gave good results with soils that did not contain rapidly spreading fungi such as species of *Rhizopus* and *Mucor* (3). Dilution plates from soils that contained these fungi, however, even with sodium propionate (an antifungal agent [7]) in the medium, were covered quickly by spreading colonies of *Rhizopus* that obscured *Trichoderma* colonies and hindered the counting.

The objective of this study was to find new antimicrobial agents that would inhibit spreading fungi without affecting *Trichoderma* spp. and to improve media TME and TME-ben10 for accurate counts of *Trichoderma* spp. from soils.

MATERIALS AND METHODS

Three wild strains and three benomyl-tolerant biotypes of *Trichoderma* were used in these studies. Strains WT-6 (*T. harzianum* Rifai) and T-1 (*T. viride* Pers. ex S. F. Gray) were obtained from H. D. Wells, Tifton, GA, who gave these designations. Strain 433-19 (*T. hamatum* (Bon.) Bain.) was isolated and identified by M. Dunn, Soilborne Diseases Laboratory. The benomyl-tolerant biotypes TMP-R1 and WT-6-24 of *T. harzianum* and biotype T-1-R4 of *T. viride* were developed by the senior author through ultraviolet light irradiation and selection (4,5). Conidia of wild strains and biotypes were obtained from 8-day-old cultures grown on V-8 juice agar at 25 C under continuous fluorescent light by adding a few milliliters of sterile water to the cultures and gently rubbing the surface with a cotton-tipped applicator. Conidia were counted in a hemacytometer and added to soil at 1 and 4×10^3 conidia per gram before dilutions were made.

TME and TME-ben10, both without sodium propionate, were used as the two basal media. In preliminary tests, the following antimicrobial agents were added to the two media to improve their selectivity by reducing or eliminating rapidly spreading fungi without affecting *Trichoderma* counts: hymexazol (Tachigaren 70% wettable powder, Sankyo Co., Yasu, Shiga-Ken, Japan), pentachloronitrobenzene (PCNB), metalaxyl 50% wettable powder, 2,6-dichloro-4-nitroaniline (DCNA), sodium propionate, and alkylaryl polyether alcohol (APA). The surfactant APA was previously used by VanEtten and Kolmark to inhibit radial growth of fast-growing *Fusarium solani* (6). Hymexazol, PCNB, metalaxyl, and DCNA were used at 0, 25, 50, 100, and 200 μg a.i./ml of medium; sodium propionate (SP) at 500; and APA at 0, 0.5, 1.0, and 2.0 ml/L.

The soil used was a Rumford sandy loam (pH 6.2) from Beltsville with a high population of *Rhizopus* spp. The soil did not contain any detectable natural population of *Trichoderma* spp. After addition of conidia to 1-kg soil batches, the soil was mixed thoroughly in a Hobart mixer, and 1:200 dilutions were prepared by suspending the equivalent of 1 g of air-dried soil in 199 ml of sterile tap water and shaking the suspensions by hand for 1 min. One-milliliter aliquots were removed from the containers while the liquid was agitated by a magnetic stirrer and spread on the media (six plates per replicate). The plates were incubated at 25 ± 2 C under continuous fluorescent light, and colonies were counted after 5–7 days. Four replicates were used throughout, and the experiments were done twice.

RESULTS AND DISCUSSION

In preliminary tests, DCNA, hymexazol, and metalaxyl did not inhibit *Rhizopus* spp. and *Mucor* spp. even at 200 μg a.i./ml. PCNB reduced the number and size of colonies of spreading fungi but, even at 25 μg a.i./ml, also reduced the number of colony-forming units (CFU) of *Trichoderma* (3). Although PCNB has been recommended as an ingredient for isolation of *Trichoderma* spp. from soil (1,2), we discontinued its further experimental use in our tests. Sodium propionate, and especially APA, reduced colony size and numbers of rapidly growing fungi such as *Rhizopus* without affecting *Trichoderma*.

A test was performed to determine the effect of increasing concentrations of APA (0, 0.5, 1.0, 2.0 ml/L) in the TME-ben10 medium on the recovery of conidia of the benomyl-tolerant biotype T-1-R4 of *T. viride* and on suppression of *Rhizopus* spp. and other rapidly spreading fungi. Aqueous suspensions of conidia were added to soil at 10 conidia

Table 1. Recovery of a benomyl-tolerant biotype (T-1-R4) of *Trichoderma viride*[y] from a soil with abundant *Rhizopus* spp. with the dilution plate method on *Trichoderma* medium E + benomyl (TME-ben10) supplemented with alkylaryl polyether alcohol (APA)

APA (ml/L)	Colony-forming units recovered (per gram of soil)	
	T-1-R4	*Rhizopus*
0	370 a[z]	2,050 ab
0.5	530 b	1,560 bc
1.0	490 b	650 c
2.0	740 c	250 d

[y] The soil was infested with 10^3 conidia of *T. viride* (T-1-R4) 24 hr before the assay.

[z] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

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per gram of soil. The conidia were mixed into the soil thoroughly, and dilutions (1:200) were made 24 hr later. The average numbers of colony-forming units recovered per gram of soil were 370, 530, 490, and 740 with APA concentrations of 0, 0.5, 1.0, and 2.0 ml/L, respectively (Table 1). The highest concentration of APA used was also the best for reducing recovery and colony size of *Rhizopus* spp. Because APA at 2.0 ml/L allowed the best recovery of *Trichoderma* (74% recovery), we used that concentration for all subsequent experiments. No attempts were made to find out why APA suppressed *Rhizopus* spp.

In addition to APA, we tested SP alone and in combination with APA. Conidia of *T. hamatum*, *T. harzianum*, and *T. viride* were added to soil at 4×10^3/g of soil, and dilutions of 1:200 were prepared 24 hr after conidia were added to soil. Sodium propionate at 500 μg a.i./ml, APA at 2.0 ml/L, and SP + APA were added to TME and TME-ben10 media after autoclaving (TME-SA and TME-ben10-SA, respectively). Dilutions (1:200) of the soil infested with wild strains of the three *Trichoderma* spp. were made on the TME medium; those of the soil infested with benomyl-tolerant biotypes were made on the TME-ben10 medium.

The combination of SP + APA in the TME medium allowed the highest number of colony-forming units of *Trichoderma* spp. and the smallest number of those of *Rhizopus* spp. and other rapidly spreading fungi to be recovered from soil (Table 2). Although small colonies of other fungi developed on the medium (Fig. 1), counting of *Trichoderma* colonies was feasible at this rather low soil dilution (1:200). Recovery of *T. hamatum* (433-19) and *T. viride* (T-1) was better than that of *T. harzianum* (WT-6). The low recovery of WT-6, however, may be attributed to strain sensitivity to the various antimicrobial agents present in the medium or to the more rapid decline of conidia of strain WT-6 than of the other strains when mixed with soil. It is of interest that conidial masses of WT-6 are white in contrast to other strains in this species.

Although recovery of the benomyl-tolerant biotypes WT-6-24 and TMP-R1 of *T. harzianum* was as good with APA as with SP + APA (Table 3), *Rhizopus* was suppressed more effectively with the mixture of the two additives than with either additive alone (Fig. 1). Recovery of biotype T-1-R4 of *T. viride* was more effective with APA than with the mixture. We noticed previously the sensitivity to SP of T-1-R4 and of some other ultraviolet light-induced biotypes of *Trichoderma*. Recovery of WT-6-24, a benomyl-tolerant biotype developed from WT-6 by irradiation, was also poor on the TME-ben10 medium with or without SP and APA.

The performance of the semiselective medium TME with and without benomyl developed for the direct isolation and enumeration of *Trichoderma* spp. from soils (3) has been satisfactory with soils that do not contain high numbers of rapidly growing fungi such as *Rhizopus* spp. With such soils, the media can be used without SP or APA. If rapidly growing fungi are a problem in the isolation and enumeration of *Trichoderma* spp. from soils, the media can be improved sufficiently by APA alone or by the addition of SP at 500 μg a.i./ml together with APA at 2.0 ml/L.

Table 2. Recovery of *Trichoderma* spp. from a soil with abundant *Rhizopus* spp. with the dilution plate method on *Trichoderma* medium E (TME) supplemented with sodium propionate (SP) and alkylaryl polyether alcohol (APA) (TME-SA)

Additives to medium TME	Colony-forming units recovered (per gram of soil)[y]			
	T. hamatum (433-19)	*T. harzianum* (WT-6)	*T. viride* (T-1)	*Rhizopus* spp.
None	620 a[z]	230 a	860 a	2,340 a
SP	1,570 b	320 a	730 a	2,100 a
APA	3,550 c	1,630 b	3,270 b	400 b
SP + APA	4,100 d	2,030 c	4,150 c	40 c

[y] Conidia (4×10^3/g of soil) of each *Trichoderma* spp. were added to soil with a natural population of *Rhizopus* spp.

[z] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 3. Recovery of benomyl-tolerant biotypes of *Trichoderam harzianum* and *T. viride* from a soil with abundant *Rhizopus* spp. by the dilution plate method on *Trichoderma* medium E + benomyl (TME-ben10) supplemented with sodium propionate (SP) and alkylaryl polyether alcohol (APA) (TME-ben10-SA)

Additives to medium TME-ben10	Colony-forming units recovered (per gram of soil)			
	T. harzianum		*T. viride*	
	WT-6-24	TMP-R1	T-1-R4	*Rhizopus* spp.
None	120 a[z]	1,580 a	1,130 a	2,100 a
SP	80 a	1,500 a	1,070 a	2,050 a
APA	820 b	3,550 b	4,650 c	640 b
SP + APA	800 b	3,120 b	3,030 b	160 c

[z] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

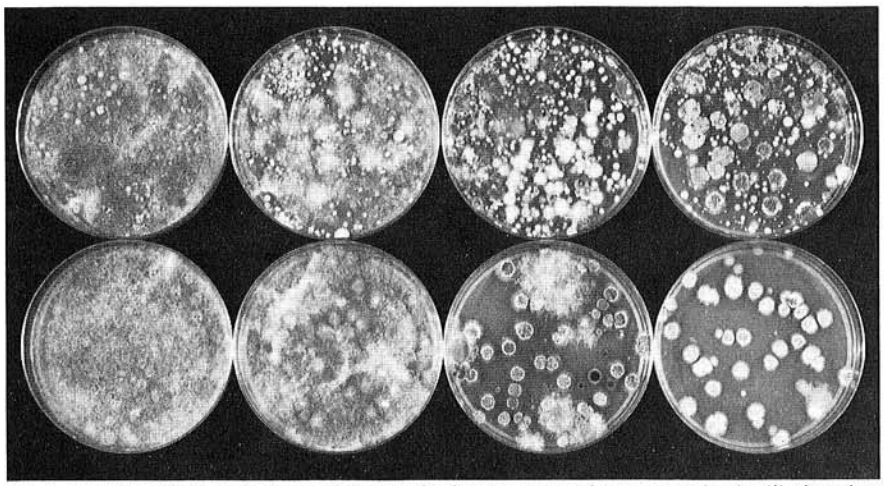

Fig. 1. Isolation of *Trichoderma viride* from soil with abundant *Rhizopus* spp. by the dilution plate method. **Upper row,** wild strain T-1 on *Trichoderma* medium E; **lower row,** benomyl-tolerant biotype T-1-R4 on *Trichoderma* medium E + ben10. **Left to right:** control, sodium propionate, alkylaryl polyether alcohol, sodium propionate + alkylaryl polyether alcohol.

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