Y. Elad et al. (1981) Phytoparasitica 9(1): 59-67.

A SELECTIVE MEDIUM FOR IMPROVING QUANTITATIVE ISOLATION OF *TRICHODERMA* SPP. FROM SOIL

Y. ELAD, I. CHET and Y. HENIS*

A *Trichoderma*-selective agar medium (TSM) was developed for quantitative isolation of *Trichoderma* spp. from soil. Selectivity was obtained by using chloramphenicol as a bacterial inhibitor, and pentachloronitrobenzene, p-dimethylaminobenzenediazo sodium sulfonate and rose-bengal as selective fungal inhibitors. The TSM also contains a low concentration of glucose which still allows relatively rapid growth and sporulation of *Trichoderma,* enabling convenient and rapid identification of *Trichoderma* colonies. All the 15 *Trichoderma* isolates tested formed colonies and grew well on this medium. Recovery of *Trichoderma* from artificially inoculated soils was high and was not affected by soil type or by other microorganisms. A positive correlation was observed betwen *Trichoderma* added to soil and counts of *Trichoderma* colonies on TSM plates. When combined with a soil pellet sampler, the selective medium was also used successfully for recovery of the indigenous *Trichoderma* population of natural soils.
KEY WORDS: Biocontrol; *Trichoderma harzianum.*

INTRODUCTION

Quantitative estimation of *Trichoderma* spp. in soil is often difficult because of the relatively rapid growth of other soil fungi on conventional agar media. In spite of the growing interest in these soil-inhabiting antagonists, no special selective medium for their isolation has been reported. However, *Trichoderma* spp. have been reported among soil fungi growing on versions of Martin's rose-bengal agar medium (9, 10, 15).

On Martin's medium, however, some *Trichoderma* isolates grow more rapidly than others, forming larger colonies which suppress the growth of other isolates, thus reducing colony counts. Furthermore, soil fungi such as *Rhizopus* spp., *Nucor* spp. and many species of imperfect fungi, often grow faster and prevent the development of *Trichoderma* colonies when dilutions of natural soil are quantitatively examined for the presence of *Trichoderma* spp.

Received April 9, 1980; received in final form Sept. 26, 1980.
* Dept. of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot.

Moubasher (11), Mughogho (12) and Smith and Dowson (14) have used soil-extract agar supplemented with rose-bengal for counting soil fungi, including Trichoderma spp. However, fungal colonies had to be marked every day and fast-growing fungi had to be transferred to other plates.

A direct counting technique for the quantitative estimation of *T. viride* conidia in barley flour inoculants was recently developed by Gindrat and Ricard (5), but it was found to be unsuitable for the estimation of *Trichoderma* in soil.

Isolates of *Trichoderma* spp. are potential biological control agents against soil-borne plant pathogens (1, 2). Two isolates of *T. harzianum* capable of hyperparasitizing the plant pathogens *Sclerotium rolfsii* and *Rhizoctonia solani* have been successfully used for the control of these pathogens under greenhouse and field conditions in natural and fumigated soils (3, 4, 6).

The purpose of this work was to develop a medium and a technique for the quantitative isolation of propagules of *Trichoderma* spp. present at any level either in natural habitats or artificially inoculated soils.

MATERIALS AND METHODS

The *Trichoderma*-selective medium (TSM) developed and used in this work consisted of the following components (g/l distilled water): $MgSO^4 \cdot 7\ H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.15; NH_4NO_3, 1.0; glucose, 3.0; chloramphenicol (Chloromycetin, Sigma Chemical Co., USA), 0.25; p-dimethylaminobenzenediazo sodium sulfonate (Dexon 60% w.p., Farbenfabrik Bayer A.G., Germany), 0.3; pentachloronitrobenzene (Terraclor 75 w.p., Olin Chemicals, USA), 0.2; rose-bengal (tetrachlorotetradiodofluorescein, BDH Chemicals Ltd., England), 0.15; agar (Difco Laboratories, USA), 20. Other media used in this work were synthetic medium (SM) (13) and Martin's rose-bengal medium (MRB) (10).

Fifteen different isolates of *Trichoderma* spp. originating from soil, wood tissue, and resting structures of plant-pathogenic fungi were used throughout this study. Of these, 13 were identified as *T. harzianum* Rifai and two as *T. viride* Rifai. They were grown on SM slants for 10 days at 20°C. Conidial suspensions were prepared by surface washing of the agar slants with 10 ml of sterile water. Aliquots (0.1 ml) of serial dilutions of the conidial suspensions were spread on the agar plates with a glass rod. Conidia of *T. harzianum* (isolate 203), *Aspergillus* sp. and *Penicillium* sp. which were obtained from SM agar slants were suspended in sterile water at a ratio of 2:1:1. Serial dilutions were used for surface plating of agar plates as described for *Trichoderma* spp. alone.

Average linear growth rates (ALG) were calculated by using the formula: ALG (mm/day) = [C5 − C1]/4, where C5 = colony diameter in mm after 5 days and C1 = colony diameter after one day of incubation. Total fungal soil population was determined as follows: 10 g of the sample was suspended in 100 ml of 0.1% agar (Difco) distilled water medium and incubated for 15 min in a rotary shaker (New Brunswick Scientific Co., USA) at 150 rpm. Serial dilutions were then made in six replicates, and

0.1 ml was pipetted into 90-mm-diameter petri dishes and spread with a glass rod on the agar surface. The plates were incubated for 5 days at 30°C.

Two soil types were tested: (i) Loamy sand soil composed of 82.3% and 2.3% silt, 15.0% clay and 0.4% organic matter; pH 7.4; moisture-holding capacity, 12.2%; and (ii) alluvial vertisol soil composed of 27% sand, 17% silt, 55.5% clay and 0.5% organic matter; pH 7.95; moisture-holding capacity, 40%.

Conidial suspensions of *T. harzianum* obtained from agar slants or from wheat bran cultures (3, 4) were used for artificial inoculation of soil samples. Conidial concentrations were determined with a hemocytometer. A soil pellet sampler, developed by Henis *et al.* (8), was used in combination with TSM as the selective medium. Fifteen soil pellets of 50 mg dry weight each were placed on every plate. *Trichoderma* colonies which developed from the soil pellets were counted after 4 days of incubation at 30°C.

RESULTS

Growth of Trichoderma *spp. isolates on TSM, MRB and SM*

Four out of the 13 *T. harzianum* (TH) isolates formed more colonies on TSM than on SM, whereas six TH isolates formed more colonies on TSM than on MRB. One out of the two *T. viride* isolates formed more colonies on TSM than on either MRB or SM (significantly different at $P = 0.05$). After 5 days of incubation, relative colony diameter on TSM and MRB, respectively, was within the range of 7.7–35.5% and 39.0–102.0% as compared with SM (61 mm). Similarly, average linear growth rate of the isolates on TSM and MRB was 8.5–50.5% and 44.0–90.0%, respectively, as compared with SM (12.9 mm/day).

Efficiency of TSM, MRB and SM in recovering Trichoderma harzianum *from artificially inoculated soil*

Natural and autoclaved soil samples inoculated with conidia of *T. harzianum* (isolate 203), were serially diluted in sterile water and the dilutions used to inoculate TSM, MRB and SM plates. After seven days of incubation, *Trichoderma* colonies were counted. Counting was impossible on SM either because colonies of germinating fungi could not be identified within 24–48 h or due to extensive growth of various fast-growing soil fungi, including *Trichoderma,* which totally masked slower growing fungi as well as each other. Counts of fungal population in soil on MRB were $(21.2 \pm 5.8) \times 10^3$ and $(83.0 \pm 7.1) \times 10^3$ for the loamy sand and alluvial soils, respectively, while on TSM, counts of all fungi other than *Trichoderma* were 2.4–4.8 times lower (Table 1). Only few fungi (*e.g. Aspergillus* sp. and *Penicillium* sp.) formed colonies larger than 3 mm in diameter. Recovery of *Trichoderma* on MRB from natural and autoclaved soils was within the range of 22–65% and 36–87%, respectively, whereas on TSM it ranged between 82 and 109% and was not affected by microorganisms existing in the natural soils. Recovery of 70–90% from both natural loamy sand and alluvial soils was achieved with TSM using wheat bran preparations of *T. harzianum* (isolate 203) as inoculum. Similarly, a positive correlation ($r = 0.996, P = 0.05$) was observed between

TABLE 1

RECOVERY OF *TRICHODERMA HARZIANUM* (ISOLATE 203) FROM ARTIFICIALLY INFESTED SOILS

Soil type	Medium[a]	Fungal population in soil (x 10^3)[b]	*T. harzianum* concn. (propagules/g soil) 500[c]	45,000	200,000	Recovery (%)
			Counts,[d] *Trichoderma*-colony-forming units/g soil (x 10^3)			
Loamy sand (natural)	MRB	21.1	0.28 a[e]	12.75 a	67.62 a	22-60
	TSM	4.4	0.47 c	40.75 d	183.25 d	87-98
Loamy sand (autoclaved)	MRB	0	0.42 b	18.25 bc	95.00 b	36-84
	TSM	0	0.48 c	43.75 d	185.25 d	82-103
Alluvial vertisol (natural)	MRB	83.1	0.27 a	15.25 ab	85.50 b	30-65
	TSM	34.9	0.45 bc	44.56 d	190.55 d	83-101
Alluvial vertisol (autoclaved)	MRB	0	0.39 b	22.00 c	132.00 c	37-87
	TSM	0	0.50 c	46.50 d	198.50 d	93-109

[a]MRB = Martin's rose-bengal medium; TSM = *Trichoderma*-selective medium.
[b]No *Trichoderma* colonies were observed at this dilution.
[c]According to counts of spore suspension with a hemocytometer.
[d]Average of six replicates.
[e]Numbers in each column followed by a common letter are not significantly different ($P = 0.05$).

the amount of *Trichoderma* propagules mixed into the two soil types and counts of colonies on TSM in this experiment (Fig. 1).

The size of *Trichoderma* colonies on TSM depended on the total number of colony-forming units (CFU) present on that plate. Thus, at concentrations of 15, 40 and 115 CFU per plate, average colony diameters (±SE) reached, respectively, 5.1 ± 1.9, 3.9 ± 0.7 and 2.5 ± 1.1 mm after 3 days' incubation, and 17.2 ± 3.9, 9.6 ± 2.5 and 5.5 ± 3.8 mm after 7 days' incubation.

Effect of Dexon on growth and sporulation of Trichoderma *spp.*

Percentage of sporulating colonies for the 15 tested *Trichoderma* isolates (3 days after incubation) on complete TSM was 12–100% as compared with 0–92% on TSM without Dexon. In ten of these isolates the difference was significant ($P = 0.05$). Similarly, average colony size for the 15 isolates was 3–100% larger on TSM. In seven of these isolates colony size on TSM was significantly larger ($P = 0.05$). Sporulation of the different isolates occurred 24–72 h earlier on the complete medium.

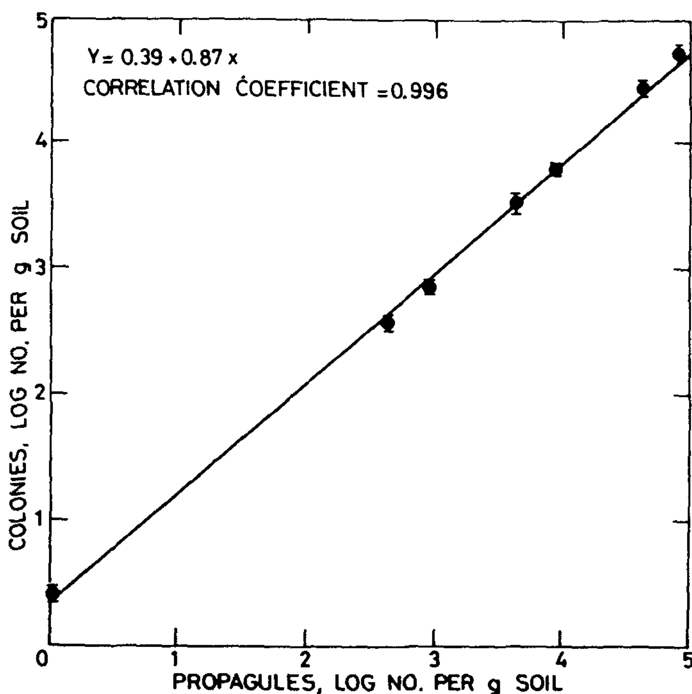

Fig. 1. Correlation between number of *Trichoderma harzianum* (isolate 203) propagules in soil (X) and their count on *Trichoderma*-selective medium (Y).

TABLE 2

GROWTH OF *TRICHODERMA HARZIANUM* (ISOLATE 203), *ASPERGILLUS* SP. AND *PENICILLIUM* SP. ON DIFFERENT MEDIA

Dilution	Medium[a]	Total colonies (no./plate)	Species ratio[b]	Colony diam. (mm ± S.E.)[d]	
				T. harzianum	*Penicillium* sp. & *Aspergillus*[e] sp.
1:10^6	MRB	8.2	0.56 a[c]	38.5 ± 3.7	20.5 ± 1.6
	TSM	7.1	0.64 b	10.0 ± 2.6	7.0 ± 3.1
1:10^5	MRB	34.7	0.34 a	19.0 ± 4.1	9.0 ± 3.2
	TSM	74.0	0.58 b	6.5 ± 1.5	4.0 ± 1.0

[a]MRB = Martin's rose-bengal medium; TSM = *Trichoderma*-selective medium.
[b]*T. harzianum* colonies per plate / *Aspergillus* sp. and *Penicillium* sp. per plate.
[c]Within each dilution, numbers followed by different letters are significantly different (P = 0.05).
[d]One week after inoculation.
[e]Isolates of these fungi which were isolated from soil and found to be able to grow on TSM.

TABLE 3

COUNTS OF *TRICHODERMA* SP. FROM SOILS CONTAINING LOW POPULATION LEVELS

Origin	Soil type	Counts of soil fungi by the dilution plate method (propagules/g soil)[a]				Counts of *Trichoderma* sp. with the pellet soil sampler, propagules/g soil (Average no.)	
		Trichoderma spp.		Other fungi			
		TSM[b]	MRB	TSM	MRB	TSM	MRB
Rehovot	Loamy sand	31 a[d]	18 a	8873 a[d]	20666 b	25.0 a[d]	0 b
Bet haShitta	Brown basaltic	–[c]	–	4866 a	43500 b	10.0 a	0 b
Tira	Brown red degrading sandy	–	–	7000 a	15110 b	10.0 a	0 b
Nezer Sereni	Brown red sandy	–	–	1500 a	2300 b	9.0 a	4.0 a
Besor	Loessial sandy	–	–	2367 a	32000 b	5.0 a	2.0 a
Hefzi Bah	Rendzina	–	–	1265 a	1967 b	7.4 a	3.0 a
Afula	Brown alluvial	–	–	8200 a	16500 b	2.0 a	0 a

[a]Soil was diluted to 1:100.

[b]TSM = *Trichoderma*-selective medium; MRB = Martin's rose-bengal medium.

[c]No *Trichoderma* colonies were observed at this dilution.

[d]Between each TSM and MRB couple within a soil type, numbers followed by a common letter are not significantly different ($P = 0.05$).

Comparative growth of Trichoderma, Penicillium, Aspergillus *and* Rhizopus *spp. on TSM and MRB*

In addition to *Trichoderma* spp., *Penicillium* sp. and *Aspergillus* sp. were also capable of growing on TSM. The linear growth on TSM and MRB of these fungi and of *Rhizopus* sp. was compared with the linear growth of *T. harzianum* (isolate 203). ALG of the tested isolates of *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp. and *T. harzianum* (isolate 203) on MRB were 2.65, 3.05, 12.50 and 5.4 mm/day, respectively, whereas on TSM rates of 1.05, 0.87, 0 and 1.98 mm/day, respectively, were recorded.

Conidia suspensions of *T. harzianum* (isolate 203), *Aspergillus* sp. and *Penicillium* sp. at a ratio of 2:1:1 were used for surface plating of MRB and TSM. Relative counts of *T. harzianum* (isolate 203) colonies were greater ($P = 0.05$) on TSM. Furthermore, relative average sizes of *Trichoderma* colonies as compared with *Aspergillus* sp. and *Penicillium* sp. colonies were larger (Table 2).

Recovery of indigenous Trichoderma *from natural soils*

Attempts to count natural populations of *Trichoderma* sp. present in different soils, using TSM and low dilution plating such as 1/50–1/10, failed. This failure was attributed to low natural populations in the soils tested.

The possibility of quantitatively assessing very low indigenous populations of *Trichoderma* sp. in natural soils was examined using a combination of TSM and the pellet soil sampler technique (8). Six out of the seven soils tested did not produce any colonies of *Trichoderma* sp. with the dilution plating method (Table 3). The general fungal populations in these soils ranged between 10^3 and 10^4 propagules per gram of soil. When the soil pellet sampler was used in combination with TSM, counts of *Trichoderma* sp. ranged from 1 to 25 propagules/g soil. When MRB was used with the soil pellet samples, four soil samples failed to show any *Trichoderma* whereas the other three yielded 2–4 propagules/g soil.

DISCUSSION

The selective medium (TSM) used in this work proved to be effective for the detection and estimation of *Trichoderma* spp. populations in both naturally infested and artificially inoculated soils. The selective effect of this medium is based on the fact that *Trichoderma* spp. are relatively tolerant to high levels of PCNB and rose-bengal, and on the capacity of *Trichoderma* spp. to grow and sporulate on media containing a low concentration of glucose. The effect of Dexon on *Trichoderma* is of special interest. Dexon (which is generally used against oomycetes) enhanced, rather than inhibited, both growth and sporulation of *Trichoderma* colonies. In the presence of Dexon, colonies of *Trichoderma* grew faster and readily developed their typical green color, which aided in their identification among other soil-borne fungi.

Satisfactory bacterial suppression was obtained by 250 μg/ml chloramphenicol.

TSM selectively inhibits *Rhizopus* spp. and *Mucor* spp. which usually spread over other fungal colonies on MBR (10) or other agar media used for counting populations of soil fungi, *e.g.* the soil-extract agar by Johnson and Curl (9), Monbasher (11), Mughogho (12), and Smith and Dowson (14). Other fungi, *e.g. Penicillium* spp. and *Aspergillus* spp., grew relatively slowly on TSM.

Fewer *Trichoderma* colonies were detected on MRB as compared with TSM and the counts on MRB were affected by the general fungal population present in natural soils. The smaller colonies of *Trichoderma* developing on TSM (as compared with MRB) do not compete with each other and are easier to count at high concentrations, so that statistical variability is diminished.

A high level of efficiency in recovery of *Trichoderma* from two different types of artificially inoculated soils was achieved with TSM. These results show that TSM combined with soil dilution plating can be used for the estimation of *Trichoderma* at relatively high population levels (>100 propagules/g soil). In order to count and isolate indigenous *Trichoderma* populations from natural soils, it was necessary to combine the selective medium and the pellet soil sampler originally developed by Henis *et al.* (8). Combination of the two techniques enabled the isolation and the quantitative estimation of *Trichoderma* populations present in seven different soils. It can be used for the quantitative estimation of *Trichoderma* populations in soils, on a large-scale basis for screening of soil for *Trichoderma* isolates, and may be of value in biological control research.

ACKNOWLEDGMENTS

This work was supported by a grant from Der Niedersachsische Minister für Wissenschaft und Kunst, West Germany. The authors wish to express their appreciation to Y. Hadar and J.S. Pullman for helpful suggestions, and to R. Guvrin, H. Harshemesh and A. Sivan for excellent technical assistance.

REFERENCES

1. Baker, K.H. and Cook, R.J. (1974) Biological Control of Plant Pathogens. W.H. Freeman, San Francisco, CA.
2. Chet, I., Hadar, Y., Elad, Y., Katan, J. and Henis, Y. (1979) Biological control of soil-borne plant pathogens by *Trichoderma harzianum. in*: Schippers, D. and Gams, W. [Eds.] Soil-Borne Plant Pathogens. pp. 585-592. Academic Press, London.
3. Elad, Y., Chet, I. and Katan, J. (1980) *Trichoderma harzianum:* A biocontrol agent of *Sclerotium rolfsii* and *Rhizoctonia solani. Phytopathology* 70:119-121.
4. Elad, Y., Katan, J. and Chet, I. (1980) Physical, biological and chemical control integrated for soil-borne diseases in potatoes. *Phytopathology* 70:418-422.
5. Gindrat, D. and Ricard, J.L. (1976) Counting techniques for *Trichoderma viride* conidia dispersed in barley flour inoculants. *Pl. Dis. Reptr* 60:321-325.
6. Hadar, Y., Chet, I. and Henis, Y. (1979) Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum. Pathopathology* 69:64-68.
7. Henis, Y. and Chet, I. (1975) Microbiological control of plant pathogens. *Adv. appl. Microbiol.* 19:85-111.

8. Henis, Y., Ghaffar, A., Baker, R. and Gillespie, S.L. (1978) A new soil-pellet sampler and its use for the study of *Rhizoctonia solani* population in soil. *Phytopathology* **68**:371-376.
9. Johnson, L.F. and Curl, E.A. (1972) Methods for Research on the Ecology of Soil-Borne Plant Pathogens. Burgess Publishing Co., Minneapolis, MN.
10. Martin, J.P. (1950) Use of acid, rose-bengal and streptomycin in the plate method for estimating soil fungi. *Soil Sci.* **69**:215-232.
11. Moubasher, A.M. (1965) Selective effects of fumigation with carbon disulphide on the fungus flora. *Trans. Br. mycol. Soc.* **46**:338-344.
12. Mughogho, L.K. (1968) The fungus flora of fumigated soils. *Trans. Br. mycol. Soc.* **51**: 441-459.
13. Okon, Y., Chet, I. and Henis, Y. (1973) Effect of lactose, ethanol and cycloheximide on the translation pattern of radioactive compounds and on sclerotium formation in *Sclerotium rolfsii. J. gen. Microbiol.* **74**:251-258.
14. Smith, N.R. and Dowson, U.T. (1944) The bacteriostatic action of rose-bengal in media used for plate counts of soil fungi. *Soil Sci.* **58**:467-471.
15. Tsao, P.H. (1970) Selective media for isolation of pathogenic fungi. *A. Rev. Phytopath.* **8**: 157-186.