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SHORT COMMUNICATION

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A novel method for the identification and enumeration of microorganisms with potential for suppressing fungal plant pathogens

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Abstract This paper describes a method that allowed counting of both the total culturable and antagonistic microorganisms in a given source such as compost. *Fusarium solani*, used as the test fungus, was spread-plated on quarter-strength (1/4) potato dextrose agar (PDA), its surface was exposed in a laminar flow for 4 h and then another layer (2–3 mm thick) of 1/4 PDA was poured over it, on which an appropriate dilution of a compost sample was spread-plated. Microorganisms in the compost samples appeared first, and were counted as total culturable organisms. Plates were further incubated until *F. solani* grew through the upper layer of PDA (generally in 4–8 days) and covered the whole plate including most of the microbial colonies, except for a few which had a halo around them. These were counted as antagonistic, and they were isolated and purified for further studies. The population of bacteria in the six specific compost samples (called Biodynamic or BD preparations by organic farmers) ranged from 3.45 $\log_{10}$ (in BD502) to 8.59 $\log_{10}$ (in BD504) per gram of materials. The population of antagonistic bacteria was counted for three of the six compost samples, and ranged from 3.24 $\log_{10}$ (in BD502) to 6.90 $\log_{10}$ (in BD500). Of the 67 bacterial isolates showing a halo that were assembled from different sources, 17 suppressed at least 1 of the 4 plant pathogenic fungi against which these were evaluated using the dual culture method.

Keywords Two-layer method · Dual plate method · Enumeration · Antagonistic microorganisms · *Fusarium solani* · Biodynamic preparations

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Introduction

Food crops (grains, fruits, vegetables) grown without the use of chemical fertilizers and pesticides are generally termed as "organic food". A recent UN survey found that farmers in at least 130 countries produce organic food commercially on over 7 million ha (International Trade Centre 1999) of land. Demand for organic food is growing rapidly, particularly in the developed world; the area sown to organic food in the European Union increased 35-fold between 1985 and 1999 (Halweil 2000). Farmers growing organic food use alternative sources of fertilizers and pesticides. Microorganisms with the ability to suppress plant pathogenic fungi and insect pests are potentially important alternatives to chemical pesticides and have been studied by many researchers (Chet and Inbar 1994; De Weger et al 1995; Heimpel and Angus 1959). Organic farmers have reported reduced incidence of diseases and insect pests (Fukuoka 1993; USDA 1980). Such farmers in Karnataka, India, in 1998, stated that their crops generally had less disease incidence than those of their neighbors following mainstream agricultural practices. It was hypothesized that some of the alternatives to chemicals they used had a high population of microorganisms that suppressed the growth of disease-causing fungi. Efforts to verify this hypothesis led to the development of a laboratory method for direct identification and enumeration of microorganisms having the potential to suppress disease-causing fungi (antagonistic microorganisms) in the presence of other naturally occurring microorganisms, which is described here.

Materials and methods

Potato dextrose agar (PDA), a routine medium known to allow the growth of most bacteria, almost all fungi and some actinomycetes, was used. Samples of six different composts, available commercially as biodynamic or BD preparations (different herbs as raw material), and used by some organic farmers in Karnataka and Tamil Nadu, India, were obtained from Kurunji Organic Foods (Genguvarpatti 625 203, Tamil Nadu, India). Each type of compost was prepared following a specific protocol. The different composts

were important components of a crop husbandry practice known as biodynamic agriculture (Proctor et al. 1997). Biodynamic agriculture is an organic farming system that utilizes fermented herbal and mineral preparations as compost additives and field sprays (Carpenter-Boggs et al. 2000). The preparations used in this study were BD 500 which is decomposed cow-dung, and BD 502–506 which are fermented herbs. The other three types were: compost prepared from fallen leaves and kitchen waste (taken from backyard of a house where no chemical fertilizer or pesticides had been used for at least 10 years), termitaria soil from a dead tree trunk, and material from the axils of leaves of *Billbergia* spp. (This ornamental plant has grass-like leaves having a space at its axils. When growing in a garden, particularly under trees, its axils have been noticed to have a natural deposition of well decomposed plant debris and dead bodies of insects). Three replications for each of the nine composts were used. The data, transformed to log scale, were analyzed as one-way ANOVA taking the nine composts as treatments.

Two-layer method

Approximately 30 ml 1/4 PDA was poured into a sterilized petri-plate (95 mm diameter) and allowed to dry for 45 min, in a laminar flow. A 4- to 5-day-old sporulating culture of *F. solani* grown on 1/4 PDA plate was scraped, suspended in 5 ml sterile water using a Tissumizer Mark II (from Tekmar, Cincinnati, Ohio) blender. The fungal suspension (0.1 ml) was spread-plated on the previously prepared 1/4 PDA plates. The plates were surface-dried in a laminar flow for 4 h. A second layer of 1/4 PDA (20 ml) was then poured on top of the first layer. Care was taken that the temperature of the molten PDA was 45°C. The plates were again surface-dried in a laminar flow for 45 min. An appropriate dilution of the likely source of antagonistic microorganisms was spread-plated on top of the second layer. Appropriate dilutions were used to enable the counting of microorganisms on the plates. The plates were incubated at 28±2°C and scored for different types of microorganisms.

Colonies of microorganisms showing a halo were isolated and purified on 1/4 PDA and assigned a number prefixed with BCB (Biological Control Bacteria) or BCF (Biological Control Fungi). Care was taken to pick up those colonies which showed a distinct difference in morphological characters on the top layer of 1/4 PDA. Sixty seven of the bacterial isolates were studied for their effect on four other plant pathogenic fungi by the dual culture method (Huang and Hoes 1976). These fungi were *Rhizoctonia bataticola* (causes dry root rot of chickpea), *Sclerotium rolfsii* (collar rot of chickpea), *Fusarium oxysporum* (chickpea wilt) and *Aspergillus flavus* (aflatoxin of groundnut). The first three were obtained from S. D. Singh, and *A. flavus* from R. P. Thakur, ICRISAT.

In the dual plate method, the test fungus was suspended in sterile water and inoculated at the center of a 1/4 PDA plate and the four different BCBs (log phase) were spotted at four areas of the plate equidistant from the center. Considering the relative rapidity of growth of these pathogenic/test fungi (slow growers) and the BCBs (fast growers) on 1/4 PDA, fungi were inoculated 1 day before the bacterial inoculation.

To verify that the pathogenic fungus plated on top of the lower layer grew through the upper layer of PDA, thin sections of agar were periodically cut through the two layers (top to base), placed on glass slides and stained with Fluor Solution A (Polysciences, Washington, Pa.). This chemical selectively stains different structures (mycelium, spores) of fungi. Observations were then made under a UV-fluorescent microscope (Olympus, Vanox, Japan).

Results

Some bacteria and fungi did not grow well on the PDA obtained from HiMedia Laboratories, Mumbai, India. Its use was therefore discontinued. All studies reported here were performed on laboratory-prepared PDA (Johnston and Booth 1983) after inconsistencies were noted using commercially available PDA (from HiMedia Laboratories, Mumbai, India). Some bacteria grew very fast on full-strength PDA (pH 5.5) but at a normal rate on 1/4 strength PDA (pH 5.7). Fungi also grew well on 1/4 PDA.

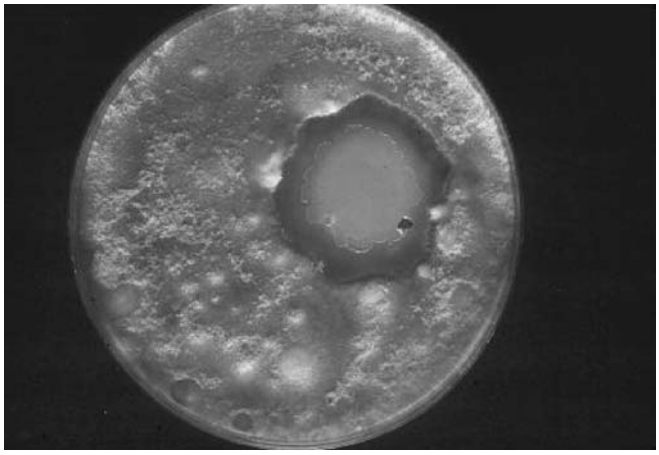

Fig. 1 Growth of the test fungus from the lower agar layer to the upper agar layer by day 8. Note one large and four small colonies of bacteria having a halo. More bacterial colonies have growth of the fungus above them, but can still be seen

Within 12–18 h, bacterial colonies were visible on top of the second layer of the two-layer plates. Fungal colonies appeared about 72 h after inoculation. During day 3 to day 5 the fungus (*F. solani*) plated on the lower layer grew through the upper agar layer. In about another 4 days, it grew over all the bacterial/fungal colonies (formed on the upper layer) except those antagonistic to it (Fig. 1). These colonies had a clear halo around them and were regarded as potential antagonists of the fungus. Microorganisms from colonies with a clear halo (>2 mm wide) were purified for further studies.

Observations under UV-fluorescent microscope of the stained two-layered agar slices at different intervals showed mycelium growing through the upper layer of 1/4 PDA. Absence of fungal growth on the top of second layer for at least the first 2 days was also indicative of the fact that subsequent growth of *F. solani* on top of the two-layer plates was indeed due to the inoculum placed in the lower layer.

Of the nine different sources of microorganisms studied (Table 1), the biodynamic preparation BD504 had the highest population of bacteria (8.59 $\log_{10}$ g^{-1} compost). BD502 had the lowest population of bacteria (3.45 $\log_{10}$ g^{-1} compost) but a high population of fungi (5.3 $\log_{10}$ g^{-1}). There were no detectable populations of fungi in five of the nine sources at the dilutions used in the study (Table 1). Interestingly, the apparently dry soil of termitaria was rich in both bacteria (6.54 $\log_{10}$ g^{-1} soil) and fungi (4.72 $\log_{10}$ g^{-1} soil). The population of antagonists was not counted in three of the nine materials because these three were processed before the two-layer method was fully developed. In the other six, the count of

Table 1 Microbial population in different composts (* the dilutions used for spread plating did not produce any fungal colonies, *NC* not counted, *BD* biodynamic preparation, *SE* effective standard error). *Data in parentheses* are percentage of colonies with halo

Source	Total microbial population (log_{10} g^{-1})		Population of antagonists (log_{10} g^{-1})		Total no. of colonies on two replicate plates used for counting
	Bacteria	Fungi	Bacteria	Fungi	
BD 500	7.35	*	6.90[a]	*	45 (36)
BD 502	3.45	5.30	3.24[a]	3.98[a]	97 (26)
BD 503	7.10	*	NC	NC	152 (NC)
BD 504	8.59	*	NC	NC	203 (NC)
BD 505	6.63	*	NC	NC	137 (NC)
BD 506	4.91	4.26	4.28	4.22	527 (13)
Backyard compost	6.65	*	5.69[a]	*	90 (11)
Billbergia sp.	6.52	5.60[a]	6.08[a]	5.60[a]	111 (12)
Termite soil	6.54	4.72[a]	5.01[a]	2.74[a]	81 (32)
SE	0.080	0.059	0.409	1.328	
CV%	1.9	1.7	12.1	45.4	

[a] Counted from plates with <30 colonies, therefore data should be used with caution. Data from plates of lower dilutions were not collected because there was poor expression of the halo due to a very high population of other microorganisms

bacteria was generally higher than that of fungi. BD500 had the highest (6.90 log_{10} g^{-1} soil) and BD502 the lowest (3.24 log_{10} g^{-1} soil) population of bacteria. The antagonistic fungal population was highest (5.60 log_{10} g^{-1} soil) in organic material collected from leaf axils of *Billbergia* spp. and lowest (2.74 log_{10} g^{-1} soil) in termitaria soil (Table 1).

A total of 1,443 colonies (ranging between 45 in BD500 to 527 in BD506) were observed (on the plates used for counting microorganisms) from the nine samples. Of these colonies (total number 161), 11–32% exhibited halos around them. Of these, 67 bacterial colonies with the largest halo (>2 mm) were picked up for further studies. All 67 were evaluated for their ability to suppress four different plant pathogenic fungi (see Materials and methods) using the dual culture method. Of the 67 isolates, 17 suppressed at least one of the four disease-causing fungi. Eight (BCB 69, 74, 91, 97, 103, 106, 111, 116) of the 17 suppressed *R. bataticola*, 7 (BCB 75, 111, 116, 117a, 122, 123, 135) suppressed *A. flavus* and 5 (BCB 85, 98, 114, 116, 117b) suppressed *S. rolfsii*. Two isolates (BCB 111 and BCB 116) suppressed three (*A. flavus*, *R. bataticola* and *S. rolfsii*) of the four pathogens.

Discussion

The success of this method depended on the ability of the fungus *S. rolfsii* spread-plated on the lower layer to grow through the upper layer of agar medium and grow uniformly. Our experience indicated that many aerobic microorganisms are able to grow within an agar-based medium of the pour-plate method. The aerobic bacterium *Bacillus subtilis* grew well and could be counted using pour-plate methods. Also, all the other fungi (*Aspergillus flavus*, *Aspergillus awamori*, *Metarhizium anisopliae, Trichoderma viride* and *Beauveria bassiana*) that we tried, grew through the upper layer. This was confirmed by microscopic examination of the sliced sections of the two layers of agar at different intervals after spread-plating a suspension of a given fungal strain. Care, however, was required that the fungal inoculum spread-plated on the surface of the lower layer was dry enough, and did not dislodge and rise to the surface of the second layer at pouring. This would potentially affect the counting of microorganisms spread-plated on the surface of the second layer.

The results reported here suggest that the two-layer method enabled the counting of the population of antagonists in compost samples—which is not feasible with the dual culture method. In addition, we counted the total population of culturable microorganisms in these samples (Table 1). This method enabled us to isolate morphologically distinct bacterial strains for further studies, and not only in composts; we could also count the total culturable microbial population in soil samples (unpublished studies). As was confirmed in subsequent studies, the population counts using the two-layer method matched well with those on PDA plates (data not shown). A high population of antagonists (>1,000 per g compost) was indeed present (Table 1) in the composts used by organic farmers in Karnataka and Tamil Nadu, India, and may be the reason for the low incidence of diseases and insect pests. This will need confirmatory studies.

The two-layer method allows the use of two different media in the upper and lower layers. In an unpublished study, we used glucose-cassamino acids-yeast extract (GCY) medium in the lower layer for growing *A. flavus* and 1/4 PDA as the upper layer. This combination of the two different media was used for identification and enumeration of antagonistic microorganisms in the rhizosphere soil of groundnut.

To obtain dependable counts of microorganisms in a sample of soil or compost, a plate should have only 30 to 300 colonies (Ross 1983). It sometimes required a repeat to fulfill this condition because the population of antagonists will only be a fraction of the total count on a given plate. In some cases it was still not possible to reach this minimum of 30 colonies per plate because the high

population of other microorganisms (without halo) per plate perhaps did not allow the expression of antagonists.

Most of the 67 isolates that showed a halo with the two-layer method did not show a clear zone when grown besides *F. solani* using the dual culture method (a microorganism antagonistic to another, forms a characteristic zone of no growth in a dual culture method when they are grown beside each other on a culture plate). However, *F. solani* did not grow over the colonies of these strains generally noticed with cultures not having halos in the two-layer method. It seems that the mechanism of action of these isolates (exhibiting a halo in the two-layer method but no zone formation in the dual culture method) is different to just secretion of some toxin into the medium and needs further study.

After mastering the method, it was possible for a technician to plate at least 50 soil/compost samples in a week and complete the observations/isolation of promising microorganisms in the following week. Besides counting the total population of microorganisms (bacteria, fungi and actinomycetes), the two-layer method also allowed counting of potentially antagonistic microorganisms in a soil or compost sample which was not possible with any other existing method.

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