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Split-agar assay of antifungal soil microbial metabolites

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ABSTRACT

Soil microorganisms suppress soil borne plant pathogenic fungi through various mechanisms. There is no appropriate, integrated method to easily quantify soil health in terms of disease control ability. A novel assay to quantify the ability of soil to inhibit fungal pathogens is described. The technique is easy to use routinely in soil biology investigations for soil quality testing as it offers an integrated expression of suppressiveness as actidione equivalents per gram of soil. Soil samples were inoculated into liquid growth medium and incubated; supernatants were filter sterilized and assayed in split agar against *Macrophomina phaseolina* to record colony radius. The antifungal activity of the soils varied widely ranging from 0.02 to 2.80 mg actidione equivalents g^{-1} soil, of which 81–98% was heat labile. The test will be a useful aid in decision support for reducing the use of chemical control agents and promote sustainable farming practices.

1. Introduction

Sustainable development requires us to maintain soils in a productive and healthy state. Global efforts have been underway for long to devise various tests for the physical, chemical and biological indicators of soil quality. While the first two are fairly standardized, the concept and methods of a soil biological test have remained as a complex goal. Various fractions of soil organic matter, microbial biomass, soil respiration and soil enzymatic tests have been proposed and tested as indicators of soil biological quality but till date there is no easy test that can be used for assaying soil health, for example ability of soil to suppress plant diseases. There is thus a need for a simple method to quantify the anti-fungal antibiosis potential of soils to assay soil suppressiveness quantitatively.

Soils vary in their inherent ability to inhibit the growth of pathogenic fungi (Alabouvette, 1986). This ability is influenced by many abiotic (pH, nitrogen content, etc.,) and biotic (composition of soil microbiota) factors, the latter being more influential (Bonilla et al. 2012). Soil microflora suppress fungal pathogens through a variety of mechanisms like competitive exclusion, antagonism and induction of host plant defense mechanisms (Mazzola, 2002). Antagonism has been most investigated since the population of antagonists can be built up in soils to improve soil suppressiveness. Antagonism to phytopathogenic fungi is usually improved by inoculating microorganisms producing antifungal metabolites (Mazzola, 2007). Strategies adopted by microorganisms to overcome competition in natural (soil) or artificial (culture media) ecosystems include production of antimicrobial compounds, higher growth rates, higher affinity for essential nutrients (e.g., iron binding by siderophore production) among others. Of these, antibiosis has been the most routinely used assay. A classical method of studying antibiosis in soils is the crowded plate technique (Cappuccino and Sherman, 2008) where antagonists are obtained by inducing competition among soil microbes. This method has been adapted and modified by Rupela et al. (2003) to obtain fungal antagonists from soil. Although such methods are very useful to screen for antagonists in soil, but they are not suitable for quantification purpose as the clearing zones are counted without taking into consideration their diameter. This makes comparative assessments of soil suppressiveness difficult. Quantification of antagonists may be achieved by extracting the anti-fungal metabolites from soils and analysis by gas chromatography or HPLC (Chuankun et al. 2004), but this requires more time and expertise and is not useful to quantify unknown metabolites. For routine assays, there is a need for a simple method to quantify the antifungal *anti*biosis potential of soils. We report the development of a method to quantify the potential to elaborate anti-fungal metabolites by various soils when inoculated and incubated in culture broths. The principle is similar to crowded plate but the competition is induced here in broth than in petri plates and the anti-fungal activity is quantifiable. Soil-water extracts have very low amount of antifungal compounds and large amounts would need to be vacuum concentrated to reduce the volumes for the

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assay. To overcome this, the potential antimicrobial production by antagonists was amplified by incubation in broth. Secondly, soil suspensions only show up the amount of antimicrobials produced until the point of sampling but the actual potential of the soil to produce antifungal compounds can be known only by an incubation assay.

2. Materials and methods

2.1. Soil samples

Nine soil samples, belonging to two different soil orders, Entisols and Vertisols, with varying cropping histories were sampled to assess the antagonistic behavior of the soils towards a plant pathogenic fungus *Macrophomina phaseolina*. The salient properties of the soils are given in Table 1. The Entisols of Hanumangarh district of Rajasthan include farmers' fields cropped to cowpea and citrus orchards under long term organic and conventional chemical management. The organic management included application of fermented cattle manures where as conventional management included fertilizers and pesticides at recommended doses. The Vertisols of Guntur, Andhra Pradesh include soils from farmers' fields cropped to chili and black gram under two different levels of intensive chemical farming using a range of chemical fertilizers and pesticides.

2.2. Metabolite production by soil microorganisms

Five gram of soil was added to 50 ml of Mueller-Hinton broth (Atlas, 1995) (pH 7.0) and incubated for 7 days at 28 °C under shaking at 125 × g. The competition for nutrients among the soil microorganisms favors the proliferation of those that can suppress others through production of antimicrobial metabolites in the broth. Incubation of seven days was kept to ensure production of antimetabolites from the maximum number of microorganisms (of different growth rates) in the soil inoculum. The broth was centrifuged at 10,000 × g for 30 min to allow the soil particles and microorganisms to settle. The supernatant was filter sterilized by passing through a 0.20 μm pore sized cellulose acetate syringe filter (Advantec MFS Inc., Japan) and stored at 4 °C. Sterile Mueller-Hinton broth without soil inoculation was treated similarly and served as the control.

2.2.1. Plate Assay

Sterile glass slides of 84 × 25 mm were placed in the centre of the sterile petriplates of 90 mm diameter so as to divide the plate in two equal compartments (Fig. 1). The left half (A) was filled with 10 ml of molten Sabouraud's dextrose medium (pH 5.6) containing 1% agar (Atlas, 1995). The medium was allowed to solidify and the glass slide was removed carefully without disturbing the gel. The right half (B) of the plate was then filled with a mixture of 5 ml of molten Sabouraud's dextrose medium with 2% agar and 5 ml of the supernatant obtained above (final concentration of agar is 1%). The medium was then allowed to solidify and the plates were kept for 2 h to allow the soil microbial metabolites to diffuse from compartment B to compartment A. All the operations were carried out under aseptic conditions in a laminar flow clean air work station. For quantification of anti-fungal activity, different concentrations of actidione (Cycloheximide, Hi-Media Laboratories, India) solutions ranging in concentration from 10 to 0.0001 mg ml^{-1} (serial dilutions) were taken and in incorporated in place of the supernatant in compartment B. Double distilled water served as a blank (Fig. 1).

Macrophomina phaseolina is a plant pathogen causing charcoal rot in many plant species (Smith and Carvil, 1977). It was used as a test fungus to assess the antifungal activity of the soils. *M. phaseolina* was grown on Sabouraud's dextrose medium and small discs were taken with a cork borer and placed at the centre of the outer edge of A-compartment and the plates were incubated at 28 °C. The plates were observed periodically for 96 h and the radius of the fungal growth obtained was measured in the test plates as well as the controls and actidione standards.

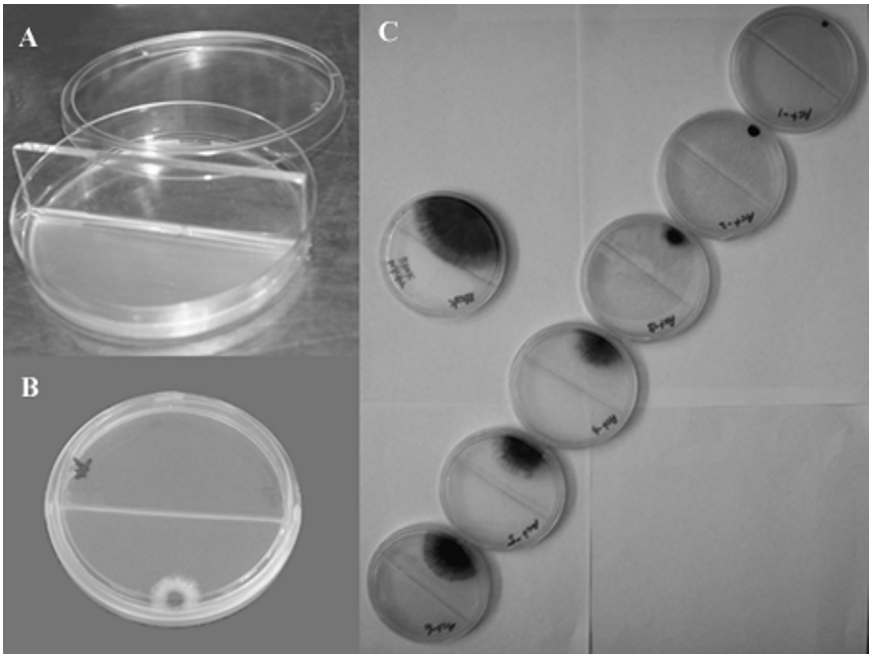

Fig. 1. Steps in preparation of split-agar plates. a) Sabouraud's dextrose medium with 1% agar in half plate bounded by glass slide. b) Plate with soil suspension incorporated Sabouraud's agarin second half showing inhibition of *M. phaseolina* inoculated on first half of the plate. c) Plates with actidione incorporated Sabouraud's agar in second half, showing linear response of growth inhibition of *M. phaseolina* (inverse of colony radius) to increasing actidione concentration.

Antifungal activity was calculated as the difference in radius (cm) of fungal colony of control (least inhibition) and supernatant. Similar data obtained for the difference between water blank and actidione standards were used to plot a standard curve to relate concentrate of actidione with inverse of fungal growth radius. The antifungal activity of the supernatants of soil incubated broth was similarly assayed and expressed by the radius of the fungal colony and expressed as actidione (cycloheximide) equivalents. Straight line relations obtained were then used to read off the actidione equivalents of soil that served as a measure of the antifungal activity. The antifungal metabolite containing supernatants were also autoclaved and the antifungal activity was assessed as described above in order to assess the heat lability of the metabolites.

During the development of the method, different volumes of the broth (0.5, 1, 2 and 5 ml); different methods of supernatant incorporation viz., circular wells of different diameters; rectangular wells in the centre of the plate; inoculation of fungus on four sides or two sides of the plate, etc., were also tested. Finally, 5 ml of the supernatant and mixing of the supernatant with molten medium was found to be the most robust of all the methods.

3. Results

The soils tested exhibited varied antifungal abilities. The quantity of antifungal metabolites produced ranged from 0.02 to 2.80 mg actidione equivalents g^{-1} soil (Table 1). All the broth supernatants obtained by inoculating different soils were in the pH range of 8.5–8.8. The organic and conventional soils showed clear differences in their antifungal ability. The organic soils showed significantly lower antifungal activity, 21.5 fold in organic cropping and 12.7 fold in organic orchards (Table 1). The Vertisols of chili and black gram under intensive chemical management did not show any considerable antifungal activity. The chili soil CH-1 showed antifungal activity of 0.02 mg actidione equivalents g^{-1} soil whereas no antifungal activity was observed in the rest of the Vertisols. Heat treatment considerably reduced the antifungal activity (Fig. 1) of the broths ranging from 81 to 98% (Table 1). Organic cropping and orchard contained 11.2% and 12.4% of heat stable antifungal activity where as conventional cropping and orchards showed 7.5% and 11.6% heat stable antifungal activity. Fallow soil contained the highest heat stable antifungal activity of 18.9%. The normal input soil of chili, which showed weak antifungal activity, showed only 2% heat stable antifungal activity.

4. Discussion

Soil fungistasis is the ability of soils to inhibit the growth of fungal propagules. Many workers have used indirect methods for enumeration of fungal antagonists in soils like enumeration of fluorescent pseudomonads (Mazzola, 2007) or assessed the community structure of soils (Pereira et al. 2009). Direct methods include estimation of volatile fungistatic compounds either produced by pure isolates of microorganisms from soils (Fernando et al. 2005) or produced from soils directly (Chuankun et al. 2004). The present method is a simple one to estimate the antifungal capacity of soil microorganisms and also offers a convenient mode of quantitative expression of the antibiosis potential as actidione equivalents. The soils tested exhibited varied antifungal abilities. The quantity of antifungal metabolites produced varied widely over a 100 fold range among the samples (Table 1). The range indicates that the method is sensitive to detect small amounts of antifungal compounds produced by soil microorganisms. The pH of the broth incubated with different soils was in a similar range (8.5-8.8), although they showed varying antifungal activities. With some of the broth supernatants, the growth of the fungus tested was similar to that of the blank (uninoculated sterile growth medium) thus showing that pH differences are not responsible for the observed effects which were only due to anti-fungal compounds elaborated.

The organic soils showed significantly lower antifungal activity, 21.5 fold in organic cropping and 12.7 fold in organic orchards (Table 1) compared to the conventionally managed soils. This is contrary to conventional belief and some reports (Postma et al. 2008; Bonilla et al. 2012) but supported by many other scientific investigations (de Boer et al. 2003; Bonanomi et al. 2007, Grantina et al. 2011; Berendsen et al. 2012; Bonanomi et al. 2013) which show that there is increased proliferation of saprophytic microbes under organic management which tend to pre-dominate over microbial antogonists. Bonanomi et al. (2007) showed in a review

Table 1
Characteristics of soils used and the antifungal activity (AFA) of culture broths inoculated with soils before and after autoclaving.

	Soil Properties			pH of broth[a]	Radius of *M.phaseolina* colony (cm)		Actidione equivalents (mg g^{-1} soil)		% heat labile AFA	% heat stable AFA
	pH	EC	OC		Before heat treatment	After heat treatment	Before heat treatment	After heat treatment		
Entisols (Hanumangarh, Rajasthan, India)										
Conventional Cropping	8.4	0.39	0.35	8.7	0.55	1.40	2.80 ± 0.42	0.21 ± 0.04	92.5	7.5
Organic Cropping	8.4	0.27	0.55	8.7	1.60	2.30	0.13 ± 0.04	0.014 ± 0.003	88.8	11.2
Conventional Orchard	8.4	0.28	0.35	8.7	0.80	1.50	1.40 ± 0.35	0.16 ± 0.02	88.4	11.6
Organic Orchard	8.3	0.38	0.75	8.7	1.65	2.30	0.11 ± 0.01	0.013 ± 0.001	87.6	12.4
Fallow	8.3	0.30	0.40	8.7	0.75	1.30	1.53 ± 0.21	0.29 ± 0.02	81.1	18.9
Vertisols (Guntur, Andhra Pradesh, India)										
Chilli – 1	7.8	0.50	0.44	8.8	2.30	3.60	0.02 ± 0.004	0.0003 ± 0.000	98.0	2.0
Chilli – 2	6.8	0.76	0.44	8.5	4.00	4.00	0.0	0.0	0.0	0.0
Black gram – 1	7.8	0.72	0.72	8.8	3.95	4.00	0.0	0.0	0.0	0.0
Black gram – 2	7.6	0.52	0.95	8.8	4.25	4.30	0.0	0.0	0.0	0.0

EC = Electrical conductivity; OC = Organic carbon;

[a] pH of the broth- post incubation.

that 20% of the organically treated soils showed increased incidence of plant diseases. Grantina et al. (2011) showed that disease incidence increased in organically managed soils whereas all other biological parameters assessed showed improvement in organic management.

Two theories of soil fungistasis are under debate (Garbeva et al. 2011):

a) Inhibition theory, which attributes soil fungistasis to presence of inhibitory compounds, predominantly of microbial origin (Dobbs and Hinson 1953) like antibiotics (Kloepper, 2003).
b) Nutrient-deficiency theory, which attributes soil fungistasis to the deprival of nutrition of the fungal pathogens by the microbial community of soil (Lockwood and Lingappa, 1963) like siderophore production (Kloepper, 2003).

Bonanomi et al. (2013) studied the influence of 42 different organic amendments on soil fungistasis and showed that uncomposted organic manures (high in labile carbon) increased fungal disease incidence where as composted organic manures (low in labile carbon and high in lignin-like carbon compounds) reduced fungal disease incidence supporting the nutrient-deficiency theory. However they could not provide a direct proof of the theory since they did not quantify the anti-fungal metabolites in the soils which was done in the present study.

In the current study the labile carbon (data not shown) was significantly high in organically managed soils and the antifungal activity was significantly lower compared to conventionally managed soils. The results prove that both the theories of soil fungistasis discussed earlier are interlinked. In soil ecosystems where labile carbon was available for a long term, the microorganisms adapted for high nutrient availability and possessing low competitive abilities would get enriched. Whereas in soil ecosystems where the microbial community is deprived of the labile carbon sources for a long term, microorganisms adapted for competition and good antagonistic capabilities would get enriched. This explains why soils amended with labile carbon rich organic manures show relief of soil fungistasis. However this observation needs further detailed investigations to understand whether it is specific to the soil type studied and agro-ecological zone or is more widespread phenomenon.

The Vertisols of chili and black gram under intensive chemical management did not show any considerable antifungal activity (Table 1). The reason for this may be related to the very high doses of chemical fertilizers and pesticides used in these soils which might have reduced the proportion of antagonistic microorganisms. This shows that intensive chemical farming reduces the antifungal activity of the soils (van Bruggen, 1995). The absence or presence in very low quantity of anti-fungal metabolites in the chili and black gram soils encouraged the growth of fungal mats in broth inoculated with these soils in stationary culture. In shaken cultures the mats were dispersed.

Heat treatment considerably reduced the antifungal activity of the broths ranging from 81 to 98% (Table 1). This shows that a large proportion of the anti-fungal activity is biotic and heat labile. This may be attributed to the loss of the heat labile antifungal metabolites including volatiles. Volatile antifungal metabolites are a major group of antifungal compounds of soils (Chuankun et al. 2004; Fernando et al. 2005). The remaining antifungal activity (2–19%) may be attributed to heat stable compounds. Yu et al. (2007) reported the production of heat stable antifungal metabolites by *Lysobacter enzymogenes*. Presence of heat stable antifungal metabolites in greater proportion in soils is of significance, particularly for tropical soils, since at high temperatures the volatile antifungal compounds are lost rapidly to environment (Gamliel and Stapleton, 1993). Thus the soils can also be studied for their capacity to produce heat stable metabolites using this method.

The organic and conventionally managed soils contained different proportions of heat stable antifungal compounds. Organic cropping and orchard contained 11.2% and 12.4% of heat stable antifungal activity where as conventional cropping and orchards showed 7.5% and 11.6% heat stable antifungal activity. The chili soil which showed weak antifungal activity, showed only 2% heat stable antifungal activity. This shows that organic management showed lower antifungal activity but showed a higher proportion of heat stable antifungal activity. Thus organic management with additional measures for disease management may prove to increase disease suppressiveness.

Thus the agar diffusion assay developed was simple and useful to measure the total antifungal activity and the proportions of heat stable and heat labile antifungal activities of soils.

5. Conclusions

We report the development of a split agar assay which is an easy technique to ascertain the anti-fungal potential of soils and provides a quick means of quantifying soil suppressiveness.

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References

Alabouvette, C., 1986. Fusarium-wilt suppressive soils from the Châteaurenard region: review of a 10-year study. Agronomie 6, 273–284.
Atlas, R.M., 1995. Hand Book of Media for environmental Microbiology. CRC Press, Boca Raton, USA.
Berendsen, R.L., Kalkhove, S.I., Lugones, L.G., Wösten, H.A., Bakker, P.A., 2012. Germination of *Lecanicillium fungicola* in the mycosphere of *Agaricus bisporus*. Environ. Microbiol. Rep. 4, 227–233.
Bonanomi, G., Antignani, V., Pane, C., Scala, F., 2007. Suppression of soilborne fungal diseases with organic amendments. J. Plant Pathol., 311–324.
Bonanomi, G., Gaglione, S.A., Incerti, G., Zoina, A., 2013. Biochemical quality of organic amendments affects soil fungistasis. Appl. Soil. Ecol. 72, 135–142.
Bonilla, N., Gutiérrez-Barranquero, J.A., Vicente, A.D., Cazorla, F.M., 2012. Enhancing soil quality and plant health through suppressive organic amendments. Diversity 4, 475–491.
Cappuccino, J.G., Sherman, N., 2008. Microbiology: A Laboratory Manual 9 Vol.. Pearson/Benjamin Cummings.
Chuankun, X., Minghe, M., Leming, Z., Keqin, Z., 2004. Soil volatile fungistasis and volatile fungistatic compounds. Soil. Biol. Biochem. 36, 1997–2004.
de Boer, W., Verheggen, P., Gunnewiek, P.J.K., Kowalchuk, G.A., van Veen, J.A., 2003. Microbial community composition affects soil fungistasis. Appl. Env. Micro. 69 (2), 835–844.
Dobbs, C.G., Hinson, W.H., 1953. A widespread fungistasis in soil. Nature 172, 197–199.
Fernando, W.G., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C., 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil. Biol. Biochem. 37, 955–964.
Gamliel, A., Stapleton, J.J., 1993. Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues. Phytopathology 83, 899–905.
Garbeva, P., Hol, W.G., Termorshuizen, A.J., Kowalchuk, G.A., De Boer, W., 2011. Fungistasis and general soil biostasis–a new synthesis. Soil. Biol. Biochem. 43, 469–477.
Grantina, L., Kenigsvalde, K., Eze, D., Petrina, Z., Skrabule, I., Rostoks, N., Nikolajeva, V., 2011. Impact of six-year-long organic cropping on soil microorganisms and crop disease suppressiveness. Šemdirbystė= Agriculture 98, 399–408.
Kloepper, J. W., 2003. A review of mechanisms for plant growth promotion by PGPR. In 6th international PGPR workshop 10, 5-10.
Lockwood, J.L., Lingappa, B.T., 1963. Fungitoxicity of sterilized soil inoculated with soil microflora. Phytopathology 58, 917–920.
Mazzola, M., 2002. Mechanisms of natural soil suppressiveness to soil-borne diseases. A Van. Leeuw 81, 557–564.

Mazzola, M., 2007. Manipulation of rhizosphere bacterial communities to induce suppressive soils. J. Nematol. 39, 213.
Pereira, P., Nesci, A., Etcheverry, M., 2009. Impact of two bacterial biocontrol agents on bacterial and fungal culturable groups associated with the roots of field-grown maize. Lett. Appl. Microbiol. 48, 493–499.
Postma, J., Schilder, M.T., Bloem, J., van Leeuwen-Haagsma, W.K., 2008. Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. Soil. Biol. Biochem. 40, 2394–2406.
Rupela, O.P., Gopalakrishnan, S., Krajewski, M., Sriveni, M., 2003. A novel method for the identification and enumeration of microorganisms with potential for suppressing fungal plant pathogens. Biol. Fert. Soils. 39, 131–134.
Smith, G.S., Carvil, O.N., 1977. Field screening of commercial and experimental soybean cultivars for their reaction to *Macrophomina phaseolina*. Plant Dis. 81, 363–368.
Van Bruggen, A.H., 1995. Plant disease severity in high-input compared to reduced-input and organic farming systems. Plant Dis. 79, 976–984.
Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J.C., Harris, S.D., Yuen, G., Li, X., Du, L., 2007. Structure and Biosynthesis of Heat-Stable Antifungal Factor (HSAF), a Broad-Spectrum antimycotic with a Novel Mode of Action. Antimicrob. Agents Ch 51, 64–72.