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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 antifungal 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.

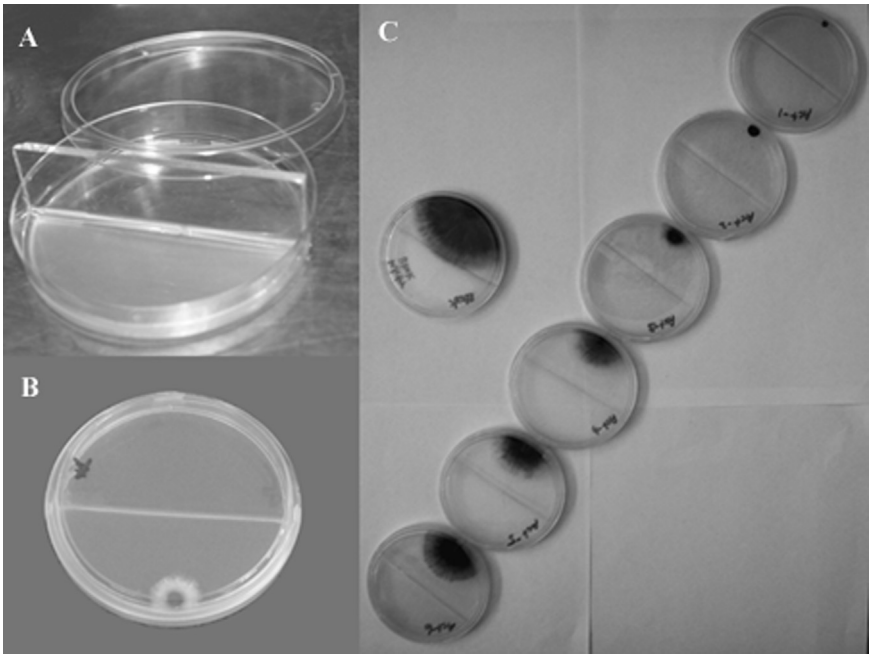

Fig. 1. Steps in preparation of split-agar plates. a) Sabouraud's dextrose medium with 1% agar in half plate bounded by glass slide. b) Plate with soil suspension incorporated Sabouraud's agarin second half showing inhibition of *M. phaseolina* inoculated on first half of the plate. c) Plates with actidione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