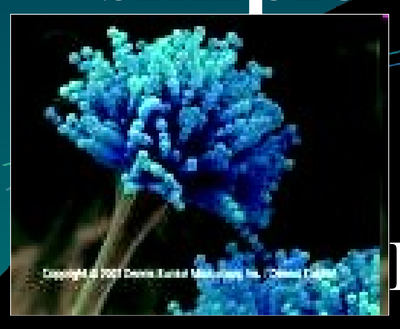
Exploring hidden dimensions of soil fungal biodiversity: A simple technique to detect soil fungi resistant to antifungal compounds



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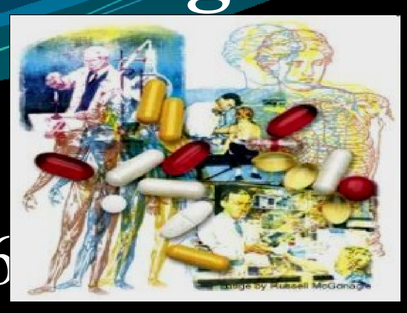

ABSTRACT

Soils are known to be ultimate and complex reservoirs of microbial diversity. The complex dimensions of bacterial and fungal diversity in tropical soils and microbial community dynamics are underexplored. Isolation techniques aimed at Actinomycetes generally employ highly selective media, powerful antibiotics and antifungal compounds to suppress undesirable bacteria and fungi. However some soil fungi may show their resistance towards these antifungal compounds. During our work to explore soil actinomycetes diversity, slides coated with Arginine Vitamin agar (AVA) incorporating a cocktail of antibiotics and antifungal compounds such as Nystatin, Cycloheximide, Terbinafin, Griseofulvin, and Fluconazole were exposed to soil environment and were retrieved at intervals of 4, 7, 15 and 28 days for detail microscopic studies of surface colonies. Along with actinomycetes the presence of unidentified aseptate and septate fungi was revealed indicating their resistance to combination and concentration of antifungals. Heat treatment of the soil was found to cause considerable decrease in fungal contamination probably due to elimination of heat labile fungi. Our results have led us to develop a simple procedure to sample the interesting and industrially useful strains of soil fungi resistant to common antifungal compounds. Some fungal strains are reported resistant to certain antifungals with resulting therapeutic failures as use of these antifungals inevitably selects resistant fungi, thereby pressing the urge for continuing and cyclical need of new antifungals (Augustin et al., 2004). This technique could prove useful to detect novel antifungal resistant strains with potential to emerge as novel human pathogens. It has not escaped our notice that the probability of such finding could also help to verify whether these fungi could utilize such antifungal compounds through use of hitherto undiscovered metabolic pathways and novel enzymes leading to identification of genes responsible for antifungal resistance.

INTRODUCTION

The successful management of invasive fungal infections continues to pose a difficult challenge to clinicians, which stimulates research directed towards the discovery of novel antifungal agents (Odds et al., 2003; Andes et al ., 2009).
Screening for novel antifungal substances can integrate the findings achieved by studies on resistance mechanisms (Sanglard, 2002).
The term "resistance" is used to describe a relative insensitivity of a microbe to an antimicrobial drug as tested *in vitro* and compared with other isolates of the same species (Loeffler and Stevens, 2003).
Pathogenic fungi have many complex mechanisms of resistance to antifungal drugs(Kontoyiannis and Lewis, 2002).
The most common mechanisms for the development of resistance involve changes in the enzymatic pathways which serve as the drug targets. For instance, changes in enzymes responsible for the biosynthesis of ergosterol, the target of azole activity, lead to azole resistance (Alexander and Perfect, 1997).
Antimycotic drugs interfere with the normal life cycle of fungi by inhibiting normal functioning of one or several vital cellular entities (Borgers, 1980). The list of antifungal agents , its activities against principle mode of action and resistance mechanisms is illustrated in the table below.

ANTIFUNGAL AGENTS: ACTIVITES AGAINST PRINCIPLE MODES OF ACTION AND RESISTANCE MECHANISMS OF FUNGAL PATHOGENS (Sanglard , 2002 and http://www2.bc.cc.ca.us/bio16/images/10-T03_Antiviral)

Antifungal	Spectrum/comments	Mode of action	Mechanism of resistance observed in clinical isolates
Antifungal drugs that inhibit cell membranes			
Polyenes			
Nystatin	molds and yeast infections are sensitive, including *Candida* spp. treating oral or gastrointestinal fungal infections.	associates with ergosterol, the main component of fungal cell membranes, forming a transmembrane channel that leads to K leakage and fungal cell death.	Alteration in specific steps of ergosterol biosynthesis
Azoles			
Fluconazole	Active against *Candida* spp and *Cryptococcus* spp, less active against *C. glabrata* and no activity against *C. krusei*; no activity against filamentous fungi	Inhibition of cytochrome P450 14α-lanosterol demethylase	Enhanced efflux by upregulation of multidrug transporter genes. Target alterations by occurrence of mutations. Alteration of specific steps in the ergosterol biosynthetic pathway
Allylamines	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of squalene epoxidase	
Terbinafine			Unknown
Griseofulvin	Molds of ringworm (Tinea)	Isolated from *Penicillium griseofulvin*, deactivates tubulin, preventing cytokinesis and segregation of chromosomes during mitosis	Unknown
Cycloheximide (Actidione)	Active against saprobic fungi, inactive against dermatophytes and systemic fungi.	Inhibits the protein synthesis (DNA-dependent RNA) of saprobic fungi eukaryotes, by binding with the 80S ribosome	Unknown

The exposure of fungal pathogens to these agents is therefore expected to give rise to growing awareness of shifts of flora to more-resistant species (Sanglard, 2002; Loeffler and Stevens, 2003).

AIM_

To develop a simple procedure to sample, the interesting and industrially useful strains of soil fungi resistant to common antifungal compounds.

MATERIALS AND METHODS

Coated slide baiting technique

Soil samples collected below the canopy of *Ficus benghalensis* L. and *Bombax ceiba* L.

↓

Slides coated with Arginine Vitamin Agar (Nonomura and Ohara, 1969).

↓

Medium incorporated with cocktail of antibiotics & Antifungal compounds such as Nystatin, Fluconazole ,Terbinafin, Griseofulvin & Cycloheximde (Total conc. 0.2mg/ml).

↓

Slides inserted in soil (in situ and ex situ treatments) at ambient temperature.

- ❖Insitu treatment had two tested variables: with and without antifungals (control)
- ❖Exsitu treatment had four tested variables: with antifungals and heat treatment, without antifungals and heat treatment (control), with antifungals and no heat treatment and without antifungals and no heat treatment(control)

↓

Slides retrieved at regular intervals of 4, 7, 15 & 28 days.

↓

Surface colonization was observed under objectives 10, 20X (Bright field(BF)and Phase Contrast(PC) Microscopy) and photographed.

↓

Percentage fungal colonization (area per field) was calculated by Digital Image Analysis (Scion Image Software)

RESULTS AND DISCUSSION

Presence of unidentified aseptate and septate fungi was observed in slides retrieved from both the soil treatments indicating - resistance to antifungal compounds.
When faced with antifungal drugs, fungal pathogens have, the capacity to overcome their inhibitory action through specific resistance mechanisms- ability to select mutants resistant to antifungal drugs, has been used (Sanglard, 2002; Loeffler and Stevens, 2003).
Colonization of fungi was found to be not specific to the host plant species.
Insitu treatment - %of fungal colonization was observed on all the days of incubation, but in lesser conc. when compared to control despite the incorporation of antifungals w.r.t the soil of *F. benghalensis.*% of fungal colonization was more when compared to control w.r.t the soil of *B. ceiba.* This is illustrated in fig. 1a & b.
Exsitu treatment - Heat treatment of the soil caused considerable decrease of fungal spores probably due to elimination of heat labile fungi. Fungal colonization was observed associated with both the soil samples, irrespective of different treatments. This is illustrated in fig. 2a & b.

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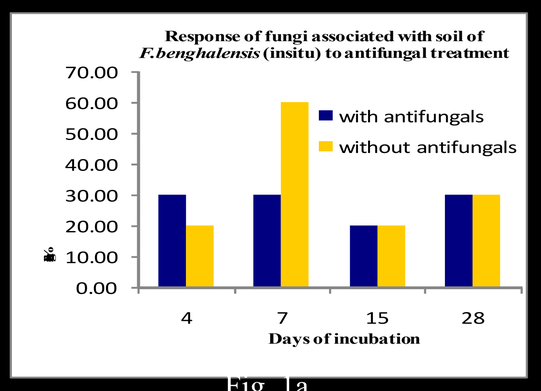

Fig. 1a

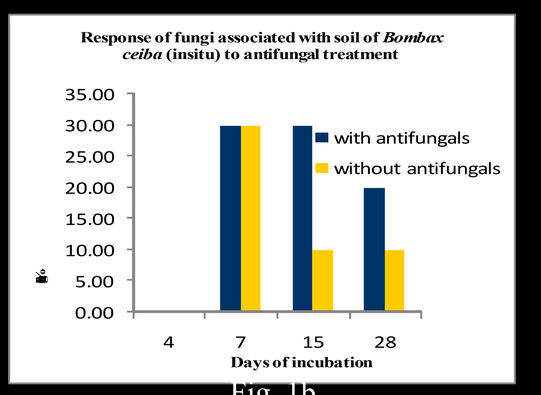

Fig. 1b

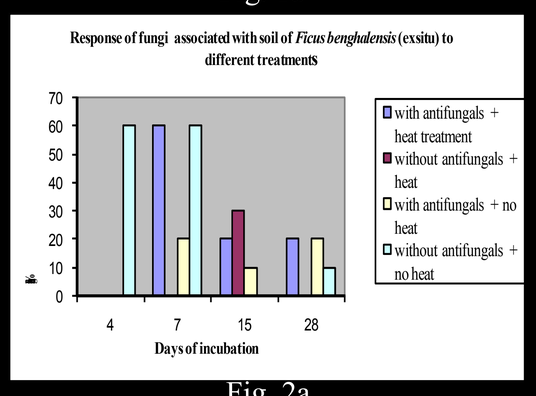

Fig. 2a

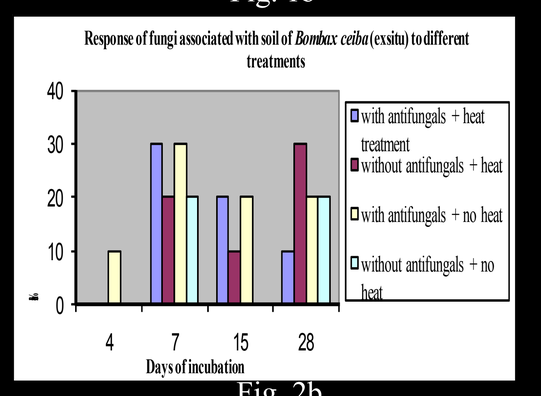

Fig. 2b

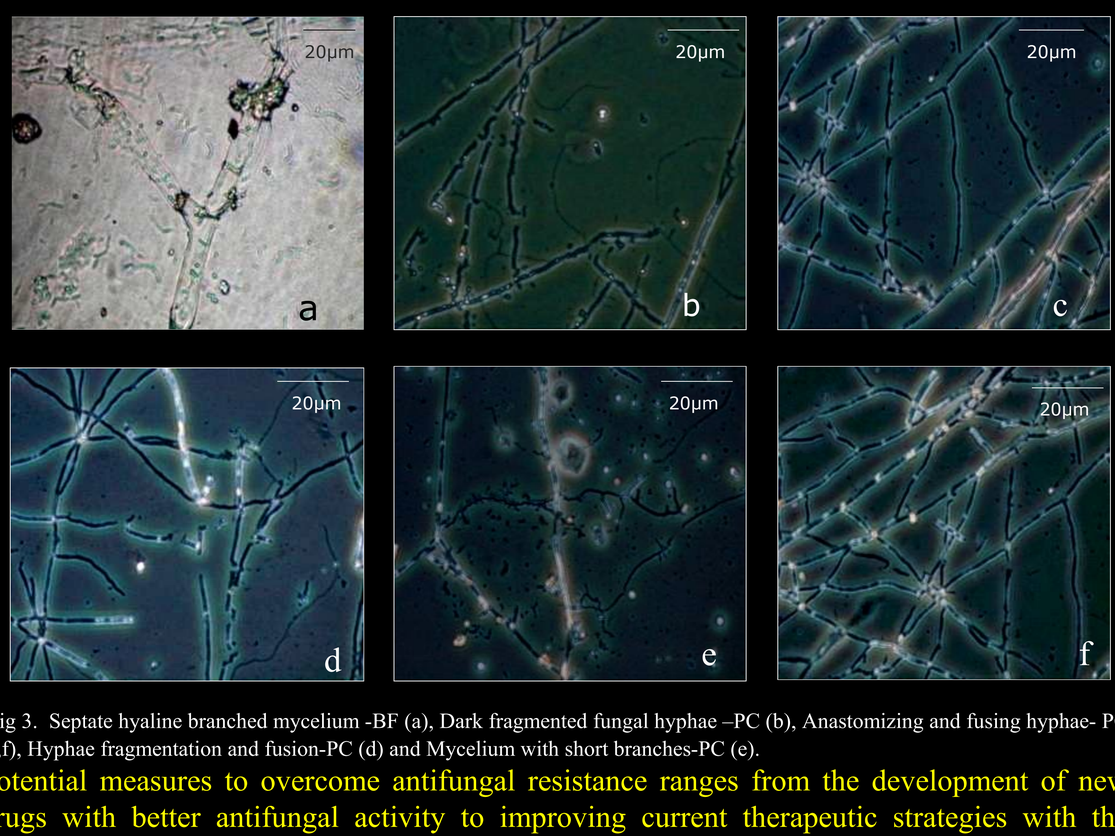

Fig 3. Septate hyaline branched mycelium -BF (a), Dark fragmented fungal hyphae –PC (b), Anastomizing and fusing hyphae- PC (c,f), Hyphae fragmentation and fusion-PC (d) and Mycelium with short branches-PC (e).

Potential measures to overcome antifungal resistance ranges from the development of new drugs with better antifungal activity to improving current therapeutic strategies with the present antifungal agents (Canuto and Rodero, 2002).
Global warming and climate change likely to release new strains of fungal pathogens where humans would have no resistance. With the approach of isolating and identifying strains of fungi resistant to known antifungal compounds & understanding the mechanisms of their resistance at cellular and molecular level, it would be easier to develop targeted antifungal drugs rationally.
Such approach may ensure advance protection to mankind likely to face novel forms of mycoses by such resistant fungi spread locally or globally.

CONCLUSIONS

This technique could prove useful to detect novel antifungal resistant strains with potential to emerge as novel human pathogens.

The probability of finding such technique could also help to verify whether these fungi could utilize such antifungal compounds through use of hitherto undiscovered metabolic pathways and novel enzymes leading to identification of genes responsible for antifungal resistance.

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