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An agar plate assay to detect cell wall active antifungal agents

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A rapid economical plate bioassay to detect inhibitors of fungal cell wall was developed using two Sabouraud agar plates seeded with *Candida albicans*. One of them was supplemented with 0.8 M sorbitol, an osmo-protectant which prevented the lysis of *C. albicans* cells by cell wall active agents. In the absence of sorbitol, an antifungal agent gave a zone of inhibition and a reduced hazy zone in the sorbitol-supplemented plate indicated the antifungal agent to be active against the cell wall. When broth samples of 550 fungal isolates were screened, one of them giving a hazy zone was identified to be papulacandin. Thus, the newly developed plate bioassay is suited for high-throughput screening for novel fungal cell wall inhibitors.

ALL over the world, fungal infections are steadily increasing in frequency and clinical importance. This trend has been attributed to the growing number of immunocompromised patients either due to aggressive treatment for illnesses or due to infection with Human Immunodeficiency Virus (HIV)[1,2]. Even more disturbing is the growing number of reports of resistant fungi against commonly used azole antifungal agents[3]. Hence there is an urgent need for new antifungal drugs with new modes of action to combat the expanding spectrum of fungal infections. The cell wall (CW) of fungi, being unique and essential for survival and not present in mammalian cells, presents an attractive target for new antifungals[2,4]. Also, agents acting on new targets are likely to circumvent specific resistance mechanisms to existing drugs[4].

A whole cell *Candida albicans* screen to identify inhibitors of fungal CW synthesis and assembly based on osmotic support and morphological characteristics of the cells has been described earlier[5]. Damage to essential CW components by any CW active agent will lyse the cells, but they will continue to grow if a suitable osmo-protectant like sorbitol is present in the medium.

Combining this rescue of growth with sorbitol and morphological characteristics of the cell formed the basis of the Sorbitol Protection And Morphology (SPAM) assay[5]. This method involves the determination of minimum inhibitory concentration (MIC) values of the test samples by broth microdilution method in 96 well plates using Yeast Nitrogen Base (YNB) and YNB supplemented with 0.8 M sorbitol. These plates are checked for growth in each well after 2 and 7 days of incubation. CW active antifungal agents gave higher MIC in the presence of sorbitol after 7 days as cells continued to grow in the medium due to osmotic protection.

This paper describes an agar plate bioassay which is a modification of the SPAM broth assay. It is a simple method mainly for the detection of CW-active antifungal agents. The assay consists of two bioassay plates of Sabouraud agar seeded with *C. albicans*, one of which is supplemented with 0.8 M sorbitol as an osmo-protectant. Any sample showing a reduced and a hazy zone of inhibition in the sorbitol-supplemented plate, resulting from osmotic protection, as against a clear zone of inhibition in the control plate due to lysis of *C. albicans* cells, was considered to have a CW-active antifungal agent.

For bioassay, the inoculum was prepared by growing *C. albicans* ATCC 10231 in Sabouraud broth overnight and adjusting the OD to 1.0 at 600 nm (using Spectronic Genesys 5, Milton Roy). One hundred micro-liters of this inoculum was added to 40 ml per plate of molten and cooled Sabouraud agar and Sabouraud agar containing 0.8 M sorbitol to prepare two bioassay plates of 15 cm diameter. Twenty-five wells of 6 mm diameter were punched in each plate and 50 µl of the test sample and appropriate solvent controls were added to each well and the plates were incubated at 37°C for 18 h.

The assay was validated using mulundocandin, a known CW-active agent[6,7] as a positive control. Antifungal agents, viz. amphotericin B, azoles, 5 fluorocytosine and terbinafine, all of which have modes of action other than inhibition of fungal CW synthesis[2] were used as negative controls (Table 1).

A clear zone was observed for mulundocandin in the Sabouraud agar plate whereas in the presence of sorbitol, it was hazy and smaller (Figure 1). The zone sizes were proportionate to the concentration of mulundocandin in

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the range of 0.4–25 µg per 50 µl. Amphotericin B and nystatin gave clear zones in Sabouraud agar plate and also in the presence of sorbitol. Other antifungal agents neither gave clear zone in Sabouraud agar nor did they show any decrease in the zone size in the presence of sorbitol. Thus, the plate assay can be used for primary screening of CW-active antifungal agents either from natural sources or from chemical synthesis programme.

After validating the assay by this agar plate method, 550 fungal isolates were screened to look for fungal CW-inhibiting agents. Each culture was inoculated into 250 ml Erlenmeyer flasks containing 50 ml seed medium composed of 0.5% glucose, 1.5% soluble starch, 1.5% soybean meal, 0.5% NaCl, 0.2% yeast extract, 0.2% $CaCO_3$, 0.1% corn steep liquor, 1.0 ml trace salt solution per liter, and pH 7.0 before autoclaving. The trace salt solution consisted of 0.7% $CuSO_4$, 0.1% $FeSO_4$, 0.8% $MnCl_2$, and 0.2% $ZnSO_4$. The flasks were incubated at 26°C for 72 h on rotary shakers at 200 rpm. Two ml of the seed culture was inoculated into sterile 100 ml of the fermentation medium composed of 3.0% glucose, 2.0% soybean meal, 0.3% NaCl, 0.25% NH_4Cl, 0.2% KH_2PO_4, 0.6% $CaCO_3$, trace salt solution 1.0 ml per liter, and pH 7.0 in 500 ml Erlenmeyer flasks. The fermentation was carried out at 26°C for 72 h on rotary shakers at 200 rpm. Equal volume of methanol, i.e. 100 ml was added to each flask and

Table 1. Agar plate assay for detecting CW-active antifungal agents

Sample	Antifungal agent	Conc. (µg/well)	Zone size (mm)	
			Sabouraud	Sabouraud + sorbitol
Positive control	Mulundocandin	0.78	23 (c)	18 (h)
		5	31 (c)	20 (hd)
Negative control	Amphotericin B	5	22 (c)	24 (c)
Negative control	Nystatin	1.56	15 (c)	21 (c)
Negative control	Fluconazole	1.56	19 (hd)	24 (vh)
Negative control	Ketoconazole	1.56	28 (hd)	28 (vh)
Negative control	5 fluorocytosine	50	25 (vh)	25 (vh)
Negative control	Terbinafine	6.25	22 (hd)	25 (d)
Fungal isolate (Y97 07633)	Methanol extract	Undiluted	19 (c)	13 (h)

Appearance of zone: c, clear; h, hazy; hd, hazy and diffused; vh, very hazy.

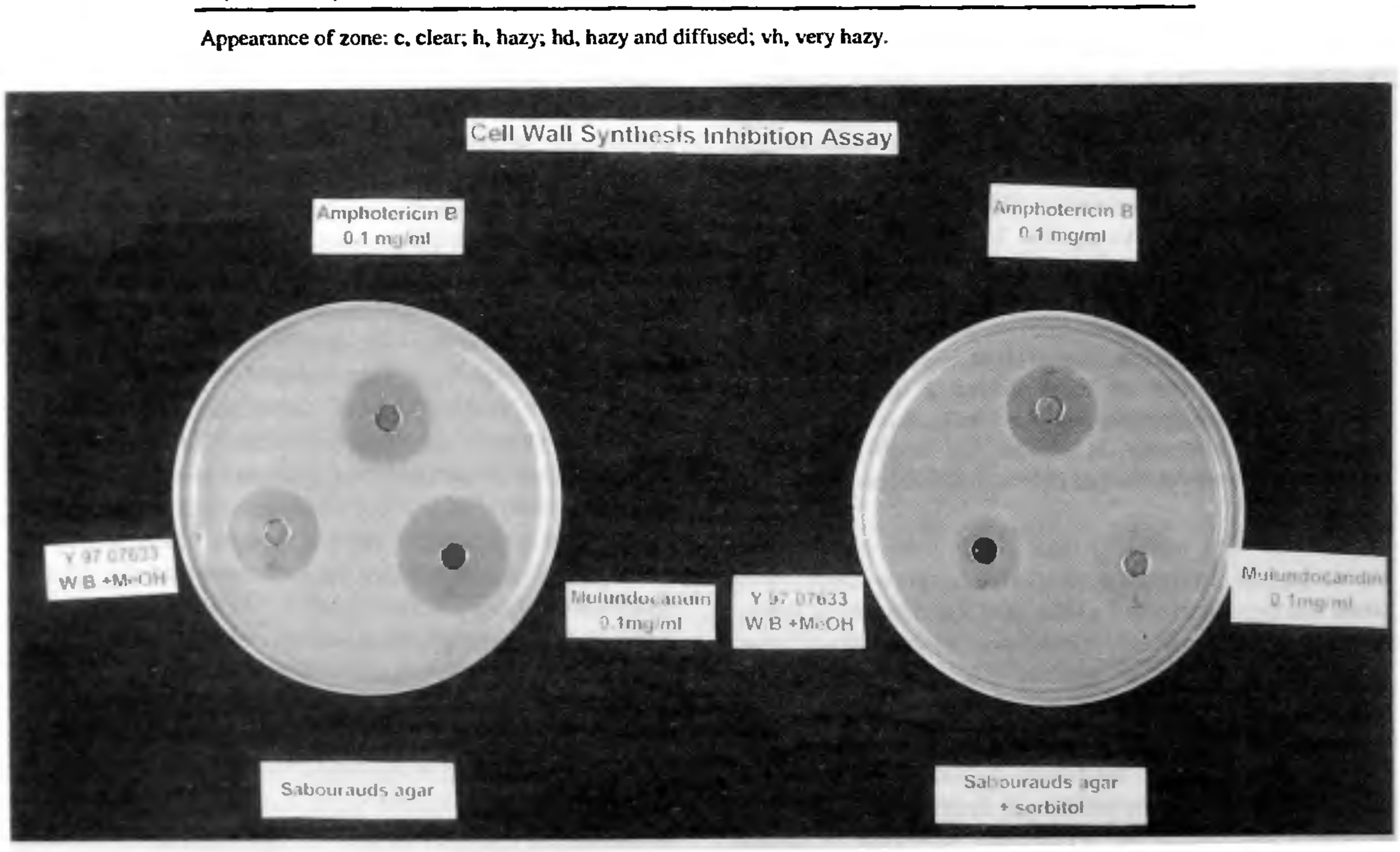

Figure 1. Sabouraud agar plate assay showing clear zone of inhibition for mulundocandin and a hazy zone for the same in the presence of sorbitol. The whole broth methanolic extract of the isolate, Y 97 07633 also shows a reduced and hazy zone in the presence of sorbitol. Amphotericin B (a noninhibitor of CW) shows clear zones in both the plates.

Table 2. MIC of papulacandin preparation against *C. albicans* by broth microdilution method using Sabouraud broth

Incubation time (days)	MIC (μg/ml)	
	Sabouraud broth	Sabouraud broth + sorbitol
2	1	4
5	2	> 64

allowed to remain on the shaker for two hours. This whole broth (W.B.) in methanol was centrifuged and 50 μl of supernatant, which was a representative of metabolites present in both culture filtrate and mycelium, was added per well in the two bioassay plates. The plates were left open in the laminar flowhood to allow evaporation of methanol from the test sample.

One of the fungal cultures, No. Y 97 07633 screened in this assay showed a reduced zone in the presence of sorbitol (Table 1), indicating the presence of a CW-active antifungal agent. The CW-active components from the fungal culture were identified by the process of dereplication[8] to be a mixture of papulacandins B and C. The papulacandin complex was enriched from the mycelium of the producing fungal strain by the use of chromatographic techniques such as Diaion HP-20, Silica gel and Sephadex LH-20. MIC of the papulacandin preparation was determined by broth microdilution method in Sabouraud broth and was found to be higher in the presence of sorbitol (Table 2), thus confirming the results of the crude extract obtained by the agar plate assay described earlier. Frost *et al.*[5] have also reported MIC values of 1.0 μg/ml for papulacandin B by SPAM.

Hence, the agar plate assay is rapid, convenient and sturdy and suited for high-throughput screening of natural products or synthetic chemical compounds, since results are available in 18 h and samples containing solvents like methanol can be tested. It can also be used for monitoring CW-active metabolites during its purification from microbial sources. Natural product or synthetic chemical samples can be tested directly by this agar plate method without knowing their MIC values. The assay replaces YNB with Sabouraud agar, which is economical and less cumbersome. The assay is neither sensitive to the density of the inoculum nor does it need the test sample to be sterile when extracted in methanol.

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Spatial variation in the capacity of soil for CH_4 uptake and population size of methane oxidizing bacteria in dryland rice agriculture

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The pattern of methane (CH_4) oxidation and population size of methane oxidizing bacteria (MOB) were studied in three different soils (rhizosphere, bulk and bare) of a dryland rice (*Oryza sativa* L. cultivar Narendra-118) field. The rhizosphere soil exhibited the strongest CH_4 oxidation activity and bare soil the weakest. MOB population size was significantly higher in the rhizosphere (671.0×10^5 cells g^{-1} soil) than in the bulk (569.0×10^5 cells g^{-1} soil) or the bare soil (49.2×10^5 cells g^{-1} soil), and NH_4^+-N concentration was highest in the bare soil (6.74 μg g^{-1} soil) followed by the bulk (5.58 μg g^{-1} soil) and rhizosphere soil (4.02 μg g^{-1} soil). Half saturation constant (K_m) and maximum oxidation rate (V_{max}) decreased significantly from the rhizosphere to bulk to bare soil and ranged from 84.01 to 5.81 μg g^{-1} dry soil and 0.62 to 0.05 μg h^{-1} g^{-1} dry soil, respectively. The rice rhizosphere not only supports a larger population of MOB but also contributes substantially to the capacity of soil for CH_4 uptake, leading to a predictable spatial pattern in CH_4 sink strength within the dryland rice ecosystem.

METHANE (CH_4) is an important greenhouse gas and plays a significant role in atmospheric chemistry[1,2]. Rice cultivation, a major anthropogenic source of methane, contributes about 20–150 Tg CH_4 yr^{-1} to the global budget[3]. Uptake by soil as a result of consumption by methane oxidizing bacteria (MOB) is a substantial sink for CH_4 (ref. 4). Even in flooded rice soil about 80 per cent of the CH_4 produced is oxidized *in situ*[5], with rhizosphere as the key site[6,7]. Dryland rice cultivation has been earlier demonstrated as a net sink for atmospheric CH_4, with rice

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