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A Preliminary Study of the Fungi Associated with Saltpans in Botswana and their Anti-microbial Properties

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ABSTRACT

The ability of fungal isolates from semi-arid saltpans in Botswana to produce secondary metabolites with antimicrobial properties was investigated. One hundred and eleven fungal isolates from 15 samples from 5 saltpans were isolated using the dilution plate method on PDA, amended with soil extracts and supplemented with streptomycin sulphate (0.3 g/l). Their antimicrobial activity was tested by confrontation and agar-well diffusion methods using attenuated *Escherichia coli*, *Staphylococcus aureus*, *Bacillus megaterium* and fungi, *Candida albicans* and *Aspergillus niger* as test organisms. *Aspergillus* spp. (26.1%), *Fusarium* spp. (18.0%), *Dendryphiopsis* sp. (13.5%), *Alternaria alternata* (7.2%) and *Phoma* spp. (5.4%), were the most common fungi isolated. In the confrontation test, almost 100% of the fungal isolates inhibited the growth of at least one test organism. Approximately 43% of the culture supernatants in malt-extract broth showed antimicrobial activity against at least one test organism in the well diffusion assays. *Candida albicans* was the least sensitive, being inhibited by only two (4.2%) fungal supernatants from the Tshane saltpan. *Bacillus megaterium* and *S. aureus* were the most sensitive and were inhibited by 81.3 and 70.8% of the fungal supernatants, respectively. *Escherichia coli* was inhibited by 12.5% and *A. niger* by 37.5% of the fungal supernatants. The most biologically active fungal isolate was Tsp22 (*Aspergillus terreus*), which inhibited the growth of all test organisms. Ten isolates showing broad activities were selected and further analysed for the minimum inhibitory concentration, which demonstrated that antimicrobial activity increased relative to increased volumes of the supernatants. Polymerase chain reaction was used to amplify the internal transcribed spacer region of the 10 selected isolates and two phylogenetic trees were created to confirm isolate identification.

Keywords: alkalophile, *Aspergillus*, *Fusarium*, halophile, secondary metabolite

INTRODUCTION

Fungi are very important in biotechnological applications, ranging from production of medicinal products, to agrochemicals and industrial products (Concepcion *et al.* 2001). Fungi from extreme environments i.e. extremophiles, which are specially adapted to their ecological niches (da Costa and Nobre 1989; Gloer 1997; Seckbach and Oren 2000) have been established as a promising source of new active biometabolites (Holler *et al.* 2000; Behal 2001). Potential habitats for novel fungi include those with extreme temperature, pH, alkalinity and low water potential, and encompass hypersaline environments, such as saline soils (Abdel-Hafez 1981; Moubasher *et al.* 1990; Guiraud *et al.* 1995), saltern ponds (Gunde-Cimerman *et al.* 2000, 2004; Butinar *et al.* 2005a, 2005b, 2005c; Cantrell *et al.* 2006), marine habitats (Holler *et al.* 2000; Raghukumar 2008) and hypersaline marine habitats (Buchalo *et al.* 1998; Kis-Papo *et al.* 2001, 2003), where only fungi with unusual ecology survive (Schulz *et al.* 2002). As natural products are adapted to a specific function in nature, the search for novel secondary metabolites now focuses on organisms that inhabit novel biotopes, such as saltpans and deserts. Therefore, the process of novel compound discovery has changed greatly (Omura 1992), and it is no longer a random search, as targets are now systematically selected and specific screenings undertaken (Hyde 2001). Fungal collection strategies are usually designed to maximise the diversity of fungal taxa in order to test organisms from a variety of niches (Hyde 2001).

Cultivating extreme environment fungi is challenging due to their slow growth and specific requirements (Pointing 2000). Enhanced isolation can be achieved by emulating the natural environment, such as with addition of salts or soil extract from the collection sites to the isolation medium (Pointing 2000). The soil extract is essential to provide micronutrients, growth factors and vitamins which do not normally exist in the commercial medium (Wollum II 1982; Warren *et al.* 2002).

Botswana is a landlocked country which lies between latitudes 18S and 27S and longitudes 20E and 30E, with an area of 582,000 km^2. The climate of Botswana is subtropical and semi-arid to arid (Dept. of Meteorological Service 2003). Mean annual rainfall varies from 650 mm in Chobe (north west) to less than 250 mm in Kgalagadi (west). Diurnal temperatures are characterised by hot days with temperatures as high as 43°C and cooler nights with temperatures as low as -8°C (Dept. of Meteorological Service 2003). The landscape is characterised by studs of saltpans and has one of the largest deserts in the world, the Kalahari Desert, which covers most of the country, spreading from western to central regions of Botswana. The saltpans are concentrated in the more arid south, and the northern and central parts of the country (Thomas and Shaw 1991). Saltpans are geological formations found in the desert. They are flat expanses of land covered with salt and other minerals. Saltpans are formed when the rate of evaporation is greater than the rate of precipitation in a lake. The salts and minerals dissolved in the lake are left behind when the water evaporates. Over the centuries, the salts accumulate creating a white surface (Owen 2005) with high salt content and alkalinity (Thomas and Shaw 1991). The Makgadikgadi saltpans, located in north central Botswana are believed to be the largest of their kind in the world, and they cover 12,000 km^2 (**Fig. 1**). They are the remains of a vast super lake that covered over 60,000 km^2 and was about 50 m deep (Owen 2005). The pan system is made up of two major saltpans being the Sua and Ntwetwe pans, and some small

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scattered saltpans such as Nxai pans. Morwamosu, Kang, Tshane and Sekoma are natural salt pans located elsewhere in the Kalahari (**Fig. 1**) that were also sampled in the present study. Among the most extreme habitats in Botswana are the man-made saltern ponds adjacent to Sua Pan (Botswana Ash (Pty) Ltd.) located to the east of Makgadikgagdi complex. The major ions are sodium (Na) and chloride (Cl) making up 21% of NaCl (Botswana Ash pamphlet, unpublished). These saltern ponds were sampled in this study although no fungi were isolated. However, other studies of saltern ponds have been successful in isolating fungi (Gunde-Cimerman *et al.* 2000, 2004; Butinar *et al.* 2005a, 2005b, 2005c; Cantrell *et al.* 2006).

The main aim of the study was to investigate the occurrence and biodiversity of fungi associated with extreme environments (desert and saltpans) in Botswana and to assess the potential of these fungi for the production of novel biologically active secondary metabolites. In order to achieve this, additional aims were to optimise the isolation of fungi associated with these extreme environments i.e. alkaline and saline soils, by using media amended with extracts from these soils. Also, to select important strains and sequence the ITS region of the ribosomal DNA (internal transcribed spacers, ITS1 and ITS2, with the 5.8S gene), to create a phylogenetic tree for definitive identification.

Fig. 1 Map of Botswana showing locations of the sample sites. Adapted from http://photo.net/travel/africa/botswana/botswana-map.gif

MATERIALS AND METHODS

Fungal isolation and media modifications

Soil samples were collected from saltpans in the southern part of Botswana i.e., Morwamosu, Tshane, Kang and Sekoma and also from Sua Pan, adjacent to the Botswana Ash Plant in Sua town, east of Makgadikgagdi complex (**Fig. 1**).

A total of 15 samples were used in this study. For each site, three replicate samples located at least 20 m apart and each consisting of three sub samples at about 80 cm apart were collected from the five saltpans. The samples were collected from the A_1 horizon using a hand spade. The samples were stored in separate ziplock sterile bags (Nasco-Pak, Whirl Park, USA) and transported to the laboratory, and kept at 4°C until analysis.

Active acidity (pH) of the soil samples was determined in 1: 2 (soil: water) suspension. The potential acidity (pH) was determined using soil: $CaCl_2$ (1: 2) according to Anderson and Ingram (1993). The pH was measured using a pH meter electrode (Fisherbrand Hydrus 300, Orion Institute).

The dilution plate method was used for isolating the fungi. Ten grams of each soil sample were suspended in 95 ml of the dilutent (8.5% NaCl in distilled water). Serial dilutions of the diluted samples were made up to 10^4, and 0.1 ml was taken from each dilution and plated on different selective agar media. The selective media were prepared by amending potato dextrose agar (PDA; Oxoid Ltd., Hampshire, UK) with soil extracts from the respective sites (Wollum II 1982; Mejanelle *et al.* 2001). The prepared media were also amended with 0.3 g/l of streptomycin sulphate (Sigma-Aldrich, Schnelldorf, Germany) to prevent bacterial growth.

For each medium two replicates were plated per dilution. The plates were incubated for 1-3 weeks or long enough for fungal colonies to develop at 25°C (Mejanelle *et al.* 2001). All fungi isolated from selective media were subcultured into both plain PDA and PDA amended with 100ml soil extract. The soil extract consisted of filtered supernatant obtained from autoclaved 500 g soil/L water (Wollum II 1982; Mejanelle *et al.* 2001). Once grown, small pieces of the fungal cultures were cut and preserved in McCartney bottles of sterile mineral oil for long term storage (Waller *et al.* 2002).

Preliminary identification of fungi was based on growth rate and colony morphology. For sporulating fungi, identification was also based on microscopic reproductive features (Ellis 1971, 1976; Carmichael *et al.* 1980; Sutton 1980; Hanlin 1990; Domsch and Gams 1993: Klich 2002). *Fusarium* spp. were identified according to Nelson *et al.* (1983) and Domsch and Gams (1993). For sterile fungi, sporulation was induced by growing them on water agar (Waller *et al.* 2002) supplemented with carnation leaves (Fisher *et al.* 1982; Kirk *et al.* 2001) and incubating the plates under ultraviolet light (short wave length-340 nm) at 25°C until the fungi sporulated. When induction of sporulation was not successful, isolates were recorded as 'sterile mycelia'. The identity of 10 fungal species showing broad antimicrobial activities which were used for minimum inhibitory volumes, were confirmed by the sequences of the ITS region (internal transcribed spacers, ITS1 and ITS2, with the 5.8S gene of the nuclear rDNA) as defined below.

Bioactivity tests

A confrontation test was used for preliminary screening of potential antagonism. Fungi that showed activity by this method were selected and used for further tests using their cultural supernatants as crude extracts. Then the supernatants of the fungal cultures were tested for antibiotic activity using the well-diffusion bioassay.

The choice of test organisms was based on the use of the strains in previous screening studies as indicated for each organism. *Staphylococcus aureus* (NCTC 0835) (Lauková 2000; Jonathan and Fasidi 2003; Madamombe and Afolayan 2003; Tepe *et al.* 2003), *Escherichia coli* (NCIMB 8545) (Schulz *et al.* 1995; Chaudhry *et al.* 2003; Jonathan and Fasidi 2003; Tepe *et al.* 2003), *Aspergillus niger* (UBCC385) (Jonathan and Fasidi 2003) and *Candida albicans* (obtained from Botswana National Health Laboratory).

Confrontation tests

For each fungal combination, three millimetre discs of fungi were placed on malt-peptone yeast extract agar (MPYA) plates (Schulz *et al.* 1995) simultaneously at opposite sides to the test organisms (yeast, *C. albicans* and fungus, *A. niger*). The plates were incubated for 7 days at 25°C. To avoid spreading of *Aspergillus* spores, a technique was used to produce a spore suspension (Pitt and Hocking 1997). The antimicrobial activity was determined by measuring the width between the edges of both colonies (Schulz *et al.* 1995; Tomita 2003). For bacterial-fungal confrontational tests, Muller-Hinton agar (MHA) (Oxoid Ltd.) was utilised. Bacterial cultures grown overnight on nutrient broth were streaked on the surface of the agar using a sterile loop, and then a plug of a fungal colony (3 mm diameter) was placed on the agar surface and incubated overnight at 37°C for 24 hrs. The antimicrobial activity of the fungal isolates was assessed by measuring the diameter of zones of inhibition of the bacterial colonies (modified from Schulz *et al.* 1995).

Preparation of the supernatants

The method described by Tomita (2003) was modified for the production of antibiotics. Plugs of agar-supporting mycelial growth were excised and transferred aseptically to 250 ml Erlenmeyer flasks containing 25 ml of antibiotic production medium, malt-peptone yeast extract broth (MPY broth) (Schulz *et al.* 1995). Larger volumes (50 and 100 ml) of antibiotic production medium were also tried but were found to be less effective. The isolates were then incubated for 6 days at 25°C in an orbital shaker (Labline, Melrose Park) set at 165 revolutions per minutes (rpm). The culture supernatants were then centrifuged at 2000 rpm for 20 min at 4°C (Eppendorf Centrifuge 5415R, Germany). The supernatants were used in the well-diffusion assay against the selected test microorganisms outlined above, and to determine the minimum inhibitory volume.

Well-diffusion bioassay

The antimicrobial activities were determined using the agar-well diffusion assay. The test was carried out as described by Jonathan and Fasidi (2003).

Bacteria and yeast strains were cultured overnight at 37°C on nutrient agar (Oxoid Ltd.) and then a suspension of the bacteria and yeast was grown in nutrient broth (Oxoid Ltd.) to give a final concentration of $OD_{560} = 1$ (Jonathan and Fasidi 2003). Then 0.1 ml of the suspension was spread on the surface of the MHA using a sterile spread bar. *Aspergillus niger* was cultured on PDA for 5 days and a spore suspension was prepared as previously described, before spreading on the MPYA agar surface. Using a cork-borer (10 mm diameter), four wells were cut into the agar per plate. The wells were then filled with 200 µl of the supernatants. The plates were left at room temperature for 3 to 4 hrs to allow for diffusion into the agar (MPYA for fungi, MHA for bacteria). The plates were incubated at 37°C for 24 hrs for bacteria and 25°C for 3 days for fungi and then examined for any zones of inhibition created. Each assay was replicated twice.

Minimum inhibitory volume (MIV) of the cultural filtrates (modified MIC)

Ten isolates were selected for this assay. Selection was based on the degree of antimicrobial activity of the supernatants as demonstrated by the size of the inhibition zones and the broad spectrum of activity as determined by the aforementioned assays. The test was carried out using different volumes of supernatants instead of determining a known concentration of the active compound(s). Different volumes of supernatants (50, 100, 150, 200 and 250 µl) were prepared. Each volume was made up to 250 µl by addition of MPY-broth used for production of supernatants. The test was carried out in triplicate and averages of the inhibition zones recorded.

The supernatants and medium (MHA) were prepared as described previously. Aliquots measuring 50, 100, 150, 200, and 250 µl were filled into prepared wells for each test microorganism that showed susceptibility to the respective metabolite. The plates were kept at room temperature for 3 to 4 hrs to allow the diffusion of the metabolite into the agar medium. The plates were then incubated at 37°C for 24 hrs for bacteria and the yeast and for 3 days for the *A. niger*. The zones of inhibition were then recorded.

DNA extraction, PCR and phylogenetic analysis

Single strain isolates were obtained for sterile cultures by culturing on water agar and picking up hyphal tips (Pitt and Hocking 1997), and single spore isolation was performed for sporulating cultures. Cultures were grown on malt extract agar (MEA; Biolab, Merck, Germany) at 25°C for 5-7 days.

DNA extraction was carried out using a modified extraction procedure (Raeder and Broda 1985), as described by Geldenhuis *et al*. (2004). Mycelia were harvested from the surface of a colony and about 0.2 to 0.3 ml of mycelia were crushed in an Eppendorf tube using the Eppendorf pestle for 3-5 min suspended in 800 µl of DNA extraction buffer (DEB) consisting of 200 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA (pH 8.0) and 0.5% sodium-dedocyl sulphate (SDS), to which 500 µl of phenol and 300 µl of chloroform added and vortexed. The suspension was centrifuged at 10,000 rpm for 60 min at 4°C to remove cell debris. The upper aqueous layer (supernatant) was transferred and additional extraction carried out using phenol: chloroform (200: 200 µl) by centrifugation for 5 min at 4°C. The supernatant was transferred and 400 µl chloroform was added and further centrifuged for 5 min. This procedure was repeated once or twice until the white aqueous interface disappeared.

The supernatant was transferred to a new tube, and DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4), and two volumes of absolute ethanol. The tubes were inverted several times and chromosomal DNA formed a little ball. The DNA was pelleted by centrifuging at 10,000 rpm for 30 min at 4°C after which ethanol was carefully removed. The pellet was then washed with 70% ethanol, centrifuged for 5 min (10,000 rpm) and then ethanol was removed. The pellets were dried at 65°C in a convection oven and resuspended in 50 µl sterile distilled water. Five microlitres of RNAse-A (Roche, Germany) (1 mg/ml) was added to the DNA solution to digest any RNA with incubation at room temperature for a few hours.

Aliquots of 10 µl of DNA sample were run in 1% agarose gel in 1X TAE buffer (0.4 M Tris, 50 nM NaOAc, 10 mM EDTA pH 7.8) using a tracking dye at 70 V for 1 h to determine the presence of DNA. The gel was then viewed using 3 µl ethidium bromide under UV light (Syngene-Bio Imaging System, Vacutec, South Africa). DNA concentrations were estimated using a spectrophotometer (UV/VIS Spectrometer Lambda 12, Perkin Elmer, Germany).

To amplify 5.8S rDNA and ITS 1 and 2 regions of the species, the internal transcribed spacer (ITS) region primers were used. These are ITS1 and ITS4 with sequences (5′-TCCGTAGGTGAA CCTGCGG-3′) and (5′-TCCTCCGCTTATTGATATGC-3′) respectively (White *et al.* 1990). A 50 µl PCR reaction mixture (master mix) was used for the amplification procedure. The reaction mixture contained 38 µl sterile water (Roche), 2 µl PCR buffer with $MgCl_2$, 2 µl of (10 mM each) deoxyribonucleotide triphosphate (DNTP mix) (Roche), and 1 µl of ITS1 and ITS4 (15 mM) (Inqaba Biotech, South Africa) each and 1 µl of *Taq* DNA polymerase (Roche). Template DNA (5 µl) of about 50-100 ng was added after DNA extraction. The thermal cycling program involved 5 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 94°C, 2 min primer annealing at 55°C, 2 min extension at 72°C. The final extension was performed for 5 min at 72°C. The template DNA was amplified in a programmed thermocycler (Techgene Ftgene2D, Cambridge, UK).

The PCR products were run in a 1.3% (w/v) agarose gel at 70 V for 1 hr. A 100 bp (XIV) marker was used and tracking dye were used. The gel was visualized under UV light (Syngene-Bio Imaging System).

Samples were submitted for sequencing to the Inqaba Biotechnical Industries (Pty) Ltd. in South Africa. Amplification for sequencing was performed in a 12.5 µl reaction volume (master mix). The master mix consisted of 10 µl PCR product, 0.5 µl exonuclease I (20 U/µl, Fermentas) and 2 µl shrimp alkaline phosphatase (1 U/µl, Fermentas). The mixture was incubated for 30 min at 37°C, after which it was activated for 15 min at 80°C. Sequence reactions were prepared with the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Perkin-Elmer, Germany) following conditions provided by the manufacturer and were analysed on a spectrumedix SCE2410 (Applied Biosystems) genetic analysis system. PCR products were sequenced with the primers used for PCR, which were ITS1 and ITS4 (White *et al.* 1990).

The sequences were edited using Chromas (Technelysium Pty Ltd., South Africa). The sequences of the ITS region were aligned with other similar sequences of fungi retrieved from the GenBank databases using Clustal W (Thompson *et al.* 1994). Phylogenetic trees were constructed from the evolutionary distance data by Neighbour-Joining analysis with the Kimura 2-parameter model (MEGA version 2.1). Statistical support was estimated by bootstrap analysis to determine the confidence values for individual branching points (5000 replicates) for the trees generated.

Table 1 †Mean ± SE soil pH of the different saltpans.

Sample site	Active acidity in water	Potential acidity in $CaCl_2$	*t*-test (p-value)
Kang	10.01 ± 0.00	10.03 ± 0.01	0.158*
Morwamosu	8.60 ± 0.03	8.56 ± 0.02	0.269*
Sekoma	9.02 ± 0.01	9.00 ± 0.00	0.251*
Sua	10.11 ± 0.00	10.09 ± 0.01	0.067*
Tshane	9.44 ± 0.01	9.39 ± 0.01	0.033

SE = standard error of the mean; † mean of three replicates. P values: *>0.05

RESULTS

Biodiversity of fungi in the saltpans

Acidity of the saltpan samples ranged from 8.6 in Morwamosu, a pan in its early stage of development, to 10.11 in Sua pan, the most developed saltpan (**Table 1**). There were no significant differences between active and potential acidity of the saltpans. The two most common fungal isolates were from *Aspergillus* and *Fusarium* genera, which accounted for 26.1 and 18.0% of the total isolates respectively. Other isolates were *Dendryphiopsis* (13.5%), *Phoma* (5.4%), *Alternaria alternata* (7.2%) and 11.7% were unidentified or sterile fungi. A detailed composition of the genera isolated is shown in **Table 2**.

Antimicrobial activities

The results for initial screening using the confrontation assays according to sample sites is summarised in **Fig. 2**. It shows levels of inhibition of the fungal isolates on the different test organisms for the different sample sites. Fungal isolates from Sekoma pan were the most inhibitory as they inhibited most of the test organisms, and fungal isolates from Kang pan were the least inhibitory. Fungal test organisms *A. niger* and *C. albicans* were least affected by the fungal isolates and were the least inhibited (**Fig. 2**).

In the well diffusion bioassays, of the 111 isolates assayed, the supernatants of 48 isolates showed activity against one or more test organisms. Gram-positive *B. megaterium* and *S. aureus* were most susceptible with inhibition by 81.3 and 70.8% of fungal isolates, respectively (**Table 3**). Generally, antimicrobial activity against *E. coli* (Gram-negative) was low (12.5%). In the fungal activity assays, *A. niger* and *C. albicans* were inhibited by 37.5 and 4.2% of the isolates, respectively. There was no consistency in inhibition ability by different isolates (i.e. strains) of the same species. Few of the isolates showed broad activity against both Gram-positive and -negative bacteria, and/or fungi. Based on this result, 10 of the isolates (in bold in **Table 3**) were selected for further antimicrobial analysis.

Table 2 Occurrence and biodiversity of fungi from saltpans in Botswana.

Fungal species	Number of colonies isolated at each site					All samples	
	SA	Tsp	Skm	Kg	MR	No. of isolates	% freq.
Actinomycetes	3	-	-	-	-	3	2.7
**Alternaria alternata*	-	3	2	-	3	8	7.2
Aspergillus						(29)	26.1
A. terreus	2	1	1	-	-	4	3.6
A. fumigatus	10	1	-	-	-	11	9.9
A. flavus	4	2	-	-	2	8	7.2
A. carneus	1	-	-	-	-	1	0.9
Aspergillus sp. 1	1	-	-	-	-	1	0.9
Aspergillus sp. 2	2	-	-	-	-	2	1.8
Aspergillus sp. 3	2	-	-	-	-	2	1.8
**Chaetomium globosum*	1	-	-	-	-	1	0.9
**Curvularia lunata*	2	-	-	-		2	1.8
**Dendryphiopsis* sp.	-	8	7	-	-	15	13.5
**Dreschlera* sp.	-	-	3	-	-	3	2.7
**Epicoccum* sp.	-	-	-	-	1	1	0.9
Fusarium						(20)	18.0
F. chlamydosporum	-	2	1	-	5	8	7.2
F. dimerum	-	1	1	-	1	3	2.7
F. sporotrichioides	-	2	1	-	-	3	2.7
Fusarium sp. 1	-	-	2	-	1	3	2.7
Fusarium sp. 2	-	-	-	-	3	3	2.7
Gilmaniella sp.	-	1	-	-	-	1	0.9
Memnoniella sp.	2	-	-	-	-	2	1.8
Penicillium cf. *glabrum*	-	1	-	-	-	1	0.9
Persiciospora sp.	-	1	-	-	-	1	0.9
Phoma						(6)	5.4
Phoma sp. 1	-	1	-	-	-	1	0.9
Phoma sp. 2	-	1	-	-	-	1	0.9
Phoma sp. 3	-	-	1	-	-	1	0.9
Phoma sp. 4	-	1	-	-	-	1	0.9
Phoma sp. 5	-	2	-	-	-	2	1.8
Trichobotrys sp.	-	-	2	-	-	2	1.8
Verticillium sp.	1	-	-	2	-	3	2.7
Unidentified						(13)	11.7
Unidentified ascomycete	-	-	-	3	-	3	2.7
*Sterile mycelium 1	-	-	-	-	2	2	1.8
Sterile mycelium 2	-	1	-	-	3	4	3.6
Sterile mycelium 3	-	-	1	-	-	1	0.9
*Sterile mycelium 4	1	-	-	-	-	1	0.9
Sterile mycelium 5	-	-	-	-	2	2	1.8
Total	32	29	22	5	23	111	100

Note: Numbers in parentheses indicate the total numbers of isolates in the given genus or group
% frequency= number of isolates of a given taxa / total number of isolates obtained x 100
Sua (SA), Tshane (Tsp), Sekoma (Skm), Kang (Kg) and Morwamosu (MR).
* represents melanised isolates

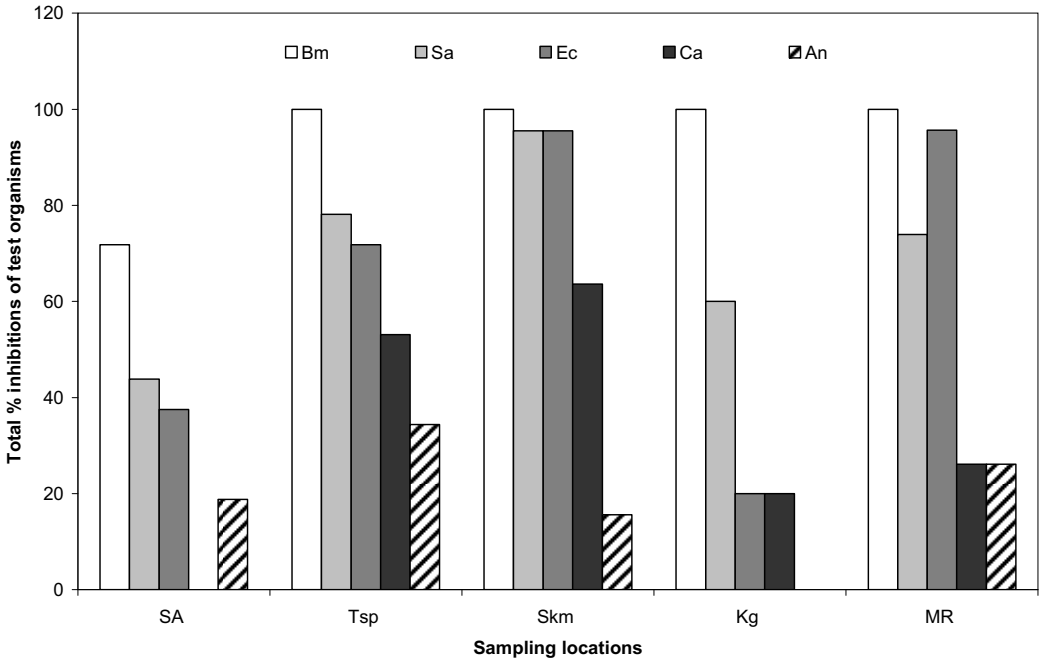

Fig. 2 Percentage inhibition of test organisms by isolates at each site using confrontation test. Test organisms: Bm-*Bacillus megaterium*; Sa-*Staphylococcus aureus*; Ec-*Escherichia coli*; Ca-*Candida albicans*; An-*Aspergillus niger*. Sampling locations: SA-Sua pan; Tsp-Tshane pan; Skm-Sekoma pan; Kg-Kang pan; MR-Morwamosu pan.

Effects of increasing supernatants volume

The isolates that inhibited the respective test organism in the well-diffusion bioassays were further tested for the minimum inhibitory volume (MIV) which is required to inhibit the particular test organism. Supernatants from the 10 selected isolates (**Table 3**) all showed activity against the tests organisms screened at highest volume (250 µl). Compared to other isolates, Tsp16 showed the highest activity at all volumes, giving the widest zone of inhibition (6.3 mm). Skm12 was least active against *B. megaterium*, showing inhibition (3.0 mm) only at 250 µl of the supernatant (**Fig. 3**). Only the supernatants of two isolates, Tsp16 and Tsp22, were active at volumes less than 100 µl. This was observed in *S. aureus* where Tsp16 and Tsp22 showed activity at values as low as 50 and 100 µl, respectively (**Fig. 4**). Tsp9 showed the highest activity at all volumes with the widest zone of inhibition of 10 mm at 200 µl. The rest of the fungal supernatants inhibited growth at volumes of 150 µl and above. Skm12 and Tsp5 had the lowest activity (active only at 250 µl), and only Tsp9, Tsp16 and Tsp22 inhibited *E. coli* at 100 µl. Only Tsp5 was inactive (**Fig. 5**) at 100 µl. Only higher volumes (200 and 250 µl) were effective against *A. niger* on all seven fungal supernatants tested (**Fig. 6**). Only two of the fungal isolates Tsp22 and Tsp5 showed antifungal activity against *C. albicans*, only at the highest volumes of 200 and 250 µl.

Molecular identification

The positively identified fungi from the Genbank database were selected for the construction of phylogenetic trees in

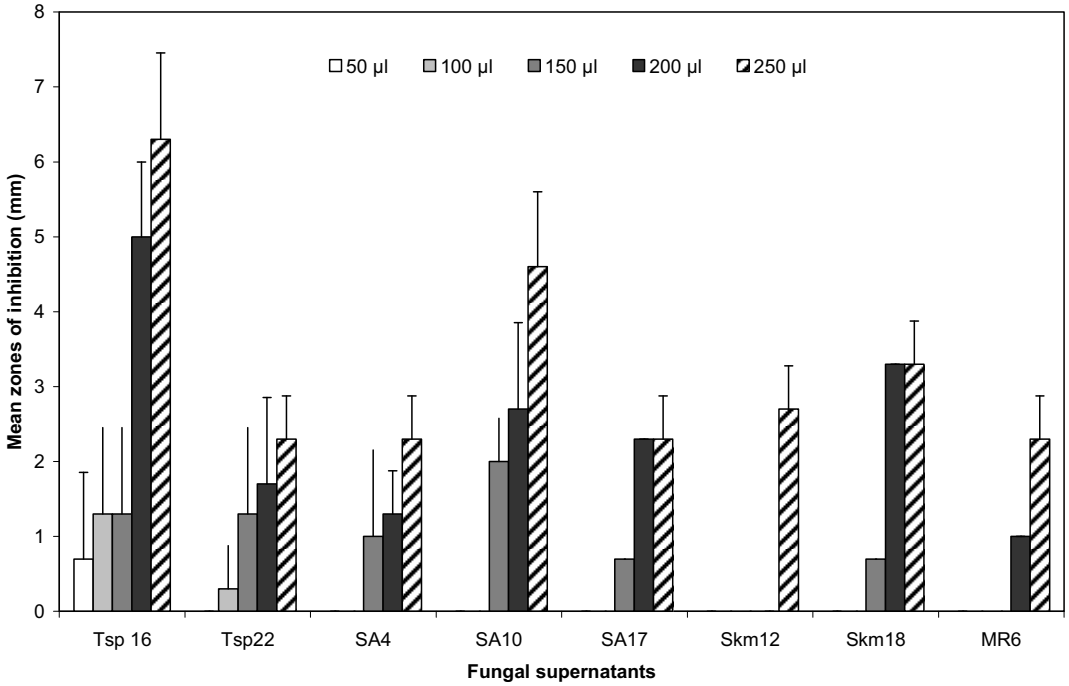

Fig. 3 Activity of fungal supernatants against *Bacillus megaterium.*

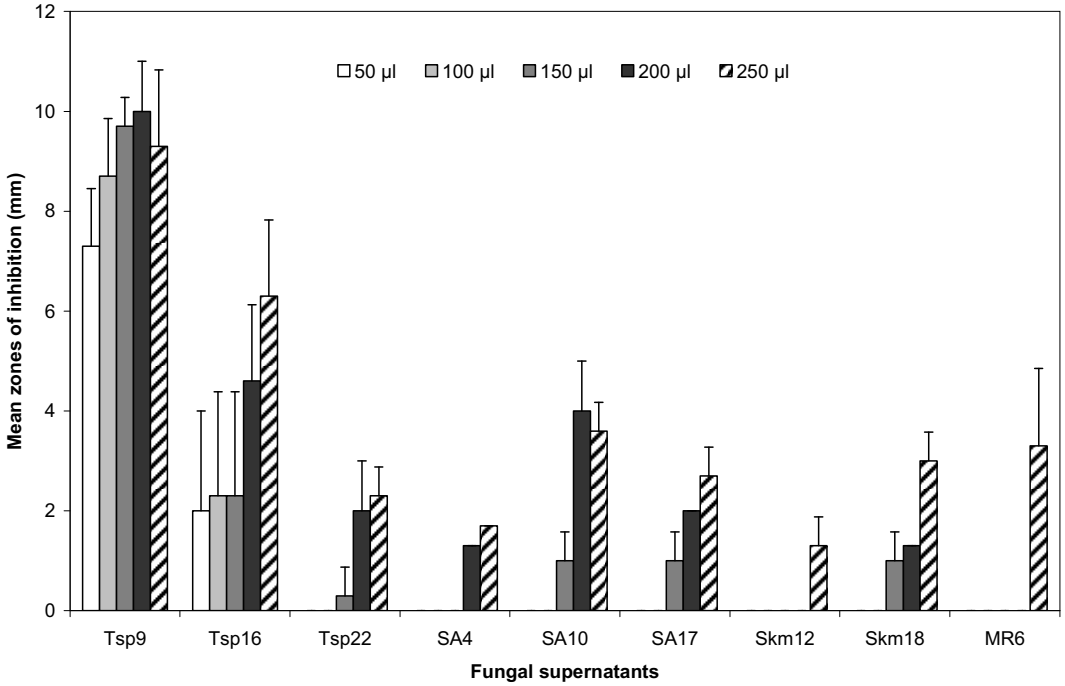

Fig. 4 Activity of fungal supernatants against *Staphylococcus aureus.*

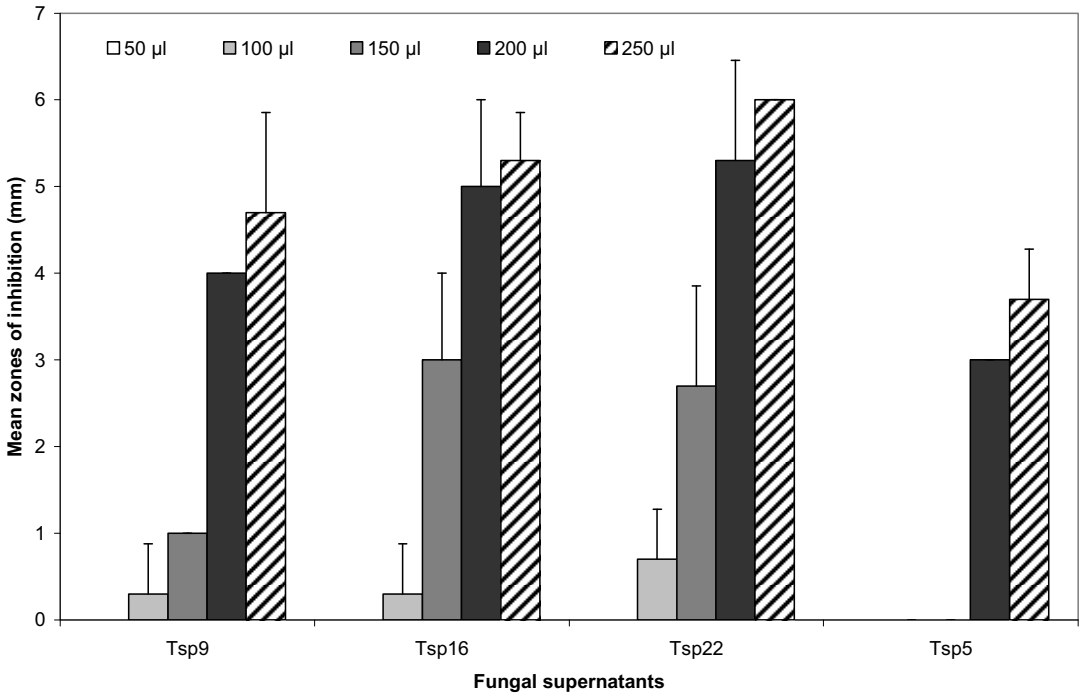

Fig. 5 Activity of fungal supernatants against *Escherichia coli.*

Figs 7 and **8** (unnamed isolates with codes only were isolated in the present study).

Eight out of 10 isolates were sequenced successfully (**Table 4**). Sequence analysis generated two phylogenetic trees as shown in **Figs. 7** and **8**. **Fig. 7** represents taxa in the Pleosporales (bitunicate ascomycetes and their anamorphs). In this tree, the sterile isolate MR6 represents a member of the Phaeosphaeriaceae (clade 1), whereas Skm12 and Tsp9 are members of the Pleosporaceae and are tentatively identified as *Dendryphiopsis* sp (clade 6). **Fig. 8** represents unitunicate ascomycetes and their anamorphs. Tsp22 and SA4, which were morphologically identified as *Aspergillus terreus*, occur in clade 1 with other strains of that species. A previously unidentified *Aspergillus* sp1 represented by Tsp16 and SA10 occurs in clade 5 with strains of *A. fumigatus.* The unidentified *Fusarium* sp. (Skm18) occurs in clade 8 with a variety of other *Fusarium* spp.

DISCUSSION

The saltpans under study were alkaline as indicated by their pH (8.56-10.34). To optimise the isolation the desired fungi (i.e. alkalo- and halotolerant), dilution plating was undertaken with media amended with soil extracts from the respective pans and isolates were also subcultured onto this media. The fungi are highly likely to be alkalo- and halotolerant as these pans are high in sodium and potassium (Thomas and Shaw 1991). None of the isolates were, however, alkalo- or halophilic as they were able to grow on normal media (PDA) without soil extracts. There have been reports of fungi isolated from saltern ponds that are halophilic, all of which are melanised species (Gunde-Cimerman *et al.* 2000), although studies of desert soils have reported fungi isolated to be mainly halotolerant (Guiraud *et al.* 1995; Moubasher *et al.* 1990). The pH of the soils from

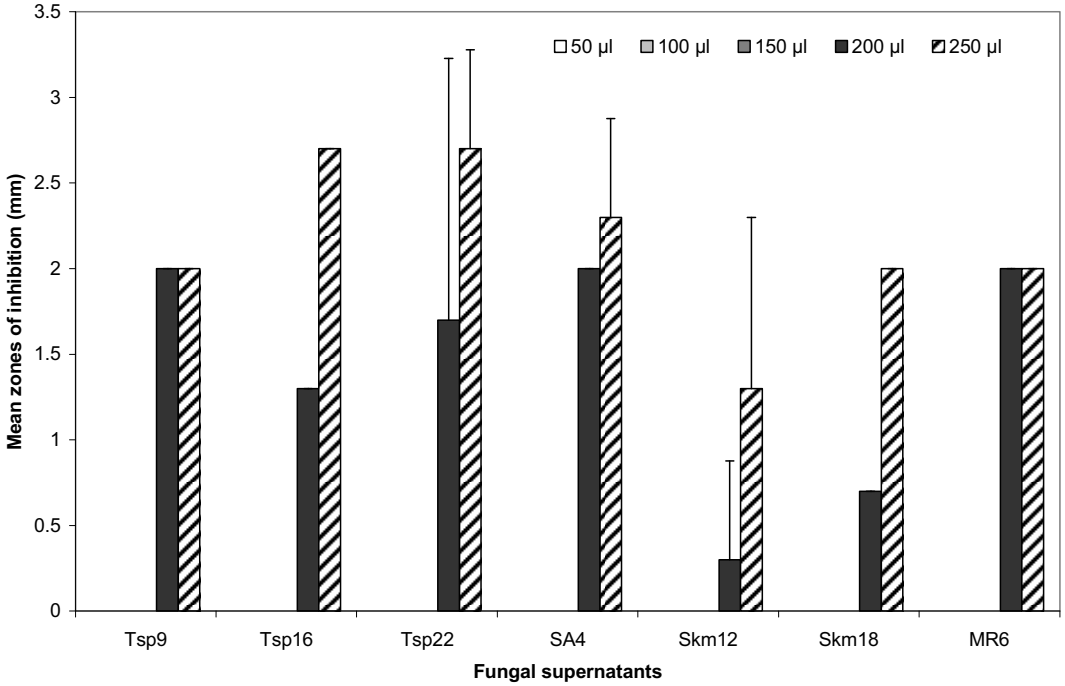

Fig. 6 Activity of fungal supernatants against *Aspergillus niger.*

the pans in the present study are much higher than those recorded by Abdel-Hafez (1981) (pH 7.05 to 7.60) from desert soils in Saudi Arabia. The alkaline nature of the saltpans is also shown by the difference between potential and active acidity of the samples. This indicates very low hydrogen and aluminium ions on the exchangeable site, which may mostly be dominated by alkaline cations such as sodium. Variable pH values from different sampling locations were recorded as these saltpans are at different stages of development.

The saltpans' alkalinity seemed to be dependent on the stage of development and may be due to the amount of basic cations and salts that have accumulated with time (Carter 1989; Thomas and Shaw 1991; McIntyre 2003).

The stage of development was depicted by the amount of vegetation cover on the pan. Pans in an early stage of development had some vegetation, e.g. Morwamosu, whereas those, more developed, had no vegetation and often had salt crusts during the dry season, e.g. Kang. The more developed the saltpan, the higher the pH. The stages of development in the increasing order were as follows, Sua and Kang pans being the most developed, followed by Tshane, Sekoma, and lastly the Morwamosu pan still in its early stage of development (Thomas and Shaw 1991).

Occurrence and biodiversity of fungi from the sampled saltpans

Despite the extreme nature of the environments sampled with high alkalinity and large fluctuations in the mean daily and annual temperatures, it was possible to isolate fungi. Fungal genera which are commonly isolated from soils were *Aspergillus*, *Fusarium*, *Penicillium* and *Phoma* (Domsch and Gams 1993). Some uncommon species such as *Dendryphiopsi*s sp., *Persiciospora* sp. and *Trichobotrys* sp. were also isolated. Surprisingly, only one species of *Penicillium* (0.9%) (**Table 2**) was encountered, even though it is ranked most common among the soil fungi (Domsch and Gams 1993). A study of halophilic fungi from the desert soil of Saudi Arabia by Abdel-Hafez (1981) recorded *Penicillium* among the most frequently represented genus (27%) of the total fungi after *Aspergillus.* Moubasher *et al.* (1990) also recorded *Aspergillus* and *Penicillium* as the most common fungi isolated in a study of desert soils and salt marshes. *Aspergillus* and its teleomorph *Eurotium* are common in studies of hypersaline waters such as the Dead Sea (Kis-Papo *et al.* 2001) and salterns (Butinar *et al.* 2005c). Other fungi recorded in the present study and also in others include *Alternaria alternata*, *Chaetomium globosum*, *Curvularia lunata* and *Dreschlera* sp. (Abdel-Hafez 1981; Guiraud *et al.* 1995).

Despite an attempt to induce sporulation in the present study (with UV light and low nutrient medium), 11.7% of the isolates remained sterile and could not be identified morphologically. This scenario is common in similar studies and the isolates are often recorded as sterile or unidentified. Holler *et al*. (2000) noted that 16% of the isolated mycelia still remained sterile after trying to induce sporulation by use of different media and culture conditions, while Okuda *et al*. (1995) recorded 23 unidentified species.

Seckbach and Oren (2000) suggested that species diversity is limited in extreme environments because of the stress challenges that these microorganisms encounter. Species diversity in the present study was generally comparable to other studies (16 genera comprising of 30 species and 6 sterile mycelia from 15 samples i.e., 1.9 species/sample). When broadly comparing the number of taxa with sample size of other studies, which isolated fungi from saline and alkaline environments, it is evident that other studies recorded a similar species abundance and diversity of fungi. Guiraud *et al*. (1995) isolated 51 genera and 20 sterile mycelia giving 106 fungal species (excluding *Penicillium* and *Aspergillus*, which were not considered even when they were isolated) from 56 samples (1.9 species/sample) from soils around the Dead Sea. Abdel-Hafez (1981) obtained 25 genera (68 species) from 40 samples (1.7 species/sample) from the desert soils of Saudi Arabia.

Some of the fungi encountered had dark pigmented hyphae and spores (melanised). Melanised fungi, in the present study, included *Alternaria*, *Dendryphiopsis* sp. and some sterile mycelium (**Table 2**). This phenomenon has also been observed in other desert soils (Guiraud *et al*. 1995) and from saltern ponds (Gunde-Cimerman *et al.* 2000; Mejanelle *et al*. 2001). Melanised fungi isolated from saltern ponds were shown to be more prevalent in higher salinities (Butinar *et al.* 2005b; Cantrell *et al.* 2006). Guiraud *et al.* (1995) suggest that melanin could be used for protection e.g. against the effects of UV light and heat, and may thus represent an adaptation to extreme environments.

Antimicrobial activities

Initial confrontation tests showed that all fungal isolates produced substances that inhibited the growth of at least one test organism as demonstrated by zones of inhibition around the test organisms. This implies that the fungal isolates produced active compounds that diffused in the agar medium and inhibited the growth of test organisms (Schulz *et al.* 1995). Confrontation tests using *Pezicula* strains carried out by Schulz *et al*. (1995) also showed that all the strains (100%) were active against other fungi. Test organism response to *A. niger* differed between the different isolates. In some cases *A. niger* inhibited the isolates instead. This was the case for isolates Skm2, MR6, SA12 and Tsp10. In other cases, *A. niger* grew over the isolates (Skm1 and Tsp8). Results by Tomita (2003) showed similar growth patterns during antifungal confrontation tests.

Bioactivity results indicated that some fungal supernatants prepared from MPY broth showed zones of inhibition against the test organisms. This is in agreement with the confrontation tests which implied that some compounds with antimicrobial properties were produced by the fungal strains and released into the medium.

Previous studies have shown that fungi from extreme habitats produce considerable amounts of antimicrobial secondary metabolites probably as a form of protection against environmental stress (Concepcion *et al.* 2001). Similarly in this study 42.3% of the isolate supernatants showed antimicrobial activity against at least one test organisms. This was higher than results obtained by Tomita (2003), who

Table 3 Biological activities of fungal supernatants against selected test organisms in the agar-well diffusion assay.

Isolate number	Species	Mean radius of inhibition measured from the well perimeter (mm)				
		Bm	Sa	Ec	Ca	An
Sua						
SA4	*Aspergillus terreus*	2.5	2	4.5	0	1
SA5	*Memnoniella* sp.	4	3.5	0	0	0
SA10	*Aspergillus fumigatus*	5.5	5.5	0	0	0
SA11	*Aspergillus fumigatus*	1	3	0	0	0
SA12	*Aspergillus carneus*	3.5	0	0	0	0
SA13	*Aspergillus fumigatus*	6	4	0	0	0
SA14	*Aspergillus fumigatus*	2.5	2.5	0	0	0
SA15	*Aspergillus fumigatus*	1.5	3	0	0	0
SA17	*Aspergillus fumigatus*	2.5	5.5	0	0	0
SA19	*Aspergillus fumigatus*	2	3	0	0	0
SA21	*Aspergillus fumigatus*	0	0	0	0	3
SA22	*Aspergillus flavus*	1	6.5	0	0	2
SA24	*Aspergillus flavus*	0	0	0	0	3
SA25	*Aspergillus flavus*	1	1.5	0	0	0
SA27	*Aspergillus terreus*	1	0	0	0	2
Tshane						
Tsp1	*Alternaria alternata*	5	2.5	0	0	2
Tsp5	*Dendryphiopsis* sp.	0	0	4	2.5	0
Tsp6	*Alternaria alternata*	2	0	0	0	0
Tsp9	*Aspergillus flavus*	0	7.5	4	0	4
Tsp14	*Dendryphiopsis* sp.	3	2	0	0	0
Tsp16	*Aspergillus fumigatus*	6.5	6.5	6	0	4
Tsp19	*Dendryphiopsis* sp.	5	0	0	0	2.5
Tsp21	*Aspergillus flavus*	0	4.5	0	0	0
Tsp22	*Aspergillus terreus*	1	1.5	6	3	2
Tsp24	*Fusarium sporotrichioides*	0	0	0	0	1.5
Tsp29	*Fusarium dimerum*	0	0	0	0	4
Sekoma						
Skm2	*Fusarium chlamydosporum*	4	0	0	0	0
Skm3	*Dreschlera* sp.	4	2	0	0	0
Skm4	*Dreschlera* sp.	4	2	0	0	0
Skm5	*Alternaria alternata*	4.5	1	0	0	0
Skm7	*Alternaria alternata*	1	3	0	0	0
Skm11	*Dendryphiopsis* sp.	0.5	0	0	0	0
Skm12	*Dendryphiopsis* sp.	1.5	0.5	0	0	4
Skm13	*Fusarium dimerum*	2	1	0	0	0
Skm15	*Trichobotrys* sp.	1	1	0	0	0
Skm16	*Aspergillus terreus*	1.5	0.5	1	0	0
Skm18	*Fusarium* sp.1	2.5	2.5	0	0	3
Skm19	*Fusarium* sp.1	0	1	0	0	4
Skm20	*Phoma* sp.3	3	2	0	0	0
Skm21	Dendryphiopsis sp.	0	0	0	0	2
Morwamosu						
MR3	*Alternaria alternata*	1	1	0	0	1
MR6	Sterile mycelium 1	1	4	0	0	4
MR8	*Fusarium* sp.2	1	1.5	0	0	3
MR13	*Alternaria alternata*	1	0	0	0	0
MR15	*Aspergillus flavus*	1.5	1.5	0	0	0
MR16	Sterile mycelium 1	1	1	0	0	0
MR20	*Fusarium* sp.2	2	3	0	0	0
MR23	Sterile mycelium 1	1	0	0	0	0
Total inhibitions (%)		39/48 (81.3)	34/48 (70.8)	6/48 (12.5)	2/48 (4.2)	18/48 (37.5)

Note: Bm-*Bacillus megaterium*, Sa-*Staphylococcus aureus*, Ec- *Escherichia coli*, Ca- *Candida albicans*, An- *Aspergillus niger*.
Isolate codes in bold indicate isolates selected for minimum inhibitory concentration (volume). Kang had only five isolates none of which showed inhibition in this assay

obtained between 10-30% of activity from supernatants of halophilic endophytes from Southeast Asia using the disc diffusion assay.

Aspergillus isolates in this study showed the most antimicrobial activity observed. This is in agreement with the ranking of *Aspergillus* in the top three positions of metabolites rich groups by Turner and Aldridge (1983), together with *Fusarium* and *Penicillium*. These three genera (*Aspergillus*, *Fusarium* and *Penicillium*) have been studied extensively for bioactive metabolites production (Concepcion *et al.* 2001). Production of bioactive metabolites by *Aspergillus* could be characteristic of the entire genus (Schulz *et al*. 1995). The potential for the overproduction of metabolites such as organic acids by the *Aspergillus* spp., has been discussed by Ruijter *et al*. (2002), who observed that these fungi have an intrinsic ability to accumulate organic acids, and there is an assumption that this ability provides the fungi with an ecological advantage.

Despite the antimicrobial activities of *Phoma* sp. described by Holler *et al*. (2000) and Yamaguchi *et al*. (2002), the different strains isolated in this study were observed to be less active. Only one isolate (Skm20) inhibited the Gram-positive test organisms (*B. megaterium* and *S. aureus*).

Generally, antimicrobial activity results against bacteria indicate that the sensitivity of test organisms varied greatly (**Table 3**). The most sensitive bacterium was *B. megaterium* (81.3%), followed by *S. aureus* (70.8%), then *E. coli* (12.5%). This agrees favourably with trends observed by Holler *et al*. (2000) who observed that test organism *B. megaterium* was more sensitive to fungal metabolites from endophytic fungi than *E. coli*.

Tsp22 identified as *A. terreus* exhibited the most antimicrobial activity inhibiting growth of all test organisms, which might indicate the production of several antimicrobial compounds. The best antifungal activity (4 mm diameter inhibition zone) was recorded in supernatants of six isolates (Tsp9, Tsp16, Tsp2, Skm12, Skm19 and MR6) against *A. niger* (**Table 3**).

The agar-well diffusion method seemed to be an effective method to use for evaluating antimicrobial activities. This is also supported by results obtained by Jonathan and Fasidi (2003) when comparing filter paper disc and agar-well diffusion. They suggested that in agar-well diffusion assay there is better contact and diffusion of the metabolite into the medium and test organisms. Whereas, in disc diffusion assay the filter paper may act as a barrier between the metabolite and the organism which may hinder proper diffusion and total release of the active compounds from the filter disc into the media. Jonathan and Fasidi (2003) also observed a similar trend but attributed it to the difference between supernatant volumes used in each case; claiming the agar-well diffusion methods emits more of the diffusible compounds into the medium than the filter disc diffusion assay.

Among the 10 fungal isolates further assayed for minimum inhibitory concentration (volume) (MIV), different metabolite supernatants reacted selectively on different tests organisms. The most effective supernatants in the MIV assay were that of Tsp9, Tsp16 and Tsp22 showing activity below 200 µl (50, 100 or 150 µl) (**Figs. 3, 4, 5** and **6**).

Generally, bioactivity was relatively increased as the supernatant volumes were increased. Volume of the supernatant used is important as noted by Jonathan and Fasidi (2003) when comparing the disc paper and well diffusion methods. Volumes were used instead of actual concentrations because the latter were not known or determined. This was beyond the scope of this study as the metabolites have to be first purified and individual compounds separated and characterised before the concentration could be determined. Purification of the active compounds could improve activity of the metabolites. Improvement in antibacterial activity was observed by Schulz *et al.* (1995) after purifying some extracts from endophytic fungi.

Failure of some isolates to react against test organisms does not necessarily mean they do not have the potential to do so, but rather could be attributed to other reasons such as medium composition, as the amount and type of metabolite produced is dependent on the conditions of fermentation of the fungal cultures (Yarbrough *et al.* 1993). Bills *et al*. (2002) gave examples, where the titres of a non-sporulating endophyte from roots of a mangrove shrub *Conocarpus erectus* (MF6232) were greatly improved; obtaining production levels of up to 700 µg/ml in shaker flasks. Also, the initial production of *Sordaria araneos* (ATCC 36386) in shaker flasks was 250 µg/ml of the metabolite sordarin, but the production was increased nearly three-fold by media manipulation. Yamaguchi *et al*. (2002) also showed that production of gentisylquinones by a *Phoma* sp. FOM-8108 from sea sand was dependent on the presence of seawater in the fermentation medium. Schulz *et al*. (1995) used four different media and found that no single medium was optimal for production of secondary metabolites by all fungal isolates. Fungi may also lose their bioactive potential due to storage and mass or frequent transfers of cultures. Yarbrough *et al*. (1993) cautioned that fungi should be stored in such a way that results are reproducible even months after the first tests, and recommends lyophilisation as the best methods for storing fungi. Although the fungi in the present study were not transferred frequently it is possible that some may not have displayed bioactivity due to the reasons discussed above.

Molecular sequence analysis

All the taxa represented in **Fig. 7** belong to the order Pleosporales (Kirk *et al.* 2001). The phylogenetic tree (**Fig. 7**) based on the ITS region allows sterile isolates such as MR6 to be broadly identified. MR6 is closely related to *Stagonospora* sp. In **Fig. 8** *Aspergillus fumigatu*s and *A. terreus* belonged to different clades branching at a low confidence level of 29%. Production of antimicrobial properties has been reported in both *A. fumigatus* and *A. terreus* (Domsch and Gams 1993). *Aspergillus fumigatus* has been reported to produce antimicrobial metabolites such as the antibiotic fumigacin, which is active against both Gram-negative and -positive bacteria. Metabolite production by *A. terreus* with antibacterial and antiviral properties has also been noted (Domsch and Gams 1993). Clade 1-6 of **Fig. 8** represent the *Trichocomaceae* and clades 7 and 8 represent the *Nectriaceae*, belonging to orders *Eurotiales* and *Hypocreales*, respectively (Kirk *et al.* 2001).

Clearly, even though very high salt concentration and alkalinity in media can inhibit fungal growth, fungi from saltpans seem adapted (Molitoris and Schaumann 1986; Grant and Horikoshi 1989). Overall, naturally occurring highly alkaline environments such as saltpans of Botswana could be considered potentially valuable sources of new alkalotolerant fungi. Most of these are ubiquitous soil fungi that seem to have the ability to survive in these environments. The most prevalent fungi were *Aspergillus*, *Fusarium*, *Dendryphiopsis* and *Phoma* species. At least 43% of fungal supernatants were active. The fungal supernatants were generally selective in their activity against the test organisms, and this indicates great potential in antimicrobial metabolites production by some fungi from extreme environments (saltpans in Botswana).

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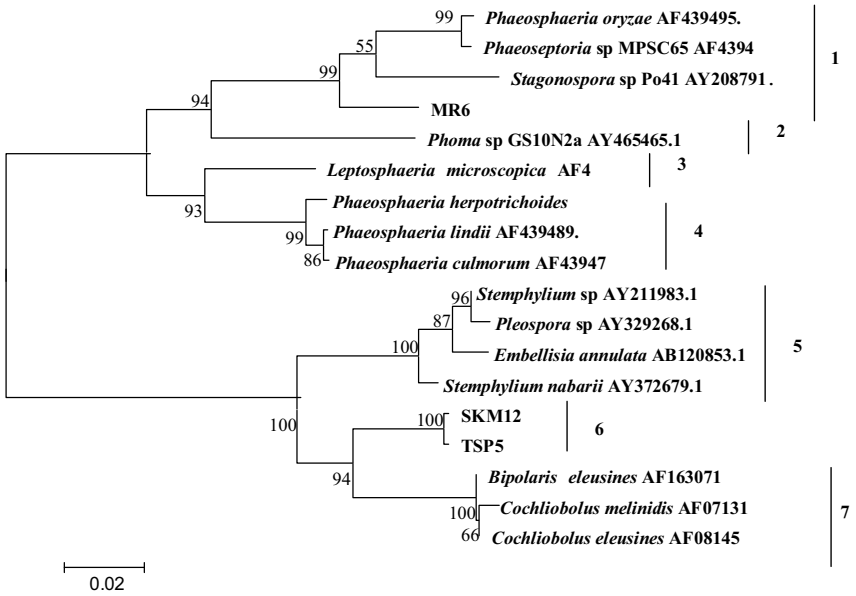

Fig. 7 Phylogenetic relationships inferred from ITS region sequences of selected isolates (Order Pleosporales) using the Neighbour-Joining analysis, Kimura 2 parameter. Bootstrap values are shown at the branches, 5000 replications. N represents clade number.

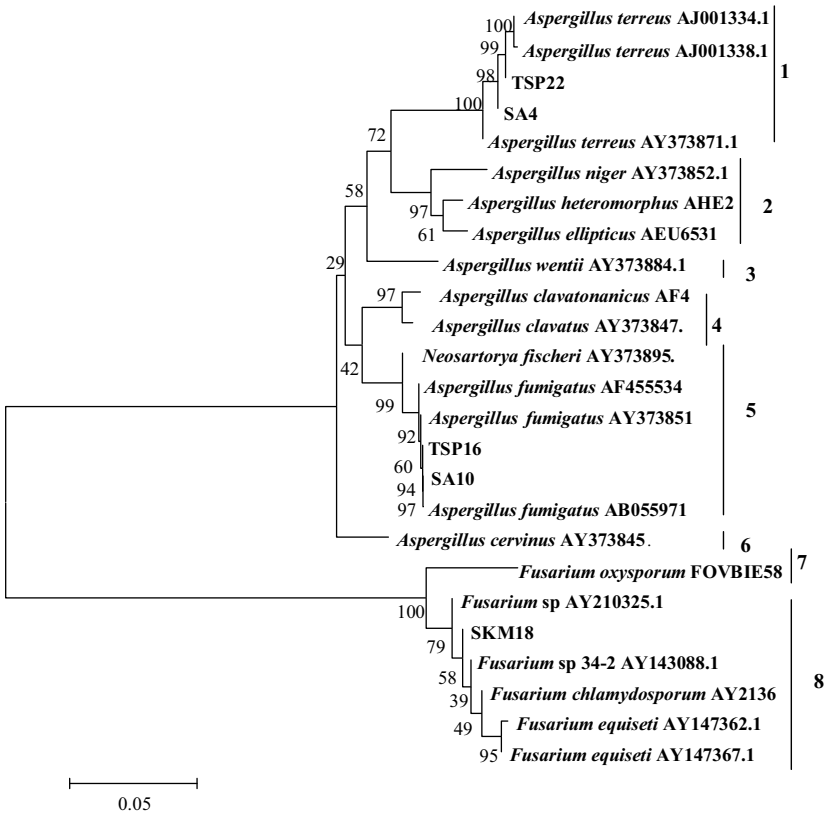

Fig. 8 Phylogenetic relationships drawn from selected isolates (anamorphs of bitunicate ascomycetes) based on ITS region sequence generated by the Neighbour-Joining analysis, Kimura 2 parameter. The numbers at the branching represent bootstrap support in 5000 replications. N represents clade number.

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