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Endophytic Fungi Occurring in *Moringa ovalifolia* in the Tsumeb Area of Namibia

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The diverse endophytic mycobiome of medicinal plants presents researchers with opportunities to discover novel species and potentially novel compounds which can be of use particularly to the medicine sector. *Moringa* species (moringa) are used as a food source and are widely used as medicinal plants in the India-subcontinent and some parts of Africa and Arabia. Basically all parts of the plant have a use spanning from dietary to water purification and medicinal purposes. Because of its extensive uses and limited works concerning the fungal endophytes of moringa in Namibia, this study was conducted to isolate and identify the fungal endophytes of *M. ovalifolia* leaves and stems. Moringa leaves and stem were surface sterilized and placed as dissected segments onto potato dextrose agar (PDA). Distinct fungi growing from the *M. ovalifolia* plant segments were subcultured and maintained on fresh PDA medium. The Internal transcribed spacer (ITS) region was amplified from the genomic DNA of the fungal isolates using primer set ITS-1 and ITS-4. The polymerase chain reaction amplified fragments were sequenced and analysed via the Basic Local Alignment Search Tool program to identify the fungal isolates. The five fungal isolates were identified as *Emericella nidulans* SCAF2, *Alternaria alternata* SCAF4, *Aspergillus sp.* SCAF9, *Peyronellaea glomerata* SCAF6 and unidentified fungal endophyte SCAF7. With a similarity percentage of 89 to its closest known relative, endophyte SCAF7 is regarded as a novel isolate. Bioactive compounds produced by the isolated endophytes could be significant for drug production.

Key words: Endophytic fungi, *Moringa ovalifolia*, medicinal plant, Namibia, Tsumeb**.**

An endophytic microorganism characteristically lives within its host during a part of or throughout its entire life cycle. Plant fungal endophytes reside in or between plant cells inside the plant tissue and cause no apparent harm to the host (Kaul *et al*., 2012). The association between the fungal endophyte and its plant host are usually commensal or mutualistic though it can become pathogenic under certain circumstances (Photita *et al*., 2004). The diverse plant endophytic fungi, estimated at 1.5 million species (Hawksworth, 2004), have been gaining importance because they produce novel compounds which may potentially be of use to medicine, agriculture and industry. Fungal endophytes from medicinal plants have been identified as a potential source for novel metabolites of medicinal significance. Many bioactive compounds with antimicrobial, cytotoxic and anticancer activities have been discovered from endophytic fungi (Zhao *et al*., 2010).

Moringa species (moringa) are angiosperms that are native to India, Pakistan, Arabia, South Asia and some parts of Africa (Anwar *et al*., 2007). There are about thirteen species in the family Moringaceae, of which *M. oleifera* is the most widely known. *M. oleifera* is cultivated throughout the tropics, subtropics and

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many African countries (Mbikay, 2012) and has an extensive number of uses. The leaves and flowers are used for culinary purposes while powder from the leaves is used as a food supplement to prevent malnutrition in children and pregnant women. *Moringa oleifera* seeds are fried and eaten, used to prepare sauces and used in water purification whereas the seed oil can be used in cosmetics (Oliveira *et al*., 1999; Bosch, 2004). *Moringa spp* are known to have medicinal properties and is used as an anodyne, antidiabetic, antihypertensive, circulatory tonic, aphrodisiac, antihelmintic, and treatment for asthma, muscle disease as well as dental caries (Bosch, 2004; Anwar *et al*., 2007; Cheenpracha *et al*., 2010; Atawodi *et al*., 2014). However, *M. ovalifolia* (which occurs only in Namibia and southern Angola) is reported to be mainly used as food by locals (Van Wyk and Van Wyk, 1997). It has not received much research attention compared to *M. oleifera*.

The multitude of beneficial uses that moringa species possess provides an opportunity to investigate the novel fungal endophytes associated with the *M. ovalifolia* population in the Tsumeb area of Namibia. Considering the medicinal and nutritional importance and a paltry knowledge about the microbiota of moringa plants in Namibia, the present study aimed to isolate and identify the fungal endophytes inhabiting the stem and the leaves of *M. ovalifolia*.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves and stems of healthy *Moringa ovalifolia* were collected from mature and healthy plants located in the Tsumeb area, Northern Namibia. The plant materials were placed in sterile bags and transported to the laboratory. The leaves and stems were washed with sterile distilled water and moved to the laminar air flow for processing.

Isolation of fungi

Leaves were surface-sterilized by submerging in 80% ethanol for 5 minutes and rinsed five times with sterile distilled water. The leaves were then sliced across the length of the lamina with a sterile blade and aseptically placed onto potato dextrose agar (PDA) media. A piece of a *M. ovalifolia* stem was surface-sterilized by submerging in 99% ethanol for 7 minutes and rinsed five times with sterile distilled water. A piece of the bark was carved off with a sterile blade and scalpel set and aseptically transferred onto PDA medium with the epidermis facing up. The surface sterilized piece of stem was dissected and pieces of its pith were transferred onto PDA media with flamed tweezers. The PDA plates were then incubated at 35±2°C for 14 days in dark conditions. Young sections from distinct fungi growing from the *M. ovalifolia* plant parts were subcultured onto fresh PDA medium and incubated for up to 10 days at 35±2ºC.

PCR amplification of Internal Transcribed Spacer

The Internal transcribed spacer (ITS) region of the 18S rRNA gene was amplified with primers ITS-1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS-4 (52 -TCCTCCGCTTATTGATATGC-32) (White *et al*., 1990). Genomic DNA of one week old fungal cultures were isolated using the Zymo Research Fungal/Bacterial DNA MiniPrep™ kit and used as the template for amplification. The polymerase chain reaction (PCR) mixture consisted of 12.5ìl Dream Taq™ Green PCR Master Mix 2x, 0.5ìM each primer, 6ìl nuclease free water and 4ìl genomic DNA. The reaction was carried out in a Bio-Rad MyCycler™ thermal cycler Thermal Cycler. The PCR cycle steps were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 45 sec, annealing at 51 °C for 30 s, extension at 72 °C for 2 min, final extension at 72 °C for 6 min and hold at 4°C. The products were separated by electrophoresis in a 1.2% agarose gel stained with ethidium bromide and visualized under UV light. The PCR fragments were purified and sequenced at Inqaba biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The Basic Local Alignment Search Tool program (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify the fungal isolates by searching for the organisms with the highest similarity to the sequences of the isolates.

RESULTS

From 18 sections (6 sections each from leaves, bark and pith) of *M. ovalifolia*, five fungi were isolated and identified as *Emericella nidulans* SCAF2, *Alternaria alternata* SCAF4, *Aspergillus sp.* SCAF9, *Leptosphaerulina sp.* SCAF7 and

Peyronellaea glomerata SCAF6 (Table 1). The endophyte *A. alternata* SCAF4 was isolated from the leaves whilst *E. nidulans* SCAF2 was the sole isolate from moringa stem pith in this particular instance (Fig. 1). *Aspergillus sp. Leptosphaerulina sp.* SCAF7 and *Peyronellaea glomerata* SCAF6 were all obtained from the bark segments of *M. ovalifolia* stem.

Table 1. Fungi isolated from *M. ovalifolia*

Isolate	*M. ovalifolia*	Organism with highest similarity to	%	Accession
code	part	isolate	Similarity	
SCAF2	leaf	*Emericella nidulans* strain DHMJ15	100	KU351171
SCAF4	pith	*Alternaria* sp. BSO7 ITS 1	100	KU351172
SCAF6	bark	*Peyronellaea glomerata* strain ZG-4-2-1	99	KU351173
SCAF7	bark	*Leptosphaerulina chartarum* strain TPL10	89	KU351174
SCAF9	bark	*Aspergillus niger* strain IHEM 22432	97	KU351175

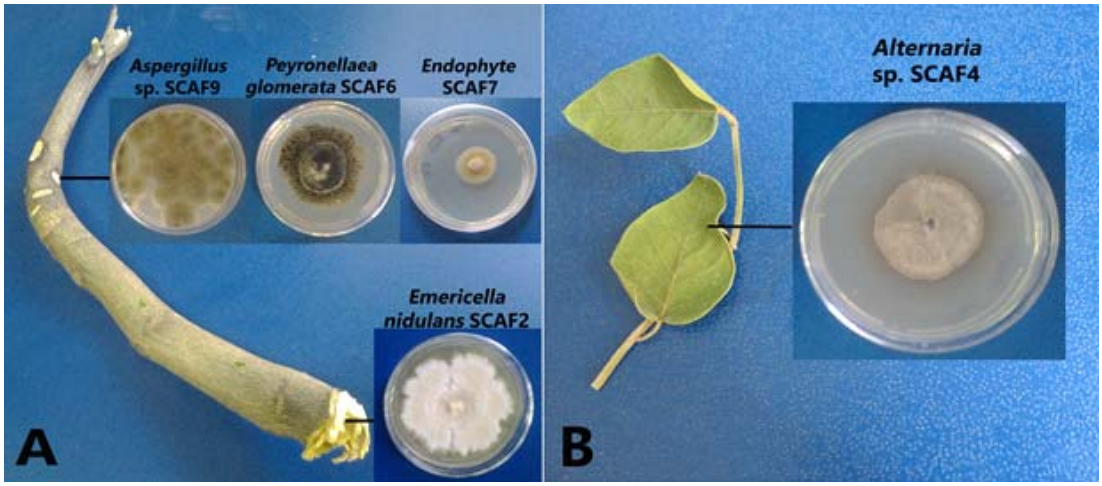

Fig. 1. A Fungal endophytes isolated from M. ovalifolia stem. B Single isolate Alternaria alternata SCAF4 from leaves of *M. ovalifolia*

DISCUSSION

The isolation of a variety of fungal endophytes from different parts of the medicinal *M. ovalifolia* is crucial for identifying novel fungal isolates which further increases the potential to discover new bioactive compounds. *Emericella* is the sexual form of the genus *Aspergillus* which has been described to produce many bioactive compounds that are antimicrobial, anti-Hepatitis C and cytotoxic to liver cancer cell lines (Hawas *et al*., 2012). An endophyte from a Mediterranean green alga *Emericella nidulans var. acristata* produced antitumor benzophenone polyketide compounds against 36 human tumor cell lines (Krajl *et al*., 2006). Given the data presented in this paper, it is understandable to imagine that the medicinal effects by *Moringa* could actually be from endophytic fungi and not only from the plant's secondary metabolites. However, this line of argument needs to be tested by further experiments.

Alternaria spp. are common occurrence of plant diseases but are known to be epiphytic and endophytic (Osono, 2002). The dematiaceous nature of *Alternaria* spp. allows them to withstand sunlight and desiccation conditions that the leaf surroundings consist of (Butler and Day, 1998). Studies by Huang *et al*. (2011) and Sadananda *et al*. (2011) described two isolates (*Alternaria* sp. ZJ9-6B and *A. alternata*) from *Aegiceras corniculatum* and *Tabebuia argentea* which produce quinones alterporriol K and lapachol,

respectively. Polyketides and bicyclic acid derivatives from an *Alternaria* sp. endophyte of the medicinal *Polygonum senegalense* exhibited cytotoxic activity against L5178Y mouse lymphoma cells (Aly *et al*, 2008). Endophytes belonging from the genera *Alternaria* and *Aspergillus* have been isolated from *Moringa* spp. in previous studies (Dhanalakshmi *et al.*, 2013; Khan *et al*. 2014). Endophytic *A. niger* isolates are known to produce quinone and cytotoxic pyrone compounds (Sadananda *et al*., 2011; Li *et al*. 2013).

The fungus *P. glomerata* has been isolated as an endophyte of a pine (*Pinus koraiensis)* and two medicinal plants, specifically *Salvia miltiorrhiza* and *Macleaya cordata* (Deng *et al*., 2011; Shan *et al*., 2012; Lou *et al*., 2013). Extracts from the *Peyronellaeae* sp. isolated from *Macleaya cordata* showed antimicrobial activity against *Agrobacterium tumefaciens, Bacillus subtilis, Escherichia coli, Pseudomonas lachrymans, Staphylococcus aureus, Staphylococcus haemolyticus, Salmonella typhimurium* and *Xanthomonas vesicatoria*. Known to cause leaf diseases in cereal grains *Pithomyces chartarum), L. chartarum* has been isolated as an endophyte from the medicinal *Tephrosia purpurea* (Suryanarayanan and Murali, 2006; Tóth *et al*, 2007; Verma *et al*., 2008; Sharma *et al*., 2013). *Leptosphaerulina* spp. are regarded as saprophytes and latent pathogens as demonstrated by Suryanarayanan and Murali (2006) when a *Leptosphaerulina* isolate from the leaves of peanut could not be regarded as pathogenic after infection studies proved otherwise. Irrespective, an endophytic *Leptosphaerulina chartarum* MA164 exhibited moderate antimicrobial activity against *Cryptococcus neoformans* ATCC90112 and *Microsporum gypseum* MU-SH4 (Sakayaroj *et al*., 2012).

CONCLUSION

For the present study five fungal endophytes from *M. ovalifolia* of the Tsumeb area in Namibia were successfully isolated and identified. To our knowledge this is a first report on the endophytic fungi cultured from *M. ovalifolia* plants of Namibia. The BLAST results indicate that the isolates SCAF7 is a novel endophytic species isolated from *M. ovalifolia.* The genera to which the isolates that are reported in the study belong are documented to be endophytic constituents of many medicinal plants. The metabolites that are produced by the endophytic fungi could be of pharmaceutical significance and if such is the case the medicinal compounds can be sourced from microbial culture rather than plant extraction thus shortening the time of drug production.

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