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Isolation and Identification of Pathogenic Fungi Associated with *Aloe zebrina* Flower Malformation- First Report

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***Aloe zebrina*, a succulent plant, is a source of energy, food and has industrial uses. This is because of its nutritional and medicinal value in treating skin cancers and diseases. The objective of this study was to isolate and identify the fungal species associated with the malformation of the *Aloe zebrina* as a primary obligatory step to control the causal agents for this disease. Samples of infected *Aloe zebrina* flowers were collected from the gardens in the campus of the University of Namibia and pure cultures of fungi were isolated from the infected flowers using Potato Dextrose Agar media. DNA was extracted using the CTAB method and was amplified using combination of primers namely, ITS1 and ITS2, ITS1 and ITS4 and ITS3 and ITS4. The PCR products were sent for automated sequencing at Inqaba Biotec Industries in South Africa. The sequences were compared to known sequences of organisms in the Genbank by using the Basic Local Alignment Search Tool (BLAST). The BLAST searches revealed the identity of different fungi to be *Nigrospora oryzae, and Arthrinium phaeospermum*. This is the first report on fungal species associated with malformation of *Aloe zebrina* flowers and further work is on testing Koch's postulates on these fungi.**

Key words: *Aloe zebrina*, Flower malformations *Nigrospora oryzae, Arthrinium phaeospermum*, Internal transcribed spacer.

Throughout Namibia especially in the northern part of the country, *Aloe zebrina*, locally known as ekundu, is a very popular vegetable. The flowers of *A. zebrina* are harvested in summer, boiled and eaten straight away or pressed into flat cakes about 15 cm in diameter, and then sun dried on the roof. These cakes, locally called *omavanda* and 'cabbage' in English, are then stored for use in the dry season when they are soaked in water, boiled, cooked, ingredients added for taste and eaten as a delicacy. The roots of *A. zebrina* contain important chemical compounds of pharmaceutical importance including isoeleutherol, aloesaponarin and aloesaponol (Jansen, 2005). In urban areas, this plant is only used as a decoration even though it has various interesting traditional uses. These uses include the use of its roots as a dye and its leaves and stems are made into a decoction taken orally by women twice a day after delivery to cleanse the female reproductive system. *Aloe zebrina* is also known as the Zebra leaf aloe and Spotted aloe (English). It is a succulent plant that belongs to the family Asphodelaceae (Jansen 2005). The genus Aloe contains about 330 species of which 274 occur on mainland Africa. Aloes are native in drier parts of Africa; however, these aloe species are widespread in southern Africa and is

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found in marshy meadows on riverbanks and dry thickets. They are common throughout their distribution range and are not threatened (Jansen 2005).

The aloe plants can be identified by being stiff and rugged, and consist mainly of a rosette of thick, large fleshy leaves (Figure 1). The *Aloe zebrina* is known for its "large oblong whitish spots" (Jansen 2005) which is arranged in irregular transverse bands. They are also characterized by their swollen flower bases and small stemless rosettes. These species are variable and flowers mostly between February and April, but some in June-July.

The medical importance of Aloes has widely been reported they are used in professional medicine (Jansen, 2005). This is because they contain over seventy five nutrients, eighteen amino acids, twenty minerals and twelve vitamins and is therefore called "a pharmacy in a plant" (Jansen, 2005). The mucilaginous gel of aloes is used as a home first aid to treat sores, wounds, burns and sunburn. It is used to treat skin cancer and skin diseases and can be used to treat diseases such as asthma, ulcer and diabetes (Davis and Moro, 1989). Dry skin conditions especially eczema around eyes and sensitive facial skin is also treated with aloe sap. Parents sometimes apply extracts from the aloe leaves on child's nails to stop nail-biting. Aloes are also used in cosmetic industries in soaps, shampoo, toothpaste, hair wash and body creams to protect skin against ultraviolet rays (Daodu, 2000). The aloe substances also soothe and moisturize the skin. Herbal drugs and drinks have also been formulated from aloe plants. These drugs and drinks help maintain a good health and are used to treat cold, cough, constipation, pile and fungal infections such as ringworm (Daodu, 2000).

The *Aloe zebrina* is of ornamental value (Jansen, 2005). It can be planted in gardens as can be seen all over Windhoek and have a potential for cultivation in arid to semi-arid, frost-free locations. It plays an important role in Namibia's flora because of its indigenous beauty (van Wyk, 1996).

Aloe zebrina has a great traditional importance to the people of Africa. The roots of *Aloe zebrina* are mainly used as a dye for the *Hyphaene* palm fibers used in basket making to give a golden-yellow colour (Jansen, 2005). The flowers of *Aloe zebrina* are pressed and boiled by the people along the Kunene River in Angola to make cake (Jansen, 2005).

Recently *Aloe zebrina* flowers has been observed to show a bizzare flower malformations. As a flowering plant it goes through the cycle of producing flowers which are bee pollinated. However, this flowering stage is disrupted when the the inflorescence becomes malformed (Figure 2). This study was therefore set to determine the causal agent for disruption of the *Aloe zebrina* life cycle, which leads to its failure to produce seeds, hence with conservation consequences. The extent of this problem is localised to a single place but it is known if it will spread to the wide areas.

Molecular identification of fungal species has been usedwidely in the literature to identify causal agents of plant diseases taking advantage of the diagnostic character of the internal transcribed spacer (ITS) regions of the ribosomal genes (Chimwamurombe and Kanyomeka 2008). Conserved primer sequences are useful for sequencing and amplification of nuclear rDNA mostly from major fungal groups. These primers are from most of the nuclear rDNA coding region. The ITS region refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. It reads from 5' to 3', this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS. During rRNA maturation, ETS and ITS pieces are excised and rapidly degraded. The ITS region has a higher degree of variation than other generic regions of rDNA due to the fact that most variation is among individual rDNA that can sometimes be observed. The ITS1 and ITS4 regions are also used mostly in laboratories. However, the greatest sequence variations in rDNA exist in the IGS region. The main objective of this study was to isolate and identify the fungi associated with causing *Aloe zebrina* flower malformation using a PCR-based approach.

MATERIAL AND METHODS

Media preparation (PDA)

Potato dextrose agar was prepared by dispensing 39 g of PDA in 1 L of distilled water. The mixture was boiled on a Bunsen burner until it

dissolved completely. It was then put in an autoclave at 121°C for 15 minutes to sterilize. After 15 minutes, the mixture was allowed to cool for 5 minutes before it was poured into Petri dishes and allowed to cool again in order to solidify.

Fungal Preparations

The affected *Aloe zebrina* flowers were cut with a sterile blade into pieces of about 2 mm to 2 mm and surface sterilized sufficiently in 70 % ethanol. The cuts were made from the top, the middle and the bottom part of the flower. Cuts were also made from the same parts of the stem. These pieces were then rinsed with sterile distilled water three times and blotted dry with sterile tissue paper. The pieces of *Aloe zebrina* were then plated on potato dextrose agar (PDA). Three pieces were placed in each plate. The plates were incubated at room temperature (25°C) for five days until mycelia growth occurred. Subculturing was done by cutting a piece of the mycelia using a sterile blade and transferring it onto fresh PDA. This procedure was repeated until a pure culture was obtained.

A single spore was aseptically isolated from each pure culture, by scraping from the mycelium using a needle and microscope then dissolving these on a drop of distilled water on a clean microscope slide. It was then left to air dry. The slide was aseptically viewed under a microscope under a high magnification to identify a single spore. The magnification was again lowered in order to isolate the spore and then it was aseptically placed on the PDA media to grow. Broth media was be prepared using Malt Extract and the different fungal isolates were inoculated onto the broth by cutting about 4 mm by 4 mm with a sterile scalpel from the single spore cultures on the PDA plates. After a week, the mycelium was collected by gravity filtration using sterilized funnels and sterile filter papers to filter the fungi from the broth media. The mycelia were then dried in an oven for 24 hours at 60°C.

DNA extraction

A sterile mortar and pestle was used to grind 2g of sample mycelium of each fungus to a fine powder in liquid nitrogen. CTAB (3000 µl), which was preheated at 65°C for 30 minutes, was added followed by another 2000µl of CTAB. This mixture (500µl) was placed in an Epperdoff tube and was incubated for 1 hour at 65°C. After that the solution was allowed to cool at room temperature, 500µl of chloroform was added and the tube was allowed to stand for 5 minutes. The mixture was centrifuged at room temperature for 20 minutes at 8000 rpm. The top layer was transferred to another tube and 1.5 times the volume of 96% ice-cold ethanol was added to the precipitate. The tube was centrifuged again at 13000 rpm for 15 minutes. The ethanol was discarded and then 100µl of 70% ethanol was used to wash the DNA by inverting the tube a couple of times. The tube was again centrifuged at 10000 rpm for 2 minutes.The ethanol was discarded and the DNA was dissloved in 75µl of TE buffer and was kept overnight at 4°C.

PCR Amplification, Sequencing and Sequence Analysis

For PCR amplification ITS1, ITS2, ITS3 and ITS4 were used and the PCR mixtures were made using a master mix of the following volumes: BioRad-Go-green buffer (12.5µl), Sterile water (5.5µl); primers (3.0µl); DNA (4.0µl) in a total volume was therefore 25µl.

The PCR running profile was: Hot start: 94°C for 4 minutes, Denaturation: 94°C for 20 seconds; Annealing: 56°C for 30 seconds; Extension: 72°C for 1 minute; Hold: products were hold at 4°C. After this products were analyzed on 1.2! agarose gel that was run at 80 Volts, 400 Amps for 45 minutes. The amplicons were then cleaned after ascertaining their integrity and send for automated sequencing in both directions to Inqaba biotec in South Africa. When the sequences were obtained they were manually edited by eye, forward and reverse were aligned by reverse complement to get regions of overlap and trimmed to obtain a single consolidated sequence for each fungal culture. After this the sequences were aligned using CLUSTAL and finally only two different sequences used to perform BLAST searches at NCBI (Yap, Frieder and Martino 1996).

RESULTS AND DISCUSSION

Two pure cultures were consistently obtained from three attempts to culture the microorganisms associated with malformation of the Aloe zebra flowers. Several ITS amplicons were obtained from PCR amplications using DNA obtained from single spore pure cultures. These were cleaned and sequenced twice in both direction using forward and reverse primers. The sequence

Fig. 1. *Aloe zebrina* in flower in a garden decoration

Fig. 2. Severely malformed flower inflorescence for *Aloe zebrina* (insert A) and a normal flower inflorescence for *Aloe zebrina* (insert B)

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Query  4    ATTGCAG-ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTA  62
            ||||||| ||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  280  ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATCAGTATTCTG  339

Query  63   GTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATCT  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  GTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATCT  399

Query  123  ACTGTACTGCAGTTCCTTAAATACCCTGCCGGAGCCATAGTTGTCCGCTGAAAAT-GATA  181
            |||||| || |||||||||| ||  || |||||| |||||||||| ||||   | | ||
Sbjct  400  ACTGTATTGTAGTTCCTTAAAGACAGTGGCGGAGCGATAGTTGTCCTCTGAGCGTAG-TA  458

Query  182  AATTTAATTCTCTGTTT-TGTAA  203
            |||||| ||||| | || |||||
Sbjct  459  AATTTATTTCTC-GCTTCTGTAA  480
```

Fig. 3. A partial display of pair wise sequence alignment. The fungus under investigation (query), *Arthrinium phaeospermum*, sequence displayed 90% sequence similarity when aligned with the subject sequence in the Genbank database. In bold are the bases showing the differences

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Query  1    AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGG  356

Query  61   CATGCCTGTTCGAGCGTCATTTCAACCCCTAAGCACAGCTTACTGTTGGGACTCTACGGC  120
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  357  CATGCCTGTTCGAGCGTCATTTCAACCCCTAAGCATAGCTTACTGTTGGGACTCTACGGC  416

Query  121  CCCCGTAGTTCCCCAAAGCCATTGGCGGAGTGGCAGTAGTCCTCTGAGCGTAGTAATTCT  180
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  CTCCGTAGTTCCCCAAAGCCATTGGCGGAGTGGCAGTAGTCCTCTGAGCGTAGTAATTCT  476

Query  181  TTATCTCGCTTTTGTTAGGCGCTGCCCCCCCGGCCGTTAAACCCCCCAATTTTTTCTGGT  240
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  TTATCTCGCTTTTGTTAGGTGCTGCCCCCCCGGCCGTTAAACCCCCCAATTTTTTCTGGT  536
```

Fig. 4. A partial display of pair wise sequence alignment. The fungus under investigation (query), *Nigrospora oryzae*, sequence displayed 98% sequence similarity when aligned with the subject sequence in the Genbank database. In bold are the bases showing the differences

information revealed that there were two main families of the amplicons, those that were 520bp in size and others that were 593bp. The 520bp amplicons showed to 90% *Arthrinium phaeospermum* (Figure 3) and showed 98% similarity to *Nigrospora oryzae* (Figure 4).

Nigrospora oryzae is commonly found on grains of rice, sorghum and maize. It is generally regarded as a weak parasite and known to be versatile (Webster 1993). This versatility implies that it can easily infect several hosts. This has evolutionary and adaptive significance as they can have a variety of hosts ensuring their survival. This is the first time *Nigrospora oryzae* is reported to occur on *Aloe zebrina* being associated with flower malformations.

Arthrinium phaeospermum species are found in the soil or in decomposing plant material. *Arthrinium phaeospermum* is disseminated by wind (Webster 1993); this has implications in the possibility of this fungus spreading to wide areas. Currently, the problem of flower malformation is localised to a small area but could potentially spread easily since the mode of spread for this fungus is by wind.

To our knowledge, this is the first report of such flower malformations on *Aloe zebrina* and in addition it a first record of the associated with two fungi on it. It remains to be investigated which of these two fungi cause the malformations by testing Koch's postulates and how it does it. It would important to determine if this co-infection is necessary to cause the malformation flowers. It could be that the one fungus is the causal agent of malformations and the other is just an opportunistic infection taking advantage of the already infected flower parts.

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