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Molecular Identification of Several Leaf Endophytic Fungi in *Tylosema esculentum* (Marama Bean) Leaves in the Otjiwarongo Area of Namibia

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Endophytes are widely defined as all organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host. These reside mostly in indigenous plant host. Indigenous plants play an important role in the ecosystem, be it by providing food or shelter to other organisms in the area or by maintaining an ecological balance. However, attack by parasitic fungi may have devastating effects. This study focused on determining the identity of endophytic fungal species associated with marama bean leaves in Otjiwarongo area. Marama is being considered for domestication for its highly nutritional value and because it grows well in dry areas. Healthy looking Marama leaves were collected for analysis, but seven months later, endophytic fungal infection was observed on leaves. Pure fungal cultures were obtained from single spore of the fungi and DNA was isolated from fungal mycelium and used as templates in PCR amplification of internal transcribed spacer (ITS) region. The amplified ITS products were electrophoresced on agarose gel and sequenced in an automated sequencer. Basic Local Alignment Search Tool (BLAST) revealed the identity of the fungi as; *Phoma sorghina, Penicillium commune, Alternaria tenuissima,* and *Alternaria alternata.* Marama is in the process of domestication, therefore it is vital to assess its natural tolerance to pest and pathogens for its eventual success as a crop.

Key Words: Endophytic fungi, Marama bean, *Tylosema esculentum*, ITS.

Tylosema esculentum known as marama beans is a wild plant that belongs to the Fabaceae family and Caesalpiniaceae subfamily (Ripper – Suhler, 1983). Marama bean plant is a wild tuberous legume, native to the Kalahari Desert and neighboring sandy regions of Botswana, Namibia and northern South Africa. It is a delicacy to the indigenous population, such as the Ovaherero, Tswana and Khoisan people. This plant is highly nutritional and is well adapted to harsh conditions and can grow well in poor quality soil (National Academy of Sciences, 2006)

Due to this important agricultural attribute, the marama plant is in a process of domestication and therefore it is important that we know and understand diseases and infections associated with this plant. Marama beans belong to *Tylosema* genus which has five known species: (1) *Tylosema fassoglense*, (2) *Tylosema esculentum* (marama bean), (3) *Tylosema argentum (*4) *Tylosema humifasum* and (5) *Tylosema angolense.* (National Academy of Sciences 2006). Marama plant can be a climber; but it grows prostate over the soil surface, sending its vinery stems in different directions, it can grow up to six meters long. Unlike most

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legumes, *Tylosema esculentum* fails to nodulate and fix nitrogen (National Academy of Sciences, 2006). In summer the marama plant vines exhibit golden yellow flowers which later become pods. The pods then turn green and ripen in late April (autumn) and become brown, woody pods with one to six large dark brown seeds inside. During winter, the plant become very dry and turns brown, but it leaves a tuber in the soils, that sprout after receiving rain. The seeds are ten to fifteen millimeter in diameter and weigh two to three grams (Ripper-Suhler, 1983).

Since the marama plant grow in the deep sandy soil of the Kalahari Desert, it has developed drought- adaptive mechanisms, such as the closure of the leaves, which maintain the greenness of the leaf under drought. Stomata closure and the shrinking of tubers. The trailing stems that creep along the ground avoid the effects of the strong destructive windstorms (Van der Maesen, 2006). The plant grow in sandy soil which has limited water-holding capacity, and is exposed to light, extreme temperatures and prolonged drought. Marama beans occur naturally in the drier areas of Southern Africa where it is harvested as a wild plant for human consumption (Amarteifio and Moholo, 1998).

Marama bean is a good protein source and is comparable to soybeans, the marama seeds have a high content of oil, which is rich in monosaturated and disaturated fatty acids and has no cholesterol. Research done at Botswana College of Agriculture revealed that marama beans are good sources of calcium, iron, zinc, phosphate, magnesium, B-vitamins and folate (Lawlor, 2004).

Just like all other plants, marama beans are vulnerable to pathogens, in fact 80% of plant diseased are caused by fungi Agrios (1997). In this research the fungi affecting the stressed marama bean leaves is known as an endophytic fungi (endophytes). Many researchers have defined endophytes in different ways. Endophytic fungi live within the host without causing any noticeable symptoms of infection or disease. (Caroll, 1988; Azevedo, 1998 and Pinto *et al.*, 2006 cited in Pimentel *et al*., 2006). Petrini (1991) defined endophytes as "all organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host". Bacon and White (2000) defined endophytes as "microbes that colonize living, internal tissues of plants without causing any immediate overt negative effects", which includes virtually any organism residing inside a plant host (Zhang *et al.*, 2006). The host plant may benefit from the presence of endophytic fungi because endophytes decrease herbivores, pathogens and various abiotic stressed resistances and tolerance to various stresses (Wilson, 1995). In return, the endophyte receives nutrients, carbohydrates and shelter from its host. Recently it has been acknowledged that the nature of the relationship varies from mutualistic to antagonistic conditions. Endophytes provide the plant with a chemical defense mechanism. Endophytic fungi also have an effect on the grazing of insect pests on plants. The presence of endophytes makes plant tissues unacceptable and unpalatable to insects such that infected plants are avoided. In addition, infected tissues may give rise to toxic effects on the insects, causing poor larval growth and development, reduction in reproductive capacity or may prevent herbivore feeding on the host; therefore the toxins produced by endophyte activity then give rise to destruction in the herbivore population (Saikkonen *et al.*, 1998). Plants infected with endophytic fungi increase plant growth, drought competitiveness, enhanced photosynthetic rates and tolerance to stressful factors such as heavy metal presence, low pH, high salinity and microbial infections (Lewis, 2004, Mandyam and Jumpponen, 2005, Waller *et al.*, 2005 and Zhang *et al.*, 2006).

Even though endophytic associations do not lead to any development of disease symptoms, they do result in some morphological and physiological changes in host tissues which increased the survival and increase the capacity of a plant to resist disease. The presence of endophytes makes the plant's appearance different. It has also been suggested that endophyte-infected plants are tolerant of water stress and recover faster than uninfected. There are suggestions that endophytes may produce growth regulators, which may alter the normal developmental pattern of the host plant (Isaac, 1992). The aim of this study is to determine the identity of the endophytic fungi associated with cold stressed marama leaves. In order to do this a

molecular technique called ITS–PCR is used. Internal transcribed spacers (ITS) are regions found in sequences of genetic ribosomal DNA of eukaryotic cells. ITS can be used for fungal identification (Kanyomeka *et al.*, 2007).

MATERIAL AND METHODS

Isolation of endophytic fungi from leaves

Healthy looking marama bean leaves were collected in Otjiwarongo area in north-central Namibia and were stored at -20°C. After 48 hours, endophytic fungal infection on marama leaves was noticed and the procedure by (Suryanarayanan *et al*., 2005) was followed to isolate endophytic fungi from the marama leaves. Pieces of 1.0cm x 1.0cm were cut from the leaf. The pieces were surface sterilized by immersing them in 75% ethanol for 20 seconds, followed by treatment with sodium hypochlorite (bleach) with 4% chlorine, for 90 seconds then rinsed with distilled water for 60 seconds. The leaves were placed on Malt Extract Agar (MEA) media. Subcultures were done until pure cultures were obtained. Single spores were isolated from the fungal mycelium and grown on MEA. A small piece of 1cm × 1cm, was cut from the growing edge of mycelium and put in Malt Extract broth, which was incubated at room temperature for two weeks. The Broth was filtered and fungal mycelium was dried at room temperature.

DNA Isolation and Polymerase Chain Reaction (PCR)

DNA extraction was done using the Promega Wizard Genomic DNA Purification kit. The DNA was visualized on an ethidium bromide stained 1 % agarose gel. Two sets of primers, ITS 1 and ITS 2 and ITS 3 and ITS 4 were used. The base sequence for: ITS1: 5´-CGTAGGTGAACCTGCG-3´; ITS2: 5´-GCTACGTTCTTCGATGC-3´; ITS 3: 5´-GCATCGATGAAGAACGCAGC-3´ and ITS4: 5´-TCCTCCGCTTATTGATATGC-3´. The PCR product contained 12.5 µl of Promega Go green (dNTPs, *Taq* polymerase, $MgCl_2$ and 10x PCR buffer), 2.0 µl of primers, 3.5 µl deionised water and 2.0 µl DNA in a total volume of 20 µl. A negative control (with no DNA) was also included. The PCR amplification conditions were set as follow: an initial temperature of 95°C for 4 minutes, followed by 32 cycles of 94°C for 30 seconds at 62 °C, the annealing phase, followed by a one minute 72°C extension phase. PCR amplified DNA was separated by electrophoresis in 1% agarose gel and viewed under UV illumination.

DNA Sequencing and analysis

The amplicons were cleaned were sent for automated sequencing to Inqaba Biotech Industries in South Africa. The amplicons were sequenced from both forward and reverse directions. The forward and reverse sequences were carefully inspected by eye for errors and later reverse complemented to produce a unified single sequence without overlaps. This was followed by BLAST searches in the NCBI Genbank database to identify any similarities.

RESULTS AND DISCUSSION

Four fungal pure cultures were consistently produced from seemingly healthy marama leaves. These were identified as *Phoma sorghina* (see Figure 3), *Penicillium commune* (see Figure 1),, *Alternaria tenuissima* (see Figure 4), and *Alternaria alternata*(see Figure 2), based on ITS sequence information that was used to interrogated NCBI Genbank database.

The BLAST search programme and local pairwise sequence alignment revealed a 100% sequence similarity to, *Phoma sorghina* (Figure 3), a 98% sequence similarity of *Penicillium commune* (Figure 1). A 100% similarity to *Alternaria tenuissima* (Figure 4) and a 98% to *Alternaria alternata* (Figure 2). *Phoma sorghina* is a parasitic seed- and soil-borne inhabitant that is generally considered as a secondary invader of suppressed or diseased plants. It is not host specific and is the cause of diseases such as leaf spot, root-rot and dying-off. Most different plant genera have been reported as hosts for the fungi, mainly the *Gramineae* (sorghum, rice and sugarcane), along with genera of *Acacia*, *Podocarpus*, *Aloe*, *Cichorium*, *Citrus* and *Eucalyptus. Phoma sorghina* produce mycotoxins (secondary metabolites that have detrimental effects on the health of animals and humans, mainly attacking various organs and causing cancer). One mycotoxin produced by *P. sorghina* is tenuazonic acid, which inhibits protein synthesis and leads to growth disorders. In 1975 it was suggested that the fungus produces another mycotoxin (as yet unidentified) that causes a disease named Onyalai

(DST/NRF Centre of Excellence in Tree Health Biotechnology, 2004). Onyalai was originally used by the Kimbundu tribe in Angola and literally translates into "blood blister". The Hallmark symptom of this disease is the formation of blood blisters in the mouth and throat. The blood platelet count of patients drastically decreases and this leads to continuous bleeding from these blisters as well as internal bleeding in severe cases. This disease was previously reported from various countries in central Africa including the northern parts of South Africa. Although Onyalai was described as a disappearing disease entity in South Africa, it is still common in Namibia where approximately 600 cases are reported annually. *Phoma sorghina* was also identified as a human and animal pathogen that causes erethematious lesions due to infection of the skin (DST/NRF Centre of Excellence in Tree Health Biotechnology, 2004).

Penicillium commune is an airborne pathogenic green mold that's able to synthesize mycotoxin cyclopiazonic. This fungus belongs to the Ascomycota phylum and subfamily Eurotiomycetes and is of major importance in the environment, food and drug production. Molds are microscopic fungi that can cause allergic reactions in sensitive individuals. They also play an important role in breaking down organic matter such as fallen leaves. They grow practically

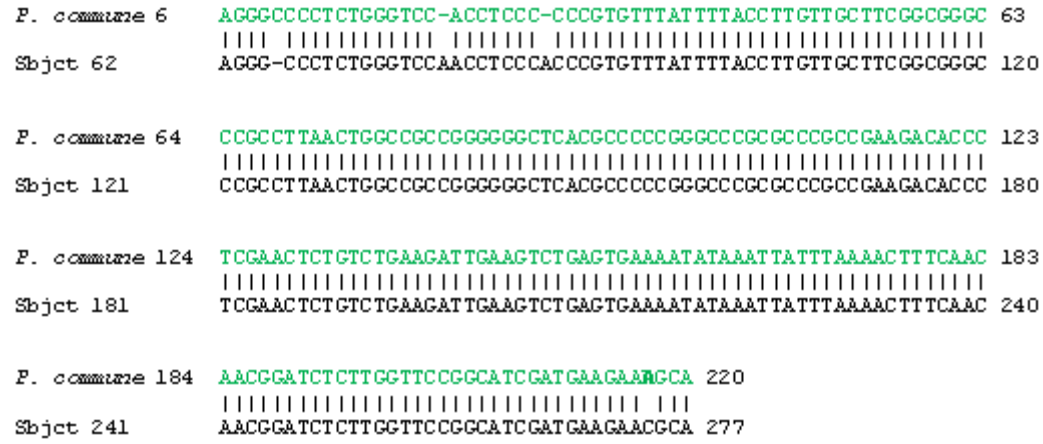

Fig. 1. Partial display of local pair wise sequence alignment of ITS regions showing 98% similarity to *Penicillium commune.* Base differences are shown in boldface type

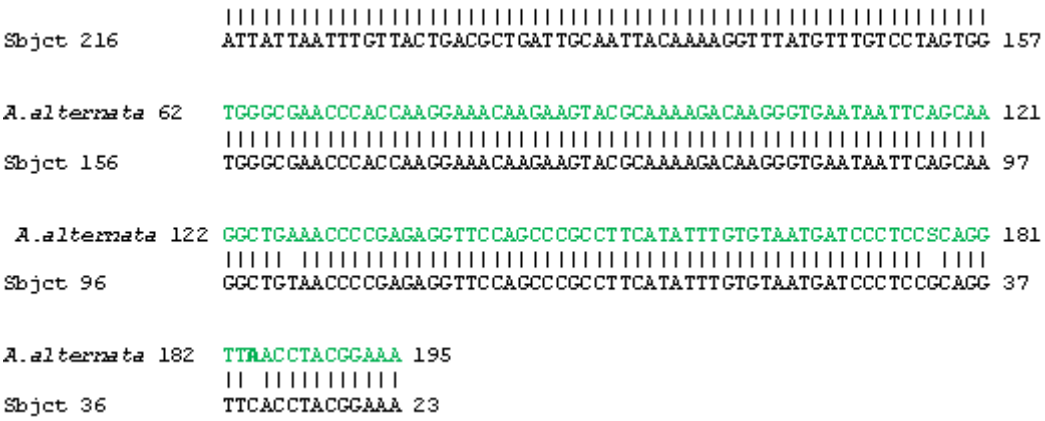

Fig. 2. Partial display of local pair wise sequence alignment of ITS regions showing 98% similarity to *Alternaria alternata.* Base differences are shown in boldface type

everywhere because they adapt to many environments and reproduce rapidly.

Alternaria alternata is a pathogenic endophyte (pathophyte) that produces host specific toxins. Fungi that produce host specific toxins are called biochemical mutants. According to Feng *et al.*, (2007), a profile of toxin production by *Alternaria alternata* was determined. *A. alternata* produced alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT) and tenuazonic acid (TeA). This fungus is one of the most common fungi associated with allergic reactions.

Alternaria tenuissima is an endophyte that is pathogenic to human and plants. According to Anderson *et al.* (2002), *A. tenuissima* can infect cereal grains and be a source of food contamination. It appears to be a cutaneous opportunistic pathogen in human; it is associated with the use of increasing immunosuppressants, immunological impairment due to organ transplants or neoplastic diseases (Feng *et al.*, 2007).It has been established that this fungus play a role in deteriorating and degradation synthetic polymeric material pollutants in the environment especially vulcanized rubbers considered as pollutants (Lugauskas *et al.*, 2004).

A plant that is infected with endophytic fungi only benefit when it is not under any type of stress, but when the plant is stressed the endophytes also try to survive and in the process may end up damaging the host plant and can also cause plant death. The three isolated endophytes, *A. tenuissima*, *A. alternata* and *P. sorghina* can be pathogenic to the plant. And this supports the idea that, endophytes can cause serious damage to the plant. However Koch's postulate needs to follow in order to verify that the isolated fungi are indeed the ones responsible for Marama leaf

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P.sorghina 6    GTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGTGG 65
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 17        GTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGTGG  76

P.sorghina 66   GTTCGCCCACCGATTGGACAAACTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAAAA  125
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 77        GTTCGCCCACCGATTGGACAAACTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAAAA 136

P.sorghina 126  CTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG  185
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 137       CTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG 196
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Fig. 3. Partial display of local pairwise sequence alignment of ITS regions showing 100% similarity to *Phoma sorghina*

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A.terrissima  2    TGAAGGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTT  61
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 3            TGAAGGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTT 62

A.terrissima  62   GCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGT 121
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct 63          GCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGT 122

A.terrissima  122  AATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGG  181
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 123          AATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGG 182

A.terrissima  182  TTCTGGCATCGATGAAGAACGCAGC  206
                   |||||||||||||||||||||||||
Sbjct 183          TTCTGGCATCGATGAAGAACGCAGC 207
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Fig. 4. Partial display of local pairwise sequence alignment of ITS regions showing 100% similarity to *Alternaria tenuissima*

infection. Koch's postulate is a four criteria method that establishes a relationship with the identified or isolated microbe and the suspected disease that it causes.

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