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Isolation and Identification of Fungal Species Associated with Gall Formation on *Acacia mellifera* in the Western Windhoek

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Acacia **is a group of shrubs and trees belonging to the subfamily** ***Mimosoideae*** **of the family, the third largest family of flowering plants. They are herbaceous and woody plant distributed mainly in the tropics and subtropics. In recent years most of the** ***Acacia*** **trees were observed to be infected by fungal species causing diseases and gall formation.** ***Acacia*** **species are economically and ecologically important for vegetation, plant products, for browsing and wood. Fungal pathogens infecting plants may lead to great destruction and even extinction of some plant species in the long run, therefore knowing the identity of such fungal pathogens enable us to develop control mechanisms to prevent the further spread of these pathogens thus conserving the** ***Acacia mellifera*** **in its natural habitat. The aim of the study was to isolate and identify the fungal species associated with gall formation on the** ***Acacia mellifera*** **in the western part of Windhoek. Fungal species were isolated from the galls on the infected twigs and grown into pure cultures using Sabouraud Dextrose Agar (SDA) and later on Malt Extract Broth (MEB) as growth media. DNA was extracted from dry mycelia and conserved internal transcribed spacer regions of the fungal species were amplified using the ITS3 and ITS4 primers. DNA amplicons were sequenced and compared to sequences of known organisms in the GenBank using nucleotide BLAST (Basic Local Alignment Search Tool). The BLAST search program revealed the identity of fungal species isolated consistently cultured as an isolate of** ***Phoma glomerata*** **(96 % homology). This organism has previously been reported as common Ascomycota plant pathogen causing dieback disease in most** ***Acacia*** **species. However, further work of testing Koch's postulates is needed to verify whether** ***Phoma glomerata*** **is directly causing the formation of galls on the** ***Acacia mellifera.***

Key Words: *Acacia mellifera*, Gall formation, ITS, PCR, Fungal pathogens, BLAST.

Acacia mellifera belongs to the family *Fabaceae – Mimosoideae*. They are found throughout Namibia on hard-surfaced sandy soils, rocky hillsides, dry rivers and loamy soils. They are most abundant and dominant in the central highlands. *Acacia mellifera* is an invasive species that causes bush encroachment in many overgrazed areas, especially in central Namibia. There are two subspecies in Namibia the *Acacia mellifera mellifera* and the *Acacia mellifera detinens* they are characterized by their leaves and flowers. (Curtis and Mannheimer, 2005)

The *Acacia mellifera* is sometimes mistaken with the *Acacia senegal*, because of its shape. *Acacia mellifera* is a source of edible gum. Its wood is used for building huts and their branches for fencing. The wood is also used for fuel and charcoal. Different parts of the tree are used for medicine to cure malaria, syphilis and for

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sterility. It provides shelter for animals from the hot sun. (Curtis and Mannheimer, 2005). Pathogenic micro organisms also interfere with the ability of the plant cells to carry out their functions properly, they utilize the nutrients and cause a shortage of nutrients for the plant and this leads to diseases. Diseases caused by fungi are known as infectious diseases. Different fungi infect plant species either as spores which are easily dispersed by wind, water or by insects, or by physical means which is through wounds resulting from wind, animal and insect activities or by direct penetration. (Agrios, 1997)

Fungal parasites often penetrate directly through an undamaged host surface, but sometimes they use natural openings such as the stomata which they locate by recognizing host surface signals. Infection is preceded by production of specialized pre-penetration structures such as the hyphopodia, mycelium, sporophores, spores and fruiting bodies. The pre-penetration structures serve to anchor the fungus to the host surface, usually by secretion of a mucilaginous medium. Fungi cause local or general symptoms on their host, some cause excessive growth or infected plants or plant parts (Agrios, 1997).

In recent years most of the *Acacia* trees are observed to be infected by fungal species causing diseases and pod malformation. According to Holz, *Acacia mellifera spp. detinens* as well as the *Acacia karoo* have been exposed to a disease since 1986. A disease caused by fungi has been identified as dieback disease. This disease was caused by four fungi the *Phoma glomerata, Phoma cava, Phoma eupyrena* and *Cytosperma chrysoperma.* The four fungi were isolated from *Acacia mellifera* seeds and identified. This problem occurred mostly in the Northern Namibia.

Phoma glomerata is most effective in areas of heavy soils, due to moisture stress, causing dieback by as much as 80 % over the past five years. There are several records of dead plants, mostly from the central parts of the country but also the south (Curtis and Mannheimer 2005). Fungal species infecting and causing diseases in Acacias are mainly *Atelocuada digitata* which causes rusts in the *Acacia koa* and *Acacia mangium. Ganoderma philippii* causes root rot in the *Acacia mangium*. The first symptom indicative for fungal infection is discoloration, the heartwood becomes purple/black and the sapwood becomes green/brown (Mohammed *et.al* 2006). The main objective of this project was to isolate and identify the fungal species associated with gall formation on the *Acacia mellifera* using molecular methods based on PCR and sequence analysis of the ITS region, which will help to develop control and prevention mechanisms to prevent spread of these pathogens.

MATERIALS AND METHODS

Sample collection

Samples of the diseased *Acacia mellifera* (see figure 1) were collected from several sites in Windhoek West in the Khomas region (Central Namibia).

Pure culture isolation

The galls on the diseased *Acacia mellifera* were surface sterilized by cutting into small pieces (4mm x 4mm) and immersed in 70 % ethanol for 2 minutes and then rinsed with distilled water and excess water was removed by placing the pieces of the plant tissue on towel paper. These pieces were then inoculated with the cut edges onto the plates containing the SDA medium. The plates were left at 25°C for a week before the fungi could be sub-cultured. After a week the fungi were sub-cultured and sub-culturing was done until pure cultures were obtained. Single spore cultures were made from the respective pure cultures; these cultures were all inoculated into sterile Malt Extract Broth (MEB). The fungi were left until they were fully grown within 3 weeks. The resulting mycelia were filtered and air dried for a week.

DNA extraction

The fungal genomic DNA was isolated from 2g of individual mycelia isolates using the modified CTAB method. DNA samples were stored at 4°C for further use. DNA was resolved in 1% agarose gel in TBE buffer and visualized by ethidium bromide staining and UV illumination.

PCR amplification

Primer pairs: ITS3 with ITS4 were used during PCR amplification. PCR was run using 12.5 µl of Biorad Go-green *Taq* solution, 3 µl of primer pairs, 4 µl of the DNA sample and 5.5 µl of sterile water in a total volume of 25µl. A negative control of sterile water was used with no DNA. The PCR

amplification profile was as follows. The initial denaturation was at 94 °C for 4 minutes, followed by denaturation at 94 °C for 20 seconds; annealing was done at 58 °C for 30 seconds for both primer sets and the extension was done at 72 °C for 1 minute and then for 10 minutes. The PCR products were held at 4 °C. The PCR products were run on a 1 % agarose gel and viewed under UV light.

DNA sequencing and sequence analysis

The PCR products were cleaned and sequenced by the Inqaba Biotec Industries in South Africa using an automated sequencer. Primer pairs used during DNA amplification ITS3 and ITS4 were used for sequencing in both forward and reverse directions. The sequences were used to perform BLAST searches in the NCBI GenBank database. The sequences with the highest base similarity were chosen thus having the same bases as the query sequence.

RESULTS AND DISCUSSION

DNA extracted from single spore culture was amplified by PCR using ITS3 and ITS4 primers which resulted in a product with a clear band of about 540 bp. The resulting product was sequenced in both direction and analyzed for homology with those of other species found in the database. The identity of the fungus was revealed as *Phoma glomerata* .

Fig. 1. Galls on the diseased *Acacia mellifera* twig

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Query 7     ATTGCAGA-TTCAGTGAAT--TCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCA  63

            |||||||| ||||||||||  ||||||||||||||||||||||||||||||||||||||
Subjct 241 ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCA  300

Query  64   TGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGG-GTTGGGTGTTT  122
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Subjct301  TGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTT  36

Query  123  GTCTCGCCTCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCAG  182
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||| |
Subjct361  GTCTCGCCTCTGCGTGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGG  420

Query  183  AGCGCAATACATCTGGCGCTTTGCACTCATAACGACGACATCCAAAAGTACATTTTTTAC  242
            |||||| ||||||| ||||||||||||||||||||||||| ||||||||||||||||| ||
Subjct421  AGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTT-AC  479

Query  243  AATCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA  296
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||
Subjct480  ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA  533
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Fig. 2. A display of a partial pair wise local sequence alignment. *Phoma glomerata* (subject) sequence aligned against the subject sequence in the GenBank database, with 96 % similarity and 4% mismatched using BLAST search program. Sequence variations are indicated in bold

Sequence analysis for homology of the ITS regions of the *A. mellifera* fungus revealed high homology with the ITS region of rDNA from *Phoma glomerata* with 96% nucleotide homology. Thus allowing us to declare that *Phoma glomerata* is associated with gall formation on the *Acacia mellifera*. *Phoma* species are commonly considered as contaminants, they may rarely cause infections in humans. They are associated with allergic responses and with human infection. In plants, *Phoma* species are common pathogens. The fungi can cause a condition known as *Phoma* blight, characterized by a withering and fading of the leaves of the plant. The blight will eventually kill the plant, and it can spread to other plants and trees in the surrounding area (Jacobs and Nall, 1997). According to Dijksterhuis (2007) *Phoma glomerata* is also associated with cheese spoilage.

The positive identification of disease-causing organisms in diseased tissues is an important part for diagnosis leading to effective treatment and control. We have verified the long-held view that ITS-PCR method for fungal identification is a rapid, accurate and a reliable means by which pathogens can be effectively detected and identified. However, it is still important of carry out tests to confirm Koch‘s postulates on the cause –effect relationship between galls and *Phoma glomerata*.

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