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Isolation and Identification of Fungi Associated with Gall Formation on *Acacia reficiens* in the Numas River Area of Namibia

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Acacia **are a genus of shrubs and trees and are widespread around the world mainly in Africa and Australia. They belong to the family of leguminous plants.** ***Acacia reficiens*** **is a small tree or bush that can grow up to 8m in height.** ***Acacia reficiens*** **are used for felling and charcoal production, their leaves are browsed by various animals, their bark and roots are used medicinally. In recent years,** ***Acacia*** **species in Namibia are being attacked by fungal species. It is of great importance to identify these pathogens so that mechanisms of inhibition can be designed to prevent their further spread** ***in vivo*** **and thus protect and conserve these tree species in their natural habitats. The aim of this study was to isolate and identify fungal species that are associated with gall formation on** ***Acacia reficiens*** **in the Numas river area in Namibia using morphological and molecular methodologies based on PCR and ribosomal DNA of the Internal Transcribed Spacer region (ITS region). In the study, infected** ***Acacia reficiens*** **branches were sampled and pure cultures were made. DNA was extracted from them and a Polymerase Chain Reaction (PCR) was done to amplify the extracted DNA. The PCR products were sequenced by the Sanger method and the identity of the fungal species was obtained by using the BLAST search. There was a 98%, 99% and 98% similarity between the subject and query sequences. The isolated fungi were identified to be** ***Periconia sp., Sporomielle isomera*** **and** ***Gliocladium cibotii*****. These are first reports on** ***Acacia reficiens*** **in Namibia. However, Koch's postulates and further studies must be done to determine the pathogenicity of these fungi and to verify whether they are associated with gall formation on the** ***Acacia reficiens*** **in the Numas river area in Namibia.**

Key words: *Acacia reficiens*, gall formation, ITS, pathogenic fungi, Namibia.

According to the *Acacia* encyclopedia, 2008, there exist about 1300 *Acacia* species in the world, mainly in Africa and in Australia. Of these species, 950 are native in Australia and the remainder is widespread around the world in dry tropical to warm temperature regions. *Acacia* tree species are the most common trees in Namibia. This may be because they are well adapted to the arid conditions that predominate in Namibia (Chimwamurombe & Kanyomeka, 2008). *Acacia reficiens* is an *Acacia* species that can grow up to 8 metres in height. It is also known as Rooihaak or Wawra. It is common amongst the different ethnics groups of Namibia and is called by different names amongst these groups, e.g. the Ovambo tribe call it *enos*, the Herero tribe call it *omungondo* and the Damara tribe call it *no's* (Palgrave, 2002).

Acacia reficiens are found in various habitats around Namibia but they are predominant on plains. They are found in various habitats

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around Namibia but they are predominant on plains. These trees are found in the North West region in dry rivers and on hills. In the central west region, they are found along rivers and in the Cuvelai, they are found around pans and oshana's. In the North Central plateau and central highlands they are found on hills. These trees mostly grow on gravel or rocky substrate. They are used for felling and charcoal production,their leaves, flowers, bark and branches are browsed by a variety of animals, for e.g springboks, giraffes and elephants . their bark and roots are used medicinally and the gum it produces is edible (Curtis and Mannheimer, 2005). *Acacia reficiens* species are similar to *Acacia Luderitzii* but these may be distinguished by fewer pinna pairs and leaflets that lack hairs along the margins on *A. Luderitzii*. Some farmers have often confused the two Acacia species and have thus referred to them as 'basterkameel' (Curtis and Mannheimer 2005).

In recent years,some of the *Acacia* species have been attacked by fungal pathogens causing disease like symptoms on theses trees.(Chimwamurombe and Kanyomeka, 2008). Galls have been noticed to have formed on the branches of *Acacia reficiens* in the Numas river area. The causal agent of these galls is not known but it is suspeced that the cause may be a fungus (Chimwamurombe pers. comm.). These galls may have a negative effect on the trees because fungi can be parasatic, they can utilize the recourses of the plant for their own needs, causing damage, diseases or even the death of plants.

For this reason,diagnostic methods are required to reveal the identity of the causal agent. The main objective of the study is to isolate and identify the fungal species associated with gall formation on *Acacia reficiens* using using morphological and molecular methods based on ITS-PCR techniques.

The ITS region is considered as a powerful diagnostic tool and it is used in the identification of different and closely related species. When the ITS region of the known species is compared with the ITS region of the organism in question, this reveals the identity of the organism. The constant areas of this region are used in the design of primers and so these can be used over a range of related organisms (Nevajas in Ilonga 2008).

According to Madigan and Martinko (2006) PCR by definition is a method used for the amplification of a specific DNA sequence in vitro by repeated cycles of synthesis using specific primers and DNA polymerase. The polymerase chain reaction can multiply DNA molecules by up to a billion fold in the test tube, yielding large amounts of specific genes for a host of applications in molecular biology. PCR technology is a relatively new technique that has many applications in recombinant DNA research. Molecular identification of fungal species can be done easily with great rapidity and accuracy y sequencing of specific DNA regions, foe e.g. the internal transcribed spacer region of ribosomal DNA isolated from fungi (Chimwamurombe and Kanyomeka 2008).

MATERIAL AND METHODS

Collection of sample and isolation

Infected *Acacia reficiens* branches were collected from the Numas river area in the Namib Desert on the 29 April 2008. The size of 2-3 mm pieces were cut from the galls from *Acacia reficiens* branches. These were surface sterilized by immersing them in 70% alchohol for 2 minutes and then rinsing them 3 times with distilled water. The cut galls were inoculated onto the Malt Extract Agar media on the plates where the cut edges faced downwards. The fungi were left for 7 days before subculturing was done. Subculturing was done until pure cultures were obtained (Chimwamurombe and Kanyomeka, 2008). Single spores of each fungus were immersed into Malt Extract broth and were left to grow for 3 days. These were then filtered using sterilized funnels and filtered paper.The mycelium was air dried for 3 days (Ilonga, 2008). DNA was extracted using the CTAB method.

PCR amplification and Fungal identification

The primers ITS 1 and 2 were used.The PCR was run using 2µl mix solution.It was prepared by mixing 12.5ul of Master mix, 3ul ITS 1 and 2, 5.5ul double distilled water and 4ul of DNA. Denaturation was done at 94°C and 30 cycles was done at 94°C for 30 seconds. Annealing was done at 57°C for 30 seconds. Extension was be done at 72 °C for 1 minute and was held at 4° C. Negative control reactions without any template DNA were carried out silmaltaneously using the

ITS primers PCR protocol. The PCR was repeated several times.

The ITS PCR products were send to Inqaba Biotec Industries in South Africa for sequencing, where the DNA nucleotides were put in order to enable the identification of the fungi. Once the DNA sequence was known, it was compared with the existing DNA sequences for its identification. The obtained DNA sequence was alligned against the known DNA sequence on the Genbank Database,using the computer programme known as the BLAST search. The fungi were identified by using percentage base scores where their DNA sequences were compared to the known DNA sequences on the programe.

RESULTS AND DISCUSSION

Three fungi were isolated from the galls on the *Acacia reficiens* branches. The fungal DNA was extracted from the different fungi that were isolated. The DNA was viewed under UV illumination. The fungal DNA was amplified by PCR, using an annealing temperature of 57°C and ITS 1 and ITS 2. 2% agarose gel electrophoresis was used and the amplified DNA was viewed under UV illumination. The 3 DNA sequences from the isolated fungi aligned with subject known sequences in the Genbank database by using the BLAST search. The fungi from this study were identified to be *Gliocladium cibotii, Sporormiella isomera and Periconia sp.*

Fungus 1

Gliocladium cibotii

Genes for 18S rRNA, ITS 1, 5.8S rRNA, ITS2, rRNA, partial and complete sequence
Length 565 base-pairs

(Photograph by Uzabakiriho J.D. 2009)

Fig. 1. Galls on *Acacia reficiens* branches

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Query  1    CGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCG**G**AAACCCCAACACGAACAC  60
            ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  126  CGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACAC  185

Query  61   TGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  TGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGG  245

Query  121  ATCTCTTGGTTCCGGCATCGATGAA**A**AACGCA**A**CGA  156
             |||||||||||||||||||||||| |||||| |||
Sbjct  246  ATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA  281
```

Fig. 2. On pair wise sequence alignment, the fungus under investigation (query), *Gliocladium cibotti*, sequence displaying 98% sequence similarity when aligned with the subject sequence in the Genbank database. In bold are points of base differences

Fungus 2

Sporormiella isomera

Genes for 18S rRNA, ITS 1, 5.8S rRNA, ITS2, rRNA, partial and complete sequence
Length 506 base-pairs

Fungus 3

Periconia sp.

Genes for 18S rRNA, ITS 1, 5.8S rRNA, ITS2, rRNA, partial and complete sequence
Length 540 base-pairs

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Query  1    CCTGTCGAGATAGAACCCTTGCCTTTTTGAGTACCTTTTCGTTTCCTCGGCAGGCTCGCC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57   CCTGTCGAGATAGAACCCTTGCCTTTTTGAGTACCTTTTCGTTTCCTCGGCAGGCTCGCC  116

Query  61   TGCCAATGGGGACCCCCAAAAAACACTTTGCAGTACCTGTAAACAGTCTGAACAAACTTT  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  TGCCAATGGGGACCCCAAAAAAACACTTTGCAGTACCTGTAAACAGTCTGAACAAACTTT  176

Query  121  AAAAATTAAAACTTTCAACAACGGATCTCATGGTTCTGGCATCGATGAAGAACGCAGC  178
           |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  177  AAAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC  234
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Fig. 3. On pair wise sequence alignment, the fungus under investigation (query), *Sporormiella isomera*, sequence displaying 99% sequence similarity when aligned with the subject sequence in the Genbank database. In bold are points of base differences

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Query  1    TGTGTACCCTTCCATCGCTTCCTCGGCGGGCTCGCCCGCCGGCAGGAACCAAACAAACCC  60
           |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  69   TGTGTACCCTTCCATCGCTTCCTCGGCGGGCTCGCCCGCCGGCAGGAACCAAGCAAACCC  128

Query  61   TTTGCATCCTACGCAAAAACTTCTGATAACCACCTAAATAAGTCACAACTTTCAACAATG  120
           ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  129  TTTGCATCCTACGCGAAAACTTCTGATAACCACCTAAATAAGTCACAACTTTCAACAATG  188

Query  121  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC  155
           ||||||||||||||||||||||||||||||||||||
Sbjct  189  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC  223
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Fig. 4. On pair wise sequence alignment, the fungus under investigation (query), *Periconia sp.*, sequence displaying 98% sequence similarity when aligned with the subject sequence in the Genbank database. In bold are points of base differences

To our knowledge this is the first study done on the *Acacia reficiens* and a first report for the three fungi occurring on this host. *Periconia sp.* are endophytic fungi that are both parasitic and saprotrophic on plant material (www. caltexmoldserices.com/sectionmold/periconia_sp/). These species are rarely found growing indoors, they are found in soil, in blackened herbaceous stems, leaf spots, grasses, rushes and sedges (www.iaqinfo.com/fglossary p html). However, several studies of plant diseases caused by these fungal species on plants have been carried out. An example is the Milo's disease that is caused by *Periconia circinata;* it was first discovered in 1924 in Chilicothe Texas growing on sorghum plants. Another similar case was found recently in USA (Scheffer, 1997).This information suggest that these fungi are versatile, meaning they can grow on different plant species and cause diseases.

On the other hand, *Gliocladium* species are fungi that are similar to *Penicillium* species and reported to be allergenic (www. caltexmoldserices. com/sectionmold/periconia_sp/). A study that was carried out in the United Kingdom in 1998 showed that these organisms are biological control agents. They attack already established pathogens and they are able to colonize potential infection courts such as growing roots and wounds (Harman and Kubicek, 1998).

While *Sporormiella* species are from the

Sporomiaceae family and these are coprophilous, in other words they are dung fungi (Krys and Wedin, 2009). There is little literature on the pathogenicity of this species on plants but the species from this genus are known to produce antimicrobial compounds. An example is, in 1995 four tetrazines were isolated from *Sporormiella tetetispora*. These are new amino acid- derived bioactive metabolites (Wang *et al*, 1995). With this information in hand, we can assume that this fungal species contains antimicrobial agents that are effective against other microorganism in a symbiotic relationship with the host plant. The three fungi have however never been reported as causal agents of plant galls, either as individuals or in a three-some synergism.

The next step in this investigation is to test Koch's postulates. This will be carried out to verify whether the gall formation on *Acacia reficiens* is blamed on which of the isolated fungi. *Acacia reficiens* are important indigenous species in Namibia and thus they must be protected to conserve them in their natural habitats. Further studies should also be done to know the identity of these fungi on a species level and to study their mode of action and biological activities in order to inhibit further spread to other plant species and humans and thus conserve the Namibian ecosystem.

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