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Isolation and Characterization of Fungi Associated with Disease Symptoms on *Ziziphus mucronata* Leaves and *Phaseolus vulgaris* Pods in Windhoek, Namibia

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Detection of phytopathogens that are involved in causing disease symptoms in plants and crops is of prime importance as a key step in disease treatment and management. *Ziziphus mucronata* is a species endemic in temperate and tropical climates and used traditionally in the treatment of infectious diseases. The common bean (*Phaseolus vulgaris)* is a rich source of nutrients for the human diet. Just like most crops, it is not immune to fungal diseases and reports had been received of *P. vulgaris* showing signs on disease. The aim of this study was to isolate and characterize the fungal species associated with the disease symptoms in the *Z. mucronata* and *P. vulgaris.* Fungal species where isolated from surface-sterilised symptomatic bark of *Z. muconata* and fresh green bean pods. These where grown on petri dishes containing Potato Dextrose Agar and incubated at room temperature. Pure cultures where then obtained by transferring small segments of fungal growth to a new petri dish that contained PDA. During DNA extraction the pure cultures where first homogenized using liquid nitrogen and then the rest of the extraction carried out as stipulated in the Zymo extraction kit. The Nanodrop was used for quantifying the DNA and amplification of the conserved Internal Transcribed Spacer (ITS) region of Ribosomal RNA genes was carried out using ITS1 and ITS2. The PCR products were sequenced at Inqaba Biotech Industries in South Africa. The obtained sequences were then compared by alignment with known sequences in the Genbank using Basic Local Alignment Search Tool (BLAST). The BLAST searches were able to reveal the fungi isolated from the *Z. mucronata* as *Fusarium penzigii* and *Fusarium dimerum* while the fungi isolated from *P. vulgaris* shown to be *Phoma destructiva*, with 100 %, 95% and 100% sequence similarity respectively. The next step in this work is carry out Koch's postulates to determine which of this fungi is the causal agent of the observed diseases symptoms in order to start a targeted diseases management programme.

Keywords: *Ziziphus mucronata, Phaseolus vulgaris*, Internal Transcribed Sequence, alignment, BLAST, *Fusarium dimerum, Fusarium penzigii, Phoma destructiva.*

The *Ziziphus mucronata* also known as the Buffalo thorn is a small tree with a spreading canopy that has characteristic zigzag young twigs with two significant thorns at the node, with one facing forward and the other backwards. *Z. mucronata* is a multipurpose tree with a variety of traditional importance such as being believed to protect from lighting in Botswana (Mazibuko, 2007). (Mazibuko, 2007) stated that many parts of the plant are used in ethno medicine with the tannins playing a role in dysentery treatment and

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the roots being used in East Africa to treat snake bites, also went on to say that these properties have been attributed to the peptide alkaloids and antifungal properties isolated from the bark and leaves.

The common bean (Phaseolus *vulgaris*) also called the green bean is a herbaceous legume that has its origin in South and Central America, it is now cultivated worldwide for its high nutritious value (Earth, 2011). The activity of bacteria found in its root nodules is able to confer nitrogen fixing properties to the bean. Due to poor management, the beans may be exposed to high moisture and temperature during storage and harvest, this may provide a suitable environment for fungal growth which might lead to contamination by mycotoxins (Scussel, 2000).

This research aimed to address the problem of lack of information on identity of plant diseases in *Z. mucronata* and common green beans. Fungi cause a wide range of crop and plant diseases worldwide. The *Ziziphus mucronata* is one of the important ethno medical plants in Africa and Asia. It has medical properties such as antitumor, anticancer and anti-diabetic. There have been various reports on green beans showing signs of diseases and a few studies have been conducted to follow up these reports hence the reason behind this study focusing on fungi causing diseases on the common green beans.

MATERIALS AND METHODS

Sampling Area

Symptomatic plant tissue from *Z mucronata* was collected from the University of Namibia while the diseased green beans samples where obtained in local green grocer shops. To minimize destruction of the plant, nondestructive sampling was carried out.

Obtaining and Sterilization of Samples

Branches that showed disease symptoms where obtained from the tree and fresh green beans pods where also obtained. The symptomatic tissues where then surface sterilized according to the procedure used in (Garas, *et al*., 2012).

Isolation of Fungi

After the sterilization, a piece of crop tissue from the bean pod and from the *Ziziphus mucronata* stem with fungal growth on it was cut and transferred onto an agar plate containing Potato Dextrose Agar. The fungi was then incubated at room temperature of 25^{o}C and left to grow for 5 days. Sub culturing was performed where required and after pure cultures of the fungi where obtained from single spores, these cultures had to be stored in 30% glycerol.

DNA Extraction and PCR Amplification

DNA was then extracted from the pure cultures using the ZYMO Fungal DNA extraction kit according to the manufacturer's manual. Gel electrophoresis was carried out so as to confirm the presence or absence of fungal DNA which was evident from the bands. Further quantification was then carried out using a Nano drop. PCR was carried out with the following PCR mixtures: Buffer (12.5µl), DNA (4µl), sterile water (5.5µl) and primers (3µl) giving a total master mix mixture of 25µl. The Amplification was done using ITS1 and ITS2 primers at a primer concentration of 0.8uM with the following profile: Pre-denaturation of DNA, 94°C for 4 minutes, Denaturation of target DNA; 94ºC for 20 seconds,

Primer annealing; 59ºC for 30 seconds, Primer elongation; 72ºC for 1 minute, Final elongation; 72ºC for 10 minute and Final hold at 4ºC The amplicons where then sent to Inqaba Biotech Industries for automated sequencing of the ITS region.

DNA Sequence Analysis

ITS 1 and ITS 2 primers that had been used for amplification were used for sequencing in both forward and reverse directions with the obtained sequences being used to perform Blast searches in the NCBI Genbank. The integrity of the sequences was ensured by eye inspection.

RESULTS AND DISCUSSION

Fungal cultures where obtained from tissue of the *Z. mucronata* showing symptoms of infection and sub culturing was carried out until pure cultures were obtained as depicted in figures 2 and 3. *Fusarium dimerum* could be identified from the plates due to the morphological orange color it grew with.

DNA was then extracted from the pure cultures and Gel electrophoresis was run and a

Fig. 1. Showing a *Ziziphus mucronata* tree at the University of Namibia showing symptoms of disease by the yellowing and loss of leaves

Thermo Scientific NANADROP 2000c a product of MET supplied by Bio Dynamics used to quantify the DNA.

The obtained amplicons were then cleansed and sequenced. After BLASTN the fungi responsible for the disease symptoms in the *Z. mucronata* where revealed by 100% and 94% similarity as *Fusarium penzigii* and *Fusarium dimerum* respectively. While *Phoma destructiva* was revealed to be the isolate from *P. vulgaris.* (Maier, et al., 2014) who carried out one of the few available studies on diseases of the *Z. mucronata* concluded that *Coniodictyum chevalieri* was responsible for the diseases symptoms that where observed in the *Z. mucronata* found in the Kruger National Park in South Africa. The authors also went on to state that *C. chevalieri* has a unique

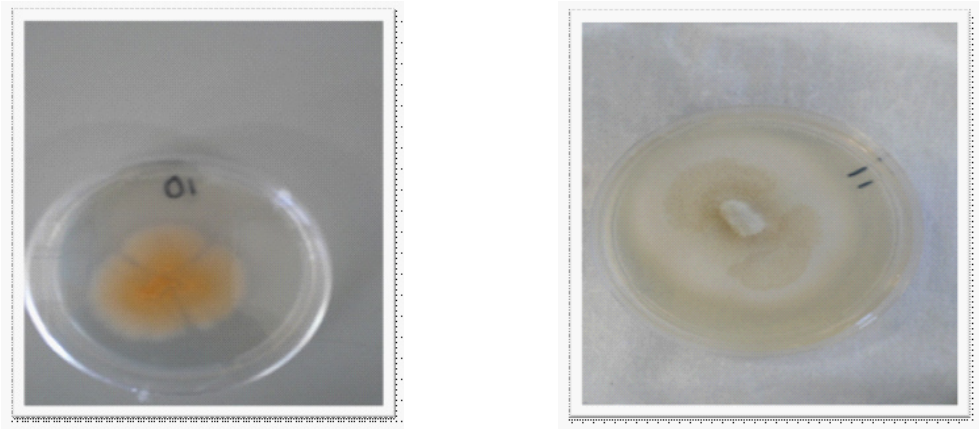

Fig. 2. Showing morphological appearance of isolates from *Z. mucronata*. (A- *Fusarium dimerum* and B-*Fusarium penzigii*)

Fig. 3. Showing morphological appearance of isolates from *P. vulgaris.* With both C and D having come from *Phoma destructiva*

biology, regarding its biogeography, ecology and host specificity, as it only infects *Z mucronata.*

Most studies that have been carried out on the *Z. mucronata* have been focused on mostly its ethno medical importance and a few studies have been documented on the diseases that affect the plant. Fusaruim species have proved to the causal agents of most plant and crop diseases not neglecting their effects on certain opportunistic infections in humans as indicated in (Salah, *et al*., 2015) where they are indicated to be found in wounds, blood, nails, cornea, urine and skin.

Fusarium dimerum had been believed to be decaying plant saprothrophs, take (Gerlach & Nirenberg, 1982) who were unaware of *F. dimerum* causing any plant diseases when they carried out their studies. The findings of this study thus refutes that belief as it has shown that the fungi is also associated with disease symptoms in plants. It is also now known as a species complex normally

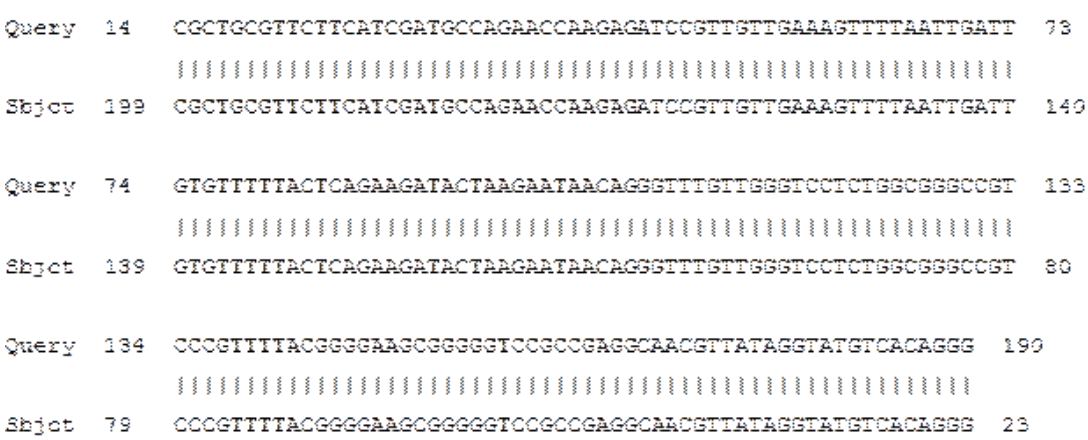

Fig. 4. Partial display of local pairwise sequence alignment of ITS regions showing 100% similarity to *Fusarium penzigii* (Query) sequence that was against the subject sequence in the GenBank database

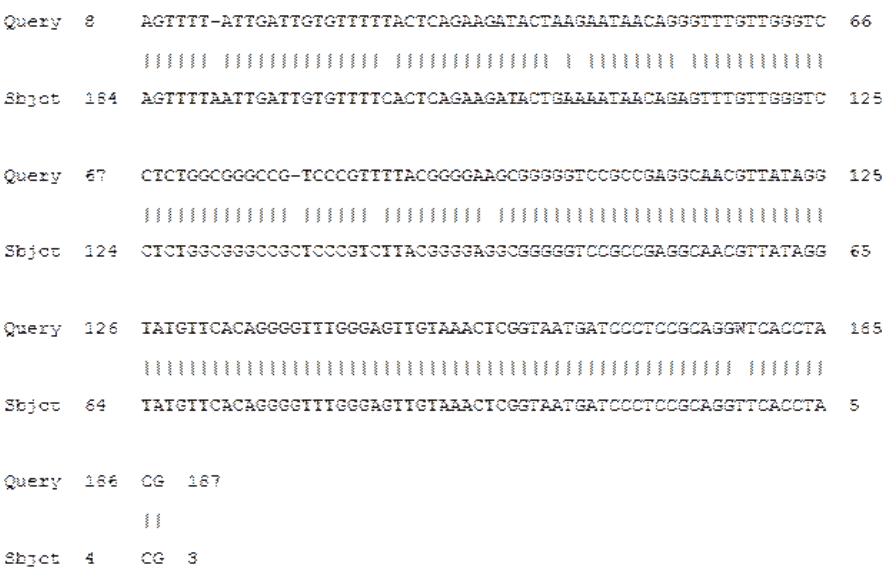

Fig.5. Partial display of local pairwise sequence alignment of ITS regions showing 95% similarity to *Fusarium dimerum* (Query) sequence that was against the subject sequence in the GenBank database and 5% mismatch

Fig.6. Partial display of local pairwise sequence alignment of ITS regions showing 100% similarity to *Phoma destructiva* (Query) sequence that was against the subject sequence in the GenBank database

abbreviated (FDSC) which contains at least 12 lineages, of which *F. dimerum sensu stricto*, *F. delphinoides*, and *F. penzigii* are common distinct species according to (van Diepeningen, *et al*., 2014), (Josef, *et al*., 2008) also goes on to support this claim by stating that *Fusarium dimerum* is known to comprise of at list 12 phylogenetically distinct species and is only known for its anamorph.

Fusarium penzigii was named in honor of an Italian mycologist by the name of Albertus Giulio Ottone Penzig. Just like the *F. dimerum* species complex it is widely known to be a soil and dead plant substrata pathogen or as an agent of trauma-related eye infections of humans so this report is one of the rare ones that indicates its role in causing plant diseases.

The results showed that *Phoma destructiva* was responsible for the disease symptoms on the green beans. This observation is one that has not been widely documented in literature as *P. destructiva* has mostly been reported to cause disease symptoms in tomatoes. Phomopsis blight of tomatoes is known to be caused by the fungi *Phoma destructiva,* it causes rot spots that are usually sunken ,light colored at margins and leathery, the fungus also produced pcynidia in dark portions of the tomato (Wani , 2011).

This study was limited in that there was not enough time available to collect the seedlings of the *Ziziphus mucronata* and carry out Koch's postulates so as to test the obtained species for pathogenesis. The results of this study support the hypothesis that fungi was responsible for the symptoms observed on the *Ziziphus mucronata* and the *Phaseolus vulgaris.* With the fungi suspected to be responsible being isolated and characterized to the species level which was the main objective of this study. It can thus be concluded that *Z. mucronata* is susceptible to infection by *Fusarum penzigii* and *Fusarium dimerum* while the common green beans is susceptible to *Phoma destructiva* infection. To be able to confidently point out the isolated fungi as the casual agents, a couple of more experiments should be designed and further extensive studies should be carried on the isolates.

CONCLUSION

The results of this study supported the hypothesis that fungi was responsible for the symptoms observed on the *Ziziphus mucronata* and the *Phaseolus vulgaris.* With the fungi suspected to be responsible being isolated and characterized to the species level which was the main objective of this study. It could thus be concluded that *Z. mucronata* was susceptible to infection by *Fusarum penzigii* and *Fusarium dimerum* while the common green beans was susceptible to *Phoma destructiva* infection.

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