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Isolation and Identification of Culturable Fungal Species Associated with Disease in *Vachellia tortilis* in Namibia

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***Vachellia tortilis* is a tree belonging to the family Fabaceae that inhabits high temperature and dry environments of the world. It is ecologically and economically significant amongst others of the genera *Vachellia* and *Senegalia*. It is native to Namibia and several other countries. Various parts of the *V. tortilis* plant are used for different therapeutic purposes both in the traditional and pharmacological settings. However, *V. tortilis* is vulnerable to pathogenic infection to which they lack natural resistance and little is known regarding the pathogens responsible for such infections. The aim of the study was to isolate and identify the fungal species associated with twig dieback disease in *V. tortilis*. Pure cultures from diseased *V. tortilis* were grown using potato dextrose agar (PDA) and DNA was subsequently isolated and later amplified in a PCR reaction using ITS1 and ITS4 primers. Sequencing and BLAST search revealed the identity of the isolates as; *Penicillium rubefaciens, Penicillium herbarum, Trichoderma longibrachiatum* and *Trichoderma harzianum*. Results indicated that *P. herbarum* was associated with disease symptoms in *A. tortilis, T. longibrachiatum and T. harzianum* were antagonistic fungi while the effect of *P. rubefaciens* on *V. tortilis* remained unknown. It is recommended that further investigations using Koch's postulates should be performed on *V. tortilis* using the isolates.**

Keywords: *Penicillium rubefaciens, Penicillium herbarum, Trichoderma longibrachiatum, Trichoderma harzianum,* Namibia, *Vachellia tortilis*.

Acacia, Vachellia and *Senegalia* species largely inhabit high temperature and dry environments of Africa. A total of about 1200 species of of these genera have been identified and are prominent in Arabia, Australia and Africa (Baldwin et al., 1999; Yadav et al., 2013). *Acacia, Vachellia and Senegalia* species are of economic importance because they are used to produce various materials such as tannin, gum and timber (Ibrahim and Aref, 2000; Yadav et al., 2013). They also provide valuable fodder for livestock and are also ecologically important in the ecosystems within they occur.

Vachellia tortilis is a tree that grows up to 21 m tall belonging to the family Fabaceae. It is a significant species amongst others of the genus *Vachellia* and is native to Namibia, Angola, Botswana, Egypt, Eritrea, Ethiopia, Iran, Israel, Kenya, India, Pakistan, Mozambique, Qatar, Saudi Arabia, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda, United Arab Emirates, Zambia and Zimbabwe (Wickens et al, 1995). *V. tortilis* contains important properties that are beneficial commercially and medicinally. Various parts of the *V. tortilis* plant are used for different

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therapeutic purposes both in the traditional and pharmacological settings.

Traditionally, *V. tortilis* plant parts are described to have the following uses; seed for food, leaves and fruits for fodder, flower for forage, wood for fuel and timber, bark for dyestuff (tanning), gum as a food additive, and whole plant for soil fertility (Fterich et al., 2012; Anderson, 1993; Baldwin et al., 1999; Abdallah et al., 2008; Oba, 1998; Satya and Jindal, 1994; Yadav et al., 2013). Pharmaceutical uses of the *V. tortilis* plant include; polysaccharide isolated from gum exudates for diabetes mellitus, stem bark for fungal diseases and infectious diseases (mouth and dental), bark tannins for diarrhea, wood for dry cough, root for cough and diphtheria, root bark for malaria, aqueous extract for hyper cholesterol and inflammation, and methanol extracts for leishmanial and parasitic diseases (Abdallah and Merito, 2013; Bisht et al., 2013; Kigondu et al.,2009; Maregesi et al., 2008; Maregesi et al., 2007; Alharbi and Azmat, 2011; Nguta and Mbaria, 2013; Njoroge and Bussmann, 2006; Tunon et al., 1995).

Although *V. tortilis* has a wide variety of uses, it remains vulnerable to infection from an increasing number of pathogens to which they lack natural resistance. Information regarding the entire pathogens associated with disease in *V. tortilis* will provide a basis for assessing the condition of the disease and develop appropriate breeding programs to minimise losses. Hence, the main aim of the study was to isolate and identify the fungal species associated with twig dieback disease in *V. tortilis*.

MATERIALS AND METHODS

Isolation of fungi

A piece of diseased *V. tortilis* stem was surface-sterilized by submerging in 99% ethanol for 7 minutes and rinsed five times with sterile distilled water. The surface sterilized stem was dissected in a laminar flow and pieces of its pith were transferred using flamed tweezers onto potato dextrose agar (PDA) for growth. In addition, white basidiomycete buds that were attached to the periderm of the diseased *V. tortilis* bark were plucked with flamed tweezers and placed onto potato dextrose agar (PDA) and left to grow. The PDA plates were then incubated at 35±2°C for 14 days. After which subcultures were created by aseptically transferring distinct fungi growing from the *V. tortilis* plant parts on the PDA media onto fresh PDA media and incubated for 10 days at 35±2°C.

DNA extraction

Fungal genomic DNA was extracted using the Zymo Research Fungal/Bacterial DNA MiniPrep™ kit according to the manufacturer's protocol. The extraction was performed on one week old fungal cultures and the DNA was stored at 4°C for later use as the template for amplification. After successful isolation, the DNA (7µl) was mixed with 2µl of 6X loading dye and run on a 1.5% agarose gel prepared in 1X TAE buffer and viewed using UV illumination.

PCR amplification and Sequence analysis

The DNA was amplified in 50 µl PCR reaction volumes using ITS-1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS-4 (52 -TCCTCCGCTTATTGATATGC-32) primers according to White et al. (1990) with minor modifications. The PCR reaction was carried out in a Bio-Rad MyCycler™ thermal cycler and the mixture contained 4 µl of template DNA, 2 µl of a 0.5 µM concentration of ITS1 primer, 2 µl of a 0.5 µM concentration of ITS4 primer, 17 µl of nuclease free water and 25 µl of 2x Dream *Taq* master mix which contained: Dream *Taq* DNA polymerase, 2x Dream *Taq* buffer, dATP, dCTP, dGTP and dTTP of 0.4 mM each, and 0.4 mM $MgCl_2$. The PCR reaction profile consisted of initial denaturation temperature of 94 ºC for 4 min, followed by 40 cycles of denaturation temperature at 95 ºC for 1 min, annealing temperature of 55 ºC for 1 min, and an extension temperature at 72 ºC for 45 seconds. The final extension was then performed at 72 ºC for 10 min and lastly the PCR products were held at 4 ºC**.** The products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

The PCR fragments were purified and sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The obtained sequences were then edited in BioEdit (Biological Sequence Alignment editor for Windows 99/98/NT/2K/XP/7) sequence alignment editor software (Hall, 1998). BLAST searches were then perform in the NCBI GenBank database and the sequences

possessing the highest similarity with the query sequence were chosen reflecting the identity of the isolates in question.

RESULTS

The culturing of *V. tortilis* sections of the stem and bark resulted in the growth of four morphologically distinct pure colonies (**fig.1 and fig. 2**). PCR was performed using ITS1 and ITS4 primers and electrophoresis results showed expected ITS amplicons of about 1450 bp.

A BLAST search using the *A. tortilis* ITS sequences revealed the identities of the isolates as; *Penicillium rubefaciens*, *Phoma herbarum*, *Trichoderma longibrachiatum* and *Trichoderma harzianum*. The isolates were obtained from the *A. tortilis* as follows; *P. rubefaciens* from the *Acacia* pith, *P. herbarum* from the *Acacia* bark, and *T. longibrachiatum* and *T. harzianum* from the *basidiomycetes* on the bark.

DISCUSSION

The results revealed the identity of the isolates from the diseased *V. tortilis* as *P. rubefaciens, P. herbarum, T. longibrachiatum* and *T. harzianum.* All these fungi have not been reported previously on this plant species in Namibia. *Penicillium* and other fungus such as *Aspergillus* and *Talaromyces* (*Eurotiomycetes*) are amongst the genera that are commonly found indoors (Amend et al. 2010; Houbraken et al. 2011).

Penicillium species have been reported (Frisvad et al. 2004; Frisvad and Samson 2004) to be linked to bio deterioration in foods especially fruits and producing various mycotoxins. *Penicillium* species have also been positively used in the food industry for the production of cheese (Giraud et al. 2010; Visagie et al., 2014), fermented sausages (Lopez-Díaz et al., 2001; Ludemann et al.,

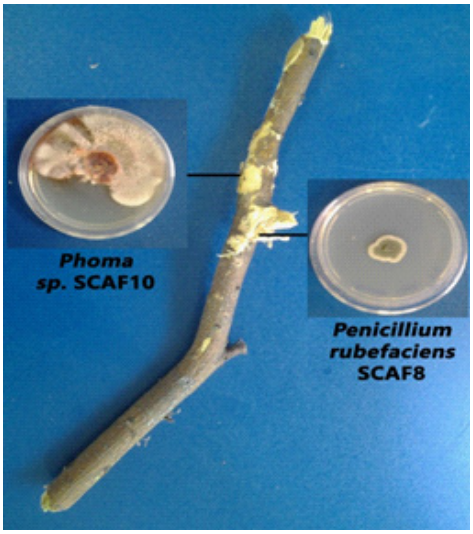

Fig. 2. Fungal isolates from diseased *A. tortilis* stem grown on PDA media

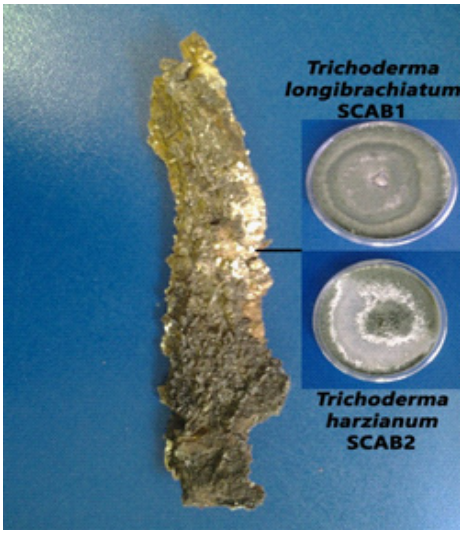

Fig. 1. White basidiomycete from diseased *A. tortilis* bark grown on PDA media

Table 1. The bacteria isolates and their corresponding identity retrieved from NCBI

Fungal isolate	Plant Host	NCBI Identity	% Identity	Accession Number
F8	*V. tortilis*	*Penicillium rubefaciens*	100	KR905616.1
F10	*V. tortilis*	*Phoma herbarum*	99	KP144996.1
B1	*V. tortilis*	*Trichoderma longibrachiatum*	100	KP326575.1
B2	*V. tortilis*	*Trichoderma harzianum*	99	LN714612.1

2010), and medical industry for the production of penicillin (Visagie et al., 2014). *Penicillium, Eurotium* and *Cladosporium* species have also been isolated from the cork (outer layer of the *Quercus suber* L. tree.) by Barreto et al. (2012) and they argued that these fungal species colonize the cork alongside different kinds of fungi inhabiting different parts of the tree and surrounding soil. Depending on the fungal species, some form a parasitic relationship while others form a saprobiotic one in which case the cork provides the relevant nutrients for the fungal growth (Barreto et al., 2012). Moreover, *Penicillium* occurs in diverse environments that include soil, vegetation, air, indoor environments and in food (Visagie et al., 2014). To our knowledge, this is the first report on the presence of *P. rubefaciens* in *A. tortilis*. The effect of *P. rubefaciens* on *A. tortilis* remains unknown and further investigations to assess if this species is the primary disease causing pathogen on *A. tortilis* are vital.

The *Phoma* genus is diverse and composed of species that inhabit a wide range of environments such as soil and plants. A substantial amount of the species of this genus are saprophytic with some being opportunistic (Irinyi et al., 2007). In addition, most coelomycetes are associated with plants and in some cases are primary pathogens (Sutton, 1980). According to Boerema et al. (2005), approximately 2000 species of *Phoma* have been isolated and characterized. Furthermore, Garas et al. (2012) labeled *Cytosperma chrysoperma* and *Phoma* species such as; *P. glomerata, P. cava* and *P. eupyrena* as pathogens responsible for dieback disease in *V. mellifera* and *V. karoo*.

P. herbarum species have been isolated from fruit trees in which they infect the leaves and twigs (Valiuskaite, 2002) and sapwood of healthy and declining scots pine trees (Giordano et al., 2009). Giordano et al. (2009) revealed that *P. herbarum*, *Thanatephorus cocumeris* and *Mucor plumbeus* species are associated with the existence of mistletoes on scots pine trees. A mistletoe is a parasitic plant that affects the stand structure by inducing crown losses and tree mortality but also has some ecological importance in the survival of some trees because it functions as a carbon sink (Cullings et al., 2005; Mueller and Gehring, 2006). Giordano et al. (2009) further argued that mistletoe on a tree influences the diversity of endophytic mycoflora inhabiting that particular tree and declining scots pine trees possess different endophytic mycobiota in their sapwood compared to that of healthy trees. However, *P. herbarum* has been designated as the primary pathogen leading to twig dieback in *A. mearnsii* after its consistent isolation from diseased *A. mearnsii* trees (Gibson, 1979; Olembo, 1972; Roux et al., 1995). Gibson (1979), Olembo (1972) and Roux et al. (1995) further argued that *P. herbarum* caused fresh infections through wounds. The present study did not observe the presence of mistletoe on the *V. tortilis* tree but can infer that *P. herbarum* is associated with disease in the *V. tortilis*.

Trichoderma species are widely found in the soil and root ecosystems and are used as biological control agents against a variety of plant pathogens especially fungi (Mulaw et al., 2013). These species are found in a diverse range of habitats ranging from soil and growing on wood, bark, and other fungi and numerous substrates (Druzhinina et al., 2011). This report is the first one on Trichoderma occurring in *V. tortilis* in Namibia. The commonly used *Trichoderma* species as biological controls are *T. harzianum*, *T. atroviride*, *T. viride*, *T. virens* and *T. koningii.* These species are antagonistic microorganisms capable of preventing the growth of pathogens in the soil and plant (Singh et al., 2014). However, the effect of *Trichoderma* species on non-target organisms is less understood although it is relevant for their effective use. *Trichoderma* species have found prominent use because they prevent increase of the pathogens, produce enzymes that degrade the cell wall of pathogens, produce antibiotics capable of destroying pathogens, induce the development of the plant, and induce plant defensive mechanisms (Singh et al., 2014). Druzhinina et al. (2011) explained that the antagonistic properties of parasitizing and killing other fungi are common in *Trichoderma* species and have thus earned them widespread use as biological control organisms. Studies have revealed that *T. longibrachiatum* and *T. harzianum* are used as biological agents against fungal infection in plants (B³aszczyk et al., 2014; Howell, 2003; Limon et al., 2004; Mohd Zainudin et al., 2008; Mulaw et al., 2013).

The isolation of *T. longibrachiatum* and *T. harzianum* in *A. tortilis* in the present study suggests the relationship between the *A. tortilis,*

and *T. longibrachiatum and T. harzianum* species is either commensal or mutualistic because of their antagonistic properties. It was revealed that *P. herbarum* is associated to twig dieback disease in *V. tortilis* while the effect of *P. rubefaciens* on *V. tortilis* remains unknown. In addition, pathogens have the ability to form synergistic relationships which have been detected in various plant species (Lamichhane and Venturi, 2015). Synergistic relationships range from fungi-fungi interaction, bacteria-bacteria interaction, virus-virus interaction, and mixed interactions such as fungi-bacteria interaction. Synergism justifies the existence of plant diseases in which more than a single pathogen is involved in the infection process (Lamichhane and Venturi, 2015). Synergism and its effects go undetected in monocultures and it probably occurs frequently in plant diseases. Glen et al. (2009) revealed fungi-fungi synergism in *A. mangium* infected with root rot disease in which the pathogens responsible were *Ganoderma philippii, Ganoderma mastoporum, , Ganoderma austral,* and *Amauroder marugosum.* Consequently, it is possible that some of the isolates in this study were opportunistic pathogens that benefit from synergism. Moreover, with the current data set, it is not possible to draw firm conclusions that *P. herbarum* is the primary cause of the twig dieback although it is associated with twig dieback as reported in previous studies (Gibson, 1975; Olembo, 1972; Roux et al., 1995) because of the possible existence of other fungal species that are not or rarely detected in culture based methods.

CONCLUSIONS

The study aimed at isolating and identifying the culturable fungal species associated with twig dieback disease in *V. tortilis* in which case four fungal species were successfully isolated and identified as; *P. rubefaciens, P. herbarum, T. longibrachiatum* and *T. harzianum*. Of the four isolates, *P. herbarum* was detected as the specie associated with the observed disease in *A. tortilis. T. longibrachiatum and T. harzianum* were identified as antagonistic fungi with the ability to protect the *V. tortilis* tree against pathogenic fungi but with the potential to be pathogenic depending on the circumstances. However, the effect of *P. rubefaciens* on *V. tortilis* remained unknown. It is therefore recommended that further; investigations using Koch's postulated should be performed on *V. tortilis* using the isolates, studies should be performed using more informative community analysis molecular techniques that are able to detect the entire community and circumvent the limitations of culture based methods; and microbial synergistic relationships leading to disease in *V. tortilis* should be investigated in order to deduce whether the twig dieback disease is caused by a single primary pathogen or it is a result of complex interactions.

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