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Full Length Research Paper

Mycorrhiza classes in the African Blackwood. A case of Babati, Kilosa and Kilwa district in Tanzania

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A study was conducted to assess classes of mycorrhiza fungi dominating the association with *Dalbergia melanoxylon* a species of highly valued wood in the world, but not propagated. A total of 120 kg of soil and 120 root cuttings were sampled from Kilwa, Kilosa and Babati district to isolate mycorrhizal fungi which might be useful for regeneration of *D. melanoxylon*. Sampling was conducted on October 2010. Processing was done by soaking the root pieces in 1.79M KOH, and 0.1M hydrochloric acid. Staining was done by using 0.05% Trypan blue and de-stained in 14:1:1 lactic acid: glycerol: water and mounted on slides for observation using a compound microscope at 400X. Soil samples were soaked in water overnight and the mycorrhiza fungi were separated from their mantle using a stereo microscope at x50. Separated mycorrhiza were incubated in both lactophenol cotton blue and toluidine blue and observed using a compound microscope at 400X. Twenty six Ectomycorrhizae were isolated from soil rhizosphere resembled only 2 species *Inocybe* and *Laccaria* sp and 18 Endomycorrhizae were isolated from root pieces resembled only 1 species of *Glomus*. It was confirmed that the dominating classes of mycorrhizal fungi in *D. melanoxylon* are Agaricomycetes in the soil rhizosphere of the plant and Glomeromycetes in the roots of the plant. Future research should investigate mycorrhiza population and the genetic diversity of identified species for improvement of *D. melanoxylon* propagation.

Key words: Mycorrhiza association, *Dalbergia melanoxylon*, Ectomycorrhiza, Endomycorrhiza.

INTRODUCTION

Mycorrhizal association with plants

Mycorrhizae are symbiotic associations formed between roots of some plant species and fungi. The relationships are characterized by effecting bi-directional movement of moisture and nutrients where carbon assimilated by the plant flows to the fungus and inorganic nutrients absorbed by fungus move to the plant, thereby providing a critical linkage between the plant root and the soil. Nutrients delivered by the mycorrhizal fungi to the plant roots are metabolized by the plant leading to improved plant growth and reproduction (Verma et al., 2010). Healthy plants containing mycorrhiza are often more competitive and more tolerant to environmental stresses, diseases, pathogens and drought than plants that have no mycorrhizal association. *Dalbergia melanoxylon* is one of the tolerant species due to improved water and mineral uptake. Ectomycorrhiza are reported also to produce Plant Growth Regulators (PGRs) in their association with plants which plays a regulatory role between the fungi and the roots. These PGRs include Indole-3-Acetic Acid (IAA), ethylene and Pyrrolizidine Alkaloids (PAs). This is yet to be verified in *D. melanoxylon* (Oehl et al., 2005).

Evidence of the effects of mycorrhiza to plant growth include re-establishment of plants in disturbed and degraded lands, increased nutrients acquisition, improvement of soil structure by aggregating soil particles, production of plant growth regulators, improvement in plant water relations and drought resistance, improvement of resistance to pathogens and reduction of environmental pollutants (Oehl et al., 2005). Classification of mycorrhizae as put by Brundrett et al. (1996) includes Ectomycorrhizae (ECM), Endomycorrhizae (Vesicular Arbuscular mycorrhizal (VAM), Orchid myc
Arbutoid mycorrhiza

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belong to the classes Basidiomycetes and Ascomycetes that dominate temperate countries. These are characterized by having sheath of hyphae (mantle) around the roots that form a network of hyphae termed hartig net. Ectomycorrhizae are typically formed in roots of woody plants (Agerer, 2006). VAM belongs to class Zygomycetes that dominate miombo woodlands and are characterized by having branching mycelia through the soil connected to the roots of the host plant forming arbuscules (Brundrett et al., 1996). Orchid mycorrhizae belong to class Basidiomycetes dominating many parts of the world in orchidaceous plants and are characterized by forming intercellular coils or hypha aggregates within host tissues (Bayman, 2006). Ericoid mycorrhizae belong to order Ericales and are confined to the northern and southern hemisphere and are characterized by having septate hyphae to facilitate penetration into intracellular host plant root (Massicotte et al., 2005b). Arbutoid mycorrhizae or Ectoendomycorrhizae are similar to ectomycorrhizae by forming sheath, but they belong to the genus *Arbutus* (Oehl et al., 2005).

D. melanoxylon Guill & Perr is a flowering plant that belongs to the family Leguminoseae and sub-family Papilionoidea (IUCN, 2008). The plant is a native to dry regions of Africa and the wood is highly valued because it is used in wood wind instruments, clarinets, oboes, and pipes. *D. melanoxylon* has fine grained heartwood, resistant to insect attack making it among the most valuable timber tree in Africa (Bekele, 2007).

Status of *D. melanoxylon* in Tanzania

The IUCN red list (IUCN, 2008) uses three categories to evaluate conservation status of plant species (Lower Risk, Threatened and Extinct category). Lower risk spp may be the Least concern (Lc), Near threatened (nt) and Conservation dependent (cd). Threatened spp may be vulnerable species (Vu), endangered species (EN) and Critically endangered (CR) and the extinct spp may either be Extinct in the wild (EW) or Total extinct (EX). An abundant species is categorized as of least concern, while an over harvested species near to extinction is categorized as endangered. *D. melanoxylon* in Tanzania is classified as Lower risk / Near threatened meaning that it is neither endangered nor of least concern. However it may be considered near threatened if propagation efforts are not instituted. The current conservation status of *D. melanoxylon* shows that is threatened in Kenya, extinct in Burkina Faso and therefore need conservation attention in Tanzania (Bekele, 2007).

Previous and recent propagation Studies on *D. melanoxylon* in Tanzania

The IUCN, (2008) reported that *D. melanoxylon* on one side have low regeneration ability while in the other hand is over harvested such that it is threatened in African countries like Kenya and categorized as Low/Risk near threatened in Tanzania. Previously, studies by Tanzania Tree Seed Agency (TTSA) (1995) reported low seed viability of less than 30% in *D. melanoxylon* which is also associated with low seed germination and that the growth rotation period of the species is 70 to 100 years to attain a harvestable age. These reports are supported by recent studies conducted by Amri (2008) and Amri, (2010). A study by Amri (2008) reported that *D. melanoxylon* has low seed viability, seed germination and seedling rotational period. In a related study seed viability varied with different time of seed harvesting. On the other hand, Amri (2010) reported an increased rooting ability of 70% stem cuttings using root promoting hormones (IBA). All these studies did not employ the advanced plant-production techniques in *D. melanoxylon*. This study intended to assess the mycorrhizal classes that dominate the association with *D. melanoxylon* so that can be used to improve propagation of the species.

Objective of the research

To improve *D. melanoxylon* propagation.

Specific objective

To assess the dominating mycorrhiza classes in *D. melanoxylon* association for propagation.

MATERIALS AND METHODS

Isolation of Varscular Arbuscular and Ectomycorrhiza (VAM and ECM)

Soil samples from soil rhizosphere and short terminal roots were collected from *D. melanoxylon* trees in Kilwa, Kilosa and Babati Districts of Tanzania using standard methods as described by Brundrett et al. (1996). The criteria used to select the three sites were prevalence of dense populations of *D. melanoxylon*, habitat heterogeneity and their different geographical locations in Tanzania. A total of 120 kg of soil samples were collected within 5 mm from their associated roots of the selected trees, whereby 20 kg of the sampled soil were used for isolation and identification of mycorrhiza. A total of 120 root terminals were extracted from the top soil layer (approx. 15 cm) of a tree soil-root zone by tracing them from the point of attachment on the stem before severing them from their stems using a Machete. A total of 20 root terminals of the collected roots were used for isolation and identification of mycorrhiza. The samples were enclosed in polythen
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Salaam for assessment.

Guidelines for characterization and identification of mycorrhizae

Suggested guidelines include linking mycorrhizae to fruit body, comparing observations with published description of previously identified types, applying characters used in fruit body taxonomy (Brundrett et al., 1996). The techniques for mycorrhizae examination include: (i) Selection of materials focusing on fresh, recently matured mycorrhizae (ii) Preparation where mycorrhizae samples are soaked in water overnight and cleaned in gently running water (iii) Microscopic separation where a stereo dissecting microscope at X50 magnification is used to examine mycorrhizae covered by water in a petri dish (iv) Microscopic observation where mycorrhizae are mounted on slides in both lactophenol cotton blue and toluidine blue for 10 to 15 s before observed by Olympus microscope at 400X magnification. Clearing and staining of VAM mycorrhizal roots can be done by using procedures recommended by (Kormanik and Mc Graw, 1982). When mycorrhizae are characterized, referenced samples are preserved in 2% glutaraldehyde and stored in a fridge at 4°C.

Identification of varscular arbuscular mycorrhiza (Endomycorrhiza)

Five (5) terminal roots of *D. melanoxylon* from each site of about 10 cm long were cut into 1 cm pieces. The pieces were cleared in 1.79 M KOH for 10 min, maintained in water bath at 90°C for 30 min, rinsed three times in distilled water before soaking in 0.1M hydrochloric acid for 2 h to soften and acidify the tissues for easing penetration of staining reagents. Soaked root pieces were then stained in acidic glycerol solution containing 0.05% Trypan blue for 30 min while incubated in a water bath at 90°C for contrast. The root pieces were de-stained overnight in 14:1:1 lactic acid: glycerol: water to remove stains of other cells and tissues not belonging to mycorrhiza tissue and for more contrast (Kormanik and Mc Graw, 1982). De-stained roots were mounted on slides for observation. Mycorrhizas were observed using a compound microscope; model Olympus CH30 RF200 at 400X. Identification was based on comparing observed results to published descriptions of previously identified mycorrhiza types in the website http://invam.caf.wvu.edu/fungi/taxonomy/species.ID.htm. Features of the spores were usually used to identify *Glomalean* types in accordance with (Kormanik and Mc Graw (1982). Other descriptors which were used to compare and confirm the identity of endomycorrhiza fungi included appearance of arbuscules and vesicles if they appear coiling within a plant cell (intracellularly) confirms a VAM.

Identification of Ectomycorrhiza

Soil samples were soaked in water overnight, and then were cleaned by passing them through gently running water. Mycorrhiza were separated from their mantle under water in petridish using a stereo microscope at X50. Separated mycorrhiza were incubated in both lactophenol cotton blue and toluidine blue dyes for 10 to 15 s for contrast to ease microscopic observation in which slides with 5 g each of soaked soil were used for this microscopic observation using Olympus CH30 RF200 at 400X. Identification was based on comparing observed results to published descriptions of previously identified mycorrhiza types in the (Bound.) Pat. ITE manual and (Fr: Fr) Gillet. ITE manual. Descriptors which were used to compare and confirm the identity of ectomycorrhiza fungi included: appearance of mantle surface, associated hyphae, strands, sclerotia and emanating hyphae in accordance with Brundrett et al. (1996).

RESULTS

Mycorrhiza fungi associated with *D. melanoxylon*

In total, three species of mycorhiza fungi from two different classes were found associated with *D. melanoxylon*. Two (ECM) mycorrhiza species *Inocybe sp* and *Laccaria* species (Plates 1 - 2) were identified from soil rhizosphere and one species of *Glomus* from the root. A total of 26 mycorrhizae isolated from 20 kg of soil rhizosphere based on the general appearance and characteristics of dark brown colour, fairly straight, slender, infrequently branched with hyphae emanating on the mantle surface and abundant clamp connections, resembled only 2 species of Ectomycorrhiza while 18 Mycorrhiza isolated from 20 root pieces resembled *Glomus.*

General characteristics observed for *Inocybe sp*

Mycorrhizas were short and stubby, with frequently irregular branching pattern. The loose fraggly hyphae were occasionally found around the base of mycorrhiza. Detailed features observed for *Inocybe* sp indicated that mycorrhizas were brown, (dark red brown), without strands and sclerotia. Emanating hyphae had abundant clamp connections and elbow-like protrusions. These features were confirmed in (Fr: Fr) Gillet. ITE as *Inocybe petiginosa*. In this particular study, *Inocybe sp* mantle looked larger since it was surrounded by loosely cotton hyphae while that identified in the ITE were slender and not surrounded by loosely cotton hyphae.. *Inocybe* sp is found in the class Agaricomycetes, Order Agaricales and family Cortinariace
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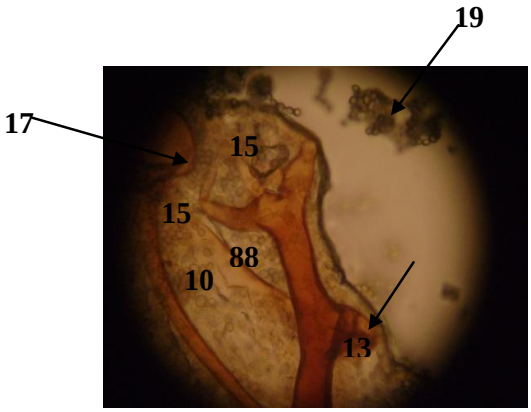


Plate 1. Isolated ectomycorrhiza. resembling morphotype of *Inocybe petiginosa* that stained dark brown with the following features: **10**: Loosely cotton hyphae, **13**: Emanating hyphae with elbow-like protrusions, **15:** Branching hyphae with clamp connections, **17:** Irregular branching pattern, **19:** Loosely, fraggly hyphae around the base of the Mycorrhizae **88:** Mantle.

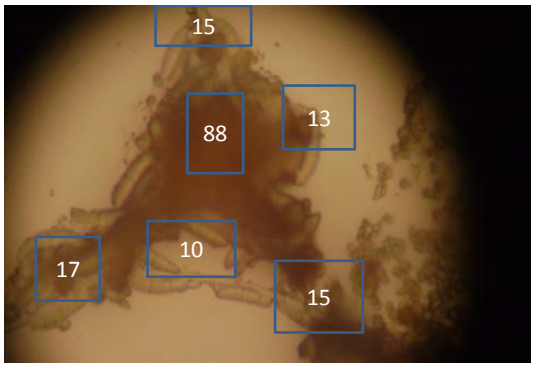


Plate 2. Isolated ectomycorrhiza resembling morphoyype of laccaria proxima that stained dark brown with the following features : **10** : Loosely cotton hyphae, **13** : Emanating hyphaewith elbow-like protrusions, **15** : Branching hyphae with clamp connections, **17** : Irregular Branching pattern, **19** : Loosely, Fraggly hyphae around the base of the Mycorrhizae, **88** : Mantle.

commonly associated with *Picea sitchensis* and *Betula pendula* found mostly in higher altitudes and temperate regions.

General characteristics observed for *Laccaria sp*

Mycorrhiza fungi were fairly long and sinuous, with frequent irregularly spaced short branches, loose straggly hyphae which were frequently close to the mantle surface without strands and sclerotia. Mantle edges were loosely formed becoming compacted in the older mycorrhiza. Emanating hyphae had abundant large irregularly formed clamp-connections and elbow-like protrusions. These features were confirmed in (Bound.) Pat. ITE for *Laccaria proxima*. In this particular study, mycorrhizae mantle looked larger due to the presence of loosely cotton hyphae surroundings unlike that identified in the ITE that looked slender due to the absence of loosely cotton hyphae. *Laccaria* Agaricomycetes, or

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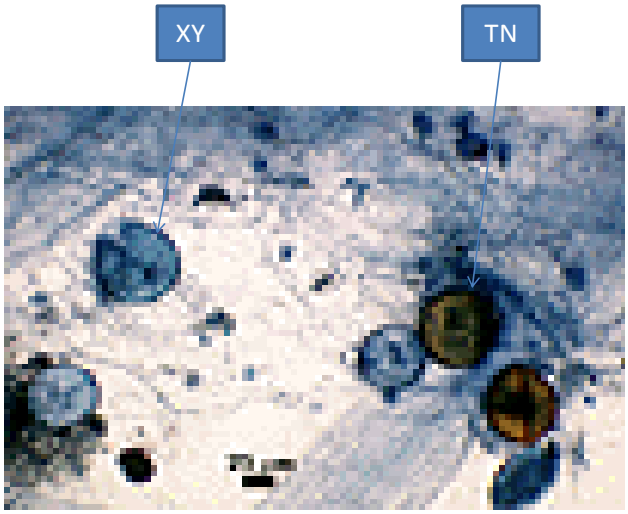


Plate 3. Appearance of spore in stained root resembling spores of Glomus versifome stained blue and brownish yellow : **XY** : Glomus spore stained blue, **TN** :Glomus spores stained brownish yellow.

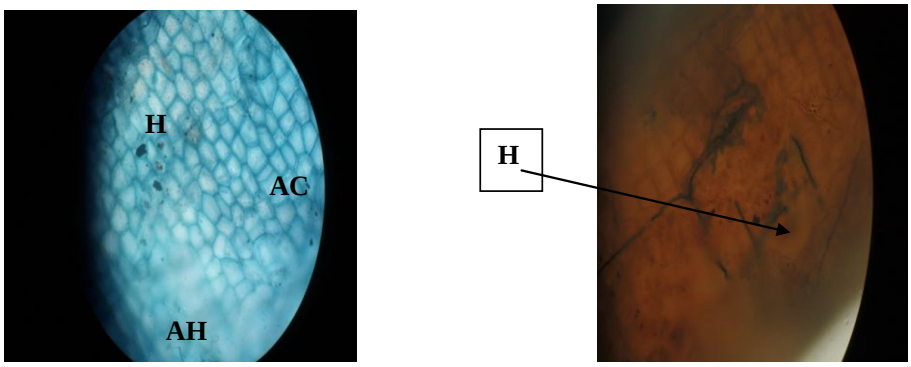


Plate 4. A stained root cortex cell with *Glomus* spore features**:** "**H**" branching hyphae ramified along the root cortex, **AC**: Dense and compacted arbuscules, **AH:** Intricately branched haustoria arbuscules in the cortex cell . **Plate 5**: Magnified H structure of plate 4.

Tricholomataceae in accordance to Biodiversity Global Information Facility (BGIF). *Laccaria proxima* is commonly associated with *Pinus sylvestris*, *Picea sitchensis* and *Betula pendula*.

Glomus sp,* the varscular arbuscular associated with *D. melanoxylon

Identified VAM are as shown in plates 3 - 5. Mycorrhiza spores identified in plate 3 resembled *Glomus* sp in general appearance and characteristics.

General characteristics observed for *Glomus* species

Hyphae were non septate and ramified within the cortical cells. Arbuscules were intricately branched haustoria in cortical cells. Detailed features for the identified *Glomus* sp indicated that a species had relatively straight hyphae that ramified along the root cortex and often producing "H" branches which resulted in simultaneous growth in two directions. These hyphae "H" usually stained relatively dark. Arbuscules were dense and compact, had intracellularly developed mantle with cell to cell hyphae. These features and spores were confirmed for *Glomus versiforme* using the Invarm website (http://invam.caf.wvu.edu/fungi/taxonomy/species.ID.htm) in the reference accession IT104 in accordance with Kormanik and Mc Graw (1982). *Glomus versiforme* is found in the Glomeromycetes; o

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(Oehl et al., 2005) and is frequently found in association with sugar cane, grape and some grains such as wheat, barley, maize and sorghum (Oehl et al., 2005).

Discussion

Like other woody and tropical trees being dominated by mycorrhizal fungi (Kanyagha, 2008). Mycorrhiza fungi have been investigated in *D. melanoxylon* through this study and confirmed the presence of them which may be responsible for keeping *D. melanoxylon* adaptive to a wide range of environments including natural regeneration through saplings from root suckers. A high environmental adaptive ability of *D. melanoxylon* seem to be contributed by its association with two ectomycorrhiza species, *Inocybe* and *Laccaria* and one endomycorrhiza species, *Glomus*. These mycorrhizal associations are involved in the formation of high hyphae network connections in the roots which facilitates absorption of a range of nutrients from the soil and increasing penetration ability of the roots into the soil (Oehl et al., 2005). *Glomus* exist in the environment both as spore and hyphae which can form dense networks called mycelia, though most of *Glomus* biomass occur within roots of host plants. *Glomus* is believed to exist in all terrestrial habitats colonized by vascular plants and may form an endosymbiotic relationship with 70 - 90% of extant vascular plants. The *Glomus*-plant symbiosis plays an important role in the economic sectors involving the growth of plants (Oehl et al., 2005). So inoculating *Glomus* in propagation of *D. melanoxylon* as obtained from the results of this study will enhance growth and regeneration of the species. *Glomus* sp are successful in producing spores in the soil around their host plant root that become a source of association by penetrating their host roots forming hyphae network connections and protrusions that increases the surface area for improved moisture and nutrients acquisition. This has contributed significantly to the success and high adaptability of the species (Oehl et al., 2005). Inoculating host plant seedlings with Glomus in the nursery and transplanted *D. melanoxylon* will therefore increase adaptive ability of the species for successful propagation. *Glomus* is an obligate biotroph, meaning that it requires a living photoautotropic host to complete their life cycle and produce the next generation of spores. The species are also entirely asexual (Oehl *et al.*, 2005). The two ectomycorrhiza species found associated with *D. melanoxylon* are the main source of spore in the soil rhizosphere around *D. melanoxylon* roots. They are well known to mobilize and convert nutrients into their available form for plant absorption (Oehl et al., 2005) hence probably they are related to the high adaptability of *D. melanoxylon* in a wide range of habitat including the poor soil environments of Kilwa, Babati and Kilosa (Kanyagha, 2008).

Conclusion and recommandation

It is now evident that *D. melanoxylon* is associated with two classes of mycorrhizal fungi, one class in soil rhizosphere (Agaricomycetes) and one class in root cortex cell (Glomeromycetes). The presence of mycorrhiza fungi in roots and soil rhizosphere of the species is responsible for the relatively higher adaptive ability of *D. melanoxylon* to a wide range of habitat. It is recommended that future researchshould investigate further the mycorrhiza population and the genetic diversity of *Glomus*, *Inocybe* and *Laccaria* in *D. melanoxylon,* their multiplication and integration into the soil, proper inoculums type and proper time of inoculating them for improvement of *D. melanoxylon* viability, germination and seedling growth rate.

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