IMPROVING PROPAGATION SUCCESS OF *DALBERGIA MELANOXYLON* (AFRICAN BLACKWOOD) IN TANZANIA (I): CHARACTERIZATION OF MYCORRHIZA ASSOCIATED WITH *D. MELANOXYLON* (AFRICAN BLACKWOOD) IN TANZANIA

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ABSTRACT

Dalbergia melanoxylon *Guill & Perr is a plant with quality wood in the World and therefore is over harvested for timber while its regeneration capacity is very low. The propagation techniques such as tissue culture or use of mycorrhiza have not been investigated which instigated conduction of this study by investigating the presence of mycorrhiza that might be useful for its regeneration. Some 120kg of soil and 120 roots of* D. melanoxylon *from Kilwa, Kilosa and Babati were sampled to identify mycorrhiza related with regeneration of the species. Cleaning the tissues was done by soaking the root pieces in 1.79M KOH, and 0.1M Hcl. Staining was done using 0.05% Trypan blue and de-staining in 14:1:1 lactic acid: glycerol: water and mounting on slides for observation. Soil was soaked in water overnight and the mycorrhiza was separated using a stereo microscope at 50X. Separated mycorrhiza were incubated for observation using a compound microscope at 400X. Twenty six (26) ECM resembling* Inocybe *and* Laccaria sp *were isolated from the root pieces and 18 VAM resembling* Glomus species *were isolated from soil. It is recommended that future research should investigate proper inoculum types and time for inoculating the species in nurseries for propagation.*

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

INTRODUCTION

Mycorrhiza is a symbiotic association between a fungus and roots of a vascular plant. In mycorrhizal association, the fungus colonizes the host plant's roots, either intracellularly as in arbuscular mycorrhizal fungi (VAM), or extracellularly as in ectomycorrhizal fungi (ECM). They are an important component of soil life and soil chemistry (Harley and Smith 1983, Jackson and Mason 1984, Kanyagha 2008). The relationships are known to affect the bi-directional movement of moisture and nutrients whereby carbon assimilated by the plant flows to the fungus and inorganic nutrients absorbed by fungus move into the plant. In this way, a critical linkage between the plant root and soil is formed. Nutrients delivered by the mycorrhiza fungi to the plant roots are metabolized by plants leading to improved plant growth including regeneration (Munyanziza *et al*. 1994). Plants having mycorrhizal associations are often more competitive and more resilient and tolerant to environmental stresses including diseases, pathogens and drought compared to plants that do not have such association. Ectomycorrhiza have been reported to produce Plant Growth Regulators (PGRs) including IAA, ethylene and Pyrrolizidine Alkaloids (PAs) in their association with plants which play a regulatory role between fungi and roots (Smith and Read 1997). These regulators are yet to be verified in *Dalbergia melanoxylon* (Smith and Read 1997).

Classification of mycorrhiza includes Ectomycorrhiza (ECM), Endomycorrhiza (Vascular arbuscular mycorrhiza (VAM), Orchid mycorrhiza, Ericoid and Arbutoid mycorrhiza (Brundrett *et al*. 1996). ECM or sheathing mycorrhiza belongs to the classes Basidiomycetes and Ascomycetes that dominate temperate countries. These are characterized by having sheath of hyphae (mantle) around the roots to form network of hyphae termed hartig net. Ectomycorrhiza are typically formed between woody plant roots. VAM belong to class Zygomycetes which are known to dominate the miombo woodlands and are characterized by having branching mycelia through the soil connected to the roots of host plant forming arbuscules. ECM can also form vesicles. Orchid mycorrhizae belong to class Basidiomycetes that dominate many parts of the World in orchidaceous plants and are characterized by forming intercellular coils or hypha aggregates within their host tissues. Ericoid mycorrhizae belong to order Ericales that are found mostly in the northern and southern hemisphere and are characterized by septate fungal hyphae which intracellularlly penetrate the host plant root. Arbutoid mycorrhizae (Ectondomycorrhiza) belong to the genera Arbutus and are similar to ectomycorrhiza by forming sheaths.

Plant preference for mycorrhiza seem to follow both taxonomic and ecological line, for example, all Pine plants family is associated with Ectomycorrhiza, while the Rose plants family is associated with VAM. Ericales are associated with ECM while VAM and most of tropical trees are associated with both VAM and ECM (Munyanziza *et al*. 1994).

Dalbergia melanoxylon Guill. & Perr. is a flowering plant native to the seasonally dry regions of Africa (IUCN 2008). This species has one of the most valuable timber in the World and therefore is over harvested for wood, carvings, timber, music instruments and furniture while its natural regeneration is low and attains a harvestable age at 70 to 100 years.

Dalbergia melanoxylon is wide spread in tropical Africa from Senegal and Cote d'Ivore in the West, to Kenya and Ethiopia in the East, and extending South to South Africa. It is found in at least 26 sub-Saharan countries (Nshubemuki 1993). In Tanzania, *Dalbergia melanoxylon* is distributed in T_4, T_5, T_6, T_8 floristic regions which are Shinyanga and Mwanza (T_4), Itigi and Dodoma (T_5), Muheza and Matombo (T_6) and Mtwara (T_8) (Redhead and Temu 1981). The species is not abundant in all regions where it is found and inhabit rainforest and open miombo woodlands of Tanzania in which rainfall is often marginal and drought periods are usual. Such areas are environmentally fragile and their vitality depends on a delicate balance between populations within them. *Dalbergia melanoxylon* has adapted well to this marginal existence and its long growing period is an out come of this adaptation to harsh conditions (Arbonnier 2004). The adaptation also covers the way it regenerates mostly by forming sapling regeneration in areas where roots gets wounded as it winds through rough rocky soil (Arbonnier 2004). Regeneration and adaptive ability of *D. melanoxylon* is thought to be contributed by mycorrhizal associated with it.

The low regeneration ability is contributed by low seed viability and seed germination and slow growth rate (Amri 2008, Washa and Nyomora 2011). *Dalbergia melanoxylon* is classified as Lower Risk / near threatened in the IUCN red listing that is, it is neither endangered nor of least concern but nearly threatened if propagation efforts on the species not are taken immediately. The propagation of the *D. melanoxylon* species by advanced techniques such as tissue culture and use of mycorrhiza fungi have not been investigated as reported by (Readhead

and Temu 1981) which instigated conduction of this study. The main objective of this research was to investigate the presence of VAM and ECM in *Dalbergia melanoxylon* found in Tanzania, identify them and establish how they can be employed in improving the species propagation techniques.

MATERIAL AND METHODS

Isolation of mycorrhiza fungi (VAM and ECM)

Soil samples from soil rhizosphere and short root terminals were collected from *Dalbergia melanoxylon* 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 *Dalbergia melanoxylon*, habitat heterogeneity and their different geographical locations in Tanzania. A total of 120kg of soil samples were collected within 5mm from their associated roots of the selected sampling trees from which 20kg 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 polythene bags and transported in cool boxes at 10^{o}C to the Botany Department, University of Dar es Salaam for assessment.

Identification of VAM

Short terminal roots of *D. melanoxylon* from each site were cut into 1cm pieces. The pieces were cleared in 1.79M KOH for 10min, maintained in water bath at 90^{o}C for 30 min, rinsed three times in distilled water before soaking in 0.1M hydrochloric acid for 2 hours 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^{o}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 non mycorrhizal tissues 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. Features of the spores were usually used to identify Glomalean types in accordance with (Kormanik and Mc Graw (1982).

Identification of ECM

Soil samples were soaked in water overnight, and then were cleaned by passing them through gently running water. Mycorrhizas were separated from their mantle under water in Petri dish using a stereo microscope at x 50 of XSZ – 3G. Separated mycorrhiza were incubated in both lactophenol cotton blue and toluidine blue dyes for 10 to 15 seconds for contrast to ease microscopic observation. About 5gms each of soaked soil were used on slides for this microscopic observation. Identity of the EMF was determined based on published descriptions of different mycorrhiza types by (Brundrett *et al* 1996) by comparing appearance of the mantle surface, associated emanating hyphae and sclerotia.

RESULTS

Mycorrhiza fungi associated with *D. melanoxylon*

In total three species of mycorhiza fungi were found associated with *D. melanoxylon*. Two (ECM) mycorrhiza species *Inocybe sp* and *Laccaria* species as shown in Plates 1 and 2 respectively were identified. A total of 26 mycorrhizae isolated from 20kg of soil rhizosphere based on the general appearance and characteristics of dark brown colour, fairly straight, slender, infrequent branching 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*

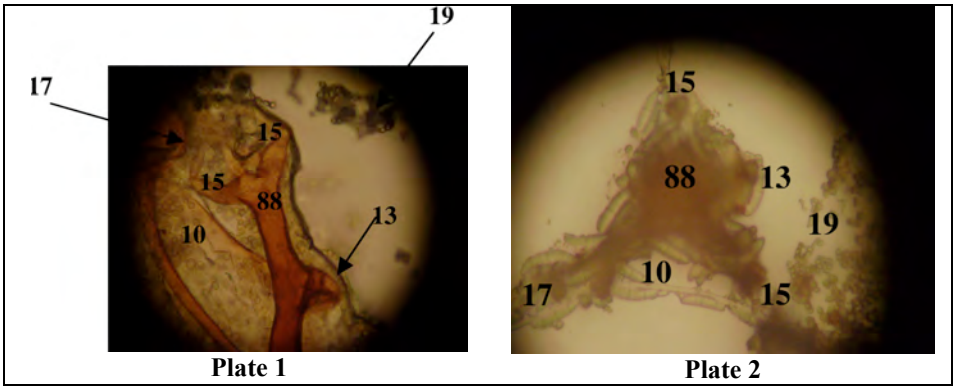

Plate 1 **Plate 2**

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 Basidiomycotina, Order Agaricales and family Cortinariaceae. *Inocybe petiginosa* is 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 proxima* is found in the Class Basidiomycotina, Order Agaricales and the family Tricholomataceae and it is commonly associated with *Pinus sylvestris*, *Picea sitchensis* and *Betula pendula*.

Glomus sp,* the VAM associated with *D. melanoxylon

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

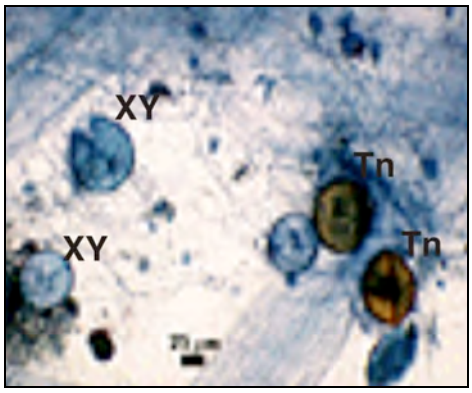

Plate 3:

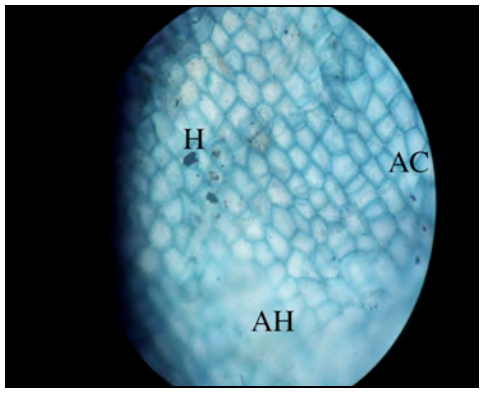

Plate 4:

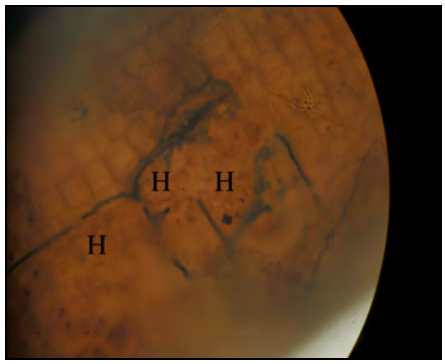

Plate 5:

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, spores and other information found confirmed for *Glomus versiforme* (http://invam.caf.wvu.edu/fungi/taxonomy/species.ID.htm) and in the reference accession IT104 in accordance with (Daniels and Trappe 1979). *Glomus versiforme* is found in the Phylum Glomeromycota, Class Glomeremycetes; Order Glomerales, Family Glomaceae and Genus *Glomus* (Sedaghati 2002) and is frequently found in association with sugar cane, grape and some grains such as wheat, barley, maize and sorghum (Sedaghati 2002

DISCUSSION

Like other woody and tropical trees which were found associated with mycorrhizal fungi (Ingleby *et al* 1990; Kanyagha 2008), mycorrhiza fungi has been found in *D. melanoxylon* for the first time in this study where it was confirmed that their presence was responsible for keeping *D. melanoxylon* adaptive to a wide range of environments including natural regeneration through saplings from root suckers. The high environmental adaptive ability of *D. melanoxylon* seem to be contributed by its association with the 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 (Borowics 2001).

Inocybe petiginosa and *Laccaria proxima* have been reported to have a broad host range and an extensive geographical distribution worldwide (Mason *et al*. 1987). This is in agreement with the current findings since the two ectomycorrhiza species were identified in Kilwa, Kilosa and Babati which are found in different ecological zones of Tanzania. The efficiency of the two ectomycorrhiza species in creating functional symbiotic relation to the host plants is indicated by the adaptive ability and abiotic stress tolerance of *D. melanoxylon*. Sexual reproduction which maintains availability of their spores ensures their continuous presence in the soil and hence a sustained symbiotic relation throughout the life of the host plant.

Glomus exists in the environment both as spore and hyphae which can form dense network called mycelia, though most of *Glomus* biomass occurs within the 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 plants growth (Modjo *et al* 1986) so inoculating the media with *Glomus* during propagation of *D. melanoxylon* as obtained from the results of this study may speed 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 the improved moisture and nutrients acquisition. Possibly inoculating *Glomus* in the nursery and transplanted *D. melanoxylon* may increase the adaptive ability of the species for successful propagation. *Glomus* is an obligate biotroph, meaning that it requires a living photoautotropic host to complete its life cycle and produce the next generation of spores. *Glomus* species are also entirely asexual (Modjo *et al*. 1986). *Glomus* begins as a spore produced outside or inside the host root, are able to germinate without a host plant. If the spore is not already in the root, a germination tube is formed which grows through the soil until it finds a host root. *Glomus* penetrates the roots and grows between root cells or penetrates the cell wall and grows within root cells to form arbuscules. The arbuscules are tree-shaped subcellular structures that form and connect plants to the hyphal network of the fungi (Modjo *et al* 1986, Sylvia 2000, Kanyagha 2008). Arbuscules are the main site for nutrient exchange between *Glomus* and its symbiotic plant partner. Soil may contain over 100 meters of hyphae per cubic centimeter (Sylvia 2000). This network of hyphae is designed to increase the plants uptake of important nutrients such as phosphates and water. In exchange for nutrients and water, plants supply *Glomus* with carbohydrates it needs to survive (Sylvia 2000).

Host specificity in VAM has led to link a positive correlation in VAM diversity and plant diversity. For example VAM have been reported to be associated with Rose family plants, Ericales plant and most of tropical trees (Munyanziza *et al*. 1994).

The two ectomycorrhiza species *Inocybe petiginosa* and *Laccaria proxima* 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 (Sylvia 2000) hence probably they are related to the high adaptability of *D.melanoxylon* in a wide range of habitat including the poor soil environments like those in Kilwa, Babati and Kilosa. The EMF spores found in the soil rhizosphere are also known to forms hyphae network connection to the host plant (Sylvia 2000). This may also on the other hand enable a directional flow of materials from the fungi to *D. melanoxylon* roots and vice-versa which contributes to its success.

CONCLUSION AND RECOMMENDATIONS

It is now evident that*Dalbergia melanoxylon* form mycorrhizal association and the presence of mycorrhiza fungi in roots and soil rhizosphere of the species are responsible for the relatively higher adaptive ability of *D. melanoxylon* to a wide range of habitat. Propagation of *D. melanoxylon* in new areas using the two ectomycorrhiza species and the *Glomus* species can possibly be accomplished by introducing the fungus in the new areas using soil rhizosphere around *D. melanoxylon* roots mixed with *D. melanoxylon* root fragments to improve natural regeneration. However the proper time for introducing mycorrhiza in new *D. melanoxylon* plantations is another area which needs investigation. Mycorrhiza could possibly also be used to acclimatize *D. melanoxylon* propagules from micropropagation using an appropriate protocol, again this needs further applied research.

It is recommended to future researchers to investigate the mycorrhiza population and the genetic diversity of *Glomus*, *Inocybe* and *Laccaria* in *D. melanoxylon,* their multiplication and integration into the soil media, proper inoculums type and the appropriate time of inoculation for *D. melanoxylon* seedling growth rate improvement.

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