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Effect of Inoculation with Mycorrhizae on Growth Parameters of Dombeya torrida, Leucaena leucocephala and Tephrosia vogelii

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Abstract
The present work evaluated the impact of arbuscular mycorrhizal (AM) fungus on growth. Three species of trees, *Tephrosia vogelii*, *Leucaena leucocephala* and *Dombeya torrida* were treated with fragments of arbuscular mycorrhizae and grown in greenhouse. The aim of the present work was to determine whether arbuscular mycorrhizal symbiotic association with these tree species would enhance their growth. Data on height, stem diameter and number of leaves were taken after every two weeks for a period of three months. Mycorrhizal plants demonstrated higher vigor with *Tephrosia vogelii* giving a mean height of 57.50 cm, 0.50 cm diameter and 9.33 number of leaves which were significantly different from the control plants at $p \leq 0.05$ according to least significant difference (LSD). The biomass was also taken at the end of 90 days of growth and the same trend was exhibited. Mycorrhizal plants also showed root nodules with mycorrhizal arbuscles. This work contributes to our understanding of the effects of AM symbiosis on the accumulation of certain micronutrients in valuable woody tree species.
Keywords: arbuscular mycorrhizae, *Tephrosia vogelii*, *Leucaena leucocephala*, plant growth

1. Introduction

Mycorrhizal fungi are naturally occurring organisms that occur within the roots of most plants forming symbiotic relationships with nearly all plants. The fungi is not a pest or parasite but it forms a non-pathogenic mutualistic symbiotic association with soil bound fungi and the roots of higher plants. It obtains organic nutrition chief among them carbohydrates, amino acids, vitamins and plant growth substances from plants. This also provides a perfect ecological niche that is necessary for fungal growth and development including the completion of the sexual cycle (Sandeep *et al*., 2015). These symbiotic associations of arbuscular mycorrhizae and plant roots are widespread in the natural environment and improves the survival and growth of most plants in natural communities (Lipnicki, 2015). They provide a wide range of benefits to the plants. These include improved nutrition by improving the uptake of nutrients such as phosphorus and nitrogen as well as improved tolerance to environmental extremes of the deficiencies of these elements (Mahanty *et al*., 2016), enhanced resistance to soil-borne pests and disease and their negative effects, improved resistance to drought by supplying water to the host (Al-Karaki *et al*., 2004; Chitarra *et al*., 2016), tolerance of low pH and heavy metals (Emamverdian *et al*., 2015) and formation of better soil structures and also reduce the soil pollution. In return, up to 20% of plant-fixed carbon in the form of sugars is transferred to the fungus. This symbiotic relationship does not affect the plants, as they produce excess carbon. In fact, lack of water and nutrients is more often the limiting factor to plants' growth and establishment. Uptake of trace elements, such as zinc, copper, boron, and molybdenum, is also thought to be enhanced by mycorrhizae (Ibiang *et al*., 2017). They function as a collection system for their host plants, accessing moisture and nutrients up to 50 times more efficiently than non mycorrhizal plants. Nutrient transport occurs through symbiotic structures inside plant root cells known as arbuscles. Arbuscles are branched hyphae, found within the root cells and this is where nutrient exchange takes place between fungi and the host plant (Afzal *et al.,* 2011; Oruru and Njeru, 2016). As roots develop, a condition for inoculation by AM fungus improves and the carbohydrates are used by AM fungus for growth (extension of the hypha). Arbuscular mycorrhizae is an unexploited potential biofertilizer in forest nurseries which can be utilized for quality tree seedling production.

Root colonization by AM fungus is a unique area that has justified the potential of it as bioprotectant and as biofertilizer (Berruti *et al*., 2015) providing protection to plants from parasitic fungi and nematodes and also increase plant growth and yield (Wei *et al*., 2016). These mycorrhizal fungi may increase plant tolerance to biotic and abiotic stresses (Abdelmoneim *et al*., 2014). One of the unique characteristics of AM fungi is to significantly increase the surface area of coverage. This is as a result of production of extensive hyphae which enable plants grow under relatively harsh conditions, such as drought stress (Chitarra *et al*., 2016) and nutrient deficiency (Mahanty *et al*., 2016).

Mycorrhizae can be divided into three categories as follows.

1.1 Ectomycorrhizae

This category of mycorrhizae is very uniform in appearance, and biologically identical despite having literally thousands of different species fungi, in the Ascomycota and Basidiomycota. It is referred to as ecto- because the fungal symbiont does not invade the cell protoplasm. However, the fungus does form a thick sheath around the

root tip and mycelium also grows between the cells of the cortex forming the so-called Hartig net. The infected roots are very distinctive, forming short, paired, branches (Glassman *et al*., 2015).

In this type of mycorrhizae, the fungal sheath that forms around the secondary root tips accumulate minerals from the decomposing litter, before they are able to pass into the deeper mineral layers of the soil where they would be unavailable to the roots. Thus, these mycorrhizal fungi are also decomposers as well (Averill *et al*., 2014). The fungus obtains simple carbohydrates that are produced by the plant but which the plant does not use. So it appears that these carbohydrates may be produced by the plant specifically for the fungus since they are not utilized by the plant.

Although we can grow the mycelium of many ecto-mycorrhizae fungi in an artificial medium, e.g. agar, they often grow slowly and not as well as in soil. It has been demonstrated that unknown growth factors exuded by the roots seems to stimulate mycelial growth. There is undoubtedly many more factors involved, with regards to growth of the fungi, that are yet unknown. Formation of fruiting bodies in artificial media also has never been accomplished. This was the reason why cultivation of truffles, e.g. *Tuber melanosporum*, which form mycorrhizae, requires planting of the host trees that have been inoculated with the fungus in order to obtain fruit bodies (Cram and Dumroese, 2012).

1.2 Endomycorrhizae

Although far less conspicuous because they do not produce large fruiting bodies, such as mushrooms, this category of mycorrhizae is far more common than the ectomycorrhizal type. Generally, it can be said that plants that do not form ecto-mycorrhizae will be the ones that form endomycorrhizae. However, because of the absence of a macroscopic fruit bodies, the presence of endomycorrhizae is more difficult to demonstrate. Because of the lack of visibility, this group was considered to be rare until a method was devised that could readily detect such fungi in the soil and demonstrate that they are in fact very common (Talbi *et al*., 2014).

There are several categories of endomycorrhizae. The only common feature that they all share is that the mycelium of the fungal symbiont will gain entry into the host, root cells by cellulolytic enzymes. Unlike the ectomycorrhizae, roots which are infected with mycorrhizal fungi do not differ morphologically from those that are not infected, i.e. root hairs are present and sheath is not formed around the root tip. However, the type of association that is formed between the host and fungus vary a great deal in the different categories of endomycorrhizae (Chafi *et al*., 2012).

1.3 Arbuscular Mycorrhizae

This category of mycorrhiza can be found throughout the world, but more abundant in the tropics than in temperate regions, and is associated with more plants than any of the other categories of mycorrhizae. The name of this type of mycorrhizae comes from the distinct structures called arbuscles that can be seen inside the cells of infected roots. These structures can be recognized by their branched tree-like appearance. Another structure that can be frequently observed are the rounded vesicles. The vesicles and arbuscles contain the stored minerals that are needed by the plant. These structures lyse in the root cells and in this way the minerals become available to the plant. There is also extensive mycelium in the soil, but do not appear to be organized in any fashion (Berruti *et al*., 2014).

1.4 Tree species

Tephrosia vogelii is native to tropical Africa and it belongs to the family Fabaceae with the common name being fish bean. It is a soft, woody branching herb or small tree with dense foliage, 0.5-4 m tall. It is a known nitrogen-fixing species, cultivated for green manure (Reddy, 2016). It is also planted as a windbreak and as a temporary shade crop. *Leucaena leucocephala* (Leguminosae) is a small, leguminous and native to tropical parts of Kenya, now widely distributed in other parts of the world thriving well only in alkaline soils, however, they fail in strong acidic soils. It is a value multipurpose legume used for forage, can provide firewood, timber, human food, green manure, shade and erosion control (Kuppusamy *et al*., 2014). *Dombeya torrida* is a deciduous shrub or much-branched forest tree 12-15 m tall, sometimes up to 25 m high with a shady umbrella crown and a trunk diameter of about 50 cm. it is also a high value tree species planted for timber, apiculture whereby it is recommended for planting to increase honey production. It is considered one of the best nectar producing trees so a good place to put bee-hives (Yoshimasa, 2014). Bees collect pollen and nectar throughout the day from its flowers. It is also used as fodder as the leaves are browsed by cattle. It has medicinal value as its root bark is used for wound dressing (Ndwigah, 2013). It is a fibre tree with the bark being used for making cloth and string or rope. Finally it can provide fuel as it is a good source of firewood and charcoal. These three tree species were selected with the aim of increasing the tree coverage. The objective of this study was to evaluate the effects of mycorrhizae on parameters i.e. height, girth and biomass of three plants; *Dombeya torrida*, *Leucaena leucocephala* and *Tephrosia vogelii*.

2. Materials and methods

This study was conducted at Egerton University, under glasshouse conditions.

2.1 Collection of Mycorrhizae and its multiplication

Permanent tree plots were established in Botanic Garden of Egerton University, and all trees ≥15 cm diameter at breast height were tagged, measured, and identified (Henkel 2003; McGuire 2008). Root samples were collected from three individuals of *Dombeya torrida* in the permanent plots by tracing lateral roots from the bases of adult trees and collecting fine roots occurring in the top 10 cm of soil. Roots were collected from the four cardinal directions of each selected tree. They were then transported on the same day to the laboratory for further processing. The root samples were surface sterilized with 70% ethanol and were rinsed in three changes of deionized distilled water. They were cut into small pieces approximately 1 cm length. These were placed on Potato Dextrose Medium (PDA) in Petri-plates and incubated for 7 days at 25 ± 2 ^{o} C. Fungal mycelia appearing on the root pieces were sub-cultured on fresh PDA medium until a pure culture was obtained. Identification was done using micro and macro characteristics.

2.2 Collection of seeds

The seeds of *Dombeya torrida, Tephrosia vogelii* and *Leucinae leucocephala* were collected from the forest in Botanic Garden of Egerton University. Twenty four mature trees of these species were tagged with unique numbers. There were eight trees for each species. Small pieces of papers were cut and labelled with the unique numbers tagged on the trees. These papers were folded properly and put in a beaker and mixed thoroughly and three pieces for each species were randomly picked. It was from these trees that the seeds were collected. There were 90 seeds in total with 30 seeds for each species. The seeds were dried properly under shade to the appropriate moisture.

2.3 Experiment 1

Dombeya torrida, Tephrosia and *Leucinae* seeds were treated with the mycorrhizae mycelia and planted in the pots containing the sandy soil. The growth medium, the sandy soil used was sieved through a 2 mm mesh and autoc1aved for 40 minutes at 121 °C to kill native mycorrhizal endophytes. Pots with seeds without mycorrhizae were used as control. The planting pots were sterilized using 70% ethanol. There were three replicates for each seed and a completely randomized design was used. The pots were mounted on metallic benches 25-30 cm high in a greenhouse at ambient air conditions. Watering was done daily at field capacity early in the morning. To allow free draining several 2-3 mm holes were made on the base of each pot. Observations were made occasionally and data on the number of leaves, diameter and height were taken after every two weeks after germination. After a growth period of three months, the plants were harvested by flooding the pots with water to loosen the roots and ensure the roots are not fragmented. They were separated into leaves, stems and roots, washed in tap water and the flesh weight taken. The dry weight was determined after drying the materials at 80 °C in an oven for 24 hours.

2.4 Experiment 2

The growth medium was treated as in experiment 1 above. Soil collected around the roots of mature *Dombeya torrida* tree species together with root fragments containing mycorrhizae was added into pots in the ratio of 3:1 (i.e. 3 parts of sandy soil and 1 part of the mycorrhizal soil). Arbuscular mycorrhizal fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Dombeya torrida*) containing spores, hyphae and mycorrhizal root fragments. *Dombeya torrida, Tephrosia vogelii* and *Leucinae leucocephala* seeds were planted in the pots. Pots with sandy soil only were used as control. There were three replicates for each seed and a control. The pots were put in the green house and watered on daily basis. After three months, the plants were harvested and fresh and dry weight taken as mentioned above.

2.5 Presence of mycorrhizae in the experimental plants

After harvesting the plants, roots were separated and cleaned of soil and organic matter with water and placed in formalin acetic acid the same day for later analysis. Roots were cleared with 10% KOH and alkaline H_2O_2 and subsequently stained with trypan blue according to the protocol of Giovannetti and Mosse (1980). The presence / absence of AM structures was noted for each species. In the green house, the mycorrhizae structures were identified by the presence of a mantle, visible with the naked eye. Cross- sections in the laboratory were made to confirm the presence of a Hartig net. Positive AM colonization was recorded only if arbuscles and/or hyphal coils were observed. Approximately 10 cm of root segments were examined for each tree species.

2.6 Statistical analysis

All data were analyzed by ANOVA. Means were compared based on least significant differences ($p \leq 0.05$).

3. Results

3.1 Shoot and root growth

All the seeds except those of *Dombeya torrida* had a 100% germination. None of the *Dombeya torrida* seeds germinated and probably the seeds were not mature enough when they were collected or they were probably infested with pathogens. The treatments had a significant effect on the biomass of leaves, stems and roots of *Tephrosia vogelii* and *Leucaena leucocephala* (Table 1) whereas no significant differences were observed in plants in the experiment 1 and non-mycorrhizal (control) plants. The treated plants did not show any signs of leaf spots and rusts unlike the control plants and those in experiment 1 (Plate 1). Experiment 2 plants showed root nodules where the mycorrhizae were contained for both species. The control plants did not form the nodules (Plate 3). The plants treated with the soil that contained mycorrhizal fragments attained the highest height, diameter and number of leaves in comparison with the control (Table 2) and there was a trend to increase in the volume of roots.

Table 1: Effects of AM fungi inoculation on shoot and roots fresh weight (gm) of *Tephrosia* and *Leucaena* species after 90 days

Species	Treatment	Roots	Stems	Leaves
Tephrosia vogelii	Mycorrhizal plants	3.9a	1.43a	3.13a
	Non mycorrhizal (Control)	1.72b	0.6b	1.36b
Leucaena leucocephala	Mycorrhizal plants	1.36a	0.80a	0.91a
	Non mycorrhizal (Control)	0.59b	0.55b	0.44b

Values represent mean of six plants. Means in the columns for each plants species followed by same letter are not significantly different ($P \leq 0.05$) according to LSD mean separation.

Table 2: Plant height, Diameter and leaf number of *Tephrosia* and *Leucaena* species inoculated with mycorrhizal species and the control plants after 90 days of growth.

Species	Treatment	Height (cm)	Diameter (cm)	No. of Leaves
Tephrosia vogelii	Mycorrhizal plants	57.50a	0.50a	9.33a
	Non mycorrhizal (Control)	29.23b	0.28c	7.67b
Leucaena leucocephala	Mycorrhizal plants	16.35a	0.32a	10.67a
	Non mycorrhizal (Control)	12.80b	0.25c	8.5b

Letters followed by the same letter on the columns for each species are not significantly different $P \leq 0.05$) according to LSD mean separation.

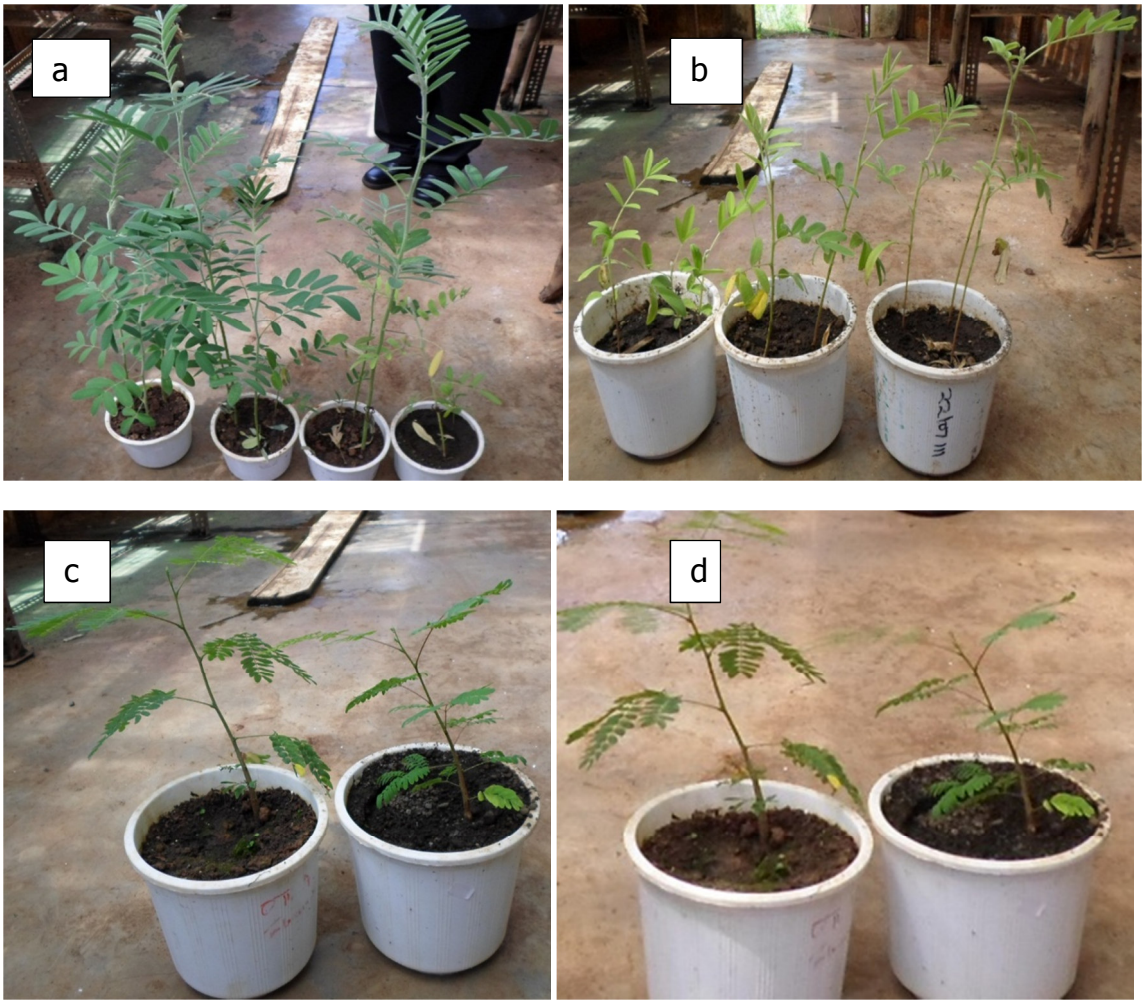

Plate 1 a *Tephrosia vogelii* with mycorrhizal fragments and control plant; b *Tephrosia vogelii* treated with mycorrhizal mycelia and control plants; c *Leucaena leucocephala* with mycorrhizal fragments and d control *Leucaena leucocephala*

3.2 Presence of mycorrhizae in the experimental plants

Mycorrhizae structures were found in plants of experiment 2 whereby the sandy soil was supplemented with soil collected around the rhizosphere of mature *Dombeya torrida*. Visible root nodules were present and especially on *Tephrosia vogelii*. However, no mycorrhizal structures were detected in the cleared and stained roots of plants in experiment 1 or control plants.

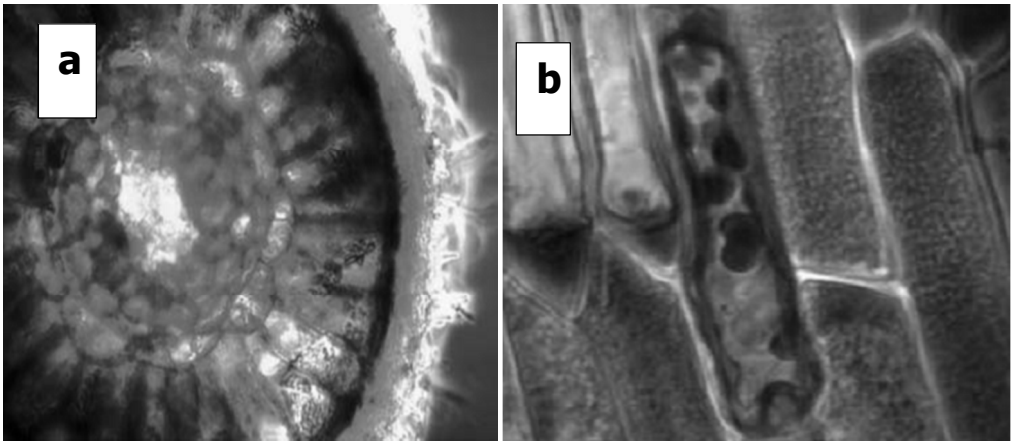

Plate 2 (a) Hartig net and (b) arbuscles in *Tephrosia vogelii* at 400× observed from the root nodules.

Plate 3: *Tephrosia vogelii* a treated plant with root nodules and b the control; *Leucaena leucocephala* d treated with nodules and c control plant.

4. Discussion

Mycorrhizal inoculation enhanced the shoot and root growth of *Tephrosia vogelii* and *Leucaena leucocephala* used in this study. The most effective in increasing the vigour was a multi-strain AM fungi inoculant obtained from the rhizosphere of mature *Dombeya torrida* tree species. The shoot and root biomass for both species in mycorrhizal plants increased more than the control plants. This is because the fungus hyphae formed in the roots were able to capture the simple nitrogen from the organic matter of the soil, and facilitate the access to the available macro-and micronutrients, stimulating plant growth (Bücking and Kafle, 2015).

Results of artificial inoculation vary greatly depending on the host, species or strain of mycorrhizal fungi, and environmental conditions of the seedlings. The mycelial that were isolated and inoculated on the seeds did not perform better than the multi-strains present in the rhizosphere soil obtained from *Dombeya torrida* tree species. This demonstrates the high performance of many mycorrhizal species acting synergistically as shown in our results. The complexity of interactions between mycorrhizal associations and environmental conditions requires careful testing of a particular mycorrhizal inoculant to ensure a positive benefit to cost ratio before

operational use. This was probably due to lack of fruiting bodies in the mycelia of the mycorrhizae grown in the artificial media as arbuscular mycorrhizae can only form the fruiting bodies when grown in association with the host (Bzdyk *et al*., 2016).

Arbuscular mycorrhizae are well known to improve the plant growth and the general health (Auge, 2001). The mycorrhizae improves resistance to plant for various stress factors and facilitates relationship between the mycorrhizal symbiont and the plant, to ensure that it will be highly responsive to management practices (AgbeNiN, 2011). Our results demonstrated that AM fungus colonized plants are less infected by pathogens and show lower disease incidence than the non-mycorrhizal plants (Dilbo *et al*., 2015). Several reported evidence of AM fungus inoculation as a means of biological control against soil-borne diseases (Harrier and Watson, 2004; Idoia *et al*., 2004) but only few authors have reported the role of AM fungus against shoot or stem diseases (Vestberg *et al*., 1994). Arbuscular mycorrhizae fungus established symbiosis with host plants, the host plants get benefited from this mutualistic relationship in terms of improved growth and reduced incidence of diseases (Krishna *et al*., 2005; Barrett *et al*., 2014). This could be attributed to better compensation for the damage caused by the pathogen (Nogales *et al*., 2009) through increased capacity for nutrient uptake by the AM fungus and plant association, which may allow host plants to be more vigorous, and consequently more resistant or tolerant of pathogen attacks (Azcón-Aguilar *et al*., 2002).

Arbuscular mycorrhizal fungi are influenced by the properties of soils and in turn they themselves have an impact on soil quality (Barrett *et al*., 2009). The major contribution of AM fungus in the soil is to aggregate the particles together (Rillig and Mummey, 2006) as they produce the glycoprotein glomalin (Wright Upadhyaya, 1998) which has a long lasting stability in the soil (Rillig *et al*., 2001) and acts as glue for soil particles. The fungal hyphae also help to improve the soil particles. The AM fungus mycelium delivers carbon rich compounds and other bioactive signals further away from the root, thereby stimulating microbial activity in more remote sites (Barto *et al*., 2012). This explains why the mycorrhizal plants performed better than the control.

5. Conclusion

The conclusion from this work is that mycorrhizal inoculation in trees production may represent a useful tool to overcome both inhibition of apical activity and stunted growth of plantlets and to reduce chemical inputs during tree production.

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