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Influence of Arbuscular Mycorrhizal Fungi on Rooting Ability of Auxin Treated Stem Cuttings of *Dalbergia melanoxylon* (Guill and Perr.)

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ABSTRACT

In efforts to optimize vegetative propagation of *Dalbergia melanoxylon,* the effects of inoculation of Arbuscular Mycorrhizal Fungi (AMF) on rooting ability of *D. melanoxylon* stem cuttings treated with auxin Indole-3-Butyric Acid (IBA) were investigated. Three inoculum levels of AMF namely control, 1:1 and 1:2 (v/v) ratio of inoculum to propagation substrate, respectively and two levels of cutting types designated as middle and basal cutting positions were used. Rooting parameters in terms of rooting percentage, root number, root length and root dry weight were evaluated. Analysis of variance revealed that the effect of AMF inoculation was significant ($p<0.001$) only for percent rooting and root dry weight among the rooting parameters evaluated. The effect of cutting type and the interactive effect of inoculation and cutting type were insignificant for all studied rooting parameters. Comparisons of means by Duncan's Multiple Range Test (DMRT) for the different treatments revealed that the quantity of inoculum significantly ($p<0.05$) influenced high performance in all rooting parameters evaluated. The highest percentages in rooting was (67.28%) for the cuttings from basal position. The study has revealed a strong AMF influence on rooting ability of auxin treated stem cuttings in *D. melanoxylon*. It was concluded that for vegetative propagation, AMF inoculum should be used for auxin treated cuttings to produce high quality planting stock material for the afforestation and conservation programme for *D. melanoxylon*.

Key words: Arbuscular mycorrhizal fungi, auxin, *Dalbergia melanoxylon*, inoculation, vegetative propagation

INTRODUCTION

Arbuscular Mycorrhizal Fungi (AMF) are the largely prevalent type of ecologically vital root fungal symbionts in higher plant species, being essential for sustainable growth and development and the survival of several land plants (Lone *et al*., 2015). The AMF are very important for improvement of soil quality through their beneficial effects on host plant physiology and for the soil ecology interactions, thus improving soil structure and stability (Hallett *et al*., 2009; Husna *et al*., 2015). Other useful effects of arbuscular mycorrhizal fungi symbiotic association have been reported on the growth of plants through enhanced uptake of macro and micronutrient, improved plant resistance against biotic and abiotic stress and valuable alternations of beneficial plant growth regulators (Watts-Williams *et al*., 2014). *Dalbergia melanoxylon* (Guill. and perr.) known as African Blackwood which belongs to the family Leguminosae is a small, heavily branched deciduous tree. African Blackwood presently exploited for international trade is the most precious heartwood in the world and one of the world's most expensive timbers (Ball, 2004). Valuable products derived from *D. melanoxylon* coupled with high exploitation pressure to its natural populations have already reached the point of threatening its natural habitats (Jenkins *et al*., 2002; Ball, 2004). Population genetic diversity reported its provenances are also threatened due to the lack of proper conservation measures (Amri *et al*., 2009a). Presently *D. melanoxylon* is categorized as Lower Risk/near threatened (LR/nt) species on the red list of threatened species on the International Union for Conservation of Nature (IUCN) (World Conservation Monitoring Centre, 1998). In spite of its economic importance, natural regeneration of *D. melanoxylon* through seed germination is limited, some most recent efforts to optimize its regeneration through vegetative propagation have been reported (Amri *et al*., 2009b, 2010; Washa *et al*., 2012). However, the interactive effects of arbuscular micorrhizal fungi and auxin like Indole-3-Butyric Acid (IBA) on the rooting ability of stem cuttings have not yet been reported in *D. melanoxylon*.

Inoculation with mycorrhizal fungi on IBA treated stem cuttings can improve rooting ability and quality of stem cuttings with advantageous effect of facilitating seedlings to survive the transplantation shock at large number (Fatemeh and Zaynab, 2014; Yang *et al*., 2014). This study was carried out to establish the influence of arbuscular mycorrhizal fungi on increasing rooting ability of IBA treated stem cuttings of *D. melanoxylon*.

MATERIALS AND METHODS

Spore sampling, identification and inocula preparation: Roots and rhizospheric soil samples *of D. melanoxylon* were collected in triplicate of 100 g of dry substrate for isolation of arbuscular mycorrhizal fungi from an undisturbed sites for *D. melanoxylon* at Ubena 06°36'S latitude, 38°09'E longitude and Mkundi 06°40'S latitude 37°39'E longitude at an altitude of 350 and 475 m, respectively. Selection of sites was based on previously reported high genetic diversity for these populations (Amri *et al*., 2009a). Roots were traced from stem base until they led to ultimately fine roots of individual trees separated by the distance of 30 m. The separation of AMF spores was carried out by wet sieving and decanting method (Gerdemann and Nicholson, 1963). Morphological characters in terms of shape, size, ornamentation, color and number of wall layers and presence or absence of subtending hyphae were compared with descriptions of fungal genera (INVAM, 1998) http://www.invam.caf.wvu.edu/fungi/taxonomy/classification.htm.

To obtain the inoculum, identified spores of dominant AMF genera were multiplied in steam sterilized soil:sand (1:1 v/v) mixture as substrate in 20 cm diameter pots planted with 40-50 seeds per pot of pearl millet (*Pennisetum glaucum*) as a host plant. Cultures were grown in a greenhouse for 3 months to increase the inocula. Plants were watered as required. Inocular produced consisted of rhizospheric soil containing AMF spores, hyphae and mycorrhizal root fragments which were then used for stem cuttings inoculation.

Stem cuttings collection and preparation: Fresh stem cuttings of *D. melanoxylon* were collected from the same site locations for populations of *D. melanoxylon* where roots and rhizospheric soil samples were collected. Cuttings were taken from shoots of mature plants then enclosed in polythene bags, held on ice and transported to the Department of Science and Laboratory Technology, ready for propagation in a non-mist plant propagators. The influence of cutting type from the stock shoot was investigated. In preparation of stem cuttings from the shoots, three cuttings positions were assigned starting at node 4 from the apex of the shoots as apical, followed by middle and basal positions sequentially down the shoots of donor plant. Cuttings from apical position were not used in rooting experiments, only middle and basal positions cuttings were used as they are considerable woody tissue containing abundant carbohydrate reserves. Stem cuttings were cut having a length of 10 cm and diameter range of 0.8-1.4 cm.

Experimental design, inoculation and propagation: The experiment was set up in 3×2 factorial design representing three inoculum levels and two levels of cuttings types. Three inoculum levels used were control, 1× and 2× treatments of Arbuscular Mycorrhizal Fungi (AMF) where 1× and 2× created by 1:1, 1:2 (v/v) ratio of inoculum to propagation substrate, respectively. Two levels of cuttings types used were the middle and basal positions from the stock shoots. The experiment was arranged in a randomized block design each treatment level contained 20 cuttings with three replications such that a total of 360 cuttings were used for the whole experiment. Non inoculated stem cuttings served as controls. All stem cuttings were treated with auxin Indole-3-Butyric Acid (IBA) at 300 ppm prior to inoculation based on earlier findings by Amri (2002) on trial for determination of optimal IBA concentration. Steam sterilized sand was used as rooting substrate placed in a non-mist propagator as described by Leakey *et al.* (1990). Humidity in the propagators was maintained at 88±2% and maximum and minimum day-night temperature at 31±1 to 25±1°C, respectively. Whenever the propagator was opened for inspection, mist spraying was applied to raise the relative humidity inside the propagator.

Observations and evaluation of rooting and sprouting parameters: Observations on rooting experiments were made twice a week and whenever the propagator was opened cuttings were sprayed with fine jet of water to maintain humidity. Data collections were in terms rooting and sprouting parameters. Rooting parameters evaluated were rooting percentage, root number, length of the longest root and root dry weight per cutting. A cutting was considered rooted if it has at least one primary root >1 mm long. Sprouting parameters were also evaluated in terms percentage cuttings sprouts, percentage cuttings survived, shoot number and shoot height per cutting. Evaluation of both rooting and sprouting parameters were made after 60 days from the start of the experiment in the non-mist plant propagator. For measurements of root dry weight, roots were washed, removed sand and oven dried at 70°C for 72 h and weighed.

Data analysis: Data analysis was done using GenStat Discovery Edition 4 Release computer software package. Analysis of variance (ANOVA) procedures were used to test for significant effect of treatments, followed by Duncan's Multiple Range Test (DMRT) for comparisons of means of different treatments. Correlation coefficients (Pearson) were also determined in order to know the strength of linear relationship among rooting and sprouting parameters as dependent variables. Before analysis in order to improve assumptions of normality, data in terms of percentages were converted by arc-sine transformation, whereas in terms of numbers were converted by square root transformation.

RESULTS

The dominant AMF genera identified from roots and rhizospheric soil sample were the *Glomus* sp. The identification in terms of features, spores and other information was confirmed for *Glomus versiforme* (http://invam.caf.wvu.edu/fungi/taxonomy/species.ID.htm). Analysis of variance for the rooting parameters evaluated revealed that the effect of AMF inoculation was significant ($p<0.001$) only for percent rooting and root dry weight while for other rooting parameters namely root number and root length was insignificant (Table 1). The effect of cutting

Table 1: Analysis of variance for the effect of arbuscular mycorrhizal fungi inoculation and cutting types for evaluated rooting parameters of *Dalbergia melanoxylon*

		Mean squares			
Sources of variation	df	Rooted cuttings (%)	Root No. cutting	Root length (cm)	Root dry weight (g)×10^{-4}
Replications	2	138.89^{ns}	1.09^{ns}	0.44^{ns}	0.38^{ns}
Inoculation	2	1293.06*	4.09^{ns}	3.42^{ns}	18.39*
Cutting position	1	272.22^{ns}	1.94^{ns}	0.05^{ns}	0.55^{ns}
Cutting position×inoculation	2	18.06^{ns}	0.35^{ns}	0.15^{ns}	1.72^{ns}

^{ns}: Not significant, *Significant at p<0.001, df: Degree of freedom, Rep: Replicate blocks

Table 2: Analysis by DMRT for the effect of arbuscular mycorrhizal fungi inoculation and cutting type for evaluated rooting parameters of *Dalbergia melanoxylon*

	Rooting parameters				
Arbuscular mycorrhizal fungi inocula	Cutting types	Rooted cuttings (%)	Roots No. per cutting	Root length (cm)	Root dry weight(g)×10^{-3}
Control	Middle	36.76±1.32^{a}	5.08±0.32^{a}	5.21±0.37^{a}	5.02±0.34^{a}
	Basal	38.35±1.24^{a}	5.11±0.44^{a}	5.43±0.15^{a}	5.67±0.36^{a}
1×	Middle	46.73±1.22^{ab}	5.47±0.13^{a}	6.03±0.16^{a}	6.33±0.37^{a}
	Basal	58.34±2.22^{c}	6.40±0.84^{ab}	6.23±0.54^{a}	7.67±0.58^{ab}
2×	Middle	61.71±1.32^{c}	6.23±0.45^{ab}	6.38±0.14^{a}	8.66±0.82^{ab}
	Basal	67.28±2.41^{c}	7.17±0.64^{ab}	6.63±0.60^{ab}	9.02±0.71^{b}

Means indicated by the same letter on the column are not statistically different at p<0.05, DMRT: Duncan's multiple range test

type from the stock shoot and the interactive effect of AMF inoculation and cutting type were all insignificant for rooting percentage, root number, length of the longest root and root dry weight (Table 1).

Means separation by Duncan's Multiple Range Test (DMRT) for the effect of AMF inoculation and cutting type was significant ($p<0.05$) for rooting percentage, root number and root dry weight. The DMRT revealed that the effect of AMF inoculation and cutting type was insignificant for root length (Table 2). The highest rooting percentage achieved was 67.28±2.41% for the basal cutting position followed by middle cutting position which had 61.71±1.32% for all treated with doubled quantity of inoculums while lowest rooting percentage was 36.76±1.32% for non-inoculated (control) cuttings from middle cutting position (Table 2). Basal cutting position treated with doubled (2×) quantity of inoculum had highest root number (7.17±0.64) and the lowest root number was in the control for middle cutting position (5.08±0.32). The highest root dry weight was ($9.02\pm0.71\times10^{-3}$ g) for basal cutting position treated with doubled quantity of inoculums followed by the value ($8.66\pm0.82\times10^{-3}$g) for middle cutting position treated with the doubled inoculums. The lowest root dry weight was $5.02\pm0.34\times10^{-3}$ g for the control middle cutting position (Table 2). In general, the combination use of AMF inoculation and IBA treated stem cuttings in enhancing rooting ability of *D. melanoxylon* was more pronounced in terms of high rooting percentage, number of primary roots and root dry weight than the control (the use of IBA alone).

Results for the correlation analysis of rooting and sprouting parameters (Table 3), revealed that all rooting parameters; rooting percentage, number of roots, root length and root dry weight were positively and significantly correlated ($p<0.01$). Also high and significant positive correlations were observed between rooting parameters and percentage of cuttings that survived (rooted and health unrooted cuttings) at the end of experiment. Low significant positive correlation ($p<0.05$) was observed between shoot height and rooting parameters (Table 3). No significant correlations were observed between shoot number and most of rooting parameters. Low significant negative correlations ($p<0.05$) were observed between rooting parameters and number of sprouted shoots per cutting (Table 3). The number of shoots depended on the number of nodes per cutting.

Table 3: Correlation coefficients of rooting and sprouting parameters for *Dalbergia melanoxylon* stem cuttings

Correlation parameters	Rooted (%)	Root No.	Root length (cm)	Root weight (g)	Callused (%)	Sprouted (%)	Shoot No.	Shoots height (cm)	Survived (%)
Rooted (%)	-								
Root number	0.67 **	-							
Root length	0.62 **	0.84**	-						
Root weight (g)	0.48**	0.87**	0.82**	-					
Callused (%)	0.43*	0.39*	0.16*	0.33*	-				
Sprouted (%)	-0.18 *	-0.34 *	-0.19*	-0.29 *	-0.35 *	-			
Shoot number	0.02 ^{ns}	0.04^{ns}	-0.07 ^{ns}	0.06^{ns}	0.14 ^{ns}	0.39*	-		
Shoots height (cm)	0.09^{ns}	0.21*	0.39*	0.35*	0.37*	0.31*	0.06 ^{ns}	-	
Survived (%)	0.62**	0.49**	0.39*	0.21*	0.31*	-0.05 ^{ns}	-0.09 ^{ns}	0.04 ^{ns}	-

**Significant at p<0.01, *Significant at p<0.05, ^{ns}: Not significant

DISCUSSION

In this study, the combination use of auxin and AMF inoculation for vegetative propagation of *D. melanoxylon* stem cuttings has revealed improved rooting performance in rooting percentage and other rooting parameters. The findings of this study for vegetative propagation of *D. melanoxylon* through the interactive effect of AMF and IBA is contrary to the previous reports for for vegetative propagation through the use of auxin alone or the use of AMF alone (Amri *et al*., 2010; Washa *et al*., 2012). Although, high good rooting percentage has been reported for the use of AMF alone by Washa *et al.* (2012) for AMF inoculation of non-auxin treated stem cuttings of *D. melanoxylon* from juvenile donor plants, in this study vegetative propagation has been optimized through the use of other middle and basal cutting position treated with auxin (IBA) prior to AMF inoculation. Although these cutting positions from *D. melanoxylon* are considerable to be woody tissue with plentiful carbohydrate reserves needed for root initiation, yet have earlier been reported to be not easy to root and revealed a low rooting ability compared to the juvenile stem cutting (Amri *et al*., 2010).

The valuable effect of the applied IBA is to promote mobilization of carbohydrate reserve through activating energy for root initiation (Nanda and Anand, 1970). The use of IBA has revealed good results for vegetative propagation of other plant species (Kilkenny *et al*., 2012; Giri and Tamta, 2013; Kebede *et al*., 2013). Consequently the hard to root IBA treated mature stem cuttings can be considerably be enhanced with the high rooting ability when inoculated with AMF as revealed in this study where AMF inoculated and IBA treated cuttings had significant higher rooting performance than the non-inoculated stem cuttings. The levels of inoculums seem also to be very important for enhancing rooting ability. In this study high improved rooting performance was revealed for the cuttings with high level of AMF inoculums. It has previously been reported that high levels of AMF inoculums can promote optimal rooting of stem cutting of plant species (Scagel *et al*., 2003). Comparable studies for use of AMF on vegetative propagation through stem cutting have previously been reported to produce significantly higher rooting and growth parameters than untreated cuttings which acted as a control (Druege *et al*., 2006; Wang *et al*., 2008; Oseni *et al*., 2010). The advantages of root colonization by mycorrhizal fungi are considered to be greatest when colonization occurs in early stage of development during plant growth (Soliman *et al*., 2012). In propagation of plants from stem cuttings, this means that useful benefits from mycorrhizal colonization would be obtained if inoculum is applied during adventitious root formation (Yang *et al*., 2014).

The interaction of auxin and AMF is very important in root initiation of cuttings as compared to cuttings treated with IBA only or with inoculated non-IBA treated cuttings. Study by Fuscon (2014) has lately reported the interactive role of auxin play and arbuscular mycorrhizal fungi and their host during the pre-colonization phase as being important in the process of lateral root formation. In addition this also enhances changes in the release of carbohydrates to the roots and modulation of phytohormone concentration resulting in increased root system branching (Kilkenny *et al*., 2012; Schott *et al*., 2013). Apart from the application of synthetic auxin, natural plant auxin play essential role as signals during the establishment of AMF symbiosis particularly for the development of lateral roots which are the preferred infection sites for the fungi (Ludwig-Muller and Guther, 2007).

The findings from this study for interactive effects of IBA and AMF inoculation for increased rooting ability is in agreement with others studies (Nowak and Nowak, 2013; Fatemeh and Zaynab, 2014). Studies have previously portrayed that auxins, such as IBA assist the colonization of a host by increasing the number of lateral roots as preferred colonization sites for the fungi during early growth development (Jentschel *et al*., 2007; Fu *et al*., 2015). The inoculum used in this study was processed from rhizospheric soil instead of using commercially available arbuscular mycorrhizal fungi. Schwartz *et al*. (2006) argued against the use of commercially available AMF inoculum and recommend local inoculum production for reforestation of the tropical trees. The beneficial use locally available inoculums is that when inoculated rooted cuttings are transplanted into the field, they can easily establish within the pre-existing fungal community compared to the commercially available AMF which often have difficulty in establishing within the pre-existing fungal community (Allen *et al*., 2005). Furthermore, commercially available AMF may become fast spreading invaders out-competing the local fungal community as they are considered to be exotic fungi.

Inoculation with mycorrhizal fungi is thought to be an appropriate practice for producing high quality nursery stocks. Mycorrhizal fungi are important due to their highest ability to increase growth and establishment with good results in increasing efficiency in plant nutrients uptake from infertile soils, increase water uptake and increased drought resistance in plants (Van der Heijden *et al*., 2008; Soliman *et al*., 2012; Nadeem *et al*., 2014; Sarkar *et al*., 2015). The beneficial effect of inoculation at initial stage of plant development is that it can promote AMF symbiosis resulting to increase plant growth in the nursery and improving performance after planting in the field (Kung'u *et al*., 2008; Wang *et al*., 2008). Furthermore through the formation of symbiotic associations with plant roots, AMF provide a variety of ecological functions affecting plant flowering, with subsequent changes to the pollinator community and can be more productive under growing conditions where nutrients and water may be limiting productivity (Cahill *et al*., 2008; Chandrasekaran *et al*., 2014). Also AMF facilitates higher water absorption and nutrient uptake in plants, which in turn and helps to combat various diseases, increase torelance to abiotic stresses and enhances plant growth (Querejeta *et al*., 2003; Bisht *et al*., 2009; Bati *et al*., 2015).

In this study, rooting quality in terms of the number of roots, root length, root dry weight and rooting percentage were all positively correlated. High root numbers and root weights per rooted cuttings suggest well-developed root system which is a good indicator of field performance (Davis and Jacobs, 2005). It is well documented that AMF stimulates the biomass of the root system, which leads to high nutrient uptake in soils for plants (Fan *et al*., 2008; Verbruggen *et al*., 2013; Soliman *et al*., 2012; Sarkar *et al*., 2015). Transplanting stem cuttings stock with good root system may give better adaptation in the field and thus ultimately resulting in good performance, survival and growth. The importance of long root system is that it should allow the uptake of nutrients outside the initial exhaustion zone (Clark *et al*., 2000).

Negative associations amongst the sprouting and rooting parameters were observed in this study. Hartmman *et al.* (2002) have previously reported that when propagating trees by stem cuttings the negative correlation for rooting and sprouting parameters is attributed by the shoot acting as a strong opposing sink for carbohydrates. Though sprouting parameters may exhibit a important role in seedling growth and establishment, however, studies by Puri and Thompson (2003) have unveiled that shoot with emerging buds are not good indicator of root initiation in vegetative propagation, because an early differentiation and growth of leaf buds is dependent on carbohydrate assimilates available in the cuttings. Furthermore during early growth of rooted cutting, energy provision might be intensely influenced towards roots, therefore shoot growth might reduce root development because of competition for nutrient reserve between roots and shoots (Davis and Jacobs, 2005). Thus onset shoot might be considered as good sign of metabolic activity occurring within a cutting rather than being correlated to root appearance in stem cuttings during vegetative propagation.

CONCLUSION

This study has revealed that the combination use Arbuscular Mycorrhizal Fungi (AMF) and auxin Indole-3-Butyric Acid (IBA) in vegetative propagation of *D. melanoxylon* through stem cuttings can significantly increase rooting performance of middle and basal cutting positions which are considered as difficult to root mature stem cuttings. Also the beneficial use of mycorrhizal fungi may include enhancing best performance of rooted cuttings when transplanted in the field. Vegetative propagation by stem cuttings in *D. melanoxylon* may solve not only the problem of inadequate seed supply due to infrequent flowering, low seed viability, poor germination and slow growth of its seedlings rapid, but also will reduce time taken by the plant to reach maturity age. Propagation through stem cuttings will ensure conservation, availability and sustainable exploitation this economically important plant. Domestication of *D. melanoxylon* will not only decrease pressure due to over exploitation of this natural resource in the wild but also will be a means to preserve its genetic diversity.

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