Micropropagation of *Dalbergia melanoxylon* Guill. & Perr.: A Threatened Tree Species

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Abstract

Dalbergia melanoxylon is an economically and ecologically important timber yielding tree species. It is propagated through seeds, but is not successful because of poor seed germination that limits its multiplication. Therefore, the objective of this study was to develop efficient micropropagation protocol for this species. Cotyledonary nodes were used as explants. The highest (85.33%) explants initiated shoots on Murashige and Skoog (MS) medium containing 2.0 mg/l 6-benzyl amino purine (BAP) and 1.0 mg/l α-Naphthalene acetic acid (NAA) of the culture revealed greatest shoot multiplication (6.12 ± 0.13) from nodal segments. The shoots rooted best (92%) on MS medium containing 1.0 mg/l NAA with 5.89 ± 0.47 roots per microshoot. Plantlets were planted in pots containing sterilized forest soil and sand mixture (1:1) and acclimatized in greenhouse. After four weeks of acclimatization in greenhouse, 84.6 % of plants survived. This protocol could be useful for large-scale multiplication and conservation of this ecologically and economically important multipurpose tree.

***Key words*:** African Blackwood, *Dalbergia melanoxylon*, *In vitro* seedlings, Threatened species

{**Citation:** Francis Kiondo, Tileye Feyissa, Patrick A. Ndakidemi, Miccah Seth, Kusirieli Lema. Micropropagation of *Dalbergia melanoxylon* Guill. & Perr.: A threatened tree species. American Journal of Research Communication, 2014, 2(12): 43-52} www.usa-journals.com, ISSN: 2325-4076.

Introduction

African Blackwood is the common name for *Dalbergia melanoxylon* Guill. & Perr. which belongs to family Fabaceae, sub-family Papilionoideae. It is also known as African ironwood, African ebony, zebrawood or Mpingo in Swahili. Its timber has an international high demand and price due to its excellent qualities of having high strength and density (Malimbwi *et al.*, 2000). *D. melanoxylon* is resistant to insect attack and therefore valued for use in musical instruments, principally woodwinds and particularly clarinets (Arbonnier, 2004). A large amount of Tanzania' stocks of *D. melanoxylon* are found in open Miombo woodlands that covers about two thirds although is able to grow in a variety of conditions (Nshubemuki, 1994). It is a nitrogen fixing species that improves soil fertility (Högberg, 1986, Amri *et al.*, 2009). In order to meet the high market demand, many of the trees growing naturally have been haphazardly logged either legally or illegally resulting in a significant decline in its population (Boga *et al.*, 2012).

D. melanoxylon is generally raised from seeds with not only poor germinations but also has low natural regeneration which is contributed by its slow growth rate, low seed viability and do not retain their viability for more than one year (Mbuya *et al.*, 1994, Msanga, 1998, Amri *et al.*, 2009, Washa *et al.*, 2012). Due to over-exploitation and lack of vegetative propagation methods, this economically and ecologically valuable species has resulted in a decrease in size of its natural stand (Jenkins *et al.*, 2002) which ultimately has led to its inclusion in the list of International Union for Conservation of Nature (IUCN) Red List as Lower Risk/near threatened species (Schatz, 2009).

This tree species has fine grained dark-coloured heartwood that can reach densities of 1.1g cm^{-3} (Malimbwi *et al.*, 2000) which is resistant to insect attack and therefore valued for use in musical instruments, principally woodwinds and particularly clarinets but also wooden flutes, oboes and pipes (Arbonnier, 2004). Locally, in Tanzania this tree species is used for making woodcarvings (makonde) for tourists (Jenkins *et al.*, 2002).

Msanga (1998), Alkhalifa (2006) and (Washa *et al.*, 2012) made efforts to improve seed germination through various pre-sowing treatments but with little success. Since this vegetative propagation did not show improvement, therefore there was a need to use micropropagation

technique to ensure sustainable supply of the raw material and strengthen the economy of both the government and manufacturers of various products from the plant species.

This study was conducted to develop an *in vitro* propagation protocol for *D. melanoxylon* using seedling explants.

Materials and methods

The seeds of *D. melanoxylon* (Figure 1A.) were procured from the Tanzania Tree Seed Agency (TTSA). After unwrapping from the pods, the seeds were thoroughly washed under tap water and few drops of liquid detergent for 10 min and then rinsed three times with distilled water. This was followed by disinfection in 70% ethanol under sterile condition for 2 min prior to surface sterilization of the seeds with 20% (v/v) sodium hypochlorite containing 20 drops of Tween 20 per litre for 10 min with gentle shaking. Remaining of the sterilant on the seeds was removed by subsequently washing five times with sterile distilled water. The seeds were cultured on growth regulators-free MS (Murashige and Skoog, 1962) medium. The *in vitro* germinated seedlings were the source of explants in the following experiments.

Shoot initiation and multiplication

Initially, the sterilized seeds were cultured on growth regulator-free MS medium containing 3% (w/v) sucrose for germination. The pH of the medium was adjusted to 5.8 using 1N solution of NaOH before gelling with 0.3% (w/v) agar (Phytagel^{TM}) and autoclaved at 121°C for 20 min. The seeds were cultured on the culture medium in 25 x 150 mm glass culture tubes containing 15 ml semi-solid medium and covered with caps and parafilm. One seed was cultured per test tube to reduce the risk of contamination. All cultures were incubated in a growth room under photoperiod of 16 h at 50 µmol $m^{-2}s^{-1}$ light intensity provided by cool-white fluorescent tubes at 25±2 °C. Cotyledonary nodes and shoot tips obtained from *in vitro* derived seedlings were used as explants in this experiment.

To obtain optimum growth regulators concentration for optimal shoot multiplication medium, 1.0-1.5 cm long nodal segments from 4-week-old seedlings of *D. melanoxylon* were cultured on

the MS medium containing various concentrations of BAP alone or in combination with NAA or Indole-3-butyric acid (IBA). For multiple shoot proliferation, MS medium supplemented with BAP (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) and IBA (0.1, 0.3, 0.5, 0.7 and 1.0 mg/l) and NAA (0.1, 0.3, 0.5, 0.7 and 1.0 mg/l) were used (Table 1). Nodal explants from 30-day-old seedlings of *D.melanoxylon* were cultured on MS medium with different concentrations (0.0-2.5 mg/l) of BAP to induce multiple shoots. Multiple shoots obtained from previously established cultures were further subcultured in the multiplication medium. After 4 weeks of culture, number of shoots and length per explants were recorded.

In vitro rooting and acclimatization

For rooting, shoots with three or four expanded leaves from multiplication cultures were cultured on full-strength MS medium containing various concentrations of NAA (0.0, 0.1, 0.3, 0.5, 0.7 and 1.0 mg/l) or IBA (0.0, 0.1, 0.3, 0.5, 0.7 and 1.0 mg/l). Number of rooted microshoots, number of roots per shoot and lengths of the roots were recorded after six weeks of culture. When adequate rooted shoots were obtained, the plantlets were washed carefully in running tap water to remove the traces of agar and were transferred to pots containing sterilized forest soil and sand mixture (1:1) for 2 weeks. The pots were covered with transparent polythene bags to ensure high humidity and maintained in a growth room. The polythene bags were completely removed after two weeks of planting when new leaves started emerging. Initially, the plantlets were irrigated with water that contains polyfeed fertilizer (inorganic salts) as starter for growth and later with water containing finisher fertilizer. Successfully acclimatized plantlets under culture room conditions were then transferred to greenhouse.

Statistical analysis

In our study, results on the percentage of shoot initiation, number of shoots per explant and rooting are expressed as mean ± standard errors (SE) of the three independent experiments. Data were statistically analyzed using one-way analysis of variance (ANOVA) and the significant differences between means were assessed by Fisher's LSD test at the $P \leq 0.05$ level of probability. All statistical analyses were performed with STATISTICA 8 version program.

Results and discussion

The sterilized seeds of *D. melanoxylon* were germinated within 3 weeks of culture on growth regulators-free MS medium (Figure 1A; 1B). Single shoot (Figure 1C) was observed from each axil of the cotyledonary nodal explant when cultured on growth regulators-free MS medium. Related result of single shoot was reported on *Leucaena leucocephala* (Dhawan and Bhojwani, 1985) and *Sterculia urens* Roxb. (Hussain *et al.*, 2007).

Supplementation of BAP increased number of shoots per explants (Figure 1D). Multiple shoots were induced within 2-4 weeks of culture on shoot induction medium and the highest number of shoots per responsive explants (3.56 ± 0.19) was observed on MS medium supplemented with 1.5 mg/l of BAP (Table 1). However, increasing the concentration of BAP beyond the optimal level reduced number of shoots as well as average shoot length. Incorporation of BAP alone has been reported for efficient production of multiple shoots in some other plant species (Rathore *et al.*, 2008) on *Terminalia bellerica*, (Reddy *et al.*, 2012) on *Asclepias curassavica*, (Ndoye *et al.*, 2004) on *Balanites aegyptiaca* (L.) Del., (Gokhale and Bansal, 2009) on *Oroxylum indicum*, (N'doye *et al.*, 2012) on *Adansonia digitata* and (Pandey and Jaiswal, 2002) on *Terminalia arjuna* Roxb.

Addition of NAA improved the rate of shoot proliferation. The best response (85.33%) of shoot formation and total number (6.12 ± 0.13) of shoots per explants were obtained on MS medium containing 2.0 mg/l BAP in combination with 1.0 mg/l NAA whilst highest shoot length (4.14 ± 0.08 cm) was obtained on MS medium containing 1.0 mg/l BAP within 30 days of culture (Table 2). This was considered to be the optimal growth regulator combination in shoot multiplication and shoot length for *in vitro* propagation of *D. melanoxylon* among all treatments using seedlings. Results obtained in this study showed consistency with other studies where BAP and NAA promoted the proliferation and multiplication of shoots in number of plants such as *Capparis decidua* Forsk. (Deora and Shekhawat, 1995), *Dalbergia latifolia* (Swamy *et al.*, 1992, Boga *et al.*, 2012), *Bauhinia variegate* (Singh *et al.*, 2013) and *Dalbergia sissoo* Roxb. (Sahu *et al.*, 2014).

Rooting of shoots was observed in full strength medium supplemented with NAA or IBA (Figure 1E and Table 2). *In vitro* raised shoots from multiplication induced roots on full strength MS

medium supplemented individually with NAA and I BA (Table 2). Full-strength MS mediun containing 1.0 mg/l NAA induced rooting in 92% of the shoots in 6 weeks. NAA at 1.0 mg/l produced a maximum number of roots (5.89 ± 0.47) compared to IBA at 1.0 mg/l which produced 2.39 ± 0.31. Explants cultured onto growth regulators-free MS medium failed to produce roots.

NAA and IBA have been widely used as root induction PGRs in both *in vitro* and *ex vitro* conditions (Al-Saqri and Alderson, 1996, Tavares *et al.*, 1996, Rathore *et al.*, 2008, Soundy *et al.*, 2008, Saffari and Saffari, 2012). NAA proved to be better as compared to IBA with regard to all rooting parameters. The primary roots were thick, which helped in establishing the plantlets in the soil. Under hardening conditions, 84.6% of the plants were successfully established in the greenhouse after 4 weeks (Figure 1F).

Table 1 Influence of different PGRs on shoot multiplication from nodal explants of *Dalbergia melanoxylon* culture on MS medium after 4 weeks

S/N	Growth regulators (mg/l)		% of Responsive explants	No. of shoots/explant	Shoot length/explant (cm)
M0		-	94	1.00 ± 0.00c	1.69 ± 0.08b
M1	BAP	0.5	88	2.12 ± 0.11b	2.22 ± 0.08d
M2		1.0	81.33	2.75 ± 0.11d	4.14 ± 0.08a
M3		1.5	90.67	3.56 ± 0.19a	3.09 ± 0.13c
M4		2.0	98.67	3.01 ± 0.18d	3.03 ± 0.13c
M5		2.5	92	2.76 ± 0.18d	2.05 ± 0.06d
M6	BAP + NAA	2.0 + 0.25	89.33	3.96 ± 0.12b	5.84 ± 0.08a
M7		2.0 + 0.50	93.33	4.24 ± 0.09b	3.53 ± 0.17b
M8		2.0 + 1.0	85.33	6.12 ± 0.13a	3.21 ± 0.13b
M9	BAP + IBA	2.0 + 0.25	90.67	3.07 ± 0.13b	2.69 ± 0.13b
M10		2.0 + 0.50	88	3.41 ± 0.14b	4.01 ± 0.17a
M11		2.0 + 1.0	92	4.29 ± 0.17a	3.03 ± 0.17b

*Means within a column with the same letter are not significantly different based on Fisher's LSD test at the P = 0.05 level of probability; (-) denotes absence of growth regulator.

Table 2 Effects of IBA and NAA on *in vitro* rooting of the microshoots of *D. melanoxylon*.

S/N	Growth regulators (mg/l)		% of Responsive Explants	No. of roots/explant	Root length (cm)
RF0		0	0.0	0.0 ± 0.00e	0.0 ± 0.00c
RF1	MS + NAA	0.1	54.67	1.20 ± 0.06d	1.59 ± 0.06ab
RF2		0.3	76	1.64 ± 0.08cd	1.71 ± 0.05b
RF3		0.5	78.67	2.08 ± 0.24c	1.69 ± 0.04b
RF4		0.7	80	2.93 ± 0.23b	1.68 ± 0.04b
RF5		1.0	92	5.89 ± 0.47a	1.55 ± 0.03a
RF6	MS + IBA	0.1	44	1.07 ± 0.06b	1.45 ± 0.06a
RF7		0.3	57.33	1.39 ± 0.09b	1.44 ± 0.06a
RF8		0.5	62.67	1.43 ± 0.10b	1.38 ± 0.06a
RF9		0.7	65.33	1.47 ± 0.18b	1.40 ± 0.05a
RF10		1.0	69.33	2.39 ± 0.31a	1.22 ±0.04a

*Means within a column with the same letter are not significantly different based on Fisher's LSD test at the P = 0.05 level of probability.

Conclusion

In conclusion, the present study describes for the first time, an efficient micropropagation protocol for *D. melanoxylon* from cotyledonary node explants. BAP was proved to be effective for improving shoot number and shoot length either alone or in combination with IBA or NAA. However, 1.5 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l BAP + 1.0 mg/l NAA were found to be most the optimum combination of the treatments with regard to number of shoots and length of axillary shoots of *in vitro* established culture respectively. Thus, this protocol could be useful for large-scale multiplication and conservation of this ecologically and economically important multipurpose tree. Besides, plant regeneration through *in vitro* propagation will bring some advantages, such as for clonal propagation and genetic improvements including genetic transformations.

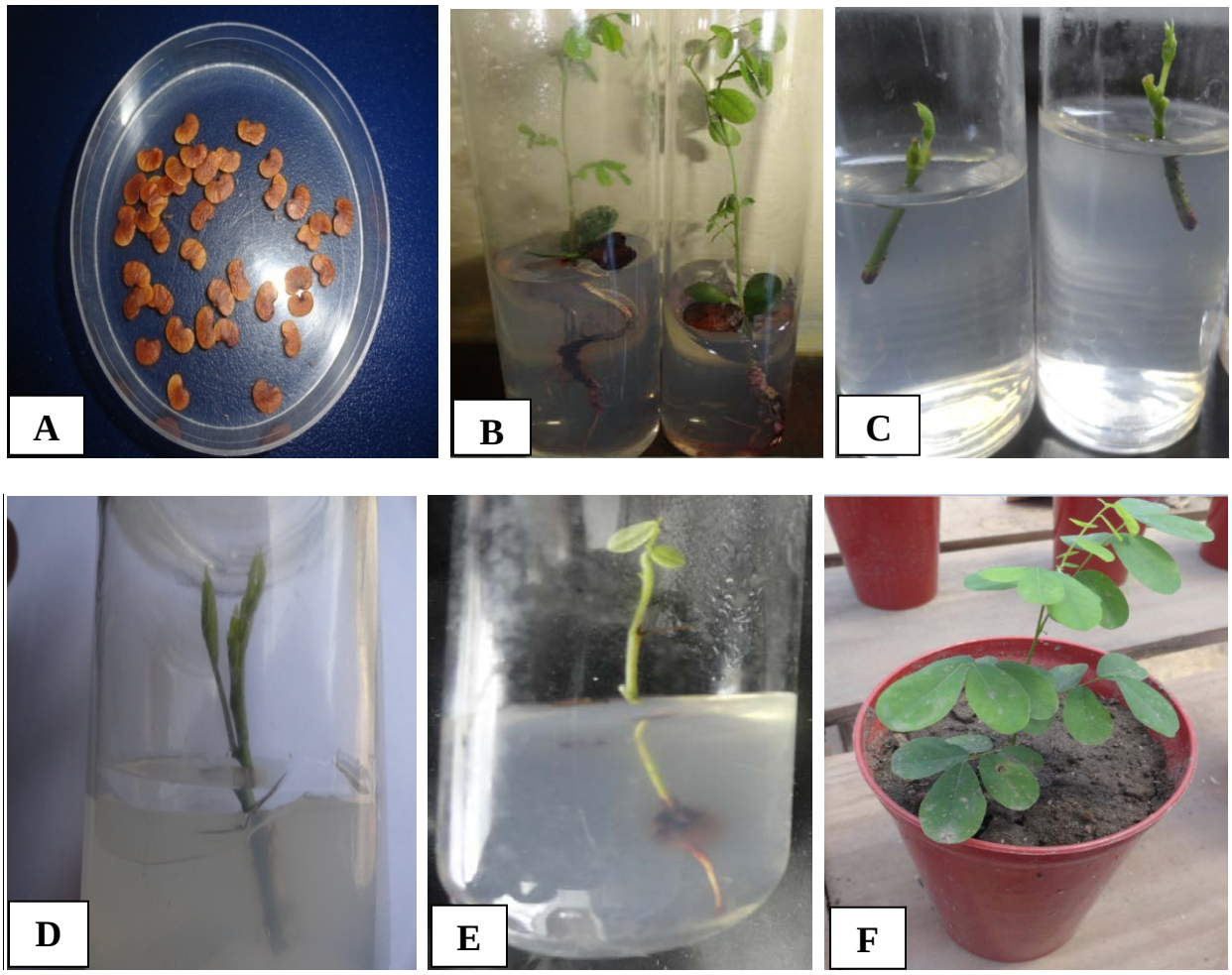

Figure 1: Various growth stages of *D. melanoxylon*: (**A**) Seeds of *D. melanoxylon*; (**B**) Seedlings on growth regulators-free MS medium; (**C**) Single shoot initiation from a seedling nodal explants on growth regulators-free MS medium; (**D**) Proliferation of multiple shoots on MS containing 2.0 mg/l BAP in combination with 1.0 mg/l NAA after 10 days of culture; (**E**) *In vitro* rooting on MS medium containing 1.0 mg/l NAA and (**F**) An acclimatized plant.

Acknowledgements

Special thanks go to School of Life Science and Bio-Engineering of Nelson Mandela African Institution of Science and Technology, Tanzania for its incredible efforts and support during the course of this study. We are grateful to the Mikocheni Agricultural Research Institute (MARI)

for making lab facilities accessible to this research and for other institutional support. This research is funded by the Commission for Science and Technology (COSTECH) of the government of Tanzania.

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