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In Vitro REGENERATION OF *Eucalyptus camaldulensis* DEHNH. PLANTLETS FROM MICRO-CUTTINGS

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Abstract
Clonal propagation of Eucalyptus camaldulensis through stem cuttings is limited by a range of problems such as low root frequency, loss of rooting competence and poor rooting quality which can be overcome through plant tissue culture. Hence, efficient method of plantlets regeneration was developed for the species with a view to enhance its propagation. Shoot tips and nodal segments of E. camaldulensis were inoculated on MS media supplemented with various growth regulators, BAP (0, 0.5 to 2mg/L) for shoot regeneration and NAA (0, 0.5 to 2mg/L) for root induction. Results indicated that, the medium without BAP (control) gave significantly higher shoot length (2.7, 3.4cm) while plantlets of other media supplemented with BAP (0.5 to 2mg/L) had comparable shoot lengths at 4 and 8 Weeks After Inoculation (WAI) respectively. Similarly, highest but comparable average number of leaves (17.6) were obtained from control while, highest number of adventitious shoots (5.2) obtained, were from medium supplemented with 0.5 mg/L BAP which was not significantly different from other treatments and control (3.2) at 8 WAI. The effect of NAA on root induction showed that, MS medium supplemented with 0.5 mg/L NAA, produced highest average number of roots (8.6 and 9.8 /plantlets) and longest root length (0.74 and 2.53 cm) which were significantly different (P ≤ 0.05) from control and other media supplemented with NAA at 2 and 4 WAI respectively.

Key Words: *Adventitious shoots, clonal propagation, root induction, shoot regeneration*

Introduction

Eucalyptus camaldulensis Dehnh. is an exotic tree species to Nigeria. The species belongs to family Myrtaceae, originated from Australia and has adapted to different climatic conditions, from tropical to arid and temperate zones, and a wide range of rainfall zones (Butcher *et al*., 2009). It is a fast-growing multipurpose tree used for the provision of timber and essential oil (Eucalyptus oil) (Rahim *et al*., 2003). In Northern Nigeria, it is used for electric poles, firewood, landscaping, windbreak and aesthetic purposes (Ekhuemelo *et al*., 2017). In South Africa, it is used for ornamental purposes, phytoremediation of mining sites, provisioning of nectar and pollen for bees (Hirsch *et al*., 2019). *E. camaldulensis* also found uses in traditional medicine as its leaves and barks are used for treatment of ailments such as typhoid and malaria fever, cough, stomach upset and fungi infection (Ekhuemelo *et al*., 2017). The species has a gestation period of about

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7–10 years and may produce a million or more seeds annually (Colloff, 2014).

Its numerous uses has made it one of the world most planted trees and a prominent candidate in tree breeding programmes worldwide (Girijashankar, 2012). As such, several efforts have been made to mass propagate the species through seeds and vegetative methods but with its limitations. In the nursery, *E. camaldulensis* is susceptible to various fungi and a range of pests and diseases which affects its seed development and growth, causing damping-off and leaf diseases (Wingfield *et al*., 2008). Moreover, the most common and widely used stem-cutting method suffers due to intrinsic genetic and physiological limitations. This is evident in rapid loss of rooting competence, intra-clonal variation and poor rooting quality thereby hindering field deployment (Xavier *et al*., 1997; Sachs *et al*., 1988,). Though, this method was improved upon by using mini-cuttings grown hydroponically and acclimatized with the mixture of ecosand, vermiculite and leaf compost which improved rooting percentage and sapling survival (Bindumadhava *et al*., 2011). Nonetheless, biotechnological approach remains the best option for enhanced plant biomass production, altered fertility, biotic and abiotic stress resistance improvement (Girijashankar, 2012). In view of the above and considering the advancement in application of plant tissue culture techniques in forestry such as production of large numbers of uniform propagules at a cost effectives compared with conventional methods, the present work sought to develop efficient method of plantlets regeneration for *Eucalyptus camaldulensis* with a view to mass produce the species.

Materials and Methods

Study Location

The research was conducted in the Plant Tissue Culture Laboratory of Biotechnology Department, Forestry Research Institute of Nigeria located on the latitude 07º23'18″ N to 07º23'43″N and longitude 03º51'20″E to 03º53'43″E (Figure 1). The location is found at 199m above sea level. The climate is West African monsoon which is characterized by wet and dry season (bimodal rainfall distribution). The wet season begins in April and continues through October with two peaks in June and September. The mean annual rainfall is 1548.9mm, average annual temperature is 25.9ºC and the relative humidity is 71.8%.

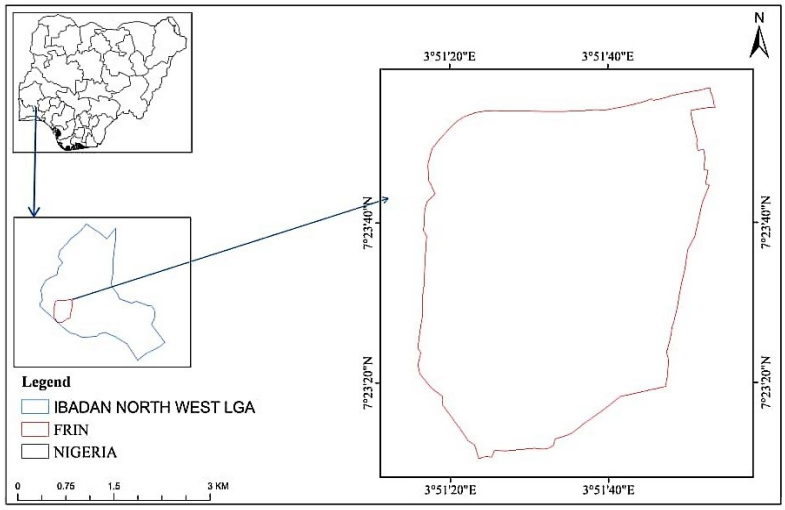

Fig. 1: Map of the study location

Experimental Design and Treatments

The experiment was conducted in two phases, Shoot regeneration and plantlets multiplication. Shoot regeneration included varying levels of Benzyl Amino Purine (BAP) in MS basal medium in order of A (Control, 0.0mg/L), B (0.5mg/L), C (1.0mg/L) D, (1.5mg/L) and E (2.0mg/L) with five replicates each. The root induction consisted five levels of α-Naphthalene Acetic Acid (NAA) (control, 0.0mg/L), 0.5mg/L, 1.0mg/L, 1.5mg/L and 2.0mg/L) with 8 replicates each in MS basal medium. All the treatments were laid out in completely randomised design. Further sub-culturing was done using freshly prepared MS medium supplemented with 0.5mg/L NAA for generation of plantlets with good root and shoot formation.

Media Preparation and Explant Inoculation

Murashige and Skoog basal medium used for the experiment was prepared using 34.43 g MS powder/litre (M5501, SXS5501015A). The media were supplemented with growth regulators according to the treatments mention above. The pH was adjusted to 5.8, gelled with 8.5g of agar, dispensed at 20 ml/tube and sterilized at 121^{o}C and 15 psi for 15minutes. The shoot tips and nodal segments of *E. camaldulensis* plantlets generated were sub-cultured for shoot regeneration while the plantlets produced were thereafter sub-cultured for root induction.

Data Collection and Analysis

The variables collected included the number of shoots, shoot length, number of roots and number of leaves for the first shoot regeneration. These were collected at 4 weeks interval starting from 4 weeks after inoculation (WAI). Root length and number of roots were collected for root induction stage at interval of 2 weeks starting from 2 WAI. The data were subjected to analysis of variance while means were separated with Fisher's protected least significant difference (L.S.D) at $p \leq 0.05$

Result and Discussion

Shoot Regeneration

The results of shoot regeneration of *E. camaldulensis* as affected by different concentrations of BAP in MS basal medium is shown in Table 1. Analysis of variance indicates that there was significant difference ($P \leq 0.05$) in the shoot length, number of leaves and number of roots but for number of adventitious shoots of the plantlets regenerated at successive growth weeks, of which there was no significant difference. The medium without BAP (TRT A: control) gave significantly higher shoot length (2.7cm, 3.4cm), average number of roots (2, 3.8) and average number of leaves (14.4, 17.6) than other treatments at 4 and 8 WAI respectively (Table 1 and Plate 1). Other media supplemented with BAP (TRT B to E) had comparable effects on shoot length while number of roots followed similar trends as the media with BAP did not produce any roots compared with control at both periods of observations (Plate 1). Although, the number of roots induced from medium devoid of growth regulators were very few with small diameters, this necessitated further sub-culturing into root induction media.

These results indicated that *E. camaldulensis* had sufficient endogenous cytokinins capable of eliciting morphological growth in the species plantlets in terms of shoot length, roots and leave formation. Several tree species propagated *in vitro* have been observed with this characteristics. Ndoye *et al*. (2003) reported that *Balanites aegyptiaca* shoot tips and nodal segment explants, inoculated on MS medium without BAP or Kinetin gave 100 % shoot formation and comparable shoot lengths to those

inoculated on media supplemented with the growth regulators. Similarly, culturing of hypocotyl segments of *Melia azedarach* on MS medium without plant growth regulators (PGRs) was reported to have 80% regenerated vegetative buds and normal shoots (Handro and Floh, 2001). Rooting of *Azadirachta indica* explants (axillary buds and nodal segments) was also obtained on MS medium without PGRs as reported by Rodríguez and Ortiz (2001). In addition, the observed better shoot regeneration of *E. camaldulensis* in present study also corroborated the findings of Chand and Singh (2004) on inoculated encapsulated nodal segments of *Dalbergia sissoo* which produced plantlets on half-strength MS medium without PGRs,

For multiple shoot induction, highest but not significant number of adventitious shoots (5.2) were generated from media supplemented with 0.5 mg/L BAP (Table 1) at 8 WAI. This depicts that the BAP concentration is optimum for the species multiple shoot production. The lowest number of shoots (3.2) from control indicated that the endogenous cytokinins was rather low while lower number of shoots observed across MS media supplemented with higher BAP concentrations (1.0 to 2.0mg/L) compared to MS medium supplemented with 0.5mg/L BAP, showed that those concentrations were too high and becoming toxic thereby limiting the shoot proliferation (Plate 1). Similar phenomenon was observed for *Ceratonia siliqua.* The MS medium supplemented with higher BAP (1 and 2mg/L) was reported to stimulate shoot formulation but with shorter length compared to those obtained from MS medium added 0.5mg/L BAP, 0.1 mg/L IBA and 0.5mg/L GA_3 (Naghmouchi, *et al*., 2008). Moreover, the results were closely related to that of Wachira (1997), who obtained highest number of shoots for *Eucalyptus grandis* on MS medium supplemented with 0.4mg/L BAP and 1.0mg/L IBA. Nonetheless, Girijashankar (2012) reported this position, stating categorically that plant growth regulators such as 2, 4-D, kinetin and zeatin were avoided for the direct regeneration of *E. camaldulensis*, while only BAP and NAA combination were found more appropriate to achieve shoot competence, the combination of which has NAA sparingly.

Table 1: Effect of BAP on Shoot Growth of *E. camaldulensis* at Successive growth weeks

TRT	Shoot length (cm)		Number of Roots		Number of leaves		Number of Adventitious Shoots	
	4 WAI	8 WAI	4 WAI	8 WAI	4 WAI	8 WAI	4 WAI	8 WAI
A	2.7	3.4	2	3.8	14.4	17.6	2	3.2
B	1.3	1.46	0	0	13.8	16.6	2.8	5.2
C	1.22	1.36	0	0	10.4	12.6	3	4.8
D	1.08	1.18	0	0	9	12.8	3.6	4.8
E	1.26	1.36	0	0	10.4	12.4	3.4	3.8
L.S.D @ $p \leq 0.05$	0.38*	0.44*	1.38*	1.41*	3.39*	4.64	0.16	3.34

*means significant at $p \leq 0.05$. TRT: treatments. A (control; 0.0 mg/L), B (0.5 mg/L), C (1.0 mg/L) D, (1.5 mg/L) and E (2.0 mg/L BAP)

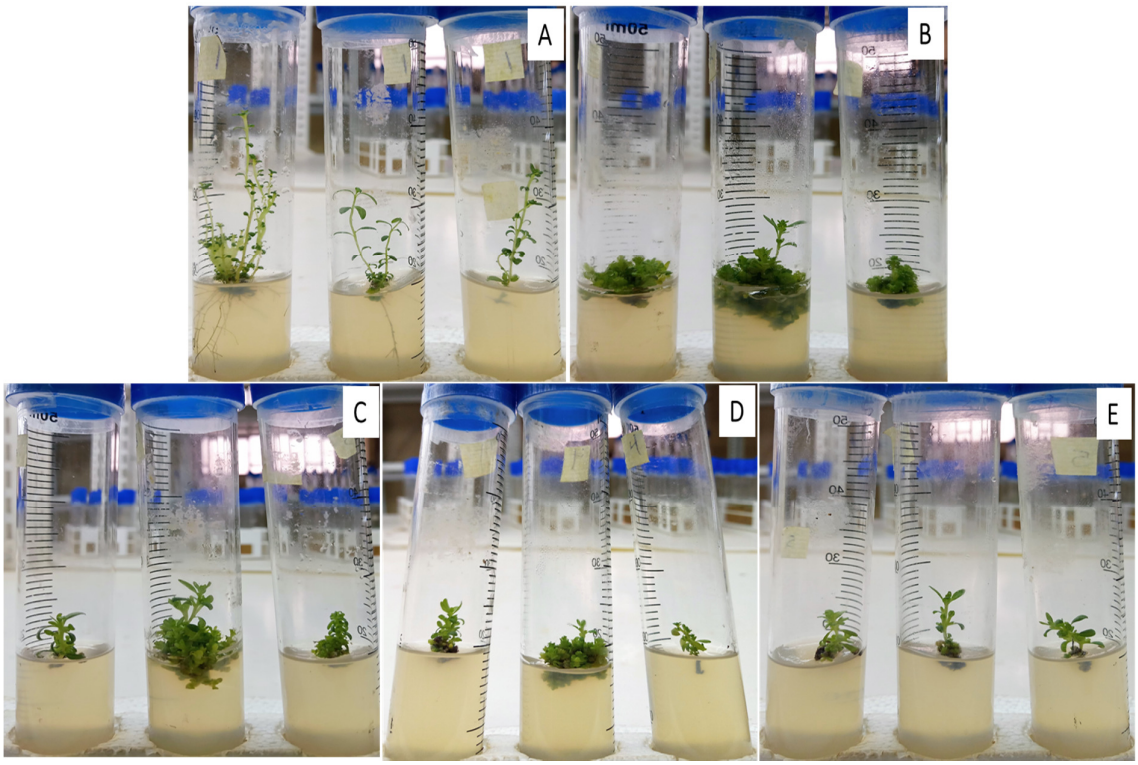

Plate 1: Effect of BAP on Shoot regeneration of *E. camaldulensis* at 8 WAI

Root Induction

The effect of NAA was assessed on the root induction of *E. camaldulensis* plantlets. Analysis of variance showed that there was significant different ($P \leq 0.05$) among the treatments for the root length and the number of roots produced at successive growth weeks (Table 2). Treatment B (MS medium supplemented with 0.5mg/L NAA) produced significantly higher ($P \leq 0.05$) average number of roots (8.63/plantlets) than the control and other media supplemented with NAA in the range of 1 to 2mg/L at 2 WAI. Moreover, there was no significant different ($P > 0.05$) among the other treatments and control during the same period. At 4 WAI, treatment B having 9.75 average number of roots, was significantly higher than the Control (4) and treatment C (3.75) (MS medium complemented with 1.0 mg/L NAA) while it was closely related to treatments D (6.63) and E (6.13) (Plate 2). The results of the root length followed similar trends at 2 and 4 WAI. The longest root length (0.74 and 2.53cm) produced by treatment B were significantly higher ($P \leq 0.05$) than control and other treatments (Table 2 and Plate 2). The medium without NAA had the least (0.27 and 0.38cm) at both periods of observation. These results confirmed the production of roots in the medium without BAP at shoot regeneration stage while it also emphasised the importance of auxin supplement for good quality root induction in *E. camaldulensis*. This result was similar to that of Chisha-Kasumu *et al*. (2006) on *Pterocarpus angolensis* when roots were initially induced on MS medium without PGRs, but more uniform and efficient roots were later formed in the presence of 5-20 µM Indole Butyric Acid (IBA). The results also corroborated with the findings of Nourissier and Monteuuis (2008) that either NAA or IBA can enhance the quality of the roots

produced just as visible rooting was achieved from the fifth day onwards when MS medium was supplemented with 0.5mg/L of NAA in their study. Conversely, the opinion of Shanthi *et al.* (2014) differs on the rooting of *E. camaldulensis*, as most root developed in the shoot medium supplemented with BAP.

Table 2: Effect of NAA on rooting of *E. camaldulensis* at Successive growth weeks.

TRT	Number of Roots		Root length (cm)	
	2 WAI	4 WAI	2 WAI	4 WAI
A	3.00	4.00	0.27	0.38
B	8.63	9.75	0.74	2.53
C	3.25	3.75	0.48	0.83
D	4.75	6.63	0.35	0.55
E	4.75	6.13	0.38	0.80
L.S.D @ $p \leq 0.05$	3.75*	4.08*	0.23*	0.94*

*means significant at $p \leq 0.05$. TRT: treatments. A (control; 0.0 mg/L), B (0.5 mg/L), C (1.0 mg/L) D, (1.5 mg/L) and E (2.0 mg/L NAA)

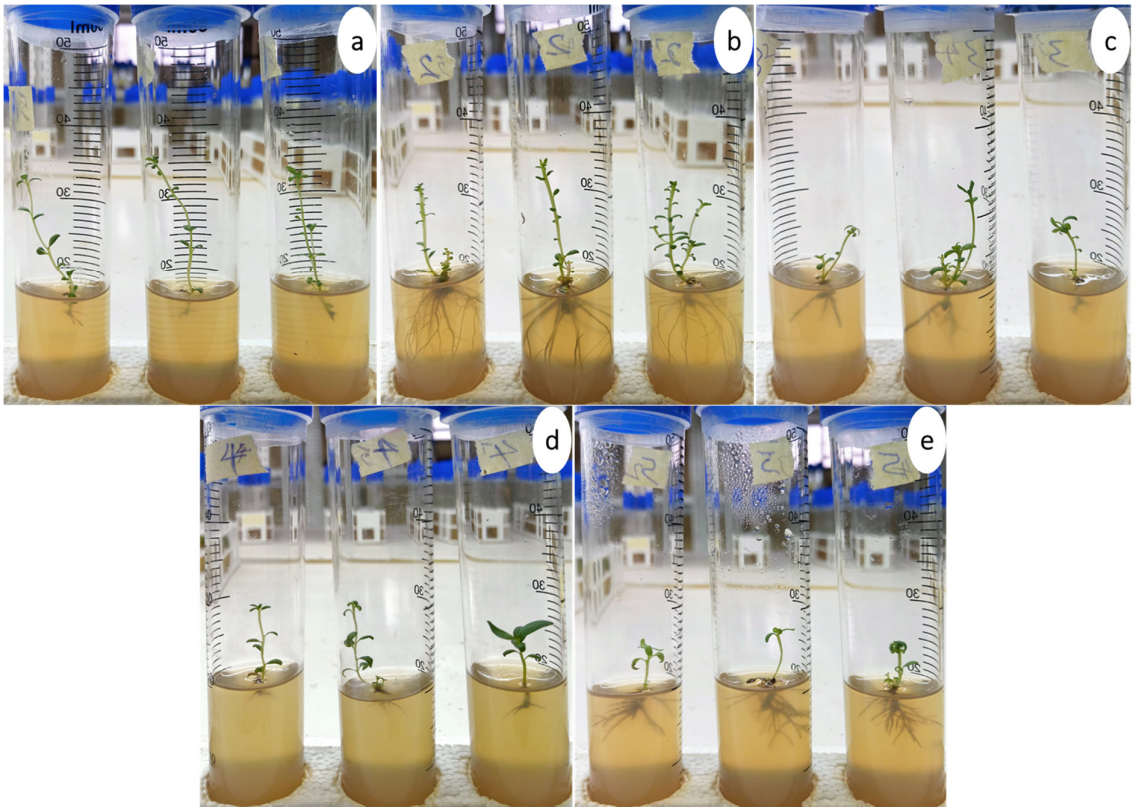

Plate 2: Effect of NAA on Root induction of *E. camaldulensis* at 4 WAI
A (control; no NAA), B (0.5mg/L NAA), C (1.0mg/L NAA) D, (1.5mg/L NAA) and E (2.0mg/L NAA)

Conclusion

The production of large numbers of propagules of *Eucalyptus camaldulensis* is necessary in order to ensure its conservation and sustainability of its economic importance. Hence, this study was conducted to facilitate *in vitro* propagation of the species. Findings

showed that plantlets of *E. camaldulensis* can be adequately regenerated on MS basal medium without growth regulators under 8 weeks whereas, multiple shoot production and good root formation require addition of 0.5mg/L BAP and 0.5mg/L NAA respectively as the optimum. This developed protocol is hereby recommended for mass propagation of the species.

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