Plant Cell Culture & Micropropagation

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In vitro shoot regeneration of *Milicia excelsa* (Welw.) C.C. Berg

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ABSTRACT

The need for mass propagation and genetic breeding of tree species with economic importance, such as *Milicia excelsa,* requires an *in vitro* regeneration protocol. Different concentrations of Gibberellic acid (GA_3) (0, 1, 2, 3 and 4 mg/L) and 6-benzylaminopurine (BAP) (0.0, 0.5, 1.0, 1.5 and 2 mg/L) were added to Murashige and Skoog medium for seed germination and shoot regeneration of the species. The treatments were laid out in completely randomised design. Each set of GA_3 and BAP treatments were composed by 20 and 6 replicates, respectively. Observations made on cultured seeds include germination percentage at 4 weeks interval while shoot length, number of shoots, number of roots and leaves collected on regenerated shoots at 2 weeks interval were subjected to analysis of variance and means separated with Fisher's protected Least Significant Difference (L.S.D) at $p \leq 0.05$. Results showed that using GA_3 positively influenced the seed germination as early germination occurred in the media with GA_3, in comparison to the treatment without plant growth regulator (control). Nonetheless, 2.0 mg/L GA_3 was observed to be the optimum, having the highest germination percentage of 30% at 4 Weeks after Inoculation (WAI). The results of the BAP effects on the shoot regeneration showed that 0.5 mg/L was the optimum for best shoot growth, having the average number of leaves (6.5), axillary shoots (1.8) and highest shoot length (3.75 cm) in comparison to the control treatment. In conclusion, these results provide insight on *in vitro* propagation of *Milicia excelsa* and the techniques employed could be adopted for its shoot multiplication.

Index terms: *In vitro* seed germination; micropropagation; multiple shoots; moraceae.

INTRODUCTION

Increasing global resource consumption and reliant on the natural forest resources necessitate the development of more timber plantations as they have the potential to produce more quantity of wood per acre than natural forest (De Wit; Hoogzaad; Von Daniels, 2020; Food and Agriculture Organization – FAO, 2020; Sedjo, 1999). *Milicia excelsa* (Welw.) C.C. Berg belongs to Moraceae family, is an economic tree species for plantation establishment. The species grows up to 50 meters high and sheds its leaves annually. It is dispersed across tropical Africa and its range extends from Guinea-Bissau in the West to Mozambique in the East (Orwa et al. 2009). It is an important source of commercial timber and primarily gotten from natural forests (Ugwu; Omoloye, 2015). Its wood is used for tanks and barrels, sliced veneer, construction works, shipbuilding, framework, trucks and indoor joinery.

In spite of *M. excelsa* commercial value and importance, there is little or no report on *in vitro* propagation. The species is listed as near threatened by the World Conservation Monitoring Centre (1998) as its natural regeneration cannot keep-up with it timber demand in Africa. *M. excelsa* seeds are observed with low and erratic germination as well as poor dispersal appendages (Chimezie, 2021). Moreover, its cultivation is hindered by quick loss of seed viability on storage and when seeds germinate, the young shoots are often susceptible to attacks by gall-forming *Phytolyma lata* which in many cases kills the seedlings (Agyeman et al. 2010). Mixed planting has been a means of managing pest attack on the species while some pest-resistant genotypes have also been reported (Cobbinah; Wagner, 1995; Ofori; Cobbinah, 2007; Ugwu; Omoloye, 2017). Hence, there is a need to apply suitable *in vitro* regeneration techniques for the species improvement and mass propagation of its well-adapted genotypes.

https://doi.org/10.46526/pccm.2022.v18.180
Received in June 16, 2022 and approved in August 15, 2022
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Successful application of this propagation techniques on several tropical woody tree species have been well documented (Harry; Thorpe, 1994; Pijut et al. 2012; De-Souza et al. 2017). Moreover, plant tissue culture strategies are also been used for conservation of threatened species (Mok; Ho, 2019; Pitekelabou; Aïdam; Kokou, 2015). Nonetheless, exogenous application of plant growth regulators such as auxin, gibberellin and cytokinins play significant roles in the actualisation of plant tissue culture objectives (Pandey; Tamta 2012; Arab et al. 2014; Mohamad, 2022). Consequently, this study aims at developing a simple and efficient protocol for *in vitro* shoot regeneration of *M. excelsa* in the presence of GA_3 and BAP towards its mass propagation.

MATERIAL AND METHODS

The study was conducted at Tissue Culture Laboratory of the Department of Biotechnology, Forestry Research Institute of Nigeria.

Milicia excelsa seedlings were generated from seeds inoculated on Murashige and Skoog (MS) basal medium supplemented with different concentrations (1.0, 2.0, 3.0 and 4.0 mg/L) of gibberellic acid (GA_3). Shoot regeneration was obtained on MS basal medium supplemented with different concentrations (0.5, 1.0, 1.5 and 2 mg/L) of 6-benzylaminopurine (BAP). No plant growth regulators have been added in the control treatment. The experimental design was completely randomized with 20 and 6 repetitions, respectively.

The MS medium was prepared using 34.43 g powder/litter (M5501) (Murashige; Skoog, 1962). The pH of the media was adjusted to 5.8, gelled with 8.5 g agar, dispensed 20 ml/tube and sterilized at 121° C and 15 psi for 15 minutes. Freshly collected seeds of *M. excelsa* were sterilised by soaking in 70% ethanol, 10% hypochlorite solution and fungicide solution (5 g/L Z-force + 5 g/L cibaplus + 0.4 g/L amoxicillin) for 2, 10 and 30 minutes respectively. Each steps was followed by rinsing with sterilized distilled water three times then, the seeds were placed on sterilized petri-dish laid filter paper and inoculated at 3 seeds/tube.

The shoot tips and nodal segments of 8 weeks-old seedlings generated (Figure 1a) were sub-cultured for shoot regeneration. Explant of about 1.5 cm was place in each culture tubes. The *in vitro* culture were placed in growth room under 16/8hrs light/dark photoperiod and 20 ± 2° C. The room was illuminated by arrays of cool white light emitting diode (LED) (20 watts, 6000 kelvin).

Data collected include germination percentage on cultured seeds at 4 weeks interval starting of 4 Weeks After Inoculation (WAI) while shoot length, number of shoots, number of roots and number of leaves were collected on regenerated shoots at 2 weeks interval starting of 2 WAI. The data related to culture initiation was subjected to descriptive analysis while those related to shoot regeneration were subjected to analysis of variance using SPSS (Edition 20) and means separated with Fisher's protected least significant difference (L.S.D) at $p \leq 0.05$.

RESULTS AND DISCUSSIONS

Culture initiation

Germination percentage

The results of GA_3 effects on *in vitro Milicia excelsa* seed germination revealed that the germination percentage among the treatments were different across successive weeks. Germination had started as early as 4 WAI and MS medium supplemented with 4.0 mg/L GA_3 having maximum percentage germination of 10%, followed by 5% in the presence of 3.0 mg/L GA_3 while other lower concentrations and control did not positively influence germination at the period (Figure 2). By 12 WAI, 30% of germination was observed in medium added 2.0 mg/L GA_3, followed by 25% in medium supplemented with 4.0 and 3.0 mg/L GA_3 while control medium had no germination.

These results identified that GA_3 had positive influence on early *in vitro* germination of *M. excelsa* and the 2.0 mg/L concentration was the optimum given. Seeds did not germinate in the GA_3 absence even at 12 WAI. It has been reported that delay in natural germination of *M. excelsa* seeds could be due to dormancy imposed by sticky, oily substances which surround the seeds while un-pre-treated seeds might take up to four weeks before germination (Nzekwe; Ubani; Ajuziogu, 2013). Moreover, endogenous factors such as gibberellic acid plays a vital role in breaking seed dormancy by stimulating growth (Gupta; Chakrabarty, 2013). This justified the use of exogenous GA_3 in the

present study to break the seed dormancy and aid quick growth as evident in the results presented here (Figure 2). Previous reports on *in vitro* seed germination of *M. excelsa* are scarce. Hence, the maximum *in vitro* germination of 30% obtained in the present study corroborated to Mohammed et al. (2020) that obtained lesser maximum germination of 19% from the pre-treated seeds of the species grown under laboratory conditions.

Shoot regeneration

Shoot length and root length

The results of shoot and root lengths of the regenerated *Milicia excelsa* plantlets as affected by BAP concentrations are presented in Figure 3. Analysis of variance indicated that there was no significant difference ($p > 0.05$) among BAP concentrations for the shoot and root lengths compared with control at successive growth weeks. Shoot length ranged from 3.75 cm in medium supplemented with 0.5 mg/L BAP to 3.03 cm in control medium. Meanwhile, all media supplemented with BAP tended to present higher shoot length over control at 4 WAI.

On the other hand, the root development in regenerated *M. excelsa* plantlets contrasted with shoot growth. It was observed that no visible root was formed in any BAP-treatment while few roots were induced in control (Table 1, Figure 1b-f). The root length in the absence of BAP was 0.4 cm at 4 WAI (Figure 3). These results indicate that available endogenous auxin in *M. excelsa* plantlets was not enough to overcome the opposing effects of BAP exogenous on root formation. Thus, the results suggest and emphasise the need for external supply of auxin for good root formation in the species regeneration protocols (Ofori et al. 1996).

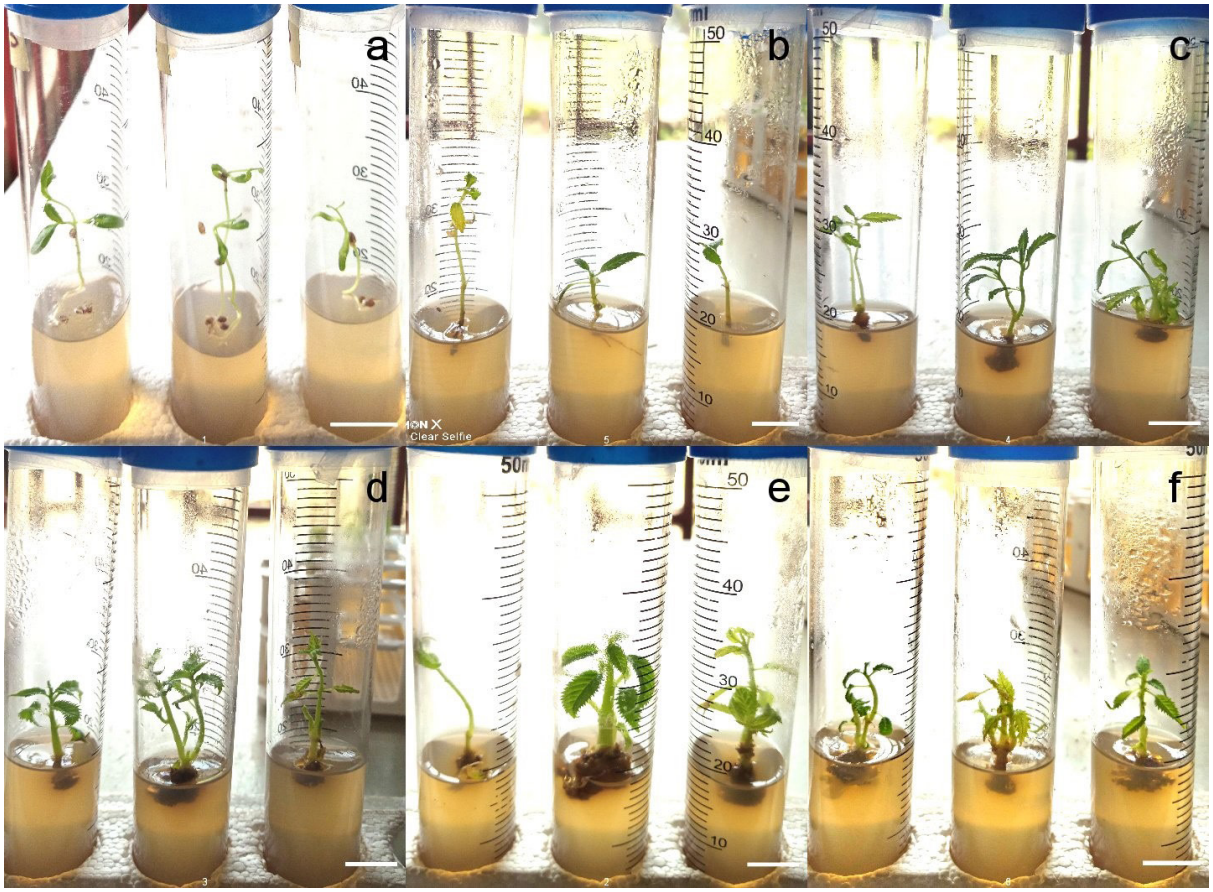

Figure 1: *In vitro* germination of *Milicia excelsa* at different *in vitro* conditions**. a:** Seedlings at 8 WAI; **b-f**: Plantlets in MS basal medium supplemented with BAP at 4 WAI. **b:** 0.0 mg/L BAP; **c:** 0.5 mg/L BAP; **d:** 1.0 mg/L BAP; **e:** 1.5 mg/L BAP and **f:** 2.0 mg/L BAP. Bar = 1.07 inches.

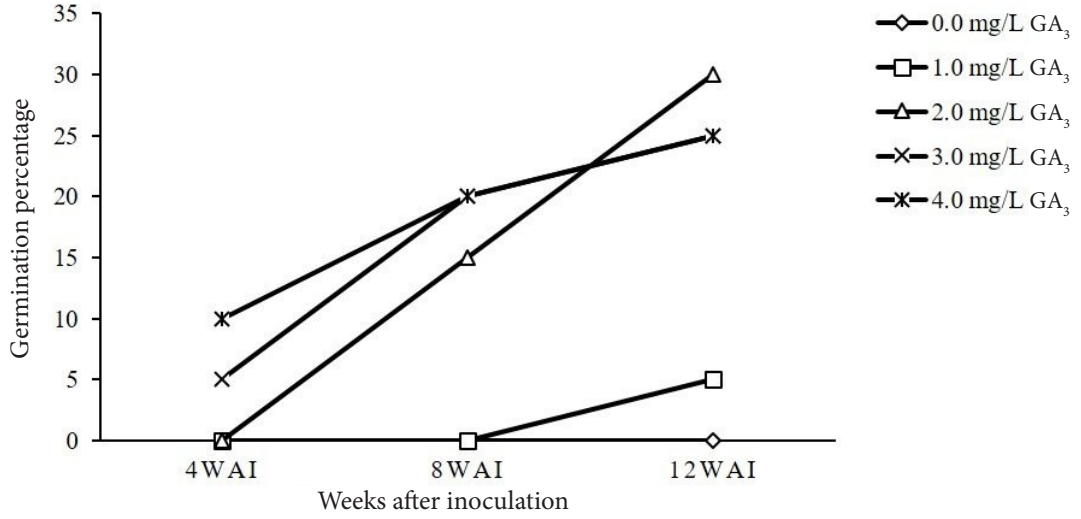

Figure 2: Effects of GA_3 on *in vitro* germination of *Milicia excelsa* seeds at 4, 8 and 12 weeks after inoculation (WAI).

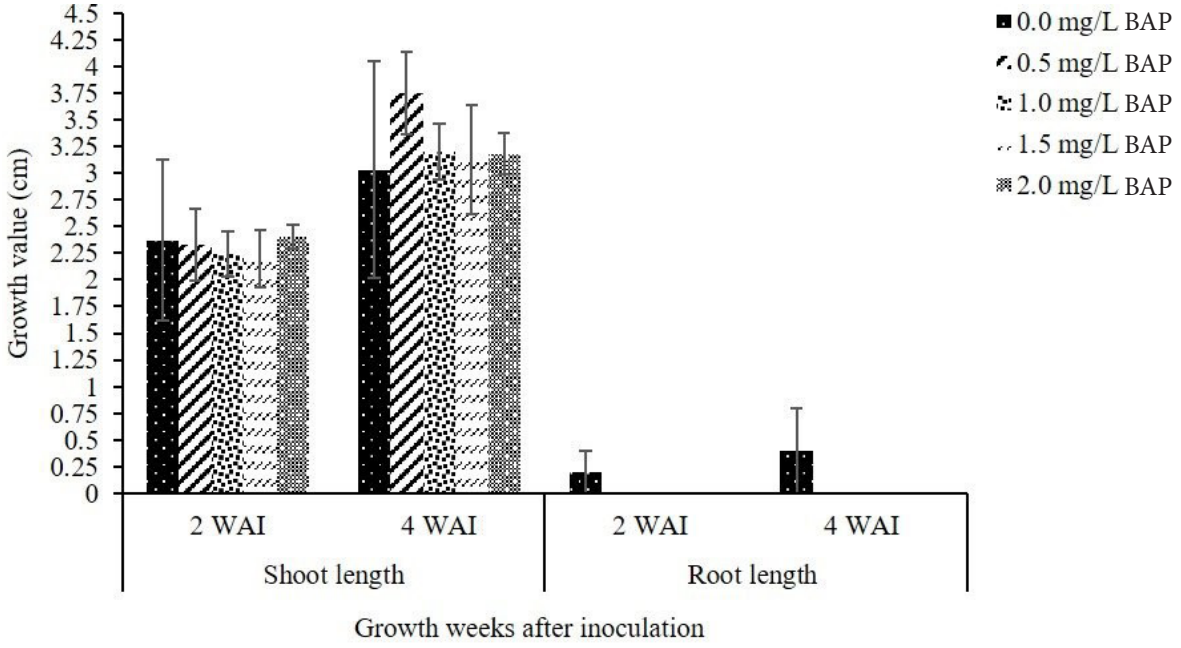

Figure 3: Effects of BAP on shoot and root lengths of *Milicia excelsa* plantlets at 2 and 4 weeks after inoculation (WAI).

Number of leaves and axillary shoots

The results of effects of BAP on number of leaves and axillary shoots of *Milicia excelsa* plantlets are presented in Table 1. There was no significant difference ($p > 0.05$) among the treatments for the parameters at 2 and 4 WAI. The highest average number of leaves (7.6) was obtained in medium supplemented with 1.0 mg/L BAP at 4 WAI, followed by 6.7 leaves in the presence of 0.5 and 2.0 mg/L BAP, while the least (4.0) was obtained in the absence of BAP.

The number of axillary shoots at 4 WAI ranged from 1.2 shoots in medium supplemented with 1.0 mg/L BAP to 2.2 shoots in the presence of 2.0 mg/L BAP (Figure 1b-f). No axillary shoots were induced in control medium.

Table 1: Effects of BAP on number of leaves, roots and axillary shoots of regenerated plantlets of *Milicia excelsa* at 2 and 4 WAI.

BAP (mg/L)	Number of leaves		Number of roots		Number of shoots	
	2 WAI	4WAI	2 WAI	4WAI	2 WAI	4WAI
0.0	4.0±0.0	4.0±0.57	1.3±1.33	1.3±1.33	0.0±0.0	0.0±0.0
0.5	4.0±0.0	6.7±0.76	0.0±0.0	0.0±0.0	0.3±0.21	1.8±0.75
1.0	4.0±0.0	7.6±1.63	0.0±0.0	0.0±0.0	0.2±0.20	1.2±0.8
1.5	3.0±0.73	6.3±1.91	0.0±0.0	0.0±0.0	0.0±0.0	2.0±0.73
2.0	4.5±0.62	6.7±0.67	0.0±0.0	0.0±0.0	0.0±0.0	2.2±0.75
SEM @ $p \leq 0.05$	0.23	0.58	0.15	0.15	0.64	0.34

WAI: Weeks after inoculation; SEM: standard error of mean.
Values in the Table represent means ± standard error.

These results emphasized the importance of an exogenous cytokinins source such as BAP for multiple shoot production in the species. This was evident in higher shoot length and number of leaves observed in medium supplemented with BAP compared with control medium (Figure 3 and Table 1). Moreover, observed was better shoot performance by shoot length and number of leaves in the presence of 0.5 mg/L and 1.0 mg/L BAP, respectively. Probably, BAP concentration beyond 1.0 mg/L was in excess and could have limit shoot growth. This was evident in highest number of axillary shoots obtained from 2.0 mg/L BAP but with stunted growth (Figure 1f). These results correlates that of Lin, Wagner and Cobbnah (1998) who stated that the use of Benzyladenine at 0.5 mg/L influenced optimum shoot multiplication of *Milicia excelsa* in a basal medium.

CONCLUSIONS

The present study provides insight on *in vitro* propagation of *Milicia excelsa.* In the presence of plant growth regulators, seeds were observed to be a good starting material for culture establishment while shoot regeneration was also achieved. Notably, application of 2.0 mg/L GA_3promoted the best *in vitro* seed germination while optimum shoot regeneration was enhanced using 0.5 mg/L BAP. Hence, the methods employed could be adopted for *in vitro* multiplication of the species while further studies are required to optimize root induction and acclimatization.

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