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In-vitro propagation of *Picralima nitida* (Stapf) through embryo culture

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Embryo abortion in wide crosses and seed dormancy has hampered the mass propagation of selected tree germplasm from the wild. An *in vitro* plant regeneration protocol was successfully established in *Picralima nitida* (Stapf), a medicinal tropical plant, by culturing excised embryo from mature seeds collected from the wild. Seeds were sterilized and dissected; embryos were raised *in vitro* in 11 different media compositions comprising pure Murashige and Skoog (MS) and MS media supplemented with varying concentrations of cytokinins labeled A, B, C, D, E, F, G, H, I, J and K. Media type was significant on metrical traits of sprouts ($P \leq 0.05$). One hundred percent survival of explants were obtained in media J while MS basal medium (K) had the lowest survival of 40%; and mean number of leaves (1.4). Number of roots per sprout, shoot and root length responses were lowest in medium H with values of 0.13, 0.79 and 0.15 cm, respectively. Overall best growth parameters in shoot (3.38 cm) and root lengths (3.75 cm) were obtained in medium F. Mass propagation of *P. nitida* can be accomplished in MS media supplemented with 0.05 mg/L kinetin and 0.01 mg/L naphthalene acetic acid (NAA).

Key word: Excised embryo, *In vitro* culture, sprouts, metrical traits, *Picralima nitida.*

INTRODUCTION

The increasing demand for forest products as a result of rapid population increase as well as deforestation activities associated with expansion of infrastructural facilities all combine to erode the genetic base of most medicinal plants. Most tropical forest tree species exist in the wild state. The regeneration and long term conservation of these species are at the mercy of the vagaries of nature and conservation-shy but profit-driven herb collectors. Many species have been harvested to near extinct status such as *Enantia chlorantha* (Gbadamosi, 2002) and *Prunus africana* (Tchounjdeu, 1989). *Picralima nitida* (Stapf) is a tropical small bushy tree of the Family Apocynaceae. Picralima is derived from the Greek word “bitter’; it has large glossy leathery leaves, conspicuous white flowers and large orange-coloured fruits (Keay, 1989). The seed is an object of commerce in the local market, and it is collected from the wild thus its availability has been severely threatened. Extract of its seeds, fruit rind and stem bark demonstrated anti-malarial activity (Iwu and Klayman, 1992); antimicrobial effect (Fakeye et al., 2002); as well as anti-inflammatory and analgesic actions (Duwiejuna et al., 2002).

It is used in traditional medicine in the treatment of inflammation, otitis, pulmonary bronchitis and venereal diseases. Mahendran and Narmatha (2009) opined that plant tissue culture and micropropagation techniques play an important role in conservation programmes and management of botanical collections. Embryo culture was used as a method of regenerating this species for domestication purpose.

MATERIALS AND METHODS

Seed source and sterilization

Mature fruits of *P. nitida* were collected from J4 Forest Reserve, Ijebu-Ode, Southwest, Nigeria (Latitude 6° 49[1] N; Longitude 3° 52[1] E). The fruits were washed thoroughly in sterilized distill water and

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Plate 1. Mature seeds of *P. nitida* in the pod.

Plate 2. Excised embryo of *P. nitida.*

cut open with long sharp knife (Plate 1); thereafter, seeds were extracted using hand and washed with liquid detergent under running water several times to remove the slime from the fruit rind. Seeds were later disinfected in 70% mercuric chloride for 10 min followed by three to five rinses in sterilized distill water.

Media preparation

The media prepared were Murashige and Skoog (MS) (1962) basal medium supplemented with different concentrations of cytokinins like 6-benzylamino-purine (BAP) (0.05, 0.075, 0.1, 0.125, and 0.15 mg/L) plus 0.01 mg of α-naphthalene acetic acid (NAA); kinetin (0.05, 0.075, 0.1, 0.125 and 0.15 mg/L) plus 0.01 mg of α-naphthalene acetic acid (NAA); and pure MS sample. The 11 different media were adjusted to pH 5.7 with NaOH or HCl and later autoclaved at 120°C for 30 min: A, MS + 0.05 mg/L BAP + 0.01 mg/L NAA; B, MS + 0.075 mg/L BAP + 0.01 mg/L NAA; C, MS + 0.10 mg/L BAP + 0.01 mg/L NAA; D, MS + 0.125 mg/L BAP + 0.01 mg/L NAA; E, MS + 0.15 mg/L BAP + 0.01 mg/L NAA; F, MS + 0.05 mg/L KIN + 0.01 mg/L NAA; G, MS + 0.075 mg/L KIN + 0.01 mg/L NAA; H, MS + 0.10 mg/L KIN + 0.01 mg/L NAA; I, MS + 0.125 mg/L KIN + 0.01 mg/L NAA
J – MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and K, pure MS sample.

Seed dissection and inoculation

The embryos were excised along with some endosperm in some cases using sterilized surgical blade under the lamina flow. One excised embryo (Plate 2) was inoculated in each test tube. The cultures were incubated at 26 ± 2°C under a 16 h photoperiod with cool-white fluorescent.

Experimental design and data analysis

Survival percentage was recorded after four weeks of culture by dividing the number of sprouted embryo by the total number of inoculated embryo under each media. Number of roots, length of root, length of shoot and number of leaves were assessed at four weeks interval for three months. Each treatment had 15 replicates. Data were subjected to analysis of variance (ANOVA) and means separation tests were performed by the Duncan's multiple range test (Duncan, 1995) using the SAS 9.1 (SAS, 1999).

RESULTS

Survival of embryo *in-vitro*

Embryo germination was recorded two weeks after inoculation in media F. At four weeks after inoculation, the highest embryo germination of 100% was observed in media J; this was followed by media C, E, F and G (93.3%). The lowest germination percentage of 40% was observed in MS basal medium (K). There were high mortality of sprouted embryo in media B, H, I and especially K at the 12th week of inoculation (Figure 1).

Development of shoot

Single shoot formation was obtained in most media from the 4th week after inoculation. There were significant differences ($P \leq 0.05$) in shoot length (SL) of sprouted embryo, however, assessment period (AP) and the interaction of AP and media were not significant ($P \leq 0.05$) on shoot length (Table 1). The mean SL of seedlings in media H, K, B and I were significantly different ($P \leq 0.05$) from embryo sprouts in media G, E, J and F (Table 2, Figure 2). The highest mean SL of 3.38 cm was obtained in embryo sprout inoculated on media F, this was followed by media J with a mean SL value of 2.64 cm. The lowest mean SL of 0.79 cm was recorded in medium H (Table 2).

Development of root

Root formation was observed in most media tested from the 4th week except in media H. Multiple roots were

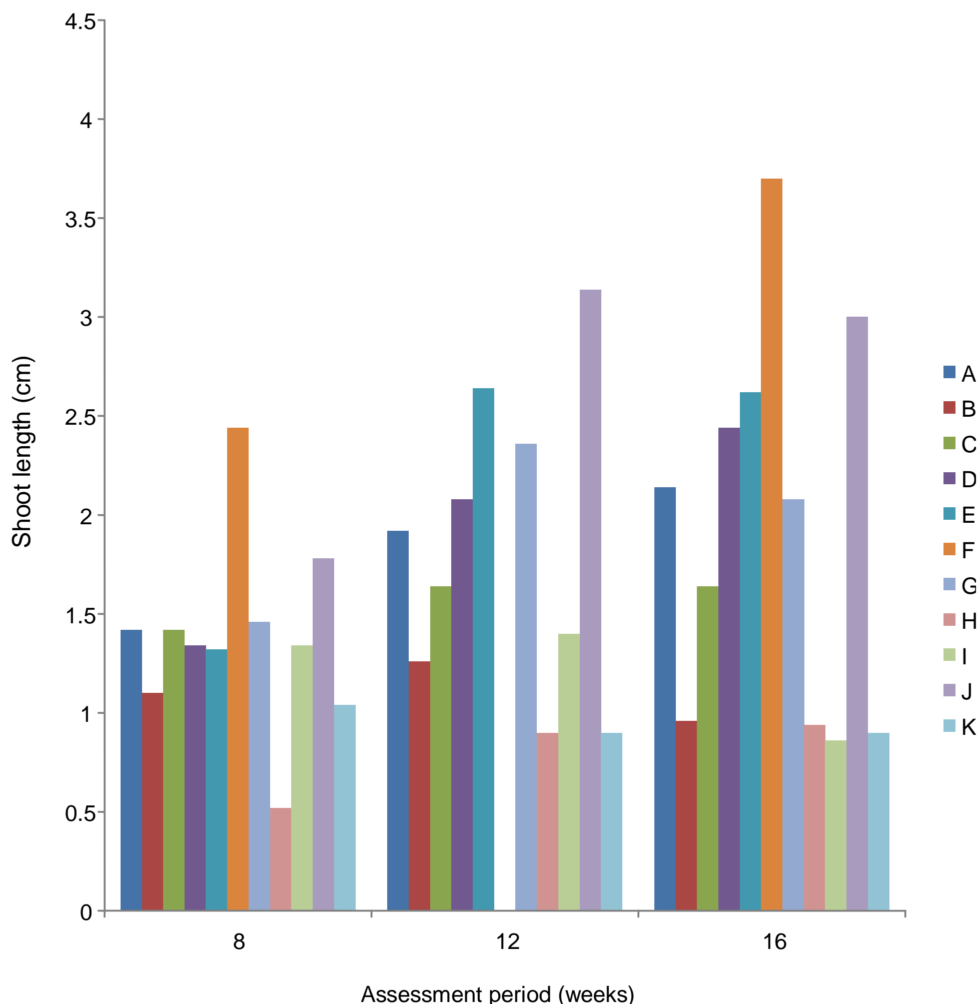

Figure 1. Shoot length of *P. nitida* under different media across the assessment period. **A,** MS + 0.05 mg/L BAP + 0.01 mg/L NAA; **B,** MS + 0.075 mg/L BAP + 0.01 mg/L NAA; **C,** MS + 0.10 mg/L BAP + 0.01 mg/L NAA; **D,** MS + 0.125 mg/L BAP + 0.01 mg/L NAA; **E,** MS + 0.15 mg/L BAP + 0.01 mg/L NAA; **F,** MS + 0.05 mg/L KIN + 0.01 mg/L NAA; **G,** MS + 0.075 mg/L KIN + 0.01 mg/L NAA; **H,** MS + 0.10 mg/L KIN + 0.01 mg/L NAA; **I,** MS + 0.125 mg/L KIN + 0.01 mg/L NAA; **J,** MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and **K,** pure MS sample.

Table 1. Summary of analysis of variance for growth variables of embryo culture sprout of *P. nitida* in different media.

SV	df	Shoot length		Root length		Number of roots		Number of leaves	
		MS	F	MS	F	MS	F	MS	F
Media (M)	10	9.08	13.37*	14.58	7.42*	5.88	3.74*	3.65	4.31*
Assessment period (AP)	2	6.66	9.99*	3.03	1.54^{ns}	0.71	0.45	0.46	0.54^{ns}
M x AP	20	0.67	0.98^{ns}	1.12	0.57^{ns}	0.26	0.16^{ns}	0.78	0.92^{ns}
Error	131	0.68		5.45		1.60		0.83	
Total	163								

*Significantly different at P≤0.05; ns- Not significantly different at P≤0.05.

obtained in embryo sprouts on media A, C, E, F, I and J (Plates 3 and 4). The effect of media type was significant (P≤0.05) on number and length of root, but assessment period and the interaction of AP and media were insignificant on root formation (Table 1). The highest mean number of roots (2.27) and root length (3.75 cm)

Table 2. Means + standard errors of growth variables of embryo culture sprouts of *P. nitida* in different media.

Media type	Shoot length (cm)	Media type	Root length (cm)	Media type	Number of roots	Media type	Number of leaves
H	0.79^{a}±0.09	H	0.15^{a}±0.14	H	0.13^{a}±0.09	K	1.4^{a}±0.39
K	0.95^{a}±0.26	I	1.35^{b}±0.23	B	0.6^{ab}±0.13	B	1.6^{ab}±0.21
B	1.11^{ab}±0.19	B	1.37^{b}±0.31	K	0.8^{abc}±0.27	I	1.6^{ab}±0.21
I	1.2^{ab}±0.17	A	1.4^{b}±0.34	D	0.8^{abc}±0.17	H	1.8^{abc}±0.21
C	1.57^{bc}±0.08	D	1.45^{b}±0.41	G	0.87^{abc}±0.19	C	2.0^{abcd}±0.21
A	1.83^{cd}±0.15	G	1.55^{bc}±0.28	I	0.87^{abc}±0.13	G	2.13^{bcde}±0.13
D	1.95^{cd}±0.18	K	1.61^{bc}±0.48	J	1.2^{bcd}±0.17	J	2.4^{cdef}±0.21
G	1.96^{cd}±0.13	C	2.55^{de}±0.35	E	1.53^{cde}±0.13	E	2.4^{cdef}±0.27
E	2.19^{ef}±0.27	J	2.72^{e}±0.29	A	1.67^{cde}±0.57	A	2.53^{def}±0.22
J	2.64^{f}±0.37	E	2.84^{ef}±0.32	F	1.87^{de}±0.47	F	2.73^{ef}±0.28
F	3.38^{g}±0.36	F	3.75^{f}±0.49	C	2.27^{e}±0.51	D	2.87^{f}±0.26

*Mean values with the same letter in a column are not significantly different at P≤0.05. **A,** MS + 0.05 mg/L BAP + 0.01 mg/L NAA; **B,** MS + 0.075 mg/L BAP + 0.01 mg/L NAA; **C,** MS + 0.10 mg/L BAP + 0.01 mg/L NAA; **D,** MS + 0.125 mg/L BAP + 0.01 mg/L NAA; **E,** MS + 0.15 mg/L BAP + 0.01 mg/L NAA; **F,** MS + 0.05 mg/L KIN + 0.01 mg/L NAA; **G,** MS + 0.075 mg/L KIN + 0.01 mg/L NAA; **H,** MS + 0.10 mg/L KIN + 0.01 mg/L NAA; **I,** MS + 0.125 mg/L KIN + 0.01 mg/L NAA; **J,** MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and **K,** pure MS sample.

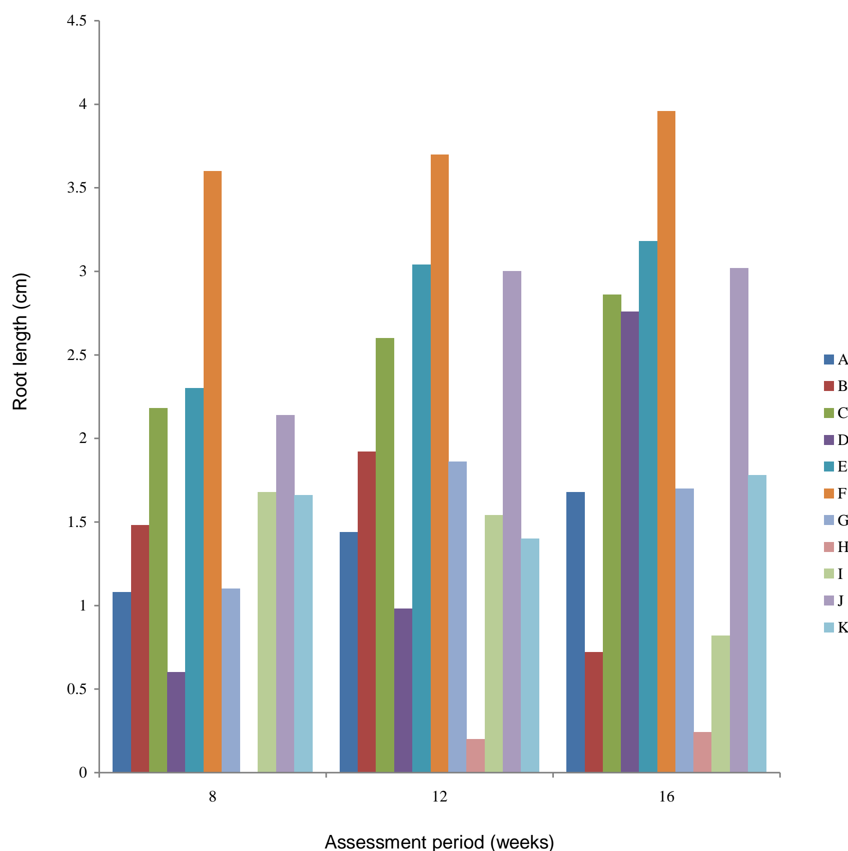

Figure 2. Root length of *P. nitida* under different media across the assessment period. **A,** MS + 0.05 mg/L BAP + 0.01 mg/L NAA; **B,** MS + 0.075 mg/L BAP + 0.01 mg/L NAA; **C,** MS + 0.10 mg/L BAP + 0.01 mg/L NAA; **D,** MS + 0.125 mg/L BAP + 0.01 mg/L NAA; **E,** MS + 0.15 mg/L BAP + 0.01 mg/L NAA; **F,** MS + 0.05 mg/L KIN + 0.01 mg/L NAA; **G,** MS + 0.075 mg/L KIN + 0.01 mg/L NAA; **H,** MS + 0.10 mg/L KIN + 0.01 mg/L NAA; **I,** MS + 0.125 mg/L KIN + 0.01 mg/L NAA; **J,** MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and **K,** pure MS sample.

Plate 3. Sprouted embryo in different MS media.

Plate 4. *In vitro* regenerated plantlets of *P. nitida.*

were obtained from embryo sprouts on media C and F, respectively; this was followed by sprouts on medium F with mean number of roots of 1.87 and medium E for root length (2.84 cm), respectively. The lowest mean number of roots (0.13) and root length (0.15 cm) were recorded in embryo sprouts in media H (Table 2, Figures 2 and 3). There were significant differences in the mean number of roots and root length among embryo sprouts on media H, I, B, D and G and those on media F.

Development of leaves

There were significant differences in the effect of media on leaf formation and development in *P. nitida* (Plates 3 and 4). However, assessment period and interaction of AP and media were not significant on leaf formation and development (Table 1). The highest mean number of leaves (2.87) was obtained in embryo sprout on media D, this was followed by media F with a mean number of leaves of 2.73; the lowest mean value of 1.4 was obtained in media K. There were significant differences in mean number of leaves of embryo sprouts inoculated on media K, B, I, H and C and those on media F and D (Table 2 and Figure 4).

DISCUSSION

Tropical forest tree seeds are known to be recalcitrant with attendant rapid loss of viability. Yet, germination is the onset of every regeneration effort. Seed dormancy often limits plant growth at the early stages which may be injurious to the overall development of such plants. The aseptic culture of plant parts and organs has increasing importance in the mass regeneration and conservation of plant genetic resources. *In vitro* culture of embryo helps to circumvent embryo abortion which may result from genetic incompatibility between the developing embryo and the endosperm during inbreeding and sometimes after hybridization. Bonga and Aderkas (1992) surmised that *in vitro* culture of embryo is sometimes practiced even when normal embryo development and germination are not a problem as seen in *Cocos nucifera*. Also, when embryos are required for planting, *in vitro* cultures of embryos would be easier to handle than the intact nuts (Assy Bah, 1986). Excised embryos germinated within two weeks after inoculation as compared to untreated seeds of the species that germinated 27 days after sowing. Gbadamosi and Oni (2004) had observed that for species with “durian-epigeal” germination; the length of time that it takes to free the apex of the germinating seedling from the encapsulating seed coat determine the growth vigour of such seedlings. A similar result was obtained by Samson et al. (2006) who reported substantial improvements in terms of rapidity and embryo/plantlet regeneration frequencies in *Coffea* spp.

The type and concentrations of growth regulators play an important role during *in vitro* propagation of plant species, hence, the need to screen media types; Mangal et al. (2009) submitted that the establishment of microbe-free, axenic cultures is a major constrain on propagation methods. In the present study, embryo developed best into single shoot plants on MS supplemented with low concentration of cytokinin; El-Agamy (2009) reported that cytokinins are usually used in the micropropagation media to stimulate axillary shoot proliferation. *P. nitida* had optimal shoot and root development in MS media containing 0.05 mg/L KIN + 0.01 mg/LNAA in this study; Mahendran and Narmatha (2009) obtained a similar result for *Satyrium napalense*. On the other hand, Faheem et al. (2011) reported an optimal production of

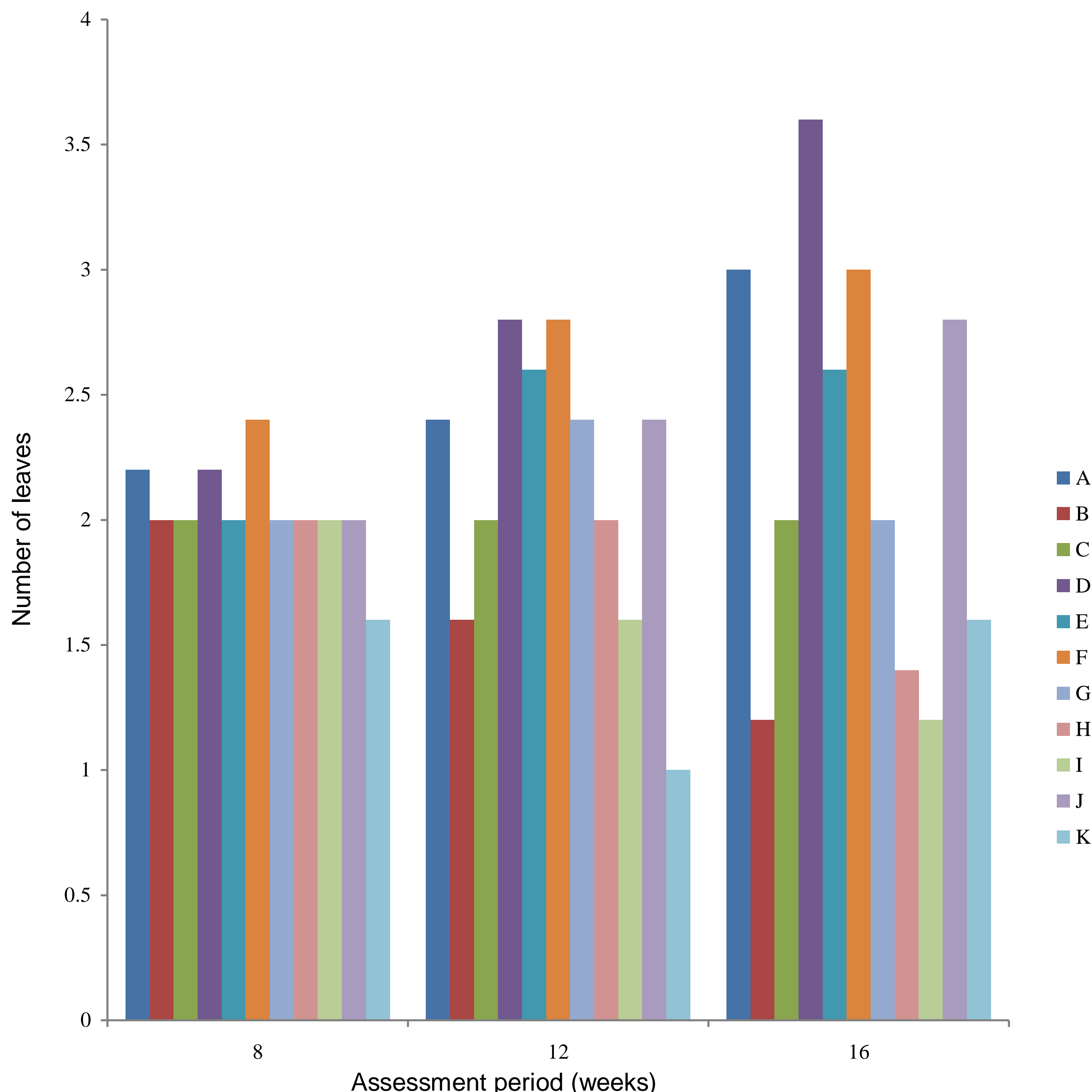

Figure 3. Number of leaves of *P. nitida* under different media across the assessment period. **A,** MS + 0.05 mg/L BAP + 0.01 mg/L NAA; **B,** MS + 0.075 mg/L BAP + 0.01 mg/L NAA; **C,** MS + 0.10 mg/L BAP + 0.01 mg/L NAA; **D,** MS + 0.125 mg/L BAP + 0.01 mg/L NAA; **E,** MS + 0.15 mg/L BAP + 0.01 mg/L NAA; **F,** MS + 0.05 mg/L KIN + 0.01 mg/L NAA; **G,** MS + 0.075 mg/L KIN + 0.01 mg/L NAA; **H,** MS + 0.10 mg/L KIN + 0.01 mg/L NAA; **I,** MS + 0.125 mg/L KIN + 0.01 mg/L NAA; **J,** MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and **K,** pure MS sample.

maximum number of shoots in MS containing BAP + NAA combination; Okere and Adegeye (2011) obtained similar result for *Khaya grandifoliola*. Contrarily, Kaviani et al. (2011) reported an optimal single effect of KIN 2 mg/L without NAA on shoot formation but combined 1 mg/LNAA + 2 mg/LKIN for rooting in *Matthiola incana*. These underscore the importance of devising species specific combination of plant growth regulators in the *in-vitro* regeneration protocol for plant species.

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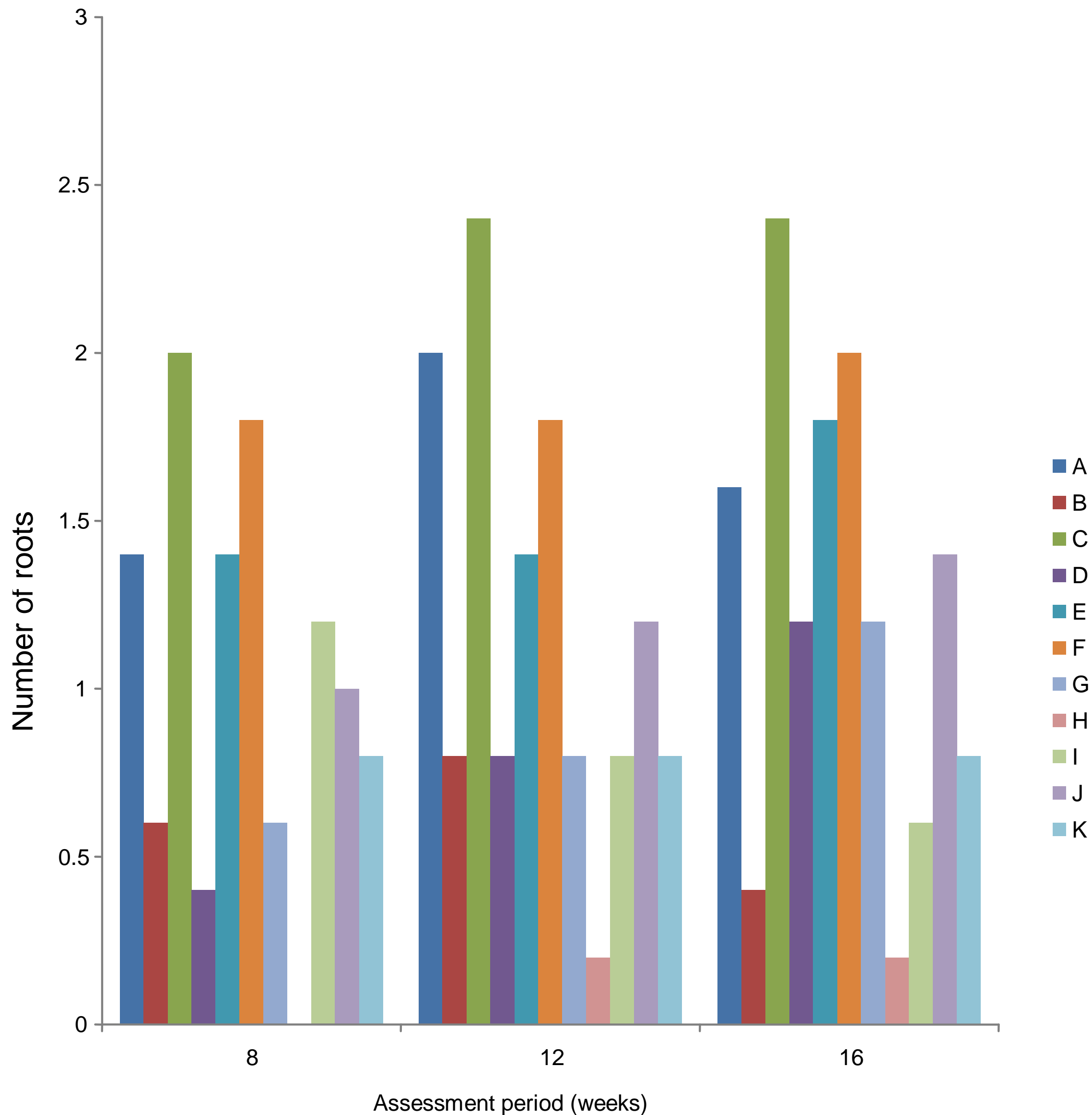

Figure 4. Number of Roots of *P. nitida* under different media across the assessment period. **A,** MS + 0.05 mg/L BAP + 0.01 mg/L NAA; **B,** MS + 0.075 mg/L BAP + 0.01 mg/L NAA; **C,** MS + 0.10 mg/L BAP + 0.01 mg/L NAA; **D,** MS + 0.125 mg/L BAP + 0.01 mg/L NAA; **E,** MS + 0.15 mg/L BAP + 0.01 mg/L NAA; **F,** MS + 0.05 mg/L KIN + 0.01 mg/L NAA; **G,** MS + 0.075 mg/L KIN + 0.01 mg/L NAA; **H,** MS + 0.10 mg/L KIN + 0.01 mg/L NAA; **I,** MS + 0.125 mg/L KIN + 0.01 mg/L NAA; **J,** MS + 0.15 mg/LKIN + 0.01 mg/L NAA; and **K,** pure MS sample.

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