



In vitro germination and buds induction and proliferation from excised embryos of rattan (*Laccosperma secundiflorum* Wendl and *Eremospatha macrocarpa* Wendl)

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1 SUMMARY

Laccosperma secundiflorum and *Eremospatha macrocarpa* are two rattan species particularly recalcitrant to regeneration by seed. This study aims to propose efficient micropropagation media for rattan seedlings regeneration. Excised embryos derived from mature seeds were placed onto seven basal and modified germination media. The obtained shoot buds were then transferred on six micropropagation media for subsequent multiple buds formation. Results showed that the embryos of both species have germinated after 20 days of incubation, whatever the germination medium. Embryos cultured onto MS basal media containing gibberellins (GA₃) 3.46 10⁻⁴ g l⁻¹ and 3.46 10⁻³ g l⁻¹ respectively, developed normally, compared to those without any growth regulator, regardless the species. Concerning the process of micropropagation, collar regions cultured on MS medium supplemented with 4 mg l⁻¹ BAP + 1 mg l⁻¹ IBA or NAA exhibited the highest frequencies of shoots regeneration and the highest average number of shoots (five shoots) per explants for *L. secundiflorum*. With *E. macrocarpa*, 1 mg l⁻¹ IBA or NAA + 2 mg l⁻¹ BAP gave the best results (three shoots per explants).

2 INTRODUCTION

The stems of the climbing palms and rattans of tropical humid Africa are an important natural resource mainly used for the production of cane furniture (Oteng-Amoako and Obiri-Darko, 2002; Zouzou, 2004; Sunderland, 2012). They also play an important role in the manufacture of household commodities in many rural areas (Dransfield, 1992; Sunderland, 2001). There is a great demand of rattan furniture for local as well as international markets (Zoro Bi and Kouakou, 2004a). African rattans have currently been harvested from wild populations, but nowadays,

these are rapidly depleting due to an overexploitation and also to the strong development of industrial and urban activities (Sunderland, 2001; Zoro Bi and Kouakou, 2004a; Kouassi *et al.*, 2009). The most seriously threatened species are *Laccosperma secundiflorum* (P. Beauv.) Kuntze and *Eremospatha macrocarpa* Mann & Wendl, from the Areaceae Palm family, particularly prized due to their high economic value in West Africa and particularly in Côte d'Ivoire (Zoro Bi and Kouakou, 2004a). The harvest of mature plants leads to seed scarcity



and consequently to a decrease of rattan populations through natural germination (Kouassi *et al.*, 2009), notwithstanding limited capacity. In seedbed and nursery condition, *L. secundiflorum* and *E. macrocarpa* seeds were observed to germinate between 13 to 64 weeks with a germination rate of 26 and 36 percent respectively for the two species (Sunderland *et al.* 2001; Kouakou *et al.*, 2008; 2009a). In natural condition, rattans also propagate vegetatively by suckers which can hardly ensure the survival of the species because seedlings are destroyed during canes harvest (Zoro Bi and Kouakou, 2004b; Kouassi *et al.*, 2009; 2014). In Côte d'Ivoire, Zoro Bi and Kouakou (2004b) and Kouakou *et al.* (2009a) have initiated vegetative propagation trials in nursery, from suckers. Although plants can be produced by these techniques, however their number remains too limited and the production cost too high for a large-scale utilization. Moreover, such methods are quite destructive for the natural populations. To circumvent these natural regeneration difficulties, tissue culture has been initiated with Asian rattans giving raise to *in vitro* shoots and plantlets formation. Multiple shoots production and plantlets formation of *Calamus manillensis* H. Wendl. have been reported by Pantena *et al.* (1984). In *Calamus mannan* L., Aziah *et al.* (1985) initiated *in vitro* shoots from cultured embryo further to callus formation while Yusoff (1989) and Goh *et al.* (1997, 2001) obtained several plantlets and multiple shoots from excised embryos. Similar results were observed *in vitro* by Padmanaban and Ilangovan (1989) and by

Dekkers and Rao (1987) from zygotic embryos of *Calamus rotang* L. and *Calamus. trachycoleus* Becc. The basal media commonly used for these investigations derived from Murashige and Skoog-MS (1962), Gamborg-B5 *et al.* (1968) and White (1963). Addition of Benzyl-aminopurine (BAP), Naphthalene-acetic acid (NAA) or Indole-butyric acid (IBA), with concentrations ranging between 2 and 6 mg l⁻¹ was necessary to inducing the formation of multiple shoots (Kouakou *et al.* 2009b; Kumar *et al.*, 2012; Verma and Singh, 2012). Eight (8) to 12 weeks were necessary to obtain shoots or plantlets. The rooted plantlets were successfully acclimatized in pots containing sterilized soil and sand mixture with 75% survival rate in the field conditions (Kundu and Sett, 2000; Verma and Singh, 2012). Several hectares of tissue-culture-issued rattan plants were planted in Malaysia (Goh *et al.*, 1997; Bacilieri *et al.*, 1998; Ali and Barizan, 2001). Unfortunately, tissue culture of African rattans is less advanced. Kouakou *et al.* (2009b) obtained preliminary results by using axillary buds of *L. secundiflorum* seedlings. However, contaminations and tissue browning remained, as for other species, severe hindrances (Kouakou *et al.*, 2009b, Onuoha *et al.*, 2011). Moreover, medium composition and culture conditions are extremely important in tissue culture of Arecacea Palms (Paranjothy, 1993; Jameel and Al-Khayri, 2011). Against this background, the objective of this present work was to optimize *in vitro* techniques for mass propagation of *L. secundiflorum* and *E. macrocarpa*.

3 MATERIALS AND METHODS

3.1 Plant material: Excised embryos of two rattan species, *L. secundiflorum* and *E. macrocarpa*, were used for this study. Mature fruits were collected from three *L. secundiflorum* individuals growing in evergreen swampy forest of “Iles Eotilé” Adiaké, covering 2 ha in the South-East of Côte d'Ivoire (5°9'24"N latitude, 3°16'42"W longitude), and from more than 10 *E. macrocarpa* individual in “Forêt classée de la haute Dodo” Tabou, in the South-West of Côte d'Ivoire (4°41'21"N latitude, 7°06'13" W longitude). The

collected fruits were transferred to the laboratory at University Nangui Abrogoua (Abidjan, Ivory Coast) and stored in sealed plastic bags at 23–25°C, 35–50% RH, during 4 days. Such storage conditions favour fruits fermentation and facilitate seeds extraction. The seeds were sealed in jute bags and air-transported to the laboratory of tropical crop husbandry and horticulture of Gembloux Agro-Bio Tech (University of Liège, Belgium) where micropropagation experimentations were achieved.



3.2 Methods

3.2.1 *In vitro* germination trial: After peeling off the mesocarp, the fleshy sarcotesta was thoroughly scrubbed off and the stony endosperm, including the embryo, was washed under tap water and then immersed for 30 min in disinfection aqueous solution of 3.6% (w/w) NaOCl containing 2–3 drops of Tween 20 followed by three rinsing in sterile distilled water. Embryos were then carefully excised, washed in sterile distilled water and placed on different culture media. All these steps were performed under aseptic conditions of a laminar flow cabinet. Germination of the excised embryos was

assessed on seven different media formulated from three basal media commonly used as mentioned in literature (Paranjothy, 1993; Goh *et al.*, 2001). These were MS salt medium with Gamborg vitamins, designated as MSO (Murashige and Skoog, 1962), B5 medium (Gamborg *et al.*, 1968), and SH medium (Shenk and Hildebrandt, 1972). Beside differences in vitamins and minerals, calcium content of MS (440 mg l⁻¹) is almost three times higher than in the other two media (150 mg l⁻¹ and 200 mg l⁻¹ for B5 and SH, respectively). The composition of the germinated media is indicated in table 1.

Table 1: Composition of germinated media tested on rattan excised embryos

Media	Composition		
	Basal media	Vitamins	Phytohormones
M1	MS salt	Gamborg vitamins	-
M2	MS salt	Gamborg vitamins + 0.5 g l ⁻¹ casein hydrolysate	0.5 g l ⁻¹ BAP
M3	MS salt	Gamborg vitamins + 0.5 g l ⁻¹ casein hydrolysate	1 g l ⁻¹ BAP
M4	MS salt	Gamborg vitamins + 0.5 g l ⁻¹ casein hydrolysate	3.46 10 ⁻³ g l ⁻¹ GA ₃
M5	MS salt	Gamborg vitamins + 0.5 g l ⁻¹ casein hydrolysate	3.46 10 ⁻⁴ g l ⁻¹ GA ₃
M6	Gamborg medium (B5)	-	-
M7	Shenk and Hildebrandt (SH)	-	-

For all the media, 30 g l⁻¹ of sucrose were used as carbon source, 8 g l⁻¹ agar (Invitrogen select agar) as solidifying agent and the pH was adjusted to 5.8 with 0.1 N KOH. Media were dispensed in 25 x 150-mm culture tubes (15 ml per tube) and autoclaved for 30 min at 121°C and 1 x 10⁵ Pa (1.1 kg cm⁻²). One embryo was inoculated per tube. Three replicates were performed by media, each consisting of one rack with 20 explants. Cultures were incubated in growth chamber at 28 ± 2°C and 60 - 75% humidity for eight weeks under total darkness as reported by Padmanadhan and Ilangovan (1989) and Goh *et al.* (2001). Embryos were considered germinated when the emerging radicle was approximately 2.5 mm long (Padmanadhan and Krishnan 1989; Kouakou *et al.*, 2009a). Germination was

recorded daily and the final germination rate was assessed after eight weeks of culture.

3.2.2 Shoots induction and proliferation: The medium inducing the highest rate of actively developed embryos was selected to promoting shoots induction keeping the same composition like during the germination step. The whole plantlet on this medium and measuring 2 - 3 cm has been selected for shoots proliferation. These seedlings were incubated in growth chamber at 28 ± 2°C, 75% relative humidity (RH), and illuminated with cool-white fluorescent lights (PPFD: 25 μmol/m²/s) under a 14/10 h (day/night) photoperiod. One month later, under laminar air flow cabinet, the developing seedlings at two leaf stage were sectioned and the radicle and leaves were discarded to only keep the 1 cm long collar regions. These were used as explants

for shoot induction on six different media supplemented with different combinations of cytokinin (BAP) and auxin (NAA, IBA). For the two species, three replicates were performed per medium, consisting of one rack with 20 explants (collar region). One explant was inoculated per tube. The culture conditions were the same as indicated previously. After 12 weeks of culture, (i) the percentage of explants inducing shoots with 0.5 cm height and (ii) the mean number of shoots produced per explants were recorded.

4 RESULTS

4.1 *In vitro* germination: The excised embryos of *E. macrocarpa* and *L. secundiflorum* swelled during the first week (7 days after incubation) on the seven media tested. With the treatments M1, M6 and M7 (respectively MSO, B5 and SH) without any growth regulators, embryos swelled slowly and formed abnormal seedlings. All embryos (100%) germinated after one month of incubation whatever the species.

3.2.3 Statistical analyses: Data were analyzed using SAS version 6 (SAS, 1999) statistical software. After checking the normal distribution of data by using Kolmogorov-Smirnov test, One-way analysis of variance (ANOVA1) was performed for assessing the influence of various media on the two traits. When the null hypothesis of ANOVA1 was rejected, multiple comparisons using the Least Significant Difference (LSD) test were carried out to test for significant pairwise differences between media. All LSD tests were carried out at $\alpha = 0.05$ significance level.

Embryos incubated on media (M4 and M5) containing GA_3 developed normally (Figure 1A and 2A). Those grown on media M2 and M3, with BAP, germinated but they could not further develop into whole plantlets (Figure 1B and 2B). Seedlings were turgid on the M3 medium, while those on M6 and M7 (medium B5 and SH, respectively) were stunted and exhibited necrotic tissues (Figure 1C and 2C).

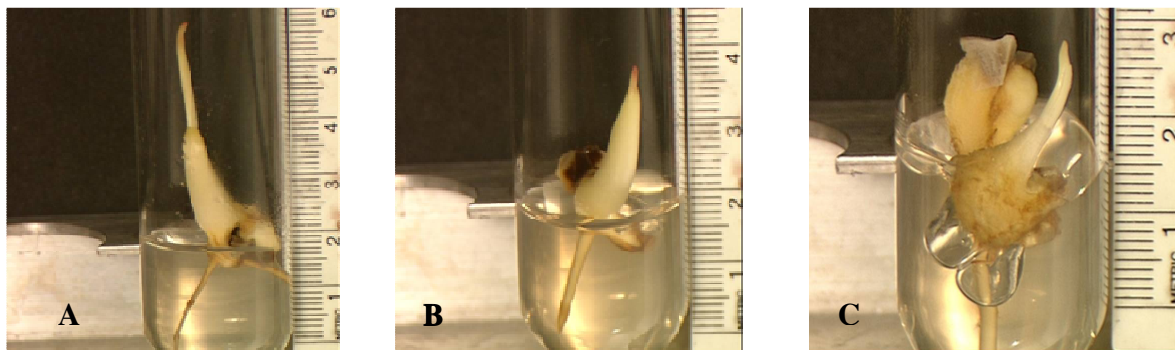


Figure 1: *Vitro* plants (30 day-old) of *Eremospatha macrocarpa* (G. Mann & H. Wendl.) H. Wendl. A, B, C: seedlings from M5 ($MSO + 3.46 \cdot 10^{-3} \text{ g l}^{-1} GA_3$), M3 ($MSO + 1 \text{ mg l}^{-1} BAP$), M6 (B5) culture media, respectively. **MSO:** Murashige and Skoog basal medium, **B5:** Gamborg basal medium

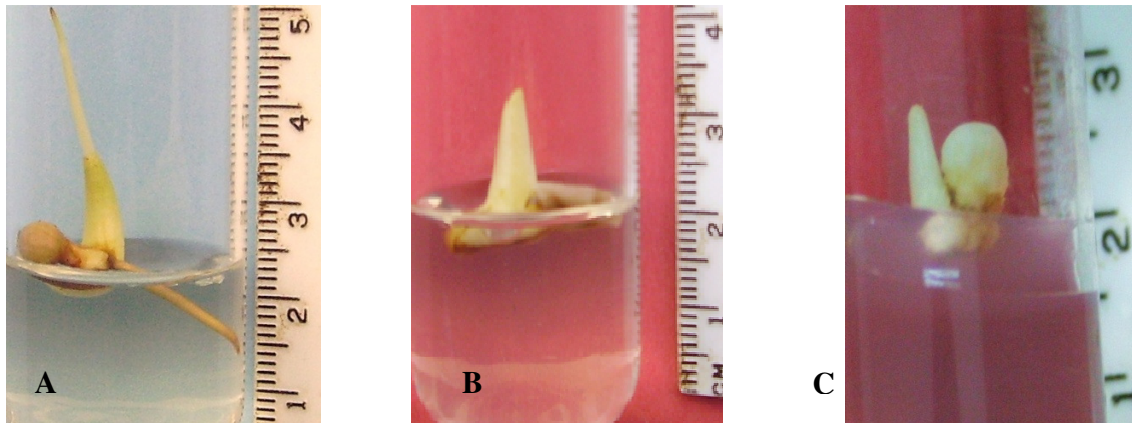


Figure 2: *Vitro* plants (30 day-old) of *Laccosperma secundiflorum*(P. Beauv) Kuntze
A, B, C: seedlings from M5 (MS0 + $3.46 \times 10^{-3} \text{ g l}^{-1} \text{ GA}_3$), M3 (MS0 + $1 \text{ mg l}^{-1} \text{ BAP}$), M7 (SH), culture media, respectively

MSO: Murashige and Skoog basal medium, SH: Shenk and Hilderbrandt medium

4.2 Shoot induction: Shoot induction is characterized by the formation of 'eyes' or buds at the collar region of explants (Figure 3). The mean number of shoots per explants and the mean percentage of explants inducing shoots, after 12 weeks culture, are reported in the table 2. The data showed that shoots induction is significantly influenced by the hormonal

combination, regardless of the species ($P < 0.001$). The percentages of explants with shoots varied from 44 to 89%, with *L. secundiflorum*. The highest percentages were obtained when BAP (4 mg/l) was associated with NAA or IBA. However, with *E. macrocarpa*, 2 mg l^{-1} BAP in combination with NAA or IBA exhibited the highest mean percentages (89%)

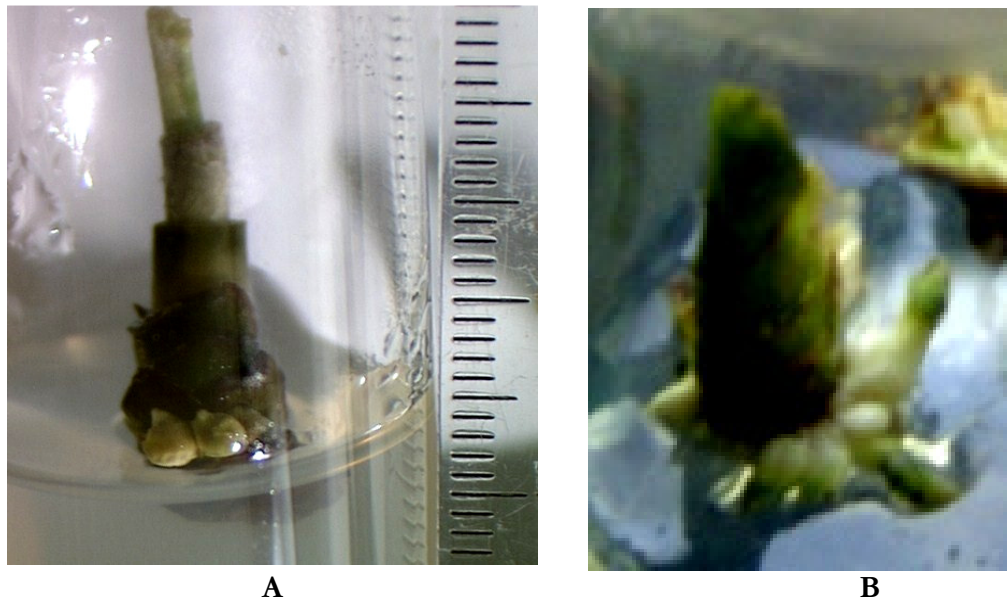


Figure 3: Formation of 'eyes' or buds at the collar region of explants,
A: *Laccosperma secundiflorum* (P. Beauv.) Kuntze, B: *Eremospatha macrocarpa* (G. Mann & H. Wendl.)



Figure 4: Plantlets of *Laccosperma secundiflorum* (P. Beauv.) Kuntze developed from axillaries buds cultivated on MS basal medium with B5 vitamins + 2 mg l⁻¹ BAP + 1 mg l⁻¹ IBA

Concerning the mean number of shoots per explants, the two species responses were significantly influenced by BAP concentration ($P < 0.05$). Indeed, 4 mg l⁻¹ BAP + 1 mg l⁻¹ IBA expressed the highest average number of shoots (5) per explants in *L. secundiflorum*. With *E. macrocarpa*, three (3) shoots per explants were

produced when BAP (2 or 4 mg l⁻¹) was combined with 1 mg l⁻¹ NAA or IBA. Whatever the species, 8 mg l⁻¹ BAP induced the lowest number of shoots characterized by hyperhydricity. Regardless of the species, these shoots, once transplanted on 2 mg l⁻¹ BAP + 1 mg l⁻¹ IBA develop into plantlets (Figure 4).

5 DISCUSSION

5.1 *In vitro* germination: The rapid germination of excised embryos revealed by this study indicates that difficulties of seed germination mentioned in literature are not related to the embryo (Bradbeer, 1988; Bewley, 1997). This result confirms the presence of probable mechanical barrier mentioned by Kouakou *et al.* (2009a) in the two species. Germination would be facilitated by the absence of seed testa between the embryo and the water available in the culture medium and the presence of growth regulators. Water and oxygen diffusion, essential for germination, takes place without hindrance (Finch-Savage *et al.* 2006; Debeaujon *et al.*, 2007, Nonogaki *et al.*, 2007). The best developing of plantlets cultivated on MS medium, with respect to the other two media could be due to its high mineral content, precisely

in calcium. Indeed, in the MS basal medium, the calcium content is twice and four time higher than in the SH and B5 basal medium respectively. Calcium is a component of cell walls and a cofactor for several enzymes involved in ATP hydrolysis. This ATP hydrolysis provides essential energy for cell metabolism (Kumar and Loh, 2012; Roberta, 2013). The low level of calcium in the basal media B5 and SH could explain the difficulty of germination and plantlet development (George and De Klerk, 2007). Padmanadhan and Ilangovan (1989) and Paranjothy (1993) have mentioned that B5 and SH basal media are not suitable for *in vitro* germination of many Asian rattans whatever hormonal combination. The positive effect of GA₃ on embryos germination has been widely discussed in the literature. Indeed, enzymatic



studies by Shepley *et al.* (1972), Feurtado and Kermodé (2007) and Gubler *et al.* (2008) have shown that, when some dormant seeds such as barley (*Hordeum vulgare* L.) are treated with GA₃, the germination process is preceded by a high activity of α -amylase synthesis in the embryos. This plant hormone could induce the synthesis of α -amylase, which will hydrolyze polysaccharides into small molecules. These monosaccharides are used to achieve embryo growth and thereby accelerate the process of germination and the healthy development of seedlings (Shepley *et al.*, 1972; Gubler *et al.*, 2008).

5.2 Effect of BAP combined with auxin on multiple shoots production: The fungal and viral contaminations of culture media and the high content of phenolic compounds and its accumulation in culture media which affects viability of explants and survival percentage particularly, in palm tissue culture, (Titov *et al.*, 2006; Onuoha *et al.*, 2011; Mustafa *et al.*, 2013) have been avoided by the use of excised embryos. BAP is the most widely used cytokinin for multiple shoots induction of rattan (Yusoff, 1989; Goh *et al.*, 2001; Kouakou *et al.*, 2009b) and many other species such as *Hibiscus* (Samanthi *et al.*, 2004). According to Herath *et al.* (2004), a single exposure of *Hibiscus cannabinus* L. explants to a low concentration of BAP improve the development of the shoot apex and causes cells that would normally remain quiescent to multiply

repeatedly and give rise to supernumerary vegetative buds. In our trials, multiple shoots were developed from the explants of both species (Table 2). The results show that the explants have a great potential to produce shoots. However, this ability, regardless of the type of auxin combined, decreases with the high concentrations of BAP ($> 4 \text{ mg l}^{-1}$). These results confirm reports of Bingshan *et al.* (2000), Verma and Singh (2012) respectively, with *Calamus* spp and *Acorus calamus* L. two Asian rattan species. Indeed, these authors have highlighted a better shoot production for BAP concentrations ranging from 1 to 6 mg l^{-1} , then a decrease in shoot production at 8 mg l^{-1} . According to our results and those of Gunawan (1995) and Bingshan *et al.* (2000), concentrations of BAP $> 4 \text{ mg l}^{-1}$ did not increase the number of shoots induced by explants in rattan. However, the high levels of BAP lead to a hyperhydricity of explants. The action of cytokinins is more noticeable in tissue culture when there are combined with auxins. However, this cytokinin/auxin ratio, for an optimal shoot induction, should be adjusted according to the species (George, 2007; Staden *et al.*, 2007; Abahmane, 2013). Cytokinins are involved in protein synthesis but high concentrations of BAP cause a disruption of cellular metabolism (Ziv and Chen, 2007). This could explain the hyperhydricity of shoots on these media.



Table 2: Effect of BAP added with NAA or IBA into MS basal medium on shoots production from *Laccosperma secundiflorum* and *Eremospatha macrocarpa* explants (collar region) 12 weeks after incubation

		<i>Laccosperma secundiflorum</i>		<i>Eremospatha macrocarpa</i>	
Auxines	BAP (mg/l)	Mean percentage (\pm SD) of explants bearing shoots	Mean number (\pm SD) of shoots per explants	Mean percentage (\pm SD) of explants bearing shoots	Mean number (\pm SD) of shoots per explant
NAA (1 mg/l)	2	83.33 \pm 0.0 ^b	2.28 \pm 1.32 ^b	88.89 \pm 19.25 ^b	2.00 \pm 1.37 ^b
	4	86.89 \pm 9.62 ^b	2.66 \pm 1.49 ^b	72.22 \pm 9.62 ^b	2.44 \pm 1.67 ^b
	8	44.44 \pm 19.24 ^a	0.72 \pm 0.96 ^a	33.33 \pm 0.0 ^a	0.66 \pm 1.03 ^a
IBA (1 mg/l)	2	77.77 \pm 9.22 ^{ab}	2.11 \pm 1.45 ^b	78.89 \pm 19.25 ^b	2.16 \pm 1.15 ^b
	4	88.89 \pm 9.62 ^b	4.44 \pm 1.97 ^c	61.11 \pm 9.62 ^b	1.94 \pm 1.69 ^b
	8	61.11 \pm 9.62 ^a	0.89 \pm 0.90 ^a	22.11 \pm 9.72 ^a	0.44 \pm 0.92 ^a

Means followed by same letters are not significantly different at the 5% level (Least Significant Difference LSD test)



6 CONCLUSION

Evidence of micropropagation of *L. secundiflorum* and *E. macrocarpa* with excised embryos has been demonstrated here for the first time. Nonetheless, the procedure developed in this work would need considerable improvement before it could be adopted for large-scale production. The determination of efficient conditions of culture such as temperature, relative humidity and a further improvement of

culture medium composition is required to increase the mean number of shoots produced per explant that is necessary for better and faster regeneration of uniform plants from desired genotypes. Studies on the survival and growth of the plantlets under nursery and field conditions and others methods such as somatic embryogenesis of immature excised embryos must be investigated.

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