



Revista Árvore

ISSN: 0100-6762

r.arvore@ufv.br

Universidade Federal de Viçosa  
Brasil

Souza Souza Pádua, Marlúcia; Viela Paiva, Luciano; Texeira da Silva, Luis Gustavo; Coutinho Silva, Luciano; Cristina Stein, Vanessa

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Revista Árvore, vol. 38, núm. 6, noviembre-diciembre, 2014, pp. 1095-1102

Universidade Federal de Viçosa  
Viçosa, Brasil

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## IN VITRO DEVELOPMENT AND ACCLIMATIZATION OF DENDEZEIRO (*Elaeis guineensis*)<sup>1</sup>

Marlúcia Souza Souza Pádua<sup>2</sup>, Luciano Viela Paiva<sup>3</sup>, Luis Gustavo Texeira da Silva<sup>4</sup>, Luciano Coutinho Silva<sup>5</sup> e Vanessa Cristina Stein<sup>6</sup>

**RESUMO** – Fruits and almond from the *dendzeiro*, oil palm belonging to the *Elaeis* genus, are widely used for the production of cooking oils or for the pharmaceutical and cosmetic industries. In the last decade, this oil palm also emerged as a promising source for commercial biofuel production. This study evaluated the effect of different culture media, MS (MURASHIGUE AND SKOOG) and Y3 (EEUWENS) and carbohydrates during *in vitro* germination of zygotic embryos, the effect of growth regulators GA<sub>3</sub>, NAA and BA *Ponin vitro* seedling development, and the survival rate of acclimatized seedlings of Manicoré hybrid (*Elaeis oleifera* x *E. guineensis*). Zygotic embryos were inoculated on MS and modified Y3 media, supplemented with different sucrose concentrations (30, 45, and 60 gL<sup>-1</sup>) or sorbitol (36 gL<sup>-1</sup>), and the germination rate was evaluated after 30 days. Subsequently, seedlings were transferred to modified Y3 culture medium supplemented with different GA<sub>3</sub> concentrations (3.5 and 7 mgL<sup>-1</sup>) or without it, combined or not with 1 mgL<sup>-1</sup> of NAA, 5 mgL<sup>-1</sup> of BAP. The highest germination percentage of germinated embryos (92%) was observed in MS medium supplemented with 36 gL<sup>-1</sup> sorbitol. Culture media supplemented with growth regulators GA<sub>3</sub>, NAA and BAP promoted greater shoot length than control media. Rooted seedlings showed high survival percentage (85%) during acclimatization.

**Palavras-chave:** Germination; Carbohydrates; Growth regulators.

## DESENVOLVIMENTO IN VITRO E ACLIMATIZAÇÃO DE PLÂNTULAS DE DENDEZEIRO

**ABSTRACT** – Frutos e amêndoas de dendzeiro, uma palmeira pertencente ao gênero *Elaeis*, são amplamente usados para a produção de óleos culinários ou para as indústrias farmacêutica e de cosméticos. Na última década esta palmeira oleífera também emergiu como fonte promissora para a produção comercial de biocombustíveis. Os objetivos deste trabalho foram avaliar o efeito de diferentes meios de cultura, MS (MURASHIGUE; SKOOG) e Y3 (EEUWENS) e de carboidratos na germinação *in vitro* de embriões zigóticos, o efeito dos reguladores de crescimento GA<sub>3</sub>, ANA e BAP no desenvolvimento das plântulas *in vitro* e a taxa de sobrevivência dessas quando aclimatizadas do híbrido Manicoré (*Elaeis oleifera* x *E. guineensis*). Embriões zigóticos foram inoculados em meio de cultivo MS e Y3 modificado, suplementados com diferentes concentrações de sacarose (30, 45 e 60 gL<sup>-1</sup>) ou sorbitol (36 gL<sup>-1</sup>) e, após 30 dias a taxa de germinação foi avaliada. Posteriormente, as plântulas foram transferidas para meio de cultivo Y3 modificado suplementado com diferentes concentrações de GA<sub>3</sub> (3,5 e 7 mgL<sup>-1</sup>) ou sem, combinadas ou não com 1 mgL<sup>-1</sup> de ANA, 5 mgL<sup>-1</sup> de BAP. A maior porcentagem de embriões germinados (92%) foi obtida em meio de cultivo MS suplementado com 36 gL<sup>-1</sup> de sorbitol.

<sup>1</sup> Recebido em 11.12.2013 aceito para publicação em 02.10.2014.

<sup>2</sup> Programa de Pós-Graduação em Biotecnologia Vegetal, Universidade Federal de Lavras, UFLA, MG – Brasil. E-mail: <marluciabio@yahoo.com.br>.

<sup>3</sup> Departamento de Química, Universidade Federal de Lavras, UFLA, MG – Brasil. E-mail: <luciano@dqi.ufla.br>.

<sup>4</sup> Mestrado em Biotecnologia Vegetal, Universidade Federal de Lavras, UFLA, MG – Brasil. E-mail: <lgustavots@gmail.com>.

<sup>5</sup> Doutorado em Fisiologia Vegetal, Universidade Federal de Lavras, UFLA, MG – Brasil. E-mail: <lucoutsilva@yahoo.com.br>.

<sup>6</sup> Unidade especial de Ciências Biológicas, Universidade Federal de Goiás, UFG, MG – Brasil. E-mail: <vanessa.stein@hotmail.com>.

*Os meios de cultivo suplementados com reguladores de crescimento GA<sub>3</sub>, ANA e BAP promoveram maior comprimento da parte aérea que os meios sem reguladores. As plântulas com raiz apresentaram a maior porcentagem de sobrevivência (85%) durante a aclimatização.*

*Keywords: Germinação; Carboidratos; Reguladores de crescimento.*

## 1. INTRODUCTION

Petroleum-based fuels are the main source of energy in the world, but increased environmental concerns and the predictions of its extinction increases the need for new energy sources, preferably renewable ones (TASHTOUSH et al., 2003; SOARES et al., 2011; RAE; BRADLEY, 2013). Biofuel production using oil plants is a good alternative for a renewable and biodegradable fuel. Brazil stands out in this scenario due to its large territorial dimensions, edaphic and climatic diversity and its many exploitable plant species for this purpose (SOARES et al., 2011).

Among various species of oil plants, palm trees are the most promising for biodiesel production (NUNES et al., 2008; SOARES et al., 2011). Particularly, the oil palm (*Elaeisguineensis* Jacq.) commonly known as “dendê” stands out as one of the most productive oil crops. Yielding on average of oil production about 6.000 kg/hectare, which corresponds to 1.5 times the yield of coconut oil, twice the olive oil, and more than ten times the soybean oil production, this oil palm is a promising source for commercial biofuel production (FURLAN et al., 2003; LIN et al., 2009).

Besides the prospect of the use of dendê oil for biofuel production, two other types of oils are already exploited for other purposes. One oil type is extracted from the fruit pulp and the other from the almond (BOARI, 2008; KONAN et al., 2005). The fruit pulp oil is widely used as a cooking oil and the almond oil is used by the pharmaceutical, cosmetic and perfumery industries (BOARI, 2008; FURLAN et al., 2003; KONAN et al., 2005).

Due to the economic importance of oil palm, breeding studies have developed hybrids with economic traits of interest. Crossing between the African species *E. guineensis* and the American *E. oleifera* generated the hybrid BRS Manicoré, obtained by Brazilian company Embrapa-Amazônia Ocidental.

The main features inherited from *E. oleifera* are resistance to lethal yellowing (COLLARES, 2011) a disease associated with phytoplasmas (BEAKBANE et al., 1972),

small size and bioenergy oil quality, while high oil production was inherited from *E. guineensis* (COLLARES, 2011). Thus, this hybrid presents a great commercial interest, generating a high demand for seeds, which is the main form of oil palm propagation. However, *E. guineensis* displays seed dormancy with germination occurring within 1-3 years, besides the low germination rate (no more than 30%) (MARTINE et al., 2009; LUIS et al., 2010). These factors hinder the process to obtain seedlings on a commercial scale and consequently the implementation of new crops.

In order to accelerate seedling production on a commercial scale, culture of zygotic embryos can be favorable for reducing germination time and obtaining vigorous, healthy seedlings (SOARES et al., 2011). The culture medium composition, source and amount of carbohydrates in *in vitro* culture are determinant factors for increased *in vitro* embryo germination (HU; FERREIRA, 1998; REGO-OLIVEIRA et al., 2003; TRUONG et al., 2013).

In addition, carbohydrates are commonly used in tissue culture (sucrose, fructose, glucose, galactose etc.), sorbitol is also added as an energy source in the culture medium for oil palm embryos germination, moreover, this sugar promoted 100% germination of *Elaeisguineensis* Jacq. var. Tenera somatic embryos (TECHATO; HILAE, 2007).

*In vitro* environment normally has high relative humidity, low light intensity and reduced gas exchange, resulting in low rates of transpiration and photosynthesis of micropropagated plants (SHIN et al., 2013). During the acclimatization in a greenhouse, some morphological, anatomical and physiological changes will occur, making plants able to grow in this new environment. This process may cause stress in plants thus limiting seedling production. Thus, physiological features as well as acclimatization conditions are extremely important to successful large-scale production of seedlings (BARBOZA et al., 2006). The aims of this study were: (i) to evaluate the *in vitro* germination of BRS Manicoré hybrid (*Elaeisguineensis* x *E. oleifera*) zygotic embryos in two culture media

supplemented with different carbohydrate sources; (ii) evaluate the effect of growth regulators on *in vitro* seedling development; and (iii) evaluate the survival rates of plants during acclimatization phase.

## 2. MATERIAL AND METHODS

### 2.1. Plant material

The trial was performed at the Central Laboratory of Molecular Biology, Federal University of Lavras, state of Minas Gerais, Brazil. Immature fruits of *Elaeisoleifera* x *E. guineensis* hybrid BRS Manicoré were provided by Denpasa Company, state of Pará, 90 to 100 days after pollination.

Fruits were first washed in sodium hypochlorite (1.25% active chlorine), opened with the aid of a vice grip to remove epicarp, mesocarp and endocarp. The almonds were extracted and rinsed in tap water before decontamination in a laminar flow hood by immersion in 70% alcohol for 30 seconds following by plunge for 20 min into 100 mL of a sodium hypochlorite solution (1.25% active chlorine) containing three drops of Tween. Subsequently, the almonds were rinsed three times in sterile water, five minutes each, with continuous stirring.

### 2.2. Effect of different culture media and sucrose concentrations on germination of zygotic embryos

Decontaminated embryos were isolated from the almonds and inoculated in Petri dishes containing 45 mL of culture medium. MS (MURASHIGE; SKOOG, 1962) and modified Y3 (EEUWENS, 1976) culture media were tested with different carbohydrate concentrations: sucrose (30, 45, 60 gL<sup>-1</sup>) and sorbitol (36 gL<sup>-1</sup>) and a control without carbohydrate.

Embryos were maintained under a 16-hour photoperiod at 26 ± 2 °C for 30 days after inoculation and then germination rate was evaluated. The experiment was conducted in a 2x5 factorial arrangement of treatments in a randomized block design, with five replicates of five embryos each per treatment. Data analysis was performed by Kruskal-Wallis test (1952), considering  $P \leq 0.05$ .

### 2.3. Effect of growth regulators on *in vitro* seedlings development

To evaluate the effect of growth regulators on *in vitro* development, we transferred 30-day old seedlings, grown in Y3 medium, supplemented with

30 gL<sup>-1</sup> sucrose to Magenta® jars containing 50 mL of Y3 medium (EEUWENS, 1976), without amino acids (modified Y3 medium). The medium was supplemented with 3.5 or 7 mgL<sup>-1</sup> GA<sub>3</sub> (gibberellic acid) and absence or 1 mgL<sup>-1</sup> NAA (naphthaleneacetic acid) combined with absence or 0.5 mgL<sup>-1</sup> BAP (6-Benzylaminopurine). As an energy source, 30 gL<sup>-1</sup> sucrose was used, the media was gelled with 0.6% agar, and pH was adjusted to 5.8. After inoculation, seedlings were maintained in a growth room chamber with white light (photon irradiance of 40 μmol m<sup>-2</sup>s<sup>-1</sup>) under 16-hour photoperiod at 27 ± 2 °C.

Seventy-five days after incubation, the following parameters were analyzed: shoots length, number of leaves, presence of roots, root length, fresh and dry weight of shoots and roots.

The experiment was conducted in a randomized block design with five replications of five seedlings each per treatment. Data were subjected to analysis of variance (ANOVA) and means were compared by Tukey test, at 5% probability, using SISVAR program (FERREIRA, 2008).

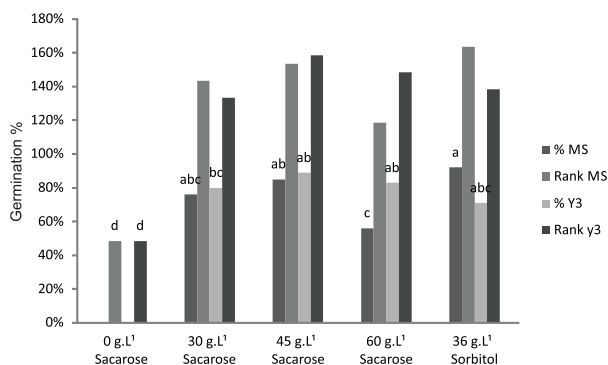
### 2.4. Acclimatization

For acclimatization, 100 seedlings presenting roots and 100 seedlings presenting no roots, all grown in Y3 medium supplemented with 30 gL<sup>-1</sup> sucrose for 90 days were used. Seedlings were washed in running water to remove medium residues and transplanted into 56 cm<sup>3</sup> polyethylene tubes containing pine bark substrate and the tubes were transferred to a greenhouse. After 60 days, survival rate of seedlings was assessed. Surviving seedlings were considered as acclimatized.

## 3. RESULTS

The addition of sucrose at different concentration or sorbitol to the culture medium was essential for oil palm embryos germination. As can be seen in Figure 1, treatments without sugar (control) presented no embryo germination.

High germination rate of zygotic embryos was observed in the medium supplemented with carbohydrates (Figure 1). Embryos cultured on MS medium containing 36 gL<sup>-1</sup> sorbitol presented 92% germination, however, it was not statistically different either from treatments on MS medium containing sucrose at concentration 30 gL<sup>-1</sup> with 76% germinated embryos



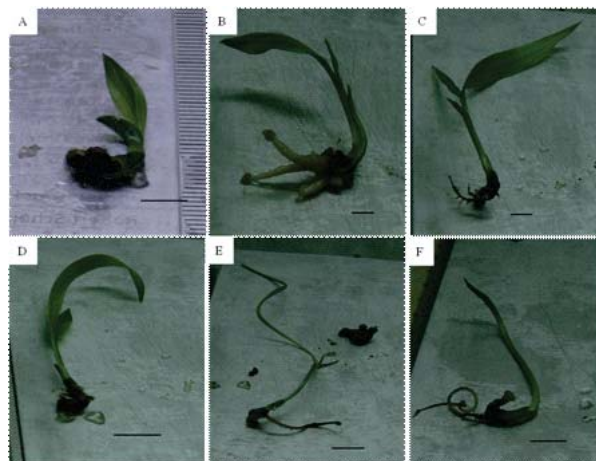
**Figure 1** – In vitro germination percentage of *E. oleifera* x *E. guineensis* Jacq. hybrid Manicoré embryos inoculated on MS medium supplemented with carbohydrates. Means followed by the same letter do not differ statistically by Kruskal-Wallis test at 5%.

**Tabela 1** – Porcentagem de germinação in vitro de embriões de *E. oleifera* x *E. guineensis* híbrido Manicoré, inoculados nos tratamentos com o meio de cultivo MS suplementado com carboidratos. Médias seguidas pela mesma letra não diferem estatisticamente pelo teste de Kruskal-Wallis a 5%.

or at concentration 45 gL<sup>-1</sup> with 85% germination, or from treatments with Y3 medium supplemented with sucrose at all concentrations.

Regarding to seedling growth, the use of growth regulators promoted a favorable effect to the parameter shoot length (SL) making them different from the control. On one hand, the longest shoot (6.2 cm) was observed using 7.0 mg L<sup>-1</sup> GA<sub>3</sub> (Table 1). On the other hand, the longest root (3.6 cm) was observed on the control treatment (Table 1). Number of leaves and the presence

of roots presented no significant differences among treatments (Table 1). Although seedlings were more elongated in the presence of GA<sub>3</sub>, there was a reduced leaf area compared with seedlings grown in the other treatments, thus, this regulator negatively affected leaf area in *in vitro* explants (Figure 2).



**Figure 2** – In vitro germinated seedlings of *E. oleifera* x *E. guineensis* hybrid Manicoré 75 days old. A - Seedling in Y3 culture medium without growth regulators; B - 5.37 μM NAA; C - 2.21 μM BAP; D - 5.37 μM NAA x 2.21 μM BAP; E - 10 μM GA<sub>3</sub>; and F - 20 μM GA<sub>3</sub>. Bars = 1 cm.

**Tabela 2** – Plântulas germinadas de *E. oleifera* x *E. guineensis* híbrido Manicoré in vitro com 75 dias de cultivo. A - Plântula em meio de cultivo Y3, ausência de reguladores de crescimento; B - 5,37 μM de ANA; C - 2,21 μM de BAP; D - 5,37 μM de ANA x 2,21 μM de BAP; E - 10 μM de GA<sub>3</sub>; e F - 20 μM de GA<sub>3</sub>. Barras = 1 cm.

**Table 1** – Average values for shoot length (SL), leaf number (LN), root presence (RP) and root length (RL) of *E. guineensis* x *E. oleifera* seedlings after 45 days in culture with growth regulators.

**Tabela 1** – Valores médios do comprimento da parte aérea (CPA), número de folhas (NF), presença de raiz (PR) e comprimento de raízes (CR) de plântulas de *E. oleifera* x *E. guineensis*, após 45 dias de cultivo na presença dos reguladores de crescimento.

Treatment	SL (cm)	LN	R (%)	RL (cm)
Control	2.0 b	1.9 a	32 a	3.6 a
1.0 mgL <sup>-1</sup> NAA	4.0 ab	2.3 a	34 a	1.6 b
0.5 mgL <sup>-1</sup> BA	4.4 a	1.9 a	30 a	2.1 b
1.0 mgL <sup>-1</sup> NAA + 0.5 mgL <sup>-1</sup> BA	3.9 ab	2.4 a	20 a	2.4 ab
3.5 mgL <sup>-1</sup> GA <sub>3</sub>	4.7 a	1.8 a	38 a	2.7 ab
7.0 mgL <sup>-1</sup> GA <sub>3</sub>	6.2 a	1.9 a	20 a	1.8 b
CV (%)	42.1	41.2		29.2

Means followed by the same letters in columns do not differ significantly by Tukey test at 5% probability.

As médias seguidas pelas mesmas letras nas colunas não diferem significativamente entre si, pelo teste de comparação de médias de Tukey a 5% de probabilidade.



Regarding to seedlings biomass, we found no significant differences in fresh matter of shoots among treatments. However, when the roots were analyzed, an increase in the biomass of roots grown in the presence of auxins was observed. Addition of growth regulator auxin in combination with cytokinin increased shoot dry matter in seedlings, however, this association did not similarly favor either root growth or dry matter (Table 2).

#### 4. DISCUSSION

The germination rates obtained in this study (92%) are highly significant when compared to results obtained with the conventional germination method (approximately 30%), which is based on direct seed germination without embryo rescue (MARTINE et al., 2009). Our results for germination BRS Manicoré hybrid (*Elaeis guineensis* x *E. oleifera*) using sorbitol as carbon source (92%) confirm the high rate germination reported by Te-chato and Hilae (2007) with somatic embryos of *E. guineensis* hybrid Tenera.

Similar germinated rates of embryos (80%) in Y3 medium supplemented with carbohydrates were found by Cardoso et al. (2010) when testing MS and Y3 media, either full or with half-strength. These trials showed higher germination rates (85.18%) in full Y3 medium for *E. guineensis* hybrid CN514. Similar results were also obtained in zygotic embryos of peach palm (*Bactris gasipaes*) when evaluating MS, Y3 and WH culture media (WHITE, 1942), where the highest

germination rates (95.5%) were found in Y3 medium. However, these values were not statistically different from those obtained on MS medium (STEINMACHER, 2005).

Our results are also in agreement with Hu and Ferreira (1998), which highlight the need for an exogenous source of sugars when embryos are immature, as they have no source of nutrients in their cotyledons. Nunes et al. (2008) observed that germination rate of immature embryos of jatropha (*Jatropha curcas* L.) increased proportionally to increase of sucrose in the culture medium, with the highest rate (83.68%) observed in the concentration 60 g L<sup>-1</sup> sucrose. Pereira et al. (2006a) evaluated germination of mature and immature embryos of *Astrocaryum mulei* with increasing concentrations of sucrose, concluding that the culture medium supplemented with 15 g L<sup>-1</sup> resulted in higher germination rates of mature embryos while 30 g L<sup>-1</sup> provided better results for immature embryos.

Angelo et al. (2007) also evaluated different sources of carbohydrates such as glucose and sucrose associated with solid and liquid MS media for oil palm embryos and obtained better results with liquid MS medium supplemented with 20 g L<sup>-1</sup> or 30 g L<sup>-1</sup> glucose. Rego-Oliveira et al. (2003) assessed supplementation of culture medium with glucose, maltose and sucrose at different concentrations, concluding that the treatment with 60 g L<sup>-1</sup> sucrose was the most suitable for embryo germination of oncidiums (*Oncidium varicosum*).

**Table 2** – Shoot and root biomass of *E. guineensis* hybrid Manicoré seedlings maintained for 75 days in *in vitro* culture medium supplemented with growth regulators. Mean values for shoots fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), and root dry weight (RDW) of seedlings of dendezeiro 75 days

**Tabela 2** – Biomassa de parte aérea e de raiz de plântulas de dendezeiro híbrido Manicoré aos 75 dias de cultivo *in vitro*, em meio de cultura suplementado com reguladores de crescimento. Valores médios de massa fresca de parte aérea (MFA), massa seca de parte aérea (MSA), massa fresca de raiz (MFR) e massa seca de raiz (MSR) de plântulas de dendezeiro aos 75 dias.

Tratamentos	SFW(mg)	SDW(mg)	RFW(mg)	RDW(mg)
Control	300 a	21 c	20 b	4 bc
5.37 µM NAA	19 a	34 ab	71 a	12 ab
2.21 µM BA	20 a	27 bc	42 ab	8 abc
5.37 µM NAA+ 2.21 µM BA	29 a	41 a	26 b	7 abc
10 µM GA <sub>3</sub>	17 a	24 bc	21 b	14 a
20 µM GA <sub>3</sub>	19 a	26 bc	18 b	3 c
CV (%)	48.58	29.37	70.00	78.29

Means followed by the same letters in columns do not differ significantly by Tukey test at 5% probability.

As médias seguidas pelas mesmas letras nas colunas não diferem significativamente entre si, pelo teste de comparação de médias de Tukey a 5% de probabilidade.

The need for a smaller amount of sucrose in the culture medium may favor acclimatization future steps, as high concentrations can cause both structural and functional alterations in organs (POSPÍSILOVÁ et al., 1999). Therefore, our results are favorable since high germination rates were also obtained at the lowest concentration of sucrose assessed (30 g L<sup>-1</sup>) in both Y3 and MS culture media (Figure 1).

Regarding the effect of growth regulators on seedling growth, although GA<sub>3</sub> provided a better shoots growth, the leaf area was reduced. Growth of seedlings in culture medium supplemented with gibberellins was not accompanied by leaf area expansion, thus requiring a balance of regulators in the culture medium. This fact may be related to the role of gibberellins, which generally promote cell elongation (TORRES et al., 1998) and probably promoted a higher stem growth at the expense of leaf expansion.

There was no increase in number of leaves in seedlings grown in the medium supplemented with BAP, although this growth regulator is related to leaf formation (TORRES et al., 1999). In addition, we found no significant root formation in medium with NAA auxins, although it is related to root formation (ASSIS; TEIXEIRA, 1998).

Similar results were found by Pereira et al. (2006b), who evaluated shoot length of cat's claw (*Uncaria tomentosa*) after 45 days of cultivation. The authors observed higher shoot length using GA<sub>3</sub>, although there were no statistical differences in the absence of this regulator. Ledo et al. (2001) evaluated *in vitro* seedling growth of açai palm (*Euterpe oleracea*) with NAA and BAP regulators and reported higher shoot length in the medium with NAA (2,68 µM) plus BAP (1,11; 1,55; 2,22 µM). Reis et al. (2008) evaluated shoot length of lemon balm (*Melissa officinalis*) on MS medium in the presence and absence of BAP and concluded that shoot length was higher with BAP.

Regarding biomass, different results were obtained by Villa et al. (2005), who observed that shoot dry weight of blackberry (*Rubus fruticosus*) was higher in the culture medium supplemented with 1 mg L<sup>-1</sup> BAP.

As for acclimatization, previous rooting of seedlings is crucial for successful process. According to Costa et al. (2008), high survival rates are found during the acclimatization phase, when *in vitro* elongation and rooting occur in most species. Similar survival rates were also

found by Thuzar et al. (2011) in oil palms over six centimeters long acclimated in substrates of sand, coconut husk and carbonized rice bran (1:1:1). Steinmacher (2005) also found that seedlings of peach palm (*Bactris gasipaes*) under six cm long obtained from *in vitro* culture had a lower survival rate.

Further studies are needed on the hormonal balance of auxins and cytokinins to optimize shoot and root development of oil palm seedlings.

## 5. CONCLUSION

Supplemental carbohydrate sources are essential to promote high *in vitro* germination rates of zygotic embryos of oil palm hybrid BRS Manicoré.

Supply of growth regulators promoted higher shoot growth.

Rooted seedlings are more apt to acclimatization phase since they had 85% survival rate, which demonstrates the need for root system formation.

## 6. ACKNOWLEDGEMENT

To the undergraduate student Daniella Cristina de Souza Borges for the contribution in the experiments and to the funding agencies FAPEMIG, CNPq e CAPES.

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