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Influence of culture medium and age of zygotic embryos on *in vitro* germination of *Elaeis guineensis* Jacq.

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Oil palm cultivation has acquired economic importance due to rising demand for vegetable oils used in food, pharmaceutical, cosmetic and most recently, in biofuel industries. However, the commercial production increase of oil palm is limited by plantlet production, as it primarily uses seeds that require a long germination period and have low germination rates. In this study we evaluated the effect of embryo age (time after pollination), culture media and presence of carbohydrates on *in vitro* germination of zygotic embryos of oil palm (*Elaeis guineensis* Jacq.) Manicoré hybrid. Embryos at 80, 90, 100 and 110 days post-anthesis were inoculated in MS medium or modified Y3 medium, with or without 3% sucrose. Media were renewed after 30 days of inoculation, and plantlets were kept for 45 days until collection for histological analyses. The embryos did not germinate in medium without sucrose. Embryos of 100 days post-anthesis in MS medium showed the best result for germination rate (88%), which did not differ statistically from Y3 medium. As for the parameters, 90-day embryos showed better results for number and length of roots in Y3 medium. It was concluded that 90-day embryos cultivated in Y3 medium generate plantlets in better conditions to be transferred to acclimatization.

Key words: Oil palm (*Elaeis guineensis* Jacq.), germination, sucrose, Y3 medium, MS, anatomy, embryo culture.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a monocot perennial and monoecious plant from the Arecaceae family. It is also an oilseed plant with a long life cycle (Morcillo et al., 2007). This crop has been economically important due to

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high oil yield of improved genotypes, approximately 6,000 kg ha^{-1} (Boari, 2008). It is even much higher than soy, the most widely cultivated oilseed crop, which produces approximately 500 kg ha^{-1}. Another relevant factor is the variety of oils produced, which may be intended for food, pharmaceutical, cosmetic, and biofuel production (Boari, 2008; Mya et al., 2010). Biofuel has enhanced interest in oil palm due to the need for fuel production through alternative and renewable energy sources, which contributes to environmental preservation, and also because fossil fuels are finite resources (Ghassan et al., 2003).

Scientific research on oil palm has enabled the generation of highly adapted and productive plants, such as the interspecific Manicoré hybrid from crossing between *E. guineensis* and *Elaeis oleifera.* This hybrid produces a large amount of oil, yielding approximately 30 tons per cluster / ha / year and is resistant to lethal yellowing, an anomaly of yet unknown cause that affects oil palm crops causing major damage (Campos, 2011). Brazil currently has 95,000 hectares intended for oil palm cultivation and approximately 75 million hectares of land suitable for palm growing (Agrianual, 2012). In this context, there is government action to stimulate culture expansion for approximately seven years, and production is estimated at 12 million seeds per year (Collares, 2011). This increasing demand must be accompanied by commercial-scale availability of plantlets. As it is a monocot plant, this species cannot be propagated by microcuttings. Propagation is usually by seeds that require a long time to germinate (1 to 3 years) and show low germination rates (approximately 30%) (Martine et al., 2009; Luis et al., 2010). Thus, there is need for developing techniques that improve germination and reduce time for establishing new plantings. Tissue culture can be used as a tool for optimizing embryo germination of *E. guineensis* Jacq through isolation and cultivation under appropriate conditions (Torres et al., 1998). Composition of culture medium is a major factor influencing resumption of *in vitro* embryo development, and it is necessary to establish an osmotic and nutrient balance suitable for embryo regeneration (Suranthran et al., 2011). Among the components in culture medium, sucrose stands out as the most commonly used carbohydrate source, which also regulates medium osmolarity. Immature embryos usually require carbohydrate sources for their development since they have no reserves, and the younger the embryo, the higher the osmolality required (Hu and Ferreira, 1998). Besides germination, proper development of anatomical structures of *in vitro* plantlets is of utmost importance for successful acclimatization. Development of shoot and root systems reflect the acclimatization ability of plantlets and can be assessed by histological analysis.

This study evaluated the effect of embryo age (time after pollination), culture medium, and presence of carbohydrates on embryo germination of *E. guineensis* Jacq. In addition, we analyzed plantlet development using anatomical parameters.

MATERIALS AND METHODS

Plant material

The trials were conducted at the Central Laboratory of Molecular Biology and Laboratory of Plant Anatomy, Federal University of Lavras, Brazil. Immature fruits of *E. guineensis* Jacq. Manicoré hybrid, at different days after pollination (80, 90, 100 and 110), were provided by Denpasa Company, state of Pará, for germination experiments.

Disinfestation and *in vitro* germination

Fruits were washed with sodium hypochlorite (1.25%) and then broken using a vice grip to remove epicarp, mesocarp and endocarp, and expose almonds. Almonds were washed with water and immersed in 70% alcohol for 30 s under laminar flow. Then, they were transferred to sodium hypochlorite solution (1.25%) containing three drops of Tween per 100 ml of solution and washed three times in autoclaved distilled water under continuous stirring. After disinfection, embryos were isolated from almonds, inoculated in MS medium (Murashige and Skoog, 1962) and modified Y3 medium (Eeuwens, 1978) without the addition of amino acids, supplemented or not with 3% sucrose, pH adjusted to 5.7 ± 0.1, and solidified with agar 0.6% (w/v). After inoculation, embryos were kept for 30 days, in 16 h photoperiods at 26 ± 2°C. Later they were transferred to Magenta jars containing the same culture medium, where they remained for 45 days under the same conditions mentioned above. Germination rate and embryo size were evaluated at 30 days after inoculation in embryos showing at least some radicles. After 75 days of embryo inoculation, plantlets were assessed for the following parameters: number of leaves, shoot size, presence of roots, and size of root system.

Anatomy of plantlets

After 75 days of germination, *in vitro* culture plantlets were collected and fixed in FAA70 (Johansen, 1940) for 48 h, then transferred to 70% ethanol and kept until analysis. Transverse sections of leaves and roots were carried out in table microtome according to the method described by Melo et al. (2007). Sections were cleared in 50% sodium hypochlorite for 10 min, washed twice in distilled water for 10 min, and stained with safranin solution and Astra blue (safrablau 7:3) for 30 s. Then, sections were rinsed in distilled water and mounted on slides with 50% glycerol (Kraus and Arduin, 1997). Slides were observed and photographed in photonic microscope coupled to a digital camera. These photomicrographs were used for measuring anatomical parameters in an image analysis program (ImageTool - UTHSCSA) calibrated with the same microscopic ruler and photomicrographed at the same magnifications. Four measurements were taken at each anatomical feature per replicate. Leaves were evaluated for the following characteristics: epidermal thickness of abaxial and adaxial surfaces, mesophyll thickness, cuticle thickness, hypodermal thickness, sclerenchyma thickness, phloem thickness, and diameter of metaxylem cells in the midrib. The roots were evaluated for epidermal thickness, cortex thickness, exodermis thickness, endoderm thickness, total root diameter, vascular cylinder diameter, metaxylem cell diameter, phloem thickness, total root area, and vascular cylinder area using the ImageJ® program. We used a completely randomized design with two types of culture medium (MS and Y3), two concentrations of

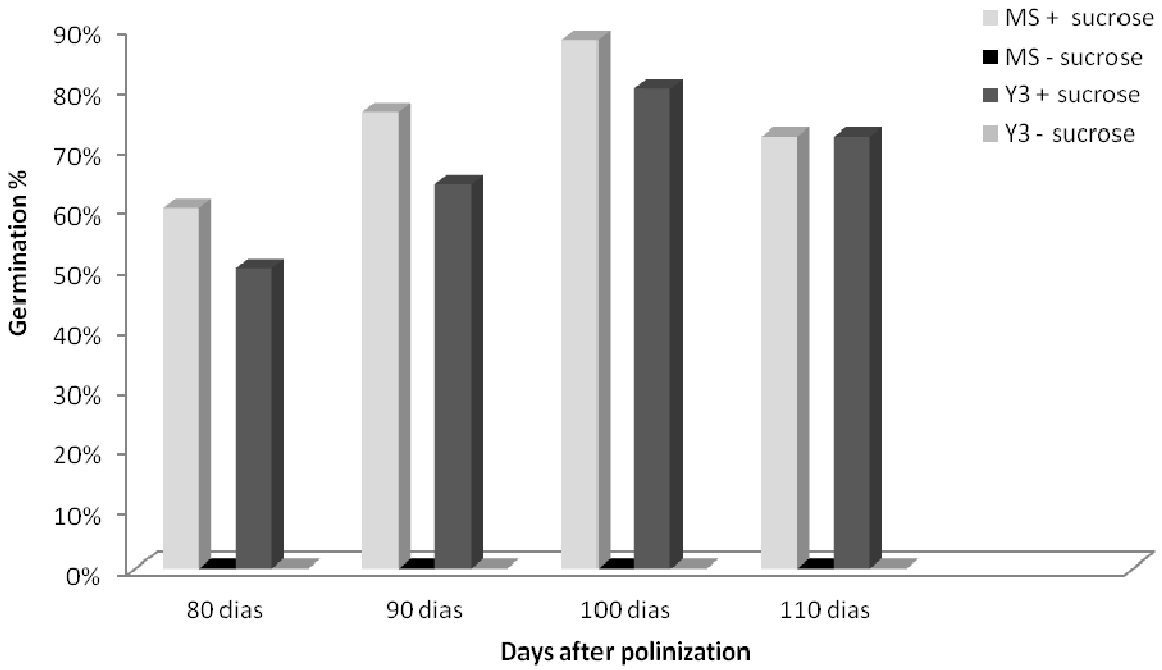

Figure 1. *In vitro* germination percentage of *E. guineensis* Jacq. hybrid Manicoré embryo with 80, 90, 100 and 110 days after anthesis, inoculated in different culture medium.

sucrose (presence or absence in the medium) and four embryo ages (80, 90, 100, 110 days) after pollination in five replicates, each containing five embryos. The data were subjected to analysis of variance, and means were compared by Tukey test at 5% probability using SISVAR ® program (Ferreira, 2008).

RESULTS AND DISCUSSION

Germination of embryos

Embryo germination of *E. guineensis* Jacq. Manicoré hybrid was influenced by culture medium composition, sucrose in the medium and embryo age, with statistical differences occurring between treatments. It was found that 80, 90, 100 and 110-day embryos cultivated on MS and Y3 media with addition of sucrose germinated, whereas those grown in the same culture media without addition of sucrose showed no germination (Figure 1). The highest germination rate (88%) was obtained in 100-day embryos inoculated with MS medium and sucrose. However, this rate did not differ significantly from treatments containing 90 and 110-day embryos in the same culture medium (72 and 76% germination respectively). Similar behavior was found in embryos in modified Y3 medium, with no statistical differences between treatments containing 100 and 110-day embryos. Carbohydrate sources were essential for embryo development of palm oil BRS Manicoré hybrid, regardless of growth stages in MS and Y3 culture media. Carbohydrate sources in medium influenced various metabolic processes, affecting growth and maintaining proper osmolarity for embryo development (Hu and Ferreira, 1998). Cardoso et al. (2010) evaluated germination of CN 470 oil palm hybrid in MS and full Y3 medium and found the highest germination rate (85.18%) in the treatment in MS medium.

Torres et al. (2005) found that addition of sucrose to culture medium was essential for embryo growth and development of *Heliconia rostrata*. Pereira et al. (2006) evaluated embryo germination of *Uncaria guianensis* (Wild) DC. and found 100% germination with 15 g L^{-1} sucrose, regardless of concentration in MS medium. Nunes et al. (2008) assessed different concentrations of sucrose and physiological stages of fruit ripening in germination of Jatropha and found that a higher concentration of sucrose (60 gL^{-1}) provided a higher number of germinated immature embryos (83.68%). Ribeiro et al. (2011) obtained no roots or leaf sheaths in plantlets from embryos of mature fruits grown in medium without sucrose, and associated with result of lack of carbohydrate reserves sufficient for full plant development.

In addition to *in vitro* germination, plantlet acclimatization has been limiting micropropagation of many species, especially palm trees. Transferring plants from a protected, sterile environment with carbohydrates and saturated humidity to non-sterile, low humidity environments without carbohydrates has been leading to plant loss, low growth rate and extended period in obtaining completely acclimatized plants (Souza Júnior et al.,

Table 1. Seedlings length - cm (SL) after 30 days and shoot length - cm (ShL), leaf number (LN), roots number (RN) and root length - cm (RL) of *Elaeis guineensis* Jacq. hybrid Manicoré seedlings after 75 days on MS medium are supplemented with sucrose and Y3.

Age (days)	Culture medium	SL (cm)	ShL (cm)	LN	RN	RL (cm)
80	MS	0.91^{B}	1.37^{B}	1.37^{B}	0.12^{A}	0.37^{A}
90		0.96^{B}	2.05^{A}	2.12^{A}	0.00	0.00
100		1.24^{A}	1.56^{B}	0.62^{B}	0.25^{A}	0.33^{A}
110		0.80^{B}	1.36^{B}	1.87^{A}	0.00	0.00
80	Y3	1.13^{a}	2.00^{a}	2.00^{a}	0.12^{b}	0.15^{b}
90		0.67^{b}	1.27^{b}	2.62^{a}	0.50^{a}	0.53 ^{b}
100		0.79^{b}	1.12^{b}	0.50^{b}	0.62^{a}	2.91^{a}
110		0.79^{b}	2.22^{a}	2.50^{a}	0.12^{b}	0.07^{b}

Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

2001). Thus, as structural changes that may occur in plantlets are detected, it is possible to optimize and even control *in vitro* conditions to maximize plant survival during acclimatization (Apóstolo et al., 2005).

In this study, we analyzed some growth parameters such as size of germinated embryos, size of shoots, number of leaves, presence of roots, and root size (Table 1). Y3 medium provided better conditions for parameters related to root formation. It should probably be related to the higher amount of salts in MS medium and the different nitrogen source, compared with Y3 medium. According to Assis and Teixeira (1998), the influence of culture medium on rooting is related to carbon-nitrogen relationship. Moderate nitrogen deficiency is usually more beneficial to rooting than excessive or even adequate levels. According to Ribeiro et al. (2011), the higher concentration of salts in MS medium (100%) inhibited root growth in oil palm plantlets thus confirming Ferreira et al. (2002), who assessed embryonic axes of cupuassu (*Theobroma grandiflorum* Schum.) in different salt concentrations and found that high nutrient availability may prevent plantlet rooting.

Anatomy of plantlets

Anatomical differences in leaves were also found in treatments in MS and Y3 media associated with different ages after pollination (Figure 2). With regard to epidermal thickness of abaxial and adaxial surfaces of plantlets grown in MS medium, all ages showed developed epidermis. Only plantlets from 100 day post-anthesis embryos showed lower results for both parameters, and the same behavior was found in Y3 medium. As for cuticle deposition, plantlets obtained from embryos at 110 days post-anthesis in MS medium and from 90-day embryos in Y3 medium showed better results. Regarding hypodermis, no statistical differences were found between treatments (Table 2). Leaf cuticle thickness can have different functions relating to leaf protection and resistance, such as reducing herbivore digestibility, limiting entry of pathogens and water and nutrient exudation, and also facilitates acclimatization of *in vitro* plants (Akin and Robinson, 1982; Rathi, 1998). Cuticle thickness varies with environmental conditions (Taiz and Zeiger, 2004), and lack of epicuticular wax on leaves of *in vitro* plants occurs as a result of high humidity in the culture flask. Rapid dehydration of *in vitro* plants transferred to a greenhouse is correlated, among other factors, with low deposition of cuticles and epicuticular wax, and high reduction of leaf mesophyll (Machado and Biasi, 2011).

With regard to leaf development, mesophyll, sclerenchyma and phloem thickness, and metaxylem diameter showed good results with plantlets from 110 day embryos in MS medium and 90 day embryos in Y3 medium, except phloem thickness (Table 3). Increase in leaf thickness, especially cell elongation or addiction is associated with decrease in mesophyll resistance to carbon dioxide (Nobel, 1977) and correlated with increase of potentially limiting factors to photosynthesis such as Rubisco, electron carriers and stomatal conductance (Bjorkman,1981). Chazdon and Kaufman (1993) found that photosynthetic capacity was correlated with mesophyll thickness in two species of *Piper* (Piperaceae). Development of plant support systems is important for acclimatization, as reduced carrying capacity (sclerenchyma and collenchyma) can limit this process (Campostrini and Otoni, 1996). Soares (2003) compared *in vitro* and *ex vitro* Ingá (*Inga vera*) leaves and reported that lack of support tissues in leaves of micropropagated plants makes plants less resistant and fragile looking. With regard to rooting, plantlets from 90 and 110 day embryos in MS medium showed no roots, whereas plantlets from 80 and 100 day embryos in MS medium and from 80, 90, 100 and 110 day embryos in Y3 medium showed rooting, thus it was possible to evaluate anatomical parameters (Figure 3). Treatments containing

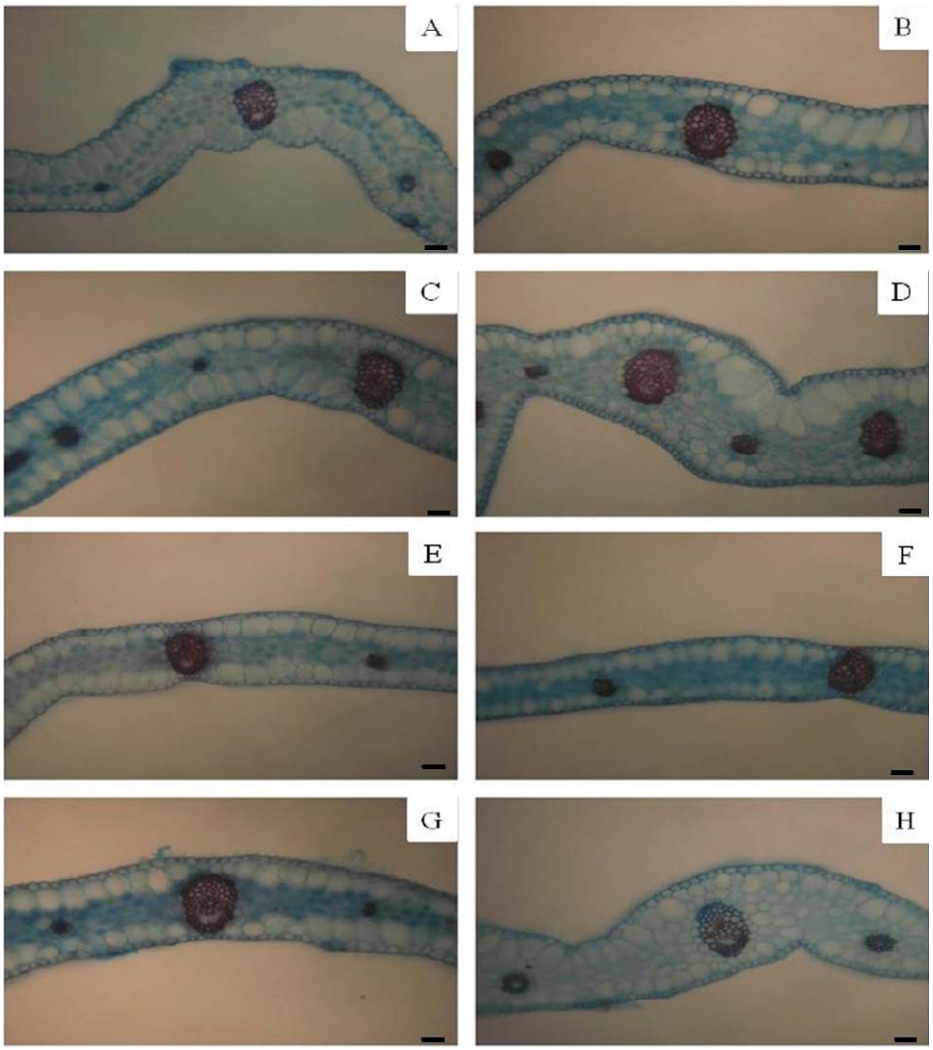

Figure 2. Photomicrographs of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages. Leaves of seedlings from embryos with 80, 90, 100 and 110 days after pollination were maintained on MS culture medium (A, C, E and G respectively) or Y3 culture medium (B, D, F and G respectively). Bars = 100 µm.

Table 2. Morophometric and anatomical characteristics of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	DET (µm)	BET (µm)	CT (µm)	DHT (µm)	BHT (µm)
80	MS	17.77^{A}	20.78^{AB}	8.33^{AB}	51.66^{A}	65.92^{A}
90		17.09^{A}	18.93^{B}	9.03^{A}	51.64^{A}	73.11^{A}
100		13.98^{B}	15.02^{C}	7.24^{B}	47.86^{A}	67.85^{A}
110		18.57^{A}	22.36^{A}	9.11^{A}	53.32^{A}	68.04^{A}
80	Y3	16.74^{ab}	18.59^{a}	5.95^{c}	49.75^{a}	63.89^{b}
90		18.50^{a}	21.28^{a}	8.60^{a}	42.25^{a}	86.62^{a}
100		14.83^{b}	15.51^{b}	7.12^{bc}	45.54^{a}	68.79^{b}
110		17.28^{a}	21.44^{a}	8.33^{a}	45.30^{a}	59.66^{b}

Adaxial epiderms thickness (DET) and abaxial epiderms thickness (BET) and cuticle thickness (CT), adaxial hypodermis thickness (DHT) and abaxial hypodermis thickness (BHT). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

Table 3. Morophometric and anatomical characteristics of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	MT (µm)	SE (µm)	FT (µm)	MD (µm)
80	MS	88.08^{AB}	58.79^{B}	46.05^{AB}	49.28^{A}
90		74.69^{B}	50.52^{AB}	35.16^{C}	38.04^{B}
100		89.71^{AB}	58.79^{AB}	57.86^{A}	43.88^{AB}
110		99.94^{A}	70.46^{A}	57.05^{AB}	45.72^{A}
80	Y3	95.54^{ab}	78.34^{a}	52.83^{b}	52.54^{a}
90		153.90^{c}	100.84^{b}	40.28^{a}	49.06^{ab}
100		81.55^{a}	69.37^{a}	37.86^{a}	40.98^{c}
110		110.74^{c}	73.44^{a}	39.88^{a}	42.35^{bc}

Mesophyll thickness (MT), sclerenchyma thickness (SE), phloem thickness (FT) and metaxylem diameter (MD). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

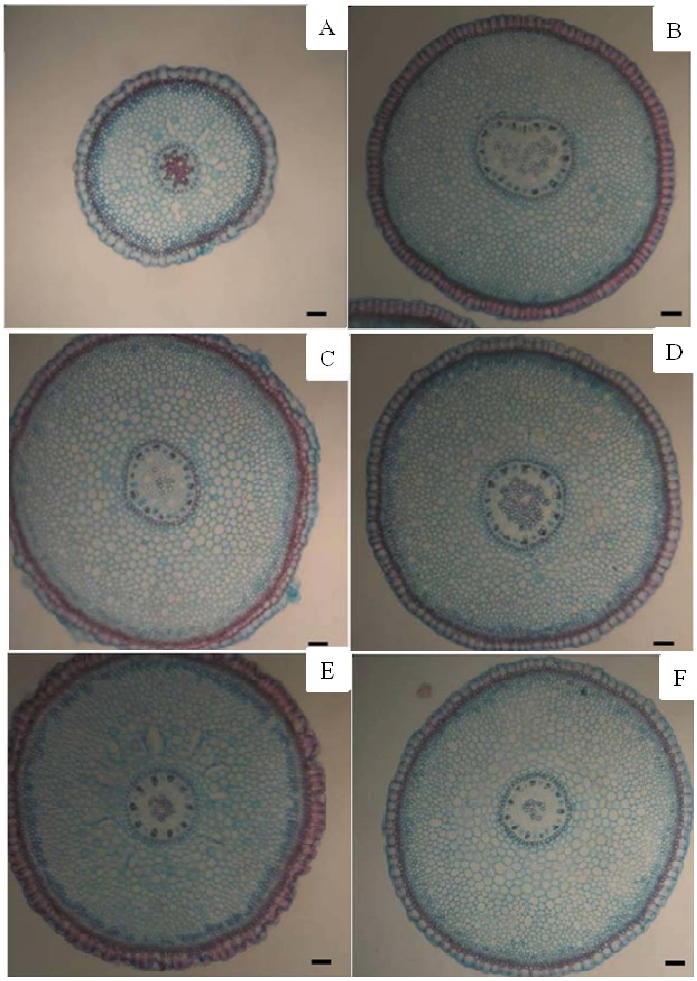

Figure 3. Photomicrographs of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages. Roots of seedlings from embryos with 80 and 100 days after pollination were maintained on MS culture medium (A and B respectively) and from embryos with 80, 90, 100 and 110 days after pollination maintained on Y3 culture medium (C, D, E and F) respectively). Bars = 100 µm.

Table 4. Morophometric and anatomical characteristics of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	RTA (mm^2)	RD (µm)	RET (µm)
80	MS	0.75^{B}	978.07^{B}	52.86^{A}
90		0.00	0.00	0.00
100		1.60^{A}	1430.10^{A}	48.54^{A}
110		0.00	0.00	0.00
80	Y3	1.84^{a}	1.53^{a}	45.49^{b}
90		1.68^{b}	1.46^{b}	50.65^{ab}
100		1.83^{a}	1.53^{a}	55.41^{a}
110		1.65^{b}	1.45^{b}	42.92^{c}

Root total area (RTA) and root diameter (RD), root epidermis thickness (RET). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

Table 5. Morophometric and anatomical characteristics of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	CT (mm)	VCA (mm^2)	VCD (mm)
80	MS	0.28^{B}	0.02^{B}	0.19^{B}
90		0.00	0.00	0.00
100		$O.41^{A}$	0.12^{A}	0.40^{A}
110		0.00	0.00	0.00
80	Y3	0.49^{a}	0.09^{b}	0.34^{a}
90		0.44^{b}	0.10^{a}	0.37^{a}
100		0.45^{b}	0.08^{b}	0.32^{b}
110		0.49^{a}	0.06^{b}	0.29^{c}

Cortex thickness (CT), vascular cylinder area (VCA) and vascular cylinder diameter (VCD). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

MS medium associated with ages 100 and 80 days after pollination did not differ statistically for the parameters analyzed in roots: total root area, total root diameter, cortex thickness, vascular cylinder area, and vascular cylinder diameter (Tables 4 and 5). In Y3 medium, total root area, total root diameter and epidermal thickness excelled in 100 day embryos. Best results for cortex thickness were obtained with 80 and 110 day embryos. The highest results for vascular cylinder area and diameter were found with 90 day embryos, and vascular cylinder diameter did not differ statistically from 80 day embryos (Tables 4 and 5).

Results for exodermis and endoderm thickness, metaxylem diameter, and phloem thickness in plantlet roots in MS medium were not statistically different (Table 6), while plantlets from 80 day embryos in Y3 medium showed greater exodermis and endodermis thickness. Results did not differ statistically in plantlets from 90 day embryos (Table 6), which also showed greater metaxylem diameter. Vascular cylinder area, vascular cylinder diameter, and metaxylem diameter may affect acclimatization process to a greater extent, and showed better results in Y3 medium with 90 day embryos. They are also critical in the conduction of water and nutrients to the entire plant.

According to Jesus et al. (2010), root hydraulic conductivity is directly related to vascular cylinder diameter, thus its good development ensures adequate supply of water and minerals to shoots.

Conclusion

Sucrose is essential for germination of immature embryos of *E. guineensis* Jacq. Manicoré hybrid. MS medium with zygotic embryos at 90, 100 and 110 days post-pollination can provide high germination rates, likewise Y3 medium with embryos at 100 and 110 days post-anthesis. The treatment in Y3 medium with zygotic embryos at 90 days post-anthesis was outstanding in number and length of roots, as well as in anatomical parameters of leaves and roots associated with the best conditions for plantlet

Table 6. Morophometric and anatomical characteristics of roots from of *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	EXT (µm)	ENT (µm)	MD (µm)	FT (µm)
80	MS	33.22^{b}	16.80^{b}	18.56^{b}	38.63^{b}
90		0.00	0.00	0.00	0.00
100		36.91^{b}	17.19^{b}	17.89^{b}	37.92^{b}
110		0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
80	Y3	51.50^{c}	18.58^{b}	16.47^{a}	37.29^{b}
90		35.69^{a}	17.12^{ab}	19.33^{b}	38.56^{bc}
100		45.10^{b}	15.80^{a}	17.45^{a}	41.52^{c}
110		35.25^{a}	17.35^{ab}	16.48^{a}	34.16^{a}

Exodermis thickness (EXT), endoderm thickness (ENT), metaxylem diameter (MD) and phloem thickness (FT).
Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

acclimatization.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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