In vitro rescue of interspecific embryos from *Elaeis guineensis* x *E. oleifera* (Arecaceae)

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Abstract: The African oil palm (*Elaeis guineensis*) is the most effective oil producer in tons per hectare. Nevertheless, its increasing cultivation in Latin America is harmed by the "lethal yellowing". Genetic resistance to this anomaly can be found in the germplasm of American oil palm or caiaué (*E. oleifera*), a native species from the Amazon rainforest. However, the procedures adopted to induce seeds of *E. guineensis* to germination frequently result mild for interespecific hybrids. Embryo *in vitro* cultivation can be a viable option. This work was aimed initially to test liquid MS *medium* supplemented with different glucose or sucrose concentrations for the *in vitro* cultivation of zygotic embryos from *E. guineensis* x *E. oleifera* controlled pollinations. Additionally we investigated different compost mixtures to acclimatize the regenerated hybrid plantlets. Concentrations of 10, 20 and 30g/L of both sugars were tested on flasks containing five mature zygotic embryos, with 15 repetitions per treatment in a total of 450 explants. The number of embryos displaying shoots and radicles at least 2mm in length per experimental unit was evaluated during phase one of *in vitro* cultivation. Plantlets displaying shoots and radicles were transferred to phase two of *in vitro* cultivation and subsequently to acclimatization, under 70% shading with manual water supply. The experiments of acclimatization were conducted with 130 plantlets randomly distributed in pure horticultural compost, 3:1 or 1:1 compost:sand mixtures and each plantlet was defined as an experimental unit. Data were submitted to ANOVA, t test and analyzes of correlation ($p \leq 0.05$). Highest emergence rates were 97% for shoots and 73% for radicles, observed in MS *medium* supplemented with 20g/L (110mM) of glucose. This sugar in concentrations of 20 or 30g/L provided balanced shoot/root development, and this was considered one of the reasons for the higher frequency of plantlet establishment. The survival percentage was 55% after the first 43 days of acclimatization and by the fourth month, 66 plants developed simultaneously longer shoot and root systems in pure horticultural compost. In conclusion, radicle development was an impairment to plantlet establishment and was overcame under *media* with glucose above 110mM. Acclimatization could benefit from an extended period of *in vitro* development. Rev. Biol. Trop. 59 (3): 1081-1088. Epub 2011 September 01.

Key words: Amazon flora, palms, oil plants, dendê, caiaué, embryo culture, plant breeding.

Currently, breeding programs in Latin America explore the interspecific hybridization between the African (*Elaeis guineensis* Jacq.) and the American oil palm or caiaué [*E. oleifera* (Kunth) Cortés]. African oil palm is the cultivated oil plant that presents the highest yields of palm oil, used for cosmetics (Fiorese 2008, Natura 2010), food and biofuel and has social (Homma *et al*. 2000, Fiorese 2008) and ecologic relevance (Veiga *et al*. 2000). American oil palm has been introduced into breeding programs as a source of resistance to the "lethal yellowing", an anomaly that destroyed thousands of acres of Latin American plantations, and also due to its slower shoot growth rate that allow exploitation for an extended period and to the production of palm oil with higher

content of unsaturated fatty acids (Barcelos *et al*. 2000, Lopes *et al*. 2008).

However, interspecific hybrid seeds induced to germinate by heat and imbibition (Nunes *et al*. 1998) only gave rise to approximately 30% plantlets on average (R.N.V. Cunha & R. Lopes 2008, pers. comm.), far below the 80% obtained for African oil palm seeds (Nunes *et al*. 1998), and slightly below the 50% observed for seeds of American oil palm.

In vitro cultivation can be useful in determining which would be the appropriate conditions to facilitate the development of somatic embryos in the future and in other applications too. This technology was used to optimize germination of avocado genotypes inflicted by precocious fruit abscission (Sánchez-Romero *et al*. 2007). Besides, immature embryos from *Hybiscus* interspecific crosses that did not produce normal fruits were recently rescued *in vitro* (van Laere *et al*. 2007).

Specifically concerning species that have oil as the principal reserve present in the seeds, plantlets of *Arabidopsis thaliana* were established *in vitro* independently of the glyoxalate cycle as long as exogenous sugars were provided (Eastmond *et al*. 2000). Sugar concentrations from 30 up to 90mM were sufficient to promote the germination of *A. thaliana* seeds in the presence of exogenous ABA, but root elongation occurred only under higher sugar concentrations (Finkelstein & Lynch 2000, Finkelstein & Gibson 2001). *In vitro* culture of zygotic embryos was used to rescue hybrids from *Brassica napus* x *B. juncea* and to improve the efficacy of interspecific breeding, thus overcoming the abortion of hybrid embryos caused by the differences in the number of chromosomes between the two species (Zhang *et al*. 2003). *In vitro*, *B. napus* isolated embryos absorbed hexoses faster than sucrose. The activities of hexokinases and sucrose synthase increased *in vitro* during embryo development and sucrose synthase was four fold more active than invertase (Hill *et al*. 2003). *In vitro* cultivation of *tenera* immature embryos produced by crossing *E. guineensis* types *dura* and *pisifera* was reported by Aberlenc-Bertossi *et al*. (2003), although the rescue of interspecific hybrids from intercrosses of *E. guineensis* and *E. oleifera* has not been evaluated.

The objectives of the present work were to evaluate sucrose and glucose as carbon sources to rescue mature hybrid embryos produced by controlled pollination of *E. guineensis* x *E. oleifera* and compost mixtures for acclimatization of the hybrid plantlets obtained *in vitro*.

MATERIAL AND METHODS

Plant material: Fruits were produced by controlled pollinations of selected *Elaeis guineensis* plants with pollen from selected plants of *E. oleifera*, in the Rio Urubu Experimental Station, Rio Preto da Eva, Amazonas, Brazil, 2°35' S - 59°28' W, at 200m in elevation. Fruits from progenies labeled as CI1488 and CI1603 were collect 150 days after pollination and stored at 20°C for 156 and 143 days, respectively. After removal of fruit tissues, seeds were washed with detergent, treated for 10min in 50% (v/v) commercial bleach (2-2.5% active chlorine) and washed in distilled autoclaved water. Embryos were immediately extracted by carefully sectioning the endosperm, taken to an aseptic environment, immersed in 5% commercial bleach (2-2.5% active chlorine) for 5min, washed three times in distilled autoclaved water and inoculated in culture *media*.

***In vitro* cultivation:** Embryos from progenies CI1603 and CI1488 were cultivated over cellulose acetate membranes on liquid MS basal salts and vitamins (Murashige & Skoog 1962) supplemented with 10, 20 or 30g/L sucrose or 10, 20 or 30g/L glucose, under 26±2°C and irradiated with 24μmol/m^2s of photosynthetically active photons for 16h photoperiods. The emergence of shoots and radicles with at least 2mm in length was scored up to 50 days. Experiments were conducted in a completely randomized design, with each experimental unit defined as a flask with five embryos and 15 repetitions per treatment, what made 450 mature embryos inoculated *in vitro*. Raw data -consisting of the average number of

embryos exhibiting emerged shoots and radicles per experimental unit- were transformed to $(x+0.5)^{1/2}$ for ANOVA analyzes and the subsequent Tukey tests. These procedures comprised phase one of *in vitro* cultivation (P1VC). Following P1VC, plantlets that exhibited shoots and radicles were transferred to half strength MS salts, supplemented with agar (8g/L), sucrose (30g/L) and active charcoal (2.5g/L). Plantlets were maintained in this *medium* for 60 days, under the conditions of temperature and irradiation described previously, until acclimatization. These procedures constituted phase two of *in vitro* cultivation (P2VC).

Acclimatization: During *ex vitro* transfer, plantlets were evaluated and randomly distributed in 200cm^3 plastic tubes with drainage holes fitted in square cages and filled with commercial horticultural compost (constituted principally of milled wood) pure or mixed 3:1 or 1:1 with sand. Plantlets in tubes were maintained under greenhouse conditions with approximately 70% shading and manual water supplying and evaluated 43, 100 and 125 days after transfer. At transfer and in the final evaluation, roots and leaves were counted along with the measurement of the longest root and the longest leaf per plant. For intermediary evaluations only the number of leaves and the length of the longest leaf were recorded. Data from plantlets damaged during transfer were excluded from further analyzes. Each plantlet constituted an experimental unit and the experiments were conducted with 43/44 repetitions per treatment. ANOVA analysis for acclimatization was performed from raw data (Kruskal-Wallis one way analysis of variance on ranks) and the medians were compared by Dunn test. The Mann-Whitney ranks sum test was applied to compare the medians of groups of data recorded for different evaluation days. Spearman's ranks order correlation was used to test the correlation among groups of data. Data from different evaluations were compared by the t-test. All the statistical analyzes were performed with SigmaStat v. 2.0 (Fox *et al*. 1995).

RESULTS

Elaeis guineensis x *E. oleifera* hybrid embryos displayed no significant differences in average values observed for shoot emergence per experimental unit under distinct glucose treatments (n=45, F=2.337, p=0.115) but significantly higher averages (n=45, F=13.742, p<0.001 and Tukey, p<0.05) were observed for radicle emergence under glucose 20 (110mM) and 30g/L (165mM) (Table 1).

For cultivation in sucrose-containing *media* the average values observed for radicle (n=45,

TABLE 1
Average values per experimental unit (absolute numbers) unit and the percentage of *Elaeis guineensis* x *E. oleifera* interspecific hybrid plantlets bearing shoots and radicles at least 2mm long in 50 days of in vitro cultivation (P1VC)

Glucose		Shoot		Radicle		Shoot + radicle (%)
g/L	mM					
30	165	4.533	a	3.333	a	66.66
20	110	4.867	a	3.367	a	73.34
10	55	4.933	a	1.467	b	29.34
Sucrose						
30	87	3.533	a	1.677	a	33.54
20	58	2.400	b	0	b	0
10	29	2.800	ab	0.200	b	4.00

Values followed by the same letters in columns are statistically similar (Tukey test, p≥0.05).

F=5.441, p=0.010) and shoot (n=45, F=21.899, p<0.001) emergence were highest for the highest concentration tested. There was no significant difference between 30g/L (87mM) and 10g/L (29mM) for shoot emergence (Tukey, p≥0.05), but radicle emergence was significantly higher in 30g/L sucrose (Table 1).

It became evident that germinating oil palm interspecific embryos exhibited higher shoot emergence frequencies in comparison to radicle emergence, independently of the sugar source in the cultivation *media* (Table 1). Although radicle development had not been evaluated quantitatively until the end of P2VC and transference to acclimatization, it could be observed that it was the principal barrier to plantlet establishment in P1VC. Indeed from the results obtained under glucose-containing *media*, it was noticed that alleviation of the impairment in root elongation was achieved in 20g/L (110mM) or highest concentration of this sugar. Shoot emergence was similarly frequent for plantlets maintained in *medium* containing 10 (55mM), 20 (110mM) or 30g/L (165mM) glucose, however during the 50 days of P1VC, the percentage of plantlets displaying developed shoots and radicles simultaneously was only significantly increased in *media* containing 20g/L (110mM) glucose or higher (Table 1). Accordingly, among sucrose treatments, increase in the emergence of radicles with subsequent development was observed almost exclusively for the highest concentration (30g/L or 89mM, Fig. 1), despite it has not been as high as in glucose 20 and 30g/L (t-test, p<0.05).

Most plantlets germinating under 10 and 20g/L sucrose and 10g/L glucose could not be forwarded to P2VC due to the observed staggered root development. Consequently these three treatments were eliminated from acclimatization experiments.

From P1VC, 130 plantlets were transferred to the greenhouse. Data collect during *ex vitro* transfer revealed the absence of significant influence of culture *media* used during P1VC (20 or 30g/L glucose or 30g/L sucrose) on the number of leaves per plant (n=130, H=0.0361, p=0.982), on the lengths of the longest roots (n=118, H=0.719, p=0.698) or on the longest leaves (n=130, H=1.171, p=0.425).

At transfer, a correlation between the lengths of the longest leaf and the longest root was significant (Spearman, n=118, r=0.330, p<0.001). Nevertheless, this inclusive result was highly influenced by the values observed for plantlets cultivated in 20 or 30g/L glucose during P1VC (Spearman, n=75, r=0.405, p<0.001), since the correlation between shoot and root lengths was not significant under cultivation on 30g/L sucrose (Spearman, n=43 r=0.203, p=0.191). This result is in agreement with the higher frequency of plantlets showing radicle development in 20 or 30g/L glucose than in 30g/L sucrose along P1VC (and Table 1).

Plants surviving up to the second evaluation under greenhouse conditions 43 days after transfer corresponded to 55% (71 plants).

Fig. 1. Some of the interspecific hybrid plantlets of *Elaeis guineensis* x *E. oleifera* after 39 days of *in vitro* cultivation over celulose acetate membranes in liquid MS *medium* containing sucrose 30g/L (89mM).

This result was similar to the 58.33% survival observed for coconut plantlets (Lédo *et al*. 2007). From the total, 24 plants were transferred to pure horticultural compost, 24 to 3:1 compost:sand and 23 to 1:1 compost:sand mixtures. Only five plants died after the second evaluation.

Plantlets gained on average 0.04cm/day in length of the longest leaf during the first 43 days in the greenhouse. Subsequent progress was significantly better (t-tests, $p<0.05$) comparing data registered upon *ex vitro* transfer *versus* subsequent evaluations. It was observed the increase in the number of leaves and the lengthening of the longest leaf (Table 2), with gains of 0.123cm/day between the second and third evaluations and 0.110cm/day from the third to the final evaluations were observed. A correlation between the length of the longest leaf and the number of leaves not observed at transfer was established by the second evaluation (Spearman, n=71, r=0.778, $p<0.001$) and persisted through the experiment.

Distinct compost mixtures had no influence on shoot development until the final evaluation, when the number of leaves was higher for pure commercial compost (n=66, H=11.23, p=0.004). Root length followed a similar pattern and was equally high in pure compost or 3:1 compost:sand mixture (n=66, H=7.66, p=0.022).

DISCUSSION

The phase of reserve (including sucrose and proteins) accumulation ends approximately 110 days after pollination, when the acquisition of tolerance to desiccation is initiated, for seeds of *Elaeis guineensis* type *tenera*. About 120 days after pollination, water loss stops, embryos preserve a water content slightly higher than the values commonly observed for orthodox species and the seed remains dormant until fruit abscission, which can last 60 additional days or for longer periods (Aberlenc-Bertossi *et al*. 2003). Besides sucrose accumulation, abscisic acid (ABA) and late embryogenesis abundant (LEA) proteins are possibly involved in this process and the acquired desiccation tolerance is likely to be lost after germination (Aberlenc-Bertossi *et al*. 2003). This process could be somewhat different for interspecific hybrids, since *E. oleifera* seeds have relatively lower viability and the combination of genomes from two species can produce novel phenotypes for a massive number of traits. For instance, different *Arabidopsis* landraces or ecotypes vary in their degree of seed dormancy (Bentsink *et al*. 2006). Nevertheless, *E. guineensis* remains the most suitable reference for the study of interspecific hybrids, and since fruits were collect 150 days after pollination and stored, they were considered to be dormant.

TABLE 2
Number of leaves (NL), length of the longest leaf (LL) and the longest root (LR) observed for *Elaeis guineensis* x *E. oleifera* interspecific hybrid plantlets during acclimatization

Data		At transfer	43 DAT	100 DAT	125 DAT
NL	mean ± SD	1.22 ± 0.52	1.73 ± 0.56	2.65 ± 0.68	3.15 ± 0.81
	min - max	1.0 - 4.0	1.0 - 3.0	1.0 - 4.0	1.0 - 4.0
LL (cm)	mean ± SD	6.48 ± 2.28	7.57 ± 3.35	13.93 ± 5.79	17.05 ± 6.03
	min - max	1.5 - 13.0	1.0 - 20.0	1.0 - 23.5	4.0 - 27.0
LR (cm)	mean ± SD	1.82 ± 1.81	NE	NE	11.33 ± 6.55
	min - max	0.2 - 11.3			1.0 - 28.0

OBS: DAT = days after transfer for acclimatization. NE = not evaluated.

The hormone ABA can accumulate in roots of dormant species just after emergence (Kende & Zeevaart 1997), is involved in embryo dormancy (Koornneef *et al*. 2002) and in the inhibition of invertase activity and root elongation, which are complex signal transduction processes orchestrated by sugars, water and light (Bewley 1997, Finkelstein & Gibson 2001). The impairment in root elongation is gradually supplanted while the radicle becomes a photosynthate sink (Finkelstein & Gibson 2001), and this physiological mechanism is possibly coordinated with the accumulation of sufficient sucrose in shoots. Embryos of *E. guineensis* type *tenera* exposed to light display chlorophyll accumulation in the *haustorium* due to chloroplast development, and subsequent starch accumulation (Rabéchault & Cas 1974). In plants, *haustorium* functions to transfer reserves from the endosperm to the germinating embryo (Carvalho 2000) and is not exposed to light. In the present study, greening of *haustoria* was observed. Accumulation of starch would also agree with the availability of hexoses in the shoots while root invertases are inhibited by ABA. Efficient reserve accumulation in the shoots and transfer to the radicles, among other factors (as the presence of light during germination), would account for the observation of highest frequencies of plantlets showing normal root development in glucose 20 and 30g/L *media*, when compared to 10g/L of the same sugar, and in the highest concentration of sucrose compared to the lower concentrations tested (Table 1). The optimum absorption and metabolism of glucose in the shoots (Hill *et al*. 2003) would compensate for a possible inhibition of root invertases following emergence (Finkelstein & Lynch 2000).

During the first 43 days under acclimatization process, hybrid plantlets dying were presumably those with a higher number of leaves or younger leaves, since no records of plants bearing four leaves at the second evaluation were observed, despite plants at that stage were observed at transfer. In the same period, a reduction in the average length of the longest leaf was not observed (Table 2). Plantlets bearing more leaves would lose higher contents of water due to the higher number of stomata and thinner superficial wax layer (Abdelouahhab & Hughes 1995), thus demanding more absorption from an insufficiently developed root system.

To attain better results in future studies, embryos at different maturation stages will be evaluated as reported for avocado (Sanchéz-Romero *et al*. 2007) and the effects of growth regulators on germination will be tested (Aberlenc-Bertossi *et al*. 2003, Lédo *et al*. 2007). Despite the higher percentage of plantlets obtained *in vitro* in comparison to the average number of interspecific hybrid seeds germinating from progeny pools, the loss of plantlets during acclimatization was of concern. This losses could be reduced by a longer P2VC, by pruning of the roots and by the use of an automated system to control the moisture in the greenhouse. Germination rates and subsequent plantlet development may, in addition, be influenced by embryo genotype, and therefore, different results may be found in future experiments.

In conclusion, interspecific zygotic embryos of *E. guineensis* x *E. oleifera* succeeded in metabolize the glucose provided in the *media* for *in vitro* culture, and displayed the highest frequencies of emerged and developed roots when this sugar concentration reached 110mM or more (20 or 30g/L glucose). A balanced shoot/root development was observed only for plantets rescued from these two treatments, by the end of P2VC and transfer to acclimatization took place. From embryos cultivated in 20g/L glucose, 76% developed normal shoots and roots simultaneously at the end of P1VC. The highest tested concentration of sucrose produced the highest frequency of plantlets bearing developed shoots and radicles. After four months in the greenhouse, plants grown solely in horticulture compost displayed simultaneously higher number of leaves and longer roots.

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RESUMEN

Elaeis guineensis es el productor de aceite más eficaz en toneladas por hectárea, su cultivo, cada vez mayor en América Latina, se ha visto perjudicado por el "amarilleamiento letal". La resistencia genética a esta anomalía se puede encontrar en el germoplasma de la palma aceitera americana o caiaué (*E. oleifera*), una especie nativa de la selva amazónica. Sin embargo, los procedimientos adoptados para inducir la germinación de las semillas de *E. guineensis* frecuentemente produce resultados modestos para híbridos interespecíficos. El cultivo de embriones in vitro puede ser una opción viable. En este trabajo se probó el medio líquido MS complementado con diferentes concentraciones de glucosa o sacarosa en el cultivo in vitro de embriones cigóticos de *E. guineensis* x *E. oleifera* originados de polinización controlada. Además se investigaron diferentes mezclas de compost para aclimatar los híbridos regenerados. Las concentraciones de 10, 20 y 30 g/L de ambos azúcares se probaron en frascos que contenían cinco embriones cigóticos maduros, con 15 repeticiones por tratamiento y un total de 450 explantes. El número de embriones que muestran brotes y radículas de al menos 2mm de longitud por unidad experimental se evaluó durante la primera fase de cultivo in vitro. Las plántulas que mostraron brotes y radículas fueron trasladadas a la segunda fase de cultivo in vitro y, posteriormente, se aclimataron, por debajo de 70% de sombra con el suministro manual de agua. Los experimentos de aclimatación se llevaron a cabo con 130 plántulas distribuidas al azar en el compost hortícola puro, compost 3:1 o 1:1: mezclas de arena y cada plántula se definió como una unidad experimental. Los datos fueron sometidos a un análisis de varianza, prueba t y análisis de correlación ($p \leq 0.05$). Las tasas más altas de emergencia fueron 97% y 73% para brotes y radículas respectivamente, en el medio MS complementado con 20g/L (110mM) de glucosa. Este azúcar en concentraciones de 20 o 30g/L permitió un desarrollo balanceado de brotes/desarrollo de raíces, que fue considerado como una de las razones de la alta frecuencia de establecimiento de las plántulas. El porcentaje de supervivencia fue de un 55% después de los primeros 43 días de aclimatación y por el cuarto mes, 66 plantas desarrollaron simultáneamente hojas largas y un sistema radical en el compost hortícola puro. En conclusión, el desarrollo radicular fue un impedimento para el establecimiento de plántulas y se superó en el medio con glucosa por encima de 110mM. La aclimatación podría beneficiarse con un largo período de desarrollo in vitro.

Palabras clave: flora amazonica, palmas, aceite de palma, cultivo de embriones, cultivo de plantas.

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