In vitro protocol optimization for development of interspecific hybrids of oil palm (*Elaeis oleifera* (H.B.K) Cortés x *Elaeis guineensis* Jacq.)

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ABSTRACT

The oil palm (*Elaeis guineensis*) is the major source of plant oil in the world. Nevertheless, is susceptible to the fatal yellowing disease, which is affecting the oil palm production in Pará state, Brazil. To overcome this problem, interspecific hybrids of *Elaeis oleifera* x *Elaeis guineensis* is a viable alternative. Some protocols for in vitro embryo rescue were already established, but it is necessary to optimize it. The aim of this paper was to optimize the in vitro protocol for culture of embryos from interspecific hybrids of oil palm (*E. oleifera* x *E. guineensis*). Disinfection embryos were carried out with 1 and 2% NaOCl for 20 or 40 minutes. Embryos were germinated in vitro on the presence of 0, 0.1, 0.2 and 0.25% activated charcoal and Embryos were cultured in vitro on the presence of 2.4-Dichlorophenoxyacetic acid (2.4-D) at 375, 500 and 625 µM for callus induction. Activated charcoal (0.25%) is necessary to reduce oxidation in the embryos. Seed disinfection can be suitable reached with 1% NaOCl for 20 minutes. Different varieties have different ideal levels of 2.4-D for callus induction. The best level of 2.4-D for callus induction is 375 and 625 µM for the varieties SJ-167 and SJ-165, respectively.

Key words: Activated charcoal, phenolic oxidation, embryo rescue, callogenesis

INTRODUCTION

The oil palm (*Elaeis guineensis* jacq.) belongs to Areccaceae family. It is native of the West Africa. In Brazil, its dispersion happened in the century XV through the slaves’ traffic. This species is the major source of plant oil in the world, nevertheless is susceptible to the fatal yellowing disease (Alves et al., 2011). On the other hand, the caiaué (*Elaeis oleifera* (HBK) Cortés) is native from Brazil, more specifically of the Amazon region, and the main characteristics is low yield, high quality of oil and tolerance about some diseases, especially against fatal yellowing disease. Nowadays, in Brazil, the Pará state is a major producer of oil palm with about 220160.000 ton per year (Alves, 2007). Nevertheless, in the last years, fatal yellowing disease is becoming a barrier to the development of oil palm plantation. The main symptom of this disease is a yellowish at the basal of youngest leaves, which causes the plant death in few period of time, and its etiologic agent is unknown. The obtaining of interspecific hybrids between *E. oleifera* and *E. guineensis* can be an alternative viable to overcome the problem of the fatal yellowing disease. Despite being geographically isolated, these two species are cross-compatible and fertile hybrids can be easily obtained (Hardon and Tan 1969; Amblard et al., 1995). However, these interspecific hybrids (*E. oleifera* x *E. guineensis*) have a problem with regard to the development of embryo, resulting sometimes in seed abortion (Viegas and Muller, 2000). Nevertheless, tissue culture can overcome this problem with regard to the development of the embryo; these embryos can be rescued before to be aborted. Moreover, the possibility for the
propagation in wide scale using tissue culture is the main alternative to the development of interspecific hybrids \((E. \textit{oleifera} \times E. \textit{guineensis})\) in areas affected by fatal yellowing disease (Teixeira et al., 1993; Alves, 2007). Therefore, the aim of this paper was to optimize the \textit{in vitro} protocol for culture of embryos from interspecific hybrids of oil palm \((E. \textit{oleifera} \times E. \textit{guineensis})\).

**MATERIAL AND METHODS**

**Plant Material**
The experiments were carried out at laboratory of Biotechnology and Genetic Resources of Embrapa-Amazonia Oriental, Pará, Brazil. It was used seeds originated from crosses between \((Elaeis \textit{oleifera} \times Elaeis \textit{guineensis})\) from Germplasm bank of Embrapa – Amazonia ocidental, Manaus, Brazil. In order to establish the disinfection and oxidation tests were used the variety SJ-165. In order to induce calli were used the varieties SJ-165 and SJ-167.

**Embryos cultured in different levels of activated charcoal**
The disinfection of the embryos was accomplished in agreement with the protocol established by Alves et al. (2011). The embryos was excised of the seeds and transferred immediately to flask culture with 50ml of culture medium containing a half strength MS medium (Murashige and Skoog, 1962) added with sucrose (3%). The treatments consisted of: 0, 0.1, 0.2 and 0.25% of activated charcoal. In the first week, the embryos were cultivated in the dark. The length stem and root number of germinated embryos was evaluated after 30 days of \textit{in vitro} culture.

**Embryos disinfection**
The seeds were washed with distilled and sterile water for five times until do not exist any type of visible dirt. After this washing, the seeds were placed in a laminar air flow chamber followed by immersion in 70% alcohol for 2 minutes and after, followed to the treatments: 1 and 2% NaOCl for 20 or 40 minutes, and after this immersion the embryos were washed for four times with distilled and sterile water. After the disinfection the embryos were removed from seeds and inoculated on the germination medium. The germination medium was composed with MS medium supplemented with sucrose (3%) and activated charcoal (0.25%). In the first week, the embryos were cultivated in the dark. The percentage of contamination and number of the embryos germinated was evaluated after 30 days of \textit{in vitro} culture.

**Callus induction**
The sterilized embryos of the varieties (SJ-165) and (SJ-167) were inoculated within flask culture containing 25 ml of basal medium, which was constituted with: \(\frac{1}{2}\) MS medium, added with sucrose (3%) and activated charcoal (0.25%). The treatments were: 375, 500 and 625 \(\mu\)M 2.4-D for both varieties. The cultures were incubated in the dark for 12 weeks. Each 30 days the calli were subcultured in the same medium during this period.

**Statistical analysis and culture conditions**
The experimental design was completely randomized with twelve replicates of three embryos. The data was submitted to the normality analysis (Lilliefors’s test) and, analysis of variance (ANOVA) followed by regression analysis (data from quantitative treatments) or Tukey’s test (data from qualitative treatments), both at a \(P<0.05\). All statistical analyses were done following the procedures of the software BIOSTAT 3.0 (from Federal University of Pará). Variables from counting were transformed to \(\sqrt{x + 0.5}\) and variables from percentage were transformed to \(\arcsin \sqrt{\frac{x}{100}}\).

All the mediums were solidified with 0.2% phytagel. The pH of all medium was adjusted to 5.8 with 0.1 NaOH or HCl before phytagel was added. The medium were autoclaved at 121°C for 15 min. Cultures were maintained in growth chamber at \(27\pm2^\circ\)C and photoperiod of 16 hours under a light intensity of 25 \(\mu\)M.m\(^{-2}\).s\(^{-1}\) obtained with white fluorescent lamps.

**RESULTS AND DISCUSSION**

**Embryos cultured in different levels of activated charcoal**
Independently of the concentration of activated charcoal used, it was observed significant influence on the results obtained. The embryos cultured in presence of activated charcoal showed a better development in the length stem and root number (Table 1).
Table 1. Effect of activated charcoal in culture medium containing half strength of Murashige and Skoog (1/2 MS) on length of stem and root number in interspecific hybrids of oil palm (Elaeis guineensis x Elaeis oleifera).

| Culture medium       | Length of stem (cm) | Number of root |
|----------------------|---------------------|----------------|
| With charcoal        | 3.7a                | 2.4a           |
| Without charcoal     | 1.7b                | 1.7b           |
| CV%                  | 15.4                | 8.7            |

Means followed by the same letter in columns are not significant by Tukey’s test (p < 0.05).

Perennials plants such as oil palm are rich in substances originated of the secondary metabolism, such as polyphenolic compounds and when these substances are in in vitro culture, tissues suffer oxidation due to polyphenolic oxidase enzyme and to avoid this process, is necessary some substance such as activated charcoal, that removes these compounds in culture medium (Cattelan et al., 2007; Alves, 2007). The activated charcoal can adsorb phenolic compounds and their products of oxidation, such as quinones, avoiding the oxidation process. (Teixeira et al., 1993; Alves, 2007).

In the regression analyses (Figure 1), it was observed a negative linear effect, and when the activated charcoal concentration increases, the percentage of oxidation decreases, being the minor percentage of embryos with oxidation found in 0.25% activated charcoal. Similar results were obtained with açaí palm (Euterpe oleraceae) using 0.2% of activated charcoal that there was suitable reduction in oxidation process (Cavalcante, 2001).

Activated charcoal in medium eliminates undesirable compounds, which aid to promote the normal development the plant in vitro, mainly in the first stages of growth (Freitas et al., 2009; Souza et al., 2009). Nevertheless, the increase of activated charcoal level into culture medium can be prejudicial, because the higher concentration could adsorb other substances of the culture medium, such as growth regulators, what could lead the undesired effects to the in vitro culture (Teixeira et al., 1993; Pullman et al., 2005; Alves, 2007).

Figure 1 - Effect of Different concentrations of activated charcoal on percentage of oxidation of oil palm embryos (Elaeis guineensis x Elaeis oleifera) after 30 days of culture.

Embryos disinfection
In the disinfection of the oil palm seeds, the contamination rate varied from 22 to 28% and there are not statistical differences among the treatments (Figure 2).

Figure 2 - Percentage of axenic embryos of oil palm (Elaeis oleifera x Elaeis guineensis) germinated in vitro under four different treatments (T1- 1% NaClO for 20 minutes; T2- 1% NaClO for 40 minutes; T3 – 2% NaClO for 20 minutes; T4 - 2% NaClO for 40 minutes) after 30 days of culture.
The embryos originated in the disinfection process varied from 67% to 90% of germination rate (Figure 2). The number of germinated embryos showed statistical differences in all treatments, varied from 0.88 to 1.18.

Table 2. Effects the time and levels of sodium hypochlorite (NaClO) in the disinfection of oil palm (Elaeis oleifera x Elaeis guineensis) seeds.

| Time       | NaClO 1% Contamination% | Not Contamination% | NaClO 2% Contamination% | Not Contamination% |
|------------|--------------------------|--------------------|--------------------------|--------------------|
| 20 minutes | 22 (10) a                | 78 (35) a          | 22 (9) a                 | 78 (36) a          |
| 40 minutes | 28 (13) a                | 72 (32) a          | 22 (10) a                | 78 (35) a          |

Means followed by the same letter in columns are not significant by Tukey’s test ($p < 0.05$).

For the number of embryos germinated the best results were T$_1$ and T$_3$ (Table 3). The time exposition of NaOCl is more harmful for embryos than the level. Similar results were found in murmuru (Astrocharyum ulei) which about 85.9% of germination of the zygotic embryos were obtained with the same concentration of sodium hypochlorite, however with minor exposition time which was 10 minutes (Pereira et al., 2006).

Table 3. Number of embryos of oil palm (Elaeis oleifera x Elaeis guineensis) germinated in four different treatments (T1- 1% NaClO for 20 minutes; T2- 1% NaClO for 40 minutes; T3 – 2% NaClO for 20 minutes; T4 - 2% NaClO for 40 minutes) after 30 days of culture.

| Treatments | Means of germinated embryos |
|------------|-----------------------------|
| T1         | 1.18a                       |
| T2         | 1.17a                       |
| T3         | 1.02c                       |
| T4         | 0.88d                       |

Means followed by the same letter in columns are not significant by Tukey’s test ($p < 0.05$). CV%=10. 25

**Callus Induction**

Callus initiation was observed within 4–6 weeks after inoculation of the embryos in medium for callus induction. In generally, the calli showed two patterns; white pattern and another with yellow appearance (Figure 3).

The two varieties showed different responses in *in vitro* culture. For the variety (SJ-165) in ½ MS medium, the best percentage of embryogenic callus occurred in the culture medium supplemented with 625 µM 2.4-D (30%), followed of 500 µM (11.60%) and 375 µM 2.4-D with 3.0% only.

However, for the variety (SJ-167), the results were quite opposite to the SJ-165, the best level was 375 µM 2.4-D which obtained 31.6% of calli, followed of 500 µM (19.60%) and 625 µM 2.4-D (8.3%) (Figure 4). For the variety SJ-165, the increase of concentration of 2.4-D promotes higher calli rate. However, for the variety SJ-167; the increase of the levels of 2.4-D decreases the calli rate.

The culture of callus can result in shoots or somatic embryos; however, it is necessary the exogenous supplementation with plant growth regulators for the adequate adjust between auxins and cytokinins (Nogueira et al., 2007; Santos et al., 2010).

**Figure 3** - Two different patterns of callus formed. The first callus shows the white pattern and the second the yellow appearance pattern.
Figure 4 - Percentage of calli formed in different concentration of 2.4-D in the varieties SJ-165 and SJ-167.

These differences between varieties, could reflect the genotype x environment interaction, that are the different responses obtained by genotypes in relation the same environmental conditions or when the same genotype responses differentially of accord with environment where itself find (Obisesan and Fatunla, 1983). The tissue culture is an important tool in genetic breeding to identification of genotypes that show high phenotype stability, or in general, genotypes that had great adaptability, being able to produce in different media culture (Cruz and Regazzi, 1997). In oil palm, callus was obtained with a minor concentration of 2.4-D (113 µM) in culture medium free of activated charcoal, however, the absence of the activated charcoal increased the oxidation percentage. (Rajesh et al., 2003). In oil palm cultured with the presence of activated charcoal in the culture medium was necessary increases fifty times (50x) the 2.4-D concentration to obtain the same result (Teixeira et al., 1993).

CONCLUSION
Activated charcoal (0.25%) is necessary to reduce oxidation in the embryos. Seed disinfection can be suitable reached with 1% NaOCl for 20 minutes. Different varieties have different ideal levels of 2.4-D for callus induction. The best level of 2.4-D for callus induction is 375 and 625 µM for the varieties SJ-167 and SJ-165, respectively.

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