INDUCTION AND MAINTENANCE OF EMBRYOGENIC CHARACTERISTICS OF CALLUS OF THE OIL PALM HYBRID MANICORÉ

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ABSTRACT – Large-scale oil palm propagation (*Elaeis guineensis* Jacq.) is difficult due to its unique apical meristem. In this context, micropropagation allows the multiplication of seedlings in vitro and the storage of germplasm elites. This study aimed to induce embryogenic calluses from leaves of oil palm plants in low concentrations of auxins and to observe the maintenance of these characteristics during in vitro cultivation. Calluses were induced in 0.5 cm leaf explants in Y3 culture medium supplemented with Picloram (4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid) or 2,4-D (2,4-dichlorophenoxyacetic acid), at concentrations of 0, 1, 3, 6, and 9 mg L^{-1}. The callus with embryogenic appearance was subcultured and evaluated regarding maintenance of embryogenic characteristics by cytochemical analyses. The best treatment for induction of calluses was composed of 1mg.L^{-1} of Picloram, which led to 30% callus formation. The calluses were classified into4 types, based on color and morphology. The cells of calluses with nodular and beige appearance have embryogenic characteristics, and the embryogenic potential of the cell masses was maintained over the 20 months of cultivation. This differentiated adaptation to the protocol can allow the advance in the mass propagation of oil palm through tissue culture, indicating the importance of investigating the topics proposed by the research.

Keywords: Elaeis guineensis, Micropropagation, Cytochemical analysis.

INDUÇÃO E MANUTENÇÃO DAS CARACTERÍSTICAS EMBRIOGÊNICAS DE CALO DO DENDEZEIRO HÍBRIDO MANICORÉ

*RESUMO – O difícil processo de propagação em grande escala do dendezeiro (**Elaeis guineensis** Jacq.) ocorre devido ao seu meristema apical único. Nesse contexto, a micropropagação permite a multiplicação de mudas in vitro e o armazenamento de germoplasma. O objetivo deste estudo foi induzir calosidades embriogênicas de folhas de dendezeiros em baixas concentrações de auxinas e observar a manutenção dessas características durante o cultivo in vitro. Calos foram induzidos em explantes de folhas de 0,5 cm em meio de cultura Y3 suplementado com Picloram (ácido 4-amino-3, 5,6-tricloro-2-piridinocarboxílico) ou 2,4-D (ácido 2,4-diclorofenoxiacético), nas concentrações de 0, 1, 3, 6 e 9 mg L^{-1}. O calo com aparência embriogênica foi subcultivado e avaliado quanto à manutenção das características embriogênicas por meio de análises citoquímicas. O melhor tratamento para indução de calosidades foi composto por 1mg. L^{-1} de Picloram, o que levou à formação de calosidades de 30%. Os calos foram classificados em 4 tipos, com base na cor e na morfologia. As células das calosidades com aspecto nodular e bege apresentaram características embriogênicas, e o potencial embriogênico das massas celulares foi mantido ao longo dos 20 meses de cultivo. Essa adaptação diferenciada ao protocolo pode permitir o avanço na propagação em massa do dendê por cultura de tecidos, indicando a importância da investigação dos temas propostos pela pesquisa.*

Palavras-Chave: Elaeis guineensis, Micropropagação, Análise citoquímica.

1.INTRODUCTION

Oil palm is a species of great economic importance, used in the cosmetics, food, and pharmaceutical industries, and as an alternative for biofuel production (Low et al., 2015). Oil palm is one of the most productive of oleaginous species, and due to growing world demand for vegetable oils, it is estimated that it will reach 240 million tons by 2050 (Corley, 2009).

Oil palm has two species that stand out in economic terms, *Elaeis guineensis* Jacq is of African origin and *Elaeis oleifera* Kunth of American origin. *Elaeis guineensis* characterized by high production of oil per bunch and *E. oleifera* by resistance to fatal yellowing disease, a lethal anomaly of an abiotic nature (Murphy, 2014) that attacks oil palm plantations, causing major damage. Crossing these species gave rise to the hybrid BRS Manicoré, which inherited resistance to fatal yellowing and high oil production. In addition, this hybrid has small sized plants, and this feature can ease collection of bunches, which causes less damage to the plant and extends its useful life. Another feature is that it has less saturated oil, with high olein content, which favors production of high-quality biodiesel (Barcelos et al., 2015).

Seedling production does not meet demand from producers due to the fact that they are mainly produced by seeds. Oil palm seed germination is a difficult process because dormancy and lower-than-expected germination rates (maximum seed germination (50%) was recorded in the case of chipping and scarification) (Murugesan et al., 2015; Luiset al., 2010; Cui et al., 2020).Despite the importance of these aspects, advances in germination metabolism have been very limited in oil palm. Oil palm seeds have a mixed physical-physiological dormancy mainly due the embryo if low degree of development in mature seeds. In practice, it means that there is a physical barrier for embryonic structures to pierce the micropylar endosperm region; and a physiological barrier, governed by hormonal signals that need to be removed to allow germination (Cui et al., 2020).

Tissue culture techniques such as somatic embryogenesis can aid in large-scale production of seedlings. This process occurs without the fusion of gametes, called asexual embryogenesis in which somatic embryos are developed from somatic or haploid cells, similar to zygotic embryos. At the end, a complete plant is formed. This technique allows large scale propagation of clones in reduced time and space under good plant health conditions (Parveezet al., 2015).

The formation of calluses and somatic embryos continues to be a major obstacle in oil palm tissue cultures. The average rate of somatic embryogenesis indirect obtained from leaf explants ranges from 3% to 6% (Low et al., 2008). Somatic embryogenesis in oil palm has been widely studied and successfully applied in plant production. However, induction of embryogenic calluses occurs at low percentages, and in addition, research studies use high concentrations of auxins in the culture medium (Scherwinski-Pereira et al., 2010; Scherwinski-Pereira et al., 2012; Balzon et al., 2013). High concentrations of plant growth regulators (PGRs), associated with numerous subcultures, can generate plant somaclonal variation (Bairu et al., 2011). Somaclonal variation may be a result of genetic and epigenetic modification (Larkinand Scowcroft, 1981). In oil palm, somaclonal variation may appear in a heterogeneous manner and in variable intensity among clones, and also among the flowers of a single palm tree. This results in partial or complete flower sterility, depending on the severity of the abnormality, and is known in the oil palm as mantled fruit. This can be observed at six years of age of the plant, causing a decline in fruit and oil production (Jaligot et al., 2000).Somatic embryogenesis in oil palm causes around 5% of plants to have somaclonal variations (Rival et al., 1999).

Consequentially the limited availability of explants, the difficult of somatic embryo initiation, proliferation and regeneration increasing the risk for somaclonal variation and several ways to improve the efficiency of the tissue culture method and to reduce the risk of somaclonal variation must be investigated. These include the use of alternative, such as different explants and propagation techniques and the detection of the mantled abnormality in an early stage (Weckx et al., 2019).

The use of cytokinins is also often associated with the occurrence of somaclonal variations (Eeuwens et al., 2002). Depending on the plant species, some types and concentrations of PGRs might include a higher risk for somaclonal variation (Weckx et al., 2019). For that reason, it is important to reduce the concentration

of PGRs used in the process of somatic embryogenesis (Jaligot et al., 2000; Mgbeze and Iserhienrhien, 2014). Reducing these PGRs can induce calluses with lower embryogenic potential, so cytological monitoring of calluses is required. Toluidine Blue can be useful for general staining and identification of phenolic compounds (O'Brien and McCully, 1981). Through cytochemical tests, using dyes such as toluidine blue, it is possible to observe embryogenic traits in callus cells, such as small, isodiametric cells with large nuclei and cell clusters (Moura and Motoike, 2009; Pádua et al., 2013). Dyes, such as Lugol, allow detection of starch granules, which provide energy for the formation and development of somatic embryos (Moura and Motoike, 2009).

The aim of this study was to induce embryogenic calluses from leaves of oil palm plants in low concentrations of auxins and to observe the maintenance of these characteristics during in vitro cultivation.

2.MATERIAL AND METHODS

2.1 Plant Material

This study was conducted at the Central Molecular Biology Laboratory of the University Federal of Lavras (Federal University of Lavras), Minas Gerais, Brazil.

Unripe fruits of the *E. guineensis* x *E. oleifera* hybrid Manicoré were provided by the Denpasa company, based in the state of Para, in the north of Brazil. The fruits (collected around 90 to 100 days after pollination) were washed in sodium hypochlorite (1.25%) and broken to remove the epicarp, mesocarp, and endocarp, exposing the coconut kernels. These kernels were washed in water and placed in a laminar flow cabinet for disinfestation. The kernels were immersed in 70% ethanol for 30 seconds, placed in sodium hypochlorite (1.25%) containing 3 drops of Tween, and then washed three times in sterile distilled water under constant shaking. After disinfestations, the embryos were isolated and inoculated in Petri dishes (100x20 mm)containing 20 ml of modified Y3 culture medium (Eeuwens, 1976), without the addition of amino acids, and supplemented with 45 g L^{-1} of sucrose and 0.6% (w/v) of agar (Sigma Aldrich), and pH was adjusted to 5.7 ± 0.1 with HCl (1N) or NaOH (1N). The inoculated embryos were kept in a light condition with photoperiod of 16 hours at 26 ± 2 °C for germination and were subcultured (transferred to a new culture medium without weighing) every 30 days.

2.2 Somatic Embryogenesis

For induction of embryogenic calluses, fragments (approximately 0.5 cm) of plant leaves of the *Elaeis guineensis* x *Elaeis oleífera* hybrid Manicoré i*n vitro* were used. The explants were inoculated with the adaxial part of the leaf in contact with the Y3 culture medium (Eeuwens, 1976) supplemented with Picloram or 2,4-D, at concentrations of 0, 1, 3, 6, and 9 mg L^{-1}. The culture media were supplemented with sucrose (3%) and solidified with agar (0.6%) (Sigma Aldrich), and the pH was adjusted to 5.7 ± 0.1 with HCl (1N) or NaOH (1N). After inoculation, the explants were kept in a growth chamber in the dark at a temperature of 27 ± 2 °C.

The experiment was conducted in a completely randomized design with 12 replications with 10 explants in each dish, for a total of 120 explants for each treatment. After 90 days, the percentage, morphology, and color of the callus were evaluated.

Different types of calluses were obtained, which were classified in four types: Type 1 (elongated and translucent), Type 2 (watery and translucent appearance), Type 3 (yellow and nodular in shape), and Type 4 (white and globular). Embryogenic calli were selected according to reports in the literature, which indicate that yellow and nodular calluses are embryogenic(Silva et al., 2014; Balzon et al., 2013;Pádua et al., 2013). Four months after inoculation, these calluses type 3 (yellow and nodular in shape) were subcultured, in the same culture medium supplemented with 1mg L-1Picloram every 30 days and their development was monitored through cytochemical analyses up to 9 months of cultivation. These calluses were kept in the same culture medium for 20 months and evaluated by cytochemical analyses to helps to visualize the possible formation of embryos.

2.3 Cytochemical Analysis of Calluses

The calluses were fixed in FAA (formaldehyde, acetic acid, and ethanol) for 72 hours and transferred to 70% ethanol. After fixing, calluses were placed in a 50% ethanol + resin solution overnight and were

then transferred to pure resin for 48 h. Finally, they were embedded in Leica® resin according to the manufacturer's protocol. Embedded samples were sectioned with a thickness of 5 mm using a rotary microtome and stained with 0.05% toluidine blue solution or Lugol solution. The stained cross sections were then mounted on slides and observed with a photonic Zeizz Scope.A1 microscope with attached camera (Sony).

3.RESULTS

3.1 Callus induction

Callus induction was observed in all treatments to which PGRs were added. The treatment with 1 mg L-1Picloram had higher percentages of explants with calluses (30%) compared to the other treatments evaluated (Figure 1).

The callus originated from different locations on the leaf explants and exhibited different features and was classified as Type 1, Type 2, Type 3, and Type 4. Type 1 callus cells are elongated and translucent (Figure 1A) and Type 2 have a watery and translucent

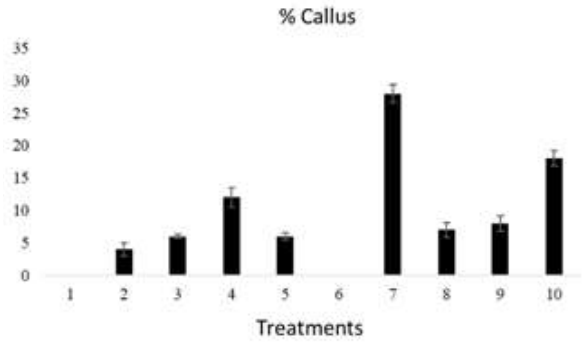

Figure 1 – Percentage of formation of callus in leaf explants of oil palm (*Elaeis guineensis* Jacq) hybrid Manicoré evaluation after 30 days, induced in culture medium Y3 under different concentrations of plant growth regulators (PGRs). T1 - control treatment without added PGRs. T2 - 1 mg L^{-1} 2,4-D. T3 - 3 mg L^{-1} 2,4-D. T4 - 6 mg L^{-1} 2,4-D. T5 - 9 mg L^{-1} 2,4-D. T6 - control with no added PGRs. T7 - 1 mg L^{-1} Picloram. T8 - 3 mg L^{-1} Picloram. T9 - 6 mg L^{-1}Picloram. T10 - 9 mg L^{-1} Picloram.

Figura 1 *– Porcentagem de formação de calos em explantes foliares de hibrido Manicoré (Elaeis guineensis Jacq) após 30 dias, induzida em meio de cultura Y3 sob diferentes concentrações de reguladores de crescimento vegetal (PGRs). T1 – tratamento controle sem adição de PGRs. T2 - 1 mg L^{-1} 2,4-D. T3 - 3 mg L^{-1} 2,4-D. T4 - 6 mg L^{-1} 2,4-D. T5 - 9 mg L^{-1} 2,4-D. T6 – controle sem PGRs adicionados. T7 - 1 mg L^{-1} Picloram. T8 - 3 mg L^{-1} de Picloram. T9 - 6 mg L^{-1} Picloram. T10 - 9 mg L^{-1} Picloram.*

appearance. Both of them arose around the wound that was made in the leaf explant. Type 3 is yellow and nodular in shape, and Type 4 is white and globular; both originated on the abaxial surface of the leaf explants.

Treatment with culture medium supplemented with 1 mg L^{-1}Picloram produced more callus, regardless of cell type (Figure 2). In this treatment, the highest percentage of Type 1 callus was also observed, in 21% of the explants, and the highest percentage of Type 2 appeared in 4% of the explants for this hybrid. Equal percentages (3%) of Type 3 callus induction were obtained in the treatments with Picloram at the concentration of 1 mg L^{-1} and 9 mg L^{-1}. Type 4 was induced in culture media supplemented with 6 mg L^{-1} 2.4-D (T4) (2%), in 1 mg L^{-1}Picloram (T7) (3%), and in 9 mg L^{-1} of Picloram (T10) (3%).To observe the embryogenic characteristics and maintenance of these characteristics during *in vitro* cultivation the callus were consequently evaluated as histological characteristic. Due the low amount callus was not performed evaluation to see how much the callus percentage increased.

3.2 Cytochemical analysis

The calluses at four and five months of cultivation exhibited at histological cross section small, isodiametric cells, with prominent nuclei, in the process of cell division (Figure 3A and 3C), and the presence of starch (Figure 3B and 3D), characteristic of embryogenic cells yellow and nodular in shape.

In calluses from six to seven months of cultivation, it was possible to observe at histological cross section regions with small and isodiametric cells and also regions with large cells without a nucleus and of irregular shape (Figure 4A and 4C) and, in some cells, the presence of phenolic compounds (Figure 4A (arrows) and 4C), which are non-embryogenic characteristics. In the region that exhibited small cells, starch could be observed (Figure 4B and 4D), which did not occur in large cells.

At eight months of cultivation, the formation of invaginations (Figure 5A), starch grain (Figure 5B) could be observed at histological cross section, which probably means the beginning of formation of somatic embryos and individualization of somatic embryos (Figure 5C). In the ninth and ten months, these embryos

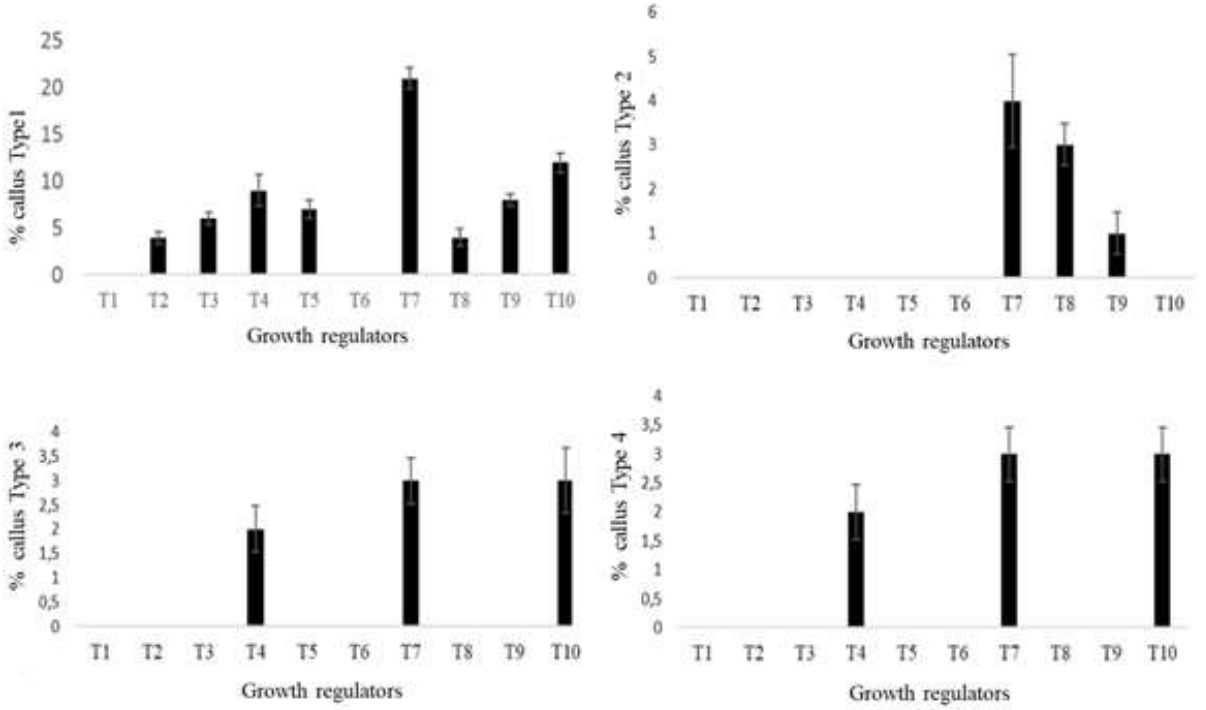

Figure 2 – Percentage of formation of different types of callus in leaf explants of oil palm (*Elaeisguineensis* Jacq.) hybrid Manicoré induced under different concentrations of growth regulators. A) Type 1 (callus cells are elongated and translucent), B) Type 2 (callus cells are watery and translucent), C) Type 3 (callus cells are beige and nodular in shape, and D) Type 4 (callus white and globular).

***Figura 2** – Porcentagem de formação de diferentes tipos de calos em explantes foliares de dendê (**Elaeis guineensis** Jacq.) Híbrido Manicoré induzido sob diferentes concentrações de reguladores de crescimento. A) Tipo 1 (as células do calo são alongadas e translúcidas), B) Tipo 2 (as células do calo são aquosas e translúcidas), C) Tipo 3 (as células do calo são de forma bege e nodular e D) Tipo 4 (branco do calo e globular).*

began the process of individualization because, around these cells, the release of large unviable cells from the cluster of cells with embryogenic characteristics such as meristems development (Figure 5C and 5E) and also presence of starch grains (Figure 5D and 5F).

Cytochemical analyses of calluses kept in culture medium for 20 months showed that the calluses, in spite of differences in color, maintained embryogenic characteristic during this period.

The embryogenic calli were morphologically separated into two regions in regard to color and texture, one white and spongy (Figure 6A circle) and the other beige and globular (Figure 6E circle). The white region (Figure 6A circle) had small cells, forming clusters, intensely stained with toluidine blue region (Figure 6B and 6C) and phenolic compounds (Figure 6B and 6C arrows) and starch in the outermost cells of the cell cluster (Figure 6D). Both regions had proembryos, and the region that was white and spongy had large cells around the formation of the proembryos containing starch (Figure 6B and 6D).

The proembryogenic callus with beige and nodular appearance (Figure 6E circle) had more individualized embryos of the callus cell(Figure 6F and 6H) in relation to the white-colored callus cell (Figure 6B), as well as large cells around them being freed from the proembryos (Figure 6F and 6G). The proembryos exhibited initial formation of procambium and protoderm tissues like (Figure6B and 6F) and the presence of phenolic (Figure 6Band 6G arrows).

4.DISCUSSION

In all treatments containing PGRs induced callus, being the treatment with 1 mg L^{-1}Picloram stood out from the others. The auxin Picloramis also reported by other authors as efficient in the formation

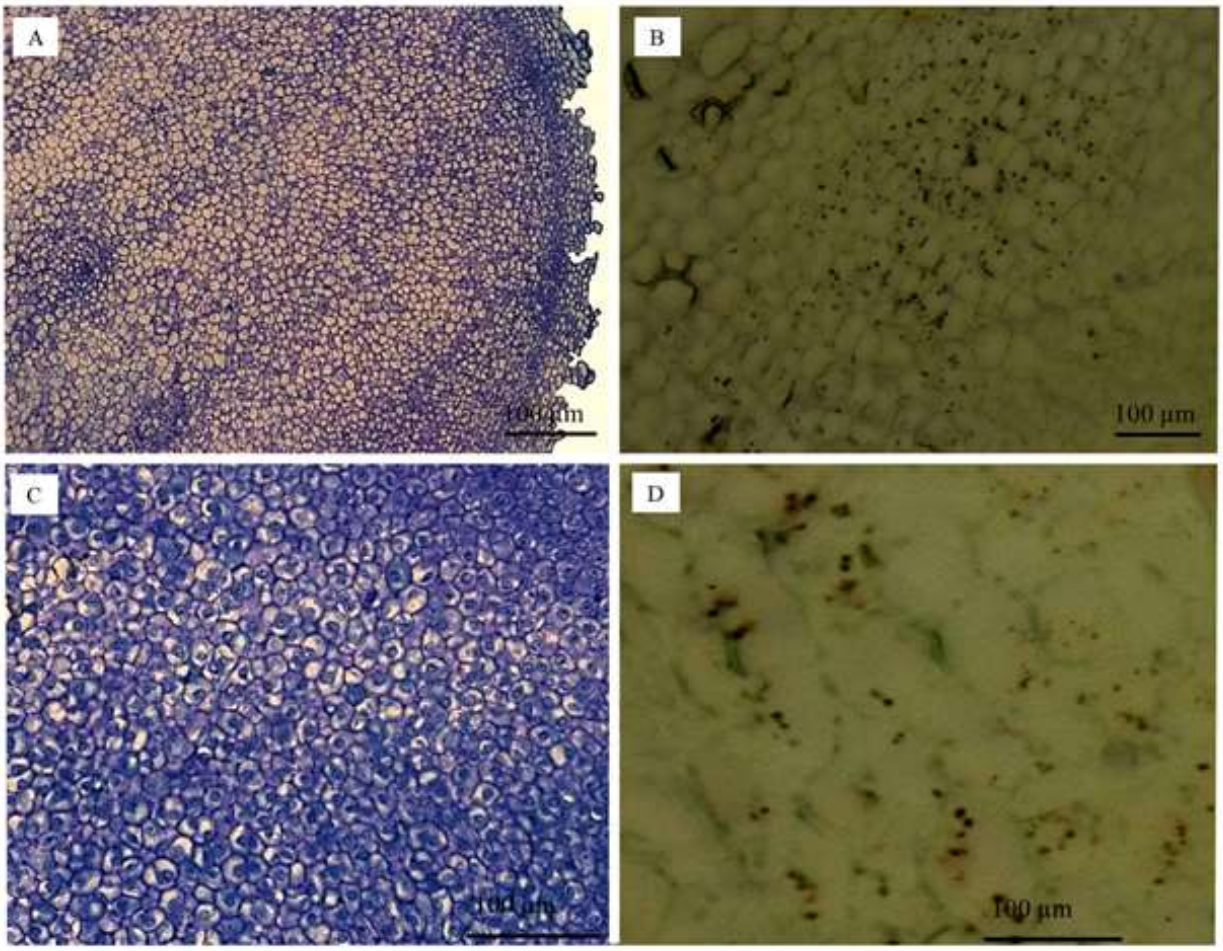

Figure 3 – Oil palm hybrid *Elaeisguineensis* Jacq. Manicoré. calluses with white and spongy appearance at 4 months of cultivation on Y3 culture medium supplemented with 1mg L^{-1}Picloram. (A) Callus cells at 4 months; after induction, stained with toluidine blue, (B) stained with Lugol indicating the presence of starch (arrows). (C) Callus culture cells at 5 months of cultivation; after induction, stained with toluidine blue. Arrows indicate cells with nuclei and division, (D) presence of starch (arrows).

Figura 3 *– Híbrido* ***Elaeis guineensis*** *Jacq. Manicoré. Calosidades com aparência branca e esponjosa aos 4 meses de cultivo em meio de cultura Y3 suplementado com 1mg L^{-1} de Picloram. (A) Células calosas aos 4 meses; após indução, corado com azul de toluidina, (B) corado com Lugol indicando a presença de amido (setas). (C) Células de cultura de calos em 5 meses de cultivo; após indução, corado com azul de toluidina. As setas indicam células com núcleo e divisão, (D) presença de amido (setas).*

of embryogenic calluses of oil palm (Scherwinski-Pereira et al., 2010; Silva et al., 2014; Pádua et al., 2013; Balzon et al., 2013; Vilela et al., 2019; Yarra et al., 2019). The first somatic embryos and regenerated plants (clones) of the *Pisifera variety* of African oil palm in Brazil obtained leaf explants were reported by Almeida et al. (2020). In this study, the leaf explants showed high levels of oxidation, starting at 90 days of cultivation (above 80%) and was observed that callus formation occurred during or after an oxidation event of the explant. In our experiment it was also observed oxidation, but this fact did not block the formation of calluses.

Callus originated from different locations on the leaf explants exhibited different features. Type 1 and Type 2 callus arose around the wound that was made in the leaf explant and Type 3 and Type 4 was originated on the abaxial surface of the leaf explants. Sumaryono and Riyadi(2011) observed for *E. guineensis*, leaves explants can improved somatic embryo production and uniformity (Sumaryono et al., 2007) and to decrease floral abnormality (Sumaryono and Riyadi, 2011).

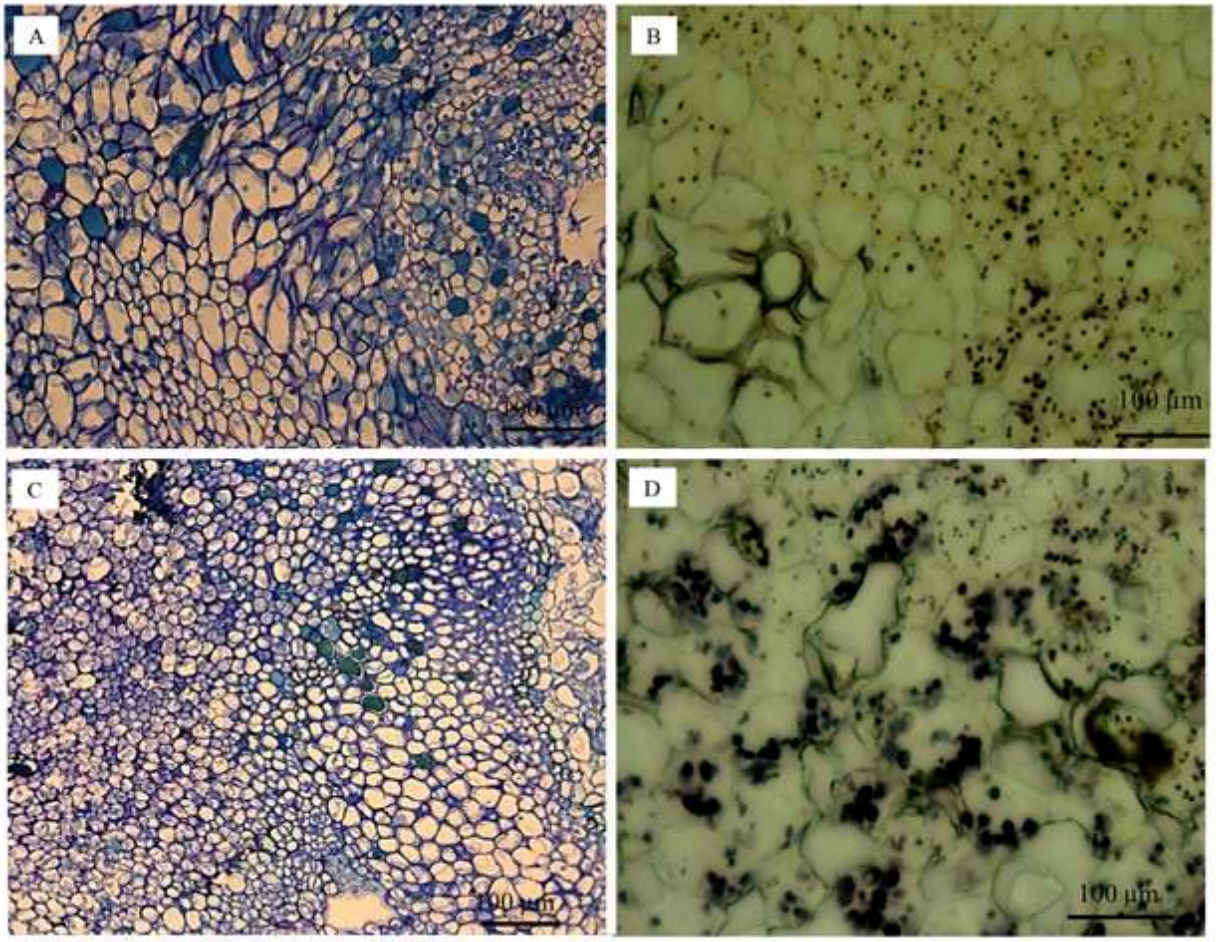

Figure 4 – Oil palm hybrid *Elaeis guineensis* Jacq. Manicoré. calluses with white and spongy appearance at 6 months of cultivation on Y3 culture medium supplemented with 1mg L^{-1}Picloram(A)stained with toluidine blue and presence of phenolic compounds (arrows), (B) Presence of starch (arrows). (C) Callus cells at 7 months; stained with toluidine blue and presence of phenolic compounds (arrows), and (D) presence of starch (arrows).

Figura 4 *– Híbrido* ***Elaeis guineensis*** *Jacq. Manicoré. calosidades com aspecto branco e esponjoso aos 6 meses de cultivo em meio de cultura Y3 suplementado com 1mg L^{-1} Picloram (A) corado com azul de toluidina e presença de compostos fenólicos (setas), (B) Presença de amido (setas). (C) células calosas aos 7 meses; corados com azul de toluidina e presença de compostos fenólicos (setas) e (D) presença de amido (setas).*

In this context, Type 3 callus, obtained on 3% of the treatment, showed embryogenic characteristics and were undercultured. This rate of embryogenic callus was not consistent with results of Páduaet al.(2013) in a study involving callus induction in the hybrid oil palm Tenera, in which Type 3 calluses exhibited embryogenic characteristics in 9% of explants with calluses in the culture medium with 1 mg L^{-1}Picloram. From this, it may be inferred that the formation rate of calluses with embryogenic potential depends on the genotype.

Type 3 calliwere yellow and nodular in shape and, for oil palm, yellow and nodular calluses have embryogenic characteristics (Silva et al., 2012;Balzon et al., 2013; Pádua et al., 2013, Pádua et al., 2018). Moreover, these morphological embryogenic characteristics are also observed in other palms, such as, date palm (Aslam et al., 2011) macauba palm (Moura et al., 2010), and peach palm (Steinmacher et al., 2011).

Therefore, four months after inoculation the Type 3 yellow and nodular calluses were selected and

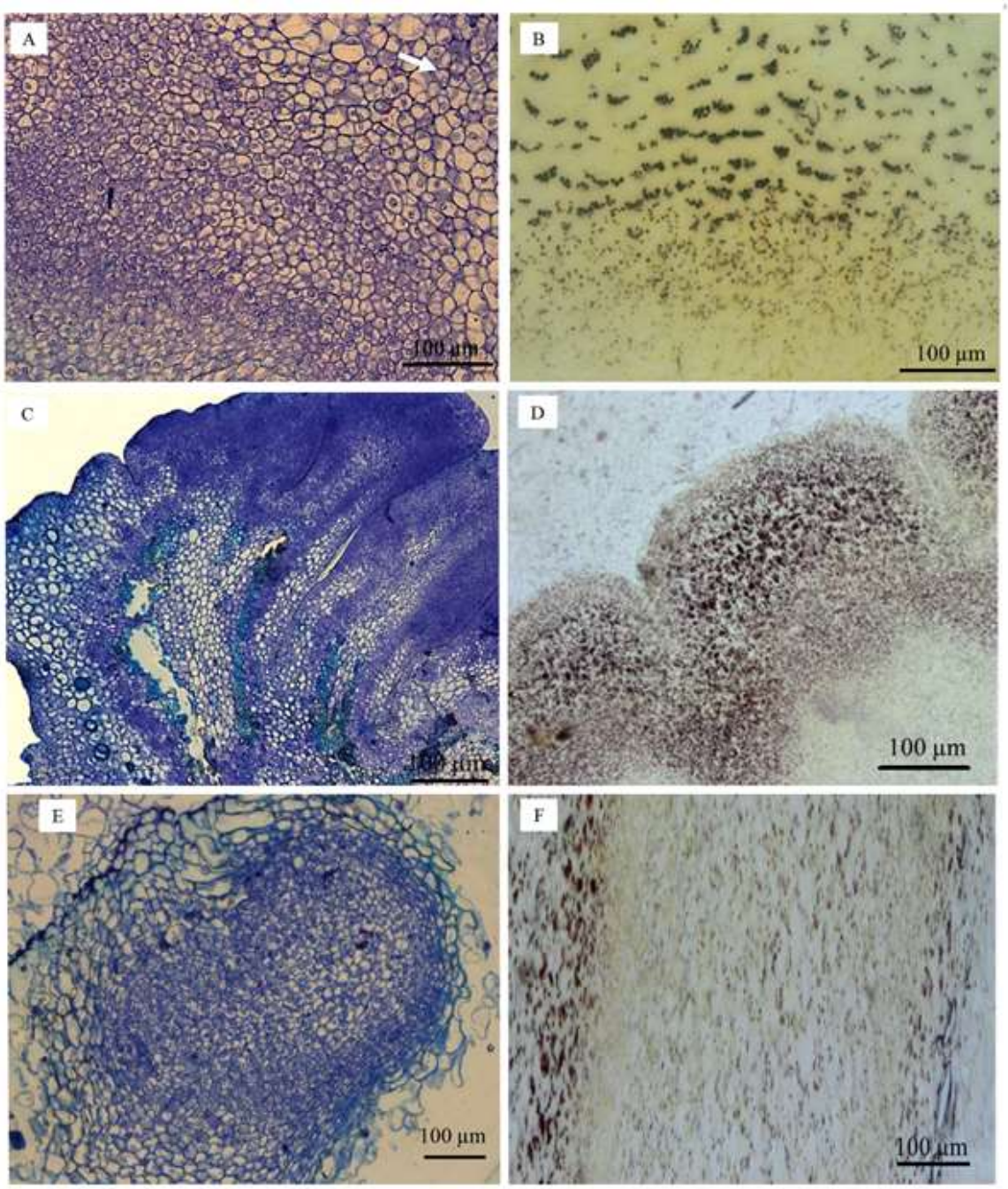

Figure 5 – Oil palm hybrid *Elaeisguineensis* Jacq. Manicoré. calluses with white and spongy appearance at 8 months of cultivation on Y3 culture medium supplemented with 1mg L^{-1}Picloram;(A) stained with toluidine blue and (B) presence of starch (arrows). (C) Cell clusters, strongly stained at 9 months of cultivation with toluidine blue and (D) Presence of starch (arrows). (E) Organized cell clusters, strongly stained at 10 months of cultivation with toluidine blue and (F) Presence of starch.

Figura 5 *– Híbrido* ***Elaeisguineensis*** *Jacq. Manicoré. Calosidades com aspecto branco e esponjoso aos 8 meses de cultivo em meio de cultura Y3 suplementado com 1mg L^{-1} Picloram; (A) corado com azul de toluidina e (B) presença de amido (setas). (C) Aglomerados de células, fortemente corados aos 9 meses de cultivo com azul de toluidina e (D) Presença de amido (setas). (E) aglomerados celulares organizados, fortemente corados aos 10 meses de cultivo com azul de toluidina e (F) Presença de amido.*

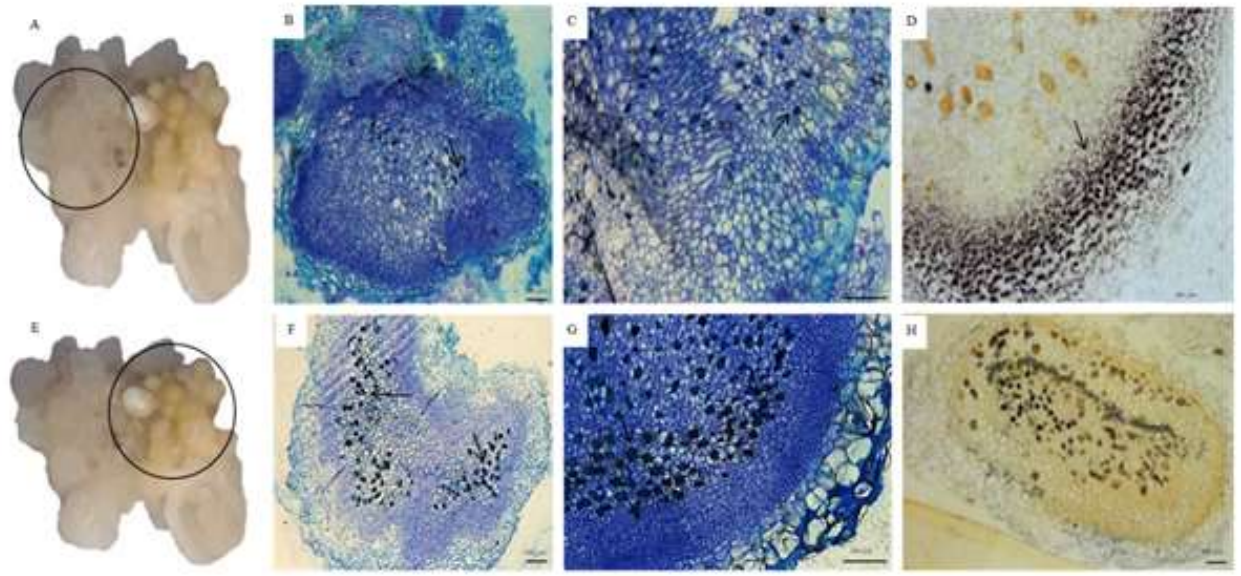

Figure 6 – Oil palm hybrid *Elaeisguineensis* Jacq. Manicoré calluses with white and spongy appearance at 20 months of cultivation on Y3culture medium supplemented with 1mg L^{-1}Picloram (A)Proembryos stained with toluidine blue with cells showing phenolic compounds (B). Proembryos showing cells with phenolic compounds (arrows) and tracheary elements (C). Presence of starch (arrows) (D). Oil palm calluses with nodular and beige appearance at 20 months of cultivation (E). Proembryos stained with toluidine blue showing cells containing phenolic compounds (arrows). Initial formation of procambium (Pc) (F). Proembryos stained with toluidine blue showing cells containing phenolic compounds (arrows) and large cells surrounding. Initial formation of procambium (Pc) and protoderm (Pt) (G). Presence of a small amount of starch (arrows). Initial formation of procambium and protoderm(H).

Figura 6 *– Híbrido* ***Elaeis guineensis*** *Jacq. Manicoré. Calosidades com aparência branca e esponjosa aos 20 meses de cultivo em meio de cultura Y3 suplementado com 1mg L^{-1} Picloram (A) Proembriões corados com azul de toluidina com células apresentando compostos fenólicos (B). Proembriões mostrando células com compostos fenólicos (setas) e elementos traqueais (C). Presença de amido (setas) (D). Calos de dendê com aspecto nodular e bege aos 20 meses de cultivo (E). Proembriões corados com azul de toluidina mostrando células contendo compostos fenólicos (setas). Formação inicial de procâmbio (Pc) (F). Proembriões corados com azul de toluidina mostrando células contendo compostos fenólicos (setas) e células grandes ao redor. Formação inicial de procâmbio (Pc) e protoderme (Pt) (G). Presença de pequena quantidade de amido (setas). Formação inicial de procâmbio e protoderme (H).*

subcultured, in the same culture medium supplemented with 1mg L^{-1} Picloram every 30 days and their development was monitored through cytochemical analyses up to 9 months of cultivation. These calluses were also kept in the same culture medium for 20 months and evaluated by cytochemical analyses.

The calluses at four and five months of cultivation exhibited characteristic of embryogenic cells such as isodiametric cells, with prominent nuclei, in the process of cell division. Small, rounded cells with evident nuclei and starch granules are considered to have embryogenic potential, in contrast with large irregular cells, which are in the process of cell death (Pádua et al., 2013, Pádua et al., 2018).

However, in calluses from six to seven months of cultivation, it was possible to observe regions with embryogenic and regions with non-embryogenic characteristics. Respectively, regions with small and isodiametric cells and regions with large cells without a nucleus proeminent and of irregular shape and presence of phenolic compounds. The presence of phenolic compounds with greenish blue color is due to the metachromatic reaction of toluidine blue (Corredoira et al., 2015; Pelegrini et al., 2013). These results were also observed by Steinmacheret al.(2011) in the vacuoles of the callus cells during somatic embryogenesis of oil palm. Phenolic secretions inhibit the development of the embryos (Kouassi et al., 2017).Accumulation of polyphenols and consequent oxidation products usually modifies the composition of the culture medium and absorption and inhibit the growth of explants, not infrequently causing their death (Van Winkle et al., 2003).

The regions with embryogenic characteristics were also observed in other work of oil palm calluses, which afterwards regenerated plants (Balzon et al., 2013) meaning that these characteristics are important as signs of embryogenic potential and were maintained even partially during subculture.

The beginning of formation of somatic embryos was visualized from eight months of cultivation, invaginations could be observed, which probably means the beginning of formation of somatic embryos and individualization of somatic embryos and presence of starch in cells.When used at low concentrations of PGRs (BAP adenine derivative cytokinin) coupled with high rates of subcultures, leads to an efficient protocol with limited oxidative browning, allowing the establishment of embryogenic cells and the multiplication of somatic embryos in date palm (Abohatem et al., 2011).Consequentially, in this work the eight months of cultivation may also reduce oxidative browning and provide the development of embryogenic callus.

Starch accumulation in embryogenic cells or in neighboring cells is related to the acquisition of embryogenic competence (Balzon et al., 2013; Silva et al., 2014;Lim et al., 2018). The starch produced in cells provides high levels of ATP, which is the energy source for cells and they are used in cell metabolism for intense cell division and subsequent development of embryos (Silva et al., 2014;Lim et al., 2018). The presence and amount of starch can vary depending on the phase of embryo development because, during cell division and embryo development, this compound (starch) is consumed (Balzon et al., 2013).In embryogenic cells of *Elaeisguineensis* (Steinmacher et al., 2011; Pádua et al., 2013) storage of starch granules during embryogenesis is commonly observed. In oil palm, starch accumulation was observed in callus cells as of 45 days of cultivation, suggesting that this accumulation is a strong indicator of cells with high embryogenic competence (Silva et al., 2014). In the proembryo formation phase in the callus cell, there was an accumulation of starch granules in the large cells adjacent to centers of cell division. These characteristics were also observed by Silva et al., (2012) and, Almeida et al., (2020) during the formation of oil palm somatic embryos, confirming this to be an energy source for embryo development (Martin et al., 2000).

Histochemically, oil palm meristematic zone cells showed starch grains, but no reserve proteins. In the morpho-anatomical and histochemical analyses of the callus types, the yellowish nodular callus analyzed during induction was the one that presented the greatest starch densificationand followed the route for the differentiation of calli and somatic embryos (Gomes et al., 2017;Almeida et al., 2020)as also observed on this study.

In the nine and ten months were observed the development of this proembryosshowing likely meristematic region and began the process of individualization. Oil palm cells calliare characterized as meristematic by the presence of small, rounded cells with dense cytoplasm and apparent nucleus and nucleolus (Silva et al., 2014;Gomes et al., 2017) and center of meristematic activity are observed where the cells were smaller, than in other parts of the callus, and more intensely stained(Gomes et al., 2017). Likewise, in *Cocos nucifera* L., a meristematic region with intense cell division strongly stained by toluidine blue was observed, which gave rise to somatic embryos after subcultures.

The process of proembryos individualization was also observed, around these cells, the release of large unviable cells from the cluster of cells with embryogenic characteristics such as meristems development and also presence of starch grains. Somatic embryos of eucalyptus globulus induced in a culture medium with Picloram, disintegration of the cell around the proembryo was likewise observed, which later developed and regenerated plants (Corredoira et al., 2015).

Consequentially, the calluses maintained embryogenic potential during 20 months de cultivation,showing the development of proembryos exhibited initial formation of meristem protoderm and procambium. The formation of protoderm was observed in the globular embryo stage in *Anthurium andraeanum* (Silva et al., 2012) and initial procambium formation occurred only when the somatic embryos were in the most advanced globular phase (Silva et al., 2014;Gomes et al., 2017). *Acrocomia aculeata*(Jacq.) Lodd. Mart. Macauba palm embryos, all the meristematic tissues could be observed: protoderm, ground meristem, and procambium,indicating greater differentiation of the embryos (Moura et al., 2010).

Procambium formation was also observed in studies by Silva et al. (2012), Gomes et al., (2017) in oil palm calluses and by Corredoiraet al. (2015) in *Eucalyptus* spp. calluses, in which these authors note differentiation of the procambium in tracheary elements and the absence of starch in these cells, which corresponds to information that the presence of starch precedes embryo formation (Silva et al., 2012).In embryogenic calluses of macauba at 60 days of cultivation, the meristematic regions began to differentiate in meristematic nodules, similar to those which we call pro embryos in this study, and they developed into globular pro embryos at 75 days, and the protoderm was observed in them. Some of these somatic embryos contained a starch reserve in the cortical parenchyma (Moura et al., 2010). The small amount of starch observed in this experiment at 20 months of cultivation may be due to hydrolysis of the starch so as to provide the high levels of ATP necessary for the divisions and differentiation of the procambium (Silva et al., 2012).

Heterogeneity of color and appearance were observed in these calluses after 20 months of cultivation; a white-colored region with a spongy appearance and another region that was yellow with a nodular aspect were observed and collected for cytochemical analyses. He et al. (2009) also observed morphological changes in calluses of *Jatropha curcas* - green calluses became yellow and then brown; however, these changes occurred more quickly (over approximately two weeks for each change in color) than the changes observed in this study.

Finally, several ways to improve the efficiency of the tissue culture method and to reduce the risk of somaclonal variation are described for tissue culture of oil palm, such as the use of alternative explants and propagation techniques, the introduction of specific embryo maturation treatments and the detection of the mantled abnormality in an early stage. These methods have not yet been fully explored and the development of an efficient oil palm micropropagation protocol is needed to keep up with the increasing demand for palm oil in a sustainable way (Weckx et al., 2019)

In this work, we described the induction of embryogenic calluses at low concentrations of PGRs from leaf explants. The cells of calluses with nodular and beige appearance have embryogenic characteristics, and the embryogenic potential of the cell masses was maintained over the 20 months of cultivation. However, the regeneration and somaclonal variation must be evaluated on further experiments. Understanding the underlying molecular mechanisms of *in vitro* plant regeneration and propagation is important for detecting the sources of abnormalities in regenerated plants (Azizi et al., 2020).

5.CONCLUSION

In this work, it was possible to induce embryogenic calluses from leaves of oil palm plants in low concentrations of auxins. The cells of calluses with nodular and beige appearance have embryogenic characteristics, and the embryogenic potential of the cell masses was maintained over the 20 months of cultivation. Early identification of embryogenic characteristics cells would increase efficiency of oil palm somatic embryogenesis. The embryogenic cells can be distinguished from non-embryogenic cells based on morphological characteristics. This different adaptation to the protocol can allow advance on mass propagation of oil palm by tissue culture, indicating the importance of the investigation of several of the proposed research topics.

6. AUTHOR CONTRIBUTIONS

Marlúcia Souza Pádua Vilela – Execução de todas as etapas, cultura de tecidos, análises histológicas e redação do artigo.Jéssica de Castro e Andrade – análises histológicas e redação do artigo. Luciano Vilela Paiva – Orientação durante todo o experimento. Raíssa Silveira Santos – cultura de tecidos, preparo de meios de cultura, inoculação e manutenção dos explantes e calos. Vanessa Cristina Stein – análise dos dados e redação do artigo. Patrick Callegari Magnani Santos Alves – preparação do material para a inoculação in vitro.

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