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Establishment, Multiplication, and Biochemical Analysis of Embryogenic Lines of the Amazonian Palm *Euterpe precatoria* Mart. under Suspension Culture

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Abstract: The palm *Euterpe precatoria* holds great social, cultural, and environmental importance. The heart of palm and the fruit are the main products used for industrialization due to their energetic properties. Thus, the aim of this study was to establish a suspension cultivation protocol for the species using different explant sources. For this, eight lineages of *E. precatoria* embryogenic calluses were tested, with five in liquid medium Murashige and Skoog (MS) with 5 µM Picloram and three for comparison in semisolid medium MS with 20 µM Picloram and 5 µM 2iP. The growth curve was obtained by weighing the calli from 60 to 180 days of cultivation. The Gompertz model was applied, and growth kinetics were evaluated. At 100 days, the contents of total soluble sugars (TSSs) and total soluble proteins (TSPs) were determined. Principal components (PCA) were measured. According to the analysis of the data, the cultivation of *E. precatoria* lineages in liquid medium was successfully carried out, and the establishment was achieved. The model can be considered adequate since the R^2 values found describe more than 90% of the growth kinetics of the lineages. In the liquid system, lineages L1 (from leaf explants and multiplied in semisolid medium—SM), L2 (from leaf explants and multiplied in SM), and L6 (from zygotic embryo explants and multiplied in liquid medium—LM) showed the shortest time to double the biomass accumulation. Multivariate analysis reveals a significant increase in masses in liquid cultures, represented by lineages L6 and L2. There was statistical difference in the amount of TSSs extracted; the highest TSS levels were observed in lineages cultivated in LM. The protein content found was very low, showing statistical differences among the lineages. In this work, the establishment and multiplication of embryogenic calli of *E. precatoria* are described for the first time, and they emerge as viable alternatives for the vegetative propagation of the species.

Keywords: Arecaceae; *Euterpe* spp.; açaí-solteiro; liquid medium; Picloram; cell suspension; total soluble sugars; proteins

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1. Introduction

Palms belong to the Arecaceae family, one of the most abundant families worldwide, distributed primarily in the tropics but also found in subtropical regions [1]. Some palm species form clumps while others have a single stem, such as *Euterpe precatoria*, also known as "açaí-solteiro", due to its single stem (trunk) [2,3].

At the apex of the *E. precatoria* trunk lies the heart of palm, which is utilized in Brazilian cuisine and internationally. It has been a dietary staple for decades and long

considered the most economically valuable product of *Euterpe* species, including *E. edulis* and *E. oleracea* [4,5]. However, it is the pulp of the fruit, from which "açaí wine" is extracted, that constitutes the most economically valuable product of açaí palm species, especially *E. oleracea* and *E. precatoria*, due to their nutritional properties. The fruits are rich in minerals such as calcium, magnesium, potassium, and phosphorus, as well as vitamins B1, C, and E. They also contain essential fatty acids, carotenoids, anthocyanins, and phenolic compounds [6–9]. In 2022, Brazil produced 1,699,588 tons of açaí [10].

Due to these characteristics, the demand for the fruits has increased considerably, necessitating techniques to improve plantlet quality, particularly in terms of faster production and more productive, disease-resistant varieties that promote uniformity in the production system. Additionally, as a single-stem species, *E. precatoria* cannot be vegetatively propagated by conventional techniques, preventing the cloning of desirable individuals, as in mass selection programs. This significantly limits the fixation of genetic gains over long selection cycles during crop breeding programs [11,12].

Thus, micropropagation represents an important tool for overcoming many of these problems through cloning via somatic embryogenesis (SE). Once mastered, cloning through somatic embryogenesis can provide the continuous production of propagative materials, with the advantage of being able to be carried out at any time of the year and in small physical spaces using a small amount of initial material. Research using SE for plant regeneration has been conducted in *E. precatoria* by Ferreira et al. [12,13], who established somatic embryogenesis protocols from different types of explants (zygotic embryo and leaf tissue).

However, optimizing protocols to make the technique more efficient, reproducible, and capable of generating material on a large scale is a constant challenge in various species where the technique has already been developed, especially in the Arecaceae family, which is notoriously composed of species considered recalcitrant to in vitro culture [14]. To date, there are no records of *E. precatoria* cultivation in liquid production systems, highlighting the need for additional studies to explore the potential of this species. Cultures in liquid consistency medium have already been successfully developed for economically important palm species such as *Elaeis guineensis* [15,16] and *Phoenix dactylifera* [17–19].

The use of liquid medium cultures favors the establishment of suspension cultures with a consequent increase in the production of embryogenic materials. This fact can facilitate the obtaining of cloned plants and favor the multiplication and maintenance of cultures. In this context, just as SE has made significant advances through suspension cultures in crops such as those mentioned above, this research reports a reliable protocol for *E. precatoria* cultures in liquid medium, which can serve as an initial step toward improving the process for the clonal plantlets production of this species. Additionally, to understand the biochemical and physiological changes that occurred during the multiplication of the embryogenic calluses obtained, biochemical analyses were also carried out.

In this context, this study aimed to establish *Euterpe precatoria* suspension cultures from different explant sources, analyze growth and multiplication kinetics, and biochemically and anatomically evaluate the materials under these culture conditions.

2. Materials and Methods

2.1. Plant Material, Induction, and Multiplication of Embryogenic Callus in Semisolid Medium

This research was conducted at the Tissue Culture Laboratory II of Embrapa Genetic Resources and Biotechnology, located in Brasília, Federal District, Brazil. The plant material used in the experiments was obtained from adult individuals of *Euterpe precatoria* collected in native forests in the state of Acre, Brazil. Embryogenic calli originating from immature inflorescence segments, zygotic embryos, and leaf tissues were induced and multiplied according to Ferreira [12,13,20], as described below:

2.1.1. Induction and Multiplication of Leaf Explant Calli

Calli originating from leaf tissues were induced from explants extracted from the apical region of the palm (heart of palm), containing immature, unexpanded, and chlorophyll-free leaves. For this purpose, the basic Murashige and Skoog (MS) medium [21] was supplemented with the auxin 4-amino-3,5,6-trichloropicolinic acid (Picloram) at three concentrations (450, 675, and 900 μM), in addition to 30 g L^{-1} sucrose, 0.5 g L^{-1} hydrolyzed casein, 0.5 g L^{-1} cysteine, 0.5 g L^{-1} glutamine, 2.3 g L^{-1} Phytagel gelling agent (Sigma, St. Louis, MO, USA), and 2.5 g L^{-1} activated charcoal.

After the induction of primary calli, these were transferred to a new semisolid multiplication medium, characterized by MS medium exactly as described above, except by the reduction in the concentration of the auxin Picloram (20 μM) and of the activated charcoal (1.5 g L^{-1}), and the addition of 5 μM 2-isopentenyl adenine (2iP).

2.1.2. Induction and Multiplication of Inflorescence Explant Calli

Segments of immature inflorescences, with unopened spathes, were inoculated on MS basic culture medium, supplemented with 2.5 g L^{-1} activated charcoal, EDTA iron, and vitamins at the original concentration of the MS medium. To this medium were added 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} cysteine, 0.5 g L^{-1} hydrolyzed casein, 2.3 g L^{-1} Phytagel gelling agent (Sigma, St. Louis, MO, USA), and 450 μM 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 45 μM 2iP.

Following the induction of primary calli, a portion was transferred to a semisolid multiplication medium, as detailed in the subsequent section.

2.1.3. Induction and Multiplication of Zygotic Embryo Explant Calli

Zygotic embryos from mature fruits were cultivated in a medium containing Y3 salts—Eeuwens and Blake medium [22]—supplemented with EDTA iron and vitamins at the original concentration of the MS medium, and 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} cysteine, 0.5 g L^{-1} hydrolyzed casein, and 2.3 g L^{-1} Phytagel gelling agent (Sigma, St. Louis, MO, USA) were added, in addition to the Picloram auxin at a concentration of 13.57 μM.

The calli obtained from the induction were transferred to a multiplication medium, maintaining the basic MS medium, adding 2.5 g L^{-1} activated charcoal, EDTA iron, and vitamins at the original concentration of the MS medium, and 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} cysteine, 0.5 g L^{-1} hydrolyzed casein, 20 μM Picloram, and 5 μM 2iP.

Before adding the gelling agent and activated charcoal to the media for each explant above, the pH was adjusted to 5.8 ± 0.1 and then autoclaved at 121 °C and 1.5 atm pressure for 20 min. The induction and multiplication of embryogenic calli occurred in disposable Petri dishes (90 × 15 mm), containing approximately 25 mL of medium, and sealed with transparent PVC film (Lusafilm R105®, Guarulhos, SP, Brazil). In all cases, the materials were cultured in a growth room with no light and a controlled temperature of 25 ± 2 °C.

Subcultures were performed every 30 days, for up to 180 days of cultivation in semisolid medium, checking the percentages of establishment, oxidation (darkening), and contamination.

An illustrative scheme of callogenesis for the establishment and multiplication of cellular aggregates in semisolid and liquid media is shown in Figure 1.

2.2. Weighing of Embryogenic Calluses in Semisolid Medium

The materials used to measure the multiplication of the embryogenic lines obtained in semisolid medium were derived from leaf tissues, immature inflorescences, and zygotic embryos. Each evaluated lineage consisted of ten replicates, each formed by a Petri dish (15 × 90 mm).

To assess the multiplication of friable embryogenic calli of the above lines in their respective semisolid multiplication media, the mass of the calli was weighed with an initial mass equivalent to 0.500 g in Petri dishes, regularly tared for weighing on a precision balance

(Figure 2). Weighing of the calluses under cultivation was performed every 30 days to obtain the growth curve of the calli in semisolid medium, with the first measurement taken 60 days after inoculation and the last evaluation at 180 days from the start of multiplication.

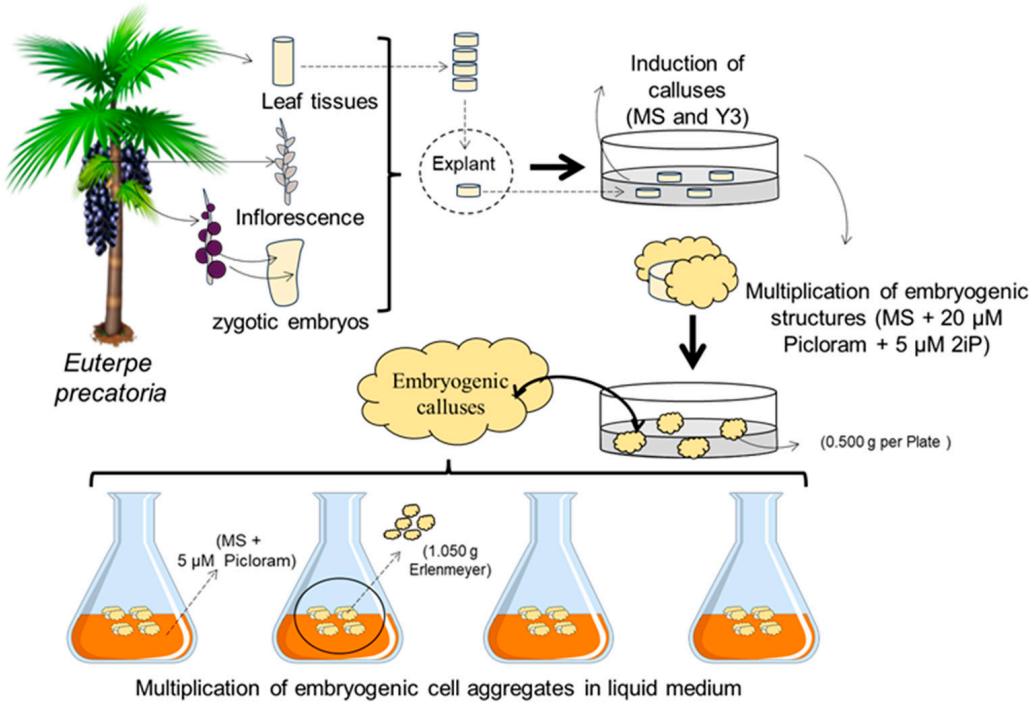


Figure 1. Illustrative scheme of callogenesis for the establishment and multiplication of cellular aggregates in semisolid (MS medium supplemented with 20 µM Picloram and 5 µM 2iP) and liquid (MS supplemented with 5 µM Picloram) media.

2.3. Establishment and Multiplication of Embryogenic Calluses in Liquid Medium

The embryogenic calli derived from immature leaflets, segments of immature inflorescences, and zygotic embryos, with friable consistency (easily fragmented), white or light yellow, were fragmented with the aid of tweezers and a no. 11" scalpel blade on filter paper placed inside Petri dishes (15 × 90 mm) (Figure 2).

Once weighed, the embryogenic lines with an average weight equivalent to 1.050 g were inoculated into 125 mL Erlenmeyer flasks (Figure 2), filled with 35 mL of liquid culture medium, consisting of MS medium salts and vitamins, supplemented with 5 µM Picloram, 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 0.1 g L^{-1} asparagine, and 0.1 g L^{-1} hydrolyzed casein. After inoculation, the flasks were capped with aluminum foil (Thermoprat) and sealed with transparent PVC film. After 24 h of rest, the Erlenmeyer flasks containing the propagative materials were placed on an orbital shaker at 100 rpm, with a 16 h photoperiod in a growth room at 25 ± 2 °C, under cool white fluorescent lamps and a light radiation of 50 µmol m^{-2} s^{-1}. Subcultures were performed every 30 days for up to 180 days of cultivation in a liquid medium, checking the percentage of establishment (material survival). To obtain the growth curve, weighing was performed every 30 days, starting 60 days after inoculation.

2.4. Characterization of Lineages

In general, eight types of propagative materials, all originating from the somatic material of adult plants, were evaluated and referred to as embryogenic lineages. Three of these lineages were obtained from leaf explants (L450, L675, and L900), named according to the concentration of auxin (450, 675, and 900 µM of Picloram) used in the callus induction phase, and according to the culture medium. Thus, L1, L2, and L3 were denoted as the lineages cultivated in liquid medium obtained from the immature leaflets of adult

plants initially cultivated in semisolid medium at concentrations of auxin at 450, 675, and 900 µM of Picloram, respectively. Lineages L5 and L6 were considered the materials cultivated in liquid medium, initially induced in semisolid medium from inflorescences and zygotic embryos, respectively. Lineages L4, L7, and L8 were considered lineages obtained from immature leaflets, inflorescences, and zygotic embryos, respectively, cultivated in a semisolid medium.

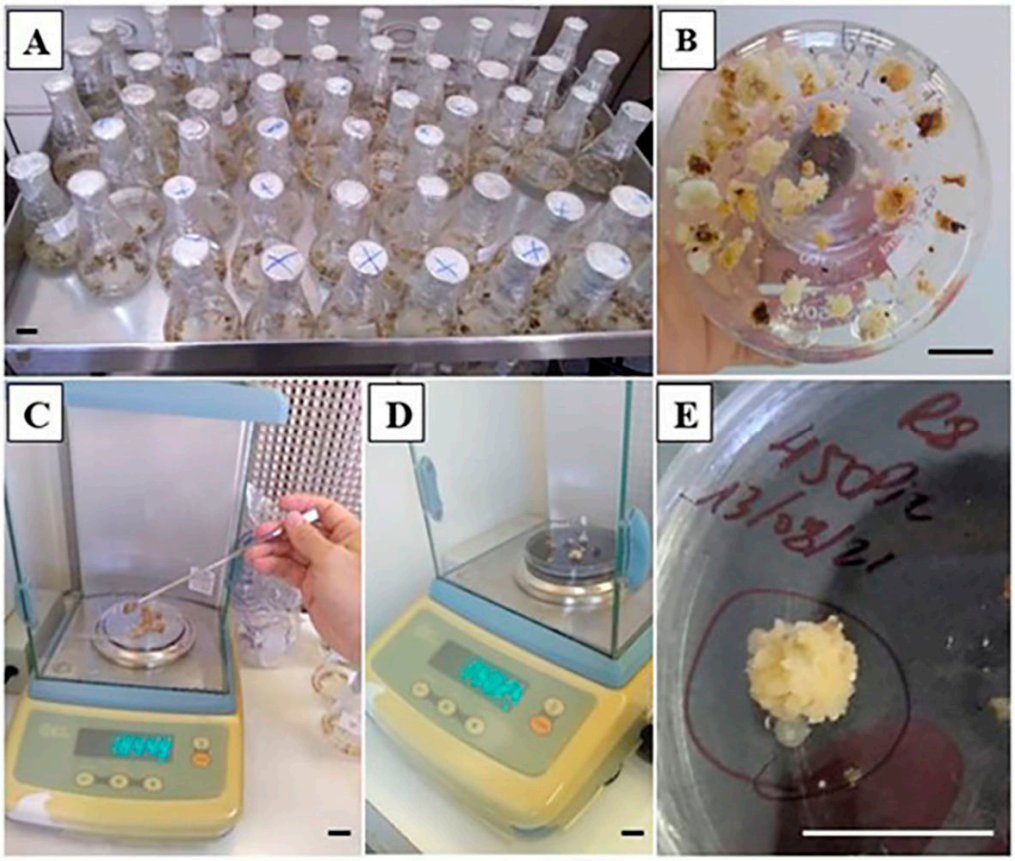


Figure 2. Aspects of embryogenic friable calli of *Euterpe precatoria* inoculated in liquid medium. (**A**) Lineages inoculated in a liquid medium, supplemented with 5 µM Picloram, under agitation; (**B**) friable callus used for establishing suspended cultures in a liquid medium; (**C**) weighing of calli cultivated in a liquid medium; (**D**) weighing of calli cultivated in a semisolid medium; (**E**) morphological aspect of embryogenic calli with friable consistency and color ranging from yellow to beige inoculated in semisolid medium. Bars = 1 cm.

2.5. Design and Statistical Analysis of Weighing Semisolid Medium vs. Liquid Medium

An entirely randomized experimental design was adopted in an 8 × 2 × 6 factorial scheme (eight embryogenic lineages × two cultivation systems × six cultivation periods), totaling 96 treatments. Each treatment consisted of ten repetitions (ten flasks). The dataset obtained was subjected to linear regression analysis, and when significant, the means were compared by Tukey's test at the 5% probability level using the statistical software SISVAR 5.8 [23].

2.5.1. Gompertz Modeling

Modeling was performed by applying nonlinear regression analysis using the Gompertz mathematical model using GraphPad Prism 9.0 software.

$$Y = a * e^{(-be^{-c*t})} \quad (1)$$

in which Y = dependent variable (g); t = cultivation time (days); "a", "b", and "c" = integration constants.

From the constants, the kinetic growth parameters of the suspensions were estimated.

2.5.2. Kinetic Parameters

μmax = maximum specific growth rate of cells (d^{-1}), calculated according to the formula below:

$$\boldsymbol{\mu max} = \boldsymbol{a} * \boldsymbol{c} \quad (2)$$

λ = duration of the lag phase;

$$\lambda = \frac{\boldsymbol{b} - \mathbf{1}}{\boldsymbol{c}} \quad (3)$$

G = time for cell generation or duplication:

$$\boldsymbol{G} = \frac{\boldsymbol{ln}(\mathbf{2})}{\boldsymbol{\mu max}} \quad (4)$$

2.5.3. Principal Component Analysis (PCA)

The application of multivariate statistics through principal component analysis (PCA) aimed to allow for the grouping or not of lineages regarding developmental similarities. For this purpose, GraphPad Prism 9.0 and R version 4.2.1 software were used. The use of each software occurred according to the specific needs of each analysis. For this step, the "FactorMiner" package was used.

2.6. Biochemical Characterization

For the biochemical analysis of embryogenic lineages of *E. precatoria*, the extraction and quantification of total soluble sugars (TSSs) and total soluble proteins (TSPs) present in embryogenic calli multiplied in liquid and semisolid medium (Figure 3) were performed, according to the methodology adapted from Gomes et al. [24], at 100 days of cultivation.

2.6.1. Total Soluble Sugars (TSSs)

For the determination of total soluble sugars (TSSs), the samples were washed three times in ultrapure water to remove culture medium residues, instantly frozen in liquid nitrogen, and lyophilized for 72 h. The extraction of sugars was carried out according to the methodology adapted from Gomes et al. [24]. In total, 10 mg of dry samples were inoculated in 2 mL microtubes, and subjected to four extraction cycles in 0.5 mL of 80% (v/v) methanol, at 80 °C in a water bath for 20 min. In each extraction, the samples were vortexed and then centrifuged at 10,000 rpm for 10 min. The supernatants were collected and added to new microtubes until reaching a volume of 2 mL. The pellets remaining in the first microtubes were stored in an ultra-freezer at −80 °C for future use.

Next, the samples were dried in a speed vac at 40 °C, and the dry product was resuspended in 500 µL of ultrapure water, following the Dubois [25] protocol. Duplicates of each sample were prepared for the quantification of total soluble sugars. Thus, in test tubes (25 × 150 mm), 50 µL of the supernatant samples, plus 450 µL of ultrapure water, followed by 500 µL of 5% (v/v) Phenol and 2500 µL of concentrated sulfuric acid (H_2SO_4), were pipetted.

The tubes were left to stand until they reached equilibrium with room temperature (approximately 15 min). Then, the absorbances of the samples were read in glass cuvettes in a spectrophotometer (Shimadzu UV-1800) at a wavelength of 490 nm. All readings were based on the glucose standard curve (1 mg/1 mL), with increasing concentrations of 0, 8, 16, 32, 64, 128, and 258 µg.

The obtained dataset was subjected to Analysis of Variance (ANOVA), and when significant, the means were compared by Tukey's test at a 5% probability level using the statistical software SISVAR 5.8 [23].

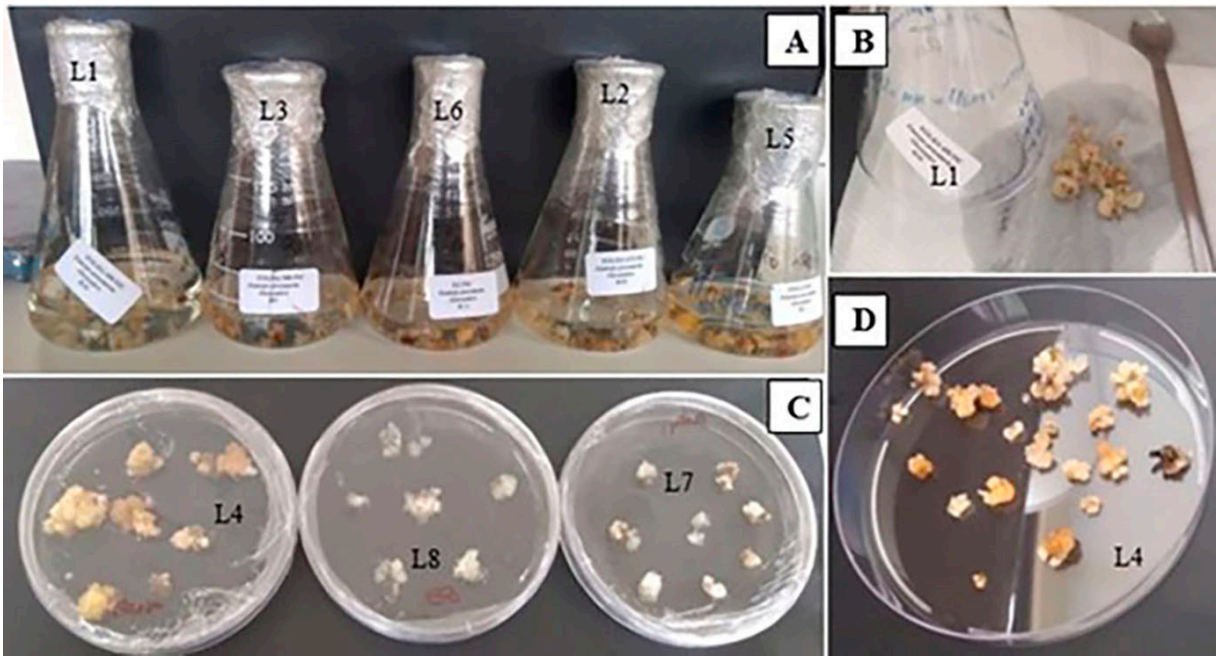


Figure 3. Calli and embryogenic lineages used in the biochemical characterization grown in liquid and semisolid medium of *Euterpe precatoria* Mart. (**A**). Lineages in liquid medium at 100 days of cultivation (L1, L3, L6, L2, and L5, respectively). (**B**,**D**). Aspect of the material inoculated in liquid medium. (**C**) Lineages in semisolid medium at 100 days of cultivation (L4, L7, and L8, respectively). L1, L2, and L3 lineages of calluses multiplied in liquid medium obtained from immature leaves cultivated in semisolid medium at concentrations of 450, 675, and 900 µM of Picloram, respectively. L5 and L6 lineages of calluses multiplied in liquid medium induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

2.6.2. Total Soluble Proteins (TSPs)

For the extraction of total soluble proteins (TSPs), a protocol adapted from Gomes et al. [24] was used. Thus, 300 mg of fresh sample of plant materials was ground in liquid nitrogen in a previously frozen porcelain crucible. Subsequently, 3 mL of potassium phosphate buffer (100 mM and pH 7.5) were added. Then, the samples were centrifuged at 14,000 rpm for 20 min at a temperature of 4 °C, and the supernatants were collected and placed in new microtubes. After the extraction procedure, the quantification of TSPs was performed according to the Bradford method. Thus, 5 µL of the samples were pipetted into 96-well microplates (Elisa), and then 250 µL of 50% Bradford were added for five minutes at rest. After this period, the absorbances of the samples were checked in a spectrophotometer at 595 nm. The analyses were carried out in triplicate, based on the standard curve of bovine serum albumin (BSA), with progressive dosages of 20, 40, 60, 80, and 100 µg mL^{-1}.

The obtained dataset was subjected to Analysis of Variance (ANOVA), and when significant, the means were compared by Tukey's test at a 5% probability level using the statistical software SISVAR 5.8 [23].

2.7. Anatomical Characterization

To evaluate the embryogenic potential of the lineages, the cell aggregate samples were collected after 100 days of cultivation for anatomical analysis. The process was conducted by Silva-Cardoso [26].

3. Results

3.1. Initial Establishment of Calli in Liquid Medium and Multiplication of Aggregates and Cells in Suspension

The cultivation of *E. precatoria* lineages in liquid consistency medium was successfully carried out, and the establishment was achieved. At 30 days of cultivation, all inoculated embryogenic lineages could be considered established, as growth was apparent during this period. The lineages showed 100% survival and negligible levels of oxidation throughout the experiment.

At 45 days, the first structures appeared in lineages inoculated in liquid medium, these being the formation of new cellular aggregates (Figure 4).

The growth is characterized by explants forming friable materials, both by the presence of isolated cells and by the presence of microcalli. Visibly, in the flasks of lineages L1 and L2, these structures were more evident, although they also appeared in flasks of lineages L3 and L6. The most present colorations were of white calli and in shades of beige, which remained throughout the experiment period (Figure 5). This behavior opens up new perspectives, paving the way not only for the use of cell suspensions for the mass production of *E. precatoria* plants via somatic embryogenesis but also for the production of secondary metabolites in the species. It is emphasized that these results record the first time that the formation of cellular aggregates in the suspension has occurred for the species *Euterpe precatoria*.

The development of new friable callogenic masses among lineages cultivated in liquid medium was even more noticeable at 60 days of cultivation. Based on weight measurements between cultivation in liquid and semisolid media, it was verified that the results obtained were satisfactory for the increase in mass among lineages cultivated in liquid medium as they showed similar or superior growth in mass increment compared to lineages cultivated in semisolid medium, especially in lineages of material originating from leaf and zygotic embryo.

This fact can be observed in lineages from immature leaves of *E. precatoria*, cultivated in liquid medium (L1, L2, and L3), which showed similar or superior behavior regarding mass increment when compared to lineage L4, which was cultivated in semisolid medium (Figure 6).

Among the immature leaf lineages, both in liquid and semisolid media, lineage L2 showed the highest mass increments at all cultivation times, although lineage L1 showed statistical similarities to L2. These lineages surpassed the mass increment in treatment L4 in semisolid medium, with statistical differences observed in the analysis of mean breakdown within each time level (Table 1).

Among the lineages originating from immature inflorescence plant material (L5—liquid medium and L7—semisolid medium), better behavior was observed in mass increment when cultivated in semisolid medium, highlighting significant statistical differences by Tukey's test at a 5% significance level. In liquid medium, this lineage showed the lowest mass increment in the experiment (Table 1).

The cultures originating from zygotic embryos (L6) cultivated in liquid medium stood out in the mass increment results at all cultivation times. Conversely, the aggregates from inflorescences (L7) stood out among lineages cultivated in semisolid medium (Table 1). Based on Table 1, for each period, there are changes in the behavior of the lineages throughout the experiment, with significant differences found in all the analyzed periods. Overall, a more significant increment in fresh weight was verified from 120 days of cultivation. At 180 days, lineage L6 stood up with 2.66 g, although it was not statistically different from other lineages, such as L2, L7, and L8.

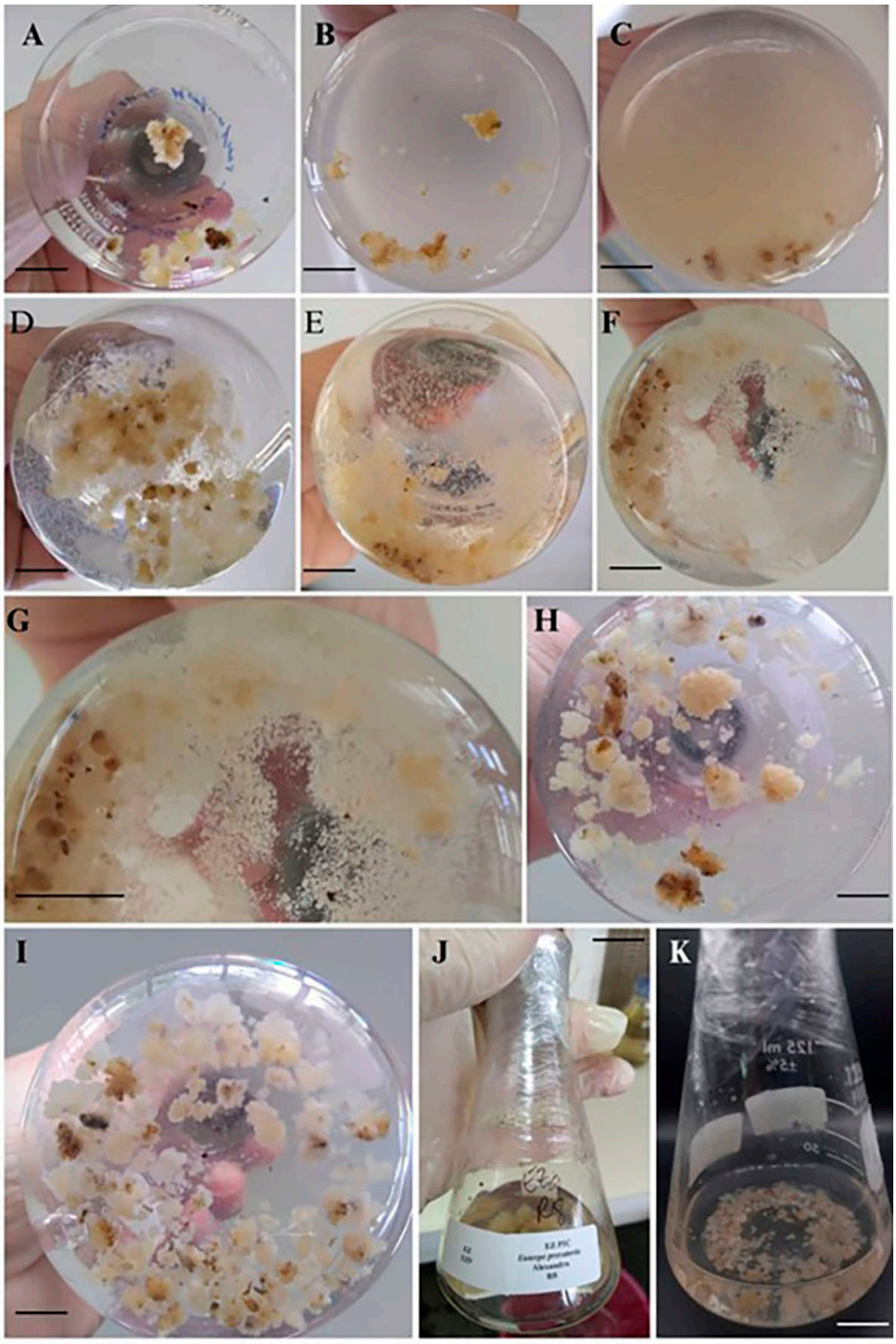
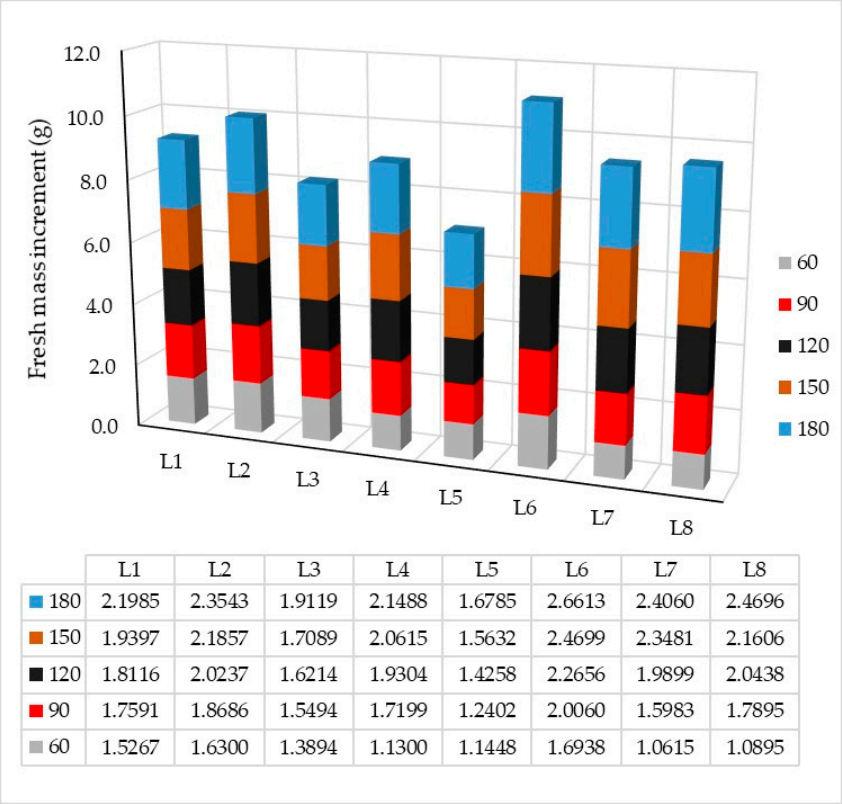


Figure 4. Development of cellular aggregates from different embryogenic lineages of *Euterpe precatoria* at different cultivation times in liquid medium. (**A**) Morphological aspects of calli on the day of inoculation (zero time in lineage L1). (**B**,**C**) Presence of isolated cells in suspension at 45 days (B = L1—lineage obtained from leaf explant cultivated in semisolid medium with 450 µM of Picloram, and C = L2—lineage obtained from leaf explant cultivated in semisolid medium with 675 µM of Picloram); (**D**–**G**) Presence of microcalli of the L2, note biomass increase; H. Detail of microcalli of the L2 at 60 days; (**H**) Calli of the L6 (lineage induced in semisolid medium from zygotic embryos) at 90 days; (**I**) Calli of the L6 at 180 days. (**J**,**K**) Representation of one of the repetitions and repotting of calli of the L6. Bars = 1 cm.



	L1	L2	L3	L4	L5	L6	L7	L8
180	2.1985	2.3543	1.9119	2.1488	1.6785	2.6613	2.4060	2.4696
150	1.9397	2.1857	1.7089	2.0615	1.5632	2.4699	2.3481	2.1606
120	1.8116	2.0237	1.6214	1.9304	1.4258	2.2656	1.9899	2.0438
90	1.7591	1.8686	1.5494	1.7199	1.2402	2.0060	1.5983	1.7895
60	1.5267	1.6300	1.3894	1.1300	1.1448	1.6938	1.0615	1.0895

Figure 5. Mean growth of cellular aggregates of *E. precatoria* in cell suspension medium under industrial shaker at 100 rpm and in semisolid medium as a function of the cell lineage. L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants cultivated in semisolid medium at auxin concentrations of 450, 675, and 900 µM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryo, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

Table 1. Mass increment (g) in cellular aggregates of *E. precatoria* cultivated in cell suspension medium under industrial shaker at 100 rpm and in semisolid medium as a function of the cell lineage at each cultivation time.

Lineages Cultivated	Fresh Mass (g) and Growing Period (Days)				
	60	**90**	**120**	**150**	**180**
L1	1.52 AB c	1.75 AB bc	1.811 BCD abc	1.93 CDE ab	2.19 BC a
L2	1.63 A c	1.86 AB bc	2.02 AB abc	2.18 ABC ab	2.35 AB a
L3	1.38 ABC bc	1.54 BC ab	1.62 CD ab	1.70 DE ab	1.91 BD a
L4	1.13 BC c	1.71 AB b	1.93 ABC ab	2.06 BCD ab	2.14 BC a
L5	1.14 BC cd	1.24 C bcd	1.42 D abc	1.56 E ab	1.67 D a
L6	1.69 A c	2.00 A bc	2.26A ab	2.46 A a	2.66 A a
L7	1.06 C d	1.59 BC c	1.98 ABC bc	2.34 AB ab	2.40 AB a
L8	1.08 C c	1.78 AB b	2.04 AB b	2.16 ABC ab	2.46 AB a

Note: Different uppercase letters indicate differences between lineage within each time level. Different lowercase letters indicate differences in the analysis of time breakdown within each lineage level by Tukey's test, 5% significance. L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants cultivated in semisolid medium at auxin concentrations of 450, 675, and 900 µM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

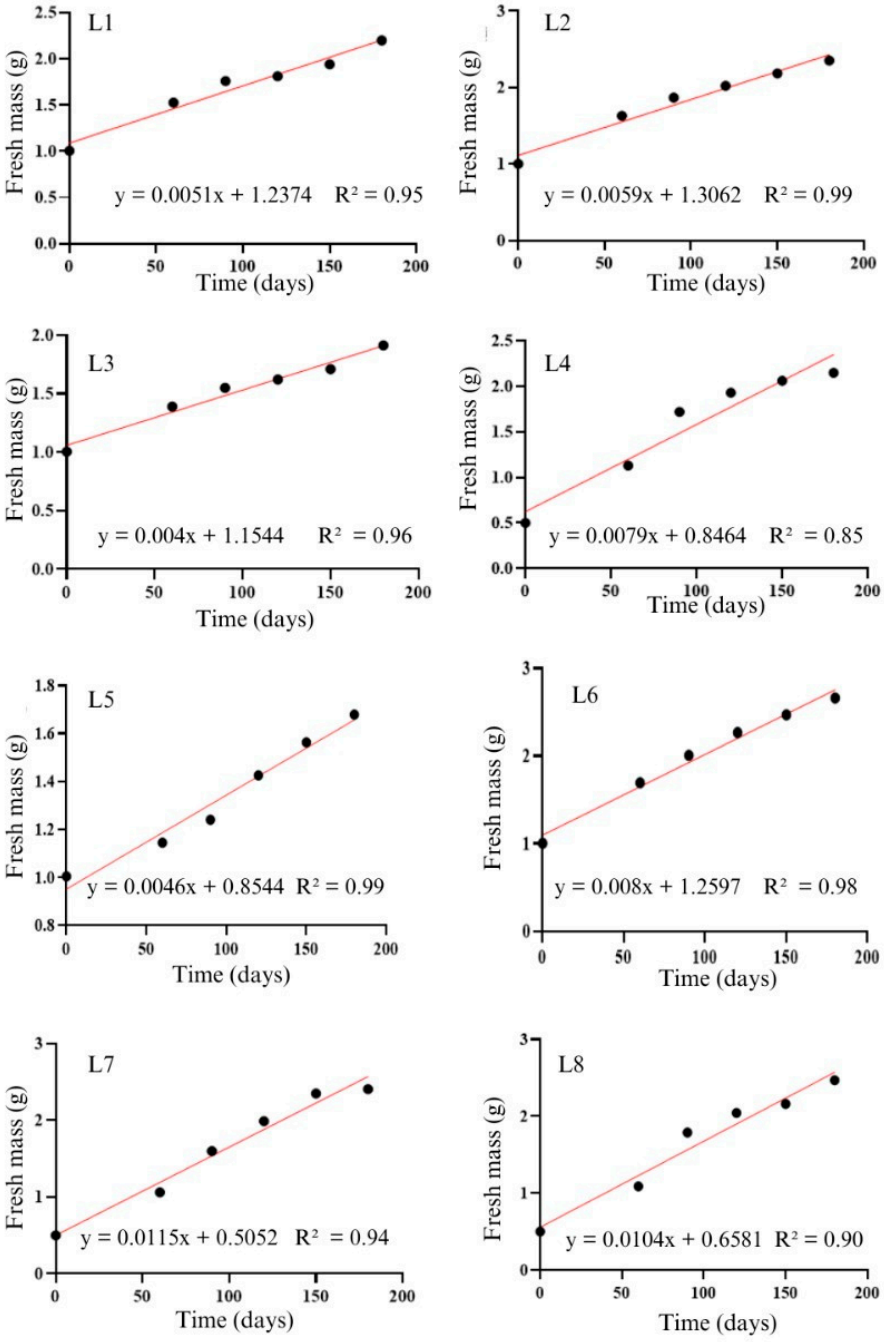


Figure 6. Linear regression analysis of the growth cellular aggregates of *E. precatoria* Mart. cultivated in liquid medium under an industrial shaker at 100 rpm and in semisolid medium as a function of the cell lineage. Note: L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants initially cultivated in semisolid medium at auxin concentrations of 450, 675, and 900 μM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

Another important result can be evidenced by regression analysis, which also verified significant growth in mass increment (g) in lineages as a function of cultivation periods. The linear behavior of each lineage is shown in Figure 6.

In the linear analysis, it is observed that the points corresponding to the masses obtained in the experiment are either on or very close to the line drawn for all lineages.

This result indicates that the deviations were very low in relation to the adjusted regression line. The correlation coefficients found range from 0.85 to 0.99, demonstrating a consistent and well-defined relationship between weight and growth time. Thus, the correlation is directly positive, indicating that as the cultivation period increases, there is a corresponding increase in the biomass production of *E. precatoria* cellular aggregates. This underscores a very strong correlation between growth time and the observed increase in mass.

3.2. Growth Kinetics of Cellular Aggregates and Application of the Gompertz Mathematical Model

The application of the mathematical model allows for a more characteristic view of the growth of cellular aggregates (calli) in their respective culture media.

The model can be considered adequate, as the R^2 values found describe more than 90% of the growth kinetics of the lineages (Table 2).

Table 2. Gompertz model constants ($Y = a{*}e^{(-be^{-c*t})}$) of *E. precatoria* suspensions cultivated in cell suspension medium under an industrial shaker at 100 rpm and in semisolid medium as a function of the cell lineage at each cultivation time.

Parameters	Lineages							
	L1	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**
a	2.552	2.672	2.328	2.405	2.04	3.235	3.18	2.911
b	−0.09	−0.031000	−0.1899	0.51238	0.008210	0.1559	0.69143	0.626376
c	0.00902	0.01084	0.00732	0.01599	0.019654	0.00988	0.01169	0.01326
R^2 (%)	0.97	0.99	0.98	0.98	0.99	0.99	0.98	0.97

Note: L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants initially cultivated in semisolid medium at auxin concentrations of 450, 675, and 900 µM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

Through the constants found in the Gompertz model, the results of the kinetic parameters were obtained. In the results, it can be observed that the data for the duration of the lag phase λ (day) of growth are negative. Thus, this confirms what was observed in the curves of the lineages: the absence of the lag phase λ (day) (Table 3).

Table 3. Kinetic parameters of *E. precatoria* Mart. suspensions cultivated in cell suspension medium under an industrial shaker at 100 rpm and in semisolid medium as a function of the cell lineage at each cultivation time; µmax = maximum specific cell growth rate (d^{-1}); λ = lag phase time, and G = generation time (days).

Parameters	Lineages							
	L1	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**
µmax (d^{-1})	0.0229	0.0289	0.0169	0.0383	0.0102	0.0319	0.0371	0.0388
λ (dia)	−120.878	−95.0695	−162.516	−30.5	−180.72	−85.4472	−26.4019	−28.1818
G (dia)	30.1	23.9	40.8	18.0	53.6	21.7	18.6	17.9

Note: L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants induced in semisolid medium at auxin concentrations of 450, 675, and 900 µM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

The results showed significant variations among the genetic lineages in terms of growth and metabolism. It was found that the cell generation time was shorter among the lineages cultivated in the semisolid system (L8, L4, and L7). The observed maximum specific growth rate (µmax) was ±0.0388 d^{-1} for Lineage L8, with a generation time (G) of 17.9 days until the cultures reached their peak biomass duplication. Lineage L4 and L7

presented μmax of ±0.0383 and ±0.0371 d^{-1}, taking on average approximately 18 and 18.6 days, respectively, for the cultures to reach their peak biomass duplication.

Among the lineages cultivated in the liquid system, lineages L6, L2, and L1 had an average time of 21.7, 23.9, and 30.1 days to double the biomass accumulation, with a maximum specific growth rate (μmax) on average of ±0.0319, ±0.0289, and ±0.0229 d^{-1}. The lineages with lower values were L3 and L5, both for maximum specific growth rate (μmax) and for generation time (G) d^{-1}.

3.3. Principal Component Analysis (PCA)

The two principal components explained 84.08% of the total data variation, indicating that the PCA was well adjusted, allowing for an observation of the trend in callus development in the suspension. PC1 represented 68.62% of the relationship, with the separation of lineages into clusters. Positive correlations between the lineages cultivated in semisolid medium formed a grouping and were plotted in both quadrants on the right side. Other parameters influenced growth and separated the lineages cultivated in liquid medium but were also plotted in both quadrants on the right side (Figure 7).

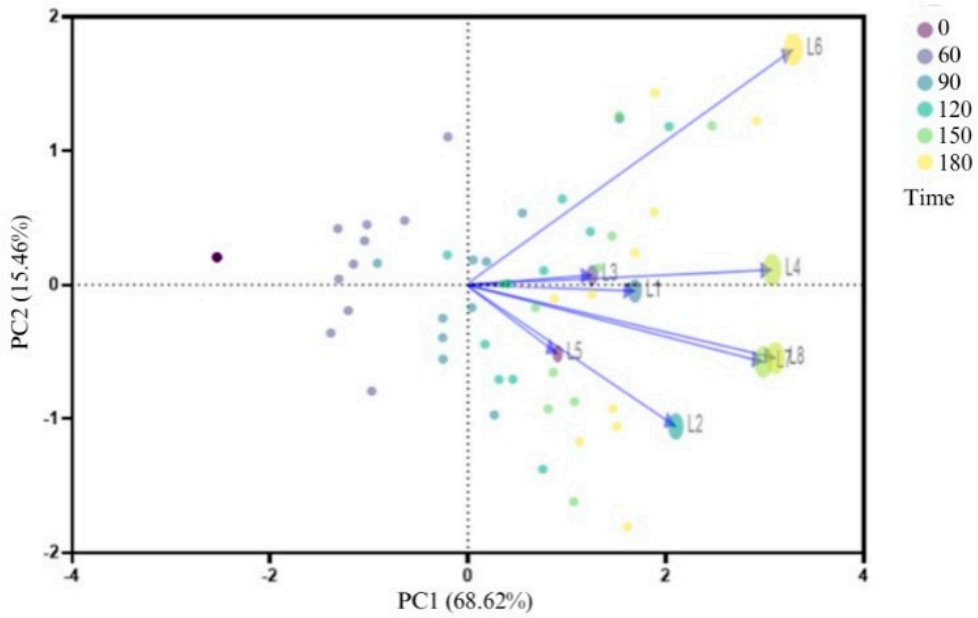


Figure 7. Biplot PC1 × PC2 on the biomass increment variables over the cultivation time in *Euterpe precatoria* Mart. lineages cultivated in liquid and semisolid medium. R = (84.08%). Note: L1, L2, and L3 lineages multiplied in liquid medium obtained from immature leaves of adult plants initially cultivated in semisolid medium at auxin concentrations of 450, 675, and 900 μM of Picloram, respectively. L5 and L6 lineages multiplied in liquid medium and induced in semisolid medium from inflorescences and zygotic embryos, respectively. L4, L7, and L8 lineages originating from immature leaves, inflorescences, and zygotic embryos, respectively, induced and multiplied in semisolid medium.

Lineage L6, derived from zygotic embryo, exhibited a higher vector when compared to the other lineages, indicating a distinct behavior regarding in vitro suspension culture. The positioning of the vector with larger and isolated angles indicates that the correlations with the other lineages were not very strong, although the results highlight a very consistent proportion of the lineage. Thus, the principal component analysis compared with the biomass increment results highlights the prominence of this lineage for suspension culture (Figures 7 and 8).

Figure 8. Morphological aspects of the best *Euterpe precatoria* Mart. lineages cultivated in liquid medium at the end of the experiment. (**A**–**C**). Calli belonging to lineage L2 originating immature leaves initially cultivated at 675 µM of Picloram. (**D**–**F**) Calli belonging to lineage L1 originating from immature leaves initially cultivated at 450 µM of Picloram. (**G**–**I**) Calli belonging to lineage L6 originating from zygotic embryo initially cultivated in Y3 medium. Barrs = 1 cm.

The principal component analysis also reveals another standout behavior regarding in vitro suspension culture, which is observed in lineage L2. For this lineage, this fact is also evidenced by the more isolated positioning of the vector compared to the other lineages derived from immature leaves cultivated in the suspension culture. The vector position of lineage L2 indicates a more distant angle compared to lineages L1 and L3, reflecting the difference in biomass increment gain by lineage L2 relative to lineages L1 and L3. The morphological aspects of lineages L1 and L2 during multiplication in liquid medium are shown in Figure 8.

Regarding the lineages cultivated in semisolid medium, a high positive correlation is observed between L7 (INFLO) and L8 (EZ). The vectors are at equidistant angles to lineages L7 and L8. Thus, the grouping of the samples presents similar growth behaviors among the lineages cultivated in the semisolid system. Another similar lineage was L4.

Thus, based on the observed correlation results, there are lineages that show stronger associations with each other. Patterns can be identified in the relationships between the studied lineages, with the strongest groupings within their respective cultivation system. Therefore, within each system, there were also different patterns and associations. These results highlight the significance of the cultivation system effects on the biomass increment (g) in the lineages within each time. Thus, the medium in which each lineage is cultivated promotes interaction between lineages and cultivation time.

3.4. Biochemical Characterization and Levels of Total Soluble Proteins (TSPs) and Total Soluble Sugars (TSSs) of E. precatoria

There was a statistical difference in the amount of TSSs extracted from the lineages by Tukey's test at the 5% significance level (Figure 9). The highest TSS levels were observed in lineages cultivated in liquid medium. The TSSs in lineages cultivated in liquid medium varied between 517.41 and 700 μg mg^{-1} of dry weight, with higher levels observed in lineages derived from immature leaves (L1, L2, L3, and L6) (Figure 9).

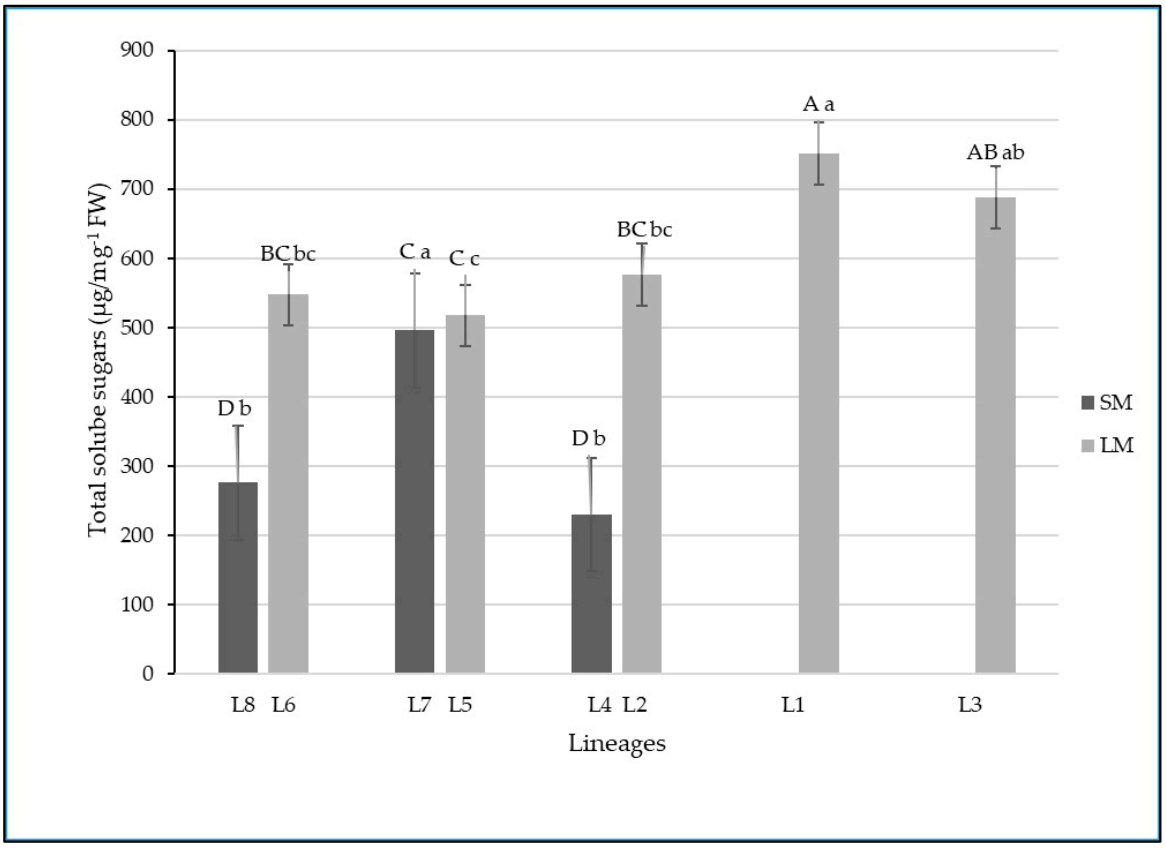


Figure 9. Total soluble sugars (TSSs) of cell aggregates in suspension and in semisolid medium from different lineages of *Euterpe precatoria* Mart. Different lowercase letters indicate differences in lineages within each medium, and different uppercase letters indicate differences between lineages by Tukey's test at the 5% probability level. Bars represent standard error. Note: L4, L7, and L8 represent lineages from immature leaves, immature inflorescences, and zygotic embryos, respectively, in semisolid medium (SM), and L1, L2, and L3 represent lineages from immature leaves, immature inflorescences, and zygotic embryos, respectively, in liquid medium (LM); L5 represents lineages from immature inflorescences in liquid medium and L6 represents lineages from zygotic embryos in liquid medium.

When comparing the lineages with the same origin (from the same type of explant), it is verified that, overall, those multiplied in a liquid medium presented higher quantities of TSSs (Figure 9).

The results of the biochemical analyzes show that two lineages exhibited the highest amounts of protein reserves, L6 (0.243 µg of Prot/mg FW) and L5 (0.233 µg of Prot/mg FW), both cultivated in liquid medium (Figure 10). Lineage L8 was statistically similar to the above-described lineages, as the reserve levels were very close to these lineages (0.231 µg of Prot/mg DM). Lineage L7 was statistically similar to lineage L8.

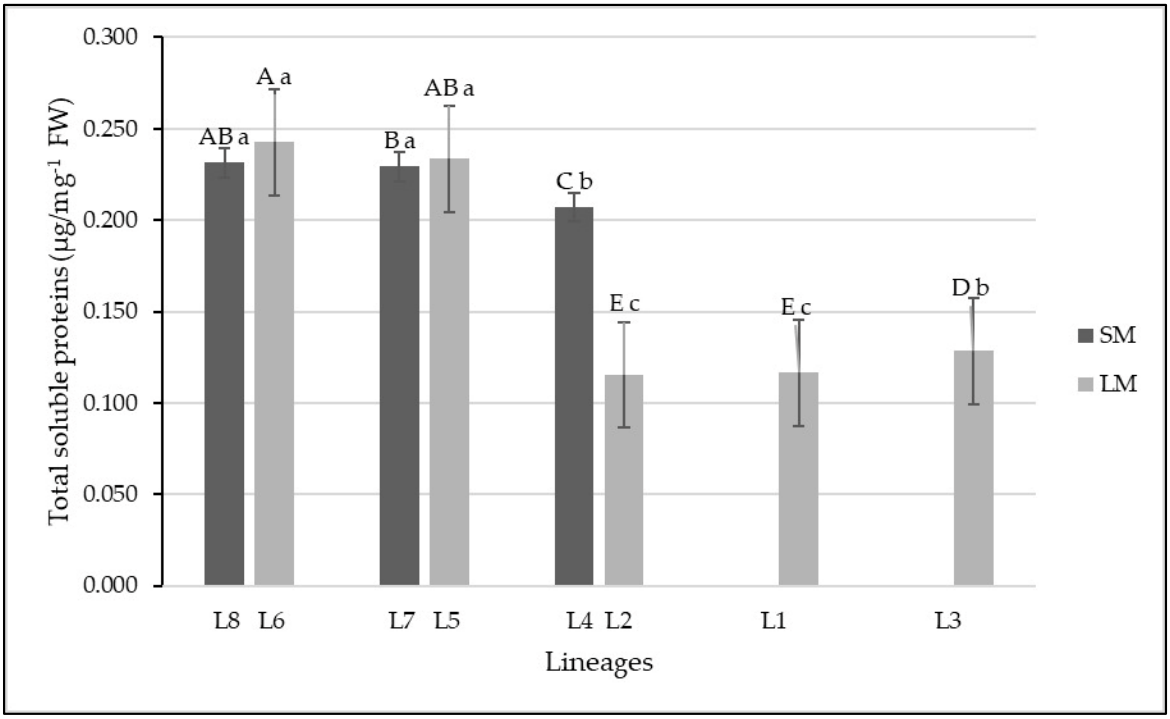


Figure 10. Total soluble proteins (TSPs) of aggregates in suspension and in semisolid medium in genetic lineages of *Euterpe precatoria*. Different lowercase letters indicate significant differences in lineages within each medium, and different uppercase letters indicate differences between lineages by Tukey's test at 5% probability level. Bars represent standard error. Note: L4, L7, and L8 represent lineages from immature leaves, immature inflorescences, and zygotic embryo, respectively, in semisolid medium (SM), and L1, L2, and L3 represent genetic lineages from immature leaves, immature inflorescences, and zygotic embryo, respectively, in liquid medium (LM); L5 represents lineages from immature inflorescences in liquid medium and L6 represents lineages from zygotic embryo in liquid medium.

3.5. Anatomical Analysis

Anatomical analysis revealed that the calli of all lineages had cells with embryogenic characteristics, and some lineages were developing somatic embryos, such as L1, L2, and L3, after 100 days of cultivation in a liquid medium (Figure 11).

The anatomical sections show the presence of cells with embryogenic characteristics, proembryos, and some embryos at different stages of development (globular and torpedo), some exhibiting protoderm and clear procambial cords (Figure 11). Somatic embryos originating from repetitive cycles of somatic embryogenesis were also observed (Figure 11H–J). Additionally, there was evidence of the unicellular origin of somatic embryos, given the visualization of structures similar to suspensors (Figure 11J), which increases the friability of the material, giving greater ease of detachment.

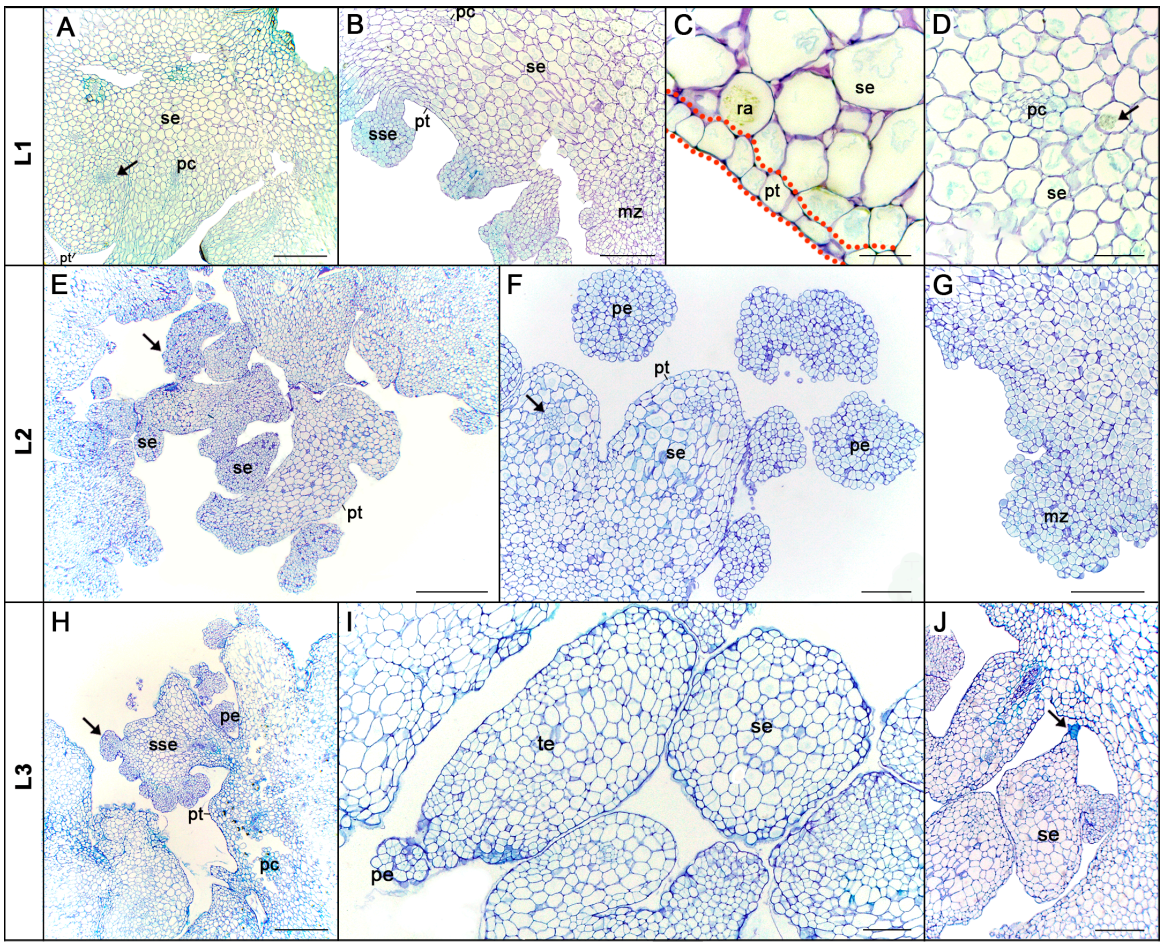


Figure 11. Anatomical sections of cell aggregates from *Euterpe precatoria* from different lineages (L1, L2, and L3) after 100 days of cultivation in a liquid medium. (**A**) Somatic embryo with protoderm and procambium; (**B**) Probable secondary somatic embryo; note vacuolated cells and a callogenic zone with more meristematic cells; (**C**) Somatic embryo with protoderm (dashed) and raphides. (**D**) Detail of somatic embryo with procambium and raphides (arrow); (**E**) Aggregates of vacuolated cells, isolated somatic embryos, and clusters of meristematic cells (arrow); (**F**) Somatic embryos with protoderm and procambium (arrow); notice isolated proembryos; (**G**) Area with cells undergoing cell division. (**H**) Secondary embryogenesis and beginning of tertiary somatic embryo formation (arrow); (**I**) Globular somatic embryo at a torpedo-like stage; (**J**) Somatic embryo with a structure similar to the suspensor (arrow). Abbreviations: (mz) meristematic zone; (pc) procambium; (pe) proembryo; (pt) protoderm; (se) somatic embryo; (sse) secondary somatic embryo; (te) torpedo somatic embryo. Bars: A, E, H = 500 µm; J = 250 µm; B, F, G = 200 µm; D, I = 100 µm; and C = 50 µm.

4. Discussion

4.1. Establishment of Calli in Liquid Medium and Multiplication of Embryogenic Aggregates in Suspension

Using lineages obtained from different types of explants (immature leaves, immature inflorescences, and zygotic embryo), suspension culture was achieved. The lineage derived from zygotic embryo (L6) obtained an expressive mass increment (g) compared to other explants. The lineages originated from leaf explants (L1, L2, L3) obtained a similar mass increment, and among the lineages originating from inflorescences, lineage L7 stood out for this variable.

The type of callus was crucial for the success of the establishment. The friable white or slightly yellowish callus (Figures 1, 3 and 4) (high fragmentation ease) was established in the liquid medium. It is noteworthy that this type of callus is essential for the establishment

of cell suspension cultures, where, in liquid medium under agitation, cells disperse and develop synergistically [27–29].

The establishment of lineages in liquid medium enables a biotechnological improvement in research developed with *E. precatoria*, making the first report in *E. precatoria*. This allows for the initiation of other studies, such as research related to the production of secondary metabolites [30–32], and enhances the multiplication of embryogenic propagules and the somatic embryo-to-plant conversion rate [33–37]. Additionally, the results obtained can support species' genetic breeding programs and the conservation of genetic resources through the cryopreservation of embryogenic lineages. It is important to note that lineages cultivated in semisolid medium also showed efficiency in callus multiplication, especially in lineages L7 and L8.

Despite its importance, the use of liquid medium for multiplication and in vitro regeneration in palms is still in its infancy [38]. Some researchers have been increasingly striving towards efficient protocols for propagation via somatic embryogenesis in palms using liquid medium, such as *Cocos nucifera* L. by Kong et al. [38], *Phoenix dactylifera* L., by Abohatem and Baazis [39], *Elaeis guineensis* Jacq. by Panggabean et al. [40], and *Euterpe edulis* by Mello et al. [41], to subsequently achieve efficient plant regeneration.

It should be noted that, to increase the success in the multiplication of embryogenic lineages in liquid media, different factors can be optimized. Ramchandra et al. [42] analyzing callus induction for the establishment of the cell suspension culture of Cumin (*Cuminum cyminum* L.) found the optimum number of cells to establish a well-developed cell suspension culture; otherwise, cell division may not occur. In addition to quantity, the size of the aggregate should also be considered, as it can also influence the results [42]. However, the size of the aggregates in the suspension may vary from species to species and general cultivation conditions [14].

Understanding these mechanisms is crucial to optimizing biomass production and compounds of interest in plant biotechnology applications. For example, the amount of liquid medium used is important. The lineages were inoculated in 35 mL of liquid medium. The amount of medium can limit the growth of suspension cultures and favor hyperhydration [43].

Another important factor to be considered is that lineages cultivated in semisolid medium were kept in the dark, and there is no information about the development of liquid medium cultures in the dark for this species. Future studies may elucidate the behavior of these lineages regarding the ideal luminosity for propagule multiplication.

The growth of lineages in liquid medium was not affected by slight oxidation, which did not compromise the embryogenic potential of the cultures. Specific compounds released by cultures in suspension medium can act in the prevention of biological molecules' oxidation, thereby avoiding the chain reaction [44].

The analysis of the production of fresh mass revealed that all lineages, regardless of the consistency used for multiplication and the explant of origin, showed an increase in callogenic mass after 180 days of cultivation by carrying out monthly subcultures. Future studies are necessary to further optimize the multiplication of the embryogenic masses of this palm by improving the factors discussed above (amount of initial inoculum, amount of medium), as well as the composition of the cultivation medium.

4.2. Gompertz and Growth Kinetics

Based on the data provided for the lineages, we can characterize the growth pattern in terms of metabolism as follows: μmax (d^{-1})—the maximum specific growth rate of cells varies among lineages, with values ranging from 0.0102 to 0.0388 d^{-1}. This indicates that different lineages have different growth capacities even under ideal conditions, with lineage L8 showing the highest specific growth rate and lineage L5 showing the lowest.

The negative values ranged from −180.72 to −26.4019 days, indicating the absence of the lag phase among lineages. Puad and Abdullah [45] reported a growth curve of suspension cells from *Ficus deltoide* without the lag phase and suggested that sampling

frequencies could be increased to visualize this phase. However, this is not uncommon in the suspension culture, especially during the establishment of protocols [46].

The generation time (G) varied among lineages, with values ranging from 17.9 to 53.6 days. This represents the time required for the cell population to double. Thus, lineages with shorter generation times (L8, L4, L7, L6, and L2) are replicating more rapidly than those with longer generation times (L5, L3, and L1). This suggests that under certain conditions, lineage L8 has the potential to grow more rapidly than other lineages. Among the lineages cultivated in liquid medium, lineages L6 and L2 showed greater efficiency in liquid medium growth.

The growth correlations found (Table 2) constitute another indicative tool that the protocol used is suitable for the multiplication of the embryogenic materials of *E. precatoria* in liquid medium. All lineages described the model and provided a satisfactory description of *E. precatoria* cell growth under in vitro conditions, with statistical confidence above 97.00% (Tukey's test). Thus, the Gompertz model stood out in successfully demonstrating the growth of different lineages in relation to the medium and cultivation system. The specific regression coefficient (R^2) data, obtained by comparing the experimental results with the predicted ones, are noteworthy. The innovative contribution of this study lies in achieving high precision in growth parameters for lineages through the successful application of the Gompertz model.

The Gompertz model proved to be robust compared to conventional approaches in experiments conducted by Martinez et al. [47], who examined the kinetics and modeling of cell growth for the potential induction of anthocyanins in in vitro cultures of *Taraxacum officinale* G.H. Weber ex Wiggers (Dandelion). The authors concluded that using mathematical models to understand the increase in and concentration of anthocyanins in *Taraxacum* calli continued to be an effective strategy for anticipating productions and reactions under varied environmental conditions. Mavituna et al. [48], applying modeling to understand changes in the distribution of aggregate sizes of plant cells during the suspension growth of *Capsicum frutescenscultura*, found that the allocation of computational models has potential for predictive use in designing experiments in plant cell cultures. Several other authors have used this model to better understand the patterns involved in the callus growth process in different plant species, such as *Glycine wightii* [49], *Jatropha curca* [50], and *Barringtonia racemose* [51].

4.3. Growth Correlations among Lineages Evaluated by Principal Component Analysis

The application of multivariate statistics through principal component analysis (PCA) allowed us to observe the interactions between lineages. The suspensions analyzed in the present study presented distinct groups regarding developmental similarities (Figure 8). Lineages cultivated in semisolid medium (L4, L7, and L8) formed one group. Lineages cultivated in liquid medium showed distinct patterns among themselves. The opposite position between lineages L6 and L2 clearly indicates a very low correlation, although both have the highest mass increment (g) among lineages cultivated in liquid medium.

Ferreira et al. [12] evaluated Y3 medium as the best option for multiplying embryogenic calli from the zygotic embryo of *E. precatoria* in semisolid medium. In this study, the PCA results show that the cultivation in the suspension of embryogenic lineages from zygotic embryo can be performed in MS medium supplemented with 5 µM Picloram. This fact can be observed in the PCA by the plotting the position of lineage L6, clearly showing the positive effect of the mass increment yield (g) of the lineage (Figure 8).

The liquid medium showed different embryogenic responses, with more responsive and isolated lineages, such as lineages L6 (from zygotic embryo) and L2 (from immature leaves), and less responsive lineages, such as lineage L5 (from immature inflorescences).

In *Phytolacca americana* L., popularly known as pokeberry (erva-de-tintureiro), principal component analysis (PCA) and hierarchical cluster analysis (HCA) revealed different response patterns between semisolid and liquid media supplemented with naphthaleneacetic acid (NAA). The researchers used liquid and semisolid media and treatments with

auxins. The MS media (semisolid and liquid) supplemented with indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), at concentrations of 0.5, 1, 2, and 4 mg/L [44].

4.4. Biochemical Analysis

Significant differences were observed in TSS levels among the lineages, presenting distinct levels in reserve content. Lineages cultivated in liquid medium showed higher TSS levels compared to lineages cultivated in semisolid medium (Figure 10). Additionally, some lineages that exhibited the higher quantities of TSSs, such as L2 and L6, presented expressive growth in terms of mass increment.

The concentration, sensitivity, and transport characteristics of plant tissues regarding different endogenous sugars can influence, both directly and indirectly, the regulation of cell division and differentiation processes in developing cultures, playing a crucial role in controlling their morphogenesis [52]. Carbohydrates play a fundamental role in providing both carbon skeletons and energy for the synthesis of organic compounds essential to cellular metabolism. This function can elucidate what occurs during the transition in development phases, highlighting the importance of carbohydrates as an energy source [53], which is necessary for maintaining the cell growth of embryogenic cultures. The cell cycle requires a high content of carbon to supply the ATP necessary for cell metabolism [54].

According to Gomes et al. [23], this characteristic is relevant for promoting the growth and development of calli and cells since, during certain stages of embryogenic development, the composition of soluble sugars in explant tissues tends to play a more significant role than the total amount of soluble sugars, often regulating specific phases of the process. For example, Bartos et al. [52] found a positive relationship in the acquisition of embryogenic competence when the levels of soluble sugars glucose and fructose decreased during somatic embryogenesis in *Coffea arabica* L.

Another important factor is that under in vitro conditions, explants tend to depend mainly on external sources of nutrients, and the accumulation of reserves is not a prominent characteristic in their metabolism [55]. Cell aggregates in the suspension are subject to a high availability of nutrients and carbon essential for growth [56]. In addition, under suspension conditions, the effects of plant growth regulators are often concentration-dependent, and their concentrations are maintained uniformly in an agitated system, reducing the negative effects of toxins generally released by tissues [38]. These factors favor cell growth.

Previous studies [24,57–60] reported oscillation in TSS levels about the growth phases of cultures. At each stage preceding a growth phase, researchers found that the TSS levels vary greatly. Some studies showed both a decline and increase in TSS levels over the cultivation period, which can be attributed to factors such as the type of explant, growth phase and developmental stage, culture medium, and cultivation time.

Specifically, a study conducted with calli of *Byrsonima intermedia* A. Juss. showed results in both mass increment and TSS levels lower than those found in this study. TSS levels ranged from 80 μg mg^{-1} of dry mass at the beginning of the experiment to 10 μg mg^{-1} of dry mass at the end of the cultivation time. The experiment lasted 120 days, with analyses performed every ten days of cultivation. The second highest TSS level occurred at 70 days of cultivation, which was the ideal time for repotting the materials, and this did not hinder the development of the cultures [59].

TSS levels in the oil palm *Elaeis guineensis* Jacq. were similar to the results found in this research for lineages cultivated in solid medium and lower when compared to lineages cultivated in liquid medium. Gomes et al. [24] found higher TSS levels during the stages of acquisition and development of somatic embryogenesis in this palm. They noted significant differences depending on the cultivation time (0 to 360 days), with variations in total levels of soluble sugars during somatic embryogenesis. Researchers observed that, at the initial moment of somatic embryogenesis induction, the TSS levels in explant tissues decrease rapidly, unlike during the maturation and regeneration stages, where concentrations of

total soluble sugars in cultures gradually increase. Bartos et al. [52] also found much lower TSS levels in *Coffea arabica* embryogenic calli, with an average of 60 μg mg^{-1} of dry mass.

For proteins (Figure 10), the levels verified in this study were very low. Even though the analysis point was at 100 days of cultivation, such low TSP (total soluble protein) levels were unexpected, as, according to the growth curves, the studied lineages presented a trend in growth—mass increment. The formation of new cell aggregates requires events of cell division. The cell cycle, in turn, requires the synthesis of diverse new proteins responsible for morphological and biochemical alterations in the cell during the mitotic process [61]. These results, however, can be a consequence of calluses specificities, and cultivation conditions, or could be related to the protocol used.

In the biochemical analysis of *Jatropha curcas* L. calli, a species of economic importance in biodiesel production, Santos et al. [53] presented biochemical patterns very similar to the results of this research, with high levels of TSSs and very low levels of TSPs. Another important finding in this study was the lowest levels of TSPs coincided with the highest levels of reducing sugars (RSs). According to this work, high levels of RSs can induce chemical modifications in proteins, favoring Amadori and Maillard reactions.

The Amadori reaction involves the binding of a reducing sugar, such as glucose, to an amino acid, while the Maillard reaction involves the binding of a reducing sugar to a primary amine. These reactions are important steps in the non-enzymatic glycation of proteins, occurring when sugars non-enzymatically bind to amino acid residues in protein [62]. In the glucose reaction (or other reducing sugars), it binds to a lysine or arginine residue in the protein, forming an initially reversible product known as the Amadori product [63].

This reaction is the first step in the formation of advanced glycation end products (AGEs), which are irreversible compounds resulting from the modification of proteins by sugars. In terms of protein synthesis, non-enzymatic glycation can interfere with the structure and function of proteins. When proteins are modified by glycation, their three-dimensional structure can be altered, affecting their normal biological function [64].

In the context of in vitro plant culture, where protein synthesis is crucial for plant growth and development, the occurrence of the Amadori reaction and the formation of AGEs can negatively affect metabolic and physiological processes [64]. This could have implications for the quality of plants grown in vitro, including biomass yield, resistance to biotic and abiotic stresses, and nutritional quality [65]. Factors such as cultivation time, temperature, high humidity, and system pH can favor Amadori and Maillard reactions [66]. In in vitro culture, the presence of a high-humidity environment is common, favoring such reactions. This can result in protein inactivation, leading to a reduction in protein levels in tissues [53].

Signaling proteins play a crucial role in cell communication and in the regulation of cell growth and differentiation. Plant hormones, such as auxins and cytokinins, are known to influence the formation and growth of embryogenic calli, acting through specific signaling pathways mediated by proteins. Studies have shown that modulating the concentration and distribution of auxins and cytokinins can significantly affect the formation and morphology of embryogenic calli [67].

Modifications in the composition of the culture medium are fundamental for improvements in protein synthesis, which may induce cellular metabolism and thus favor the growth and cellular development of lineages. Factors such as levels of auxins and cytokinins, levels of vitamin supplements, and amino acids have been tested in other studies. Alwael et al. [68] found that the addition of abscisic acid (ABA) to the suspension improved protein levels in the cultivation of *P. dactylifera* L.

The results obtained enabled the evaluation of the dynamic of the reserves in the lineages at 100 days of cultivation, allowing us to verify that, overall, the liquid medium proportioned the higher accumulation of TSSs, which likely favored the higher cell growth in compared to the semisolid medium. However, the reserves analyzed could not be used as markers of the growth potential of these lineages, owing to the wide variation observed.

4.5. Anatomical Analysis

The anatomical analyses revealed the embryogenic potential of the evaluated lineages and allowed for the verification of the differentiation of the somatic embryos in some of them after 100 days of cultivation in a liquid medium supplemented with 5 µm of Picloram (Figure 11). It should be noted that low concentrations of auxin can induce the formation of somatic embryos in palm trees [2,25].

The proembryos observed are similar to those reported in *E. precatoria* by Ferreira et al. [12], typical of friable calluses. Additionally, there was evidence of the unicellular origin of somatic embryos, given the visualization of structures similar to suspensors (Figure 11J), which increases the friability of the material, making detachment easier. The unicellular origin has been associated with the somatic embryogenesis of *E. precatoria* [12,13].

Taking into account the results obtained, after 90 and 100 days of cultivation, it is now possible to transfer the callus with somatic embryos to maturation media and, subsequently, germination media, with the purpose of plant regeneration.

5. Conclusions

Friable embryogenic calli of *Euterpe precatoria* can be established and multiplied in liquid medium. However, the multiplication potential of embryogenic calli in liquid medium is influenced by the lineage used. In this work, lineages L2 (immature leaves) and L6 (zygotic embryo) exhibit higher multiplication rates and shorter response times. The growth kinetics described by Gompertz modeling can be used to elucidate the growth in suspension cultures of this species. Lineages cultivated in the liquid medium have higher sugar levels than those in the medium, although the protein content in the investigated lineages was low. These biochemical variables could not serve as reliable markers of growth potential due to the wide variation observed. Anatomical analyses underscore the embryogenic potential of the studied lineages. Future studies are necessary to elucidate the role of the reserves studied during the subsequent steps of the redifferentiation, maturation, and germination of the somatic embryos originating from these lineages.

The cultivation of *Euterpe precatoria* in an in vitro liquid system appears promising and can also be used for vegetative propagation and obtaining standardized raw materials, such as secondary metabolites for pharmaceutical and medicinal purposes, among others. Optimization of mineral salt concentrations in the nutrient medium will be the subject of future research to achieve even higher productivity in the lineages. The events described in this study pave the way for new investigations aimed at improving callus multiplication and eventually converting somatic embryos into regenerated *E. precatoria* plants.

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