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Effect of nutrients starvation in the cell cycle synchronization of cell suspension cultures of *Jatropha curcas* L.

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ABSTRACT

The knowledge about the processes that control cell cycle activity in *J. curcas* has not yet been addressed. Cell suspension cultures offer an attractive alternative to carry out this kind of studies in a simplified biological system. Nevertheless, the cell suspensions exhibit a high degree of heterogeneity concerning cell cycle distribution; therefore, synchronization methods must be previously established for this plant species. In the present research, cell suspensions of *J. curcas* were used to assess phosphorus, nitrogen or sucrose starvation as cell cycle synchronization approaches. Cell suspension cultures were established from the endosperm of seeds using a method for the enzymatic breakdown, without a previous callogenesis stage. The cell suspensions reached a maximum growth rate of 0.15 d^{-1} and a doubling time of 4.8 d. Microscopy analyses showed amyloplasts in some of the cells; however, oil and protein bodies were not observed. Regarding cell cycle synchronization, the results indicated that phosphorus or nitrogen starvation did not affect the cell-cycle arrest in the G0/G1 phase. However, the sucrose depletion for three days induced an accumulation of 67% of the cells in the G0/G1 phase. This effect was reversed with the addition of a carbon source. Moreover, these culture conditions generated a population of cells (approx. 5%) with a higher DNA content (3n > 6n), which was interpreted as the activation of an endoreplication process. Our findings suggest that nutrient starvation can affect in a differential way the cell synchronization process.

1. Introduction

During the last years, *Jatropha curcas L.* (*Euphorbiacea*e) has been highlighted as an important source of vegetable oils for the fuels industry; this has encouraged companies and universities to develop numerous research projects to improve the oil yields in this oleaginous plant [1–3]. So far, related studies have not been addressed on these species to study the mechanisms that control the cell cycle activity in somatic and specialized cells formed during organogenesis. For instance, there is a lack on research that allows establishing the correlation and regulation of cell expansion and division with the accumulation of storage compounds (such as oils, proteins, and carbohydrates) during the development of the *J. curcas* seeds. These processes have shown great complexity in other plant species [4,5].

Studies related to the cell cycle, using the whole plant, have found some limitations. They involve the enormous complexity of the tissues, the slow cellular division of some of them, the asynchrony of most of the somatic cells that compose them, and the fact that in many cases, only a cell fraction is cycling [6]. Thus, for more than three decades, plant cell suspension cultures have been proposed, as an alternative to studying several complex processes in a more simplified biological model [7–9]. In these cultures, cells grow in an aqueous medium where most of the culture conditions are under control, and synchronization treatments are more homogeneous than those on a plant tissue [10,11]. Thus, allowing to obtain a higher number of cells in the same phase of the cell cycle, to know the specific processes that are carried out in each one of them.

Cell synchronization consists in arresting the highest number of cells

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in a specific phase of the cell cycle with a subsequent release, to continue cycling in a coordinated way [12]. Cell synchronization is achieved through different strategies ranging from physical methods, such as light [13] and temperature [14], to the use of chemicals (e.g., hydroxyurea and aphidicolin) that interfere with specific processes of the cell cycle [12,15] and depletion of nutrients from the culture medium [16].

Synchronization by nutrient starvation has shown several advantages when compared to other methods. For instance, it is non-toxic as some chemical inhibitors, and cell cycle progression can be quickly restored when nutrients are re-added [11,17,18]. Phosphorus, nitrogen or sucrose starvation has been used for synchronization of different plant cell suspension cultures [11,16,19,20]. Sucrose and phosphorous starvation, particularly this last one, for specific time periods and subsequent supplementation have allowed the arrest of a high percentage of cells in G1 and some in G2 phases of the cell cycle [16]. For example, for *A. thaliana*, phosphorous depletion has allowed obtaining 70–80% of cells in G1 phase; these values improved with the combination of chemical inhibitors [11]. In contrast, sucrose depletion for a maximum time of 27 h generated only a partial synchronization since longer times can induce autophagy and cell death [11,16].

Different studies in *J. curcas,* including our studies, have been performed using cell suspensions [21–26]. These investigations have shown substantial progress in the protocols to establish cell cultures under *in vitro* conditions, likewise, the possibility of using this system as a simplified model to carry out different studies such as terpene production [27], heavy metal accumulation [28], or drug biotransformation [29]. Nonetheless, synchronization methods for *Jatropha* cell suspensions have not been evaluated yet, which has limited the outset of studies related to the cell cycle in this species.

Therefore, this research aimed to characterize *J. curcas* cells cultivated under *in vitro* conditions and to evaluate the effect of phosphorus, nitrogen or sucrose starvation on cell cycle synchronization. The results achieved in our study showed that phosphorus and nitrogen depletion arrests the cells in the transit from S to G2. Instead, sucrose starvation induced the cell cycle arrest in the G0/G1 phase, in more than half of the cellular population. These findings suggest that nutrients starvation can affect the *Jatropha* cell suspension in different ways and that it is necessary to evaluate other culture conditions to improve the protocols of cell cycle synchronization.

2. Materials and methods

2.1. Plant material, establishment, and maintenance of cell suspension culture

For this study, ripe fruits of *J. curcas* with a yellow-green coloration in the exocarp were used. The collection was carried out in March 2015, in the sub-region of Bajo-Cauca in Antioquia Department, Colombia. The cell suspension cultures were established from the endosperm of seeds, applying an enzymatic breakdown method developed in our laboratory (Patent U 7,7772,002 B1). The cells released from the explant were transferred to the MS2 culture medium (Online Resource 1, Table 1) and subcultured every eight days (d) for two years. During subcultures, the spent medium was withdrawn, and 30 mL of decanted cells were transferred to 70 mL of fresh culture medium in 250 mL Erlenmeyer flasks with cotton plugs to enable gas exchange. The suspension cultures were kept in a rotary incubator shaker under orbital agitation of 80 rpm at 29 ± 2 °C in darkness. A sieving process was performed every eight weeks to decrease the aggregates size in suspensions; the cells were suspended in a sterile 500 mL Nalgene filter unit fitted with a stainless-steel membrane with a pore size of 500 µm (Spectra Mesh®). The cells sieved (with an aggregate size < 500 µm) were recovered and subcultured as described above. Once the cell suspensions were established, three of them were selected to perform this study, and they were called as BRA-1, BRA-2, and BRA-3.

2.2. Determination of the culture growth curve

Kinetic parameters were measured for the three-month-old cell suspensions BRA-1, BRA-2, and BRA-3. Approximately 200 mL of cells from each culture were used to prepare the inoculum; cells were washed several times with fresh MS2 culture medium, using the Nalgene filtration system with a 20 µm nylon membrane (Spectra Mesh®), to obtain concentrated cell suspensions. For each experimental unit, 4 mL of washed cells were transferred to 16 mL of the MS2 culture medium in 100 mL Erlenmeyer flasks. The suspension cultures were incubated at 29 ± 2 °C, 80 rpm in darkness. The growth kinetics was carried out during 17 d; three Erlenmeyer flasks were taken from each culture every 3 d. Biomass was frozen at −20 °C until further processing. The biomass was recovered by filtration, dried at 70 °C in a convection stove for 48 h, and the dry weight was quantified by gravimetry. Based on the data obtained above, the specific cell growth rate (µ) was calculated by plotting the natural logarithm of biomass against time: the slope of the linear part of the kinetics, which corresponds to exponential phase was defined as µ. The doubling time (Dt) was calculated from the µ value.

2.3. Storage compounds and cell viability

Cells growing under in vitro conditions were characterized according to the presence of storage compounds, using some specific stains to recognize amyloplasts, oil bodies, and protein bodies, with microscopy techniques. To identify amyloplasts, 300 µL of cells and 700 µL of a solution of Lugol (0.06% w/v of Potassium Iodide + 0.02% w/v of Iodine) were mixed. For protein bodies, 2 mL of cells were stained with 4 mL of Amido Black (1% w/v Amido Black 10B + 7% w/v Acetic acid) during 1 min. Then, the excess dye was removed by repeatedly washing with 0.04% acetic acid, using cotton to absorb the dye in each wash. The presence of storage lipids was observed by mixing 600 µL of cells with 200 µL of Nile red (20 µg/mL of Nile red in acetone, SIGMA®). Likewise, cell viability was verified by mixing 500 µL of cells with 25 µL of FDA (5 mg/mL of Fluorescein Diacetate in acetone, SIGMA®), the mix was incubated 30 s in darkness. Viability was recorded as the percentage of live aggregates from at least six observations per sample of the same culture in different fields (Neubauer chamber), comparing the observations for each sample under the optical and fluorescence microscopy.

The observation and analysis of cells were carried out with a Nikon Eclipse 80i microscope (Nikon®), provided with a phase contrast system for 40X objectives. A mercury lamp and a filter set were used to observe the fluorescence in all the staining. The red fluorescence in the cells stained with Nile Red was observed in an excitation range of 528–553 nm and an emission of 590–650 nm. The green fluorescence in the cells stained with FDA was observed with an excitation range of 465–495 nm and emission of 515–555 nm. The images were captured with a DS-F1 CCD camera and the analysis was performed using the software NIS-elements Basic Research (4.30.02 version, Nikon®).

2.4. Cell cycle synchronization

2.4.1. Phosphorus and nitrogen starvation

Phosphorus, nitrogen, or sucrose starvation were assessed for synchronizing the cell suspensions of *J. curcas* by arresting a high proportion of cells in G1 with the subsequent release from blocking and the progression of the cell cycle. Growth kinetics was first performed for every synchronization experiment. Cells were grown in a culture medium with all the nutrients, or in a phosphate- or nitrogen-free culture medium to evaluate the effect of these nutrients depletion. The BRA-2 suspension culture, which showed a higher rate of cell division, was used as inoculum. Approximately 300 mL of a middle exponential phase cell suspension (7 d after the previous subculture), with a cell aggregate size < 500 µm and cell viability close to 90% was used. The cells were washed five times with the phosphorus- or nitrogen-free MS2

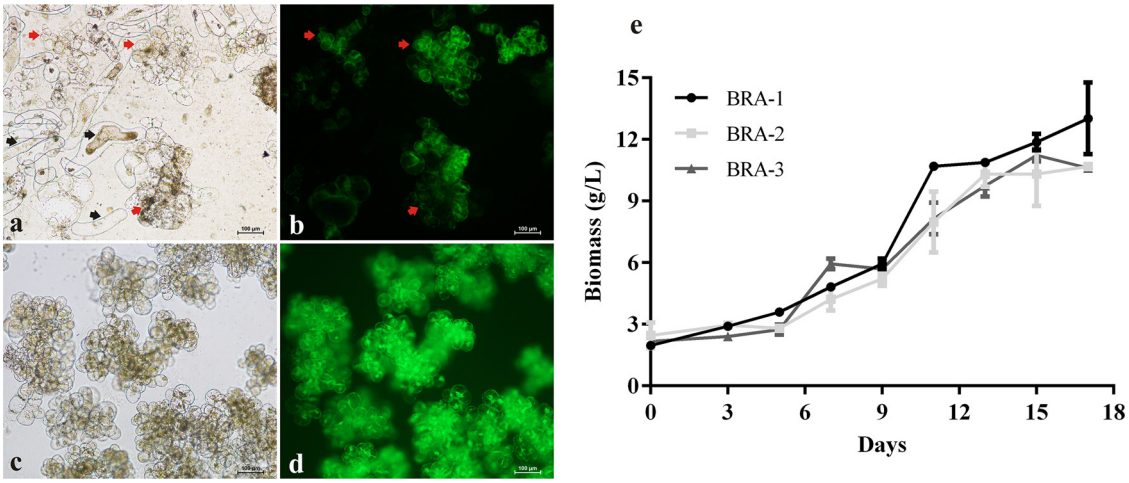

Fig. 1. Morphological aspect of *J. curcas* cell in suspension and growth kinetics. Cells observed under optical, and fluorescence microscope after FDA stain: (a–b) Cells cultured during 15 (c–d), and 45 d (e). Growth kinetics of BRA-1, BRA-2 and BRA-3 suspensions; the graph shows the values obtained from dry biomass (g/L) *vs.* time. The bars correspond to the standard deviation of two replicates. Black arrows indicate the endosperm cells highly plasmolyzed, and red arrows indicate the daughter cells, divided from the endosperm. The bars show 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

culture medium, using Nalgene filtration system with a 20 μm nylon membrane.

For each experimental unit, 1.5 mL of the washed cells were transferred to 8.5 mL of the MS2 culture medium in 100 mL Erlenmeyer flask. The suspension cultures were incubated at 29 ± 2 °C, 80 rpm in darkness. Growth kinetics was performed during 14 d, three Erlenmeyer flasks were taken every 2 d, and dry weight was quantified by gravimetry. For cells cultured with all the nutrients (control), both phosphorus and nitrogen were added to each Erlenmeyer flask from a stock solution, with a final phosphorus concentration of 170 mg/L (KH_2PO_4) and nitrogen of 2500 mg/L (KNO_3). For cells grown without nitrogen (0 mg/L Nitrogen), phosphorus was added, and for cells grown without phosphorus (0 mg/L phosphorus), nitrogen was added.

New growth kinetics was performed, taking into account the results of the last experiment, and using the same conditions previously described. In this assay, the cells were cultivated during 8 d, and on days 5, 6 and 7, three experimental units were taken from each culture condition for quantifying the percentage of cells within the different phases of the cell cycle by flow cytometry.

2.4.2. Sucrose starvation

The sucrose depletion was assessed by experiments similar to those carried out for nitrogen and phosphorus. First, growth kinetics was performed with cells growing in a medium with all the nutrients (control), or without sucrose but supplemented with 3% w/v of polyethylene glycol (PEG) as an osmotic regulator (PEG, MW: 3000 g/mol, EMD Millipore). Cells were washed with a sucrose-free medium, and the experiment was carried out as described here above. Growth kinetics was performed during 4 d, three Erlenmeyer flasks were taken every day, and dry weight was quantified by gravimetry.

From this experiment outcomes, new growth kinetics was then performed using the same conditions described above. In this assay, the cells were cultivated for 3 d, and three experimental units were taken every day to quantify the percentage of cells within different phases of the cell cycle by flow cytometry. On the third day of kinetics, the cells grown without sucrose and supplemented with 3% PEG, were washed and re-suspended in a culture medium with sucrose, using an inoculum of 30%.

The comparison of cell percentage in each phase of the cell cycle under different culture conditions over time was carried out using ANOVA with a p-value < 0.05 (with Tukey's test), using the statistical program Prism 6.0 version 6.03.

2.4.3. Flow cytometry

To determinate the cell cycle distribution in cell suspensions of *J. curcas*, a protocol was established to isolate the nuclei and quantify DNA content by flow cytometry, based on previously described protocols [30–32]. The isolated nuclei were stained with 7.5 mg/mL Propidium Iodide (PI), and nuclear DNA content was measured using an LSR Fortessa Flow cytometer (BD Biosciences, CA, USA). The aggregates were excluded using the pulse Area versus the peak Height of fluorescence at 620 nm, and the reading threshold was defined with non-stained nuclei. For each sample, at least 10,000 events were read, from which an average of 3000–5000 was within the region of unique events. Each point in the charts represents the average of four biological replicates processed independently. The cell cycle progression was analyzed by FlowJo7.6.2 software (Tritar Inc., San Carlos, California, USA), using the Watson Pragmatic model.

3. Results

3.1. Establishment and growth kinetics of cell suspensions

Cell suspension cultures of *J. curcas* were established from the endosperm of seeds using a method of enzymatic breakdown, without a previous step for callus induction. Fifteen-day-old cultures were formed by endosperm cells and their daughters; the first ones died quickly, and therefore they were removed from every subculture by a sieving process (Fig. 1a, b). In the first stage of cultures, the cell viability, estimated by FDA staining, percentages were not higher than 50%. The cells showed different shapes: elongated, isodiametric and spherical, with sizes from 20 μm to 150 μm. The cellular aggregates at this stage were formed by 5–10 cells (Fig. 1a, b). Approximately three months after cultures were established, higher percentages of cell viability were found, with an isodiametric or spherical likely cell morphology, with prominent nuclei and a cellular size from 50 μm to 100 μm (Fig. 1c, d). The rise in cell division rates generated an increase of aggregates size from 200 μm to 2000 μm; for this reason, the sieve process was essential to maintain a suitable nutrients availability and oxygen exchange in all cells (Fig. 1c, d).

The results of growth kinetics of the three *J. curcas* cultures showed a very similar growth pattern during the 17 d of culture (Fig. 1e). Cell growth was initially slow during the first 4 d, defined as the latency phase; subsequently, accelerated growth was recorded between days 5 and 13, considered as the exponential phase; then, the cell division rate

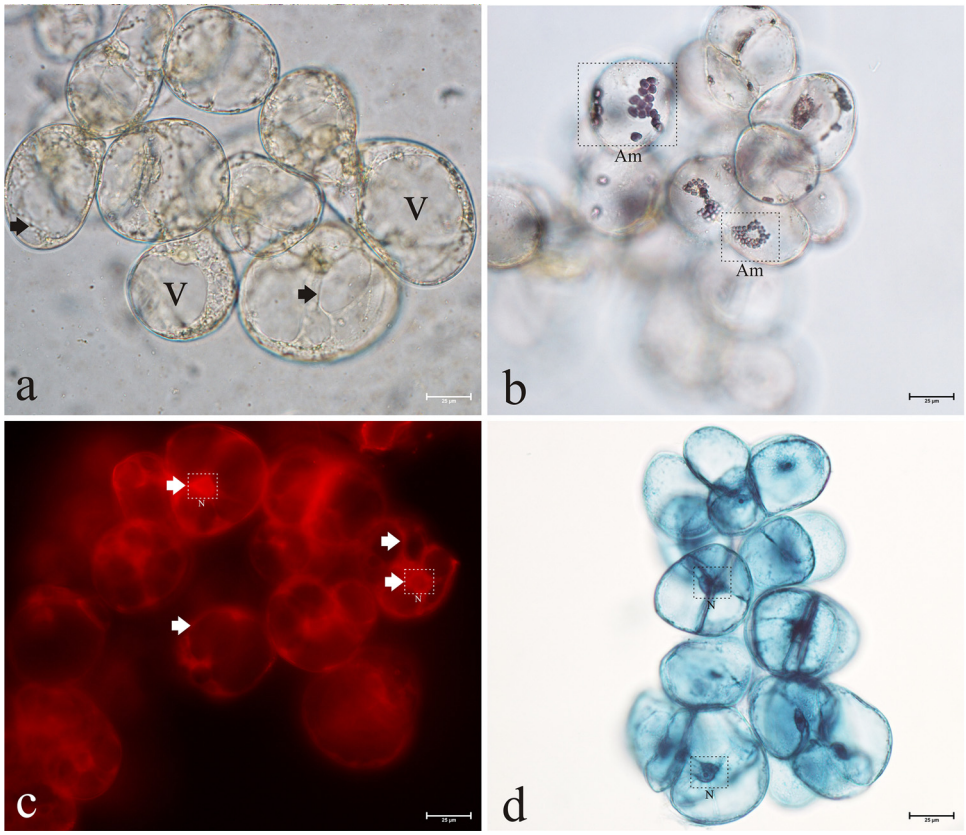

Fig. 2. Microscopy analysis of three-month-old J. curcas suspensions. (a) Cells observed under the optical microscope. (b) Cells storing starch in amyloplasts (Am), stained with a Lugol solution. (c) Cells stained with Nile red to observe lipidic structures. (d) Cells stained with Amido Black to reveal proteins. Black arrows indicate the cytoplasmic bands. White arrows point the cellular membranes. N: nuclei, V: vacuoles. The bars show 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was reduced, corresponding to the stationary phase. From this growth kinetics, the specific cell growth rate and the doubling time were calculated for the three cultures BRA1, BRA2, and BRA3: growth rates were 0.12, 0.15, and 0.13 d^{-1}; and doubling times were 5.6, 4.8, and 5.29 d respectively. With a 2 g/L inoculum, a dry biomass value between 11 g/L and 13 g/L was obtained at the end of the kinetics, showing a 6–7 times increase in biomass.

3.2. Storage compounds under in vitro conditions

The morphological characteristics of cells growing *in vitro* are a critical aspect of knowing how they respond to different culture conditions. Besides, they allow having a first approach to the metabolites they can accumulate in their cytoplasm. The cells of *J. curcas* were characterized by having prominent vacuoles and reduced cytoplasm, noticed as cytoplasmic bands (Fig. 2a). Lugol staining showed the presence of cells with amyloplasts (Fig. 2b), although these did not seem to be a predominant organelle in all cells (Fig. 2a). Regarding Nile red staining, the presence of oil bodies was not found, and it was only possible to observe the plasma, vacuolar and nuclear membranes (Fig. 2c). Also, cells stained with Amido Black revealed the absence of protein bodies in the cytoplasm and only allowed to observe the presence of a significant amount of protein in the nucleus (Fig. 2d).

3.3. Nitrogen starvation induces an arrest of cells in the S phase of the cell cycle

Phosphorus, nitrogen or sucrose starvation conditions were assessed as methods for cell synchronization to obtain a more homogenous population in *J. curcas* cell suspensions, regarding cell cycle distribution. For cells grown without phosphorus (0 mg/L phosphorus) or nitrogen (0 mg/L nitrogen), there was a sharp decrease in biomass produced during the whole kinetics (Fig. 3a). From days 4–12, the values of dry biomass for both culture conditions were similar, in a range of 1.8 g/L and 2.5 g/L. At the end of kinetics, there was a biomass increase to 4.36 g/L in phosphorus-free suspensions and 3.8 g/L in nitrogen-free suspensions. Furthermore, cells grown with all nutrients (control) showed higher values of dry biomass: 3.4 and 10.6 g/L on days 4 and 14, respectively.

Concerning cell cycle distribution, the percentage of cells in each phase was recorded from cell suspensions cultured with all nutrients, without phosphorus or nitrogen, in days 5, 6 and 7 of the growth kinetics. In cultures with all nutrients, the percentages of G0/G1-phase cells during the three days of sampling did not show significant differences, remaining around 40% (Fig. 3b). For S or G2 phases, a significant change was found only on day 7, when a decreased percentage of S- and an increased percentage of G2-phase cells were observed (Fig. 3c and d).

Regarding cells grown without phosphorus, the percentage of G0/G1-phase cells was close to 40%, which did not show significant changes. However, a significant increase was found in the fraction of S-phase cells by day 5, with the concomitant reduction of G2-phase cells. Similarly, in cells grown without nitrogen, the depletion of this nutrient did not affect the G0/G1 arrest, with a cell accumulation remaining around 40%. Nevertheless, a significant increase in the percentage of S-phase cells was found, rising from 38.7% to 57.2% on days 5 and 7, respectively.

Taking into account all these results, it is clear that phosphorus or nitrogen starvation during 7 d generates a reduction in the cell division rate and a slight decrease in the percentage of cell viability with values close to 80% (Online resource 1, Fig. 1). However, the depletion of these nutrients did not affect the G0/G1 cell arrest; instead, a higher number of cells in the S to G2 transition was observed. Histograms (Fig. 3e–g) show the distribution of the cell cycle on day 7 of kinetics, where the cellular asynchrony of the suspension grown in different culture conditions is evident.

It should be emphasized that the statistical analysis was performed comparing the percentages of cells in the different phases versus the

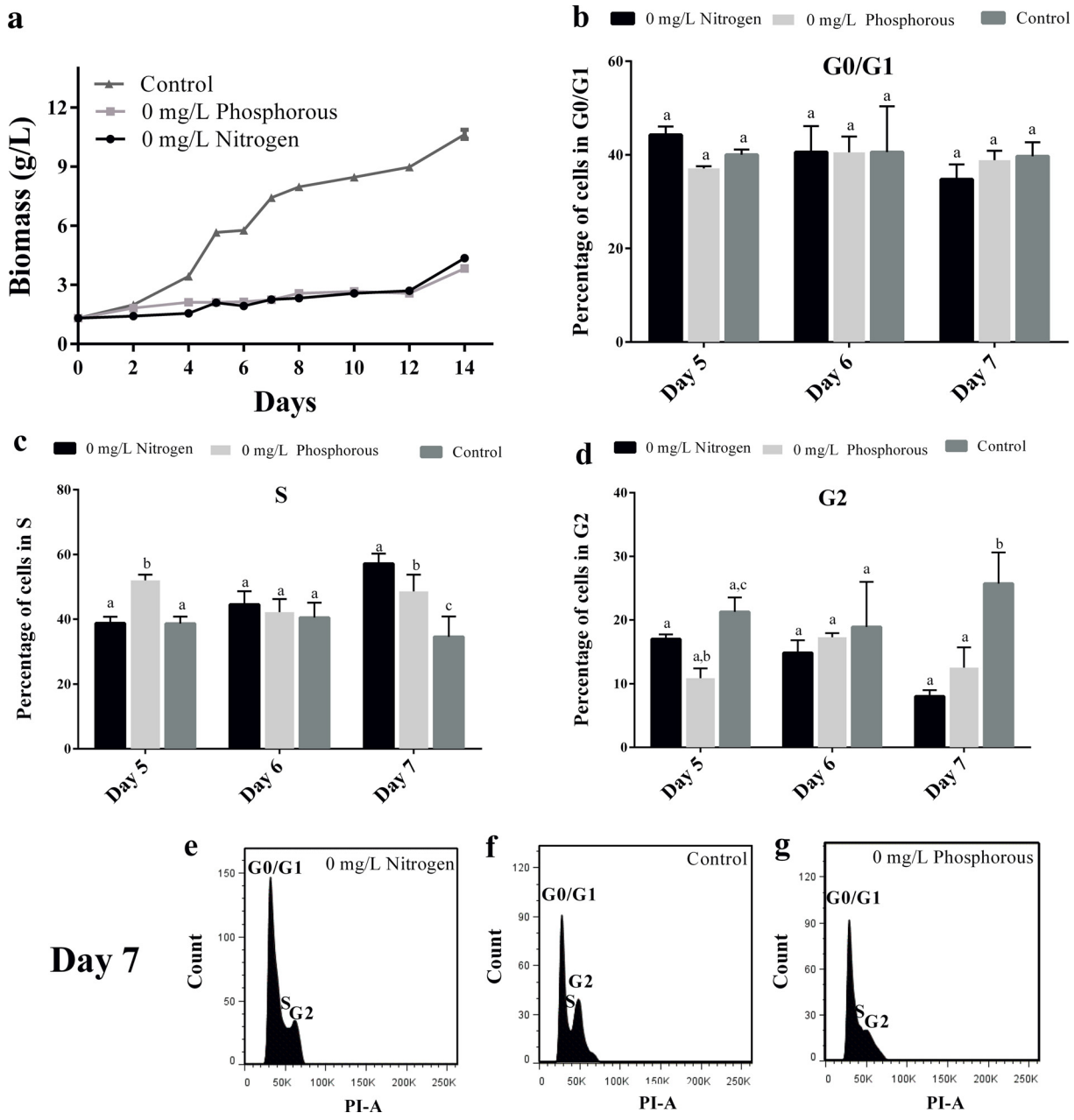

Fig. 3. Effect of phosphorus and nitrogen starvation on cell cycle distribution. *J. curcas* cell suspensions were cultured with all nutrients (control), without phosphorous (0 mg/L phosphorous) or nitrogen (0 mg/L nitrogen). (a) Growth kinetics during 14 d. (b–d) Cell cycle distribution during days 5, 6, 7. (e–g) DNA content histograms show the distribution of cell populations in each phase of the cell cycle on day 7. Statistical analyses were performed comparing the percentages of cells in each phase of the cell cycle in every culture condition and for each day of sampling, but there was no interaction between times. The different letters indicate significant statistical differences ($p < 0.05$, HSD Tuckey Test) in each group of samples. The dates are the averages ± the standard error of four replicates.

culture condition, for each day of sampling.

3.4. Sucrose depletion induces an arrest of cells in G0/G1

For the experiments related to sucrose, a decrease in the growth rate was found in cells grown under sucrose starvation (0 g/L sucrose), recording 1.2 g/L of dry biomass at the end of the kinetics. This value is low when compared to cells cultured with all nutrients, which reached 4.9 g/L of dry biomass on day 4 (Fig. 4a). Cell cycle distribution was recorded only for the first three days of kinetics since longer times negatively affected cell viability (Online resource 1, Fig. 2). In the sucrose-free cultures, a significant increase in the percentage of cells arrested in the G0/G1 phase was found during the three days of sampling, showing 43.6% on day 1 and 66.1% on day 3 (Fig. 4b). Concomitant reductions of cell percentages in S and G2 phases were observed (Fig. 4c–d).

Aiming to evaluate how long cells were arrested in G0/G1 and if this blocking was reversible, the cells cultured without sucrose for 3 d were washed and re-suspended in a medium with all nutrients. The cell cycle distribution in the first 6 h did not show significant changes and 66% of cells remained in the G0/G1 phase (Fig. 4e). However, at 12 and 24 h, the cell population in this phase was reduced to 47%, with an increased percentage of cells in the S and G2 phases (Fig. 4e). But again at 48 h, the cell cycle distribution was very similar to the one observed at 0 h, showing a higher peak in the G0/G1 phase when compared to the other ones (G0/G1:67%, S: 12.5%, G2:17%), thus obtaining again a partially synchronized population.

In cell suspensions grown without sucrose, a low percentage of cells

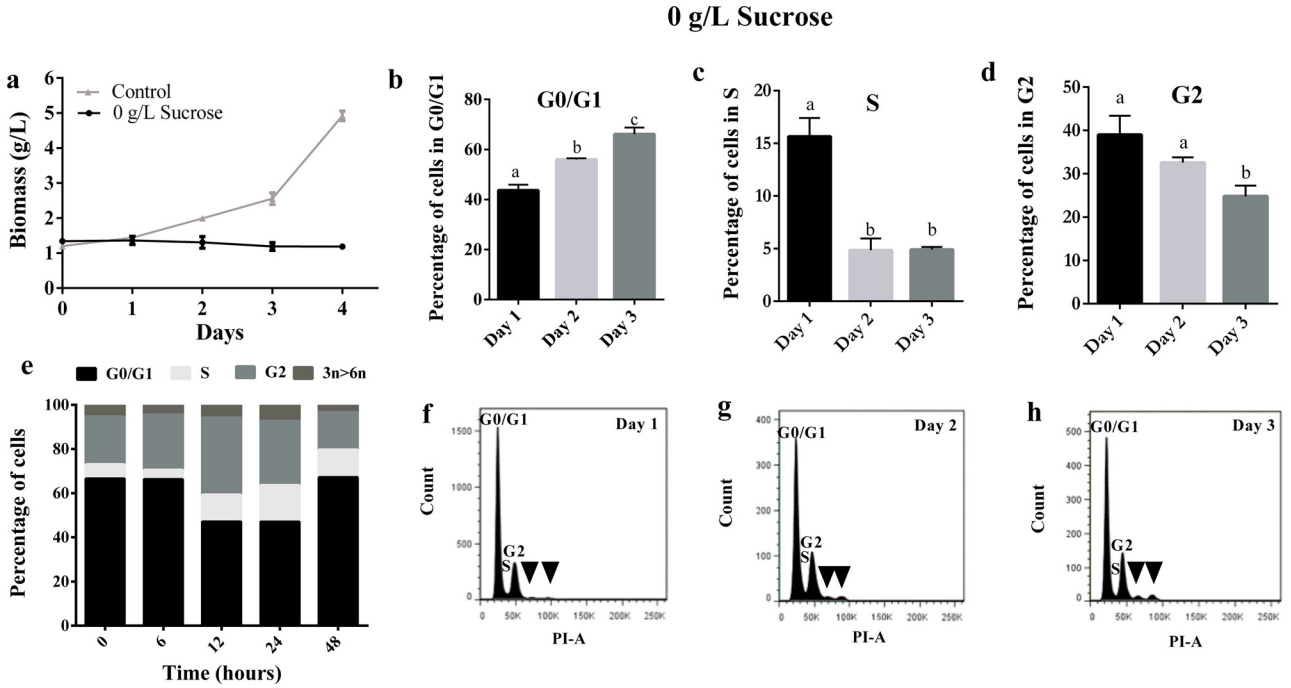

Fig. 4. Effect of sucrose starvation on the *J. curcas* cell cycle distribution. *J. curcas* cell suspensions were cultured with all nutrients (control) and without sucrose + 3% PEG (0 mg/L sucrose). (a) Growth kinetics during 4 d. (b–d) Cell cycle distribution in days 1, 2, 3. (e) Cell cycle distribution after re-suspending cells in a medium with all nutrients; "(3n > 6n)" key represents the endocycling cell populations. (f–h) DNA content histograms show the distribution of cell populations in each phase of the cell cycle on day 1, 2 and 3. Black arrows indicate the endocycling cells. Statistical analyses were performed comparing the percentages of cells in each phase of the cell cycle in every culture condition and for each day of sampling, but there was no interaction between times. The different letters indicate significant statistical differences ($p < 0.05$, HSD Tuckey Test) in each group of samples. The bars are the averages ± the standard error of four replicates.

with DNA content > 6n (~5%) was observed, which was attributed to the activation of an endoreplication process. These cells were not taken into account in the analysis of cell cycle distribution (Fig. 4e), yet, they are shown in the histograms (Fig. 4f–h).

4. Discussion

4.1. Establishment of cell suspensions without a previous callogenesis stage

In this research, the *J. curcas* cell suspensions were established directly from the seeds' endosperm, without a prior callogenesis stage, getting cells that reached a high percentage of viability and a high cell division rate (Fig. 1a–d). This methodology was first developed by our laboratory (Patent U7.7772.002 B1), given that the reported publications, so far, use a callogenesis stage [21,24–28,33–35]. The suspensions of *J. curcas*, established in this study, registered a maximum growth specific rate of 0.15 d^{-1} and a minimum doubling time of 4.8 d. Similar results have been reported by other authors [21,26,28], that together with our results show that this species may present high cell division rates when cultivated *in vitro*.

Through microscopic observations, the presence of amyloplasts was found in some cells (Fig. 2b). Similar findings have been reported in research studies on this [21] and other plant species, where the starch accumulation is very heterogeneous [36], and depends on culture conditions [37]. Regarding storage lipids (e.g., triacylglycerols) in the *J. curcas* cell suspensions, the presence of oil bodies was not observed. Lipidic reserves are not a usual feature in plant cell suspensions; however, in some species, the accumulation of these compounds has been described [38,39]. It means that for getting triacylglycerols accumulation in the *J. curcas* cell suspensions, it is necessary to evaluate other culture parameters, such as the addition of abscisic acid or osmotic agents (e.g., PEG, mannitol or sucrose) at high concentrations, among several culture conditions.

4.2. Phosphorous, nitrogen or sucrose depletion affects differently the cell cycle synchronization

In these research outcomes, *J. curcas* cell suspensions did not show any significant change in the percentage of G0/G1-phase cells when they were cultured without phosphorous or nitrogen, reaching maximum values of 45%, which were similar to values found in the control culture (Fig. 3b). For other plant suspensions, phosphorous starvation has shown to be successful. For instance, tobacco suspensions cultured during 5 d without phosphorous reached 90% of cells arrested in the G1 phase [40], this assessment time was similar to the one tested in our study; nevertheless, *J. curcas* suspensions did not accumulate in that phase. For cell cultures of *Taxus,* much more prolonged times have been required to synchronize the cells [14]. However, for *Arabidopsis* cells, a period of 72 h was enough to arrest 70% of cells in the G0/G1 phase [11]. The more prolonged periods of phosphorous or nitrogen starvation for *J. curcas* cells showed that this is not an alternative option, since these higher times affected cell viability, especially when nitrogen was removed. For the latter, depletion had a severe impact on cell viability, probably due to the importance of nitrogen as a constituent of molecules and compounds required in the physiological processes that are being carried out during cell division and growth. Besides, cells may need longer times to re-enter into the cell cycle, once the nitrogen source is added [20].

The cell arrest between the S/G2 phase transition observed under nitrogen and phosphorus depletion showed cell cycle progression even under the absence of both nutrients. Nevertheless, the growth kinetics showed conflicting results (Fig. 3a), because the growth rates of cells cultured without these nutrients were twice lower than the control cells. Partial synchronization and cell cycle progression of plant cells under nutrients depletion have been described before [19], as well as the observation that nutrients like phosphorous are stored in vacuoles and transferred again to the cytoplasm when it is depleted in the culture medium [41]. This evidence allows suggesting that in this study, the *J.*

curcas cells used nitrogen and phosphorous reserves to proceed with the most important cellular processes, such as cell cycle progression. Moreover, it is important to highlight that cellular growth involves two processes: cell division and expansion. It is clear that cell division was not strongly affected when cells grew without one of the nutrients; yet, it is suggested that cell expansion processes were stopped entirely, as it is evidenced by the biomass yield data.

In spite of the fact that the phosphorus and nitrogen starvation did not affect the synchronization of *J. curcas* cell suspensions, it allowed us to determine the cell cycle distribution through the growth kinetics. The results showed that cells found in the G1 phase or the non-division state G0 (Quiescence), progressed to the synthesis phase (S) (Fig. 3b, c). For instance, by day seven nitrogen-free cultures recorded only 34.8% of cells in the G0/G1 phase and 66% in the others (S: 57.8%, G2: 8.0%). These results suggest that more than half of the population are continually cycling. This feature is valuable for the establishment of a plant suspension as a cell line, and particularly for the performance of cell cycle studies or the synthesis of metabolites associated with biomass production.

Regarding sucrose depletion results, it was possible to prove that a high percentage of the cell population can be arrested in the G0/G1 phase, rising from 43% to 67% in 24–72 h (3 d) (Fig. 4b). It was also found that arrest is reversible and the cells continue cycling (Fig. 4e). Similar results were reported in *A. thaliana* cell suspensions, this being why low synchronization percentages were obtained when cells were cultured without sucrose during 24 [16] or 27 h [11]. These results were improved, in both studies by the addition of chemical substances like aphidicolin. This strategy was not used in the current research. Instead, the sucrose starvation time was increased up to 72 h, adding PEG into the medium, which performed an essential role as osmotic agent, keeping high percentages of cell viability during the three days of starvation and allowing more than half of the population to accumulate in the G0/G1 phase.

In this context, it is critical to keep in mind that getting a high percentage of cells in a specific phase of the cell cycle, also depends on other suspension features, such as high rates of cell division [11,17] and cell size uniformity. Culture dilution is also a crucial step for achieving high rates of synchronization [16]. For *J. curcas* cell suspensions, these parameters have not been fully established yet; this being why the evaluation of different culture variables could significantly improve the rate of cell growth. Likewise, it is necessary to develop methodologies to establish more homogeneous cultures concerning cell size, to have higher uniformity in the cell population, and thus increase the percentages of cells arrested in the G0/G1 phase, to get a highly-synchronized population.

4.3. Sucrose depletion generates endocycling cells

Flow cytometry analyses conducted throughout this research did not show cell populations with higher DNA content, even in nitrogen or phosphorus-free culture media (Fig. 3). Opposite results had been reported in tobacco cell suspensions, for which the phosphorus depletion generated binucleate cells [19]. However, when *J. curcas* suspensions were grown without sucrose, cells with higher DNA content were found; such population was thought to correspond to endocycling cells (Fig. 4e–h) which are different from aggregated nuclei.

In plants, sucrose plays an essential role in several biological processes [42]. For instance, it acts together with the auxins as signaling molecules that control the cell cycle progression [43]. It is clear so far that several phytohormones, in particular, auxins, have a direct effect on the activation or repression of endoreplication [44]. For example, in *Arabidopsis*, a low concentration of auxins induce endocycling cells [45]. But, in *Medicago truncatula* seeds the cell cycle progression and triggering of endoreplication depend on the stage of seed development and the type of auxins [46]. Nevertheless, the switch of the endoreplication process under sucrose starvation conditions is less clear.

A strong correlation between sucrose starvation and the arrest in the G1 phase has been widely studied, to understand the cell autophagy processes [47,48]. However, these studies have left aside the possible endoreplication events that may occur. The results of the present study show that sucrose depletion can lead to endocycling cells generation, suggesting that some cells skip the entire M phase and in consequence avoid chromosome segregation and cell division.

The induction of endopolyploidy in plant cells is not an unusual process. It may be an ordinary and necessary behavior for some morphogenetic processes, such as cell expansion [49], cell differentiation (it can support the gene expression upregulation and capacity in specialized cells) [50,51], trichomes [52] and endosperm development [53]. Likewise, this process has been correlated with some stress conditions [51,54]; but also, it may only act as a response to the plasticity that characterizes plant cells [44,51].

In *J. curcas* suspensions, the generation of endocycling cells could be a response to the stress conditions generated by the lack of sucrose, which is involved in hundreds of processes such as cell signaling, provision of a source of energy, protection against oxidative stress, and mainly being a precursor for the synthesis of macromolecules. However, mechanisms that regulate the triggering of the endocycling process in cell suspensions have not been addressed yet, and research is needed on the subject to clarify these cellular responses.

5. Conclusions

The present research shows that the establishment of *J. curcas* cell suspensions without a callogenesis stage is feasible by directly using the seeds' endosperm as explant. This strategy might reduce the establishment time and genetic and epigenetic changes that could be generated during callogenesis process. Now it is clear that phosphorous and nitrogen starvation do not have an effect on the cell cycle arrest at the G1 phase, although a higher percentage of S-phase cells was observed, probably associated to the reduced fractions in G2 and G1 phases. Additionally, we found that sucrose starvation allows a partial synchronization, which could not be maintained for a more extended period because cell viability may be affected. Nonetheless, once sucrose was added, the cells remained synchronized during the first 6 h, an effect that was not observed at 12 and 24 h; however, strikingly at 48 h, there was a high percentage of cells in the G0/G1 phase. These results open the possibility to start studies related to the control of cell cycle for *J. curcas* in an area of knowledge that so far has not been addressed on this species. These cell suspension cultures could be used as a biological model for oleaginous plants, and to perform research that allows the understanding of the correlation between the cell cycle and the accumulation of storage compounds during seeds development.

Competing interests

The authors declare no competing financial interests.

Authorship

All authors have made substantial contributions to the construction of this study. Laura Carmona, Mauricio Rojas and Aura Urrea, proposed and developed the original project, designed the experiments, acquisition of all the data, analysis and interpretation of them. Lucia Atehortua, as the manager of the laboratory facilitated the infrastructure resources y supervised the experiments.

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Conflict of interest statement

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cpb.2018.03.002.

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