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Callus induction and chemical characterization of cell suspension cultures of jojoba (*Simmondsia chinensis* L.)

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ABSTRACT

Jojoba (*Simmondsia chinensis* L.) oil is also known as liquid wax or fixed oil. It is an important metabolite of jojoba having commercial importance in cosmetics as well as a potential biofuel source. We presented an efficient system for *in vitro* establishment of cell suspension cultures (CSC) from proliferating friable calluses. For this purpose, cotyledon, internode, and leaf explants were cultured on MS medium + 1, 2, 4, 6, 8 or 10 µM 2, 4-Dichlorophenoxyacetic acid (2, 4-D), α-Naphthalene acetic acid (NAA) alone or in combination with 1 or 2 µM N^6-benzylaminopurine (BAP) or Kinetin. Results demonstrated that 100% healthy, friable and variegated calluses were obtained on 8 µM, 10 µM 2, 4-D or 2, 4-D 10 µM + 2 µM BAP and represented as callus lines (CL) CL-1, CL-2 or CL-3, respectively, after 38 days. One-gram callus tissue per CL was then immersed in the respective liquid medium and agitated on an orbital shaker at 60-70 rpm under the growth room conditions (25 ± 2 °C, 16 h light period) for the preparation of CSC. After 15 days, CSC was sieved and large clumps were removed. Growth measurement of CSC was determined by cell counting, packed cell volume (PCV) and cell viability. The highest number of viable cells was obtained at 2.57 OD with CL-3, where PCV was highest (0.35 ml) on CL-1 of 38 days old calluses. 2,3,5-Triphenyltetrazolium chloride was a reliable approach for the determination of cell viability of CSC.

Keywords: 2,3,5-Triphenyltetrazolium chloride, callus, cell suspension culture, cell viability, jojoba, packed cell volume.

INTRODUCTION: Potential metabolites reserved by plant parts are important sources for current medication for the treatment of various ailments. Amongst, some metabolites have immense value in the commercial industry including cosmetics and confectionaries. For instance, fixed oil is used in the cosmetics and lubricant industry. Jojoba (*Simmondsia chinensis* L.) is one such plant of fixed oil sanctuaries of seeds. This is a small bush inhabited in deserts and in dry climatic regions of the world and in various areas of Bahawalpur, Multan, and other zones of Pakistan (Aftab *et al.*, 2008). Jojoba oil has a golden color, therefore, this is also known as "Desert Gold". Plant tissue culture is an important horticultural approach for enhanced *in vitro* metabolites production under ideal culture conditions for "Desert Gold". Variable in vivo procedures have been devised to get oil from seeds and whole plants. For example, the oil may be extracted via direct extraction from seeds, leaves, whole plants or using *in vitro* tissues like calluses and somatic embryos. Plant cells store the same products on maturity as well as when aggregated on the culture medium. Besides, some plants release their products when cells become isolated in the liquid culture medium. Cell suspension culture (CSC) is an authentic system to achieve single-cell metabolites. Research on SCS has been reported (Silva and Menéndez-Yuffá, 2006; Tan *et al.*, 2010; Dwivedi *et al.*, 2016; Parra *et al.*, 2017). Optimizing the culture conditions of CSC is a prerequisite to achieve such results. Type and the concentration of plant growth regulators (PGRs) are recognized before the experimentation. For this purpose, auxin 2, 4-dichlorophenoxyacetic acid (2, 4-D) is typically used for friable and soft callus induction and establishment of CSC (Kong *et al.*, 2020). Isolated plant cells divide and multiply faster in a liquid medium as compared to a solid medium which have more mortality rate whereby needs to determine their viability status. Mathur and Shekhawat (2013) demonstrated that plant cell viability may be maintained by maintaining the optimum level of PGRs in the medium and may be increased by increasing the concentration and culture time. Division of labor increases the cell biomass production which may be determined by Packed Cell Volume (PCV) (Dwivedi *et al.*, 2016) for estimation of the total biomass of the cultured cells. Cell counting and chemical methods such as Evans Blue (EB) and 2,3,5-triphenyl tetrazolium chloride (TTC) are suitable methods for the determination of the cell viability status of CSC. TTC is more appropriate for determining cell growth while staining with Flouricine Diacetate tended to overestimate it. Regrowth is more sensitive and consistent when using the TTC approach to estimate the viability status of cultured cells. Maintenance of optimum CSC conditions is an initial phase and may be considered as independent research.

OBJECTIVES: the present study aimed to investigate the most appropriate culture conditions for callus induction and establishment and determination of cell viability of jojoba CSC.

MATERIALS AND METHODS

Culture conditions for callus induction: Murashige and Skoog (1962) (MS) medium was prepared as a general method, added 0.8% bacteriological agar (Oxoid, UK) and 3% sucrose (Fischer, Germany), and ~10 mL medium was poured into glass vessels (Pyrex® Germany) and autoclaved at 104 kPa and 121°C for 10 min. Seeds were surface sterilized with 15% v/v commercial bleach (6% active chlorine) for 15 min. Then the seeds were rinsed with 70% ethanol for 35 sec. and finally washed with sterile distilled water 4-5 times and inoculated on the agar-solidified medium for germination. Fifteen days old green leafy cotyledons were excised to one half and cultured on MS medium supplemented with 1, 2, 4, 6, 8 or 10µM 2, 4-

Dichlorophenoxyacetic acid (2,4-D) or α-Naphthalene acetic acid (NAA) alone or in combinations 1+1, 2+1, 4+1, 6+2, 8+2 or 10+2 µM (2, 4-D or NAA + N^6-benzylaminopurine-BAP or Kinetin-Kin) in the dark at 25 ± 2 °C. Treatments produced healthy and friable calluses were designated as callus lines (CL).

Cell suspension cultures (CSC): Cell suspension cultures (CSC) were prepared from 40 days old 1g callus mass obtained from CL in the liquid culture medium (50mL) having the same concentrations in which CL was obtained as stated above, in 250 mL capacity Erlenmeyer flasks. The flasks were capped with non-absorbent cotton and aluminum foil and placed on the orbital shaker (OPTIMA OS-752, Indonesia), and spun at 100 rpm under culture conditions for 15 days. Then the aliquot was passed through a 750µm mesh-size sieve to separate the clumps and the sieved material was again agitated by adding fresh liquid medium up to 50 ml at 100 rpm for another 15 days.

Growth measurements of cell suspension cultures: cell counting: Cells of suspension cultures of CL were counted by taking 10µl aliquots on the glass slide and placed over with glass-cover and observed under a compound microscope at 10X. Further detail of cell morphology was also observed under high power (40X) of the microscope.

Packed cell volume (PCV): The packed cell volume or residual volume per CL of the cell debris was determined by taking 10 ml aliquots of each sample and centrifuged (Sorvall RC-5B, UK) at 2000 rpm for 5 minutes. Then the centrifuged samples were poured into plastic graduated tubes (Fisherbrand®) for 30 min. to settle down the cells and calculated for PCV.

Cell viability*:* To determine viable cells showing the protoplasmic streaming and a visible nucleus, the following methods were used.

Tetrazolium test: The 2, 3, 5-triphenyl tetrazolium chloride (TTC) was used for viability assay. This method measures the presence of respiring cells. Chemicals required for this test included TTC stock solution, 50 mM phosphate buffer, 50% methanol containing 1% sodium dodecyl sulfate (SDS) (M-SDS). TTC stock solution was prepared by taking 0.2 g salt in 10 ml sterile phosphate buffer (50 mM), pH 7.5. Phosphate buffer (50 mM) was prepared by dissolving 0.87g K_2HPO_4 and 0.68g of KH_2PO_4 in 100 mL distilled water pH 7.5. One mL sample of cell suspension was washed 3 times aseptically with 50mM phosphate buffer and added TTC to make a final volume of 2.5 ml and incubated under the dark conditions at 25°C for 8 h. The TTC was reduced to formazan solubilized in 1.5mL 50% methanol containing 1% SDS at 60°C for 30 min. These samples were centrifuged at 1875 rpm for 5 min and then the supernatant was collected and taken optical density (OD) at 485 nm by spectrophotometer (UV-4000, Germany). The high absorbance value indicated the presence of more viable cells taken after different periods.

Evans blue (EB) test: The stock solution of EB was prepared by dissolving 100 mg salt in 10mL sterile distilled water and stored at 4°C in dark. One percent SDS in 50% methanol was prepared as mentioned in the previous section. Two drops of EB stock solution was added in 1mL suspension culture and incubated at room temperature. After 15 min the samples were washed with distilled water to remove unbound dye. The samples were then solubilized in M-SDS at 60°C for 30 min and repeated three times. The supernatant was collected, pooled, and centrifuged at 1875 rpm for 15 min and then diluted up to 7 mL and OD was quantified at 600 nm by spectrophotometer (UV-4000, Germany).

RESULTS

Establishment of CSC from calluses: Highest (100%) callus induction was obtained at 8 µM or 10 µM 2, 4-D from both cotyledons as well as leaf explant (figure 1A-F).

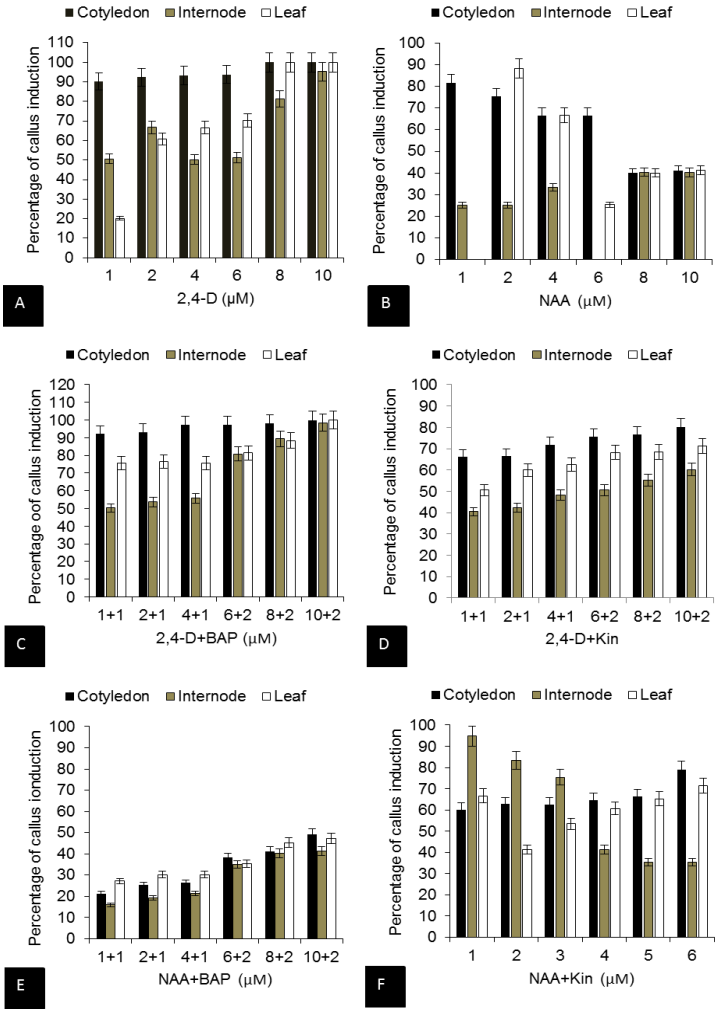

Figure 1: Effect of 36 treatments of the individual as well as the combinations of cytokinins and auxins (A-F) on the formation of callus from explants of jojoba after 40 days of initial culture. Each value is the mean of three replicates of three independent experiments. Bars over the columns are ±SE.

Similarly, cent percent callus induction was also achieved at 10 µM 2, 4-D + 2 µM BAP from the same explants, after 40 days of culture. These treatments were then designated as CL-1, CL-2, and CL-3, respectively. There is a strong linkage between the treatments and callus induction from cotyledon explant (figure 2).

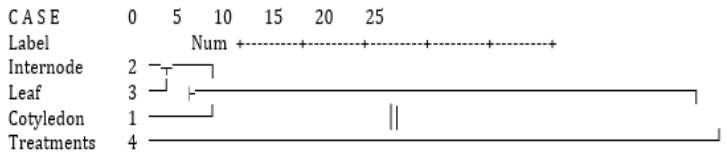

Figure 2: Dendrogram using average linkage (4) of treatments with the callus induction percentage from different explants (1, 2, 3). Rescaled distance cluster combine.

The trend of callus formation was seen towards the combination of 2, 4-D, and NAA whereas the highest and closest in terms of parentage was observed with 2, 4-D, and BAP (figure 3).

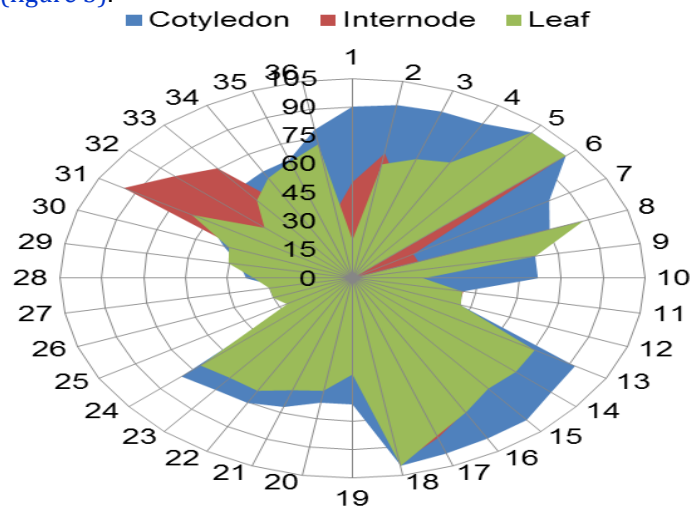

Figure 3: The trend of callus induction percentage as indicated by different colors towards the specific serial number of treatments (1-36) as given in figure 1.

Calluses on CLs were cultured in the respective liquid fresh media and incubated under the same culture room conditions. Suspension culture's growth remarkably changed, oblong, spiral, and small suspension cells were observed on CL-1 after 11, 21, or 38 days (figure 4A,B,C), respectively. Little difference (in terms of elongation and aggregation cell clumps) in cell shapes was recorded with CL-2 after the same culture periods (figure 4D,E,F). The density of CSC was higher indicated a greater number of cells was present on CL-3 (figure 4 G, H,I) as compared to other lines.

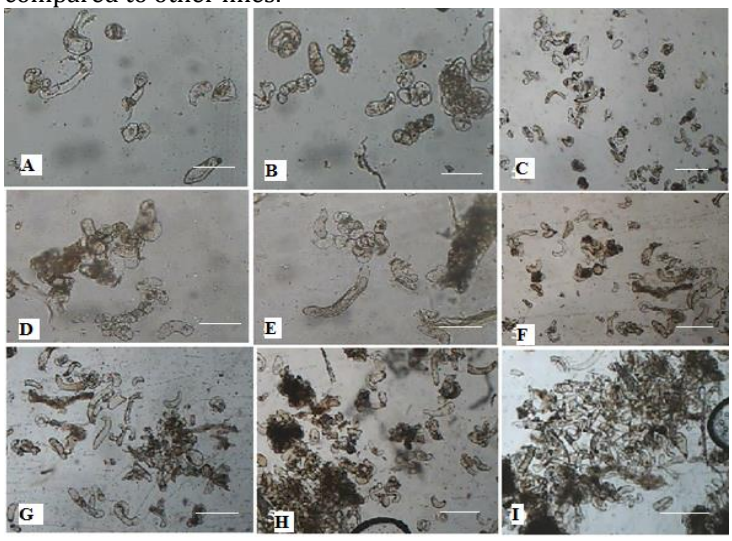

Figure 4: Morphology of 11, 21, and 38 days old cell suspension cultures from friable callus of jojoba. A,B,C) Oblong and small suspension cells were observed on CL-1 (8 µM 2,4-D). D,E,F) Long aggregated cells on CL-2 (2,4-D 10 µM) whereas profusely multiplied cells were detected on CL-3 (2,4-D 10µM+BAP 2µM).

The number of viable cells was significantly improved with the passage of culture time. We obtained the highest 230 number of viable cells on CL-3 after 38 days of culture as compared to 210 and 200 cells after 21 and 11days, respectively (figure 5).

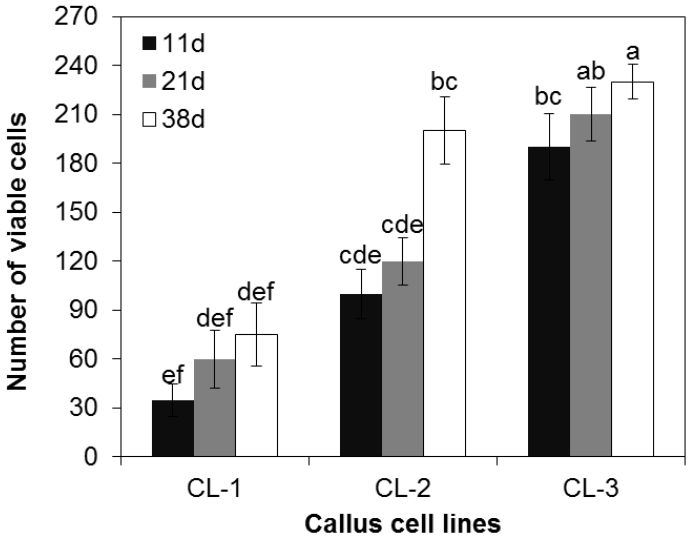

Figure 5: Counting the number of viable cells of jojoba suspension cultures of different lines (CL-1: 2, 4-D 8 µM, CL-2: 2, 4-D 10 µM, CL-3: 2, 4-D 10 µM + 2 µM BAP). Bars indicate ±SE and significantly different results are indicated by different small letters as determined by DMR test p<0.05.

This was followed by CL-1 and CL-2 for the production of cells in suspension. The figure 6 showed absorbance values obtained as the result of EB and TTC after 11, 21, and 38 days. The highest absorbance was obtained with CL-3 by TTC as compared to EB of 21 days old cells. After this period, the number of viable cells was decreased as determined by TTC. Absorbance was recorded lowest with CL-2 throughout the experiment.

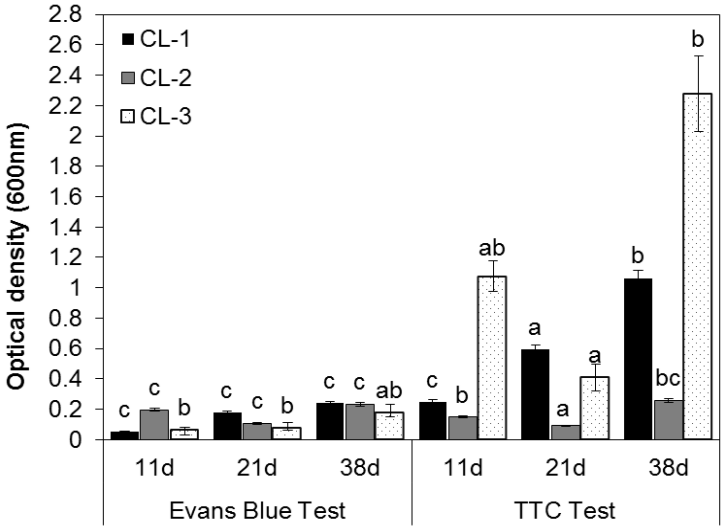

Figure 6: Quantitatively determined cell viability of jojoba suspension cultures. Bars over each column represent the ±SE of the mean. Different letters over vertical bars represent significantly different results as determined by DMR test p<0.05.

Packed cell volume (PCV) was however higher with CL-1 followed by CL-2 and CL-3 after the successive culture periods of 11, 21, or 38 days. The figure 7 showed that PCV was significantly increased with CL-1 with time. The rate of PCV was highest (0.35 mL) with CL-1 of 38-day old incubated cells. It was reduced subsequently with CL-2 (0.10 ml) and further decreased with CL-3.

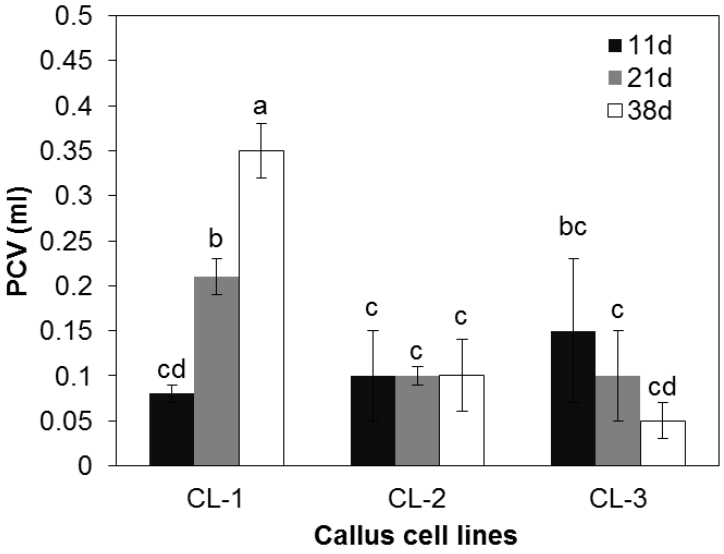

Figure 7: Determination of PCV of jojoba suspension cultures. Bars indicate ±SE and significantly different results are indicated by different small letters as determined by DMR test $p<0.05$. (CL-1: 2, 4-D 8 µM, CL-2: 2, 4-D 10 µM, CL-3: 2, 4-D 10 µM + 2 µM BAP).

DISCUSSION

The callus is an appropriate source for single-cell separation for studying the biochemical and molecular aspects. For this purpose, friable callus is usually selected for the preparation of healthy CSC. In the nutrient-rich medium, medicinal plants in CSC commonly exude metabolites for sustainable growth of cells. Generally, plant growth regulators play a vital role in maintaining suspension cell viability. We observed that a greater number of viable cells was obtained with a higher amount of auxin 2, 4-D with the increase of incubation period with CL-3. In contrast, higher levels of picloram (auxin) seldom support the cell growth and viability in CSC of various plants (Mishiba *et al.*, 2001). Tan *et al.* (2010) reported that cytokinin alone is not enough for viable growth of CSC. This may be improved by using the combination of both auxin and cytokinin as reported in the present study. Other than PGRs, various precursor compounds including biotin improved the growth and suspension cell viability of *Theobroma cacao* (Parra *et al.*, 2017).

The extent of the percolated compounds depends upon the whole biomass production from tissues under investigation. We reported that the rate of PCV was increased from 11 to 38 days in CL-1 as reported in *Stevia rebaudiana* by Dwivedi *et al.* (2016). Moreover, we demonstrated that 8 µM 2, 4-D was good enough for high biomass production. A similar approach has also been adopted by Tan *et al.* (2010) using 2, 4-D. In the present study, with the increasing dose of 2, 4-D from its optimum level (8 µM), PCV decreased in other cell lines containing cytokinin. These results demonstrated that a higher amount of growth regulators is detrimental to producing low PCV biomass. The small amount of 2, 4-D (0.27 µM) + BAP (0.27 µM) + ascorbic acid (0.06 µM) improved PCV by increasing the inoculum density of CSC of *Stevia rebaudiana* (Mathur and Shekhawat, 2013).

In the present study, Evan's blue test did not produce color and was not optimal as compared to TTC. Similarly, Evan's blue was unable to stain the whole viable cells and protoplasts of *Coffea Arabica* cv. Catimor (Silva and Menéndez-Yuffá, 2006). On the other hand, Evan's blue stained the live cells that varied from 94.6% to 87% from the first week to the third week, respectively, indicate that CSC of *Satureja khuzistanica* was successfully established (Sahraroo *et al.*, 2016). Double staining with Evan's blue and acetocarmine was a good indicator for the determination of *Chamaecyparis thyoides* somatic embryos achieved in CSC (Ahn *et al.*, 2017). Relative TTC content in terms of OD was increased by increasing the time duration of CSC in the present study. Our results are in agreement with other reports (Mikuła *et al.*, 2006; Silva and Menéndez-Yuffá, 2006; Silver, 2011). Silva and Menéndez-Yuffá (2006) reported 3-[4,5-Dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was slightly more effective than TTC for the determination of cell color reaction. Fungal chitosan antibacterial activity was efficiently determined by the color reaction of TTC (Silver, 2011). Cryopreserved cell suspension viability of *Gentiana* spp. reflects that TTC was an efficient spectrophotometric reduction method for the determination of shortly incubated plant cells (Mikuła *et al.*, 2006). TTC is an efficient and more reliable method that should be handled carefully for the determination of cell viability (Duncan and Widholm, 2004).

CONCLUSION: In conclusion, the present study demonstrated that CL-3 was the most effective treatment for callus induction from leaf explants as well as good enough for friable callus biomass production. Moreover, the number of viable cells and TTC activity of CSC was highest with CL-3 in older cultures. We recommend the use of TTC for an efficient determination of cellular respiring growth of jojoba suspension cultures. According to our knowledge, this is the first report on CSC in jojoba.

CONFLICT OF INTERESTS: Authors have no conflict of interest

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