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Cell Viability of *Byrsonima intermedia* A Juss Calli

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Abstract: *Byrsonima intermedia* A Juss. is a species with pharmacological properties from the Brazilian Cerrado that shows difficulties related to sexual propagation. Research on cell viability may provide useful information for the selection of cells with embryogenic potential during the callus culture. Within this context, our research is aimed at establishing the cell viability of calli from *Byrsonima intermedia* leaf segments. The calli went through three subculture phases, of 60 days each, in MS medium with 0.09 M sucrose, 0.6% agar, pH 5.8 and 4.52 μM 2,4-D. The calli were stored in dark conditions and samples were collected every 10 days from each subculture for viability tests with fluorescein 3,6-diacetate (FDA) and 2,3,5-triphenyltetrazolium chloride (TTC). The staining methods allowed quantifying cell viability in each subculture. The best results from the FDA tests were obtained at 21, 25 and 29 days for the first, second and third subcultures respectively, with 53.86%, 61.88% and 53.73% viable cells. Regarding the TTC test, the largest absorbance values were obtained at 21, 27 and 28 days for the first, second and third subcultures respectively. Fluorescence and spectrophotometry analyses were efficient for determination of cell viability during callus cultivation period.

Key words: Cell viability, fluorescein 3,6-diacetate, 2,3,5-tripheniltetrazolium chloride, subculture, tissue culture, native plant.

1. Introduction

The use of stains in order to determine cell viability generates important information for the selection of cells with embryogenic potential. Those cells are small, isodiametric and have a dense cytoplasm [1-5].

The stains fluorescein 3,6-diacetate (FDA) and 2,3,5-triphenyltetrazolium chloride (TTC) are widely employed in the determination of cell viability [6-8]. Only live cells can convert non-fluorescent FDA to fluorescein, a fluorescent compound, through the action of an esterase [9]. The main advantage of this method is the speed at which cells can be spotted under fluorescence microscopy. TTC is also a vital dye which is reduced to a deep red color compound, triphenylformazan, or just “Formazan”, after being inserted in the mitochondrial electron transport chain [10]. Formazan can be extracted through ethanol and spectrophotometrically quantified [11]. While FDA is more efficient for cell counts under the microscope, TTC is more efficient in the quantification of cell viability at larger cell aggregates [12] and can be easily applied to wild germoplasm [13].

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Another advantage to this approach is that it only requires small amounts of plant material (callus) for analysis, instead of a classical callus growth curve which needs a large amount of material (as well as being overly destructive), aiming to take fresh as well as dry weights.

The combined use of both techniques, microscopy fluorescence and spetrophotometry, may allow us to develop a simple and practical method for determining cell viability. The identification of the timing and availability of larger cell viability during at the callus phase could turn this approach into a tool that can be used in studies aimed at somatic embryogenesis, making those studies more swift and efficient. Given the problems with the sexual propagation of *Byrsonima intermedia*, tissue culture techniques have been employed aiming at the production of somatic embryos in calli from leaf segments [14-16]. Within this context, our research aimed at employing FDA and TTC in the quantification of the cell viability of *Byrsonima intermedia* calli.

2. Material and Methods

2.1 Plant Material

Byrsonima intermedia leaf segments from *in vitro* germinated seedlings were inoculated in test tubes containing 10 mL of MS culture medium [17] supplemented with 4.52 µM 2,4-D and 0.09 M sucrose. The medium was solidified using agar at 0.6% and pH was corrected to 5.8 before autoclaving the medium at 121 °C for 20 minutes.

The explants were maintained in the dark during 60 days, the period of callus induction. After that period, the calli went through three successive subculture phases: first subculture (SB1), second subculture (SB2) and third subculture (SB3) of 60 days each.

For each subculture, sampling for the viability tests was carried out in a random fashion every 10 days from inoculation. We took between 1.5 and 2.0 g of calli (fresh weight), which comprised the final subculture sample. This final sample was carefully homogenized and the fractions were separated for the cell viability analyses with FDA and TTC.

2.2 FDA Assays

Each sample containing 1 g calli was homogenized with a 10 mL solution of 0.6 M mannitol and 0.03 M $CaCl_2$, pH 5.8 [1] for 30 minutes at 90 rpm, at a temperature of 27 ± 2 °C. We filtered the samples in the absence of light with a 100 µm-pore filter, took out 980 µL of the suspension, and added 20 µL of a 5 µg mL^{-1} FDA solution, producing an end FDA concentration of 0.1 µg mL^{-1}. After 5 minutes we made the cytological preparations and carried out the count of viable embryogenic cells under a fluorescence microscope Olympus BX 60, employing a WIBA filter for that end.

We counted 1,000 cells for each subculture per analysis day, 200 per glass slide. We tallied the percentage of elongated and isodiametric cells during the running of subcultures and plotted the cell viability curve.

2.3 TTC Assays

For each day of analysis, we prepared five samples of 100 mg, homogenized in a test tube with 3mL of TTC reagent at 0.6% (p/v), and prepared in a buffer phosphate solution, pH 7.4. The mixture was incubated for 24 hours, in the dark, at 28 °C. After this period, we added 7 mL ethanol 95% (v/v) to the tube. We extracted the red-colored compound, Formazan, by incubating the tubes in boiling water for four minutes. After the extraction of Formazan, the samples were centrifuged twice at 6,000 rpm for a period of 20 minutes to reach the solid separation phase. The supernatant fraction was set aside for the absorbance readings using a Beckman spectrophotometer, model DU®640B, at 490nm wavelength according to Benson [18].

2.4 Experimental Design and Statistical Analyses

We set the experiment in a completely randomized

block design, using the split plot model, in a factorial scheme (3 subcultures × 7 sampling days). The experimental plot comprised of 75 test tubes for each subculture. The software SISVAR [19] was used for the analysis of variance of the untransformed data. Regression or averages tests (Tukey's test) were applied to the means at a 5% significance level.

3. Results

3.1 Maximum Cell Viability for the Three Subcultures — Fluorescence Assays

The FDA tests showed significant differences among the subcultures (*P = 0.0003*), according to the Tukey's test (Fig. 1).

For the first subculture, the largest percentage of isodiametric cells, 53.86%, was achieved at 25 days. For the second subculture, the largest percentage was obtained at 29 days, with 61.88% viable cells. For the third subculture, 53.73% viable cells were achieved at 21 days. These viability values were obtained through the derivative of the equation for each subculture (Fig. 2).

3.2 Maximum Cell Viability for the Three Subcultures — Absorbance Assays

The TTC assay results showed significant differences among the absorbance values of the three subcultures (*P = 0.0000*), according to the Tukey's test at a 5% nominal significance level (Fig. 3).

Despite the differences regarding maximum absorbance values among the three subcultures, as well as the timing of occurrence of such values, the factor 'sampling day' was significant (*P = 0.0000*) for the subcultures. We show the general response of the sampling day for the three subcultures on Fig. 4. The derivative of the general regression equation for the sampling day indicates that the largest absorbance value (0.5689) was observed at 25 days. These results suggest an intermediary date for the maximum viability values with TTC, in comparison with the data for each subculture (Fig. 5). Knowing the maximum

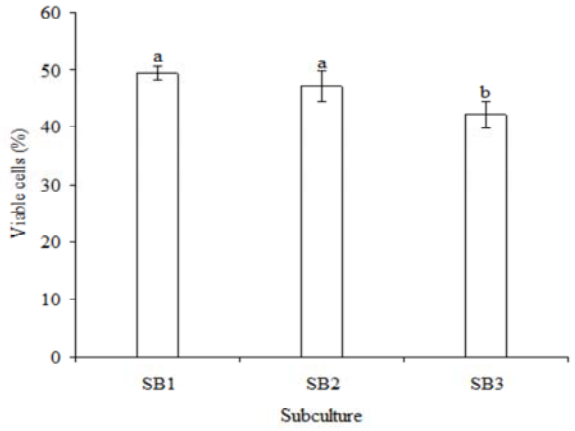

Fig. 1 Overall mean of the number of viable cells (isodiametric), stained with FDA, for the first (SB1), second (SB2) and third (SB3) subcultures.
Bars ±SE with the same letter are not statistically different, according to the Tukey's test at a 5% significance level.

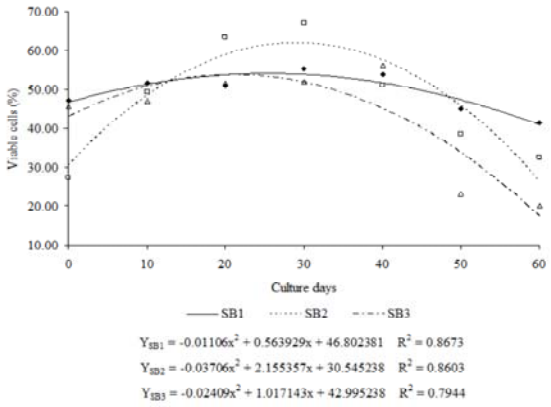

Fig. 2 Cell viability curve according to the FDA test for the first (SB1), second (SB2) and third subculture (SB3).
Each curve point represents the percentage of viable (isodiametric) cells in a sample of 1,000 cells.

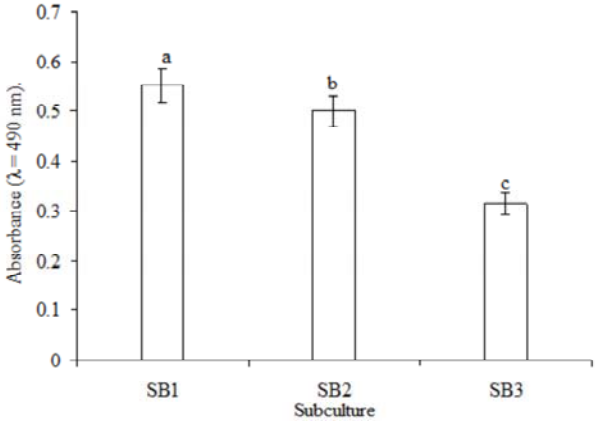

Fig. 3 Overall absorbance mean for the three subcultures (SB1, SB2 and SB3).
Bars ±SE with the same letter are not statistically different, according to the Tukey's test at a 5% significance level.

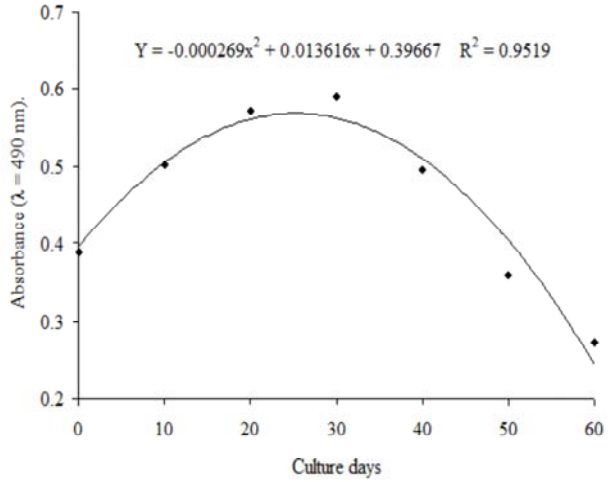

Fig. 4 General regression curve of absorbance values against sampling days for the three subcultures.

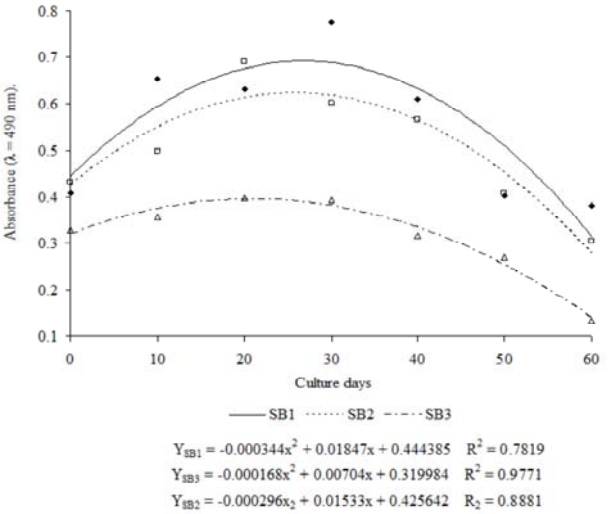

Fig. 5 Cell viability according to TTC test for the first (SB1), second (SB2) and third (SB3) subcultures.

viability values through the TTC test for each subculture is relevant, given it may help in the decision-making process regarding the time length of each subculture.

According to the derivative of the equations for each subculture, we can see that maximum viability occurred at 27, 28 and 21 days, given the maximum absorbance values (0.6924; 0.6190 and 0.3938) for the first, second and third subculture respectively (Fig. 5).

4. Discussion

4.1 Cell Viability through FDA Test

When the FDA molecules, a nonpolar and non-fluorescent substance, contact live cells, they suffer hydrolysis by plasmatic membrane-bound esterases, which remove acetate molecules. Such reaction leads to a build-up of fluorescein, a polar and fluorescent molecule, in the cytoplasm. Given its polarity, fluorescein stays inside the cells, since they cannot go through the plasmatic membrane and, when excited by a light source, they emit a green fluorescent light. Such feature allowed us to spot and counting viable and non-viable cells under a fluorescence microscope in a swift and reliable fashion (Fig. 6). Along these lines, elongated cells (Fig. 6a), although alive, were counted as non-viable, and isodiametric cells (Fig. 6b) as viable for embryonic development.

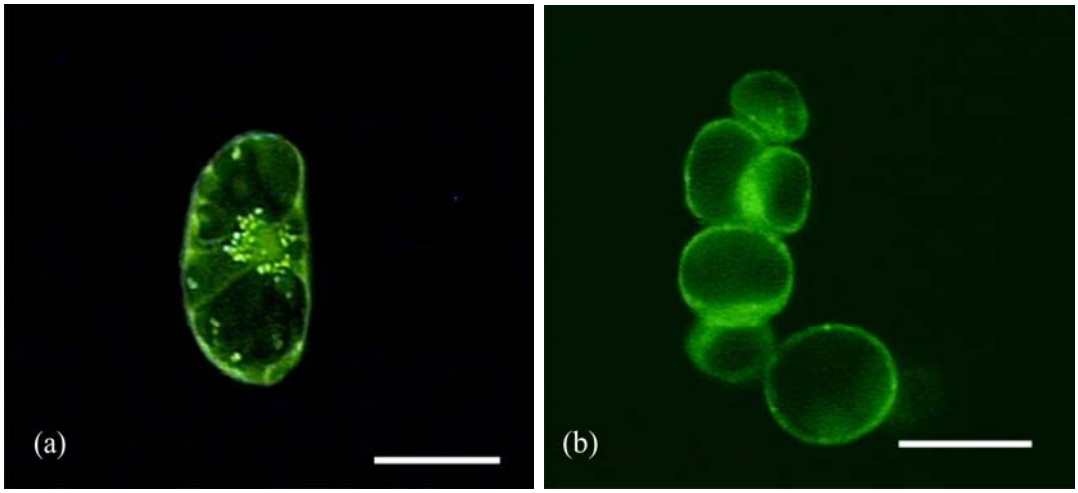

Fig. 6 *Byrsonima intermedia* callus cells stained with FDA as seen under the fluorescence microscope. (A) Elongated cell in a 20 × magnification (bar = 20 µm). (B) Isodiametric cells in a 10 × magnification (bar = 50 µm).

The use of FDA as a tool to measure cell viability has been reported by several authors for different purposes: *Picea abies* protoplasts [1], leaf alfafa protoplasts [2], *Arabidopsis thaliana* protoplasts [20], tomato cell suspension [21], *Coffea arabica* cv Catimor protoplast and cell suspensions [22], *Arabidopsis thaliana* programmed cell death [23], integrity of membranes as a measure cell viability of *Vitis vinifera* during the development of monocarpic tissues in the fruit formation [24], maize root cells [25], *Arabidopis* root cells [8], *Musa spp* protoplasts [26], bamboo protoplasts [27], rice leaf sheath sections [7], bacterial viability [28]. In our experiments, FDA was successfully applied to the callus culture and demonstrated a good ability to detect maximum cell viability. Even when compared to the *Bysonima intermedia* callus growth curve [29], strong similarities were observed, over time, with the higher cell viability using FDA and the growth phase (exponential) supposed to be the most active in metabolism as well as in cell division.

4.2 Cell Viability Employing TTC

The use of TTC tests as a viability measure has also been reported by several authors: defrosting effect on cryopreserved *Solanum tuberosum* cells [30], embryogenic cultures of several cryopreserved plant species [31], *Cyclamen persicum* pro-embryogenic masses [32], *Agrostis scabra* roots submitted to different temperature stress [33], *Carica papaya* pollen viability [34], *Brassica napus* seedling [35], seedling thermotolerance among 30 cultivars of wheat [36], heat tolerance mechanisms in wheat operating at cellular level [13], *Zea mays* callus culture [37], somatic embryos after cryopreservation [38] and bacterial viability [28].

TTC is a water-soluble substance that is converted into Formazan when it goes through the mitochondrial electron transport chain [10]. Formazan is insoluble in water, but it is soluble in ethanol [11]. The TTC test, therefore, not only detects the presence of live cells, but indicates their respiratory metabolism status as well. The higher the number of live cells and the larger their metabolic activity, the larger the amount of Formazan produced. Therefore, this test also shows the degree of tissue viability [39]. Since dead cells do not convert TTC into Formazan, this is a reliable test to measure cell viability. This test provide us important information about the respiratory metabolism during the callus culture along the three subcultures (Figs. 3-5)

4.3 First Subculture (SB1)

The FDA and TTC tests demonstrated that the maximum viability for the first subculture took place on the 25th and 27th day, showing 53.86% of viable cells and 0.6924 of maximum absorbance value respectively. This culture period corresponds to the exponential phase of the growth curve of *B. intermedia* calli [29]. This phase is characterized by rapid cell division [40, 41]. Differently from the second and third subcultures, the largest number of viable cells stained with FDA took place two days earlier than the maximum cell viability as indicated by the TTC test.

The fact above may indicate that the induction period, when calogenesis was stimulated in leaf explants during 60 days before the start of the first subculture, may have been too long. Such an extended induction period may have triggered an increase of the lag phase in this first subculture. The lag phase is distinguished by the lack of cell divisions, acquisition of competence for the start of a new division cycle, as well as a significant increase of cell metabolism [29, 40, 41]. Such features of the lag-phase, when considered in conjunction with an extension of its time length, could explain the fact that the first subculture was the one which showed the highest viability via the TTC test, and this viability took place after the highest viability day as shown by the FDA test.

The auxin factor may also bear a relation to the results above, since the subculture period is long (60

days), and auxins rapidly degrade in the culture medium [2, 42]. Although the 2,4-D growth regulator has great stability in the culture medium, and it does not suffer oxidation, its conjugation and degradation rate may be quite high when absorbed by a tissue [42].

The fact that the cell viability measured through the FDA test starts its decline earlier in this subculture reinforces the idea that the callus induction period was too long, something that suggests that cell viability assays must also be carried out during the induction phase of such process.

4.4 Second Subculture (SB2)

For the second subculture, the results point that the largest viability indexes by the FDA and TTC tests took place on the 29th and 28th culture days, showing 61.88% of viable cells and a maximum absorbance value of 0.6190, respectively. Although the absorbance value is 10.61% smaller when compared to the first subculture the maximum viability period was very close between the two tests. Besides, the cell viability measured by the FDA test is 12.96% larger in relation to the first subculture.

Both viability measures are comprised near the median region of the exponential phase of callus growth [29]. Such phase is distinguished by rapid cell division [40, 41], that may explain the largest percent of viable cells stained by FDA taking place soon after the day with largest viability shown by the TTC test.

4.5 Third Subculture (SB3)

For the third subculture, the largest viability indexes by the FDA and TTC tests took place at 21 days, at the start of the exponential phase and at the end of the lag phase, showing 53.73% of viable cells stained with FDA and a maximum absorbance value of 0.3938, respectively. In this subculture, the largest viability measured through the FDA test is 13.17% smaller in relation to the second subculture. Regarding the TTC test, there was a reduction in cell viability of 36.38% and 43.13% in comparison to the second and first subcultures respectively.

The fact that this subculture showed the lowest viability as measured by the TTC test, mostly during the interval between the end of the lag phase, when cell metabolism should be higher, and the start of the exponential phase [29], when cell divisions start, reveals a clear reduction in the number of viable cells, as well as in the respiratory metabolism probably caused by cell differentiation and death [1].

5. Conclusions

Fluorescence and spectrophotometry analyses are efficient for the determination of cell viability during callus cultivation periods.

Respiratory metabolisms increase with successive subcultures, leading to higher cell differentiation as well as cell death, according to an analysis using 2,3,5-triphenyltetrazolium chloride.

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