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Characterization of Pro-embryogenic Calli and Somatic Embryogenesis of *Byrsonima intermedia* A. Juss.

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Abstract: *Byrsonima intermedia* A. Juss. is a species from the Brazilian Cerrado that produces edible fruits and, in common with other species from the *Byrsonima* genus, has pharmacological potential. Previous attempts to propagate the species through conventional methods showed difficulties. Thus, the purpose of this work was to characterize pro-embryogenic masses of *Byrsonima intermedia* callus, aiming for their *in vitro* propagation through somatic embryogenesis. Leaf segments from *in vitro* germinated seedlings were employed as explants for callus production. The calli were then subcultured and exposed to dyes to fulfill their embryogenic potential. Digitalizations of the cytological preparations were made in order to measure the area that was stained by both Aceto-Carmine and Evans-Blue, using image tool software. Somatic embryos were induced after treatments with 1-naphthaleneacetic acid (NAA). The percentages of double-colored areas (by Aceto-Carmine and Evans-Blue) were calculated and the data were analyzed by using the Skott-Knott test ($P \leq 0.05$) and, the embryogenic callus, as well as the formation of somatic embryos were analyzed by using the Krsuskal-Wallis rank test ($P \leq 0.05$). The results show that double coloration is effective at identifying cells showing embryogenic potential. Early callus subculture phases show a larger percentage of embryogenic area (83%). Somatic embryos were induced by using high auxin level.

Key words: Double staining, aceto-carmine, evans-blue, somatic embryogenesis, native plant, auxin.

1. Introduction

Byrsonima intermedia A. Juss is a medicinal species that produces edible fruits, native to the Brazilian Cerrado, a highly endangered biome due to anthropic activities [1-3]. The species shows germination problems and has a slow emergence of its seedlings [4, 5].

Given the difficulty with the sexual propagation of species, the production of *in vitro* seedlings is an option, both for repopulating the flora in affected areas as well as for medicinal use [6-11]. In order to achieve propagation of native species, researchers have been employing the technique of somatic embryogenesis with a large number of species, and such propagation can be obtained either in a direct or indirect way, the former not requiring a callus production phase [12-14].

Determining the embryogenic potential of callus cultures of a given species during *in vitro* culture can provide important information to research projects aimed at achieving somatic embryogenesis.

The use of staining techniques can make the differentiation between cells with embryogenic potential and non-viable ones easier, with the help of dyes such as Aceto-Carmine and Evans-Blue [15].

Auxins are involved in somatic embryo induction and initiation. It has been suggested that auxin is necessary for embryogenic aggregate formation from

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single cells expressing totipotency [16]. In many species, cultivation of explants in a medium with a relatively high auxin concentration may trigger the process of initiating embryogenesis. The subsequent development of somatic embryos occurs after the transfer of callus to a culture medium with low concentration of auxin, or in its absence [17-19], because after initiation, auxins inhibit embryo development [20].

In this context, the purpose of the work described here was to induce and characterize the pro-embryogenic masses by employing a double staining approach and to induce somatic embryos from callus of *Byrsonima intermedia*.

2. Materials and Methods

2.1 Callus Induction

According to protocol developed by Nogueira et al. [21], the authors used 0.25 cm^2 leaf segments from *in vitro* germinated *B. intermedia* seedlings as the starting explants for embryogenic callus induction. Explants were inoculated in test tubes containing 10 mL MS culture medium [22], supplemented with 4.52 μM 2, 4-D and 0.09 M sucrose. The medium was solidified by using agar at 0.6% and pH was corrected to 5.8 before autoclaving the medium at 121 °C during 20 min.

Prior to inoculation, cuts were performed in the abaxial side of the explants, the side in direct contact with the culture medium. Explants were incubated in the dark for 60 days, at a temperature of 25 ± 2 °C, the period of callus induction.

At the end of the induction period, every 60 days according to the growth curve described by Nogueira et al. [5], calli were transferred to a fresh culture medium identical to the one used for callus induction. This procedure was repeated three times, thus defining the three subcultures (SB1, SB2 and SB3), of 60 days each, which were regarded as the treatments.

2.2 Staining Analyses

At the end of each subculture, a 50 mg sample of fresh tissue from the callus was collected and lightly macerated with the help of a glass rod on top of a laboratory watch glass. After maceration, three drops of the Evans-Blue (EB) dye at 0.1% were added, and left to react for three minutes. After that period, the excess of dye was removed and three drops of Aceto-Carmine (AC) dye at 2% was added, and left to react for another three minutes [23, 24]. Lastly, the excess of dye was removed and the cell mass was spread on a glass slide and analyzed under a bright field microscope (Olimpus BX 60). Five glass slides for each of the three subcultures were prepared and, for each slide, five digitalizations were made. The pro-embryogenic masses were analyzed with the help of a light microscope under 10 and 40 × magnification levels.

The best fields were digitalized and the size of the stained areas was measured with the aid of the Image Tool software. Calibration was carried out by using a digitalization of a micrometric-marked glass slide provided by the equipment manufacturer at the same magnification levels used in the photographs [25]. The whole area was stained with AC and the area stained with EB was measured. From these values, the authors calculated the percentage of the area stained with each dye for subsequent statistical analyses.

2.3 Development of Embryogenic Callus

Calli from the third subculture (SB3), after 60 days under dark, were transferred to a MS inductive medium (MSI) with 1-Naphthaleneacetic acid (NAA) in high concentration (265.52 and 537.06 μM) or without NAA (control). The media was supplemented with 0.09 M sucrose, 0.1% activated charcoal and solidified with 0.7% agar. The pH was adjusted to 5.8 prior to sterilization at 121 °C for 20 min. After inoculation calli were maintained in a growth room chamber for 60 days at 25 ± 2 °C, under 35 μmol m^{-2} s^{-1} irradiance and 16 hours photoperiod.

After NAA treatments, the pro-embryonic calli were transferred to MS expression medium (MSE), growth regulator free, with 0.09 M sucrose, 0.7% agar, 0.1% activated charcoal, pH 5.8. The pro-embryogenic calli were maintained in a growth room chamber for 60 days at 25 ± 2 °C, under 35 μmol m^{-2} s^{-1} irradiance and for a 16 h photoperiod. After 60 days the formation of embryonic callus as well as somatic embryos were evaluated.

2.4 Experimental Design and Statistical Analyses

The experiment was set in a completely randomized design. For the analysis of stained areas, 25 replicates for each treatment were used. For somatic embryo induction, 10 replicates per treatment were used. The free statistical software SISVAR [26] was employed for the analysis of variance of the untransformed data on callus double staining and the averages were compared through the Skott-Knott test. For the evaluation of embryo formation, the free statistical program [27] was used and the averages were analyzed through the Kruskal-Wallis test.

3. Results and Discussion

3.1 Double Staining with Aceto-Carmine and Evans-Blue

It was observed that the AC dye reacted strongly with the first subculture (SB1) with 83% of the cells stained in red (Table 1). In this subculture, there was a preponderance of small isodiametric cells (Fig. 1a). Small clusters of isodiametric cells were also detected in this subculture, thus reinforcing its embryogenic potential (Fig. 1b).

Meristematic cells have an isodiametric shape and embryogenic callus is mostly made up by cells with meristmatic features, with relatively small sizes and dense cytoplasm [13, 15, 22, 28]. It should be emphasized that it was possible to note the start of the production of globular structures by the end of the first subculture period (Fig. 1c).

The second subculture (SB2) showed 69% of its cells stained with AC (Table 1). In this subculture, there was an increase in reaction with EB, which that can be seen at the borders of the pro-embryogenic masses (Fig. 1d). In this subculture, the presence of small clusters of isodiametric cells was noted and also that of elongated cells which reacted to the EB dye, (Fig. 1e), with overall staining at 31% (Table 1). Small embryos at a globular stage were also produced at the surface of the callus at the end of the 60 culture days (Fig. 1f).

The authors observed a trend of increased staining by EB in the second subculture, which was even clearer in the third subculture, which showed 68% reaction to this dye (Table 1). In this subculture, there was a predominance of blue-stained cells, pointing at a cell redifferentiation from the callus (Fig. 1g). High production of globular embryos was observed at the callus surfaces at the end of the culture period (Fig. 1h).

According to Steiner et al. [15], the use of AC and EB makes it possible to discriminate embryogenic cells. Therefore, the test for the identification of pro-embryogenic masses provides important information about the quality of these cultures. The pro-embryogenic masses basically show two cell types. The first group is made up of small isodiametric cells that have a dense cytoplasm. Such cells are reactive to the AC dye, producing a reddish color. The other group is made up by non-embryonic cells, which

Table 1 Percentage of areas stained with Aceto-Carmine (AC) and Evans-Blue (EB) in *Byrsonima intermedia* callus cultivated in MS medium supplemented with 4.52 μM of 2, 4-D.

Subculture	AC (%)*	EB (%)*
SB1	83 a	17 c
SB2	69 b	31 b
SB3	32 c	68 a

* Means followed by the same letter in the columns are not statistically different according to the Scott-Knott test ($P \leq 0.05$).

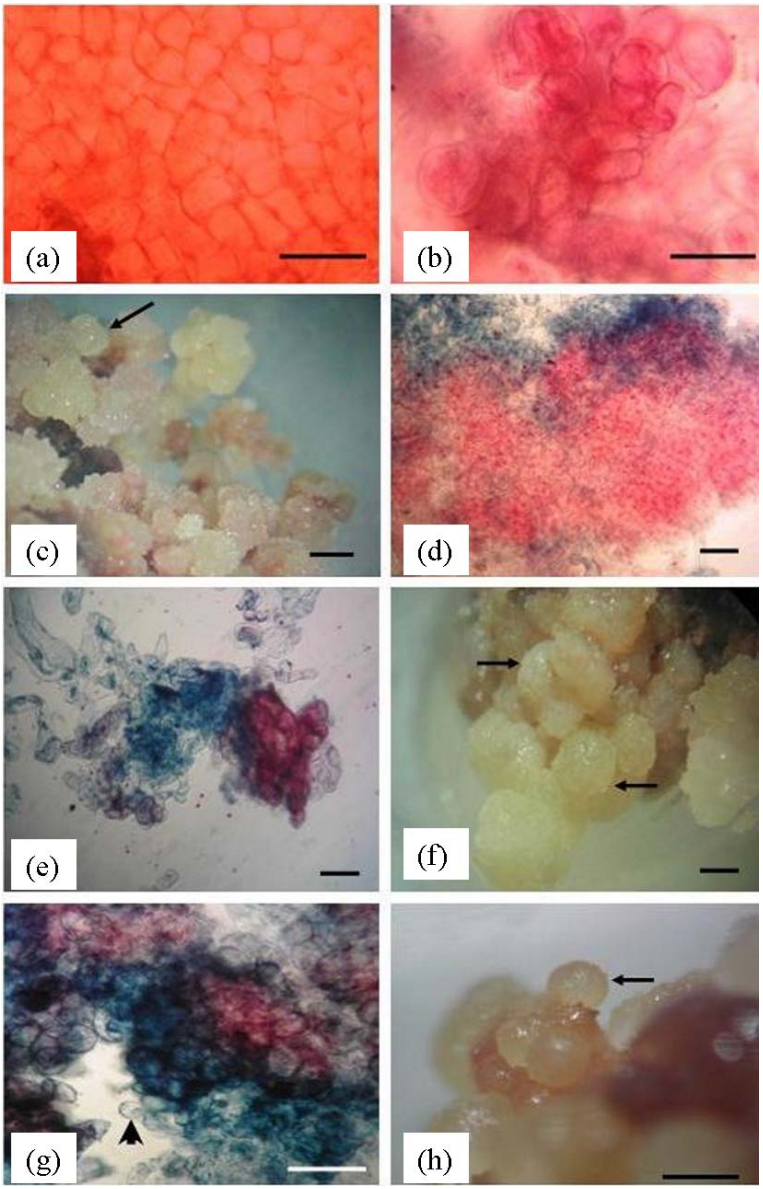

Fig. 1 Cell masses from the first (a, b, c), second (d, f) and third (g and h) subcultures. Cells heavily stained with Aceto-Carmine (a, b). (a) Isodiametric cells at a 40× magnification; (b) Production of clusters of isodiametric cells at a 40 × magnification; (c) Production of globular structures at the callus surface at the end of the 60-day period (arrow); (d) Cell mass reacting both to Aceto-Carmine and Evans-Blue at a 10 × magnification; (e) Strong reaction to Evans-Blue with very elongated cells which were reactive to Evans-Blue at a 20 × magnification; (f) Large production of globular structures at the callus surface at the end of the 60 days (arrow); (g) Isodiametric cells reacting strongly to the Evans-Blue dye (arrow), photograph at a 20 × magnification; (h) Large production of embryos at a late globular stage at the callus surface at the end of the 60 days (arrow). Bar: a and b = 50 µm; c = 15 mm; d = 100 µm; e = 50 µm; f = 2.5 mm; g = 100 µm; h = 5 mm.

are elongated, vacuolated and permeable to EB, thus producing a bluish color.

The viability of *Coffea arabica* cell suspension cultures was determined by Gatica-Arias et al. [29] using EB. The non-viable cells showed a deep blue coloration, while the viable cells had an isodiametric shape, showing prominent nucleus and nucleolus, a dense cytoplasm and lack of coloration from this dye.

The positive reaction to the AC may show chromosomal integrity [30], and cells with dense cytoplasm are considered meristematic, something that reinforces the theory of chromosomal integrity.

These cells have the potential for embryogenic development [15], which would explain the strong reaction in the first subculture (Figs. 1a and 1b).

The results showed that, as the calli were progressively subcultured, there was a declining reaction to AC, and at the same time that the reaction to EB increased. According to Hetherington and Fry [31] and Bhargava et al. [32], the reaction to this dye takes place on those cells which have sustained damage to their cell membrane. Elongated cells reacted more strongly to this dye (Fig. 1e). Highly vacuolated cells are also elongated, and that vacuolization is the first sign of cell death [33]. Cells which are going through the process of cell death do show ruptures at their plasmatic membranes, which then allows staining with EB [32, 33].

In the third subculture, it was observed that even isodiametric cells showed reaction to EB (Fig. 1g). This reaction was the opposite of the one in the first subculture, where this cell type strongly reacted to AC. The smallest reaction percentage to AC was observed in this subculture where 32% cells were stained with a red color (Table 1).

According to George et al. [13], an auxin supply is needed for the multiplication of pro-embryogenic masses. If the auxin source is reduced or completely eliminated from the culture after an embryogenesis inducing period, the embryos may then start their development. Auxin controls the basic process of cell elongation and division [13, 34, 35]. The production of globular embryos on the callus surfaces may be related to the decrease in the activity of the 2, 4-D auxin, thus making the occurrence of cell polarization possible. Globular embryos were produced in the three subcultures, but more strongly so in the third one (Fig. 1h). An increased formation of embryogenic structures on the third subculture of callus was observed by Nogueira et al. [36] on the *B. intermedia* callus cultivated on MS medium with 4.52 μM 2, 4-D through ultrastructural analysis, agreeing with the results found in this present work.

The fact that isodiametric cells also showed blue staining may also be related to the auxin factor, not only through a reduction of its activity during the subculture, but also because of the subculture process in itself. According to Smertenko et al. [37], the decrease in growth regulator levels also leads to a process of degradation of pro-embryogenic masses.

This kind of reaction may indicate that continuing to subculture *B. intermedia* pro-embryogenic masses up to a third subculture may promote the increase of cell death, possibly of programmed cell death. Programmed cell death commonly occurs during embryonary development [33, 38]. The above mentioned reaction may also point to a viability reduction due to the successive subcultures, leading even to the death of isodiametric cells with embryogenic potential.

3.2 Embryogenic Callus and Somatic Embryos Induction

During the induction of pro-embryogenic callus, cultivated on MSI media with high NAA concentration for 60 days under light, the authors did not observe the formation of later stage somatic embryos. However, after one passage through MSE media, growth regulator free, with light for 60 days, the authors observed the formation of somatic embryos (Fig. 2) as well as embryogenic callus. The use of high NAA concentrations on MSI media were statistically significant (P = 0.03987) according to the Kruskal-Wallis test for the formation of somatic embryos on MSE media. The highest percentage of embryo formation (30%), corresponding to a rank of 18.5, was observed at 537.06 μM of NAA concentration. No embryos were formed when MSI was used with the NAA concentration at 0 or 268.52 μM (Fig. 2).

The embryogenic callus formed on MSE media showed a friable structure, yellowish white in color, with small darkened areas (Fig. 3a). They also had small nodular structures, identified as somatic embryos in the initial stage of development (Fig. 3a).

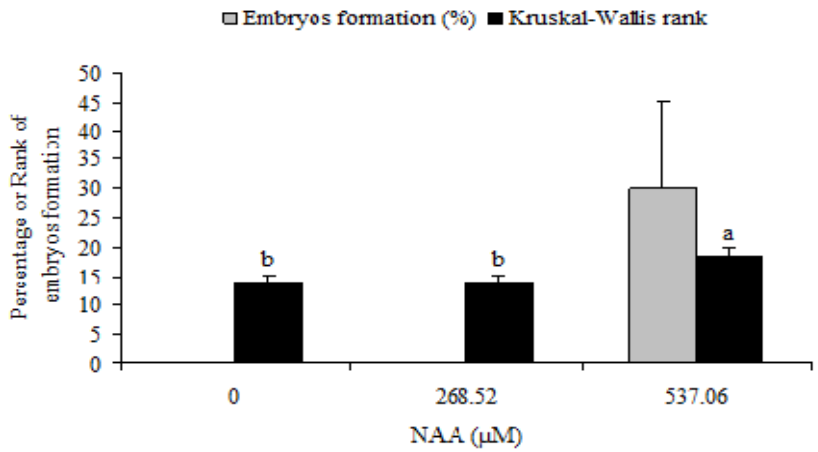

Fig. 2 ***Byrsonima intermedia*** **somatic embryo formations in a MS expression medium (MSE), growth regulator free, after 60 days inoculation. Callus were previously cultivated in a MS inductive medium (MSI) with high NAA concentrations or without that auxin (control) for 60 days. For both, the media were supplemented with 0.09 M sucrose, 0.7% agar, pH 5.8 and 0.1% activated charcoal and the calli were maintained at 25 ± 2 °C, under 35 µmol m^{-2} s^{-1} irradiance and 16 h photoperiod. Means (± SE) followed by the same letter do not differ according to Kruskal-Wallis test ($P \leq 0.05$).**

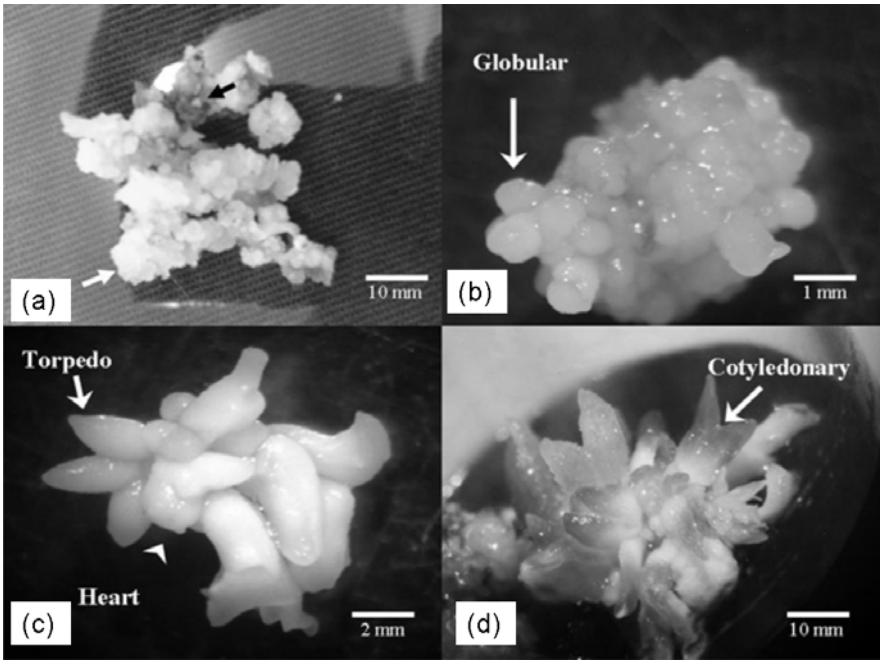

Fig. 3 ***Byrsonima intermedia*** **embryogenesis cultured on MS expression media (MSE), 60 days after inoculation. Somatic embryogenesis was induced through calli cultivation on MS inductive media (MSI) with high concentrations of NAA (537.06 µM). (a) Embryogenic callus showing darkened areas (black arrow) and small nodular structures (white arrow); (b, c, d) Somatic embryos at different development stages: (b) Globular; (c) Heart (arrowhead) and torpedo (arrow); (d) Cotyledonary.**

These characteristics were also observed in other species such as *Avena sativa* [39], *Pfaffia tuberosa* [40] and *Zea mays* [41], with only minor differences in color from white to yellow. Nodules found on the calli of *Hevea brasiliensis* could be identified as embryogenic units that developed up to the pro-globular embryos stage [42]. In our study, the different NAA concentrations used on MSI media, or its absence, was not statistically significant (P = 0.1971) according to the Kruskal-Wallis test,

considering the embryogenic callus formation.

We found different developmental stages of somatic embryos as globular (Fig. 3b), heart and torpedo (Fig. 3c) and cotyledons (Fig. 3d) at the end of 60 days of culture on MSE media. According to Gray [43], somatic embryos formed from pro-embryonic complexes tend to develop asynchronously. Our results demonstrate that the use of NAA on MSI media favors somatic embryo development (Figs. 2, 3c). This result is in agreement with Zimmerman [44], who reported that NAA is one of the most effective auxins for somatic embryogenesis induction. The same was observed by Flores et al. [40] working with *Pfaffia tuberousa* who found a positive NAA effect on nodular embryogenic callus induction from root explants.

The auxins, including the NAA, for the most models of embryogenesis induced *in vitro*, are regarded as the substances responsible for triggering the processes of dedifferentiation (indirect model) by changing the determination and granting new skills to the responsive cells present in the explants [45].

The effect of auxins on somatic embryo development is primarily inhibitory, and manifests itself following the globular stage [18]. In the presence of auxin 2, 4-D, somatic embryos only develops to the pro-embryo stage [13]. The later development stages as globular, heart and cotyledon could only occur in the auxin-free culture medium. The transfer of embryogenic cultures to a medium without growth regulators is typically required for somatic embryo production [46] and such a transfer is essential for the formation of somatic embryos in our study.

4. Conclusions

Double coloration with Aceto-Carmine and Evans-Blue is effective in the identification of cells with embryogenic potential in different subcultures of *Byrsonima intermedia* callus.

The use of the auxin NAA at a high concentration was efficient to promote the somatic embryogenesis in callus obtained from leaf of *in vitro Byrsonima intermedia* plantlets.

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