Original Research Article

Influence of 2,4-D growth regulator and foliar explant source on the *Coffea arabica* embriogenic callus

ABSTRACT

Aims: In order to obtain more productive and resistant Coffea arabica varieties researchers in Brazil have been conducting experiments on elite plant propagation through in vitro techniques, such as somatic embryogenesis. This large-scale multiplication method presents a great potential for exploration, which would enable coffee propagation maximization. The aim of the current work was to compare the effect of different 2,4-D concentrations present in two pre-established methodologies for obtaining embryogenic callus of Coffea arabica, and to evaluate whether the foliar explant source can interfere on somatic embryogenesis.

Place and Duration of Study: The experiments were conducted in the Central Laboratory of Molecular Biology at Federal University of Lavras, (Brazil), between 2016 and 2017.

Methodology: The callus of C. arabica cv. Catiguá MG2 obtained from two renown methodologies and from two explant sources: seedling leaves cultivated both in vitro and inside greenhouse. Potentially embryogenic callus quality was verified under light microscopic morphological analysis whereas the quantity was determined by variance analysis.

Results: After five months culturing, all treatments presented potentially embryogenic callus. These callus were histologically characterized by having a homogeneous tissue, which consisted of small isodiametric cells and aggregates displaying a dense cytoplasm and clear nucleus. Moreover, small and numerous starch grains were noted.

Conclusion: The in vitro grown plant leaf explants showed a higher potentially embryogenic callus percentage whenever inoculated in a culture medium containing a lower 2,4-D concentration. Morphological characteristics may be used as structural markers to select structural embryogenic cultures.

KEY WORDS*: histological analysis, morphological characterization, somatic embryogenic*

INTRODUCTION

Global coffee output for 2017/18 is preliminarily estimated at 158.78 million bags, 0.7% higher than last year. Arabica production is provisionally estimated at 97.32 million bags, 1.1% lower than last year due to an expected reduction in output from Colombia and Brazil [1]. Moreover, Coffea arabica produces the best beverage with the least caffeine content, although it is very susceptible to diseases such as rust (*Hemileia vastatrix*) and antracnosis of unripened beans (*Colletotrichum kahawaen*) [2]. This predicament has stimulated genetic transformation [3](Etienne *et al*, 2002), by enabling the development of cultivars, which are better adapted to the various stresses and agronomic interest [4]. However, the spreading of a resistant plant material depends on the clonal propagation efficiency as well as *in vitro* regeneration [5].

One of the *in vitro* propagation possibilities would be the indirect somatic embryogenesis where the embryos grow from cells known as callus, which originates from explant cell dedifferentiation. Such process depends on the use of growth regulators to induce tissue differentiation and embryogenic callus formation [6]. Indirect response to

growth regulators action requires a high auxin/cytokinin ratio for the non-differentiated callus formation under an initial medium culture and a low ratio in order to induce embryogenic callus during succeeding cultivations [7].

Somatic embryogenic in *Coffea* displays distinct callus during the induction phase. The primary callus nodules refer to compact globular formations, which occur less frequently in most explant edges. Embryogenic callus are those characterized by being granular cell clusters and are easily distinguished with a creamy-yellowish color [8].

One of coffee research focuses is determining a protocol that enables commercially *in vitro* plant propagation of *C. arabica*. Even though the plant regeneration technic by somatic embryogenesis is well established for some coffee varieties [9], it is important to conduct thorough analysis of current protocols, which may help to differentiate the efficiency in obtaining potentially embryogenic callus for each cultivar. Histological analysis made under photonic and electronic microscopy help distinguish differentiating characteristics among cells as well as to infer about the embryogenic potential of the plant material.

The aim of the current work was to compare the effect of different 2,4-D concentrations present in two pre-established methodologies for obtaining embryogenic callus of *Coffea arabica,* and to evaluate whether the foliar explant source can interfere on somatic embryogenesis.

2. MATERIAL AND METHOD

2.1 Disinfestation and Inoculation

The source of explants used were that of leaves of plants cultivated *in vitro* as well as inside greenhouses. Leaves of *Coffea arabica* cv. Catiguá MG2 were collected early in the morning and taken for disinfection under a laminar flow hood. Disinfection consisted of a 70% alcohol treatment for 2 to 3 minutes. These leaves were then treated with 2.4 % sodium hypochlorite for 10 minutes, followed by 3 washes in sterile distilled water. After the disinfection, a scalpel was used to cut the leaves into 0.5 cm^2 segments that were inoculated into Petri dishes containing gel culture medium. The leaf segments were placed with their adaxial portion onto the medium. The plates were then kept in dark at 24 to 27 ^oC.

For *in vitro* cultivated plants, the leaves did not undergo disinfection but followed all of the other phases mentioned before.

2.2 Culture medium for callus induction

In order to evaluate the effect of different 2,4-D concentrations on the formation of embryogenic callus, a culture media compositions described by Boxtel and Berthouly [10] and Teixeira [11] were used. On the protocol published by Boxtel and Berthouly, the leaf segments were initially cultivated on a primary callus-inducing medium, which contained 2.3 µM of 2,4-D. One month later, the explants were transferred to an embryogenic callus-inducing medium having 4.5 µM of 2,4-D. Following the protocol published by Teixeira, the leaf segments were also initially cultivated on the primary callus-inducing medium, but with 20 µM of 2,4-D. One month later, the explants were transferred to an embryogenic callus-inducing medium having 10 µM of 2,4-D.

2.3 Statistical analysis

The cultivation of leaf explants taken from seedlings in both *in vitro* (inv) and in greenhouse (cv) environment associated with the methodologies proposed by Boxtel and Berthouly (BB) and Teixeira (T) resulted into four treatments, namely: BBinv, BBcv, Tinv and Tcv. These treatments were analyzed every month for six months based on two parameters: primary callus formation and embryogenic callus number obtained. Callus growth was evaluated according to the percentage area it covered on the explant leaf surface as grades 1, 2, 3 and 4, which respectively corresponded to 25, 50, 75, and 100%.

An entirely randomized experimental design was adopted, with five repetitions per plate and eight plates per treatment. The data underwent variance analysis by applying SISVAR software [12], and the means were compared by the Tukey test (P = .05).

2.4 Histological analysis

The embryogenic callus samples were fixed in FAA 50% for 48 hours in room temperature, then separately dehydrated in a graded ethylic series (60, 70, 80, 90 and 100%) during 1 hour. Next, the samples were infiltrated for 24 hours with 1:1 epoxi resin (Historesin® Leica) and ethanol solution, then 24 later they were infiltrated in pure resin. After infiltration, the samples were embedded and cut 3µm thick each by employing a manual rotatory mycroton and stained with a 0,05% toluidine blue and a lugol solution then observed under a light microscope (Zeiss, Axio Scope).

3. RESULTS AND DISCUSSION

3.1 Callus evaluation

At the end of the first month, primary callus formation was observed for all treatments with intense cellular proliferation on the explants edges.

During a four-month period, all four treatments underwent comparative analysis for each monthly cultivation, regarding primary callus growth time, as a function of the explant leaf area covered by the callus. The analysis showed that the BBcv treatment presented primary callus having the most significant growth. Tiv and BBinv treatments showed the least significant primary callus growth (Figure 1).

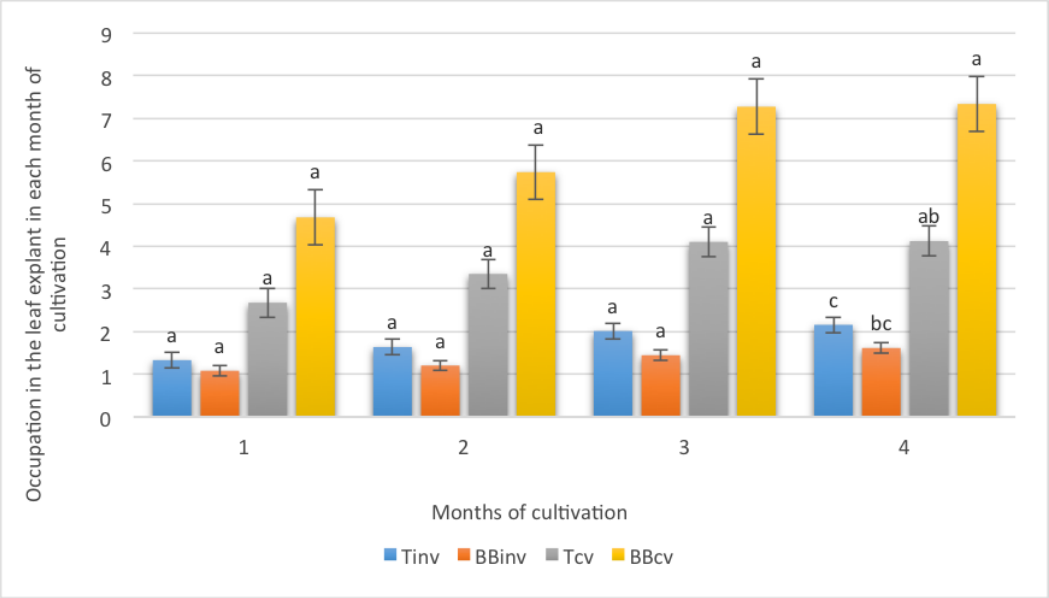

Figure 1 – The assessment of foliar primary growth callus occupation by comparing each treatment during four months cultivation.

After a four-month period of cultivation in an embryogenic callus induction medium it was possible to observe, under stereoscopic microscopy, the first yellow region formation over primary callus, hence, corresponding to the beginning of the embryogenic sector formation. Four months later, it was possible to observe through the naked eye, the growth of embryogenic sectors having 1 to 3 mm in length, displaying granular texture and intense yellowish color. At the end of the fifth month, the leaf explants showed friable yellowish callus having embryogenic potential in all four treatments. Besides these yellow callus, those brown and watery white ones were characterized, as well. Nevertheless, only the friable yellowish ones were collected and used in the histological analysis experiment.

Up until six months of cultivation, the embryogenic sectors continued to grow, thus reaching lengths of 8 to 10 mm. After this period, there was decrease in growth as well as a change of color from intense yellow to grayish-yellow. These changes suggest callus aging, possibly due to the quality and quantity of the medium culture and/or an increase of growth inhibitors produced by the original explant [11].

In regard to the number of potentially embryogenic callus, the BBinv treatment produced the most significant percentage (20)%, followed by the Tinv treatment. BBcv treatment presented the least significant percentage of potentially embryogenic callus (Figure 2).

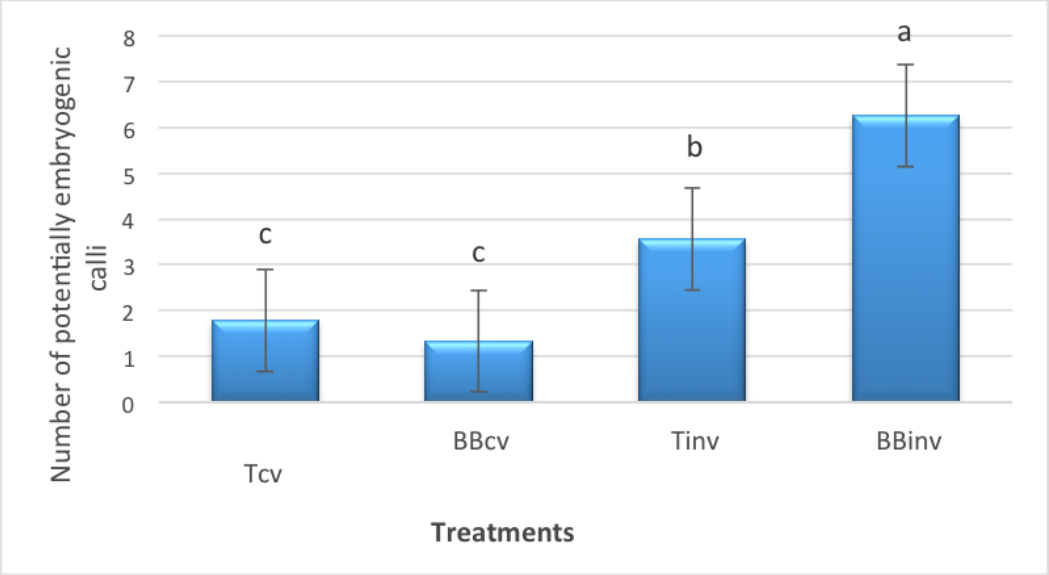

Figure 2 – Comparative graphic among the four treatments according to the presence of potentially embryogenic callus after five months of cultivation in secondary medium. Averages followed by the same letter do not differ statistically, by Tukey test (*P* = .05).

In the current work, Tiv and BBinv treatments presented primary callus with the smallest growth in relation to explant leaf covering although having produced the largest number of potentially embryogenic callus. The rising of embryogenic callus is directly related to the primary callus size, in other words, the smaller the primary callus the greater the chance of having embryogenic callus, as there occurs less competition between the induction of embryogenic sector formation and the growth of primary callus [11, Unpublished results Barros, PMC, 2012, University of Brasília, Brazil].

The results of this work regarding embryogenic callus obtainment are the same as those of pioneer works. Embryogenic callus were obtained by applying 5.4 µM-auxin (ANA) for *C. arabica* genotypes; hence, the authors have reported a 20% formation of potentially embryogenic callus [13]. As for the *C. canephora*, authors confirm a 20.8% of callus with embryogenic potential, by using 2 µM of 2,4-D [14].

Later works carried out with coffee Mundo Novo and Catuaí cultivars presented 8% and 100% potentially embryogenic callus, respectively [11]. These authors report that the 20µM concentration of 2,4D was essential to get a substantial increase of the embryogenic sectors formation frequency. Nevertheless, the best results regarding the formation of potentially embryogenic callus for Catiguá MG2 were presented by the protocol published by Boxtel and Berthouly [10], which used the 2.3 µM concentration of 2,4-D. It is suggested that the formation of embryogenic callus may be influenced by the genotype, which in turn is influenced by the concentration of the tested auxin.

Leaf explants from *in vitro* plants showed to be a more promising source for the formation of embryogenic callus when compared to those from explants cultivated in greenhouse, regardless of the tested protocol.

The best results for the cv. Rubi (*c. arabica* L.) regarding callus formation were obtained for 2 µM concentration of 2,4-D, [15]. In order to evaluate the effect of growth regulators on callus induction from *C.arabica*

cv. Catuaí Vermelho, it was also reported that 2 μM [1] concentration gave better results to produce pro-embrioids [16].

In the present study, the protocol having the smallest 2,4-D concentration on the induction of both primary callus (2.3 μM) and of embryogenic callus (4.5 μM) was the most efficient on the embryogenic callus formation.

The use of 2,4-D auxin is very frequent on the callus induction. Such regulators affect cell division initialization and control the processes of cell growth and elongation. They also participate in RNA metabolism by inducing transcription of RNA that de-codify proteins for growth, which could induce to disoriented cell proliferation at high dosages [17]. The 2,4-D is undoubtedly the most appropriate auxin for callus induction and maintenance [18]. In the current work it was verified that 2,4-D behaved both isolated and associated to BAP. We were able to achieve the best results for the obtainment of embryogenic callus when 2,4-D was associated to BAP.

3. 2 Morphological characteristics of different types of callus

During callus induction phase, both explants from greenhouse and those from *in vitro* cultivation presented different types of callus. Heterogenous primary callus were observed, thus, characterizing their visibly fast growth (Figure 3A). Nodular primary callus were present either on most explant edges or mostly frequently in portions of the edges displaying compact globular formations (Figure 3B). A potentially embryogenic callus with yellowish color and granular cell aggregates easily detachable and a friable aspect were noted (Figure 3C). Another kind of callus of watery aspect and translucent was also observed (Figure 3D).

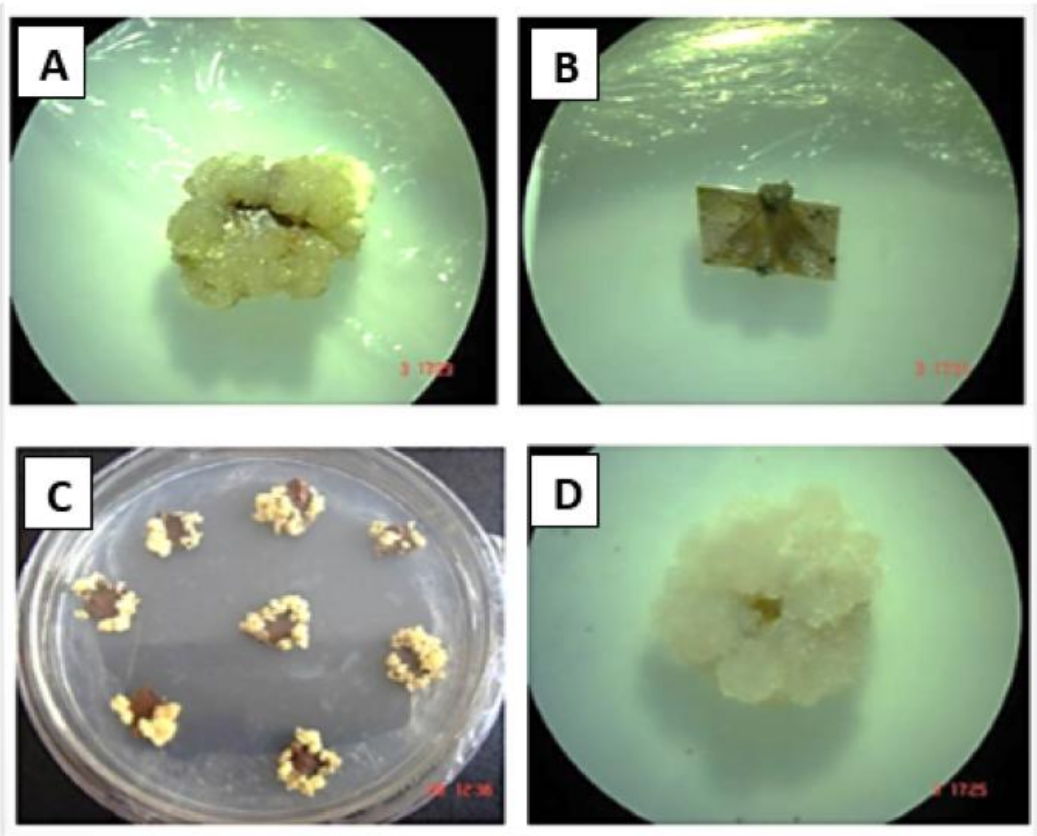

Figure 3 – Morphological characterization of different types of callus as observed during indirect somatic embryo formation in *Coffea arabica* cv. Catiguá MG2 from leaf explants cultivated both in greenhouse and *in vitro*. A) Heterogenous primary callus with amorphic formations. B) Nodular primary callus. C) Potentially embryogenic callus aggregates. D) Non-embryogenic callus.

This same characteristics were described in various other works on embryogenic callus for *C. canephora* [19] and *C. arabica* [20, 21].

3.3 Histological analysis

Potentially embryogenic callus originated from plants cultivated either *in vitro* or in greenhouse, displayed intense yellowish color and friable aspect. This material first underwent staining with blue toluidine then histological sections, hence, enabling the observation of small isodiametric cells (15 to 25 µm in cell diameter), surrounded by few non-embryogenic cells, with larger size than embryogenic cells due to the presence of large vacuoles. In some embryogenic regions, it was verified cells displaying clear nucleus undergoing cell division. The friable yellowish callus corresponded to a highly homogenous tissue, constituted by small cell aggregates similar to pro-embryogenic masses (Figure 4).

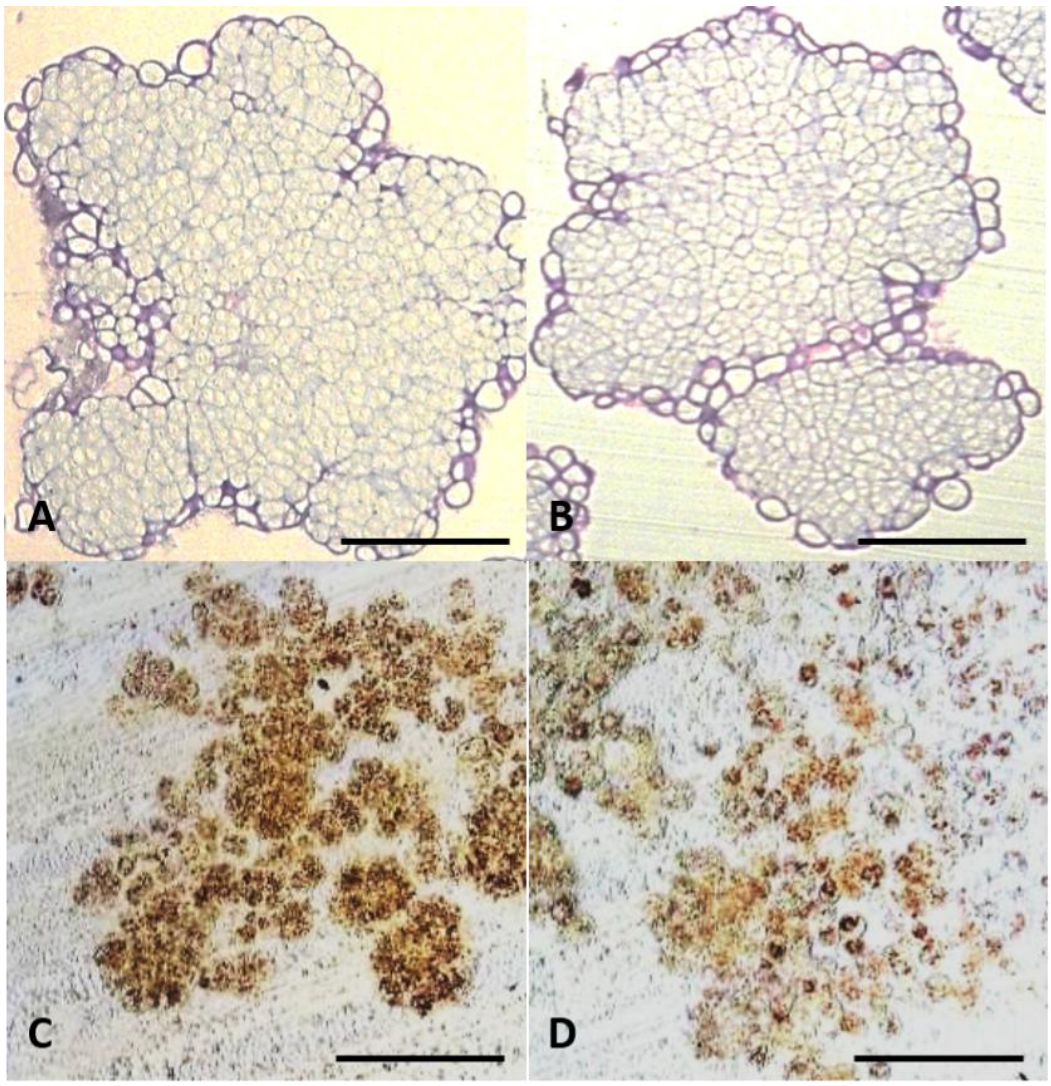

Figure 4 – Histological sections of potentially embryogenic callus from *in vitro* (A and C) and greenhouse (B and D) plants. A and B show histological sections stained with Blue Toluidine. C and D show histological sections stained with lugol. Bar: 50 µm.

While analyzing histological sections of *C. arabica* cv. Catuaí vermelho, researchs observed similar results to the current work regarding cellular diameter, it was verified 15-25 µm diameters for embryogenic cells and 39-48 µm for non-embryogenic ones [Unpublished results Barros, PMC, 2012, University of Brasília, Brazil]. Studies with *C. arabica* cv. Caturra Rojo showed embryogenic cells with diameters between 15 and 10 µm [6; 20].

The cells also presented a high nucleus-cytoplasm relation and a bulky central nucleus, with varying cell wall thickness (Figure 4) as normally noted for embryogenic cells [22, 23].

Morphologically, embryogenic cells are small, isodiametric, homogenous cells, showing only an extremely thin primary wall, dense cytoplasm and small vacuole. These characteristics are primordial for a cellular division of this tissue, because smaller size enables greater cell per tissue volume as well as faster cell divisions. Isodiametric cells optimize division because they do not have a larger cell axis. The cell wall be thin, not thick and lignified, as it helps cell division by providing a greater nutrient influx. And a small vacuole that allows more space for the organelles to be replicated during cell divisions [24].

In the case of histological slides stained with lugol, all the section from potentially embryogenic callus, regardless of leaf explant origin, presented a large number of stained cells, hence, evidencing the presence of small starch grains.

Similar results to the current work have also identified starch grains on macaúba (*Acrocomia aculeate*) [25], callus of ingazeiro (*Inga edulis*) [26], pupunha (*Bactris gasipaes*) [27] and on embryogenic suspensions of *C. arabica* cultivar Catiguá MG2 [28]. The authors report that starch presence on embryogenic cells indicate the same embryogenic formation as found on this present work.

Starch storing, either in embryogenic or adjacent cells, is a phenomenon that frequently indicates embryogenic formation [19]. Neverthenless, the probable starch role in the somatic embryogenesis process is not clear yet. It would be appropriate that starch storing be related to the weak mitotic activity of the cells that store it since generally embryogenic cells under greater mitotic activity has less starch storing [29].

4. CONCLUSIONS

The more vigorous the primary callus is the smaller is the embryogenic formation sectors frequency.

Embryogenic sector growth rate decreases with cultivation time, which is correlated with the color change from intense yellow to grayish yellow.

Explants obtained from *in vitro* cultivated plants presented the greatest development of potentially embryogenic callus when these were inoculated into culture medium having smaller 2,4-D concentration.

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