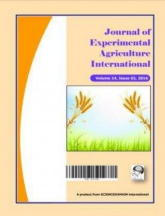

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Influence of 2,4-D Growth Regulator and Foliar Explant Source on *Coffea arabica* Embryogenic Calli

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KGL, LFT, FVB, JCA and DEL designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors KGL and LVP managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: With the goal of obtaining *Coffea arabica* varieties with greater productivity and resistance, researchers in Brazil have been conducting experiments on elite plant propagation through *in vitro* techniques, such as somatic embryogenesis. This large-scale multiplication method has great potential for exploration and could enable maximization of coffee propagation. The aim of the current work was to compare the effects of different 2,4-D concentrations and two pre-established methodologies for obtaining embryogenic calli of *Coffea arabica*, and to evaluate whether the foliar explant source can affect somatic embryogenesis.
Place and Duration of Study: The experiments were conducted in the Central Laboratory of

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Molecular Biology at the Federal University of Lavras, (Brazil), between 2016 and 2017.
Methodology: The calli of *Coffea arabica* cv. Catiguá MG2 were obtained using two well-known methodologies and from two explant sources: seedling leaves cultivated both *in vitro* and in a greenhouse. The quality of potentially embryogenic calli was verified by morphological analysis by light microscopy, and the quantity was determined by analysis of variance.
Results: After five months of culture, all treatments generated potentially embryogenic calli. The calli were histologically characterized as having a homogeneous tissue consisting of small isodiametric cells and aggregates displaying a dense cytoplasm and clear nuclei. Numerous small starch grains were also noted.
Conclusion: The plant leaf explants grown *in vitro* showed a high percentage of potentially embryogenic calli after inoculation in a culture medium containing a low 2,4-D concentration. Morphological characteristics may therefore be used as structural markers to select embryogenic cultures.

Keywords: Coffea arabica; histological analysis; morphological characterization; somatic embryogenesis.

ABBREVIATIONS

2,4-D : 2,4-dichlorophenoxyacetic acid
BAP : 6- benzylaminopurine
NAA : naphthaleneacetic acid
BBinv : treatment Boxtel and Berthouly/in vitro
BBcv : treatment Boxtel and Berthouly/ greenhouse
Tinv : treatment Teixeira/in vitro
Tcv : treatment Teixeira/greenhouse

1. INTRODUCTION

Preliminary estimates for global coffee output for 2017/18 are 158.78 million bags, or 0.7% higher than 2016/17. Arabica production is provisionally estimated at 97.32 million bags, or 1.1% lower than 2016/17 due to an expected reduction in output from Colombia and Brazil [1]. *Coffea arabica* produces the best beverage with the least caffeine content, but it is very susceptible to diseases such as rust (*Hemileia vastatrix*) and anthracnosis (*Colletotrichum kahawaen*) of the unripened beans [2]. This vulnerability has stimulated the use of genetic transformation [3] to enable the development of cultivars that are better adapted to various stresses and that are of greater agronomic interest [4]. However, the dissemination of resistant plant material depends on its clonal propagation efficiency, as well as its capacity for *in vitro* regeneration [3,4,5].

One of the *in vitro* propagation possibilities is indirect somatic embryogenesis, whereby embryos are grown from cells that originate from callus derived from the dedifferentiation of cells from an explant. This process depends on the use of growth regulators to induce tissue differentiation and embryogenic callus formation. A high auxin/cytokinin ratio is needed in the initial culture medium for the formation of non-differentiated callus, and a low ratio is required to induce embryogenic callus during succeeding cultivations [5,6,7].

Somatic embryogenesis in the *Coffea* genus begins with distinct calli during the induction phase. The primary callus nodules assume compact globular formations that occur infrequently along most explant edges. Embryogenic calli are characterized by granular cell clusters and are easily distinguished by their creamy yellowish color [8,3,4].

One area of coffee research focuses on the development of protocols that enable the commercial *in vitro* plant propagation of *C. arabica*. Plant regeneration by somatic embryogenesis is a well-established technique for some coffee varieties [9], but a thorough analysis of current protocols is needed to determine the efficiency of obtaining potentially embryogenic calli for the various cultivars. Histological analysis by light and electron microscopy can aid in establishing characteristics that differentiate various cells and in determining the embryogenic potential of the plant material.

The aim of the current work was to compare the effect of different 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations used in two pre-established methodologies for obtaining embryogenic calli of *C. arabica* and to evaluate whether the choice of foliar explant source affects somatic embryogenesis.

2. MATERIALS AND METHODS

2.1 Disinfestation and Inoculation

The source of explants used in this study were leaves of plants cultivated *in vitro* or inside greenhouses. Leaves of *C. arabica* cv. Catiguá MG2 were collected early in the morning and disinfected under a laminar flow hood. Disinfection consisted of a 70% alcohol treatment for 2 to 3 minutes, followed by 2.4% sodium hypochlorite for 10 minutes, and then 3 washes in sterile distilled water. After disinfection, a scalpel was used to cut the leaves into 0.5 cm^2 segments, which 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 27ºC.

For *in vitro* cultivated plants, the leaves were not disinfected, but all the other steps were as described above.

2.2 Culture Medium for Callus Induction

The effect of different 2,4-D concentrations on the formation of embryogenic callus was evaluated using the culture medium compositions described by Boxtel and Berthouly [10] and Teixeira [11]. As described by Boxtel and Berthouly, the leaf segments were initially cultivated on a primary callus-inducing medium, which contained 2.3 µM 2,4-D. One month later, the explants were transferred to an embryogenic callus-inducing medium containing 4.5 µM 2,4-D. As described by Teixeira, the leaf segments were also initially cultivated on the primary callus-inducing medium, but with 20 µM 2,4-D. One month later, the explants were transferred to an embryogenic callus-inducing medium containing 10 µM 2,4-D.

2.3 Statistical Analysis

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

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

2.4 Histological Analysis

The embryogenic callus samples were fixed in 50% FAA (50% Ethanol, 10% formalin, 5% acetic acid) for 48 hours at room temperature, then separately dehydrated in a graded ethanol series (60, 70, 80, 90, and 100%) for 1 hour. The samples were infiltrated for 24 hours with a 1:1 epoxy resin (Historesin® Leica) and ethanol solution, and then infiltrated in pure resin. After infiltration, the samples were embedded, cut at 3µm thickness on a manual rotatory microtome, and stained with 0.05% toluidine blue or Lugol solution and viewed 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 monthly comparative analysis in terms of primary callus growth time as a function of the explant leaf area covered by the callus. The BBcv treatment showed the most significant primary callus growth after a four-month period. The Tiv and BBinv treatments showed the least primary callus growth (Fig. 1).

After a four-month period of cultivation in the embryogenic callus induction medium, stereoscopic microscopy revealed the first formation of a yellow region over the primary calli, corresponding to the beginning of the embryogenic sector formation. Four months later, observation with the naked eye revealed the growth of embryogenic sectors 1 to 3 mm in length that displayed a granular texture and intense yellowish color. At the end of the fifth month, the leaf explants in all four treatments showed friable yellowish calli having

embryogenic potential. Brown and watery white calli were also observed in addition to the yellowish ones. Only the friable yellowish ones were collected and used for histological analysis.

For up to six months of cultivation, the embryogenic sectors continued to grow, reaching lengths of 8 to 10 mm. After this period, growth decreased and the color changed from intense yellow to grayish-yellow. These changes suggested callus aging, possibly due to the quality and quantity of the culture medium and/or an increase in growth inhibitors produced by the original explant [11,13].

The numbers of potentially embryogenic calli were largest for the BBinv treatment (20%), followed by the Tinv treatment. The BBcv treatment produced the smallest percentage of potentially embryogenic calli (Fig. 2).

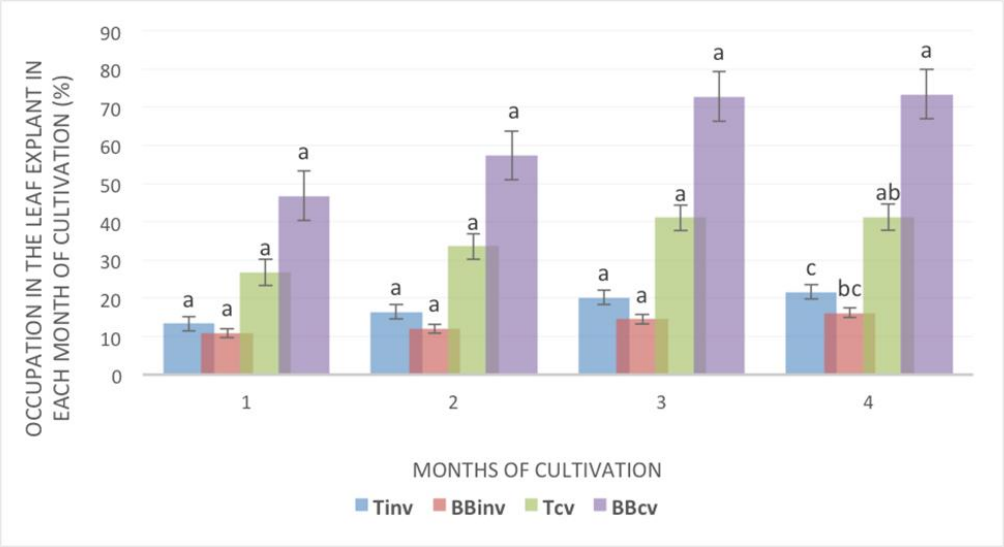

Fig. 1. Assessment of primary growth of foliar calli by comparing each treatment during four months of cultivation

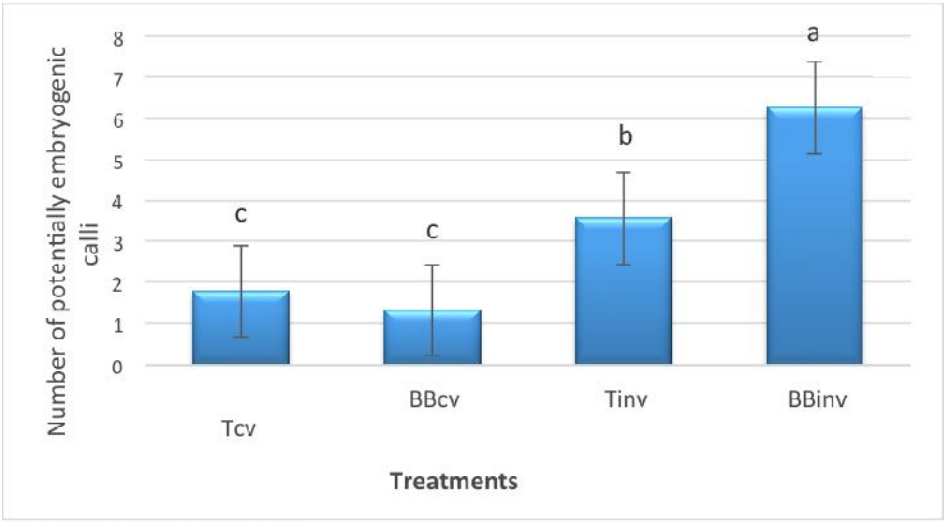

Fig. 2. Comparison of the four treatments and the presence of potentially embryogenic calli after five months of cultivation in secondary medium. Averages followed by the same letter do not differ statistically according to the Tukey test (*P* = .05)

In the current work, the Tinv and BBinv treatments yielded the smallest growth of primary calli in terms of the coverage of the explant leaf, but these treatments produced the largest numbers of potentially embryogenic calli. This increase in embryogenic callus production was directly related to the primary callus size; i.e., a smaller primary callus had a greater chance of being an embryogenic callus, as less competition occurred between the induction of the 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 the numbers of embryogenic calli obtained agree with those of earlier work. For example, embryogenic calli were obtained by applying 5.4 µM-auxin NAA (naphthaleneacetic acid) to *C. arabica* genotypes. The authors reported a 20% formation of potentially embryogenic calli [14]. For *C. canephora*, previous researchers have confirmed 20.8% of the calli had embryogenic potential following treatment with 2 µM 2,4-D [15].

Later work carried out with the coffee cultivars Mundo Novo and Catuaí confirmed the production of 8% and 100% potentially embryogenic calli, respectively [11]. A 20 µM concentration of 2,4-D was essential to obtain a substantial increase in the formation of embryogenic sector. The best results in terms of the formation of potentially embryogenic calli were obtained for Catiguá MG2 using the protocol published by Boxtel and Berthouly [10] and 2.3 µM 2,4-D. This finding suggested that the formation of embryogenic calli may be influenced by the plant genotype as well as by the auxin concentration.

Leaf explants from *in vitro* plants appeared to be a more promising source for the formation of embryogenic calli when compared to explants cultivated in the greenhouse, regardless of the treatment protocol.

The best results in terms of callus formation were obtained for the Rubi (*C. arabica*) cultivar with 2 µM 2,4-D, [16]. A 2 µM concentration also resulted in the production of pro-embrioids in *C. arabica* cv. Catuaí Vermelho [17].

In the present study, the smallest 2,4-D concentrations for the induction of both primary calli (2.3 µM) and embryogenic calli (4.5 µM) were also the most efficient at inducing embryogenic callus formation.

The auxin 2,4-D is very frequently used for callus induction. Auxin-type regulators affect the initialization of cell division and control the processes of cell growth and elongation. They also induce transcription of RNA that codes for proteins needed for growth, and they can cause disoriented cell proliferation at high dosages [18]. Other research has characterized 2,4-D as the most effective auxin for callus induction and maintenance [19]. In the current work, 2,4-D functioned both in isolation and in association with 6-benzylaminopurine (BAP). We obtained the best results for embryogenic callus formation when 2,4-D was supplied together with BAP.

3.2 Morphological Characteristics of Different Types of Calli

During the callus induction phase, the explants from the greenhouse and from *in vitro* cultivation generated different types of calli. Heterogeneous primary calli, characterized by their fast growth, were observed (Fig. 3A). Nodular primary calli were present on most explant edges or most frequently in portions of the edges that displayed compact globular formations (Fig. 3B). Potentially embryogenic calli with a yellowish color and granular cell aggregates that were easily detachable and friable were noted (Fig. 3C). Another type of callus was translucent and had a watery aspect (Fig. 3D).

These same characteristics were described in previous work on embryogenic calli of *C. canephora* [3] and *C. arabica* [13,20].

3.3 Histological Analysis

Potentially embryogenic calli that originated from plants cultivated either *in vitro* or in the greenhouse displayed an intense yellowish color and had a friable aspect. Staining of histological sections with toluidine blue revealed small, isodiametric cells (15 to 25 µm diameter), surrounded by a few non-embryogenic cells, which were larger in size than embryogenic cells due to the presence of large vacuoles. In some embryogenic regions, cells with clear nuclei were undergoing cell division. The friable yellowish calli corresponded to a highly homogenous tissue, consisting of small cell aggregates similar to pro-embryogenic masses (Fig. 4).

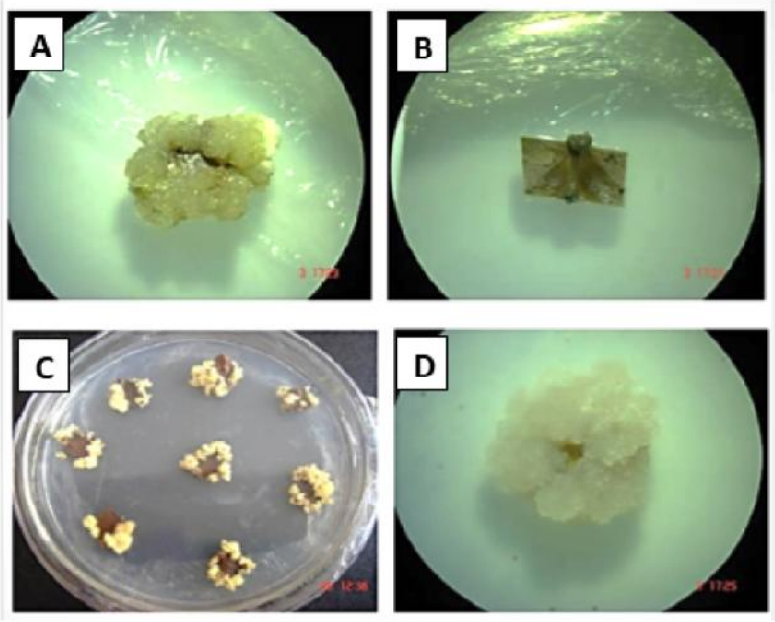

Fig. 3. Morphological characterization of different types of calli observed during indirect somatic embryo formation in *C. arabica* cv. Catiguá MG2 from leaf explants from obtained from a greenhouse or *in vitro*. A) Heterogeneous primary callus with amorphic formations. B) Nodular primary callus. C) Potentially embryogenic callus aggregates. D) Non-embryogenic callus

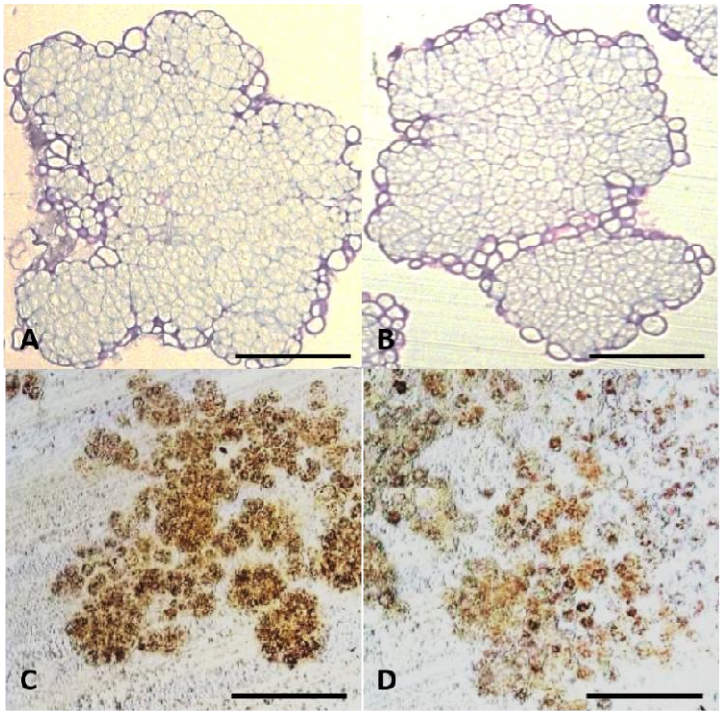

Fig. 4. Histological sections of potentially embryogenic calli from *in vitro* (A and C) and greenhouse (B and D) plants. A and B show histological sections stained with toluidine blue. C and D show histological sections stained with Lugol. Bar: 50 µm

Analysis of histological sections of *C. arabica* cv. Catuaí vermelho revealed similar results to the current work in terms of cell diameters, which were 15–25 µm 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 had a high nucleus to cytoplasm ratio, a bulky central nucleus, and varying cell wall thickness (Fig. 4), as typically found in embryogenic cells [21,22].

Morphologically, embryogenic cells are small, isodiametric, homogeneous cells that have an extremely thin primary wall, dense cytoplasm, and a small vacuole. These characteristics are primordial for cell division of this tissue, because a smaller size enables a greater number of cells per tissue volume as well as faster cell divisions. Isodiametric cells optimize cell division because they do not have a large cell axis. The cell wall should be thin, not thick and lignified, as this helps cell division by providing a greater nutrient influx. A small vacuole allows more space for the replication of organelles during cell division [23].

The histological slides stained with Lugol showed the presence of small starch grains in a large number of stained cells in all the sections from potentially embryogenic calli, regardless of leaf explant origin.

Similar results to those in the current work were presented for starch grains in macaúba (*Acrocomia aculeate*) [24], in callus of ingazeiro (*Inga edulis*) [25] and pupunha (*Bactris gasipaes*) [26], and in embryogenic suspensions of *C. arabica* cultivar Catiguá MG2 [27]. The presence of starch in embryogenic cells indicates the same embryogenic formation as found in the present work.

Starch storage, either in embryogenic or adjacent cells, is a phenomenon that frequently indicates embryogenic formation [3]. Nevertheless, the role of starch in the somatic embryogenesis process is not yet clear. Starch storage could be related to the weak mitotic activity of the cells that store it, since embryogenic cells with greater mitotic activity generally have less starch storage [28].

4. CONCLUSIONS

A more vigorous primary callus gives a smaller frequency of embryogenic formation sectors.

The growth rate of the embryogenic sectors decreases with cultivation time and is correlated with a color change from intense yellow to grayish yellow.

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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