

## Early Somatic Embryogenesis in *Heliconia chartacea* Lane ex Barreiros cv. Sexy Pink Ovary Section Explants

Cláudia Ulisses<sup>1\*</sup>, Terezinha Rangel Camara<sup>2</sup>, Lilia Willadino<sup>2</sup>, Cynthia Cavalcanti de Albuquerque<sup>3</sup> and Júlio Zoé de Brito<sup>4</sup>

<sup>1</sup>Universidade Federal Rural de Pernambuco; Unidade Acadêmica de Garanhuns; Av. Bom Pastor s/n; 55.296-901; Garanhuns - PE - Brasil. <sup>2</sup>Universidade Federal Rural de Pernambuco; Recife - PE - Brasil. <sup>3</sup>Universidade Estadual do Rio Grande do Norte; Mossoró - RN - Brasil. <sup>4</sup>Centro de Tecnologias Estratégicas do Nordeste; Recife - PE - Brasil

### ABSTRACT

The present work evaluated the development of embryogenic callus from transversal ovary sections. The experiments were carried out under two experimental regimes using combinations of IAA (0; 5.71; 8.56; 11.42; 14.27 $\mu$ M) and 2,4-D (0; 13.57; 18.10; 22.62 $\mu$ M) or combinations of 2,4-D with BA (0; 4.43; 6.65; 8.87; 11.09 $\mu$ M). Assessments were made of anatomical aspects of the callus and for the presence of embryogenic structures using cytochemical and histological analyses and stereomicroscopic and scanning electronic microscopic observations. Treatments with 2,4-D and IAA produced friable calluses demonstrating cellular acquisition of morphogenetic competence as well as the formation of pro-embryogenic sectors. The expression of embryogenic program could be observed, with proembryogenic cell clusters developing into globular embryos. These results offer the possibility of using new types of explants for culturing helicones that avoid the growth of endophytic bacteria.

**Key words:** Callogenesis, growth regulator, histology, scanning electron microscopy

### INTRODUCTION

Ornamental tropical plants have attracted the interest of international flower markets due to their beauty, exotic nature, and the post-harvest persistence of their inflorescences. Brazil has a large contingent of native plant species that fit these requirements, with the Sexy Pink cultivar of *Heliconia chartacea* Lane ex Barreiros being among the most valued for exportation.

Helicones have a subterranean rhizome from which new buds are commonly removed for propagation (Castro, 1995), although this vegetative propagation system yields reduced number of plants and offers serious risks of pest

and disease dissemination. Micropropagation has emerged as an important alternative for providing large-scale production of helicon plants with high standards of quality, but the micropropagation of helicones via shoot tips presents problems linked to endophytic contamination that can limit explant development (Nathan et al., 1992; Atehortua et al., 1999; Dias and Rodrigues, 2001; Rodrigues, 2005) so that the use of floral explants may represent a viable alternative (Atehortua et al., 1999).

Plant genetics and biotechnology is highly dependent on the use of in vitro culture techniques, and among the various systems available, somatic embryogenesis offers the opportunity for the production of true-to-type plants by clonal

\* Author for correspondence: claudia@nlink.com.br

propagation and is a useful tool for basic research on totipotency and the fundamental processes of plant morphogenesis (Gaj, 2004).

Ovary sections were used in the present work to establish in vitro cultures of *H. chartacea* Lane ex Barreiros cv. Sexy Pink without endophytic contamination and to evaluate the induction and development of embryogenic callus.

## MATERIALS AND METHODS

The ovaries from immature inflorescences of the Sexy Pink cultivar of *Heliconia chartacea* with one closed and four open bracts were used as explant sources. These explants were washed with pure detergent, rinsed with running water, immersed in alcohol (70%) for one minute, followed by two immersions in calcium hypochlorite solution (a 3% solution for 20 minutes and another at 1.5% for 15 minutes, both containing three drops of 20% Tween) and finally submitted to three rinses with sterile distilled water to eliminate the disinfecting agents. Cross and transversal sections of the ovaries were then made, measuring approximately 0.7 cm in diameter and 0.2 cm in thickness, and these were inoculated into test tubes (2.5 x 15 cm) containing 10 mL of MS culture medium (Murashige and Skoog, 1962).

Culture medium was prepared containing either 0; 13.57; 18.10 or 22.62  $\mu\text{M}$  2,4-D with either 0; 5.71; 8.56; 11.42 or 14.27  $\mu\text{M}$  IAA, in accordance with Valencia and Atehortua (1999). In a second series of assays, these same varying concentrations of 2,4-D were combined with 0; 4.43; 6.65; 8.87 or 11.09  $\mu\text{M}$  BA. Both assays were conducted using 10 replicates per treatment. The basic MS medium was supplemented in all cases with 30  $\text{g.L}^{-1}$  sucrose, 0.2  $\text{g.L}^{-1}$  glycine, and 2  $\text{g.L}^{-1}$  phytigel. The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes. All inoculated material was kept in the dark for 90 days at 25 $\pm$ 2 °C.

Evaluations of callus material were undertaken at 30, 60 and 90 days after inoculation with the aid of a stereomicroscope. The induction frequency (%) of the callus was calculated by: (the number of explants forming calluses/total number of explants) x 100. A non-parametric statistical test was applied to the induction frequency of calluses based on the Qui-square distribution, and arcsen $\sqrt{\%$  transformed data was compared by Tukey's test.

Calluses that presented nodular surfaces and a yellow coloration were selected after 90 days in the induction medium and samples were prepared for cytochemical and histological analyses. The remaining calluses were transferred to MS medium supplemented with 30  $\text{g.L}^{-1}$  of sucrose, 2  $\text{g.L}^{-1}$  phytigel, and 4.43  $\mu\text{M}$  BA in order to induce a somatic conversion of the embryos into plants. The calluses were kept for 60 days at 25 $\pm$ 2°C with a 16 hr photoperiod at a light intensity of 50  $\mu\text{mol.s.m}^{-2}.\text{s}^{-1}$ .

Callus derived from the induction medium were stained with Evans blue (0.1%) and acetic carmine (2%) to visualize the somatic pro-embryos, as described by Guerra et al. (1999) and were examined and photographed using an optical microscope (Carl Zeiss).

Callus that presented nodular structures on their surfaces were selected for histological studies, and were dehydrated, infiltrated using automatic histo-technical equipment, and embedded in paraffin wax on a Tissue-tec apparatus. Semi-thin (4-6 $\mu\text{m}$ ) sections were cut on a horizontal rotary microtome (Slee Technik) for general examination, and then stained with hematoxylin and eosin (Johansen, 1940).

Samples to be viewed by scanning electron microscopy were fixed in 3% glutaraldehyde, dehydrated in ethylic alcohol series (50, 70, 90 and 100%), dried using a critical point CO<sub>2</sub> dryer, and then gold coated (300A°). A scanning electron microscope (Hitachi S800-10kV) was used for examination and photographing the callus material.

## RESULTS AND DISCUSSION

Ovary sections were observed to swell after 30 and 60 days of treatment with IAA+2,4-D and BA+2,4-D, which is characteristic of the callus-induction phase in which the explant swells and then develops into a friable callus (Yadav and Rajam, 1998). Ninety days after inoculation, the explants growing in IAA+2,4-D demonstrated higher frequencies of callus formation than explants in BA+2,4-D (Table 1). Yellowish callus 5-7 mm in diameter were obtained in treatments with IAA and 2,4-D. These callus showed smooth, compact, globular embryogenic structures that were easily distinguishable from the rest of callus.

These globular embryogenic structures were quite numerous and covered most of surface of the callus that was not in direct contact with the medium (Fig. 1A). Similar results have been reported for coconut (Sáenz et al., 2006) and papaya sections (Almeida et al., 2001).

Embryogenic callus can be obtained from floral shoots of *Heliconia stricta* after incubation in MS medium supplemented with 13.57 to 22.62  $\mu\text{M}$  2,4-D and 5.71 to 14.27  $\mu\text{M}$  IAA for four to six months (Valencia and Atehortua, 1999). This induction period is longer than that seen in the present work, in which the development of embryogenic callus and somatic embryos were seen after only three months of in vitro cultivation. The results of this simultaneous use of two auxin sources to induce embryogenic callus was quite different from that reported by other authors (Kurten et al., 1990; Mathew and Philip, 2003) who used combinations of auxins and cytokinins. The callus formed in treatments with BA and 2,4-D appeared whitish and aqueous. A majority of the BA treatments demonstrated harmful effect on callus development in the Sexy Pink cultivar. Similar results were reported by Martini et al. (2001), who observed an inhibiting action of the BA on induction and proliferation, in orchid (*Gongora quinquenervis*) callus. According to Williams and Maheswaran (1986), BA acts strongly in inducing cell polarity and favors the differentiation process of embryogenic callus material.

No callus development was observed without auxin treatment (control). In the treatment without 2,4-D but with 5.71  $\mu\text{M}$  IAA, a low frequency of callus formation was seen that was inhibited by increasing IAA levels (Table 1).

2,4-D is capable of setting off processes of de-differentiation (indirect models) and re-differentiation (direct models), thus altering cellular determination and endowing new competencies to responsive cells present in the explants (Nowak and Shulaev, 2003). The pro-embryogenic cellular mass cultivated in the presence of auxin is able to synthesize the proteins necessary for forming a globular-stage embryo (Zimmerman, 1993).

Proteins are one of the molecular bases of the embryogenic cells formation (Liu et al., 2006).

Roots were observed to grow from pseudostem

fine sections of *Heliconia psittacorum* when these were cultivated in the presence of 10  $\mu\text{M}$  of 2,4-D, whereas callus formation occurred only at higher concentrations (40 and 80  $\mu\text{M}$ ) (Goh et al., 1995).

It has been demonstrated that 2,4-D is more effective than IAA in promoting embryogenic callus development in *Feijoa sellowiana*, *Carya illinoensis*, and *Fragaria* sp. (Cruz et al., 1990; Rodriguez and Wetzstein, 1998; Flores et al., 2003). On the other hand, IAA specifically induced the development of somatic embryos of *Paulownia tomentosa* (Radojevic, 1979). The different results that are induced by different auxin sources are apparently related to the genetic constitution of the plant material (Hu and Ferreira, 1998; Sakhanokho et al., 2005).

The callus in most treatments with IAA+2,4-D demonstrated cells with reduced sizes, nucleolus not clearly defined, dense cytoplasmic content and intense metabolic activity (as indicated by the deep red color observed with added acetic carmine, Fig. 1B). Cells that demonstrated these characteristics were considered embryogenic cells (Durzan, 1988; Mathew and Philip, 2003; Kim et al., 2003; Sáenz et al., 2006) and staining with acetic carmine indicated the presence of reserve substances that would favor tissue development (Guerra et al., 1999).

The combination of IAA and 2,4-D also promoted the development of yellowish nodules, especially in the treatments with 8.56  $\mu\text{M}$  IAA plus 13.57  $\mu\text{M}$  2,4-D; 11.42  $\mu\text{M}$  IAA plus 22.62  $\mu\text{M}$  2,4-D; and 14.27  $\mu\text{M}$  IAA plus 22.62  $\mu\text{M}$  2,4-D. Histological analyses of this callus material revealed heavily stained cells located on the surface of the callus. Transverse sections showed that these structures consisted of meristematic cells surrounded by a protoderm. Some globular embryogenic structures became larger, while others seemed to fuse and acquire an elongated appearance. The inner tissue of the callus consisted mostly of parenchyma cells and vascularized zones (Fig. 1C). Hervé et al. (2001) likewise observed the presence of a protuberance on the superficial layer of the callus in *Eucalyptus gunnii* Hook, which they interpreted as a region of pro-embryogenic cells.

**Table 1** - Induction frequencies of callus formation from ovary sections of *H. chartacea* Lane ex Barreiros cv. Sexy Pink after 90 days in MS medium with different combinations of IAA+2,4-D or BA+2,4D ( $\mu\text{M}$ ).

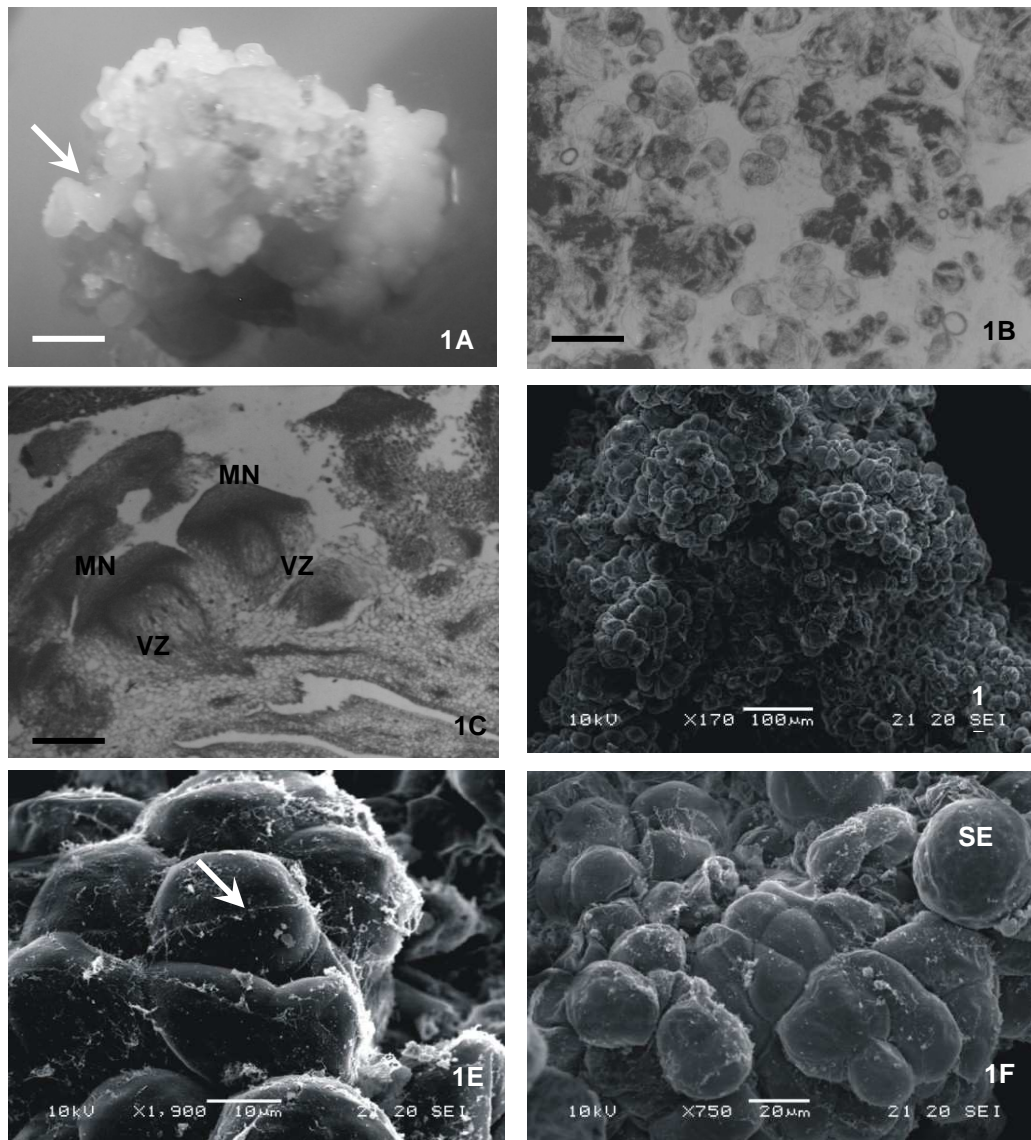
Treatments ( $\mu\text{M}$ )	Frequency of formation callus (%)	Treatments ( $\mu\text{M}$ )	Frequency of formation callus (%)
0 IAA + 0 2,4-D	0 b	0 BA + 0 2,4-D	0 b
0 IAA + 13.57 2,4-D	100 a	0 BA + 13.57 2,4-D	100 a
0 IAA + 18.10 2,4-D	70 ab	0 BA + 18.10 2,4-D	100 a
0 IAA + 22.62 2,4-D	100 a	0 BA + 22.62 2,4-D	100 a
5.71 IAA + 0 2,4-D	30 ab	4.43 BA + 0 2,4-D	0 b
5.71 IAA + 13.57 2,4-D	80 ab	4.43 BA + 13.57 2,4-D	20 ab
5.71 IAA + 18.10 2,4-D	100 a	4.43 BA + 18.10 2,4-D	40 ab
5.71 IAA + 22.62 2,4-D	80 ab	4.43 BA + 22.62 2,4-D	100 a
8.56 IAA + 0 2,4-D	0 b	6.65 BA + 0 2,4-D	0 b
8.56 IAA + 13.57 2,4-D	60 ab	6.65 BA + 13.57 2,4-D	20 ab
8.56 IAA + 18.10 2,4-D	100 a	6.65 BA + 18.10 2,4-D	0 b
8.56 IAA + 22.62 2,4-D	60 ab	6.65 BA + 22.62 2,4-D	0 b
11.42 IAA + 0 2,4-D	0 b	8.87 BA + 0 2,4-D	0 b
11.42 IAA + 13.57 2,4-D	100 a	8.87 BA + 13.57 2,4-D	20 ab
11.42 IAA + 18.10 2,4-D	100 a	8.87 BA + 18.10 2,4-D	0 b
11.42 IAA + 22.62 2,4-D	100 a	8.87 BA + 22.62 2,4-D	20 ab
14.27 IAA + 0 2,4-D	0 b	11.09 BA + 0 2,4-D	0 b
14.27 IAA + 13.57 2,4-D	100 a	11.09 BA + 13.57 2,4-D	0 b
14.27 IAA + 18.10 2,4-D	100 a	11.09 BA + 18.10 2,4-D	0 b
14.27 IAA + 22.62 2,4-D	100 a	11.09 BA + 22.62 2,4-D	0 b

Averages followed by different letters on each column, differ among themselves at the 5% level of probability by the Tukey's Test.

Callus submitted to treatment with 8.56 $\mu\text{M}$  IAA plus 13.57 $\mu\text{M}$  2,4-D demonstrated the onset of organogenic processes, with the presence of a chlorophyll-bearing structures above the callus tissue. Although organogenic competence is usually regulated by cytokinins (Inoue et al., 2000; Rai and McComb, 2002), this structure did not continue organogenic development when transferred to growth medium with 4.43 $\mu\text{M}$  BA. Significant differences in in vitro organogenic responses have been observed when the mineral composition, vitamins or sugar sources of the medium are altered (Peres, 2002). Plant growth regulators, however, still remain the most critical components of the culture medium. Competence in organogenic processes can be understood as the ability to respond to the hormonal stimuli necessary for the induction organ development. The competence failure of a tissue might therefore reflect the absence of receptors (proteins) that

interface with growth regulators to induce organogenic processes in those cells.

After the callus-induction phase, the yellowish friable callus demonstrated cellular acquisition of morphogenetic competence during which pro-embryogenic sectors were formed (Yadav and Rajam, 1998). The images obtained by scanning electron microscopy show embryogenic tissue with several pro-embryos (Fig. 1D) in the 14.27 $\mu\text{M}$  IAA plus 22.62 $\mu\text{M}$  2,4-D treatment. A close-up of this embryogenic tissue shows two spherical cells at the time of cytokinesis (Fig. 1E) and demonstrates how the daughter cells of the embryogenic tissue remain as a coherent tissue (Fig. 1F), like was observed by Sondahl et al. (1979) in coffee embryogenic callus. The expression of embryogenic program could subsequently be observed when the proembryogenic clusters developed into the first visible globular embryos (Yadav and Rajam, 1998).



**Figure 1** - General aspect of the callus with embryogenic cells, and histological sections of callus derived from transversal ovary sections of cv. Sexy Pink after 90 days of treatment with  $14.27 \mu\text{M}$  IAA plus  $22.62 \mu\text{M}$  2,4-D *in vitro* culture: **(1A)** Somatic embryos on the surface of the callus (arrows) (Bar = 1.25 mm); **(1B)** Pro-embryogenic cells with densely stained cytoplasm indicating high metabolic activity (Bar =  $1 \mu\text{m}$ ); **(1C)** Cross-section of the callus showing meristematic nodules (MN) and vascularized zones (VZ) (Bar =  $25 \mu\text{m}$ ). **(1D)** General view of the grouping of pro-embryos in calluses as viewed using scanning electron microscopy (SEM); **(1E)** Close-up of embryogenic tissue shows two spherical cells at the time of cytokinesis (arrow) under SEM; **(1F)** Close-up (SEM) of a region with a cylindrical shaped somatic embryo (SE) demonstrating how the daughter cells of the embryogenic tissue remain as a coherent tissue.

A cylindrical-shaped embryo in a more advanced stage than the globular phase is shown in Fig. 1F. Our results indicate that combinations of two sources of auxin (natural IAA and synthetic 2,4-D) are more efficient in inducing embryogenic calluses than combinations of auxin (2,4-D) and cytokinin (BA), and also confirmed the existence of embryogenic cells and the outstanding importance of auxins in embryogenic callus induction in the species under study.

High 2,4-D concentrations can affect somatic embryo development and plantlet regeneration. According to Franco et al. (2006), somatic embryo expression is possible when 2,4-D is suppressed or replaced by weaker auxins (such as NAA in low concentrations). However, in the present work, somatic embryos did not show any potential for conversion into plants when treated with BAP without 2,4-D. In spite of the high numbers of somatic embryos produced in many systems there was only a low or zero frequency of embryo conversion into plants. A number of different strategies were tested to stimulate embryo conversion and to improve the efficiency of plant regeneration, including the addition of gibberellic acid (Kim et al, 1997) or polyamines, especially spermine (Kevers et al., 2002).

To our knowledge, there have been no previous studies using explants from ovaries to induce somatic embryogenesis in heliconas. These results indicate an alternative source of explants for *in vitro* culturing of heliconas without any apparent contamination from endophytic bacteria. We were able to induce the expression of an embryogenic program in these explants in spite of the fact that the embryos failed to generate new plants.

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## RESUMO

Este trabalho teve como objetivo avaliar a resposta de secções transversais de ovários e o desenvolvimento de calos embriogênicos. O experimento constou de dois ensaios. No primeiro avaliou-se combinações entre AIA (0; 5.71; 8.56; 11.42; 14.27 $\mu$ M) e 2,4-D (0; 13.57; 18.10; 22.62 $\mu$ M) e no segundo avaliou-se as concentrações de 2,4-D supracitadas, combinadas com concentrações de BA (0; 4.43; 6.65; 8.87; 11.09 $\mu$ M). Os calos formados foram avaliados quanto à presença de estruturas embriogênicas utilizando-se estereomicroscópio, microscópio eletrônico de varredura, além de análises citoquímicas e histológicas. Combinações entre 2,4-D e AIA induziram a formação de calos friáveis com setores pró-embriogênicos, refletindo a aquisição de competência morfogenética. Posteriormente foi observada a expressão do programa embriogênico quando massas pró-embriogênicas desenvolveram-se formando embriões somáticos. Esses resultados apresentam uma alternativa para a utilização de um tipo de explante que possibilita o cultivo *in vitro* de helicônia sem o desenvolvimento de bactérias endofíticas.

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