DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

Maturation of *Anthurium andraeanum* cv. Eidibel somatic embryos from nodal segments

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Abstract Maturation of somatic embryos of *Anthurium andraeanum* cv. Eidibel from embryogenic callus was evaluated. Following induction of embryogenic calli from nodal segments, tissues were transferred to 125-mL Erlenmeyer flasks containing 25 mL liquid medium, with 0, 4.52, or 9.05 μM 2,4-dichlorophenoxyacetic acid and 0, 0.47, or 2.32 μM kinetin. Callus cultures were maintained in a dark growth room at 25±2°C. At 45 d, the mass of embryogenic calli, number of primary and secondary somatic embryos, and percentage browning were evaluated. Nonparametric tests were used to evaluate color, texture, and somatic embryo development. The highest yield of somatic embryos was in the medium with 0.47 μM kinetin. Calli were friable, with a lower yield of secondary somatic embryos, and have minimal browning. Histology revealed polar globular somatic embryos and mature somatic embryos with defined apical and root meristematic zones, axillary buds, and primary leaves. These are important features for converting somatic embryos into plantlets.

Keywords AA2 medium · Kinetin · Liquid medium · Pierik medium · Somatic embryogenesis

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Introduction

Anthuriums belong to the Araceae family and the genus *Anthurium* Schott. Most of the 600 species in this genus are ornamental plants and many of them are herbaceous epiphytes native to the warm regions of tropical America (Castro *et al.* 2004; Tombolato *et al.* 2004; Nhut *et al.* 2006; Liendo and Mogollón 2009; Maira *et al.* 2010). Nearly 130 species can be found in Brazil (Castro *et al.* 2004; Tombolato *et al.* 2004) and all are protogynous, bisexual monocots (Winarto *et al.* 2010).

Anthuriums have been widely used in Brazilian floriculture, although they are commercially grown worldwide as cut flowers and potted plants. *Anthurium* inflorescences may easily last 20 d after harvest when cultivated for cut flower purposes (Assis *et al.* 2011).

Propagation of *Anthurium* occurs both sexually and asexually. Asexual propagation, by clump division or cuttings, potentially spreads pests and diseases and limits the number of available plants, affecting the value of the final product. Sexual propagation through seeds is a slow process and the resulting plants show wide variation in vigor, size, and productivity, and the spathes differ in color, shape, and size (Hamidah *et al.* 1997; Tombolato *et al.* 2004; Viégas *et al.* 2007). In vitro propagation is an important technique for overcoming these hurdles and is a suitable method for the production and genetic manipulation of anthuriums (Kuehnle *et al.* 1992; Fuzitani and Nomura 2004; Viégas *et al.* 2007; Maira *et al.* 2010).

Anthuriums have been propagated in vitro using several tissues and organs including the leaf, petiole, spadix, spathe,

apical meristem (Geier 1990; Matsumoto and Kuehnle 1997; Nhut *et al.* 2006; Yu *et al.* 2009), axillary bud (Kunisaki 1980), fruits or seeds (Santos *et al.* 2005; Schiavinato *et al.* 2008; Maira *et al.* 2010), and anther (Winarto *et al.* 2010; Winarto *et al.* 2011a; Winarto *et al.* 2011b). Anthuriums typically regenerate via indirect organogenesis. Following culture of the leaf blade from young leaves, callus is formed, followed by the formation of adventitious buds (Pierik *et al.* 1974; Pierik 1975; Tombolato and Quirino 1996; Nhut *et al.* 2006; Atak and Çelik 2009; Liendo and Mogollón 2009).

Additional explant types can also be used for the successful in vitro culture of the plant, such as leaves, nodal and internodal segments from indirect organogenesis (Te-chato *et al.* 2006), or even explants from the petiole for genetic transformation (Kuehnle and Sugii 1991; Zhao *et al.* 2010).

Somatic embryogenesis of anthuriums has been described using explants from leaves, petioles, nodal and internodal segments, and roots of in vitro-grown plants (Kuehnle *et al.* 1992; Hamidah *et al.* 1997; Duquenne *et al.* 2007; Bautista *et al.* 2008; Fitch *et al.* 2011). Somatic embryogenesis and plant recovery have been problematic and inefficient, demonstrating that the maturation, germination, and conversion into plants are limiting the application of somatic embryogenesis for the micropropagation of this plant (Sivanesan *et al.* 2011).

The present work aimed to evaluate and determine the culture medium composition, concentration of growth regulators, and growth conditions for maturation of somatic embryos of *Anthurium andraeanum* cv. Eidibel, aided by the histological analysis of somatic embryo development.

Materials and Methods

Plant material. In vitro-grown *A. andraeanum* Lindl cv. Eidibel plantlets from the indirect organogenesis of young leaves were kindly provided by the Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil) and were used as starting plant material. Nodal segments containing a single bud were subcultured every 30 d. Glass flasks (220 mL) were filled with 30 mL Pierik culture medium (Pierik 1976) supplemented with 4.44 µM 6-benzyladenine (BA) and 0.54 µM α-naphthaleneacetic acid (NAA) and solidified with 6.5 g L^{-1} agar (Merck KGaA, Darmstadt, Germany) with a pH of 5.8. Five explants were inoculated into each flask, which were placed in a growth room at 25±2°C with a 16-h photoperiod at an irradiance of 36 µmol m^{-2} s^{-1}.

Induction and proliferation of embryogenic cultures. Nodal segments of *Anthurium* cv. Eidibel were inoculated into Petri dishes measuring 90×15 mm (J. Prolab, Curitiba, Brazil) containing 25 mL Pierik medium to induce embryogenesis. The pH was adjusted to 5.8 prior to autoclaving at

121°C for 15 min and the cultures were maintained in a dark growth room at 25±2°C. For the proliferation stage, nine embryogenic calli were subcultured to each Petri dish. Five successive subcultures were performed at 45-d intervals to achieve sufficient calli to start experiments on the maturation of somatic embryos, stabilization of embryogenic capacity, and proliferation of cell lines.

Maturation of somatic embryos. Embryogenic calli, weighing approximately 90 mg (fresh mass) each, were aseptically inoculated into 125-mL Erlenmeyer flasks containing 25 mL of either Pierik medium (containing inorganic carbon only) or AA2 (Abdullah *et al.* 1986) medium modified to contain 300 mg L^{-1} L-glutamine as an organic nitrogen source and with 0, 4.52, or 9.05 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0, 0.47, or 2.32 µM kinetin (KIN). Cultures were maintained on an orbital shaker (100 rpm) in a dark growth room at 25±2°C.

Statistical procedures. The treatments were arranged in a completely randomized design in a 2×3×3 factorial arrangement with two nutrient media (Pierik or AA2), three concentrations of 2,4-D (0, 4.52, or 9.05 µM), and three concentrations of KIN (0, 0.47, or 2.32 µM). Five Erlenmeyer flasks were taken as replicates and each experimental unit consisted of five calli per flask, resulting in 25 explants per treatment. After 45 d of growth, the morphogenetic responses were evaluated as the fresh mass of embryogenic calli, production of somatic embryos (number of somatic embryos per callus), production of secondary somatic embryos (number of secondary somatic embryos per callus, in cases where the previously induced embryogenic calli had produced new calli), and percentage of callus browning (Table 1). The results were analyzed using SAS statistical software.

Table 1. Treatments for morphogenetic responses of fresh mass of embryogenic calli, production of primary somatic embryos, production of secondary somatic embryos, and percentage of callus oxidation (browning) of anthurium (*Anthurium andraeanum* cv. Eidibel) in Fig. 1

Code	Treatment
T1	Nutrient medium (Pierik or AA2) without growth regulators
T2	Medium+0.47 µM KIN
T3	Medium+2.32 µM KIN
T4	Medium+4.52 µM 2,4-D
T5	Medium+4.52 µM 2,4-D and 0.47 µM KIN
T6	Medium+4.52 µM 2,4-D and 2.32 µM KIN
T7	Medium+9.05 µM 2,4-D
T8	Medium+9.05 µM 2,4-D and 0.47 µM KIN
T9	Medium+9.05 µM 2,4-D and 2.32 µM KIN

KIN kinetin

For the nonparametric evaluations, grades from 1 to 3 for callus color (1=light yellow, 2=dark yellow, and 3=brown) and for the development of somatic embryos (1=absence of mature somatic embryos, 2=mature embryos, and 3=germinated embryos) and from 1 to 4 for the texture of embryogenic calli (1=friable, 2=semi-friable, 3=compact, and 4=highly compact) were applied to the 18 treatments (Table 2).

The count data, such as those from somatic embryo production and secondary somatic embryo production, were transformed into $\sqrt{x + 0.5}$, whereas the percentage of oxidation was converted into arcsin $\sqrt{x}$. These data were evaluated using analysis of variance (ANOVA), followed by Tukey's test at a significance level of 5% to compare the means. For further evaluations, the data were likewise analyzed using ANOVA and Tukey's test.

For the nonparametric evaluations, such as somatic embryo color, texture, and development, the Kruskal–Wallis test at a significance level of 5% was used.

Histological procedures. The anatomic aspects of the somatic embryos were evaluated after fixation of the samples in Karnovsky (1965) solution (2.5% glutaraldehyde, 4.0% paraformaldehyde, 3.0% sucrose, and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer [pH 6.8]), under refrigeration. The fixed samples were dehydrated in an alcohol series (10% to 95%)

and embedded in methacrylate (HistoResin, Leica Instruments, Heidelberg, Germany). Serial sections from 5 to 8 μm in thickness were made with the aid of an automated rotary microtome (model RM2155; Leica Microsystems Inc., Deerfield, IL) featuring disposable steel razors. After sectioning, the sections were mounted in Permount and then stained with pH 4.0 toluidine blue (O'Brien and McCully 1981) for 10 min.

Structural analysis of the samples was performed using an optical microscope (Olympus AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo system and digital camera (Spot Insight Color 3.2.0; Diagnostic Instruments Inc., New York, USA). Other images were obtained through stereoscopic microscopy (Olympus SZX), using an image recording system (Olympus E-330).

Results and Discussion

Data analysis. The maturation of anthurium (*A. andraeanum*) cv. Eidibel somatic embryos depended upon several factors, including the nutrient medium and concentrations of 2,4-D and KIN. Despite the fact that the embryogenic calli remained agglomerated, somatic embryos developed normally. Although some embryos developed from globular to mature stages, some embryogenic calli simply proliferated, producing more calli with globular embryos, referred to here as secondary somatic embryogenesis.

Analysis of variance showed that the interactions between the sources of variation in the nutrient medium (Pierik or AA2) and the concentration of 2,4-D and KIN were statistically significant at the 5% probability level, using an *F* test, for the following parameters: the fresh mass of embryogenic calli, for which there was an effect only of the nutrient medium; the production of somatic embryos, for which there was no interaction between factors; the production of secondary somatic embryogenesis, for which there were differences depending on the nutrient medium, the concentration of KIN, and the interaction between the nutrient medium and 2,4-D; and the percentage of browning, for which all factors were significant except the nutrient medium.
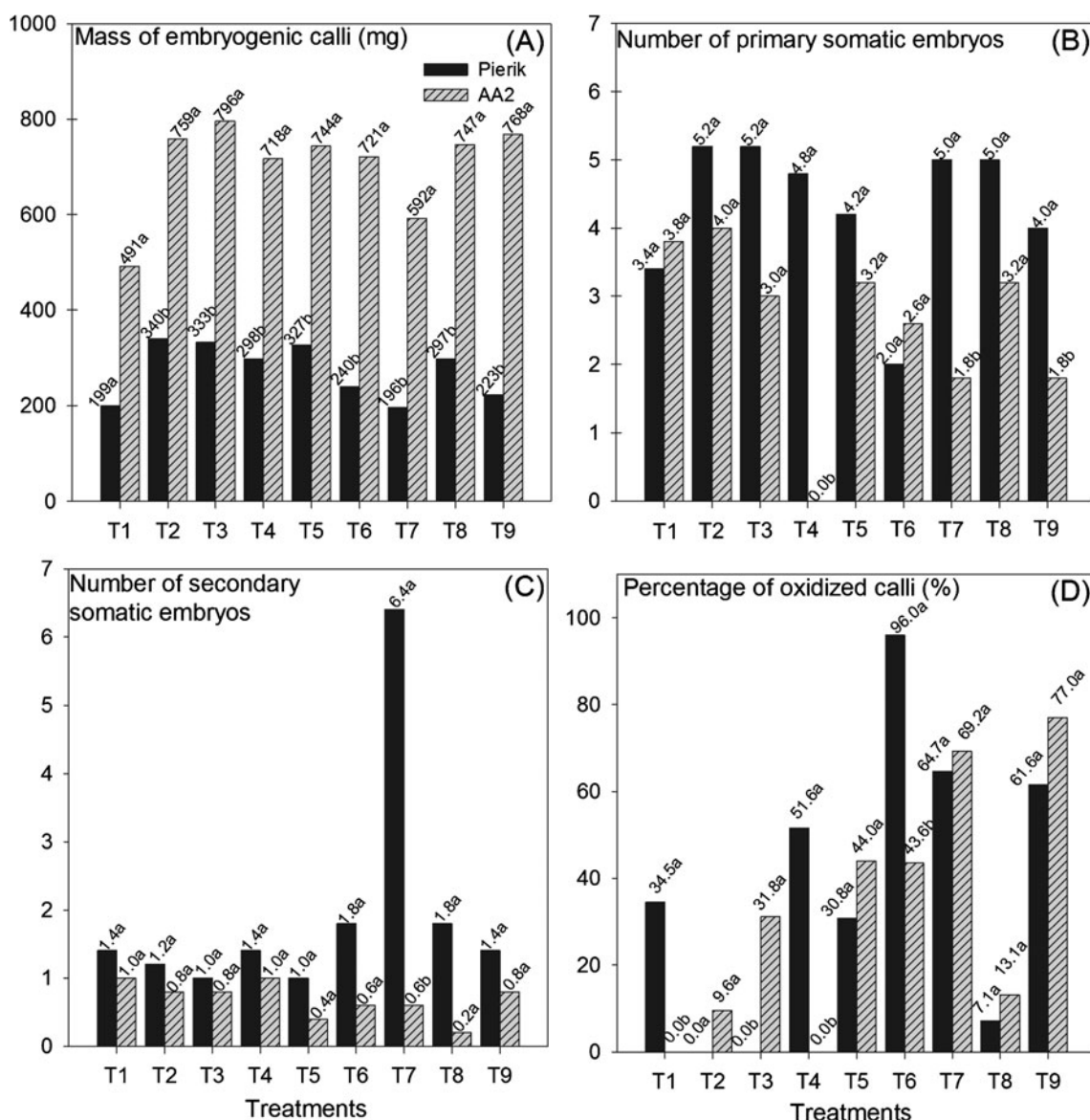
The nutrient medium influenced the development of the fresh mass of embryogenic calli after 45 d of growth. The highest means of fresh mass were observed with the AA2 medium, in which there was great swelling of the embryogenic calli (Fig. 1*A*).

The most suitable nutrient medium for the production of somatic embryos was Pierik medium, producing more embryos per callus piece than most of the treatments with AA2 medium (Fig. 1*B* and Table 1), chiefly T2 (5.2), T3 (5.2), T4 (4.8), T7 (5.0), T8 (5.0), and T9 (4.0). However, only media T4 (4.8), T7 (5.0), and T9 (4.0) resulted in a significantly

Table 2. Treatments for nonparametric evaluations of callus color, texture of embryogenic callus, and development of somatic embryos of anthurium (*Anthurium andraeanum* cv. Eidibel) in Fig. 2

Code	Treatment
T1	Pierik without growth regulators
T2	Pierik+0.47 μM KIN
T3	Pierik+2.32 μM KIN
T4	Pierik+4.52 μM 2,4-D
T5	Pierik+4.52 μM 2,4-D and 0.47 μM KIN
T6	Pierik+4.52 μM 2,4-D+2.32 μM of KIN
T7	Pierik+9.05 μM 2,4-D
T8	Pierik+9.05 μM 2,4-D and 0.47 μM KIN
T9	Pierik+9.05 μM 2,4-D and 2.32 μM KIN
T10	AA2 without growth regulators
T11	AA2+0.47 μM KIN
T12	AA2+2.32 μM KIN
T13	AA2+4.52 μM 2,4-D
T14	AA2+4.52 μM 2,4-D and 0.47 μM KIN
T15	AA2+4.52 μM 2,4-D and 2.32 μM KIN
T16	AA2+9.05 μM 2,4-D
T17	AA2+9.05 μM 2,4-D and 0.47 μM KIN
T18	AA2+9.05 μM 2,4-D and 2.32 μM KIN

KIN kinetin

Figure 1. Fresh mass (*A*), production of primary somatic embryos (*B*), production of secondary somatic embryos (*C*), and percentage of oxidation (browning) (*D*) of embryogenic calli of anthurium (*A. andraeanum* cv. Eidibel) in different treatments (Table 1), evaluated after 45 d of in vitro growth. Values in (*A*–*C*) are per callus. Means followed by a *different letter* within the same treatment are different by the Tukey's test at a 5% probability level.

better embryo production on the Pierik medium. The only treatments that showed better embryo production on the AA2 medium, although not significantly different (Fig. 1*B*), were T1 (3.8) and T6 (2.6), due to the large amount of browning found in these treatments when the Pierik medium was used (Fig. 1*D*).

Sané *et al*. (2006) and Moura *et al*. (2008) used culture media without growth regulators for maturation of somatic embryos of *Phoenix dactylifera* and *Acrocomia aculeata*, respectively. Moura *et al*. (2009) induced maturation and germination of *A. aculeata* of the somatic embryos merely with the addition of activated charcoal to the culture medium. In many embryogenic systems, the transfer of somatic

embryos to a culture medium without growth regulators favors their maturation and conversion into plants (Sivanesan *et al*. 2011). Growth regulators have clearly played a crucial role in eliciting responses (Chitra Devi and Narmathabai 2011). Bautista *et al*. (2008) observed anthurium (*A. andraeanum* Lindl.) somatic embryo maturation using a BA-containing medium. Cytokinins were responsible for the development of somatic embryos in lily (*Lilium longiflorum*) (Nhut *et al*. 2001) and the rubber tree *Hevea brasiliensis* (Muell.) (Kumari Jayasree *et al*. 1999) where the highest number of developing somatic embryos was obtained using a medium supplemented with KIN and NAA. Chitra Devi and Narmathabai (2011) used a

Murashige and Skoog (MS) medium (Murashige and Skoog 1962) without growth regulators for 1 mo for somatic embryo development, followed by transfer to a BA-containing culture medium for 4 d for the further maturation of somatic embryos of *Desmodium motorium*. These results are similar to our findings, where the Pierik medium with 0.47 μM KIN (T2, Table 1) led to adequate production of primary somatic embryos, reduced the production of secondary somatic embryogenesis, and minimized the browning of the explants, demonstrating an ability to promote the maturation of embryos. Deo *et al*. (2010) also observed maturation and germination of *Colocasia esculenta* var. *esculenta* in media with low concentrations of BA and indoleacetic acid.

There was a statistically significant difference in the production of secondary somatic embryos only in T7, which consisted of Pierik medium with 9.05 μM 2,4-D (6.4 secondary somatic embryos per callus) and had markedly higher production than AA2 medium with the same concentration of 2,4-D (0.6 per callus) (Fig. 1C). Hence, it is clear that the induction of embryogenic calli continued when high concentrations of 2,4-D were added to the culture medium, resulting in increased production of embryogenic calli and reduced maturation of the somatic embryos.

Yang *et al*. (2000) and Deo *et al*. (2010) observed high induction rates of somatic embryos using 9.05 μM 2,4-D and 4.52 μM BA for asparagus (*Asparagus officinalis* L.) and 9.05 μM 2,4-D for *C. esculenta* var. *esculenta*, respectively. The present work led to similar outcomes: use of the Pierik medium with 9.05 μM 2,4-D led to a high frequency of induction of secondary somatic embryos. However, one of the probable causes of low conversion rates of somatic embryos in several species may result from residual 2,4-D, which may prevent embryo development and promote proliferation (Cangahuala-Inocente *et al*. 2007; Konieczny *et al*. 2010). Pan *et al*. (2010) suggested that 2,4-D may even inhibit the initiation of somatic embryos of citrus.

Browning was observed at high levels with T6 (96%) when the Pierik medium with 4.52 μM 2,4-D and 2.32 μM KIN was used, compared to the modified AA2 medium containing 300 mg L^{-1} L-glutamine and the same concentrations of growth regulators (Fig. 1D). Aslam *et al*. (2008) stated that the glutamine concentration used here was ideal for the maturation of somatic embryos of *Catharanthus roseus* L. (G.) Don.

The highest levels of browning were also obtained on Pierik medium for treatments T1 and T4 (34.5% and 51.6%, respectively). Unlike the other treatments, browning of the embryogenic calli grown in Pierik medium was not observed with 0.47 μM (T2) and 2.32 μM (T3) KIN, treatments which had the largest production of somatic embryos (Fig. 1B, D).

The color and texture of embryogenic calli have been used as visual indicators of highly regenerable plant material. Embryogenic calli produced during the induction phase of somatic embryogenesis (Fig. 3A) showed a color and texture similar to the calli formed in the culture medium with 9.05 μM 2,4-D (T7; Table 1 and Fig. 2A,B), confirming the values obtained for the production of secondary somatic embryogenesis in these treatments (Fig. 1C).

Less desirable color scores were obtained for T6 (Pierik medium with 4.52 μM 2,4-D and 2.32 μM KIN) and T18 (AA2 medium with 9.05 μM 2,4-D and 0.47 μM KIN), in which brown calli predominated. However, the lowest sum for this parameter was in the Pierik medium with 9.05 μM 2,4-D (T7), in which a dark yellow-colored calli was produced during the embryo induction phase (Fig. 2A and Table 2).

The production of secondary somatic embryos was highest in the Pierik medium with 9.05 μM 2,4-D (T7; Fig. 1C). The effect of this medium on somatic embryogenesis was also seen in the nonparametric parameter color: dark yellow calli were found (Fig. 2A), just as in the calli formed during the induction of somatic embryogenesis (Fig. 3A). Thus, a concentration of 9.05 μM 2,4-D is recommended for the induction of embryogenic calli in nodal segments of *A. andraeanum* cv. Eidibel. This finding is accordance with Rai *et al*. (2007) working with guava, *Psidium guajava*, who found an increase in the induction of embryogenic calli when they were maintained on media with 4.52 or 9.05 μM 2,4-D. Winkelmann *et al*. (2006) and You *et al*. (2011) induced secondary somatic embryogenesis in cyclamen, *Cyclamen persicum* Mill., on a culture medium with 9.05 μM 2,4-D and 0.89 μM BA.

The least desirable texture scores were found with T17 (AA2 medium with 9.05 μM 2,4-D and 0.47 μM KIN), T18 (AA2 medium with 9.05 μM 2,4-D and 2.32 μM KIN), and T6 (Pierik medium with 4.52 μM 2,4-D and 2.32 μM KIN), in which the calli were very compact (Fig. 2C). The most desirable texture scores were observed with T2 (Pierik medium with 0.47 μM KIN), T3 (Pierik medium with 2.32 μM KIN), and T7 (Pierik medium with 9.05 μM 2,4-D), indicating the treatments with the most friable embryogenic calli (Fig. 2B).

The highest scores for somatic embryo development were found with treatments T11 (AA2 medium with 0.47 μM KIN) and T12 (AA2 medium with AA2+2.32 μM KIN), followed by T2 (Pierik medium with 0.47 μM KIN), in which the embryos developed from the maturation stage until germination (Figs. 2C and 3B–E). In the present study, the use of the lowest tested concentration of KIN (0.47 μM) gave the greatest embryo development.

The biggest difference between the Pierik and AA2 culture media is the nitrogen source. The Pierik medium, widely applied for the genus *Anthurium*, has a modified composition of MS salts (Murashige and Skoog 1962) and the nitrogen source is inorganic. The AA2 medium, on the other hand, has been used for somatic embryogenesis of rice, *Oryza sativa*, and the nitrogen source is organic.
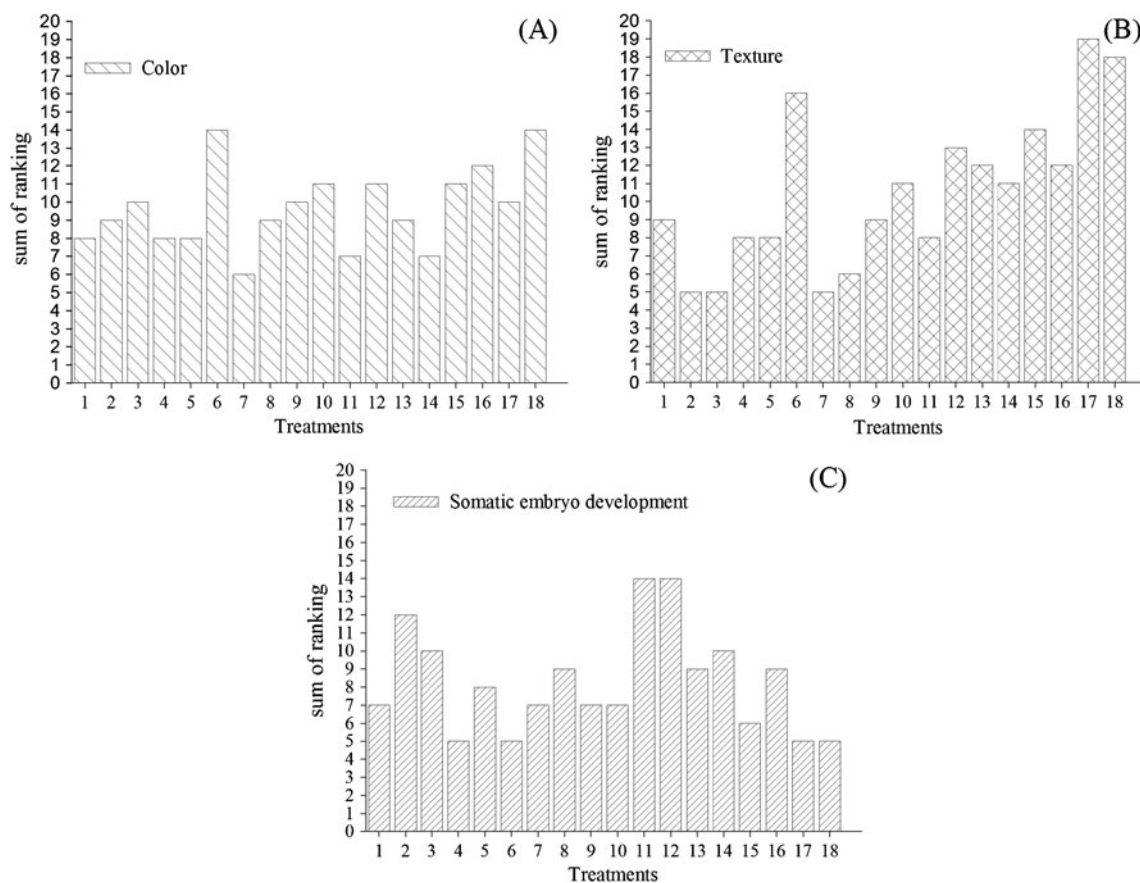
Figure 2. Kruskal–Wallis test for the ranked data of color (*A*), texture (*B*), and embryonic development (*C*) of embryogenic calli of anthurium (*A. andraeanum* cv. Eidibel) in different treatments (Table 2), evaluated after 45 d of in vitro growth.

Morpho-physiological aspects. After 45 d of growth, histological analyses demonstrated the beginning of the formation of somatic embryos (Fig. 3*F*), which were observed in the initial stage of development and showed quite limited evidence of a protoderm. Steinmacher *et al.* (2007) also observed somatic embryos of peach palm, *Bactris gasipaes*, with the same characteristics.

In the embryogenic calli grown on Pierik medium with 0.47 μM KIN, histological analysis revealed globular structures with well-delimited protoderms (Fig. 3*G*). The procambium was also observed, indicating the presence of a closed vascular system and demonstrating signals of polarization (Fig. 3*H*) and the presence of raphides (Fig. 3*I*).

Yu *et al.* (2009), working with embryogenic calli of anthurium (*A. andraeanum* cv. Valentino), also reported a well-defined protoderm and procambium, indicating the presence of a closed vascular system. The proliferation and further development of the somatic embryos proceeded once there was no connection with the mother tissue (Keng *et al.* 2009).

Most of the somatic embryos formed on Pierik medium with 0.47 μM KIN showed polarization, although embryos were observed in different stages of development (Fig. 3*C–E*).

Histological sections during the last development stage of the embryos showed the complete conversion into plants, as evidenced by the presence of primary leaves, apical and root meristem zones, a closed vascular system, an axillary bud, and raphides (Fig. 3*J*) and, hence, displaying the attributes of whole plants (Fig. 3*K–L*).

Figure 3. Maturation of embryogenic cultures of anthurium ▶ (*A. andraeanum* cv. Eidibel), grown on Pierik medium. (*A*) Initial explant (day 0): embryogenic callus cultivated with 10 μM of NAA. (*B–J*) After 45 d of in vitro growth on Pierik medium with 0.47 μM of KIN. (*B*) Embryogenic calli inoculated into Erlenmeyer flasks. (*C*) Embryogenic calli with somatic embryos at different developmental stages. (*D*) Somatic embryos at different stages: globular, coleoptile, juvenile vegetative, and mature. (*E*) Final explant: germinated embryogenic calli with complete maturation. (*F*) Embryogenic callus with initial formation of a somatic embryo (*Eb*) and Callus (*Cl*). (*G*) Meristemoids (*Mt*) and somatic embryos in the globular stage with a well-defined protoderm (*Pt*). (*H*) Somatic embryo in an intermediate developmental stage with a well-defined protoderm and procambium (*Pc*). (*I*) Somatic embryo in an advanced development stage with a defined protoderm and raphide (*Rp*). (*J*) Complete maturation of the somatic embryo into a plant, showing evident apical and root meristematic zones, a vascular system (*Vs*), an axillary bud (*Ab*), primary leaves (*Fp*), and raphides. Shoot apical meristem (*SAM*); root apical meristem (*RAM*). (*K–L*) Plants acclimatized under artificial light and room temperature. *Bars*=200 μm (*A, C, D, E, K*); 10 mm (*B, L*); 300 μm (*F, G, H, I, J*).
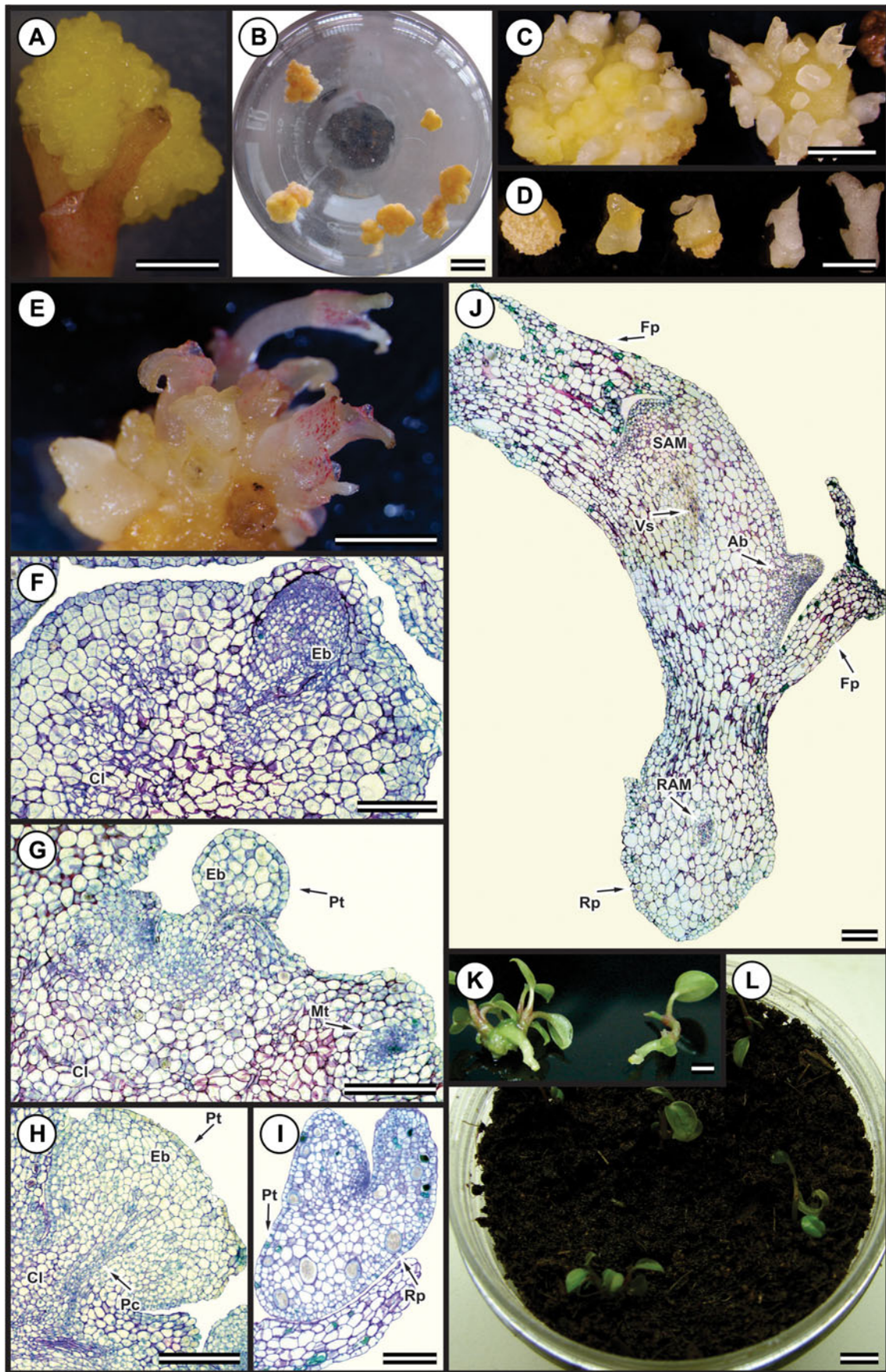
In conclusion, this work resulted in an efficient protocol for the maturation of somatic embryos of *A. andraeanum* cv. Eidibel, in which the Pierik medium with 0.47 μM KIN favored embryo development. The presence of a protoderm, procambium, closed vascular system, primary leaves, apical and root meristem zones, and an axillary bud in the mature somatic embryos was demonstrated. The findings of this study suggest new possibilities for the genetic transformation, germplasm preservation, cryopreservation, synthetic seed production, and rapid propagation of *A. andraeanum*.

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