



Somatic embryogenesis in anthurium (*Anthurium andraeanum* cv. Eidibel) as affected by different explants

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ABSTRACT. This study establishes a protocol for the induction of somatic embryogenesis in *Anthurium andraeanum* cv. Eidibel. The experiment was arranged in a completely randomized 5 x 5 x 5 factorial design using five explant types (whole leaves, half leaves; petiole; nodal segments and root segments) from *in vitro* plantlets; five auxins: indole-3-acetic acid (IAA), α -naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloropicolinic acid (Picloram); at five concentrations (0, 2.5, 5, 7.5 and 10 μ M), with five replications using five Petri dishes. The cultures were maintained in a growth room at $25 \pm 2^\circ\text{C}$ in the dark. The explant type was investigated for the induction of somatic embryogenesis in anthurium cv. Eidibel, and nodal segments were shown to be the most suitable explant for this process. After 60 days in culture, the highest number of embryogenic calli was recorded for the nodal segments cultured in NAA (5, 7.5 and 10 μ M), 2,4-D (10 μ M) and Picloram (7.5 and 10 μ M). The histological analysis confirmed the presence of embryos with established polarization, procambium, ground meristem and protoderm in the nodal segments cultured in Pierik medium containing 10 μ M NAA. After the conversion of the somatic embryos into plantlets, these plantlets were acclimatized transferred to *in vivo* conditions and grown into normal plants.

Keywords: auxins, nodal segments, somatic embryos.

Embriogênese somática em antúrio (*Anthurium andraeanum* cv. Eidibel) afetada por diferentes explantes

RESUMO. O objetivo foi estabelecer um protocolo para indução de embriogênese somática em *Anthurium andraeanum* cv. Eidibel. O experimento foi arranjado em delineamento inteiramente casualizado, com esquema fatorial 5 x 5 x 5, cinco explantes (folha inteira e seccionada ao meio; pecíolo; segmento nodal e de raiz); cinco auxinas (AIA, ANA, AIB, 2,4-D e Picloram) em cinco concentrações (0,0; 2,5; 5,0; 7,5 e 10 μ M) e cinco repetições, consistindo de cinco placas de Petri, mantidas em sala de crescimento a $25 \pm 2^\circ\text{C}$, no escuro. Após 60 dias de cultivo, a maior produção de calos embriogênicos foi registrada nos segmentos nodais cultivados na presença de ANA (5; 7,5 e 10 μ M), 2,4-D (10 μ M) ou Picloram (7,5 e 10 μ M). As análises histológicas confirmaram a presença de embriões com polaridade definida, procâmbio, meristema fundamental e protoderme em segmentos nodais cultivados em meio Pierik com 10 μ M ANA. Depois da conversão dos embriões somáticos, as plantas foram aclimatizadas e transferidas para as condições *in vivo*, com crescimento normal das mesmas.

Palavras-chave: auxinas, segmentos nodais, embriões somáticos.

Introduction

Anthurium Schott is a large neotropical genus belonging to the Araceae family. The majority of the 600 species included in this genus are ornamental. These plants are grown for their brightly colored flower spathes and ornamental leaves. Many of the plants are herbaceous epiphytes native to the warm regions of tropical America, and approximately 130

species are found in Brazil. *Anthurium andraeanum* Linden has been used widely in floriculture and landscaping, and among the tropical ornamentals, it is the second most traded species in the world, surpassed only by orchids (CASTRO et al., 2004; TOMBOLATO et al., 2004).

Propagation using seeds is a lengthy process, resulting in heterogeneous plants, which may vary in vigor, size and productivity and exhibit notable

variations in the color, shape and inflorescence size. On the other hand, asexual propagation is achieved by clump division or the use of single node cuttings along with the division of suckers or offshoots, which allows the spread of pests and diseases and limits the number of available plants (HAMIDAH et al., 1997; TOMBOLATO et al., 2004; VIÉGAS et al., 2007). Therefore, *in vitro* propagation has been used extensively as an important method for the mass production of *A. andraeanum* clones allowing for the uniformity of the plants (FUZITANI; NOMURA, 2004; MAIRA et al., 2010).

The anthurium has been propagated via indirect organogenesis from various explants, such as leaves (ATAK; ÇELİK, 2009; BEYRAMIZADE et al., 2008; NHUT et al., 2006; PIERIK, 1975; PIERIK et al., 1974; PUCHOOA, 2005; TENG, 1997; VIÉGAS et al., 2007; YU et al., 2009), petioles (KUEHNLE; SUGIL, 1991; YU et al., 2009), axillary buds (KUNISAKI, 1980), fruits (SANTOS et al., 2005), seeds (MAIRA et al., 2010; SCHIAVINATO et al., 2008), roots (CHEN et al., 1997) or anthers (WINARTO et al., 2010, 2011a and b). However, traditionally, young leaf sections have been used to establish calli and the further differentiation of adventitious shoot buds *in vitro* (ATAK; ÇELİK, 2009; NHUT et al., 2006; PIERIK, 1975; PIERIK et al., 1974; TOMBOLATO; QUIRINO, 1996).

Currently, the anthurium species are propagated *in vitro* using somatic embryogenesis from leaves (BAUTISTA et al., 2008; DUQUENNE et al., 2007; HAMIDAH et al., 1997; KUEHNLE et al., 1992; TECHATO et al., 2006), petioles (FITCH et al., 2011; HAMIDAH et al., 1997; KUEHNLE et al., 1992), nodal and internodal segments and root explants (FITCH et al., 2011).

Somatic embryogenesis has a number of advantages over other techniques because its high level of automation (bioreactors) allows large-scale plant production and the reduction of labor input which reduces the cost per unit produced significantly. In addition, the synchronization of somatic embryo development can be achieved, resulting in a high level of clonal uniformity and genetic conformity, which makes somatic embryogenesis an essential method for the improvement of the majority of economically important plants (LU et al., 2011; NINKOVIĆ et al., 2010; PINTO et al., 2011; SIVANESAN et al., 2011; VON ARNOLD et al., 2002; YANG et al., 2010; YEUNG, 1995). The method is also useful for various applications, such as artificial seed production, germplasm conservation (cryopreservation), and transgenic plant development (BAKHSHAIIE et al., 2010; FITCH et al., 2011; KONIECZNY et al., 2010; QUIROZ-FIGUEROA et al., 2006).

In this study, somatic embryogenesis in *A. andraeanum* cv. Eidibel is induced to evaluate the morphogenetic responses relative to the explant source and the type and concentration of auxin. In addition, the development of the embryogenic calli produced is examined histologically.

Material and methods

Experimental site and plant material

The Agronomic Institute of Campinas (IAC) provided *A. andraeanum* of cv. Eidibel plantlets that were established *in vitro* from the indirect organogenesis of a young leaf-derived callus. These *in vitro* regenerated plantlets were multiplied by the successive subculture of single-bud nodal segments every four weeks. The explants, five in total, were inoculated into 220-mL glass jars containing 30 mL of Pierik medium (PIERIK, 1976) supplemented with 4.44 μM BA and 0.54 μM NAA; the pH was adjusted to ± 5.7 prior to autoclaving and the medium was solidified using 6.5 g L⁻¹ agar (Merck®, Germany). The cultures were maintained in a growth chamber at 25 $\pm$ 2°C, 36 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and a 16-hour photoperiod.

Embryogenic cultures

Single-bud nodal segments (Figure 3B), petiole (Figure 3C), root segments without the apex (Figure 3D), and whole leaves (Figure 3E) and half leaves sectioned perpendicular to the midrib (Figure 3F) were obtained from the *in vitro* *A. andraeanum* cv. Eidibel (Figure 3A) clones. The explants measured approximately 1.0 cm in length, and the leaf explants were measured based on the midrib length. The leaf explants were cultured with the adaxial surface facing up, whereas the petiole and the nodal and root segments were inoculated on to the medium horizontally. The explants were placed in 90 x 15 mm Petri dishes containing 25 mL of Pierik medium supplemented with 20 g L⁻¹ sucrose and 6.5 g L⁻¹ agar, the pH was adjusted to 5.8, and the medium was autoclaved at 121°C for 15 minutes. The medium was supplemented with different concentrations (0, 2.5, 5, 7.5, 10 μM) of the auxins IAA, IBA, NAA, 2,4-D and Picloram. The cultures were maintained in a growth chamber at 25 $\pm$ 2°C in the dark for 60 days.

Statistical analysis

The treatments were arranged in a completely randomized 5 x 5 x 5 factorial design using five explant types (whole leaves, half leaves, petioles, nodal segments and root segments), five auxins (IAA, NAA, IBA, 2,4-D and Picloram) at five concentrations

(0, 2.5, 5, 7.5 and 10 μM) plus five control plates (consisting of the five explants types cultured on the same medium but lacking growth regulators), with five replications. Each replication consisted of five Petri dishes containing nine explants per dish, totaling 45 explants per treatment. After 60 days, the morphogenetic responses were evaluated for the production of the somatic embryogenesis of shoots and roots. The data were processed and analyzed using SAS software (SAS, 2001).

The homogeneity, normality and independence of the data were assessed using Bartlett, Kolmogorov-Smirnov and Durbin Watson's tests, respectively. The count data, such as the occurrence of embryogenic calli, shoots and roots were transformed to $\sqrt{x+0.5}$ and were examined by analysis of variance. The means were compared using Tukey's test at a 5% probability level.

A regression analysis related to the growth regulator concentrations in which the quantitative data were significant by Tukey's test for the production of embryogenic calli was performed. The significance of the regression was assessed using the F test, whereas the significance of the coefficients was assessed using the 't' test. The regression adjustment was performed using the adjusted determination coefficient (R^2). The data applied to the adjustment of regressions were transformed as $\sqrt{x+0.5}$. The regression analyses were performed using the statistical software SISVAR (FERREIRA, 2003).

Histological analyses

The histochemical analyses of the embryogenic calli were performed using double staining with acetocarmine (2%) and Evan's blue (0.1%) (DURZAN, 1988), and Lugol solution (0.1%) was used for starch detection (JENSEN, 1962).

The anatomical aspects of the somatic embryogenesis were assessed by fixing the samples in Karnovsky (1965) solution (2.5% glutaraldehyde, 4% paraformaldehyde, 3% sucrose, and 5 mM CaCl_2 in 0.1 M cacodylate buffer) under refrigeration. The samples were dehydrated using a series of increasing concentrations of ethanol solutions (10 to 95%) and were embedded in methacrylate resin (Historesin Leica® Instruments, Heidelberg, Germany). The thin sections (5-8 μm) were cut using a rotary automatic microtome (RM 2155, Leica Microsystems Inc., Deerfield, USA) equipped with disposable steel blades and were stained in toluidine blue, pH 4.0 (O'BRIEN; McCULLY, 1981), for 10 minutes. After drying, the glass slides were mounted in Permount® synthetic resin (Daigger, USA).

The structural analysis and photography were performed using an Olympus AX70TRF microscope (Olympus Optical, Japan) equipped with a U-Photo camera system (Spot Insightcolour 3.2.0, Diagnostic Instruments Inc., USA). The remaining photographs were taken using an Olympus SZX stereo microscope with a coupled with a camera system (Olympus E-330).

Results and discussion

Data analysis

The induction of somatic embryogenesis in *A. andraeanum* cv. Eidibel is affected by three main factors: the source of the explants and the type and concentration of the auxin. In this study, we observed differences in the morphogenetic responses among the explants tested regarding the development of embryogenic calli, shoots and roots. The most competent explants for the induction of embryogenic calli were derived from the petioles and the nodal segments; the nodal segments were the most responsive (Figure 4A). In contrast, the whole leaf, sectioned leaf and root segments did not produce calli with embryogenic capacities, becoming oxidized after five weeks in culture. However, the root explants produced roots, even after oxidization.

The growth responses under the *in vitro* conditions can vary with the explant type, and genotype is a major factor influencing the development and the physiology of explants in tissue culture (KRISHNARAJ; VASIL, 1995). Vasudevan and Van Staden (2011) reported that shoot-tip explants of leopard orchid (*Ansellia Africana* Lindl.) responded better in terms of shoot length and leaf number than nodal segments. In anthurium, the callus formation was significantly affected by the genotype, and the type and color of callus obtained from each cultivar differed (TE-CHATO et al., 2006).

Similar results were obtained by Pierik et al. (1979) in experiments testing callus formation in leaf explants from 38 genotypes of *A. andraeanum*; 31 of the genotypes demonstrated satisfactory callus production, four genotypes gave little callus formation and three genotypes demonstrated no response. Nhut et al. (2006) also tested leaf explants from ten cultivars of *A. andraeanum* and determined that after 30 days in culture, two cultivars formed calli and after 60 days, 8 of the 10 cultivars demonstrated callus production. Therefore, because of the genotype-dependence of the morphogenetic responses, not all *Anthurium* species can be propagated *in vitro*.

The analysis of variance showed a significant triple interaction for the sources of the variation (explant type and the type and concentration of the auxin), using the F test for the independent variables.

Although the Kolmogorov-Smirnov and Bartlett's tests were significant for shoot production, the Durbin Watson's test was not significant at a 5% significance level. For the variables for embryogenic callus and root production, all tests were significant at 5% significance level. Thus, because at least two of the assumptions were not fulfilled, the data for all the variables were transformed to $\sqrt{x+0.5}$, and the analyses were rerun on the transformed data at 5% significance level.

There was no induction of somatic embryogenesis, or the induction was relatively low, using the media supplemented with IAA and IBA, regardless the concentration, for all explant types tested. The nodal segment was the most responsive explant type for embryogenic callus induction, showing the highest means on media containing NAA, Picloram and 2,4-D (Figure 1A; Table 1). In contrast, Kuehnle et al. (1992) demonstrated that using a modified half-strength MS medium (MURASHIGE; SKOOG, 1962) supplemented with 6.79 μM 2,4-D and 2.33 μM 6-furfuryl aminopurine (kinetin), the somatic embryo production was higher in the leaf explants compared with the petioles from *A. andraeanum* hybrids.

For calli production affected by NAA, the cubic model ($R^2 = 99.75\%$) for the regression displayed the greater adjustment of transformed data for the callus yield, whereas the quadratic ($R^2 = 99.75\%$) and linear model ($R^2 = 84.69\%$) fitted better to the callus yield as affected by 2,4-D and Picloram, respectively (Figure 2). With the exception of the regression coefficient b1 for 2,4-D, all the remaining regression coefficients were significant ($p < 0.05$). The regression analysis indicated that the maximum average values for the induction of embryogenic calli were achieved at 10.0 μM NAA, 2,4-D and Picloram (Figure 2A, B and C, respectively).

We observed that the highest frequencies of embryogenic callus induction from nodal segments occurred only on medium supplemented with NAA (5, 7.5 and 10 μM), 2,4-D (10 μM) and Picloram (7.5 and 10 μM) (Figure 1Aa, Ad and Ae; Table 1). Biologically, 10 μM NAA (4.6) was more efficient at establishing embryogenic cultures from nodal segments of

A. andraeanum cv. Eidibel (Figures 1Aa, 2A and 4G), despite the higher numbers of embryogenic calli obtained using 7.5 μM Picloram (6.0) (Figure 1Ae; Table 1). NAA was also used for the induction of somatic embryogenesis in transformed explants of *Medicago truncatula* on medium supplemented with 10 μM NAA and 4 μM BA (NOLAN et al., 2009). In *Eucalyptus camaldulensis*, lower levels of 2,4-D (0.45, 2.26, 4.52 μM) induced loose and whitish calli, whereas at higher concentrations (9.05 μM), the callus was hard (PRAKASH; GURUMURTHI, 2010). However, the same authors reported that a friable nodular callus was formed on basal medium containing NAA (10.75 or 16.13 μM).

Prakash and Gurumurthi (2010) reported that the frequency of callus induction and the type of callus generated varied depending on the explant and type and level of auxin used. In this study, after 45 days in culture, the whole leaves plated on medium containing 10 μM 2,4-D produced a yellowish-white callus mass in only one of the nine explants plated (Figures 1Ad and 2B; Table 1). Different results were reported in a study by Bautista et al. (2008), demonstrating reporting that leaf segments of *A. andraeanum* var. Lambada produced calli after 30 days in culture. Hamidah et al. (1997), testing three explant types (leaves, petioles and roots) of *A. scherzerianum*, also observed that leaf explants were more responsive than petioles and roots after 45 days in culture. Yu et al. (2009) tested leaf and petiole explants of *A. andraeanum* cv. Valentino and observed higher callus production in the leaf explants 30 days after inoculation. In these studies, the petioles produced calli at both ends of the explants (HAMIDAH et al., 1997; YU et al., 2009), which is similar to our observations for petiole and nodal segments of the cultivar Eidibel (Figure 4B).

The inoculation of nodal segments on medium supplemented with 10 μM 2,4-D increased the frequency of embryogenic cultures (Figure 1Ad; Table 1). A number of studies have indicated a key role for auxins, especially 2,4-D, in somatic embryogenesis. The auxin 2,4-D is considered the most efficient for the induction of higher frequencies of somatic embryogenesis in anthuriums (BAUTISTA et al., 2008; HAMIDAH et al., 1997; KRISHNARAJ; VASIL, 1995; KUEHNLE et al., 1992). However Sivanesan et al. (2011) demonstrated a gradual reduction in the frequency and number of somatic embryogenesis with increased concentrations of 2,4-D.

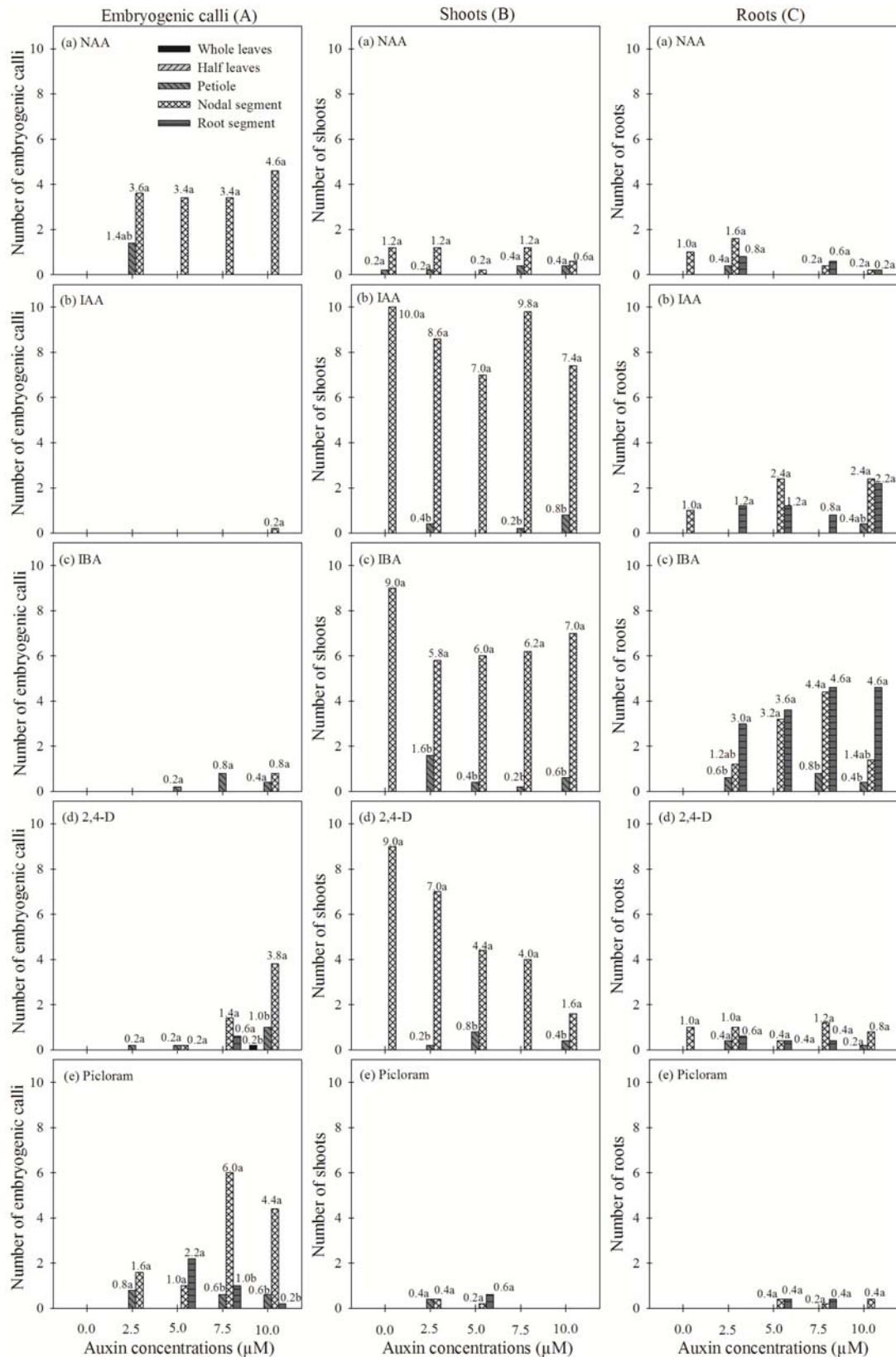


Figure 1. Mean number of embryogenic calli (A), shoots (B) and roots (C) from different sources of explants of *Anthurium andraeanum* cv. Eidibel, as affected by auxin type and concentration, after 60 days of culture *in vitro*. Means followed by the same letter on different auxin concentration are not significantly different according to Tukey test ($p \leq 0.05$). Coefficients of variation (CV) were 27.29% (A), 20.71% (B) and 31.37% (C).

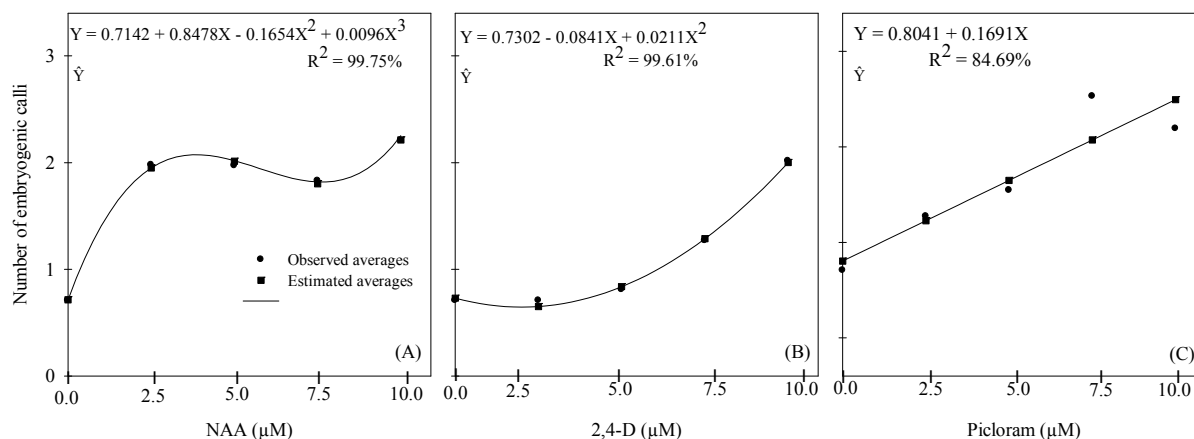


Figure 2. Regression analysis for embryogenic calli as affected by auxins concentrations (0.0; 2.5; 5.0; 7.5; 10.0) (A) NAA; (B) 2,4-D and (C) Picloram from transformed data.

According to Lema-Rumińska (2011), 2,4-D in the culture medium did not influence the ploidy levels of regenerated cactus (*Copiapoa tenuissima* Ritt. forma *monstruosa*) tissue. However, the prolonged maintenance of embryogenic calli on medium containing 2,4-D may induce epigenetic changes affecting the embryogenic potential of the cultures. It has been shown that somatic embryos become auxin-habituated when maintained in the presence of this growth regulator for long periods, leading to the loss of maturation capacity and the ability of the embryos to convert into plants. Accordingly, Pan et al. (2010) determined that 2,4-D also exerts a negative effect on the growth and development of plant tissues and organs cultured *in vitro*. Moreover, the authors suggest that 2,4-D inhibits somatic embryo initiation at least in part, by inducing osmotic stress in callus cells. Cangahuala-Inocente et al. (2007) demonstrated that in a somatic embryogenesis system, the reduction in the conversion rates of somatic embryos may be caused by the residual effects of the 2,4-D used in the induction phase and that these effects can be minimized by replacing the auxin with Picloram.

Picloram, at concentrations of 7.5 and 10 μM, induced higher frequencies of embryogenic calli in nodal segments than in the other explants (Figures 1Ae and 2C; Table 1). Picloram has been used successfully to induce embryogenic callus formation in peach palm (*Bactris gasipaes*), macaw palm (*Acrocomia aculeata*), and spring snowflake (*Leucojum vernum* L.) among other species (MOURA et al., 2008, 2009, 2010; PTAK, 2010; STEINMACHER et al., 2007). Picloram also induces somatic embryogenesis in date palm (*Phoenix dactylifera* L.), although a high frequency of hyperhydricity and oxidation was observed in these cultures (OTHMANI et al., 2009).

The highest means of shoot production means were obtained from the nodal segments, which were higher than the shoot production means in the other explant types in most treatments. This superiority is likely due to the proliferation of shoots from the pre-existing axillary buds, even when grown in medium in the absence of growth regulators. The highest shoot production occurred in the nodal segments inoculated on medium supplemented with IAA and IBA (Figures 1Bb, Bc and 3J; Table 1). However, in medium supplemented with 2,4-D, a reduction in the shoot production rate was observed with increasing concentrations of this auxin (Figure 1Bd; Table 1). In media supplemented with Picloram and NAA, the shoot production was low compared with the other treatments (Figure 1Ba and Bc; Table 1).

In most treatments, the highest mean of root production was observed in the nodal segments and in the root explants themselves. IBA was more effective at in producing roots than the other auxins. The highest means were achieved at concentrations of 5 and 7.5 μM IBA for nodal segments and 2.5, 5, 7.5 and 10 μM IBA for the root explants (Figure 1Cc; Table 1). The highest means for media supplemented with IAA were recorded at concentrations of 5 and 10 μM for the nodal segments and 2.5, 5 and 10 μM for the root explants. The explants cultured on medium containing NAA, 2,4-D or Picloram (Figure 1Ca, 1Cd and 1Ce; Table 1) demonstrated low root production compared to the treatments with IBA or IAA. Therefore, root production was lower in the treatments that stimulated the formation of embryogenic calli, i.e., with the addition of NAA, 2,4-D and Picloram.

Table 1. Standard deviation of embryogenic calli, shoots and roots from different sources of explants of *Anthurium andraeanum* cv. Eidibel, as affected by auxin type and concentration, after 60 days of in vitro culture.

Auxin/Explant	Standard deviation		
	Embryogenic calli	Shoot	Root
0.0 μ M NAA/Pt	-	$\pm$ 0.4472	-
0.0 μ M NAA/NS	-	$\pm$ 4.2779	$\pm$ 0.5477
2.5 μ M NAA/Pt	$\pm$ 1.1402	$\pm$ 0.4472	$\pm$ 0.5477
2.5 μ M NAA/NS	$\pm$ 1.8166	$\pm$ 1.3038	$\pm$ 1.3416
2.5 μ M NAA/Rt	-	-	$\pm$ 0.8366
5.0 μ M NAA/NS	$\pm$ 0.5477	$\pm$ 0.4472	-
7.5 μ M NAA/Pt	-	$\pm$ 0.5477	-
7.5 μ M NAA/NS	$\pm$ 2.7928	$\pm$ 0.8367	$\pm$ 0.8944
7.5 μ M NAA/Rt	-	-	$\pm$ 0.8944
10.0 μ M NAA/Pt	-	$\pm$ 0.8944	-
10.0 μ M NAA/NS	$\pm$ 2.6077	$\pm$ 0.8944	$\pm$ 0.4472
10.0 μ M NAA/Rt	-	-	$\pm$ 0.4472
0.0 μ M IAA/NS	-	$\pm$ 4.2779	$\pm$ 0.5477
2.5 μ M IAA/Pt	-	$\pm$ 0.5477	-
2.5 μ M IAA/NS	-	$\pm$ 0.866	-
2.5 μ M IAA/Rt	-	-	$\pm$ 1.7889
5.0 μ M IAA/NS	-	$\pm$ 1.5811	$\pm$ 1.1402
5.0 μ M IAA/Rt	-	-	$\pm$ 1.3038
7.5 μ M IAA/Pt	-	$\pm$ 0.4472	-
7.5 μ M IAA/NS	-	$\pm$ 1.9203	-
7.5 μ M IAA/Rt	-	-	$\pm$ 0.8292
10.0 μ M IAA/Pt	-	$\pm$ 0.8366	$\pm$ 0.8944
10.0 μ M IAA/NS	$\pm$ 0.4472	$\pm$ 0.5477	$\pm$ 1.1402
10.0 μ M IAA/Rt	-	-	$\pm$ 2.0494
0.0 μ M IBA/NS	-	$\pm$ 4.2779	-
2.5 μ M IBA/Pt	-	$\pm$ 1.1402	$\pm$ 0.8944
2.5 μ M IBA/NS	-	$\pm$ 1.0954	$\pm$ 1.0954
2.5 μ M IBA/Rt	-	-	$\pm$ 1.5811
5.0 μ M IBA/Pt	$\pm$ 0.4472	$\pm$ 0.8944	-
5.0 μ M IBA/NS	-	$\pm$ 1.5811	$\pm$ 3.4187
5.0 μ M IBA/Rt	-	-	$\pm$ 2.0616
7.5 μ M IBA/Pt	$\pm$ 0.8367	$\pm$ 0.4472	$\pm$ 0.8367
7.5 μ M IBA/NS	-	$\pm$ 0.8366	$\pm$ 2.0736
7.5 μ M IBA/Rt	-	-	$\pm$ 2.5099
10.0 μ M IBA/Pt	$\pm$ 0.8944	$\pm$ 0.5477	$\pm$ 0.8944
10.0 μ M IBA/NS	$\pm$ 1.0954	$\pm$ 1.0	$\pm$ 1.5166
10.0 μ M IBA/Rt	-	-	$\pm$ 2.4083
0.0 μ M 2,4-D/NS	-	$\pm$ 4.2779	$\pm$ 0.5477
2.5 μ M 2,4-D/Pt	$\pm$ 0.4472	$\pm$ 0.4472	$\pm$ 0.5477
2.5 μ M 2,4-D/NS	-	$\pm$ 1.2247	$\pm$ 1.2247
2.5 μ M 2,4-D/Rt	-	-	$\pm$ 0.8944
5.0 μ M 2,4-D/Pt	$\pm$ 0.4472	$\pm$ 0.8366	-
5.0 μ M 2,4-D/NS	$\pm$ 0.4472	$\pm$ 2.7019	$\pm$ 0.5477
5.0 μ M 2,4-D/Rt	-	-	$\pm$ 0.5477
7.5 μ M 2,4-D/NS	$\pm$ 1.6733	$\pm$ 2.4495	$\pm$ 1.6432
7.5 μ M 2,4-D/Rt	$\pm$ 0.8944	-	$\pm$ 0.5477
10.0 μ M 2,4-D/HL	$\pm$ 0.4472	-	-
10.0 μ M 2,4-D/Pt	$\pm$ 0.7071	$\pm$ 0.8944	$\pm$ 0.4472
10.0 μ M 2,4-D/NS	$\pm$ 2.1679	$\pm$ 1.9494	$\pm$ 1.7889
2.5 μ M Picloram/Pt	$\pm$ 0.8367	$\pm$ 0.8944	-
2.5 μ M Picloram/NS	$\pm$ 2.1909	$\pm$ 0.5477	-
5.0 μ M Picloram/NS	$\pm$ 1.9235	$\pm$ 0.4472	$\pm$ 0.5477
5.0 μ M Picloram/Rt	$\pm$ 0.5477	$\pm$ 0.5477	$\pm$ 0.5477
7.5 μ M Picloram/Pt	$\pm$ 0.8944	-	-
7.5 μ M Picloram/NS	$\pm$ 1.8708	-	$\pm$ 0.4472
7.5 μ M Picloram/Rt	$\pm$ 1.4142	-	$\pm$ 0.5477
10.0 μ M Picloram/Pt	$\pm$ 0.5477	-	-
10.0 μ M Picloram/NS	$\pm$ 1.5166	-	$\pm$ 0.5477
10.0 μ M Picloram/Rt	$\pm$ 0.4472	-	-

The concentration and type of auxin used directly influences rhizogenesis. Different auxins can induce rooting and the most commonly used are IBA, IAA and NAA. Other auxins, including 2,4-D (CHEN et al., 2010; LEMA-RUMIŃSKA, 2011; YANG et al., 2010; ZHANG et al., 2010; SIVANESAN et al., 2011) and Picloram (MOURA

et al., 2008, 2009, 2010; OTHMANI et al., 2009; PTAK, 2010; STEINMACHER et al., 2007), are important growth regulators that are used in the majority of embryogenic cell and tissue culture systems in most plant species. NAA is commonly used in isolation media, although when added at concentrations above a few tenths of a milligram, this auxin tends to stimulate callus formation in a manner similar to that observed in this study for the cultivar Eidibel.

Morpho-histology

The whole leaf, sectioned leaf and root explants, regardless of the type and concentration of auxin, did not adequately form embryogenic calli or shoots. Many of these explants became yellowish and/or were oxidized after five weeks in culture (Figure 3G-H), the swelling of the buds, the development of roots in nodal segments (Figure 3J) and the proliferation of roots in root segments (Figure 3L). After five weeks in culture, the was swelling and oxidation of nodal segments and petiole explants was observed, leading to the formation of pale yellow and semi-friable primary calli with radial growth (Figure 3I and K).

The histochemical analyses confirmed the embryogenic nature of the calli (Figure 4B) by double staining with acetocarmine and Evans blue. The calli reacted strongly with the acetocarmine, revealing their embryogenic characteristics (Figure 4C). The Lugol test revealed large quantities of starch grains, corroborating that the calli are embryogenic structures (Figure 4D).

The histological sections were prepared from calli formed in the nodal segments that were maintained for 60 days in Pierik medium supplemented with NAA, Picloram and 2,4-D. The analysis of these sections demonstrated that the calli developed at different times and in different manners. The somatic embryos formed on the Pierik medium containing 10 μ M of 2,4-D contained procambium and exhibited a well-defined delimitation of the protoderm layer (Figure 4E). In contrast, the embryogenic calli formed on 10 μ M Picloram (Figure 4F) produced embryos in early developmental stages, displaying well-defined protoderm but no signs of vascularization. On the other hand, the somatic embryos produced in the Pierik medium supplemented with 10 μ M NAA demonstrated procambium, the delimitation of the protoderm layer and early signs of polarization (Figure 4G), which are important indicators of somatic embryo maturation and conversion into plants (Figure 4H-K).

Embryo polarity can be affected by an increase in endogenous auxin levels caused by high concentrations of exogenous auxin in the culture medium (FISCHER; NEUHAUS, 1996). Filippi et al. (2001) induced embryogenic calli from banana (*Musa* spp.) explants on medium supplemented with 5 and 10 μ M Picloram; however the embryos were not functional, demonstrating that the quality of embryos produced is essential for their successful conversion into plants.

The genotype, explant type, growth phase, and the type and concentration of exogenous growth regulators markedly affected the acquisition of embryogenic competence in *Acca sellowiana* (GUERRA et al., 2001). Our study corroborates this finding, considering that the induction of embryogenic cultures in *A. andraeanum* cv. Eidibel varied with the explant type and the concentration and type of growth regulator.

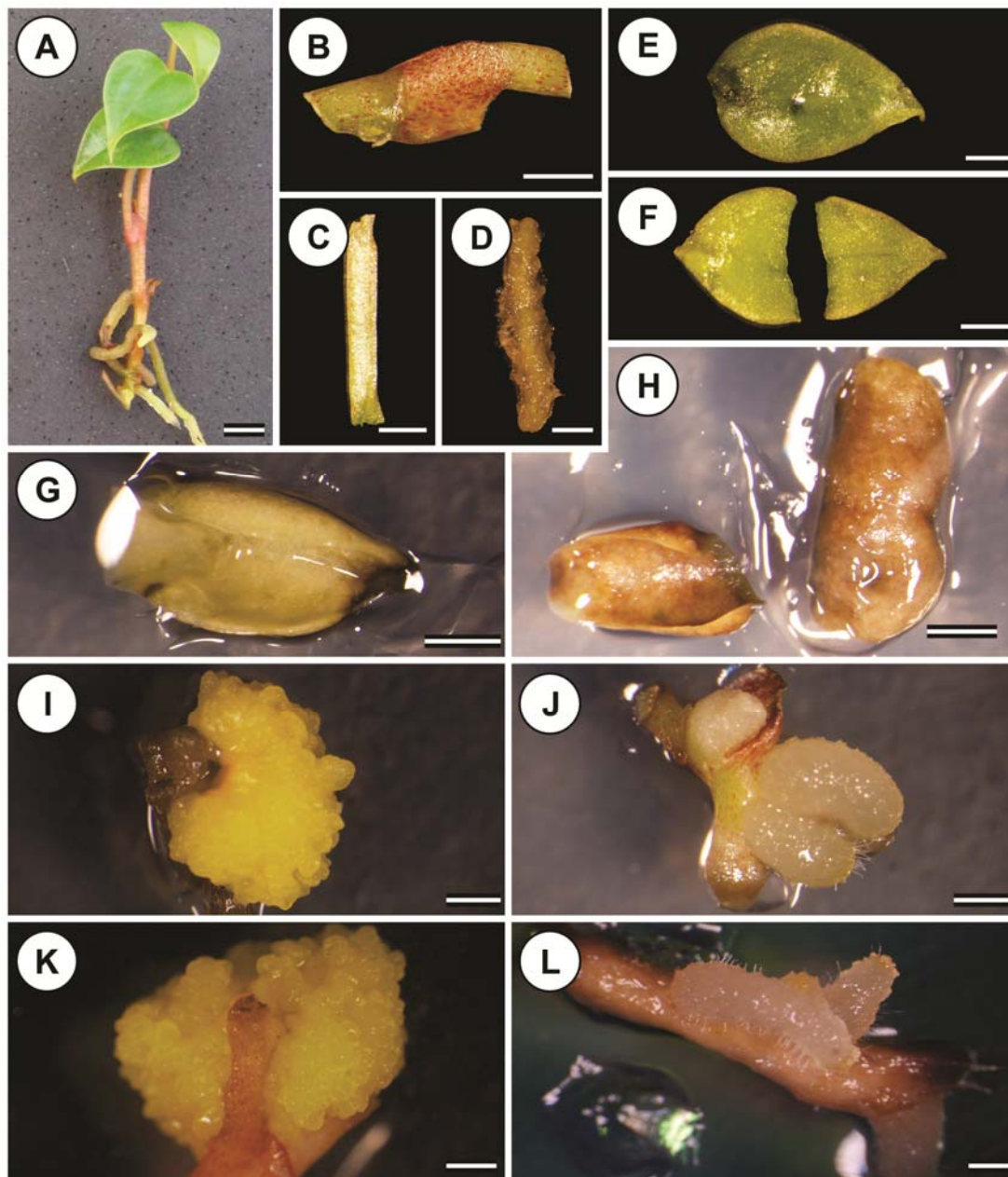


Figure 3. Embryogenic calli from *Anthurium andraeanum* cv. Eidibel explants cultured on Pierik medium, after 60 days in culture. (A) *In vitro* plantlet used as source of explants. (B) Nodal segment. (C) Petiole explant. (D) Root segment. (E) Whole leaf. (F) Sectioned leaf. (G) Whole leaf becoming oxidized. (H) Sectioned leaf becoming oxidized. (I) Petiole forming embryogenic calli on Pierik medium with 2.5 μ M NAA. (J) Nodal segment forming roots and shoots on Pierik medium with 7.5 μ M IBA. (K) Nodal segment forming embryogenic calli on Pierik medium with 10 μ M NAA. (L) Root segment forming roots on Pierik medium with 10 μ M IBA. Bars: A: 5 mm, C - L: 1 mm.

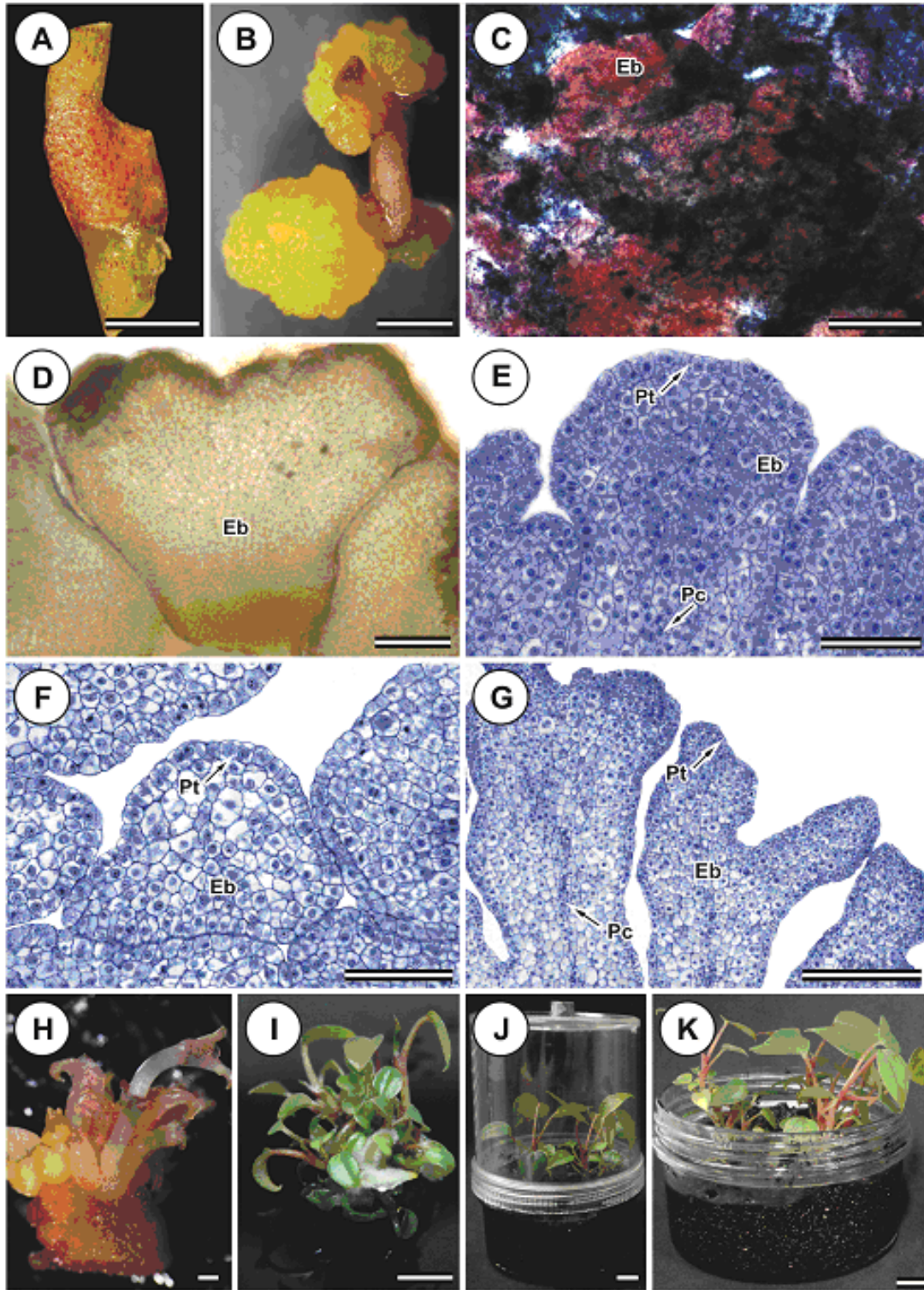


Figure 4. Somatic embryogenesis in nodal segments of *Anthurium andraeanum* cv. Eidibel. (A) Nodal segment cultured in Pierik medium with 10 μ M NAA. (B) Nodal segment forming embryogenic calli on Pierik medium with 10 μ M NAA, after 60 days of culture *in vitro*. (C) Embryogenic culture, on Pierik medium with 10 μ M NAA, with proembryogenic cells stained with acetocarmine. (D) Embryogenic culture, on Pierik medium with 10 μ M NAA, stained with Lugol solution showing starch grains. (E-G) Cross sections of embryos at 60 days of culture *in vitro*. (E) Somatic embryos on Pierik medium with 10 μ M 2,4-D, forming procambium and protoderm. (F) Initial embryo development on Pierik medium with 10 μ M Picloram, showing protoderm. (G) Somatic embryos on Pierik medium with 10 μ M NAA, forming procambium and protoderm, after 60 days of culture *in vitro*. (H) Embryogenic calli showing fully mature embryos. (I) Plantlets converted from somatic embryos (J-K) Plants acclimatized under artificial light and room temperature. Eb: embryos; Pc: procambium; Pt: protoderm. Bars: A, H: 500 μ m; B, D: 200 μ m; C: 300 μ m; E, F, G: 150 μ m; I, J, K: 10 mm.

Conclusion

In this study, we developed a highly efficient protocol for the induction of somatic embryogenesis in anthurium (*Anthurium andraeanum* cv. Eidibel) using nodal segments from *in vitro* established plantlets that were cultured in Pierik medium supplemented with 10 μ M NAA. This auxin resulted in a well-defined procambium, a clear delimitation of the protoderm layer and signs of polarization, which are important features for further maturation of the somatic embryo and the effective conversion into plants. This protocol is useful not only for large-scale vegetative propagation but also for genetic transformation or synthetic seed production and can be combined with other biotechnological approaches.

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