Chapter 24

Somatic Embryogenesis in *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae)

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Abstract

This chapter deals with the features of somatic embryogenesis (SE) in *Araucaria angustifolia*, an endangered and native conifer from south Brazil. In this species SE includes the induction and proliferation of embryogenic cultures composed of pro-embryogenic masses (PEMs), which precede somatic embryos development. *A. angustifolia* SE model encompasses induction, proliferation, pre-maturation, and maturation steps. Double-staining with acetocarmine and Evan's blue is useful to evaluate the embryonic somatic structures. In this chapter we describe *A. angustifolia* SE protocols and analyzes morphological features in the different SE developmental stages.

Key words Conifers, Forest biotechnology, Germplasm conservation, Plant cell culture, Plant physiology, Somatic embryogenesis

1 Introduction

The Brazilian pine *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae) is a native conifer with relevant economic importance in Brazil, representing the most exploited timber source until the 1970s [1]. Uncontrolled exploitation of the high-quality wood has led to the species classification as critically endangered in the International Union for the Conservation of Nature and Natural Resources Red Book [2]. In the last years, it has been suggested for *A. angustifolia* conservation integrated ex situ and in situ strategies to conserve genetic resources [3]. In addition, the maintenance of ex situ seed banks is not feasible for recalcitrant seeds, such as *A. angustifolia* requiring the use of in vitro techniques to germplasm conservation [4].

Biotechnological tools have a large potential in breeding and biodiversity conservation programs for woody species [5].

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In this sense, somatic embryogenesis (SE) has been successfully applied for somatic cells and viable embryos obtaining, in a morphogenetic process closely related to the natural process of zygotic embryogenesis (ZE) [6]. SE in *A. angustifolia* is a complex and multifactorial pathway that includes induction and proliferation of embryogenic cultures (EC), composed of proembryogenic masses (PEMs) preceding somatic embryo formation [1, 5, 7]. *A. angustifolia* SE model encompasses two cycles. The cycle A consists in induction, proliferation and pre-maturation steps. Induction is characterized by EC formation in zygotic embryo apex (Fig. 1a), which is retrieved and in vitro cultivated in both auxin and cytokinin presence (Fig. 1b) or in plant growth regulator (PGR)-free culture medium [8–10]. Through double-staining analysis with acetocarmine and Evan's blue, it is possible to identify in the PEMs the presence of two typical conifer cells: embryogenic cells and suspensor-like cells (SCs) [11–13]. During proliferation step, PEMs evolve through three specific developmental stages, PEM I, II, and III, evaluated by the abundance of embryogenic cells and SCs [10–12].

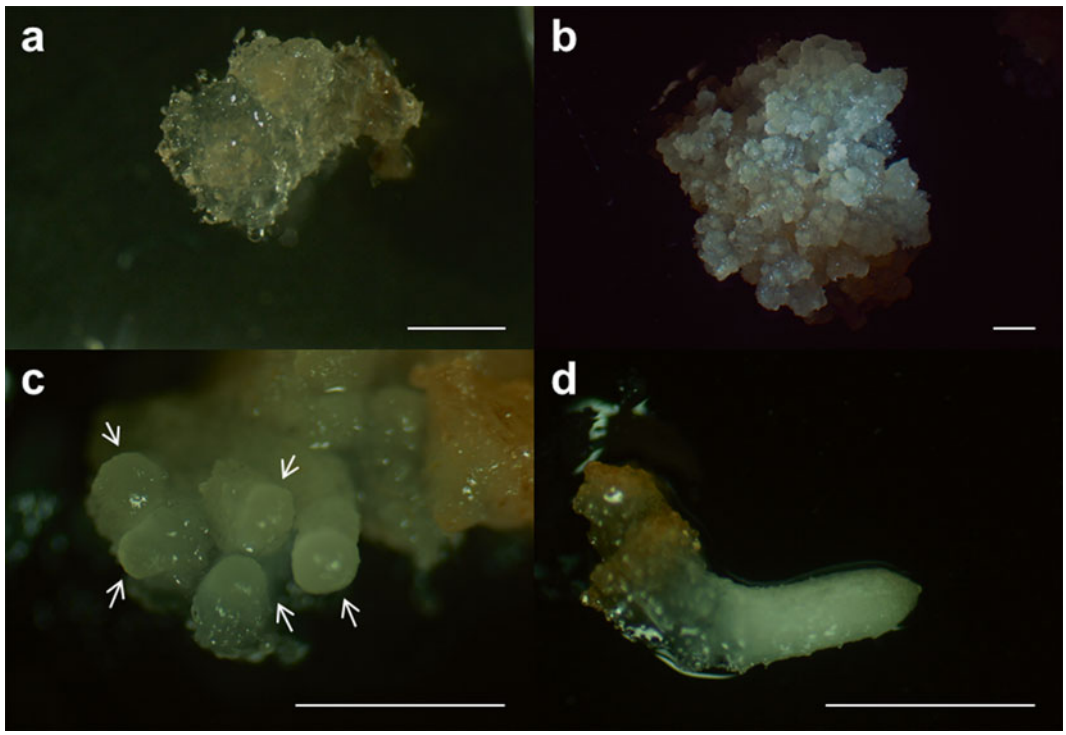

Fig. 1 Morphological aspects of *Araucaria angustifolia* embryogenic cultures. (**a**) Embryogenic callus 30 days after somatic embryogenesis induction. (**b**) Embryogenic callus during multiplication cycles in gelled culture medium. (**c**) Callus with globular-staged somatic embryos during maturation cycle. (**d**) Torpedo-staged somatic embryo after 90 days in maturation culture medium (*arrows* indicate globular-staged somatic embryos). Bar, 2 mm

PEMs-to-early somatic embryo transition is a central event in conifers SE [6]. In *A. angustifolia* SE, pre-maturation step is the starting point of the early SE polarization and individualization from PEM III [7]. The trigger for this process is the PGR removal of culture medium, followed by maltose and PEG supplementation [11, 14]. Early somatic embryos arise when compact clusters of embryogenic cells grow from PEM III with two regions, the dense globular embryonal mass (EM) in the apical part, and suspensor (S) in the basal part [5]. After pre-maturation step, in the cycle B, starts the maturation phase, where early somatic embryos (Fig. 1c) are able to develop in late somatic embryos (Fig. 1d). Late somatic embryos formation can be achieved when the early embryos are capable to respond to the new specific signals with osmotic and hormonal adjustment during maturation step [3, 7, 11]. The initiation of early somatic embryo formation can be observed with the embryonic cell group increase, while the elongated suspensor cells undergo programmed cell death [9, 13–15]. The early somatic embryo development marks the beginning of structural differentiation with the protoderm formation around the early somatic embryo followed by the meristem determination (root and shoot apical meristems). After that, the somatic embryos obtained can be converted into plantlets. Thus, the approach of this chapter is to describe SE protocols and describe morphological features of SE developmental stages in *A. angustifolia*.

2 Materials

Prepare all solutions using distilled water and analytical grade reagents. Prepare all stock solutions at room temperature. All stock solutions can be autoclaved excepting solutions containing vitamins and amino acids.

2.1 Plant Material and Surface Sterilization

1. Immature zygotic embryos of *A. angustifolia* excised of seeds collected from female cones in December.
2. 70 % (v/v) ethanol.
3. 2 % sodium hypochlorite.
4. Sterile distilled water.
5. Glass flasks.

2.2 Stock Solutions of the Induction and Proliferation Culture Medium

1. BM-macrosalt solution [16], 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 12.07 g NH_4NO_3, 18.20 g KNO_3, 2.72 g KH_2PO_4, 4.93 g $MgSO_4{\cdot}7H_2O$, 5.13 g $Mg(NO_3)_2{\cdot}4H_2O$, 1 g $MgCl_2{\cdot}6H_2O$, and 4.72 g $Ca(NO_3)_2{\cdot}4H_2O$, transfer to the beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.

2. BM-microsalt solution 200× [16]: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 1.59 g $MnSO_4 \cdot H_2O$, 2.82 g $ZnSO_4 \cdot 7H_2O$, 3.1 g H_3BO_3, 0.83 g Kl, 25 mg $CuSO_4 \cdot H_2O$, 25 mg Na2MoO4·5H2O, and 25 mg $CoCl_2 \cdot 6H_2O$. Transfer to glass beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.
3. BM-amino acid solution 100×: Add 5 mL of distilled water to a 10 mL glass beaker. Weigh 1 g L-glutamine, 0.5 g casein, 1 g myoinositol, transfer to beaker and solubilize. Make up to 10 mL with water. Prepare just before use, do not stock.
4. Fe-EDTA solution 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 187.2 mg $Na_2EDTA \times 2H_2O$ and 139 mg $FeSO_4 \times 7H_2O$, transfer to beaker and solubilize. Make up to 1000 mL with water. Store at 4 °C.
5. Vitamins and glycine solution 500×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 500 mg thiamine HCl, 250 mg pyridoxine HCl, 250 mg nicotinic acid, 1 g glycine, add to the beaker and solubilize. Make up to 1000 mL with water. Store aliquots of 2 mL microtubes at −20 °C.

2.3 Stock Solutions of the Pre-maturation and Maturation Culture Medium

1. MSG-macrosalt solution [17], 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 29 g NH_4NO_3, 38 g KNO_3, 8.8 g $CaCl_2 \cdot 2H_2O$, 3.4 g KH_2PO_4, 7.4 g $MgSO_4 \cdot 7H_2O$, and 14.9 g KCl. Transfer to the beaker and solubilize. Make up to 1000 mL with water. Store at 4 °C.
2. MSG-microsalt solution [17], 200×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 3.38 g $MnSO_4 \cdot H_2O$, 1.72 g $ZnSO_4 \cdot 7H_2O$, 1.24 g H_3BO_3, 0.16 g Kl, 5 mg $CuSO_4 \cdot H_2O$, 50 mg $Na_2MoO_4 \cdot 5H_2O$, and 5 mg $CoCl_2 \times 6H_2O$, transfer to the beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.
3. MSG-amino acid solution 100×: Add about 5 mL of distilled water to a 10 mL glass beaker. Weigh 1.46 g L-glutamine, 0.1 g myoinositol, and transfer to the beaker and solubilize. Make up to 10 mL with water. Prepare just before use, do not stock.
4. Fe-EDTA solution 20×: Use the same solution described in Subheading 2.2.
5. Vitamins and glycine solution 500×: Use the same solution described in Subheading 2.2.

2.4 Other Stock Solutions

1. 1000 μM 2,4-Dichlorophenoxyacetic acid (2,4-D): Weigh 22.10 mg of 2,4-D and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve 2,4-D. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).

2. 1000 μM 6-benzylaminopurine (BAP): Weigh 22.50 mg of BAP and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve BAP. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).
3. 1000 μM kinetin (KIN): Weigh 21.50 mg of KIN and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve KIN. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).

2.5 Culture Medium Supplements

1. Sucrose.
2. Maltose.
3. Phytagel®.
4. Gelrite®.
5. Polyethylene glycol 3350.
6. Polyethylene glycol 4000.
7. Reduced L-glutathione.
8. Abscisic acid (ABA).
9. Activated charcoal.

2.6 Culture Medium Preparation (See Note 2)

2.6.1 Induction Culture Medium (BMi)

1. To prepare 1 L of BMi add 30 g of sucrose to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, 5 mL of Fe-EDTA stock solution, 5 mL of 2,4-D stock solution, 2 mL of BAP stock solution, and 2 mL of KIN stock solution.
2. At this step, PGR-free culture medium is also used for SE induction.
3. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Autoclave for 15 min at 121 °C.
4. Wait the autoclaved mixture temperature to reach 40 °C. In the laminar air flow cabinet, add the filter-sterilized (*see* **Note 3**) solution containing 10 mL of BM-amino acid stock solution and 2 mL of vitamins and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.

2.6.2 Proliferation Culture Medium (BMp)

1. To prepare 1 L of BMp add 30 g of sucrose to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, 5 mL of Fe-EDTA stock solution, 2 mL of 2,4-D stock solution, 0.5 mL of BAP stock solution, and 0.5 mL of KIN stock solution.

2. Cultures induced in PGR-free culture medium can be multiplied either in the culture medium described in 1, or in PGR-free culture medium.
3. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Alternatively the EC can be multiplied in liquid medium. Autoclave for 15 min at 121 °C.
4. Wait the autoclaved mixture temperature to reach 40 °C. In the laminar airflow cabinet, add the filter-sterilized (*see* **Note 3**) solution containing 10 mL of BM-amino acid stock solution and 2 mL of vitamin and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.
5. For gelled culture medium, shake the solution to homogenize the mixture while warming. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (1 L of culture medium provides ~40 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.
6. For liquid culture medium, shake the solution to homogenize the mixture. Distribute 50 mL of the mixture into a sterile 250 mL Erlenmeyer flask. Close and seal with Parafilm®.

2.6.3 Pre-maturation Culture Medium 1 [14]

Recently, two pre-maturation protocols have been described for *A. angustifolia* SE and both of them can be successfully applied [14, 18].

1. To prepare 500 mL of pre-maturation culture medium (MSGpm1), add 45 g of maltose and 35 g of PEG 3350–200 mL of water in a 500 mL glass beaker and stir on a magnetic stirrer. Add 25 mL of MSG-macrosalt stock solution, 2.5 mL of MSG-microsalt stock solution, 2.5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 494 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8. Autoclave for 15 min at 121 °C in a 750–1000 mL Erlenmeyer flask.
3. Wait the temperature to reach 40 °C and add the filter-sterilized (*see* **Note 3**) solution containing 5 mL of MSG-amino acid stock solution, 1 mL of vitamin and glycine stock solution, and 1.53 g of reduced L-glutathione in the laminar airflow cabinet. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization. This procedure should be done preferably in the dark to prevent reduced L-glutathione degradation.

4. Shake the solution to homogenize the mixture. Distribute 2 mL of the mixture by pipetting into sterile 12-well culture plate (500 mL of culture medium provides ~20 multiwell culture plates). Close and seal with Parafilm®.

2.6.4 Pre-maturation Culture Medium 2 [18]

1. To prepare 500 mL of pre-maturation culture medium (MSGpm2), add 15 g of sucrose, 35 g of maltose, 45 g of PEG 4000, and 1.5 g of activated charcoal to 200 mL of water in a 500 mL glass beaker and stir on a magnetic stirrer. Add 25 mL of MSG-macrosalt stock solution, 2.5 mL of MSG-microsalt stock solution, and 2.5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 494 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.7. Add 1.5 g of Gelrite® and autoclave for 15 min at 121 °C in a 750–1000 mL Erlenmeyer flask.
3. In the laminar airflow cabinet, wait the temperature to reach 40 °C and add the filter–sterilized (*see* **Note 3**) solution containing 0.73 g of L-glutamine, and 1 mL of vitamin and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.7 before filter-sterilization.
4. Shake the solution to homogenize the mixture while warm. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (500 mL of culture medium provides ~20 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.

2.6.5 Maturation Culture Medium

1. To prepare 1 L of maturation culture medium (BMm), add 90 g of maltose, 70 g of PEG 3350 and 1.5 g of activated charcoal to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, and 5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Autoclave for 15 min at 121 °C.
3. Wait the temperature to reach 40 °C and add the filter-sterilized (*see* **Note 3**) solution containing 31.7 mg of ABA (*see* **Note 4**), 10 mL of BM-amino acid stock solution, and 2 mL of vitamin and glycine stock solution in the laminar flow cabinet. Adjust the solution pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.
4. Shake the solution to homogenize the mixture while warm. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (1 L of culture medium provides ~40 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.

2.7 Cytochemical Analysis

1. 2 % carmine: Dissolve 2 g carmine in 100 mL acetic acid 45 % (v/v). Boil in reflux condenser for 3 h. Cool at room temperature and filter with filter paper.
2. 0.05 % (w/v) Evan's blue: Dissolve 1 g Evan's blue in 100 mL distilled water.
3. Slides and cover glass.
4. Light microscope.

2.8 Other Useful Materials

1. Scalpel, forceps.
2. Spirit burner.
3. Magnetic stirrer.
4. pH meter, autoclave.
5. Bottles, Petri dishes, Erlenmeyer flasks, glass beaker, 12-well culture plate.
6. Parafilm®.
7. Syringe, sterile syringe filters Chromafil®, filter paper.
8. Analytical balance.
9. 0.5 N sodium hydroxide (NaOH), 0.5 N hydrochloric acid (HCl).
10. Incubator chamber, laminar flow cabinet, stereomicroscope.

3 Methods

All the procedures described below must be performed in laminar flow cabinet, with sterilized instruments.

3.1 SE Induction

1. Only immature seeds of *A. angustifolia* with globular-staged zygotic embryos are used, in order to induce SE. Surface sterilize seeds in a glass beaker with 70 % ethanol for 5 min. Remove ethanol and add 2 % sodium hypochlorite for 20 min. Remove sodium hypochlorite and wash seeds three times with autoclaved distilled water. All solutions must be added in enough volume to cover the seeds into the beaker.
2. With the aid of a stereomicroscope, scalpel, and forceps on a sterilized Petri dish, excise the immature zygotic embryo and inoculate into the induction culture medium. Cultures are maintained in BOD incubator chamber at 24 ± 2 °C.

3.2 EC Proliferation

After 30-day culture in BMi culture medium, EC is generally obtained. During proliferation step, EC are composed by PEMs, maintained in repetitive multiplication cycles for an undetermined period of time. At this point, EC proliferation can be achieved with or without PGR supplementation. Proliferation can also be performed in gelled or liquid culture medium.

1. To perform the subculture for gelled BMp culture medium, friable and translucent EC should be removed from the BMi medium with the aid of a forceps and transferred to fresh gelled BMp culture medium. Colonies of cells should be mixed during the process of subculture to promote uniform distribution of nutrients contained in culture medium. The subculture procedure must be performed every 21 days to fresh gelled BMp culture medium and can be done indefinitely. Cultures are maintained in BOD incubator chamber at 24 ± 2 °C.
2. For liquid BMp culture medium, about 500 mg of friable and translucent EC should be removed from the BMi medium with the aid of a forceps and transferred to a fresh liquid BMp medium. The subculture procedure must be performed every 15 days to a fresh liquid BMp culture medium and can be done indefinitely. This procedure is realized with the aid of "Cell Dissociation Sieve" (Sigma-Aldrich), 80 mesh screens. Capture the EC by pouring the culture medium with EC in proliferation in the "Cell Dissociation Sieve." With the aid of a forceps, take 500 mg of EC and transfer to a new flask. Cultures are maintained in an orbital shaker at 90 rpm, at 24 ± 2 °C in the dark.

3.3 EC Pre-maturation 1

Pre-maturation is an important step in conifers SE, and it was recently applied to *A. angustifolia* protocol [14, 18]. In this step, the transition of PEMs to early somatic embryos is observed.

1. After proliferation step, repeat the same procedure described above (*see* Subheading 3.2) to capture the EC. Transfer about 50 mg of EC with the aid of a forceps to a 12-well culture plate containing 2 mL MSGpm1 per well.
2. The plates should be incubated in an orbital shaker at 90 rpm in the dark. Cultures are maintained in a growth room at 24 ± 2 °C for 15 days.

3.4 EC Pre-maturation 2 [18]

1. After proliferation step, repeat the same procedure described above (Subheading 3.2, **step 2**) to capture the EC. Transfer 100 mg of EC with the aid of a forceps to a sterile filter paper disc (Ø 80 mm). Transfer the filter paper with the cultures to Petri dish containing MSGpm2 culture medium.
2. Cultures are maintained in a growth room at 24 ± 2 °C for 30 days.

3.5 Early Somatic Embryo Maturation

1. For somatic embryos maturation, about 500 mg of EC containing early somatic embryos is transferred with the aid of a forceps to BMm culture medium.
2. Petri dishes are maintained in BOD incubator chamber at 24 ± 2 °C for 60 days. One subculture should be performed at day 30 in culture to a fresh BMm culture medium.

3.6 Morphological and Cytochemical Analysis Procedure

The quality of cultures is evaluated by double staining under light microscope based on acetocarmine and Evan's blue staining [19]. This double-staining analysis reveals the presence of the two typical embryonic conifer structures: the embryogenic cells, which are isodiametric and densely cytoplasmic, reacting in red with acetocarmine, and the suspensor-like cells, which are vacuolated and reacts in blue to Evan's blue [20].

1. Take an aliquot of 50 mg of EC and transfer to a watch glass.
2. Add a drop of 1 % acetocarmine (w/v) to the sample, gently mix, and wait for 1 min.
3. Carefully remove the acetocarmine with the aid of toilet paper.
4. Drop 0.05 % Evan's blue (w/v) to the sample, gently mix and wait 1 min.
5. Carefully remove the Evan's blue with the aid of toilet paper.
6. Drop 1 mL of sterile distilled water.

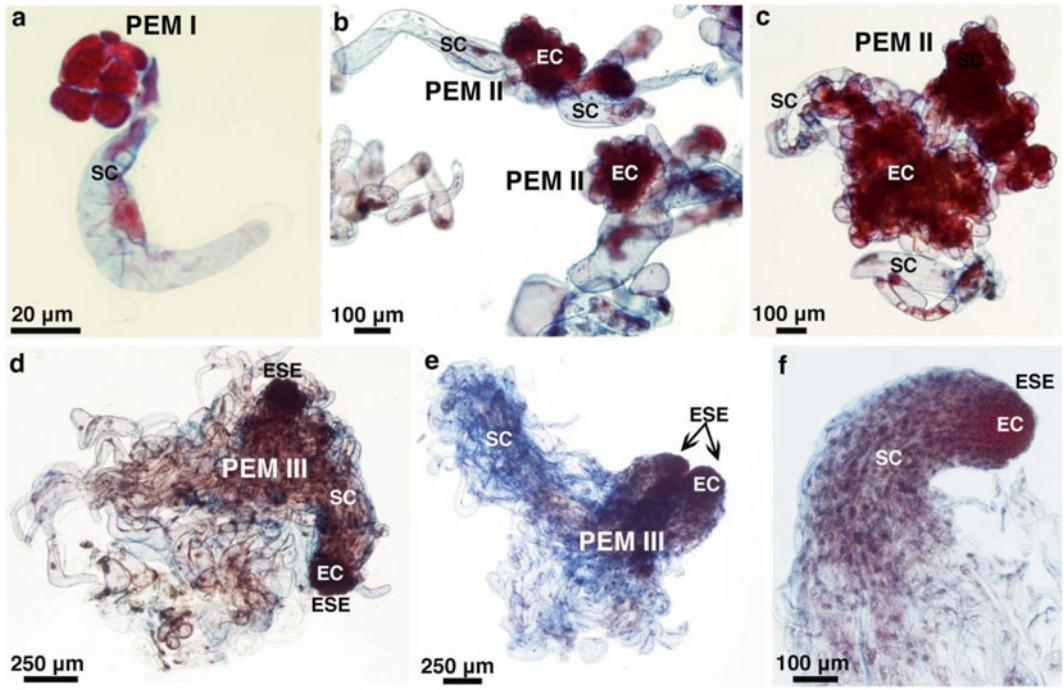

Fig. 2 *Araucaria angustifolia* embryogenic cultures morphological and cytochemical analysis with acetocarmine and Evan's blue. (**a**–**c**) Proembryogenic masses at PEM I stage (**a**), PEM II stage (**b**) and PEM III stage (**c**). (**d**) PEM III-staged embryogenic cells starting polarization and individualization process. (**e**) Early somatic embryos (ESE) individualized and polarized. (**f**) Globular-staged ESE. EC, embryogenic cells stained with acetocarmine; SC, suspensor-like cells stained with Evan's blue

7. Drop with a pipette an aliquot on a slide glass, and then visualize in the light microscope.
8. Analyze and quantify the presence of PEM I (Fig. 2a), PEM II (Fig. 2b), PEM III (Fig. 2c), and early somatic embryos (Fig. 2e, f) as well as the presence of SCs (Fig. 2a) and embryogenic cells (Fig. 2a, b).

4 Notes

1. The PGR stock solutions can be autoclaved for 15 min to decrease bacterial and fungal contamination, and improve the solubilization.
2. Culture medium should be prepared at least 3 days before the inoculation procedure. This is the required period to ensure that there was no fungal or bacterial contamination during the culture medium preparation.
3. Filter-sterilization is made with the aid of a syringe and sterile Syringe filters Chromafil® (Macherey-Nagel), with PTFE membrane, 0.20 μm pore size into the laminar flow cabinet.
4. Abscisic acid cannot be maintained in stock solution. Weigh the abscisic acid with the aid of a analytical balance, add 200 μL of NaOH 1 M to dissolve ABA, and then add the vitamins, amino acids, or other stock solutions you need to filter-sterilize.

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