

## SOMATIC EMBRYOGENESIS AND MORPHOANATOMY OF *Ocotea porosa* SOMATIC EMBRYOS

### EMBRIOGÊNESE SOMÁTICA E MORFOANATOMIA DE EMBRIÕES SOMÁTICOS DE *Ocotea porosa*

Luciana Luiza Pelegrini<sup>1</sup> Luciana Lopes Fortes Ribas<sup>2</sup> Erika Amano<sup>3</sup>  
Marguerite Quoirin<sup>4</sup>

#### ABSTRACT

*Ocotea porosa* seeds have strong tegument dormancy, recalcitrant behavior, low and irregular germination and that makes its natural propagation difficult. The aim of this study was to establish a protocol of regeneration of *Ocotea porosa* from somatic embryogenesis. Immature embryonic axes were inoculated on WPM culture medium supplemented with 2,4-D (200  $\mu\text{M}$ ) combined or not with hydrolyzed casein or glutamine (0.5 or 1  $\text{g l}^{-1}$ ), during 90 days. The repetitive embryogenesis was induced on medium with 2,4-D (22.62  $\mu\text{M}$ ) combined with 2-iP (2.46  $\mu\text{M}$ ) followed by transfer to culture medium with hydrolyzed casein or glutamine (1  $\text{g l}^{-1}$ ) during 90 days. The maturation of somatic embryos was tested in culture medium containing NAA (0.5  $\mu\text{M}$ ) and 2-iP (5; 10 and 20  $\mu\text{M}$ ). The highest percentage of somatic embryos induction (8.3%) was observed in WPM culture medium containing 200  $\mu\text{M}$  2,4-D and 1  $\text{g L}^{-1}$  hydrolyzed casein and the development of somatic embryos occurred indirectly. Repetitive somatic embryogenesis was promoted in WPM medium containing hydrolyzed casein or glutamine. However, the culture medium containing hydrolyzed casein promoted the maintenance of embryogenic capacity for more than two years. During the maturity phase, there was a low progression of globular embryos to cordiform and torpedo stages. The different ontogenetic stages of somatic embryos of *Ocotea porosa* were characterized by histological studies.

**Keywords:** Embryogenic mass; hydrolyzed casein; glutamine; histology.

#### RESUMO

*Ocotea porosa* apresenta sementes com forte dormência tegumentar, comportamento recalcitrante, baixa germinação e irregularidade, o que dificulta a propagação natural. O objetivo deste trabalho foi estabelecer um protocolo de embriogênese somática para *Ocotea porosa*. Eixos embrionários zigóticos imaturos foram inoculados em meio de cultura WPM suplementado com 2,4-D (200  $\mu\text{M}$ ) combinado ou não com caseína hidrolisada ou glutamina (0,5 ou 1  $\text{g l}^{-1}$ ) durante 90 dias. A embriogênese repetitiva foi induzida em meio contendo 2,4-D (22,62  $\mu\text{M}$ ) combinado com 2-iP (2,46  $\mu\text{M}$ ) seguido de transferência para meio de cultura com caseína hidrolisada ou glutamina (1  $\text{g l}^{-1}$ ), durante 90 dias cada. A maturação dos embriões somáticos foi testada em meio de cultura contendo ANA (0,5  $\mu\text{M}$ ) e 2-iP (5, 10 ou 20  $\mu\text{M}$ ). A maior porcentagem de embriões somáticos induzidos (8,3%) foi observada em meio de cultura WPM contendo 200  $\mu\text{M}$  de 2,4-D e 1  $\text{g l}^{-1}$  de caseína hidrolisada e o desenvolvimento de embriões somáticos ocorreram de forma indireta. A embriogênese somática repetitiva foi promovida em meio de cultura WPM contendo caseína hidrolisada ou glutamina, no entanto, a caseína hidrolisada promoveu a manutenção da competência embriogênica por mais de dois anos. Na fase de maturação, houve baixa progressão dos embriões globulares para os estádios

1 Bióloga, Dr<sup>a</sup>. pelo Programa de Pós-Graduação em Produção Vegetal, Setor de Ciências Agrárias, Universidade Federal do Paraná, CEP 81531-990, Curitiba (PR), Brasil. Bolsista Reuni. lupelegrini@hotmail.com

2 Bióloga, Dr<sup>a</sup>, Professora Associada II do Departamento de Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná, CEP 81531-970, Curitiba (PR), Brasil. llfribas@ufpr.br

3 Bióloga, Dr<sup>a</sup>, Professora Adjunto do Departamento de Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná, CEP 81531-980, Curitiba (PR), Brasil. erika.amano@ufpr.br

4 Engenheira Agrônoma, Dr<sup>a</sup>, Professora Associada do Departamento de Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná, CEP 81531-990, Curitiba (PR), Brasil. mquoirin@ufpr.br

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cordiforme e torpedo. Os diferentes estádios ontogenéticos de embriões somáticos de *Ocotea porosa* foram caracterizados pelo estudo histológico.

**Palavras-chave:** massa embriogênica; caseína hidrolisada; glutamina; histologia.

## INTRODUCTION

*Ocotea porosa* (Liberato Barroso), known as “imbuia”, belongs to the *Lauraceae* family and is native to the Mixed Ombrophilous Forest (Araucaria Forest) where it was heavily exploited due to the high quality and worldwide value of its hardwood, that is exported in large quantities for luxury furnishing manufactures (CARVALHO, 2003). As a result, this species is an endangered one and is quoted in the official list of endangered Brazilian flora species, in the vulnerable category (IBAMA, 1992) and in the Red List of Parana, in the rare category (SEMA, 1995).

The sexual propagation of *Ocotea porosa* at its natural occurrence area is difficult, due to the strong tegumentary dormancy and its irregular germination (CARVALHO, 2003). Moreover, the seed viability is short by virtue of being a recalcitrant species, showing high level of humidity (CARVALHO, 2003). Another limiting factor for its vegetative propagation is the low response of cuttings to the induction of adventitious roots (4%) (INOUE and PUTTON, 2007).

Somatic embryogenesis is a process by which the somatic cells, under inductive conditions, generate embryogenic cells, which undergo a series of morphological and biochemical alterations, resulting in formation of somatic embryos (SANTOS et al., 2002; KOMAMINE et al., 2005). Somatic embryogenesis is a model for the study of morphological, physiological, molecular characteristics and biochemical events that occur during the embryogenesis in higher plants. Beyond that, it has potential biotechnological applications, such as artificial seed production and propagation of elite plants or genetically modified plants (SANTOS et al., 2002; QUEIROZ-FIGUEROA, 2006).

The maturation is considered a fundamental step in the development of the embryo for subsequent conversion and for the seedling development (PERAN-QUESADA et al., 2004). During maturation, embryo cells undergo various physiological changes, which become evident by the deposit of storage materials, the repression of germination and

the acquisition of desiccation tolerance (JIMÉNEZ, 2005; VAHDATI et al., 2008). The cell expansion and accumulation of reserve substances are crucial to stimulate the conversion process (GARCIA-MARTIN et al., 2005). Although in some woody plants, the maturation and the germination of somatic embryos have been obtained in media which lack plant growth regulators (SANTA-CATARINA et al., 2001; CANHOTO et al., 2005; SHI et al., 2009), in most cases, the application of specific treatments is necessary (RIBAS, 2000). Abscisic acid and osmotic stresses are known to be important factors for the seed maturation in many angiosperms (RAI et al., 2011; VAHDATI et al., 2008). The addition of reduced nitrogen in the maturation medium is a requirement to ensure maturation of somatic embryos (MERKLE et al., 1995). Compared to other inorganic nitrogen sources the nitrogen in reduced form of amino acids such as glutamine, asparagine and arginine is rapidly absorbed and incorporated into the carbon skeletons for the metabolism and protein synthesis (LEA, 1993). Hydrolyzed casein is an excellent source of reduced nitrogen containing a mixture of up to 18 amino acids, particularly glutamine (GEORGE and DE KLERK, 2008).

In somatic embryogenesis, the histological and morphological analysis of the plant material may be critical for the results interpretation and a help to increase the efficiency of protocols. Histological analysis can be used to identify and to characterize the formation of monopolar and bipolar structures as well as the cellular changes, analyzing the quality and development of these structures (PEREZ-NUÑEZ et al., 2006).

For many forest species, including several members of Lauraceae family, like *Laurus nobilis* (CANHOTO et al., 1999), *Persea americana* Mill (SANCHEZ-ROMERO, 2006), *Ocotea catharinensis* (VIANA and MANTELL, 1999) and *Ocotea odorifera* (SANTA-CATARINA et al., 2005), somatic embryogenesis induction protocols have been established. However, for *Ocotea catharinensis*, Santa-Catarina et al. (2005) found difficulties in optimizing the conversion stage of somatic embryos into plants.

The aim of this study was to establish a protocol of somatic embryogenesis of *Ocotea porosa* from immature zygotic embryonic axes.

## MATERIAL AND METHOD

Zygotic embryonic axes from immature *Ocotea porosa* seeds, collected at Campina Grande do Sul, Paraná state, Brazil, in February 2009, were used as explants. The seed disinfestation was performed in a laminar flow chamber, through immersion in ethanol 70% (v/v) for 5 min, followed by 20 min immersion in a sodium hypochlorite solution (NaOCl) 4% (v/v) supplemented with 0,1% Tween® 20. After that, the seeds were rinsed five times with sterile water.

### Induction of somatic embryos

The immature embryonic axes (0.2 to 0.4 cm) were inoculated in an induction medium consisting of WPM "Woody Plant Medium" (LLOYD and McCOWN, 1980) vitamins and salts, supplemented with sucrose (20 g l<sup>-1</sup>), activated charcoal (1.5 g l<sup>-1</sup>), Vetec® agar (4 g l<sup>-1</sup>) and 2,4-D (2,4-dichlorophenoxyacetic acid) (200 µM) alone or combined with hydrolyzed casein or glutamine (0.5 and 1.0 g l<sup>-1</sup>). The explants were kept in the dark and, after 90 days, they were evaluated for embryogenic mass forming somatic embryos, friable mass callus (not embryogenic), yellowish white callus, blackish callus (brown or black) and necrosis of immature zygotic embryonic axes. The evaluation was performed every 30 days.

### Repetitive embryogenesis

The somatic embryos obtained in the experiment of induction were transferred to the same culture medium supplemented with 2,4-D (22.62 µM) and 2-iP (2-isopentyladenine) (2.46 µM) for secondary somatic embryogenesis induction. Three subcultures were performed at 30-day intervals. After this period, the cultures were transferred to WPM culture medium without plant growth regulator, supplemented with sucrose (20 g l<sup>-1</sup>), agar Vetec® (3.5 g l<sup>-1</sup>) and hydrolyzed casein or glutamine (1 g l<sup>-1</sup>). Three subcultures were carried out at 30-day intervals and were evaluated after 90 days. The number of globular, cordiform, torpedo and cotyledonary embryos was recorded. These cultures were maintained during three years, being transferred to a fresh medium every 30 days.

### Maturation of somatic embryos

Somatic embryos formed through secondary somatic embryogenesis in culture media containing hydrolyzed casein (1 g l<sup>-1</sup>) were transferred to WPM media supplemented with sucrose (20 g l<sup>-1</sup>), agar Vetec® (3.5 g l<sup>-1</sup>) and 2-iP (5, 10 e 20 µM) combined with NAA (naphthaleneacetic acid) (0.5 µM). After 30 days the percentage of callus developing embryogenic mass and the average number of globular, heart shaped and torpedo embryos was recorded.

### Culture conditions

The pH of all culture media was adjusted to 5.8 prior to autoclaving. Test tubes (25 x 150 mm) containing 10 ml of culture media were used. The cultures were placed in the dark at 27±2°C (day) and 18±2°C (night).

### Experimental design and statistical analysis

Experimental design was completely randomized with four replications and 10 embryonic axes per plot. The data were subjected to Kruskal-Wallis test at 5% of probability, using the STATISTICA program, version 7.1.

### Morphoanatomic studies

Samples were fixed using glutaraldehyde (2%) and paraformaldehyde (4%) buffered with 0.2 M phosphate buffer, at pH 7.2 for 96 hours (KARNOVSKY, 1965). After that, they were dehydrated in an alcoholic series with a duration of 60 min for each treatment. The samples were embedded in Historesin® (Leica, Heidelberg). Serial sections were performed on a rotation microtome with a thickness of 7µm. The sections were stained with toluidine blue O (O'BRIEN et al. 1965), observed under optical microscope and then photographed with a digital camera.

## RESULTS AND DISCUSSION

### Induction of somatic embryos

Immature zygotic embryonic axes of *Ocotea porosa* showed different responses when cultured *in vitro*. The expression events of somatic embryogenesis occurred between the 30<sup>th</sup> and 40<sup>th</sup>

day after inoculation on the culture medium and the induction of somatic embryos occurred indirectly.

After 90 days, the mean percentage of embryogenic mass forming somatic embryos varied between 4.2% and 8.3%, without any statistical difference between treatments ( $P = 0.9149$ ) (Table 1). On the culture medium containing 200  $\mu\text{M}$  2,4-D, the percentage of embryogenic mass forming somatic embryos was 6.3% and the formation of a globular embryo was visible. When 200  $\mu\text{M}$  2,4-D was combined with 1 g l<sup>-1</sup> hydrolyzed casein this percentage was 8.3% and two globular somatic embryos were developed per embryogenic mass (Figure 1c) differing of treatments containing 1 g l<sup>-1</sup> glutamine (Table 1). However, in the medium containing 200  $\mu\text{M}$  2,4-D and 1 g l<sup>-1</sup> glutamine, after 90 days the percentage was smaller (4.2%) and two globular embryos and one cordiform shape were observed (Table 1). In the present study, the maximum percentage of somatic embryos induction was 8.3%. Similar results were found with *Ocotea odorifera* that presented a mean percentage of 6.5% (SANTA-CATARINA et al., 2001). Thus, the low percentage of primary somatic embryogenesis induction from zygotic embryos seems to be characteristic of *Ocotea* genus. Pinto et al. (2002) also reported that primary somatic embryogenesis was less efficient than secondary somatic embryogenesis in *Quercus suber*.

The induction of friable mass callus occurred only in immature zygotic embryonic axes cultured in WPM medium alone or containing hydrolyzed casein without any statistical difference between treatments ( $P = 0.0643$ ) (Table 1; Figure 1b). The induction of yellowish white or dark callus was observed first after 20 days of induction in the different treatments. However, the lowest percentage of yellowish white callus was observed in the immature zygotic embryonic axes cultured in medium supplemented with 200  $\mu\text{M}$  2,4-D and 1 g l<sup>-1</sup> glutamine without differences with other treatments ( $P = 0.9166$ ). Regarding the induction of blackish callus (Figure 1a), the highest percentages were observed in immature zygotic embryonic axes cultured in WPM medium containing 200  $\mu\text{M}$  2,4-D combined with 0.5 or 1.0 g l<sup>-1</sup> hydrolyzed casein, but no significant difference was observed between the treatments ( $P = 0.0973$ ). The highest percentage of necrosis of zygotic embryonic axes (67.1 and 64.8) was observed in treatments in which glutamine was added (0.5 and 1 g l<sup>-1</sup> respectively) which were statistically different from other treatments ( $P = 0.0241$ ) (Table 1). The presence of dark callus is likely to be due to the fact that this species exhibits elevated quantity of phenolic compounds, which oxidize. This result was also found for *Ocotea catharinensis* (VIANA and MANTELL,

TABLE 1: Response of immature zygotic embryonic axes of *Ocotea porosa* cultured in WPM culture media supplemented with sucrose (20 g l<sup>-1</sup>), activated charcoal (1.5 g l<sup>-1</sup>), agar (4.0 g l<sup>-1</sup>) and 2,4-D (200  $\mu\text{M}$ ) combined or not with hydrolyzed casein (HC) or glutamine (GLN), after 90 days.

TABELA 1: Resposta dos eixos embrionários zigóticos imaturos de *Ocotea porosa* cultivados em meio de cultura WPM suplementado com sacarose (20 g l<sup>-1</sup>), carvão ativado (1,5 g l<sup>-1</sup>), ágar (4,0 g l<sup>-1</sup>) e 2,4-D (200  $\mu\text{M}$ ) combinado ou não com caseína hidrolisada (HC) ou glutamina (GLN), após 90 dias.

Amino acids (g l <sup>-1</sup> )	Percentage				
	EMSE <sup>1</sup>	FMC <sup>2</sup>	YWC	BC	NEZA
Control	6.3 <sup>ns</sup>	6.3 <sup>ns</sup>	9.4 <sup>ns</sup>	40.6 <sup>ns</sup>	37,5 <sup>b</sup>
0.5 g l <sup>-1</sup> HC	0.0	28.5 <sup>ns</sup>	7.8 <sup>ns</sup>	53.1 <sup>ns</sup>	10,6 <sup>bc</sup>
1.0 g l <sup>-1</sup> HC	8.3 <sup>ns</sup>	16.3 <sup>ns</sup>	7.4 <sup>ns</sup>	54.6 <sup>ns</sup>	13.4 <sup>bc</sup>
0.5 g l <sup>-1</sup> GLN	0.0	0.0	5.4 <sup>ns</sup>	27.5 <sup>ns</sup>	67.1 <sup>a</sup>
1.0 g l <sup>-1</sup> GLN	4.2 <sup>ns</sup>	0.0	3.6 <sup>ns</sup>	27.4 <sup>ns</sup>	64.8 <sup>a</sup>

Wherein: EMSE = embryogenic mass forming somatic embryos; FMC = friable mass callus; YWC = yellowish white callus; BC = blackish callus (brown or black); NEZA = necrosis of immature zygotic embryonic axes. Means followed by the same letter in the column do not differ significantly by Kruskal-Wallis test at 5% of probability. <sup>1</sup>The statistical analysis was done with treatments 1, 3 and 5. <sup>2</sup>The statistical analysis was done with treatments 1, 2 and 3.

1999). According to Benson (2000) and Martin; Madassery (2005), phenolic compounds are usually seen as deleterious substances during culture *in vitro* and exudation and the oxidation of these substances can adversely affect the explants, causing browning and necrosis. However, in some cases such as persimmon (*Diospyros kaki*) the mass em-

bryogenic formation occurred from oxidized callus. (CARVALHO et al., 2004).

### Repetitive embryogenesis

During the repetitive embryogenesis phase, primary somatic embryos turned dark but, even with

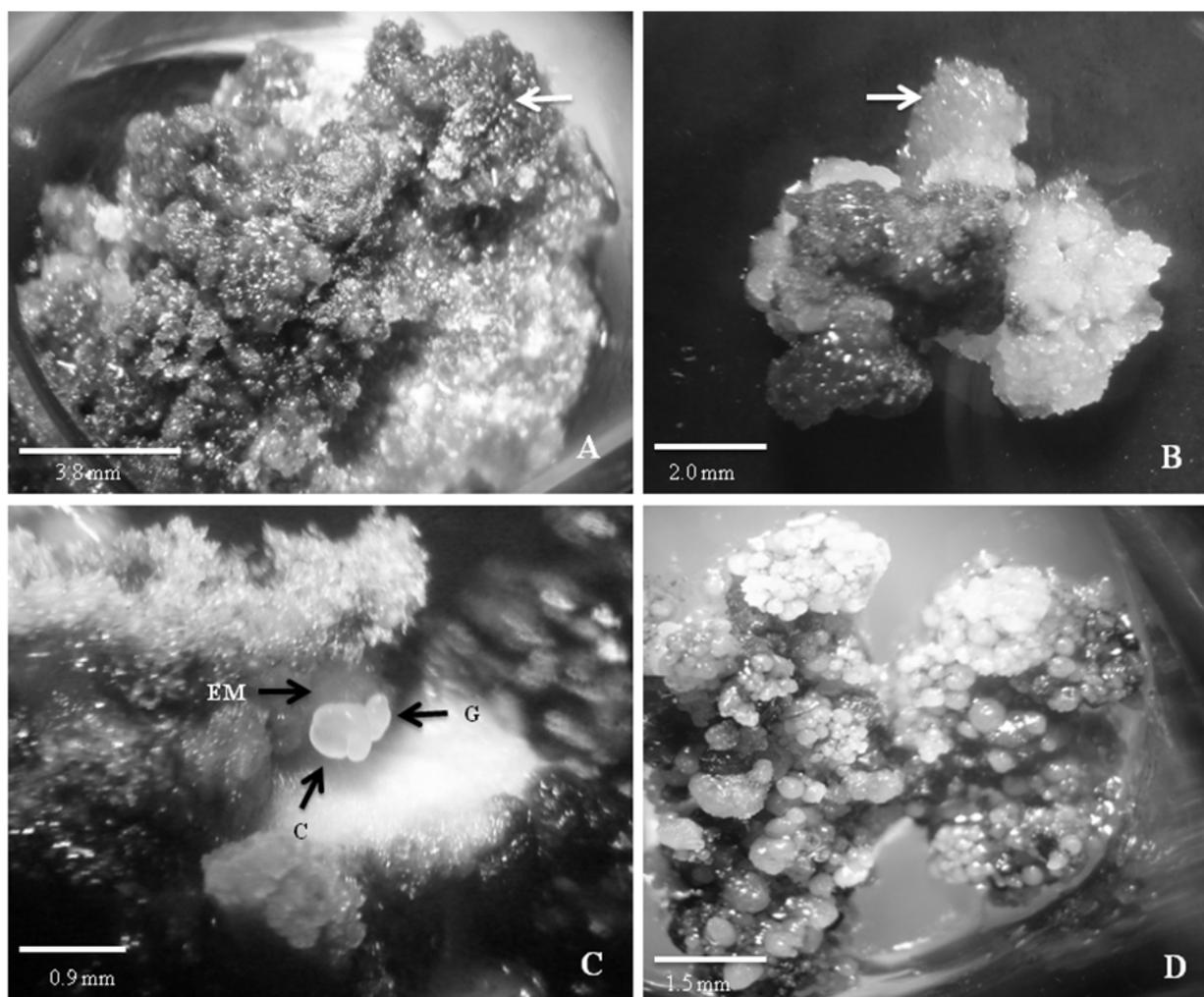


FIGURE 1: A) Morphological appearance of blackish callus on culture medium containing 2,4-D (200  $\mu\text{M}$ ) after 60 days (arrow). B) Friable mass callus (arrow) on culture medium containing 200  $\mu\text{M}$  2,4-D combined with 0.5 g l<sup>-1</sup> hydrolyzed casein, after 90 days. C) Somatic embryo at globular (G) and cordiform (C) stage (arrow) in culture medium with 200  $\mu\text{M}$  2,4-D + 1 g l<sup>-1</sup> hydrolyzed casein after 90 days. EM: embryogenic mass. D) Globular embryos induced by repetitive embryogenesis in culture medium containing 22.62  $\mu\text{M}$  2,4-D and 2.46  $\mu\text{M}$  2-iP.

FIGURA 1: Aspecto morfológico de calo escuro cultivado em meio de cultura contendo 2,4-D (200  $\mu\text{M}$ ) após 60 dias (seta). B) Calo com massa friável (seta) em meio de cultura contendo 200  $\mu\text{M}$  de 2,4-D combinado com 0,5 g l<sup>-1</sup> de caseína hidrolisada, após 90 dias. C) Embrião somático em estágio globular (G) e cordiforme (C) (seta) cultivado em meio de cultura com 200  $\mu\text{M}$  de 2,4-D + 1 g l<sup>-1</sup> de caseína hidrolisada, após 90 dias. EM: massa embriogênica. D) Embriões globular induzidos por embriogênese repetitiva em meio de cultura contendo 22,62  $\mu\text{M}$  de 2,4-D e 2,46  $\mu\text{M}$  de 2-iP.

a necrotic appearance, the formation of new structures in culture medium with 2,4-D (22,62  $\mu\text{M}$ ) and 2-iP (2,46  $\mu\text{M}$ ) was induced. After three months, on the calluses subcultured at 30-day intervals in media with 2,4-D (22.62  $\mu\text{M}$ ) and 2-iP (2.46  $\mu\text{M}$ ), an average of 76.9 globular, 3.4 cordiform, 1.5 torpedo and one cotyledonary embryos was formed per callus (Figure 1d). These results corroborate those of Moura-Costa et al. (1993) which reported the darkening of somatic embryos of *Ocotea catharinensis* that, after being subcultivated, formed new globular structures.

After this period, the embryos were subcultured in media containing 1 g l<sup>-1</sup> of hydrolyzed casein or glutamine without plant growth regulators. The addition of 1 g l<sup>-1</sup> hydrolyzed casein promoted an average formation of 61.8 new globular and 0.5 cordiform embryos per callus at the end of every subculture ( $P = 0.0433$ ) (Table 2). When 1 g l<sup>-1</sup> glutamine was added into the media, the induction of globular somatic embryos was lower (30.4 embryos) than the one in the presence of HC and other stages of development were not observed (Table 2).

In the present study, it was shown that hydrolyzed casein was more effective than glutamine as a source of amino acids for the induction of new somatic embryos through repetitive embryogenesis, as well as to maintain the embryogenic competence (dates not shown). The amino acids are important substances for primary assimilation of nitrogen and for protein synthesis (ORTIZ-LOPEZ et al., 2000). According to Morcillo et al. (1999) and Garin et al. (2000) the glutamine combined or not with hydrolyzed casein can be used at different stages of somatic embryogenesis. The addition of glutamine and/or hydrolyzed casein to the culture media could increase the level of proliferation of calluses and somatic embryos, as these substances

provide great amount of reduced organic nitrogen (GEORGE, 1996).

During repetitive embryogenesis the new somatic embryos are formed from others and some species keep this embryogenic competence for many years (PINTO et al., 2008). Embryogenic competence was observed, for example, in *Quercus suber* (HERNÁNDEZ et al., 2003), *Vitis rupestris* (MARTINELLI et al., 2001), *Ocotea odorifera* (SANTA-CATARINA et al., 2001), *Ocotea catharinensis* (MOURA-COSTA et al., 1993) and *Aspidosperma polyneuron* (RIBAS et al., 2000). Litz et al. (1995) and Tulecke et al. (1995) reported that the embryogenic capacity could be maintained in *Mangifera indica* and *Juglans regia* through repetitive embryogenesis for more than 5 and 9 years, respectively. After the evaluation period (90 days), the cultures remained in WPM culture medium supplemented with 1 g l<sup>-1</sup> hydrolyzed casein, showing that the embryogenic competence was maintained and the new embryos were multiplied for more than one year. Considering the low induction of somatic embryos from zygotic embryonic axes, the repetitive embryogenesis may be an important system of mass propagation, as high production rates of embryos were reached.

### Maturation of somatic embryos

The cultures, which consisted of globular somatic embryos obtained on culture media containing hydrolyzed casein and transferred to maturation media containing 2-iP (5, 10 and 20  $\mu\text{M}$ ) combined with NAA (0.5  $\mu\text{M}$ ), showed different morphogenetic responses (Table 3). The percentage of embryogenic mass was similar in all treatments, without any statistical difference ( $P = 0.8446$ ). The maturation media containing 2-iP combined with NAA promoted continuous prolif-

TABELA 2: Repetitive somatic embryogenesis from immature zygotic embryonic axes of *Ocotea porosa* during three subcultures.

TABLE 2: Embriogênese somática repetitiva a partir de eixos embrionários zigóticos imaturos de *Ocotea porosa* durante três subcultivos.

Treatments (1 g l <sup>-1</sup> )	Average number of embryos/callus			
	Globular	Cordiform	Torpedo	Cotyledonary
Hydrolyzed casein	61.8 <sup>a</sup>	0.5	0.0	0.0
Glutamine	30.4 <sup>b</sup>	0.0	0.0	0.0

Means followed by same letter in column do not differ significantly by Kruskal-Wallis test at 5% of probability.

TABLE 3: Effect of 2-iP combined with NAA on the maturation of somatic embryos of *Ocotea porosa* after 30 days of culture on WPM culture media.TABELA 3: Efeito do 2,iP combinado com ANA na maturação de embriões somáticos de *Ocotea porosa* após 30 dias de cultivo em meio de cultura WPM.

2-iP ( $\mu\text{M}$ )	Embryogenic mass (%)	Average number of embryos/callus		
		Globular	Cordiform	Torpedo
5	85.0 <sup>ns</sup>	30.7 <sup>ns</sup>	0,5 <sup>ns</sup>	1,5 <sup>ns</sup>
10	92.5 <sup>ns</sup>	37.2 <sup>ns</sup>	1,0 <sup>ns</sup>	1,0 <sup>ns</sup>
20	90.0 <sup>ns</sup>	36.2 <sup>ns</sup>	2,8 <sup>ns</sup>	3,0 <sup>ns</sup>

Wherein: <sup>ns.</sup> = not significant according to Kruskal-Wallis test at 5% probability.

eration of embryogenic mass and globular somatic embryos.

During maturation phase, the somatic embryos develop from initial stages to final stages. However, in the present conditions of maturation, the percentage of embryos developing to cordiform and torpedo ontogenetic stages was low and no embryo at cotyledonary phase was seen, revealing an asynchronous development, once globular somatic embryos were formed (Table 3). The average number of globular somatic embryos varied few and without any statistic difference between treatments ( $P = .6084$ ); the same was observed for the average number of cordiform and torpedo somatic embryos ( $P = 0.2437$  and  $P = 0.4835$  respectively). According to Guerra et al. (1999), during the maturation phase, it is necessary to disrupt the repetitive cycles of cell division and to provide physiological, biochemical and environmental stimuli for cellular differentiation, then the cycles of development and maturation give rise to a large number of mature somatic embryos of high quality and capable of turning into plants. However, in the present study, a combination of 2-iP and NAA was not effective for maturation of *Ocotea porosa* somatic embryos. Carvalho et al. (2004) also reported that the progression of somatic embryos of persimmon (*Diospyros kaki*) cultures in media containing 2-iP combined with IBA occurred in an asynchronous way. Guerra and Handro (1998), obtained mature somatic embryos of *Euterpe edulis* in media containing 2-iP and NAA and, at the same time, managed to maintain the embryogenic competence culture. Santa-Catarina et al. (2001), observed the development of *Ocotea odorifera* somatic embryos in culture media without plant growth regulator.

Preliminary test using hydrolyzed casein ( $1 \text{ g l}^{-1}$ ) combined with NAA ( $0.5 \mu\text{M}$ ) and 2-iP (5, 10 or  $20 \mu\text{M}$ ) was also tested in the maturation of

somatic embryos of *Ocotea porosa*. However, the results were similar to those found when the NAA and 2iP alone were utilized. Thus, it is recommended to test other concentrations of hydrolyzed casein or other amino acids in order to increase the percentage as well as synchronization of the somatic embryo maturation.

In the maturation media, some abnormal somatic embryos were formed and, above them, globular somatic embryos. The formation of abnormal somatic embryos during the secondary somatic embryogenesis is probably due to the duration of contact with the medium containing 2,4-D. Titon et al. (2007) also reported lack of synchronization in the development and the formation of abnormal somatic embryos of *Eucalyptus grandis*.

### Morphoanatomical studies

Structures obtained in WPM medium containing 2,4-D ( $22.62 \mu\text{M}$ ) combined with 2-iP ( $2.46 \mu\text{M}$ ) presented histodifferentiation of somatic embryos (Fig. 2). In the Figure 2a, two distinct regions were observed: a region consisting of embryonic isodiametric cells, with dense cytoplasm, prominent nucleus and stained dark nucleoli showing the formation of pro-embryos (Fig. 2a arrow) with dividing cells (Fig 2b arrow). The inner region consisted of cells with large vacuole content featuring blue-green color through the metachromatic reaction with toluidine blue, indicating the presence of phenolic compounds. In *Myrciaria aureana* also, embryogenic explants were rich in phenolic compounds that had accumulated primarily in a sub-superficial zone between layers of cells of the peripheric region and storage parenchyma (MOTOIKE et al., 2007). As in the present study, the meristematic activity was observed near the zone rich in phenolic compounds where the cells

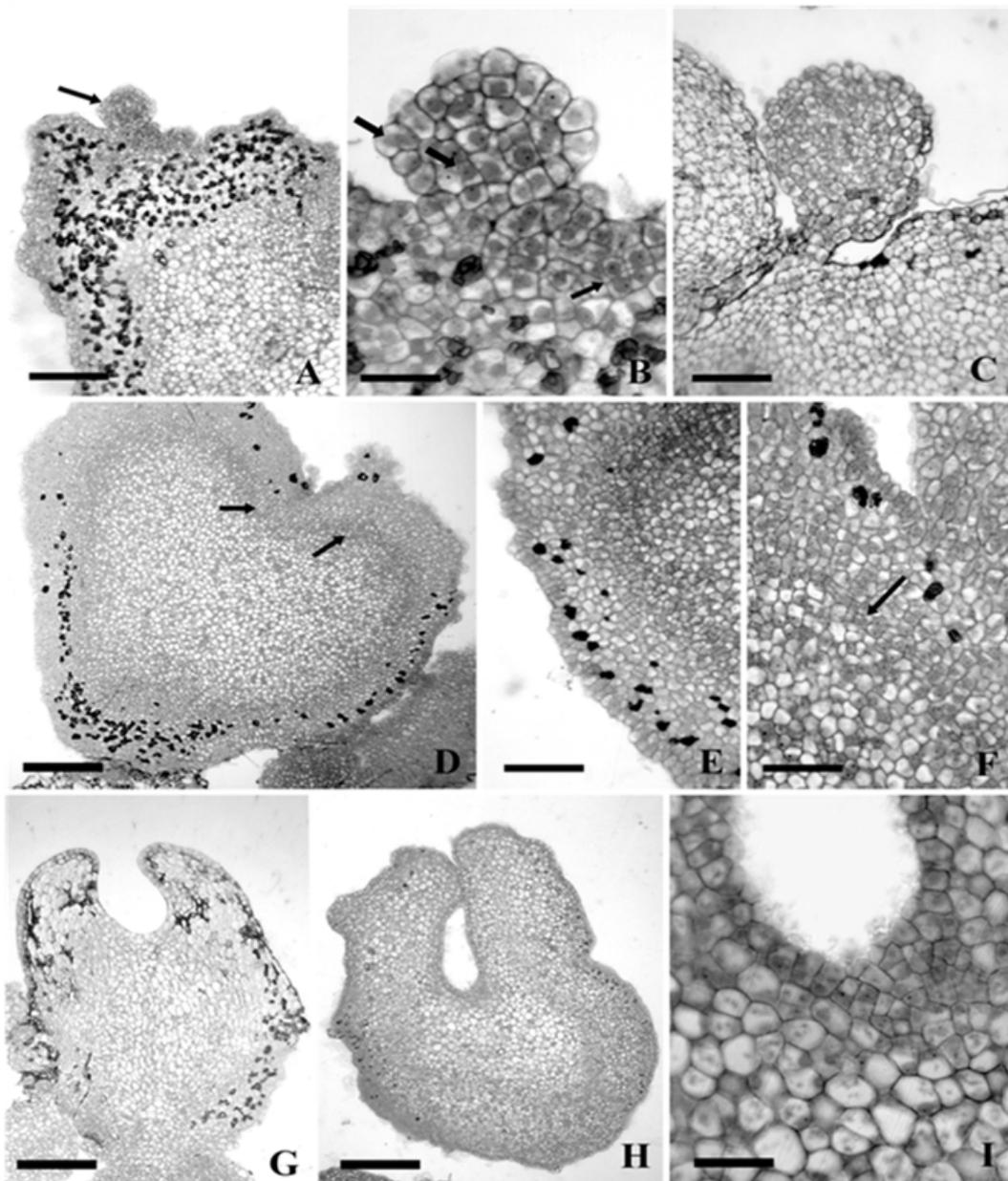


FIGURE 2: Developmental stages of somatic embryos of *Ocotea porosa*. A) Longitudinal section of the pro-embryo (eg arrow), bar: 150  $\mu\text{m}$ . B) Longitudinal section with isodiametric cells with large nuclei, prominent nucleoli and anticlinal and periclinal division (arrow), bar: 30  $\mu\text{m}$ . C) Globular somatic embryo, bar: 100  $\mu\text{m}$ . D) Longitudinal section of cordiform embryo, bar: 65  $\mu\text{m}$ . E) Defined protoderm, bar: 50  $\mu\text{m}$ . F) Early formation of the procambium of cordiform embryo (arrow) bar: 50  $\mu\text{m}$ . G) Longitudinal section of torpedo embryo, bar: 40  $\mu\text{m}$ . H) Longitudinal section of somatic embryo cotyledonary, bar: 100  $\mu\text{m}$ . I) Anticlinal and periclinal division and early organization of the apical meristem, bar: 20  $\mu\text{m}$ .

FIGURA 2: Estádios de desenvolvimento dos embriões somáticos de *Ocotea porosa*. A) Secção longitudinal do pró-embrião (seta), barra: 150  $\mu\text{m}$ . B) Secção longitudinal com células isodiamétricas com núcleo grande, nucléolo proeminente e divisão anticlinal e periclinal (seta). C) Embrião somático globular, barra: 100  $\mu\text{m}$ . D) Secção longitudinal do embrião cordiforme, barra: 65  $\mu\text{m}$ . E) Protoderme definida, barra: 50  $\mu\text{m}$ . F) Início da formação do procâmbio do embrião cordiforme (seta), barra: 50  $\mu\text{m}$ . G) Secção longitudinal do embrião torpedo, barra: 40  $\mu\text{m}$ . H) Secção longitudinal do embrião somático cotiledonar, barra: 100  $\mu\text{m}$ . I) Divisão anticlinal e periclinal e organização do meristema apical, barra: 20  $\mu\text{m}$ .

divided rapidly, forming somatic embryos from cells of the peripheric region. Histological sections confirmed the formation of various stages of somatic embryos of *Ocotea porosa* (Figure 2).

A globular structure with protoderm and a suspensor was observed, characterizing the first stage of somatic embryo development (Fig. 2c). The transition from globular to cordiform stage was marked by cellular elongation and cotyledon early growth (Fig. 2d). In this phase a defined protoderm (Fig. 2e) was observed, as well as cell division leading to procambium formation (Fig. 2f arrow). In Figure 2g, the differentiation of procambial track with elongated and vacuolated cells and the elongation of the cotyledons are visible, characterizing the torpedo stage. In this phase, the longitudinal section shows bilateral symmetry. At that stage, it was evident that deposition of phenolic compounds in the outer layers caused the degradation of cells. Figure 2h shows a somatic embryo at cotyledonary stage. Organized meristematic tissues are also present, initiating the formation of a shoot apical meristem (Fig. 2i) but root apical meristem is not observed. Although it was possible to characterize the cotyledonary stage of the embryo, the presence of the entire vascular system was not confirmed. According to Dodeman et al. (1997), in order to complete somatic embryogenesis, the formation of meristems must occur. However, histological analysis showed that somatic embryos of *Ocotea porosa* did not finish their development. Although somatic embryos have been characterized up to cotyledonary stage, these structures are not considered bipolar, since no difference was observed at the root apical meristem. Ribas et al. (2000), also observed the absence of differentiation of the meristem in somatic embryos of *Aspidosperma polyneuron*.

## CONCLUSIONS

For the first time, we demonstrated that somatic embryogenesis of *Ocotea porosa* can be induced from immature zygotic embryonic axes on WPM medium containing 200  $\mu\text{M}$  2,4-D alone or combined with 1 g l<sup>-1</sup> hydrolyzed casein. Somatic embryos were maintained and proliferated on hormone-free WPM medium supplemented with hydrolyzed casein (1 g l<sup>-1</sup>) via repetitive embryogenesis. The maturation of the embryos was low and asynchronous. Thus, the results of this study indicate that somatic embryogenesis of *Ocotea porosa* is feasible and this repetitive embryogenesis system

will provide a source of somatic embryo production, ensuring the optimization of a complete protocol for the regeneration of this species.

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