

## AUXIN PULSE IN THE INDUCTION OF SOMATIC EMBRYOS OF *Eucalyptus*

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**ABSTRACT** – The objective of this study was to evaluate the effect of auxin pulse intervals on the induction of somatic embryos of *Eucalyptus grandis* x *E. urophylla* and to describe the embryogenic behavior of callus under the effect of auxinic stress. Cotyledons were inoculated in culture medium containing 207.07 µM picloram, a treatment considered as auxin pulse. Explants that were in the auxin pulse treatment were transferred to semisolid or liquid medium containing 20.71 µM picloram after one, two, four or eight days of auxin pulse. In a second experiment, explants that were on auxin pulse treatment were transferred to semi-solid medium containing 20.71 µM picloram after one, two or three days of auxin pulse. Auxiliary picloram pulse treatments (207.02 µM) can be used as an initial source of stress for the acquisition of embryogenic competence. The oxidation of cotyledonary explants may be considered as an indication of the formation of embryogenic calli. The presence of pectins in peripheral regions of somatic pro-embryos can be considered as a marker of somatic embryogenesis in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla*.

**Keywords:** Auxin stress; Micropropagation, *Eucalyptus grandis* x *Eucalyptus urophylla*.

## PULSO DE AUXINA NA INDUÇÃO DE EMBRIÕES SOMÁTICOS DE EUCALIPTO

**RESUMO** – O objetivo do estudo foi avaliar o efeito de intervalos de pulsos de auxina na indução de embriões somáticos de *Eucalyptus grandis* x *E. urophylla* e a descrição do comportamento embriogênico de calos sob efeito do estresse auxínico. Cotilédones foram inoculados em meio de cultura contendo 207,07 µM de picloram, tratamento considerado como pulso de auxina. Explantes que estavam no tratamento de pulso de auxina foram transferidos para meio semissólido ou líquido, contendo 20,71 µM de picloram, após um, dois, quatro ou oito dias de pulso de auxina. Num segundo experimento, explantes que estavam no tratamento de pulso de auxina foram transferidos para meio semissólido contendo 20,71 µM de picloram, após um, dois ou três dias de pulso de auxina. Tratamentos com pulso de auxina picloram (207,02 µM) podem ser utilizados como fonte de estresse inicial para aquisição da competência embriogênica. A oxidação de explantes cotiledonares pode ser considerada um indicio de formação de calos embriogênicos. A presença de pectinas em regiões periféricas de pró-embriões somáticos pode ser considerada como marcador de embriogênese somática em explantes cotiledonares de *Eucalyptus grandis* x *E. urophylla*.

**Palavras-Chave:** Estresse auxínico, Micropropagação, *Eucalyptus grandis* x *Eucalyptus urophylla*.



## 1. INTRODUCTION

Somatic embryogenesis was developed with the purpose of achieving high and rapid rates of in vitro multiplication of plant species. In this sense, research advances aim to provide its use, not only as a research technique, but with a commercial scale application (Pinto et al., 2009).

In the forest area, the adoption of somatic embryogenesis aims its use, among other applications, towards homogeneous and large-scale production of clonal seedlings (Varis et al., 2018) as a vegetative propagation technique within forest improvement programs, or as a technique for rejuvenating clones for purposes of clonal forestry (Xavier et al., 2013).

However, low levels of embryogenic initiation were observed in *Eucalyptus* species (Muralidharan and Mascarenhas, 1995; Pinto et al., 2002), the inability of somatic embryos to reach full maturity and germinate (Muralidharan and Mascarenhas, 1995) and the occurrence of somaclonal variation (Jain, 2006) have limited their adoption as a commercial technique.

Among the several factors that interfere with somatic embryogenesis propagation, it is known that high doses of auxin are essential as an initial trigger in the acquisition of cellular competence, causing dedifferentiation and embryogenic redifferentiation (Nic-Can et al., 2016; Nic-Can and Loyola-Vargas, 2016; Krishnan and Siril, 2017; Kaur et al., 2018; Grzyb and Mikula, 2019). However, continuous exposure of the explant to the growth regulators, especially auxins, may have negative effects on the morphology of in vitro propagated plants, such as hyperhydricity, dwarfism, fasciation, structure distortion or somaclonal variation (Faisal et al., 2012).

A strategy to use the auxin dosage required to induce somatic embryos, even in recalcitrant species such as *Eucalyptus*, without causing great damage to the propagated seedling, would be the use of auxin pulse treatments.

Treatments of 2,4-D (*dichlorophenoxyacetic acid*) pulse were used to induce somatic embryos in plant species such as carrot (*Daucus carota*) (Kitamiya et al., 2000), potato (*Solanum tuberosum* L.) (Sharma et al., 2017), and papaya (*Caric papaya* L.) (Koehler

et al., 2013), and with auxin ANA (naphthaleneacetic acid) in string bean (*Vigna unguiculata* L.) (Aasim, 2010).

Pulses of different auxins also promoted, morphogenesis of adventitious roots in *Eucalyptus grandis* (Picoli et al., 2006). However, in the literature, there are no reports on auxin pulse treatments in the induction of somatic embryos of *Eucalyptus* species.

The objective of the present study was to evaluate the effect of different auxin pulse intervals on the induction of somatic embryos of *Eucalyptus grandis* x *E. urophylla*, as well as to describe the embryogenic behavior of callus under the effect of auxinic stress.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

In order to obtain the explants used in the present study, hybrid seeds of *Eucalyptus grandis* x *E. urophylla* from APS 13 of the company Gerdau, located in Três Marias-MG, were collected in January 2011. For disinfestation, the seeds were previously washed in running water and, in a horizontal laminar flow chamber, immersed in 70% alcohol for 30 seconds and then in 5% sodium hypochlorite (2.5% active chlorine) for 15 minutes, with 4 drops of tween 20 detergent being added every 100 mL of solution. After the disinfestation treatment, the seeds were washed six times in autoclaved water. In order to obtain the cotyledons, the already disinfested seeds were placed to germinate in disposable sterile petri dishes (90 x 15 mm) containing 30 mL of pre-autoclaved semi-solid culture medium at 120 °C and 1 atm pressure for 20 minutes. The culture medium used contained 50% of the salts and vitamins MS (Murashige and Skoog, 1962), 1.5% sucrose, 50 mg.L<sup>-1</sup> myo-inositol and 2,8 g.L<sup>-1</sup> de Phytigel® and adjusted pH to 5.8 ± 0.01. The seeds were maintained for 10 days in a culture room at 24 ± 1 °C and a 16-hour photoperiod with an irradiance of 33 μmol m<sup>-2</sup> s<sup>-1</sup> (quantified by a radiometer (LI-COR®, LI-250A Light Meter), supplied by two tubular fluorescent lamps (Special Daylight, 40W, Osram, Brazil)

### 2.2. Induction of somatic embryos

Cotyledons were used as explants and inoculated in a 250 ml erlenmeyer flask containing 20 mL of MS medium liquid with 100% salts and vitamins, 100

mg.L<sup>-1</sup> myo-inositol, 3% sucrose and pH adjusted to  $5.8 \pm 0.01$ , previously autoclaved at 120 ° C and 1 atm pressure for 20 minutes. 207.07 µM 4-amino-3,5,6-trichloropicolinic acid (picloram), a treatment considered as auxin pulse, was added to the culture medium.

The erlenmeyers, containing 20 explants (cotyledons) each, were kept in the culture room in the dark at a temperature of  $24 \pm 1$  °C, with an agitation of 50 rpm to maintain oxygenation in the culture medium for the auxin pulse treatment. Based on this material, two experiments were performed, as described below.

### 2.1.1. Experiment 1

Explants (cotyledons) that were in the auxin pulse treatment were transferred to sterile disposable Petri dishes (60 x 15 mm) containing 10 mL of semisolid MS medium or to 125 mL erlenmeyers containing 10 mL of liquid MS medium. The culture medium used in this step (somatic embryo induction medium) was prepared with 100% of the salts and vitamins, 100 mg.L<sup>-1</sup> myo-inositol, 3% sucrose, 2,8 g.L<sup>-1</sup> Phytigel (for semi-solid medium) and 20.71 µM picloram, pH adjusted to  $5.8 \pm 0.01$ , previously autoclaved at 120°C and 1 atm pressure for 20 minutes.

The transfer of the explants from the auxin pulse medium to the somatic embryo induction medium was performed after one, two, four or eight days of auxin pulse. The control treatment was also performed, where the explants were inoculated directly into the somatic embryo induction medium, without auxin pulse pre-treatment.

All explants were kept in the culture room in the dark at a temperature of  $24 \pm 1$  °C and shaking at 50 rpm (liquid medium) to maintain oxygenation in the culture medium.

A completely randomized design was used in a 2x5 factorial scheme, consisting of two types of culture medium (semi-solid or liquid) and five auxin pulse pretreatment times (zero, one, two, four and eight days), with six replicates and 10 explants per plot.

After 30 days of induction, the percentages of callogenesis, friable callus and callus containing somatic embryos (embryogenic callus) were evaluated. The presence or absence of oxidation of

the explants per callus was also evaluated at 30 days. Samples were collected for light microscopy analysis and for live photographic documentation performed by means of a digital camera (Olympus E-330) coupled to a microscope binocular stethoscope.

### 2.1.2. Experiment 2

Explants (cotyledons) that were in the auxin pulse treatment were transferred to sterile disposable Petri dishes (60 x 15 mm) containing 15 mL of semi-solid MS medium, prepared with 100% of the salts and vitamins, 100 mg.L<sup>-1</sup> of 3 g of sucrose, 2,8 g.L<sup>-1</sup> of Phytigel® and 20,71 µM of picloram, pH adjusted to  $55,8 \pm 0,01$ , pre-autoclaved at 120°C and 1 atm pressure for 20 minutes

The transfer of explants from the auxin pulse medium to the somatic embryo induction medium was performed after one, two or three days of auxin pulse. All the explants were kept in the culture room in the dark at a temperature of  $24 \pm 1$  °C.

A completely randomized design with three treatments (one, two or three days of auxin pulse) with 10 replicates and 10 explants per plot was used.

At 30, 37 and 44 days of induction, the percentages of oxidation, callogenesis, of frail calli and explants containing somatic embryos (embryogenic calli) were evaluated. The mean number of somatic pro-embryos and the mean number of somatic pro-embryos per embryogenic callus were also evaluated. Sampling was performed for light microscopy analysis and for live photographic documentation performed by means of a digital camera (Olympus E-330) coupled to a microscope binocular stethoscope

### 2.3. Data analysis

The homoscedasticity of the data was analyzed by the Cochran test at 5% probability and the residues normality by the Shapiro-Wilk test at 5% probability. For the statistical analysis of the data, the variances were compared through the F test at 5% probability and the means were compared by the Tukey test at 5% probability. Oxidation data, due to the binomial distribution (0 = absence and 1 = presence), were analyzed for all in vitro growth variables, using the logit link function and the generalized linear model. The model was chosen based on the significance of the coefficients, using the z test at 7% of probability.

Statistical analyses were performed using the statistical program R, version 3.0.3 (R Core Team, 2018) and the ExpDes package (Experimental Designs) (Ferreira et al., 2014). In addition to the statistical analysis, a descriptive analysis of the data was performed through means and standard deviations.

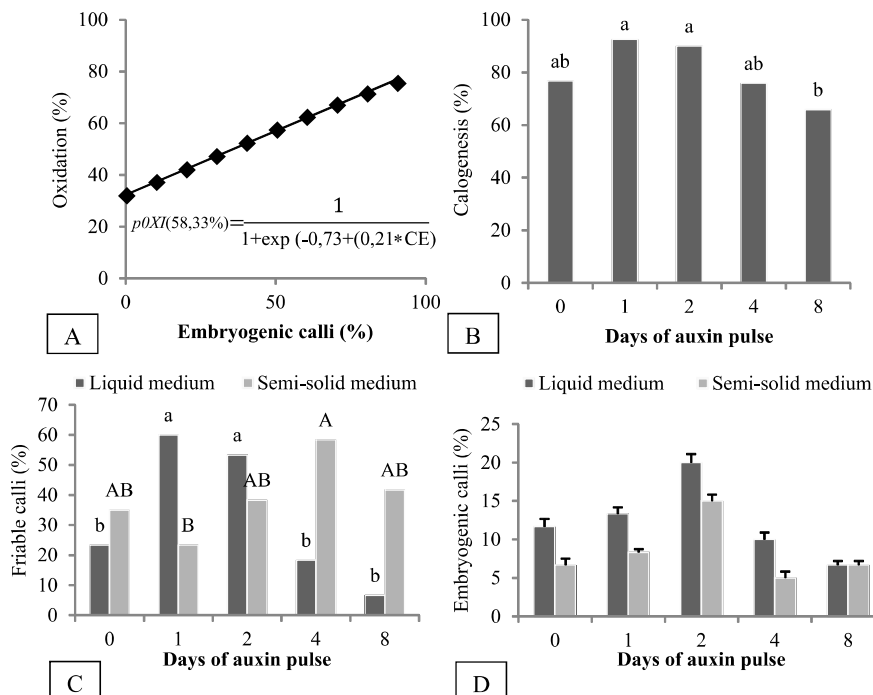
#### 2.4. Anatomical and histochemical analysis

Samples of embryogenic calli were collected for glutaraldehyde solution (Karnovsky, 1965) modified - 2.5% glutaraldehyde, 4% paraformaldehyde, 3% sucrose, 5  $\mu$ M CaCl<sub>2</sub> in 0.1M cacodylate buffer pH 6.8), for at least one week, dehydrated in ethanol series and included in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). To obtain cross sections with 5  $\mu$ m thickness, an automatic feed rotary microtome (RM 2255, Leica Microsystems Inc., Deerfield, USA) was used. For structural

characterization, the sections were stained for 10 minutes in toluidine blue pH 4.0 (O'Brien and McCully, 1981). For histochemical characterization, the sections were stained for 10 minutes in Ruthenium Red (Johansen, 1940) to evidence pectins. The slides were assembled with Permount and the images captured using a photomicroscope (AX70TRF, Olympus Optical, Tokyo, Japan) equipped with the U-Photo system.

### 3. RESULTS

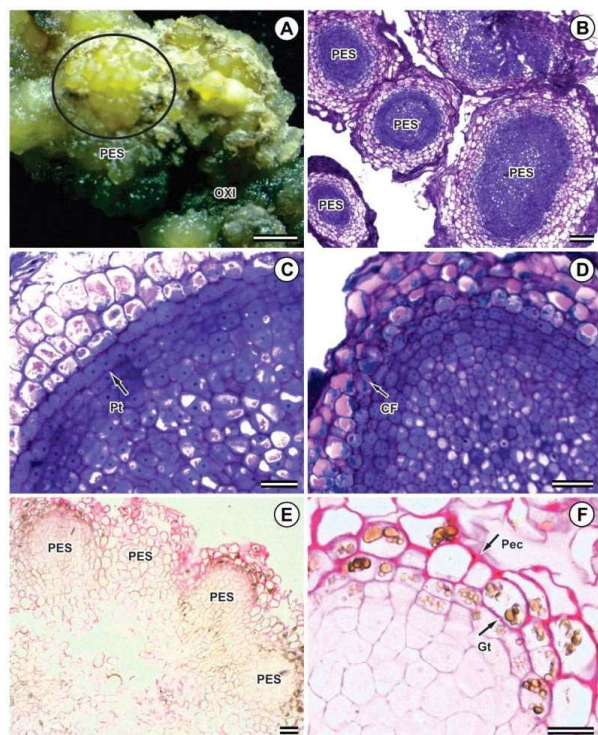
The relationship between the probability of occurrence of oxidation in the callus and the percentage of embryogenic calli was positive and increasing according to the logit binding function at 7% probability. It was observed that the increase in the percentage of embryogenic callus reflects in a greater probability of occurrence of oxidation in the callus (Figure 1A). In addition, embryogenic callus



**Figure 1** – Probability of oxidation (%) in relation to the percentage of embryogenic calli, according to the Logit binding function (A), percentage of calogenesis in relation to days of auxin pulse (B), percentage of friable calli (C) and percentage of embryogenic calli (D) in relation to the days of auxin pulse and the type of culture medium of *Eucalyptus grandis* x *E. urophylla* explants at 30 days. Averages of the same lowercase letter [between the pulse days (B) for the liquid medium (C)] and uppercase [between the pulse days for the semi-solid medium (C)] do not differ by the 5% Tukey test. The bars represent the standard deviations.

**Figura 1** – Probabilidade de oxidação (%) em relação ao porcentual de calos embriogênicos, segundo a função ligadora Logit (A), porcentual de calogênese em relação aos dias de pulso de auxina (B), porcentual de calos friáveis (C) e porcentual de calos embriogênicos (D) em relação aos dias de pulso de auxina e ao tipo de meio de cultura, de explantes de *Eucalyptus grandis* x *E. urophylla* aos 30 dias. Médias contempladas com a mesma letra minúscula [entre os dias de pulso (B) para o meio líquido (C)] e maiúscula [entre os dias de pulso para o meio semissólido (C)] não diferem entre si pelo teste Tukey a 5%. As barras representam os desvios padrões.

was observed with a large part of the oxidized tissue and the presence of indirectly formed somatic pro-embryos (with callogenesis in the explant, preceding the induction of pro-embryos) (Figure 2A).



**Figure 2** – Photograph captured by stethoscopy (A), longitudinal sections obtained by photomicroscopy of light (B, C, D, E and F) during the induction of embryogenic cultures in cotyledons of *Eucalyptus grandis* x *E. urophylla*, obtained at the time of 30 (C), 37 (B, D, E e F) e 44 (A) days of induction, submitted to treatments of 1 day (E and F), 2 days (A, B e D) and 3 days (C) of auxin pulse with 50µM picloram. Cross sections submitted to toluidine blue (structural analysis) (B, C and D) and ruthenium red, with positive reaction for pectins (E and F). CF = phenolic cord; Gt: Oil droplets; Oxidação Pec = Pectin; PES = somatic pro-embryos; Pt: Protoderm. 100 µm bars (B); 50 µm (D and E); 20 µm (C and F); 1 mm (A).

**Figura 2** – Fotografia capturada por estetoscopia (A), seções longitudinais obtidas por fotomicroscopia de luz (B, C, D, E e F) durante a indução de culturas embriogênicas em cotilédones de *Eucalyptus grandis* x *E. urophylla*, obtidas nos tempos de 30 (C), 37 (B, D, E e F) e 44 (A) dias de indução, submetidas aos tratamentos de 1 dia (E e F), 2 dias (A, B e D) e 3 dias (C) de pulso de auxina com 50µM de picloram. Seções transversais submetidas ao azul de toluidina (análise estrutural) (B, C e D) e ao vermelho de rutênio, com reação positiva para pectinas (E e F). CF= cordão fenólico; Gt: Gotículas de óleo; OXI: Oxidação Pec= Pectina; PES= pró-embriões somáticos; Pt: Protoderme. Barras 100 µm (B); 50 µm (D e E); 20 µm (C e F); 1 mm (A).

Significant differences were found between the types of culture medium used (Test F) and also between auxin pulse days for the callogenesis percentage characteristic (Figure 1B). In the semi-solid medium, the percentage of callogenesis was 96%, and in the liquid medium, 64%. In relation to the number of days of auxin pulse, one and two days of auxin pulse were found to have significant mean averages in callogenesis percentage over eight days of auxin pulse (Figure 1B).

For the liquid culture medium, one and two days of auxin pulse provided higher percentages of friable calli than the other auxin pulse intervals (Figure 1C). For the semi-solid culture medium, the average percentage of friable callus at four days of auxin pulse was significantly higher than the average of friable calluses at one day of auxin pulse.

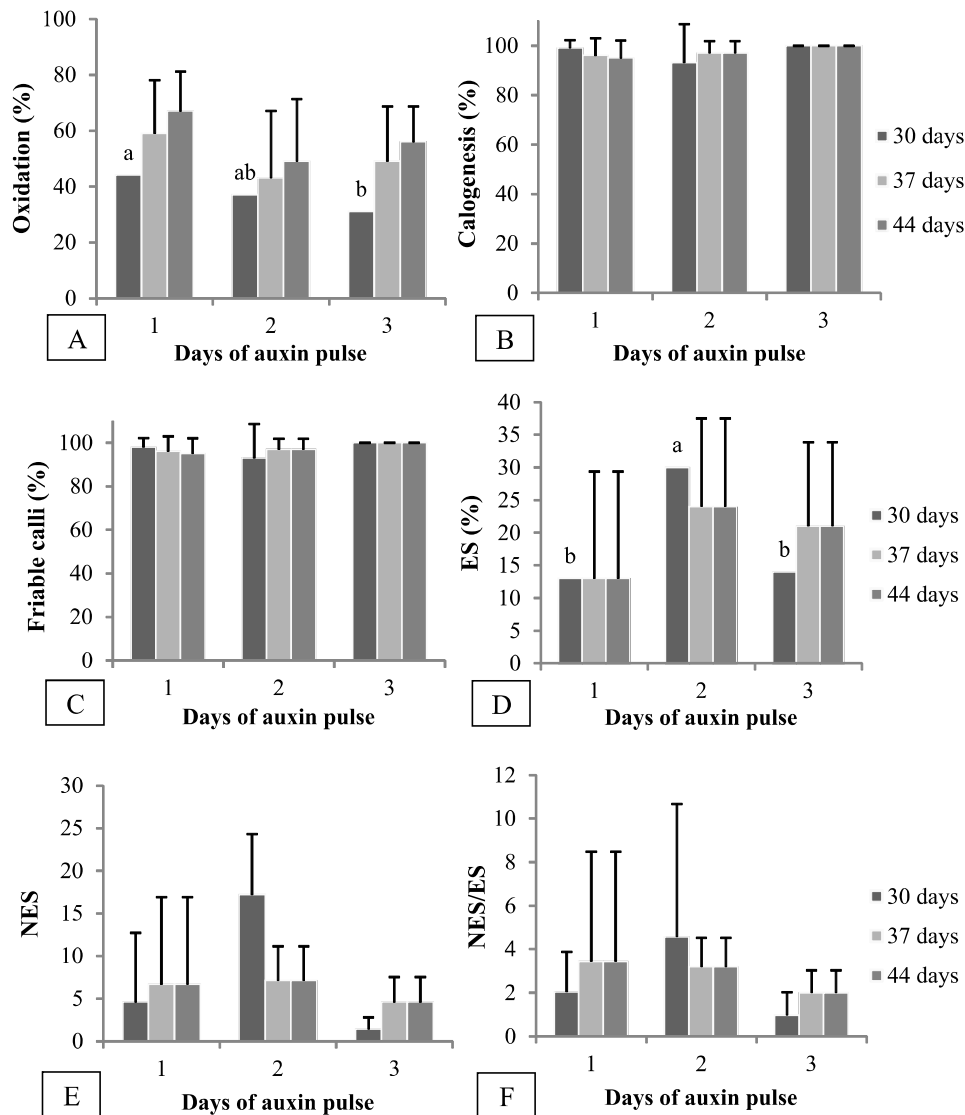
For the percentage of embryogenic calli, no significant differences were found between the interaction and between the treatments. However, a similar trend between the two types of culture medium (liquid and semi-solid) can be observed in Figure 1D, where there is a growth in the percentage of embryogenic calluses up to the two-day auxin pulse interval, followed by the decrease of this characteristic from that interval.

In general, the somatic pro-embryos formed in this study look shiny and yellowish and are in the globular stage. Embryogenic calli are friable and oxidized (Figures 2A, 2B and 2E).

In some somatic pro-embryos, protoderm differentiation was observed, characterized by adjacent cells (Figure 2C), as well as by the presence of a phenolic cord around this structure (Figure 2D).

At 30 days of evaluation, the average oxidation relative to one day of auxin pulse was higher than the mean of oxidation at three days of auxin pulse (Figure 3A). This same behavior remained at 37 and 44 days of evaluation, with the percentage of oxidation decreasing as the interval of days of auxin pulse increased (Figure 3A).

For the percentages of callogenesis and percentage of friable calli, mean values close to 100% were observed in all auxin pulse treatments and in all evaluation intervals (Figures 3B and 3C).



**Figure 3** – Percentage of oxidation (A), percentage of calogenesis (B), percentage of friable callus (C), percentage of embryogenic callus somatic embryos (ES%) (D), number of somatic pro-embryos (NES) (E) and the mean number of embryogenic callus somatic embryos (F) in relation number of days of auxin pulse of *Eucalyptus grandis* x *E. urophylla* explants at 30, 37 and 44 days. The bars represent the standard deviations of the patterns.

**Figura 3** – Porcentual de oxidação (A), porcentual de calogênese (B), porcentual de calos friáveis (C), porcentual de calos embriogênicos (ES%) (D), número médio de pró-embriões somáticos (NES) (E) e o número médio de pró-embriões somáticos por calos embriogênico (F) em relação número de dias de pulso de auxina de explantes de *Eucalyptus grandis* x *E. urophylla* aos 30, 37 e 44 dias. As barras representam os desvios padrões das médias.

In the evaluation at 30 days for the percentage of embryogenic callus (% ES), the mean was significantly higher with two days of auxin pulse in relation to the other days tested at 30 days of evaluation (Figure 3D). For the other evaluation dates, 37 and 44 days, this same behavior was observed, with averages of two days of auxin pulse higher than the means at one

or three days of auxin pulse for the percentage of embryogenic calluses (% ES) (Figure 3D).

For the average number of somatic pro-embryos (NES) and the mean number of somatic embryos by embryogenic callus (NES / ES), similar behavior was observed in the three evaluations performed at 30, 37 and 44 days of induction. As the interval from

one to two days of auxin pulse increased, there was an increasing trend of NES and NES/ES, and, after increasing the auxin pulse from two to three days, there was a decreasing trend of NES and NES/ES (Figures 3E and 3F). Figures 3E and 3F allow to observe the occurrence of high magnitude standard deviations for the NES and NES/ES characteristics. This situation was already expected, since the NES and NES/ES characteristics did not follow a normal distribution ( $p < 0.05$  by the Shapiro-Wilk test). In both NES and NES/ES, the occurrence of discrepant data was observed in all the evaluation intervals, for example, the presence of up to 32 globular somatic pro-embryos (Figure 2A) in a single explanted treated with two days of auxin pulse at 44 days of induction per evaluation.

#### 4. DISCUSSION

The formation of embryogenic calli in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla* is usually accompanied by the oxidation of callogenic tissue, as observed in the present study, which was positive and increasing between the percentage of embryogenic calli and the occurrence of oxidation in the callus.

In some non-woody species, callus oxidation causes absence of the embryogenic process and death of the explant (Takamori et al., 2015). However, in some other species, woody or otherwise, the oxidation of callogenic tissue preceding or concomitant to the formation of somatic embryos can be observed, as in the somatic embryogenesis of *Eucalyptus grandis* x *E. urophylla* (Moura et al., 2017), *Vitis* (Martínez et al., 2015) and *Mondia whitei* (Baskaran et al., 2015). Oxidation may occur when the excised explant produces secondary metabolites when in contact with the culture medium, producing a brownish color in the tissue.

In the present study, the induction of somatic embryogenesis in *Eucalyptus grandis* x *E. urophylla* proved to be asynchronous, since in all the evaluation times of the second experiment (30, 37 and 44 days) it was possible to observe the beginning of dedifferentiation of the cotyledon explant for the formation of embryogenic calli.

A similar aspect occurred during the ontogenetic study of somatic embryogenesis of *Euterpe oleracea* from zygotic embryos, and the somatic embryo induction at different stages of maturation was

observed, as well as the formation of secondary somatic embryogenesis (Scherwinski-Pereira et al., 2012). Some plant cells may acquire competence at different stages of tissue exposure to the culture medium plus growth regulators. When cells acquire meristematic activity again, they are called competent cells (Rocha et al., 2012).

From the results of the first experiment it was observed that the morphogenic responses of the *Eucalyptus grandis* x *E. urophylla* explants were higher when using semisolid culture medium, relative to the liquid medium. Within the range of days of the auxin pulse tested, between zero and eight days, there was an increasing tendency of occurrence of embryogenic callus up to two days of pulse, and decreasing from that value. For this reason, we chose to use the semi-solid medium and the auxin pulse interval of one to three days in the second experiment conducted in this study, to prove the results and to perform a more detailed analysis, with data collection and samples in three induction intervals.

The mean induction of embryogenic calli was higher in the two-day auxin pulse interval, compared to the other two intervals tested in the second experiment, both at 30 days and at 37 and 44 days of evaluation, agreeing with the results of the first experiment.

According to some studies that have also used the pulse of growth regulators in plant morphogenesis, the duration of the pulse treatment may last a few hours or a few days (Sharma et al., 2007; Zaytseva et al., 2016).

Often, pulse treatment is used to minimize negative effects on the morphology of plants propagated in vitro, where there is usually continuous exposure to growth regulators. This exposure may result in plants with hyperhydricity, dwarfism, fasciation, structure distortion (Faisal, 2012) or somaclonal variation, and pulse treatment may reduce plant exposure time to the growth regulator (Aasim et al., 2010).

Auxin pulse treatments can also be used to accelerate the morphogenic process, as observed in somatic potato embryogenesis using 2,4-D pulses (Sharma et al., 2007). However, in the present study, auxin pulse treatment was tested as a trigger for cell stress, causing dedifferentiation and embryogenic redifferentiation (Fehér, 2015).

In carrot zygotic embryogenesis, they indicate high auxin concentration after fertilization (Ribinicky et al., 2002), which emphasizes the importance of temporary alterations in endogenous auxin levels for the expression of cell totipotency (Fehér, 2015). These observations suggest that the endogenous auxin pulse may be one of the primary signs for the induction of somatic embryogenesis (Fehér et al., 2003), and, under controlled conditions, it may also be achieved with exogenous auxin pulse treatments.

Some authors have observed this interference factor for the auxin pulse in carrots (Kitamiya et al., 2000) and potato (Sharma et al., 2007), where the 2,4-D pulse was able to provide the necessary stimuli for induction of somatic embryogenesis. However, in these same studies, new components were required in the culture medium and/or new culture conditions for the maturation and germination of somatic embryos (Karmaiya et al., 2000; Sharma et al., 2007).

Thus, although the pulse of picloram was the initial trigger for induction of somatic pro-embryos in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla*, new studies must be carried out to continue the embryogenic process in the species, with maturation, germination and acclimatization of the seedlings formed. For example, the bipolarity of embryogenic structures, a process that evidences the formation of a somatic embryo (Mendéz-Hernández et al., 2019), is the name of the structures of this study as somatic pro-embryos. One must also consider the genetic factor, since explants from seeds were used that came from several different genetic materials. Some authors have already verified the existence of genetic control in somatic embryogenesis in some species such as palm oil (Corrêa et al., 2015) and eucalyptus (Pinto et al., 2007).

By the Ruthenium Red test performed in the present study, pectin accumulation was observed in the peripheral regions of the somatic pro-embryo, regions where it was possible to observe the beginning of the formation of the protoderm, characterized by adjacent cells.

Pectins are the major components of the primary cell walls of all terrestrial plants (Pilarska et al., 2013). In studies with *Medicago arborea* (Endress et al., 2009), pectin content differences were observed in embryogenic and non-embryogenic callus cells. Evidence regarding the specific distribution of pectin

epitopes during somatic embryogenesis is scarce (Pilarska et al., 2013), but embryogenic callus-proliferative cells have been shown to contain large amounts of reactive pectins (Xu et al., 2011). The presence of pectins in peripheral regions of somatic pro-embryos could then be considered as a marker of somatic embryogenesis in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla*.

Also in the histochemical test with Ruthenium Red, the accumulation of oil droplets inside cells located in the peripheral region of the somatic pro-embryo was observed in some embryogenic calli. These droplets of oil may be used by somatic pro-embryos as reserve substances, since in dicotyledons mainly lipids and proteins are found as initial sources of reserve (Rocha et al., 2012) essential for somatic embryos because they do not have a connection with maternal tissue (Pila Quinga et al., 2018).

## 5. CONCLUSIONS

- Pulse treatment of auxin picloram (207.02 µM) can be used as an initial source of stress for the acquisition of embryogenic competence in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla*;
- Mean induction of embryogenic callus was higher in the two-day auxin pulse interval, compared to the other two intervals tested at both 30 days and at 37 and 44 days of evaluation;
- Oxidation of cotyledonary explants of placed *Eucalyptus grandis* x *E. urophylla* may be considered an indication of formation of embryogenic calli;
- The presence of pectins in peripheral regions of somatic pro-embryos can be considered as a marker of somatic embryogenesis in cotyledonary explants of *Eucalyptus grandis* x *E. urophylla*.

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