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ENDOGENOUS QUANTIFICATION OF ABSCISIC ACID AND INDOLE-3-ACETIC ACID IN SOMATIC AND ZIGOTIC EMBRYOS OF *Nothofagus alpina* (Poepp. & Endl.) Oerst.

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Abscisic acid (ABA) and indole-3-acetic acid (IAA) participate in the propagation of plants by somatic embryogenesis, causing polar structural differentiation of the embryo. The goal of the assay was to compare endogenous levels of ABA and IAA between somatic embryos (SE) and zygotic embryos (ZE) of *Nothofagus alpina* (Poepp. & Endl.) Oerst. In this study, a somatic embryo maturation assay involving the addition of varying concentrations of exogenous ABA was performed on cotyledonary-stage of *N. alpina*. Furthermore, the endogenous levels of ABA and IAA were quantified in the immature ZE, the mature ZE, and the embryonic axis of a mature embryo of *N. alpina*. The current study utilized high performance liquid chromatography (HPLC) for quantification. The maturation treatments performed did not present significant differences in the endogenous ABA levels in SE. However, significant differences did exist in levels of ABA and IAA between SE submitted to the different maturation treatments and mature ZE of *N. alpina*. The application of exogenous ABA to the culture medium increased endogenous ABA levels, therefore, increasing the number of germinated somatic embryos. Thus, the plant conversion process was also successfully completed in somatic embryos of *N. alpina*.

Key words: Somatic embryogenesis, maturation, germination, HPLC.

Nothofagus alpina (Poepp. & Endl.) Oerst., "raulí", is a native species of the Chilean forest. It has the greatest potential for commercial use due to its rapid growth and its quality wood (Gutiérrez, 2000). *Nothofagus alpina* also has the ability to diversify Chilean forestry production. Nonetheless, the growing problem of a deteriorating, shrinking agricultural landscape in Chile, coupled with the industry preference of exotic forest species of faster growth (Donoso and Lara, 1995) establish the need to apply biotechnological tools to *N. alpina* in order to potentiate its internal characteristics, increase its production, and preserve it as a natural resource of Chile (Pérez, 1998).

Among the available techniques of massive propagation, somatic embryogenesis is considered by some researchers as the most preferred method (Cevallos *et al*., 2002). This regeneration technique of *in vitro* plant tissue culture involves a process by which somatic cells of the donor plants are "reprogrammed." These cells maintain the genotype of the donor plant by following an identical pattern of development as that of an embryo coming from a zygotic origin (Merkle and Dean, 2000; Celestino *et al*., 2005).

In "raulí" it has been possible to obtain somatic embryos from mature seeds by conditioning proembryogenic masses with a high dose of auxins and cytokinins during the induction phase. The multiplication and maintenance of the clonal line has been achieved by secondary somatic embryogenesis (SSE), which is apparently unlimited in time and provides an even greater multiplying potential (Castellanos *et al*., 2005).

The main problem in the somatic embryogenesis process is the efficient maturation of the embryos, in general, somatic embryo germination is altered by culture conditions (embryo induction and maturation); therefore, it generally results in different degrees of development (germination) and plantlet development (Vahdati *et al*., 2008). Therefore, it is necessary to apply maturation treatments in order to maximize the development of embryos in later stages (Corredoira *et al*., 2003; Miguel *et al*., 2004). In general, it is sought to achieve a synchronic and quality production of the embryos, so that they resemble the mature zygotic embryos (ZE) of the species (Celestino *et al*., 2005).

There are numerous morphological and biochemical similarities observed in developing somatic and ZE

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(Tereso *et al*., 2007). Consequently, culture sequences should include a maturation promotion phase of the somatic embryos (SE) before germination to resolve differences encountered mainly between the embryonic development and maturation processes (Palada-Nicolau and Hausman, 2001).

In general, studies have shown that abscisic acid (ABA) is the main regulator of SE maturation. ABA had a positive effect in the control of SSE in *Quercus suber* L. (García-Martín *et al*., 2005) and plays a role in the accumulation of reserve substances (Gupta and Grob, 1995). ABA has been recognized as a factor for promotion of normal development and maturation of somatic embryos and their uniformity in *Quercus ilex* L. and *Juglans regia* L. (Mauri and Manzanera, 2003; Vahdati *et al*., 2008). Studies show a decrease in fresh weight (FW) and SSE in treatments where ABA was added can be explained by a possible osmotic besides the effect and ABA's capability to inhibit premature germination and control SSE (Bentsink and Koornneef, 2008; Manoj *et al*., 2008)

ABA plays a fundamental role in both somatic embryogenesis and zygotic embryogenesis. At the beginning and middle stages of seed development, an increase in ABA levels controls and regulates protein synthesis, promoting desiccation tolerance. ABA levels are abundant during medium and advanced seed stages as well, promoting the accumulation of mRNA, which encodes reserve proteins known as LEA (Late-embryogenesis-abundant) (Dodeman *et al*., 1997; Azcón-Bieto and Talón, 2000; Finkelstein *et al*., 2002; Von Arnold *et al*., 2002; Pandey *et al*., 2008).

A few studies point out that indole-3-acetic acid (IAA) is an important factor for describing the behavior that ABA presents in the embryogenesis process. The biosynthesis of IAA increases during the development of the ZE until the early maturation phase (Von Arnold *et al*., 2002). However, Hansen and Grossmann (2000) report that auxins could induce *de novo* synthesis of endogenous ABA in *Galium aparine* L. Miller *et al*. (1994) postulate that the developmental differences observed in the germination rate between SE and ZE is attributed to the lack of maternal signs by somatic tissue. These maternal signs allow *de novo* synthesis of ABA and other regulators necessary for development.

Therefore, the main objective of this study was to evaluate the effectiveness of exogenous ABA on the maturation of SE and compare it to endogenous ABA levels of SE and ZE of *N. alpina* with the ultimate goal of obtaining quality SE for germination.

MATERIALS AND METHODS

Plant material

Plant material from the embryogenic line *N. alpina* RaC-01 (raulí cotyledonary explants 01) was induced from mature seeds, which were obtained through controlled pollination (Castellanos *et al*., 2005). After inducing embryogenic calli and producing SE, the cultures were transferred to maintenance medium prepared with the minerals and vitamins solution, broadleaved tree medium (BTM) (Chalupa, 1983), plus growth regulators 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) both at a concentration of 0.5 µM, supplemented with 30 g L^{-1} sucrose and 7.0 g L^{-1} agar agar (Merck, Darmstadt, Germany). Every 28 d, cultures were placed in fresh medium, alternating subcultures in BTM medium with growth regulators and BTM medium without regulators. The explants remained in the latter condition until the assays began.

Starting material

The starting material for each one of the maturation assays consisted of proembryogenic masses (PEM) of the clonal line RaC-01 containing SE in the cotyledonary stage. Between 40-70 mg (FW) of PEM was picked up at the beginning of the maturation treatment. The initial fresh weight of each explant was recorded.

For ABA quantification, immature embryos were extracted from seeds and mature embryos from seeds. To evaluate ABA storage inside the mature embryo, the embryonic axis and the cotyledons were isolated in order to obtain the best parameter of endogenous ABA levels between SE and ZE.

Somatic embryo maturation assay

Four maturation treatments were carried out with the addition of ABA in concentrations of 0, 7.5, 11.3, and 18.9 µM (T1, T2, T3 and T4, respectively) in the culture medium. In each case, the mineral solution BTM was used as a basal medium, plus 60 g L^{-1} sucrose and 7.0 g L^{-1} agar agar. The culture was kept in continuous darkness at 25 ± 1 ºC day temperature and 22 ± 1 ºC at night. After 3 wk of culture, the fresh weight increase (FWI) of the PEM was evaluated and the final weight was recorded. In addition, SSE incidence was evaluated for each treatment.

Somatic embryo germination assay

After the maturation phase, PEM were subcultured in basal BTM without growth regulators for a period of 3 wk. During the germination phase, 10 samples of PEM under maturation treatment were randomly isolated, leaving SE in the cotyledonary stage within the isolated PEMs. These SE were later cultured in BTM medium with 25% (v/v) diluted macronutrients, supplementing with 30 g L^{-1} sucrose, 7.0 g L^{-1} agar agar, and 0.3 µM GA_3. GA_3 was sterilized by filtration and applied to the medium after being autoclaved at 121 °C at 1 atm for 20 min. The culture was kept in darkness for the first 7 d and later under a 16:8 h photoperiod, at a temperature of 25 ± 1 °C at day and 22 ± 1 °C at night, for 3 wk.

ABA and IAA quantification

Free ABA and IAA extractions for the comparison of endogenous ABA levels between somatic embryos (SE) and zygotic embryos (ZE) were carried out with some modifications by the method proposed by Materán *et al*. (2009). A sample of 100 mg of fresh tissue was obtained from mature ZE, immature zygotic embryos (Ie), and embryonic axes isolated from mature embryos (Aze) of *N. alpina*. Fresh tissue of *N. alpina* (100 mg) was also sampled from cotyledonary stage SE isolated from PEM submitted to different maturation treatments.

Tissue was homogenized in liquid nitrogen and resuspended in 10 mL of 80% v/v methanol. This solution was kept under constant agitation of 150 rpm at 4 °C for 12 h. Afterwards, the samples were filtered with hydrophobic paper of 0.22 μm porosity, and the extract was concentrated with a rotary evaporator at 50 °C to eliminate the methanol. It was added 5 mL deionized water to the solution, and the pH was adjusted to 2.0. An extraction procedure was performed four times with 10 mL ethyl acetate, forming a liquid phase that contained conjugated ABA and IAA and an organic phase that contained free ABA an IAA. Once again, the organic phase (free ABA and IAA) was concentrated with a rotary evaporator at 50 °C until a completely dried residue was obtained. Finally the residue was resuspended in 300 μL absolute methanol and maintained at 80 °C until quantification.

ABA and IAA standard curve calibration

In order to set the standard curve for ABA and IAA, a solution of ABA (Sigma, Steinheim, Germany) and IAA (Merck, Darmstadt, Germany) of 99% purity at a concentration of 25 μg mL^{-1} was prepared for each case. For the curve calibration, successive dilutions were prepared with 0.4, 0.6, 0.8, and 1.0 μg mL^{-1} ABA and with 0.5, 1.0, 1.5, and 2.5 μg mL^{-1} IAA.

HPLC quantification of ABA and IAA

The extract resuspended in 300 μL of absolute methanol in the extraction phase was filtered in 0.22 μm hydrophobic paper. The filtrate was later quantified using HPLC (SPD-M10 Avp N°c20903702918 Shimadzu, Tokyo, Japan) with a diode array detector SPD- M 10 Avp, control system SCL-10 AVP and a FCV-10 AL vp pump (Shimadzu, Tokyo, Japan). ABA and IAA were separated in a BioRad HPLC column RP-18 (Lichrospher, Darmstadt, Germany) 100 (250 mm) at a temperature of 30 °C with a 0.8 mL min^{-1} flow. To complete the quantification process, the chromatograms obtained from the samples quantification were compared with the chromatograms of ABA and IAA standards.

Variables evaluated

For the maturation phase, the initial and final weight of the PEM under maturation treatments were recorded (Figure 1a) for an increase in fresh weight (mg) after 3 wk of culture. Furthermore, the incidence per treatment of secondary somatic embryogenesis (SSE) was evaluated (Figure 1b) and expressed in percentage (%). The germination phase of SE in cotyledonary stage (Figure 1c) was evaluated after 4 wk.

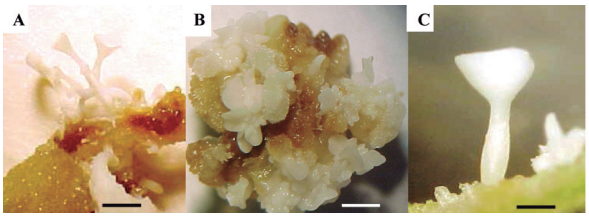


Figure 1. Different aspects of proembryogenic masses (PEM) of *Nothofagus alpina*. A) PEM subjected to maturation treatments, bar: 2 mm; B) PEM presenting secondary somatic embryogenesis, bar: 5 mm; C) Somatic embryo in cotyledonary stage, bar: 2 mm.

Because SE germination was affected by culture conditions (embryo induction and maturation), different grades of germination or plantlet development were present. Therefore, adequate classification criteria of SE germination described by Vahdati *et al*. (2008) were used. Germination was evaluated according to the following: Caulynary presence only; radicle presence only; and caulynary and radical presence.

Experimental design

A completely random experimental design was established. In the maturation phase, the experimental unit was composed of a 9 cm diameter Petri dish containing 6 PEM. Four replicates of units were performed for each treatment. In the ABA and IAA quantification phase, three extractions of 100 mg of fresh tissue were carried out per maturation treatment ZE, Ie, Aze.

In a similar manner, an experimental unit was also constituted by a test tube containing PEM with SE in the cotyledonary stage during the germination phase. For each treatment, 10 replicates were carried out.

In every case, the treatment effect was analyzed by means of ANDEVA, followed by the Tukey test at $p \leq 0.05$ to compare maturation phase means. Before analyzing the data, the raw data from ABA quantification was reduced by a squared root; however, the results display the original data untransformed. Moreover, the Dunnett test for multiple comparisons was applied to the quantification phase in order to describe the effect of ABA in the maturation treatments compared to endogenous ABA found in the ZE (control).

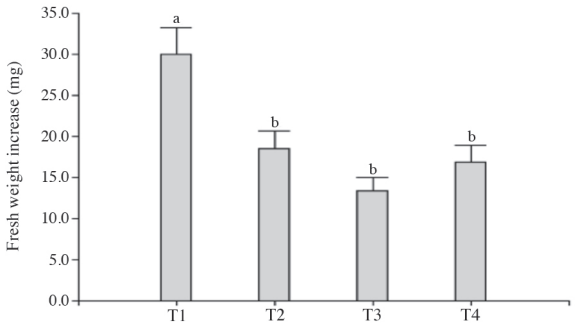
RESULTS AND DISCUSSION

Somatic embryo maturation assay

During the maturation phase of the PEM, a significantly smaller fresh weight increase ($p < 0.0019$) (FWI) was observed in all three exogenous ABA concentrations applied to the culture medium when compared to the

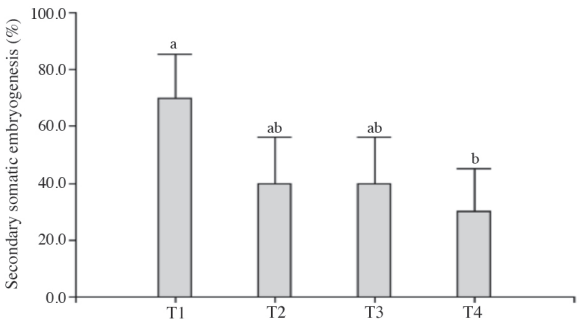
control (Figure 2). These results agree with those obtained by García-Martín *et al*. (2005). Their results indicated a significant decrease of the fresh weight as exogenous ABA concentration increased in the culture medium. It remains inconclusive whether ABA has influence as an osmotic regulator on the medium that maintains the SE. The FWI, according to studies performed in *Q. ilex* and *J. regia*, was related to culture conditions and perhaps, even to the high concentrations of sucrose applied to the medium (between 30 and 90 g L^{-1}). Those high concentrations of sucrose would lead to the accumulation of reserve substances in the embryos, contributing to their maturation (Mauri and Manzanera, 2003; Vahdati *et al*., 2008).

The SSE response does not present significant differences amongst culture treatments where ABA was added (Figure 3). However, significant differences did exist between them and the control, observing a decrease in SSE frequency when ABA was applied. The control of SSE has been previously reported to enter a maturation phase or to accumulate reserve substances in several studies such as *Q. ilex* (Mauri and Manzanera, 2004), *Castanea dentata* (Marshall) Borkh. (Robichaud *et al*., 2004), *Q. suber* (García-Martín *et al*., 2005) and *J. regia* (Vahdati *et al*., 2008). The decrease in FW and SSE in treatments where ABA was added can be explained by a possible osmotic besides the effect and ABA's capability to inhibit premature germination and control SSE (Bentsink and Koornneef, 2008; Manoj *et al*., 2008). This generates a greater number of cell divisions along with immature embryo formation in the control treatment, which is reflected in a greater FW in the absence of ABA (T1), matching the results found in *Q. ilex* (Mauri and Manzanera, 2004).



Distinct letters over the bars indicate differences according to Tukey test (P < 0.05).

Figure 2. Effects of abscisic acid (ABA) treatments in fresh weight increase (FWI) of somatic proembryogenic masses of *Nothofagus alpina*. Treatments: T1: control; T2: 7.5 µM ABA; T3: 11.3 µM ABA; T4: 18.9 µM ABA.

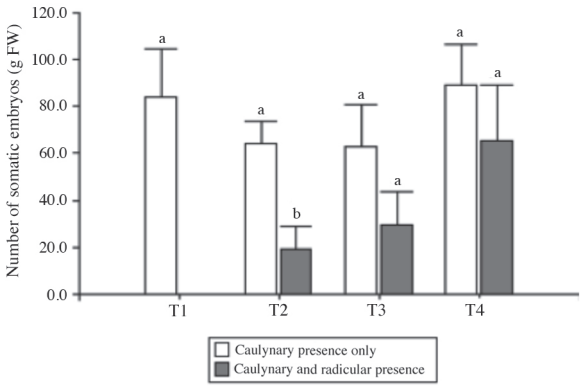


Distinct letters over the bars indicate differences according to Tukey test (P < 0.05).

Figure 3. Effects of abscisic acid (ABA) treatments on secondary somatic embryogenesis (SSE), in somatic embryos of *Nothofagus alpina*. Treatments: T1: control; T2: 7.5 µM ABA; T3: 11.3 µM ABA; T4: 18.9 µM ABA.

Somatic embryo germination assay

Somatic embryo germination is altered by culture conditions (embryo induction and maturation); therefore, it generally results in different degrees of development (germination) and plantlet development. When evaluating the number of germinated SE per gram of fresh weight, according to the categories described earlier, we observed that SE with root presence cannot be obtained (Figure 4) and that the majority of SE presented only shoot development (Figure 5a, 5b). There were no significant differences observed between the ABA concentrations added to the medium.



Distinct letters over the bars indicate differences according to Tukey test (P < 0.05).

Figure 4. Number of somatic embryos presenting developed shoot pole and root (plantlet). T1: control; T2: 7.5 µM abscisic acid (ABA); T3: 11.3 µM ABA; T4: 18.9 µM ABA.

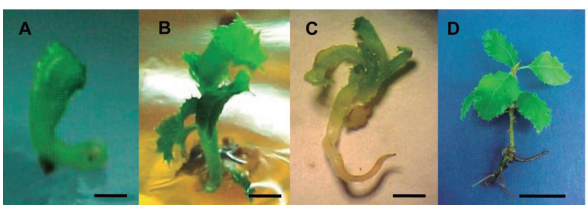


Figure 5. Germinated somatic embryos and subjected to maturation treatments. A) Somatic embryo presenting shoot development, bar: 2 mm; B) somatic embryo presenting only shoot development after 3 wk of culture, bar: 3 mm; C) embryo with shoot and root development, bar: 3 mm; and D) plantlet, bar: 8 mm.

The greater number of SE germinated (65 SE g^{-1} FW) with root and shoot presence (Figure 5c) were obtained when adding 18.9 µM ABA to the culture medium. This agrees with what is reported for *J. regia*, where 41% SE conversion to plantlet was obtained (Vahdati *et al*., 2008). However, a larger number of SE obtained in this study only presented shoot development, which could be caused by germination inhibition of the embryos that were submitted to high concentrations of ABA. The literature suggests that it is more appropriate to apply only short pulses of ABA in early stages of embryo development to achieve an appropriate maturation and subsequent conversion to plant (Figure 5d) (Manoj *et al*., 2008).

Numerous morphological and biochemical similarities of somatic and zygotic embryogenic processes indicate that culture sequences should add a maturation promotion phase of SE before germination, for which the information of the ZE that is to be mimicked is needed (Schmidt *et al*., 2006).

ABA and IAA quantification

The HPLC results (Figure 6a) showed that the standard curves obtained for ABA and IAA had retention times of 5.9 and 7.3 min, respectively. The extraction process was appropriate for the tissue (Figure 6b).

The results of the endogenous quantification of ABA and IAA obtained from Ie, ZE, and Aze of *N. alpina* (Figure 7), verified the proposal based on the findings of Karssen *et al*. (1983) and several other authors (Quatrano *et al*., 1997; Finkelstein *et al*., 2002) in *Arabidopsis thaliana* (L.) Heynh. Their studies indicated that lower levels of ABA and greater levels of IAA were present during embryogenesis (Ie). This behavior is reverted when maturation begins with the inhibition of premature germination, increasing ABA levels in the middle developing stage of the seed (ZE), which are later decreased during the desiccation period.

The greater levels of IAA (24 µg g^{-1}) found during the immature phase of the seed or during embryogenesis are a result of greater cellular division and expansion, which decrease as the seeds enters the middle phase of development, triggering the synthesis of reserve proteins (Azcón-Bieto and Talón, 2000; Von Arnold *et al*., 2002). The inhibition of premature germination was observed in *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. and mediated by the increase of ABA levels, which were greater in the endosperm than in the embryo (Etienne *et al*., 1993).

When comparing endogenous ABA levels between mature ZE and isolated embryonary axis, we observed that there was a significant decrease in ABA levels as in IAA levels (Figure 7). Bianco *et al*. (1997) reported similar results in *Pseudotsuga menziesii* (Mirb.) Franco, where the embryonic axis had 20 times less levels of endogenous ABA when compared to the whole seed. This is attributed to the fact that the regulators are synthesized in greater proportion in zones closer to the embryonic axis (cotyledons), preventing the loss and oxidative degradation of ABA. This phenomenon also contributes to a decrease of this hormone in isolated embryos (Kong *et al*., 1997). As also observed in Figure 7, the mature ZE composed of the embryonary axis and the cotyledons is the most ideal indicator for comparing endogenous ABA and IAA levels with the SE.

According to the results obtained, significant differences did exist between ABA levels in ZE (22 µg g^{-1}) and in SE from treatments where 18.9 µM ABA was added to the medium (T4) (Figure 8). Alemanno *et al*. (1997) proposed that the immaturity of the SE is based on deficient levels of endogenous ABA and thus, a

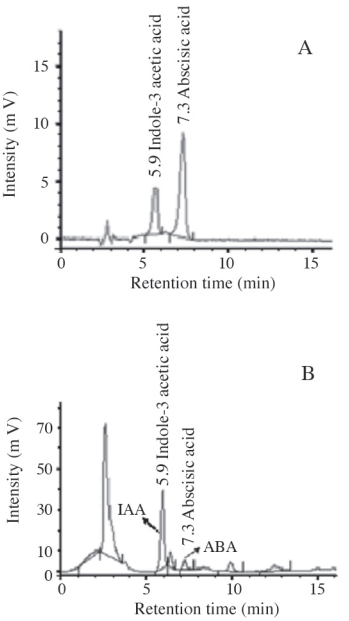
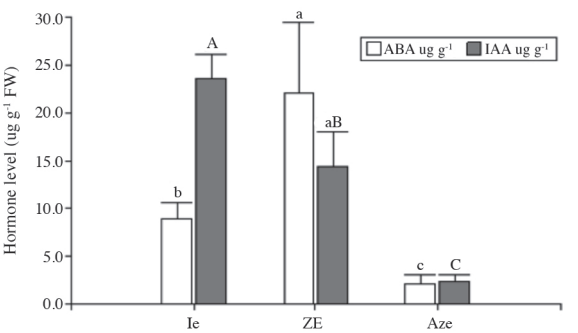
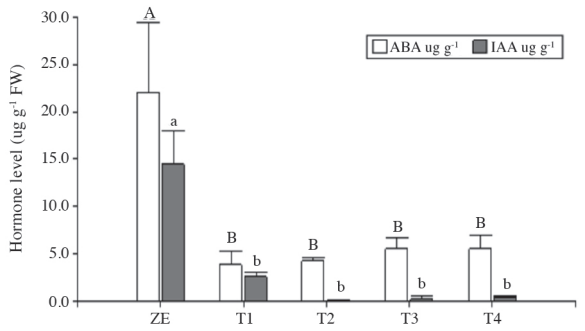


Figure 6. Chromatograms that indicate the retention time of abscisic acid (ABA) and indole-3-acetic acid (IAA), according to the standard used (A) and concentrations of ABA and IAA obtained from samples of immature zygotic embryos (Ie) of *Nothofagus alpina* (B).



Distinct letters over the bars indicate differences according to Dunnett test ($P < 0.05$).

Figure 7. Endogenous levels of abscisic acid (ABA) and indole-3-acetic acid (IAA) in the immature embryo (Ie), mature zygotic embryo (ZE), and the embryonary axis (Aze) of *Nothofagus alpina*. The values represent an average of $n = 3$ ± standard error.



Distinct letters over the bars indicate differences according to Dunnett test ($P < 0.05$).

Figure 8. Endogenous levels of abscisic acid (ABA) and indole-3-acetic acid (IAA) contained in somatic embryos (SE), compared to zygotic embryos (ZE) of *Nothofagus alpina*. Treatments: T1: control; T2: ABA 7.5 µM; T3: ABA 11.3 µM; T4: ABA 18.9 µM. The values represent an average of $n = 3$ ± standard error.

lack of reserve proteins, which allow maturity and later, germination of the embryos. In *Q. suber* it was determined that the addition of 0.9 µM ABA to the culture medium promoted the maturation of SE, presenting a similar role to endogenous ABA (García-Martín *et al*., 2005). Thus, it was determined that the amount of endogenous ABA during secondary embryogenesis was similar to what was observed in an immature stage, demonstrating that secondary embryos were generated from primary embryos under low levels of ABA.

The differences between ZE and SE may be the result of the absence of a maternal source, which could emit signals to induce ABA synthesis in an *in vitro* culture (Miller *et al*., 1994). Hence, the addition of ABA to the culture medium during the SE initial stages may simulate certain signals that would allow the accumulation of reserve proteins, generating embryos similar to those obtained in a zygotic manner.

As it can be appreciated in Figure 8 no significant differences existed in the quantification of ABA between the control (T1) and treatments T2, T3, T4 where ABA was applied. This has been reported in some studies in conifers, where it has been demonstrated that SE were capable of synthesizing endogenous ABA at low concentrations in response to exogenous ABA or in the absence of the latter (Kong and Yeung, 1995; Kong and Von Aderkas, 2007). Therefore, the timing of the addition of exogenous ABA to the culture medium appears to be vital in the maturation induction process of SE. In consequence, the maturation process of SE must be studied for each species and/or embryogenic system (globular-torpedo-cotyledonary), so as to manage the concentrations that would allow it to mimic, as much as possible, the natural conditions that promote embryogenic maturation. This would achieve germination and plant conversion rates at acceptable levels.

CONCLUSIONS

From this study we concluded that the addition of ABA to the culture medium is necessary to decrease the incidence of secondary somatic embryogenesis. At the same time, low levels of endogenous ABA that were found in immature zygotic embryos (embryogenesis) of *N. alpina* were later observed to increase significantly in the mature zygotic embryos. In turn, mature zygotic embryos presented significantly greater levels of endogenous ABA when compared to somatic embryos in the cotyledonary stage submitted to different concentrations of exogenous ABA. Finally, the number of germinated somatic embryos per gram of tissue was found to increase significantly when ABA was applied to the culture medium.

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Cuantificación endógena de ácido abscísico y ácido indol-3 acético en embriones somáticos y cigóticos de *Nothofagus alpina* (Poepp. & Endl.) Oerst. El ácido abscísico (ABA) y el ácido indol 3 acético (IAA) participan en el proceso de propagación de plantas mediante embriogénesis somática, ya que permiten la diferenciación de la estructura polar del embrión, órganos y regiones meristemáticas de éste. En este estudio se llevó a cabo un ensayo de maduración de embriones somáticos en estado cotiledonar con la adición de diferentes concentraciones de ABA exógeno, además se determinaron niveles endógenos entre ZE inmaduro, ZE maduro, y eje embrionario aislado desde el embrión maduro para luego comparar niveles endógenos de ABA e IAA en embriones somáticos (SE) y cigóticos (ZE) de raulí, *Nothofagus alpina* (Poepp. & Endl.) Oerst. La cuantificación se realizó mediante cromatografía líquida de alta eficiencia (HPLC). Los tratamientos de maduración estudiados incrementan los niveles endógenos de ABA en SE no existiendo diferencias significativas entre las diferentes concentraciones estudiadas. Al comparar los niveles de ABA e IAA endógeno entre SE sometidos a los diferentes tratamientos de maduración y los ZE maduros de *N. alpina*, se observan niveles significativamente mayores en SE. La aplicación de ABA exógeno al medio de cultivo aumentó significativamente el número de SE germinados, lo que permite optimizar el proceso de conversión a planta de SE de raulí.

Palabras clave: embriogénesis somática, maduración, germinación, HPLC.

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