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Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos

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Abstract

Knowledge of the relationship between indole-3-acetic acid (IAA) and abscisic acid (ABA) is relevant to control the development and the maturation of cork oak (*Quercus suber* L.) somatic embryos. The addition of 1 μM ABA to the culture medium significantly promoted somatic embryo maturation and increased both fresh and dry matter without affecting the relative water content. This effect was parallel to the pattern of variation observed in the endogenous ABA level, which increased from the immature to the mature stage. Endogenous ABA content during the occurrence of secondary embryogenesis was similar to that of the immature stage, showing that embryos with lower ABA levels produced secondary embryos. In contrast, IAA showed the highest concentration during early embryo development and decreased afterwards. Only in somatic embryos subjected to 1-week desiccation followed by stratification at 4 °C for 2 weeks, was a moderate increment of endogenous IAA content observed. IAA and ABA showed opposite levels during the development and maturation of cork oak somatic embryos and characterised specific stages of the embryonic development.

Abbreviations: ABA – abscisic acid; ACN – acetonitrile; DW – dry weight; FW – fresh weight; IAA – indole-3-acetic acid; MS – Murashige and Skoog medium; RWC – relative water content; SH – Schenk and Hildebrandt medium; TW – turgid weight

Introduction

Cork oak (*Quercus suber* L.) is a woody species supplying raw material for the cork industry. Conventional vegetative propagation of this species is very difficult and, therefore, the propagation of selected trees by somatic embryogenesis could be a good alternative. Cork oak somatic embryogenesis has been obtained from zygotic embryos (Bueno et al., 1992; Manzanera et al., 1993), and somatic embryo germination has been studied (García-Martín et al., 2001; González-Benito et al., 2002).

Initiation and proliferation of embryogenic cultures are usually obtained by exogenous application of auxin (e.g., Mauri and Manzanera, 2003). This plant growth regulator is usually removed during the following stage, allowing embryo histo-differentiation to proceed (von Aderkas et al., 2001). This treatment applied to most

somatic embryos resembles the pattern observed in developing seeds, where the biosynthesis of indole-3- acetic acid (IAA) increases throughout embryo development until early maturation (see review by von Arnold et al., 2002 and references therein).

Another major plant growth regulator affecting seed development is abscisic acid (ABA). Kermode (1995) postulated that precocious germination was prevented by ABA of maternal origin. ABA also promotes the accumulation of reserve substances (Liao and Amerson, 1995; Cailloux et al., 1996), promotes maturation (Black, 1991) and reduces the frequency of abnormal morphologies (Etienne et al., 1993a) and secondary embryogenesis (Nuutila et al., 1991). Characterisation of gene expression during embryo development, maturation and germination has led to the identification of a group of genes expressed abundantly in later stages of embryogenesis until seed maturation.

These genes are activated by ABA. Besides, some studies have also revealed that specific genes interact and play a major role in regulating maturation, including desiccation tolerance, sensitivity to ABA and expression of storage proteins (see review by von Arnold et al., 2002 and references therein). The synthesis and deposition of storage and late embryogenesis abundant (LEA) proteins during embryogenesis are usually regulated by ABA- and water-stress-induced gene expression (Dodeman et al., 1997).

Nevertheless, the interrelations between auxin and ABA should be further explored. The fact that auxin induces the accumulation of endogenous ABA in *Galium aparine*, mainly from *de novo* synthesis, shows that ABA appears to function as a further hormonal second messenger of auxin (Hansen and Grossman, 2000). In contrast, in *Arabidopsis thaliana*, ABA reaches its peak level when the IAA level begins to rise in the developing fruit and its levels decline thereafter against a background of rising levels of IAA (Müller et al., 2002).

Desiccation of somatic embryos prior to germination improves the quality of the germinated embryos (Tang et al., 2000; Pond et al., 2002). To enhance desiccation tolerance, cold treatments have been used in mature somatic embryos of *Picea glauca* (Pond et al., 2002). Similarly, cold treatments have been applied to cork oak somatic embryos to obtain higher germination rates (García-Martín et al., 2001). Desiccation is related to the decrease of ABA endogenous content (Lelu et al., 1995; Find, 1997). Understanding the relationship between chilling, desiccation and the hormonal regulation of cork oak somatic embryo development and maturation is needed to prepare mature embryos for efficient germination.

The objective of this work is to expand the knowledge of endogenous auxin and ABA levels in somatic embryos and of the effect of exogenous ABA on the regulation of maturation. This would help to obtain high quality somatic embryos of this species for germination.

Material and methods

Source of somatic embryos

Small immature somatic embryos, i.e., translucent embryos up to 2–3 mm long induced on leaves of a 2-month old *Q. suber* seedling from Salamanca (Spain), were obtained according to the protocol established by Fernández-Guijarro et al. (1995). The embryogenic line remained productive, by means of secondary embryogenesis, on medium without growth regulators and monthly subculture. This clone of somatic embryos was used as the source for all the experiments carried out in this work (named stock cultures). Throughout this work, embryos were classified as immature (cotyledonary, translucent and 2–3 mm long), mature (length greater than 8 mm, opaque ivory-coloured, and with well formed cotyledons) and proliferating (showing embryogenic tissue around the hypocotyl, without clear development of secondary embryos).

Application of ABA to immature somatic embryos for 1 week

Immature somatic embryos of cork oak were cultured in 100 ml glass flasks (Magenta Corp.) with 30 ml SH medium gelled with 6 g l^{-1} agar (Sigma, Type E) (pH 5.8 prior to autoclaving). SH medium included macronutrients of Schenk and Hildebrandt (1972), and micronutrients, iron, cofactors and vitamins of MS medium (Murashige and Skoog, 1962). Sucrose concentration was 90 mM. Embryos were incubated in a chamber at 25 ± 1 °C with a 16 h light/8 h dark photoperiod

and an irradiance of 50 μmol m^{-2} s^{-1} provided by cool-white plus Grolux fluorescent lamps. ABA was added to the medium prior to autoclaving in the following concentrations: 0 (control), 0.01, 0.1 and 1 mM. Somatic embryos were cultured on these media for 1 week and subsequently they were transferred to basal SH medium without growth regulators. Sixty somatic embryos were cultured per treatment (12 flasks per treatment with 5 embryos per flask). The percentages of maturation and secondary embryogenesis were recorded 30 days after transfer to ABA-free medium.

Continuous culture in the presence of ABA

Immature somatic embryos were subjected to the following treatments: 0 (control), 0.1, 1 and 10 μM ABA added to SH agar-solidified medium with 90 mM sucrose. Embryos were cultured for four months with monthly transfer onto the same fresh medium. Incubation took place at 25 $\pm$ 1 °C and a 16 h/8 h light/dark photoperiod (50 μmol m^{-2} s^{-1} irradiance). Twenty Magenta flasks (approx. 590 ml capacity with 45 ml medium) with 10 somatic embryos each were used per treatment. At the end of the fourth month of culture, the fresh weight (FW), turgid weight (TW), dry weight (DW) and relative water content (RWC) of mature embryos were recorded. To promote germination, the mature embryos were subjected to partial desiccation. This procedure was carried out by placing one embryo in each of the nine open small glass wells contained inside a Magenta flask with 5 ml distilled sterile water in the bottom. Embryos were maintained in such condition for 7 days at 25 °C in the dark. Afterwards, they were kept for 1 month in fresh SH medium at 4 °C in darkness (Fernández-Guijarro et al., 1995). Subsequently, embryos were germinated on SH medium with 90 mM sucrose and solidified with 6 g l^{-1} agar, with a photoperiod of 16 h/8 h and a 50 μmol m^{-2} s^{-1} irradiance and a thermoperiod of 25 °C/15 °C. The germination percentage was recorded 90 days after embryos were set to germinate.

Quantification of endogenous ABA and IAA content by HPLC and GC-MS

Endogenous levels of ABA and IAA were studied in somatic embryos isolated from stock cultures at different developmental stages (immature, mature and proliferating), and in mature embryos subjected to several partial desiccation/cold treatments. Those treatments were: partial desiccation at 4 °C for 1 or 2 weeks, or partial desiccation for one week at 25 °C and subsequent storage in fresh medium at 4 °C for 2 or 3 weeks. Partial desiccation was carried out as described in the previous section. None of these variously treated embryos had been exposed to exogenous ABA prior to the determination of their endogenous ABA levels.

Extraction was based on Chen's et al. (1988) method. Samples of 1 g fresh tissue were immersed in liquid nitrogen and ground with a mortar. For both ABA and IAA extraction the buffer used was: 35% 200 mM imidazol buffer (pH 7) and 65% isopropanol. Extraction buffer (4 ml) was added, plus 100 ng deuterated ABA (D-ABA) and 100 ng radiolabelled $^{13}C_6$-IAA as internal standards. The mixture was spun in a vortex and left for 1 h at 4 °C. Then, the mixture was centrifuged at 2000 $\times$ *g* for 5 min at 4 °C. The pellet was washed with 0.5 ml extraction buffer and centrifuged as before. This process was repeated twice. The supernatants of all three centrifugations were mixed and the propanol fraction was evaporated in a flash evaporator (rotavapor) at 40 °C until volume was reduced to 1/3 of the initial one. The sample was then diluted in 41 ml bi-distilled water. The samples were kept on ice during processing. The pH of the extract was adjusted to 2.5 with 1 N HCl and the extract was dispensed in a C_{18} reverse-phase column of 0.3 g (Fisher). The column was pre-conditioned by flowing 5 ml each of hexane, methanol, bi-distilled water and acetic acid 1% in this order. The column was washed with 5 ml HPLC-pure water. Then, the sample was eluted with 5 ml acetonitrile (ACN). The eluent was evaporated in a flash evaporator (rotavapor) at 40 °C and resuspended with 4 ml imidazole hexane, ethyl-acetate, ACN and methanol. The sample was recovered with 6 ml methanol and 2% acetic acid. Then, it was desiccated in a rotavapor (40 °C), resuspended by three rinses of 80 μl (a total of 240 μl) ACN 30% in water with 1% acetic acid. Radioactive labels were ^{3}H-ABA and ^{3}H IAA (39,000 dpm). HPLC was previously calibrated with ABA and IAA standards, and solutions were degasified in vacuum. The column was conditioned with 5 ml ACN. Then, 200 μl samples were injected. Elution occurred at a constant flow rate of 25 ml min^{-1}. Polarity progressively increased

with 90% ACN solution (89.1/9.9/1, v/v/v of ACN, water and acetic acid). The mobile phase was ACN in a gradient from 25 to 87% in water and acetic acid, over 15 min. The flow rate was 0.75 ml s^{-1}. Then the column was conditioned with 100% ACN progressively reduced to 25%.

In these conditions, both ABA and IAA were monitored at 10.8 min with a UV absorbance detector by adjusting absorbance to 254 nm and 0.1 V^{-1}. Separation was carried out at room temperature. The sample was recovered in 1 ml aliquots. Radioactivity label of each fraction was measured in a scintillation chamber. Fractions with high radioactivity were mixed, desiccated in a rotavapor at 40 °C and resuspended in 100 ml methanol. Then, the samples were methylated with 1 ml diazomethane, left at room temperature for 5 min and desiccated under nitrogen flow. Samples were stored at −20 °C until use.

The ion source worked at 70 eV and 200 °C and interphase at 280 °C. The stationary phase capillar column (Econo-Cap SE-54, Alltech Associates, Inc.) was 30 cm long, with 0.25-mm internal diameter, packed in poly-diphenyl-dimethyl-siloxane (0.25 μ coat) on melted silica. The mobile phase was He with a constant 50 ml min^{-1} flow. Furnace temperature was programmed for a linear increase from 140 to 240 °C in 20 min (5 °C/min slope). HPLC-purified and methylated ABA and IAA were resuspended in 20 μl ethyl-acetate (3 rinses of 10, 5 and 5 μl) and injected in the gas chromatograph. Three 2 μl aliquots of each HPLC-purified sample were injected. The most abundant ions were monitored, i.e., 130 and 189 m/z for the methylated IAA, 136 and 195 m/z for the methylated $^{13}C_6$-IAA, 134 and 190 m/z for the methylated ABA and 138 and 194 m/z for the methylated $^{2}H_3$-ABA. All samples were analysed in triplicate and the measures were repeated three times.

Statistics

For statistical analysis, treatments were compared by analysis of variance, which was carried out using the General Linear Model procedure of the computing statistical program package SAS (SAS Institute, Inc., 1985). Means of the studied variables were compared at the end of the observation period with Scheffé test.

Results

Application of ABA to immature somatic embryos for 1 week

Maturation of immature embryos and secondary embryogenesis were inhibited by the application of 1 mM ABA for 1 week. Other ABA treatments (0.01 and 0.1 mM) significantly increased the maturation percentage in comparison to the control or 1 mM (Figure 1). Besides, the occurrence of secondary embryogenesis was inhibited by ABA.

Continuous culture in the presence of ABA

Continuous culture with ABA in the medium significantly affected ($p < 0.006$) the maturation of immature embryos (Figure 2); 1 μM ABA was the best treatment for maturation. Few cases of precocious germination were recorded, being lower ($p < 0.0000$) in 10 μM ABA than in all other treatments. The fresh and turgid weight of somatic embryos cultured on 10 μM ABA were lower than in the other treatments (Figure 3). Dry weight was significantly higher in embryos cultured with 0.1 and 1 μM ABA than both with 10 μM ABA and without ABA (control) (Figure 3), while no differences were observed for RWC, which ranged from 0.92 to 0.98. No significant differences in the germination rate of mature embryos, obtained in presence or not of ABA, were observed among

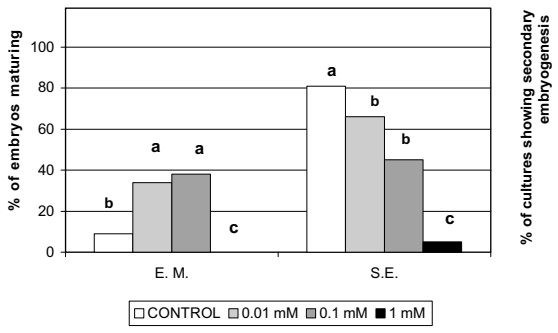

Figure 1. Developmental pattern followed by immature embryos after 1-week culture with different ABA concentrations (0.01, 0.1 and 1 mM), and in medium lacking ABA (Control) after 30 additional days in control medium. (EM = embryos maturing; SE = secondary embryogenesis). For the same variable, means with different letters were significantly different (Scheffé Test; $p < 0.05$). Number of embryos per treatment = 60.

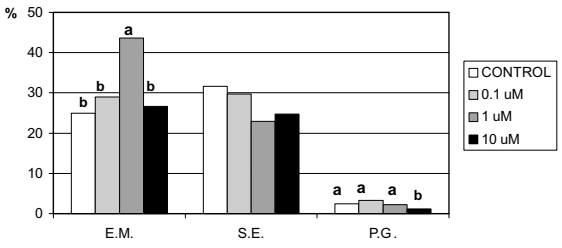

Figure 2. Developmental pattern followed by the embryos after the fourth culture cycle with different ABA concentrations (0.1, 1 and 10 μM) and in medium lacking ABA (Control). (EM = embryos maturing; SE = secondary embryogenesis; and PG = precocious germination). For the same variable, means with different letters were significantly different (Scheffé Test; $p < 0.05$). Number of embryos recorded per treatment = 200.

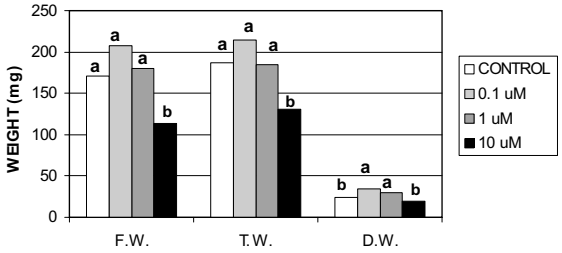

Figure 3. Embryo weight after the fourth culture cycle with different ABA concentrations (0.1, 1 and 10 μM) and in medium lacking ABA (Control). (FW = fresh weight; TW = turgid weight and DW = dry weight). For the same variable, means marked with different letters indicate significant differences (Scheffé Test; $p < 0.05$). Number of embryos per treatment = 90.

treatments. Between 90 and 100% of the germinating embryos showed root elongation and between 30 and 50% of them developed both radicle and plumule.

Quantification of endogenous ABA and IAA content by HPLC and GC-MS

Endogenous ABA content (ng/g FW) significantly increased from small immature to mature embryos (Figure 4). Proliferating embryos showed a similar ABA content to that of the immature stage embryos. The level of ABA was lower in embryos subjected to pre-germination treatments (partial desiccation and storage at 4 °C). In contrast, the endogenous level of IAA was highest in immature somatic embryos, during the early development phase, and decreased in mature embryos and in those subjected to pre-germination treatments.

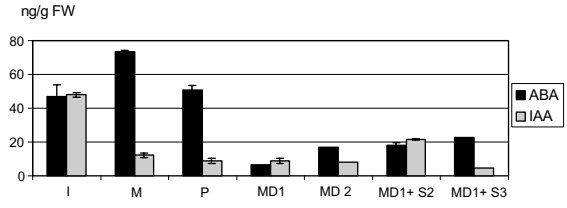

Figure 4. ABA and IAA endogenous content (ng/g fresh weight, FW) in different developmental stages of cork oak somatic embryos (I = small immature; M = mature; P = proliferating; MD1 = mature desiccated for 1 week at 4°C; MD2 = mature desiccated for 2 weeks at 4 °C; MD1 + S2 = mature desiccated for one week at 25 °C and stored in fresh medium at 4 °C for 2 weeks; and MD1 + S3 = mature somatic embryos desiccated for one week at 25 °C and stored in fresh medium at 4 °C for 3 weeks). Samples were analysed in triplicate and the measures were repeated three times.

Discussion

The exogenous addition of ABA to cultures of cork oak somatic embryos efficiently reduced the rate of unwanted secondary embryogenesis and favoured maturation. Similarly, the application of 1 μM ABA favoured the maturation of holm oak (*Quercus ilex*) somatic embryos (Mauri and Manzanera, 2004). However, embryos exposed to a high ABA concentration (1 mM) showed no maturation and barely any secondary embryogenesis. Concentrations of 0.01–0.1 mM promoted maturation when applied for one week. With longer periods of culture in ABA, lower concentrations were more efficient in promoting maturation (1 μM). With those longer periods, 10 μM ABA slowed the FW and DW increase of embryos.

The endogenous content of ABA in cork oak somatic embryos increased from the immature to the mature stage (Figure 4). This pattern has also been observed in zygotic (Finch-Savage, 1992) and somatic embryos (von Aderkas et al., 2001) of other species. Endogenous ABA content was lowered in cork oak embryos subjected to partial desiccation and cold treatments. In other woody species, such as in *Picea abies* (Find, 1997) and in *Larix* × *leptoeuropaea* (Lelu et al., 1995), lower ABA levels were also recorded after desiccation treatments.

In *Hevea brasiliensis*, the endogenous IAA concentration was lower in somatic than in zygotic embryos (Etienne et al., 1993b). These authors suggested that high levels of IAA were necessary

for adequate histodifferentiation in early stages of development, while ABA is needed in later stages for maturation. The results obtained with cork oak somatic embryos are in accordance with those studies. However, it should be noticed that mature embryos engaged in forming secondary embryos showed low levels of endogenous IAA. In embryogenic cells of alfalfa, an early peak of IAA accumulation, at the end of the second or third day of culture, correlated with the early activation of the division cycle (Pasternak et al., 2002). Ribnicky et al. (2001) also reported an increase in endogenous IAA synthesis associated with embryogenesis.

The addition of ABA to the medium efficiently promoted somatic embryo maturation and DW increase. The ABA-containing medium possibly mimics the maternal effect on the development of zygotic embryos (Groot et al., 1991, Belefant-Miller et al., 1994).

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