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Changes in water status and proline and abscisic acid concentrations in developing somatic embryos of pedunculate oak (*Quercus robur*) during maturation and germination

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Summary Somatic embryos of oak (*Quercus robur* L.) were matured on P_{24} media differing in gel strength (0.8, 0.9 and 1.0% (w/v) agar). Viscosity and osmotic potential ($\Psi_{\pi,medium}$) of the media were determined. Developing cotyledonary embryos were analyzed at maturity Stages I–III for water content, osmotic potential ($\Psi_{\pi,embryo}$) and concentrations of abscisic acid (ABA) and proline. Proliferation of embryogenic tissue, germination rates and the number of embryos formed were also determined in order to relate embryo quality to physiological parameters. Viscosity increased with agar concentration, a phenomenon apparently related to water availability. Many Stage III embryos with high germination potentials were obtained on P_{24} medium containing 1.0% agar. Embryo water content decreased progressively from 94 to 80% during embryo maturation. Stage I and II embryos that matured on media containing 0.8 or 0.9% agar had similar values of $\Psi_{\pi,embryo}$, whereas $\Psi_{\pi,embryo}$ of Stage III embryos that matured on medium containing 1.0% agar was significantly lower, although $\Psi_{\pi,medium}$ was unaffected by gel strength. Stage III embryos showed a nearly 16-fold increase in proline concentration and a 50% decrease in ABA concentration compared with Stage I embryos. We conclude that tissue water status and a complex relationship between ABA and proline concentrations, modulated by medium gel strength, are important factors in the maturation process and the quality of oak somatic embryos.

Keywords: Fagaceae, forest tree, hardwood, micropropagation, somatic embryogenesis, tissue culture.

Introduction

Interest in somatic embryogenesis of woody plant species has increased during the last several years, both as a method for genetic manipulation and as a procedure for mass propagation of superior forest tree genotypes. The advantages of somatic embryogenesis include high multiplication rates, the potential for scale-up in liquid culture (e.g., bioreactors) and the ability to produce artificial seeds (Prewein and Wilhelm 2003). Advances in synthetic seed technology offer prospects for using somatic embryos (SEs) in commercial plant production (Zoglauer et al. 2003). In addition, cryopreservation provides a means of storing germplasm until its phenotypic traits can be evaluated in adult trees under field conditions (Park et al. 1998). Feasible somatic embryogenesis systems have been developed for the mass propagation of several tropical woody species, including sandalwood (*Santalum* spp.), coffee (*Coffea arabica* L.) and *Acacia* spp. (Rugkhla and Jones. 1998, Vengadesan et al. 2002, Etienne and Bertrand 2003, Santana et al. 2004). The development of similar systems for temperate broad-leaved forest tree species has proceeded more slowly, and oak (*Quercus* spp.) is probably one of the most well-developed systems (Wilhelm 2000). A major limitation, the inability to initiate embryogenic cultures from mature oak trees, has recently been overcome (Toribio et al. 2004). Despite numerous studies on the physiological regulation of the development of oak SEs (e.g., Cvikrova et al. 1998, 2003, Sunderlikova and Wilhelm 2002), the low conversion rate continues to be the major bottleneck in oak somatic embryogenesis. Somatic embryo maturation is closely associated with developmental, physiological and biochemical changes. Abscisic acid (ABA) plays important roles during embryogenesis and germination (Finkelstein et al. 2002, Nambara and Poll 2003), including supporting the accumulation of storage proteins in the embryo, inhibiting precocious germination and promoting embryo maturation by suppressing the formation of aberrant embryo structures (Roberts et al. 1990, Kapik et al. 1995, White et al. 2000, Senger et al. 2001).

Proline, a compatible solute (Yancey et al. 1982), accumulates in drought-stressed plants. Proline synthesis is regulated by ABA (Hare et al. 1999). Recently, Abraham et al. (2003) suggested that proline accumulation in *Arabidopsis* is the result of the simultaneous activation of biosynthesis and inactivation of degradation pathways during osmotic stress, controlled by ABA. Attempts to stimulate the in vivo environment by modifying the composition of the maturation medium resulted in increases in storage compounds and desiccation tolerance of the SEs (Leal et al. 1995). Little is known about how

medium composition influences water status within the plant. Metabolic processes are regulated by turgor pressure. Water status is important for SE development, and the absence of water triggers the transition from maturation to germination (Johnson et al. 1997). Most studies on water relations parameters have focused on conifer embryos (Dumont-BeBoux et al. 1999), and little is known about SE water relations during development in woody angiosperm species.

Our objective was to relate embryo quality and germination ability with physiological parameters in order to identify the optimal conditions for maturation and germination of SEs. Media differing in gel strength were characterized based on osmotic potential and viscosity. Water status as well as concentrations of proline ([proline]) and abscisic acid ([ABA]) of developing SEs at defined stages of maturity were analyzed and related to germination rate.

M. rials and methods

Plant material and culture conditions

The embryogenic culture line P28H9, which was chosen because of its high genetic stability (Endemann et al. 2001), was obtained from immature zygotic embryos of *Quercus robur* L. by the protocol of Endemann and Wilhelm (1999). The culture line was maintained by repetitive embryogenesis on proliferation medium comprising P_{24} medium (Teasdale 1992) supplemented with 3% (w/v) sucrose and 0.9 μM 6-benzylaminopurine (BA), and solidified with 0.8% (w/v) agar (Daishin, Brunschwig Chemie, Amsterdam, The Netherlands). Cultures were transferred to fresh medium at 5-week intervals.

For maturation, hormone-free P_{24} media containing 3% (w/v) sucrose and agar concentrations ranging from 0.8 to 1.0% were used (Table 1; M1–M3). About 0.3 g of embryogenic tissue (globular, heart-shaped, cotyledonary stages of SEs) was weighed into a petri dish (10 replications per treatment with two repetitions). After 5 weeks, proliferation of embryogenic tissue was determined by the increase in fresh mass, the well-formed cotyledonary SEs were counted and the number of SEs per gram fresh mass was calculated.

Cotyledonary SEs were grouped by morphology during three stages of maturity. Stage I cotyledonary SEs were immature, transparent-white and < 5 mm long. Stage II cotyledonary SEs were mature, white-yellow and ~5 mm long. Stage III cotyledonary SEs were mature, yellow-green and 6–10 mm long.

For germination, the P_{24} medium contained 3% (w/v) sucrose, 0.8% (w/v) agar, 0.9 μM BA and 0.1 μM indole-3-butyric acid (IBA). The SEs were scored after 4 weeks for germination (root/shoot formation). Germination capacity was also evaluated for SEs at each maturation stage (I–III).

Cultures were incubated in plastic petri dishes (90 × 15mm) containing 20 ml of medium (pH 5.9) that had been previously autoclaved at 121 °C for 20 min. The cultures were kept in a 16-h photoperiod (50 μmol m^{-2} s^{-1} white light from TL-D 36W/33 (Philips, Eindhoven, The Netherlands)) at 25 ± 1 °C. Data were analyzed by Duncan's test and by a separate 2 × 2 contingency χ^2 test based on a significance level of $\alpha = 0.05$.

Characterization of media

Viscosity The viscosity (η; mPa·s) of a liquid or gaseous substance depends on its internal friction (τ) and the shear gradient (γ) of its fluidity: $\eta = \tau/\gamma$. Media differing in gel strength (0.7 to 1.0% (w/v) agar) were analyzed with a rotation viscosimeter (Haake Rotovisco RT20, Thermo Electron, Dreieich, Germany) with respect to their pseudoplasmatic fluidity. Each medium was tested six times by removing the upper parts of the gelled medium in 20-ml petri dishes at 1 day and 5 weeks after preparation. Measurements were made at 20 °C at a distance (d) of 0.105 mm. Values taken at a defined measuring point in the rheogramm ($\gamma = 10\ s^{-1}$) were used to compare all measurements.

Medium osmotic potential The media were frozen and stored at –20 °C until analyzed. After thawing at room temperature, media were centrifuged at 10,000 rpm for 2 min and the supernatant used to determine medium osmotic potential ($\Psi_{\pi,medium}$) (six samples per treatment) with a digital microosmometer (Vogel, Giessen, Germany). We determined the freezing point depression of the solutes, which was directly proportional to the osmolality of the liquid samples (solute concentration expressed in mOsm kg^{-1}). The osmometer was calibrated with distilled water and 300 mOsm natrium chloride solution. A multiplication factor (–2478.9) for an appropriate temperature was used to convert osmolality (Osm kg^{-1}) to osmotic potential (Ψ_π; MPa) at 25 °C (George 1993, Dumont-BeBoux et al. 1999).

Table 1. Proliferation, maturation and germination media for somatic embryogenesis of oak (*Quercus robur*). Viscosity and osmotic potential (Ψ_π) of the various maturation media are shown. Values of Ψ_π are means ± standard error. Within a column, values followed by the same letter are not significantly different ($\alpha = 0.05$) according to Duncan's test. Abbreviations: BA = 6-benzylaminopurine; and IBA = indole-3-butyric acid.

Medium	Supplements	Viscosity (MPa·s)		Osmotic potential (MPa)
		One day	Five weeks	
Proliferation P (control)	0.8% Agar, 0.9 μM BA	–	–	–
Maturation M0	0.7% Agar	7,492 a	8,352 a	–
Maturation M1	0.8% Agar	9,368 b	11,060 b	–0.365 ± 5.16 a
Maturation M2	0.9% Agar	12,917 c	13,917 c	–0.369 ± 0.00 a
Maturation M3	1.0% Agar	17,200 d	18,583 d	–0.364 ± 3.72 a
Germination	0.8% Agar, 0.9 μM BA, 0.1 μM IBA	–	–	–

Abscisic acid in somatic embryos

Abscisic acid was determined in complete SEs, including axis, cotyledons and root pole region, of small immature Stage I SEs, mature Stage II embryos and large Stage III embryos derived from SE cultures on maturation media M1 and M3. The SEs (about 0.5 g; 2–10 per sample, depending on size) were frozen in liquid nitrogen, extracted in methanol and purified by ether partitioning and column chromatography on Polyclar AT as described by Cvikrova et al. (1998). Abscisic acid was analyzed by gas chromatography with an HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with an ECD OV-1 column. Tritiated (±)-ABA was used to quantify ABA in the samples.

Proline in somatic embryos

We determined L-proline in white-yellow SEs (Stage II) cultured on M1 medium as well as yellow-green SEs cultured on M2 and M3 media (Stage III) according to the Bates method (Bates 1973). Somatic embryos (about 0.5 g) were frozen in liquid nitrogen and stored at –20 °C. Proline was precipitated with 5 ml of 3% (w/v) 5-sulfosalicylic acid dihydrate (Merck, Whitehouse Station, NJ), released by autoclaving at 121 °C for 20 min and colored by the addition of 2,2-dihydroxy-1,3-indanedione (ninhydrin) (Fluka, Buchs, Switzerland). The heated ninhydrin–proline complex (96 °C, 65 min) was mixed with 4 ml of toluene (Merck). The condensation product was measured spectrophotometrically (DU 640, Beckman, Fullerton, CA) at 519 nm with purified L-proline (ICN Biomedicals, Aurora, OH) as a standard. The proline concentration was calculated on a fresh mass basis according to Bates (1973).

Somatic embryo osmotic potential

Somatic embryos were harvested from proliferation and maturation media. Somatic embryos (about 0.5 g) cultured on either proliferation or maturation media were collected in 1.5-ml Eppendorf tubes (Greiner, Kremsmuenster, Austria), immersed in liquid nitrogen and stored at –20 °C until analyzed. After thawing at room temperature for 30 min, sap was extracted by mashing the SEs and collected by centrifugation at 10,000 rpm for 2 min. Embryo osmotic potential ($\Psi_{\pi,embryo}$) was determined as described for $\Psi_{\pi,medium}$.

Statistical analyses for ABA, proline and osmotic potential

Six samples (SEs in Stages I–III) from each maturation treatment with one repetition were statistically analyzed by Duncan's test with a significance level of $\alpha = 0.05$.

Water content of somatic embryos

The water content of proliferating embryogenic tissue containing a mixture of all embryogenic stages (globular, heart-shaped and cotyledonary embryos) cultured on proliferation, M1 or M3 media was determined gravimetrically after drying for 105 °C for 48 h. Single cotyledonary SEs were collected from maturation medium (M1–Stage I, M2–Stage II and M3–Stage III) and the water content determined (six samples each with 10 SEs per stage) with one repetition. Data were analyzed by the Duncan test based on a significance level of $\alpha = 0.05$.

Results

Medium viscosity

Medium and gel strength increased with increasing agar concentration. Viscosity also increased with increasing age of the medium (Table 1). The lowest η (7,492 mPa·s) was measured in 1-day-old medium containing 0.7% agar, increasing to 8,352 mPa·s after a 5-week storage period at room temperature. The highest viscosity (18,583 mPa·s) was measured in 5-week-old medium containing 1.0% agar. Medium viscosity was highly correlated with gel strength (Pearson correlation coefficient = 0.98).

Medium osmotic potential

Medium osmotic potential was not significantly affected by agar concentration with M1–M3 media having similar $\Psi_{\pi,medium}$ values of about –0.365 MPa (Table 1). The slight differences in $\Psi_{\pi,medium}$ among media were probably associated with hydrolysis of sucrose to monosaccharides during autoclaving.

Maturation and germination of somatic embryos

At the beginning of maturation, Stage I embryos were translucent and < 5 mm in length. During maturation, these embryos differentiated into white-yellow Stage II embryos 5 mm long. At the completion of maturation, the Stage III embryos were yellow-green and 6–10 mm long. Proliferation rates of embryogenic tissue were similar on control (P) medium, M1 and M2 media, whereas the proliferation rate was significantly lower on M3 medium, which contained 1.0% agar. Although the control and M1 media produced a large number of SEs (1.04 and 0.87 g_{FW}^{-1}, respectively), mean germination frequency was low (25 and 29%, respectively), because many (42 and 33%, respectively) of the SEs remained at Stage I (Figure 1, Table 2). Medium M2 yielded only 0.55 g_{FW}^{-1}, but mean germination frequency was 91.0% because 64% of the SEs developed to Stage II and few Stage I embryos remained (20%). Culture of SEs on M3 medium significantly ($P < 0.04$) enhanced both maturation and germination, with 46% of the SEs reaching Stage III (1.68 g_{FW}^{-1}) and achieving a germination frequency of 80%.

The germination frequency of SEs differed significantly ($P < 0.001$) with maturation stage (I–III). Only 25% of Stage I SEs germinated, whereas 58% of Stage II SEs formed either a root or shoot and the highest germination frequency of 72% was found in Stage III SEs (Figure 2).

Somatic embryo osmotic potential

The agar concentration in the maturation medium had a significant effect ($P < 0.001$) on the osmotic potential of maturing SEs. The $\Psi_{\pi,embryo}$ was significantly more negative for mature Stage III SEs on M3 (–0.845 MPa; Table 3) than for Stage II SEs on M2 (–0.648 MPa) or immature Stage I SEs cultured on M1 medium (–0.701 MPa).

Water content of somatic embryos

The agar concentration in the proliferation and maturation media significantly affected the water content of the embryogenic

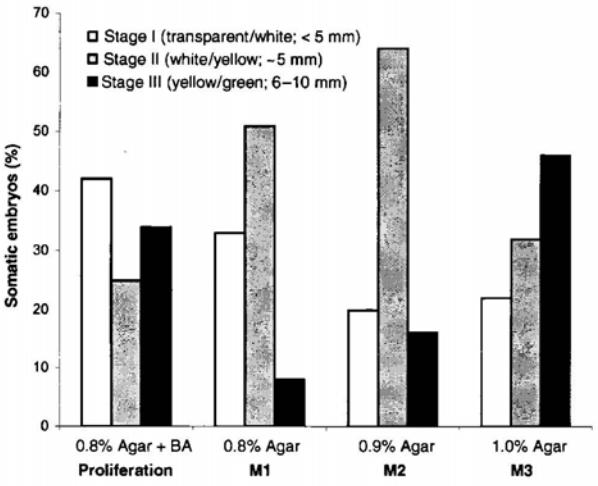

Figure 1. Formation (%) of oak cotyledonary somatic embryos (Stages I–III) following culture on maturation and proliferation media. Abbreviation: BA = 6-benzylaminopurine.

tissue. The SEs cultured on proliferation and M1 media had similar water contents (86.8%), whereas SEs cultured on M3 medium had significantly lower water content (82.3%; $P < 0.001$) (Figure 3A). The water content of SEs decreased during embryo maturity from 94.3% at Stage I to 80.7% at Stage III (Figure 3B).

Proline concentration of somatic embryos

The highest [proline] (0.47 ng g_{FW}^{-1}) was found in green Stage III SEs on M3 medium with 1.0% agar. Yellow Stage II SEs that matured on M1 medium had significantly ($P < 0.01$) lower [proline] (0.03 ng g_{FW}^{-1}). Yellow-green Stage III SEs cultured on M2 medium with 0.9% agar reached a [proline] of 0.21 ng g_{FW}^{-1} (Table 3).

Abscisic acid concentration of somatic embryos

Abscisic acid concentration decreased from the mid to the late maturation stages of SEs. Among SEs, the Stage I SEs cultured on M1 medium contained the highest [ABA] (0.33 µg g_{FW}^{-1}; $P < 0.001$) (Table 3). Beginning with late maturation, the [ABA] in Stage II SEs cultured on M3 medium decreased to 0.21 µg g_{FW}^{-1}. The lowest [ABA] (0.13 µg g_{FW}^{-1}) was measured in mature, Stage III SEs cultured on M3 medium.

Discussion

Increasing the gel strength of the maturation medium with 1.0% agar promoted the maturation and subsequent germination of oak SEs, as has been shown previously with several other oak embryogenic culture lines (Wilhelm et al. 1996, 1999, Sunderlikova and Wilhelm 2002). The greatest number (27) of Stage III SEs, with the highest germination capacity (96.3%), was obtained on M3 medium, which contained 1.0% agar (Table 2, Figure 1). The low germination frequency of Stage I SEs (25%) is probably associated with the incomplete

Table 2. Proliferation of *Quercus robur* embryonic tissue fresh mass (mean ± standard error), somatic embryo formation, number and germination frequency (%). Within a column, values followed by the same letter are not significantly different ($\alpha = 0.05$) according to Duncan's test. Abbreviation: BA = 6-benzylaminopurine.

Maturation					Somatic embryos			Germination			
Medium	Embryonic tissue fresh mass (g)	No. somatic embryos per gram fresh mass	No. somatic embryos per petri dish	No. somatic embryos per treatment	Stage I Transparent/white; < 5 mm	Stage II White/yellow; ~5 mm	Stage III Yellow/green; 6–10 mm	Stage I (%)	Stage II (%)	Stage III (%)	Total (%)
P: 0.8% Agar + BA	2.31 ± 0.078 a	1.04 b	2.41	53 b	22	13	18	23	38	17	25 a
M1: 0.8% Agar	2.33 ± 0.090 a	0.87 ab	2.45	49 ab	16	25	8	0	24	100	29 a
M2: 0.9% Agar	2.26 ± 0.091 a	0.55 a	1.25	25 a	5	16	4	60	100	100	91 b
M3: 1.0% Agar	1.76 ± 0.067 b	1.68 b	2.95	59 b	13	19	27	46	79	96	80 b

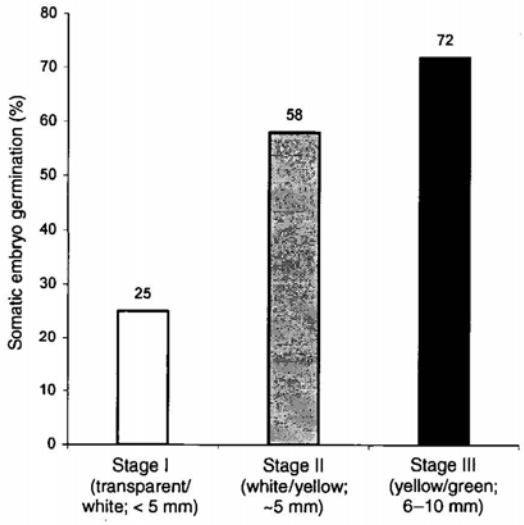

Figure 2. Germination frequency (%) of oak cotyledonary somatic embryos at maturity stages I–III. Data were significantly different at $\alpha = 0.05$, as determined by the separate 2×2 contingency χ^2 test.

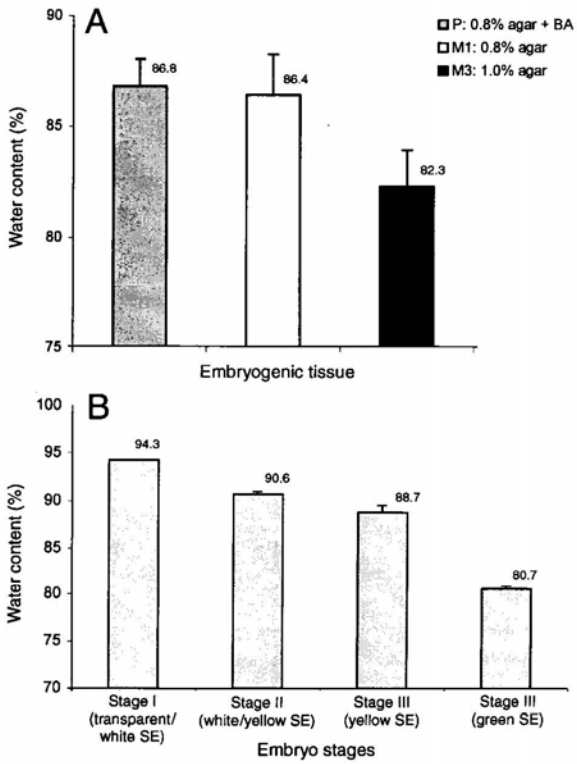

Figure 3. Water content (%) of (A) oak embryogenic tissue cultured on proliferation and maturation media (P, M1, M3) and (B) oak somatic embryos at maturity stages I–III. Abbreviation: BA = 6-benzylaminopurine.

maturation process, because the more mature Stage III SEs had a mean germination frequency of 72% (Figure 2). In other somatic embryogenesis systems, hormone-free media have improved SE maturation. In developing SEs of Norway spruce (Filanova et al. 2000), omitting plant growth regulators during maturation induces two waves of programmed cell death or apoptosis, which ensure normal progression of somatic embryogenesis and correct embryonic pattern formation, respectively. However, no significant differences in the proliferation of embryogenic oak tissue were observed on control and M1 media, regardless of the addition of cytokinin, perhaps indicating some type of "autoembryogeny" (Merkle 1995). Therefore, it must be concluded that the main factor governing the development of high quality oak SEs is the gel strength. These results are consistent with those of Klimaszewska and Smith (1997) and Klimaszewska et al. (2000), who found that an increase in gel strength promoted SE maturation in *Pinus strobus* L.

Medium viscosity increased with increasing agar concentration, reflecting reduced water availability for the developing embryos, and reduced water content of the Stage III embryos. On the other hand, the osmotic potential of the medium gelled with 0.8–1.0% agar remained constant. These experiments suggest that the primary effect of increased agar concentration on the maturation of oak SE was to restrict the availability of water to the cultures. Somatic embryos derived from media with 0.8 and 0.9% agar had a higher osmotic potential than SEs derived from the medium with 1.0% agar. The improved maturation of oak SEs derived from medium with 1.0% agar is associated with physiological markers. The Stage III SEs were characterized by lower osmotic potential, reduced water content, decreased [ABA] and increased [proline].

The lower osmotic potential of mature oak SEs is analogous

Table 3. Mean concentrations (± standard error) of L-proline and (±)-abscisic acid (ABA) and mean osmotic potentials of *Quercus robur* somatic embryos cultured on various media. Within a column, values followed by the same letter are not significantly different ($\alpha = 0.05$) according to Duncan's test.

Maturation medium	Physiological parameters		
	L-proline concentration (ng g_{FW}^{-1})	ABA concentration (μg g_{FW}^{-1})	Osmotic potential (MPa)
M1: 0.8% Agar	0.03 ± 0.043 a (Stage II)	0.33 ± 0.003 a (Stage I)	−0.701 a
M2: 0.9% Agar	0.21 ± 0.221 ab (Stage III)	–	−0.648 a
M3: 1.0% Agar	0.47 ± 0.461 b (Stage III)	0.21 ± 0.004 b (Stage II) 0.13 ± 0.003 b (Stage III)	−0.845 b

to the findings of Xu et al. (1990), who observed that more negative osmotic potential during early and late stages of embryo development coincided with low [ABA]. As reported by Finkelstein et al. (1985), a low osmotic potential may influence the regulation of storage protein biosynthesis and the latter stages of embryo development. For example, osmotic potential may serve as a physiological parameter for defining the maturity status of the SE. Somatic embryos matured on 1.0% agar preferentially formed Stage III embryos with high germination potential; these treatments are thus seen to complete the maturation process by stimulating a weak dehydration process. Osmotic treatments are known to promote embryo maturation by increasing accumulation of storage reserves in both angiosperm and conifer species (Linossier et al. 1997, Stasolla et al. 2002). Similarly, Roberts et al. (1990) emphasized the influence of low osmotic potential on the regulation of storage protein biosynthesis and embryo maturation in interior spruce. The physiological data are confirmed by the molecular investigations of the different expression patterns of storage protein genes and heterologous probes for *Lea* genes in oak SEs (Sunderlikova and Wilhelm 2002). A higher transcript accumulation of the storage protein legumin and Em- and dehydrin-like genes in *Q. robur* L. embryos matured on 1.0% agar medium was observed compared with control medium.

A low [ABA] was found in Stage III SEs. Similarly, Cvikrova et al. (1998) reported significantly lower [ABA] in converting SEs with ivory or light green cotyledons of *Q. petraea* L. (99 ng g_{FW}^{-1}) compared with non-converting embryos with deep green cotyledons (137 ng g_{FW}^{-1}). A decrease in [ABA] was also observed in SEs and seeds of *Vitis* during the later stages of embryogeny (Rajasekaran et al. 1992). A decrease in [ABA] is also considered to be a prerequisite for embryo germination at the onset of seedling development. Abscisic acid regulates the course of embryo maturation. Osmotic treatments with agents such as sorbitol and polyethylene glycol (PEG) decrease [ABA] in maturing SEs of several species and inhibit precocious germination (Find 1997).

As with SEs, Finch-Savage et al. (1992) reported that in zygotic embryos of oak, water status acts as a factor controlling embryo development and is modified by ABA. A high [ABA] resulted in high quantities of matrix-bound water, whereas decreasing tissue water content corresponded to a decline in [ABA]. According to Xu et al. (1990), the internal amounts of osmoticum in developing alfalfa embryos are more important for germination than ABA, imparting different metabolic processes in the embryo.

Among maturing oak SEs, proline accumulated during Stage III, at which time water content is reduced, dormancy is broken and embryos begin to germinate. A weak dehydration effect induces an accumulation of proline, whereas [ABA] remains low. The mild water stress imposed on oak SEs is reflected by an increase in [proline] similar to that reported for other vegetative tissues. According to Brugiere et al. (1999), proline serves as a nitrogen source in tobacco leaves and is a key metabolite that is synthesized in response to water stress.

We conclude that tissue water status is an important factor influencing the maturation process and hence the production of high-quality oak SEs. The presence of 1.0% agar in the medium reduced water availability and stimulated a shift in the developmental program, from proliferation of early embryos to the maturation of somatic embryos. Although we tested only one oak embryogenic culture line, the results obtained are generally applicable to other genotypes (Sunderlikova and Wilhelm 2002) and to other *Fagaceae* species such as *Castanea sativa* Mill. (U. Sauer and E. Wilhelm, unpublished observations). We conclude that a complex relationship exists among cell water content, [ABA], [proline] and somatic embryogenesis.

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References

Abraham, E., G. Rigo, G. Szekely, R. Nagy, C. Koncz and L. Szabados. 2003. Light dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis*. Plant Mol. Biol. 51:363–372.

Bates, J.S. 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207.

Brugiere, N., F. Dubois, A.M. Limami, M. Lelandais, Y. Roux, R.S. Sangwan and B. Hirel. 1999. Glutamine synthetase in the phloem plays a major role in controlling proline production. Plant Cell 11:1995–2012.

Cvikrova, M., J. Mala, J. Eder, M. Hrubcova and M. Vagner. 1998. Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol. Biochem. 36:247–255.

Cvikrova, M., J. Mala, M. Hrubcova, J. Eder, J. Zon and I. Machackova. 2003. Effect of inhibition of biosynthesis of phenylpropanoids on sessile oak somatic embryogenesis. Plant Physiol. Biochem. 41:251–259.

Dumont-BeBoux, N., P. von Aderkas, N.J. Livingston and R.E. Percy. 1999. Water relation between parameters in conifer embryos: methods and results. *In* Somatic Embryogensis in Woody Plants. Eds. S.M. Jain, P.K. Gupta and R.J. Newton. Vol. 4. Kluwer Academic Publishers, Dordrecht, pp 149–168.

Endemann, M. and E. Wilhelm. 1999. Factors influencing the induction and viability of somatic embryos of *Quercus robur* L. Biol. Plant. 42:499–504.

Endemann, M., K. Hristoforoglu, T. Stauber and E. Wilhelm. 2001. Assessment of age-related polyploidy in *Quercus robur* L. somatic embryos and regenerated plants using DNA flow cytometry. Biol. Plant. 44:339–345.

Etienne, H. and B. Bertrand. 2003. Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. Tree Physiol. 23:419–426.

Filanova, L.H., P.V. Bozhkov, V.B. Brukhin, G. Daniel, B. Zhivotovsky and S. von Arnold. 2000. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. J. Cell Sci. 113:4399–4411.

Finch-Savage, W.E., H.A. Clay, P.S. Blake and G. Browning. 1992. Seed development in the recalcitrant species *Quercus robur* L.: water status and endogenous abscisic acid levels. J. Exp. Bot. 43: 671–679.

Find, J.I. 1997. Changes in endogenous ABA levels in developing somatic embryos of Norway spruce (*Picea abies* (L.) Karst.) in relation to maturation medium, desiccation and germination. Plant Sci. 128:75–83.

Finkelstein, R.R., K. Tenbarge, J.E. Shumway and M.L. Crouch. 1985. Role of ABA in maturation of rapeseed embryos. Plant Physiol. 78:630–636.

Finkelstein, R.R., S.S.L. Gampala and C.D. Rock. 2002. Abscisic acid signaling in seeds and seedlings. Plant Cell 14:15–45.

George, E.F. 1993. Plant propagation by tissue culture. Part 1, the technology. Exegetics, Somerset, U.K., pp 327–336.

Hare, P.D., W.A. Cress and J. van Staden. 1999. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J. Exp. Bot. 50:413–434.

Johnson, R.W., P.S. Asokanthan and M. Griffith. 1997. Water and sucrose regulate canola embryo development. Physiol. Plant. 101: 361–366.

Kapik, R.H., R.J. Dinus and J.F.D. Dean. 1995. Abscisic acid and zygotic embryogenesis in *Pinus taeda*. Tree Physiol. 15:485–490.

Klimaszewska, K. and D.R. Smith. 1997. Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol. Plant. 100:949–957.

Klimaszewska, K., M. Bernier-Cardou, D.R. Cyr and B.C.S. Sutton. 2000. Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cell Dev. Biol. Plant 36:279–286.

Leal, I., S. Misra, S.M. Attree and L.C. Fowke. 1995. Effect of abscisic acid, osmoticum and desiccation on 11S storage protein gene expression in somatic embryos of white spruce. Plant Sci. 106:121–128.

Linossier, L., P. Veisseire, F. Cailloux and A. Coudret. 1997. Effects of abscisic acid and high concentrations of PEG on *Hevea brasiliensis* somatic embryos development. Plant Sci. 124:183–191.

Merkle, S.A. 1995. Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tissue Cult. Biotech. 1: 112–121.

Nambara, E. and M.A. Poll. 2003. ABA action and interactions in seeds. Trends Plant Sci. 8:213–217.

Park, Y.S., J.D. Barret and J.M. Bonga. 1998. Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control and stability of cryopreserved clones. In Vitro Cell Dev. Biol. Plant 34:231–239.

Prewein, C. and E. Wilhelm. 2003. Plant regeneration from encapsulated somatic embryos of pedunculate oak (*Quercus robur* L.). In Vitro Cell Dev. Biol. Plant 39: 613–617.

Rajasekaran, K., J. Vine and M.G. Mullins. 1992. Dormancy in somatic embryos and seeds of *Vitis*: changes in endogenous abscisic acid during embryogeny and germination. Planta 154:139–144.

Roberts, D.R., B.S. Flinn, D.T. Webb, F.B. Webster and B.C.S. Sutton. 1990. Abscisic acid and indole-3-butyric acid regulation of maturation and accumulation of storage proteins in somatic embryos of interior spruce. Physiol. Plant. 78:355–360.

Rugkhla, A. and M.G.K. Jones. 1998. Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. J. Exp. Bot. 49:563–571.

Santana, N., M.E. Gonzalez, M. Valcarcel et al. 2004. Somatic embryogenesis: a valuable alternative for propagating selected robusta coffee (*Coffea canephora*) clones. In Vitro Cell Dev. Biol. Plant 40:95–101.

Senger, S., H.P. Mock, U. Conrad and R. Manteuffel. 2001. Immunomodulation of ABA function affects early events in somatic embryo development. Plant Cell Rep. 20:112–120.

Stasolla, C., L. Kong, E.C. Yeung and T. Thorpe. 2002. Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. In Vitro Cell Dev. Biol. Plant 38: 93–105.

Sunderlikova, V. and E. Wilhelm. 2002. High accumulation of legumin and *Lea*-like mRNAs during maturation is associated with increased conversion frequency of somatic embryos from pedunculate oak (*Quercus robur* L.). Protoplasma 220:97–103.

Teasdale, R. 1992. Formulation of plant culture media and applications thereof. International Publication No. WO 92/07460, Patent No. Europe: 92902531.0, Forbio PTY, Queensland, Australia.

Toribio, M., C. Fernández, C. Celestino, M.T. Martínez, M.C. San-José and A.M. Vieitez. 2004. Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell Tissue Organ Cult. 6:283–287.

Vengadesan, G., A. Ganapathi, S. Amutha and N. Selvaraj. 2002. In vitro propagation of *Acacia* species—a review. Plant Sci. 163: 663–671.

White, C.N., W.M. Proebsting, P. Hedden and C.J. Rivin. 2000. Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiol. 122:1081–1088.

Wilhelm, E., A. Burg, M. Berenyi, M. Endemann and R. Rodler. 1996. Plantlet regeneration via somatic embryogenesis and investigations on *Agrobacterium tumefaciens* mediated tranformation of oak (*Quercus robur*). *In* Somatic Cell Genetics and Molecular Genetics of Trees. Eds. M.R. Ahuja, W. Bourjan and D. Neale. Kluwer Academic Publishers, Dordrecht, pp 125–130.

Wilhelm, E., M. Endemann, K. Hristoforoglu, C. Prewein and M. Tutkova. 1999. Somatic embryogenesis in oak (*Quercus robur* L.) and production of artificial seeds. *In* Proc. Applications of Biotechnology to Forest Genetics. Eds. S. Espinel and E. Ritter. Bifor 99, Vitoria-Gasteiz, Spain, pp 213–225.

Wilhelm, E. 2000. Somatic embryogenesis in oak (*Quercus* spp.) In Vitro Cell Dev. Biol. Plant 36:349–357.

Xu, N., M. Coulter and J.D. Bewley. 1990. Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. Planta 182:382–390.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science 217:1214–1222.

Zoglauer, K., U. Behrendt, A. Rahmat, H. Ross and Taryono. 2003. Somatic embryogenesis—the gate to biotechnology in conifers. *In* Plant Tissue Culture—100 Years Since Gottlieb Haberlandt. Eds. M. Laimer and W. Rücker. Springer-Verlag, Wien, pp 175–203.