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Storage lipid dynamics in somatic embryos of Norway spruce (*Picea abies*): histochemical and quantitative analyses

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Summary Adequate storage compounds are a prerequisite for successful development during the later stages of somatic embryogenesis; however, the critical amount of reserves below which somatic embryos fail to mature and germinate has not been determined. We analyzed storage lipids during Norway spruce (*Picea abies* (L.) Karst.) somatic embryogenesis. As maturation progressed, lipids, which were stored as lipid bodies in the cytoplasm, were localized first in suspensor cells of the early embryos, and later in the embryonic root pole, superficial layers of the hypocotyl and in cotyledons. The concentration of total lipids exhibited marked variation, with values peaking during cotyledon development and then decreasing during maturation. Linoleic (18:2), oleic (18:1), palmitic (16:0) and 5,9-octadecenic (5,9-18:2) acids were the most abundant fatty acids in embryos. As embryos developed, linoleic acid concentration increased slightly, whereas oleic acid concentration decreased. Oleic acid was the most prominent component of the fatty acid spectrum in isolated dormant zygotic embryos and megagametophytes. Addition of 5% polyethylene glycol to the medium during somatic embryo maturation caused a shift in the fatty acid spectrum toward that of zygotic embryos. During maturation, changes in the exogenous carbohydrate supply had no significant effect on total lipid concentration in mature embryos. A marked decrease in lipid concentration was detected during desiccation, indicating the importance of adequate lipid reserves during this developmental stage. The lipid content of zygotic embryos differed considerably with harvest year and location, suggesting that zygotic embryo data cannot be an indicator of somatic embryo quality.

Keywords: embryo maturation, fatty acid composition, histochemistry, oil bodies, zygotic embryo.

Introduction

Somatic embryogenesis refers to the process in which somatic cells are induced to form bipolar structures similar to zygotic embryos. It is a powerful experimental model for studying plant embryogenesis. The technique has been used to mass produce plants that are not readily propagated by classical methods and to propagate endangered plant species. Somatic embryogenesis comprises several phases: formation of the proembryogenic mass with early embryo, maturation, desiccation (if necessary), germination and conversion. During maturation, besides structural changes, embryos accumulate storage compounds necessary for post-embryonic growth. The main storage compounds are carbohydrates, lipids and proteins. Both the amount and composition of storage compounds that accumulate during maturation are essential for further embryonic development and can be used to assess the competence of a somatic embryo to complete subsequent developmental stages (e.g., *Picea glauca* (Moench) Voss (Attree and Fowke 1991, Attree et al. 1992)).

Our previous studies, which focused on the dynamics of storage saccharide concentration in somatic embryos of *Picea abies* (L.) Karst., revealed low carbohydrate accumulation in mature somatic embryos (Lipavská et al. 2000). Nevertheless, these carbohydrates provide an important source of carbon and energy during distinct phases of somatic embryo development. During maturation, minor starch reserves accumulate that are mobilized during the desiccation phase (H. Konrádová and H. Lipavská, Charles University, Prague, unpublished data). Raffinose family oligosaccharides (RFOs) appear during desiccation of somatic embryos of *P. abies* (Kumstýřová et al. 2000) and *Picea mariana* (Mill.) BSP (Bomal et al. 2002). Raffinose family oligosaccharides function in sugar transport, membrane protection and acquisition of desiccation tolerance (Crowe et al. 1992, Turgeon et al. 1993 and Black et al. 1999, respectively). Based on the relatively high RFO concentration at the end of the desiccation phase, the rapid degradation of RFOs during the first hours of germination and the complete elimination of RFOs within 3 days of germination, Kumstýřová et al. (2000) postulated that, although RFOs do not meet all energy demands during germination, they serve as an energy source during the early germination of Norway spruce (*P. abies*) somatic embryos. In addition to carbohydrates, pro-

teins (e.g., in *P. abies* (Stabel et al. 1990, Hakman 1993)) and lipids (e.g., in *P. abies* (Feirer et al. 1989), *Abies alba* Mill. (Kovać and Kregar 1989), the *P. glauca engelmanni* complex (Carrier et al. 1999) and *Pinus taeda* L. (Stone and Gifford 1999)) are important during somatic embryogenesis.

Storage lipids are mainly represented by triacylglycerols (TAGs). Biosynthesis of TAGs is an energy-consuming process that takes place in many cell compartments (Voelker and Kinney 2001). Storage lipids represent up to 80% of the dry mass of storage tissues in many plants. In seeds, storage lipids are usually localized in endosperm tissue and cotyledons, and, in conifers, they are preferentially deposited in the megagametophyte. In many conifers, storage lipids comprise 30–50% of the seed dry mass (e.g., *A. alba* (Kovač and Kregar 1989), *P. abies* (Feirer et al. 1989), *P. taeda* (Krasowski and Owens 1993)). Lipids are the most important storage compounds for embryo development in Norway spruce and silver fir, comprising about 50 and 48% of the fresh mass of seeds, respectively (Kovač and Kregar 1989, Feirer et al. 1989, Attree et al. 1992).

As in angiosperms, there are substantial amounts of linolenic (18:3), linoleic (9,12-18:2) and palmitic (16:0) acids in the fatty acid (FA) spectrum of conifers. In addition, special groups of C20 polyunsaturated and C18 delta-5-polyunsaturated FAs occur exclusively in conifers (Jamieson and Reid 1972). However, little is known about the dynamics and composition of storage lipids in somatic embryos of conifers, particularly in Norway spruce.

To complement a comprehensive study on the carbohydrate status of Norway spruce somatic embryos during maturation (Lipavská et al 2000, Gösslová et al. 2001, Konrádová et al. 2002, 2003), we investigated the location and accumulation dynamics of storage lipids, and how they are affected by osmotic stress and carbohydrate supply during the maturation phase. For comparison, the lipid status of mature, naturally developed zygotic embryos was determined.

Materials and methods

Plant material

Zygotic embryos Seeds were harvested from cones collected from forest stands in the Bohemian-Moravian Highlands in 1999, in central Bohemia in February 2001, in northeastern Moravia (Beskydy Mountains) in January–February 2002 and November 2003, and in North Moravia in 2004. Only healthy trees were selected for cone harvest. For seeds harvested in 1999 and 2004, germination under standard test conditions was 51% and 92%, respectively. For the samples from years 2001–2003, estimated germination ranged between 60 and 70%. Zygotic embryos and megagametophytes were isolated and analyzed separately. Before germination of zygotic embryos, seeds were stored at –18 ± 2 °C. Seeds were sterilized with 10% commercial bleach for 5 min, followed by 2 min in 70% ethanol, then washed with distilled water and gently dried with sterile filter paper. The sterilized seeds were stratified in paper bags at 4 °C in darkness for 4 weeks. Stratified seeds were then imbibed in distilled water at 4 °C for about 24 h. Seeds were germinated by incubation on wet filter paper in the dark at 25 °C for about 5 weeks.

Somatic embryos An embryogenic culture of *Picea abies* (line AFO 541, from Dr. Bercetche, AFOCEL, France) was grown on GD medium (Gupta and Durzan 1986). Proliferation medium was supplemented with 5 μM 2,4-dichlorophenoxyacetic acid, 2 μM kinetin, 2 μM 6-benzylaminopurine (all Sigma) and 30 g l^{-1} sucrose (Lachema, Brno, Czech Republic). The maturation medium contained 20 μM abscisic acid (Sigma) and lacked auxins and cytokinins. Medium containing 5% (w/v) polyethylene glycol-4000 (PEG) was prepared by combining (after partial cooling) separately autoclaved medium and PEG solution. Embryogenic cultures were maintained by weekly subculture as described by Svobodová et al. (1999). Five samples were analyzed at each harvest. The mean number of somatic embryos per sample ranged between 40 and 60 according to the stage of embryo development.

To study the effects of changes in sugar supply during a 6-week period of somatic embryo maturation, the embryogenic cultures were grown on rafts (Osmotek, Rehovot, Israel) floating on liquid medium. Four media and subculture intervals were used: (1) medium with 3% sucrose and a one-week subculture interval; (2) medium with 1.57% glucose and 1.57% fructose and a one-week subculture interval; (3) medium with 3% sucrose and subculture every second, fourth and seventh day of each week to limit sucrose hydrolysis in the medium; and (4) medium with 3% sucrose for the first two days, 2.25% sucrose, 0.375% glucose and 0.375% fructose for the next two days, and 1.5% sucrose, 0.75% glucose and 0.75% fructose for the last three days of each week to mimic sucrose hydrolysis occurring during the one-week subculture on 3% sucrose.

Partial desiccation and germination treatments

Somatic embryos were isolated from cultures after a 6-week maturation period and gradually desiccated over 3 weeks at 20 °C with a 16-h photoperiod by placing the embryos on dry filter paper in a small open petri dish within a larger, sealed petri dish containing wet filter paper. Embryos were germinated on GD medium supplemented with 1% (w/v) sucrose, 0.8% (w/v) agar and 0.4% (w/v) charcoal (Sigma), without growth regulators, at 25 °C with a 16-h photoperiod.

Histochemical study

Lipid bodies in free-hand sections of fresh material were stained with Sudan Red 7B–PEG (Fluka) according to Brundrett et al. (1991), and visualized with a binocular microscope (Olympus BX 50, digital camera Olympus E 10).

Lipid concentration analysis

Samples (minimum dry mass of 30 mg) were mixed with 0.5 ml of 1:1 (v/v) methanol:chloroform, vortexed and filtered. The extraction was repeated three times and the extracts combined. After solvent evaporation in a fume chamber, total lipid mass was determined.

Fatty acid composition

The FA methyl esters in the lipid extracts were separated on an AHP 5995 gas chromatograph performance system (Agilent, Czech Republic). The methyl esters were prepared according to Metcalfe and Wang (1981). The samples were injected onto a 25 m × 0.25 mm × 0.1 m Ultra-1 capillary column (Supelco, Czech Republic) (injector and detector temperatures of 300 °C) under a temperature program of: 5 min at 50 °C, increasing at 10 °C min^{-1} to 320 °C and 15 min at 320 °C. Helium was the carrier gas at a flow of 0.52 ml min^{-1}. Methyl esters were identified by comparison of their retention times with those of standards (kit RM-7, Supelco).

Results and discussion

For successful somatic embryogenesis, determination of the optimum duration of the maturation phase, is essential. In previous studies, we found that, after 6 weeks of maturation, somatic embryos exhibited well developed structure (Svobodová et al. 1999), carbohydrate status (Lipavská et al. 2000, Gössl ová 2001) and activity of key enzymes of carbohydrate metabolism (Konrádová et al. 2002), closely resembling the corresponding characteristics of zygotic embryos. After three weeks of slow desiccation, somatic embryos had a high germination rate. Prolonging the maturation period to 8 weeks impaired both embryo structure and germination efficiency (data not shown).

In both somatic embryos and zygotic embryos, storage lipids were detected as lipid bodies dispersed freely in the cytoplasm. Histochemical analysis of somatic embryogenic cultures revealed the following developmental pattern of lipid deposition. During Weeks 1 and 2 of maturation, lipid bodies were located in undifferentiated cells of the embryonal-suspensor mass and in the suspensor cells of the early embryos. In Week 4 of maturation, when isolation and staining of single embryos were possible, storage lipids had accumulated in the embryonic root poles. Thereafter, lipid bodies accumulated in the surface layers of the hypocotyls and in cotyledons (Figure 1). At the end of the 6-week maturation period, lipid bodies were also present in the central part of the hypocotyl and in the apical region of the embryo. Histochemical analysis of isolated mature zygotic embryos showed a similar localization of lipid bodies as that found for fully mature somatic embryos cultured for 6 weeks on maturation medium. Staining revealed significant amounts of storage lipids in the megagametophyte

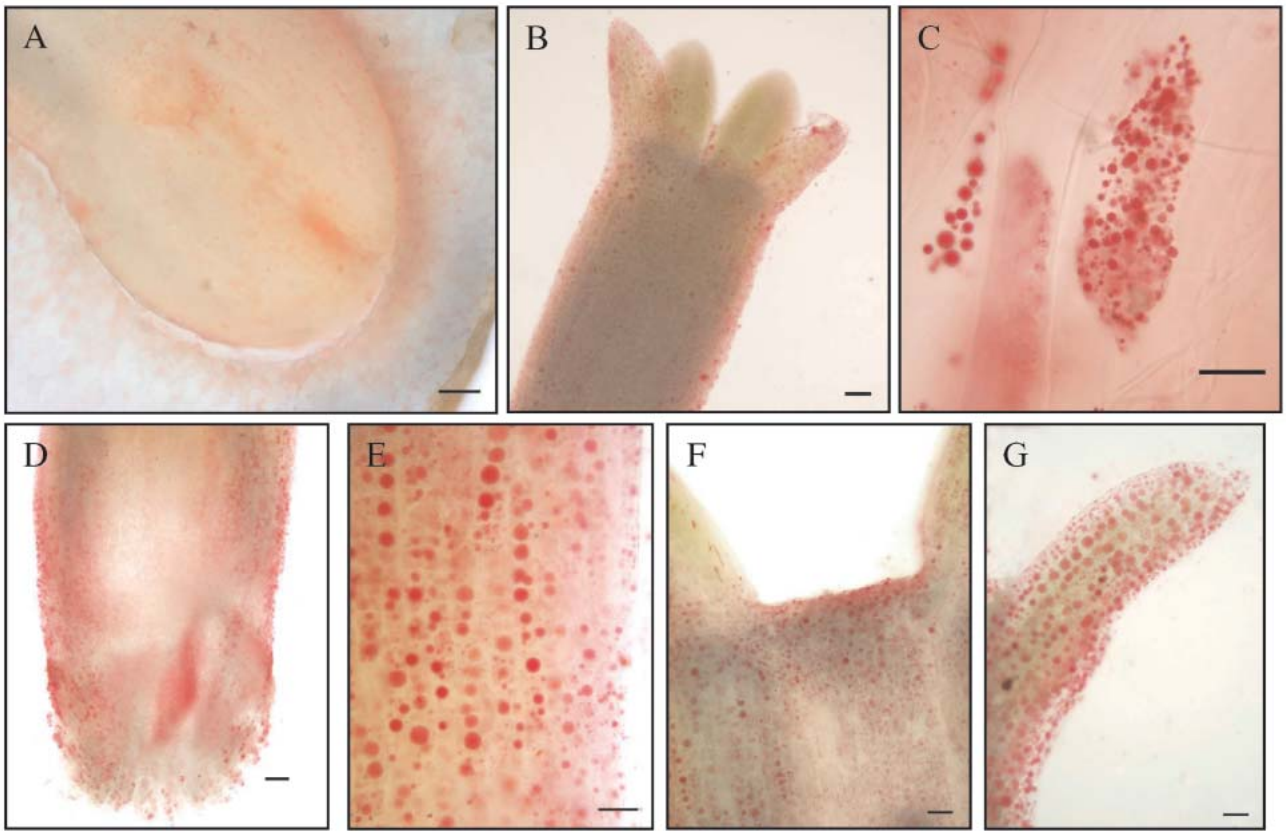

Figure 1. (A) Zygotic and (B–G) somatic *Picea abies* embryos. (A) Detail of cotyledons and apical meristem surrounded by the megagametophyte. (B) Somatic embryo at Week 5 of maturation. (C) Lipid bodies inside the cells of the embryonal suspensor mass at Week 1 of maturation. (D) Root pole at Week 4 of maturation. (E) Surface layers of the hypocotyls at Week 4 of maturation. (F) Detail of the apical meristem and cotyledons at Week 5 of maturation. (G) Detail of a fully developed cotyledon at Week 6 of maturation. Scale bars = 100 µm (A, B), 20 µm (C), 100 µm (D), 20 µm (E) and 50 µm (F, G).

tissue surrounding the zygotic embryos (Figure 1). Based on histochemical studies, Joy et al. (1991) identified a precise pattern of storage compound accumulation during somatic embryogenesis in white spruce (*Picea glauca*): starch accumulated first, followed by lipids and proteins. They found that starch accumulated in the proximal part of the suspensor during early somatic embryogenesis, and in the shoot pole and cortex at the cotyledonary stage of development. Lipid bodies accumulated acropetally in the late filamentous stage of somatic embryogenesis (corresponding to Week 2 of maturation in our study). The gradients in reserve deposition during somatic embryo development indicate that physiologically different regions are present in developing embryos, and that they may be required for establishing patterns of tissue differentiation, as has been suggested to occur during apical meristem formation in zygotic embryos (Yeung et al. 1998).

After 6 weeks of culture on maturation medium lacking PEG, the dry-mass lipid concentration of mature somatic embryos was nearly 80 μg mg^{-1}, increasing to about 350 μg mg^{-1} in the presence of 5% PEG (Figure 2). Mean fresh mass of individual zygotic embryos was 0.67 mg (with a mean water content of 35.7%), whereas the mean fresh mass of individual mature somatic embryos was 2.41 mg. The water contents of mature somatic embryos varied between 54 and 95% depending on developmental stage and medium osmotic potential (Table 1).

Total lipid concentration increased during the first weeks of somatic embryo maturation, then gradually decreased during late maturation (Figure 2). Starch deposition dynamics followed a similar course (data not shown). The maximum concentrations in both cases correlated with the appearance of cotyledons, suggesting that storage compounds contributed to cotyledon formation.

The accumulation of adequate reserves in conifer somatic embryos is vital, because post-germination growth occurs in the absence of the haploid megagametophyte, which is the major storage organ in conifer seeds. Insufficient stored reserves, especially lipids, have been correlated with reduced viability of somatic embryos during the desiccation and germination phases (Attree et al. 1992). As shown in Figure 3, lipid concentrations in isolated zygotic embryos varied significantly with harvest year and locality (dry mass between about 20 and 218 μg mg^{-1}). Given the large variation in the amounts of storage lipids in zygotic embryos, we cannot conclude that somatic embryos generally have lower lipid concentrations than zygotic embryos (Figures 2 and 3), as has been reported for several conifer species (Feirer et al. 1989). The variation in zygotic embryo lipid concentrations suggests somatic embryos can cope with varying lipid concentration. Norway spruce embryos also contain proteins and carbohydrates, suggesting that a shortage of one type of storage compound could be compensated for by the presence of another, or that distinct phases of embryonic development are dependent on the supply of specific storage compounds, or both (Lipavská and Konrádová 2004). However, we found that the FA spectrum and its dynamics were stable under all of the experimental conditions tested.

The FA composition of TAGs in the major oil crops (coconut, olive, sunflower, rapeseed and soybean) is limited to C16

Table 1. Water content of *Picea abies* somatic embryos cultured on medium with or without polyethylene glycol-4000 (PEG). Measurements were made on the embryonal-suspensor mass (Weeks 1–4) and isolated embryos (Weeks 5–6).

	Water content (%)	
	0% PEG	5% PEG
Maturation week		
1	94.2	90.8
2	94.3	89.9
3	93.9	90.1
4	95.1	88.3
5	78.9	75.2
6	74.6	73.2
After 3 weeks of desiccation	54.3	54.1
After 3 weeks of germination	90.5	91.3

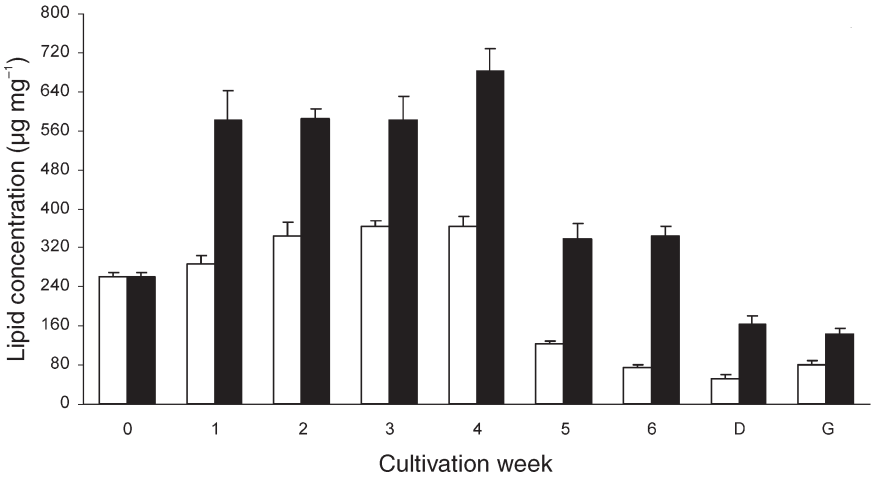

Figure 2. Dry-mass lipid concentration of *Picea abies* somatic embryos cultured on medium lacking (open bars) or containing 5% (filled bars) polyethylene glycol-4000. Week 0: proliferation of embryonal-suspensor mass. Weeks 1–4: maturation of embryonal-suspensor mass. Weeks 5–6: maturation of isolated embryos. D: Week 3 of desiccation. G: Week 1 after germination. Bars denote standard deviations for $n = 5$.

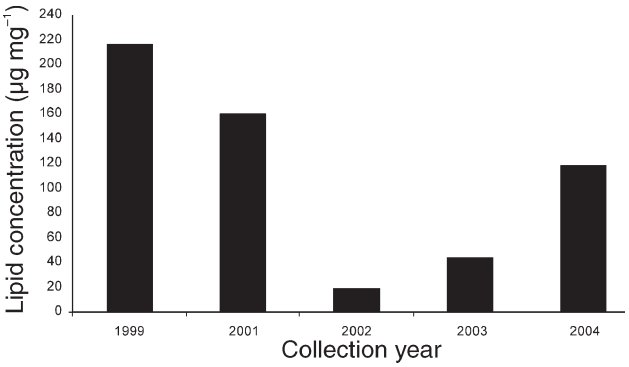

Figure 3. Dry-mass lipid concentration of isolated *Picea abies* zygotic embryos from different localities and collection years: 1999, Bohemian-Moravian Highlands; 2001, mid Bohemia (Prague west); 2002 and 2003, Beskydy Mountains; and 2004, North Moravia.

and C22 acyl chains with up to three double bonds (Töpfer et al. 1995). For conifers, many authors have documented the similarity in FA spectra between somatic and zygotic embryos (*Picea glauca*, Attree et al. 1992; *Picea glauca engelmanni* complex, Carrier et al. 1997). Palmitic, oleic, linoleic and linolenic acids and the characteristic groups of C20 polyunsaturated and C18 delta-5-polyunsaturated FAs are generally the predominant FAs in conifers (Jamieson and Reid 1972, Carrier et al. 1997, Wolff et al. 2000). In both somatic and zygotic Norway spruce embryos, we detected the following FAs: palmitic (16:0), palmitoleic (16:1), hexadecadienic (16:2), stearic (18:0), oleic (18:1), 7-octadecenic (7-18:1), linoleic (9,12-18:2), 5,9-octadecadienic (5,9-18:2), linolenic (18:3), arachidonic (20:0) and eicosenic (20:1) acids (Figures 4 and 5). The most abundant FAs in somatic embryos were: linoleic (18:2), oleic (18:1), palmitic (16:0) and 5,9-octadecadienic (5,9-18:2) acids. Linoleic (18:2) acid comprised about 30–50% and oleic acid (18:1) about 20–30% of total FA concentration. As somatic embryos developed, the linoleic acid concentration increased slightly, whereas the concentration of oleic acid decreased (Figure 4). In contrast, oleic acid was most prominent in the FA spectrum of isolated zygotic embryos and in megagametophytes (Figure 4).

Both lipid concentration and FA spectrum can be influenced by specific cultivation conditions. Abscisic acid added to the cultivation medium during maturation increases the storage lipid concentration in developing somatic embryos in many coniferous species (e.g., Norway spruce (Hakman and von Arnold 1988, Feirer et al. 1989), white spruce (Attree et al. 1992) and hybrid larch (*Larix* × *eurolepis* A. Henry) (von Aderkas et al. 2002)). A cold-induced stress treatment (4 °C) improved the maturation process and increased storage lipid accumulation in somatic embryos of *Prunus avium* L. (Reidiboym-Talleux et al. 2000). Attree et al. (1992) noted a significant increase in storage lipids in white spruce somatic embryos cultured on medium containing 7.5% PEG, and Misra et al. (1993) reported that PEG enhanced protein deposition in somatic embryos of white spruce. We found that osmotic stress induced by 5% PEG increased lipid concentrations in somatic embryos in all developmental phases (Figure 2).

The developmental stage of maturing zygotic soya embryos can be determined based on the FA spectrum composition (Dahmer et al. 1991), indicating that lipid composition may have potential as a diagnostic marker of somatic embryo quality. Linoleic acid (18:2) was the predominant FA in seeds of loblolly pine (Janick et al. 1991) and white spruce (Attree et al. 1992). In our zygotic Norway spruce embryos, linoleic (18:2) and oleic (18:1) acids were the most abundant FAs. We observed a high 18:1/18:2 ratio in zygotic embryos and found that PEG increased the 18:1/18:2 fatty acids ratio in somatic embryos, indicating that osmotic stress induced by PEG shifts the FA spectrum in somatic embryos towards that of zygotic embryos (Figure 5).

Although PEG-treated somatic embryos had increased lipid concentrations, the PEG treatment suppressed germination, as has been reported in the majority of conifer species tested (Kong and Yeung 1995, Klimaszewska et al. 1996, Find 1997, Bozhkov and von Arnold 1998). It remains to be determined if germination of PEG-treated somatic embryos can be improved

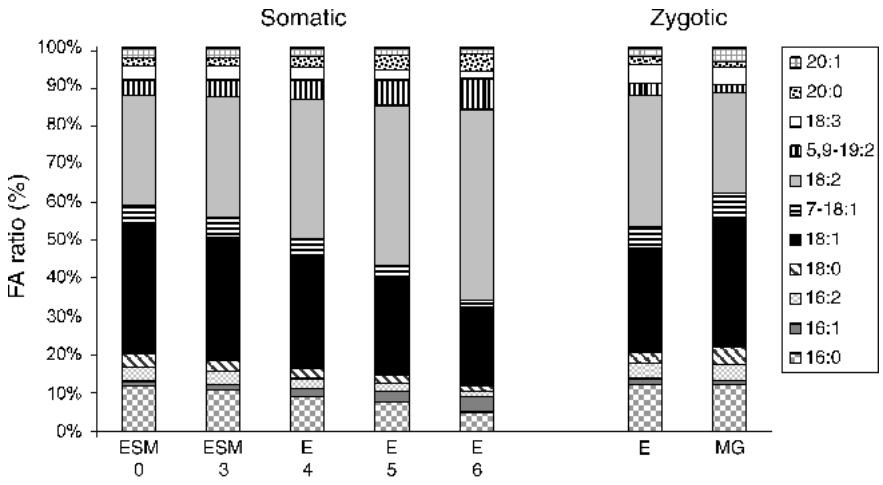

Figure 4. Fatty acid (FA) ratios in maturing somatic embryos compared with isolated zygotic embryos and megagametophytes. Abbreviations: ESM = embryonal-suspensor mass; E = isolated embryos; and MG = megagametophytes. Numbers 0–6 denote weeks of maturation.

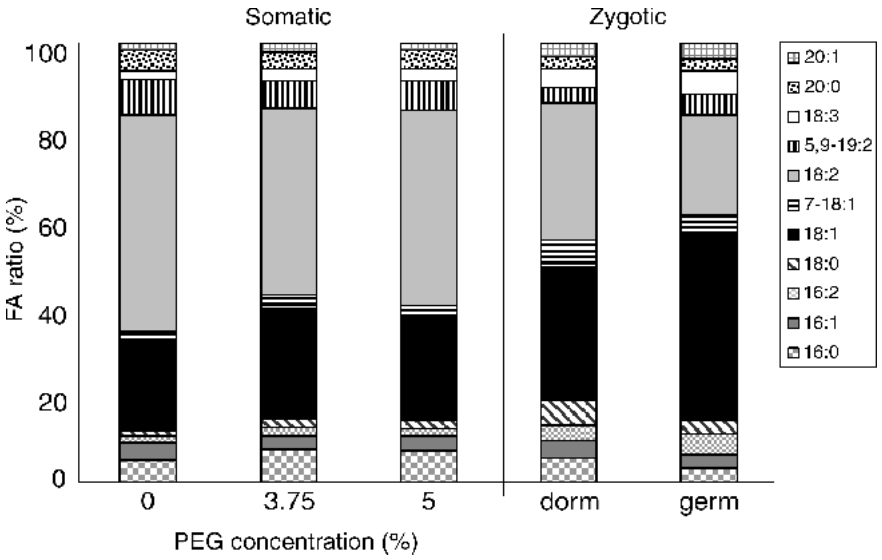

Figure 5. Fatty acid (FA) ratios in mature somatic embryos ($n = 5$) cultivated on maturation medium containing 0, 3.75 or 5% polyethylene glycol-4000 (PEG), and in isolated fully mature dormant (dorm) and germinating (germ) zygotic embryos.

by subjecting them to different conditions during post-maturation phases. Previously, we showed that the PEG application influences the structure of somatic embryos (Svobodová et al. 1999). Total length of the embryos, length:width ratio and length of the root cap increased when somatic embryos were incubated with 3.75% PEG. However, 7.5% PEG in the culture medium resulted in ruptures in the embryo center and rows of dead cells in the cortex. In our study, 5% PEG concentration was selected as a compromise between positive effects on storage compound deposition and negative effects on embryo structure.

Changes in carbohydrate availability during embryo development may influence embryo yield and improve vigor during the post-maturation phases (Gibson 2005, Iraqi et al. 2005).

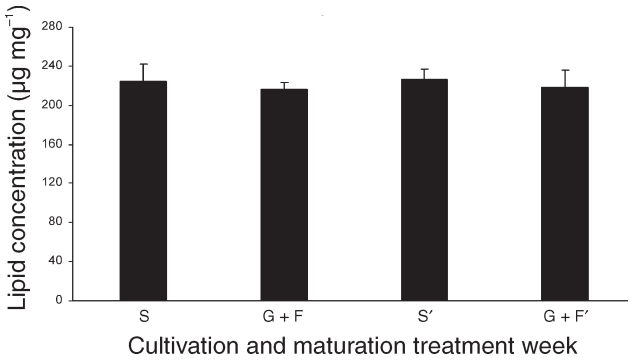

Figure 6. Effects of exogenous carbohydrate supply on dry-mass lipid concentrations in mature somatic embryos at Week 6 of maturation. Treatments: S = liquid maturation medium supplemented with 3% sucrose; G + F = liquid maturation medium supplemented with 1.57% glucose and 1.57% fructose; S′ = S but with transfer to fresh medium every second, fourth and seventh day of each week; and G + F′ = liquid maturation medium supplemented with 3% sucrose for the first 2 days, 2.25% sucrose, 0.375% glucose and 0.375% fructose for the next 2 days, and 1.5% sucrose, 0.75% glucose and 0.75% fructose for the last 3 days of each week. Bars denote standard deviations for $n = 5$.

Iraqi and Tremblay (2001*a*, 2001*b*) showed that alterations in carbohydrate supply significantly influenced starch and protein deposition in *Picea mariana* and *P. glauca*. We used a similar experimental design to determine if lipid storage in embryogenic cultures of *Picea abies* is influenced by changes in exogenous sugar availability during maturation; however, in our study, somatic embryos were cultivated on liquid medium. The type of medium (liquid or solid) influences somatic embryo cultures, precluding comparison of our data with data obtained for somatic embryos cultured on solid medium. We found no significant differences in lipid concentrations in response to changes in carbohydrate supply in the culture medium (Figure 6), nor did we detect changes in endogenous carbohydrate concentration or composition in response to changes in exogenous sugar availability (data not shown).

Many authors have documented significant anatomical and metabolic differences in somatic embryogenesis not only among conifer species, but also among different genotypes of the same species and even within genotypic lines (Feirer et al. 1989, Flinn et al. 1991, Tillman-Sutela et al. 1995, Reidiboym-Talleux et al. 1999, Pullman et al. 2003). For example, an unrelated Norway spruce line, C110 (derived in IEB, Czech Republic), exhibited lower lipid concentrations and lower responses to PEG treatments than line AFO 541 studied here. These lower values were consistent with differences in other biochemical characteristics (e.g., no RFO accumulation after cold treatment), indicating an overall difference in biochemical status. Thus, further studies are needed to determine if it is possible to manipulate lipid deposition in conifers to promote embryo yield and germination.

The natural model represented by zygotic embryogenesis shows high variability in lipid accumulation, indicating that treating maturing somatic embryos so they accumulate large amounts of storage lipids may not enhance their success in completing subsequent developmental stages. We conclude that the presence in maturing somatic embryos of specific FAs in appropriate ratios is crucial, because lipids, in concert with

other storage compounds, support development, with each storage compound having a prominent role during distinct phases of embryogenesis.

Acknowledgments

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