Mortality of transplanted somatic seedlings at the stage of acclimatization is often high and likely due to rapid change in environmental conditions. To investigate the potential of in vitro acclimatization of somatic seedlings before soil transfer, somatic seedlings of white spruce (Picea glauca [Moench] Voss) were germinated on a liquid medium supplemented with sucrose. After 6 weeks in germination, sucrose was omitted from the medium for a supplementary 6 weeks at which time somatic seedlings were acclimatized in vitro in their germination tubes before transfer to soil. In vitro acclimatization of somatic seedlings was realized by transferring the test tubes containing the germinated somatic seedlings to the greenhouse for 9 days. During this period, the culture tube lids of acclimatized somatic seedlings were lifted progressively increasing air exchange between the tube and the greenhouse whereas, for non-acclimatized somatic seedlings the culture tubes were maintained closed during in vitro acclimatization. In vitro acclimatized somatic seedlings had higher asymptotic net photosynthesis ($P_n$) at light saturation than non-acclimatized seedlings (6 versus 4.5 μmol m$^{-2}$ s$^{-1}$). At the end of the in vitro acclimatization period, a lower rate of epidermal transpiration was also observed for acclimatized seedlings (3.85 versus 4.75% h$^{-1}$). Microscopic observations showed that starch granules were more abundant in needles of acclimatized somatic seedlings than in non-acclimatized somatic seedlings, probably as a result of their greater photosynthetic capacity. Needles from acclimatized somatic seedlings also showed more epicuticular wax projections than needles from non-acclimatized somatic seedlings. These structural changes may help somatic seedlings to restrict epidermal water loss and stomatal aperture.

Introduction

The use of somatic embryogenesis as an efficient commercial technique in forestry is still limited by its relatively high production costs and low survival rate of seedlings during ex vitro transplanting. During in vitro culture, somatic seedlings are grown under unnatural conditions such as high relative humidity, low light intensity, relatively constant temperature, large fluctuations in CO$_2$ and limited space. Due to these highly stressful conditions, in vitro plantlets can develop some abnormalities in the epidermal wax, stomata and roots leading to an inability of the plantlet to control excessive epidermal transpiration, which is considered to be the main cause of seedling mortality following transfer to soil (Ziv 1995). Wardle et al. (1983) observed that high relative humidity during in vitro growth inhibited surface wax production on Brassica oleracea L. plantlets.

To improve the rate of survival and physiological functioning of somatic seedlings, Ziv (1995) suggested that, for ex vitro acclimatization, the procedures such as gradual decrease in relative humidity (RH), removal of sugars and increase of CO$_2$ and light intensity should be applied during the in vitro acclimatization period. Different techniques were applied to decrease RH

Abbreviations – ABA, abscisic acid; PPFD, photosynthetic photon flux density; RH, relative humidity; RWC, relative water content.
including uncapping of culture vessels, use of dessicants, a ventilation system, and permeable membranes (Ripley and Preece 1986, Tanaka et al. 1992). During ex vitro acclimatization, somatic seedlings are generally transferred under mist conditions with a gradual decrease in relative humidity (Khlifi and Tremblay 1995). This type of acclimatization is still a limiting step in moving towards large-scale production. Malfunctioning of the water balance as well as poor development of the photosynthetic apparatus are the major constraints when somatic seedlings are transferred to regular greenhouse conditions (Preece and Sutter 1991).

Several thousands of white spruce, black spruce and hybrid larch somatic seedlings representing several genotypes have been produced in our laboratory since 1996 using in vitro acclimatization in the greenhouse. Our hypothesis has been that the period of in vitro acclimatization was sufficient to increase net photosynthesis and induce development of normal amounts of wax which decrease epidermal transpiration. In vitro acclimatization consisted of a gradual transition of somatic seedlings from in vitro to greenhouse environmental conditions by progressively uncapping the test tubes during 9 days prior the transfer to soil. The objectives of this study were: (1) to evaluate the effect of in vitro acclimatization on survival of somatic seedlings after transfer to soil; (2) to follow the rate of net photosynthesis of Picea glauca somatic seedlings during the period of in vitro acclimatization; (3) to compare light saturation curves at the end of in vitro acclimatization between acclimatized and non-acclimatized somatic seedlings, and (4) to evaluate the effects of in vitro acclimatization period on epidermal transpiration, epicuticular wax and starch accumulation.

Materials and methods

Plant materials and growth conditions

Embryogenic tissue lines originated from controlled crosses of selected white spruce genotypes by the Canadian Forest Service-Quebec Region (Beaulieu 1996). The embryogenic lines used in this study, G-305, G-369 and G-362, had been maintained by subculturing every 14 days on HLM-1 medium (Tremblay 1990) for a maximum of 6 months before maturation. Somatic embryos were matured according to Isabel et al. (1993) with 45 μM abscisic acid (ABA). After 5 weeks of maturation, normal mature somatic embryos were transferred onto Sorbarod TM plugs (Baumgartner, Papier SA, Lausanne, Switzerland) saturated with liquid Campbell and Durzan’s salts half strength (Campbell and Durzan 1975) supplemented with 1.5% sucrose (w/v) (Khlifi and Tremblay 1995). Every 2 weeks, fresh liquid medium was added, but after 6 weeks, the sucrose was removed from the medium.

In vitro acclimatization and growth conditions

Test tubes containing germinated somatic seedlings showing normal and well-developed epicotyl were transferred to the greenhouse. One-half of the somatic seedlings were submitted to a gradual acclimatization by letting increasingly greater air exchanges between the test tubes and the greenhouse during 9 days prior to transplanting. On the first day, somatic seedlings were transferred to the greenhouse operated with a day/night temperature of 25/18°C, a RH of 35–45% and a 17 h photoperiod. From days 2–5, the culture tube lids were lifted halfway in order to permit a progressive adaptation of somatic seedlings to the greenhouse environment. From days 6–8, the culture tubes were completely uncapped and somatic seedlings were directly exposed to greenhouse conditions. On day 9, the somatic seedlings were transplanted to soil as regular seedlings. The other half of somatic seedlings (non-acclimatized) had their culture tubes closed during the in vitro acclimatization period were used as a control.

Survival after in vitro acclimatization

For both acclimatized and non-acclimatized seedlings and for each clone (G-305, G-369 and G-362), more than 100 somatic seedlings were transferred to Styroblock® (Beaver Plastics Ltd, Edmonton, Canada) cavities (45 cavities per block, 340 cm³ per cavity) filled with a moistened mixture of peat and vermiculite (3/1, v/v). After 3 months, the rate of survival of acclimatized and non-acclimatized somatic seedlings was evaluated.

Net photosynthesis

Measurements of net photosynthesis (Pn) were carried out on days 1, 6, 8 and 9 of the period of in vitro acclimatization using a portable open-mode gas analyser system with a cylindrical coniferous cuvette (Model LCA-4; Analytical Development Company, Hoddesdon, UK). On each sampling day, six somatic seedlings of white spruce (line G-305) were selected randomly from both in vitro acclimatized and non-acclimatized somatic seedlings. The total needle area was measured for each sample used in gas exchange measurements, as described in details by Lamhamedi et al. (2000).

At the end of the 9-day in vitro acclimatization, the relationship between Pn and photosynthetic photon flux density (PPFD) was determined using the model described by Hanson et al. (1987). PPFD varied between 0 and 1000 μmol m⁻² s⁻¹ by varying the distance between the cuvette and light source, and by covering the cuvette with a black nylon screen or with aluminium paper (Lamhamedi et al. 1994). The three parameters (B1, B2 and B3) of this model (Eqn 1) were determined using the non-linear procedure in SAS (SAS Institute Inc., Cary, NC, USA). A Hougaard’s measure of skewness also was used to assess linearity of the estimator of each parameter (Draper and Smith 1981, Ratkowsky 1990) using multiple measurements that were arbitrarily chosen by varying photosynthetic photon flux density (PPFD).
The contribution of light to limitation of photosynthesis was calculated for both acclimatized and non-acclimatized somatic seedlings (Jones 1995) using \( (P_{n, \text{max}} - P_n)/P_{n, \text{max}} \), where \( P_{n, \text{max}} \) is the rate of net photosynthesis at light saturation, calculated as the limit of the function \( P_n = f(PPFD) \), when \( PPFD \) tends towards infinity. \( P_n \) is the assimilation rate under non-saturating light calculated at \( PPFD = 550 \, \text{mol m}^{-2} \, \text{s}^{-1} \) using empirical models developed for both acclimatized and non-acclimatized somatic seedlings.

Epidermal transpiration

At the end of the period of in vitro acclimatization, 10 somatic seedlings from both the acclimatized and non-acclimatized groups were used to estimate epidermal transpiration. Shoots of somatic seedlings (line G-305) were cut under water and allowed to fully hydrate overnight by placing their cut ends in culture tubes containing distilled water and sealed in plastic bags. Next morning the shoots were placed into home-made Plexiglas\textsuperscript{\textregistered} dessicators (56 cm long, 29 cm wide, 46 cm high). Relative humidity of 85\% ± 2\% was generated using saturated Na\textsubscript{2}CO\textsubscript{3} salt solution (Rockland 1960, Bomal and Tremblay 1999). A fan was used to maintain homogeneity of relative humidity within the dessicator. The weight loss of each sample was determined (± 0.1 mg) every 30 min for 10 h. Epidermal transpiration rates were determined by construction of curves of water loss versus time (Quisenberry et al. 1982). Theoretically, these curves should have three distinct phases: (1) an initial steep linear decline in relative water content associated with stomatal transpiration; (2) a curvilinear phase where the effects of closing stomata are important; and finally (3) a linear portion indicative of epidermal transpiration. The transition point between the curvilinear and linear regions of the curve was determined from the change in the slope and the significance of the coefficient of determination of the linear region, as suggested by Schulte and Hinckley (1985). The rate of epidermal transpiration was calculated by linear regression as the slope of water-loss over time (Snedecor and Cochran 1989). Statistical differences in epidermal transpiration between the two treatments (acclimatized versus non-acclimatized) were performed using an analysis of covariance according to Snedecor and Cochran (1989).

Tissue processing for microscopy

At the end of the period of in vitro acclimatization and for each clone (G-305, G-369 and G-362), composite samples (20–30 needles) were collected from acclimatized and non-acclimatized somatic seedlings. For each clone, the middle portions of 3–4 needles that had been selected randomly, were cut in small fragments (2 mm long), placed in a fixative solution consisting of 3\% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2. Samples were submitted to a gentle vacuum (1/2 atmosphere) for 5 h then placed overnight at 4°C. After three 20 min washes in the buffer, they were post-fixed overnight at 4°C in 1\% osmium tetroxide containing 2 mM K ferrocyanide and 6\% sucrose in cacodylate buffer. They were thereafter washed with the buffer, dehydrated in a graded series of ethanol, and embedded in Epon (Polysciences, Halifax, Canada).

Semi-thin sections were stained with 0.1\% toluidine blue and examined under a Reichert-Jung Polysar microscope (Reichert-Jung Polysar, Vienna, Austria). Photographs were taken with a Technical Pan Estar (Kodak, Toronto, Canada) film.

For transmission electron microscopy, ultra-thin sections were deposited on nickel grids and stained with uranyl acetate and lead citrate. Observations were made with an electron microscope (JEOL, Model 1200X; Jeol Tokyo, Japan).

For scanning electron microscopy, three needles from two different genotypes (G-305 and G-369) were fixed with osmium tetroxide vapours for 48 h and fastened to specimen holders with double-sided silver tape. They were thereafter coated with gold (4 × 1 min.) using a sputter-coater instrument (Semprep 2; Nanotech, Manchester, UK). Observations were carried out with a scanning electron microscope (JEOL, JSM-35; Jeol). The structure of the waxes was characterized following the classification and terminology of plant epicuticular waxes defined by Barthlott et al. (1998).

Results

Survival after in vitro acclimatization

After 9 days of in vitro acclimatization followed by transfer into soil without supplementary care, 95–100\% of these somatic seedlings (G-305, G-369 and G-362) survived. Furthermore, they resume growth immediately without any sign of transplanting shock. No sign of dessication could be observed on the needles after in vitro acclimatization. Non-acclimatized somatic seedlings dehydrated rapidly and 100\% mortality was observed.
Epidermal transpiration

Acclimatized and non-acclimatized somatic seedlings of *Picea glauca* showed different water retention curves (Fig. 1). The rates of water loss (% h\(^{-1}\)), controlled by epidermal transpiration, are indicated by the slopes of regression. Covariance analysis showed that these rates differed significantly (*P* < 0.05) among acclimatized and non-acclimatized somatic seedlings, with, respectively, 3.85 versus 4.75% h\(^{-1}\). In fact, as the needles dehydrated, acclimatized somatic seedlings showed more effective restriction of epidermal water loss than those of non-acclimatized somatic seedlings. After 8 h of transpiration, the relative water content (RWC) was higher in acclimatized somatic seedlings.

Net photosynthesis during in vitro acclimatization and photosynthetic light-response models

During 9 days of in vitro acclimatization, both acclimatized and non-acclimatized somatic seedlings showed positive values of *P\(_n\)* (Fig. 2). On days 6, 8 and 9, acclimatized somatic seedlings maintained significantly higher values of *P\(_n\)* than non-acclimatized seedlings. This difference in *P\(_n\)* was not attributed to changes in the root system since the latter was similarly well developed in both acclimatized and non-acclimatized somatic seedlings.

At the end of in vitro acclimatization, acclimatized and non-acclimatized somatic seedlings showed different fitted *P\(_n\)* responses to the PPFDs (Fig. 3). In comparison with non-acclimatized somatic seedlings, acclimatized somatic seedlings had a higher asymptotic *P\(_n\)* at saturation (6 versus 4.5 μmol m\(^{-2}\) s\(^{-1}\)), a lower dark respiration rate (−1.42 versus −1.04 μmol m\(^{-2}\) s\(^{-1}\)) and a higher compensation point (83 versus 79 μmol m\(^{-2}\) s\(^{-1}\)). In addition, in vitro acclimatization increased light use efficiency by 29% in comparison with non-acclimatized somatic seedlings (0.0190 versus 0.0147).

Light and electron microscopy

One of the main differences observed at the light microscope level between leaf samples was the accumulation of
starch grains in mesophyll cells from acclimatized somatic seedlings (Fig. 4). Starch occurred in chloroplasts as large granules which in many cases appeared to fill the cell cytoplasm (Fig. 4A, arrows). In contrast, in non-acclimatized somatic seedlings, starch grains were rare and mesophyll cells consisted of a thin layer of cytoplasm with distinct chloroplasts along the cell wall (Fig. 4B). Another difference observed between acclimatized and non-acclimatized somatic seedlings was an apparent thickening of the outermost epidermal walls in the former.

Electron microscope observations further confirmed the differences in starch distribution between the two types of samples (Fig. 5). Indeed, needles from acclimatized samples displayed chloroplasts with numerous large and electron-lucent starch grains (Fig. 5A) whereas those from non-acclimatized samples showed roundish chloroplasts that were generally devoid of starch deposits (Fig. 5B).

Under scanning electron microscopy, wax projections were much more abundant on needles from acclimatized somatic seedlings than from non-acclimatized needles (Fig. 6). On acclimatized somatic seedlings, these projections formed membraneous platelets distributed as rosettes along the needle surface and forming long threads over the stomata (Fig. 6A). In contrast, the needle surface of non-acclimatized samples appeared smoother with a few thread-like wax projections present over stomata (Fig. 6B).

Discussion

A 9-day in vitro acclimatization was sufficient to insure plant survival and growth after transfer to soil. Our acclimatization protocol induced marked physiological and anatomical changes in *Picea glauca* somatic seedlings (Figs. 1–6). In vitro acclimatized somatic
seedlings exhibited greater maximum $P_n$ rates, higher light use efficiency, accumulation of starch granules and epicuticular wax than non-acclimatized somatic seedlings. Furthermore, excised shoots of acclimatized somatic seedlings exhibited a lower epidermal transpiration than non-acclimatized somatic seedlings. The values of epidermal transpiration are indicative of the significant effect of in vitro acclimatization on the ability of somatic seedlings to adapt rapidly to new environmental conditions. It is reasonable to assume that the lower epidermal transpiration on acclimatized somatic seedlings resulted from the presence of well-developed epicuticular waxes which may retard water loss, making in vitro acclimatized somatic seedlings less susceptible to water stress after transfer to soil. A second physiological adaptation observed during in vitro acclimatization concerns the control of stomatal closure. The value of RWC at which stomata closed was significantly increased in in vitro acclimatized somatic seedlings. The high RWC observed in acclimatized somatic seedlings (Fig.1) suggests that the well-developed epicuticular waxes, shown by electron microscopy (Fig.6) might play an important role in the maintenance of plant water balance just after transfer to soil from in vitro conditions to the greenhouse. An insufficient epicuticular wax formation and its effects on epidermal transpiration observed for non-acclimatized somatic seedlings was consistent with observations reported for several species including mature trees (Baig and Tranquillini 1976, Wardle et al. 1983, Kozai et al. 1992, Ziv 1995, Kerstiens 1996a, Jenks and Ashworth 1999). However, a recent study (Lamhamedi et al. 2000) showed that after one growing season under greenhouse conditions, somatic and zygotic seedlings of white spruce showed similar epicuticular wax features.

The photosynthetic ability of both acclimatized and non-acclimatized somatic seedlings on day 1 suggests the establishment of autotrophic growth at the end of germination. The autotrophic growth was established in response to the removal of sucrose from the medium 6 weeks after beginning the germination of white spruce somatic embryos. It is well established that the presence of high concentrations of sucrose in the culture medium lead to dramatic decreases in phototrophic development and to less efficient acclimatization of plants grown under in vitro conditions (Kozai 1991, Kozai et al. 1992, Serret and Trillas 2000). Considerably less information is available regarding the photosynthetic response of coniferous somatic seedlings during in vitro acclimatization. From our results, it appears that the photosynthetic performance of acclimatized somatic seedlings can be viewed as a response to the removal of sucrose and also to increased PPFD levels at the end of in vitro acclimatization when culture tubes were completely uncapped (Fig.2). This agrees with previous results (Evers 1982, Kozai 1991, Ziv 1995, Kozai et al. 1997) showing that sucrose levels and light intensity affect net photosynthesis of in vitro plants, lower concentrations of sucrose being positively correlated with higher photosynthetic capacity. Evers (1982) measured $P_n$ of Douglas-fir in vitro plants and found that the in vitro maximum $P_n$ reached the same levels as reported for trees. In contrast, for both in vitro acclimatized and non-acclimatized $P. glauca$ somatic seedlings, the rates of $P_n$ were lower than those recorded on newly transplanted zygotic seedlings (Marsden et al. 1996). Low photosynthesis rates have also been reported for plants under in vitro conditions (Grout 1988). During the period of in vitro acclimatization, $P_n$ increased significantly (Fig.2) probably as a result of improved stomatal function due to the small vapour pressure deficit gradient between the intercellular leaf space and the saturated vessel atmosphere (Blanke and Belcher 1989).

The presence of starch grains in the chloroplasts of acclimatized somatic seedlings (Figs 4 and 5) can be directly related to current photosynthesis. Using steady-state labelling with $^{14}$C, it was demonstrated that most starch in chloroplasts of needles of 1-year-old balsam fir and 26-year-old Douglas-fir was generated from current photosynthesis (Little 1970, Webb and Kilpatrick 1993). The positive effect of current net photosynthesis on
starch accumulation in mesophyll cells of white spruce in the present study is consistent with recent studies on Gardenia jasminoides Ellis and Nicotiana tabacum L. cv. (Serret and Trillas 2000, Kadlecˇek et al. 2001). Furthermore, current photosynthesis is considered to be the critical factor in establishing root growth (van den Driessche 1987).

The progressive uncapping of the culture tubes during in vitro acclimatization simultaneously induces several changes in the seedling micro-environment, particularly the relative humidity, the temperature, the light quality and quantity and the vapour pressure deficit. From the results of this study, it seems that the formation of epicuticular waxes is induced by the decreased of RH around the seedlings during in vitro acclimatization. Accumulation of epicuticular waxes can be viewed as a rapid adaptation of boreal species to harsh environmental conditions. Grantz (1990) reported that wax deposition on guard cells and other cells of the stomatal complex was absent at high humidity, and induced at low humidity. This accumulation is also attributed to other environmental factors as well as to plant genetics (Vanhinsberg and Colombo 1990, Kerstiens 1996a, Jenks and Ashworth 1999). Epicuticular waxes increase light reflectance, reducing the energy absorbed by the needles, and consequently decreasing the temperature of needles (Jenks and Ashworth 1999). On the other hand, a possible decrease in needle temperature is also favoured by the ability of cuticles to absorb water molecules associated with the polysaccharides of the cuticular membrane (Kerstiens 1996b). Such decreases in temperature at the needle surface can be advantageous for acclimatized somatic seedlings by leading to a reduction in the rate of respiration and transpirational losses, and to more active photosynthesis. In contrast, an increase in needle temperature can arise in non-acclimatized somatic seedlings without well-developed epicuticular waxes. This situation results in a high vapour pressure gradient between the needle surface and the surrounding air, thus increasing stomatal transpiration and the possibility of water loss through the poorly developed cuticle.

In vitro acclimatization directly in culture vessels was found to improve the photosynthetic capacity of somatic seedlings by influencing the three parameters ($B_1$, $B_2$ and $B_3$) determined by empirical models. From the results of empirical models of the relation $P_{\text{g}}-\text{PPFD}$, it appears that an increase of PPFD at the end of the period of in vitro acclimatization increases the photosynthetic capacity of somatic seedlings. Our results are in agreement with those reported by Whish et al. (1992) showing that a decrease of RH during in vitro culture increased plant survival after transfer to soil. The protocol described here for in vitro acclimatization of P. glauca somatic seedlings allows a greater rate of survival (> 95%). The same protocol was applied to several thousands of seedlings in our laboratory with similar success on P. mariana, P. abies and hybrid larches (unpublished data). In vitro acclimatization induced greater accumulation of epicuticular waxes suggesting that these structural changes may be some of the strategies used by seedlings to restrict epidermal water loss and stomatal aperture. Acclimatization under in vitro conditions contributes to a reduction in the labour costs associated with the handling of non-acclimatized seedlings. These results of in vitro acclimatization will also prove useful for the production of somatic seedlings on a large scale.

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References

Baig MN, Tranquillini W (1976) Studies on upper timberline: morphology and anatomy of Norway spruce (Picea abies) and stone pine (Pinus cembra) needles from various habitat conditions. Can J Bot 54: 1622–1632

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