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ORIGINAL ARTICLE

Effects of abscisic acid and an osmoticum on the maturation, starch accumulation and germination of *Picea* spp. somatic embryos

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Abstract The aim of this study was to investigate whether ABA, sucrose or Phytagel applied to the maturation medium at different concentrations affected the growth, development and starch content of somatic embryos of *Picea abies* and *Picea omorika*. Embryogenic tissues of both spruce species were placed on maturation medium supplemented with various doses of ABA (10, 20, 40 and 80 μM), sucrose (17, 34 and 68 g/L) or Phytagel (4, 6 and 8 g/L). Our results showed that ABA and the osmoticum had a significant effect on the production and maturation of somatic embryos of both spruce species. Supplementing the medium with low concentrations of ABA (10 μM) or sucrose (17 g/L) resulted in the precocious germination of embryos. Adding sucrose to the maturation medium at the highest tested concentration (68 g/L) improved the growth of the radicles of the embryos of both spruce species during the germination stage. Moreover, the intensity of the growth of the hypocotyls and radicles of *P. omorika* germinating embryos depended on the Phytagel concentration applied to the maturation medium. The starch content and the starch accumulation pattern of *Picea abies* and *P. omorika* somatic embryos were dependent on the concentration of ABA, sucrose or Phytagel in the maturation medium. In general, during development, the *P. abies* somatic embryos accumulated a much greater amount of starch than *P. omorika* embryos did. The effects of ABA and osmoticum on the starch accumulation pattern of *Picea abies* and *P. omorika* somatic embryos are first reported in this paper.

Keywords ABA · Phytagel · Somatic embryos · Spruce · Starch · Sucrose

Abbreviations

ABA	Abscisic acid
IBA	Indolile-3-butyric acid
KOH	Potassium hydroxide
LED	Light-emitting diode
LM	Litvay medium
ME	Margara medium
Picloram	4-Amino-3,5,6-trichloropicolinic acid

Introduction

Somatic embryogenesis is the most useful micropropagation method for conifers (Bonga et al. 2010). Plant breeding using the somatic embryogenesis technique is a multistep process that requires the development of efficient protocols for each developmental stage of the somatic embryos (Garin et al. 2000; Hogberg et al. 2001; Szczygieł et al. 2007; Hazubska-Przybył et al. 2008; Lelu-Walter and Pâques 2009; Deo et al. 2010). The fundamental problems associated with this propagation method are the following:

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the loss of the embryogenic potential of the obtained cultured lines shortly after their establishment, the proper maturation of the somatic embryos and their limited ability for germination, conversion into plants and adaptability to natural conditions (Hazubska and Szczygieł 2003; Szczygieł et al. 2007; Lelu-Walter and Pâques 2009). Moreover, the somatic embryogenesis process of conifers is often highly genotype dependent, and the development of universal propagation protocols is associated with several difficulties (Kong and von Aderkas 2007; Hazubska-Przybył and Bojarczuk 2008; Bonga et al. 2010; Teixteira da Silva and Malabadi 2012). Only by overcoming these difficulties can the somatic embryogenesis method be used more widely.

Many investigations of the somatic embryogenesis of coniferous species showed that abscisic acid (ABA), osmotically active agents and gelling agents significantly impacted the maturation and germination of somatic embryos (Hakman and von Arnold 1988; Gutmann et al. 1996; Teyssier et al. 2011; Teixteira da Silva and Malabadi 2012). ABA and osmotic stress are the key factors that determine the proper maturation of somatic embryos. Both factors also play an important role in the accumulation of reserve materials, such as proteins, lipids and carbohydrates, in the developing embryos and in the synchronisation of their development during the maturation stage (Misra 1994; von Aderkas et al. 2002; Kermode 2005; Businge et al. 2013). Moreover, ABA promotes the development of somatic embryos from embryogenic tissues, and during their maturation stage, ABA triggers and enhances their tolerance to desiccation and inhibits precocious germination (Kermode 2005; Rai et al. 2011).

Sucrose is one of the osmotically active agents that is most frequently used during the maturation of conifer somatic embryos. Sucrose is a source of carbon and energy and may participate in regulating the expression of genes that affect the embryonic maturation process (Lipavská et al. 2000; Iraqi and Tremblay 2001; Iraqi et al. 2005). This compound is often added to the culture medium to improve the maturation rate of somatic embryos (Lema-Rucińska et al. 2013) and to increase the content of storage materials, such as starch and oligosaccharides, in the embryos (Kępczyńska 2006).

Sucrose participates in many of the metabolic processes of plants. Sucrose is synthesised in source tissues and is transported to sink tissues, where it is stored or utilised (Sauer 2007; Wind et al. 2010). Changes in the levels of synthesis, transport and degradation of sucrose affect the growth, development and physiology of plants (Wind et al. 2010).

Phytagel is the gelling agent that is most commonly applied in conifer embryogenic cultures (Kim et al. 1999; Klimaszewska et al. 2000). Some studies showed that supplementing culture media with Phytagel at the appropriate concentration had a positive effect on the development and growth of somatic embryos of certain coniferous species (Garin et al. 2000; Klimaszewska et al. 2000; Lelu-Walter and Pâques 2009; Teyssier et al. 2011; Morel et al. 2014).

During plant embryogenesis, reserve materials, including starch, are accumulated (Kermode 2005). The process of starch accumulation in the form of characteristic grains during the somatic embryogenesis of conifers has been described (Misra 1994; Iraqi and Tremblay 2001). This process was particularly intense after the embryogenic tissue was placed on maturation medium supplemented with ABA (Misra 1994). Starch grains began to appear in the cells of early, immature somatic embryos, and their content changed as embryonic development progressed (Gutmann et al. 1996). At the beginning of development, the number and size of the starch grains increased, whereas during the cotyledonary stage of development, the starch content gradually decreased as it was mobilised by the growing embryo (Salopek et al. 1997; Hazubska-Przybył et al. 2008).

P. abies and *P. omorika* occur naturally in Europe. Both of these spruce species are often planted in parks and gardens because of their decorative qualities. Between the mid-1980s and the early 1990s, the successful application of the somatic embryogenesis method for the micropropagation of both spruce species was achieved (Hakman et al. 1985; Chalupa 1985; Budimir and Vujičić 1992). Thereafter, *P. abies* became the model species for the study of various aspects of conifer somatic embryogenesis (Hakman and von Arnold 1988; Lipavská et al. 2000; Malá et al. 2009; Sun et al. 2011; Bříza et al. 2013; Hazubska-Przybył et al. 2013). Few studies have investigated the somatic embryogenesis of *P. omorika* (Tramisak-Milaković et al. 1999; Leljak-Levanić et al. 2009; Hazubska-Przybył et al. 2010), a more unique spruce species because of its endemic and relict character (Vujičić and Budimir 1995; Ballian et al. 2006). To date, the results of research on the propagation of both spruce species via the somatic embryogenesis technique are not entirely satisfactory; thus, this method cannot be applied on a wider scale. Therefore, there is a need to develop efficient protocols for the most highly productive in vitro micropropagation of *P. abies* and *P. omorika* using this technique.

The aim of this study was to determine the effect of ABA, sucrose and Phytagel at various concentrations on the growth, development and starch content of *Picea abies* and *P. omorika* embryos obtained via somatic embryogenesis.

Materials and methods

Embryogenic tissue origin

The somatic embryogenesis of mature zygotic embryos taken from seeds of *Picea abies* and *P. omorika* that were

collected from trees growing in the Experimental Forest 'Zwierzyniec' near Kórnik (provenance Serwy) and in the Kórnik Arboretum (52°15′N, 17°04′E), respectively, was induced. The zygotic embryos (explants) were cultured on half-strength LM medium (½ LM; Litvay et al. 1985) supplemented with 9 μM 2,4-D and 8.8 μM BA for 8 weeks to induce the production of embryogenic tissues (ETs). Subsequently, the ETs were allowed to proliferate on medium supplemented with 9 μM Picloram and 4.5 μM BA.

Maturation of the somatic embryos

To obtain somatic embryos, pieces of actively growing *Picea abies* and *P. omorika* ETs were placed on growth regulator-free ½ LM medium supplemented with 1 % activated charcoal (Sigma) and 34 g/L sucrose and were incubated for 7 days. Next, the ETs of both of the spruce species were transferred to maturation medium containing different concentrations of abscisic acid (ABA), sucrose or Phytagel and were incubated for 7 weeks.

During the first 3 weeks somatic embryos exhibited precotyledonary (from globulary to torpedo) stage. In the 5th week embryos started to reach the cotyledonary stage. In the next 2 weeks they continued their growth entering late-cotyledons stage. C refers simply to cotyledonary stage. Somatic embryos at late-cotyledons stage were taken to begin the plantlet conversion. Each developmental stage is supported by a representative picture (Supplemental Fig. 1).

Experiment I: effect of abscisic acid

Embryogenic tissues of *Picea abies* and *P. omorika* were placed on medium containing IBA (1 μM), sucrose (34 g/L), Phytagel (6 g/L) and ABA at different concentrations, namely, 10, 20, 40 and 80 μM. This experiment was repeated three times for *Picea abies* and two times for *P. omorika*. Three replicates were used per each treatment.

Experiment II: effect of sucrose

Embryogenic tissues of both spruce species were placed on medium containing IBA (1 μM), ABA (20 μM), Phytagel (6 g/L) and sucrose at different concentrations, namely, 17, 34 and 68 g/L. This experiment was repeated three times for *Picea abies* and *P. omorika*. Three replicates were used per each treatment.

Experiment III: effect of phytagel

Embryogenic tissues of *Picea abies* and *P. omorika* were placed on medium containing IBA (1 μM), ABA (20 μM) and sucrose (34 g/L) and Phytagel at different concentrations, namely, 4, 6, 8 g/L. This experiment was repeated two times for *Picea abies* and three times for *P. omorika*. Three replicates were used per each treatment.

The pH of the media was adjusted to 5.8 prior to autoclaving at 121 °C and 100 kPa for 20 min. The growth regulators (ABA and IBA-indole-3-butyric acid) and L-glutamine were filter-sterilised and were added to the media after autoclaving.

ETs were dispersed on Whatman number 2 filter papers, which were placed on the maturation media. The cultures were placed under blue and red LED light (light intensity of 35 μM m^{-2} s^{-1}; 16-h photoperiod) at 22 ± 1 °C. After 5 weeks, all produced somatic embryos from globular to cotyledonary stage (P) and cotyledonary somatic embryos (C) per 1 g of embryogenic tissue was determined. Finally, the percentage of somatic embryos capable of maturation was assessed. Three Petri dishes each (90-mm diameter) containing 200 mg of embryogenic tissue were used per variant.

Analysis of starch content

The starch content of ETs (zero treatment) was determined before transferring them to maturation medium and that of the somatic embryos was determined at the precotyledonary (3 weeks of cultivation on maturation medium), cotyledonary (5 weeks of cultivation) and late-cotyledonary (7 weeks of cultivation) stages. The latter embryos had the ability to conduct photosynthesis due to the presence of chlorophyll. Somatic embryos subjected to each treatment were collected for analysis.

The starch content was determined according to the method of Huber and Israel (1982). ETs and somatic embryos were both extracted using 80 % ethanol. The pellet obtained by centrifugation (at 4 °C, 15,000*g* for 20 min) was suspended in 2 ml of 0.2 N KOH and placed in boiling water for 30 min. After cooling, the pH of the mixture was adjusted to 5.5 using 1 M acetic acid. An equal volume of a solution of amyloglucosidase (400 units/ml in 9.1 M citrate buffer, pH 5.5) was added. After an incubation (at 45 °C for 4 h), the glucose concentration of the supernatant was determined according to the modified method of Somogyi (1952).

Germination of the somatic embryos

Cotyledonary somatic embryos of each variant of both of the tested spruce species obtained in three above-described experiments were removed from the ½ LM at the 5th week of culture and were transferred to ME medium (Margara 1977) lacking growth regulators but supplemented with sucrose (20 g/L) to allow germination. They were cultured for 2 weeks in darkness (first phase) and then, for the next

2 weeks, in blue and red LED light (second phase; light intensity and photoperiod as above) at 22 ± 1 °C. After each phase, the length of the hypocotyls and radicles was measured to determine the germination capacity of the somatic embryos. Three Petri dishes containing 60 embryos per variant were used to test the germination capacity. Each experiment was repeated twice. Somatic embryos were treated as germinated when the radical started to protrude (Kępczyńska 2006).

Statistical analysis

The number of produced (P) and cotyledonary (C) somatic embryos and the percentage of mature embryos (calculated as the number of cotyledonary embryos/all produced embryos on the maturation medium × 100) were analysed using a one-way ANOVA and a post hoc Tukey test ($p < 0.05$). Bliss transformation of the maturation frequency data was performed before the statistical analysis was conducted. The data concerning the germination of somatic embryos and the starch content were analysed using the Kruskal–Wallis and Dunn's tests ($p < 0.05$). The homogeneity of the variances was verified using Levene's test (STATISTICA software, StatSoft Polska, Kraków, 1995–2005).

Results

Maturation of the somatic embryos

Experiment I: effect of abscisic acid

The ABA concentration in the maturation medium had no effect on the production of precotyledonary somatic embryos of *P. abies*. In contrast, the ABA concentration affected the number of *P. abies* embryos observed at the cotyledonary (C) stage (Table 1). The application of 40 µM ABA was most beneficial for the formation of embryos at the cotyledonary stage. In the case of *P. omorika*, the ABA concentration had a significant effect on both the production and maturation of somatic embryos. The least favourable concentration of ABA was 40 µM. The most effective results were obtained using 10 µM ABA (78 P/g ET and 50 C/g ET).

ABA treatment during the maturation period affected the ability of the immature somatic embryos of *P. abies* to reach the cotyledonary stage. The highest maturation frequency (60 %) accompanied by the highest number of produced (506 P/g ET) and cotyledonary-stage (327 C/g ET) embryos was observed when 40 µM ABA was applied (Table 1). Significant differences in the maturation frequency of the *P. omorika* embryos were also observed. The best results (64 %) were obtained using the lowest concentration of ABA (10 µM) (Table 1). However, during the experiment, we observed that the somatic embryos of both spruce species showed a tendency to precocious germination when matured on the lowest dose of ABA (data not shown). Increasing the dose of ABA to 20 µM (Control) inhibited the precocious germination of the somatic embryos and led to a slight decrease in the number of somatic embryos that reached the cotyledonary stage of development (Table 1).

Experiment II: effect of sucrose

The presence of sucrose in the maturation medium at concentrations of 17, 34 or 68 g/L significantly affected the number of obtained somatic embryos of both spruce species (Table 2). The highest number of *P. abies* somatic embryos (463 P/g ET) was obtained from ETs cultured in the presence of sucrose at 34 g/L. Additionally, the highest number of cotyledonary-stage embryos (185 C/g ET) was observed on this medium. The sucrose concentration in the maturation medium did not affect the ability of P. *abies* somatic embryos to undergo maturation, with the average maturation frequency being approximately 43 % independent of the sucrose concentration. In contrast, the highest number of *P. omorika* somatic embryos (272 P/g ET and 220 C/g ET) was observed upon applying sucrose at 17 g/L. Furthermore, in this spruce species, the sucrose concentration had a significant effect on the maturation frequency (Table 2). The highest maturation frequency (81 %) was observed when 17 g/L sucrose was used, and the lowest maturation frequency (49 %) was observed when sucrose was used at 68 g/L. Unfortunately, the somatic embryos that matured on medium containing the lowest concentration of sucrose (17 g/L) had a tendency towards precocious germination. This concentration of sucrose also proved to be unfavourable for the proper development of *P. abies* somatic embryos, which germinated too early.

Experiment III: effect of Phytagel

The application of Phytagel at three different concentrations had a significant effect on the ability of *P. abies* ETs to produce somatic embryos (Table 3). The highest number of *P. abies* somatic embryos (430 P/g ET) was observed on maturation medium supplemented with Phytagel at 4 g/L, and the highest number of *P. omorika* somatic embryos (355 P/g ET) was observed on medium supplemented with Phytagel at 6 g/L. The number of cotyledonary somatic embryos produced was also the highest using these two concentrations of Phytagel. In both cases, the Phytagel dose had a significant effect on the number of cotyledonary

Table 1 Effect of ABA supplied at various concentrations in ½ LM medium on the production and maturation of *Picea abies* and *P. omorika* somatic embryos

Species	ABA concentration (μM)	Number of somatic embryos[a]		Maturation frequency (%)
		Different stages of embryos	C	
Picea abies	10	424 ± 40.6 a	214 ± 32.6 ab	50 ± 5.2 ab
	20[b]	429 ± 36.3 a	212 ± 44.0 ab	47 ± 7.1 ab
	40	506 ± 57.9 a	327 ± 74.0 a	60 ± 8.4 a
	80	456 ± 40.1 a	177 ± 21.1 b	41 ± 5.8 b
P. omorika	10	78 ± 7.2 a	50 ± 0.5 a	64 ± 2.4 a
	20[b]	55 ± 12.1 b	32 ± 9.2 b	53 ± 7.3 b
	40	27 ± 10.5 c	9 ± 2.8 d	34 ± 8.5 c
	80	57 ± 17.5 ab	18 ± 4.1 c	37 ± 6.2 c

The mean values ($n = 9$ for *P. abies* and $n = 6$ for *P. omorika*, ±SE) indicated with the same letters were not significantly different at $p < 0.05$. Different stages of embryo include globular, torpedo and cotyledonary stage embryos. C refers to somatic embryos at the cotyledonary stage

[a] Per 1 g of fresh weight of ET

[b] Control

Table 2 Effect of sucrose at various concentrations in ½ LM medium on the production and maturation of *Picea abies* and *P. omorika* somatic embryos

Species	Sucrose concentration (g/L)	Number of somatic embryos[a]		Maturation frequency (%)
		Different stages of embryos	C	
Picea abies	17	403 ± 85.3 ab	180 ± 41.0 a	43 ± 2.7 a
	34	463 ± 52.7 a	185 ± 25.6 a	41 ± 2.9 a
	68	321 ± 55.7 b	160 ± 36.3 a	46 ± 3.5 a
P. omorika	17	272 ± 35.3 a	220 ± 28.8 a	81 ± 1.8 a
	34	198 ± 49.5 ab	154 ± 39.7 a	79 ± 3.5 a
	68	105 ± 25.1 b	67 ± 17.3 b	49 ± 10.3 b

The mean values ($n = 9$, ±SE) indicated with the same letters were not significantly different at $p < 0.05$. Different stages of embryo include globular, torpedo and cotyledonary stage embryos. C refers to somatic embryos at the cotyledonary stage

[a] Per 1 g of fresh weight of ET

somatic embryos. Significant differences in the maturation frequency of both spruce species were observed in the presence of different concentrations of Phytagel; the addition of 6 g/L Phytagel resulted in the highest maturation frequency.

Analysis of starch content

Experiment I: effect of abscisic acid

The concentration of ABA in the maturation medium significantly and differentially affected the starch accumulation patterns of somatic embryos of *P. abies* and *P. omorika*. The fluctuation in the starch content of maturing and mature *P. abies* embryos was milder than that of *P. omorika* embryos. The starch content of the majority of 3-, 5- and 7-week-old somatic embryos was nearly 2-fold higher than that of the embryogenic tissues (Fig. 1). It was not possible to determine one specific concentration of ABA that led to the highest rate of starch accumulation; however, the older were the *P. omorika* embryos, the higher was the ABA concentration needed for the acquisition of the highest starch content. The differences between the starch contents of embryos at different stages of their development that had been treated with ABA at various concentrations were small.

The pattern of starch accumulation in the somatic embryos of *P. omorika* was more dependent on the concentration of ABA in the medium. The precotyledonary-stage embryos had the highest starch content of the embryos treated with 20 μM ABA, which was more than 2-fold higher than that of the ETs (Fig. 1). The cotyledonary-stage somatic embryos had the highest starch content (more than 3-fold higher than that of the ETs) among

Table 3 Effect of Phytagel at various concentrations in ½ LM medium on the production and maturation of *Picea abies* and *P. omorika* somatic embryos

Species	Phytagel concentration (g/L)	Number of somatic embryos[a]		Maturation frequency (%)
		Different stages of embryos	C	
Picea abies	4	430 ± 17.4 a	239 ± 16.2 a	56 ± 2.9 b
	6	268 ± 49.7 b	181 ± 37.6 b	67 ± 3.7 a
	8	334 ± 27.8 b	206 ± 18.8 ab	62 ± 1.5 ab
P. omorika	4	308 ± 41.1 a	122 ± 22.4 b	56 ± 1.1 b
	6	355 ± 51.7 a	228 ± 33.7 a	65 ± 4.6 a
	8	323 ± 26.1 a	201 ± 22.8 a	61 ± 3.7 a

The mean values ($n = 6$ for *P. abies* and $n = 9$ for *P. omorika*, ±SE) indicted with the same letters were not significantly different at $p < 0.05$. Different stages of embryo include globular, torpedo and cotyledonary stage embryos. C refers to somatic embryos at the cotyledonary stage

[a] Per 1 g of fresh weight of ET

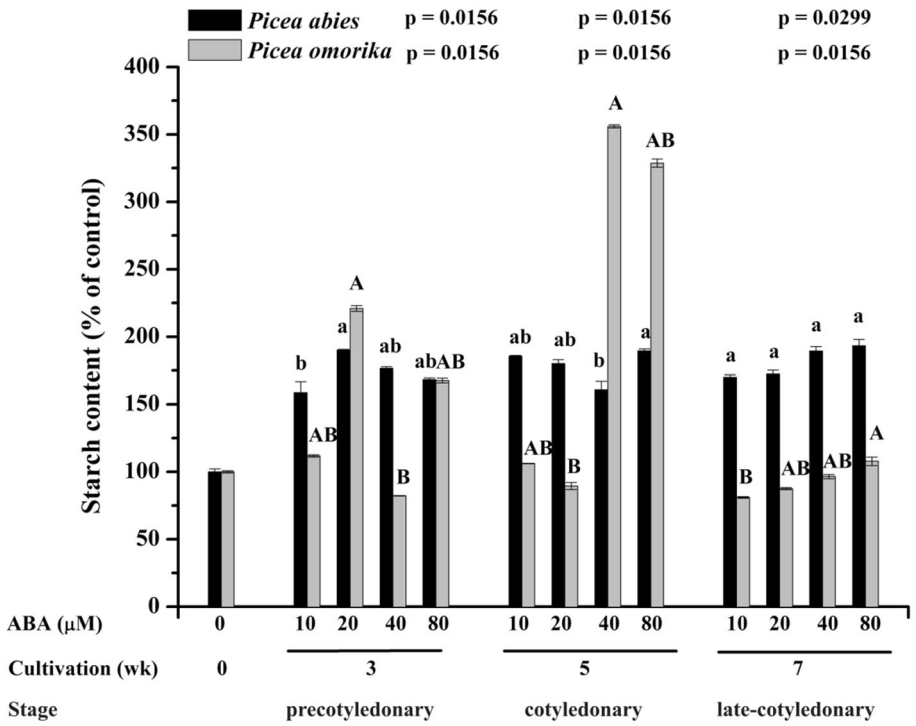

Fig. 1 Starch content of embryogenic tissues (0 weeks of cultivation, not treated with ABA) and somatic embryos (3, 5 and 7 weeks of cultivation) of *Picea abies* and *P. omorika* during their maturation in the presence of ABA at different concentrations. *Upper and lower case letters* relate to *P. omorika* and *P. abies*, respectively. *Bars* are mean ± standard error (SE). Means followed by *same letters* in the *bar* are not significantly different ($P = 0.05$) using Kruskal–Wallis and Dunn's tests

the embryos treated with 40 μM ABA. In the late cotyledonary-stage embryos, the starch content was reduced and was slightly lower than that of the ETs. Finally, embryos treated with 10 μM ABA exhibited similar starch levels from 0 to 7 weeks of cultivation.

Experiment II: effect of sucrose

The concentration of sucrose applied to the maturation medium significantly affected the starch-accumulation ability of *P. abies* somatic embryos at 3 and 5 weeks of cultivation and of *P. omorika* somatic embryos at 5 and 7 weeks of cultivation (Fig. 2). Comparing two species at each treatment, *P. abies* somatic embryos accumulated significantly more starch than the *P. omorika* somatic embryos, with the exception of the 3-week-old embryos of both species, in which the starch contents were equivalent (Fig. 2). Greatly increased starch accumulation occurred in both *P. abies* and *P. omorika* embryos that matured in the presence of sucrose at a concentration of more than 17 g/L. The highest starch content, which was approximately 28-fold higher than that of the ETs, was observed in the cotyledonary-stage *P. abies* somatic embryos that had developed in the presence of 68 g/L of sucrose. In the late

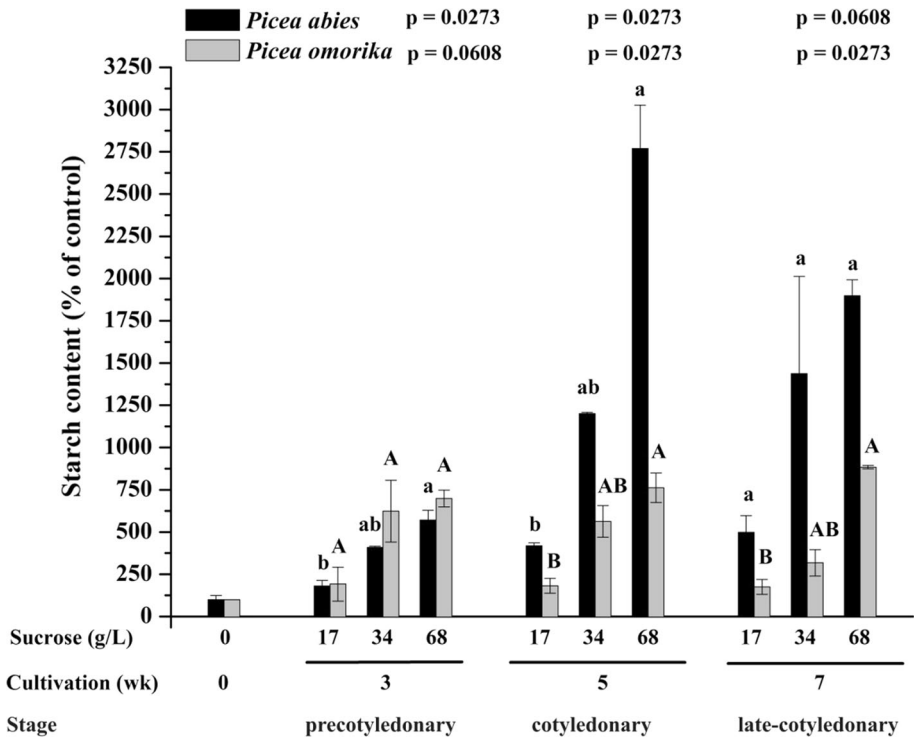

Fig. 2 Starch content of embryogenic tissues (0 weeks of cultivation, not treated with sucrose) and somatic embryos (3, 5 and 7 weeks of cultivation) of *Picea abies* and *P. omorika* during maturation in the presence of sucrose at different concentrations. *Upper and lower case letters* relate to *P. omorika* and *P. abies*, respectively. *Bars* are mean ± standard error (SE). Means followed by *same letters* in the *bar* are not significantly different ($P = 0.05$) using Kruskal–Wallis and Dunn's tests

cotyledonary-stage embryos, this value was 1.5 times lower.

Notable changes in the starch content were observed when the *P. omorika* somatic embryos were treated with sucrose at 68 g/L during maturation. At the 3rd week of culture, the starch content of the somatic embryos was 7-fold higher than that of the ETs, and it increased continuously up to the 7th week of culture (Fig. 2).

Experiment III: effect of phytagel

The pattern of starch accumulation in the 3-, 5- and 7-week-old somatic embryos of the two spruce species varied depending on the dose of Phytagel applied to the maturation medium. The starch content of the developing somatic embryos was significantly higher than that of the ETs (Fig. 3). In 3-week-old *P. abies* somatic embryos grown in the presence of Phytagel at 6 g/L, the starch content was found to be more than 3-fold higher than that of the ETs. In the following weeks, the starch content of these embryos decreased. However, in the embryos that were incubated on medium supplemented with Phytagel at 4 or 8 g/L, the starch content was further increased at the 5th week of culture and was decreased at the 7th week.

P. omorika somatic embryos contained the greatest amount of starch at the precotyledonary stage (Fig. 3). Later, in the majority of the embryos, the starch content continuously decreased with development or did not change (7th week, 8 g/L Phytagel).

Germination of the somatic embryos

Experiment I: effect of abscisic acid

Germination of *P. abies* somatic embryos was conducted for 2 weeks in darkness (first phase) and for the next 2 weeks in light (second phase). The most intensive hypocotyl and radicle growth was observed in the presence of 80 µM ABA (Table 4). During the second phase of germination, the hypocotyls and radicles extended for 15.1 mm and 5.3 mm, respectively. The other ABA concentration did not significantly affect the growth of the hypocotyls and radicles of *P. abies* somatic embryos during either phase of germination. Similarly, the hypocotyl to radicle (H:R) length ratio was not significantly altered by the tested concentrations of ABA. The best synchronisation of hypocotyl and radicle growth was obtained using 20 µM ABA (Control-0), in which the H:R ratio was 2.6. For technical reasons, this experiment was not conducted using *P. omorika* somatic embryos.

Experiment II: effect of sucrose

Applying sucrose to the maturation medium proved to significantly affect the growth of the radicles, particularly during the second phase of germination of *P. abies* somatic embryos. The presence of sucrose at 68 g/L led to the radicles growing up to an average of 7.9 mm (Table 5). In contrast, the growth of the hypocotyls in each sucrose-dose

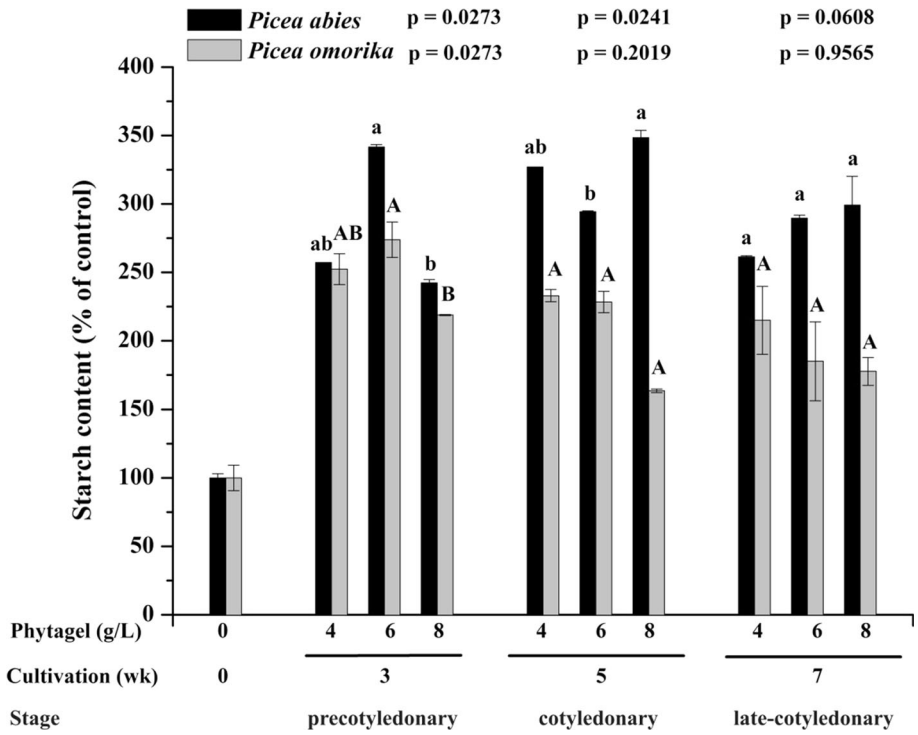

Fig. 3 Starch content of embryogenic tissues (0 weeks of cultivation, not treated with Phytagel) and somatic embryos (3, 5 and 7 weeks of cultivation) of *Picea abies* and *P. omorika* during maturation in the presence of Phytagel at different concentrations. *Upper and lower case letters* relate to *P. omorika* and *P. abies*, respectively. *Bars* are mean ± standard error (SE). Means followed by *same letters* in the *bar* are not significantly different ($P = 0.05$) using Kruskal–Wallis and Dunn's tests

Table 4 Effect of ABA at various concentrations in the maturation medium on the length of the hypocotyls (*H*) and radicles (*R*) of germinating *Picea abies* somatic embryos at 2 and 4 weeks of culture

ABA concentration (μM)	Darkness (2 weeks)		Light (next 2 weeks)		*H:R*
	Length (mm)				
	H	*R*	*H*	*R*	
10	12.6 ± 0.2 a	3.0 ± 0.2 a	14.1 ± 0.1 a	3.8 ± 0.1 a	3.7 ± 0.1 a
20	11.1 ± 1.8 a	4.1 ± 0.1 a	12.4 ± 2.0 a	4.7 ± 0.0 a	2.6 ± 0.4 a
40	10.2 ± 0.6 a	3.0 ± 0.8 a	11.2 ± 0.6 a	3.8 ± 1.1 a	3.5 ± 1.0 a
80	13.7 ± 0.4 a	4.4 ± 0.4 a	15.1 ± 0.3 a	5.3 ± 0.5 a	2.9 ± 0.2 a
p value	0.1473	0.1952	0.1462	0.1450	0.2362

The mean values in the columns are not significantly different at $p < 0.05$

treatment was comparable (13.5 mm on average). Somatic embryos matured in the presence of 68 g/L of sucrose were characterised by better synchronisation of the hypocotyl and radicle growth compared with that of embryos matured in the presence of lower concentrations of sucrose.

Significant differences in the radicle growth of germinating *P. omorika* somatic embryos were observed after growth in darkness and light. Embryos that matured on medium supplemented with 68 g/L of sucrose produced longer radicles during both phases of germination than did embryos treated with sucrose at lower concentrations (Table 5). The growth of somatic embryos treated with 34 g/L of sucrose was perfectly synchronised because the H:R ratio was 1.0. Applying sucrose at other concentrations led to much more intense growth of the hypocotyls than of the radicles.

Experiment III: effect of Phytagel

The Phytagel dose in the maturation medium did not significantly affect the dynamics of germination of *P. abies* somatic embryos (Table 6). Despite some differences in the growth of hypocotyls and radicles of individual variants during the first phase of germination of the somatic embryos, during the second phase, the growth of the hypocotyls and radicles was very similar regardless of the Phytagel dose applied. The approximately 3-fold faster growth rate of the hypocotyls led to poor growth synchronisation.

Differences between hypocotyl and radicle growth were observed during both phases of germination of the *P. omorika* embryos. Somatic embryos treated with 6 or 8 g/L of Phytagel produced longer radicles during the first

Table 5 Effect of sucrose at various concentrations in the maturation medium on the length of the hypocotyls (*H*) and radicles (*R*) of germinating somatic embryos of *Picea abies* and *P. omorika* at 2 and 4 weeks of culture

Species	Sucrose concentration (g/L)	Darkness (2 weeks)		Light (next 2 weeks)		*H:R*
		Length (mm)				
		H	*R*	*H*	*R*	
Picea abies	17	11.1 ± 0.7 a	2.6 ± 0.1 a	13.2 ± 1.0 a	4.0 ± 0.2 b	3.3 ± 0.2 a
	34	12.1 ± 0.2 a	2.9 ± 0.2 a	13.9 ± 0.1 a	5.0 ± 0.5 ab	2.8 ± 0.3 ab
	68	11.8 ± 0.2 a	4.6 ± 0.3 a	13.8 ± 0.3 a	7.9 ± 2.0 a	1.9 ± 0.4 b
	p value	0.2522	0.0549	0.7390	0.0380*	0.0439*
P. omorika	17	4.9 ± 0.8 a	2.6 ± 0.2 b	5.2 ± 0.8 a	3.2 ± 0.5 b	1.8 ± 0.5 a
	34	4.1 ± 0.3 a	3.4 ± 0.1 ab	4.5 ± 0.3 a	4.4 ± 0.1 ab	1.0 ± 0.1 a
	68	9.7 ± 0.4 a	4.3 ± 0.2 a	10.9 ± 0.4 a	5.1 ± 0.2 a	2.1 ± 0.1 a
	p value	0.0594	0.0265*	0.0608	0.0265*	0.2463

Within each species, the mean values in the columns were significantly different at $p < 0.05$ (*) or were not significantly different

Table 6 Effect of Phytagel at various concentrations in the maturation medium on the length of the hypocotyls (*H*) and radicles (*R*) of germinating somatic embryos of *Picea abies* and *P. omorika* at 2 and 4 weeks of culture

Species	Phytagel concentration (g/L)	Darkness (2 weeks)		Light (next 2 weeks)		*H:R*
		Length (mm)				
		H	*R*	*H*	*R*	
Picea abies	4	12.2 ± 0.3 a	3.9 ± 0.6 a	14.4 ± 0.3 a	5.5 ± 0.8 a	2.8 ± 0.5 a
	6	12.9 ± 0.4 a	3.9 ± 0.2 a	14.7 ± 0.6 a	5.3 ± 0.4 a	2.8 ± 0.2 a
	8	12.3 ± 0.1 a	4.2 ± 0.1 a	14.1 ± 0.3 a	5.2 ± 0.0 a	2.7 ± 0.1 a
	p value	0.4937	0.5814	0.6583	0.7066	0.6658
P. omorika	4	4.0 ± 0.1 a	1.4 ± 0.1 a	4.7 ± 0.2 a	2.7 ± 0.8 a	2.1 ± 0.5 a
	6	3.0 ± 0.2 b	1.8 ± 0.1 a	3.5 ± 0.2 b	3.0 ± 0.5 a	1.2 ± 0.2 a
	8	3.6 ± 0.1 ab	1.8 ± 0.1 a	3.9 ± 0.1 ab	2.6 ± 0.2 a	1.5 ± 0.1 a
	p value	0.0382*	0.0484*	0.0428*	0.8645	0.3123

Within each species, the mean values in the columns were significantly different at $p < 0.05$ (*) or were not significantly different

phase of germination than did embryos treated with the lowest concentration of Phytagel, although the differences were not significant (Table 6). During the second phase of germination, the longest radicles were detected in embryos that had matured in the presence of 6 g/L of Phytagel, indicating good synchronisation of the growth of both plant organs.

The somatic embryos of the two spruce species clearly differed in terms of their development. The *P. omorika* embryos were characterised by more synchronised hypocotyl and radicle growth than that of *P. abies* embryos. However, the lengths of the hypocotyls and radicle of these germinating embryos generally did not exceed 10 mm and 5 mm, respectively, in contrast to the those of the germinating *P. abies* somatic embryos (Tables 5, 6), in which the growth of these plant organs was up to 3 times more extensive.

Discussion

The development of somatic embryos of various coniferous tree species is affected mainly by the concentrations of ABA, sucrose or Phytagel (osmoticum) in the maturation medium (Klimaszewska et al. 2000; Iraqi and Tremblay 2001; Teixteira da Silva and Malabadi 2012; Nolan et al. 2014). The concentrations of these components may also be crucial for the further development of the embryos into plants.

Our research showed that the concentration of ABA or sucrose had a significant effect on the production and

maturation of *P. omorika* somatic embryos. In *P. abies* somatic embryos, this effect was less substantial. The difference in the response of the embryos to the ABA concentration may arise from the greater sensitivity of *P. omorika* embryos to the presence of this plant hormone in the maturation medium or from different levels of endogenous ABA in the somatic embryos of the two spruce species. The highest number of *P. omorika* somatic embryos was obtained when the lowest concentration of ABA (10 μM) or sucrose (17 g/L) were applied to the maturation medium. Furthermore, the application of ABA or sucrose at these concentrations contributed to the precocious germination of both *P. omorika* and *P. abies* somatic embryos. Most of these embryos had developed hypocotyls, cotyledons and radicles as soon as 3 weeks of cultivation in the presence of 10 μM ABA or 17 g/L sucrose (data not shown). Moreover, chloroplasts were observed in the hypocotyls and cotyledons of these embryos. In contrast, higher concentrations of these components prevented the precocious germination of the somatic embryos of both spruce species (data not shown). According to the data in the literature, ABA was added to the medium at concentrations ranging from 1 to 80 μM to obtain properly developed somatic embryos of conifers (Gjuleva and von Arnold 1999; Garin et al. 2000; Klimaszewska et al. 2000; Iraqi and Tremblay 2001). Harry and Thorpe (1991) applied 40 μM ABA to avoid the precocious germination of *Picea rubens* somatic embryos. In contrast, *Picea glauca* and *P. mariana* somatic embryos did not exhibit a tendency towards precocious germination when 12 μM ABA was applied to the maturation medium (Attree et al. 1990). The sensitivity to ABA differs within the *Picea* genus and differs even more strongly for more distant species. For example, applying ABA at low concentrations (0–1 μM) stimulated the elongation of *Copiapoa tenuissima* Ritt. forma *mostruosa* embryos during the globular stage, whereas applying ABA at high concentrations (10–100 μM) inhibited their growth (Lema-Rumińska et al. 2013).

Similar to the case for ABA, the concentration of sucrose affected the proper development of the somatic embryos. Applying the highest concentration of sucrose (68 g/L), decreased the rates of production and maturation of somatic embryos, particularly *P. omorika* embryos compared with the results obtained using the lowest sucrose concentration (17 g/L). Despite the similar maturation frequency of embryos of both spruce species, the precocious germination of somatic embryos did not occur upon applying sucrose at 68 g/L. The optimal sucrose concentration in the maturation medium of *Picea* sp. was reported to be 30 g/L (von Arnold and Hakman 1988; Hakman and von Arnold 1988; Attree et al. 1991). Our results confirmed this finding because optimal development and production of *P. abies* and *P. omorika* somatic embryos occurred upon applying 34 g/L of sucrose. Iraqi and Tremblay (2001) demonstrated that 6 % sucrose positively affected the maturation of *Picea mariana* and *P. glauca* somatic embryos by increasing the levels of soluble and insoluble proteins and contributing to the development of more epicotyls. The effect of the sucrose concentration on the maturation of somatic embryos depended mainly on the genotype of the cell line (Garin et al. 2000). For example, applying higher concentrations of sucrose (263 and 350 mM) increased the number of *Pinus strobus* somatic embryos of one line, whereas the number of embryos of the second line was reduced.

In vitro developmental cell fate is governed by factors such as the genetic composition, stress and plant-growth regulators (Lema-Rumińska et al. 2013). Hoth et al. (2002) identified 1354 *Arabidopsis thaliana* genes that were either up- or down-regulated following ABA treatment (Hoth et al. 2002). Subsequently, Akihiro et al. (2006) identified 27 genes in rice cultures, including starch biosynthesis-related genes, for which expression was induced by combined sucrose and ABA treatment, highlighting the importance of establishing the sucrose and ABA concentrations required for optimal embryonic development, maturation and germination. Moreover, the sugar/osmoticum levels modulate the abscisic acid-independent expression of stress-responsive genes (Déjardin et al. 1999). Chromatin is assumed to integrate stress, hormonal, and developmental pathways, leading to the activation of the embryogenic programme (Fehér 2014). Light-mediated transcriptomic changes might explain the inhibition of hypocotyl growth and the subsequent deficiency of radicle and hypocotyl growth synchronisation because the expression of the chromatin remodelling factor ENHANCED PHOTOMORPHOGENIC1, previously known as PICKLE, which is a repressor of photomorphogenesis in *A. thaliana*, was specifically repressed in the hypocotyls by light exposure (Jing et al. 2013).

The Phytagel concentration in the maturation medium affected the production of somatic embryos of *Picea abies* and the maturation of both of the tested spruce species. The highest maturation frequency was obtained upon applying 6 g/L of Phytagel. Some reports suggested that increasing the concentration of a gelling agent from 4 to 8 g/L (Teyssier et al. 2011) or 9 g/L (Morel et al. 2014) promoted the maturation of the somatic embryos of some conifers. Garin et al. (2000) demonstrated that the Phytagel concentration was critical for the maturation of somatic embryos of five tested embryogenic lines of *Pinus strobus*. The authors obtained a higher efficiency of embryonic maturation when Phytagel was applied at 1.0 % compared with 0.6 %. A similar effect was observed by Klimaszewska et al. (2007) for various pine species when

using Phytagel at 12 g/L and by Lelu-Walter and Pâques (2009) for a hybrid larch when using Phytagel at 8 g/L. Increasing the concentration of Phytagel in the maturation media allowed these authors to obtain well-developed somatic embryos capable of germination. High concentrations of gellan gum in the maturation medium were also required for somatic embryos of *Pinus pinaster* to reach the cotyledonary developmental stage (Morel et al. 2014). Such a phenomenon was not observed in the two *Picea* species that we tested. The Phytagel concentration might modulate the endogenous ABA content in maturing somatic embryos. Pine embryos maturing in the presence of 9 g/L Phytagel contained more endogenous ABA compared with that of embryos incubated in the presence of 4 g/L Phytagel (Morel et al. 2014). Therefore, a Phytagel-induced increase in the endogenous ABA level may explain why the hypocotyls of *P. omorika* embryos were the longest specifically when the lowest concentration of Phytagel was used.

Analysis of the starch content of the somatic embryos of *P. abies* and *P. omorika* showed that the ABA concentration significantly affected their starch-accumulation pattern during various stages of embryonic development. Notable variations in the starch content were observed particularly in 5-week-old *P. omorika* somatic embryos that matured in the presence of high concentrations (40 or 80 μM) of ABA. In these embryos, the starch content was more than 3-fold higher than that of embryos treated with the lower concentrations of ABA (10 and 20 μM). However, 2 weeks later, embryos treated with the lower or higher concentration of ABA had similar starch contents. Most likely, during this period, the accumulated starch was rapidly hydrolysed for the further development of the embryos. The effect of the ABA concentrations on the starch contents was more pronounced in *P. omorika* embryos, similar to the relation between the ABA concentration and the formation and maturation of embryos. These results suggested that *P. omorika* somatic embryos were definitely more sensitive to the high concentration of ABA than were *P. abies* embryos. ABA did not affect the starch accumulation of the somatic embryos of *Larix* x *leptoeuropaea* (von Aderkas et al. 2002), whereas the starch accumulation of somatic embryos of *Camellia sinensis* increased according to the concentration of ABA applied (Sharma et al. 2004).

The higher concentrations of sucrose in the medium supported starch storage by both *P. abies* and *P. omorika* developing somatic embryos; the higher the concentration of sucrose used, the higher the level of starch in the somatic embryos, irrespective of their developmental stage. Iraqi et al. (2005) also observed a higher rate of starch accumulation in tissues of *Picea mariana* maturing on 3 and 6 % sucrose-containing media than in tissues matured on a medium containing 1 % sucrose. Additionally, Businge et al. (2013) found that *Picea abies* somatic embryos treated with 3 % sucrose contained a high level of sucrose and other water stress-related compounds, including raffinose and late embryogenesis abundant protein. Increased starch accumulation was reported when greater sucrose concentrations were added to the maturation medium. The highest starch content was observed when *P. abies* were grown on medium containing 68 g/L of sucrose. Businge et al. (2013) observed that the starch content of *P. abies* somatic embryos cultivated on 3 % sucrose ranged from 160 to 220 mg/g DW, depending on the cell line. In our experiments, the starch content of *P. abies* somatic embryos cultivated with 34 g/L of sucrose reached a maximum of 40 mg/g FW (data not shown). In contrast, their starch content was 120–125 mg/g FW when the osmoticum was 4–8 g/L of Phytagel (data not shown).

P. abies somatic embryos accumulated much more starch than did *P. omorika* embryos during maturation at the same concentration of sucrose. In our opinion, this result may be due to the lower demand of *P. omorika* somatic embryos for this type of storage material compared with that of *P. abies* somatic embryos. Despite this consideration, our results suggested that the concentration of sucrose in the medium affected the dynamics of the accumulation and hydrolysis of starch during the development of somatic embryos of both spruce species. For example, *Picea abies* somatic embryos maturing on medium supplemented with sucrose at 17 or 34 g/L accumulated starch continuously from 3 to 7 weeks of culture, whereas embryos maturing on medium supplemented with 68 g/L of sucrose began to hydrolyse starch after 5 weeks of culture. These results are in perfect agreement with the findings of Lipavská et al. (2000) that in *P. abies* somatic embryos, the starch content increased during approximately 5–6 weeks of cultivation on maturation medium and then, the starch content clearly decreased during weeks 7–8 of cultivation. Compared with this pattern, the pattern of accumulation and mobilisation of starch in *P. abies* somatic embryos that matured in the presence of 68 g/L of sucrose appeared to be more appropriate for embryonic development. This hypothesis was confirmed by our observation that during the germination of *P. abies* somatic embryos, the embryos derived from the cultures supplemented with 68 g/L of sucrose produced better developed roots and were characterised by the improved synchronisation of the hypocotyl and root growth compared with those of embryos that matured at a lower concentration of sucrose.

Our study showed that manipulating the osmoticum in the medium through applying various concentrations of Phytagel also affected starch accumulation in the cells of maturing somatic embryos of both spruce species. For

example, *P. omorika* somatic embryos contained much more starch when matured on medium containing Phytagel at the lower concentrations (4 or 6 g/L). At the highest concentration of Phytagel (8 g/L), the starch-accumulation process was inhibited. This result suggested that too high a level of an osmoticum in the medium limited the transport of sucrose from the medium to the somatic embryo, and therefore, the level of starch reserves in the developing embryos was reduced.

The *P. abies* somatic embryos accumulated significantly more starch than did the *P. omorika* embryos matured in the presence of the same concentrations of Phytagel. This finding is the same as was observed in the case of embryos matured in the presence of various concentrations of sucrose. Recent research has shown that the concentration of Phytagel significantly affected the maturation of the somatic embryos of some conifer species (Garin et al. 2000; Klimaszewska et al. 2007; Lelu-Walter and Pâques 2009; Teyssier et al. 2011). However, the published literature did not address whether this component of the maturation medium affected starch accumulation in the somatic embryos. Our results provide additional knowledge about the effect of the Phytagel concentration on the physiology of maturation of conifer somatic embryos.

Some authors reported that ABA or osmotic-stress treatment during the maturation of conifer somatic embryos affected their ability for further development (Hogberg et al. 2001). In our study, we did not observe a stimulatory or inhibitory effect of ABA on the germination of *P. abies* somatic embryos (the experiment was not conducted using *P. omorika* somatic embryos). However, in the case of osmotic-stress treatment, we noticed that the concentration of both sucrose and Phytagel in the maturation medium had an effect, in some cases, on the germination of *Picea abies* and *P. omorika* somatic embryos. Although in our experiments, the presence of sucrose at the highest concentration in the medium reduced the abilities of the tested line for the production and maturation of somatic embryos, during germination, sucrose at the highest concentration improved the radicle growth of embryos of both spruce species. We observed that the growth of hypocotyls and radicles of germinating *P. omorika* embryos was dependent on the Phytagel dose in the maturation medium.

Our findings are partially consistent with the results obtained by other authors (Iraqi and Tremblay 2001; Lelu-Walter and Pâques 2009). Additionally, our study showed that the effect of the osmoticum on the germination of spruce somatic embryos was also dependent on the tree species and the genotype of the embryogenic-tissue line.

In general, our studies demonstrated that the response of spruce embryogenic-tissue lines to the concentration of the three tested components in the maturation medium was highly dependent on the species. The concentration of both ABA and the osmoticum in the medium affected the pattern of starch accumulation in the developing embryos of the tested spruce species. These factors may be the keys to the further development of plant embryos. Based on our findings, the quality of the *Picea* sp. somatic embryos was enhanced by adding 68 g/L of sucrose or 6 g/L of Phytagel to the maturation medium. Adding sucrose or Phytagel to the cultivation medium at these concentrations improved the starch-accumulation process and radicle growth, whereas the ABA dose during the maturation stage was less important. The presented results provide new information on the effects of ABA and osmoticum on the starch accumulation pattern of *Picea abies* and *P. omorika* somatic embryos.

Author contribution statement THP conceived and designed research. THP conducted experiments. EMK and ER performed starch content analysis. THP and EMK analysed data. THP, EMK and KB wrote the manuscript. All authors scrutinized and corrected the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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