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Structure of embryogenic tissues and accumulation of storage materials in somatic embryos of *Picea abies* and *P. omorika*

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Abstract: Microscopic analysis of embryogenic tissues of *Picea abies* and *P. omorika* showed that the tissues differed in proportions of various types of proembryogenic masses, and in presence/absence of early somatic embryos, which suggests that they differed in embryogenic potential. On the basis of this study, embryogenic competence of tissues can be assessed at early stages of somatic embryogenesis, and thus the most efficient lines can be selected (one *Picea abies* and two *P. omorika*). In the course of development of somatic embryos embryogenic tissue was placed on a medium with abscisic acid (ABA), which affects an increase in osmotic pressure at the stage of maturation of somatic embryos and finally on the regeneration of cotyledonary embryos. In this stage of embryos development, storage materials were accumulated, which are used in specific metabolic processes during embryo development.

Additional key words: spruce, proembryogenic masses, development, starch grains, lipid bodies

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Introduction

Embryogenic tissue is a special type of plant tissue, able to produce embryos without fertilization. It can derive from various explant types. In coniferous tree species, it is usually derived from seeds, as a result of culture of immature or mature zygotic embryos, in specific physicochemical conditions. Such embryogenic tissue, deriving from the given explant, is termed an embryogenic cell line, to emphasize its genetic specificity.

Embryogenic cell lines of a given coniferous tree species usually vary in structure and ability to produce somatic embryos. On the basis of these differences, embryogenic cell lines are divided into two groups (A and B), depending on the structure and developmental stage of proembryogenic masses (PEMs) (Egertsdotter and von Arnold 1993, 1998; Filonova et al. 2000). The type of PEMs found in embryogenic lines indicates their competence to produce properly developed somatic embryos.

On the basis of morphological structure and pattern of cell development, Filonova et al. (2000) distinguished three developmental stages of cell aggregates: PEM I, PEM II and PEM III, and early somatic embryos. PEM I is a cell aggregate composed of a small number of cells with dense cytoplasm, accompanying a single vacuolated cell that tends to elongate, like suspensor cells in cell lines of type A. PEM II is a similar cell aggregate, but has more than one vacuolated cell. PEM III represents a larger group of meristematic cells, with very numerous vacuolated suspensor cells.

Parallel to the development of the proembryos present in proliferating embryogenic cell lines, storage materials are accumulated in the form of starch, lipids, and proteins. Storage materials are necessary for proper functioning of many metabolic processes, associated with the development of somatic embryos to the cotyledonary stage. Intensification of the accumulation of storage materials, in proembryos and precotyledonary stages of development of somatic embryos, is triggered by contact of embryogenic tissue with a medium supplemented with abscisic acid (ABA) (Misra 1994).

Aims of this study were: (1) to analyse selected cell lines of *Picea abies* and *P. omorika* in respect of their structure and potential for production of somatic embryos, and (2) to identify what storage materials are accumulated in cells of globular and cotyledonary somatic embryos of those spruce species.

Our earlier experiments resulted in production of somatic plantlets of *Picea abies* and *P. omorika* (Hazubska-Przybył and Bojarczuk 2008). This enables the application of the somatic embryogenesis method for genetic and breeding research or for nursery production of these two spruce species.

Materials and methods

Structure of embryogenic tissues and their ability to produce somatic embryos

To detect the presence of proembryos in embryogenic tissue of *P. abies* and *P. omorika*, initiated from mature zygotic embryos in medium BM-3 (Gupta and Durzan 1986), we applied staining with acetocarmine (Gupta and Durzan 1987). Nuclei of the embryogenic regions of proembryos are then stained red. Stained fragments of embryogenic tissue were observed under a light microscope (Carl Zeiss Jena or Zeiss Axioskop 20) and micrographs were taken (microscopic camera MC 80 DX). If proembryos were detected, embryogenic tissue was cultured in a proliferation medium (BM-3).

In three cell lines of *P. abies* (a–c) and six lines of *P. omorika* (a–f), under a microscope, we assessed the frequency of individual types of PEMs (Filonova et al. 2000) in tissues of the studied spruce species. Embryogenic cell suspension was prepared earlier according to the method described by Tramisak-Milaković et al. (1999). In brief, 10–20 mg of embryogenic tissue was stained for several minutes in 500 ml of acetocarmine solution, in 1-ml Eppendorf tubes. Next, the material was rinsed with 0.5 ml of distilled water. Then again 0.5 ml of distilled water was added to the tube and it was shaken for 1 minute, to prepare a cell suspension. For microscopic observations, we collected three replications of 10 ml of embryogenic cell suspension. The number of each PEM type was counted in the volume in each sample, and next their mean percentage contributions in tissues of the given line were calculated as was described by Tramisak-Milaković et al. (1999).

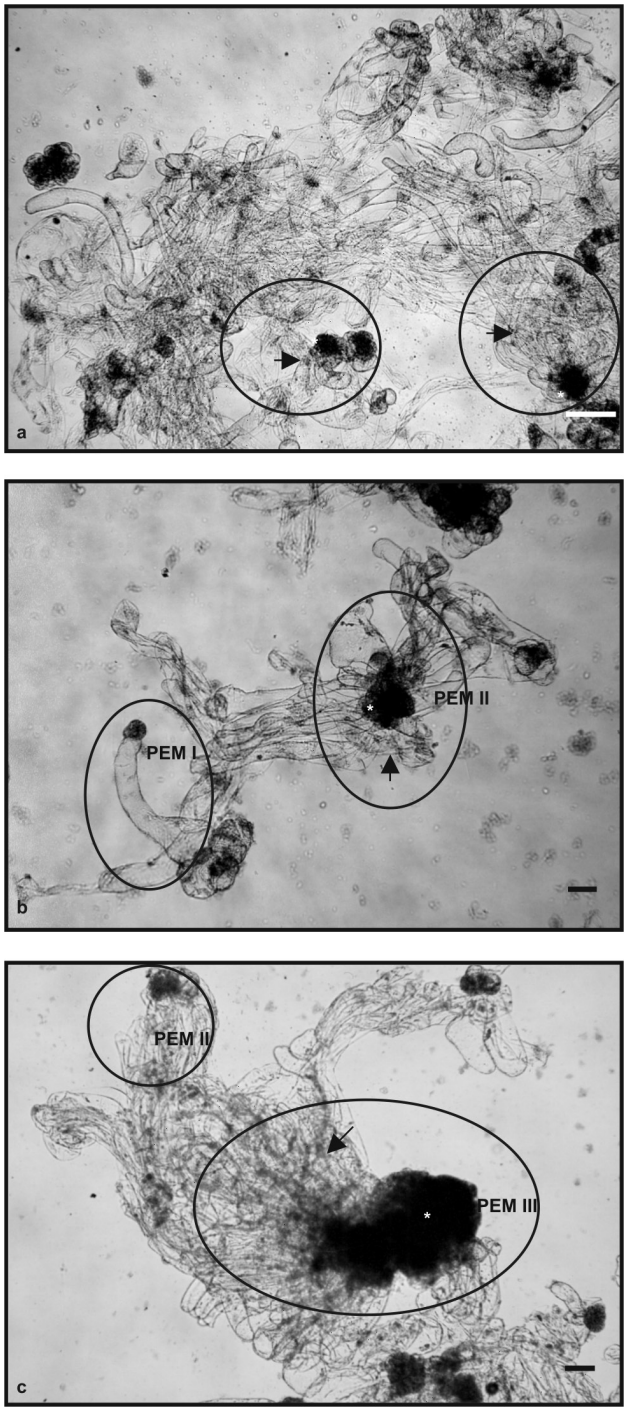

Fig. 1. Embryogenic tissue of *Picea abies*: (a) proembryos with typical embryogenic regions (*) and suspensors (arrow), (b) proembryogenic masses PEM I and PEM II, (c) proembryogenic masses PEM II and PEM III. Scale bar = 50 μm

Storage materials accumulated in cells of somatic embryos

To identify what storage materials are accumulated in cells of somatic embryos of *P. abies* and *P. omorika*, globular and cotyledonary embryos were collected from tissues cultured in BM-3 medium (Gupta and Durzan 1986) with 20 μM ABA and 1 μM IBA. The embryos were placed in a modified Karnovsky's fixative (Karnovsky 1965), including 3% paraformaldehyde (PFA) and 3% glutaraldehyde (GA) at a ratio of 1:1, at pH 6.8. To improve the preservation of cell membranes, 5 mM calcium chloride was added to the mixture (Harris and Oparka 1994). The material was fixed for 2 hours at room temperature, rinsed in 0.05 M cacodylate buffer for three changes of 15 minutes each, and stored overnight in a refrigerator. Next, the material was fixed for 2 hours in 1% OsO_4 (osmium tetroxide), rinsed in 0.05 M cacodylate buffer for three changes of 10 minutes each, and contrasted in 2% water solution of uranyl acetate at pH 5.0 for 2 hours. The material prepared in this way was dehydrated through an ethanol series: 80%, 90%, 96% (for 10 minutes each), and three times in 100%. The material was imbedded in low-viscosity epoxy resin, according to the method described by Spurr (1969). The resin was composed of 9.2 ml of VCD (vinyl cyclohexene dioxide), 4.5 ml of DER 736 (diglycidyl ether of polypropylene glycol), 25.5 ml of NSA (nonenyl succinic anhydride) and 0.5 ml of DMAE (dimethylaminoethanol). To enable polymerization of the resin, the material was placed in an incubator, at 45°C for 24 hours. After this time, the temperature was raised to 60°C, and after another 24 hours, to 75°C. Next the material was sectioned on a microtome (Reichert Ultracut S, Leica) into ultrathin sections (about 70 nm thick) by means of diamond knives.

The sections prepared in this way were placed on copper mesh and contrasted with uranyl acetate and lead citrate (for 7 minutes each). Storage materials accumulated in embryo cells were observed under a transmission electron microscope (JEM 1200 EX, JEOL Co.), at an accelerating voltage of 80 keV.

Results

Structure of embryogenic tissues and their ability to produce somatic embryos

After staining of embryogenic tissues of *P. abies* and *P. omorika* with acetocarmine, characteristic structures were visible, composed of embryogenic regions and suspensor cells, i.e. the so-called proembryos or PEMs (Figs 1a and 2a).

The analysis of frequencies of individual types of proembryogenic structures in embryogenic tissues of *P. abies and P. omorika* showed that the studied lines were heterogeneous (Figs 1 and 2).

Embryogenic tissues of the studied species differed in proportions of individual types of PEMs. Embryogenic lines of *P. abies* were composed mostly of PEM I (Fig. 1 b), which were much more numerous than PEM II, PEM III (Fig. 1b, c) and early somatic embryos (Table 1). In contrast, embryogenic lines of *P. omorika* were composed chiefly of PEM II (Fig. 2c). The proportion of PEM III in embryogenic tissues was similar in all the lines of the studied spruce species (5.5–13.3%, Table 1). Embryogenic lines of *P. abies* (except line a) and *P. omorika* contained early somatic embryos (Table 1, Fig. 1c, Fig. 2d–f). These analyses revealed some differences in proportions of PEM I and PEM II in embryogenic tissues of *P. omorika,* which were divided into two groups. The first group included lines a, b and d, where the proportion of PEM I was similar to the proportion of PEM II (Table 1). The second group was composed of lines c, e and f, where the percentage of PEM I was much lower than

Table 1. Proportion (%) of various types of proembryogenic masses (PEMs) and early somatic embryos in the volume of embryogenic tissues of selected lines of *Picea abies* and *P. omorika*

Species	Line	Developmental stage of PEMs			Early somatic embryos (%)
		PEM I (%)	PEM II (%)	PEM III (%)	
Picea abies	a	72.3	22.2	5.5	0
	b	60.0	16.0	8.0*	16.0
	c	76.6	11.7	8.8*	2.9
P. omorika	a	29.7	29.6	11.1*	29.6
	b	33.4	20.0	6.6*	40.0
	c	3.1	67.8	12.9*	16.2
	d	38.9	44.5	12.9*	3.7
	e	20.0	56.7	13.3*	10.0
	f	8.6	71.4	8.6*	11.4

* PEM III with large embryogenic regions and elongated suspensor cells

the percentage of PEM II. In the observed embryogenic tissues of the studied spruce species, the structure of PEM I was usually poorly polarized (Fig. 1a, Fig. 2b). PEM III, with large embryogenic regions and long suspensor cells, were found in two lines of *P. abies*: b and c (Table 1, Fig. 1c), and in all six lines of *P. omorika* (Table 1, Fig. 2a–f). *P. abies* line a contained PEM III, but with a poorly developed embryogenic region and generally short suspensor cells (Table 1, Fig. 1c), which visually differed from long suspensor cells observed in above-mentioned spruce lines.

Storage materials accumulated in cells of somatic embryos

As a result of our ultrastructure study of the cortex cells of globular somatic embryos of *P. abies*, numer-

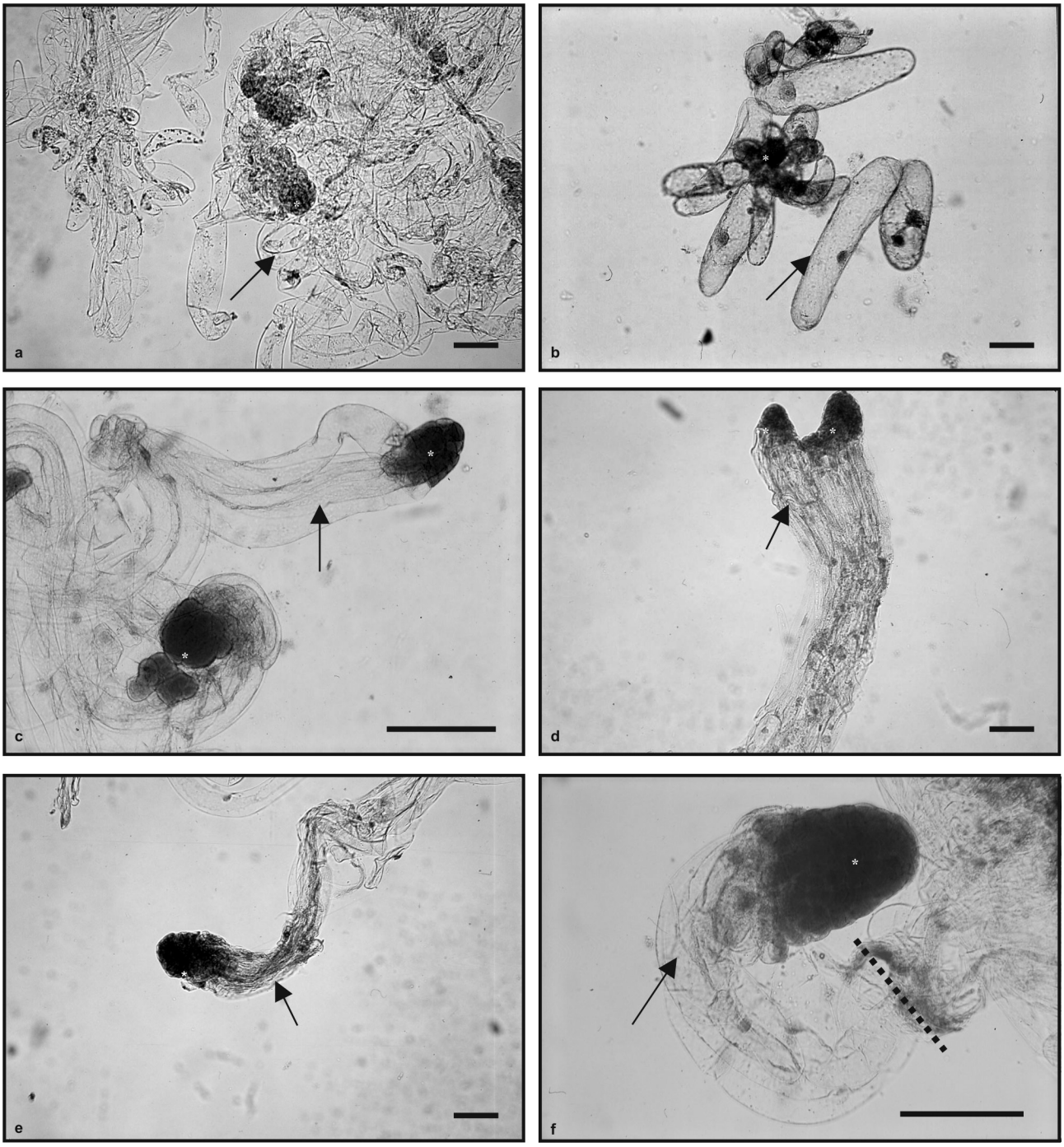

Fig. 2. Embryogenic tissue of *Picea omorika*: (a) proembryos with visible embryogenic regions (*) and suspensors (arrow), (b) poorly polarized PEM I, (c) PEM II, (d) two proembryos sharing a common suspensor, (e) formation of an immature somatic embryo, (f) an early somatic embryo, with a well-defined embryogenic region (*), separated (---) from a suspensor (arrow). Scale bar = 50 μm

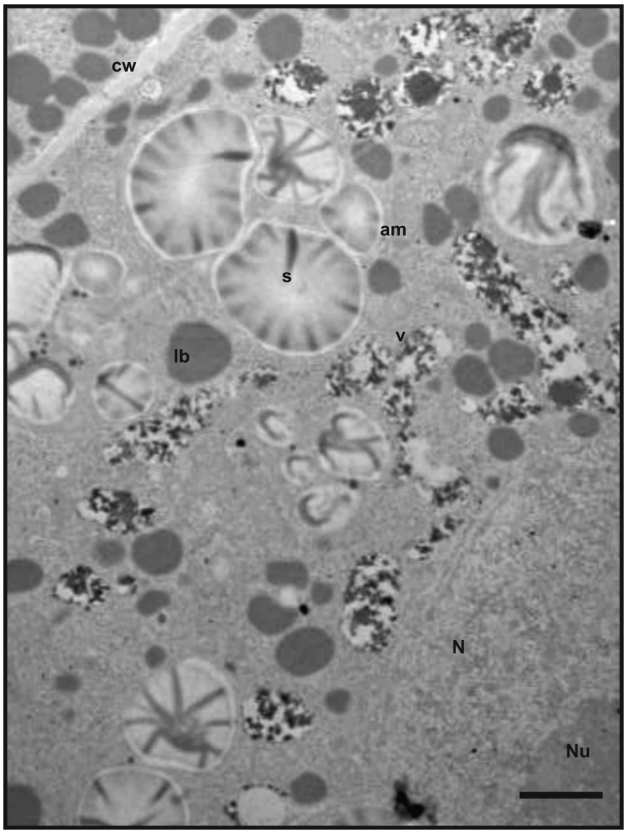

Fig. 3. Fragments of two cells of a globular somatic embryo of *Picea abies*; (cw) cell wall, (N) fragment of a nucleus, (Nu) nucleolus, (am) amyloplasts, (s) starch grains, (lb) lipid bodies, and (v) vacuoles, containing fluffy osmiophilic material. Scale bar = 1 μm

ous amyloplasts (am) were found, with large, usually single starch grains (s), ca. 1–2 mm in diameter (Fig. 3), visible in cross sections. Abundant lipid bodies (lb) were present in cells. In contrast, in cells of cotyledonary somatic embryos of this spruce species, large amyloplasts were found, but usually each with

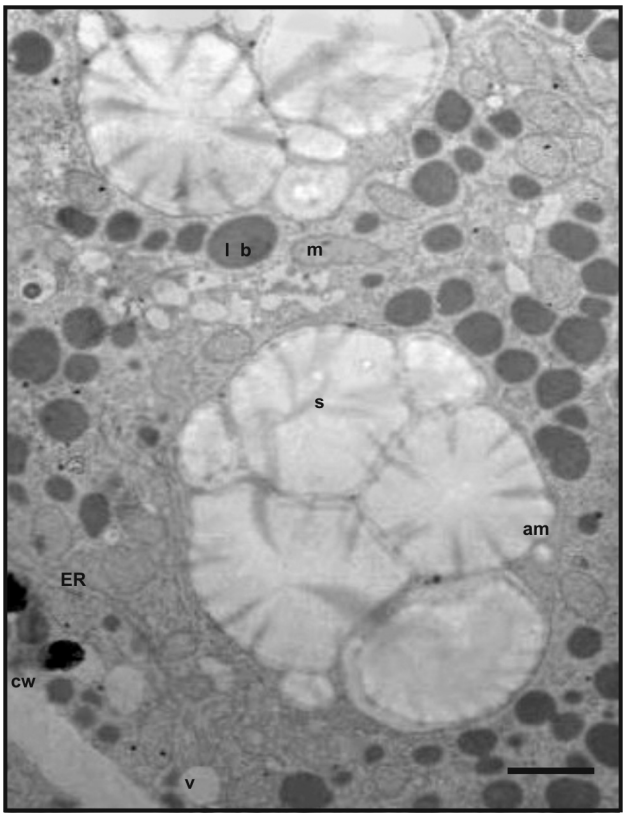

Fig. 4. Fragments of two cells of a cotyledonary somatic embryo of *Picea abies*; (cw) cell wall, (am) amyloplasts, (s) starch grains, (lb) lipid bodies, (m) mitochondria, (v) vacuoles, (ER) endoplasmic reticulum. Scale bar = 1 μm

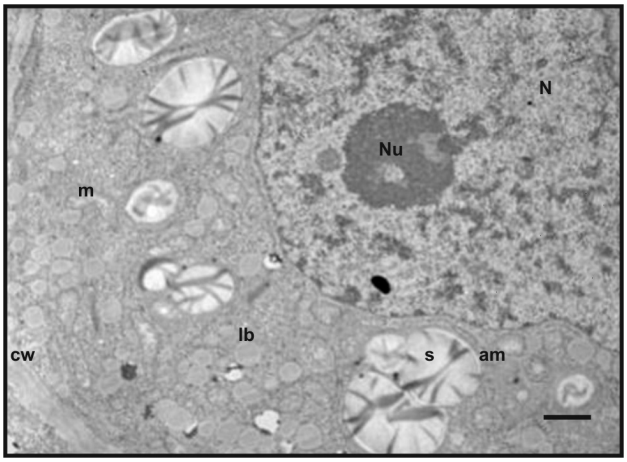

Fig. 5. Fragments of two cells of a globular somatic embryo of *Picea omorika*; (cw) cell wall, (N) nucleus, (Nu) nucleolus, (am) amyloplasts, (s) starch grains, (m) mitochondria, (lb) lipid bodies. Scale bar = 1 μm

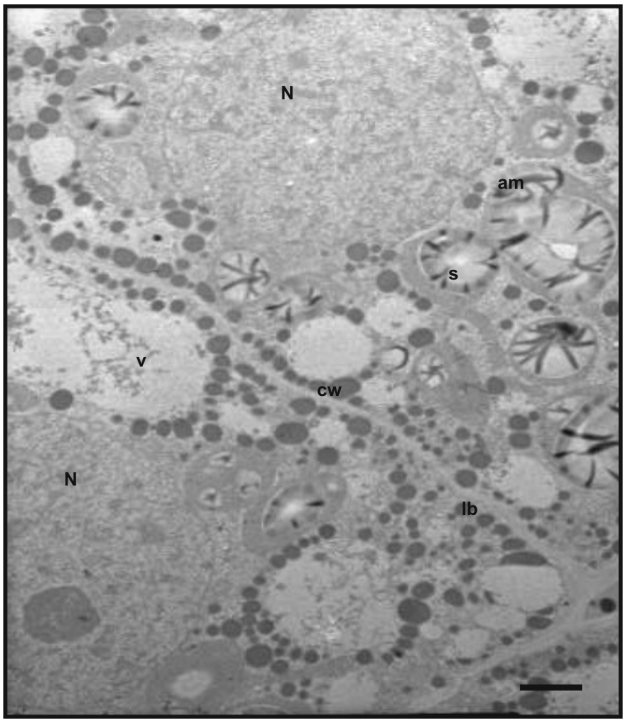

Fig. 6. Fragments of three cells of a cotyledonary somatic embryo of *Picea omorika*; (cw) cell wall, (N) nuclei, (am) amyloplasts, (s) starch grains, (v) vacuoles, containing fluffy osmiophilic material, (lb) lipid bodies. Scale bar = 2 μm

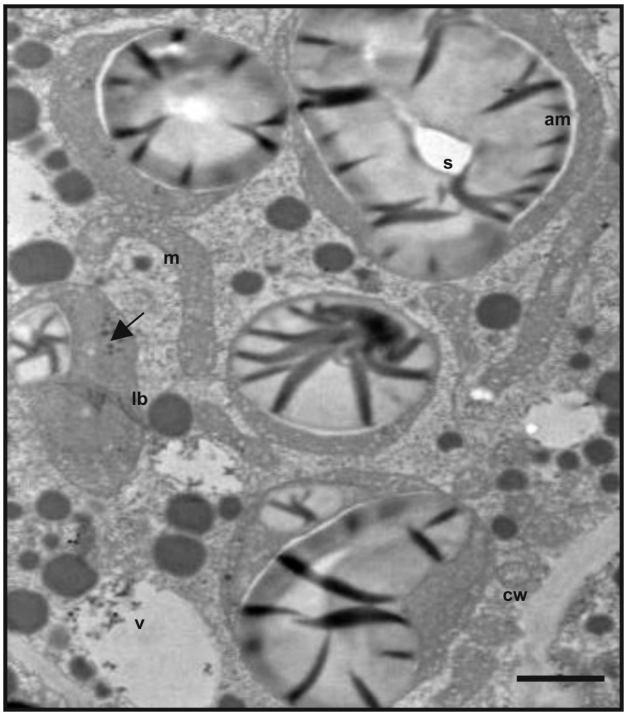

Fig. 7. Amyloplasts (am) in cells of a cotyledonary somatic embryo of *Picea omorika*; (cw) cell wall, (s) in section, single starch grains within amyloplasts, (lb) lipid body, (m) mitochondrion, (v) vacuoles. Scale bar = 1 μm

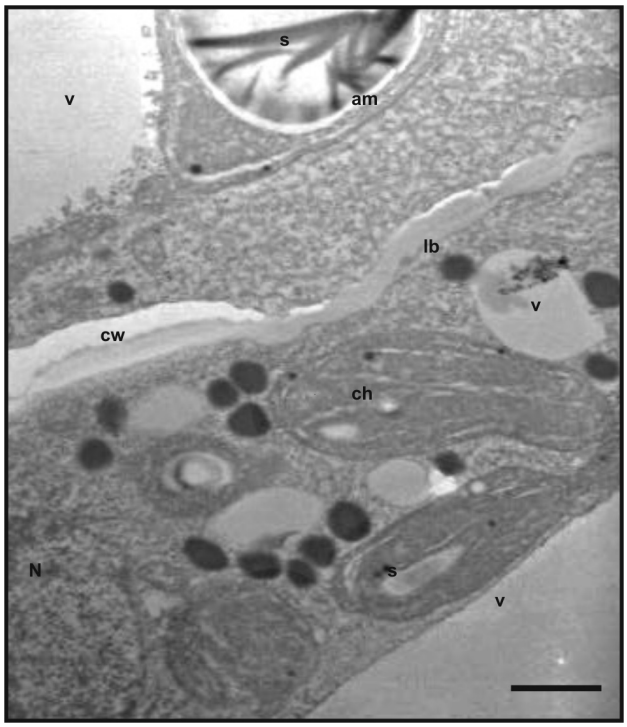

Fig. 8. Lens-shaped chloroplasts (ch) in single cells of a cotyledonary somatic embryo of *Picea omorika*; (cw) cell wall, (am) amyloplast, (lb) lipid body, (N) nucleus, (s) starch, (v) vacuoles, containing fluffy osmiophilic material. Scale bar = 1 μm

several large starch grains (Fig. 4), often >2 mm in diameter, and abundant lipid bodies as well.

Similarly, in cells of globular somatic embryos of *P. omorika*, starch grains and lipid bodies were visible (Fig. 5). We observed small differences in starch distribution in globular embryos of *P. omorika*, where most of amyloplasts, with one or three starch grains, were found in the zone underlying the protoderm (Fig. 5). Cells of cotyledonary somatic embryos of *P. omorika* contained usually numerous vacuoles (v), filled mostly with lipid bodies as well as amyloplasts with single starch grains (Fig. 6). In those cells, two types of plastids were observed: amyloplasts and chloroplasts. Amyloplasts were present in most of cells and contained usually single starch grains, visible in sections of plastid (Fig. 7). Chloroplasts were rare, found in single cells, and also contained single, but very small starch grains (Fig. 8).

In vacuoles of cells of somatic embryos, in both spruce species, we observed fluffy osmiophilic material (Figs 3, 6 and 8).

Discussion

Structure of embryogenic tissues and their ability to produce somatic embryos

Embryogenic tissue of conifers can be easily distinguished on the basis of its colour and general appearance. However, these features are insufficient to determine its embryogenic competence, i.e. ability to produce somatic embryos. To investigate the differences between embryogenic and nonembryogenic tissues, Wann et al. (1987) analysed biochemically tissues of *P. abies*, initiated from immature zygotic embryos. As a result, those authors found that embryogenic competence of the studied tissues was strongly correlated with the rate of ethylene production by those tissues, as well as with glutathione concentration and the total number of reducers in individuals tissues. Their study showed that ethylene production was faster in nonembryogenic than in embryogenic tissues. An analysis of those factors during tissue growth helps to determine if the tissue is embryogenic or not.

A simpler method enabling an assessment of the potential for production of somatic embryos from embryogenic tissues is their observation under a microscope (Jalonen and von Arnold 1991; Tramisak-Milaković et al. 1999; Filonova et al. 2000; Salajova and Salaj 2005) . Results of microscopic observations of embryogenic tissues, morphology of somatic embryos, their potential for differentiation and further development, reported by Jalonen and von Arnold (1991), enabled a classification of the tissues into three groups: tissues of group A1 (polar type) and A2 (solar type), which were able to produce somatic embryos, and group B (undeveloped type),

which did not produce somatic embryos when cultured on a medium with ABA. Those tissues can be distinguished already at the stage of initiation, by means of microscopic observations. In later studies, Egertsdotter and von Arnold (1993, 1995, 1998) divided embryogenic cell lines into two groups A and B, on the basis of differences in types of extracellular proteins released to the media, conditioning the later development of somatic embryos.

On the basis of the detailed analysis of the structures found in embryogenic tissues, conducted by Filonova et al. (2000), three types of PEMs (PEM I, PEM II and PEM III) and early somatic embryos were distinguished. On the basis of the structure of the least organized stage, e.g. PEM I, present within embryogenic tissues and showing a clear polarization (meristematic cells vs. vacuolated cell), those authors concluded that such tissues have a potentially higher embryogenic competence. Those authors observed structures of this kind in embryogenic lines of type A, which as a result of culture on a medium with ABA produced properly developed, mature somatic embryos. In embryogenic lines of type B, apart from PEM I with a clearly polarized structure, also some others were present, where such a structure could not be distinguished (meristematic cells vs. vacuolated cell). During culture on the medium with ABA, those tissues did not produce somatic embryos, or produced abnormally developed embryos, incapable of further development. Those authors also showed that the clearer is the polarization of PEM II and PEM III, the more likely they are to produce embryos that are visible in proliferating embryogenic tissues as early somatic embryos.

Microscopic observations described in presented paper revealed that embryogenic tissues differed in respect of structure, both between the studied spruce species and within species. Most of them contained PEM I, usually poorly polarized, which suggests that the studied tissues had a low embryogenic competence. Similarly to Filonova et al. (2000), we observed PEM III within embryogenic tissues often in association with PEM II. PEM III formed large groups of embryogenic cells with long, numerous suspensor cells, or small aggregates of meristematic cells surrounded by short suspensor cells. Sometimes, from single PEM III structures, early somatic embryos developed.

On the other hand, Tramisak-Milaković et al. (1999) indicated an association between the embryogenic potential of cell lines of *P. omorika* and the frequency of somatic embryos of type C, which are approximately equivalent to PEM III (Filonova et al. 2000). Tramisak-Milaković et al. (1999) distinguished three categories of PEMs in *P. omorika*, on the basis of cell number: type A, composed of 1–5 cells (approximately equivalent to PEM I); type B, composed of 6–20 cells (approximately equivalent to PEM II); and type C, composed of >20 cells (approximately equivalent to PEM III). Type C was considered by those authors as the first stage of development of somatic embryos. They studied two types of embryogenic tissues of *P. omorika*, which were previously distinguished on the basis of their phenotype. The first type of embryogenic tissues was characterized by white colour, so they were marked as W. These tissues contained mostly PEMs of type A, and relatively rarely of type C. PEMs of type C, although infrequent, consisted of a well-defined embryogenic region and elongated suspensor cells, which seems to indicate a high embryogenic competence. In contrast, the second type of embryogenic tissues was characterized by brown colour (B). Those tissues contained mostly PEMs of type C, which consisted of a small embryogenic region and short suspensor cells. After placing both types of embryogenic tissues on media with abscisic acid, B tissues showed a higher potential for production of somatic embryos than W tissues, although their PEMs of type C were rather poorly developed, but more numerous than the other types. In that case, proportions of individual types of PEMs exerted a decisive influence on the embryogenic competence of the tested tissues. Their study showed that the embryogenic competence of tissues is evidenced not only by the presence of PEMs but also a specific structure (meristematic cells vs. vacuolated cell), as well as frequency of PEMs.

Results of this study show that embryogenic tissues of the studied species differed mostly in proportions of PEM I and PEM II. PEM I usually prevailed in embryogenic tissues of *P. abies*, while PEM II in embryogenic tissues of *P. omorika*, which indicates that the latter are more advanced in development. The proportion of PEM III was much lower in all the lines of the studied spruce species, as compared to PEM I and PEM II. This suggests a low embryogenic competence of the analysed tissues. In the studied embryogenic lines, the structure of PEM I was usually poorly polarized, which may also indicate their low potential for production of somatic embryos. However, the presence of PEM III with a well-developed embryogenic region and elongated suspensor cells in some embryogenic lines of both spruce species, suggests that those lines are more predisposed to produce somatic embryos, as compared to the lines containing poorly developed PEM III. Our observations indicate that the studied lines of both spruce species usually belonged to group B, because they were dominated by poorly polarized PEM I. Embryogenic lines with poorly developed, infrequent PEM III, but with no early somatic embryos (the stage of the embryos between PEM III and globular stage of development), were probably unable to produce somatic embryos. Most of the lines analysed in this study had also infre-

quent, well-developed PEM III and early somatic embryos, which were probably able to develop into normal embryos, capable of germination. This is evidenced by the fact that somatic embryos of *P. abies* and *P. omorika* were produced as a result of this experiment (Hazubska-Przybył and Bojarczuk 2008).

Proper evaluation of embryogenic competence of individual embryogenic tissues, on the basis of their structure, would enable a quick assessment of the quality of the generated tissues, and selection of the most valuable ones already at the stage of induction from explants. Such a procedure would help to shorten markedly the time of culture, and to reduce labour intensity, associated with the selection of desirable embryogenic lines. Thus it is extremely important to analyse differences in the structure of embryogenic tissues of various coniferous tree species, produced via somatic embryogenesis. Our results indicate that somatic embryogenesis is useful for spruce propagation *in vitro*. We selected one line (b) of *Picea abies* and 3 lines (a, b, c) of *P. omorika*, which produced an embryogenic tissue with somatic embryos. The tissue of these lines was characterised by PEM III with large embryogenic regions and elongated suspensor cells as well as higher percentage of early somatic embryos.

Storage materials accumulated in cells of somatic embryos

Transfer of embryogenic tissue from the proliferation medium onto a medium containing ABA, leads to differentiation of somatic embryos. The structural and physiological changes observed then within PEMs, are clear symptoms of tissue development. ABA plays an important role in the process of maturation of somatic embryos (Bozhkov et. al. 2002), affecting e.g. synthesis and accumulation of storage materials. Many reports show that during development of somatic embryos in media containing ABA, abundant lipid bodies, protein bodies, and starch are accumulated in them (Hakman and von Arnold 1988; Hakman et al. 1990; Flinn et al. 1991b; Joy et al. 1991; Salopek et al. 1997; Kong et al. 1999; Lippert et al. 2005). Those substances are accumulated as reserves, both in zygotic and in somatic embryos (Attree and Fowke 1993), and are later released during embryo development. During embryo differentiation, storage proteins are accumulated at the final stages of their development. Starch is accumulated earliest, in plastids, and next some lipid bodies appear (Misra 1994).

In the studied globular and cotyledonary somatic embryos of *P. abies* and *P. omorika* we detected starch grains, within amyloplasts, and abundant lipid bodies in the cytosol. In Norway spruce, in the course of development of cotyledonary somatic embryos number of starch grains in amyloplasts increased, and some of them remained similar in size to the starch in cells of globular somatic embryos. In somatic embryos of Serbian spruce, the number and size of starch grains in cells were similar in both developmental stages. A similar amount of starch was observed also in cells of globular embryos, in both spruce species. According to the report by Salopek et al. (1997), the greatest accumulation of starch in meristematic cells of somatic embryos of Serbian spruce, was observed at the globular and torpedo stage, while the starch content of cotyledonary embryos was lower. Some symptoms of this process were observed in cotyledonary somatic embryos of *P. omorika*, where in single cells the first chloroplasts appeared. They contained very small starch grains, which suggests that storage materials were gradually used by the developing embryo.

The amount of lipid bodies in cells of somatic embryos of both studied spruce species was similar at the globular and cotyledonary stages of development.

Apart from starch grains and lipid bodies, also vacuoles with fluffy osmiophilic material were present in cells of somatic embryos of both spruce species. This may reflect the process of accumulation of immature protein bodies, which are the third type of storage materials accumulated in maturing somatic embryos of spruces. However, at this stage of research, it is uncertain if they are truly protein bodies.

The analysis of biochemical processes (e.g. accumulation of storage materials) taking place during formation of somatic embryos of coniferous trees, is extremely important when preparing procedures of plant propagation via somatic embryogenesis. As reported by Misra (1994), application of the traditional method of evaluation of development and condition of developing somatic embryos on the basis of morphological characters is very often insufficient. The processes taking place at successive stages of somatic embryogenesis can be investigated in detail by the use of more advanced methods, such as biochemical and molecular analyses (Klimaszewska et al. 2004; Lippert et al. 2005). It has turned out that normal morphology of somatic embryos does not always guarantee their proper development. According to Misra (1994), some defects can be detected within apparently normal embryos only after detailed analyses of somatic embryos at the biochemical and molecular level.

Many earlier studies indicated that ABA concentration, as well as the kind and amount of osmoregulatory factors in media, affect the accumulation of storage reserves during somatic embryogenesis in coniferous tree species (Hakman et al. 1990; Joy et al. 1991; Flinn et al. 1993; Misra 1994). An analysis of the process of accumulation of storage materials, particularly of protein and lipid bodies (which can be used, e.g., as biochemical markers for comparing the processes of differentiation of somatic and zygotic embryos) enables an appropriate modification of cul-

ture conditions, allowing production of high-quality somatic embryos (Hakmann et al. 1990; Flinn et al. 1991a). Our preliminary cytological research proved the differences in content of the storage materials in somatic embryos cells of *Picea abies* and *P. omorika*. In further study special attention should be paid to identification of the biochemical changes (mostly analysis of proteins) associated with development of somatic embryos of various coniferous tree species.

Conclusions

1. Microscopic analysis of embryogenic tissues of *P. abies* and *P. omorika* enables an assessment of their embryogenic competence at early stages of somatic embryogenesis, and consequently the most efficient lines can be selected. The most efficient lines for *Picea abies* was line b and for *P. omorika* line a, b and c.
2. In the course of development of somatic embryos of the studied spruce species, storage materials are accumulated within their cells in the form of starch grains and lipid bodies, which are used in specific metabolic processes.

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