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Dedicated to Prof. dr. MERCEDES WRISCHER
on the occasion of her 70th birthday.

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EFFECTS OF ABSCISIC ACID AND CARBOHYDRATES ON THE MATURATION OF *PICEA OMORIKA* (PANČ.) PURK. SOMATIC EMBRYOS

TAMARA TRAMIŠAK-MILAKOVIĆ[2], SNJEŽANA MIHALJEVIĆ[2], SIBILA JELASKA[1, 2]*

[1] University of Zagreb, Faculty of Science, Department of Molecular Biology, Rooseveltov trg 6

[2] Institute Ruđer Bošković, Department of Molecular Genetics, Bijenička 54, HR-10000 Zagreb, Croatia

In eight embryogenic tissue lines of omorika spruce the roles of carbohydrates and abscisic acid on embryo maturation were examined. Sucrose, glucose and maltose (3 % and 6 %) with abscisic acid (20, 40, 60 μM) were screened for optimal combination with respect to embryo maturation. The **B** lines, which expressed a brown tissue phenotype, achieved superior embryo maturation on 3 % glucose and 40 μM abscisic acid. The **W** lines with a white and translucent tissue phenotype showed the best maturation capacity on 3 % sucrose or 3 % maltose with 20 or 40 μM ABA. Sixty μM ABA inhibited embryo maturation. Media containing 6 % of either sucrose, glucose and maltose produced fewer mature embryos compared to media with 3 % of the same sugars. The **W** lines had almost three times lower maturation capacity in relation to the **B** lines.

Key words: *Picea omorika*, omorika spruce, somatic embryogenesis, abscisic acid, embryo maturation, carbohydrates

Introduction

Since first reported for Norway spruce, *Picea abies* (L.) Karst., (HAKMAN and von ARNOLD 1985) considerable progress has been made in achieving somatic embryogenesis in conifers and improving the protocols for embryo maturation

* Corresponding author: E-mail: sibila@hazu.hr

(BECWAR et al. 1987, DURZAN and GUPTA 1987, BOULAY et al. 1988, DUNSTAN et al. 1988, HAKMAN and von ARNOLD 1988, ROBERTS et al. 1990, TAUTORUS et al. 1991).

Nevertheless, plant regeneration from conifer somatic embryos is still a problem and only a few authors established plantlets in soil (BECWAR et al. 1989, WEBSTER et al. 1990). One of the critical step is the maturation process. It affects the viability of the mature somatic embryos obtained, especially their ability to germinate and to produce plantlets. There is evidence that ABA is an important medium component for the maturation of somatic embryos of Norway spruce (HAKMAN et al. 1985, DURZAN and GUPTA 1987, BOULAY et al. 1988, DUNSTAN et al. 1988, HAKMAN and von ARNOLD 1988, ROBERTS et al. 1990, GUTMANN et al. 1996). ROBERTS et al. (1990) report the effects of ABA on the later stages of somatic embryo maturation, storage protein accumulation and the quality of somatic embryos. Furthermore, it has been shown that medium constituents such as the kind and concentration of the sugar added (TREMBLAY and TREMBLAY 1991, LELU et al. 1994) also play a predominant role in conifer somatic embryo development.

Somatic embryogenesis in *P. omorika* has been accomplished by using shoot explants (BUDIMIR and VUJIČIĆ 1992) and, in our laboratory, in cultures of mature zygotic embryos (KOLEVSKA-PLETIKAPIĆ et al. 1995).

The aim of our work was to assess the effect of different carbohydrates, in interaction with abscisic acid, on embryo maturation in omorika spruce. Emphasis was placed on investigating possible correlations of maturation, ABA concentration used in the maturation medium, and the genotype of the tested embryogenic tissue lines.

Materials and methods

Plant material

The experiments were carried out with eight omorika spruce cell lines, which had been in culture for more than two years.

Tissue cultures were initiated from mature seeds of open-pollinated trees of omorika spruce (*Picea omorika* (Panč.) Purk.), collected from a natural locality near Višegrad, Bosnia and Hercegovina, (1988–1989) and supplied by the Forestry Institute, Jastrebarsko, Croatia. Seeds were stored at 4 °C until use.

The zygotic embryos were removed from surface-sterilized seeds and placed on induction culture medium. Ten mature embryos were cultured in one 90×20 mm Petri dish. The Petri dishes were sealed with parafilm and incubated at 25±1 °C in the dark. The induction of embryonal-suspensor masses (ESMs) was achieved on modified LP medium (von ARNOLD and ERIKSSON 1981) from which amino acids and sugars, with the exception of sucrose, were omitted, and Zn-EDTA was replaced by $ZnSO_4 \times 7H_2O$. The induction medium was supplemented with 1 % sucrose. 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5.0 µM benzyladenine (BA) and 0.6 % agar (Sigma^R). The pH of the medium was adjusted to 5.8–6.0 before autoclaving at 121 °C, 110 kPa, for 20 min.

Embryogenic white and mucilaginous tissues (ESMs) were initiated from embryos as previously described by KOLEVSKA-PLETIKAPIĆ et al. (1995) and

maintained on proliferation medium, i.e. 1/2 LP containing 5.0 μM 2,4-D, 2.5 μM BA, 1 % sucrose and 0.6 % agar at 25±1 °C. Cultures were subcultured at 14-to-21-day intervals.

During two years, several dozens of embryogenic tissue lines were established. Embryogenic tissues from individual seeds were subcultured separately on maintenance medium. The stock culture lines were kept in the dark at 25±1 °C.

Histological staining

ESMs were stained with 2 % acetocarmine as described by GUPTA and DURZAN (1987). Cells were examined under a light microscope and photographed. The lines used in this study differed by their morphogenetic and histogenetic characteristics. On the base of their tissue colour we classified the lines into two classes: white (**W**) and brown (**B**). The characteristics of these two classes are described in the Results section.

For microscopical examination of proembryogenic structures, specimens of fresh tissue were squashed in 2 % acetocarmine. Depending on the number of embryogenic cells per cluster we have distiguished three sizes of proembryogenic structures: size A (1–5 cells), size B (6–20 cells) and size C (>20 cells), (Fig. 1). The lines were characterized by the frequency of each of the three cluster sizes. The number of each size of proembryogenic structures was determined in 10 μL of suspension. Ten to 20 mg of tissue was squashed in 2 % acetocarmine, diluted with 500 μL distilled water in a one-mL Eppendorf tube and vigorously agitated for 10 s. Each counting was repeated three times.

The growth rate of the lines was determined as the increase of fresh weight within two-week intervals, over a period of 6 months. Tissue was weighed before transfer to fresh medium. Inocula weighed 100–200 mg. The growth index was calculated using the formula:

$GI = W_1 - W_0/W_0$; where W_0 is the weight in g at the start and W_1 at the end of the subculture period.

Maturation of somatic embryos

The standard maturation medium consisted of 1/2 LP salts supplemented with 3 % sucrose and 20 μM (±)cis-trans-abscisic acid (ABA). The ESMs, about 500 mg, containing stage 1 embryos (VON ARNOLD and HAKMAN 1988) were transferred onto maturation medium. During the ABA treatment (4–6 weeks), the cultures were not transferred to fresh medium.

The maturation experiments were carried out on lines maintained for two and more years in culture: four lines, class **B** (**B**B19, **B**C33, **B**C67, **B**C90), and four lines, class **W** (**W**C14, **W**D24, **W**C64, **W**D73). The media were solidified with 0.6 % Agar-agar and pH adjusted to 5.8–6.0 before autoclaving at 121 °C. For each combination of sugar type and concentration with ABA concentration, five tissue pieces (5×100 mg) of each line were placed in a Petri dish and incubated in the dark at 25±1 °C. After 4–6 weeks of culture, the mature somatic embryos were counted under a dissecting microscope. The maturation capacity of the lines was defined as the number of mature embryos per gram of tissue fresh weight.

The influence of various carbohydrate sources (sucrose, glucose, maltose), their concentration (3 % and 6 %, w/v) and combination with three concentrations of ABA (20, 40 and 60 μM) on somatic embryo maturation was tested in both classes (**W** and **B**) of tissue lines.

The results were evaluated statistically. The values presented are arithmetic means of three replicate experiments (3×5) followed by the standard errors (±S.E.) of the mean.

Results

Induction of embryogenic tissue

Callus proliferation from mature zygotic embryos occurred within two weeks of culture on induction medium, mostly in the hypocotyl area of embryo explants. After two months of induction, white, translucent, embryogenic tissue could be distinguished from the green nonembryogenic callus. Two-month-old embryogenic tissue showed a morphology typical for the embryonal-suspensor mass in *Picea* sp. The proembryogenic structures consisted of intensely aceto-carmine-stained small cells with dense cytoplasm and highly vacuolated, elongated suspensor-like cells (Fig. 1). Induction of ESMs was significantly influenced by the genotype. Callus proliferation was observed on 346 (87 %) mature embryo explants from which 127 (38 %) ESMs were selected and maintained. During two years in culture the number of established ESMs was reduced.

Morphological and anatomical characteristics of ESMs

Established embryogenic tissue lines were classified into two classes: **W** lines with white translucent tissue and **B** lines with brown-coloured tissue. Tissue of both classes was heterogeneous, composed of different sizes of proembryogenic structures. In the **W** lines the size A was the most frequent and the size C was rare, while in the **B** lines the most frequent was the size C (Tab. 1). The organization of the proembryogenic structures size C varied in the two classes. In the **W** lines the size C was composed of large lumps of embryogenic cells and many long and vacuolated suspensor cells. In the **B** lines the size C consisted of tiny embryogenic cells and short, often brown-coloured suspensor cells. Also, there were differences in the growth rates of the embryogenic tissue of the two classes. The growth rate in the **B** lines was lower than that of the **W** lines (Fig. 2).

Maturation of somatic embryos

Embryo stages were determined according to von ARNOLD and HAKMAN (1988). The capacity of mature somatic embryo formation in four **B** lines (**B**B19, **B**C33, **B**C67, **B**C90) and four **W** lines (**W**C14, **W**D24, **W**C64, **W**D73) was determined on the standard maturation medium: 1/2 LP with 3 % sucrose and 20 μM ABA. Up to the second week of culture, no visible changes occurred in the tissues. During the third and fourth weeks, numerous somatic embryos at the globular and torpedo stages (stage 2) with smooth domes became visible in the **B** lines. At the same time, globular and torpedo stage (2) embryos were rarely observed in the **W** lines. The maximum production of mature stage 3 embryos was achieved during the sixth week of culture, but varied according to the cell line. The maximum number of mature embryos in the **B** lines was about two times that observed in the **W** lines (Tab. 2).

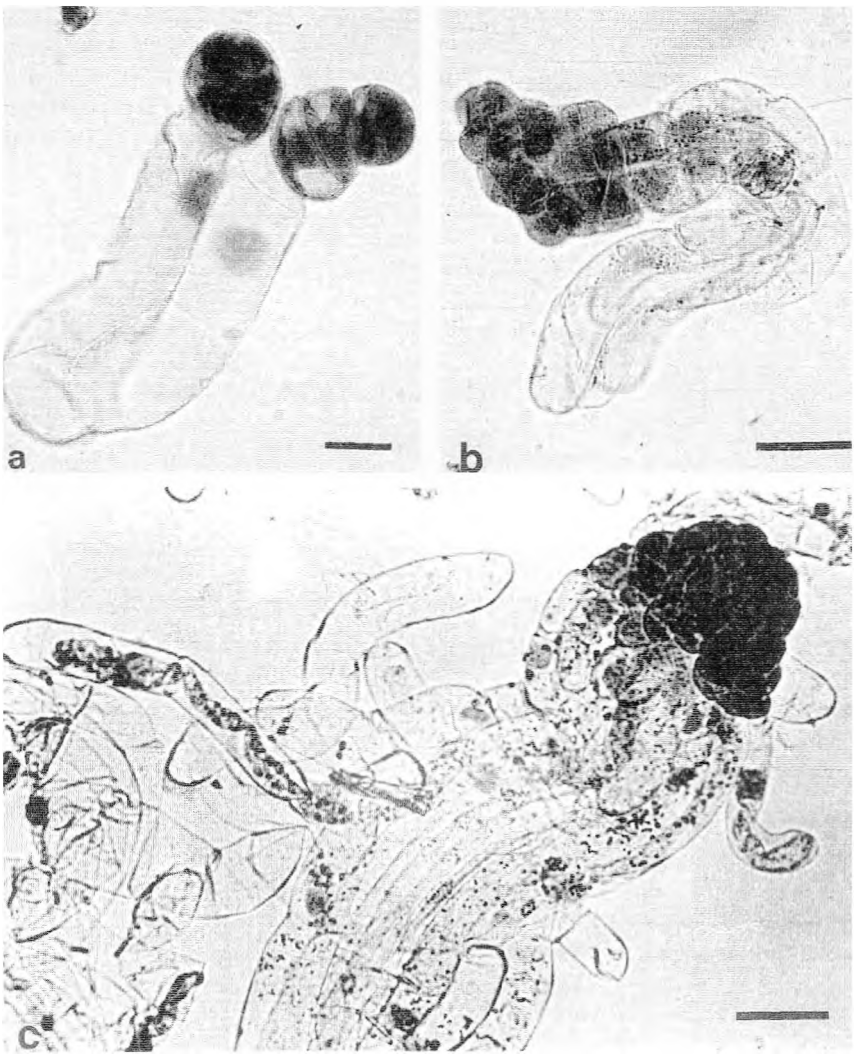

Fig. 1. Proembryogenic structures in tissue culture of *Picea omorika* grown on proliferation medium 1/2 LP with 5.0 μM 2,4-D, 2.5 μM BA and 1 % sucrose.
a) Size A structure (1–5 celled somatic proembryos with early embryonal mass).
b) Size B structure (6–20 celled proembryos).
c) Size C structure (composed of more than 20 cells) presents stage 1 somatic embryo. Bars in a–c represent 20, 50, 50 μm, respectively.

The effects of different sugars and ABA concentrations on the maturation capacity

We tested sucrose, glucose and maltose in two concentrations (3 % and 6 %), combined with three concentrations of ABA (20, 40, 60 μM), for their effects on embryo maturation. After 6 weeks on maturation medium the number of mature embryos was determined (Tab. 2). The **B** lines maximally (highest number of mature embryos) responded to media with 3 % glucose, while the maturation capacity of the **W** lines was optimally expressed on the standard medium with

Tab. 1. Frequency (mean ± SE) of proembryogenic structures classified by their size (see text for details) in *Picea omorika* tissue lines B (brown phenotype) and W (white phenotype). The presence of clusters of a certain size is presented ($\times 10^{-4}$) in grams tissue of fresh. Size C represents stage 1 somatic embryos and the embryogenic potential of the line. The medium was 1/2 LP supplemented with 5.0 µM 2,4-D, 2.5 µM BA and 1 % sucrose.

Lines	BB19	BC33	BC67	BC90
Size				
A	0.14±0.01	0.15±0.02	0.11±0.01	0.13±0.01
B	0.38±0.02	0.18±0.01	0.24±0.01	0.16±0.02
C	1.05±0.12	0.33±0.09	0.59±0.01	0.47±0.03
Lines	WC14	WD24	WC64	WD73
Size				
A	3.44±0.09	9.23±0.21	1.74±0.01	3.40±0.15
B	1.66±0.03	4.42±0.21	2.56±0.14	1.26±0.16
C	0.23±0.01	1.09±0.23	4.21±0.09	1.60±0.20

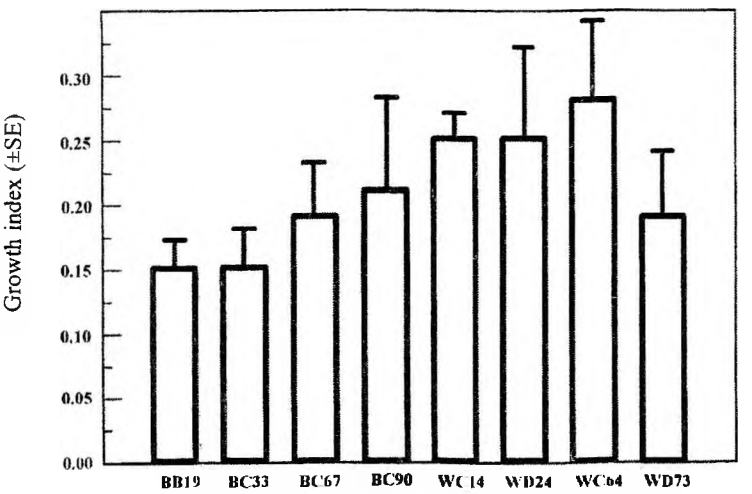

Fig. 2. Mean relative growth rates of maintained *Picea omorika* embryogenic tissue lines, classes **B** and **W**, for 12 subsequent two-week growth periods. The lines were cultured on the proliferation medium 1/2 LP supplemented with 5.0 µM 2,4-D, 2.5 µM BA and 1 % sucrose.

Tab. 2. Number (per gram fresh weight) of mature somatic embryos (stage 3) of *Picea omorika* produced on different carbohydrates and ABA concentrations. Each number represents the mean of 20 replicates (4 lines × 5). Basal medium was 1/2 LP.

			No. of mature stage 3 embryos/g	
Carbohydrate	%	ABA (μM)	Phenotypes **B**	Phenotypes **W**
Sucrose	3	20	76	42
		40	124	75
		60	96	43
Sucrose	6	20	32	36
		40	61	4
		60	8	4
Glucose	3	20	182	29
		40	253	18
		60	64	18
Glucose	6	20	65	10
		40	140	11
		60	31	8
Maltose	3	20	2	10
		40	29	98
		60	14	52
Maltose	6	20	0	37
		40	17	3
		60	2	12
Sucrose + Maltose	3	20	86	22
	3	40	33	13
	3	60	108	5
Glucose + Maltose	3	20	113	4
	3	40	118	25
	3	60	70	20

3 % sucrose or 3 % maltose and 20–40 μM ABA. The highest sugar concentration examined (6 %) decreased embryo maturation in all the tested lines. In the **B** lines combination of 3 % sucrose with 3 % maltose, or 3 % glucose with 3 % maltose allowed a significantly higher number of embryos to mature than on the medium with 6 % of a single sugar (sucrose, glucose or maltose). Increasing the ABA concentration from 20 to 40 μM resulted in higher numbers of mature embryos, whereas 60 μM ABA prevented maturation. The inhibitory effect of the high ABA concentration was enhanced in combination with 6 % sugar. On all media tested the maturation capacity of the B lines was higher than that of the **W** lines, except on media supplemented with maltose (Tab. 2).

Discussion

The purpose of this work was to assess the optimal combination of ABA and carbohydrate treatment to improve somatic embryo maturation in omorika spruce. The ability of ABA to promote the maturation of spruce somatic embryos, and to improve their development, has been well documented (BECWAR et al. 1987, BOULAY et al. 1988, DUNSTAN et al. 1988, von ARNOLD and HAKMAN 1988, ROBERTS 1991). However, the appropriate ABA concentration remains to be determined experimentally as it depends on the plant species, genotype, and even on the composition of the proliferation medium. In the eight established omorika spruce ESM lines, which were originally induced in mature zygotic embryos and maintained on the same proliferation medium for two and more years, we could distinguish two different tissue phenotypes. One phenotype (**B**) had brown tissue, a moderate growth rate and a high proportion of stage 1 embryos relative to the small proembryo clusters. The other phenotype (**W**) had white and translucent tissue, a higher growth rate and a lower frequency of stage 1 embryo clusters.

Noticeable differences were found among omorika spruce lines with respect to their response to the composition of the maturation medium. BECWAR et al. (1987) support observations that regeneration, developmental and maturation capacities vary among the genotypes of Norway spruce. It has also been indicated that adjustment of the maturation protocol for each individual genotype is required for the optimization of mature embryo production (WEBSTER et al. 1990).

In our experiments, for optimum maturation of omorika spruce somatic embryos, high levels of exogenous ABA (20–40 μM) had to be applied. Endogenous ABA levels have been found to be extremely low in somatic embryos matured for four weeks on ABA-minus medium, but could be raised up to 250 nmol g^{-1} DW by exogenous supply of 40–60 μM ABA (LABEL and LELU 1994). In our study, we used racemic mixtures of ABA sterilized by autoclaving. White spruce suspension-cultured tissues do not metabolise the R-(-)-ABA enantiomer (DUNSTAN et al. 1991); it may thus be assumed that only half the amount of added ABA was active.

During the maturation stage, ABA was shown to promote the accumulation of storage proteins and lipids in-conifer embryos (FEIRER et al. 1989, HAKMAN et al. 1990, ROBERTS et al. 1990, SALOPEK et al. 1997). For interior spruce (ROBERTS et al. 1990, WEBSTER et al. 1990), white spruce (DUNSTAN et al. 1991) and red spruce (HARRY and THORPE 1991) ABA concentrations of 20–40 μM were found to promote the normal development of embryos into plants. The optimal ABA concentration varied among black spruce cell lines (TREMBLAY and TREMBLAY 1995).

In a detailed study of the carbohydrate requirements during maturation of black and red spruce, it was shown that 175 mM (corresponding to 6 % sucrose) of either sucrose, fructose, glucose, maltose, or cellobiose supported embryo development (TREMBLAY and TREMBLAY 1991). For black spruce, sucrose gave a significantly higher number of mature embryos than the monosaccharides and disaccharides tested. However, an important difference between autoclaved and filter-sterilized sucrose was obtained, the latter producing twice as many

embryos in black spruce. For red spruce, no significant difference was observed among the carbohydrates tested but it was noticed that the highest numbers of embryos were obtained with cellobiose and maltose in the maturation medium.

The results of our experiments show the same kind of inconsistencies. The brown phenotypes produced the highest number of mature embryos on 3 % glucose, while the white phenotypes responded optimally to 3 % sucrose or 3 % maltose. Media containing 6 % of any of the sugars examined inhibited embryos maturation.

Von ARNOLD (1987) found that an increased sucrose concentration was important for further somatic embryo development in *Picea abies* and that the effect was not due to increased osmotic pressure. However, LU and THORPE (1987) reported that increasing osmolarity enhanced development and maturation of the embryos in *Picea glauca*, and in interior spruce low levels of mannitol (2–6 %) promoted the formation of globular embryos (ROBERTS 1991).

The two phenotypes of omorika spruce embryogenic lines could be compared with Norway spruce embryogenic cell lines A and B described by EGERTSDOTTER and von ARNOLD (1993, 1995). The authors consider that only well developed somatic embryos will undergo the maturation process and differentiate into mature embryos. They showed that the presence of specific extracellular proteins and the arabinogalactan protein fractions are active components for stimulating the embryos to develop further.

This study revealed that embryo maturation is a complex process controlled by many factors. To achieve the most efficient maturation of somatic embryos we combined three factors (embryogenic line, ABA, sugar) in order to sufficiently exploit the embryogenic potential of various omorika spruce lines.

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