GLASNIK INSTITUTA ZA BOTANIKU I BOTANIČKE BAŠTE UNIVERZITETA U BEOGRADU
BULLETIN DE L'INSTITUT ET DU JARDIN BOTANIQUES DE L'UNIVERSITE DE BEOGRAD
Tom XXIX, 1995, Beograd

UDC 588.14:577.175.1:582.475(497.11)
Original scientific paper

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DEVELOPMENT OF SOMATIC EMBRYOS AND EMBRYOGENIC CAPACITY IN *PICEA OMORIKA* (PANČ.) PURK. CULTURE*

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Budimir, S., Vujičić, R., Ćulafić, Lj. (1995): *Development of somatic embryos and embryogenic capacity in Picea omorika (Panč.) Purk. culture.* – Glasnik Instituta za botaniku i botaničke bašte Univerziteta u Beogradu, Tom XXIX, 43 - 50.

In *Picea omorika* seedling explant culture, after induction on medium supplemented only with cytokinin, cells from epidermal or subepidermal cotyledon layers gave rise to embryogenic tissue. Somatic embryos seems to be of single cell origin. Embryogenic tissue was induced on explants from five elite genotypes (seed families). One genotype with an induction frequency of 19% was superior to other four tested. On abscisic acid-containing medium somatic embryos underwent maturation. During precotyledonary stage of development starch accumulation from suspensor to the apical dome was observed. On the cotyledonary stage distinct meristemous were well defined and hypocotyl-shoot axis was formed.

* Dedicated to Prof. Mirjana Nešković on the occasion of her 45 th anniversary of scientific work

Key words: *Picea omorika* (Panč.) Purk., somatic embryogenesis, genotype, embryo development, abscisic acid.

Ključne reči: *Picea omorika* (Panč.) Purk., somatska embriogeneza, genotip, razviće embriona, abscisinska kiselina.

INTRODUCTION

Serbian spruce, *Picea omorika* (Pančić) Purk. is a Tertiary relic species, endemic to the Balkan Peninsula. At present times it resides on a limited number of about 30 localities distributed along the central part of the Drina river (Fukarek, 1951). *Picea omorika* is usually reproduced by seeds and only a few reports indicate the possibility of vegetative reproduction by grafting or cuttings (Gajić, 1994). The regeneration of *Picea omorika* can be also achieved by application of tissue culture methods (Budimir & Vujičić, 1990).

One of the most effective method of *in vitro* vegetative reproduction in conifer species, is by somatic embryogenesis (Sutton et al., 1993). The somatic embryogenesis is a process by which somatic cells of an explant are able to undergo all developmental stages of a zygotic embryo. One of the advantages of vegetative reproduction via somatic embryogenesis are well defined shoot and root apical meristems. In order to achieve a successful induction of somatic embryogenesis, a detailed protocol must be worked out for each species. The efficiency of method can further be enhanced using a phenotype with proven high potential for somatic embryogenesis.

Results which allow practical application of somatic embryogenesis, have already been achieved for a number of conifer species and in particular species of *Picea* (Tautorus, Fowke & Dunstan, 1991; Attree & Fowke, 1993; Vujičić & Budimir, 1995). Somatic embryogenesis in *Picea omorika* has been reported by Budimir & Vujičić (1992). It was demonstrated that somatic embryogenesis in the culture of Serbian spruce was induced when cytokinin, as a sole growth factor was present in the nutrient medium. The induction frequency of somatic embryogenesis was found to vary between 0-8%, most probably because a mixture of seed from various trees was used for obtaining seedling-derived explants.

The aim of the present study was to investigate the effect of genotype on the induction frequency of somatic embryogenesis, using seed families of known origin.

MATERIAL AND METHODS

Plant material

Seeds of *Picea omorika* used in the experiments collected from natural population were stored at 4°C for 18 months, the seeds collected from five open-pollinated elite trees were stored at 4°C for 48 months. The seeds were washed in running tap water for 24 h, surface disinfected with 30% H_2O_2 containing a drop of Tween 20 for 30 min, and then rinsed three times with sterile water.

The seeds were placed to germinate in Petri dishes on the medium containing 2 g l^{-1} glucose and 6 g l^{-1} agar. Shoot apices bearing 4-8 mm long cotyledons (shoot explants) were excised under sterile conditions. Ten explants per dish were placed horizontally on the surface of the culture medium.

Culture medium

Von Arnold & Eriksson (1981) modified basal medium (BM), consisting of salts, vitamins and sugars (90 mM sucrose), was used. Amino acids were omitted. Media were solidified with 7 g l^{-1} agar. The pH of the medium was adjusted to 5.7 prior to autoclaving for 25 min, at 115°C.

For the induction of somatic embryogenesis, explants were grown on BM with 22.5 μM benzyladenine (BA) for 2 weeks and then transferred to growth regulator-free medium for 4 to 6 weeks. Embryogenic tissue that developed on the explant was isolated and grown on BM to which 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) puls 4.5 μM BA and 30 mM sucrose were added. Cultures were kept in darkness. Subculturing to a fresh medium was carried out every two weeks.

For further embryo development parts of embroygenic tissue were transferred to Petri dishes on filter paper (Whatman No 2) supports on the surface of the solidified BM with 12 μM abscisic acid (ABA) for 4 to 8 weeks. Single mature embryos with developed cotyledons were transferred to half-strength BM without growth regulators for root growth.

Culture conditions

The seeds were germinated and all cultures maintained at $25 \pm 2^{\circ}C$ under a 16 h photoperiod, at a photon flux density of 5.1 μmol m^{-2} s^{-1} provided by white fluorescent tubes (Tesla, Pancevo, 65W, 4500 K), unless stated differently.

Microscopy

(a) Fresh material – small pieces of embryogenic tissue were placed on a glass slide, stained with 0.5% acetocarmin, and pressed gently with a cover glass. The entire preparation was then observed and photographed under Jenamed, Carl Zeiss photomicroscope.

(b) Paraffin – tissue was fixed in formalin-acetic acid-ethanol (FAA) for 24 h, dehydrated in graded ethanol, and embedded in paraffin wax at $57^{\circ}C$. Sections 5 μm thick were stained with haematoxylin.

(c) Plastic – tissue was fixed in 3% phospahte-buffered glutaraldehyde, pH 7.2, for 2 h, and postfixed in 2% phosphate-buffered OsO_4 for 2 h. Samples were dehydrated in graded ethanol, and embedded in Araldite (Serva, Heidelberg). Sections 1 μm thick were stained with methylene blue.

RESULTS AND DISCUSSION

Origin of somatic embryos

In the shoot apex culture of *Picea omorika* the cotyledon elongation was the only morphologically observable response, after two weeks on the induction BM supplemented with 22.5 μM BA. Within the following three weeks on hormone-free medium, whole explant or only its basal region become swollen with nodular appearance. Histological analysis of the cotyledon confirmed that first cell divisions occurred in epidermal and a few subepidermal layers. Anticlinal cell divisions were frequently present in epidermal layers, while in subepidermal layers (Fig. 1) mostly periclinal divisions occurred. Further cell divisions resulted in formation of a callus tissue, which caused disruption of epidermal/subepidermal layers, the neighbouring cells being loosely associated. By the end of fourth week the white glossy, mucilaginous, embryogenic tissue with polarized structures and small cell aggregates was found on the disrupted epidermal tissue (Fig. 2). The cells (Fig. 3), probably formed after an equal division of single superficial cells, are believed to be initials of a somatic proembryo. One of the two cells enlarged first and then divided transversely, which resulted in an

early filamentous somatic proembryo formation (Fig. 4). Further cell divisions of the upper cell, the embryo initial cell, resulted in an immature embryo formation. Immature embryos consist of small group of apical, densely protoplasmic cells, the apical dome, supported by a highly vacuolated and elongated suspensor cells (Fig. 5).

Similar results were obtained by Nagmani, Becwar & Wann (1987) in *Picea abies* and *Picea glauca*. The authors suggested that the origin of somatic embryos can be traced to singel cells in hypocotyl region, of immature embryos and that the first unequal cell division resulted in an early proembryo with the embryonal and suspensor initial. However, Mo & Von Arnold (1991) observed that in seedling explants of *Picea abies*, embryogenic structures could differentiate from epicotyl, hypocotyl and cotyledons. The authors found that embryos originated from nodules that were formed either from epidermal, subepidermal or cortical cells. Some embryogenic cultures might also differentiate directly from single epidermal cells.

Effect of genotype on induction frequency

Seeds collected from five elite trees were used in order to determine the effect of genotype on embryogenic capacity. Seeds originated from a parent tree were designated as seed family. The induction of embryogenic tissue formation was achieved as described above. The frequency of induction obtained among seeds from different families, varied in the range of 1-19%. Seed family No 2, with induction frequency of 19% was significantly superior to the other four families tested. The induction frequency among these four seed families varied in the range of 1.3-6.3% (Table 1).

Tab. 1. – Genotype effect on induction of somatic embryogenesis in Picea omorika

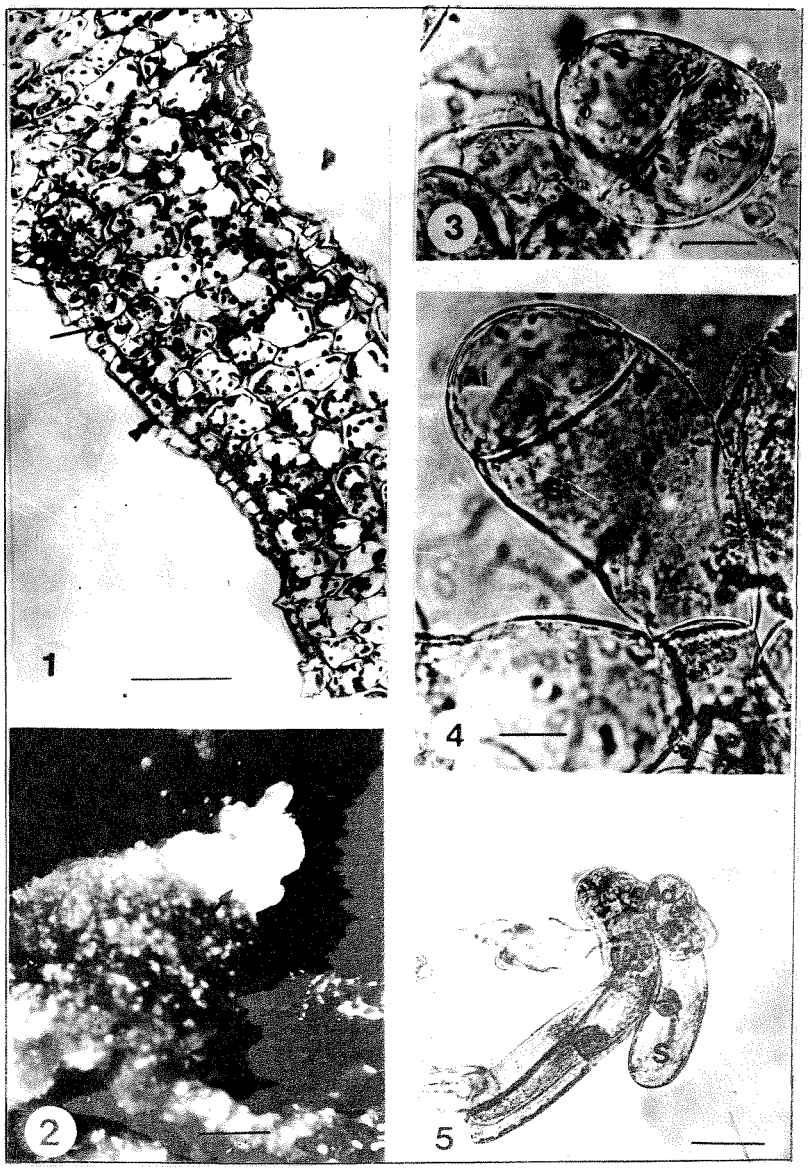
Seed family	No. of explants cultured	Induction frequencies (%)
1	80	6.3
2	107	18.7*
3	125	1.6
4	96	5.2
5	154	1.3

*Induction frequency is significantly different based on confidence intervals on the level $P < 0.05$.

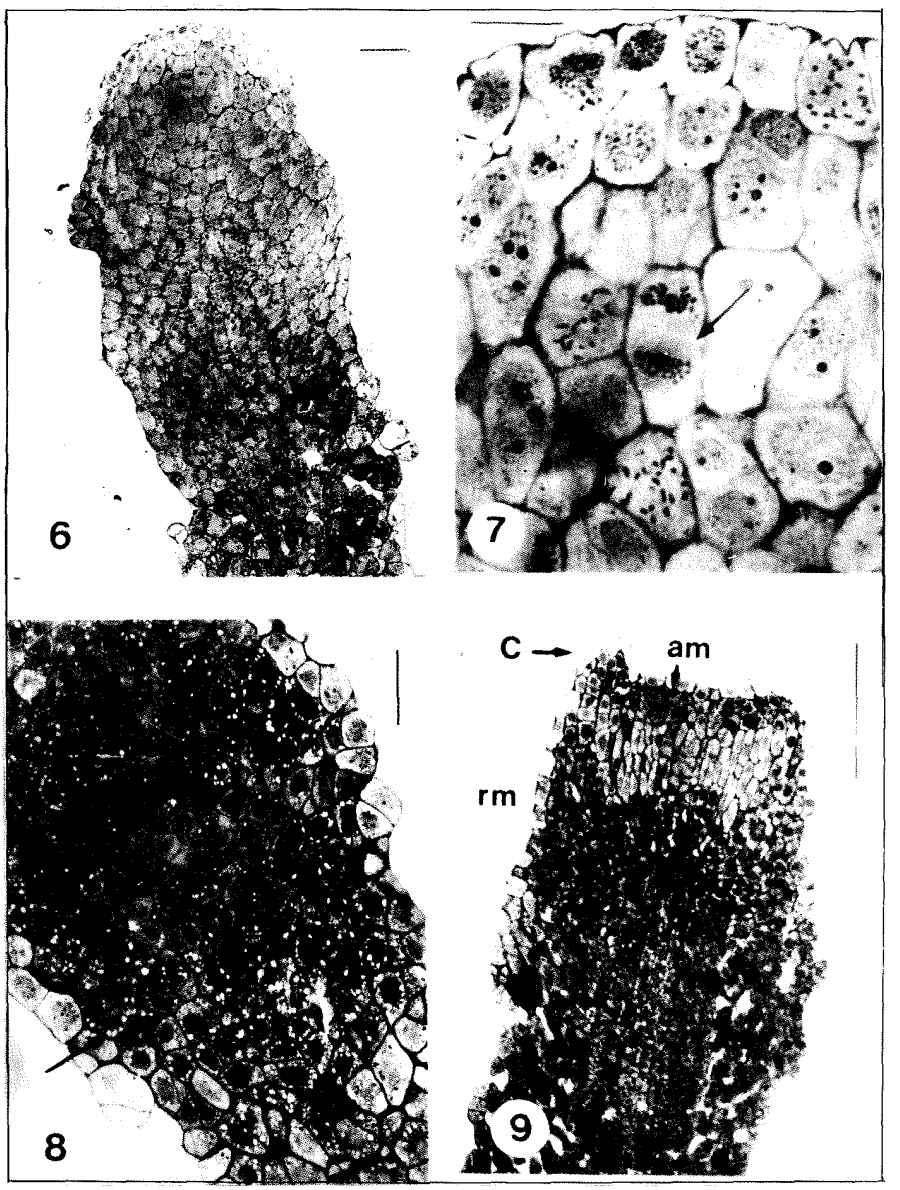
The varied induction ability demonstrated by different genotypes, suggests that in *Picea omorika*, genetic component is implicated in the capacity for embryogenic tissue formation. Cheliak & Klimaszewska (1991) also reported that in *Picea mariana*, among 20 genotypes tested, 85% gave rise to somatic embryogenesis. The induction frequencies varied from 0-17%. Jain, Dong & Newton (1989) suggested that genotype, controlled pollination and pre-conditioning of explants are of tremendous importance in the initiation and enhancement of somatic embryogenesis.

Development of somatic embryos

Isolated embryogenic tissue proliferated when grown on the medium supplemented with 9 μM 2,4-D and 4.5 μM BA (Budimir & Vujičić, 1992). Histochemical analysis of embryogenic tissue with acetocarmin, showed the presence of numerous filamentous, immature embryos. The mechanism of embryo multiplication



Figs. 1. – Longitudinal section of cotyledon with anticlinal cell divisons (double arrowhead) in epidermal, and periclinal cell divisions (arrowhead) in subepiermal cell layers. Bar = 100 μm; 2. – Embryogenic structures (arrow) protruding from the basal part of the cotyledon. Bar = 1 mm; 3. – Two-celled somatic proembryo. Bar=20 μm; 4. – Filamentous somatic proembryo with apical initial (Ai) and suspensor ini-tial (Si). Bar = 20 μm; 5. – Immature somatic embryo with defined apical dome (Ad) and suspensor (S). Bar = 50 μm



Figs. 6. – Longitudinal section of an embryo at the precotyledonary stage. Bar = 50 μm; 7. – Dividing cells (arrow) in the apical dome of somatic embryo. Bar = 50 μm; 8. – The basal part of somatic embryo with starch grains (arrow) in the cells. Bar=50 μm; 9. – Longitudinal section of an embryo at the cotyledonary stage; c - cotyledons, am - apical meristem, rm - root meristem. Bar = 20 μm.

is still unclear. The authors observed that embryos could arise by the mechanism similar to the cleavage polyembryogenesis in zygotic embryos (data not shown). With regular two-week subculturing the embryogenic potential of the tissue was retained for more than two years.

Further development of somatic embryos was observed after exposure to abscisic acid (Budimir & Vujičić, 1992). When grown on the medium containing 12 μM of abscisic acid, single somatic embryos were enlarged, and protruded from mucilaginous tissue. Histological examination of precotyledonary embryos (Fig. 6) showed that embryo enlargement was the result of intensive cell divisions. Cell divisions were observed in the apical dome as well as in the suspensor, the most frequent divisions, however, were found in the apical dome (Fig. 7). A gradient of accumulated starch grains from the suspensor towards the apical dome was prominent on longitudinal sections of the embryo. The cells in the suspensor region contained an abundance of large starch grains (Fig. 8). Similarly in *Picea glauca* culture, starch accumulation began at the proximal zone of the suspensor, continued to the peripheral regions and in the developing embryo. After polysaccharide accumulation, lipid and protein deposition was observed (Joy et al., 1991).

In *Picea omorika* culture structural differentiation of embryos occurred at the cotyledonary stage of embryo development (Fig. 9). Shoot apex with defined shoot meristem attained slightly convex shape. Provascular elements could be distinguished bellow the apex. The root initial consisting of meristematic cells was formed at the opposite side of the shoot apex. Root initial gave rise to cells that divided transversally forming a massive root cap.

An inhibory effect of abscisic acid (12 μM) on embryo multiplication, and at the same time the stimulatory effect on embryo maturation, was also described for a number of other conifer species (Becwar, Noland & Wann, 1987; Von Arnold & Hakman, 1988; Webster et al., 1990) as reviewed by Vujičić & Budimir (1995).

The present results show that the induction of somatic embryogenesis from embryonic (seedling) explants is possible in a range of genotypes of Serbian spruce. The somatic embryos seem to differentiate from single superficial cells of cotyledons. Our studies are now focusing on somatic embryogenesis induction in explants isolated from more mature plant material.

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Rezime

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RAZVIĆE SOMATSKIH EMBRIONA I EMBRIOGENI KAPACITET U KULTURI *PICEA OMORIKA* (PANČ.) PURK.

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U kulturi isklijalih biljaka *Picea omorika*, nakon indukcije na hranljivoj podlozi sa citokininom, ćelije epidermalnog i subepidermalnih slojeva kotiledona formiraju somatske embrione. Somatski embrioni su verovatno jednoćelijskog porekla. Nezreli somatski embrioni imaju diferenciran embriogeni region, građen od meristemskih ćelija i region suspenzora građen od izduženih, vakuoliziranih ćelija. Embriogeno tkivo je indukovano na eksplantatima poreklom od pet različitih genotipova (familija semena). Jedina familija semena sa frekvencijom indukcije od 19% bila je izrazito bolja od ostale četiri ispitivane familije. Kada se embriogeno tkivo izoluje sa eksplantata i dalje gaji na hranljivoj podlozi sa auksinom i citokininom dolazi do formiranja novih somatskih embriona. Embrioni mogu nastati procesom koji je označen kao „cleavage" poliembriogeneza. Na hranljivoj podlozi sa abscisinskom kiselinom somatski embrioni sazrevaju. Za vreme prekotiledarnog stupnja razvića uočeno je da dolazi do akumulacije skroba i to prvo u ćelijama suspenzora, a zatim i u ostalim delovima embriona. Na kotiledonarnom stupnju razvića apikalni meristemi postaju uočljivi, a duž ose embriona počinju da se diferenciraju vaskularni elementi.