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BRIEF COMMUNICATION

Developmental histology of organogenic and embryogenic tissue in *Picea omorika* culture

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Abstract

The histological events during adventitious bud and somatic embryo development in explants from *Picea omorika* seedlings cultivated *in vitro* have been examined. Meristematic activity in the superficial layers of cotyledons led to bud primordia formation. Bud primordia had characteristic zonal organization with needle primordia arising on the flanks of the meristem. Somatic embryo initial was formed on the cotyledon surface after an unequal transversal division of a cell. The smaller, apical cell gave rise to apical dome and later on secondary, filamentous suspensor while the larger vacuolated cell presented primary suspensor.

Additional key words: Serbian spruce, conifers, organogenesis, somatic embryogenesis.

Increasing need for reforestation have lead to establishment of improved programs for tree propagation. Micropropagation through organogenesis or somatic embryogenesis has been reported in number of conifer species. Initiation of organogenic or embryogenic tissue in conifer species is generally achieved from juvenile tissue as reviewed by Bonga and Durzan (1987), Attree and Fowke (1991) and Stasolla *et al.* (2002).

Serbian spruce, *Picea omorika* (Pančić) Purk. is tertiary relic and endemic conifer that is native only within a restricted area in Yugoslavia. As a decorative tree *P. omorika* is successfully grown in cities, and has been planted in many European countries. The regeneration of Serbian spruce from mature zygotic explants (Kolevska-Pletikapić and Buturović-Derić 1995) and from seedling derived explants (Budimir and Vujičić 1992) has been achieved. In seedling explants culture of *Picea omorika*, adventitious buds, needle-like organs, embryogenic and callus tissues were formed under the same culture conditions. The aim of this study was to analyze the origin and cell division pattern during early stages of adventitious bud and somatic embryo development. The study is important in a view of the need to identify cells associated with induction processes related to the formation of structures capable of organized growth and finally developing into seedlings.

Seeds of *Picea omorika* collected from open pollinated trees and stored at 4 °C were used for experiments. Seeds washed for 24 h under running tap water were further surface disinfected with 25 cm^3 of 30 % H_2O_2 containing a drop of *Tween 20* for 20 min, and rinsed three times with sterile water. The seeds were placed to germinate in Petri dishes on medium containing 2 g dm^{-3} glucose and 6 g dm^{-3} agar (*Torlak*, Belgrade, Serbia and Montenegro). After 21 d shoot apices bearing cotyledons 4 - 8 mm long (shoot explant) were excised.

For the induction of adventitious buds and embryogenic tissue shoot explants were placed on a modified LP medium (Von Arnold and Eriksson 1981) consisting of salts, vitamins, sugars (90 mM sucrose) and 22.5 μM benzyladenine (BA). Cultures were grown on this medium for 2 weeks and than transferred to growth regulator-free medium for another 4 - 6 weeks (Budimir and Vujičić 1992). Cultures were maintained at 25 °C

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Abbreviations: LP - Von Arnold and Eriksson medium; BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid.
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under a 16-h photoperiod at photon flux density of 5.1 μmol m^{-2} s^{-1} provided by white fluorescent tubes (*Tesla*, Pančevo, Serbia and Montenegro, 65 W, 4500 K). Developed embryogenic tissue was further isolated from the explant and grown on LP medium supplemented with 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.5 μM BA and 30 mM sucrose. Media were solidified with 7 g dm^{-3} agar. The pH of the media was adjusted to pH 5.7 prior to autoclaving for 25 min, at 115 °C.

For paraffin sections organogenic tissue was fixed in formalin:acetic acid:ethanol (10:5:85) for 24 h, dehydrated in graded ethanol and embedded in paraffin wax at 57 °C. Sections 5 μm thick were stained with Harris's haematoxylin (Johansen 1940). For semi-thin sections embryogenic tissue was fixed in 3 % phosphate-buffered glutaraldehyde, pH 7.2 for 2 h and postfixed in 2 % OsO_4 for 2 h. Samples were dehydrated in graded ethanol and embedded in *Araldite*. Sections 1 μm thick were stained with methylene blue. For squash preparation small pieces of embryogenic tissue were placed on glass slide and stained with 1 % acetocarmine. Material was observed and photographed under *Jenamed* photomicroscope (*Carl Zeiss*, Jena, Germany).

In the shoot apex culture of *Picea omorika* after two weeks on medium supplemented with 22.5 μM BA, the only morphologically observed response was the cotyledon elongation. Within the following three weeks on hormone-free medium tissue proliferation has occurred and whole explant become swollen and nodular in appearance. Subsequently explant surface was overgrown by abundant greenish callus tissue, adventitious buds and foliar structures. At the rim of cotyledons or at their base a small mass of mucilaginous, translucent embryogenic tissue was also readily distinguishable.

Histological analyses of the cotyledons, six weeks after culture initiation, confirmed the presence of various developmental stages of adventitious buds. Thus it was possible on the same explant to trace the origin and cell division pattern during bud formation. Cell divisions were observed throughout the cotyledon (Fig. 1), but the cells in superficial layers contained prominent nuclei and cell divisions appeared to be more frequent, so that regions of meristematic activity could be distinguished (Fig. 2). In these meristematic zones, both anticlinal and periclinal cell divisions occurred frequently. This led to protrusion of meristematic domes over the surface of the explant (Fig. 3). These meristematic domes presented apical meristems of bud primordia with distinct zonal organisation. Periclinal cell divisions on the flanks of the bud meristem further resulted in needle formation.

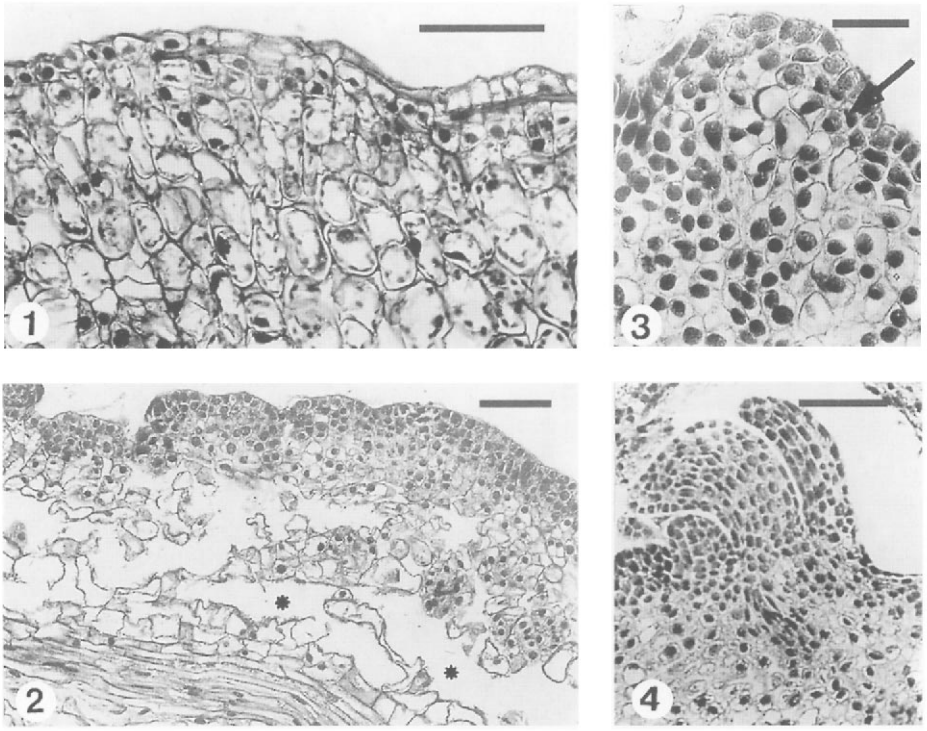

Fig. 1. Longitudinal section through cotyledon with pronounced mitotic activity in the subepidermal and subjacent region. *Bar* = 100 μm.

Fig. 2. Longitudinal section through nodular segment of cotyledon. Note the meristematic centers in superficial regions of explant and formation of large intercellular spaces in deeper layers (*). *Bar* = 100 μm.

Fig. 3. Median longitudinal section through apical meristem of bud primordia. Apical meristem was characterized by distinct cytohistological zonation and periclinal cell divisions at the flanks of the meristem (*arrow*). *Bar* = 50 μm.

Fig. 4. Median longitudinal section through adventitious shoot with needle primordia. *Bar* = 100 μm.

Although shoots had organized apical meristem and leaf primordia (Fig. 4), further shoot elongation was arrested because most of the explant surface was overgrown by callus tissue. Abundant callus tissue originated from cell divisions in deeper layers of cotyledon. Within the callus tissue two kinds of cell division pattern were observed. Namely organized cell divisions led to the bud primordia formation, and divisions in various planes resulted in growth of undifferentiated tissue (not shown).

On the squashed preparations of mucilaginous, embryogenic tissue small cell aggregates, dissociated highly vacuolated cells and immature embryos at various stages of development have been observed. Proembryo consisted of smaller, apical cell and larger, suspensor cell (Fig. 5). The two-cell aggregate was formed after an unequal cell division of cell in superficial layers of cotyledon. Early somatic embryo was composed of spherical apical dome subtended by long suspensor (Fig. 10). On the agar-solidified medium supplemented with 2,4-D (9 μM) and BA (4.5 μM), continuous proliferation of embryogenic tissue was sustained. Microscopic analyses showed that regular cell division pattern occurred during embryo development. Transversal cell divisions in the apical initial were often followed by longitudinal divisions, giving raise the cylindrical appearance of apical dome (Figs. 6 - 8). The basal cells of the apical dome gradually vacuolated and elongated forming secondary suspensor (Fig. 8). In the enlarging apical dome, cell divisions were more frequent at the dome periphery and were often occurring in a periclinal plane. With regular subculturing of embryogenic tissue long-term maintenance was possible.

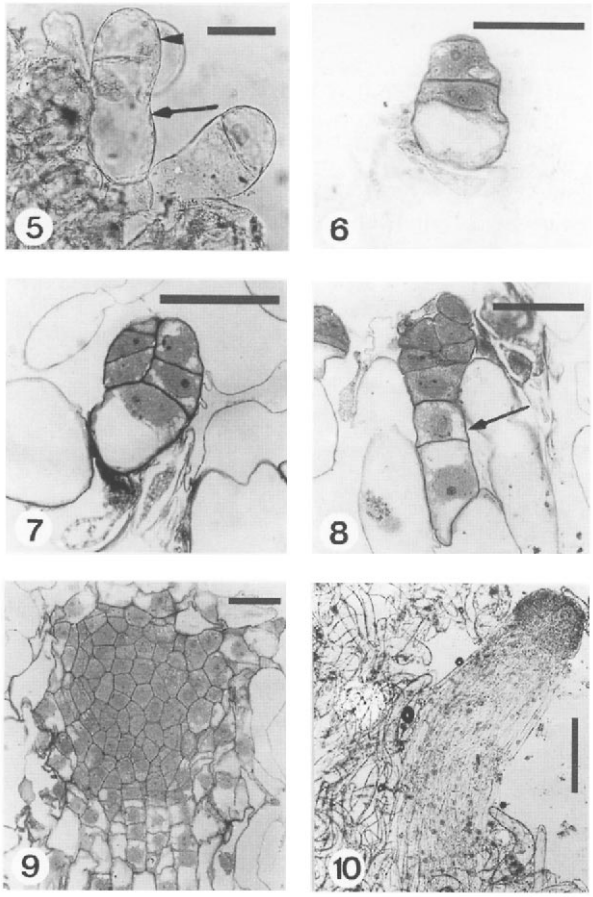

Fig. 5. Bipolar somatic proembryo composed of apical initial (*arrowhead*) and primary suspensor cell (*arrow*), arising from superficial cotyledon tissue. *Bar* = 50 μm.
Fig. 6. Somatic proembryo, grown on auxin and cytokinin medium, composed of apical, cytoplasm rich cells and vacuolated suspensor cell. *Bar* = 50 μm.
Fig. 7. Somatic proembryo composed of two files of cells in the apical dome and vacuolated primary suspensor cell. *Bar* = 50 μm.
Fig. 8. Filamentous, somatic embryo. Note cell elongation and vacuolation of secondary suspensor cells (*arrow*). *Bar* = 50 μm.
Fig. 9. Cross section of a somatic embryo. Note the frequent cell divisions at the periphery of the apical dome. *Bar* = 50 μm.
Fig. 10. Early somatic embryo composed of globular apical dome and long filamentous suspensor, surrounded with elongated, highly vacuolated cells. *Bar* = 200 μm.

In the seedling explant culture of *P. omorika*, two different cell types, epidermal and subepidermal cells of cotyledons and callus tissue cells, responded to induction treatment resulting in adventitious bud formation. In both cases organized cell divisions proceeded within the meristemoids, resulting in bud primordia formation. Corresponding developmental sequences have also been described in *Pinus radiata* (Yeung *et al.* 1981), *Picea abies* (von Arnold and Hawes 1988) and *Abies amabilis* (Kulchetscki *et al.* 1995) culture. These indicate that during organogenesis, under *in vitro* conditions, similar control mechanisms operate.

Little is known about the origin and development of embryogenic tissue in seedling explants of conifers. Somatic embryos in *P. omorika* culture originated from superficial cotyledon cells. Embryos were formed after an unequal cell division. Mo and von Arnold (1991) have found that somatic embryos can differentiate from nodular structures in the epidermal layers or in the cortex region of seedling but also from single epidermal cells. In *Picea abies* and *Picea glauca* culture (Hakman and Fowke 1987, Nagmani *et al.* 1987) somatic embryos derived from single elongated and vacuolated cell after an unequal division. Somatic embryo initials or frequently more advanced embryos in *Picea omorika* (Vujičić and Budimir 1995) as in some other conifer species (Nagmani and Bonga 1985, Von Arnold and Woodword 1988, Jasik *et al.* 1995) can also arise at the apical end of embryos by cleavage polyembryogenesis. Even though the results of the first stages of somatic proembryo formation in conifers are somewhat contradictory depending on the type of the explant or culture conditions the further events during embryo development are more similar, as was described for *Picea glauca* (Hakman and Fowke 1987), *Picea abies* (Nagmani *et al.* 1987) and *Pinus nigra* (Jasik *et al.* 1995). For Norway spruce embryos, abscisic acid was added to trigger further development of embryos

(Svobodová *et al.* 1999). The study also verified the necessity of the presence of osmotic stress induced by polyethylene glycol (PEG-400) for successful embryo maturation.

Although there are obvious differences between the developmental pattern of adventitious buds and somatic embryos, this study has shown that in *P. omorika* they could originate from the same type of cells. Adventitious buds may also develop indirectly from callus tissue, while somatic embryos can arise by secondary somatic embryogenesis defined as cleavage polyembryogenesis.

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