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Induction of adventitious buds on the cotyledons of *Abies concolor* × *Abies grandis* hybrid seedlings

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Abstract

Possibilities of adventitious buds induction on the cotyledons obtained from sterile seedlings of *Abies concolor* × *Abies grandis* hybrid were investigated. The following variables influencing bud induction and their further development were studied: the effect of explant age, the effect of different growth regulators and their concentrations and duration of their application. The most suitable explants proved to be the cotyledons of 7 d old seedlings. The most efficient cytokinin was benzylaminopurine (5 mg l^{-1}) in combination with napthaleneacetic acid (0.01 mg l^{-1}). The most optimal duration of treatment was 17 to 21 d culture of explants on induction medium. Shoot growth was achieved on basal medium to which 14 mg l^{-1} spermidine was added.

Introduction

In vitro induction of adventitious buds in conifers was described on various explants including mature embryos, needle fascicles, cotyledons and hypocotyls. However representatives of the genus *Abies* belong to the most conservative trees in this to respect (Boulay 1987). The best known work demostrating the importance of suitable explant selection for the regeneration of conifers is that by Aitken *et al.* (1981). The authors proved experimentally that in *Pinus radiata* the cotyledons of aseptically growing seedlings are the most suitable material for organogenesis. This explant is at present most frequently used for *in vitro* induction of adventitious buds aimed at obtaining the numerous regenerants of conifers.

In the present study three main factors were considered important in achieving bud hybrid: (1) timing of explant removal from the seedling, (2) application of different phytohormones and their concentrations, (3) duration of phytohormones treatment.

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Material and methods

The hybrid seeds of *Abies concolor* x *Abies grandis* were obtained by artifical pollination of the fir species in Arboretum Mlyňany located in the western Slovakia. Seeds were surface-sterilized in 70 % ethanol (m v^{-1}) for 30 s followed by 15 min treatment in 0.1 % $HgCl_2$ (m v^{-1}). After 36-48 h imbibition in sterile water seeds were once again sterilized in 6 % H_2O_2 (6 min). Isolated embryos were grown on modified Murashige and Skoog medium containing 1/2 concentration of macroelements, full concentration of microelements, 1/4 content of KNO_3 and NH_4NO_3, 250 mg l^{-1} inositol, 2.5 mg l^{-1} thiamin - HCl, 20 g saccharose and 1 % agar (*Kobe*). This medium served as a basal medium in all described experiments. For the induction of adventitious buds the cotyledon whorls of 6, 7, 10, 12 and 20-d old seedlings, were placed onto the basal media containing cytokinins (6-benzyl-aminopurine - BAP, zeatin - ZEA, kinetin - KIN) and auxins (naphtaleneacetic acid - NAA, indolylbutyric acid - IBA, indol-3-yl acetic acid - IAA), supplemented with spermidine (S). The phytohormone treatments in this experiment studied are shown in Table 1.

Table 1. Phytohormones used for induction of adventitious buds on cotyledons *.A. concolor* x *A. grandis*. (a) the effect of various cytokinins in combination with NAA; (b) the effect of various concentrations of BAP combined with NAA; (c) the effect of various auxin and spermidine in the presence of BAP.

Cytokinin [mg l^{-1}]		Auxin [mg l^{-1}]	
BAP	5	NAA	0.01
ZEA	5	NAA	0.01
BAP + KIN	5 + 5	NAA	0.01
BAP	1 - 10	NAA	0.01
BAP	5	NAA	0.05
BAP	5	NAA	0.1
BAP	5	S	14
BAP	2	IAA	2
BAP	5	IAA	2
BAP	3	NAA + IBA	0.25 + 0.125

The effect of phytohormones treatment duration on bud induction and its further development was studied on media containing BAP (5 mg l^{-1}) and NAA (0.01 mg l^{-1}) on which the cotyledons were cultured for 56, 28, 21 or 17 d, respectively. After this period the explants were transferred to hormone-free medium containing only 14 mg l^{-1} spermidine, because this medium was the most efficient one in our last experiments (Vooková *et al.* 1989). Induction of adventitious buds were scored after 56 d of cultivation. The explants were transferred to the same fresh medium every 4 weeks. The effect of pulse treatment with high concentration of BAP on bud induction was investigated, too. The cotyledons have been soaked in induction medium containing 25 mg l^{-1} BAP for 2 h. After this treatment they were placed on hormone-free agar medium containing 14 mg l^{-1} spermidine. After several weeks of growth, shoots longer than 1 cm were detached from the parent tissue and cultured

separately. Each combination of growth hormones was tested at least twice. Approximately 30 explants were used for each treatment.

Induction of adventitious buds was scored as the percentage of cotyledon fascicles on which buds were produced. The results were statistically evaluated using *t*-test.

Results and discussion

Cotyledons of seedlings placed onto the medium with phytohormones elongated, turned green and thickened after several days in culture. The growth apex assumed globular shape. Whether organogenesis appeared, it became evident after *ca.* 4 to 6 weeks of culture when adventitious needles or shoots appeared both on the growth apex and on individual cotyledons (Fig. 1 and 2).

Significant differences were observed in survival and organogenesis induction when explants of various ages were cultivated. Cotyledons from 20, 12 and 10 d old

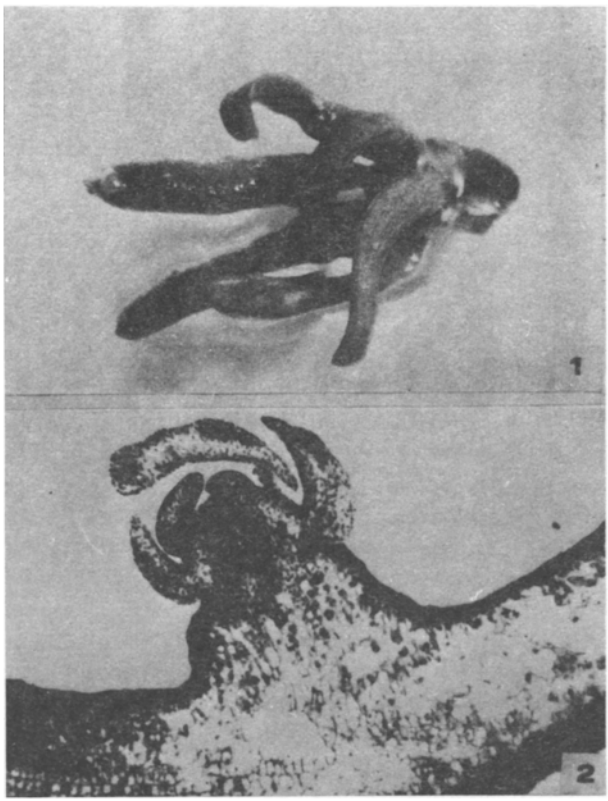

Fig. 1. Adventitious buds formed on cotyledons after cultivation on medium with 5 mg l^{-1} BAP and 0.01 mg l^{-1} NAA.
Fig. 2. Longitudinal section of a cotyledon with adventitious bud (× 50).

seedlings in culture necrotized earlier and induction of adventitious structures was either very sporadic, or none at all (in 20 and 12 d old cotyledons). Organogenesis appeared in 10 d old seedlings cotyledon culture with the highest frequency (38.4 %) on the medium containing 5 mg l^{-1} BAP and 14 mg l^{-1} spermidine. However, induced adventitious buds were not viable and together with the parent tissue had necrotized during subsequent period. The most suitable for adventitious bud induction were cotyledons of 7 d old seedlings which produced the highest number of buds capable of further development. These results were similar with those reported by Thorpe (1986), Aitken-Christie (1984) and Aitken-Christie *et al.* (1985) for *Pinus radiata* as well as with those by Toivonen and Kartha (1988) for *Pinus glauca* cotyledons. It is known, that epidermal and subepidermal cells of cotyledons predisposed to form shoots are active in division. In the older cotyledons of *Pinus radiata* the stomatal complex was fully developed, and epicuticular wax covered the entire cotyledon surface (Aitken-Christie *et al.* 1985). Older cotyledons have reduced lipid and protein levels and may therefore have insufficient reserves for organogenetic process, which, in part, has a high energy requirement (Thorpe 1980). Králík and Šebánek (1987) found out that after planting the acorns or seeds (*Quercus robur* L. and *Aesculus hippocastaneum* L.) and their swelling, an increase in the level of gibberelins was exhibited in the cotyledons, which was accompanied with the growth of radicle and plumula. Probably, 7 d old cotyledons have been still in the stage of increased level of phytohormones which may affect the shoot forming ability of the cotyledons.

The most efficient cytokinin tested for induction of adventitious buds was BAP, whereas ZEA stimulated rather the production of axillary shoots in cotyledonary axils. On the other hand, KIN was little efficient in inducing organogenesis. BAP in lower concentrations (1 to 3 mg l^{-1}) was not efficient enough which is demonstrated by the overgrowing of apical and rarely also of axillary shoots. Conversely, BAP in 4 and 5 mg l^{-1} concentrations stimulated the production of numerous adventitious buds on the surface of cotyledons and inhibited the overgrowing of apical shoots. Similarly as in *Pinus radiata* (Aitken-Christie 1984, and others), the highest induction of adventitious buds in *Abies concolor* × *Abies grandis* was achieved on medium containing 5 mg l^{-1}. Higher BAP concentrations (6, 8 and 10 mg l^{-1}) did not prove efficient in adventitious bud induction. They did not bring the expected increase of bud induction, on the contrary, the organogenesis droped. As a rule, the induced buds developed slowly and the explants necrotized rapidly (Table 2). The results obtained by several authors have shown that cytokinin concentration required for the induction of morphogenesis varies widely depending on the species, type and age of explant. Therefore, the optimal concentration for bud formation must be verified experimentally for each individual case.

Stimulating effect of BAP on the induction of organogenesis occurred in the presence of auxin, NAA being the most suitable. Of all the NAA concentrations tested it was 0.01 mg l^{-1} that best suited the production of well growing buds. Higher concentrations induced increased callogenesis, disintegration of cotyledonary fascicles and faster necrotization of individual cotyledons. IAA together with BAP induced formation of adventitious buds, however, only on a small number of explants. The combined treatments by the auxins IBA and NAA resulted in increased

callogenesis of explants. Another factor principally influencing adventitious bud induction and development was the duration of treatment with growth substances. The results are given in Table 3.

Table. 2. The effect of various BAP concentrations combined with 0.01 mg l^{-1} NAA on adventitious bud induction on 7 d old seedling cotyledons.

BAP [mg l^{-1}]	Bud induction [%]
2.00	10.00**
4.00	38.64
5.00	48.99
6.00	24.70**
8.00	8.93**
10.00	6.49**

** highly significant difference when comparing induction on medium with 5 mg l^{-1} BAP.

Table 3. The time cultivation effect of 7 d old seedling cotyledons on medium containing 5 mg l^{-1} BAP and 0.01 mg l^{-1} NAA on adventitious bud induction (the differences between the above given values were not significant).

Culture duration [d]	Bud induction [%]
56	44.50
28	52.17
21	54.51
17	48.99

In longer cultures (28 d) on induction medium containing cytokinin and auxin adventitious buds have been induced on 52.2 % of explants. But the further development of buds was mostly suppressed, or they produced only slowly growing shoots with short and broad needles which did not elongate even during long-term cultivation on hormone free medium. Similar result was achieved also in the embryos of *A. concolor* x *A. grandis* (Vooková *et al.* 1989). On the other hand, buds produced during 21 and 17 d treatment with phytohormones developed well, and the shoots, that appeared, did elongate (Fig. 3).Buds induced at 17 d cytokinin treatment were numerous (1 to 18 buds on 1 cotyledon) and their further growth was the most rapid. The BAP exposure time is critical in obtaining growth competent shoots (Amerson *et al.* 1985). After *ca.* 5 months of culturing they were separated from parent explant and cultivated individually (Fig. 4). Pulse (2 h) treatment by high concentration of cytokinin (25 mg l^{-1} BAP) did not bring expected results. Cotyledon fascicles thickened but no organogenesis appeared and explants necrotized rapidly.

Using this method, Bornman (1983) achieved not only the induction of a larger number of buds on the cotyledons of *Picea abies* but also shortening of culture period for obtaining results.

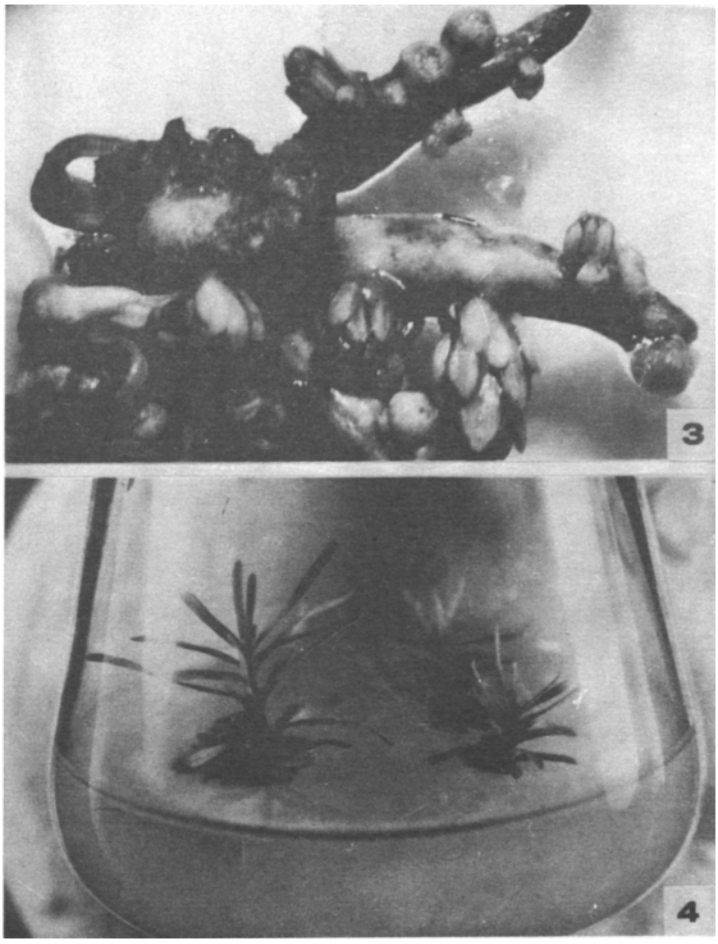

Fig. 3. Elongating buds on cotyledon explant after 21 d of culture.
Fig. 4. Cultivation of separated adventitious shoots.

We achieved growth of shoot on basal medium without growth substances, to which spermidine was added. The role of polyamines in various growth and developmental processes in a plant is known. It has appeared that exogenous application of spermidine induces cell division. Spermidine stimulated both DNA synthesis and a limited amount of mitotic activity in oat leaf protoplasts (Kaur-Sawhney *et al.* 1981). Polyamines can serve as "second messengers", mediating the effects of recognized hormones (Galston 1983).

Comparing the efficiency of inducing adventitious buds capable of producing shoots on embryos and cotyledons of seedlings of coniferous species, the results point in favour of the cotyledons. According to Bornman and Jansson (1982) cotyledons are suitable explants because they retain more of the embryogenic properties in their tissue. This means that they contain high levels of nutrients and are, owing to their anatomy, responsive to treatment.

Additional studies are under way to optimize technique for rooting the shoots and hardening these plantlets for field experimentation.

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