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In vitro propagation of European larch (*Larix decidua* Mill.)

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ABSTRACT: Methods of *in vitro* propagation of *Larix decidua* are described in the paper. The influence of explant source, tree age, genotype, composition of nutrient media and phytohormones on *in vitro* propagation of *Larix decidua* has been investigated. Needles, isolated vegetative buds, shoot tips, zygotic embryos and cotyledons were used as initial explants. Axillary and adventitious bud cultures were used for fast *in vitro* shoot multiplication. Root induction was stimulated on low salt medium supplemented with auxin. After rooting and hardening off, micropropagated trees were planted in experimental plots. The growth of micropropagated trees was comparable with the height and diameter growth of trees originated from seeds.

Keywords: *Larix decidua*; *in vitro* propagation; larch micropropagation; shoot multiplication; root induction; field growth of micropropagated trees

European larch (*Larix decidua* Mill.) is an important timber tree species, valued for fast growth and good wood properties. Trees of European larch posses many desirable attributes, are very hardy and show a great stress tolerance. They grow over wide cooler regions and their altitudinal range extends from lowlands to the mountains. As deciduous trees they are more resistant to air pollution than evergreen conifers. Larches are light and space demanding trees, tolerant of low temperature and often occupy sites unsuitable for other trees.

Larch trees have a low transpiration capacity in winter. The deciduous habit confers a significant advantage to larch in reduced susceptibility to winter desiccation damage. Their great demand for light is associated with the relatively low leaf mass and area. Larches are resistant to wind, snow, fire and air pollution. Their height and diameter growth is rapid, however, each tree needs light and space. Larch wood is very durable, of high technical value. Larch timber has good strength properties and larch pulp is suitable as raw material for paper with high tearing strength and can be used for high-quality printing paper by mixing it with short-fibered hardwoods. Larch trees combine rapid growth with high wood quality and good fibre characteristics.

Under the influence of different ecological conditions, larch trees differentiated into various ecotypes. Within the species, various provenances occurr and in climatic adaptation larches differentiated into mountainous and lowland ecotypes. Larch trees show considerable variability in stem and branching form, timber production, wood quality and disease and stress resistance. Rapid propagation of selected superior trees is important for mass production of trees with desirable genetic traits. At present, European larch is propagated mostly by seeds, however, seed germination is low and good seed harvests are not frequent. Vegetative propagation of larch is important, because of its potential to propagate valuable genotypes. Forest yield could be enlarged significantly by propagation of selected genotypes with improved growth rates, valuable wood quality and high disease resistance. Conventional practices of larch propagation are inadequate for rapid multiplication of selected genotypes. *In vitro* propagation of selected larch genotypes will contribute to the formation of more productive and resistant forests.

The main objectives of *in vitro* larch propagation relate to the desirability of cloning trees or hybrids that possess high growth rates and are more resistant to diseases and environmental stresses. *In vitro* technologies represent new approaches to overcome existing problems. The application of *in vitro* methods offers new prospects for fast propagation of selected genotypes and provenances. The method can be used for propagation of elite genotypes which do not produce seeds in sufficient quantity and for fast production of more resistant genotypes and can provide planting stock in a short time.

In past years various techniques for *in vitro* propagation of European larch have been tested, using isolated buds, nodal segments, zygotic embryos and leaf segments as initial explants. Plant regeneration and micropropagation of European larch has been achieved (CHALUPA l983, l985, l989, l990, l99l). *In vitro* multiplication depends on the control of organogenesis, which is influenced by internal and external factors. In this study, results obtained in

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recent experiments with *in vitro* propagation of European larch are described.

MATERIALS AND METHODS

Plant material

Tissue cultures of European larch (*Larix decidua* Mill.) have been initiated from explants taken from juvenile parts of plants. Embryos, shoot tips, needles, nodal segments and vegetative buds were used as initial explants. Shoots taken from trees 6 years old were used for experiments. Needles and nodal segments were rinsed in tap water for 20–30 minutes and surface sterilized by successive immersion in calcium hypochlorite solution (7.5% w/v) for 20 minutes and in mercuric chloride solution (0.1% $HgCl_2$) for 20–30 minutes, followed by rinses in sterile water. After sterilization, segments were washed three times in sterile distilled water and placed on agar nutrient medium. Seeds were surface sterilized in mercuric chloride solution for 20–40 minutes, followed by rinses in sterile distilled water. The embryos were aseptically excised from seeds and placed on nutrient medium.

Culture media and conditions

Explants were cultured on WPM (LLOYD, MC COWN 1980), BTM (CHALUPA 1983), MS (MURASHIGE, SKOOG 1962) , QL (QUOIRIN, LEPOIVRE 1977), or GD (GRESSHOFF, DOY 1972) medium. The basal medium was supplemented with glutamine (100–200 mg/l) and casein hydrolysate (100–200 mg/l) and 20–30 g/l sucrose. The media were solidified with Difco Bacto agar (7 g/l) or Gelrite (2.5 g/l) and adjusted to pH 5.7 before sterilization by autoclaving at 121°C for 20 min. Growth regulators and glutamine were filter-sterilized. Low salt nutrient media were used for stimulation of adventitious shoot formation and shoot elongation. Low salt media were also used for root initiation and root elongation. Growth and morphogenesis of larch cultures were regulated by the composition of nutrient medium and growth regulators in the medium.

Growth regulators added to nutrient medium included 6-benzylaminopurine (BAP) or 6-benzylamino-9-(2-tetrahydropyranyl)-9H-purine (BPA) or thidiazuron (TDZ) and auxin indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA).

Cultures were grown in growth cabinets at 23°C ± 1°C with 16 h photoperiod under the cool white fluorescent light with a photon flux density of 30–40 μmol/m^2/s. Each treatment involved 25–30 explants and was repeated at least twice. The explants were cultured in 100 ml flasks containing 30 ml of nutrient medium. Explants were cultured for 5–6 weeks and were transferred on fresh medium every 2–3 weeks.

Initiation of adventitious buds and shoot multiplication was stimulated by combinations of cytokinin and auxin in

Fig. 1. Formation of needles and shoots developed from adventitious buds induced on cultured isolated vegetative bud of *Larix decidua*

Fig. 2. Elongated shoots developed from adventitious buds induced on a shoot tip of *Larix decidua*

Table 1. Formation of adventitious shoots on *Larix decidua* needles (needles were cultured 4 weeks on WPM supplemented with BAP and 6 weeks on WPM supplemented with IBA 0.1 mg/l)

BAP (mg/l)	Needles with induced adventitious buds (%)	Needles with adventitious shoots (%)
2	40	36
3	46	44
4	38	33

culture medium. In rooting experiments, excised microshoots 15–25 mm long were used. Low salt media (WPM, BTM, QL, GD) were used for *in vitro* rooting.

RESULTS

In vitro shoot multiplication by axillary bud cultures

Axillary bud cultures are often used for shoot multiplication of broadleaved forest tree species (CHALUPA 1983) and were also used for European larch micropropagation (CHALUPA 1983, 1985, 1989, 1991). The main advantages of this method are that cultures are genetically stable and multiplication rates are high. Nodal segments and shoot tips of European larch placed on agar nutrient medium, produced elongated shoots within 5–6 weeks. New shoots reached the length of 25–30 mm and were cut into 2 to 3 nodal segments and shoot tips. The nodal segments exhibited formation and growth of axillary shoots in nutrient medium containing a low concentration of auxin. Of tested media, nutrient medium WPM, QL, and BTM supplemented with a low concentration of auxin (IBA 0.1–0.2 mg/l) stimulated growth of new shoots from nodal segments and shoot tips. Within 6–8 weeks new shoots reached a length of 20–40 mm and were cut into 2–3 nodal segments and shoot tips, which were used for further multiplication. Shoots from apical buds produced 2–3 axillary buds within 7–8 week culture period and shoots from axillary buds produced 1–2 buds. Axillary bud proliferation rate differed significantly among provenances and clones.

In vitro shoot multiplication by adventitious bud cultures

Adventitious buds were induced on embryos, cotyledons, needles, shoot tips and isolated vegetative buds cultured on WPM or QL medium, supplemented with cytokinin (BAP or BPA 1–3 mg/l) or TDZ (0.05 to 0.2 mg/l) or on media containing BAP (1 mg/l) plus TDZ (0.1 mg/l). Cultured cotyledons produced adventitious buds frequently. On average 70–85% cultured cotyledons produced adventitious buds within 3–5 weeks. Cotyledons with induced buds were trasferred to media lacking cytokinin and after trasfer to the fresh WPM or QL medium supplemented with a low concentration of auxin (IBA 0.1–0.2 mg/l), shoot development was promoted. Each cotyledon produced 2–4 shoots.

Induction of adventitious buds on needles was significantly affected by needle age, young needles formed adventitious buds in higher frequency. The adventitious buds were formed mostly on the basal part of needles and number of adventitious buds decreased from the base to the apex of needles. Needles were cultured on WPM or QL medium supplemented with cytokinin (BAP or BPA 2–4 mg/l, or TDZ 0.2–0.4 mg/l) for 4–6 weeks. On average, 38–46% of cultured needles produced adventitious buds (depending on the clone – Table 1). Needles with induced adventitious buds were transferred to WPM lacking cytokinin and containing a low concentration of auxin (IBA 0.1 mg/l) where shoots developed. Shoots 0.5–1 cm long were separated and cultured individually. Needles with induced adventitious buds produced 1–4 shoots. Elongated shoots were used for further multiplication or for rooting.

Adventitious shoots were also induced on isolated vegetative buds. WPM and QL medium supplemented with BAP (2–4 mg/l) promoted formation of adventitious buds on 44–52% of explants (Table 2). Shoot elongation was stimulated on WPM or QL medium supplemented with a low concentration of auxin (IBA 0.1–0.3 mg/l). Explants with induced buds produced 3–5 shoots.

Adventitious buds and shoots were also induced on shoot tips immersed in agar nutrient medium supplemented with cytokinin. The upper part of the shoot tip was immersed in agar medium and the lower part of the segment projected above the agar surface. Shoot tips were cultured in inverted position in WPM or QL medium supplemented with BAP (1–4 mg/l) or with TDZ

Table 2. Formation of adventitious shoots on *Larix decidua* vegetative buds (buds were cultured 4 weeks on WPM supplemented with BAP and 6 weeks on WPM supplemented with IBA 0.1 mg/l)

BAP (mg/l)	Vegetative buds with induced adventitious buds (%)	Vegetative buds with adventitious shoots (%)
2	44	40
3	52	45
4	46	42

Fig. 3. Rooted microshoot of *Larix decidua*. Shoot developed from an adventitious bud induced on a needle

(0.1–0.3 mg/l) for 3 to 4 weeks. After the transfer on the medium lacking cytokinin, where shoot tips were cultured in normal position, shoots developed from induced adventitious buds.

Also shoot tips soaked in a cytokinin solution and then cultured on an agar nutrient medium produced adventitious buds and shoots. Shoot tips were soaked in BAP (10–50 mg/l) or TDZ solution (1–3 mg/l) for 3 to 6 hours. After this hormonal treatment, the shoot tips were cultured on WPM or QL medium lacking cytokinin. Within 6 to 7 weeks new shoots elongated from induced buds. Shoot tips with induced buds produced 2–5 new shoots which were used for further multiplication (Table 3).

Rooting of microshoots

Rooting of microshoots consisted of two-step process. Microshoots selected for rooting, were first placed on the medium containing auxins and after the hormone treatment, shoots were transferred to medium lacking growth regulators. Low salt media were used for root induction. The microshoots selected for rooting were placed on WPM (half strength) containing auxins (NAA 2mg/l plus IBA 2 mg/l). After 20–30 days of the hormone treatment, the shoots were transferred on media lacking growth hormones, where roots elongated. Rooting percentages varied among clones and ranged between 47–83%. Rooted microshoots were trasplanted into pots. After hardening off outside, the micropropagated trees were planted in the field.

Field performance of *in vitro* propagated trees

After rooting and hardening off micropropagated trees were trasferred to experimental plots. The planting was usually done in early summer. At the end of the first growing season, the planted micropropagated trees attained a height of 27–45 cm. The survival of micropropagated trees planted in experimental plots was high and was comparable with the survival of trees originated from seeds. Small differences in height growth of trees originated from different explant sources were observed. Micropropagated trees planted in experimental plots survived the winter without losses and next spring continued in growth. Even in the following years the growth of *in vitro* propagated trees was comparable with the height and diameter growth of trees originated from seeds.

DISCUSSION

European larch is propagated mostly by seed. Low seed germination and growing importance to propagate the most valuable genotypes of larch, focused the attention to the *in vitro* propagation methods. In last decades considerable progress has been achieved in propagation of forest trees by *in vitro* methods. *In vitro* propagation of trees is a promising method for fast propagation of selected superior genotypes. The importance of tissue cultures as new propagation methods of forest trees continues to grow.

Experiments with *in vitro* propagation of larch showed that new technologies can be successfully used for fast propagation of valuable larch genotypes (CHALUPA 1983, 1985,1989, 1991; BONGA 1984; BONGA, VON ADERKAS 1988; CHALUPA, ALDEN 1988; KLIMASZEWSKA 1989a,b; LALIBERTÉ, LALONDE 1988; NAGMANI, BONGA 1985).

Table 3. Induction of buds and shoot formation on *Larix decidua* shoot tips [shoot tips were soaked in BAP solution for 3 hours and then cultured on agar WPM supplemented with IBA (0.1 mg/l)]

BAP solution (mg/l)	Shoot tips with induced new shoots (%)	Number of new shoots
10	75	2.5 ± 1.7
20	82	3.2 ± 1.8
40	84	2.8 ± 1.6

A system based on micropropagation of larch by *in vitro* methods was improved and used for production of larch plants for field tests. Experiments presented in this paper are a continuation of our previous investigations.

The efficiency of shoot multiplication was affected by the medium composition and concentration of external growth regulators. Low concentration of cytokinin and auxin stimulated axillary bud development and shoot multiplication. The initiation of adventitious bud formation on needles and isolated buds was stimulated by combination of higher concentration of cytokinin and low concentration of auxin. Adenine types cytokinin and thidiazuron effectively induced adventitious bud formation on needles and isolated buds. Low concentration of auxin stimulated shoot development from induced adventitious buds. Rooting experiments showed that microshoots, after auxin treatment, produce roots in high frequency.

Experiments demonstrated that micropropagated larch trees can be successfully trasplanted into soil, hardened and grown in the field. *In vitro* propagated trees exhibited normal growth and appearance. Height and diameter growth of trees produced from explants was rapid and after four years of growth on experimental plots, dimensions of micropropagated trees were comparable with dimensions of trees produced from seeds. The high multiplication rates obtained using axillary and adventitious bud cultures suggest the possibility of using this methods in commercial production. Successful regeneration of larch trees from needles, axillary buds and shoot tips allows propagation of new productive varieties and genotypes. Larch trees with desirable genetic traits might be produced by propagation of existing superior genotypes and by propagation of hybrids obtained by sexual crossings.

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In vitro rozmnožování modřínu opadavého (*Larix decidua* Mill.*)*

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ABSTRAKT: Experimenty byly zaměřeny na vypracování vhodných metod pro rychlé *in vitro* rozmnožování modřínu opadavého (*Larix decidua).* Byl zjišťován vliv auxinů a cytokininů na indukci adventivnich a axilárních pupenů a jejich další vývin

v prýty. Prýty rozmnožené *in vitro* byly zakořeněny a po otužení byly stromky vysazeny na venkovní pokusné plochy. Rozměry stromků vypěstovaných *in vitro* a rostoucích na venkovních pokusných plochách se významně nelišily od rozměrů stromků vypěstovaných ze semen.

Klíčová slova: *Larix decidua*; *in vitro* rozmnožování modřínu; indukce adventivních pupenů a kořenů; růst *in vitro* vypěstovaných stromků na pokusných plochách

Modřín opadavý (*Larix decidua* Mill.) patří k našim významným jehličnatým lesním stromům, ceněným zejména pro rychlý růst, odolnost ke znečištěnému ovzduší a velmi dobré technické vlastnosti dřeva. Pro zachování odolných genotypů s vynikajícími vlastnostmi je třeba dosáhnout jejich rychlého rozmnožování. Reprodukce lesních stromů metodami *in vitro* má velký význam pro záchranu odolných a rychle rostoucích populací lesních dřevin. Cílem rozmnožování lesních stromů těmito metodami je dosáhnout zlepšení genetických vlastností pěstovaných porostů rozmnožením odolných a produktivních genotypů.

Naše experimenty byly zaměřeny na vypracování vhodných metod pro rychlou reprodukci modřínu opadavého. Byl zjišťován vliv auxinů a cytokininů na indukci adventivních a axilárních pupenů a jejich další vývin v prýty a zakořenění vypěstovaných prýtů. K založení kultur byly použity jednak explantáty z juvenilního materiálu (embrya, semenáčky), jednak prýty z juvenilních částí dospělých stromů. Jako počáteční explantáty byly použity jehlice, axilární pupeny, vrcholové špičky prýtů, kotyledony a embrya. Explantáty byly po sterilizaci pěstovány na agarových živných médiích (WPM, QL, BTM, MS, GD médium) za kontrolovaných vnějších podmínek v růstových komorách (při teplotě 22–24 °C).

Nízké koncentrace auxinu v agarovém živném médiu stimulovaly vývin a prodlužování prýtů z axilárních a adventivních pupenů. Vytváření adventivních pupenů bylo stimulováno na kotyledonech, jehlicích, vegetativních pupenech a vrcholcích prýtů při jejich pěstování na živných médiích obsahujících vyšší koncentrace cytokininů. Cytokininy adeninového typu a thidiazuron stimulovaly vytváření adventivních pupenů na jehlicích a na izolovaných pupenech.

Prýty rozmnožené *in vitro* byly zakořeněny na živných médiích obsahujících nízké koncentrace auxinu. Bylo dosaženo vysokého procenta zakořenění prýtů vypěstovaných *in vitro*. Po otužení byly stromky vypěstované *in vitro* vysazeny na venkovní pokusné plochy. Výškový a tloušťkový růst stromů vypěstovaných *in vitro* byl rychlý a jejich rozměry se po několikaletém pěstování na pokusných plochách významně nelišily od rozměrů stromů vypěstovaných ze semen.

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