Changes in Development of European Larch (*Larix decidua* Mill.) Vegetative Buds Induced by Plant Hormones

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Abstract

Effects of exogenously applied auxins and cytokinins on morphogenesis of European larch vegetative buds collected from 40-year-old tree were investigated using plant tissue culture techniques. Explants were prepared as short segments with axillary buds cut from the current year twigs or one-year-old twigs and were cultivated on modified MS medium. Different variants of nutrient medium were featured by different content of phytohormones: abscisic acid, auxins (indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid), cytokinins (kinetin and 6-benzylaminopurine) and gibberellin (gibberellic acid-3). Abscisic acid negatively affected organogenesis in larch vegetative buds. Negative effect of auxins on the development of basal needles was significant after larch buds were cultivated *in vitro* for 25 days but after 75 days the tendency was observed that the number of explants with newly developing shoots increases with increasing concentration of auxins. Auxins strongly increased callus formation on larch explants. Cytokinins blocked elongation of needles and induced formation of long-shoot primordia (structures able to form new meristems and to develop adventitious buds). Positive effect of cytokinins on viability of larch buds was significant in dark-grown explants, while large amounts of cytokinins have decreased the viability of light-grown explants (but less significantly). Gibberellin was able to promote the development of axial needles in the shoot apex zone.

Key words: larch, bud development, cytokinins, auxins, gibberellin, phase change

Introduction

European larch (*Larix decidua* Mill.) is one of introduced conifer species in Lithuania. It can take more significant economic importance. European larches grow faster than trees of native conifer species in Lithuania: Norway spruce and Scots pine (Туминаускас, Раманаускас 1983). Larch species are recognized as important for timber production (because of its rot resistance, larch wood is especially valuable for posts, transmission poles, railroad ties), habitat or food for wildlife, watershed protection, enviromental forestry, and also for ornamental purposes (Rudolf 1974).

It is important to investigate methods allowing propagation of superior European larch trees. Vegetative micropropagation by using plant tissue techniques *in vitro* could help to keep valuable properties of certain genotype in new plants. This occurs regularly in poplar and other broadleaf cultivation but is rare in conifer cultivation (John 2002). One of the major problems facing the implementation of clonal forestry is phase change that can be defined as the series of changes that occur when a tree passes from the juvenile phase in which there is the ability to initiate adventitious structures and general absence of flowering to the mature phase where flowering is common and the ability to initiate adventitious structures is lost or dramatically reduced (Wareing 1959). Maturation is not only an interesting developmental phenomenon, but a major concern to those wishing to use tissue culture for propagation or application of biotechnological methods to forest trees. Commercially desirable genotypes of conifers can normally be identified as "superior" only after they have reached their adult phase. Not earlier trees can be ranked reliably according to their economic traits such as long-term growth, straightness and wood properties (Zhang *et al.* 2003). But it is very difficult to regenerate plants from tissues of conifer trees once they have passed the embryonic or seedling stage. Furthemore, without

reversal of maturation, clonal propagules (if obtainable) from mature trees will not exhibit the period of rapid growth associated with a juvenile phase. Juvenile behavior of seedling explants may be easily lost, but mature characteristics appear quite persistent (Greenwood *et al.* 1989). In contrast to juvenile larch cultures, mature plant material is characterized by slower shoot growth and enhanced formation of short shoots (Ewald 1998). The short-shoot buds show a large quantity of basal-needle primordia and there are no axial-needle primordia, in contrast to the long-shoot buds featured by a visible shoot apex with axial-needle primordia (Remphrey and Powell 1984). Because of their disability to elongate short shoots cannot be used as a material for rooting, therefore they are not desirable in micropropagation. Phytohormonal treatment (cytokinins combined with auxins) is necessary wishing that short shoots would be able to elongate (Kretzschmar 1993). But fine results inducing short shoot elongation *in vitro* were obtained with juvenile plant material, while cultivation of short shoots formed on adult tree is more problematic. Even if they form roots (spontaneously or when induced), they do not start to elongate (Ewald 1998). According to these facts it is important to find ability to manipulate the mature phase in vegetative buds of larch. Reports about the role of phytohormones in regulation of phase change suggest that enrichment of nutrient medium with plant hormones may at least give some knowledge about the control of maturation in particular tree species. In a common view of hormonal signal transduction in plants, a particular signal activates a signalling cascade that recruits specific transcription factors (Vogler and Kuhlemeier 2003). These trancription factors activate downstream executor genes, which in turn carry out the required response. Also biosynthesis of one kind of plant hormones can be regulated by transcription factors (both positive and negative) induced by another. The question, whether the ratios of abscisic acid, gibberellins, auxins and cytokinins play a primary role in causing maturational change (Haffner *et al.* 1991), or changes in relative concentrations of plant hormones are, like morphological characteristics, symptoms of a more fundamental driving force (Greenwood 1995), remains still open.

According to high relevance between various physiological proceesses in plants that can be more or less strictly regulated by different phytohormones it was important to adjust some basic points. The aim of our research was to fix changes in development of European larch vegetative buds induced by different phytohormones in various concentrations and combinations.

Materials and methods

In our research the investigation of two widely used in propagation of plant tissue cultures types of phytohormones – cytokinins and auxins – has been chosen for the main task. The concentrations of these phytohormones that were found suitable for micropropagation of other woody plant species (as aspens) (Gradeckas *et al.* 2001) were applied for cultivation of adult larch buds trying to fix attention firstly on morphological features probably related with phase change in investigated buds, as formation of adventitious structures or elongation of shoot axis. For wider understanding, some experiments were carried out on abscisic acid and gibberellin but combined with auxin treatment according to the data about the role of auxins in regulation of gibberellin (Ross *et al*. 2000) and abscisic acid (Rodrigo and García-Martínez 1998) content. Since major seasonal changes are characteristic of naturally occuring concentrations of latter phytohormones (Tanino 2004), these experiments were carried out in different seasons.

Abbreviations

ABA – abscisic acid
BAP – 6-benzylaminopurine
2,4-D – 2,4-dichlorophenoxyacetic acid
GA – gibberellic acid (gibberellin)
IAA – indole-3-acetic acid

Plant material and growth conditions. The current year twigs and one-year-old twigs were collected from the lower one-third of the crown of 40-year-old European larch tree (dates of collection differ in different experiments). After removal of needles the twigs were cut into short pieces (1-2 cm, each segment with an unburst vegetative bud). Segments of twigs were soaked for 3 minutes in 75 % ethyl alcohol and then for 4 minutes in 0.1 % solution of silver nitrate. After sterilization larch explants were prepared as follows: all tissues except green bud meristems were removed from woody cores using sterile pincers. Bare buds were cultivated in glass test-tubes (height 15 cm, diameter 2 cm) under regulated light and temperature regime. The following growth conditions were kept: white-light photoperiod of 16 h and 20 ºC temperature. Modified MS nutrient medium (Murashige and Skoog 1962) containing 24 g/l sucrose (pH 5.5 before autoclaving) enriched with different concentrations of phytohormones (obtained from ICN Biomedicals GmbH, Germany) was used for cultivation of explants.

Treatment with abscisic acid. Axillary buds of the current year twigs harvested in the first decade of August were cultivated on MS nutrient medium enriched with auxins (0.2 mg/l IAA and 0.02 mg/l 2,4-D) and different concentrations of abscisic acid (ABA):

a) 2 mg/l;
b) 8 mg/l.

MS medium with IAA and 2,4-D but without ABA was used as a control variant. Each variant contained 30 explants. In culture we counted explants with developmental features characterized as: proliferating tissues (after 12, 20 and 30 days), organogenesis (after 20 and 30 days) and shoot development (after 30 days).

Treatment with auxins. Axillary buds of the current year twigs harvested in the second decade of October were cultivated on MS nutrient medium enriched with different concentrations of auxins. Three variants were tested:

a) IAA 0.05 mg/l, 2,4-D 0.01 mg/l;
b) IAA 0.2 mg/l, 2,4-D 0.02 mg/l;
c) IAA 0.6 mg/l, 2,4-D 0.04 mg/l.

Each variant contained 36 explants. Morphological changes of explants were assessed after 25 days and then after 75 days.

Treatment with cytokinins. Two variants were tested by investigating the effect of cytokinins (axillary buds were collected in the second decade of October):

a) kinetin 0.15 mg/l, BAP 0.05 mg/l;
b) kinetin 2.25 mg/l, BAP 0.75 mg/l.

Viability and morphogenesis of dark-grown explants treated with cytokinins (the same concentrations) also was tested (larch explants were cultivated in absence of light for 20 days). Each variant contained 24 explants. Viability (featured by greening) and morphological changes of explants were assessed after 30 days in both cases. Further development of explants that have formed long shoot primordia on nutrient medium with cytokinins during the first subculture was observed on nutrient medium without phytohormones or on medium enriched with auxins (IAA 0.2 mg/l, 2,4-D 0.02 mg/l).

Combined treatment with cytokinins and auxins. Vegetative buds of one-year-old twigs harvested in the first decade of January were cultivated on MS nutrient medium enriched with auxins (0.24 mg/l IAA and 0.03 mg/l 2.4-D) or cytokinins (2.25 mg/l kinetin and 0.75 mg/l BAP, in the medium with cytokinins used for the second subculture the amounts were reduced to 1.8 mg/l kinetin and 0.6 mg/l BAP). Four variants of combined phytohormonal treatment (in two subcultures) were used:

Variant 1. 1st subculture: kinetin 2.25 mg/l, BAP 0.75 mg/l; 2nd subculture: kinetin 1.8 mg/l, BAP 0.6 mg/l;

Variant 2. 1st subculture: kinetin 2.25 mg/l, BAP 0.75 mg/l; 2nd subculture: IAA 0.24 mg/l, 2.4-D 0.03 mg/l;

Variant 3. 1st subculture: IAA 0.24 mg/l, 2,4-D 0.03 mg/l; 2nd subculture: kinetin 1.8 mg/l, BAP 0.6 mg/l;

Variant 4. 1st and 2nd subcultures: IAA 0.24 mg/l, 2.4-D 0.03 mg/l.

Each variant contained 36 explants. Morphological changes, including callus formation, elongation of needles and formation of long-shoot primordia (structures very similar to the apex of new-developing long shoot in germinating bud before elongation), were assesssed after two subcultures (1st subculture continued for 15 days and 2nd subculture continued for 25 days). Further development of explants was observed during the third subculture on nutrient media with different concentrations of auxins (either with 0.06 mg/l IAA and 0.01 mg/l 2.4-D or with 0.6 mg/l IAA and 0.04 mg/l 2.4-D).

Treatment with gibberellin. Axillary buds of the current year twigs harvested in the first decade of March were cultivated on MS nutrient medium enriched with the following concentrations of phytohormones:

a) 0.08 mg/l gibberellin (GA_3)
b) 0.08 mg/l GA_3 and 0.32 mg/l IAA.

Morphological changes in explants, including development of basal and axial needles, were assessed after 22 days. Each variant contained 18 explants.

Control and statistics. MS nutrient medium without phytohormones was used for the control in all cases, except investigation of ABA effect (here explants of the control group were cultivated on MS medium with 0.2 mg/l IAA and 0.02 mg/l 2.4-D).

Bias of values expressed in percentage (S_p) was used for statistical verification of reliability of obtained results. It was calculated by the formula:

$$S_p = \pm\sqrt{p(100-p)/n}$$

here p – value of an analysed parameter expressed in percentage, n – sample size.

Results

Effect of ABA treatment. Abscisic acid in applied concentrations had negative effect on organogenesis in vegetative buds of larch (Table 1). 2 mg/l ABA in the nutrient medium have decreased the rate of organogenesis. During 30 days one sixth of the explants in the control group have formed shoots with well-expressed developmental pattern while this rate has been decreased by treatment with 2 mg/l ABA. 8 mg/l ABA in the nutrient medium had more significant negative effect on the development of larch buds and totally blocked organogenesis. The number of buds with proliferating tissues was significantly smaller in the group of explants treated with 8 mg/l ABA after 12 days in culture. But even a large amount of ABA

Table 1. Development of European larch buds under ABA treatment

Period of cultivation	Morphological changes	Control	ABA 2 mg/l	ABA 8 mg/l
12 days	Proliferating tissues (%)	76.7±7.7	84.6±7.1	53.1±8.8
20 days	Proliferating tissues (%)	90.0 ±5.5	88.5±6.3	78.1±7.3
	Organogenesis (%)	30.0±8.4	15.4±7.1	-
30 days	Proliferating tissues (%)	90.0±5.5	88.5±6.3	84.4±6.4
	Organogenesis (%)	33.3±8.6	19.2±7.7	-
	Shoot development (%)	16.7±6.8	7.7±5.2	-

had no significant negative effect on the viability of explants. After 30 days in culture the rates of buds with proliferating tissues were close in all three variants. The buds with proliferating tissues, that were not able to start organogenesis, formed only callus tissue. In general, explants of the control group have formed more plentiful callus than those treated with abscisic acid.

Effect of auxin treatment. Auxins have induced noticeable changes in the development of larch buds prepared from the current year larch twigs in October (Table 2). Auxins had negative effect on normal development of both axial and basal needles. The number of buds that have sprouted long needles was significantly decreased by auxins. After 25 days in culture the most significant difference from the control group has been observed in the group of explants treated with 0.6 mg/l IAA and 0.04 mg/l 2.4-D. There was not any explant in this group that has developed long needles from formed primordia, whereas a half of explants in the control variant had normally elongating needles. Smaller amounts of auxins also had strong negative effect on the development of larch needles. During further cultivation the number of shoots with green needles slightly decreased in the control group of explants and in the group of explants treated with 0.05 mg/l IAA and 0.01 mg/l 2,4-D, while the rate of shoots with browning apical zones (languished axial needles or their primordia) increased. But on the nutrient medium with 0.6 mg/l IAA and 0.04 mg/l 2,4-D the number of explants with well-developed needles increased in time. The buds that did not sprout elongating needles formed only very short needles or did not develop entirely and kept browning. After 25 days the best rate of the viability has been observed among explants treated with 0.05 mg/l IAA and 0.01 mg/l 2,4-D. During this period the viability of explants was decreased by larger amounts of auxins. One feature of developing larch vegetative buds has significantly increaced by any concentration of auxins: most explants were able to develop only short needles. These structures often were similar to long-shoot primordia and, as we observed during their further development, not only by morphological appearance (reduced elongation of needles, their circular disposition), but also functionally. During 25 days almost three quarters of explants showed reduced development of needles on the medium with 0.05 mg/l IAA and 0.01 mg/l 2.4-D. This rate was slightly smaller on the medium with 0.6 mg/l IAA and 0.04 mg/l 2,4-D, and significantly smaller on the medium with 0.2 mg/l IAA and 0.02 mg/l 2,4-D, because of decreased viability of explants. As showed morphological changes of explants that have been assessed after 75 days in culture, most of these explants with decreased elongation of needles either stopped developing, or were able to induce development of the shoot apical zone leading to elongation of the shoot axis (Figure 1). New-induced shoot formation was the only feature in development of larch vegetative buds that had a tendency to increase with increasing concentration of auxins. Between the first and the second assessments of morphological changes the viability of explants, treated with 0.05 mg/l IAA and 0.01 mg/l 2.4-D, decreased to the most considerable degree. In oth-

Table 2. Development of European larch buds under auxin treatment

Variants of the nutrient medium	Explants with visible shoot apical zone		Well-expressed development of needles (%)		Browning explants (%)	
	Reduced development of needles after 25 days(%)	Newly developing shoot apical zones after 75 days (%)	After 25 days	After 75 days	After 25 days	After 75 days
Control	23.5±7.3	5.9±4.0	50.0±8.6	44.1±8.5	23.5±7.3	41.2±8.4
IAA 0.05 mg/l, 2,4-D 0.01 mg/l	73.5±7.6	12.1±5.7	11.8±5.5	6.1±4.2	14.7±6.1	75.8±7.5
IAA 0.2 mg/l, 2,4-D 0.02 mg/l	47.2±8.3	22.9±7.1	5.6±3.8	5.6±3.8	41.7 ±8.2	62.9±8.2
IAA 0.6 mg/l, 2,4-D 0.04 mg/l	66.7±7.9	32.4±8.0	-	11.8±5.5	30.6±7.7	52.9±8.6

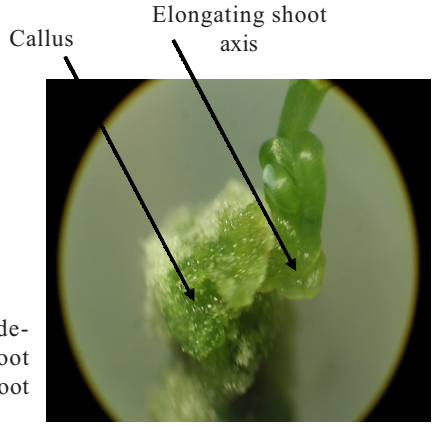

Figure 1. New-developing larch shoot with elongating shoot axis

er variants the rates of a decrease in viability of explants were significant, but not so dramatic.

Effect of cytokinin treatment. The effect of cytokinins (Table 3) was mainly characterized by total formation of long-shoot primordia (buds with clearly visible shoot apex, Figure 2). All explants of the current year twigs treated with each of the tested concentrations of cytokinins were able to form long-shoot primordia. Some effects of cytokinins on the viability of larch explants were observed. Large amounts of exogenous cytokinins (2.25 mg/l kinetin and 0.75 mg/l BAP) decreased the viability of light-grown explants but the difference was not very significant. Small amounts (0.15 mg/l kinetin and 0.05 mg/l BAP) had no effect on the viability of explants, only induced formation of long-shoot primordia. In contrast, the viability of explants grown in the absence of light for 20 days was positively affected by treatment with cytokinins. Only less than one-third of explants remained viable (not totally brown or languished) in control group after cultivation in the absence of light. But their morphological appearance was different from that light-grown explants of the control group. Normal development of short shoots from lateral buds typical of explants cultivated in the light without treatment with plant hormones was lost. Only very short colourless needles developed from buds of dark-grown larch explants of the control group. Exogenous cytokinins were able to increase the viability of larch explants during cultivation in the absence of light, as compared to the control group. The significance of this effect depended on the concentration of cytokinins in the nutrient medium. All buds treated with cytokinins started to form long shoot primordia, but some of them kept browning. Only less than one quarter of dark-grown explants treated with 0.15 mg/l kinetin and 0.05 mg/l BAP had green long-shoot primordia. In this context, positive effect of a large amount of cytokinins on the viability of long-shoot primordia after dark-treatment was significant. The results were opposite with light-grown explants. All viable explants had green long-shoot primordia when treated with small amounts of kinetin and BAP, and significantly smaller part of explants were able to maintain green shoot apexes under treatment with large amounts of cytokinins.

Table 3. Development of European larch buds under cytokinin treatment

Variants of the nutrient medium	Cultivation in the light		Cultivation in the dark	
	Viable explants (%)	Explants with green long-shoot primordia (%)	Viable explants (%)	Explants with green long-shoot primordia (%)
Control	82.6±7.9	-	30.4±9.6	-
Kinetin 0.15 mg/l, BAP 0.05 mg/l	82.6±7.9	82.6±7.9	44.0±9.9	24.0±8.5
Kinetin 2.25 mg/l, BAP 0.75 mg/l	69.6±9.6	60.9±10.2	62.5±9.9	45.8±10.2

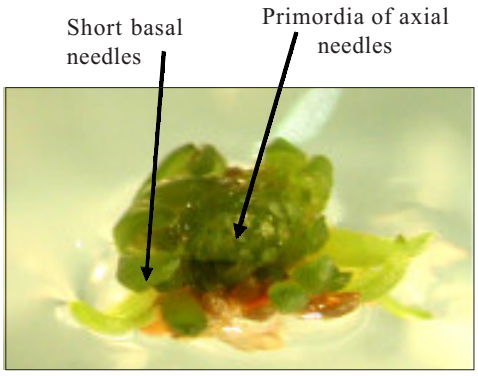

Figure 2. Long-shoot primordium of larch

Investigating further development of long-shoot primordia formed during the first subculture we observed that medium concentrations of auxins (0.2 mg/l IAA and 0.02 mg/l 2.4-D) had negative effect on the formation of adventitious buds. Only 6.7% explants started to form adventitious buds on nutrient medium with auxins during 25 days in the second subculture. Vice versa, 46.2% long-shoot primordia were able to start the formation of adventitious buds (Figure 3) on the medium without phytohormones. Callus formation was strongly increased by auxins (86.7%). 53.9% long-shoot primordia formed callus on the medium without plant hormones, too, but callus was not so plentiful as formed under treatment with IAA and 2.4-D. In general, further development of explants on nutrient

medium without phytohormones during the second subculture was various. Some explants that had not formed adventitious buds languished, but some of them developed long needles around long shoot primordium. Other explants developed either many small adventitious buds or several larger structures, similar to developing long shoots. Explants that had been cultivated on the medium with auxins and had formed plentiful callus lost their viability and kept browning.

Effect of combined treatment with cytokinins and auxins. The results obtained after larch buds had been cultivated under combined treatment with cytokinins and auxins are shown in Figure 4. Cytokinin caused total changes in the development of vegetative buds from the one-year-old twigs that were similar to the changes induced on explants from the current year twigs: elongation of needles was blocked and the formation of long-shoot primordia was induced on almost all treated explants. The development of needles blocked by cytokinins was restored during succeeding subcultures and one-third of ex-

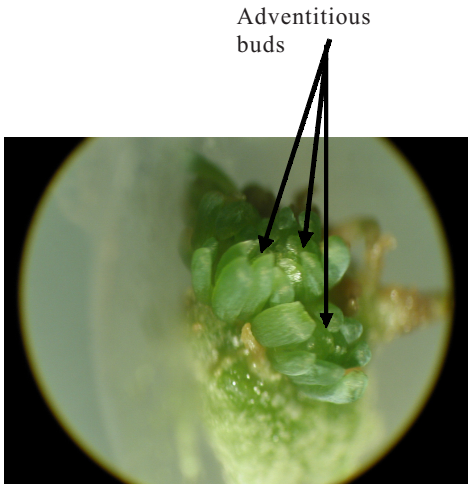

Figure 3. Adventitious buds formed on the long-shoot primordium

plants grown on the medium with cytokinins during the first subculture had normally developed basal needles after the second subculture on the medium with auxins). Exogenous cytokinins had no significant effect on callus induction. The formation of callus tissue was strongly increased by auxins. Interestingly, auxins were able significantly to increase callus formation only after some time of cultivation in vitro. The rate of callus formation on explants treated with auxins only in the first subculture had no difference from that of the control group but this rate strongly increased when auxins were used in both or only in the second subcultures. Auxins had slight neg-

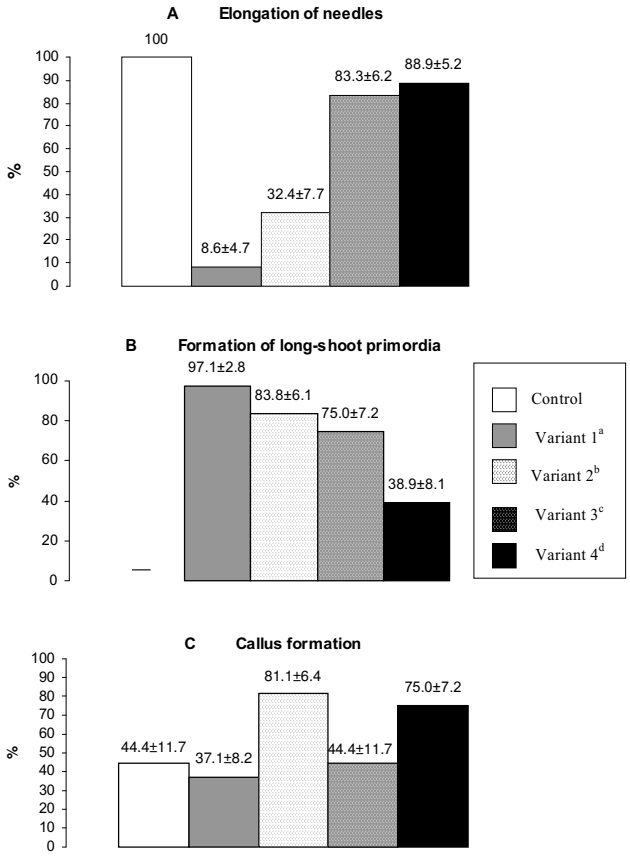

Figure 4. Development of European larch buds under combined cytokinin and auxin treatment: A. Elongation of needles; B. Formation of long-shoot primordia; C. Callus formation

[a] Kinetin 2.25 mg/l, BAP 0.75 mg/l for 15 days and kinetin 1.8 mg/l, BAP 0.6 mg/l for 25 days

[b] Kinetin 2.25 mg/l, BAP 0.75 mg/l for 15 days and IAA 0.24 mg/l, 2,4-D 0.03 mg/l for 25 days

[c] IAA 0.24 mg/l, 2,4-D 0.03 mg/l for 15 days and kinetin 1.8 mg/l, BAP 0.6 mg/l for 25 days

[d] IAA 0.24 mg/l, 2,4-D 0.03 mg/l for 15 days and then for 25 days

ative effect on elongation of basal needles as morphology of explants was assessed after two subcultures but we should mention that at the start of bud development explants on the medium with auxins showed more expressed development of axial needles and the expansion of basal ones was somewhat delayed. Auxins induced the formation of long-shoot primordia on the part of explants but this effect was not so total as that of cytokinins. Cytokinins were able to induce the formation of long-shoot primordia on the major part of explants also when used only in the second subculture.

During the third subculture explants of one year old twigs were cultivated on the media with different concentrations of auxins (either with 0.06 mg/l IAA and

0.01 mg/l 2.4-D or with 0.6 mg/l IAA and 0.04 mg/l 2.4-D). 27.8% of explants treated with cytokinins (2.25 mg/l kinetin and 0.75 mg/l BAP) during the first subculture and with auxins (0.24 mg/l IAA and 0.03 mg/l 2.4-D) during the second one started to develop elongating shoots on nutrient medium with small concentrations of auxins (0.06 mg/l IAA and 0.01 mg/l 2.4-D).

Effect of gibberellin treatment. Gibberellin (GA_3) had negative effect on the development of basal needles (Table 4). The rate of explants with normally developing short shoots has significantly decreased. GA_3 treatment both alone and supported with auxin treatment strongly increased the development of axial needles. All viable buds in these variants showed well-expressed development of needle primordia in the apical zone. Significant role of exogenous auxin was observed only in assessing the development of basal needles that was more significantly prevented by combined treatment with GA_3 and IAA than with GA_3 alone. It should be mentioned that even the explants able to develop basal needles after phytohormonal treatment did not do this so fast as explants of the control group. Neither GA_3 nor IAA had negetive effect on the viability of explants. Of interest is the fact that one explant on the medium containing both gibberellin and auxin started developing an adventitious shoot from callus.

Table 4. Development of European larch buds under gibberellin treatment

Variants of the nutrient medium	Developing basal needles (%)	Developing axial needles (%)	Browning explants (%)
Control	88.9±7.4	38.9±11.5	11.1±6.2
GA_3 0.08 mg/l	61.1±11.5	94.4±5.4	5.6±5.4
GA_3 0.08 mg/l; IAA 0.32 mg/l	38.9±11.5	94.4±5.4	5.6±5.4

Discussion and conclusions

Aspects of cytokinin effect on shoot development. Effect of cytokinins on the development of European larch buds observed during our research can be discussed as similar to reversion of vegetative buds from mature to juvenile phase because cytokinins did not allow normal development of short shoots: elongation of needles was blocked and structures able to form new organ primordia developed. Other reports about the effect of cytokinins on conifer buds suppose this suggestion. Cytokinins together with small concentrations of auxin induced elongation of larch short shoots (Kretschmar 1993) and the formation of adventitious buds (Ewald *et al.* 1997). Corresponding results have been obtained by researching another conifer species: exogenous cytokinin caused the adult buds of *Pinus radiata* to revert to juvenile bud morphology *in vitro* (Zhang *et al.* 2003). Both *Pinus radiata* and larch mature trees produce vegetative buds with different morphological characteristics and so-called adult characteristics of buds are mainly featured by reduced development of apical meristems that can be restored by exogenous cytokinins *in vitro*. Another significant effect of cytokinins observed during our research was the increase in viability of dark-grown larch explants. Cytokinins are known to promote greening by affecting abundance of the enzyme that catalyses the penultime step in chlorophyll biosynthesis (Kusnetsov *et al.* 1998). Interestingly, although some authors conclude that this reaction is light-dependent exclusively in angiosperms and light-independent in other plants (also in gymnosperms) (Fujita 1996, Schoefs and Franck 2003) but we were able to assess the viability of dark-cultivated larch explants only after some days of light-treatment as larch buds cultivated on the nutrient medium with cytokinins started greening when illuminated and others did not.

Aspects of negative effect of auxins on shoot development. The effect of abscisic acid (ABA) on explants harvested in August was similar to the effect of larger concentrations of auxins that was initially observed on explants harvested in October. Large amounts of endogenous ABA possibly served as mediator of negative response to auxins in the larch explants harvested in October, as it was reported that the buds of woody plants accumulate largest amounts of ABA namely in October (Tanino 2004) and some authors suggest the role of auxin in maintenance of ABA content (Shimizu-Sato and Mori 2001). This presumption can be supported by the data that exogenous auxin increases ABA content in pea ovaries and inhibition of auxin transport from apical shoot decreases ABA level (Rodrigo and García-Martínez 1998). The possibility that auxin may prevent development of needles via accumulation of another stress-hormone ethylene whose biosynthesis is well-known to be induced by IAA (Chen *et al.* 2005) seems uncertain, according to the data that long-term inhibition of leaf blade expansion by exogenous auxin was also observed in bean and *Arabidopsis* and even inhibition of ethylene biosynthesis was not able to rescue from negative effect of auxin (Keller *et al.* 2004).

Aspects of positive effect of auxins on shoot development. Elongation of the shoot axis in larch buds seemed to be related to the effect of exogenously applied auxins. Individual buds either had ability to response to auxin signalling via elongation of the shoot axis or not and this ability occured only after some time of cultivation *in vitro*. The slow response sug-

gests about more complicated way of auxin effect, possibly by regulating accumulation of another kind of phytohormones – gibberellins. Auxins seem to play an important role in increasing the amount of bioactive gibberellins (Ross *et al*. 2000). The latter phytohormones promote stem elongation and leaf expansion (Olszewski *et al.* 2002). Knowing about the monooxygenases that may affect as key enzymes both in gibberellin synthesis and ABA degradation (Kushiro *et al.* 2004), suggests that activity of such enzymes might be increased under favourable growth conditions, leading to low ABA level but high level of gibberellin precursor (Rademacher 2000). Further, as long-day photoperiod promotes late steps in the pathway of gibberellin biosynthesis (Wu *et al.* 1996), auxins are involved in up-regulation of production of bioactive gibberellins (Ross *et al*. 2000). Short-day treatment, on the contrary, is known as a factor that negatively affects the height growth of conifer seedlings, additionally, it improves frost hardiness (Konttinen *et al.* 2003). This suggests that auxin may play a positive role in regulation of shoot growth only after decrease of ABA level.

Possible manipulations with phytohormones wishing to establish juvenile phase. Finally, the phytohormone content suitable for establishing juvenile phase in larch vegetative buds should be discussed. Although exogenous auxins were able to promote elongation of the shoot axis, this kind of phytohormones should be used carefully. During our research auxins mostly affected the development of larch explants in the way similar to stress factors, possibly via signal mediated by reactive oxygen species, since auxin signalling is known as a factor increasing the content of reactive oxygen (Joo *et al.* 2001). Additionally, callus formation promoted by auxins is an undesirable phenomenon trying to achieve clonal larch plantlets via organogenesis, although another method based on the formation of somatic embryos from emryogenic callus can be used for larch micropropagation and a large amount of synthetic auxin 2.4-D combined with cytokinin BAP should be used for induction of embryogenic callus on immature zygotic embryos (Klimaszewska 1989). In our case, working with vegetative buds of adult larch, auxins seem helpful in selection of the buds able to start long-shoot development (possibly the shoot apical meristems with majority of cells able to maintain autonomous gibberellin biosynthesis may be sellected in this way) but the necessity to use auxins is not so obvious, according to some nuances of cytokinin effect. Cytokinins induce proliferation of meristemic tissue, also of that in the shoot apex. If a larch bud contains some cells in the shoot apical zone able to synthesize gibberellin, multiplication of these cells might be the reason for so-called rejuvenation that occurs as a consequence of cytokinin treatment. Cytokinin treatment without application of other phytohormones may even be sufficient for long-shoot development, since it induces the formation of adventitious buds that can be recognized by new-formed needle primordia and these new-formed primordia show that vegetative larch buds can be characterized as auxin-autonomous, according to the data that auxins are the only phytohormones that can directly induce the formation of organ primordia (Reindhardt *et al.* 2000). This suggests that developing vegetative larch buds contain a sufficient amount of endogenous auxins and should be able to synthesize bioactive gibberellins even without application of exogenous auxins. If we conclude that juvenile morphology in larch vegetative buds depends on the autonomy in gibberellin biosynthesis, this speculation might explain the difficulties trying to propagate buds from especially old larch trees (Kretschmar and Ewald 1994). These buds might have a very reduced quantity of meristemic cells able to start autonomous gibberellin biosynthesis and even under cytokinin treatment the possibility that after proliferation these meristems would be able to form the shoot axis remains minimal. Wishing to lift efficiency of adult larch micropropagation via vegetative buds, the search for more reliable methods is required.

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ИЗМЕНЕНИЯ РАЗВИТИЯ ВЕГЕТАТИВНЫХ ПОЧЕК ЛИСТВЕННИЦЫ ЕВРОПЕЙСКОЙ (*LARIX DECIDUA* MILL.) ПОД ВЛИЯНИЕМ ФИТОГОРМОНОВ

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Резюме

Изучено развитие вегетативных почек 40-летнего дерева лиственницы европейской в культуре тканей. Экспланты с боковыми почками, приготовленные из ветвей нынешнего или прошлого года, их питание *in vitro* (на MS среде) было дополнено разнообразными концентрациями фитогормонов: абсцисной кислоты, ауксинов (индолилуксусной кислоты и 2,4-дихлорфеноксиуксусной кислоты), цитокининов (кинетина и бензиламинопурина) или гиббереллина. Абсцисная кислота имела отрицательный эффект на органогенез в вегетативных почках лиственницы. Ауксины задерживали развитие основных игл (через 25 дней развития почек в культуре тканей), но через 75 дней количество эксплантов с новообразованными побегами возрастало по мере повышения концентрации ауксинов в среде. Ауксины также стимулировали образование каллуса. Цитокинины остановили удлинение игл и определили образование зачатков длинных побегов. На этих структурах могли образоваться новые меристемы и добавочные почки. Положительный эффект цитокининов на жизнеспособность почек лиственницы был значительный для эксплантов, которые развивались в темноте. Между тем при нормальных условиях (в свете) высокие концентрации цитокининов уменьшали количество жизнеспособных эксплантов. Гиббереллин способствовал развитию осевых игл на верхушечной зоне побега.

Ключевые слова: лиственница, развитие почек, цитокинины, ауксины, гиббереллин, обмен фаз