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Continuous micropropagation of juvenile larch from different species via adventitious bud formation

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Abstract

A continuous propagation of juvenile larch *in vitro* was based on adventitious bud formation and different cytokinin combinations were tested concerning their effectiveness to induce elongation of adventitious buds. Zeatin (1.5 mg dm^{-3}) combined with kinetin (0.15 mg dm^{-3}) was found to be the best. Development and elongation of buds was achieved on a modified LP-medium. Using this system it was possible to propagate different larch species (*Larix decidua, L. gmelinii* and *L. sukaczewii f. multiramosus*) continuously. Shoots were successfully rooted and transferred to the soil.

Additional key words: *Larix*, regeneration, tissue culture.

Introduction

One of the tasks micropropagation is aimed at the multiplication of restricted seed lots of valuable larch material either from controlled pollination experiments or from limited seed sources in natural stands as well as from very rare genotypes.

Micropropagation of larch of different age was reported for several species (Diner *et al.* 1986, Diner and Karnosky 1984, Bonga and Aderkas 1988, Bonga and Pond 1991, Zhihua *et al.* 1991, Ellis *et al.* 1991, Karnosky *et al.* 1993, Nasu and Itahana 1995). Only in a few cases a continuous propagation process was established. The elaboration of a system for serial propagation of larch seedlings *in vitro* based on the cut of elongated long shoots into shoot tips and bud bearing stem segments in regular cycles on phytohormone free nutrient media, using the capacity of larch to sprout, was already described by Hübl and Zoglauer (1991) and Kretzschmar (1993) and for

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adult larch by Kretzschmar and Ewald (1994). Although reliable micropropagation methods of different juvenile larch species via adventitious buds were elaborated (Chalupa 1983, 1985, 1989, 1991, Diner *et al.* 1986, Diner and Karnosky 1984) only a very few hints were given concerning the repeatability of this process. Based on a system elaborated for Norway spruce (Ewald and Süß 1993), experiments were carried out to develop similar conditions for a repeatable formation of elongating adventitious buds of larch.

Materials and methods

Plants: Seeds of European larch (*Larix decidua* Mill.) from Germany, seeds of Dahurian or Kuril larch (*Larix gmelinii*) of the DaXingan Mountain area (China) harvested at an elevation of 700 - 1300 m above sea level, and seeds of Sukaczev larch (*Larix sukaczewii f. multiramosus* Put.), characterized by a broadleaved tree-like compact crown, harvested near Magnitogorsk (Russia) were used.

In case of heavily contaminated seeds from *L. gmelinii* dry seeds were immersed in 70 % ethanol for 1 min. Seed scales were opened with sterile scalpels and discarded. The embryo was removed with sterile scalpels out of the endosperm and placed on the nutrient media. Seeds of other larch species were sterilized for 5 min with a 0.25 % solution of mercuric chloride and rinsed three times with sterile water. Sterilized seeds were placed onto a sterile basic medium (BEMB) containing macroelements used by Von Arnold (1981 - LP) with a reduced concentration of ammonium nitrate (2.5 mM). The microelement composition was used according to Boulay (1979). This medium, without plant growth regulators, was supplemented by 100 mg dm^{-3} polyvinylpyrrolidone (PVP). After one day embryos were removed out of the endosperm and placed on adventitious bud induction media.

Induction of adventitious buds and subculturing of the lines: Embryos were induced on a half-strength MCM-medium (Bornman 1983) with 100 mg dm^{-3} arginine, 100 mg dm^{-3} PVP and different combinations of zeatin and kinetin, 2iP (6-(γγ-dimethylallylamino)purine) and IBA (indole-3-butyric acid) for four weeks during the first induction period. Within the following 2 subcultures (7 - 8 weeks) clusters of adventitious buds were subcultured on a medium without plant growth regulators (BEMB). One propagation cycle consisted of the induction period and two subcultures for the development of induced buds. Afterwards, the bud clusters were divided into single explants. The formed adventitious buds were divided into three groups according to their size and counted. Three size ranges (length of the shoot axis formed) were defined: A = 0 - 3 mm, B = 3 - 6 mm and C = > 6 mm. In the following cycles the induction period was reduced to three weeks. The control treatment was placed on the same medium without phytohormones.

To find out cytokinin combinations effective in stimulating the adventitious bud formation without preventing their later elongation 11 embryos of European larch per treatment were used. The following treatments were tested (phytohormone concentrations given in mg dm^{-3}): control (no phytohormones), treatment 1 (0.05 kinetin + 0.5 zeatin), treatment 2 (0.1 kinetin + 1.0 zeatin), treatment 3

(0.15 kinetin + 1.5 zeatin), treatment 4 (0.05 kinetin + 0.5 zeatin + 0.2 IBA), treatment 5 (0.1 kinetin + 1.0 zeatin + 0.2 IBA), treatment 6 (0.2 2iP). Number and size of adventitious buds formed were counted during and after 3 cycles. The average numbers of adventitious buds formed per clone (mean value including standard deviation) were calculated. During the third cycle the number and size of adventitious buds formed was determined depending on the size of the adventitious bud used for induction (A, B, C). A coefficient of induction efficiency for buds of a certain size was calculated as the number of buds formed of a given size range (A, B or C) divided by the number of buds of the size range they derived from. This coefficient was calculated summing up the results of all treatments (1 - 6) containing cytokinins. Distribution of adventitious buds formed in selected treatments was tested with Fisher-test.

To confirm these results for another larch species twenty embryos of *Larix gmelinii* were excised and cultured with treatment 3 in the way described for *L. decidua*. During the third propagation cycle the formation of adventitious buds depending on the size of the bud used for induction was determined. The propagation efficiency of buds different in size range (A, B, C) were calculated as already described. Adventitious bud formation of four clone lines was determined for one propagation cycle and at least fourty explants of size A. The coefficients for induction efficiency were calculated.

To test the process with a limited seed source of a rare larch species 28 embryos of *Larix sukaczewii f. multiramosus* Put. (Fig. 3*A*) harvested from the cones in September 1994 were used (Putenikhin 1995, Ewald *et al.* 1995). The propagation method applied was the same as already described with induction treatment 3 during 4 cycles (7 months). Only adventitious buds of size A were used for repeated induction steps. The other buds were subcultured on nutrient medium BEMB until they reached a length of 10 mm. Then they were treated as described for elongation. The number of adventitious buds in different size ranges was counted after the first induction period and after seven months. Rooting of shoot material formed from single clone lines was tested in an additional experiment.

Elongation of shoots: Shoots with an axis longer then 10 mm after the third cycle were cultured on a medium without plant growth regulators (BEMB) but with an enhanced concentration of ammonia nitrate (7.5 mM) in order to support shoot elongation. After shoots had reached a length of 30 mm the explants were transferred on hormone free medium B1 (Boulay 1979) which contained 1 mM l-glutamine and 3 % saccharose (Hübl and Zoglauer 1991).

The cultures were kept during induction and shoot elongation at 23 °C under continuous red light (*Narva* tubes *LS 65 red W 93*, 650 nm peak emission, irradiance 30 µmol m^{-2} s^{-1}). All nutrient media were solidified with 0.4 % gelrite and pH adjusted to 5.8 (BEMB, B1) or 6.4 (MCM) before autoclaving.

Rooting of shoots and transfer to the soil: Shoot tips of approximately 40 mm in length were used. They were induced with 2 mg dm^{-3} naphthalene acetic acid (NAA) on a reduced nutrient medium according to Murashige and Skoog (1962) (Kretzschmar and Ewald 1994). During root induction and development the

temperature was reduced to 17 °C. The cultures were kept under a photoperiod of 16 h in white light (*Narva* tubes *LS 40 white*, irradiance 30 μmol m^{-2} s^{-1}). After 2 weeks the shoots were placed into JIFFY-7 peat pellets and stored in little plastic greenhouses. Rooted shoots were counted and plants were transferred to the greenhouse after 3 months.

Results and discussion

A lack of cytokinins lead to a reduced survival of European larch embryos during three propagation cycles (Table 1). The treatment with 2iP was the best treatment concerning the survival of clone lines, because all explants survived. The most efficient cytokinin combinations concerning the number of adventitious buds formed were those with higher amounts of zeatin and kinetin (treatment 2 and 3) followed by the treatment with 2iP. Although the efficiency according to the mean value of adventitious buds formed per explant is higher in treatment 2 the standard deviation

Table 1. Formation and elongation of adventitious buds formed on *Larix decidua* embryos after repeated propagation cycles (mean ± S.E.; distribution of adventitious buds between treatment 2 and 3 after the third cycle was tested with Fisher test; * - significantly different distribution at $P < 0.05$).

Growth regulators [mg dm^{-3}]	Cycle	Surviving clones [number]	Adventitious buds [number per surviving clones]	Adventitious buds per size range [mm] 0 - 3	3 - 6	> 6
Control	1	2	0.5	1	0	0
	2	1	3	0	2	1
	3	1	0	0	0	0
Treatment 1:	1	8	5.9 ± 7.5	23	24	0
0.5 zeatin+0.05 kinetin	2	6	19.5 ± 20.2	64	27	26
	3	6	22.8 ± 26.4	90	22	31
Treatment 2:	1	6	6.0 ± 1.9	21	12	9
1.0 zeatin+0.1 kinetin	2	5	33.4 ± 24.8	138	18	16
	3	5	83.4 ± 82.6	397	0	20
Treatment 3:	1	7	12.7 ± 9.1	49	22	16
1.5 zeatin+0.15 kinetin	2	7	33.3 ± 30.1	161	34	38
	3	7	81.1 ± 70.6	377	75*	115*
Treatment 4:	1	10	3.6 ± 2.2	13	15	8
0.5 zeatin+0.05 kinetin+0.2 IBA	2	9	17.4 ± 18.8	124	15	21
	3	9	16.7 ± 28.1	83	54	13
Treatment 5:	1	6	5.5 ± 2.3	24	4	4
1.0 zeatin+0.01 kinetin+0.2 IBA	2	5	12.8 ± 6.8	51	4	9
	3	5	37.0 ± 31.3	170	8	7
Treatment 6:	1	11	8.9 ± 5.0	58	19	21
2.0 2iP	2	11	22.9 ± 17.8	140	50	54
	3	11	39.2 ± 35.6	317	22	14

is lower in treatment 3. This led to the conclusion that treatment 3 is able to stimulate a larger part of all explants (clones) more efficiently. On contrary the high value of standard deviation in treatment 1 and 4 is an expression that only some clones were induced very efficiently because phytohormone combinations were not optimal for most of the clones. Supplementing the media with IBA increased callus formation which reduced the formation of adventitious buds during the third cycle. Comparing the distribution of the buds formed in different size ranges between treatment 3 and 4 we found a significant difference ($P < 0.05$). This meant that treatment 3 was able to form a higher amount of larger buds. Testing the efficiency of different bud sizes concerning their ability for a repeated induction the smallest buds (size A, 0 - 3 mm) were the most effective ones. On average two new buds of size A were formed per bud when size A was used as explant (Fig. 1). Although it was possible to stimulate buds longer then 3 mm in size the propagation rates were lower. Rooting of adventitious bud derived shoots of *Larix decidua* was possible as described for shoots of the same species produced by the elongation of axillary buds (Kretzschmar 1993, Kretzschmar and Ewald 1994). Plants from two adventitious bud forming clone lines were produced and transferred to the greenhouse.

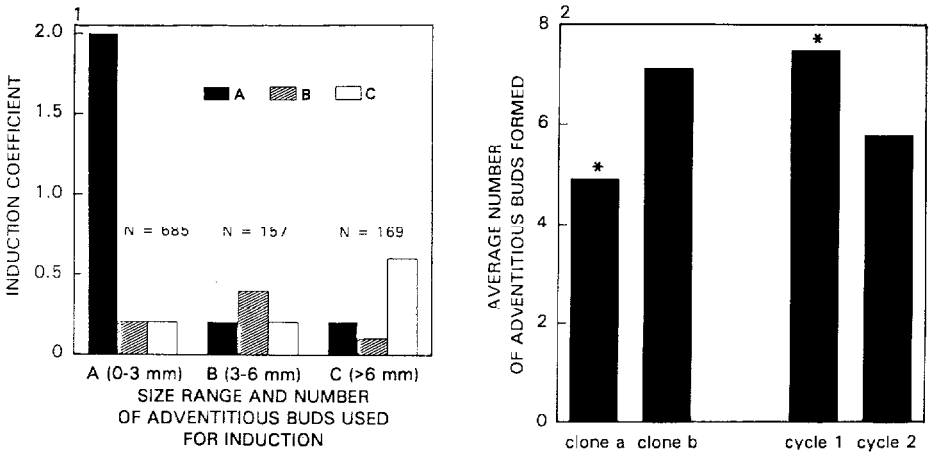

Fig. 1. Influence of the size of buds used for cytokinin mediated adventitious bud induction on the formation of buds in different size ranges.

Fig. 2. Influence of the clone and the propagation cycle on the average number of adventitious buds formed by two *L. gmelinii* clones during 2 propagation cycles. During the first cycle 20, during the second cycle 100 adventitious buds of size A were used for induction. The data were compared with Tukey-test. Columns for the clone or the cycle effect with an asterisk are significantly different at $P = 0.05$.

As well as for *L. decidua* also for *L. gmelinii* clone lines were established (Fig. 3*B*). Based on the results of the first experiment only treatment 3 (1.5 mg dm^{-3} zeatin + 0.15 mg dm^{3} kinetin) was used for induction. The efficiency for induction of new buds was as shown for *L. decidua* best for buds of the smallest size (A). From one hundred size-A-buds of six clones each bud formed 2.25 size A buds on average.

Fig. 3. Steps of plant regeneration: *A* - rare form of Sukaczev larch, characterized by a broadleaved tree-like compact crown; *B* - adventitious buds of *L. gmelinii* (× 5); *C* - elongated shoots of *L. gmelinii*; *D* - rooted shoots of *L. gmelinii*.

Using four selected clones 3.8 ± 2.5, 6.0 ± 2.7, 6.6 ± 2.8 and 9.1 ± 5.4 adventitious buds of size A were formed per size-A-bud. The average numbers of buds which were larger then 3 mm (size range B, C) did not exceed values of 0.5. A propagation efficiency is depending on clone line (Fig. 2). Although the propagation rates in total differed for the first and second cycle the relation between propagation rates of the two clones was similar. Elongation of buds (Fig. 3*C*) and rooting of shoots longer than 40 mm was possible also for *L. gmelinii* in the same way as for *L. decidua*. Plants of three clones were succesfully transferred to the greenhouse (Fig. 3*D*).

Four embryos of *L. sukaczewii* were able to produce more then 200 small adventitious buds (0 - 3 mm) during seven months. Four embryos produced more then 100, four more than 50, eight embryos more then 10 and only two embryos refused to produce adventitious buds of this size at all. These results are summarized in Table 2. The percentages of rooted shoots from three clones were 21.4, 41.6 and 55.9 respectively. From a total of 85 shoots 41 shoots rooted. After root formation plants were acclimatized and transferred to the greenhouse.

Table 2. Number and size of adventitious buds formed from embryos of *L. sukaczewii* after four repeated propagation cycles over a period of 7 months.

Embryos used [number]	Cycle	Explants forming buds [number]	Buds in different size ranges [number explant^{-1}] 0 - 3 mm	3 - 6 mm	> 6 mm
28	1st	25	4.7 ± 3.8	3.6 ± 5.9	1.7 ± 2.2
22	4th	20	105.2 ± 85.1	14.0 ± 24.0	9.8 ± 24.2
Total number of adventitious buds formed			2730	280	196

The repeated formation of elongating adventitious buds is one precondition to develop a system for the continuous propagation this way. Using highly effective cytokinins to induce a large amount of adventitious buds, as often practised, was shown to be a contradiction concerning the later elongation of adventitious buds formed (Ewald and Süß 1993). A zeatin/kinetin combination in a 10:1 relation was shown to be effective for larch as well as it was for spruce. The optimal size of adventitious buds for such a process was found to be 0 - 3 mm that means the smaller a bud the easier it was to induce it anew to form again adventitious buds. The elaborated system was used for more than 2 years (appoximately 14 cycles) with clones of different larch species. This method was supplementing an already existing *in vitro* propagation system for juvenile larch based on the elongation of axillary buds (Hübl and Zoglauer 1991, Kretzschmar 1993). Serial propagation of juvenile larch material via axillary bud formation without phytohormones was possible also with those shoots which derived from adventitious buds. This enlarges the *in vitro* propagation capacity for these species because it is now possible to combine these two methods to get a proper explant stock for continuous propagation. Additionally the formation of adventitious buds offers the possibility to stimulate a larger part of genotypes from the beginning compared with the method of axillary shoot formation. Nevertheless the propagation system via axillary buds is preferred for final

multiplication steps because the lack of cytokinins promotes lignification of the material as well as the later root induction and development. Using a restricted seed lot of valuable larch material showed the potential of the propagation method also for the conservation of rare material.

The ability to induce a repeatable organ formation might be a step on the way to multiply so far recalcitrant larch material by vitalization successively. It can be also a precondition to apply methods of gene transfer to a certain selected material.

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