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Shoot Regeneration from Cambial Tissue Culture of European Larch (*Larix decidua*)

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유럽낙엽송의 형성층조직 배양으로부터 줄기의 재분화

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Adventitious shoots were induced from cambial tissue cultures of 3-year-old seedlings using BLG mineral salts medium supplemented with 10 mM glutamine and 30 mM sucrose. The optimum growth regulator level for bud induction was 4.5 μM BA which produced average 25.5 shoots per cambium segment. Induced buds were elongated on GD medium supplemented with 30 mM sucrose followed by LMG medium supplemented with 30 mM sucrose for further shoot elongation. Elongated shoots were rooted on half-strength GD medium containing 0.54 μM NAA with the frequency of 20%. This system proved the high morphogenic potential of cambial tissue in larch.

Key words: Micropropagation, conifer, organogenesis

INTRODUCTION

Larch (*Larix*) species and hybrids are important conifers that are grown for lumber, poles and pulp as well as ornamental purposes in northern temperate zone forests across northeastern Asia, North America and Europe. Among the principal commercial larch species are European larch, Japanese larch, tamarack, Western larch, Dahurian larch and Siberian larch. Hybrid larches have shown heterosis in growth, stem form and wood mechanical properties as compared to parental plants (Paques, 1989).

Traditional breeding and large-scale production of hybrids for larch are hindered by the poor seed yields obtained. Empty seeds often account for two thirds or more of the seed obtained following controlled pollinations (Hall and Brown, 1977; Kosinski, 1987; Shin and Karnosky, 1995). The relatively small cone sizes and the early seasonal flowering phenology which often leads to low-temperature injury to flowers also contribute to the difficulty in breeding these trees. Therefore, *Larix* species and hybrids are good candidates for vegetative propagation via micropropagation.

While conifers have generally been more difficult to handle *in vitro* than have hardwood trees, larches are unique set of conifers in that they are highly amenable to micropropagation systems. Larches have been regenerated from juvenile and mature tissues via organogenesis and embryogenesis (Klimaszewska, 1989a,b) and haploid plants from female gametophyte tissue (Von Aderkas and Bonga., 1988; Pattanavibool *et al.*, 1995).

Materials used for cell and tissue cultures of larch were mostly embryonal or *in vitro* germinated young seedlings. Cambial tissues which lead diameter growth of woody plants can be excellent material for the *in vitro* micropropagation because of their meristematic activity. Thus, micropropagation using cambial tissue culture can provide an additional tissue culture system for larch.

In this paper, we will report the shoot regeneration from cambial tissues through the intermediate callus stage in European larch.

MATERIALS AND METHODS

Plant material and cambium preparation

Stems (0.7 - 1.0 cm in diameter) of 3-year-old greenhouse-grown European larch seedlings were cut in 3 cm pieces, washed with commercial detergent and rinsed with running tap water for 1 h. Stem pieces were first surface-sterilized by 70% ethanol by soaking 1 min and rinsed 3 times with sterilized distilled water. Then, they were soaked for 5 min in 10% aqueous commercial bleach and rinsed 3 times with sterilized distilled water. Bark was removed from the stem by scrubbing with scalpel, and cambial tissues were excised from the stem. Excised cambial tissues were dissected about 5 × 5 mm segments and placed onto the bud induction medium. Ten segments were placed in each Petri dish and this was repeated 3 times.

Bud induction

The basal medium for bud induction was 1% agar solidified BLG mineral salts medium (Brown and Lawrence, 1968) supplemented with glutamine (10 mM), sucrose (30 mM). To determine the optimum growth regulator levels for bud initiation from the cambial tissues, 0.22, 2.25, 4.5, 22.5 and 45 μM BA (6-benzyladenine) alone or in combination with 0.54 μM NAA (α-naphthalene acetic acid) were incorporated into the medium. Petri dishes were then sealed with parafilm and incubated at 20°C under constant 4500 lux mixed cool-white fluorescent (70%) and incandescent (30%) illumination for 4 weeks.

Shoot elongation

When the height of buds reached to about 3 mm, the whole segment was divided into 3 to 4 sections and transferred to shoot elongation medium. Evaluation was made at this time by scoring the number of shoots induced from the cambial segments. Either half strength GD or full strength GD mineral salts medium (Gresshoff and Doy, 1972) supplemented with 30 mM sucrose was used. On this medium, cultures were incubated for 6 weeks with 2 successive 3 week interval subcultures under the same conditions as descibed above. After this first elongation step, each shoot was excised and transferred onto the 1% agar solified LMG (Litvay *et al.*, 1981) mineral salts medium supplemented with 30 mM sucrose and incubated for 2 successive 2 week interval subcultres to elongate shoots further. The heights of shoots were measured at the end of the second subculture.

Rooting of shoots

For rooting of these shoots, shoot bases were trimmed to remove browned tissue, and inserted in 1% agar solidified, half-strength GD medium containing 0.54 μM NAA and 30 mM sucrose. Sealed plates are incubated for 2 weeks at 20°C under the same conditions described above. Plantlets are then transferred to half-strength GD medium with 30 mM sucrose and no NAA. When the secondary roots were developed, plantlets were moved to a soilless mix (peat:perlite:vermiculite=1:1:1) in a mist chamber in the greenhouse for shoot and root growth. After 3 weeks in mist chamber, they were moved out of the mist chamber and grown in normal greenhouse conditions.

RESULTS AND DISCUSSION

Since the first *in vitro* culture of larch was reported by Sterling (1944) using immature European larch embryos, organogenesis and embryogenesis from various tissue and organ sources of larches have been reported (Diner *et al.*, 1986: Klimaszewska, 1989a,b: Von Aderkas and Bonga, 1988: Pattanavibool *et al.*, 1995). Cambial tissue, however, which has meristematic activity has not been used for inducing organogenesis from larch. In our study, cambial tissue has proven its excellent organogenic potential *in vitro*.

Within 10 days of culture, cambial segments swollen and started to form callus. Calli formed on the segments were green, compact and glossy in appearance (Fig. 1A). Sometimes white and friable calli were developed from the green calli, but these white ones failed to produce buds (Fig. 1A). In most cases where organogenesis or embryogenesis were obtained in conifers, the calli induced were white type (Gupta and Durzan, 1987: Hakman and Von Arnold, 1985: Nagmani and Bonga, 1985: Von Aderkas and Bonga, 1988) and calli induced were distingished between morphogenic white callus and nonmorphogenic green callus (Tautorus *et al.*, 1990). In these previously reported studies, the types of source materials were mostly megagametophyte, or embryonal materials such as immature embryos or suspensors and they were cultured under the dark conditions. In contrast to these embryonal materials, cambium used in our study is rich in

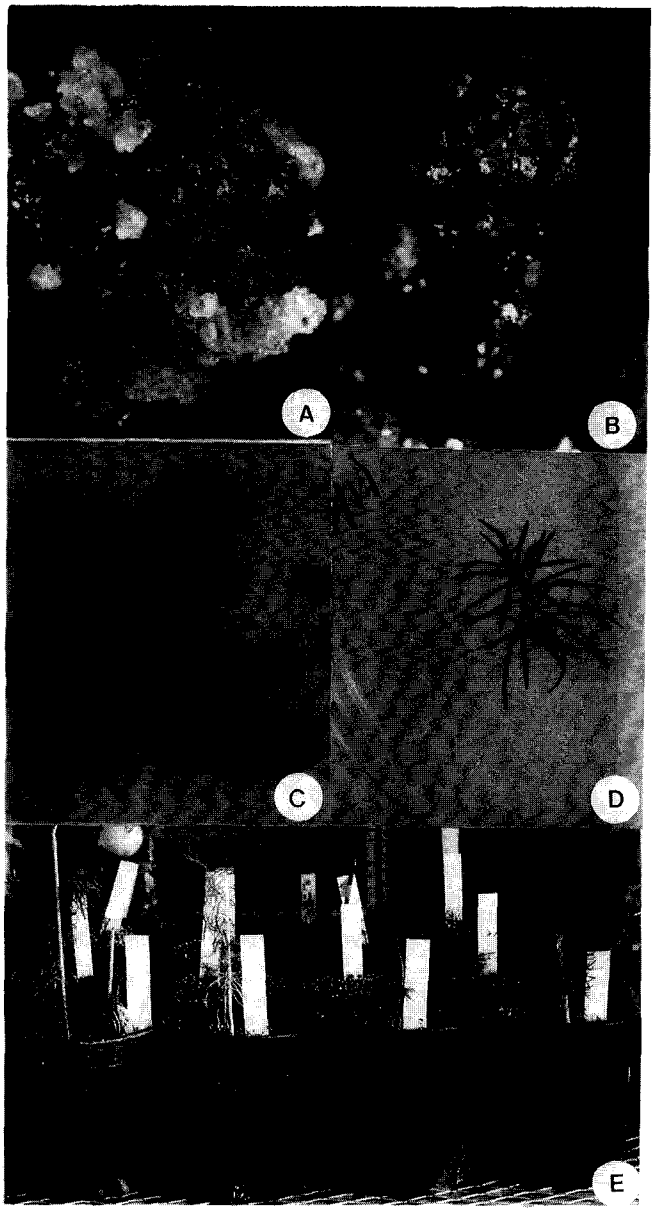

Figure 1. Adventitious buds developing from cambial segments of *Larix decidua* after 3 weeks of culture.
A: buds initiating from green, compact callus derived from the cambial tissue;
B: developing bud; C: clusters of buds; D: rooted shoot; E: growing shoots in the greenhouse.

chlorophylls and cultured under continuous light condition. Thus, differences in materials and culture conditions seemed to cause the difference in the morphology of calli. As stated above, most embryogenic and organogenic calli derived from conifer materials were white and translucent (Ruaud *et al.*, 1992; Krogstrup, 1986; Schuller *et al.*, 1989). Krogstrup (1986) stated that green callus obtained from Norway spruce was nonmorphogenic and delayed embryogenesis in which

Table 1. Growth regulator concentration effects on adventitious bud initiation from cambial tissues of *Larix decidua*.

Growth regulator concentration (μM) BA	NAA	Number of cambial segments tested	Average number of buds formed per segment
0.22		30	4.07 ± 3.28* a**
2.25		30	11.00 ± 8.79 b
4.50		30	25.53 ± 8.48 c
9.0		30	3.47 ± 2.64 d
22.5		30	0.67 ± 0.97 ed
0.22	0.54	30	4.20 ± 2.91 ed
2.25	0.54	30	5.87 ± 2.83 ed
4.50	0.54	30	17.27 ± 2.58 ed
9.0	0.54	30	2.73 ± 2.15 ed
22.50	0.54	30	1.33 ± 1.67 e
0.0	0.0	30	0.00 e

*Standard deviation
**Means followed by the same letters are not significantly different (α=0.05, Tukey's multiple comparison test).

nonembryogenic green callus occasionally produced somatic embryos after several subcultures was reported from black spruce (Tautorus *et al.*, 1990). However, green and compact calli obtained in our study revealed morphogenic ability without delay.

Small bud primordia started to develop after 3 weeks of culture. The medium supplemented with 4.5 μM BA was the most effective and induced an average of 25.53 buds per segment (Table 1). Many of the cambial segments that developed shoots produced them in large numbers (Fig. 1C). Because the shoots are often crowded and needles tangled, it was generally difficult to determine the exact number of shoots per segment. Higher BA concentrations than this resulted in both a decline in bud initiation and an increase of tissue chlorosis. Addition of NAA to the medium did not promote bud induction. No difference was noted in morphology of the buds initiated by different concentrations of BA. Laliberte and Lalonde (1988) reported the morphologies of shoots induced from the short shoot bud-derived callus of hybrid larch are different depending on the concentrations of BA added in the induction medium.

In most calli, 4 to 5 localized organogenic centers were created, leading to extensive bud proliferation. This type of bud development is similar to those reported from cotyledons of young conifer seedlings and immature female strobili of larch (Bonga, 1984; David, 1982; Von Arnold and Eriksson, 1985). As buds grew, these centers seemed to be connected to others, finally developing a cluster of buds on a whole callus. Buds were produced mostly on the callus developed from the cambial segments while some were formed directly on the cut

Table 2. Effects of half and full-strength GD media on elongation of adventitious shoots.

Medium	Number of shoots measured	Average stem height (cm)
Half GD	20	2.54 ± 0.40* a**
Full GD	20	1.78 ± 0.11 b

*Standard deviation
**Means followed by the same letters are not significantly different (α=0.05, two sample t-test).

edges. Buds were developed into shoots on the elongation medium. As shown in Table 2, half strength GD was better than full strength GD in shoot elongation. LMG or LP medium has been reported to promote developing adventitious buds of European larch (Diner *et al.*, 1986). However, prolonged culture periods (over 4 weeks) on these media are known to cause shoot chlorosis.

Rooting of tissue culture propagules are notoriously difficult in conifers. According to Amerson *et al.*, (1988), continuous exposure of adventitious shoots to a low level of auxin (0.5 μ M NAA) yielded only 17% rooting, whereas the same 0.5 μ M NAA gave 50% rooting when supplied as a 12-day pulse. In our study, 2 week culture on 0.54 μM NAA supplemented in half strength GD medium followed by another 2 week culture on NAA free half strength GD medium gave 20% of rooting rate (Fig. 1D). Prolonged culture on NAA containing rooting medium has known to cause stunted root growth (Amerson *et al.*, 1988). The rooting rate we obtained was low when compared as those of other studies (Amerson *et al.*, 1987; Webb *et al.*, 1988). Diner *et al.*, (1986) reported 2 types of shoots developed from the cotyledon culture of European larch. One is rootable type and the other is bushy type which is difficult to root. In our study, most shoots produced were bushy type. Although what causes different shoot types develop and why their rootabilities are different are not known, the type of shoots obtained in our study may be the reason for poor rooting rate as indicated by Diner *et al.*, (1986). Spontaneous rooting was also observed with very low frequency.

Our system using cambium of 3-year-old plants has an advantage over young seedlings or embryonal marerials used so far in larch micropropagation. Since larch is one of the fast growing conifers, early selections for certain characteristics can be made for 3-year-old plants. Thus, micropropagation using these selected plants will facilitate breeding of larch.

적 요

3년생 유럽낙엽송의 실생묘로부터 형성층조직을 분리하여 부정아를 유기하였다. BLG배지에 4.5μM의 BA가 첨가된 배지에서 형성층 절편체 당 약 25개의 부정아가 형성되어 시험된 여러 가지 BA와 NAA농도 중 최고의 발생율을 보였다. 유기된 부정아는 1/2 GD배지와 LMG배지에서 10주 후 약 2.5 cm의 신장을 보였다. GD배지에서는 이보다 낮은 약 1.8 cm의 신장을 나타내었다. 발근은 NAA가 0.54 μM 함유된 1/2 GD배지에 2 주간 배양하였을때 20% 정도로 나타났다. 발근된 shoot들은 NAA가 함유되지 않은 1/2 GD 배지에서 뿌리를 신장시킨 후 온실로 옮겨져 순화되었다. 본 실험의 결과 그동안 낙엽송의 조직배양시료로 사용되지 않았던 형성층 조직의 뛰어난 형태형성능을 보여줌으로써 기내 급속대량증식 뿐 아니라 향후 낙엽송의 체세포 변이주 선발이나 형질전환 및 그와 관련된 연구분야에 유용하게 사용될 수 있을 것으로 사료된다.

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