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Efficient plant regeneration and micropropagation from callus derived from mature zygotic embryo of *Larix gmelinii*

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Abstract

Calli derived from mature zygotic embryos and seedling explants of *Larix gmelinii* were used to investigate for organogenesis. After 4 weeks of culture on half-strength DCR medium containing BA, numerous adventitious buds formed on the callus tissues which were derived from zygotic embryos, but none formed on calli generated from seedling explants. Rates of callus proliferation and adventitious bud formation were higher when the callus was transferred to medium supplemented with activated charcoal. To induce shoot elongation, multiple shoots were separated and transferred to medium with 0.05% activated charcoal. To induce adventitious root formation, elongated shoots of 1.5-2.0 cm in length were cultured in half-strength MS medium containing 0.3 mg l^{-1} NAA and 0.3 mg l^{-1} IBA. To assess genetic homogeneity and somatic variation, chromosomes of regenerated plantlets were observed. No numerical or structural changes to the chromosomes were found in regenerated plantlets using this system.

Key words: Callus induction, Chromosome, Conifer, *Larix gmelinii*, Micropropagation, Organogenesis, Plant regeneration.

Abbreviations

BA, benzyladenine; NAA, naphthaleneacetic acid; IBA, indole-3-butyric acid; IAA, indoleacetic acid; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; DCR, Douglas fir cotyledon revised (Gupta and Durzan, 1985); MS, Murashige and Skoog (1962); BFC, bud-forming capacity; AC, activated charcoal.

The Dahurian larch *Larix gmelinii* (Rupr.) Kuz. is a tree species that is native to northeast China and widely distributed in Russia, China, Japan, and Korea. Larch is now the major coniferous tree species in the plantation forests of northeast China. The dominant breeding method for this species is seeding, since larch is not conducive to vegetative propagation methods such as grafting or the rooting of cuttings. There is an urgent need to develop effective protocols for larch plant regeneration by tissue culture, not only to supplement the transplantation of nursery-grown seedlings, but also to propagate selected elite trees. The technique of micropropagation can produce large numbers of genetically identical plants at an affordable cost, but it has technical limitations that must be overcome before its use in conventional tree-improvement programs.

Organogenesis, the most popular regeneration technique for many plant species, has the potential for use in the mass propagation of superior and genetically engineered forest tree genotypes, both conifers and hardwood species (Tang, 2001). Organogenesis methods have been reported for more than thirty species of conifer, but plant regeneration frequencies have been very low (Kaul and Kochhar, 1985; Gladfelter and Phillips, 1987; Laliberter and Lalonde, 1988; Attree and Fowke, 1993; Goldfarb *et al.*, 1996). No plant regeneration has been reported from *L. gmelinii* callus, although there have been some reports of regeneration from larch axillary buds (Bonga, 1997; Diner, 1995) and hybrid larch cotyledons (*L. gmelinii* × *L. leptolepis*) (Kuromaru and Satoh, 1987). In this paper, we report the development of a protocol for efficient plant regeneration of *L. gmelinii* by organogenesis from callus of mature zygotic embryo.

Mature seeds of *L. gmelinii* procured from Inner

Mongolia were used for all experiments. Prior to culturing, the seeds were surface-sterilized with 70% ethanol and 2% sodium hypochlorite and washed with sterile distilled water. To soften the seed coats and to eliminate non-viable, empty seeds, the seeds were soaked in sterile distilled water at 4℃ for 48 hours, and mature embryos were excised from the female gametophytes. Healthy and viable embryos were chosen for callus induction.

Two basal media, Douglas-fir cotyledon revised (DCR) medium (Gupta and Durzan, 1985) and MS medium (Murashige and Skoog, 1962), were used in this study. All media contained 2% sucrose, were solidified with 0.8% agar or 0.3% gellan gum, and were adjusted to pH 5.75 with 1N KOH or 1N HCl prior to autoclaving (120℃, 1.2 kg cm^{-2}) for 20 min. Cultures were maintained at 23 ± 2℃ under light of 11.7 μmol m^{-2} s^{-1} from cool white fluorescent tubes.

For callus induction, mature zygotic embryos were cultured in Petri dishes (90 × 20 mm) containing half-strength DCR medium supplemented with BA (0.0, 2.5, 5.0, 10 or 20 μM), and embryos were transferred onto fresh medium once per week. After one day in culture under the above conditions, the plumular axes of mature zygotic embryos became red in color, and red calli formed on their surface after 5 days. The red callus is very soft and friable. **Fig. 1A** shows an embryo and callus that had been cultured for 1 week on medium containing 5.0 μM BA. After 3 weeks in culture, prime adventitious bud formation could be observed under a microscope (**Fig. 1B**), and the adventitious buds could be observed by eye after 4 weeks (**Fig. 1C**). Fifteen embryos were cultured in each of four separate Petri dishes, a total of 60 healthy embryos. The experiment was repeated 3 times. The percentages of embryos that produced callus after 1 month were 75.0, 76.19, 75.9 and 71.43 in half-strength DCR medium containing 2.5, 5.0, 10 and 20 μM BA, respectively (**Fig. 2**). The percentages of embryo explants that formed adventitious buds on surface of their calli after 8 weeks were 60.0, 67.73, 64.21, and 62.5 in medium with 2.5, 5.0, 10 and 20 μM BA, respectively (**Fig. 2**).

After 2 months in culture, calli on which adventitious buds or green hard-spots could be observed were separated into about 1 cm × 1 cm, and these callus blocks were transferred onto half-strength DCR medium either supplemented with activated charcoal (0.05% or 0.1%) or lacking it. Multiple shoots were separated and mean numbers of shoots on calli of each embryo were calculated. The bud-forming capacity (BFC) was calculated using the following formula (Capuana and Gianni, 1995; Harry and Thorpe, 1994; Pulido *et al.*, 1994; Saborio *et al.*, 1997):

$$BFC = \frac{(\text{mean number of shoots per explant}) \times (\%\ \text{explants that formed buds})}{100}$$

Although the percentages of callus induction and bud formation show no significant difference among media with 2.5, 5.0, 10 and 20 μM BA (**Fig. 2**), the growth of calli and adventitious buds was the best in culture on medium with 5 μM BA, so the number of adventitious buds formed on medium supplemented with 5 μM BA was higher than those of other media. The average numbers of adventitious buds on calli per embryo were 9.4, 17.3, 11.6, and 5.8 on medium with 2.5, 5.0, 10 and 20 μM BA, respectively (**Fig. 3A**).

Adventitious buds formed in large numbers from proliferating calli when the cultures were transferred onto medium containing activated charcoal (**Fig. 1D**). On average, 26.2 adventitious shoots formed on calli per embryo on medium containing 0.05% activated charcoal, and 48.8 adventitious shoots formed on medium containing 0.1% activated charcoal (**Fig. 3A**). The greatest number of shoots, 128, occurred on calli produced from an embryo explant that was initially cultured on medium containing 20 μM BA (data not shown). The greatest BFC of 33.05 was obtained from explants cultured for 2 months on medium containing 5.0 μM BA and then subcultured for 2 months on medium containing 0.1% activated charcoal (**Fig. 3B**).

Cultures of seedling explants generated three types of callus: red, green, and yellow. These callus types were transferred to either DCR or MS medium containing or lacking various hormones (BA, NAA, 2, 4-D, and IBA) in an attempt to induce differen-

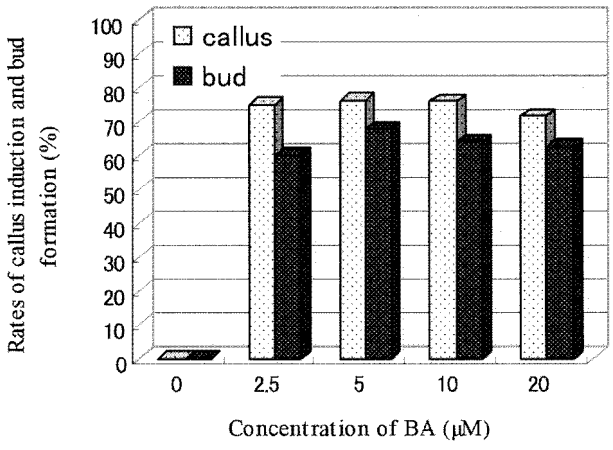

Fig. 2 The effects of BA (benzyladenine) on callus induction and adventitious bud formation from calli of *Larix gmelinii* embryo explants. The value indicated by each bar is the average of three independent experiments with 60 healthy embryos.

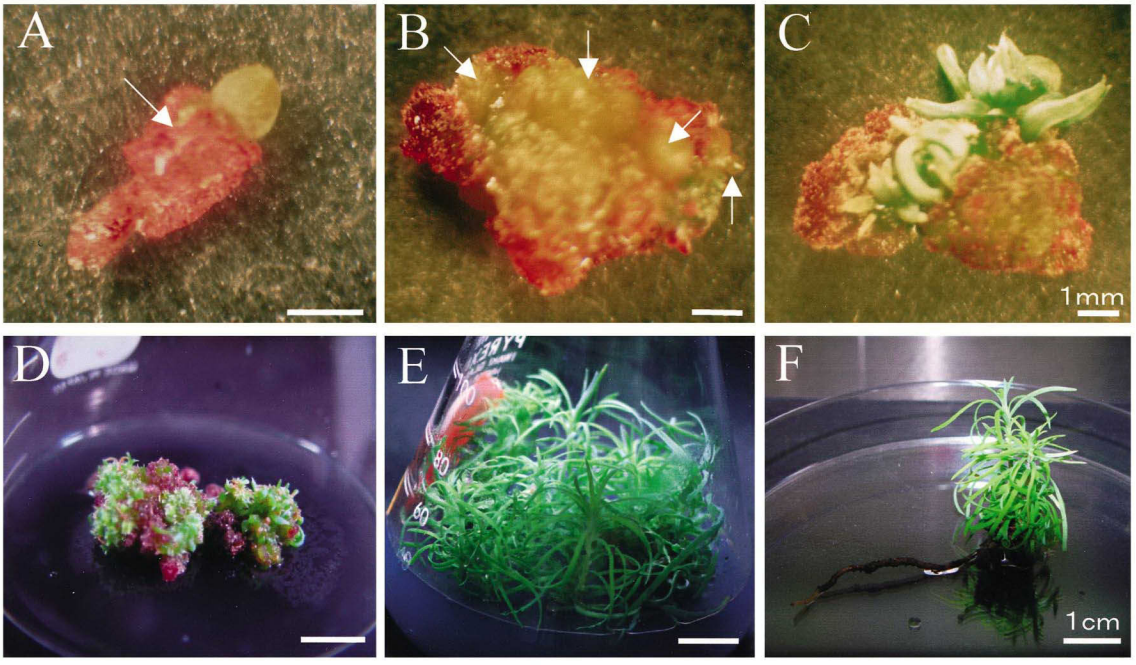

Fig. 1 The process of plant regeneration from calli derived from embryo explants of *Larix gmelinii*. A : An embryo and callus (indicated by arrow) cultured for 1 week on half-strength DCR medium supplemented with 5 μM BA. B : Formation of primary buds (indicated by arrows) after 3 weeks of culture. C : Developing adventitious buds after 1 month of culture. D : Developing adventitious buds and proliferating calli cultured for 2 weeks on medium containing 0.1% activated charcoal. E : Elongated adventitious shoots cultured for 1 month on medium containing 0.05% activated charcoal. F : Plantlet with well-developed roots.

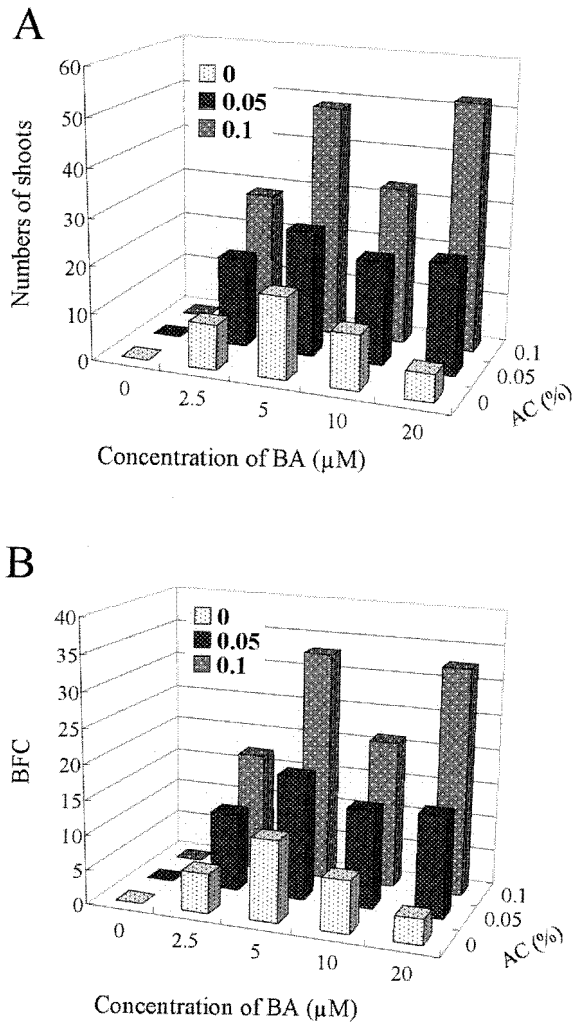

Fig. 3 Effects of BA (benzyladenine) and activated charcoal on adventitious shoot formation. A: Numbers of adventitious shoots formed on calli per embryo. B: Bud-forming capacity. Values represent the numbers of shoots cultured for 2 months on media with various concentrations of BA and subsequently subcultured for 2 months on media with various concentrations of activated charcoal.

tiation, but no adventitious bud formation was observed from these calli (data not shown).

After about 4 months of culture, multiple buds were separated and transferred to half-strength DCR medium supplemented with activated charcoal to induce elongation. Medium containing 0.05% activated charcoal gave the best results for adventitious bud elongation. Although many of the buds withered and turned yellow when cultured on medium with 0.1% activated charcoal for more than 3 months, it was the best medium for bud propagation. **Fig. 1E** shows multiple shoots that were cultured for 2 months on medium containing 0.1% activated charcoal, and then subcultured for 1 month on medium containing 0.05% activated charcoal.

Fridborg *et al.* (1978) showed that culture on medium with low concentrations of activated charcoal promotes the elongation of adventitious buds due to its adsorption of phytohormones and other phenolic substances. Activated charcoal has been used to promote shoot elongation in cultures of several pine species (Pulido *et al.*, 1990; Murithii *et al.*, 1991; Puido *et. al.*, 1994; Mathur *et al.*, 1999). In these studies, multiple buds were separated and transferred to medium supplemented with 0.05% activated charcoal, on which the adventitious buds elongated. We transferred proliferating callus pieces on which bud formed to medium supplemented with 0.1% or 0.05% activated charcoal, and observed the effects. Medium with 0.1% activated charcoal promoted adventitious bud formation more strongly than that containing 0.05% activated charcoal (**Fig. 3A, B**). Elongation of larch adventitious buds was best promoted by culture on medium containing 0.05% activated charcoal, confirming previous reports (Pulido *et al.*, 1990; Murithii *et al.*, 1991; Pulido *et al.*, 1994; Mathur *et al.*, 1999).

After 5-6 weeks, adventitious shoots of about 1.5-2.0 cm in length were subjected to one of the following four rooting treatments. **(1)** Shoots were cultured on half-strength MS medium supplemented with NAA (0, 0.1, 0.3, 0.5, 0.7, or 1.0 mg l^{-1}) and IBA (0, 0.3 0r 0.5 mg l^{-1}). **(2)** Shoots were cultured on half-strength DCR medium supplemented with NAA (0, 0.1, 0.3, 0.5, 0.7, or 1.0 mg l^{-1}). **(3)** Shoots were cultured on half-strength DCR medium supplemented with NAA (0.3 mg l^{-1}), IBA (0.3 mg l^{-1}) and activated charcoal (0, 0.05, or 0.1 %), as well as 2% sucrose and 0.3% gellan gum. **(4)** Shoots were pretreated for 3-6 hours with a combination of NAA (1 mM) and IBA (1 mM) or IAA (1 mM) and IBA (1 mM), transferred to a sterile mixture of sand, peat, perlite and vermiculite (1:1:1:1), and irrigated once per week with a quarter strength DCR liquid medium.

Roots formed on adventitious shoots after 2 months. Most commonly, a single adventitious root developed from each adventitious shoot, but occasionally secondary root development was observed (**Fig. 1F**). The highest rooting frequency, 45.5%, was observed with shoots cultured on MS medium supplemented with 0.3 mg l^{-1} NAA and 0.3 mg l^{-1} IBA. The rooting frequency was 29.0% on shoots cultured on DCR medium supplemented with 0.3 mg l^{-1} NAA and 0.3 mg l^{-1} IBA. On MS medium supplemented with 0.3 mg l^{-1} NAA, the rooting frequency was 22.2% (**Table 1**). While rooting frequency was improved on the medium with a combination of NAA and IBA, no rooting was

Table 1 Effects of various auxins on root formation from *Larix gmelinii* adventitious shoots.

Medium	Phytohormone	Concentration (mgl^{-1})	No. of shoots with root / No. of shoots (%)
MS	NAA	0.0	0 / 36 (0)
		0.1	0 / 36 (0)
		0.3	8 / 36 (22.2)
		0.5	0 / 36 (0)
		0.7	0 / 33 (0)
		1.0	0 / 33 (0)
	NAA, IBA	0.3 each	15 / 33 (45.5)
		0.5 each	0 / 26 (0)
DCR	NAA	0.0	0 / 32 (0)
		0.1	0 / 32 (0)
		0.3	0 / 32 (0)
		0.5	0 / 28 (0)
		0.7	0 / 32 (0)
		1.0	0 / 24 (0)
	NAA, IBA	0.3 each	9 / 31 (29.0)

Table 2 Chromosome numbers of mother plants and regenerated plantlets of *Larix gmelinii* (2n=24).

Object	No. of plant	
	2n=24 (%)	Others (%)
Mother plants	50 (100)	0 (0)
Regenerated plantlets	156 (100)	0 (0)

observed on medium only with IBA (data not shown). Clearly, NAA is an effective growth regulator for the rooting of the larch adventitious shoots, because no rooting was observed on medium without NAA. Adventitious shoots cultured on medium containing activated charcoal failed to root. Plantlets regenerated on the rooting media (**1**), (**2**) and (**3**), were transferred to a sterile mixture of sand, peat, perlite, and vermiculite (1:1:1:1) in plastic pots and grown in a greenhouse. Many plantlets turned yellow and died, after 1 month, the survival of plantlets was only about 50%. Shoots directly cultured in a sterile mixture of sand, peat, perlite, and vermiculite (**4**) showed a 25.6% rooting frequency. In this case, no difficulties were encountered in the transfer of plantlets to the greenhouse. All regenerated plantlets were cultivated in the greenhouse.

To determine the level of genetic homogeneity and somatic variation in plantlets regenerated by organogenesis, we observed the chromosomes of cells from the roots and shoots of regenerated plantlets and the roots of the mother plants. Chromosomes were visualized as described previously (Lee and Ono, 1999) from 50 mother plants and 156 regenerated plantlets (**Table 2**). The somatic chromosome number of the *L. gmelinii* donor plants was 2n = 24 (**Fig. 4A**). Only the normal karyotype of 2n = 24 was observed in both the mother plants and the regenerated plantlets, and the karyotypes of the regenerated plantlets were similar to those of the mother plants (**Fig. 4A, Fig. 4B**). No numerical or structural changes in the chromosomes were found in the regenerated plantlets.

There have been some reports of pine plant regeneration *via* direct organogenesis from embryo explants. With these methods, adventitious buds formed directly on the surfaces of the embryos or cotyledons, but the numbers of adventitious buds formed were low, probably because the embryo and cotyledon are very small in most pine species. Regeneration of about six buds per embryo was observed for both *Pinus wallichiana* (Mathur and Nadgauda, 1999) and *P. banksiana* (Harry and Thorpe, 1994), and regeneration of about 14 adventitious buds per cotyledon was observed for *P. canariensis* (Pulido *et al.*, 1994). Using our method, a large number of adventitious buds regenerated from calli of *Larix* embryo explants. The number of adventitious buds regenerated on callus per embryo averaged about 50, ranging to a high of 128. This demonstrates that the frequency of plant regeneration *via* indirect organogenesis was very high in comparison to methods described in other reports, including *Larix* species. To our knowledge, this is the highest number of adventitious buds formed per explant that has been reported for a *Larix* regeneration protocol.

This is the first report of efficient plant regeneration and micropropagation from callus derived from mature zygotic embryo of *L. gmelinii*. These results suggest that organogenesis from callus derived from mature zygotic embryo may be used routinely for clonal micropropagation and genetic

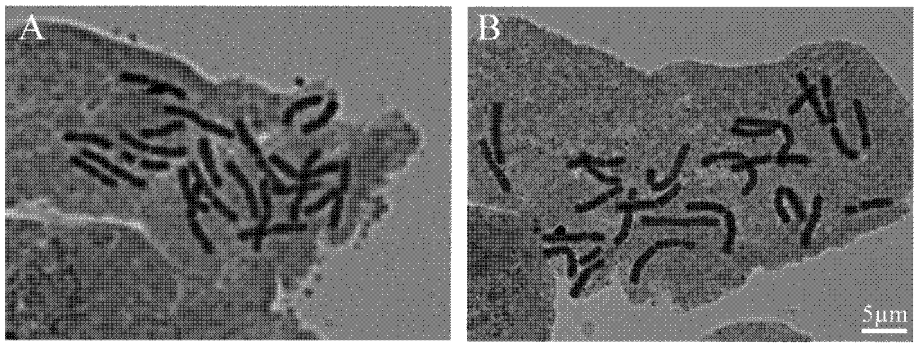

Fig. 4 Metaphase chromosomes in root tip cells of *Larix gmelinii* (Rupr.) Kuz. A: Mother plant (2n = 24). B: Regenerated plantlet (2n = 24).

transformation studies of larch species.

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