

An Efficient Procedure for Plant Regeneration from Long-term-cultured Callus Tissue of Hybrid Poplar (*Populus sieboldii* Miq. × *Populus grandidentata* Michx.)

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Plant regeneration *via* shoot differentiation was achieved using calli derived from leaf or stem explants of four genotypes of hybrid poplar (*Populus sieboldii* Miq. × *P. grandidentata* Michx., Y-63, Y-78, Y-79 and Y-102). The callus was induced and maintained in the presence of 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). A high concentration of 2,4-D permitted the proliferation of friable calli from explants and supported the active growth of homogeneous callus tissue during subculture. In all the genotypes tested, more than 60% of the calli from explants regenerated shoot-buds when transferred to a modified Linsmaier and Skoog medium with 2,4-D omitted and containing 0.2 to 2.0 mg/l zeatin. However, as the calli were repeatedly subcultured, the frequency of shoot regeneration under these conditions was reduced to less than 2% within a year. To improve the shoot regeneration from these calli, they were first incubated for two weeks on a medium without any growth regulators, and then cultured in the presence of 0.6 mg/l zeatin. With this procedure, the calli from a genotype Y-63 differentiated shoot-buds with an efficiency of 36%, which was markedly higher than the value obtained for calli grown continuously, without any change in the zeatin concentration. With a similar procedure, shoot regeneration from calli was possible for all the genotypes tested. The regenerated shoots differentiated roots when grown in a medium supplemented with a low concentration of auxin, and developed into mature plants after transfer to soil in a greenhouse.

Introduction

The application of developing technologies such as somatic cell fusion or genetic modification of plant cells by gene transfer to plant improvement is largely dependent on whether an efficient regeneration system can be established. In recent years, tissue culture of woody plants has been examined extensively for micropropagation¹⁾, and such technology has been widely incorporated into studies of tree transformation.

The genus *Populus*, including hybrid poplar, is important for wood and pulp production, and for roadside trees. Among *Populus* species, hybrid poplars have been examined for their regeneration

from stem explants²⁾, calli³⁻⁵⁾ and protoplasts^{6,7,14)}. A few reports have also described plant regeneration from established cell lines or aged callus tissues of *Populus sieboldii* × *P. grandidentata*^{3,4)}. On the other hand, the cells show various degrees of recalcitrance to shoot or embryo regeneration by prolonged culture with repeated transfer, which inevitably restricts the utilization of established cell lines or tissues maintained *in vitro*. In cultures of hybrid poplar calls, a high concentration (2 mg/l) of 2, 4-dichlorophenoxyacetic acid (2, 4-D) supports the active growth of friable callus, but severely reduces the frequency of shoot regeneration from calli during repeated subculture. However, we considered that manipulation of the culture system might improve the efficiency of shoot differentiation.

In this paper, we describe a procedure for shoot differentiation and subsequent plant regeneration from calli of *Populus sieboldii* × *P. grandidentata*, maintained for over a year by repeated subculture in the presence of a high concentration of 2, 4-D. The system would extend the choice of source materials and the availability of cell lines cultured *in vitro* for genetic modification of hybrid poplar cells.

Materials and Methods

1. Plant materials

Four genotypes of hybrid poplar, *Populus sieboldii* Miq. × *P. grandidentata* Michx., (Y-63, Y-78, Y-79 and Y-102), were vegetatively propagated at the Breeding Station, Kitakami Afforestation Branch, Touhoku Pulp Co. Ltd., Iwate, Japan⁸⁾.

2. Media

The media used were: LS, Linsmaier and Skoog medium (1965)⁹⁾; WS, Wolter and Skoog medium (1966)¹⁰⁾; LSB5, a medium consisting of inorganic salts of LS, B5 vitamins¹¹⁾ and 2% sucrose; mLSB5, a medium consisting of modified LS inorganic salts, in which the concentration of NH_4NO_3 was reduced from 21 mM to 10 mM and KNO_3 increased from 19 to 25 mM, B5 vitamins and 2% sucrose. All these media were supplemented with 0.4 g/l Casamino acids (certified grade, Difco, Detroit, Michigan, USA), adjusted to pH 5.7-5.8 with 1 M KOH and solidified with 0.8% (w/v) agar. The media were sterilized at 120°C for 8 min., except for 6-[4-hydroxy-3-methyl-but-2-enylamino] purine (zeatin), which was filter-sterilized and added to the media just before gelling.

3. Induction and maintenance of callus

Callus was induced from expanded leaves and young stems of plants grown in a greenhouse. Excised leaves and stems were dipped in 70% ethanol for a few seconds and then surface-sterilized with 0.25% sodium hypochlorite solution. After 2 min. and 5 min. of sterilization for leaves and stems, respectively, they were washed extensively in sterilized water. The sterilized leaves were cut into rectangular pieces about 8 mm long, and the stems were cut into 5-mm segment lengths using a surgical blade. These explants were placed on 90-mm Petri dishes containing 30 ml of LS or LSB5 medium supplemented with 2 mg/l 2, 4-D. The cultures were maintained at 25°C under continuous light of $5 \mu\text{E m}^{-2}\text{s}^{-1}$ from cool white fluorescent tubes. The proliferated calli were detached from the original tissue after 3 to 4 weeks of culture and subcultured thereafter at regular intervals of 3 weeks, using LSB5 containing 2 mg/l 2, 4-D.

4. Shoot regeneration

Stem- and leaf-derived calli maintained for at least one year were subjected to experiments for organogenesis. To test the capacity for shoot regeneration, three nutrient media of different composition, *i. e.* LSB5, WS and mLSB5, were employed. The regeneration was carried out using medium containing zeatin and lacking growth regulators. In some experiments zeatin was re-

placed by 6-furfurylaminopurine (kinetin) and benzyladenine (BA). The calli divided into pieces of approximately 80 mg in fresh weight were placed 90-mm Petri dishes containing 30 ml of medium, and the cultures were maintained at 25°C under a 16-h light/8-h dark photoperiod of $40 \mu\text{Em}^{-2}\text{s}^{-1}$ from white fluorescent tubes. Regeneration frequencies were scored at 5 weeks of culture on regeneration medium, on the basis of the percentage of calli differentiating visible shoot-buds that had been placed on the regeneration medium. The other conditions are described in **Results and Discussion**.

Once regenerated shoots had expanded leaves, they were excised from the callus and transferred to mLSB5 supplemented with 0.05 mg/l α -naphthaleneacetic acid (NAA) in order to differentiate adventitious roots. When the regenerated plantlets had grown to a sufficient size, they were transplanted to soil in a humidified chamber and then grown in a greenhouse.

5. Histology

All specimens for histology were made according to the procedure described by Jensen¹²). Sections 8 μm thick were cut with a microtome and stained with safranin followed by counterstaining with fast green.

Results and Discussion

Each of the tissue explants of hybrid poplar placed on the medium containing 2, 4-D showed signs of callus proliferation within seven days. On the LS and LSB5 media, the calli first appeared around the cut end of the explant, and then friable, yellowish-green cell clumps proliferated throughout almost all of the tissue fragments. More than 60% of these calli produced shoot-buds when transferred to the mLSB5 medium containing 0.2, 0.6 and 2.0 mg/l zeatin. Shoot regeneration also occurred at a comparative efficiency, using media containing 0.6 mg/l of kinetin or BA. Profuse calli produced on explants were transferred to fresh LSB5 medium for subculture. After several passages in subculture, the calli grew vigorously and increased 20–30-fold in fresh weight within three weeks. Among the media tested, LS and LSB5 in the presence of 2 mg/l 2, 4-D supported the best growth of the callus and yielded tissue with a homogeneous appearance after subculture (**Fig. 1-A**).

For shoot regeneration from subcultured calli, the basal components of LSB5, WS and mLSB5 were employed in the presence of 0.6 mg/l zeatin and absence of growth regulators. The morphogenic responses of calli from Y-63 are summarized in **Table 1**. The callus transferred to hormone-free LSB5 showed poor tissue growth and generated no shoots or roots. In the case of LSB5 medium supplemented with zeatin, extensive growth of green callus was observed, but no shoots or roots differentiated. The calli transferred to hormone-free WS medium showed very slow tissue growth and did not differentiate organs. Zeatin added at 0.6 mg/l to WS medium had no positive effect on the induction of organs. On the hormone-free mLSB5, callus proliferated slowly and no shoots regenerated, whereas almost all calli differentiated roots. On the other hand, on mLSB5 supplemented with zeatin, dark green callus grew extensively, and differentiation of shoot-buds occurred after about two weeks in a few cases; 2% of calli that had been transferred to the regeneration medium differentiated visible shoots. No positive effect of kinetin and BA on organogenesis was found at concentrations ranging from 0.02 to 2.0 mg/l, irrespective of the media tested. Thus, although the shoot-forming ability of hybrid poplar callus declined in culture containing a high concentration of 2, 4-D, exogenous zeatin was effective in inducing shoot-buds from these calli. In addition, comparison of the nutritional ingredients of the media indicated that an excess of NH_4NO_3 was inhibitory for shoot-bud regeneration. These results agreed with

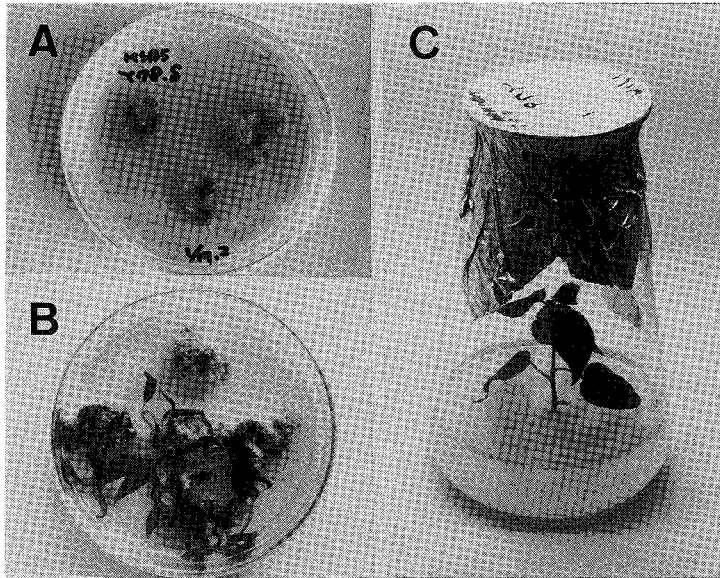


Fig. 1 Growth and differentiation of callus in *Populus sieboldii* × *P. grandidentata*. Stem-derived calli (A) of Y-78 were incubated for two weeks on hormone-free mLSB5 medium followed by subculture in the presence of 0.6 mg/l zeatin (B); the calli differentiated shoots. Shoots detached from the calli developed into plantlets on medium supplemented with a low concentration of auxin (C).

Table 1. Morphogenic responses of stem-derived callus of *Populus sieboldii* × *P. grandidentata* on different culture media*¹.

Media	Zeatin* ²	Growth responses	
		Callus growth	Organogenesis
LSB5	—	Slow	No
LSB5	+	Extensive (green callus)	No
WS	—	Slow	No
WS	+	Slow	No
mLSB5	—	Slow	Roots
mLSB5	+	Extensive (green callus)	Shoots

*¹ The genotype used was Y-63.

*² The calli were cultured in the presence of 0.6 mg/l zeatin (+) or in the absence of growth regulators (—).

previous reports on other clones of hybrid poplar (*Populus sieboldii* × *P. grandidentata*, Y-79, Y-100 and Y-102)^{3,4}.

Since we had confirmed that shoot regeneration occurred in callus culture, subsequent experiments were designed to improve the frequency of shoot regeneration. **Table 2** shows the effects of subculture during regeneration culture on the efficiency of shoot differentiation. When the calli were incubated for two weeks on mLSB5 hormone-free medium and then cultured on the same composition medium but containing 0.6 mg/l zeatin, a marked improvement of regeneration frequency was found (**Table 2** and **Fig. 1-B**). Tests employing different combinations of zeatin application revealed an appreciable increase in the regeneration frequency only when the callus tissue was first cultured on a hormone-free medium, followed by incubation in the presence of

Table 2. Effect of transfer during regeneration culture on the frequency of shoot differentiation in the hybrid poplar, *Populus sieboldii* × *P. grandidentata**¹ ($n=5$).

Application of Zeatin* ²		Shoot differentiation (Mean % ± SE)
Before transfer	After transfer	
+	(without transfer)	2±2
-	(without transfer)	0
-	-	0
-	+	36±4
+	+	8±2

*¹ Stem-derived calli of Y-63 were used. The calli were incubated for two weeks before transfer followed by incubation for an additional three weeks.

*² A basal medium of mLSB5 was employed in the presence of zeatin at 0.6 mg/l (+) or in the absence of growth regulator (-).

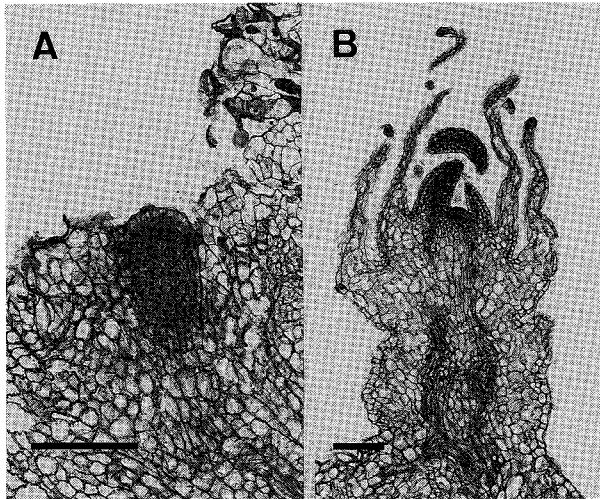


Fig. 2 Histology of shoot-bud differentiation in *Populus sieboldii* × *P. grandidentata* callus cultures.

Stem-derived calli of Y-63 cultured on mLSB5 containing 0.6 mg/l zeatin were harvested on day 20 (A) and day 30 (B). The tissue was processed for histology as described in **Materials and Methods**. Bars indicate 100 μm.

zeatin. From these data, it is evident that the improvement of shoot regeneration is due not only to the transfer itself but also the change of zeatin concentration. This implies that callus growth preceding the morphogenesis and differentiation of shoot-bud primordia requires different optimal hormone conditions. In addition, there is a possibility that the breakdown or release of intracellular 2, 4-D, which leads to enhancement of shoot differentiation, is promoted by callus transfer; aged callus seems to be more sensitive to exogenous auxins than callus produced in primary culture. It has been reported that the number of shoots was increased by transfer during regeneration culture of *Populus alba*¹³⁾ and *P. alba* × *P. grandidentata*⁵⁾.

Histological micrographs are presented in **Fig. 2**. In the late phase of callus development at 20 days after transfer, organization of meristematic tissue composed of small, cytoplasm-rich cells was found at the periphery of the callus tissue (**Fig. 2-A**). Thin sections of developing shoots revealed tissue continuity at their proximal ends with callus tissue (**Fig. 2-B**), indicating that

regeneration occurs not through somatic embryogenesis but through shoot organogenesis.

When differentiated shoots were transferred to medium containing a low concentration of NAA, they easily formed adventitious roots at their cut ends (**Fig. 1-C**). The plantlets that had developed roots were then transplanted to soil in pots, and grown to mature plants in a greenhouse. In four genotypes of hybrid poplar calli, maintained by subculture, shoot regeneration was achieved by an essentially similar procedure, although the frequency ranged from 6% to 56%, depending on the genotype and cell line. We were unable to detect any appreciable differences in regeneration frequency between stem-derived and leaf-derived callus. This procedure was also applied to protoplast-derived callus of Y-63, and plantlets were also regenerated; details will be reported elsewhere¹⁵⁾. The present results indicate that the method we have described is efficient for shoot regeneration from long-term-cultured poplar callus, suggesting that the cells sustain their competence for a considerably long period in culture.

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《和文要約》

長期間継代培養した交雑ヤマナラシ(*Populus sieboldii* Miq. × *Populus grandidentata* Michx.) カルスにおける効果的な植物体再生法

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交雑ヤマナラシ(*Populus sieboldii* Miq. × *P. grandidentata* Michx., Y-63, Y-78, Y-79, Y-102)の若い茎と葉から 2.0 mg/l 2,4-dichlorophenoxyacetic acid 存在下でカルス誘導し、継代培養によって維持した。カルスは柔軟で均質な組織として旺盛に生長した。初代培養において組織片に生じたカルスは、zeatin, kinetin あるいは benzyladenine を含む修正 Linsmaier と Skoog の培地上で効率よく茎葉を分化したが、1年以上継代維持したカルスから同様の方法で茎葉分化を試みた場合、茎葉を分化したものは移植片の2%以下であった。そこで、継代培養によって維持されたカルスを用い、茎葉分化と植物体再生法の改良を試みた。このようなカルスから効率よく茎葉を誘導するには、まず組織片を生長調節物質を含まない修正 Linsmaier と Skoog の培地で2週間培養し、その後 0.6 mg/l の zeatin を添加した培地に移し培養を続けるという方法が有効であった。この方法により、供試した全てのクローンに由来するカルスにおいて茎葉が分化し、植物体が復元された。さらに、誘起された形態形成が胚分化でなく茎葉分化であるという組織学上の知見も得られた。