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An Improved System for Shoot Regeneration from Stem Explants of Lombardy Poplar (*Populus nigra* L. var. *italica* Koehne)

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ABSTRACT

We developed a system for the regeneration of Lombardy poplar (*Populus nigra* L. var. *italica*) shoots from internodal stem explants. Using this system, shoots regenerated from 87% of the stem explants placed on Murashige and Skoog (MS) medium supplemented with 0.1 mg/L indole-3-acetic acid and 0.5 mg/L benzylaminopurine without undergoing callus formation. About 80% of the *in vitro* regenerated shoots developed roots on MS medium supplemented with 0.5 mg/L indole-3-butyric acid and 0.02 mg/L 1-naphthylacetic acid. Well-rooted seven-to eight-week-old regenerated plants could be transferred to soil for further growth and the survival rate of such plants after three weeks was 88%. The protocol presented here is simple and economical because it does not rely on pre-incubation in callus induction medium or repeated subculture in shoot induction medium containing *trans*-zeatin, an expensive substance. The *in vitro* regeneration system presented here could be used for evaluation of radiation sensitivity for Lombardy poplar tissues.

Keywords: Lombardy Poplar; Shoot Regeneration; Stem Explants; Auxin and Benzylaminopurine; Radiation Sensitivity

1. Introduction

Poplars are of growing scientific importance due to their small genome size, short rotation cycle, ability to undergo *in vitro* regeneration, and ease of transformation and vegetative propagation. These important traits, coupled with the existence of a library of full-length enriched expressed sequence tags [1] and availability of the genome sequence of *Populus tricocarpa*, have established poplar as a model system in molecular genetics [2-5] and tree physiology [6-8] studies. Furthermore, these rapidly growing plants, which have the potential to enhance wood supplies for the plywood, hardboard, pulp, and paper industries, represent a commercially important resource [9].

In vitro plant regeneration of Lombardy poplar [11] or its close hybrids [10,12-14] was achieved from leaf or stem explants. These methods consists of two steps of tissue culture stages; first, stem or leaf explants were incubated on the medium containing high concentration of plant hormone auxin (in most case, it is called callus inducing medium, CIM) for several days then transferred to shoot induction medium (SIM) that dominantly contains plant hormone cytokinin. The system is also available for *Agrobacterium*-mediated gene transformation [10,11,15]. In an early study of Lombardy poplar regeneration by Mohri *et al.* [15], 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) was added to CIM for calli induction. Then, four weeks old calli developed in CIM were repeatedly subcultured in SIM containing 2 mg/L trans-zeatin and 0.2 mg/L benzylaminopurine (BA), followed by re-transfer to root induction medium (RIM). In later by Nishiguchi *et al.* [11], much simple regeneration method had been developed for Lombardy poplar regeneration, without callus formation. But, still they relied on pre incubation of explants in CIM (0.5 mg/L 2,4-D and 1 mg/L BA) for 6 days. There after, pre incubated explants in CIM were subcultured in SIM (0.5 mg/L zeatin and 0.1 mg/L BA) for several times, followed by re-transfer to RIM. However, in our experience, we were unable to perform efficient shoot regeneration with these protocols. Thus, we tried to re-optimize shoot regeneration protocol for Lombardy poplar by using different types of auxins and cytokinins at various concentrations. Here, we report a simple shoot regeneration protocol from stem explants of Lombardy poplar that is charac-

terized by unrequisite of pre-incubation step in CIM.

2. Materials and Methods

2.1. Plant Materials, Growth Condition and Surface Sterilization

Lombardy poplar plants were propagated in our laboratory from cuttings of poplars obtained from the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan. Donor plants were grown in pots containing a 1:1 mixture of vermiculite and Metro-Mix 350 (Scotts-Sierra Horticultural Products Company, Marysville, OH) at 26˚C under fluorescent tubes (90 $\mu E \cdot m^{-2} \cdot s^{-1}$, 16-h photoperiod). The relative humidity was kept at 70%. The plants received weekly applications of Hyponex solution (1:1000) and were watered daily.

In preliminary experiments, surface sterilized explants (1 cm) from leaves, petioles, and stems were used to examine regeneration proficiency to a range of different concentration of 2,4-D, indole-3-acetic acid (IAA), naphthylacetic acid (NAA), thidiazuron (TDZ), zeatin and BA, singly or in combination. But, efficient and reproducible regeneration were obtained only with stem explants in preliminary experiments. Thus, only internodal stem explants were used for remaining experiments of this study. Internodal stem explants were excised from newly growing branches of 3- to 4-month-old donor plants, cut to 1-cm segments, and divided vertically into halves (**Figure 1A**). Surface microorganisms were removed from the explants by thorough washing under running water for 20 min, followed by treatment with sodium hypo-chlorite solution (15%) for 20 min. The explants were then washed three times for 20 min each with sterile water.

2.2. Culturing Tissues and Shoot Regeneration

For plantlet regeneration, the surface-sterilized stem explants were cultured on Petri dishes containing Murashige and Skoog (MS) basal medium [16] supplemented with 30 g/L sucrose, 1 ml/L vitamin B5, 3 g/L Gelrite (Wako, Osaka, Japan) and various concentrations of IAA, NAA and/or cytokinin (BA) (**Table 1**). The pH of media was adjusted to 5.8 prior to autoclaving at 121˚C for 20 min. The explant cultures were maintained at 26˚C under 16-h light and 8-h dark intervals in a growth chamber and subcultured in every 2 weeks.

2.3. Root Induction

For root induction, regenerated shoots were separated and cultured on MS medium supplemented with 30 g/L sucrose, 1 ml/L vitamin B5, 2 g/L Gelrite, 4 g/L agar and various concentrations of indole-3-butyric acid (IBA) and

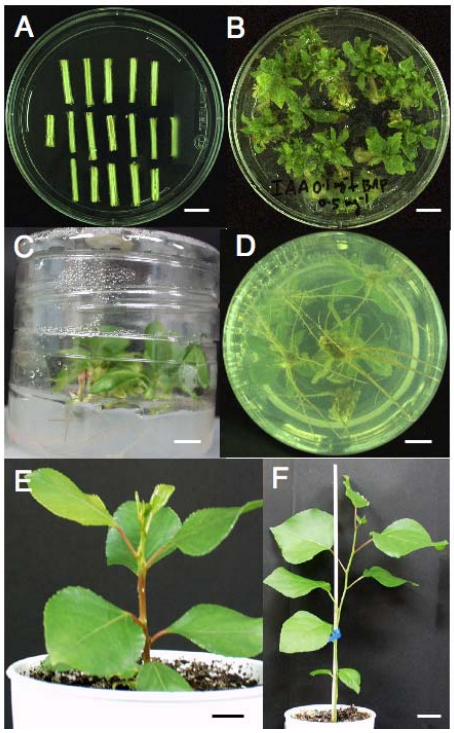

Figure 1. Direct plant regeneration of Lombardy poplar. (A) Vertically divided internodal stem segments were used as starting materials; (B) Shoot regeneration on medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BA; (C) and (D) Root development [side view (C) and bottom view (D)] in seven-to-eight-week-old *in vitro* raised plants on MS medium supplemented with 0.5 mg/L IBA, 0.02 mg/L NAA, 4 g/L agar and 2 g/L Gelrite; (E) Regenerated plant growing in a soil mixture of vermiculite and Metro-Mix 350 (1:1); (F) Well-established and hardened *in vitro* raised *Populus* plant growing in soil mixture. Bars indicate 1 cm (A)-(E) and 2 cm (F).

Table 1. Direct shoot regeneration from internodal stem explants of Lombardy poplar.

Treatment (mg/L)				Shoot induction (%)[a]	Number of shoots per explant[a]
IAA	NAA	2,4-D	BA		
0	0	0	0	0	0
0	0	0.5	0.5	0	0
0.1	0	0.5	0.5	0	0
0.1	0	0	0.1	46 ± 2.31	1.87 ± 0.11
0.1	0	0	0.2	51 ± 2.40	1.96 ± 0.07
0.1	0	0	0.5	87 ± 3.71	3.87 ± 0.09
0.2	0	0	1	87 ± 4.81	3.63 ± 0.08
0.5	0	0	0.5	45 ± 3.71	2.13 ± 0.12
0	0.1	0	0.5	79 ± 4.37	3.18 ± 0.10
0	0.2	0	0.5	83 ± 3.33	3.55 ± 0.07
0	0.5	0	0.5	39 ± 4.80	2.05 ± 0.07

[a]Results represent means ± standard errors (SEs) of at least three replicated experiments. Each experiment contained 40 to 50 explants. Measurements were taken after five weeks of culture.

NAA. Again, the pH of the medium was adjusted to 5.8.

2.4. Irradiation of Tissues

For investigation of radiation dose response relationship on viability test and growth proficiency of regenerated shoots, stem explants were excised to 1 cm, surface sterilized, divided vertically into halves and cultured on medium with 0.1 mg/L IAA and 0.5 mg/L BA in Petri dishes. One week after from culture, Petri dishes containing stem segments were subjected for irradiation with a range of doses (0 Gy to 30 Gy). The explants were exposed to 320 MeV carbon ions accelerated by the azimuthally varying field cyclotron [22,23] or gamma rays. Gamma rays obtained from ^{60}Co source maintaining at Japan Atomic Energy Agency (Takasaki, Japan). Irradiation time for gamma rays was fixed to 30 min. Shoots above 1 cm in length at five weeks after irradiation were counted as regenerated shoots. Regeneration ratio was calculated with dividing the number of stem explants that produce regenerated shoots by total number of stem explants irradiated. The regeneration ratio of non-irradiated control stems was plotted as 100%.

3. Results and Discussion

3.1. Direct Shoot Regeneration

In preliminary experiments, we cultured explants from leaves, petioles, and shoots to medium supplemented with different concentrations of 2,4-D, IAA, NAA, TDZ, zeatin and BA, singly or in combination (data not shown). Leaf and petiole explants did not develop any robust calli or shoots on any of the media mentioned above. On the other hand, stem explants developed calli on medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L BA; however, no shoots emerged from these calli until five weeks of culture (**Table 1**). Interestingly, we found that direct shoot regeneration occurred when stem explants were cultured on medium supplemented with NAA and IAA in combination with BA (**Table 1** and **Figure 1(B)**).

Over 87% of the stem explants produced at least one shoot on medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BA. The average number of shoots per explant was 3.87 ± 0.09 (**Table 1**). A further increase of IAA to 0.2 mg/L or 0.5 mg/L and BA to 1 mg/L failed to improve the regeneration efficiency. Thus, we used 0.1 mg/L IAA and 0.5 mg/L BA for the direct regeneration of Lombardy poplar in this study. Application of the synthetic auxin analog NAA yielded similar results as IAA. Over 83% of the explants induced shoots on medium supplemented with 0.2 mg/L NAA and 0.5 mg/L BA, and the average number of shoots per explant was 3.55 + 0.07 (**Table 1**). Once again, an increased NAA concentration (0.5 mg/L) failed to improve the regeneration efficiency (**Table 1**). In Eastern Cottonwood (*Populus deltoides* Bartram ex Marsh.), addition of cytokinin (1 mg/L zeatin) alone resulted in the most efficient shoot regeneration from stem explants in woody plant medium (WPM) [17]. However, we found that cytokinin alone could not stimulate the growth of calli or shoots in Lombardy poplar and an optimal combination of auxin and cytokinin (BA) was required for direct shoot regeneration. This indicates that the optimal hormone concentration for direct shoot regeneration in poplar is genotype specific. Alternatively, the difference in response might be for the reason of different basal medium, although there is no major difference between the composition of WPM and MS. Thus, for efficient regeneration, appropriate combination and concentration of growth regulators need to be determined precisely.

3.2. Root Induction

To optimize root induction conditions, the directly regenerated shoots were cultured on media containing IBA and NAA. In agreement with a report [11], we found that a combination of 0.5 mg/L IBA and 0.02 mg/L NAA was the most effective dose for root development in Lombardy poplar (**Figure 1(C)** and **1(D)**), although 0.5 mg/L IBA alone could induce roots in 50% of the regenerated shoots (**Table 2**). Doubling the concentration of IBA and NAA in the medium did not improve the root induction efficiency (**Table 2**). The average number of roots per shoot in the presence of 0.5 mg/L IBA and 0.02 mg/L NAA was 5.2 ± 0.34 (**Table 2**).

We separated the well-rooted plants that were approximately seven weeks old from the culture medium, transferred them to pots containing a 1:1 mixture of vermiculite and Metro-Mix 350 and incubated them at 26˚C under a 16/8 h light/dark regimen. **Figure 1(E)** shows a regenerated plant growing in soil mixture. Initially, the potted plants were covered with clear plastic bags to protect them from any direct physical stress, and the plants

Table 2. Root induction in the regenerated shoots.

Treatment (mg/L)		Root induction (%)[a]	Number of roots per shoot[a]
IBA	NAA		
0	0	0	0
0.5	0	50.00 ± 4.33	3.6 ± 0.24
0	0.02	0	-
0.5	0.01	60.83 ± 4.41	3.67 ± 0.12
0.5	0.02	78.33 ± 3.00	5.2 ± 0.34
1	0.04	75.83 ± 3.63	5.1 ± 0.31

[a]Results represent means ± SEs of at least three replicated experiments. Each experiment contained 40 to 50 regenerated shoots. Measurements were taken after seven weeks of culture.

were unwrapped after 7 - 10 days. The *in vitro* raised poplar plants grew well and showed a high percentage of survival rates (88%) at three weeks of growth in soil. Healthy plants were transferred to larger pots with fresh soil. Well-established and hardened plants were allowed to grow (**Figure 1(F)**) in an incubator with weekly applications of Hyponex solution (1:1000).

3.3. Test of Radiation Sensitivity of Tissues

The direct shoot regeneration protocol developed in this study can be used to estimate radiation sensitivity of the tissues, which is initial requirement for a strategy of tissue-culture-mediated screening mutants with ionizing radiation. **Figure 2** represents dose response relationship curves for carbon ion-beam (**Figure 2(a)**) and gamma-ray (**Figure 2(b)**) irradiation on viability test and growth proficiency of *in vitro* regenerated Lombardy poplar shoots. No visible decrease in growth of regenerated shoots was found up to the irradiation dose of 2.5 Gy and 5 Gy for ion beams and gamma rays, respectively. Hence, irradiation with these doses to stem explants of Lombardy poplar hardly affects survival ability of regenerated shoots. It is relevant to mention that now a day, ionizing radiation is attracting increasing attention as a new mutagen [22,23]. Various novel mutants of a wide variety of plants, which include *Arabidopsis thaliana*, *Lotus japonicus*, tobacco, rice, verbena, rose, carnation, *Torenia*, petunia, and Hinoki cypress, have been obtained via ion-beam irradiation [24]. Similarly, soybean mutant lacking lipoxygenases was identified by gamma-ray irradiation [25]. Thus, using this strategy, isolation of mutants would be achievable in poplar, as well.

4. Conclusion

We found that shoot regeneration from Lombardy poplar stem segments can be achieved by a simple procedure, in which pre-culture in CIM is not necessary. Similar procedure is reported in Eastern Cottonwood (*P. deltoides*) [17-19] and male Himalayan poplar (*P. ciliata*) [20]. No callus induction in this method might be another advantage, because it is well known that plants regenerated *via* a callus phase may differ from the mother plant due to somaclonal variations [21]. The present protocol is also economical, because it results high frequency (87%) direct shoot regeneration without *trans*-zeatin, an expensive substance that have used in the previous protocols of

Table 3. Comparison of regeneration systems in Lombary poplar (*Populus nigra* cv. *italica*) and related species.

Genetic background	No. of media used for shoot regeneration*	Name of media	Growth regulators (mg/L)	Explant tissue type	Incubation period (week)	Reference
Populus nigra L. var *italica* Koehne	1	SIM	IAA (0.1) + BA (0.5)	Stem	8	**Present study**
		RIM	IBA (0.5) + NAA (0.02)			
Populus nigra L. var *italica* Koehne	2	CIM	2,4-D (0.5)	Stem	10	[15]
		SIM	*t*-zeatin (2.0) + BA (0.2)			
		RIM	IBA (0.5) + NAA (0.02)			
Populus nigra L. var *italica* Koehne	2	CIM	2,4-D (0.5) + BA (1.0)	Stem	13	[11]
		SIM	*t*-zeatin (0.5) + BA (0.1)			
		RIM	IBA (0.5) + NAA (0.02)			
Populus nigra L.	2	CIM	NAA (0.2) + BA (0.5)	Leaf	13~	[10]
		SIM	NAA (0.05) + BA (0.5)			
		RIM	None			
Populus nigra × *P. pyramidales*	2	CIM	NAA (0.5) + BA (1.0)	Leaf	INT	[14]
		SIM	NAA (0.25) + BA (0.6)			
		RIM	NAA (0.1) +BA (0.1)			
Populus nigra var thevestina × (*P. diversifolia* + *P. tomentosa*)	2	CIM	IAA (0.1) + BA (0.5)	Leaf	INT	[12]
		SIM	NAA (0.01) + BA (0.5)			
		RIM	IAA (0.8)			
Populus nigra var thevestina	2	CIM	IBA (0.1) + BA (0.5)	Leaf	INT	[13]
		SIM	IBA (0.1) + BA (1.0)			
		RIM	IBA (1.0)			

*RIM is not included. INT: Information not traceable.

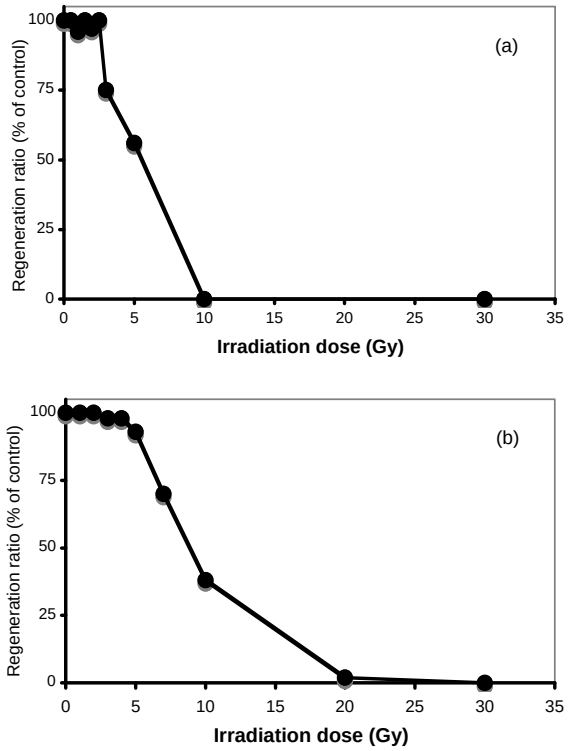

Figure 2. Dose response relationship curves for carbon ion-beam (a) and gamma-ray (b) irradiation on regeneration ratio of *in vitro* raised Lombardy poplar. Shoots above 1 cm in length at five weeks after irradiation were counted as regenerated shoots. Regeneration ratio was calculated with dividing the number of stem explants that produce regenerated shoots by total number of stem explants irradiated. The regeneration ratio of non-irradiated control stems was plotted as 100%.

Lombardy poplar regeneration (**Table 3**) [11,15,17]. Our method is able to produce and perpetuate a large number of disease-free Lombardy poplar plants, and will thus benefit physiological and genetic studies of hardwood plants by providing a constant supply of competent and efficient plant materials.

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