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Research Article

High-frequency regeneration by stem disc culture in selected clones of *Populus euramericana*

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Abstract An efficient regeneration protocol for stem disc culture of *Populus euramericana*, which is important species for bioenergy resource in agroforestry, was established. The number of explants that were obtained and the number of explants that regenerated varied with the genotypes. However, in all the genotypes, stem disc culture produced more regenerated shoots than did in axillary bud culture. A comparison of the effects of cytokinin type and concentration on shoot regeneration in different explants (i.e., petiole, leaf, and root segments of *P. euramericana*) revealed that a concentration of 0.002 mg l^{-1} thidiazuron (TDZ) used on petiole segments resulted in the greatest shoot regeneration (95.83%). The hormonal requirements for the greatest shoot regeneration in the three explant types varied. Different concentrations of $AgNO_3$ and $CoCl_2$ were added separately to the medium to stop the yellowing and subsequent necrosis of the regenerated shoots. Lower concentrations (3 and 5 mg l^{-1}) of these compounds improved shoot regeneration and elongation, compared with the control. The in vitro-regenerated shoots were transferred to rooting medium and subsequently acclimatized. The highly efficient regeneration system of *P. euramericana* reported here can be used for mass propagation of this recalcitrant for regeneration, economically important tree species.

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Abbreviations

TDZ	Thidiazuron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea)
BA	6-Benzylaminopurine
IBA	Indole-3-butyric acid
PPFD	Photosynthetic photon flux density
pH	Hydrogenion concentration

Introduction

Populus euramericana is a hybrid of *P. deltoides* and *P. nigra.* Recently it was selected as a candidate for planting in high salinity areas, as it tolerates these conditions well. It is also a fast-growing species, so it is regarded as an economically important source of bioenergy (Wullscheleger et al. 2002). Due to these qualities, *Populus* is now the leading choice of the U.S. Department of Energy for woody bioenergy crops (Kang et al. 2009). *Populus* trees have been the subject of several biotechnology studies because they grow quickly, may be propagated sexually or asexually, and show potential for interspecific crosses (Tsvetkov et al. 2007). Poplar is also a model subject for biotechnology studies because it is easy species to regenerate in vitro and because the full genome of *P. trichocarpa* has been released (Tuskan et al. 2006). However some of the species belonging to this family are recalcitrant in regeneration, and *P. euramericana* is one of them (Sellmer et al. 1989; Colman & Ernst 1990; Noel et al. 2002).

The purpose of the present study was to establish a highly efficient regeneration system for clonal mass propagation of *P. euramericana* that can also be used during genetic transformation of selected clones of this species. Stem disc culture

has been studied as an alternative to axillary bud culture to regenerate shoots. A secondary aim of this study was to examine the effect of exposure of thin layers of lateral meristem in the stem sections to the regeneration medium. Elo et al. (2009) reported that although many regulatory mechanisms controlling the development and function of root and shoot apical meristems are known, our knowledge of similar processes in lateral meristems, including the vascular cambium, is still limited.

Materials and Methods

Plant materials

Stems approximately 1 meter in length (diameter 5 ~ 10 mm) were taken from 5 clones of *P. euramericana* Guinier (*P. deltoides* × *P. nigra*): 'Dorskamp,' 'ECO-28,' 'I-476,' 'Venziano,' and 'Ay48' growing at the Korea Forest Research Institute. The stems were then cut into segments 5 ~ 7 cm in length and washed with running tap water for 30 min. Next, they were treated with 70% ethanol for 30 sec and then with 0.2% $HgCl_2$ for 25 min. After receiving a final treatment with NaOCl for 15 min, they were thoroughly rinsed with sterilized water 3 ~ 5 times.

Stem disc culture and shoot regeneration

To prepare stem discs for culture, 1 ~ 2 mm thick cross sections of the sterilized stem were cut. An approximately 3~5-cm length of stem node with a single axillary bud was selected as a control. The stem discs were transferred onto the regeneration medium with 10 stem-disc sections per petri dish (87 x 10 mm). For nodal culture, a single node (3 ~ 5 cm) was transferred into a test tube (15 x 20 mm) containing the regeneration medium. The regeneration medium consisted of ½ MS (Murashige and Skoog, 1962) supplemented with zeatin (Duchefa, The Netherlands) 0.5 mg l^{-1} and 2% (w/v) sucrose.

Effects of cytokinin type and concentration and explant type

To study the effect of cytokinin type and concentration on regeneration, basal medium consisting of ½ MS and sucrose at 20 g l^{-1} was supplemented with 6-benzylaminopurine (BA; Duchefa, The Netherlands) (0.1 ~ 1.0 mg l^{-1}), zeatin (0.1 ~ 1.0 mg l^{-1}), or thidiazuron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea; TDZ) (Duchefa, The Netherlands) 0.002 ~ 0.1 mg l^{-1}. To study the effect of the type of explant on regeneration, petiole, leaf, and root segments of plantlets of clone 'I-476' that were grown in vitro were used. The plantlets were cultured for 4 weeks.

Effect of $AgNO_3$ and $CoCl_2$ on shoot development

To study the effect of $AgNO_3$ and $CoCl_2$ on shoot development, petioles of plantlets of clone'I-476' that were grown in vitro were used as explants. $AgNO_3$ and $CoCl_2$ were added at concentrations of 0, 3, 5, 10, and 15 mg l^{-1} separately to the basal medium, which consisted of ½ MS and sucrose at 20 g l^{-1} concentration. The plantlets were cultured for 4 weeks.

Acclimatization

In vitro-regenerated shoots were transferred into ½ MS medium containing Indole-3-butyric acid (IBA) 0.05 mg l^{-1} and 2% (w/v) sucrose for rooting. After the plantlets had been cultured for 4 weeks, a number of plantlets about 5 cm tall with at least 5 leaves were selected. These plantlets were then transferred into plastic pots (diameter 10 cm) containing artificial soil mixture [perlite:peatmoss:vermiculite = 1:1:1 (v:v:v)], and grown for 8 weeks under 80% relative humidity at 100 ~ 150 μmol m^{-2} s^{-1} Photosynthetic photon flux density (PPFD) for acclimatization in a greenhouse.

Statistical analysis

Statistical analysis was performed according to the SAS system (Version 6.21, SAS Institute Inc., Cary, NC 27513, USA). Means and standard errors were used throughout and the statistical significance between mean values was assessed using ANOVA or Duncan's multiple range tests at $P < 0.05$. All the experiments were repeated 3 times.

Results

Stem-disc culture

The 5 clones of *P. euramericana* ('Dorskamp,' 'ECO-28,' 'I-476,' 'Venziano,' and 'Ay48') produced different numbers of explants from the 30-cm stem. 'Venziano' showed the greatest number of explants per 30-cm stem, nearly 70, compared with the average 50 explants from all the clone types (Fig. 1A). The number of explants showing regeneration also varied, with 'Venziano' showing the greatest response. The average response of all the clone types was 40 per 30 cm. stem (Fig. 1B). Irrespective of the clone type, the stem disc culture produced more explants than did nodal culture, and more shoots were obtained from the stem disc section than

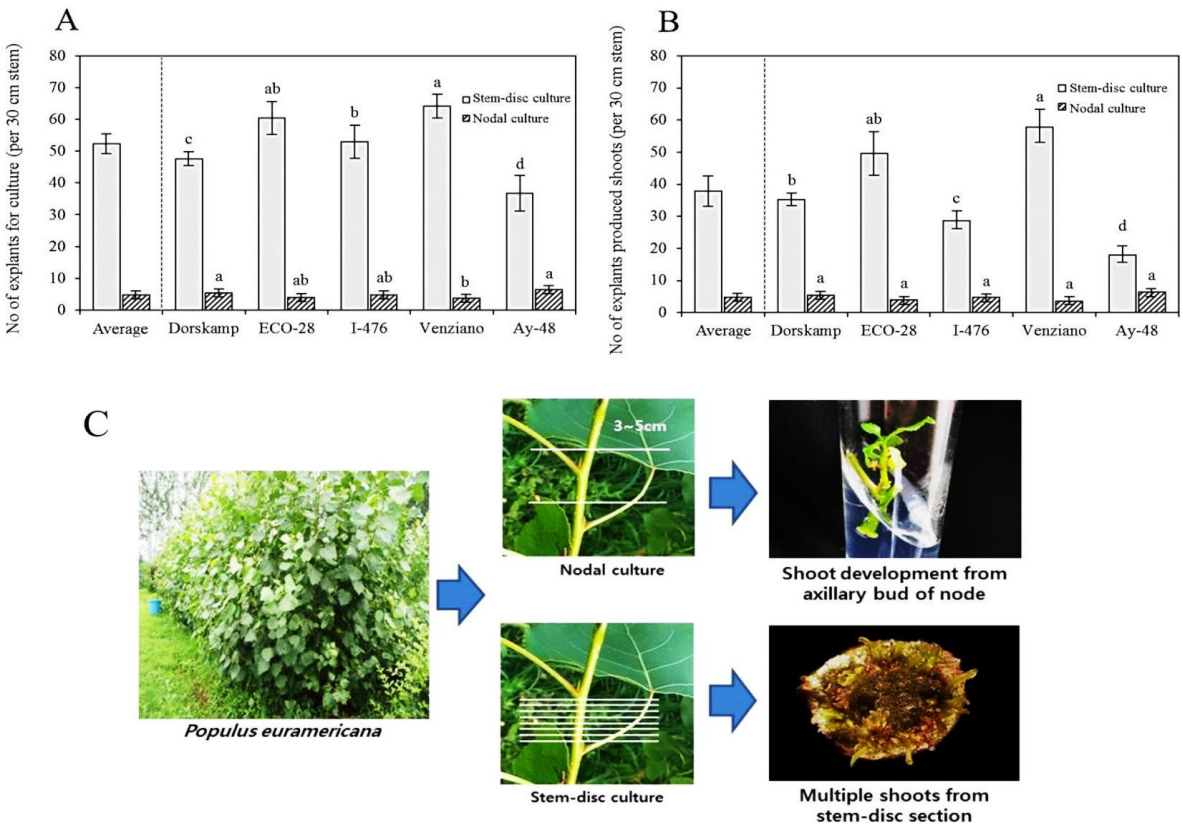

Fig. 1 Effects of genotypes and explant types for efficient shoot regeneration in *Populus euramericana*. A. Number of explants can be obtained from 30 cm length stem in conventional nodal culture and stem-disc culture. B. Number of shoots regenerated from two types of explants, nodes and stem-discs. C. Shoot regeneration process from nodes and stem-discs cultures

Table 1 Effects of different types and concentrations of cytokinins on shoot regeneration from various tissues of stem-disc culture derived plantlets in *P. euramericana* 'I-476'after 4 weeks of culture

Cytokinin			No. of shoots (explant^{-1})			Percentage of regeneration (%)		
BA (mg l^{-1})	TDZ (mg l^{-1})	Zeatin (mg l^{-1})	Petiole	Leaf	Root	Petiole	Leaf	Root
0	0	0	0.00f[z]	0.00d	3.42efg	0.00g	0.00d	20.83cd
0.1	0	0	6.25d	3.04c	6.08bc	50.00cd	4.17d	45.83ab
0.2	0	0	8.29bc	5.63b	7.46ab	75.00ab	29.17bc	50.00a
0.5	0	0	2.42e	3.00c	3.25efg	37.50de	0.00d	4.17d
1.0	0	0	1.00f	3.04c	2.00g	0.00e	4.17d	0.00d
0	0.002	0	5.29ab	5.54bc	8.79a	95.83a	33.33b	50.00a
0	0.004	0	10.04a	7.13a	3.38efg	91.67ab	41.67ab	16.67cd
0	0.02	0	4.67ef	3.00c	2.00g	29.17def	0.00d	0.00d
0	0.1	0	1.00f	2.00c	1.78g	0.00g	0.00d	0.00d
0	0	0.1	4.75ef	3.04c	4.83cde	41.67d	4.17d	25.00bcd
0	0	0.2	1.25f	2.00c	5.92bcd	12.50fg	0.00d	37.50abc
0	0	0.5	1.08f	3.00c	4.67cde	8.33fg	0.00d	33.33abc
0	0	1.0	1.17f	2.70c	3.17fg	16.67efg	0.00d	8.33d
0.2	0.02	0	8.46bc	7.54a	2.00g	91.67ab	54.17a	0.00d
0.2	0	0.1	10.13a	5.33bc	4.58cde	83.33ab	12.50cd	25.00bcd
0.2	0	0.2	7.58cd	5.58bc	4.71cde	70.83bc	33.33b	45.83ab

[z]Mean separation within columns by Duncan's multiple range test at 5% level.

from the axillary bud culture (Fig. 1C).

Effect of cytokinin and explant type

The effect of cytokinin type and concentration were examined using the clone 'I-476,' as it was a good material for bioenergy source with relatively low lignin content among the 5 clones (data not shown). Shoot regeneration was observed after 7 days of culture on the regeneration medium. A concentration of 0.2 mg l^{-1} BA produced the greatest number of shoots per explant, irrespective of the explant type. A concentration of 0.004 mg l^{-1} TDZ induced the greatest number of shoots (10.04) with petiole as the explant. A concentration of 0.1 mg l^{-1} zeatin induced the greatest regeneration when petiole was used (41.67%). When a combination of cytokinins was used, a combination of 0.2 mg/l BA and 0.02 mg l^{-1} TDZ resulted in the greatest shoot regeneration (91.67%) from the petiole (Table 1).

Effect of $AgNO_3$ and $CoCl_2$

Shoots that had regenerated from explants started to turn yellow after about 6 weeks on the regeneration medium containing cytokinins, and subsequently they showed necrosis (data not shown). Thus, regenerated shoots with explants were transferred on the ½ MS medium containing $AgNO_3$ and $CoCl_2$ separately. Lower concentrations (3 and 5 mg l^{-1}) of these compounds promoted shoot regeneration and elongation, compared with the control (Table 2). Of these two, $CoCl_2$ at a concentration of 3 mg/l resulted in better shoot regeneration (91.11%) and shoot elongation (100%).

Discussion

In the present study, the five different clone types of *P. euramericana* showed different shoot regeneration potential. Similar findings were reported for strawberry by Passey et al. (2003), who found that different cultivars respond differently during adventitious shoot regeneration. Interestingly, irrespective of the clone type used, the stem disc culture yielded more regenerated shoots than did nodal culture. Similarly, Nayak et al. (2002) reported an efficient micropropagation system for

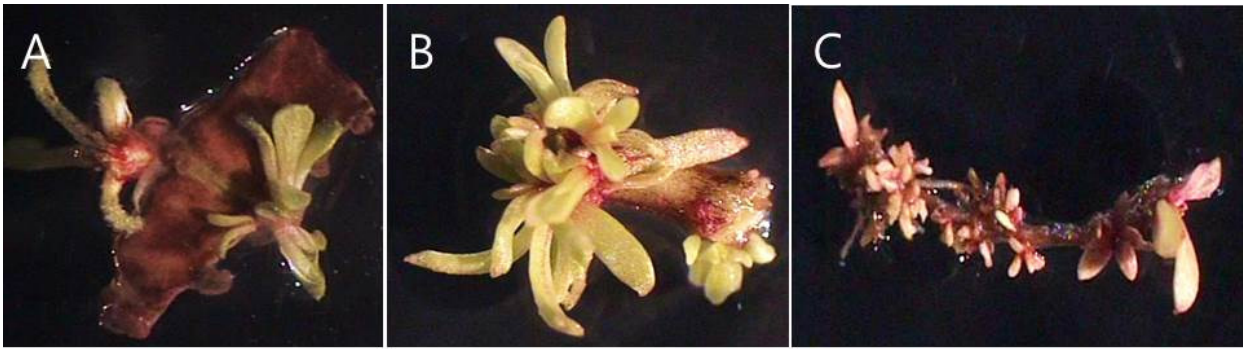

Fig. 2 Adventitious shoot formation from various explants of *P. euramericana* on ½ MS medium with 0.2 mg l^{-1} BA and 0.1 mg l^{-1} zeatin from (A) leaf segment, (B) petiole, and (C) root segment

Table 2 Effect of $AgNO_3$ and $CoCl_2$ concentrations on shoot regeneration of *P. euramericana* 'I-476' after 4 weeks of culture

Treatment (mg l^{-1})		Shoot regeneration		Shoot elongation	
		No. of shoots (explant^{-1})	Shoot regeneration (%)	No. of shoots (explant^{-1})	Shoot elongation (%)
Control	0.0	15.88ab[z]	86.67ab	5.19ab	80.17b
$AgNO_3$	3.0	15.50ab	71.11ab	7.95ab	95.24ab
	5.0	17.47ab	80.00ab	7.63ab	90.45ab
	10.0	16.58ab	73.33ab	6.39ab	91.67ab
	15.0	9.96b	66.67bc	5.79ab	91.67ab
$CoCl_2$	3.0	17.14ab	91.11a	6.53ab	100.00a
	5.0	26.13a	82.22ab	8.54a	97.62ab
	10.0	9.00b	55.56c	5.92ab	87.22b
	15.0	8.00b	13.33d	5.56ab	84.62b

[z]Mean separation within columns by Duncan's multiple range test at 5% level.

Cymbidium aloifolium and *Dendrobium nobile* using thin cross sections of protocorm-like bodies.

Stem disc culture exposes more layers of cambium to the hormones. In the present study, shoot regeneration from the cambium region could be seen in the stem disc culture (Fig. 3A, 3B). Although several studies have explored the behavior of the two primary meristems at the root and shoot apexes, little attention has been paid to the lateral meristem (cambium), which forms the bulk of the biomass of woody plants. The cambium refers to one or several layers of initials that are analogous to the stem cells proposed for other meristems (Larson 1994). Several researchers have focused on the molecular nature of these positional cues (Doerner 2003). During active cell division in the cambium of *P. tremula,* microarray studies have indicated differential expression of more than 100 genes in a thin cambial zone (Schrader et al. 2004). Further, Laux (2003) proposed that that the fate of stem cells and their derivatives depends on their positional cues rather than their cell lineage.

Among the three types of explants examined in the present study, (petiole, leaf, and roots of in vitro-grown plantlets of clones of *P. euramericana*), petiole was the best explant, as it resulted in the greatest shoot regeneration and elongation. The hormonal requirements for the three explant types for maximum shoot regeneration also varied. Similarly, Kulkarni et al. (2001) found that different explant types have different hormonal requirements for shoot regeneration in *Withania somnifera.*

In the present study, 0.2 mg l^{-1} BA resulted in good shoot regeneration in all the explant types (Table 1). Khattab (2011) observed a beneficial effect of 0.2 mg l^{-1} BA on shoot regeneration in *Populus* hybrids. A concentration of 0.002 mg l^{-1} TDZ resulted in the greatest shoot regeneration (95.83%) from petiole. Similarly, Sherif and Khattab (2011) reported maximum shoot regeneration from stem internode explants of *Populus* hybrids at a low TDZ concentration.

The regenerated shoots showed yellowing and necrosis after a few weeks of culture on the cytokinin medium. Nadel et al. (1992) also observed yellowing of leaves in *P. trichocarpa.* Thus, the compounds $AgNO_3$ and $CoCl_2$ were added to the basal medium. Both of these compounds promoted shoot regeneration at low concentrations as they not only stopped the yellowing of leaves but also increased shoot regeneration and elongation. The yellowing of leaves is known to occur due to ethylene production during in vitro culture (Ferrante and Francini 2006). $AgNO_3$ and $CoCl_2$ are known to absorb ethylene and have proved to be beneficial during in vitro culture of several species.

Thus, the present study shows that for highly efficient shoot regeneration of *P. euramericana,* stem disc culture is a better option than nodal culture. Furthermore, among the different

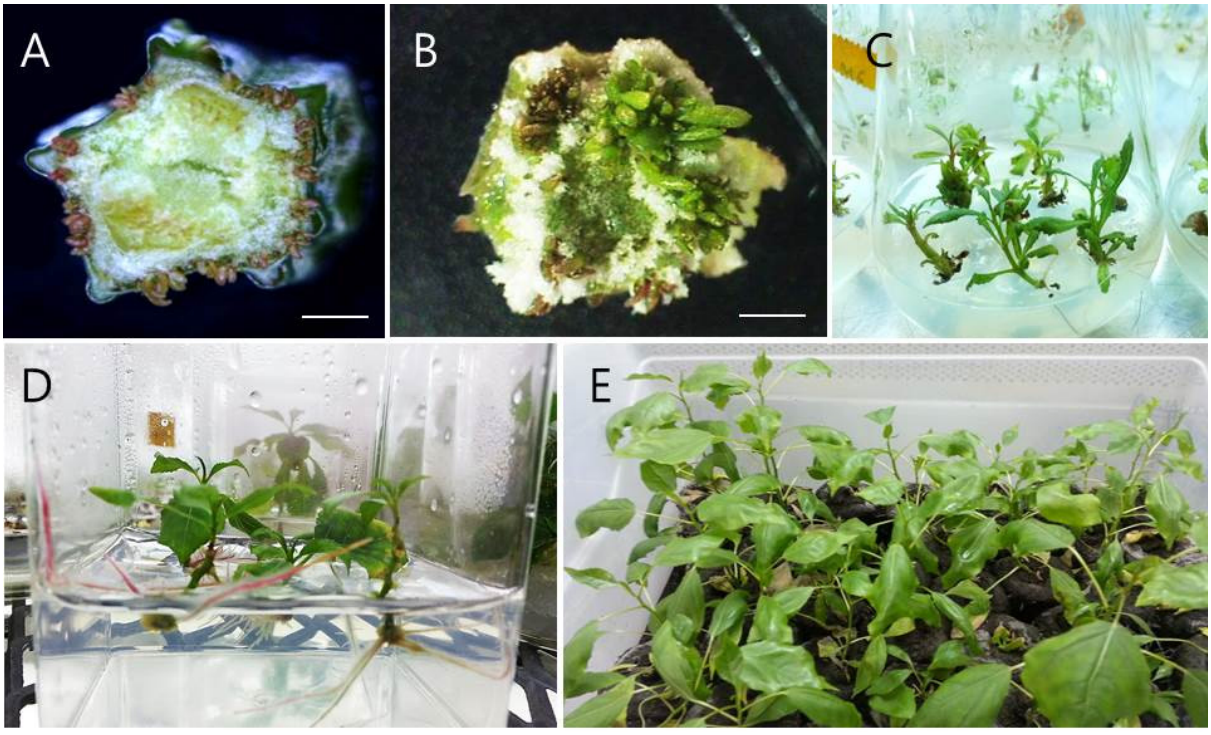

Fig. 3 Efficient shoot regeneration from stem-disc culture of *P. euramericana* 'I-476.' Shoot regeneration from cambium region of stem-disc cultured on ½ MS medium containing 0.5 mg l^{-1} zeatin after (A) 2 weeks and (B) 4 weeks. (C) Shoot elongation after 4 weeks on CoCl2-containing medium. (D) Rooting of regenerated shoots. (E) Acclimatized plants (scale bar = 3 mm)

explant types, petiole produced the best response during shoot regeneration. Also, during shoot regeneration, a combination of the cytokinins BA and TDZ gave better results than the individual cytokinins. The problem of yellowing and necrosis of the in vitro-regenerated shoots was eliminated by using $AgNO_3$ and $CoCl_2$. Therefore, proper manipulation of the medium can result in highly efficient in vitro shoot regeneration in *P. euramericana*. The high percentage of regenerated shoots obtained from stem disc culture through proper medium manipulation resulted in healthy plantlets (Fig. 3A ~ E). The highly efficient regeneration system of *P. euramericana* reported here can be used for mass propagation of this recalcitrant, economically important tree species.

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