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In vitro shoot regeneration of *Populus deltoides*: effect of cytokinin and genotype *

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Abstract. Treatment differences were observed in the *in vitro* adventitious shoot regeneration response from internodal explants of three genotypes of *Populus deltoides* cultured on media supplemented with five concentrations each of the cytokinins 6-benzyladenine, 2-isopentyladenine, and zeatin. For each of the three genotypes, the greatest number of shoots were consistently regenerated on media containing the cytokinin zeatin. Tissue necrosis resulted when explants from any of the three genotypes were cultured on media supplemented with 6-benzyladenine. A zeatin concentration by genotype interaction was also observed. Genotypic differences in shoot regeneration were observed for 16 genotypes of *Populus deltoides* when cultured on medium supplemented with 0.5 mgL^{-1} zeatin. Six genotypes were highly recalcitrant and failed to regenerate shoots. The percent of explants regenerating was greater than 50% for four genotypes.

Abbreviations : WNA: modified Woody Plant Media; BA: N^6-benzyladenine; 2-iP: 2-isopentenyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; IBA: indole-3-butyric acid; MS: Murashige Skoog (1962) medium; NAA: 1-naphthaleneacetic acid; PAR: photosynthetically active radiation.

Introduction

Plant biotechnology methods such as genetic transformation, somaclonal cell selection, somatic cell hybridization, and haploid production require reliable and efficient adventitious shoot regeneration procedures. The failure of cells and tissue to regenerate shoots is the major limitation preventing the application of plant biotechnology to forest tree species (Ledig and Sederoff 1985, Riemenschneider et al. 1988).

The genus *Populus* has been successfully utilized for *in vitro* shoot regeneration (for review see Ahuja 1987), protoplast culture (Russell and McCown 1986), genetic transformation (Fillatti et al. 1987), and somaclonal cell selection (Michler and Bauer 1988; Ostry and Skilling 1988). However, the majority of the success in the cell culture of poplars has occurred with species or hybrids incorporating species of the Leuce section (aspens and white poplars). Successful cell and tissue culture of species of the Aigieros group (the cottonwoods) has been limited.

Populus deltoides is an economically important species of the Aigeiros group of poplars and is extensively cultivated (Dickmann and Stuart 1983). Successful *in vitro* shoot regeneration of *Populus deltoides* has been reported from explants of immature embryos (Kouider et al. 1984) and anthers (Uddin et al. 1988). Nonembryonic tissues of *Populus deltoides* and interspecific hybrids of *Populus deltoides* have been recalcitrant to attempts at *in vitro* shoot regeneration and shoot culture (Sellmer et al. 1989). Douglas (1984) reported the *in vitro* production of buds and shoots from internodal stem explants of *Populus deltoides*; however, only cursory results were presented. Adventitious shoots have also been regenerated from calli originating from leaf disks of *Populus deltoides*, but the procedure was very laborious and involved multiple transfers (Prakash and Thielges 1988). Although these reports are encouraging, the development of a reproducible and efficient *in vitro* shoot regeneration system is a prerequisite if plant biotechnological techniques such as genetic transformation and somaclonal cell selection are to be applied to *Populus deltoides*.

In this report, we detail protocols for an efficient and reproducible *in vitro* shoot regeneration system for *Populus deltoides*. We also show that the cytokinin used to induce shoot regeneration and the genotype of the explant tissue are critical to the success of the system.

Materials and Methods

Plant Tissue: Stem cuttings from 16 clones of *Populus deltoides* Bartr. ex Marsh (eastern cottonwood) were collected from the stoolbed (established in Lincoln, NE, in 1983, and cut back annually) during January, 1988, and rooted in a soilless medium of 1:1 peat:vermiculite in the greenhouse. The 16 clones were originally part of a regional study of genetic variation in eastern cottonwood (Ying and Bagley 1976). The ramets were grown in the greenhouse under a 24 h photoperiod. All plants were fertilized weekly with a complete nutrient solution (Ross 1974).

After 8 weeks of growth in the greenhouse, stem sections consisting of the apical 9 internodes were removed from the stock plants. The first two apical internodes were removed and discarded. The remaining shoot segments consisting of 7 internodes were placed in a solution of 1.0% Tween-20 and agitated for 15 min to remove foreign debris. Stem segments were then rinsed 3 times in distilled water and placed in a 20% solution of Clorox for 20 min. The stem sections were rinsed 5 times with sterile double distilled water, aseptically dissected into 5 mm internodal explants, and inserted vertically with the basal end in a solidified medium.

Media: All internodal stem explants were cultured on a modified version of Woody Plant Medium (Lloyd and McCown 1980) designated WNA. The modifications included the substitution of $Ca(NO_3)_2$ at a concentration of 2000 mgL^{-1}, NH_4NO_3 at 1650 mgL^{-1}, and the addition of 500 mgL^{-1} casein hydrolysate. Preliminary experiments established that this modified version of Woody Plant Medium resulted in increased viability of *Populus deltoides* internodal explants. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121° C and 1 $kgcm^{-2}$ for 20 min. The medium was solidified with 0.25% (w/v) Gelrite (Scott Laboratories, Carson, CA). All media were supplemented with 500 mgL^{-1} of carbenicillin (Sigma Chemical Company, St. Louis, MO) for the first 30 d in culture to control systemic bacterial contamination. The phytohormones and carbenicillin were added as filter sterilized solutions after autoclaving.

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Cytokinin Treatments: The cytokinins BA, 2iP and zeatin were added to WNA medium at 0.25, 0.5, 1.0, 2.0, and 4.0 mgL^{-1}. Fifteen explants from each of three genotypes (10, 177, 174) were cultured on each cytokinin treatment plus a phytohormone-free control, and each treatment was replicated 3 times for a total of 48 treatment combinations (3 cytokinins by 5 concentrations of each cytokinin by 3 genotypes plus one basal control for each genotype). Internodal sections were cultured in 100 mm sterile polystyrene Petri dishes (each dish contained 20 ml of medium) sealed with parafilm. Cultures were maintained in the dark at 25°C for 10 d and then transferred to a 16 h photoperiod at 25°C under cool white fluorescent lamps (15$\mu Em^{-2} s^{-1}$ PAR). Explants were subcultured after 30 d. After 60 d in culture, the number of regenerated shoots on each explant and the number of explants regenerating shoots were determined for each treatment.

Genotype Effects: Based on the results from the cytokinin treatments, 16 genotypes were cultured on WNA medium supplemented with 0.5 mgL^{-1} zeatin. Twenty-five internodal stem explants from each genotype were cultured per 100 mm sterile polystyrene Petri dish containing 20 ml of medium, and sealed with parafilm. Each genotype treatment was replicated 3 times. The number of shoots regenerating from each explant and the number of explants regenerating shoots were determined for each genotype after 60 d in culture. Culture conditions were as described for the cytokinin treatments.

Data Analysis: Analysis of variance was performed using the general linear models procedure of SAS (SAS Institute 1985) with the mean number of shoots from the zeatin treatments and the genotype treatments serving as the dependent variable. Concentration and genotype means were compared using the Duncan's multiple range test (α=0.05). Because the shoot regeneration response for the BA and 2iP treatments was so minimal, they were not analyzed statistically.

Results and Discussion

Cytokinin study

The effect of the type of cytokinin on mean *in vitro* shoot regeneration is shown in Table 1. For each of the 3 genotypes the greatest number of shoots were consistently regenerated on medium with zeatin. Internodal explants cultured on WNA medium supplemented with BA browned and rapidly became necrotic. The mean overall shoot regeneration response for explants cultured on BA supplemented WNA medium ranged from 0.0 (1.0, 2.0, 4.0 mgL^{-1} BA) to 0.89 (0.5 mgL^{-1} BA) shoots per explant with 0% (1.0, 2.0, 4.0 mgL^{-1} BA) to 11.8% (0.25 mgL^{-1}) of the explants regenerating. The mean number of shoots regenerated on 2iP supplemented WNA medium ranged from 0.02 (4.0 mgL^{-1} 2iP) to 0.36 (0.25 mgL^{-1} 2iP) shoots per explant with 1.5% (4.0 mgL^{-1} 2iP) to 22.2% (0.25 mgL^{-1} 2iP) of the explants regenerating. When explants were cultured on zeatin supplemented WNA medium, the mean number of shoots regenerated ranged from 1.43 (4.0 mgL^{-1} zeatin) to 2.41 (0.5 mgL^{-1} zeatin) shoots per explant with 25.2% (2.0 mgL^{-1} zeatin) to 57.0% (0.25 mgL^{-1} zeatin) of the explants regenerating. The overall mean number of shoots regenerated on basal medium was 0.26 shoots per explant with 17.7% of the explants regenerating (Table 1).

This study demonstrates the superiority of zeatin as a shoot inducing cytokinin in the *in vitro* induction of adventitious shoots from internodal stem explants of *Populus deltoides*. Although BA has been utilized in shoot regeneration from immature embryos (0.50 mgL^{-1}) (Kouider et al. 1984), anther calli (1.0 mgL^{-1}) (Uddin et al. 1988) and leaf disk calli (0.23 mgL^{-1}) (Prakash and Thielges 1988) of *Populus deltoides*, our results suggest a phytotoxic response of *Populus deltoides* stem internodal explants to BA. Internodal explants cultured on BA supplemented media consistently browned and became necrotic. BA concentrations greater than 0.50 mgL^{-1} resulted in tissue death in all genotypes and in most cases lower concentrations of BA reduced shoot regeneration relative to the control treatment. Phytotoxicity of BA has been reported in shoot cultures of *Salix* (Bergman et al 1985), a second genus in the family Salicaceae. Since BA is the most commonly employed cytokinin in tissue culture, BA toxicity may well be one of the factors that has limited tissue culture success of *Populus deltoides*. The failure to establish shoot cultures of a *Populus deltoides* x *Populus nigra* hybrid on Woody Plant Medium supplemented with BA (Sellmer et al. 1989) may be related to the phytotoxicity of BA to *Populus deltoides*.

Because zeatin produced the best shoot regeneration response compared to BA and 2iP, the zeatin results were further analyzed. The effect of the genotype on shoot regeneration of the internodal explants cultured on zeatin supplemented shoot regeneration medium was statistically significant (Table 2). When averaged across all treatments, genotype 10 regenerated significantly more shoots than genotypes 174 and 177 (summary data not shown). A significant zeatin concentration by genotype interaction was also evident (Table 2). For example, the greatest number of regenerated shoots produced for genotype 10 was 5.78 shoots per explant at a zeatin concentration of 1.0 mgL^{-1}, while the highest percent of explants regenerating (97.7%) was on WNA medium supplemented with 0.5 mgL^{-1} zeatin. Genotype 174 regenerated the greatest mean number of shoots per explant (1.56) and the highest percent of explants regenerating shoots (71.1%) when cultured on medium with 0.25 mgL^{-1} zeatin. Genotype 177

Table 1. Effect of cytokinins on shoot regeneration from internodal stem explants of three genotypes of Populus deltoides.

		Genotype							
		10		174		177		Total	
Cytokinin	Concentration mgL^{-1}	Mean number of shoots per explant (S.E.)[1]	Percent[2]	Mean number of shoots per explant (S.E.)	Percent	Mean number of shoots per explant (S.E.)	Percent	Mean number of shoots per explant (S.E.)	Percent
BA	0.25	0.289 (0.289)	4.4%	0.622 (0.256)	22.2%	0.111 (0.022)	8.9%	0.340 (0.121)	11.8%
	0.50	0.089 (0.058)	6.6%	0.022 (0.022)	2.2%	0.156 (0.124)	6.7%	0.889 (0.044)	5.2%
	1.00	0.000 (---)[3]	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%
	2.00	0.000 (---)	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%
	4.00	0.000 (---)	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%	0.000 (---)	0.0%
2iP	0.25	0.511 (0.124)	26.7%	0.578 (0.182)	40.0%	0.000 (---)	0.0%	0.363 (0.111)	22.2%
	0.50	0.556 (0.124)	26.7%	0.267 (0.139)	20.0%	0.000 (---)	0.0%	0.274 (0.097)	15.5%
	1.00	0.622 (0.347)	21.9%	0.133 (0.077)	8.8%	0.089 (0.059)	6.7%	0.281 (0.135)	14.8%
	2.00	0.245 (0.178)	8.9%	0.067 (0.038)	4.4%	0.133 (0.067)	13.3%	0.148 (0.062)	8.9%
	4.00	0.022 (0.022)	2.2%	0.000 (---)	0.0%	0.022 (0.022)	2.2%	0.015 (0.010)	1.5%
Zeatin	0.25	5.111 (1.554) a[4]	93.3%	1.556 (0.523) a[4]	71.1%	0.088 (0.022) cd[4]	6.6%	2.252 (0.883) a[4]	57.0%
	0.50	5.600 (0.308) a	97.7%	1.333 (0.267) ab	57.7%	0.289 (0.118) c	17.7%	2.407 (0.822) a	41.5%
	1.00	5.778 (1.057) a	82.2%	0.378 (0.124) c	24.4%	0.267 (0.102) cd	17.7%	2.141 (0.960) a	41.5%
	2.00	3.556 (1.051) ab	31.1%	0.177 (0.177) c	6.6%	0.711 (0.097) b	37.7%	1.482 (0.608) a	25.2%
	4.00	2.733 (1.598) ab	15.5%	0.599 (0.267) bc	22.2%	0.955 (0.059) a	40.0%	1.430 (0.573) a	25.9%
Control	0.00	0.555 (0.190) b	28.9%	0.178 (0.058) c	17.8%	0.044 (0.023) d	4.4%	0.259 (0.096) b	17.7%

[1] Each value represents the mean (standard error of the mean) of three replications after 60 days in culture.
[2] Each value represents the percent of explants regenerating at least one shoot for three replications.
[3] --- Not calculated
[4] Means followed by different letters are significantly different according to Duncan's Multiple Range Test (α=0.05).

regenerated the greatest mean number of shoots per explant (0.96) and the highest percent of explants regenerating (40.0%) when cultured on WNA medium supplemented with 4.0 mgL^{-1} zeatin (Table 1).

Table 2. Results of analysis of variance for the mean number of shoots regenerated from internodal stem explants for the cytokinin study and for the gentotype study.

ANOVA Tables				
Source of Variation	DF	MS	F value	P > F
Cytokinin study				
Total	53	---	--	-
Model	19	10.8138	8.12	0.0001
Genotype	2	67.3982	50.62	0.0001
Zeatin concentration	5	5.7366	4.31	0.0038
Replication	2	0.8372	0.63	0.5394
Genotype x Zeatin concentration interaction	10	4.0310	3.03	0.0076
Error	34	1.3315	--	-
Genotype study				
Model	17	4.0927	27.68	0.0001
Genotype	15	4.6249	31.28	0.0001
Replications	2	0.1015	0.69	0.5120
Error	27	0.1479	---	---

Genotype study

Because 0.5 mgL^{-1} zeatin produced the greatest overall mean number of regenerated shoots in the previous experiment this concentration of zeatin was utilized in the study of the effect of genotype on *in vitro* shoot regeneration. The mean number of shoots regenerated per explant varied significantly by genotype (Table 2), and ranged from 4.28 for genotype 10 to 0.0 for genotypes 53, 54, 297, 298, 177, and 301 (Table 3). The mean shoot regeneration responses of genotypes 10, 175, 171 and 179 were each significantly different; furthermore, these four genotypes were significantly different from the remaining 12 genotypes. The percent of explants regenerating was highest for both genotypes 10 and 175 (100 and 95.9% respectively).

Table 3. Effect of genotype on mean number of shoots regenerated from internodal stem explants of Populus deltoides cultured on 0.5 mgL^{-1} zeatin.

Genotype	Mean number of shoots per explant[1]	Standard Error of the mean	Percent[2]
10	4.280 a[3]	0.400	100.0%
175	3.574 b	0.266	95.5%
171	2.107 c	0.338	61.0%
179	1.080 d	0.624	50.8%
174	0.316 e	0.101	10.4%
12	0.307 e	0.141	22.9%
296	0.307 e	0.157	18.7%
300	0.160 e	0.106	9.3%
178	0.077 e	0.077	4.8%
56	0.027 e	0.013	2.7%
53	0.000 e	---[4]	0.0%
54	0.000 e	---	0.0%
297	0.000 e	---	0.0%
298	0.000 e	---	0.0%
177	0.000 e	---	0.0%
301	0.000 e	---	0.0%

[1] Each value represents the mean of three replications (after 8 weeks in culture).
[2] Each value represents the percent of explants regenerating at least one shoot for three replications (after 8 weeks in culture).
[3] Means followed by different letters are significantly different according to Duncan's Multiple Range Test (α=0.05).
[4] --- not calculated

Douglas (1984) reported that when 8 mm internodal stem explants of *Populus deltoides* were cultured on phytohormone-free MS medium, a mean of 8.4 buds plus shoots per explant were formed with 100% of the explants responding; however, only 1 or 2 of the buds developed into shoots. The regeneration response of the control treatment of the current study differs from that reported by Douglas (1984) in that a mean of 0.26 shoots were regenerated per explant and 17.7% of the explants regenerated. Since Douglas (1984) did not distinguish between buds and shoots, a direct comparison of responses is difficult and may well account for the difference observed in our study. We commonly observed multiple bud development on control explants but most buds failed to develop into shoots. In addition, the discrepancy may well be the result of genotype differences because Douglas (1984) cultured only one genotype and we demonstrate in this study that the genotype of the explant has a significant effect on adventitious shoot regeneration.

Prakash and Thielges (1988) reported the formation of multiple shoots from *Populus deltoides* leaf disk calli. Multiple shoots were induced from leaf disk calli on WPM medium supplemented with 1µM (0.23 mgL^{-1}) BA. Comparison of the current study with Prakash and Thielges (1988) is difficult since shoot numbers were not reported and only one genotype was utilized. Our reported method is technically simpler to utilize however, since multiple callus inducing media transfers are not required. In addition, Prakash and Thielges (1988) used leaf disk explants from which adventitious shoots were regenerated. Results in our laboratory have demonstrated that internodal stem explants respond much better to *in vitro* manipulation than do leaf disks, nodal stem sections, shoot tips, and root sections of Populus deltoides (results not shown). The increased viability of internodal stem section explants may have contributed to the development of a simpler tissue culture system.

Genotypic effects are well established in tissue culture responses (Abe and Futsuhara 1986; Dietert et al. 1982; Kurtz and Lineberger 1983; Skvirsky et al. 1984). Genotypic control of shoot regeneration has also been observed for *Populus tremula* and *Populus tremuloides* (Ahuja 1983). Our study also demonstrates the importance of explant genotype in *in vitro* shoot regeneration. In our study, 6 of the 16 genotypes were completely unresponsive, while for 4 of the 16 genotypes, greater than 50% of the explants produced adventitious shoots. Therefore, genotype must be considered when developing an *in vitro* shoot regeneration system for *Populus deltoides*. The significant genotype by zeatin concentration interaction also indicates that genotypes have different physiological requirements for *in vitro* shoot regeneration.

Because of the long generation intervals and large size of forest trees, genetic improvement has been limited. The application of recombinant DNA technology may therefore play a significant role in tree improvement through gene transfer. Prerequisite to gene transfer is the development of a reliable and efficient adventitious shoot regeneration method (Ledig and Sederoff 1985). Successful *Agrobacterium* mediated gene transfer has been reported for the interspecific poplar hybrid *Populus alba* x *grandidentata* (Fillatti et al. 1987). In this system, a regeneration response of 1 to 3 shoots from 35% of control (non-cocultivated) leaf disk explants was sufficient for initiation of transformation experiments. By comparison, the internodal shoot regeneration system that we report produces 5.6 shoots per explant with 97.7% of the explants regenerating (genotype 10 on 0.50 mgL^{-1} zeatin). This suggests that the number of shoots regenerated with our system should be sufficient for the application of gene transfer techniques. In addition, regenerated shoots are easily rooted on WNA media supplemented with 1.0 mgL^{-1} IBA. Rooted plants have been acclimated and established in the greenhouse. This regeneration procedure also results in a low level of somaclonal variation. This laboratory has utilized this regeneration procedure for over one year (>10,000 shoots regenerated) and only three somaclones have been identified. Furthermore, the regeneration response of the 16 genotypes has remained consistent and repeatable (Coleman and Ernst, in preparation). Regenerated shoots from 4 genotypes have also been maintained for over one year as stable shoot cultures (Coleman and Ernst, in preparation).

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