

Full Length Research Paper

Direct and indirect plant regeneration from various explants of eastern cottonwood clones (*Populus deltoides* Bartram ex Marsh.) with tissue culture

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***Populus* species are important resource for certain branches of industry and have special roles for scientific study on biological and agricultural systems. Plant regeneration via direct and indirect organogenesis of four *Populus deltoides* Bartram ex Marsh. ssp. *deltoides* × *Populus deltoides* Bartram ex Marsh. ssp. *deltoides* hybrid clones (89 M 011, 89 M 044, 89 M 048, 89 M 066) and *P. deltoides* ssp. *deltoides* clone (Samsun) were investigated. Direct organogenesis was established from nodes and internodes on woody plant medium (WPM) supplemented with cytokinins and/or auxins. The 89 M 011 clone gave the highest percentage (100%) of regeneration on WPM with 1 mg/l zeatine from internode explants. Indirect organogenesis via callus phase was obtained from nodes and petioles on WPM supplemented with different concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D). The nodes part of the 89 M 066 clone gave the highest rate of generative callus (100%) on WPM supplemented with 2 mg/l 2,4-D. Indirect shoots were obtained from the node callus on WPM with cytokinins. There was root formation from directly regenerative shoots which were cultured on WPM or Murashige and Skoog Basal Medium (MS) containing different ratios of indole butyric acid (IBA). Rooted seedlings *in vitro* were successfully acclimatized. Data on *in vitro* study were subjected to statistical evaluation. The *in vitro* regeneration system will allow this study to set reliable procedures for the genus and clones.**

Key words: Poplar, tissue culture, regeneration, organogenesis.

INTRODUCTION

The poplar is an important model for woody perennial biotechnology because it is amenable to *in vitro* culture and genetic engineering through *Agrobacterium*-mediated transformation (Jehan et al., 1994; Gozukirmizi et al., 1998; Confalonieri et al., 2000; Han et al., 2000; Dai et al., 2003). There are now numerous techniques available for rapid and extensive multiplication of elite and desirable plant species *in vitro*. Clonal multiplication and genetic manipulation of the poplar and its clones has now

become a major subject of investigation through tissue culture, because investigators are developing interest on improvement of the biomass production of available resources of fast growing trees. In addition, hybrid cottonwoods, aspen and other poplar species (genus *Populus*) are highly valued by the pulp and paper industry for their fast growth and high quality fiber.

Populus deltoides was introduced to Europe in the mid-1700s from North America. In Turkey, it is an exotic species of poplar and was introduced in the late 1960s from the USA. As a female parent, *P. deltoides* is used with Turkey's natural black poplar for a crossbreeding programme. On the other hand, poplar biomass production will be increased with culturing *P. deltoides*

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clones and hybrids that can be grown rapidly (Tunctaner, 1989).

Vegetative propagation of trees is an effective way to capture genetic gain (Park and Bonga, 1992) and produce large amounts of plant material. Cloning can be achieved by grafting and rooting of cuttings, micro-propagation or somatic embryogenesis (Bonga and Von Aderkas, 1992). There are several studies on *in vitro* organogenesis with *Populus* spp. including *P. deltoides* or its hybrids (Rutledge and Douglas, 1988; Sellmer et al., 1989; Coleman and Ernst, 1990; Noël et al., 2002; Yadav et al., 2009). However, with several of the known *Populus* genotypes, it was found that they are hard to perpetuate and difficult to work with for *in vitro* culture studies (Yadav et al., 2009).

The aim of this study was to develop a reliable system for plant regeneration with or without an intervening callus phase. The influence of different clones, growth regulators and explant types on regenerative capacity was investigated.

MATERIALS AND METHODS

Source of plants

The original stock plant material, four *P. deltoides* ssp. *deltoides* × *P. deltoides* ssp. *deltoides* clones (89 M 011, 89 M 044, 89 M 048 and 89 M 066) and one *P. deltoides* ssp. *deltoides* non-hybrid clone (Samsun), was provided by the Ministry of Forests, Poplar and Fast Growing Forest Trees Research Institute, Kocaeli, Turkey. Two-year-old dormant rootstock plants were harvested with terminal branches including fresh buds in early spring.

Sterilization procedures

The initial branches, including fresh buds of 5 different clones, were cut into 10 cm pieces, washed with running tap water for 3 h and then disinfected in 70% ethanol (v/v) for 1 min, followed by 4% Ca-hypochloride treatment for 15 min and 8% hydrogen-peroxide for 10 min. The explants were finally rinsed three times in sterile distilled water for 20 min each and dried on sterile filter paper for 30 min. After the procedure, the disinfected branches were cut into segments that sized up to culture and explant types and cultured.

Media and incubation conditions

The explants used were planted for direct and indirect regeneration on woody plant medium (WPM) (Lloyd and Mc Cown, 1981) or Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) and WPM for rooting. The media used for all purposes were supplemented with 9 g/l agar and pH was adjusted 5.2 for WPM and 5.8 for MS before autoclaving. The plant growth regulators were filter sterilized and added to media after autoclaving. The culture on Petri dishes containing 20 ml medium with 6 explants or magenta vessels containing 50 ml medium with 4 explants was set in a climate chamber at 25°C with a 16 h photoperiod provided by cool white fluorescent light at 50 $\mu\text{mol}\cdot\text{s}^{-2}\cdot\text{m}^{-1}$ and 70% humidity.

Direct organogenesis

For direct organogenesis after disinfecting, the initial branches of all

five clones (Samsun, 89 M 011, 89 M 044, 89 M 048 and 89 M 066) were cut into 15 mm segments including node parts and 10 mm segments including internodes. The explants were placed horizontally on Petri dishes containing WPM with 1 mg/l zeatine; WPM with 0.5 mg/l N⁶-Benzyladenine (BA) and 0.5 mg/l zeatine; WPM with 0.2 mg/l BA and 0.01 mg/l α -Naphthalene acetic acid (NAA) or WPM with 1 mg/l BA and 0.1 mg/l NAA. The cultures were maintained for 3 weeks in a growth chamber and sub-cultured at the end of every 3 weeks in the same media.

Indirect organogenesis

For indirect organogenesis from four hybrid clones (89 M 011, 89 M 044, 89 M 048, 89 M 066), regenerative calli were induced from node and petiole explants, obtained from directly regenerative plants in *in vitro* culture, on WPM with 1 mg/l 2,4-Dichlorophenoxy acetic acid (2,4-D) or WPM with 2 mg/l 2,4-D for each clone after 3 weeks for the node and 4 weeks for petiole explants. Indirect shoot regeneration from the node callus was induced on WPM with 0.2 mg/l BA or WPM with 1 mg/l zeatine after 3 weeks.

Rooting and acclimatization condition

Directly regenerative shoots 2 to 3 cm long with 5 to 6 leaves were induced to root *in vitro* by culture on agar-solidified WPM with 0.5 mg/l indole butyric acid (IBA), WPM with 2 mg/l IBA and MS with 2 mg/l IBA in glass tubes. After 2 weeks, shoots that had produced roots were recorded and transferred to sterile compost with ratio (v/v/v) of organic fertilizer: sand: peat (1:2:2; 3:1:0 or 2:2:1) in flowerpots in a greenhouse.

Experimental design and statistical analysis

Tests were conducted in randomised block design with four replicates, each replicate being calculated as percentage value. All data were evaluated using the analysis of variance (ANOVA) (Minitab for Windows) and the groups that showed variance were then, subjected to Duncan's multiple range test with a significance value at $P < 0.05$. Before statistical calculation, percentages of the data were transformed by arcsine \sqrt{x} .

RESULTS

Direct organogenesis

New axillary and adventitious buds were induced on the internodes and node explants. According to the data, WPM with 1 mg/l zeatine was statistically found to be the most efficient medium from internodes for direct regeneration when clones were undervalued. WPM with 0.5 mg/l BA and 0.5 mg/l zeatine was the second most efficient medium. WPM with 0.2 mg/l BA and 0.01 mg/l NAA and WPM with 1 mg/l BA and 0.1 mg/l NAA ranked as the third and fourth significantly effective groups. It was noted that all media used gave a response to the parts and clones used (Table 1). But WPM supplemented with 1 mg/l zeatine gave the highest rate and in addition had stronger and healthier shoots than the other media. On the media, 89 M 011 (100%) and 89 M 066 (60%) gave the highest rate of regeneration capacity from

Table 1. Effects of clones and medium on direct organogenesis from internode explants of clones in 3 weeks *in vitro****.

Clone	Shoot Regeneration in WPM with plant growth regulator (%)				Mean*
	1 mg/l zeatine	0.5 mg/l BA+ 0.5 mg/l zeatine	0.2 mg/l BA+ 0.01 mg/l NAA	1 mg/l BA+ 0.1 mg/l NAA	
Samsun	57	48	38	29	43.12 ^{b*}
89M011	100	90	77	35	75.89 ^a
89M044	43	35	32	8	29.72 ^b
89M048	54	38	46	38	44.27 ^b
89M066	60	60	20	15	38.75 ^b
Mean**	62.99 ^{a**}	54.45 ^{ab}	42.90 ^b	25.05 ^c	

*Means within the column indicate that the media means for each clone having a different letter were significantly different at $P < 0.05$; **means within line indicate that the clone means for each media having a different letter were significantly different at $P < 0.05$; ***clones and media used separately were found significantly important but clones \times medium interaction did not indicate significant importance for regeneration.

Table 2. Effects of clones on direct organogenesis from node explants in WPM with 1 mg/l zeatine in 3 weeks *in vitro**.

Clone	WPM with 1 mg/l Zeatine (%)
Samsun	20
89M011	19
89M044	16
89M048	9
89M066	30

*Regeneration data did not indicate significant difference between clones.

internodes. But 89 M 066 was observed to have healthy-looking shoots.

After the study on internodes, node explants of five clones were cultured in WPM with 1 mg/l zeatine to obtain the most efficient clone for direct regeneration from nodes (Table 2). Although, the data did not show significant difference in clones, 89 M 066 gave the highest percentage of regeneration (30%) and the 89 M 048 hybrid clone gave the lowest result (9%).

Indirect organogenesis

Indirect organogenesis via callus phase was tested with four clones. To obtain callus, node or petiole explants were used. WPM with 2 mg/l 2, 4-D statistically gave the highest rate of regenerative callus formation when clones were undervalued in both explant types (Table 3).

89 M 066 in particular was found to be the best for callus proliferation from nodes and petioles on WPM with 2 mg/l 2, 4-D, with ratios of 100 and 80%, respectively. Although, no clone indicated significant importance, regenerative callus capacity from node explants was higher than that of petioles in WPM with 2 mg/l 2,4-D. So the study went on to shoot regeneration from node callus

on WPM with 0.2 mg/l BA or 1 mg/l zeatine. According to the data, WPM with 1 mg/l zeatine gave a higher percentage of indirect shoot rates from node callus (85 to 100%) than WPM with 0.2 mg/l BA (20 to 60%) for all clones. Data indicated that clones and media are significantly important (Table 4). Also, indirect shoot regeneration from callus gave the best results with 89 M 066 for both of the media.

Rooting and acclimatization conditions

Directly regenerated shoots of four clones on WPM with 1 mg/l zeatine, 2 to 3 cm long with 5 to 6 leaves were induced to form roots on WPM with 0.5 mg/l IBA, WPM with 2 mg/l IBA and MS with 2 mg/l IBA (Table 5). All media were used with plant growth regulators activated to rooting with high value after two weeks. Although the data did not show statistical difference on the media, observation revealed that WPM with 0.5 mg/l IBA gave the strongest and healthiest roots after several weeks. 89 M 066 showed statistical importance for rooting. All rooted plants were adapted to sterile compost with the ratio of 2:2:1 (v:v:v) organic fertilizer:sand:peat that gave the best (90 to 100%) adaptation ratio in the plant growth chamber. All adapted plants continued to adapt in the greenhouse at a 100% ratio.

DISCUSSION

Although, there are several studies about *in vitro* direct or indirect regeneration of poplar (Kolevska-Pletikapić and Besendorfer, 1989; Lee-Stadelmann et al., 1989; Vinocur et al., 2000) or cottonwood hybrids (Rutledge and Douglas, 1988; Sellmer et al., 1989; Han et al., 1994; Jehan et al., 1994; Han et al., 2000; Bajrovic et al., 2001), there are still difficulties for tissue culture regeneration of the poplar.

Table 3. Callus proliferation from node and petiole explants of clones.

Clone	Callus proliferation rate (%) from node after 3 week		Callus proliferation rate (%) from petiole after 4 week	
	WPM with growth regulator		WPM with growth regulator	
	1 mg/l 2,4-D	2 mg/l 2,4-D	1 mg/l 2,4-D	2 mg/l 2,4-D
89 M 011	18	95	12	78
89 M 044	20	95	22	60
89 M 048	15	90	15	64
89 M 066	22	100	20	80
Mean	18,75 ^{b*}	95 ^a	17.25B ^{**}	70.50 ^A

*Means within lines having different small letters were significantly different at $P < 0.05$ indicating node explant and media mean interaction; ** means within lines having different capital letters were significantly different at $P < 0.05$ indicating interaction between the petiole explants and media means on regeneration.

Table 4. Shoot regeneration from node callus of clones after 3 weeks *in vitro*.

Clone	Indirect shoot regeneration rate (%) from node callus	
	WPM with growth regulator	
	0.2 mg/l BA	1mg/l zeatine
89 M 011	35b ^{*BC**}	85 ^{aB}
89 M 044	20 ^{bC}	100 ^{aA}
89 M 048	40 ^{bB}	95 ^{aA}
89 M 066	60b ^A	100 ^{aA}

*Means within lines having different small letters were significantly different at $P < 0.05$ indicating media differences on the same clone; **means within columns having different capital letters were significantly different at $P < 0.05$ indicating clone differences on the same medium.

Table 5. Rooting of directly regenerated plants *in vitro* after 2 weeks.

Clone	Rooting rate in WPM or MS with plant growth regulator (%)			
	WPM with 0.5 mg/l IBA	WPM with 2 mg/l IBA	MS with 2 mg/l IBA	Mean
89 M 011	100	100	100	100a [*]
89 M 044	100	80	95	91,67 ^b
89 M 048	95	90	90	91.67 ^b
89 M 066	100	100	100	100 ^a

*Means within columns having different letters were significantly different at $P < 0.05$ indicating clone differences.

In this study, direct and indirect regeneration systems of the poplar were successfully developed. According to these results, clone differences were observed in both regeneration types, that is, direct and indirect regeneration. The highest direct shoot regeneration from internodes of the 5 clones was obtained with 89 M 011 in WPM with 1 mg/l zeatine (100%), while the lowest results were obtained from 89 M 044 in the same medium (43%). As emphasized by Gozukirmizi et al. (1998) strong genotype dependence for *in vitro* regeneration was also observed. According to the study, nine *Populus tremula* clones were tested for direct regeneration, with the Dursunbey genotype giving the best results. Similarly, Coleman and Ernst (1990) showed that the adventitious

shoot regeneration competence states of *P. deltoides* were genotype dependent. Rutledge and Douglas (1988) studied 12 commercial clones of the poplar *in vitro*. They emphasized that, the genotype of culture had a greater influence on development of shoot-producing cultures than the medium composition.

The other critical points for the tissue culture procedure are media and plant growth regulator types. Solid WPM for direct and indirect regeneration was used and solid MS and WPM for rooting. According to Gozukirmizi et al. (1998), shoot regeneration of *P. tremula* on WPM with 1 mg/l zeatine was found to be 50% more efficient than with aspen culture medium (ACM) (with 0.5 mg/l BA and 0.02 mg/l NAA) and MS (with 0.05 mg/l NAA and 2.25

mg/l BA). Similarly, Han et al. (1994) used WPM with 5 μ M trans-zeatine for direct shoot regeneration in their study on the F2 family of *Populus trichocarpa* \times *P. deltoides*. Noël et al. (2002) used WPM without growth regulator for *P. trichocarpa* \times *P. deltoides* (*P. \times interamericana*) and 1/2 MS for *P. deltoides* \times *Populus nigra* (*Populus euramericana*) hybrids. According to the study results, WPM with 1 mg/l zeatine was the first, WPM with 0.5 mg/l BA and 0.5 mg/l zeatine was the second and WPM with 0.2 mg/l BA and 0.01 mg/l NAA was the third most successful medium for direct regeneration from internode explants. For node explants, 89 M 066 clones gave numerically the best result on WPM with 1 mg/l zeatine.

Comparison with internode or node explant types for direct regeneration in WPM with 1 mg/l zeatine, internodes gave a higher rate of shoot regeneration than nodes under the same conditions in all five clones in this study. According to a study (Rutledge and Douglas, 1988), twelve commercial clones of poplar were cultured *in vitro* from meristem tips, shoot tips and nodal segments with the result that shoot-producing cultures were obtained from 4, 32 and 70% of meristem tips, shoot tips and nodal segments within 12, 6 and 4 weeks, respectively. Similarly, in this study, the nodal culture directly produced organogenesis in 3 weeks. Yadav et al. (2009), studied direct plant regeneration from leaf and root segments and internodes of the eastern cottonwood (*P. deltoides*). The study showed that, direct plant regeneration was at the high frequencies of 92% in internodes, 88% in leaf segments and 43% in root segments.

For indirect organogenesis via callus phase, nodes and petiole explants were used in the first step. Callus formation ratios on WPM with 2 mg/l 2,4-D from nodes and petioles were 95 to 100 and 60 to 80%, respectively depending on the clones. Media means showed statistical difference on each explant type. Indirect shoot regeneration from node callus in WPM with 1 mg/l zeatine (85 to 100%) was statistically higher than in WPM with 0.2 mg/l BA (20 to 60% depending on the clones used). At this stage, media and clone interaction occurred. In all clones, 89 M 066 gave the highest callus-forming rate as well as indirect regeneration capacity from both nodes and petioles. Tomovic and Kolevska-Pletikavic (1991) studied plant regeneration from callus tissue cultures of poplar hybrids (*Populus alba* \times *Populus grandidentata*, *P. alba* \times *P. alba*) and *Populus trichocarpa* and *P. deltoides*. Organogenic calli were obtained from leaves, stems and roots for poplar hybrids (first group) and from immature embryos for *P. trichocarpa* and *P. deltoides* (second group). The authors stated that, a high frequency of callus induction was achieved in the first group from leaf culture (93 to 100% of inoculated explants). In the second group, when immature embryos were used, calli were induced on 73 to 83% of inoculated explants. Jehan et al. (1994) studied ontogenesis and ploidy level of plantlets regenerated from *P. trichocarpa* \times

deltoides cv. Hunnegem root, leaf and stem explants. According to their result, the best hormonal conditions for callus induction from excised petioles or internodes were 10 μ M NAA or 10 μ M 2,4-D. Transfer of the callus initiated on 2,4-D onto MS medium containing 10 μ M NAA and 5 μ M BAP led to bud regeneration. The hormone level for callus induction (10 μ M 2,4-D) is near the study's result (2 mg/l 2,4-D). Dai et al. (2003) studied an efficient regeneration and transformation system for two elite aspen hybrid clones (*Populus canescens* \times *Populus grandidentata* and *Populus tremuloides* \times *Populus davidiana*). In the study, calli were induced from *in vitro* leaf explants on modified MS medium and WPM. The calli regenerated into shoots on WPM medium supplemented with 2 mg/l zeatin or 0.01 mg/l thidiazuron. In this study, calli regenerated on WPM with 1 mg/l zeatin.

For rooting, there are several studies that support the study's results. Han et al. (1994) found the rooting ratio for *P. trichocarpa* \times *P. deltoids* to be 67% on WPM with 10 μ M IBA. Gozukirmizi et al. (1998) found that, WPM with 0.5 mg/l IBA induced rooting after 2 weeks at 100% for *P. tremula*. According to these results for four clones as shown in Table 5, rooting was statistically affected by clones. 89 M 011 and 89 M 066 gave the highest rooting rate. Rooting on WPM with 0.5 mg/l IBA (95 to 100%), WPM with 2 mg/l IBA (80 to 100%) and MS with 2 mg/l IBA (90 to 100%) gave similar results depending on the clones and did not indicate statistical differences. Chalupa (1974) transplanted the rooted poplar into a mixture of soil, peat and perlite (3:1:1 v/v/v) in a growth cabinet. In this study, the rooted shoots were transplanted into a different mixture of organic fertilizer: sand: peat. A ratio of 2:2:1 (v/v/v) gave the best (90 to 100%) adaptation ratio in the plant growth chamber. All adapted plants continued to adapt in the greenhouse at a ratio of 100%.

This study developed a rapid and efficient regeneration method for *P. deltoides* and their hybrid clones. Therefore, the regeneration systems allowed us to set dependable regeneration procedures. Results presented here are expected to be helpful for biotechnological studies for poplar improvement.

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