Romanian Biotechnological Letters *Vol. 17, No. 1, 2012*

ORIGINAL PAPER

Somatic Embryogenesis of *Populus deltoides*

Received for publication, February 12, 2011
Accepted, June 1, 2011

AYSUN CAVUSOGLU[1*], ZELIHA IPEKCI-ALTAS[2], KASIM BAJROVIC[3], NERMIN GOZUKIRMIZI[4], AHMET ZEHIR[5]

[1]Kocaeli University, Arslanbey Agricultural Vocational School, TR-41285 Kartepe, Kocaeli, Turkey, Tel:00 90 262 3513281, Fax:00 90 262 351 32 83, e-mail: cavusoglu@kocaeli.edu.tr*

[2]TUBITAK, Research Institute for Genetic Engineering and Biotechnology, TR-41470 Gebze, Kocaeli, Turkey

[3]Institute for Genetic Engineering and Biotechnology, Gajev trg 4/1, BH-71000 Sarajevo, Bosnia and Herzegovina

[4]Istanbul University, Molecular Biology and Genetics Department, TR-341134 Vezneciler, Istanbul, Turkey

[5]Marmara University, Biology Department, TR-34472 Goztepe, Istanbul, Turkey

Abstract

Node, internode, and leaf explants of Populus deltoides Bartr. ex Marsh. × Populus deltoides Bartr. ex Marsh. hybrid poplar clone (89 M 066) tissue culture regenerated plantlets were used for direct and indirect somatic embryogenesis. Direct somatic embryos were differentiated on node, internode and leaf explants on Murashige and Skoog basal medium (MS) supplemented with N^6-Benzyladenine (BA) and 2,4-Dichlorophenoxy acetic acid (2,4-D). The best somatic embryogenesis observed on MS medium supplemented with 0.05 mg/l BA and 5 mg/l 2,4-D with internodes explants in darkness within 3 weeks. Embryogenic calli formation for the first step of indirect regeneration were obtained on node, internode and leaf explants on MS with 2,4-D or MS with 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) and Coconut Water (CW). Internode explants gave the best result (100%) for embryogenic calli formation on MS with 0.8 or 1 mg/l TDZ with 2% CW after 3 weeks. Subsequent transfer of internode-derived embryogenic calli on the same media resulted in somatic embryogenesis within 2 weeks with the frequency of 33% on MS with 0.8 mg/l TDZ with 2% CW . Further development of embryos to the torpedo stage was obtained by subculturing on the same medium. There after, germination of the direct somatic embryos were achieved on Woody Plant Medium (WPM) supplemented with 1 mg/l zeatine in 3 weeks. Data in the study were subjected to statistical evaluation. These systems could be useful for rapid clonal propagation via somatic embryogenesis of eastern cottonwood clones.

Keywords: *Populus deltoides*, Poplar, Eastern Cottonwood, Tissue culture, Regeneration, Somatic embryogenesis.

Introduction

Research areas such as micropropagation, somatic embryogenesis, genetic engineering, marker-aided selection, and molecular diagnostics are merging with traditional forest biological studies to help identify and produce better-suited trees for agroforestry plantings (N.B. KLOPFENSTEIN & al. [1]). The development of methods for *in vitro* culture and genetic engineering has increased the possibility of producing poplar genotypes improved in insect pest resistance, herbicide tolerance, growth rate and wood quality, or reduction in undesirable traits (M. CONFALONIERI & al. [2]). *Populus deltoides* Bartr commonly known as Eastern cottonwood belongs to the family *Salicaceae*, having two genera (*Salix* and *Populus*) with 485 species (G. SINGH [3]). Most of the species are used for timber, pulp and paper industry or for afforestation and ornamental purpose. Some of the species have medicinal and aromatic properties. In recent years, somatic embryogenesis has been studied with some gymnospermous and angiospermous woody species. In most of the studies, somatic embryo initiation started with embryonic or embryo-derived tissue i.e. seed, anther,

immature fruit explants and few embryos were obtained (C.F. POPESCU [4], S.A. MERKLE & al., [5], A. ONAY & al. [6], P. DAS & al. [7], S. AGARWAL & al. [8]). Somatic embryogenesis have been used for several species of poplar such as *Populus nigra* × *Populus maximowiczii* (Y.G. PARK & al.[9]), *Populus ciliata* (G.S. CHEEMA [10]) and *Populus alba* × *Populus grandidentata* (C.H. MICHLER & al. [11]). The present work was carried out to establish an efficient method for direct and indirect somatic embryogenesis from various vegetative explant sources of *P. deltoides* × *P. deltoides* clone. The results on somatic embryogenesis could provide a large scale propagation of these trees and it can possibly be used in genetic manipulation.

Materials and methods

Plant materials: The original stock plant material, *P. deltoides* × *P. deltoides* clone (89 M 066) 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. For direct and indirect somatic embryogenesis node, internode and leaf explants were originated from *in vitro* directly regenerated plantlets.

Media and incubation condition for direct somatic embryogenesis: Stem with node parts 0.5-1 cm, internodes 1 cm in length and leaves 0.5-1 cm^2 were aseptically placed laterally on petri dishes containing 20 ml solid medium with 5-6 explants or magenta vessels containing 50 ml solid medium with 3-4 explants, were set in climate chamber at 25^0C, 70% humidity in darkness without any interval at light phase. All explants were planted on MS basal medium (T. MURASHIGE & al. [12]). The media used for all purposes were supplemented with 30 g/l sucrose, 9 g/l agar and pH was adjusted 5.8 before autoclaving. The media were added: 0.1 mg/l BA and 0.5 mg/l 2,4-D, 0.01 mg/l BA and 5 mg/l 2,4-D, 0.05 mg/l BA and 4 mg/l 2,4-D or 0.05 mg/l BA and 5 mg/l 2,4-D. The most successful explants observed were internodes with first step of embryoids. For maturity of the embryos, the internodes were transferred to MS medium with 0.05 mg/l BA and 5 mg/l indole-3-acetic acid (IAA) or MS with 0.01 mg/l BA and 5 mg/l IAA at the same climate chamber in darkness for a week. For germination of the directly obtained internode somatic embryos Woody Plant Medium (WPM) (G. B. LLYOD & al. [13]) (supplemented with 20 g/l sucrose, 9 g/l agar and pH was adjusted 5.2 before autoclaving) added 0.5 mg/l BA or 1 mg/l zeatine and MS medium supplemented with 0.5 mg/l BA were tested. Germination studies of the direct embryoids were maintained in growth chambers at 50 $\mu mols^{-2}m^{-1}$ cool-white fluorescent light with 16 h, darkness with 8 h photoperiod in 2-3 weeks.

Media and incubation for indirect somatic embryogenesis via calli phase: For induction of embryogenic callis, explants were placed on MS with 0.5 mg/l 2,4-D, MS with 0.8 mg/l TDZ and 2 % CW or MS with 1 mg/l TDZ and 2% CW added media at the climate chamber at 25^0C, 70% humidity in darkness in 3 weeks. Subsequently, embryogenic calli obtained from the internode calli were subcultured on plant growth regulator(PGR)-free MS media, MS with 0.8 mg/l TDZ and 2% CW or MS with 1 mg/l TDZ and 2% CW for induction and further development of somatic embryos to torpedo and cotyledonary stages. The cultures were maintained in darkness.

Experimental design and statistical analysis: Tests were conducted in Radomised 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 the Duncan's multiple range test with a significance value at $P<0.05$. Before the statistically calculation, percentage of the data were transformed by arcsine $\sqrt{x}$.

Results

Direct somatic embryogenesis was induced on the node, internode and leaf explants from directly regenerated shoots of the hybrid poplar, *P. deltoides* × *P. deltoides* clone *in vitro*. While MS with 0.05 mg/l BA and 5 mg/l 2,4-D gave statistically the highest regenerative response (5% from nodes, 10% from leaves and 40% from internodes) for all used explants; MS with 0.1 mg/l BA and 0.5 mg/l 2,4-D did not give any response for direct somatic embryos from nodes or leaves (Table 1). Internode explants showed statistically the highest response for all used media as shown in Table 1. After fixing the most regenerative explants, internode were transferred on to the two different solid media (MS with 0.01 mg/l BA and 5 mg/l IAA or MS with 0.05 mg/l BA and 5 mg/l IAA) for the maturity for the embryo development step in the climate chamber in darkness, 4-6 embryoids/internode were observed on MS with 0.05 mg/l BA and 5 mg/l IAA medium in a week. In this step MS medium with 0.01 mg/l BA and 5 mg/l IAA did not activate embryo progression on internodes. Shoot regeneration from the internode embryos at mature step, the embryos were transferred on solid WPM with 0.5 mg/l BA, MS with 0.5 mg/l BA or WPM with 1 mg/l zeatine. While plant regeneration was not obtained from the first and the second media, the WPM with 1 mg/l zeatine gave the highest shoot (80%) from embryoids at photoperiod (16 h in light, 8 h in darkness) in 3 weeks.

Indirect somatic embryogenesis via callus phase, nodes, internodes and leaves that were obtained from directly regenerated shoots *in vitro* were planted on MS with 0.5 mg/l 2,4-D, MS with 0.8 mg/l TDZ and 2% CW or MS with 1 mg/l TDZ and 2 % CW media in darkness to obtain embryogenic calli (Table 2). Statistically the best results were observed on both MS with 0.8 mg/l TDZ and 2% CW or MS with 1 mg/l TDZ and 2% CW media with a ratio of 100% from internode explants after 3 weeks, whereas the callus induction frequency from nodes were reduced to 50% on MS with 0.8 mg/l TDZ and 2% CW or MS with 1 mg/l TDZ and 2% CW media. Embryonic leaf callus was only obtained on MS with 0.8 mg/l TDZ and 2% CW. Embryonic calli were not induced on MS with 0.5 mg/l 2,4-D medium with any tested explants.

To induce somatic embryos on the internode callus, MS with 0.8 mg/l TDZ and 2% CW or MS medium with 1 mg/l TDZ and 2% CW or plant PGR-free MS media were tested (Table 3). Somatic embryos were obtained on MS with 0.8 mg/l TDZ with 2% CW medium with the frequency of 33% and showed statistically different group, however the induction frequency was reduced to 5% on MS with 1 mg/l TDZ and 2% CW media and no embryos were obtained on PGR-free MS medium. For further development of somatic embryos to torpedo stage was obtained by subculturing the embryogenic internode callus on the same medium after 2 weeks in darkness.

Table 1. Effects of media on direct somatic embryogenesis from hybrid *P. deltoides* × *P. deltoides* clone in darkness within 3 weeks.***

MS medium with plant growth regulators (mg/l)	Somatic embryoids development rate per used explants (%)			
BA+2,4-D	Leaf	Node	Internode	Mean*
0.1+0.5	0	0	3	1c
0.01+5	5	3	20	9,33b
0.05+4	8	4	25	12,33ab
0.05+5	10	5	40	18,33a
Mean**	5,75b	3b	22a	

*Means within the column indicate that the explants means for each medium, having a different letter was significantly different at P<0.05.
**Means within the line indicate that the medium means for each explants, having a different letter was significantly different at P<0.05.
***The medium and explant types separately were found significantly important but medium×explant interaction did not indicate significant importance for direct somatic embryogenesis.

Table 2. Embryogenic calli proliferation from different explants of hybrid *P. deltoides* × *P. deltoides* clone in darkness within 3 weeks.

MS medium with plant growth regulators (mg/l) and additives	Embryogenic callus rate per used explants (%)		
	Leaf	Node	Internode
MS with 0.5 mg/l 2,4-D	0a*B**	0aC	0aB
MS with 0.8 mg/l TDZ + 2% CW	30cA	50bA	100aA
MS with 1 mg/l TDZ + 2% CW	0cB	20bB	100aA

*Data within lines having different lowercase letters were significantly different at P<0.05 indicating explant differences on each medium.
**Data within columns having different capital letters were significantly different at P<0.05 indicating media differences on each explant.

Table 3. Indirect somatic embryoid formation from internode derived calli in darkness within 2 weeks.

MS medium with plant growth regulators (mg/l) and additives	Somatic embryoids rate on internode callus per used explants (%)
MS-PGR free	0c*
MS with 0.8 mg/l TDZ +2% CW	33a
MS with 1 mg/l TDZ +2% CW	5b

*Data within column having different letters were significantly different at P<0.05 for indicating medium differences on somatic embryogenesis from internode callus

Discussion

The results of this work demonstrated that *in vitro* grown *P. deltoides* × *P. deltoides* hybrid's nodes, leaves, and internodes posses the competence, regenerate direct somatic embryos. Simultaneous regeneration of direct somatic embryos was also observed on leaves of *Populus nigra* × *Populus maximowiczii* (Y.G. PARK & al. [9]), and of *Populus alba* × *Populus grandidentata* (NC-5339) (C.H. MICHLER & al. [11]). When 0.05 mg/l BA and 5 mg/l 2,4-D added the MS medium, initiation of embryos were started. In the other studies, importance of cytokinins and auxins were stated for embryo initiation or maturity (C.H. MICHLER & al. [11], V. M. VERMA & al. [14], I. O. AHN & al. [15]). In the study obtained directly initial embryos on internodes transferred to maturity step medium, MS with 0.05 mg/l BA and 5 mg/l IAA or MS with 0.01 mg/l BA and 5 mg/l IAA as interval phase in darkness. The second media gave a high response while in the first media no embryos maturity was observed. Some of the researchers stated that IAA or other auxin type have a role on embryo maturation from embryogenic callus each derived from different plant genus and tissue part (S. A. MERKLE [16], C.F. POPESCU [4], C.H. MICHLER & al. [17], V. A. BAPAT & al. [18], S. KINTZIOS & al. [19]). Darkness is a key factor for some genus until germination step of direct or indirect somatic embryogenesis that is similar to the study (C.H. MICHLER & al. [11], V. M. VERMA & al. [14], S.A. MERKLE & al., [5], R. R. TERMIGNONI & al. [20], B. CUENCA & al. [21]). Germination of direct somatic embryos from internodes was achieved only on WPM supplemented with 1 mg/l zeatine. The other germination media used

did not give any response. W. DENG & al. [22] obtained that transgenic calli embryos germinated to form plantlets on ½ strength WPM medium for *Populus tomentosa*. I. TSVETKOV [23] transferred to WPM with 0.2 mg/l BAP, the immature embryos derived somatic embryos for germination step of *Quercus robur* L.

Indirect somatic embryogenesis via callus phase was tested on leaf, node and internode explants. Internode explants statistically gave the highest embryogenic callus rate on both of two media; MS with 0.8 mg/l TDZ and 2% CW or MS with 1 mg/l TDZ and 2% CW. The calli of internodal segments was lasted to turned embryogenic step on MS with 0.8 mg/l TDZ and 2% CW and MS with 1 mg/l TDZ and 2% CW. The embryogenesis occured with a ratio of 33% and 5% respectively. Both of the two steps, embryogenic callus formation and embryo initiation were maintained in darkness.

There are a few reports stated internode callus derived somatic embryogenesis. M. OSTRY & al. [24] studied on *Populus nigra* var. *betulifolia* Torr. × *Populus trichocarpa* Torr. & Gray; B. CUENCA & al. [21] on *Quercus robur* L., V. A. BAPAT & al. [18] on *Santalum album*. Importance of TDZ for indirect embryogenesis at different stages is reported in some study on different woody species (S. A. MERKLE & al. [25], A. ONAY & al. [6], I. O. AHN & al. [15]).

Effect of Coconut Water (CW) to induction of somatic embryogenesis was mentioned in other studies. S. BANDYOPADHYAY & al. [26], and S. S. BHOJWANI & al. [27], used CW for somatic embryogenesis of *Eucalyptus sp.* and *Datura sp.* respectively. Darkness was another key factor for indirect somatic embryogenesis in the study as well as the other studies as mentioned above.

Populus species are as important as woody plants for pulp and paper industry or ornamental purposes and nearly become a model plants in forestry and genetic manipulation of trees. One of the micropropagation methods, somatic embryogenesis, requires further investigations on species-based due to mass propagation and stability genes. According to our search, this study has been the first for somatic embryogenesis of *Populus deltoides* × *Populus deltoides*.

Acknowledgements

The study was a part of A. Cavusoglu PhD thesis partially supported by Marmara University, Research Funds (Project No:1999-Fen-26). The author is grateful to TUBITAK-MAM Genetic Engineering and Biotechnology Institute (Kocaeli, Turkey) Directory in whose laboratory the study was performed.

References

1. N. B. KLOPFENSTEIN, J. G. KERL , The potential of biotechnology in temperate agroforestry practices, *Agroforestry Systems*, 32, 29-44, (1995)
2. M. CONFALONIERI, A. BALESTRAZZI, S. BISOFFI, D. CARBONERA, *In vitro* culture and genetic engineering of *Populus* spp.:synergy for forest tree improvement, *Plant Cell, Tissue and Organ Culture*, 72, 109-138, (2003)
3. G. SINGH, Plant Systematics.An integrated Approach, Science Publishers, Inc. Enfield, New Hampshire 03748, USA, (2004)
4. C. F. POPESCU, Somatic embryogenesis and plant development from anther culture of *Vitis vinifera* (L.), *Plant Growth Regulation*, 20, 75-78, (1996)
5. S. A. MERKLE, M. T. HOEY, A. A. WATSON-PAULEY, E. SCHLARBAUM, Propagation of *Liriodendron* hybrids via somatic embryogenesis, *Plant Cell, Tissue and Organ Culture*, 34, 191-198, (1993)

6. A. ONAY, C. E. JEFFREE, M. M. YEOMAN, Somatic embryogenesis in cultured immature kernels of Pistachio, *Pistacia vera* L, *Plant Cell Reports,* 15, 192-195, (1995)
7. P. DAS, S. SAMANTARAY, A. V. ROBERTS, G. R. ROUT, *In vitro* somatic embryogenesis of Dalbergia sissoo Roxb.-a multipurpose timber-yielding tree, *Plant Cell Reports*, 16, 578-582, (1997)
8. S. AGARWAL, K. KANWAR, D. R. SHARMA, Factors affecting secondary somatic embryo maturation in *Morus alba* L., *Scientia Horticulturae*, 102, 359-368, (2010)
9. Y. G. PARK, S. H. SON, *In vitro* organogenesis and somatic embryogenesis from punctured leaf of *Populus nigra* × *P. maximowiczii*, *Plant Cell, Tissue and Organ Culture*, 15, 95-105, (1988)
10. G. S. CHEEMA, Somatic embryogenesis and plant regeneration from cell suspension and tissue cultures of mature himalayan poplar (*Populus ciliata*), *Plant Cell Reports*, 8, 124-127, (1989)
11. C. H. MICHLER, E. O. BAUER, High frequency somatic embryogenesis from leaf tissue of *Populus* spp., *Plant Science*, 77, 111-118, (1991)
12. T. MURASHIGE, F. SKOOG, A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Plant Physiology*, 15, 473-479, (1962)
13. G. B. LLYOD, B. H. MC COWN, Commercially feasible micropropagation of mointain laurel, *Kalmia latifolia*, by use of shoot-tip culture, *Proc. Int. Plant Prop. Soc*., 30, 421-427, (1981)
14. V. M. VERMA, J. J. CHO, Plantlet development through somatic embryogenesis and organogenesis in plant cell cultures of *Colocasia esculenta* (L.) Schott., *Asia Pacific Journal of Molecular Biology and Biotechnology*, 18(1), 167-170, (2010)
15. I. O. AHN, B. V. LE, C. GENDY, K. T. T. VAN, Direct somatic embryogenesis through thin cell layer culture in *Panax ginseng*, *Plant Cell, Tissue and Organ Culture*, 45, 237-243, (1996)
16. S. A. MERKLE, Strategies for dealing with limitations of somatic embryogenesis in hardwood trees, *PlantTissue Culture and Biotechnology*, 1(3), 112-121, (1995)
17. C. H. MICHLER, E. O. BAUER , Somatic embryogenesis in plant cell cultures of *Populus*, *In Vitro Cell. Dev. Biol.*, 23(3), part II: 46 A, (1987)
18. V. A. BAPAT, P. S. RAO, Sandalwood plantlets from 'Synthetic seeds', *Plant Cell Reports*, 7, 434-436, (1988)
19. S. KINTZIOS, C. MANOS, O. MAKRI, Somatic embryogenesis from mature leaves of rose (*Rosa* sp.), *Plant Cell Reports*, 18, 467-472, (1999)
20. R. R. TERMIGNONI, P. J. WANG, C. Y. HU, Somatic embryo induction in *Eucalyptus dunnii*, *Plant Cell, Tissue and Organ Culture*, 45, 129-132, (1996)
21. B. CUENCA, M. C. SAN-JOSÉ, M. T. MARTINEZ, A. BALLESTER, A. M. VIEITEZ, Somatic embryogenesis from stem and leaf explants of *Quercus robur* L., *Plant Cell Reports*, 18, 538-543, (1999)
22. W. DENG, K. LUO, Z. LI, Y. YANG, A novel method for induction of plant regeneration via somatic embryogenesis, *Plant Science*, 177, 43-48, (2009)
23. I. TSVETKOV, Somatic embryogenesis and regeneration of plantlets in common oak (*Quercus robur* L.), *Biotechnology and Biotechnological Equipment*, 12 (1), 51-55, (1998)
24. M. OSTRY, W. HACKETT, C. MICHLER, R. SERRES, B. MC COWN, Influence of regeneration method and tissue source on the frequency of somatic variation in *Populus* to infection by *Septoria musiva*, *Plant Science*, 97, 209- 215, (1994)
25. S. A. MERKLE, K. A. NEU, P. J. BATTLE, R. L. BAILEY, Somatic embryogenesis and plantlet regeneration from immature and mature tissues of sweetgum (*Liquidambar styraciflua*), *Plant Science*, 132, 169-178, (1998)
26. S. BANDYOPADHYAY, K. CANE, G. RASMUSSEN, J. D. HAMILL, Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species-*Eucalyptus nitens* and *E. globulus*, *Plant Science*, 140, 189-198, (1999)
27. S. S. BHOJWANI, M. K. RAZDAN, Plant tissue culture: theary and practise. Revised Edition, Elsevier Science, Armsterdam, 297-335, (1996)