Direct shoot regeneration from leaf, root and stem internode segments of male poplar trees and the molecular analysis of variant regenerated plants

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Abstract: A regeneration protocol has been developed by using thidiazuron (TDZ) with a high frequency of *in vitro* leaf, root and stem internode induction in male (*Populus alba, Populus tremula* L. and *Populus tremula* L. x *Populus. tremuloides* "Michx"). There were differences in average number of shoots among the different sources of explants, the stem internode explants regenerated shoots more effectively than leaf and root explants. In contrast to root and stem internode explants, leaves had poor regeneration abilities in the case of *P. tremula* and *P. tremula* x *P. tremuloides* "Michx". The highest frequency of adventitious shoot formation was (8.2, 39.2 and 38.3 shoots /explant) for *Populus alba, Populus tremula* L. and *Populus tremula* L. x *Populus tremuloides* "Michx" respectively on a medium containing 0.02 µM TDZ when stem internode explants were cultured. Higher TDZ concentrations significantly stimulated further elongation in the newly formed shoots on the three *Populus* species. *In vitro* regenerated plants were genetically analyzed using RAPD fingerprints. The presence of specific loci in the regenerated plants indicated that no genetic variation existed in the regenerated plants.
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Key words: *In vitro* regeneration, *Populus alba*, *Populus tremula, Populus tremula* x *Populus tremuloides* "Michx", male trees, TDZ, RAPD.

1. Introduction

Poplar (Populus species) are important elements of the riparian ecosystems and target of scientific interest (Tsvetkov et al. 2007). Male clones in some Populus species tended to have longer internodes, higher plant dry weight and heavier wood as compared to the female clones (Khosla and Deol 1984). Recent development in somatic hybridization and direct gene transfer are techniques of interest to extend the genetic variability in Populus. There have been extraordinary developments in molecular genetics and genomics of trees in recent years. These advances were most striking in Populus species and hybrids (Busov et al. 2005) a result of a broad international consensus regarding its value as a scientific and technological model for woody perennial plants (Wullschleger et al. 2002 and Brunner *et al.* 2004). Maximum regeneration rates are the key to successful genetic transformation (Confalonieri *et al.* 2003). Among the possible initial explants root, leaf and stem internode have proven to be a suitable starting point for elaboration of organogenetic systems for *in vitro* regeneration in different species, forest ones inclusive (George 1993**;** Sunpui and Kanchanapoom 2002**;** Shimada *et al.* 1997). *In vitro* cultures of *Populus have* been successfully initiated from several sources of explants including leaf, root and stem (Chaturvedi *et al.* 2004; Tsvetkov *et al.* 2007; Marco *et al.* 2008). This is not the case for male *populus* where this kind of work is limited, especially in aspen and hybrid aspen.

RAPDs are powerful tools for fingerprinting individuals in *Populus* (Kiss *et al.* 2001; Liu and Furnier 1993 and Lu *et al.* 2006). In the present study, RAPD fingerprints from the PCR were used for clonal identification in poplar.

We describe efficient direct shoot regeneration from leaf, root and stem internode explants suitable for genetic transformation, emphasizing the importance of TDZ concentration variation which induced shoot regeneration in the three male poplar species under study. Detection of any probability of somaclonal variations in the *in vitro* regenerated plants was also accomplished.

2. Materials and Methods

2.1 Plant materials

The plant materials used in these experiments were kindly provided by the Institute for Forest Genetics, Grosshansdorf, Germany. It has already been introduced into the culture so that in this paper we only conducted the procedures of regenerated plants from *in vitro* leaf, root and stem internode segments.

Leaf, root and stem internodes [10-15 mm in length (root tips excised) were obtained from stabilized *in vitro* culture of male (*Populus alba, Populus tremula* L. and *Populus tremula* L. x *Populus tremuloides* "Michx"] and used as initial

explants. The explants were distributed separately on Petri dishes (92 x 16 mm) containing 30-35 ml of MS medium (Murashige and Skoog 1962) basic salts and vitamins supplemented with 2.0 % (w/v) sucrose and 6.0 gl^{-1} agar. The pH of the medium was adjusted to 5.7 with NaOH solution (0.1 N) or HCl (0.1 N). The medium was autoclaved for 20 min at 121 C° and different concentrations of thidiazuron (TDZ) (0.0, 0.005, 0.01, 0.02, 0.04 and 0.08 µM) (for root and stem internodes explants) and or (0.0, 0.005, 0.01and 0.02 µM) (for leaf explant), were added to the medium after being autoclaved and cooled to 47 °C. Growth regulator free medium was used as a control. The explants were placed on Petri dishes with adaxial surface in contact with the medium. Every Petri dish was inoculated with six explant discs, each treatment contained ten replicates (Petri dishes). After eight weeks, the average number of regenerated shoots per explant, average length of the longest shoot, explant weight and callus percentage were calculated.

2.2 Genomic DNA extraction

DNA was extracted from fresh leaves of three *in vitro* derived plants which produced from leaf, root and stem internodes separately of the three *populus* species as well as control plants using a standard CTAB extraction procedure (Wolff *et al.* 1994), modified after (Saghai-Maroof *et al.* 1984). Cleaning with ammonium acetate was necessary. Samples were diluted with half the volume of 7.5 M, cold ammonium acetate, cooled in a fridge for 15 min, followed by spinning for 15 min at 5000 rpm. The supernatant was taken and two volumes of cold 96% ethanol gently mixed and left for 30 min in a freezer. After spinning for 15 min, the precipitate was taken, and 500 µl of cold 70 % ethanol was added for washing. The supernatant was removed and the precipitate left to air-dry at room temperature for 10-20 min, and then dissolved in a suitable volume of TE buffer. DNA concentration was determent by NanoDrop 3300 (Thermo Scientific).

2.3 Random Amplified polymorphic DNA (RAPD)

RAPD analysis was performed in 25 µl volume reactions according to Wolff and Peters-Van (1993). A reaction mixture (17.5 ng genomic DNA, 12.5 REDTaq Ready Mix (Sigma) [20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3mM $MgCl_2$, 0.002% gelatin, 0.4 mM mix dNTP(dATP, dCTP, dGTP, dTTP) and 0.06 unit/ µl Taq DNA polymerase] and 0.4 pmole) was prepared for each primer sufficient for all samples plus one negative control in which water was added instead of DNA. All reagents were centrifuged and kept on ice during the preparation of the master mix. Amplifications were carried out in a Mastercycler gradient programmed according to Wolff (1996) [the initial denaturation for 3 min at 94 °C was followed by 45 cycles of denaturation (30 sec. at 94 °C), annealing (45 sec. at 36 °C), extension (1.5 min at 72°C)]. PCR products were analyzed by gel electrophoresis on 1.4% agarose gel prepared in 0.5 X TBE buffers, DNA ladder (Fermentas) was used as a standard with molecular sizes of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The gel was stained with ethidium bromide for 20 min and examined using UV cabinet unit and photographed with a Polaroid camera connected to a computer system with analytical software (GelDocu Advanced version).

Ten different oligonucleotide random primers used for RAPD analysis were:-

(A1) 5'AGACGTCCAC3'
(A2) 5ACGCGCATGT3'
(A3) 5'AATGGCGCAG3'
(A4) 5'GAATCGGCCA3'
(A5) 5'GGGAGACATC3'
(A6) 5'GGAAGTCGCC3'
(A7) 5'ACGCGCATGT3'
(A8) 5'GGTCGGAGAA3'
(A9) 5'CCTACGTCAG3'
(A10) 5' CTGACCAGCC3'

2.4 Statistical analysis

Experiments were set up in completely randomized design. Data were statistically analyzed using ANOVA / MANOVA of Statistica 6 software (Statsoft, 2001), the significance of differences among means was carried out using the Least Significant Test (L.S.D) at $p = 0.05$.

3. Results and Discussion

3.1 Plant regeneration

Regeneration of poplar is the process of micropropagating plants using a small amount of plant tissue and reproducing an entire plant. It is based on the theory of totipotency which states that each cell has everything needed to reproduce an entire plant (Steward, 1968), it is a useful mean of production of plantlets with a lower risk of genetic instability than by other methods. The preliminary experiment using leaf, root and stem internode segments cultured on hormone-free MS basal medium revealed that all explants did not regenerate shoots effectively and became pale and necrotic after 6-8 weeks.

After 1-2 weeks in the culture media contain (0.005, 0.01 and 0.02 µM) TDZ the leaf explants remained green and small shoots were formed only at the margin of the leaf explants Table (1) and Fig (1) in the case of *populus tremula* and *populus tremula* x *populus tremuloides* "Michx". Small granulated callus formed at the margin of the leaves in the case

of *populus alba*, most leaves were induced to form good callus on the MS medium enriched with TDZ. In the beginning, callus growth was rather slow but after 4-weeks culture period the speed of callus formation was fast and small green nodules could be seen. Eight weeks after initial cultures, numerous adventitious shoot formations took place readily from these leaves. In contrast no callus was formed on the leaf margin of *populus tremula* and *populus tremula* x *populus tremuloides* "Michx". **(**Table 1). TDZ promoted production of granular masses of tissues and numerous shoot primordia on the surface of leaf explants of *populus tremula* and *populus tremula* x *populus tremuloides* "Michx". The results showed that TDZ stimulated shoot regeneration and the number of adventitious shoots from leaves explants varied depending on the concentrations of TDZ and the plant species. Considering the positive effect of TDZ on shoot induction, shoot height and explants fresh weight, at optimal TDZ concentration for shoot regeneration from leaf explants were (5.4, 18.2 and 29.1 shoots/ explant) in the case of *populus alba*, *populus tremula* and *populus tremula* x *populus tremuloides* "Michx" respectively. Leaf tissue has been studied and shown to have the greatest regeneration capacity of *Populus* species (Chaturvedi *et al*. 2004; Mingozzi *et al.* 2008).

TDZ at 0.02 µM proved to be the best treatment for direct shoot regeneration since it provide (8.2, 39.2 and 38.3 shoots per explant) from stem internodes of *populus alba*, *populus tremula* and *populus tremula* x *populus tremuloides*, respectively. When TDZ concentration was increased, a reduction in the regeneration capacity was noticed. Stem internodes did not produce shoots on media containing (0.005 and 0.01 µM) TDZ in the three poplar species (Table 2). The highest concentration of TDZ (0.08 µM) induced formation of significantly longer shoots (3.0 cm), with shoot length progressively increasing together with rising of the TDZ concentration in the case of *populus alba* (Table 2). No callus was formed by the stem internode explants in the case of *populus tremula* x *populus tremuloides* "Michx".

The data presented in Table (3) showed that regeneration frequency of root segments as well as callus formation were strongly affected by the thidiazuron concentration in all three species. However, the maximum number of regenerated shoots (5.8, 39.3 and 35.3 shoots/ explant) were obtained when the root segments were cultured on MS medium supplemented with 0.02, 0.01 and 0.02 µM TDZ on *populus alba*, *populus tremula* and *populus tremula* x *populus tremuloides* "Michx", respectively. TDZ was reportedto stimulate shoot organogenesis from root explant of *Populus deltoids* (Chaturvedi *et al.* 2004).

All TDZ- treated cultures yielded healthy shoots in all explant types. The results emphasize the importance of TDZ and suggest that a reasonable TDZ concentration induces shoot regeneration. Thidiazuron (TDZ), a urea-derived cytokinin, is a potent cytokinin for woody plant tissue culture (Huetteman and Preece, 1993) and is extensively used for the induction of shoot regeneration in several plant species (Li *et al.* 2000; Mohan and Krishnamurthy, 2002; Liu *et al.* 2003). It exhibits strong cytokinin-like activity in several bioassays (Mok *et al.* 1982; Mok and Mok, 2001). The inhibition of cytokinin oxidase-mediated degradation of endogenous cytokinins by TDZ is sufficient to account for this extremely high activity. Capelle *et al.* (1983) reported that TDZ stimulated the conversion of cytokinin nucleotides to the biologically more active ribonucleosides. These results indicate that the type of explant is highly important in establishing an efficient regeneration system as reported by Uranbey *et al.* (2005). It could be observed that adventitious shoots frequency of the stem internode explants had regenerated shoots more effectively than leaves and root explants.

3.2 RAPD analysis

Generally, it is important to make sure that the regenerated plants were genetically true-to-type of their donor plants with respect to genetic fidelity for species conservation (Quiala *et al.* 2009). In order to know if there is any aberration in the regenerated plants, the RAPD marker system was employed for this purpose. Three regenerated plants from leaf, root and stem internodes from each species were randomly selected from *in vitro* derived explants as well as control donor plants. Two primers (A6 and A7) of the ten primers tested gave bands in RAPD analysis. The three regenerants and the control shared a large proportion of their RAPD markers (Fig. 4 A and B), which suggests homology among the regenerants and control plant and during our regeneration system no genetic changes had occurred. Results of the present study are in agreement with the results of Lin *et al.* (1993) and Cassells and Curry (2001) that RAPD is useful for establishing a genetic basis for somaclonal variation in poplar. RAPD analysis has been revealed to be a potential marker for distinguishing genetic variation in plant other than poplar (Raimondi *et al.* 2001; S˘us˘ek *et al.* 2002).

Table (1): Effect of different concentrations of TDZ on adventitious shoot regeneration from leaf explants of *Populus alba*, *Populus tremula* and *Populus tremula* x *Populus tremuloides* "Michx"

TDZ (µM)	*P. alba*				*P. tremula*				*P. tremula x P. tremuloides*			
	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %
0.00	0.0b*	0.0b	0.0b	0.0b	0.0b	0.0c	0.0b	0.0	0.0b	0.0b	0.0c	0.0
0.005	3.8a	1.6a	0.5a	70.0a	0.0b	0.0c	0.0b	0.0	0.0b	0.0b	0.0c	0.0
0.01	5.4a	1.7a	0.5a	67.5a	14.7a	1.2b	0.17b	0.0	27.5a	1.8a	0.5b	0.0
0.02	3.9a	2.4a	0.5a	64.3a	18.2a	2.6a	0.69a	0.0	29.1a	2.2a	0.9a	0.0

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

Table (2): Effect of different concentrations of TDZ on adventitious shoot regeneration from internode explants of *Populus alba*, *Populus tremula* and *Populus tremula* x *Populus tremuloides* "Michx"

TDZ (µM)	*P. alba*				*P. tremula*				*P. tremula* x *P. tremuloides*			
	No. of shoots/ explant	Longest shoot (cm))	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %
0.00	0.0c*	0.0c	0.0a	0.0b	0.0c	0.0c	0.0c	0.0b	0.0c	0.0b	0.0b	0.0
0.005	0.0c	0.0c	0.0a	0.0b	0.0c	0.0c	0.0c	0.0b	0.0c	0.0b	0.0b	0.0
0.01	0.0c	0.0c	0.0a	0.0b	0.0c	0.0c	0.0c	0.0b	0.0c	0.0b	0.0b	0.0
0.02	8.2a	2.0b	2.5a	50.0a	39.2a	2.3a	1.2a	0.0b	38.3a	1.8a	1.1a	0.0
0.04	2.4b	2.1b	1.2a	100.0a	18.5b	1.5b	1.0ab	70.8a	32.2ab	1.8a	0.89a	0.0
0.08	1.3b	3.0a	2.0a	100.0a	20.8b	1..3b	0.78b	70.0a	28.4b	1.7a	0.72a	0.0

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

Table (3): Effect of different concentrations of TDZ on adventitious shoot regeneration from root explants of *Populus alba*, *Populus tremula* and *Populus tremula* x *Populus tremuloides*"Michx"

TDZ (µM)	*P. alba*				*P. tremula*				*P. tremula* x *P. tremuloides*			
	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %	No. of shoots/ explant	Longest shoot (cm)	Explant fresh weight (g)	Callus %
0.00	0.0c*	0.0c	0.0b	0.0c	0.0d	0.0b	0.0b	0.0c	0.0c	0.0c	0.0b	0.0b
0.005	2.3bc	0.4bc	0.1b	0.0c	26.0b	1.8a	0.4a	0.0c	24.4a	1.7b	0.9ab	0.0b
0.01	3.9ab	1.3b	0.4b	21.4c	39.3a	2.1a	1.1a	0.0c	31.3a	2.6a	1.3ab	0.0b
0.02	5.8a	2.8a	2.1a	75.0b	33.0a	2.5a	1.2a	8.3abc	35.9a	2.8a	1.7a	0.0b
0.04	1.7bc	2.3a	2.2a	100.0a	38.6a	2.3a	1.9a	17.9ab	31.0a	1.7b	1.5a	8.3b
0.08	0.0c	3.0a	3.0a	100.0a	18.0c	2.4a	1.9a	25.0a	26.0a	2.0ab	1.1ab	58.3a

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

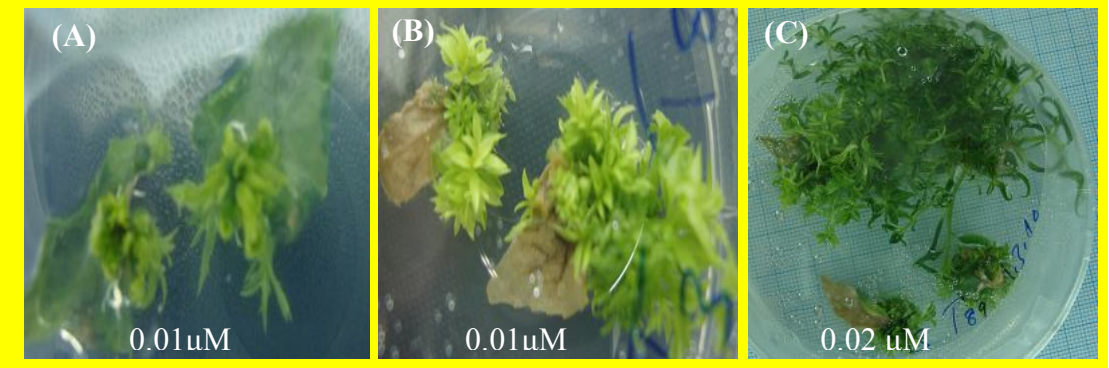

Fig (1): Direct shoot regeneration obtained from leaves on MS medium supplemented with TDZ . (A and B) development of shoot initials on leaves of *P. tremula* x *P. tremuloides* after 4 and 8 weeks in culture, respectively. (C) Adventitious shoots formed on leaves of *P. tremula* x *P. tremuloides* eight weeks after the initial culture.

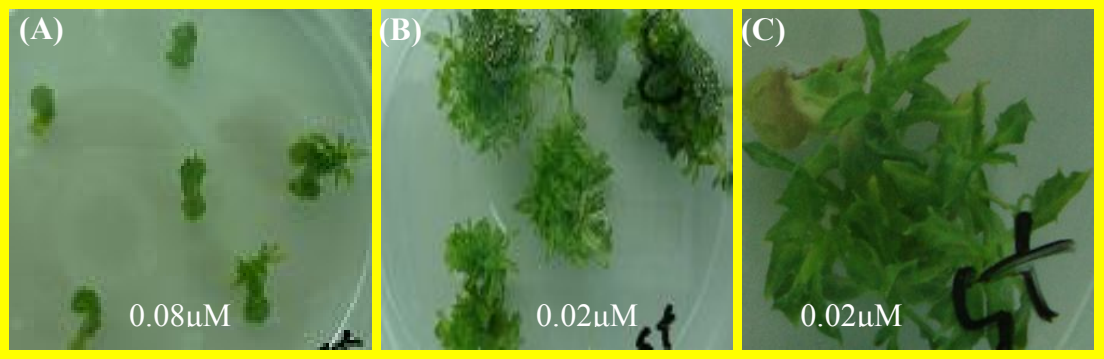

Fig (2): Direct shoot regeneration obtained from stem internode explans on MS medium supplemented with TDZ. (A and B) Adventitious shoots of *P. tremula* x *P. tremuloides* after 4 and 8 weeks in culture, respectively and (C) Adventitious shoots of *P. alba* after eight weeks.

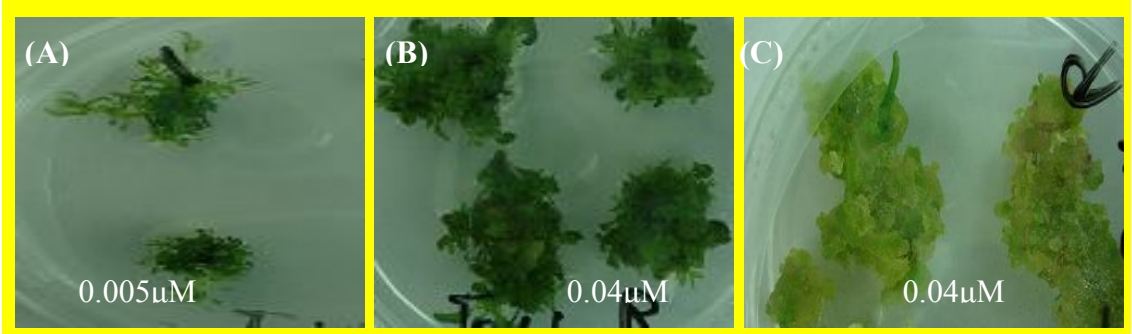

Fig (3): Adventitious shoot regeneration from root explans on MS medium supplemented with TDZ. (A and B) Adventitious shoots of *P. tremula* after 4 and 8 weeks in culture, respectively and (C) Callus formation and development of shoot initials of *P. alba* after eight weeks.

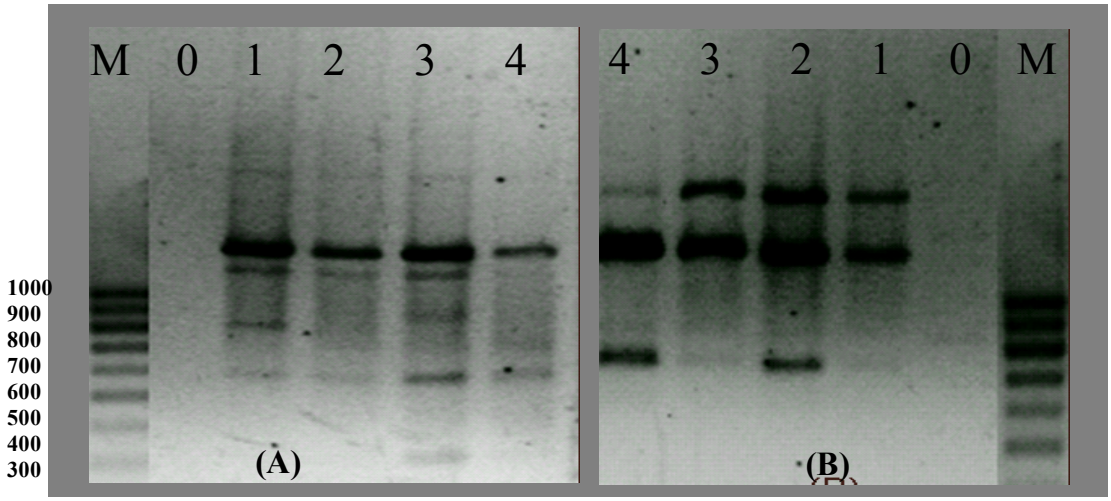

Fig (4): (A and B) Electrophoresis pattern of DNA amplification products obtained after direct shoot regeneration from stem internode and root explans of *P. tremula* x *P. tremuloides* and *P. tremula*, with primers A6 and A7, respectively, Lanes 2, 3 and 4 refer to sample plants, 1 is the control plant, 0 refers to the negative control of PCR and M refers to the DNA marker.

4. Conclusion

Data presented in this study showed a striking improvement of adventitious shoot regeneration in MS medium amended with TDZ which greatly increased adventitious shoot from leaf, stem internode and root explants of different poplar species under study, but regeneration was genotype-dependent. Furthermore, while the same tissue was used in three species, a differentiated response was obtained. There were differences in average number of shoots among the sources of explants in all poplar species. The stem internode explants regenerated shoots more effectively than leaf and root explants. In contrast, leaf had poor regeneration abilities in the case of *populus tremula* and *populus tremula* x *populus tremuloides* "Michx". In conclusion this research demonstrated that direct plant regeneration of the three male poplar trees through culture of leaf, root and stem internode segments could be obtained easily. The rapid clonal propagation described here may be useful for the development of gene transfer system and for micropropagation of genetically stable plants from theses important male trees.

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References

Brunner A.M., Busov V.B. and Strauss S.H. 2004. The poplar genome sequence: functional genomics is a keystone plant species. Trends Plant Science. 9: 49-56.

Busov V. B., Amy M. B., Richard M., Sergei F., Lisa G., Sonali G., and Steven H. S. 2005. Genetic transformation: a powerful tool for dissection of adaptive traits in trees. New Phytologist. 167 (1) 9–18.

Capelle, S.C., Mok, D.W.S., Kirchner, S.C. and Mok, M.C. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6 (d2-isopentenyl) [8-'4C] adenosine in callus tissue of *Phaseolus lunatus* L. Plant Physiol. 73: 796-802.

Cassells A.C. and Curry R.F. 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell, Tissue and Organ Culture. 64: 145–157.

Chaturvedi, H.C., Sharma, A.K. Agha, B.Q. Jain M. and Sharma M. 2004. Production of cloned trees from *Populus deltoids* through *in vitro* regeneration of shoots from leaf, stem and root explants and their field cultivation. Indian J. of Biotechnology. 2: 203-208.

Confalonieri M., Balestrazzi A., Bisoffi S., Carbonera D. 2003. *In vitro* culture and genetic engineering of *Populus* species: synergy for forest tree improvement. Plant Cell, Tissue and Organ Culture. 72:109–138.

George E.F. 1993. Plant Regeneration by Tissue Culture, 2nd Ed., Exegetics Ltd., Part I, inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. 81: 8014-8018.

Huetteman C.A. and J.E. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell, Tissue and Organ Culture. 33: 105-119.

Kiss J., Kondrák M., Törjék O. , Kiss E., Gyulai G., Mázik-Tökei K. and Heszky L.E. 2001. Morphological and RAPD analysis of poplar trees of anther culture origin Euphytica. 8 (2) 213-221.

Khosla, P. K. and Deol, G. S. 1984. Growth response of Male and Female clones of *Populus Ciliata* "Wall." Ex royle to nitrogen fertilizer .Proc. Indian main. Sci. Acad. B50 (6). 603-606.

Li H., Murch S.J. and Saxena P.K. 2000. Thidiazuron induced de novo shoot organogenesis on seedlings, etiolated hypocotyls and stem segments of Huang-qin. Plant Cell Tissue and Organ Culture. 62: 169-173.

Lin D., Hubbes M. and Zsuffa L. 1993. Differentiation of poplar and willow clones using RAPD fingerprints. Downloaded from http://treephys.oxfordjournals.org on August 15, 2010.

Liu Z. and Furnier G.R. 1993. Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. TAG Theoretical and Applied Genetics. 87 (1-2) 97-105.

Liu C.Z., Murch S.J. Demerdash E.L. and Saxena P.K. 2003. Regeneration of the Egyptian medicinal plant *Artemisia judaica* L. Plant Cell Report. 21: 525-530.

Lu Z., Wang Y., Peng Y., Korpelainen H. and Li C. 2006. Genetic diversity of *Populus cathayana* Rehd populations in Southwestern China revealed

by ISSR markers. Plant Science. 170 (2) 407-412.

Marco M., Paul M. and Scott M. 2008. Adventitious shoot regeneration from leaf explants of eastern cottonwood *Populus deltoides* cultured under photoautotrophic conditions Tree Physiology. 29: 1- 11.

Mingozzi M., Montello P. and Merkle S. 2008. Adventitious shoot regeneration from leaf explants of Eastern cottonwood *Populus deltoides* cultured under photoautotrophic conditions. Tree Physiology. 10 :1-11.

Mok D. and Mok M 2001. Cytokinin metabolism and action. Annual Review of Plant Physiology and Plant Molecular Biology. 52:89-118.

Mok M.C, Mok D.W.S., Armstrong D.J., Sudo K., Isogai Y. and Okamato T. 1982. Cytokinin activity of 7V-phenyl- N-l ,2,3-thidiazol-5-ylurea (thidiazuron). Phytochemistry. 21: 1509-1511.

Mohan M.L. and Krishnamurthy K.V. 2002. Somatic embryogenesis and plant regeneration in pigeonpea. Biol. Plant. 45: 19-25.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Quiala E., Jesús M., Grecia M., Manuel de F., Maité C., Alina C., Naivy P., Raúl B. and Britta K. 2009. *In Vitro* propagation of *Pilosocereus robinii* (Lemaire) Byles *et* Rowley, endemic and endangered cactus. J. PACD. 11: 18−25.

Saghi-Maroof M. A., Soliman K. M., Jorgensen R. A. and Allard R. W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian

inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. 81: 8014-8018.

Shimada T., Matsushita T. and Otani M. 1997. Plant regeneration from leaf explants of *Primula cuneifolia* var. *hakusanensis*, "Hakusankozakura". Plant Biotechnol. 14: 47–50.

Statsoft Inc. 2001. STATISTICA fur Windows (software-system fur Datenanalyse) Version 6. http:/www.statisoft.com.

Steward F.C. 1968. Growth and Organization in Plants. P. 546. Addison-Wesley Pub. Co., Reading, MA.

Sunpui W. and Kanchanapoom K. 2002. Plant regeneration from petiole and leaf of African violet (*Saintpaulia ionantha* Wendl.) cultured *in vitro*. J. Sci. Technol 24(3) 357-364.

S˘us˘ek A., Javornik B. and Bohanec B. 2002. Factors affecting direct organogenesis from flower explants of *Allium giganteum*. Plant Cell, Tissue and Organ Culture. 68: 27–33.

Tsvetkov J., Hausman F. and Jouve L. 2007. Thidiazuron-induced Regeneration in Root Segments of White Poplar (*P. alba* L.). Bulgarian Journal of Agricultural Science. 13: 623-626.

Uranbey S., Sevimay C.S. and Özcan S. 2005. Development of high frequency multiple shoot formation in Persian Clover (*Trifolium resupinatum* L.). Plant Cell, Tissue and Organ Culture. 80: 229-232.

Wolff K 1996. RAPD analysis of sporting and chimerism in *Chrysanthemum*. Euphytica. 89: 159-164.

Wolff, K. and Peters-Van Rijn J. 1993. Rapid detection of genetic variability in *Chrysanthemum* (*Dendranthema grandiflora* Tzvelev) using random primers. Heredity 71: 335-341.

Wolff K., Peters-Van Rijn J. and Hofstra H. 1994. RFLP analysis in *Chrysanthemum* I. Probe and primer development. Theoretical and Applied Genetics. 88: 472-478.

Wullschleger S.D., Jansson S. and Taylor G. 2002. Genomics and forest biology: *Populus* emerges as the perennial favorite. Plant Cell. 14: 2651-2655.

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