Effect of Different Media and Growth Regulators on the *in vitro* Shoot Proliferation of Aspen, Hybrid Aspen and White Poplar Male Tree and Molecular Analysis of Variants in Micropropagated Plants

Salah Khattab

Suez Canal University, Faculty of Agriculture, Department of Horticulture, Ismailia- Egypt
khattabfar@yahoo.de

Abstract: Among other *in vitro* factors like temperature and light, concentration of plant growth regulators and medium constituents are two of the most important aspects of successful micropropagation. With the aim of optimization of *in vitro* multiplication of *Populus alba* L., *Populus tremula* L. and *Populus tremula* L. *x Populus. tremuloides* Michx , the effect of MS and WPM media with various concentrations of BAP and 2iP was studied. The following multiplication parameters were monitored: number of shoots regenerated/explant, explant height, and explants weight were determined. MS medium proved to be the most effective one, resulting in better and morphologically superior microshoots as compared to WPM medium in the case of *Populus alba*. However in *Populus tremula* and *Populus tremula x Populus Tremuloides* the highest number of shoots was found when grown on WPM medium. In all three poplar lines, the highest shoot multiplication was obtained on MS and WPM media supplemented with BAP at (0.1 and 0.2 mgl^{-1}). Very poor multiplication was achieved on media with 2iP. Shoot tips were isolated and induced to root on MS medium supplemented with IAA, IBA and/or NAA (0.0, 0.1, 0.2 and 0.4 mg l^{-1}). About 90% of the rooted plantlets tested have successfully established in soil. *In vitro* derived plants were genetically analyzed using RAPD fingerprints. RAPD analysis confirmed that all the *in vitro* derived plant which tested were genetically identical to their donor plants, suggesting the absence of detectable genetic variation in the regenerated plants.

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1. Introduction:

Forest trees are of great environmental and economic importance and also display remarkable developmental traits (Groover *et al*., 2004). Fast-growing poplar trees are widely used in a variety of climate zones for applications to stabilize soils, and to decrease windblown dust and the vertical migration of pollutants (Schnoor, 2000) *Populus* species are economically and ecologically important because of their suitable wood properties (i.e for paper making and timber), fast growth rate and high biodiversity of organisms dependent on living or decaying trees of *Populus* (Ranua, 1996, 2001; Rautio *et at*., 2001; Karl**,** 1988). *Populus* has been the second most used tree genus in biotechnology studies in general (after *Pinus*) and the most used in genetic modification worldwide (Marchadier and Sigaud, 2005). Aspen and white poplar belong to the genus ***Populus*,** section *Populus*, family Salicaceae. They are dioecious, with male and female flowers (in catkins) occurring on separate trees.

Hybrid aspen grow even faster than European aspen and can reach a height of 20 meters in just 25 years (Hynynen and Karlsson, 2002). White poplar (*Populus alba* L.) have been introduced and widely used in commercial scale in a number of countries from Europe, North Africa, Near and Middle East during the second half of the twentieth century (Confalonieri *et al*., 2000). *Populus alba* is a species important for its resistance to disease, dryness, and sea breezes, and it has been widely used as a designated source of hybrid poplars (Chiba, 1971). Male clones in some *Populus* spp. tended to have longer internodes, higher plant dry weight and heavier wood as compared to the female clones (Khosla and Deol**,** 1984).

Poplar trees propagated through sucker shoots that arise from horizontal roots, and their rapid growth (Bradshaw *et al*., 2000). Aspen (*Populus tremula* and *P. tremuloides*) are, however, difficult to root from woody cuttings (Ahuja, 1983). Therefore, an efficient *in vitro* propagation system for aspen is highly needed. Recently, many techniques have been developed to detect and identify genetic variations of vegetatively propagated plants (Ruibal-Mendieta and Lints, 1998). Molecular techniques such as RAPD, for instance, are a quick and reliable method that could significantly detect small genetic changes in plants (Williams *et al*., 1990). RAPD analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Rani *et al*.

1995, Soniya *et al*. 2001). According to results of Kiss *et al*., (2001), Liu and Furnier (1993) and Lu *et al*., (2006) RAPDs are powerful for fingerprinting individuals in *Populus*. The main aim of the present study was to find an efficient and simple method of *in vitro* clonal propagation using shoot tip explant for producing large numbers of male (aspen, hybrid aspen and white poplar) trees plants for forest plantations and for further phytoremidiation studies and detect if it was somaclonal variations in *in vitro* derived plants with the aim to monitor the uniformity of plants multiplied *in vitro*.

2. Material and Methods

2.1 Plant material

The plant material used in my experiments was kindly provided by the Institute for Forest Genetics, Grosshansdorf, Germany. It had already been introduced into the culture so that in this paper I only conducted the procedures of shoot multiplication, rooting of shoots and their *ex vitro* acclimatization.

The following clones were used: W52 (*Populus tremula* L.), (*Populus alba* L.) and the hybrid aspen clone T89 (*Populus. tremula* L. x *Populus tremuloides* Michx.).

2.2 Culture medium

Shoot proliferation medium: Shoot tips and stem cuttings were used as explants for shoot multiplication. The explants were cut into small pieces (about 10-15 mm long). Then explants were inoculated aseptically on MS (Murashige and Skoog, 1962) and WPM woody plant media (Lloyd and McCown 1980). Media supplemented with either 6-Benzyladenopurine (BAP) or 2-isopentenyladenine (2iP) varying concentrations (0.0, 0.1, 0.2 and 0.4 mgl^{-1}) were prepared for shoot proliferation. The MS and WPM media were supplemented with 2% (w/v) sucrose and solidified with 0.6% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 1.2–1.3 kg/cm^2 pressure for 20 min. 400 ml culture vessels (containing 60 ml of medium) were used to maintain shoot cultures. Every jar contained two explants. The cultures were incubated under growth room conditions (22 ± 2 °C, 16 h photoperiod and light intensity of 4000 lux provided by cool white fluorescent lamps (Phillips TLM 40W/33RS). After 4 weeks of plant culture the data were recorded on number of shoots regenerated/explants, explants height and explants weight. Each treatment had ten replicates.

2.3 Rooting and transplantation

For root formation, shoots developed on MS and WPM multiplication media with different levels of BAP or 2iP were transferred and cultured in 400 ml jars containing 60 ml full strength MS basic medium supplemented with 2% (w/v) sucrose, 6 g/L agar with different concentrations of 3-indolebutyric acid (IBA), indole acetic acid (IAA) or naphthalene acetic acid (NAA) at (0.0, 0.1, 0.2 and 0.4 mgl^{-1}). Every jar was inoculated with four shoots (about 10-15 mm long). The shoots were maintained for 4 weeks under the same culture conditions as for development of shoots. After this time, the number of root, root length, plant weight and plant height were recorded. Each experiment was repeated three times and ten replications per treatment were taken into account. Rooted plantlets were taken out from the culture flasks and carefully washed with tap water to remove agar and they were transplanted to pots filled with sterilized mixture of compost and sand (1:1) and grown for 4 weeks in greenhouse conditions to determine the percentage of plants that survived. The potted plants were irrigated with distillation water twice a day for 4 weeks. Established plantlets were then transferred in 30 cm pots.

2.4 Genomic DNA extraction

DNA was extracted from fresh leaves of five *in vitro* derived plants 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 determend by NanoDrop 3300 (Thermo Scientific)

2.5 Random Amplified polymorphic DNA (RAPD)

RAPD analysis was performed in 25 µl volume reactions according to Wolff and Peters Van Rijn (1993). A reaction mixture (17.5 ng genomic DNA, 12.5 REDTaq ReadyMix (Sigma) [20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3mM $MgCl_2$, 0.002% gelatin, 0.4mM mix dNTP (dATP, dCTP, dGTP, dTTP) and 0.06 unit/ µl Taq DNA polymerase] and 0.4 p mole was prepared for each primer sufficient for all samples plus one negative control to 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 were used for RAPD analysis (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' and (A10) 5' CTGACCAGCC3' were used for RAPD.

2.6. 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 Shoot multiplication

From our previous work, we found that shoot multiplication from shoot tips yielded much higher number of shoots than from stem cuttings. Shoot tips proved an effective explant for micropropagation, and provided more than one explant. Therefore, we used shoot tips to find optimal culture conditions for proliferation. The growth rate of the plant material of three poplars under *in vitro* conditions was proportionate to the multiplication rate in both types of multiplication mediums.

Among two cytokinins tested, BAP proved to be more effective than 2iP for initiating shoots per explant (Table 1). From this experiment it was evident that best result obtained from WPM medium amended with BAP (0.1 and 0.2 mgl^{-1}) which produced 51.3 and 28.3 shoots/explant in the case of *Populus tremula* and *Populus tremula x Populus tremuloides*), respectively. In *Populus alba* BAP at 0.2 mgl^{-1} in MS medium seemed to be the best formula, since it facilitated a high rate of proliferation (14 shoots/explant) and shoot development without altering their elongation. Besides, no callus formation occurred at all BAP concentrations. Further increase in the concentrations of BAP had no effects on the number of multiple shoots of *Populus alba* and *Populus tremula* grown on MS or WPM (Table1). In contrast, with *Populus tremula x Populus tremuloides*, the elevated level of BA had stimulating effects on the total number of regenerated plantlets which grown on MS. Micropropagation is, thus, similar to the traditional method of vegetative propagation using cuttings has the distinct advantage of producing greater number of identical plants in a much shorter time (Barakat, 2008). The positive effect of BAP on the capacity to induce plant regeneration in *Populus alba* has been reported previously Pintaric' (2008) reported a range from 5.36 and 5.86 shoots per explant after 28–35 days from culture on MS and WPM media, respectively.

When the present results on this species were compared with the previous studies it was seen that each species needs appropriate culture medium, with appropriate concentration of growth regulators. *In vitro* shoot multiplication had been reported in *Populus spp* by using stem cutting as explant (Špela *et al*; 2009, Rahman and Rajora, 2001, Sung *et a*l., 1991).

3.2 Root formation

Data presented in Table (2) and Fig. (1D and E) show the effect of IAA, IBA and /or NAA on the *in vitro* rooting of proliferated shoots of the *Populus alba*, *Populus tremula* and *Populus tremula x Populus. tremuloides*. Root formation was 100% in response to the application of IAA, IBA and /or NAA to the culture media. The MS basic medium without IAA, IBA and /or NAA also revealed root formation. No callus formation was observed in the cultures. The MS medium with IBA (0.4 mgl^{-1}) provides the highest number of roots per explants (9.5 roots/explants in *Populus alba*)**,** Table (2). However, the highest number of roots per plant (7.0 and 6.4) were recorded in IAA (0.2 $mgl^{-1)}$ and NAA (0.1 mgl^{-1}) in the case of *Populus tremula* and *Populus tremula x Populus. tremuloides*, respectively**.** Further increase in the concentrations of IAA, IBA and /or NAA had no effects on the number of roots in the case of *Populus tremula.* The maximum root growth was recorded on MS medium with 0.1 mgl^{-1} IBA, 0.4 mgl^{-1} IAA and 0.1 mgl^{-1}NAA in the case of *Populus alba, Populus tremula* and *Populus tremula x Populus. tremuloides*, respectively. Although excessive auxin is commonly characterized by callus formation, no callus formation was detected in rooting stages of *Populus alba* and *Populus tremula.* The absence of callus at shoot base is an important observation

because it can be excluded that auxin treatments were supplied in improper high supplements (Nerman *et al*., 2009).

Table (1): The effect of medium type and cytokinin concentrations on shoot induction, elongation and plant fresh weight of *Populus alba, Populus tremula* and *Populus tremulax Populus tremuloides*

			P. alba			*P. tremula*			*P. tremula x P. tremuloides*		
Medium	Growth regulators mgl^{-1}		No of shoots/ explant	Length of the longest shoot (cm)	explant fresh weight (g)	No of shoots/ explant	Length of the longest shoot (cm)	explant fresh weight (g)	No of shoots/ explant	Length of the longest shoot (cm)	explant fresh weight (g)
	BA	2iP									
	0.0	0.0	1.5d	5.5b	0.39bcd	2.3d	8.7abc	1.5cd	5.0f	8.7ab	1.3ab
	0.1	0.0	7.75bc	3.25cd	0.30cd	16.8cd	7.6bcd	1.4cd	8.2ef	6.5bcd	1.2bc
MS	0.2	0.0	14.0a	4.67bc	0.51bcd	19.8c	11.1d	2.9ab	14.8cd	6.7bc	1.7a
	0.4	0.0	12.0ab	5.0bc	0.79bcd	15.7cd	6.2bcd	1.4cd	24.8ab	4.3def	0.84b
	0.0	0.1	1.0d	5.0bc	0.63bcd	4.7cd	4.7de	0.4d	5.7f	9.1a	0.93bcd
	0.0	0.2	1.0d	5.0bc	2.28a	8.0cd	8.2bc	2.0bc	5.4f	6.7ab	0.68cd
	0.0	0.4	1.0d	3.4cd	0.3cd	6.3cd	6.3bcd	1.0cd	2.7f	6.3bcde	0.7bcd
	0.0	0.0	1.0d	7.67a	1.21b	1.0d	11.3a	1.5cd	6.2f	5.2cde	0.6d
	0.1	0.0	11.25a	4.75bc	1.26b	51.3a	6.9bcd	1.7cd	16.4cd	3.6ef	0.8bcd
WPM			b								
	0.2	0.0	4.75cd	4.25bc	0.19d	21.3bc	6.3cde	1.6cd	28.3a	3.7ef	1.4ab
	0.4	0.0	8.25bc	2.38d	0.48bcd	33.5b	4.5e	1.1cd	19.3bc	2.8f	0.9bcd
	0.0	0.1	1.0d	5.33b	0.20cd	7.3cd	8.9abc	1.7bcd	12.3de	8.5cde	1.2b
	0.0	0.2	4.25cd	4.25bc	0.97bc	5.8cd	8.8abc	1.9bc	7.3ef	7.7ab	1.1bc
	0.0	0.4	1.0d	4.33bc	0.49bcd	4.0d	10.8a	3.1a	6.5f	4.9cde	0.9bcd

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 IAA, IBA and NAA on number of roots , root length, Plant height and Fresh weight of *Populus alba, Populus tremula* and *Populus tremula x Populus tremuloides*

			P. alba				*P. tremula*				*P. tremula x P. tremuloides*			
Growth regulators mgl^{-1}			Root number	Length of the Longest root (cm)	Plant height (cm)	Plant Fresh weight (g)	Root number	Length of the Longest root (cm))	Plant height (cm)	Plant Fresh weight (g))	Root number	Length of the Longest root (cm)	Plant height (cm)	Plant Fresh weight (g)
IAA	IBA	NAA												
0.0	0.0	0.0	2.6b	2.0c	4.5de	0.4b	2.2b	5.2a	7.5bc	1.5ab	0.7d	0.8bc	7.3ab	0.7bcd
0.1	0.0	0.0	3.25b	6.0ab	7.0bcd	0.5b	4.7ab	3.7a	7.9bc	0.6c	1.0d	0.4c	6.7ab	0.5cd
0.2	0.0	0.0	3.33b	6.0ab	5.5de	0.8ab	7.0a	5.6a	8.8ab	1.2b	2.0cd	0.7bc	5.0d	0.54bcd
0.4	0.0	0.0	5.0ab	8.25a	8.8abc	1.04ab	6.5a	5.3a	10.3a	1.5ab	0.2d	0.6c	6.4abcd	1.6a
0.0	0.1	0.0	6.0ab	2.0c	11.0a	1.5ab	3.3b	3.9a	9.0ab	1.3b	1.6cd	0.7c	7.1ab	0.82b
0.0	0.2	0.0	6.2ab	2.8bc	9.4ab	1.7a	3.5b	3.7a	7.1bc	1.9a	2.9c	1.1bc	6.4abcd	0.8bc
0.0	0.4	0.0	9.5a	2.5bc	5.5cde	1.7ab	3.3b	3.4a	7.3bc	1.0bc	4.4b	2.4a	6.5abc	0.45d
0.0	0.0	0.1	4.5ab	0.5c	3.7e	0.9ab	3.1b	3.2a	6.8c	1.2bc	6.4a	2.9a	5.5cd	0.7bcd
0.0	0.0	0.2	4.0b	2.0bc	5.0de	0.26b	3.4b	3.2a	10.2a	1.2bc	0.7d	0.3c	7.8a	0.45cd
0.0	0.0	0.4	3.30b	4.3bc	7.3bcd	1.0ab	2.8b	2.9a	6.5c	0.9bc	1.0cd	1.9ab	5.8bcd	0.6bcd

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

3.3 Acclimatization

The rooted plantlets were transferred to a mixture of Sand: Peat-moss (1:1, v/v) in the portrays for further development and hardening (Fig.1F). Humidity was maintained by covering the trays with rigid plastic cover and frequently spraying of water. Similar process of maintaining humidity was practiced for hardening (Jasrai *et al*., 1999; Rolf and Ricardo 1995). Plants transferred to the field after 4 weeks showed good growth. Almost 90% of the

regenerated plants of the three species survived and showed a vigorous growth.

3.4 RAPD analysis

Maintaining genetic stability in regenerated plants is essential for species conservation (Quiala *et al.* 2009). In order to confirm whether somaclonal variation was detectable in the regenerated plants. . RAPD was employed to analyze the genetic fidelity of five plants from each species randomly selected from *in vitro* derived plants as well as control donor plants. Two primers (A6 and A7) of the ten primers tested gave bands in RAPD analysis. Fig 2 shows the *in vitro* derived plants shared the same banding patterns as those of the donor plants, implying that they were possibly genetically identical to each other. Generally, it is important to make sure that the regenerants were genetically true-to-type of their donor plants with respect to genetic fidelity. In order to know if there is any aberration in the regenerated plants, the RAPD marker system was employed for this purpose. This system has been revealed to be a potential marker for distinguishing genetic variation (Piccioni *et a*l; 1997; Raimondi *et al*; 2001). These results confirm that all of the regenerants showed genetic stability in our regeneration system. Therefore, we can conclude that direct regeneration from shoot tip explants did not induce any somaclonal variation that has been depicted in other explants-mediated culture (Cassells and Curry, 2001; S˘us˘ek *et al*; 2002).

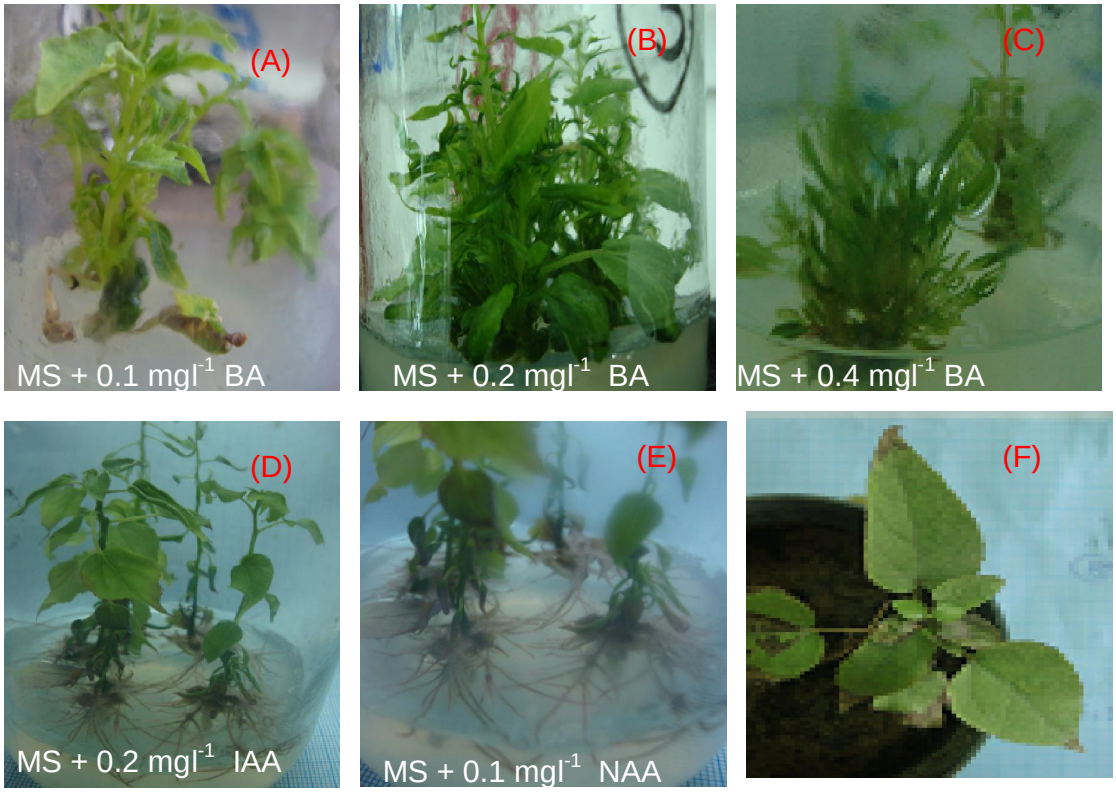

Figure (1): Modified micropropagation method of *Populus spp*., (A, B and C) Shoot regeneration of *Populus alba*, *Populus tremula* and *Populus tremula x Populus. Tremuloides* respectively. (D and E) Root formation of *Populus tremula* and *Populus tremula x Populus. Tremuloides* respectively, (F) transplantation of regenerated plantlets of *Populus tremula x Populus. Tremuloides* in plastic pots after eight weeks.

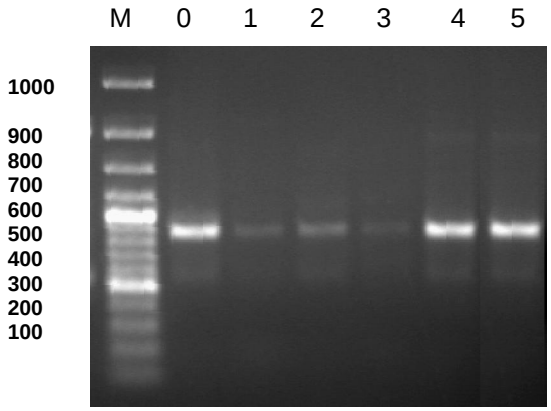

Fig. (2): RAPD pattern of aspen with primer A6 (1, 2, 3, 4 and 5) refer to sample plants, (0) is the control plant of aspen and (M) refers to the DNA marker

4. Conclusions

Plant regeneration through *in vitro* shoot tip is a rapid and simple method for clonal and mass propagation of male aspen, hybrid aspen and white poplar tree. Using this technique, regenerated plants were obtained within 4 weeks at an average of 51.3 and 28.3 shoots/explant in the case of *Populus tremula* and *Populus tremula x Populus tremuloides*), and (14.0 shoots/explant) in the case of *Populus alba.* In the current work I used two different media to induce shoots from poplar shoot tips explants. These media, with various combinations of cytokinin and auxin, triggered direct shoot organogenesis, for forest plantations and for further phytoremidiation studies. Maintaining genetic stability in regenerated plants is essential for conservation of endangered species.

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Correspondence author

Salah Khattab

Suez Canal University, Faculty of Agriculture, Department of Horticulture, Ismailia- Egypt

khattabfar@yahoo.de

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