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MOLECULAR CHARACTERIZATION AND *IN VITRO* PROPAGATION BEHAVIOR OF *POPULUS ALBA* L. PLANTS UNDER EFFECT OF SLOW GROWTH CONSERVATION

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Abstract

This work was aimed to identify the optimum osmotic stress condition that needed for *in vitro* short- term preservation protocol and detection of genetic variation of micropropagated *Populus alba* L. plants. The experiment was carried out to examine the micropropagation ability of *Populus alba* L. shootlets under the effect of different strengths of culture medium, sucrose and sorbitol (as osmotic stress was firstly used with *Populus alba* L. plants concentrations as slow growth treatments during various conservation periods (2, 4 and 6 months) to asses an *in vitro* protocol for conservation of the genetic diversity of the species. For maintaining survived shootlets (100%) for 4 months of conservation period, full or half strength of culture medium enriched with 30g/l sucrose could be used. Replacing sucrose by sorbitol (30 g/l) added to half strength of MS led to the highest percent of rooting after 6 months conservation. *In vitro* shootlet regrowth ability were obtained with full or half MS containing sucrose 30g/l followed by half MS containing sorbitol 30g/l. The highest rooting percent and roots number were recorded for *in vitro* resurvived shootlets which were cultured on half strength MS supplemented with sucrose 30g/l for 6 months conservation. Based on ISSR and SCoT marker analysis, the highest percentage value of similarity (81%) was between the shootlets cultured on half strength of MS medium supplemented with 30g/l sucrose and those cultured in the same medium supplemented with 30g/l sorbitol. The data obtained from this study revealed that ISSR and SCoT molecular markers have been proved to be more accurate in genetic diversity studies because of their high reproducibility and great power for the detection of polymorphism.

Key words : *Populus alba* L., *in vitro*, micropropagation, conservation, ISSR, SCoT.

Introduction

The great environmental and economic importance features of forest trees show the remarkable development (Groover *et al*., 2004).

In Egypt, *Papulus alba* can grow well under salinity level in compressed soil. The trees grow rapidly and make significant gains with low farming costs and service operations. The management plan aims to increase and preserve the genetic resources of poplar trees that can be used in wood and round wood to reduce imported timber. Thus, the tissue culture technique is an effective method to allow the rapid propagation of very little plant material for genetic diversity *ex situ* conservation (Fay, 1994 and Jain, 2012). The short and medium term preservation is among the different *in vitro* conservation techniques. For lengthen the subculture period, short-term conservation is applied by reducing the rate of plant growth. Moreover, the genetic stable regenerated and successful propagated plants from cultures are prerequisites for *in vitro* conservation (Rao, 2004).

In many woody plant species, preservation *in vitro* using slow growth can be achieved by reducing the nutrient concentration in the culture medium by 25 or 50% (Moriguchi and Yamaki, 1989) or supplementing osmoticum (Wilkins *et al*., 1988). Soluble carbohydrates (Sugar solutions) such as sucrose, mannitol, sorbitol and glucose are non- ionic substances which can produce low osmotic potentials and are different according to each plant type (Chawla, 2002). The disadvantages of repeated subcultures can be avoided using the safe, slow growth storage of culture *in vitro* that can be observed during

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their growth and return subculture *in vitro* (Withers, 1991). Jain (2012) described the advantages of *in vitro* conservation using osmotic stabilizers including high multiplication rate of plants.

Recently, with the progress in plant molecular biology, different molecular marker techniques such as RAPD, ISSR, AFLP, RFLP, functional gene targeted markers and start codon-targeted (SCoT) marker have been employed and widely used in evaluation of genetic diversity and phylogeny relationships between closely related cultivars and different plant species including orchids (Bhattacharyya *et al*., 2013) wheat (Etminan *et al*., 2016) and *Quercus infectoria* (Rahmani *et al*., 2015). SCoT is a novel molecular marker, offers a simple DNA-based marker. It includes the short conserved region in the plant genes surrounding the ATG (Rahmani *et al*., 2015) initiation of translation. SCoT has been successfully used to assess genetic diversity, identifies cultivars and detects map quantitative trait loci (QTL). No previous studies have been reported to assess the genotypic differences and relationships in treating *Populus alba* L. using SCoT markers. This study is the first one to employ SCoT as molecular markers. The main objective of this study evaluates the most suitable osmotic stabilizer material used for conserved genetic resources of economic important woody plants under laboratory condition and detected this genetically.

Materials and Methods

The experimental study was carried out during the years 2016 and 2017 on *Populus alba* L. at Tissue Culture Technique lab., Central laboratories, Department of Ornamental Plants and Woody Trees, National Research Centre (NRC), Egypt to examine some slow growth conditions (culture medium strength, sucrose type and concentration) affected the micropropagation ability during three consecutive periods (2, 4 and 6 months) to asses an *in vitro* protocol for conservation of the genetic diversity of the species.

Explant source and disinfection

The explants were taken from 2 years old seedlings in the nursery of Timber Trees Dept., Hort. Res. Inst., and Agriculture Research Center, Egypt.

The shoot tip explants were washed in soapy water using septal soap with shaking for 20 min., and then washed with running tap water for one hour. The explants were then immersed in different solutions under aseptic conditions in a laminar air flow cabinet as follows: 70% (v/v) commercial ethanol for 30 Sec., Clorox (NaOCl, 5.25% free chlorine) at concentrations of 15% (v/v) for 10 min., mercuric chloride (MC) at concentrations of 0.2 (w/v) for 5 min. The explants were rinsed three times with sterile distilled water after each disinfection treatment.

Culture media

The explants were cultured for conservation experiment on a basal MS medium (Murashige and Skoog, 1962) at two strengths (Full and half strengths) and two types of sugars (Sucrose and sorbitol) at two concentration (30 and 60 g/l) enriched with 0.5 mg/l of benzyl amino purine (BAP) and indole- 3- butyric acid at 0.1 mg/l. MS culture medium was solidified by the addition of 0.7% agar prior to autoclaving at 1.2 kg/cm2 for 15 min. The pH of the culture medium was adjusted to 5.8. Culturing was done in 200 ml glass jars containing 25 ml of the medium.

Incubation condition

Cultures were incubated for 2, 4 and 6 months under controlled conditions in the growth chamber. The incubation temperature was 24±2°C controlled by a "Power" air conditioner. The photoperiod was 16 hours

Table 1: ISSR and SCoT primers and their sequences used in this study.

Primer	Sequences (5' to 3')	Primer	Sequences (5' to 3')
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'	ISSR- 19	5'-HVHTCCTCCTCCTCCTCC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'	SCoT -1	5'-CAACAATGGCTACCACCA-3'
ISSR- 3	5'-ACACACACACACACACYT-3'	SCoT -2	5'-CAACAATGGCTACCACCC-3'
ISSR- 4	5'-ACACACACACACACACYG-3'	SCoT -3	5'-CAACAATGGCTACCACCG-3'
ISSR- 11	5'-ACACACACACACACACYA-3'	SCoT -4	5'-CAACAATGGCTACCACCT-3'
ISSR- 12	5'-ACACACACACACACACYC-3'	SCoT -5	5'-CAACAATGGCTACCACGA-3'
ISSR- 13	5'-AGAGAGAGAGAGAGAGYT-3'	SCoT -11	5'-AAGCAATGGCTACCACCA-3'
ISSR- 14	5'-CTCCTCCTCCTCCTCTT-3'	SCoT -12	5'-ACGACATGGCGACCAACG-3'
ISSR- 15	5'-CTCTCTCTCTCTCTCTRG-3'	SCoT -13	5'-ACGACATGGCGACCATCG-3'
ISSR- 16	5'-TCTCTCTCTCTCTCTCA-3'	SCoT -14	5'-ACGACATGGCGACCACGC-3'
ISSR- 18	5'-HVHCACACACACACACAT-3'	SCoT -16	5'-ACCATGGCTACCACCGAC-3'

light/8 hour darkness, controlled automatically. Illumination intensity was 3000 Lux from cool fluorescent lamps.

Experiment procedure

Eight conservation treatments for three periods (2, 4 and 6 months) were examined as follow:

T_1-Full strength of MS (FMS) + Sucrose 30g/l (control)

T_2-FMS + Sucrose 60g/l

T_3-FMS+ Sorbitol 30g/l

T_4-FMS+ Sorbitol 60g/l

T_5- Half strength of MS (HMS) + Sucrose 30g/l

T_6-HMS+ Sucrose 60g/l

T_7-HMS+ Sorbitol 30g/l

T_8-HMS+ Sorbitol 60g/l

At the end of each conservation period (2, 4 and 6 months), the morphological parameters of micropropagated shootlets [Survival percent of explants, number of shootlets/explant and length of shootlets (mm)] and roots [Rooting percent, the number of roots/shootlet and length of roots (mm)] were recorded then returned to subculture for two months to determine their regrowth ability by recording the same mentioned parameters after slow growth treatments.

Extraction of genomic DNA

Extraction of Genomic DNA was carried out using Qiagen Kit cat no # 69104 (Qiagen Sciences, Maryland, USA) according to the manufacturer's instruction manual

ISSR and SCoT reactions

Twelve ISSR and ten SCoT primers were used to detect the polymorphism of three samples of *Populus alba* L., these primers were synthesized by Metabion Corp., Germany. The primer sequences are listed in table 1. The PCR amplification reactions were carried out as mentioned by Adawy *et al*. (2002).Reactions were performed in 25 µl volume composed of 1x reaction buffer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of primer, 0.5 unit of *Taq* polymerase (Qiagen Ltd., Germany) and 50 ng of template DNA, in sterile distilled water.

ISSR and SCoT amplification

PCR amplification of the DNA was performed in a Perkin Elmer thermal cycler 9700. The temperature profile in the different cycles was as follows: an initial strand separation cycle at 94°C for 5 min followed by 40 cycles comprised of a denaturation step at 94°C for 1min, an annealing step at 45°C for 1 min and an extension step at 72°C for 1.5 min. The final cycle was a polymerization cycle for 7 min at 72°C. However, for SCoT, the same PCR condition was employed except an annealing step was at 50°C for 1 min**.** PCR products were mixed with 5 µl gel loading dye and resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) in 1 × TBE buffer at 120 volts. A 100bp DNA ladder was used as molecular size standard. PCR products were visualized under UV light and documented using a ™XR + Gel Documentation System (Bio-Rad).

Data analysis

The banding patterns generated by SCoT and ISSR marker analyses were compared to determine the genetic relatedness of control and two treatments. Clear and distinct amplification products were scored, as 1 for presence and 0 for absence of bands. Bands of the same mobility were scrod as identical. The genetic similarity coefficient between control and treatments were estimated according to Dice coefficient PAST program**.**

Statistical analysis

The data were analyzed using a randomized complete block design with 3 replicates per treatments. The treatments means were compared for significance by new multiple range tests at the 0.05% level of probability (Duncan D.B.,1955) using COSTATV-63.

Results and Discussion

Micropropagation ability

The *in vitro* cultures of *Populus alba* L. shootlets respond to different strengths of culture medium, sucrose and sorbitol concentrations as slow growth treatments during various conservation periods (2, 4 and 6 months) was shown in (table 2). The survival percent and number of shootlets formed per explants were significantly highest (100% and 8.07, respectively) when the explants were cultured on full strength of MS culture medium containing 30g/l sucrose while, pronounced decline in these values (40.42% and 2.09, respectively) were observed on full MS medium containing 60g/l sorbitol. Reducing medium strength to half strength enriched with 30g/l sucrose resulted in the longest shootlets (59.48 mm). It can noticed that both survival rate and number of shootlets formed per explants were reduced with increasing conservation period to 6 months, reached the lowest ones (56.13% and 3.86, respectively) while, the opposite trend was observed with length of shootlets which was maximum (47.75mm) after 6 months. Also, the interaction effect between slow growth treatments and conservation period was significant. During 2 months period of conservation, the survival percent was highest (100%) with full or half strength of MS supplemented with 30g/l sucrose or sorbitol

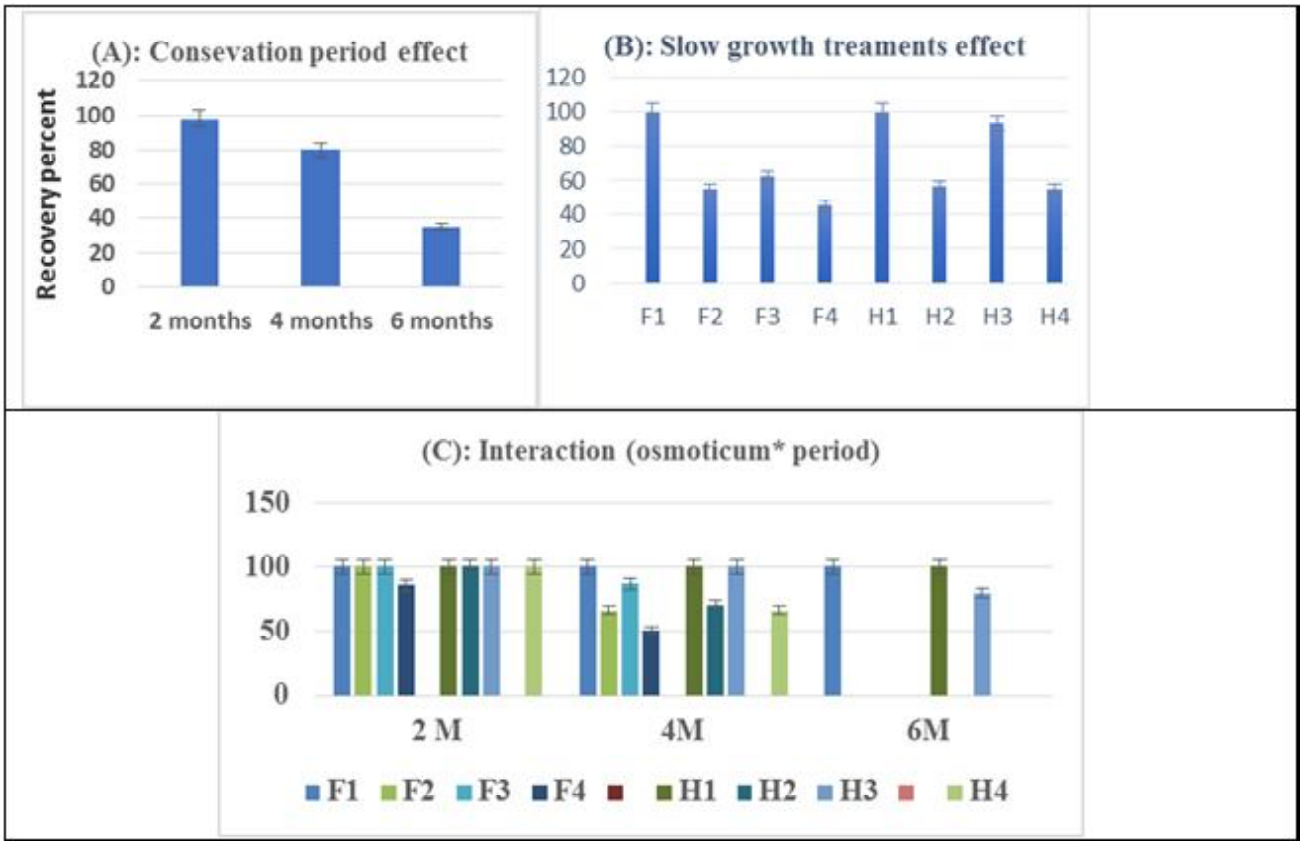

Fig. 1 : A, B, C : Effect of culture medium strength, sugar type and concentration on recovery ability of *Populus alba* L. after different conservation periods (2, 4 and 6 months).

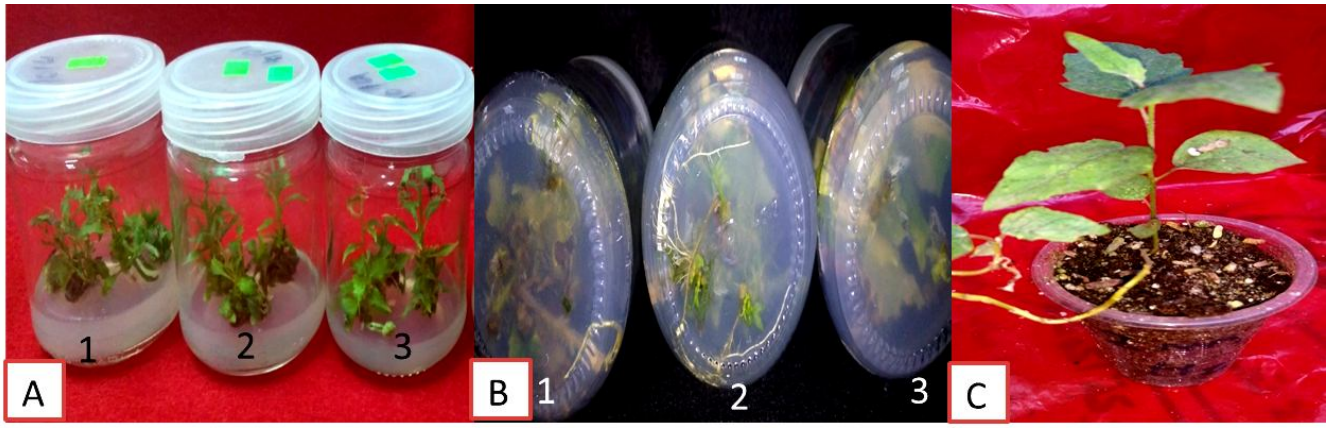

Fig. 2 : *In vitro* shoots propagation of *Populus alba*; (A): Full strength of MS (FMS) + Sucrose 30g/l , (B): Rooting of shootlets cultured in vitro ,(B,1): Half strength of MS (HMS) + Sucrose 30g/l ,(B,2) HMS+ Sorbitol 30g/l and (C): Acclimatized plants to greenhouse.

as well as half strength of MS containing 60 g/l sucrose. With increasing the conservation period to 4 months, full or half strength of culture medium enriched with 30g/l sucrose resulted in the same result for survival (100%). It seems from these results that reducing the sugar concentration to 30 g/l was better significantly for maintaining survived shootlets.

It seems from the present study that the highest recovery percent (100%) was obtained with full or half MS and reducing the sugar concentration to 30 g/l was better significantly for maintaining survived shootlets. The internal carbohydrate source is suggested to have an important role in morphogenesis of several woody species as *Populus alba* L. These results are in accordance with those of Lambardi *et al*. (2006), who mentioned the supplementation of osmotic and modifying the strength of culture medium suppressed the *in vitro* culture growth under normal conditions incubation. Moreover, Pruski *et*

Table 2 : Effect of culture medium strength, sugar type and concentration on micropropagation ability of *Populus alba* L. plants under different conservation periods (2, 4 and 6 months).

Character	Survival (%)				Number of shoots/explant				Length of shoots (mm)			
Period (Month) / Treatment	2 M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)
FMS+ Sucrose 30g/l	100	100	100	100	8.06	7.90	8.25	8.07	20.14	66.91	66.97	51.34
FMS+ Sucrose 60g/l	88.41	76.63	67.52	77.52	5.00	4.90	5.77	5.22	18.47	32.93	39.93	30.45
FMS+ Sorbitol 30g/l	100	66.54	32.77	66.44	4.28	3.06	3.1	3.48	13.17	20.10	19.94	17.74
FMS+ Sorbitol 60g/l	88.29	32.98	0.0	40.42	4.28	2.00	0.0	2.09	17.19	17.23	0.0	11.48
HMS+ Sucrose 30g/l	100	100	67.31	89.10	5.60	4.28	3.66	4.51	31.33	73.36	73.75	59.48
HMS+ Sucrose 60g/l	100	88.12	80.33	89.49	5.72	3.10	2.72	3.85	13.20	28.05	42.00	27.75
HMS+ Sorbitol 30g/l	100	88.79	68.22	85.67	4.00	4.34	5.35	4.57	14.14	40.62	94.74	49.85
HMS+ Sorbitol 60g/l	77.58	57.22	32.89	55.90	2.29	2.10	2.03	2.14	13.33	21.22	44.67	26.41
Period means (B)	94.28	76.29	56.13		4.91 a	3.96 b	3.86		17.62	37.55	47.75	
$LSD_{0.05}$	A= 1.82 B= 1.11 A*B= 3.36				A= 0.31 B= 0.19 A*B= 0.57				A= 1.06 B=0.63 A*B= 1.96			

FMS means full strength of MS culture medium. HMS means half strength of MS culture medium.

Table 3 : Effect of culture medium strength, sugar type and concentration on *in vitro* rooting ability of *Populus alba* L.under different conservation periods (2, 4 and 6 months).

Character	Rooting (%)				Number of roots/shootlet				Length of roots (mm)			
Period (Month) / Treatment	2 M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)
FMS+ Sucrose 30g/l	33.27	67.30	70	56.86	1.03	5.21	7.5 a	4.58	29.93	48.66	86.31	54.97
FMS+ Sucrose 60g/l	49.55	50	50.67	50.07	1.47	2.28	3.00	2.25	17.20	22.13	60.00	33.11
FMS+ Sorbitol 30g/l	30	33.27	55	39.42	1.00	2.03	4.09	2.38	41.72	50.10	94.17	62.00
FMS+ Sorbitol 60g/l	0	0	0	0	0	0	0	0	0	0	0	0
HMS+ Sucrose 30g/l	44.30	77.74	77	66.35	4.27	5.05	6.10	5.14	35.23	54.96	85.00	58.40
HMS+ Sucrose 60g/l	32.77	33.67	55	40.48	1.03	1.13	1.37	1.18	17.47	20.03	82.02	39.84
HMS+ Sorbitol 30g/l	34.43	34.37	87.89	52.23	2.06	3.72	5.33	3.71	17.00	33.06	83.22	44.43
HMS+ Sorbitol 60g/l	40.07	44.30	66.92	50.43	1.30	1.50	2.00	1.60	22.47	25.00	30.49	25.98
Periods mean (B)	33.05	42.58	57.81		1.52	2.62	3.67 a		22.63	31.74	65.15	
$LSD_{0.05}$	A= 1.79 B= 1.10 A*B= 3.31				A= 0.19 B= 0.12 A*B= 0.35				A= 1.36 B=0.83 A*B= 2.51			

FMS means full strength of MS culture medium. HMS means half strength of MS culture medium.

al (2000) recorded the effect of carbohydrates on growth and physiological processes of plants during all *in vitro* culture cycles, act as carbon source and osmotic regulators in the culture medium for tissues. The addition of osmotic such as sorbitol, to the medium has proved efficient for reducing growth rates of different plant species. These osmotic retard plant growth by reducing cell mineral uptake through differences in osmotic pressures thereby (Thompson *et al*., 1986). In addition, Ahmed and Anjum (2010) attributed the reducing survival of stored shoots for longer periods to nutrients depletion.

Root initiation

The tabulated data in table 3 indicate the *in vitro* root initiation of *Populus alba* L. shootlets under effect various osmotic treatments for conservation periods 2, 4

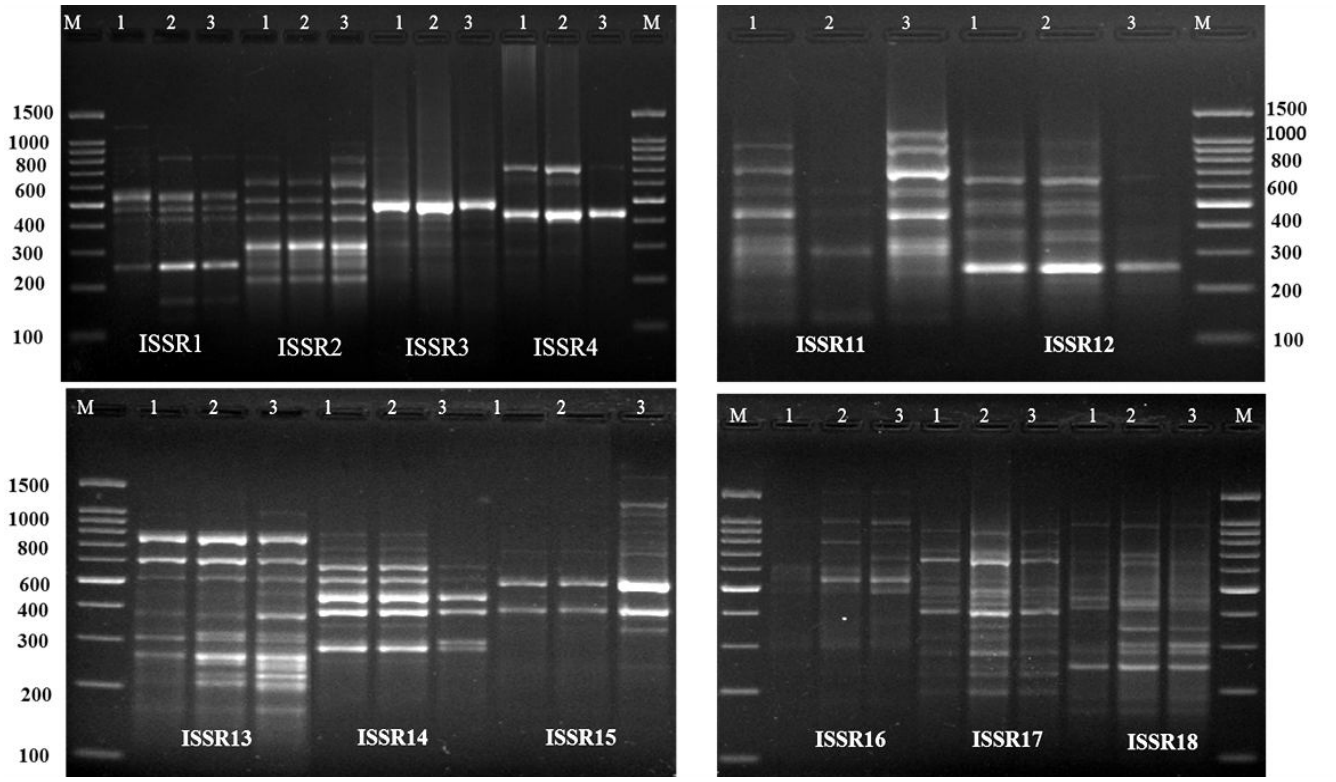

Fig. 3 : ISSR profile for the control and two treatment of *Populus alba* L. (Lane 1-3), M 100 bp DNA ladder; (1): Half strength of MS (HMS) + Sucrose 30g/l, (2): HMS+ Sorbitol 30g/l and (3):Full strength of MS (FMS) + Sucrose 30g/l.

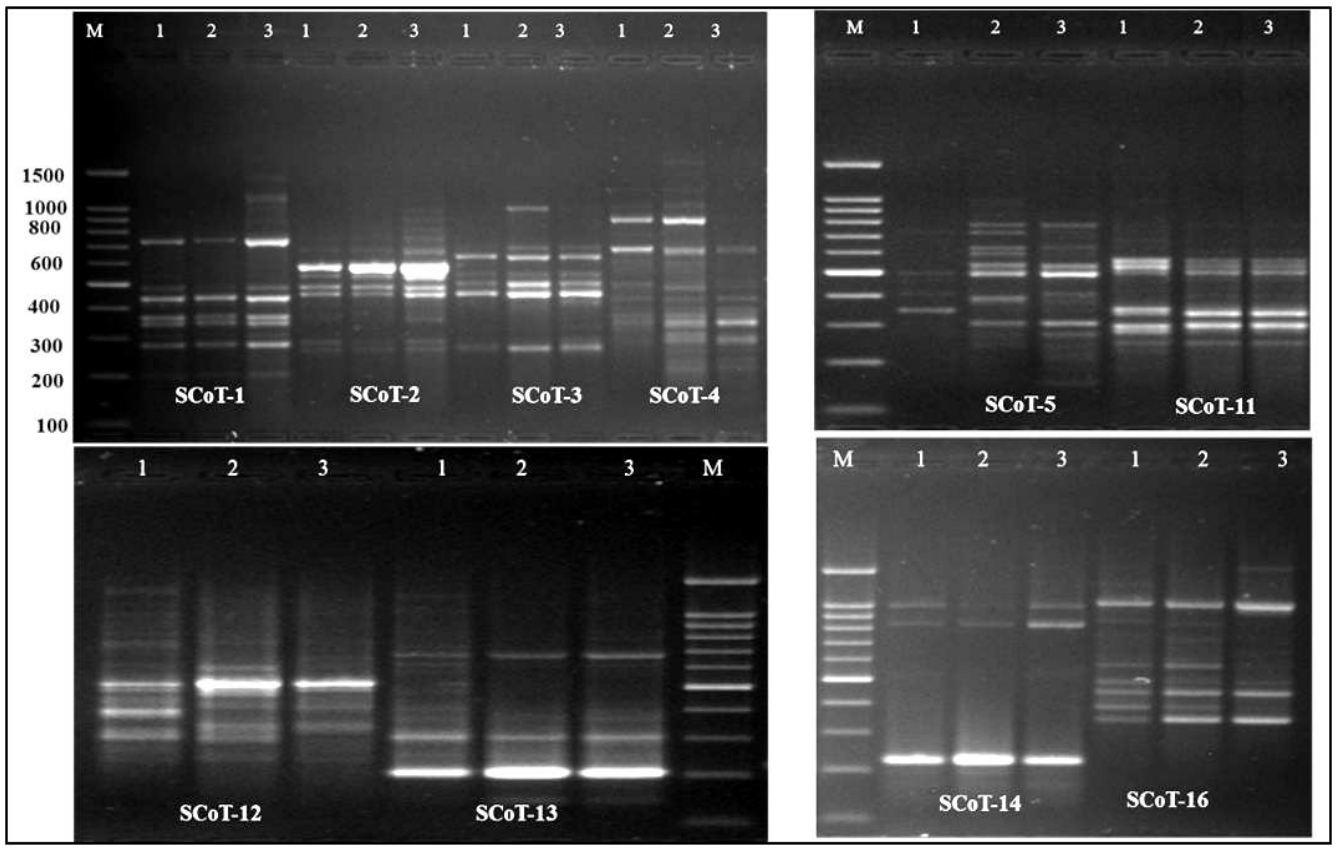

Fig. 4 : SCoT profile for the control and two treatment of *Populus alba* L. (Lane 1-3), M 100 bp DNA ladder; (1): Half strength of MS (HMS) + Sucrose 30g/l, (2): HMS+ Sorbitol 30g/l and (3):Full strength of MS (FMS),Sucrose 30g/l.

and 6 months. It can be observed that using half strength of MS medium supplemented with lower concentration of sucrose caused the highest rooting percent and roots number per shootlets (66.35% and 5.14, respectively). However, the interaction significant effect of osmotic treatments and conservation period showed that replacing sucrose by sorbitol (30 g/l) added to half strength of MS led to the highest percent of rooting (87.89%) after 6 months conservation. Full MS supplemented with sucrose 30 g/l gave the highest number of roots (7.5) for 6 months conservation. The longest roots (94.17 mm.) were obtained with sorbitol (30 g/l) added to full MS strength. It can notice also, that all rooting characters were maximum at longest conservation period (6months). The confirmed results by Bertrand-Desbrunais *et al*. (1992) reported that low sucrose at low temperature promotes root initiation. George *et al*. (2013) observed the interaction effect of culture medium strength and incubation period on root induction that was accelerated after 8 months on full strength MS compared to ½ and ¼ strength MS.

Regrowth ability

Micopropagated shootlets were transferred into the culture multiplication medium after each conservation period (2, 4 and 6 months) to detect their regrowth ability under effect of slow growth treatments. The results (fig.1: A, B, C) declared that the recovery ability of explants was decreased with increasing the conservation period. The highest recovery percent (100%) was obtained with full or half MS containing sucrose 30g/l followed by half MS containing sorbitol 30g/l (93.27%) as well as, *in vitro* shoots regrowth (shootlets number and length) was best on these treatments (table 4).The same mentioned treatments had promotion significant effect (high recovery percent after 6 months) when interacted with conservation period. While, other treatments caused explants dying. In this share, Hana *et al*. (2012) found that survival and re-growth of plantlets were decreased as the concentration of sucrose increased in the medium. Also, Edirisinghe *et al*. (2017) could slow the explants growth through culture medium salt concentration, sucrose and sorbitol thus, half strength of MS containing 15g/l sucrose and 1% sorbitol was favored for conservation of banana explants. They mentioned that lowering the minerals in the culture medium reduced the growth rate and this might attributed to low availability of nutrients in the medium.

Rooting ability

The demonstrated data in table 5 revealed that the rooting percent and roots number of *in vitro* shootlets

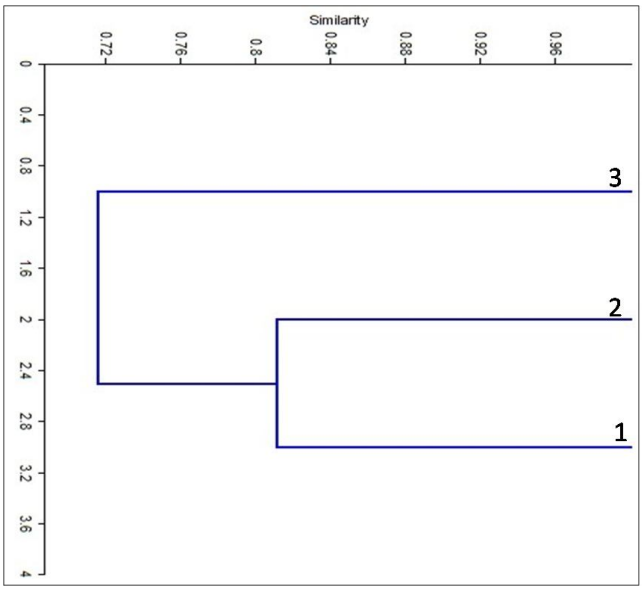

Fig. 5 : UPGMA Dendrogram indicating the genetic relationships between control and two treatment of *Populus alba* generated based on ISSR and SCoT markers, (1): Half strength of MS (HMS) + Sucrose 30g/l, (2): HMS + Sorbitol 30g/l and (3): Full strength of MS (FMS),Sucrose 30g/l.

cultured on half strength MS supplemented with sucrose 30g/l for 6 months conservation were highest (35.18% and 1.21) significantly as compared to other treatments. While full strength MS supplemented with sucrose 30g/l for 6 months resulted the longest roots (24.18 mm). Opposite results were obtained by Hana *et al*. (2012), observed that no roots developed during the storage period of *Capparis spinosa* plant and this could be due to the presence of osmoticum such sucrose, sorbitol, mannitol or glucose.

Molecular characterization of micropropagated of *Populus alba* L. using ISSR and SCoT molecular marker

In this study, two types of molecular marker were successfully used; inter-simple sequence repeats (ISSR) and Start Codon Targeted (SCoT) for genetic diversity and relationship analysis of control explants propagated on (FMS + Sucrose 30g/l) and two treatments of *Populus alba* L. 12 ISSR and 10 SCoT primers were used. The first amplified 117 bands and the second amplified 102 loci at different molecular length.

ISSR and SCoT analysis

Populus alba L. explants propagated on (FMS) + Sucrose 30g/l (control) and only the best two treatments which were able to propagate for six months were subjected to ISSR analysis. Table 6 and fig. 3 shows that 36 polymorphic bands and 13 unique bands were

Table 4 : Effect of culture medium strength, sugar type and concentration on regrowth ability of *Populus alba* L. after different conservation periods (2, 4 and 6 months).

Character	Number of shoots/explant				Length of shoots (mm)			
Period (Month) / Treatment	2M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)
F: FMS+ Sucrose 30g/l	8.31	5.47	4.70	6.10	13.51	22.30	34.22	23.34
F2: FMS+ Sucrose 60g/l	9.97	3.93	0	4.63	18.55	10.12	0	9.56
F3: MS+ Sorbitol 30g/l	6.60	3.57	0	3.39	22.09	12.92	0	11.67
F4: MS+ Sorbitol 60g/l	7.90	6.06	0	4.65	17.99	8.28	0	8.75
H1: MS+ Sucrose 30g/l	11.5	6.38	3.61	7.17	30.11	26.09	16.10	24.10
H2: MS+ Sucrose 60g/l	9.21	7.10	0	5.43	25.31	19.47	0	14.93
H3: MS+ Sorbitol 30g/l	6.34	6.06	5.02	5.81	23.67	21.34	20.58	21.86
H4: MS+ Sorbitol 60g/l	6.03	4.03	0	3.36	20.61	19.74	0	13.45
Periods mean (B)	8.21	5.33	1.67		21.48	17.53	8.86	
$LSD_{0.05}$	A= 0.37 B= 0.23 A*B= 0.69				A= 0.78 B= 0.48 A*B= 1.44			

FMS means full strength of MS culture medium. HMS means half strength of MS culture medium.

Table 5 : Effect of culture medium strength, sugar type and concentration on *in vitro* rooting ability of resurvived *Populus alba* plantletss after different conservation periods (2, 4 and 6 months).

Period (Month) / Treatment	2 M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)	2M	4M	6M	Treat. means (A)
FMS+ Sucrose 30g/l	13.49	19.36	29.97	20.93 b	0.6	1.03	2.09	1.24	14.43	20.28	24.18	19.63
FMS+ Sucrose 60g/l	22.09	0	0	7.36	0.67	0	0	0.22	25	0	0	8.33
FMS+ Sorbitol 30g/l	0	0	0	0	0	0	0	0	0	0	0	0
FMS+ Sorbitol 60g/l	0	0	0	0	0	0	0	0	0	0	0	0
HMS+ Sucrose 30g/l	22.33	27.13	35.18	28.21	1.64	1.0	1.0	1.21	11.38	15.17	24.89	17.15
HMS+ Sucrose 60g/l	16.50	10.41	0	8.97	0.75	0.30	0	0.35	22.13	12.68	0	11.60
HMS+ Sorbitol 30g/l	33.17	21.40	0	18.19	2.77	1.02	0	1.26	13.98	8	0	7.33
HMS+ Sorbitol 60g/l	0	0	0	0	0	0	0	0	0	0	0	0
Periods mean (B)	13.45	9.79	8.14		0.80	0.42	0.39		10.87	7.02	6.13	
$LSD_{0.05}$	A= 1.59 B= 0.97 A*B= 2.93				A= 0.07 B= 0.04 A*B= 0.12				A= 0.68 B= 0.42 A*B= 1.26			

FMS means full strength of MS culture medium. HMS means half strength of MS culture medium.

amplified by ISSR primers. The obtained results showed that ISSR-18 and ISSR-19 have amplified the maximum number of bands (14) and indicated that primers ISSR-18 and ISSR-19 more abundant in the *Populus alba* L. genome than the ISSR-4. The highest percentage of polymorphism (80%) was detected with the primer ISSR-12, these data could be used for marker assisted selection. It has been successfully utilized for reflecting the genetic diversity and revealing a remarkable molecular discrimination between the control and the two selected treatments of *Populus alba* L.

SCoT as a second type of molecular marker was used. Ten SCoT primers succeeded to amplify a total of 102 bands, 31 bands of them were polymorphic and 25 were unique. 14 bands and the highest polymorphism 93% were achieved by SCoT -5 (table 7 and fig. 4). Dendrogram was conducted based on ISSR and SCoT markers analysis. Table 8 shows that the similarity matrix

Table 6 : ISSR primers names and statistical analysis.

No	Name of primer	Monomorphic bands	Polymorphic bands	Number of unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	ISSR-1	4	3	2	9	56	123-1213	0.7
2	ISSR-2	7	1	0	8	13	202-842	0.1
3	ISSR-3	6	0	0	6	0	264-543	0.1
4	ISSR-4	3	2	0	5	40	264-755	0.9
5	ISSR-11	3	6	1	10	70	125-1196	0.7
6	ISSR-12	2	8	0	10	80	197-950	0.7
7	ISSR-13	9	3	1	13	31	155-297	0.9
8	ISSR-14	6	4	0	10	40	268-297	0.9
9	ISSR-15	4	0	5	9	56	230-333	0.6
10	ISSR-16	4	2	3	9	56	273-510	0.7
11	ISSR-18	10	4	0	14	29	186-510	0.9
12	ISSR-19	10	3	1	14	29	159-510	0.9
Total		68	36	13	117			8.1
Average		5.6	3	1.08	9.75	41.6		0.675

Table 7 : SCoT primers names and statistical analysis.

No	Name of primer	Monomorphic bands	Polymorphic bands	Number of unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	SCoT-1	7	0	4	11	36	202-1360	0.8
2	SCoT -2	7	1	3	11	40	258-868	0.8
3	SCoT -3	5	1	3	9	45	258—1036	0.7
4	SCoT -4	5	7	1	13	62	202-1148	0.8
5	SCoT -5	1	7	6	14	93	140-816	0.6
6	SCoT- 11	5	2	2	9	44	201-346	0.8
7	SCoT-12	4	7	1	12	67	546-816	0.8
8	SCoT-13	5	2	2	9	44	189-346	0.8
9	SCoT-14	3	1	0	4	25	192-1026	0.9
10	SCoT-16	4	3	3	10	60	330-1520	0.7
Total		46	31	25	102			7.7
Average		4.6	3.1	2.5	10.2	51.6		0.77

Table 8 : The Similarity matrix based on of ISSR and SCoT markers analysis of two *Populus alba* treatment and control.

Similarity matrix between control and two treatment t of *Populus alba L.*			
Name	**HMS+ Sucrose 30g/l**	**HMS+ Sorbitol 30g/l**	**FMS+ Sucrose 30g/l**
HMS+ Sucrose 30g/l	100		
HMS+ Sorbitol 30g/l	81	100	
FMS+ Sucrose 30g/l	70	**75**	100

of control and two treatments of *Populus alba* L. The highest percentage of similarity (81%) was recorded between samples from half strength of MS supplemented with 30g/l sucrose and those of half strength MS supplemented with 30g/l sorbitol. Fig. 5 revealed that two distinct clusters were generated, cluster 1 includes full strength of MS supplemented 30g/l sucrose and cluster 2 includes half strength of MS supplemented with 30g/l sucrose and half strength MS supplemented with 30g/l sorbitol. Cluster analysis also revealed low genetic variation among these treatments.

In the current study, ISSR and SCoT molecular markers were used to reflect the genetic diversity between control and treatment in propagated *Populus alba* L. plants under osmotic stress data shows in tables 6, 7 and 8 and fig. 3, 4 and 5, these results in agreement with Etminan *et al*. (2018), who also used twenty-one and twenty selected ISSR and SCoT primers of molecular Markers to study the genetic diversity and relationship analysis of nine *Salvia* species. The results showed that both techniques were effective to assess the genetic diversity; SCoT markers can be used as a reliable and informative technique for evaluation of genetic diversity and relationships among *Salvia* species in Iranian wild Salvia populations. Another study, Yousefi *et al*. (2015) also used an ISSR marker to differentiate between fourteen accessions of *Thymus* species including *T. daenensis*, *T. kotschyanus* and *T. vulgaris* have been genetically fingerprinted by Inter simple sequence repeat (ISSR) markers. The 20 ISSR primers amplified 334 fragments of which 325 were polymorphic. Number of amplified fragments ranged from 4 to 30 and their size was 200-2800 bps. The analyzed ISSR markers created sufficient polymorphism and reproducible fingerprinting profiles and provided a powerful and reliable molecular tool for detecting genetic variation and relationships. Bhattacharyya *et al*. (2013) used Start Codon Targeted (SCoT) marker to reveal the genetic diversity of *Dendrobium nobile* L indl., an endangered medicinal orchid species collected from different parts of Northeast India, suggested the effectiveness of SCoT marker system to estimate the genetic diversity of *D. nobile* and that it can be seen as a preliminary point for future research on the population and evolutionary genetics of this endangered orchid species of medicinal importance.

Conclusion

Populus alba L. explants could be conserved *in vitro* without serious losses on full or half MS containing sucrose 30g/l followed by half MS containing sorbitol 30g/l. Increased sugar concentration led to loss explants cultures. ISSR and SCoT molecular marker were the most powerful and accurate techniques to evaluate genetic diversity and relationship analysis between different treatments.

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