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Regeneration and genetic conservation of the endangered *Astragalus trigonus* plant

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Astragalus trigonus as an important medical plant had been used as traditional medicine, which improve the resistance to viral infections, hepatoprotective, heart tonic, nephritis and diabetes. For the purpose of genetic conservation of the *Astragalus trigonus*, an efficient and repeatable *in vitro* propagation system from the hypocotyl explants was developed. Murashige and Skoog medium (MS) included various concentration and /or of a-naphthaleneacetic acid (NAA), benzylamino-purine (BAP), thidiazuron (TDZ) and kinetin (KIN) was used for shoot initiation. The number of shoot/explants was high (14 shoots) in the presence of 0.5 mg/l NAA and 0.5 mg/l BAP. Elongated shoots were successfully rooted in MS medium supplemented with 1.0 mg/l NAA. High similarity percentage was detected among the *in vitro* regenerated plant with the mother plants as reveled by the molecular marker analysis; random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR). The data obtained from both of RAPD and ISSR analyses showed 35.2 % polymorphism among the regenerated and their mother plants. The protocol in this study can be applied to conserve the endangered *Astragalus trigonus*.

Keywords: ***Astragalus trigonus*, *in vitro* regeneration, molecular markers, genetic conservation.**

INTRODUCTION

Plants consider one of the main source of products used in the pharmaceutical, flavor, fragrance, and pesticide industries (Flores *et al*., 1999). Regrettably, the increasing human population led to extinct of many plant species and destruction of their habitats as well as their commercial photochemicals over-collection (Flores *et al*., 1999). Consequently, different countries developed different strategies to memorize the important species, mostly the endangered and rare plant species (Flores *et al*., 1999). Tissue culture techniques presented efficient and reasonable alternative for plant *in vitro* propagation (Vicente *et al*., 2011). Many different methods for *in vitro* culture were used in the recent years as important protocol for *Astragalus trigonus* germplasm preservation and mass multiplication (Vicente *et al*., 2011). The obtaining of true to type plants is the main objective of tissue culture, however, this technique can led to some genetic aberration usually known as ‘somaclonal variations’. So it’s very important to screen the plantlets obtained from micropropagation for their clonal fidelity (Goyal *et al*., 2015). From many years the *Astragalus* species are known to be good source of immune stimulant, hepatoprotective and activation agent (Tang and Eisenbr 1992). This genus included more than 2000 species, so it considered from the largest genus of the family Leguminosae (Akan *et al*., 2008). *Astragalus* roots are used in China as

traditional medicine, due to their antioxidant, antidiabetic and antineoplastic properties of their compounds (polysaccharides, saponins and isoflavonoids) (Bedir *et al.* 2000). Somaclonal variation is sort of a genetics spontaneous change that can produced by the *in vitro* regeneration of plants (Larkin and Scowcroft, 1981). RAPD and ISSR, different types of molecular marker, are dependable in detect the genetic material variation (Othmani *et al.,* 2010). RAPD technique was used for the determination of the genetic variation (Othmani *et al*., 2010). This investigation was aimed to develop an efficient tissue culture system for the endemic *A. trigonus* plant in order to ensure plants conservation and to produce the secondary metabolite products.

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MATERIALS AND METHODS

Plant material

The wild population of Northern West Coast; Matrouh, Ras El-Hekma, Egypt were used to gathered the seeds of *A.trigonus* **(Fig. 1 A).**

Seed sterilization and germination

The surface of the seeds were sterilized by solution of 0.5% (v/v) sodium hypochloride (NaOCl) for 20 min, after that the seeds were washed four times with d H_2O. For seeds germination, half-strength MS medium (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar were used. Thirty-day old seedlings were used for hypocotyl provided **(Fig. 1 B).**

Shoot induction and multiplication

MS medium with NAA, BAP, TDZ and KIN combination and MS without plant growth regulators (as control) have been used for shoot induction (Table 1). Media were containing 30 g/l sucrose, adjusted to pH 5.8 before gelling with 8% agar. Survived explants percentage, explants forming shoots percentage, mean number of shoots/explant and mean length of shoots were recorded after one month of culturing on the suitable medium.

For multiplication, the explants were sub-cultured five times on MS medium containing 0.5 mg/l NAA+0.5 mg/l BAP (the suitable medium for the establishment stage) after 25-30 days. Shoots obtained in each subculture were divided into clusters of approximately 4-5 small shoots and subcultured on the fresh medium. Mean number and length of axillary shoots/explant were recorded after 6 weeks of each subculture

Table 1: The different combinations of plant growth regulators used in the establishment of shoot induction.

Combination	Concentrations of plant growth regulators (mg/l)			
	NAA	BAP	TDZ	KIN
1	0.0	0.0	0.0	0.0
2	0.0	1.0	0.0	0.5
3	0.0	2.0	0.0	0.5
4	0.0	3.0	0.0	0.5
5	0.0	4.0	0.0	0.5
6	0.5	0.5	0.0	0.0
7	0.5	1.0	0.0	0.0
8	0.5	1.5	0.0	0.0
9	0.5	2.0	0.0	0.0
10	0.0	0.0	0.5	0.5
11	0.0	0.0	1.0	0.5
12	0.0	0.0	1.5	0.5
13	0.0	0.0	2.0	0.5

Rooting and acclimatization

The 2-3 cm shoots were transferred to MS medium supplemented with different concentration (0, 0.5, 1.0 and 2.0 mg/l) of NAA or IBA for root induction. After one month, the root percentage was calculated and plantlets were transferred into pots filled with a mixture of sand and peat moss (1:1 v/v). Pots were wrapped with transparent polyethylene bags and placed in a greenhouse. Seven days later, the covers were removed gradually within thirty days. The survived transplants percentage was recorded.

Studying the genetic variation among the regenerated plantlets and their mother plants.

Detection of genetic variation among *A. trigonus* and the regenerated plantlets was conducted using RAPD and ISSR marker experiments. Rogers and Bendich (1985) method were used for DNA isolation. A set of 18 RAPD 10-mer primers (Operon Technology, Inc., Alameda, CA, USA) and 7 ISSR primers (procured from British Columbia University) were used in the detection of somaclonal variation **(Table 2)**.

RAPD and ISSR amplification condition

RAPD and ISSR amplification were conducted using the same conditions. A reaction mixture of 20 µl containing 10 ng DNA, 200 mM dNTPs, 1 mM primer, 0.5 units of Red Hot *Taq* polymerase (AB-gene Housse, UK) and 2 µl of 10-X *Taq* polymerase buffer (AB-gene Housse, UK) was used. The PCR profile was as follows: Preheat at 94 ^{o}C for 5 min, followed by 40 cycles of 1 min at

.

Table (2): RAPD and ISSR primers name and sequence used to study the genetic variation between the mother plant and there generated plantlets

RAPD primers					
	Primer name	**Sequence (5` - 3`)**		**Primer name**	**Sequence (5` - 3`)**
1	OPA.18	AGGTGACCGT	10	OPG.14	GGATGAGACC
2	OPB.04	GGACTGGAGT	11	OPF.04	GGTGATCAGG
3	OPC.02	GTGAGGCGTC	12	OPK.04	CCGCCCAAAC
4	OPC.11	AAAGCTGCGG	13	OPK.10	GTGCAACGTG
5	OPD.05	TGAGCGGACA	14	OPK.12	TGGCCCTCAC
6	OPD.08	GTGTGCCCCA	15	OPP.09	GTGGTCCGCA
7	OPD.20	ACCCGGTCAC	16	OPM.15	GACCTACCAC
8	OPE.04	GTGACATGCC	17	OPL.04	GACTGCACAC
9	OPE.08	TCACCACGGT	18	OPQ.12	AGTAGGGCAC
ISSR primers					
	Primer name	**Sequence (5` - 3`)**		**Primer name**	**Sequence (5` - 3`)**
1	UBC 08	AGCGAGAGAGACAGAGC	5	UBC 62	AGCAGCAGCAGCAGCAGC
2	UBC 24	TCTCTCTCTCTCTCTCG	6	UBC 67	GGCGGCGGCGGCGGCGGC
3	UBC 26	ACACACACACACACACC	7	UBC 73	GACAGACAGACAGACA
4	UBC 28	TGTGTGTGTGTGTGTGA			

94 ºC; 1 min at 32, 42 ºC (for RAPD and ISSR, respectively) and 2 min at 72 ºC and a final extension at 72 ºC for 10 min. 2% (w/v) agarose gel in 1X TAE buffer were used to separate the PCR product and visualized by staining with ethidium bromide with 1 kb DNA ladder (Bio Basic, Canada).

Statistical analysis

The experiments were repeated three time and treatments consisted of at least 10 replicates. Values were expressed as mean of ± standard error. Data were subjected to completely randomized design and analyzed using analysis of variance (ANOVA). The means were compared using Duncan's multiple range tests ($P \leq 0.05$) using CoStat 6.4 software (CoHort Software, Pacific Grove USA) (Duncan, 1955).

RESULTS AND DISCUSSION

In vitro propagation

Culture establishment and multiplication of shoots

For culture establishment, explants were transferred to MS medium include 0.5 mg/l of NAA and different concentration of BAP (0.0, 1.0, 1.5, 2.0, 3.0 and 4.0 mg/l) and combinations or separates of TDZ (0.5, 1.0, 1.5 and 2.0 mg/l) and KIN (0.5 mg/l). The MS medium containing 0.5 mg/l BAP and 0.5 mg/l NAA showed the highest mean number of shoots (14 shoots/explant) and also the highest mean length of shoots (10 cm) as shown in **Table (3)**. Followed by the concentration of 1.0 mg/l BAP with the same NAA concentration, the mean number and length of shoots culture (11 shoots of 9.5 cm) were decreased (**Fig. 1 C).** Shoot initiation started after 13 days from culturing and the multiple shooting formation started after 20 days from subculturing.

In the present study, the combination between 0.5 mg/l BAP and 0.5 mg/l NAA was examined for shoot multiplication for five successive subcultures; each subculture took 21-30 days. The results showed that the second and third subcultures achieved the highest mean number of shoots (18 shoots), but the second subculture recorded the highest obtained mean length of shoot of 12 cm, as showed in **Table (4).**

Rooting and acclimatization

The shoots were evaluated for inducing roots on MS medium containing IBA (0.0, 0.5, 1.0 and 2.0 mg/l) or NAA (0.0, 0.5, 1.0 and 2.0 mg/l), individually or in combination in addition to MS medium without auxin. Rooting percentage varied and ranged between 73.3 and 100%. MS medium include 1.0 mg/l IBA registered 100% rooting, also this concentration gave the highest mean root number (5.4 roots), in addition to the maximum shoot height of 12 cm. Among the two tested auxins, IBA was more favorable for the induction of root than NAA **(Table 5 and Fig. 1 D).** The obtained results are in agreement with those on *Astragalus membranaceus* (Hou and Jia, 2004).

Table 3: *In vitro* establishment of *Astragalus trigonus* cultured on MS medium supplemented with NAA, BAP, TDZ and KIN.

Combinations	Concentrations of plant growth regulators (mg/l)				% of SE	% of EFG	MNS / explant	MSL (cm)
	NAA	BAP	TDZ	KIN				
1	0.0	0.0	0.0	0.0	100	100	3.1 j	8.2 c
2	0.0	1.0	0.0	0.5	100	100	9.5 c	9.0 abc
3	0.0	2.0	0.0	0.5	100	100	7.0 g	8.8 bc
4	0.0	3.0	0.0	0.5	100	100	4.8 h	8.1 c
5	0.0	4.0	0.0	0.5	100	100	3.5 ij	8.0 c
6	0.5	0.5	0.0	0.0	100	100	14.0 a	10.0 a
7	0.5	1.0	0.0	0.0	100	100	11.0 b	9.5 ab
8	0.5	1.5	0.0	0.0	100	100	9.0 cd	9.0 abc
9	0.5	2.0	0.0	0.0	100	100	8.0 ef	9.5 ab
10	0.0	0.0	0.5	0.5	100	100	8.5 de	9.5 ab
11	0.0	0.0	1.0	0.5	100	100	7.1 fg	8.9 bc
12	0.0	0.0	1.5	0.5	100	100	6.7 g	8.5 bc
13	0.0	0.0	2.0	0.5	100	100	4.0 hi	8.0 c

MNS: Mean number of shoots, MLS: Mean length of shoots, SE: survival explants and EFG: Explants Forming Growth. (Results were taken after 6 weeks of culture

Table 4: *In vitro* multiplication of *Astragalus trigonus* cultured on MS medium supplemented with 0.5 mg/l NAA + 0.5 mg/l BAP.

Subculture	0.5 mg/l NAA + 0.5 mg/l BAP	
	MNS	MLS
1st	15 b	10 bc
2nd	18 a	12 a
3rd	18 a	9 c
4th	12 c	11 ab
5th	11 c	9 c

MNS: Mean number of shoots and MLS: Mean length of shoots. (Results were taken after 6 weeks of culture)

Table (5): *In vitro* rooting of *Astragalus trigonus* cultured on MS medium supplemented with auxins.

IBA	NAA	Rooting %	MNR/Shoot	MSH
0.0	0.0	73.3 e	3.0 d	10.5 bcd
0.0	0.5	86.6 c	4.0 cd	10.0 d
0.0	1.0	73.3 e	4.0 cd	11.2 abcd
0.0	2.0	93.0 b	4.0 cd	11.7 ab
0.5	0.0	80.0 d	4.8 abc	11.5 abc
0.5	0.5	86.6 c	4.5 abc	10.3 cd
0.5	1.0	86.6 c	5.0 abc	12.0 a
0.5	2.0	93.3 b	4.6 abc	12.0 a
1.0	0.0	100 a	5.4 a	12.0 a
1.0	0.5	93.3 b	5.0 abc	10.8 abcd
1.0	1.0	93.3 b	5.2 ab	11.8 a
1.0	2.0	93.3 b	4.0 cd	12.0 a
2.0	0.0	73.3 e	4.0 cd	10.5 bcd
2.0	0.5	80.0 d	4.3 bc	11.0 abcd
2.0	1.0	86.6 c	4.5 abc	12.0 a
2.0	2.0	80.0 d	4.0 cd	11.5 abc

MNR/S: Mean number of roots/shoot. MSH: Mean shoot heights.

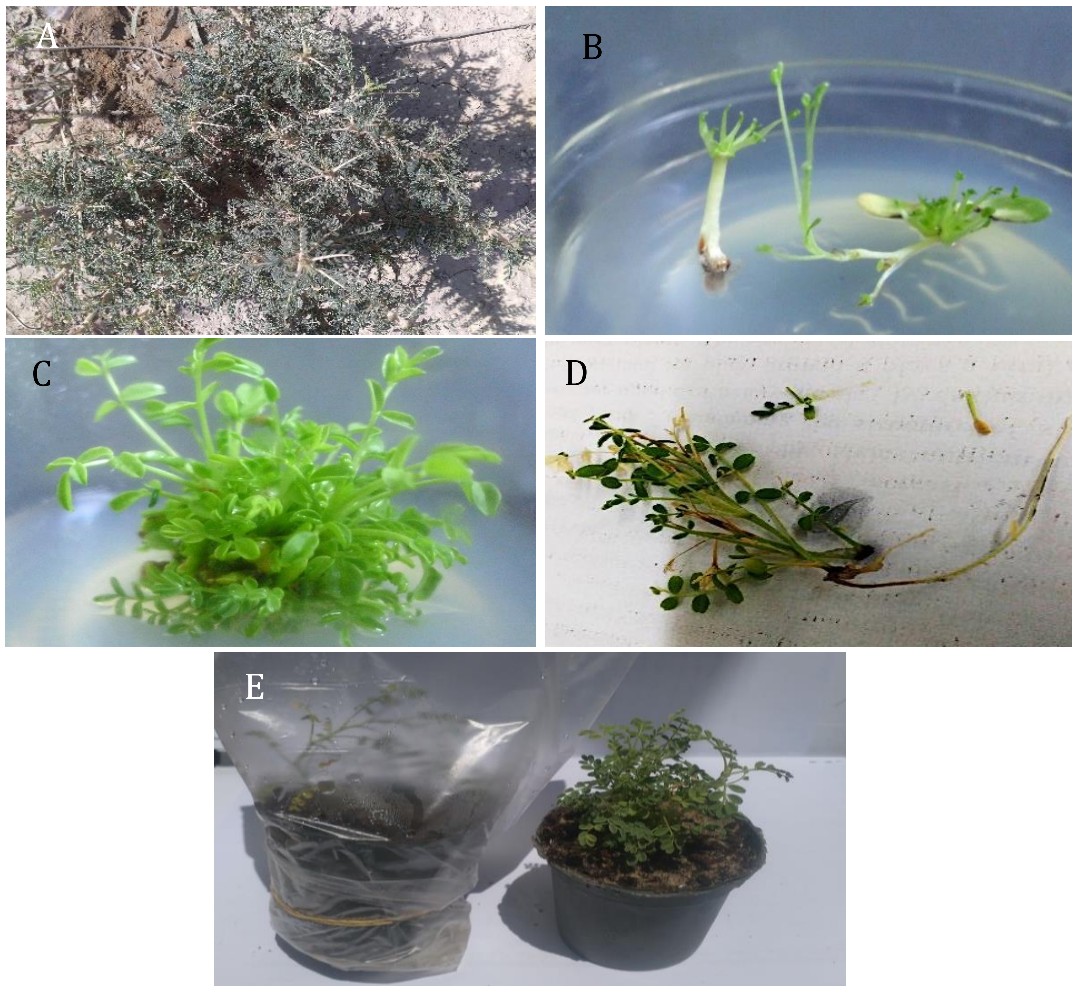

Figure 1. *In vitro* propagation of *Astragalus trigonus* plant: (A) Naturally grown plant used to collect seeds, (B) formed shoots from sterilized seeds, (C) shoot multiplication stage, (D) rooting stage and (E) acclimatization stage.

Esmaeili *et al.* (2016) showed that, the 0.3 mg L^{-1} IBA concentrations recorded the best lateral rooting development in *Astragalus adscendens*. The difference from using IBA in root formation may be replied on different species. For acclimatization, plantlets were planted on 8 and 16 cm pots containing 1:1 (v:v) mixture of peat and perlite. Plantlets transferred to 8 cm pots showed more healthy development and higher survival rates, 60% **(Fig. 1 E)**. After thirty days in greenhouse, the plants were transferred to larger pots containing the same soil composition and were irrigated with tap water and were grown till maturity under greenhouse conditions.

Genetic variation between mother plant and regenerated *A. trigonus* plantlet

The production of true-to-type plant consider one of the major aims of plant commercial micropropagation. (Giri *et al.,* 200). One important source of genetic variation is the somaclonal variation as same as the induction of mutation by chemical and physical mutagens. This type of variation have been observed in many plant. (Jain, 1998). Somaclonal variation could be heritable (genetic) and non-heritable (epigenetic) due to it is unpredictable in nature. Genetic stability studies is requisite to assess the potential usefulness of the tissue culture techniques (Armstrong and Phillips, 1988). Also it is important to develop a methods for easily and rapidly screened to reveal any genetic difference from non tissue culture derived controls (Raimondi *et al*., 2001).

In this study, genetic stability was examined among of mother plant and plantlets regenerated from *in vitro* propagation of *Astragalus trigonus* by RAPD using 18 random primers. The 18 primers generated 195 bands ranging in size from 10,000 bp to 250 bp (Figure 2). The data present in Table 5 and Figure 2 show that the regenerated plants produced 32.5% polymorphism and shared 128 bands (67.5% similarity). The similarity percentage between the regenerated plants and their mother plant ranged from 11.1 to 66.6% for the primers OPE-C-11 and OPE-A-11, respectively. The similarity percentages between the regenerated plants derived from *A. trigonus* with their mother plants ranged from 88.9 to 33.4% for the OPE-C-11 and OPE-A-11 primers, respectively. Seven ISSR primers were used to study genetic stability between *A. trigonus* mother plant and their generated plantlets. Thirty-eight bands were generated ranging in size from 10000 bp to 250 bp (Figure 3). The data presented in Table 5 and Figure 3 show 50% polymorphism and sharing in 19 bands (50.0% similarity).

The similarity percentages between the regenerated plantlets and their mother plant ranged from 37.5 and 60.0% for the primers UBC 62 and UBC 28, respectively. Many different causes could led to RAPD and ISSR variation including point mutations, the insertion or deletion of sequence or transposable elements and loss/ gain of a primer annealing. Since, with single base change in the primer annealing site is obviously may cause the appearance or disappearance of RAPD and ISSR bands. Therefore, (Othmani *et al*., 2010) suggested that the conditions of tissue culture can induced different amount of genetic variation for *in vitro* plants production. The genetic similarity percentage between *A. trigonus* mother plant and the regenerated plants which obtained by the analysis of RAPD and ISSR indicated that the protocol used in this study is favorable and can be used for genetic conservation of plants.

Table (6). Combined polymorphism between RAPD and ISSR analysis.

	Total scorable band	No. Monomorphic band	No. Polymorphic bands	%polymorphism
RAPD	195	128	63	32.3
ISSR	38	19	19	50
Combined data	**233**	**147**	**82**	**35.2**

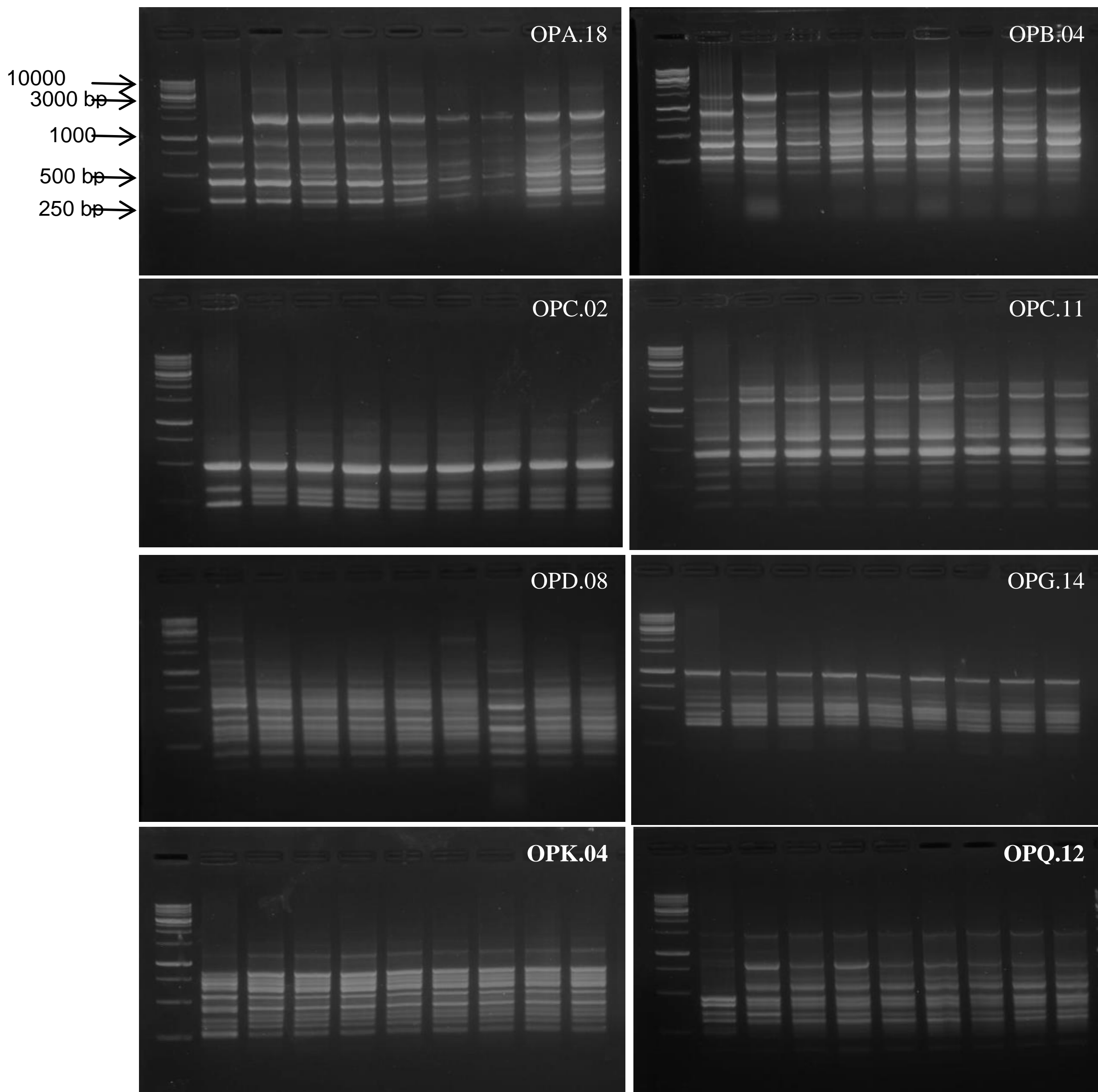

Figure 2. RAPD profile of *in vitro* regenerated *A. trigonus* plantlets in comparison with their mother plant. M: 1 kp Plus DNA ladder, T: mother plant, Lanes 1 to 8: *in vitro* regenerated *A. trigonus* plantlets.

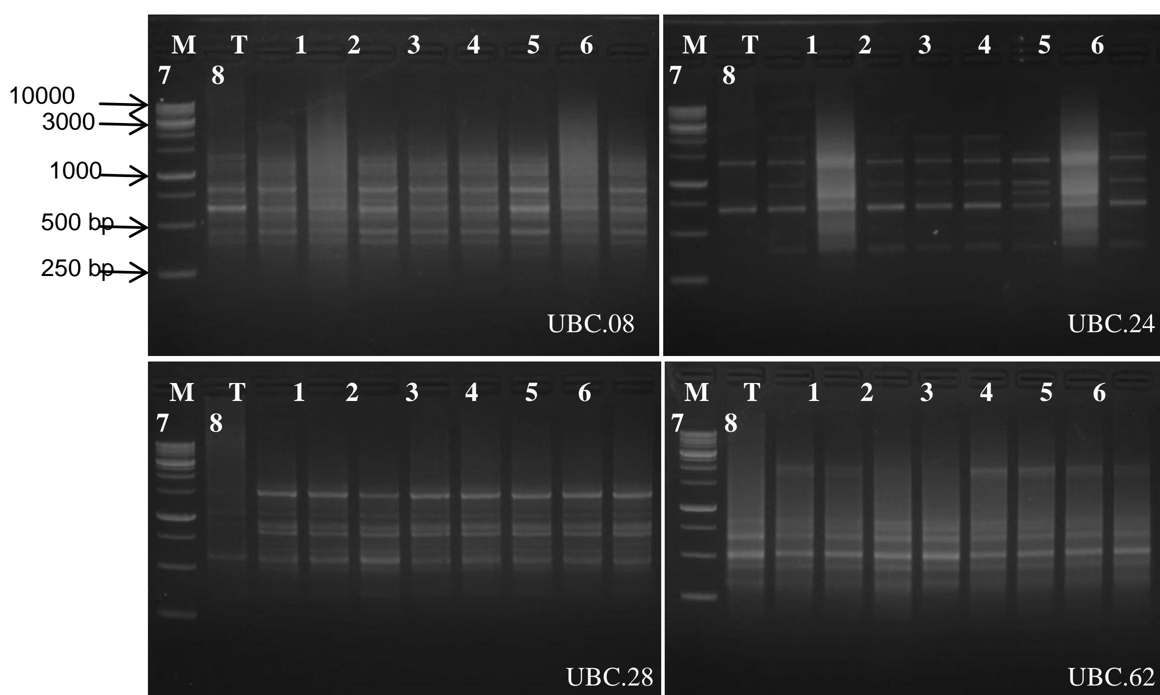

Figure 3. ISSR profile of *in vitro* regenerated *A. trigonus* plantlets in comparison to their mother plant. M: 1 kp Plus DNA ladder, T: mother plant, Lanes 1 to 8: *in vitro* regenerated *A. trigonus* plantlets.

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ISSR	38	19	19	50
Combined data	**233**	**147**	**82**	**35.2**

The combined data between both RAPD and ISSR analyses showed that the polymorphism percentage was 35.2 % among the regenerated and their mother plants **(Table 6)**.

CONCLUSION

In the present study, a successful and efficient micropropagation, conservation protocol and clonal propagation of *Astragalus trigonus* has been developed and described. The used of RAPD and ISSR primers showed that there was practically low variability among the *in vitro* produced plantlets and the mother plant. Therefore, it can be concluded that the *in vitro* raised plants can be used in conservation of endangered medicinal plants.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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