Micropropagation of Several Genotypes of *Argania spinosa* L. to Selection Superior

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ABSTRACT

This study was intended to find out the well-defined protocol easily for *in vitro* propagation on different genotypes of *Argania spinosa* due to its difficulty in traditional propagation. In this respect, the highest survival percentage of shoot tips (100%) was found at 25% clorox for surface sterilization. While, genotype 3 was cultured on ¾ MS medium formed the highest number of leaves and number of shoots. For rooting, MS medium contained 3.0 mg/l NAA was suitable for genotype4. Plantlets after root development exhibited 79.66% survival in pots filled with mixture of peat moss: vermiculite (1:1, v/v) at genotype4. Inter simple sequence repeat (ISSR) analysis detected similarity to the mother tree in the *in vitro* regenerated plants from the shoot tips, that the direct and indirect regeneration protocol will be useful for *Argania spinosa* tree production.

Keywords: Argan,-Shoot tip- Genotype.

INTRODUCTION

Argan (*Argania spinosa* L.) is an endemic tree species of south Morocco, growing in arid habitats. Argan (Sapotaceae) is cultivated for oil production, which was extracted from the fruits (El Kharrassi et al., 2018). Argan tree is monoecious and exhibits high genetic diversity. Argan oil is uses as a anti-wrinkles (Msanda, 1993). Argan propagate tradiontary by seeds, stem cuttings or grafting. Propagation by seeds is least used because of the long juvenile period and don't production of true-to-type plants of argan (Nouaim et al., 2002 and Al-Menaie et al., 2007). The stem cuttings for argan propagation were hindered by difficulty of rooting. The rooting depends on genotype and the cutting type (Nouaim et al. 2002, Taoufq et al., 2011 and Metougui et al., 2017).

Argan oil contains saponin that derivatives have been as protective agents against infective fungi. Argan nuts contain three kernels from which argan oil is extracted with a 30–55% yield (Bowyer et al., 1995). The leaves were used for forage that complemented by the energetic leftovers obtained after the oil preparation. Trees are also used for fuel production (Collier and Lemaire, 1974). Argan oil contain fatty acids which are essential for normal growth and development, and reduce the risk of heart disease and improve cognitive functions in old age (Ibarguren et al., 2014).

Tissue culture could be used for the commercial exploitation of natural products from plants for increasing demand (Rao and Ravishankar, 2002, Lee et al., 2010, Schmid and Zu¨lli, 2012 and Pavlovic´ and Radotic´,2017).

The surface sterilization was soaking the explants in 6% sodium hypochlorite solution plus 0.1 ml of Tween 80 with continuous shaking then rinsed three times with sterilized water (Lamaoui et al., 2019). MS medium was suitabled for the proliferation of *Argania spinosa* (Boonne et al., 1992).Shoot were formed on MS medium contained BA and GA_3. The highest number of shoots per explant was observed on medium containing 1 mg/l BA and 2 mg/l GA_3 (Amghar et al., 2021a). The highest rooting percentages were recorded on MS medium by reducing the ammonium nitrate

concentration and added 1.5 mg/l indole butyric acid, 0.5 mg/l naphthalene acetic acid, 2 mg/l $AgNO_3$ and 160 mg/l putrescine (Amghar et al., 2021b).

The highest root length of argan seedling was formed on half-strength of MS medium contained 1 mg/l GA_3 and 1 mg/l BA (Koufan et al., 2018). For Argan plantlets acclimatization, moisture must be reduced from 100% to 80% during three - six weeks period to avoid plant mortality (Nouaim et al., 2002).

Tissue culture produces homogenized and genetically stabilized plants. Somaclonal variation can be occurred at DNA level, clonal stability can be assessed by studying chromosome numbers, isozyme profile and PCR-based molecular markers like inter simple sequence repeat (ISSR) Devi et al. (2013) and Rathore et al. (2014).

Due to its economic value, the aims of the current study were to develop of efficient micro- propagation protocol of *Argania spinosa* plant using different types of media as well as using different plant growth regulators for initiation, multiplication and rooting stages and acclimatization using different genotypes of *Argania spinosa*.

MATERIALS AND METHODS

The current study was conducted in the Plant Biotechnology Dept., Genetic Engineering and Biotechnology Institute, Sadat City University. Practical part was conducted in the Laboratory of Tissue Culture, Horticulture Research Institute, Agriculture Research Center (ARC), Ministry of Agriculture, Giza, Egypt. The current research was conducted during the period from 2015 to 2022.

Plant materials

Preparation of plant materials:

Plant materials were obtained from El-Nada Farm, Alex. Desert Road (Km 70). Healthy shoots of *Argania spinosa* were excised from the mother plants and collected from the farm and transferred directly to the laboratory with 5-8 cm in length. The explants were immediately soaked in soap water for 10 min followed by washing in running tap water for 30 min. Then they were prepared by removing leaves and excising about 1.5-2.0 cm of shoot tips containing the apical meristems as well as lateral buds and axillary buds were also used as explants.

Preparation of culture media and incubation condition:

MS medium complete medium with vitamins and CHU medium was supplemented with sucrose (30 g/l). The pH was adjusted to 5.7±0.1 using KOH or HCl (1N). Agar (agar-agar) at the rate of 7 g/l was added to the medium. Aliquotes of 25 ml of the prepared MS medium were dispensed into 200 ml culture jars or 50 ml into 400 ml jars. The media were then autoclaved at 121 ºC and 1.5 kg/cm^2 for 21 min. All prepared media were incubated at room temperature for 5 days.

Experimental treatments:

1. Surface sterilization of explants:

Explants were submerged with the commercial bleach Clorox containing the active ingredient sodium hypochlorite (NaOCl at 5.25%) at the rates of 15, 25, 35 and 45% for 15 minutes, followed by rinsing in sterilized water 3 times. The explants were cultivated on MS medium under aseptic conditions. Cultured jars were transferred to the growth room and incubated for one month (16/8 light/dark) and were incubated at 24 C ± 1. The replicate was contained three jars. Each jar contains one explant. The recorded data were: survival percentage, contamination percentage and mortality percentage. Data were recorded after one month.

These experiments were tested to determine the best concentration of sodium hypochlorite and the best explant growth to genotype 4. Then, all genotypes will sterilize with the best concentration for the best explant.

2. Multiplication stage:

Both MS (Murashigeand Skoog,1962) and CHU (Chu et al., 1975) media were tested at 3 rates of media salt strengths (full strength, three quarters strength and half strength), to determine the best salt strength and medium type for genotypes 1, 2, 3 and 4 of *Argania spinosa* growth. All media were contained 1.5 mg/l Kin (6-Furfuryl-aminopurine). Cultured jars were transferred to the growth room and incubated for 6 weeks for every subculture at 24 ºC ± 1, under 16/8 light/dark photoperiod. The recorded data were: number of leaves, shoot length (cm) and number of shoots, these parameters were determined in successive 3 subcultures.

3. Rooting stage:

For rooting of the genotypes 1, 2, 3 and 4 of *Arganiaspinosa*, MS medium were prepared contained 0.0, 1, 2, 3 and 4 mg/l of either IBA (Indole Butyric Acid) or NAA (Naphthalene Acetic Acid). Activated charcoal at 0.0 and 3.0 g/l was added to all media in order to improve root formation. Shoots were cultured for ten weeks. Number of roots, root length (cm), shoot length (cm) and number of leaves were determined at the end of the rooting experiment.

4. Acclimatization stage:

In vitro plantlets were taken out of the jar, and then washed under tap water followed by soaking in the fungicide Rhizolix at the rate of 1g/l for 10 min. Plantlets were planted in 5 cm diameter pot filled with vermiculite alone, peat moss: vermiculite (1:1, v/v), fine sand: vermiculite (2:1, v/v) and fine sand: vermiculite: culture soil (2:1:3, v/v/v). Plantlets were irrigated with tap water and kept under plastic tunnel under greenhouse conditions at 25℃ and 95% RH. Tunnels were uncovered every day for a period of 15 min. Plantlets were fertilized after 14 days with commercial compound fertilizer (kristalon 20:20:20+ T.E.) at 0.25 g/l for one week and 0.5 g/l thereafter. Data were recorded after one month. The following data were recorded: shoot length (cm), number of leaves and survival percentage (%).

5. Molecular genetics identification:

The DNA marker system used was inter simple sequence repeat (ISSR).

DNA extraction: Total genomic DNA was extracted and purified from 0.1 g of freeze dried powered samples (Dellaporta et al., 1983**)**. DNA present in the supernatant was precipitated according to the protocol, re-dissolved in sterile, distilled water and quantified.

Statistical analysis:

The experimental design was complete randomized complete randomized design (CRD), with three replicates with analysis of variance to show statistical differences between treatments using analysis of variance (ANOVA) using COSTAT statistical package. The differences between means were compared using least significant difference (L.S.D.) test at a probability level 0.05 **(**Steel and Torrie, 1980**)**.

Abbreviations: CHU= CHU medium, Kin= Kinetin.

BA = 6-Benzylaminopurine, IBA =Indole-3-butyric acid, NAA= Naphthalene Acetic Acids.

RESULTS AND DISCUSSION

1. Effect of Clorox Concentrations on Surface Sterilization of *Argania spinosa*:

Results calculated in **Table (1)** indicate that clorox at 25% gave the highest percentage value of explants survival (100%) as compared with the other concentrations on shoot tips. The highest survival of axillary bud (100%) was found at 35% clorox. The percentages of mortality and contaminated explants (0.0%) were recorded when the axillary bud immersed in same concentration (35% clorox). But, the highest survival percentage of shoot tips (100%) was found at 25% clorox and the lowest mortality percentage (0.0%) or contamination percentage (0.0%).

Table (1).Effect of clorox concentration on percentages of survival, mortality and contamination of *Argania spinosa*explants.

Clorox®	Survival %			Mortality %			Contamination %		
	Explant		Mean (A)	explant		Mean (A)	Explant		Mean (A)
	Shoot tip	axillary bud		Shoot tip	axillary bud		Shoot tip	axillary bud	
15%	100	100	100	0	0	0	33.33	100	66.67
25%	100	100	100	0	0	0	0	66.67	33.33
35%	66.67	100	83.33	33.33	0	16.67	0	0	0
45%	0	33.33	16.67	100	66.67	83.33	0	0	0
Mean (B)	66.67	83.33		33.33	16.67		8.33	41.67	
LSD =	Colorox (A) = 34.45 Explant (B) = 24.36 A x B = 48.71			Colorox (A) = 34.45 Explant (B) = 24.36 A x B = 48.71			Colorox (A) = 34.45 Explant (B) = 24.36 A x B = 48.71		

On the other hand, the results showed that increasing the concentration of clorox increased the mortality percentage and decreased the survival or contamination percentage of explants.

The current results are in harmony with those obtained on *Arganiaspinosa* by Lamaoui et al.(2019).

2. Effect of media type (MS and CHU)with different salt strength (Full, three quarters and half) on genotypes 1, 2, 3 and 4 during multiplication stage:

2.a. The first subculture:

The results calculated in **Table (2)** demonstrate that the survival shoot tips were planted on MS and CHU media. Furthermore, MS treatment at full strength gave a positive effect on the number of leaves or shoots and shoots length which gave 56.08 leaves, 3.92 shoots and 4.71 cm, respectively as compared with other strength.

Half-strength of MS medium has been used in a variety of tissue culture systems to promote growth and development of *Arganiaspinosa***(**Koufan et al., 2018).

The results for genotypes also in Table (2) showed that genotype 3 gave the highest number of leaves or shoots and shoot length (54.06 leaves, 3.72 shoots and 5.04 cm, respectively) when compared to other genotypes.

The interaction between the media with different salt strengths and genotypes 1, 2, 3 or 4 explained that the highest leaf number and shoot number was recorded on MS at three quarters strength on genotype 3, which gave 80.33 leaves and 6.33 shoots, respectively. The highest shoot length was obtained on MS at full strength on genotype 3, which gave 6.60 cm.

2.b. The second subculture:

The results recorded in **Table (3)** show the same trend as in Table (2). MS treatment at full strength gave the largest leaf number and longest shoot (68.58 leaves and 5.31 cm, respectively) as compared with other strength. While, the highest shoot number was recorded on MS at three quarters strength which gave 5.83 shoots.

Results obtained here are in harmony with those obtained elsewhere when MS medium were used for the proliferation of *Arganiaspinosa***(**Boonne et al., 1992).

The data for genotypes demonstrated that genotype 3 gave the highest leaf number, shoot length and shoot number (70.71 leaves, 5.73 cm and 4.89 shoots, respectively) as compared to other genotypes.

The interaction between the media type with different salt strength and genotypes 1, 2, 3 or 4 in **Table (3)** showed that the highest number of leaves and number of shoots were obtained on MS at three quarters strength on genotype 3, which gave 80.33 leaves and 6.33 shoots, respectively. The longest shoot length was obtained on MS at full strength which gave 6.60 cm on genotype 3.

Table (2). Effect of media type (MS and CHU)with different salt strength (Full, three quarters and half) on number of leaves or shoots and shoot length during the 1st subculture at multiplication stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

Media (Salt strength)	Number of leaves					Shoot length					Number of shoots				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4	
Full MS	40.00	57.33	66.00	61.00	**56.08**	3.60	3.97	5.97	5.30	**4.71**	4.00	3.67	4.33	3.67	**3.92**
Three quarters MS	37.67	53.67	68.67	61.33	**55.34**	3.83	4.00	5.40	4.80	**4.51**	3.33	3.67	4.33	3.33	**3.67**
Half MS	33.67	51.67	46.67	40.00	**43.00**	2.97	3.10	4.57	3.90	**3.64**	2.67	3.00	2.67	2.33	**2.67**
Full CHU	36.70	44.67	50.33	41.33	**43.25**	2.80	3.07	5.40	4.37	**3.91**	2.67	4.33	3.33	2.67	**3.25**
Three quarters CHU	34.70	41.00	55.33	44.00	**43.75**	3.03	3.40	4.70	3.70	**3.71**	2.33	3.67	5.00	3.00	**3.50**
Half CHU	30.30	39.67	37.33	33.00	**35.08**	3.23	3.80	4.20	3.30	**3.63**	1.67	2.33	2.67	2.33	**2.25**
Mean (B)	**35.50**	**48.00**	**54.06**	**46.78**		**3.24**	**3.56**	**5.04**	**4.23**		**2.78**	**3.44**	**3.72**	**2.89**	
LSD $_{0.05}$=	**Media (A) = 2.78 Genotypes (B) = 2.27 A x B = 5.57**					**Media (A) = 0.24 Genotypes (B) = 0.20 A x B = 0.49**					**Media (A) = 0.50 Genotypes (B) = 0.41 A x B = 1.00**				

Table (3). Effect of media type (MS and CHU)with different salt strength (Full, three quarters and half) on number of leaves or shoots and shoot length during the 2nd subculture at multiplication stage of Argania spinosa genotypes 1,2,3 and 4.

Media (Salt strength)	Number of leaves					Shoot length (cm)					Number of shoot				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
Full MS	52.00	71.00	74.00	77.33	**68.58**	4.47	4.50	6.60	5.67	**5.31**	5.33	6.00	5.67	5.67	**5.67**
three quarters MS	49.00	66.00	80.33	78.00	**68.33**	4.40	4.77	6.50	5.17	**5.21**	4.67	4.67	6.33	5.33	**5.25**
Half MS	35.67	62.00	68.33	66.67	**58.17**	4.13	4.33	5.10	4.40	**4.49**	4.00	4.00	4.67	3.67	**4.09**
Full CHU	49.00	56.00	71.33	70.33	**61.67**	3.47	3.87	5.83	4.63	**4.45**	3.67	5.33	4.67	5.00	**4.67**
Three quarters CHU	47.00	51.33	73.00	68.00	**59.83**	3.80	3.87	5.43	4.20	**4.33**	3.33	4.33	5.67	5.33	**4.67**
Half CHU	40.33	50.00	63.00	56.67	**52.50**	4.07	4.13	4.93	3.47	**4.15**	2.67	3.00	4.33	3.67	**3.42**
Mean (B)	**45.50**	**59.39**	**71.67**	**69.50**		**4.06**	**4.25**	**5.73**	**4.59**		**3.94**	**4.56**	**5.22**	**4.78**	
LSD $_{0.05}$=	**Media (A) = 2.80 Genotypes (B) = 2.29 A x B = 5.61**					**Media (A) = 0.20 Genotypes (B) = 0.16 A x B = 0.40**					**Media (A) = 0.49 Genotypes (B) = 0.40 A x B = 0.98**				

2.c. The third subculture:

The results recorded in **Table (4)** show the same trend as in **Tables (2 and 3)**. MS and CHU media at full strength gave largest leaf number (79.67 leaves). While, the highest shoot number and shoot length were obtained on MS at three quarters strength which gave 8.00 shoots and 5.20 cm, respectively.

Results obtained here are in harmony with those obtained elsewhere when the highest rooting of *Argania spinosa* were observed on low concentration of ammonium nitrate in MS medium (Amghar, et al. 2021b).

The results also for genotypes showed that genotype 3 gave the highest leaf number, shoot length and shoot number (78.83 leaves, 6.29 cm and 6.17 shoots, respectively) as compared to other genotypes.

The interaction between the media with different salt strength and genotypes 1, 2, 3 or 4 showed that in **Table (4)** the highest leaf number was obtained on MS medium at full or three quarters strength on genotype 3, which gave 88.67 leaves. While, the highest shoot length was observed on full strength of MS medium on genotype 3, which gave 7.40 cm.The highest shoot number was recorded on three quarters strength of MS medium on genotype 3, which gave 8.33 shoots.

3. Effect of IBA or NAA concentrations and activated charcoal on genotypes 1, 2, 3 and 4 during rooting stage:

3.a. Effect of IBA concentrations:

The results recorded in **Table (5)** reveal that IBA caused an increase in root, leaf number and root, leaf length after ten weeks. The highest number of roots and root, shoot length was found at 4.0 mg/l IBA, which gave 2.50 roots, 4.11 cm and 8.63 cm, respectively. While, the highest leaf number (41.25 leaves) was obtained on medium contained 3.0 mg/l IBA also when compared with the zero-level (control).

The data for genotypes showed that genotype 3 gave the highest root, leaf number and root, shoot length(1.33 roots, 37.40 leaves, 2.21 cm and 7.87 cm, respectively) as compared to others genotypes.

The interaction between IBA and genotypes showed that the highest root number (3.33 roots) was found at 4.0 mg/l IBA on genotype 2. The highest root length and leaf number were recorded at 3.0 mg/l IBA, which gave 4.47 cm and 42.67 leaves, respectively on genotype 4. The highest shoot length (9.17 cm) was found at 4.0 mg/l IBA on genotype 4.

The obtained data are in agreement with those obtained by Amghar, et al., (2021a).They reported that the highest rooting of *Argania spinosa* was showed on medium contained IBA.

3.b. Effect of IBA with activated charcoal:

The data showed in **Table (6)** indicate that IBA caused an increase in root, leaf number and root, shoot length at medium with activated charcoal after ten weeks. The highest root, leaf number and root length was found at 4.0 mg/l IBA and activated charcoal, which gave 3.67 roots, 41.33 leaves and 4.80 cm, respectively. While, the longest shoot length 9.46 cm was obtained on medium contained 3.0 mg/l IBA and activated charcoal.

The results for genotypes showed that genotype 3 gave the highest root, leaf number and shoot length (2.07 roots, 38.20 leaves and 8.54 cm, respectively) as compared to others genotypes. While, the highest root length (3.23 cm) was recorded at genotype 4.

For the interaction between IBA concentrations plus activated charcoal and different genotypes revealed that the highest root, leaf number (4.67 roots and 43.67 leaves, respectively) was recorded at genotype 3 on 3.0 mg/l IBA. The longest root length was noted on 4.0 mg/l IBA at genotype 4, which gave 5.57 cm. The highest shoot length (9.77 cm) was observed on medium with 3.0 mg/l IBA at genotype 2.

Table (4). Effect of media type (MS and CHU)with different salt strengths (Full, three quarters and half) on number of leaves or shoots and shoot length during the 3rd subculture at multiplication stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

Media (Salt strength)	Number of leaves					Shoot length (cm)					Number of shoots				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4	
Full MS	60.67	81.00	88.67	88.33	**79.67**	5.13	5.20	7.40	6.13	**5.97**	6.33	6.67	7.67	8.00	**7.17**
Three quarters MS	52.33	76.67	88.67	86.33	**76.00**	5.10	5.30	6.87	5.73	**5.75**	5.00	6.33	8.33	7.67	**6.83**
Half MS	47.00	73.33	71.00	69.00	**65.08**	4.23	4.23	5.70	4.87	**4.76**	4.67	5.33	4.67	4.67	**4.84**
Full CHU	55.33	68.33	79.33	79.00	**70.50**	3.90	3.87	6.30	5.00	**4.77**	4.67	6.67	6.33	6.00	**5.92**
Three quarters CHU	56.33	62.00	79.33	76.33	**68.50**	3.90	4.03	5.90	4.70	**4.63**	4.00	5.33	5.67	5.67	**5.17**
Half CHU	47.67	57.00	66.00	63.33	**58.50**	4.37	4.23	5.60	4.00	**4.55**	3.33	3.67	4.33	4.00	**3.83**
Mean (B)	**53.22**	**69.72**	**78.83**	**77.06**		**4.44**	**4.48**	**6.29**	**5.07**		**4.67**	**5.67**	**6.17**	**6.00**	
LSD 0.05=	**Media (A) = 2.39; Genotypes (B) = 1.95 A x B = 4.78**					**Media (A) = 0.19; Genotypes (B) = 0.16 A x B = 0.38**					**Media (A) = 0.50; Genotypes (B) = 0.41 A x B = 1.00**				

Table (5).Effect of IBA (mg/l) on roots, leaf number and root, shoot length during rooting stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

IBA (mg/l)	Number of roots					Root length (cm)					Shoot length (cm)					Number of leaves				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	4	
Control	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	6.20	6.70	6.80	6.17	**6.47**	30.67	27.67	32.33	28.00	**29.67**
1.0	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	6.97	7.30	7.23	7.07	**7.14**	30.67	33.00	32.67	34.33	**32.67**
2.0	0.00	0.33	1.00	0.67	**0.50**	0.00	0.87	2.47	2.37	**1.43**	7.60	8.03	7.87	7.97	**7.87**	38.67	37.00	38.67	36.67	**37.75**
3.0	1.67	2.67	3.00	2.33	**2.42**	3.87	3.60	4.27	4.47	**4.05**	7.93	8.27	8.57	8.40	**8.29**	39.67	41.00	41.67	42.67	**41.25**
4.0	2.00	3.33	2.67	2.00	**2.50**	3.87	4.03	4.30	4.23	**4.11**	8.20	8.27	8.87	9.17	**8.63**	40.67	39.67	41.67	42.33	**41.09**
Mean (B)	**0.73**	**1.27**	**1.33**	**1.00**		**1.55**	**1.70**	**2.21**	**2.21**		**7.38**	**7.71**	**7.87**	**7.76**		**36.07**	**35.67**	**37.40**	**36.80**	
L.S.D 0.05=	**IBA (A) = 0.54; Genotypes(B)= 0.45 A x B = 1.43**					**IBA (A) = 0.92; Genotypes(B) = 0.77 A x B = 2.44**					**IBA (A) = 0.37; Genotypes(B)= 0.31 A x B = 0.97**					**IBA (A) = 2.87; Genotypes(B) = 2.41 A x B = 7.60**				

Table (6). Effect of IBA (mg/l) with activated charcoal on roots, leaf number and root, shoot length during rooting stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

IBA (mg/l)	Number of roots					Root length (cm)					Shoot length (cm)					Number of leaves				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	4	
0.0 + AC	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	6.60	7.10	6.70	6.57	**6.74**	29.00	28.67	31.00	31.00	**31.00**
1.0 +AC	0.00	0.00	0.67	0.33	**0.25**	0.00	1.03	1.47	1.07	**0.89**	7.50	7.73	7.53	7.20	**7.49**	35.33	33.67	34.67	32.67	**32.67**
2.0 +AC	1.00	1.00	1.33	1.33	**1.17**	2.10	3.27	4.10	4.13	**3.40**	8.13	8.73	7.83	8.20	**8.22**	39.33	39.33	39.33	37.33	**37.33**
3.0 +AC	2.67	3.33	4.67	3.67	**3.59**	3.80	4.37	5.13	5.37	**4.67**	9.50	9.77	9.17	9.40	**9.46**	38.67	42.67	43.67	39.00	**39.00**
4.0 +AC	2.33	2.67	3.67	3.00	**3.67**	3.97	4.47	5.20	5.57	**4.80**	9.30	9.37	8.97	9.40	**9.26**	40.00	41.00	42.33	41.33	**41.33**
Mean (B)	**1.20**	**1.40**	**2.07**	**1.67**		**1.97**	**2.63**	**3.18**	**3.23**		**8.15**	**8.04**	**8.54**	**8.21**		**36.47**	**37.07**	**38.20**	**36.27**	
L.S.D 0.05=	**IBA (A) = 0.81; Genotypes(B) = 0.68 AxB = 2.14**					**IBA (A) = 1.18; Genotypes(B)= 0.99 AxB = 3.13**					**IBA (A) = 0.51; Genotypes(B) = 0.43 AxB = 1.36**					**IBA (A) = 2.92; Genotypes(B) = 2.45 AxB = 7.74**				

3.c. Effect of NAA concentrations:

The data noted in **Table (7)** revealed that NAA caused an increase in roots, leaf number and root, shoot length after ten weeks. The highest roots, leaf number and root, shoot length were found when 3.0 mg/l NAA was used, which gave 1.58 roots, 39.92 leaves, 2.97 cm and 8.58 cm, respectively.

The results for genotypes showed that genotype 3 exhibited the highest root number and root length (0.67 roots and 1.60 cm, respectively) as compared to others genotypes. The highest shoot length and leaf number were found when NAA was used, which gave 8.22 cm and 36.93 leaves, respectively on genotype 4.

For the interaction between NAA concentrations and different genotypes, it showed that the highest number of roots, root length, shoot length and number of leaves were found when 3.0 mg/l NAA was used, which gave 1.67 roots, 3.73 cm, 9.20 cm and 43.33 leaves, respectively on genotype 4.

Equally noticeable, root induction of *Arganiaspinosa* was obtained when shoots were grown in half strength medium contained IBA plus NNA at equal concentrations (5 mg/l) and 5 g/l activated charcoal(Bousselmame, et al., 2001).

3.d. Effect of NAA with activated charcoal:

For NAA concentrations, data in **Table (8)** demonstrated that root, leaf number and root length after ten weeks were achieved at a medium containing 3.0 mg/l NAA plus activated charcoal. The highest root, leaf number and root length were gave 2.00 roots, 42.17 leaves and 4.53 cm, respectively. But, medium contained 4.0 mg/l NAA plus activated charcoal was most suitable for shoot length which gave 8.36 cm.

Also results for genotypes showed that genotype 4 gave the highest root number and root, shoot length were found on medium with activated charcoal, these value were 2.35 roots, 2.35 cm and 8.17cm, respectively. Meanwhile, medium supplemented with activated charcoal was the most suitable for number of leaves of genotype 2, which gave 38.40 leaves.

For the interaction between NAA with activated charcoal and genotypes, the data showed that the highest root, leaf number and root shoot, length (2.33 roots, 44.67 leaves, 5.40 cm and 8.80 cm, respectively) were found at 3.0 mg/l NAA with activated charcoal on genotype 4.

4. Effect of different acclimatization media on genotypes 1, 2, 3 and 4 during acclimatization stage:

Data recorded in **Table (9)** show that the plantlets grew with a healthy appearance during acclimatization stage and the longest shoot was achieved by transplanting of plantlets in pots containing vermiculite alone, peat moss: vermiculite (1:1, v/v), fine sand: Vermiculite (2:1, v/v) and fine sand: vermiculite: culture soil (2:1:3, v/v/v). After four weeks, no abnormalities in physical appearance or growth habits were observed on the transplanted plantlets. The highest shoot length (9.77 cm) was achieved by transplanting of the plantlets to pots containing fine sand: vermiculite: culture soil (2:1:3, v/v/v) at genotype 4. The highest leaf number (43.67 leaves) was recorded by transplanting of the plantlets to pots containing peat moss: vermiculite (1:1, v/v) at genotype 3. The best percentage of survival plantlets (79.66 %) was observed when plantlets were cultured in a mixture of peat moss: vermiculite (1:1, v/v) at genotype 4.

Similar results were reported on *Argania spinosa* by Nouaim, et al. (2002).

Table (7). Effect of NAA (mg/l) on root, leaf number and root, shoot length during rooting stage of *Argania spinosa genotypes* 1, 2, 3 and 4.

NAA (mg/l)	Number of roots					Root length (cm)					Shoot length (cm)					Number of leaves				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
Control	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	6.83	6.53	6.73	6.83	**6.73**	31.67	30.00	29.33	30.00	**30.25**
1.0	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	7.57	7.30	7.90	7.63	**7.60**	34.67	32.33	33.33	34.00	**33.58**
2.0	0.00	0.33	0.33	0.00	**0.17**	0.00	1.27	0.93	0.00	**0.55**	7.93	8.07	7.73	8.47	**8.05**	37.33	35.00	37.67	36.00	**36.50**
3.0	1.33	1.67	1.67	1.67	**1.58**	1.97	2.53	3.63	3.73	**2.97**	7.93	8.30	8.90	9.20	**8.58**	37.00	41.33	38.00	43.33	**39.92**
4.0	1.67	0.67	1.33	1.33	**1.25**	2.20	1.33	3.43	3.57	**2.63**	8.10	8.63	8.63	8.97	**8.58**	34.33	42.67	39.33	41.33	**39.42**
Mean (B)	**0.60**	**0.53**	**0.67**	**0.60**		**0.83**	**1.03**	**1.60**	**1.46**		**7.67**	**7.77**	**7.98**	**8.22**		**35.00**	**36.27**	**35.53**	**36.93**	
L.S.D $_{0.05}$=	**NAA (A) = 0.76** **Genotypes(B) = 0.63** **AxB = 2.00**					**NAA (A) = 1.21** **Genotypes(B) = 1.02** **AxB = 3.22**					**NAA (A) = 0.64** **Genotypes(B) = 0.53** **AxB = 1.69**					**NAA (A) = NS** **Genotypes(B) = NS** **AxB = NS**				

Table (8). Effect of NAA (mg/l) with activated charcoal on root, leaf number and root, shoot length during rooting stage of *Argania spinosa genotypes* 1, 2, 3 and 4.

NAA (mg/l)	Number of roots					Root length (cm)					Shoot length (cm)					Number of leaves				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
0.0 + AC	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	7.70	7.30	7.33	7.47	**7.45**	32.67	32.00	31.67	29.00	**31.33**
1.0 +AC	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	**0.00**	7.73	7.77	7.37	7.90	**7.69**	34.33	35.33	36.33	35.33	**35.33**
2.0 +AC	0.67	0.33	0.67	0.33	**0.50**	2.10	1.43	1.53	1.27	**1.58**	8.10	8.17	8.07	8.20	**8.14**	40.00	41.00	38.00	38.67	**39.42**
3.0 +AC	2.00	1.33	2.33	2.33	**2.00**	4.20	3.40	5.10	5.40	**4.53**	8.20	7.57	8.30	8.80	**8.22**	39.00	43.00	42.00	44.67	**42.17**
4.0 +AC	2.00	1.00	2.00	2.00	**1.75**	4.10	3.43	4.50	5.10	**4.28**	8.10	8.10	8.73	8.50	**8.36**	40.00	40.67	41.67	41.33	**40.92**
Mean (B)	**0.93**	**0.53**	**1.00**	**0.93**		**2.08**	**1.65**	**2.23**	**2.35**		**7.97**	**7.78**	**7.96**	**8.17**		**37.20**	**38.40**	**37.93**	**37.80**	
L.S.D $_{0.05}$=	**NAA (A) = 0.76** **Genotypes(B) = 0.63** **AxB = 2.00**					**NAA (A) = 1.35** **Genotypes(B) = NS** **AxB = 3.59**					**NAA (A) = 0.72** **Genotypes(B) = NS** **AxB = 1.92**					**NAA (A) = 3.49** **Genotypes(B) =NS** **AxB = 9.27**				

Table (9).Effect of different acclimatization media on shoot length, leaf number and survival percentages during acclimatization stage of *Argania spinosa genotypes* 1, 2, 3 and 4.

Acclimatization media	Shoot length (cm)					Leaf number					Survival (%)				
	Genotypes					Genotypes					Genotypes				
	1	2	3	4	Mean (A)	1	2	3	4	Mean (A)	1	2	3	4	Mean (A)
Vermiculite alone	9.50	8.20	7.50	6.70	**7.98**	35.33	33.67	34.67	35.33	**34.75**	32.7	26.9	49.5	49.9	**39.74**
Peat moss: Vermiculite (1:1, v/v)	9.17	7.83	7.20	6.57	**7.69**	38.67	42.67	43.67	38.67	**40.92**	39.0	73.8	70.9	79.7	**65.84**
Fine sand: Vermiculite (2:1, v/v)	9.40	8.13	7.50	6.60	**7.91**	39.33	39.33	39.33	39.33	**39.33**	37.3	59.4	60.7	69.9	**56.83**
Fine sand: Vermiculite: culture soil (2:1:3, v/v/v)	9.77	8.73	7.73	7.10	**8.33**	29	28.67	31	29	**29.42**	31	55.3	56	68.2	**52.63**
Mean (B)	**9.46**	**8.22**	**7.48**	**6.74**		**35.58**	**36.09**	**37.17**	**35.58**		**35**	**53.85**	**59.28**	**66.92**	
L.S.D $_{0.05}$=	**Media (A) = 0.39; Genotypes (B) = 0.39; A x B = 0.79**					**Media (A) = 1.88; Genotypes (B) = 1.88; A x B = 3.73**					**Media (A) = 2.51; Genotypes (B) = 2.51; A x B = 5.03**				

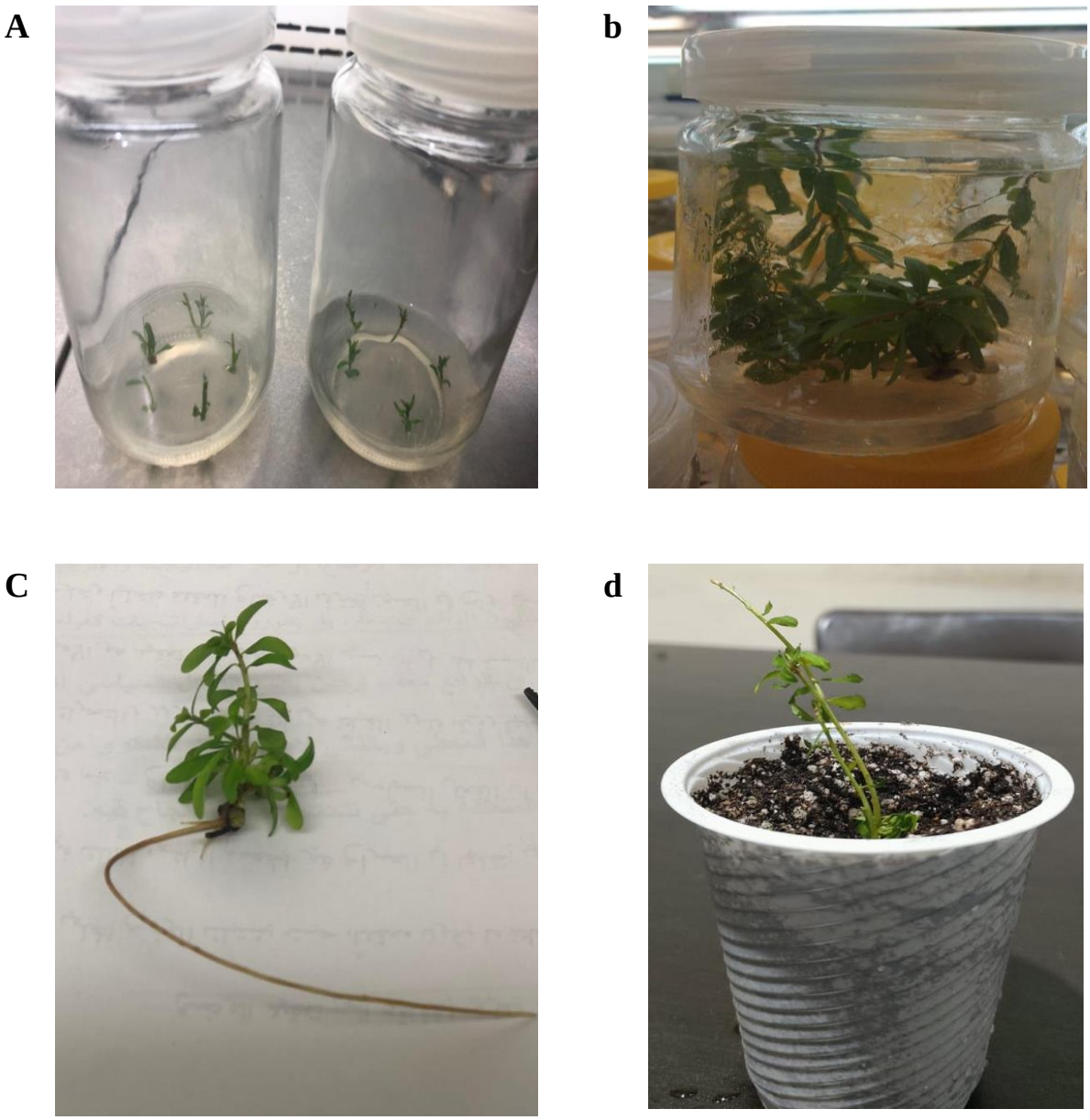

Fig. (1). Micropropagation of *Arganiaspinosa* (L.)

a. Establishment stage. **b. Multiplication stage.**

c. Rooting stage. **d. Acclimatization stage.**

5. ISSR analysis of mother plants and *in vitro*argan plants

As shown in **Table (10),** the similarities between the tested samples were differenced. In sample (1mother) was semiarid with samples 2 mother, 3 mother and 4 mother with ratio 80%, 80%, and 80% respectively and with samples 1 direct, 2 direct, 3 direct, and 4 direct with 73%, 78%, 68%, and 78% respectively and with 1 indirect, 2 indirect, 3 indirect, and 4 indirect with 66%, 70%, 71%, and 78% respectively. In case of sample (2mother), table showed that there was semiarid with 3 mother and 4 mother with 97%, and 82% respectively and with 1 direct, 2 direct, 3 direct, and 4 direct with 79%, 85%, 74%, and 85% respectively, and with 1 indirect, 2 indirect, 3 indirect, and 4 indirect with 74%, 70%, 68%, and 79% respectively. In sample (3mother), data revealed the similarity was 76% with sample 4mother and 69%, 81%, 80%, and 76% with samples 1 direct, 2 direct, 3 direct, and 4 direct and that was 80%, 85%. 74%, and 76% with samples 1

indirect, 2 indirect, 3 indirect and 4 indirect respectively. The results also showed there was semiarid between sample (4mother) and samples 1 direct, 2 direct, 3 direct, and 4 direct with 79%, 84%, 75%, and 93% respectively and was 74%, 82%, 80%, and 90% with samples 1 indirect, 2 indirect, 3 indirect, and 4 indirect.

Sample (1direct), the obtained results showed that ratio of similarity with 2 direct, 3 direct, and 4 direct was 93%, 80%, and 76% respectively and was 86%, 85%, 86%, and 74% with samples 1 indirect, 2 indirect, 3 indirect, and 4 indirect respectively. Furthermore in sample (2direct) was semiarid with 3 direct, and 4 direct with 95%, and 83% and that was 91% with all samples 1 indirect, 2 indirect, and 3 indirect and in sample 4 indirect was 86% . Moreover, in sample (3direct), sample was semiarid with sample 4direct with ratio 73% and with samples 1 indirect, 2 indirect, 3 indirect, and 4 indirect was 80%, 85%, 74%, and 75% respectively. Results showed in case of sample (4direct) the similarity was 80%, 79%, 80%, and 93% with samples 1 indirect, 2 indirect, 3 indirect, and 4 indirect respectively.

Data showed also the similarity between sample (1indirect) and 2 indirect, 3 indirect and 4 indirect was 94%, 86%, and 74% respectively. While, that was 94% and 79% in sample (2indirect) with 3 indirect, and 4 indirect, respectively. Finally, data appeared the similarity was 86% between sample 3 indirect and sample 4 indirect.

Table (10). ISSR analysis of DNA extracted from *in vivo* and *in vitro* produced argan plants.

	1 mother	2 mother	3 mother	4 mother	1 direct	2 direct	3 direct	4 direct	1 indirect	2 indirect	3 indirect	4 indirect
1 mother	**100%**	**88%**	**80%**	**80%**	**73%**	**78%**	**68%**	**78%**	**66%**	**70%**	**71%**	**78%**
2 mother	**88%**	**100%**	**97%**	**82%**	**79%**	**85%**	**74%**	**85%**	**74%**	**70%**	**68%**	**79%**
3 mother	**80%**	**97%**	**100%**	**76%**	**69%**	**81%**	**80%**	**76%**	**80%**	**85%**	**74%**	**76%**
4 mother	**80%**	**82%**	**76%**	**100%**	**79%**	**84%**	**75%**	**93%**	**74%**	**82%**	**80%**	**90%**
1 direct	**73%**	**79%**	**69%**	**79%**	**100%**	**93%**	**80%**	**76%**	**86%**	**85%**	**86%**	**74%**
2 direct	**78%**	**85%**	**81%**	**84%**	**93%**	**100%**	**95%**	**83%**	**91%**	**91%**	**91%**	**86%**
3 direct	**68%**	**74%**	**80%**	**75%**	**80%**	**95%**	**100%**	**73%**	**80%**	**85%**	**74%**	**75%**
4 direct	**78%**	**85%**	**76%**	**93%**	**76%**	**83%**	**73%**	**100%**	**80%**	**79%**	**80%**	**93%**
1 indirect	**66%**	**74%**	**80%**	**74%**	**86%**	**91%**	**80%**	**80%**	**100%**	**94%**	**86%**	**74%**
2 indirect	**70%**	**70%**	**85%**	**82%**	**85%**	**91%**	**85%**	**79%**	**94%**	**100%**	**94%**	**79%**
3 indirect	**71%**	**68%**	**74%**	**80%**	**86%**	**91%**	**74%**	**80%**	**86%**	**94%**	**100%**	**86%**
4 indirect	**78%**	**79%**	**76%**	**90%**	**74%**	**86%**	**75%**	**93%**	**74%**	**79%**	**86%**	**100%**

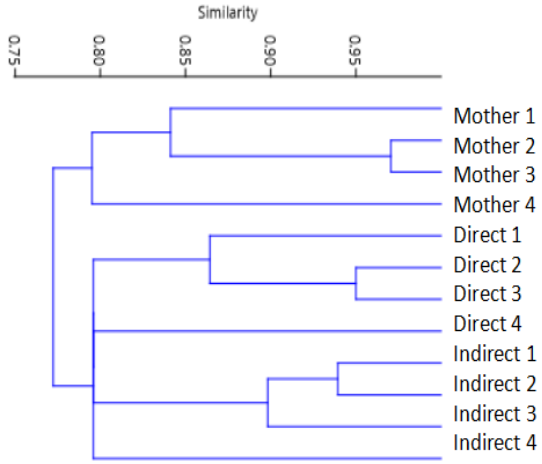

Fig.(2).Dendrogram illustrating the genetic distance for Argan genotypes based RAPD data.

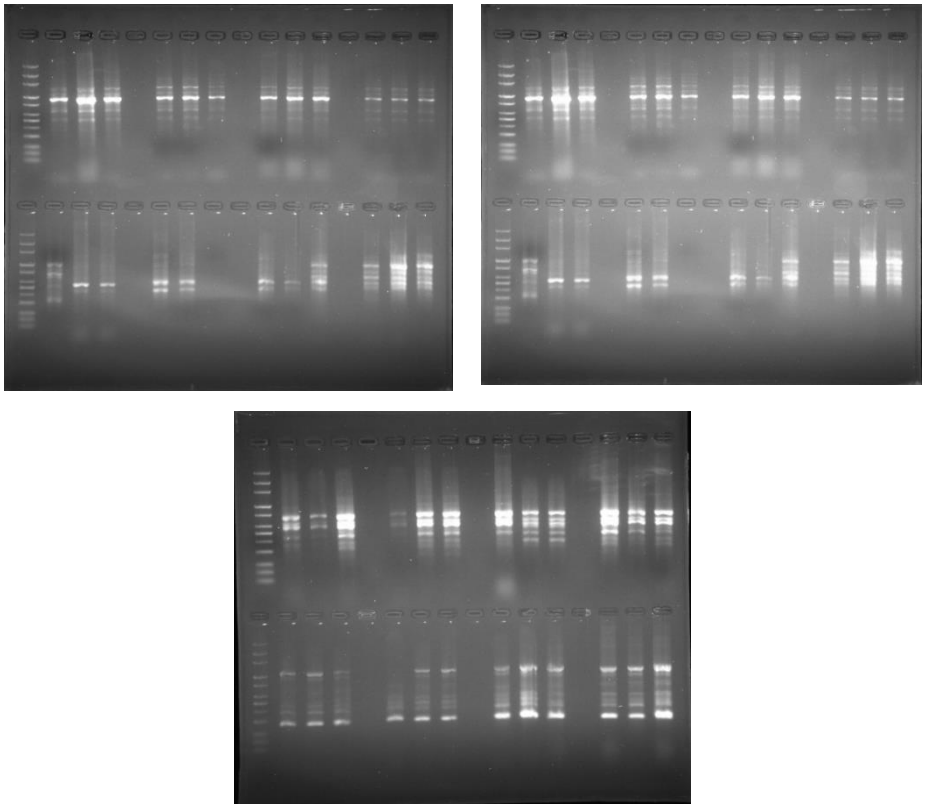

Fig. 3: Gel electrophoresis of ISSR fragments generated by primer

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الملخص العربى

التكاثرالدقيق لعدة طرز وراثية من نباتL *Argania spinosa* لإنتخاب المتفوق

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1- قسم بحوث نباتات الزينة وتنسيق الحدائق – معهد بحوث البساتين -مركز البحوث الزراعية - الجيزة -مصر.

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3- قسم بحوث الحدائق النباتية -معهد بحوث البساتين -مركز البحوث الزراعية – الجيزة –مصر.

الهدف من هذه الدراسة هو تحديد انسب بروتوكول للإكثار المعملي لعدة طرز وراثية مختلفة من نبات *Argania spinosa* نظرًا لصعوبة طرق الإكثار التقليدي. وقد تم التوصل إلى أفضل معاملة للحصول على أعلى نسبة بقاء للبراعم الطرفية (100%) وذلك عند استخدام الكلوروكس بتركيز 25% للتعقيم السطحي. بينما تم زراعة الطراز الوراثي 3 على بيئة MSبقوة ثلاثة أرباع لتعطى أعلى عدد من الأوراق والأفرع.وبالنسبة للتجذير في المعمل كانت البيئة المضاف إليها 3.0 ملجم/لتر NAAللطراز الوراثي 4 هي الاحسن لزراعة النباتات الصغيرة لنمو الجذور. وبالنسبة لمرحلة الأقلمة كانت نسبة بقاء النباتات هى 79.66% للنباتات المزروعة في أصص بلاستيكية مملوءة بخليط من البيت موس : الفيرميكوليت (1:1، حجم/حجم) بإستخدام الطراز الجيني 4. وتم إستخدام تحليل تكرار التسلسل البسيط (ISSR) لتوضيح التشابه مع الشجرة الأم للنباتات التي تم إكثارها في المعمل من البراعم الطرفية، وأتضح أن بروتوكول الإكثار المباشر مفيد لإنتاج شجرة *Argania spinosa*.