Multiplication and Control of Vitrification During Culture Establishment of *Argania spinosa* L.

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ABSTRACT

The aim of this study was to reach a well-defined protocol to *in vitro* propagate of *Argania spinosa* and overcome vitrification phenomenon. In this respect, the highest number of shoot was found at 2.0 mg/l Kin at genotype 3 for multiplication stage. MS medium supplemented with 100 mg/l calcium pantothenate was suited for to eliminate vitrification at all genotypes. For callus induction, MS medium supplemented with 1.5 mg/l NAA when incubated in the light or darkness was favorable to callus induction. MS medium supplemented with 0.5 mg/l GA_3 + 1.5 mg/l BA led to increases shoot initiation.

Keywords: Micropropagation- In vitro - Tissue culture –Argan– Callus induction.

INTRODUCTION

This multipurpose tree is increasingly required due to its ecological interest and its socio-economic value (Le Polain and Waroux, 2013, Lizard et al., 2017 and Mateille, et al., 2016).Argan tree effectively protects the soil against rain or wind-induced erosion and maintains soil fertility (Morton and Voss, 1987).Oleic acid is the major fatty acid in argan oils (Charrouf and Guillaume, 1999).Polyphenols extracted from virgin argan oil have anti-proliferative on human prostate cancer cell lines (Bennani et al., 2007).

Callus induction from somatic tissues used technology for basic research and for industrial applications (Bourgaud et al., 2001, Rao and Ravishankar, 2002, Duclercq et al., 2011, Tito et al., 2011, Schmid and Zu¨lli, 2012 and Pavlovic and Radotic, 2017).

Callus development process required the successful sterilization way that prevents economic losses (Leifert et al., 1989 and Trehan et al., 2017).

Callus initiation of *Argania spinosa* occurred by addition of 1.0 mg/l NAA and 1.0 mg/l 2,4-D. The axillary buds were the most responsive with the highest callus induction (Lamaoui et al., 2019).

Using 1-naphthaleneacetic acid (NAA) at 1 mg/l and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) was the best efficient combination for callus induction from different explants of argan (Lamaoui et al. , 2015).

The germination seeds were cultured in 1/2MS medium supplemented with 1 mg/l IAA and 1 mg/l BA. After 2 months of culture, young leaves were collected from the shoots and cultured in callus induction medium (Koufan et al., 2020).

Adventitious shoot buds were performed on MS medium supplemented with different combinations of BAP and GA_3. The best medium was observed that containing 1 mg/l BAP and 2 mg/l GA_3 (Amghar et al., 2021).

The highest percentage of shoots that was cultured on an MS medium containing 1 mg/l Kin and 0.5 mg/l IAA. The better elongation of argan plants was recorded on MS medium supplemented with 1.5 mg/l of GA_3 (Aizer et al., 2019).

Hyperhydricity is caused some certain anatomical, morphological, physiological, and metabolic disturbances. Hyperhydricity reduces the efficiency of plants micropropagation, the quality of seedlings and

prevents the adaptation of plants in greenhouse. The main factors influencing the level of hyperhydricity of plants *in vitro* are the mineral and hormonal composition of a medium, cultivation conditions and the aeration of cultivation vessels. Based on these factors, various approaches are proposed to eliminate hyperhydricity, such as the use of exogenous additives (Polivanova and Bedarev, 2022). Compared to normal plants, hyperhydric plants also exhibited damaged membranes and cell structure, reduction of cell wall thickness, more intercellular spaces, and collapse of vascular tissues (Gao et al., 2018). Disorders associated with hyperhydricity appear on leaves but also on stems and roots (Ziv, 1991, Saher et al., 2004 and Fernandez-García et al, 2008).The use of the medium with a reduced content of mineral salts or MS medium with low salt strength leads to a decrease in hyperhydricity of clove and cucumber shoots (Ziv et al., 1987).The results of scanning electron microscopy showed that in the tissues of hyperhydric plants exhibited extensive intercellular spaces (Sonrizaand Lilian, 1997).

High concentration of cytokinins enhances ethylene biosynthesis, which can cause hyperhydricity, impaired shoot proliferation and apical necrosis (Žd'árská et al., 2013 and Liu et al., 2017).

Thus, the study aims to develop an efficient procedure for the micropropagation establishment of the economically important *Argania spinosa* tree and overcome vitrification phenomenon in micropropagation protocol.

MATERIALS AND METHODS

1. Plant materials and sterilization:

This study was carried out 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 experiments were conducted throughout 2015 - 2022.

Shoots were obtained from El-NADA Farm, Alex. Desert Road (Km 70). Shoot tips of *Argania spinosa* were excised from the mother plants were collected from the farm and transferred to the laboratory. Shoot tips were immediately soaked in soap under running tap water for 30 min, then transferred to a laminar air flow-cabinet, where submerged with the commercial bleach Clorox containing the active ingredient sodium hypochlorite (NaOCl at 5.25%) at 25% for 15 minutes. Shoot tips were cultured on MS medium under aseptic conditions. Cultured jars were transferred to the growth room and incubated for one month under 16/8 light/dark photoperiod at 24 ºC ± 1.

2. Multiplication stage:

At the multiplication stage, two experiments were initiated to determine the most suitable medium for four genotypes. An initial experiment was carried out using BA, Kin and 2iP at 0.0, 1.0, 1.5, 2.0 and 2.5 mg/l. 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. Shoot length (cm), number of leaves and number of shoots were recorded for each genotypes 1, 2, 3 and 4 of*Arganiaspinosa* growth.

As cultures showed a vitrification problem because of repeated reculturing on media supplemented with cytokinins especially BA, cultures were transferred to media containing calcium pantothenate at 0.0, 50, 75 and 100 mg/l or calcium chloride at 0.0, 83, 166, 244 and 332 mg/l to eliminate vitrification. Therefore, the study of vitrification requires a comprehensive approach, and measures aimed at its elimination should be complex and species-specific.

Callus induction was induced in dark or light when transferred to media treatments supplemented with NAA at 0.0, 0.5, 1.0 and 1.5 mg/l or 2,4-D at 0.0, 0.5, 1.0 and 1.5 mg/l. For shoot initiation, NAA or GA_3 at 0.5 mg/l and Kin or BA at 0.5, 1.0 and 1.5 mg/l were added to MS medium.

- Statistical analysis:

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

Abbreviations: MS = Murashige&Skoog medium, $CaCl_2$= calcium chloride, Kin = Kinetin = 6-Furfuryl-aminopurine, BA = BAP = 6-benzyladenine = 6-benzylaminopurine.

RESULTS AND DISCUSSION

1. Effect of different concentrations of BA, Kin and 2iP during the first subculture on multiplication stage of *Argania spinosa*: For BA, Kin and 2iP at multiplication stage during the first subculture, data calculated in **Table (1)** show that the best concentration of cytokinins was 2.5 mg/l Kin that produced 69.42 leaves, 5.08 cm and 5.67 shoots, respectively.Results obtained here are in harmony with those obtained elsewhere when MS medium plus 1 mg/l Kin and 0.5 mg/l IAA were used to shoot formation of *Argania spinosa* (Aizer et al., 2019).

For genotypes in **Table (1)** recorded that genotype 3 gave the highest number of leaves, shoot length and number of shoots was recorded on medium, which gave 55.95 leaves, 4.75 cm and 3.92 shoots, respectively.

For the interaction between cytokinins and genotypes after the first subculture reported that the highest number of leaves, shoot length and number of shoots were found on medium supplemented with 2.5 mg/l Kin on genotype 3, which gave 74.00 leaves, 5.50 cm and 6.00 shoots, respectively.

2. Effect of different concentrations of BA, Kin and 2iP during the second subculture on multiplication stage of *Argania spinosa*:

The data recorded in **Table (2**) after the second subculture showed that the highest number of leaves, shoot length and number of shoots was found on 2.0 mg/l Kin, which gave 83.42 leaves, 5.99 cm and 6.33 shoots, respectively.The results for genotypes **Table (2)** showed that genotype 3 increased number of leaves, shoot length and number of shoots. The highest number of leaves, shoot length and number of shoot were gave 65.05 leaves, 4.98 cm and 4.62 shoots, respectively.

For interaction between cytokinins and genotypes showed that the highest number of leaves, shoot length and number of shoots were obtained on medium supplemented with 2.0 mg/l Kin on genotype 3, which gave 90.00 leaves, 6.20 cm and 6.67 shoots, respectively.

3. Effect of different concentrations of BA, Kin and 2iP during the third subculture on multiplication stage of *Argania spinosa*:

Data recorded that shoots were not formatted when cultured on medium supplemented with BA. All BA concentrations did not form any shoots with subculturing. Notably, BA increased the vitrificationwhen compared to the other cytokinins. In the same trend, the lowest amount of callus induction was achieved under high BA concentration combined with low auxin levels (Lamaoui et al., 2019).

For Kin and 2iP at multiplication stage, data calculated in **Table (3)** show that the best concentration of Kin was 2.0 mg/l that produced the highest number of leaves, shoot length and number of shoots which recorded (88.58 leaves, 6.39 cm and 9.08 shoots, respectively) after the third subculture.

The results for genotypes also in **Table (3)** showed that genotype 3 gave the highest number of leaves, shoot length and number of shoots was recorded on medium, which gave 70.74 leaves, 5.60 cm and 7.37 shoots, respectively after the third subculture.

The interaction between the Kin and 2iP at different concentrations and different genotypes 1, 2, 3 or 4 showed that the highest number of leaves, shoot length and number of shoots was recorded on medium supplemented with 2.0 mg/l Kin on genotype 3, which gave 95.00 leaves, 7.07cm and 10.67 shoots, respectively after the third subculture.

Table (1). Effect of MS medium with different concentrations of BA, Kin and 2iP (mg/l) on number of leaves, shoot length and number of shoots during the 1st subculture at multiplication stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

Cytokinins (mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4	
Control	23.00	21.00	27.67	21.00	**23.17**	5.80	5.97	6.07	5.97	**5.95**	1.33	1.00	1.33	1.00	**1.17**
1.0 BA	49.00	41.67	55.33	50.67	**49.17**	3.87	3.87	4.47	4.10	**4.08**	3.33	3.67	3.33	3.33	**3.42**
1.5 BA	52.33	45.67	60.67	57.00	**53.92**	4.03	4.20	4.77	4.77	**4.44**	4.67	4.33	4.33	4.67	**4.50**
2.0 BA	52.00	48.67	68.00	60.00	**57.17**	4.03	4.13	4.97	5.10	**4.56**	4.67	5.00	4.67	4.33	**4.67**
2.5 BA	62.00	53.67	70.00	65.33	**62.75**	3.70	4.50	4.99	4.70	**4.39**	5.33	4.67	5.00	4.67	**4.92**
1.0 Kin	43.33	43.33	48.67	45.67	**45.25**	4.27	4.00	4.17	4.03	**4.12**	3.00	3.00	3.33	3.00	**3.08**
1.5 Kin	51.67	54.33	62.33	62.00	**57.58**	4.43	4.30	4.80	4.40	**4.48**	4.33	3.67	4.00	4.33	**4.08**
2.0 Kin	58.00	64.67	71.67	67.33	**65.42**	4.67	4.70	5.50	4.87	**4.93**	5.00	5.33	5.67	5.67	**5.42**
2.5 Kin	61.67	70.67	74.00	71.33	**69.42**	4.73	5.00	5.50	5.10	**5.08**	5.67	5.33	6.00	5.67	**5.67**
1.0 2ip	35.00	34.67	38.67	37.00	**36.34**	3.50	3.30	3.47	3.30	**3.39**	2.33	2.33	2.67	2.33	**2.42**
1.5 2ip	39.67	41.00	44.00	46.67	**36.33**	3.73	3.80	3.73	3.60	**3.72**	3.67	3.00	3.00	3.33	**3.25**
2.0 2ip	43.67	46.67	49.33	49.00	**42.83**	3.93	4.00	4.40	4.13	**4.12**	4.33	3.33	3.67	3.67	**3.75**
2.5 2ip	52.00	51.00	57.00	55.00	**47.17**	4.30	4.40	4.60	4.33	**4.41**	4.67	3.67	4.00	3.67	**4.00**
Mean (B)	**47.95**	**47.46**	**55.95**	**52.92**		**4.27**	**4.37**	**4.75**	**4.55**		**3.96**	**3.72**	**3.92**	**3.81**	
$LSD_{0.05}$ =	**Growth regulators (A) = 2.59 Genotypes (B) = 1.43 AxB = 5.17**					**Growth regulators (A) = 0.25 Genotypes (B) = 0.14 AxB = 0.50**					**Growth regulators (A) = 0.43 Genotypes (B)= 0.24 AxB = 0.87**				

Table (2). Effect of MS medium with different concentrations of BA, Kin and 2iP (mg/l) on number of leaves, shoot length and number of shoots during the 2nd subculture at multiplication stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

Cytokinins (mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4	
Control	20.00	21.00	27.67	21.00	**22.42**	5.90	5.57	6.07	5.97	**5.88**	1.33	1.00	1.33	1.00	**1.17**
1.0 BA	68.00	67.67	72.00	72.33	**70.00**	4.10	4.27	4.27	4.40	**4.26**	4.33	4.00	4.67	4.33	**4.33**
1.5 BA	76.67	72.00	64.67	67.67	**70.25**	4.83	4.73	4.63	4.47	**4.67**	4.67	4.67	4.67	4.67	**4.67**
2.0 BA	67.67	66.00	64.33	63.67	**65.42**	3.90	4.37	4.13	3.87	**4.07**	3.67	4.33	4.33	3.33	**3.92**
2.5 BA	64.67	54.33	52.00	54.67	**56.42**	3.97	4.20	4.20	4.07	**4.11**	3.67	3.67	4.00	4.00	**3.83**
1.0 Kin	71.33	70.33	75.67	72.33	**56.42**	4.57	4.87	4.97	5.13	**4.88**	4.67	4.33	4.67	4.33	**4.50**
1.5 Kin	74.67	74.00	81.33	78.33	**72.42**	5.27	5.20	5.13	5.73	**5.33**	5.33	5.33	5.33	5.33	**5.33**
2.0 Kin	79.67	79.00	90.00	85.00	**77.08**	5.83	5.97	6.20	5.77	**5.99**	6.00	6.33	6.67	6.33	**6.33**
2.5 Kin	77.67	77.67	86.67	82.00	**83.42**	5.23	5.63	6.13	6.00	**5.75**	5.67	6.00	6.33	6.00	**6.00**
1.0 2ip	44.67	42.67	47.00	45.33	**44.92**	4.27	4.30	4.37	4.20	**4.28**	4.00	4.33	4.33	4.00	**4.17**
1.5 2ip	49.67	48.67	54.67	52.33	**51.33**	4.73	4.77	4.47	4.60	**4.64**	4.33	4.00	4.67	4.33	**4.33**
2.0 2ip	55.00	54.00	61.00	58.33	**57.08**	4.97	4.80	5.07	5.20	**5.01**	4.67	5.00	5.00	5.33	**5.00**
2.5 2ip	61.33	60.67	68.67	67.00	**64.42**	5.03	4.80	5.13	5.30	**5.07**	4.67	4.67	5.67	5.00	**4.75**
Mean (B)	**62.38**	**60.62**	**65.05**	**63.08**		**4.82**	**4.88**	**4.98**	**4.96**		**4.31**	**4.39**	**4.62**	**4.46**	
$LSD_{0.05}$ =	**Growth regulator (A) = 2.56 Genotypes (B) = 1.42 AxB = 5.12**					**Growth regulator (A) = 0.31 Genotypes (B) = 0.17 AxB = 0.62**					**Growth regulator (A) = 0.51 Genotypes (B) = 0.28 AxB = 1.03**				

Table (3). Effect of MS medium with different concentrations of Kin and 2iP (mg/l) on number of leaves, shoot length and number of shoots during the 3rd subculture at multiplication stage of *Argania spinosa* genotypes 1, 2, 3 and 4.

Cytokinins (mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
Control	22.67	26.33	32.00	28.67	**27.42**	6.17	6.20	6.33	6.17	**6.22**	1.67	1.00	1.67	1.33	**1.42**
1.0 Kin	75.33	75.33	74.00	72.67	**74.33**	4.50	5.00	5.13	5.10	**4.93**	6.33	5.67	7.00	6.67	**6.42**
1.5 Kin	80.33	77.33	82.00	82.67	**80.58**	5.03	5.57	5.37	5.40	**5.34**	7.33	6.67	8.33	7.67	**7.50**
2.0 Kin	83.33	83.67	95.00	92.33	**88.58**	5.03	5.50	7.07	6.03	**5.91**	8.00	7.67	10.67	10.00	**9.08**
2.5 Kin	83.00	83.00	90.67	91.33	**87.00**	5.33	6.43	6.47	6.73	**6.24**	8.33	7.33	10.33	9.33	**8.83**
1.0 2ip	54.67	52.33	53.67	52.00	**53.17**	4.13	4.27	4.63	4.53	**4.39**	5.33	5.00	5.67	5.33	**5.33**
1.5 2ip	61.67	60.33	62.33	61.67	**61.50**	4.67	4.40	4.90	5.23	**4.80**	6.00	5.67	7.00	6.67	**6.33**
2.0 2ip	66.33	64.67	72.00	71.33	**68.58**	4.67	5.03	5.20	5.37	**5.07**	6.33	6.67	7.67	7.33	**7.00**
2.5 2ip	70.33	72.00	75.00	75.00	**73.08**	5.00	5.13	5.30	5.63	**5.27**	6.67	6.67	8.00	7.67	**7.25**
Mean$_{0.05}$ (B)	**66.41**	**66.11**	**70.74**	**69.74**		**4.95**	**5.28**	**5.60**	**5.58**		**6.22**	**5.81**	**7.37**	**6.89**	
LSD =	**Growth regulator (A) = 2.73** **Genotypes (B) = 1.82** **AxB = 5.46**					**Growth regulator (A) = 0.33** **Genotypes (B) = 0.22** **AxB = 0.65**					**Growth regulator (A) = 0.60** **Genotypes (B) = 0.40** **AxB = 1.201.19**				

4. Effect of different concentrations of Calcium Pantothenate during the first subculture after vitrification of *Argania spinosa*:

Data in **Table (4)** showed that shoots were cultured on medium without calcium pantothenate (control treatment) gave shoot suffered from vitrification. The best calcium pantothenate concentration in suppressing vitrification was 100 mg/l which gave the highest number of leaves, shoot length and number of shoots (71.65 leaves, 5.00 cm and 5.40 shoots, respectively).Furthermore, it was observed that the best genotype in suppressing vitrification was genotype 3 when calcium pantothenate was used which gave 63.5 leaves, 4.77 cm and 4.75 shoots.

The interaction between calcium pantothenate at different concentrations and genotypes 1, 2, 3 or 4 showed that the highest number of leaves, shoot length and number of shoots was recorded on medium supplemented with 100 mg/l calcium pantothenate on genotype 3, which gave 76.80 leaves, 5.30 cm and 5.80 shoots, respectively.

5. Effect of different concentrations of calcium pantothenate during the second subculture after vitrification of *Argania spinosa*:

The results for calcium pantothenate also in **Table (5)** showed that 100 mg/l calcium pantothenate gave the highest number of leaves, shoot length and number of shoots (80.00 leaves, 5.43 cm and 6.85 shoots, respectively) as compared to other concentrations.The results for genotypes showed that genotype 3 gave the highest number of leaves, shoot length and number of shoots which gave 68.80 leaves, 5.13 cm and 5.80 shoots, respectively.

The interaction between calcium pantothenate and genotypes found that the highest number of leaves, shoot length and number of shoots was demonstrated on medium supplemented with 100 mg/l calcium pantothenate on genotype 3, which gave 81.20 leaves, 5.80 cm and 7.60 shoots, respectively.

The results are in line with Leshem et al. (1988) on the presence of BA in the medium can cause vitrification.

6. Effect of different concentrations of calcium chloride during the first subculture to control vitrification of *Argania spinosa*:

The results recorded in **Table (6)** show that 83 mg/l the best calcium chloride concentration in suppressing vitrification was produce the highest number of leaves, shoot length and number of shoots which gave 73.42 leaves, 4.76 cm and 4.42 shoots, respectively.Result was observed also that the best genotype in suppressing vitrification was genotype 3 recorded the highest number of leaves, shoot length and number of shoots which gave 60.87 leaves, 4.49 cm and 3.60 shoots, respectively.

The interaction between Calcium chloride at different concentrations and different genotypes showed that the highest number of leaves, shoot length and number of shoots was found on medium supplemented with 83 mg/l Calcium chloride on genotype 3, which gave 76.00 leaves, 4.97 cm and 4.67 shoots, respectively.

7. Effect of different concentrations of calcium chloride during the second subculture to control vitrification of *Argania spinosa*:

The results showed in **Table (7)** note that the best calcium chloride concentration in suppressing vitrification was 83 mg/l with the highest number of leaves, shoot length and number of shoots which gave 75.33 leaves, 4.98 cm and 5.50 shoots, respectively.Result was observed also that the best genotype in suppressing vitrification was genotype 3 with the highest number of leaves, shoot length and number of shoots which gave 63.93 leaves, 4.44 cm and 4.47 shoots, respectively.

The interaction between calcium chloride at different concentrations and genotypes showed that the highest number of leaves, shoot length and number of shoots was found on medium contained with 83 mg/l calcium chloride on genotype 3, which gave 78.00 leaves, 5.20 cm and 6.00 shoots, respectively.

In the same trend, an increase of calcium concentration in the medium was an effective measure for eliminating vitrification in tissue culture for some tree species (Singha et al., 1990).

Table (4).Effect of different concentrations of calcium pantothenate (mg/l) on number of leaves, shoot length and number of shoots during the 1st subculture to control vitrification of *Argania spinosa* genotypes 1, 2, 3 and 4.

CaPantothenate(mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
Control	36.80	37.40	41.00	42.8	39.50	3.84	3.82	3.92	3.76	**3.84**	2.40	2.60	2.60	2.40	**2.50**
50	65.40	64.20	64.60	64.4	64.65	3.86	4.18	4.60	4.24	**4.22**	4.00	4.40	5.20	4.60	**4.55**
75	70.00	69.80	71.60	70.6	70.50	4.56	4.48	5.26	4.90	**4.80**	4.60	5.20	5.40	5.60	**5.20**
100	68.80	70.20	76.80	70.8	71.65	4.94	4.78	5.30	5.00	**5.00**	4.40	5.60	5.80	5.80	**5.40**
Mean (B)	**60.25**	**60.4**	**63.5**	**62.15**		**4.30**	**4.32**	**4.77**	**4.48**		**3.85**	**4.45**	**4.75**	**4.60**	
$LSD_{0.05}$ =	**CaPantothenate(A) = 3.05 Genotypes (B) = 3.05 AxB = 6.10**					**CaPantothenate(A) = 0.28 Genotypes (B) = 0.28 AxB = 0.55**					**CaPantothenate(A) = 0.50 Genotypes (B) = 0.50 AxB = 0.99**				

Table (5).Effect of different concentrations of calcium pantothenate (mg/l) on number of leaves, shoot length and number of shoots during the 2nd subculture to control vitrification of *Argania spinosa* genotypes 1, 2, 3 and 4.

CaPantothenate(mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	(A)
Control	36.80	37.40	41.00	42.8	**39.50**	3.84	3.82	3.92	3.76	**3.84**	2.40	2.60	2.60	2.40	**2.50**
50	71.20	72.60	73.80	72.8	**72.60**	4.38	4.62	5.08	4.98	**4.77**	5.40	5.00	6.00	5.60	**5.50**
75	77.80	79.60	79.20	79.6	**79.05**	5.16	5.30	5.72	5.38	**5.39**	6.20	6.00	7.00	7.00	**6.55**
100	81.00	79.60	81.20	78.2	**80.00**	5.08	5.18	5.80	5.64	**5.43**	6.20	6.40	7.60	7.20	**6.85**
Mean (B)	**66.70**	**67.30**	**68.80**	**68.35**		**4.62**	**4.73**	**5.13**	**4.94**		**5.05**	**5.00**	**5.80**	**5.55**	
$LSD_{0.05}$=	**CaPantothenate (A)= 3.02 Genotypes (B) = 3.02 AxB = 6.04**					**CaPantothenate(A)= 0.42 Genotypes (B) = 0.42 AxB = 0.84**					**CaPantothenate(A)= 0.53 Genotypes (B) = 0.53 AxB = 1.07**				

Table (6).Effect of different concentrations of calcium chloride(mg/l) on number of leaves, shoot length and number of shoots during the 1st subculture to control vitrification *Argania spinosa* genotypes 1, 2, 3 and 4.

$Ca\ Cl_2$ (mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean (A)	Genotypes				Mean (A)	Genotypes				Mean (A)
	1	2	3	4		1	2	3	4		1	2	3	4	
Control	36.67	35.00	43.67	41.00	**39.08**	4.10	4.13	4.27	4.20	**4.18**	2.33	2.67	2.67	2.67	**2.58**
83	71.00	72.33	76.00	74.33	**73.42**	4.63	4.70	4.97	4.73	**4.76**	4.33	4.33	4.67	4.33	**4.42**
166	64.33	67.67	66.33	66.67	**66.25**	4.50	4.37	4.67	4.50	**4.51**	3.67	3.33	4.00	4.00	**3.75**
244	60.33	60.67	62.33	60.67	**61.00**	4.17	4.20	4.43	4.43	**4.31**	3.33	3.33	3.67	3.33	**3.42**
332	54.00	52.67	56.00	55.67	**54.58**	4.23	4.10	4.13	4.10	**4.14**	3.00	3.00	3.00	3.00	**3.00**
Mean (B)	**57.27**	**57.67**	**60.87**	**59.67**		**4.33**	**4.30**	**4.49**	**4.39**		**3.33**	**3.33**	**3.60**	**3.47**	
LSD $_{0.05}$=	**$Ca\ Cl_2$(A) = 2.39 Genotypes (B) = 2.14 AxB = 4.78**					**$Ca\ Cl_2$ (A) = 0.21 Genotypes (B) = 0.19 AxB = 0.43**					**$Ca\ Cl_2$ (A) = 0.21 Genotypes (B) = 0.19 AxB = 0.43**				

Table (7).Effect of different concentrations of calcium chloride(mg/l) on number of leaves, shoot length and number of shoots during the 2nd subculture to control vitrification *Argania spinosa* genotypes 1, 2, 3 and 4.

$Ca\ Cl_2$ (mg/l)	No. of leaves					Shoot length (cm)					No. of shoots				
	Genotypes				Mean	Genotypes				Mean	Genotypes				Mean (A)
	1	2	3	4	(A)	1	2	3	4	(A)	1	2	3	4	
Control	36.67	35.00	43.67	41.00	**39.08**	4.10	4.13	4.27	4.20	**4.18**	2.33	2.67	2.67	2.67	**2.58**
83	73.33	74.67	78.00	75.33	**75.33**	4.77	4.90	5.20	5.03	**4.98**	5.33	5.00	6.00	5.67	**5.50**
166	67.33	68.67	72.33	72.33	**70.17**	4.33	4.67	4.47	4.40	**4.47**	4.33	4.67	5.33	5.00	**4.83**
244	63.67	63.33	65.00	64.33	**64.08**	4.07	4.33	4.23	4.17	**4.20**	3.67	4.33	4.67	4.33	**4.25**
332	61.67	60.00	60.67	58.67	**60.25**	3.97	4.07	4.03	3.87	**3.98**	3.33	3.00	3.67	3.33	**3.33**
Mean (B)	**60.53**	**60.33**	**63.93**	**62.33**		**4.25**	**4.42**	**4.44**	**4.33**		**3.80**	**3.93**	**4.47**	**4.20**	
LSD $_{0.05}$=	**$Ca\ Cl_2$(A)= 2.23 Genotypes (B) = 1.20 AxB = 4.47**					**$Ca\ Cl_2$ (A) = 0.15 Genotypes (B) = 0.13 AxB = 0.30**					**$Ca\ Cl_2$ (A) = 0.48 Genotypes (B) = 0.43 AxB = 0.97**				

8. Effect of light or dark with different concentrations of NAA or 2,4-D on callus induction of *Argania spinosa*:

Results presented in **Table (8)** show that NAA at 1.5 mg/l was better than 0.5 or 1.0 mg/l NAA and 0.5, 1.0 or 1.5 mg/l 2,4-D at light or darkness in callus induction percentage. Result indicatedthat callus induction percentage was markedly increased under darkness. Darkness was better than light in improving callus induction percentage. The highest callus induction percentage was found on NAA at 1.5 mg/l, which gave 100 percentage. The highest fresh weight was found on1.5 mg/l 2,4-D, which gave 0.699 g at light. The highest number of days till callus induction was found on 0.1 mg/l NAA (58±2 days) al light. The callus was yellow at darkness and green at light.

These results are in line with others obtained with different concentration, The maximum response of callus induction was occurred by the addition of 1.0 mg/l NAA and 1.0 mg/l 2,4D to medium (Lamaouiet al., 2019).

Table (8). Effect of darkness or light and different concentration of NAA or 2,4-D (mg/l) on callus induction (%), fresh weight of callus (g), number of days till callus induction and callus color of *Argania spinosa* after 10 weeks of culture.

Treatments		Callus induction %	Fresh weight of callus	No. of days till callus induction	Callus color
Darkness and light	Growth regulators				
Darkness	Control	-	-	-	-
Darkness	0.5 NAA	83.0	0.0661	54±2	Yellow/friable
Darkness	1.0 NAA	92.6	0.1425	56±2	Yellow/friable
Darkness	1.5 NAA	100.0	0.1982	54±2	Yellow/friable
Darkness	0.5 2,4-D	-	-	-	-
Darkness	1.0 2,4-D	-	-	-	-
Darkness	1.5 2,4-D	56.6	0.43	54±2	Yellow/friable
Light	Control	-	-	-	-
Light	0.5 NAA	78.0	0.2542	56±2	Green/friable
Light	1.0 NAA	88.0	0.3794	58±2	Green/friable
Light	1.5 NAA	100..0	0.6046	56±2	Green/friable
Light	0.5 2,4-D	-	-	-	-
Light	1.0 2,4-D	-	-	-	-
Light	1.5 2,4-D	52.0	0.699	56±2	Green/friable
LSD $_{0.05}$=		**3.64**	**0.110**		

9. Effect of different concentration of NAA or GA_3 with Kin or BA on shoot initiation of *Argania spinosa*:

Results recorded in **Table (9)** show that GA_3 was better than NAA on shoot initiation percentages. Result demonstrated that shoot initiation percentages increased with GA_3 with BA. The highest shoot initiation percentages were found on 0.5 mg/l GA_3 plus 1.5 mg/l BA, which gave 80 percentage. The highest shoot length was recorded on medium supplemented with 0.5 mg/l GA_3 and 0.5 mg/l BA (1.65 cm). The highest number of days till shoot initiation was noted on medium supplemented with 0.5 mg/l NAA and 1.5 mg/l Kin which gave shoots after 66±2 days.

Similar results were reported by Aizer et al. (2019) on *Argania*, as the

addition of 1.5 mg/l GA_3 to MS medium greatly induced a better elongation of plants.

Table (9). Effect of different concentration of NAA or GA_3 with Kin or BA (mg/l) on the percentage of shoot initiation (%), number of days till shoot initiation and shoot length of *Argania spinosa* after 8-10 weeks of culture.

Growth regulators	Shoot initiation (%)	No. of days till shoot initiation	Shoot length (cm)
0.5 NAA + 0.5 Kin	-	-	-
0.5 NAA + 1.0 Kin	-	-	-
0.5 NAA + 1.5 Kin	20	66±2	1.2
0.5 NAA + 0.5 BA	-	-	-
0.5 NAA + 1.0 BA	20	62±2	1.4
0.5 NAA + 1.5 BA	40	60±2	1.6
0.5 GA_3 + 0.5 Kin	-	-	-
0.5 GA_3 + 1.0 Kin	20	64±2	1.0
0.5 GA_3 + 1.5 Kin	60	62±2	1.26
0.5 GA_3 + 0.5 BA	40	62±2	1.65
0.5 GA_3 + 1.0 BA	60	60±2	1.50
0.5 GA_3 + 1.5 BA	80	64±2	1.32
$LSD_{0.05}$=	**6.18**		**0.16**

REFERENCES

Aizer, N., Abdellatif, N.,Saidi, F. and Chaouia, C. (2019).Initiation of micropropagation by microbouturage of Arganier (*Arganiaspinosa* L. skeels).AgroBiologia, 9(2):1628-1635.

Amghar, I., Diria, G.,Boumlik, I., Gaboun, F., Iraqi, D., Labhilili, M., Mentag, R., Meziani, R., Mazri, M. A.,Ibriz, M. and Abdelwahd, R. (2021).An efficient regeneration pathway through adventitious organogenesis for the endangered *Argania spinosa* (L.) skeels.Vegetos, 34(2):355-367.

Bennani, H., Drissi, A., Giton, F., Kheuang, L., Fiet, J .and Adlouni, A. (2007). Antiproflative effect of polyphenols and sterols of virgin argan oil on human prostate cancer cell lines. Cancer Detect Prev., (31):64–69.

Bourgaud, F., Gravot, A.,Milesi, S. and Gontier, E. (2001). Production of plant secondary metabolites: A historical perspective, Plant Sci., (161): 839– 851.

Charrouf, Z and Guillaume, D (1999): Ethnoeconomical, ethnomedical and phytochemical study of *Argania spinosa* (L.) Skeels. J Ethnopharmacol, (67):7–14.

Duclercq, J., Sangwan-Norreel, B.,Catterou,V. and Sangwan, R.S. (2011). De novo shoot organogenesis: from art to science, Trends Plant Sci., (16): 597–606.

Fernandez-García, N.,Piqueras A. and Olmos E. (2008). Sub-cellular location of H_2O_2, peroxidases and pectin epitopes in control and hyperhydric shoots of carnation. Environ. Exp. Bot., (62): 168–175.

Gao, H., Li, J., Ji H., An, L. and Xia, X. (2018).Hyperhydricity-induced ultrastructural and physiological changes in blueberry (*Vaccinium* spp.). Plant Cell, Tissue and Organ Cult., (133): 65–76.

Koufan, M., Belkoura, I., Mazri, M. A., Amarraque, A., Essatte, A., Elhorri, H., Zaddoug, F.

and Alaoui, T. (2020).Determination of antioxidant activity, total phenolics and fatty acids in essential oils and other extracts from callus culture, seeds and leaves of *Argania spinosa* (L.)Skeels. Plant Cell, Tissue and Organ Culture, (141):217–227

Lamaoui, M., Aissam, S., Wahbi, S., Chakhchar, A., Ferradous, A,, El Moousadik, A., Ibnsouda-Koraichi, S., Filali-Maltouf, A. and El Modafar, C. (2015). Anti-oxidant activity in *Arganiaspinosa* callus selected under water stress conditions. J HorticSciBiotechnol, 90(2):127–134.

Lamaoui, M., Chakhchar, A., Benlaouane, R., El Kharrassi, Y., Farissi, M.,Wahbi, S. and El Modafar, C. (2019).Uprising the antioxidant power of *Arganiaspinosa* L. callus through abiotic elicitation.ComptesRendusBiologies, 342(1/2): 7–17.

Le Polain, Y. and Waroux, Y. (2013).De´gradationenvironnementa leetde´veloppement e´ conomiquedansl'arganeraied'Aoulouz (Maroc), Science et ChangementsPlanetaires – Secheresse, 24(1):29-38.

Leifert, C.,Waites, W.M. and Nicholas, J.R. (1989). Bacterial contaminants of micropropagated plant cultures. J. Appl. Bacteriol., (67): 353–361.

Leshem, B.,Warker, E. and Shalev D.P. (1988).The effect of cytokinins on vitrification in melon and carnation. Ann. Bot., (62): 271–276.

Liu, M., Jiang, F., Kong, X.,Tian,J., Wu, Z. and Wu, Z. (2017). Effects of multiple factors on hyperhydricity of *Allium sativum* L. Sci. Hortic., (217): 285–296.

Lizard, G.,Filali-Zegzouti, Y. and Midaoui, A.E. (2017).Benefits of argan oil on human health. Int. J. Mol. Sci., 18 e1383, doi:10.3390/ ijms18071383.

Mateille, T., Tavoillot, J., Martiny, B., Dmowska, E., Winiszewska, G., Ferji, Z.,Msanda, F. and El Mousadik, A. (2016). Aridity or low temperatures: What affects the diversity of plant-parasitic nematode communities in the Moroccan argan relic forest? Appl. Soil Ecol., (101): 64–71.

Morton, J.F. and Voss, G.L., (1987).The argan tree (*Arganiasideroxylon*, sapotaceae), a desert source of edible oil. Economic Botany, (41): 221–233.

Pavlovic´, M. and Radotic´, K. (2017).Cultured Plant Stem Cells as a Source of Plant Natural Products, in: Animal and Plant Stem Cells, Springer, Cham, pp. 211–216.

Polivanova, O.B. andBedarev, V.A. (2022): Hyperhydricity in Plant Tissue Culture. Plants, (11): 3313.

Rao, S.R. and Ravishankar, G.A. (2002). Plant cell cultures: chemical factories of secondary metabolites, Biotechnol. Adv., (20) :101–153.

Saher, S.,Piqueras, A.,Hellin, E. and Olmos, E. (2004).Hyperhydricity in micropropagated carnation shoots: The role of oxidative stress. Physiol. Plant., (120): 152–161.

Schmid, D. and Zu¨lli, F. (2012). Use of Plant Cell Cultures for a Sustainable Production of Innovative Ingredients, SO¨ FW, p. 138.

Singha, S., Townsend, E.C. and Oberly, G.E. (1990). Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydoniaoblonga* Mill.) shoots *in vitro*. Plant Cell, Tissue and Organ Cult., (23): 135–142.

Sonriza M.Rasco and Lilian F. Patena (1997).*In vitro* shoot vitrification (hyperhydricity) in shallot (*Allium cepa* var. gr. aggregatum) Philipp. J. Crop. Sci., (22): 14–22.

Steel, R.G. and Torrie, J.H. (1980).Principles and Procedures of Statistics.A Biometrical Approach. McGraw. Hill Book Company, New York, USA., 633 p.

Tito, A., Carola, A., Bimonte, M., Barbulova, A., Arciello, S., De Laurentiis, F., Monoli, I., Hill, J., Gibertoni, S.,Colucci, G. and Apone, F. (2011). A tomato stem cell extract, containing antioxidant compounds and metal chelating factors, protects skin cells from heavy metal induced damages. Int. J. Cosmet. Sci., (33): 543–552.

Trehan, S.,Michniak-Kohn, B. and Beri, K. (2017). Plant stem cells in cosmetics: current trends and future directions, Future Sci. OA 3 FSO226, doi:10.4155/fsoa-0026.

Žd'árská, M.,Zatloukalová, P.,Benítez, M.,Šedo, O.,Potěšil, D.,Novák, O.,Svačinová, J.,Pešek, B.,Malbeck, J. and Vašíčková, J. (2013). Proteome analysis in arabidopsis reveals shoot- and root-specific targets of cytokinin action and differential regulation of hormonal homeostasis. Plant Physiol., (161): 918–930.

Ziv, M. (1991).Quality of micropropagated plants–vitrification*in Vitro* Cell. Dev. Biol.Plant, (2):64–69.

Ziv, M., Schwartz, A. and Fleminger, D. (1987). Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated in vitro; implications for hardening. Plant Sci., 52: 127–134.

الملخص العربى

التحكم في تزجيج الأجزاء النباتية وتكاثرها أثناء إنتاج *Argania spinosa* L

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الهدف من هذه الدراسة هو التوصل إلى بروتوكول جيد للإكثار المعملى لنبات*Argania spinosa*. تم الحصول على أكبر عدد من الأفرع عند اضافه 2.0 ملجم/لتر كينتين للطراز الجيني 3 وذلك في مرحلة التضاعف. وبيئة MSالمضاف إليها 100 ملجم / لتر بانتوثينات الكالسيوم كانت مناسبه للتغلب على التزجج في جميع الطرز الجينية للنبات. بينما كانت بيئة MS المضاف إليها 1.5 ملجم/لتر NAAوتحضينه في كلا من الضوء أو الظلام مناسبه لتشجيع تكوين الكالس. أدى إضافة 0.5 ملجم/لتر 1.5 +GA_3 ملجم/لتر BA إلى البيئة الي زيادة الأفرع المتكونة من الكالس.