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PLANT PRODUCTION

In vitro Clonal Propagation of "Al-Belehi" Pomegranate (*Punica granatum* L.)

Abdelrahman S. A. Al-Wasel
Dept. of Horticulture and Forestry, College of Agriculture and Veterinary Medicine, King Saud University, AlQassim, Saudi Arabia

Abstract. *In vitro* clonal propagation of pomegranate (*Punica granatum* L.) cv Al-Belehi was achieved by culturing nodal segments from one-year-old plants grown in the greenhouse on three different Murashige and Skoog (MS) media (solid, liquid and double-phase). Solid MS medium alone or with 0.5 or 1.0 mg naphthalene acetic acid (NAA) produced the highest number of bursting buds and the longest shoots. The highest shoot proliferation rate occurred on auxin-free media containing 2 or 4 mg 6-benzyladenine (BA) (3.33 and 3.4 shoots/explant, respectively). Thidiazuron (TDZ), especially at high levels, resulted in a notable reduction in shoot number and shoot length. The optimal combination of growth regulators was 0.5 mg BA and 0.1 mg NAA. Considerable adventitious rooting occurred on half strength MS salts. The highest rooting percentage (92%) was obtained with 0.5 mg NAA. Above 80% of the rooted shoots were transferred successfully to the soil.

Introduction

Pomegranate (*Punica granatum* L.) is one of the most common fruit trees grown in Saudi Arabia. It is almost grown and adapted to most parts of the country. It thrives under arid and semi arid conditions and it is relatively capable of tolerating salinity and drought. It is a deciduous tree, and mainly cultured for fruit production and as an ornamental and medicinal plant [1, 2].

Hardwood cuttings, suckers or air-layering are the common methods practiced for propagation of pomegranate since sexual propagation does not give rise to true to type progenies [2, 3]. However, the propagation of pomegranate by cuttings or other conventional vegetative methods requires a large number of stock plants which means also a lot of space in the greenhouse. Over the last few years, tissue culture technique has been successfully utilized to obtain a large number of woody fruit trees (rootstocks and varieties) by stimulating the development of axillary buds *in vitro* [4; 5, pp. 15-26; 6, pp. 51-139] and through morphogenesis [7, pp. 214-314]. Successful *in vitro*

propagation of some pomegranate cultivars through shoot tips, axillary buds or morphogenesis was reported [8-12; 13, pp. 215-217; 14; 15].

Thus, the present study was conducted to develop a protocol for *in vitro* propagation of pomegranate cv AL-Belehi. This protocol may be utilized in future studies for improving pomegranate through tissue culture technique.

Material and Methods

Establishment of aseptic culture

Initial explants were obtained from one-year-old rooted cuttings which were prepared from mature trees grown at the College of Agriculture and Veterinary Medicine, King Saud University, Research Station at Al-Qassim, Saudi Arabia. Nodal segments were prepared from the new growth after stripping all leaves. To remove the phenolic compounds, the explants were agitated for one hour in sterilized distilled water and then agitated for one hour in antioxidant solution containing 150 mg/ℓ of each of ascorbic acid, citric acid and polyvinylpolypyrrolidone (PVP). Surface-sterilization was carried out for 10 min. with 10% Clorox solution (5.25% sodium hypochlorite) with 2 drops/ℓ of tween 20 and rinsed 3 times with sterilized distilled water. Then, the explants were implanted on 3 different MS media [16]; solid, liquid, and double phase (solid phase + liquid phase on the top of the solid phase); containing 30 g/ℓ sucrose, 7 g/ℓ agar (Micro Agar, DUCHEF Biochemicals, the Netherlands) and a combination of 0.0, 0.3, and 0.6 mg/ℓ TDZ and 0.0, 0.5, and 1.0 mg/ℓ NAA. All explants were cultured in Magenta GA7 (Sigma Chemical Company, Plant Culture, St. Louis, Missouri, 63178, USA) containers containing 50 ml of the solid or the liquid phase media. For the double phase media, 10 ml of the liquid media were poured on the top of 50 ml solid phase media, after the inoculation of the explants. Membrane raft (Sigma Chemical Company, Plant Culture, St. Louis, Missouri, 63178, USA) was used with the liquid media to hold explants in place. Six Magenta, each with one explant, were assigned for each treatment. The Explants in the solid and double phase media were transferred to other locations within the same magenta after 24 hours to save them from the adverse effects of the exudated phenols.

Shoot proliferation and rooting

Shoots of about 15-20 mm in length from the above experiment were excised and then cultured in 15 x 150 mm tubes containing 15 ml of MS medium supplemented with 30 g/ℓ sucrose, 7 g/ℓ agar, 10 mg/ℓ gentamicin sulphate and various levels of BA (0.0, 0.5, 2, 4 mg/ℓ) and NAA (0.0, 0.1, 0.5 mg/ℓ) or TDZ (0.5, 1.5, 2.5, 3.5, 4.5 mg/ℓ) and NAA (0.0, 0.1 mg/ℓ). Ten test tubes were assigned for each treatment.

For rooting, 15-20 mm long shoots were transferred to 15 x 150 mm tubes containing 15 ml of half strength MS salt supplemented with 30 g/ℓ sucrose, 7 g/ℓ agar, 10 mg/ℓ gentamicin sulphate and 0.1, 0.5, 1.0, and 2.0 mg/ℓ of indole-3-acetic acid

(IAA), indole-3-butyric acid (IBA), or NAA. Twelve test tubes were assigned for each treatment.

The pH of all media was adjusted to 5.7 prior to autoclaving for 20 minutes at 121° C. All cultures were incubated in 16 hour light/ 8 hour dark cycle at 25± 2° C and were illuminated with fluorescent lights with an intensity of 2500-3000 lux.

The rooted shoots were acclimatized by transferring plantlets to small plastic pots filled with sterile perlite. They were covered with plastic bags for a week to maintain high humidity and then they were transferred to the greenhouse under the mist.

Shoot numbers, shoot length, callus diameter, root number, and root length were recorded after 6-8 weeks. These data were subjected to analysis of variance using WINKS statistical data analysis program [17]. Separation of treatment means was performed using Newman-Keuls test.

Results

In general, all explants cultured on the three different MS media without growth regulators gave higher number of bursting buds and longer shoots. However, the highest number of bursting buds, with almost all buds grew out, and the longest shoots (15.43 mm) were promoted on the hormone-free solid phase media, followed by solid media supplemented with 0.5 or 1.0 mg NAA (Fig. 1a) (Tables 1, 2 and 3). In contrast, larger callus were formed on the double phase media with or without growth regulators (Fig. 1b). The largest calli which were 5.35 and 6.07 mm in diameter were significantly induced by 0.3 and 0.6 mg/L TDZ, respectively (Tables 1, 2 and 3).

The highest shoot proliferation rate was brought about by 2 and 4 mg BA, but there were no significant differences among the BA levels (Fig. 2). With the exception of 0.1 mg NAA at 0.5 mg BA, the addition of NAA significantly reduced the number of shoots (Table 4). TDZ, especially at the highest concentration, exhibited lower shoot number. The highest proliferation rate was observed with 0.5 and 1.5 mg TDZ, irrespective of the presence of NAA (Table 5). None of the developed shoots arose from callus; they all originated from the base of the microshoot or from the pre-formed buds.

Regarding the shoot length BA alone or with 0.1 mg NAA gave significantly longer shoots, 40.0 and 35.3 mm, respectively. No significant differences were observed among TDZ levels and the shoot height varied between 0.4 and 0.9 mm. Although the TDZ with NAA produced the largest callus diameter, no significant differences were observed among all the combinations of TDZ and NAA (Table 5). BA gave smaller callus diameter and no callus formation was induced by 0.0 or 0.1 mg NAA without

Table 1. Effects of medium phase and TDZ on culture establishment of pomegranate cv Al-Belehi *in vitro*

	TDZ (mg)									
	0.0	0.3	0.6		0.0	0.3	0.6	0.0	0.3	0.6
	Shoot no.			Ave.	Shoot length (mm)			Callus diameter (mm)		
Medium										
Solid	1.79	.87	0.77	1.14A[1]	15.43Aa[2]	1.73Ab	2.00Ab	0.00A	2.73Ba	3.00Ba
Liquid	1.18	0.33	0.27	0.62B	3.29Ba	1.00Aa	0.27Ab	0.59Aa	0.93Ca	1.20Ca
Double Phase	1.13	0.12	0.29	0.51B	3.38Ba	0.24Ab	0.29Ab	1.44Ab	5.35Aa	6.07Aa
Ave.	1.34a	0.43b	0.43b							
Level of Significance[w]										
Medium	*									
TDZ	*									
Medium x TDZ	NS					*			*	

[w] NS not significant, * Significant by Newman-Keuls at the 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

[2] Means within rows bearing different upper case letters are significantly different at the 5% level.

Table 2. Effects of medium phase and NAA on culture establishment of pomegranate cv Al-Belehi *in vitro*

	NAA (mg)											
	0.0	0.5	1.0		0.0	0.5	1.0		0.0	0.5	1.0	
	Shoot no.			Ave.	Shoot length (mm)			Ave.	Callus diameter (mm)			Ave.
Medium												
Solid	1.14	1.24	1.00	1.14A[1]	4.43	7.47	7.18	6.38A	2.50	1.82	1.27	1.91C
Liquid	0.69	0.67	0.50	0.62B	1.75	2.07	1.00	1.60B	1.25	0.93	0.50	0.89B
Double Phase	0.60	0.56	0.38	0.51B	2.13	1.25	0.63	1.32B	4.07	3.88	4.75	4.23A
Ave.	0.80a[2]	0.83a	0.58a		2.71a	3.71a	2.44a		2.58a	2.23a	2.28a	
Level of Significance[w]												
Medium			*				*				*	
NAA			NS				NS				NS	
Medium x NAA			NS				NS				NS	

[w] NS not significant, * Significant by Newman-Keuls at the 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

[2] Means within rows bearing different upper case letters are significantly different at the 5% level.

Table 3. Effects of TDZ and NAA on culture establishment of pomegranate cv Al-Belehi *in vitro*

	NAA(mg)										
	0.0	0.5	1.0	0.0	0.5	1.0		0.0	0.5	1.0	
TDZ(mg)	Shoot no.			Shoot length (mm)			Ave.	Callus diameter (mm)			Ave.
0.0	0.93A[1]b[2]	1.75Aab	1.31Aab	4.93	9.81	5.94	6.89A	0.73	0.75	0.63	0.703
0.3	0.56Aa	0.50Ba	0.15Ba	1.44	0.83	0.54	0.94B	3.31	2.50	3.69	3.17A
0.6	0.93Aa	0.21Bb	0.14Bb	1.79	0.43	0.21	0.81B	3.71	3.57	2.86	3.38A
Ave.				2.71a	3.71a	2.44a		2.58a	2.23a	2.28a	
Level of significance[w]											
TDZ					*				*		
NAA					NS				NS		
TDZ x NAA		*			NS				NS		

[w] NS not significant, * Significant by Newman-Keuls at the 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

[2] Means within rows bearing different upper case letters are significantly different at the 5% level.

Table 4. Effects of BA and NAA on proliferation rate of pomegranate cv Al-Belehi *in vitro*

	NAA (mg)									
	0.0	0.1	0.5	0.0	0.1	0.5	0.0	0.1	0.5	
BA (mg)	Shoot no.			Shoot length (mm)			Callus diameter (mm)			Ave.
0.0	0.71B[1]a[2]	1.67ACa	1.13Aa	9.40Ba	13.90Ba	7.70Aa	0.00	0.00	0.75	0.25B
0.5	2.80Aa	3.13Aa	0.88Ab	40.00Aa	35.30Aa	7.80Ab	4.13	3.00	6.38	4.50A
2.0	3.33Aa	1.67ACb	1.11Ab	8.80Ba	3.60Ba	9.30Aa	3.56	4.78	3.00	3.78A
4.0	3.40Aa	1.00BCb	1.90Ab	9.20Ba	3.30Ba	5.90Aa	6.70	4.33	4.90	5.31A
Ave.							3.89a	3.03a	3.80a	
Level of significance[w]										
BA								*		
NAA								NS		
BA x NAA		*			*			NS		

[w] NS not significant, * Significant by Newman-Keuls at the 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

[2] Means within rows bearing different upper case letters are significantly different at the 5% level.

Table 5. Effects of TDZ and NAA on proliferation rate of pomegranate cv Al-Belehi *in vitro*

	NAA (mg)								
	0.0	0.1		0.0	0.1		0.0	0.1	
TDZ (mg)	Shoot no.		Ave.	Shoot length (mm)		Ave.	Callus diameter (cm)		Ave.
0.5	2.30	2.50	2.40	8.00	9.00	8.50	10.50	12.90	11.70A[1]
1.5	2.10	2.50	2.30	7.00	7.00	7.00	10.10	11.90	11.00A
2.5	1.80	1.40	1.60	7.00	9.00	8.00	11.80	10.50	11.15A
3.5	1.10	1.80	1.45	6.00	8.00	7.00	7.00	12.80	9.90A
4.5	1.56	0.90	1.20	8.00	4.00	5.79	4.44	7.30	5.95B
Ave	1.78	1.82		7.20	7.40		8.77b[2]	11.08a	
Level of significance[w]									
TDZ		NS		NS			*		
NAA		NS		NS			*		
TDZ x NAA		NS		NS			NS		

[w] NS not significant, * Significant by Newman-Keuls at the 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

[2] Means within rows bearing different upper case letters are significantly different at the 5% level.

Table 6. Effects of auxins (IAA, IBA, NAA) on rooting induction and development of pomegranate cv Al-Belehi *in vitro*

Growth Regulator	Concentration (mg/L)	Shoot height (mm)	Callus diameter	Root no.	Root length (mm)	Rooting %
IAA	0.1	38.10	0.0e[1]	0.78c	0.86e	14.30
	0.5	36.71	1.86de	1.36bc	7.43abcde	50.00
	1.0	41.36	2.64cd	1.93bc	6.64bcde	64.30
	2.0	33.00	2.85cd	0.92bc	5.00bcde	38.50
IBA	0.1	51.10	4.45c	6.18ab	12.64abc	72.70
	0.5	30.25	6.10b	3.83abc	3.30cde	41.70
	1.0	43.73	8.18a	7.18a	7.00abcde	72.30
	2.0	28.46	6.46b	2.00abc	1.64de	27.30
NAA	0.1	44.27	0.0e	4.18abc	17.00a	82.00
	0.5	44.00	0.0e	7.00a	13.75ab	92.00
	1.0	33.20	0.0e	4.20abc	11.80abcd	80.00
	2.0	36.44	0.0e	5.33abc	8.78abcde	77.80
Level of significance[w]		NS	*	*	*	

[w] NS not significant, * Significant by Newman-Keuls at 5% level.

[1] Means within columns followed by different lower case letters are significantly different at the 5% level.

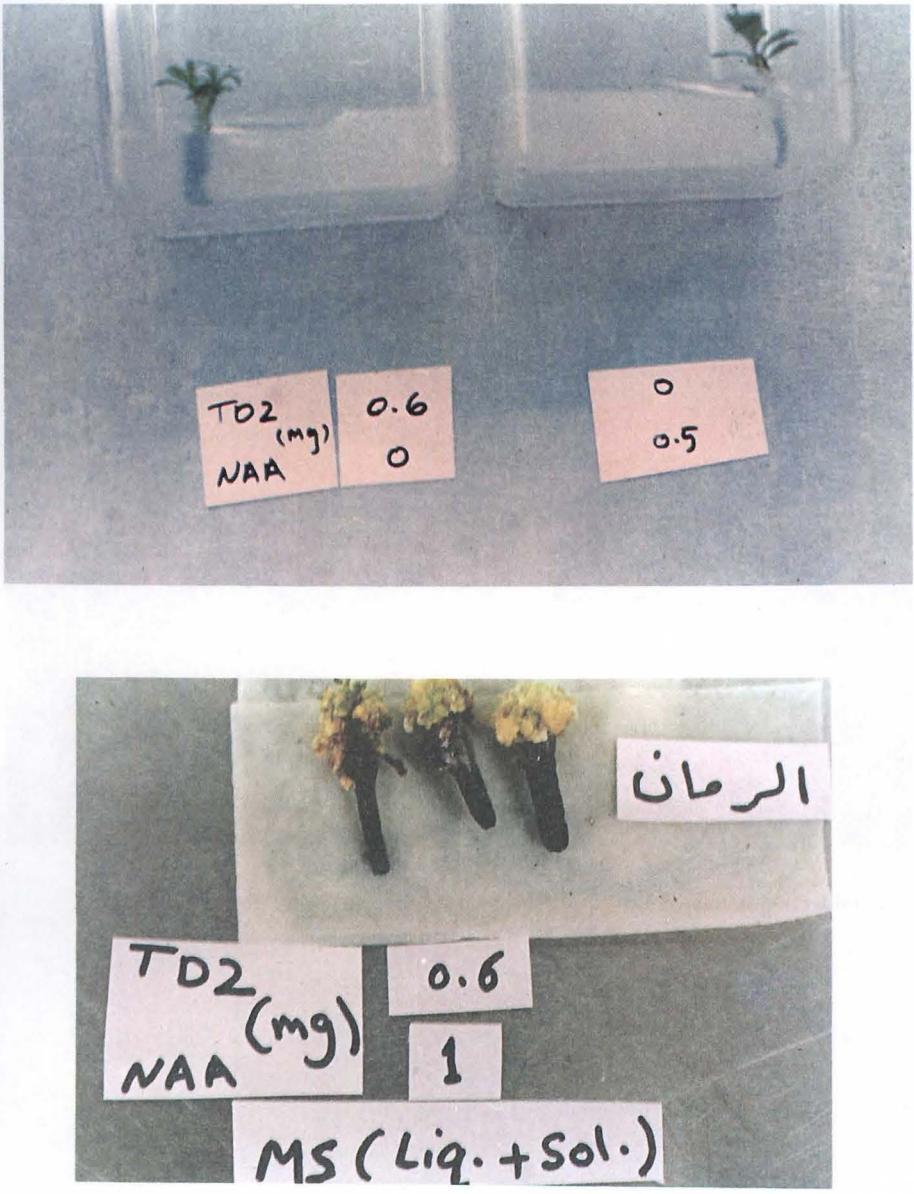

Fig. 1. Establishment of aseptic culture of Al-Belehi pomegranate (*Punica granatum* L.) *in vitro* using nodal segments as explants. A, bursting buds in solid MS medium at different levels of TDZ and NAA. B, Callus formation on double phase MS medium and no shoots developed from buds.

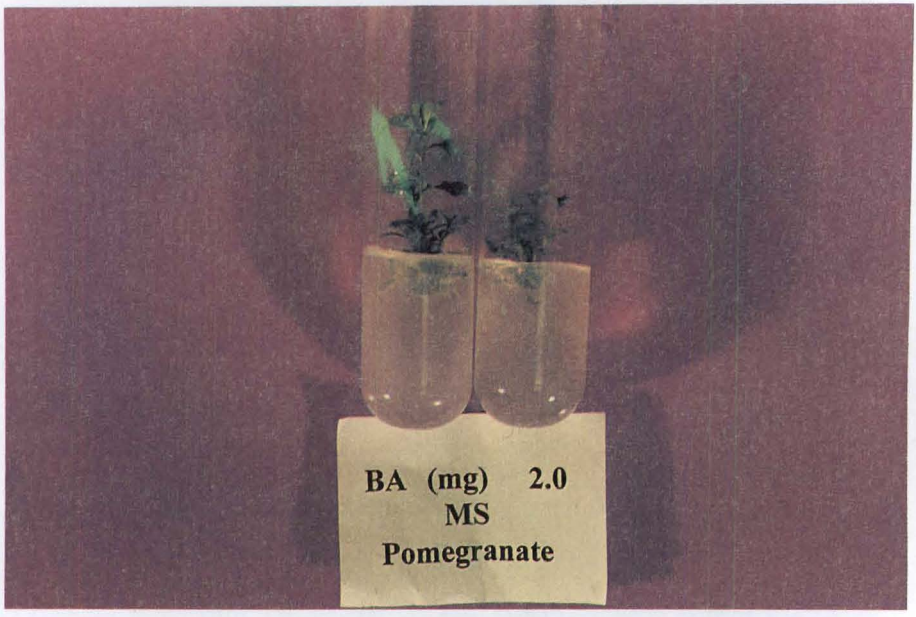

Fig. 2. Proliferation of pomegranate on MS medium supplemented with 2 mg/ℓBA.

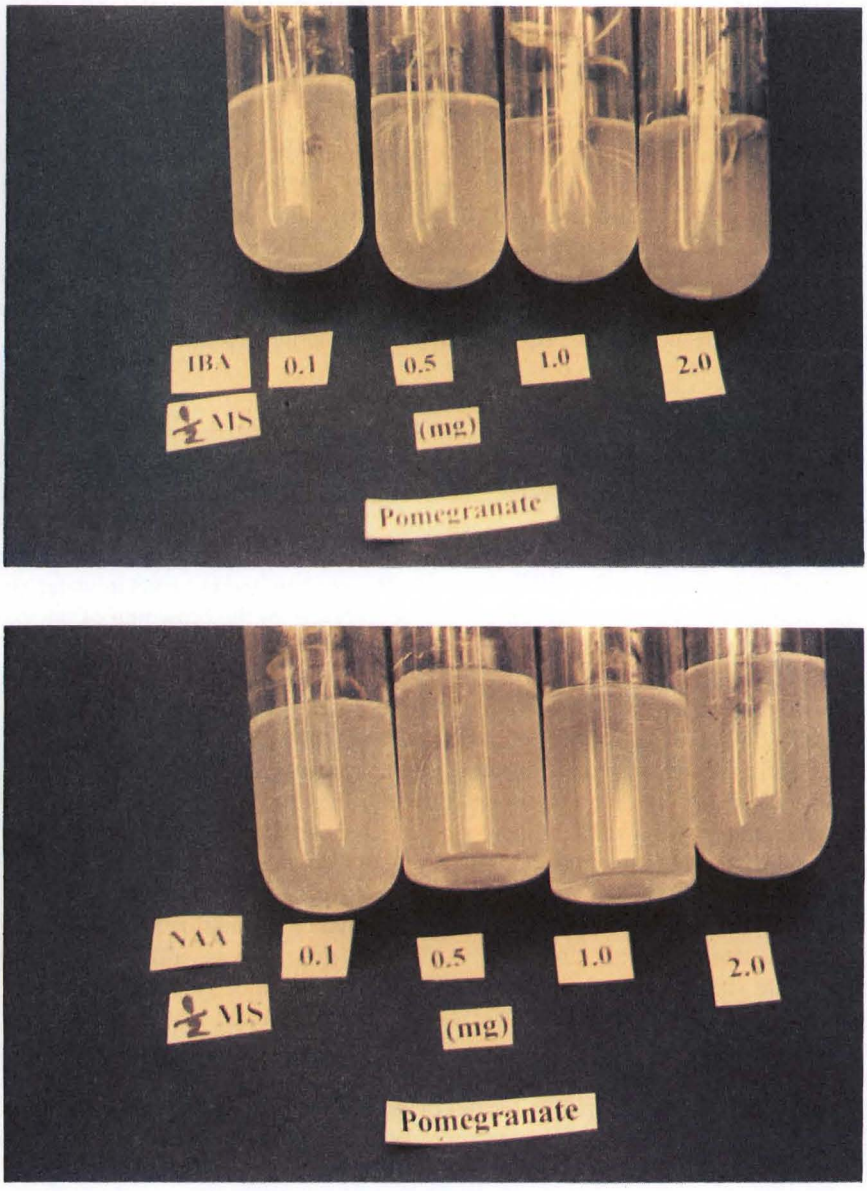

Fig. 3. Rooted microshoots on half strength MS medium supplied with different auxin types. A, Microshoots rooted on different levels of IBA. B, Microshoots rooted on different levels of NAA.

BA. The larger callus diameters were exhibited by 4 mg BA without NAA and by 0.5 mg BA with 0.5 mg NAA (Table 4).

The highest rooting percentage (77.8 - 92%) was obtained on half strength MS salts supplemented with NAA (Fig. 3b). The best NAA level was 0.5 mg. All NAA levels yielded a high number of roots (4.2-7.0 roots/microshoot) and long roots (8 - 17 mm) (Table 6). IBA showed high rooting percentage (> 72%) and root number, 6.2 and 7.2 roots/microshoot at 0.1 and 1.0 mg IBA, respectively (Fig 3a and Table 6). IAA gave the lowest rooting percentage and root number. Callus induction occurred with all IAA and IBA concentrations, except 0.1 mg IAA. The largest callus diameter was significantly observed at 2 mg IBA. No callus formation was observed at all NAA concentrations. More than 80% of the plantlets survived the transfer to the greenhouse.

Discussion

One of the main problems confronting the successful establishment of most of the woody plants *in vitro* is the effective removal of the phenolic compounds (discoloration of the medium and browning or blackening of the explants) [18, pp. 168-189]. This study showed that the elimination of the phenols is also necessary for the establishment of pomegranate cv Al-Belehi culture *in vitro*. Pre-soaking the explants in water and then in the antioxidant solution prevented the occurrence of the browning of the explants. Thus, this method was found to be effective in reducing the necrosis of the cultured tissue. Mahishi *et al.* [13] reported that the addition of PVP-360 to the media and transferring the explants to fresh media, 4-6 hours after initial inoculation, is effective in the establishment of the pomegranate selection (HS-4). Similarly, the removal of the phenols from guava was done by pre-soaking the explants in the antioxidant solution [19, pp. 235-243]. In the present study, culturing the initial explants of Al-Belehi pomegranate in solid media without or with 0.5 or 1.0 mg NAA not only resulted in highest number of bursting buds but also promoted shoot elongation.

The Cytokinin BA was better for pomegranate proliferation than the TDZ, which reduced the shoot number and also resulted in short shoots. TDZ may be useful in pomegranate improvement since it stimulated dramatically callus formation. The optimal BA concentrations that gave high proliferation rate were 2 and 4 mg, and the combination of 0.5 mg BA and 0.1 mg NAA. However, shoot elongation was decreased at the two highest BA levels and 0.5 mg NAA. Likewise, Zhang and Stolz [14] found the optimal shoot number of pomegranate cv Nana was induced by 0.2 or 0.4 mg BA and 0.2 mg NAA whereas higher concentrations of BA and NAA resulted in the reduction of shoot elongation.

High rooting percentage, root number and root length were achieved with half strength MS media with NAA. These results agreed with the findings obtained with other pomegranate cultivars [9; 13, pp. 215-217]. However, the optimal NAA level in

this study was different from theirs, indicating that most likely a genotype-dependent response. The auxin NAA produced profuse rooting with good vascular connections, because it did not induce callus formation which resulted in poor connections between the microshoots and the roots that may develop only from callus.

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التكاثر الخضري للرمان صنف " البليهي" في الأنابيب

عبد الرحمن بن صالح بن عبدا لرحمن الواصل

قسم البساتين والغابات، كلية الزراعة والطب البيطري، جامعة الملك سعود، القصيم، المملكة العربية السعودية

ملخص البحث. تم إكثار الرمان صنف البليهي عن طريق زراعة الأنسجة وذلك بزراعة براعم أبطية مأخوذة من نباتات عمرها سنة نامية في البيوت المحمية، على ثلاث بيئات مختلفة من بيئة موراشيجي وسكوج (صلبة، سائلة، وشبه صلبة). وقد أثبتت النتائج أن بيئة موراشيجي وسكوج الصلبة الخالية من الهرمونات أو المضاف إليها نفثالين حمض الخليك (NAA) شجعت أعلى عدد من البراعم على التفتح والنمو، وكذلك أعطت أطول النموات الخضرية. وكان أعلى معدل تضاعف للنموات الخضرية علي البيئات المحتوية علي تركيز ٢ أو ٤ مجم من هرمون البنزيل أدينين (BA) بينما سببت التركيزات العالية من هرمون الثيديزرون (TDZ) نقصا ملحوظا في عدد وأطوال النموات الخضرية المتكونة. وجد أيضا أن التوليفة المثلي للهرمونات كانت ٠,٥ مجم بنزيل أدنين و ٠,١ مجم نفثالين حمض الخليك.

حدثت أعلي نسبة تجذير (٩٢٪) للنموات الخضرية على نصف تركيز أملاح بيئة موراشيجي وسكوج والمزودة بـ ٠,٥ ملجم نفثالين حمض الخليك. كما تم أقلمة اكثر من ٨٠٪ من النباتات المجذرة بنجاح في البيوت المحمية.