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WORTHY MICROPROPAGATION PROTOCOLS OF SEVEN CULTIVARS OF POMEGRANATE (PUNICA GRANATUM L.) CULTIVARS IN THE PROVINCE OF DUHOK, KURD ISTAN RIGION OF IRAQ.

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ABSTRACT:

An efficient attempt was conducted to increase the mass production of seven local cultivars of pomegranate (Punica granatum L.) Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and Dwarf pomegranate cultured in the Kurdistan region of Iraq from October 2021 to February 2023. Micropropagation technique was applied using eight combinations of plant growth regulators (PGRs) added to two media (MS and WPM). The results indicated that immersing explants in 70% Ethyl alcohol (EtOH) for (2 minutes) followed by immersion in (2.5%) Sodium Hypochlorite (NaOCl) plus 3 drops of tween 20 for 13 mins. was the more suitable combination for explant sterilization. MS and WPM media salts supplemented together with eight combinations of PGRs were carried out to evaluate the more suitable combination for the initiation stage. The obtained data clarified that the ½ strength of the MS medium (1/2 MS) supplemented with 0.5 BAP plus 0.5 mg/l Kinetin increases the pomegranate explant initiation response percentage. WPM basal medium was the preferred media to be used in the multiplication stage. The impact of different levels and protocols of Cytokinins, Auxins, and Gibberellins were investigated by eight protocols including using BAP, GA3, and adenine sulfate. The outcomes indicated that adding both concentrations of BAP (0.5 and 1.0 mg/l) separately supported by GA3 and adenine sulfate in amounts of (0.3 and 30 mg/l) respectively again were the most suitable protocol that affected the multiplication of regenerated shoots when compared to using BAP alone. Valuable results were found in rooting response after 6 weeks by using ½ strength of MS media along with two levels of Auxins (NAA, and IBA). Moreover, the visual observation records that around 85-90% of rooted cultures were successfully acclimatized by transferring the cultures from in vitro to the ex-vitro environment. Finally, Dwarf cultivars exceed all the other cultivars in studded parameters.

KEYWORD: Punica granatum L., WPM, MS, BAP, IBA, NAA, Pomegranate micropropagation.

1. INTRODUCTION

The term "pomegranate" is derived from two Latin words, pomum, which means "an apple," and granatus, which means "full of seeds." The pomegranate (*Punica granatum* L.) is a member of the family Punicaceae, which also includes both *Punica granatum* and *Punica protopunica*. Tropical and subtropical areas are where it is primarily grown. According to Kumar *et al*. (2017), it first appeared in Iran and then expanded to the Mediterranean areas of Asia, Africa, and Europe. Pomegranates, “an economically significant fruit crop,” are grown commercially as fresh fruit. It is very nutritional, high in proteins, lipids, fibers, carbohydrates, and ions e.g., ferrous, and calcium, in addition, to the antioxidants such as tannins, phenols, and pigments. Due to the ability to treat illnesses like leprosy and dyspepsia, traditional treatments for diarrhea, dysentery, and intestinal parasites include the fruit’s peel and tree’s bark (Pal *et al.*,2014).

A suitable pomegranate planting material can be produced in large quantities using an *In vitro* propagation process, making commercialization possible. Micropropagation in pomegranate can initiate by regenerating existing meristems, adventitious meristems, and somatic embryogenesis (Guruanna *et al.*, 2017). Several explants such as leaf segments (Omura *et al.*,1987and Murkute *et al*., 2002) and cotyledon explants (Raj & Kamlesh, 2008 and Kanwar *et al.*, 2010) were the source of regenerated callus. Therefore, the current investigation was carried out to find out a valuable and reproducible combination of huge mass production of healthy propagules of a pomegranate via tissue culture technique avoiding the seasonal barrier by using different concentrations and combinations of plant growth regulators by selecting the more suitable one and its combination levels for pomegranate shoots and roots proliferation and acclimatization to develop a reliable and successful micropropagation protocol for pomegranate mass production.

2. MATERIAL AND METHODS

2.1. Explant surface sterilization and initiation

The Scientific Research Center of the College of Science at the University of Duhok was the site of the current investigation, from October 2021 to February 2023 to study the micropropagation of seven different pomegranate cultivars, Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and dwarf pomegranate in the province of Duhok, Kurdistan region of Iraq. Explants of (7 cultivars) of pomegranate, as mentioned above, were collected in the middle of October 2021 by taking healthy juvenile shoot cuttings containing many nodes and about 1-2 cm with 2-3 nodes were selected as a source of initial micropropagation experiments. Running water (tap water) was used for washing the explants before sterilization, in separate jars with a few drops of local liquid detergent for 30 minutes.

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The jars were then transferred to the sterilized zone in the laminar flow cabinet to start the different protocols for sterilization by dipping them in (70%) (EtOH) for (2 minutes), proceeding by soaking them in sodium hypochlorite (2.5% NaOCl) with three drops of tween twenty for 13, 15, and 20 min., the explants then were three times swilled in sterilized distilled water to remove the effect of sterilant. The improper explants were removed followed by cutting a small distance from both sides of healthy undamaged explants. Suitable size explants (about 1 to 2cm long with at least two nodes) were dried on sterilized filter papers and prepared to culture in the initiation medium.

The initial stage started by using 2 kinds of growth media MS (Murashige and Skoog, 1962), and woody plant medium WPM (LIoyd & McCown, 1980) in four protocols for each medium. MS full-strength salts supplemented first with 0.5mg/l of BAP (Benzyl amino purine) and 0.5mg/l Kinetin (medium A), second (medium B) with 1.0 mg/l of BAP and kinetin 0.5 mg/l. The same combinations were tested by using half-strength of MS salts (medium C and D) respectively. The similar four combinations were examined regarding the second initial medium (WPM) (medium E, F.G, and H). Three explants were cultured per jar, each treatment includes 5 replicates. A controlled growth room ($25 \pm 2C^{O}$) was used for the incubation of the cultures and photoperiodic cycles of 16 hours of light and 8 hours of dark which offers around 2000-2500 lux of light intensity, the observations responses parameters (shoot initiation response, healthy explants and contaminated explants %) were recorded after six weeks of incubation.

2.2. Multiplication of Shoots

WPM basal medium was utilized after establishing aseptic cultures to select and evaluate the best combination in the multiplication stage. The influence of variable concentrations protocols of Cytokinins, Gibberellins, and Auxins were investigated: BAP alone, in three concentrations of (0, 1.0, and 2.0 mg/l) of BAP, identical concentrations of BAP combined enrichment by 0.1mg/l NAA and same levels of BAP were repeatedly augmented by 0.3 mg /l of GA_3. Another combination was selected for shoot proliferation by adding 1, 0.3 and 30 mg/l of BAP, GA_3 and adenine sulfate respectively to improve and enhance the most suitable combination. All the cultures in the above combinations and protocols included three explants per jar with five replicates (jars) of each treatment, and the cultures were then kept under controlled conditions in the culture room. After six weeks in culture, the shoots number, average length of shoot, highest shoot length, and leaves number parameters were recorded.

2.3. Shoots Rootings

Proper uniform single shoots were excised and chosen from all seven cultivars for the formation of roots utilizing basal MS medium used in three combinations: (1/2 MS) medium without Auxin as control treatment. (1/2 MS) combined with 0.5 mg/l (IBA) once and supported with 0.5 mg/l on Naphthalin Acetic Acid (NAA). Each treatment included three replicates with three plants for each jar. After six weeks in culture, the rooting percent, number of roots, and average length of roots were measured.

2.4. Acclimatization of Plantlets

The plantlets were treated with (0.1%) of Benlate fungicide solution for 5 minutes after being thoroughly washed with tap water to remove any potential contaminants from the roots. The plantlets were then transferred to pots containing an autoclaved mixture of peat moss, loam, and Styrofoam in the ratio of (1:1:0.5) (v:v:v), which were then placed in sterile boxes and covered with polyethylene bags to maintain high relative humidity. For fifteen days, the potted plants were kept in an incubator. To promote growth during the first week, the plantlets were foliar sprayed with MS liquid media at a quarter salt strength (1/4 MS). The plastic bag suffered numerous punctures in the second week.

After that, the cover was gradually opened until the plantlets were free of it, allowing them to gradually acclimate. The pots then were covered with polyethylene bags and grown in culture in a growth room at 25°C with a photoperiod (16 hours) of roughly 2000 lux illumination each day. The obtained data for all studied parameters were then subjected to SPSS programs (SPSS, 2019) to statistically analyze using one-way ANOVA, two-way ANOVA, and three-way ANOVA, in addition to the descriptive statistics for the studied parameters. The means of the studied parameters were estimated by using the Duncan test (Duncan, 1955).

3. RESELTS

3.1. Explant surface sterilization and initiation

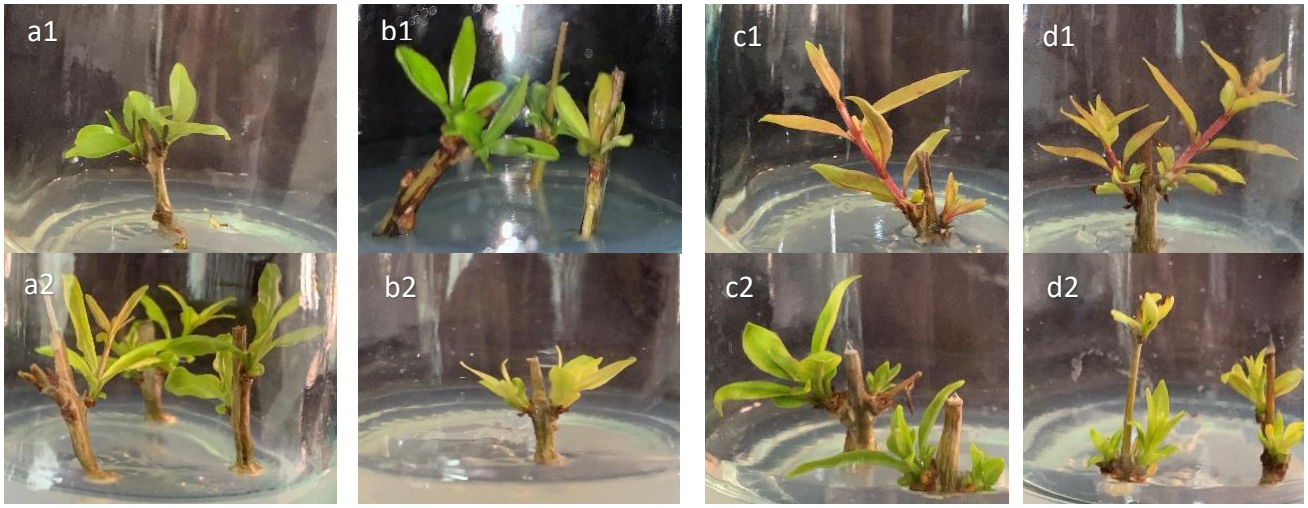
Visual observations showed that using (70%) ethanol for 2 minutes followed by soaking the explants in sodium hypochlorite (NaOCl) solution (2.5%) with three drops of tween twenty for 13 min. was more suitable combination for explant sterilization as shown in Table (1), which refers the ratio of healthy explants grown in MS media that Dwarf cultivar record the highest (100%) healthy uncontaminated explants followed by Diala cultivar with 95%, while Masafik, Halapja, Armishte, Radisho and Melisse that recorded (75, 60, 55, 50 and 45%) respectively. Back to Table (2), the ratio of the healthy explant grown in WPM medium was as followe: Dwraf (100%) > Diala (85) > Radisho (80) > Masafik (75%) >Armishte (61%) > Halapja (36.5%) > Melisse (20%).

All the initiation experiments were carried out using the previous sterilization protocol to obtain the healthy responded explants. The results revealed that the highest response for initiation (80%) was recorded in Masafik explants cultured in medium (C) and the lowest response (25%) was shown in medium (D). while (66.6%) in medium A and (1.3%) in medium (C) in the Melisse cultivar (Figure 1). Moreover, Radisho cultivar records (53.3%) in medium (A) and a lower ratio (6.6%) in medium (D). Regarding the Armishte cultivar the highest (41.3%) response was shown in medium (B) and the lowest (20%) was in both (C and D) media. Diala shows a high response (80%) in both (C) and (D) media and the lowest (66.6%) was in media (A) and (B). Meanwhile, the highest response (46.6%) was found in Halapja cultivar grown in media (A and D), and the lowest ratio (40%) was seen in (B and C) media. Finally, the Dwarf shows that medium (B) records (60%) responded explants and the lowest (11%) was in medium (D).

Table 1: **The effect of full and (1/2 MS) media and PGRs on pomegranate parameters at initiation stage after six weeks of incubation.**

Parameters	Cultivars	F.S + 0.5 BAP + 0.5 mg/l Kinetin (A)	F.S + 1.0 BAP + 0.5 mg/l Kinetin (B)	H.S + 0.5 BAP + 0.5 mg/l Kinetin (C)	H.S + 1.0 BAP + 0.5 mg/l Kinetin (D)
Shoot initiation response (%)	Masafik	60.0 b	60.0 b	80.0 a	25.0 c
	Melisse	66.6 a	20.0 d	1.30 d	26.0 c
	Radisho	53.3 c	40.0 c	20.0 c	6.6 f
	Armishte	33.23 f	41.3 c	20.0 c	20.0 d
	Diala	66.6 a	66.6 a	80.0 a	80.0 a
	Halapja	46.6 d	40.0 c	40.0 b	46.6 b
	Dwarf	40.0 e	60.0 b	20.0 c	11.0 e
Healthy explants (%)	Masafik	80 b	60 b	80 b	80 b
	Melisse	81.33 b	40.0 c	20.0 d	40.0 d
	Radisho	80.67 b	60.0 b	20.0 d	40.0 d
	Armishte	60.0 c	60.0 b	40.0 c	60.0 c
	Diala	100.0 a	100.0 a	100.0 a	80.0 b
	Halapja	60.67 c	40.0 c	80.00 b	60.0 c
	Dwarf	100.0 a	100.0 a	100.0 a	100.0 a
Contaminated explants (%)	Masafik	20.0 b	40.0 b	20.0 c	20.0 c
	Melisse	20.0 b	60.0 a	80.0 a	60.0 a
	Radisho	20.0 b	40.0 b	80.0 a	60.0 a
	Armishte	40 a	40 b	60 b	40 b
	Diala	.00 c	.00 c	.00 d	20.0 c
	Halapja	40.0 a	60.0 a	20.0 c	40.0 b
	Dwarf	.00 c	.00 c	.00 d	.00 d

The different letters in the same column refer to significant differences between the means.



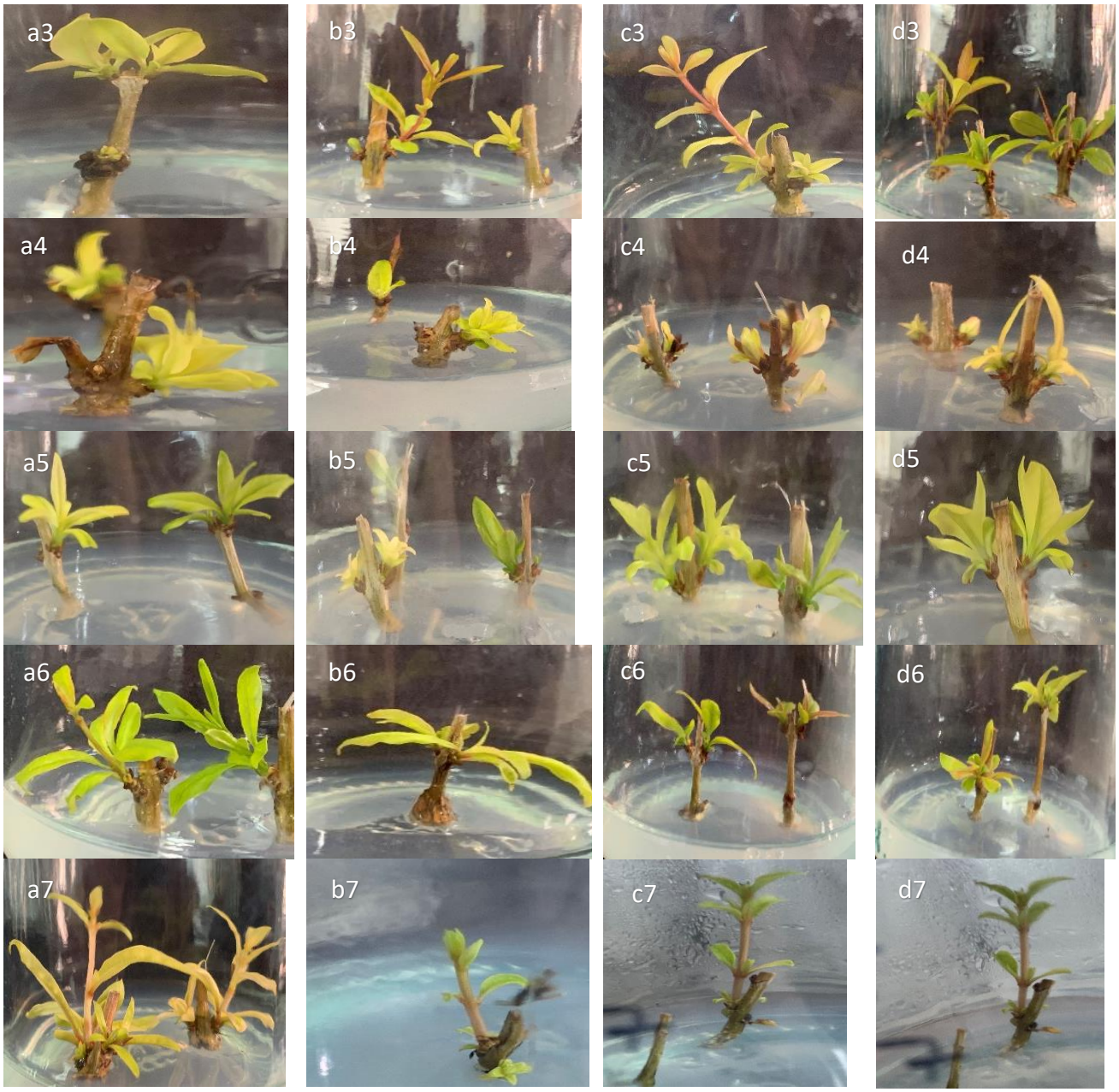


Figure (1): Full and ½ MS strength salts and PGRs in initiation stage (%) of pomegranate after 6 weeks in culture, where 1,2,3,4,5,6, and 7 are the Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and dwarf cultivars respectively.
a: F.S + (0.5mg/l) BAP + (0.5 mg/l) Kinetin, b: F.S + (1.0 mg/l) BAP + (0.5mg/l) Kinetin, c: H.S + (0.5 mg/l) BAP + 0.5 mg/l Kinetin, d: H.S + (1.0mg/l) BAP + (0.5mg/l) Kinetin

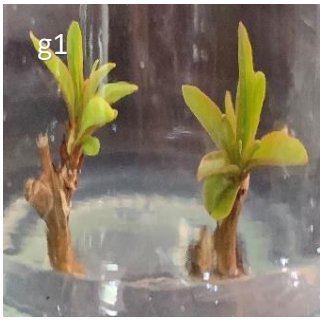
The data in Table (2) shows the effect of WPM medium with the four PGRs combinations as mentioned previously with (1/2 MS). The results revealed that the highest response for shoot initiation (86%) was recorded when Masafik explants cultured in medium (E) and the lowest response (26%) (13%) in (E, F, and H) media. Regarding the Armishte cultivar the highest shoot initiation response (26%) was shown in medium (F) and the lowest (6.6%) was in (H) medium. Diala shows a high response (66.6%) in medium (G) and the lowest (20%) was in medium (E). Meanwhile, the highest response (40%) was found in Halapja cultivar grown in medium (H), and the lowest ratio (12.67%) was seen in (F) medium (Figure 2). Finally, the Dwarf cultivar shows that the (F) medium records (26%) responded explants and the lowest (20%) was in (E, G, and H) media.

was shown in medium (F). While (20%) was recorded in medium (H) and no response was found in media (E, F, and G) with Melisse cultivar. Moreover, Radisho cultivar records (33.3%) in medium (G) and a lower ratio

Table 2: The effect of Full and (1/2 WPM) media and PGRs on pomegranate parameters at initiation stage after six weeks of incubation.

Parameters	Cultivars	F.S + 0.5 BAP + 0.5 mg/l Kinetin (E)	F.S + 1.0 BAP + 0.5 mg/l Kinetin (F)	H.S + 0.5 BAP + 0.5 mg/l Kinetin (G)	H.S + 1.0 BAP + 0.5 mg/l Kinetin (H)
Shoot initiation response (%)	Masafik	86.0 a	26.0 b	40.0 b	66.6 a
	Melisse	.00 d	.00 d	.00 e	20.0 d
	Radisho	13.0 c	13.0 c	33.3 c	13.0 e
	Armishte	13.0 c	26.0 b	20.0 d	6.6 f
	Diala	20.0 b	40.0 a	66.6 a	53.0 b
	Halapja	13.0 c	12.67 c	33.3 c	40.0 c
	Dwarf	20.0 b	26.0 b	20.0 d	20.0 d
Healthy explants (%)	Masafik	100.0 a	60.0 b	60.0 c	80.0 b
	Melisse	20.0 d	20.0 d	20.0 e	20.0 e
	Radisho	60.0 c	100.0 a	100.0 a	60.0 c
	Armishte	66.6 b	60.0 b	80.0 b	80.0 b
	Diala	40.0 e	100.0 a	100.0 a	100.0 a
	Halapja	33.3 f	33.3 c	40.0 d	40.0 d
	Dwarf	100.0 a	100.0 a	100.0 a	100.0 a
Contaminated explants (%)	Masafik	.00 f	40.0 c	40.0 c	20.0 d
	Melisse	80.0 a	80.0 a	80.0 a	80.0 a
	Radisho	40.0 d	.00	.00 e	40.0 c
	Armishte	33.3 e	40.0 c	20.0 d	20.0 d
	Diala	60.0 c	.00 d	.00 e	.00 e
	Halapja	66.6 b	66.6 b	60.0 b	60.0 b
	Dwarf	.00 f	.00 d	.00 e	.00 e

The different letters in the same column refer to significant differences between the means.



No initiation response was found in (e2 and f2)

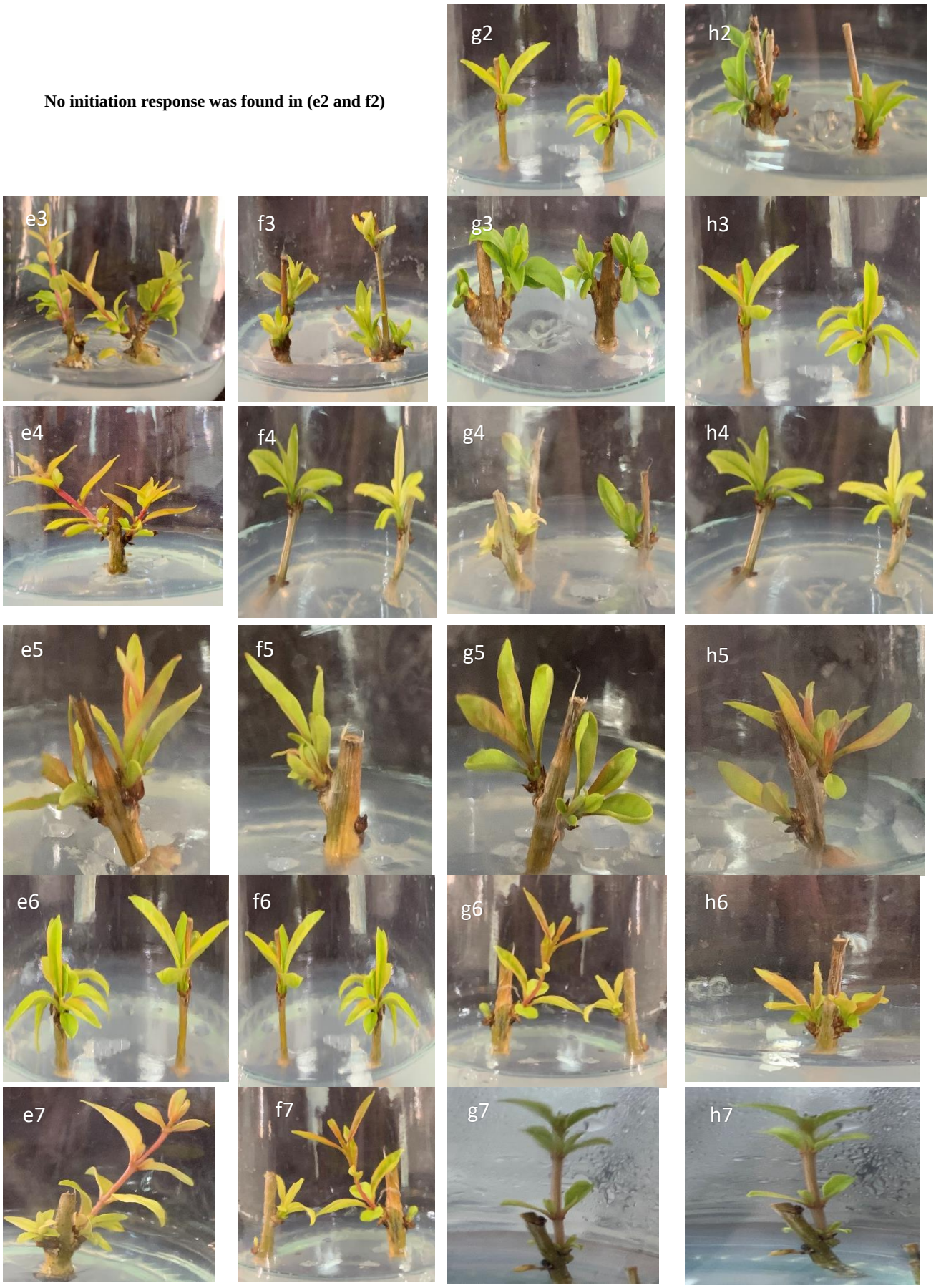


Figure (2) Effect of Full and half-strength WPM salts with PGRs in initiation stage (%) of pomegranate after 6 weeks in culture, where 1,2,3,4,5,6, and 7 are the Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and Dwarf cultivars respectively.
e: F.S + (0.5mg/l BAP) + (0.5mg/l Kinetin), f: F.S + (1.0mg/l BAP) + (0.5mg/l) Kinetin, g: H.S + (0.5mg/l) BAP + (0.5mg/l) Kinetin, h: H.S + (1.0mg/l BAP + 0.5mg/l) Kinetin.

3.2. Shoot Multiplication

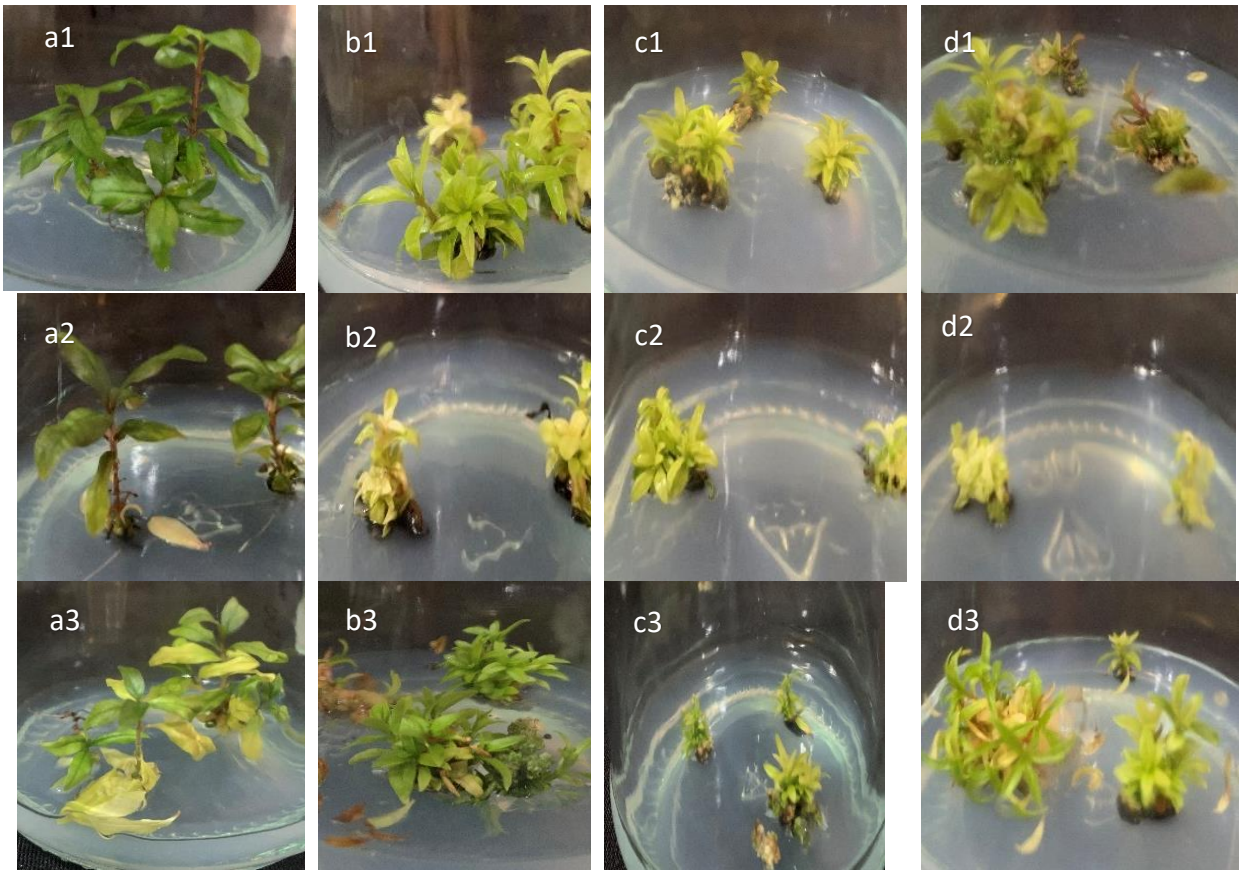
The findings in Table (3) demonstrated that non-significant differences were seen between all the cultivars in their highest shoots length except the Dwarf cultivar which showed a highly significant difference with all other cultivars as a result of growing in different PGR treatments (Figure3). Meanwhile, it was obvious that the Dwarf cultivar proceeds with the other cultivar in this parameter.

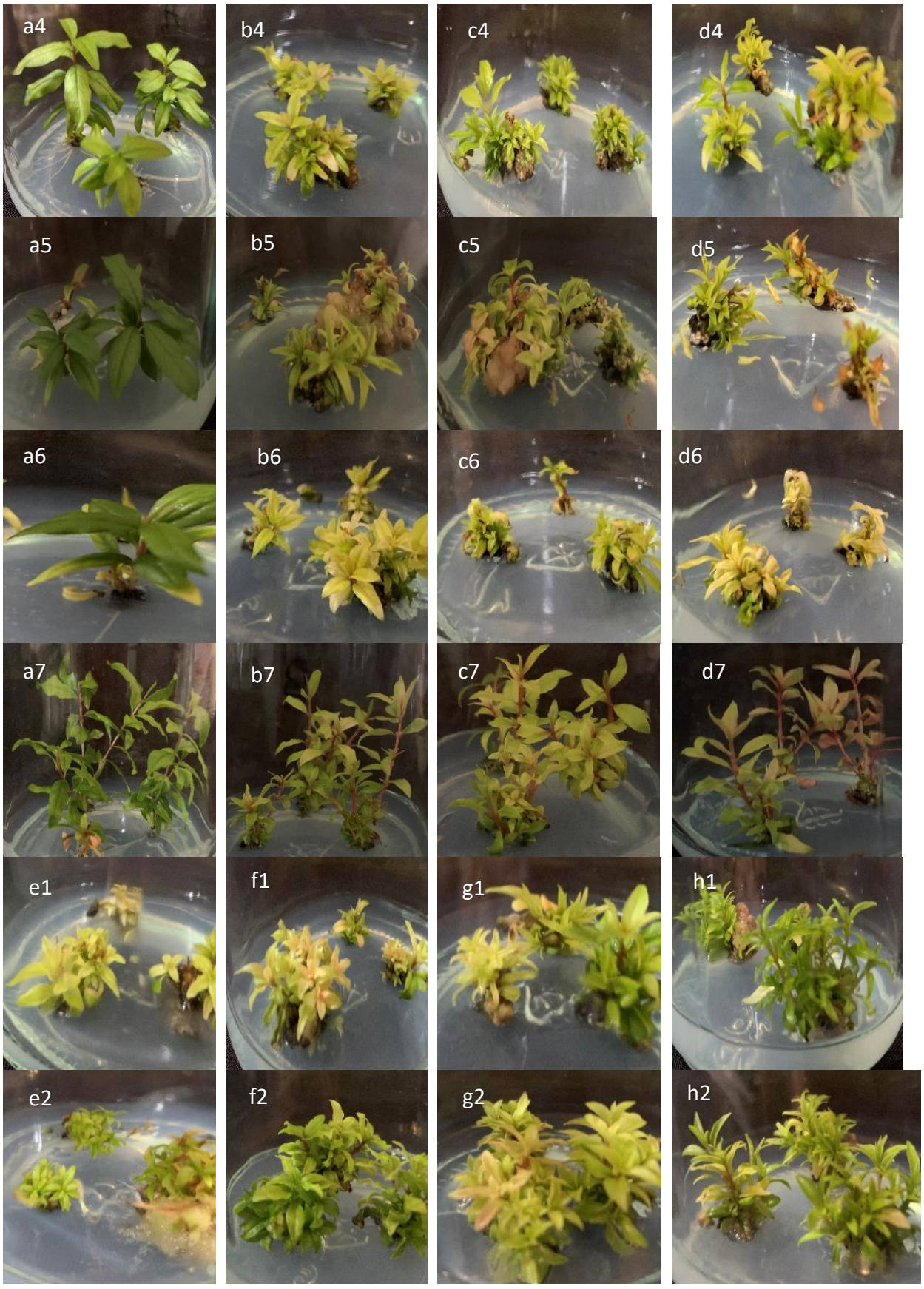
To estimate the influence of variable combinations of PGRs, outcomes indicated that adding (0.5 and 1.0 mg/l) of BAP supported by (0.3mg/l) GA_3 was the more suitable protocol that affected the highest shoot length although non-significant differences were noticed among most of these protocols. Therefore, a highest shoot length (3.9 cm) was seen in dwarf cultivar shoots grown in (0.5mg/l) BAP and (0.3 mg/l) GA_3, and the shorter one (1.01cm) was found in the same medium in Melisse cultivar. Furthermore, the shortest shoot length (0.59 cm) was recognized in Masafik cultivar when grown in WPM supplemented with 2 mg/l of BAP alone.

Table 3: The effect of PGRs on shoot length (cm) of pomegranate at multiplication stage after 6 weeks of incubation.

Cultivars	PGR treatments								Cultivars effect
	(0.0) mg/l BAP	(1.0) mg/l BAP	(2.0) mg/l BAP	(1.0) BAP +(0.1) mg/l NAA	(2.0) BAP + (0.1) mg /l NAA	(0.5 BAP + 0.3) mg/l GA_3	(1.0) BAP + (0.3) mg/l GA_3	(1.0)BAP+(0. 3) GA_3 + (30) mg/l Adenine	
Masafik	1.1 lm	0.72 nm	0.59 o	0.62 no	0.66 no	1.33 k	1.46 ij	1.31 k	**0.974 b**
Melisse	1.62 h	0.78 nm	0.7 n	0.62 no	0.73 mn	1.01 l	1.54 hi	1.41 jk	**1.051 b**
Radisho	2 f	1.43 j	0.78 mn	0.86 m	0.66 no	1.19 l	1.43 j	1.8 g	**1.268 b**
Armishte	1.78 g	0.83 m	0.63 no	0.98 lm	0.76 n	1.99 f	1.46 hj	0.81 m	**1.155 b**
Diala	1.56 h	0.68 n	0.83 m	0.77 mn	0.59 o	1.32 k	1.71 gh	1.2 l	**1.085 b**
Halapja	1.2 l	0.79 mn	0.59 o	0.67 n	0.87 m	2.5 e	1.14 l	1.1 lm	**1.108 b**
Dwarf	2.81 c	2.37 e	2.43 e	2.4 e	2.65 d	3.9 a	3.83 a	3.34 b	**2.968 a**
The effect of PGR treatments	**1.83a**	**1.15 a**	**0.99 b**	**1.05 b**	**1.04 b**	**1.99a**	**1.85 a**	**1.61 a**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

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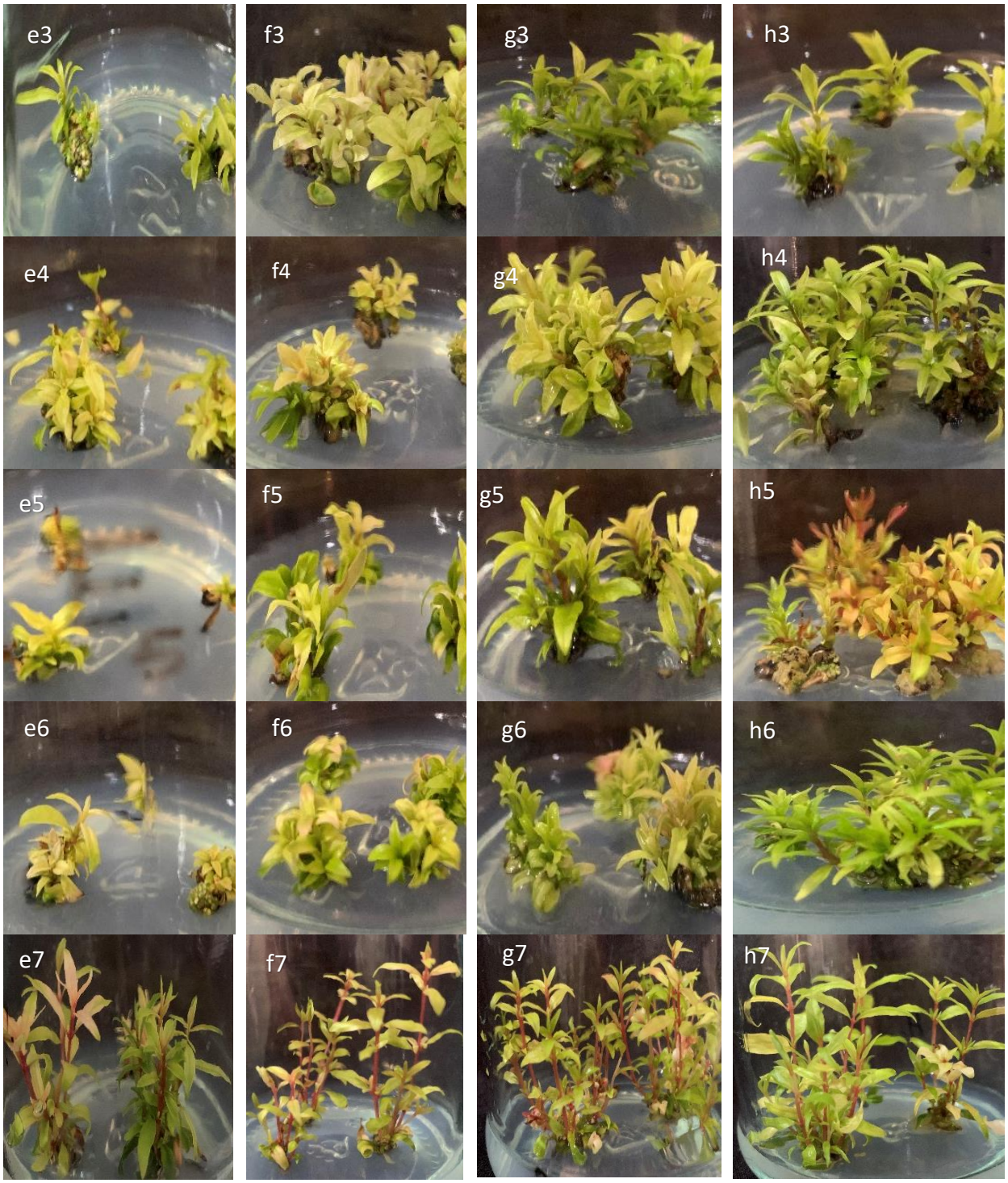

Figure (3) Effect of different growth regulators multiplication stage of pomegranate after 6 weeks in culture, where 1,2,3,4,5,6, and 7 are the Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and dwarf cultivars respectively.
a: (0.0mg/l) BAP, b: (1.0mg/l) BAP, c: (2.0mg/l) BAP, d: (1.0mg/l) BAP + (0.1mg/l) NAA, e: (2.0mg/l) BAP + (0.1mg /l) NAA, f: (0.5mg/l) BAP + (0.3mg/l) GA_3, g: (1.0mg/l) BAP + (0.3mg/l) GA_3, h: (1.0mg/l) BAP + (0.3GA_3 + 30 mg/l) Adenine sulphate

The outcomes of Table (4) clarify that the highest average shoots length (1.57 cm) was obtained by the control treatment (WPM salt-free of PGRs) which surpasses the other treatments. Moreover, non-significant differences were found between Melisse, Armishte and Diala, but significant differences were noticed with Masafik, Radisho, Diala, and Dwarf cultivars that record the highest average length (1.98 cm) compared with (0.71 cm) in Halapja regenerates shoots and the lowest average shoot length (0.32 cm) was shown in Masafik cultivar cultured in WPM medium enriched by (1.0 mg/l) BAP. Besides, the data detected that the Dwarf cultivar exceeds the other cultivars and the more effective protocol was culturing the shoots in WPM media plus (1.0 mg/l) BAP enrichment with (0.3mg/l) AG_3.

Table 4: The effect of WPM medium supplemented with PGRs on pomegranate cultivars shoot length (cm) at multiplication stage after 6 weeks of incubation.

Cultivars	PGR treatments								Cultivars effect
	(0.0) mg/l BAP	(1.0) mg/l BAP	(2.0) mg/l BAP	(1.0) BAP +(0.1) mg/l NAA	(2.0) BAP + (0.1) mg /l NAA	(0.5 BAP + 0.3) mg/l GA_3	(1.0) BAP + (0.3) mg/l GA_3	(1.0) BAP +(0.3) GA_3 + (30) mg/l Adenine sulphate	
Masafik	1.25 hi	0.32 o	0.44 m	0.42 m	2.44 c	0.83 k	0.86 k	0.62 l	**0.898 c**
Melisse	1.62 f	0.45 m	0.51 m	0.47 mn	0.48 mn	0.69 k	1.08 j	0.86 k	**0.77 d**
Radisho	1.802 e	0.72 k	0.6 l	0.64 l	0.52 m	1.71 ef	0.95 j	0.96 j	**0.99 b**
Armishte	1.67 ef	0.43 n	0.46 n	0.54 m	0.47 m	1.3 hi	0.82 k	0.5 lm	**0.77 d**
Diala	1.4 h	0.5 mn	0.7 kl	0.6 m	0.45 m	0.69 l	0.98 j	0.75 k	**0.75 d**
Halapja	1.13 i	0.48 mn	0.43 m	0.49 mn	0.53 n	1.46 g	0.61 l	0.56 lm	**0.71 e**
Dwarf	2.09 d	1.48 g	1.44 g	1.69 ef	1.38 h	2.67 b	2.97 b	2.14 d	**1.98 a**
The effect of PGR treatments	**1.57 a**	**0.62 e**	**0.66 e**	**0.69 e**	**0.9 d**	**1.33 b**	**1.18 c**	**0.91 d**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

The consequences of the Table (5) revealed almost significant differences between cultivars, where Armishte cultures create the highest number (5.39) of branches and Masafik cultivar gained the least number (4.16). Concerning the most effective and valuable combination of PGRs, there were non-significant variations between the cultivars in the control treatment, but it seems that the branch number start to increase with the increment of BAP level in growing media. Furthermore, WPM supported by (1.0mg/l) BAP plus (0.3mg/l) GA_3 shows an almost higher number of branches (10.77) in Melisse cultivar followed (10.33) in Diala cultivar grown in (0.5mg/l) BAP and (0.3 mg/l) GA_3 protocol. Moreover, the lowest branch number (1.0) was found in the free of PGRs (control) treatment in Melisse cultivar.

Table 5: The effect of WPM medium supplemented with PGRs on pomegranate cultivars branches number/explant at multiplication stage after 6 weeks of incubation.

Cultivars	PGR treatments								Cultivars effect
	(0.0) mg/l BAP	(1.0) mg/l BAP	(2.0) mg/l BAP	(1.0) BAP +(0.1) mg/l NAA	(2.0) BAP + (0.1) mg /l NAA	(0.5 BAP + 0.3) mg/l GA_3	(1.0) BAP + (0.3) mg/l GA_3	(1.0) BAP +(0.3) GA_3 + (30) mg/l Adenine sulphate	
Masafik	1.44 mn	2.66 kl	2.11 l	2.33 l	3.77 k	6.66 g	6.17 h	8.11 e	**4.16 f**
Melisse	1 n	3.66 k	2.89 kl	2.22 l	4.11 j	5.44 i	10.77 a	7.99 e	**4.76 d**
Radisho	1.1 mn	4.89 j	3.33 k	3.89 jk	3.44 k	3.66 k	5.33 i	7.99 e	**4.20 e**
Armishte	1.33 m	6.11 h	4.55 j	5 i	4.22 j	7.44 f	8.99 c	5.44 i	**5.39 a**
Diala	1.33 m	5.33 i	6.55 g	3.89 jk	1.77 m	10.33 b	6.33 g	4.88 j	**5.05 b**
Halapja	1.22 mn	5.88 i	3.33 k	3.78 k	3.77 k	8.78 d	7.33 f	4.99 ij	**4.89 c**
Dwarf	2 lm	4.89 j	5.55 i	3.22 k	4.33 j	7.11 fg	6.89 h	4.33 j	**4.79 d**
The effect of PGR treatments	**1.35 g**	**4.77 d**	**4.04 e**	**3.04 f**	**3.63 e**	**7.06 b**	**7.4 a**	**6.25 c**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

The results of Table (6) elucidate that there were dense and intensive (42.6) in the Dwarf cultivar which varies significantly with all other cultivars while Masafik showed less amount in their leaves number (27.88). Focusing on the data of Table (6) and Figure (3), it was clear that BAP affects leaf morphogenesis when compared to the control treatment which showed low numbers of leaves (12.84) that differs almost significantly from all other PGRs combinations that recorded the highest leaves number (47.5) when (1.0mg/l) BAP plus (0.3mg/l) GA3 were used, followed by (0.5mg/l) BAP + (0.3 mg/l) GA_3 then (1.0mg/l) BAP + (0.3mg/l) GA_3 + (30mg/l) Adenine sulfate.

Table 6: The effect of WPM medium supplemented with PGRs effects on pomegranate cultivars leaves number/ explant at multiplication stage after 6 weeks of incubation.

Cultivars	PGR treatments								Cultivars effect
	(0.0) mg/l BAP	(1.0) mg/l BAP	(2.0) mg/l BAP	(1.0) BAP +(0.1) mg/l NAA	(2.0) BAP + (0.1) mg /l NAA	(0.5 BAP + 0.3) mg/l GA_3	(1.0) BAP + (0.3) mg/l GA_3	(1.0) BAP +(0.3) GA_3 + (30) mg/l Adenine sulphate	
Masafik	10.78 o	19.47 m	19 m	14.33 n	25.33 k	40.87 h	44.53 g	48.74 f	**27.88 e**
Melisse	12.22 o	21.55 l	22.67 l	16.33 m	25.33 k	30.66 ij	63.11 b	55.33 d	**30.9 c**
Radisho	10.11 o	31.33 ij	19.78 m	25 k	21.89 l	30.11 ij	39.77 hi	62.99 b	**30.12 c**
Armishte	17.33 n	32.66 ij	26.33 ij	33.66 i	30.88 ij	53.55 e	64.88 a	38.87 hi	**37.27 b**
Diala	14.11 n	33.0 ij	41.55 gh	22 k	11.44 o	42.89 g	42 g	29.89 k	**29.61 d**
Halapja	8.89 p	43 h	18 m	28.32 kl	22.89 l	58.89 c	33.66 ij	29.78 k	**30.43 c**
Dwarf	16.44 n	59 c	51.77 e	34.33 i	43.11 h	56.88 c	44.55 g	34.89 i	**42.6 a**
The effect of PGR treatments	**12.84 g**	**34.29 d**	**28.44 e**	**24.85 f**	**25.84 f**	**44.94 b**	**47.5 a**	**42.93 c**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

3.3. Root formation

3.3.1. Rooting response percentage (%)

Valuable results were found in rooting response following 6 weeks in culture by using (½) MS salt strength media with two levels of Auxins (Table 7 and Figure 4). On one hand, Masafik, Melisse and Diala propagules record 100% rooting using (1/2 MS) media free of Auxins (control treatment) which differs significantly from other cultivars, while Radisho records the lowest response (66.6%). On the other hand, the data showed that four cultivars got a (100%) rooting ratio (Masafik, Melisse, Armishte, and Halapja) by adding (0.5mg/l) NAA. To illustrate the effect of the IBA, the outcomes clarify that the micro-shoots of Masafik, Melisse, and Armishte recorded (100%) rooting, while only (55.5%) of Halapja microshoots were rooted when (1/2 MS) medium supplemented with (0.5 mg/l) IBA. plantlets stimulate root induction. Therefore, it was clear that Masafik and Melisse proceed with the other cultivars in root formation while the dwarf one showed that adding both types of Auxins to the medium cultures inhibits root induction (Figure 4).

Table 7: The effect of (1/2 MS) medium and Auxins on rooting response percentage (%) at rooting stage of pomegranate cultivars after 6 weeks of incubation.

Cultivars	PGR treatments			
	Free of auxin (control)	(0.5 mg/l) NAA	(0.5 mg/l) IBA	Cultivars effect
Masafik	100 a	100 a	100 a	**100 a**
Melisse	100 a	100 a	100 a	**100 a**
Radisho	66.6 d	66.6 d	66.6 d	**66.6 f**
Armishte	88.8 b	100 a	100 a	**96.27 b**
Diala	100 a	88.8 b	66.6 d	**85.13 c**
Halapja	77.7 c	100 a	55.5 e	**77.73 d**
Dwarf	88.8 b	66.6 d	66.6 d	**74 e**
The effect of PGR treatments	**88.84 a**	**88.86 a**	**79.33 b**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

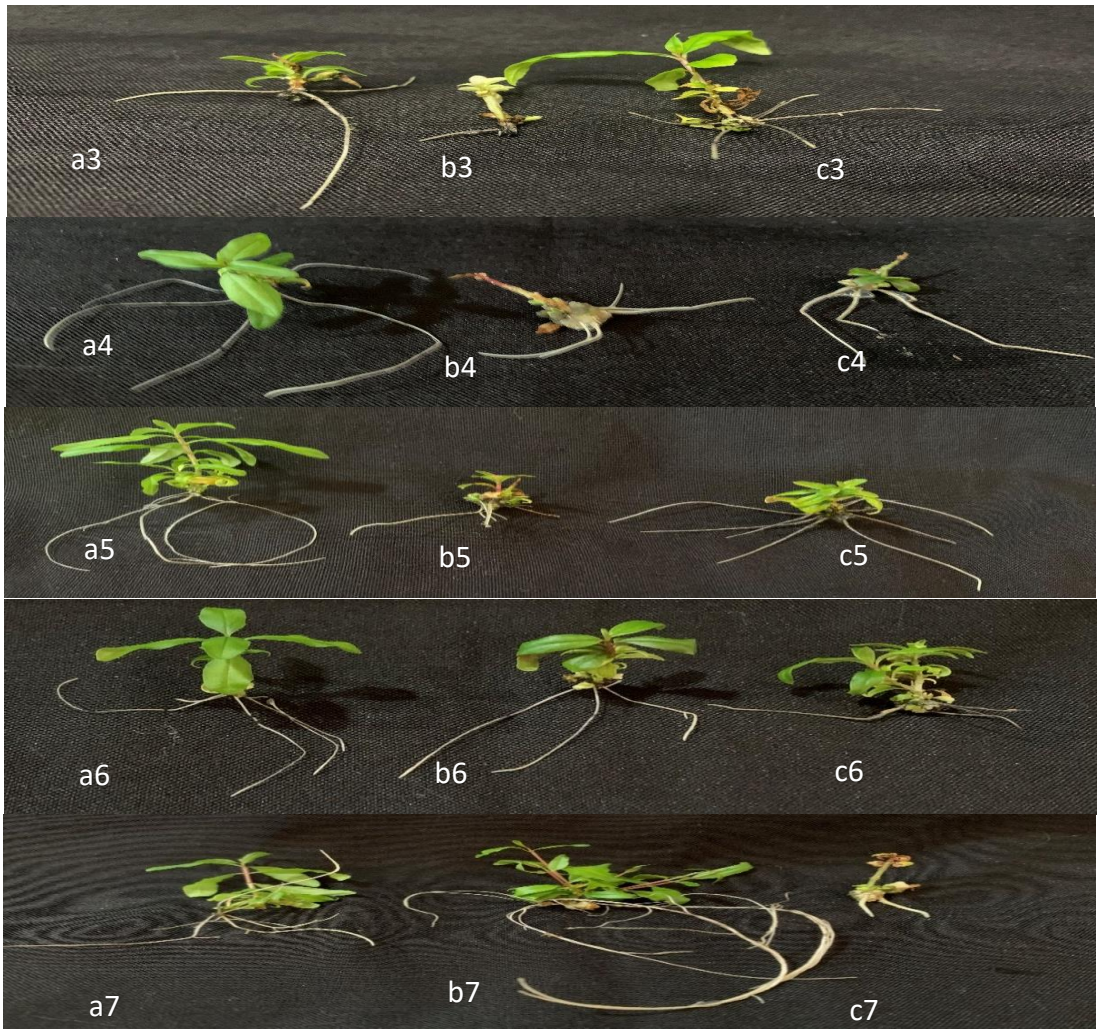

Figure: 4 The effect of (1/2 MS) medium and auxins on rooting response percentage (%) at rooting stage of pomegranate cultivars after 6 weeks of incubation, where 1,2,3,4,5,6, and 7 are the Masafik, Melisse, Radisho, Armishte, Diala, Halapja, and dwarf cultivars respectively. A: Free of auxin (control), B:(0.5mg/l) NAA, C: (0.5mg/l) IBA

3.3.2. Roots number (Roots/shoot)

To investigate the role of both NAA and IBA inserted to half strength of MS media on root number, the results of Table (8) and Figure (4) elucidate that NAA was more valuable and caused a significant increase in roots number per shoot, while IBA inhibit roots number comparing to treatment of control. In more detail, it was shown that supplementing (0.5mg/l) of NAA in culture medium caused a significant excess in root numbers of Diala cultivars and gained the highest number (4.89/roots/shoot) followed by Armishte (4.22 roots/shoot) Masafik and Halapja (3.33 and 33.3 roots/shoot) respectively that differs significantly from other cultivars especially Radisho that records the lower number of roots (1.78 roots/shoot).

Table 8: The effect of (1/2 MS) medium and auxins on roots number at rooting stage of pomegranate cultivars after 6 weeks of incubation.

Cultivar Type	PGR combinations			
	Free of auxin (control)	(0.5 mg/l) NAA	(0.5 mg/l) IBA	Cultivars effect
Masafik	3 cd	3.33 cd	2.17 f	**2.83 b**
Melisse	2 fg	3 cd	2.33 ef	**2.44 c**
Radisho	1.33 hi	1.78 fgh	0.89 i	**1.33 d**
Armishte	2.33 ef	4.22 b	3.67 bcd	**3.41 a**
Diala	3.78 bc	4.89 a	2.22 f	**3.63 a**
Halapja	2.22 f	3.33 cd	1.56 gh	**2.37 c**
Dwarf	4.11 b	2.44 ef	1.22 i	**2.59 bc**
Effect of PGR combinations	**2.68 b**	**3.28 a**	**2.01 c**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

3.3.3. Roots average length (cm)

Unexpected and opposite behavior appeared in the root's average length after six weeks in cultures using both NAA and IBA separately that control free of auxin treatment exhibit the highest length average as a result of the effects of auxins enrichment (Table 9). In addition, it was obvious that adding (0.5 mg/l NAA and 0.5 IBA) caused a significant decrease (1.84 cm) and (2.05cm) respectively compared with (2.89 cm) in the control treatment. Besides that, the highest average length of root (3.75 cm) was found in Dwarf plantlets that differ significantly from all other cultivars especially the Radisho cultivar which shows a weak response and records the shorter (0.8 cm) root average length (Table 9).

Table 9: The effect of (1/2 MS) medium and auxins on roots average length (cm) at rooting stage of pomegranate cultivars after 6 weeks of incubation.

Cultivars	PGR treatments			
	Free of auxin (control)	(0.5mg/l) NAA	(0.5mg/l) IBA	Cultivars effects
Masafik	3.29 d	2.70 ef	1.70 i	**2.56 b**
Melisse	2.49 f	2.28 gh	3.18 d	**2.65 b**
Radisho	0.94 j	0.99 j	0.47 k	**0.8 f**
Armishte	2.89 de	1,89 hi	2.03 fgh	**2.46 c**
Diala	3.66 c	1.73 i	0.97 j	**2.12 d**
Halapja	2.16 gh	2.33 fg	0.54 jk	**1.68 e**
Dwarf	4.78 b	0.98 j	5.49 a	**3.75 a**
The effect of PGR treatments	**2.89 a**	**1.84 c**	**2.05 b**	

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly.

3.4. Acclimatization of plantlets

The visual observation records that around (85-90%) of rooted cultures were successfully acclimatized by transferring the cultures from indoor to outdoor environments. Two reasons were behind the loss of propagated plantlets, first, the high light conditions in the field and greenhouse compared to artificial light provided in the growth room, and second, starting the photosynthesis process to synthesize the source of sugar. Moreover, controlling the amount of humidity as the result absence of thickened cuticle layer that protects the plant from drought. Following these steps of gradual hardening, there is an agreement with some reports in pomegranate that have been transferred to a field open-air environment.

Figure (5) Different stages of Acclimatization

4. DISCUSSION

Sodium hypochlorite and ethanol exhibit a successful effect in surface sterilization, which is the most commonly used as surface sterilant; other chemicals have also been tried successfully (Singh *et al.*, 2010 and Prajwala *et al.*, 2021). Many researchers have recommended using mercuric chloride ($HgCl_2$) for surface disinfestation, which records a good survival percentage for the explants in pomegranate, in addition to their high effectiveness in totally removing bacteria and fungi from the explants.

Applying a disinfectant at a low concentration for a short time is one of the most crucial aspects of explant sterilization. Commercial bleaching using NaOCl, for example, is a common practice in plant tissue and has proven effective in getting rid of various pollutants, such as yeasts, fungi, and bacteria, as well as preventing explant browning; in addition to being simple to remove from the explant (Salehi, 2006).

The compositions of the culture medium have a crucial role in *In vitro* morphogenesis. Different culture media and

growth regulators combinations were used to establish the micropropagation protocol. Thus, in the present investigation, the MS medium showed a better response on culture establishment when compared to the WPM medium. These results agree with Valizadeh Kaji *et al*., 2013; Singh & Patel, 2016; Mulaei *et al.*, 2020 and Singh *et al*., 2022 who used MS medium for plant culture initiation. Further, a half-MS has a more influential role in the establishment stage than full-MS strength, as Singh *et al*. (2010) reported by using a half-strength MS medium for meristem cultured. This may result due to nutrient media components that are more suitable for pomegranate species and cultivars and offers suitable demands for the initiation stage.

In vitro plant and development are affected fundamentally by the type of culture media, and its nutrient composition, in addition to the plant growth regulators, which is a crucial control for *in vitro* morphogenesis (Danial *et al*., 2021). Although the basic requirements of *in vitro* culture are the same as those of whole plants grown in the natural open environment, the optimal growth is influenced by the composition of the culture media that promotes the metabolism of all activities in the plant tissues under controlled conditions that may differ depending on specific genera (Kumar *et al*., 2017).

Multiplication of pomegranate shoots using a woody plant medium is well documented (Valizadeh Kaji, 2013 and Eshaghi Saroyi *et al*., 2020). The beneficial effect of BAP was found to be 1.4 mg/l (Baghdad Abadia *et al*., 2020), while Desai *et al*., (2018) reported a 1mg/l BAP level as optimal concentration for shoot multiplication, and an average of 11.21 shoots were developed from each explant at this level. These results agree with those published by Khosh-Khui *et al*., 1984, utilizing a WPM medium supported by 5 mg/l Kinetin. Similar findings have been submitted regarding the beneficial effect of cytokinin (BAP) for pomegranate shoot proliferation using an MS medium (Patil *et al*., 2011; Mulaei *et al*., 2020 and Choudhary *et al*., 2022). The data obtained in apical dominance as a result of the precence of cytokinins in removing the apical dominance in the buds (Mohammed & Al-Younis, 1991; Al-Rifae'e & Al-Shobaki, 2002; Danial *et al* ., 2019).

Many references (Jones, 1985 and Baraldi, *et al*., 1988) highlight the critical role that cytokinins and auxins play in the formation of shoot and root tissues in plants. On the other hand, according to these studies, using BA in experiments may control the cell cycle and cell division, stimulate the growth of auxiliary and adventitious shoots, control differentiation, and prevent the formation of roots (Taiz and Zeiger, 2002). Similarly, using BAP has been shown to significantly increase the development of axillary and adventitious buds as well as the foliar growth of shoot tip cultures (Abeyaratneb and Lathiff, 2002). This investigation confirmed these observations, which indicated that adding the cytokinin benzylaminopurine (BAP) to the culture medium has an essential role in shoot proliferation which accelerate cell division and stimulates the synthesis of RNA nucleic acid, then raising the enzyme and the protein inside the cells, which increases the buds and consequently shoot proliferation rate.

Additionally, as noted in this study (Gonbad *et al*., 2014), the interaction between cytokinins and GA_3 is crucial for woody plant shoot multiplication and elongation. The outcomes for the combination of BA and GA_3 revealed that adding BA alone had a more detrimental effect on shoot multiplication features than combining it with GA_3.

Rooting is the pre-final stage of micropropagation before transferring the plantlets to the open-air environment. This step aims to stimulate the establishment of fully developed plantlets. Therefore, Auxins are the most suitable PGRs mainly used for adventitious root formation and inducing cell division through tissue culture technique. In nature, the hormones of this group are involved with stem and internodes elongation, apical dominance, leaf abscission, root induction, and tropism, (Mohammed & Al-Younis, 1991; Al-Rifae'e & Al-Shobaki 2002 and Kumar *et al*., 2017). Thus, IBA auxin is considered the most important and the most effective of all auxin types, followed by NAA, that commonly used for *in vitro* root induction.

IAA, on the other hand, was the least efficient despite being natural and having a high sensitivity to light. In general, plantlets generate more roots when placed in a rooting media with low salt concentration (Drazeta, 1997). The optimum method for root inducing in pomegranates, according to various researchers, is to employ 0.5 mg/l NAA or IBA (Patil *et al*., 2011; Baghdad-Abadi *et al*., 2020; Maheswari *et al*., 2023 a,b). Contrarily, half-strength MS medium with 0.5 NAA or IBA demonstrated the highest adventitious rooting percentage in *Punica granatum*, which is consistent with the findings of this study (Desai *et al*., 2018; Bachake *et al*., 2019; Mulaei *et al*., 2020; *Kabir et al*., 2021). The outcomes result of (Hatzilazarou *et al*.,2003) were similar to those in this study in that the IBA concentrations were the best for obtaining root, although they used WPM medium instead of MS medium.

Concerning acclimatization, controlling the amount of humidity due to the absence of the thick layer of the cuticle, followed by the other steps of gradual hardening, are the primary limits of successful acclimatization. The current results are in agreement with the suggestions of many researchers in pomegranate plants which finally transferred to open-air field conditions (Bachake *et al* ., 2019; Mulaei *et al*., 2020 ; Singh *et al*., 2022 and Maheswari *et al* ., 2023a)

5. CONCLUSION

In the present study, a successful *in vitro* propagation system was developed for seven cultivars of pomegranate (*Punica granatum* L.) cultivated in Kurdistan region of Iraq using MS and WPM media. The regenerated plants were successfully transferred to the soil. This protocol can serve as an efficient method for the rapid propagation and many other biotechnology aspects for future studies.

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