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Research Article
Optimization of Development of Pomegranate (*Punica granatum* L.) Varieties from Microclonal Propagation

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Abstract

Background and Objective: Besides, increasing cost-efficiency of MS and WPM nutrient media for the regeneration of explants and rhizogenesis of microshoots was planned by introducing changes in the composition of phytohormones. Studying the effect of a supramolecular complex of glycyrrhizinic and salicylic acids on the rhizogenesis of microshoots was aimed in terms of economic benefits. The research was aimed at optimizing *in vitro* microclonal propagation of some pomegranate varieties (*Punica granatum* L.). **Materials and Methods:** August, Kazake-anar, Achikh-dona and Tuyatish varieties of local pomegranate were selected in the study. Various concentrations of sterilants were used for the surface sterilization of explants. Explants were grown in MS and WPM media containing various concentrations of cytokinin, auxin and gibberellic acid. The supramolecular complex was used for microshoot rhizogenesis. Moreover, optimal conditions for acclimatization in microplants were developed. **Results:** The addition of gibberellic acid into MS and WPM nutrition media did not result in significant differences in the microshoot length. But the addition speeded up the formation of buds. Only earlier development of buds in some varieties were observed. The supramolecular complex used in this work at 0.15 and 0.17 mg L^{-1} led to several-fold improvement in root number and length. No significant changes were observed between MS and WPM culture media. Economically utilizing the supramolecular complex was about 45 times cheaper than the mixture of IBA and IAA that was added to MS and WPM nutrition media. **Conclusion:** An enhanced cost-efficiency of the technology that enables obtaining pomegranate microshoot during the whole year.

Key words: Pomegranate, *in vitro* growth, supramolecular complex, phytohormones, microclonal propagation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Punica protopunica Balf. is one of two species of the genus *Punica*, known as the Socotran pomegranate, is an endemic species found in the Socotra Archipelago in the northwestern part of the Indian Ocean and considered to be the ancestor of the pomegranate[1]. Pomegranate (*Punica granatum* L.) is a deciduous shrub native to Iran[2]. Pomegranate (*Punica granatum* L.) has long been localized and widespread in the Caspian Region and Northern Turkiye[3]. For many years, people have selected native plants for their desired traits. The pomegranate tree has been cultivated since ancient times and has gained significance in the historical and cultural life of mankind. Recent researches were devoted to the transmission of traits from generation to generation, genetic variability, genome, transcriptome and metabolome studies. Recent researches were also carried to develop new resistant ones[4].

The pomegranate tree is widely distributed in tropical and subtropical regions due to its resistance to abiotic factors. Wild, semi-wild, cultivated, traditional and ornamental varieties are kept in germplasm collections in many pomegranate-growing countries[5]. The average summer temperature of 24.0-27.7 °C in Central Asia makes it possible to grow pomegranates in large plantations[6]. In Uzbekistan, pomegranates are propagated mainly by traditional methods, using hardwood parts of a plant, i.e., cuttings[7]. Traditional methods of pomegranate propagation are not suitable for providing a large amount of planting material at the same time, because it is too slow for plant reproduction and the availability of planting material is limited throughout the year[8]. The traditional method of pomegranate propagation is time-consuming and laborious and does not ensure disease-free plants. The *in vitro* method of propagation is the only perspective of plant tissue culture that can avoid these problems[9].

Browning problems linked with the exudation of phenolics has a harmful effect on the growth and development of explants at the initial stage of *in vitro* cultivation of woody plants[10-12], which might affect regeneration and rhizogenesis[13]. In order to prevent the harmful effects of phenol exudation on explants *in vitro*, many studies have been carried out: Preserving in an antioxidant solution for a certain period of time, adding antioxidants to the nutrient medium, keeping in the dark, subculturing, etc.[14]. Browning of explants and nutrient medium is the main problem of pomegranate, which is caused by a large amount of exudation of phenols. This is especially observed in mature explants[15]. Phenols are common chemical compounds among plant substances that have an aromatic ring containing one or more hydroxyl components[16]. Micropropagation is used to avoid these problems. The process consists of mass propagation of terminal and lateral shoots and petioles under *in vitro* condition[17]. Microclonal propagation of pomegranate is carried out using existing meristems, root meristems and somatic embryogenesis regeneration[18].

Initially, the development of a large number of axillary shoots and plant regeneration from cotyledon nodes of pomegranate by adding single cytokinin (BAP) to MS medium was reported. The addition of 2.3-23.0 µM benzyladenine (BA) or kinetin (Kn) to the (MS) medium had a significant effect on the development of microshoots from cotyledon segments of Ganesh pomegranate cultivar[19]. Microclonal propagation of Iranian pomegranate varieties "Malas Saveh" and "Yousef Khani" in two different artificial nutrient media such as Woody plant medium (WPM) and Murashige and Skoog (MS) medium and under the influence of different growth regulators was studied. In the proliferation phase, various concentrations (2.3, 4.7, 9.2 and 18.4 µM) of kinetin and 0.54 µM NAA were used and the WPM medium was found to be more effective than the MS medium[9].

In this research, optimization of growing in MS[20] and WPM[21] nutrient media and selection of optimal media were carried out and the influence of plant growth regulators (BAP, NAA and GA_3) on pomegranate explants was studied. Besides, we studied the effects of the supramolecular complex of glycyrrhizic and salicylic acids on the rhizogenesis of microshoots. This study was carried out in microclonal *in vitro* propagation of local pomegranate varieties from the terminal and lateral buds using various concentrations and combinations of plant growth regulators and the supramolecular complex to grow pomegranate on a large scale without seasonal barriers to create a complete protocol for the cultivation of pomegranate seedlings.

MATERIALS AND METHODS

Pomegranate varieties: The research was conducted in the first and second quarters of 2022 at the Transgenomics and Tissue Culture Laboratory of the Center of Genomics and Bioinformatics of Uzbekistan Academy of Sciences. Experimental varieties-August, Kazake-anar, Achikh-dona and Tuyatish were selected from the collection available at the Surkhandarya Research Station of the Scientific Research Institute of Horticulture, Viticulture and Winemaking named after M. Mirzayev.

Plant materials and surface sterilization: Explants were selected from August, Kazake-anar, Achikh-dona and Tuyatish varieties of 2 years old pomegranate grown in greenhouse conditions (Fig. 1a-b). Terminal and lateral bud explants of the various samples, selected for the study, were collected in special containers (Phyto Technology Lab. USA). The explants were cleaned of excess parts in a laboratory condition and kept in a fungicide solution (fundazol 0.02%) for 1 min and under running water for 10 min.

Surface sterilization was performed in a laminar box (HF safe LC. China) under aseptic conditions. The effect level of several chemical compounds for surface sterilization of explants at different time intervals was studied (Table 1). In all variants of the experiment (Control, A_1 and A_2), 50 explants were selected for surface sterilization. Because burning was observed in some explants as a result of the effect of sterilization agents, observations and statistical analyses were made on 40 explants.

Nutrient medium and its content: Two different nutrient media MS and WPM were used in the study (Table 1 and 2). The composition of the medium was prepared according to literature data[20,21]. The pH value of the prepared nutrient medium was adjusted to 5.8 using 0.1 N NaOH solution. Nutrient media were divided into 1000 mL heat-resistant conical flasks and sterilized in an autoclave at 120°C under a pressure of 0.75-1.0 atm for 20 min. The sterile medium was equally divided by 50 mL into 500 mL bottles.

Explant regeneration (bud proliferation): The stage of regeneration (proliferation) of explants was carried out on the basis of various concentrations and combinations of three different phytohormones in two various nutrient media (1.0 mg L^{-1} BAP +0.1 mg L^{-1} NAA and 1.0 mg L^{-1} BAP +0.1 mg L^{-1} NAA+0.5 mg L^{-1} GA_3) (Table 2). The 0.5 cm part of the explants was inserted into the nutrient medium and placed in an upright position. Explants were kept in the dark for the 1st week of cultivation, then grown under a white lamp light at the condition of room temperature 25±1°C and humidity 85±5%, photoperiod 16/8. From the 28th to the 30th day of the observations, the microshoots formed in the explants, depending on the size (higher than 2.0 cm), were transplanted to a new nutrient medium and grown under the above photoperiod conditions. The explants by 30 pieces were selected for each nutrient medium to study shoot organogenesis in explants.

Root formation and rooting development in microshoots (rhizogenesis): Microshoots were grown in a culture medium of 14 000 mg L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 800 mg L^{-1} agar-agars. The supramolecular complex in 0.15 and 0.17 mg L^{-1} doses was used as an inducer[22] for rhizogenesis (Table 3). The explants were kept in the nutrient medium containing the supramolecular complex preparation until they were transplanted from the rhizogenesis stage to the non-sterile (substrate) conditions, that is, re-subculture was not performed. In the 5th week of cultivation, the

Table 1: Surface sterilization and the duration of the process

Number	Components		
Control	5 min H_2O_2 (3%)	10 sec C_2H_5OH (96%)	10 min NaClO (2%)+Twin-20+H_2O (Sterill)
A_1	5 min H_2O_2 (3%)	15 sec C_2H_5OH (96%)	15 min NaClO (2%)+Twin-20+H_2O (Sterill)
A_2	5 min H_2O_2 (3%)	20 sec C_2H_5OH (96%)	20 min NaClO (2%)+Twin-20+H_2O (Sterill)

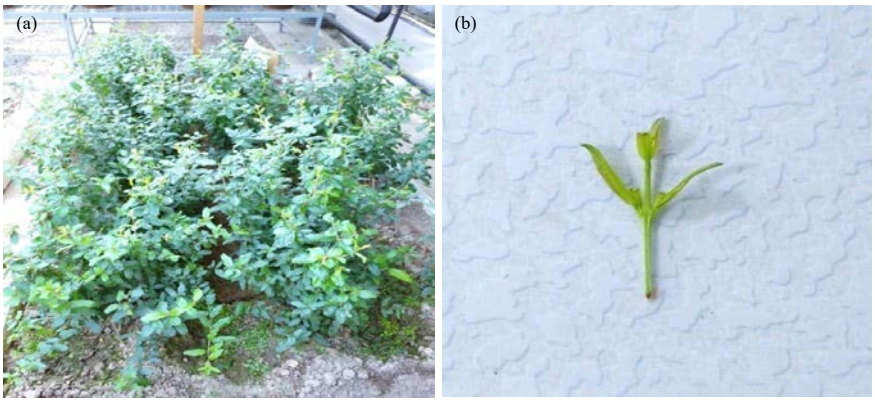

Fig. 1(a-b): Initial material, (a) Two-year-old seedlings of experimental varieties and (b) Selected explant

Table 2: Chemical composition of MS and WPM nutrient media, selected for shoot organogenesis in pomegranate explants

	Concentration (mg L^{-1})			
Components of the nutrient medium	MS_1	MS_2	WPM_1	WPM_2
NH_4NO_3	1,650.0	1,650.0	400.0	400.0
KNO_3	1,900.0	1,900.0	990.0	990.0
$MgSO_4{\cdot}7H_2O$	370.0	370.0	180.7	180.7
KH_2PO_4	170.0	170.0	170.0	170.0
$CaCl_2$	440.0	440.0	72.5	72.5
$FeSO_4{\cdot}7H_2O$	27.8	27.8	27.8	27.8
Na_2EDTA	36.7	36.7	37.3	37.3
H_3BO_3	6.2	6.2	6.2	6.2
$MnSO_4{\cdot}5H_2O$	22.3	22.3	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	8.6	8.6	8.6	8.6
KJ	0.83	0.83	-	-
$Na_2MoO_4{\cdot}\ 2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4{\cdot}\ 5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}\ 6H_2O$	0.025	0.025	-	-
Myo-inositol	100.0	100.0	100.0	100.0
Aminobenzoic acid	0.5	0.5	-	-
Thiamine HCl	0.1	0.1	0.1	0.1
Pyridoxine HCl	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Glutamine	50.0	50.0	-	-
Glycine	2.0	2.0	2.0	2.0
BAP	1.0	1.0	1.0	1.0
NAA	0.1	0.1	0.1	0.1
GA_3	-	0.5	-	0.5
Agar-agar	8,000	8,000	8,000	8,000
Saccharose	14,000	14,000	14,000	14,000
Activated carbon	200	200	200	200

Table 3: Chemical composition of MS and WPM nutrient media selected for root formation and development in microshoots

	Concentration (mg L^{-1})					
Nutrient medium composition	Control	MS_1	MS_2	Control	WPM_1	WPM_2
NH_4NO_3	1,650.0	1,650.0	1,650.0	400.0	400.0	400.0
KNO_3	1,900.0	1,900.0	1,900.0	990.0	990.0	990.0
$MgSO_4{\cdot}7H_2O$	370.0	370.0	370.0	180.7	180.7	180.7
KH_2PO_4	170.0	170.0	170.0	170.0	170.0	170.0
$CaCl_2$	440.0	440.0	440.0	72.5	72.5	72.5
$FeSO_4{\cdot}7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8
Na_2EDTA	36.7	36.7	36.7	37.3	37.3	37.3
H_3BO_3	6.2	6.2	6.2	6.2	6.2	6.2
$MnSO_4{\cdot}5H_2O$	22.3	22.3	22.3	22.3	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6
KJ	0.83	0.83	0.83	-	-	-
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025	-	-	-
Myo-inositol	100.0	100.0	100.0	100.0	100.0	100.0
Aminobenzoic acid	0.5	0.5	0.5	-	-	-
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Glutamine	50.0	50.0	50.0	-	-	-
Glycine	5.0	5.0	5.0	5.0	5.0	5.0
Supramolecular complex	-	0.15	0.17	-	0.15	0.17
Agar-agar	8,000	8,000	8,000	8,000	8,000	8,000
Saccharose	14,000	14,000	14,000	14,000	14,000	14,000
Activated carbon	200	200	200	200	200	200

rooting percentage, root number and length of each microshoot were quantitatively evaluated. In all cases, 30 microshoots were selected for each nutrient medium containing supramolecular complex preparation.

Transplantation and adaptation of microplates to sterile (soil) conditions: Microplants with morphologically proper root systems were transplanted to biohumus+coconut husk+sand (2: 1: 1) substrate. The substrate sterilized in an autoclave was placed in 8×7 cm plastic containers and microplants were planted there. To maintain high humidity, plastic containers were closed with special light-transmitting lids and kept for 20 days in the conditions of an artificial climate station (phytotron) with relative humidity (70-75%), temperature (25±2°C), under 16/8 light and dark photoperiodic cycle. Taking into account a high level of transpiration due to the relative infirmity of microplants, the slow development of leaf tissue cells and the wide axil of leaves, special lids were opened, artificially moistened and closed at certain time intervals. After 20 days, the acclimatized microplants were planted on a substrate of field soil+sand (1: 1) distributed in 18×18 cm disposable polyethylene containers and transferred to greenhouse conditions. The survival rate of microplants was analyzed on the 30th day after transplanting into the greenhouse.

Data analysis and measurements: The experiment for the stage of the proliferation of explants was carried out using four cultivars, two different nutrient media (MS and WPM) and different concentrations of three types of phytohormones and was statistically evaluated. Significant differences were illustrated by the time of shoot formation, number and length of shoots for explants of each variety as a result of the effect of phytohormone concentrations. The stage of rhizogenesis in microsystems was carried out without phytohormones using the supramolecular complex. Data were analyzed using the ANOVA program.

RESULTS AND DISCUSSION

Surface sterilization stage: In this experiment, the bacterial and fungal infestation of explants was studied and various concentrations of sterilization agents were used. The A_1 variant (5 min H_2O_2 (3%)+15 sec C_2H_5OH (96%)+15 min NaClO (2%)+Twin-20+H_2O) with explants free from fungal infestation did not reveal significant differences compared to the control (5 min H_2O_2 (3%)+10 sec C_2H_5OH (96%)+10 min NaClO (2%)+Twin-20+H_2O). Similar results were observed in the explants of the A_1 variant which were free from bacterial damage.

In experimental option A_2 (5 min H_2O_2 (3%)+20 sec C_2H_5OH (96%)+20 min NaClO (2%)+Twin-20+H_2O) a significant difference was observed relative to the control option, i.e., the percentage of explants free of fungal and bacterial infestation in all experimental varieties showed a high index. In the August and Tuyatish varieties a bacterial infestation, while, in the Achikh-dona variety a fungal infestation was not observed. There was not a significant difference between the A_1 option and the control option (Table 4).

The $HgCl_2$ and NaClO are the most widely used surface sterilant in the microclonal *in vitro* reproduction of pomegranate plants. In this study, surface sterilization of explants was effectively performed and achieved a 65% survival rate of explants in sterilization of pomegranate leaf axil buds using a combination of NaClO and Na merthiolate for 20 min. The results obtained in that work[23] were higher than others. Mulaei *et al.*[24] achieved the highest rate (90.58%) of survival of axillary buds using 0.1% $HgCl_2$ solution for 10 min. Similar results were obtained in our work. In our work, the most optimal choice for surface sterilization of pomegranate explants was the A_2 variant.

Nutrient medium: There were no significant differences between experimental varieties and nutrient media (MS and WPM). However, the plants grown in the WPM medium

Table 4: Effect of surface sterilization agents on the damage of explants of experimental varieties

Pomegranate varieties	Variants	Explants not infected with bacteria	p-value	Explants not infected with fungi	p-value
August	Control	0.77±0.066	0.422	0.72±0.071	0.008
	A_1	0.95±0.034		0.80±0.064	
	A_2	1.00±0.000		0.92±0.042	
Kazake-anar	Control	0.82±0.060	0.014	0.77±0.066	0.116
	A_1	0.92±0.042		0.82±0.060	
	A_2	0.97±0.025		0.95±0.034	
Achikh-dona	Control	0.82±0.060	0.013	0.70±0.073	0.316
	A_1	0.90±0.048		0.85±0.057	
	A_2	0.97±0.025		1.00±0.000	
Tuyatish	Control	0.80±0.064	0.318	0.72±0.071	0.318
	A_1	0.92±0.042		0.82±0.060	
	A_2	1.00±0.000		0.97±0.025	

differed morphologically, possibly due to the number and length of buds resulting from bud formation time. Further, both the MS and WPM nutrient media were used for explant rhizogenesis (Table 3). For micropropagation of pomegranate mostly full-strength and half-strength MS, WPM and B5 nutrient media were used[15]. In this work, we aimed at selecting the optimal culture media for local pomegranate genotypes.

Effect of growth regulators on regeneration of explants (proliferation of buds): The explants that survived during the surface sterilization stage were transplanted into a cytokinin medium from the 2nd week of cultivation. In explants with terminal and lateral buds, the shoot regeneration was formed after 14 days. The number and length of shoots were checked in the 6th week of cultivation. Explants grown in MS_2 (1.0 mg L^{-1} BAP+0.1 mg L^{-1} NAA+0.5 mg L^{-1} GA_3) medium were not morphologically significantly different from explants grown in MS_1 (BAP 1.0 mg L^{-1}+NAA 0.1 mg L^{-1}) medium. The difference was on shoot formation day which was observed only in explants of the August variety (Fig. 2 and 3(a-h)).

Verma *et al.*[25] studied the effect of subculture on microbuds proliferation induced by callus induction of leaf explants of pomegranate variety Kandhari Kabuli in solidified MS medium supplemented with 9.0 µM BAP, 2.5 µM kinetin and 1.5 µM GA_3. They found enhanced number (I-1.20, II-3.24, III-4.07 and IV-3.92) and length (I-1.16, II-2.12, III-2.91 and IV-2.70) of microshoots. Besides, the authors showed shoot organogenesis by the addition of 10 µM BAP and 2.5 µM NAA to MS nutrient medium that produced adventitious shoots (42.95%) from explants. Parmar *et al.*[26] showed a higher rate (68.21%) of direct organogenesis from hypocotyl segments of pomegranate variety Kandhari Kabuli using different concentrations of plant growth regulators BAP (1.0-2.5 mg L^{-1}) and NAA (0.5-1.5 mg L^{-1}) in MS medium and observed the formation of 3.18 shoots per explant. The results obtained in this work were in correspondence with these above discussed data. Our results are also compatible with previous protocols in other varieties of the pomegranate, where the auxins such as BAP and NAA or IAA had a significant morphogenetic reaction in MS nutrient medium[27-29]. Shoot elongation depends on the synergistic effect of cytokinin and GA_3[30]. But in our study, no significant changes were observed in the effect of GA_3 on the length of the shoot. It may be because of not enough concentration of GA_3.

No significant differences were observed in experimental explants in WPM_2 (1.0 mg L^{-1} BA P +0.1 mg L^{-1} NAA+0.5 mg L^{-1} GA_3) nutrient medium compared to the explants grown in WPM_1 (BA P 1.0 mg L^{-1}+NAA 0.1 mg L^{-1}) nutrient medium. The shoot formation in explants of August and Achikh-dona varieties, grown in the WPM_2 nutrient medium, was several days earlier compared to the WPM_1 nutrient medium. The length of explants formed as a result of the organogenesis of shoots in the WPM_2 nutrient medium was of a higher level in August and Kazake-anar varieties compared to explants in the WPM_1 nutrient medium. No significant differences were observed in Achik-dona and Tuyatish varieties (Fig. 2 and 4(a-h)).

The addition of GA_3 to MS_2 and WPM_2 nutrient media resulted in relatively wide nodes in explants. It did not cause significant differences in the explants. El-Agamy *et al.*[31] investigated the optimum nutrient medium by propagating Manfalouty and Nab El-Gamal varieties of pomegranate on three different nutrient media: MS, Nitsch & Nitsch and WPM. In their case, the tallest microshoots were grown in (average 5.10-4.58 cm) WPM medium. The number and length of microshoots in this work correspond to their results.

ValizadehKaji *et al.*[9] studied Iranian pomegranate varieties Malas Saveh and Yousef Khani in basal WPM and MS nutrient media using different plant growth regulators. Plants grown in WPM nutrient medium had higher shoot proliferation (51.42±32.81), shoot length (2.15±1.31) and the number of leaves (6.97±3.66) compared to MS nutrient media. In this work, micro shoots grown in WPM nutrient medium were slightly morphologically different compared to microshoots in MS medium. However, there was no significant difference between microshoots.

Effect of supramolecular complex on rhizogenesis of microshoots: The rhizogenesis of explants was studied in different concentrations of MS and WPM nutrient media containing the supramolecular complex. The addition of 0.15 mg L^{-1} concentration of the complex to Rhizo-MS_1 nutrient medium resulted in an average root number of 1.36-2.14 and an average length of 1.50-2.19 cm in explants. This indicator was 2-3 times higher than the control. As a result of the addition of 0.17 mg L^{-1} of the complex to the Rhizo-MS_2 nutrient medium, the number and length of roots in the explants of the experimental varieties were 3-5 times higher than in the control (Fig. 5(a-h)) and 2-2.5 times higher than the explants grown in the Rhizo-MS_1 nutrient medium. Thus, significant differences were determined among these variants. The results of the explants grown in the WPM nutrient medium were similar with those in the MS nutrient medium.

The addition of 0.17 mg L^{-1} of the supramolecular complex to the WPM_2 nutrition media increased the root length 3-5 times compared to control (Fig. 6(a-h)). In

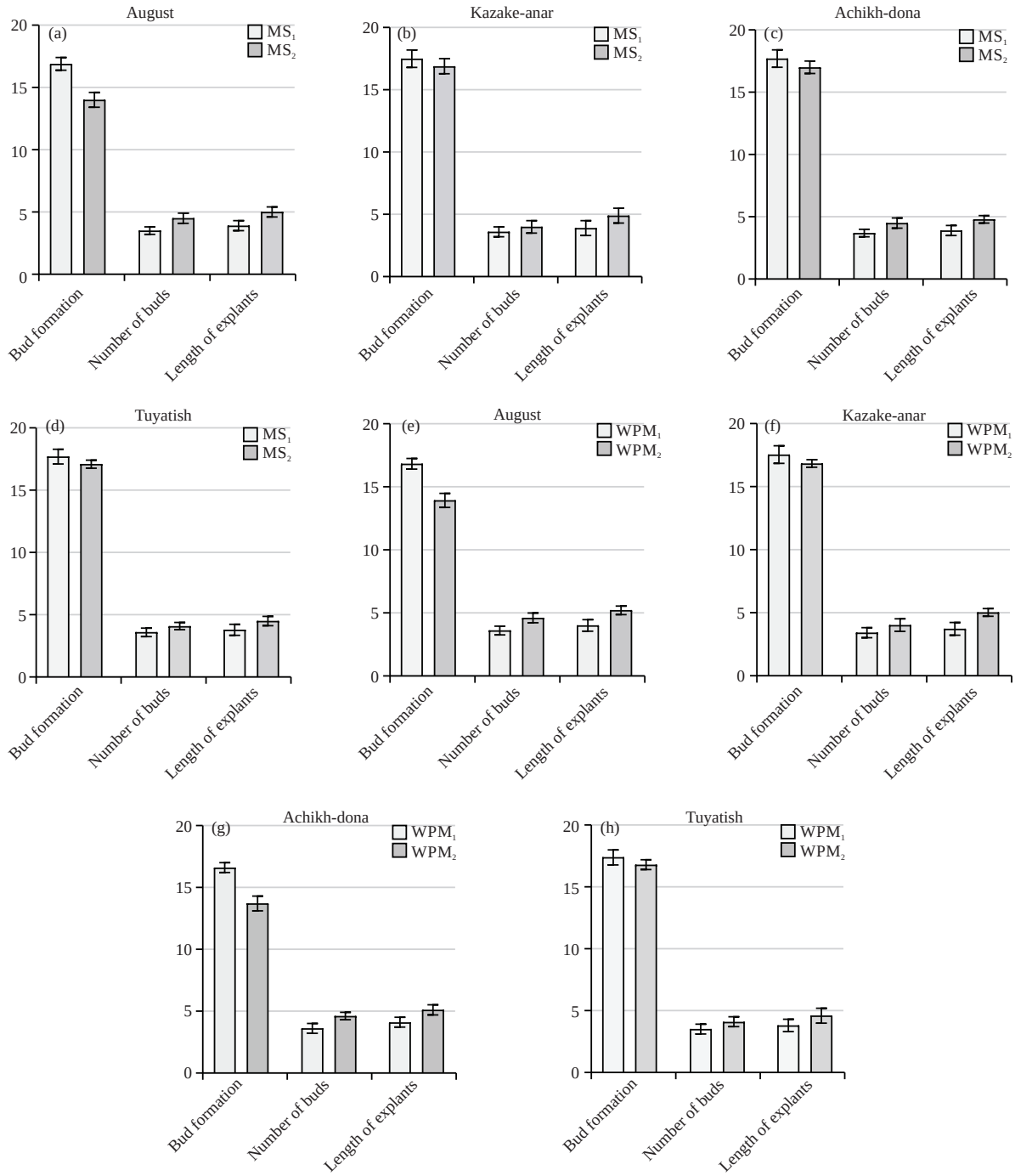

Fig. 2(a-h): Effects of phytohormones with different compositions and concentrations on shoot organogenesis of some pomegranate cultivars in MS and WPM nutrient media. MS_1 and WPM_1 media contained 1.0 mg L^{-1} BAP+0.1 mg L^{-1} NAA, MS_2 and WPM_2 media contained 1.0 mg L^{-1} BAP+0.1 mg L^{-1} NAA+0.5 mg L^{-1} GA_3

*p<0.5 in all cases except for August (0.520) and Kazake-anar (1.00) varieties in MS and Achikh-dona (0.866) variety in WPM. Error bars mean standard deviation

Rhizo-MS_2 and Rhizo-WPM_2 nutrient media containing 0.17 mg L^{-1} of the complex, the rhizogenesis stage showed the highest rate in all varieties. Due to the lack of addition of growth regulators to the control variant, rhizogenesis in these explants was of low level: The average number of roots made 0.23-0.33 and average length was 0.11-0.18 cm.

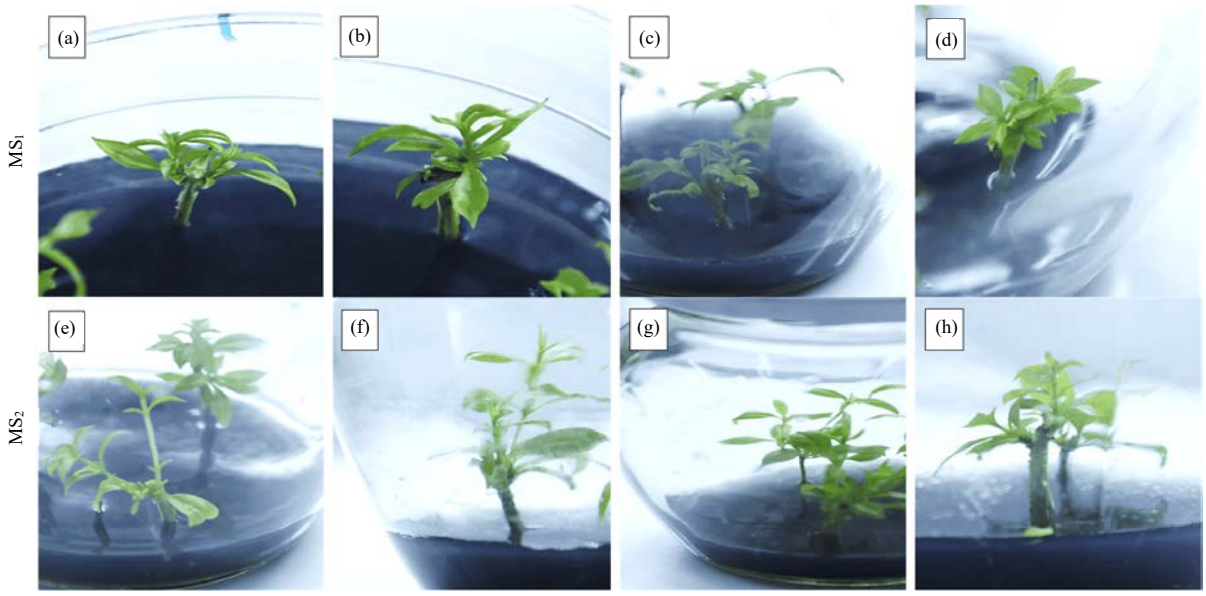

Fig. 3(a-h): Shoot growth and development in explants of experimental cultivars in MS_1 and MS_2 nutrient media, (a, e) August, (b, f) Kazake-anar, (c, g) Achikh-dona and (d, h) Tuyatish

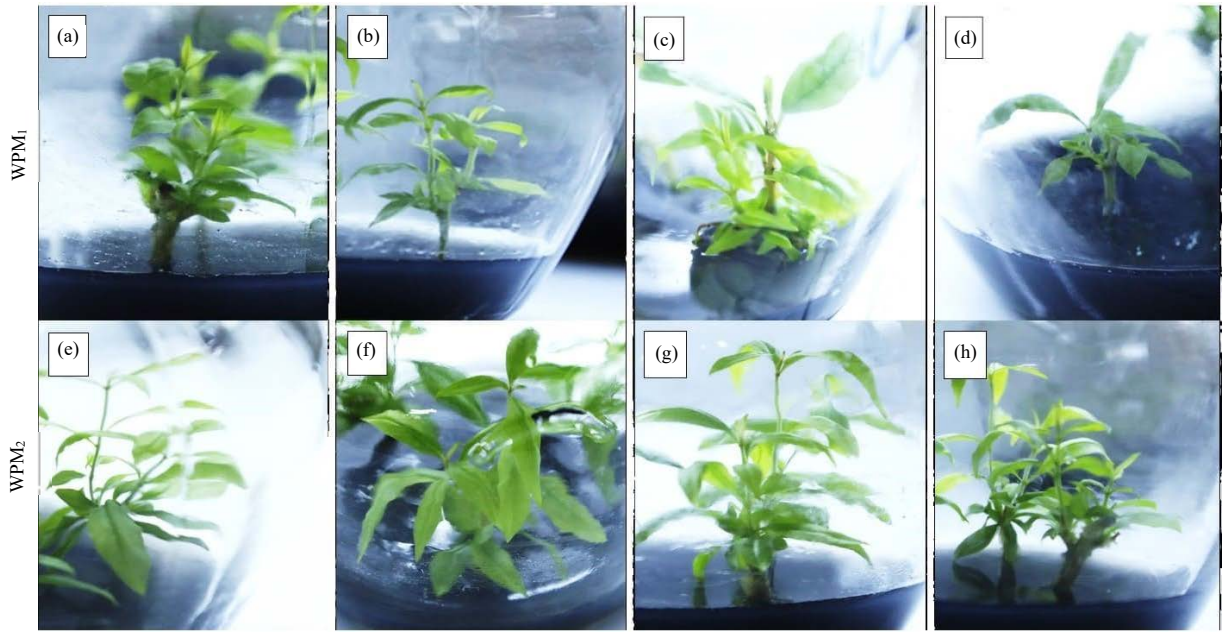

Fig. 4(a-h): Shoot growth and development of explants of experimental cultivars in WPM_1 and WPM_2 nutrient media, (a, e) August, (b, f) Kazake-anar, (c, g) Achikh-dona and (d, h) Tuyatish

Bachake *et al.*[32] found out that root formation and development in microshoots grown in WPM nutrient medium required less days compared to microshoots grown in MS nutrient medium. The addition of 1.0 mg L^{-1} NAA to WPM medium was found to increase the number of primary roots per microshoot (5.0) and the percentage of rooting (76%) compared to other concentrations.

Dessoky *et al.*[33] studied and achieved the root formation and development of Taify and Yemeni varieties of pomegranate, grown in Taif Province of Saudi Arabia, by

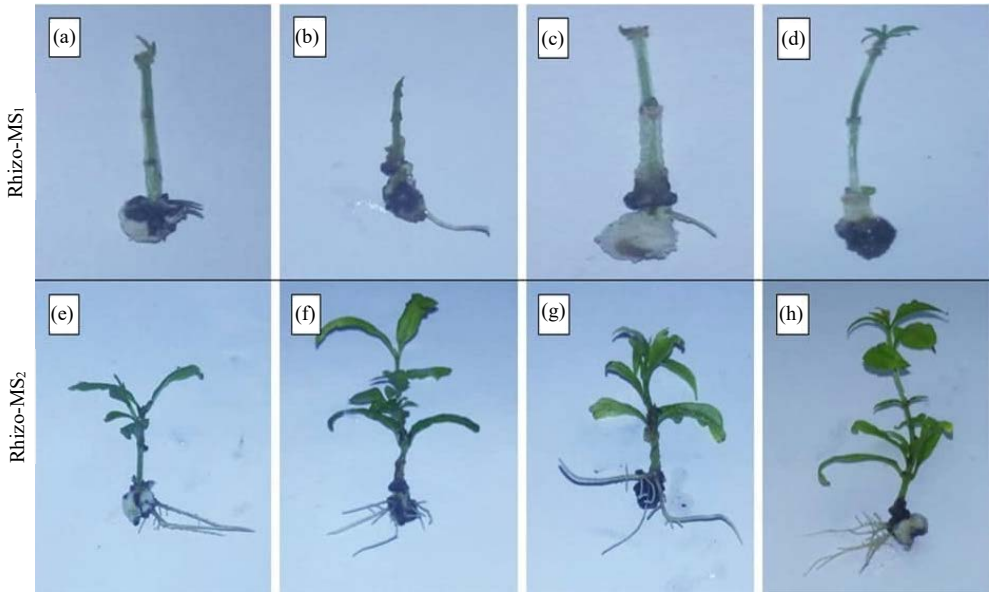

Fig. 5(a-h): Effect of supramolecular complex preparation (0.17 mg L^{-1}) on rhizogenesis of explants of experimental varieties, (a, e) August, (b, f) Kazake-anar, (c, g) Achikh-dona and (d, h) Tuyatish

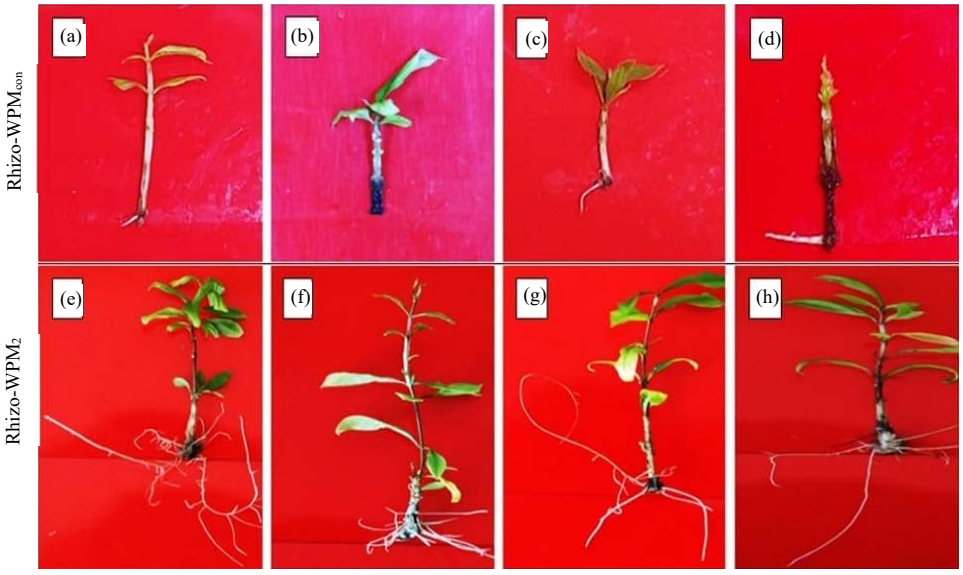

Fig. 6(a-h): Effect of supramolecular complex preparation (0.17 mg L^{-1}) on rhizogenesis of explants of experimental varieties, (a, e) August, (b, f) Kazake-anar, (c, g) Achikh-dona and (d, h) Tuyatish

adding 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} IBA to MS nutrient medium *in vitro*. The results in the varieties were 83.3 and 79.6%, respectively. These auxin compounds were the most effective in terms of shoot number and root length per explant.

Singh *et al.*[34] studied rooting and development of cotyledon and nodal explants of Ganesh pomegranate in half and full-strength MS medium and white's plant nutrient medium with different concentrations of NAA (0.1, 0.2, 0.5 and 0.8 mg L^{-1}) and supplemented with 200 mg L^{-1} activated carbon under *in vitro* condition. Rhizogenesis in microshoots resulting from the regeneration of explants showed the highest root number (4.17) and root length (3 and 87 cm). These results explain various approaches can result in optimum outcomes in different varieties.

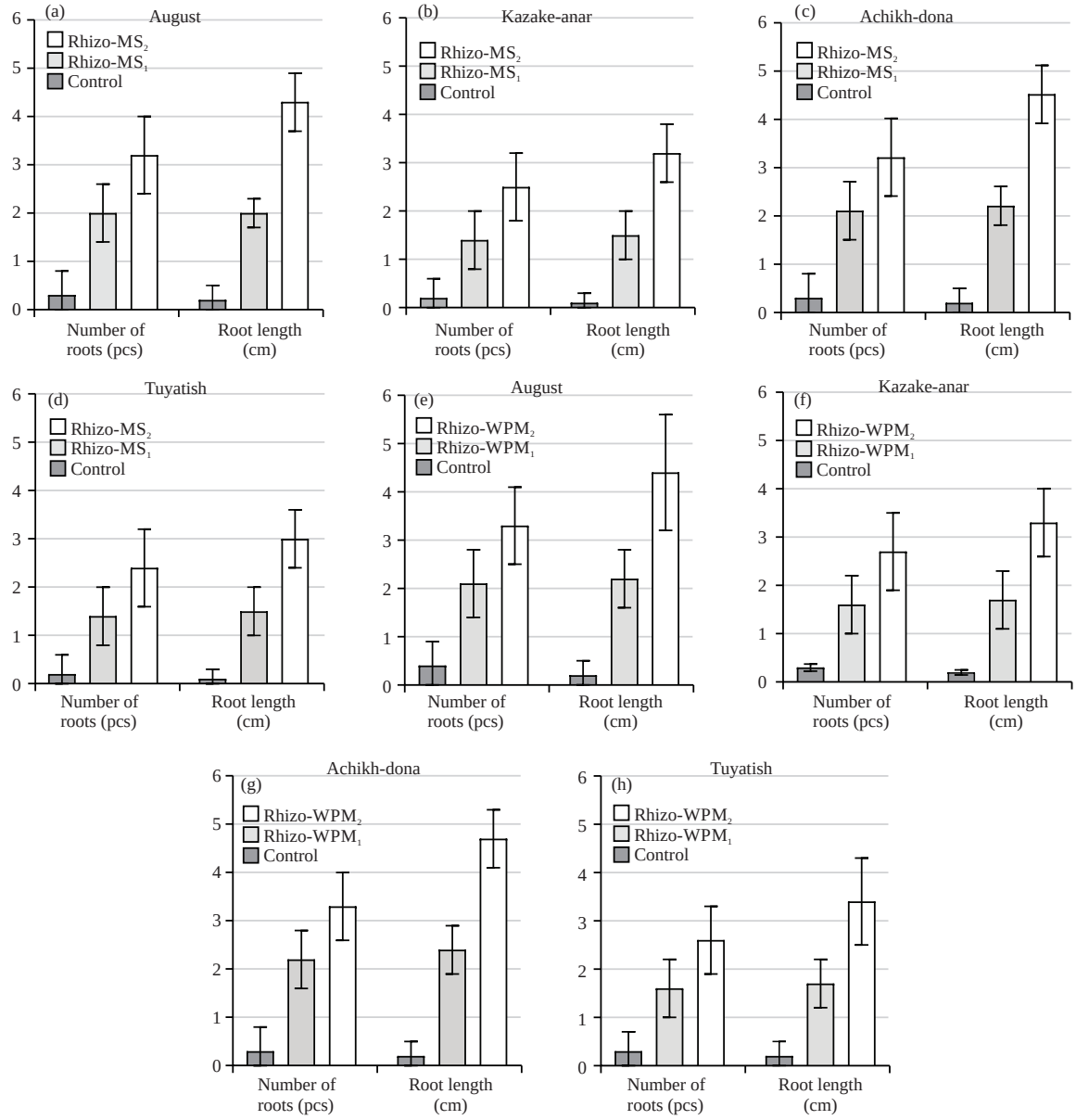

Fig. 7(a-h): Effect of different concentrations of the supramolecular complex drug on the rhizogenesis of some pomegranate varieties in MS and WPM nutrient media. The MS_1 and WPM_1 contained 0.15 mg L^{-1} of the supramolecular complex, MS_2 and WPM_2 contained 0.17 mg L^{-1} of the supramolecular complex

*p<0.005 in all cases. Error bars mean standard deviation

Patil *et al.*[35] reported that MS medium containing 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} IBA showed the highest rooting rate. The highest mean rooting response was recorded in MS medium containing 0.5 mg L^{-1} NAA (97%) or 0.5 mg L^{-1} IBA (97%). Root length was recorded from 0.3 to 3.4 cm in a nutrient medium containing IBA and 1.3-3.2 cm in a medium containing NAA. However, thick root formation was observed in a medium containing 0.5 mg L^{-1} IBA. Current results were in agreement with these outcomes that the roots of all the explants of the experimental cultivars grown in the MS medium were thicker than those grown in the WPM medium (Fig. 7(a-h)).

No significant differences were observed in the rhizogenesis of the explants grown in nutrient media (Rhizo-MS_2, Rhizo-WPM_2). However, explants grown in the WPM_2 medium differed in root system thinness compared to explants grown in the Rhizo-MS_2 medium (Fig. 6). The cuticular layer was found well developed in thin roots[36]. The

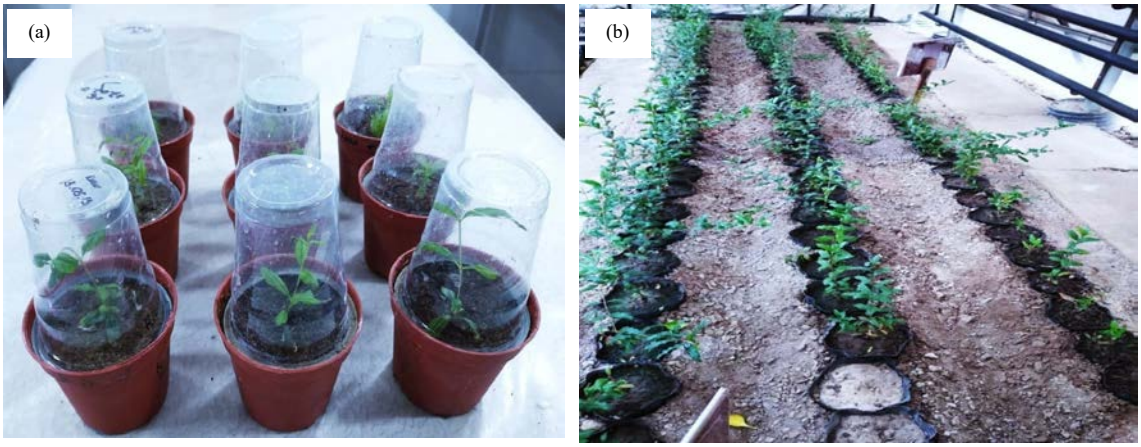

Fig. 8(a-b): Adaptation of experimental varieties to non-sterile conditions, (a) Phytotron and (b) Greenhouse

development of the cuticular layer had a positive effect on the growth and development of microplants in non-sterile conditions. This can be linked with plant genotypes that require further research. The development of Kazake-anar and Tuyatish varieties was relatively slow. Based on the conducted studies and literature analysis, it was assumed that the varieties require different nutrient media or auxins. However, it was found that the growth and development of varieties are not low and this may be related to the genotype of the plant[18,37].

Our group has been carrying out a scientific project on *in vitro* propagation of local varieties of pomegranate (*Punica granatum* L.) and the development of healthy and disease-free varieties. In the course of the project, various nutrient media were tested for *in vitro* reproduction of local genotypes. Various studies on quantitative changes and additions to all components of nutrient media are being conducted and growth and development phases in explants are being studied. Several types of plant hormones (cytokinin and auxin) have been tested and positive results have been achieved. However, imported plant hormones cost expensive. Therefore, we developed a strategy of using plant hormone substitutes such as the supramolecular complex of glycyrrhizic and salicylic acid that lower the cost of developed seedlings several times. The cost efficiency of the used supramolecular complex was about 45 times greater than classical approach that utilizes phytohormones. This approach would enable to lower the expenses and thus lead the rooting of microshoots of pomegranate varieties into practice.

Transplantation of microplants to non-sterile soil condition and their adaptation: Microplants showed a survival rate of 95% in biohumus+coconut husk+sand (2: 1: 1) substrate. Successfully acclimatized plants were transplanted to a substrate of field soil+sand (1: 1) and grown in a greenhouse for 30 days (Fig. 8(a-b)). There was no death of plants in greenhouse conditions.

The microplants grown in the greenhouse conditions were morphologically different compared to the phytotron conditions, i.e., the leaves were large and wide, the intensity of greenness was high, the stems were branched, etc. Such a difference in the growth and development of microplants may be due to the change of climatic factors from artificial to natural, the expansion of the growing area and the implementation of additional agrotechnical measures such as tillage, fertilization and irrigation.

CONCLUSION

In this study, the effect of phytohormones on the regeneration of explants during the *in vitro* propagation of some local pomegranate genotypes was investigated and the optimal amount of glycyrrhizin and salicylic acid supramolecular complex on the rhizogenesis of microshoots was determined. The addition of the 0.17 mg L^{-1} supramolecular complex to the nutritional medium (Rhizo-MS_2 and Rhizo-WPM_2) resulted in 3-5 times higher root number and length in explants compared to the control. About 45 times greater cost efficiency of the complex promotes using it in the practice of rooting while developing microshoots.

SIGNIFICANCE STATEMENT

We developed *in vitro* propagation of local varieties of pomegranate (*Punica granatum* L.) varieties of Uzbekistan. The selected surface sterilants in this study reduced the

bacterial and fungal damage of explants of the experimental varieties by several times compared to the control. The optimal effect of the type and concentration of phytohormones on the regeneration of explants was determined. The effect of the supramolecular complex of glycyrrhizic and salicylic acids on the rhizogenesis of microshoots was established. The addition of a 0.17 mg L^{-1} dose of the supramolecular complex to MS and WPM nutrient media showed several times greater levels of rhizogenesis of microshoots compared to the control.

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