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Study of Direct Adventitious Shoot Regeneration in Pomegranate (*Punicagranatum* cv. MalasSaveh) through Cotyledonary Explants

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ABSTRACT

Punicagranatum cv. Malassaveh is one of the most desirable commercial cultivars in Iran. In this study some experiments were conducted to introduce the best hormonal treatments for direct adventitious shoot regeneration from mentioned cultivar via cotyledonary explants. For this purpose, both liquid and agarMurashige and Skoog (1962) (MS) medium supplemented with BA at 2, 4.5, 9, 13 µM alone and in combination with 5.5 µM NAA or 5 µM 2,4-D were used. The highest number of shoot per explant 8.2 and 7.9 were obtained in MS liquid and agar medium supplemented with 13µM BA and 5.5 µM NAArespectively, although there was no significant differences between two medium. The highest shoot length 1.65 cm was obtained from MS liquid medium supplemented with13µM BA and 5.5 µM NAA as well. The highest rooting was obtained in half-strength MS containing 2 mg/l IBA with 3.1 roots per explant. Rooting plantlets were transferred to Jiffy and acclimatized after 35 days successfully.

Keywords: Shoot regeneration; Cotyledon; Pomegranate.

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INTRODUCTION

Pomegranate (*Punicagranatum*L.) is generally known in a distinct family (Punicaceae), which comprises only one genus (*Punica*) and only two species, *P. granatum*and *P. proptopunica*, (Popenoe, 1974). Pomegranate is native to Iran and perhaps some surrounding areas. is a multipurpose species having both pharmaceutical and nutritional significance, fruit is high in vitamin C, potassium andantioxidant polyphenols, a good source of fiber and low in calories (Fuhrman & Aviram, 2007).

Pomegranate trees are conventionally propagated via cutting, a highly desirable technique, however, this method is not very efficient because of contamination, non-uniformity of produced saplings and highly cost of human and space, besides, it requires about 1 year to grow sapling. Genetic improvement of fruit trees, in general, by conventional breeding methods is a slow and difficult process due to long life cycle. In this respect, the introduction of agronomically important traitsby genetic transformation could be a rapid alternative method with minimum destruction of genetic integrity of the elite commercial cultivars. A desirable requirement for successful genetic transformation is the development of an efficient method to regenerate plants adventitiously in a reasonably high frequency from explants such as leaf, stem, hypocotyl or cotyledon segments (Shao *et al*., 2003; Sharon *et al*., 2000; Terakami *et al*., 2007). Besides, some programs such as identification and propagation of commercial cultivars with preserving their desirable traits, producing plant materials and high number of saplings in short time, study of biotic and abiotic stress resistances, production of useful secondary metabolites, are also required to utilize the high effective tissue culture techniques. In vitro plantlet regeneration through adventitious shoot formation from explants has been reported in a number of fruit species (Zimmerman & Swarts, 1994).

Rapid clonal propagation has been documented in pomegranate from shoot tip (Mahishi *et al*., 1990), nodal segments and cotyledonary nodes (Naik *et al*., 1999, 2003). Some reports have been indicatedplantlet regeneration through somatic embryogenesis (Bhansali, 1990; Nataraja & Neelambika, 1996).Shoot organogenesis has been reported in pomegranate from callus derived from anther wall (Moriguchi *et al*., 1987) or leaf segment (Omura *et al*., 1987). Adventitious shoot regeneration has also been reported from cotyledon explant in *Punicagranatum*cv. Ganesh (Naik & Chand, 2003) and in wild pomegranate (Murkut *et al*., 2002; Kanwar *et al*., 2010) in the presence of ethylene inhibitors.

In this study, an efficient method for direct adventitious shoot regeneration from cotyledonary explants without any subsequent subcultures with rapid organogenesis and high number of shoot regenerationin order to utilize in *Punicagranatum*cv. Malas Savehbreeding and gene transformation projects is reported.

MATERIALS AND METHODS

Explant preparation

Seeds from immature pomegranate (Punicagranatum L. cv. MalasSaveh) fruits were collected, washed free of juicy testa, kept under running tap water for 1 h, soaked for 24 h followed by a 30 sec treatment with 70% (v/v) ethanol and finally rinsed with distilled water. Seeds were sterilized with 20% (v/v) commercial bleaching solution (containing 5% active NaHClO) for 20 min and rinsed 5-6 times with distilled water.The shell of surface-sterilized seeds was removed under aseptic condition, then naked seeds were established in 125 mL screw-capped glass containing PGRs-free MS medium. Two to three days old developed cotyledons without proximal ends used as explantswere cut into 5-10 mm^2pieces and placed on the medium.

Culture medium and growth condition

In order to study the callus induction, just agar medium and for shoot regeneration studies, both liquid and agarMurashige and Skoog (1962) medium scontaining 30 mg/l sucrose were used. Allkind of mediums were further supplemented with 2, 4.5, 9, 13 µMbenzyladenine (BA) alone or in combination with5.5µMnaphthaleneacetic acid (NAA) or 5 µMdichlorophenoxyacetic acid (2,

4-D) and PGRs-free MS medium was used as control treatment. The pH of the medium was adjusted to 5.8 by adding 1 N HCl or NaOH. After adding 8 mg/l agar (in agar medium), the medium was autoclaved (121ºC and 1.5 atm) and then dispensed into 125 mL screw-capped glasses and 12cm diameter petridishes for liquid and agar medium respectively. Cultures were maintained at 25±1ºC, 2500 Lux light density provided by cool white fluorescent and 70% relative humidity.

For comparison of callus induction studies in light and dark condition, agar mediums were divided into two groups, one was kept under dark condition and another kept under 16 h photoperiod and2500 Lux light density, both groups were maintained at 25±1ºC and 70% relative humidity. Data including amount and frequency of inducing callus were collected after 4 weeks and presented in Table 1 and 2.

In order to study the effects of mediums physical condition on shoot regeneration, small pieces of callus produced on agar medium were subcultured on liquid medium of the same composition and placed on shaker with 90 RPM. All cultures (agar and liquid) were grown at 25±1ºC under 16 h photoperiod, 2500 Lux light density and 70% relative humidity as described above. Data on shoot regeneration, regeneration frequency, mean number of shoot per explant and shoot length was recorded after 8 weeks (from the date of explant establishment) as shown in Table2.

Rooting and acclimatization

Shoots, 1.5-2.5 cm long, were excised and transferred to half-strength MS medium (1/2 MS) supplemented with 1, 2 mg/l IBA and 1, 2 mg/l NAA. Number of roots per shoot and root length was recorded after 3 weeks as presented in Table 3.

Plantlet with well-developed roots were removed from the culture medium and the roots were washed gently with distilled water then transferred to capped plastic box containing disinfected jiffy with 1/1000 (w/v) fungicide and were covered with polyethylene bags to maintain high humidity for 10 days. Covers were gradually removed for acclimatization.

Statistical analysis

All experiments were conducted in a completely randomized design (CRD). Each treatment consisted of 12 replicates. Data on percentage of explant forming shoots, shoot number per explant and shoot length were collected after 8 weeks, root number and length were collected after 3 weeks, and the means were calculated and $(X+0.05)^{1/2}$transformation was performed. For significant treatment effects, Student-Newman-Keul (SNK) test ($P<0.05$) was used to compare the means. All transformed data are presented in original values. Whole experiment was replicated twice.

RESULTS AND DISCUSSION

Callus induction

Callus was initiated after 10 days from cut ends of explants and covered whole surface of explants within 4 weeks. Callus induction in light condition was more than those were in dark condition and mediums supplemented with NAA showed more callus induction (Table 1). The highest frequency of callus induction (100%) was obtained in medium supplemented with 13 µM BA and 5.5 µM NAA, in control treatment callus was not induced in explants (Table 2). This callus was compact, green in color, and fast growing, while callus induced in medium supplemented with 2,4-D was friable and cream in color (Figure 1). Murkute *et al*. (2002) have obtained highest callus induction (76.4%) in MS medium supplemented with 4.44 µM BA and 2.69 µM NAA, Kanwar *et al*. (2010) have obtained 81% of callus induction in MS supplemented with 9 µM BA and 21 µM NAA. Thus the results of the present investigation showed higher frequency of callus induction (100%) from cotyledonary explant as compared to earlier reports. Foughat *et al*. (1997) reported callus induction in MS supplemented with 9.26 µM kinetin, 21.52 µM NAA and 15% coconut water. There are many reports which introduced cotyledon pieces as

best explant for callus induction in Punicagranatum (Jaidka & Mehra, 1986; Foughat *et al*., 1997; Murkute *et al*., 2002; Naik & Chand, 2003; Kanwar *et al*., 2010)

Shoot regeneration

Data presented in Table 2 reveals that the highest percentage of shoot regeneration in explant (90%) was obtained in MS medium supplemented with 13 µM BA and 5.5 µM NAA. Jaidka and Mehra (1986) reported highest ferequency of shoot regeneration of 80% from the same explant. Murkute *et al*. (2002) reported 62.4% shoot bud regeneration frequency on MS medium containing 1 mg/l BA and 0.5 mg/l NAA. Naik and Chand (2003) and Kanwar *et al*. (2010) also reported 57% and 63% of shoot regeneration frequency in explants. In comparison with other results, present investigation showed more regeneration frequency.

Highest number of shoots (8.2) per explant was obtained on liquid MS mediumsupplemented with 13 µM BA and 5.5 µM NAA. The mean number of shoots in this treatment was not significantly different with the same treatment on agar medium which was (7.9) per explant, while there was significant difference between control and these treatments. The average of shoot length in mentioned treatment was 1.65 and 1.48 cm in liquid and agar medium respectively (Figure 1).

Statistical analysis showed BA singularly had significant effect on shoot bud differentiation from the explant, as increase in BA concentration led to more shoot regeneration, whereas there was no shoot bud differentiation in medium free of BA. Presence of BA seems to be essential to induce shoot bud formation, however, BA alone could induce adventitious shoot buds but at a frequency which was unacceptably low, moreover, BA had no influence on shoot length (Table 2). Our results were in accordance with Jaidka and Mehra (1986), Murkute *et al*. (2002), Naik and Chand (2003) and Kanwar *et al*. (2010) which reported BA as the most important PGR in adventitious shoot regeneration. In comparison with previous reports, various optimum concentration of BAmay be due to cultivar differences, endogenous levels of PGRs and even orientation of explants on culture medium.

Naik and Chand (2003) and Kanwar *et al*. (2010) also reported the importance of BA in combination with NAA to induce shoot bud differentiation, in their reports the highest number of shoots has been obtained in the presence of ethylene inhibitors such as $AgNO_3$ or AVG while in present study no ethylene inhibitors were used, it could be due to cultivar differences and explant preparation techniques. Aside from this point, in present investigation callus induction and shoot regeneration medium was the same which resulted in timesaving, laborsaving and material saving.

NAA had significant effect on shoot regeneration, as in treatments free of NAA there was no shoot regeneration or at least low number of shoot were obtained, 2, 4-D, on the other hand, had no desirable effect on shoot regeneration, as BA in combination with 2, 4-D was not proper composition of PGRs in order to adventitious shoot regeneration, because no shoot regeneration were observed in those treatments (Table 2).

Shoot buds usually arise from a group of meristematic cells called meristemoids or nodules, which give rise to leaf primordia and the apical meristem. In a favouring shoot regeneration medium, meristemoids appear and the developing buds develop procambial strands, which become connected with the pre-existing vascular tissue present in the explant or callus. Meristemoids are considered to arise in areas that accumulate starch, which is believed to serve as an energy source for shoot bud differentiation.Organ formation depended on the auxin/cytokinin ratio and not on their absolute concentrations (Murashige & Skoog, 1962). Synergistic effects of BA and NAA led to induce cell division, form nodular meristemoids and finally adventitious shoot bud regeneration. The endogenous levels of these PGRs may vary considerably among different plant species so that a PGR may be suboptimal, optimal or superoptimal for shoot regeneration. The response of a plant species to an exogenous PGR would, therefore, depend mainly on the endogenous level of that PGR.

Physical condition of medium (liquid or agar) has a marked influence on shoot regeneration; in some cases liquid medium was superior, while in others it was drastically inferior to agar medium. In present study, shoot regeneration percentage, shoot number and length in liquid medium was

more than agar mediumalthough there was no significant difference between two mediums (Table 2). Theonly difference was the time of regeneration beginning that was occurred just one week after callus establishment in liquid medium while it was 16 days for agar medium, this difference could be due to appropriate availability and adequate absorption of nutrients, especially sucrose as an energy source, in liquid medium. Generally, it is concluded that agar medium was superior in this respect because the more phenol exudations were released in liquid medium which led to medium browning and explants destruction eventually, besides, in agar medium callus induction and shoot bud regeneration was occurred in one medium and it is not required that induced callus is transferred to the liquid medium.

Rooting of shoots and acclimatization

Highest number of roots per shoot (3.1) was obtained in half-strength MS medium supplemented with 2 mg/l IBA which was found to be the best and significant as compared to all other treatments, there was no significant difference in root length between treatments (Table 3). The regenerated shoots were become well-rooted in 3 weeks (Figure 1). Addition of an auxin to the medium was essential to induce rooting in the regenerated shoots. The IBA has been reported as the best rooting auxin by Naik *et al*. (1999) which was in accordance with our result, however, in some other reports NAAhas been introduced the bestauxin (Moriguchi *et al.,* 1987; Omura *et al*., 1987; Mahishi *et al*., 1991; Kantharajah *et al*., 1998; Naik & Chand, 2003). Plantlets with fully expanded leaves and well developed roots were successfully acclimatized in 35 days and over 70% of plantlets survived.

Table1: Callus induction in different treatments

BA+NAA(µM)	Callus induction	
	Light	Dark
2+5.5	+++	+
4.5+5.5	+++	+
9+5.5	+++	++
13+5.5	+++	++
BA+2,4-D(µM)		
2+5	+	+
4.5+5	++	+
9+5	+	+
13+5	+	.

0: without callus induction, +: low callus induction in cut ends, ++: high callus induction in cut ends, +++: callus induction in cut ends and surface

CONCLUSION

On the basis of present investigation it is concluded that cotyledon excised from immature seeds are the best explants for callus induction and plantlet regeneration. In this study we have successfully developed a protocol for adventitious plant regeneration which differs from earlier organogenesis reports in which the explants as well as the cultivars were different (Omura *et al*., 1987; Moriguchi *et al*., 1987; Kantharajah *et al*., 1998) and the percentage and the average number of shoots per explant were very low compared to the result of this study. Additionally, in this investigation the frequency of callus induction and percentage of shoot regeneration in explant were more than the last reports (Murkute *et al*., 2002; Naik & Chand 2003; Kanwar *et al*., 2010), although in their reports highest number of shoot regeneration were obtained in the presence of ethylene inhibitors. In our study callus induction and regeneration occur in one medium so it makes present protocol more economic by shortening regeneration duration, labor and material saving. This protocol seems to be promising and may help in Punicagranatum improvement program through genetic transformation because high frequency of plantlet regeneration in this method increases the achievement possibility of transformed plantlets. The probability of

undesirable somatic variation is decreased because subsequent subcultures were unnecessary, besides it provides high responsive explants with thin cell wall which are appropriate for introgression of useful genes mediated by *agrobacterium* or other vectors.

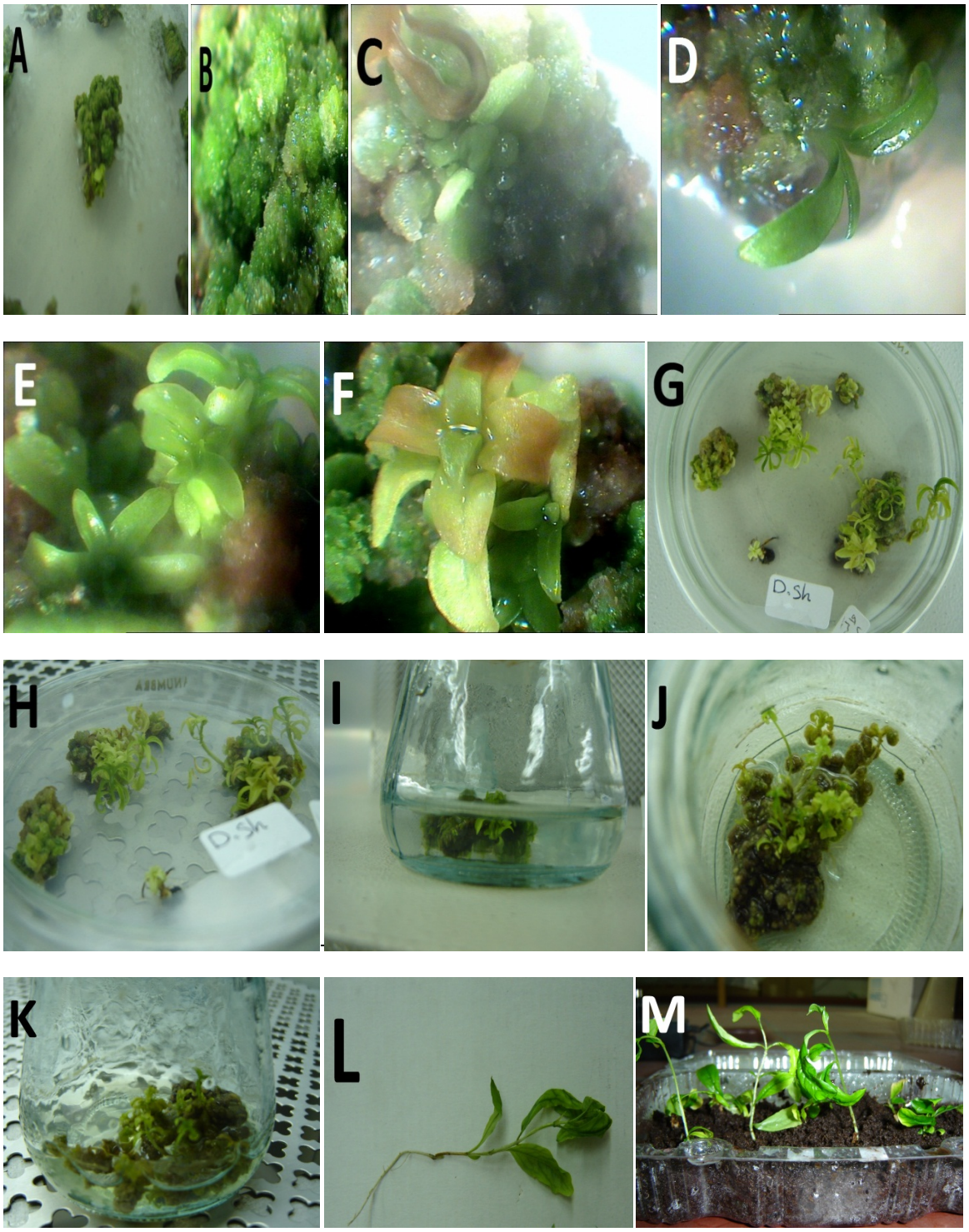

Fig.1: A, B: callus induction in medium supplemented with NAA C, D, E, F: appearance of meristemoids, adventitious bud formation and shoot regeneration G, H: highest shoot regeneration in agar medium I, J, K: highest shoot regeneration in liquid medium L, M: rooting and acclimatization of plantlet.

Table2: Effect of different plant growth regulators combination on callus induction, frequency of shoot regeneration, mean number of shoot per explant and shoot length

Treatment	%Callus induction	%Shoot regeneration		Shoot num./Explant*		Shoot length*	
BA+NAA (µM)		Agar	Liquid	Agar	Liquid	Agar	Liquid
2+5.5	70%	60% b	60% b	2.2 c	3.6 c	1.27ab	1.31ab
4.5+5.5	70%	70% b	70% b	5.4 b	6.1 b	1.53 a	1.64 a
9+5.5	80%	60% b	70% b	5.2 b	6.0 b	1.33ab	1.58 a
13+5.5	100%	90% a	90% a	7.9 a	8.2 a	1.58 a	1.65a
BA (µM)							
2	-	-	-	-	-	-	-
4.5	-	-	-	-	-	-	-
9	20%	20% c	20% c	0.4 d	0.9 d	0.80 c	0.80 c
13	20%	20% c	30% c	0.5 d	1.1 d	0.90 c	1.11bc
BA+2,4-D (µM)							
2+5	30%	-	-	-	-	-	-
4.5+5	40%	-	-	-	-	-	-
9+5	30%	-	-	-	-	-	-
13+5	30%	-	-	-	-	-	-
Control	-	-	-	-	-	-	-

*Means within a column having same letter are not significantly different according to Student-Newman-Keul (SNK) ($P<0.05$).

Table3: Effect of IBA and NAA on root number and length

Treatment	Root number*	Root length (cm)*
IBA =1	2 b	1.08 a
IBA =2	a3.1	1.18 a
NAA =1	1.5 b	0.98 a
NAA =3	2 b	1.43 a

*Means within a column having same letter are not significantly different according to Student-Newman-Keul (SNK) ($P<0.05$).

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