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# Callus Induction and Organogenesis of Seven Local Pomegranate (*Punica granatum* L.) Plants Derived from Seeds.

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**ABSTRACT**

Seven local cultivars of pomegranate (*Punica granatum* L.) - Masafik, Melisse, Radisho, Armishte, Sahraban, Halapja, and Dwarf pomegranate - were cultivated in the Kurdistan region of Iraq from October 2020 to February 2023. The purpose was to test their callus induction and organogenesis to establish efficient protocols for mass production and future molecular studies. The results showed that treating the seeds by soaking them in water and a salicylic acid solution separately for 24 hours increased seed germination in some cultivars. It was observed that the leaves responded better than the cotyledons for callus induction. Furthermore, the combination of MS salts plus 1.5 $mgL^{-1}$ BAP supplemented with 1.5 $mgL^{-1}$ NAA significantly increased callus induction. In addition, Armishte and Shahraban explants exhibited positive responses for callus induction, followed by Masafik, Radisho, Melisse, Halapja, and Dwarf. The combination of 3.0 $mgL^{-1}$ BAP with 0.5 $mgL^{-1}$ NAA resulted in a higher percentage of shoot organogenesis and regenerated plantlets derived from callus. Conversely, 2.0 $mgL^{-1}$ BAP + 0.5 $mgL^{-1}$ NAA and 1.0 $mgL^{-1}$ BAP + 0.5 $mgL^{-1}$ NAA protocols showed lower responses, respectively.

***Keywords*:** Regeneration, *In vitro*, Organogenesis, Calus, *Punica granatum*.



## INTRODUCTION

Pomegranate is a fruit rich in polyphenolic compounds that benefit heart health. It can help reduce blood pressure and chest pain and expel worms from the intestines [1,2]. Additionally, it has anti-diabetic properties [3]. Studies suggest that pomegranate may have anticancer effects, particularly for certain types of cancer, such as prostate cancer. It has been observed to limit the spread of tumors and slow their growth. Furthermore, pomegranate may help protect brain health and prevent conditions like Alzheimer's disease and Parkinson's by reducing oxidative damage and increasing the survival of brain cells [4,5,6,7].

*In vitro* regeneration protocol can be used to produce suitable pomegranate planting material on a large scale, which will make commercialization achievable. Micropropagation in pomegranate can be initiated through the regeneration of existing meristems and/or regeneration from adventitious meristems, or regeneration by somatic embryogenesis [8]. Protocols have been developed for the regeneration of callus, which is the major outcome of plant tissue culture that based on the principle of totipotency, using several explants, such as leaf segments [9] and cotyledon explants. [10] as well as the plant can regenerate new organ and even an entire copy of themselves after they are wounded which is means organogenesis. Thus, the present study established callus induction and organogenesis to develop a reliable and successful micropropagation protocol for pomegranate mass production derived from seeds and future molecular studies.

## Materials and methods

Seeds of seven cultivars of pomegranate *in vitro* culture were used in callus induction and plant regeneration**.** The experiments were carried out in plant biotechnology laboratories of Scientific Research Center, College of Science, Duhok University.

## Seed surface sterilization

The mature seeds of the seven cultivars were isolated from ripe fruits. They were gently pressed between filter paper to remove the juicy pulp and then kept under running tap water for 30 minutes. Subsequently, they were dried at room temperature for two weeks. The seeds were then surface sterilized in running tap water for 15 minutes, transferred to a laminar

flow cabinet, immersed in 70% ethanol for one minute, and soaked in a 2.5% NaOCl ratio for 13 minutes with three drops of Tween 20. Finally, they were rinsed for three minutes with sterilized distilled water three times. Any damaged seeds by sterilant and isolation were removed, and the undamaged ones were ready for culturing. To find a healthy source for callus induction, three experiments were designed using MS salts as a basic medium as follows:

(A): Untreated seeds were directly cultured after sterilization. (B): Seeds soaked in sterilized distilled water for 24 hours to soften the seed coat, followed by a longitudinal incision along the seed coat. And (C): Drenched seeds in 100mg/l salicylic acid solution for 24 hours with an incision along the seed coat.

Three seeds were cultured in each jar with three replicates, and cultures were kept in the dark growth room at 25±2°C for one week. After that, they were subjected to a photoperiod of 16 hours in light conditions with 2000 lux of light intensity and eight hours in the dark at the same temperature for three months. After that, the response result was recorded (Figure 1).

Figure (1) I*n vitro* pomegranate seed germination on MS medium

**Callus induction**

After 60 days in culture, 30-day-old seedlings were used as an explant source. Cotyledons and leaves of germinated seeds were used as explants for callus induction. The cotyledons were split transversely into two halves, and the leaf margins were removed and cut into small pieces. All these explants were cultured in selected MS media containing different concentrations of plant growth regulators as follows: A: MS media supplemented with 1.5 $mgl^{-1}$ BAP and 1.5 $mgl^{-1}$ NAA. B: MS media supported with 2 $mgl^{-1}$ BAP and 1 $mgl^{-1}$ NAA. And C: MS media containing 1 $mgl^{-1}$ Kinetin with 1 $mgl^{-1}$ NAA. Three explants per jar with three replicates were cultured in a growth room at 25±2°C with a controlled photoperiod of 16 hours of light with light intensity around 2000 lux and 8 hours of darkness.

**Remediation of phenolic exudation**

To address the issue of explant browning caused by the oxidation of phenols during the initiation stage, two treatments were employed: first, quickly transferring explants to a new medium (subculturing), and second, adding 100 $mgl^{-1}$ of polyvinylpyrrolidone (PVP) to the growth media.

**Organogenesis of the Callus**

For the regeneration of the callus, the callus that was created from the explants was gathered and then separated into small pieces weighing 1 gram each for shoot regeneration. Various protocols for callus organogenesis were proposed, including the use of MS medium supplemented with different combinations of BAP (1.0, 2.0, 3.0) $mgL^{-1}$ and NAA 0.5 $mgL^{-1}$. Three callus pieces were cultured in each jar with three replicates. The cultures were maintained in a growth room for eight weeks to encourage callus differentiation and shoot proliferation. The resulting shoots were subsequently transferred to the same favorable multiplication media.

**Statistical analysis**

The data collected was analyzed using SPSS programs (SPSS, 2019) to conduct statistical analysis, including one-way ANOVA, two-way ANOVA, and three-way ANOVA, as well as descriptive statistics for the parameters under study using CRD design. The means of the parameters were estimated using the Duncan test [11].

**Results and discussion**

**Seed germination**

The results presented in Table 1 indicate no significant differences or effects due to the treatments in Masafik and Radisho. However, seeds treated with salicylic acid solution showed a significant 66.6% increase in germination compared to other treatments in Melisse. Additionally, the seed germination of Armeshte, Sahraban, and Dwarf cultivars increased in both treated seeds compared to the control. An exception was observed in Halapja seeds, which showed 100% germination when soaked in water, while germination decreased in those soaked in salicylic acid and the control.

Table (1): Effect of Full-strength MS in seed germination for the seven cultivars of pomegranate after 60 days in culture.

| Cultivar Type | Seed treatment | | |
|---|---|---|---|
| | Untreated (control) | Soaked in water | Soaked in Salicylic acid |
| Masafik | 33.30 c | 33.30 c | 33.30 c |
| Melisse | 0.00 d | 0.00 d | 66.67 b |
| Radisho | 33.30 c | 33.30 c | 33.30 c |
| Armishte | 44.40 a | 66.60 b | 66.67 b |
| Sahraban | 33.30 b | 100.00 a | 100.00 a |
| Halapja | 22.20 c | 100.00 a | 33.30 c |
| Dwarf | 0.00 d | 33.30 c | 33.30 c |
| Effect of treatment | 23.79c | 52.357 b | 52.3619 b |

Overall means with different letters for Treatments (Horizontal) differed significantly according to Duncan's multiple range test at 5% level

Seed dormancy is a significant hurdle for plant establishment and reproductive growth in important plant species like pomegranate. This dormancy is controlled by genetic factors and environmental cues [12]. Seed priming has become a common seed treatment in the past thirty years. It aims to increase the germination rate, achieving uniformity and sometimes total germination for the seeds [13]. Pomegranate seeds have a low and irregular germination rate, making it difficult to propagate and make seedlings available through the seeds. The hard seed coat inhibits water entry and gaseous exchange [14]. Water is generally used to overcome dormancy at the embryonic level, resulting in positive effects on seed germination [15]. Additionally, acid treatment of pomegranate seeds could significantly increase germination percentage and speed. This treatment is necessary before culturing to induce test permeability for water [14]. Various pre-sowing seed treatments have been used in *in vitro* pomegranate seed cultures to increase the germination rate and reduce the time between seed culture and seedling emergence. One of these treatments involves soaking seeds in sterilized distilled water for 24 hours to improve germination performance [16,17].

## Callus induction

The results from Table 2 show the impact of using different combinations of plant growth regulators (PGRs) and types of explants on callus formation for seven different cultivars. The data indicate significant differences based on the type of explant used. Specifically, leaf explants demonstrated a higher response rate (49.7%) for callus formation compared to cotyledons (28.03%) (see Figure 2). Additionally, the combination of MS salts with 1.5 $mgl^{-1}$ BAP and 1.5 $mgl^{-1}$ NAA led to a significant increase in callus induction to 44.4%, compared to the other two protocols, especially the 1.0 $mgl^{-1}$ kin + 1.0 $mgl^{-1}$ NAA which resulted in a 33.3% induction rate. In terms of cultivar type, Armishte and Sahraban showed the highest positive response to callus induction at 57.4%, followed by Masafik (50%), Radisho (35.2%), Melisse (33.3%), Halapja (27.8%), and Drawf (11.1%).

Table (2): Effect of MS supplemented with PGRs. and different types of explants on callus induction response (%) of pomegranate after 60 days in culture

| Cultivar Type | Type of explant | PGRs Protocols | | | Effect of cultivar | Effect of explant type |
|---|---|---|---|---|---|---|
| | | 2.0 mg/l BAP + 1.0 mg/l NAA | 1.5 mg/l BAP + 1.5 mg/l NAA | 1.0 mg/l kin + 1.0 mg/l NAA | | |
| Masafik | Cotyledons | 0 j | 100 a | 22.2 h | 50 b | Cotyledons |
| | Leaves | 88.8 b | 33.3 g | 55.5 e | | |
| Melisse | Cotyledons | 0 j | 66.6 d | 11.1iI | 33.3 c | 28.03 B |
| | Leaves | 88.8 b | 0 j | 33.3 g | | |
| Radisho | Cotyledons | 0 j | 66.6 d | 0 j | 35.2 c | |
| | Leaves | 100 a | 11.1 i | 33.3 g | | |
| Armishte | Cotyledons | 0 j | 100 a | 33.3 g | 57.4 a | Leaves |
| | Leaves | 77.7 c | 33.3g | 100 a | | |
| Sahraban | Cotyledons | 0 j | 100 a | 22.2 h | 57.4 a | |
| | Leaves | 77.7 c | 44.4 f | 100 a | | |
| Halapja | Cotyledons | 0 j | 44.4 f | 0 j | 27.8 d | 49.7 A |
| | Leaves | 88.8 b | 0 j | 33.3 g | | |

| | | | | | |
|---|---|---|---|---|---|
| Dwarf | Cotyledons<br>Leaves | 0 j<br>22.2 h | 22.2 h<br>0 j | 0 j<br>22.2 h | 11.1 e |
| Effect of treatment | | 38.9 b | 44.4 a | 33.3 c | |

Overall means with different letters for Cultivar (vertical) and for Treatments (Horizontal) differed significantly according to Duncan's multiple range test at 5% level

Figure (2) shows callus induction from leaf explant in pomegranate

The investigation revealed that the leaf explant was a successful source for callus induction in Pomegranate when using MS medium supplemented with BAP and NAA. These findings align with those of [18, 19, and 20]. The variations in response to different combinations of cytokinins and auxins, as well as cytokinin levels and explant types, may be attributed to the differing physiological activities and metabolism of each plant hormone, as well as their translocation through the explant. Additionally, the culture media plays a significant role [21, 22, and 23]. Overall, most research indicates that a combination of cytokinins and auxins is essential for callus induction in *Punica granatum* L.

**Remediation of phenolic exudation**

The successful method for preventing explant browning, caused by the oxidation of phenols, is to quickly transfer the explants to a new medium (subculturing). This method has proven to be more effective in reducing browning compared to adding 100 $mgl^{-l}$ of polyvinylpyrrolidone (PVP) to the growth media.

The main issue in woody plants is the exudation of phenols, which is indirectly stimulated by factors such as plant age, biotic and abiotic stresses. In plant tissue culture medium, the oxidation of exuded phenolic substances and secondary metabolites from the cutting surface of the explants causes browning or darkening, posing a significant challenge in the establishment stage of sterile cultures. This can hinder nutrient uptake and lead to the production of free radicals through oxidation reactions, ultimately resulting in explant death [24,25]. Browning is a serious problem in pomegranates, which contain a high level of phenolic compounds. One of the different approaches to overcome the harmful effects of browning is to utilize various absorbents and antioxidants such as activated charcoal and polyvinylpyrrolidone (PVP) to prevent the oxidation of molecules and control the browning of the growth medium [26,27].

**Shoots organogenesis**

Nine callus pieces were cultured using different combinations of PGR protocols. The results in Table 3 showed that 3.0 $mgl^{-L}$ BAP combined with 0.5 mg/l NAA resulted in a higher percentage (20.6%) of callus initiation for organogenesis, while only 1.59% of callus pieces initiated organogenesis when using 1.0 $mgl^{-1}$ BAP with 0.5 $mgl^{-1}$ NAA. Additionally, four of the studied cultivars (Masafik, Milesse, Radisho, and Dwarf) exhibited similarly low responses for shoot organogenesis (3.7%), which differed significantly (see Figure 3) from Halapja (7.4%), Sahraban (11.1%), and Armishte, which showed the highest shoot organogenesis response at 18.5%.

Table (3): Effect of MS and different types of PGRs on organogenesis response (%) induced from the callus of pomegranate after 60 days in culture

| Cultivar Type | PGRs Protocols | | | Effect of cultivars |
|---|---|---|---|---|
| | 1.0 mg/l BAP + 0.5 mg/l NAA | 2.0 mg/l BAP + 0.5 mg/l NAA | 3.0 mg/l BAP + 0.5 mg/l NAA | |
| Masafik | 0 e | 0 e | 11.1 d | 3.7 d |
| Melisse | 0 e | 0 e | 11.1 d | 3.7 d |
| Radisho | 0 e | 0 e | 11.1 d | 3.7 d |
| Armishte | 11.1 d | 0 e | 44.4 a | 18.5 a |
| Sahraban | 0 e | 0 e | 33.3 b | 11.1 b |
| Halapja | 0 e | 0 e | 22.2 c | 7.4 c |
| Dwarf | 0 e | 0 e | 11.1 d | 3.7 d |
| Effect of treatment | 1.59 b | 0 c | 20.6 a | |

Overall means with different letters for Cultivar (vertical) and Treatments (Horizontal) differed significantly according to Duncan's multiple range test at 5% level

The results suggest that moderate levels of cytokinin are necessary for shoot formation from pomegranate callus. These findings align with [17], who achieved a high percentage of callus organogenesis in dwarf pomegranate leaf segments cultured in MS medium supplemented with 3 $mgl^{-1}$ BAP and 0.5 $mgl^{-1}$ NAA. Conversely, [19] found that 1.0 or 2.0 $mgl^{-1}$ BAP in combination with 2.0 $mgl^{-1}$ NAA induced shoots from leaf callus in pomegranate. Additionally, [17] reported that pomegranate callus differentiated on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA.

**Number of regenerated shoots (%)**

The investigation of the effects of different combinations of PGRs indicates that the percentage rate of regenerated shoots derived from callus organogenesis is shown in Table 4 and Figure 3. The number of regenerated shoots initiated from the callus was calculated using the following equation:"

Regeneration percentage (%) = Amount of shoots/ number of callus pieces × 100

The research findings showed that the most effective combination of plant growth regulators (PGRs) added to MS cultures for regeneration was 3.0 $mgl^{-1}$ BAP combined with 0.5 $mgl^{-1}$ NAA, which resulted in a 41.2% success rate. In contrast, the 2.0 $mgl^{-1}$ BAP + 0.5 $mgl^{-1}$ NAA protocol had a 0% success rate, and the 1.0 $mgl^{-1}$ BAP + 0.5 $mgl^{-1}$ NAA protocol had a 7.9% success rate. Additionally, all cultivars showed a response for regeneration.

Armishte (33.3%) > Sahraban (29.6%) > Dwarf (22.2 %) >Melisssse (11.1%) > Halapja (7.4%) = Masafik (4.7%) >Radisho (3.7%).

Table (4): Effect of MS and different types of PGRs on numbers of regenerated shoots (%) from the callus of pomegranate after 60 days in culture.

| Cultivar Type | PGRs Protocols | | | Effect of cultivars |
|---|---|---|---|---|
| | 1.0 mg/l BAP + 0.5 mg/l NAA | 2.0 mg/l BAP + 0.5 mg/l NAA | 3.0 mg/l BAP + 0.5 mg/l NAA | |
| Masafik | 0 h | 0 h | 22.2 f | 7.4 e |
| Melisse | 0 h | 0 h | 33.3 e | 11.1 d |
| Radisho | 0 h | 0 h | 11.1 g | 3.7 f |
| Armishte | 55.5 c | 0 h | 44.4 d | 33.3 a |
| Sahraban | 0 h | 0 h | 88.8 a | 29.6 b |
| Halapja | 0 h | 0 h | 22.2 f | 7.4 e |
| Dwarf | 0 h | 0 h | 66.6 b | 22.2 c |
| Effect of treatment | 7.9 b | 0 c | 41.2 a | |

Overall means with different letters for Cultivar (vertical) and Treatments (Horizontal) differed significantly according to Duncan's multiple range test at 5% level

Figure (3): Different stages of shoot regeneration derived from the callus of the seventh cultivars respectively.

The previous results align with numerous studies on pomegranate. [16] demonstrated the highest regeneration rate of 81.9%, with an average of 16.47 shoots per explant when cultured in MS medium containing 3.0 $mgl^{-1}$ BAP + 0.5 $mgl^{-1}$ NAA. [18] reported the highest number of shoots (7 shoots/explants) from callus obtained in MS medium supplemented with 5 $mgl^{-1}$ BA + 0.1 $mgl^{-1}$ NAA, and the highest plantlet regeneration from leaf callus occurred in a medium containing 5.0 $mgl^{-1}$ BA + 0.1 $mgl^{-1}$ NAA. Additionally, [28] noted that the highest plantlet regeneration percentage of 60% and over 4 shoots per explant from another callus was achieved in MS medium supplemented with 3.0 $mgl^{-1}$ BAP + 3.0 $mgl^{-1}$ NAA. These outcomes can be attributed to the hormonal regulation of cytokinin and auxin balance, which plays a crucial role in controlling cell division and differentiation in tissue culture [29].

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# انتاج الكالس والتشكل العضوي من البذور لسبعة أصناف محلية لنبات الرمان *Punica granatum*. L.

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الخلاصة

سبعة أصناف محلية لنبات الرمان وهي مسافيك، ميليسي، راديشو، ارمشتي، شهربان، حلبجة إضافة الى الرمان القزمي المزروعة في إقليم كوردستان العراق كانت مصدرا للبذور المستخدمة في هذه التجارب باستخدام تقنية الاكثار الدقيق بهدف اختبار قابليتها على انتاج الكالس والتشكل العضوي لاختيار بروتوكول كفء للإنتاج الكمي لنبات الرمان والاستخدام اللاحق في الدراسات الجزئية . أظهرت النتائج ان غمر البذور لمدة 24 ساعة في الماء وحامض السالساليك وبشكل مستقل قد ساهم في زيادة نسبة انبات بذور بعض الأصناف. كما لوحظ بان استجابة الأوراق كانت أفضل من استجابة الأوراق الفلقية في انتاج الكالس. ومن ناحية أخرى فقد وجد بان تقسيم الكالس وإعادة زرعه على نفس بيئته (***Subculturing***) قد اظهر فعالية أكبر في اختزال اكسدة الكالس وموته وتحوله الى اللون البني ***Browning*** من إضافة 100 ملغم / لتر ***polyvinylpyrrolidone (PVP)***. كما بينت النتائج ان التداخل المؤلف من املاح الوسط الغذائي ***MS*** المدعم بإضافة 1.5 ملغم/ لتر بنزايل امينو بيورينل ***BAP*** مضافا اليه 1.5 ملغم / لتر الاوكسين نفثالين حامض الخليك ***NAA*** قد أدى الى زيادة معنوية في انتاج الكالس فضلا عن ذلك ، فان استجابة الصنفين ارمشتي وشهربان كانت إيجابية بدرجة اكبر تلتها الأصناف راديشو ، ميليسي ، حلبجة و الصنف القزمي. ومن ناحية أخرى فقد اظهر التداخل الهرموني استخدام 3.0 ملغم / لتر بنزايل امينو بيورين المدعم بإضافة 0.5 ملغم / لتر نفثالين حامض الخليك النسبة الأعلى لتشكل تكوين الافرع الخضرية وتوالد النبيتات المتكونة من الكالس، وعلى العكس من ذلك فقد وجد ان التداخل الذي تضمن استعمال كل من التداخل 2 ملغم/ لتر بنزايل امينو بيورين مضافا اليه 0.5 ملغم / لتر نفثالين حامض الخليك والتداخل 1.0 ملغم/ لتر بنزايل امينو بيورين المدعم بإضافة 0.5 ملغم / لتر نفثالين حامض الخليك قد اظهر استجابة أدنى على التوالي.

الكلمات المفتاحية: انتاج الكالس، الاكثار الدقيق، تشكل الأعضاء، ***Punica granatum***، نبات الرمان.