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EFFICIENT STERILIZATION AND CULTURE OF SEEDLESS PUNICA GRANATUM L. (MOLLAR DE ELCHE) FOR CLONAL MICROPROPAGATION IN VITRO

Urazboev Azizbek Narzullo ogli [1],
Olimov Avazbek Bobosher ogli [1],
Valieva Shoira Abduvositovna [2]
[1] Master's student of the Fruit Growing program, Joint Education Faculty, at the Tashkent State Agrarian University and the Northwest A&F University of China.
[2] Senior Lecturer, Department of Fruit Growing and Viticulture, Faculty of Fruit, Vegetable and Viticulture, Tashkent State Agrarian University.
Corresponding author: azizbekorazboyev005@gmail.com

ABSTRACT

This study aimed to develop an efficient in vitro clonal micropropagation protocol for the seedless pomegranate cultivar Punica granatum L. (‘Mollar de Elche’). The experiments were conducted in 2024 at the scientific-experimental stations of the Scientific Research Institute of Horticulture, Viticulture, and Winemaking named after Academician Mahmud Mirzayev in Uzbekistan. A multi-step surface sterilization procedure was evaluated using different concentrations and exposure durations of sodium hypochlorite (NaOCl). The most effective treatment—1% NaOCl for 15 minutes—produced up to 97% contamination-free cultures with no observable tissue browning. For shoot induction, Murashige and Skoog (MS) medium supplemented with 1.0 mg/L BAP and 0.01 mg/L NAA yielded the highest shoot proliferation (6 shoots per explant) and maximum shoot length (7.1 cm). The results highlight the effectiveness of optimized hormonal combinations and sterilization protocols for large-

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scale propagation of elite seedless pomegranate cultivars, contributing to sustainable horticultural development in new climatic zones.

Keywords: Punica granatum L., Mollar de Elche, micropropagation, sterilization, BAP, NAA, tissue culture.

INTRODUCTION

The pomegranate (Punica granatum L.) is a historically significant deciduous fruit crop that has been cultivated for millennia, offering considerable varietal diversity and a wide array of uses for consumers (Preece & Moersfelder, 2016; Chater et al., 2018; Morton, 1987). Its native range spans from Iran to the Himalayan regions of northern India, while present-day cultivation is predominantly concentrated in Mediterranean and Middle Eastern countries (Jbir et al., 2008; Melgarejo et al., 2009). Levin (2006) identified three principal centers—primary, secondary, and tertiary—as well as five major macro-centers of origin and genetic diversity: the Middle Eastern, Mediterranean, Eastern Asian, American, and South African regions. As a species adapted to temperate climates, pomegranate requires prolonged periods of high summer temperatures for optimal fruit ripening.

Although pomegranate can be propagated from seed, commercial cultivation typically relies on hardwood cuttings ranging from 25 to 50 cm in length to minimize the genetic variability inherent in seed-grown plants. Traditional propagation methods, however, are limited by factors such as low seed viability, seasonal dependency, and the slow pace of vegetative multiplication. While air layering offers an alternative approach, grafting techniques have shown poor success rates (Morton et al., 1987). As a result, conventional breeding programs tend to be slow, labor-intensive, and inefficient. To overcome these limitations, biotechnological tools—such as tissue culture and molecular genetics—are increasingly being integrated into breeding strategies (Lichtenstein & Draper, 1985).

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Several in vitro regeneration protocols have been successfully developed for Punica granatum L., utilizing explants like leaf tissues (Murkute et al., 2002), cotyledons (Kanwar et al., 2010), and anthers (Moriguchi et al., 1987).

Among modern cultivars, ‘Mollar de Elche’ stands out as the most sought-after variety in Spain due to its sweet taste, soft and chewable arils, and distinctive sensory qualities (Cano-Lamadrid et al., 2017). This cultivar is recognized globally for its exceptional flavor profile and high antioxidant content, making it one of the most valued pomegranate varieties worldwide (Manera et al., 2013). Its soft seeds enhance edibility, further increasing its popularity as a ‘superfruit’.

Given its seedlessness, ‘Mollar de Elche’ is predominantly propagated through vegetative means. Although these methods are used in commercial settings, they tend to be laborious, time-consuming, and heavily reliant on seasonal conditions (Krul & Mowbray, 1984). In contrast, micropropagation techniques offer a promising alternative, enabling the large-scale production of genetically uniform plants in a controlled environment.

The current study is focused on establishing an efficient sterilization and in vitro culture protocol for the seedless pomegranate cultivar ‘Mollar de Elche’, with the aim of facilitating its clonal propagation through micropropagation techniques.

MATERIALS AND METHODS

This study focuses on the in vitro micropropagation of seedless pomegranate (Punica granatum L., cv. 'Mollar de Elche'), emphasizing the essential stages of clonal propagation using artificial nutrient media. The primary objective of this research is to develop an optimized tissue culture protocol that ensures high explant survival, genetic uniformity, and efficient shoot regeneration, with the potential to support the expansion of elite pomegranate cultivars in Uzbekistan. The study was conducted in 2024 at the scientific-experimental stations of the Scientific Research Institute of Horticulture, Viticulture, and Winemaking named after Academician Mahmud Mirzayev.

The plant material consisted of young, actively growing meristematic shoot tips collected from 'Mollar de Elche' pomegranate plants grown on calcareous soils.

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Explants were selected from soft, healthy shoot segments during the March–April vegetative growth period to ensure high regeneration potential.

To eliminate microbial contamination and establish clean in vitro cultures, a multi-stage sterilization protocol was applied:

- Initial Washing: Explants were rinsed under running tap water for 20 minutes to remove dust and surface debris.

- Distilled Water Rinse: This was followed by three rinses with sterile distilled water.

- Surface Sterilization: All operations were carried out under aseptic conditions in a laminar airflow cabinet. Explants were first immersed in 70% ethanol for 30 ± 2 seconds for surface disinfection.

- Chemical Disinfection: Explants were then treated with sodium hypochlorite (NaOCl) solutions at concentrations of 1.0% and 2.0% for two different exposure times—10 and 15 minutes—to determine the optimal balance between sterilization efficiency and tissue viability.

- Final Rinsing: Following sterilization, explants were rinsed three times with sterile distilled water to remove all chemical residues, thereby reducing the risk of phytotoxicity during culture initiation.

The success of sterilization treatments was evaluated based on contamination levels observed during a 14-day incubation period.

The explants were cultured on Murashige and Skoog (MS) basal medium, which is widely used in plant tissue culture due to its high nutrient content and suitability for a broad range of species, including pomegranate. MS medium supports vigorous shoot proliferation due to its balanced macro- and micronutrients, especially nitrogen and potassium.

To enhance shoot induction and elongation, various concentrations and ratios of plant growth regulators were tested—specifically 6-Benzylaminopurine (BAP) as a cytokinin and Naphthalene acetic acid (NAA) as an auxin. The balance between these

hormones was optimized to encourage direct shoot organogenesis while minimizing unwanted callus formation.

The pH of the culture medium was set at 5.7 ± 0.1 before autoclaving at 121°C for 20 minutes. This pH level ensured maximum nutrient solubility and favorable conditions for explant development during the in vitro culture phase.

RESULTS AND DISCUSSION

The effectiveness of surface sterilization treatments using different concentrations of sodium hypochlorite (NaOCl) and exposure durations on meristem segments of seedless Punica granatum L. (‘Mollar de Elche’) is summarized in Table 1. The aim was to achieve a high percentage of clean cultures while minimizing tissue browning and contamination.

Table 1.

Effect of various NaOCl concentrations and four exposure times on disinfection of seedless Punica granatum L. (‘Mollar de Elche’) meristem segments

Treatment	NaOCl (1%)			NaOCl (2%)		
	10 min	15 min	Mean	10 min	15 min	Mean
Clean culture (%)	91-94	93-97	92-95.5	85-91	78-87	81-89
Contamination (%)	6-9	3-7	4.5-8	4-6	3-5	3.5-5.5
Browning (%)	0	0	0	5-9	10-17	7.5-13

Treatment with 1% NaOCl for 15 minutes resulted in the highest percentage of clean cultures (93–97%), with negligible contamination levels (3–7%) and no browning observed. Similarly, 1% NaOCl for 10 minutes also provided excellent results, yielding clean culture rates between 91–94% and contamination between 6–9%, again with zero browning. The mean clean culture percentage for the 1% NaOCl

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treatments ranged from 92% to 95.5%, indicating that this concentration is both effective and non-damaging to the explants.

In contrast, treatments with 2% NaOCl showed a moderate decrease in clean culture rates and a noticeable increase in browning. Specifically, the 10-minute exposure to 2% NaOCl resulted in 85–91% clean cultures and 4–6% contamination, with browning rates ranging from 5–9%. The situation worsened with 15-minute exposure, where clean cultures dropped to 78–87%, and browning increased significantly to 10–17%. The mean browning percentage for 2% NaOCl treatments ranged from 7.5% to 13%, suggesting that higher NaOCl concentration and longer exposure times may cause oxidative damage and phenolic browning in meristem tissues.

Overall, 1% NaOCl for 15 minutes emerged as the optimal sterilization treatment, ensuring maximum culture cleanliness with no adverse effects on tissue viability or appearance. Higher concentrations, while somewhat effective against contamination, had a clear negative impact on explant quality.

The influence of varying concentrations of 6-Benzylaminopurine (BAP) in combination with Naphthalene acetic acid (NAA) on shoot induction from meristem segments of seedless Punica granatum L. (‘Mollar de Elche’) was evaluated using Murashige and Skoog (MS) basal medium with a constant pH of 5.7. Parameters such as time to bud swelling, number of shoots per explant, and average shoot length were recorded to assess the morphogenic response.

Table 2.

Effect of different BAP concentrations on shoot induction in MS medium in seedless Punica granatum L. ('Mollar de Elche') explants

Amount of growth substances (mg/l)	pH index	Bud swelling (day)	Shoot branching (pcs)	Length of branched plants (cm)

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BAP	NAA				
0,0	0,0	5,7	28-24	2	2,6
0,5	0,01	5,7	15-18	3	4.7
	0,1	5,7	12-17	4	5,2
1,0	0,01	5,7	10-14	6	7.1
	0,1	5,7	10-12	5	6,2
2,0	0,01	5,7	9-15	5	4,6
	0,1	5,7	10-15	4	3,9

In the control treatment (0.0 mg/L BAP and NAA), explants showed slow and weak development, with bud swelling occurring after 24–28 days, and only 2 shoots being induced, averaging 2.6 cm in length. This reflects the limited regenerative potential in the absence of exogenous hormonal stimulation.

Introduction of 0.5 mg/L BAP significantly improved shoot initiation. In combination with 0.01 mg/L NAA, explants showed bud swelling within 15–18 days, producing 3 shoots with an average shoot length of 4.7 cm. When the NAA concentration was increased to 0.1 mg/L, further improvement was observed, with bud swelling occurring slightly earlier (12–17 days), and the number of shoots and shoot length increased to 4 and 5.2 cm, respectively. This indicates that low-to-moderate levels of NAA enhance the shoot elongation effect of BAP, possibly by improving cellular division and vascular differentiation.

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The highest shoot induction efficiency was observed at 1.0 mg/L BAP combined with 0.01 mg/L NAA, where bud swelling occurred within 10–14 days, and the number of shoots peaked at 6 per explant, with a maximum shoot length of 7.1 cm. This suggests that a balanced cytokinin-to-auxin ratio at these concentrations promotes both organogenic competency and elongation. When the NAA concentration was increased to 0.1 mg/L, the shoot number slightly decreased to 5, and shoot length was recorded at 6.2 cm, indicating that higher levels of NAA might begin to shift cellular development away from shoot induction toward other morphogenic pathways.

At the highest BAP concentration (2.0 mg/L), shoot induction efficiency declined. Although bud swelling remained relatively early (9–15 days), shoot numbers dropped to 4–5, and shoot lengths decreased to 3.9–4.6 cm across both NAA levels. This suggests that excessive cytokinin levels can lead to hyperhydricity, vitrification, or growth inhibition, particularly when combined with increased auxin concentrations.

The most favorable response for in vitro shoot induction in 'Mollar de Elche' pomegranate was achieved with 1.0 mg/L BAP + 0.01 mg/L NAA, yielding the highest number of shoots and greatest shoot elongation within the shortest time frame. These results indicate that a lower auxin-to-cytokinin ratio supports optimal shoot morphogenesis, and that careful calibration of growth regulator concentrations is crucial for successful micropropagation.

The findings further confirm that NAA, when used in small concentrations, effectively complements BAP in enhancing shoot initiation and development, while higher levels may suppress shoot elongation or induce undesired morphogenic responses.

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	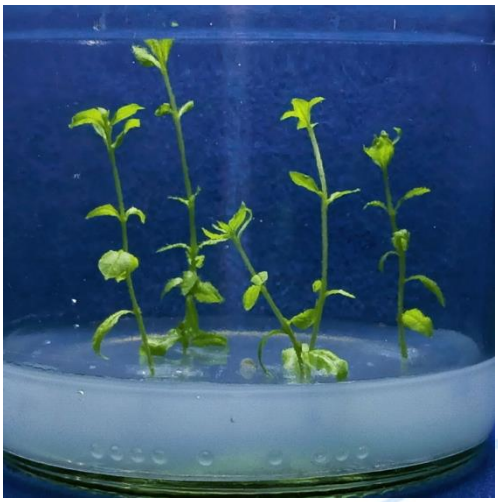
Vegetative parts of seedless pomegranate 'Mollar de Elche' are placed in the MS medium.	**Shoots developing on MS medium containing 1 m/g-1L BAP and 0.01 m/g-1L NAA.**

Successful in vitro micropropagation critically depends on two major factors: (1) effective explant surface sterilization to establish aseptic cultures, and (2) optimized hormonal balance to promote organogenic responses such as shoot initiation and elongation. In this study, both these aspects were investigated using meristematic explants of seedless Punica granatum L. (‘Mollar de Elche’) to develop an efficient and reproducible micropropagation protocol.

The results of surface sterilization treatments demonstrated that both sodium hypochlorite (NaOCl) concentration and exposure duration significantly affected culture cleanliness, contamination levels, and tissue viability. The best results were obtained with 1% NaOCl for 15 minutes, yielding up to 97% clean cultures with no visible browning or phytotoxic effects. This confirms the findings of previous studies that suggest low to moderate concentrations of NaOCl are effective in eliminating surface microbes while preserving tissue viability. In contrast, higher NaOCl concentrations (2%) and longer exposure times (15 min) resulted in increased browning and reduced viability, indicating possible oxidative damage to the delicate meristematic tissues. These observations support the importance of optimizing sterilization parameters for each specific genotype and explant type.

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In the second phase of the experiment, the influence of various concentrations of BAP (a cytokinin) in combination with NAA (an auxin) was assessed for shoot induction. The hormonal balance played a crucial role in determining not only the number of shoots formed per explant but also the time of bud swelling and the length of regenerated shoots.

Among the tested combinations, 1.0 mg/L BAP + 0.01 mg/L NAA was found to be the most effective, leading to early bud swelling (10–14 days), maximum shoot proliferation (6 shoots per explant), and longest shoot length (7.1 cm). These results are consistent with the general understanding that cytokinins promote shoot organogenesis, and that a small amount of auxin supports shoot elongation by enhancing vascular differentiation and cellular expansion. Interestingly, increasing the NAA concentration to 0.1 mg/L led to a slight reduction in shoot number and length, indicating a shift in hormonal balance that may have promoted root primordia or callus formation instead of direct shoot regeneration.

Similarly, increasing BAP concentration to 2.0 mg/L led to a decline in both shoot number and elongation, likely due to supraoptimal cytokinin levels causing hormonal imbalance, vitrification, or stress-induced inhibition. These results highlight the necessity of hormonal fine-tuning, as both the concentration and ratio of BAP to NAA must be carefully controlled to achieve high-efficiency shoot multiplication in woody plant species like pomegranate.

The current findings are in line with earlier research on pomegranate and other woody perennials, where low to moderate cytokinin levels, in combination with trace auxins, have been shown to effectively induce shoot proliferation. While previous studies have commonly used IBA (Indole-3-butyric acid) as the auxin, the current study demonstrates that NAA can be an effective alternative for shoot induction when used in carefully optimized concentrations.

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CONCLUSION

The present study successfully established an efficient and reproducible in vitro micropropagation protocol for the seedless pomegranate cultivar Punica granatum L. (‘Mollar de Elche’), a highly valued fruit variety known for its sensory qualities and commercial significance. Through systematic optimization of explant sterilization and shoot induction protocols, key parameters influencing culture success were identified.

The optimal surface sterilization treatment was determined to be 1% sodium hypochlorite (NaOCl) for 15 minutes, which provided the highest percentage of contamination-free cultures (up to 97%) with no browning or tissue damage. This highlights the importance of using moderate disinfectant concentrations and carefully timed exposures to ensure both cleanliness and explant viability.

In the shoot induction phase, the most favorable morphogenic response was observed on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L BAP and 0.01 mg/L NAA, which yielded early bud swelling (within 10–14 days), the highest shoot proliferation (6 shoots per explant), and maximum shoot elongation (7.1 cm). These results underscore the importance of fine-tuning the cytokinin-to-auxin ratio for promoting direct organogenesis in woody fruit crops like pomegranate.

The use of NAA as an effective auxin alternative to IBA further demonstrated the flexibility of hormonal combinations for shoot induction in this species. The established protocol provides a solid foundation for large-scale clonal propagation of ‘Mollar de Elche’ under controlled conditions and may contribute significantly to the commercial expansion and genetic conservation of elite pomegranate cultivars in regions such as Uzbekistan.

Future research may focus on optimizing rooting and acclimatization stages, as well as extending the protocol to other high-value, seedless pomegranate genotypes to further support the development of sustainable pomegranate production systems.

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