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Development of an Efficient In Vitro Propagation Protocol for the Elite Bidah Pomegranate (*Punica granatum* L.) Landrace in Saudi Arabia

Nada M. Alattas [1], Khalid M. AL-Rohily [1], Saheed O. Anifowose [1], Abdulmoneem Al-Khamees [1] and Abdalrhaman M. Salih [1,2,*,†]

[1] The National Research and Development Center for Sustainable Agriculture (Estidamah), Riyadh Techno Valley, Riyadh 12373, Saudi Arabia; olaidesaeed@hotmail.com (S.O.A.)
[2] Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
* Correspondence: abdalrahamanm@gmail.com
† This author contributed significantly to this work.

Abstract

The Bidah pomegranate (*Punica granatum* L.) is an elite landrace native to Wadi Bidah in the Al-Baha region of Saudi Arabia. This cultivar is distinguished by its unique taste, high yield, genetic component, and phytochemical profile. In addition, its resilience to harsh environmental conditions aligns closely with national priorities in sustainable agriculture and food security. This study articulated the successful micropropagation of the Bidah pomegranate cultivar using both direct and indirect in vitro approaches. Phenolic exudation was effectively mitigated through sequential subculturing onto a plant growth regulator (PGR)-free medium. Shoot proliferation was highest for Murashige and Skoog (MS) medium supplemented with 3 mg/L benzyladenine (BA), resulting in a 95% shoot induction rate, which was significantly higher than those obtained with the other investigated media. Callus induction and bioreactor-based mass production were maximized on Woody Plant medium (WPM) containing 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), yielding significantly higher biomass than MS medium. Rooting of regenerated shoots was successfully achieved on half-strength MS (1/2 MS) medium supplemented with 3 mg/L 1-naphthaleneacetic acid (NAA). The hardening of plantlets was conducted by transferring the rooted shoots into pots containing a soil mixture of vermicompost and perlite. The acclimatization process was performed under controlled greenhouse conditions, where the photosynthetically active radiation (PAR) averaged 4530.5 µmol m^{-2} s^{-1}, air temperature was maintained at 22 °C, relative humidity (RH) averaged 74.5%, and the pad-wall cooling rate was 2695.56 L. Additionally, the genetic fidelity was investigated using genome size estimation. The in vitro propagation method offers significant advantages over conventional techniques, enabling efficient plant reproduction, genetic conservation, and the obtaining of mass plant material for different uses. Furthermore, genetic enhancement through in vitro selection and the application of CRISPR-Cas9 gene editing technologies can be performed.

Keywords: Bidah pomegranate; phenol exudation control; micropropagation; genome size estimation

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1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit belonging to the Punicaceae family [1]. It is a shrub or small tree native to Iran and the Himalayas in the northern part of

India, but is now widely cultivated throughout the Mediterranean region, the Middle East, and other arid or semi-arid regions [2,3]. Besides its nutritional value, pomegranate holds cultural and medicinal value, with a long history of use in traditional medicine worldwide. For instance, pomegranate materials are used to treat different types of diseases such as diarrhea, dysentery, and various infections [4]. Furthermore, many studies have demonstrated that pomegranate exhibits strong antioxidant, anti-inflammatory, antimicrobial, and anticancer activities [5]. Additionally, it contributes to the prevention and management of several risk factors, including hypertension, hypercholesterolemia, oxidative stress, inflammation, and hyperglycemia [6,7].

Additionally, the pomegranate plant can grow in various agro-climatic environments, and its long post-harvest shelf life makes it a valuable crop with significant economic potential [8,9]. Moreover, various parts of the pomegranate plant, including seeds, juice, peel, bark, leaves and flowers, contain high levels of bioactive compounds such as polyphenols, flavonoids, tannins, and anthocyanins. These phytochemical compounds contribute to a wide range of medicinal and economic benefits [3,9]. Consequently, market growth is being driven by an increasing demand for pomegranate-derived products (juice, powder, and extracts) within the food, beverage, nutraceutical, and cosmetic industries [1].

In Saudi Arabia, pomegranate cultivation has a long history of being practiced, particularly in the Al-Baha region, where it provides significant income for local farmers and supports rural economies [10]. Local cultivars of pomegranate, such as the Bidah cultivar, has been found to have unique natural compounds, aligning with the national objectives for agricultural innovation and food security [9]. Moreover, pomegranate demonstrates resilience to the hot and dry climate of Saudi Arabia, exhibiting tolerance to abiotic stresses such as salinity and drought, which are prevalent in the region [10,11]. In this context, pomegranate trees can live up to seventy years and produce fruit between 15 and 20 years old. This promotes its consideration as a sustainable crop for long-term agricultural planning. However, conventional propagation methods of pomegranate trees face several challenges. For commercial production, sexual propagation via seeds is not popular for farmers due to genetic variability, which results in inconsistent fruit quality and an extended juvenile phase of 4 to 5 years before fruiting. Thus, seed propagation is unsuitable for the multiplication of elite cultivars and is generally confined to breeding programs or rootstock production [12]. Asexual or vegetative propagation methods such as stem cuttings and layering are more commonly used for pomegranate cultivation. However, these methods also present difficulties. For instance, stem cutting often shows poor rooting and high rates of death, which are affected by the time of year and the health status of the mother plant [12,13]. Air layering provides more reliable outcomes but requires specialized skills and optimal environmental conditions. Additionally, both stem cutting and layering may facilitate the transmission of diseases from mother plants, complicating efforts to obtain disease-free plants [14]. Moreover, the rooting of hard wood cuttings and the establishment of a new plant requires one year [15,16].

On the other hand, in vitro propagation stands as an essential modality for the conservation and mass propagation of plant species. Despite its diverse benefits and applications, micropropagation remains constrained without corresponding efforts in large-scale commercial cultivation [17]. Thus, micropropagation approach can be used as alternative method for accelerating the reproduction of pomegranate for conservation and getting mass production for different applications. In this context, significant progress has been made in the in vitro propagation of pomegranate cultivars, with several studies reporting improved regeneration efficiency, enhanced shoot proliferation, and stable clonal fidelity across different genotypes [1–3]. Collectively, these advancements underscore the increasing feasibility of using tissue culture for mass propagation, conservation, and trait

stabilization in pomegranate. However, these protocols cannot be universally generalized, as micropropagation efficiency is strongly influenced by cultivar-specific responses, explant type, physiological age, and environmental conditions. Therefore, the main objective of this research was to develop an efficient micropropagation protocol for elite pomegranate grown in Saudia Arabia, mainly the Bidah cultivar. Hence, explants of a two-year-old pomegranate were inoculated on different types of media with the supplement of different combinations of plant growth regulators (PGRs).

2. Materials and Methods

2.1. The Explants Collection and Disinfection

A Bidah pomegranate donor plant (10 seedlings) was collected from the Bidah area, the Al-Bahah region of Saudi Arabia, in 2022, following the international standards, guidelines, and legislation. The Bidah pomegranate represents an elite cultivar with unique traits (high yield, sweet taste and deep red color) grown in Saudia Arabia [18]. The cuttings of nodal segments containing at least one axillary bud were used as explants. Then, the nodal segments were washed well to remove unwanted debris and disinfected using the following protocol [9]. The explants were immersed in 50% (v/v) Clorox solution for 20 min, followed by 0.1% mercuric chloride for three minutes, and then in 70% ethanol for one minute. They were subsequently washed three times with sterile distilled water under the sterile conditions of a Class-II-B2-biological-safety-cabinet (Biobase, Jinan, China).

2.2. Phenolic Exudation Control

To control the high release of phenolic compounds from Bidah pomegranate explants, pre-treatment strategies were employed. Firstly, explants were soaked in adsorbing agents including polyvinylpolypyrrolidone (PVPP), citric acid and ascorbic acid at concentrations ranging from 0.2% to 2% for durations between 2 and 24 h. The treatment was conducted both in adsorbent solutions and in nutrient media. Following this, explants were inoculated into nutrient media without PGRs and subjected to two subculturing cycles at 24 h intervals. Subsequently, the explants were transferred to shoot induction media containing different concentrations of BA for bud breaking and shoot induction.

2.3. Shoot Induction

The cuttings were inoculated into different types of media; Woody Plant medium (WPM) [19], Murashige and Skoog medium (MS) [20], CHU (N6) medium [21] and Gamborg's B5 medium [22]. These media were supplemented with different concentrations of plant growth regulators (PGRs), sucrose as a carbon source (1–3%), and 4–7 g/L of agar, and the pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min. For each treatment one explant (three jars per treatment) was cultured under laminar conditions. The plant cultures were incubated for three months under optimal growth conditions (humidity 39%, temperature 20.6 °C ± 1, and 16/8 h illumination periods with 40 µmol m^{-2} s^{-1} light intensity).

2.4. Shoot Multiplication

For shoot multiplication of Bidah pomegranate, different concentrations (0.1, 0.2, 0.4, 0.5, 1.0, 1.5, 2.0, 3 mg/L) of BA were supplanted to the WP and MS media, and the sub-cultured shoots were incubated under the above-mentioned conditions.

2.5. Callus Induction and Shoot Regeneration

For callus induction and formation in Bidah pomegranate, nodal segments were cultured on MS and WP media containing 20 and 30 g/L sucrose and 7 g/L agar for medium solidification and supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D).

2.6. Bioreactor System for Callus Masspropagation

The resulting callus was subsequently transferred to a RITA® bioreactor system (Vitropic, Montpellier, France), a temporary immersion bioreactor (TIB) for mass propagation using the same medium (200 mL/U) composition without the solidifying agent. For shoot induction from the established callus, MS and WP media were supplemented with BA at concentrations of 0.5 and 1.0 mg/L. Then, the cultures were incubated for three months.

2.7. Roots Induction

To optimize root induction in Bidah pomegranate, the induced shoots (originated directly from microcuttings) were cultured in half-strength MS (1/2 MS) medium containing 0.5 g of activated charcoal (AC). This medium was supplemented with different concentrations of α-Naphthaleneacetic acid (NAA) and Indole3-butyric acid (IBA) to evaluate their efficacy in promoting root initiation and development.

2.8. Hardening and Acclimatization

The transplantation of in vitro regenerated plantlets has been conducted as follows. The well-developed Bidah pomegranate plantlets cultured for 12 weeks in a nutrient medium (MS) were subjected to a hardening phase, with Bidah pomegranate plantlets approximately 10 cm in height gently removed from the rooting medium and rinsed with distilled water to eliminate any residual and unwanted particles from the roots. Then, the plantlets were transferred to pots containing a soil mixture of vermicompost and perlite in a 1:1 ratio. Each pot was covered with a transparent plastic cap to maintain high humidity and minimize transplant shock for 15 days. The plantlets were maintained in a greenhouse at the Estidamah Center (Riyad city) under controlled environmental conditions. The average photosynthetically active radiation (PAR) was 4530.5 µmol m^{-2} s^{-1}, the air temperature was 22 °C, the relative humidity (RH) averaged 74.5%, and the pad-wall cooling rate was 2695.56 L, and the survival rate of the plant was estimated.

2.9. Genome Size Estimation

The nuclear DNA content (2C DNA content) of Bidah pomegranate was measured according to Doležel et al.'s method [23,24]. A minimum of five thousand propidium iodide-stained nuclei were estimated using a flow cytometer FACS Muse cell analyzer (Sigma, St. Louis, MO, USA). A minimum flow rate (0.12 µL/s) of the capillary was set to ensure accuracy of the results. *Solanum lycopersicum* (2C = 1.96 pg.) was provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic, and was used as the standard for the estimation of the genome size of Bidah pomegranate. The histograms generated in the Muse cell analyzer were computerized, and further analysis was conducted for genome size estimation. The 2C DNA content of Bidah pomegranate was measured according to the following formula [25]:

$$2C\ DNA\ content = \frac{\text{Fluorescence mean intensity of Bidah pomegranate}}{\text{Fluorescence mean intensity of standard}} \times \text{2C DNA content of standard}$$

The number of base pairs per haploid genome was calculated based on the equivalent of 1 pg. DNA = 965 megabase pairs [26].

2.10. Statistical Analysis

The data generated from shoot proliferation and callus induction experiments, each conducted with three replicates, were analyzed using one-way analysis of variance, which is a comparison based on a completely randomized design. The Duncan test was used to separate means and to determine the significant differences at $p < 0.05$ using SPSS Version 20–32 bit (IBM, New York, NY, USA).

3. Results and Discussion

3.1. Phenolic Exudation and Seasonal Intervals

The phenolic exudation is a significant challenge in the mass propagation of economically important woody plants. The releasing of phenolic content of these plants causes browning of the nutrient medium and subsequent explant necrosis, resulting in explant death. This problem is primarily associated with the high degree of lignification in woody tissues and the accumulation of secondary metabolites that diffuse into the medium. It has been reported that this browning is primarily attributed to the enzymatic oxidation of phenolic substances by polyphenol oxidases and peroxidases [27,28]. Phenol exudation is one of the major challenges of in vitro regeneration in woody plants. Therefore, several strategies are commonly employed to mitigate this problem. For instance, phenolic exudation can be minimized through adsorbing agents, antioxidant treatments, frequent subculturing and sealing explant cut ends with paraffin wax [28]. In this study, different concentrations of absorbing agents including PVPP, citric acid, ascorbic acid and activated charcoal were evaluated to alleviate explant browning and to induce bud breaking of Bidah pomegranate explants. However, the addition of these agents did not result in any significant reduction in phenolic exudation (Table 1 and Figure 1A). Interestingly, when explants were inoculated into nutrient media without PGRs and subjected to two subculturing cycles within 48 h intervals, a notable decrease in phenolic content was observed (Figure 1B). This reveals that, in the case of the Bidah pomegranate cultivar, the use of PGR-free medium combined with regular subculturing plays a more crucial role in reducing phenolic exudation than the application of antioxidant agents. On the other hand, the timing of Bidah pomegranate explant cutting is also particularly important. For example, the cuttings taken in late March and early April show faster bud break compared to those taken during other times of the year, making this period ideal for collecting explants of Bidah pomegranate for mass propagation. In contrast, the cuttings of Bidah pomegranate during July and August exhibited a slower response and higher levels of phenolic secretion. This might be due to the increase in temperature during these months. It has been reported that strong seasonal variation in plant regeneration frequency was observed [29]. Pomegranate trees enter a new growth phase at the end of June, providing another opportunity for collecting small and actively growing cuttings. The buds of younger cuttings tend to break faster than those of older ones. However, the overall growth and development of older cuttings is faster than that of younger cuttings from Bidah pomegranate. On the other hand, older shoots produced significantly more vigorous and elongated shoots. The faster response of younger explants can be attributed to higher endogenous cytokinin levels and reduced cell wall lignification, which lower the threshold for axillary bud activation. Conversely, older shoots possess fully differentiated vascular bundles facilitating nutrient transport and structural support for sustained shoot elongation post-break.

Table 1. The effect of antioxidant agents (concentration, application method, and exposure duration) on phenolic exudation of Bidah pomegranate.

Antioxidant Agent	Concentration (*w*/*v* %)	Application Method	Duration	Observed Effect
PVPP	0.2, 0.5, 1.0 and 2.0	Pre-soaking	2 & 24 h	None
Citric acid	0.2, 0.5, 1.0 and 2.0	Pre-soaking	2 & 24 h	None
Ascorbic acid	0.2, 0.5, 1.0 and 2.0	Pre-soaking	2 & 24 h	None
Activated charcoal	0.2, 0.5, 1.0 and 2.0	Supplemented to nutrient medium	1 month	None
PVPP	0.2, 0.5, 1.0 and 2.0	Supplemented to nutrient medium	1 month	None

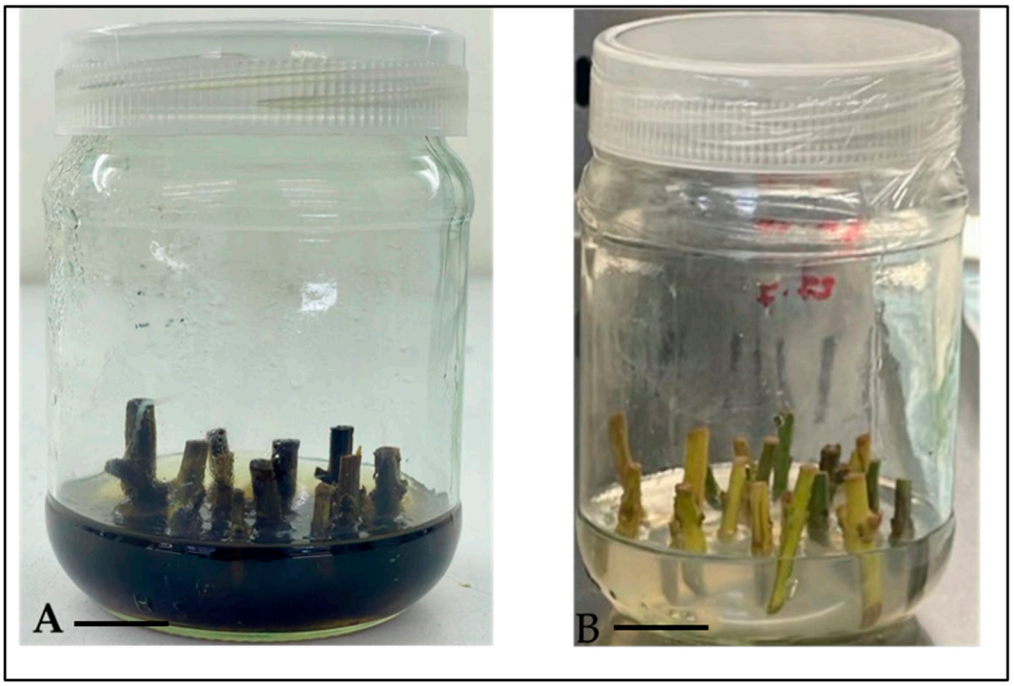


Figure 1. (**A**) Phenolic exudation and (**B**) suppression of phenol exudation by subjecting to two subculturing cycles within 48 h intervals into nutrient media without PGRs (Scale bar = 1 cm).

3.2. Shoots Induction and Multiplication

For Bidah pomegranate shoot induction, different types of nutrient media (WPM, MS, N6 and B5) were tested. These media were supplemented with various concentrations of BA (0.1–3 mg/L). The initial obtained results showed that the bud breaking and shoot indication of Bidah pomegranate required a high concentration of BA (3 mg/L). This finding matches with the previous report, which stated that the bud breaking of pomegranate required a high concentration of cytokinin [28]. For this reason, the study focused on incorporating 3 mg/L of BA into the different media. Among the different nutrient media, MS medium recorded the highest percentage of shoot induction (95%), followed by WPM 49%, N6 22% and B5 1% (Table 1). Conversely, WPM showed remarkable results in the number of induced shoots (Table 2). For the multiplication of induced Bidah pomegranate shoots, various concentrations of BA were added to the MS medium. The results demonstrated that 0.5 mg/L of BA induced shoot proliferation significantly among the investigated concentrations (Figure 2). Optimizing in vitro shoot proliferation is a complicated task, as it is influenced by interactions between many factors as well as the genotype of the targeted cultivar [30]. Therefore, each cultivar may require distinct nutrient media, plant growth regulators (PGRs), and environmental conditions.

Table 2. Effect of nutrient media type supplemented with 3 mg/L BA on shoot and bud induction rate of Bidah pomegranate.

Nutrient Media	Number of Explants	Induction Rate %	Number of Buds/Explants
MS	162	95 ± 2.0 [a]	2 ± 0.1 [b]
WPM	162	48 ± 4.0 [b]	3 ± 0.5 [a]
N6	162	22 ± 2.3 [c]	2 ± 0.2 [b]
B5	162	8.0 ± 1.0 [d]	2 ± 0.0 [b]

The data presents the number of explants used and the average number of the induction rate of the shoot, induced buds ± standard deviation (SD). [a,b,c,d] Means within the same column with different superscripts differ significantly ($p < 0.05$).



Figure 2. Sequential steps of in vitro shoot induction, regeneration and multiplication of Bidah pomegranate: (**a**) Induced shoots established in the culture medium and (**b**) stages of shoot development and subsequent multiplication (Scale bar = 1 cm).

3.3. Callus Induction, Mass Production and Shoot Regeneration

For callus induction and formation from Bidah pomegranate nodal segments, 0.5 mg/L concentration of 2,4-D supplemented to Woody Plant medium (WPM) showed the best results of callus induction and multiplication, followed by Murashige and Skoog medium with the same concentration of 2,4-D. For the mass production of callus, the same medium composition was used on the bioreactor system. In addition, the obtained results demonstrated that WPM, with the addition of 0.5 mg/L of 2,4-D, recorded significant results (95 ± 5.0[a] g/U) of fresh callus and induced a friable texture with light green, green and white pigmentation. In contrast, MS medium produced lower callus biomass (46 ± 3.0[a] g/U) (Figure 3) and induced a compact texture with green, white and brown colors. For shoot regeneration from induced callus, MS and WPM with the addition of BA at 0.5 mg/L and 1.0 mg/L concentrations were used. WPM supplemented with 1.0 mg/L of BA showed good result of shoot regeneration from callus (Figure 4). However, no positive result was recorded on MS medium. The findings of this study underscore the critical role of medium composition and plant growth regulators in optimizing callus induction and shoot regeneration from Bidah pomegranate nodal segments. WPM provides a more favorable nutrient for callus initiation and formation compared to MS medium.

This may be attributed to WPM's lower salt concentration and tailored formulation for woody plants, which aligns well with the physiological needs of pomegranate. In addition, a significant increase in callus biomass has been observed in the bioreactor system. In this regard, it has been stated that the controlled environment of the bioreactor system provides optimal conditions for plant growth that likely enhanced nutrient uptake and aeration, thereby contributing to the higher yield of callus material [28,29]. These findings hold significant value for commercial applications, where robust and efficient callus induction is pivotal to downstream processes such as genetic transformation, metabolite extraction, somatic embryogenesis, and large-scale propagation. In terms of shoot regeneration, the use of BA at 1.0 mg/L in WPM proved to be effective, while MS medium failed to support shoot development under similar conditions for the bioreactor system. This reinforces the importance of medium specificity not only for callus induction but also for organogenesis. The responsiveness of callus to BA in WPM may reflect better hormonal synergy or improved cytokinin uptake in this medium. The lack of regeneration in MS could be due to its higher ionic strength or suboptimal hormonal balance for shoot induction in this cultivar. Overall, the study presents a robust and reproducible protocol for callus induction and shoot regeneration of Bidah pomegranate, with clear advantages for using WPM.

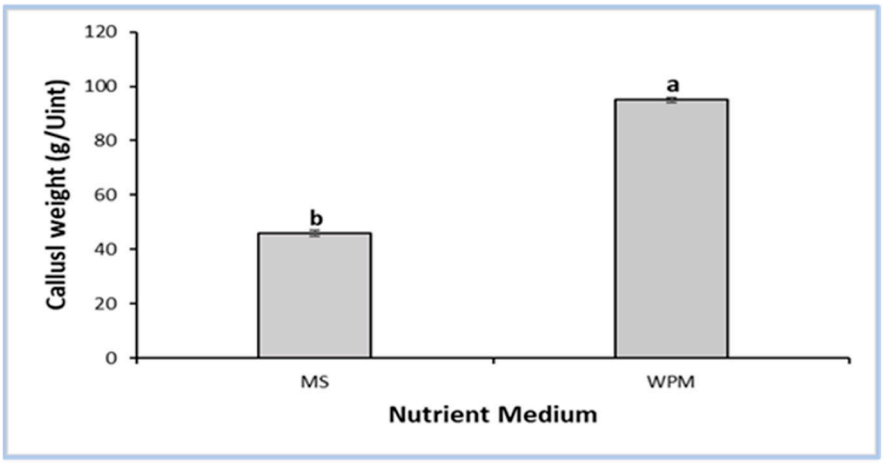


Figure 3. Effect of MS and WP media on callus production in a bioreactor system. [a,b] Means within the same column with different superscripts differ significantly ($p < 0.05$).

3.4. Roots Induction

The data presented in Table 2 demonstrate the impact of the auxins (NAA and IBA) on root induction in Bidah pomegranate cultured in half-strength MS (1/2 MS) medium. Among the tested concentrations of auxins, NAA at 3.0 mg/L exhibited the most pronounced effect, inducing root formation in 65% of the inoculated shoots. At this concentration, the average number of roots per shoot was 4.0 ± 0.0 (Table 3 and Figure 5a), indicating consistent and vigorous root development. In contrast, IBA at the same concentration (3.0 mg/L) did not induce any root formation in the Bidah pomegranate (Table 3). Additionally, NAA treatment resulted in the formation of four roots per shoot (Figure 5a), whereas IBA induced only a single root in Bidah pomegranate (Figure 5b). In contrast to previous studies where PGR-free WPM induced efficient rooting in the Bhagva pomegranate cultivar [1], optimal rooting for both Malas Saveh and Yusef Khani cultivars required half-strength WPM supplemented with 5.4 µM NAA [2]. Furthermore, a maximum rooting rate of 86% for regenerated shoots of pomegranate was achieved using half-MS medium supplemented with 1.0 mg/L IBA [28].

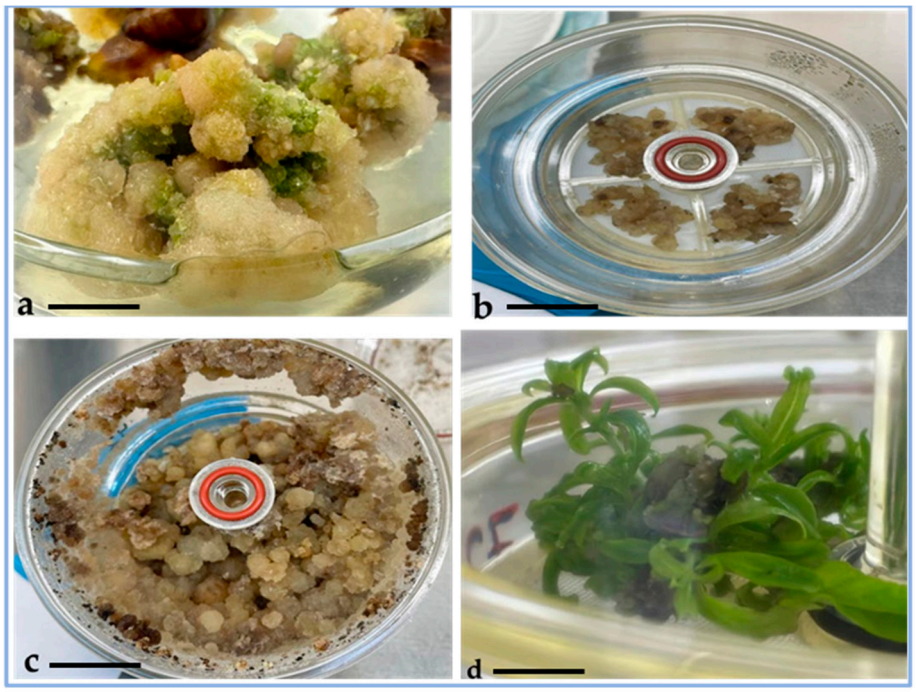


Figure 4. Callus induction, mass production and shoot regeneration of Bidah pomegranate: (**a**) Callus induction, (**b**) callus inoculated into the bioreactor system, (**c**) callus multiplication into the Rita bioreactor system for two months, and (**d**) shoot regenerated from callus materials (Scale bar = 1 cm).

Table 3. The effect of PGRs on root induction of Bidah pomegranate landrace % and the average number of induced roots on half-strength MS medium.

PGRs Concentration (mg/L)	Percentage of Rooted Seedlings %	Number of Induced Roots/Seedling
NAA (1.0)	30	3.0 ± 0.0^{b}
NAA (2.0)	-	-
NAA (3.0)	65	4.0 ± 0.0^{a}
IBA (1.0)	30	1.0 ± 0.0^{c}
IBA (2.0)	25	1.0 ± 0.0^{c}
IBA (3.0)	-	-

The data present the average number of callus weight ± standard deviation (SD). ^{a,b,c} Means within the same column with different superscripts differ significantly ($p < 0.05$).

3.5. Hardening of Plantlets

Acclimatization of woody plants in plant tissue culture (PTC) is the critical transitional phase in which in vitro grown plantlets are gradually shifted from a sterile, high-humidity, sugar-supplemented environment to natural ex vitro conditions [31]. This process enables the development of functional stomata, cuticle formation, lignification, and autonomous photosynthesis, preparing the plantlets to withstand ambient fluctuations in light, temperature and humidity. Here in this study, after successful rooting of shoots, the plantlets of Bidah pomegranate regenerated via direct organogenesis were gently washed with distilled water to remove any residual material from the rooting system. Next, the plantlets were transferred to pots containing a soil medium composed of vermicompost and perlite in a 1:1 ratio. Each pot was covered with a transparent plastic cap to maintain high humidity and minimize transplant shock during the initial acclimatization phase. During the initial phase, plantlets were irrigated directly into the pots. As the plantlets established, the plastic

caps were gradually removed to facilitate acclimatization to field conditions. Following this hardening process, the plants achieved an 80% survival rate (Figure 6).

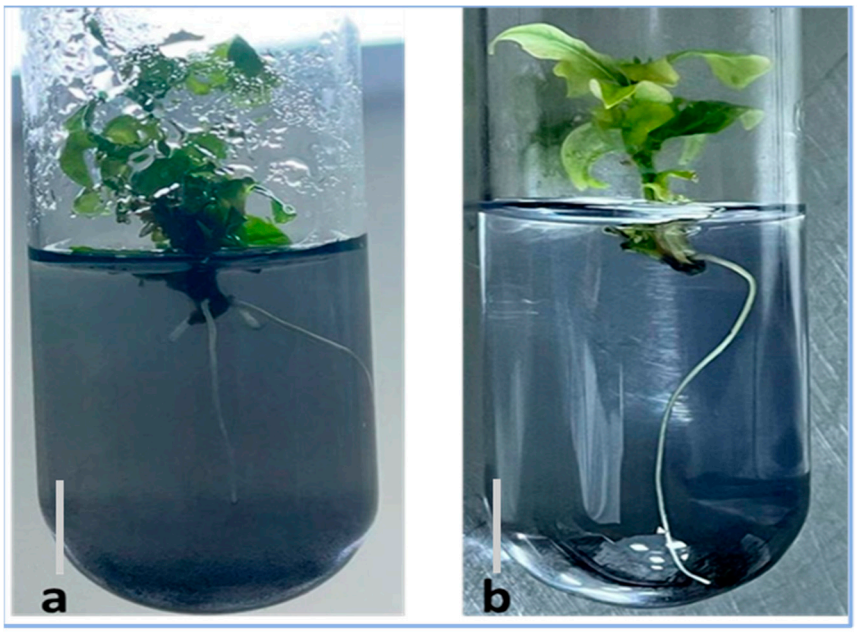


Figure 5. Effect of plant growth regulators (PGRs) on root induction in Bidah pomegranate: (**a**) Naphthaleneacetic acid (NAA) and (**b**) Indole-3-butyric acid (IBA) (Scale bar = 1 cm).



Figure 6. Hardening stage of Bidah pomegranate plantlets: (**a**) Bidah pomegranate plantlets at one month of age showing initial acclimatization and early leaf expansion; (**b**) Bidah pomegranate plantlets at three months demonstrating enhanced stem elongation and increased leaf number; (**c**) Bidah pomegranate plantlets at eight months; (**d**) Bidah pomegranate plantlets at fourteen months exhibiting full hardening characteristics, including lignified stems, mature foliage, and a well-developed branching system.

3.6. Genome Size Estimation

One of the major challenges in pomegranate cultivation is the propagation of true-to-type plants [32]. Plant tissue culture (PTC) provides an effective platform for clonal propagation [33], yet it requires genetic-fidelity assessment to ensure that the regenerated plants remain genetically true-to-type [34]. Somaclonal variation in micropropagated plants can be revealed through phenotypic characterization, cytogenetic examination, DNA-based molecular marker analysis, RNA-seq profiling and bisulfite sequencing [35]. Hence, the genetic fidelity of in vitro regenerated plantlets compared to the mother plant was assessed by estimating the genome size of the mother plant compared to the plantlets (callus-based materials). Flow cytometric profiling demonstrated that the in vitro regenerated plants maintained genetic stability, as shown in Figure 7 and Table 4. The 2C DNA content of the donor plants was 2.01 ± 0.06 pg, whereas that of the indirect regenerated plants was 2.01 ± 0.05 pg, showing no significant variation from the donor parent (Figure 7 and Table 4). Conversely, the genetic fidelity of the directly regenerated in vitro plantlets was not assessed, given that direct organogenesis is generally regarded as a pathway that yields true-to-type plants [36,37]. Moreover, genetic fidelity will be investigated using molecular markers such as simple sequence repeat (SSR), inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) before distributing the regenerated plantlets to the farmers.

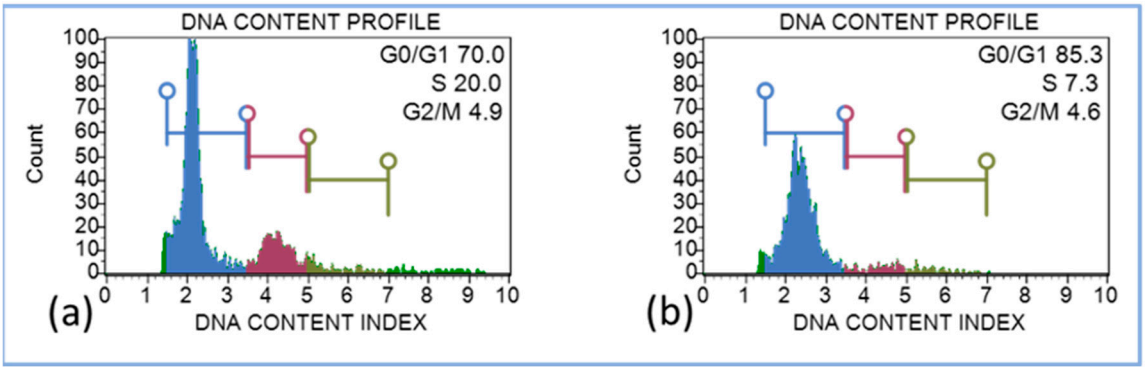


Figure 7. Flow cytometry histogram: (**a**) Mother plant (2.0 ± 0.05 pg.) and (**b**) callus-based plant (2.01 ± 0.06 pg.) using the MB01 buffer.

Table 4. Genome size of Bidah pomegranate mother plant and callus-based plantlet.

Source of Plant	2c DNA (pg.)	Genome Size (Mbp.)	Coefficient of Variation (%)
Mother plant	2.0 ± 0.05 [a]	1956	2.5
Callus-based plantlets	2.01 ± 0.06 [a]	1965.78	2.9

The data presented the average of three replicates ± standard deviation (SD). Each column is followed by letters of significant difference at $p \leq 0.05$ using Duncan's multiple range test.

4. Conclusions

To date, no reports have documented the in vitro propagation of the Bidah pomegranate cultivar. This study articulated the first efficient micropropagation protocol for the Bidah pomegranate landrace in Saudi Arabia using both direct and indirect organogenesis approaches. Bidah pomegranate phenolic exudation was successfully mitigated through sequential subculturing of explants into plant growth regulator (PGR)-free medium. However, antioxidants (citric acid, ascorbic acid, Ac and PVPP) pre-treatments did not show any positive impact. Among the tested media, MS medium proved to be the most effective for shoot induction and proliferation, whereas WPM was superior for callus induction, shoot multiplication and shoot regeneration in the bioreactor system. A higher root induction rate was obtained with half-strength MS medium containing 3 mg/L NAA. The genetic fidelity

of the regenerated plantlets was confirmed by genome size estimation, demonstrating a high degree of genetic stability. Overall, the developed protocol offers a reliable and scalable platform for conservation, large-scale propagation and phytochemical applications. Moreover, the developed protocol can be used for genetic improvement of the Bidah pomegranate cultivar. This work establishes the first comprehensive and efficient in vitro propagation system for this elite landrace, providing a strong foundation for advanced biotechnological interventions, including genome editing and transformation.

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Abbreviations

PGRs	Plant Growth Regulators
PVPP	Polyvinylpolypyrrolidone
WPM	Woody Plant medium
MS	Murashige and Skoog medium
2,4-D	2,4-dichlorophenoxyacetic acid
IBA	Indole 3-butyric acid
AC	Activated charcoal
NAA	α-Naphthaleneacetic acid
PTC	Plant Tissue culture
BA	Benzyladenine
BAP	6-Benzylaminopurine
2c DNA	Nuclear DNA content
RH	Relative humidity
PAR	Photosynthetically active radiation

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