Advancements in Micro-Propagation and Bio-Priming: Critical Factors for Pomegranate Plant Quality

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Abstract: Pomegranate (*Punica granatum* L.) is a highly lucrative fruit crop, particularly suited for arid and semi-arid regions that are frequently affected by climate variability, water scarcity for irrigation, and various soil-related challenges. In recent years, pomegranate cultivation has seen remarkable growth, especially in India, driven by its valuable nutraceutical benefits, adaptability to diverse environments, high market demand, and considerable returns on investment. As the global demand for pomegranate increases, the large-scale production of superior-quality planting material has become a critical priority. Diseases such as bacterial blight, wilt, and those transmitted through saplings or potting media pose significant threats to sustainable pomegranate production. Thus, there is an urgent need for the development of quality planting material (QPM) to prevent the spread of these pathogens. Micro-propagation provides a promising approach, allowing for the production of disease-free elite saplings in large quantities, which is essential for ensuring the long-term viability of pomegranate cultivation.

Keywords: Bio-priming, *In vitro*, nutraceutical, Punica granatum.

Introduction

Pomegranate is an ancient fruit crop that has been domesticated by humans and is recognized as a highly profitable species in semi-arid tropical regions. Its remarkable adaptability and resilience enable the crop to sustain fruit production, effectively mitigating the challenges posed by climatic fluctuations and soil-related constraints characteristic of semi-arid environments [1]. The exceptional nutraceutical and therapeutic benefits of pomegranate have garnered widespread global consumer interest, leading to a substantial demand for its fruits, value-added products, and dietary supplements. On a global scale, pomegranate is cultivated across approximately 0.5 million hectares, yielding around 6 million tons annually. In India, pomegranate is grown on approximately 0.234 million hectares, with an annual production of 2.85 million metric tons. There has been considerable expansion in both the area under cultivation and overall production in recent years. Notably, India has experienced a nearly threefold increase in cultivated area and a fourfold rise in production over the past two decades. Additionally, pomegranate exports from India are estimated at 67,000 metric tons. Over the last decade, the average annual increase in pomegranate cultivation in India has been about 19,300 hectares, necessitating the production of approximately 15 million healthy saplings annually to meet this expansion [2]. While traditional propagation methods are commonly employed for the production of pomegranate saplings, these approaches do not guarantee the generation of high-quality, disease-free, or blight-resistant plants. Bacterial blight, wilt, and other diseases transmitted through saplings or potting media pose significant challenges to the sustainable production of pomegranate. The adoption of in vitro techniques for clonal propagation has facilitated the rapid multiplication of superior pomegranate genotypes, ensuring the production of pathogen-free plants, devoid of the pathogens responsible for blight and wilt diseases. The overarching goal is to expand pomegranate cultivation globally, which requires large-scale, disease-free clonal propagation of elite genotypes through micropropagation methods.

Materials and Methods

Micropropagation: Micropropagation is a plant tissue culture method employed to generate plantlets, wherein small, aseptic sections of plant tissues or organs are cultivated in sterile vessels containing specific culture media. These cultures are maintained under controlled environmental conditions to facilitate the development of new plantlets.

There are five distinct stages in micro-propagation:

1. Stage 0: It involves the selection and maintenance of elite and healthy mother plant of a commercial variety and explant selection and pretreatments

2. Stage I: In vitro culture establishment

3. Stage II: In vitro shoot proliferation

4. Stage III: In vitro rooting

5. Stage IV: hardening or acclimatization

The origin of explants, the type of explants, and the pretreatments applied to them are critical factors influencing the establishment of in vitro cultures and the success of the micropropagation protocol for pomegranate. Not all explants exhibit the same regenerative potential. In commercial micropropagation, meristems, shoot tips, and nodal buds are commonly used as explants for efficient plant regeneration [3-9]. Alternatively, in vitro regeneration can also be achieved using other explants such as leaf segments and cotyledons [10-13], anthers [14]. Alternatively, regeneration can occur through embryogenesis using a variety of seedling explants, including petals and immature zygotic embryos [10] were also reported. Stage 0 involves the selection of healthy mother plants and their treatment to promote the production of more responsive explants that are suitable for establishment in contamination-free cultures. For successful culture initiation, appropriate explants are chosen, sterilized, and transferred onto nutrient media. Nodal segments are typically soaked in a fungicide solution (Dithane M-45 and/or Carbendazim) at concentrations of 0.1-0.2% for 30-45 minutes, followed by treatment with an antibiotic solution (streptomycin at 0.05-0.1%) for 10-20 minutes. Finally, surface sterilization is performed for 5-10 minutes to prevent most microbial infections [6,7]. The most commonly used surface sterilants for pomegranate explants are mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl). Prior to surface sterilization, the explants are thoroughly rinsed under running tap water [15].

In vitro Culture Establishment

In vitro propagation of pomegranate is primarily conducted using modified Murashige and Skoog (MS) medium, as well as Woody Plant Medium (WPM) [2,9]. The cytokinin to auxin ratio plays a crucial role in regulating shoot proliferation during pomegranate micropropagation. Among cytokinins, 6-Benzylaminopurine (6-BAP), kinetin, and supplements such as adenine sulfate are commonly employed to promote culture establishment and shoot proliferation. For auxin supplementation, α-naphthaleneacetic acid (NAA) is frequently used. [14,16,2]. The highest proliferation rate (10-15 shoots per explant) for Bhagwa nodal segments at the establishment stage was observed on MS medium supplemented with 1.8 mg/l BAP, 0.9 mg/l NAA, 1 mg/l silver nitrate (AgNO3), and 30 mg/l adenine sulfate. [6]. Two types of basal media, MS and WPM, have been utilized for the culture establishment of nodal segments and shoot tips from pomegranate cultivars 'Malas Saveh' and 'Yousef Khani' [17]. Media Browning The establishment of woody plants such as pomegranate is often hindered by medium browning caused by the leaching of secondary metabolites like phenols. To mitigate this issue, various strategies can be employed, including the use of juvenile explants or new growth flushes during their active growth phase, culturing in darkness, frequent transfer of inoculants to fresh medium (rapid sub-culturing), culturing in liquid media with antioxidants, or soaking explants in water or antioxidant solutions prior to inoculation. Additionally, supplementing the medium with adsorptive agents such as activated charcoal and polyvinylpyrrolidone (PVP) can help control browning. In the case of pomegranate cultivar 'Ganesh,' shoot tips and nodal segments are obtained in vitro through rapid sub-culturing to manage medium browning. [12]. Rapid subculturing on the first- and third-day post-inoculation has been recommended for the in vitro propagation of pomegranate cultivar 'Mridula,' using shoot tips and nodal segments from mature trees as explants [3]. The phenol exudation could be reduced if the cut edges of nodal segments are sealed with sterile wax [8]. In vitro rooting was best obtained on half strength MS medium containing 500mg/l of activated charcoal [18].

Shoot Proliferation

The extent and rate of multiplication are influenced by the duration and concentration of auxins and cytokinins in the multiplication medium. Among various plant species, the cytokinin 6-benzylaminopurine (BAP) is the most commonly used growth regulator for promoting shoot regeneration. A comprehensive protocol for in vitro regeneration of pomegranate (Punica granatum L.) was developed using cotyledonary nodes derived from axenic seedlings. [14]. A

medium supplemented with 9.0 µM BA resulted in the highest number of shoots, with an average of 9.8 shoots per explant [14]. The combination of MS medium with 2.0 mg/l BAP produced the highest number of shoots per explant (1.73), while the MS medium supplemented with 2.5 mg/l BAP resulted in the greatest shoot length and leaf number [15].MS medium supplemented with 2.0 mg/l 6-benzylaminopurine (BAP) and 1.5 mg/l naphthalene acetic acid (NAA) yielded the highest shoot regeneration frequency (67.89%), along with an average shoot length of 3.62 cm and an average of 5.38 shoots per explant [19]. Optimal hormonal treatments for direct adventitious shoot regeneration in cotyledonary explants were identified under both liquid and solidified Murashige and Skoog (1962) media. [20].

Photoperiod and Light Intensity Effects

Different light intensities, maintained at a constant temperature of 26±2°C with a 16/8-hour light/dark cycle in the incubation room, were tested. The highest number of shoots per explant and the longest shoot length were observed at a light intensity of 3000 lux [8].

In vitro Rooting

During stage III, the proliferating shoots are transferred to a rooting medium, which represents the final in vitro stage before the plantlets are moved to ex vitro conditions. The aim of this stage is to produce fully developed plantlets that can begin to grow. Auxins play a central role in in vitro root induction, with IBA being the most effective among various auxins. In contrast, IAA, despite being a natural auxin, proved to be the least effective due to its susceptibility to light degradation. Rooting media typically have low salt concentrations. For the rooting of microshoots in cultivars 'Malas Yazdi' and 'Shirine Shahvar,' half-strength WPM medium supplemented with 0.54 µM NAA was found to be the most effective. [21] Half-strength MS medium supplemented with 0.1 mg/l IBA proved to be the most effective in inducing rooting of microshoots. In vitro shoots cultured in half-strength MS medium with 1.0 mg/l IBA developed strong roots (80%) within 8-10 days. [22]. Half strength MS medium supplemented with 0.5g/l of IBA showed good invitro rooting [12]. According to Singh and Khawale, the MS medium supplemented with 2.0mg/l IBA, 200mg/l and40g/l sucrose promoted better root quality [23]. When compared to control, rot number per shoot increased considerably after a brief dip of in vitro produced shoot in 1000mg/l IBA [24] and was 7.4. The root regeneration

frequency was 80.12% in half strength MS medium with 0.1mg/l NAA and 0.02% activated charcoal and was found to be more efficient.

Hardening/ Acclimatization

Hardening is the process of acclimatizing in vitro-grown plantlets to natural outdoor conditions by gradually exposing them to diffused light in humid chambers. This step is essential for ensuring the long-term survival and establishment of the plantlets. The ex vitro survival rate of 'Ganesh' cultivar plantlets was reported to be 68% when transplanted into vermicompost. [12]. The plantlets of cultivar 'G137' exhibited the highest survival rate (89%) and required the shortest time for field transfer, taking only 35 days [8]. The use of a glass jar with a polypropylene cap filled with a wet peat: Soilrite® mixture (1:1) proved to be the most effective method, resulting in the highest plantlet survival rate of 86.5% for in vitro-raised 'G-137' plants. [23]. The rooted micro-cuttings achieved an 80% survival rate when transferred to a mixture of vermiculite (60%), perlite (30%), and cocopeat (10%) on a volume/volume basis. [25]. Acclimatizing regenerated plantlets in plastic cups filled with autoclaved peat moss resulted in an 85% survival rate [19]. Pots filled with a sterilized cocopeat-perlite mixture and covered with polythene bags were effectively used for the hardening of in vitro rooted plantlets. The plantlets were maintained at 25±1ºC under artificial light (50 µmol m^-2 s^-1) for 3-4 weeks to ensure proper acclimatization [10].

In India, the primary hardening of in vitro-raised rooted pomegranate plantlets is typically performed in nursery trays or net pots, where they are acclimatized under low tunnel polyhouses with high relative humidity. Bio-priming involves the use of plant-beneficial microbes and/or their formulations, which are inoculated into the rhizosphere and/or phyllosphere of in vitro-raised plants or saplings during the in vitro, ex vitro, or nursery stages. This process aims to enhance field survival and improve the performance of the inoculated saplings. [26,9]. Bioprimed or biohardened plantlets demonstrate improved survival rates, increased root and shoot biomass production, enhanced photosynthetic activity, and better nutrient absorption [27-33,9].

Conclusion

Significant advancements have been made in developing efficient, reproducible, and reliable in vitro plant regeneration systems for pomegranate. To support the rapid expansion of pomegranate cultivation, the large-scale production of Quality Planting Material (QPM) is essential for ensuring sustainable production.

References

1.Mahishi D.M., Muralikrishna A., Shivashankar G and Kulkarni R.S (1991). Shoot tip culture method for rapid clonal propagation of pomegranate (*Punica granatum* L.). In Horticulture-New Technologies and Applications, Springer, Dordrecht, pp. 215-217.

2. Kumar R, Jokhar ML, Verma R and Jat H.R. (2017). Pomegranate micropropagation - a review. International Journal of Pure and Applied Bioscience 5(5): 1138-1149.

3. Chaugule R.R, More T.A, Kamble A.B. and Karale A.R .(2007). Studies on micropropagation in pomegranate (Punica granatum L.). In: recent trends in horticultural biotechnology (Vol I) and II-ICAE National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies, Vellanikkara, Kerala, India, 10-12, pp. 195-199.

4. Murkute A.A., Patil S and Singh SK (2004). In vitro regeneration in pomegranate cv. Ganesh from mature trees. Indian Journal of Horticulture 61(3): 206- 208

5. Naik S. K, Pattnaik S and Chand P.K. (1999). In vitro propagation of pomegranate (Punica granatum L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Scientia Horticulturae 79(3-4): 175-183.

6. Patil V.M., Dhande G.A., Thigale D.M., Rajput J.C (2011). Micro-propagation of pomegranate (Punica granatum L.) 'Bhagava' cultivar from nodal explant. African Journal of Biotechnology 10(79): 18130-18136.

7. Singh J, Gupta S, Khoshe P (2014). In vitro regeneration of pomegranate (Punica granatum L.) from nodal explant. International Journal of Advances in Pharmacy, Biology and Chemistry 3(3): 734-736.

8.Singh N.V., Singh S.K., Patel V.B (2007). In-vitro axillary shoot proliferation and clonal propagation of 'G 137'pomegranate (Punica granatum). Indian Journal of Agricultural Science 77(8): 505-508.

9. Singh N.V, Karimi H.R, Babu K.D, Sharma J. (2021). Propagation techniques and nursery management. In: Zamani Z, Sarkhosh A, *et al*. (Eds.), The Pomegranate: Botany, Production and Uses. CABI- Wallingford OX10 8DE, United Kingdom, pp.196-224.

10.Kanwar K, Joseph J, Deepika R (2010). Comparison of in vitro regeneration pathways in Punica granatum L. Plant Cell Tissue Organ Culture 100: 199-207.

11.Guranna P, Hosmani I, Sathyanarayana R, Hegde R, Hipparagi K (2017). Micropropagation in pomegranate (Punica granatum L.) cv. Bhagwa through indirect organogenesis and assessment of genetic fidelity by RAPD marker. Biotechnology Journal International 20(3): 1-8.

12. Murkute A.A and Patil S. (2003). Exudation and browning in tissue culture of pomegranate. Agricultural Science Digest 23(1): 29-31.

13. Raj D and Kanwar K (2010). In vitro regeneration of Punica granatum L. plants from different juvenile explants. Journal of Fruit and Ornamental Plant Research 18(1): 5-22.

14.Naik S.K., Pattnaik S, Chand P.K (2000). High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (Punica granatum L.). Scientia Horticulturae 85(4): 261- 270.

15.Kalalbandi B.M, Waskar D.P, Khandare V.S, and Gorad D.S (2014). Micropropagation studies on pomegranate var. Bhagwa. Indian Journal of Horticulture 71(4): 564-566.

16. Mulaei S., Jafari A., Shirmardi M. and Kamali K. (2020). Micropropagation of arid zone fruit tree, pomegranate, cvs. ‘Malase Yazdi’ and ‘Shirine Shahvar. International Journal of Fruit Science 20(4): 825-836.

18. Deepika R. and Kanwar K. (2010). In vitro regeneration of *Punica granatum* L. plants from different juvenile explants. Journal of Fruit Ornamental Plant Research 18(1): 5-22.

19.Parmar N., Kanwar K., and Thakur AK. (2015). High efficiency plant regeneration from cotyledon explants of pomegranate (*Punica granatum* L.) cv. Kandhari Kabuli. Vegetos 28(2): 160-165.

20.Soukhak F., Khalighi A. and Ghaemmaghami S.A. (2012). Study of direct adventitious shoot regeneration in pomegranate (Punica granatum cv. Malas Saveh) through cotyledonary explants. International Journal of Agricultural Science and Research 2(3): 19-26.

21.Kaji B.V., Ershadi A and Tohidfar M. (2013). In vitro propagation of pomegranate (*Punica granatum* L.) Cv.'Males Yazdi'. Albanian Journal of Agricultural Sciences 12(1): 1-5.

22.Naik S.K. and Chand P.K (2011). Tissue culture-mediated biotechnological intervention in pomegranate: a review. Plant Cell Reports 30(5): 707- 721.

23.Singh S. and Khawale R.N (2006). Plantlet regeneration from the nodal segments of pomegranate (Punica granatum L.) cv. Jyoti. Plant Biotechnology and its Applications in Tissue Culture 105-113.

24.Golozan A.B and Shekafandeh A. (2010). Effects of plant growth regulators on pomegranate (*Punica granatum L.* cv. Rabbab) shoot proliferation and rooting. Advances in Horticultural Science 24(3): 207-211.

25.Bonyanpour A and Khui M.K. (2013). Callus induction and plant regeneration in Punica granatum L. 'Nana' from leaf explants. Journal of Central European Agriculture 14(3): 75-83.

26.Kajla S., Poonia A., Kharb P. and Duhan J.S. (2013). Role of Biotechnology for Commercial Production of Fruit Crops. In: Salar RK, Gahlawat SK, *et al*. (Eds.), Biotechnology: Prospects and Applications. Springer, New Delhi, India. pp. 27-37.

27.Puthur J.T., Prasad KVSK., Sharmila P and Pardhasaradhi P. (1998) .Vesicular arbuscular mycorrhizal fungi improves establishment of micropropagated Leucaena leucocephala plantlets. Plant Cell Tissue Organ Culture 53: 41-47.

28. Rupnawar BS, Navale AM (2000) Effect of VA-mycorrhizal inoculation on growth of pomegranate layers. Journal of Maharashtra Agricultural University 25(1): 44-46.

29.Singh NV, Singh SK, Singh AK, Meshram DT, Suroshe SS, *et al*. (2012) Arbuscular Mycorrhizal Fungi (AMF) induced hardening of micropropagated pomegranate (Punica granatum L.) plantlets. Scientia Horticulturae 136: 122-127.

30.Bharose AA, Khandagale DJ Damse DN (2014) Regeneration media standardization of pomegranate (Punica granatum L.) cv. Bhagava from Shoot Tip, Cotyledonary Node, Hypocotyls and Leaves. Vegetos 27(2): 338-342.

31.Chauhan RD, Kanwar K (2012) Biotechnological advances in pomegranate (Punica granatum L.). In Vitro Cellular & Developmental Biology-Plant 48(6): 579-594.

32.Gadapa M, Singh G. (2022). Recent trends in pomegranate propagation: A review. The Pharma Innovation SP-11(5): 405-414.

33.Singh P. and Patel R.M (2014). Factors influencing in vitro growth and shoot multiplication of pomegranate. The Bioscan 9(3): 1031-1035.