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Review Article

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Pomegranate Micropropagation - A Review

Ravi Kumar[1], M. L. Jakhar[2], Rajani Verma[1] and Hari Ram Jat[1]

[1] Research scholar & [2] Professor

Department of Plant Breeding and Genetics

SKN College of Agriculture, SKN Agriculture University, Jobner

*Corresponding Author E-mail: bishnoiravi@ymail.com

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ABSTRACT

Conventional method of propagation of pomegranate is time consuming and tiresome. It does not ensure disease free and healthy plants. It has several limitations like low success, very slow propagation, new plants require one year for establishment. In vitro technique is the only prospect of plant tissue culture that has the potential to circumvent these problems. Hence, there lies an ample scope for large scale multiplication of desired genotypes using micropropagation. In vitro propagation has been attempted in different pomegranate growing countries to propagate similar type and virus free plants.

Key words: *Micropropagation, Pomegranate, In vitro, Meristem culture, Virus free plants.*

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family "Punicaceae" having two species *viz.*, *P. granatum* and *P. protopunica*. It is native from Iran and spread throughout the Mediterranean region of Asia, Africa and Europe[32]. It has 2n=2x=16, 18 chromosomes[42] .It is suitable fruit crop for arid and semi arid regions of the world. In India, it is cultivated over 1.43 lakh ha with a production of 17.74 lakh tones and productivity of 9.88 tones/ha. Pomegranate is mainly cultivated in the states of Maharashtra, Gujarat, Karnataka, Tamil Nadu, Uttar Pradesh, Haryana, and Andhra Pradesh[3].

It is commercially cultivated for its delicious fruits. A fully matured fruit is highly nutritive and rich source of protein, fat, fibre, carbohydrate *etc*. The fruit are rich in Fe, Ca, and antioxidant component like phenol, pigments and tannins. Apart from its demand for fresh fruits and juice, the processed products like pomegranate wine, pomegranate tea and candy are also gaining importance in world trade. The pomegranate fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols and a fair source of iron. In addition, the tree is also valued for its pharmaceutical properties. It is used for treating dyspepsia and considered beneficial in treating leprosy. The rind of the fruit and the bark of pomegranate tree are used as a traditional remedy against diarrhea, dysentery and intestinal parasites. Fruit rind, bark of stem and root are widely used for tannin production[14].

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Plant tissue culture forms the backbone of plant biotechnology, which is comprised of micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future[20]. Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts made in developing nations. Plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cell, tissue and organ under sterile and controlled environmental conditions[23]. In India, tissue culture research began nearly five decades ago with the first report on production of test tube fertilization[17].

Pomegranate is conventionally propagated by hard wood and soft wood cuttings. But, this traditional propagation method has several limitations like low success, very slow propagation, new plants require one year for establishment, not ensure disease-free and healthy plants. In addition, this method is a very time-consuming and labor-intensive process[19]. Hence, there lies an ample scope for large scale multiplication of desired genotypes using micropropagation. *In vitro* propagation has been attempted in different pomegranate growing countries to propagate similar type and virus free plants.

MICROPROPAGATION

Micropropagation is a plant tissue culture technique used for production of plantlets, in which the culture of aseptic small sections of tissues and organs in vessels with defined culture medium and under controlled environmental conditions.

The process of micropropagation in pomegranate involves 5 distinct stages

- **Stage-0:** Identification of elite mother plants/parental material.
- **Stage-I:** Selection of suitable explants, their sterilization and transfer to nutrient medium for establishment / initiation of a sterile culture explant.
- **Stage II:** Proliferation of shoots (direct and indirect shoot proliferation via callus) from the explant on medium.
- **Stage III:** Transfer of shoots to a rooting medium.
- **Stage IV:** Transfer of plants to polythene bags for hardening and finally field transfer.

Stage-0: This stage consists of identification of mother plants which should be healthy and their preparation in such a way that they provide more responsive explants suitable for establishment in contamination free cultures.

Stage-I: Explant: The source of explants has been considered a critical variable for *in vitro* culture in pomegranate. All explants are not equal in terms of regenerability. It is likely that different selective pressures would be exerted against different explants. This could result in different frequencies and spectrums of multiplication/regeneration among plants from different explants.

In this stage cultures are initiated from various kind of explants such as meristem, shoot tips, nodal buds, inter-nodal segments leaves *etc.* Meristem, shoot tips and nodal buds are most prefer for commercial micro propagation. These explants were used to enhance axillary branching, because such explants have preformed dormant vegetative meristematic buds such as nodal segments[6]. Meristems < 0.2mm (or) 0.2-0.4mm are devoid of pathogens and thus result in the production of disease, virus free plants through micro propagation.

Deepika and Kanwar[10,19] developed reliable and reproducible protocols to get healthy and well formed plants from juvenile explants of the pomegranate cv. 'Kandhari Kabuli'. Calli were initiated from cotyledon, hypocotyl, leaf and internode sections excised from 30 days old *in vitro* germinated seedlings. The best media for callus induction from cotyledon, hypocotyl, internode and leaf explants were MS medium supplemented with 13.0 μM NAA and 13.5 μM BA, 13.0 μM

NAA and 18.0 µM BA, 5.0 µM IBA and 9.0 µM BA, 8.0 µM NAA and 9.0 µM kinetin, respectively. The highest percentage of callus was obtained from cotyledon explants (85.50) followed by hypocotyl (79.67), internode (79.47) and leaf (75.48) explants. The calli thus obtained showed differentiation on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA. Cotyledon derived callus showed the highest regeneration rate (81.97%, with mean number of 16.47 shoots per explant) followed by hypocotyl, internode and leaf derived calli.

Bharose *et al.*[4] induced highest (60%) embryogenic callus from hypocotyl explants on full MS + 2, 4-D (0.5 mg/l) + kinetin (0.5 mg/l).

Kalalbandi *et al.*[16] studied in 'Bhagawa' pomegranate, micropropagation undertaken using different explants from fruiting mother plants. Shoot tip was found the best explant for culture establishment and microbial contamination (14.28%).

Surface sterilants: Successful disinfection of explants is a pre-requisite for *in vitro* culture and often involves a standard set of treatments. In pomegranate meristem culture, depending on meristem size most of the microbes are expected to be eliminated because it is free from endophytic microbes. Washing the plant material intensively in running tap water before the surface sterilization process. In pomegranate $HgCl_2$ and NaOCl are the most widely employed surface sterilants.

Damiano *et al.*[9] could successfully sterilize axillary bud segments using a combination of NaOCl and Na methiolate for 20 min resulting in 65% explant survival.

Kalalbandi *et al.*[16] showed the maximum survival (90.58%) and minimum microbial contamination (9.52%) on surface sterilization with 0.1% mercuric chloride for 10 min.

Browning: Establishment of *in vitro* culture of several plant species, especially woody plants, is greatly hampered by the lethal browning of explant and culture medium at initial establishing stage due to leaching of phenolic substances and secondary metabolites from cut surface. Explants and medium browning is a major problem in pomegranate due to the exudation of high amount of phenols, especially in mature explants[26].

The various techniques employed to overcome the harmful effects of browning attempts to either neutralize or avoid the buildup of toxic substances in the medium. The different approaches are culture of juvenile explants, or new growth flushes during the active growth period, culture in darkness, transfer to explant in fresh medium at short intervals (Rapid sub-culturing), culture in liquid medium inclusion of antioxidants in the culture medium, or soaking explants in water or solutions containing antioxidants prior to inoculation, use of absorbing agents such as activated charcoal (AC), polyvinyl pyrrolidone (PVP) *etc.*[44], use of low salt media and optimum proper growth regulators, sealing the cut ends with paraffin wax[5] and drying the explant under laminar airflow.

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation reactions can produce free radicals. Free radicals can cause damage or cell death. Antioxidants remove free radical intermediates and inhibit other oxidation reactions.

Murkute *et al.*[24] regenerated shoot tips and nodal segments of pomegranate cv. 'Ganesh' *in vitro* by rapid sub-culture to control media browning.

Chaugule *et al.*[8] attempted *in vitro* propagation of pomegranate cv. 'Mridula' using shoot tips and nodal segments of mature trees; rapid sub-culture on the first and third day after inoculation was suggested.

Singh *et al.*[33] sealed the cut edges of nodal segments with sterile wax to reduce phenol exudation; this lead to a high regeneration rate. In general, the most commonly used methods to reduce browning in pomegranate tissue culture included the use of adsorbents (e.g., activated charcoal or polyvinylpyrrolidone (PVP), the addition of antioxidants to culture medium or soaking explants in antioxidant solution, frequent sub-culture of explants to fresh medium or a combination of these methods[1,2,21,24,28,39,41].

Deepika and Kanwar[10,19] achived *in vitro* rooting best on half strength MS medium containing 500 mg/l of activated charcoal.

Singh and Patel[37]conducted the study to identify the most suitable types of nodal explants and browning control treatment for *in vitro* regeneration of pomegranate. Murashige and Skoog (MS) medium containing 1.0 mg/L BAP + 0.5 mg/L NAA was used commonly for all the treatments tested. Result revealed that the intensity of browning was increased with increased position and the length of explants. Minimum browning intensity was observed in 1st nodal explants having 1.5 cm length. However, explants of 3rd node with 2.5 cm length registered higher establishment (68.5%) and growth of explants. Furthermore, the most effective browning control was observed in subculturing of nodal explants twice, at the first day and third day of inoculation, which also found better in establishment of explants followed by activated charcoal 200 mg/L into the medium. Maximum length of shoots (3.9 cm) was recorded in 1st position of node with 2.5 cm length of explants.

Media: Selected explants are surface sterilized and aseptically cultured on a suitable medium. Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. Media compositions are therefore formulated considering specific requirements of a particular culture system.

Agamy *et al.*[1] cultured shoot tips of 2-3 cm long of two pomegranate cultivars (Manfalouty and Nab El-Gamal) on three different media at full strength, namely Murashige and Skoog (MS), Nitsch & Nitsch (NN) and Woody Plant Medium (WPM). The plantlets grown on WPM were found to be significantly better in average survival (100 and 60%), plantlet height (5.10 and 4.58 cm) and average leaves number per shoot (11.3 and 10.0) for Manfalouty and Nab El-Gamal pomegranate cvs., respectively compared to other media. The two cultivars grown on WPM containing 1.0 mg/l BA had significantly the highest proliferation rate (6.8 and 5.8 shoot/explant) compared to 1.0 mg/l kinetin which produced the least value of proliferation rate (2.2 and 2.8 shoot/explant) for both investigated cultivars, respectively. The same trend was found concerning the average leaves number in response to BA and kinetin treatments. NAA at 0.25 mg/l significantly produced the highest rooting response (100% as an average for HSW and FSW) for Nab El-Gamal pomegranate cv., while 0.25 mg/l IBA induced the highest value of rooting (85% as an average) for Manfalouty pomegranate cv. IBA significantly increased the average number of roots compared to NAA treatments on both strengths of woody plant medium. The average roots length of the plantlet grown on HSW medium was longer (5.82 and 2.46 cm) than those grown on FSW medium (5.32 and 2.03 cm) in Manfalouty and Nab El-Gamal cvs., respectively.

Patil *et al.*[30] developed reliable and reproducible protocols to get healthy and well formed plants from nodal explants of the pomegranate (*Punica granatum* L) cv. 'Bhagava'. Nodal segments were cultured on two different media at full strength Murashige and Skoog (MS) and Woody Plant Medium (WPM). The media was prepared as a basal medium supplemented with 0.2 to 2 mg/l 6-benzylaminopurine (BAP), 0.1 to 1 mg/l 1-naphthalacetic acid (NAA), 0.5 to 2.5 mg/l silver nitrate (AgNO3) and 10 to 50 mg/l adenine sulphate for establishment stage. The nodal explants grown on MS medium containing 1.8 mg/l BAP, 0.9 mg/l NAA, 1 mg/l silver nitrate and 30 mg/l adenine sulphate had the highest proliferation rate (10 to 15 shoots/explants) in establishment stage. The same trend was found concerning the maximum leaves numbers (15 to 20 leaves/explants) on proliferation medium containing 0.4 mg/l BAP and 0.3 mg/l NAA. The plantlets grown on MS medium were found to have better survival compared to

WPM medium. 0.5 mg/l NAA and 0.5 mg/l IBA showed equal rooting response in both the medium, whereas thick root formation was observed in the medium containing IBA.

Kaji *et al.*[15] investigated the influence of two basal medium, WPM and MS, and different plant growth regulators on micropropagation of the Iranian pomegranate cultivars, 'Malas Saveh' and 'Yousef Khani'. For proliferation stage, media supplemented with different concentrations (2.3, 4.7, 9.2 and 18.4 µM) of kinetin along with 0.54 µM NAA was used. WPM proved to be more efficient medium compared to MS. The best concentrations of kinetin were 4.7 µM for 'Malas Saveh' and 9.2 µM for 'Yousef Khani', resulting in the highest number of shoots per explants, shoot length and leaf number. For both cultivars, half-strength WPM medium supplemented with 5.4 µM NAA was most effective for rooting of shoots.

Stage II: Cytokinin: Effective explants from stage I are sub-cultured on to a fresh medium. The time and concentration of auxins and cytokinins in multiplication medium is an important factor affecting the extent of multiplication. Cytokinins are adenine derivatives which are mainly concerned with cell division, modification of apical dominance and shoot differentiation in the tissue culture. The cytokinin 6- benzylamino-purine (BAP) is the most commonly used growth regulator for shoot regeneration in a variety of plants. Naik *et al.*[28] presented a complete protocol for *in vitro* regeneration of pomegranate (*Punica granatum* L.), a tropical fruit tree, using cotyledonary nodes derived from axenic seedlings. Shoot development was induced from cotyledonary nodes on Murashige and Skoog (1962) medium supplemented with 2.3±23.0 mM benzyladenine (BA) or kinetin (Kn). Both type and concentration of cytokinin significantly influenced shoot proliferation. The maximum number of shoots (9.8 shoots/explant) was developed on a medium containing 9.0 mM BA. Shoot culture was established by repeatedly sub-culturing the original cotyledonary node on a fresh batch of the same medium after each harvest of the newly formed shoots. *In vitro* raised shoots were cut into nodal segments and cultured on a fresh medium for further multiplication. Thus, from a single cotyledonary node about 30±35 shoots were obtained in 60 days. Singh and Khawale[39,41] reported that semi-hardwood nodal segments of pomegranate cv. 'Jyoti' established best when cultured on half-strength MS medium supplemented with 1.0 mg/l BA + 1.0 mg/l Kin along with 200 mg/l activated charcoal (AC). Axillary buds were aseptically removed from the established cultures and transferred onto proliferation medium. Multiple shoot proliferation occurred on MS medium supplemented with various concentrations of BA, Kin and 40 mg/l adenine sulphate (Ads) (ranging from 1.3 to 3.4 shoots/explant). For shoot elongation and rooting, MS medium containing 2.0 mg/l indole-3-butyric acid (IBA), 200 mg/l AC and 40 g/1 sucrose was best. Golozan and Shekafandeh[13] reported an effective procedure of pomegranate (*P. granatum* L. Rabbab) micropropogation. Nodel segments were used as explants. A comparison between three types of cytokinin (BA, TDZ and kin) on shoot proliferation indicated that 2 mg / lit. BA produced the highest shoot number (3.5 shoots per explant) and shoot length (6.1 cm). Kalalbandi *et al.*[16] studied in 'Bhagawa' pomegranate, micropropagation undertaken using different explants from fruiting mother plants. Maximum number of shoots per explant (1.73) was recorded in treatment MS + BAP 2.0 mg/l, while maximum shoot length and number of leaves were observed on medium containing MS + BAP 2.5 mg/l. Singh and Patel[34] obtained maximum proliferation of shoot (78.25 %), number of shoot (3.75) per explants and shoot length (3.06 cm) on MS medium supplemented with 1. 0 m g/l B A P + 1. 0 mg/l kinetin with 40 mg/l adenine sulphate in medium. Parmar *et al.*[29] supplemented MS media with 2.0 mg/l 6-benzylaminopurine (BAP) and 1.5 mg/l naphthalene acetic acid (NAA) was optimal for shoot regeneration, which resulted into highest frequency of shoot regeneration (67.89%) along with maximum average

number of shoots formed per explant (5.38) and average shoot length (3.62 cm). Shoot multiplication and elongation took place on the same medium.

Interactions of auxins and cytokinins: The ratio of auxins and cytokinins is important with respect to morphogenesis in the culture system. For embryogenesis, callus initiation and root initiation the requisite ratio of auxins to cytokinin is high, while the reverse leads to axillary and shoot proliferation. In pomegranate, a combination of cytokinin (s) and auxin has generally been employed for culture initiation and shoot proliferation/ multiplication. For indirect regeneration, different combinations are effective depending upon the genotype. Zhang and Stoltz[1]supplemented MS medium with 1.0 µM -naphthaleneacetic acid (NAA) and 2.0 µM BA for maximum shoot regeneration on terminal shoot explants in a dwarf pomegranate genotype. Drazeta[11] compared the micropropagation in different pomegranate cultivars 'Slatki Barski', 'Serbetas', 'Konjski Zubi' and 'Dividis'. Most shoots formed on medium containing 1 mg/l BA and 0.1 mg/l NAA, but shoots were hyperhydric, so subsequent cultures were transferred onto medium containing BA at 0.5 mg/l and 0.1 mg/l NAA. Fougat *et al.*[12] also obtained good success on MS medium supplemented with 0.5 mg/l kinetin (Kin), 1.0 mg/l BA and 500 mg/l CH (cyclo-heximide).Naik *et al.*[27] compared shoot proliferation on an elite pomegranate cultivar 'Ganesh' using nodal stem segments excised from a mature tree. They tried three cytokinins, *viz.* BA, zeatin riboside (ZR) or thidiazuron (TDZ), and found that the highest number of shoots developed on a medium containing 2.0 mg/l ZR, while TDZ was least effective Murkute *et al.*[24] attempted *in vitro* regeneration in pomegranate cv. 'Ganesh' using mature tree explants like shoot tip and nodal segments. Shoot proliferation was obtained best on MS basal medium supplemented with 1.0 mg/l BA + 0.5 mg/l NAA. Singh *et al.*[33] developed an efficient protocol for *in vitro* clonal propagation of pomegranate cv. 'G137' using nodal segments and shoot tips of mature trees. Culture establishment was better on MS medium supplemented with 2.0 mg/l BA + 0.1 mg/l NAA and 0.5 mg/l gibberellic acid (GA3). The highest number of shoots per explant and longest shoot length were recorded on MS medium containing 1.0 mg/l BA, 1.0 mg/l Kin and 0.1 mg/l NAA. Chaugule *et al.*[8] attempted *in vitro* propagation of pomegranate cv. 'Mridula'. Shoot tip and nodal segment explants collected from mature tree were cultured on MS medium supplemented with NAA and BA at different concentration and combinations. To control phenolic browning, sub-culturing was undertaken at first and third day after inoculation. The MS medium supplemented with 0.4 mg/l NAA + 1.0 mg/l BA showed the highest percentage of shoot differentiation, i.e. 77.77% and 81.25% response from shoot tip and nodal segment explants, respectively. Damiano *et al.*[9] showed good shoot multiplication onto a basal Quorin and Lepoivre medium supplemented with BA (0.4 mg/l) and IBA (0.05 mg/l). Nodal segments were important for regeneration of 'Bhagava' on MS-based medium in the presence of 0.2–2 mg/l BA, 0.1–1 mg/l NAA and 0.5–2.5 silver nitrate ($AgNO_3$)[30] .Soukhak *et al.*[43] reported the best hormonal treatments for direct adventitious shoot regeneration in cotyledonary explants under both liquid and solidified Murashige and Skoog (1962) medium supplemented with BA at 2, 4.5, 9, 13 µM alone and in combination with 5.5 µM NAA or 5 µM 2,4-D. The highest number of shoot per explant 8.2 and 7.9 were obtained in MS liquid and agar medium supplemented with 13µM BA and 5.5 µM NAA respectively, although there was no significant difference between two medium. The highest shoot length 1.65 cm was obtained from MS liquid medium supplemented with13µM BA and 5.5 µM NAA as well. Bonyanpour and Khui[7] placed leaf explants of a local cultivar of dwarf pomegranate on Murashige and Skoog (1962) (MS) medium supplemented with various concentrations of 6-benzyl adenin (BA) and naphthalene acetic acid (NAA) for callus

induction. After 40 days, maximum callus induction was observed on a media containing 1 mg/l BA and 0.2 to 0.4 mg/l NAA. However, the highest callus growth was obtained on a medium containing 1 mg/l BA and 1 mg/l NAA. The highest number of shoots (7 shoots per explants) was obtained by transferring the calli to the media containing 5 mg/l BA with 0.1 mg/l NAA. Maximum shoot proliferation was observed when shoots were cultured on woody plant medium (WPM) supplemented with 5 mg/l kinetin (Kin).Singh *et al.*[38] observed the maximum percentage establishment of cotyledonary node explants on Murashige and Skoog (MS) medium + 1.0 mg/l 6-benzylaminopurine (BAP) + 0.5 mg/l naphthaleneacetic acid (NAA). However, MS medium fortified with 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal exhibited maximum multiplication rate for the first two subcultures. The maximum frequency of multiple shoots in cotyledonary explants (86.33 %) was observed on treatment MS + 1.0 mg/l BAP + 1.0 mg/l kinetin + 200 mg/l activated charcoal. Singh *et al.*[34] cultured reliable and reproducible protocols to get healthy and well formed plants from nodal explants of the pomegranate (*Punica granatum* L) nodal segments on M.S. media at full strength. Murashige and Skoog media was prepared as a basal medium supplemented with organic acids and vitamins. The pH of the prepared media was adjusted between 5.6 to 5.8 and agar-agar was added as 8.0 g/l for media solidification. For establishment stage, BAP 0.2 to 2.0 mg/l, NAA 0.1 to 1.0 mg/l, BAP 0.1 to 0.5 mg/l and NAA 0.1 to 0.5 mg/l were tested.

Effect of photoperiod and light intensity: Photoperiodism is the physiological reaction of organisms to the length of day or night. Photoperiodism can also be defined as the developmental responses of plants to the relative lengths of the light and dark periods. Hence, it should be emphasized that photoperiodic effects relate directly to the timing of both the light and dark periods. Singh and Patel[34] tested various level of light intensity by keeping the constant temperature 26± 2°C and 16/8 h light/dark period in the incubation room. The maximum number of shoots per explants and highest length of shoots was recorded under 3000 lux intensity.

Stage III: Rooting: Shoots proliferated during stage II are transferred to a rooting medium. This phase is designed to induce the establishment of fully developed plantlets. Auxins are mainly concern with inducing cell division. In nature the hormones of this group are involved with such activities as elongation of stem, internodes, tropism, apical dominance, abscission and rooting. Different factors favoring root initiation have been tried. The auxin IBA was found the most effective of all auxin types. In contrast, IAA though being natural was least effective as it got degraded due to light. The availability of IBA induces primary / secondary roots where as NAA induces root hairs. In general rooting medium has low salt. It is the last period *in vitro* before transferring the plantlets to *ex vitro* conditions.Drazeta[11] found that MS medium supplemented with 0.1 mg/l IBA was most effective for rooting. Fougat *et al.*[12] got good rooting success on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l Kin and 15% coconut water (CW). Naik *et al*.[27] reported that half strength MS medium containing 1.0 mg/l IBA induced good rooting (80%) on *in vitro* derived shoots in 8–10 days. From each shoots, 3–4 roots developed from complete plantlet. Kantharajah *et al.*[18] showed that lower salt level in culture medium had beneficial effect on *in vitro* rooting. They obtained both highest rooting with higher number of roots per micro-shoot on WPM supplemented with 2 mg/l NAA. Naik *et al.*[28] reported that addition of auxin to the media was essential to include rooting on *in vitro* regenerated shoots. Root initiation occurred within 10–15 days in half-strength MS medium supplemented with 0.54–5.4 µM α-NAA. The highest number of roots (10.33 roots/shoot) was formed in medium containing 0.54 µM. Murkute *et al.*[24] got efficient rooting on half-strength MS basal medium supplemented with either NAA or IAA at 0.5 mg/l. Singh and Khawale[39,41]suggested the role of AC during

rooting with higher level of sucrose, i.e. MS medium containing 2.0 mg/l IBA, 200 mg/l AC and 40 g/1 sucrose which also resulted in improved root quality. Chaugule *et al.*[8] suggested supplementation of auxin at 0.5 mg/l to be optimum irrespective of their type. Singh *et al.*[33] could induce good rooting (70.37%) with dual auxin, i.e. IBA and NAA supplemented to the rooting medium. Damiano *et al*.[9]suggested that the effective auxin level ranged from 0.75 to 2.0 mg/l either synthetic or natural (IBA or IAA) for cv. 'Mridula'. Golozan and Shekafandeh[13] reported a quick dip of *in vitro* derived shoots in 1000 mg/lit IBA for significantly increased average root number per shoot (7.4), in comparison with control (1.2). Rooted shoots after acclimatization were transferred to soil with a survival rate of 92%. Raj and Kanwar[31]developed reliable and reproducible protocols from juvenile explants of the pomegranate cv. 'Kahdhari Kabuli'. *In vitro* rooting was best on half-strength MS medium containing 500 mg/l of AC. Even though several basal media such as MS, WPM, White's and B5 in full or half-strength have been used with varying success, 1/2 MS has been the most effective for pomegranate tissue culture. Three auxins (IAA, IBA and IPA (indole-3-propionic acid)), when supplemented to White's medium, were useful for root induction in the fruit cv. 'Ganesh' and 'Muskat'[22,28]. Bonyanpour and Khui[7] cultured shoots for rooting experiments on WPM medium containing 0.2 mg/l indol butyric acid (IBA) had the maximum root percentage (100%) and good root growth (2.06 cm mean length and 2 roots in each explants).Singh *et al.*[38] recorded, the maximum number of root/shoot (4.17) and root length (3.87 cm) from *in vitro* rooting of regenerated shoot in half strength MS medium supplemented with 0.5 mg/l NAA + 200 mg/l activated charcoal. Kalalbandi *et al.*[16] got the best rooting with maximum number of roots per shoot (4.00) and root length (3.72 cm) on half-strength MS medium along with NAA 8 mg/l. Singh *et al.*[34] reported maximum number of root/shoot on MS medium containing 0.50 mg/L NAA (97%) and 0.50 mg/L IBA (95%).Parmar *et al.*[29] reported half strength MS medium containing 0.1 mg/l NAA and 0.02% activated charcoal most effective for rooting of shoots resulting into 80.12% root regeneration frequency. Yang and Ludders[46] noted that dwarf pomegranate var. 'Nana' did not require any auxin to root while AC promoted rhizogenesis with[21,39.41] or without[10,19,10] a growth regulator supplement.

Stage IV: Hardening: Hardening refers to the process of acclimating plants from indoor temperatures to the outdoors. The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Direct transfer of tissue culture raised plant to field is not possible due to high rate of cosseted environment with a very high humidity, varied light and temperature condition and being protected from the attack of microbial and other agents. Transfer of plantlets to soil is the most critical step in micro propagation. The plantlets are maintained under highly protected conditions in *in vitro* i.e. high humidity, low irradiance, low CO_2 levels and high sugar content. This is also called acclimatization phase. Acclimatization is defined as the climatic or environmental adaptation of a plant. In conventional acclimatization, the main effort of environment control in the acclimatization stage is to keep the relative humidity high particularly at an early stage of acclimatization. The high humidity could be generally achieved by covering the plantlets with plastic film under shade together with frequent misting. Shading is necessary since the strong solar light itself may directly damage the plantlets and also the fluctuating solar light intensity with time leads to fluctuation in temperature and relative humidity and hence an excess water loss from the plantlets[40] .

As for most horticultural crops, the success of tissue culture is determined by the ability of regenerated plants to be transferred from *in vitro* substrates to soil with as little loss and variability as possible. Successful acclimatization of *in vitro*-regenerated plantlets has been reported in pomegranate

using different substrates, albeit with differing rates of success. Mahishni *et al.*[21] obtained over 80% success in hardening using potting mixture comprising 1:1:1 (v/v) peat: perlite: sand. Yang *et al.*[45] standardized the micropropagation and transplantation of a valuable and rare pomegranate cv. 'Ruanzi'. They were successful in getting higher rooting > 90% with good plantlet survival upon their transfer to glasshouse conditions. Al-Wasel[2] could acclimatize 80% of plantlets in perlite under mist in a greenhouse. Naik *et al.*[27] reported 68% ex vitro survival of cv. 'Ganesh' plantlets when transferred to vermicompost which later gave 80% survival upon transfer to soil. Plantlets with well developed roots were successfully acclimatized and eventually established in soil. The survival of the plantlets after transfer to vermicompost was 60%, while it was 70% when transferred to soil[28]. Murkute *et al.*[24] achieved 50% survival when the plantlets were transferred to vermicompost + soil mixture (1:1) and hardened in mist chamber. Singh *et al.*[33] reported highest survival (89%) with minimum duration (35 days) to field transfer for plantlets in cv. 'G-137'. The different stages of micropropagation were standardized for pomegranate cv. 'G-137'.

Different *in vitro* hardening methods were compared by Singh and Khawale[39]. The strategy comprising glass jar with polypropylene cap filled with moistened with peat: Soilrite® (1:1) was found most effective which gave the highest (86.5%) plantlet survival. The hardened plantlets were successfully transferred to greenhouse and ultimately to the field. Soukhak *et al.*[43] transferred rooting plantlets to Jiffy and acclimatized after 35 days successfully. Singh *et al.*[35] used arbuscular mycorrhizal fungi (AMF) to harden pomegranate plantlets by averting transplantation shock of unfavorable *ex vitro* environmental conditions. Maximum survival was registered with Glomus mosseae (91.33% and 89% at 60 and 90 DAI (days after inoculation), respectively) inoculated plantlets followed by G. manihotis (89% and 87.33% at 60 and 90 DAI, respectively). The AMF-infected plants grew faster and had better physiological and biochemical parameters. Bonyanpour and Khui[7] cultured rooted plantlets in a soil mixture containing vermiculite (60%), perlite (30%) and coco peat (10%) v/v. After 2 months, 80% of plants survived and transferred to the greenhouse. Singh *et al.*[38] reported that *In vitro* grown plantlets having 5 to 6 cm length of shoot transferred to vermicompost + soil (1:1v/v) media kept in net house showed better survival of plantlet (85.50%) within 11.75 days. Bharose *et al.*[4] acclimatized well rooted plantlets of pomegranate cv. Bhagava on the medium containing sand: soil: FYM (1:1:1) and kept in the greenhouse. Kalalbandi *et al.*[16] obtained hardening of rooted plantlets ideal with maximum survival of plantlets (71.72%) on medium containing soil + sand (1:1; v/v). Parmar *et al.*[29] transferred regenerated plantlets to plastic cups containing autoclaved peat moss and successfully acclimatized with 85% survival rate.

Advantages of Micropropagation in Pomegranate

- Rapid multiplication of pomegranate plants threw micropropagation and produced plants (clones) are genetically similar. A single explant can be produce several thousand plants within a short period of time. These plants are true to type.
- All plants (clones) produced threw *in vitro* culturing are healthy and almost free from the pathogens.
- Tissue cultured plants produce early fruiting as compare to the seed plants.
- Required only limited number of explants.
- Easier transport.
- Rejuvenation of old varieties.
- It can be carried out throughout the year independent of seasons.

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