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In vitro propagation techniques in pomegranate (*Punica granatum* L.): A review

K Antony Prajwala, P Subbaramamma and M Viswanath

Abstract
Pomegranate was commercially propagated through hardwood cuttings or by air layering. However the supply of true to type planting material was scarce due to several limitations like low multiplication rate, requirement of large quantity of propagating material, rapid expansion of area under pomegranate cultivation and use of very slow propagation methods. Establishment of new plantlets require more time and are easily prone to pests and disease attack can leads to non- availability of quality plantlets through-out the year. *In vitro* technique has the potential to solve the problems and supply of quality planting material through-out the year. So, there was a ample scope for large scale multiplication of desired genotypes within a short time through micro propagation technique in pomegranate. In this review we had reported various steps followed in micro propagation techniques *viz*., selection of explants type, effective explants sterilization methods chemicals, preservation of phenol exudation use of efficient media for culture establishment and shoot proliferation use of growth regulators and effective photoperiod etc involved in regeneration of pomegranate plantlets through *in vitro* culture. The hardening of plantlets by using different rooting media association with VAM fungi was also discussed.

Key words: *In vitro* propagation, explant type, sterilization media composition, growth regulators and hardening

Introduction
Pomegranate (*Punica granatum* L.) is an economically important fruit crop of the tropical and subtropical regions of the world. The name Pomegranate is derived from two Latin words Pomum meaning apple and granatus meaning full of seeds. It belongs to the family Punicaceae, which comprises only one genus (Punica) and two species; *P. granatum* and *P. protopunica*.It is originated from Iran and is spread throughout the Mediterranean regions of Asia, Africa and Europe (Kumar *et al.*, 2017) [22]. The pomegranate is a small tree, it can grows to a height of 20 or 30ft. It is having, a glossy and leathery leaves and it bears flowers with red, fleshy, tubular calyx which persists on the fruit. Fruit is nearly round with tough outer membrane, which is typically light or deep pink or rich red in color and the interior is separated by membranous walls to in chambers, with sacs filled with sweetly acidic, juicy, red, pink or whitish pulp or aril (Maurya *et al.*, 2018) [25].

Pomegranate is commercially cultivated for fresh consumption of fruits. It is highly nutritive and is rich in proteins, fats, fiber, carbohydrates, minerals like Fe, Ca and antioxidant components like phenols, pigments and tannins. Apart from its high demand for fresh fruits and juice, the processed products like pomegranate wine, tea and candy are also gaining more importance in the world trade. The tree is also valued for its pharmaceutical properties because it cures diseases like dyspepsia and leprosy. The rind of the fruit and the bark of pomegranate tree are also used as a traditional remedy against diarrhea, dysentery and intestinal parasites. Fruit rind, bark of the stem and the roots are widely used in tannin production (Pal *et al.*, 2014) [27].

Pomegranate has the ability to withstand harsh and adverse climatic conditions. The versatile adaptability, hardy nature, low maintenance cost, production of steady but high yields, better keeping quality, fine table and therapeutic values have lead to a steady increase in the area and production of pomegranate in India. In India, it is cultivated in an area of over 2.09 Lakh ha with a production of 24.42 Lakh MT (NHB 2017). It is commercially grown in Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Tamil Nadu and Rajasthan states. Maharashtra is the major pomegranate growing state in India and is considered as the "*pomegranate basket of India*". Maharashtra contributes more than 70% of the total area (1.36 Lakh ha) under pomegranate cultivation followed by Karnataka and Andhra Pradesh.

Propagation of Pomegranate through seeds results has results in heterozygous population, which leads to wide variations in tree and fruit characteristics. Pomegranate is commercially propagated to get true to type planting material either by hardwood cuttings or by air layering. However, the supply of true to type planting material is scarce due to certain limitations like low multiplication rate of planting material, requirement of large quantity of propagating material, rapid expansion of area under pomegranate cultivation use of very slow propagation methods. Planting material easily prone to the pest and disease attack and requirement of one year time for establishment of new plants have lead to the non-availability of quality planting material through-out the year.
Recently tissue culture technique has been exploited more in pomegranate for mass multiplication of elite, robust, healthy plantlets in a short time and are available throughout the year. *In vitro* propagation methods in pomegranatehas been reported by several workers in the following steps.

Selection of explants

The selection of explant has been considered as one of the important step in pomegranate *in vitro* propagation technique. All types of explants are not equal in terms of their regenerability. It may vary based on ontogeny of the mother plant, explant type, explant position on mother plant, metabolic status and genetic constitution of the explant (Zulfiqar, 2009) [48]. The type of explants used in optimizing the tissue culture protocol in pomegranate leaf blade, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root. It may be due to variation in the levels of endogenous hormones present in the plant parts (Kumar and Jakhar, 2018) [23].

In vitro propagation methods in pomegranatehas been reported by several workers i.e. use of different types of explants *viz.*, shoot tip (Murkute *et al.*, 2004), nodal cuttings (Desai *et al.*, 2018) [10], leaf segments (Bonyanpour and Khosh-Khui, 2013) [6] and cotyledonary leaves *etc.*(Suhasini *et al.*, 2017) [45].

Deepika and Kanwar (2010) [9] conducted an experiment to develop reliable and reproducible protocols to get healthy and well developed plantlets from juvenile explants of the pomegranate (*Punica granatum* L.) cv. 'Kandhari Kabuli'. The highest callus percentage was obtained from cotyledon explants (85.50) followed by hypocotyl (79.67), internode (79.47) and leaf (75.48) explants excised from 30 days old *In vitro* germinated pomegranate seedlings.

Maximum multiple shoot induction in pomegranate (*Punica granatum* L.) was noticed with nodal segment explants (76.3%) than with stem and meristem segment explants (Devidas *et al.*, 2017) [11].

Satheesh and Sridharan (2015) [34] stated that auxillary shoot tips (82%) showed the highest regeneration response, followed by young nodal (63%) and mature nodal (21%) explant micropropagation process in pomegranate.

Axillary second nodal segment showed superior response to per cent aseptic culture establishment (34.44) as compared to shoot tip, first nodal segment, axillary bud of first and double nodal segments were used as explants in pomegranate micro propagation (Suhasini *et al.*, 2017) [45].Meristem, shoot tips and nodal buds were mostly preferred in commercial micro propagation. The explants were used to enhance the axillary branching, because the explants have dormant vegetative meristematic buds (Kumar *et al.*, 2017) [22]. In most of the *In vitro* propagation studies, juvenile explants were used to carry out the experiments as they have higher organogenic competence as compared to the mature explants in pomegranate (Kanwar *et al*., 2008; Kanwar *et al*., 2010) [19, 18]

Surface sterilization of explants

Surface sterilization of an explant is an essential step in plant tissue culture. Mortality of the plant tissue was highly noticed in plant tissue culture if the surface of the explants were easily contaminated by the microorganisms present in the external environment. The surface of the explants used in plant tissue culture were sterilized thoroughly with different chemicals like *viz*., ethanol, sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$) and plant preservatives etc to remove microbial contamination. The plant material was intensively washed in running tap water before the start of surface sterilization process to reduce the microbial infection drastically. Though other chemicals have been tried with good success, the mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) are the most widely employed as surface sterilents. The effective sterilization may vary based on nature of the explants used (Singh, 2018) [36].Desai *et al.* (2018) [10] conducted an investigation on micropropagation protocol in pomegranate variety 'Bhagwa' by using axillary buds as explants. The treatmentcombination of 2000 mgl^{-1} Carbendazim-50% (14 min), 1000 mgl^{-1} Cefotaxime (12 min), 1000 mgl^{-1} Kanamycin (12 min), 1000 mgl^{-1} Streptocycline (14 min) and 1000 mgl^{-1} HgCl2 (2 min) showed the least contamination percentage (21%). Any reduction either in the concentrations of the sterilants used or in duration of the treatment had resulted in higher contamination percentages.

Golozan and Shekafandeh (2010) [15] stated that treatment of nodal explants with 10% Chlorax solution+70% Ethanol + warm water at 40ºC + Streptomycin 100 showed 0% of fungal and bacterial contamination may lead to easy shoot proliferation and early rooting of nodal explants in pomegranate (*Punica granatum* L.) cv. Rabbab. Gorad *et al.* (2018) [16] stated that surface sterilization of shoot tip explants with 0.1 per cent mercuric chloride for 10 minutes had showed the maximum survival percentage (90.58%) and less contamination percentage (9.42%) in *In vitro* propagation of shoot tip explants in pomegranate.

Suhasini *et al.* (2017) [45] reported that successful establishment of aseptic culture in Pomegranate Cv. Bhagwa by surface sterilization of second nodal segments with mercuric chloride ($HgCl_2$) @ 0.01% for 3 min. (24.99%) followed by incubation on full strength of MS medium. The maximum survival (90.58%) and minimum microbial contamination percentages (9.52%) were observed by surface sterilization of explants with 0.1% mercuric chloride for 10 min in micropropagation of Pomegranate var. Bhagwa (Kalalbandi *et al.*, 2014) [17].

Phenol exudation and its control

In tissue culture, excision of explants during preparation for culturing had stimulate the phenolic exudation. Phenol exudation was the main problem in woody plants. The production of phenolic compounds was indirectly stimulated by several factors like age of the plant and duration of biotic and abiotic stresses. The oxidation of exuded phenolics can cause darkening or browning of the media, which blocks the uptake of nutrients, ultimately leads to death of the explants. The exudation of phenolic compounds from explants was minimized by use of different absorbents and antioxidants

(Ahmad *et al.*, 2013) [1].

The different approaches were practiced while culturing of juvenile explants or flushes of new growth during active growth period, *viz*. culturing in darkness, transfer of explant to fresh medium with short intervals, culturing in liquid medium containing anti-oxidants in culture media, or soaking of explants in water or solutions containing antioxidants prior to inoculation, use of absorbing agents, such as activated charcoal (AC), polyvinyl pyrrolidone (PVP), (Wang *et al.* 1994) [47], use of low salt media and sealing off the cut ends with paraffin wax (Bhat and Chandel 1991) [5] and drying off the explant in laminar airflow.

A study was conducted on exudation and browning in the pomegranate tissue culture (Murkute *et al.*, 2003) [26] with several treatments including the use of adsorbents and antioxidants along with different concentrations of activated charcoal (adsorbent), ascorbic acid (antioxidant) and sub culturing. The treatments *viz*., sub culturing of explants thrice, at an interval of 24 h had controlled the browning in all the explants and showed the best results.

In pomegranate tissue culture propagation, treatment of explants with distilled water, Ascorbic acid, Polyvinylpyrrolidone (PVP) and activated charcoal (AC) and subculturingof explants.

The best results were noticed by transfer of explants regularly at an interval of 24 h duration. The exudation, browning and survival percentage were more with explants soaked in Ascorbic acid (*viz*., 50–75%, 25–50% and 10–50%, respectively) than with explants soaked in distilled water (50%, 50–75% and 20–40%, respectively). Addition of PVP to the media had resulted in less exudation but percent survival of the explants were very poor (0–10%). The survival percentage was ranged from 10% to 50% if the explants were cultured on the medium supplemented with the activated charcoal (Desai *et al.* 2018) [10].

Singh and Patel (2016) [38] reported that control of *in vitro* browning in pomegranate by using different treatments. Minimum browning intensity was observed with 1st nodal explants having 1.5 cm in length. However, the explants of 3rd node with 2.5 cm in length had registered the higher establishment percentage(68.5%) and growth of the explants. Furthermore, the most effective method for control of browning was observed with subculturing of nodal explants twice, at first and third day of inoculation and better establishment of explants were noticed with the addition of activated charcoal @ 200 mg/L into the medium. Chaugule *et al*. (2007) [7].

stated that sub culturing of shoot tip and nodal segment explants selected from mature tree of pomegranate cv. 'Mridula' on first and third day of inoculation was proved to be best than addition ofdifferent concentrations of activated charcoal, ascorbic acid to media and subculturing of explants for one to two days. Singh *et al*. (2007) [35] showed that sealing off the cut edges of nodal segments with sterile wax reduced the phenol exudation and lead to the higher percentage of culture establishment.

In general, the most commonly used methods to reduce browning in pomegranate tissue culture are the use of adsorbents (e.g., activated charcoal or polyvinylpyrrolidone (PVP), the addition of antioxidants to culture medium or soaking explants in antioxidant solutions, frequent sub-culturing of explants to fresh medium or a combination of these methods were proved to be best (Kumar *et al.*, 2017) [22].

The explants were soaked in different combinations of anti-oxidant chemicals like ascorbic acid and citric acid (100 mgL^{-1} and 200 mgL^{-1})

for 10 and 15 min. The pre-treatment of explants with 100 mgL^{-1} of each of citric acid and ascorbic acid had registered the least browning intensity with 0.10 ± 0.06 sprout development between 9 to 15 days of culturing (Patel *et al.*, 2018) [29].

Media composition for culture establishment and shoot proliferation

Types of media

Growth and morphogenesis of plant tissues under *in vitro* conditions are largely governed by the composition of culture media. Although the basic requirements essential for cultured plant tissues are similar to those of whole plants, but in practice, nutritional components required for promoting optimal growth of a plant tissue under laboratory conditions may vary with respect to the particular species. Different media compositions are formulated by considering the specific requirements of a particular culture system under laboratory conditions, i.e. White's medium for root culture, MS medium for organogenesis and regeneration, B5 medium for cell suspension and callus culture, N6 medium for cereal anther culture, Nitsch's medium for anther culture(Kumar and Jakhar, 2018) [23].

In vitro propagation of pomegranate cv. manfalouty and Nab El-gamal in three different media at full strength, namely Murashige and Skoog (MS), Nitsch & Nitsch and WPM (El-Agamy *et al.*, 2009) [13]. WPM produced the tallest plantlets (5.10 and 4.58 cm average), with an average (10.67) number of leaves and average height of 4.84 cm of $explant^{-1}$ in both (Manfalouty and Nab El-Gamal) the cultivars respectively followed by MS medium,whereas NN medium had recorded the lowest values for the above parameters. On the other hand WPM and NN media were showed an approximately opposite trend for intermodal length. NN medium had produced significantly the highest average internodal length (0.73 cm), while the WPM had gave the least value (0.53 cm).

Patil*et al.* (2011) [31] conducted an experiment on micropropagation of pomegranate (*P. granatum* L.) cv 'Bhagava'in two different media (MS and WPM). MS medium had proved to produce the explants with best vegetative growth characteristics as compared to WPM medium. The micropropagation of Iranian pomegranate cultivars, (*viz*., 'Malas Saveh' and 'Yousef Khani') in basal medium (*viz*. WPM and MS) and in different plant growth regulators. The plantlets produced in WPM medium were apparently more vigorous, in terms of the percentage of shoot proliferation (51.42±32.81), length of shoots (2.15±1.31), and number of leaves (6.97±3.66)$plantlets^{-1}$ as compared to MS medium (ValizadehKaji *et al.*, 2013) [46]. The*In vitro* rooting of proliferated shoots took less number of days if cultured on Woody Plant medium (WPM medium) than cultured on half strength MS medium (irrespective of type and concentration of growth regulator used in the media).The number of roots $shoot^{-1}$ (5.00) and the rooting percent (76%) were found high in WPM medium supplemented with NAA @ 1.0 mg L^{-1} (Bachake *et al.*, 2019) [4].

Composition of growth regulators

Plant growth regulators are organic compounds other than nutrients, but influenced the growth and development of plants, since phyto-hormones are the key compounds in tissue culture studies. In pomegranate a combination of cytokinins and auxins have been employed for culture growth initiation,

shoot proliferation and multiplication.

Addition of auxins to the culture media has been found essential for obtaining the good rooting. Different combinations of plant growth regulators are used while preparation of culture media was found effective in regeneration of callus tissue depending upon the type of explants used, condition of the culture media prepared and selection of genotype (Sing *et al.*, 2010 and Gasper *et al.*, 1996) [42, 14].The pomegranate cv Bhagwa nodal segment explants had performed better than meristem tip explants. The nodal segment explants proliferated more and produced the maximum number of shoots (76.3±1.7%). On an average 6.29±0.13 shoots explant^{-1} were produced on MS media supplemented with BAP (3 mg L^{-1}) than supplemented with Kinetin (1 to 5 mg L^{-1}). The newly formed shoots were elongated more on MS media with variable levels of GA_3 (0.1 to 0.5 mg/l).The maximum shoot elongation over initial shoot length (on an average 4.57 cm) was noticed with GA_3 @ 0.4 mg/l (Devidas *et al.*, 2017) [12].

The leaf explants of *Punica granatum* L. '*Nana'* were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyl adenin (BA) and naphthalene acetic acid (NAA) for callus induction. Maximum callus induction was observed after 40 days on a culture media containing (1 mg L^{-1}) BA and (0.2 to 0.4 mg L^{-1}) NAA. However, the highest callus growth was obtained on a medium containing 1 mg L^{-1} of BA and 1 mg L^{-1} of NAA. The highest number of shoots (7 shoots explants^{-1}) were obtained by transferring the calli to the media containing (5 mg L^{-1}) BA and (0.1 mg L^{-1}) NAA. Maximum shoot proliferation was observed by culturing the shoots on woody plant medium (WPM) supplemented with 5 mg L^{-1} BA (Bonyanpour and Khosh-Khui, 2013) [6].

The *In vitro* shoot formation and elongation of dwarf pomegranate were more (6.6 shoots culture^{-1}) on medium supplemented with fixed levels of BA (2.0 µM) and1 µMNAA than varied doses of NAA (*viz*., 0, 0.5, 2.0, 4.0, 8.0 and 16.0 µM). Fixed levels of NAA (2.0 µM) and (1 µM) BA in culture media produced more number of shoots (5.2 shoots culture^{-1}) than varied doses of BA *viz*., (0, 0.5, 2.0, 4.0, 8.0 and 16.0 µM). NAA at fixed (2.0 µM) and BA at other concentration in culture media had resulted in production of fewer shoots and at higher concentrations of BA showed more callus production (Zhang and Stoltz., 1991).

In vitro propagation of Pomegranate cv. Al-Belehi on culture media was supplemented with BA (0.0, 0.5, 2, 4 mgL^{-1}) and NAA (0.0, 0.1, 0.5 mgL^{-1}) or TDZ (0.5, 1.5, 2.5, 3.5, 4.5 mgL^{-1}) and NAA (0.0, 0.1 mgL^{-1}). The highest shoot proliferation rate was observed on auxin-free media containing 2 or 4 mg of 6-benzyladenine (SA) (3.33 and 3.4 shoots/explant, respectively). Thidiazuron (TDZ), especially at higher levels had resulted in a notable reduction in both shoot number and shoot length. The supplementation of media with optimal combination of growth regulators was 0.5 mg BA and 0.1 mg NAA (AI-Wasel, 1999) [2].The culturing of pomegranate cotyledonary segments on woody plant medium (WPM) supplemented with different concentrations of BA ranged from 8-13 µM L^{-1} in combination with 5.5 µM L^{-1} NAA were tried for callus induction. All concentrations of BA and NAA were tried in the experiment had showed the significant influence on callus growth under *in-vitro* conditions. The WPM medium containing BA (9.0 µM L^{-1}) and NAA (5.5 µM L^{-1}) was proved to be the superior medium for induction of maximum callus growth at 14 days after initiation (DAI), with maximum fresh weight (0.50 g) of green, friable callus with highest (5 mm) diameter and maximum (5 per cent) callus dry weight at 40 DAI (Sonone and Kshirsagar., 2016) [43].

Parmar *et al.* (2015) [28] stated that the high plant regeneration efficiency from cotyledon explants of pomegranate (*Punica granatum* L.) cv. Kandhari Kabuli cultured on MS medium supplemented with 2.0 mgL^{-1} 6- benzylaminopurine (BAP) and 1.5 mg L^{-1} of naphthalene acetic acid (NAA). This medium had the highest frequency of shoot regeneration (67.89%) along with maximum average number of shoot formation (5.38) and the average shoot length (3.62 cm) explant^{-1}.

In vitro rooting

In vitro clonal Propagation of Pomegranate (*Punica granatum* L.) cv. Al-Belehiwas carried out on half strength MS culture media supplemented with varied doses(0.1, 0.5, 1.0, and 2.0 mg/l) of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) andNAA to study the intiation of rooting. The highest adventitious rooting percentage was observed on half strength MS media (92%) supplemented with 0.5 mg L^{-1} of NAA (AI-Wasel, 1999) [2].

In vitro propagation of cotyledonary and nodal explants of pomegranate cv. Ganesh cultured on half and full strength MS media and white's plant media supplementedwith different concentrations of NAA (0.1, 0.2, 0.5 and 0.8 mgL^{-1}) along with activate charcoal (@200 mg/l) for rooting. *In vitro* rooting of regenerated shoot was found in explants cultured on half strength MS medium supplemented with 0.5 mg L^{-1} of NAA + 200 mg L^{-1} of activated charcoal and recorded the maximum number of roots (4.17) and root length (3.87 cm) shoot^{-1} (Singh *et al.*, 2013) [40]. Raja *et al.* (2008) [32] conducted an experiment on *in vitro* propagation of pomegranate (*P.Granatum*) explants cultured on media supplemented with different concentrations of (0.01 mg/l to 1 mg L^{-1}) IAA and IBA for *in vitro* rooting. The explants cultured on medium supplemented with 0.5mg L^{-1} of IAA had produced the healthy, highly branched, thick and long roots.Kumari *et al.* (2015) [24] and Kanwar *et al.* (2010) [18] signified that the transfer of*in vitro* grown shoots to rooting media (consists of half the strength of solid MS medium supplemented with 0.04% of activated charcoal) showed the well-developed root system at 4 weeks after culturing and resulted in 80% rooting with an average root length of 2.5 cm and an average root numer of 2–3 roots/micro shoot.

Apart from hormonal treatment, the root induction in pomegranate explants were practiced through biotechnological approaches like injection of explants with *A. rhizogenes*. The root induction was earlier (35-40 days) in explants injected with *A. rhizogenes* than treatment of explants with hormones (55-60 days). It was indicated that the root induction was 1-3 weeks earlier in explants injected with *A. rhizogenes* as compared to the induction of rooting in explants treated with harmones. The number of roots formed by the explants injected with *A. rhizogenes* were more than the double the number of roots formed by the explants treated with hormones (7). The per cent increase in number of roots in explants injected with *A. rhizogenes* was 128.5% more over hormonal treatment ofexplants in pomegranate (Patil and Borkar, 2015) [30].

Effect of photoperiod

The photoperiod is the duration of light and darkness in a 24 h cycle. Generally, plant growth and development are affected

by both internal (genotype and plant hormones) and external factors (light intensity, light duration, light quality, temperature and moisture supply). The interaction of light intensity with internal factors could directly affect plant growth. The suitable light intensity and duration could give the best results in production of the product (Soontornchainaksaeng *et al.* 2001) [44]. Kumar *et al.* (2018)[23] carried out an investigation on callus induction, shoot bud induction and shoot regeneration of leaf and shoot apex explants by subjecting to different photoperiod regimes (16:8, 14:10, 12:12 and 8:16). The explants subjected to 14:10 h photoperiod regime with a light intensity of 3000 lux and incubated at 25±2°Cwas found best in shoot bud induction, callus differentiation and de novo shoot development out of all the photoperiod regimes tested in pomegranate callus culture. Singh and Patel (2014) [39] tested the various levels of light intensity by keeping the temperature (26± 2°C) constant. The maximum number of shoots explant^{-1} and the longest shoot of explants were recorded by subjecting the explants to 16/8 h light/ dark period at 3000 lux light intensity in an incubation room.

Hardening of plantlets

The term acclimatization is defined as adaptation of an organism, especially a plant, to a new set of environmental at conditions (Conover and Poole 1984) [8]. In conventional method of acclimatization, the main effort is environmental control on plantlet. During the acclimatization stage, 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 was necessary since the strong solar light may itself directly damage the plantlets. The fluctuating light intensity with time lead to variation in temperature and relative humidity can cause an excessive loss of water from the plantlets (Singh *et al.*, 2010) [42]. *In vitro* propagation of cotyledonary nodal explants of pomegranate cv. Ganesh plantlets were acclimatized in different rooting media like, vermicompost, leaf mould soil alone and also in combination of vermicompost: soil (1:1v/v), vermicompost: leaf mould: soil (1:1:1v/v) packed in plastic pots. The explants grown in a combination ofvermicompost + soil (1:1v/v) media and kept in net house were showed better survival percentage (85.50%) up to 11.75 days (Singh *et al.*, 2013) [40].Singh and Khawale (2016) [38] conducted an experiment on Plantlet regeneration from nodal segments of pomegranate (*Punica granatum*) cv. Jyoti and were acclimatized into external environment in different *in vitro* hardening methods. The plantlets kept inglass jar with polypropylene cap filled with moistened peat: Soilrite (1:1) were found most effective and recorded the highest (86.5%) plantlet survival. The success rate of plantlets developed through tissue culture methods can effectively improve by proper hardening methods by using bioagents like arbuscular mycorrhizal fungi (Rupnawar and Navale, 2000) [33]. Many fruit crops develop a symbiotic mycorrhizal relationship and exhibited a high degree of dependence on this symbiosis for normal development and improved field performance (Aseri *et al.*, 2008) [3]. Furthermore, AMF can mitigate the effects of extreme variations in temperature and water stress by improving the uptake of water and nutrients through an increased exploration of rhizosphere area (Krishna *et al.*, 2006) [20]. Singh *et al.* (2012) [37] conducted a study on hardening of micropropagated pomegranate plantlets by using four strains of arbuscular mycorrhizal fungi (AMF) (*viz*., Glomus mosseae, Acaulospora laevis, Glomus manihotis and a mixed AMF strain) as biohardening agents to improve the survival and growth of an *in vitro* raised pomegranate plantlets. Plantlets inoculated with *G. mosseae* recorded the highest survival percentage (90.40% and 88.00% at 60 and 90 DAI, respectively) and per cent root colonization (47.40 and 87.60 at 60 and 90 DAI, respectively). The predominant effect of *G. mosseae* was evident by an increase in plant height (24.96 and 30.50 cm at 60 and 90 DAI, respectively) and root length (23.42 and 27.68 cm at 60 and 90 DAI, respectively) of the inoculated plantlets.

Conclusion

Clonal propagation using micropropagation technique is feasible in pomegranate. This technique can be used for mass multiplication of elite, robust, healthy plantlets in a short time and are available throughout the year. Different types of explants *viz.*, shoot tip, nodal cuttings, leaf segments and cotyledonary leaves *etc* had been employed in pomegranate micro propagation, of these shoot tip and nodal cuttings have been reported to be the best explants for regeneration of pomegranate plantlets. The problem of browning has been reduced by using an antioxidant (ascorbic acid) or absorbent (PVP and activated charcoal), incubation in the dark or frequent transfer to fresh media. PGR play an important role in culture establishment, multiplication and rooting of plantlets.

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