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REVIEW

# Tissue culture-mediated biotechnological intervention in pomegranate: a review

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**Abstract** The past 30 years have witnessed a series of systematic biotechnological advances made in pomegranate. These encompass optimization and establishment of in vitro culture techniques including micropropagation, somatic embryogenesis, synthetic seed production, plant regeneration via callus-mediated shoot organogenesis, adventitious shoot regeneration, anther culture, tetraploid induction and genetic transformation. This review attempts to provide a comprehensive account on the tissue culture-mediated biotechnological interventions made in pomegranate aimed at complementing conventional programmes for improvement of this nutraceutically important fruit crop.

**Keywords** Pomegranate (*Punica granatum* L.) · Micropropagation · Somatic embryogenesis · Synthetic seed · Organogenesis · Anther culture · Genetic transformation

**Abbreviations**

| | |
|---|---|
| AC | Activated charcoal |
| AS | Adenine sulfate |
| AVG | Aminoethoxyvinylglycine |
| BAP | 6-Benzylaminopurine |
| $B_5$ | Gamborg's $B_5$ (1968) medium |
| CH | Casein hydrolysate |
| CW | Coconut water |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| $GA_3$ | Gibberellic acid |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| IPA | Indole-3-propionic acid |
| Kin | Kinetin |
| MS | Murashige and Skoog (1962) medium |
| NAA | Naphthaleneacetic acid |
| PGRs | Plant growth regulators |
| PVP | Polyvinylpyrrolidone |
| WPM | Lloyd and McCown (1980) woody plant medium |
| ZR | Zeatin riboside |

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## Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known fruit trees of the tropics and sub-tropics, cultivated for its delicious edible fruits. In addition, the tree is also valued for its pharmaceutical properties. In 'Unani' system of medicine, pomegranate fruit extracts are served as a remedy for diabetes (Saxena and Vikram 2004). Fruits are also used in Ayurvedic system of medicine as an antiparasitic agent (Naqvi et al. 1991), a blood tonic (Lad and Frawley 1986) and to heal diarrhea and ulcers (Caceres et al. 1987). Recent studies show that extracts of all parts of the fruit possess therapeutic properties (Lansky and Newman 2007), while that of the bark, roots, and leaves have proven medicinal benefits too (Naqvi et al. 1991). The most therapeutically beneficial pomegranate constituents are

ellagic acid, ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones. Over the last decade, studies indicate that pomegranate is a potent antioxidant and its therapeutic properties include treatment and prevention of cancer, cardiovascular disease, diabetes, erectile dysfunction, dental conditions and protection from ultraviolet radiation. Other potential applications include infant brain ischemia, Alzheimer's disease, arthritis and obesity (Julie Jurenka 2008). Also the stem and root bark, leaves and fruit rind are a good source of secondary products, such as tannins and dyes (Anonymous 1982).

The lone genus *Punica* under family Punicaceae comprises two species i.e. *Punica granatum* L. (pomegranate) and *Punica protopunica* Balf. f. The latter one is relatively less known and it originated in the Socotra Island of the Democratic Republic of Yemen adjacent to Red Sea. On the other hand, *Punica granatum* L. is believed as native to Iran (Stover and Mercure 2007) stretching the area between Iran and the Himalayas in northern India or indigenous to Turkey (Ercisli et al. 2007). Pomegranate has been cultivated and naturalized since ancient times throughout the mediterranean region of Asia, Africa and Europe. It is grown commercially in Tunisia, Turkey, Egypt, Spain, Morocco, Iran, Afghanistan, India and to some extent in China, Japan, Russia and the United States, particularly California (Kumar 1990). However, these two species are sometimes classified in different genera because of difference in habitats (Omura 1991). During the systematic development of *Punica granatum* L., several varieties were generated from the species including *P. granatum* L. var. nana which is a dwarf variety, and more valuable for its ornamental characteristics of bearing numerous small flowers and seeds (Shao et al. 2003). A highly valuable feature of the dwarf pomegranate is that it bears fruit within a year of sowing seed or cutting (Omura 1991).

Advances in biotechnological applications, in particular tissue culture of pomegranate were first reviewed by Omura (1991). Since then, a number of in vitro studies have been carried out on pomegranate such as micropropagation through shoot proliferation by means of meristem culture, somatic embryogenesis, synthetic seed production and utilization, plant regeneration via shoot organogenesis either indirectly through callus cultures or directly by adventitious organogenesis in explants, induction of altered ploidy and genetic transformation. In this review, the contemporary information on in vitro studies in pomegranate is presented. The review also indicates how present biotechnological components, in general and tissue culture in particular, might affect the direction of future research aimed at pomegranate improvement. The attempted and possible biotechnological interventions in pomegranate through tissue culture are depicted in Fig. 1.

## Problems associated with tissue culture of pomegranate

### Browning of media

High phenolic exudation owing to activation of explant and media browning has made it hard for workers dealing with pomegranate. Mostly when mature plant parts such as

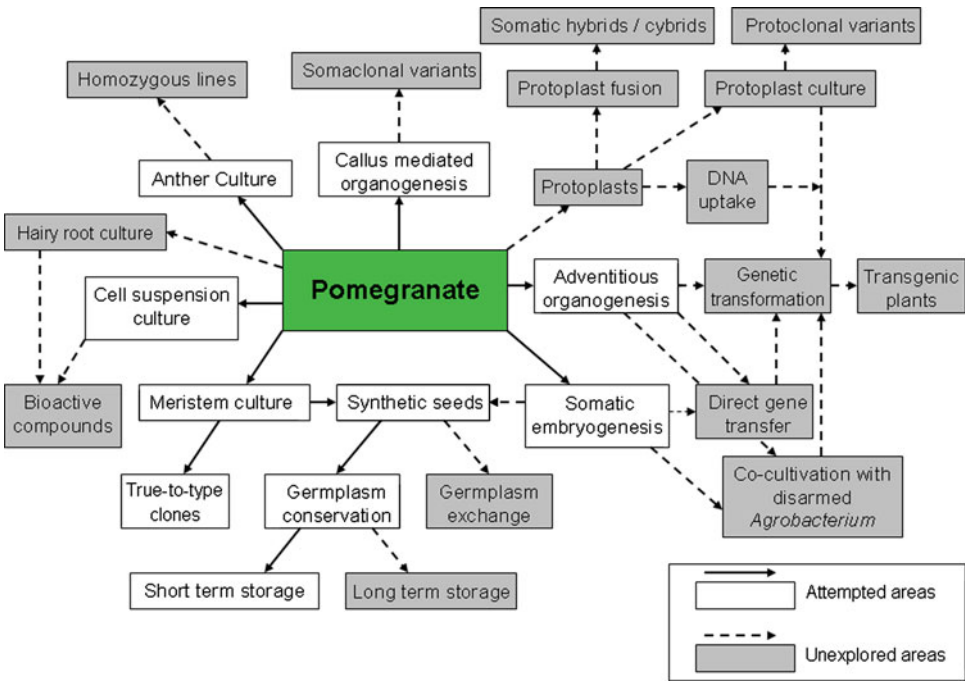


**Fig. 1** Biotechnological intervention in pomegranate through tissue culture

nodal explants, shoot tips, leaf segments are used for culture establishment of pomegranate, the browning is encountered and the elimination of browning is necessary for the establishment of cultures. The methods commonly used to overcome the harmful effect of browning in pomegranate included the use of adsorbing agents such as activated charcoal or polyvinylpyrrolidone (PVP), addition of antioxidants to the culture medium or soaking the explants in antioxidant solution, transfer of explants to fresh medium at frequent intervals or a combination of approaches with varying degree of success (Mahishi et al. 1991; Naik et al. 1999; Al-Wasel 1999; Murkute et al. 2003, 2004; Singh and Khawale 2006; El-Agamy et al. 2009).

### In vitro recalcitrance

The inability of plant cells, tissues and organs to respond to tissue culture is often referred to as 'in vitro recalcitrance' (Benson 2000). Morphogenic responsiveness of explants cultured in vitro, is largely a net positive result of interplay between the innate physiological status, precisely the endogenous levels of phytohormones of the donor tissue and the influence of the exogeneously added plant growth regulators (PGRs) in the culture medium. Reports (Moriguchi et al. 1987; Omura et al. 1987a; Naik and Chand 2003) showed that pomegranate is a difficult species, if not a recalcitrant one, at least for in vitro regeneration through organogenesis. Of the several factors responsible for in vitro recalcitrance of a plant species, production of ethylene in in vitro culture is a key factor. Efforts were made to ameliorate this problem in pomegranate using ethylene inhibitors namely silver nitrate or aminoethoxyvinylglycine (AVG) (Naik and Chand 2003; Kanwar et al. 2010).

## In vitro propagation

### Multiple shoot proliferation through meristem culture

Conventional methods of propagation of pomegranate through stem cutting is time consuming and tedious as it requires about 1 year to raise the saplings (Anonymous 1982). Micropropagation is the only aspect of plant tissue culture that has the potential to circumvent these problems in pomegranate. The composition of nutrient medium, concentration and permutation–combination of plant growth regulators and environmental ambience in culture, genotype and physiological status of the donor plant play a pivotal role in micropropagation. In addition, the success of micropropagation depends on the selection of a suitable plant part to serve as the starting experimental material. Till date, shoot tips or nodal segment from mature trees/shoot cultures or cotyledonary nodes from in vitro raised seedlings have been the appropriate choice of explants for micropropagation of pomegranate. In general, nodal segments were more responsive as compared to shoot tips.

#### *Mature nodal explants/shoot tips*

The nutritional requirement for optimal bud break and multiple shoot proliferation in axillary/apical meristems from mature trees has been reported to vary with pomegranate cultures (Table 1). Media compositions have a key role in morphogenesis; Murashige and Skoog (1962) (MS) in full salt strength was the most commonly used basal medium for pomegranate micropropagation while half-strength MS (1/2 MS), woody plant medium (WPM; Lloyd and McCown 1980) and $B_5$ (Gamborg et al. 1968) are useful in some cultivars. 6-Benzylaminopurine (BAP) has been the most commonly used plant growth regulator (PGR) either alone or in combination with a low concentration of cytokinin kinetin (Kin) or auxin (naphthaleneacetic acid, NAA). The superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones, such as zeatin within the tissue (Malik et al. 2005). Zeatin riboside (ZR) was reported to induce bud break and axillary shoot proliferation in mature nodal segments of an elite cultivar Ganesh (Fig. 2a; Naik et al. 1999). Besides, gibberellic acid ($GA_3$) and indole-3-butyric acid (IBA) in combination with BAP were also helpful in promoting shoot development from apical explants of two Egyptian cultivars; namely, Manfalouty and Nab-El-gamalusing (El-Agamy et al. 2009). Several growth adjuvants, such as casein hydrolysate (CH; Mascarenhas et al. 1981), coconut water (CW; Sharon and Sinha 2000) or adenine sulfate (AS, Singh and Khawale 2006) have also proven useful for specific cultivars. The addition of adsorbents, such as PVP (Mahishi et al. 1991) or activated charcoal (Singh and Khawale 2006; El-Agamy et al. 2009) facilitated shoot development too. In several cultivars, it was necessary to transfer the cultures to a fresh medium of a modified composition for promoting shoot elongation (Table 1).

#### *Axenic cotyledonary node explants*

An alternative explant for in vitro propagation has been the cotyledonary node, a juvenile meristematic part from axenic seedlings grown from in vitro germinated seeds, as reported in a number of fruit tree species. In addition, cotyledonary node-based plant regeneration could be an efficient strategy to obtain somaclonal variants of pomegranate possessing agronomically desirable traits (Naik

**Table 1** Clonal propagation through in vitro shoot proliferation

| Clone type/cultivar/variety | Explant | Mature/juvenile | Results/response | Medium + plant growth regulators | | Reference(s) |
|---|---|---|---|---|---|---|
| | | | | Shoot multiplication | Rooting | |
| Fruit clone cvs. 'Ganesh' and 'Muskat' | Nodal segments of mature tree | M | Multiple shoot formation, shoot elongation, rooting, plantlet formation | MS + BAP (1.0 mg/l) + Kin (0.5 mg/l) + CH (500 mg/l) ↓ Liquid MS without CH or with CH (100 mg/l) | White's medium + IAA (2.0 mg/l) + IBA (2.0 mg/l) + IPA (2.0 mg/l) | Mascarenhas et al. (1981) |
| Fruit clone (pomegranate selection; HS-4) | Shoot tips | M | Shoot formation, shoot elongation, rooting, plantlet formation | MS + BAP (8.0 mg/l) + NAA (1.0 mg/l) + PVP (600 mg/l) ↓ Modified WPM ($CaCl_2$ 440 mg/l) + IAA (6.0 mg/l) + Kin (0.6 mg/l) | MS + NAA (3.0 mg/l) + AC (3 g/l) | Mahishi et al. (1991) |
| Ornamental clone (dwarf pomegranate var. 'nana') | Nodal segments from plant grown in the green house | M | Axillary shoot proliferation | Modified MS + BAP (2.0 μM) + NAA (1.0 μM) | – | Zhang and Stoltz (1991) |
| Fruit clone cv. 'Wonderful' | Nodal cuttings from in vitro shoot cultures | J | Axillary shoot proliferation, rooting and plantlet regeneration | 1/2 MS + BAP (0.5 mg/l) + NAA (0.1 mg/l) | WPM + NAA (2.0 mg/l) | Kantharajah et al. (1998) |
| Fruit clone cv. 'Al-Belehi' | Nodal segments of one-year-old plant grown in the green house | M | Shoot proliferation, rooting and plantlet regeneration | MS + BAP (2.0 or 4.0 mg/l) | 1/2 MS + NAA (0.5 mg/l) | Al-Wasel (1999) |
| Fruit clone cv. 'Ganesh' | Nodal segments from mature tree | M | Axillary shoot proliferation, shoot elongation, rooting and plantlet formation | MS + ZR (2.0 mg/l) ↓ MS + ZR (1.0 mg/l) | 1/2 MS + IBA (1.0 mg/l) ↓ 1/2 MS | Naik et al. (1999); Naik and Chand (2001) |
| Fruit clone cv. 'Ganesh' | Cotyledonary nodes | J | Multiple shoot regeneration, rooting and plantlet production | MS + BAP (9.0 μM) | 1/2 MS + NAA (0.54 μM) ↓ 1/2 MS | Naik et al. (2000) |
| Fruit clone cv. 'Ganesh' and 'Kabul' | Cotyledonary nodes | J | Multiple shoot initiation, shoot elongation, rooting and plantlet formation | $B_5$ + BAP(1.0 mg/l) + Kin (1.0 mg/l) + NAA (0.05 mg/l) (for cv. 'Ganesh') and $B_5$ + BAP (0.5 mg/l) + NAA (0.05 mg/l) (for cv. 'Kabul') ↓ $B_5$ + NAA (0.05 mg/l) + BAP (0.5 mg/l) + CW (10%) | $B_5$ + IAA (3.0 mg/l) | Sharon and Sinha (2000) |
| Fruit clone cv. 'Ganesh' | Shoot tip and nodal segments | M | Multiple shoot, rooting and plantlet formation | MS + BAP (1.0 mg/l) + NAA (0.5 mg/l) | 1/2 MS + NAA or IAA (0.5 mg/l) | Murkute et al. (2004) |

**Table 1** continued

| Clone type/cultivar/variety | Explant | Mature/juvenile | Results/response | Medium + plant growth regulators | | Reference(s) |
|---|---|---|---|---|---|---|
| | | | | Shoot multiplication | Rooting | |
| Fruit clone cv. 'Jyoti' | Nodal segments of mature tree | M | Axillary shoot proliferation, rooting and plantlet formation | 1/2 MS + BAP (1.0 mg/l) + Kin (1.0 mg/l) + AC (200 mg/l) ↓ 1/2 MS + BAP (1.0 mg/l) + Kin (1.0 mg/l) + AS (40 mg/l) | MS + IBA (2.0 mg/l) + AC (200 mg/l) | Singh and Khawale (2006) |
| Fruit clone cvs. 'Manfalouty' and Nab-El-gamal | Shoot tip from mature tree | M | Shoot proliferation, rooting and plantlet formation | WPM + $GA_3$ (5.0 mg/l) + BAP (0.1 mg/l) + IBA (0.02 mg/l) + AC (3.0 g/l) ↓ WPM + BA (1.0 mg/l) | 1/2 WPM + IBA (0.25 mg/l) (for Manfalouty) 1/2 WPM + NAA (0.25 mg/l) (for Nab-El-gamal) | El-Agamy et al. (2009) |

AC activated charcoal, AS adenine sulfate, BAP 6-benzylaminopurine, $B_5$ Gamborg's $B_5$ (1968) medium, CH casein hydrolysate, CW coconut water, $GA_3$ gibberellic acid, IAA indole-3-acetic acid, IBA indole-3- butyric acid, IPA indole-3-propionic acid, Kin kinetin, MS Murashige and Skoog (1962) medium, NAA naphthaleneacetic acid, PVP polyvinylpyrrolidone, WPM Lloyd and McCown (1980) woody plant medium, ZR zeatin riboside, – data not available

et al. 2000). Naik et al. (2000) were the first to report a high-frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate cv. Ganesh using MS with a single cytokinin supplement (BAP) (Fig. 2b). Subsequently in the same year $B_5$ medium containing a combination of two cytokinins (BAP and/or Kin) along with an auxin supplement (NAA) was found to be effective for cv. Ganesh and cv. Kabul (Sharon and Sinha 2000).

## Somatic embryogenesis

Somatic embryogenesis has enormous potential for clonal multiplication, synthetic seed production and genetic transformation. To date, both direct and indirect somatic embryogenesis and plant regeneration of pomegranate has been reported from five different laboratories using different explants (Table 2). Zygotic embryos are composed of embryogenically competent cells (pre-embryogenic determined cells, PEDCs) which hold the potential to be induced to follow the embryogenic programme (Sharp et al. 1980). In pomegranate, immature or mature zygotic embryos from unripened or ripe fruits, respectively, have been utilized for the induction of somatic embryogenesis (Jaidka and Mehra 1986; Raj Bhansali 1990; Kanwar et al. 2010). Besides, hypocotyl, cotyledon, stem and leaf segment of 20–25-day seedlings as well as root segments or whole petals have also been used (Jaidka and Mehra 1986; Nataraja and Neelambika 1996; Sinha and Sharon 1997). MS has been the commonly used basal media barring one report in which $B_5$ was useful (Table 2). Several auxins (indole-3-acetic acid [IAA], IBA, NAA, 2,4-dichlorophenoxyacetic acid [2,4-D]) and cytokinins (Kin, BAP) either used singly or in combinations proved promotive for different clones. It is noteworthy that 2,4-D, which is usually effective for induction of somatic embryo in several other plant species (Rai et al. 2010) is not the best choice for pomegranate. A 6-week dark incubation period was essential for embryogenic callus induction (Kanwar et al. 2010) similar to that reportedly required for zygotic embryos from unripened fruits (Raj Bhansali 1990). The addition of higher level of sucrose (6%) on MS medium was essential for the germination of somatic embryos as compared to that (4%) required for embryoids from petal-derived callus (Nataraja and Neelambika 1996). In another case, the removal of 2, 4-D and inclusion of activated charcoal (0.3%) in the culture media was essential for the germination of mature somatic embryos to complete plantlets (Raj Bhansali 1990).

Interestingly, a low proportion (15%) of immature embryos showed precocious germination as reported by Raj Bhansali (1990) in a fruit clone pomegranate. Thus, a critical analysis of factors influencing plant retrieval from somatic embryos is necessary (Jimenez 2005). In pomegranate lowering of the basal medium strength and/or elevation in

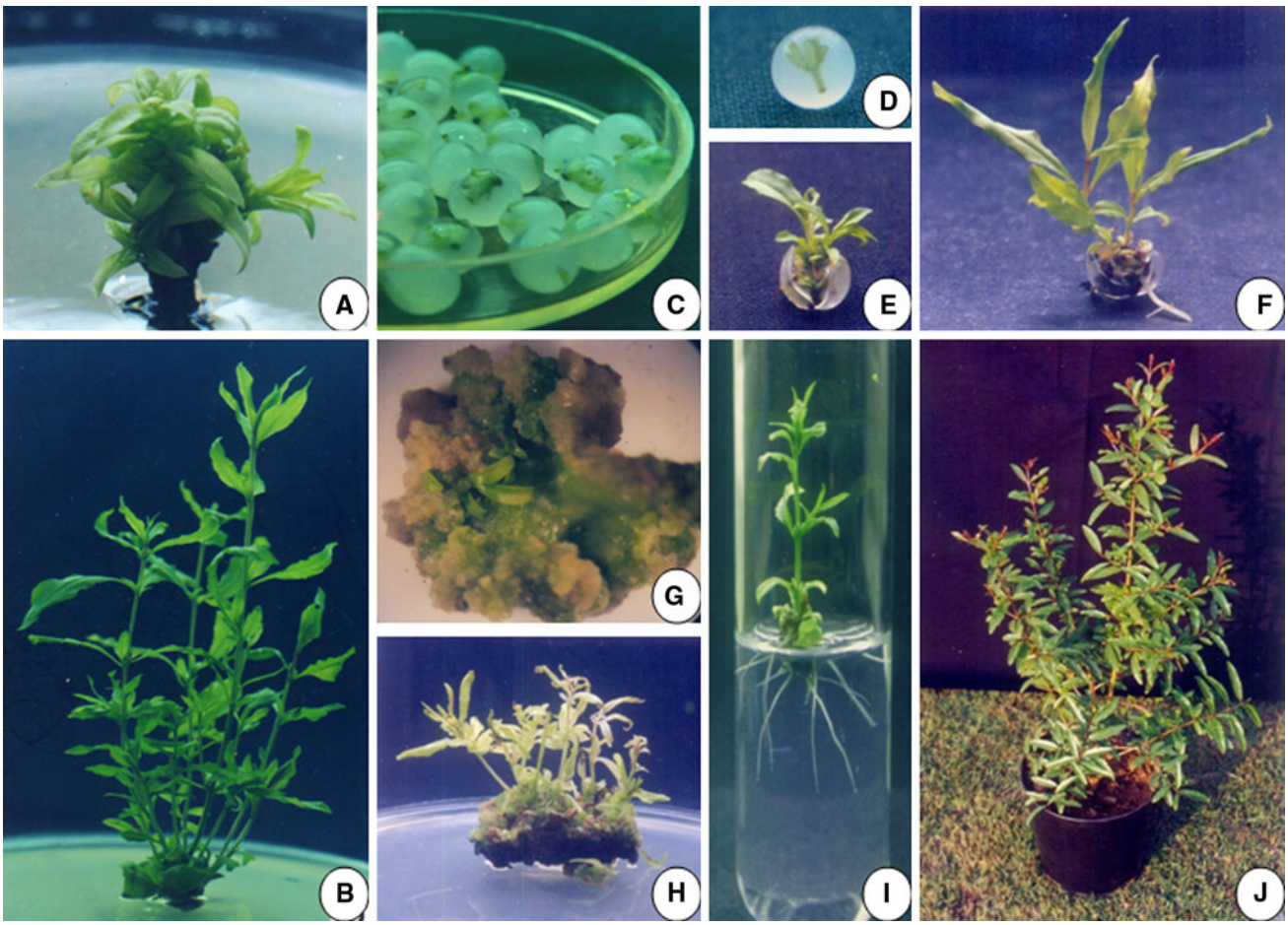


**Fig. 2** **a** Development of multiple shoots from a nodal explant in MS + 2.0 mg/l ZR after 25 days of culture, **b** prolific shoot development from a cotyledonary node after 30 days of culture in MS + BA (9.0 μM), **c** a group of freshly prepared synthetic seeds, **d** single synthetic seed, **e** sprouting of an encapsulated nodal segment, **f** germination of an encapsulated nodal segment (source **b**) in MS, **g** a callus undergoing shoot organogenesis, **h** adventitious shoot regeneration from a cotyledon explant in the regeneration medium following transfer from an $AgNO_3$ (20 μM) supplemented medium, **i** a well-rooted shoot in 1/2 MS, j a 4-month-old plant established in garden soil

sucrose strength were essential for germination of somatic embryos. Invariably in these reports information on percent embryo-to-plant conversion was either lacking (Jaidka and Mehra 1986; Nataraja and Neelambika 1996) or the germination frequency of somatic embryo was very low, i.e. only 4.45% (Kanwar et al. 2010). One of the major limitations to profitably exploiting somatic embryogenesis in pomegranates has been the poor germination rate; the commercial application of somatic embryogenesis will be successful only when the germination rate of somatic embryos is high up to 80–85% (Rout et al. 2006). Further studies are, therefore, needed in pomegranate to improve the maturation and germination of somatic embryos for plant conversion.

## Synthetic seed production

Since the very inception of the concept of synthetic seed technology, somatic embryos are considered as the choiced propagules for preparation of synthetic seeds. However, improper maturation and/or germination of somatic embryos have been one of the major bottleneck for the utilization of synthetic seeds. This has underpinned a new perspective in synthetic seed technology: the use of non-embryogenic unipolar meristematic plant propagules, such as shoot apices, axillary buds or nodes. Considering the inadequate information or inordinately low embryo-to-plant conversion rate in pomegranate, Naik and Chand (2006) were prompted to use in vitro nodal segment for production of synthetic seeds of pomegranate. They prepared synthetic seeds by encapsulating nodal segments from in vitro shoot cultures derived from mature nodal explants (source A) or axenic cotyledonary nodes (source B) in calcium alginate hydrogel containing MS medium supplemented with 4.4 μM BAP and 0.54 μM NAA. A combination of 3% sodium alginate and 100 mM calcium chloride was most suitable for formation of ideal synthetic

**Table 2** Somatic embryogenesis and embryo-to-plant conversion

| Clone type/ cultivar/variety | Explant | Results/response | Medium + plant growth regulators | | | | References |
|---|---|---|---|---|---|---|---|
| | | | Callus | Induction of somatic embryo | Maturation of somatic embryos | Germination of somatic embryos | |
| Fruit clone cv. 'kandhari anar' | Parts of 20-25 day-old axenic seedlings and young embryos | Callus, somatic embryo and plantlets | MS + sucrose (2.0%) + NAA (4.0 mg/l) + Kin (2.0 mg/l) + CW (15%) | MS + sucrose (2.0%) + NAA (2.0 mg/ l) + BAP (2.0 mg/l) | – | – | Jaidka and Mehra (1986) |
| Fruit clone | Segment of immature zygotic embryos along with cotyledon from unripened fruit | Callus, somatic embryogenesis and plantlet regeneration | RBM-I media (1/2 MS + 30% sucrose + 500 mg/l L-glutamine + 500 mg/l CH + 500 mg/l inositol) + Kin (1.0 mg/l) + BAP (2.0 mg/l) + 2,4-D (5.0 mg/l) | RBM-I media + Kin (2.0 mg/ l) + BAP (2.0 mg/l) + 2,4-D (2.5 mg/l) | RBM-I media + Kin (2.0 mg/ l) + BAP (2.0 mg/l) + 2,4-D (0.5 mg/l) | 1/2 MS + sucrose (30%) ↓ 1/2 MS + sucrose (30%) + AC (0.3%) | Raj Bhansali (1990) |
| Fruit clone cv. 'Jyoti' | Whole petal | Callus, somatic embryo and plantlets | MS + sucrose (2.0%) + IAA (1.0 mg/l) or IBA (1.0 mg/l) | | | 1/2 MS + sucrose (4%) + IAA (1.0 mg/l) or IBA (1.0 mg/l) + BAP (1.0 mg/l) | Nataraja and Neelambika (1996) |
| Fruit clone cv. 'Ganesh' | Root segments | Callus, somatic embryo and plantlet | $B_5$ + 2,4-D (–) | $B_5$ + BAP (0.5 mg/l) | – | $B_5$ + NAA (0.01 mg/ l) + BAP (0.5 mg/ l) + Kin (2.0 mg/l) | Sinha and Sharon (1997) |
| | | Direct somatic embryogenesis and plantlet | | $B_5$ + Kin (2.0 mg/l) | – | | |
| Fruit clone (Elite wild pomegranate) | Zygotic embryo from ripened fruit | Embryogenic callus, somatic embryo, maturation and germination of somatic embryo | MS + sucrose (3%) + NAA (21 μM) + BAP (9.0 μM) + CW (15%) | | MS + sucrose (6%) | | Kanwar et al. (2010) |

AC activated charcoal, BAP 6-benzylaminopurine, $B_5$ Gamborg's (1968) medium, CH casein hydrolysate, CW coconut water, 2,4-D 2,4-dichlorophenoxyacetic acid, IAA indole-3-acetic acid, IBA indole-3-butyric acid, Kin kinetin, MS Murashige and Skoog (1962) medium, NAA naphthaleneacetic acid, –data not available

seeds (Fig. 2c, d). Sprouting (shoot development) of encapsulated nodal segments of both the sources was observed, but one step germination, i.e. both shoot and root formation was achieved, only with encapsulated nodal segments of source B (Fig. 2e, f). The encapsulated nodal segments could be stored up to 30 days at 4°C without marked loss of sprouting ability. Since a desirable feature of synthetic seeds is their ability to retain viability in terms of sprouting/germination even after a period of storage, the synthetic seed system developed by Naik and Chand (2006) could facilitate transfer of sterile and elite materials in the form of synthetic seeds to extension centers and laboratories of distance places while maintaining their viability. Nevertheless, keeping in view of the innate variability among seedling-derived nodal explants, future attention on improvement in preservation strategies for synthetic seeds should be focused more on those derived from nodal explants from in vitro shoot cultures of mature trees.

## Adventitious organogenesis

### *Organogenesis in de-differentiated culture*

De-differentiation is a fundamental prerequisite that triggers cells to become competent so as to be enabled to respond to new physiological and environmental stimuli. The most commonly used inductive signals for onset of de-differentiation are PGRs, especially auxins and cytokinins. Two most common de-differentiated culture modes which hold the potential for biotechnological exploitation are derived from well-differentiated plant organs via in vitro cultures either in form of callus grown in an usually agar-solidified medium or callus-derived cell suspension cultures using an agitated liquid medium.

*Callus-mediated shoot organogenesis*. Establishment of callus cultures and obtention of plants regenerated from calli via shoot organogenesis hold a potential for the production of useful somaclonal variants. Somaclonal variations offer promises to result in alterations in a wider range of plant characteristics of horticultural significance, including plant height, flowering time, fruit yield, tolerance to abiotic or biotic stress, etc. (Jain 2001). Callus culture and subsequent shoot bud regeneration in pomegranate using different explants has been reported from different laboratories (Table 3). As per literature in pomegranate, callus-mediated plant regeneration is the second most documented method next to axillary shoot proliferation. MS was the most preferred medium for callus culture. The best combination of PGRs for establishment of callus culture was found to be NAA and BAP followed by NAA and Kin at different concentration. However, for inducing shoot regeneration from callus culture the combination of NAA and BAP or BAP alone was essential (Fig. 2g; Naik and Chand unpublished). Inclusion of $GA_3$ and $AgNO_3$ in addition to BAP-NAA combination was helpful in achieving the highest number of shoots (8.23) per callus clump and longest shoot average (1.93 cm) in an elite wild fruit clone (Kanwar et al. 2010). Differentiation of isolated leaves was a common phenomenon in callus culture and interestingly occasional differentiation of a floral shoot with a greenish white flower at the top of the callus has been reported (Jaidka and Mehra 1986). Omura et al. (1987b) made a series of systematic investigations to standardize various factors influencing adventitious shoot bud formation from callus cultures of dwarf pomegranate (*Punica granatum* L. var. nana). These factors encompassed explant age and size, basal medium, plant growth regulators, pH, culture vessels and illumination.

*Cell suspension-mediated shoot organogenesis*: Cell suspension cultures offer advantages over stationary cultures in being more effective for isolation of mutant cell lines, somaclonal variants and for production of important phyto-compounds of commercial relevance. The singular report on plant regeneration from cell suspension culture of pomegranate was that by Omura et al. (1990). Cell suspension culture of dwarf pomegranate var. nana Pers. was initiated and established from nodular and compact green calli derived from leaf segments on MS media supplemented with 5 μM BAP and 1 μM NAA. The development of cells into colonies was maximum on MS medium supplemented with a combination of 2.0 μM BAP and 1.0 μM NAA. Adventitious shoot buds were developed from calli derived from suspension cells on 1/2 MS supplemented with 2.0 μM BAP. Cell suspension plated at initiation stage produced cell colonies, 64% of which differentiated into shoot buds. However, there was a retrogression in shoot bud formation in cell colonies obtained from suspension cultures experiencing advanced age growth (3–12 months); shoot bud development was drastically reduced to 0.8% in calli derived from 1 year-old suspensions. This was clearly indicative of a gradual age-dependent loss of totipotent ability of cells in long-term suspension cultures.

### *Direct adventitious shoot organogenesis in explants*

Plant regeneration via adventitious shoot organogenesis occurring directly in non-meristematic explants, without intervening callus, is preferred to that through de-differentiated cultures. Plants obtained via callus or cell suspension cultures sometimes contain polyploid or mixoploid cells that are genetically unstable, whereas those adventitiously generated directly from explants (mature/seedling source) are genetically stable (Krul and Myerson 1980). Development of an efficient method to regenerate plants adventitiously in a reasonably high frequency directly from explants is a desirable requirement for high-throughput

**Table 3** Callus-mediated shoot organogenesis and plant regeneration

| Clone type/cultivar/variety | Explant | Mature/juvenile | Results/response | Medium + plant growth regulators | | | References |
|---|---|---|---|---|---|---|---|
| | | | | Callus induction/proliferation | Shoot regeneration | Rooting | |
| Fruit clone cv. 'Kandhari anar' | Cotyledon, stem, Hypocotyl, shoot tip and leaf segments of 20-25 day-old axenic seedlings and zygotic embryos from seeds | J | Callus, shoot bud differentiation and plantlet formation | MS + NAA (4.0 mg/l) + Kin (2.0 mg/l) + CW (15%) | MS + NAA (2.0 mg/l) + BAP (2.0 mg/l) | – | Jaidka and Mehra (1986) |
| Ornamental clone (dwarf pomegranate cv. 'Issaizakuro') and fruit clone (normal endemic) | Leaf segments | M | Callus, shoot regeneration, rooting and plantlet formation | MS[a] + BAP (5.0 μM) + NAA (0.5 μM) MS[a] + BAP (1.0 μM) + NAA (0.5 μM) | 1/2 MS + BA (2.0 μM) 1/2 MS + BAP (2.0 μM) | 1/2 MS + NAA (0.1 μM) | Omura et al. (1987a) |
| Ornamental clone (dwarf pomegranate var. 'nana') | Segments of leaf and stem internode | M | Callus, shoot regeneration, rooting and plantlet regeneration | MS + BAP (10.0 μM) + NAA (1.0 μM) | 1/2 MS + BAP (2.0 μM) | 1/2 MS + NAA (0.01 or 0.1 μM) | Omura et al. (1987b) |
| Ornamental clone (dwarf pomegranate var. 'nana') | In vitro regenerated leaf and stem segments | J | Callus, shoot formation, rooting and plantlet formation | MS + BAP (0.5 mg/l) + NAA (0.5 mg/l) (for leaf) and MS + BAP(0.5 mg/l) + NAA (1.0 mg/l) (for stem explant) | MS + BAP (0.5 mg/l) + NAA (0.5 mg/l) (for leaf) and MS + BAP (0.5 mg/l) + NAA (1.0 mg/l) (for stem explant) | 1/2 MS | Yang and Ludders (1993) |
| Fruit clone cv. Ganesh | Cotyledon and leaf | J | Callus, shoot regeneration, rooting and plantlet formation | MS + NAA (4.0 mg/l) + Kin (2.0 mg/l) + CW (15%) | MS + NAA (2.0 mg/l) + BAP (2.0 mg/l) | – | Foughat et al. (1997) |
| Fruit clone cv. 'Wonderful' | Axenic leaf segments | J | Callus, shoot regeneration, rooting and plantlet formation | MS + BAP (1.0 mg/l) | MS + BAP (1.0 mg/l) | WPM + NAA (2.0 mg/l) | Kantharajah et al. (1998) |
| Fruit clone | Axenic seedling-derived cotyledon and hypocotyl segments | J | Callus, adventitious shoots, rooting and plantlet regeneration | 1/2 MS + BA (1.0 mg/l) + NAA (0.1 mg/l) | 1/2 MS + BAP (1.0 mg/l) + NAA (0.1 mg/l) | 1/2 MS + NAA (0.5 mg/l) | Amin et al. (1999) |
| Fruit clone cv. 'Ganesh' | Cotyledon segments | J | Callus, shoot bud differentiation, rooting and plantlet regeneration | MS + BA (1.0 mg/l) + NAA (0.5 mg/l) | MS + BAP (1.0 mg/l) + NAA (0.5 mg/l) | 1/2 MS + IBA (1.0 mg/l) | Murkute et al. (2002) |

**Table 3** continued

| Clone type/cultivar/variety | Explant | Mature/juvenile | Results/response | Medium + plant growth regulators | | | References |
|---|---|---|---|---|---|---|---|
| | | | | Callus induction/proliferation | Shoot regeneration | Rooting | |
| Fruit clone (Elite wild pomegranate) | Cotyledon segments | J | Callus, shoot regeneration, rooting and plantlet formation | MS + NAA (21.0 μM) + BA (9.0 μM) | MS + BAP (8.0 μM) + NAA (6.0 μM) + $GA_3$ (6.0 μM) + $AgNO_3$ (24 μM) | 1/2 MS + AC (0.02%) | Kanwar et al. (2010) |
| Fruit clone cv. 'Kandhari Kabuli' | Cotyledon segments | J | Callus, shoot regeneration, rooting and plantlets | MS + NAA (13.0 μM) + BA (13.5 μM) | MS + BAP (9.0 μM) + NAA (2.5 μM) | 1/2 MS + AC (500 mg/l) | Deepika and Kanwar (2010) |

AC activated charcoal, BAP 6-benzylaminopurine, CW coconut water, $GA_3$ gibberellic acid, IAA indole-3-acetic acid, IBA indole-3- butyric acid, IPA indole-3-propionic acid, Kin kinetin, MS Murashige and Skoog (1962) medium, MS[a] Modified MS (devoid of nicotinic acid, pyridoxine–HCl and glycine), NAA naphthaleneacetic acid, WPM Lloyd and McCown (1980) woody plant medium, – data not available

genetic transformation (Naik and Chand 2003). To date, direct shoot organogenesis in pomegranate has been rarely reported. Jaidka and Mehra (1986) were the first to report direct regeneration (without intervention of callus) of shoots and whole plants from cotyledon, leaf and stem explants of pomegranate cv. Kandhari anar. The regeneration of shoots and whole plants was achieved on MS or Nitsch's (1969) medium supplemented with 2.0 mg/l NAA and 2.0 mg/l BAP. Later a reproducible protocol was developed by Naik and Chand (2003) for adventitious shoot organogenesis followed by complete plant regeneration from the hypocotyl and cotyledon explants of pomegranate (cv. Ganesh).

Of several factors responsible for in vitro recalcitrance of a plant species, production of ethylene in in vitro culture is a key factor. Naik and Chand (2003) investigated the role of two ethylene inhibitors $AgNO_3$ and AVG in enhancing the regenerative potential of the explants. They showed that the addition of ethylene inhibitors, such as $AgNO_3$ (20 μM) or AVG (10 μM) to the 'regeneration medium' (MS + 8.9 μM + 5.4 μM NAA + 10% CW) markedly enhanced the regeneration, frequency as well as number of shoots obtained per cotyledon explant. The stimulatory effect of ethylene inhibitors were tissue specific or explant dependent, as of the two types of explant used the promotive effect was observed only in case of cotyledons (Fig. 2h). In addition, the use of a ethylene releasing compound 2-chloroethylphosphonic acid by Naik and Chand (2003) further support the hypothesis that ethylene is a key factor in the down-regulation of cell differentiation and indicates that enhanced shoot regeneration from cotyledons of pomegranate is closely associated with reduced ethylene synthesis and/or action.

## Rooting of in vitro regenerated shoots

Rooting of shoots derived from in vitro cultures is a prerequisite to make possible their physiological establishment in soil. NAA or IBA has been the most commonly used auxin for promoting rooting of in vitro regenerated shoots of pomegranate. In pomegranate, basal media formulations, such as MS, WPM, White's and $B_5$ in full or half strength have been attempted with varying success (Tables 1, 3). In majority of reports, 1/2 MS has been most effective. A combination of three different auxins; namely, IAA, IBA and IPA as a supplement to White's medium was useful for root induction in the fruit clones cv. Ganesh and Muskat (Mascarenhas et al. 1981). Since then, NAA or IBA have been the most commonly used auxin for promotion of rooting of in vitro shoots. In cv. Ganesh, there was requirement for transfer of rooted shoots to an auxin free 1/2 MS medium in which the primary roots elongated within 5–7 days during which a few more roots were also

developed (Fig. 2i; Naik and Chand 2003). In dwarf pomegranate var. nana, rooting was reportedly achieved without any auxin (Yang and Ludders 1993). Activated charcoal has also played a promotive role in rhizogenesis with (Mahishi et al. 1991; Singh and Khawale 2006) or without (Kanwar et al. 2010; Deepika and Kanwar 2010) a growth regulator supplement.

### Acclimatization and soil establishment

The key to final success of tissue culture is the ability of regenerated plants to be transferred from the culture medium to the soil and acclimatized to the free-living autotrophic conditions at low cost along with negligible mortality. Successful acclimatization of the in vitro-regenerated plantlets has been reported in pomegranate using different potting substrates with varying degree of success. Vermiculite was used by Omura et al. (1987a) for acclimatization of normal endemic and dwarf pomegranate plantlets in the green house. Sharon and Sinha (2000) reported the establishment of plantlets (cv. Ganesh and Kabul) in the pots filled with either vermiculite or a 1:1 mixture of vermiculite and soil in the net house. They found 100% survivability of plantlets in both the potting medium when 80% RH, 14–18 klux light intensity and temperature below 30°C were constantly maintained inside the net house. A sterile mixture of peat: soilrite (1:1) moistened with 1/4 MS macro and micro-salts without organics facilitated a maximum survival (78.7%) of plantlets (Singh and Khawale 2006). The survival of plantlets was 94.12% in a potting mix of sterilized loamy soil and sand (1:1) of which 67.85% survived upon subsequent transfer to loamy soil (Kanwar et al. 2010). Vermicompost was used by Naik et al. (1999, 2000) and Naik and Chand (2003) for acclimatization of plants (cv. Ganesh) before establishment in soil (Fig. 2j). 60–68% of plantlets survived following transfer to vermi-compost and 75–84% of these plants transferred to soil survived. There was 80% success in establishment of the plantlets in perlite under mist in green house (Al-Wasel 1999). Provision of misting (overhead sprinkler irrigation; 2 s duration 5 min intervals) inside a greenhouse was helpful in achieving 100% survival of in vitro-raised plants of var. nana (Yang and Ludders 1993).

## In vitro induction of altered ploidy

### Anther culture towards production of haploids

In vitro androgenesis via anther culture or microspore culture is an efficient technique for generating haploids that offer advantages, such as shortening of breeding period and production of homozygous diploid lines through chromosome doubling. In pomegranate, only two preliminary reports are available on anther culture; unfortunately, neither of them could demonstrate the production of haploid plants. Moriguchi et al. (1987) were the first to report anther culture in pomegranate (dwarf pomegranate cv. Issaizakuro). They cultured anthers having microspores at the uninucleate to binucleate stage in the dark at 28°C. Of the three different basal media tested, MS and Miller (1965) were superior to Nitsch and Nitsch (1969). About 20% of the anthers cultured in dark at 28°C remained alive (yellow white) after 30 days of culture and produced calli on MS or Miller (1965) media each supplemented with 5.0 μM BAP and NAA. Shoot bud differentiation was induced in these calli within 3 weeks of culture on 1/2 MS medium containing 0.5 μM NAA and 2.0 μM BAP. Unfortunately, all the regenerated shoots had a diploid chromosome number of 18. Histological studies confirmed that the calli originated from somatic tissues of the anther wall (tapetal cells) instead of microspores resulting from microsporogenesis. Mascarenhas et al. (1988) attempted to obtain haploid plantlet via anther culture of cultivars Ganesh and Muskat. Green, compact nodular callus with embryoid-like structures were formed on Nitsch (1969) medium containing Kin (0.5 mg/l), IAA (0.5 mg/l) and BAP (0.2 mg/l). Only roots were regenerated on Nitsch medium supplemented with 0.5–1.0 mg/l IAA, whereas leaves developed on Nitsch medium supplemented with BAP (0.1–4.3 mg/l). Till date, there is no report from that laboratory on complete haploid plant production from embryoids.

### In vitro production of tetraploids

Polyploidization, including chromosome doubling has been used as a breeding tool in horticulture for obtaining new ornamental characteristics such as larger and more heavily textured flowers, a longer or delayed flowering period, a more compact growth habit, etc. Pomegranate is a diploid species possessing $2n = 2x = 16(18)$ chromosomes (Smith 1976). Shao et al. (2003) reported the production of tetraploid plants in a dwarf pomegranate var. nana by colchicine treatment of in vitro-raised shoots. Shoots cultured on MS medium supplemented with 10 mg/l colchicine, 1.0 mg/l BAP and 0.1 mg/l NAA for 30 days produced stable tetraploids at a high frequency (20%). In vitro-generated tetraploid plants were with shorter roots, wider and shorter leaves than diploid ones. The level of ploidy and its stability was confirmed by flow cytometry. The tetraploid plants flowered normally after acclimatization, but possessed with new ornamental characteristics, such as darker leaves and larger flowers with increased diameter and decreased length as compared to diploids.

Besides, tetraploid plants produced more pollen grains per anther, but exhibited lower pollen viability than the diploids. Being semi-fertile, such tetraploid pomegranate plants will be valuable breeding parents for crossing with diploids aiming at producing seedless triploids (Shao et al. 2003). In the contingent event of failure following interploid hybridization excised triploid embryos can be rescued via in vitro culture and triploid pomegranates can be raised. Endosperm culture could be another worth-attempting strategy for producing triploid plants of pomegranates. Triploids can be multiplied either by conventional method of cuttings or by micropropagation. Seedless fruit setting in the triploid pomegranates by stimulative parthenocarpy will require the pollination stimulus following pollination with viable and compatible pollen from a diploid source. Alternatively, parthenocarpic fruit set can also be induced in triploids by exposure to low temperatures or treatment with plant growth regulators, such as auxins, gibberellic acid or cytokinins ([2-chloro-4-pyridyl]-*N*-phenyl urea; CPPU) which has been successful for several fruit crops, including watermelon (Maroto et al. 2005).

## In vitro induction of mutants

Omura et al. (1987b) were the first to have examined the effect of gamma-ray irradiation to induce mutants from leaf segments of dwarf pomegranate var. nana. From the second day after culture, the segments were irradiated with gamma rays 250 R/h to a total of 4–64 kR. Exposure to 64 kR severely inhibited callus and bud formation and reduced the number of shoots appearing on each segment to 2.2. In the green house, the variations in leaf shape and growth habit were observed in the plants regenerated from irradiated leaf segments. The frequency of induction of variants was high for leaf shape. There were variations in leaf shape between narrow, slender, thready (thread like), round, lanceolate, curly and wrinkled; the slender and curly leaf types occurred more frequently. Variations in growth habit consisted of more dwarfed, upright plants with larger leaves. They also reported a high frequency of pollen sterility; complete sterility being observed in 19.7% of the plants.

## Genetic transformation

To date, there is a single report on successful genetic transformation of pomegranate (dwarf ornamental var. nana) by Terakami et al. (2007) using *Agrobacterium tumefaciens*. Two different bacterial strains were employed for transformation, LBA 4404 and EHA 105, both harboring the binary factor pBin19-*sgfp* which carries the selectable marker neomycin phosphotransferase (*npt*II) gene driven by the *nos* promoter and the synthetic reporter green fluorescent protein (*gfp*) gene driven by CaMV 35S promoter. Transient GFP activity (up to 39%) was detected in adventitious shoots regenerated from leaf segments and co-cultivated with EHA 105 in particular. The transformed (kanamycin resistant) plant regenerants revealed GFP fluorescence in all organs (stem, leaf and mature fruits), but were phenotypically normal as the donor. Molecular analysis by PCR and PCR-Southern blotting demonstrated the integration of the transgene into the transformed plant genome. The transgene stability in the germ line was substantiated by GFP assay of T1 seedlings which followed the typical Mendelian monohybrid segregation ratio of 3:1.

## Conclusion and future prospects

Till date good progress has been made regarding the development of rapid, reproducible and reliable in vitro plant regeneration systems for pomegranate. The synthetic seed technology involving nutrient-alginate encapsulation developed for pomegranate could be useful in germplasm distribution and exchange. At the same time, efforts should be made to develop an optimized cryopreservation system which could be useful for long-term storage of elite pomegranate germplasm. Establishment of callus cultures and obtention of plants regenerated from calli or cell suspensions via shoot organogenesis hold a potential for the production of novel somaclonal variants. Somaclonal variations offer promises to result in alterations in a wider range of plant characteristics of horticultural significance. In consideration of the proven therapeutic value of pomegranate, its cell suspension cultures and *Agrobacterium rhizogenes*-transformed hairy root cultures could be scaled-up using bioreactors so as to serve as a fast growing, renewable source of bioactive compounds including ellagic acid, ellagitannins (punicalagins), punnicic acid, flavonoids, anthocyanidins, anthocyanins and estrogenic flavonols and flavons of pharamaceutical relevance.

Efforts are to be intensified towards obtaining androgenic haploids in pomegranate via in vitro culture to be followed by diploidization aiming at production of isozygous purelines for breeding purposes. In addition to anther culture, attention may also be addressed to in vitro androgenesis in isolated microspores, the latter being a homogenous haploid population whose further development in culture is independent of that of the sporophytic tissues. It is imperative to optimize factors influencing culture competence, such as the genotype, physiological condition of the donor plant, developmental stage of anther/microspores at culture, composition of the nutrient medium, especially the carbon source, PGRs and their

concentrations in addition to in vitro environmental components, particularly abiotic stress pretreatments. Further success in this direction may be achieved by combining signal transduction and functional genomics approach. In addition, microspore culture under optimal conditions would serve as an useful tool for investigating the biochemical and molecular events underlying gametophytic-to-sporophytic transition in pollens, genetic transformation, in vitro selection for gametoclonal variants/mutants and gameto-somatic hybridization towards production of triploids.

Protoplast-to-tree protocol is to be developed for pomegranates in order that novel protoclonal variants of breeder's choice can be generated. Asymmetric somatic hybrids can be produced following intergeneric protoplast fusion, thus making it possible for introgression into pomegranate of the desirable gene(s) from allied fruit tree species. On the other hand, a regenerable protoplast culture system can facilitate further genetic manipulations, particularly by exploiting freshly isolated protoplasts as recipients of heterologous DNA encoding target agro-horticultural traits of significance via electroporation/PEG-induced membrane permeabilization.

*Agrobacterium*-mediated genetic transformation system by explant co-cultivation could be a rapid alternative method with minimum destruction of genetic integrity of the elite commercial cultivars. Considering the remarkable development of efficient in vitro plant regeneration system via adventitious shoot formation directly from explants (Naik and Chand 2003), efforts are needed to optimize efficient transformation system for fruit clone pomegranates. The development of an efficient transformation protocol either involving disarmed-engineered *Agrobacterium* as a vector or a suitable direct vectorless method, such as particle gun bombardment of selected propagules or somatic embryos could be useful in genetic improvement of this important fruit tree through introgression of useful candidate genes especially those which confer abiotic or biotic stress tolerance in elite pomegranates. Introduction of floral meristem activating genes from *Arabidopsis* (AGL 20 AP1, LFY; Blazquez and Weigel 2000; Borner et al. 2000) or dwarfing genes from wild pomegranate could be overwhelmingly profitable from horticultural stand point. In essence, biotechnological intervention has opened up new vistas for genetic improvement, hitherto unachievable through conventional breeding, of this nutraceuticallly important fruit tree crop.

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## References

Al-Wasel ASA (1999) In vitro clonal propagation of "Al-Belehi" pomegranate (*Punica granatum* L.). J King Saud Univ 11:3–14

Amin MN, Islam MN, Azad MAK (1999) Regeneration of plantlets in vitro from the seedling explants of pomegranate (*Punica granatum*). Plant Tissue Cult 9:53–61

Anonymous (1982) A dictionary of indian raw materials and industrial products. vol VIII (Ph-Re), CSIR Publication, New Delhi, India

Benson EE (2000) In vitro recalcitrance: an introduction. In Vitro Cell Dev Biol Plant 36:141–148

Blazquez MA, Weigel D (2000) Integration of floral inductive signals in *Arabidopsis*. Nature 889–892

Borner R, Kampmann G, Chandler J, Gleissner R, Wisman E, Apel K, Melzer S (2000) A MADS domain gene involved in the transition to flowering in *Arabidopsis*. Plant J 24:591–599

Caceres A, Giron LM, Alvarado SR, Torres MF (1987) Screening of antimicrobial activity of plants popularly used in Guatemala for treatment of dermatomucosal diseases. J Ethnopharmacol 20:223–237

Deepika R, Kanwar K (2010) In vitro regeneration of *Punica granatum* L. plants from different juvenile explants. J Fruit Ornam Plant Res 18:5–22

El-Agamy SZ, Mostafa RAA, Shaaban MM, El-Mahdy MT (2009) In vitro propagation of Manfalouty and Nab El-gamal pomegranate cultivars. Res J Agric Biol Sci 5:1169–1175

Ercisli S, Agar G, Orhan E, Yildirim A, Hizarci Y (2007) Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punica granatum* L.) growing in Southern Anatolia Region in Turkey. Biochem Syst Ecol 35:764–769

Foughat RS, Pandya SB, Ahmad T, Godhani PR (1997) In vitro studies in pomegranate (*Punica granatum* L.). J Appl Hortic Navsari 3:23–29

Gamborg OL, Miller RA, Ojima KI (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Jaidka K, Mehra PN (1986) Morphogenesis in *Punica granatum* (Pomegranate). Can J Bot 64:1644–1653

Jain SM (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153–166

Jimenez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul 47:91–110

Julie Jurenka MT (2008) Therapeutic applications of pomegranate (*Punica granatum* L.): a review. Alt Med Rev 13:128–144

Kantharajah AS, Dewitz I, Jabbari S (1998) Auswirkungen unterschiedlicher Nahrmedien, Wachstums-regulatoren und Explantatquellen auf die in vitro-Vermehrung von Granatapfel (*Punica* granatum L.) [The effect of media, plant growth regulators and source of explants on in vitro culture of pomegranate (*Punica granatum* L.). Erwerbsobstbau 40:54–58

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

Krul WR, Myerson J (1980) In vitro propagation of grape. In: Zimmerman RH (ed) Proceedings of the conference on nursery production of fruit plants through tissue culture-application and feasibility. Agric Res Sci Educ Admin, USDA, Beltsville, pp 35–43

Kumar GNM (1990) Pomegranate. In: Nagy S, Shaw PE, Wordowski WF (eds) Fruits of tropical and subtropical origin. Florida Science Source, Inc, USA, pp 328–347

Lad V, Frawley D (1986) The yoga of herbs. Lotus Press, Santa Fe, pp 135–136

Lansky EP, Newman RA (2007) *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol 109:177–206

Lloyd GB, McCown BH (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. Proc Plant Prop Soc 30:421–427

Mahishi DM, Muralikrishna A, Shivashankar G, Kulkarni RS (1991) Shoot tip culture method for rapid clonal propagation of pomegranate (*Punica granatum* L.). In: Prakash J, Pierik RLM (eds) Horticulture-new technologies and applications: proceedings of the international seminar on new frontiers in horticulture. Kluwer Academic Publishers, Dordrecht, pp 215–217

Malik SK, Chaudhury R, Kalia RK (2005) Rapid in vitro multiplication and conservation of *Garcinia indica*: a tropical medicinal tree species. Sci Hortic 106:539–553

Maroto JV, Miguel A, Lopez-Galarza S, San Bautista A, Pascual B, Alagarda J, Guardiola JL (2005) Parthenocarpic fruit set in triploid watermelon induced by CPPU and 2, 4-D applications. Plant Growth Regul 45:209–213

Mascarenhas AF, Gupta PK, Kulkarni VM, Mehta U, Iyer RS, Khuspe SS, Jagannathan V (1981) Propagation of trees by tissue culture. In: Rao AN (ed) Proceedings of COSTED symposium on tissue culture of economically important plants. Singapore Institute of Biology, Bangalore, pp 175–179

Mascarenhas AF, Nair S, Iyer RS, Gupta PK (1988) Genetic improvement of fruit crops through tissue culture. In: Genetic manipulation in crops—Proceedings of the international symposium on genetic manipulation in crops, the third international symposium on haploidy; the first international symposium on somatic cell genetics in crops (Beijing)—1984. Cassell Tycooly, pp 41

Miller CO (1965) Evidence for the natural occurrence of zeatin and derivatives compounds from maize which promote cell division. Proc Natl Acad Sci USA 54:1052–1058

Moriguchi T, Omura T, Matsuta N, Kozaki I (1987) In vitro adventitious shoot formation from anthers of pomegranate. Hortic Sci 22:947–948

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murkute AA, Patil S, Patil BN, Mayakumari MS (2002) Micropropagation in pomegranate, callus induction and differentiation. South Indian Hortic 50:49–55

Murkute AA, Patil S, Mayakumari (2003) Exudation and browning in tissue culture of pomegranate. Agric Sci Digest 23:29–31

Murkute AA, Patil S, Singh SK (2004) In vitro regeneration in pomegranate cv. Ganesh from mature trees. Indian J Hortic 61:206–208

Naik SK, Chand PK (2001) In vitro cloning of a medicinally important fruit tree *Punica granatum* L. by nodal culture. In: Sahoo S, Ramesh DB, Rao YR, Debata BK, Mishra VN (eds) Conservation and utilization of medicinal and aromatic plants. Allied Publishers Limited, New Delhi, pp 313–322

Naik SK, Chand PK (2003) Silver nitrate and aminoethoxyvinylglycine promote in vitro adventitious shoot regeneration of pomegranate (*Punica granatum* L.). J Plant Physiol 160:423–430

Naik SK, Chand PK (2006) Nutrient-alginate encapsulation of in vitro nodal segments of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. Sci Hortic 108:247–252

Naik SK, Pattnaik S, Chand PK (1999) In vitro propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Sci Hortic 79:175–183

Naik SK, Pattnaik S, Chand PK (2000) High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). Sci Hortic 85:261–270

Naqvi SA, Khan MS, Vohora SB (1991) Antibacterial, antifungal, and antihelminthic investigations on Indian medicinal plants. Fitoterapia 62:221–228

Nataraja K, Neelambika GK (1996) Somatic embryogenesis and plantlet from petal cultures of pomegranate, *Punica granatum* L. Ind J Exp Biol 34:719–721

Nitsch JP (1969) Experimental androgenesis in *Nicotiana*. Phytomorphology 19:389–404

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Omura M (1991) Pomegranate (*Punica granatum* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 16, Trees III. Springer, Berlin, pp 44–61

Omura M, Matsuta T, Moriguchi T, Kozaki I (1987a) Adventitious shoot and plantlet formation from cultured pomegranate leaf explants. Hortic Sci 22:133–134

Omura M, Matsuta T, Moriguchi T, Kozaki I, Sanada T (1987b) Establishment of tissue culture methods in dwarf pomegranate (*Punica granatum* L. var. *nana*) and application for induction of variants. Bull Fruit Tree Res Stn 14:17–44

Omura M, Matsuta N, Moriguchi T, Kozaki I (1990) Suspension culture and plantlet regeneration in dwarf pomegranate (*Punica granatum* L. var. nana Pers.). Bull Fruit Tree Res Stn 17:19–33

Rai MK, Asthana P, Singh SK, Jaiswal VS, Jaiswal U (2010) Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. Trees Struct Funct 24:1–12

Raj Bhansali R (1990) Somatic embryogenesis and regeneration of plantlets in pomegranate. Ann Bot 66:249–253

Rout GR, Mohapatra A, Jain SM (2006) Tissue culture of ornamental pot plant: a critical review on present scenario and future prospects. Biotechnol Adv 24:531–560

Saxena A, Vikram NK (2004) Role of selected Indian plants in management of type 2 diabetes: a review. J Altern Complement Med 10:369–378

Shao J, Chen C, Deng X (2003) In vitro induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell Tissue Organ Cult 75:241–246

Sharon M, Sinha S (2000) Plant regeneration from cotyledonary node of *Punica granatum* L. Ind J Plant Physiol 5:344–348

Sharp WR, Sondahl MR, Caldas LS, Maraffa SB (1980) The physiology of in vitro asexual embryogenesis. Hortic Rev 268–310

Singh SK, Khawale RN (2006) Plantlet regeneration from the nodal segments of pomegranate (*Punica granatum* L.) cv. Jyoti. In: Kumar A, Roy S (eds) Plant Biotechnology and its applications in tissue culture. IK International Pvt. Ltd., New Delhi, pp 107–113

Sinha S, Sharon M (1997) Somatic embryogenesis and plant regeneration from roots of Punica granatum L. Hortic Sci 32:545–546

Smith PM (1976) Minor crops. In: Simmonds NW (ed) Evolution of crop plants. Longman, New York

Stover E, Mercure EW (2007) The pomegranate: a new look at the fruit of paradise. Hortic Sci 42:1088–1092

Terakami S, Matsuta N, Yamamoto T, Sugaya S, Gemma H, Soejima J (2007) Agrobacterium-mediated transformation of the dwarf pomegranate (Punica granatum L. var. nana). Plant Cell Rep 26:1243–1251

White PR (1943) A hand book of plant tissue culture. Jacques Cattell Press, Lancaster

White PR (1963) The cultivation of animal and plant cells. Ronalds Press, New York

Yang Z, Ludders P (1993) Organogenesis of Punica granatum L. var. nana. Angew Bot 67:151–156

Zhang B, Stoltz LP (1991) In vitro shoot formation and elongation of dwarf pomegranate. Hortic Sci 26:1084