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Pomegranate Tissue Culture and Biotechnology

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ABSTRACT

In vitro regeneration systems in pomegranate have been established basically for two factors, i.e. a need for an inexpensive and efficient method for clonal propagation of elite genotypes and for application of modern genetic improvement methods for efficient *de novo* recovery of plants from cell cultures. Pomegranate cell and tissue culture is not easy and regeneration from existing meristems (shoot tip and nodal bud) and vegetative and reproductive plant parts have also been attempted with some noteworthy success. Reliable procedures now exist for micropropagation, organogenesis, somatic embryogenesis, and other regeneration procedures *in vitro*. However, the exploitation of these regeneration pathways for genetic transformation has been slow. Besides *in vitro* approaches, molecular marker technology has opened up new vistas for pomegranate breeding and germplasm management strategies. Nowadays these are in vogue for germplasm characterization, progeny analysis, etc. In this review an effort has been made to consolidate the state-of-the-art cell and tissue culture and biotechnological potential and applications, advancements and future implications in pomegranate improvement and propagation.

Keywords: micropropagation, molecular markers, *Punica granatum*

CONTENTS

INTRODUCTION

The pomegranate is one of the oldest known edible fruits. It has a mention in the Bible and the Koran and is often associated to fertility. It is native to Persia and perhaps some surrounding areas. It was cultivated in ancient Egypt and early in Greece, Italy and Iraq. Later, it spread into Asian countries like Turkmenistan, Afghanistan, Iran, India, China, etc.), North Africa and Mediterranean Europe (Melgarejo and Martínez 1992). It is one of the most potential fruit crops of the world owing to it multi-faceted properties and uses. Now, India occupies first position in pomegranate area and production globally (Jadhav and Sharma 2007). Maharashtra is a leading state for production of pomegranate and is contributing more than 65% of its total production in India. As far as export is concerned, Iran secured first rank with an annual export of 60,000 tonnes followed by India (35,176 t) (Holland and Bar-Ya'akov 2008; Chandra and Jadhav 2009). There are several improved pomegranate varieties, which are under cultivation world over but still a lot need to be done by utilizing the modern tools of biotechnology, which would ease its breeding for

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evolving desirable genotypes and also aid in genetic conservation.

Pomegranate is usually propagated by hardwood cutting obtained after annual pruning. Generally cuttings of 25-30 cm long of pencil thickness excised from previous season growth are rooted. However, soft-wood cuttings can be rooted where mist facilities exist. In this way pomegranate is typically grown as self-rooted plants. Hence, there lies an ample scope for large scale multiplication of desired genotypes using *in vitro* means. Micropropagation has been attempted in different pomegranate-growing countries. Though the attempts are limited but still it draws a lot of attention of researchers the world over.

CELL AND TISSUE CULTURE

Several *in vitro* techniques have been tried in the recent past in pomegranate. The different factors, which directly or indirectly influence the success in *in vitro* regeneration, have been dealt with in this review.

Selection of parent material

For *in vitro* tissue culture, apparently healthy plants are selected for excising the explants (Debergh and Maene 1981). Always, the plant material of proven horticultural traits should be selected for micropropagation. This can be either a selected phenotype (a specific tree or a plus tree) or the source of elite seed. However, most of the symptomless plants are infected endogenously with bacterial and fungal spores causing hindrance in their culture initiation. Torres (1988) suggested that attention should be given to make sure that the stock plant is disease-free, preferably grown in either growth chamber or glasshouse for collection of explants.

Selection of explants

For enhanced axillary branching, such explants are selected which have preformed dormant vegetative meristematic buds such as nodal segments (Bhojwani and Razdan 1983). Hence, judicious selection of such explants helps in achieving the eventual success in micropropagation. Use of juvenile material as a source of explant for micropropagation has been reported in many woody plants including fruit trees. In pomegranate, too different explants have been tried for their *in vitro* regeneration however; shoot tips and axillary bud or nodal segments have been most widely employed by different workers. Apical shoot tips excised from field-grown plants have been successfully cultured *in vitro*. However, the glasshouse grown plants have shown to give better response owing to low phenolic exudation in medium and low fungal and microbial contaminants. Meristem could be successfully cultured on half-strength Murashige and Skoog (1962) MS medium supplemented with 2.0 mg/l benzylaminopurine (BAP) and 30 g/l sucrose. Liquid medium with filter paper wicks were also helpful in establishing cultures (Singh and Khawale 2003).

Micropropagation

In vitro establishment of shoot tips and lateral nodes, shoot elongation and mass multiplication of axillary buds are essential steps in micropropagation. Unlike several other woody species, pomegranate is identified as a moderately difficult-to-micropropagate species due to problems associated with high phenol exudation and attendant medium browning, explant necrosis, systemic or latent microbial contamination and *in vitro* recalcitrance of the tissues. In pomegranate, both direct and indirect regeneration pathways have been attempted for its *in vitro* propagation.

Surface sterilization of explants

Successful disinfection of explants is a pre-requisite for *in vitro* culture and often involves a standard set of treatments, which vary with the explant type and plant species in question. Contamination in tissue culture can originate from two sources, either through carry over of microorganisms present on the explant surface or in the tissue itself (endophytic microorganisms). Although in meristem culture, depending on meristem size most of the microorganisms are expected to be eliminated, whereas in leaf, petiole and stem explants, the infection is carried over to the cultures. Washing the plant material intensively in running tap water before the beginning of surface sterilization process drastically reduced the microbial infection. Though $HgCl_2$ and NaOCl are the two most widely employed surface sterilants other chemicals have also been tried with good success. Damiano *et al.* (2008) could successfully sterilize axillary bud segments using a combination of NaOCl and Na methiolate for 20 min which gave good explant survival (65%).

Phenol exudation and its control

Establishment of *in vitro* culture of several plant species, especially woody plants, is greatly hampered by the lethal browning of explant and culture medium. The various techniques employed to overcome the harmful effects of browning attempts to either neutralize or avoid the buildup of toxic substances in the media. The different approaches are culture of juvenile explants, or new growth flushes during the active growth period, culture in darkness, transfer of explant to fresh medium at short intervals, culture in liquid medium inclusion of anti-oxidants in the culture media, 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. (Weatherhead *et al.* 1978; Wang *et al.* 1994), use of low salt media and optimum proper growth regulators, sealing the cut ends with paraffin wax (Bhat and Chandel 1991) and drying the explant under laminar airflow.

It has been shown that, nodal segments collected during active growth period showed minimum amount of phenolic exudation and were maximum responsive to micropropagation, culture of explants in darkness at 5^0C during the first phase (6-8 days) of culture initiation reduced browning and improved culture establishment (Wang *et al.* 1994). In pomegranate too fast sub-culture of explants onto fresh medium, use of anti-oxidants, culture of juvenile plant parts etc. are employed to manage the phenolic problem in its tissue culture. Murkute *et al*. (2004) attempted *in vitro* regeneration in pomegranate cv. 'Ganesh' using mature trees explants like shoot tip and nodal segment explants. They suggested fast sub-culturing to control media browning. Chaugule *et al*. (2007) attempted *in vitro* propagation of pomegranate cv. 'Mridula'. Shoot tip and nodal segment explants were selected from mature tree and they suggested fast subculturing at first and third day after inoculation. Singh *et al*. (2007) showed that sealing of cut edges of nodal segments with sterile wax reduced the phenol exudation and lead to high percentage of culture establishment (**Fig. 1**).

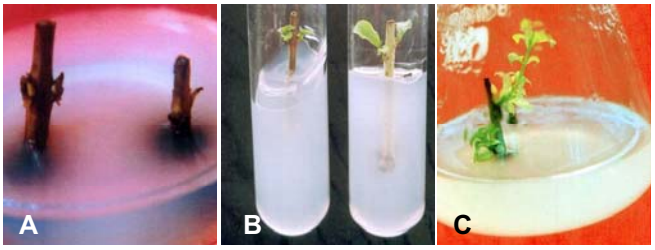

Fig. 1 Comparative performance of phenol controlling techniques in pomegranate *in vitro* culture initiation. (**A**) Control, (**B**) Wax-sealed explants, (**C**) sprouted explant.

Media composition for shoot proliferation

Various mineral salt formulations have been used for *in vitro* culture. However, full-strength mineral salts are not always optimum (Thorpe *et al*. 1991). Modifications with respect to different constituents like phyto-hormones, sucrose, agar concentrations and other additives like PVP, AC, coconut milk (CM), etc. are usually done in order to ensure better *in vitro* response (Thorpe *et al.* 1991). Plant growth regulators are not nutrients, but they influence growth and development of plants. They are generally produced naturally in plants; hence they are added selectively to culture media. Since phyto-hormones are the key compounds in tissue culture studies; hence a good deal of effort is required to determine their optimum levels (Skoog and Miller 1957). The cytokinin 6-benzylamino-purine (BAP) is the most commonly used growth regulator for shoot regeneration in a variety of plants. In some cases, mixed cytokinins have been used in combination for shoot regeneration systems. In pomegranate too a combination of cytokinin(s) and auxin has been employed to for culture initiation and shoot proliferation/multiplication; and addition of auxin has been found essential for obtaining good rooting. For indirect regeneration, different combinations are effective depending upon the genotype.

Zhang and Stoltz (1991) suggested supplementing the medium with 1.0 µM α-naphthaleneacetic acid NAA and 2.0 µM BA for maximum shoot regeneration on terminal shoot explants in a dwarf pomegranate genotype. Drazeta (1997) compared the micropropagation in different pomegranate cultivars 'Slatki Barski', 'Serbetas', 'Konjski Zubi' and 'Dividis'. Best production of shoots was achieved on medium containing 1 mg/l BAP and 0.1 mg/l NAA, but shoots exhibited vitrification, hence subsequent cultures were suggested to be transferred onto a medium containing BAP at 0.5 mg/l and 0.1 mg/l NAA. Fougat *et al.* (1997) also obtained good success on MS medium supplemented with 0.5 mg/l kinetin, 1.0 mg/l BA and 500 mg/l CH (cyclohexiimide).

A comparative study on shoot proliferation was attempted by Naik *et al.* (1999) 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. Later, Naik *et al.* (2000) obtained high frequency axillary shoot proliferation and plant regeneration from established cotyledonary nodes. Shoot development was induced on nodes upon culture on MS medium supplemented with 2.3 to 23.0 µM BA or kinetin. Both type and concentration of cytokinin significantly influenced the shoot proliferation. The maximum number of shoots (9.8 shoots/explant) was developed on a medium containing 9.0 µM 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. For further multiplication, *in vitro* raised shoots were then cut into nodal segments and cultured onto fresh medium. Thus, from a single cotyledonary node about 30-35 shoots were obtained in 60 days.

Singh and Khawale (2003) 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 BAP + 1.0 mg/l kinetin along with 200 mg/l AC (**Table 1**). Axillary buds were aseptically removed from the established cultures and transferred on to the proliferation medium. Multiple shoot proliferation occurred on MS medium supplemented with various concentrations of BAP, kinetin and 40 mg/l adenine sulphate (Ads). For shoot elongation and rooting, MS medium containing 2.0 mg/l IBA, 200 mg/l AC and 40 g/l sucrose was found the best.

Murkute *et al*. (2004) 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 BAP + 0.5 mg/l NAA. Again, Singh *et al.* (2007) 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 BAP + 0.1 mg/l NAA and 0.5 mg/l GA_3. The highest number of shoots per explant and longest shoot length were recorded on MS medium containing 1.0 mg/l BAP, 1.0 mg/l kinetin and 0.1 mg/l NAA. Chaugule *et al*. (2007) 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 BAP 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 BAP 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.* (2008) showed good shoot multiplication onto a basal Quorin and Lepoivre medium supplemented with BA (0.4 mg/l) and IBA (0.05 mg/l).

In vitro rooting

Different factors favouring root initiation have been tried. 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. In pomegranate, Drazeta (1997) found that MS medium supplemented with 0.1 mg/l IBA was most effective for rooting. However, Fougat *et al.* (1997) also got good rooting success on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l Kin kinetin and 15% CW (coconut water). Naik *et al.* (1999) reported that half-strength MS medium containing 1.0 mg/l indole-3-butryric acid IBA induced good rooting (80%) on *in vitro* derived shoots in 8-10 days. From each shoot, 3-4 roots developed to form a complete plantlet. Again, Naik *et al.* (2000) reported that addition of auxin to the media was essential to induce rooting on *in vitro* regenerated shoots. Root initiation occurred within 10-15 days in half-strength MS medium supplemented with 0.54 to 5.4 µM α-naphthalene acetic acid (NAA). The highest number of roots

Table 1 Effect of cytokinins and adenine sulphate on shoot proliferation in pomegranate.

Treatment (mg l^{-1})	Proliferation (%)	Days to shoot proliferation	No. of shoots per explant	Mean length of shoots (cm) at 30 days
BAP-0.5	60.4 (51.0)	22.4	1.7	1.2
BAP-1.0	53.5 (59.0)	20.8	2.1	1.8
BAP-2.0	56.4 (37.1)	28.4	1.9	0.9
Kin-0.5	45.8 (42.6)	26.7	1.6	1.0
Kin-1.0	59.6 (50.5)	29.5	1.3	0.9
Kin-2.0	28.7 (32.3)	31.4	0.8	0.5
BAP-0.5 + Kin-0.5 + Ads-40	75.6 (60.4)	19.6	2.9	1.5
BAP-1.0 + Kin-1.0 + Ads-40	89.8 (71.3)	21.3	3.4	2.2
BAP-2.0 + Kin-2.0 + Ads-40	51.7 (45.9)	33.3	1.3	0.75

BAP = 6-benzylamino purine; Kin = kinetin; Ads = Adenine sulphate
Source: Singh and Khawale 2003

(10.33 roots/shoot) was formed in medium containing 0.54 µM NAA.

Kantharajah *et al*. (1998) 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. Singh and Khawale (2003) suggested the role of AC during rooting with higher level of sucrose, i.e. MS medium containing 2.0 mg/1 IBA, 200 mg/1 AC and 40 g/1 sucrose which also resulted in improved root quality. Murkute *et al*. (2004) got efficient rooting on half-strength MS basal medium supplemented with either NAA or IAA at 0.5 mg/l.

In contrast, Chaugule *et al*. (2007) suggested supplementation of auxin at 0.5 mg/l to be optimum irrespective of their type. Singh *et al.* (2007) could induce good rooting (70.37%) with dual auxin, i.e. IBA and NAA supplemented to the rooting medium. However, Damiano *et al.* (2008) 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'.

Hardening of plantlets

The term acclimatization is defined as the climatic adaptation of an organism, especially a plant, which has been moved to a new environment (Conover and Poole 1984). In conventional acclimatization, the main effort of environmental 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.

Naik *et al.* (1999) reported 68% *ex vitro* survival of the plantlets in cv. 'Ganesh' 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 (Naik *et al.* 2000).

Mahishni *et al.* (1991) obtained over 80% success in hardening using potting mixture comprising 1: 1: 1 (v/v) peat: perlite: sand. Yang *et al*. (1991) 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. Different *in vitro* hardening methods were compared by Singh and Khawale (2004). 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 (**Table 2**). Murkute *et al*. (2004) achieved 50% survival when the plantlets were transferred to vermicompost + soil mixture (1: 1) and hardened in mist chamber. Again, Singh *et al*. (2007) reported highest survival (89%) with minimum duration (35 days) to field transfer for plantlets in cv. 'G-137'. The different stages of micropropagation standardized for pomegranate cv. G-137 by Singh *et al*. (2007) is shown in **Fig. 2**.

Plantlet bio-hardening using arbuscular mycorrhizal fungi

The beneficial interaction between arbuscular mycorrhizal fungi (AMF) and horticultural crops have been well documented (Menge 1983). Mycorrhizal inoculation of *in vitro* propagated transplants has proven to be effective in respect to tolerance to transplant stress and improvement of growth and mineral nutrient status. The earlier the AMF mycelia develop, the sooner the host micropropagated plants benefit from the symbiosis (Branzanti *et al.* 1992). Inoculation of AMF at the start of the acclimatization period or even during *in vitro* culture (Brazanti *et al.* 1992; Mathur and Vyas 1999) has been demonstrated to be beneficial. Singh (2007) attempted the use of AMF in hardening to avert transplantation shock of unfavorable *ex vitro* environmental conditions of pomegranate plantlets. He found that the maximum survival was registered with *Glomus mosseae* (91.33 and 89.00% at 60 and 90 DAI (days after inoculation), respectively) inoculated plantlets followed by *G. manihotis* (89.00 and 87.33% at 60 and 90 DAI, respectively). The mycorrhizal plants showed faster growth, with better physiological and biochemical parameters.

Table 2 Effect of different hardening methods on acclimatization of *in vitro* rooted plantlets.

Method	Survival (%)	
	30 days	45 days
I. Plastic pots with polythene cover (Peat; soilrite: 1:1)	45.8 (42.6)*	36.8 (37.3)
II. Plastic pots with inverted beaker (Peat: soilrite; 1:1)	68.9 (56.1)	50.4 (45.2)
III. Glass jar with polypropylene cap (Peat: soilrite; 1:1)	86.5 (67.6)	78.7 (62.5)
CD at 5%	11.2	8.9

* transformed data
Source: Singh and Khawale 2004

Fig. 2 Stages of *in vitro* micropropagation in pomegranate cv. 'G137'.

Somatic embryogenesis and shoot bud organogenesis

In order to exploit cell and tissue culture for genetic manipulation of pomegranate, it is essential that regeneration system of plants from cell cultures be very efficient and should have originated from vegetative tissue. Studies that have focused on the embryogenic response in pomegranate have depended on the morphogenic potential of different vegetative tissues. Regeneration of plants *via* organogenesis, i.e., *de novo* induction of either shoot or root meristems from cultured explant or cell cultures is of practical significance in developing protocols for employing genetic engineering.

Table 3 Some early attempts on *in vitro* studies in pomegranate.

Explant(s)	Medium	Success	Reference
Leaf segment	MS + 5.0 µM BAP + 0.5 µM NAA	Adventitious shoots	Omura *et al*. 1987a
Root, internode, sepal	MS + 5.0 µM BAP + 0.5 µM NAA	Adventitious shoots	Omura *et al*. 1987b
Anther	MS + 10.0 µM BAP + 5.0 µM NAA	Adventitious shoots	Moriguchi *et al*. 1986
Different parts of young seedling	NS 4.0 mg/l NAA + 2.0 mg/l Kin + 15 Coconut milk (v/v)	Embryogenesis	Jaikada and Mehera 1986

BAP = 6-benzylamino purine; Kin = kinetin

Indirect shoot organogenesis and somatic embryogenesis in pomegranate have been reported by Jaidka and Mehra (1986). They observed that MS medium supplemented with 4.0 mg/l NAA + 2.0 mg/l kinetin and 15% CW was most effective for callus induction from seedling explants, i.e. root, hypocotyl, stem, shoot tip and leaves in cv. 'Kandhari'. Embryo-like structures were observed with 2.0 mg/l NAA + 2.0 mg/l BAP. Omura *et al.* (1987a, 1987b) reported adventitious shoot bud formation on leaf segment of dwarf pomegranate cv. 'Nana'. They were also successful in obtaining plantlet regeneration from suspension culture derived from leaf callus. The best response was registered with addition of 2.0 µM BAP and 1.0 µM NAA (Omura *et al.* 1990). Bhansali (1990) obtained somatic embryogenesis on cotyledonary tissue. Embryogenic cell clusters proliferated vigorously with regular sub-culturing on RBM-II medium containing 1 µM kinetin, 2 µM BAP and 5 µM 2,4-dichlorophenoxy-acetic acid (2,4-D). Developmental stages of somatic embryos were expressed on sub-culturing with a low level of 2,4-D (2.5 µM). Embryogenic initials cells were small, round to oval, thick-walled, contained dense cytoplasm which stained with acetocarmine and were usually attached to non-embryogenic cells. Embryo maturation was obtained on RBM-III and IV media to produce young seedlings on the initiation of the first long tap root. Some of the earliest attempts made are listed in **Table 3**.

Yang and Ludders (1993) demonstrated organogenesis in *P. granatum* L. var. 'Nana' using callus derived from leaf and stem explants of *in vitro* cultured shoots. Leaf segment and stem explants were initially cultured on modified MS basal medium supplemented with the following growth regulators, i.e. (1) BA, zeatin, kinetin or 2-iso pentanyladenine (2iP) at 0.1-1.5 mg/l and (2) IAA, IBA or NAA at 0.1-1.0 mg/l + 0.5 mg/l BA. Adventitious shoot elongation was stimulated on MS basal medium supplemented with 0.5 mg/l BA and 0.1 mg/l IBA. Elongated shoots rooted easily on half-strength MS medium. Nataraja and Neelambika (1996) were successful in getting somatic embryos from cultured petals on MS medium supplemented with 5.0 mg/l BAP and 5.0 mg/l IAA.

Fougat *et al.* (1997) compared four types of explant (leaf, shoot tip and nodal segment from field-grown trees and cotyledons from laboratory-grown seedlings) for *in vitro* regeneration in cv. 'Ganesh' on MS medium supplemented with various growth regulator combinations. Exudation of phenolics was overcome by pre-treating the explants for 2-3 days with varying concentrations of antioxidant compounds such as polyvinyl pyrrolidone and ascorbic acid. Callus growth and induction from cotyledon and leaf explants were best on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l kinetin and 15% CW. Subculturing on MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l BA resulted in fast-growing nodular callus followed by good shoot proliferation. Enhanced axillary branching of nodal segments and proliferation on shoot tip meristems was achieved on MS medium supplemented with 0.5 mg/l kinetin, 1.0 mg/l BA and 500 mg/l casein hydrolysate. Rooting was best in shoots derived from all explant sources, on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l kinetin and 15% CW.

Kantharajah *et al*. (1998) studied the combination effect of media, plant growth regulators and explant source on *in vitro* culture of pomegranate. The effects of plant growth regulators (BAP and NAA) and media (MS, 1/2MS, W (White's) and WP (Woody Plant) on *in vitro* propagation of cv. 'Wonderful' were investigated. Callus cultures were initiated from leaf and nodal explants obtained from aseptically cultured shoots. Callus initiation and growth was best on MS basal medium containing either 1 mg/l BAP (leaf explant) or 1 mg/l BAP + 0.4 mg/l NAA (nodal explant). Leaf calli initiated on MS and WP media regenerated shoots about 8 weeks after incubation on MS basal medium supplemented with 1 mg/l BAP. On nodal cuttings, the best adventitious bud formation was observed on half-strength MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA. This medium also promoted good proliferation along with formation of longest shoots. The highest rooting and average number of roots/explant were induced on WP medium supplemented with 2 mg/l NAA. Similarly, a complete protocol was demonstrated by Naik *et al.* (2000) for *in vitro* regeneration using cotyledonary node derived from axenic seedlings, shoot development was induced from cotyledonary nodes on MS medium supplemented with 2.3-23.0 µM BA or kinetin. Naik *et al.* (2003) observed that the addition of ethylene inhibitors like $AgNO_3$ (10-40 µM) and aminoethoxyvinylglycine (AVG) (5-15 µM) to the MS medium containing BAP and NAA markedly enhanced the regeneration frequency as well as number of shoots per explant of pomegranate. However, this procedure could not easily be repeated due to the oxidation of phenols following explanting and subsequent death of the tissue.

Murkute *et al*. (2002) proposed a method for the callus induction and differentiation using leaf segment and cotyledon explants of cv. 'Ganesh'. Cotyledon explant was found to be almost free of leaching of phenolic compounds. Callus induction was obtained on MS basal medium supplemented with BAP and NAA. Cotyledon explant responded extremely better than leaf segment. Profuse callus induction, proliferation and shoot differentiation was obtained in MS + 1.0 mg/l BAP + 0.5 mg/l NAA and good rooting was obtained in 1/2 MS + 1.0 mg/l IBA. Later, Chaugule *et al.* (2005) attempted callus culture for rapid regeneration using leaf segment and cotyledon explants on MS medium supplemented with BAP with or without NAA. The MS medium supplemented with BAP alone did not respond for callus initiation. The cotyledon was found to be most responsive (78.94%) for callus induction on MS medium fortified with 0.4 mg/l NAA + 1.0 mg/l BAP. The minimum duration for callus induction from leaf segment was 8.8 days, whereas cotyledons recorded 10.0 days. Callus initiation occurred at the cut ends of explants. Callus weight and proliferation was highest on MS medium containing 0.4 mg/l NAA + 1.0 mg/l BAP. However, the callus derived from the leaf segment showed greater dry weight than those derived from the cotyledon. There was poor somatic embryo induction confirming recalcitrant nature of pomegranate. Recently, Kanwar *et al*. (2010) cultured cotyledonary explants, excised from *in vitro* germinated seedlings. They found solid MS medium supplemented with 21 µM NAA and 9 µM BA gave over 80% callusing. Shoot organogenesis was obtained on MS medium supplemented with 8 µM BA, 6 µM NAA, and 6 µM gibberellic acid (GA_3). However, adding 24 µM silver nitrate ($AgNO_3$) to this medium markedly enhanced shoot regeneration frequency (63%) and mean number of shoots per explant (11.26) and length of shoots (2.22 cm). Rooting was achieved best, i.e. number of roots/shoot (4.32), and mean root length (2.71 cm) when regenerated shoots were transferred onto half-strength MS medium supplemented with 0.02% AC. They also found that zygotic embryos excised from seeds collected at

16 weeks following full bloom, when incubated on MS medium containing 30 g/l sucrose, 15% CW, 21 µM NAA, and 9 µM BA, produced highest frequency of embryogenic callus clumps with globular embryos, and mean number of both globular and heart-shaped embryos per callus clump. Subjecting zygotic embryo explants to six-week dark incubation period was essential for embryogenic callus induction, and these were subsequently transferred to 16 h photoperiod for further growth and development of somatic embryos. Germination of somatic embryos was observed when these were transferred to MS medium was supplemented with 60 g/l sucrose.

Synthetic seed

The synthetic seed technology has been developed to use somatic embryos and/or other micropropagules as seed analogues successfully in the field or greenhouse, and their mechanical planting at a commercial level. The technology provides method for preparation of seed analogues called synthetic seeds or artificial seeds from the micropropagules like somatic embryos, axillary shoot buds, apical shoot tips, embryogenic calli as well as protocorm or protocorm-like bodies. For the last fifteen years, intensive research efforts have been made on synthetic seed production in a number of plant species (Ara *et al*. 2000).

However, in pomegranate, Naik and Chand (2006) were successful in encapsulating nodal segments from *in vitro* proliferated shoot cultures or axenic cotyledonary nodes. These tissues could then be encapsulated in calcium alginate hydrogel containing M medium supplemented with 4.44 µM BAP and 0.54 µM NAA. Of various concentrations of sodium alginate (1-6%) and the complexation solution of calcium chloride (50-125 mM), a combination of 3% sodium alginate and 100 mM calcium chloride was most suitable for formation of ideal synthetic seed. Morphogenic response of encapsulated nodal segments was the highest in MS medium augmented with 4.44 µM BAP and 0.54 µM NAA. One step germination, i.e. both shoot and root formation was possible only with encapsulated nodal segments on MS (1/2) MSS and natural soil + (1/2) MSS. Encapsulated nodal segments stored up to 30 days at 4°C were capable of sprouting.

Somaclonal variation and *in vitro* selection

There has been significant consumer resistance towards adoption of new cultivars with transgene in many countries of the world. For several years, researchers have attempted to use applied physiology in order to resolve genetic problems in different established cultivars, e.g., susceptibility to diseases, tree shape and size, fruit quality etc. Conventional fruit breeding has consequently focused on developing new cultivars that are largely indistinguishable from traditional selections with respect to fruit size, appearance, taste, flavour and overall quality. Somaclonal variation is defined as genetic and phenotypic variation among clonally propagated plants of a single donor clone (Larkin and Scowcroft 1981). Somaclonal variations could be of great value to the breeders as it is an efficient tool to create variations. Single gene mutations may result in the alteration of a significant horticultural trait and therefore, may give rise to the best available variety *in vitro* with improved targeted character. Despite the advantages that somaclonal variations proffer, it has been unable to leave a significant impression on breeding *via* development of advantageous off-types of existing selection, which lack in some useful attributes. In pomegranate, this approach can be exploited for evolving desirable variants especially those with tolerance to biotic and abiotic stresses.

In vitro mutagenesis

Although mutation breeding has made a significant impact on crop breeding during latter half of the twentieth century, production of useful off-types of existing cultivars has not been successful; however, naturally occurring variation due to somatic mutation can occur in pomegranate on the basis of variation within clonal population/natural seedling population. The different merits of this approach can be exploited successfully. It can be exploited using culture filtrates produced by pathogenic fungi and bacteria, abiotic stresses like NaCl/polyethylene glycol to select cell lines that possess resistance to the pathogens *in vitro* or abiotic stresses and can also be induced. However, the former approach can be used if a highly morphogenic (i.e., embryogenic) suspension culture is challenged with the culture filtrate, whose effect at the whole plant level is the same as it is at the single cell.

In vitro induced mutation followed by selection is an efficient approach for addressing a specific breeding problem of perennial trees for which there are both an effective selection agent and a highly embryogenic regeneration protocol. Unfortunately, there are very few appropriate selection agents that can be used in this manner. Shao *et al*. (2003) attempted *in vitro* induction of tetraploids in pomegranate var. 'Nana' by colchicine treatment of shoots propagated *in vitro*. Shoot segments cultured on MS medium supplemented with 10 mg/l colchicine along with 1.0 mg/l BAP and 0.1 mg/l NAA for 30 days produced tetraploids at a high frequency (20%). Shoots treated by 5,000 mg/l colchicine for 96 h produced three morphological mutants having narrow leaves, which were later confirmed as mixoploids that separated into diploids and tetraploids after further subculture. *In vitro* tetraploid plants had shorter roots, wider and shorter leaves than the diploid ones. Tetraploid pomegranate plants grew and flowered normally in pots, but possessed flowers with increased diameter and decreased length compared to diploids. The number of pollen grains per anther was higher in tetraploids, but the viability of pollen decreased significantly. Tetraploidy can lead to evolution of genotypes with larger fruits with improved quality.

Anther culture and haploidy

Haploidy has several uses in plant breeding as homozygous diploids have several advantages added to it. Doubled haploid (DH) systems have several attractive features for inducing and fixing mutations. Doubled haploidy provides the fastest route to homozygosity with the greatest fidelity. The DH system themselves provide an opportunity to target haploid as well as doubled haploid cells for mutation treatment and capture the mutation in a homozygous, pure line, heterozygous (Vegetatively propagated species) genotypes (Szarejko and Forster 2007). Moriguchi *et al.* (1987) cultured anthers of pomegranate on M medium containing 5 or 10 µM BA and 1 or 5 µM NAA. The callus was produced upon transfer onto MS medium containing 2.0 µM of BA and 0.5 µM of NAA which also lead to good shoot regeneration.

Genetic transformation

Genetic transformation is one of the potent methods for improving existing pomegranate cultivars for specific horticultural traits using embryogenic cultures. To date, the different horticultural traits that has been targeted for improvement by genetic transformation has been the control of pests and diseases in pomegranate. Terakami *et al*. (2007) attempted genetic transformation in a dwarf pomegranate species (*Punica granatum* L. var. *'Nana'*). *In vitro* regenerated adventitious shoots derived from leaf segments were inoculated with *A. tumefaciens* strain EHA105 harbouring the binary vector pBin19-sgfp, containing neomycin phosphotransferase (*npt*II) and green fluorescent protein (*gfp*) gene as a selectable and visual marker, respectively. After cocultivation, the inoculated adventitious shoots were cut into small pieces to induce regeneration, and then selected on MS medium supplemented with 0.5 µM NAA, 5 µM BAP, 0.3% gellan gum, 50 mg/l kanamycin and 10 mg/l meropenem. Putative transformed shoots were regenerated after

6-8 months of selection. PCR and PCR-Southern blot analysis revealed the integration of the transgene into the plant genome. Transformants bloomed and bore fruits within 3 months of potting, and the inheritance of the transgene was confirmed in T_1 generation. The advantage of the transformation of dwarf pomegranate was shown to be the high transformation rate, which can successfully be exploited in other desirable genotypes with any desirable traits.

APPLICATION OF MARKER TECHNOLOGY

The use of molecular markers, which comprise isozyme and DNA markers can be used for cultivar identification. Another promising application could be marker-assisted selection (MAS) to expedite the breeding programmes. This technology has been applied in pomegranate for diversity analysis and germplasm management. The different morphological approaches like traditional taxonomy based on flavour, petal colour, pericarp colour, seed colour, rigidity of pit etc. give variable results hence have got limited use for the purpose of crop improvement using the above traits.

Molecular marker techniques overcome several limitations of the morphological and biochemical marker systems since they are not affected by environmental or plant developmental stage and can detect variation at the DNA level (Tingey and del Tufo 1993). The technique called Randomly Amplified Polymorphic DNA-PCR uses randomly amplified polymorphic DNA to quickly scan an entire genome using single short arbitrary primers (Welsh and McClelland 1990; Williams *et al*. 1990). Since the abundance of priming sites for short primers follows a known statistical distribution, no prior knowledge of the genome is required. This allows previously uncharacterized genomes or sets of closely related but phenotypically distinct cultivars to be quickly and easily scanned for polymorphisms using a simple PCR based laboratory technique, which only requires a small amount of genomic DNA. When the RAPD technique is performed at standard low annealing temperatures between 35 and 38°C though, band reproducibility can be problematic. Therefore, by increasing the annealing temperature to over 46°C, the high annealing temperature RAPD (HAT-RAPD) technique has been shown to provide a higher degree of reproducible polymorphism (Anuntalabhochai *et al*. 2000). This method has been successfully used to characterize the ancestry of different tropical fruits. To overcome the difficulties with band reproducibility encountered using standard RAPD, a technique called sequence characterized amplified regions (SCARs) can be used to amplify single bands corresponding to genetically defined loci (Paran and Michelmore 1993). Using a primer pair with a length over 16 nucleotides eliminates the complexity of band patterns found using the shorter arbitrary RAPD primers. SCAR markers are similar to Sequence-Tagged Sites (STSs) used as landmarks for physical mapping of the human genome (Olson *et al*. 1989), and have been used extensively in crop species for mapping and marker-assisted selection. The different attempts made in employment of marker system in pomegranate are presented hereunder.

Biochemical markers

These marker systems are though tedious but are normally used to study the genetic diversity at protein/enzyme level. Though these markers suffer from reproducibility and other demerits but are still applied in conjunction with DNA markers. Jalikop and Kumar (1990) described a monogenic marker (designated R) which was suitable for estimating the extent of natural cross-pollination, since it enables heterozygous progenies to be visually distinguished from homozygous recessive progenies at the seedling stage. The dominant gene (present in cv. 'Ganesh') confers red and the recessive gene (cv. 'Kabul Yellow') yellow pigmentation of the petiole base, leaf margin, flower bud and fruit rind. In pollination experiment with four randomly located 'Kabul Yellow' plants surrounded by 'Ganesh', the 'Kabul Yellow' progeny showed 13% cross pollination. The new marker technique is compared with previous methods of assessing cross pollination.

DNA/molecular markers

Molecular approaches offer an efficient alternative tool to conventional breeding. It is a very useful tool for characterizing the genetic diversity and deciphering the relatedness among different cultivars or species of *Punica*, for identifying gene(s) of commercial interest, improvement through gene transfer technology, creation of variations in existing cultivars *in vitro*, overcoming reproductive isolation barrier *via* protoplast fusion etc.

1. Randomly amplified polymorphic DNA (RAPD)

This technique uses arbitrary 10-base primers to amplify the random portions of the genome (Welsh *et al*. 1990; William *et al*. 1990). The fragments produced are easily visualized on ethidium bromide stained gel, and polymorphism can be detected between the amplification products of different individuals. RAPD are however, inherited as dominant markers, requires large number of fragments to be identified and screened, but it is inexpensive and readily adaptable technique for routine germplasm fingerprinting and evaluation of genetic relationship between genotypes and construction of genetic linkage maps (Lu *et al*. 1998).

Sarkosh *et al*. (2006) employed RAPD markers to determine the genetic diversity level among 24 Iranian pomegranate genotypes. One hundred decamer random primers were used of which 16 showed reliable polymorphic patterns. These primers produced 178 bands, of which 102 were polymorphic. Cluster analysis using Jaccard's similarity coefficient and UPGMA revealed that the highest and lowest similarities detected between genotypes were 0.89 and 0.29, respectively. At a similarity of 60%, the genotypes were divided into four sub-clusters. Cophenetic correlation coefficient between similarity matrix and cophenetic matrix of dendrogram was relatively high (r = 0.9) showing the goodness of fit of the dendrogram.

Yang *et al*. (2007) analysed 25 pomegranate cultivars using RAPD markers. Twelve out of 128 arbitrary 10-mer primers were used for amplification. A total of 110 bands were obtained, and the average bands per random primer were 9.17. The degree of genetic diversity was 71.8%. The genetic distance between cultivars was 0.027 to 0.342. A phylogenetic tree constructed showed that the genetic background of pomegranate germplasm resources in Yunnan province in China was complex and difficult to be classified. Similarly, Zamani *et al*. (2007) studied the genetic relationships in 24 Iranian pomegranate genotypes. Measurements of 28 fruit characteristics together with RAPD marker data were used. Among 113 random decamer primers tested, 27 showed good amplification and polymorphism, and a total of 158 RAPD markers were produced. Estimates of genetic relationships, using Jaccard's similarity coefficient, ranged from 0.30 - 0.88. Grouping by fruit characteristics were compared with the results from RAPD analysis did not produce any significant correlation. This means that information based on fruit characteristics was not sufficient for genetic discrimination in pomegranate; however, RAPD markers proved useful.

Sheidai *et al*. (2007) studied the genetic variations in eleven pomegranate genotypes using 15 RAPD markers. Thirteen primers were found polymorphic producing a total of 173 bands out of which 73 bands were common in all the cultivars, while six bands were specific, which can be used in the cultivar discrimination. Primers OPB12 and OPA13 produced the highest number of polymorphic bands (12 bands out of 16 = 0.75% and 11 bands out of 25 = 0.44%), while primers OPR15 and OPA15 produced the least number of polymorphic bands (2 out of 12 = 0.16%). Different similarity coefficients determined among the cultivars studied, showed the highest value of similarity between culti-

vars 'Khatooni' and 'Anbari' as well as between 'Khatooni' and 'Atabaki' (r = 0.94), while the lowest value of similarity was estimated between the cultivars 'Sefid' and 'Bihaste' as well as 'Sefid'and 'Khatooni' (r = 0.62).

Molecular analysis was also carried out by Durgac *et al*. (2008) employing fruit characteristics and RAPD band patterns in highly diverse six local cultivars from Hatay, Turkey. The principle component analysis of 18 quantitative fruit characteristics revealed that fruit weight, aril number/ fruit, peel colour and soluble solids/acid ratio are important traits for discriminating the cultivars. The UPGMA cluster of fruit characteristics indicated that 'Katrbas' and 'Kannar' were similar to each other and they were separated from rest of the cultivars. Twenty-two RAPD primers generated total of 106 reproducible bands, 22% of which were polymorphic. The UPGMA dendrogram of RAPD data showed that 'Tatl-nar' and 'Serife' were very closely related, while 'Incekabuk' was distinct from the other cultivars. Discrepancies were detected between morphological and molecular data. Therefore, it was inferred that diversity among the fruit characteristics were not good indication of genetic relatedness, while molecular tools were effective to study such similarities. Jambhale *et al*. (2007) conducted the molecular characterization to distinguish four popular pomegranate cultivars, i.e. 'Ganesh', 'G-137', 'Mridula' and 'Phule Bhagawa'. RAPD analysis using 12 oligonucleotide random primers, generated 156 amplicons, of which 29 were monomorphic (19%) and 127 were polymorphic (81%). All the primers produced typical banding profiles for each of the cultivar, suggesting usefulness of the technique in DNA fingerprinting and cultivar identification. The genetic distance was estimated as 1.243, which is high in comparison to the morpho-agronomical difference, suggesting a broad genetic base of the crop.

Sheidai *et al*. (2008) studied the genetic variations in ten pomegranate cultivars using 40 RAPD primers, which generated 2,050 bands in total. Two hundred and fifteen bands were polymorphic (about 10.50%) and 1,835 bands were monomorphic (80.50%). Primers H02 and R01 produced the highest number of polymorphic bands (13 bands, 0.63%), while primer R15 produced no polymorphism. Specific bands were observed in some of the cultivars, which may be used for differentiating cultivars. Different similarity coefficients determined among the cultivars studied, showed the highest value of similarity between 'Bihasteh' and 'Berit' (r = 0.86), while the lowest value of similarity occurred between the cultivars 'Alakshirin' and 'Bihasteh' (r = 0.36). Different clustering methods showed distinctness of the pomegranate cultivars studied. The grouping of the cultivars did not correlate with the cytogenetic clustering.

2. Sequence related amplified polymorphism (SRAP)

Zhang *et al*. (2008) applied sequence related amplified polymorphism (SRAP) to analyze 23 Chinese pomegranate genotypes (*Punica* spp.) using 7 pairs of primers. About 1,380 bands were amplified, of which 662 were polymorphic, and the polymorphic percentage of bands was 48%. A dendrogram, constructed by UPGMA method showed that the range of genetic distance among 23 genotypes was 0.0769 to 0.4131. The genotypes could be divided into five sub-clusters (A, B, C, D and E) at the genetic distance of 0.33. Four white single-petal flower types clustered into group A, while the genotypes with single-petal flower and sweet flesh cultivars in group B. The peel of cultivars in group C was all non-red, and four ornamental and flesh types of single-petal flower with different floral colour and leaf shapes were clustered into group D. One ornamental type, *P. granatum* var. *nigra*, was significantly different and clustered into group E. Genotype 'Qingpi' could be distinguished from all the rest based only on the lack of ME4/ EM4-800 bp fragment, and Yichenghong and Heyinruanzi lacked ME4/EM4-480 bp fragment. 'Yichenghong', 'Baipisuan' and 'Daqingpi' genotypes could be identified as they lacked ME2/EM3-120 bp fragment.

3. Simple sequence repeats (SSRs)

Simple sequence repeats or SSRs, also known as microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, analytical simple and readily transferable. Microsatellites are reported to be more variable than RFLPs or RAPDs, and have been widely utilized in plant genomic studies. The SCAR marker shows that it is possible to characterize a specific variety of pomegranate, which could allow an unambiguous method to register new and modified varieties and reduce infringement upon previously developed varieties. The development of a SCAR primer provides a proof of concept that the development of variety specific SCAR markers is an effective and feasible method to characterize hybrid varieties. In addition, new SCAR markers could be developed to characterize varieties which could further protect the rights of plant breeders. As a method to provide ancestry identification, the SCAR primer allows a cost effective morphologically independent characterization of genotypes.

4. Inter-simple-sequence-repeats (ISSRs)

It is a relatively novel technique and has proven to be a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in several species, including fruit trees (Gonzalez *et al.* 2002). Simple sequence repeats or SSRs, also known as microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, analytical simple and readily transferable. Microsatellites are reported to be more variable than RFLPs or RAPDs, and have been widely utilized in plant genomic studies (Weber 1990; He *et al.* 2003). Koohi-Dehkordi *et al*. (2007) suggested SSRs or microsatellite markers are an excellent tool for cultivar identification and for the evaluation of genetic distances. A study was conducted to construct microsatellite-enriched libraries from pomegranate and to identify microsatellite-containing sequences. Two genomic libraries enriched for AG and GT repeats were constructed from 'Malase Yazd'. The enrichment was efficient and was 51% in the GA library and 47% in the GT library. Primer pairs were designed for 15 microsatellite-containing sequences. Five out of 15 primer pairs amplified the expected size fragment and were polymorphic in 29 genotypes. These SSRs could be used to analyse genetic relationships among pomegranate accessions and for cultivar identification.

Narzary *et al*. (2009) studied the genetic diversity in the Western Himalayan regions of Jammu and Kashmir, Himachal Pradesh and Uttarakhand States using ISSR markers. Forty-nine accessions representing eight populations from two regions were analysed. Seventeen ISSR primers resulted in 268 polymorphic bands, with 87.01% polymorphism. The results indicate that the ISSR method is sufficiently informative and powerful to assess genetic variability in pomegranate, and that patterns of genetic variability observed among populations of wild pomegranate from the Western Himalaya differ considerably.

5. Amplified fragment length polymorphism (AFLP)

Yuan *et al.* (2007) studied the genetic diversity among 85 pomegranate cultivars from six geographical populations from Shandong, Anhui, Shaanxi, Henan, Yunnan, and Xinjiang provinces, China by fluorescent AFLP markers. A total of 135-185 polymorphic loci were amplified by 8 pairs of primers at the species level. An average of 158.25 polymorphic loci was amplified for each primer combination. The polymorphism percentage ranged from 62.5 to 86.11, and the average polymorphism was 73.26%. The genetic diversity was highest for the Henan population, followed by the Xinjiang, Shaanxi, Anhui, Shandong and Yunnan populations. The genetic differentiation coefficient between the

populations was 0.2018, which indicated that gene differentiation was mainly within the population. The genetic differentiation between the populations accounted for 20.18% of the total variation. Gene flow between the populations was found to be 1.9027 based on the genetic differentiation coefficient between the populations, indicating that there was moderate gene flow between the populations.

Yuan *et al*. (2008) studied the genetic diversity of 25 pomegranate genotypes of Shandong Province, China using fluorescent-AFLP marker techniques with eight pairs of *Eco*RI/*Mse*I primer combinations. The number of polymorphic loci was found to range from 70 to 126, with an average of 90.25. The mean percentage of polymorphic loci was 41.78%. All average observed number of alleles, average effective number of alleles, average Nei's gene diversity index, and average Shannon's information index were 1.4178, 1.1874, 0.1133 and 0.1752, respectively. Cluster analysis performed using UPGMA method based on Lei and Li similarity coefficients divided the 25 pomegranate cultivars into four groups at 0.786 level of similarity coefficient. Similarly, Jbir *et al*. (2008) employed AFLP analysis of DNA to characterize 34 pomegranate genotypes using a combination of six primers. A total of 327 markers were scored with a mean of 57.5. The high percentage of polymorphic bands (ppb) of 94.7 and the resolving power (Rp) collective rate value of 129.14 were scored. Data proved that the tested primers were informative to discriminate among cultivars and to survey the genetic diversity in this fruit crop. It has been assumed that the local pomegranate germplasm is characterized by a typically continuous genetic diversity. The derived dendrogram proved that cultivars are clustered independently from their geographical origin and their denomination. In addition, AFLP permitted the generation of a nearly unlimited number of molecular markers that are reliable in differentiating the cultivars and/or the polyclonal varieties.

Recently, to asses the genetic diversity among 12 pomegranate landraces collected from three locations in Jordan, Awamleh *et al*. (2009) conducted AFLP analysis. Eight primer combinations generated a total of 1,433 bands with an average of 14.9 bands per landrace. *Mse*I+CTG and *Eco*RI +ATG primer combinations have the highest ability to discrimination the landraces; they revealed 265 bands with an average of 22.1 band/landrace. The polymorphism that detected by individual primer combinations ranged from 56.7 to 100%. The average genetic similarity ranged from 0.46 to 0.87 among the twelve tested landraces. The highest similarity was recorded between the landraces Qrati and Khdari1, Táeafi and Helow Khashabi. Landrace Zeglabi showed broad diversity comparing to the other landraces.

Comparative evaluation of DNA markers

Different marker systems have some advantages and it is better to revalidate the diversity data using two or more marker systems. Wang *et al*. (2007) identified the genetic relationship among pomegranates available in Taiwan, seedling and cutting derived plants introduced from China, Singapore and United States, were with internal transcribed spacer (ITS) sequences of rDNA, ISSR and RAPD markers. Sequences of 5.8S rRNA and ITS in eight samples of five lines showed no differences among these lines. RAPD analysis revealed 22 polymorphic bands produced by 6 primers with 29.72% polymorphism. One specific band was found only in the Singapore line. According to UPGMA clustering in RAPD polymorphic bands the 30 pomegranate lines could be divided into five groups with their similarities of 71%. Dinucleotides motif: (GA)n sequence presented major part of pomegranate genome through ISSR analysis. Based on UPGMA clustering of ISSR polymorphic bands, these lines could be divided into four groups with their similarities of 92%. The result of ISSR clustering analysis coincided with the origin of the individual pomegranate lines. It was suggested that ISSR and RAPD markers could be used on identifying part of the pomegranate lines.

CONCLUSION AND FUTURE THRUSTS

Pomegranate cultivation is still confronted with several problems like bacterial blight, wilt, fruit borer, fruit cracking etc. in India that modern breeding programmes must try to resolve. The future of this fruit depends on the selection of high quality cultivars with soft seeds and fruits resistant to bacterial blight, cracking and fruit borers (Tous and Ferguson 1996; Sharma *et al.* 2006). It is here where plant biotechnology can make a dent. Micropropagation of elite pomegranate genotypes has proven to be partly successful and most of the success achieved so far is through organogenesis. Being woody in nature the culture of elite material has pervasive microbial contamination of most tissues and the activation of polyphenolaxidases following explanting of tissues, surface disinfestations and culture conditions. On the other hand, *de novo* regeneration, i.e. somatic embryogenesis need to be exploited more vigorously for its application of genetic engineering and synthetic seed production, *in vitro* conservation etc. A limited genetic transformation attempt has being used to target specific production problems in the popular cultivars without altering the major horticultural traits. There needs to be renewed focus on organogenesis using vegetative tissues like leaf explant; ensuring explanting year-round. Furthermore, cryopreservation of embryogenic cultures has to be exploited been and need to be perfected so that it can be used to back up field germplasm collections. Diversity analysis and marker-assisted selection will go a long way in fastening the pomegranate breeding and germplasm management. In conclusion, it evident that several limitations are imposed in the tree and fruit characteristics (Levin 1990; Jalikop and Kumar 1990) and thus modern approaches and tools need to be explored for pomegranate breeding.

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