A REVIEW ON *IN VITRO* APPROACHES TOWARDS POMEGRANATE IMPROVEMENT IN INDIA

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Abstract
Pomegranate (Punicagranatum L.) is highly delightful edible fruits which mostly grown in the tropical and subtropical regions of the world. Pomegranate not only cultivate as edible fruit but also for pharmaceutical and ornamental uses. During the past years, protocols for in vitro propagation were established for number of fruit trees. For woody fruit trees, conventional breeding techniques find quite difficult, slow and time consuming. Therefore it is an emerging prerequisite to establish efficient protocols for regeneration and somatic embryogenesis which is applicable in genetic transformation technology. In present review article we try to focus on in vitro approaches towards improvement of pomegranate using most significant and reputed research articles which were published in University Grant Commission (UGC) approved journals.

Keywords: Pomegranate, *Punicagranatum* L., *in vitro*

Introduction

Pomegranate has beautiful red flower, therefore it grows in parks and gardens (Deepika and Kanwar, 2010). There is one consideration that pomegranate originated in Iran and North India (Stover, and Mercure, 2007). In the world, India has second rank with 7,90,000 tonnes productivity per annum (Sadegh, 2015). All the states of India cultivate the pomegranate (from Kanyakumari to Kashmir) but commercially cultivated only in Maharashtra. Other states like Gujarat, Rajasthan, Karnataka,Tamil Nadu, Andhra Pradesh, Uttar Pradesh, Punjab and Haryana also planted at small-scale and total area under cultivation is 1,00,000 ha which yielding 4,50,000 tonnes fruit per year (Patil et al. 2011). Pomegranate can be grown on variety of soils from acidic sandy loam to alkaline calcareous soils even in drought area (Sepulveda et al 2000). For cultivation of pomegranate, India has all favourable condition but production is very less because of lack of disease free plant material of selected variety. Therefore there is urgent need to develop suitable method of large scale propagation of selected variety (Desai et al 2018). During the past decades, protocols for*in vitro* propagation were established for number of fruit trees (Bajaj, 1986; Hutchinson and Zimmerman, 1987).Regenerations of plantlets were achieved through *in vitro* organogenesis, somatic embryogenesis and calluses (Omura et al. 1987). For woody fruit trees, conventional breeding techniques find quite difficult, slow and time consuming due to heterozygosity and long duration between successive crosses with developing hardwood cuttings(Sriskandarajahet al. 1994; Naiket al. 1999; Naik and Chand 2003; Singh et al. 2007).
Therefore it is an emerging prerequisite to establish efficient protocols for regeneration and somatic embryogenesiswhich is applicable in genetic transformation technology. In present review article we try to focus on *in vitro* approaches towards improvement of pomegranate using most significant and reputed research articles which were published in University Grant Commission (UGC) approved journals.

Material and methods

For preparation of present review, we studied several research papers, review articles and books. We daily visited to our college library and studied available literature. We also access several online journals. Among these we select significant and reputed research articles and efforts were made for review on *in vitro* approaches towards pomegranate improvement in India.

In vitro studies

Naiket al. (1999) described a rapid and effective system for *in vitro* clonal propagation of important cultivar Ganesh by using nodal stem explants (collected from mature tree). Surface sterilized (by aqueous solution of 5% liquid detergent Laboline and 0.1% $HgCl_2$ for 5 min. with 5-6 time washing with autoclaved distil water) nodal stem segment (1 to 1.5 cm) and inoculate on Murashige and Skoog (1962) (MS) medium supplemented with 0.5 to 3.0 mg/Lzeatinriboside (ZR)or benzyladenine (BA) or 0.01 to 0.5 mg/L thidiazuron (TDZ).Naiket al. (1999) reported that during their investigation, 95% of the explants (surface sterilized with Laboline and $HgCl_2$) were found free from microbial contamination but they face the problem of browning of culture

medium within three days of inoculation of explants. First browning was observed at cut ends of the explants and it was progressively spread into the culture medium. They also observed the extreme browning problem in explants (older shoots) than younger shoots which resulted into inhibition of shoot development and explants died by the end week. The browning problem of *in vitro* cultures was reported due to oxidation of phenolic substances which released by cut ends explants by polyphenol oxidase or peroxidase. Some other researcher suggest solution to this problem by using absorbing agent like activated charcoal or polyvinylpolypyrrolidoneinin the culture medium while in some cases its overcome by inoculation of explants on fresh medium or seal the cut ends of explants with paraffin wax (Broome and Zimmerman, 1978; Weatherhead et al. 1978; Lloyd and McCown 1980; Gupta et al., 1980 Amin and Jaiswal, 1988; Bhat and Chandel, 1991; Samina et al. 2014). Naik et al. (1999) illuminate to this browning problem by transferring explants to the new fresh MS medium fortified with same composition after 24 hour interval and problem was completely solved after two successive subculturing and normal shoot development observed.

Shoot development occurs in the nodal explants induced by bud break on MS medium fortified with cytokinin while at the same time shoot development not observed in phytohormone free MS medium.The medium fortified with BA (1 mg/L)and ZA(2 mg/L) showed bud break in explants with 93% and 85% respectively within 12-15 days of inoculation. Within 4-5 days shoot elongation observed by transferring shoots on MS medium containing 1 mg/L (lower concentration) ZR. This observation of Naiket al. (1999) in agreement with other fruit tree plants like guava(Amin and Jaiswal, 1988) and mulberry (Pattnaik et al. 1996) which suggest BA was the most operative than other cytokinins for bud breaking and shoot development.Naik et al. (1999) also inoculate nodal explant on TDZ (0.01 to 0.5 mg/L) for development of shoot. They observed maximum number of bud break (63%) at 0.05 mg/L but shoot was unsuccessful to elongate. Though some researchers widely used TDZ for woody plants regeneration and achieved shoot proliferation (Huetteman and Preece, 1993)but in the investigation of Naik et al. (1999), it shows poor results by reduction in shoot development frequency with higher concentration of TDZ but elongation of shoot (3.6 cm in 10 days) achieved successfully when shoots transferred on MS media fortified with 0.5mg/L BA.WhenNaiket al. (1999) transferred excised shoots on half strength MS medium (without auxin), induction of roots not observed even after one month. For rooting purpose they used 0.5 to 2 mg/Lauxins (IAA and IBA) but maximum number (86%) of rooting observed on half strength MS medium fortified with 1.0 mg/L IBA within 8-10 days. With elongation of primary roots, few more new roots observed (within 5-7 days); when rooted shoots were inoculate on auxin-free half-strength MS medium. Some other researchers also reported that IBA is best for rooting in other tree plants like *Artocarpusheterophyllus*(Rahman and Blake, 1988) and *Morusnigra* (Yadav et al. 1990).

From past few decades considerably major efforts were carried out for tissue culture techniques in tree propagation. Across the world various researchers try to develop efficient and reliable protocols for *in vitro* regeneration through organogenesis or somatic embryogenesis using different kinds of explants(Hutchinson and Zimmerman, 1987; Litz and Jaiswal, 1991; Grosser, 1994; Zimmerman and Swartz, 1994).
In another investigation Naik et al.(2000) also try to focus on high frequency axillary shoot proliferation and regeneration from cotyledonary nodes, as result they developed *in vitro*regeneration protocol. They achieved shoot development on MS medium supplemented with 2.3 to 23.0 µM BA and KIN from cotyledonary nodes. BA and KIN and their concentrations considerably influences shoot proliferation. Naik et al. (2000) was achieved maximum number of shoots per explant on MS medium supplemented 9 µM BA. On same medium subculturing shoot cultures were established. For further multiplication they cut the shoot into nodal segments and inoculated on fresh medium. Within 60 days,30 to 35 shoots were developed from single cotyledonary nodes. Root induction was observed on half strength MS medium fortified with 0.054 to 5.4 µM NAA from *in vitro* regenerated shoots and maximum number of shoots were observed on 0.54 µM NAA.

Naik et al. (2003) also try to examine the impact of ethylene inhibitors like silver Nitrate ($AgNO_3$) and Aminoethoxyvinylglycine (AVG) on adventitious shoot organogenesis. They first time focused on stimulatory effects of these inhibitors on adventitious shoot organogenesis encouraging to high frequency plant regeneration from explant like cotyledon segments derived from axenic cultures of pomegranate. They used MS medium supplemented with 8.9 µmol/L BA + 5.4 µmol/L NAA with range of $AgNO_3$ (10-15 µmol/L) or AVG (5-15 µmol/L). After shoot differentiation, explants with shoot clumps transferred on same medium without $AgNO_3$ or AVG. For investigation of interaction, explants like cotyledon grown on medium with or without 20-60µmol/L 2-chloroethylphosphonicacid (CEPA). This chemicals were prepared separately, filtered and added to the autoclaved medium. $AgNO_3$(20 µmol/L) or AVG (10 µmol/L) was found favourable when added to the shoot regeneration medium by considerably improves the percentage and number of shoots per cotyledon explants but at the same time $AgNO_3$and AVG did not show promotive effect on hypocotyl explants. Among the different concentrations of $AgNO_3$ and AVG, Naik et al (2003) suggested the 20 µmol/L $AgNO_3$showed 57% shoot bud regeneration while 20 µmol/L AVG showed 53 % shoot bud regeneration from cotyledon. For further investigation of effect of ethylene on shoot regeneration Naik et al. (2003) also exogenously applied CEPA (an ethylene releasing compound). By applying CEPA they resulted that promotive effect of ethylene inhibitors on shoot

regeneration was reversed by CEPA. In case of AVG medium, the frequency of shoot regeneration (cotyledon) declined gradually (23%) while at the same time $AgNO_3$initiated shoot less affected by CEPA.

Murkute et al. (2004) were attempted for rapid *in vitro* multiplication by using nodal segment and shoot tip explants of cultivar Ganesh. They obtained direct regeneration of shoots without formation of callus from shoot tip and nodal segments. They achieved shoot differentiation on MS basal medium fortified with BAP with IAA or NAA. They found that shoot buds were not formed on BAP alone or with addition of coconut water (10% v/v) but on MS medium supplemented with 1 mg/L BAP and 0.5 mg/L NAA shoot differentiation occurred. This occurs due to combine effect of cytokinins and auxins on cell elongation. So they consider that absolute values hormones were important than ratio between auxin and cytokinin (Broome and Zimmerman, 1978; Murkute et al. 2004). After six to twelve days of inoculation, shoot tip and nodal explants developed shoot buds on nodes. They obtained 1 to 2 shoots on MS medium fortified with 1 mg/L BAP and 0.5 mg/L NAA within 6 to 8 days on shoot tip and 1 to 3 shoots bloomed from nodal segment. Maximum shoot height obtained on MS medium fortified with1 mg/L BAP and 0.5 mg/L NAA from shoot tip (3.19 cm) and nodal segment (3.11 cm). Murkute et al. (2004) correlate their results with Zhang and Stoltz (1991), as application of BAP and NAA in combination. They observed maximum rooting (66.4%) on ½ MS medium fortified with 0.5 mg/L NAA within 8 to 9 days. With this results Murkute et al. (2004) succeeds for rapid *in vitro*regeneration of cv. Ganesh by using nodal segment and shoot tip as explants.

Recently Desai et al. (2018) reported the efficient micropropagation protocol for variety Bhagwa using axillary shoot buds as explants. Pomegranate contains phenolics which is result of secondary metabolism. They also face the problem of phenolic exudation. *In vitro* growth of explants restricted by exudation of phenolics. (Desai et al 2018). They solve this problem by using five treatments of distilled water, Ascorbic acid, Polyvinylpyrrolidone (PVP), activated charcoal. They suggest the best way for escape from phenolic exudation, by transferring explants to fresh medium at regular interval (24h). They also suggest that by soaking of explants in distilled water, exudation (50%), browning (50-75%) and survival (20-40%) improved while in case of soaking of explants in Ascorbic acid was 50–75% (exudation), 25–50% (browning)and 10–50%(survival) were recorded. They also try to addition of Ascorbic acid in media but Ascorbic acid unsuccessful to control phenolic exudation while addition of PVP to media decreases the rate of exudation but survival of percentage also affected (upto 10%). In case of activated charcoal they achieved the survival percentage from 10 to 50%.

For shoot induction, two different media (MS and WPM) fortified with different combinations of BAP (0.5 to 4.0 mg/L) and NAA (0.1 to 0.5 mg/L)were used by Desai et al. (2018). To identify the best shoot induction medium they transferred the explants to same medium combinations for three subsequent subcultures and observations were made by number of shoots and leaves and length of shoots. After using both the media, they suggest WPM is best medium for shoot induction than MS medium. They recorded the sprouting percentage on MS (30-80%)and WPM (40-80%) as well as they also recorded the bud break duration on MS (10 days) and WPM (7 days). They achieved highest number of shoots (2.75 ± 0.89), number of leaves (3.71 ± 0.25) and length of shoots (1.76 ± 0.51cm) on MS medium fortified with 0.5 mg/L BAP and 0.2 mg/L NAA while on WPM medium supplemented with 1 mg/L BAP maximum number (5.71 ± 1.23) of shoots, number of leaves (0.58 ± 0.33 to 5.17 ± 0.56) were achieved.For multiple shoot induction they used different cytokinins like BAP and KIN, among these they achieved highest number of shoots (11.21 ± 0.14) and shoot length (6.54 ± 0.85 cm) on 1 mg/L BAP while highest number of leaves (25 ± 0.36) obtained on 0.5 mg/L BAP. At the same time KIN has moderate effect on number of shoots while highest number of leaves (10.41 ± 0.84) and length of shoot (4.20 ± 0.20 cm) was reported on media supplemented with 0.5 mg/L Kin. Developed shoots showed 100% rooting on various rooting medium with various concentrations of phytohormones. The WPM medium (without any plant growth regulators) developed highest number (5.60 ± 0.74) and length (6.78 ± 0.52 cm) of roots. They also try ½ WPM medium which resulted into 4.80 ± 0.80 roots. Thus, with tremendous efforts Desai et al. (2018) succeeds in development of an efficient micropropagation protocol of variety Bhagwa.

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