



RESEARCH ARTICLE

Micropropagation of Pomegranate (*Punica Granatum L.*) ‘Ganesh’ Cultivar from Nodal Explants.

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ABSTRACT

The present study was carried out to develop a rapid *in vitro* multiplication protocol using nodal explants. Complete plantlets have been successfully regenerated from node explants. The explants were incubated on Murashige and Skoog (MS) culture medium supplemented with different combinations of 6-benzyl amino purine (BAP) Indole acetic acid (IAA) and kinetin. Multiplication of node explants was achieved on BAP while from other combinations of growth hormone, The response of various growth regulators was investigated. The combination of showed the highest rate of shoot induction (100%) and a shoot regenerated about 6 shoots per explants when cultured on MS media supplemented with BAP (4 μM) within 2 weeks. The multiple elongated shoots were obtained on MS basal medium combination of Activated charcoal and growth hormones. Well-developed roots were achieved on MS medium in combination with BAP and IAA as well as 80% of plantlets were survived in the soil successfully in the hardening process. A robust and optimized protocol will be helpful for the mass production of this economical important fruit.

Abbreviations: BAP-6-Benzyl Amino Purine, IAA- Indole Acetic Acid, IBA Indole 3- Butyric Acid MS-Murashige and Skoog medium, LSD- Least Significant Differences, CRD- Completely Randomized Design

Keywords: Auxins, Cytokinins, *Punica granatum*, Micropropagation, Rooting

INTRODUCTION

Pomegranate is a fruit-bearing deciduous small tree and belongs to the family Punicaceae. It is an economically important plant which is growing tropical and sub-tropical regions of the world due to its delicious edible traits with pharmaceutical and ornamental usage (Jayesh and Kumar 2004). Pomegranates are rated a super fruit because of its excellent medicinal capabilities and numerous health advantages (Silva *et al.* 2013). Pomegranate fruit includes ellagitannins, notably punicalagins anthocyanins, flavonols, and flavonoids, among other beneficial components (Salgado *et al.*, 2012; Yuan *et al.*, 2018). Pomegranate is valued highly for its delicious edible fruits are rich in sugars, vitamins, polysaccharides, polyphenols, and minerals (Ferrara *et al.*, 2014). Because of its widespread use in the pharmaceutical and food industries, the demand for high-quality pomegranate planting material is increasing.

Pomegranates are propagated commercially through stem cuttings by vegetative propagation. These methods are time-consuming and labor-intensive, and they have other drawbacks such as a low success rate and the fact that new plants need a year to develop. As a result, plantlets are not available throughout the year. Furthermore, this conventional method of propagation does not offer disease-free and healthy plants (Kanware *et al.*, 2010). Despite a large rise in pomegranate planting acreage, the fruit's production is severely hindered by diseases and insect pest attacks (Pathania *et al.*, 2019). In recent years, the bacterial blight and wilt diseases causing enormous

losses of the crop and propagation through conventional methods does not ensure diseases-free healthy plantlets. Therefore, to increase production, quick propagation methods are required. Seeds are not a highly dependable means of propagation since they cause population heterozygosity. Furthermore, employing hardwood and softwood cuttings for plant propagation

does not guarantee the development of disease-free clones (Desai *et al.*, 2018). In the large-scale propagation, the use of micropropagation techniques for over the past decades has increased significantly. The tissue culture has many benefits over the old propagation techniques, including uniform quality, seasonal independence, and rapid mass production of disease-free material and in vitro culture is the only mass propagation technique of healthy plants over a short period of time. The aim of this study was to investigate the effect MS medium with different concentrations of BAP, KIN on shoot induction, proliferation and elongation stage, and auxins (IAA) on rooting stage of node explants Then, the major purpose of

this research was the development of a micropropagation protocol via node explants for the most popular cultivars Ganesh, under cultivation in India.

MATERIALS AND METHODS

Plant material and explant preparation

This work conducted by using nodal segment explants from newly emerged shoots, containing three to four node, each were collected from 3 years mature mother plant of pomegranate cv. Ganesh from Institute of biosciences and Technology, MGM University, Aurangabad. Leaves were removed. The excised nodes were washed thoroughly in running tap water for about 30 minutes to remove traces dust and dirt. Explants were shifted in laminar air flow hood and followed by three times rinsing in sterile distilled water. Nodal segments were further soaked in Streptomycin solution (200 mg/L) treatment was also given to explants for 10 min. and then washed by sterile distilled water. Finally, 0.1 per cent mercuric chloride solution for 8 min. was used to treat these explants followed by three times washes with sterile distilled water for complete sterilization of nodal explants.

Inoculation

Sterilized explants were inoculated on shoot induction media. After establishing transferred explants on proliferation media for growth, completely proliferated and elongated explants were then transferred to rooting media.

Culture media

MS medium with different concentration and combination of growth hormones were tested for micropropagation of pomegranate cultivar 'Ganesh'. Media was prepared as a basal medium supplemented with organic acids and vitamins. Sucrose was added at 30.0 g/L and myoinositol at 0.1 g/L. The pH of the prepared media was adjusted between 5.8 and agar-agar (Hi-Media) was added as 8.0 g/L for media solidification. For shoot induction stage, BAP 2 to 8 μ M/L, IAA 0.2 to 0.6 μ M/L, silver 50 μ M/L and adenine sulphate were used while for proliferation stage, BAP 0.4 to 0.8 and Kin 2 to 6 μ M/L and IAA 0.1 to 0.5 μ M/L were tested. Also, for rooting stage, two different auxins; IBA and IAA were tested at 0.2, 0.4 and 0.6 μ M/L on MS medium at full strength

Statistical analysis

All the experiments were setup in the completely randomized design (CRD) and repeated three times, each treatment consisted of 50 explants and the means separation were done according to Least Significant Differences (LSD) at 5% level.

RESULTS

Effect of BAP on shoot bud induction

Nodal explants on MS medium supplemented various concentration of plant growth regulators were inoculated, it reacted by an approach of shoot bud break at different levels of BAP with 100 % frequency. At BAP (4 μ M) maximum shoot bud induction 6 shoots per explants was recorded, followed by 4 buds at BAP (6 μ M) (Fig.1) raising the level of BAP in the node explant promoted shoot bud induction up to (4 μ M). However, higher levels of BAP had an inhibitory influence on bud induction (Table 1).

Table 1. Effect of PGR on treatment of shoot estimation of cotyledonary nodal explant of Pomegranate

Treatment	Concentration (μ m/L)
T ₀	MS
T ₁	MS + BAP (2 μ m/L) + Kin (2 μ m/L)
T ₂	MS + BAP(4 μ m/L) + Kin (4 μ m/L)
T ₃	MS + BAP (6 μ m/L) + Kin (6 μ m/L)
T ₄	MS + BAP (8 μ m/L) + Kin (8 μ m/L)
T ₅	MS + BAP (10 μ m/L) + Kin (10 μ m/L)

Effect of KIN on proliferation of shoots

Results for the nodal explants grown on MS medium with different concentration of KIN 1 to 4 μ M/L indicated that the highest average growth response (97%) was recorded on MS medium co KIN 1, containing 0.2 μ M/L IAA, whereas five to six shoots per explants having highest shoot length (1.2 cm) was recorded at same concentration.

Table 2. Effect of BAP +KIN+IAA and full strength of medium on the shoot initiation response in Pomegranate

Treatment	Concentration(μ m/L)
T ₀	MS
T ₁	MS + BAP (2 μ m/L) + Kin (1 μ m/L) + IAA (0.2 μ m/L)
T ₂	MS + BAP(4 μ m/L) + Kin (2 μ m/L) + IAA (0.2 μ m/L)
T ₃	MS + BAP (6 μ m/L) + Kin (3 μ m/L) + IAA (0.2 μ m/L)
T ₄	MS + BAP (8 μ m/L) + Kin (4 μ m/L) + IAA (0.2 μ m/L)
T ₅	MS + BAP (10 μ m/L) + Kin (6 μ m/L) + IAA (0.2 μ m/L)

Effect of BAP and IAA in proliferation medium on leaf induction (Table3)

For the nodal explants grown on MS medium with concentration of 0.1 to 0.5 μ M/L of BAP and IAA, the data in Table 4 shows that the highest average maximum leaf

number (12 to 218) was recorded on MS medium containing 4 μ M/L BAP and 0.2 μ M/L IAA. Similarly, 98 and 96% explants showed maximum leaf formation in proliferation medium having same concentration of BAP and IAA, respectively (Figure A-H).

Table 3. Morphogenic effect of various concentrations of cytokines (BAP/KIN) and auxins (IAA) with ADS and activated charcoal added in medium

Treatment	Concentration (μ m/L)
T ₀	MS+ Activated charcoal +ADS
T ₁	MS + AC + ADS+BAP(2 μ m) +KIN(2 μ m) + IAA (0.5 μ m)
T ₂	MS + AC + ADS+BAP(4 μ m) +KIN(4 μ m) + IAA (1 μ m)
T ₃	MS + AC + ADS+BAP(6 μ m) +KIN(6 μ m) + IAA (1.5 μ m)
T ₄	MS + AC + ADS+BAP(8 μ m) +KIN(8 μ m) + IAA (2 μ m)
T ₅	MS + AC + ADS+BAP(10 μ m) +KIN(10 μ m) + IAA (2.5 μ m)



Fig. A : Explant of Pomegranate

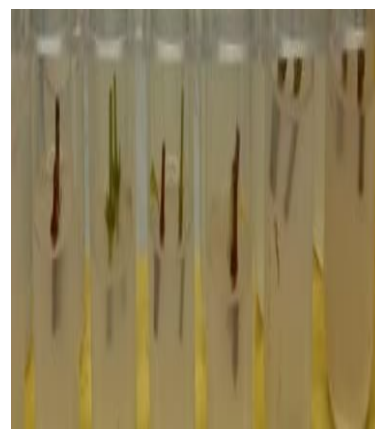


Fig. B: Explant inoculated from MS medium with BAP and Kinetin (2 to 10 μ M) conc.,



Fig. C: Explant estimation on MS medium with BAP and KIN conc.,



Fig. F: Shoot bud proliferation in nodal segment explant on MS medium supplement with BAP + KIN + IAA,



Fig. D: Explant initiation on MS medium with BAP +KIN+IAA conc.,



Fig. G: Shoot bud proliferation in shoot apex explant on medium supplemented with activated charcoal + Adenine sulphate,



Fig. E: Shoot bud proliferation in nodal segment explant on MS medium supplement with BAP + KIN + IAA

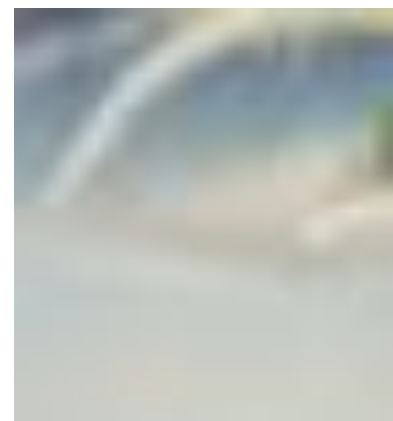


Fig. H: *In vitro* rooting on full strength MS medium with BAP+KIN+IAA

Effect of adenine sulphate on Shoot induction

The nodal explants grown on MS medium with different concentration of adenine sulphate (10 to 50 mg/L), five to six shoots per explants was recorded in the medium having 50 mg/L adenine sulphate (Figure 3). The data in Table 1 shows that the highest average growth response (97%) was recorded on MS medium containing 50 mg/L adenine sulphate.

Effect of IAA in MS medium on rooting

The MS medium containing 0.2 µM/L IAA showed the highest rooting response. The data in Table 2 shows that the highest average rooting response was recorded on MS medium containing 0.2 µM/L IAA (97%). However, thick root formation was observed in medium containing 0.2 µM/L IBA. Root length of 0.3 to 3.4 cm was recorded on medium containing IBA.

DISCUSSION

In this study, young nodes were used as explants. While an explant gets older, the regeneration capacity decreases. In the same study, in banana, using young suckers as explants was effective during the establishment stage (Strosse et al., 2006). Similarly, Yildiz (2012) reported differences in regeneration capacity of seedlings in different ages that used as microcutting in flax (*Linum usitatissimum* L.) In pomegranate, HgCl₂ and NaOCl are the most widely employed surface sterilants. In this research, HgCl₂ was effective in surface sterilization as Kalalbandi et al. (2014) reported by using 0.1% HgCl₂ for 10 min, minimum microbial contamination and the highest survival (90.58%) was observed. According to Naik et al. (1999) and Murkute et al. (2004), a common disinfectant in sterilization of pomegranate explants was 0.1% mercuric chloride.

We were successfully developed a protocol for the micropropagation of pomegranate (*P. granatum* L.) "Ganesh" Cultivar. MS medium proved to produce best vegetative growth characteristics. These findings are contrast to Samir et al. (2009), they found that WPM is best for vegetative growth compared to MS and NN medium.

IAA and BAP combinations were rewarding in many fruit tree species (Zimmerman and Swartz, 1994). Synthesis and activities of auxin, cytokinins and ethylene are thought to be closely related (Klee and Romano, 1994). Ramesh et al. (2006) also reported that the addition of adenine sulphate (60 mg/L) along with other growth regulators was the most effective in inducing shoot multiplication. as well as adenine sulphate promotes shoot multiplication (Shrivastava and Banerjee, 2008).

In this study, we used BAP (cytokinin), IAA (auxin), adenine sulphate. The highest number of shoot per explants was observed on MS medium containing 4 µM/L BAP, 0.2 µM/L IAA and 50 mg/L adenine sulphate with sucrose (30%) MS medium when different levels of alone BAP and IAA were tried, MS medium showed significant proliferation response in 2 µM/L KIN. Same trend was found in the maximum number of leaves on proliferation medium containing 2 µM/L KIN and 0.2 µM/L IAA. Singh and Patel (2014) showed the best result at multiplication stage in medium containing 1.0 µM/L -1 BAP + 1.0 µM/L -1 kinetin. IBA-derived auxin has strong roles in various aspects of root development, including regulation of root apical meristem size, root hair elongation, lateral root development, and formation of adventitious roots. Gao et al 2022 shown that IAA promoting adventitious root formation in tea plants.

In this experiment, when proliferated shoots were subjected to *in vitro* rooting and shoot elongation in Half strength MS medium containing IAA at 0.2 µM/L respectively with 30% sucrose, However, thick root formation was observed in media containing 0.4 µM/L IBA. Similarly, to this finding, rooting in regenerated shoots from cotyledon derived callus cultures of *P. granatum* L. cv. 'Ganesh' was observed in half strength MS medium supplemented with IBA by Murkute et al. (2004)

In this study, cytokinins (BAP/KIN) and auxins (IAA) were applied to nodal segment, explants. Under MS media supplemented with 4.0 µM/L BAP nodal segment explants had the most shoot proliferation. These findings are similar with those published in pomegranate by Kalalbandi et al., (2014). In our findings it also observed that cut end of explants leads to browning of the

medium and reduced explants development. To avoid and minimize this problem, various workers tried the use of PVP, Activated charcoal and silver nitrate solution etc. and such compounds have been suggested to control phenol exudation (Murkute et al., 2002).

CONCLUSION

Our study focused on using a appropriate explants with particular concentration and combination of plant growth regulators which can play an important ingredient for better multiple shoot initiation and rooting ability to form into complete plantlets of pomegranate Ganesh cultivar. Moreover, the findings would be useful for development of direct organogenesis protocol in this species, genetic improvement through somaclonal variation and production of disease-free plant material.

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