

An *in vitro* regeneration system for pomegranate (*Punica granatum* L. cv. Bhagwa) from leaf explants

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Abstract: The pomegranate cultivar Bhagwa (*Punica granatum* L. cv. Bhagwa) is the most popular commercial cultivar in India. Traditional propagation methods are beset with many difficulties that restrict their large-scale multiplication. Thus, the current study was carried out to establish a reliable protocol for genotype-specific *in vitro* propagation of pomegranate cultivar Bhagwa from the leaf explants via indirect somatic embryogenesis and organogenesis. The leaf explants were cultured in Murashige and Skoog (MS) or Woody Plant Medium (WPM) supplemented with various concentrations and combinations of 6-Benzylaminopurine (BA) and 1-Naphthaleneacetic acid (NAA) for callus induction (BA (0,1,2,3,4,5 mg l⁻¹) and NAA (0, 0.1, 0.2, 0.3, 0.4, 0.6 mg l⁻¹)). 100% callus induction was observed in MS medium supplemented with 1 mg l⁻¹ BA and 0.2 mg l⁻¹ NAA. The best callus growth was observed on MS medium supplemented with 1 mg l⁻¹ BA and 1 mg l⁻¹ NAA combination after 30 days. MS media containing 5 mg l⁻¹ BA with 0.4 mg l⁻¹ NAA produced the highest number of shoots (4 per explant). Among the treatments tested, shoot proliferation was highest on Woody Plant Medium (WPM) supplemented with 5 mg l⁻¹ Kinetin (KT) and on an average produced seven shoots. The excellent root growth (3.9 cm mean length) and rooting per cent (91.1%) were obtained on WPM medium comprising 0.2 mg l⁻¹ Indole-3-butyric acid (IBA). Eighty percent of the rooted plantlets were hardened and established in the soil.

Keywords: Callus induction; embryogenesis; micropropagation; *Punica granatum* L. cv. Bhagwa; root induction; shoot induction.

Abbreviations: AC_activated charcoal; BA_6-benzylaminopurine; DMRT_Duncan Multiple Range Test; IBA_indole-3-butyric acid; KT_kinetin; MS_Murashige and Skoog; NAA_naphthaleneacetic acid; PVP_polyvinylpyrrolidone; TALEs_transcription activator-like effectors; WPM_Woody Plant Medium; *Xcp_Xanthomonas citri* pv. *punicae*

Introduction

The pomegranate (*Punica granatum* L.) is considered a nutritionally valuable fruit crop due to its antioxidative and anti-inflammatory functions. In India, pomegranate is an emerging crop with 2.6 lakh hectares under cultivation, producing 23.15 lakh million tons (National Horticulture Board. Government of India, 2024). The cultivar Bhagwa is the most sought after among the farmers due to its many desirable traits like high yield and attractive uniform colored big fruits with deep red arils, long shelf life and higher prices in the market compared to other varieties (Prabhuling and Huchesh, 2018). Although India has favorable conditions for cultivating pomegranates, the production is limited due to the scarcity of suitable, disease-free planting materials.

Traditionally, pomegranates are propagated through stem cuttings (hard and soft wood) or air layering (Chandra and Dhinesh Babu, 2010). These methods are time-consuming and labor-intensive, and the new plants require a year to develop. Further, employing the conventional propagation method does not guarantee disease-free and healthy plants (Kanwar et al., 2010). Propagation by seeds is not dependable since they cause population heterozygosity (Chauhan and Kanwar, 2012). Therefore, micropropagation method is considered as an alternative to supplement the traditional ways to satisfy the demand for healthy planting material and to increase pomegranate production.

In recent years, the bacterial blight disease of pomegranate caused by *Xanthomonas citri* pv. *punicae* (*Xcp*) has caused significant economic losses, hampering the crop's sustainable cultivation and raising concerns among pomegranate growers. The disease is primarily transmitted through infected planting material, emphasizing the need for propagation methods to produce disease-free and healthy pomegranate plants (Kumar et al., 2010). Xanthomonads in general, are found to induce disease by targeting host genes termed as susceptibility genes, using its endogenous transcription activator-like effectors (TALEs). Customizing the CRISPR-Cas9 tool offers a potential solution to enhance resistance against bacterial blight by targeting TALE binding elements in the promoter region of susceptibility genes (Sivaraman et al., 2022). The

efficacy of CRISPR-based gene editing in pomegranate plants relies significantly on an efficient genotype-specific *in vitro* propagation method from easily available explants.

Being woody, the micropropagation of pomegranates has proven to be partly successful (Naik et al., 1999). Pomegranate *in vitro* culture is significantly affected by explants. The phenolic exudation from the cut surface of explants, tissue browning and microbial contamination (systemic or latent) make it challenging to establish *in vitro* culture in pomegranate (Singh et al., 2010). Chauhan and Kanwar, 2012 reviewed the various treatments that were attempted to address these limitations, including the culture of juvenile explants, culture in darkness, rapid sub-culturing, culture in the liquid medium, soaking of explants in water or solution containing antioxidants and the use of antioxidants such as activated charcoal (AC), polyvinylpyrrolidone (PVP) and citric acid in the culturing medium to reduce phenolic exudation, but the effectiveness of these methods vary within cultivar types and the physiological conditions of plants

Indirect somatic embryogenesis and organogenesis are two key regeneration pathways in plants that rely on the formation of callus, an undifferentiated mass of cells. In indirect somatic embryogenesis, somatic cells dedifferentiate into an embryogenic callus, which forms pro-embryonic masses that eventually develop into somatic embryos capable of forming complete plants. While this process takes longer due to the callus phase, it results in a higher number of regenerated plantlets and is widely used in crop species. Indirect organogenesis, on the other hand, involves the formation of a pluripotent callus, from which shoots or roots regenerate without forming somatic embryos. Regeneration of pomegranate plantlets *in vitro* has been reported through either organogenesis from calli obtained from cotyledons, leaf segments (Murkute et al., 2004; Deepika and Kanwar, 2010) and anthers (Moriguchi et al., 1987) or through embryogenesis from different seedling explants (Jaidka and Mehra, 1986), petals (Nataraja and Neelambika, 1996) and immature zygotic embryos (Deepika and Kanwar, 2010). But, these protocols have limitations of low shoot generation and fewer seedlings. Micropropagation of the pomegranate cultivar Bhagwa through axillary shoot proliferation from nodal segments, shoot tips (Murkute et al., 2004), and cotyledonary nodes (Suhasini et al., 2017) has been reported. Guranna et al. (2017) reported the regeneration of the cultivar Bhagwa through indirect organogenesis using pooled calli obtained from different plant parts (shoot tip, nodal segment, leaves and petal segments), the efficacy of the leaf explant for pomegranate micro propagation could not be ascertained due to the nature of this study. The present study was undertaken to develop an *in vitro* regeneration protocol for developing complete plantlets from the leaves of cultivar Bhagwa through indirect somatic embryogenesis and organogenesis. Since leaves are the most plentiful source of explants, an efficient regeneration system from leaves through somatic embryogenesis can help in the genetic improvement of plants through genetic transformation or gene editing.

Results

Callus induction and somatic embryogenesis

The leaf explants that were transferred to fresh medium at 24 hr intervals for three consecutive days showed better establishment and higher survival rates compared to the control, where explants were maintained without such transfers (Fig 1a.). Leaf explants in all treatments with BA initiated callus in about 12-14 days after culture, as observed through routine visual monitoring of the cultures. The whole surface of the leaves was covered with callus within 5-6 weeks (Fig 1b.). In the present study, callus induction was observed only when BA was included along with NAA, whereas explants cultured with NAA alone did not initiate callus, indicating that the presence of cytokinin (BA) was necessary for callus induction. Among all treatments, MS medium containing 1 mg l⁻¹ BA and 0.2 mg l⁻¹ NAA had the maximum callus induction (100%) (Table 1). Adding BA to the medium increased the callus induction rate, while a further increase in BA levels decreased this trait. 1 mg l⁻¹ of both BA and NAA was the best combination of cytokinin and auxin for callus growth, as it resulted in callus with the highest fresh weight of 0.67g and dry weight of 0.087g (Table 2, Fig 1c.). Different somatic embryo development stages, i.e., globular, heart, torpedo and cotyledonary, were observed on the embryogenic callus under a stereo microscope (Fig 2.). The somatic embryos appeared either individually or in tightly clustered groups, with a pale yellow to green coloration. Among these, globular embryos were the most predominant. Among the various concentrations tested, BA and NAA at 1 mg l⁻¹ was found to be the optimum concentration for embryogenesis. These embryos were subsequently subcultured onto fresh WPM medium for further proliferation. After six weeks on hormone-free WPM medium, complete plantlet formation was achieved from the somatic embryos.

Shoot regeneration

The first shoot regenerated after three weeks in MS medium containing 5 mg l⁻¹ BA with 0.4 mg l⁻¹ NAA (Fig 3a.). Shoot induction was not observed on hormone-free media. Also, shoots could not be regenerated on media containing only auxin (NAA), indicating that the presence of cytokinin (BA) is critical for shoot induction. MS media produced more shoots and longer shoots compared to WPM. The sprouting percentage for MS ranged from 24.44-86.66%, while it ranged from 19.99-73.33% for WPM. MS supplemented with 5 mg l⁻¹ BA and 0.4 mg l⁻¹ NAA showed the highest number of shoots; the longest was from MS supplemented with 5 mg l⁻¹ BA and 0.2 mg l⁻¹ NAA (Fig 3b-c.). In the case of WPM, augmentation with 5 mg l⁻¹ BA with 0.2 mg l⁻¹ NAA recorded the maximum number of shoots. The detailed results for the different treatments are shown in Table 3.

Shoot proliferation

Among the two cytokinins (BA and KT) tested for multiple shoot proliferation, medium supplemented with 5 mg l⁻¹ KT showed the highest number of shoots and maximum shoot length. After two subsequent subcultures, the total

Table 1. Effects of BA and NAA on the percent of callus induction from leaf explants of pomegranate cultivar Bhagwa on MS and WPM media after 30 days.

MS						
NAA(mgl ⁻¹)	BA(mgl ⁻¹)					
	0	1	2	3	4	5
0	0	53.33 l	91.66 cde	90 def	28.33 n	31.66 n
0.1	0	58.33 kl	55 l	46.66 m	45 m	43.33 m
0.2	0	100 a	96.66 abc	81.66 gh	73.33 i	26.66 n
0.3	0	93.33 bcd	85 fg	76.66 hi	73.33 i	66.66 j
0.4	0	98.33 ab	96.66 abc	86.66 efg	78.33 hi	65 j
0.5	0	88.33 def	81.66 gh	61.66 jk	56.66 kl	48.33 m
WPM						
NAA (mgl ⁻¹)	BA(mgl ⁻¹)					
	0	1	2	3	4	5
0	0	51.66 kl	88.33 cde	83.33 ef	26.66 n	28.33 n
0.1	0	56.66 jk	53.33 jkl	43.33 m	41.66 m	45 m
0.2	0	88.33 cde	98.33 a	76.66 gh	71.66 hi	25 n
0.3	0	91.66 bcd	83.33 ef	80 fg	76.66 gh	66.66 i
0.4	0	93.33 abc	95 ab	83.33 ef	71.66 hi	66.66 i
0.5	0	86.66 de	83.33 ef	58.33 j	53.33 jkl	50 l

Parameters have been recorded after 30 days of transfer into callus growth media. Data are in the form of mean ±SE. Means with the same letter along the column are not significantly different at p <0.05.

Table 2. Effects of BA and NAA on callus growth from leaf explants of pomegranate cultivar Bhagwa after 30 days.

BA (mgl ⁻¹)	NAA (mgl ⁻¹)	Fresh weight (g)	Dry weight(g)
0	0	0.13±0.026 d	0.016±0.005 d
0	0.5	0.19±0.081 d	0.023±0.005 d
0	1	0.16±0.02 d	0.07±0.017 bc
0.5	0	0.153±0.047 d	0.02±0.01 d
0.5	0.5	0.423± 0.058 bc	0.046±0.005 bc
0.5	1	0.36±0.069 bc	0.05±0.017 bc
1	0	0.337±0.032 c	0.026±0.015 cd
1	0.5	0.463±0.035 b	0.063±0.023 b
1	1	0.677±0.105 a	0.087±0.005 a

Parameters have been recorded after 30 days of transfer into callus growth media. Data are in the form of mean ±SE. Means with the same letter along the column are not significantly different at p<0.05.

number of shoots produced in WPM medium containing 5 mgl⁻¹KT was seven (Table 4). An increase in BA or KT levels up to 5 mgl⁻¹ enhanced the number of shoots produced, while a further increase in either BA or KT concentration did not affect the shoot multiplication rate.

Rooting and hardening

Root initiation was observed 10-12 days after transfer, and profuse rooting was obtained within four weeks (Fig 3d.). The WPM medium containing 0.2 mgl⁻¹ IBA showed the highest rooting percentage (91.10%), producing the greatest number of roots per shoot with an average root length of 3.91 cm (Table 5, Fig 3e.). However, the rooting ability declined with higher auxin concentrations, as increasing IBA to 0.5 mgl⁻¹ reduced rooting to 68.88%, while 0.2 mgl⁻¹ NAA resulted in 55.55% rooting and further increase to 0.5 mgl⁻¹ lowered it to 48.88%. Average growth of the plantlets was observed in the first week after transfer to pots, and subsequently, they showed good development and high survival rates. Eighty percent of the rooted plantlets survived after two weeks of transplantation to the soil (Fig 3f.). After successful primary hardening in the growth chamber, the plantlets were transferred to the polyhouse.

Discussion

This study has established an *in vitro* propagation protocol for pomegranate *via* indirect somatic embryogenesis and organogenesis from leaf explants of Bhagwa. Pomegranate is an understudied plant with limited tools for genetic studies and pomegranate cultivation is plagued by pests and diseases, and therefore, for functional genomics and genetic improvement studies, an *in vitro* propagation method is a much-needed tool. For several years, pomegranate tissue culture has been actively pursued to develop regeneration protocols and genetic transformation studies. However, pomegranate seems to be a difficult plant material for tissue culture response, and efforts to micro-propagate pomegranate have met with varied success. The main problem in the tissue culture of pomegranate is the development of axenic cultures, phenol

Table 3. Effects of two different media (MS and WPM) along with various combinations of cytokinins and auxins on *in vitro* shoot induction.

BA (mg l ⁻¹)	NAA (mg l ⁻¹)	MS			WPM		
		% Sprouting	No. of shoots/ explant	Average length of shoots (cm)	% Sprouting	No. of shoots/ explant	Average length of shoots (cm)
0	0	0	0	0	0	0	0
1	0	24.44 g	1.22±0.19 i	0.48±0.29 cdef	19.99 j	0.99±0.33 i	0.46± 0.12def
2	0	46.66 de	2.33±0.33 ef	0.46±0.08 cdef	35.55 fgh	1.77±0.19 efgh	0.83±0.18 abcd
3	0	42.22 def	2.10±0.50 fg	0.48±0.18 cdef	31.10 ghi	1.55±0.38 ghi	0.25±0.14fg
4	0	33.33 fg	1.66±0.33 ghi	0.29±0.28 ef	42.22 def	2.33±0.33 cde	0.71±0.20 abcde
5	0	31.11 fg	1.48±0.18 hi	0.25±0.23 ef	24.44 ij	1.22±0.19 hi	0.35±0.30efg
0	0.2	0	0	0	0	0	0
1	0.2	39.99 ef	1.77±0.19 gh	0.47±0.38 cdef	37.77 efg	1.88±0.19 efg	0.47±0.26def
2	0.2	48.89 de	2.44±0.19 ef	0.49±0.41 cdef	42.22 def	2.11±0.19 def	0.55±0.40cdef
3	0.2	53.32 d	2.66±0.33 e	0.51±0.32 cdef	48.88 cde	2.22±0.69 cde	0.65±0.22bcde
4	0.2	73.33 bc	3.66±0.33 bcd	1.09±0.25 bc	59.99 bc	2.99±0.33 b	0.74±0.30 abcde
5	0.2	79.99 bc	3.99±0.33 b	1.84±0.47 a	73.33 a	3.88±0.19 a	1.07±0.15 a
0	0.4	0	0	0	0	0	0
1	0.4	51.08 de	2.55±0.19 ef	0.77±0.56 cde	46.66 def	2.33±0.33 cde	0.49±0.17def
2	0.4	68.88 bc	3.44±0.19 cd	0.79±0.56 cde	46.66 def	2.33±0.33 cde	0.49±0.28def
3	0.4	71.10 bc	3.55±0.19 bcd	0.95±0.54 cde	53.33 cd	2.66±0.33 cd	0.89±0.18 abc
4	0.4	75.55 bc	3.77±0.50 bc	1.03±0.59 cd	64.33 ab	3.21±0.50 ab	0.93±0.13 ab
5	0.4	86.66 a	4.66±0.33 a	1.66±0.49 ab	73.33 a	3.66±0.33 a	1.02±0.13 ab
0	0.6	0	0	0	0	0	0
1	0.6	42.22 def	2.10±0.50 fg	0.35±0.28 def	26.63 hij	1.33±0.33 ghi	0.46±0.25def
2	0.6	53.33 d	2.67±0.33 e	0.48±0.44 cdef	51.10 cd	2.77±0.38 bc	0.45±0.14def
3	0.6	64.44 c	3.22±0.19 d	0.61±0.39 cdef	59.99 bc	2.99±0.57 b	0.47±0.26def
4	0.6	66.66 c	3.33±0.33 cd	0.53±0.40 cdef	48.88 cde	1.99±0.33 ef	0.47±0.09def
5	0.6	71.10 bc	3.55±0.19 bcd	0.47±0.31 cdef	44.44 def	1.77±0.50 efgh	0.43±0.10ef

Parameters have been recorded after eight weeks of transfer in shoot induction media. Data are in the form of mean ±SE. Means with the same letter along the column are not significantly different at p< 0.05.

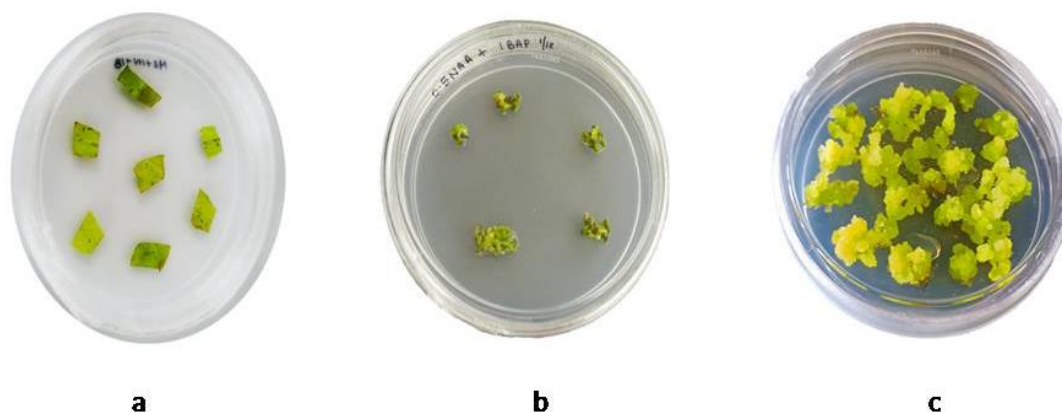


Fig 1. Different stages in the *in vitro* regeneration of *Punica granatum* L. cv. Bhagwa; a) Leaf explants transferred to fresh media after three continuous transfers; b) Callus initiation from leaf explants on MS medium after 12-14 days of culture; c) Growth of callus on MS medium supplemented with 1 mg L⁻¹BA and NAA after 30 days of culture.

leaching, and browning of tissue explants (Naik et al., 1999). Although somatic embryogenesis from seedlings (Jaidka and Mehra, 1986) or petals (Nataraja and Neelambika, 1996) has been documented in a few pomegranate cultivars, information regarding the conversion of somatic embryos into plantlets is lacking.

Micropropagation of the pomegranate cultivar Bhagwa has been explored using axillary shoot tip, nodal buds and seeds (Suhasini et al., 2017; Desai et al., 2018). Leaves are excellent sources of explants and enable genotype-specific propagation as opposed to cotyledonary leaves. Tissue browning caused by phenolic exudation from the cut surface of leaf segments was overcome by repeatedly transferring the explant into fresh media at 24 hrs. Out of different treatments used for callus induction, the MS media supplemented with 1 mg l⁻¹BA and 0.2 mg l⁻¹ NAA resulted in the highest callus induction percentage. The growth of the callus was measured in terms of fresh and dry weight. The highest explant for the fresh and dry weight of callus was observed in the MS medium supplemented with 1 mg l⁻¹BA and NAA. These results are similar to those obtained by Bonyanpour and Khosh-Khui (2013) and Guranna et al. (2017) in which the highest callus growth from the leaf segments of dwarf pomegranate Nana and cultivar Bhagwa was observed in MS supplemented with 1 mg l⁻¹BA and NAA.

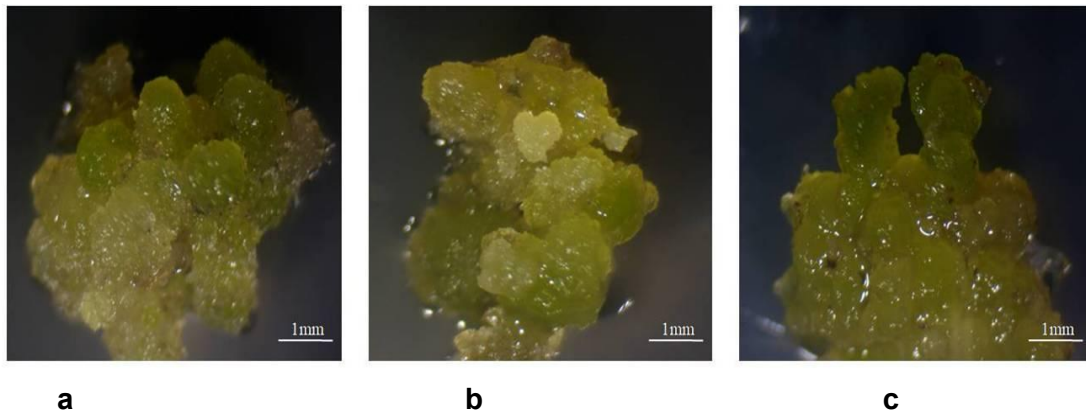


Fig 2. Different stages of somatic embryogenesis viewed under a stereo microscope; a) Globular-shaped somatic embryo; b) Heart-shaped somatic embryo; c) Torpedo and early cotyledonary stage of embryo.

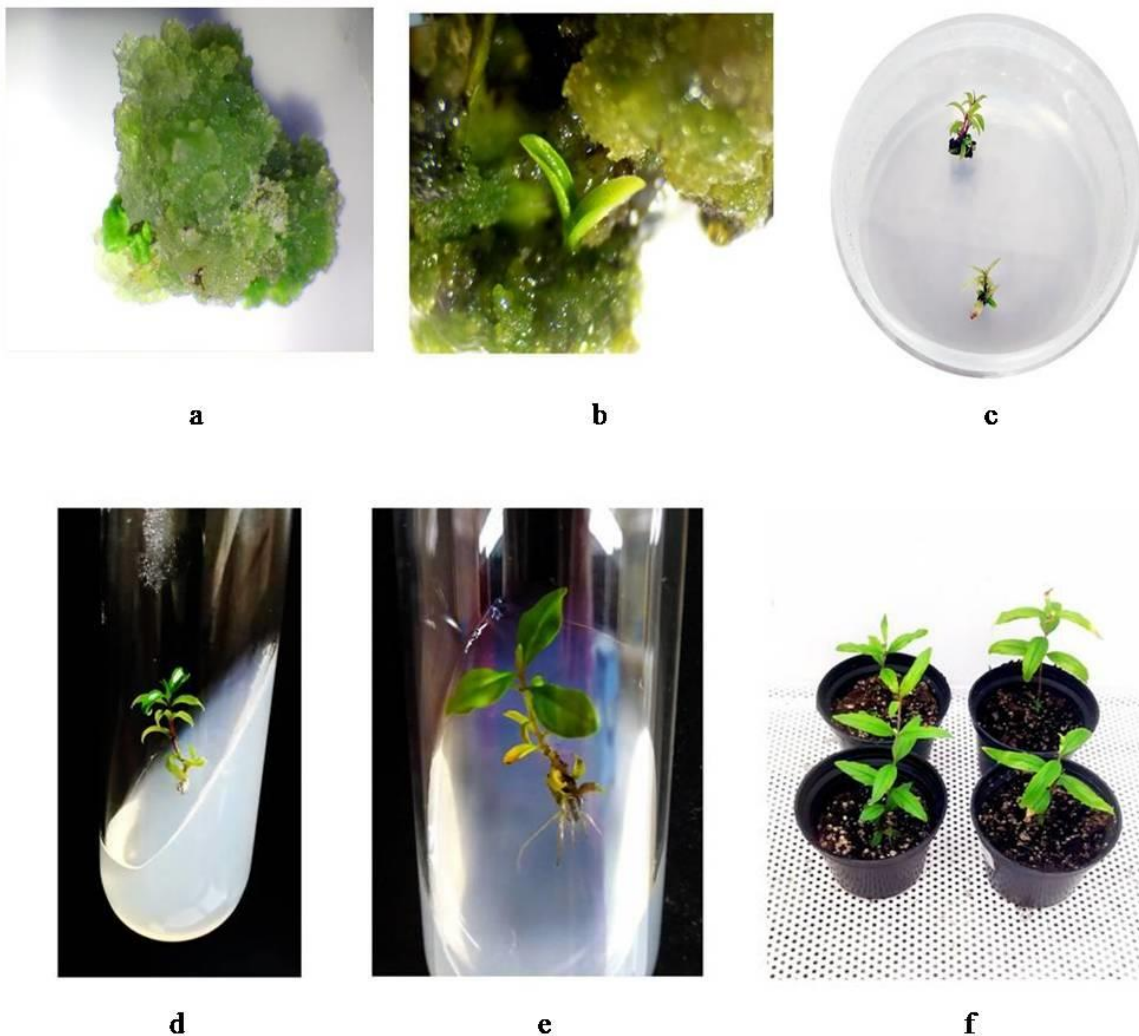


Fig 3. Different stages in the *in vitro* regeneration of *Punica granatum* L. cv. Bhagwa; a) Shoot initiation on MS medium; b) Shoots developed from the callus on MS medium; c) Regenerated shoots on MS with 5 mg l⁻¹ BA and 0.4 mg l⁻¹ NAA; d) Root initiation from shoots on rooting media; e) Well-rooted shoots on WPM medium containing 0.2 mg l⁻¹ IBA; f) Two week old transplanted plantlets in pots.

The results indicate that the higher cytokinin to auxin ratio promotes dedifferentiation and callus initiation in Bhagwa. In contrast, maximum callus growth was observed on MS medium containing 1 mg l⁻¹ BA and 1 mg l⁻¹ NAA, suggesting that a balanced auxin-to-cytokinin ratio enhances cell division and proliferation. Together, these results clearly demonstrate that the specific auxin-to-cytokinin ratio is critical for both callus induction and subsequent growth in pomegranate cultivar Bhagwa.

Different stages of embryo development, i.e., globular, heart, torpedo and cotyledonary stages, were observed on the

Table 4. Effects of various concentrations of BA and KT on multiple shoot induction.

BA (mg l ⁻¹)	KT (mg l ⁻¹)	MS		WPM	
		No. of shoots	Average length of shoots (cm)	No. of shoots	Average length of shoots (cm)
0	0	1.33±0.33 e	1.43±0.49e	1.10±0.50 f	1.44±0.13f
1	0	2.33±0.33 cd	1.71±0.16de	3.21±0.50 de	1.81±0.10de
2	0	2.88±0.50 cd	1.94±0.05bcd	3.44±0.50 de	1.94±0.10cd
3	0	3.11±0.84 c	1.93±0.11bcd	3.77±0.19 d	2.02±0.16c
4	0	4.55±0.50 b	2.09±0.09 abc	4.66±0.33 c	2.06±0.10 bc
5	0	4.77±0.69 b	2.24±0.12 ab	5.55±0.50 b	2.25±0.12 b
0	1	1.88±0.50 de	1.70±0.16de	2.88±0.50 e	1.71±0.14e
0	2	1.99±0.33 de	1.80±0.14cd	3.10±0.69 de	1.96±0.11cd
0	3	2.77±0.50 cd	1.99±0.14 abcd	3.77±0.19 d	2.07±0.09bc
0	4	4.66±0.66 b	2.16±0.11 ab	5.33±0.33 bc	2.24±0.07 b
0	5	5.77±0.50 a	2.33±0.08 a	7.21±0.50 a	2.47±0.09 a

Parameters have been recorded after four weeks of transfer in shoot multiplication media. Data are in the form of mean ±SE. Means with the same letter along the column are not significantly different at p<0.05.

Table 5. Effects of various concentrations and combinations of NAA and IBA on *in vitro* root induction.

WPM (Half-H / Full-F)	Plant growth regulators		Rooting (%)	No of roots	Average length of roots (cm)
	NAA (mg l ⁻¹)	IBA (mg l ⁻¹)			
H	0	0	46.63 ef	2.33±0.33 efg	1.78±0.32 f
H	0.2	0	39.97 fg	1.99±0.33 fg	1.12±0.21 g
H	0.5	0	28.88 g	1.77±0.19 g	2.69±0.17 cd
H	0	0.2	51.10 def	2.55±0.50 ef	2.1±0.28 ef
H	0	0.5	53.33 def	2.55±0.19 ef	3.80±0.26 a
H	0.2	0.2	48.88 ef	2.44±0.38 efg	2.13±0.24 ef
H	0.5	0.5	48.88 ef	2.44±0.19 efg	3.92±0.13 a
F	0	0	68.87 c	3.33±0.33 cd	3.64±0.25 ab
F	0.2	0	55.55 de	2.77±0.50 de	3.16±0.49 bc
F	0.5	0	48.88 ef	2.44±0.50 efg	2.51±0.40 de
F	0	0.2	91.10 a	4.88±0.19 a	3.91±0.11 a
F	0	0.5	68.88cd	3.33±0.33 bc	2.53±0.44 def
F	0.2	0.2	82.22 ab	3.99±0.33 b	3.65±0.24 ab
F	0.5	0.5	73.33 bc	3.77±0.19 bc	2.37±0.19 de

Parameters have been recorded after four weeks of transfer into rooting media. Data are in the form of mean ±SE. Means with the same letter along the column are not significantly different at p < 0.05.

embryogenic callus. The appearance of globular structures marked the first sign of embryogenesis, indicating their early development on MS medium supplemented with 1 mg l⁻¹BA and 0.5 mg l⁻¹NAA. Some callus tissues showed heart-shaped embryos representing the early differentiation of the shoot and root meristems. Concurrently, torpedo and cotyledonary stage embryos were also observed on further subculturing into MS medium supplemented with 1 mg l⁻¹BA and 1 mg l⁻¹ NAA. The initially pale-yellow cotyledons gradually turned green and eventually germinated. After two more weeks in culture, the regenerated embryos developed shoots and roots, ultimately forming complete plantlets after six weeks on hormone-free WPM medium.

We obtained a high percentage of shoot regeneration (86.66%) and the maximum number of shoots on MS medium with a high BA level and a low NAA level (BA 5 mg l⁻¹ and NAA 0.4 mg l⁻¹). Adding NAA along with BA to the medium enhanced the regeneration response significantly. Also, there was no sign of shoot induction when calli were cultured in a medium devoid of BA. Maximum shoot length (1.84 cm) was observed on MS supplemented with BA 5 mg l⁻¹ and NAA 0.2 mg l⁻¹. Guranna et al. (2017), observed maximum shoot induction, number of shoots and shoot length in cultivar Bhagwa when calli were cultured on MS basal media containing BA 2 mg l⁻¹, NAA 0.1 mg l⁻¹ and GA3 0.50 mg l⁻¹. Omura et al. (1987), reported maximum shoot regeneration and shoot growth occurred at a moderate BA (2 µM) and low NAA (0.1 µM) in dwarf pomegranate 'Nana'. These differences may be related to the difference in cultivars. Murkute et al. (2004), suggested using MS medium to establish explants and shoot regeneration in pomegranate. Contrarily, El-Agamy et al. (2010) reported that using WPM for culture establishment and shoot growth in Manfalouty and Nab El-Gamal pomegranate cultivars. In our observation, MS medium is best for shoot induction in Bhagwa compared to WPM medium, whereas WPM was more efficient than MS for shoot multiplication. Also, the plantlets produced in WPM were found to be more vigorous than those in the MS medium. Comparison between KT and BA showed that KT was more effective than BA in shoot proliferation and produced more shoots on the pomegranate cultivar Bhagwa. This is in contradiction to reports by Omura et al. (1987), that 5 µM of BA was more effective than the same concentration of KT and zeatin for shoot proliferation of dwarf pomegranate (98% bud formation and 4.2 shoots per explants). Also, Naik et al. (1999) reported BA was more effective than KT for shoot

proliferation in cultivar Ganesh using MS medium supplemented with 9 μm BA.

IBA was more effective compared to NAA in terms of the frequency of rooted shoots and the number of roots formed per shoot. Of the various concentrations of IBA tested, 0.2 mg l^{-1} was the best treatment, producing a mean number of 4.88 roots per shoot at 91.10% frequency. Consistent with our results, Bonyanpour and Khosh-Khui (2013) reported that WPM medium with a low level of IBA produced maximum rooting in the semi-dwarf pomegranate cultivar. Guranna et al. (2017) reported the highest percent rooting and maximum number of roots per plantlet in the cultivar Bhagwa when microshoots were cultured on full-strength MS medium supplemented with a higher concentration of IBA (3 mg l^{-1}). More than 80 % of the rooted plantlets were successfully acclimatized and transferred to the soil. Acclimatized plants showed normal growth, and no signs of morphological variation were observed.

Comparison with previous studies using nodal or apical explants of pomegranate cultivar Bhagwa indicates that the leaf explants used in the present study exhibited markedly superior regeneration responses. Earlier reports on micropropagation of this cultivar using nodal or shoot-tip explants achieved limited shoot proliferation, typically ranging from 1 to 2 shoots per explant (Kalalbandi et al., 2014; Uma Maheswari et al., 2023), while Bachake et al. (2019) obtained moderate proliferation (3.8 shoots per explant) from shoot tips under modified MS media with multiple additives. Guranna et al. (2017) observed superior shoot proliferation (up to eight shoots per explant) using pooled calli derived from multiple explants rather than evaluating regeneration from specific explant types, and achieved only 72.5% rooting. Patil et al. (2011) reported higher proliferation (10–15 shoots per explant) using complex media supplements such as adenine sulfate and silver nitrate, which increases cost and may reduce reproducibility. Moreover, their protocol relied exclusively on nodal explants and direct adventitious shoot regeneration, limiting its applicability for somatic embryogenesis, callus-mediated propagation, and genetic transformation. In contrast, leaf explants of *Punica granatum* cv. Bhagwa in our study produced 100% callus induction and high shoot regeneration frequency (86.66%), producing up to seven shoots per explant, indicating a markedly higher regenerative competence compared to most nodal and apical explant studies. Moreover, excellent rooting (91.1%) and survival (80%) were obtained during acclimatization. Thus, under the optimized culture conditions and for the Bhagwa genotype, our study indicate leaf explants as superior alternative to apical or nodal explants in callus induction, shoot multiplication, rooting, and plantlet establishment. Being abundant and readily accessible, they provide a reliable, genotype-specific system suitable for large-scale clonal propagation, genetic transformation and genome editing studies in *Punica granatum* cv. Bhagwa.

Materials and methods

Tissue culture medium and condition

MS and WPM medium (Himedia, India), along with various combinations of plant growth regulators, were tested for the micropropagation of the pomegranate cultivar Bhagwa. Both the media were supplemented with 3% sucrose and 0.8% Agar (Himedia, India). The pH of the medium was adjusted to 5.6–5.8 before adding agar and was autoclaved at 121°C. All the cultures were maintained at a temperature of $25 \pm 2^\circ\text{C}$ with a photoperiod of 16hrs light at 1500 lux intensity and 8 hrs of dark.

Explant preparation and surface sterilization

Healthy and disease-free leaves of *Punica granatum* L. cv. Bhagwa were collected from approximately ten-year-old plants maintained in a polyhouse. Prior to explant collection, the plants were pruned and shifted to a growth chamber under controlled conditions to promote new growth, and freshly grown, fully expanded young leaves were selected as explants. The excised leaves were thoroughly rinsed with running tap water to minimize surface microbial contamination before sterilization. Surface sterilization of leaves was performed under aseptic conditions in an ESCO Class II Biosafety Cabinet (BSC). Leaves were initially treated with 0.1% aqueous solution of Tween-20 for 30 seconds, followed by 3–4 washes with sterile water (1 minute each). They were then treated with 70% ethanol for 30 seconds, and 1% sodium hypochlorite (Himedia, India) for 2 minutes, and finally washed 4–5 times with sterile distilled water (1 minute each).

Callus induction, growth and somatic embryogenesis

Two separate experiments were conducted to study callus induction and callus growth. After sterilization, the leaf explants were excised into segments of 0.5–1 cm in size and placed in abaxial orientation on MS or WPM medium supplemented with various concentrations of BA (0, 1, 2, 3, 4, 5 mg l^{-1}) and NAA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg l^{-1}) for callus induction. All treatments were replicated thrice, with a total of 20 leaves used per treatment. Each replication consisted of 20 leaf segments derived from these 20 original leaf explants. Cultures were maintained at $25 \pm 2^\circ\text{C}$ and subjected to a 16hrs light (1500 lux light intensity emitted from cool-white fluorescent lamps) and 8hrs dark photoperiod. Cultures were transferred to fresh medium every 24 hrs for three consecutive days to reduce browning and phenolic exudation. After that, they were maintained by regular subcultures at two-week intervals on fresh media for four weeks, and the percentage of callus induction was recorded. For the callus growth experiment, uniformly developed calli obtained from the best induction treatments were cut into approximately 0.5 cm segments and transferred onto MS medium supplemented with different concentrations of BA and NAA (0, 0.5, and 1 mg l^{-1}). These cultures were maintained under the same growth conditions to assess callus proliferation and fresh weight increase. Treatments were maintained for four weeks, and callus fresh and dry weights were measured. Different developmental stages of somatic embryos were recorded after six weeks of culture. All the embryo stages were photographed using a stereo microscope (Euromex Microscopen B.V. Netherlands). The somatic embryos were cultured for two weeks on the same media and then transferred to hormone-free WPM to promote their conversion into plantlets.

Shoot induction and multiplication

The *in vitro*, shoot induction was tested in two different media viz. MS and WPM, along with varying combinations of BA (0, 1, 2, 3, 4, 5 mg l⁻¹) and NAA (0, 0.2, 0.4, 0.6 mg l⁻¹). The callus pieces (about 0.5–1 cm) were cultured, and the treatments were subcultured on the same medium every two weeks. After eight weeks, the number and length of the regenerated shoots in each treatment were recorded. Regenerated shoots of about 1–1.5 cm in length were cultured on MS and WPM medium supplemented with various concentrations of BA and KT (0, 1, 2, 3, 4, 5 mg l⁻¹) for shoot proliferation. The total number of shoots and length was recorded after four weeks. All treatments were maintained under a 16 hrs photoperiod with 1500 lux light intensity emitted from cool-white fluorescent lamps.

Rooting

For root initiation, regenerated shoots, measuring around 2–4 cm in length, were excised aseptically and transferred to WPM media (Full and half-strength) fortified with various concentrations of NAA and IBA (0, 0.2, and 0.5 mg l⁻¹). Cultures were kept for four weeks, and the rooting percentage, the number of roots and the lengths of roots were recorded.

Hardening

The healthy *in vitro* rooted plantlets were carefully removed from the culture tubes and washed with distilled water to remove the adhering media. The roots were then immersed in 1% bavistin for 30 secs and rinsed in distilled water. The rooted plantlets were transferred to pots containing a sterile mixture of soilrite and vermicompost in a ratio of 1:1. The plants were covered with polyethylene bags to maintain humidity and kept in the growth chamber. The enclosed polyethylene bags were sequentially perforated each day and completely removed after two weeks. The plants were watered daily with sterile water and acclimatized for two–three weeks in a growth chamber until the plants were ready for transfer to the poly house.

Statistical analysis

The data generated from the experiments conducted for various stages, viz. callus induction, shoot induction, multiplication, rooting and hardening, were examined periodically and statistically analyzed by Duncan Multiple Range Test (DMRT) using SPSS software at P < 0.05 for each experiment.

Conclusions

The present study demonstrated that the leaf explant could be a better alternative to apical or nodal explants to regenerate *Punica granatum* L. cv. Bhagwa via indirect somatic embryogenesis and organogenesis. MS medium augmented with 1 mg l⁻¹ BA and NAA proved best for callus growth. A higher concentration of BA (5.0 mg l⁻¹) was most suitable for shoot regeneration from callus, and IBA was more effective in promoting root induction than NAA. The regenerated plants are phenotypically normal; therefore, this method provides an alternative to the current practice of micro propagation through axillary shoots and also opens up the possibility of functional genomic studies in pomegranate.

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Author Contributions

Conceptualization, G.A.; methodology, G.A.; formal analysis, G.A, and S.S.; investigation, G.A., and S.S.; data curation, G.A., and S.S.; writing-original draft preparation, S.S.; review and editing, G.A.; project administration, G.A.; funding acquisition, G.A. All authors read and approved the final manuscript.

Declaration of interest

The authors declare no conflict of interest.

Data Availability

All data supporting the study are available within this paper.

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