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Micropropagation in Pomegranate (*Punica granatum* L.) cv. 'Bhagwa' through Indirect Organogenesis and Assessment of Genetic Fidelity by RAPD Marker

Prabhuling Guranna[1*], Iranna Hosamani[2], Raghavendra Sathyanarayana[3], Rashmi Hegde[3] and Kulapati Hipparagi[4]

[1]Center for Horticulture Biotechnology, University of Horticultural Sciences, Bagalkot 587 104, Karnataka, India.
[2]Department of Horticulture, Government of Karnataka, India.
[3]Department of Biotechnology and Crop Improvement, University of Horticultural Sciences, Bagalkot 587 104, Karnataka, India.
[4]Department of Fruit Science, College of Horticulture, University of Horticultural Sciences, Bagalkot 587 104, Karnataka, India.

Authors' contributions

This work was carried out in collaboration with all authors. Author PG designed the study, managed the literature searches and wrote the first draft of the manuscript. Author IH performed the micropropagation study and statistical analysis. Author RH managed the RAPD marker study. Authors RS and KH managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Pomegranate (*Punica granatum* L.) is an ancient fruit crop belonging to the family *Punicaceae*. Conventionally, pomegranate is propagated through air layering, hard wood and semihard wood cuttings. Recently micropropagation is gaining popularity due to rapid mass propagation of disease free plants in short period of time and small space. Rapid regeneration of plants by

**Corresponding author: E-mail: gprabhuling@gmail.com;*

micropropagation can be achieved by indirect organogenesis involving induction of callus with exogenous application of plant growth regulators. In the present study nodal segment was found superior for induction of callus when cultured on MS basal medium consisting of BAP 5 mg/l + NAA 0.4 mg/l. Early shoot initiation, more number of shoots per explant and maximum shoot length was noticed when proliferated calli were cultured on MS basal medium containing BAP 2 mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l. Early *in vitro* root initiation, highest per cent rooting and maximum number of roots per plantlet were recorded when microshoots were cultured on full strength MS medium supplemented with IBA 3 mg/l. RAPD marker analysis revealed 90.66 and 9.33 % monomorphic and polymorphic bands, respectively among tissue culture regenerated plantlets of pomegranate.

Keywords: Bhagwa; callus induction; shoot proliferation; operon primers; RAPD maker.

ABBREVIATIONS

MS: Murashige and Skoog's Medium, BAP: 6-Benzylaminopurine; NAA: Naphthalene-3- acetic acid; IAA: Indole -3-acetic acid, GA_3: Gibberellic acid; IBA: Indole-3-butyric acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid; RAPD: Random amplified polymorphic DNA.

1. INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the oldest known fruit trees of the tropics and sub-tropics belonging to the family *Punicaceae*. The cultivation of pomegranate by mankind as a fruit crop dates back to antiquity and the usage of pomegranate is deeply embedded in human history with references in many ancient cultures about its use in food and medicine [1]. Pomegranate is considered native to Iran, Afghanistan and Southern Pakistan's Baluchistan region to the Himalayas in Northern India. India is the world's leading country in pomegranate production. The estimated global cultivated area under pomegranate is around 3.00 lakh ha and production is 3.00 million tonnes.

Conventionally pomegranate is propagated through air layering, hard wood and semihard wood cuttings. However, this traditional propagation method does not ensure production of disease-free and healthy plants and it is tedious as it requires about one year to raise the saplings [2]. Advances in biotechnological applications, in particular tissue culture of pomegranate were first reviewed by [3]. Since then, a number of *in vitro* studies have been carried out on pomegranate such as micropropagation through shoot proliferation by means of meristem culture, somatic embryogenesis, synthetic seed production and utilization, plant regeneration *via* shoot organogenesis either indirectly through callus cultures or directly by adventitious organogenesis in explants. Therefore, micropropagation is the best method to overcome these difficulties. Further rapid regeneration of plants can be achieved by indirect organogenesis involving callus with exogenous application of plant growth regulators. In the present study, therefore, efforts were made to optimise the protocol for micropropagation in pomegranate (*Punica granatum* L.) cv. 'Bhagwa' through indirect organogenesis.

2. MATERIALS AND METHODS

2.1 Source of Plant Material

The explants for the experiment were collected from two year old healthy and vigorously growing mother plant of pomegranate cv. 'Bhagwa' (Figs. 1 and 2) grown at fruit orchard, University of Horticultural Science, Bagalkot. The type of the explants used for present study includes shoot tip: apical portion of 2-3 cm was collected from the current season growth; nodal segment: nodal segment of 3-4 cm length was taken from mature tree of current season shoot; leaf segment: leaf segment of 1-2 cm^2 from green expanded leaves obtained from new flush and petal segment: petal segment with 2-3 cm^2 size isolated from fully developed unopened flower bud.

2.2 Surface Sterilization

Isolated explants were cleaned under running tap water for about 15 to 20 minute. Then were washed 3-4 times with distilled water containing few drops of 0.1% Tween-20 for 10 min. The explants were soaked in fungicide 1 gm/l Dithane M-45 and 500 mg/l cetrimide solution for 20 and 25 min, respectively followed by 4-5 times washes with sterile distilled water. Later explants

were treated with 500 mg/l streptocycline for 20 minute and washed four to five times with sterile distilled water. Finally explants were surface sterilized with 100 mg/l mercuric chloride ($HgCl_2$) for 1 minute (Shoot tip, Petal and leaf segment) and 2-3 minute (Nodal segment), respectively followed by four to five washes with sterile distilled water under laminar air flow cabinet.

2.3 Callus Induction

The selection of explants and proper growth regulators to induce callus is an important aspect in plant tissue culture technology. In the present study, to identify a suitable explants type and growth regulator combination for induction of callus, four different explants *viz.,* shoot tip, nodal segment, leaf segment and petal explants along with ten different concentration of MS medium +BAP 1-5 mg/l + NAA 0.40 mg/l and MS+2,4- D 1-5 mg/l were tried. Surfaced sterilised explants were transferred to Baby jars bottles (275 ml) containing 25 ml of MS medium (Table 1) and cultures were incubated under standard culture conditions of 25 ± 2^0 C, 70 % RH and photoperiodic cycle of 16 hr light and 8 hr dark period for 4 weeks. The callus quality was observed and good quality *in vitro* induced calli were pooled together after second subculture cycle.

2.4 Callus Proliferation

Good quality *in vitro* induced calli were pooled together and placed onto proliferation medium containing BAP 0.5 - 2 mg/l + NAA 0.25 - 1.5 mg/l at different concentrations for Callus proliferation. Cultures were incubated under standard culture conditions as above for 4 weeks (Table 1).

2.5 Shoot Proliferation

The *in vitro* proliferated very good and good quality of green colour calli were pooled together and placed for adventitious shooting on MS medium supplemented with BAP 0.5 - 2 mg/l , NAA 0.1- 1 mg/l , Kinetin 0.5 - 1 mg/l, GA3 0.5 mg/l and Adenine sulphate 40 mg/l at different concentrations. Cultures were incubated under standard culture conditions as above for 4 weeks (Table 1).

2.6 *In vitro* Rooting

Individual micro-shoots were obtained by cutting the multiple shoot clumps using sterilized scalpel and cultured on half and full strength ½ MS medium containing NAA 0.5 - 3 mg/l, IBA0.5 - 3 mg/l and MS medium containing NAA 0.5 - 3 mg/l, IBA 0.5 - 3 mg/l at different concentrations and 3 g/l activated charcoal. Cultures were incubated under standard culture conditions as above for 4 weeks (Table 1).

2.7 Assessment of Genetic Fidelity by Molecular Marker

Genetic stability of regenerated plantlets were analysed using PCR based RAPD marker. DNA samples were extracted from leaves samples of regenerated plantlets and mother plants. PCR amplification was carried out using operon primers. Amplified products were separated by agarose electrophoresis.

Fig. 1. Mother plant of Pomegranate cv. 'Bhagwa'

Fig. 2. Different explants of Pomegranate: (a) shoot tip; (b) nodal segment; (c) leaf segment: (d) petal segment

Completely randomized design (CRD) was employed for the experiments. The data were subjected to ANOVA as suggested by [4]. Critical difference values were tabulated at one per cent probability where "F" test was significant.

Table 1. Different stages and media composition involved in indirect organogenesis

Sl. no.	Stage	Media composition
1	Callus induction	MS + BAP1 - 5 mg/l + NAA 0.4 mg/l, MS + 2,4-D 1-5 mg/l
2	Callus proliferation	MS+ BAP 0.5 -2 mg/l +NAA 0.25-1.5 mg/l
4	Shoot proliferation	MS+BAP 1 -2 mg/l + NAA 0.1-0.5 mg/l, MS+BAP 0.5 -1 mg/l + Kinetin 0.5 -1 mg/l + Adenine sulphate 40 mg/l, MS+ BAP 1-2 mg/l+ NAA 0.1 mg/l + GA_3 0.5 mg/l
5	*In vitro* rooting	MS + NAA 0.5 - 3 mg/l, MS + IBA 0.5 - 3 mg/l

3. RESULTS AND DISCUSSION

3.1 Callus Induction

There were significant differences among the different explants with respect to callus quality (Table 2 & Fig. 3). Among them nodal and leaf segment induced very good quality of callus (++++) when cultured on MS medium supplemented with BAP 5 mg/l + NAA 0.4 mg/l. Meanwhile, MS medium supplemented with same ingredients recorded good quality of callus (+++) when shoot tip was used as explant. Good quality of callus has fireable cells, green in colour and has growing buds. The MS media provided with BAP 4 mg/l + NAA 0.4 mg/l, BAP 3 mg/l + NAA 0.4 mg/l induced good callus quality in shoot tip, leaf segment and nodal segment explants, while poor quality of callus (+) noticed in MS medium supplemented with 1,2,3, and 4 mg/l 2,4 D with shoot tip, nodal and leaf segment. Inhibition of dedifferentiation leads to poor quality of callus, which has hard, discoloured and no growing buds. Meanwhile MS medium + 2,4-D 5 mg/l recorded medium callus good quality (++). Probably this may be due to higher cell dedifferentiation, higher chlorophyll formation and protein synthesis resulting in good quality and regenerable calli (Table 2). Whereas, petals failed to produce callus. This may be attributed to higher sensitivity of petal explants to $HgCl_2$ treatment which might have led to the bleaching activity of chloride atoms resulting in death of cells.

3.2 Callus Proliferation

Among the different treatments, MS basal media supplemented with BAP 1.0 mg/l + NAA 1.0 mg/l resulted in early callus proliferation with very good quality (Table 3 & Fig. 4). This may be attributed to use of moderate level of cytokinin and auxin, which determines the organogenesis in callus [5]. Opined that moderate levels of cytokinin and auxin result in proliferation of callus and lower levels causes root formation. Similar results were obtained by [6,7] in pomegranate and [8] in *Viola patrinii* (medicinal herb).

3.3 Adventitious Shoot Proliferation

Superior shoot proliferation was observed with MS basal media containing BAP 2.0 mg/l+ NAA 0.1 mg/l+ GA_3 0.50 mg/l (Table 4 & Fig. 5). This may be attributed to the higher level of cytokinin to auxin levels resulting in *de novo* formation of

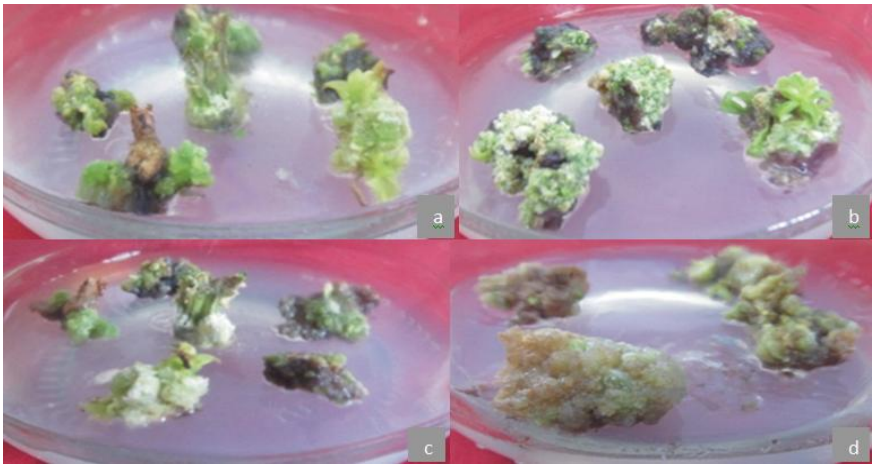

Fig. 3. Callus quality: (a) Very good; (b) good; (c) medium: (d) Poor

Table 2. Effect of type of explants and growth regulators on callus quality

Type of explants/Growth regulators	Callus quality			
	Shoot tip	Nodal segment	Leaf segment	Petal
MS+BAP 1 mg/l + NAA 0.40 mg/l	++	++	++	-
MS+BAP 2 mg/l + NAA 0.40 mg/l	++	++	++	-
MS+BAP 3 mg/l + NAA 0.40 mg/l	+++	+++	+++	-
MS+BAP 4 mg/l + NAA 0.40 mg/l	+++	+++	+++	-
MS+BAP 5 mg/l + NAA 0.40 mg/l	+++	++++	++++	-
MS+2,4-D 1 mg/l	+	+	+	-
MS+2,4-D 2 mg/l	+	+	+	-
MS+2,4-D 3 mg/l	+	+	+	-
MS+2,4-D 4 mg/l	+	+	+	-
MS+2,4-D 5 mg/l	+	++	++	-

++++: Very good, +++: good, ++: medium, +: Poor, - : No Callus

Table 3. Effect of growth regulators on callus proliferation

Treatments	Days taken for callus growth	Fresh weight (gm)	Diameter (cm)	Callus quality	Callus colour
MS+ Control	28.33	0.79	1.50	++	*
MS+BAP 0.50 mg/l + NAA 0.25 mg/l	27.50	1.12	1.74	+++	*
MS+BAP 0.50 mg/l + NAA 0.50 mg/l	26.17	1.66	1.93	+++	**
MS+BAP 1.0 mg/l + NAA 0.50 mg/l	25.33	2.11	2.40	+++	**
MS+BAP 1.0 mg/l + NAA 1.0 mg/l	19.33	2.21	2.82	++++	***
MS+BAP 1.5 mg/l + NAA 0.5 mg/l	21.83	2.16	2.27	+++	***
MS+BAP 1.5 mg/l + NAA 1.5 mg/l	20.92	0.92	2.44	+++	**
MS+BAP 2.0 mg/l + NAA 0.00 mg/l	22.75	0.84	2.12	+++	**
S.Em ±	0.52	0.04	0.04		
CD@1%*	2.06	0.16	0.13		

**Critical difference values were tabulated at one Percent where F test p<0.01 was significant*

*++: Medium, +++: Good, ++++: Very good. *: light yellow colour, **: light green, ***: Green colour*

Table 4. Effect of growth regulators on shoot proliferation

Treatments	Days taken for shoot initiation	Number of shoots per explants	Number of leaves per shoot	Length of shoot (cm)
MS+BAP 1mg/l + NAA 0.25 mg/l	27.75	1.65	6.58	1.75
MS+BAP 1mg/l + NAA 0.5 mg/l	25.31	3.38	8.94	2.15
MS+BAP 2 mg/l + NAA 0.5 mg/l	24.56	4.44	10.31	2.36
MS+BAP 0.5 mg/l + Kinetin 0.5 mg/l + Adenine sulphate 40 mg/l	20.75	5.38	11.75	3.16
MS + BAP 1mg/l+ Kinetin 0.5 mg/l + Adenine sulphate 40 mg/l	18.44	5.56	14.19	3.72
MS + BAP 1 mg/l + Kinetin 1 mg/l + Adenine sulphate 40 mg/l	17.93	6.13	15.19	4.20
MS + BAP1mg/l + NAA 0.1 mg/l + GA_3 0.5 mg/l	19.69	7.44	13.31	6.58
MS + BAP 2 mg/l + NAA 0.1mg/l + GA_3 0.5 mg/l	17.06	8.13	13.06	7.32
S.Em ±	0.44	0.25	0.36	0.12
CD@1%*	1.75	0.99	1.42	0.41

**Critical difference values were tabulated at one Percent where F test p<0.01 was significant*

adventitious buds and proliferating shoot. Better shoot growth may be due to the involvement of GA_3 in elongation of shoot. Similar findings was obtained by [9] in apple, they reported that highest multiplication and elongation of shoots was obtained on MS medium containing BAP 2

mg/l + GA_3 0.04 mg/l + NAA 0.02 mg/l. [10] reported maximum shoot induction, number of shoots and shoot length in pomegranate when leaf callus cultured on MS medium supplemented with BAP 1.5 mg/l + Kinetin 0.5 mg/l and NAA 0.25 mg/l.

Fig. 4. Callus proliferation on BAP 1.0 mg/l+ NAA 1.0 mg/l medium

3.4 Root Regeneration

The minimum days for root initiation, highest percentage of rooting, maximum number of roots and minimum days taken for root initiation was recorded on full strength MSB + IBA 3 mg/l, while highest root length was observed on ½ MS+ IBA 3.0 mg/l (Fig. 6 & Table 5). This may be attributed to higher effectiveness of IBA in adventitious rooting as it might have absorbed and utilised very efficiently as compared to NAA. The results are conformity with reports of [11,12] in papaya.

Fig. 5. Shoot proliferation on BAP 2.0 mg/l+ NAA 0.1 mg/l+ GA_3 0.5 mg/l medium

Fig. 6. *In vitro* rooting of microshoots on IBA 3 mg/l medium

Table 5. Effect of auxin and media strength on *in vitro* root regeneration

Treatments	Days taken for root initiation	Per cent rooting	Length of longest root (cm)	Number of roots per explants
Full strength MS+NAA 0.5 mg/l	26.63	13.50	0.58	1.18
Full strength MS +NAA 1 mg/l	23.75	22.50	0.77	1.35
Full strength MS+NAA 2 mg/l	24.50	20.00	1.43	2.23
Full strength MS+NAA 3 mg/l	23.00	57.50	1.81	2.69
Full strength MS+IBA0.5 mg/l	23.13	22.50	0.54	1.18
Full strength MS+IBA 1 mg/l	23.50	27.50	0.76	1.20
Full strength MS+IBA 2 mg/l	21.88	37.50	2.10	2.18
Full strength MS+IBA 3 mg/l	24.38	60.00	2.86	2.68
Half strength MS+NAA 0.5 mg/l	20.88	15.00	0.68	2.83
Half strength MS+NAA 1 mg/l	25.88	25.00	0.80	2.63
Half strength MS+NAA 2 mg/l	22.50	40.00	0.80	2.70
Half strength MS+NAA 3 mg/l	24.63	57.50	1.12	3.00
Half strength MS+IBA0.5 mg/l	22.63	30.00	0.48	2.65
Half strength MS+IBA 1 mg/l	24.38	47.50	0.71	1.68
Half strength MS+IBA 2 mg/l	22.13	61.00	2.18	2.90
Half strength MS+IBA 3 mg/l	20.25	72.50	2.59	3.95
S.Em ±	0.74	1.88	0.04	0.08
CD@1%*	2.84	7.46	0.20	0.32

**Critical difference values were tabulated at one Percent where F test p<0.01 was significant*

3.4.1 Assessment of genetic fidelity

In tissue culture propagation, indirect regeneration through callusing results in genetic instability of plantlet. Therefore these plantlets were subjected to assessment of genetic fidelity by RAPD marker because of its simplicity and cost effectiveness. A total of 30 primers screened, 12 primers gave amplification and these 12 primers were employed to asses genetic stability (Table 6, Figs. 7 & 8). The number of amplification product was primer dependent and ranged from 2-13. The primer OPA 04 amplified the maximum number of fragments (13) followed by (9) with OPD 18. The total number of amplified bands was 75, out of which 68 bands were monomorphic (90.66 %) and 7 bands were polymorphic (9.33 %). This

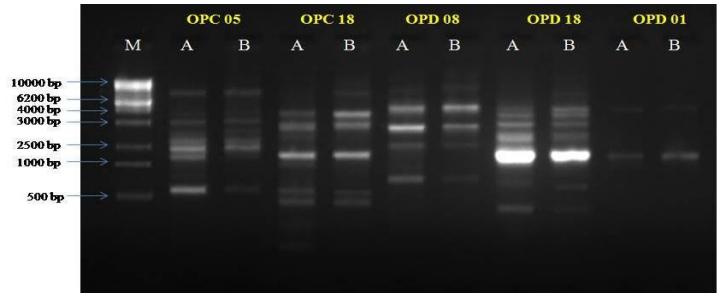

Fig. 7. RAPD gel profile generated with OPC 05; OPC 18; OPD 08; OPD 18 & OPD 01. Lane M: 1 kb ladder; A: Mother plant; B: *In vitro* regenerated plants

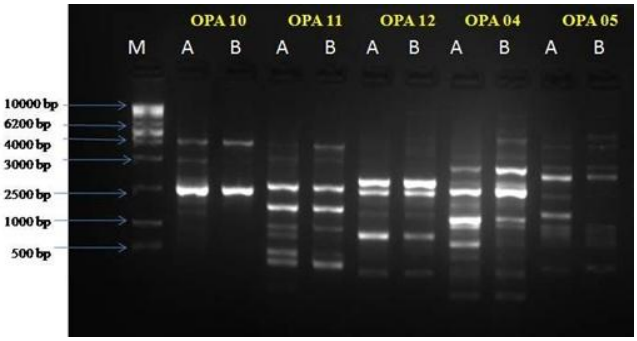

Fig. 8. RAPD gel profile generated with OPA 10; OPA 11; OPA 12; OPA 04 & OPA 05. Lane M: 1 kb ladder; A: Mother plant; B: *In vitro* regenerated plant

Table 6. List of primers, their sequences and number of amplified fragments generated by operon primers

Primer name	Sequence 5'-3'	Monomorphic band	Polymorphic band	Total scorable band
OPA 10	GTGATCGCAG	3	1	4
OPA 11	AAAGCTGCGG	8	0	8
OPA 12	TGTCATCCCC	6	0	6
OPA 04	AATCGGGCTG	12	1	13
OPA 05	AGGGGTCTTG	7	0	7
OPA 17	GACCGCTTGT	1	4	5
OPC 07	GTCCCGACGA	2	0	2
OPC 05	GATGACCGCC	7	1	6
OPC 18	TGAGTGGGTG	6	2	8
OPD 08	GTGTGCCCCA	6	0	6
OPD 18	GAGAGCCAAC	8	1	9
OPD 01	ACCGCGAAGG	2	0	2
Total		68	7	75

might be attributed to higher rate of cell division during callus formation in response to higher levels of BAP and 2, 4 - D. [13] opined that genetic variation of *in vitro* raised plant in pomegranate may be related to somaclonal variation. Somaclonal variation among microprapagated plantlets of tea cv. T-78 was detected through RAPD markers by [14]. In *Clivi miniata*, [15] observed somoclonal variation between young petal and ovaries derived *in vitro* plant and mother plant.

4. CONCLUSION

The present study established efficient protocol for micropropagation of pomegranate cv. 'Bhagwa' through indirect organogenesis with 90.66% genetic fidelity which could be used for scaling-up production of tissue culture plantlets and development of transgenic plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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