Research Article

In Vitro Callus Induction and Regeneration from Leaf Explants in (*Punica Granatum* L.)

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Abstract

Leaf explant of pomegranate were placed on Murashige and Skoog Medium (MS medium, 1962) supplemented with different concentration of cytokinins and auxins for callus induction. Maximum callus induction was observed on a medium containing 1.5 mg/l BAP followed by 1.5 mg/l Kn and IAA with 100 per cent frequency. The highest regeneration of plantlets from callus was obtained upon subculture of calli on the medium containing 1.0 mg/l and 2.0 mg/l NAA. *De novo* regenerated plantlets induced rooting at 1.0 mg/l IBA and successfully transferred to field after proper hardening.

Keywords: Pomegranate, Callus induction, Organogenesis, Tissue culture, Micropropagation

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Introduction

Pomegranate (*Punica granatum* L.) belongs to the family "Punicaceae". It is native from Iran and spread throughout the Mediterranean region of Asia, Africa and Europe [1]. It has 2n=2x=16, 18 chromosomes [2]. At global level India is the world's largest producer of pomegranates followed by Iran, Turkey, Spain, Tunisia, Morocco, Afghanistan, China *etc*. Over the last one decade, the country has registered sizeable increase in both area and production. The export of pomegranate from India has upsurged by 3.5 times during this period. There has been marked shift towards the consumption of pomegranate worldwide owing to its several nutritive, nutraceutical and medicinal properties. Pomegranate has wider adaptability ranging from normal soil type, saline soil and drought condition. Although pomegranate can be grown under varied climatic conditions of the country, it performs better in semi-arid and arid regions. In India, pomegranate is commercially cultivated in Maharashtra, Karnataka, Gujarat, Andhra Pradesh, Telangana, Madhya Pradesh, Tamil Nadu and Rajasthan. At present, Maharashtra is the leading state in acreage covering about 68.7 per cent of the area and 70.2 per cent of total production under pomegranate [3].In India, it is cultivated over 2.16 lakh ha with a production of 27.95 lakh tones and productivity of 12.94 tones/ha [4].In Rajasthan, it is cultivated over 2857 ha area with production of 10379 tones and productivity of 3.63 tones/ha. Jalore, Chittorgarh, Barmer, Bhilwara and Jodhpur are major pomegranate producing district in Rajasthan. Out of these districts Jalore is leading district with 847 ha area, 3134 tones production and 3.7 tones/ha productivity [5].

Pomegranate is commercially propagated by stem cuttings (Hardwood cutting) or by air layering. These methods are time-consuming, labour-intensive process and it has other limitations like low success rate and new plants require one year for establishment. This results in non-availability of plantlets throughout the year. Further, this traditional propagation method does not ensure disease free and healthy plants [6]. Pomegranate cell and tissue culture is not easy though regeneration from existing meristems (shoot tip and nodal bud), vegetative and reproductive plant parts have been attempted with some noteworthy success. The present investigation has been undertaken to establish reliable protocol for callus induction and organogenesis under *in vitro* conditions to produce true to type plants.

Material and Methods

The present research work was conducted on *Punicagranatum*cv. Sindhuri. Leaves were used as explant and obtained from healthy trees grown at Department of Plant Breeding and Genetics, S.K.N. College of Agriculture, Jobner. Leaf explant was sterilized by using different surface sterilization agents. Explant was washed thoroughly in running tap water for 20 minutes, these were again washed with liquid detergent (RanKleen) for ten minutes with vigorous shaking. After washing with detergent, explant was again washed with running tap water to remove any trace of detergent for 5 minutes. Finally explants were surface sterilized with 0.1 per cent $HgCl_2$ in a laminar air flow cabinet for 1-2 minutes.

Induction of callus

Leaf segments were placed on MS medium supplemented with different concentration of cytokinins (BAP/Kn0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l and auxins (NAA/IBA0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) alone and BAP (1.0 and 2.0 mg/l) + NAA/IAA (1.0 and 2.0 mg/l) and Kn (1.0 and 2.0 mg/l) + NAA/IAA (1.0 and 2.0 mg/l) in combinationfor callus induction.

Organogenesis

Once the secondary stock of callus was established from leaf explant, were used to initiate experiments on organogenesis. Small pieces of callus were cultured on MS medium supplemented with various concentration of cytokinin - BAP/Kn (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 mg/l) and Auxin - NAA/IBA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 mg/l) singly and cytokinin -BAP/Kn (1.0 and 2.0mg/l) and auxin - NAA/IBA (1.0 and 2.0 mg/l) in combinations.

Rooting

The micro propagated and regenerated shoots were subjected to different levels of auxins (IBA/NAA: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.5 mg/l.) for the induction of roots.

Results

Callus induction

When leaf explants were inoculated on medium supplemented with different concentration of plant growth regulators, it responded differently. The maximum callus (0.85 g) proliferation was reported at 1.0 mg/l BAP + 1.0 mg/l NAA with 100 per cent frequency (**Figure 1**). The semi-compact greenish yellow callus induced at the cut ends of the leaf explants was potent during subculture and used for shoot morphogenesis (**Tables 1** and **2**).

Figure 1 Callus induction in leaf explant on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA

Organogenesis

The slight to profuse semi-compact greenish yellow callus was also induced at the cut end of leaf on the MS supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA within 23 – 25 days of inoculation with 100 per cent frequency (**Figure 2**). This callus culture were maintained through serial subculture at an interval of 30 days and also assessed for shoot morphogenesis.

In the present investigation shoot morphogenesis was observed upon subculture of stock callus (derived from leaf explant and sub-cultured on 1.0 mg/l BAP + 1.0 mg/l NAA) on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l BAP + 2.0 mg/l NAA after 15 – 20 days of incubation (**Figures 3** and **4**). The reproducibility of shoot morphogenesis from callus was 40 per cent at the medium supplemented with 2.0 mg/l BAP, whereas, the reproducibility of shoot morphogenesis was 60 per cent on sub-culture at 1.0 mg/l BAP + 2.0 mg/l NAA. Thus for shoot regeneration from callus derived from leaf explant (1.0 mg/l BAP + 1.0 mg/l NAA) subculture on 1.0 mg/l BAP + 2.0 mg/l NAA found most responsive for shoot bud regeneration.

Table 1 Morphogenetic effect of various concentrations of cytokinins (BAP/Kn)and auxins (IBA/NAA) added singly in the MS medium leaf explant.

Concentration (mg/l)	Callus			Callus			
	Response (%)	Days taken for callus initiation	Fresh callus weight (g)	Concentration (mg/l	Response (%)	Days taken for callus initiation	Fresh callus weight (g)
BAP				**Kn**			
0.5	90	27.9	0.55 (+)	0.5	70	31.2	0.59 (+)
1.0	100	27.1	0.58 (+)	1.0	100	32.7	0.62 (++)
1.5	100	24.9	0.77 (+++)	1.5	100	31.0	0.70 (+++)
2.0	90	25.8	0.62 (++)	2.0	100	33.1	0.61 (++)
2.5	80	26.3	0.59 (+)	2.5	100	30.7	0.61 (++)
3.0	70	27.0	0.54 (+)	3.0	100	29.5	0.55 (+)
IBA				**NAA**			
0.5	100	26.0	0.60 (++)	0.5	70	29.0	0.52 (+)
1.0	100	26.8	0.62 (++)	1.0	70	29.1	0.54 (+)
1.5	100	27.7	0.64 (++)	1.5	80	28.5	0.55 (+)
2.0	100	27.3	0.75 (+++)	2.0	90	28.9	0.60 (++)
2.5	100	27.2	0.64 (++)	2.5	100	27.5	0.63 (++)
3.0	100	26.1	0.62 (++)	3.0	100	28.9	0.65 (++)

+=Slight callus, ++=Medium callus, +++=Profuse callus

Table 2 Morphogenetic effect of various concentration of cytokinin (BAP/Kn) and auxin (NAA/IAA) added in combination in the MS medium on leaf explants

Concentration (mg/l)	Callus			Callus			
	Response (%)	Days taken for callus initiation	Fresh callus weight (g)	Concentration (mg/l)	Response (%)	Days taken for callus initiation	Fresh callus weight (g)
Leaf explants							
BAP	**NAA (1.0 mg/l)**			**BAP**	**IAA (1.0 mg/l)**		
1.0	100	1.0	0.85 (+++)	1.0	100	25.7	0.70 (+++)
2.0	100	2.0	0.59 (+)	2.0	100	25.2	0.73 (+++)
BAP	**NAA (2.0 mg/l)**			**BAP**			
1.0	100	1.0	0.60 (++)	1.0	100	27.0	0.62 (++)
2.0	100	2.0	0.63 (++)	2.0	100	26.8	0.65 (++)
Kn	**NAA (1.0 mg/l)**			**Kn**	**IAA (1.0 mg/l)**		
1.0	100	25.1	0.57 (+)	1.0	100	25.0	0.67 (++)
2.0	100	25.0	0.60 (++)	2.0	100	25.9	0.58 (++)
Kn	**NAA (2.0 mg/l)**			**Kn**			
1.0	100	27.0	0.58 (+)	1.0	100	24.8	0.57 (+)
2.0	100	26.0	0.53 (+)	2.0	100	24.9	0.58 (+)

+=Slight callus, ++=Medium callus, +++=Profuse callus

Root induction

Three to five centimeters long shoots were excised individually from the proliferated shoot clumps and cultured on rooting medium. In present investigation, root induction was assessed in the MS media supplemented with different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.5 mg/l) of auxins (IBA and NAA). Majority of *in vitro* shoots developed roots within 20 – 22 days of incubation.

IBA and NAA both showed different morphology of roots as well as root frequency. Very thin and long roots were observed at 0.1 – 0.4 mg/l IBA, whereas, thin and medium long roots were observed at 0.5 – 0.8 mg/l IBA. Thick and small roots were observed at 1.0 mg/l and 1.5 mg/l IBA. Root frequency ranged from 20-100 per cent. Highest roots were observed at 1.0 mg/l IBA with 100 per cent frequency (**Figure 5**).

Figure 2 Sub culture of leaf derived callus culture at 1.0 mg/l BAP + 1.0 mg/l NAA.

Figure 3 Shoot morphogenesis at 2.0 mg/l BAP in leaf derived callus

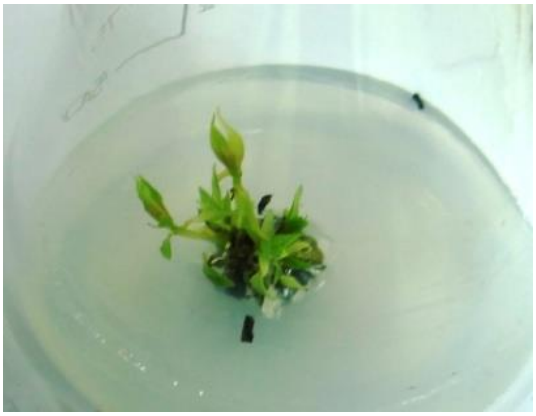

Figure 4 Shoot morphogenesis at 1.0 mg/l BAP + 2.0 mg/l NAA in leaf derived callus

Figure 5 Root induction in shoot buds at 1.0 mg/l IBA

Figure 6 Hardening of *in vitro* developed pomegranate plantlets

Thin and medium long roots were observed at 0.1 – 0.3 mg/l NAA, whereas, thick and medium long roots were observed at 0.4 and 0.9 – 1.5 mg/l NAA. Thick and small roots were observed at 0.5 – 0.8 mg/l NAA. Root induction frequency ranged from 20 – 100 per cent. Highest roots were observed at 0.5 mg/l NAA with 100 per cent frequency (**Table 3**, **Figure 6**).

Table 3 Effect of auxins (IBA/NAA) added singly in the MS medium for root induction in *in vitro* proliferated shoots in pomegranate cv. Sindhuri.

Concentrations (mg/l)	IBA				NAA			
	Root induction	No. of roots/ plant	Root length (cm)	Root morphology	Root induction	No. of roots/ plant	Root length (cm)	Root morphology
0.1	+ (20)	2.5	6.7	Very thin and long root	+ (30)	3.2	5.8	Thin and medium long root
0.2	+ (30)	3.2	6.2	Very thin and long root	+ (40)	3.4	5.9	Thin and medium long root
0.3	+ (40)	3.4	6.3	Very thin and long root	+ (40)	3.5	5.7	Thin and medium long root
0.4	+ (40)	3.6	6.0	Very thin and long root	++ (70)	4.0	5.5	Thick and medium long root
0.5	+ (50)	3.6	5.7	Thin and medium long root	+++ (100)	4.1	4.5	Thick and small root
0.6	+ (50)	3.5	5.8	Thin and medium long root	++ (90)	3.9	4.6	Thick and small root
0.7	+ (50)	3.5	5.6	Thin and medium long root	++ (80)	3.6	4.6	Thick and small root
0.8	+ (55)	3.8	5.2	Thin and medium long root	++ (70)	3.6	4.7	Thick and small root
0.9	++ (70)	3.8	5.0	Thick and medium long	+ (40)	3.2	5.0	Thick and medium long root
1.0	+++ (100)	4.2	4.3	Thick and small	+ (30)	3.1	5.1	Thick and medium long root
1.5	++ (70)	4.1	4.8	Thick and small	+ (20)	3.0	5.2	Thick and medium long root

(+)=Slight roots, (++)=Medium roots, (+++)=Profuse roots

Discussion

In current investigation addition of cytokinins in combination with auxins inducedcallusin the explant. Profuse callus was observed at 1.0 mg/l BAP + 1.0 mg/l NAA in leaf explant with 100 per cent frequency (Table 2 and Figure 1). Murkute*et al*., [7] also reported significant role of BAP and NAA in combination for differentiation of callus in

cotyledon and leaf explant of pomegranate. These observations are contrary with the findings of Thirupathy *et al.*,[8] for callus induction in *Tefrosiahookeriana*from leaf, node and internode explants in MS media supplemented with 0.25 mg/l BAP + 2.0 mg/l 2, 4-D. This is might be due to difference in genera and kind of explants used in the particular study. Similarly, Fougat*et al.,* [9] observed callus induction from cotyledon and leaf explants on MS media supplemented with 4.0 mg/l NAA + 2.0 mg/l Kn in pomegranate cv. Ganesh. Jarzina*et al*., [10] reported callus induction in leaf explants in five different varieties of hemp on MS medium supplemented with 1.0 mg/l Kn + 0.5 mg/l NAA. These results were also contrary to the present findings because of difference in the genera, species and kind of explant used for the study.

In the present investigation *de novo* shoot regeneration was achieved on the medium supplemented with 2.0 mg/l BAP or 1.0 mg/l BAP + 2.0 mg/l NAA with 60 per cent frequency. Karthaet *al*. [11] observed that BAP in combination with NAA induced multiple shoot in *Arachis hypogeal.* In *Cyamopsistetragonoloba*shoot tip explants proliferated multiple shoot buds on a medium supplemented with BAP with IAA/IBA [12]. BAP (0.1 mg/l) and NAA (0.5 mg/l) combination was found to be highly effective in inducing adventitious shoots from callused cotyledon tissues of *H. tuberosum* [13].

Semi compact, greenish yellow callus derived/proliferated from cut ends of the leaf on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA did induce *de novo* shoots from callus upon subculture on the MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l BAP + 2.0 mg/l NAA after 15 – 20 days of incubation (Figure 3 and Figure 4). The reproducibility of shoot morphogenesis from callus was 40 per cent at the medium supplemented with 2.0 mg/l BAP, whereas, the reproducibility of shoot morphogenesis was 60 per cent on sub-culture at 1.0 mg/l BAP + 2.0 mg/l NAA. Thus for shoot regeneration from callus derived from leaf explant (1.0 mg/l BAP + 1.0 mg/l NAA) subculture on 1.0 mg/l BAP + 2.0 mg/l NAA found most responsive for shoot bud regeneration.

Our results are in agreement with many reports such as Bhat et al., [14] in brinjal, Munshi *et al*., [15] in Tagetes erecta, Mitrofanova et el., [16] in apricot where they reported high frequency of shoot regeneration in callus cultures in the medium supplemented with BAP and NAA in combination.

To standardize the induction of roots in *in vitro* regenerated shoots, MS medium supplemented with 0.1 – 1.5 mg/l levels of auxins (IBA/NAA) in the present study. Root induction was observed at all the levels of IBA and NAA with 20 – 100 per cent frequency (Table 3). Maximum rooting was observed at 1.0 mg/l IBA and 0.5 mg/l NAA with 100 per cent frequency. Naik*et al.,* [17], Helaly *et al*., [18] in pomegranate and Ali *et al*., [19] in guava found similar results (1.0 mg/l IBA) to our study.

Results of present investigation were similar to Drazeta, [20](0.1 mg/l IBA), Naik*et al*.,[21] (0.54µM NAA), Parmer *et al*., [22] (0.1 mg/l NAA) in pomegranate, Choudhri *et al.*,[23] (2.5 mg/l IBA), Lizarraga *et al.*,[24] (0.1 mg/l IBA), Meneguzzi *et al*., [25](1.5 mg/l IBA) in apple, Prabhuling *et al*., [26] (2.0 mg/l IBA) in Jamun. However in our study maximum root induction was observed at 1.0 mg/l IBA. This might be due to difference in genera, species or in plant growth regulator used.

Results of present investigation were also similar to the reports of Choudhury and Mukunelan [27] in *Aloe vera* and Mouhamad *et al.,* [28] in *Sesbania grandiflora.* They advocated role of auxins in root induction. They observed maximum root on the medium supplemented with 0.1 mg/l IAA. However in this study maximum root induction was observed at 1.0 mg/l IBA. This might be due to difference in genera, species or in plant growth regulator used or culture conditions utilized.

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