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Choudhary R
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Jakhar ML
Professor, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Kumar R
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Jat H
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Shekhawat K
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Kumawat S
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

Choudhary RN
Associate Professor, Commissionerate College Education Rajasthan, Jaipur, Rajasthan, India

Correspondence
Choudhary R
Research Scholars, Department of Plant Breeding and Genetics, SKN Agriculture University, Jobner, Rajasthan, India

In vitro callus induction from different explants in Pomegranate (*Punica granatum* L.)

Choudhary R, Jakhar ML, Kumar R, Jat H, Shekhawat K, Kumawat S and Choudhary RN

Abstract
Leaf, Shoot apex and Nodal segment 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 in leaf explant with 100 per cent frequency. In shoot apex explants, maximum callus induction was observed on 2.0 mg/l BAP followed by 2.0 mg/l Kn with 100 per cent frequency. Maximum callus induction in Nodal segment was observed on 2.0 mg/l BAP followed by 2.0 mg/l IAA with 100 per cent frequency.

Keywords: pomegranate, callus induction, tissue culture, micropropagation

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]. Pomegranate was domesticated in 2000 BC and was one of the first five fruit crops (date palm, fig, olive, grape and pomegranate) domesticated by mankind. Pomegranate has different ecotypes *viz.,* cultivated (*Punica granatum* L.), wild types (*Punica protopunica*) and ornamental forms (Japanese Dwarf pomegranate - *Punica granatum* var. Nana). Pomegranate has great adaptability to saline soil and drought conditions.
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 [3]. 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 of Rajasthan. Out of these districts Jalore is leading district with 847 ha area, 3134 tones production and 3.7 tones/ha productivity [4].
Pomegranate is commercially propagated by stem cuttings (Hardwood cutting) or by air layering. These methods are time consuming, labor 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 [5].
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.
In vitro culture of pomegranate are greatly affected by explants. Thus, the present investigation has been undertaken to suggest a reliable protocol for *in vitro* culture to produce true to type and virus free plants

Material and Methods
The present research work was conducted on *Punica granatum*. Leaves, shoot apexes and nodal segments were used as explants and obtained from healthy trees grown at Department of plant Breeding and Genetics, S.K.N. College of Agriculture, Jobner. Explants were sterilized by using different surface sterilization agents. Explants were 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, explants were 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 2-5 minutes according to explant.

Induction of callus

Leaf, shoot apex and nodal segments were placed on MS medium supplemented with different concentration of cytokinins (BAP/Kn 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mg/l and auxins (IAA/2, 4–D 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mg/l) for callus induction.

Results

Callus induction

When nodal segment explants were inoculated on medium supplemented with different concentration of plant growth regulators, it responded differently. After 20-24 days of incubation, slight light green with yellowish appearance, semi compact callus was also induced at the base of nodal segment explants incubated at different level of BAP (1.0-5.0 mg/l). Highest fresh callus weight (0.84 g) was observed at 2.0 mg/l BAP level (Fig. 1) (Table. 1).

In BAP supplemented medium, shoot apex explants started to grow within 10 days of incubation on MS medium. Likewise nodal segment explants shoot bud and callus induction was observed with 100 per cent frequency at all the level of BAP in shoot apex explants. After 17-20 days of incubation, slight light green, semi compact callus was proliferated at the base of shoot apex explants incubated at different levels of BAP (1.0-5.0 mg/l). Highest fresh callus weight (0.83 g) was observed at 2.0 mg/l (Fig. 2) (Table 2).

Light green, semi compact callus induction was started at the base of shoot apex explants within 16 -20 days of inoculation at all the levels of Kn (Table 2). Maximum callus induction (0.80 g) was observed at 2.0 mg/l kn with 100 per cent frequency (Fig. 3).

The response of leaf explants was different from nodal segment and shoot apex, only callus proliferated from the cut ends of leaf explants. Callus induction was started within 25 - 30 days of inoculation. Light green, semi compact callus was induced at 1.0 – 5.0 mg/l BAP (Table 3). Highest callus weight (0.88 g) was observed at 1.5 mg/l BAP with 100 per cent frequency followed by 2.0 mg/l BAP (0.79 g) (Fig. 4).

Discussion

In current investigation addition of cytokinins in Table 1- 2 revealed that slight to profuse callus induction was observed in leaf, shoot apex and nodal segment explants within 15 – 30 days of incubation in all the explants under the influence of BAP and Kn. Callus differentiation was more under BAP in comparison to Kn supplemented media. These results are in agreement with those of Omura *et al*. [6] who obtain high frequency of callus induction in leaf segments of dwarf pomegranate at 5 µM BAP.

In the present investigation auxins evoked significant different response in different explants of pomegranate as cytokinins. Auxins (IAA/2, 4-D) induced shoot bud and callus in nodal segment and shoot apex whereas, only callus proliferation was observed in leaf explants. Callus induction was also observed by Fougat *et al.* [7] in cotyledon and leaf explants of pomegranate.

Type of explant is one of the important factors in optimizing the tissue culture protocol. Type of explants like leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root explant significantly effect on tissue culture process of plants. The *in vitro* response of an explant is influenced by several factors such as organ from which it is derived, the physiological state of the explant and its size. Orientation of explant on the medium and the inoculation density may also affect shoot bud differentiation, callus induction, root induction and organogenesis Khan *et al*. [8], Sujatha and Mukta [9], Tyagi *et al*. [10], Gubis *et al*. [11], Alagumanian *et al*. [12], Ali and Mirza [13] and Kumar *et al*. [14]. This may be due to the different level of endogenous plant hormones present in the plants parts.

In the present investigation nodal segment, shoot apex and leaf explants were subjected under cytokinins (BAP/Kn) and auxins (IAA/2,4 -D). Leaf explant was found best for callus induction under the influence of 1.5 mg/l BAP (Table 1 and Fig 4). Among the different explants maximum shoot proliferation was obtained in nodal segment explants under MS medium supplemented with 2.0 mg/l BAP. These results coincide with the results of Kalalbandi *et al.* [15] reported in pomegranate.

Further, results of present investigation revealed that shoot apex started callus induction within 17 - 20 days and leaf explant started callus induction within 23 – 28 days. However, after start of callus induction in both the explants, the rate of callus differentiation was more in leaf explants in comparison to shoot explants. These finding indicates strong role of explants for callus differentiation Deepika and Kanwar, [16] and Kalalbandi *et al*. [17].

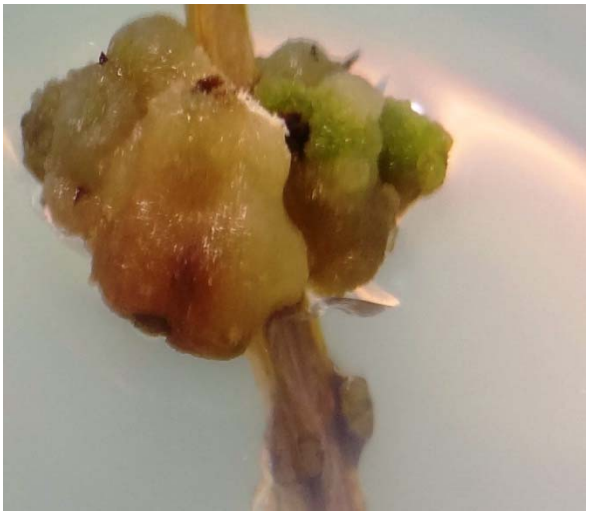

Fig. 1: Induction of callus in nodal segment explant on MS medium supplemented with 2.0 mg/l BAP

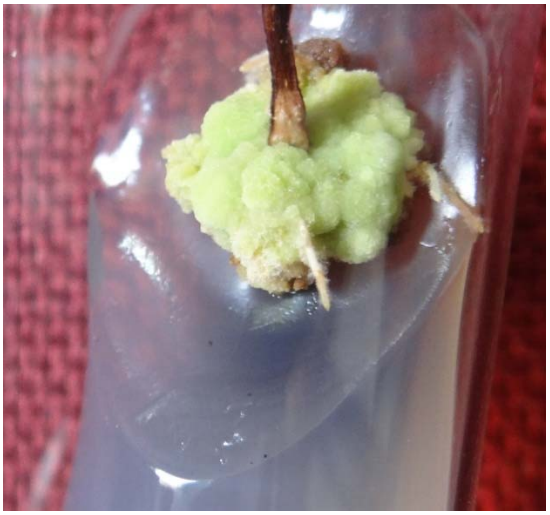

Fig 2: Callus induction in shoot apex explant on MS medium supplemented with 2.0 mg/l BAP

Fig 3: Callus induction in shoot apex explant on MS medium supplemented with 2.0 mg/l Kn

Fig 4: Callus induction in leaf explant on MS medium supplemented with 1.5 mg/l BAP

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

Concentration (mg/l)	Callus			Concentration (mg/l	Callus		
	Response (%)	Days taken for callus initiation	Fresh callus weight (g)		Response (%)	Days taken for callus initiation	Fresh callus weight (g)
BAP				**Kn**			
1.0	100	20.5	0.74 (++)	1.0	100	21.5	0.73 (++)
1.5	100	21.0	0.77 (+++)	1.5	100	21.2	0.76 (+++)
2.0	100	20.6	0.84 (+++)	2.0	100	22.6	0.71 (++)
2.5	100	22.1	0.77 (+++)	2.5	100	23.2	0.68 (+)
3.0	100	24.0	0.72 (++)	3.0	100	23.3	0.65 (+)
4.0	100	23.2	0.66 (+)	4.0	100	24.0	0.62 (+)
5.0	100	21.5	0.62 (+)	5.0	100	25.0	0.59 (+)
IAA				**2,4-D**			
1.0	100	22.6	0.70 (++)	1.0	100	21.5	0.73 (++)
1.5	100	21.3	0.72 (++)	1.5	100	20.2	0.74 (++)
2.0	100	23.7	0.77 (+++)	2.0	100	23.6	0.76 (+++)
2.5	100	24.3	0.70 (++)	2.5	100	22.4	0.70 (++)
3.0	100	25.1	0.68 (+)	3.0	100	25.3	0.69 (+)
4.0	100	23.0	0.59 (+)	4.0	100	22.3	0.61 (+)
5.0	100	24.0	0.56 (+)	5.0	100	24.2	0.55 (+)

(+) = Slight callus, (++) = Medium callus, (+++) = Profuse callus, (-) = No response

Table 2: Morphogenetic effect of various concentrations of cytokinins (BAP/Kn) and auxins (IBA/NAA) added singly in the MS medium Shoot apex explant.

Concentration (mg/l)	Callus			Concentration (mg/l	Callus		
	Response (%)	Days taken for callus initiation	Fresh callus weight (g)		Response (%)	Days taken for callus initiation	Fresh callus weight (g)
BAP				**Kn**			
1.0	100	18.1	0.75 (+++)	1.0	100	16.1	0.73 (++)
1.5	100	17.5	0.76 (+++)	1.5	100	16.9	0.75 (+++)
2.0	100	17.2	0.83 (+++)	2.0	100	17.2	0.80 (+++)
2.5	100	19.5	0.77 (+++)	2.5	100	18.0	0.73 (++)
3.0	100	18.6	0.71 (++)	3.0	100	18.2	0.69 (+)
4.0	100	18.4	0.65 (+)	4.0	100	19.0	0.66 (+)
5.0	100	19.0	0.61 (+)	5.0	100	19.2	0.65 (+)
IAA				**2,4-D**			
1.0	100	15.1	0.66 (+)	1.0	100	15.2	0.71 (++)
1.5	100	15.9	0.73 (++)	1.5	100	14.8	0.72 (++)
2.0	100	16.3	0.75 (+++)	2.0	100	15.4	0.77 (+++)
2.5	100	17.1	0.71 (++)	2.5	100	18.2	0.72 (++)
3.0	100	17.3	0.69 (+)	3.0	100	18.3	0.66 (+)
4.0	100	18.1	0.59 (+)	4.0	100	17.4	0.65 (+)
5.0	100	18.3	0.66 (+)	5.0	100	17.5	0.59 (+)

(+) = Slight callus, (++) = Medium callus, (+++) = Profuse callus, (-) = No response

Table 3: 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			Concentration (mg/l	Callus		
	Response (%)	Days taken for callus initiation	Fresh callus weight (g)		Response (%)	Days taken for callus initiation	Fresh callus weight (g)
BAP				**Kn**			
1.0	100	27.8	0.73 (++)	1.0	100	26.2	0.73 (++)
1.5	100	27.6	0.88 (+++)	1.5	100	25.8	0.79 (+++)
2.0	100	25.3	0.79 (+++)	2.0	100	27.1	0.76 (+++)
2.5	90	26.8	0.72 (++)	2.5	90	28.0	0.71 (++)
3.0	80	25.3	0.69 (+)	3.0	80	26.5	0.68 (+)
4.0	70	27.4	0.61 (+)	4.0	70	28.3	0.59 (+)
5.0	70	28.0	0.59 (+)	5.0	70	29.0	0.58 (+)
IAA				**2,4-D**			
1.0	100	26.0	0.71 (++)	1.0	100	26.5	0.70 (++)
1.5	100	25.0	0.79 (+++)	1.5	100	25.1	0.78 (+++)
2.0	100	26.1	0.77 (+++)	2.0	100	27.1	0.74 (++)
2.5	90	27.2	0.71 (++)	2.5	90	26.2	0.70 (++)
3.0	80	25.5	0.68 (+)	3.0	80	26.5	0.67 (+)
4.0	70	27.4	0.58 (+)	4.0	70	28.4	0.59 (+)
5.0	70	28.0	0.56 (+)	5.0	70	29.0	0.57 (+)

(+) = Slight callus, (++) = Medium callus, (+++) = Profuse callus, (-) = No response

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