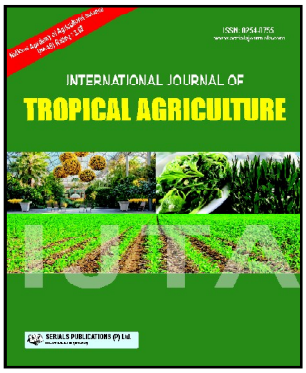

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Studies on Callus Induction in Pomegranate cv. Bhagwa

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Abstract: Pomegranate (*Punica granatum* L.) is one of the oldest known fruit trees of the tropics and sub-tropics belonging to the family *Punicaceae*. It is regenerated through tissue culture directly by axillary bud proliferation or by callus mediated adventitious bud proliferation. Callus-mediated plant regeneration is the second most documented procedure next to axillary shoot proliferation. Hence successful induction of callus is essential prerequisite for various callus mediated downstream application such as mass multiplication of disease free plantlets, production of useful somaclonal variants, regeneration of transgenic plants and production of secondary metabolites. The selection of suitable explants and proper growth regulators is paramount aspect in callus culture technology. In the present study suitable explants type, concentration and combination of growth regulator were tried for induction regenerable callus in Pomegranate cv. Bhagwa. Nodal segment was found superior for induction of callus followed by leaf segment and shoot tip. Among the growth regulators BAP 5 mg/l + NAA 0.40 mg/l induced very good callus both quantitatively and qualitatively.

Keywords: Callus, Nodal explants, Mass multiplication, Auxins, BAP, Bhagwa.

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 fruit has a good consumer preference for its refreshing, sweet juicy and attractive arils. There is a growing demand for good quality fruits and processed products in both domestic and international market [17]. The tree is conventionally propagated through stem cuttings and air layrings, but the method is time consuming and tedious. Propagation by seed is not preferred because of the resulting variability in tree and fruit characters [18]. In addition recent years the bacterial blight disease caused by *Xanthomonas*

compestris pv. *punicae* and fungal wilt caused by *Ceratocysis fimbriata* causing dramatic yield losses [23 and 10]. To overcome these problems, tissue culture of pomegranate through axillary bud proliferation is found to be satisfactory. The technique provided many advantages over the conventional methods of vegetative propagation. It ensures true to type of plants with uniform quality and rapid mass production of disease free planting materials [19]. Several studies have been conducted on micropropagation of pomegranate trees over the past two decades. Protocols have been developed for *in vitro* regeneration of pomegranate through axillary and adventitious bud proliferation methods. But both the methods have prose and cons, recalcitrant nature of woody plants, higher chance of microbial contamination and polyphenol exudation limits the efficacy of axillary bud proliferation. In addition it is time-consuming, labour-intensive and expensive process.

Several studies were dedicated on adventitious bud proliferation *via* leaf segments [16 and 17], cotyledons [12 and 9], anthers [17], or through embryogenesis from various seedling explants [7] and petals [14]. Callus-mediated plant regeneration is the second most documented method next to axillary shoot proliferation. Establishment of callus cultures and plants regenerated from calli *via* shoot organogenesis hold a potential for the production of useful somaclonal variants. Somaclonal variations offer promises to result in alterations in a wider range of plant characteristics of horticultural significance, including plant height, flowering time, fruit yield, tolerance to abiotic or biotic stress. In spite of that, currently the bacterial blight disease caused by *Xanthomonas compestris* pv. *punicae* and fungal wilt caused by *Ceratocysis fimbriata* causing dramatic yield losses [10] both the disease are transmitted mainly through planting material. Besides the mass multiplication healthy plantlets, introduction of resistance genes against both disease through genetic transformation requires callus mediated tissue culture techniques.

In addition pomegranate has both medicinal and therapeutic properties hence secondary metabolite production through callus culture is open niche to carryout pharmaceutically important active molecule. It is evidenced from the various previous research (MS) medium was the most preferred medium for callus culture. The best combination of plant growth regulators for establishment of callus culture was found to be 2.4.D, NAA and BAP followed by NAA and Kinetin at different concentration. Keeping these facts in view, the present research entitled studies on callus induction in Pomegranate cv. Bhagwa was carried out with the objective to find suitable explants and growth regulators for induction of regenerable callus.

MATERIALS AND METHODS

Preparation of Explants

The present investigation was carried out at Centre for Horticulture Biotechnology, Directorate of Research, UHS, Bagalkot- 587 104. The plants materials for *in vitro* culture establishment were collected from three years old healthy and vigorously growing mother plant of pomegranate cv. Bhagwa (Figure 1) grown at fruit orchard, University of Horticultural Science, Bagalkot. The various explants *viz*., shoot tip (2-3 cm), nodal segment (3-4 cm), leaf segment (1-2 cm^2) and petals (Figure 2) were used for establishment of *in vitro* cultures. The shoots of current season growth were taken from the healthy and vigorously growing mother plant, then washed thoroughly with potable tap water and soaked in a detergent solution containing 2–3 drops of Tween-20 for 20 min. Later different explants were excised and dipped in a solution containing Indofil M-45 1000 mg/l (Indofill Industries Limited, Mumbai) + K-Cycline 500 mg/l (Karnataka Antibiotics and Pharmaceuticals Limited, Bengaluru) for 45 minutes fallowed by 4-5 washes with sterile distilled. Further, explants were treated with Citrimide 500 mg/l (Himedia) for 30 minutes and washed with sterile distilled water for 6-7 times under laminar air flow

cabinet. Finally explants were surface sterilized with mercuric chloride ($HgCl_2$) 1000 mg/l for 1 minute for shoot tip, petal and leaf segment and 2-3 minute for nodal segment, respectively. Later explants were washed thoroughly with sterile distilled water for 4-5 times.

Media and Culture Condition

For callus induction, the explants were cultured on Murashige and Skoog [MS] medium supplemented ten with different concentrations and combinations of growth regulators MS medium + BAP 1-5 mg/l + NAA 0.40 mg/l and MS medium + 2,4 – D 1-5 mg/l were tried. Growth regulators were added and the pH of the medium was adjusted to 5.8 with NaOH/HCl (0.1N) before autoclaving. The chemicals and growth regulators used were analytical grade and purchased from Himedia Pvt. Ltd. Mumbai, India. The culture bottles were incubated in culture room at 25 ± 2°C temperature and 70-80% RH with 16 hours light and 8 hours dark period.

Collection of Data and Statistical Analysis

After incubation, callus induction was observed regularly and day of initiation was recorded. The final data was recorded after four weeks including number

Figure 1: Mother plant of Pomegranate *cv.* Bhagwa

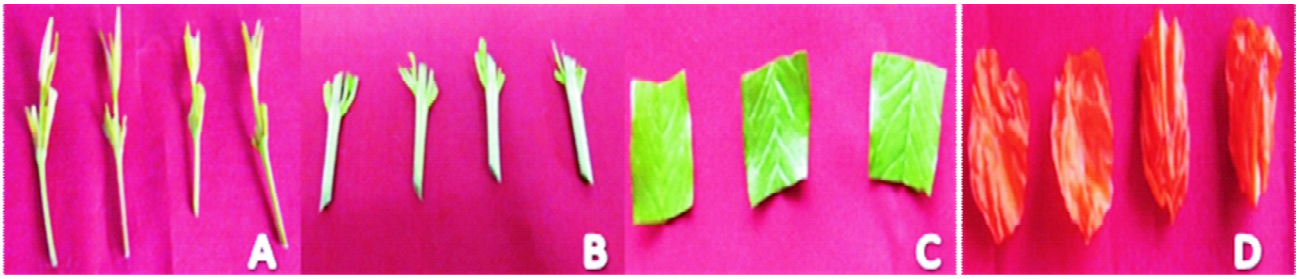

Figure 2: Explants used for callus induction in pomegranate *cv.* Bhagwa: (A) Shoot tip; (B) Nodal segments; (C) Leaf segment; (D) Petals.

of days taken for callus induction, per cent of callus induction and callus quality. Callus quality was recorded based on visual observation [++++: very good calli, (completely regenerable), +++: Good calli with many white islets, ++: Medium calli yellowish white, +: Poor calli formed is not capable of regeneration (dull yellow/brown) and finally callus weight (gm)]. Factorial complete randomized design (FCRD) was employed for experiments. The data in percentages were transformed to arc sine values for statistical analysis. Data observed zero value were transformed to square root transformation and analyzed as factorial experiment [5].Critical difference values were tabulated at one per cent where "F" test was significant

RESULT AND DISCUSSION

Among the different type of explants, shoot tip produced early callus (28.92 days) which was followed by nodal segment (30.10 days) and leaf segment (37.96 days), whereas, petals failed to produce callus. The failure of petal explants to induce the callus may be probably due to the tender nature of the petal explants more sensitivity to mercury chloride treatment, possibly the bleaching activity of chloride atoms present in $HgCl_2$ resulted in death of cell subsequently no response for callusing.

This difference in response for days taken for callusing among the different explants might be due to the fact that, the shoot tip and nodal segment contains meristems in leaf axils which might have lead to early cell dedifferentiation and callus induction. Delayed callus induction in leaf segment may be due to presence of differentiated tissue which is physiologically less active observations are in accordance with report [6].

The result of effect of explants type and growth regulators on per cent callus induction are presented in Table 2. Highest per cent of callus induction was

Table 1
Effect of type of explants and growth regulators on days taken for callus induction in pomegranate cv. Bhagwa

	Days taken for callus induction				
Type of explants / Growth regulators	*Shoot tip*	*Nodal segment*	*Leaf segment*	*Petal*	*Mean*
MSB + BAP 1 mg/l + NAA 0.40 mg/l	37.38	36.88	41.10	–	28.84
MSB + BAP 2 mg/l + NAA 0.40 mg/l	34.13	34.50	40.20	–	27.21
MSB + BAP 3 mg/l + NAA 0.40 mg/l	30.75	32.50	39.95	–	25.80
MSB + BAP 4 mg/l + NAA 0.40 mg/l	28.50	30.25	37.05	–	23.95
MSB + BAP 5 mg/l + NAA 0.40 mg/l	26.70	29.30	36.05	–	23.01
MSB + 2,4-D 1 mg/l	29.75	29.00	38.00	–	24.19
MSB + 2,4-D 2 mg/l	27.38	28.13	37.50	–	23.25
MSB + 2,4-D 3 mg/l	26.10	27.60	37.13	–	22.71
MSB + 2,4-D 4 mg/l	25.25	27.10	36.88	–	22.31
MSB + 2,4-D 5 mg/l	23.30	25.75	35.75	–	21.20
Mean	28.92	30.10	37.96	–	
	Type of explants (E)	*Growth regulators (G)*	*Interaction (E XG)*		
S. Em ±	0.23	0.30	0.61		
CD@ 1%	0.74	1.14	2.40		

– : No callus induced in petal. MSB: MS basal media.

observed in leaf segment (64.75 %) followed by nodal segment (56.50 %) and shoot tip (50.75 %). Whereas petal showed no (0.00 %) callus induction. Probably, presence of endogenous growth factors might have led to higher dedifferentiation of cells than the nodal segment and shoot tip explants. Similar findings were also reported in strawberry [1] and pomegranate [2].

The effect of different levels of auxins (NAA and 2, 4-D), cytokinin (BAP) and their combinations on days taken for callusing and per cent of callus induction were also studied (Table 1 and 2). Of the various concentrations of auxins, cytokinin and their combinations tried, MS basal medium containing 2, 4-D, 5 mg/l exhibited minimum time with highest percentage of callus induction (69.38). This might

Table 2
Effect of type of explants and growth regulators on per cent callus induction in pomegranate *cv.* Bhagwa

	Per cent of callus induction				
Type of explants/Growth regulators	*Shoot tip*	*Nodal segment*	*Leaf segment*	*Petal*	*Mean*
MSB + BAP 1 mg/l + NAA 0.40 mg/l	15.00 (22.50)*	15.00 (22.50)*	20.00 (26.26)*	0.00 (0.28)*	12.50 (17.96)*
MSB + BAP 2 mg/l + NAA 0.40 mg/l	17.50 (24.67)	17.50 (24.67)	40.00 (39.23)	0.00 (0.28)	18.75 (22.21)
MSB + BAP 3 mg/l + NAA 0.40 mg/l	20.00 (26.56)	20.00 (26.56)	60.00 (50.76)	0.00 (0.28)	25.00 (26.04)
MSB + BAP 4 mg/l + NAA 0.40 mg/l	20.00 (26.56)	20.00 (26.56)	60.00 (50.76)	0.00 (0.28)	25.00 (26.04)
MSB + BAP 5mg/l + NAA 0.40 mg/l	40.00 (39.23)	60.00 (50.76)	100.00 (89.71)	0.00 (0.28)	50.00 (35.00)
MSB + 2,4-D 1 mg/l	52.50 (46.44)	77.50 (61.71)	60.00 (50.76)	0.00 (0.28)	47.50 (39.80)
MSB + 2,4-D 2 mg/l	80.00 (63.43)	80.00 (63.43)	60.00 (50.76)	0.00 (0.28)	55.00 (44.48)
MSB + 2,4-D 3 mg/l	80.00 (63.43)	80.00 (63.43)	80.00 (63.43)	0.00 (0.28)	60.00 (47.64)
MSB + 2,4-D 4 mg/l	85.00 (67.50)	95.00 (80.63)	87.50 (69.38)	0.00 (0.28)	66.88 (54.45)
MSB + 2,4-D 5 mg/l	97.50 (83.39)	100.00 (89.71)	80.00 (63.43)	0.00 (0.28)	69.38 (59.20)
Mean	50.75 (46.37)	56.50 (51.00)	64.75 (55.48)	0.00 (0.28)	
	Type of explants (E)	*Growth regulators (G)*	*Interaction (E XG)*		
S. Em ±	0.63	1.52	2.00		
CD @ 1%	2.49	4.60	7.85		

*Figures in parenthesis are arc sin transformation. MSB: MS basal media.

be attributed to more potentiality of 2, 4-D in increasing rate of cell division resulting in early callus formation and increased callus induction. These findings are in agreement with previous reports in *Curcuma longa* L. [15], *Pythium graminicolum* [4], *Curcuma aromatic* [11], *Phyllanthus emblica* [8] and *Citrus jambhiri* [20 and 21].

In the present study, significantly maximum weight of callus (Table 3) was observed in nodal segment (1.49 gm) followed by leaf segment and shoot tip (0.98 gm). Lowest weight was noticed with shoot tip (0.45 gm), while, petal shown no response (0.00 gm) for callus weight. This may be due to callus induced by nodal segment may contain more of

Table 3
Effect of type of explants and growth regulators on callus weight (gm) in pomegranate cv. Bhagwa

	Weight of callus (gm)				
Type of explants/Growth regulators	*Shoot tip*	*Nodal segment*	*Leafsegment*	*Petal*	*Mean*
MSB + BAP 1 mg/l + NAA 0.40 mg/l	0.16 (0.81)*	1.02 (1.23)*	1.13 (1.27)*	0.00 (0.71)*	0.58 (1.00)*
MSB + BAP 2 mg/l + NAA 0.40 mg/l	0.18 (0.82)	1.08 (1.25)	1.26 (1.32)	0.00 (0.71)	0.63 (1.02)
MSB + BAP 3 mg/l + NAA 0.40 mg/l	0.19 (0.82)	1.18 (1.29)	1.30 (1.33)	0.00 (0.71)	0.66 (1.04)
MSB + BAP 4 mg/l + NAA 0.40 mg/l	0.23 (0.85)	1.28 (1.33)	1.40 (1.37)	0.00 (0.71)	0.73 (1.06)
MSB + BAP 5 mg/l + NAA 0.40 mg/l	0.30 (0.89)	1.68 (1.47)	1.71 (1.48)	0.00 (0.71)	0.92 (1.14)
MSB + 2,4-D 1 mg/l	0.50 (0.99)	1.54 (1.42)	0.53 (1.01)	0.00 (0.71)	0.64 (1.03)
MSB + 2,4-D 2 mg/l	0.64 (1.06)	1.64 (1.46)	0.54 (1.01)	0.00 (0.71)	0.70 (1.06)
MSB + 2,4-D 3 mg/l	0.70 (1.09)	1.79 (1.51)	0.60 (1.04)	0.00 (0.71)	0.77 (1.09)
MSB + 2,4-D 4 mg/l	0.74 (1.11)	1.85 (1.53)	0.66 (1.07)	0.00 (0.71)	0.81 (1.10)
MSB + 2,4-D 5 mg/l	0.87 (1.16)	1.92 (1.55)	0.69 (1.09)	0.00 (0.71)	0.87 (1.13)
Mean	0.45 (0.96)	1.49 (1.40)	0.98 (1.20)	0.00 (0.71)	
	Type of explants (E)	*Growth regulators (G)*	*Interaction (E XG)*		
S. Em ±	0.007	0.011	0.022		
CD @ 1%	0.027	0.041	0.085		

*Figures in parenthesis are square root transformation. MSB: MS basal media.

lignin and tannin than callus produced by leaf and shoot tip consequently resulting in maximum weight of callus. Among different growth regulators BAP 5.0 mg/l + NAA 0.40 mg/l recorded maximum callus weight with very good callus quality (++++) (Table 4 and Figure 4 A). This may be due to higher cell dedifferentiation, higher chlorophyll formation and proteins synthesis resulting in maximum callus weight with good quality and high regeneration (Figure 3 A). The results are in line with [10] in pomegranate and peepal tree [22].

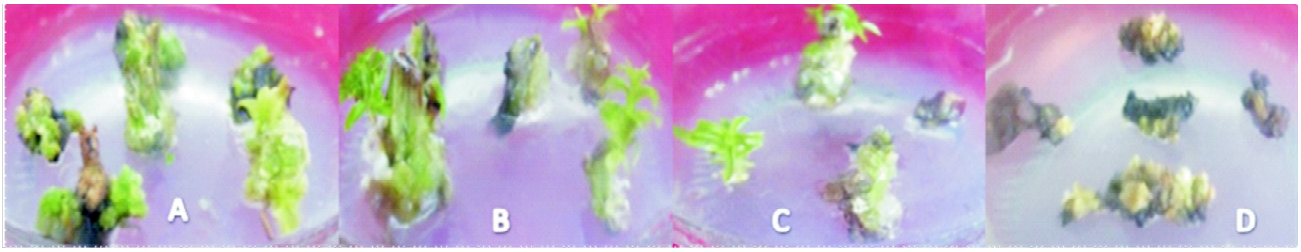

Figure 3: Nodal explants showing callus induction on MS medium containing- (A) BAP 5 mg/l + NAA 0.40 mg/l; (B) BAP 4 mg/l + NAA 0.40 mg/l; (C) BAP 3 mg/l + NAA 0.40 mg/l; (D) 2,4-D 5 mg/l in pomegranate cv. Bhagwa

Table 4
Effect of type of explants and growth regulators on callus quality in pomegranate cv. Bhagwa

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

++++: Very good, +++: good, ++: medium, +: Poor, –: No Callus. MSB: MS basal media.

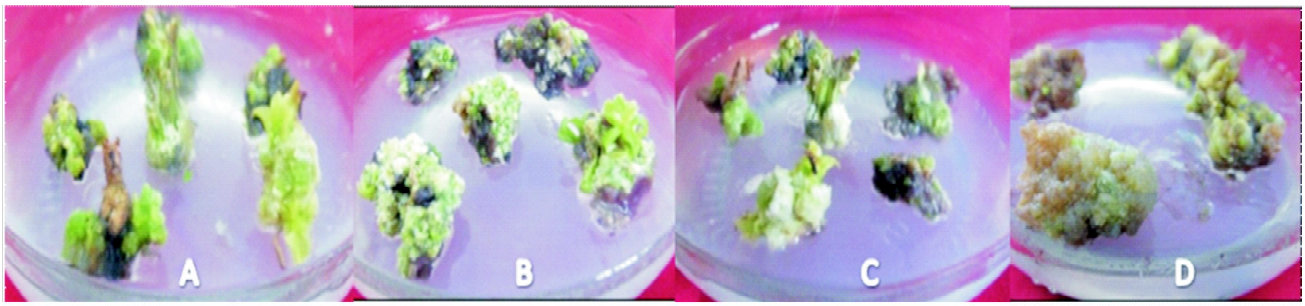

Figure 4: Callus quality- (A) ++++: Very good; (B) +++: Good; (C) ++: Medium; (D) +: Poor

CONCLUSSION

It is evident that the good quality regenerable callus can be obtained by transferring nodal segment onto the MS medium supplemented with BAP 5 mg/l + NAA 0.40 mg/l. This protocol could be useful for the production of desirable somaclonal variants, transgenic plant and secondary metabolites besides mass multiplication of superior genotypes.

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