

Original Research Article

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Recent Advances in Plant Regeneration from Callus Cultures of Bael [*Aegle marmelos* (L.) corr.] and Projection of its Economic and Social Benefits

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ABSTRACT

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Callus cultures were established from cotyledon explants from mature fruits of *bael* cvs. viz., Local, Gonda Selection and Mirzapuri on Knop's medium supplemented with different combinations of phytohormones. The maximum callus induction (76.66%) were observed in cv. Gonda Selection on Knop's medium supplemented with 2mg l^{-1} NAA, 2.0mg l^{-1} 2,4-D & 0.5mg l^{-1} KIN. However, maximum organogenesis (62.50) was observed in cv. Local on Knop's medium supplemented with 1.0mg l^{-1} BAP. Maximum number of plantlets per calli (3.85) was observed in cv. Mirzapuri from cotyledon derived callus on Knop's medium containing 1.0mg l^{-1} BAP. No plantlets were observed in all the three cvs. on Knop's basal medium.

Introduction

The Bael (*Aegle marmelos* corr.) belongs to the family Rutaceae, to which also belong wood apple, lemon and oranges. It is also called Bengal quince. The *bael* fruit has a hard shell and the pulp contains funnels which are filled with mucilage. The leaves are astringent, febrifuge, expectorant, and are reported to have hypoglycaemic and antiasthmatic properties (Nambiar, *et al.*, 2000). It is native to India and grows wild all over India. The *bael* is considered to have

many medicinal properties and is effective in the treatment of dysentery. It also makes an excellent of squash. The fruits are a good source of minerals and vitamins (Morton, 1987) and all parts of the plant (i.e. stem, bark, roots, leaves and fruit) are used in Ayurvedic medicine (Jayaweera, 1982). The unripe and ripe fruits are useful for curing diarrhoea, dysentery, and stomachalgia (Warrier *et al.*, 1996). It can stand on swampy and alkaline soil and is propagated usually by seeds, root suckers and budding. Seedlings have long phase of juvenility and the first

crop is obtained very late. Root suckers formations is rare occurrence and the budding which is slow, difficult and season bound.

Micro propagation techniques have been widely used for the propagation of several plant species during the past years. Protocols have also been developed for *in vitro* propagation of a number of fruit trees (Bajaj, 1986; Hutchinson and Zimmerman, 1987). Arya *et al.*, (1981) reported callus formation and some organogenesis from cultures developed from cotyledon and hypocotyl explants. Plant regeneration *via in vitro* methods has been reported in *A. marmelos* from different explants, i.e., cotyledonary node (Nayak *et al.*, 2007), root segments (Bhati *et al.*, 1992), nucellus (Hossain *et al.*, 1994), and single-node segments (Ajithkumar and Seeni, 1998). Therefore, attempts have been made for rapid multiplication of *bael* cvs. through callus culture form cotyledon explants.

Materials and Methods

Plant material

Cotyledon explants were excised from mature fruits which were collected from 14-15 years old trees of *bael* cvs. Viz., Local, Gonda Selection and Mirzapuri growing at experimental orchard of the Department of Horticulture, CCS Haryana Agricultural University, Hisar. The explants were collected and culture in the months of December, January and February (2001-2002). Seeds were separated from the fruits and thoroughly washed in tap water with few drops of teepol. Cotyledons were removed from seeds and 4-7mm diameter cotyledon explants were prepared by removing embryo axis and then treated with 70 per cent aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1 per cent aqueous solution of HgCl₂ (w/v) for 2 minutes. Finally, the explants were

thoroughly washed in sterilized single distilled water to remove all the traces of mercuric chloride. All the disinfection operations were carried out in the horizontal laminar flow cabinet.

Culture media

The Knop's (1865) basal medium with some modifications was used for callus induction as well as plant regeneration. Cotyledon explants without embryo axis cultured on Knop's medium supplemented with 3%(w/v) sucrose 100mg l⁻¹ inositol and different combinations of BAP, KIN, NAA and 2,4-D. The data on per cent explants showing callus induction, organogenesis and mean number of plantlets through intermediate stage of callus were recorded after seven weeks of culture initiation. The media were melted at 1.2kg cm⁻² pressure for 15 minutes in the autoclave. Then the culture bottles with lid and the flasks were closed with cotton plugs and sterilized.

Culture conditions

All the cultures were maintained at 25 ±2⁰C under 16/8 hours cycle of light (2000 lux fluorescent tubes)

Callus culture

Callus was induced by culturing cotyledon explants on Knop's medium supplemented with various combinations of BAP, KIN, NAA and 2, 4-D. Small portions (50-60mg) of the primary callus that green from the cut end and the abaxial surface were removed from the original explants and subcultured into fresh medium after every 20-25 days of callus growth and maintenance of callus cultures.

Production of *in vitro* plants

Number of plantlets obtained through intermediate stage of callus was transferred to

half and full strength Knop²'s medium supplemented with different concentrations and combinations of growth regulators.

Results and Discussion

Callus Induction

Callus induction was observed in cotyledon explants on various media formulations within 10-21 days of inoculation. Callus induction was seen usually at cut end and on the abaxial surface of explants. On the basis of all the three cvs. used in the present investigation, cv. Gonda Selection showed maximum callus induction (76.66%) on K₁₇ medium followed by (74.44%) on K₁₅ medium in cv. Mirzapuri whereas, cv. Local showed maximum callus growth (66.66%) on K₁₅ and K₁₆ media. Minimum callus growth (25.55%) was recorded on K₁₃ medium in cv. Mirzapuri. No callusing was observed on K₀ medium in all the three cvs. (Fig 1 & Table 2). The growth regulators requirements for callus initiation and organogenesis varied between the three cvs. were studied. Standardization of media compositions for callus induction and establishment of cultures of the experiment crops is first step in the application of any tissue culture technique. The specific combination and concentration of growth regulators. Nutrients and incubation conditions modify the normal physiology of explants and induce de-differentiation and redifferentiation of tissues. Thus, it is necessary to understand the nutrient requirements and physical factors influencing callus induction. In the present investigation, callus induction was observed in all the media tested except Knop's medium without growth regulators in bael. Cotyledon explants after seven weeks of culture revealed maximum percentage of callus induction in cv. Gonda section on Knop's medium supplemented with 0.5mg l⁻¹ KIN, 2.0mg l⁻¹ NAA & 2.0 mg l⁻¹ 2, 4-D followed by cv. Mirzapuri on Knop's

medium containing 0.2 mg l⁻¹ BAP & 1.0 mg l⁻¹ 2, 4-D (Fig 1 and Table 2). Hossain *et al.*, (1994b) reported maximum callus (95.2%) on MS medium supplemented with 5 mg l⁻¹ NAA and 1 mg l⁻¹ KIN. The differential response is due to difference between media with different combinations of growth regulators. Moreover, the choice of genotype beside explant is also very important which plays a definite role in callus induction. M.S. Hazeena and G.R. Sulekha (2008) reported that Murashige and Skoog (MS) medium supplemented with benzyl adenine (2.2 μM) and 2,4-dichlorophenoxy acetic acid (2.26 μM) recorded the highest growth score for callus induction and proliferation. Pranita Jamdhade and Narayan Pandhure (2016) observed maximum callus induction on Murashige and Skoog (MS) medium supplemented with 2,4-D (2.0 mg/l) alone and combination of 2,4-D (0.5 mg/l) +KIN (2.0mg/l) + NAA (1.0 mg/l).

Per cent Organogenesis from various calli

On the basis of all the three cvs. used in the present investigation, cv. Local showed maximum organogenesis (62.50%) on K3 medium followed by cv. Mirzapuri (57.14%) on K12 medium (Refer Plate 1 & 2) whereas, Gonda Selection showed maximum organogenesis (50.00%) on K3 and K12 Media. Minimum organogenesis (12.50%) was observed in cv. Gonda Selection on K7 medium in Fig 2 and Table 3. The responses of different media combination on number of plantlets obtained through intermediate stage of callus have been presented in Fig 3 and Table 4. On the basis of results, it is clear that among three cvs., cv. Mirzapuri (refer Plate 3) showed maximum mean number of plantlets per calli (3.85) on K12 medium followed by 3.42 and 3.00 in cvs. Local and Gonda Selection on same media. However, minimum mean numbers of plantlets per calli (0.66) were observed in cv. Mirzapuri on K10

medium. The data on mean shoot length using cotyledon explants of different cvs. on different combination of media are presented in Fig 3 and Table 4. Among all the three cvs. maximum mean shoot length (1.54 cm) was observed in cv. Local on K3 medium followed by cv. Mirzapuri (1.53cm) and Gonda Selection (1.36cm) on K1 and K3 media respectively. Minimum mean shoot length (0.83) was observed in cv. Gonda Selection on K7 medium. Percentages of rooting in plantlets obtained through intermediate stage of calli are presented in Fig 4 and Table 5. Regeneration of complete plants from single callus and tissues is of

great importance for the application of biotechnology in crop improvement. Application of biotechnology techniques (in vitro mutant selection and protoplast fusion to cell culture) had limitation in many crop species because of the instability to regenerate plants. Only few species have been exploited fully to such studies. In present organogenesis studies, among all the three cvs., cv. Local showed best organogenesis on Knop's medium containing 1.0 mgL⁻¹ BAP followed by cv. Mirzapuri at 1.0Mgl-1 BAP,1.0 mgL⁻¹ KIN & 0.5 mgL⁻¹ NAA. No organogenesis was observed on Knop's basal medium (Fig. 2 and Table 2).

Table.1 List of media tested for callus induction/ shoot regeneration in different explants of *bael*

xop	Media	Basal medium	Growth regulators (mgL ⁻¹)			
			Cytokinin		Auxin	
			BAP	KIN	NAA	2,4-D
1	K ₀	Knop's	0.00	0.00	0.00	0.00
2	K ₁	Knop's	0.25	0.00	0.00	0.00
3	K ₂	Knop's	0.50	0.00	0.00	0.00
4	K ₃	Knop's	1.00	0.00	0.00	0.00
5	K ₄	Knop's	2.00	0.00	0.00	0.00
6	K ₅	Knop's	0.00	0.25	0.00	0.00
7	K ₆	Knop's	0.00	0.50	0.00	0.00
8	K ₇	Knop's	0.00	1.00	0.00	0.00
9	K ₈	Knop's	0.00	2.00	0.50	0.00
10	K ₉	Knop's	0.50	0.50	0.50	0.00
11	K ₁₀	Knop's	0.50	1.00	0.50	0.00
12	K ₁₁	Knop's	1.00	0.50	0.50	0.00
13	K ₁₂	Knop's	0.20	1.00	0.50	0.00
14	K ₁₃	Knop's	0.20	0.00	0.00	0.20
15	K ₁₄	Knop's	0.20	0.00	0.00	0.50
16	K ₁₅	Knop's	0.00	0.00	0.00	1.00
17	K ₁₆	Knop's	0.50	0.50	5.00	0.00
18	K ₁₇	Knop's	0.50	0.50	2.00	2.00

Table.2 Effect of different media combination on *in vitro* callus induction of *bael cvs.* from cotyledon explants

Media	Per cent shoot regeneration		
	Local	Gonda selection	Mirzapuri
K ₀	0	0	0
K ₁₃	54.44±2.93	51.11±1.11	25.55±2.93
K ₁₄	65.55±2.93	51.11±5.87	51.11±1.11
K ₁₅	62.22±2.22	56.66±3.33	74.44±2.93
K ₁₆	48.88±1.11	63.33±3.33	45.55±2.93
K ₁₇	57.77±2.22	76.66±3.33	56.66±3.33

± SE

Table.3 Effect of different media combination on per cent Organogenesis from various calli induced of *bael cvs.*

Media	Per cent organogenesis		
	Local	Gonda Selection	Mirzapuri
K ₀	0	0	0
K ₂	42.85	28.57	33.33
K ₃	62.50	50.00	50.00
K ₆	28.57	14.28	16.66
K ₇	37.50	12.50	33.33
K ₁₀	42.85	28.57	16.66
K ₁₂	57.14	50.00	57.14

± SE

Table.4 Effect of different media combination on mean number of plantlets from various calli induced of *bael cvs.*

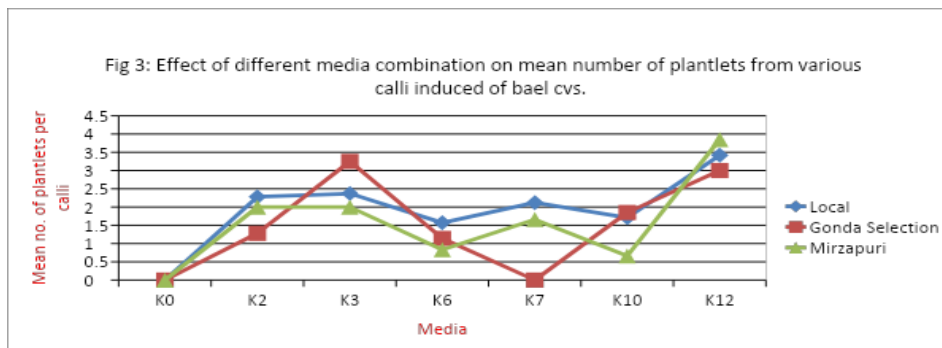
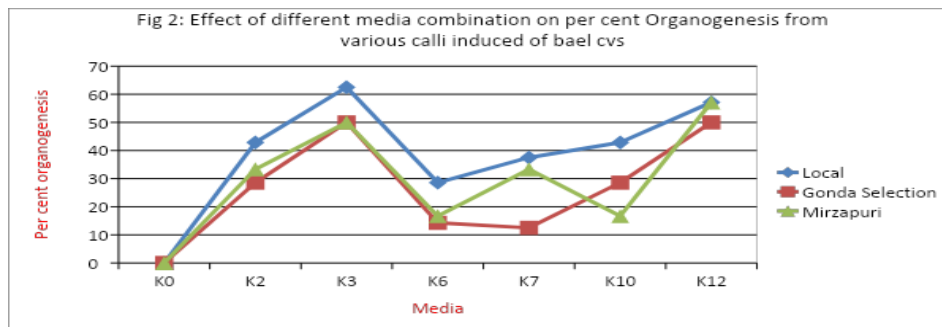
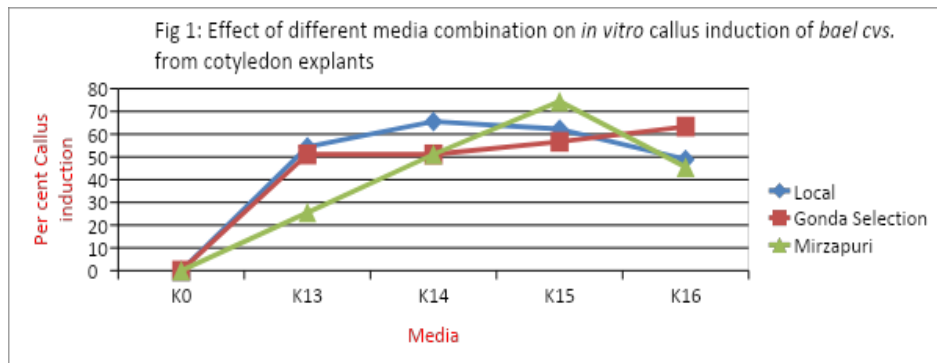
Media	Mean number of plantlets per calli		
	Local	Gonda selection	Mirzapuri
K ₀	0	0	0
K ₂	2.28±1.12	1.28±0.89	2.00±1.29
K ₃	2.37±0.80	3.25±1.46	2.00±1.36
K ₆	1.57±1.06	1.14±1.14	0.83±0.83
K ₇	2.12±1.14	1.12±1.12	1.66±1.17
K ₁₀	1.71±0.91	1.85±1.24	0.66±0.66
K ₁₂	3.42±1.39	3.00±1.19	3.85±1.66

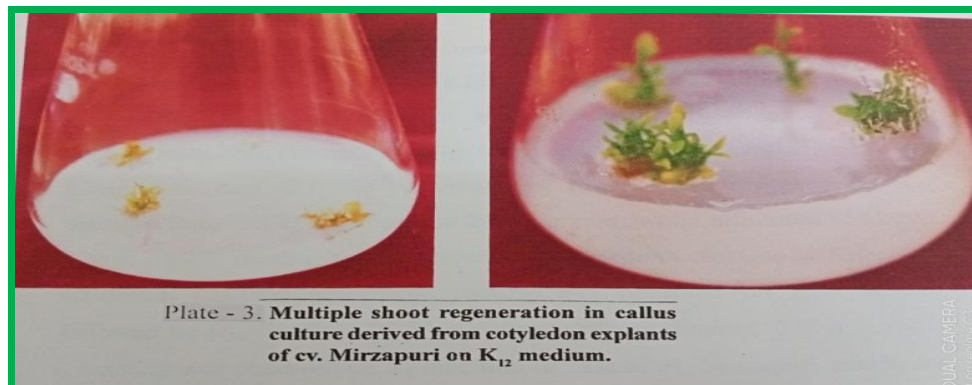
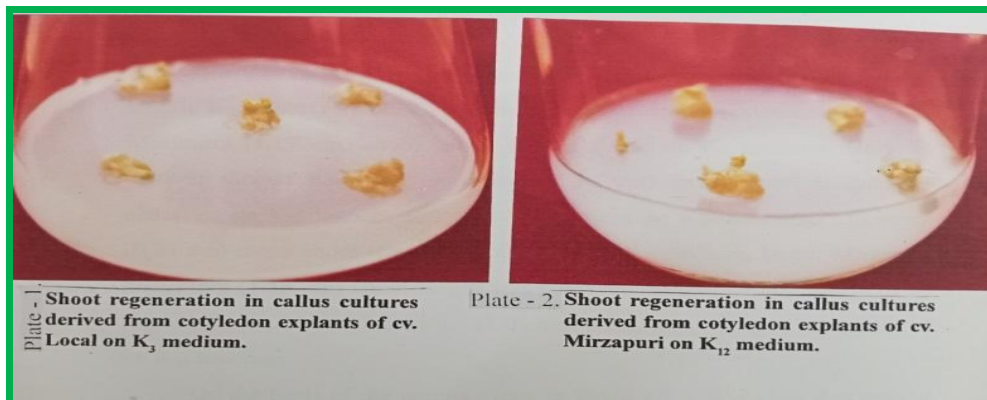
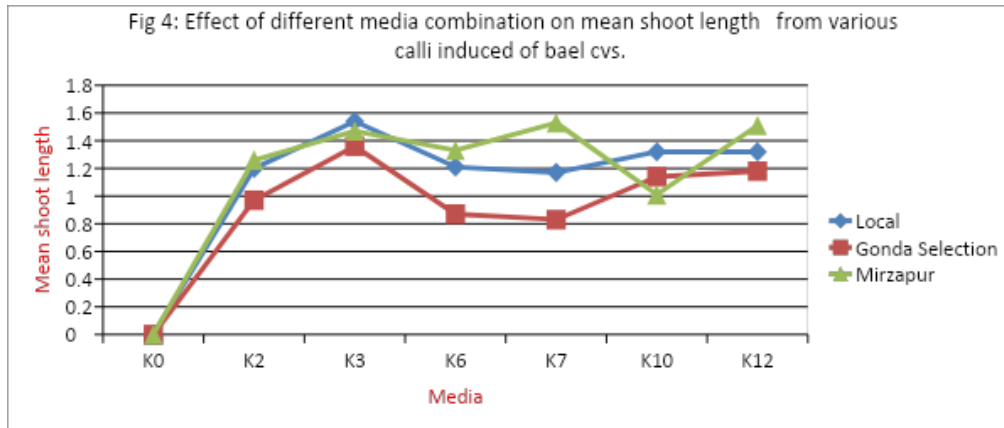
± SE

Table.5 Effect of different media combination on mean shoot length from various calli induced of *bael cvs.*

Media	Mean shoot length(cm)		
	Local	Gonda selection	Mirzapuri
K ₀	0	0	0
K ₂	1.20±0.22	0.97±0.18	1.26±0.18
K ₃	1.54±0.15	1.36±0.16	1.47±0.19
K ₆	1.21±0.18	0.87±0.13	1.33±0.35
K ₇	1.17±0.17	0.83±0.10	1.53±0.21
K ₁₀	1.32±0.20	1.14±0.16	1.01±0.37
K ₁₂	1.32±0.14	1.18±0.10	1.51±0.14

± SE





Arya *et al.*, (1981) reported shoot development from meristemoids occurred only when they were transferred to BAP and KIN alone or BA+NAA, whereas KIN was quite suitable for shoot induction from cotyledonary explant callus in bael. Generally, low auxin and high cytokinin concentration in the medium resulted in induction of shoot morphogenesis in the present study. M.S. Hazeena and G.R. Sulekha (2008) reported that shoot

regeneration response from the callus was best on MS medium containing 8.8 μM benzyl adenine and 2.85 μM indole-3- acetic acid. Pranita Jamdhade and Narayan Pandhure (2016) was observed maximum induction of somatic embryogenesis and shoot induction from cotyledon explant on Murashige and Skoog (MS) medium supplemented with BAP(2.0 mg/l) + NAA (0.5mg/l).

Number of plantlets obtained through intermediate stage of callus

Maximum mean number of plantlets per calli were observed in cv. Mirzapuri from cotyledon explant derived callus on Knop's medium having 1.0 mg l⁻¹ BAP, 1.0 mg l⁻¹ KIN & 0.5 mg l⁻¹ NAA followed by cv. Local on media containing 1.0 mg l⁻¹ BAP, 1.0 mg l⁻¹ KIN & 0.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BAP. No plants were observed in all the three cvs. on Knop's basal medium (Fig.3 and Table 4). It might be due to higher concentration of cytokinin which enhances the shoot initiation and auxin elongates the shoot primordial. Varghese *et al.*, (1993) reported that low concentration of BAP resulted in less development of shoots (3.2), but higher concentration promoted greater number (11.82) of shoots. However, BAP in combination with NAA resulted in the percentage of shoot formation in bael. Similarly, Rao and Lee (1982) achieved multiple shoot formation in calophyllum, Eugenia and Fragaria in presence of BAP. This was in contrast to the present investigation could be due to differences in genotypes and variation in endogenous level of growth regulators in cotyledon explants.

Mean shoot length

Knop's medium supplemented with 1.0 mg l⁻¹ BAP produced better shoot length in cvs. Local and Mirzapuri (Fig. 4 and Table 5). Hossain *et al.*, (1994a) reported maximum mean shoot length (7.2) on MS medium supplemented with 5 mg l⁻¹ BAP+0.1 mg l⁻¹ NAA+1.0 mg l⁻¹ GA3 in bael. This difference could be genotype and growth regulators used.

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