



P-ISSN: 2349-8528

E-ISSN: 2321-4902

[www.chemijournal.com](http://www.chemijournal.com)

IJCS 2020; 8(4): 218-222

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Received: 18-05-2020

Accepted: 22-06-2020

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## Challenges in micropropagation of Bael [*Aegle marmelos* (L.) Corr.] from leaf Disk Explants and methods for its adoption amongst progressive farmers and farm women

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**DOI:** <https://doi.org/10.22271/chemi.2020.v8.i4c.9692>

**Abstract**

The present study was performed to define tissue culture techniques for micropropagation of *bael* cvs. through leaf disk explants from 25 days old *in vitro* grown plantlets on Knop's medium supplemented with 0.5 mg<sup>l</sup><sup>-1</sup> BAP. Leaf disk explants were cultured on various concentrations of growth regulators (BAP, KIN and NAA). Knop's medium supplemented with 1.0 mg<sup>l</sup><sup>-1</sup> BAP, 0.5 mg<sup>l</sup><sup>-1</sup> KIN and 0.5 mg<sup>l</sup><sup>-1</sup> NAA produced maximum percentage of shoot regeneration (46.66%) and maximum per cent multiple shoot (33.33%) was found in cv. Mirzapuri on Knop's medium supplemented with 1.0 mg<sup>l</sup><sup>-1</sup> BAP+0.5 mg<sup>l</sup><sup>-1</sup> KIN +0.5 mg<sup>l</sup><sup>-1</sup> NAA. Maximum mean number of shoots per explants (1.20) was recorded in cv. Mirzapuri at 1.0 mg<sup>l</sup><sup>-1</sup> BAP+0.5 mg<sup>l</sup><sup>-1</sup> KIN+0.5 mg<sup>l</sup><sup>-1</sup> NAA whereas maximum shoot length (0.95cm) was observed in cv. Mirzapuri on Knop's medium supplemented with 1.0mg<sup>l</sup><sup>-1</sup> BAP+0.5mg<sup>l</sup><sup>-1</sup> KIN+0.5mg<sup>l</sup><sup>-1</sup> NAA.

**Keywords:** Micro propagation, tissue culture, growth regulators, Knop's medium, leaf Disk, Explants

**Introduction**

*Aegle marmelos* Corr. locally called Bael and occupies an important place among minor fruits in India. It is grown throughout India as well as in Sri Lanka, Pakistan, Bangladesh, Myanmar, Thailand and most of the Southeast Asian countries. It is very hardy, subtropical, deciduous trees that can thrive in various soil-climate conditions and can tolerate alkaline soil and is not injured by temperatures as low as -7<sup>o</sup> C. In India, there is no organized orcharding of these fruit. It grows mainly wild or in temple garden. All the parts of tree, including bark, stem, leaves, root and fruit at any stage of maturity and ripening have some use or other. The medicinal uses of this fruit were reported by Sebastian and Bhandari (1984). Anti-diarrheic activity of "Bael" root was studied by Pitre and Srivastava (1987)<sup>[11]</sup>. Owing to such valuable properties, it has become necessary to have plants of superior cultivars available for more exploitation.

The availability of genuine plant material limiting its cultivation is the main problem. It is usually propagated by seeds, suckers and by patch budding. The conventional method of propagation is very slow and season bound. Recently, regeneration of multiple shoots through organogenesis was achieved from seedling leaves (Islam *et al.*, 1993)<sup>[6]</sup>, Cotyledons (Hossain *et al.*, 1994)<sup>[2]</sup>, nucleus from developing fruits (Hossain *et al.*, 1993)<sup>[3]</sup> and root tips of intact seedlings (Islam *et al.*, 1996)<sup>[5, 7]</sup>. Micropropagation was the first biotechnological method applied in production. Maciej *et al.*, (1986)<sup>[10]</sup> commented that, at first, micropropagation was introduced as a commercial horticulture, though it was not a biotechnological method, the term biotechnology was adopted later. Economic analysis revealed that the micropropagation operations needs as high as 69% of intensive labour expenses. Watad *et al.* (1999)<sup>[17]</sup> suggested for scale up and use of mechanization technology for the expansion of commercial micropropagation. Challenges due to contaminations in the form of fungus and bacteria pose problems. Cobrado and Fernandez (2016)<sup>[1]</sup> highlighted common fungi contamination affecting tissue-cultured abace during initial stage micropropagation. Shi *et al.*, (2019)<sup>[14]</sup> developed a handy method to remove bacterial contamination.

Explants (Solim *et al.*, 2017) [15] from leaf were cultured at abaxial and adaxial positions in solid medium along with adequate supplements. Tomar *et al.*, (2008) [16] found that higher the multiplication rate lower will be the cost. Less number of stages in micropropagation will always lower the Tissue culture plant cost. In this study, an attempt has been made for *in vitro* leaf disc culture in bael cultivars.

## Materials and Methods

### Plant material

Seeds were removed from mature fruits of bael cvs. *viz.* Local, Gonda selection and Mirzapuri and washed thoroughly under running tap water with few drops of teepol. Cotyledons were separated from seeds and surface disinfected with 70 per cent aqueous solution (v/v) for 30 seconds followed by 0.1% aqueous solution of mercuric chloride; HgCl<sub>2</sub> (w/v) plus 2 drops of teepol in 100 ml solution for 2 minutes and then 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. Leaf disk explants were excised from *in vitro* raised plantlets from cotyledons with embryo axis on

Knop's medium supplemented with 0.5 mg l<sup>-1</sup> BAP from the middle portion of the unfolded leaf lets.

**Culture media:** The Knop's (1865) [8] basal medium with some modifications was used for plant regeneration. Leaf disk explants cultured on Knop's medium (1865) [8] supplemented with 3% sucrose (w/v), 100 mg l<sup>-1</sup> inositol, various concentrations and combinations of BAP, KIN and NAA. The data were recorded on per cent shoot regeneration, multiple shoot regeneration, mean number of multiple shoots per explants and mean shoot length (cm) after 7 weeks of culturing on half and full strength Knop's based media supplemented with various concentrations and combinations of BAP, IBA and NAA. The pH of the media was adjusted to 5.7 with 1N HCl and 1N NaOH and after that 0.8 per cent agar-agar was added to the medium. The media were melted at 1.2 kg cm<sup>2</sup> pressure for 15 min. in the autoclave. The melted were dispensed in 100 ml culture bottles and 100 ml or 150 ml conical flasks then the culture bottles with lid and the flasks with cotton plugs were closed and sterilized at 1.2 kg cm<sup>2</sup> pressure for 15 minutes. Then the culture bottles with lid and the flasks were closed with cotton plugs and sterilized.

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

Sr. No.	Media	Basal medium	Growth regulators (mgL <sup>-1</sup> )			
			Cytokinin		Auxin	
			BAP	KIN	NAA	2,4-D
1	K <sub>0</sub>	Knop's	0.00	0.00	0.00	0.00
2	K <sub>1</sub>	Knop's	0.25	0.00	0.00	0.00
3	K <sub>2</sub>	Knop's	0.50	0.00	0.00	0.00
4	K <sub>3</sub>	Knop's	1.00	0.00	0.00	0.00
5	K <sub>4</sub>	Knop's	2.00	0.00	0.00	0.00
6	K <sub>5</sub>	Knop's	0.00	0.25	0.00	0.00
7	K <sub>6</sub>	Knop's	0.00	0.50	0.00	0.00
8	K <sub>7</sub>	Knop's	0.00	1.00	0.00	0.00
9	K <sub>8</sub>	Knop's	0.00	2.00	0.50	0.00
10	K <sub>9</sub>	Knop's	0.50	0.50	0.50	0.00
11	K <sub>10</sub>	Knop's	0.50	1.00	0.50	0.00
12	K <sub>11</sub>	Knop's	1.00	0.50	0.50	0.00
13	K <sub>12</sub>	Knop's	0.20	1.00	0.50	0.00
14	K <sub>13</sub>	Knop's	0.20	0.00	0.00	0.20
15	K <sub>14</sub>	Knop's	0.20	0.00	0.00	0.50
16	K <sub>15</sub>	Knop's	0.00	0.00	0.00	1.00
17	K <sub>16</sub>	Knop's	0.50	0.50	5.00	0.00
18	K <sub>17</sub>	Knop's	0.50	0.50	2.00	2.00

### Culture conditions

All the cultures were maintained at 25±2<sup>0</sup>C under 16/8 hours cycle of light (2000 lux fluorescent tubes) and leaf disk explants were cultured with their abaxial surface touching the medium.

### Production of *in vitro* plants

Number of plantlets obtained through full strength Knop's medium supplemented with different concentrations and combinations of growth regulators.

## Results and Discussion

### Effect of different media combination on per cent shoot regeneration

The per cent shoot regeneration leaf disk explants of *bael* cvs. varied with Knop's medium (Fig.1 and Table 2). The growth regulators BAP and Kinetin when tested individually and in combination of BAP and Kinetin with 0.5 mg l<sup>-1</sup> NAA at various concentrations in Knop's medium produced varying effects. Leaf disk explants were placed abaxial on media tried

in all the three cvs. Leaf disk explants from 25 days old *in vitro* raised plantlets gave the maximum per cent shoot regeneration (46.66) in cv. Mirzapuri at 1.0 mg l<sup>-1</sup> BAP+0.5 mg l<sup>-1</sup> KIN+0.5 mg l<sup>-1</sup> NAA followed by cv. Gonda selection 36.15 per cent on same medium. Minimum per cent shoot regeneration (13.33) was observed in cv. Local on same medium. The data also revealed that in cvs. Local, the maximum (23.33) shoot regeneration was observed on K<sub>12</sub> medium followed by 16.66 per cent shoot regeneration on K<sub>4</sub> medium. Minimum (13.33%) shoot regeneration was observed in K<sub>11</sub> medium. No shoot regeneration was observed on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>5</sub>, K<sub>6</sub>, K<sub>7</sub>, K<sub>8</sub>, K<sub>9</sub> and K<sub>10</sub> media in cv. Local. Cultivar Gonda Selection showed maximum 36.15 per cent shoot regeneration from K<sub>11</sub> medium followed by 23.8 per cent shoot regeneration on K<sub>12</sub> medium. No shoot regeneration was observed on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>, K<sub>4</sub>, K<sub>5</sub>, K<sub>6</sub>, K<sub>7</sub> and K<sub>8</sub> media in cv. Gonda Selection. Whereas, in cultivar Mirzapuri maximum shoot regeneration (46.66%) was observed on K<sub>11</sub> medium followed by 43.33 per cent shoot on K<sub>3</sub> and K<sub>10</sub> media. Minimum 33.33 per shoot regeneration

was observed on K<sub>7</sub> and K<sub>9</sub> media. No shoot regeneration was found on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>5</sub> and K<sub>6</sub> media (Fig. 1 and Table2). While Islam *et al.* (1993)<sup>[6]</sup> reported remarkably varied shoot regeneration when leaves of 15-40 days old seedling placed on MS medium supplemented with 1.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> IAA and 53.9 per cent shoot regeneration was observed from 25 day old seedling in *bael*.

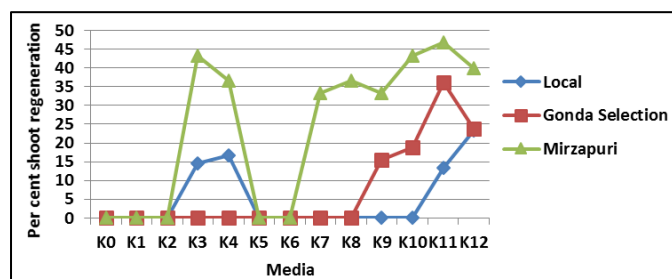


Fig 1: Effect of different media combination on per cent shoot regeneration of bael cvs. From leaf disc explants.

Table 2: Effect of different media combination on per cent shoot regeneration of bael cvs. from leaf disc explants.

Media	Per cent shoot regeneration		
	Local	Gonda selection	Mirzapuri
K <sub>0</sub>	0	0	0
K <sub>1</sub>	0	0	0
K <sub>2</sub>	0	0	0
K <sub>3</sub>	14.44±2.93	0	43.33±3.33
K <sub>4</sub>	16.66±3.33	0	36.66±3.33
K <sub>5</sub>	0	0	0
K <sub>6</sub>	0	0	0
K <sub>7</sub>	0	0	33.33±3.33
K <sub>8</sub>	0	0	36.66±3.33
K <sub>9</sub>	0	15.55±2.93	33.33±3.33
K <sub>10</sub>	0	18.88±1.11	43.33±3.33
K <sub>11</sub>	13.33±3.33	36.15±3.88	46.66±3.33
K <sub>12</sub>	23.33±3.33	23.80±3.15	40.00±5.77

± SE

### Effect of different media combination on per cent multiple shoots

Maximum per cent multiple shoot (33.33%) was found in cv. Mirzapuri on Knop's medium supplemented with 1.0 mg l<sup>-1</sup> BAP+0.5 mg l<sup>-1</sup> KIN +0.5 mg l<sup>-1</sup> NAA followed by 23.33 per cent multiple shoots in cv. Local on Knop's medium containing 1.0 mg l<sup>-1</sup> BAP+1.0 mg l<sup>-1</sup> KIN+0.5 mg l<sup>-1</sup> NAA. Minimum per cent multiple shoot regeneration (9.44%) was observed in cv. Gonda Selection on Knop's medium supplemented with 0.5 mg l<sup>-1</sup> BAP+0.5 mg l<sup>-1</sup> KIN +0.5 mg l<sup>-1</sup> NAA medium. The data also revealed that in cv. Local, the maximum (23.33%) multiple shoots were observed on K<sub>12</sub> medium followed by 20 per cent multiple shoots on K<sub>11</sub> medium. Minimum (13.33%) multiple shoots were observed on K<sub>4</sub> medium. No solitary and multiple shoots were observed on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>5</sub>, K<sub>6</sub>, K<sub>7</sub>, K<sub>8</sub> K<sub>9</sub> and K<sub>10</sub> media. In cultivar Gonda Selection, maximum 21.79 per cent multiple shoots were observed on K<sub>11</sub> medium followed by 12.77 per cent multiple shoots on K<sub>10</sub> medium. Minimum (9.44%) multiple shoots were observed on K<sub>9</sub> medium No solitary and multiple shoots were observed on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>, K<sub>4</sub>, K<sub>5</sub>, K<sub>6</sub>, K<sub>7</sub> and K<sub>8</sub> media in cv. Gonda Selection. Whereas, in cultivar Mirzapuri maximum multiple shoots (33.33%) was observed on K<sub>11</sub> medium followed by 23.33 per cent multiple shoots on K<sub>3</sub>,

K<sub>10</sub> and K<sub>12</sub> media. Minimum 13.33 per multiple shoots were observed on K<sub>4</sub> and K<sub>7</sub> media. No solitary and multiple shoots were found on K<sub>0</sub>, K<sub>1</sub>, K<sub>2</sub>, K<sub>5</sub>, K<sub>6</sub> and K<sub>8</sub> media. The variation in response may be due to different endogenous level of growth regulators. (Fig. 2 and Table 3). Pozoga *et al.*, (2019)<sup>[12]</sup> were obtained, 2 shoots grown with 3.5 culturable nodes on each, and in 70% reduced light, 2 new shoots were grown with 6 culturable nodes on each, using a half-strength MS medium containing 0.5 mg/L BAP in standard light conditions.

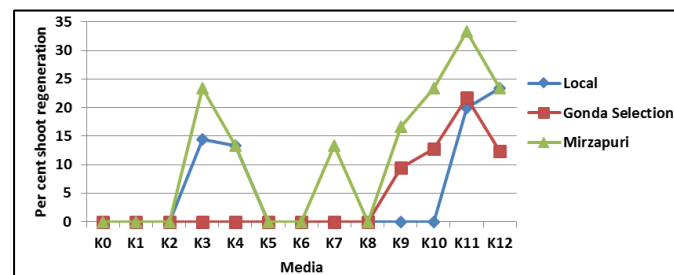


Fig 2: Effect of different media combination on per cent multiple shoots of bael cvs. from leaf disc explants

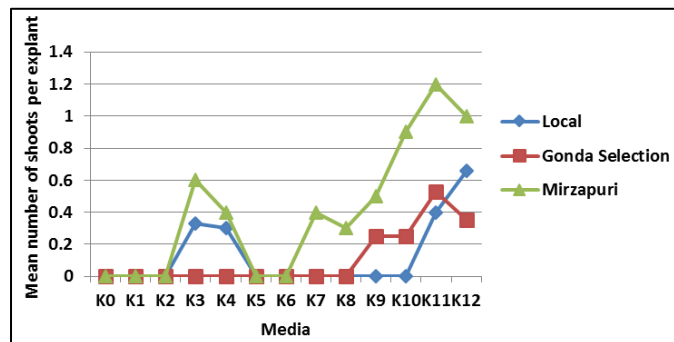
Table 3: Effect of different media combination on per cent multiple shoots of bael cvs. from leaf disc explants.

Media	Per cent multiple shoot		
	Local	Gonda selection	Mirzapuri
K <sub>0</sub>	0	0	0
K <sub>1</sub>	0	0	0
K <sub>2</sub>	0	0	0
K <sub>3</sub>	14.44±2.93	0	23.33±3.33
K <sub>4</sub>	13.33±3.33	0	13.33±3.33
K <sub>5</sub>	0	0	0
K <sub>6</sub>	0	0	0
K <sub>7</sub>	0	0	13.33±3.33
K <sub>8</sub>	0	0	0
K <sub>9</sub>	0	9.44±0.55	16.67±6.77
K <sub>10</sub>	0	12.77±3.64	23.33±3.33
K <sub>11</sub>	20.00±5.77	21.79±4.31	33.33±3.33
K <sub>12</sub>	23.33±3.33	12.38±3.90	23.33±3.33

± SE

### Effect of different media combination on mean number of shoots

Solitary as well as multiple shoots were observed in all the three cvs. Maximum mean number of shoots per explants (1.20) was recorded in cv. Mirzapuri at 1.0 mg l<sup>-1</sup> BAP+0.5 mg l<sup>-1</sup> KIN+0.5 mg l<sup>-1</sup> NAA followed by local (0.66) on knop's medium supplemented with 1.0 mg l<sup>-1</sup> BAP+1.0 mg l<sup>-1</sup> NAA. Minimum mean number of shoots per explants (0.25) were recorded in cv. Gonda Selection on Knop's medium containing 0.5 mg l<sup>-1</sup> BAP+0.5 mg l<sup>-1</sup> KIN+0.5 mg l<sup>-1</sup> NAA and Knop's medium having 0.5 mg l<sup>-1</sup>+1.0 mg l<sup>-1</sup> KIN+0.5 mg l<sup>-1</sup> NAA media (Fig. 3 and Table4). In general, younger tissue has been observed to be more responsive in tissue culture than older tissue. In this case our results is in contrast from those of Kumar and Seeni (1998)<sup>[9]</sup> who found that whole leaf were regenerative and produced 11.3 shoots and buds at frequencies of 100% in seven weeks. Islam *et al.*(1993)<sup>[6]</sup> reported single shoot produced from more than 20 days older explants on MS medium supplemented with 1.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> IAA. This difference may be due to genotype or growth regulators used.



**Fig 3:** Effect of different media combination on mean number of shoots per explant of *bael* cvs. from leaf disc explants.

**Table 4:** Effect of different media combination on mean number of shoots per explant of *bael* cvs. from leaf disc explants.

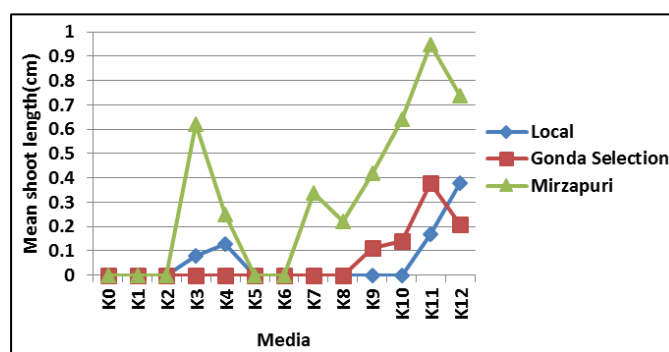
Media	Mean number of shoots per explant		
	Local	Gonda selection	Mirzapuri
K <sub>0</sub>	0	0	0
K <sub>1</sub>	0	0	0
K <sub>2</sub>	0	0	0
K <sub>3</sub>	0.33±0.23	0	0.60±0.26
K <sub>4</sub>	0.30±0.30	0	0.40±0.22
K <sub>5</sub>	0	0	0
K <sub>6</sub>	0	0	0
K <sub>7</sub>	0	0	0.40±0.22
K <sub>8</sub>	0	0	0.30±0.15
K <sub>9</sub>	0	0.25±0.17	0.50±0.30
K <sub>10</sub>	0	0.25±0.17	0.90±0.45
K <sub>11</sub>	0.40±0.40	0.53±0.26	1.20±0.53
K <sub>12</sub>	0.06±0.38	0.35±0.16	1.00±0.53

± SE

#### Effect of different media combination on mean shoot length

Maximum shoot length (0.95cm) was observed in cv. Mirzapuri on Knop's medium supplemented with 1.0mg<sup>-1</sup> BAP+0.5mg<sup>-1</sup> KIN+0.5mg<sup>-1</sup> NAA followed by 0.38 in cvs. Local and Gonda Selection on Knop's medium containing 1.0mg<sup>-1</sup> BAP+1.0 mg<sup>-1</sup> KIN+0.5 mg<sup>-1</sup> NAA and 1.0mg<sup>-1</sup> BAP+0.5 mg<sup>-1</sup> KIN+0.5mg<sup>-1</sup> NAA media, respectively. Minimum mean shoot length (0.11) were observed in cv. Gonda Selection on Knop's medium having 0.5 mg<sup>-1</sup> BAP+0.5 mg<sup>-1</sup> KIN+0.5mg<sup>-1</sup> NAA (Fig. 4 and Table 5). Kumar and Seeni (1988) [9] recorded maximum mean shoot length (5.2cm) on MS medium supplemented with 2.5 mg<sup>-1</sup> BAP and 1.0mg<sup>-1</sup> IAA from nodal segments of mature plant in bael. The difference in shoot length may be due to the physiological states of the buds on different regions of a stem. Among all the three cvs., cv. Gonda Selection produced maximum per cent rooting (43.89%) of regenerated shoots on full strength Knop's medium supplemented with 15.0 mg<sup>-1</sup> IBA followed by 43.33 in cv. Local on same medium. Regenerated shoots were excised and place on Knop's medium supplemented with concentration of IBA and in combination of BAP, IBA and NAA at different concentration for root induction. Differences in genotypic responses to root initiation in "bael" were observed. Similarly, Hossain *et al.*, (1993) [3] reported 50-60% rooting responses of "bael" in 1-2 mg<sup>-1</sup> IBA supplemented media in bael. However, Kumar and Seeni (1998) [9] reported that 90% micro

shoots of bael formed roots on MS medium containing IBA (10.0 mg<sup>-1</sup>) in bael. This was in contrast to the per cent root formation in bael may be due to genotype and growth regulators used. Knop's medium supplemented with 15mg<sup>-1</sup> IBA produced 3.0 & 2.46 mean number of shoots per plantlet in cvs. Gonda Selection and Local, respectively. Similarly, Hossain *et al.*, (1993) [3] reported mean number roots per micro shoot (3.3) on MS medium containing 4.9 OM IMA in "bael". However, Hossain *et al.* (1994a) [2] reported maximum mean number roots per micro shoot (3.5) in "bael" on MS medium containing 0.5 mg<sup>-1</sup> IBA and 0.5 mg<sup>-1</sup> NAA in a separated experiment when another genotype was used. The variation may be due to the genotype and endogenous levels of growth regulators. Further, it was observed that mean shoot length (1.31 cm) in cv. Local was higher on full strength Knop's medium supplemented with 15 mg<sup>-1</sup> IBA and 1/2 Knop's medium supplemented with 15 mg<sup>-1</sup> IBA and it was not significantly different from mean shoot length (1.25 cm) and (1.24 cm) observed in cvs. Mirzapuri and Gonda Selection on 1/2 Knop's medium containing 15 mg<sup>-1</sup> IBA and full strength Knop's medium having 10 mg<sup>-1</sup> IBA, respectively. Hossain *et al.* (1993) [3] reported 3.1 cm mean root length on Ms medium containing 4.9 OM IBA in bael. In separate experiment, maximum mean root length (3.5cm) was observed on MS medium containing 0.5 mg<sup>-1</sup> IBA and 0.5 mg<sup>-1</sup> NAA when another genotype was used (Hossain *et al.* 1994a) [2]. This difference may be due to the genotype and growth regulators used.



**Fig 4:** Effect of different media combination on mean shoot length of *bael* cvs. from leaf disc explants.

**Table 5:** Effect of different media combination on mean shoot length of *bael* cvs. from leaf disc explants.

Media	Mean shoot length(cm)		
	Local	Gonda selection	Mirzapuri
K <sub>0</sub>	0	0	0
K <sub>1</sub>	0	0	0
K <sub>2</sub>	0	0	0
K <sub>3</sub>	0.08±0.05	0	0.62±0.13
K <sub>4</sub>	0.13±0.07	0	0.25±0.11
K <sub>5</sub>	0	0	0
K <sub>6</sub>	0	0	0
K <sub>7</sub>	0	0	0.34±0.14
K <sub>8</sub>	0	0	0.22±0.11
K <sub>9</sub>	0	0.11±0.06	0.42±0.15
K <sub>10</sub>	0	0.14±0.07	0.64±0.12
K <sub>11</sub>	0.17±0.07	0.38±0.12	0.95±0.09
K <sub>12</sub>	0.38±0.09	0.21±0.08	0.73±0.11

± SE



**Plate 1:** Shoot regeneration from disk explants of cv. Gonda selection on K<sub>11</sub> medium



**Plate 2:** Multiple shoot regeneration from Leaf disk explants of cv. Mirzapuri on K<sub>11</sub> medium

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