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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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 l⁻¹ BAP. Leaf disk explants were cultured on various concentrations of growth regulators (BAP, KIN and NAA). Knop’s medium supplemented with 1.0 mg l⁻¹ BAP, 0.5 mg l⁻¹ KIN and 0.5 mg l⁻¹ 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 l⁻¹ BAP+0.5 mg l⁻¹ KIN +0.5 mg l⁻¹ NAA. Maximum mean number of shoots per explants (1.20) was recorded in cv. Mirzapuri at 1.0 mg l⁻¹ BAP+0.5 mg l⁻¹ KIN+0.5 mg l⁻¹ NAA whereas maximum shoot length (0.95cm) was observed in cv. Mirzapuri on Knop’s medium supplemented with 1.0mg l⁻¹ BAP+0.5mg l⁻¹KIN+0.5mg l⁻¹ 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°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) [11]. 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) [6], Cotyledons (Hossain et al., 1994) [2], nuclleus from developing fruits (Hossain et al., 1993) [3] and root tips of intact seedlings (Islam et al., 1996) [5, 7]. Micropropogation was the first biotechnological method applied in production. Maciej et al., (1986) [10] commented that, at first, micropropogation was introduced as a commercial horticulture, though it was not a biotechnological method, the term biotechnology was adopted later. Economic analysis revealed that the micropropogation operations needs as high as 69% of intensive labour expenses. Watad et al. (1999) [17] suggested for scale up and use of mechanization technology for the expansion of commercial micropropogation. Challenges due to contaminations in the form of fungus and bacteria pose problems. Cobrado and Fernandez (2016) [1] highlighted common fungi contamination affecting tissue-cultured abace during initial stage micropropogation. Shi et al., (2019) [14] developed a handy method to remove bacterial contamination.
Explants (Solim et al., 2017) from leaf were cultured at abaxial and adaxial positions in solid medium along with adequate supplements. Tomar et al., (2008) found that the 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 of HgCl₂ (w/v) for 30 seconds followed by 0.1% aqueous solution of mercuric chloride; HgCl₂ (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⁻¹ BAP from the middle portion of the unfolded leaflets.

Culture media: The Knop’s (1865) basal medium with some modifications was used for plant regeneration. Leaf disk explants cultured on Knop’s medium (1865) supplemented with 3% sucrose (w/v), 100 mg l⁻¹ 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⁻²pressure 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⁻² pressure for 15 minutes. Then the culture bottles with lid and the flasks were closed with cotton plugs and sterilized.

| Sr. No. | Media | Basal medium | Cytokinin Growth regulators (mg l⁻¹) | Auxin | 2,4-D |
|---------|-------|--------------|-------------------------------------|-------|-------|
| 1 | K₀ | Knop’s | 0.00 | 0.00 | 0.00 | 0.00 |
| 2 | K₁ | Knop’s | 0.25 | 0.00 | 0.00 | 0.00 |
| 3 | K₂ | Knop’s | 0.50 | 0.00 | 0.00 | 0.00 |
| 4 | K₃ | Knop’s | 1.00 | 0.00 | 0.00 | 0.00 |
| 5 | K₄ | Knop’s | 2.00 | 0.00 | 0.00 | 0.00 |
| 6 | K₅ | Knop’s | 0.00 | 0.25 | 0.00 | 0.00 |
| 7 | K₆ | Knop’s | 0.00 | 0.50 | 0.00 | 0.00 |
| 8 | K₇ | Knop’s | 0.00 | 1.00 | 0.00 | 0.00 |
| 9 | K₈ | Knop’s | 0.00 | 2.00 | 0.50 | 0.00 |
| 10 | K₉ | Knop’s | 0.50 | 0.50 | 0.50 | 0.00 |
| 11 | K₁₀ | Knop’s | 0.50 | 1.00 | 0.50 | 0.00 |
| 12 | K₁₁ | Knop’s | 1.00 | 0.50 | 0.50 | 0.00 |
| 13 | K₁₂ | Knop’s | 0.20 | 1.00 | 0.50 | 0.00 |
| 14 | K₁₃ | Knop’s | 0.20 | 0.00 | 0.00 | 0.20 |
| 15 | K₁₄ | Knop’s | 0.20 | 0.00 | 0.00 | 0.50 |
| 16 | K₁₅ | Knop’s | 0.00 | 0.00 | 0.00 | 1.00 |
| 17 | K₁₆ | Knop’s | 0.50 | 0.50 | 5.00 | 0.00 |
| 18 | K₁₇ | Knop’s | 0.50 | 0.50 | 2.00 | 2.00 |

Culture conditions

All the cultures were maintained at 25±2°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⁻¹ 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⁻¹ BAP+0.5 mg l⁻¹ KIN+0.5 mg l⁻¹ 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. The data also revealed that in cvs. Local, the maximum (23.33) shoot regeneration was observed on K₂ media followed by 16.66 per cent shoot regeneration on K₄ medium. Minimum (13.33%) shoot regeneration was observed in K₁₁ medium. No shoot regeneration was observed on K₀, K₆, K₁₀, K₁₁, K₁₂, K₁₃, K₁₄, K₁₅, K₁₆ and K₁₇ media in cv. Local. Cultivar Gonda Selection showed maximum 36.15 per cent shoot regeneration from K₁₁ medium followed by 23.8 per cent shoot regeneration on K₂ medium. No shoot regeneration was observed on K₀, K₁, K₃, K₅, K₆, K₇, K₈ and K₁₀ medium in cv. Local. Cultivar Mirzapuri maximum shoot regeneration (46.66%) was observed on K₁₁ medium followed by 43.33 per cent shoot on K₁ and K₁₀ media. Minimum 33.33 per cent shoot regeneration...
was observed on K7 and K9 media. No shoot regeneration was found on K6, K1, K2, K3, K4 and K6 media (Fig. 1 and Table 2). While Islam et al. (1993) [6] reported remarkably varied shoot regeneration when leaves of 15-40 days old seedling placed on MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L IAA and 53.9 per cent shoot regeneration was observed from 25 day old seedling in bael.

Maximum per cent multiple shoot (33.33%) was found in cv. Mirzapuri on Knop’s medium supplemented with 1.0 mg/L BAP+0.5 mg/L KIN +0.5 mg/L NAA followed by 23.33 per cent multiple shoots in cv. Local on Knop’s medium containing 1.0 mg/L BAP+1.0 mg/L KIN+0.5 mg/L 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 BAP+0.5 mg/L KIN +0.5 mg/L NAA medium. The data also revealed that in cv. Local, the maximum (23.33%) multiple shoots were observed on K12 medium followed by 20 per cent multiple shoots on K11 medium. Minimum (13.33%) multiple shoots were observed on K9 medium. No solitary and multiple shoots were observed on K6, K1, K2, K3, K4, K5 K6 and K9 media. In cultivar Gonda Selection, maximum 21.79 per cent multiple shoots were observed on K11 medium followed by 12.77 per cent multiple shoots on K10 medium. Minimum (9.44%) multiple shoots were observed on K6 medium. No solitary and multiple shoots were observed on K9 medium. No solitary and multiple shoots were observed on K6, K1, K2, K3, K4, K5 K6 and K9 media in cv. Gonda Selection. Whereas, in cultivar Mirzapuri maximum multiple shoots (33.33%) was observed on K11 medium followed by 23.33 per cent multiple shoots on K3, K10 and K12 media. Minimum 13.33 per multiple shoots were observed on K4 and K7 media. No solitary and multiple shoots were found on K9, K1, K2, K3, K5, K6 and K9 media. The variation in response may be due to different endogenic level of growth regulators. (Fig. 2 and Table 3). Pozoga et al., (2019) [12] were obtained, 2 shoots grown with 3.5 cultivable nodes on each, and in 70% reduced light, 2 new shoots were grown with 6 cultivable nodes on each, using a half-strength MS medium containing 0.5 mg/L BAP in standard light conditions.

Effect of different media combination on per cent multiple shoots

Solitary as well as multiple shoots were observed in all the three cv.s. Maximum mean number of shoots per explants (1.20) was recorded in cv. Mirzapuri at 1.0 mg/L BAP+0.5 mg/L KIN+0.5 mg/L NAA followed by local (0.66) on knop’s medium supplemented with 1.0 mg/L BAP+1.0 mg/L 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 BAP+0.5 mg/L KIN+0.5 mg/L NAA and Knop’s medium having 0.5 mg/L+1.0 mg/L KIN+0.5 mg/L NAA media (Fig. 3 and Table 4). 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 Seeini (1998) [9] 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) [6] reported single shoot produced from more than 20 days older explants on MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L IAA. This difference may be due to genotype or growth regulators used.

![Fig 1: Effect of different media combination on per cent shoot regeneration of bael cvs. From leaf disc explants.](image1.png)

![Fig 2: Effect of different media combination on per cent multiple shoots of bael cvs. from leaf disc explants](image2.png)

![Table 3: Effect of different media combination on per cent multiple shoots of bael cvs. from leaf disc explants](table3.png)
shoots of bael formed roots on MS medium containing IBA (10.0 mg/l) 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/l 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/l IBA and 0.5 mg/l 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/l IBA and 1/2 Knop’s medium supplemented with 15 mg/l 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/l IBA and full strength Knop’s medium having 10 mg/l 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/l IBA and 0.5 mg/l NAA when another genotype was used (Hossain et al. 1994a) (2). This difference may be due to the genotype and growth regulators used.

**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/l BAP+0.5mg/l KIN+0.5mg/l NAA followed by 0.38 in cvs. Local and Gonda Selection on Knop’s medium containing 1.0mg/l BAP+1.0 mg/l KIN+0.5 mg/l NAA and 1.0mg/l BAP+0.5 mg/l KIN+0.5mg/l NAA media, respectively. Minimum mean shoot length (0.11) were observed in cv. Gonda Selection on Knop’s medium having 0.5 mg/l BAP+0.5 mg/l KIN+0.5mg/l 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/l BAP and 1.0mg/l 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/l IBA followed by 43.33 in cv. Local on same medium. Regenerated shoots were excised and placed 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/l IBA supplemented media in bael. However, Kumar and Seeni (1998) (9) reported that 90% micro
Plate 1: Shoot regeneration from disk explants of cv. Gonda selection on K11 medium

Plate 2: Multiple shoot regeneration from Leaf disk explants of cv. Mirzapuri on K11 medium

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