UK Journal of Pharmaceutical and Biosciences
Available at www.ukjpb.com
ISSN: 2347-9442

Regeneration of Aegle *marmelos* (l.) Through Enhanced Axillary Branching from Cotyledenory Node

Asha Gupta[1]*, Tessy Thomas[1], Shagufta Khan[2]

[1]Department of Botany, Govt. P.G. College, Bhopal (MP)- 462022, India
[3]GrowTips Biotech, Saket Nagar, Bhopal (MP)- 462024, India

Article Information
Received 22 February 2018
Received in revised form 9 April 2018
Accepted 10 April 2018

Keywords:
Axillary proliferation,
Medicinal tree,
Cotyledonary nodes,
Micropropagation

Corresponding Author:
E-mail : growtips_200254@yahoo.com
Mob.: +919826016389

Abstract

A complete protocol is standardized for *invitro* micropropogation of *Aegle marmelos* for the first time using cotyledonary nodes derived through *invitro* raised seedlings.Higher percentage of direct multiple shoots were regenerated from cotyledonary nodal segments through forced axillary branching. The cotyledonary nodes of *invitro* raised seedlings were used as explants for shoot formation on MS Medium supplemented with Cytokinins (BAP) and Auxins (NAA), either alone or in combinations. Maximum (80%) shoots having shoot length of 2-3 cm were achieved on MS medium fortified with BAP (1.0 mg/l) and NAA (0.5mg/l).By repeating sub culturing of the cotyledonary node on shoot multiplication medium followed by shoot elongation medium after each harvest of the newly formed shoots. Thus, from a single cotyledonary node, about 20-25 shoots were obtained. Shoots formed in vitro were best rooted on MS medium supplemented with 1.0-2.0 mg/l Napthalene acetic acid(NAA),

1 Introduction

Aegle marmelos (L.) Corr. belongs to Family Rutaceae is a spiny species medicinal tree mainly distributed wildly throughout India. Commonly known as Bael fruit is in high demand for its religious and pharma-ceutical purposes[1]. Every parts of the tree have tremendous nutritive and medicinal properties are used in ayurvedic preparation for various ailments. Several workers investigated different parts of *A. marmelos* has reported the plant contains coumarins, alkaloids, triterpenes, sterols and essential oils[2].

There are many investigations about antibacterial and antifungal importance of essential oils has reported. The bael fruits contains marmelosin, which is laxative, diuretic, being used in many Indian drugs[3]. Has a wide therapeutic value in the treatment of diabetes, anaemia, fractures, healing of wound, swollen Joints, high blood pressure, Jundice, diarrhoea, troubles during pregnancy and typhoid. Fruits and roots also shows antiamoebic and hypoglycemic activities[4]. The leaves contain alkaloid aegeline is a potent antiasthamatic agent[5]. Green leaves shows anti-inflamatory, antipyretic and analgesic properties, leaves juices are used for the treatment of bronchitis, and the decoction of root barks has also been used as anti-malarial drug[6]. It is reported that the leaves also helps in controlling pollution by absorbing foul gases from the atmosphere and keep it clean and salubrious and can be grown under various wasteland suitably. Due to heavy unrestricted exploitation and demand in ayurvedics and pharmaceuticals industries the natural occurrence of this important medicinal plant has been markedly depleted. Thereby it is included in the red listed medicinal tree[7]. However, its commercial orcharding is not expanding at a faster pace due to severe shortage of planting material.

Conventionally the plant propagation of *Aegle marmelos* (Inarching, budding and soft wood grafting) is season bound and slow. The seeds are mostly very small in number and have poor potential to germination. *In vitro* micropropagation technology can be beneficially employed in mass multiplication of elite bael plant varieties[8]. To accomplish in this contest an attempt was made to standardize an efficient protocol for tissue culture so as to conserve the species *in vitro*. Although there

are many techniques have been reported for propagation of *Aegle marmelos by using in vitro raised* seedling explants, *i.e*. from roots[9], from hypocotyl[10] cotyledonary node[11] and excised leaf explants[12] have more been concentrated on *in vitro* grown seedling explants may be due to more efficiency to regenerate through juvenile tissues.

Therefore the present investigation has been carried out to establish the most appropriate protocol for *invitro* regeneration and mass propagation of *A. marmelos* of M.P. region.

2 Materials and Methods

2.1 Collection of Plant material

Unripe fruits of *A. marmelos* were collected from an elite tree growing in the Sehore district area. Immature white seeds were isolated from the fruits and used as explants material for the present experiments.

After removing the mucilaginous coat the seeds, were washed three to four times with plain water and then with liquid soap solution followed by washing with tap water. Further surface sterilization treatment was conducted in a laminar air flow chamber. Seeds were dipped into 0.1% (w/v) freshly prepared mercuric chloride solution for 5 minutes, and then washed with 4-5 times in sterile double distilled water and are then inoculated aseptically in different composition medias.

To obtain the *in vitro* seed germination surface-sterilized seeds *Aegle marmelos* of were inoculated aseptically on basal media namely MS[13] (Murashige and Skoog, 1962). Seeds were also inoculated on the concentration of half strength MS media containing 3% sucrose and gelled with 0.8% agar having pH 5.7 in culture bottles. The cultures incubated at 24-25^{o}c under dark for 10 days and under fluorescent light with a 16/8 light/dark photoperiod.

2.2 Shoot Regeneration Experiment

Cotyledonary nodes (CNs), excised from 1-2 cm length *in vitro* raised seedlings and were taken for shoot development experiment on MS medium supplemented with varied concentration of cytokinins like BAP (0.5-2.0 mg/l), and Kinetin (0.5mg/l-1.0 mg/l) alone and with combination. The pH of all medium was adjusted to 5.8 before autoclaving. The cultures were incubated in a culture room at 25 ± 2^{o}c under sixteen hours photo period provided by cool white fluorescent tubes (Phillips India). After shoot initiation from CNs of seedlings, the initiated cultures were sub culture in the fresh medium for profuse multiplication of shoots on different higher concentration of cytokinin in combination with NAA and additive Adenine sulphate. The growth responses of explants were studied at weekly interval. The parameters were taken as the number of shoots initiated and multiplied, the height of regenerated and multiplied shoots and the callus developed.

2.3 Root Induction and Root Growth

Multiplied shoots developed in the presence of different growth regulators (cytokinins) and additives generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium, which is different from the shoot multiplication medium, especially in its hormonal composition. The shoot were sub cultured for further growth and after attainment to a height of 2 cm the shoots were transferred to rooting media and number of days taken for root initiation was observed and recorded in the medium containing different concentration of auxins like IAA, NAA, IBA and Activated charcoal (AC). The growth parameter was observed as percentage of root initiate, length in cms and number of roots formed.

2.4 Data scoring

Data were recorded on percentage of response, shoot number, shoot length after 4 weeks of culture on MS basal medium with or without addition of plant growth regulators (PGRS). Similarly, data were also recorded on percentage of root induction, root numbers, and root length after 4 weeks of culture in the root induction medium. Data was analyzed statistically using analysis of variance (ANOVA) for a completely randomized design. Ten replicates were used for every treatment and repeated thrice.

3 Results and Discussion

In vitro micropropagation of woody tree species shows some difficulties due to slow growth, formation of phenolic exudates in the culture medium, long complex life cycles and great genetic variations.

So it is essential to standardize an efficient regeneration protocol to establishment of given species under *in vitro* condition. Standardization begins with use of explants and various media and different hormonal concentration to different explants. Various previous studies reported that cytokine: auxin ratio is the deciding factor in the establishment of efficient reproducible protocol, Higher cytokine and low auxin concentration helps to multiple shoot regeneration.

The method of *in vitro* seed germination and use of seedling explants could be exploited for the microprapogation of woody species to preserve intrinsic genetic variability and also prove useful for obtaining sterile explants as juvenile tissues shows better regenerability potential in the technique.

Initiation and Adventitious shoot Regeneration

In all the subsequent experiments, MS medium has been frequently used for the *in vitro* micropropagation of a large number of plants[14,15]. In the present study isolated cotyledonary nodes (CNs), from *in vitro* raised seedling shows shoot induction in every medium tried for shoot development experiment. Incorporation of a cytokinin in the medium greatly affected the induction of axillary shoot proliferation from cotyledonary nodes.

Higher cytokine with the combination of low auxins concentration helps to multiple shoot proliferation. Shoot formation was stimulated by the media with various concentrations cytokinin (BAP) either alone with combination with different concentrations of Auxin (NAA). Maximum shoots per CNs, was achieved on the frequency or regeneration (70%) and the average mean 6.8 ± 0.10 shoots per CN , having 6.4 ± 0.13 of length developed from explants were maximum on MS medium containing BAP (1.0 mg/l) and NAA (0.5mg/l) within 6 weeks (Table 1).

Table 1: Effect of Different growth regulators for shoot Initiation in *Aegle marmelos*

MS Medium +PGR (mg/l)	% of Shoot Initiation	Mean no. of shoots/ explants ± SE	Mean length of shoots(cms) ± SE
0.5 BAP	45%	2.0 ±0.16	1.85 ± 0.02
1.0 BAP	50%	2.2 ±0.56	2.2 ± 0.06
0.5 BAP+0.1 KN	60%	1.6 ±0.01	1.67 ± 0.08
1.0 BAP+0.1 KN	60%	5.4 ± 0.11	1.8 ± 0.04
1.0 BAP+0.5NAA	70%	6.8 ± 0.10	6.4 ± 0.13

Further subculture of shoots on the fresh medium could not enhance shoot elongation. These results are similar to earlier reports where cytokinins such as BAP, zeatin, and kinetin have been shown to stimulate shoot proliferation while inhibiting shoot elongation[16,17]. To improve shoot multiplication and elongation an additive AS was incorporated in combination with 1.0 mg/l BAP and 0.5 NAA. Incorporation of 20 mg/l AS induced a significant increase (80%) of regeneration response having the maximum mean number of 19.9±0.76 of mean length of 8.5± 0.06 elongated shoots within 40 to 45 days culture (Table 2). Multiple shoots obtained were regenerated into 2-3 clumps after each subcultured in the fresh multiplication media having additional substance (AS- 20mg/l) with the formation of about 20-25 number of shoots. Multiple shoot formation from the young shoot explants of *Clitoria ternatea* on 0.5mg/l with the addition of lower concentration of NAA or IAA has also been reported by Kalamni and Michael Gomez (2002)[18].

The result shows the addition of small amount of AS were effective in *Aegle marmelos* production and elongation. For the root induction on regenerated shoots addition of auxins in the medium was essential. Maximum percentage of root initiated occurred within 18-20 days culture on both MS half and full strength medium supplemented with 2.0 mg/l of NAA (Auxin). However 50 to 60 percent shoots were rooted on medium contains higher concentration of NAA. (Table.3). While IAA widely used as rooting hormones also shows good results as compared to IBA. As IBA auxin has proved the mostly used growth inducing hormone for the woody plants both under in vivo and in vitro conditions[19].

Table 2: Effect of Different growth regulators for Multiplication of shoot in *Aegle marmelos*

MS Med.+PR (mg/l)	% of shoot Multipl.	Mean no. of shoot produced ±SE	Mean shoot length(cms)± SE
0.5 BAP	50%	10.2±0.96	9.5± 0.08
1.0BAP+0.5NAA	70%	13.74± 0.12	8.2± 0.06
1.0 BAP+1.0 KN	60%	12.9±0.16	7.5±0.17
1.0 BAP+0.5 NAA+ 20 AS	80%	19.9±0.76	8.5± 0.06
2.0BAP+1.0NAA	50%	8.9±0.16	6.1± 1.30

Table 3: Effect of Different growth Regulators on Root Inducton in *Aegle marmelos*

MS Medium +PGR (mg/l)	Days req. for root induction	Root formation(%)	Roots morphology
MS 1/2 + 1.0 IBA	22-25	30	Short, Healthy
MS 1/2 + 2.0 IBA	20-22	20	Short, Healthy
MS 1/2 +1.0 NAA	18-20	20	Long, thin
1.0 NAA	20	60	Long, thin
2.0 NAA	18	70	Long, thin
0.5 IAA	16-18	40	Long, thick
1.0 IAA	17	40	Short thick

Each values represents mean ±SE calculated from three separate experiments each with 10 replicates per treatment

4 Conclusion

In vitro micropropogation contribute the best and efficient of many important and economically important plants as the rate of conventional method are very low in woody plants.

The present studies have been successful in micropropagation protocol by using CN segment from axenic seedlings. The fact may be attributed that the use of juvenile tissues to increase enzymatic activity at the cotyledonary node zone as also observed in case of bean[20]. Successful shoot regeneration from cotyledon node segments of *A. marmelos* has been reported earlier[21- 24]. The regeneration system can be adopted for mass production due to readily available immature seed embryos maybe a starting point for the development of genetic

transformation technologies in this important medicinal tree species.

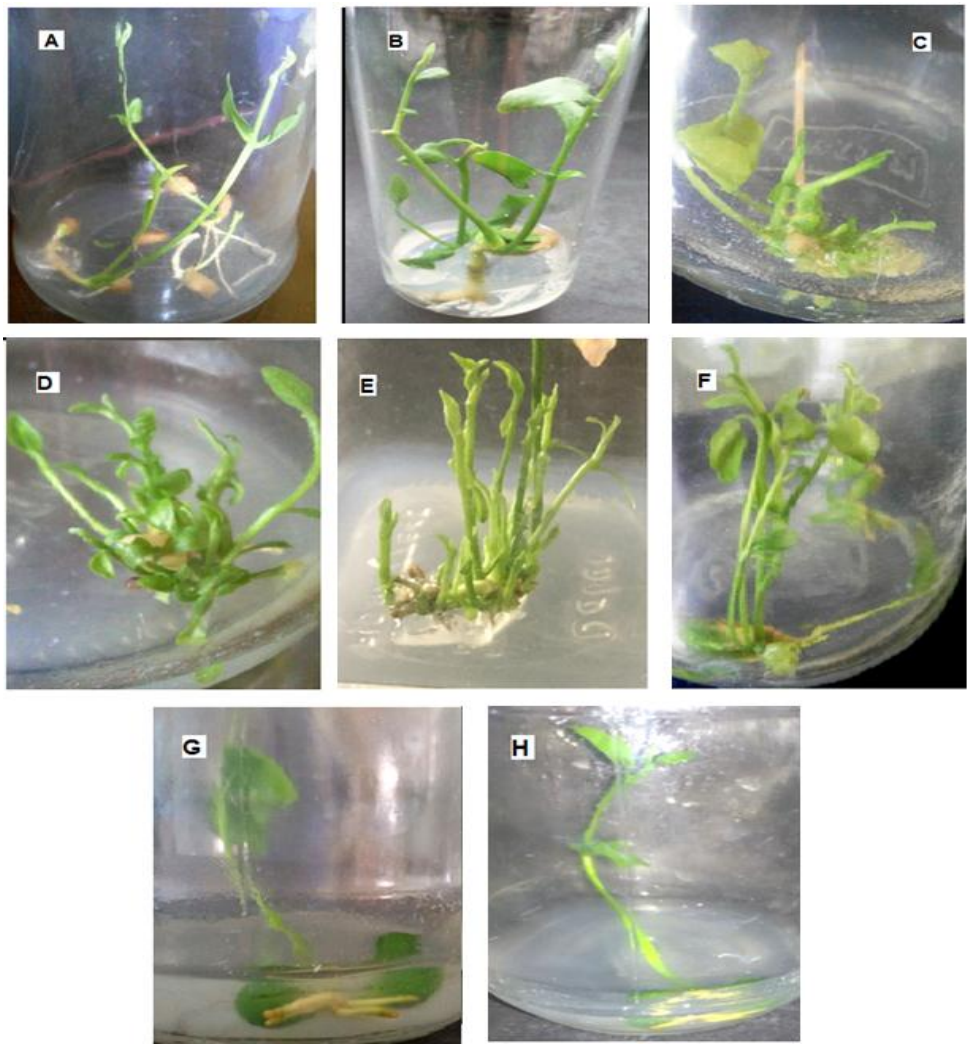

Fig. 1 (A) invitro raised seedlings, of *Aegle marmelos* on MS media (B,C) Shoot proliferation on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA (D-F) Shoot multiplication on MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA (G, H) In vitro rooting on MS media supplemented with 1.0-2.0 mg/l NAA

5 Conflict of interests

None

6 Authors contributions

AG and TT design and carried out research work and SK drafted the manuscript.

7 References

1. Hazeena MS, Sulekha GR. Callus induction and Plantlet regeneration in *Aegle marmelos* (L.) Corr. using cotyledon explants. Journal of Tropical Agriculture. 2008; 46(1-2): 79–84.
2. Karawya MS, Mirchom YW, Shehuata IA. Sterols, triterpenes, coumarins and alkaloids of *Aegle marmelos* correa, Cultivated in Egypt. Egyptian Journal of Pharmaceutical Science. 1980; 21: 239-248.
3. Pati, Muthukumar M. Genetic transformation of bael (*Aegle marmelos.* Corr.) Biotechnology of Neglected and Underutilized Crops. 2013.
4. Ponnachan PTC, Paulose CS, Panikkar KR. Effect of leaf extract of *Aegle marmelos* in diabetic rats. Ind.J. Exp. Biol. 1993; 31: 345-347.
5. Haravey SK. A preliminary communication of the action of *Aegle marmelos* (Bael) on heart. 1968; Indian J. Med. Res. 56:327.
6. Mishra BB, Singh DD, Kishore N, Tiwari VK, Tripathi V. Antifungal constituents isolated from the seeds of *Aegle marmelos.* Phytochemistry. 2010; 71:230-234.
7. Ravikumar K, Ved k. *Aegle marmelos,* 100 Red- listed medicinal plants of conservation concern in southern India, foundation for revitalization of local healthTraditions, Bangalore. 2000; pp: 41-43.

8. Devi P, Gopal RM, Settu A. Plant regeneration of Aegle mormelos, corr. from cotyledon explants through *in vitro* studies. J. Nat. Prod. Plant Resour. 2014; 4(2):52-55.

9. Bhati R, Sekhwatt NS, Arya HC. *Invitro* regeneration of plantlets from root segments of *Aegle marmelos*. Indian Journal of Experimental Biology. 1992; 30: 844-845.

10. Hossain M, Islam R, Islam A, Joarder OI. Direct organogenesis in cultured hypocotyls explants of *Aegle marmelos* corr. Plant Tissue Culture. 1995; 5: 21-25.

11. Arumugam A, Rao MV. *In vitro* production of plantlets from cotyledonaryode cultures of *Aegle marmelos* L. Corr. Advances in Plant Sciences. 1996; 9: 181-186.

12. Islam R, Hossain M, Joarder OI, Karim MR. Adventitious shoot formation on excised leaf explants of invitro growth seedlings of of *Aegle marmelos*. Corr. Journal of Horticultural Science. 1993; 68:495-498.

13. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum. 1962; 15: 473-495.

14. Bouza L, Jacques M, Miginiac E. *In vitro* propagation of Paeonia suffruticosa 'Andr. C.V. Mme de Vatry': Developmental effects of exogenous hormones during the multiplication phase. Sci Hort. 1994; 57: 241–251.

15. Purohit SD, Singhvi A. Micropropagation of Achras sapota through enhanced axillary branching. Sci Hort. 1998; 76: 219–229.

16. Brassard N, Brissette L, Lord D, Laliberte S. Elongation, roting and acclimatization of micropropagated shoots from mature material of hybrid larch. Plant Cell Tiss. Org Cult. 1996; 44: 37–44.

17. Figueiredo SFL, Albarello N, Viana VRC. Micropropagation of Rollinia mucosa (Jacq.) Baill. In Vitro Cell Dev Biol Plant. 2001; 37: 471–475

18. Kalamani A, Michael Gomez S. *In vitro* propagation studies in Clitoria spp. International Journal Mendel. 2002; 19(2): 29-30.

19. Bonga JM. In tissue culture in forestry (eds Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff Publishers, Dordrecht, Vol.1, 1987; pp.249-271.

20. Davis BD. Effects of sugars on α-amylase activity in pea embryonic axes. Am. J. Bot. 1983; 70(6):821-826.

21. Manikkannan T, Pradeep K, Chand. Adventitious plantlet regeneration from different explants of *Aegle marmelos* (L.) Corr .2013; 7(37): 2761-2768.

22. Puhan PP, Rath SP. *Invitro* propagation of *Aegle marmelos* (L.) Corr., a medicinal plant through axillary bud multiplication. Advances in Bioscience and Biotechnology. 2012; 3: 121-125.

23. Annapurani S, Priya R. Antimutagenic, antitumourogenic and antigenotoxic effects of polyphenols extracts of selected medicinal plants. Ind. J. Nutr. Diet. 1999; 36: 431-435.

24. Pattnaik S, Subramanyam VR, Kale C. Antibacterial and antifungal activity of ten essential oils in vitro. Microbios. 1996; 86: 237-246.