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In vitro clonal multiplication of *Aegle marmelos* (L.) Corr. through cotylodonary node culture

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Abstract

Clonal multiplication of *Aegle marmelos* was achieved through *in vitro* culture. Cotyledonary nodes from one month old *in-vitro* grown seedlings of *Aegle marmelos* were cultured on MS medium supplemented with BAP, Kn, and IBA either alone or in combination. The highest regenerative response was observed on medium containing 2.5 mg/L BAP and 0.5 mg/L Kn from node and shoot tip within 8-10 days. When regenerated shoots were subcultured on MS medium supplemented with 0.2 mg/L BAP and 0.02 mg/L Kn, maximum number of multiple shoots were observed after third phase of subculture. Poor response was found using MS medium supplemented with Kn only. *In vitro* induced shoots were transferred into root induction medium consisting of half-strength MS supplemented with auxins, IAA, IBA or NAA. Rooting was best in medium supplemented with 1.0 mg/L IBA. Rooted plantlets were acclimatized and transferred to the soil with 96% survival rate.

Keywords: *Aegle marmelos;* Clonal multiplication; Explant; MS media

Introduction

Aegle marmelos Correa (Bael) is a medicinal tree belongs to the family Rutaceae. It is popularly known as Bilva, Bilwa, Bel, Kuvalam, Koovalam, or Beli fruit, Bengal quince, Stone apple and Wood apple. The fruits of the wild trees considerably smaller than those of the cultivated types grown in the plains (Parmar *et. al.,* 1982). The tree grows wild in dry forests on hills and plains of central Southern India, Burma, Pakistan, Bangladesh, Srilanka, Northern Malaya, Java and Philippine Islands (Pati *et. al*., 2008). Fruits are digestive, stomachic, laxative, astringent and tonic. They are used in constipation and dysentery. Unripe fruits are particularly useful in diarrhoea and dysentery (Ghani, 1998). The fruits of *Aegle marmelos* contain Furocoun Marin Marmalosin, which is responsible for its medicinal properties (Dixit and Dutt, 1932). The alkaloids Aegiline present in the leaf is a patent antiasthamatic agent (Haravey, 1968). Leaf extract of *Aegle marmelos* has been used as antispermatogenic (Sur *et. al.,* 1999), to cure jaundice (Gupta *et. al*., 1999) and to enhance wound-healing activity (Jaswanth *et. al.,* 2001). Gummy substance around the seeds serves as an adhesive, as varnish for pictures and adds brilliancy to water-colour paints and wood is used as timber (Ambasta, 2000).

Aegle marmelos is an out breeder and is routinely propagated by seeds for cultivation (Singh *et. al.,* 1976). Seeds have short viability and are prone to insect attack. Seedlings from seeds show slow growth and are liable to diseases and pest in the initial stage. The seeds are recalcitrant and cannot be stored for longer periods under normal storage conditions. Vegetative propagation through root suckers is slow, difficult and cumbersome (Ajithkumar and Seeni, 1998). Moreover, propagation by method such as grafting and cutting are not feasible for commercial propagation. Since *Aegle marmelos* is a cross pollinated plant, maintenance of varietal purity is one of the important problems (Hossain *et. al.,* 1993). All parts of these trees *viz* root, leaf, trunk, fruit and seeds are used for medicine as the plant is very important for medicinal value. It is understood from the above discussion that conventional method of propagation either through seed or by root cuttings and layers are associated with problems like seasonal seed production (takes one year), pest problems and low percentage of seed germination (Ajithkumar and Seeni, 1998; Hossain *et. al.,* 1993). This problem can be overcome by non conventional approaches like *in vitro* propagation methods, for true to type clones without any genetic change.

Successful micropropagation has been easily achieved with seed derived explants of *Aegel marmelos* (Hossain *et. al,* 1993; Islam *et. al.,* 1993) and rapid clonal multiplication through *in vitro* axillary shoot proliferation has been estab-

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lished by Ajithkumar and Seeni (1998). But in their experiment limited number of shoots was found to be induced. Here in the present investigation, we report a callus-free micropropagation system for producing vigorous numbers of multiple shoots from cotyledonary nodal explants through *in vitro* micro propagation of *Aegle marmelos.*

Materials and methods

Seed of *Aegle marmelos* (Bael) were collected from the best quality and large size mature field grown bael fruits. For seed germination, seeds were first washed with detergent under running tap water for 3 - 5 min. Floating seeds were considered to be empty and discarded. Later the seeds were dipped in 70% alcohol for 30 sec, followed by washing with distilled water. Then the seeds were surface sterilized with 0.1% (w/v) mercuric chloride for 10 min. with continuous shaking. Finally it was washed five times with sterilized distilled water. Seed coat was removed from the surface sterilized seeds and then inoculated in conical flask containing 2% agar-gelled MS (Murashig and Skoog, 1962) medium with 3% sucrose for supporting seed germination and seedling development. Shoot tips and nodal segments were excised from 15-20 days old seedlings. For shoot induction all explants were cultured on MS medium with various hormonal supplements namely BAP, Kn, NAA, IAA. All *in vitro* seedlings and cultures were maintained under illumination on a 16h photoperiod at 25 ± 20°C.

For induction of roots, regenerated shoots (3.5 - 4.5 cm long) were excised and transferred to MS medium supplemented with different concentrations of IAA, IBA and NAA. After the development of sufficient root plantlets were transferred to small plastic pots containing sterilized soil. The pots were covered with polythene bags to maintain high humidity and after 50 days the plantlets were transferred to larger pots.

Results and discussion

The *in vitro* experiments of the present study initially involved the establishment of nodal segments and shoot tip explants in aseptic cultures from the germinated seeds. *In vitro* or *ex vitro* seed germination of *Aegle marmelos* is little difficult, so that, germination rate is very poor (Ajithkumar and Seeni, 1998). The frequency of seed germination was enhanced after inoculation of the seeds onto MS medium without seedcoat. The percentage of seed germination of *A. marmelos* with seed coat and without seed coat was 10% and 80% respectively. The rapid clonal multiplication of shoot, proliferation of leaf, development of roots for plantlet formation and finally the establishment of plantlets under *ex-vitro* condition were observed in this investigation.

Earlier reports demonstrated that plant regeneration was possible through apical shoot bud, node, internodes, leaf and nucellar tissues explants (Pati *et. al*., 2008; Arumugam *et. al.,* 2003; Ajithkumar and Seeni, 1998; Hossain *et. al.,* 1993; Dass *et. al.,* 1999). Ajithkumar and Seeni (1998) reported that nodal segments were more responsive than apical shoot tips. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of a stem (Vieitez *et. al*., 1985).

In the present study, different concentration of BAP, Kn, IAA, NAA were used singly or in combination with MS medium to observe their effect on initiation and multiple shoot formation. MS supplemented with different concentration of BAP (0.5-2.5 mg/L) was tried for shoot regeneration from both the explants. It was observed that BAP alone in MS medium did not show optimum response toward shoot regeneration. Though Ajithkumar and Seeni (1998) obtained multiple shoot in case of single node, whole leaf, shoot tip and inter node explants. Pati *et. al*. (2008) cultured shoot tip on MS supplemented with BAP and IAA and got 9.67 micro shoots / explant. Hossain *et. al.* (1993) also reported same combination for multiple shoot in case of nucellar tissues explant. In the present investigation, same combinations were tried but the number of shoots per explant was comparatively low. A maximum of 5-6 shoots per explant were observed.

Hossain *et. al.* (1993) reported the maximum frequency of adventitious bud proliferation in MS medium with BAP and NAA. In present study, combination of BAP and NAA in MS medium did not show the desired results.

The combined effect of BAP and Kn was studied on MS medium to determine their effect on shoot regeneration. Best response towards regeneration was observed when BAP and Kn were used combinedly. Among the different treatment combinations, best response towards shoot regeneration was found on MS medium with 2.5 mg/L BAP and 0.5 mg/L Kn and days of shoot initiation was 8-10 days only (Table I, Figs. 1a, 1b). The explants that regenerated shoots, were subcultured on same combination of medium in two times. In this medium combination, maximum mean number of shoot/explant was 22.5 (Table I). It was observed that in the above mentioned hormone supplemented medium the number

Table I. Effect of different concentration of hormone on multiple shoot regeneration from nodal segment and shoot tip explant of *Aegle marmelos* Corr

BAP	Kn	IAA	NAA	No. of explants inoculation	Days of shoot initiation	Average no. of shoot/explants
0.5				50	14-18	2.25
1.0				50	14-16	3.0
2.0				50	15-18	3.75
2.5				50	12-16	4.25
	0.5			50	16-20	3.0
	1.0			50	15-18	4.25
	2.0			50	16-20	4.75
	2.5			50	16-18	4.25
0.5		0.1		50	12-18	3.75
1.0		0.5		50	13-18	5.2
2.0		1.0		50	12-19	4.5
2.5		2.0		50	14-18	5.6
0.5			0.1	50	14-18	3.0
1.0			0.5	50	14-20	5.25
2.0			1.0	50	13-19	4.0
4.0			2.0	50	12-16	3.5
5.2						
0.5	0.1			50	12-16	15.2
1.0	0.5			50	11-16	12.5
2.0	0.1			50	11-16	18
2.5	0.5			50	10-16	22.5

of multiple shoots was not increased. For multiple shoot formation and development the regenerated explants were transferred to medium containing a lower concentration of BAP and Kn for maximum multiple shoot formation. Maximum number of multiple shoots was obtained in MS medium with 0.2mg/L BAP and 0.02 mg/L Kn. After six to seven subculture maximum number of multiple shoots were obtained (Fig. 1c). The maximum mean number of shoot per explants was 84.5. The multiple shoots obtained from the lower concentration of BAP and Kn supplemented media produce few leaves. For the development of leafy shoots the initially developed shoots were transferred on MS medium without any hormonal supplements, where shoots were elongated with leaf expansion (Fig.1c).

Induction of healthy roots from the regenerated shoots is an essential part for stable development of plantlets and as such regenerated shoots were cultured on full as well as half the strength of MS supplemented with IAA and IBA for root induction. It was observed that half-strength of MS with 1.0mg/L IBA was effective for root induction (95%) (Table II, Fig. 1d). Pati *et. al.* (2008) observed best response on

Table II. Effect of different concentrations of auxins in ½ strength of MS medium on root induction of *in vitro* raised shoots of *Aegle marmelos* Corr

Name of the hormone	Concentrations (mg/L)	% of shoots responding to root induction	Days to root induction	Mean no of roots/ shoots
IAA	0.2	-	-	-
	0.5	-	-	-
	1.0	60	35-40	10-15
IBA	0.2	-	-	-
	0.5	-	-	-
	1.0	95	15-20	15-20
NAA	0.2	-	-	-
	0.5	80	42 - 49	8 - 10
	1.0	65	47 - 62	5-10

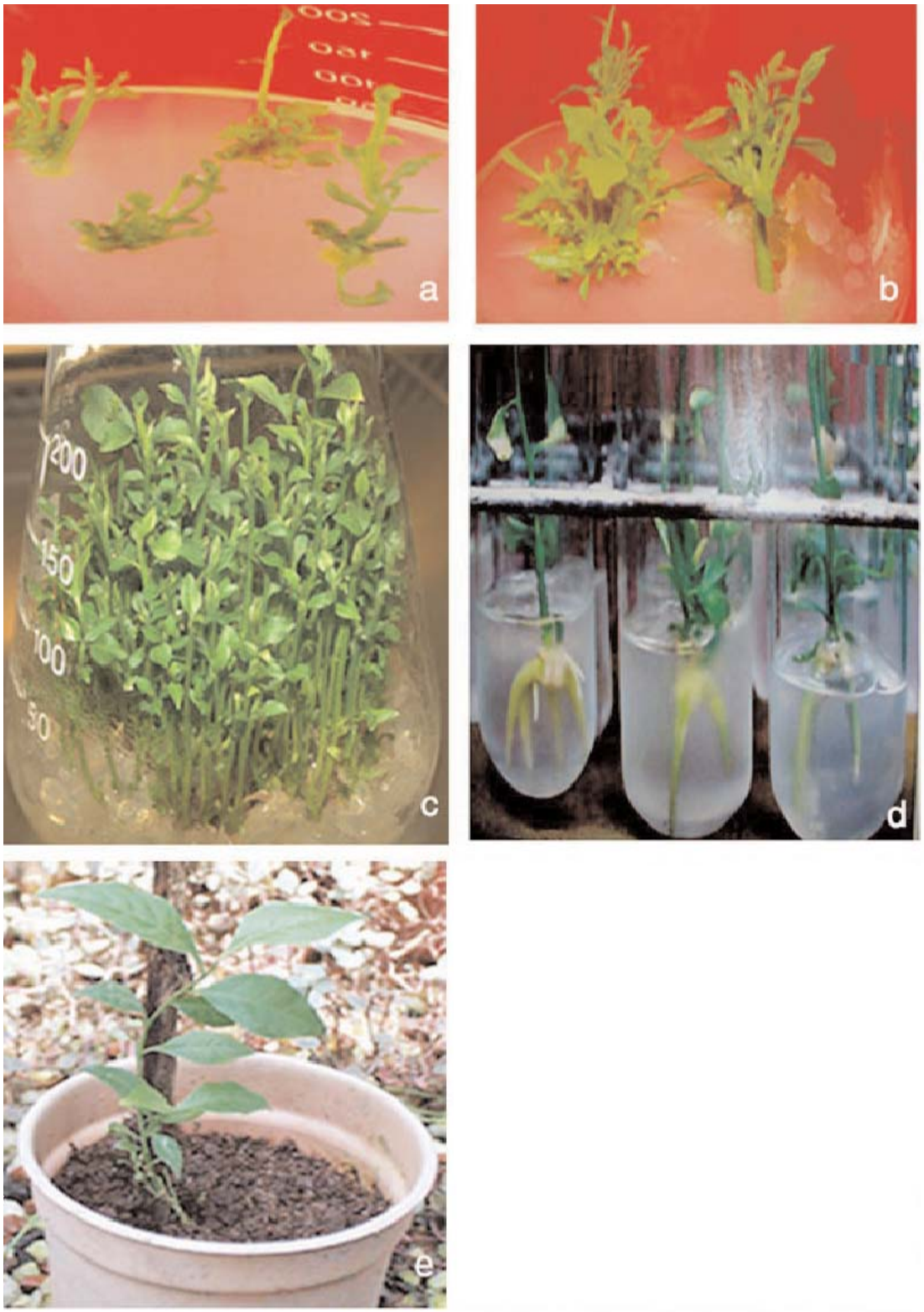

Fig. 1a) *In vitro* regenerated shoots from shoot tip and nodal explant. b) Multiple shoot initiation from shoot tip and nodal explant in MS media with 2.5 mg/L BAP and 0.5 mg/L Kn. c) Vigorous number of multiple shoot formation from both explant on MS medium with 0.2 mg/L BAP and 0.02 mg/L Kn. d) Root induction on MS media with 1.0 mg/L IBA. e) Transplanted Plantlet of *Aegle marmelos* in pot

Table III. Survival rate of transplanted plantlets using different combinations of soil and its supplements

Treatments	Ratio	Days old	Total plantlets	Survival of plantlets	Percentage of survival
Soil	-	10	25	20	80%
Soil and sand	5:1	10	10	7	70%
	5:2	10	15	9	60%
	4:1	10	10	6	60%
	3:1	10	20	14	70%
	3:2	10	25	20	80%
	1:1	10	15	9	60%
Soil, sand, cowdung	1:1:1	10	25	24	96%
	2:1:1	10	20	15	75%
	2:2:1	10	20	14	70%
	1:2:1	10	10	9	90%
	1:2:2	10	25	20	80%

half-strength MS medium supplemented with IBA and IAA (100%). Ajithkumar and Seeni (1998) got best rooting response in presence of IAA (70%) or IBA (90%) with half - strength of MS medium. After sufficient development of roots, the plantlets were successfully transplanted into small plastic pots containing sand, soil and cowdung (1:1:1) (Fig. 1e). The survival rate of the transplanted plantlets was found to be about 96% (Table III). Following proper acclimatization the plantlets were established in field condition. The *in vitro* regeneration protocol described here is easily reproducible, produces maximum number of multiple shoots, and requires minimum hormonal supplements and genotype independent. Moreover, the regeneration of plantlets achieved without the intervention of callus and this clearly indicates the possibility of obtaining true to type plantlets and it can be get within 8-12 days. The technique described here appears to be readily adaptable for maximum multiple shoots formation of *Aegle marmelos* Corr.

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