Regeneration of plantlets from *in vitro* cultured seed of endangered *Aegle marmelos* (L.) Corr. Rutaceae

Article Received on 15 Dec 2016

Accepted on: 13 Feb 2017

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ABSTRACT

A tissue - to - plant regeneration system was developed for *Aegle marmelos* (L.) Corr. (bael tree, family Rutaceae) achieved using seed from mature fruit. It is one of the most important tree with religious importance and several medicinal uses. This tree when normally propagated using seeds, exhibit morphological and biochemical variations within the population due to its heterozygous nature. Developing, *in vitro* technique for propagation from elite tree will help in preserving genotype qualities in raised population. In the present study, efforts were made to find out the suitable media composition for micropropagation of *Aegle marmelos* tree using hypocotyle and leaf primordia as explant material. All the experiments were carried out in Murashige and Skoog's (MS) media using different phytohormone concentrations to record callogenic and morphogenic responses. The regeneration studies indicated that the following combinations of hormones with MS media containing KIN with BAP (1.5:1.5 mg/L) and 2, 4-D with KIN (1:1 mg/L) is most suitable for shoot induction and calli formation. These calli later developed shoots when transferred to MS medium for root initiation. In the establishment stage, 10 individual phytohormone treatments (Auxin/Cytokinin) and 24 combination treatments (Auxin + Cytokinin) were used.

Keywords: Auxin, Bael, Cytokinin, MS medium, Rutaceae

INTRODUCTION

Aegle mermelos (L.) Corr., (Rutaceae) is an armed spiny popular medicinal plant in the Ayurvedic and Siddha systems of medicines used to treat a wide variety of ailments. In India, this plant is known as "Bael Tree". It is mostly found in tropical and subtropical region and in hilly tracts up to 1300 m elevation (Anonymous 2003, Raghu A V *et al.* 2007). *A. marmelos* is naturally distributed in India, Myanmar and Sri Lanka and widely cultivated in Southeast Asia and Tropical Africa. It is a medium sized deciduous thorny tree with its roots, bark, leaves and fruits of high medicinal value. Bael fruit is a heterozygous out-breeding plant that can be easily

propagated by seeds. However, the seedlings show great variation in form, size, texture and quality of rind, number of seeds (Prematilake D P *et al.* 2006) and is cited as one of the red-listed medicinal species of South India (Ravikumar and Ved 2000) due to its overexploitation in Ayurvedic medicines. Micropropagation using tissue culture is an alternative method used to multiply mainly vegetative propagated crops. A tissue-to-plant regeneration system is thus an important pre-requisite for application of this technology for *A. marmelos*. Propagation through tissue culture is a viable alternative in this species. Several workers have reported *in vitro* propagation of *A. marmelos* using axillary bud multiplication (Arumugam and Rao 1996, Islam *et al*. 1994, Hossain *et al*. 1994a, Varghese *et al*. 1993, Ajithkumar and Seeni 1998), nucellar calli (Hossain *et al*. 1994b) and from leaf explants (Islam *et al*. 1993). In addition, this technology can be applied in crop improvement through induced variations and mutations. Within this context, the objective of this study was to develop an efficient method of plant regeneration system using vegetative plant tissues of *A. marmelos*.

MATERIALS & METHODS

Explant preparation and surface sterilization:

Ripe fruits were collected from a 20-year-old tree of *A. marmelos* grown in the Shiva temple garden. Seeds were removed from the fruits with the help of scalpel and washed in tap water and with tween-20 for 5 min than three times washed with sterile distilled water in laminar air flow again sterilized with 0.1 % $HgCl_2$ for 2 min and then three times washed with sterile distilled water. Finally, the seeds were thoroughly rinsed in sterile double distilled water for at least four times under aseptic conditions. The sterile seeds were aseptically inoculated on to sterile petri-plate, containing sterile distilled water and cotton. After 12-14 days' seeds were germinated under aseptic conditions.

Culture media and initiation:

Culture media consist of MS (Murashige and Skoog 1962) complemented with 3% (w/v) sucrose, 0.8% (w/v) agar and different combination of auxin and cytokinin (growth regulators). The pH of the medium is adjusted to 5.8 before gelling with agar and autoclaved at 121ºC at 15 lbs pressure for 20 minutes. The surface sterilize explants were inoculating on the above media under aseptic conditions.

When seeds were germinated, cotyledons, leaf primordia, hypocotyledon and root were obtained. These were cultured on agar - solidified MS medium containing sucrose (3% w/v) and different combination of auxin and cytokinin for callus and shoot formation. The cultures were incubated at 25°C ± 2°C and photoperiod of 16 hrs light and 8 hrs in dark with 50-55% relative humidity.

Callus culture:

Collection of explants

Explants of *Aegle marmelos* were collected from Shiva temple of Rajkot and maintain at the campus of Shree M. & N. Virani Science College, Rajkot. Seeds were collected as explants from this grown plant whenever required during the work.

Sterilization of explants

First of all, seeds were removed out from the fruit. The explants were surface sterilization with tween-20 for 5 min than three time wash with sterile D/W in laminar air flow again sterilize with 0.1% $HgCl_2$ for 2 minutes and then three times wash with sterile D/W.

Seed germination

The sterile seeds were aseptically inoculated on to sterile petri-plate, containing sterile distilled water and cotton. After 12-14 days' seeds were germinated under aseptic conditions. Cultures were incubated in culture room under controlled conditions of light and temperature for 24 hrs dark at 25±2°C.

Inoculation

The explants cotyledon (from seed germination) were cut in to an appropriate size with the help of scalpel and forceps and aseptically inoculated in to a culture tube containing MS media supplemented with various concentration of auxin and cytokinin (Table 1).

Growth hormones	Concentration of plant growth hormone (mg/L)
2,4-D + BAP	0.5:1.0
2,4-D + BAP	1.0:0.5
2,4-D + BAP	1.0:1.0
2,4-D + BAP	1.5:1.0
2,4-D + BAP	1.0:1.5
2,4-D + KIN	1.0:1.0
2,4-D + KIN	0.5:1.0
2,4-D + KIN	1.0:0.5
2,4-D + KIN	1.5:1.5
2,4-D + KIN	1.0:1.5
IAA+ KIN	0.5:1.0
IAA+ KIN	1.5:1.0
IAA+ KIN	3.0:1.0
IAA+ KIN	3.0:0.5
IAA+ KIN	1.0:0.5
IAA+ BAP	1.0:1.0
IAA+ BAP	1.5:1.0
IAA+ BAP	3.0:1.0
IAA+ BAP	3.0:0.5
IAA+ BAP	1.0:0.5
NAA+ BAP	0.5:1.0
NAA+ BAP	1.0:0.5
NAA+ BAP	1.0:1.0
NAA+ BAP	1.5:2.0
NAA+ BAP	1.0:1.5
NAA+ KIN	3.0:0.5
NAA+ KIN	0.5:1.0
NAA+ KIN	1.0:1.0
NAA+ KIN	3.0:1.0
NAA+ KIN	1.5:1.0

Table 1: Different concentration of auxin and cytokinin for callus induction and shoot formation from callus

Incubation:

Culture was incubated in culture room under controlled conditions of light and temperature of 24 hr dark at 25±2°C.

Sub culture:

Calli were sub-cultured on the MS basal medium supplemented with specific hormone concentration for organogenesis.

Shoot formation:

The Callus was grown on the MS basal medium and transferred for shoot formation. Various concentration of BAP, 2, 4-D, IAA, NAA and KIN were tested out for shoot induction.

The explants hypocotyls (from seed germination) were cut in to an appropriate size with the help of scalpel and forceps and aseptically inoculated in to a culture tube containing MS media supplemented with various concentration of auxin and cytokinin (Table 2).

Growth Hormones	Concentration of plant growth hormone (mg/L)
2,4-D + KIN	0.5:2.0
2,4-D + KIN	0.5:2.5
BAP + KIN	1.0:0.5
BAP + KIN	1.0:1.0
BAP + KIN	1.5:1.0
BAP + KIN	1.0:1.5
BAP + KIN	1.5: 1.5
BAP + KIN	2.0: 0.5
BAP + KIN	0.5: 2.0
BAP + KIN	2.0:1.0
BAP + 2,4-D	2.0:0.5
BAP + 2,4-D	2.5:0.5
BAP + KIN	1.5:2.0
BAP + KIN	2.0:1.5
BAP + KIN	3.0:0.5
BAP + KIN	0.5:3.0
BAP + KIN	3.0:1.0
BAP + KIN	1.0:3.0
BAP + KIN	3.0:1.5
BAP + KIN	1.5:3.0
BAP + KIN	3.0:2.0
BAP + KIN	2.0:3.0

Table 2: Different concentration of hormone for shoot formation

RESULTS

Seed germination:

Seeds after sterilization were inoculated on the sterile petri-plate under aseptic condition. Seed germination was observed after 12-14 days which were kept at 25±2°C. Out of 150 seeds, 121 seeds were germinated within 12-14 days i.e. 80.66% germination. The seeds in which no response was obtained got contaminated or less response for germination and discarded.

Callus Culture:

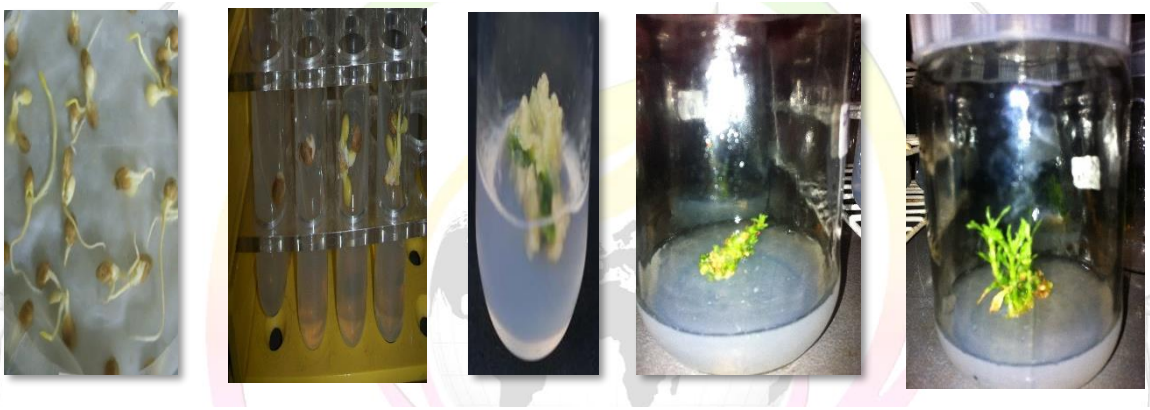
Seeds and cotyledon were inoculated on the callusing medium containing of MS medium supplemented with 3mg/L NAA and 1mg/L BAP and 3% sucrose (Figure 1).

Different concentration of BAP, 2, 4-D, NAA, IAA and KIN were used for the callus induction. Callus induction was observed almost within 4-6 weeks after inoculation and initially callus was white in color which later became cream-brown in color (Table 3). It can be estimated that low concentrations of IAA did not favour better callus induction. Visual observation also indicated that explants responded better for callus induction on MS media. Among the different concentrations and combination tested, MS medium enriched with NAA and BAP stimulated the best response in terms of callus induction. The callus was transferred to the organogenesis media.

Media composition (MS)	Concentration of plant growth hormone (mg/L)	No. of days required for callus formation	callus growth
2,4-D + BAP	1.0:1.0	45	+ + + +
2,4-D + BAP	1.5:1.0	30	+ + +
2,4-D + KIN	1.0:1.0	32	+ + + +
2,4-D + KIN	1.5:1.5	45	+ + + +
IAA+ BAP	0.5:1.0	50	+ +
IAA+ BAP	1.0:1.0	40	+ + + +
IAA+ KIN	0.5:1.0	35	+ + + +
NAA+ BAP	3.0:1.0	30	+ + + + +
NAA+ BAP	1.5:2.0	35	+ + + +

(++) Poor growth, (+++) Moderate growth, (++++) Good growth, (+++++) Very good growth

Table 3: Effect of different concentration of auxin and cytokinin for callus induction.



A **B.** **C.** **D** **E.**

Figure 1. *In vitro* propagation of *Aegle marmelos* A & B: Seed germination in different culture media; C. Calli growth from cotyleledons; D. Direct shoot regeneration from callus; E: Clusters of shoot buds from calli

Micropropagation:

Various concentrations of NAA, IAA, 2,4-D, KIN and BAP were used for shoot multiplication of *A. marmelos* from callus and hypocotyls on the establishment media. The results were recorded in Table 4 (Figure 1).

Callus and hypocotyls were inoculated on the shooting medium containing of MS medium supplemented with 2mg/L BAP and 0.5mg/L 2,4-D, 2.5mg/L BAP and 0.5mg/L 2,4-D, and 3% sucrose.

Different concentration of BAP, 2, 4-D, NAA, IAA and KIN were used for the callus induction. Callus induction was observed almost within 20 days after inoculation (Table 4). Among the different concentrations and combination tested, MS medium enriched with BAP and 2,4-D stimulated the best response in terms of shoot bud formation. The shoot was transferred to the organogenesis media for sub-culture.

Media composition (MS)	Concentration of plant growth hormone (mg/L)	No. of days required for shoot formation	Shoot growth
BAP + 2,4-D	2.5:0.5	25	+ + + + +
BAP + KIN	1.0:0.5	30	+ +
BAP + KIN	1.0:1.0	35	+ + +
BAP + KIN	1.0:1.5	20	+ + +
BAP + KIN	1.5:1.5	25	+ + + +
BAP + 2,4-D	2.0:0.5	20	+ + + + +
BAP + KIN	0.5:1.5	28	+ + +
BAP + KIN	1.5:0.5	20	+ + +
BAP + KIN	1.5:2.0	20	+ + + +
BAP + KIN	2.0:1.5	25	+ + + +

(++) Poor growth, (+++) Moderate growth, (++++) Good growth, (+++++) Very good growth

Table 4: Effect of different concentration of auxin and cytokinin for shoot formation

DISCUSSION

Explant collected during October - November was found to be ideal because 80.66% explant shows in vitro condition. BAP and KIN also were found very effective for the shoot induction. Calli formation were observed within four to six weeks on cotyledon culture on MS medium supplemented with 3.0mg/L NAA + 1.0 mg/L BAP. The callus induction always preceded by swelling of the explants Rekha Warrier *et al.* (2010). From the above result it is obvious that NAA, KIN and BAP at many of the concentrations either singly or in combinations were very effective in the induction of callus and shoots which might be because of its chemically stable, low mobility in the plant and prolonged action nature Zimmerman R (1985), Puspasree Puhan *et al*. (2011).

Shoot formation were observed within 15-30 days on hypocotyledon culture on MS medium supplemented with 2.0 mg/L BAP + 0.5 mg/L 2,4-D and 1.5 mg/L BAP + 1.5 mg/L KIN (Puspasree Puhan *et al*. 2011, Zimmerman 1985).

ACKNOWLEDGMENT

The author is grateful to Department of Biotechnology, New delhi, India for providing financial assistance.

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