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SHORT COMMUNICATION

AN EFFICIENT MASS PROPAGATION PROTOCOL FOR *Aegle marmelos* (L.) Corr. THROUGH *IN VITRO* GENERATED SHOOT TIPS

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ABSTRACT

Aegle marmelos* (L.) Corr. commonly known as 'beli' in Sri Lanka is an important fruit tree with extensive medicinal uses in indigenous medicinal systems. The plant is conventionally propagated by seeds which have short viability and low germination percentage. Vegetative propagation through root suckers is slow and challenging. Root being the major medicinally valuable part, destructive harvesting poses a serious threat to the sustenance of the tree. Therefore, the aim of this study was to develop an efficient mass propagation protocol for *A. marmelos* through *in vitro* generated shoot tips. Shoot tips taken from three weeks old *in vitro* grown seedlings of *A. marmelos* were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) (1.0 mgL^{-1} or 2.0 mgL^{-1}), kinetin (0.5 mgL^{-1} or 1.0 mgL^{-1}), and thidiazuron (TDZ) (0.25 mgL^{-1} and 0.5 mgL^{-1}) either alone or in combination. The highest mean number of shoots per shoot tip (16.73) and highest mean total length per shoot tip (10.58 cm) were observed on MS medium containing 1.0 mgL^{-1} BAP and 1.0 mgL^{-1} kinetin. In this study poor response of shoot proliferation was observed when using MS medium supplemented with TDZ alone. Shoot formation was optimum in the fifth week from establishment. *In vitro* derived shoots were transferred to root induction medium consisting of half-strength MS medium supplemented with three different concentrations of indole-3-butyric acid (IBA) (1.0, 1.5, 2.0 mgL^{-1}) with or without 5% activated charcoal. Highest rooting was achieved in the medium supplemented with 1.0 mgL^{-1} IBA with activated charcoal. Rooted plantlets were acclimatized using coco pellets and transferred to the soil with 80% survival rate.

Keywords: *Aegle marmelos* (L.) Corr, BAP, *In vitro* culture, Kinetin, MS medium, Shoot tip culture, TDZ

INTRODUCTION

Aegle marmelos (L.) Corr. commonly known as Bael is an important medicinal as well as a fruit tree belongs to the family Rutaceae with extensive uses in ayurvedic, *unani* and traditional medicine systems throughout the Indo-Malayan region. It is a medium sized deciduous tree (Arumugam *et al*. 2003) which grows in dry forests on hills and plains of central southern India, Burma, Pakistan, Bangladesh, Sri Lanka, Northern Malaya, Java and Philippine Islands (Pati *et al*. 2008).

Leaves, fruits, stem and roots of this tree at all stages of maturity are used as ethno-medicines against human ailments as they are rich sources of numerous secondary metabolites (Badam *et al*. 2002). Many of these compounds including skimmianine, aegelin, lupeol, cineole, citral, citronella, cuminaldehyde, eugenol, marmesimine, marmelosin, luvagngetin, qurapten, psoralen, marmelide, fagarine, marmin and tannin have been proved to be biologically active against various major and minor diseases (Maity *et al*. 2009) ranging from asthma, anemia, fracture, healing wounds, swollen joints, diarrhea, high blood pressure and troubles during pregnancy (Choudhary *et al*. 2017). Numerous therapeutic values of the tree have been identified as anti-diabetic, hepatoprotective, antimicrobial, analgesic, anti-inflammatory, antipyretic, anti-cancer, radioprotective, antispermatogenic, antiulcer and anti-thyroid (Sharma *et al*. 2011).

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Though *A. marmelos* is conventionally propagated by seeds and root suckers, seeds have shown short viability and produce slow growing seedlings which are liable to diseases and pests in the initial stage (Akter *et al*. 2013). Vegetative propagation through root suckers is also slow and difficult (Ray and Chatterjee 1996). Due to unrestricted, extensive exploitation to meet growing demands by the pharmaceutical industries tied with inadequate cultivation and insufficient reforestation, this important medicinal plant species has been markedly depleted from their natural habitats (Puhan and Rath 2012). Therefore, shifting for a non-conventional propagation technique as *in vitro* culture is important for the mass production of this important medicinal tree.

There has been an increased interest in *in vitro* culture techniques in recent years which offer a feasible tool for mass propagation and germplasm conservation of rare, endangered, aromatic and medicinal plants, as well as forest trees where the lifecycle takes a longer period. *In vitro* propagation of tree species provides several advantages over conventional propagation as high speed of propagation, reduced space requirements, independency from climatic and seasonal variations and year-round production.

Though some protocols have been introduced for the mass propagation of this plant, most of the protocols suggest ex-plants like nodal segments (Akter *et al*. 2013; Warrier *et al*. 2010), leaves and twigs (Pathirana *et al*., 2020) which are difficult to collect from full grown trees and success rate is low due to contaminations and phenolic browning. *In vitro* generated shoot tips of *A. marmelos* can be easily obtained by establishing seeds in MS medium. The process is efficient and effective than using ex-plants from field grown plants. Hence, the aim of this study was to develop an efficient *in vitro* protocol for the mass propagation of this important medicinal tree through *in vitro* generated shoot tips.

MATERIALS AND METHODS

Seed of *A. marmelos* (Bael) were collected from field grown plants in Faculty of Agriculture, University of Ruhuna with large fruits containing sweet and soft pericarp. For seed germination, seeds were first washed with detergent (Teepol) under running tap water for 3 - 5 min. Floating seeds were considered to be empty and discarded. Later the seeds were dipped in a fungicide solution (0.6 mgL^{-1} Topsin) for 30 min, followed by washing with distilled water. Then the seeds were surface sterilized with 5% (v/v) Clorox® for 5 min with continuous shaking and rinsed off with sterilized distilled water. Finally, seeds were treated with 70% ethyl alcohol for 30 seconds and washed off with sterilized distilled water. Seed coats were removed from the surface sterilized seeds and then they were inoculated into the culture vessels containing MS (Murashige and Skoog 1962) medium. After three weeks of culture, shoot tips were excised from *in vitro* seedlings. For shoot induction, all explants were cultured on MS media supplemented with four different combinations of BAP and kinetin (T1: 1 BAP + 0.5 Kin; T2: 1 BAP + 1 Kin; T3:2 BAP + 0.5 Kin; T4: 2 BAP + 1 Kin (mgL^{-1})) with two different levels of TDZ (0.25 TDZ; T6: 0.5 TDZ (mgL^{-1})). All cultures were maintained under illumination on a 16 hrs. photoperiod at 25 ± 2^0C. The study was conducted at the plant tissue culture laboratory, Department of Crop Science, Faculty of Agriculture, University of Ruhuna during 2019 – 2020.

For the induction of roots, regenerated shoots (3.5 - 4.5 cm long) were excised after eight weeks and transferred to half strength MS medium supplemented with three different concentrations (1.0, 1.5, 2.0 (mgL^{-1})) of IBA with or without 0.5 % (w/v) activated charcoal. After the formation of sufficient roots (3-5) plantlets were transferred to coco pellets and maintained under high humidity (80-95% RH). After 50 days, the plants were transferred to pots containing a medium with soil, sand and compost (1:1:1).

All experiments were arranged according to the Completely Randomized Design (CRD) with 20 replicates. Number of newly formed shoots per shoot tip and total length of newly generated shoots per shoot tip were recorded to select the best plant growth regulator

combination for shoot multiplication. Number of roots and length of roots were observed and average length of roots and percentage of root formation were calculated for the selection of best plant growth regulator combination for the root induction. Data were analyzed using ANOVA and mean separation was done with DMRT (Duncan's Multiple Range Test).

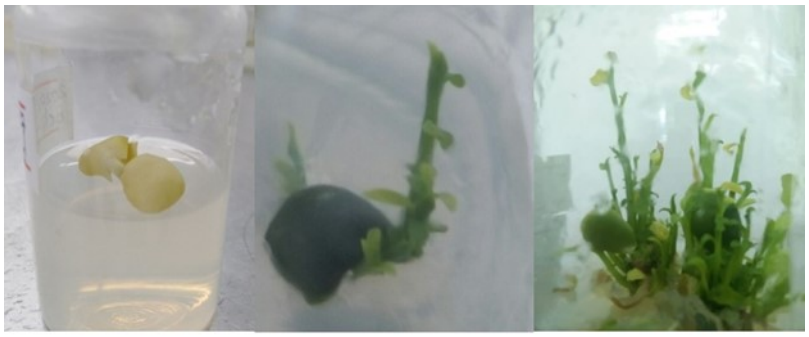
RESULTS AND DISCUSSION

The present experiment initially involved the seed germination of *A. marmelos* seeds under *in vitro* conditions. *Ex vitro* germination as well as *in vitro* germination of *A. marmelos* seeds found to be very poor according to previous studies (Ajithkumar and Seeni, 1998). The rate of seed germination was enhanced by inoculating the seeds onto MS medium after removing seed coat and splitting cotyledons to expose embryo (Fig. 1).

Plant growth regulators should be added to the culture medium to induce multiple shoots in *in vitro* generated shoot tips. According to previous studies, cytokinins stimulate cell division of plant tissues which promotes axillary shoot growth generating multiple shoots from explants (Gray and Jayasankar, 2005) while inhibiting root formation (Shkolnik-Inbar and Bar-Zvi 2010). BAP is identified as the most effective and reliable cytokinin by vast majority of past studies (Khatri *et al*., 2019). At the same time, kinetin and TDZ are two other *cytokinins* which showed promising results in many studies (Akter *et al*., 2013; *Novikova et al*., 2020; Warrier *et al*., 2010). Therefore, four different combinations of BAP and kinetin were tested with two different levels of TDZ to identify the best plant growth regulator or concentration for shoot formation of *A. marmelos.*

The significantly highest mean number of shoots per shoot tip (16.7) was recorded in MS medium supplemented with 1.0 BAP mgL $^{-1}$ and 1.0 mgL^{-1} kinetin (Figure 2 a) ($P<0.05$). Number of shoots per shoot tip was significantly lower in the treatments supplemented with TDZ alone ($P<0.05$). At the same time, highest total shoot length (10.58 cm) was observed in MS medium supplemented with 1.0 BAP mgL^{-1} and 1.0 mgL^{-1} kinetin which was not significantly different from the treatment supplement with 2.0 BAP mgL^{-1} and 0.5 mgL^{-1} kinetin (Figure 2 b) ($P<0.05$). All other treatments showed significantly lower values ($P<0.05$). The highest shoot formation was observed after fifth week from culture initiation (Figure 3 c).

Half strength MS medium supplemented with three different concentrations of IBA were tested with and without activated charcoal to identify the most favorable treatment for root induction of *A. marmelos* (Table 1). A significantly higher percentage (80%) of root induction, mean number of roots (4.68) and



a b c

Figure 1 a: Established *A. marmelos* seed in MS medium after seed coat removal and splitting; b: Germinating seed after a week; c: Shoot formation after 3 weeks of seed culture

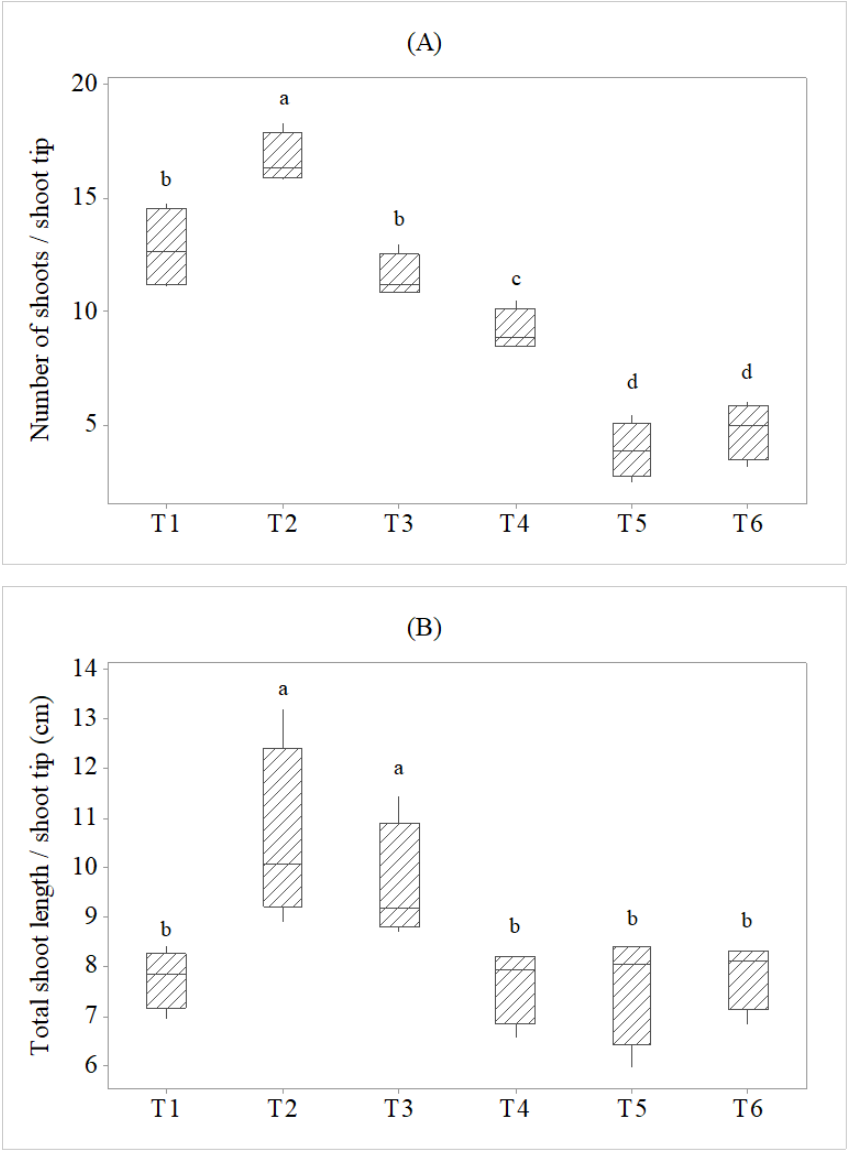


Figure 2: (A): Number of shoots per shoot tip and (B): total shoot length per shoot tip on different MS media (T1: 1 BAP + 0.5 Kin; T2: 1 BAP + 1 Kin; T3:2 BAP + 0.5 Kin; T4: 2 BAP + 1 Kin; T5: 0.25 TDZ; T6: 0.5 TDZ (mgL^{-1}))

mean length of roots (1.75 cm) were observed in half strength MS medium with 1.0 mgL^{-1} IBA and 0.5% activated charcoal (Table 1) ($P<0.05$). Lowest values in all tested parameters were observed in half strength MS medium with 2.0 mgL^{-1} without activated charcoal. Other two treatments with activated charcoal also showed significantly higher values in root induction percentage and mean number of roots than all treatments without activated charcoal ($P<0.05$).

Rooting of regenerated shoots is crucial for a successful acclimatization process of *in vitro* grown plants. Auxins proved to be responsible for root induction of shoots and hence adding of auxins singly or in combinations to the rooting medium have been practiced for many plant species (Gopi *et al*., 2006). According to the literature, IBA proved to be more successful in root induction of various medicinal plants (Chandra *et al*., 2006). Activated charcoal has been used in many *in vitro* root induction studies to prevent

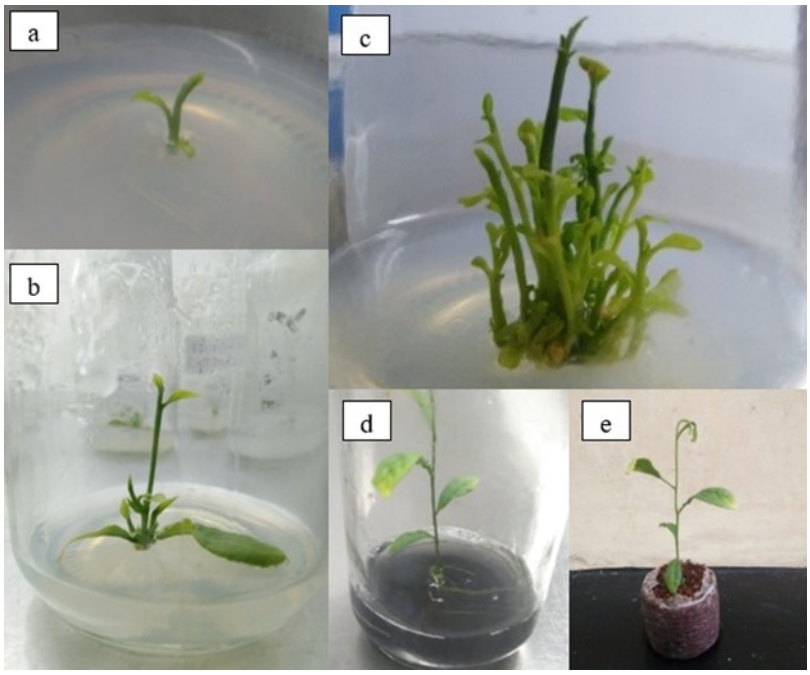


Figure 3: *In vitro* propagation of *A. marmelos.* a: An *in vitro* generated shoot tip established in shoot proliferation medium; b: The cultured shoot tips after one week; c: Shoot formation from shoot tip after 5 weeks; d: A rooted shoot ready for acclimatization; e: An *in vitro* generated plantlet in a coco pellet.

oxidation of polyphenols and reduce the light effect (Puhan and Rath, 2012).

In vitro generated plantlets were transferred to coco pellets and acclimatized to the normal environment with 80% survival rate. All the plantlets were morphologically similar to the mother plants.

CONCLUSION

According to the results of present study, it can be concluded that *A. marmelos* can be successfully micro propagated by *in vitro* generated shoot tips. Multiple shoots were regenerated in MS medium with 1 mgL^{-1} BAP and 1 mgL^{-1} kinetin while roots in regenerated shoots were formed in half strength MS medium with 1.0 mgL^{-1} IBA and 0.5 % activated charcoal. This *in vitro* propagation protocol for *A. marmelos* is useful for mass production of plants for commercial purposes and for further studies.

Table 1: Effect of half strength MS medium containing different concentrations of IBA with or without activated charcoal on root induction of *in vitro* raised shoots of *A. marmelos*

IBA (mgL^{-1})	Activated charcoal (%)	Root induction (%)	Number of roots per shoot	Average Length of roots per shoot (cm)
1.0	-	35 ^{c}	1.42^{c}	1.28^{b}
1.5	-	30 ^{d}	1.35 ^{c}	1.24 ^{b}
2.0	-	15 ^{d}	1.26^{c}	1.25^{b}
1.0	0.5	80 ^{a}	4.68 ^{a}	1.75^{a}
1.5	0.5	65 ^{b}	2.98^{b}	1.32^{b}
2.0	0.5	60 ^{b}	2.66 ^{b}	1.36^{b}

AUTHOR CONTRIBUTION

DLCKF, SANPH and HNA conceptualized and designed the study. DLCKF and SANPH performed the experiments, analyzed and interpret the data. DLCKF, SANPH and HNA contributed in drafting the manuscript and DLCK and HNA critically revised the manuscript.

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