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AN EFFICIENT *IN VITRO* PROPAGATION PROTOCOL FOR *MORINDA CITRIFOLIA* L., AN IMPORTANT MEDICINAL PLANT

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ABSTRACT
An efficient protocol was developed for *in vitro* propagation of *Morinda citrifolia* L., a traditional folk medicinal tree with pharmacological value and low seed germination capacity. A source of several phytochemicals and potent anti-microbial activities, development of a micropropagation protocol, will serve as a tool for exploiting the production of phytochemicals *in vitro*. The effect of different concentrations of plant growth regulators such as cytokinins (BAP and Kinetin) on shoot proliferation and auxins (IBA and NAA) and different strength of MS medium (Full, ½ and ¼ MS) on rooting were investigated. Cytokinin BAP promoted shoot proliferation more favourably than kinetin. Endogenous auxin levels indicated that rooting was induced without the exogenous supply of growth regulators and quarter strength MS basal media was sufficient for rooting of microshoots. Hardening and acclimatization of the plantlets were successfully with over 96%, exhibiting normal development. Synthetic seeds of *M. citrifolia* were also produced using nodal segments and shoot tips with sodium alginate, as a method for germplasm conservation.

KEYWORDS: Synthetic seeds, *In vitro* regeneration, Noni, plant growth regulators, rhizogenesis.

INTRODUCTION
The traditional folk medicinal plant *Morinda citrifolia* L., is popularly known as Noni or Indian Mulberry grows in tropical and subtropical regions of the world. It is an ever green medium sized tree (3-10 m) bearing flowers and fruits throughout the year belongs to the family Rubiaceae. Due to the increasing demand in pharmaceutical industry, wild populations of this species are becoming increasingly scarce and without any pre-treatment the seeds of this plant show a low germination percentage because of the presence of extremely tough cellulose layers on the seed coat (Nelson 2005).

The antibacterial, antiviral, antifungal, antitumor, antidiabetic, analgesic, anti-inflammatory, immune enhancing activity of *M. citrifolia* have already been reported (Wang et al. 2002; Mathivanan et al. 2006; Surendiran et al. 2006). All the plant parts are used in the treatment of various diseases and disorders. The fruits of Morinda have a history of use in the pharmacopoeias of Pacific Islands and South East Asia (Morton 1992). It has more than 150 neutraceuticals, several vitamins, minerals, micro and macro nutrients that help the body in different ways from cellular level to organ level. The major micronutrients identified in Noni plant are phenolic compounds, organic acids and alkaloids (Wang and Su 2001). Ascorbic acid (Morton 1992; Shovic and Whistler 2001) and provitamin A (Dixon et al. 1999) are the vitamins reported in the fruit. The global popularity of Noni has increased dramatically in the past decade (Dixon et al. 1999; Clatchey 2002).

The main objective of this study was (i) to develop an efficient micropropagation method, (ii) to study the effect of growth regulators and medium strength on propagation and rooting, (iii) to provide a reliable source of *M. citrifolia* plants to replenish declining population in the wild and to make use in pharmaceutical industry and (iv) production of artificial seeds from nodal and shoot tip explants for medium term conservation.

MATERIALS AND METHODS
Seeds of *M. citrifolia* were collected from a tree (4-6 years) growing in Kozhikode district of Kerala (India). The fruit of *M. citrifolia* is oval and fleshy and its colour ranges from green to yellow. The seeds taken from ripened fruit were surface sterilized with a few drops of Tween-20 and 1% (v/v) $HgCl_2$ for 15 min and then washed three times in sterile water.

The seeds were germinated in test tubes containing 10 ml MS basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose (Hi media, India) and 0.6% (w/v) agar-agar (Hi media, India). The pH of

the medium was adjusted to 5.8 before autoclaving at 121ºC for 20 min. All cultures were maintained under a 16 hours photoperiod provided by cool white fluorescent tubes (Philips, India) and 8 hours dark period at a temperature of 25 ± 2ºC.

After one month of germination, roots were discarded, and the entire shoot (Fig. 2a) was subcultured onto fresh MS basal media to obtain enough shoots for the proliferation assays. The effect of different concentrations of (cytokinins) BAP and Kinetin (0.5mg/l- 2 mg/l), was studied. After 4 weeks, the proliferation rate of both BAP and Kin in different concentrations was scored. The shoot proliferation was evaluated by counting the total number of shoots/culture and the increased height of the shoot in nodal and apical shoot explants.

To evaluate the effect of auxins and different strength of MS media on rooting, individual shoots cultured in BAP and Kin were transferred to full strength, half strength, quarter strength and MS full strength media with IBA and NAA (0.5mg/l - 2 mg/l). After 4 weeks of culture, rooting was expressed in terms of the rooting frequency, the root number, root length and the increased height of the shoot.

Plantlets with well developed roots were placed in (10-cm-diameter) plastic pots containing sterilized coco peat. To maintain humidity, the potted plantlets were covered with polypropylene cover, which were gradually opened after 4 weeks and completely removed after 6 weeks. The potted plantlets were kept in the greenhouse at 26 ± 2ºC and natural light. The percentage survival rate of the plants and their morphology were recorded after 2 months. In the proliferation and rooting assays, single shoots were inoculated in 10 test tubes, each containing 10 ml of culture medium and capped with cotton plug. Data are presented as mean ± standard deviation of three replicates with ten shoots each.

Production of artificial seeds

Shoot tips and nodal segments were used as explants for the artificial seed production. Shoot tips and nodal explants 3-4 mm long were aseptically excised from *in vitro* cultured plants. Sodium alginate beads were produced by encapsulating the explants in MS liquid medium with sodium alginate. 100 ml MS (liquid) basal medium was prepared and added 5g of sodium alginate to it in a 250 ml conical flask. With a glass rod mix the alginate in the solution. Alginate was partially dissolved and it was then kept aside. During autoclaving alginate was completely dissolved. $CaCl_2$ (25g) was dissolved separately in 100 ml distilled water. Another 100ml basal MS liquid is kept aside to wash the encapsulated beads.

The nodal segments with active buds and shoot tips from *in vitro* grown plants were dipped in gel solution of 5% sodium alginate prepared in liquid nutrient medium. These propagules are then picked by the micropipette or spatula and dropped into a solution of 25% calcium chloride. After 30 minutes each explant become a hardball encoated by alginate. Then the $CaCl_2$ solution is decanted and the beads are washed 3-4 times in the nutrient medium.

RESULT AND DISCUSSION

Establishment of aseptic shoot cultures

The nodal segments and shoot tips from seedlings were cultured in different concentrations of cytokinins (BAP and Kinetin) for shoot multiplication (Fig. 2b). Nodal segments showed highest proliferation rate in MS media than shoot tips. The resident meristem of node can be induced more easily and effectively than that of shoot tips. So shoot tips were a poor substitute for nodes due to its increased apical dominance (Nair and Seeni 2000). The preference of the nodal explants over the apically dominant shoot tips is well documented in *Fagus* (Vieitez et al. 1993) and *Chestnut* (Sanchez et al. 1997). 2 mg/l of BAP in MS medium produce highest number of shoots (2 shoots). Maximum number of shoots produced from nodal segments is 2. In the study of Sreeranjini & Siril (2014) maximum number of shoots, shoot length and shoot forming capacity in *M. citrifolia* is seen in media supplemented with BAP. Saini & Patel (2015) also showed maximum length of shoots (2.33 cm) and number of shoots/ explants (2 shoots) in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/NAA. But in the present study highest shoot length is achieved from shoot tip explants with 1 mg/l kinetin (3.3 cm) than nodal explants.

Table 2 Shoot initiation from node and shoot tip explants after 4 weeks of incubation (±SD).

Media	Concentration	Shoot tip -Length increased (cm) (Mean ± SD)	Node- Length increased (cm) (Mean ± SD)	Number of shoots (Mean ± SD)
MS + BAP	0.5	1.4 ± 0.5	2.3 ± 0.7	0.5 ± 0.5
	1.0	0.8 ± 0.5	1.7 ± 0.8	1.2 ± 0.4
	1.5	0.4 ± 0.3	1.2 ± 0.6	2 ± 0
	2.0	1.0 ± 0.25	2.5 ± 0.5	2 ± 0
MS + Kin	0.5	1.7 ± 0.5	0.3 ± 0.2	0.5 ± 0.2
	1.0	3.3 ± 0.8	0.4 ± 0.1	0.9 ± 0.4
	1.5	1.0 ± 0.5	0.2 ± 0.1	0.5 ± 0.5
	2.0	2.2 ± 0.5	0.5 ± 0.1	1.1 ± 0.5

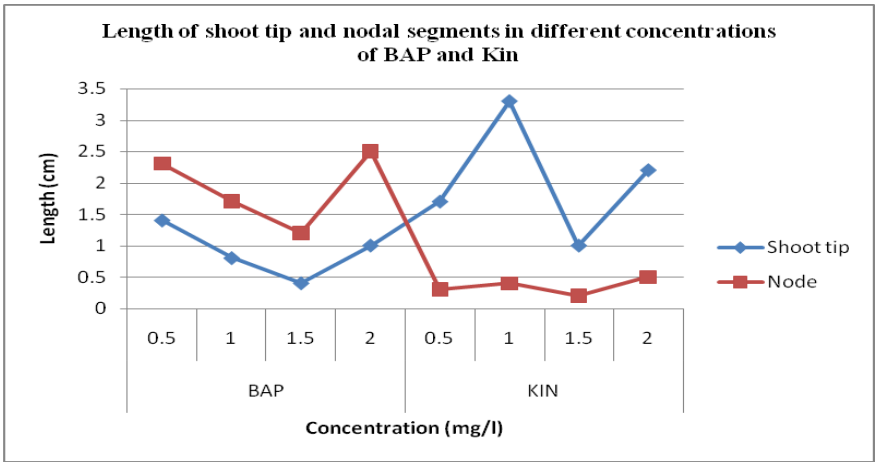

Fig.1 Effect of different concentrations of cytokinins (BAP and Kin) in the proliferation of *M. citrifolia* shoots.

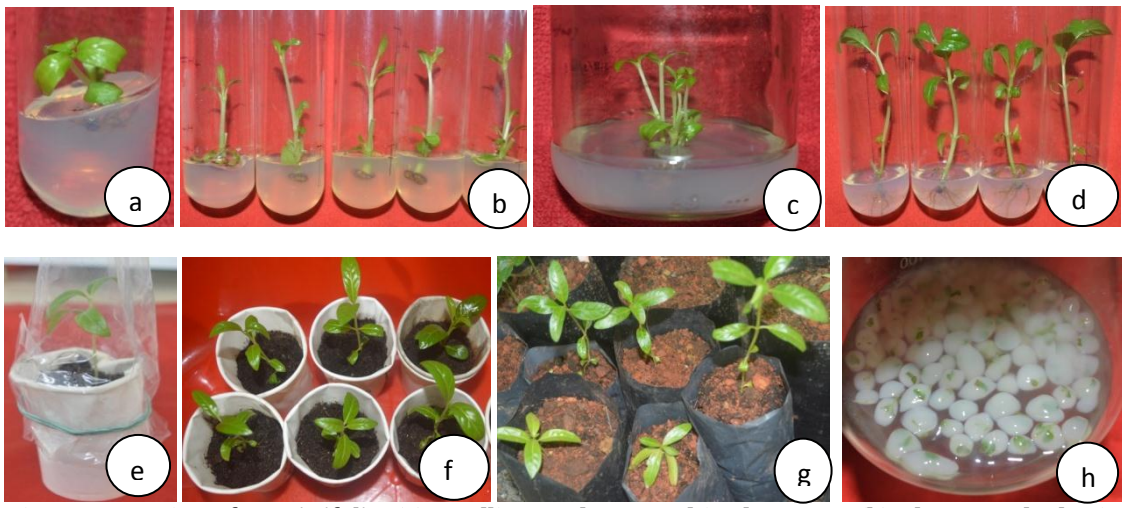

Fig. 2 Micropropagation of *M. citrifolia*. (a) Seedling explants used in the assays (b) Shoots at the beginning of proliferation phase (c) Shoots at the end of proliferation phase (d) Rooted shoots after 4 weeks in culture (e) Rooted shoot in cocopeat covered with Polypropylene cover (f) Acclimatized plant after 2 months (g) Acclimatized plants for planting in the field (h) Artificial seeds in MS liquid.

Callus formation is seen at cut ends in all concentrations of cytokinins after a period of 4 weeks. After 2 to 3 subculture of the nodal segments with callus at their base in different concentration of BAP and Kin (Table 3) produce multiple shoots. The highest proliferation rates were observed in MS medium supplemented with 1 mg/l BAP (5 ± 1.3) (Fig. 2c).

Table. 3 Multiple shoots induction in different concentration of BAP and Kin in culture media.

Medium	Concentration	Number of shoots	Number of shoots (Mean ± SD)
MS + BAP (After 2-3 subculture with callus)	0.5 mg/l BAP	2-3 shoots	3 ± 0
	1 mg/l BAP	5-6 shoots	5 ± 1.25
MS + KIN (after 2-3 subculture with callus	0.5 mg/L KIN	1-2 shoots	2 ± 1.0
	1mg/l KIN	3-4 shoots	3 ± 0.2

BAP is superior to kinetin in producing multiple shoots in both shoot tip and nodal segments (Fig.1), but maximum shoot length is achieved from shoot tip explants treated with Kinetin. Vankova et al. (1991) described the effectiveness of BAP over Kinetin in triggering the synthesis of endogenous cytokinins and in uptake and metabolization of nutrients. Shekhawat et al (2015) has reported the superiority of BAP over Kinetin for bud break as well as shoot growth and multiplication in *M. citrifolia*. From the study, it is observed that nodal explants are superior to shoot tips in producing multiple shoots and shoot tips are superior to nodal explants in increasing shoot length, in a period of 4 weeks. In nodal segments new axillary buds are produced but in case of shoot tips, only the elongation of shoot length was

observed. Thus apical dominance seems to hinder the precocious production of multiple shoots.

Root initiation, *in vitro*

High root induction frequency was obtained in auxin–free media, particularly in ¼ MS (Fig. 2d). Hundred percent rooting is seen in full, ½ and ¼ MS. Root initiation starts from 8th day of culture in total strength MS medium. In ½ and ¼ MS, root development is observed from the 10th day of culture. Highest number of roots, root length and increased shoot length is observed in ¼ MS without any PGR. Media with IBA and NAA (0.5mg/l-2mg/l) shows slow root initiation only after 14 days (Table 4). Thus endogenous levels of auxins lead to root induction on supplementation with basal nutrients itself.

The time course of the rooting process was faster in auxin free media (full, ½, ¼ MS) where root emergence started after 8-10 days (100% rooting) of the initiation of experiment. In the *in vitro* propagated shoots, endogenous concentration of auxin present in the plant is efficient for root development rather than exogenous auxin. Majority of the *in vitro* rooting studies on *M. citrifolia*, use IBA and NAA at various concentrations or in combinations for root induction (Subramani et al. 2007; Saini & Patel 2015). But in this study, best rooting is achieved in ¼ MS without using any PGRs. Root development without using PGR is advantageous as unnecessary usage of growth regulators may lead to variations in the regeneration pathway, at the same time it reduces the cost and increase the survival rate of the plants while hardening. In the present study, an intermediate callus phase is seen at the basal cut ends of shoots when placed in rooting media IBA and NAA (0.5-2.0 mg/l), which is not desirable for the conduction and absorption of nutrients. In all concentrations of IBA (0.5-2.0 mg/l) roots are produced from callus where as NAA produce roots from callus only in one concentration (2.0 mg/l) within a period of 4 weeks. Others produce only callus at the basal cut ends (0.5-1.5 mg/l).

Without the presence of exogenous auxins, *in vitro* root formation relies on endogenous auxin synthesized in the shoot apex and transported downwards to create an auxin gradient required for root induction. This established gradient of auxin allows cells to maintain information about their growth and development past the initial signals that caused cell differentiation, while changes in the auxin gradient allow the plant to control its development (Grieneisen et al. 2007).

Table. 4 Root induction from shoots after 4 weeks of incubation in different strength of basal MS and auxins (IBA & NAA) (± SD).

Medium	Concentration	Root length (mean ±SD) (cm)	Rooting frequency	Callus	Rooting %	Plant height increased (mean±SD) (cm)	Number of roots/shoot
Basal MS	Full strength	2 ± 0.2	8 days	No callus	100%	0.7 ± 0.3	7.5 ± 2.2
½ MS	Half strength	2.6 ± 0.2	10 days	No callus	100%	1.4 ± 0.1	10.0 ± 2.8
¼ MS	Quarter strength	2.9 ± 0.1	10 days	No callus	100%	2.3 ± 0.4	11.3 ± 2.0
IBA	0.5 mg/l	1.3 ± 0.4	21 days	++	32%	0.3 ± 0.5	3 ± 1.3
	1.0 mg/l	2.0 ± 0.3	15 days	++	66%	0.43 ± 0.7	6 ± 2.1
	1.5 mg/l	1.9 ± 0.4	14 days	+++	71%	1.2 ± 0.8	7 ± 1.6
	2.0 mg/l	2.2 ± 0.2	19 days	++++	100%	1.8 ± 0.5	10 ± 1.0
NAA	0.5 mg/l	_	_	++	No root	0.2 ± 0.2	-
	1.0 mg/l	_	_	++	No root	0.2 ± 0.3	-
	1.5 mg/l	_	_	++	No root	0.4 ± 0.5	-
	2.0 mg/l	2.0 ± 0.4	17 days	+++	60%	1.2 ± 0.4	6 ± 2.0

Four-week-old rooted shoots were removed from the culture vessels and transferred into pots containing sterilized coco peat, soil and cocopeat + soil (1:1) (Table 5). In the present study the transferred shoots grow well in the potting mixture of coco peat (96%) (Fig.2e). Similar results are seen in the study of Saini & Patel (2015) that the maximum per cent survival of plantlets (95.25%) was reported in coco peat which gives better grip for the roots and ample aeration. These results are consistent with earlier finding of Subramani et al. (2006) in noni. The *in vitro* produced plantlets did not show any morphological variation and ninety six percent of regenerated plantlets were successfully acclimatized to *ex vitro* conditions (Fig. 2f & g).

Table. 5 Growth response of hardened plants in survival percentage, plant height and number of leaves after a period of 2 months.

Potting mixture	Survival % of hardened plants	Number of leaves increased	Plant height increase (cm)
Coco-peat	96%	4.2 ± 1.5	3.04 ± 0.37
Soil	75%	2.8 ± 1.2	1.6 ± 0.43
Coco-peat + soil	82%	3.3 ± 1.4	2.4 ± 0.35

Artificial seed production

Artificial seed production is an important method for the conservation of medicinal plant species, which are difficult to regenerate through conventional methods and natural seeds. High conversion ability and compact size are indispensable features for synthetic seeds (Nieves et al. 1995). Synthetic seeds have multiples advantages such as easy handling and transportation, potential long-term storage, higher scale-up capacity, uniformity in production, potential for automation of the whole production process, seeding of clonal varieties and may provide a means for maintenance of elite germplasm (Singh et al. 2007).

In the present investigation the nodal segments and shoot tips explants of *M. citrifolia* coated with sodium alginate were kept in storage under growth chamber at 20 ± 2°C with liquid MS medium (Fig.2h). Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is inert, non toxic, cheap and can be easily handled (Endress 1994, Jaiswal et al. 2008). At the end of 30 days storage period, artificial seeds were immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds were regrown under in vitro conditions on nutrient media for shoot development and root induction. The survival percentage of encapsulated shoot tips and nodal explants decreased significantly after 4 weeks. The alignate matrixes ruptured and shoot tips and roots emerged from the capsule after 3 weeks in MS basal medium. The plantlets grew vigorously and were comparable to the plants developed from non-encapsulated explants grown under identical conditions. All the plantlets exhibited new growth and normal morphological characteristics under *ex vitro* conditions.

CONCLUSION

In this study an efficient protocol was developed for *M. citrifolia*, an important medicinal plant with high pharmacological value. Shoots showed high proliferation capacity with BAP. Nodal segments are seen as superior to shoot tips in producing multiple shoots. Rooting is significantly influenced by the endogenous auxin levels present in the plant. Highest root number and root length were attained in ¼ MS medium. So the regenerated plantlets could be used to replenish declining population in the wild. Through artificial seed production the germplasm of *M. citrifolia* can be conserved and whenever we need them we can regenerate from the synthetic seeds.

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