Plant Knowledge Journal
ISSN: 2200-5390
EISSN: 2200-5404
1(2)46-51 (2012)

Southern Cross Publishing Group
AUSTRALIA

Conservation and *in vitro* multiplication of highly endangered Indian traditional medicinal plant (*Morinda reticulata* Gamble) through nodal explants

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Abstract

Morinda reticulata Gamble is a highly endangered medicinal plant of Southern Western Ghats of India. Since conventional propagation methods are not effective, *in vitro* propagation was attempted for the first time using nodal explants. Year long experiment on responses of apical bud and nodal explants revealed that summer season (March – May) induced early bud break and faster shoot regeneration than in the other seasons. MS medium supplemented with 6-benzylaminopurine (BAP) and kinetin (KN) were tested at various concentrations ranging from 2.2μM – 46 μM. The maximum response of explants (62.7%) with longest mean shoot length (6.7 cm) was obtained with 4.4 μM BAP. The number of shoots per explant never increased beyond four even at higher levels of cytokinin However, healthy microshoots with uniform shoot development from the nodal segments led to the establishment of a large number of shoots. A combination of 0.12 mM silver nitrate with 22 μM BAP resulted in 4-5 shoots per nodal explant. However, the shoots were smaller with shorter internodes. Shoots regenerated in presence of BAP were converted into plantlets by *in vitro* rooting using 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and BAP. About 80% of the micropropagated plants were acclimatized for field conditions.

Keywords: *Morinda reticulata*, Shoot regeneration, Micropropagation, Conservation.

Abbreviations:

$AgNO_3$	Silver nitrate
BAP	6- Benzylaminopurine
IBA	Indole-3-butyric acid
KN	Kinetin
NAA	1-Naphthalene acetic acid
PG	Phloroglucinol

Introduction

Morinda reticulata Gamble (Rubiaceae) is an evergreen woody climber, distributed in wild forest of Southern Western Ghats in India. Its geographical distribution covers Agasthyamalai Mountain and adjoining areas of Tamilnadu and Kerala states in southern part of India. It is an arborescent climber, growing to a height of 20 m. It forms an important component in a variety of herbal formulation in Indian system of traditional medicines for its wide range of medicinal properties (Ijinu et al., 2011). Extracts of various parts of *Morinda* sp. are used as laxatives in traditional medicines of India due to the abundance of anthraquinone derivatives (Temiyaputra et al., 2008). Decoction of leaf, stem and root of this species is also used to relieve stomach ailments. Powdered leaves of *M. reticulata* mixed with rice is used for purification of blood. Fresh leaves of *M. reticulata* are used for healing leucorrhoea and back pain (Ijinu et al., 2011). Reliable conservation measures as suggested by Henry et al., (1984) are needed to prevent the destruction of the habitat of *M. reticulata* due to large scale deforestation and indiscriminate collection. Depletion of the natural population has led to the declaration of *M. reticulata* as an endangered species by International Union for Conservation of Nature (Gopalan and Henry, 2000). Unfortunately, conventional methods could not be applied for restoration of *M. reticulata* due to lack of rooting, seed germination and susceptibility of stem cuttings to soil borne pathogens. Therefore the present work was undertaken in order to develop an *in vitro* culture method for large scale propagation of *M. reticulata*. This study investigates the influence of seasons and *in vitro* culture conditions on shoot regeneration from apical bud and nodal explants of *M. reticulata*. This is the first report demonstrating the *in vitro* regeneration and multiplication of *M. reticulata*.

Results

Effect of cytokinin on shoot regeneration

Nodal explants of *M. reticulata* from actively growing shoots of a thirty year old mother plant (Figs. 1a - e) responded to various concentrations of BAP and KN either in combination or alone with varying degrees of sprouting ranging 9.8 – 62.7% (Table 1). The highest percentage of sprouting (62.7%) occured with 4.4 μM BAP. Any further increase in

levels of BAP and KN either alone or in combination reduced the sprouting percentage significantly. Shoots regenerated from nodal explants on medium containing either 4.4 μM BAP or 4.6 μM KN produced the longest shoots with 6.7 cm and 5.6 cm length respectively with a large number of microshoots (Figs. 2a, b). Shoots regenerated on these media produced significantly longer internodes. Higher concentrations of BAP and KN each at 22 μM induced 2 – 4 slender shoots per nodal explant (Figs. 2 c, d). Of the various treatments, 4.4 μM BAP was optimal for regeneration of healthy shoots as the nodal segments cultured on this medium had produced the longest shoots with 3 – 4 nodes per shoot. Highest concentrations of BAP (44 μM) and KN (46 μM) induced profuse calli without any regeneration of shoots.

Effect of seasons on bud-break and shoot regeneration

Apical buds and nodal explants established in the summer season, particularly during May produced the maximum sprouting (73.3%). Bud break was occurred within one week of culture and most of the cultures had produced an average of 3.3 and 4.3 shoots per explants from apical bud and nodal explants respectively (Table 2). In contrast, explants of both types cultured during intermediate season (December – February) and South West Monsoon season (June – August) took a longer duration (2-3 weeks) for bud break. Recovery from microbial contamination was higher in nodal explants than apical buds in all the seasons. In addition, nodal explants were amenable for early bud break and shoot regeneration than apical bud. Decline in growth of microshoots followed by browning of tissues progressing from the basal end of the shoot was observed in many cultures. Of the four seasons, summer which spreads from March – May was suitable for culture establishment due to maximum response of explants with early bud break and shoot regeneration.

Effect of $AgNO_3$ and PG on shoot proliferation

Evaluation of $AgNO_3$ and PG was carried out with apical bud and nodal segments to optimize their concentrations for shoot regeneration. MS medium supplemented with 4.4 μM and various concentrations of $AgNO_3$ induced higher percentage of sprouting in nodal segments than in apical buds. Presence of $AgNO_3$ at 0.06 mM, 0.12 mM and 0.24 mM induced 76.5%, 97.8% and 57.8% sprouting respectively in nodal explants. Sprouting was reduced significantly when higher concentrations of $AgNO_3$ (0.36 mM and 6 mM) were used. Nodal explants responded for early bud break and faster regeneration than apical buds (Fig. 2e). This experiment also confirmed that $AgNO_3$ was more effective with nodal explants than with apical buds as the latter one showed slower development of shoots in presence of $AgNO_3$ (Fig. 2f). Presence of 0.12 mM AgNO3 was more effective for regeneration of shoots from nodal explants (Fig. 3a). A similar experiment with PG did not enhance sprouting in nodal and apical bud explant (Fig. 3b). Combination of 0.12 mM $AgNO_3$ either with 4.4 μM BAP or with 4.6 μM KN induced the highest sprouting with 73.8% and 60.7% respectively. Shoots grown on these combinations produced longer shoots ranging from 2.3 – 2.7 cm. Each nodal explant produced 2 – 3 shoots in many of the cultures. Higher concentrations of BAP(22 μM) and KN (23 μM) with 0.12 μM $AgNO_3$ induced 4 - 5 shoots per node but with reduced shoot length. Increase in leaf size of micropropagated shoots was often influenced by the presence of $AgNO_3$.

Rooting and ex vitro establishment

The highest percentage of rooting (41.2%) was recorded when shoots were cultured in medium containing 0.53 μM NAA and 2.2 μM BAP whereas the latter alone induced 24.5% rooting with significantly improved root length (Fig. 4a). Shoots cultured on medium containing 0.53 μM NAA and 0.49 μM IBA seperately resulted in 14.6% and 9.8% rooting respectively without any significant differences in root length. Combination of 0.53 μM NAA and 2.2 μM BAP induced 5 – 6 roots/shoot but in other treatments, the number of roots was significantly reduced. Combination of auxins did not induce any roots. Of the 160 plantlets with well developed shoot and roots transferred to *ex vitro* condition, 132 (82.5%) were hardened within 2 months with the development of new shoots (Figs. 4b, c).

Discussion

M. reticulata is one of the important woody climbers in India (Gopalan and Henry, 2000) and is an essential ingredient in many herbal formulations due to its wide range of medicinal properties (Ijinu et al., 2011). *M. reticulata* is at the verge of extinction due to the indiscriminate removal of roots for herbal medicines (Ijinu et al., 2011). Attempts at propagation of this plant through seeds and nodal cutting were never successful. Although, *in vitro* methods were successfully applied for the conservation of several rare Indian medicinal plant species (Fay, 1992; Chaturvedi, 2007; Kapai, 2010) there was no attempt to propagate *M. reticulata.* The present work demonstrates the possibility for cloning *M. reticulata* by *in vitro* culture using nodal segments. Regeneration of shoots in *M. reticulata* was influenced more by seasons than by growth regulators as reported in many of the woody species (Ganesh and Sreenath, 2000; Kavitha et al., 2009)) Nodal segments collected and cultured during summer season were significantly more responsive than those during the other seasons. Though, *M. reticulata* is a fast growing climber, regeneration of shoots was slower with only a few number of shoots even at higher levels of cytokinins as reported in other woody climbers (Komalavalli and Rao, 2000; Karuppusamy et al., 2006). However, a large number of shoots were established when nodal segments were cultured on MS medium with 4.4 μM BAP during summer season. In many species, combination of $AgNO_3$ and PG was found to enhance shoot regeneration (Mallika et al., 1996; Mercy et al., 2010; Siwach and Gill, 2011). But in this study, nodal and apical bud segments did not respond for PG. Early bud break and shoot regeneration was influenced only in the presence of $AgNO_3$. A combination of BAP and $AgNO_3$ was beneficial in early regeneration of shoots in *M. reticulata*. Difficulty in induction of roots from nodal cuttings of *M. reticulata* by conventional method was overcome in the present work. Root induction did not occur when NAA, IAA and IBA were combined. Instead presence of NAA and IBA separately in the medium induced 14.6% and 9.8% rooting respectively. Highest percentage of rooting was induced by a combination of 0.53 μM NAA and 2.2 μM BAP. Generally cytokinins used for multiplication of shoots are withdrawn during *in vitro* rooting since they inhibit root induction. Paradoxically, 2.2 μM BAP alone has induced rooting in this study. A similar finding was reported during *in vitro* rooting of *Lens culinaris*, where roots were induced by BAP without auxins (Polanco and Ruiz, 1997). Our study also revealed

Table 1. Effect of different concentrations of BAP and KN on shoot proliferation in cultured nodal explants of *Morinda reticulata*. Nodal explants were cultured on MS medium for 60 Days.

Cytokinins (μM)		Sprouting (%)	Shoot length (cm)	No. of shoots/ Explants	Internodal length (cm)	No. of nodes/shoot
BAP	KN					
0.0	0.0	19.7^{j}	2.3^{j}	1.0^{i}	1.4^{h}	1.0^{i}
2.2	0.0	30.0^{i}	3.3^{h}	1.2^{g}	1.7^{f}	1.1^{h}
0.0	2.3	31.1^{h}	4.1^{e}	1.1^{h}	1.8^{e}	2.1^{d}
2.2	2.3	38.7^{f}	4.8^{c}	1.1^{h}	2.0^{c}	1.9^{e}
4.4	0.0	62.7^{a}	6.7^{a}	2.0^{f}	2.1^{b}	3.6^{a}
0.0	4.6	40.1^{e}	5.6^{b}	2.5^{e}	2.3^{a}	2.1^{d}
4.4	4.6	51.3^{b}	4.2^{d}	2.8^{d}	1.9^{d}	2.2^{c}
22.0	0.0	34.8^{g}	3.9^{f}	3.7^{a}	1.3^{i}	1.7^{g}
0.0	23.0	43.7^{d}	3.4^{g}	3.1^{c}	1.5^{g}	2.6^{b}
22.0	23.0	48.4^{c}	2.9^{i}	3.4^{b}	1.1^{j}	1.8^{f}
44.0	0.0	9.8^{l}	***	***	***	***
0.0	46.0	11.8^{k}	***	***	***	***

* Data represents the mean values of 30 explants in each treatment with three replications and analysis are made using Duncan's new multiple range test. Figures with same superscripts are not significantly different at $p < 0.05$. *** indicate the enlargement of axillary buds without proliferation.

Fig 1. (a) 30 year old conserved plant of *M. reticulata* in Sanjeevani Vanam (South India). **(b)** Actively growing shoots from mother plant. (**c-e)** Flower buds, unripe and ripe fruits of *M. reticulata.*

that healthy shoots that are suitable for *in vitro* rooting were induced only in the presence of 4.4 μM BAP or in combination with 0.12 mM $AgNO_3$. Higher levels of cytokinins only induced 4 – 5 slender shoots/nodal segment that were not suitable for *in vitro* rooting. This study describes a simple and reliable protocol (Fig. 5) for *in vitro* multiplication of *M. reticulata*, thus paving the way for restoration of this important medicinal plant from the threat of extinction.

Materials and methods

Plant material

A 30-year-old climber of *M. reticulata* in Sanjeevani Vanam, Department of Social Forestry, Kulathupuzha in Kollam District of Kerala (Southern part of India) was used as the mother plant for this study. The mother plant was healthy and exhibited active vegetative growth for collection of young shoots for establishment of aseptic apical bud and nodal explants throughout this study.

Explant preparation and culture conditions

Apical bud and nodal segments (1.5 cm) were cut from the actively growing twigs and thoroughly washed under tap water for 10 – 15 min. Both the explants were separately surface sterilized with 0.1 % $HgCl_2$ (Hi-Media, Mumbai, India) for 5 min followed by rinsing with sterile distilled water. Nodal segments were trimmed using sterile surgical blade under the solution containing 0.1% each of ascorbic acid and citric acid (British Drug House, Chennai, India), and blotted on sterile filter paper before implanting the explants on sterile nutrient media. MS medium (Murashige and Skoog, 1962) supplemented with sucrose (3%) become the basic media. The media was augmented with various growth regulators like BAP, KN, NAA, IAA and IBA (Hi-Media, Mumbai, India) in different concentrations ranging from (0.49 μM – 46 μM) depending upon the experimental design (Table 1 – 4). A range of concentrations of $AgNO_3$ and PG (British Drug House, Chennai, India) was also used for optimizing the culture conditions for shoot proliferation. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, Mumbai, India). Molten medium was dispensed into 200 ml screw-capped glass jars and 25 × 150 mm culture tubes (Borosil, Chennai, India) and capped with cotton plugs before sterilization at 121°C for 20 min. All cultures were maintained at 25±2°C and kept under a 16 h photoperiod provided by cool white fluorescent tubes (Phillips, Mumbai, India) with a light intensity of 40 μmol m^{-2} s^{-1}. The number of explants cultured in each treatment ranged from 30 - 60 and the culture period was 30 - 60 d depending upon the experimental design.

Selection of optimal concentration of $AgNO_3$ and PG

Dissected apical bud and nodal explants were vertically implanted in culture tube containing various combinations of media (Table 1) and maintained for 60 d with a subculture after 30 d of inoculation. Data on the recovery, percentage of

Table 2. Effect of explanting seasons on bud break and shoot regeneration in apical bud (A) and nodal (N) explants of *Morinda reticulata*. Explants were cultured on full strength MS medium supplemented with BAP (1mg/l) for 45 days. Data represents mean values of 30 explants.

Response	Intermediate (Dec – Feb)		Summer (Mar – May)		South – West (Jun – Aug)		North – East (Sep – Nov)	
	A	N	A	N	A	N	A	N
Number of responded cultures	2 (6.6)	3 (10.0)	11 (36.6)	22 (73.3)	3 (10.0)	12 (40.0)	3 (10.0)	18 (60.0)
Number of cultures turned brown	14 (46.6)	14 (46.6)	10 (33.3)	5 (16.6)	9 (30.0)	10 (33.3)	10 (33.3)	3 (10.0)
Bud break (Weeks)	2-3	2-3	1	1	2-3	2-3	1-2	1-2
Shoots/ explant	1.10± 0.31	1.30±0. 48	3.30± 0.48	4.3± 0.5	1.50± 0.52	1.70± 0.48	2.30± 0.48	2.60± 0.51

* Figures in parentheses are percentages.

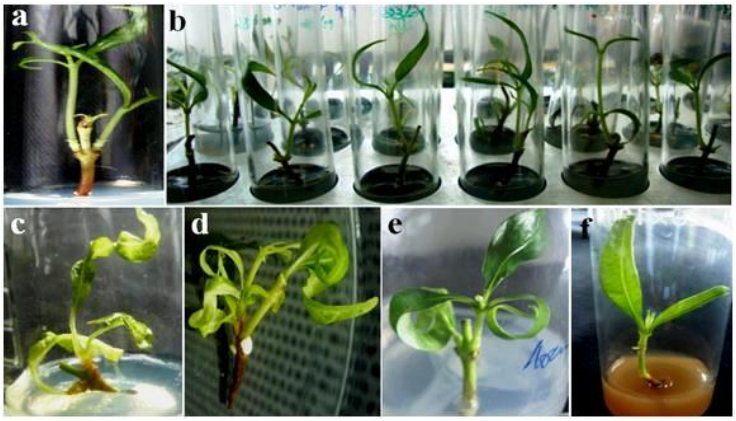

Fig 2. (a) Response of nodal explant for regeneration of shoots during primary culture on MS medium with 4.4 µM BAP. (b) Establishment of microshoots from nodal segments during summer season. (c, d) Development of weaker single and low frequency multiple shoots in presence of 22 µM BAP). (e) Early bud break and shoot regeneration from nodal segment on MS medium supplemented with 4.4 µM BAP. (f) apical bud showing enlarged leaves with weaker shoot in presence of 0.12 mM $AgNO_3$.

response, shoot length, number of shoots per explant and internodes were recorded. To determine the effect of $AgNO_3$ and PG on shoot induction, both the types of explants were cultured on MS medium containing 4.4 µM BAP and with various concentrations of $AgNO_3$ and PG ranging from 0.06 mM – 8 mM (Fig. 3). Response of apical bud and nodal explants in the above treatments were recorded after 45 d of culture and data on shoot proliferation was analyzed. The optimum concentration of $AgNO_3$ (0.12 mM) identified from the previous experiment was once again combined with nine different combinations of BAP and KN (Table 3) and tested for shoot multiplication. Nodal explants inoculated in these combinations were observed after 45 d of inoculation and sub cultured onto fresh media of the same composition. Response of explants for bud break, shoot regeneration and multiple shoot developments were recorded.

Response of explants in different seasons

Apical bud and nodal explants were collected during four different seasons, namely summer (March – May), South-West Monsoon (June – August), North – East Monsoon (September – November) and Intermediate (December – February) and cultured on MS medium supplemented with 4.4 µM BAP and 0.12 mM $AgNO_3$. Responding explants were maintained on the same medium for 45 d with a subculture after two weeks of inoculation. Response of apical bud and nodal explants for bud break and shoot regeneration was recorded for analysis. This experiment was repeated for three subsequent years.

Rooting and acclimatization

Nodal segments with one or two shoots were transferred to half strength MS medium supplemented with different combinations of NAA, IAA, IBA and BAP (Table 4) and cultured for 45 d. Percentage of root induction, root length and the number of roots per shoot were recorded. Shoots with well developed roots were removed from the culture vessels, rinsed gently with sterile water to remove medium adhering the roots and planted in polybags containing a mixture of sterile soil, sand and manure (6:2:1). Plants were maintained under natural shade by covering the plant individually with small polybags for providing humidity. Frequent watering was carried out to maintain soil moisture. After 45 d of acclimatization, healthy plants were transferred to earthen pots containing the above soil for field establishment.

Statistical analysis

All of the experiments were carried out with three replications, each with 30 samples. The effect of different treatments on various parameters was quantified and the level of significance was determined by analysis of variance (ANOVA) using SPSS version 11.0 and level of differences

Table 3. Effect of $AgNO_3$ in combination with cytokinins on shoot proliferation in cultured nodal explants of *Morinda reticulata*. Nodal explants were cultured on MS medium for 45 Days.

Media (µM))			Sprouting (%)	Shoot length (cm)	No. of shoots/ explant	Leaf size (cm)	
BAP	KN	$AgNO_3$				Length	Width
0.0	0.0	0.0	21.8^{i}	1.1^{f}	1.1^{h}	1.3^{h}	0.3^{c}
0.0	0.0	0.12	44.1^{e}	1.7^{d}	1.2^{g}	1.9^{g}	0.3^{c}
4.4	0.0	0.12	73.8^{a}	2.7^{a}	2.3^{d}	2.9^{c}	0.5^{b}
0.0	4.6	0.12	60.7^{b}	2.3^{b}	1.7^{f}	2.2^{e}	0.3^{c}
4.4	4.6	0.12	52.3^{c}	2.1^{c}	1.8^{e}	2.1^{f}	0.3^{c}
22.0	0.0	0.12	42.5^{g}	1.1^{f}	4.3^{a}	3.8^{a}	0.7^{a}
0.0	23.0	0.12	46.3^{d}	1.2^{e}	3.5^{c}	3.0^{b}	0.5^{b}
22.0	23.0	0.12	43.6^{f}	0.9^{g}	3.8^{b}	2.8^{d}	0.5^{b}
44.0	0.0	0.12	31.1^{g}	***	***	***	***
0.0	46.0	0.12	27.6^{h}	***	***	***	***

*Data represents the mean values of 30 explants in each treatment with three replications and analysis are made using Duncan's new multiple range test. Figures with same superscripts are not significantly different at $p < 0.05$. *** indicate the enlargement of axillary buds without proliferation.

Table 4. Effect of auxins and cytokinins on root induction in cultured nodal explants of *Morinda reticulata*. Nodal explants were cultured on MS medium for 45 days

Auxins (µM)			Cytokinin (µM)	Rooting (%)	Root length (cm)	No.of roots/ explant
NAA	IBA	IAA	BAP			
0.00	0.00	0.00	0.0	0.0	0.0	0.0
0.53	0.00	0.00	0.0	14.6^{c}	1.4^{c}	3.2^{b}
0.00	0.49	0.00	0.0	9.8^{d}	1.3^{c}	2.3^{c}
0.00	0.00	0.57	0.0	0.0	0.0	0.0
0.00	0.00	0.00	2.2	24.5^{b}	3.5^{a}	2.2^{c}
0.53	0.49	0.00	0.0	0.0	0.0	0.0
0.53	0.00	0.57	0.0	0.0	0.0	0.0
0.53	0.00	0.00	2.2	41.2^{a}	2.7^{b}	5.4^{a}
0.00	0.49	0.57	0.0	0.0	0.0	0.0
0.00	0.49	0.00	2.2	0.0	0.0	0.0

* Data represents the mean values of 30 explants in each treatment with three replications and analysis are made using Duncan's new multiple range test. Figures with same superscripts are not significantly different at $p < 0.05$.

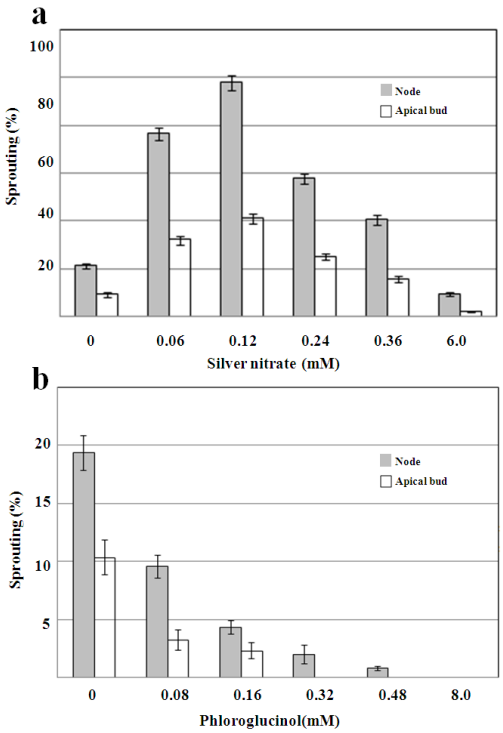

Fig 3. (a) Response of nodal and apical bud explants of *M. reticulata* in various concentrations of Silver nitrate. (b) Response of nodal and apical bud explants of *M. reticulata* in various concentrations of Phloroglucinol.. Note the increase in percentage of sprouting in nodal explants. 4.4 µM BAP was common in both experiments.

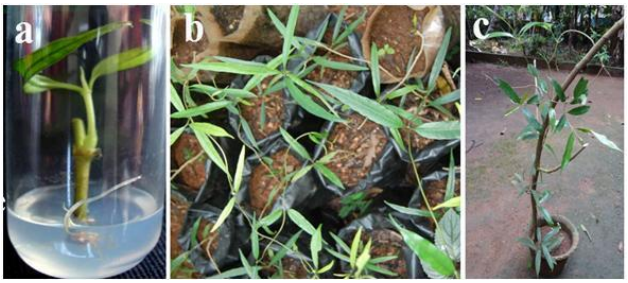

Fig 4. (a) Induction of roots from microshoots of *M. reticulata* on MS medium containing 0.53µM NAA and 2.2µM BAP.(b) Semi hardened micropropagated plants in poly bags.(c) Fully hardened plant *M. reticulata* for field planting.

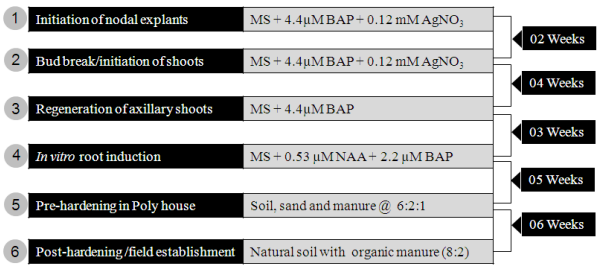

Fig 5. Sequential steps involved in micropropagation of *M. reticulata*. This protocol is reproducible only when nodal explants are cultured during active phase of vegetative growth of mother plants from March – May (Summer season)

between the treatments were assessed by Duncan's New Multiple Range Test (DMRT) at $p < 0.05$.

Conclusion

This is the first report on plant regeneration of *M. reticulata* utilizing nodal segments. As this endangered species is medicinally important, the present protocol is valuable for conservation and restoration.

Acknowledgements

The first author acknowledges Department of Biotechnology, Manonmaniam Sundaranar University for providing facilities for carrying out this work. The first author expresses his gratitude to Mr. T. Joy, Sanjeevani Vanam, Division of Social Forestry, Government of Kerala, India, for providing plant materials, and Dr. P.S. Udayan, Sree Krishna College, Guruvayur, Kerala, for authenticating the plant specimen. This work is a part of research work by the first author for Ph.D Degree.

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