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Enhanced micropropagation protocol of *Morinda citrifolia* L. through nodal explants

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ABSTRACT

We report an improved *in vitro* propagation protocol for *Morinda citrifolia* L. in the present communication using nodal segments as explants from a 4 year old plant. Murashige and Skoog (MS) medium was used to inoculate the sterilized explants. Under laboratory conditions, 4.6 ± 0.48 shoots were regenerated from the nodal meristem of an explant on the combination of MS medium fortified with 4.0 mg/L 6-benzylaminopurine (BAP). Semi-solid (with agar) and liquid MS medium was used for the multiplication of shoots *in vitro*. Combined effect of BAP and kinetin in shoot multiplication was reported favorable in semi-solid (10.6 ± 0.17 shoots per vessel) and liquid MS medium (11.4 ± 0.47 shoots per vessel). The shoots were rooted *in vitro* on half-strength agar-gelled MS medium supplemented with 1.0 mg/L indole-3 butyric acid (IBA) and 44.30 roots per shoot were observed from the cut ends of the shoots. All the shoots were also rooted by *ex vitro* method and maximum 36.12 roots per shoot were induced by treating the shoots with 300 mg/L IBA for 5 min. The *in vitro* rooted and *ex vitro* treated plantlets were transferred to the soilrite containing paper cups for hardening in the greenhouse. The plantlets were shifted to nursery bags after 4 weeks. Finally the hardened plantlets were planted in the field with 100% rate of survival under natural conditions. The *ex vitro* rooting was reported more advantageous than *in vitro* rooting in terms of cost, time and percent survival of plantlets.

1. Introduction

Morinda citrifolia L. (Indian mulberry or Noni) belongs to the family Rubiaceae, is an important medicinal, economically valuable and dye yielding plant. It is native to South-East Asia to Australia (Dixon et al., 1999; Fleming, 2000). The plant is growing in the south India and the Andaman and Nicobar Islands of India. Noni is a small tree or large shrub and grows in dry and wet areas, acidic as well as in alkaline soils, and even in infertile soil conditions.

The fruits of *M. citrifolia* find place in ancient medicinal texts of Ayurveda and Siddha in India. It is a traditional remedy to treat broken bones, deep cuts, bruises, sores and wounds in India (Rethinam and Sivaraman, 2007). Noni has reported to have a broad range of therapeutic values against cancer, infection, arthritis, diabetes, asthma, hypertension and pain (Kamavalli and Rao, 1997; Dixon et al., 1999; Yanine et al., 2006). The fruit is a natural product and has a broad range of immune-enhancing effects, smooth muscle stimulatory activity, histaminergic effects, antibacterial, antiviral, anthelmintic anti-tubercular, anti-inflammatory, analgesic, antioxidant and hypotensive effects (Wang and Su, 2001; Wang et al., 2002; McClatchey, 2002; Furusawa et al., 2003). The root, stem, bark, leaves, flowers, and fruits of the Noni plant are involved in various combinations in almost 40 known and recorded herbal remedies. Additionally, the fruits were eaten as food supplement to improve health (Abbott, 1992).

The primary commercial products from Noni include beverages (fruit juice, juice drinks), fruit powders (for manufacture of reconstituted juice or juice drink products made from dried ripe or unripe fruits), toiletries (lotions, soaps, *etc.*), oil (from seeds), and leaf powders (for encapsulation of pills) (Wang et al., 2002).

The roots and root bark of *M. citrifolia* have been used to get dye (yellow/red anthraquinone) to color traditional and ceremonial clothes by Polynesians for over 2000 years (Nelson, 2001; McClatchey, 2002; Palu et al., 2004). Noni is used in India to make the morindone dye, which is sold under the trade name "Suranji". Morindone is used for the dyeing of cotton, silk and wool in the

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shades of red, chocolate or purple. The coloring matter is found principally in the root bark and collected when the plants reach four to 5 years of age. The active substance is extracted as the glucoside known as morindin, this upon hydrolysis produces the dye (Singh and Tiwari, 1976).

The biodiversity of flora on the earth are abolishing day-by-day due to over population, urbanization and industrialization (Arora and Bhojwani, 1989; Purohit and Tak, 1992; Sudha and Seeni, 1994). Since, the root bark of Noni contains dye, the well grown plants are generally uprooted, and due to this activity the entire population of *M. citrifolia* is facing threat in natural conditions (Das and Mondal, 2012; Sharma and Thokchom, 2014).

Noni plants are conventionally propagated through seeds or by stem or root cuttings. However, seeds have a problem of seed dormancy/hard seed coat (water repellant) thus limiting its commercial cultivation and the survival of cuttings was only 83.3% in Noni propagation (Chandra and Sagar, 2013). However, the planting of extensive new orchards of vegetatively propagated clones of trees has sometimes been limited by pathogens. Furthermore, propagation by root cutting in majority of trees is often characterized by a rapid loss of rooting capacity of the cutting with increasing age of parent plant (Rai et al., 2010). Further, there is relatively high degree of genetic and morphological variability in the fruits and leaves within the species of *M. citrifolia* (Wang et al., 2002).

Plant tissue culture technique is an alternate source of mass cultivation of rare, endemic, threatened and endangered plants (Sahoo and Chand, 1998; Prakash et al., 1999). Micropropagation has reported for *in vitro* mass propagation of many economically and medicinally valuable plants (Pattnaik and Chand, 1996). Subramani et al. (2007) and Wei et al. (2006) studied the effect of plant growth regulators on *M. citrifolia*. Gajakosh et al. (2010) reported organogenesis from shoot tips and leaf explants of Noni and, Sreeranjini and Siril (2014) multiplied shoots of *M. citrifolia in vitro*. Most of the earlier *in vitro* work on *M. citrifolia* were conducted with embryos or explants collected from seedlings. Therefore, it is an urgent need to develop a rapid plantlet regeneration protocol from mature plant of *M. citrifolia* for providing continuous supply of better source of elite plant to be used as standard material in the field of drug research as well as in manufacturing of drugs. An improved micropropagation method has been developed using agar gelled and liquid medium and the shoots were rooted efficiently using *in vitro* as well as *ex vitro* methods in present investigation.

2. Material and methods

2.1. Explant sterilization, medium and culture conditions

The selected species *M. citrifolia* was collected from Shakthi Auro, Pondicherry, East-Coast Region of South India. The fresh sprouts were collected from 4 year old mature and healthy plant. The fresh and young nodal segments (2–3 cm long) with 1–2 nodes were used as explants. The explants were cleaned, dressed and washed with help of 0.1% Tween® 20 (Sigma–Aldrich, India) for five min. These were surface sterilized with 0.1% (w/v) Bavistin solution (Systemic fungicide, BASF India Ltd.) for 10 min and then with 0.1% (w/v) $HgCl_2$ solution for 5–6 min in the laminar hood. The sterilized explants were washed with autoclaved distilled water for 7–8 times.

MS basal medium (Murashige and Skoog, 1962) was used for the present study. Agar-gelled (0.8% agar) and without agar (liquid) MS medium was prepared with help of macro and micro nutrients, amino acids, vitamins and sucrose (3%) as sources of carbon. Cultures were maintained at $25 \pm 2\,^{\circ}C$ and 40–50 $\mu mol\,m^{-2}\,s^{-1}$ Spectral Flux Photon (SFP) light intensity under 12:12 h light:dark photo regimes.

2.2. Establishment and multiplication of cultures

The sterilized nodal explants were inoculated vertically on the medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and kinetin (ranging from 1.0–6.0 mg/L) alone or in combination with IAA (0.1–1.0 mg/L). The regenerated shoots from the nodal meristems of explants were further multiplied on MS semi-solid (with agar) as well as in liquid media (without agar) in 250 mL culture flask. The media were fortified with BAP and kinetin (0.1–3.0 mg/L) and IAA (0.1–1.0 mg/L) for multiplication of shoots in cultures. Each flask bearing one regenerated shoot allowed for 5 weeks of incubation in growth room. The number and length of the shoots were measured after 3rd and 4th cycle of shoots multiplication.

Experiments were also conduced to test the effect of liquid medium (without agar) on the growth and development of shoot (shoots number and length) in cultures and to reduce the cost of production of shoots with same concentrations of plant growth regulators and culture conditions. The cultures with liquid medium were kept on gyratory shaker at 90 rpm without filter paper on any other type of support.

2.3. Induction of roots from the shoots in vitro

For root induction, long (4–5 cm) and healthy shoots were selected from the multiplication stage of the cultures. The unwanted parts were removed from the shoots and placed on the semi-solid full strength, half- strength and 1/4th strength MS medium augmented with indole-3 butyric acid (IBA) and indole-3 acetic acid (IAA) concentrations (ranging from 0.5 to 5.0 mg/L). The cultures were maintained under normal light conditions for 2 days for pre root induction and then maintained at $25 \pm 2\,^{\circ}C$ temperature in diffused light (15–20 $\mu mol\,m^{-2}\,s^{-1}$ SFP) field for 5 weeks. After 5 weeks, the *in vitro* rooted plantlets were carefully taken from the culture tubes and washed with sterilized distilled water. The response (%) of root induction, number and length (cm) of roots and callus significance were measured.

2.4. Ex vitro rooting of the shoots

Experiments were carried out for *ex vitro* induction of roots from the shoots of *M. citrifolia*. Rooting and hardening together could be possible using this method. The cut ends of the *in vitro* raised shoots (3–4 mm) were dipped in different concentrations of IBA, IAA or α-naphthalene acetic acid (NAA) (50–500 mg/L solutions) for 5 min and then transferred to eco-friendly paper cups (size 150 mL; Vandana Paper Products, Chennai, India) containing 55 gm sterile Soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India). These were moistened with 10 mL of one-forth strength of MS basal salts by the interval of 1 week in the greenhouse. The cups were covered with transparent disposable plastic cups (size 200 mL; Swastik PolyPack, Chennai, India). These were kept in the greenhouse and maintain at $25 \pm 2\,^{\circ}C$ temperature.

2.5. Hardening and acclimatization of plantlets

The *in vitro* rooted plantlets were removed from the culture vessels and washed with sterilized distilled water to remove the adhered nutrient agar from the roots. These plantlets were planted in paper cups containing Soilrite® and covered with transparent plastic cups. The rooted plantlets were maintained in the greenhouse for 5 weeks. The plantlets were transferred to nursery bags contains red soil, garden soil, Soilrite® and Vermi-compost (1:1:1:1) after 5 weeks. These were maintained in the greenhouse