



***In vitro* regeneration of *Moringa oleifera* Lam.: A medicinal tree of family Moringaceae**

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(Received: May 2019; Revised: August 2019; Accepted: August 2019)

Abstract

A protocol is developed for somatic embryogenesis and organogenesis from nodal segments and shoot-apices of *Moringa oleifera* Lam. Callusing and multiple shoots induction occurred in nodal segments (0.5-1.0 cm) after 2 weeks of culture on Woody Plant Medium, 1981 (WPM) containing N⁶-benzyladenine (BA), alone or in combinations with various concentrations of α -naphthalene acetic acid (NAA). The concentration of BA at 4.44 μ M was found optimum for multiple shoots formation, resulting in 3.22 \pm 0.17 shoots per explants. WPM was enriched by adding certain additives to minimize yellowing and shedding of leaves in proliferated shoots. Maximum roots induction (86.6%) was observed in enriched WPM containing 2.46 μ M IBA. Somatic embryos were developed from the calli obtained from nodal segments on enriched WPM supplemented with IBA and BA. Histological studies were carried out for studying the pattern of somatic embryogenesis. Multiple shoots formed from shoot apices (1.0-1.5 cm) cultured on enriched WPM (with or without IBA) after 2 weeks of culture. Root induction occurred simultaneously. The regenerated plantlets were gradually transferred to half strength and later to quarter strength liquid WPM, for 10 days, before transferring to pots. Approximately, 70% survival rate was recorded from *in vitro* grown plantlets on transfer to pots. Somatic embryogenesis in *M. oleifera* from nodal explants is reported for the first time in this communication.

Key words: Drumstick, embryogenesis, *in vitro* propagation, nodal segments, organogenesis, shoot induction.

Introduction

Moringa oleifera Lam. (syn. *M. pterygosperma* Gaertn), commonly known as horseradish tree, drumstick, the miracle tree, ben oil tree, malunggay is a widely cultivated tree species of monogeneric family

Moringaceae (Olson 2002). It is a tropical, soft wooded, fast growing, perennial tree, found commonly in the western and sub-Himalayan areas of the Indian subcontinent (Wealth of India 1962; Morton 1991; D'Souza and Kulkarni 1993). It is a natural source of nutrition in various parts of the world (Palada 1996). Flowers and fruits of this species are sources of amino acids, glucose, sucrose, citric acid, mallic acid, succinic acid, fumaric acid and oxalic acid. Flowers are used as stimulant, aphrodisiac, abortifacient, and cholagogue. Leaves are a good source of α -, γ - and δ tocopherols, quercetin, beta-sitosterol, caffeoylquinic acid, and kaempferol. All parts of the plants are useful in treating hematological and hepatorenal disorders, gastrointestinal infections and cardiovascular diseases. *Moringa* has robust pharmacological properties (D'Souza and Kulkarni 1993; Gebhardt et al. 2008; Sultana and Anwar 2008; Choudhary et al. 2016). It has been used in fighting malnutrition also. Thus, this species has tremendous potential for commercial utilization (Salem 2016).

Propagation of plants/trees through tissue culture method using different ex-plant has long been practiced for conservation of plants including endangered species and for enhancing selection efficiency of desirable traits at cellular level (Bhojwani and Rajdan 1996; Dang et al. 2011; Huynh et al. 2017; Heidary et al. 2018; Pandey et al. 2019). *M. oleifera* is traditionally propagated by cuttings or seeds. Seedling propagated plants show reduced growth rate and vary in phenotype as well as genotype. One of the approaches to easily cultivate this species is through tissue culture or micropropagation technique

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(Stephenson and Fahey 2004). Somatic embryogenesis applied to secondary metabolites producing plants has been directed either to massive propagation of quality materials or one of the strategies used for genetic improvement (Loyola et al. 2016). Efficient methods for plant production via somatic embryogenesis have been worked out for many medicinal and tree species such as *Dalbergia sissoo* (Singh et al. 2002; Singh and Chand 2003), *Hardwickia binata* (Chand and Singh 2001), *Pterocarpus marsupium* (Chand and Singh 2004), *Cunninghamia lanceolata* (Zhou et al. 2017) and *Pinus sylvestris* (Abrahamsson et al. 2017). Stephenson and Fahey (2004) have observed multiple shoot formation from nodal segments in *M. oleifera* and *M. stenopetala*. Gayathri et al. (2015) studied the effect of cyanobacterial extracellular product on *in vitro* regeneration from nodal explants in *M. oleifera*. *In vitro* shoots regeneration and multiplication from shoot tips and axillary meristem in *M. oleifera* have been reported earlier (Islam et al. 2005; Forster et al. 2013; Avilla-Trevino et al. 2017). However, somatic embryogenesis has not been reported in this species from vegetative explants. Devendra et al. (2012) reported somatic embryogenesis from zygotic embryos in this species. But, no histological evidences for embryogenesis provided. The broad research and endeavour for commercialization of drumstick tree and its items has prompted a critical requirement for its preservation from dietary, pharmacological, ethnobotanical and biotechnological perspective (Gupta et al. 2018). Therefore, a study was conducted to develop a reproducible protocol for somatic embryogenesis and multiple shoot proliferation from nodal and shoot apices of *M. oleifera*.

Materials and methods

Plant material, sterilization and explant preparation and culture conditions

Mature dried pods of *M. oleifera* were hand-plucked from the tree grown in Devi Ahilya University campus, Indore. Seeds were isolated from the soaked (distilled water for 24 h) pods, followed by washing three to four times in distilled water. Isolated seeds were treated for 15 min with 0.1% (v/v) tween-20 solution, followed by with 20% sodium hypochlorite solution (Hi-media). Seeds were rinsed with 70% (v/v) ethanol for 1 min followed by washing with distilled water. Final sterilization was carried by using 0.1% (w/v) mercuric chloride solution for 20 min and washing with sterile distilled water.

Water agar medium (0.8%, pH 5.8) was used to germinate 4-5 sterile seeds per plate (100 mm x 25 mm). The plates were incubated at 24-28°C with a relative humidity 55-65%, light intensity of 40 mmol m⁻² s⁻¹ and 16-h light/8-h dark period. Germinated seedlings, 5-7 days old were transferred to WPM basal medium. After 20 days, nodal segments and shoot apices were excised and used as explants. Cultures were transferred to a fresh medium after every 3 weeks.

Callus induction and shoot proliferation from nodal explants

Nodal segments (0.5-1 cm) from 20-day-old seedlings were cultured on WPM supplemented with various concentrations of BA (1.11, 2.22, 3.33 or 4.44 µM) alone or in combination with NAA (2.64 or 5.28 µM). Each treatment consisted of fifteen explants and all the experiments were repeated thrice. After 40-45 days of culture shoot proliferation (no. of shoots/explant) was observed. *In vitro* regenerated shoots (2.5-3 cm) were transferred to WPM enriched with additives like silver nitrate, charcoal, proline and lysine for elongation and proliferation. The mean value for shoot length was observed before transfer to the rooting medium (enriched WPM containing IBA 1.23-4.92 µM).

Protocol for minimizing yellowing and shedding of leaves in proliferated shoots

Yellowing and shedding of leaves from regenerated shoots derived from nodal explants were observed during subsequent subcultures. To overcome the problem, the medium was enriched by supplementing activated charcoal (500 mg L⁻¹), silver nitrate (5 mg L⁻¹), proline (1035 mg L⁻¹), m-inositol 200 mg L⁻¹, glycine 4 mg L⁻¹, along with double concentration of vitamins (pyridoxine HCl 1 mg L⁻¹, thiamine HCl 2 mg L⁻¹, nicotinic acid 1 mg L⁻¹).

Somatic embryogenesis from nodal explants

After 4 weeks of transfer of nodal cultures on enriched WPM supplemented with IBA (1.23, 2.46 or 4.52 µM) and/or BA (1.11 or 2.22 µM), the callus from nodal explants were observed for the presence of somatic embryos. The somatic embryos obtained were maintained either on the same medium or on WPM basal medium. The nodal segments showing direct somatic embryogenesis on the inoculation medium were maintained on the same medium for 2 passages. The germinated somatic embryos, both direct and indirectly developed embryos were transferred to

enriched WPM supplemented with NAA and BA for shoots proliferation. Some of the nodal explants directly formed shoots and somatic embryos simultaneously. The calli excised from the basal portion of *in vitro* regenerated shoots were cultured on enriched WPM containing PGRs (Plant Growth Regulators), at lower concentrations, IBA (1.23 μM) in combination with BA (1.11 or 2.22 μM).

Multiple shoots formation, elongation from shoot apices, root formation and acclimatization

Shoot apices (1.5-2 cm) were inoculated on enriched WPM containing NAA alone (2.64 or 5.28 μM), or in combination with BA (1.11, 2.22, 3.33 or 4.44 μM) to induce multiple shoot proliferation. For each experiment minimum fifteen shoot apices were used and the experiments was repeated thrice.

Regenerated shoots, 3-4 cm long, having 2-3 nodes and a minimum of 10-12 leaves were selected for *in vitro* root induction. These shoots were cut off at the base and transferred to enriched WPM containing either IBA (1.23, 2.46 or 4.52 μM) or IAA (2.85 or 5.70 μM) for root induction. In case of multiple shoots proliferation from shoot apices, the individual shoots were cut off from the plantlets and were transferred to enriched WPM containing either IBA (1.23, 2.46 or 4.52 μM) or IAA (2.85 or 5.70 μM) for root induction. Rooted plants were shifted to half strength and then to quarter-strength liquid WPM for 10 days, for each treatment. Plants with well developed roots systems (4-5 cm) were transferred to plastic pots filled with autoclaved soil and compost mixture in equal amount (1:1). These pots were covered with perforated polythene to maintain humidity. Plants were subsequently transferred to polybags (25 cm diameter X 40 cm height) containing soil, peat moss and compost mixture (1:1:1). Plant survival was assessed after one month of transfer.

Data recording and analysis

The experiments were completely randomized design (CRD) with three replicates of each experiment and at least fifteen explants per replicate. All the experiments were repeated three times. Percentage response for callus formation was recorded after 2 weeks of inoculation. The percentage of explants forming shoots was recorded during 3rd and 5th week of culture and mean number of shoots were determined after 5th weeks of culture. Percent root formation was recorded during 7-9 weeks of culture. Data were analyzed with one-way ANOVA using 5.01

Graphpad Prism software (*GraphPad Software Inc., La Jolla, CA, USA*). Results were analyzed for mean, standard error values, significant differences, using Student- Newman-Keuls Multiple Comparison Test: compare all pairs of a column. Significance was determined at $P \leq 0.01$ (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$). Results were analysed in terms of mean and standard blunder esteems for each analysis pursued by huge contrasts and essentialness was resolved at $P \leq 0.01$.

Histological studies

The histology of nodular embryogenic calli was performed to study the morphogenesis and development of differentiating cell masses. For histological examinations, the embryogenic calli were fixed in mixture of formaldehyde/glacial acetic acid/ ethanol (FAA, 5: 5: 90, v/v/v) for 24 h, dehydrated through a graded tertiary butyl alcohol (TBA) series, for 24 h each and embedded in saturated paraffin wax. Embedded material were sectioned at 5-7 μM thickness with rotary microtome. Paraffin wax was expelled by xylene preceding tissues rehydration in a graded ethanol series and staining the tissue with 1.0% (w/v) safranin. Tissues were washed in water to evacuate excess stain and subsequently dehydrated in a graded ethanol series. Photos were taken with a binocular Labovision E GLASS (Germany) compound microscope.

Results and discussion

Shoots proliferation from nodal explants

New shoots emerged from the nodal segments cultured on medium fortifying with 1.11, 2.22, 3.33 or 4.44 μM BA, either alone or with 2.64 or 5.28 μM NAA. Bud break was observed from nodal segments after 20-25 days of culture. Differentiating clumps were observed on the swelled nodal explants within 3 weeks of culture (Fig. 1A and B). These small green protuberances elongated on transfer to WPM basal medium containing BA alone or in combination with NAA and number of shoots per explants increased in subsequent days. After 10-15 days of transfer of nodal cultures to enriched WPM containing BA 4.44 μM , maximum response for shoot proliferation (73.33 ± 1.15) and the elevated mean number of shoots per explants (3.22 ± 0.17) were recorded (Fig. 1 C-E). BA was found most suitable cytokinin for bud break in our study. Addition of NAA to the media along with BA at higher concentration was found to impede mean value of shoots per explant in this species. Shoot lengths were

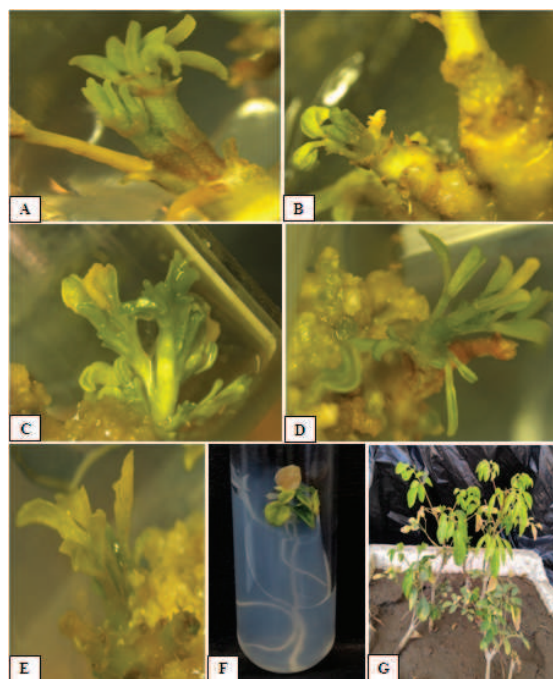


Fig. 1. Multiple shoots formation from nodal segments and roots development in *in vitro* regenerated shoots of *M. oleifera*. (A-B) *In vitro* shoots regeneration from nodal segments on WPM containing 4.44 μM BA; (C-E) Multiple shoots regeneration from nodal derived callus on enriched WPM containing BA alone along with 2.64 μM NAA; (F) Roots differentiation in *in vitro* regenerated shoots; g. *In vitro* regenerated plantlets transferred to pots

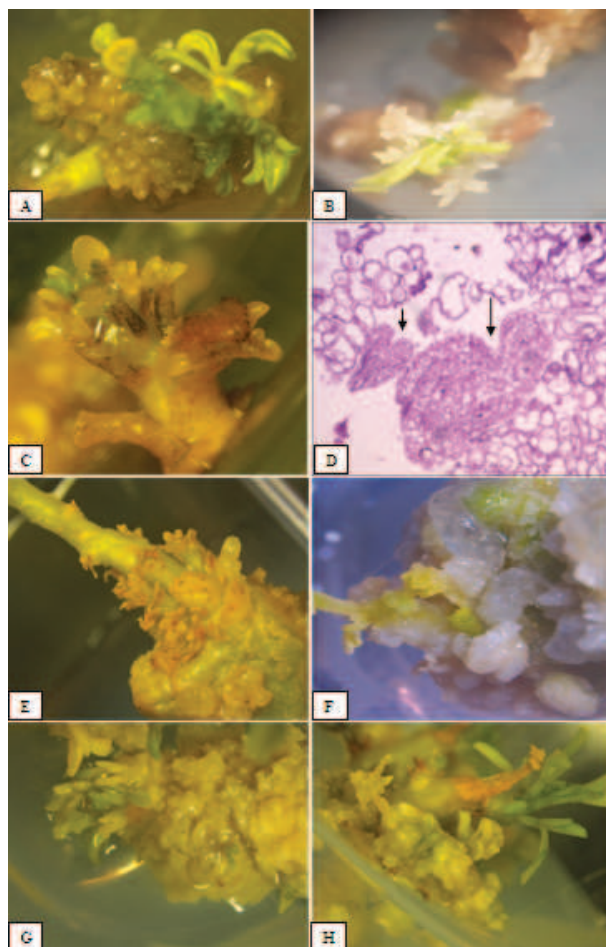


Fig. 2. Somatic embryogenesis from nodal segments in *M. oleifera*. (A-C) Direct somatic embryogenesis along with organogenesis from nodal segments on WPM containing 1.23 μM IBA and 1.11 μM BA; (D) Longitudinal section showing initiation of shoots and somatic embryo in five weeks old cultures; (E) Somatic embryogenesis along with organogenesis at the basal portion of *in vitro* regenerated shoots on enriched WPM containing only 4.44 μM BA; (F) Somatic embryos formed from calli on enriched WPM containing BA (4.44 μM); (G-H) Nodular calli and somatic embryos formation in repeated subcultures

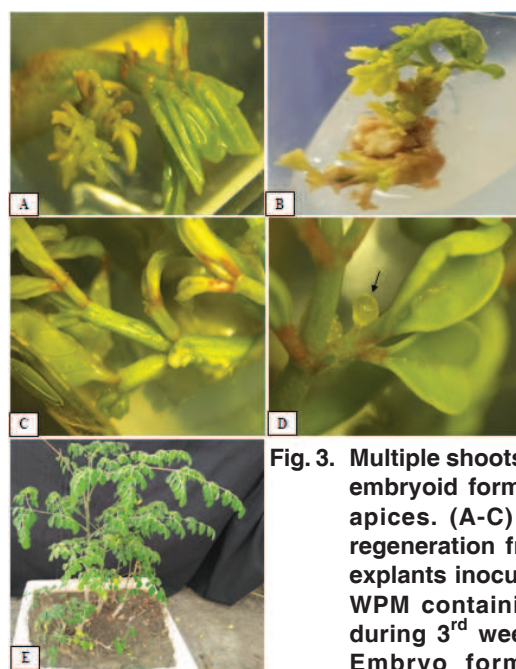


Fig. 3. Multiple shoots proliferation and embryoid formation from shoot apices. (A-C) Multiple shoots regeneration from shoot apices explants inoculated on enriched WPM containing NAA and BA during 3rd week of culture; (D) Embryo formation on WPM without growth regulators; (E) Plantlets transferred to pots

recorded after 8th weeks of culture and maximum length of shoots (3.38 ± 0.18) were observed on enriched WPM supplemented with 4.44 μM BA (Table 1).

Somers et al. (2003) observed that a higher concentration of BA in general, inhibits further development and growth of shoot buds upon continued proliferation of explants on the same medium. Lower concentrations of BA

Table 1. Effect of different PGRs at various concentrations on shoot regeneration and mean number of shoots/explants from nodal segments (after 35 days of culture) of *M. oleifera*. One way ANOVA statistical analysis (P dⁿ 0.05) was performed (mean ± SE)

Growth regulators in enriched WPM (µM)	% responding explants ±S.E.	Mean no. of shoots/explants ±S.E.	Shoots length (cm)±S.E.
BA (1.11)	21.42±0.33 ^a	1.17±0.16 ^a	1.75±0.50 ^a
BA (2.22)	31.56±0.58 ^b	1.19±0.12 ^a	1.98±0.39 ^b
BA (3.33)	67.27±0.88 ^c	2.98±0.27 ^b	2.25±0.07 ^c
BA (4.44)	73.33±1.15 ^d	3.22±0.17 ^c	3.38±0.18 ^d
NAA (2.64)+ BA (4.44)	71.51±0.57 ^e	2.94±0.26 ^d	2.13±0.03 ^b
NAA (5.28)+ BA (4.44)	67.44±0.57 ^f	2.28±0.08 ^e	2.10±0.07 ^b

Values are mean ± S.E of three independent experiments, each with a minimum of 15 replicates. Mean number followed by the same letters in each column do not differ significantly from one another (P ≤ 0.05) according to Student-Newman-Keuls Multiple Comparison Test

and addition of auxin, such as NAA resulted in multiple shoot proliferation. Marfori (2011) examined the effect of various cytokinins in inducing bud break from nodal explants, namely BA, Kinetin (Kn), thiadiazuron (TDZ) and reported BA (5.28 µM) to be optimal (4.6 axillary shoots per explants) in inducing bud break from nodal explants in *M. oleifera*.

Direct embryogenesis from nodal explants

Nodal segments cultured on enriched WPM medium containing 1.23 µM IBA and 1.11 µM BA, developed globular somatic embryos without any intervening callus stage. These somatic embryos developed in clusters and about 30-35 embryos per nodal segment were observed (Fig. 2A-C). An interesting observation was recorded that germination of somatic embryos into shoots along with proliferation of somatic embryos occurred repeatedly and simultaneously in cultures. Secondary somatic embryogenesis was also observed on the same medium. Formation of somatic embryos was confirmed by histological studies, showing their development from the epidermal cells (Fig. 2D). Longitudinal section displayed an apical tip with two cotyledons. Study of serial longitudinal sections of torpedo-shaped embryos demonstrated a bilateral symmetry with cotyledon formation. Variation in the type and concentration of the plant growth regulators

in the medium direct the explants to follow various routes of organogenesis or embryogenesis. The signaling molecule controls plant morphogenesis via its activity gradients, by intercellular auxin transport (Mravec et al. 2008). Direct embryogenesis generally occurs in the cultures from immature embryos. The explants capable of direct embryogenesis seem to carry pre-embryogenic determined cells (PEDC). These induced cells can be maintained and multiplied in the embryonic state under appropriate culture conditions (Bhojwani and Razdan 1996).

Organogenesis and embryogenesis from calli developed from nodal segments

Callus formation was observed at the basal portion of nodal explants. Response varied with respect to the form of callus developing on the culture medium with respect to change in concentration of plant growth regulators (PGRs). The callus formed was distinguishable on the basis of texture and color, into type I – white compact callus, granular in texture and type II - greenish white and compact, nodular calli (Table 2). During the 6th week of culture of nodal segments on enriched WPM fortified with 4.44 µM BA alone or in combination with NAA, the compact nodular calli formed at the basal portion of nodal segments which turned to golden yellow. These organized calli

Table 2. Effect of different PGRs on the percentage response for callus formation from nodal segments inoculated on WPM. One way ANOVA statistical analysis (P dⁿ 0.05) was performed (mean ± SE).

Growth regulators in WPM (µM)	% Callus induction	Type of callus
BA (1.11)	51.11 ± 0.333 ^a	White friable, granular
BA (2.22)	62.22 ± 0.882 ^b	White & Compact
BA (3.33)	73.33 ± 0.577 ^c	White & Compact
BA (4.44)	100.00 ± 0.00 ^d	White & Compact, Nodular
NAA (2.64) + BA (4.44)	93.33 ± 0.577 ^e	Greenish White, Compact, Nodular
NAA (5.28) + BA (4.44)	80.00 ± 0.577 ^f	Greenish White, Compact, Nodular
NAA (2.64)	42.22 ± 0.33 ^g	Green and compact
NAA (5.28)	33.33 ± 1.0 ^h	Green and compact

Values are mean ± S.E of three independent experiments, each with a minimum of 15 replicates. Different letters represent significant difference (P dⁿ 0.05) according to Student-Newman-Keuls Multiple Comparison Test

eventually developed into nodular embryogenic structures (NES) on subsequent transfer. From this organised calli, shoots and somatic embryos developed. Somatic embryos were globular, cotyledonary and heart shaped. Proliferation of shoots, and embryogenesis occurred simultaneously on medium containing 4.44 μM BA (Fig. 2E) or IBA 1.23 μM along with BA 1.11 μM (Fig. 2F). It was observed in the cultures quite often that calli developed from the nodal explants, formed somatic embryos and these embryos if continued to be maintained on WPM containing 1.23 IBA μM and 1.11 μM BA, revert back to nodular calli and formed somatic embryos on the medium enriched with 1.23 IBA μM and 1.11 μM BA (Fig. 2G and H).

We observed embryogenesis and shoots multiplications simultaneously from nodal segments and also from the calli in *M. oleifera*. Embryo initiation in plants is not restricted to the fertilized egg cell or zygote (Radoeva and Weijers 2014). Embryogenesis may be initiated from microspores, pollen grains as well as from somatic cells, tissues or organs (Feher 2015, 2016). Small differentiating cell patches may opt the pathway of organogenesis and others may prefer to become embryogenic and even other cells may not re-differentiate and continue to retain callus status (Loyola et al. 2016).

Shoot proliferation from shoot apices

Bud proliferation occurred when shoot apices were inoculated on WPM basal medium supplemented with NAA 2.64 or 5.28 μM along with 4.44 μM BA (Fig. 3A) after 20-22 days. Compact white calli formed at the basal portion of cultured explants (Fig. 3B). Enriched WPM supplemented with NAA (2.64/5.28 μM) along with BA (4.44 μM) led to induction multiple adventitious shoots (Fig. 3C). On an average, 12-15 adventitious shoot buds were formed on medium containing 2.64 μM NAA along with 4.44 μM BA. Globular embryos also developed on the basal portions of leaf axils occasionally (Fig. 3D) after 2-3 weeks. In *M. oleifera*, yellowing of leaves and leaf fall from regenerated plantlets were observed frequently. It was controlled by adding activated charcoal (500 mg L^{-1}), silver nitrate (5 mg L^{-1}), proline 1035 mg/L and by doubling additives such as m-inositol 200 mg L^{-1} , pyridoxine HCl 1 mg L^{-1} , thiamine HCl 2 mg L^{-1} , nicotinic acid 1 mg L^{-1} , glycine 4 mg L^{-1} in WPM. After 3 weeks of culture on the enriched WPM, shoots (3-5 cm) were shifted to medium for roots proliferation. Stephenson and Fahey (2004) reported clonal propagation in *M. oleifera* and

M. stenopetala from seeds and observed 4.7 shoots per cultured seeds on MS medium enriched with 4.44 μM BA along with 2.88 μM GA_3 . Saini et al. (2012) reported production of 9.0 ± 1.0 shoots per nodal explants on MS medium supplemented with BA (4.44 μM).

Root formation and acclimatization

Various concentrations of IAA (1.42-2.85 μM), IBA (1.23-4.92 μM) alone or in combination with BA (1.11-2.22 μM) and enriched WPM without growth regulators were used for roots induction of *in vitro* regenerated shoots derived from nodal segments. Roots formation occurred in WPM supplemented with IBA 1.23 or 2.46 μM (Fig. 1F). Maximum roots (86.66 \pm 0.33%) were observed in *in vitro* regenerated shoots in enriched WPM containing IBA 2.46 μM followed by 73.33 \pm 0.33% roots formation in enriched WPM enriched with IBA 1.23 μM . Presence of BA was found inhibitory for roots induction from *in vitro* regenerated shoots. Enriched WPM without any PGRs was also used for roots induction in proliferated shoots.

Shoot apices inoculated on enriched WPM with or without IBA (1.23-4.92 μM) resulted in direct roots regeneration. Roots formation were observed in enriched WPM without any plant growth regulator and also on medium supplemented with IBA 1.23 or 2.46 μM . Maximum roots induction (60.00 \pm 0.57%) were observed in shoot apices cultured on WPM containing IBA 1.23 μM . The roots regeneration frequencies decreased with increase in IBA concentration in the medium. Effect of media and growth regulators on roots formation from shoot apices and microshoots from nodal segments on enriched WPM are summarized in Table 3. Plants with roots (3-4cm in length) were acclimatized and transferred to pots containing

Table 3. Effect of growth regulators on roots formation in microshoots derived from nodal segments and shoot apices of *M. oleifera* on enriched Woody Plant Medium. Values are mean \pm S.E of three independent experiments, each with a minimum of 15 replicates

IBA (μM)	% rooting \pm S. E.	
	Nodal segments	Shoot apices
0.00	0.00	33.33 \pm 0.33
1.23	73.33 \pm 0.33	60.00 \pm 0.57
2.46	86.66 \pm 0.33	40.00 \pm 0.00
4.92	—	13.00 \pm 0.33

autoclaved mixture of soil, sand and manure (2:1:1). Survival rate of regenerated plantlets on transfer to pots was nearly 70 % (Fig. 3E). The response of differentiation of nodal explants varied when the proliferated shoots were transferred to enriched WPM. Multiple branching in proliferated shoots was observed on enriched WPM containing NAA and BA. Somatic embryogenesis was also achieved in nodal derived calli when transferred to WPM containing IBA 1.23 μM + BA 1.11 μM . Roots induction were accomplished on medium containing 2.46 μM IBA. Results of this study revealed that rapid propagation of *M. oleifera* by tissue culture method is executable and several plantlets can be regenerated from one nodal and shoot apices explants.

This is the first report on organogenesis and somatic embryogenesis from shoot and nodal apices of *M. oleifera* an important medicinal plant belonging to family Moringaceae.

Authors' contribution

Conceptualization of research (SC, AP); Designing of the experiments (AP, OV); Contribution of experimental materials (SC); Execution of field/lab experiments and data collection (AP, OV); Analysis of data and interpretation (SC, AP, OV); Preparation of manuscript (SC, AP, OV).

Declaration

The authors declare no conflict of interest.

Acknowledgement

Authors are thankful to University Grants Commission, New Delhi for providing financial assistance for this work under UGC-CAS (SAP) Program.

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