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In Vitro Shoot Organogenesis and Regeneration of Plantlets from Nodal Explants of *Murraya koenigii* (L.) Spreng. (Rutaceae), a Multipurpose Aromatic Medicinal Plant

Shiwali Sharma • Anwar Shahzad* • Mohammad Anis

Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202 002, India

Corresponding author: * **shahzadanwar@rediffmail.com**

ABSTRACT

A protocol for high frequency shoot organogenesis and plantlet establishment has been developed through nodal explants of *Murraya koenigii*. The response of horizontally or vertically placed explants on culture media was also evaluated. Among 2 orientations of nodal explants, vertical placement was more responsive for shoot induction and multiplication than horizontal placement. Between 2 cytokinins, 6-benzyladenine (BA) and kinetin (KIN) tested, BA (5.0 µM) induced a maximum of 6.60 ± 0.40 shoots/explant. The supplementation of α-naphthalene acetic acid (NAA) with BA did not improve regeneration efficiency. Growth in the regenerating tissue was improved significantly within the same incubation period at an optimal concentration of BA (5.0 µM). An improvement in shoot multiplication was noticed in subsequent passage of subculturing up to the 3rd subculture passage with a maximum of 15.20 ± 0.37 shoots/explant recorded. *In vitro* rooting in microshoots was best obtained on half-strength MS medium supplemented with indole-3-butyric acid (IBA) (1.0 µM) on which 90% of cultured microshoots produced healthy roots. Plantlets that developed were hardened and 95% of plants established in natural soil showed normal morphological characteristics.

Keywords: explant orientation, *in vitro* rooting, micropropopagation, microshoot, plantlet establishment

INTRODUCTION

Murraya koenigii (L.) Spreng. (Rutaceae), commonly known as curry leaf tree, is an aromatic medium-sized tropical tree native to India, distributed in hot and damp places. It is a multipurpose tree. Every part of the plant is very useful, but cultivated mainly for its aromatic leaves which are the rich source of vitamin A, B and C, minerals, carbohydrates, proteins, amino acids and alkaloids. Leaves used as a good source of spice. As a reputed herbal medicine in the Ayurvedic and Unani system of medicine, the plant is used as a tonic, stomachic, febrifuge, anti periodic, stimulant, anti emetic, anti dysenteric and against bites of poisonous animals and against skin eruptions (Anonymous 2003). Aqueous extract of the plant exhibited a strong anti fungal activity (Pandey and Dubey 1997). Freshly prepared aqueous extract of the leaves may be prescribed for dietary therapy and drug treatment for controlling diabetes mellitus and in the treatment of gastrointestinal problems. The root juice can be used to relieve pain associated with kidneys (Anonymous 2003; Shah and Juvekar 2006). It has antioxidant and antidiabetic activities as well (Khan *et al*. 1995).

Conventional propagation occurs by root suckers. Poor seed viability, low germination rate are the obstacles for propagation of the plant through seeds. Rapid and large scale propagation is a prerequisite to meet the pharmaceutical needs and for effective conservation of this multipurpose medicinal plant. Micropropagation offers a great potential for large scale multiplication of a number of aromatic, medicinal and commercial plant species *in vitro* (Shahzad *et al*. 2007; Sharma *et al*. 2009; Sahai *et al*. 2010). Although micropropagation of *M. koenigii* through axillary bud culture had been reported previously (Bhuyan *et al*. 1997; Rout 2005), but they showed slow rate of shoot proliferation and late root induction along with callusing. So there was an urgent need to establish an efficient protocol for micropropagation of this multipurpose plant. The present communication describes a simple and efficient procedure for the *in vitro* propagation of *M. koenigii* with high frequency shoot proliferation and *in vitro* rooting without callusing from the nodal segments.

MATERIALS AND METHODS

Plant material

Nodal explants (4.0-5.0 cm) were collected from a 10-year old plant of *M. koenigii* growing in the Department of Botany, A.M.U. The source tissues were washed under running tap water for 30 min, followed by soaking in 5% (v/v) Teepol (Qualigens, India) and ascorbic acid, an antioxidant (Qualigens) for 15 min each by continuous stirring, followed by surface sterilization with 70% (v/v) alcohol and 0.1% (w/v) $HgCl_2$ (Qualigens) for 2-3 min under aseptic conditions. After 5-6 rinses with sterilized double distilled water (DDW), explants were cut into small pieces (1.0-2.0 cm) and finally inoculated on culture media either in a horizontal or a vertical orientation.

Media and culture conditions

The basal medium MS (Murashige and Skoog 1962) with 3% (w/v) sucrose (Qualigens) and 0.8% (w/v) agar (Qualigens) was used throughout the experiment. For proper sterilization medium was autoclaved at 121°C, 15 lbi^2 for 20 min. The pH was adjusted to 5.8 using 1 N HCl and 1 N NaOH before adding 0.8% (w/v) agar. The cultures were incubated at 25 ± 2°C, 16-h photoperiod at 50 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by cool white light (Philips, India) and 55 ± 5% relative humidity.

Effect of orientation and culture establishment

To study the effect of orientation on culture establishment, nodal explants were placed horizontally and vertically on agar-solidified MS medium. The MS medium supplemented with various concen-

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trations of cytokinins such as 6-benzyladenine, BA (0.0, 1.0, 2.5, 5.0 and 10.0 µM) and kinetin, KIN (0.0, 2.5, 5.0 and 10.0 µM) either individually or in combination with α-naphthalene acetic acid, NAA (0.5 µM) were investigated to optimize the hormonal requirement for multiple shoot induction through nodal explants. All plant growth regulators (PGRs) were purchased from Sigma-Aldrich, St. Louis, MI, USA.

Multiplication and proliferation for long-term establishment

For further multiplication and long-term maintenance the regenerating tissues (0.5-0.8 cm) grown on optimum treatment were subcultured on the same medium every 4 weeks.

Root induction and plant acclimatization

The regenerated microshoots (3.0-4.0 cm) were transferred to full- and half-strength MS medium supplemented with different concentrations of auxins such as indole-3-butyric acid, IBA, indole-3-acetic acid, IAA and α-naphthalene acetic acid, NAA (0.0, 1.0 and 2.5 µM) individually. The plantlets were transferred to pots containing sterilized Soilrite™ after washing the roots under running tap water to remove agar and kept under high humidity (95%) for an initial 2 weeks. After 4 weeks, acclimatized plantlets were transferred to pots containing garden soil and sand in equal quantities.

Statistical analysis

All experiments were conducted with a minimum of 10 replicates per treatment. The experiments were repeated in triplicate. These were analyzed statistically using SPSS v. 12 (SPSS Inc., Chicago, USA). The significant differences among means were assessed using Tukey's at $P = 0.05$. Results are expressed as the mean ± SE of 3 experiments.

RESULTS AND DISCUSSION

It was evident from *in vitro* studies that inherent capacity of living plant cell to express their morphogenetic potentiality could be released by exogenous stimulus of active chemicals in the ambient medium. Regeneration potential of cultured explants evidently depends upon a large number of factors such as explants orientation, temperature, light, humidity, nutrients and endogenous levels of phytohormones (Yae *et al*. 1987; Reddy and Vaidyanath 1990; Farooq *et al*. 2002). Therefore, an experiment was conducted to compare the effect of orientation of explant on the number and length of proliferated axillary bud.

Effect of orientation and culture establishment

In the present study, of the two explant orientations, the one placed vertically was more responsive to maximum shoot bud induction and multiplication than the one placed horizontally. The responsiveness of the vertically placed explant to adventitious shoot regeneration over the horizontal placement might be because of channelized movement of nutrition through vascular tissues upward. In the horizontally placed explants, the whole surface received nutrients supply which caused callusing and phenolic exudation. This in turn, adversely affected a proper nutritional requirement for the initiation of direct shoot bud organogenesis leading to improper sprouting of axillary buds.

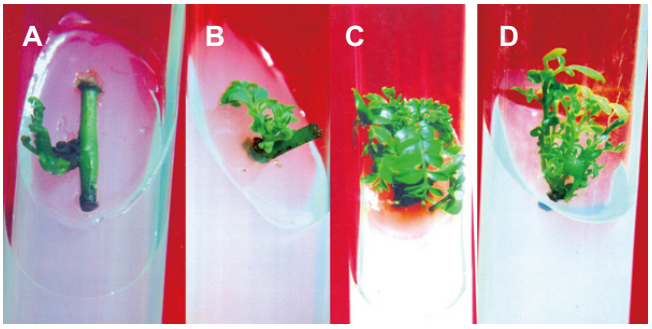

Fig. 1 (**A**) Multiple shoot bud induction from nodal segment placed horizontally on MS medium + BA (5.0 µM) and callusing at the cut end of the explants – 4-weeks-old culture. (**B**) Axillary shoot proliferation from nodal segment placed vertically on MS medium + BA (5.0 µM) - 2 weeks old culture. (**C**) Multiplication of shoots and expansion of leaves from nodal segment placed vertically on MS medium + BA (5.0 µM) – 4 weeks old culture. (**D**) Proliferation of shoots on MS medium + BA (5.0 µM) after 3rd subculture passage – 6-weeks-old culture.

Horizontal placement

The initiation of axenic cultures from horizontally placed explants was difficult due to excessive exudation of phenolic compounds even after presoaking of nodal explants in an antioxidant solution (ascorbic acid) to control leaching of the phenolics in the medium. Nodal explants failed to sprout on basal MS medium without any PGRs. The addition of BA induced a few shoot buds/explant. Initially, nodal segments enlarged and swelled at the axillary region wherein differentiation of shoot buds were noticed which were organized into deformed shoots (**Table 1, Fig. 1A**).

Vertical placement

On MS basal medium the explants failed to induce shoot regeneration, while on all cytokinin augmented media regeneration of shoots occurred. The percentage response, average number of shoot buds/explant, average number of shoots formed/explant and average shoot length varied considerably with the type of growth regulators used at different concentrations (**Table 2**). The explants showed first response by initial enlargement of the explants with axillary bud break within 2 weeks of incubation (**Fig. 1B**). The optimum concentration for shoot bud induction and regeneration of shoots for each cytokinin was different. In the present study, 5.0 µM BA was more effective as induced 6.60 ± 0.40 shoot buds/explant with healthy growth (in shoots) attained a length of 4.30 ± 0.07 cm after 4 weeks of incubation (**Fig. 1C**). The higher concentration of BA was found inhibitory considerably in term of all the parameters. Inhibitory effect of higher concentration of BA on shoot formation had also been reported in *Albizia chinensis* (Sinha *et al.* 2000), *Pterocarpus marsupium* (Husain *et al*. 2005) and *Tylophora indica* (Sahai *et al*. 2010).

When KIN used as the sole cytokinin (2.5, 5.0 and 10.0 µM), no significant response was observed. Explants grown on BA supplemented medium showed better growth and elongation and was found to be more responsive to BA than

Table 1 Effect of 6-benzyladenine (BA) added to MS medium on *in vitro* shoot organogenesis through nodal explants in *M. koenigii* after 4 weeks of culture in a horizontal position.

		Position of explants: Horizontal		
Treatment	**% Response**	**Mean* No. of shoot buds/explant**	**Mean* No. of shoots/explant**	**Mean* shoot length (cm)**
BA (0.0 µM)	0	0.00 ± 0.00 c	0.00 ± 0.00 b	0.00 ± 0.00 c
BA (1.0 µM)	20	2.00 ± 0.32 b	1.00 ± 0.32 b	0.98 ± 0.10 b
BA (2.5 µM)	30	1.00 ± 0.32 bc	1.00 ± 0.32 b	1.20 ± 0.05 b
BA (5.0 µM)	50	4.00 ± 0.32 a	4.00 ± 0.32 a	3.40 ± 0.08 a

*Values are in mean ± SE of 10 replicates. Mean followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test.

Table 2 Effect of 6-benzyladenine (BA) and kinetin (KIN) added to MS medium on *in vitro* shoot organogenesis through nodal explants in *M. koenigii* after 4 weeks of culture in a vertical position.

Position of explants: Vertical				
Treatment	**% Response**	**Mean* No. of shoot buds/explant**	**Mean* No. of shoots/explant**	**Mean*shoot length (cm)**
BA (0.0 µM)	60	2.20 ± 0.37 d	2.00 ± 0.55 c	3.00 ± 0.07 bc
BA (1.0 µM)	60	4.00 ± 0.32 bc	3.80 ± 0.37 b	3.18 ± 0.12 bc
BA (2.5 µM)	80	5.40 ± 0.24 ab	4.80 ± 0.20 b	3.38 ± 0.06 b
BA (5.0 µM)	95	6.60 ± 0.40 a	6.60 ± 0.40 a	4.30 ± 0.07 a
BA (10.0 µM)	80	5.80 ± 0.37 a	4.80 ± 0.37 b	2.78 ± 0.12 c
KIN (2.5 µM)	40	4.00 ± 0.32 bc	3.60 ± 0.24 bc	1.40 ± 0.09 c
KIN (5.0 µM)	40	3.60 ± 0.40 c	3.20 ± 0.37 bc	1.84 ± 0.10 d
KIN (10.0 µM)	80	4.00 ± 0.32 bc	4.00 ± 0.31 b	2.08 ± 0.06 cd

*Values are in mean ± SE of 10 replicates. Mean followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test.

Table 3 Effect of different concentration of 6-benzyladenine (BA) added to MS medium with 0.5 µM α-naphthalene acetic acid (NAA) on *in vitro* shoot organogenesis through nodal explants in *M. koenigii* after 4 weeks of culture in a vertical position.

Treatment	**% Response**	**Mean* No. of shoot buds/explant**	**Mean* No. of shoots/explant**	**Mean*shoot length (cm)**
BA (2.5 µM)	65	4.00 ± 0.32 b	3.00 ± 0.00 b	1.46 ± 0.05 c
BA (5.0 µM)	70	6.20 ± 0.20 a	6.00 ± 0.00 a	2.48 ± 0.10 a
BA (10.0 µM)	60	6.00 ± 0.45 a	5.20 ± 0.37 a	2.10 ± 0.07 b

*Values are in mean ± SE of 10 replicates. Mean followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test.

KIN. The superiority of BA for inducing shoot multiplication also reported in many woody tree species including *Swartzia madagascariensis*, *Dalbergia sissoo* and *Sesbania rostrata* (Berger and Schaffner 1995; Pradhan *et al*. 1998; Jha *et al*. 2004).

To assess the synergistic effect of auxin and cytokinin for morphogenic response in nodal explants, low level of NAA (0.5 µM) in conjunction with different concentrations of BA (2.5, 5.0 and 10.0 µM) were tried. However, this combination was not found beneficial because huge callusing from basal ends was favored. A maximum of 6.00 ± 0.00 shoots/explant were differentiated on MS medium supplemented with BA (5.0 µM) and NAA (0.5 µM) with 70% success within 4 weeks (**Table 3**). The inhibitory effect of cytokinin together with auxin over cytokinin alone for shoot induction was also documented in *Simmondsia chinensis* (Agarwal *et al*. 2002), *Pterocarpus marsupium* (Husain *et al*. 2005) and in *Amygdalus communis* (Akbaş *et al*. 2009). This was in contrast with the results reported in *Wrightia tinctoria* (Purohit *et al*. 1994) and *Acacia catechu* (Kaur *et al*. 1998) where a maximum number of shoot buds was initiated in a BA + NAA combination medium and in *M. koenigii* (Rout 2005) where MS medium containing 2.5 mg/l BA + 50 mg/l adenine sulphate + 0.25 mg/l IAA was most responsive.

Multiplication and proliferation for long term establishment

Further, shoot multiplication experiment was carried out with the application of BA (5.0 µM) only. The regenerating tissues (0.5-0.8 cm) were regularly subcultured on fresh medium with a passage of 4 weeks. It was observed that subculturing had a significant effect on *in vitro* shoot proliferation. The shoot proliferation efficiency increased considerably from the 1st-3rd subculture passage and thereafter a significant reduction was noticed in the subsequent subcultures (**Table 4, Fig. 1D**). As far as the literature was concerned, there was no report available on prolonged maintenance of *M. koenigii* culture. The present study is of considerable importance for the maintenance of the primary tissues in organogenic state up to the 15th subculture passages. However, in *Vitex negundo*, Ahmad and Anis (2007) maintained maximum multiplication rate up to the 5th subculture passage and Sahoo *et al.* (1998) maintained only up to the 2nd subculture passages. While, an increasing effect on shoot number after successive subculture had been reported in *Eclipta alba* by Borthakur *et al.* (2000).

Table 4 Effect of subsequent subculturing on shoot proliferation from vertically placed nodal explants of *M. koenigii* on 6-benzyladenine (BA, 5.0 µM) after every 4 weeks of culture.

Subculture passage	**Mean* No. of shoots**
1st	7.80 ± 0.37 b
2nd	15.00 ± 0.83 a
3rd	15.20 ± 0.37 a
4th	9.80 ± 0.86 b

*Values are in mean ± SE of 10 replicates. Mean followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test.

Root induction and acclimatization

Healthy shoots (3.0-4.0 cm long) were transferred to rooting medium consisting of half- and full-strength MS medium with and without different auxins (**Table 5**). Effect of auxins on root induction and root length was examined in 4 weeks old cultures. Half-strength and full-strength MS basal media failed to induce rooting. However, the additions of auxins to nutrient media lead to root induction. Half-strength MS basal medium with auxins proved to be more efficient for root induction as compared to full-strength nutrient medium. The positive effect on rooting of a decrease in mineral concentration can be explained by the reduction in nitrogen concentration of the culture medium (Driver and Suttle 1987; Moncousin 1991). Efficiency of half-strength culture medium for rooting had also been reported in *Melia azedarach* (Shahzad and Siddiqui 2001), *Pterocarpus marsupium* (Husain *et al*. 2005) and *Acacia sinuta* (Shahzad *et al*. 2006). Amongst the treatments tested, 1.0 µM IBA in half-strength MS basal medium induced root

Table 5 Effect of nutrient strength and various concentration of auxin on *in vitro* root induction from *in vitro* raised microshoots of *M. koenigii* after 4 weeks of culture.

Treatment	**Mean* days to rooting**	**Mean* root length (cm)**
MS	0.00 ± 0.00 e	0.00 ± 0.00 d
MS + IBA (1.0 µM)	0.00 ± 0.00 e	0.00 ± 0.00 d
MS + IBA (2.5 µM)	0.00 ± 0.00 e	0.00 ± 0.00 d
MS + IAA (1.0 µM)	0.00 ± 0.00 e	0.00 ± 0.00 d
MS + IAA (2.5 µM)	0.00 ± 0.00 e	0.00 ± 0.00 d
MS + NAA (1.0 µM)	12.00 ± 0.32 c	0.70 ± 0.03 c
MS + NAA (2.5 µM)	11.20 ± 0.37 c	0.84 ± 0.05 c
½ MS	0.00 ± 0.00 e	0.00 ± 0.00 d
½ MS + IAA (1.0 µM)	8.80 ± 0.37 d	0.92 ± 0.50 c
½ MS + IAA (2.5 µM)	13.60 ± 0.50 ab	1.02 ± 0.10 c
½ MS + NAA (1.0 µM)	15.00 ± 0.31 a	0.68 ± 0.70 c
½ MS + NAA (2.5 µM)	11.80 ± 0.37 c	4.60 ± 0.40 b
½ MS + IBA (1.0 µM)	8.00 ± 0.31 d	6.80 ± 0.07 a
½ MS + IBA (2.5 µM)	12.4 ± 0.50 bc	0.52 ± 0.07 cd

*Values are in mean ± SE of 10 replicates. Mean followed by the same letter within columns are not significantly different (P = 0.05) using Tukey's test.

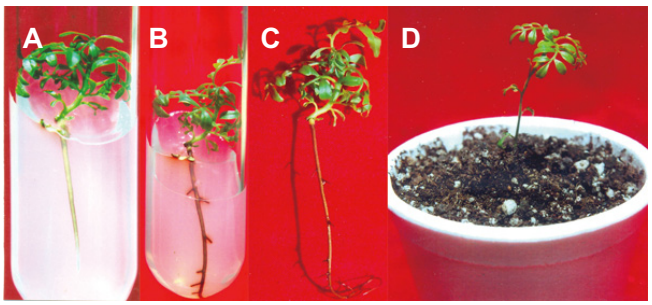

Fig. 2 (**A**) *In vitro* root initiation in regenerated microshoot on half-strength MS medium + IBA (2.5 µM) - 2 weeks old culture. (**B**) Elongated root on half-strength MS medium + IBA (2.5 µM) - 4-weeks-old culture. (**C**) A healthy plantlet of *M. koenigii* with root length of 4.5 cm. (**D**) An acclimatized *M. koenigii* plant.

initiation within 1 week of transfer (**Fig 2A**, **B**). The stimulatory effect of IBA on root induction had been well documented in *M. koenigii* (Lalitha *et al*. 1997), *Melia azedarach* (Shahzad and Siddiqui 2001), *Olea europaea* (Santos *et al*. 2003), *Sesbania drummondii* (Cheepala *et al.* 2004) and *Acacia sinuata* (Shahzad *et al*. 2006). The variation in rooting response with NAA, IAA and IBA might be because of the endogenous level of hormones in the regenerated shoots.

Plantlets with healthy leaves and well developed root system were removed from culture medium and transferred to pot containing soilrite (**Fig. 2C, 2D**). Hardening and acclimatization of *in vitro* raised plantlets was carried out according to the procedure explained in the materials and methods. A 95% survival rate was obtained when acclimatized plantlets were transferred to green house. Plantlets developed by the present protocol exhibited normal morphology with respect to growth habit and leaf shape when compared with the mother plant.

CONCLUDING REMARKS

The present study is important in terms of providing an efficient and reproducible protocol for enhanced multiplication of shoots from nodal segments of *M. koenigii*, as a good multiplication rate is the most important factor in rendering propagation cost effective. In the present protocol, the regeneration frequency was maintained up to the 15th subculture passage without loss of morphological response ensuring a continued supply of germplasm. Thus, the establishment of the present protocol for *in vitro* propagation and direct regeneration of *M. koenigii* offers a potential system for improvement, conservation and mass propagation of this multipurpose medicinal plant.

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