International Journal of Plant Developmental Biology ©2011 Global Science Books

In Vitro Propagation of *Murraya koenigii* L. Spreng (Curry Leaf Plant) through Adventitious Shoot Proliferation from Internode Explants

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ABSTRACT

Murraya koenigii (L.) Spreng, commonly known locally as "curry patta" or "mitha neem" in India, is a valuable medicinal plant known for its biochemical and aromatic properties. Adventitious regeneration, which is a pre-requisite in most genetic transformation studies using *Agrobacterium* and ballistics, needs to be developed as a protocol for micropropagation of *M. koenigii*. This paper presents a procedure for the rapid, high frequency regeneration of *M. koenigii* plantlets from internode explants via adventitious shoot formation. The concentration of plant growth regulators (PGRs) in liquid MS medium exhibited a discrete role in the efficacy of adventitious shoot induction. N6-benzyle adenine (BA), kinetin, adenine sulphate and indole-3-acetic acid (IAA) in combination were the most effective PGRs for adventitious shoot induction. Murashige and Skoog (MS) liquid medium with 9.29 µM kinetin, 13.317 µM BA, 2.854 µM IAA and 70 mg/l adenine sulphate yielded the maximum number (18) of shoot buds from internode explants. The number of shoots was further increased (27.30) after sub-culturing them into semi-solid (containing 8 g/l agar-agar) MS medium fortified with similar concentrations and combinations of PGRs. Most *in vitro* shoots (2.5-3.0 cm long), rooted (90%) on semi-solid MS medium containing 19.68 µM indole-3-butyric acid within 28-30 days. The rooted plantlets were transplanted into pots containing a mixture of soilrite (mixture of peat moss + vermiculite + perlite in a 1: 1: 1 ratio that was mixed with natural soil in the ratio of 1: 1) at 70-80% relative humidity and 28 ± 2°C for hardening. 85% of *in vitro*-raised plantlets survived under field conditions.

Keywords: adenine sulphate, "mitha neem", multiplication, plant growth regulators, plant regeneration

INTRODUCTION

Murraya koenigii (Linn) Spreng, commonly known as "curry patta" or" mitha neem" (curry leaf tree) in India, belongs to the Rutaceae family. Its aromatic leaves, which contain essential oil, are used as condiments (Gantait *et al*. 2011). The fresh leaves of *M. koenigii* showed antimicrobial, mosquitocidal and topo-isomerase inhibition activities (Russel *et al.* 1999); *M. koenigii* leaf extracts showed an antioxidant property (Tachibana *et al.* 2001). Previous studies have been reported on plant regeneration from stem segments of curry leaf tree (Iyer and Gopinath 1999). Nodal cuttings from matured curry leaf plants cultured on woody plant medium (WPM) supplemented with 4.4 µM 6-benzyl adenine (BA) and 4.6 µM kinetin (Kn) produced 12-30 multiple shoots in a period of 8 weeks (Nirmal Babu *et al.* 2000). Multiple shoots regenerated from nodal segments of *M. koenigii* on MS basal medium containing BA (2.5 mgl^{-1}), indole-3-acetic acid (IAA) (0.25 mgl^{-1}) and adenine sulfate (AS; 25 mgl^{-1}) (Rout 2005). Protocols for micropropagation have been reported through seedling explants on MS medium containing 5.0 mgl^{-1} BA (Bhuyan e*t al*. 2004) and through *in vitro* leaf explants on 6.6 µM BA with 2.9 µM IAA (Mathew and Prasad 2007). Reports on somatic hybridization between *Citrus* and *M. paniculata* via protoplast electrofusion (Guo and Deng. 2004) and *in vitro* flowering from shoot and cotyledonary explants of *M. paniculata* are available (Taha 1997). Medicinal plants are valuable sources of pharmaceutical products. Conventional propagation methods take a long time for multiplication due to the poor rate of fruit set, poor germination and also clonal variation through seeds. Naturally growing plants are the main sources of phyto-pharmaceutical preparations. Due to the increasing demand of crude drugs, medicinal plants are being overexploited and disappearing from their natural habitats. With the availability of advanced biotechnological methods, there is a need to update the available protocols of *M. koenigii*, especially using non-meristematic tissues for enhanced micropropagation and also model cultures for any desired genetic transformation in the yields.

The present investigation reports the first efficient protocol for high frequency regeneration of *in vitro* plants of *M. koenigii* through adventitious shoot formation from internode explants.

MATERIALS AND METHODS

Internode segments of *M. koenigii* (plants identified by senior author) were collected from naturally growing plants in Ajmer, Rajasthan, India. The explants were first washed twice with liquid soap (Labolene, India), rinsed thoroughly with distilled water, treated with 0.1% (w/v) mercuric chloride for 5-6 min to surface sterilize then washed 5 times for 3 min each with autoclaved distilled water. The surface sterilized explants were cut into 20-25-mm pieces. The explants were inoculated on MS medium (Murashige and Skoog 1962) supplemented with various concentrations and combinations of plant growth regulators. MS liquid and semi-solid medium supplemented with 4.43 to 19.97 µM BA, 32 to 13.93 µM 6-furfuryl amino purine (FAP) and 0.57 to 4.56 µM IAA and 81.44 to 244.34 µM AS. MS medium supplemented with 8.87 µM BA, 4.64 µM FAP with 81.44 µM AS were used for multiplication and elongation of adventitious shoots.

The regenerated adventitious shoots (25-30 mm) were excised and sub-cultured on MS medium containing different concentra-

Received: 26 January, 2010. Accepted: 8 April, 2011.

Short Communication

Table 1 Direct adventitious shoots regeneration from internode explants of *Murraya koenigii* (L.) on MS liquid medium with various plant growth regulators.

BAP (µM)	Kinetin (µM)	IAA (µM)	ADS (µM)	Shoot regeneration (%) (Mean ± SD)	Number of shoots/explant (Mean ± SD)	Length of shoots (mm) (Mean ± SD)
4.43	2.32	0.57	81.44	40 ± 1.41 a	3.8 ± 0.74 df	20.0 ± 0.12 b
6.65	3.48	1.14	81.44	55 ± 1.41 bc	9.6 ± 0.48 gh	23.5 ± 0.48 c
8.87	4.64	1.71	135.74	65 ± 1.41 cd	13.6 ± 0.48 ef	26.0 ± 0.16 d
11.09	8.13	2.28	135.74	70 ± 2.28 cd	14.8 ± 0.74 ef	27.0 ± 0.06 d
13.31	9.29	2.85	190.04	85 ± 1.41 abc	18.2 ± 0.74 bc	29.0 ± 0.14 e
15.53	10.45	3.42	190.04	82 ± 1.67 abc	13.8 ± 0.74 ef	21.0 ± 0.10 b
17.75	11.61	3.99	244.34	80 ± 1.41 abc	8.6 ± 0.80 gh	18.0 ± 0.10 f
19.97	13.93	4.56	244.34	75 ± 1.89 cd	6.4 ± 0.48 ij	10.0 ± 0.10 g

$P < 0.05$; Each value represents the mean ± standard deviation (SD) of 10 replicates per treatment in three repeated experiments. **ADS**: adenine sulphate, **BA**: N^6-benzyl adenine, **FAP**: 6-furfuryl amino purine, **IAA**: indole-3-acetic acid, **MS**: Murashige and Skoog.

tions of IBA (4.92 to 34.14 µM) for root induction. Sucrose was added to the media as the carbon source at 30 g/l. The pH of media was adjusted to 5.8 with 1N NaOH and agar-agar (Hi-Media, Mumbai, India) was added at 8 g/l for semi-solid media.

Glass bottles (200 ml) with polypropylene lids were used as culture vessels for the initiation and multiplication of cultures. Each glass jar/bottle was filled with 25 ml of media. Liquid media was poured into 25 mm × 150 mm Borosil test tubes into which a Whatman filter paper (Grade 40) bridge was placed to hold explants in a vertical position. All the media, glassware, forceps and knife were autoclaved at 121°C under 20 psi for 20 min.

The cultures were incubated in a growth room under a 16-h photoperiod at 2000-2500 lux and 25°C. The experiments were carried out in a completely randomized design with 10 replicates per treatment and each experiment was repeated three times. Mean values were subjected to analysis of variance (ANOVA) and statistical significances between means were assessed using Duncan's multiple range test (DMRT) at $P < 0.05$.

RESULTS

Adventitious shoot regeneration

Internode explants were cultured in liquid MS medium with combinations of BA, FAP, IAA and adenine sulphate at different concentrations (**Table 1**). Adventitious shoot regeneration was observed only in liquid MS medium. Shoot formation occurred at all the concentrations of PGRs but shoot number varied at different concentration of PGRs. On MS liquid medium supplemented with BA (4.43 µM), FAP (2.23 µM), IAA (0.57 µM) and ADS (81.44 µM) a mean of 3.8 ± 0.74 shoots regenerated in 40% of explants; the *in vitro* shoots attained an average length of 20.00 ± 0.12 mm (**Table 1**). Increasing the concentration of PGRs enhanced the number of shoots in explants and promoted higher percentage of explants producing shoot induction (**Fig. 1**).

A maximum of 85% of explants showed adventitious shoot regeneration with a mean of 18.2 ± 0.74 shoots 29.0 ± 0.12 mm long when grown on MS liquid medium supplemented with BA (13.31 µM), FAP (9.29 µM), IAA (2.85 µM) and ADS (190.04 µM). Increasing the concentration of PGRs in MS liquid medium up to 19.97 µM BA, 13.93 µM FAP, 4.56 µM IAA and 244.34 µM ADS reduced the percentage of shoot induction to 43% with only a mean of 6.4 ± 0.48 shoots regenerating from each explant.

Shoot multiplication

Adventitious shoot buds initiated within 18 to 21 days from the date of inoculation of explants. Shoot buds reached 29 mm in length after 30 days' incubation in the growth room. Multiplication of *in vitro* shoots was achieved by repeated harvesting of shoots and re-culturing the same explants on fresh semi-solid MS medium containing 8.87 µM BA, 4.64 µM FAP and 81.44 µM ADS (**Fig. 2**). The number of shoots increased every 30 days; a maximum of 42 ± 0.89 shoots were produced from 120 days-old explants. Regeneration potential was reduced in 150 days-old explants, which yielded 9.03 ± 0.32 shoots.

Table 2 Efficacy of MS medium with different concentrations of IBA in the induction of roots from *in vitro* derived shoots.

IBA (µM)	Rooting (%)	Root length (mm) (Mean ± SD)
4.92	25	25.0 ± 1.41 ab
9.84	60	28.0 ± 1.41 cd
14.76	80	30.0 ± 1.09 de
19.68	85	35.0 ± 0.89 ef
24.60	95	38.0 ± 1.41 gh
29.52	90	36.0 ± 1.41 ef
34.14	65	27.62 ± 1.06 cd

$P < 0.01$; Each value represents the mean ± standard deviation (SD) of ten replicates per treatment in three repeated experiments. **IBA**: indole-3-butyric acid

Rooting

Well-elongated healthy shoots (at least 29 mm long), grown on shoot induction or shoot multiplication media, rooted on rooting medium. Root induction was not observed on shoots transferred to MS medium free of PGRs. IBA, when supplemented in MS medium, induced roots (**Table 2**). IBA at different concentrations showed different responses in terms of percentage and growth of roots *in vitro*. The maximum percentage (95%) of rooting was achieved on MS medium supplemented with 24.60 µM IBA (**Fig. 3**). Low level of rooting (25%) was observed on MS medium containing 4.92 µM IBA in regenerated shoots after 3 weeks and roots recorded up to 25.0 mm in length. At higher concentrations of IBA (34.14 µM), 65% of shoots rooted, but callus was also formed.

Establishment of plantlets

In vitro plantlets were hardened in small earthen pots containing a mixture of Soil Rite (peat moss: perlite: vermiculite; 1: 1: 1) at 70-80% relative humidity and 28 ± 2°C for 21 days (**Fig. 4**). Survival rate was 65-70% in hardened plantlets. These plants were then transferred to field conditions (**Fig. 5**).

DISCUSSION

A review on the micropropagation of aromatic plants has been updated recently in the literature (Gantait *et al*. 2011). Earlier studies on the micropropagation of *Murraya koenigii* reported using juvenile (Iyer and Gopinath 1999) and seedling explants (Rani *et al.* 2010). Bhuyan *et al*. (2004) micropropagated *M. koenigii* by axillary proliferation using intact seedlings on MS basal medium. Combinations of 4.4 µM BA and 4.65 µM Kn (Nirmal Babu *et al*. 2000) and 2.5 mg/l BA, 0.25 mg/l IAA and 0.25 mg/l ADS (Rout 2005) were essential for shoot multiplication. In our study, liquid MS medium with higher concentrations of BA (13.31 µM), Kn (9.29 µM) and ADS (190.04 µM) was used to induce direct adventitious shoots. Reports are available on multiple shoot regeneration from different types of explants, such as nodal explants (Nirmal Babu *et al*. 2000; Rout 2005), seedling explants (Rani *et al*. 2010) and *in vitro* leaf explants

Fig. 1 Adventitious shoot bud regeneration on MS liquid medium containing BA (13.31 µM) + kinetin (19.29 µM) + IAA (2.85 µM) + adenine sulfate (190.04 µM).

Fig. 2 Adventitious shoot multiplication on MS + BA (8.87 µM) + kinetin (4.64 µM) + adenine sulfate (81.44 µM).

Fig. 3 Rooting in adventitious shoots on MS medium containing IBA (24.60 µM).

(Iyer and Gopinath 1999; Bhuyan *et al*. 2004; Mathew and Prasad 2007) for induction of multiple shoots on MS medium supplemented with various concentrations and combinations of PGRs. Reports are scanty on the regeneration of adventitious shoots from internode explants of *M. koenigii*. This study reports for the first time that ADS in liquid MS medium plays an important role in the induction of adventitious shoots from non-meristamatic tissues (internode) where pre-existing shoot primordia are not available, indicating that the physico-chemical conditions (liquid medium and higher concentration of ADS) support the induction of meristematic activities in non-meristematic tissues of internode explants, resulting in the regeneration of

Fig. 4 Plantlets under greenhouse conditions (1 week).

Fig. 5 Well-hardened *in-vitro*-induced *M. koenigii* plantlets in the nursery (4 weeks).

adventitious shoots which facilitate fast availability of cultures of *M. koenigii* as may be required for genetic manipulation. The protocol described in this study enables the production of more than 100 plants from a single explant. The results reported could also be useful for meeting raw material demands of pharmaceutical industries for isolation of target compounds from *M. koenigii*.

ACKNOWLEDGEMENTS

Authors wish to thank Dr. Jaime A. Teixeira da Silva for linguistic improvements and Dr. Vinod Joshi, principal scientist, Desert Medicine Research Center (DMRC), Jodhpur, for editing the manuscript.

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