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Rapid Plantlet Regeneration System from Cotyledon and Leaf Explants of Curry Leaf Tree (*Murraya koenigii* L. Spreng)

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ABSTRACT

In the present study a highly efficient and reproducible procedure for the large scale propagation of *M. koenigii* is described. Rapid plantlet regeneration system via adventitious shoot proliferation from cotyledon and leaf explants was developed. The best results were observed on modified MS medium supplemented with BAP 12.75 µM, Kinetin 8.98 µM and ADS 152.74 µM. On this medium 91.0 ± 0.59 percent cotyledon explants showed an average number 8.09 ± 0.02 shoots whereas 6.3 ± 0.07 shoots were regenerated leaf explants. The highest shoot multiplication rate in cotyledons (4.9 ± 0.09 fold) and leaf explants (2.8 ± 0.07 fold) were achieved on MS basal medium augmented with 9.05 µM BAP, 4.46 µM Kinetin and 186.94 µM ADS. Rooting of *in vitro* shoots occurred in 3-4 weeks on transfer to MS basal medium containing 14.86 µM IBA. *In vitro* plantlets were hardened in small earthen pots containing a mixture of peat moss: perlite: vermiculite in the ratio of 1: 1: 1 at 70-80% relative humidity and 28°C for 21 days. 70% of *in vitro*-raised plantlets survived under field conditions. Standardization of an efficient *in vitro* regeneration protocol could be helpful in carrying out various genetic modifications in this economically important crop.

Keywords Murraya koenigii, Adenine sulphate, Adventitious shoots, Regeneration, Rutaceae

[I] INTRODUCTION

Murraya Koenigii, belongs to the family Rutaceae, commonly known as curry-leaf tree, is a native of India, Sri Lanka and other south Asian countries. It is found almost everywhere in the Indian subcontinent. *M. koenigii* is a plant which has various important uses in the traditional system of medicine in Eastern Asia [1]. Based on ethanomedicine, *M. koenigii* is used as a stimulant, antidysentric and for the management of diabetes mellitus [2]. The leaves of this plant contain high amount of oxalic acid, leaves also contains crystalline glycosides, carbazole alkaloids, koenigin and resin. In general, *M. koenigii* has been propagated by seeds, which is usually unreliable due to low viability and death of young seedlings under natural conditions [3]. The *in vitro* propagation methods in *Murraya Koenigii* are highly advantageous, especially using non-meristematic tissues for enhanced micropropagation for genetic improvement.

Development of regeneration protocol through adventitious shoot proliferation using non-meristematic tissue is prerequisite for germplasm conservation and the development of transgenic plants.

There are few reports on *in vitro* studies of *M. koenigii* which are restricted to *in vitro* shoot multiplication from intact seedling, inter node segments, nodal cuttings, leaf as explants [4-9]. Reports are not available on *in vitro* adventitious shoots regeneration in *Murraya koenigii* from cotyledons.

This paper presents an efficient protocol for the rapid and high frequency regeneration of *M. koenigii* plantlets via adventitious shoot formation from cotyledons and leaves.

[II] MATERIALS AND METHODS

2.1 Preparation of explant

The fruits of *Murraya koenigii* were collected from surrounding areas of Ajmer, Rajasthan, India, the seeds were carefully taken out by removing the pulp of fruits with the help of scalpel and were washed in running tap water for 15 min. Seeds were first washed with liquid detergent (Teepol; Qualigen, India) and then soaked with 0.1% solution of Bavistin fungicide (BASF, India) and rinsed with distilled water. The seeds were surface sterilized with an aqueous solution of 0.1% (*w/v*) $HgCl_2$ (Hi Media, India) for 5-6 minutes followed by four to five washes with sterile distilled water to remove all the traces of mercuric chloride. Disinfected seeds were germinated in 200 ml screw-capped glass jars containing 40 ml seed germinating half strength Murashige and Skoog (MS) basal medium devoid of plant growth regulators. Cotyledons and Leaves were excised from 60 days old seedling [10].

2.2 Culture media and experimental conditions

For induction of *in vitro* adventitious shoots, the explants were inoculated on MS medium supplemented with various concentrations and combinations of plant growth regulators. MS basal medium supplemented with 6-benzylaminopurine (BAP) 2.64 µM to17.68 µM, Kinetin 0.48 µM to 12.75 µM and Adenine sulfate (ADS) 82.35 to 243.48 µM. The *in vitro* raised shoots (40-45 mm) were excised and transferred individually on MS medium containing different concentration of indole-3-butyric acid (IBA) 2.75 to 24.65 µM for rooting. 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 ((Qualigen, India) was added at 8 g L^{-1} for semi-solid media. All the media, glassware, forceps and knife were autoclaved at 121°C and 20 psi for 20 min. Cultures were maintained at 25±1□ C, 16-h photoperiod under 35µE m^{-2} s^{-1} light intensity by white fluorescent tubes (Philips, India).

2.3 Acclimatization and field transfer

In vitro developed plantlets with 40-50 mm shoot length and strong root were washed with running tap water and were inoculated in a glass bottle 1/3 filled with a mixture of vermiculite, peat moss and perlite in equal ratio. The plantlets in the screw capped jars were kept under a hardening unit for one week and then the screw caps were removed from bottle. They were later gradually transferred to the low humidity and high light intensity zone of hardening unit in the interval of one week. The plantlets were finally transferred to poly bags and exposed to field conditions.

2.4 Statistical analysis

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 new Duncan's multiple range test (DMRT) at $P < 0.05$ [11].

[III] RESULTS

3.1 *In vitro* seed germination and explant preparation

In vitro cultured seeds showed 89% germination after 1 to 2 weeks of inoculation and attain a height of 6 to 7 cm in 4 to 5 weeks time. Seedlings of 7-8 weeks old were used to excise the

Cotyledon and Leaf for induction of adventitious shoots.

3.2 Adventitious shoot bud initiation and development

Excised intact cotyledons and leaves were inoculated on to MS basal medium augmented with or without plant growth regulators. No significant response was noted in the MS medium without growth regulators from cotyledons and leaf explants on MS medium without PGRs. Addition of plant growth hormones to the medium had a positive effect on shoot formation from both the explants (Table 1).

Various concentrations of 6- benzyl amino purine (BAP) 2.64 µM to17.68 µM and Kinetin 0.48 µM to 12.75 µM alone and in combinations with and Adenine Sulfate 82.35 to 243.48 µM were added in MS basal medium in order to achieve maximum number of fast growing shoots from explants.

Highest number of shoot induction (8.9± 0.02) was observed from 91.0 ± 0.59 percent cotyledon explants (Fig. A) on MS medium augmented with BAP (12.75 µM), Kinetin (8.98 µM) and ADS 152.74 µM. On the same MS basal medium an average of 6.3 ± 0.07 shoots were produced from 72.8 ± 0.08 percent leaf explants (Fig. B).

3.3 Shoot multiplication

In order to achieve shoot multiplication, the *in vitro* induced shoots were scooped from explants and were transferred on to the fresh MS medium containing BAP, Kinetin with Adenine sulphate in different concentrations (Table 2). On MS medium supplemented with BAP 9.05 µM, Kinetin 4.46 µM and ADS 186.94 µM compact clumps of shoots were formed from both the explants. On this medium 4.9 ± 0.04 fold and 2.8 ± 0.07 fold shoot multiplication was achieved from cotyledon and leaf explants respectively (Fig. C). Six weeks old *in vitro* shoots when attained a length of 40 mm were harvested individually and transferred on rooting media.

3.4 Rooting

In vitro developed shoots showed different responses in terms of percentage & growth of roots, when transferred to the MS basal medium augmented with IBA in the concentrations from 2.75 µM to 24.65 µM (Table 3). The highest 92.8 ± 0.53 percent and 76.2 ± 0.15 percent of rooting was observed from the cotyledons and leaf originated shoots explants respectively on IBA 14.86 µM (Fig. D).

3.5 Establishment of plantlets

In vitro plantlets were hardened in small earthen pots containing a mixture of Soil Rite (peat moss: perlite: vermiculite in the ratio of 1: 1: 1 at 70-80% relative humidity and temperature gradient of 28-36°C under green house conditions for 21 days. Survival rate was 70 percent in hardened plantlets (Fig. E). These plants were then transferred to field conditions (Fig. F).

[IV] DISCUSSION

A plant regeneration system via adventitious shoot proliferation from Cotyledons and Leaf explants of *Murraya koenigii* was successfully revealed through this investigation. The adventitious bud formation efficiency oh cultured explants showed varied response and seems to be dependent more precisely on type of explant, culture medium, concentrations and combination of PGRs. Culture medium is one of the most important key factors which influenced induction and subsequent multiplication and rooting of adventitious shoots.

WPM medium was found to be the most effective for axillary bud culture of curry leaf plant [7], as well as for the regeneration in other plant species through callus [12]. However, the present study suggested that MS medium responded better than WPM media.

The type of explant is an important factor for organogenesis in tissue culture [13]. The regeneration studies have been reported in a number of plant species using stem cuttings and intact seedling [3,5] and also reported in other species of *Citrus* and *Aegle marmelos* [14,15]. Which are close relatives of *Murraya koenigii.*

In our study Cotyledons and Leaf explants were used which do not have any apparent pre-existing meristems. Cytokinin either alone or in

combination has significant effects on shoot induction and their subsequent multiplication [16-19]. Adenine sulphate is known to be precursor of adenine during the DNA replication in cell, which supposed to be indirectly helps in the rejuvenation of plant vigor, therefore, the explants and the shoots in the adenine sulphate supplemented in MS medium exhibited rejuvenation after each sub culture [5,6]. Similar observation was noted in present investigation in which the highest shoot proliferation was recorded on BAP, Kinetin and Adenine sulphate added MS basal medium.

In general, lower concentration of adenine sulphate or without ADS the cultures could not maintain their vigor for longer time under *in vitro* conditions. Similar observation was recorded in our study that ADS at the concentration 152.74 µM favored the induction of maximum number of shoots and in higher concentration 186.94 µM showed higher rate of shoot multiplication of shoots from Cotyledons and Leaf explants of *Murraya koenigii.*

Type of auxin and their optimized concentration in the medium was found to be the critical factor in the regeneration of healthy roots. Superiority of IBA over other auxins in root formation has also been reported in other plant species such as *Cunila galoide, Clitoria ternatea* and *Cassia siamea* [20-22]. The IBA has been reported to have a stimulatory effect on root induction in many tree species including *Alnus glutinosa* and *Morus indica* [23, 24]. In present study, best rooting response in cotylodonary shoots (92.8 ± 0.53%) and 76.2 ± 0.15% in the shoots originated from leaf was achieved on MS basal medium supplemented with 14.86 µM IBA.

In general, *in vitro* raised plantlet grow in microbe free and control conditions, therefore the hardening these plants in pre requisite for their field transfer. In our study rooted plantlets were hardened prior to their field transfer. Six weeks old hardened plantlets with tap roots were transferred to poly bags containing a mixture of perlite, vermiculite and peat moss in equal ratio. The survival rate of the transplanted plantlets was 70% which was similar to the findings in *C. gigantena, Prunus* sp. and *Aegle marmelos* [25-27].

[V] CONCLUSION

High frequency of adventitious plantlet regeneration from Cotyledon and leaf explants from axenic seedling of *Murraya koenigii*, which can suffice the need of translational studies for lab to land technology. Our protocol can be incorporated into a gene transfer program of *Murraya koenigii* as well as in other members of family Rutaceae.

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Table 1. Effect of different concentrations of growth regulators in MS basal medium on multiple shoot induction from cotyledon and leaf explants of *Murraya koenigii*.

PGRs			Cotyledon			Leaf		
BAP (µM)	Kinetin (µM)	ADS (µM)	Explant response (%) for shoot initiation (Mean ± S.D.)	No. of shoots per explant (Mean±S.D)	Length of shoots in mm (Mean±S.D.)	Explant response (%)for shoot initiation (Mean ± S.D.)	No. of shoots per explant (Mean±S.D	Length of shoots in mm (Mean±S.D.)
0.00	0.00	0.00	0.0±0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
4.56	4.60	0.00	59.4±0.05aa	4.6±0.01gh	8.1±0.04df	49.4±0.06f	1.9±0.03c	5.9±0.02f
9.05	6.87	0.00	76.69±0.21cb	5.2±0.05df	10.4±0.26ad	54.7±0.02c	2.5±0.04bc	7.7±0.07df
12.75	8.98	0.00	81.4±0.06df	6.1±0.04g	13.7±0.04c	61.0±0.06b	4.2±0.21ef	10.7±0.06i
17.68	11.65	0.00	78.0±0.08g	5.6±0.02a	12.2±0.05g	56.2±0.52a	3.3±0.21b	8.7±0.03d
12.75	8.98	82.35	84.4±0.02bb	7.1±0.06b	12.2±0.05g	60.3±0.04aa	4.2±0.21ef	13.2±0.06h
12.75	8.98	139.58	88.62±0.32d	8.6±0.04df	15.8 ± 0.02cc	65.2±0.02d	5.7±0.05h	15.6±0.02fd
12.75	8.98	152.74	91.0±0.59fg	8.9±0.02h	19.3 ± 0.09ac	72.8±0.08gh	6.3±0.07g	16.4±0.09ac
12.75	8.98	186.94	84.4±0.02bb	7.1±0.06b	17.6 ± 0.07gh	65.2±0.02d	4.4±0.02cf	14.9±0.05cc
12.75	8.98	219.76	78.0±0.08g	6.8±0.07ab	15.5 ± 0.02jk	63.6±0.07i	3.9±0.05cb	12.2±0.02f

$P < 0.05$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments, PGRs plant growth regulators, BAP 6- benzylaminopurine, ADS Adenine sulphate

Table 2. Effect of different concentrations of BAP and Kinetin with ADS (Adenine sulphate) in MS basal medium on shoot multiplication from cotyledon and leaf explants of *Murraya koenigii*.

PGRs			Cotyledon	Leaf
BAP (µM)	Kinetin (µM)	ADS (µM)	Multiplication Rate (Mean ± S.D.)	Multiplication Rate (Mean ± S.D.)
2.64	0.48	82.35	2.8 ± 0.07ac	1.6 ± 0.09gh
4.56	2.72	139.58	3.4 ± 0.02bd	2.2 ± 0.02ac
9.05	4.56	186.94	4.9 ± 0.09 jh	2.8 ± 0.07ad
12.75	6.87	219.76	3.8 ± 0.05cc	2.5 ± 0.03jk
17.68	11.65	243.48	3.0 ± 0.08gh	2.2 ± 0.02ac

$P < 0.05$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments, PGRs plant growth regulators, BAP 6- benzylaminopurine, ADS Adenine sulphate

Table: 3. Effect of different concentrations of IBA in MS medium on rooting of *in vitro* adventitious shoots of *Murraya koenigii* from cotyledon and leaf explants.

IBA (µM)	Rooting (%)	
	Cotyledon (Mean ± SD)	**Leaf (Mean ± SD)**
0.0	0.0 ± 0.00	0.0 ± 0.00
2.75	25.2 ± 0.83a	18.7 ± 0.35fd
4.96	59.8 ± 1.78c	39.8 ± 0.73gh
7.64	80.6 ± 2.40f	56.2 ± 0.48bc
12.49	86.8 ± 1.00cd	68.9 ± 0.18aa
14.86	92.8 ± 0.53gh	76.2 ± 0.15ef
19.72	89.2 ± 0.83cc	65.4 ± 1.14bd
22.19	86.8 ± 1.00cd	60.8 ± 2.45ad
24.65	83.2 ± 1.13ij	56.2 ± 0.48bc

$P < 0.01$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments; PGRs plant growth regulators, IBA indole-3-butyric acid

Fig: A-F Adventitio*us* plantlet regeneration from Cotyledon and Leaf explants of *Murraya koenigii*: (A) Shoot regeneration from Cotyledon, (B) Shoot regeneration from Leaf explant, (C) Shoot multiplication, (D) Rooting, (E) Six week-old tap rooted plantlets prior to hardening, (F) Hardened field growing plants of *M. koenigii*.