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IN VITRO ADVENTITIOUS SHOOT REGENERATION FROM COTYLEDON AND HYPOCOTYL EXPLANTS OF *MURRAYA KOENIGII* (L) SPRENG

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Present study was carried out to standardize a protocol for high efficiency *in vitro* adventitious shoot regeneration from *Murraya koenigii* using cotyledon and hypocotyl explants. 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*. Direct adventitious shoot proliferation was achieved from intact seedling on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyleaminopurine (BAP) 2.64 µM to 22.21 µM, Kinetin 2.34 to 13.96 µM and Adenine sulphate (ADS) 40.72 to 244.39 µM to induce *in vitro* multiple shoots. Percentage response of cotyledon explants was 95.00 ± 0.58 which was significantly higher than the response of hypocotyl explants (76.2 ± 0.06) explant in the MS basal medium supplemented with 12.95 µM BAP, 8.98 µM Kinetin and 152.74 µM ADS. The 35-40 mm elongated shoots were cultured to MS basal medium augmented with different concentrations of indole-3-butyric acid (IBA). The maximum percentage, 84.8 ± 0.13 of rooting was achieved on MS basal medium containing 17.26 µM IBA. *In-vitro* plantlets regenerated from cotyledon and hypocotyl explants were hardened for four weeks in a green house. The hardened plantlets were transferred to field conditions. Eighty percent hardened plantlets were successfully survived under natural conditions.

Keywords : Adenine sulphate; Adventitious shoots; Cotyledons; Hypocotyl explants; *Murraya koenigii*; Rutaceae.

Introduction

Murraya koenigii (L.) Spreng, locally known as meetha neem & curry leaf plant, belongs to the family Rutaceae. Rutaceae is a large family comprising 160 genera and 1650 species largely distributed in the tropical and subtropical parts of the world [1]. The family included the genus *Citrus, Zanthoxyllum acanthopodium, Tetractomia tetranda and Murraya koenigii.* Leaf contains various phyto-constituents [2, 3] such as carbazole alkaloids and phenolic compounds in rich amount both are

responsible for antioxidant [4, 5] and many other activities of drug. Antioxidants are used in prevention of various diseases such as skin disease, cancer etc. Volatile oil is used as a fixative for soap, perfume. The leaves, bark and root of the plant are used in the indigenous medicine as a tonic, stimulant, carminative and stomachic.

In order to cater the increasing demands of herbal drug markets, conservation and commercial production of this species have become necessary. 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 nodal segments, nodal cuttings, leaf as explants [6-11].

Reports are not available on *in vitro* adventitious shoots regeneration in *Murraya koenigii* from cotyledons and hypocotyl explants. This paper presents an efficient protocol for the rapid and high frequency regeneration of *M. koenigii* plantlets via adventitious shoot formation from cotyledons and hypocotyl explants.

Material and Methods

(i) Explant preparation : Fruits of *Murraya koenigii* were obtained in the month of June to the end of July from surrounding of Ajmer, Rajasthan, India. Seeds from mature fruits were carefully taken out by removing the pulp of fruits with the help of forceps & scalpel and then washed with liquid detergent (Teepol; Qualigen, India) for 2 min. and then treated with 0.1% solution of Bavistin fungicide (BASF, India) for about 5 min. to remove fungal contaminants from the explants. The seeds were surface sterilized with 0.1% aqueous HgCl2 solution for 5-6 min. and then rinsed 4-5 times with autoclaved distilled water.

(ii) Nutrient media and culture conditions : The nutrient medium consisted of Murashige and Skoog (MS) basal medium supplemented with sucrose (3% w/v). Disinfected seeds were germinated in 200 ml screw-capped glass jars containing 40 ml seed germinating half strength MS basal medium devoid of plant growth regulators. Cotyledons and hypocotyl segments were excised from 60 days old seedling as explants.

In vitro shoots were induced on MS medium supplemented with different plant growth regulators such as 6-benzylaminopurine (BAP, 2.64 µM to 22.21 µM), Kinetin (2.34 to 13.96 µM), and Adenine sulphate (ADS) 40.72 to 244.39 µM in combination to MS basal medium. The *in vitro* raised shoots (35-40 mm) were excised and individually transferred on MS medium containing different concentration of indole-3-butyric acid (IBA, 4.90 to 27.09 µM) for rooting. Media were solidified by adding 0.8% agar powder (Qualigen, India).

The pH of media was adjusted at 5.8 and was autoclaved at temperature 121°C and 15 psi pressure for 15-20 minutes. All the cultures were incubated in a culture room maintained at 25 ± 2°C under 16/8 h light/dark cycle, 45 µM m−2 s−1 irradiance level provided by cool white fluorescent tubes. Each treatment consisted of 10 explants and was repeated thrice.

(iii) Acclimatization and field transfer : In vitro developed plantlets with 40 - 45 mm shoot length and strong tap root were washed with running tap water and were transferred into 200 ml jars 1 / 3 filled with a pasteurized mixture of vermiculite, perlite and peat moss 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. 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.

(iv) Statistical analysis : Experiments were set up in completely randomized design with 10 replicates per treatment and each experiment was repeated thrice. Mean values were subjected to analysis of variance (ANOVA) and statistically significant ($P < 0.05$) means were determined with new Duncan's Multiple- range test [12].

Results and Discussion

(i) In vitro seed germination and explant preparation : The surface sterilized seeds were inoculated on half strength Murashige and Skoog (MS) basal medium for germination. *In vitro* cultured seeds showed 87% germination after 1 to 2 weeks of inoculation and attain a height of 6 to 7 cm in 4 to 5 week. Cotyledons and hypocotyl segments were excised from 4-5 week old *in vitro* raised seedling and used as explants

(ii) Induction of Adventitious shoot and development : Excised intact cotyledons and hypocotyl segments 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 hypocotyl explants. 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) and Kinetin and Adenine Sulfate were added in MS basal medium in order to achieve maximum number of fast growing Adventitious shoots from explants.

Highest number of shoot induction (8.6 ± 0.04) was observed from 95.00 ± 0.58 percent cotyledon explants (Fig. A) on MS medium augmented with BAP (12.95 µM), Kinetin (8.98 µM) and ADS 159.32 µM. On the same MS basal medium and average of 7.4 ± 0.01 shoots were produced from 76.2 ± 0.06 percent leaf explants (Fig. B).

(iii) 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 8.59 µM, Kinetin 4.62 µM and ADS 183.62 µM compact clumps of shoots were formed from both the explants. On this medium 4.6 ± 0.03 fold and 2.8 ± 0.06 fold shoot multiplication was achieved from cotyledon and hypocotyl explants respectively (Fig. C). Six weeks old *in vitro* shoots when attained a length of 35-40 mm were harvested individually and transferred on rooting media.

(iv) Rooting : The *in vitro* raised shoots recovered from all the explants when attained a length of 40 - 45 mm. were transferred to MS basal medium augmented with or without plant growth regulators for root induction. Root induction was not observed on shoots transferred to MS medium free of PGRs. IBA, when supplemented in MS medium, induced roots. IBA at different concentrations (4.90 to 27.09 µM) showed different responses in terms of percentage and growth of roots *in vitro.* The maximum 84.8 ± 0.13 percent rooting was achieved from shoots of

cotyledon on MS medium supplemented with 17.26 µM IBA whereas on the same medium 64.4 ± 0.11% rooting was achieved from shoots of hypocotyl explants (Fig. 1D).

(v) 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 temperature gradient of 28-36℃ under green house conditions for 21 days. Survival rate was 80% in hardened plantlets (Fig.1 E). The plantlets were finally transferred to poly bags and exposed to field conditions (Fig. 1F).

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

PGRs			Cotyledons			Hypocotyl Segments		
BAP (µM)	Kinetn (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 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.64	2.34	40.72	45.4 ± 0.01ca	2.6 ± 0.05ad	7.6 ± 0.02e	27.4 ± 0.08a	2.6 ± 0.06f	3.1 ± 0.21cf
4.56	4.60	81.45	77.80 ± 0.05aa	6.2 ± 0.02cc	12.6 ± 0.05g	53.7 ± 0.02cd	5.4 ± 0.11d	6.3 ± 0.02ij
8.89	6.87	135.76	86.62 ± 0.32c	7.1 ± 0.06gh	15.8 ± 0.02k	60.4 ± 0.08bd	6.6 ± 0.08h	9.1 ± 0.08bb
12.95	8.98	159.32	95.00 ± 0.58g	8.6 ± 0.04ef	19.3 ± 0.09ij	76.2 ± 0.06gh	7.4 ± 0.01a	15.3 ± 0.05ed
17.77	11.65	217.37	89.40 ± 0.02bb	7.2 ± 0.25jk	17.4 ± 0.05ef	71.8 ± 0.02j	7.1 ± 0.07c	12.9 ± 0.07fg
22.21	13.96	244.39	87.00 ± 0.09ad	6.8 ± 0.07fd	15.5 ± 0.02d	67.9 ± 0.04e	6.2 ± 0.03h	10.2 ± 0.02i

$P < 0.05$; 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. Effects of different concentrations of BAP, Kinetin and ADS (Adenine sulphate) in MS medium on shoot multiplication from cotyledons and hypocotyl explants of *Murraya koenigii*.

PGRs			Cotyledons	Hypocotyl Segments
BAP (µM)	Kinetin (µM)	ADS (µM)	Multiplication Rate (Mean ± S.D.)	Multiplication Rate (Mean ± S.D.)
0.0	0.0	0.0	1.2 ± 0.03e	0.8 ± 0.02g
2.78	0.62	81.45	2.5 ± 0.01ad	1.2 ± 0.03h
4.27	2.54	135.76	3.1 ± 0.09cc	1.9 ± 0.01ij
8.59	4.62	183.62	4.6 ± 0.03b	2.8 ± 0.04k
12.54	6.89	217.37	3.3 ± 0.02aa	2.1 ± 0.02aj
17.38	9.21	244.39	2.9 ± 0.07f	1.9 ± 0.05bb

$P < 0.05$; 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

Fig.1.A-F Adventitio*us* plantlet regeneration from Cotyledon and Hypocotyl explants of *Murraya koenigii*: Shoot regeneration from Cotyledon, (B) Shoot regeneration from Hypocotyl explant, (C) Shoot multiplication, (D) Rooting, (E) Six week-old tap rooted plantlets prior to hardening, (F) Hardened field growing plants of *M. koenigii.*

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

IBA (µM)	Rooting (%)	
	Cotyledon (Mean ± SD)	Hypocotyl (Mean ± SD)
0.0	0.0 ± 0.0	0.0 ± 0.0
4.90	52.9 ± 1.17g	37.2 ± 0.03ed
9.98	60.7 ± 0.47ii	48.9 ± 0.08gh
12.20	65.2 ± 1.18d	56.2 ± 0.05cd
14.82	76.2 ± 0.04bc	61.7 ± 0.08h
17.26	84.8 ± 0.13cg	64.4 ± 0.11aa
19.79	80.2 ± 0.01d	59.1 ± 0.04c
22.43	78.6 ± 0.12i	49.4 ± 0.01dd
24.15	75.2 ± 0.17ef	44.8 ± 0.42ed
27.09	72.9 ± 1.17h	41.2 ± 0.06b

< 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

In the present investigation, 60 days old aseptically grown seedlings were used as a source of explants (cotyledon and hypocotyl), which do not have any apparent pre-existing meristems. The type of explant is an important factor for organogenesis in tissue culture[13]. It is well established that *in vitro* propagation of plant species is influenced by several factors, like genotype, age and source of initial tissue/organ which in turn are related to their endogenous hormonal status [14]. Cytokinin either alone or in combination has significant effects on shoot induction and their subsequent multiplication[15-18]. In the present study it was observed that BAP in combination with kinetin was more efficient for initiation and subsequent proliferation of shoot buds[9]. Similar observations were reported in several other plants such as *Feronia limonia*[19] and *Aegle marmelos*[20]. This observation is in agreement with the previous published works demonstrating BA as the most successful cytokinin for shoot organogenesis in several other systems including *Bacopa monnieri*, *Holarrhena pubescens*, *Cynodon dactylon*, *Salvia officinalis*, *Scopolia parviflora* and *Durcus carota*[21-26]. It is common to observe a relationship between BA concentrations and shoot number and shoot size[27]. 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[7, 28]. 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. Highest 95.0 ± 0.58 percent cotyledons explants responded for induction of 8.6 ± 0.04 shoots per explants and 76.2 ± 0.06 percent responses was observed in hypocotyl explants for induction

of 7.4 ± 0.01 shoots per explant on MS basal medium supplemented with BAP 12.95 µM, Kinetin 8.98 µM and ADS 152.74 µM.

Concentration and type of auxin in the medium was found to be the critical factor in producing healthy roots. The rooting methods in our study revealed that the presence of an exogenous auxin was vital for *in vitro* root induction of micro-shoots and IBA has been found to be the most effective auxin for *in vitro* rooting in *Murraya koenigii* shoots. The superior effects of IBA on the root development may be due to several factors such as its preferential uptake, transport and stability over other auxins and subsequent gene activation [29].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* [30-32]. The IBA has been reported to have a stimulatory effect on root induction in many tree species including *Alnus glutinosa* and *Morus indica*[33, 34]. The highest 84.8 ± 0.13 percent of rooting was observed from cotyledons and 64.4 ± 0.11 percent of rooting was observed from hypocotyl explants on MS medium supplemented with IBA 17.26 µM.

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. In present investigation the plants were hardened in a mixture of perlite, vermiculite and peat moss in equal ratio. Several reports are available for many plant species such as *Celastrus Paniculatus*, *Dalbergia latifolia* and *Dendrocalamus asper* in which soil, sand and composed in the ratio of 1:1:1 was used for acclimatization of micropropagated plants[35-37]. The *in vitro* plantlets developed during the study program were successfully hardened and transferred to the field where 80% plants were found healthy.

Conclusion

In the present study, a protocol has been worked out on reliable and high frequency of adventitious plantlet regeneration from cotyledons and hypocotyl explants of *Murraya koenigii,* which can suffice the need of translational studies for lab to land technology.

The advantage of this system offers interesting perspectives for the introduction of some desirable genes in this medicinally useful species by exploitation of recombinant DNA technology aimed at genetic improvement.

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