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EFFICIENT PLANTLET REGENERATION SYSTEM VIA ENHANCED ADVENTITIOUS SHOOT PROLIFERATION IN *MURRAYA KOENIGII* (L.) SPRENG.

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Rapid shoot proliferation was established by adventitious shoot formation from inter-node segments. Shoots were directly regenerated *in vitro* without callusing. The concentration of plant growth regulators (PGRs) in MS medium exhibited a discrete role in the efficacy of adventitious shoot induction. Maximum number of shoot (82.02 ± 0.08) regeneration was achieved on modified MS medium supplemented with BAP 13.01 µM, Kinetin 8.87 µM and ADS 136.52 µM. Rooting of *in vitro* shoots occurred in three to four weeks on transfer to MS medium containing IBA (19.68 µM). About 88 % of *in vitro*-raised plantlets were survived under field conditions. The protocol development in present study for rapid adventitious shoot regeneration from inter-node segments of *Murraya koenigii* could be helpful in carrying out various genetic modifications in this economically important medicinal plant.

Keywords: Adventitious shoots; Inter-node segments; Medicinal plant; *Murraya koenigii*; Rutaceae.

Introduction

Murraya koenigii (L) Spreng, popularly known as curry leaf plant, is a small aromatic tree belonging to the family Rutaceae that grows widely in Southeast Asia. Of the 14 global species belonging to the genus *Murraya*, only two are available in India, namely, *M. koenigii* and *M. paniculata*. *M. koenigii* is more popular due to its large spectrum of medicinal properties. *M. koenigii* leaves have a slightly pungent, bitter and feebly acidulous taste and these characteristics are retained even after drying. Fresh and dried curry leaves are extensively used in South Indian culinary practices for seasoning and flavouring dishes[1]. Leaves are aromatic and contain proteins, carbohydrates, fiber, minerals, carotene, nicotinic acid and vitamin C. It is rich in vitamin A. and calcium The leaves contain high amount of oxalic acid, leaves also contains crystalline glycosides, carbazole alkaloids, koenigin, resin, fresh leaves contain yellow color 2.5 percent volatile oil[2]. The aromatic components of this tree are widely utilized in the medicinal field.

The presence of several monomeric, binary carbazole alkaloids and simple furo and pyranocoumarin in various plant parts which are bioactive enabled new vistas in several scientific investigations[3-5]. These alkaloids are proved to be antimicrobial, antioxidative, antidiabetic and anti trichomonal[6-9]. Some of them showed anti carcinogenic properties in a cultured human leukemia cell line[10, 11].

The conventional method of propagation of this tree is limited to seeds only, which retain their viability for a short period. Hence, a biotechnological approach might have an advantage edging over traditional breeding of *M. koenigii* within a short period. The development of a reproducible regeneration protocol is the prerequisite for ex situ conservation and micropropagation.

There are few reports on *in vitro* studies of *M. koenigii* which are restricted to *in vitro* shoot multiplication from intact seedling, nodal cuttings, leaf as explants[12-17]. The objective of this study was to develop a protocol for the rapid and high frequency regeneration of plantlets via adventitious shoot formation from inter-node segments and to evaluate the effects of plant growth regulators on the growth performance of *Murraya koenigii in vitro.*

Material and Methods

(i) Explant preparation : Inter-node segments of *Murraya koenigii* were collected from surrounding areas of Ajmer in the month of March to the end of July. The juvenile and healthy explants were selected for the study. The explants were first washed twice with liquid detergent (Teepol; Qualigen, India) for 2min. and then treated with 0.1% solution of Bavistin fungicide (BASF, India) for about 5 min. to remove fungal contaminants from the explants. The explants were surface sterilized with 0.1% aqueous $HgCl_2$ solution for 4-5 min. and then rinsed 4-5 times with autoclaved distilled water. The surface sterilized explants were cut into inter-node 20-25 mm pieces.

(ii) Nutrient media and culture conditions : For induction of *in vitro* adventitious shoots, the explants were inoculated horizontally on MS medium supplemented with various concentrations and combinations of plant growth regulators[18]. MS medium supplemented with Benzyl amino purine (BAP) 2.67 µM to 21.56 µM Kinetin 2.96 µM to 13.56 µM and Adenine sulfate (ADS) 40.72 to 244.34 µM. 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, 2.46 to 29.52 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 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 Duncan's Multiple- range test (DMRT) using SPSS for Windows version 10.0[19].

Results and Discussion

(i) Explant preparation and induction of Adventitious shoot : Juvenile and mature inter-node segments of *M. koenigii* were inoculated aseptically on MS basal medium augmented with or without plant growth regulators.

No significant response was noted in the MS medium without growth regulators from inter-node segments on MS medium without PGRs. Addition of plant growth hormones to the medium had a positive effect on shoot formation from the explants (Table 1). Various concentrations of 6-benzyl amino purine (BAP) 2.67 µM to 21.56 µM and Kinetin 2.96 µM to 13.56 µM alone and in combinations with and Adenine Sulfate 40.72 to 217.37 µM were added in MS basal medium in order to achieve maximum number of fast growing shoots from explants.

Highest number of shoot induction (6.9 ± 0.06) was observed from 82.02 ± 0.08 percent Inter-node explants (Fig. A, B) on MS medium augmented with BAP (13.01µM), Kinetin (8.87 µM) and ADS 136.52 µM.

Table 1. Effect of different concentrations of BAP and Kinetin with ADS (Adenine sulphate) in MS medium on shoot induction from Inter-node segments of *Murraya koenigii.*

S. No.	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.)
1	0.0	0.0	0.0	0.0±0.00	0.0 ± 0.00	0.0 ± 0.00
2	2.67	2.96	40.72	61.02±0.04ac	4.6±0.01gh	8.1±0.04df
3	4.43	4.64	81.45	66.69±0.21cb	5.2±0.05df	10.4±0.26ad
4	8.87	6.87	105.76	73.40±0.06df	6.1±0.04g	13.7±0.04c
5	**13.01**	**8.87**	**136.52**	**82.02±0.08cc**	**6.9±0.06b**	**15.2 ± 0.06gh**
6	17.75	11.65	186.07	78.00±0.08g	5.6±0.02a	12.2±0.05g
7	21.56	13.56	217.37	68.09±0.29cb	5.1±0.05df	10.9±0.02d

$P < 0.05$; 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

(ii) Shoot multiplication : In order to achieve shoot multiplication, the *in vitro* induced cluster of 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). Six weeks old 40-45 mm shoots were excised from the inter-nodal explant and the same explant was re - cultured onto the fresh semi solid medium for shoot

induction medium MS + BAP 9.35µM, Kinetin 4.56 µM and ADS 186.07 µM. On this medium 4.8 ± 0.02 fold shoot multiplication was achieved. (Fig. C).

(iii) Rooting : Six weeks old *in-vitro* shoots when attained a length of 40 - 45 mm were harvested individually and transferred on rooting media. Root induction was not observed on shoots transferred to MS medium free of PGRs. (Table 3).

Table 2. Effect of different concentrations of BAP and Kinetin with ADS (Adenine sulphate) in MS basal medium on shoot multiplication from Inter-node segments of *Murraya koenigii*.

2	**BAP (µM)**	**Kinetin (µM)**	**ADS (µM)**	**Multiplication Rate (Mean ± S.D.)**
1	2.67	0.48	81.45	2.8 ± 0.07ac
2	4.56	2.96	136.52	3.4 ± 0.02bd
3	**9.35**	**4.56**	**186.07**	**4.8 ± 0.02 bb**
4	13.01	6.87	217.37	3.8 ± 0.05cc
5	17.75	8.87	244.34	3.0 ± 0.08gh

$P < 0.05$; 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 Inter-node segments.

S. No.	**IBA (µM)**	**Rooting (%) of adventitious shoots (Mean ± SD)**
1	0.00	0.00
2	2.46	19.0 ± 1.00 a
3	4.92	25.2 ± 0.83 f
4	9.84	59.8 ± 1.78 d
5	12.35	73.8 ± 0.13ef
6	14.76	84.8 ± 0.83 cc
7	**19.68**	**91.4 ± 0.02 g**
8	24.65	89.6 ± 1.14df
9	29.52	80.6 ± 2.40 h

$P < 0.01$; 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

(*iv) 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. These plants were then transferred to field conditions where 88% survival rate was observed (Fig. E, F).

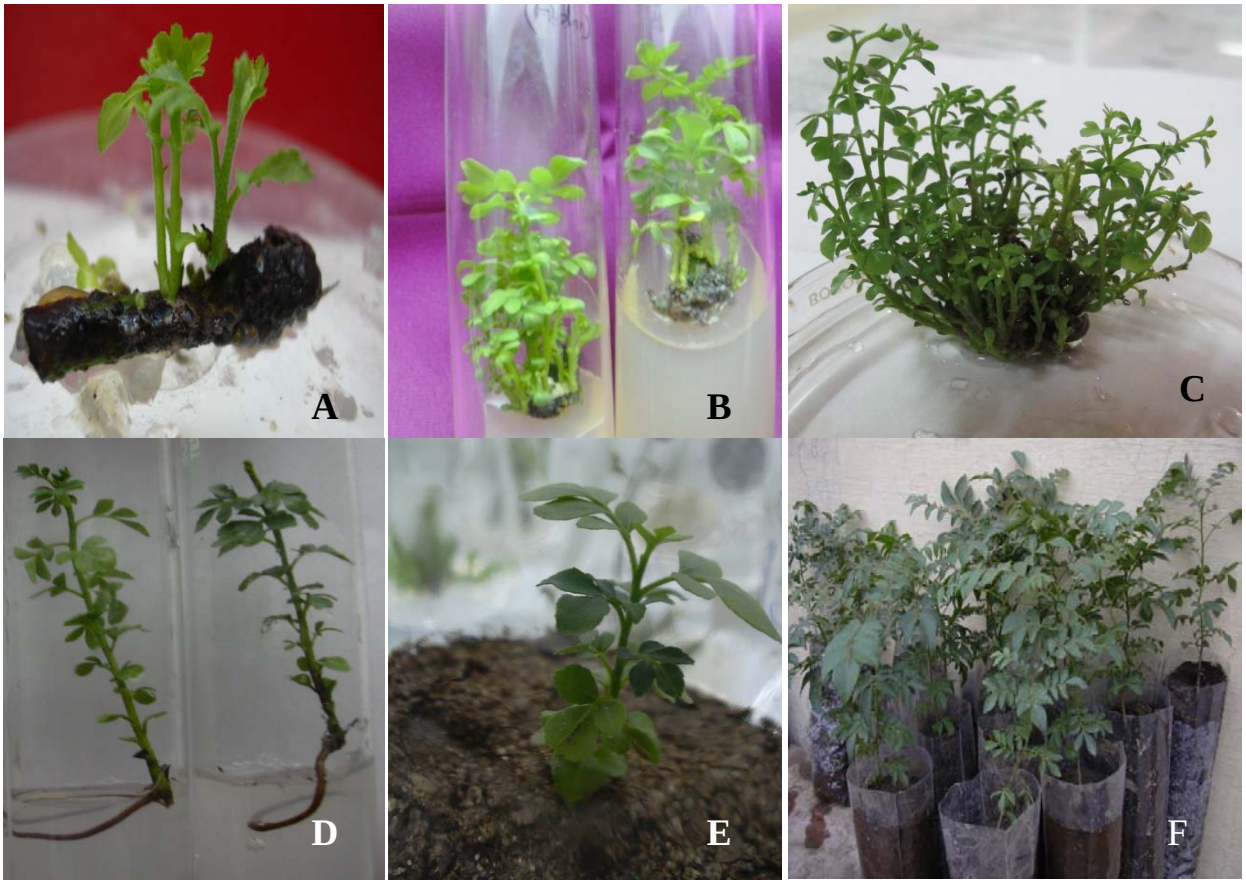

Fig.1. A-F Adventitio*us* plantlet regeneration from Inter-node segments of *Murraya koenigii*: (A, B) Shoot regeneration from Inter-node segment (C) Shoot multiplication (D) Rooting, (E) Six week-old tap rooted plantlets prior to hardening, (F) Hardened field growing plants of *M. koenigii.*

A regeneration system via adventitious shoot proliferation from Inter-node segments of *Murraya koenigii* was successfully revealed through this investigation. The type of explant is an important factor for organogenesis in tissue culture[20].

The matured inter-node segments were used to induce adventitious shoots. The adventitious bud formation efficiency of cultured explants showed varied response and seems to be dependent more precisely on type of explant, culture medium, concentrations and combination of PGRs for subsequent multiplication and rooting of adventitious shoots. Inter-node explants were used which do not have any apparent pre-existing meristems. The important observation of this study was that, the explant showed better response when they inoculated horizontally on to the surface of nutrient medium.

Reports are available that Cytokinin either alone or in combination has significant effects on shoot induction and their subsequent multiplication[15,21-23]. Similar observation was noted in present investigation in which the highest shoot

proliferation was recorded on MS medium augmented with BAP (13.01 µM), Kinetin (8.87 µM) and Adenine sulphate (136.52 µM). 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* [24-29]. It is common to observe a relationship between BA concentrations and shoot number and shoot size[30].

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[13, 31].

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 auxin in root formation has also been reported in other plant species such as *Cunila galoide*, *Clitoria ternatea* and *Cassia siamea*[32-34]. The IBA has been reported to have a stimulatory effect on root induction in many tree species including *Alnus glutinosa* and *Morus indica*[35, 36]. The highest 91.4 ± 0.02 percent of rooting was observed from Inter-node explants on MS medium supplemented with IBA 19.68 µM.

The *in vitro* plantlets developed during the study program were successfully hardened and transferred to the field where 88% plants were found healthy.

Conclusion

In present study, the protocol developed for high frequency regeneration of adventitious shoots from Inter-node explants from matured plant of *Murraya koenigii*, can be useful for translational studies for lab to land technology and can also be incorporated into a gene transfer program of *Murraya koenigii* and its relatives of family Rutaceae.

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