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In vitro plantlet regeneration from Hypocotyl and Root explants of *Murraya koenigii* (L.) Spreng.

KEYWORDS: *Murraya koenigii*, Adenine sulphate, Seedling explants, Rutaceae

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ABSTRACT *Protocol for high frequency plantlet regeneration from Hypocotyl and Root explants from in vitro raised seedlings of Murraya koenigii was developed. The surface sterilized seeds were inoculated on half strength Murashige and Skoog (MS) basal medium for germination. Explants were obtained from 7-8 weeks old axenic seedlings & cultured on modified MS medium supplemented with 6-benzylaminopurine (BAP) 13.05 µM, Kinetin (Kin) 9.15 µM and Adenine sulphate (ADS) 148.45 µM alone or in combination to induce in vitro multiple shoots. The best result observed for the average number of shoots (94.5 ± 0.05%) was from root segment explants, which was significantly higher than hypocotyls (74.0 ± 0.04%). The elongated shoots were shifted to MS basal medium supplemented with indole-3-butyric acid (IBA) 19.72 µM for root induction. Plantlets were acclimatized and established in soil where they exhibited normal growth.*

Introduction

Murraya koenigii, commonly known as curry leaf or kari patta in Indian dialects, belonging to family Rutaceae which represent more than 150 genera and 1600 species (Satyavati et al. 1987). *Murraya* comprises of about 11 species of shrubs and small trees. Its distribution ranges encompass the tropical and subtropical regions of Sri Lanka, India and South China to South-East Asia, Malaysia, New Guinea, North-East Australia and New Caledonia (Swingle and Reece 1967). Its leaves, root and bark are considered tonic, stomachic, stimulant and carminative. Concoctions made from leaves are applied externally to bruises, eruptions and the bites of poisonous animals (Anonymous 1962). Also, odoriferous essential oils from the leaves and a crystalline glycoside "koenigin" and "murrayin" from flowers are some of the highly valued products of industrial importance obtained from the curry leaf plant (Chopra et al. 1996; Joseph and Peter 1985; Nair and Nayar 1997). An alkaloid, mukonine, is also found in this plant (Chakrabarty et al. 1978).

In order to cater the increasing demands of herbal drug markets, conservation and commercial production of this species have become necessary. Since, the conventional methods of propagation are generally slow, labour intensive, requiring large number of propagules and are not very successful, use of modern methods of biotechnology need to be adopted to speed up the propagation of plants (Kyte and Kleyn, 1996).

There are only a few reports of *in vitro* studies of *M. koenigii* which are restricted to *in vitro* shoot multiplication from intact seedling (Bhuyan et al. 1997) or internodes (Hazarika et al. 1995; Joshi et al. 2011) as explants. Though seed set is poor and results in highly heterogeneous population, seed propagation is most followed (Ranganathappa et al. 2001) because of high-level recalcitrance for rooting of mature stem cuttings that could be solved only with high concentration (5000 ppm) of IBA (Ranganathappa et al. 2002). The present study was therefore, aimed to develop an efficient protocol for regeneration of plantlets from hypocotyls and root segments of the species.

Materials and methods

Explant preparation

Fruits of *Murraya koenigii* were obtained in the month of June to the end of July from surrounding areas 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 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 seeds were surface sterilized with 0.1% aqueous $HgCl_2$ solution for 5-6 min. and then rinsed 4-5 times with autoclaved distilled water.

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 (Murashige and Skoog 1962) devoid of plant growth regulators. Hypocotyls (10 to 15 mm) and root segments (10 to 20 mm) 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, 4.42 to 22.21 µM), Kinetin (Kin, 0.49 to 13.96 µM), and Adenine sulphate (ADS, 81.45 to 244.39 µM) alone or 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, 2.46 to 29.55 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.

Acclimatization and field transfer

In vitro developed plantlets with 35-40 mm shoot length

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and strong taproot 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.

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) (Gomez and Gomez 1976) using SPSS for Windows version 10.0.

Results

Multiple shoot regeneration

Root (10 to 20 mm) and Hypocotyl (10 to 15 mm) were excised from 7-8 weeks old axenic seedlings and were cultured on to MS basal medium augmented with or without plant growth regulators. No significant response was noted in the absence of plant growth regulators on hypocotyls as well as root explants. Addition of plant growth hormones to the medium had a positive effect on shoot formation from both the explants. Shoot bud formation and further development varied with the explants and type and combination of plant growth regulators used.

Highest shoot induction (8.5 ± 0.07) was found in 74.0 ± 0.04 percent hypocotyl explants on MS medium augmented with BAP (13.05 µM) , Kinetin (9.15 µM) ADS 148.45 µM, whereas on MS medium supplemented with lower concentration of BAP (4.42 µM), Kinetin (4.60 µM) and ADS 81.45 µM, only 52.6 ± 0.58% hypocotyl explants exhibited a minimum 5.8 ± 0.03 shoot per explants (Fig. a). The hypocotyl segments did not respond on MS medium without PGRs.

The root explants exhibited shoot induction (35.9 ± 0.07%) on control, MS without PGRs, medium. Highest number of shoots (8.8 ± 0.07) were induced from 94.5 ± 0.05% of root segments (Fig. b) on the MS medium supplemented with BAP 13.05 µM, Kinetin 9.15 µM and ADS 148.45 µM within 4 week (Table 1).

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

PGRs			Hypocotyl			Root		
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	35.9 ± 0.07a	3.2 ± 0.06df	3.5 ± 0.01h
4.42	4.60	81.45	52.6 ± 0.58c	5.8 ± 0.03d	7.1 ± 0.04gh	73.2 ± 0.06bc	7.2 ± 0.05gh	10.5±0.06jk
8.89	6.87	135.76	62.1 ± 0.01b	7.4 ± 0.09ac	9.6 ± 0.02k	84.2 ± 0.03cd	7.9 ± 0.09ef	14.7±0.02cd
13.05	9.15	148.45	74.0 ± 0.04gh	8.5 ± 0.07cb	14.8 ± 0.08cc	94.5 ± 0.05abc	8.8 ± 0.07bc	19.9±0.05ac
17.77	11.65	217.37	72.4 ± 0.02h	8.1 ± 0.02ef	11.7 ± 0.03bd	90.2 ± 0.02cf	8.5 ± 0.02a	18.5±0.03cd
22.21	13.96	244.39	68.7 ± 0.12ij	7.4 ± 0.09ac	9.9 ± 0.01cb	84.2 ± 0.03cd	8.0 ± 0.03b	16.9±0.07df

*P*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

Shoot multiplication

In order to achieve shoot multiplication and elongation 3-4 weeks old cultures 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 8.85 µM, Kinetin 4.66 µM and ADS 190.18 µM culture formed compact clumps of shoots from both the explants. On this medium 4.2±0.06 fold and 3.0±0.03 fold shoot multiplication was achieved from root and hypocotyl explants respectively (Fig. c).

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

PGRs			Hypocotyl	Root
BAP (µM)	Kinetin (µM)	ADS (µM)	Multiplication Rate (Mean ± S.D.)	Multiplication Rate (Mean ± S.D.)
4.42	0.49	81.45	1.6 ± 0.05 a	1.9 ± 0.02 d
6.68	2.34	135.76	1.8 ± 0.09 cd	3.0 ± 0.04 ef
8.85	4.66	190.18	3.0 ± 0.03 jk	4.2 ± 0.06 ad
11.13	6.87	217.37	1.9 ± 0.09 bc	3.2 ± 0.09 gh
13.35	9.35	244.39	1.6 ± 0.05 cc	2.8 ± 0.04 ij

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

Rooting of shoots and acclimatization of plants

The *in vitro* raised shoots recovered from all the explants when attained a length of 35-40 mm. were transferred to root induction media. No significant response was observed on shoots transferred to auxin-free medium (control) to form roots. IBA at different concentrations (2.46 µM to 29.55 µM) showed responses in terms of percentage for regeneration of root (Table 3).

The maximum percentage (95%) of rooting was achieved on MS medium supplemented with 19.72 µM IBA (Fig. d). *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 (Fig. e). Survival rate was 87% in hardened plantlets. These plants were then transferred to field conditions (Fig. f).

Table: 3. Effect of different concentrations of IBA in MS medium on rooting of *in vitro* shoots of *Murraya*

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koenigii.

IBA (µM)	Rooting (%)	
	Hypocotyl (Mean ± SD)	Root (Mean ± SD)
0.0	12.8 ± 0.03a	28.4 ± 1.34c
2.46	21.3 ± 0.30cc	43.9 ± 1.16aa
4.94	39.8 ± 0.73d	60.4 ± 1.15cb
7.39	50.9 ± 1.13e	65.4 ± 0.04bc
12.35	56.1 ± 0.15ab	74.2 ± 0.14cd
14.80	52.6 ± 0.14g	86.6 ± 1.23jk
19.72	65.4 ± 1.14h	95.2 ± 0.79gh
24.65	56.1 ± 0.15ab	87.2 ± 0.12ad
29.55	45.4 ± 1.12k	65.4 ± 0.04bc

*P*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

Discussion

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 (George 1993). Most of the hardwood species produce phenolic compounds after wounding (George and Sherrington 1984), which on oxidation gets converted into quinines which actually affect tissue blackening and inhibit new *in vitro* morphogenetic responses in plants, particularly in tree species.

In the present study it was observed that BAP in combination with kinetin was more efficient for initiation and subsequent proliferation of shoot buds (Nirmal babu et al. 2010). Similar observations were reported in several other plants such as *Feronia limonia* (Hiregoudar et al. 2003) and *Aegle marmelos* (Das et al. 2008). 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 (Rout 2005; Joshi et al 2011). In general, lower concentration of adenine sulphate or without ADS the cultures could not maintain their vigor for longer time under *in vitro* conditions.

In present study the ADS 148.45 µM in MS medium favored the induction of maximum number of shoots and in higher concentration 190.18 µM showed higher rate of shoot multiplication. Culturing of explants with inverted polarity gave maximum shoot buds than the explants inoculated in horizontal direction. This may be due to the new meristematic activity of the apical organogenic region which is in direct contact with the medium under the influence of growth regulators (Kumar et al. 2005) in *Capsicum annuum* L. In present study it was observed that both the explants when inoculated in horizontal direction gave maximum shoots.

A stimulating effect of IBA on rooting of curry leaf cuttings was also reported by Lalitha et al. (1997). The stimulatory effect of IBA on root formation has also been reported in many medicinal plants like *Ocimum basilicum* (Sahoo et al. 1997) and *Clitoria ternatea* (Barik et al. 2007). Similarly in present investigation the highest percentage of rooting was achieved on MS medium augmented with IBA 19.72 µM.

Hardening and acclimatization procedures for establishment of micropropagated plantlets were also developed for many species such as *Salvadora persica* (Mathur et al. 2008), *Peganum harmala* (Goel et al. 2009). Several reports are available for many plant species such as *Celastrus paniculatus* (Martin et al. 2004.), *Dalbergia latifolia* (Raghavaswamy et al. 1992), *Dendrocalamus asper* (Arya and Arya 1997) in which soil, sand and composed in the ratio of 1:1:1 was used for acclimatization of micropropagated plants. In present investigation the plants were hardened in a mixture of perlite, vermiculite and peat moss in equal ratio. The *in vitro* plantlets developed during the study program were successfully hardened and transferred to the field where 87% plants were found healthy.

Conclusion

We have developed an improved and viable regeneration system based on adventitious shoot proliferations from naturally non proliferating loci of plant parts such as hypocotyl and root of *M. koenigii* which can suffice the need of translational studies for lab to land technology. Furthermore, the investigation will potentially address the issues of large scale micropropagation and genetic transformation of *M. koenigii* and its close relatives.

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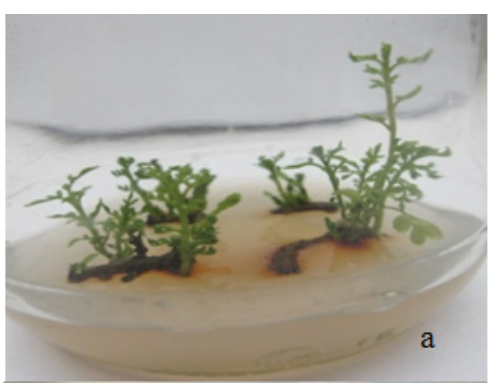

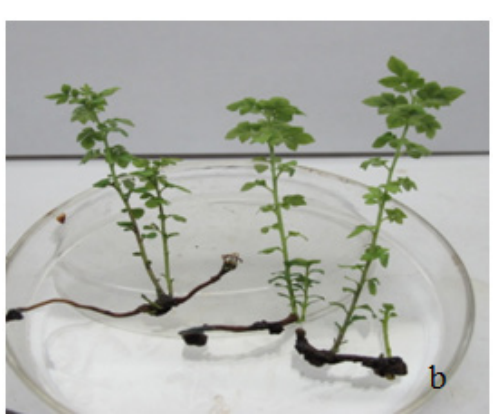

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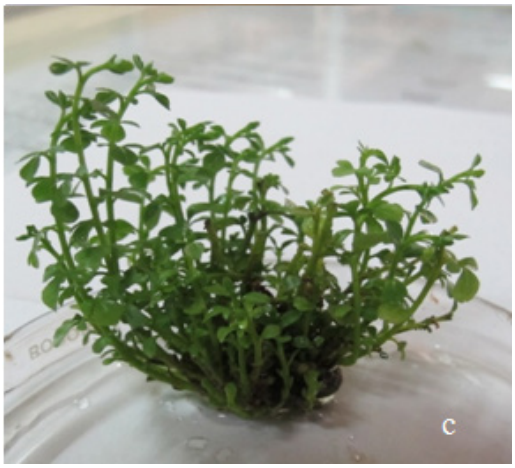

Fig. a-f ***In vitro*** **plantlet regeneration from Hypocotyl and Root segments of** ***Murraya koenigii:*** **a Shoot regeneration from hypocotyl, b Shoot regeneration from Root explant, c Shoot multiplication, d rooting, e six week-old tap rooted plantlets prior to hardening, f hardened field growing plants of** ***M. koenigii.***

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