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In vitro multiple shoot regeneration from axillary bud explant of *Murraya Koenigii* L. Spreng. An aromatic plant

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Abstract

The *Murraya koenigii* have very poor rate of multiplication through conventional method under natural conditions. Considering that the proposed plants species have proven valuable medicinal importance for their chemical properties. Micropropagation of this plant through standardized tissue culture technologies will compensate their poor propagation under natural conditions. Present investigation is mainly focused on feasible research on development of micro propagation protocol through axillary buds. Juvenile and mature stem explants of *Murraya koenigii* were inoculated aseptically on MS semi solid as well as on liquid media containing cytokinis and auxins in the different concentrations and combinations. The shoot initiation was observed on both liquid and semi solid media but in semi solid media browning of shoots and explants with medium and leaf fall and chlorosis was observed within one week of culture. Due to the excessive browning liquid media (without agar-agar) were used in all the experiments, conducted for shoot induction. The adenine sulphate was added in the concentration ranges between 13.57µM to 81.44µM. On the optimum physico-chemical conditions for induction and growth of shoots from axillary bud explant were defined, the *in vitro* shoot cultures were excised as such in the form of a cluster and were cut into two or more bunches each consisted of 4-5 shoots and each bunch was called a propagule. In this way if one mother propagule produced ten shoots after a period of 30-35 days incubation then the same propagule was cut into two daughter propagules the number of daughter propagules (4-5 shoots) achieved and from each mother propagule was presented as rate of multiplication to multiplication (M-M). At the time of sub-culture of mother propagule the elongated shoots (3-4cm) were excised individually and were transferred for rooting. The number of shoots harvested for rooting from each mother propagule was presented as rate of multiplication to rooting (M-R) in the results. The experiments for shoot multiplication were conducted in liquid as well as in semi-solid (with agar-agar) MS media but chlorosis and browning of shoots were not observed in semi solid media in multiplication experiments, therefore, only semisolid MS media were used for subsequent experimentations of multiplication. The ADS was added in concentration range of 13.57µM to 81.44µM with pre-optimized BAP concentration 13.31µM and kinetin 13.93µM. At lower concentration of ADS was not effectively enhanced the M-M and M-R. From the results of all the experiments conducted for multiplication of cultures from axillary bud explants of *Murraya koenigii*, it was found that BAP 13.31µM and kinetin 13.93µM and ADS 81.44µM in MS semisolid medium was found to be most suitable for high frequency multiplication of shoots for M-M and M-R. During the experiments for shoot regeneration from axillary bud explant it was observed that on the media in which ADS was added in some of this shoot buds were developed from the inter nodal region of stem explants.

Keywords: *Murraya koenigii*, media, *in vitro*, axillary bud, Adenine sulphate, Aromatic plant.

List of Abbreviations:

Benzylamino-purine (BAP) Kinetin (KIN) Indole 3-acetic acid (IAA) ά Naphthalene acetic acid (NAA) and 2, 4-Diclorophenoxy acetic acid (2, 4-D), ADS- Adenine sulphate Plant Growth Regulator (PGR), multiplication to multiplication (M-M), multiplication to rooting (M-R).

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1. Introduction

Murraya koenigii found throughout tropical and subtropical East Asia, from India and China to New Caledonia and Northeastern Australia (Xie et al., 2006). Curry leaf is a small tree with dark grey bark and compound leaves (Jayaweera, 1982). In India, this plant is commonly found in the outer Himalayas, from the Ravi eastwards, ascending to 5,000 feet, in Assam, Chittagong, Upper and Lower Burma. It is also found in evergreen and deciduous forests of peninsular India. The shrub is of common occurrence in Himachal Pradesh in areas lying between 800 and 1,450 metres above the sea level. Almost every part of this plant has a strong characteristic odour. A small spreading shrub, about 2.5 metres high, the main stem is dark green to brownish with numerous dots on it. Leaves, exstipulate, bipinnately compound, 30 cm long, each bearing 24 leaflets having reticulate venation. Leaflet is lanceolate, 4.9 cm long, 1.8 cm broad, having 0.5 cm long petiole. Flowers, bisexual, white, funnel-shaped, sweetly scented, stalked, complete, ebracteate, regular, actinomorphic, pentamerous and hypogynous. Inflorescence, terminal cyme, each bears 60 to 90 flowers, calyx, 5 lobed. Androecium is polyandrous, inferior, with 10 stamens, dorsifixed, arranged into circles of five each; smaller stamens, 4 mm. long whereas the longer ones, 5 to 6 mm; gynoecium, consists of 5 to 6 mm long stigma, bright, sticky; style, ovary, superior. Fruits, round to oblong, 1.4 to 1.6 cm long, 1 to 1.2 cm in diameter, fully ripe fruits are black with a very shining surface with blue pulp, the number of fruits per cluster varying from 32 to 80. Seed, one and/or more in each fruit, 11 mm long, 8 mm in diameter with spinach green in colour. Flowering starts from the middle of April and ends in the middle of May.

The leaves bark and roots of *Murraya koenigii* can be used as a tonic and a stomachache. They are also used externally to cure eruptions and the bites of poisonous animals. Strong odiferous oil occurs in the leaves and the seeds of this plant. An alkaloid, mukonine, is also found in this plant (Chakrabarty et al., 1978). The seed occupies the major part of the fruit and the edible portion is only 49.4 per cent of the whole fruit. The fruits are very sweet and are eaten fresh. They have a characteristic odour, which makes them slightly unpleasant. As revealed by the chemical composition of the fruits, they are very nutritious. The leaves are used as a spice in different curries and impart a very good flavour to the preparations. These fruits have also many medicinal properties. The branches of *Murraya koenigii* are very popular for cleaning the teeth as datun and are said to strengthen the gums and the teeth. This plant is quite ornamental due to its compound leaves. They have a distinct, spicy curry like flavor and odor. The tree prefers warm temperatures with full sun to partial shade. In nature *M. koenigii* propagates by seeds but methods of conventional propagation take a long time for multiplication because of poor rate of fruit set and/or poor germination and also clonal variations through seeds. The naturally growing plants are main sources of phyto-pharmaceutical preparation. Due to the increasing demand of crude drugs, medicinal plants are being overexploited and disappearing at an alarming rate from their natural habitats. With the availability of advanced biotechnological methods, there is a need to update the available protocols of *Murraya koenigii,* especially using non-meristamatic tissues so that not only fast micro propagation protocols, but also the model cultures for any desired genetic transformation in the yields, could be made available. The biochemical analysis especially evaluation of total phenols and flavonoids and antioxidant activity of different parts of *Murraya koenigii* would quantify their latent chemical constituent to ensure their scientific utility. Highly reactive free radicals such as superoxide radicals, hydroxyl radicals, non free radical species and oxygen species are present in biological systems from a wide variety of sources such as environmental pollutions, chemical toxins and physical stress which are associated with cellular and metabolic injury. The free radicals may oxidize nucleic acids, proteins and lipids and cause depletion of immune system, change in gene expression and induce abnormal proteins accelerating aging gastritis, cancers cardiovascular and neuro degenerative **(Ames 1983, Gey 1990, Stadtman 1992, Sun 1990, Diaz et al., 1997).**

2. Materials and Method

***In vitro* Culture Technique**

Collection and preparation of explants:

The disease free, juvenile and healthy explants were selected for carrying out study as young cells is supposed to have retained their totipotency. The nodal, axillary bud explants were collected for establishment of *in vitro* cultures. The explants were treated with tween-80 solution for 15 min. followed by several washes with tap water and finally rinsed with distilled water.

Surface sterilization of explant:

The washed explants were treated with different concentration of various sterilizing agents for different duration of treatment. The surface sterilizing agent mercuric chloride (0.1-0.5%w/v) was used. The treatment of surface sterilization with suitable agent was followed by washing of explants 4-5 times with autoclaved distilled water for removal of surface sterilizing agent from the surface of explant. The explants were also treated with some specific fungicides where fungal contaminants were observed in the explants.

Inoculations, sub culture and multiplication of propagules:

After establishment of explants aseptically in culture media these were subcultured either on same medium or on the modified medium with some changes in ingredients and growth hormones like Benzyl amino purines (BAP); Kinetin (Kin); Indole 3- acetic acid (IAA), Naphthalene acetic acid (NAA), Indole 3- butyric acid (IBA), 2,4-Dichlorophenoxy acetic acid (2,4-D). The explants were sub-cultured onto fresh media every 4-5 weeks. When the explants started to multiply, well grown micro shoots were separated with the help of a sterile scalpel under the hood and put in the same media for further multiplication. The shoot-lets derived from each explant were tracked individually to determine the total number of plants produced from single seed and their subsequent genetic identity. The *in vitro* raised cultures were further multiplied by sub-culturing them on to same or modified media.

Rooting Media:

Eight to 10 cm long aseptic shoots when raised in sufficient numbers; these were transferred to root inducing media like White's MS basal, MS ½ salt strength ¾ and ¼ strength of MS Salts for induction of roots. *In vitro* produced shoots were also transferred for rooting into the other media like B5 basal medium, WP medium and WS medium supplemented with vitamin, amino acids, and various root inducing hormones like NAA, IAA, and IBA. The physical conditions, concentrations and combination of growth regulators, vitamins, amino acids and inorganic salts of media were changed according to the need of culture.

Culture conditions:

The cultures were maintained at the temperature of 25$\pm$2°C with 16 hrs illumination of light of intensity 2000 to 2500 lux by cool fluorescent tubes and incandescent bulbs and 8 hrs dark. The temperature and light were varied according to the experiments.

Transplantation and acclimatization of the plantlets:

The regenerated plants were taken out from culture vessel and roots of the plants are gently washed to remove the agar sticking to them. The agar-agar adhered to the roots and shoots was removed carefully and gently. These plants were kept in distilled water for 15-20 minutes and washed again with distilled water for 5-6 times. The plants were then treated with systemic fungicide, carbendazim (Bavistin) 0.1% for 5 minutes and finally washed with distilled water to remove fungicide. These plantlets were transferred to earthen pots containing soil mixture. The different soil mixtures in various ratios were tried for pot transfer of plantlets. In *Murraya koenigii* the soil of natural habitat, vermiculate and gravels were used in the pots. The plants were transferred in the pots. The pots were put in a hardening unit or a green house under a gradient of temperature (30-35ºC) and relative humidity (70-45%) from pad to fan section of green house. The sun light intensity was gradually increased from pad to the fan section of hardening unit using agro-net of various grades inside the green house. During acclimatization initially the plantlets were kept near the pad section of unit and they were gradually shifted from pad end to the center of unit and then to the fan end of the unit in the interval of 10 days. After keeping micro plants in hardening unit for 30 days they were finally shifted to the shade house and then to the field conditions.

3. Results and Discussion

Results

Effect of BAP on shoot initiation: Surface sterilized juvenile stem explants of *Murraya koenigii* were inoculated aseptically on MS liquid medium containing BAP in the concentration ranging from 2.21µM to 31.07µM. The results showed that the number of shoots produced per explant varied in various concentration of BAP in MS liquid medium shoot regeneration was achieved within 10-12 days but number and further growth of micro-shoots were varied in different concentrations of BAP. On the MS medium without PGR only one shoot initiated from each axillary bud of explant. The enhancement of shoot primordial began at the BAP concentration level of 4.43uM and maximum number of explants (94.4%) showed regeneration with 4-5 of micro-shoots on MS medium containing17.75uM BAP **(Table-1)**. On the same medium the shoots attained a length of 17-18mm in 25-30 days.

Further increase in the concentration of BAP was found to be inhibitory for induction of multiple shoots and their growth.

Table 1. Effects of BAP in MS liquid medium on shoot induction from axillary bud explant of *Murraya koenigii*. (Figure- 1a and 1b)

S. No	BAP (µM)	Percentage of explant sprouted (Mean± SD)	Number of shoots per explant (Mean± SD)	Shoot length (mm) (Mean ± SD)
1	0.0	59.4 ± 0.30	1.2 ± 0.04	3.5 ± 0.07
2	2.21	65.4 ± 0.04	1.6 ± 0.05	6.6 ± 0.07
3	4.43	72.8 ± 0.58	2.0 ± 0.07	7.6 ± 0.05
4	8.87	80.6 ± 0.40	2.6 ± 0.05	11.8 ± 0.06
5	13.31	85.0 ± 0.54	3.2 ± 0.07	15.8 ± 0.09
6	17.75	94.4 ± 0.04	4.0 ± 0.07	17.7 ± 0.04
7	22.19	88.0 ± 0.54	3.4 ± 0.05	12.0 ± 0.05


A **B**

Figure 1a. Shoot Regeneration on MS liquid +17.75 µM BAP, 2b. Shoot Regeneration on MS semi solid +17.75 µM BAP

Effect of Kinetin:

The shoot formation was also achieved from juvenile explants on MS liquid medium supplemented with various concentrations of kinetin. On medium containing kinetin (2.32µM to 3.93µM) only one to three shoots were produced from each node with 10mm length **(Table-2).** The higher concentrations of kinetin (18.58µM to 27.87µM) in the medium inhibited the shoot proliferation.

Table 2. Effects of kinetin + MS liquid medium on shoot initiation from axillary bud explant of *Murraya koenigii*. (Figure-2a,b,c)

S. No.	Kinetin (µM)	Percentage of shoot initiation (Mean± SD)	No. of shoots per explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	2.32	60.0 ± 0.58	1.4 ± 0.05	5.4 ± 0.01
2	4.64	69.4 ± 0.70	2.2 ± 0.08	6.0 ± 0.07
3	9.29	76.0 ± 0.54	2.4 ± 0.08	7.2 ± 0.03
4	11.93	92.0 ± 0.39	3.0 ± 0.07	10.6 ± 0.05
5	18.58	87.6 ± 0.70	2.2 ± 0.08	8.0 ± 0.05
6	23.23	84.2 ± 0.34	2.4 ± 0.05	8.4 ± 0.03



A **B** **C**

Figure 2a. Micro shoots regeneration on MS (liquid) + Kin. (11.93µM), 2b and 2c. on MS (semi solid) + Kin. (11.93µM).

Effect of BAP + IAA/NAA:

In previous experiment BAP 17.75µM in liquid medium was found suitable for regeneration of 4.0 ± 0.70 shoots, therefore, in present experiment the concentration of BAP 17.75µM was kept constant with varied concentrations of IAA from 0.57µM to 5.7µM **(Table-3)** and NAA from 0.53µM to 5.37µM **(Table-4)**.From the results of this experiments I was observed that the combination of BAP with IAA/NAA was not found effective to enhance percentage response of explant for shoot induction and also number and growth of micro-shoots in vitro, even percentage response of explant for regeneration was decreased to 96.6 ± 1.14 on the medium containing BAP and NAA with little callusing.

Table 3. Effects of BAP (17.75µM) with IAA in MS liquid medium on shoots initiation from axillary bud explants of *Murraya koenigii*. (Figure-4)

S. No	I.A.A (µM)	Percentage of shoot initiation (Mean± SD)	No. of shoots per explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	0.57	92.8 ± 0.83	3.6 ± 0.05	17.8 ± 0.08
2	1.71	93.2 ± 0.48	3.8 ± 0.08	18.4 ± 0.05
3	2.85	98.2 ± 0.83	4.8 ± 0.08	19.4 ± 0.05
4	3.99	95.8 ± 0.83	3.8 ± 0.08	18.2 ± 0.08
5	5.70	94.6 ± 0.89	3.0 ± 0.07	17.2 ± 0.08

Table 4. Effects of BAP (13.93 µM) with NAA in MS liquid medium on shoot initiation from axillary bud explant of *Murraya koenigii*. (Figure-5)

S. No	NAA (µM)	Percentage of shoot initiation (Mean± SD)	No. of shoots per explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	0.53	90.2 ± 0.92	3.0 ± 0.07	16.2 ± 0.08
2	1.61	91.0 ± 0.58	3.4 ± 0.05	17.4 ± 0.08
3	2.68	95.2 ± 0.30	3.8 ± 0.08	18.2 ± 0.08
4	3.75	96.6 ± 0.14	4.2 ± 0.08	18.6 ± 0.03
5	4.83	94.4 ± 0.14	3.2 ± 0.03	18.4 ± 0.01
6	5.37	90.4± 0.14	2.8 ± 0.04	17.8 ± 0.09

Figure.4

Figure.5

Figure.4 Shoot regeneration with callus on BAP (17.75µM) + IAA (2.85µM), Figure-5. Multiple shoot formation with callus on BAP (17.75µM) + NAA (3.75µM).

Effect of kinetin + IAA:

Results showed that the kinetin in combination with IAA was not found as effective as BAP for induction and growth of shoots from axillary buds. The kinetin (13.93µM) with IAA (0.57µM to 5.70µM) was incorporated in the medium. The combination of Kinetin and IAA was also found ineffective for further increase of shoot numbers and their growth **(Table-5).**

Table 5. Effects of optimum kinetin (13.93 µM) + IAA + MS liquid medium on shoots initiation (regeneration) from axillary buds of *Murraya koenigii*.

S. No	I.A.A (µM)	Percentage of shoot initiation (Mean± SD)	No. of shoots per explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	0.57	92.0 ± 0.58	3.0 ± 0.07	10.6 ± 0.04
2	1.71	93.8 ± 0.30	3.4 ± 0.05	10.8 ± 0.08
3	2.85	97.0 ± 0.70	3.8 ± 0.08	11.4 ± 0.01
4	3.99	95.0 ± 0.58	3.4 ± 0.05	11.2 ± 0.08
5	5.70	92.8 ± 0.30	3.0 ± 0.07	10.8 ± 0.08

Combined effect of BAP and kinetin:

In order to further enhance the multiplication of shoot primordia from axillary buds of explants, attempts were made by incorporating BAP and kinetin in combination in MS liquid medium in the range of 2.21µM to 17.75µM and 2.32µM to 18.58µM respectively. Amongst all the concentrations of BAP with kinetin used in combinations on BAP (11.09µM) + kinetin (11.61µM) 96.8% explants **(Table-6)** exhibited shoot regeneration and their growth increased up to 18-19 mm but number of shoots (4-5) remained un-changed. The higher concentration of BAP + Kinetin was found to be inhibitory for shoot regeneration and their growth *in vitro*.

Table 6. Effects of different concentration of BAP and kinetin in MS liquid medium on shoots initiation from axillary bud explant of *Murraya koenigii*. (Figure-6)

S. No	BAP + Kinetin (µM)	Percentage of shoot initiation (Mean ± SD)	No. of shoots per explant (Mean ± SD)	Length of shoots in MM (Mean ± SD)
1	2.21 + 2.32	70.0 ± 0.54	2.0 ± 0.07	07.2 ± 0.07
2	4.43 + 4.64	76.6 ± 0.94	2.6 ± 0.05	12.4 ± 0.04
3	8.87 + 9.29	82.6 ± 0.40	2.8 ± 0.04	15.8 ± 0.04
4	11.09 + 11.61	96.8 ± 0.92	4.2 ± 0.03	18.8 ± 0.08
5	13.31 + 13.93	89.0 ± 0.58	3.0 ± 0.07	15.8 ± 0.03
6	17.75 + 18.58	85.0 ± 0.58	2.6 ± 0.05	14.2 ± 0.07

Figure.6

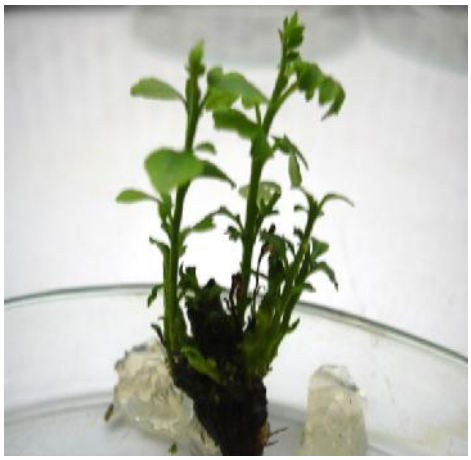

Figure.7

Figure 6. Formation of multiple shoots on MS + BAP (11.09µM) Kinetin (11.61µM), Figure-7. Multiple shoots formation on MS + BAP (11.09µM) + Kinetin (11.61µM) + IAA (2.85µM)

On the basis of results of previous experiments the BAP (11.09µM) in combination with Kinetin (11.61µM) in MS liquid medium was found suitable for optimum growth of in vitro shoots, therefore, this combination of BAP (11.09µM) +Kinetin (11.61µM**)** in MS liquid medium was used in combinations with auxins (IAA and NAA) for further experimentation to increase multiple shoots and their growth from axillary bud explants of *Murraya koenigii*

Effects of (BAP 11.09µM) + kinetin (11.61µM) + IAA:-The results obtained are presented in Table-7.This experiment was conducted to enhance the shoot numbers and their growth but 4-5 shoot were regenerated from axillary bud of each explant and growth of shoots was further decreased with little callusing at the basal end of the regenerated shoots. The maximum 14.8 ± 0.83 mm shoot growth was achieved in 30-35 days on MS medium supplemented with BAP (11.09µM) + kinetin (11.61µM) and IAA 1.71µM.Further increase in concentration of IAA moderate callusing was observed with minimal growth of shoots.

Table 7. Effects of different concentration of IAA in MS liquid medium supplemented with BAP (11.09µM) + Kinetin (11.61µM) on shoot induction from axillary bud explants of *Murraya koenigii*. (Figure-7)

S. No	I.A.A (µM)	Percentage of shoot initiation (Mean± SD)	No. of shoots per explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	0.57	97.0 ± 0.58	4.4 ± 0.01	14.0 ± 0.07 *
2	1.71	97.2 ± 0.83	4.8 ± 0.06	14.8 ± 0.08 *
3	2.85	98.2 ± 0.83	4.4 ± 0.05	13.8 ± 0.08 **
4	3.99	97.4 ± 0.14	4.0 ± 0.02	12.4 ± 0.05 **
5	5.70	96.6 ± 0.14	3.8 ± 0.08	11.0 ± 0.07 **

*Little Callusing, ** Moderate Callusing

From the results of previous experiments it was observed that addition of IAA with BAP and kinetin in MS liquid medium the percentage response of explants for shoot induction from axillary bud explant was un-changed (95% to 98%), shoot number and their growth did not enhanced. In order to further enhance the shoot numbers, growth and to get stronger shoots adenine sulphate was tested in different concentrations with optimized concentrations of BAP (11.09µM) and kinetin (11.61µM) in MS liquid media.

Effect of Adenine sulphate with BAP and Kinetin:

The adenine sulphate was added in the concentration ranges between13.57µM to 81.44µM **(Table-8).** On the medium containing lower concentrations of adenine sulphate from13.57µM to 54.29µM, shoot regeneration was poor (4-5 shoots) with little increase in the growth of shoots to 20mm in the period of 30-35 days. The MS liquid medium containing 81.44µM Adenine Sulphate with pre-optimized concentrations of BAP (11.09µM) and Kinetin (11.61µM) was found optimum for shoot regeneration potential of axillary bud explants of *M .koenigii* on which maximum number (8-9shoots) of shoots were obtained which attained a height of 42.6 ± 1.14mm.

Table 8. Effects of different concentration of Adenine sulphate with BAP (11.09µM) +Kinetin (11.61µM) in MS liquid medium on shoots induction from axillary bud explant of *Murraya koenigii*. (Figure-8)

S. No	Adenine-Sulphate (µM)	% of shoot initiation (Mean± SD)	No. of shoots per Explant (Mean± SD)	Length of shoots in MM (Mean± SD)
1	**13.57**	**91.4 ± 0.14**	**4.4 ± 0.01**	**18.0 ± 0.07**
2	**27.14**	**91.2 ± 0.83**	**4.8 ± 0.01**	**18.2 ± 0.06**
3	**40.72**	**92.2 ± 0.48**	**4.2 ± 0.03**	**18.6 ± 0.06**
4	**54.29**	**94.6 ± 0.96**	**4.8 ± 0.01**	**20.0 ± 0.02**
5	**67.87**	**98.2 ± 0.83**	**7.0 ± 0.01**	**26.6 ± 0.01**
6	**81.44**	**97.6± 0.34**	**8.8 ± 0.02**	**42.6 ± 0.05**

Figure 8. In vitro multiple shoot induction from axillary bud explant on MS (liquid) with BAP (11.09µM) + Kinetin (11.61µM) + ADS (81.44µM)

Multiplication of propagules in *Murraya koenigii*

Once the optimum physico-chemical conditions for induction and growth of shoots from axillary bud explant were defined, the *in vitro* shoot cultures were excised as such in the form of a cluster and were cut into two or more bunches each consisted of 4-5 shoots and each bunch was called a propagule. In this way if one mother propagule

produced ten shoots after a period of 30-35 days incubation then the same propagule was cut into two daughter propagules the number of daughter propagules (4-5shoots) achieved and from each mother propagule was presented as rate of multiplication to multiplication (M-M). At the time of sub-culture of mother propagule the elongated shoots (3-4cm) were excised individually and were transferred for rooting. The number of shoots harvested for rooting from each mother propagule was presented as rate of multiplication to rooting (M-R) in the results. The experiments for shoot multiplication were conducted in liquid as well as in semi-solid (With agar-agar) MS media but chlorosis and browning of shoots were not observed in semi solid media in multiplication experiments, therefore, only semisolid MS media were used for subsequent experimentations of multiplication.

Effects of BAP/kinetin

The BAP (2.21µM to 22.19µM) and kinetin (2.32µM to 23.23µM) was added in MS medium for multiplication and rooting. In the BAP added medium multiplication rate was found 2.0 ± 0.09 on 2.21BAP and the M-M rate was remained constant up to the higher concentration of BAP 22.19 µM. In this experiment the rate of multiplication to rooting (M-R) was varied. The M-R was 4.22 ± 0.83 and it enhanced to 6.28 ± 0.83 on MS medium containing BAP 17.75µM which was further decreased on higher concentration of BAP. The kinetin was also found to be in effective to increase rate of M-M and M-R and kinetin was found to be less effective as compare to BAP **(Table-9).**

Table 9. Effects of different concentrations of BAP/ kinetin in MS Semi-solid medium on shoots multiplication of *Murraya koenigii* (Figure-9)

S. No	Kinetin (µM)	BAP (µM)	Rate of multiplication to multiplication (M-M) (Mean ± SD)	Rate of multiplication to Rooting (M-R)(Mean ± SD)
1	0.0	0.0	2.0 ±0.09	4.22 ± 0.83
2	-	2.21	2.0 ± 0.07	4.24 ± 0.54
3	-	4.43	2.4 ± 0.09	5.16 ± 1.14
4	-	8.87	2.6 ± 0.04	5.04 ± 1.14
5	-	13.31	2.6 ± 0.04	6.16 ± 0.54
6	-	17.75	2.2 ± 0.03	6.28 ± 0.83
7	-	22.19	2.0 ± 0.09	5.38 ± 1.48
8	2.32	-	2.6± 0.06	2.22± 0.09
9	4.64	-	2.2 ±0.04	2.12 ±0.03
10	9.29	-	2.8 ±0.44	2.16 ±0.04
11	13.93	-	2.2 ±0.44	3.04 ±0.10
12	18.58	-	2.2 ±0.83	3.14 ±0.14
13	23.23	-	2.8 ±0.44	4.08 ±0.15

In previous experiment M-M and M-R was very poor and to enhance the rate of M-M and M-R. The IAA with optimized BAP (13.31 µM) was added in the MS medium.

Effect of IAA with BAP (13.31µM):

The addition of IAA in MS medium with BAP13.31µM for enhancement of rate of multiplication of propagule as well as rate of multiplication for rooting is shown in **Table (10).** On the medium containing IAA 0.57µM with BAP13.31µM the M-M was 2 fold and by increasing the concentration of IAA the rate of M-M was further decreased to 1.62± 0.21 and callusing on the base of shoot were observed on the same medium. M-R rate was also decreased from 4.02 ± 0.08 to 3.22 ± 0.10. Hence, the IAA in different concentration with BAP13.31µM was again not found suitable for enhancement of M-M and M-R. As we have observed in previous experiments that ADS was found suitable for induction of maximum number of shoots from axillary bud explant of *M. koenigii* the ADS was again used for multiplication of *in vitro* raised shoots with BAP13.31µM.

The addition of ADS in the medium with BAP13.31µM effectively increased the M-M rate. On lower concentration of ADS 13.57µM to 54.29µM. The mother propagule exhibited 4 to 5 fold M-M and 6 to 7 M-R but in higher concentration of ADS 67.87µM and 81.44µM the M-M rate was increased to 5.66 fold and M-R raised to 8.9 fold **(Table 10).** On the basis of results of above experiments it was observed that BAP13.31µM with ADS 81.44µM was found to be suitable for regeneration of propagules as well as the root able shoots. To develop a commercially viable protocol it was found necessary to further increase the M-M and M-R, Therefore the variable concentration of ADS with pre-optimized concentration of BAP (13.31µM) and kinetin (13.93µM) were used.

Table 10. Influence of Various concentrations of IAA and Adenine sulphate with optimum BAP (13.31µM) on shoot Multiplication of *Murraya koenigii* (Figure-10 a, b)

S. No	IAA (µM)	Rate of M-M (Mean ± SD)	Rate of M-R(Mean ± SD)
1	0.57	2.8 ±0.04	4.02 ±0.08
2	1.14	2.01 ±0.07	4.22 ±0.12
3	1.71	2.08 ±0.08	3.88 ±0.11
4	2.28	1.62 ±0.12	3.08 ±0.08
5	2.85	1.85 ±0.03	3.26 ±0.09
6	3.42	1.09 ±0.01	3.22 ±0.10
S. No.	**Adenine Sulphate (µM)**	**Rate of M-M (Mean ± SD)**	**Rate of M-R (Mean ± SD)**
1	13.57	3.0 ±0.02	6.8 ±0.08
2	27.14	3.0 ±0.03	6.28 ±0.07
3	40.72	4.0 ±0.02	6.88 ±0.09
4	54.29	4.22 ±0.05	7.22 ±0.08
5	67.87	5.10 ±0.04	8.28 ±0.09
6	81.44	5.66 ±0.06	8.92 ±0.07

Figure.9

Figure.10a

Figure.10b

Effect of ADS with BAP (13.31µM) and kinetin (13.93µM):

The ADS was added in concentration range of 13.57µM to 81.44µM with pre-optimized BAP concentration 13.31µM and kinetin 13.93µM. At lower concentration of ADS 13.57µM the M-M was 5.01±0.01 and M-R was 6.01± 0.03. The rates for M-M and M-R were significantly increased to 6.89±0.04 and 10.11±0.06 respectively in the medium containing 81.44µM ADS with BAP and kinetin. By further increasing the concentration of ADS was not effectively enhanced the M-M and M-R. The rate of results of all the experiments conducted for multiplication of cultures from axillary bud explants of *Murraya koenigii.* It was found that BAP 13.31µM and kinetin 13.93µM and ADS 81.44µM in MS semisolid medium was found to be most suitable for high frequency multiplication of shoots for M-M and M-R.

Table 11. Influence of Various concentrations of ADS with optimum BAP (13.31µM) + optimum Kin (13.93µM) on shoots Multiplication of *Murraya koenigii* (Figure-11 a, b, c)

S. No	Adenine Sulphate (µM)	Rate of M-M (Mean ± SD)	Rate of M-R (Mean + SD)
1	13.57	5.01 ±0.01	6.01 ±0.03
2	27.14	5.11 ±0.08	6.82 ±0.01
3	40.72	5.21±0.08	8.12 ±0.07
4	54.29	5.81 ±0.09	8.99 ±0.06
5	67.87	6.22 ±0.07	10.22 ±0.05
6	81.44	6.89 ±0.04	10.11 ±0.06


Figure.11a


Figure.11b


Figure.11c

Table 12. Effects of IBA + MS Semisolid medium on root induction of axillary shoots of *Murraya koenigii* (Figure-12)

S. No	IBA (µM)	% of Rooting (Mean ± SD)	Root length in MM(Mean ± SD)	Callusing
1	0.0	17.2±0.83	19.5 ± 0.83	-
2	4.92	25.4±2.30	22.5 ± 0.70	-
3	9.84	60.4±1.14	23.0 ± 1.48	-
4	14.76	74.4±1.14	23.5 ± 1.14	-
5	19.68	85.8±0.83	25.5 ± 0.83	+
6	24.60	89.8±1.0	24.5 ± 0.54	+
7	29.52	91.0±1.0	24.0 ± 0.70	+

Table 13.Effects of IBA +IAA + MS medium on root induction of axillary shoots of *Murraya koenigii* (Figure-13 a, b)

S. No	IBA + IAA (µM) (µM)	Percentage of rooting (Mean ± SD)	Root length in MM (Mean ± SD)	Callusing
1	2.46 + 0.57	33.2 ±1.92	20.0 ± 0.83	-
2	4.92 + 1.71	42.6 ±1.67	22.5 ± 1.48	-
3	7.38 + 2.85	62.6 ±1.67	23.0 ± 1.14	-
4	9.84 + 3.99	72.2 ±1.48	23.5 ± 1.58	-
5	12.30 + 5.13	95.6 ±1.14	24.0 ± 1.30	+
6	14.76 + 5.70	94.0 ±0.70	23.0 ± 0.83	+
7	17.22 + 7.13	86.0 + 0.70	20.0± 0.73	++


Figure.12


Figure.13a


Figure.13b

Table 14. Effects of different media and their nutritional strength with IBA (12.30µM) and IAA (5.13µM) on rooting and survival rate of axillary shoots of *Murraya koenigii* (Figure-14 a, b, c)

S. No	Types of culture media	% of rooting (Mean ± SD)	% of survival under *in vitro* hardening (Mean ± SD)	% of survival under ex *vitro* hardening (Mean ± SD)	% of survival under field condition (Mean ± SD)
1	MS full strength	95.4 ± 1.14	98.0± 1.39	88.0± 1.21	82.8± 1.12
2	WP full strength	93.2± 1.09	96.2± 0.13	86.2± 0.19	81.4± 0.19
3	White's full strength	94.2± 0.19	95.8± 0.19	85.6± 0.19	80.8± 0.11
4	MS ½ strength	90.2± 0.14	98.2± 0.79	88.6± 0.09	83.6± 0.13
5	WP ½ strength	89.8± 1.13	97.0± 1.14	87.0± 0.29	82.2± 0.21
6	White's ½ strength	90.2± 0.17	96.8± 0.21	86.2± 0.17	81.4± 0.24
7	MS ¼ strength	85.6± 1.19	98.8± 0.11	88.8± 0.17	92.4± 1.17
8	WP ¼ strength	86.4± 0.09	97.2± 0.13	87.4± 0.21	89.4± 0.23
9	White's ¼ strength	86.8± 0.11	97.2± 0.16	87.2± 0.21	88.2± 0.31

Figure.14a

Figure.14b

Figure.14c

***In Vitro* Rooting:**

The elongated 35 to 40mm long shoot from 30 to 35 days incubated propagules or bunches regenerated from nodal explants were incubated aseptically on different culture media containing various concentration of rooting to induce roots in them for production of complete plantlets.

Effect of IBA:

The elongated *in vitro* raised shoots (35 to 40mm) excised from propagules were inoculated on MS media containing IBA individually. The IBA was added to the MS semisolid media in the concentration ranging between 4.92µM to 34.14µM. The results obtained are presented in **Table-12.** On control medium (without PGR) the axillary shoots exhibited 17.2 ± 0.83 percentage rooting. The percentage of rooting in different concentration of IBA alone varied in axillary shoots. The rooting percentage of axillary shoots did not increased in concentration of IBA higher than 24.60 µM.

Effect of IBA + IAA:

In order to further enhance the rooting percentage in axillary shoots a combination of IBA and IAA in different concentration was used. The data are presented in **Table-13**. In this experiment IBA was added in the lower concentration ranging between 2.46µM to 17.22µM and IAA was taken in concentration ranging between 0.57µM to 7.13µM. The data revealed that the combination of IBA and IAA is suitable for induction of 95.6 ±1.14 percentage rooting in axillary bud shoots (Fig.13 a, b) on IBA 12.30µM as with IAA 5.13µM in MS medium.

Effect of culture media strength on rooting and post rooting survival:

The rooting hormone IBA (12.30µM) + IAA (5.13µM) in MS medium for axillary shoots was optimized in previous experiment and maximum rooting percentage (89.8± 0.83) was achieved in axillary shoots *in vitro*. After optimization of physico-chemical condition for rooting of axillary shoots, the axillary shoots were inoculated on the different types of media and there nutritional strength to observe further effect on rooting behaviour of shoots as well as their effect on survival rate of rooted plantlets under different stages of hardening and their field survival. The data recorded showed (**Table-14**) that the rooting behaviour of axillary shoots did not affected significantly on different media and their nutritional strength. The rooting percentage of axillary shoots was in the range of 85 to 89 percent. The rooted plantlets were first transferred to the soilrite filled in the culture bottle and kept under growth room conditions (Fig. 14a, b and c). The *in vitro* plantlets were incubated under *in vitro* hardening conditions for two weeks and then they were transferred to polybags and were kept in green house/hardening chamber in a gradient of temperature and humidity for one month and were finally transferred to the field conditions. The axillary shoots rooted on these media exhibited significant variations in survival rates during hardening stages and their field transfer. The rooting percentage of axillary shoots (**Table-14**) was between 96 to 97% on full strength salt of MS, WP and White's media. The rooted plantlets developed on full strength salts of all the types of media exhibited 94 to 96% survival under green house conditions (*ex vitro* hardening) which were further transferred to field conditions where 80% of *ex vitro* hardened plants were survived. Similarly the survival rates under different hardening conditions and in field were recorded for half strength nutrients and ¼ strength of nutrients of MS, WP and White's media and it was observed that the shoots axillary origin rooted on ¼ salt strength of MS, WP, and White's media showed maximum survival under *in vitro* hardening conditions (98%) *ex vitro* hardening conditions (88%) and field conditions (90%).

Discussion:

The microplants regeneration through matured explants is considered to one of the most promising ways for multiplying a selected clone true to its type. Such individuals are genetically similar showing the same genotypic and phenotypic characters. In order to develop a reproducible protocol (after optimization of explant type, size and surface sterilization technique) further experiments were conducted to induce multiple shoots and their establishment *in vitro*, through axillary bud proliferation. After extensive experimentation for shoot induction from axillary bud explants various combination and concentration of growth regulators were tried on semi-solid as well as liquid nutrient media and finally it was concluded that texture of media play key role for shoot induction in *Murraya koenigii*. The explants inoculated on semisolid media (with agar-agar) showed browning and leaching after 2-3 weeks they became brown due to the leach outs of phenolic compounds into the media by explant tissues. Addition of antioxidants likes ascorbic acid, citric acid and PVP could not over come the problem of browning of explant as well as the media. Leaching of toxic phenolics by the explant tissue in to the semisolid media and their accumulation in semisolid media again made available for the explant to re-absorb the phenolics which may prevent the induction of shoot from explant under *in vitro* conditions. The explants were transferred to the liquid media on blotting paper bridges. The explants showed positive response when they were inoculated on liquid MS media with various combinations and concentrations of plant growth regulators such as Kinetin, BAP, IAA and adenine-sulphate. Though reports are available on multiple shoot regeneration from stem segments of *Murraya koenigii* Bhuyan, et al., (2004) have reported to develop reproducible procedure for large-scale propagation of *Murraya koenigii* using seedling explant on MS media supplemented with 5.0mg/lit BAP. Similarly Iyer and Gopinath, (1999) have published morphogenesis of immature embryo of *Murraya koenigii*, but development of protocol through seedling and embryo is not advantageous for clonal propagation of plants.

In present investigation the shoot regeneration through matured explant of *Murraya koenigii* was achieved, which is the most advantageous for cloning. Reports are also published on plant regeneration through matured stem segments (Rout, 2005; and Hazarika, et al., 1995). In the protocol developed by Nirmal Babu et al., (2000) the 12-30 shoots were achieved on woody plant medium supplemented with 4.4µM BAP and 4.65µM Kinetin and the multiple shoot regeneration was achieved on MS medium containing 2.5mg/lit BAP, 0.75mg/lit IAA and 0.25mg/lit adenine sulphate with 3% sucrose. None of the publication reports the regeneration of *in vitro* shoots from axillary bud explants in liquid medium on blotting Paper Bridge where as the present investigation reports first, on regeneration of multiple shoots on MS liquid medium containing BAP (11.09µM), Kinetin (11.61µM) and adenine sulphate (81.44µM). The important findings were recorded during the present study was the supplementation of higher concentration of adenine sulphate (81.44µM). Promotes the regeneration and growth of shoots, as 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 vigour, therefore the explants and the shoots in the adenine sulphate supplemented liquid MS medium exhibited rejuvenation after each sub culture. It was also observed that the cultures on the medium with lower concentration of adenine sulphate or without ADS could not maintain their vigour for longer time under *in*

vitro conditions. The use of liquid medium also helps in preventing browning of explants or culture because the leach outs of explant tissue were settling down at the bottom of culture vessels which again helped in maintaining longevity of same explant to regeneration shoots in subsequent sub-culture. In present study the rate of multiplication of *in vitro* shoots are designated of propagule (clump of shoots) which is the correct approach for commercial production in tissue culture industry. Present investigation also reports for the first time, the technique for repeated harvesting of multiple shoots for same explants for a period of one year. By this technique the number of multiple shoots regeneration after each subculture was increased. After first subculture the shoot number were multiplied in the multiple of two.

Total number of shoots after lights subculture of same explants reached up to fifty eight. Present investigation also reports the multiplication of shoots in the form of propagules (each propagule consists of 4-5 shoots). Though reports are available on shoot multiplication of *Murraya koenigii in vitro* but multiplication of individual *in vitro* shoot was not achieved in present study. But shoot multiplication was achieved only when clumps of shoots (propagule) were subcultured to fresh media. This is the first report in which protocol in developed by harvesting shoots for two purposes (i) after each subculture 3-4cms elongated shoots in a propagule (cluster of shoots) were excised leaving one to two nodes at the basal portion and are transferred for rooting and propagule was again transferred on fresh media for further multiplication. In this study two directional multiplication was achieved, one, multiplication to multiplication (M-M) and second multiplication to rooting (M-R). The important finding of present investigation is use of liquid media for multiplication of micro shoots and simultaneous harvesting of shoots for rooting as well as for further multiplication which in fact reduce the cost of plant as agar-agar is one of the costly ingredients of tissue culture media. The technique developed during the present study for simultaneous harvesting of shoot clumps for multiplication (M-M) and rooting (M-R) also helpful to avoid again &again surface sterilization of explant, during the experimentations. Hence, the protocol developed in present study is cost effective reproducible and commercially viable.

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