In vitro propagation of *Murraya koenigii* by axillary bud proliferation using mature explants

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*Murraya koenigii* is an important medicinal and aromatic plant. This plant is a native species of arid and semi-arid area of Rajasthan, India. Its leaves used as an analgesic, astringents, anti-dysenteric, antioxidant, hypo-lipidemic and for regulation of fertility. An efficient and reproducible procedure for the large scale propagation of *M. koenigii* is described. High frequency axillary shoot proliferation was induced in mature nodal explants. Regeneration of shoots (8-10) achieved on Murashige & Skoog (MS) medium (liquid) supplemented with benzylaminopurine (11.09 µM), kinetin (11.61 µM) and adenine sulphate (81.44 µM). Rooting (95%) in regenerated shoots occurred in 3 - 4 weeks of transfer on MS basal medium containing 12.30 µM indole butyric acid (IBA) and 5.13 µM indole acetic acid (IAA). In vitro plantlets regenerated from axillary bud proliferation were hardened for four weeks in a green house. The hardened plantlets were transferred to field conditions and high (82 to 85%) survival rate was achieved.

**Keywords:** Curry leaf plant, *Murraya koenigii* micropropagation, nodal explant

**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. Its leaves are slightly pungent, bitter, and acidulous in taste. Fresh and dried leaves are used extensively as a flavoring agent in many Indian culinary practices. 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 pyrano coumarin in various plant parts which are bioactive enabled new vistas in several scientific investigations. These alkaloids are proved to be antimicrobial, antioxidative, anti-diabetic and anti-trichomonal. Some of them showed anti-carcinogenic properties in a cultured human leukemia cell line. The presence of numerous valuable medicinal properties and other usage established its potential demand for export. Recent phyto chemical studies have revealed the presence of antioxidant protein in leaves of *M. koenigii*. There are only a few reports of in vitro studies of *M. koenigii* which are restricted to in vitro shoot multiplication from intact seedling, inter node segments and nodal cuttings. Despite having huge beneficial value, only a few in vitro studies on micropropagation have been reported so far.

The conventional method of propagation of this tree is limited to seeds only, which retain their viability for only a short period. Hence, a biotechnological approach might have an advantage edging over traditional breeding as well as the genetic improvement of *M. koenigii* within a short period. The development of a reproducible regeneration protocol is the prerequisite for ex situ conservation and micropropagation. Present investigation reports an efficient protocol for high frequency regeneration of in vitro plants of *M. koenigii* through axillary shoot formation from repeated re-culturing of nodal explants.

**Materials and Methods**

Nodal segments of *M. koenigii* were collected from approximately 15 - 20 years old mature tree. The explants were first washed twice with Laboline (liquid soap) rinsed thoroughly with distilled water, treated with 0.1% (w/v) mercuric chloride for 5 - 6 min to surface sterilize then washed 5 times for 3 min each with autoclaved distilled water. The surface sterilized explants were cut into 20 - 25 mm pieces. The explants were inoculated on MS medium supplemented with various concentrations and combinations of plant growth regulators. MS liquid and semi-solid medium supplemented with benzylaminopurine (BAP) 2.21 µM.
to 19.97 µM, kinetin 2.32 µM to 13.93 µM and
adenine sulfate (ADS) 81.44 to 244.34 µM.

The regenerated axillary shoots (42 mm) were
excised and sub-cultured on MS medium containing
different concentrations of indole butyric acid (IBA)
2.46 µM to 14.76 µM and indole acetic acid (IAA)
0.57 µM to 5.70 µM for root induction. 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 (Hi Media, India) was added at 8 g L⁻¹
for semi-solid media.

Glass bottles (200 ml) with polypropylene lids
were used as culture vessels for the initiation and
multiplication of cultures. Each glass jar/bottle was
filled with 25 ml of media. Liquid media was poured
into 25 mm × 150 mm Borosil test tubes into which a
Whatman filter paper bridge was placed to hold
explants in a vertical position. All the media,
glassware, forceps and knife were autoclaved at
121°C and 20 psi for 20 min.

The cultures were incubated in a growth room
under a 16-h photoperiod at 2000 - 2500 lux at 25°C.
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 Duncan’s multiple range
test (DMRT) at P < 0.05.

Results

Axillary Shoot Induction

Juvenile and mature nodal explants of *M. koenigii*
collected from same tree were inoculated aseptically
on MS semi-solid as well as on liquid media-
containing cytokinin and auxin in different
concentrations and combinations. The shoot initiation
was observed on both liquid and semi-solid media but
in semi-solid media the shoot induction was poor and
browning of shoots and explants with leaf fall was
observed, therefore, liquid media (without agar-agar)
were used for axillary shoot induction.

Various concentrations of benzyl amino purine (BAP)
2.21 µM to 22.19 µM, kinetin 2.32 µM to 23.23 µM
and ADS 13.57 µM to 81.44 µM alone and in
combinations with auxin IAA 0.57 µM to 5.7 µM
were added in liquid MS medium in order to achieve
maximum number of fast growing shoots from nodal
explants.

Shoot formation occurred at all the concentrations
and combinations of PGRs but shoot number and their
growth varied at different concentration of PGRs.
After repeated experimentations with different
concentrations of BAP and kinetin combined, the MS
liquid medium supplemented with BAP 11.09 µM and
kinetin 11.61 µM was observed suitable for
regeneration of 4.2 ± 0.03 shoots with a length of 18.8
± 0.08 mm from 96.8 ± 0.92 percent explants
(Table 1) in a period of six weeks. On this medium
growth of shoots was not sufficient for root induction.
To enhance the shoot growth the ADS in different
concentrations was added in the medium containing
BAP 11.09 µM and kinetin 11.61 µM.

A maximum of 97.6 ± 0.34 of explants showed
axillary shoot regeneration (Fig. 1A) with a mean of
8.8 ± 0.02 shoots of 42.6 ± 0.05 mm length when
grown on MS liquid medium supplemented with BAP
11.09 µM and kinetin 11.69 µM and ADS 81.44 µM
(Table 1). Increasing the concentration of ADS in MS
liquid medium up to 244.34 µM did not show
significant increase in the shoot numbers and growth.

<table>
<thead>
<tr>
<th>BAP (µM)</th>
<th>Kinetin (µM)</th>
<th>ADS (µM)</th>
<th>Shoots regeneration (%) (Mean ± SE)</th>
<th>Number of shoots/ explant (Mean ± SE)</th>
<th>Length of shoots (mm) (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.21</td>
<td>2.32</td>
<td>50.00</td>
<td>40 ± 1.41 a</td>
<td>3.8 ± 0.74 df</td>
<td>20.0 ± 0.12 b</td>
</tr>
<tr>
<td>4.43</td>
<td>4.64</td>
<td>50.00</td>
<td>55 ± 1.41 bc</td>
<td>4.6 ± 0.48 gh</td>
<td>23.5 ± 0.48 c</td>
</tr>
<tr>
<td>8.87</td>
<td>9.29</td>
<td>81.44</td>
<td>65 ± 1.41 cd</td>
<td>6.6 ± 0.48 ef</td>
<td>26.0 ± 0.16 d</td>
</tr>
<tr>
<td>11.09</td>
<td>11.61</td>
<td>81.44</td>
<td>97 ± 2.28 ef</td>
<td>8.8 ± 0.02 ef</td>
<td>42.6 ± 0.05 a</td>
</tr>
<tr>
<td>13.31</td>
<td>13.93</td>
<td>135.04</td>
<td>85 ± 1.41 abc</td>
<td>7.9 ± 0.74 bc</td>
<td>29.0 ± 0.14 e</td>
</tr>
<tr>
<td>15.53</td>
<td>10.45</td>
<td>135.04</td>
<td>82 ± 1.67 abc</td>
<td>6.8 ± 0.74 ef</td>
<td>21.0 ± 0.10 b</td>
</tr>
<tr>
<td>17.75</td>
<td>11.61</td>
<td>244.34</td>
<td>80 ± 1.41 abc</td>
<td>6.6 ± 0.80 gh</td>
<td>18.0 ± 0.10 f</td>
</tr>
<tr>
<td>19.97</td>
<td>13.93</td>
<td>244.34</td>
<td>75 ± 1.89 cd</td>
<td>4.4 ± 0.48 ij</td>
<td>10.0 ± 0.10 g</td>
</tr>
</tbody>
</table>

P < 0.05; Each value represents the mean ± standard error (SE) of 10 replicates per treatment in three repeated experiments
ADS: adenine sulphate, BAP: benzylaminopurine, MS: Murashige and Skoog
Shoot Multiplication

Six weeks old 42.6 ± 0.05 mm shoots were excised from the nodal explants and the same explants was re-cultured onto the fresh semi-solid shoot induction-medium MS + BAP 11.09 µM, kinetin 11.69 µM and ADS 81.44 µM. Shoot induction from the re-cultured explants was initiated again within 18 to 21 days from date of inoculation. *In vitro* shoots attained 42 mm length after 6 weeks incubation in the growth room. Shoot multiplication was achieved by repeated harvesting of shoots and re-culturing the same explants on fresh semi-solid MS medium containing BAP 11.09 µM, kinetin 11.69 µM and ADS 81.44 µM (Table 2). The re-culture cycle was 6, 12, 18 and 24 weeks. The number of *in vitro* shoots increased every 6 weeks and a maximum 34 shoots (Fig. 1B) were produced from 24 weeks old explants. Regeneration potential of explants was reduced in 30 weeks old explants which yielded 6-7 shoots.

Rooting

Elongated healthy shoots (at least 42 mm long) harvested from explant rooted on rooting medium. Root induction was not observed on shoots transferred to MS medium free of PGRs. The indole butyric acid (IBA), when supplemented in MS medium, it induced roots (Table 3). IBA and IAA at different concentrations showed different responses in terms of percentage and growth of roots *in vitro*. The maximum percentage (95.6 ± 1.14) of rooting was achieved on MS medium it supplemented with 12.30 µM IBA and 5.13 µM IAA (Fig. 1C). Poorest rooting 33.2 ± 1.92 was observed on MS medium containing 2.46 µM IBA 0.57 µM in 3 weeks and roots recorded up to 25.0 mm in length.

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 – 36ºC under green house conditions for 21 days. Survival rate was 82-85% in hardened plantlets. These plants were then transferred to field conditions (Fig. 1D).

Discussion

Earlier studies on the micropropagation of *Murraya koenigii* reported using juvenile and seedling explants. Bhuyan et al. reported micropropagation of *M. koenigii* by axillary proliferation using intact seedlings on MS basal medium. A review on the micropropagation of aromatic plants has been updated recently in the literature. Combinations of 4.4 µM BAP and 4.65 µM kinetin and 2.5 mg/l BAP, 0.25 mg/l IAA and 0.25 mg/l ADS were essential for shoot regeneration on MS medium supplemented with BAP 11.09 µM, kinetin 11.69 µM and ADS 81.44 µM (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Re-culture cycle (week)</th>
<th>Number of shoots</th>
<th>Mean ± SE</th>
<th>Length of shoot (mm)</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>8.8 ± 0.02a</td>
<td>20.0 ± 0.12 ef</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>18.22 ± 0.32b</td>
<td>23.5 ± 0.48gh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>27.11 ± 0.08c</td>
<td>26.0 ± 0.16ij</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>34.17 ± 0.03d</td>
<td>42.6 ± 0.05cd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>6.22 ± 0.32a</td>
<td>29.0 ± 0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P* < 0.05; Each value represents the mean ± standard error (SE) of 10 replicates per treatment in three repeated experiments

### Table 3

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IBA (µM)</th>
<th>IAA (µM)</th>
<th>Rooting (%)</th>
<th>Root length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.46</td>
<td>0.57</td>
<td>33.2 ± 1.92a</td>
<td>20.0 ± 0.83ab</td>
</tr>
<tr>
<td>2</td>
<td>4.92</td>
<td>1.71</td>
<td>42.6 ± 1.67b</td>
<td>22.5 ± 1.48c</td>
</tr>
<tr>
<td>3</td>
<td>7.38</td>
<td>2.85</td>
<td>62.6 ± 1.67c</td>
<td>23.0 ± 1.14ef</td>
</tr>
<tr>
<td>4</td>
<td>9.84</td>
<td>3.99</td>
<td>72.2 ± 1.48d</td>
<td>23.5 ± 1.58ef</td>
</tr>
<tr>
<td>5</td>
<td>12.30</td>
<td>5.13</td>
<td>95.6 ± 1.14e</td>
<td>24.0 ± 1.30gh</td>
</tr>
<tr>
<td>6</td>
<td>14.76</td>
<td>5.70</td>
<td>65.3 ± 0.70c</td>
<td>23.0 ± 0.83ef</td>
</tr>
</tbody>
</table>

*P* < 0.05; Each value represents the mean ± standard error (SE) of 10 replicates per treatment in three repeated experiments

IBA: Indole butyric acid, IAA: Indole acetic acid
multiplication. In our study, liquid MS medium with optimum concentrations of BAP 11.09 µM, kinetin 11.69 µM and ADS 81.44 µM was used to induce direct axillary shoots from mature nodal explants. Reports are available on multiple shoot regeneration from different types of explants, such as nodal explants, seedling explants, inter-node explants and in vitro leaf explants of curry leaf tree for induction of multiple shoots on MS medium supplemented with various concentrations and combinations of plant growth regulators. Reports are scanty on the regeneration of axillary shoots from mature node explants of *M. koenigii*. This study reports for the first time that ADS in liquid MS medium plays an important role in the induction and growth of axillary shoots indicating that the physico-chemical conditions (liquid medium and higher concentration of ADS) support the induction of fast growing shoots. The study also reports for the first time the multiplication of shoots by repeated re-culturing of explants which facilitate fast availability of cultures of *M. koenigii* as may be required for genetic manipulation. The protocol described in this study enables the production of more than 300 plants from single explants. The results reported could also be useful for meeting raw material demands of pharmaceutical industries for isolation of target compounds from *M. koenigii*.

**References**