

Regeneration of Plantlets via Direct Somatic Embryogenesis from Different Explants of *Murraya koenigii*. (L.) Spreng

Nisha Khatik¹, Ramesh Joshi²

Plant Biotechnology Laboratory, Department of Botany, S.P.C. Government College Ajmer, Rajasthan 305001, India

Abstract: An *in vitro* plant regeneration system was developed via direct somatic embryogenesis from different seedling explants of an important medicinal plant *Murraya koenigii* (L.) Spreng Cotyledons (COT), Hypocotyl (HYP) (10 to 15 mm) and Root (RT) segments (10 to 20 mm) were excised from 60 days old seedling as explants. The somatic embryos induction was achieved on Murashige and Skoog (MS) basal medium augmented with different concentrations of 6-benzyleaminopurine (BAP) 1.33 to 8.40 μM and thidiazuron (TDZ) 1.08 to 9.82 μM . The globular embryos originated from cut ends and entire surface of the root, hypocotyl explants and margins of cotyledons within 30-40 days. The percentage of somatic embryos induction per explant was significantly higher in HYP explants (94.21 \pm 5.77%) in the MS basal medium supplemented with 6.20 μM BAP and 8.64 μM TDZ. The highest rate of conversion of torpedo, heart and cotyledonary stages from globular stage was obtained in MS medium supplemented with 8.64 μM TDZ. The matured somatic embryos were transferred to the MS basal medium without Plant Growth Regulators (PGRs). Highest 88% of the matured embryos were germinated on transfer to $\frac{1}{2}$ MS basal medium without PGR, where they grew for a further 3-4 weeks. Out of seventy six hardened plants seventy (92%) plantlets were found healthy under field conditions.

Keywords: *Murraya koenigii*, Somatic embryogenesis, Thidiazuron, Rutaceae

1. Introduction

Murraya koenigii (L.) Spreng. locally known as “Curry patta” or “Meetha neem” belonging to the family Rutaceae that grows wildy in Southern Asia. Its aromatic leaves are commonly used as the raw material for traditional medicinal formulations in India. The leaves contain essential oil and also used as a condiment. Several monomeric and binary carbazol alkaloids present in the various plant parts [1]. These alkaloids have been reported to possess antimicrobial [2], [3], mosquitocidal, topo-isomerase inhibition [4]-[7] and antioxidant properties [8], [9]. Some of the alkaloids reported in this plant have showed anti carcinogenic [10], [11] and antidiabetic properties [12].

The conventional method of propagation of *M. koenigii* is by seeds only, which retain their viability for a short period. The biotechnological approach for genetic improvement and development of regeneration protocol is prerequisite for germplasm conservation through micropropagation and the development of transgenic plants. An efficient method of somatic embryogenesis is required for genetic improvement of this valuable medicinal plant. However, *in vitro* propagation of curry leaf plant through adventitious shoot proliferation from inter-nodal cuttings was reported by Joshi *et al.* [13]. Also in *Citrus*, close relative of *M. koenigii*, methods for genetic improvement by somatic hybridization have been reported by Grosser *et al.* [14]. Although few reports are available for *in vitro* studies of this plant which are restricted to the nodal and stem segments [15], [16] and from intact seedling [17]. Paul *et al.* [18] reported a regeneration system of direct and indirect somatic embryogenesis in this plant but they have used Zygotic embryonic axis and Cotyledon as explants only. The present communication reports an efficient translational protocol for regeneration of plants through direct somatic embryogenesis from Hypocotyl (HYP), Cotyledon (COT) and Root (RT) explants.

2. Materials and Methods

Plant material, explant preparation and cultures establishment

Ripe blackish purple colour fruits collected from matured tree of *Murraya koenigii* grown in campus and surrounding area of Ajmer were washed with running tap water for 15 minutes. Seeds were removed from fruits and washed free of pulp. Seeds were first washed with liquid detergent (Teepol; Qualigen, India) and then soaked with 0.1% solution of Bavistin fungicide (BASF, India) and rinsed with distilled water. The seeds were surface sterilized with an aqueous solution of 0.1% (w/v) HgCl_2 (Hi Media, India) for 5-6 minutes followed by four to five autoclaved distilled water rinses. Disinfected seeds were germinated in 200 ml screw-capped glass jars containing 40 ml seed germinating half strength micro and macronutrient Murashige and Skoog (MS) basal medium [19] devoid of plant growth regulators. Cotyledons (COT), Hypocotyl (10 to 15 mm) and root segments (10 to 20 mm) were excised from 60 days old seedling as explants.

Culture media and experimental conditions

For induction of direct somatic embryogenesis the explants were inoculated on MS medium supplemented with 1.33 to 6.20 μM 6-benzylaminopurine (BAP) and 1.08 to 9.82 μM thidiazuron (TDZ). Somatic embryos were further transferred to another set of MS medium supplemented with 1.08 to 9.82 μM TDZ for their maturation. Thus, for germination the matured somatic embryos were sub-cultured to $\frac{1}{2}$ basal MS medium free of plant growth regulators (PGR). All the cultures were maintained regular sub-culture after three to four weeks on the same medium. Media were solidified by adding 0.8% agar powder (Qualigen, India) and 3% sucrose were added as carbon source (Qualigen, India). The pH of media was adjusted to 5.8 (\pm 0.1) before autoclaving. The media were autoclaved at 121°C Psi for

twenty minutes. Cultures were maintained at 25±1°C, 16-h photoperiod under 35µE m⁻² s⁻¹ light intensity by white fluorescent tubes (Philips, India).

Acclimatization and field transfer

In vitro developed plantlets with 40-50 mm shoot length and strong 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 from bottle. 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

All experimental data were subjected to analysis of variance (ANOVA) and significant ($P>0.05$) means were determined with Duncan's Multiple-range test (DMRT) to distinguish differences between treatments means at the $\alpha = 0.05$ level using SPSS or Windows version 16.0.

3. Results

Induction of direct somatic embryos from COT, HYP and RT explants

The somatic embryos induction was achieved on MS basal medium supplemented with different concentrations of BAP 1.33 to 8.40 µM and TDZ 1.08 to 9.82 µM. The embryos originated from cut ends as well as from surface of the root, hypocotyl and margins of cotyledons within 30-40 days. However, the frequency of responding explants and number of somatic embryos per explants varied in different concentrations of PGRs and type of the explants. The percentage of somatic embryos induction per explant was significantly higher in HYP explants (94.21±5.77%) whereas the lowest percentage of embryos (79.25±1.66%) were formed from cotyledon explants in the MS basal medium supplemented with 6.20 µM BAP and 8.64 µM TDZ (Table 1). The somatic embryos formed in these media were of globular stages (Fig. 1a) only and they could not attain the advanced stages of somatic embryogenesis i.e. heart and torpedo stages even after culturing them for 60 days in the same medium.

Table 1: Effect of different concentrations of cytokinins upon the induction of somatic embryos from COT, HYP and RT explants of *M. koenigii* on MS basal medium containing 3% sucrose and 0.8% agar.

| Sr. No. | Concentrations of PGRs (µM) | | Explants forming Somatic Embryos (%) | | |
|---------|-----------------------------|------|--------------------------------------|--------------------------|---------------------------|
| | BAP | TDZ | HYP | RT | COT |
| 1. | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2. | 1.33 | | 14.22±2.11 ^a | 18.12±3.71 ^{cd} | 16.52±3.22 ^{jkf} |
| 3. | 1.33 | 1.08 | 28.32±1.74 ^{bc} | 24.13±2.30 ^{ek} | 22.71±1.25 ^{if} |
| 4. | | 1.08 | 33.32±1.21 ^{de} | 30.17±1.79 ^{jk} | 28.55±3.35 ^{ij} |
| 5. | 1.55 | | 41.32±1.74 ^{gh} | 59.82±3.16 ^{df} | 49.91±2.89 ^{cd} |
| 6. | 1.55 | 2.16 | 48.17±2.33 ^{jk} | 42.22±1.72 ^{ab} | 49.25±1.66 ^{df} |
| 7. | | 2.16 | 55.21±5.77 ^{cde} | 49.12±1.99 ^{cf} | 48.11±2.88 ^{ab} |
| 8. | 3.11 | | 61.37±4.42 ^j | 58.29±2.98 ^d | 55.72±2.62 ^{cb} |
| 9. | 3.11 | 4.32 | 72.82±2.99 ^{eg} | 68.41±2.77 ^{cc} | 67.33±3.33 ^k |
| 10. | | 4.32 | 73.24±1.74 ^b | 69.22±1.79 ^{ac} | 68.11±2.88 ^{ab} |
| 11. | 6.20 | | 81.76±2.66 ^c | 77.52±2.67 ^{bc} | 72.76±2.51 ^{ac} |
| 12. | 6.20 | 8.64 | 94.21±5.77 ^{df} | 84.41±2.77 ^c | 79.25±1.66 ^{df} |
| 13. | | 8.64 | 82.27±3.27 ^d | 70.59±3.35 ^a | 66.77±2.76 ^{cg} |
| 14. | 8.40 | | 72.82±2.99 ^{eg} | 68.41±2.77 ^{cc} | 67.33±3.33 ^k |
| 15. | 8.40 | 9.82 | 69.32±3.31 ^{ef} | 59.82±3.16 ^{df} | 71.72±2.62 ^{cc} |
| 16. | | 9.82 | 61.37±4.42 ^j | 78.66±2.92 ^h | 72.76±2.51 ^{ac} |

*PGRs plant growth regulators, BAP 6- benzylaminopurine, TDZ Thidiazuron

*Values are expressed as mean± standard error of mean (SE) taking five explants in each experiment with three replicates. Within each group, values with different letters are indicative of significant difference at $P > 0.05$ using Duncan's multiple-range test (DMRT)

Maturation and germination of somatic embryos

The globular stages of somatic embryos did not convert into next stages. The conversion percentage varied widely from 37 to 98% depending on the concentrations of growth regulators used. The highest rate of conversion of globular stage of embryos to torpedo, heart and cotyledonary stages was obtained from MS medium supplemented with 8.64 µM

TDZ (Fig. 2) which converted the highest 98% of globular embryo into further heart, torpedo (Fig 1. b) and cotyledonary stages (Fig. 1c) of somatic embryogenesis within three to four weeks. The same basal medium when supplemented with higher concentrations of TDZ did not show any significant differences on the percentage of conversion.

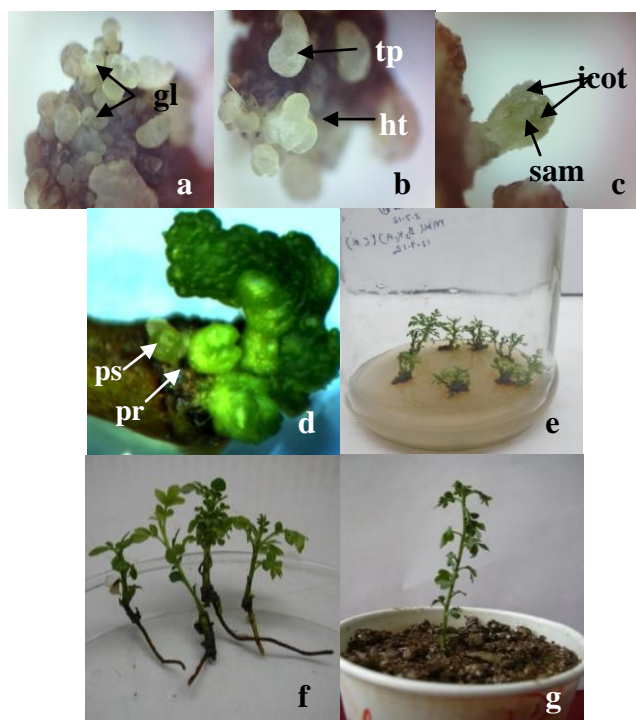


Figure: 1 [a-g] - Photomicrograph of different stages of direct somatic embryos in *Murraya koenigii*. (a): Globular (gl); (b): Torpedo(tp) and heart(ht) stages; (c): somatic embryo with shoot apical meristem (sam) and two initial cotyledons (icot); (d): germination of somatic embryo on mother tissues with primary shoot (ps) and primary root (pr) on ½ MS medium without PGRs; (e): conversion of somatic embryos in to plantlets upon culture on ½ MS basal medium without PGRs; (f): six week-old tap rooted plantlets prior to hardening; (g): hardened plantlet in nursery.

Discussion

In present study an improved regeneration system through direct somatic embryogenesis in *M. koenigii* was developed. The induction of direct somatic embryogenesis and their subsequent development are influenced by culture media, choice of explants and concentration and combinations of PGRs. Culture medium is one of the most important factors to be considered for *in vitro* plant cell culture. The MS medium is most common medium which has been reported to be used in plant regeneration via direct and indirect somatic embryogenesis [18] and by axillary and adventitious bud culture [13], [18], [15]. Alternatively B5 medium has been used for *in vitro* regeneration of *Arabidopsis thaliana* [20]. However, present study suggested that MS medium responded better than other culture media.

In general, relatively high auxin concentrations favor callus formation and induction of cell polarity. Afterwards, when somatic embryo induction stage has been achieved, it is necessary to reduce or eliminate the auxins because the embryos begin to synthesize their own auxin, possibly via an alternative pathway [21], [22]. In the species such as *Zoysia japonica* [23], *Begonia gracilis* [24] and *Oncidium* spp. [25], the use of cytokinins favors the induction of somatic embryos. In our study the BAP 6.20 μM and TDZ 8.64 μM

The matured somatic embryos, either in clusters or individually, were transferred to the MS basal medium without PGRs, for their germination. Some of the (10%) embryos germinated even when they were still attached to the mother tissues (Fig. 1d) and the highest 88% of the matured embryos were germinated on transfer to the PGR free medium where they grew for a further 4-6 weeks (Fig. 1e). Six weeks old plantlets with 5-6 cm shoots and well developed tap roots (Fig. 1f) were transferred to poly bags containing a mixture of perlite, vermiculite and peat moss in equal ratio. Out of seventy six hardened plants ninety two percent plantlets were found healthy under field conditions (Fig. 1g).

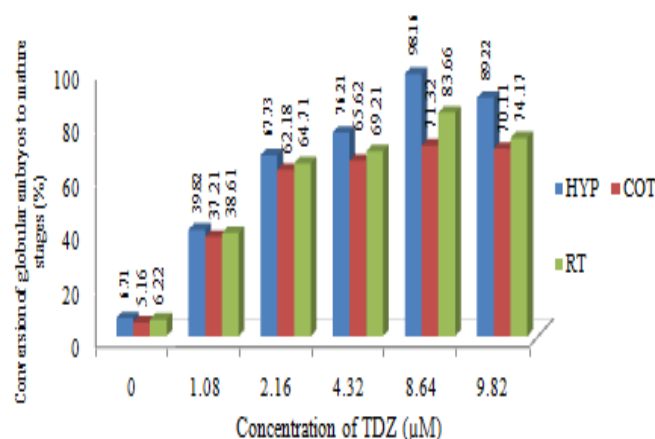


Figure: 2- Conversion of globular embryos to mature embryos (heart, torpedo and cotyledonary stages) in COT, HYP and RT explants of *M. koenigii*.

were found more effective for induction of globular embryos directly from HYP, COT and RT explants.

The TDZ a phenyl urea derivative (N-Phenyle-N1 – 1, 2, 3 – thiazol-5-ylurea) has a unique mode of action with intrinsic cytokinin like activity [26] and is known to be more effective than all adenine type cytokinins in inducing high frequency organogenesis in a number of plant species [27], [28]. Application of TDZ may increase the levels of endogenous cytokinins by inhibiting the action of cytokinin oxidase [29]. Present investigation revealed that the TDZ alone in the concentration of 8.64 μM is prerequisite for conversion of 98% of globular embryos into torpedo and heart shape embryos. The germination or conversion of somatic embryo into plantlet is difficult during somatic embryogenesis [30]. For woody species in particular, the efficiency of germination is relatively low, which restricts application of somatic embryogenesis system for commercial purpose [31].

In our study it was observed that the matured somatic embryos could not germinate and even prolonged exposure to TDZ supplemented medium resulted in their distortion. Similar results have also been reported by Murch *et al.* [32] and Khurana *et al.* [33]. Inhibition of germination of somatic embryos by TDZ may be due to its high cytokinin activity

[34]. In this study we have found that within 20 to 30 days the matured somatic embryos, recovered from HYP, COT and RT explants, were germinated on ½ MS basal medium without PGRs.

The *in vitro* plantlets developed under low light, aseptic conditions and on the media containing sample of sugar and nutrients cannot survive in the external environmental conditions when directly placed in green house or field [35], [36]. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the green house or field conditions [37]. The *in vitro* plantlets developed during the study program were successfully hardened and transfer to the field where 92% plants were found healthy.

In conclusion, we have developed an improved and viable regeneration system based on direct somatic embryogenesis from HYP, COT and RT explants 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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