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PROTOCOL FOR REGENERATION OF PLANTLETS THROUGH SOMATIC EMBRYOGENESIS FROM HYPOCOTYL OF CURRY LEAF PLANT (*MURRAYA KOENIGII* L. SPRENG)

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In the present study a highly efficient and reproducible protocol was established to regenerate direct somatic embryogenesis from hypocotyl segments (HYP) of *Murraya koenigii*. The surface sterilized seeds were inoculated on half strength Murashige and Skoog (MS) basal medium for germination. Explants were obtained from 60 days old axenic seedlings of *Murraya koenigii* and cultured on MS basal medium supplemented with different concentrations of 6-benzyleaminopurine (BAP) 0.56 to 8.89 μM and thidiazuron (TDZ) 0.48 to 9.37μM. The globular embryos originated from cut ends and entire surface of the hypocotyl explants within 35-40 days. The highest rate of conversion of torpedo, heart and cotyledonary stages from globular stage was obtained in MS medium supplemented with 7.98 μM TDZ. The matured somatic embryos were transferred to the MS basal medium without Plant Growth Regulators (PGRs). Highest 81% of the matured embryos were germinated on transfer to ½ MS basal medium without PGR, where they grew for a further 4-5 weeks.

Keywords: Hypocotyl segment; *Murraya koenigii*; Rutaceae; Somatic embryogenesis; Thidiazuron.

Introduction

Murraya koenigii L. Spreng (family: Rutaceae) is a small and strong smelling perennial shrub that commonly grows in South East Asian countries and known as curry leaf plant. This plant is widely cultivated for its leaves which possess characteristic flavor and aroma and are used as a condiment and flavoring agent in ethnic foods[1, 2]. Traditionally, the leaves of this plant are used to treat a wide range of diseases and disorders such as pain, inflammation, itching, cancer, diabetes, and blood disorders[1,3]. The leaves also contain monoterpene derived hydrocarbons and alcohols possessing antioxidant potentials *in vitro*[4]. In 2013, the research groups of Nakamura and Ma isolated six new carbazole alkaloids including karapinchamines A and B[5], N-benzyl carbazole-A, N-benzyl carbazole-B, iso-koenidine, and iso-koenigine along with fourteen other known carbazole alkaloids having hepatoprotective and anticancer properties[6]. In order to cater the increasing demands of herbal drug markets,

conservation and commercial production of this species have become necessary.

The *in vitro* propagation methods in *Murraya Koenigii* are highly advantageous, for enhanced micropropagation for germplasm conservation and the development of transgenic plants.

There are few reports on *in vitro* studies of *M. koenigii* which are restricted to *in vitro* shoot multiplication from intact seedling, inter node segments, nodal cuttings and leaf as explants[7-13]. Paul *et al.*[14] 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 study was therefore, aimed to develop an efficient protocol for regeneration of plants through direct somatic embryogenesis from Hypocotyl (10 to 15 mm) explants.

Material 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[15] devoid of plant growth regulators. Hypocotyl (10 to 15 mm) segments 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 0.56 to 8.89 µM 6- benzylaminopurine (BAP) and 0.48 to 9.37µM thidiazuron (TDZ). Somatic embryos were further transferred to another set of MS medium supplemented with 0.48 to 9.37µM TDZ for their maturation. Thus, for germination the matured somatic embryos were sub-cultured to ½ basal MS medium free of plant growth regulators (PGR).

Cultures were maintained and multiplied by sub-culturing at regular interval four weeks on the same culture 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 (±0.1) before autoclaving. The media were autoclaved at 121℃ Psi for twenty minutes. Cultures were maintained at 25±1℃, 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 determine 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.[16]

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 µM and TDZ µM.

The embryos were originated from cut ends as well as from surface of the hypocotyl segments within 35-40 days. However, the frequency of induction of somatic embryos per explants varied in different concentrations and combinations of PGRs.

The percentage of somatic embryos induction per explant was found (89.40 ± 2.77) on MS basal medium supplemented with 6.08 µM BAP and 7.98 µM TDZ (Table 1).

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

Concentrations of PGRs (µM)		Explants forming Somatic Embryos (%)
BAP	**TDZ**	**Hypocotyl**
0.00	0.00	0.00
0.56	0.48	16.02 ± 0.22^{ab}
1.25	1.04	24.11 ± 0.30^{e}
2.78	2.26	55.28 ± 5.77^{cd}
4.42	4.45	77.56 ± 2.67^{bc}
6.08	**7.98**	$\mathbf{89.40 \pm 2.77^{f}}$
8.89	9.37	78.66 ± 2.92^{jh}

*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)

The somatic embryos formed in these media were of globular stage (Fig. 1. A).

Maturation and germination of somatic embryos: The globular stages of somatic embryos did not convert into next stages on their induction medium. The conversion percentage varied widely from 42% to 89% 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 7.98 µM TDZ (Fig. 2) which converted the highest percentage of globular embryo into further heart and torpedo stages (Fig. 1. B, C) followed by the induction of cotyledon (Fig. D) within three to four weeks.

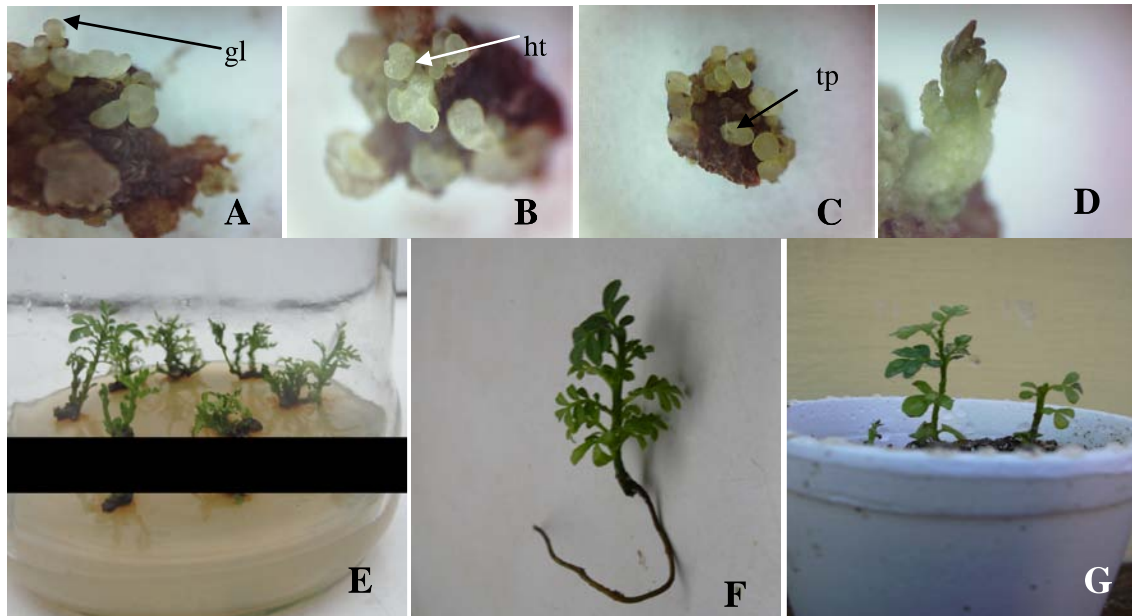

Fig. 1 A-G - Photomicrograph of different stages of direct somatic embryos in *Murraya koenigii.* (A) Globular (gl) (B) Torpedo (tp) (C) Heart (ht) stages (D) somatic embryo with shoot apical meristem and two initial cotyledons (E): conversion of somatic embryos in to plantlets upon culture on ½ MS basal medium without PGRs (F) six week-old tap rooted plantlets prior to hardening (G) hardened plantlet in nursery.

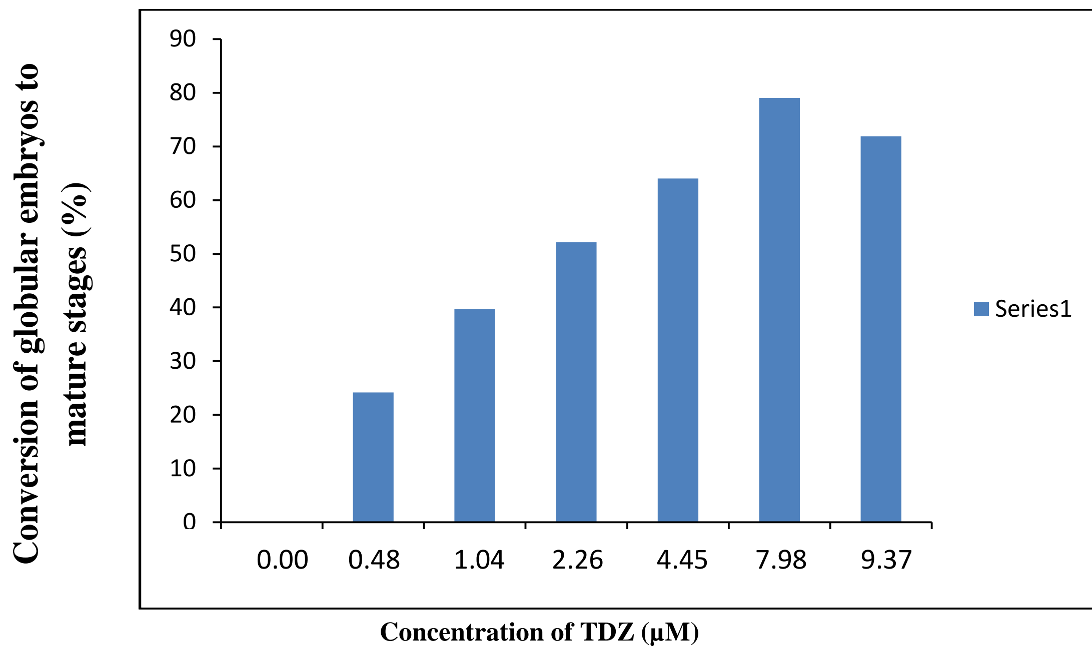

Fig. 2- Conversion of globular embryos to mature embryos (Heart, Torpedo and Cotyledonary stages) in hypocotyl explants of *M. koenigii.*

The matured somatic embryos, either in clusters or individually, were transferred to the MS basal medium without PGRs, for their germination. The highest 81% of the matured embryos were germinated on transfer to the PGR free medium where they grew for a further 4-5 weeks (Fig. 1 E). 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 (Fig. 1G).

Discussion

The MS medium is most common medium which has been reported to be used in plant regeneration via direct and indirect somatic embryogenesis[14] and by axillary and adventitious bud culture[8,14,9]. Alternatively B5 medium has been used for *in vitro* regeneration of *Arabidopsis thaliana*[17]. 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 auxin because the embryos begin to synthesize their own auxin, possibly via an alternative pathway [18,19]. In the species such as *Zoysia japonica*[20], *Begonia gracilis*[21] and *Oncidium* spp.[22], the use of cytokinins favors the induction of somatic embryos. In our study the BAP 6.08 µM and TDZ 7.98 µM were found more effective for induction of globular embryos directly from Hypocotyl explants.

The TDZ a phenyl urea derivative (N-Phenyle-N1 – 1, 2, 3 – thidiazol-5-ylurea) has a unique mode of action with intrinsic cytokinin like activity[23] and is known to be more effective than all adenine type cytokinins in inducing high frequency organogenesis in a number of plant species[24,25]. Application of TDZ may increase the levels of endogenous cytokinins by inhibiting the action of cytokinin oxidase[26]. Present investigation revealed that the TDZ alone in the concentration of 7.98 µM is prerequisite for conversion of 79% of globular embryos into torpedo and heart shape embryos. The germination or conversion of somatic embryo into plantlet is difficult during somatic embryogenesis[27]. For woody species in particular, the efficiency of germination is relatively low, which restricts application of somatic embryogenesis system for commercial purpose[28].

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.*[29] and Khurana *et al.*[30]. Inhibition of germination of somatic embryos by TDZ may be due to its high cytokinin activity[31]. In this study we have found that within 28 to 35 days the matured somatic embryos, recovered from Hypocotyl 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[32,33]. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the green house or field conditions[34]. The *in vitro* plantlets developed during the study program were successfully hardened and transfer to the field where 81% plants were found healthy.

Conclusion

We have developed an improved and viable regeneration system based on direct somatic embryogenesis from Hypocotyl 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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