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Optimization of *in vitro* conditions for induction of somatic embryos and regeneration of plantlets in *Euphorbia hirta* L.

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Abstract

Plantlet regeneration methods through somatic embryogenesis were standardized from stem explants of *Euphorbia hirta* in this report. The explants were surface sterilized with 0.1% (w/v) mercuric chloride solution. The sterilized explants were inoculated on MS (Murashige and Skoog) medium to induce somatic embryos. The explants with somatic embryos were transferred to the embryo germination and shoots elongation medium. The explants responded maximum on MS medium augmented with 0.4 mg/L 6-benzyleaminopurine (BAP) and kinetin (Kin) combined with 0.5 mg/L á-naphthalene acetic acid (NAA). About 93% response was observed when indole-3 acetic acid (IAA) was used at the place of NAA in the medium with BAP and Kin. Sucrose at 3% concentration was reported optimum for the induction of somatic embryos. Somatic embryos were differentiated into plantlets within 4 weeks on half strength MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L Kin. Maximum 83.9±0.51 plantlets (average length of 5.3±1.60 cm) per culture vessel were yielded on this medium combination after 4-5 subcultures. The induction of somatic embryos from the epidermal and subepidermal cells of the explants was confirmed by the microscopic observations of the transverse sections of explants with the embryoids. The methods were developed and the conditions were optimized for the induction of somatic embryos from the nodal explants of *E. hirta* in this study.

Keywords: *Euphorbia hirta*; somatic embryogenesis; MS medium; microscopic observations.

Introduction

Euphorbia hirta L. (family Euphorbiaceae) is an important medicinal herb, belongs to the tropical parts of the India, Bangladesh, Africa, America and Australia. The plant is popularly known as Pill bearing spurge and Basri dudhi (Hindi). It is a slender, hairy plant with profuse branches, which can spread up to the height of 40 cm (1). The plant is well acknowledged for its various medicinal properties. Traditionally, it is used for the treatment of asthma, bronchitis, ulcer, amoebic dysentery, diarrhea and dengue fever (2). The latex from the plant is used in treating hypertension, anemia, skin diseases, jaundice, edema, warts, sprains, inflammation, epilepsy and malaria (3).

Euphorbia hirta is explored for its valuable pharmacological activities like antibacterial, antifungal, antimalarial, anthelmintic, anticatarrhal, antidiarrheal, diuretic, antihypertensive, anxiolytic, anti-inflammatory, antidiabetic, antispasmodic, anticancer, antioxidant, antiasthmatic and antisyphilitic activities (2,3).

The various bioactivities of *E. hirta* are influenced by the presence of secondary metabolites such as flavonoids, polyphenols, tannins, sterols, alkaloids, glycosides and triterpenoids (4). The whole plant is reported to possess afzelin, myricitrin, rutin, quercitin,

euphorbin, gallic acid, protocatechuic acid, â-amyrin, â-sitosterol, heptacosane, nonacosane, shikmic acid, tinyatoxin, choline, camphol, rhamnose etc. The latex is endowed with inositol, taraxerol, friedelin, ellagic acid and kaempferol (5-7).

In vitro induction of somatic embryos is a preferred method of crop improvement programs (8). It provides a model system to explore the genetic basis of early differentiation events and cellular totipotency of somatic cells (9,10). The direct somatic embryogenesis has been reported in many plants like, *Prunus persica* (11), *Kalanchoe blossfeldiana* (12), *Nicotiana tobacum* (13), *Begonia pavonina* (14) etc. Somatic embryogenesis reduces the time required for complete plant regeneration which is advantageous in reduction of culture-induced genetic changes (15,16). Somatic embryogenesis has also been demonstrated in *Leptadenia pyrotechnica* (17), *Wedelia calendulacea* (18) etc. for the conservation of endangered germplasms.

The large scale production of plantlets through somatic embryogenesis would offer a stable alternative to meet the pharmaceutical demand of this plant. Barring few reports on the *in vitro* propagation of *E. hirta* to date (19), there is no report on direct regeneration through somatic embryogenesis. Therefore, the aim of the present study was to develop an efficient regeneration protocol through somatic embryogenesis and optimization of *in vitro* conditions for the induction of somatic embryos in *E. hirta*.

Materials and Methods

Explant material and surface sterilization : Different types of explants (node, internode and leaves) of *E. hirta* were procured from a three months old young emerging plant growing in the institute campus. The explants were isolated and cleaned under running tap water for 10-15 min, treated with 3% (v/v) Sodium hypochlorite (disinfectant) for 5-8 min. and rinsed five times with sterilized distilled water. The explants were subjected to a broad spectrum systemic fungicide 0.1% (w/v) Bavistin (BASF India Ltd.) for 5-6 min. Surface sterilization was carried out with 0.1% (w/v) mercuric chloride solution for 3-5 min and rinsed with sterile distilled water to remove the sterilant under aseptic conditions.

Culture medium and culture conditions : The surface sterilized explants were cultured on Murashige and Skoog (MS) medium (20) containing 0.8% agar and additives (50 mg/L ascorbic acid, 25 mg/L each of adenine sulphate, L-arginine and citric acid) with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.2-1.0 mg/L), 6-benzylaminopurine (BAP; 0.2-1.0 mg/L), kinetin (Kin; 0.2-1.0 mg/L), indole 3-acetic acid (IAA; 0.2-1.0 mg/L) and á-naphthalene acetic acid (NAA; 0.2-1.0 mg/L) (Himedia, Mumbai, India) for the induction of somatic embryos. Hormone free MS medium served as control. The pH of the medium was adjusted to 5.8 ± 0.02 with NaOH and / HCl prior to autoclaving. The media was dispensed into culture vessels and autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 15 min. All the cultures were maintained at 25±2°C with 16:8 (light:dark) photoperiod at 40–50 ìmol m^{-2} s^{-1} spectral flux photon density (SFPD) under cool white fluorescent tubes (Philips, Mumbai, India).

Induction and maturation of somatic embryos : The explants were cultured on different strengths of MS media augmented with different concentrations and combinations of growth regulators for the induction of somatic embryos. The explants were inoculated horizontally and vertically on growth medium and incubated at above mentioned *in vitro* conditions. After the induction of embryogenic masses on the surface of the explants, these were transferred to MS basal medium supplemented with growth regulators for the development and maturation of somatic embryos. The early stage somatic embryos were transferred to MS medium augmented with or without BAP and different concentrations of sucrose (1–5 % w/v) for the maturation of somatic embryos. After four weeks, the explants with somatic embryos were transferred to the multiplication medium and maintained under dark conditions at room

temperature for further multiplication of somatic embryos.

Germination of somatic embryos and elongation of shoots : The meristemoid portion with mother explants were excised and cultured on different strengths (full, ½ and 1/4th) of MS medium supplemented with different concentrations of BAP and Kin (0.25, 0.5 0.75 and 1.0 mg/L) for the germination of somatic embryos. Initially cultures were maintained in dark for a week. Once the embryogenic potential gained bythe explants on MS medium, the proembryo masses were transferred to fresh medium for further elongation of the shoots.

Photomicrography of somatic embryos : The somatic embryogenesis was further confirmed through microscopic observations. Fresh embryogenic tissues containing somatic embryos at different developmental stages were withdrawn from the cultures and fixed primarily in FAA solution (formalin: acetic acid: ethyl alcohol at 1:1:3 ratio) for 48 hrs and stored in 70% ethanol at room temperature. Free hand sections were done using a sharp razor. Sections were stained with 1% aqueous safranin (w/v) and mounted onto the glass slides. Photomicrographs of different magnifications were taken under bright light field using Olympus Stereomicroscope (SZ61) and Labomed iVu photomicroscope. The images captured were analyzed using Pixelpro software.

Acclimatization of plantlets : The well developed plantlets regenerated from somatic embryos were transferred to paper cups containing autoclaved soilrite® (Keltech Energy Limited, Bangalore, India). The plantlets were covered with a polyethylene bags with minute holes to maintain high humidity and irrigated weekly with tap water. After 4 weeks, the plantlets were shifted to nursery polybags filled with the mixture of red soil, garden soil and vermi-compost (1:1:1) for hardening. Finally, the plants were transferred to the field after two months.

Experimental design and statistical analysis: All the experiments were conducted with 20 replicates per treatment and the experiments were repeated thrice. The percentage of embryo induction and germination from stem explants was calculated. Data were recorded after 3 weeks in each experiment. The results were expressed as mean ± SE of three independent experiments. The data were analyzed statistically using one-way ANOVA and the significance in variation between responses with reference to various concentrations of hormones were assessed by Duncan's multiple range tests at a 5% probability level by SPSS version 16 (Chicago, USA).

Results

Induction and maturation of somatic embryos: Among the three types of explants used, maximum percentage of response in induction of somatic embryos was observed with inter – nodal explants than with the node and the leaf explants. The development of somatic embryos directly on the surface of explants was clear within a week of inoculation on MS medium (Fig. 1A). The surface of the explants is fully covered by somatic embryos of different stages within four weeks.

The somatic embryos formation was not recorded with the medium containing 2,4-D. Of the growth regulators used to induce somatic embryos, combination of cytokinins and auxins in the medium elicited better response. Somatic embryos were induced on all concentrations of BAP and Kin augmented with IAA or NAA. Application of NAA produced higher percentage of somatic embryos than IAA. MS medium supplemented with 0.4 mg/L each of BAP and Kin and 0.5 mg/L NAA resulted in good response of somatic embryo induction. Cent percentage response was observed on this medium combination. Maximum 92.9% induction was resulted when IAA was used at the place of NAA in the medium. Less percentage of somatic embryos were induced on BAP and Kin when incorporated alone in the medium (Table 1).

Full strength MS medium with 3% sucrose was reported optimum for the induction of somatic embryos in *E. hirta*. Lower and higher concentrations than 3% sucrose was not found

impressive in the induction frequency of somatic embryos in this study (Table 2).

Germination of somatic embryos and elongation of shoots : The germination of somatic embryos into plantlets was achieved by subculturing of mature and premature embryos to half strength MS medium containing low concentrations of cytokinins (BAP and Kin). Somatic embryos were developed into plantlets with the normal developmental stages to root and shoot differentiation within 4 weeks, when the meristemoids transferred onto half strength MS medium + additives and fortified with 0.5 mg/L BAP and 0.1 mg/L Kin (Fig. 1B).

The strength of MS medium significantly affected the regeneration of shoots from the somatic embryos. The embryos failed to germinate on full strength MS medium, even one-fourth strength of MS medium was also reported comparatively less effective in the germination of somatic embryos. The regeneration of the somatic embryos was clearly visible and green tiny shoots primordia were observed within week of transfer to the fresh medium. The green shoots emerged out from the green mass of cells after another one week (Fig. 1C-D). The regular subculturing of somatic embryos on fresh medium with the same composition and combinations yielded 83.9±0.51 plantlets per culture vessel (size 400 ml) with an average length of 5.3±1.60 cm (Fig. 1E-F and Table 3). The number of plantlets germination from the embryos was decreased gradually beyond the optimum concentrations of BAP and Kin.

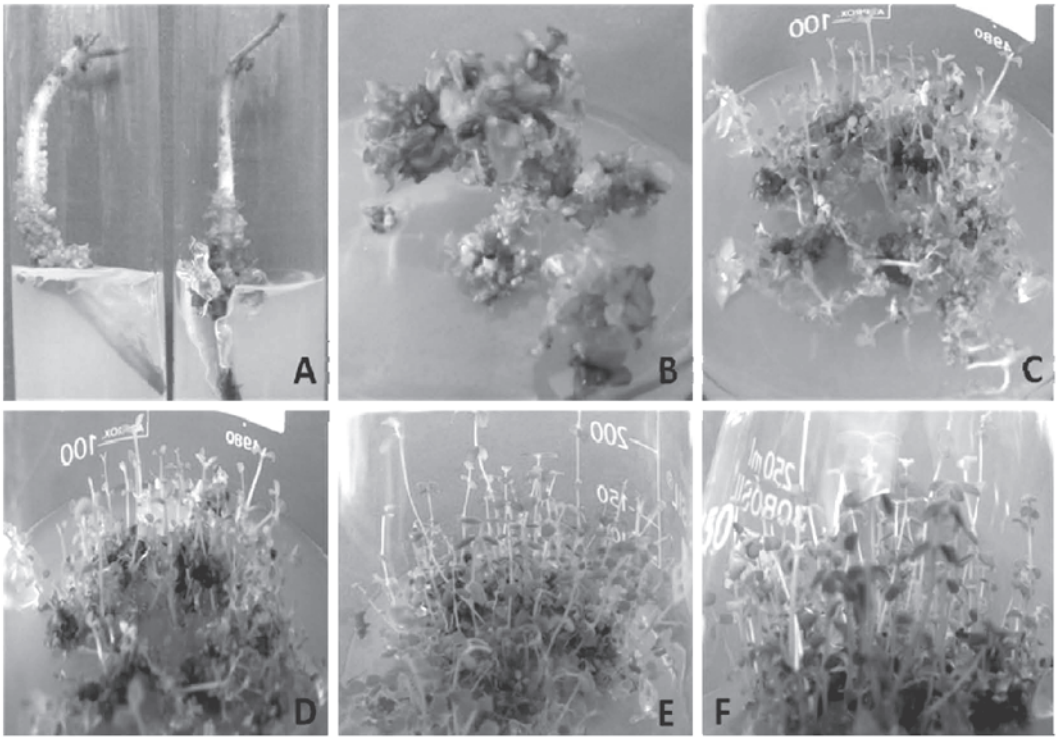

Fig. 1A. Somatic embryo on the surface of the explants. **Fig. 1B.** Germination of somatic embryos. **Fig. 1C.** Tiny green plantlets emerged out from the somatic embryos. **Fig. 1D.** Small shoots on half strength MS medium. **Fig. 1E.** Micropropagation of shoots on MS medium. **Fig. 1F.** Elongated shoots of *E.hirta*.

Maximum 15.4±0.23 shoots were germinated on hormone free MS medium.

Microscopic analysis of somatic embryos : The microscopic analysis of the cultures confirmed the induction of somatic embryos from the surface of the explants on full strength MS medium containing BAP, Kin and NAA/IAA (Fig. 2A). The clusters of green mass on explants surface showed pre-globular pro-embryos after 7 days of inoculation (Fig. 2B-C. The microscopic sections of explants tissue showed that the formation of somatic embryos occurred from the epidermal and subepidermal cells (Fig. 3A). Incubation under dark conditions promoted the development of globular somatic embryos. The globular embryos were less as compared to the bifurcated heart-shaped, and torpedo stages of embryos in the second weekof cultures (Fig. 3B). The different types of morphogenic responses were observed with time duration of the cultures. Globular and heart-shaped embryos were prominent in the first two weeks (Fig. 3C). Later at third and fourth weeks, torpedo and cotyledonary stages were prominent. The different stages of somatic embryos were detected on the same explants (Fig. 3D). Finally the mature embryos were developed into the shoots and roots.

Discussion

Somatic embryogenesis opens a promising opportunity to regenerate large number of identical

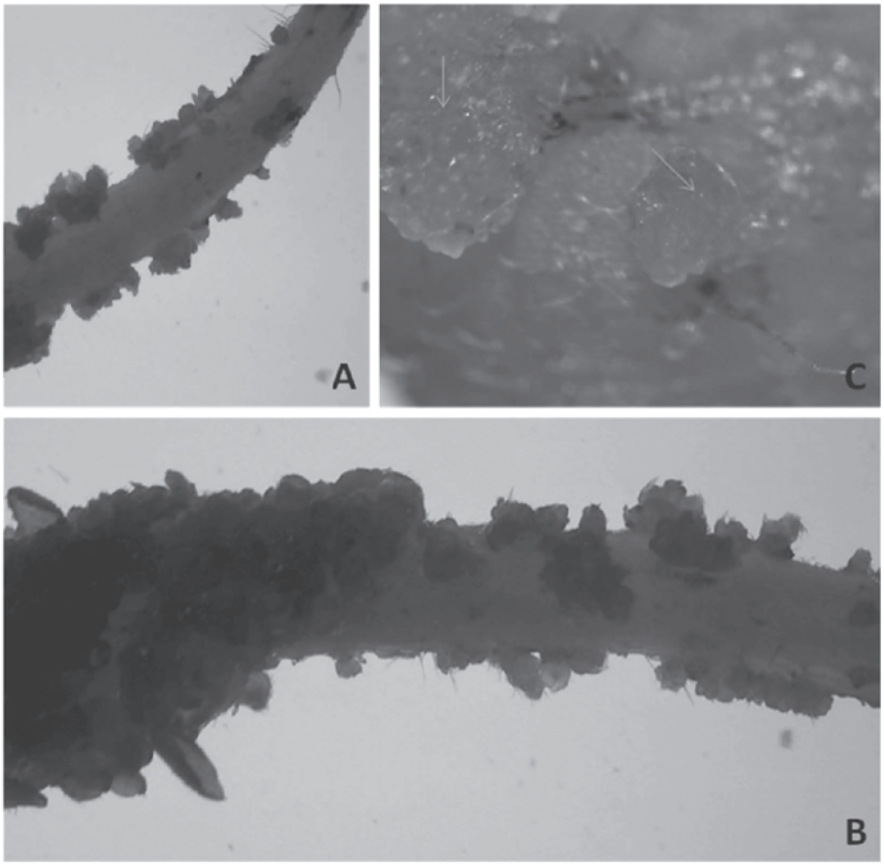

Fig. 2A. Microscopic image of the somatic embryos on the surface of the explant.
Fig. 2B. Early stages in the development of somatic embryos in *E. hirta*.
Fig. 2C. Different stages in the development of somatic embryos with the explants.

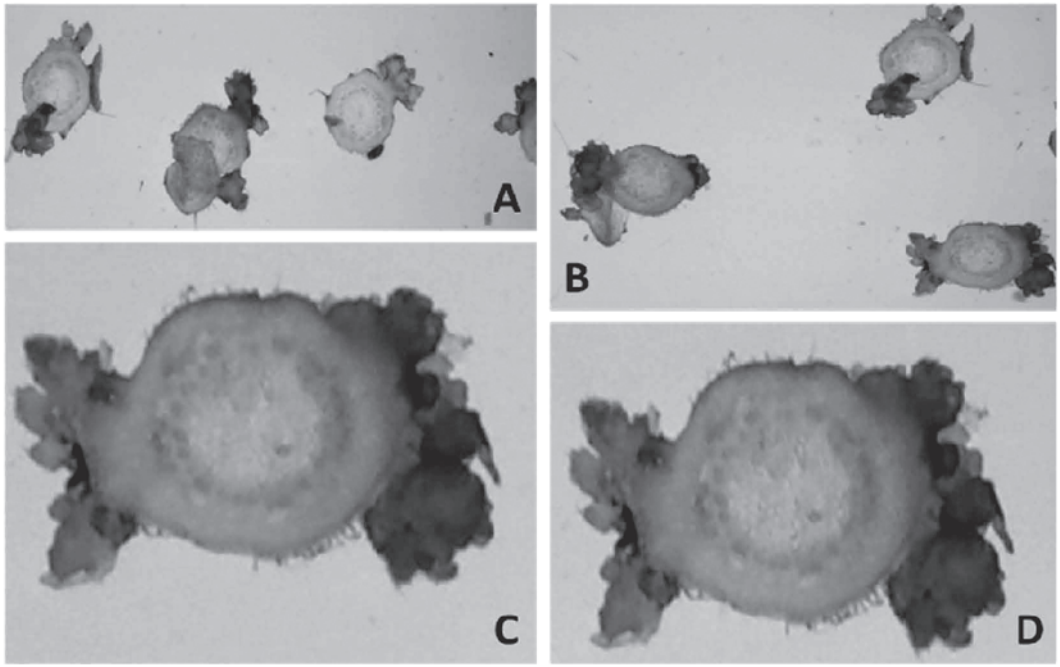

Fig. 3A. Transverse sections of the somatic embryos with the explants.
Fig. 3B. Bifurcated heart-shaped stages of embryos after two weeks of incubation.
Fig. 3C. Globular and heart-shaped embryos.
Fig. 3D. Different stages of somatic embryos after four weeks of incubation.

plantlets within short duration of time (13). Maximum percentage of response in induction of somatic embryos was observed with inter-nodal explants in this study. The response of explants on *in vitro* condition is genotype independent (21). Somatic embryos were induced from several of explants in number of Euphorbiaceae members (19). Marconi and Radice (22) reported somatic embryogenesis using leaf explants in *Codiaeum variegatum*. Immature anthers were used as explants in *Hevea brasiliensis* (23), mesophyll cells in *Euphorbia nivulia* (24), nodal segments in *Euphorbia pulcherrima* (25) and leaf and floral tissues in *Manihot esculenta* (26).

The embryos formation was not recorded with the MS medium augmented with 2,4-D but the combination of cytokinins and auxins in the medium elicited better response in this study. Martin et al. (24) induced maximum percentage of somatic embryos in E. nivulia using BAP and 2,4-D in contrast to these results. Singh *et al.* (27) reported that the presence of 2,4-D in the medium produced negligible amount of somatic embryos than the presence of BAP in *Sapindus mukorossi*. The addition of cytokinin with 2,4-D could enhance formation of the somatic embryogenesis than the 2,4-D alone (28).

The MS medium supplemented with 0.4 mg/L each of BAP and Kin and 0.5 mg/L NAA resulted in good response of somatic embryo induction. The combination of Kin and NAA induced maximum number of somatic embryos in *H. brasiliensis* (23). Somatic embryogenesis have been reported in several Euphorbiaceae members such as *M. esculenta* (29), *E. pulcherrima* (30), *E. nivulia* (24), *Jatropha curcas* (21) etc. The influence of growth regulators and culture environments were reported to be crucial in the development of somatic embryos in number of

Table 1: Effect of auxins and cytokinins on induction of somatic embryos in *E. hirta*.

BAP(mg/L)	Kin(mg/L)	IAA (mg/L)	NAA (mg/L)	Explants showing somatic embryogenesis (%)
0	0	0	0	0^{a}
0.2	-	-	-	83.9^{ij}
0.4	-	-	-	91.4^{n}
0.6	-	-	-	88.0^{m}
0.8	-	-	-	81.9^{h}
1.0	-	-	-	76.2^{f}
-	0.2	-	-	56.0^{b}
-	0.4	-	-	72.3^{e}
-	0.6	-	-	69.7^{d}
-	0.8	-	-	63.1^{c}
-	1.0	-	-	57.5^{b}
0.2	0.4	0.5	-	83.0^{hi}
0.4	0.4	0.5	-	92.9^{m}
0.6	0.4	0.5	-	89.6^{m}
0.8	0.4	0.5	-	85.0^{jk}
1.0	0.4	0.5	-	77.5^{fg}
0.2	0.4	-	0.5	86.3^{kl}
0.4	0.4	-	0.5	100^{o}
0.6	0.4	-	0.5	91.8^{n}
0.8	0.4	-	0.5	87.7^{l}
1.0	0.4	-	0.5	79.2^{g}
LSD				3.42

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Table 2: Effect of different concentrations of sucrose on induction of somatic embryogenesis.

Sucrose concentration (%)	Induction of somatic embryos (%)
1.0	43.0^{a}
2.0	51.5^{b}
3.0	93.9^{e}
4.0	70.6^{d}
5.0	66.4^{c}
LSD	2.01

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Table 3: Effect of BAP and Kin on germination of somatic embryos on half strength MS medium after 4 weeks.

Conc. of BAP (mg/L)	Conc. of Kin (mg/L)	No. of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
0	0	15.4±0.23^{a}	1.2±0.16^{a}
0.5	0.1	83.9±0.51^{b}	5.3±1.60^{e}
1.0	0.1	79.0±0.57^{h}	4.7±0.91^{de}
1.5	0.1	74.1±0.05^{g}	4.4±1.00^{d}
2.0	0.1	65.9±1.15^{f}	3.7±0.63^{c}
2.5	0.1	60.3±0.17^{e}	3.0±0.17^{b}
3.0	0.1	55.8±0.28^{d}	2.9±0.59^{b}
LSD	3.91	1.24	

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Euphorbiaceae plant species. Linossier *et al.* (31) studied the effect of ABA and high concentration of polyethylene glycol on induction of somatic embryos in *H. brasiliensis*.

Full strength MS medium with 3% sucrose was reported optimum for the induction of somatic embryos in this investigation. Rambabu *et al.* (32) induced zygotic embryos in *Givotia rottleriformis* with the same concentrations of sucrose but Konnan *et al.* (33) initiated somatic embryos with 2% sucrose. The present results are similar with the somatic embryogenesis in Rose plant, *Curculigo orchioides* and *S. mukorossi* (33, 34, 27), where maximum percentage of somatic embryos was induced with 3% sucrose in the medium.

The embryos were unable to germinate on full strength MS medium, even one-fourth strength of MS medium was also reported less effective in E.hirta. Groll *et al.* (29) suggested half and full strength MS salt strength more suitable in somatic embryogenesis of *M. esculenta*. Cheruvathur *et al.* (34) reported half strength MS medium optimum for the germination of somatic embryos in *Rhinacanthus nasutus*. Germination of somatic embryos was confirmed by the development of bipolar structures leads to the formation of shoot and root from the mass of tissues. Similar observations were recorded in *Decalepis hamiltonii* (37), *Tylophora indica* (38) and *R. nasutus* (36).

The regular subculture of somatic embryos on fresh medium with BAP yielded maximum plantlets. The essentiality of BAP in the medium for germination of somatic embryos was also reported in *Phoenix dactylifera* (39), *W. calendulacea* (18) and *S. mukorossi* (27). Combination of BAP and NAA was found optimum for morphogenesis of somatic embryos in *E. nivulia* (24).

The microscopic examinations confirmed the origin of somatic embryos from the surface of the explants. The formation of direct somatic embryos from surface of the explants was also reported in *Arabidopsis thaliana* (10). Rai *et al.* (40) observed that the induction of somatic embryos is determined by the specific tissues of explants during developmental stages. The stages of somatic embryos from meristematic region to step wise globular, heart and torpedo stages through microsocopical analysis were also reported in *P. dactylifera* (39) and *Nicotiana* sp. (13). The morphogenesis of somatic embryos and plantlet conversion has been studied in *L. pyrotechnica* (17), *Elaeis guineensis* (41) and *Hybanthus enneaspermus* (42).

Conclusion

The somatic embryos could be directly produced from the surface of the explants of *E.hirta* These can be elongated and developed into shoot and root on different strengths of MS media. The embryos were originated from the epidermal and subepidermal cells of the explants as confirmed by the microscopic observations of the sections of explants. The present investigation could be explored for the large scale plant production and germplasm conservation of multipotent medicinal plant *E. hirta*.

Conflict of interests : Authors declare that there is no conflict of interests in this publication.

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