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Somatic embryogenesis and plantlet regeneration of *Cassia angustifolia* from immature cotyledon-derived callus

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Abstract

Plant regeneration through indirect somatic embryogenesis was attempted from the immature cotyledon-derived explant of *Cassia angustifolia* Vahl. – a valuable leguminous shrub. The highest frequency (90.5 %) of somatic embryos was obtained on a Murashige and Skoog (MS) medium augmented with 10.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 μM benzyladenine (BA) with the production of a maximum of 22.8 embryos per explant, of which 35.3 % germinated on the same medium after 6 weeks of culture. A half strength MS medium without plant growth regulators facilitated better conversion of embryos into complete plantlets compared to a full strength MS medium. Regenerated plantlets were successfully acclimatized in sterile *Soilrite* and transferred to field conditions with a 70 % survival rate. Histological studies performed at different stages of embryogenesis revealed the mode of differentiation of embryos from the callus. The content of chlorophylls (*a* + *b*) and carotenoids, and the net photosynthetic rate (P_N) in the regenerated plantlets were tested during different periods of acclimatization.

Additional key words: acclimatization, auxin, carotenoids, chlorophyll, cytokinin, histology, net photosynthetic rate, senna.

Introduction

Somatic embryogenesis includes a number of characteristic events like differentiation of cells, activation of cell division and reprogramming their physiology, metabolism, and expression patterns (Komamine *et al.* 2005, Yang and Xang 2010). The first report of somatic embryogenesis was documented by Steward *et al.* (1958) and Reinert (1959) in carrot cell suspension cultures. Since then the regeneration of complete plantlets *via* somatic embryogenesis has been successfully reported in a large number of plant species (*e.g.*, Jayanthi and Mandal 2001, Martin 2004a, Sudha and Seeni 2006, Sahai *et al.* 2010, Nowak *et al.* 2012, Sala *et al.* 2013) including many leguminous plants (Chand and Sahrawat 2002, Faisal *et al.* 2008, Husain *et al.* 2010, Mishra *et al.* 2012).

Somatic embryos develops either directly from the explant (direct somatic embryogenesis, DSE) or indirectly after an intermediate callus phase (indirect somatic embryogenesis, ISE) (Sharp *et al.* 1980). DSE proved to be advantageous for the production of true-to-type clones of the plants due to the minimal changes in the genotype (Peshke and Philips 1992), however, reports on DSE are relatively rare. Various explants have been exploited for the induction of embryogenic calli but generally, immature meristematic tissues proved to be the most suitable ones, and immature zygotic embryos and cotyledons have been used for the induction of somatic embryogenesis in most legumes (Sagare *et al.* 1995, Ahmed *et al.* 1996, Gairi and Rashid 2005).

In the present investigation, efforts were made to induce somatic embryogenesis from immature cotyledon explants of senna which is a valuable legume of high medicinal value. Leaves and pods contain important alkaloids which are derivatives of anthraquinone glycosides and commonly referred as sennosides. Besides sennosides, rhein, chrysophanol, emodin, aloe-emodin, and several mono- and di-glycosides are also present in different parts of the plant.

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Abbreviations: BA - benzyladenine; Car - carotenoid; Chl - chlorophyll; 2,4-D - 2,4-dichlorophenoxyacetic acid; 2iP - 2-isopentenyl adenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; IC - immature cotyledon; Kn - kinetin; MS - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; PGR - plant growth regulator; P_N - net photosynthetic rate; 2,4,5-T - 2,4,5-trichlorophenoxyacetic acid.

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Materials and methods

Green pods of *Cassia angustifolia* Vahl. were surface sterilized with 0.1 % (m/v) $HgCl_2$ (*Qualigens*, Mumbai, India) for 5 - 6 min followed by repeated washing (4 - 5 times) with sterile double distilled water (DDW). For somatic embryogenesis, immature cotyledons (IC) were excised from semi-mature seeds. They were thoroughly washed under running tap water for about 30 min to remove adherent particles and then dipped in 1 % (m/v) *Bavistin* (*BASF*, Mumbai, India) for 25 to 30 min followed by thorough washing with 5 % (v/v) *Teepol* for 15 min. Subsequent washing with sterile DDW was done 3 - 4 times under the laminar air flow followed by a short treatment (30 - 40 s) with 70 % (v/v) ethanol.

A basal medium consisted of Murashige and Skoog (Skoog 1962; MS) inorganic salts supplemented with 3 % (m/v) sucrose and 0.8 % (m/v) agar (*Hi-media*, Mumbai, India). The pH of the medium was adjusted to 5.8 using 1 M NaOH or 1 M HCl prior to autoclaving at a temperature of 121 ºC and a pressure of 1.06 kg cm^{-2} for 20 min. All the cultures were maintained at a temperature of 24 ± 2 ºC, a 16-h photoperiod, a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40W, *Philips*, Mumbai, India), and a 50 - 60 % relative humidity. Explants were cultured on MS media containing different concentrations of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or α-naphthaleneacetic acid (NAA)] either alone or in combination with cytokinins [benzyladenine (BA), kinetin (Kn), or 2-isopentenyl adenine (2iP)] for the induction of embryogenic calli. The same media were used for somatic embryo germinations. Germinated somatic embryos were transferred to the MS basal medium or half strength MS medium without plant growth regulators (PGRs) for the maturation and production of complete plantlets with well differentiated shoot and root systems.

Well-developed plantlets were washed gently under running tap water to remove any adherent gel and transferred to *Thermocol* cups containing sterile *Soilrite*. These plantlets were covered with transparent polyethylene bags to ensure high humidity and were irrigated every 3 d with a one fourth strength MS salt solution for two weeks. Bags were then removed, and after 4 weeks, the plants were transferred to earthen pots containing sterilized garden soil and garden manure (1:1) and maintained in a greenhouse under natural day length. A set of *in vitro* regenerated plantlets was used for physiological studies. Leaf samples were collected at day 0 (control) and after 1, 2, 3, and 4 weeks of acclimatization. The chlorophylls (Chl *a* and Chl *b*) and carotenoid (Car) content were estimated in an 80 % (v/v) acetone extract by the methods of MacKinney (1941) and MacLachan and Zalik (1963), respectively. For the estimation of Chl, the absorbances were read at wavelengths of 645 and 663 nm, and for carotenoids at 480 and 510 nm on a spectrophotometer (*UV-Pharma Spec 1600*, *Shimadzu*, Tokyo, Japan). The net photosynthetic rate (P_N) was measured using a portable infra-red gas analyzer (*LICOR 6400*, Lincoln, USA) at an irradiance of 1 000 ± 5 μmol m^{-2} s^{-1}, a CO_2 concentration of 360 μmol mol^{-1}, a leaf temperature of 23 ± 2 ºC, and a relative humidity of 60 ± 5 %

For histological studies, the embryogenic calli at different developmental stages were fixed in a FAA solution consisting of formalin + glacial acetic acid + 70 % ethanol in the ratio of 5:5:90 (v/v/v) for about 24 h and then stored in 70 % ethanol. The fixed material was dehydrated through a graded ethanol-xylol series and then embedded in paraffin wax as described by Johansen (1940). The embedded tissues were cut using a *Spencer 820* rotary microtome (*American Optical Corporation*, Buffalo, USA) at 10 μm thickness, and resulting paraffin ribbons were mounted on clean glass slides. After dewaxing in a xylol-ethanol series, sections were stained with safranin and fast green followed by permanent mounting in Canada balsam. The sections were observed under an *Olympus* (Tokyo, Japan) *CH20i* microscope and photographed using a *Cannon* (Tokyo, Japan) *Power Shot 640* camera.

All experiments were conducted with a minimum of ten replicates per treatment and repeated three times. Each replicate represented one explant per culture vial. Data were analyzed statistically through one-way *ANOVA* using *SPSS v. 16* (*SPSS Inc.*, Chicago, USA). The significance of difference among means was carried out by the Duncan's multiple range test at $\alpha = 0.05$.

Results

Immature cotyledons were cultured on MS media (Fig. 1*A,B*) containing different concentrations of auxins (2,4-D, 2,4,5-T, IAA, IBA, and NAA) either alone (Table 1) or in combination of the best auxin with cytokinins (BA, Kn, and 2iP) (Table 2) to induce embryogenic calli. An MS medium without any PGR served as control medium on which explants failed to induce an embryogenic callus even after 3 weeks of incubation. Auxins stimulated swelling explants within one week of culture, and yellowish to light green calli started to differentiate from cut ends of the explant (Fig. 1*C*). The induction of globular embryoids was observed after 3 weeks of culture and later on heart shaped and torpedo embryoids were also observed (Fig. 1*D*). Cotyledonary staged embryos were most frequently seen and clearly visible with naked eye.

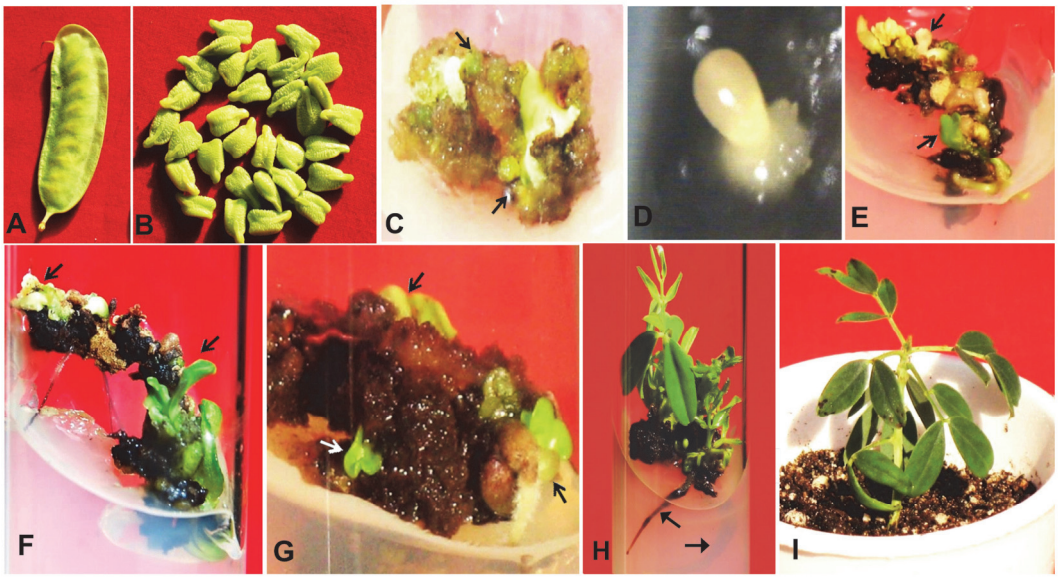

Fig. 1. Different organs used as explants for somatic embryogenesis in *C. angustifolia*: *A* - green immature pod, *B* - immature seeds. *C* - The production of an embryogenic callus from an immature cotyledon on an MS medium supplemented with 5.0 µM 2,4-D (*arrows* represent globular stage embryoids) after 3 weeks. *D* - An enlarged view of a globular embryo under a stereozoom microscope. *E* - The differentiation of mature somatic embryos on an MS medium containing 5.0 µM 2,4-D (*arrows* represent cotyledonary stage embryoids) after 6 weeks. *F* - The maturation and germination of cotyledonary stage embryoids on an MS medium containing 10.0 µM 2,4-D (*arrows* represent a germinating embryoid with proper shoot primordia) after 6 weeks. *G* - The development of somatic embryoids on an MS medium containing 5.0 µM NAA along with the germination of mature embryoids on the same medium (*arrows* represent a germinating embryoid with proper shoot and root primordia) after 5 weeks. *H* - The development of complete plantlets from germinating embryoids on transferring to a half strength MS medium (*arrows* represent a developing root) 3 weeks after transfer. *I* - Successfully acclimatized embling in sterile *Soilrite* 3 weeks after the transfer.

Table 1. The effect of different auxins on the induction of somatic embryogenesis from immature green cotyledons of *C. angustifolia.* Data recorded after 6 weeks. Means ± SE of 3 repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P > 0.05$) using the Duncan's multiple range test.

2,4-D [µM]	2,4,5-T [µM]	IAA [µM]	IBA [µM]	NAA [µM]	Response [%]	Number of embryoids [explant^{-1}]
-	-	-	-	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
1.0	-	-	-	-	26.43 ± 1.86^{e}	3.90 ± 0.37^{c}
2.5	-	-	-	-	38.30 ± 1.90^{d}	6.23 ± 0.53^{b}
5.0	-	-	-	-	65.20 ± 1.53^{b}	8.30 ± 0.62^{a}
10.0	-	-	-	-	83.90 ± 1.70^{a}	9.23 ± 0.67^{a}
-	1.0	-	-	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	2.5	-	-	-	12.76 ± 1.29^{g}	1.83 ± 0.32^{d}
-	5.0	-	-	-	19.73 ± 2.21^{f}	3.90 ± 0.20^{c}
-	10.0	-	-	-	10.26 ± 0.95^{gh}	1.53 ± 0.31^{d}
-	-	1.0	-	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	2.5	-	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	5.0	-	-	8.63 ± 2.07^{hi}	2.23 ± 0.53^{d}
-	-	10.0	-	-	5.86 ± 1.41^{i}	1.50 ± 0.28^{d}
-	-	-	1.0	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	-	2.5	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	-	5.0	-	9.63 ± 1.50^{ghi}	3.63 ± 0.23^{c}
-	-	-	10.0	-	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	-	-	1.0	00.00 ± 0.00^{j}	0.00 ± 0.00^{e}
-	-	-	-	2.5	29.46 ± 2.11^{e}	3.83 ± 0.32^{c}
-	-	-	-	5.0	42.96 ± 1.43^{c}	6.26 ± 0.40^{b}
-	-	-	-	10.0	30.30 ± 1.02^{e}	3.60 ± 0.20^{c}

Table 2. The effect of the optimal concentration of 2,4-D with different cytokinins on somatic embryogenesis from immature green cotyledons of *C. angustifolia.* Data recorded after 6 weeks. Means ± SE of 3 repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P > 0.05$) using the Duncan's multiple range test.

2,4-D [μM]	BA [μM]	K [μM]	2iP [μM]	Response [%]	Number of embryoids [explant^{-1}]	Germination [%]
10.0	1.0	-	-	90.56 ± 1.88^{a}	22.80 ± 1.59^{a}	35.33 ± 2.90^{a}
10.0	2.5	-	-	86.06 ± 0.58^{ab}	18.20 ± 1.20^{b}	30.00 ± 1.15^{ab}
10.0	5.0	-	-	83.43 ± 0.34^{b}	17.06 ± 0.74^{b}	26.00 ± 2.30^{b}
10.0	-	1.0	-	68.23 ± 0.95^{d}	13.23 ± 1.43^{cd}	12.33 ± 1.45^{cd}
10.0	-	2.5	-	78.03 ± 1.59^{c}	14.33 ± 0.76^{c}	15.66 ± 2.33^{b}
10.0	-	5.0	-	66.56 ± 1.82^{d}	11.40 ± 0.45^{d}	10.00 ± 1.15^{cd}
10.0	-	-	1.0	52.73 ± 1.75^{e}	11.73 ± 0.62^{cd}	8.66 ± 2.02^{d}
10.0	-	-	2.5	64.50 ± 2.32^{d}	13.10 ± 0.20^{cd}	11.33 ± 1.76^{cd}
10.0	-	-	5.0	50.46 ± 1.35^{e}	11.43 ± 0.31^{d}	7.66 ± 1.45^{d}

Among various concentrations of different auxins tested, the lower concentrations of 2,4-D were not much effective, and at 5.0 μM 2,4-D, about 65.2 ± 1.5 % of cultures exhibited the induction of an embryogenic callus with the differentiation of cotyledonay staged embryos after 6 weeks (Fig. 1*E*). The most effective was 10.0 μM 2,4-D, inducing embryogic calli in 83.9 ± 1.7 % of cultures, wherein a maximum of 9.2 ± 0.7 embryoids per explant were produced and a few started germinating on the same medium (Fig. 1*F*). All other auxins including 2,4,5-T produced a light brown loose and watery callus which showed a moderate embryogenic response, and lower concentrations (1.0 or 2.5 μM) of auxins failed to induce somatic embryogenesis at all. IAA and IBA at 5.0 μM concentrations were found to be the least effective and exhibited only 8.6 ± 2.1 and 9.6 ± 1.5 % responses, respectively. However, 5.0 μM NAA yielded a 42.9 ± 1.4 % response producing an average of 6.3 ± 0.4 embryoids per explant. Some embryoids started germinating on the same medium containing 5.0 μM NAA and showed the development of root and shoot apices (Fig. 1*G*) which ultimately resulted in the formation of complete plantlets (Table 1).

The optimal concentration (10.0 μM) of 2,4-D was also tested with three different cytokinins at various concentrations to produce embryogenic calli. The addition of cytokinins along with 2,4-D improved the rate of embryogenesis and also facilitated the germination of embryoids on the same medium. The best embryogenic callus was obtained on an MS medium amended with 10.0 μM 2,4-D and 1.0 μM BA, producing a maximum of 22.8 ± 1.6 embryoids per explant in 90.6 ± 1.9 % cultures. A maximum of 35.3 ± 2.9 % of the germination rate was recorded on the same medium, the cotyledons of the germinating embryoids started expanding along with the emergence of root primordia. A further increase in the concentration of BA resulted in the formation of non-embryogenic calli and the rate of embryogenesis was reduced to 83.4 ± 0.3 % on MS + 10.0 μM 2,4-D + 5.0 μM BA. Kn provided a moderate response in 78.0 ± 1.6 % cultures on a medium containing MS + 10.0 μM 2,4-D + 2.5 μM Kn, whereas 2iP proved to be less effective and exhibited a 64.5 ± 2.3 % response on a medium comprised of MS + 10.0 μM 2,4-D + 2.5 μM 2iP (Table 2).

The germinating embryoids (8-d-old) measuring 0.5 - 0.6 cm when transferred to the hormone free half strength and full strength MS media started expanding the cotyledonary leaves along with elongating the radicle 4 d after the transfer. The expansion of cotyledonary leaves and the growth of root system was more pronounced in the half strength than full strength MS medium (Fig. 1*H*). Apical shoot growth with the appearance of leaf primordia was observed 10 d after the transfer on both media. However, the growth and differentiation of shoots was better on the half strength MS medium, and after further 14 d, shoots attained a length of 2.20 cm followed

Table 3. The analysis of various photosynthetic pigments and a net photosynthetic rate on *in vitro* raised plantlets of *C. angustifolia* during the acclimatization period. Means ± SE of 3 repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using the Duncan's multiple range test.

Acclimatization [week]	Chl *a* [mg g^{-1}(f.m.)]	Chl *b* [mg g^{-1}(f.m.)]	Chl (*a*+*b*) [mg g^{-1}(f.m.)]	Car [mg g^{-1}(f.m.)]	P_N [μmol(CO_2) m^{-2} s^{-1}]
0	0.44 ± 0.04^{e}	0.16 ± 0.05^{b}	0.60 ± 0.09^{c}	0.17 ± 0.02^{c}	2.50 ± 0.28^{e}
1	0.29 ± 0.04^{d}	0.15 ± 0.03^{b}	0.45 ± 0.04^{c}	0.10 ± 0.02^{c}	1.20 ± 0.11^{d}
2	0.57 ± 0.05^{c}	0.32 ± 0.03^{a}	0.89 ± 0.08^{b}	0.29 ± 0.02^{b}	3.90 ± 0.20^{c}
3	0.83 ± 0.03^{b}	0.32 ± 0.03^{a}	1.15 ± 0.06^{a}	0.35 ± 0.03^{b}	8.16 ± 0.20^{b}
4	1.04 ± 0.02^{a}	0.35 ± 0.05^{ab}	1.39 ± 0.07^{a}	0.52 ± 0.03^{a}	9.23 ± 0.14^{a}

by 1.07 cm in the full strength MS medium. However, the survival rate was rather low, 30 and 20 % on the half strength and full strength MS media, respectively.

After six weeks, germinated embryoids (emblings) were transferred from the culture medium onto the planting substrate consisting of sterile *Soilrite* in *Thermocol* cups (Fig. 1*I*) and exhibited a 70 % survival rate. Regenerated plantlets showed normal morphological characters when compared with the *in vivo* grown plants and were further evaluated for different physiological parameters at various stages of acclimatization (0, 1, 2, 3, and 4 weeks).

The content of Chl *a* and Chl *b* initially decreased [from 0.44 to 0.29 and 0.16 to 0.15 mg g^{-1}(f.m.), respectively] and later on increased linearly with the acclimatization period. At the end of the fourth week, the content of Chl *a* and Chl *b* was 1.04 and 0.35 mg g^{-1}(f.m.), respectively. These values suggest that the plants were fully adapted to the external environment. The Car content showed similar trend. In the first week, the Car content was reduced from 0.17 to 0.10 mg g^{-1}(f.m.) and later on it increased to 0.29 mg g^{-1}(f.m.) after the second week and to 0.52 mg g^{-1}(f.m.) by the end of the fourth week (Table 3).

The net photosynthetic rate decreased, when the plantlets were transferred to the external environment, from 2.50 to 1.20 µmol(CO_2) m^{-2} s^{-1} during the first week. As soon as the plantlets adapted to the external environment, P_N increased linearly and it reached 8.16 and 9.23 µmol(CO_2) m^{-2} s^{-1} by the end of the third and fourth week of acclimatization, respectively (Table 3).

The embryogenic callus produced on MS + 5.0 µM 2,4-D showed the differentiation of globular embryoids with well-marked boundaries and distinction from the callus tissue (Fig. 2*A*). The globular embryoids grew in size and became pear shaped (Fig. 2*B*). The heart shaped embryoids were also clearly observed, having a well differrentiated boundary with the apical notch (Fig. 2*C,D*). The mature and cotyledonary staged embryoids were distinctly present in the embryogenic callus obtained on MS + 10.0 µM 2,4-D and showed well differentiated cotyledonary leaves (Fig. 2*E,F*).

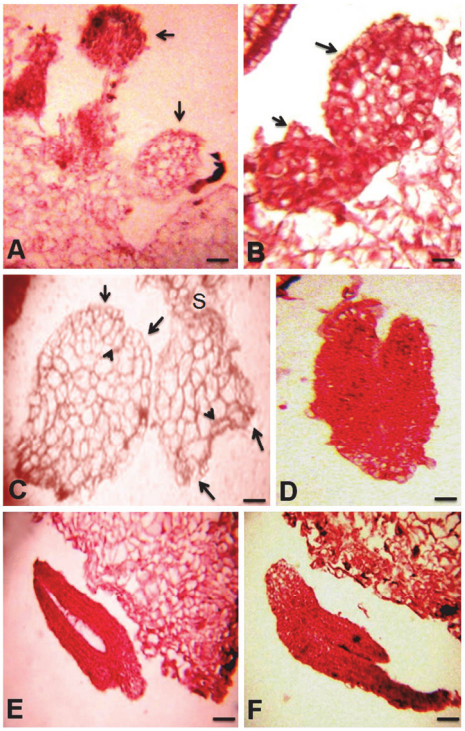

Fig. 2. Histological sections showing the differentiation of somatic embryos of *C. angustifolia* from an immature cotyledon derived callus at different developmental stages: *A* - the formation of globular embryos at the peripheral surface of callus tissue on MS + 5.0 µM 2,4-D (*bar* = 50 µm); *B* - the conversion of globular embryos to pear shaped at later stages of development on the same medium (*bar* = 30 µm); *C* - heart shaped embryos showing cotyledonary initials (*arrows*) and a differentiating apical meristem region (*arrow head*) with a suspensor like structure on MS + 5.0 µM 2,4-D (*bar* = 40 µm); *D* - a heart shaped embryo at the later stage of development (*bar* = 100 µm); *E, F* - the formation of mature, cotyledonary stage somatic embryos on MS + 10.0 µM 2,4-D (*bar* = 400 µm).

Discussion

Somatic embryogenesis is a process where a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue (Arnold *et al.* 2002). In the present study, an embryogenic callus was induced from IC of *C. angustifolia*. Callus production followed by somatic embryogenesis has been reported in several plants using different explants, such as cotyledon in *Juglans nigra* (Neuman *et al*. 1993) and leaf *in Holostemma ada-kodien* (Martin 2003). However, immature and young explants are generally more responsive than mature ones as in the cases of *Hardwickia binata* (Chand and Singh 2001) and *Dalbergia sissoo* (Singh and Chand 2003). The developmental stage of the explant has a critical influence on the ability to induce somatic embryogenesis in woody legumes, and transition of somatic cells into embryogenic cells (Trigiano *et al*. 1992).

Generally, auxin like 2,4-D is considered essential for the induction and maintenance of embryogenic cultures (Choi *et al*. 1999), however, a combination of auxin and cytokinin can be the best to induce embryogenic calli in several plants (Jayanthi and Mandal 2001, Martin 2004b). In the present investigation, various auxins were tried and amongst them, 2,4-D proved to be the best. Similarly in *Eleutherococcus sessiliflorus* and *Holostemma ada-kodien*, the role of 2,4-D in the production of embryogenic callus has been emphasized (Arnold *et al*. 2002, Choi *et al*. 2002) and the same was corroborated by our study. The capability of 2,4-D in activating the embryogenic pathway may be related to its capacity to

induce stress genes which have been shown to contribute to the cellular reprogramming the somatic cells towards embryogenesis (Kitaniya *et al*. 2002). All other auxins tested proved to be less effective than 2,4-D, whereas 5.0 µM NAA was efficient for the germination of embryoids on the same medium, although it produced a lesser number of embryoids. According to Peeters *et al*. (1991), the uptake and utilization of NAA is faster which could explain the conversion of somatic embryos.

The presence of cytokinin in the induction medium proved to be crucial for a high frequency of somatic embryos. Accordingly, the augmentation of different cytokinins with optimal concentration of 2,4-D (10.0 µM) enhanced the rate of embryogenesis and facilitated the germination of embryoids. The maximum number of embryoids was obtained on a medium containing 10.0 µM 2,4-D and 1.0 µM BA. However, in an earlier report of somatic embryogenesis in senna (Agrawal and Sardar 2007), a lesser number of embryoids were produced compared to our study. Thus, our protocol proved to be more effective for efficient embyogenesis in *C. angustifolia*. The combined favourable influence of auxin and cytokinins observed in the present study is in accordance with reports on other plants like *Rauvolfia caffra* (Upadhyay *et al*. 1992), *Echinochloa colona* (Samantaray and Rout 1997), and *Arachis hypogea* (Venkatachalam 1999). Conversely, the addition of 2,4-D alone or with BA was not favourable for somatic embryo formation in *Rauvolfia micrantha* (Sudha and Seeni 2006).

The mature somatic embryos started germinating on a cytokinin + auxin supplemented medium. Xie and Hong (2001) reported a callus induction from cotyledons of mature zygotic embryos of *Acacia mangium* in an MS medium supplemented with 2,4-D and Kn. Transferring 8-d-old germinating embryoids to a hormone free full and half strength MS media helped in the development of complete plantlets with a proper shoot and root formation within 3 weeks, although the survival percentages of embryioids on the MS basal and half strength MS media were quite low (20 and 30 %, respectively). Similar to our results, Sahai *et al*. (2010) reported a successful develop-ment of complete plantlets in *Tylophora indica* when
10-d-old germinating somatic embryoids were transferred to a half strength MS medium.

In vitro raised plantlets may exhibit abnormal morphology, anatomy and physiology (Desjardins 1995). In many plants, these abnormal characters affect the acclimatization of *in vitro* plantlets to the external environment. Therefore, the physiology of micro-propagated plantlets, in particular the photosynthetic performance during *in vitro* and *ex vitro* growth, has been the subject of several investigations (Van Huylenbroeck *et al.* 2000). Nevertheless, after transfer to *ex vitro* conditions, most plantlets develop a functional photosynthetic apparatus (Kozai 1991).

The content Chl *a*, Chl *b*, and Car showed a decreasing trend in senna during initial days after the transfer from *in vitro* to *ex vitro* conditions, but later on a linear increase was observed. Lu and Zhang (1998) and Sopher *et al*. (1999) suggested that a loss of Chl during acclimatization means that the leaves are damaged due to photoinhibition. By the end of the fourth week, the highest content of Chl *a*, Chl *b*, and Car was recorded suggesting that the plants were then fully acclimatized and adapted to the external environment. A similar trend of Chl content was observed by Kadleček *et al.* (1998), Pospíšilová *et al*. (1998), Amâncio *et al*. (1999), and Jeon *et al.* (2005). An increase in the Car content reflects the functional response of photosynthetic apparatus to the different irradiance, since the protective role of Car against photooxidative damage is well documented (Reuther 1991).

During *in vitro* culture, P_N is affected by the irradiance, the CO_2 concentration in the vessels, and the type and concentration of sugars in the medium. It usually decreases in the first days after transplantation and increases thereafter (Pospíšilová *et al*. 2009). Similar observations were recorded in the present study. P_N decreased during the first week of acclimatization and increased thereafter. The decline in P_N after the transplantation indicates that changes in climatic conditions created a stress in the regenerated plants. Further increase in the P_N is usually associated with the formation of new leaves (Kadleček *et al*. 1998, 2001, Slavtcheva and Dimitrova 2001, Guan *et al.* 2008, Dias *et al.* 2013).

The histology of the embryogenic callus produced from the immature cotyledon explants of *C. angustifolia* revealed the mode of differentiation of somatic embryos *via* indirect embryogenesis. Certain cells of the callus differentiated to produce proembryos which later developed into globular embryos having distinct boundaries and no vascular connection with the parent tissue. This criterion is one suggested by Haccius (1978) to distinguish somatic (non-zygotic) embryos from shoot buds. Globular embryos underwent several divisions and grew in size to become heart shaped having cotyledonary initials and a distinct apical meristem region. A single celled row of a suspensor-like structure or rudimentary suspensor was also observed at the heart shaped stage confirming the unicellular origin of the somatic embryo. There are only a few reports which mention the appearance of a suspensor or suspensor like structure during somatic embryogenesis (Quiroz-Figueroa *et al.* 2002, Sharma and Millam 2004). The developing heart shaped embryos further progressed towards late heart shaped or torpedo shaped embryos and finally attained the cotyledonary stage with well-developed mature cotyledons having differentiating apical meristem clearly visible.

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