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Study of Different Stages of Somatic Embryogenesis in a Medicinal Plant, Madar (*Calotropis procera*)

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ABSTRACT

An efficient protocol for *in vitro* regeneration of endangered medicinal plant *Calotropis procera* has been developed. The juvenile leaf explants were transferred to MS medium containing different combinations of PGRs. Among the various combinations of Kinetin (1.0-3.0 mg L^{-1}) and 2,4-D (1.0-5.0 mg L^{-1}) the intensity of callus induction was highest in MS medium with Kinetin (1 mg L^{-1}) and 2, 4-dichloro phenoxy acetic acid (3 mg L^{-1}). The percentage of calli forming embryos was 82.6. During organogenic callus formation, different types of calli with variation in color and texture were noticed and among them, the light green, fragile calli responded well for the induction of shoots. Among the various combinations of BAP and IAA used the differentiation was seen to be the best in the presence of 2 mg L^{-1} BAP and 1 mg L^{-1} NAA frequency of shoot regeneration was highest 75%. The shoot was transferred to MS media for root regeneration containing PGRs BAP (1-4) +NAA (1-4) mg L^{-1}. The frequency of root regeneration was 100% in MS medium containing BAP (1) + NAA (4) mg L^{-1}. The protocol was optimized by manipulations of different PGRs for enhanced multiplication. Protocol explained in this research paper provides a rapid plant regeneration system which could be used for the somaclonal variation; shoot induction and producing transgenic plants in *Calotropis*.

Key words: Madar, medicinal plant, callus, growth regulators, somatic embryos

INTRODUCTION

Medicinal plants have been the subject of man's curiosity since time immemorial. Almost every civilization has a history of medicinal plant use. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs and about 85% of traditional medicine involves the use of plant extracts. *In vitro* cell and tissue culture methodology is envisaged as a means for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large scale revegetation and for genetic manipulation studies. Combinations of *in vitro* propagation techniques and cryopreservation may help in conservation of biodiversity of locally used medicinal plants (Singh *et al*., 2009).

Calotropis procera commonly known as madar is an important medicinal shrub of family, Asclepiadaceae. *Calotropis procera* is a spreading shrub or small tree to 4 m, exuding copious milky sap when cut or broken; leaves opposite, grey-green, large upto 15 cm long and 10 cm broad, with

a pointed tip, two rounded basal lobes and no leaf stalk; flowers waxy white, petals 5, purple-tipped inside and with a central purplish crown, carried in stalked clusters at the ends of the branches; fruit grey-green, inflated, 8 to 12 cm long, containing numerous seeds with tufts of long silky hairs at one end (Brandes, 2005). Traditionally, different parts of *Calotropis* is used alone or with other medicinals (Caius, 1986) to treat common diseases such as fever, rheumatism, indigestion, cough, asthma, elephantiasis, diarrhoea, bronchitis, dyspepsia. *C. procera* was used in traditional medicine as anticancer as well as to treat leucoderma, ulcers, piles and diseases of the spleen (Jain *et al.*, 1996). The latex of *Calotropis procera* has been used in traditional medicine to treat different inflammatory diseases. The anti-inflammatory activity of Latex Proteins (LP) has been well documented using different inflammatory models. In this work the anti-inflammatory protein fraction was evaluated in a true inflammatory process by inducing a lethal experimental infection in the murine model caused by *Salmonella enterica* subsp. enterica serovar typhimurium (Lima-Filho *et al.*, 2010), ability to activate macrophages-effector cells in inflammatory and immune responses (Seddek *et al.*, 2009; Mathur *et al.*, 2009) and hepatoprotective (Setty *et al.*, 2007) and possess anti-tumor activity (Quaquebeke *et al.*, 2005), hepatoprotective activity (Quereshi *et al.*, 2007), antifungal activity and phytochemical analysis of leaves, roots and stem barks extracts of *Calotropis procera* (Hassan *et al.*, 2006) etc. The presence of alkaloids, flavonoids, cardiac glycosides as well as sterols and uscharin has been reported in the entire part of the plant (Edman, 1983; Al-Robai *et al.*, 1993; Hussein *et al.*, 1994).

Somatic embryogenesis offers an alternative and efficient protocol for plant regeneration. The technique of somatic embryogenesis has also contributed information for the genetic, morphological and physiological manipulation (Sharma *et al.*, 2010). Embryogenic tissue was identified as a target tissue for transformation by many researchers (Steward *et al.*, 1996; Maughan *et al.*, 1999). Somatic embryogenesis offers several advantages in crop improvement, as cost-effective and large-scale clonal propagation is possible using bioreactors, ultimately leading to automation of somatic seed production and development of artificial seeds. Besides, such a system could also provide a new source for use in genetic transformation.

MATERIALS AND METHODS

The year of study of this study was in July to November, 2004, in Centre for Biotechnology, University of Allahabad, Allahabad.

Plant material: Plantlets of *Calotropis procera* were obtained from Roxburg Garden of Botany Department, Allahabad University and grown in Centre for Biotechnology. All the explants were taken from these donor plants for present investigation. Leaf explants were cut with the help of scalpel and collected in a beaker containing water. It was then washed thoroughly under tap water for half an hour. For surface sterilization, chemicals such as $HgCl_2$ (0.1%), NaOCl (1%), H_2O_2 (1%) and ethanol (70%) was used. Juvenile leaves were washed thoroughly in running tap water for 30 min and then with distilled water three times (Haque *et al.*, 2009). Leaves were treated with Tween 20 solution for 4-5 min and then rinsed thoroughly with sterile distilled water. The leaves were subjected to 0.1% $HgCl_2$ for 30 sec washed with distilled water and then placed in 70% ethanol for 1 min and again washed with distilled water, followed by addition of three drops of antibiotic solution (Cefotaxime) in laminar airflow cabinet. In the antibiotic solution, all leaves were dissected into small pieces and trimmed so that maximum part can be exposed to media. All the chemicals used were purchased from Hi-media unless stated otherwise (Singh *et al.*, 2009).

Culture media and growth condition: The medium comprised of macro and micro elements according to Murashige and Skoog (1962) with 3% sucrose (Hi-Media), 100 mg L^{-1} myo-inositol (E. Merck) with 0.8% agar. The plant growth regulators used were 6-Benzylaminopurine (BAP), α-naphthalene acetic acid (NAA) and indole acetic acid (IAA). All experiments were carried out in culture tubes (150×25 mm) containing 30 mL of culture medium. The pH of media were adjusted to 5.8 prior to autoclaving at 121°C at 15 lbs pressure for 20 min. Culture were incubated under 16/8 h light/dark cycles (Haque *et al*., 2009).

Callus induction and maintenance of embryogenic calli: For callus induction juvenile leaf section (3-5 mm in length) with cut end surface in contact with culture medium were placed on MS medium supplemented with various concentrations of PGRs 2, 4-D and Kinetin. After 7 days of culture, the leaves cultured on MS basal medium supplemented with 3% (w/v) sucrose, kinetin (1.0 mg L^{-1}) and 2, 4 D (0.5 mg L^{-1}) were found to give profuse callusing and when callusing was observed in entire explant, the callus was cut into small pieces transferred to MS media having kinetin and 2, 4 D in same concentration as for callus induction. Subculturing was done after every 1-2 week. After 3-4 weeks of subculturing first shooting is observed in callus. The growth response of the calli is shown in (Table 1, 2), plant regeneration through indirect somatic embryogenesis was attempted from leaf-derived callus of *Calotropis procera* and the significant results were obtained.

Table 1: Effect of growth regulators on embryogenic callus induction from leaf segment of *Calotropis procera* (after 8 weeks inoculation)

Concentration of growth regulators (mg L^{-1})	Nature of callus	Percentage of calli forming embryos	Mean number of cotyledonary stage embryo
MS + KN			
0.5	No response	-	-
1.0	No response	-	-
1.5	No response	-	-
MS + KN +	2,4-D		
1.0 2.5	Creamy, embryogenic	73.4±0.08	5.0±0.4
1.0 3.0	Green, embryogenic	82.6±0.17	7.9±0.1
1.0 3.5	Green, embryogenic	75.3±0.33	6.2±0.5
MS KN IAA			
1.0 2.5	Creamy, friable	-	-
1.0 3.0	Creamy, friable	-	-
1.0 3.5	Creamy, friable	-	-
MS BAP 2, 4-D			
1.0 2.5	White, friable	-	-
1.0 3.0	White, embryogenic	48.0±0.12	2.3±0.7
1.0 3.5	White, embryogenic	52.2±0.66	3.1±0.3
MS BAP IAA			
1.0 0.25	No response	-	-
1.0 0.30	White, friable	-	-
1.0 0.35	White, friable	-	-
MS + 2,4-D			
2.5	White, compact	-	-
3.0	White, embryogenic	30.6±0.0	2.1±0.2
3.5	White, embryogenic	15.3±0.86	2.0±0.9

*Mean of 10 replication±SE (Standard error). The significant result were obtained Means with the same letters are not significantly different at the 5% level. The weight includes that of explants, whose initial weight per explants was. 005 g for leaf

Table 2: Growth pattern of calli on the basis of fresh weight from different number of days of *C. procera* on MS medium fortified with same growth regulator. Culture period 56 d. Mean of 10 replicates. Callus fresh mass includes the explant mass

	Fresh weight of calli (Number of days)								
Serial No.	7	14	21	28	35	42	49	56	Growth (%)
1.	0.124	0.126	0.154	0.162	0.175	0.201	0.203	0.211	70.161
2.	0.091	0.097	0.101	0.124	0.134	0.430	0.530	0.653	61.758
3.	0.112	0.125	0.136	0.144	0.158	0.167	0.178	0.186	66.071
4.	0.214	0.225	0.239	0.240	0.258	0.263	0.274	0.289	35.046
5.	0.131	0.145	0.151	0.168	0.174	0.181	0.194	0.212	61.832
6.	0.152	0.177	0.183	0.198	0.209	0.278	0.289	0.290	90.789
7.	0.306	0.319	0.322	0.337	0.340	0.352	0.376	0.386	26.143
8.	0.290	0.299	0.311	0.324	0.335	0.345	0.357	0.369	27.241
9.	0.221	0.232	0.243	0.252	0.262	0.268	0.275	0.281	27.149
10.	0.192	0.124	0.132	0.144	0.150	0.168	0.176	0.188	-2.083
Sum	1.833	1.869	1.972	2.093	2.195	2.653	2.852	2.854	101.9931
Mean	0.183	0.187	0.197	0.209	0.220	0.265	0.285	0.285	46.4104

Growth (%) = (Final weight-initial weight/Initial weight)×100

Somatic embryos at the highest frequency were induced on Murashige and Skoog (MS) medium supplemented Kinetin (1 mg L^{-1}) and 2, 4-dichloro phenoxy acetic acid (3 mg L^{-1}).

Differentiation of embryogenic callus into different stages of somatic embryogenesis: The calli were sub cultured onto fresh medium having same concentration of the growth regulators and maintained for months. After 45 days of each passage, the medium was exhausted and the calli started showing starvation. At this stage embryogenic calli were transferred into MS media containing different concentration of BAP, IAA, IBA, NAA, KN and 2, 4-D (Table 3) for inducing differentiation of these calli to somatic embryos. After 20 days calli started differentiating and showed different stages of somatic embryogenesis.

The somatic embryos of different stages were collected and stored in 4% formalin. These were dehydrated in increasing ethanol gradient and xylol and embedded in paraffin wax block. Thin sections (10 μm) were obtained using microtome. Section of the slides were dewaxed. Xylol was used for this purpose. Slides were passed through two changes of xylol (15 min in the first and 5 min in the second) so that wax was completely removed and only the tissue remained affixed to the slides. These sections were rehydrated and aqueous safranin stain was used at this stage. The slides were kept in this stain for 20-30 sec and again dehydrated.

DPX was used as a mountant. Once the DPX had dried completely, the slides were cleaned and examined under low power and high power lens. Photographs of the sections fixed to the slide were taken for studying the different stages using Leica DMLB with DC 300 camera with personal computer attached to it and the photographs were saved in the computer.

RESULTS AND DISCUSSION

The leaf disc segments (about 1 cm^2) size showed swelling of the explant and initiation of callusing from the edges and mid-rib in the first week of culture calli induction for the production of somatic embryos has been reported on MS medium supplemented with 2,4-D by various scientists in various plants of Asclepiadaceae family. Friable callus, developed from leaf and internode explants grown on Murashige and Skoog (MS) medium supplemented with 2,4-

Table 3: Effect of growth regulators on the differentiation of embryogenic callus into different stages of somatic embryogenesis after 30 days

	Days	
Conc. of growth regulator (mg L^{-1})	15	30
Stages of somatic embryogenesis		
MS + BAP		
0.5	No differentiation	No differentiation
1.0	No differentiation	No differentiation
1.5	Globular and Heart	Globular and Heart
2.0	Globular, Heart, Torpedo	Globular and Heart, Torpedo
2.5	Globular, Heart, Torpedo	Globular, Heart, Torpedo
MS + BAP + NAA		
0.5 1.0	No differentiation	No differentiation
1.0 1.0	Globular	Globular
1.5 1.0	Globular, Heart, Torpedo	Globular, heart, torpedo and cotyledonary
2.0 1.0	Globular, Heart, Torpedo	Globular, heart, torpedo and cotyledonary
2.5 1.0	Globular, Heart, Torpedo	Globular, heart, torpedo and cotyledonary
MS + BAP + IAA		
0.5 1.0	Globular	Globular
1.0 1.0	Globular	Globular
1.5 1.0	Globular	Globular
2.0 1.0	Globular	Globular
2.5 1.0	Globular	Globular
MS + KN		
0.5	No differentiation	No differentiation
1.0	No differentiation	No differentiation
1.5	No differentiation	No differentiation
2.0	No differentiation	No differentiation
2.5	No differentiation	No differentiation

dichlorophenoxyacetic acid (2,4-D), underwent somatic embryogenesis has been reported in *Ceropegia candelabrum* L. (Beena and Martin, 2003). Callus was obtained from leaf sections in Murashige and Skoog (MS) medium supplemented with α-naphthaleneacetic acid (NAA)+N^6-benzyladenine (BA) or 2,4-dichlorophenoxyacetic acid (2,4-D)+BA in *Decalepis hamiltonii* (Giridhar *et al*., 2004). Somatic embryogenesis and whole plant regeneration were achieved in callus cultures derived from hypocotyl, cotyledon and leaf explants excised from seedlings of *Gymnema sylvestre*. Embryogenic callus was induced on Murashige and Skoog (MS) medium containing 2,4-D +BA and 2% (w/v) sucrose in 6-8 weeks of culture (Ashok *et al*., 2002). Embryogenic calli were produced from inter-nodal explants of *Tylophora indica* (Burm. f.) Merrill. and the best result was achieved using Murashige and Skoog (MS) medium supplemented with 2, 4-Dichlorophenoxyacetic acid (Thomas, 2006) and an efficient procedure has been developed for inducing somatic embryogenesis from mature leaves on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) in addition with 2, 4-dichlorophenoxy acetic acid (2,4-D), particularly TDZ along with 2,4-D was very effective in inducing somatic embryos (Chandrasekhar *et al*., 2006).

The callus formation showed best response in the presence of low cytokinin and high auxin. Cytokinins like Benzyl amino purine (BAP) and Kinetin (KN) were tried. Auxins used for the

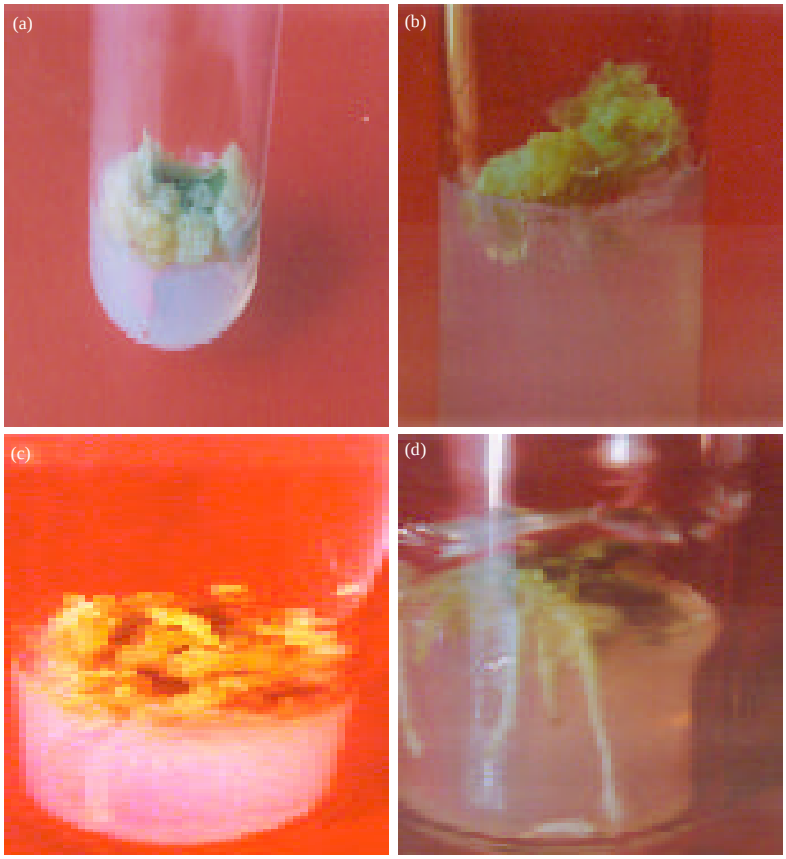

Fig. 1: Different stages of somatic embryogenesis formation in *Calotropis procera*. (a) Callus initiation from the edges, (b) Embryogenic callus, (c) Differentiating embryogenic callus and (d) Somatic embryo showing rooting

induction of embryogenic callus were Indole acetic acid (IAA) and 2, 4-dichloro phenoxy acetic acid (2, 4-D). Maximum embryogenic response per explant was observed on MS medium with Kinetin (1 mg L^{-1}) and 2, 4-dichloro phenoxy acetic acid (3 mg L^{-1}). The percentage of calli forming embryos was 82.6. Embryogenic callus response was also good with other concentrations of these two growth regulators. The percentage of calli forming embryos was 82.6. Kinetin alone and in combination with IAA was not effective in inducing the embryos. BAP along with IAA also did not show any response towards the embryo induction. However, BAP in conjunction with 2,4-D produced 48-52% embryo-forming calli. 2,4-D alone also induced 31-15% of calli forming embryos. The percentage growth of 10 replicates was 46.4106% monitored for a period of 56 days (Table 1). Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins). In the next two weeks, callus initiation was seen at the cut ends of the explant (Fig. 1a). Well-developed globular-shaped embryos were visible all over the cultured explant within six weeks (Fig. 1b, 2a). The embryo at this stage exhibited compactly arranged cells with thick cytoplasm (Fig. 3a).

The callus showed varied response when sub cultured under different growth regulator regimes. The callusing response in KN (1.0 mg L^{-1}) and 2, 4-D (2.5 mg L^{-1}) was late, the callus formed was creamy and percentage of calli forming embryo was less (73.4%). The mean number of embryos formed was 5.0. In media supplemented with low concentration of KN (1.0 mg L^{-1}) and

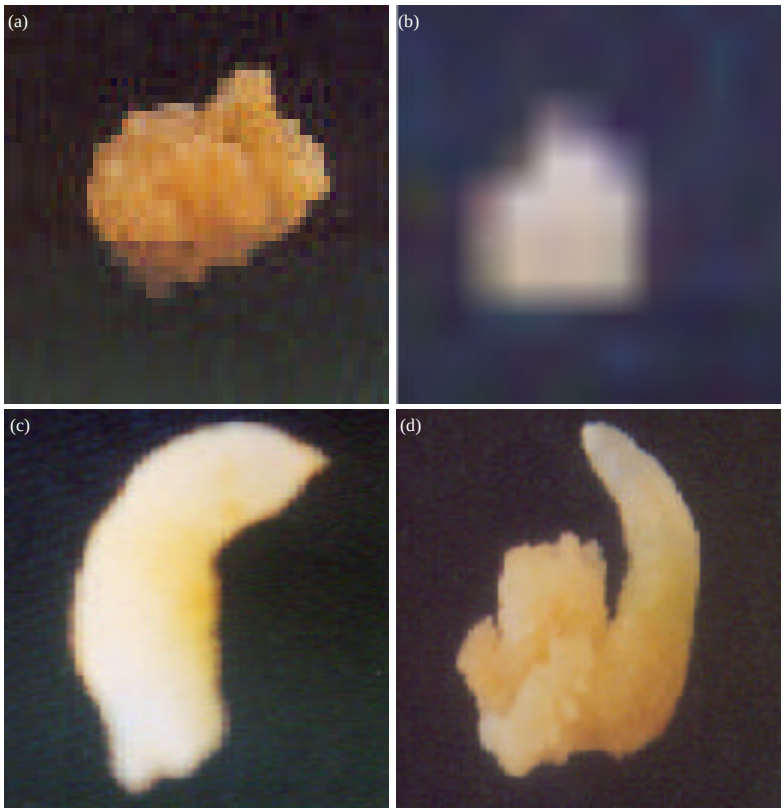

Fig. 2: Stages of somatic embryogenesis, (a) Embryogenic callus showing globular somatic embryos, (b) Heart-shaped somatic embryo, (c) Torpedo-shaped somatic embryo and (d) Cotyledonary somatic embryo

high concentration of 2, 4-D (3.0 mg L^{-1}), the embryos remained green and proliferative in nature without highest percentage of embryogenic calli formation (82.6%) and the mean numbers of embryos formed were 7.9. However, a higher concentration of 2, 4-D (3.5 mg L^{-1}) and low concentration of auxin (1.0 mg L^{-1}) did not show any signs of embryo formation till four weeks of culture after which it formed embryogenic callus. The callus induced a mean of 6.2 somatic embryos with a frequency of 75.3% of embryo induction among all the tested combinations.

Differentiation of the embryogenic callus into the different stages of somatic embryogenesis: Two months old culture of embryogenic callus was transferred onto a different media containing different concentrations of either BAP alone or in combination with NAA. The embryogenic callus was excised into small pieces and transferred to new medium. The differentiation was seen to be the best in the presence of 2 mg L^{-1} BAP and 1 mg L^{-1} NAA. High concentration of cytokinin was required with low concentration of auxin for differentiation. The ratio of BAP and NAA was 2:1 for differentiating callus into different stages (Table 3, Fig. 1c).

After two weeks of transfer to the differentiation medium, globular-shaped embryos were seen distributed throughout the callus mass (Fig. 1b, 2a, Fig. 3b). These globular-shaped embryos developed into heart shapes after a week (Fig 2b, Fig. 3c). After two weeks, heart-shaped embryos changed to torpedo shape (Fig. 2c, Fig. 3d) which finally differentiated to cotyledonary stage embryo (Fig. 2d).

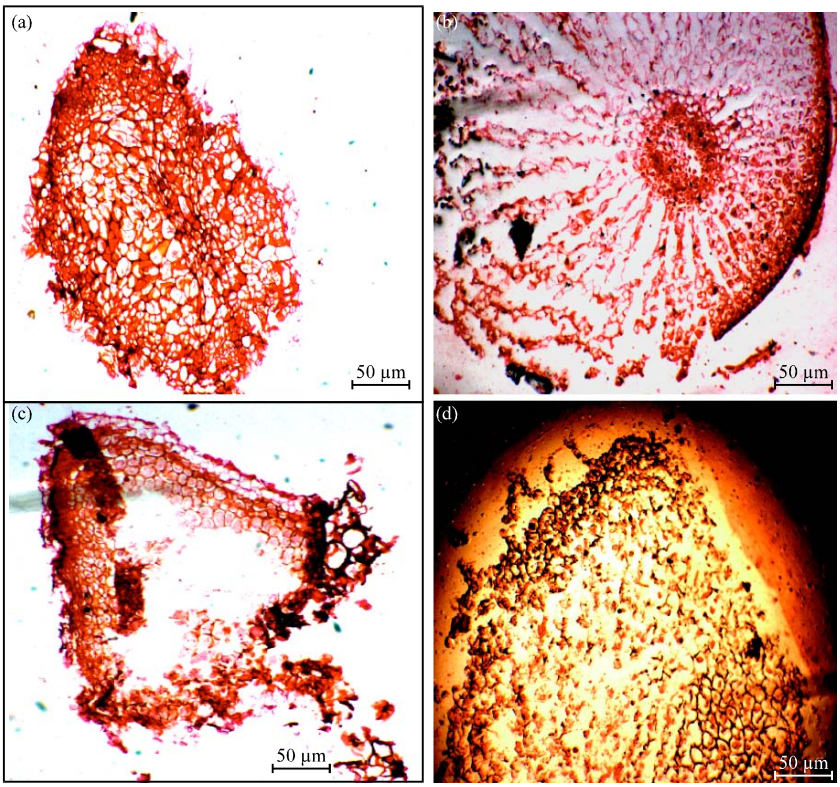

Fig. 3: Sections of different stages of somatic embryogenesis, (a) Sections of embryogenic callus, (b) Sections of Globular-shaped somatic embryo, (c) Sections of Heart-shaped somatic embryo and (d) Sections of Torpedo-shaped somatic embryo

The differentiation of embryogenic callus into different stages was maximum in BAP (2 mg L^{-1}) and NAA (1 mg L^{-1}) (Table 3, Fig. 1c). The different stages of somatic embryos were examined under stereomicroscope. Thin sections (15 µm) were cut and stained with safranin. The microscopic observation clearly revealed the different stages.

Different studies have shown that the callus is initiated and multiplied on a medium rich in auxin, which induces differentiation of localized group of meristematic cells called embryogenic clumps. Somatic embryo development generally follows the transfer of cells of callus to media lacking auxin, or with reduced level of the same auxin, or with similar or reduced levels of a weaker auxin. When transferred to a medium with low auxin or no auxin the embryogenic clumps develop into mature embryos (Chawla, 2002). The present study of somatic embryogenesis conducted in *Calotropis procera* also supports the similar results.

Germination of somatic embryos: The cotyledonary stage somatic embryos were transferred to MS medium supplemented with BAP and NAA. The embryos showed good germination in the presence of high BAP concentrations with a maximum germination at a concentration of 4 mg L^{-1} BAP without NAA. The results reveal that the shooting response of the embryo during germination was not as good as the rooting response (Fig. 1d). Shooting: rooting ratio was 2:1 in case of 4 mg L^{-1} BAP. Less shooting may be due to the incomplete maturation of the cotyledonary stage embryos. According to Krishnamurthy, somatic embryos are unipolar in development and the roots

are only adventitious by origin. The high frequency of somatic embryos in the result suggests that it might influence the endogenous level of cytokinin, auxin, abscisic acid, so as to induce the positive embryogenic response of the activated tissue. The results showed that high cytokinin concentration is required for the germination of *Calotropis procera* somatic embryos.

The results show that an efficient protocol is developed for somatic embryogenesis of *Calotropis procera*. The result confirms the generalization that the intermediate levels of auxins, either alone or in combinations with cytokinins, support initiation and maintenance of callus (Johri *et al*., 1996; Mohan *et al*., 2000).

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