International Journal of Bioassays
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Original Research Article

UNRAVELLING THE REGULATION OF SOMATIC EMBRYOGENESIS BY EXTRACELLULAR CALCIUM AND AUXIN EFFLUX BLOCKERS IN HYPOCOTYL EXPLANTS OF *ALBIZZIA LEBBECK* L.: SIMILARITY IN ACTION

Tanushri Kaul[1]*, Babu Ram[1], Saurabh Pandey[1], Chinreddy Subramanyam Reddy[1] and Malireddy K Reddy[1]
[1]Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India

Received for publication: September 17, 2014; **Revised:** September 21, 2014; **Accepted:** October 07, 2014

Abstract: Somatic embryogenesis involves developmental reprogramming and de-differentiation of somatic cells towards embryo development that is crucial for cellular totipotency in higher plants. It is a prototype for comprehension of the physiological, biochemical and molecular biological events transpiring during plant embryo development. In this study, we analyzed the role of exogenous calcium and auxin during somatic embryogenesis in hypocotyl explants of *Albizzia lebbeck* L. Light microscopic studies revealed that anterior and posterior ends of hypocotyl explants had entirely different patterns of differentiation. Scanning electron microscopic analysis of cultured hypocotyl explants exhibited globular-, heart- and torpedo-shaped somatic embryos developing from the anterior cut surface and gradually proceeding towards the other end. Surface texture of these somatic embryos showed uni-or bi-celled trichomes. Presence of exogenous calcium (1-4mM) in hormone-free B5 medium is prerequisite for induction of somatic embryos in hypocotyl explants derived from 7-d-old, light grown seedlings of *Albizzia lebbeck* L. A two-fold increase in calcium concentration from 2mM (B_5 medium) to 4mM led to doubling of somatic embryogenic response whereas, embryogenic callus was induced in explants raised on medium containing 10 or 20mM calcium. Depletion or high dosage in the medium (20 mM) induce non-embryogenic callus in explants. A similar response induced on hypocotyl explants upon treatments with NPA and PCIB, (auxin transport blockers). Results indicate that polar auxin transport or basipetal movement of IAA triggers acropetal transport of calcium. This combination of inverse fluxes acts as the primary signal for embryogenic response at the cut ends proximal to shoot apex and callusing at the cut ends distal to shoot apex of explants. Moreover, a correlation exists between NPA and PCIB-induced disruption of basipetal IAA transport and the inhibitory effect of NPA and PCIB on calcium translocation in the induction of similar morphogenetic responses.

Key Words: calcium chloride; indole-3-acetic acid; morphogenesis; naphthylphthalamic acid; parachlorophenoxyisobutyric acid: somatic embryos: tree legume: trichomes.

INTRODUCTION

The ability to manipulate morphogenetic events in forest tree species holds tremendous promise for circumventing limitations inherent in tree improvement programmes [1,2]. Somatic embryogenesis (SE) is sexual propagation process where somatic cells differentiate somatic embryos that can be used for studying the regulation of embryo development. However, SE has been viewed as a tool for massive propagation of commercial crops and as a potential model system for the study of the regulation of gene expression required for the earliest developmental events in the life of higher plants, such as the developmental mechanism of embryogenesis [3]. In addition, as the initial basis of cellular and genetic engineering, SE plays an important role in genetic transformation, somatic hybridization, and somaclonal variation. One of the major drawbacks in obtaining efficient somatic embryogenic systems in tree legumes is their inability to induce somatic embryos or low frequency of embryo production [4]. Past reports have implicated an intermediary role of calcium during plant embryogenesis. Calcium enhances embryogenic frequency in cell suspensions [5-7] whereas its deprivation arrests somatic embryo formation [8]. Enhanced concentration of calcium in the embryogenic induction media favours long-term proliferation of friable embryogenic cultures and development of somatic embryos [9]. Chelation or deprivation of exogenous Ca^{2+} arrests somatic embryo development and inhibits the accumulation of a Ca^{2+}-dependent protein kinase [10]. Thus, the role of exogenous Ca^{2+} in somatic embryogenesis provides evidence for calcium-mediated signaling. Possible role of polar auxin transport in somatic embryogenesis has also been demonstrated by the use of auxin transport blockers, leading to inability of somatic embryos to undergo morphogenetic transition to subsequent stages [11]. This implies that the ability of developing somatic embryos to maintain structural polarity is dependent on polar auxin transport.

Present investigations involve the possible role in modulation of somatic embryo induction in a legume tree species *Albizzia lebbeck* L. by calcium, auxin and auxin transport blockers in the nutrient medium. Based on these observations a similarity in the action of extracellular calcium and auxin efflux blockers is being suggested.

MATERIAL AND METHODS

Culture conditions: Seeds of *Albizzia lebbeck* L. were scarified at the non-micropylar end using sand paper followed by sterilization with teepol. Subsequently, seeds were thoroughly washed under running tap water and treated with freshly prepared chlorine water for 20min, followed by rinsing with

***Corresponding Author:**
Dr. Tanushri Kaul,
Research Scientist, Plant Molecular Biology Group,
International Center for Genetic Engineering Biotechnology,
Aruna Asaf Ali Marg, New Delhi-110067, India.

sterile distilled water. Sterilized seeds were cultured on Knop's medium solidified with 1% agar. Seedlings were raised in vitro in culture tubes and maintained under continuous illumination from fluorescent tubes (4.3 watts m^{-2} sec^{-1}) and at 25°C. Hypocotyl explants of equal length (~1cm) were excised from 7 d old seedlings and cultured on B5 medium [12] solidified with 0.9% agar in 90mm petri dishes. Petri plates were sealed with Parafilm (Parafilm "M", American National Can., USA) and kept under the above-mentioned conditions of light and temperature in an upright position to maintain the anterior-posterior flow of endogenous auxin in each of the explants as seen in seedlings or mature plants. Explants were observed for the morphogenetic changes at regular intervals. Within 20 days of culture, hypocotyl explants exhibited induction of somatic embryos.

Light Microscopy: Hypocotyl explants were fixed in FAA (formalin, acetic acid and 70% ethyl alcohol; 5:5:90) and subsequently preserved in 70 % ethanol. Dehydration of the fixed material i.e., cultured hypocotyl explants exhibiting embryogenesis, was carried out in ethanol-TBA (tertiary-butyl alcohol) series [13]. Tissue was embedded with paraffin wax. Sections of 10 µm thickness were obtained by microtomy and stained with Safranine - Fast Green for observations under compound microscope (Model: Axioscope; Zeiss, Germany).

Scanning Electron Microscopy: Hypocotyl explants were fixed by treatment with a solution of 2 % tannic acid and 4 % glutaraldehyde in 0.05 M sodium phosphate buffer, pH 6.5, for 5-6h at 4°C. Subsequently, explants were washed with 1 % osmium tetraoxide in 0.05M sodium phosphate buffer, pH 7.2, for 2-3 h at 4°C. Material was dehydrated in graded ethanol series for 5 min each and subsequently passed through 3 changes of absolute alcohol for 5 min each. Samples were sputter coated lightly with platinum-palladium for 60 s at 6 mA, 3000 V with an ion coater. Samples were examined under a scanning electron microscope (Model: Philips 501 B).

Effect of Calcium, IAA and Auxin Transport Inhibitors. Hypocotyl explants were cultured separately on B5 medium with variable concentrations of $CaCl_2.H_2O$ i.e., 0, 1, 2, 4, 10 and 20 mM. Cultures were kept under conditions of constant light and temperature as stated before. Additionally, hypocotyl explants were cultured on solid B5 medium individually containing indole-3-actetic acid (IAA; 5µM), N-1-napthylpthalamic acid (NPA; 1,10 and 20µM) and Parachlorophenoxyisobutyric acid (PCIB; 10, 20 and 50µM). For all experiments, hypocotyl explants cultured on basal B5 medium (without hormones) were used as control. Morphogenetic changes in the explants were recorded at regular intervals and final data analysed after 20 days of culture.

RESULTS

Somatic embryogenesis: Within 15 days of culture, hypocotyl explants exhibited longitudinal splitting along the exposed surface followed by the emergence of embryos (globular to dicot) at the anterior cut surface and along the split longitudinal axis. Average number of embryos per explant varied from 20 to 30 within 20 days of culture. These embryos developed into shoots without any sign of rhizogenesis. Subsequent excision and implantation of these shoots on the same medium led to differentiation of roots giving rise to complete plantlets. It was noteworthy that the anterior cut surface (proximal to shoot apex) of each hypocotyl explant gave rise to embryos whereas the posterior cut surface (distal to shoot apex) produced compact callus. In addition, a significant gradation was observed in the proliferative capacity of explants i.e. number of somatic embryos per explant derived from basal to apical regions of the hypocotyl. The basal explants (i.e. those derived from hypocotyl region nearest to root) showed maximum proliferative capacity in contrast to the apical explants (i.e. region closest to cotyledons), which exhibited least induction of somatic embryos, and callusing from the anterior and posterior ends, respectively (Fig. 1 A, B).

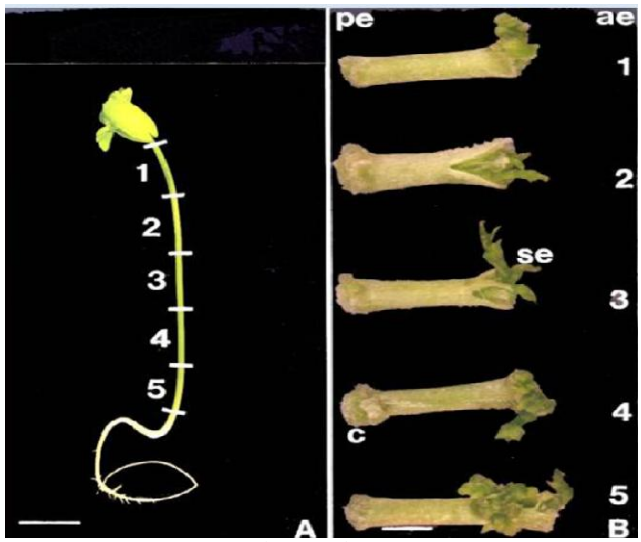

Figure 1A: Seedling (7-d-old) of *Albizzia lebbeck* L. germinated in vitro on Knop's medium. Bars across the seedling indicate the regions of hypocotyl used as explants (1 cm long).

B. Gradation in the frequency of somatic embryos developed on hypocotyl explants cultured on B5 medium. Basal explant (No. 5) exhibits maximum embryos and youngest explant (No. 1) shows the least. (ae: anterior end ; pe: posterior end; c:callus ; se: somatic embryos). Bar = 7.5 mm (A); 3.75 mm (B).

Histology and Anatomy: Light microscopic studies revealed various developmental stages of the somatic embryos from the explants. A clear distinction can be made between embryogenic and callus regions of the explants on the basis of anatomical details. The embryogenic region consists of inner small, cytoplasm-rich cells, delineated by few layers of protodermal cells (Fig. 2 A). They remain attached to parent tissue by a broad and short multi-celled stalk. Embryo development accompanies splitting of the ground tissue of the hypocotyl explant. Central region of the stalk shows a gradual development of elongated tracheidal elements. Their differentiation from the cortical cells of the parent explant is evident. Median longitudinal section through the callus region of the hypocotyl explants revealed three distinct layers of cells (Fig. 2 B). The outermost layer comprised of large cells that were highly vacuolated and loosely attached. This layer of cells gets sloughed off during sectioning due to loose arrangement of cells. Next inner layer is 10 to 15-celled thick. It is composed of small-sized and rectangular cells. The innermost core is formed of compact mass of cells with a defined pattern of arrangement. Central mass of cells gradually merges with the central cells of the anterior cut end of hypocotyl explant. Thus, the anterior and posterior ends of the explants reveal entirely different patterns of differentiation.

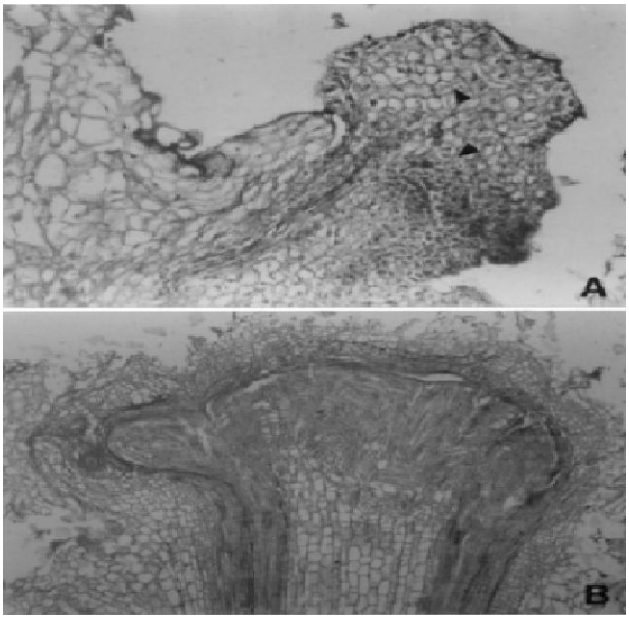

Figure 2: Light micrographs showing comparative anatomical details of embryogenic and callus regions of the hypocotyl explants. **A.** Transverse section of cut end proximal to shoot apex exhibiting somatic embryo induction (200 X). **B.** Longitudinal section of cut end distal to shoot apex showing the anatomical details of the callused region of the explant (200 X).

Scanning Electron Microscopic Analysis: Cultured hypocotyl explants exhibited a median longitudinal split with various stages of somatic embryos developing from the anterior cut surface and gradually proceeding towards the other end (Fig. 3 A). Callusing was observed at the posterior cut surface (Fig. 3 B). Different developmental stages of somatic embryos (globular, early heart-shaped, late heart-shaped, early and late torpedo-shaped embryos) can be distinctly seen (Fig. 3 C). Surface texture of embryos was rough and uni-or bi-celled trichomes were also observed (Fig. 3 D).

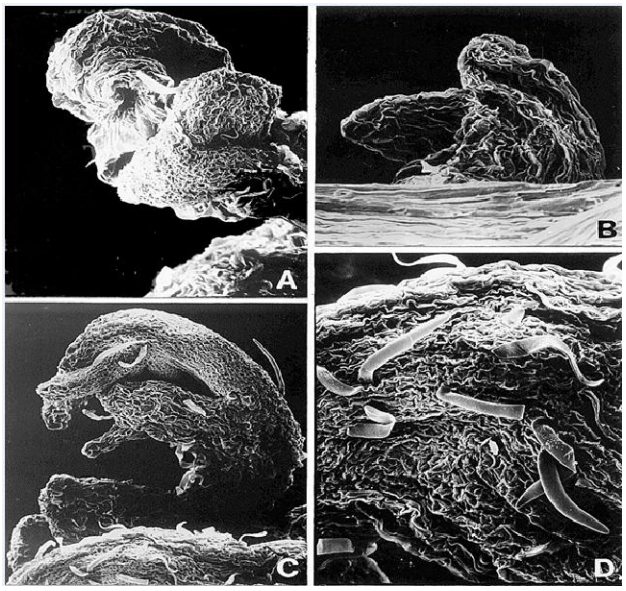

Figure 3: Scanning electron micrographs of hypocotyl explants of *A. lebbeck* L.
A. Embryogenic end showing emergence of somatic embryos from cut end proximal to shoot apex and longitudinal split margin (14 X). **B.** Enlarged view of somatic embryos (55X). **C.** Callus end showing unorganised mass of cells (16 X). **D.** Enlarged view of embryo surface showing one or two-celled trichomes (305 X).

Effect of Calcium: Hypocotyl explants from 7-d-old seedlings cultured on B5 medium supplemented with varying concentrations of $CaCl_2.2H_2O$ (0, 1, 2, 4, 10 and 20mM), exhibited differential somatic embryogenic response (Fig. 4). Calcium depletion inhibited embryo induction from the anterior cut end of the explants to a large extent (Fig. 4 A). A polarity exists in terms of embryogenic and callus ends of explants on no calcium or low calcium (up to 2mM) media (Fig. 4 A–C). Doubling of calcium concentration to 4mM led to considerable enhancement in the number and size of somatic embryos produced from the anterior cut surfaces and split margins of all explants as compared to explants cultured on B5 medium containing 2mM calcium (control; 4C).

Interestingly, the posterior cut ends of these explants also exhibited somatic embryogenesis instead of callus formation (Fig. 4D). Moreover, the response was uniform in all explants with no gradation with reference to the zone of the hypocotyl from where the explant was obtained. Higher dosage of calcium (10mM) induced an intermediate response of somatic embryogenesis intermixed with calli formation or embryogenic calli at both cut ends of all explants (Fig. 4 E). Finally, a ten-fold increase in calcium concentration in B5 medium (20mM) led to production of whitish green callus on both the cut surfaces. Explants showed uniform swelling due to callusing and suppression of somatic embryogenesis (Fig. 4F).

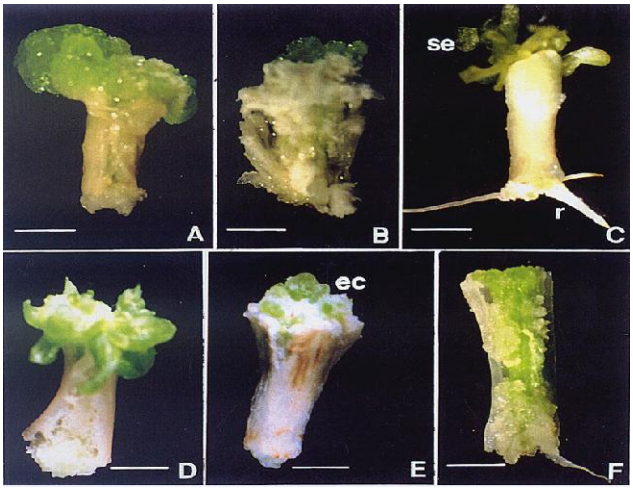

Figure 4: Comparative analysis of the basal explants (proximal to shoot – root junction) cultured on B5 medium supplemented with varying concentrations of $CaCl_2.2H_2O$.
A. Without calcium, **B.** 1mM Ca^{2+}(control), **C.** 2 mM Ca^{2+}, **D.** 4 mM Ca^{2+}, **E.** 10 mM Ca^{2+} and **F.** 20 mM Ca^{2+}. (se: somatic embryos; **ec**: embryogenic callus). **Bar**= 3.75 mm (A, B, C, D, E, F); 5 mm (D).

***Effect of IAA*:** Supplementation of B5 medium with IAA (5 µM) resulted in a marked reduction and gradation in somatic embryogenic potential of different explants of a seedling as compared to those raised on B5 basal medium. Apical explant i.e. proximal to shoot apex, elongated and produced greenish white, compact callus on both its cut surfaces (Fig. 5 A). Intermediate explants showed few somatic embryos intermixed with compact callus mass (Fig. 5 B), whereas the basal explant showed somatic embryo formation at the anterior cut surface (Fig. 5 C). Root induction was evident at the posterior cut surface in all the explants (Fig. 5 A-C).

Effect of Auxin Efflux Blockers: NPA (1µM) incorporated in B5 basal medium led to suppression of the embryogenic potential of the explants by more than 50 per cent. Meagre callusing was observed intermixed with somatic embryos at the cut surface proximal to the shoot apex, whereas the other end showed compact green callus (Fig. 6A).

Figure 5: Effect of IAA (5 µM) on hypocotyl explant embryogenesis from a single seedling.
A. Apical or youngest explant, **B**. Intermediate, **C**. Basal explant. Arrowheads indicate root induction on the anterior cut ends of basal and intermediate explants. (r: adventitious root; se: somatic embryos). **Bar**= 4.3 mm (A); 3.75 mm (B, C).

Explants cultured on B5 medium containing 10µM NPA exhibited significant suppression of embryogenesis at the cut end proximal to shoot apex (Fig. 6 B) whereas 20 µM NPA completely inhibited the embryogenic response and replaced it with light green callus at both cut ends of the explants (Fig. 6 C). Hypocotyl explants raised on PCIB (10–50 µM) –containing medium also exhibited suppression of somatic embryogenic response, at the cut end proximal to the shoot apex with very few developing embryos interspersed with callusing (10 µM) whereas the other cut end (proximal to the root-junction) exhibited green and compact callus formation (Fig. 7 D). Higher concentrations of PCIB (20 µM and 50 µM) in the culture medium led to induction of whitish, green, friable callus at the cut end (proximal to shoot apex) whereas the other cut end proximal to shoot apex did not show any significant proliferative activity (Fig. 7 E, F).

Figure 6: The effect of auxin inhibitor NPA (1-naphthylpthalamic acid) on somatic embryogenic response from hypocotyl explants cultured on B5 medium for 20 days.
A. Control (-NPA), **B**. B5 medium supplemented with 1µM NPA, **C**. 10µM NPA, **D**. 20µM NPA. (ae: anterior end; pe: posterior end; c:callus ; se: somatic embryos). **Bar** = 3.75 mm (A); 2.5 mm (B-D).

DISCUSSION

Present investigation on *Albizzia lebbeck* L. indicates an autonomous state of hypocotyl explants for induction of somatic embryos upon culture in hormone-free medium. A gradation of embryogenic response is evident in hypocotyl explants from a seedling, mature explants being more responsive than those derived from region closer to shoot apex. Although induction of somatic embryos on hormone-free medium has been recorded in the cotyledonary ex plants of *Panax ginseng* [14] and hypocotyl explants of *A. lebbeck* L. [15, 16], *A. julibrissin* [17] and *Clitorea ternatea* [18], present investigation provides detailed analyses, in terms of response versus extent of maturity of explant as well as anatomical origin of embryos. Light microscopic analysis (present work) revealed the sub-epidermal and cortical origin of somatic embryos at the anterior cut surface of explants. Initial signs of vasculature formation are visible at the base of each developing embryo, which has no apparent connection with the parent vasculature of the explant. Scanning electron microscopic analysis supplement the results obtained from light microscopic analysis. Thus, certain cells from the cut surface of explants seem to give rise to meristemoids, which later develop into globular embryos. Similar investigations have earlier been undertaken in legumes e.g. *Medicago sativa* [19], *Vigna radiata* [20] and *Passiflora cincinnata* [21].

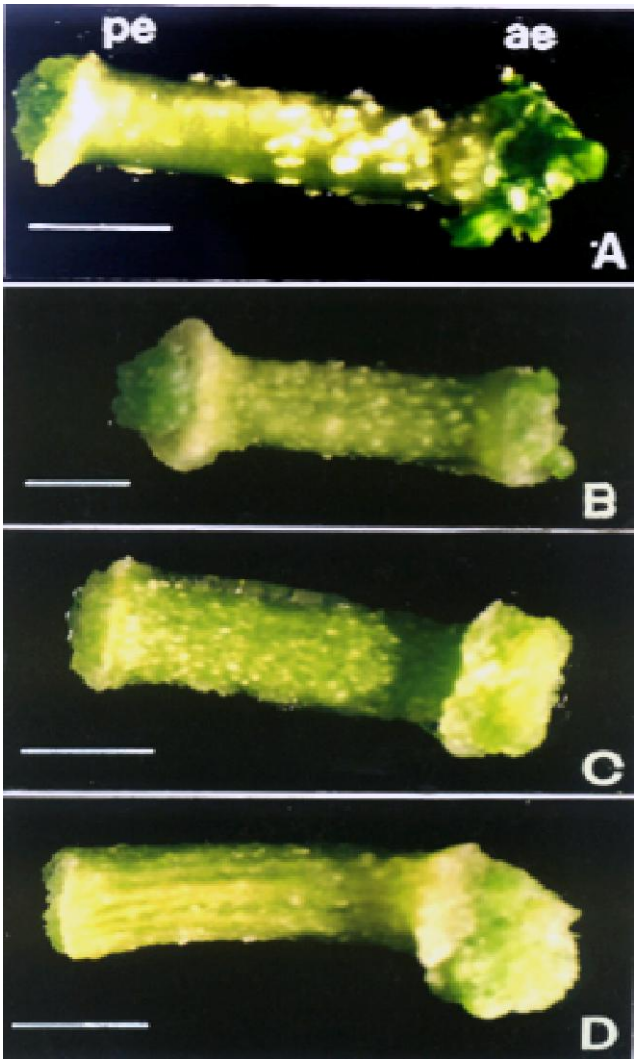

Figure 7: The effect of auxin inhibitor PCIB (parachlorophenoxyisobutyric acid) on somatic embryogenic response from hypocotyl explants cultured on B5 medium for 20 days.
A. Control (-PCIB), **B**. B5 medium supplemented with 10 µM PCIB, **C**. 20 µM PCIB, **D**. 50 µM PCIB. (ae: anterior end ; pe: posterior end; c:callus ; se: somatic embryos). **Bar** = 3.75 mm (A); 2.6 mm (B, C); 3 mm (D).

Physiological investigations undertaken in the present work indicate significant role of exogenous calcium in the induction of somatic embryos. Increasing concentrations of calcium up to 20 mM gradually leads to replacement of somatic embryos by embryogenic callus. In some plants, an upward shift in $CaCl_2$ in

culture medium results in a marked increase in the embryogenic potential [5, 22, 6, 9, 23] and calcium deprivation arrests somatic embryo formation [8]. High exogenous calcium is expected to increase $[Ca^{2+}]_{cyt}$. Calcium may have a role in the formation of protoderm, the first step towards the induction of somatic embryos [24]. Somatic embryogenesis is arrested under Ca^{2+} - chelated culture conditions, thus validating the significance of exogenous Ca^{2+} as a second messenger in the induction or regulation of this process [10]. Explanting may release Ca^{2+} from internal calcium pools temporarily and thus initiate signal for induction of somatic embryos. Calcium is essential during somatic embryogenesis for morphogenesis of undifferentiated cells into somatic embryo. Fluorescent dye assays of membrane-associated calcium and total calcium distribution exhibited variation in calcium distribution during embryogenesis, without changes in the membrane-associated calcium concentration. Moreover, functionally active calcium/calmodulin complexes have also been observed in the meristematic regions of heart and torpedo-stage embryos, thereby elucidating the crucial regulatory role of activated calcium / calmodulin in embryonal parts manifesting accelerated cell divisions. An increase in the expression of CaM mRNA has also been seen to increase upon induction of somatic embryos and remain constant thereafter. Genes coding for calcium binding protein (*MsCaM1*) also show an increase in the transcript levels after 2, 4-D treatment and preferentially accumulate at early globular stages. A novel family of calcium dependent/calmodulin independent protein kinases (CDPKs) was first characterized from soybean. Unlike calcium / calmodulin-dependent protein kinases, direct binding of calcium activates CDPKs [10].

Auxin availability (endogenous or exogenous) is also an important factor for induction of embryogenesis but inhibits development of subsequent stages. Supplementation of nutrient medium with exogenous IAA (5µM) leads to suppression of somatic embryogenic responses in hypocotyl explants of *A. lebbeck* L. (present work). In addition, it induces adventitious root and callus from cut ends (distal to shoot apex) of hypocotyl explants. Apparently, a polarity evolves in each explant due to a gradient of endogenous auxins, leading to an embryogenic response at their cut ends proximal to the shoot apex and a callus response at their cut ends distal to the shoot apex in auxin-free medium. IAA in the medium apparently disrupts the polarity of endogenous auxin in explants, leading to replacement of the embryogenic response by callus formation at the explant cut ends proximal to shoot apex. Inhibitors of polar auxin transport disrupt somatic embryogenesis implying that specific spatial auxin distribution due to auxin transport from cell to cell may be important in establishing embryonic pattern formation in plants [25]. Polar auxin transport inhibitors, such as NPA (N-1-napthylpthalamic acid) and PCIB (parachlorophenoxyisobutyric acid) block basipetal auxin transport by binding to the auxin efflux carrier complex [26]. This promotes intracellular accumulation of auxin by inhibiting carrier-mediated cellular efflux [27]. Polar auxin transport or basipetal movement of IAA triggers acropetal transport of calcium in the seedlings, which is may be simulated within each explant [28]. This combination of inverse fluxes may act as a primary signal for embryogenic response on the cut end of explants proximal to shoot apex and callusing on the end distal to shoot apex. Any disruption in this combination of inverse fluxes either by exogenous application of auxins, absence or overdose of calcium or auxin transport blockers i.e., NPA and PCIB, results in callusing in place of somatic embryogenesis. These findings indicate modulation of somatic embryogenesis in hypocotyl explants through activation of a specific pathway common to calcium and auxin at some stage of the chain of signaling events associated with the process.

ACKNOWLEDGEMENTS

We thank the Biocare grant (Department of Biotechnology) awarded to Dr. Tanushri Kaul for funding the publication and the CSIR-UGC for the fellowship to perform the above-mentioned work. We express deep regards and thanks to Prof. S.C. Bhatla, Department of Botany, University of Delhi for his suggestions and ideas to perform the aforesaid work.

REFERENCES

1. d'Ajuda, A. IUFRO Tree Biotechnology Conference: From Genomes to Integration and Delivery, BMC Proceedings 2011, 5, 7.

2. Merkle SA, Dean JFD, Forest tree biotechnology, Curr Opi Biotechnol, 2000, 11, 298-302.

3. Karami O, Aghavaisi B, Pour AM, Molecular aspects of somatic to transition in plants, J Chem Biol, 2009, 2, 177-190.

4. Merkle SA, Parrot WA, Flinn BS, Morphogenetic aspects of somatic embryogenesis, In: Thorpe TA, eds. In vitro embryogenesis in plant, Dordrecht: Kluwer Academic Publishers, 1995, 155-205.

5. Jansen MAK, Booij H, Schel JHN, de Vries SC, Calcium increases in the yield of somatic embryos in carrot embryogenic suspension cultures, Plant Cell Rep, 1990, 9, 221-223.

6. Montoro P, Etienne H, Michaux-Ferriere N, Carron MP, Callus friability and somatic embryogenesis in *Hevea brasiliensis*, Plant Cell Tissue Org Cult, 1993, 33, 331-119.

7. Montoro P, Etienne H, Carron MP, Maintainable somatic embryogenesis in *Hevea brasiliensis*, Rev Cyt Biol Vegetat Bot, 1994, 17, 113-119.

8. Overvoorde PJ, Grimes HD, The role of calcium and calmodulin in carrot somatic embryogenesis, Plant Cell Physiol, 1994, 35, 331-338.

9. Etienne H, Lartand M, Carron MP, Michaux-Ferriere N, Use of calcium to optimise long-term proliferation of friable emryogenic calluses and plant regeneration in *Hevea brasiliensis* (Mull Arg), Jour Exp Bot, 1997, 48, 129-137.

10. Anil VS, Rao KS, Calcium–Mediated Signaling During Sandalwood Somatic Embryogenesis. Role for Exogenous Calcium as Second Messenger, Plant Physiol 2000, 123, 1301-1311.

11. Schiavone FM, Cooke TJ, Unusual pattern of somatic embryogenesis in the domesticated carrot: developmental effects of exogenous auxins and auxin transport inhibitors, Cell Differen, 1987, 21, 53-62.

12. Gamborg OL, Miller RA, Ojima O, Nutrient requirements of suspension cultures of soybean root cell, Exp Cell Res, 1968, 50, 151-158.

13. Ruzin SE, Plant microtechnique and microscopy, Oxford University Press, 1999, New York.

14. Kim Y-J, Lee OR, Kim K-T, Yang D-C, High Frequency of Plant Regeneration through Cyclic Secondary Somatic Embryogenesis in *Panax ginseng*, Jour Ginseng Res, 2012, 36, 442-448.

15. Gharyal PK, Maheshwari SC, In vitro differentiation of somatic embryos in a leguminous tree, *Albizzia lebbeck* L., Naturwissenschaften, 1981, 68, 379-380.

16. Gharyal PK, Maheshwari SC, In vitro differentiation of plantlets from tissue cultures of *Albizzia lebbeck L.*, Plant Cell Tiss Org Cult, 1983, 2, 49-53.

17. Sankhla D, Davis TD, Sankhla N, Effect of gibberellin biosynthesis inhibitors on shoot regeneration from hypocotyl explants of *Albizzia julibrissin*, Plant Cell Rep, 1993, 13, 115-118.

18. Dhanalakshmi S, Lakshmanan KK, In vitro somatic embryogenesis and plant regeneration of *Clitorea ternatea,* Jour Exp Bot, 1992, 43, 312-319.

19. Xu N, Bewley JD, Contrasting pattern of somatic and zygotic embryo development in alfalfa (*Medicago sativa* L.) as revealed by scanning electron microscopy. Plant Cell Rep, 1992, 11, 279-284.

20. Mendoza AB, Hattori K, Nishimura T, Futsuhara Y, Histological and scanning electron observations on plant regeneration in mungbean cotyledon (*Vigna radiata* (L). Wilczek cultured in vitro, Plant Cell Tissue Org Cult, 1993, 32, 137-143.

21. Rocha DI, Vieira LM, Tanaka FAO, da Silva LC, Otoni WC, Somatic embryogenesis of a wild passion fruit species Passiflora cincinnata Masters: histocytological and histochemical evidences, Protoplasma, 2012, 249, 747–758.

22. Silva P, Ricardo CPP, β-Fructosidases and in vitro de-differentiation and redifferentiation of carrot cells, Phytochemistry, 1992, 31, 1507-1511.

23. Ulamin N, Sanga G, Ara N, Shah SH, Farhatullah. Effect of various concentrations of calcium chloride on callus growth and potassium nutrition of calli cultures of potato (*Solanum tuberosum*), Pak J Bot, 2013, 45, 209-214.

24. Timmers ACJ, Kieft H, Schel JHN, An immunofluorescence study on calmodulin distribution during somatic and zygotic embryogenesis of carrot (*Daucus carota* L), Acta Bot Neerl, 1995, 44, 19-32.

25. Fischer-Iglesias C, Sunderberg B, Neuhaus G, Jones AM, Auxin distribution and transport during embryonic pattern formation in wheat, Plant Jour, 2001, 26, 115-129.

26. Muday GK, Delong A, Polar auxin transport: Controlling where and how much, Trends Plant Sci 2001, 6, 535-542.

27. Geldner N, Friml J, Stierhof YD, Jurgens G, Palme K, Auxin transport inhibitors block PIN 1 cycling and vesicle trafficking, Nature, 2001, 413, 425-428.

28. Srivastava LM, Plant growth and development: hormones and environment, 2002, Academic press.

Source of support: Biocare grant, Department of Biotechnology
Conflict of interest: None Declared