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South African Journal of Botany 72 (2006) 613–618

SOUTH AFRICAN JOURNAL OF BOTANY

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Cold-enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium

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Received 29 March 2006; accepted 25 April 2006

Abstract

The possible inter-relationship between exogenous calcium during cold pretreatment-induced somatic embryogenesis was investigated using vegetative shoot apices of mature trees of *Pinus patula*. Cold pretreatment of thick (0.5–1.0 mm) apical shoot sections at 2 °C for 3 days on 0.3% activated charcoal-induced white mucilaginous embryogenic tissue on initiation medium (II). The enhanced embryogenic response of cells to cold pretreatment was dependent on the Ca^{2+} level in the pretreatment medium (I). Addition of Ca^{2+} channel blockers such as La^{3+} ($LaCl_3$) or ethyleneglycol-bis-B-aminoethyl ether-N,N,N,N-tetra acetic acid completely eliminated the cold-induced enhancement of somatic embryogenesis. These results suggest that embryogenic cells require minimal concentrations of Ca^{2+} during pretreatment for the expression of the cold-enhancement of embryogenesis in mature *Pinus patula* tissues.

Keywords: Calcium; Cold pretreatment; Somatic embryogenesis

1. Introduction

In plant cells, cold pretreatment affects several cellular processes such as cytoplasmic streaming, cell division and differentiation, cell shape determination, organelle movement and cell wall deposition (Traas et al., 1987). The temperature and duration of this pretreatment varies according to genotype (Kiviharju and Pehu, 1998). It was proposed that exposure to cold pretreatment results in a modification of cell wall and plasma membrane interactions which in turn, leads to changes in gene expression and synthesis of products that are responsible for embryogenesis (Komamine et al., 1992). Cold pretreatment of plant cells for a short period results in rapid up-regulation of gene(s), which encodes for stress-induced proteins. This may be necessary for successful embryogenesis (Janeiro et al., 1995; Bergmann et al., 1996).

In plants calcium ions (Ca^{2+}) function as a second messenger in the signal transduction of a variety of environmental stimuli (Poovaiah and Reddy, 1993; Bush, 1995). The environmental stimuli and signaling events that trigger and regulate plant embryogenesis are largely unknown (Zimmerman, 1993; Tomaszewski et al., 1994). Experiments with calcium-channel blockers and calcium chelators used in somatic embryogenesis of sandalwood and carrot indicated that the influx of exogenous calcium is essential for the initiation of somatic embryogenesis (Overvoorde and Grimes, 1994; Anil and Rao, 2000; Anil et al., 2000). These observations are suggestive of an intermediary role for Ca^{2+} during plant embryogenesis. Increasing evidence has demonstrated that the elevation of cytoplasmic free calcium $(Ca^{2+})_{cyt}$ plays an important role in the response of plants to cold pretreatment shock (Knight et al., 1996; Holdaway-Clarke et al., 2000; Knight and Knight, 2000). Such elevation of $(Ca^{2+})_{cyt}$ has been proposed to mediate a variety of physiological and developmental processes occurring at low temperature (Knight and Knight, 2000). Recently, a regulatory role involving changes in

Abbreviations: BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxy acetic acid; Ca^{2+}, calcium chloride; $(Ca^{2+})_{cyt}$, cytoplasmic free calcium; DCR, Gupta and Durzan (1985); EGTA, ethylenglycol-bis-(B-aminoethyl ether)-N,N,N,N-tetra acetic acid; La^{3+}, lanthanum chloride; NAA, naphthyl acetic acid.

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doi:10.1016/j.sajb.2006.04.001

$(Ca^{2+})_{cyt}$ during somatic embryogenesis has been reported for sandalwood (Anil and Rao, 2000). Increased exogenous calcium concentration favors somatic embryo formation in some species (Arruda et al., 2000; Etienne et al., 1997; Jansen et al., 1990; Rao, 1996; Silva and Ricardo, 1992). Therefore, one can infer that cold-enhanced somatic embryogenesis could be strongly related to cold-induced $(Ca^{2+})_{cyt}$ elevation by the influx of exogenous calcium during cold pretreatment. In the present study cold pretreatment with basal medium (I) and initiation medium (II) containing different concentrations of calcium or calcium-channel blockers $LaCl_3$ and EGTA were used to investigate the possible relationship between exogenous calcium levels during cold pretreatment and cold-enhanced somatic embryogenesis of *Pinus patula* using vegetative shoot apices of mature trees.

2. Materials and methods

2.1. Plant material and initiation of embryogenic cultures

Apical shoots (Fig. 1A) from mature 15-year-old trees of *Pinus patula* Scheide et Deppe from three genotypes (PP3, PP13, PP18) belonging to different families were collected from the Forest Orchard located at the South African Paper and Pulp Industries (SAPPI), Shaw Research Centre (MAP–998 mm; MAT–16.7 °C; Altitude–1100 m; Latitude–29° 47,899′ S; Longitude–30,248 17,826′ E) Pietermaritzburg, South Africa. The mature material was first rinsed with commercial NaOCl (household bleach containing 3.5% sodium hypochlorite as active ingredient) for 5 min and then washed 3 times with sterile distilled water. They were then surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.2% $HgCl_2$ for 2 min and rinsed 4 times with sterile distilled water. Transverse sections of approximately 0.5–1.0 mm thick were cut, using a scalpel or a sharp sterilized razor blade, from the decontaminated apical shoots for the initiation of embryogenic tissue. After pretreatment apical shoot sections were cultured individually on full strength DCR inorganic salt basal medium (Gupta and Durzan, 1985) containing 0.2 g l^{-1} polyvinyl pyrollidone (PVP) (BDH Chemicals), 1.5 g l^{-1} Gellan gum (Sigma), 90 mM maltose (Analar grade, ACE Chemicals) and 0.3% activated charcoal (Sigma) without growth regulators. The cultures were placed in 25 mm × 75 mm glass culture tubes (Borosil) containing 10 ml nutrient medium. These cultures were incubated in the dark at 2 °C for 3 days respectively (cold pretreatment basal medium (I)). After this cold pretreatment the apical shoot sections were subcultured onto full strength DCR basal medium for the initiation of embryogenic tissue. DCR basal medium containing 0.2 g l^{-1} PVP, 1.5 g l^{-1} Gellan gum, 1 g l^{-1} casein hydrosylate, 1 g l^{-1} meso-inositol supplemented with 20 μM 2,4-D, 25 μM NAA and 9 μM BA was used in the following experiments as an initiation medium (II) (Malabadi and van Staden, 2003, 2005a,b,c; Malabadi et al., 2004, 2006). The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The media were sterilized by autoclaving at 121 °C and 103 kPa for 15 min. L-glutamine and casein hydrosylate were filter sterilized and added to the media after they had cooled to below 50 °C. All cultures were maintained in the dark at 25±2 °C.

2.2. Establishment of embryogenic cell suspension cultures

Embryogenic tissue of 3 genotypes of *Pinus patula* was induced and subcultured as described previously (Malabadi and van Staden, 2003; Malabadi and van Staden, 2005a,b,c). Cell suspension cultures were initiated by selecting and transferring 1 g fresh mass of white mucilaginous embryogenic tissue to 150 ml Erlenmeyer flasks with 50 ml full strength DCR liquid basal medium supplemented with 20 μM 2,4-D, 25 μM NAA and 9 μM BA initiation medium (I) (Malabadi and van Staden, 2003, 2005a,b,c). The cultures were maintained on a horizontal rotatory shaker at 100 rpm in the dark at 25±2 °C. The suspension cultures were maintained for 2 weeks and the resulting suspension culture was settled by centrifugation for 5 min (70 ×*g*). The settled cell aggregates were resuspended in a fresh liquid DCR basal medium for further proliferation. After filtering 3 times, an embryogenic suspension culture was established, and then subcultured every week by transferring l ml of cell suspensions to the following solid DCR maintenance basal medium (Malabadi and van Staden, 2003, 2005a,b,c; Malabadi et al., 2004).

2.3. Treatment conditions

To test the role of exogenous Ca^{2+} during cold pretreatment and during initiation of embryogenic suspension cell cultures, the above full strength DCR basal medium (cold pretreatment medium (I) and during establishment of cell suspension cultures) containing calcium at different concentrations (0, 2, 4, 5 mM) was used. Calcium at 0 mM was obtained by the addition of 2 and 5 mM Calcium-channel blockers $LaCl_3$ and EGTA individually into the cold pretreatment medium (I) and during establishment of embryogenic suspension cultures lacking $CaCl_2$ respectively.

2.4. Maintenance of embryogenic cultures

The embryogenic tissue producing embryonal suspensor masses or early embryos were subcultured onto a maintenance medium. The DCR basal medium containing 120 mM maltose, 2 g l^{-1} Gellan gum supplemented with 2 μM 2,4-D, 2.5 μM NAA and 1 μM BA (maintenance medium III) (Malabadi and van Staden, 2003, 2005a,b,c) was used for this purpose. All the cultures were maintained in the dark at 25±2 °C. On this medium embryonal suspensor masses or early embryos were cultured for 30 days with 2 subcultures. The presence of embryonal suspensor masses was established using morphological and cytological observations.

2.5. Maturation of somatic embryos

After desiccation for 24 h, the partially desiccated embryogenic tissue was transferred to the maturation medium to induce cotyledonary development (Attree et al., 1995; Lelu et al., 1995; Malabadi et al., 2004; Malabadi and van Staden, 2003, 2005a,b,c). The DCR basal medium with 175 mM maltose, 80 μM ABA and 9 g l^{-1} Gellan gum (maturation medium IV) was tested for this purpose. All the cultures were placed in the dark at 25±2 °C and these were maintained for 10 to 14 weeks.

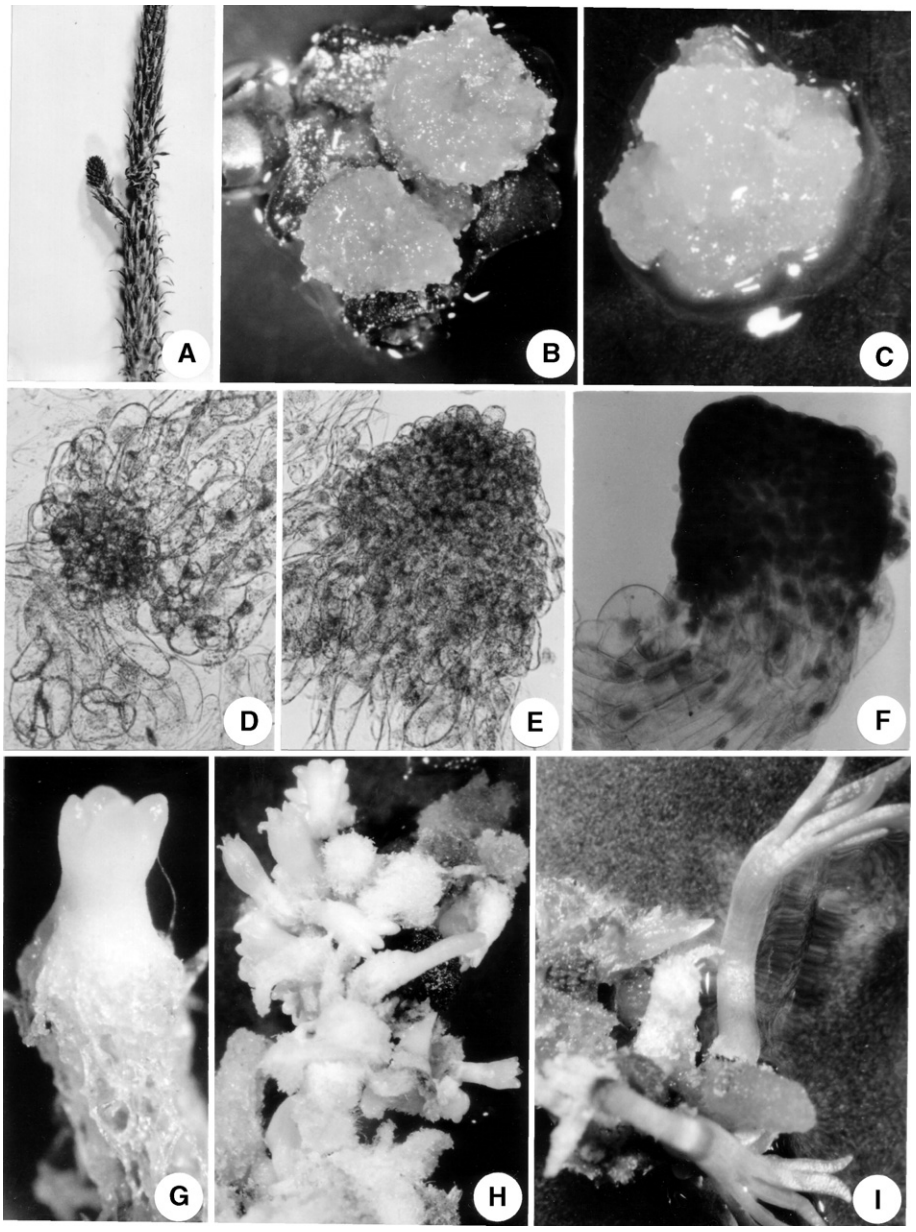

Fig. 1. Influence of exogenous Ca^{2+} on cold-enhanced embryogenesis using vegetative shoot apices of mature trees of *Pinus patula*: A. apical shoot harvested from mature trees (scale 1 cm = 0.58 cm); B. cold pretreated apical shoot section showing the initiation of white mucilaginous embryogenic callus under the influence of 4 mM exogenous Ca^{2+} (scale 1 cm = 0.14 cm); C. embryogenic callus along with the mother tissue subcultured on the maintenance medium for the further proliferation (scale 1 cm = 0.13 cm); D. elongated cells showing the sign of initiation of embryonal head with 4 mM exogenous Ca^{2+} in the cell suspension cultures (scale 1 cm = 0.02 cm); E. formation of embryonal head due to cleavage (scale 1 cm = 0.04 cm); F. distinct head formation with suspensors with acetocarmine staining by the addition of 4 mM exogenous Ca^{2+} during cell suspension cultures (scale 1 cm = 0.04 cm); G. development of somatic embryo on maturation medium (scale 1 cm = 0.13 cm); H. various developmental stages of somatic embryos as seen under the microscope (scale 1 cm = 0.1 cm); I. successful germination of somatic embryos on germination medium (scale 1 cm = 0.1 cm).

2.6. Germination and plantlet recovery

After 10 to 14 weeks of maturation in the presence of ABA, and higher concentrations of maltose and Gellan gum, advanced cotyledonary somatic embryos were taken from the cultures for germination. The germination medium (V) used was DCR basal medium with 2 g l^{-1} Gellan gum (Malabadi and van Staden, 2003, 2005a,b,c). Somatic embryos were considered germinated as soon as radicle elongation occurred. Conversion to a plantlet was based on the presence of an epicotyl. After 4 to 6 weeks on the germination medium, plantlets were transferred to vermiculite. Plantlets were placed in a growth room under a 16 h photoperiod (50 μmol m^{-2} s^{-1}) for hardening.

2.7. Statistical analysis

In all experiments each culture tube contained a single explant. Each replicate contained 50 cultures and each treatment had at least 2 replicates (100 cultures). All the experiments were repeated 3 times (total 300 cultures). Data presented in the tables

were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using Fishers pairwise comparisons using GENSTAT® release 4.21 (Rothamsted Experimental Station, United Kingdom). All statistical analysis was performed at the 5% level.

3. Results and discussion

To validate the role of exogenous Ca^{2+} as a second messenger in the induction/regulation of embryogenesis, this study investigated the necessity of a Ca^{2+} pool for embryogenesis. In the present study exogenous Ca^{2+} influenced somatic embryogenesis in all 3 genotypes of *Pinus patula*. The percentage of somatic embryogenesis was significantly affected by increasing the exogenous Ca^{2+} concentration from 0 to 4 mM on the cold pretreatment medium (I) and during the establishment of embryogenic suspension cultures. The highest percentage of explants showing embryogenesis (PP3: 8%, PP13: 10%, PP18: 3.5%) was recorded for the 3 genotypes of *Pinus patula* by the addition of 4 mM exogenous Ca^{2+} during the cold pretreatment or during cell suspension culture when compared to the controls (0 mM Ca^{2+}) (PP3: 4.5%, PP13: 3.7%, PP18: 2.0%) (Table 1). The incorporation of exogenous Ca^{2+} at a concentration of 2 mM during cold pretreatment and during the establishment of embryogenic cell suspension cultures did not have a marked effect on embryogenesis. An increase in the exogenous Ca^{2+} concentration to 5 mM significantly reduced the percentage of embryogenesis of *Pinus patula* following the cold pretreatment and during establishment of cell suspension cultures. Therefore, 4 mM exogenous Ca^{2+} was the optimum concentration for embryogenesis (Table 1). Hence, exogenous Ca^{2+} concentrations of 4 mM in the DCR basal medium during cold pretreatment supported the expression of cold-enhanced capacity for somatic embryogenesis. When the residual Ca^{2+} in the cold pretreatment medium was chelated with 2 mM EGTA, somatic embryogenesis was significantly inhibited in pretreated suspension cultures compared with the control. These results suggest that cold-enhanced embryogenesis was related to Ca^{2+} in the medium used for cold pretreatment and that an optimum concentration (4 mM) was required during cold pretreatment for the expression of cold-enhanced embryogenesis (Fig. 1D–I).

Table 1
Effect of various concentrations of exogenous Ca^{2+} on embryogenesis of *Pinus patula*

Ca^{2+} (mM)	Genotypes	Explants showing embryogenesis (%)	Somatic embryos/g fresh wt	Somatic embryos germinated/g fresh mass	Somatic embryos/g fresh mass
0	PP3	4.5±2.2c	36±2.0c	32±1.8c	29±1.5c
*(control)	PP13	7.0±1.9d	29±1.6d	26±1.5d	20±1.4d
	PP18	2.0±1.0b	22±1.8e	18±1.3e	13±1.0e
	PP3	4.4±0.3f	32±3.0f	31±0.6f	28±1.6g
2	PP13	6.8±0.3i	30±1.6h	26±1.7h	19±2.0g
	PP18	1.9±2.0j	20±2.7h	15±3.6h	12±1.0h
	PP3	8.0±2.0a	42±2.6la	37±2.4a	30±1.8a
4	PP13	10.0±0.5a	39±0.6la	33±1.8la	30±0.8a
	PP18	3.5±0.3b	30±0.89b	27±0.34b	23±2.5b
	PP3	2.5±1.1hg	20±0.8f	14±0.6g	9±0.3g
5	PP13	1.4±0.6bg	12±0.3h	6±0.7g	2±0.3g
	PP18	1.3±0.4j	4±1.1lj	4±0.3j	2±0.5j

Data recorded after 14 weeks and represents the mean±SE of at least 3 different experiments. In each column, the values with different letters are significantly different ($P<0.05$).
*Control without any exogenous Ca^{2+} in the cold pretreatment basal medium and cell suspension cultures.

Table 2
Effect of various concentrations of calcium-channel blockers $LaCl_3$ and EGTA on embryogenesis of *Pinus patula*

Calcium-channel blockers (mM)	Genotypes	Explants showing embryogenesis (%)	Somatic embryos/g fresh wt	Somatic embryos germinated/g fresh mass	Somatic embryos/g fresh mass
0	PP3	4.5±2.2a	36±2.0a	32±1.8b	29±1.5c
*(control)	PP13	7.0±1.9a	29±1.6a	26±1.5d	20±1.4e
	PP18	2.0±1.0b	22±1.8b	18±1.3d	13±1.0e
	PP3	1±0.3f	3±0.3f	0	0
La^{3+}	PP13	2±1.4g	2±0.4g	0	0
2	PP18	1±1.2f	1±0.0h	0	0
	PP3	0	0	0	0
La^{3+}	PP13	0	0	0	0
5	PP18	0	0	0	0
	PP3	2±0.2f	1±0.0g	0	0
EGTA	PP13	0	0	0	0
2	PP18	0	0	0	0
EGTA	PP3	0	0	0	0
5	PP13	0	0	0	0
	PP18	0	0	0	0

*Control without any exogenous Ca^{2+} channel blockers in the cold pretreatment basal medium and cell suspension cultures.
Data recorded after 14 weeks and represents the mean±SE of at least 3 different experiments. In each column, the values with different letters are significantly different ($P<0.05$).

To further investigate whether the incorporation of exogenous Ca^{2+} in the medium during cold pretreatment plays a role in cold-enhanced somatic embryogenesis, La^{3+} and EGTA at concentrations of 2 and 5 mM were added to the cold pretreatment medium to examine their effects on subsequent somatic embryogenesis. However, suspension cultures preincubated in the medium containing either La^{3+} or EGTA for 2 weeks displayed no growth when compared to the control. La^{3+} and EGTA at 2 and 5 mM completely negated the cold-enhanced capacity for embryogenesis. Furthermore, La^{3+} and EGTA at 5 mM greatly inhibited embryogenesis, suggesting that the influx of exogenous Ca^{2+} during cold pretreatment was also required for the expression of cold-enhanced somatic embryogenesis (Table 2). The arrest of embryogenesis with Ca^{2+}-chelated culture conditions (DCR basal medium with La^{3+} and EGTA at 2 or 5 mM) suggests that exogenous Ca^{2+} indeed is required for embryogenesis. Concomitantly, the inhibition of embryogenesis by plasma membrane Ca^{2+} channel blockers confirmed the necessity of a Ca^{2+} pool for the process of embryogenesis.

Culture treatments with Ca^{2+} antagonists further confirmed the need for an exogenous Ca^{2+} pool for embryogenesis. Ca^{2+} regulates many cellular functions in response to cold shock in plant cells thus an exogenous Ca^{2+} source is very important for cold shock signaling (Knight and Knight, 2000). Ca^{2+} also participates in the initiation of cell polarity during somatic embryogenesis (Overvoorde and Grimes, 1994; Timmers et al.,

1989, 1996). Higher Ca^{2+} concentrations in the nutrient medium favored the maintenance of embryogenic potential and production of somatic embryos in cell cultures of *D. carota* (Jansen et al., 1990), *Eucalyptus urophylla* (Arruda et al., 2000) and *Hevea brasiliensis* (Etienne et al., 1997).

La^{3+} is an inorganic, impermeable Ca^{2+} channel blocker, which specifically inhibits the influx of extracellular Ca^{2+} in response to cold treatment (Monroy and Dhindsa, 1995; Knight and Knight, 2000). It has been suggested that exogenous Ca^{2+} ions play a role as a second messenger during sandalwood somatic embryogenesis (Anil and Rao, 2000). In carrot somatic embryogenesis activated CaM has been localized to regions undergoing rapid cell division, and an increase in the level of CaM mRNA was observed during the globular and heart-shaped stages (Overvoorde and Grimes, 1994). Yet the modulator role of CaM in the regulation of embryogenesis remains unclear (Anil et al., 2000). The chelation of exogenous Ca^{2+} with EGTA or the initiation of exogenous Ca^{2+} movement across membranes with the Ca^{2+} channel blockers $LaCl_3$, nifedipine, verapamil and bepridil reduced the frequency of embryogenesis in carrot and sandalwood (Overvoorde and Grimes, 1994; Anil and Rao, 2000). Our results show that incubation of suspension cells in the medium containing La^{3+} resulted in the loss of the cold-enhanced capacity for somatic embryogenesis in *Pinus patula* suggesting that the influx of exogenous Ca^{2+} during cold pretreatment may be essential for cold-enhanced somatic embryogenesis. Although a preliminary experiment showed that somatic embryogenesis in La^{3+} –treated cultures could recover after La^{3+} was removed by washing, cold treatment of 5 mM La^{3+} for 2 weeks resulted in a significant inhibition of somatic embryogenesis. A similar observation has been reported for carrot callus growth (Yu et al., 1991). Possible reasons are the presence of a residue of La^{3+} or that La^{3+} entered cells and exerted its effect intracellularly (Quiquampoix et al., 1990).

This study presents evidence that culture conditions conducive to the initiation of embryogenic tissue and embryo development elicit a transient movement of exogenous Ca^{2+} into the cytosol of *Pinus patula* pro-embryogenic cells. Our results presented here show inter-dependence between Ca^{2+} concentrations in the pretreatment medium and cold-enhanced embryogenic response, indicating that the embryogenic cells of *Pinus patula* require minimal concentrations of Ca^{2+} during pretreatment for the maximal expression of cold-enhanced capacity for somatic embryogenesis (Fig. 1B–I).

Acknowledgements

We are grateful to the National Research Foundation (NRF), Pretoria, South Africa and the University of KwaZulu-Natal Research Fund for the financial support, in the form of a Post Doctoral Fellowship to RBM. SAPPI is thanked for providing the plant material.

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Edited by CH Bornman