Induction and maturation of somatic embryos from intact megagametophyte explants in Khasi pine (*Pinus kesiya* Royle ex. Gord.)

Hiranjit Choudhury[1,2,*], Suman Kumaria[1] and Pramod Tandon[1]

[1]Division of Plant Biotechnology, Department of Botany, School of Life Sciences, North-Eastern Hill University, Shillong 793 022, India
[2]Present address: Biotechnology Division, Department of Basic Sciences and Social Sciences, School of Technology, North-Eastern Hill University, Shillong 793 022, India

Embryogenic cultures were initiated and established in Khasi pine from intact megagametophytes. Factors affecting initiation, including genotype and developmental stages of explants, basal medium and growth regulator concentrations were investigated. Megagametophytes collected at different developmental stages from open-pollinated tree genotypes, *Pk-01*, *Pk-02*, *Pk-03*, *Pk-04* and *Pk-05*, were cultured on DCR medium supplemented with 2,4-D, NAA and BA. Megagametophytes containing zygotic embryos of 0.05–1.20 mm embryonal head size were suitable for somatic embryogenesis. Extrusion of embryogenic tissues from intact megagametophytes varied depending on the genotype, the developmental stage of the megagametophytes and media composition. Altogether 44 embryogenic lines were established with the genotype *Pk-04*. The embryogenic tissues exhibited luxuriant growth and cleavage polyembryony on maintenance medium. The proembryos failed to convert to advanced stage embryos on the maintenance medium. Maximum frequency (63.5%) of advanced cotyledonary somatic embryo (stage-III) was obtained on maturation medium with enhanced ABA (35 µM), gellan gum (5 g l^{-1}) and maltose (60 g l^{-1}) concentrations. The stage-III somatic embryos produced seedlings on germination medium.

Keywords: Conifers, megagametophytes, *Pinus kesiya*, somatic embryogenesis, zygotic embryos.

THE commercial importance of conifers as timber-yielding trees has drawn considerable attention of plant biotechnologists throughout the world to develop techniques for rapid clonal propagation of these favoured forest trees. Conifers initially received lesser attention compared to angiosperms as far as *in vitro* propagation was concerned, but since 1985 considerable efforts have been made to propagate conifers through somatic embryogenesis[1–5].

Various types of explants have been used to achieve somatic embryogenesis in conifers, including pines. Immature and mature zygotic embryos or intact megagametophytes have been used in most cases for establishing embryogenic cultures in conifers. However, the mature embryos have been reported to form embryogenic cultures at much lower frequencies. In pine species such as *Pinus mariana* and *P. taeda*, only immature zygotic embryos at the cotyledonary or pre-cotyledonary stages have been found effective for inducing somatic embryogenesis[6].

Pinus kesiya Royle ex. Gord. (Khasi pine), an economically important timber yielding tree is found in Northeast India and extends up to Myanmar, Philippines and Vietnam. It is one of the major sources of timber in the region. Some genotypes of this species have tremendous biomass potential and ole-resin prospects (2.3–2.7 kg/tapping season of tree dia 30–40 cm). Over-exploitation, unplanned developmental activities and age-old practices of 'slash and burn' cultivation have resulted in the dwindling of the Khasi pine population. Propagation of Khasi pine by conventional methods faces constraints mainly because the seed orchards show great variations and often seed germination is poor. Moreover, a large number of seedlings are destroyed by fire, low winter temperature and heavy rainfall. The vegetative propagation method used for its multiplication from favoured genotypes is rather difficult as this species reaches sexual maturity at an early stage and hence the rooting ability of cuttings decreases resulting in poor regeneration. Due to all these problems, there is an urgent need for rapid clonal propagation of *P. kesiya* through tissue-culture methods. Some efforts have been made earlier to micropropagate *P. kesiya* through organogenesis[7–10] and limited work has been done on somatic embryogenesis of this species[11–13] using apical dome sections and mature zygotic embryos as explants. The present work is focused on induction of somatic embryogenesis from intact megagametophyte explants (with immature zygotic embryos) and plantlet regeneration in *P. kesiya*. Somatic embryogenesis and plantlet regeneration from intact megagametophyte explants has not been reported so far in *P. kesiya*.

Materials and methods

Selection of explants and genotypes

Young, green, female cones from five different mature (15–20 yrs old), open-pollinated tree genotypes (desi-

gnated as *Pk-01, Pk-02, Pk-03, Pk-04* and *Pk-05*) of *P. kesiya* were collected at 15-day intervals (after fertilization from May to December) from North-Eastern Hill University Campus, Shillong, Meghalaya, India (lat. 25°34′N, long. 91°54′E, altitude 1500 m asl) and stored in cardboard boxes at 4°C for a maximum period of 15 days. The cones were cut into two halves and immature seeds were removed carefully and surface-cleansed with Citramide 1.0% (potent disinfectant) for 5 min and washed thoroughly under running tap water for 10–15 min. The seeds were then surface-sterilized with 70% ethanol followed by immersion in 0.1% mercuric chloride for 2 min and rinsed 5–6 times with sterile ultra pure water (Milli-Q water purification system) under aseptic conditions. The sterilized immature seeds were dissected and intact megagametophytes containing different stages of zygotic embryos (a–e) as defined here (Table 1) were removed and cultured. One thousand explants (200 explants for each developmental stage) were cultured at each collection date for each genotype.

Media and culture conditions

The explants were cultured in 60 × 15 mm sterile, disposable petri plates (Tarson) containing 10 ml of medium. Five explants were cultured in each petri plate and the plates were sealed with parafilm (American National Can). The basal medium used for initiation experiments was DCR[14]. The medium was solidified with 2 g l^{-1} gellan gum. The basal medium was supplemented with 5–30 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 3–18 μM α-naphthaleneacetic acid (NAA), 3–18 μM N^6-benzyladenine (BA), singly or in combination, to study the initiation of embryogenic tissues (ET) only. The combinations of plant growth regulators (PGRs) were selected on the basis of a preliminary study (Table 2). The selected medium and PGR combination for the initiation of ET, DCR basal medium containing 2 g l^{-1} gellan gum (Phytagel, Sigma), 1 g l^{-1} L-glutamine, 1 g l^{-1} casein hydrolysate, 1 g l^{-1} meso-inositol, 30 g l^{-1} maltose (Analar, Sigma) supplemented with 20.0 μM 2, 4-D, 12.0 μM NAA and 12.0 μM BA were used directly for the following experiments and the embryogenic response of different genotypes was tested on the induction medium (IM).

The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl before addition of gellan gum. The medium was then autoclaved for 15 min at 121°C and 1.05 kg cm^{-2}. L-glutamine and casein hydrolysate were filter-sterilized and added to cooled (ca. 45°C) medium. The cultures were incubated at 23 ± 2°C in the dark. The embryogenic extrusion from the micropylar end of the megagametophytes (Figure 1 *a*) was isolated after 4–6 weeks of inoculation.

The ET obtained from megagametophytes were subcultured on the same basal medium but with reduced PGRs for maintenance. On the basis of our earlier studies[12], the DCR basal medium containing 40 g l^{-1} maltose, 4 g l^{-1} gellan gum, 2.0 μM 2,4-D, 1.2 μM NAA and 0.60 μM BA (maintenance medium [PM]) was used for maintenance and proliferation of ET. In all the experiments, control was maintained and all the results were compared against the control. In case of the control, the ET were maintained on IM.

Maturation of somatic embryos

After 3–4 weeks on PM, the ET were transferred directly onto maturation medium (MM) for cotyledonary embryo

Table 1. Identification of developmental stages of zygotic embryos from genotypes *Pk-01, Pk-02, Pk-03, Pk-04* and *Pk-05* in *Pinus kesiya* during collection period

Stage of defined embryo	Size of embryonal head	Approximate number of weeks after fertilization
a	8–16 cells	5–6
b	0.05–0.2 mm	9–12
c	0.2–1.1 mm	13–17
d and e (cotyledonary embryo)	1.3–3.5 mm	19–26

Table 2. Various media combinations tested in the preliminary experiment for embryogenic extrusion and culture establishment in *P. kesiya*

Component (μM)	Medium					
	DCR$_1$	DCR$_2$	DCR$_3$	DCR$_4$*	DCR$_5$	DCR$_6$
2,4-D	5	10	15	20	25	30
NAA	3	6	9	12	15	18
BA	3	6	9	12	15	18

Media DCR$_1$ to DCR$_6$ contain DCR basal salts + 1 g l^{-1} L-glutamine + 1 g l^{-1} casein hydrolysate + 1 g l^{-1} meso-inositol.
*DCR$_4$ medium showed best result for initiation of embryogenic cultures.

development. The DCR basal medium was tested with four different combinations of gellan gum, abscisic acid (ABA) and maltose: (1) 3 g l⁻¹ gellan gum + 30 μM ABA + 40 g l⁻¹ maltose; (2) 5 g l⁻¹ gellan gum + 30 μM ABA + 40 g l⁻¹ maltose; (3) 5 g l⁻¹ gellan gum + 35 μM ABA + 60 g l⁻¹ maltose and (4) 5 g l⁻¹ gellan gum + 40 μM ABA + 60 g l⁻¹ maltose. All the cultures were kept in the dark at $25 \pm 2°C$ with 55–60% relative humidity. After 2–4 subcultures at 2 weeks interval in the above medium, the cultures were transferred to the germination medium.

Germination and plantlet recovery

After 8–10 weeks of maturation in the presence of ABA, advanced cotyledonary somatic embryos were picked from the cultures for germination. The number of cotyledonary somatic embryos formed was determined per gram ET by counting, and for each explant type three replicates were taken. The germination medium (GM) used was half-strength DCR basal medium with 2 g l⁻¹ gellan

Figure 1. Somatic embryogenesis in *Pinus kesiya* Royle ex. Gord. (Khasi pine) from intact megagametophytes (stage c) and isolated mature zygotic embryo explants. *a*, Embryogenic extrusion from the micropylar end of intact megagametophytes after 4 weeks of culture (bar: 0.5 mm). *b*, White, translucent embryogenic tissue (callus). Arrows indicate proembryos (bar: 1.0 mm). *c*, Early stage proembryos. Arrow indicates embryonal head (bar: 0.1 mm). *d–e*, Cleavage polyembryony. Arrows indicate embryonal heads (bar: 0.5–0.1 mm). *f*, A distinct early stage (stage-I) somatic embryo with dense embryonal head and many suspensor-like cells. Arrow indicates embryonal head (bar: 0.2 mm).

gum on the basis of our earlier experiment[12]. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of an epicotyl. After 6–7 weeks on the GM, plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16 h photoperiod (50 μmol m⁻² s⁻¹) for hardening.

Statistical analysis

In all experiments two replicates were maintained. Each replicate consisted of 20 petri plates, containing five explants each. Thus, for five developmental stages a total of 1000 explants were cultured in one season. The experiments were repeated twice in two seasons of cone development. Callus and proembryos were observed under inverted microscope (Leitz), while somatic embryos were observed under stereo zoom microscope (Leitz), and data were recorded on weekly basis. Data presented in the tables were analysed for significance and the differences contrasted using two-way ANOVA at 5% level.

Results

Genotype, developmental stage of explants and initiation of embryogenic cultures

The study revealed that the embryogenic extrusion and subsequent embryogenic culture establishment from various developmental stages of explants (Table 1) in IM varied considerably with different genotypes. In fact, some genotypes of *P. kesiya* did not show any embryogenic response. The genotypes *Pk-02*, *Pk-03* and *Pk-04* exhibited embryogenic response, while there was no embryogenic response in genotypes *Pk-01* and *Pk-05* (Tables 2–4). Though somatic embryogenesis was noticed with *Pk-02* and *Pk-03*, embryogenic lines could be established only with genotype *Pk-04* (44 lines), which indicates that the genotype of the parent has an important bearing on the embryogenic response. Out of 44 embryogenic lines from different developmental stages, 36 were obtained from stage-c explant alone (Table 4).

It was found that stages-b and -c (0.2–1.2 mm) zygotic embryos were highly responsive and stage-c (Figure 1 *a*) was the most appropriate for somatic embryogenesis. Though somatic embryogenesis was achieved with stages-d and -e explants, the frequency of initiation showed a declining trend (Tables 3 and 4).

Maintenance of embryogenic cultures

After 4–6 weeks of culture on IM, the ET of genotype *Pk-04* were subcultured on PM for further growth and proliferation of embryonal suspensor masses. DCR basal
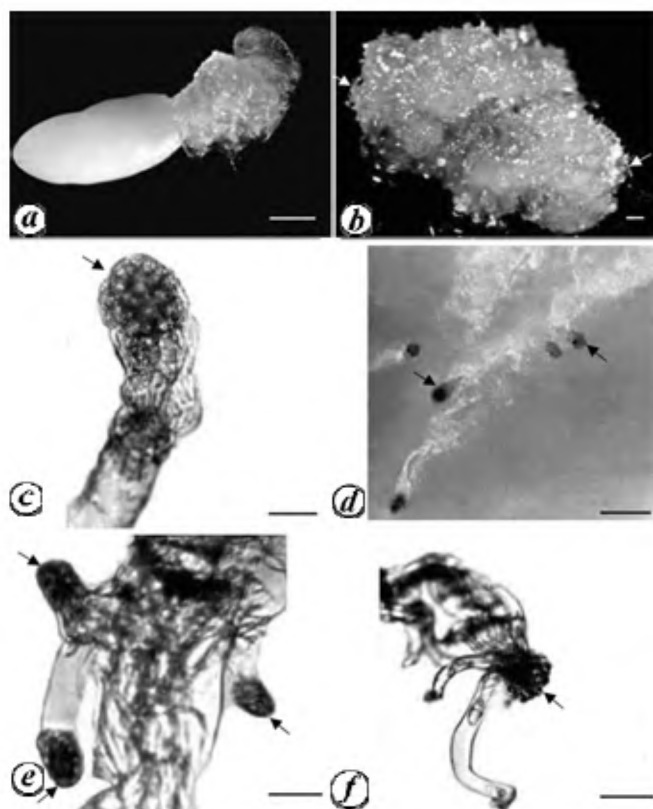
Table 3. Embryogenic responses of different genotypes of *P. kesiya* on DCR₄ medium (induction medium)

Genotype	Initiation of embryogenic extrusion from different developmental stages of the explant (%)				
	a*	b*	c*	d*	e*
Pk-01	–	–	–	–	–
Pk-02#	–	–	0.50 ± 0.68^a	1.00 ± 0.93^a	–
Pk-03#	–	–	1.50 ± 1.00^a	1.30 ± 0.79^a	–
Pk-04	–	5.00 ± 0.86^a	46.00 ± 2.31^b	4.50 ± 1.11^a	4.50 ± 0.58^a
Pk-05	–	–	–	–	–

*200 explants were cultured for each developmental stage of explants.
aMeans (±) followed by the same letter were not significantly different at $P < 0.05$.
#Genotypes *Pk-02* and *Pk-03* showed initiation of embryogenesis, but no embryogenic lines were established with these genotypes.

Table 4. Embryogenic response and somatic plantlet recovery in *P. kesiya*

Genotype	Develop-mental stage of explants	Number of explants cultured	Embryogenic extrusion (%)	Established embryogenic culture (%)	Number of embryogenic lines established	Somatic embryo $(g^{-1}$ fr wt$)$	Seedlings $(g^{-1}$ fr wt$)$
Pk-04	a	200	–	–	–	–	–
	b	200	5.00 ± 0.86^a	1.50 ± 0.58^a	3	43.00 ± 1.15	20.00 ± 1.15
	c	200	46.00 ± 2.31^b	18.00 ± 2.31^b	36	51.00 ± 1.15	29.00 ± 1.15
	d	200	4.50 ± 1.11^a	1.50 ± 1.00^a	3	35.00 ± 1.73	18.00 ± 1.15
	e	200	4.50 ± 0.58^a	1.00 ± 1.15^a	2	35.00 ± 1.15	17.00 ± 1.15

aMeans (±) followed by the same letter were not significantly different at $P < 0.05$. –, No response.

medium with 40 g l⁻¹ maltose, 4 g l⁻¹ gellan gum with reduced PGR concentrations of 2.0 µM 2,4-D, 1.2 µM NAA and 0.60 µM BA (maintenance medium [PM]) was suitable for maintenance and proliferation of ET. Maintenance of ET on IM resulted in browning of cultures. Five per cent of the embryogenic cultures from the stage-b explants proliferated for 6–8 weeks on IM but, only 1.5% cultures could be established after six months on PM. Maximum frequency (18.0%) of the established ET after six months was observed with the stage-c explants (Table 4). The frequency of established embryogenic cultures was 1.5% with stage-d, and 1.0% with stage-e explants respectively, after six months. In our study, a total of 44 embryogenic lines were established after six months in culture with stages-b–e from *Pk-04* genotype (Table 4).

Microscopic observation of the cultures showed the presence of somatic embryos at various stages of development (Figures 1 *b–f* and 2 *a*, *b*). Proembryos with dense embryonal head and suspensors of one to many elongated cells (Figure 1 *c–e*) were observed. In the initial and established cultures, somatic embryos with fused embryonal suspensor masses with embryo heads were also observed (Figure 2 *a*).

Development and maturation of somatic embryos

The proembryos and stage-I embryos which developed on maintenance medium failed to grow further until they were transferred to a medium with increased maltose,

ABA and gellan gum concentrations respectively. Out of four different combinations of media tried, DCR basal medium containing 5 g l⁻¹ gellan gum, 35 µM ABA and 60 g l⁻¹ maltose (MM) was found suitable for maturation of somatic embryos. Proembryos and stage-I embryos matured into advanced stage-II and stage-III somatic embryos on MM. Stage-I and stage-II somatic embryos consisted of an opaque, smooth and glossy embryogenic head subtended by long, translucent suspensor cells (Figures 1 *f* and 2 *a*). With majority of embryogenic lines, stage-I and stage-II somatic embryos formed advanced cotyledonary somatic embryos (stage-III; Figure 2 *b*) only when cultured on DCR basal medium devoid of growth regulators, but containing ABA (35 µM), gellan gum (5 g l⁻¹) and maltose (60 g l⁻¹). A maximum maturation frequency of 63.5% was recorded on this medium.

Germination of somatic embryos

One gram fresh weight of ET produced a maximum of 51 somatic embryos from stage-c-derived cultures, of which 36 somatic embryos were successfully germinated and produced 29 somatic plantlets. On the other hand stage-b cultures developed 43 somatic embryos per gram fresh weight of callus and 29 somatic embryos germinated to produce 20 somatic plantlets. Similarly, 35 somatic embryos each were observed in stages-d and stage-e derived ET respectively. In the case of stage d, 23 somatic embryos germinated successfully producing 18 somatic
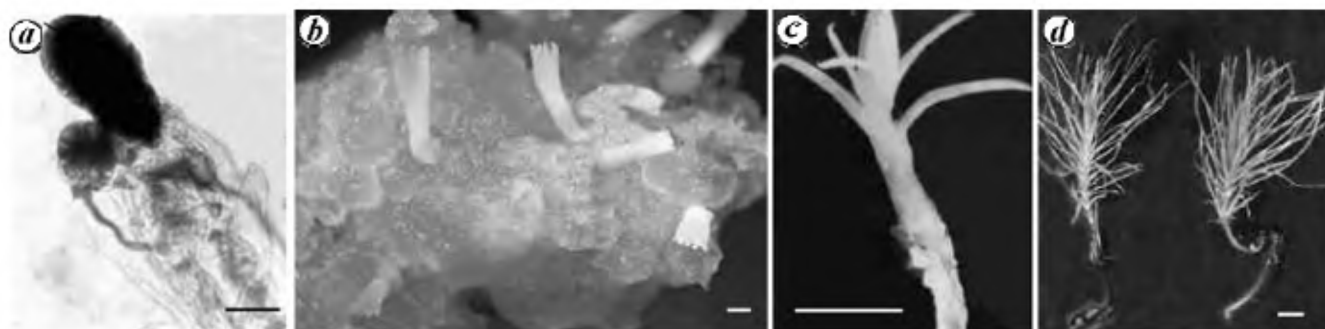
Figure 2. Somatic embryogenesis in *P. kesiya*: Maturation of somatic embryos, seedling recovery and establishment. *a*, Stage-I and stage-II somatic embryos sharing common suspensor cells (bar: 0.4 mm). *b*, Stage-III (cotyledonary) somatic embryos (bar: 1.0 mm). *c*, A well-germinated somatic seedling (bar: 1.0 cm). *d*, Healthy somatic seedlings ready for field transfer (bar: 1.0 cm).

plantlets. Seventeen somatic plantlets were obtained from 22 germinated somatic embryos with stage-e cultures. Highest percentage (80.55) of somatic seedling recovery was observed with stage-c cultures and the least seedling recovery of 68.96% was recorded with stage-b cultures (Table 4).

After 7–8 weeks of maturation, advanced cotyledonary somatic embryos (Figure 2 *b*) were harvested from the cultures for germination. The germination medium used was half-strength DCR basal medium with 2 g l^{-1} gellan gum. After 4–6 weeks, germinated somatic embryos (Figure 2 *c*) were ready to be transferred to vermiculite in a growth room for hardening (Figure 2 *d*).

Discussion

The major bottleneck in the induction of embryogenic cultures in pines has been the identification and use of the correct developmental stage regarded as a 'window'[15,16]. Harvesting of explants at the proper developmental stage of the zygotic embryo is critical for inducing somatic embryogenesis in conifers[17–19]. Identification and determination of the proper developmental stage on the basis of time of the season may not be an effective marker for induction of somatic embryogenesis, as the size of the embryos and developmental stages differed even among seeds from a single tree. The post-fertilization period can serve as an effective marker, but determination of precise time of fertilization is difficult to ascertain. Therefore, embryo size and morphology serve as better parameters for selecting responsive embryos[4]. Immature zygotic embryos at the precotyledonary stage of development in intact megagametophytes are the most responsive type of explants and embryogenic response declines drastically with cotyledonary-stage explants[6,19,20]. Immature female cones of *P. kesiya* collected at different developmental stages and examined to determine the optimum stage for initiation of embryogenic cultures, showed that intact megagametophytes containing stage-c immature zygotic embryos were suitable for somatic embryogenesis, an

observation in conformity with Arya *et al.*[4]. The embryogenic response declined with cotyledonary-stage explants in *P. kesiya*. The present study reveals a similar higher embryogenic response with precotyledonary-stage zygotic embryos and a decline in embryogenesis with mature cotyledonary zygotic embryos on IM. This result conforms to earlier reports[21–23].

Some genotypes did not show any embryogenic response. Somatic embryogenesis was noticed with *Pk-02* and *Pk-03*, but embryogenic lines could be established only with *Pk-04*, which indicates that the genotype of the parent has an important bearing on embryogenic response, as observed earlier[23,24].

The strength of gelling agents seems to play an important role in the initiation and maintenance media, as it affects water availability to the cultures. Phytagel proved to be an effective alternative gelling agent to agar in conifer somatic embryogenesis[25–28]. In the present study, the use of enhanced gellan gum in the initiation medium (2 g l^{-1}) and maintenance medium (4 g l^{-1}) formed gels of higher strength, which is associated with reduced water availability, triggering a shift in the development programme of the cultures, from proliferation of embryogenic cells with cleavage polyembryony to the development of proembryos and early-stage embryos in the maintenance medium[26].

Enhanced concentration of maltose, gellan gum and low concentration of growth regulators in PM improved the development of proembryos, which could be attributed to the reduced water availability of the medium. Our results are in full agreement with earlier reports that low water availability resulting from high gellan gum concentration promotes somatic embryo maturation in *Pinus strobus*[6,26]. The development of somatic embryos by partial desiccation treatment could affect the endogenous ABA level. This treatment might set in rapid biochemical changes, which result in the formation of specific enzymes that might play an important role in water-stress tolerance and in the maturation of somatic embryos[29].

Thus the present investigation demonstrates the induction of somatic embryogenesis, maturation and plantlet

regeneration from somatic embryos of *P. kesiya* from intact megagametophytes containing immature precotyledonary zygotic embryos. The initiation of somatic embryogenesis is dependent on the developmental stage of the explant, genotype of the parent and media composition. The somatic embryos formed on IM matured in large numbers only when transferred to a medium with higher osmoticum (maltose) and gellan gum concentration. Further efforts are required to achieve higher frequency of somatic embryogenesis using megagametophyte explants.

1. Hakman, I., Fowke, L. C., von Arnold, S. and Erikson, T., The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Sci.*, 1985, **38**, 53–59.
2. Bonga, J. M., Klimaszewska, K., Lelu, M. A. and von Aderkas, P., Somatic embryogenesis in *Larix*. In *Somatic Embryogenesis in Woody Plants* (eds Jain, S., Gupta, P. and Newton, R.), Kluwer, The Netherlands, 1995, vol. 3, pp. 315–339.
3. Norgaard, J. V., Somatic embryo maturation and plant regeneration in *Abies nordmanniana* LK. *Plant Sci.*, 1997, **124**, 211–221.
4. Arya, S., Kalia, R. K. and Arya, I. D., Induction of somatic embryogenesis in *Pinus roxburghii* Sarg. *Plant Cell Rep.*, 2000, **19**, 775–780.
5. Yildirim, T., Kaya, Z. and Isik, K., Induction of embryogenic tissue and maturation of somatic embryos in *Pinus brutia* TEN. *Plant Cell Tissue Organ Cult.*, 2006, **87**, 67–76.
6. Klimaszewska, K. and Smith, D. R., Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiol. Plant.*, 1997, **100**, 949–957.
7. Nadgauda, R. S., Nagarwala, N. N., Parasharam, V. A. and Mascarenhas, A. F., Bud break and multiple shoot formation from tissues of mature trees of *Pinus caribeae* and *P. kesiya*. *In Vitro Cell. Dev. Biol. – Plant*, 1993, **29**, 131–134.
8. Kumar, A. and Tandon, P., *In vitro* propagation of *Pinus kesiya* Royle ex. Gord. In *Advances in Plant Tissue Culture in India* (ed. Tandon, P.), Pragati Prakashan, Meerut, 1994, pp. 200–250.
9. Nandwani, D., Kumaria, S. and Tandon, P., Micropropagation of *Pinus kesiya* Royle ex. Gord. (Khasi pine). *Gartenbauwissenschaft*, 2001, **66**, S68–S71.
10. Tandon, P., Kumaria, S. and Choudhury, H., Plantlet regeneration of *Pinus kesiya* Royle ex. Gord. from mature embryos. *Indian J. Biotechnol.*, 2007, **6**, 262–266.
11. Tandon, P. and Choudhury, H., Development of an embryogenic system in *Pinus kesiya* Royle ex. Gord. In Conference on Tree Biotechnology, Lantbruks Universitet, Umeå, Sweden, 7–12 June 2003. Abstr. S2, p. 16.
12. Malabadi, R. B., Choudhury, H. and Tandon, P., Initiation, maintenance and maturation of somatic embryo from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord.) promoted by partial desiccation and Gellan gum. *Sci. Hortic.*, 2004, **102**, 449–459.
13. Malabadi, R. B., Mulgund, S. G. and Nataraja, K., Plant regeneration via somatic embryogenesis in *Pinus kesiya* (Royle ex. Gord.) influenced by triacontanol. *Acta Physiol. Plant*, 2005, **27**, 531–537.
14. Gupta, P. K. and Durzan, D. J., Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Rep.*, 1985, **4**, 177–179.
15. Mathur, G., von Arnold, S. and Nadgauda, R., Studies on somatic embryogenesis from immature zygotic embryos of chir pine (*Pinus roxburghii* Sarg.). *Curr. Sci.*, 2000, **79**, 999–1004.
16. Tautorus, T. E., Fowke, L. C. and Dunstan, D. I., Somatic embryogenesis in conifers. *Can. J. Bot.*, 1991, **69**, 1873–1899.
17. Attree, S. M. and Fowke, L. C., Micropropagation through somatic embryogenesis in conifers. In *Biotechnology in Agriculture and Forestry: High-tech and Micropropagation I* (ed. Bajaj, Y. P. S.), Springer-Verlag, Berlin, 1991, vol. 17, pp. 53–70.
18. Becwar, M. R., Nagmani, R. and Wann, S. R., Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). *Can. J. For. Res.*, 1990, **20**, 810–817.
19. Becwar, M. R., Blush, T. D., Brown, D. W. and Chesick, E. E., Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. *Plant Cell Tissue Organ Cult.*, 1991, **26**, 37–44.
20. Finer, J. J., Griebel, H. B. and Becwar, M. R., Initiation of embryogenic callus and suspension cultures of easter white pine (*Pinus strobus* L.). *Plant Cell Rep.*, 1989, **8**, 203–206.
21. Huang, J., Wei, Z. and Xu, Z., Recovery of somatic embryos and plantlets from callus of mature masson pine embryos. *Chin. Sci. Bull.*, 1995, **40**, 1196–1200.
22. Bozhkov, P. V., Ahn, I. S. and Park, Y. G., Two alternative pathways of somatic embryo origin from polyembryonic mature stored seeds of *Pinus koraiensis* Sieb et Zucc. *Can. J. Bot.*, 1997, **75**, 509–512.
23. Garin, E., Isabel, N. and Plourde, A., Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos. *Plant Cell Rep.*, 1998, **18**, 37–43.
24. Isabel, N., Jeandroz, S., Beaulieu, J., Park, Y. S., Plourde, A. and Bousquet, J., Genetic characterisation of SE initiation in white spruce. In Joint Meeting of the IUFRO Working Parties on Somatic Cell Genetics and Molecular Genetics of Trees, Cubec, Canada, 12–16 August 1997.
25. Kim, Y. W., Youn, J., Noh, E. R. and Kim, J. C., Somatic embryogenesis and plantlet regeneration from immature zygotic embryos of Japanese larch (*Larix leptolepis*). *Plant Cell Tissue Organ Cult.*, 1999, **55**, 95–101.
26. Klimaszewska, K., Cardou, M. B., Cyr, D. R. and Sutton, B. C. S., Influence of gelling agents on culture medium, gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. *In Vitro Cell. Dev. Biol. – Plant*, 2000, **36**, 279–286.
27. Klimaszewska, K., Garin, E., Cardou, M. B., Isabel, N. and Plourde, A., Effects of sugars, amino acids, and culture technique on maturation of somatic embryos of *Pinus strobus* on medium with two Gellan gum concentrations. *Plant Cell Tissue Organ Cult.*, 2000, **62**, 27–37.
28. Klimaszewska, K., Park, Y. S., Overton, C., Ian, M. and Bonga, J. M., Optimized somatic embryogenesis in *Pinus strobus* L. *In Vitro Cell. Dev. Biol. – Plant*, 2001, **37**, 392–399.
29. Rance, I. M., Tian, W., Mathews, H., Kochko, A., Beachy, R. N. and Fauquet, C., Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. *Plant Cell Rep.*, 1994, **13**, 647–651.