J. Plant Biol., Vol. 29 (3), December, 2002, pp. 301–306.

ARTICLE

Somatic Embryogenesis and Plantlet Regeneration from Mature Zygotic Embryos of *Pinus kesiya* (Royle ex. Gord.)

Chitta Ranjan Deb*,† and Pramod Tandon

Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong 793 022, India
*Present address: Department of Botany, Nagaland University, Headquarters: Lumami, Mokokchung 798 601, India

MS received 28 May 2002; accepted 20 July 2002

Embryogenic cultures were initiated at a high frequency from mature zygotic embryos (from seeds stratified at 4°C for 24 h) of *Pinus kesiya* cultured on modified Murashige and Skoog medium containing sucrose (20 g l^{-1}), 2,4-dichlorophenoxyacetic acid and α-naphthaleneacetic acid (22.6 μM and 26.85 μM respectively), benzyl adenine (11.1 μM), casein hydrolysate and myo-inositol (each at 1000 mg l^{-1}) and L-glutamine (500 mg l^{-1}). Proembryonal masses developed in 2–3 weeks upon subculture on the same medium but with 1/10th the concentration of growth regulators. Cotyledonary embryos resulted when the proembryonal masses were cultured for 4–5 weeks on basal medium containing abscisic acid (15.12 μM) and sucrose (40 g l^{-1}). The cotyledonary embryos elongated within 3–4 weeks on medium devoid of casein hydrolysate and L-glutamine but containing myo-inositol (1000 mg l^{-1}), sucrose (30 g l^{-1}) and activated charcoal (2.0 g l^{-1}) under 12 h photoperiod. In about 3–4 weeks, the embryos germinated into seedlings on modified MS medium containing sucrose (30 g l^{-1}).

Keywords: Mature zygotic embryo, *Pinus kesiya*, regeneration, somatic embryo, emblings.

Introduction

Since the first report of the induction of embryogenic cultures from immature zygotic embryos of Norway spruce (Hakman *et al.*, 1985), somatic embryogenesis and plant regeneration (Attree and Fowke, 1993; Gupta *et al.*, 1993; Nagmani *et al.*, 1993; Gupta and Grob, 1995; Barrett *et al.*, 1997; Carrier *et al.*, 1997; Guevin and Kirby, 1997; Tandon and Deb, 1998; Kim *et al.*, 1999; Arya *et al.*, 2000; Deb and Tandon, 2002) has been accomplished in several conifer species.

Pinus kesiya (Royle ex. Gord.) is an economically important early successional species, which is predominant in the subtropics (800–2000 m) of northeast India, Myanmar and the Philippines. The species is the major source of timber in khasi hills of Meghalaya where it covers about 30% of the total forest area. Genotypes with high biomass potential and oleo-resin are available in natural forests. The pine forests of this region are rapidly being depleted. The propagation of *P. kesiya* by conventional methods faces constraints mainly because the seed orchards show great variations and at times seed germination is very poor. A large number of seedlings are destroyed by fire, low winter temperature and heavy rainfall. The vegetative propagation method used for its multiplication from economically favoured genotypes is rather difficult as this pine reaches sexual maturity at an early stage, after which the rooting ability of cutting decreases resulting in poor regeneration. These problems indicate that, there is an urgent need for large-scale afforestation programme using cloning material. In this paper we describe somatic embryogenesis and plantlet regeneration using mature zygotic embryos.

†For correspondence. (e-mail: debchitta@rediffmail.com)

Materials and methods

Plant material

Mature cones were collected from open pollinated plus trees identified by the Forest Department, Government of Meghalaya during late January to March of every year. Seeds were extracted by air-drying the

cones at 25–30°C. The extracted seeds were stored at 4°C in sealed polythene bags till used.

Media

Half strength MS salts (Murashige and Skoog, 1962) with reduced ammonium nitrate (550 mg l^{-1}) and increased potassium nitrate (4460 mg l^{-1}) (mMS) and DCR (Gupta and Durzan, 1985) media, both supplemented with casein-hydrolysate (CH) and myo-inositol (0–1500 mg l^{-1} singly or in combination), L-glutamine (0–1000 mg l^{-1}), sucrose (0–30 g l^{-1}), polyvinyl pyrollidone (PVP) (200 mg l^{-1} as antioxidant) were used. Various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0–45.2 μM) and α-naphthaleneacetic acid (NAA) (0–53.68 M) singly or in combination along with 2.5 mg l^{-1} benzyl adenine (BA) were incorporated into the above media. The pH of the medium was adjusted to 5.75 and difco-bacto agar (Hi-media) (7.0 g l^{-1}) was used as the gelling agent. The media were sterilized by autoclaving at 1.06 kg cm^{-2} pressure for 15 min. L-glutamine, myo-inositol and abscisic acid (ABA) were filter sterilized and incorporated into the medium after autoclaving. About 15 ml autoclaved medium was dispensed into each test tube (size-150 × 25 mm) and cotton plugged.

Tissue culture

The seeds were treated with mercuric chloride (0.25% w/v) for 2–5 min and washed with sterilized double distilled (pure) water for 4–5 times. Seeds were further treated with 6.0% (v/v) hydrogen peroxide for 10 min and washed 4–5 times with sterilized pure water. The surface-sterilized seeds were stratified at 4°C for 0–96 h and at 25°C for a week. Embryos were dissected from stratified seeds and cultured on mMS_1 and DCR_1 media (Table 1). All the cultures were maintained at 25 ± 2°C in the dark unless indicated otherwise. For each treatment, 50 embryos were cultured and experiments were repeated thrice. After three weeks, the resulting cell masses were subcultured on the same media for two passages at 2-week intervals. The embryogenic cell masses were transferred to mMS_2 medium for 2–3 passages. The proembryonal masses and resulting proembryos were transferred to mMS_3 medium containing 0–30.24 μM ABA singly or in combination with 0–5% sucrose for embryo development and

Table 1. Formulation of media* for initiation, maintenance, maturation and germination of somatic embryos of *Pinus kesiya* derived from zygotic embryos.

Components	Media for initiation and proliferation of embryogenic cultures		Maintenance medium	Somatic embryo development and maturation medium	Germination medium
Basal medium	mMS_1**	DCR_1	mMS_2	mMS_3	mMS_4
Supplements (g l^{-1})					
Sucrose	20	20	20	40	30
CH	1.0	1.0	1.0	1.0	–
Myo-inositol	1.0	1.0	1.0	1.0	0.1
L-glutamine	0.5	0.5	0.5	0.5	–
PVP	0.2	0.2	0.2	0.2	0.2
AC	–	–	–	–	2.0
Growth regulators (μM)					
2,4-D***	22.6	22.6	2.26	–	–
NAA***	26.85	26.85	2.68	–	–
BA	11.1	11.1	1.11	–	–
ABA	–	–	–	15.12	–

*Only optimum media compositions are tabulated.
**Half strength MS salts with reduced ammonium nitrate (550 mg l^{-1}) and increased potassium nitrate (4460 mg l^{-1}).
***Concentrations optimized from initial experiment (0–45.2 μM 2,4-D and 0–53.68 μM NAA singly or in combination).

were maintained for 2–3 passages. The resultant cotyledonary embryos were transferred on to mMS_4 medium and cultures were maintained at 25 ± 2°C under a 12 h photoperiod of 60 mol m^{-2} s^{-1} photon flux provided by cool white fluorescent tubes. The elongated embryos were subsequently transferred to mMS medium containing sucrose (30 g l^{-1}) for germination.

Results

Initiation of embryogenic cultures

Within three weeks of culture on both mMS_1 and DCR_1 media, two types of cell masses were developed: a white, translucent, gelatinous embryogenic callus and a hard, greenish non-embryogenic callus. In mMS_1 medium, 79.6% type 'a' cell masses and in DCR_1 medium 57.4% type 'a' cell masses were observed (data not shown). α-naphthaleneacetic acid (NAA) alone as an auxin source was found to be superior to 2,4-D (Table 2). A 24 h stratification at 4°C exhibited optimum induction of embryogenic cultures, while, with increase in this period, a gradual decline in the initiation of embryogenic cultures was observed (Figure 1).

Table 2. Effect of different concentrations and combinations of growth regulators in mMS_1 medium* on initiation of embryogenic cultures from mature zygotic embryos.

Growth regulators (μM)		Per cent response of embryogenic culture formation (± SE**)
2,4-D	NAA	
0	0	0
11.3	0	20.40 (± 0.55)
22.6	0	29.60 (± 0.55)
33.9	0	20.40 (± 0.22)
45.2	0	18.50 (± 0.22)
0	13.42	9.25 (± 0.55)
11.3	13.42	44.0 (± 0.77)
22.6	13.42	50.0 (± 0.55)
33.9	13.42	51.85 (± 1.10)
45.2	13.42	33.50 (± 0.22)
0	26.84	50.0 (± 0.77)
11.3	26.84	51.85 (± 0.67)
22.6	26.84	79.60 (± 0.55)
33.9	26.84	42.60 (± 0.77)
45.2	26.84	29.60 (± 1.10)
0	40.26	40.70 (± 0.22)
11.3	40.26	41.90 (± 1.10)
22.6	40.26	46.30 (± 0.55)
33.9	40.26	36.50 (± 0.22)
45.2	40.26	31.50 (± 1.10)
0	53.68	20.30 (± 0.22)
11.3	53.68	35.20 (± 1.65)
22.6	53.68	33.30 (± 0.55)
33.9	53.68	27.30 (± 0.22)
45.2	53.68	26.10 (± 0.67)

*MS basal medium plus sucrose (2%), BA (11.1 μM), CH and myo-inositol (each at 1000 mg l^{-1}), L-glutamine (500 mg l^{-1}) and PVP (200.00 mg l^{-1}).

**Standard error.

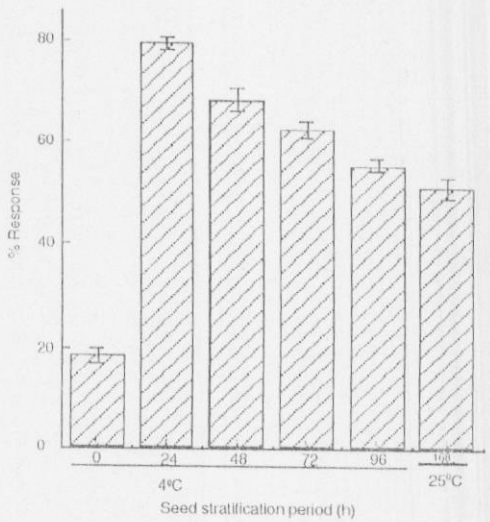

Figure 1. Effect of seed stratification period on induction of embryogenic cultures from mature zygodic embryos (details are provided in the materials and methods).

Maintenance and proliferation of embryogenic culture

Embryogenic cultures raised on initiation medium were proliferated for two subcultures where cultures almost doubled with each subculture. The polyembryonal masses (PEMs) and proembryos were formed after 2–3 passages on mMS medium containing 1/10th concentration of growth regulators of that in initiation medium (Figure 2A). The resulting proembryos failed to grow further on the same media until they were cultured specifically on development and maturation medium.

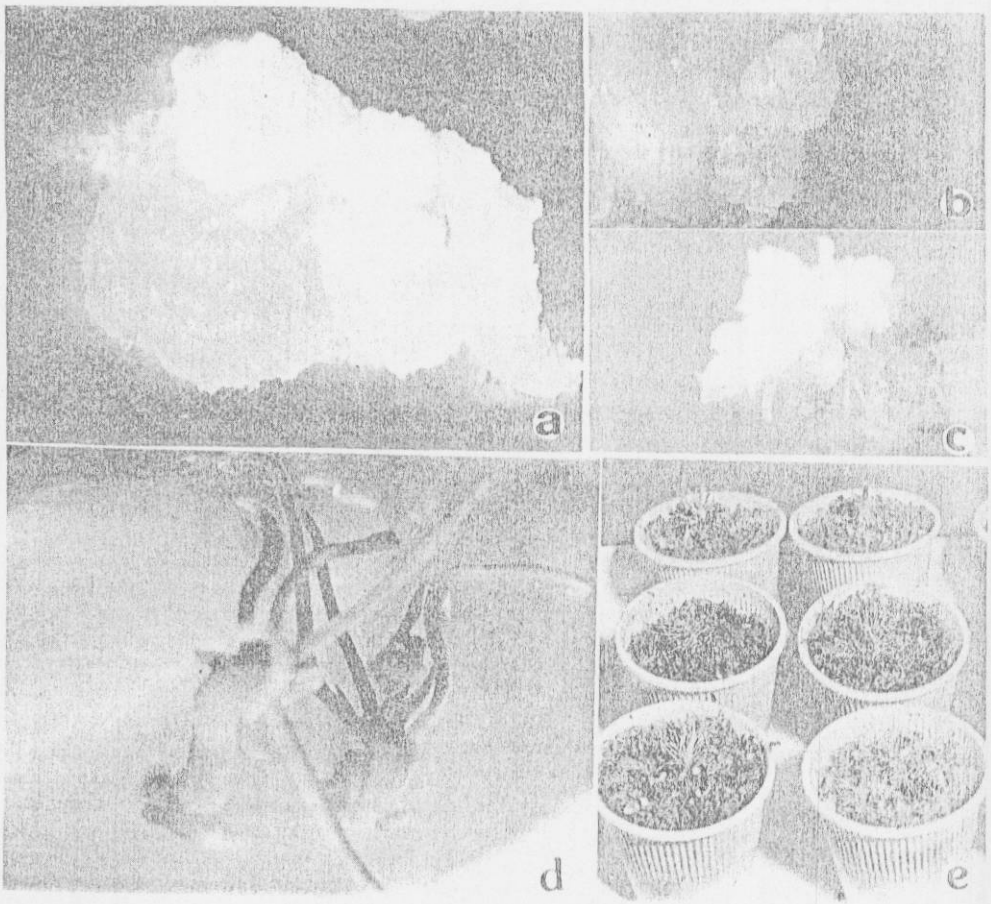

Figure 2. A. Embryogenic culture with pro-embryonal masses; B, cotyledonary stage embryo; C, germinated somatic embryos converted into emblings (somatic seedlings); D, 7–8 embryos clumped together; E, potted regenerated plantlets.

Embryo development and maturation

The PEMs and proembryos converted into cotyledonary stage embryos (Figure 2B) within four weeks of transfer to medium containing ABA (15.12 μM) and increased concentration of sucrose (40 g l^{-1}). In many cases, 7–8 embryos clumped together with fused cotyledons (Figure 2C).

Germination and plantlets regeneration

The cotyledonary stage embryos separated from the clumps started elongation within two weeks of culture on mMS_4 medium under light. The elongated embryos, upon transfer to modified mMS_4 medium, formed rooted somatic seedlings (Figure 2D). About 40.0% of the tested somatic embryos were converted

into somatic seedlings after 4–5 weeks of transfer. The plantlets were transferred to a potting mix (containing the decomposed litter of pine forest) (Figure 2E) and about 62% survival frequency was recorded.

Discussion

In the present study the stratification of seeds for 24 h at 4°C prior to dissection of embryos exhibited a pronounced promotive effect on the formation of embryogenic cultures. Increase in stratification period and in temperature resulted in poor embryogenic response. Tremblay (1990) reported better embryogenic culture induction following a 4 h imbibition of *Picea glauca* seeds. Other workers like Finer *et al.* (1989) in *Pinus strobes*, Nagmani *et al.* (1993) in *Pinus palustris*, Lelu *et al.* (1994) and Bonga *et al.* (1995) in *Larix deciduas* reported that precotyledonary to pre-germinating embryos were suitable for initiation of embryogenic cultures.

In the case of *P. kesiya*, 1/2 MS salts with reduced NH_4NO_3 and enhanced KNO_3 along with CH (1000 mg l^{-1}) and L-glutamine (500 mg l^{-1}) was found to be more effective for initiation of embryogenic cultures. This report differs from the report of Hristoforoglu *et al.* (1995), where, in a comparative study, they reported an increase in the number of *Picea abies* embryogenic lines by three times in a medium containing glutamine and asparagine than in the one containing NH_4NO_3 and KNO_3. While Norgaard (1997) reported better embryogenic culture formation of *Abies nordmanniana* on 1/2 BLG medium (Verhagen and Wann, 1989) with no NH_4NO_3, with reduced levels of KNO_3 (50.0 mg l^{-1}) and KCl (372.5 mg l^{-1}) and supplemented with L-glutamine (750 mg l^{-1}) and aspargine (50 mg l^{-1}). Like in the other conifers (Nagmani *et al.*, 1993; Li *et al.*, 1998) lower concentration of the carbon source (2.0% sucrose) was found to be more effective for initiation of embryogenic cultures of *P. kesiya*. The embryogenic cultures of *P. kesiya* could proliferate well on the initiation medium up to 5–6 weeks, while Nagmani *et al.* (1993) reported that the embryogenic cultures of long-leaf pine could not grow well on initiation medium and turned brown after one subculture.

The proembryos were converted into cotyledonary embryos on basal medium containing higher concentrations of sucrose in conjunction with ABA. It was observed that neither sucrose nor ABA singly could promote maturation of somatic embryos. A concentration of 4% sucrose and 15.12 µM ABA was found to be effective for somatic embryo maturation. Tremblay and Tremblay (1995) reported that sucrose (4–6%) in the medium could serve as an osmotic agent and as a carbon source for black spruce somatic embryo maturation. Norgaard (1997) reported that a combination of both and sucrose was more effective than either of them used singly for maturation of somatic embryos in *Abies nordmanniana*. Webster *et al.* (1990) reported that 40 µM ABA was optimum for the production of mature embryos. Vagner *et al.* (1998) reported an increase in exogenous ABA levels (5–40 µM), an increase in somatic embryo maturation of *Picea abies* was observed.

P. kesiya somatic embryos became distinct and elongated on basal medium free of growth regulators, CH and L-glutamine but containing activated charcoal (0.2%). It is believed that activated charcoal (AC) absorbed all the endogenous growth regulators and growth inhibitors and stimulated better singulation and elongation of embryos. Beewar *et al.* (1989) transferred the Norway spruce cultures with embryonal suspensor masses on hormone-free medium containing activated charcoal for one week. It was thought that activated charcoal absorbed growth regulators and caused the culture to cease cleavage polyembryony. Gupta *et al.* (1993) reported that a combination of increased osmolarity with ABA and activated charcoal produced good quality cotyledonary embryos. In *Pseudotsuga menziesii*, embryo development and maturation were promoted by amending the medium with activated charcoal (1.25 g l^{-1}) and ABA 3515.4 µM (Gupta *et al.*, 1995).

In the present study, initiation, development, maturation and germination of *P. kesiya* somatic embryos have been achieved. This opens up a great potential for large-scale propagation of this species, which is facing a rapid decline in population. Further work on increasing conversion frequency of somatic embryos is in progress.

Acknowledgements

We thank the Department of Biotechnology, Ministry of Science and Technology, Government of India

for financial support by a research grant F.No.BT/R&D/08/003/95 to Pramod Tandon.

References

Arya S, Kalia R K and Arya I D 2000 Induction of somatic embryogenesis in *Pinus roxburghii* Sarg; *Plant Cell Rep* **19** : 775–780.

Attree S M and Fowke L C 1993 Embryogeny of gymnosperms: Advances in synthetic seed technology of conifers; *Plant Cell Tiss Org Cult* **35** : 1–35.

Barrett J D, Park Y S and Bonga J M 1997 The effectiveness of various nitrogen sources in white spruce [*Picea glauca* (Moench) Voss] somatic embryogenesis; *Plant Cell Rep* **16** : 411–415.

Becwar M R, Noland T I and Wykoff J I 1989 Maturation, germination and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants; *In vitro Cell Dev Biol* **25** : 575–580.

Bonga J M, Klimaszewska K, Lelu M A and Von Aderkas P 1995 Somatic embryogenesis in *Larix*; in *Somatic embryogenesis in woody plants* (eds) S Jain, P Gupta and R Newton (The Netherlands, Kluwer Academic Publishers) vol 3, pp 315–339.

Carrier D J, Cunningham J E and Taylor D C 1997 Sucrose requirements and lipid utilization during germination of interior spruce (*Picea glauca engelmanni* complex) somatic embryos; *Plant Cell Rep* **16** : 550–554.

Deb C R and Tandon P 2002 Induction of somatic embryogenesis in khasi pine (*Pinus kesiya*) from secondary needles; *J Plant Biol* **29** : 113–118.

Finer J, Kriebel J and Becwar M R 1989 Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobes* L.); *Plant Cell Rep* **8** : 203–206.

Guevin T G and Kirby E G 1997 Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir; *Plant Cell Tiss Org Cult* **49** : 219–222.

Gupta P K and Grob J A 1995 Somatic embryogenesis in conifers; in *Somatic embryogenesis in woody plants* (eds) V Jain and R Newton (The Netherlands, Kluwer Academic Publishers) pp 81–98.

Gupta P K, Pullman G S, Timmis R, Kreitinger M, Carlson W C, Grob J and Welty E D E 1993 Forestry in the 21st century: The biotechnology of somatic embryogenesis; *Bio/Technology* **11** : 454–459.

Gupta P K, Timmis R, Timmis K A, Grob J A, Carlson W C and Welty E D E 1995 Clonal propagation of conifers via somatic embryogenesis; in *Proceedings of the IUFRO meeting on somatic cell genetics and molecular genetics of trees* (Belgium, Gent) pp 62–69.

Hakman I, Fowke L C, Von Arnold S and Eriksson T 1985 The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce); *Plant Sci* **38** : 53–59.

Hristoforoglu K, Schmitdt J and Bolharnordenkampf H 1995 Development and germination of *Abies alba* somatic embryos; *Plant Cell Tiss Org Cult* **40** : 277–284.

Kim Y W, Youn Y, Noh E R and Kim J C 1999 Somatic embryogenesis and plant regeneration from immature zygotic embryos of Japanese larch (*Larix leptolepis*); *Plant Cell Tiss Org Cult* **55** : 95–101.

Lelu M A, Klimaszewska K and Charest P J 1994 Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*; *Can J For Res* **24** : 100–106.

Li X Y, Huang F H and Gbur Jr E E 1998 Effect of basal medium, growth regulators and phytagel concentration on initiation of embryogenic cultures from immature zygotic embryos of loblolly pine (*Pinus taeda* L.); *Plant Cell Rep* **17** : 298–301.

Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassay with tobacco tissue cultures; *Physiol Plant* **15** : 473–497.

Nagmani R, Diner A M and Sharma G C 1993 Somatic embryogenesis in long leaf pine (*Pinus palustris*); *Can J For Res* **23** : 873–876.

Norgaard J V 1997 Somatic embryo maturation and plant regeneration in *Abies nordmanniana* LK; *Plant Sci* **124** : 211–221.

Tandon P and Deb C R 1998 Somatic embryogenesis and plantlets regeneration in *Pinus kesiya* (Royle ex. Gord.) (Poster); in *IX International congress on plant tissue and cell culture*, Jerusalem, Israel, 14–19 June 1998.

Tremblay F M 1990 Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*; *Can J Bot* **68** : 236–242.

Tremblay L and Tremblay F M 1995 Maturation of black spruce somatic embryos: Sucrose hydrolysis and resulting osmotic pressure of the medium; *Plant Cell Tiss Org Cult* **42** : 39–46.

Vagner M, Vondrakova Z, Strnadova Z, Eder J and Machackova I 1998 Endogenous levels of plant growth hormones during early stages of somatic embryogenesis of *Picea abies*; *Adv Hortic Sci* **12** : 11–18.

Verhagen S A and Wann S R 1989 Norway spruce somatic embryogenesis: high frequency initiation from light-cultured matured embryos; *Plant Cell Tiss Org Cult* **16** : 103–111.

Webster F B, Roberts D R, Mcinnis S M and Sutton B C S 1990 Propagation of interior spruce by somatic embryogenesis; *Can J For Res* **20** : 1759–1765.