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SHORT COMMUNICATION

Induction of Somatic Embryogenesis in Khasi Pine (*Pinus kesiya*) from Secondary Needles

Chitta Ranjan Deb[†] and Pramod Tandon*
Plant Biotechnology Laboratory, Botany Department, North-Eastern Hill University, Shillong 793 022, India
[†]Present address: Botany Department, Nagaland University, Headquarters: Lumami, Mokokchung 798 601, India

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Embryogenic cultures (88.6%) were raised from secondary needles of 5–6-week-old glasshouse-raised seedlings of *Pinus kesiya* on MS medium supplemented with 2% sucrose, 3.0 mg l^{-1} each of 2,4-dichlorophenoxyacetic acid and α-naphthaleneacetic acid and 1.0 mg l^{-1} benzyl adenine in the dark for three weeks. The embryogenic cultures proliferated well on initiation medium for three passages at two-week intervals. Proembryos and globular embryos developed on basal medium containing 1/10th level of growth regulators and subsequently on growth regulator-free medium. Cotyledonary somatic embryos were obtained on MS medium enriched with higher sucrose (4%) concentration in combination with abscisic acid (4 mg l^{-1}). The cotyledonary embryos elongated in four week upon maintaining on basal medium containing 0.4% activated charcoal under 12 h photoperiod. Somatic embryos germinated on MS medium containing 3% sucrose. About 40% conservation frequency into somatic seedlings was achieved.

Keywords: Khasi pine, *Pinus kesiya*, secondary needles, somatic embryo.

The commercial importance of conifers has drawn considerable attention of plant biotechnologists to evolve means for rapid clonal propagation of selected genotypes. In contrast to angiosperms, conifers have received lesser attention as far as the *in vitro* propagation is concerned. Since 1985 (Chalupa, 1985; Hakman *et al.*, 1985; Nagmani and Bonga, 1985) considerable efforts have been made to propagate conifers through somatic embryogenesis (Bonga *et al.*, 1995; Gupta and Grob, 1995; Gupta *et al.*, 1995; Guevin and Kirby, 1997; Norgaard, 1997; Tandon and Deb, 1998; Kim *et al.*, 1999; Arya *et al.*, 2000).

A key factor to establish successful cultures with conifer has been the choice of explants. Mature and immature zygotic embryos or intact megagametophytes have been used extensively to initiate embryogenic cultures in conifers. There are few reports of induction of embryos from cotyledons of germinated seedlings and secondary needles (Bourgkard and Favre, 1988; Attree *et al.*, 1990; Lelu and Borman, 1990; Ruaud *et al.*, 1992; Westcott, 1992). In this paper we describe induction of somatic embryogenesis in the cultures of secondary needles of khasi pine, *Pinus kesiya*.

*For correspondence. (e-mail: profptandon@yahoo.com)

Seeds of *P. kesiya* were germinated in a glasshouse under normal daylight conditions. About 20 seeds were placed equidistant in 90 mm petri dish lined with moist cotton pad. The needles were harvested from 5 to 6-week-old seedlings (after appearance of the first batch of secondary needles) and placed in water containing 100 mg l^{-1} PVP. The healthy needles were surface cleansed with 'Labklin' (a laboratory detergent) (1 : 100, v/v) for 5 min, followed by surface sterilized with mercuric chloride (0.25% w/v) for 3–5 min and washed 4–5 times with sterilized distilled water.

MS (Murashige and Skoog, 1962), half strength MS salts with reduced ammonium nitrate (550 mg l^{-1}) and increased potassium nitrate (4460 mg l^{-1}) [mMS] and DCR (Gupta and Durzan, 1985) were used for the experiments. All the media (MS, mMS and DCR) with full or half-strength salts were supplemented with 1000 mg l^{-1} casein-hydrolysate (CH), 500 mg l^{-1} L-glutamine, 1000 mg l^{-1} myo-inositol, 0–3% sucrose and 200 mg l^{-1} PVP (as anti-oxidant). In addition, the media were supplemented with 0–5 mg l^{-1} 2,4-D and NAA (singly or in combination) along with 1.0 mg l^{-1} BA. The media were gelled with 0.7% agar and the pH adjusted to 5.75 with 0.5 N NaOH. The media were autoclaved at 1.06 kg cm^{-2}

pressure for 15 min. L-glutamine, myo-inositol and abscisic acid were filter sterilized and added to the autoclaved media. About 20 ml of the autoclaved medium was poured in each petri dish (90 mm).

Five needles were cultured per petri dish and were sealed with parafilm. Fifty needles were cultured per treatment for each medium and experiments were repeated thrice. Cultures were maintained at 25 ± 2°C in the dark unless mentioned otherwise. After three weeks, the embryogenic cultures were transferred thrice at two-week intervals on fresh medium. Thereafter embryogenic cultures were maintained on optimum basal medium containing reduced growth regulators (1/10th level of initiation medium) for 2–3 passages. Subsequently the cultures were transferred to medium devoid of growth regulators and subcultured for two passages. The resulting proembryos were cultured on medium containing varying concentrations of sucrose (0–5%) and ABA (0–8 mg l^{-1}), singly or in combination for 2–3 passages for the formation of cotyledonary embryos. The cotyledonary embryos were elongated on medium free of growth regulators, CH and L-glutamine but containing 0.4% activated charcoal (AC), 100 mg l^{-1} myo-inositol and 2% sucrose under a photoflux of 60 μmol m^{-2} s^{-1} at 12 h photoperiod. The somatic embryos were transferred to medium containing 3% sucrose and maintained at 12 h photoperiod for two passages for germination.

Among the six media used for initiation of culture, MS medium was found to be most suitable followed by mMS and DCR media. In all the half strength media initiation frequency was comparatively poor. The highest callus initiation frequency of 88.6% (after three week culture) occurred on MS medium containing 2% sucrose and 2,4-D and NAA (each at 3 m gl^{-1}) (Tables 1 and 2) followed by on mMS medium with 2% sucrose (76.4%). Embryogenic cultures were white, soft and translucent (Figure 1A) while, non-embryogenic cultures were hard and greenish.

After 2–3 passages on initiation medium the cultures ceased proliferation and turned brown. The cultures were transferred and maintained on MS medium containing 1/10th growth regulators of initiation medium and subsequently on growth regulators-free medium where proembryos converted into globular embryos (Figure 1B).

Table 1. Effect of media composition and sucrose concentration on initiation of embryogenic cultures from secondary needles of *Pinus kesiya*.

Media*	Sucrose conc. (%)	% response ± SE
Half-strength MS	0	0 ± 0
	1.0	42.1 ± 2.85
	2.0	59.3 ± 2.54
	3.0	55.5 ± 2.67
MS	0	0 ± 0
	1.0	73.6 ± 2.36
	2.0	88.6 ± 2.82
	3.0	80.0 ± 2.88
Half-strength mMS	0	0 ± 0
	1.0	42.1 ± 1.84
	2.0	43.6 ± 2.60
	3.0	41.4 ± 2.10
mMS	0	0 ± 0
	1.0	55.0 ± 2.67
	2.0	76.4 ± 1.79
	3.0	67.8 ± 2.64
Half-strength DCR	0	0 ± 0
	1.0	34.3 ± 2.54
	2.0	37.8 ± 2.40
	3.0	32.1 ± 2.64
DCR	0	0 ± 0
	1.0	44.3 ± 2.02
	2.0	50.7 ± 2.64
	3.0	50.6 ± 2.02

*Additional adjuvants: CH (1000 mg l^{-1}), myo-inositol (1000 mg l^{-1}), L-glutamine (500 mg l^{-1}), PVP (200 mg l^{-1}) and plant growth regulators: 2,4-D and NAA (3.0 mg l^{-1} each) + BA (1.0 mg l^{-1}).

Immature embryos were converted into cotyledonary embryos (Figure 1B) on MS medium containing ABA (4 mg l^{-1}) and sucrose (4%) in combination. The cotyledonary somatic embryos elongated within two passages on MS medium free of any growth regulators but containing 100 mg l^{-1} myo-inositol, 2% sucrose and 0.4% AC.

The somatic embryos germinated and formed shoots (Figure 1C) on MS medium containing only 3% sucrose in about 40% cultures.

In the present study somatic embryos were induced in secondary needles from 5 to 6-week-old glasshouse-grown seedlings of *Pinus kesiya*. In *Picea*

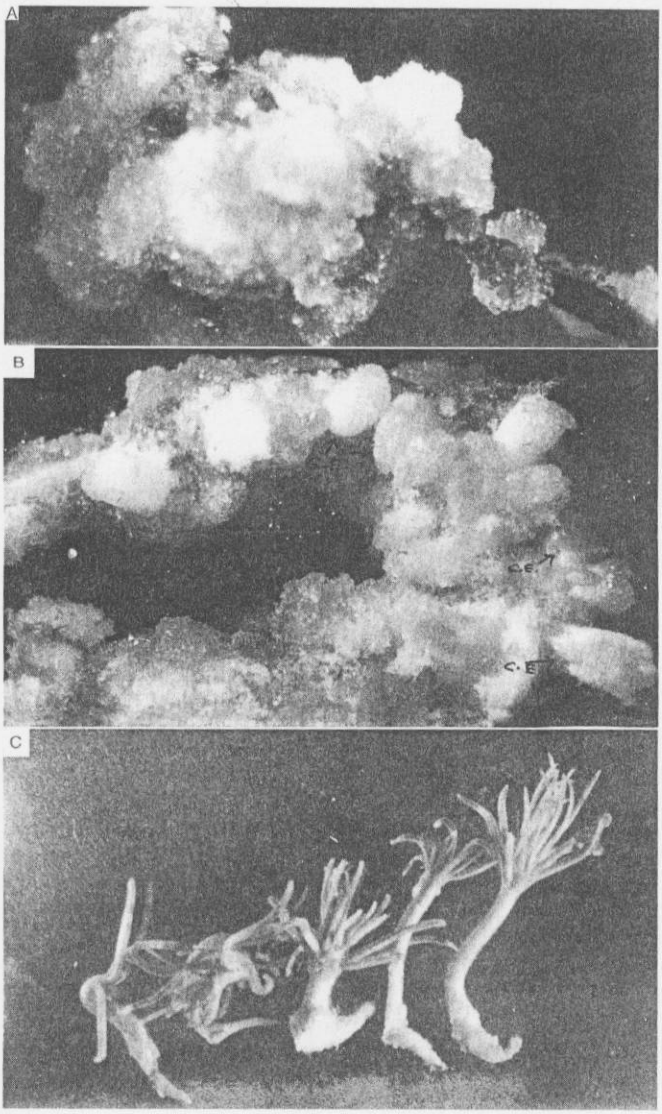

Figure 1. A, Embryogenic culture raised from cultured needle (3-week-old culture); B, globular and cotyledonary stage somatic embryos developed all over the needle; C, somatic embryos germinated into somatic seedlings.

Table 2. Effect of 2,4-D, NAA on initiation* of embryogenic cultures from secondary needles of *P. kesiya*.

BAP (mg l^{-1})	2,4-D (mg l^{-1})	NAA (mg l^{-1})	% response ± SE
1	0	0	0
1	1.0	0	26.7 ± 2.20
1	3.0	0	30.7 ± 1.63
1	5.0	0	30.7 ± 2.66
1	0	1.0	37.0 ± 2.66
1	1.0	1.0	60.0 ± 2.10
1	3.0	1.0	68.0 ± 2.49
1	5.0	1.0	46.7 ± 2.10
1	0	3.0	61.3 ± 1.33
1	1.0	3.0	65.3 ± 2.49
1	3.0	3.0	88.6 ± 1.82
1	5.0	3.0	73.3 ± 2.11
1	0	5.0	28.0 ± 2.48
1	1.0	5.0	60.0 ± 2.10
1	3.0	5.0	64.0 ± 1.63
1	5.0	5.0	44.0 ± 1.64

*MS medium with 2% sucrose.

abies embryogenic culture was established from explants excised from 7-day-old seedlings (Krogstrup, 1986; Lelu *et al.*, 1987). Embryogenic cultures were also initiated from secondary needles of *P. glauca* and *Picea mariana* (Attree *et al.*, 1990), *Picea abies* (Ruaud *et al.*, 1992) and *P. abies* (Westcott, 1992).

Among the different media tried, MS medium with 2% sucrose was found suitable for initiation of embryogenic cultures. Like other conifers (Becwar *et al.*, 1988; Nagmani *et al.*, 1993; Bonga *et al.*, 1995; Li *et al.*, 1998) in *Pinus kesiya* also lower concentration of organic carbon source was more effective.

The embryogenic cultures from secondary needles of *P. kesiya* formed proembryos on medium containing reduced levels of growth regulators. Nagmani *et al.* (1993) reduced the 2,4-D to 0.5 mg l^{-1} gradually for maintenance of long leaf pine cultures. Gupta *et al.* (1995) reported that it was necessary to reduce the growth regulator level in the maintenance medium compared to initiation medium in *P. abies*.

Many workers (Tremblay and Tremblay, 1995; Carrier *et al.*, 1997) studied the role of exogenous sucrose on somatic embryo development and maturation. ABA has been used for cotyledonary embryo development in many plant species (Skriver and Mundy, 1990) including conifers (Webster *et al.*, 1990; Vagner *et al.*, 1998). In case of *P. kesiya* better cotyledonary embryo formation was observed on medium containing both sucrose (4%) and ABA (4 mg l^{-1}) in combination. Neither sucrose nor ABA alone could promote cotyledonary stage embryo formation. The resulted cotyledonary embryos elongated on growth regulators free medium containing AC. It is believed that AC absorbed all the endogenous growth regulators and growth inhibitors. This observation is in agreement with that of other workers (Pullman and Gupta, 1991; Gupta *et al.*, 1993, 1995).

The findings described here will provide help in efforts on large-scale production of somatic embryos-derived plants in confiers.

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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, Budimir S and Fowke L C 1990 Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings from stored seeds of black and white spruce (*Picea mariana* and *Picea glauca*); *Can J Bot* **68** : 30–34.

Becwar M R, Wann S R, Johnson M A, Verhagen S A, Feirer R P and Nagmani R 1988 Development and characterization on *in vitro* embryogenic systems in conifer; in *Somatic cell genetics of woody plants* (ed.) M R Ahuja (Dordrecht, Kluwer Academic Publishers) pp 1–18.

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.

Bourgkard F and Favre J M 1988 Somatic embryos from callus of *Sequoia sempervirens*; *Plant Cell Rep* **7** : 445–448.

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.

Chalupa V 1985 Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) krast; *Commun Inst For Cech* **14** : 57–63.

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 Durzan D J 1985 Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*); *Plant Cell Rep* **4** : 177–179.

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 Erikson T 1985 The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce); *Plant Sci* **38** : 53–59.

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.

Krogstrup P 1986 Embryo-like structure from cotyledons and ripe embryos of Norway spruce (*Picea abies*); *Can J For Res* **16** : 664–668.

Lelu M A and Bornman C H 1990 Induction of somatic embryogenesis in excised cotyledons of *Picea glauca* and *Picea mariana*; *Plant Physiol Biochem* **28** : 785–791.

Lelu M A, Boulay M and Arnaud Y 1987 Formation of embryogenic calli from cotyledons of *Picea abies* (L.) krast collected from 3 to 7-day-old seedlings; *CR Acad Sci Ser* **305** : 105–109.

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 and Bonga J M 1985 Embryogenesis in subcultured callus of *Larix deciduas*; *Can J For Res* **15** : 1088–1091.

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.

Pullman G S and Gupta P K 1991 Method for reproducing coniferous plants by somatic embryogenesis using absorbent materials in the development stage media; US patent No. 5,034,326.

Ruaud J N, Bercetche J and Paques 1992 Fir evidence of somatic embryogenesis from needles of one-year-old *Picea abies*; *Plant Cell Rep* **11** : 1460–1467.

Skriver K and Mundy J 1990 Gene expression in response to abscisic acid and osmotic stress; *Plant Cell* **2** : 503–512.

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*.

Tremblay L and Tremblay F M 1995 Maturation of black spruce somatic embryos: sucrose hydrolysis and resulting