Studies on somatic embryogenesis from immature zygotic embryos of chir pine (*Pinus roxburghii* Sarg.)

Gaurav Mathur*, Sara von Arnold[§] and Rajani Nadgauda*,[#]

*Tissue Culture Pilot Plant, National Chemical Laboratory, Dr Homi Bhabha Road, Pune 411 008, India
[§]Department of Forest Genetics, Swedish University of Agricultural Science, Box 7027, S-75007, Uppsala, Sweden

Embryogenic cultures were initiated and established in chir pine using immature megagametophytes. Three different basal media with various combinations of growth regulators and other supplements were tested initially during the 1996 studies for the extrusion of embryogenic mass, which was initiated and proliferated on DCR basal medium. Variation was observed in the extrusion of embryogenic mass, mostly depending on the location, collection dates, media composition and developmental stage of megagametophytes. Three embryogenic cell lines (R-1, R-3 and R-29) were selected and used in the initial studies for maturation and conversion of somatic embryos to plants.

SOMATIC embryogenesis in conifers has been regarded as a model for large-scale propagation of tree species throughout the world. *Pinus roxburghii* Sarg. or chir pine, forms an important feature of the major flora in the lower altitudes (460–1500 m) of western Himalayas. Apart from timber and fuel-wood, indiscriminate tapping for resin and lopping for fuel-wood from chir pine, has put a great pressure on the ecology of the area[1].

Elite trees producing high quality resin are mostly selected, propagated and tapped for resin by the forest department and other non-governmental agencies by the conventional methods[2]. Our objective is propagation of selected elite varieties for high quality resin production, by initiating studies using somatic embryogenesis, in continuation with the ongoing work on other Indian pine species in our lab[3,4]. The present work describes the explant, media composition and conditions for extrusion and proliferation of embryogenic mass in *P. roxburghii*, an important Indian pine.

The immense surge in the field of conifer biotechnology in the past decade has been reviewed by several authors[5–7] and somatic embryogenesis has been suggested and proven to an extent, as a powerful tool for successful clonal propagation[8,9]. Partial or complete embryogenesis has been reported in various species of *Pinus*, viz. *P. caribaea*[10], *P. elliotii*[11], *P. lambertiana*[12], *P. nigra*[13], *P. patula*[14], *P. pinaster*, *P. sylvestris*[15], *P. strobus*[16,17] and *P. taeda*[18], describing the importance of the genus worldwide.

[#]For correspondence. (e-mail: rsn@ems.nc.res.in)

Liao and Amerson[11] have described the recalcitrance of the genus *Pinus* in terms of selection of suitable explants for initiating somatic embryogenesis and the difficulty in getting successful plantlet regeneration through tissue culture compared to other conifers. The major bottleneck in initiating embryogenic cultures of pines has been the identification of the correct developmental stage of the immature zygotic embryo known as the 'window', followed by successful maturation and conversion of somatic embryos to plants. The aim of the present study has been identification of the critical developmental stage for establishing embryogenic cultures of *P. roxburghii* by determining the correct time for collection followed by, an efficient regeneration protocol.

Immature female cones of chir pine were procured from two different locations; the Conifers Research Centre Forest Nursery, Solan, Himachal Pradesh (HP) (altitude 800 m) and Institute of Himalayan Bioresource Technology, Palampur, HP (altitude 1300 m). The weekly collections were made during the months of May till July in 1996 and 1997. The cones were transported to the laboratory in Pune and stored in cardboard boxes at 8 to 10°C till utilized (within 10 to 15 days of collection). The developmental stage of the zygotic embryo was determined by histological examination of immature megagametophytes removed from 2 to 3 cones picked out randomly from each collection.

Seeds were individually removed from the cones, cleaned and washed thoroughly in running tap water. Surface sterilization included washing with liquid detergent (Labolene®, Qualigens, India; 1% solution for 5 to 6 min), followed by 70% alcohol (2 to 3 min) and then with 0.05% $HgCl_2$ (5 min), ending with 5 to 6 washes with sterile distilled water in the laminar airflow.

The choice and response of initial explants for the induction of somatic embryogenesis in coniferous species has been very limited. Mature zygotic embryos, seedling cotyledons, cotyledonary stage immature zygotic embryos and haploid megagametophytes are some of the sources used for raising embryogenic tissue in a number of species[5,7]. In genus *Pinus*, however, the most suitable explant for successful induction of embryogenesis has been immature zygotic embryo cultured within the megagametophytes. The megagametophytes were aseptically removed from the seeds and placed horizontally on various initiation media. The number of explants tested for each treatment varied according to the number of cones collected and seeds removed from each cone.

Three different basal media, DCR[19], LP[20] and BM1 (ref. 21) were tested for the initiation of embryogenic cultures in earlier studies (in 1996). All the three basal media were supplemented with 3% sucrose, 1.0 g l^{-1} myo-inositol, 0.5 g l^{-1} CH (casein hydrolysate), 0.5 g l^{-1} L-glutamine, 50 µM 2,4-D (2,4-dichlorophenoxyacetic acid), 20 µM BA (N^6-benzyladenine), 20 µM kin (kinetin) and 0.18% gellan gum (Figure 1). The pH of all media was adjusted

to 5.8 with 1N NaOH/HCl prior to autoclaving. An average of 15 to 20 megagametophytes were cultured in each petri plate (Laxbro®, 15 × 85 mm) and all cultures were incubated at 20 ± 2°C in dark for the extrusion of embryogenic mass. Explants were cultured (60 to 100 megagametophytes per treatment in triplicate) depending on the availability of cones and the harvest of immature seeds from each cone.

On the basis of earlier studies (in 1996), the megagametophytes showed best response of extrusion and proliferation of embryogenic tissue on DCR basal medium (48.31% extrusion and 13.5% cultures established) among the three basal media tested (Figure 1), and were used in the experiments in the subsequent year (1997) (Table 1).

Variation was observed in the developmental stages of immature megagametophytes of *P. roxburghii* collected from the two provenances, Solan and Palampur (western Himalayas) (Tables 1–3). The cones from the trees in Solan (altitude 800 m) showed delayed development compared to the cones from Palampur (altitude 1500 to 1700 m) (Tables 2–4). This had a direct effect on the extrusion 'window' in the two provenances. Variation was also observed among the trees in Palampur (Tables 3 and 4). Despite this variation in the maturation stages of the developing megagametophyte, the correct stage for extrusion (Type 1, Figures 2 *a* and 3 *a*) or the 'window', appeared quite uniformly within 10 to 15 days of the fertilization.

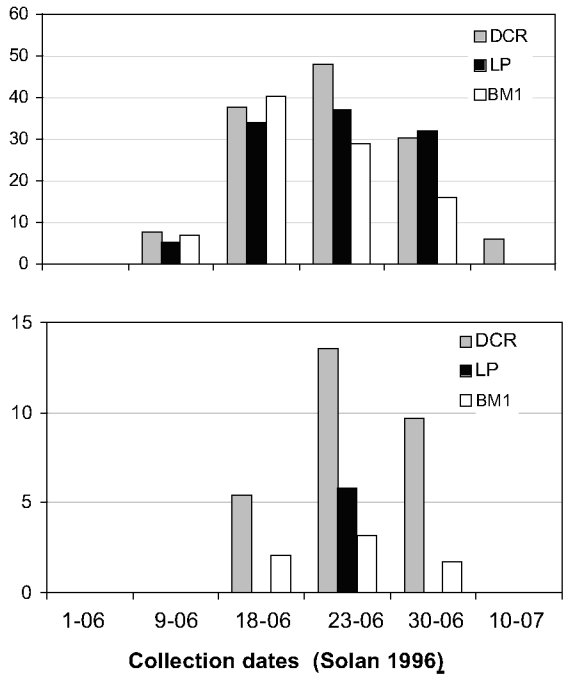

Figure 1. Effect of three different basal media (DCR, LP and BM1) on the extrusion and establishment of embryogenic cultures of *P. roxburghii*. All three basal media contained 3% sucrose + 1.0 g l^{-1} myo-inositol + 0.5 g l^{-1} L-glutamine + 0.5 g l^{-1} caesin hydrolysate + 50 µM 2,4-D + 20 µM BA + 20 µM Kin + 0.18% gellan gum. (Data from collections made in Solan during May–June 1996.)

A study of the development of megagametophytes in *P. roxburghii* before and after the fertilization was conducted in the previous year to assess the correct time of collection for the induction of somatic embryogenesis. The reference regarding the developmental stages of the zygotic embryo of *P. roxburghii* followed throughout this manuscript is from the monograph on genus *Pinus* [2].

The first indication of culture initiation is the extrusion of immature zygotic embryos through the micropylar end of megagametophyte within 10 days of culture, which was translucent to white. The morphology of the extrusions varied and was classified according to Liao and Amerson[11]. No single type of extrusion was observed to be prominent on one particular basal medium, thus implying that the variation in extrusions is related only to the developmental stage of the immature zygotic embryo. Among the four different types of extrusions that were observed during the study (Figure 3 *a–d*), Types II–IV did not give rise to embryogenic cultures (Figure 3 *b–d*). Embryogenic callus that had proliferated to 1–1.5 cm in diameter was considered as an established culture and was further multiplied by subculturing every two weeks.

It is notable that only the Type I extrusions (Figures 2 *a* and 3 *a*) survived, proliferated and established as embryogenic cultures. The proliferating cells in the embryogenic mass in pines are generally the suspensor cells[22]. Figure 2 *b* shows initial somatic embryos separated out from embryogenic mass and the embryo heads are stained red with acetocarmine. Apart from a suitable media composition (basal salts, growth regulators, organic nitrogen, and gelling agent), the induction and proliferation of embryogenic mass was observed to be sensitive to temperature. The proliferation was best observed at 20 ± 2°C and an increase in temperature by 3 to 4°C was absolutely detrimental for the proliferating cultures.

The extrusion and establishment of embryogenic mass is completely dependent on the collection of cones at the correct stage of the immature zygotic embryo. As it can be clearly observed from the present study, the time of maturation of cones varies not only within the locations

Table 1. Various media combinations tested for the extrusion and establishment of embryogenic cultures of *P. roxburghii*

Medium*	D-1	D-2	D-3	D-4	D-5#
CH (g l^{-1})	0.5	0.5	–	0.5	0.5
L-glutamine (g l^{-1})	0.5	0.5	–	0.5	0.5
2,4-D (µM)	50	50	50	50	–
BA (µM)	20	20	20	–	–
Kin (µM)	20	20	20	–	–
Phytagel (%)	0.18	–	0.18	0.18	0.18
Difco Agar (%)	–	0.8	–	–	–

*Media D-1 to D-5 contain DCR basal salts[16] + 3.0% sucrose + 1.0 g l^{-1} myo-inositol with different concentrations and combinations of growth regulators, organic nitrogen (L-glutamine and CH) and gelling agents (agar and gellan gum).
#Medium D-5 is the control devoid of growth regulators.
These combinations were used in the studies during 1997 (Tables 2–4).

(Tables 2–4), but also within the trees in one location (Tables 3 and 4). The collection schedule has to be well regularized to obtain maximum number of well-established embryogenic cultures.

The advanced cellular stages of zygotic embryos only (Type I extrusion) produce embryogenic cultures and even the type of extrusion is very much similar among different locations. In the Type II extrusions, the zygotic embryo is of very early cellular stage and unable to give rise to the embryogenic cultures. Similarly, in Type IV extrusion the zygotic embryo has matured to an early smooth paraboloid structure and is thus unable to respond and give rise to embryogenic tissue.

Three embryogenic lines (R-1, R-3 and R-29) were selected among the different cell lines that were initiated, which are presently being maintained on the same initiation medium and cultures are being utilized for maturation studies. Pre-maturation treatment for two weeks on hormone-free DCR medium containing 0.5% activated charcoal to stop the proliferation of embryogenic tissue, was observed to be sufficient before the maturation treatment. Somatic embryo resembling the early smooth paraboloid stage zygotic embryo can be seen in Figure 2 *c*, where the bullet-shaped head (0.3 mm long) with suspensor cells is visible. Cultures at this stage of development were then transferred to maturation treatments.

Table 2. Effect of growth regulators, organic nitrogen and gelling agent on extrusion and establishment of embryogenic cultures of *P. roxburghii* (Collection at Solan, 1997)

Collection date (1997)	Stage of zygotic embryo[19]	Culture medium (see Table 1)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion ± S.E. (% Established cultures)[#]				
23 May	No fertilization	No extrusion observed				
31 May	No fertilization	No extrusion observed				
13 June	Early cellular	41.12 ± 2.2 (3.8)	18.60 ± 1.54 (2.7)	24.22 ± 1.39 (0.0)	18.94 ± 3.13 (4.4)	18.04 ± 2.98 (0.0)
24 June	Mid-cellular	46.64 ± 4.58 (20.6)	25.45 ± 1.37 (11.8)	21.43 ± 0.67 (0.0)	30.60 ± 0.76 (10.4)	16.07 ± 1.81 (0.0)
8 July	Smooth paraboloid	14.22 ± 1.06 (0.0)	22.62 ± 0.98 (0.0)	16.40 ± 2.2 (0.0)	12.43 ± 1.06 (0.0)	(0.0)

[#]Data are given as % extrusion ± S.E.; Data within parentheses represent % established cultures from the extruded embryogenic tissue.

Table 3. Effect of growth regulators, organic nitrogen and gelling agent on extrusion and establishment of embryogenic cultures of *P. roxburghii* (Collection at Palampur: T1, 1997)

Collection date (1997)	Stage of zygotic embryo[19]	Culture medium (see Table 1)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion ± S. E. (% Established cultures)[#]				
2 June	Early cellular	30.03 ± 1.68 (6.8)	39.80 ± 1.44 (7.7)	25.26 ± 2.24 (0.0)	5.70 ± 1.16 (0.0)	0.0 (0.0)
10 June	Mid-cellular	49.10 ± 3.14 (26.3)	35.88 ± 1.3 (16.6)	22.03 ± 1.64 (0.0)	26.32 ± 1.17 (0.0)	32.94 ± 1.53 (0.0)
17 June	Smooth paraboloid	27.46 ± 0.72 (9.5)	33.30 ± 3.85 (7.3)	30.75 ± 0.67 (0.0)	23.31 ± 0.85 (0.0)	14.76 ± 1.13 (0.0)
24 June	Pre-cotyledonary bumps	29.48 ± 9.78 (0.0)	33.46 ± 1.01 (0.0)	0.0 (0.0)	23.73 ± 0.82 (0.0)	0.0 (0.0)
1 July	Developing cotyledons	No extrusion observed				
8 July	Germination	No extrusion, only germination of seeds observed				

[#]Data are given as % extrusion ± S.E.; Data within parentheses represent % established cultures from the extruded embryogenic tissue.

Table 4. Effect of growth regulators, organic nitrogen and gelling agents on extrusion and establishment of embryogenic cultures of *P. roxburghii* (Collection at Palampur: T2, 1997)

Collection dates (1997)	Stage of zygotic embryo[19]	Culture medium (see Table 1)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion ± S.E. (% Established cultures)[#]				
2 June	No fertilization	No extrusion observed				
10 June	No fertilization	No extrusion observed				
17 June	Early cellular	39.16 ± 0.56 (8.5)	41.83 ± 0.80 (7.3)	12.6 ± 1.08 (0.0)	26.30 ± 0.15 (0.0)	22.12 ± 1.06 (0.0)
24 June	Mid-cellular	41.40 ± 1.29 (14.6)	35.44 ± 1.11 (14.2)	24.80 ± 2.07 (0.0)	37.96 ± 2.47 (2.4)	20.91 ± 1.89 (0.0)
1 July	Smooth paraboloid	9.03 ± 1.78 (0.0)	14.90 ± 0.95 (3.3)	0.0 (0.0)	15.23 ± 1.0 (0.0)	0.0 (0.0)
8 July	Developing cotyledons	No extrusion				

[#]Data are given as % extrusion ± S.E.; Data within parentheses represent % established cultures from the extruded embryogenic tissue.

Effects of ABA (abscissic acid, Sigma Chemical Co, USA) and gellan gum were tested in the initial studies on the maturation of somatic embryos. ABA was filter sterilized and added to pre-autoclaved medium. Initial experiments suggest that a combination of 30 μM ABA with 0.25% gellan gum, among the various combinations tested, was best suited for the maturation of somatic embryos (Table 5). Klimaszweska and Smith[23] reported the

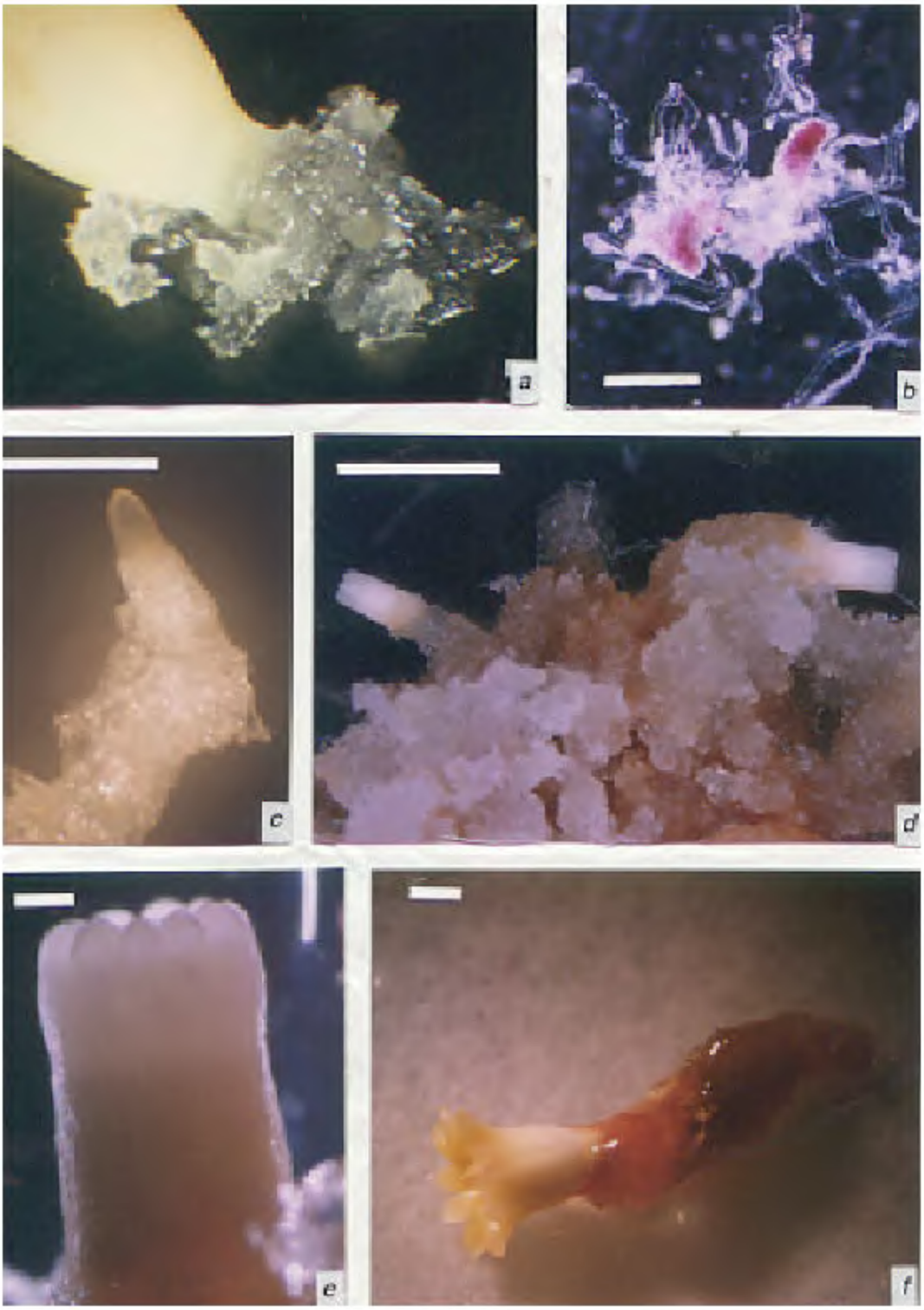

Figure 2. Initiation of somatic embryogenesis from immature zygotic embryos of *P. roxburghii* Sarg. ***a***, Type I extrusion from within the megagametophyte (bar = 1 mm); ***b***, Cellular stage somatic embryos with distinct head, stained with acetocarmine (bar = 1 mm); ***c***, Somatic embryo resembling a bullet-shaped head of zygotic embryo (bar = 1 mm); ***d***, Embryogenic culture on maturation medium showing developing somatic embryos (bar = 5 mm); ***e***, Somatic embryo showing development of pre-cotyledonary bumps (bar = 0.5 mm); ***f***, Fully developed somatic embryo (bar = 2 mm).

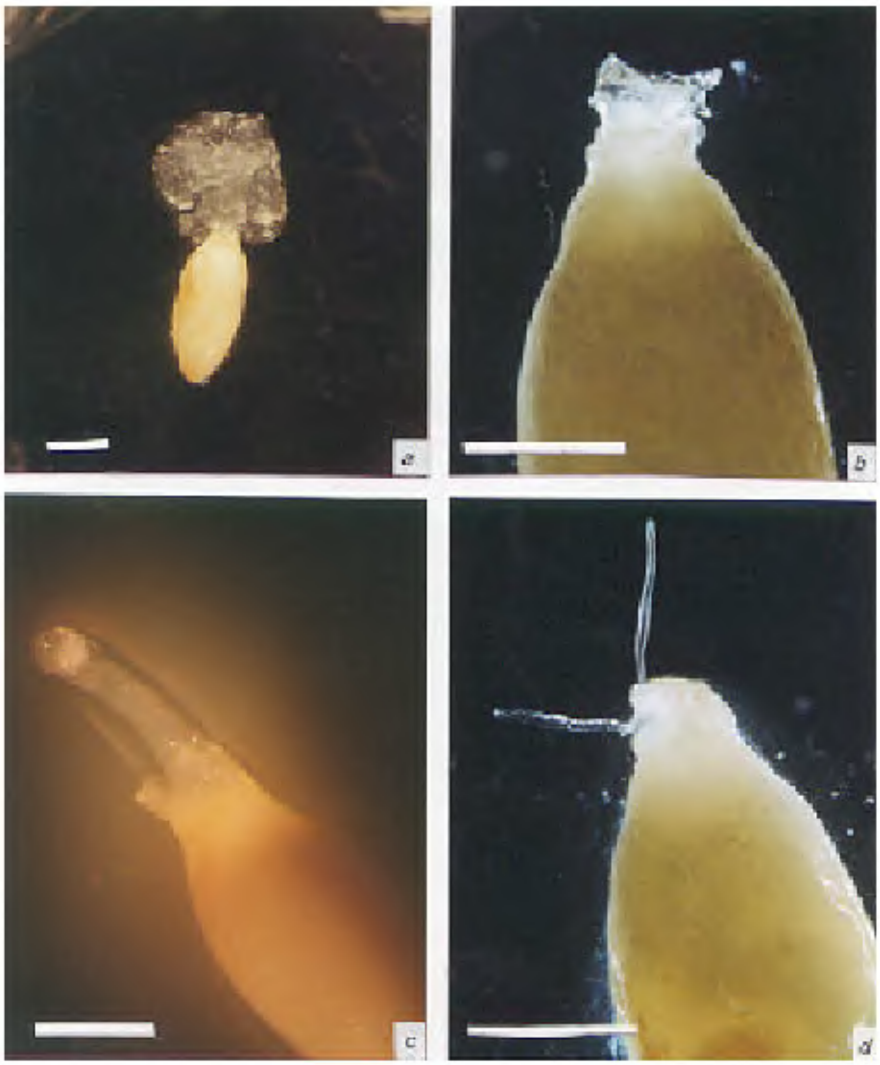

Figure 3. Different types of extrusions observed during culture initiation in somatic embryogenesis of *P. roxburghii* (bar = 2 mm). ***a***, Type I extrusion giving rise to embryogenic cultures; ***b***, Type II extrusion with individually proliferating embryonal heads; ***c***, Type III extrusion with cell mass pushed away by cord-like formation of suspensors; ***d***, Type IV extrusion with two early cellular stage embryos protruded by suspensor cells.

use of very high concentration of gellan gum (1%) and ABA (120 µM) suitable for *P. strobus* somatic embryo maturation. There are mixed reports regarding the medium suitable for maturation in *Pinus* species, but the role of ABA in the normal development is fully established[5]. Somatic embryos resembling stage-D zygotic embryos can be seen developing in Figure 2 *d* and *e*. The prominent head with pre-cotyledonary bumps or small cotyledons, and hypocotyl with a developing radicle can be clearly observed. Fully developed somatic embryos are formed on separating out from the callus and culturing them on a medium devoid of growth regulators (Figure 2*f*).

From our studies it can thus be collectively concluded that somatic embryogenesis in immature zygotic embryos is possible and is similar to the cleavage polyembryony observed in nature. The 'window' or the correct stage of the zygotic embryo is perhaps the middle cellular stage, where the embryo has not developed into a smooth paraboloid structure and this is completely independent of the different locations, differing even among the trees within the same location. But, the appearance of the Type I extrusion is very much uniform in relation to the stage of the developing zygotic embryo.

Further studies for better understanding of the developmental process are essential to fully ascertain the best media supplements and culture conditions for the maturation of somatic embryos, in order to obtain the optimum conversion percentage, to maximize the embryogenic potential and normal development of somatic embryos into plantlets. The pre-maturation and desiccation treatments are critical stages after successful establishment of

Table 5. Effect of various media on somatic embryo maturation in three established cell lines (R-1, R-3 and R-29) of *P. roxburghii*. The embryogenic culture clumps were first transferred to hormone-free half-strength DCR basal medium for one week before transferring them to maturation medium

	Embryogenic cell lines		
Medium*	R-1	R-3	R-29
10 µM ABA + 0.18% gellan gum	54 (4)#	46 (0)	38 (2)
30 µM ABA + 0.18% gellan gum	45 (7)	38 (3)	47 (4)
30 µM ABA + 0.25% gellan gum	38 (13)	47 (8)	52 (18)
0.30% gellan gum	28 (0)	35 (0)	42 (1)
Control	0 (0)	0 (0)	0 (0)

*All media contain half-strength DCR basal salts[16] + 2% sucrose + 1.0 g l^{-1} myo-inositol.
#Data represent number of clumps of embryogenic cell lines cultured on different media combinations; Data within parentheses represent the number of clumps of embryogenic tissue showing somatic embryo formation. (Weight of each clump of the embryogenic mass varied from 320–550 mg).

embryogenic tissue for the development of somatic embryos to plants.

1. Chaturvedi, A. N., *Indian For.*, 1998, **124**, 391–396.
2. Maheshwari, P. and Konar, R. N., *Pinus*, Botanical Monograph No. 7, CSIR, Delhi, 1971.
3. Nadgauda, R. S., Nagarwala, N. N., Parasharami, V. A. and Mascarenhas, A. F., *In Vitro Cell Dev. Biol. (Plant)*, 1993, **209**, 131–134.
4. Mathur, G. and Nadgauda, R., *Plant Cell Rep.*, 1999, **19**, 73–80.
5. Attree, S. M. and Fowke, L. C., *Plant Cell Tissue Org. Cult.*, 1993, **35**, 1–35.
6. Dunstan, D. I., *Can. J. For. Res.*, 1988, **18**, 1497–1506.
7. Tautorus, T. E., Fowke, L. C. and Dunstan, D. I., *Can. J. Bot.*, 1991, **69**, 1873–1899.
8. Gupta, P. K. and Grob, J., in *Somatic Embryogenesis in Woody Plants*, Kluwer Acad. Publ., The Netherlands, 1995, vol. 1, pp. 81–98.
9. Timmis, R., *Biotechnol. Prog.*, 1998, **14**, 156–166.
10. Laine, E. and David, A., *Plant Sci.*, 1990, **69**, 215–224.
11. Liao, Y. K. and Amerson, H. V., *New For.*, 1995a, **10**, 145–163.
12. Gupta, P. K. and Durzan, D. J., *Bio/Technology*, 1986, **4**, 643–645.
13. Radojevic, L., Alvarez, C., Fraga, M. F. and Rodriguez, R., *In Vitro Cell Dev. Biol. (Plant)*, 1999, **35**, 206–209.
14. Jones, N. B., van Staden, J. and Bayley, A. D., *J. Plant Physiol.*, 1993, **142**, 366–372.
15. Lelu, M. A., Bastien, C., Drugeault, A., Gouez, M. L. and Klimaszweska, K., *Physiol. Plant.*, 1999, **105**, 719–728.
16. Finer, J. J., Kriebal, H. B. and Becwar, M. R., *Plant Cell Rep.*, 1989, **8**, 203–206.
17. Garin, E., Isabel, N. and Plourde, A., *Plant Cell Rep.*, 1998, **18**, 37–43.
18. Gupta, P. K. and Durzan, D. J., *Bio/Technology*, 1987a, **5**, 147–151.
19. Gupta, P. K. and Durzan, D. J., *Plant Cell Rep.*, 1985, **4**, 177–179.
20. von Arnold, S. and Eriksson, T., *Can. J. Bot.*, 1981, **59**, 870–874.
21. Gupta, P. K. and Pullman, G. S., 1990, US Patent No. 4,957,886.
22. Becwar, M. R., Blush, T. D., Brown, D. W. and Chesick, E. E., *Plant Cell Tissue Org. Cult.*, 1991, **26**, 37–44.
23. Klimaszeweska, K. and Smith, D. R., *Physiol. Plant.*, 1997, **100**, 949–957.

ACKNOWLEDGEMENTS. We thank the staff at CRC (Shimla, HP) and Dr P. S. Ahuja (IHBT, Palampur, HP) for helping in collection of plant material. Financial support for the work provided by SIDA, Stockholm and DBT, New Delhi is duly acknowledged. We also thank CSIR for the fellowship provided to G.M.

Received 10 February 2000; revised accepted 17 July 2000