Somatic Embryogenesis in *Pinus roxburghii* Sarg.

Humyra Majeed, Azra N. Kamili and A. M. Shah

Centre of Research for Development, The University of Kashmir, Srinagar – 190 006, Jammu & Kashmir, India.

ABSTRACT

The somatic embryogenic process was investigated using mature zygotic embryos of *Pinus roxburghii* Sarg. A translucent mucilaginous embryogenic tissue was induced on half-strength MS medium supplemented with NAA (10μM). Histological examination confirmed the presence of early somatic embryos. To stimulate the maturation of these early somatic embryos, ABA (10μM) combined with sucrose (3 & 6%) was used which resulted in dessication of somatic embryos but no cotyledonary somatic embryos developed. The best results were obtained when the mature zygotic embryos were cultured in presence of NAA (10μM) and BAP (5μM). Cotyledonary somatic embryos developed were later on placed on germination medium and at present are under observation.

Keywords: Somatic embryogenesis, *Pinus roxburghii* Sarg., maturation, phyto-hormones.

Abbreviations: NAA – Napthalene acetic acid; BAP – 6 benzyl amino purine; MS (x ½) – Murashige and Skoog (half salt strength); ABA – Abscisic acid.

INTRODUCTION

Pinus roxburghii Sarg. (Chir Pine) is a slow growing, evergreen coniferous tree, widely distributed on the hills of Jammu, Punjab, H.P.,U.P. etc. It is an important coniferous species for timber, pulp and resin production.

The long life cycle and slow growth of this pine species slows the genetic gains obtained in traditional breeding programmes. For this reason, some alternative methods of tree improvement and rapid mass propagation are to be considered. With new biotechnologies being developed, the cell and tissue culture offers an opportunity for mass propagation of this pine species. Recently somatic embryogenesis is becoming one of the most efficient techniques for mass clonal propagation of the Pine species. Somatic embryogenesis is a process by which multiple embryos develop from one or more somatic (vegetative) cells in culture, leading to development of a bipolar structure possessing an embryonic head and elongated suspensors.

The genus *Pinus* in general is considered to be recalcitrant to somatic embryogenesis, however, considerable efforts have been made in this direction as is

evident from the published reports on different *Pinus* species like *Pinus lambertiana* (Gupta & Durzan, 1986), *P.radiata* (Chandler *et al.*, 1989), *P. caribaea* (Laine & David, 1990),*P. nigra* (Salajova & Salaj, 1992), *P. elliottii* (Tang *et al.*, 1997), *P.nigra* (Radojevic, 1999). The present work is an attempt in this direction and reports successful initiation and maturation of somatic embryos in *Pinus roxburghii* Sarg.

MATERIAL AND METHODS

Mature female cones were collected from trees located in S. Park (Shankaracharya Park), Srinagar in the months of September and October. Seeds were excised from cones and stored at 4°C for a chilling period of one month until the time of dissection. The chilled seeds were thoroughly washed in running tap water alongwith 0.5% v/v, Cedpol (detergent) & 1-2 drops ofTween –20 (surfactant). This was followed by their surface sterilization in 0.1% $HgCl_2$ for 15 minutes and then rinsing 3 times with sterile double distilled water. These seeds were kept for soaking in sterile double distilled water at 4°C for 36-40 hours in small flasks with their mouths properly closed. Mature zygotic embryos were aseptically dissected out at Laminar air flow and sterilized with 1% NaOCl for about 3-4 minutes, rinsed 2-3 times with autoclaved doubled distilled water and then inoculated on MS (x ½) medium (1962) supplemented with different growth adjuvants. The rest of the methodology followed was same as in our previous communications.

RESULTS

Somatic embryogenesis in embryos of *P.roxburghii* was achieved in following steps:

1. Embryogenic culture initiation
2. Embryogenic culture proliferation
3. Somatic embryo maturation
4. Somatic embryo germination

Two independent trials were given as under:

Trial 1: The culture requirements and the subsequent results in this trial are depicted in Table 1.

Table 1. Culture requirements and subsequent results of Somatic embryogenesis in *P.roxburghii* (Trial 1)

	Medium & Sucrose %	Growth regulators (µM)	Temperature (^{0}C) & Light	Results	
				Morphogenetic response*	Percentage response
Initiation	MS (x ½) 3	NAA (10)	22-25 dark (8 hrs)	Translucent, mucilaginous callus formation at radicle and hypocotyl regions	90
Proliferation	MS (x ½) 3	NAA (10)	22-25 dark (8 hrs)	Proliferation of callus and appearance of small mucilaginous nodules (5-6 per embryo)	100
Maturation	MS (x ½) 6	ABA(10)	22-25 dark 8(hrs)	--	
	MS (x ½) 3	ABA(10)	22-25 dark 8(hrs)	dessication of the embryogenic calli & development of white structures from mucilaginous nodules.	100

* Data scored after 4 weeks of culture period; Number of replicates 24

- No response.

Step-1 Initiation: For initiation of embryogenic tissue, mature zygotic embryos were cultured on MS (x ½) medium with NAA (10 µM). In the first week of culture period, the cotyledons turned green and the callus initiation occurred on radicle and hypocotyl regions of the embryo. The callus continued its proliferation, became translucent and mucilaginous in the fourth week of culture in about 90% cultures. (Table 1, Fig 1a) Acetocarmine squash preparation of this mucilaginous callus showed elongated suspensor cells(S) and clusters of highly cytoplasmic embryonic cells (EC) (Fig. 1b).

Step-2 Proliferation: These embryogenic calli were subcultured on medium used under Step-1, for maintenance and

proliferation. The calli became more mucilaginous and numerous dirty white small mucilaginous nodules apperared in the fourth week of subculture in 100% cultures. The number of nodules per embryo was 5-6 (Table 1, Fig. 1c).

Step-3 Maturation: For the maturation of somatic embryos, 50% of the calli were subcultured on MS (x ½) medium containing ABA (10 µM) + Sucrose (6%, as an osmoticum) and rest 50% on MS (x ½) medium containing ABA (10 µM) + Sucrose (3%) . It was observed that in the fourth week of subculture, no major change occurred in the cultures growing on sucrose (6 %), while as in all the cultures supplemented with 3 % sucrose, dessication of the embryogenic calli occurred but no cotyledonary somatic embryo formation was recorded (Table 1, Fig. 1d) Surprisingly the mucilaginous nodules turned into "White structures" (Fig. 1e). Germination trials could not be carried out because of altogether different response achieved in Step-3.

Trial 2: The culture requirements and the subsequent results in this trial are depicted in Table 2

Table 2. Culture requirements and subsequent results of Somatic embryogenesis in *P. rowburghii* (Trial 2)

	Medium & sucrose %	Growth regulators (µM)	Temperature (°C) & light	Results	
				Morphogenetic response*	Percentage response
Initiation	MS (x ½) 3	NAA (10) + BAP (5)	22-25 dark (8 hrs)	Slimy and yellowish embryogenic callus formation	75
Proliferation	MS (x ½) 3	NAA (10) + BAP (5)	22-25 dark (8 hrs)	Regeneration of Stage 1 & Stage 2 somatic embryos	30
Maturation	MS (x ½) 3	-	22-25 dark 8(hrs)	Cotyedonary somatic embryo formation (5 per callus)	20
Germination	MS (x ½) 3	-	22-25 dark 8(hrs)	Results awaited	

* Data scored after 4 weeks of culture period; Number of replicates 24

\- No growth harmones

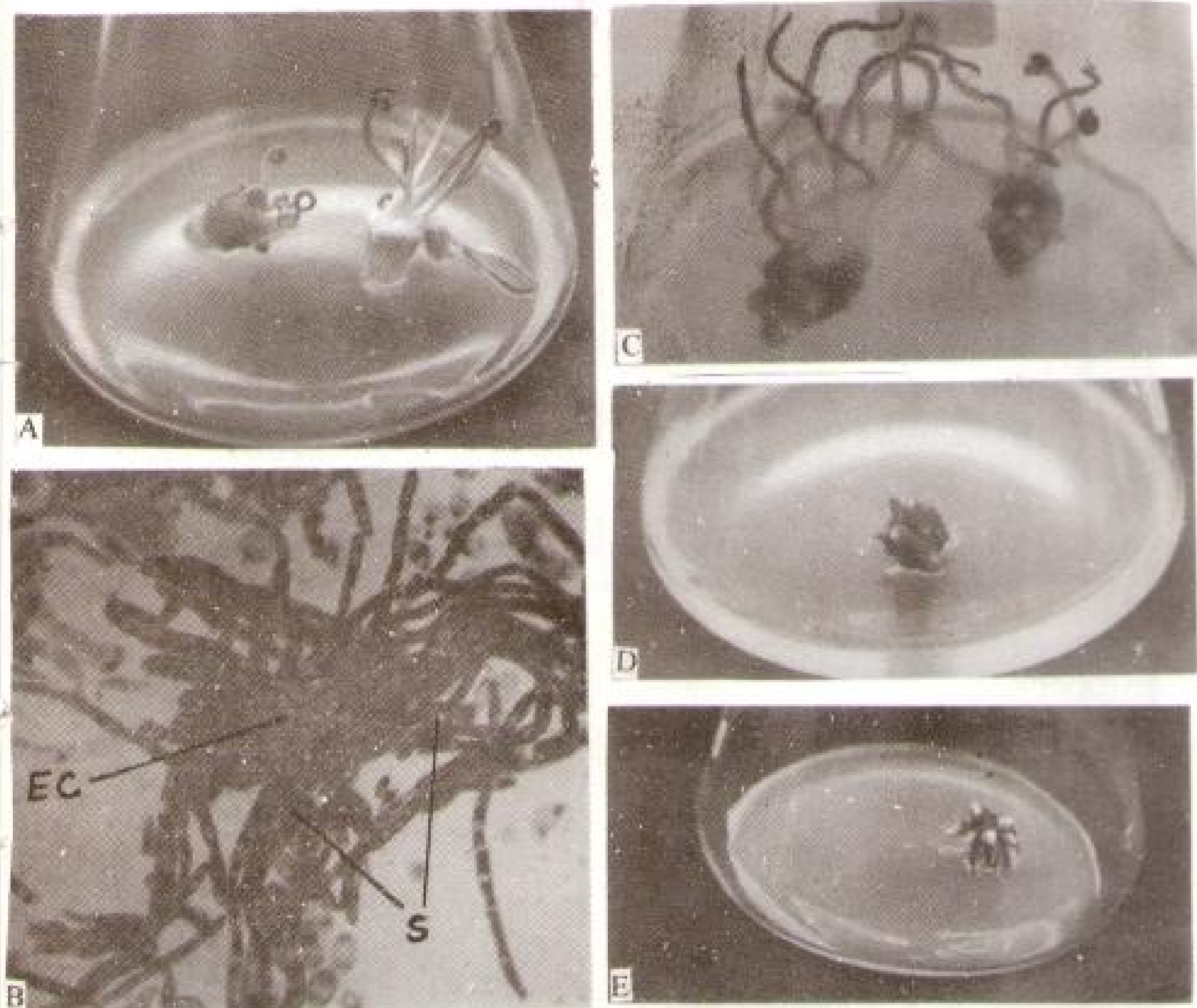

Fig 1 (a-e) Somatic embryogenesis in cultured embryos of *P.roxburghii*, (Trial I).

(a) Mucilaginous callus formation at radicle and hypocotyl regions on MS (x1/2) + NAA (10 μM) (after 4 weeks).

(b) Photomicrograph of mucilaginous tissue stained with acetocarmine depicting 2 kinds of cell populations – elongated suspensor cells (S) and clusters of highly cytoplasmic embryonic cells (EC).

(c) Mucilaginous dirtywhite nodules on the embryogenic callus (4 weeks after subculture).

(d) Dessication of embryogenic calli on MS (x1/2) + ABA (10 μM) + sucrose (3%) (after 4 weeks).

(e) Development of "White structures" from the mucilaginous nodules.

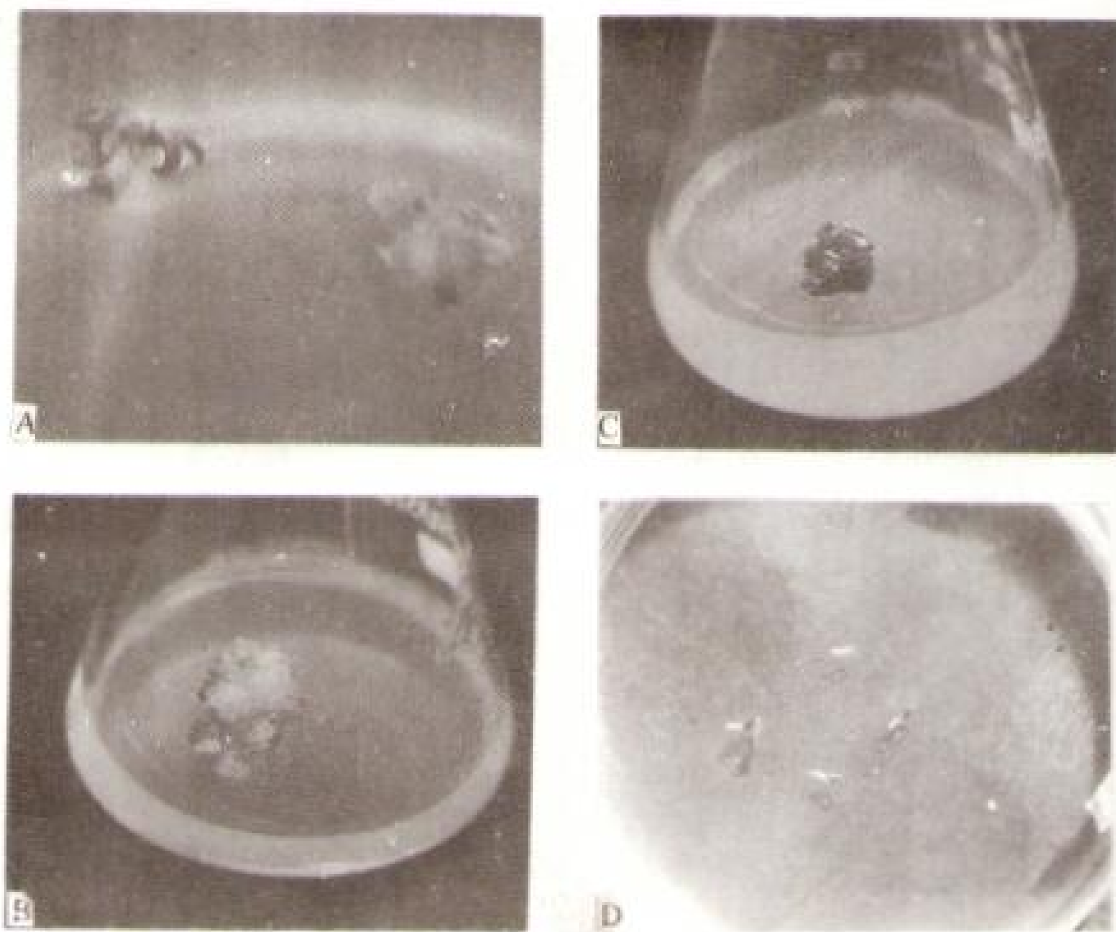

Fig 2 (a-d) Somatic embryogenesis in cultured embryos of *P. roxburghii*, (Trial 2).

(a) Slimy-yellowish callus formation all over embryo on MS (x ½) + NAA (10 µM) + BAP (5µM) (after 4 weeks)

(b) Regeneration of Stage 1 and Stage 2 somatic embryos (4 weeks after subculture).

(c) Cotyledonary somatic embryo formation on MS (x ½) basal medium (after 4 weeks).

(d) Somatic embryos on MS (x ½) basal medium for germination.

Step-1 Inititation: For initiation of embryogenic tissue, mature zygotic embryos were cultured on MS (x ½) medium fortified with NAA (10 μM) + BAP (5μM). In the first week of culture period, friable embryogenic callus formation occurred on the whole embryo but later in the fourth week, it turned slimy and yellowish in about 75% cultures (Table 2, Fig. 2a).

Step- 2 Proliferation: The calli were subcultured on the same medium for proliferation. After 4 weeks of subculture, the calli expressed their embryogenic potential and regenerated stage 1 and Stage 2 of somatic embryos in 30% cultures (Table 2, Fig. 2b).

Step 3 Maturation: For maturation of these somatic embryos (Stage 1 and 2), these calli were subcultured on MS (x ½) basal medium. Codtyleonary somatic embryo formation (5 per callus) was observed after 4 weeks of subculture in about 20% cudltures (Table 2, Fig.2c).

Step 4 Germination: The somatic embryos were **isolated** and placed on MS (x ½) basal medium for germination (Fig. 2d). Further results are awaited.

DISCUSSION

The present studies revealed that, mucilaginous embryogenic callus formation occurred from mature zygotic embryos cultured on MS medium supplemented with NAA (10 μM). This observation is in contrast with that of Chandler and Young (1995) who recorded the non-embryogenic callus formation in *Pinus radiata* on a medium supplemented with NAA. Further in present studies, for maturation of the somatic embryos, the embryogenic calli were transferred to a medium containing ABA (10 μM) + Sucrose (6%) which did not help in maturation or cotyledon differentiation and hence are contrary to those of Bercetche and Paques (1995) who also used medium containing ABA (15 μM) + Sucrose (6%) for maturation of somatic embryos in *Pinus pinaster* and reported cotyledon differentiation from these somatic embryos. In yet another trial on somatic embryogenesis, it was observed that the embryos cultured on MS medium fortified with NAA (10 μM) + BAP (5μM) proliferated to form an embryogenic callus which when subcultured on the same medium gave rise to somatic embryos. This observation is in conformity with that of Radojevic *et al.* (1999) who also used the combination of NAA and BAP for raising

the embryogenic cultures from mature zygotic embryos of *Pinus nigra*. However this result is not in agreement with those of Bozhkov *et al.* (1997); Tang *et al.* (1997) who instead of NAA used 2,4-D at varying concentrations for raising embryogenic cultures in *Pinus koraiensis* and *Pinus elliottii*. In present studies, the initiation of embryogenic calli was carried under light conditions which is in conformity with the observations of Hohtola (1995) who also recorded initiation and proliferation of embryogenic calli under light conditions in *Pinus sylvestris*. But it is contradictory to that of Kaul (1995); Newton *et al.* (1995) who recorded somatic embryogenesis under dark conditions in *Pinus strobus* and *Pinus elliottii*.

From the present results, it is concluded that there exists a great potential for micropropagation of *Pinus roxburghii* via somatic embryo formation as the same were recovered from embryogenic callus obtained after embryo culture.

ACKNOWLEDGEMENTS

The authors wish to thank MoE and Forests, New Delhi, for the financial support. Thanks are also due to Director, CORD for providing facilities to carry out these investigations.

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