Callus Formation and Shoot Bud Induction in Embryo Cultures of *Pinus roxburghii* Sarg

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ABSTRACT

Mature zygotic embryos of *Pinus roxburghii* Sarg were used as explants for establishing tissue cultures on half salt-strength MS medium (1962) supplemented with different auxin-cytokinin combinations. A combination of NAA (10μM) and BAP (10μM) favoured callus formation in the embryos. When the concentration of BAP was reduced to 5μM, again it resulted in callus formation. However, when the concentration of NAA was reduced to 5μM, and that of BAP was kept constant i.e., at 10μM, induction of shoot buds and small shoots was observed at the tip of embryonic cotyledons in 20% cultures.

Keywords: *Pinus roxburghii* Sarg, shoot bud induction, callus initiation, embryo culture.

Abbreviations: MS (x ½) - Murashige and Skoog (half salt-strength); NAA - Naphthalene acetic acid; BAP - 6 benzyl amino purine

INTRODUCTION

Pinus roxburghii Sarg, also known as Chir pine, belongs to family Pinaceae. It is a good source of timber, paper pulp and turpentine oil. The usual method of propagation is through seeds and cuttings. Being a slow grower and difficult to propagate by cuttings, it becomes important to use some alternative method of propagation. *In vitro* regeneration technique for this plant species may result in the production of multiple number of plants in a short period as is evident from the published reports on conifers (Sommer and Brown, 1979; Durzan, 1980; Smith, 1986; Amerson *et. al.*, 1988; Gleed *et. al.*, 1995; Paques and Cremiere, 1966 and Flygh *et. al.*, 1998). The present report deals with observations on morphogenetic response of excised mature zygotic embryos of *Pinus roxburghii* Sarg *in vitro* and successful shoot bud induction thereof.

MATERIAL AND METHODS

Cones were collected from Shankaracharya Park, Srinagar in the month of October. Seeds were separated from these cones and stored in a refrigerator for one month prior to the excision of embryos. Seeds were thoroughly washed with a

detergent cedpol (0.5% v/v) to which 1-2 drops of tween-20 (Surfactant) were added. This was followed by washing of seeds with running tap water for 5 minutes. The seeds were then surface sterilized with 0.1%$HgCl_2$ solution for about 15-16 minutes, washed 4 – 5 times with autoclaved double distilled water and kept for 36-40 hour soaking in a flask containing autoclaved double distilled water. The mouth of the flask was properly closed before placing in a refrigerator. These were again given a mild treatment of 0.01% $HgCl_2$ for 3-4 minutes and rinsed thoroughly 4-5 times with autoclaved double distilled water before they were aseptically dissected. The embryos taken out were given a dip in 1% NaOCl (Sodium hypochlorite) solution for about 3-4 minutes, rinsed thoroughly 2-3 times with autoclaved double distilled water and then inoculated on MS (x ½) medium (1962) supplemented with 3% sucrose, different auxin-cytokinin combinations and gelled with 0.8% agar. The pH of the medium was adjusted at 5.5 – 5.6 with 0.1 N NaOH or 0.1 N HCl. About 40 ml of the prepared medium was dispensed in each of 100 ml culture vials which were properly plugged. Medium was autoclaved at 15 lb pressure (temperature 121^{0}C) for 20 minutes. After inoculation, the cultures were incubated in culture room at a temperature of 25±3^0 C and a relative humidity ranging from 50-65% under cool white fluorescent light (3000 lux) at day-night regime of 16 hours photo period.

RESULTS

The effect of the auxin-cytokinin combinations on zygotic embryos of *Pinus roxburghii* is summarized in Table 1. MS medium supplemented with NAA (10μM) and BAP (10μM) favoured moderate callus formation of the embryos. The embryos started showing the response in the first week of culture period. The cotyledons opened and retained their original creamish colour. During the second week, callus initiation was observed on the radicle and the hypocotyl region of the embryo. The callus continued its proliferation and became friable and shining in the fourth week of culture in about 80% cultures (Fig.1). With the decrease in concentration of BAP from 10μM to 5μM, callus continued to proliferate but the initiation of callus was from radicle, hypocotyls and cotyledons. There was observed a change in colour of the callus which was initially light green and later on changed to reddish in colour. The callus was again friable but callus from hypocotyl and radicle region turned slimy and white in about 75% cultures. (Fig.2). When the medium was supplemented with 5μM

Table 1. Effect of auxin-cytokinin combinations on the zygotic embryos of *Pinus roxburghii*

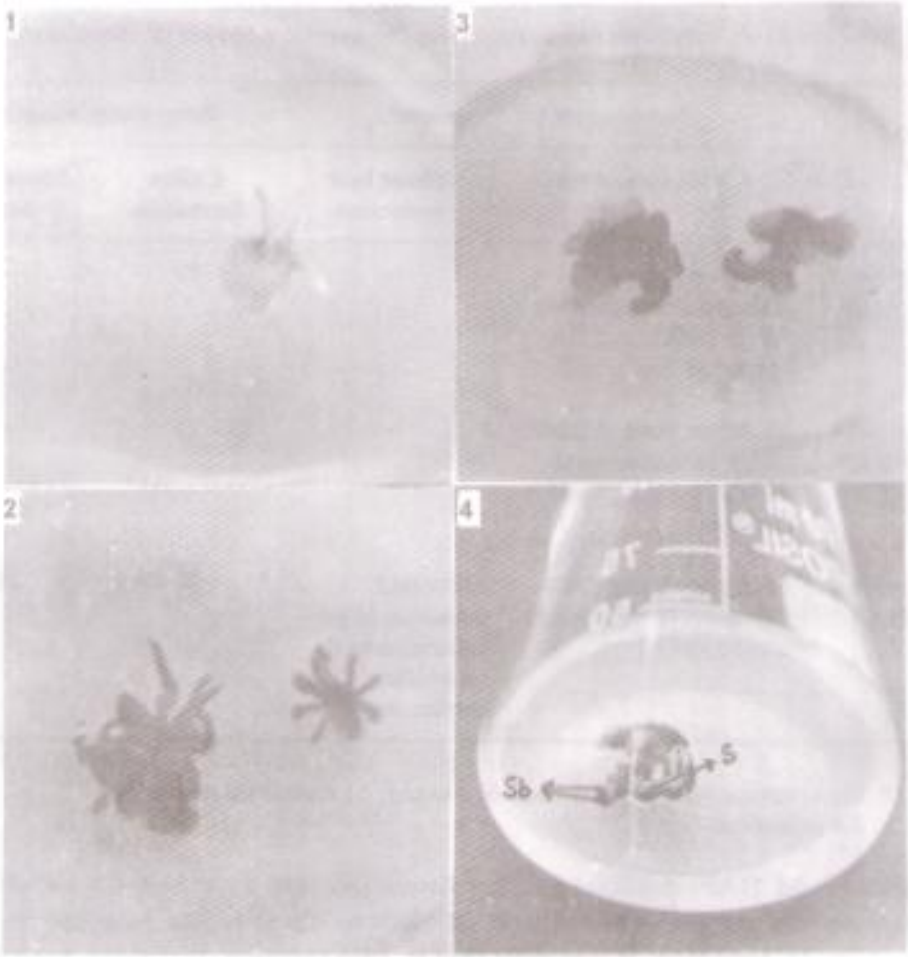
Growth medium	Morphogenetic Response*		Percentage Response	
	Callus formation	Shoot bud induction	Callus formation	Shoot bud induction
MS (x½)+NAA (10μM) +BAP (10μM)	Moderate, yellowish friable callus formation on the radicle and hypocotyl	-	80	-
MS (x½)+NAA (10μM) +BAP (5μM)	Moderate, reddish friable callus formation on the radicle, hypocotyl and cotyledon	-	75	-
MS (x½)+NAA (5μM) +BAP (10μM)	Moderate friable callus formation on the radicle and hypocotyl	Shoot bud induction (6 per culture) directly on swollen cotyledons	70	20

* Observations recorded at the end of 4 weeks. 12 replicates/ treatment
\- No induction

NAA and 10μM BAP, moderate callus formation was favoured on radicle and hypocotyl region of embryo but the cotyledons turned into needles in 70% cultures (Fig. 3). However, it was seen that in 20% cultures, there was poor callus formation on the radicle and hypocotyl. The cotyledons swelled and turned green in colour. This was followed by emergence of small shoot buds and 1mm-2mm long shoots at the tips of these swollen green cotyledons (Fig. 4). Some of the cultures are at present on the proliferation medium and trials to elongate shoot buds and shoots are in progress.

DISCUSSION

The present investigations have shown that the embryo responded differently to auxin-cytokinin combinations. It was observed that when the concentration of NAA was equal to that of BAP (10μM), there was only callusing and no shoot bud induction was observed. Use of this particular concentration of phytohormones in embryo cultures has not been reported so far in *Pinus* species but overall effectiveness of BAP alongwith NAA in inducing shoot buds in



Figs. 1-4 Morphogenetic response of embryos of *P. roxburghii* to different phytohormonal concentrations.

1. **Formation of yellowish friable callus on MS(x ½) + NAA (10μM) + BAP(10μM)**
2. **Formation of Reddish friable callus on MS(x ½) + NAA (10μM) + BAP(5μM)**
3. **Formation of Needles and friable callus on MS(x ½) + NAA (5μM) + BAP(10μM)**
4. **Induction of Shoot buds (Sb) and Shoots (S) at the tip of cotyledons on MS(x ½) + NAA (5μM) + BAP(10μM)**

embryo cultures of various *Pinus* species has been reported by Sommer *et. al.* (1975), Minocha (1980), Floh and Handrow (1986) and Vargas and Gonzatez (1992). In present studies callus formation was again favoured to get initiated and proliferated when concentration of BAP was decreased to 5μM and that of NAA was kept unchanged. This callus also failed to show shoot bud induction which is again contradictory to the observations of Bhatnagar *et al.* (1983), where shoot bud induction in *Pinus roxburghii* has been reported on swollen cotyledons in 60% cultures. In the current experiment, however, when the concentration of BAP was kept constant (10μM) and that of NAA decreased to 5μM, shoot bud

induction was observed in 20% cultures. This result is in conformity with that of Kaya and Gokce (1997) in *Pinus nigra*. However, Franco (1983) reported the same results in *Pinus oocarpa* but on a reduced concentration of NAA (2.5µM). Multiple shoots obtained from such shoot buds after elongation can be highly beneficial for producing true to type plants after successful rooting. Presently trials for elongation of shoo: buds and shoots are in progress.

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