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Plantlet regeneration from fascicular buds of seedling shoot apices of *Pinus roxburghii* Sarg

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Abstract

Shoot apices of *Pinus roxburghii* Sarg were cultured on Murashige and Skoog's medium (MS) supplemented with cytokinins [6-benzyladenine (BA), kinetin and N-benzyl-9-(2-tetrahydropyranyl) adenine (BPA)] alone and in combination with auxin, α-napthaleneacetic acid (NAA). Of the three cytokinins tested at varying concentrations, medium supplemented with 10 μM BA was found optimal in respect of explant responsiveness (97.22 %) and average number of buds induced per explant (7.42). The concentration of cytokinins in the induction medium had a profound effect on rate of elongation of induced buds on MS basal medium containing 0.5 % activated charcoal. Further, shoots induced on lower concentrations of BA increased up to 2.4 times in length in 4 weeks. Decapitation of the explant enhanced the rate of axillary bud elongation. Proliferating shoot cultures were established by sub-culturing the axillary shoots on MS supplemented with 10 μM BA. Shoots 2 - 3 cm in length were suitable for culturing as more buds were induced on them compared to longer or shorter shoots. Root primordia were induced on 70.83 % shoots when transferred to ½ MS medium supplemented with 5.0 μM NAA. Elongation of root primordia (60 %) was achieved in liquid ½ MS basal medium. The plantlets were successfully transferred to soil after hardening; the time period from initiation of shoot buds to transplantation being 20 - 22 weeks.

Additional key words: apical bud, auxins, axillary bud, chir pine, cytokinins, micropropagation, seedling.

Introduction

The genus *Pinus* belonging to family *Pinaceae* comprises nearly 94 species distributed in the northern hemisphere. *Pinus roxburghii* Sarg syn. *P. longifolia* commonly known as long leaf pine or chir pine is the most important pine among the six indigenous pines of Indian subcontinent. Micropropagation of conifers using embryonic and seedling explants has been reported successfully for numerous species through somatic embryogenesis, adventitious bud induction and axillary bud proliferation. Successful regeneration of *Pinus roxburghii* through adventitious bud induction (Murithii *et al.* 1994, Kalia *et al*. 2001), axillary bud proliferation (Parasharmi *et al*. 2003) and somatic embryogenesis (Arya *et al.* 2000, Mathur *et al.* 2000) has been reported. However, fascicular bud proliferation from seedling explants has not been reported yet. *In vitro* induction of axillary buds capable of forming shoots is a widely used method of propagation of flowering plants and forest trees. The clones derived from axillary buds maintain genetic stability with less risk of mutation that may arise during organogenesis from callus (Pierik 1987, Lin *et al*. 1991). Keeping in view the silvicultural importance of *P. roxburghii* and potential applications of micropropagation, the present study was undertaken with an objective to develop a method for induction of multiple shoots and plantlet regeneration from juvenile *i.e.* three-week-old seedling apical buds of chir pine.

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Abbreviations: BA - 6-benzyladenine; BPA - N-benzyl-9- (2-tetrahydropyranyl) adenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog's medium (1962); NAA - α-napthaleneacetic acid.

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Materials and methods

Half sib seeds of *P. roxburghii* were collected from trees possessing desirable silvicultural traits. Seeds were surface sterilized with 0.1 % $HgCl_2$ solution for 20 min followed by 10 % (m/v) hydrogen peroxide for 10 min. Sterilized seeds were rinsed thoroughly with sterile distilled water and inoculated on agar (0.8 %) and sucrose (3.0 %) medium without mineral salts for germination. Seeds germinated after 10 - 14 d of culture and developed into seedlings after three to four weeks. Shoot apices 1.5 - 2.0 cm in length along with cotyledons trimmed to a length of 1.0 cm were excised from three-week-old germinated seedlings and used as explants.

Shoot apices (1.5 - 2.0 cm) from *in vitro* germinated seedlings were cultured on Murashige and Skoog's (MS) medium (1962) supplemented with plant growth regulators for induction of fascicular buds. Effect of cytokinins 6-benzyladenine (BA), kinetin and N-benzyl-9-(2-tetrahydropyranyl) adenine (BPA) added singly to the medium was assessed at concentrations ranging from 5.0 - 25.0 μM. Combination of BA (5.0 - 25.0 μM) was tried with α-napthaleneacetic acid (NAA) at 0.50 μM and 2.5 μM concentration in the second set of experiment.

For elongation, explants with induced buds were cultured on basal medium (MS) supplemented with 0.5 % activated charcoal. Effect of decapitation of apical buds on fascicular shoot elongation was also investigated. In one set of experiment, effect of induction medium (5 - 25 μM BA) on elongation of induced axillary buds was also studied. The explants initially cultured on BA supplemented media were transferred to MS medium supplemented with 0.5 % activated charcoal and elongation was monitored for 8 - 10 weeks with subculture to fresh medium every 4 weeks.

Multiplication of *in vitro* raised axillary shoots was standardized by culturing them on MS medium supplemented with BA, kinetin and BPA alone at 5.0 - 25.0 μM concentrations and combination of BA (5.0 - 25.0 μM) and NAA (0.50 μM, 2.5 μM). Elongated axillary shoots of different lengths (< 1 cm, 1 - 2 cm, 2 - 3 cm and > 4 cm) were excised and cultured on MS medium supplemented with 10 μM BA to study the effect of size of *in vitro* raised axillary shoots on fascicular bud induction potential during subsequent shoot multiplication cycles.

For rooting, 2.0 - 3.0 cm long shoots were transferred to ½ MS medium supplemented with 2.5 - 7.5 μM NAA, indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) added singly, 2.0 % sucrose and 0.6, 0.7 or 0.8 % agar. The shoots with induced root primordia were transferred to semisolid (gelled with 0.6 % agar) and liquid ½ MS medium with filter paper bridges for elongation of roots. The rooted plantlets were washed thoroughly and transferred to liquid ¼ MS medium containing 1 % sucrose and absorbent cotton as support for hardening. Plantlets were placed in this medium for 15 - 20 d. For acclimatization, these hardened plantlets were transferred to polybags containing sand, soil and farmyard manure in 1:1:1 ratio and were supplied with ¼ MS medium without vitamins. Initially the plantlets were covered with perforated polybags to retain moisture and were kept in shade. After 1 week, the perforated polybags were removed for 1 - 2 h daily, gradually the time was increased to 5 - 6 h and then to 8 - 10 h daily. Finally, the polybags were permanently removed. After one month, acclimatized plantlets were transferred to pots containing a mixture of soil, vermiculite and farmyard manure in 1:1:1 ratio.

MS medium fortified with 3 % sucrose and 0.8 % agar was used in this study unless and otherwise stated. The pH of all the media was adjusted to 5.8 prior to autoclaving at 104 kPa for 15 min at 121 °C. The explants were cultured in 100 cm^3 conical flasks containing 30 cm^3 medium. All the cultures were maintained at 25 ± 2 °C and 16-h photoperiod with an irradiance of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. During hardening, the plantlets were maintained under culture room conditions with humidity of approximately 70 % while during acclimatization, the plantlets were shifted to net house and high humidity levels were maintained by covering them with perforated polybags.

For each experiment, 20 explants per treatment were used and each experiment was repeated twice. Observations were made fortnightly and treatment effects were quantified based on responding explants percentage and the average number of buds and length of shoots per explant. Elongation *i.e*. increase in shoot and root length was calculated as a ratio of enhanced length (final minus initial) to the initial length. The length of buds was measured using micrometer scale on *Nikon* stereozoom microscope and length of shoots was measured by graph paper. The data was subjected to univariate analysis of variance using *SPSS 10.0* software. Post hoc analysis of data was performed by Scheffe's method. In results presented in tables, values with same alphabet as superscript are not significantly different at 5 % level.

Results

Induction of fascicular buds: Multiple buds were induced in the axils of needles present on shoot apices within 3 - 4 weeks of culture on cytokinin-supplemented media (Fig. 1*A*,*B*). Explants cultured on basal medium failed to develop axillary buds but instead exhibited copious elongation. Among the cytokinins tested, BA was more effective compared to BPA and kinetin (Table 1). The most effective concentration of BA was 10 μM on

which an average of 7.42 buds were induced on 97.22 % cultured explants. Higher concentrations of BA (20 and 25 μM) in the medium, led to the development of buds in the axils of cotyledons of some explants. However, bud to shoot conversion percentage of these buds was low on transfer to elongation medium where the buds remained stunted and clustered. Development of secondary and tertiary buds (buds developing in the axils of prophylls of primary and secondary buds) was also recorded in some explants at these BA concentrations. Explants cultured on kinetin and BPA supplemented media developed fewer fascicular buds than those cultured on BA alone (Table 1). Moreover, few explants cultured on kinetin-enriched media developed abnormal buds in the axils of needles, which failed to develop into shoots. The bud length increased with increasing concentration of the cytokinin up to the optimal level and declined thereafter in each of the hormone tested.

Combination of BA and NAA was deleterious with significant decline in percentage of responding explants as well as average number of buds induced compared to media supplemented with BA alone (Table 1). Moreover, most of the explants cultured on NAA supplemented media exhibited development of callus at their bases with enhanced vitrification of induced buds. Thus, BA when added singly in the medium was the most effective plant growth regulator indicating the cytokinin specificity of *P. roxburghii* seedling explants for multiple bud induction.

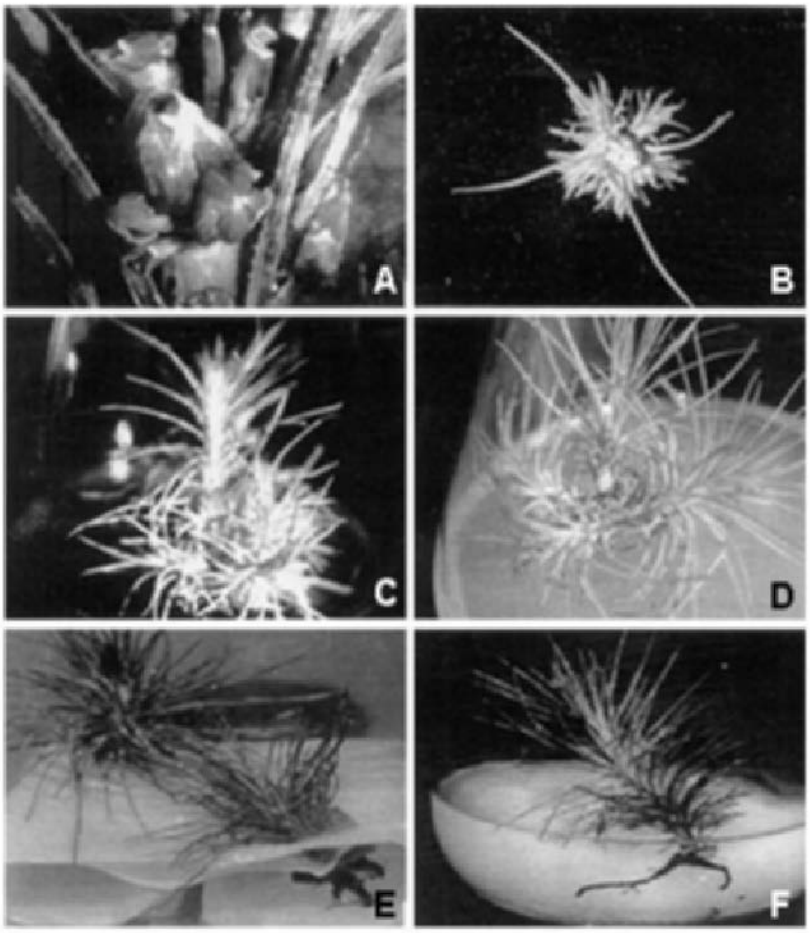

Fig. 1. *In vitro* multiplication of *Pinus roxburghii* Sarg through axillary bud proliferation from seedling shoot apices. *A* - Induction of shoot buds in needle axils after 3 weeks; *B* - decapitated explant showing axillary position of induced buds; *C* - elongation of induced axillary shoot buds on MS basal medium supplemented with 0.5 % activated charcoal (after 6 weeks of transfer to elongation medium); *D* - elongation of induced axillary shoot buds of decapitated explant on MS basal medium (after 5 weeks of transfer on elongation medium); *E* - elongation of root primordia on filter paper bridge in liquid ½ MS basal medium (after 3 weeks of transfer); *F* - hardening of *in vitro* raised plantlets in liquid ¼ MS medium containing cotton as support (after 3 weeks of transfer).

Elongation of buds: The shoot buds developing in the axils of needles were small in size and were subcultured on hormone free MS medium supplemented with 0.5 % activated charcoal for elongation, *i.e*. conversion into shoots (Fig. 1*C*). The development of shoots was further enhanced by decapitation of the mother explant (Fig. 1*D*). The excised tip of the explant was re-cultured on the basal medium for elongation and further multiplication.

Table 1. Effect of cytokinins and cytokinin-auxin combinations supplemented in MS medium on induction of axillary buds on seedling shoot apices of *P. roxburghii*. Data recorded after 4 weeks of culture. Means ± SD, $n = 20$.

Cyto-kinin	Conc. [μM]	NAA [μM]	Responding explants [%]	Number of buds
BA	5	0	87.50 ± 4.17^{ab}	5.22 ± 0.20^{cd}
	10	0	97.22 ± 4.81^{a}	7.42 ± 0.40^{a}
	15	0	93.05 ± 2.41^{a}	6.72 ± 0.43^{ab}
	20	0	90.28 ± 6.36^{ab}	5.42 ± 0.31^{bc}
	25	0	81.94 ± 6.36^{ab}	4.81 ± 0.27^{cdef}
KIN	5	0	48.61 ± 6.16^{b}	2.36 ± 0.22^{i}
	10	0	62.50 ± 7.21^{ab}	3.04 ± 0.36^{ghi}
	15	0	63.89 ± 8.67^{ab}	3.55 ± 0.41^{efghi}
	20	0	66.67 ± 4.17^{ab}	3.71 ± 0.49^{efghi}
	25	0	63.89 ± 2.41^{ab}	3.08 ± 0.14^{ghi}
BPA	5	0	73.61 ± 6.36^{ab}	2.75 ± 0.13^{hi}
	10	0	79.17 ± 7.22^{ab}	3.11 ± 0.18^{ghi}
	15	0	81.94 ± 10.48^{ab}	3.18 ± 0.11^{ghi}
	20	0	81.94 ± 6.36^{ab}	3.66 ± 0.14^{efghi}
	25	0	70.83 ± 8.33^{ab}	2.86 ± 0.30^{hi}
BA	5	0.5	76.38 ± 12.03^{ab}	3.94 ± 0.24^{defgh}
	10	0.5	88.88 ± 4.81^{ab}	4.92 ± 0.23^{cde}
	15	0.5	83.33 ± 11.02^{ab}	4.42 ± 0.27^{cdefg}
	20	0.5	81.94 ± 10.48^{ab}	3.65 ± 0.11^{efghi}
	25	0.5	75.00 ± 8.33^{ab}	4.01 ± 0.12^{cdefgh}
BA	5	2.5	72.22 ± 2.41^{ab}	3.75 ± 0.36^{efghi}
	10	2.5	70.83 ± 11.02^{ab}	4.71 ± 0.22^{cdef}
	15	2.5	62.50 ± 11.02^{ab}	4.15 ± 0.10^{cdefgh}
	20	2.5	63.89 ± 9.62^{ab}	3.56 ± 0.26^{efghi}
	25	2.5	61.11 ± 12.73^{ab}	3.48 ± 0.17^{fghi}

Induction medium was found to have a profound effect on shoot development. Shoots induced on lower concentrations of BA (5 - 15 μM) showed better elongation rates when transferred to elongation medium compared to those induced on higher BA concentrations (20 - 25 μM) which remained stunted (Table 2). More than 2.4-fold increase was observed for shoots induced on 10 μM BA. The elongated shoots were excised after 4 - 8 weeks of culture and the explants were cultured again on fresh basal medium for elongation of the remaining smaller shoot buds. Some of the explants cultured on higher BA concentrations (≥ 20 μM) when transferred to basal medium for elongation developed 1 - 3 more buds after 3 - 4 weeks.

Table 2. Effect of 5 - 25 μM BA in induction medium on elongation of axillary buds (on MS medium supplemented with 0.5 % activated charcoal) induced on seedling shoots apices of *P. roxburghii*. Data recorded after 4 weeks. Means ± SD, $n = 20$.

BA [μM]	Initial bud length [mm]	Final bud length [mm]	Fold increase
5	3.75 ± 0.24	12.20 ± 0.46	2.25^{a}
10	3.95 ± 0.28	13.45 ± 0.60	2.40^{a}
15	3.10 ± 0.25	9.65 ± 0.47	2.11^{a}
20	2.65 ± 0.25	7.60 ± 0.39	1.86^{a}
25	2.11 ± 0.14	4.45 ± 0.29	1.10^{b}

Multiplication of *in vitro* raised shoots: The elongated axillary shoots and decapitated shoot tips were cultured on cytokinin or cytokinin-auxin combination supplemented media for multiplication. It was found that the *in vitro* raised shoots exhibited a response similar to those of seedling explants towards different cytokinins (BA, BPA and kinetin). BA at 10 μM concentration was the most potent cytokinin inducing an average of 9.71 buds on 98.61 % explants. Addition of NAA was deleterious in the multiplication phase also as it resulted in reduced recovery of shoots. Study of effect of explant size on rate of shoot multiplication showed that 2 - 3 cm long shoots were most responsive towards multiplication with an average of 8.48 buds induced on 93.06 % explants followed by 1 - 2 cm and 3 - 4 cm shoots on which an average of 7.83 and 7.42 buds were induced on 83.33 and 91.67 % explants respectively (Table 3). Shoots shorter than 1 cm and longer than 4 cm were least responsive towards *in vitro* conditions. The stumps left after excision of axillary buds when cultured on BA supplemented media produced more buds. This method was highly useful to raise additional shoot crop, thus enhancing the rate of shoot multiplication.

Table 3. Effect of length of shoot cultured on MS medium supplemented with BA (10 μM) on shoot multiplication. Data recorded after 4 weeks of culture. Means ± SD, $n = 20$.

Explant size [cm]	Responding explants [%]	Number of buds
< 1	69.44 ± 6.36^{c}	6.19 ± 0.92^{c}
1 - 2	83.33 ± 4.17^{ab}	7.83 ± 0.49^{ab}
2 - 3	93.06 ± 6.37^{a}	8.48 ± 0.33^{a}
3 - 4	91.67 ± 4.17^{a}	7.42 ± 0.58^{ab}
> 4	75.00 ± 7.22^{bc}	7.19 ± 0.53^{bc}

Rooting and transplantation: Rooting was observed at low frequency on shoots cultured on basal medium during the elongation phase. An average of 7.44 root primordia developed at the base of 70.83 % shoots on ½ MS medium supplemented with 5.0 μM NAA and 0.6 % agar (Table 4). IBA at 5.0 μM concentration induced 5.59 root primordia at the base of 58.33 % shoots compared to a maximum of 4.83 root primordia developing at the base of 38.89 % shoots cultured on 7.5 μM IAA supplemented media. Thus, NAA proved better among the three auxins tested. The concentration of agar added in the medium played an important role because the shoots cultured on media gelled with 0.8 and 0.7 % agar produced less number of root primordia at the

Table 4. *In vitro* induction of root primordia on auxin-supplemented media gelled with different agar concentrations. Data recorded after three weeks of culture. Means ± SD, $n = 20$.

Auxin	Conc. [μM]	Agar conc. [%]	Responding explants [%]	Number of root primordial
NAA	2.5	0.8	34.72 ± 6.37^{bcd}	4.08 ± 0.45^{cdef}
		0.7	44.44 ± 6.36^{abcd}	4.42 ± 0.13^{cdef}
		0.6	51.39 ± 10.48^{abcd}	4.78 ± 0.18^{bcdef}
	5.0	0.8	48.61 ± 8.67^{abcd}	5.74 ± 0.31^{abc}
		0.7	63.89 ± 6.36^{ab}	6.38 ± 0.23^{ab}
		0.6	70.83 ± 11.02^{a}	7.44 ± 0.21^{a}
	7.5	0.8	40.28 ± 6.37^{abcd}	5.74 ± 0.37^{abc}
		0.7	48.61 ± 2.41^{abcd}	5.95 ± 0.64^{abc}
		0.6	56.94 ± 6.36^{abc}	6.44 ± 0.50^{ab}
IBA	2.5	0.8	29.17 ± 4.17^{cd}	3.72 ± 0.40^{def}
		0.7	40.28 ± 2.41^{abcd}	4.10 ± 0.29^{cdef}
		0.6	48.61 ± 6.36^{abcd}	4.78 ± 0.17^{bcdef}
	5.0	0.8	36.11 ± 4.82^{bcd}	4.84 ± 0.57^{bcdef}
		0.7	45.83 ± 4.17^{abcd}	5.53 ± 0.28^{abcde}
		0.6	58.33 ± 4.17^{abc}	5.59 ± 0.17^{abcd}
	7.5	0.8	26.39 ± 6.36^{cd}	4.72 ± 0.47^{bcdef}
		0.7	36.11 ± 6.36^{bcd}	5.31 ± 0.18^{bcde}
		0.6	40.28 ± 6.37^{abcd}	5.39 ± 0.76^{bcde}
IAA	2.5	0.8	19.45 ± 4.81^{d}	3.36 ± 0.13^{f}
		0.7	23.61 ± 6.36^{d}	4.24 ± 0.46^{cdef}
		0.6	27.78 ± 6.37^{cd}	4.71 ± 0.09^{bcdef}
	5.0	0.8	26.39 ± 2.41^{cd}	3.63 ± 0.11^{ef}
		0.7	29.17 ± 4.17^{cd}	4.31 ± 0.19^{cdef}
		0.6	34.72 ± 4.81^{bcd}	4.45 ± 0.30^{cdef}
	7.5	0.8	30.55 ± 8.67^{cd}	4.46 ± 0.28^{cdef}
		0.7	34.72 ± 4.81^{bcd}	4.72 ± 0.17^{bcdef}
		0.6	38.89 ± 2.41^{abcd}	4.83 ± 0.35^{bcdef}

Table 5. Effect of type of elongation medium on growth of root primordia induced at the base of *in vitro* raised shoots of *P. roxburghii* (cultured on ½ MS medium supplemented with different auxins). Data recorded after 4 weeks. Means of 20 shoots.

Induction medium	Elongation medium	Root elongation [%]	Fold increase
2.5 μM NAA	liquid	60	3.91^{a}
	semisolid	15	1.53^{b}
2.5 μM IBA	liquid	45	2.25^{b}
	semisolid	5	0.93^{c}

base of shoots with profuse callusing and no further development of these primordia into roots. Addition of 0.6 % agar instead resulted in very low or no callusing at the base of shoots. Elongation of root primordia into roots was observed in 60 % shoots with 4 fold increase in length in liquid basal medium with filter paper bridges (Table 5, Fig. 1*E*) while only 5 - 15 % shoots showed 2 - 3 fold elongation of root primordia when transferred to semisolid ½ MS basal medium.

The *in vitro* raised plantlets required hardening and acclimatization before field transfer as the non-hardened plantlets died soon after field transfer. The root system developed further in the hardening medium and elongated to 6 - 8 cm (Fig. 1*F*). The rooted plantlets after acclimatization were finally transferred to pots containing soil, vermiculite and farmyard manure in 1:1:1 ratio. The plantlets resumed growth and showed 80 % survival. The regenerated plantlets showed normal growth and morphological characteristics.

Discussion

Clonal propagation of superior selected trees is desirable in silviculture. However, the forest trees are usually difficult to propagate from mature explants, therefore, most of the studies have been done using juvenile plant material in conifers (Horgan and Aitken-Christie 1981, Baxter *et al*. 1989, Burns *et al*. 1991, Kalia *et al*. 2001, Kalia 2002, Salajová and Salaj 2005). In the present endeavour, seedlings raised from half sib seeds collected from plus trees were used for standardization of axillary bud multiplication in *Pinus roxburghii*. Loss of competence of mature explants has been attributed to progressive specialization of the tissue, which reduces the plasticity and capability of the cells to dedifferentiate (Abdullah *et al*. 1987). Moreover, many biochemical changes occur during culture of mature tissues resulting in rapid tissue browning followed by deterioration of cellular ultrastructure and necrosis. Also, high percentages of infections usually occur during culture of mature tissues (Andersone and Ievinsh 2005). Plant regeneration from axillary or quiscent meristems is a widely used method as it results in formation of genetically identical individuals. It is known that plants regenerated from axillary buds and shoot tips are genetically stable and free from somaclonal variations (Abdullah *et al*. 1986) as in pines, buds generally arise from quiescent meristems located adaxial to the juvenile needles and in the axil of cotyledons (David 1982).

The present study indicated that multiple shoots could be induced from seedling shoot apices of *P. roxburghii* on a medium containing cytokinins. The dosage of cytokinin in the culture medium was critical for axillary shoot proliferation. In the present study, BA was found to be more effective compared to other cytokinins (kinetin and BPA) used. Superiority of BA for shoot induction may be attributed to the ability of plant tissues to metabolize BA more readily than other synthetic plant growth regulators or to the ability of BA to induce production of natural hormones such as zeatin within the

tissue (Zaerr and Mapes 1982). Similar promotory effect of BA in inducing multiple shoots has been reported in several other reports on variety of pine species (Gupta and Durzan 1985, Abdullah *et al*. 1986, Lapp *et al*. 1996, Kalia *et al*. 2001). The concentration of BA used in the medium significantly influenced multiple bud formation. A maximum of 7.42 shoot buds per explant were induced on media supplemented with 10 µM BA. Lapp *et al*. (1996) also reported that the amount and type of cytokinin are important factors affecting the induction of shoots. Toxicity of BA at supra-optimal concentrations as evidenced in the present study has already been reported in *P. oocarpa* (Baxter *et al*. 1989), *P. monticola* (Lapp *et al*. 1996) and *P. roxburghii* (Parasharami *et al*. 2003). Development of secondary and tertiary buds at higher BA concentrations is in conformity with the results of Zel *et al*. (1988) in *P. sylvestris*.

Incorporation of NAA in the medium had a deleterious effect on shoot bud formation and encouraged callus formation. Similar effect of NAA was earlier reported in *P. ponderosa* (Lin *et al*. 1991). On the contrary, Kaul (1987) reported a positive role of NAA on morphogenetic responses in *P. strobus*. The variable response of different species to auxin-supplemented media may be due to different endogenous levels of auxins. The inhibition of shoot formation may be due to action of auxins accumulated at the basal end of the explants (Marks and Simpson 1994).

The shoot buds developing on the seedling explants were small in size and essentially required subculture on MS basal medium supplemented with activated charcoal for elongation. Similar observations have previously been reported in *P. roxburghii* (Parasharami *et al*. 2003) and *P. brutia* (Abdullah *et al*. 1987). Charcoal has been reported to adsorb the metabolites inhibiting morphogenesis thus supporting better growth (Fridborg *et al*. 1978). The induction medium had a profound effect on rate of bud elongation. Rate of shoot elongation was more, as high as 2.4-fold, in explants initially cultured on lower concentrations of BA as has previously been reported by Kaul (1987) and Schwarz *et al*. (1988) in *P. strobus*. Continued exposure of explants to higher BA concentrations during induction phase probably led to accumulation of cytokinins, which inhibits further shoot growth. Development of more buds during the elongation phase has also been attributed to the carry-over effect of cytokinins. Similar results were also reported in *P. sylvestris* (Zel *et al*. 1988). Decapitation of the explants having induced axillary buds had a promotive effect on rate of shoot elongation as has also been reported by Toribio and Pardos (1981) and Parasharami *et al*. (2003). This is due to the release of inhibitory effects of auxins accumulated in the apical region (apical dominance) thus supporting a faster rate of shoot elongation. Thus, removal of the elongated axillary shoots at regular intervals was necessary to allow the elongation of smaller buds. Aitken-Christie and Jones (1987) employed this hedging technique to ensure a continuous supply of radiata pine shoots.

The *in vitro* raised axillary shoots, decapitated shoot tips and the stumps left after removal of axillary buds were proliferated by repeated sub-culturing at regular intervals on cytokinin-supplemented media. Multiplication of *in vitro* raised shoots followed a trend similar to that shown by seedling shoot apices. Shoots, 2 - 3 cm long showed a better rate of axillary bud development during multiplication phase compared to longer or shorter shoots.

The efficiency of adventitious rooting is highly variable among the conifers and remains one of the key problems in plantlet regeneration *in vitro*. Maximum rooting was achieved on ½ MS medium supplemented with 5.0 µM NAA and 0.6 % agar. Concomitant with these results are the reports of Amerson and Mott (1982) and Patel *et al*. (1986) where NAA induced more percentage of rooting. Hardness of the gelled medium played an important role in elongation of the root primordia. The agar gelled media opposed the penetration of root primordia leading to radial growth of roots which dedifferentiated into callus on prolonged culture on auxin supplemented media. Mohammed and Vidaver (1988) reported that quality of roots produced on agar gelled medium was poor due to impediment of gaseous exchange.

Hardening and acclimatization of plantlets was essential for survival of plantlets under *ex vitro* conditions. For hardening, medium with reduced mineral salt and sucrose concentration was used as it probably forced the regenerants to rely on their own photosynthetic apparatus for nutrition (Kozai *et al.* 1988). During acclimatization, the plantlets were gradually shifted from high moisture environment to regular environment to gradually develop the protective system including epicuticular waxes. Such hardened and acclimatized plantlets showed better survival when transferred to *ex vitro* conditions.

The present study demonstrates a simple and efficient protocol for direct multiple shoot regeneration from half sib seedling shoot apices of *Pinus roxburghii* Sarg. Moreover, the system is rapid with the initiation of tissue culture to transplanting of regenarants to soil completed in 20 - 22 weeks. The *in vitro* cultures give a stable supply of shoots for rooting and plantlet regeneration. Commercial exploitation of developed protocol is possible, as the *in vitro* raised shoots can be employed as propagules for further multiplication obviating the dependence on field material unlike grafting and rooting methods.

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