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CLONAL PROPAGATION OF PINE TREES BY METHODS OF TISSUE CULTURE

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GRAND ABSTRACT

P. roxburghii and *P. Wallichiana* are the two major pine trees of Nepal. Two different types of approaches were taken for regeneration of *Pinus roxburghii* and *P. wallichiana*: (1). Regeneration of pine plantlets from cotyledons excised from germinating embryos (cotyledons ----> shoots -----> roots -----> plantlets) and (2) Regeneration of pine plantlets from inverted embryo (inverted embryo ----> shoots-----> roots ------> plantlets). The results of these approaches have been described here.

Regeneration of pine plantlet from cotyledons excised from germinating embryos: Cotyledons of *Pinus roxburghii*, an important forest tree of the Himalayas, were cultured under 16 hr light for 21 days on Murashige and Skoog (MS), Quoirin and Lepoivre media modified by Aitken Christie (LP), Gresshoff and Doy (GD) and Schenk and Hildebrandt (SH) media containing combinations of various concentrations of 6 - benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA). Induction of shoot buds was optimum in LP medium containing 22 μM BAP; MS, SH and GD media failed to induce shoot buds within 21 days of incubation. Presence of NAA invariably elicited formation of callus except in GD. The extent of callus formation depended upon the nature of the medium and concentration of NAA: MS medium was found most suitable for callus formation. For elongation cotyledons containing shoot buds were transferred to LP medium without BAP; inclusion of charcoal in the medium was essential for this process. Explants containing shoots can be subcultured at least for 3-4 times every 2-3 weeks in LP (half strength) without BAP and charcoal with no apparent loss in regeneration potential. After 3-4 cycles of subculture shoots elongate to attain a height of 1-1.5 cm and by the end of fourth cycle they can be separated individually. These shoots are transferred to LP 1/2 + 0.54 μM NAA (rooting media) where shoot buds root within a period of 2-3 weeks. Experiments with cotyledons from seeds germinated for various lengths of time indicated that germination for 5 days yielded cotyledons with highest regeneration potential.

Regeneration of *Pinus roxburghii* plantlets using inverted embryo technique: Embryos obtained from mature seeds of *Pinus roxburghii* germinated for 96 hr were incubated in three orientations - vertical, horizontal and inverted, in modified Quoirin and

Lepoivre media (LP) containing 22 μM N-6 benzyl amino purine (BAP) at 26°C under diffused light for 1, 3, 5, 7, 10, 14 and 21 days. These embryos were then transferred to LP medium containing 1% activated charcoal devoid of any hormone. After 21 days in charcoal, embryos were transferred to LP 1/2 for a week for greening. Individual cotyledons were dissected out and incubated individually in LP 1/2 for 2-3 weeks. The explants containing bunch of shoots were further dissected in smaller bunches and incubated in LP 1/2 for 2 to 3 weeks. This cycle of dissection followed by transfer was continued till individual buds were separated and further incubated in LP 1/2 for 3 weeks and transferred to fresh medium till shoots elongated to a height of 1 to 1.5 cm. Elongated shoots were transferred to LP 1/2 medium supplemented with 0.54 μM NAA for rooting. Rooting (30-40%) was achieved within three weeks in this medium.

Results obtained from studies suggested that (a) inverted orientation of embryo is most suitable for shoot proliferation (b) incubation in LP5 for 7 to 10 days is optimal for shoot bud induction. (c) charcoal is essential for shoot proliferation (d) about 200-300 shoots per embryo can be obtained in a period of 4 months.

Regeneration of *Pinus wallichiana* plantlets using inverted embryo: Embryos excised from seeds of *Pinus wallichiana*, germinated for seven days, were incubated in inverted orientation for 10 days in modified Quoirin and Lepoivre (LP) medium containing 44 μM 6-benzyl aminopurine (BAP). For shoot initiation BAP-treated embryos were further incubated in inverted orientation for 21 days in LP containing 1% activated charcoal. Differentiating embryos were then transferred in normal orientation to half LP for seven days for greening. Individual cotyledons were dissected and incubated individually in half LP for 2-3 weeks. The explants containing bunches of shoots were further dissected into smaller bunches and incubated in half LP for 2-3 weeks. This cycle of dissection followed by transfer was continued until individual buds were separated and grown to a height of 1-2.5 cm. All incubations were maintained under 16 h light (800-1300 lux). Day and night temperatures were 26°C and 21°C respectively. This method yields 40-50 shoots per embryo as compared to 200-300 shoots per embryo for *P. roxburghii*.

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List of Abbreviations

MS	-	Murashige and Skoog's medium
LP	-	Quoirin and Lepoiver's medium
GD	-	Gresshoff and Doy's medium
SH	-	Schenk and Hildebrandt's medium
BAP	-	6 - benzylaminopurine
NAA	-	α - naphthalene acetic acid
Zip	-	2 - isopentemylademine
PVP	-	Polyvinyl Pyrrolidon
IET	-	Inverted Embryo Technique
PAGE	-	Polyacrylamide gel electrophoresis
SDS	-	Sodium dodecyl sulphate

INTRODUCTION

In Nepal, desertification due to deforestation has posed serious problems of topsoil erosion and water losses in irrigation. Every year the monsoon causes serious soil erosion by causing heavier flooding not only in Nepal, but also in its neighbouring countries, India and Bangladesh, as many rivers tributary to the Ganges originate in Nepal. Therefore, desertification in Nepal, if not stopped in time, can become a tremendous problems for the Indian subcontinent as a whole.

P. roxburghii (Fig 1) and *P. Wallichiana* (Fig 2) are the two major pine trees of Nepal; *P. patula* being the only minor species (Howland and Howland 1984). The mixed community of these species tends to occur in marginally fertile areas like rock screes, land slides and land slips (Puri 1969) (Fig 3 & 4). So, these species are not only the most abundant pine trees of Nepal, but also are very important by virtue of being most amenable to reforestation.

Pinus roxburghii (Indian pine), previously named *P. longifolia*, is a large tree (150-180 ft. high and 7-11 ft. in diameter) and is native to the outer ranges and principal valleys of the Himalayas, usually at elevations of 1,500-7,500 ft (Dallimore and Jackson 1966). In the dry upper valleys of Western Nepal it is quite abundant even at an elevation of 9,000 ft (Stainton 1972). Its leaves are needle-like, soft, more than 15.5 cm. long and present in fascicles of three (trifoliar). It is a frost hardy species and tends to occur in conditions that are drier than average. Apart from its value as a timber tree *P. roxburghii* is of considerable commercial importance, for it is the principal resin-bearing pine of the East (Uphof 1968).

P. wallichiana (Bhutan pine or Blue pine), also known as *P. excelsa* wall, is a 50-150 ft. tall tree and is native to the temperate Himalayas at elevations of 6,000-12,000 ft. extending westward to Afghanistan & Eastward to Bhutan (Dallimore and Jackson 1966). In Western Nepal it is also found at elevation of up to 14,000 ft (Stainton 1972). Next to *Cedrus deodara* this is the most important coniferous timber tree of the Himalayas. It is much less abundant in the midlands than in other parts more sheltered from the full force

Fig 1: A *Pinus roxburghii* plant

Fig 2: A *Pinus wallichiana* plant

Fig 3: A forest of *Pinus roxburghii*

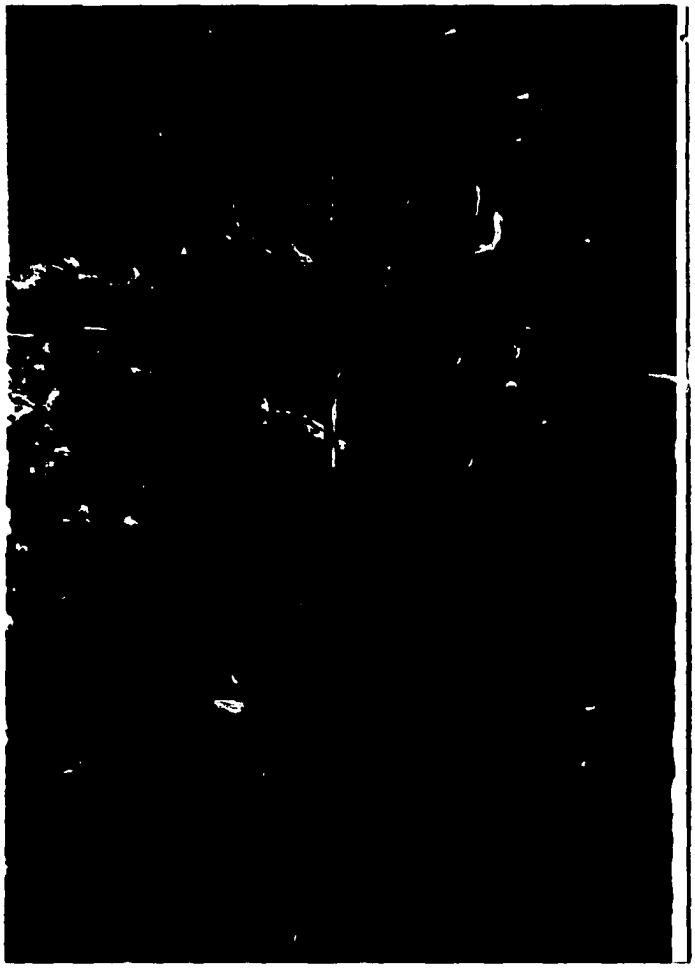


Fig 4: A forest of *Pinus wallichiana*

of rain. It is also used for production of turpentine and tar. Its leaves are needle-like, sometimes slightly bent and are present in fascicles of five (pentafoliar).

LITERATURE REVIEW OF TISSUE CULTURE RESEARCH IN PINUS SPECIES

Propagation of tree genera by conventional methods of breeding is time consuming and often inefficient owing to low seed set, poor germination and great variability. Cell culture techniques can be applied in order to overcome these barriers and may provide a short-cut in reducing the normal life cycle which may take several years. Normally in *Pinus*, the seeds mature 12 month after fertilization; two years intervene between initiation of ovules and formation of seeds (Weier *et al.* 1982). The potential of plant tissue culture techniques as a means of mass propagation of superior forest trees has been recognized for several years (Durzon and Campbell 1974, Bonga 1977, Winton 1978, Mott 1978, Sommer and Brown 1979, Mott 1981, Bonga and Durzan 1982, Biondi and Thorpe 1982, Horgan 1982, Thorpe and Biondi 1983, Aitken-Christie and Thorpe 1984, Evans *et al.* 1981).

For several species of pine trees the commercial exploitation of this potential has not materialized due to insufficient knowledge about the physiology of *in vitro* grown tissues from these tree species. Considerable progress has been made in the regeneration of gymnosperms which can be regenerated only from juvenile tissues such as embryos and seedlings. In many instances, only regeneration directly from the explant with no intervening callus growth has been reported. Regeneration is usually via organogenesis (Evans *et al.* 1981).

Literature survey indicates that out of about 90 *Pinus* species, only 13 have been the subject of report on any sort of tissue culture research. For example, shoot regeneration in *Pinus bankigsiana* (grey pine) from hypocotyl as explant (Campbell and Durzan 1975), Plantlet regeneration in *P. palustris* (long leaf pine) from embryo (Sommer *et. al.* 1975), plantlet regeneration in *P. pinaster* from cotyledons (David and David 1977), root

formation in *P. lambertiana* from hypocotyls (Greenwood), regeneration in *P. strobus* from embryos and needle follicles (Minocha 1980, Cohen 1975), plantlet and embryo regeneration in *P. elliotti* (slash pine) (Sommer and Brown 1974), regeneration in *P. mugo* from seedling (Girouard 1971), plantlet and embryo regeneration in *P. taeda* (loblolly pine) from juvenile explants (Sommer and Brown 1974, Mott and Amerson 1981), plantlet regeneration in *P. radiata* from several types of juvenile explants (Reily and Washer 1977, Aitken *et al.* 1981, Horgan and Aitken 1981), (one of the best example showing the success of tissue culture research in pine), plantlet regeneration in *P. sylvestris* from cotyledons and shoot tips (Bormann and Jasson 1980), and bud regeneration in *P. nigra* from embryos and shoot tips (Jalaska *et al.* 1981) have been reported.

Although regeneration from juvenile explants has been successful in more than 10 *Pinus* species, such process with mature explants has been demonstrated only in *Pinus pinaster* Sol., in which the regeneration from an 11 year old tree was achieved (Aitken-Christie and Thorpe 1984, David *et al.* 1979). Induction of callus, but not its morphogenesis into plantlets, has been shown in *P. strobus* (Minocha 1980), *P. lambertiana* (Greenwood), *P. resinosa* (Bonga 1974), *P. mugo* and *P. nigra* (Bonga 1981), and *P. densiflora* (Tominga and Oga 1970). The application of *in vitro* methodologies to clone select genotypes provides an opportunity to realize large genetic gains more rapidly than is possible using classical tree improvement methodologies (Durzan 1980, David 1982).

Efforts to regenerate *P. roxburghii* by using methods of tissue culture can become extremely useful in future as (a) Nepal is facing a serious problem of desertification due to erosion of topsoil and (b) chir pine forests of Nepal have been reported to suffer from infection by a fungus *Cronartium himalayense* causing stem rust (Cotter *et al.* 1987).

Before 1988, when research on tissue culture of *Pinus roxburghii* and *Pinus wallichiana* was initiated in Research Laboratory for Agriculture Biotechnology and Biochemistry (RLABB) with USAID/PSTC support literature on tissue culture of *P. roxburghii* was virtually non-existent except one report which described preliminary results on shoot bud induction in whole embryo (Bhatnagar *et al.* 1983).

PROJECT AIMS AND SPECIFIC OBJECTIVES

Aim of this project was to define the nutritional and hormonal requirements of steps involved in regeneration of plantlets (explant → shoot → root → plantlets) from explants of seeds, parts of seedling-cotyledons, hypocotyls and epicotyls and young shoot tips of *Pinus roxburghii* and *Pinus wallichiana*.

The overall aim of this project was to start a tree tissue culture laboratory for clonal propagation of superior forest trees native to the Indian subcontinent. This project had tried to define the steps involved in the clonal propagation of pine trees found abundantly in the Himalayan range. Its results can be used to rapidly multiply special families or clones from control-pollinated crosses.

APPROACH

Two different types of approaches were taken for micrpropagation of *Pinus roxburghii* and *P. wallichiana*:

1. Regeneration of pine plantlets from cotyledons excised from germinating embryos (cotyledons ----> shoots -----> roots -----> plantlets).

2. Regeneration of pine plantlets from inverted embryo (inverted embryo ----> shoots -----> roots ------> plantlets).

In the following pages these approaches and results there of have been described.

REGENERATION OF PINE PLANTLETS FROM COTYLEDONS EXCISED FROM GERMINATING EMBRYOS

ABSTRACT

Cotyledons of *Pinus roxburghii*, an important forest tree of the Himalayas, were cultured under 16 hr light for 21 days on Murashige and Skoog (MS), Quoirin and Lepoivre media modified by Aitken Christie (LP), Gresshoff and Doy (GD) and Schenk and Hildebrandt (SH) media containing combinations of various concentrations of 6 - benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA).

Induction of shoot buds was optimum in LP medium containing 22 μM BAP; MS, SH and GD media failed to induce shoot buds within 21 days of incubation. Presence of NAA invariably elicited formation of callus except in GD. The extent of callus formation depended upon the nature of the medium and concentration of NAA: MS medium was found most suitable for callus formation. For elongation cotyledons containing shoot buds were transferred to LP medium without BAP; inclusion of charcoal in the medium was essential for this process. Explants containing shoots can be subcultured at least for 3-4 times every 2-3 weeks in LP (half strength) without BAP and charcoal with no apparent loss in regeneration potential. After 3-4 cycles of subculture shoots elongate to attain a height of 1-1.5 cm and by the end of fourth cycle they can be separated individually. These shoots are transferred to LP 1/2 + 0.54 μM NAA (rooting media) where shoot buds root within a period of 2-3 weeks.

Experiments with cotyledons from seeds germinated for various lengths of time indicated that germination for 5 days yielded cotyledons with highest regeneration potential.

MATERIAL AND METHODS

Seed source and stratification procedure

Seeds of *Pinus roxburghii* (Fig 5) were obtained from Forest Development Project, Hattishar, Kathmandu in 1988 and stored at 7°C for further use. Seeds were washed in liquid detergent for 15 min. and rinsed in running water thoroughly. Washed seeds were kept at 7°C for 24 h., sterilized with 20% Clorox containing a drop of Tween 80 for 15 min and rinsed thrice with sterile water. Their testa were removed asceptically and placed on agar plates (1%) in dark at 27°C (Fig 6) for germination for required period (percentage of germination was more than 90%; seeds treated at 7°C for periods more than 24h did not germinate well (less than 50%). Cotyledons were excised on the 5th day of incubation and used as primary explants.

Culture media

The basal media tried for tissue culture were Murashige and Skoog, 1962 (MS); Schenk and Hildebrandt, 1972 (SH); Gresshoff and Doy, 1972 (GD); and Quoirin and Lepoivre medium modified by Aitken Christie and Thorpe, 1984 (modified LP), detailed composition of all media is given in Table I. Media were supplemented with 3% sucrose and 0.8% agar. All media were adjusted to a pH of 5.7 before autoclaving. Six concentrations of BAP (44, 22, 5.5, 4.4, 2.2 and 0.0 μM) and four concentrations of NAA (5.4, 2.7, 0.54 and 0.0 μM) were used for factorial experiments resulting in 28 combinations (Table II). Cotyledons were incubated in culture tubes containing media supplemented with various combinations of phytohormones for 21 days at 22-27°C under 1 photoperiod of 16h light provided by two fluorescent tubes of 1,000 lumen each. Further subcultures were done in LP Medium (half strength) containing 3% sucrose.

After 21 days cotyledons were microscopically studied for various growth parameters - elongation, swelling, roughness of the surface, shoot bud, callus etc. The criteria for shoot bud induction was roughness of the explant surface and formation of shoot bud primordia.

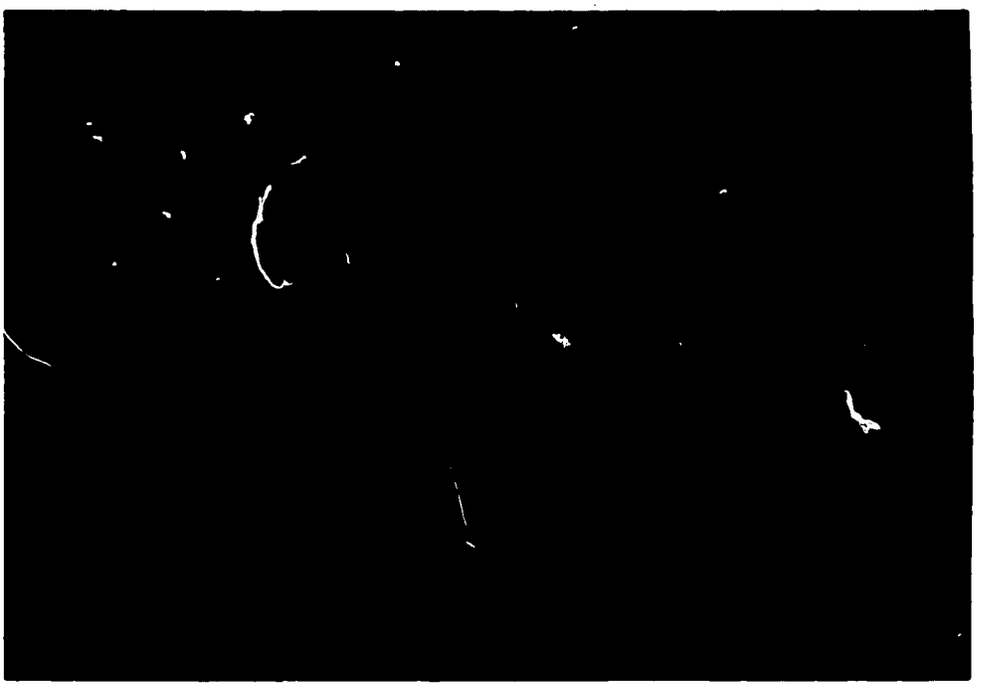


Fig 5: Bigger seeds: *P. roxburghii*

Smaller seeds: *P. wallichiana*

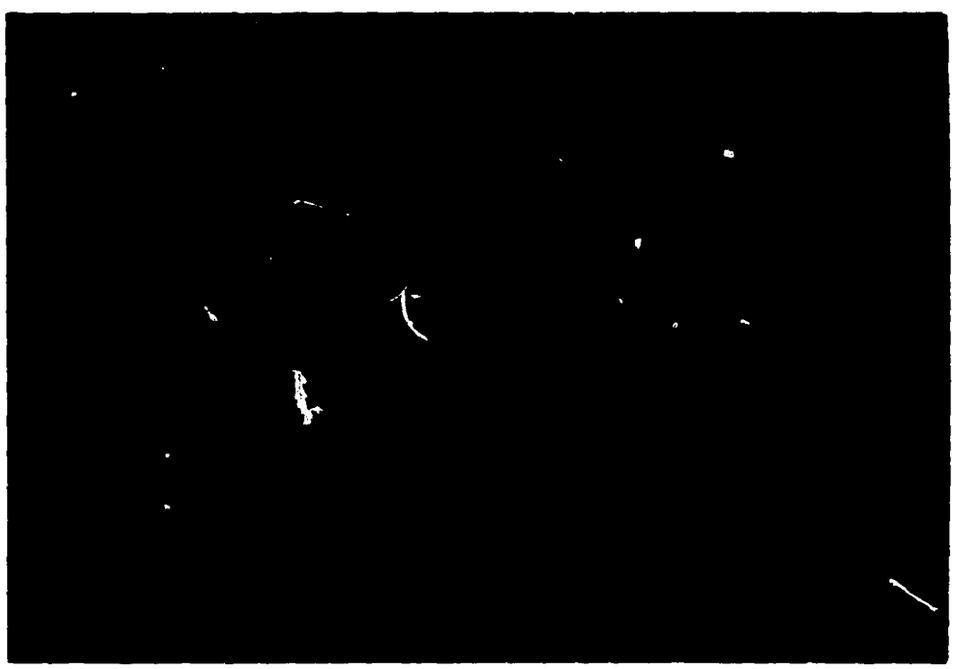


Fig 6: Seeds of *P. roxburghii* without seed coat

TABLE 1

COMPOSITION OF VARIOUS MEDIA USED FOR PINE RESEARCH

Major	MS [1] mg/lit	LP [2] mg/lit	SH [3] mg/lit	G [4] mg/lit
KNO_3	1900	1800	2500	1000
$MgSO_4$- $7H_2O$	370	360	400	250
$CaCl_2$- $2H_2O$	440	-	200	150
NH_4NO_3	1650	400	-	-
KH_2PO_4	170	270	-	-
$Ca(NO_3)_2$ $4H_2O$	-	1200	-	-
Minor				
$FeSO_4$ -$7H_2O$	27.8	30	15	30
Na_2EDTA- $2H_2O$	37.3	40	20	40
$MnSO_4$- $4H_2O$	15.6	1	15.16	15.16
H_3BO_3	06.2	6.20	5	5.0
$ZnSO_4$- $7H_2O$	08.6	8.6	1	1
KI	0.83	0.08	1	1
$CuSO_4$-$5H_2O$	0.025	0.025	0.2	0.2
Na_2MoO_4- $2H_2O$	0.25	0.25	0.2	0.2
$CoCl_2$-$6H_2O$	0.025	0.025	0.2	0.2
Vitamines				
Thiamine HCl	0.5	0.4	5	5
Nicotinic acid	0.05	-	5	5
Pyridoxine.HCl	0.5	-	0.5	0.5
Glycine	3.0	-	-	-
Myo-inositol	100	1,000	1,000	1,000
Sucrose	30,000	30,000	30,000	20,000
Agar	1%	1%	1%	1%
pH	5.7-5.8	5.7-5.8	5.7-5.8	5.7-5.8

1= Murashige and Skoog (1962); 2 = Quirin and Lepoivre (1977); 3 = Schenk and Hildebrand (1972); 4 = Gresshoff and Doy (1972)

Table II

FACTORIAL DESIGN USED FOR SHOOT INDUCTION IN PINE

NAA (µM)	BAP(µM) →	0	2.2	4.4	5.5	22	44
0		0, 0	0, 2.2	0, 4.4	0, 5.5	0, 22	0, 44
0.54		0.54, 0	0.54, 2.2	0.54, 4.4	0.54, 5.5	0.54, 22	0.54, 44
2.7		2.7, 0	2.7, 2.2	2.7, 4.4	2.7, 5.5	2.7, 22	2.7, 44
5.4		5.4, 0	5.4, 2.2	5.4, 4.4	5.4, 5.5	5.4, 22	5.4, 44

Table III

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN MS MEDIA

NAA (µM) \ BAP (µM)	0.0	2.2	4.4	5.5	22	44
0.0	ONLY ELONGATION & SWELLING					
0.54		CALLUSING AT CUT ENDS				
2.7	CALLUSING ALL ALONG THE EXPLANT SURFACE					
5.4	CALLUSING ALL ALONG THE EXPLANT SURFACE					

RESULTS AND DISCUSSION

Effect of media composition on shoot bud induction

Factorial experiments conducted in MS medium revealed that callus formation was evident in all the treatments containing NAA (Table III). Treatments devoid of NAA resulted in excessive elongation and swelling of the explants. Callus formation increased with an increase in NAA concentration. Treatments having less NAA induced callus at cut ends of explants whereas higher concentrations induced callusing all over the explant surface and no treatment could induce shoot bud formation within 21 days.

Shoot bud induction was not seen in any of the treatments in SH medium (Table IV). Callus formation restricted to the cut ends alone was induced by 2.70 μM NAA in combination with 2.20 to 22 μM BAP.

It was interesting to note that neither shoot bud nor callus formation was induced in GD medium within 21 days in any of the treatments (Table V).

LP medium yielded most favorable results among all the media tested (Table VI). Shoot buds were produced in LP medium containing BAP (4.4, 5.5 and 22 μM), thus revealing the role of cytokinin in this process. Shoot bud generating explants started swelling after establishment, followed by formation of rough texture on the surface (Fig 7). These rough spots eventually turned into small protuberances (Fig 8 & 9). In a single explant 30-40 buds were obtained after first 21 day incubation period. High BAP concentration (44μM BAP) did not increase the number of buds. Treatments with NAA alone or in combination with BAP resulted in callus induction (Fig 11) . Callus formation was not profuse and was usually restricted to the cut end regions of explants.

Explants containing shoot buds were transferred to LP medium devoid of growth hormones and containing 1% activated charcoal for further elongation of shoots (Fig 10).

Table IV

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN SH MEDIA

NAA(µM) \ BAP (µM)	0.0	2.2	4.4	5.5	22	44
0	ONLY ELONGATION & SWELLING					
0.54	CALLUS	SWELLING				
2.7	ELONGA-TION	CALLUS				ELONGATION & SWELLING
5.4		NO RESPONSE			ELONGATION	

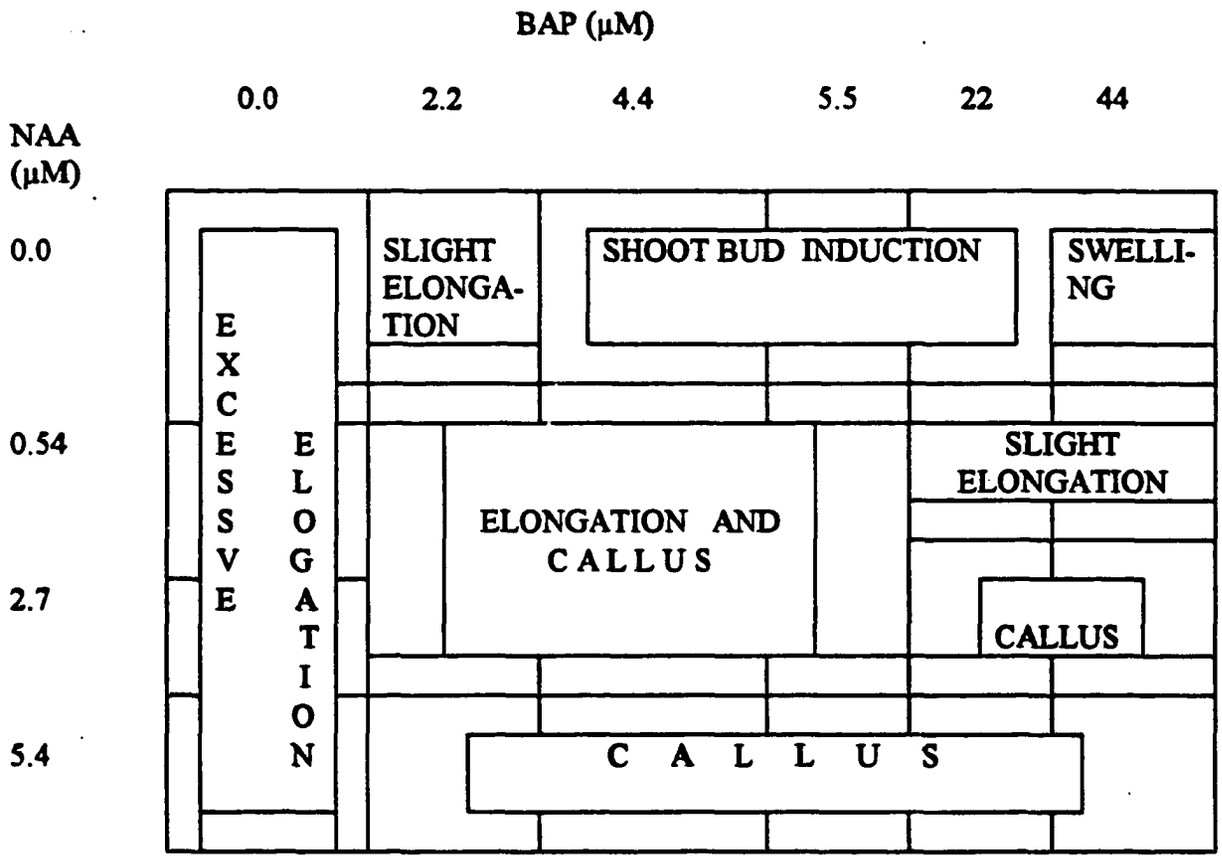
Table V

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN GD MEDIA

NAA(µM) \ BAP (µM)	0.0	2.2	4.4	5.5	22	44
0	ELONGATION & SLIGHT SWELLING					
0.54		ELONGATION & SWELLING				
2.7		ELONGATION				
5.4		ELONGATION				

Table VI

FACTORIAL EXPERIMENTS OF P. ROXBURGHII IN LP MEDIUM



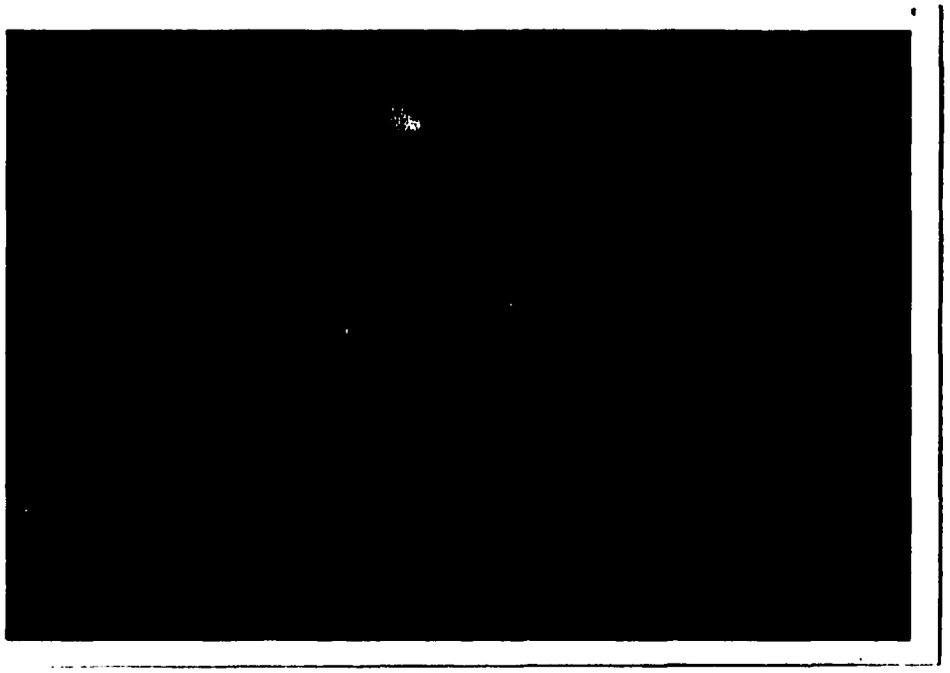


Fig 7: Shoot bud induction on LP medium from explant of *P. roxburghii*

Fig 8: Effect of 1% charcoal on buds of *P. roxburghii* raised on LP - 5.5μM BAP

Fig 9: Effect of 1% charcoal on buds of *P. roxburghii* raised on LP - 4.4μM BAP

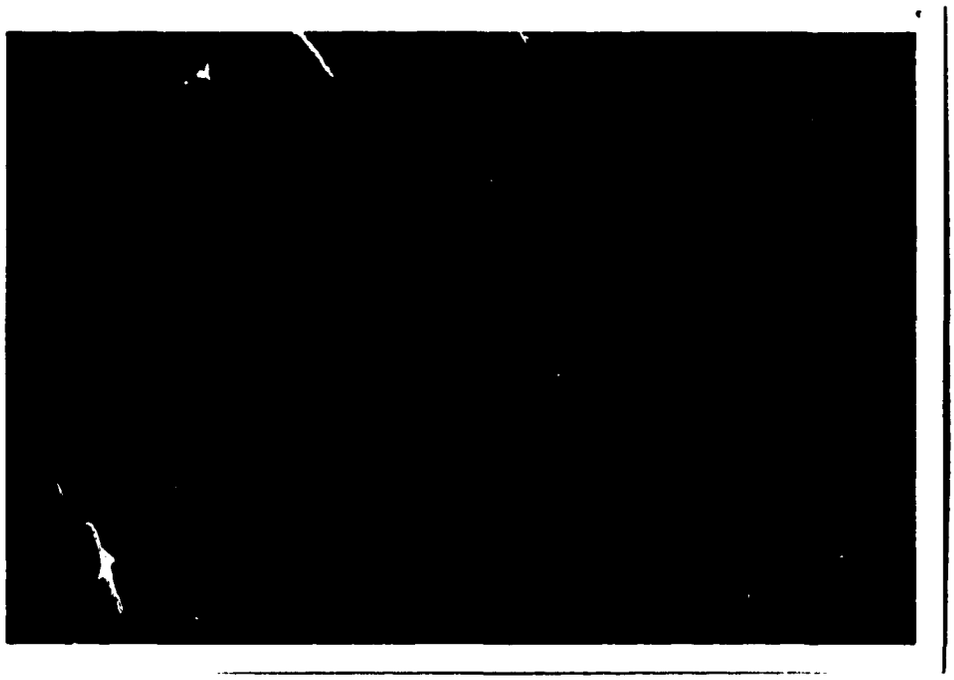


Fig 10: *P. roxburghii* shoot buds in elongation medium

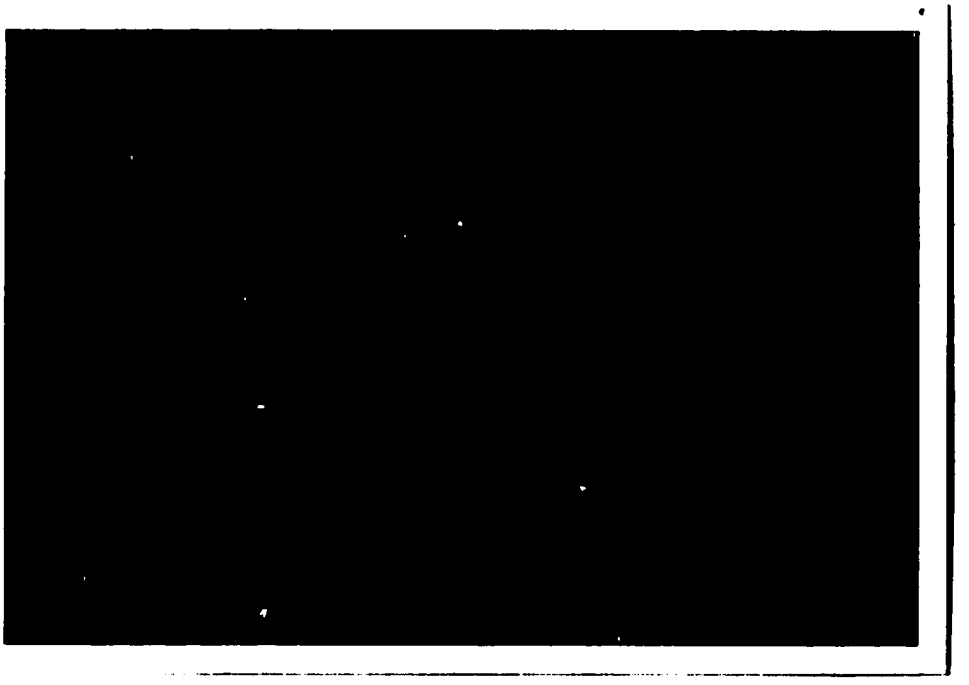


Fig 11: Callus induction from cotyledon of *P. roxburghii*

Effect of light

Light was found to show profound role in shoot bud induction. Light in the range of 600-700 lux resulted in excessive elongation and callusing sometimes. For shoot bud induction at least 1000 lux of light intensity is necessary.

Role of activated charcoal on shoot bud development

Shoot buds obtained in 4.4, 5.5, 22, 44 μM BAP were transferred to LP basal medium and LP medium with 1% activated charcoal. Most interesting results were obtained in the shoot buds of 4.4 and 5.5 μM BAP. Shoot buds raised on 4.4 μM BAP revealed better development in LP basal medium whereas shoot buds raised on 5.5, 22 and 44 μM BAP exhibited much better growth in LP basal medium supplemented with 1% activated charcoal (Fig 9) Inclusion of charcoal in the medium was not necessary if 4.4 μM of BAP was used for shoot bud induction. (Fig 8) Since this level did not induce as many shoot buds as higher levels of BAP, it was advantageous to use 22 μM for induction and include charcoal in the elongation medium. The differential effect of charcoal for 4.4 and 5.5 μM BAP clearly suggests that charcoal exerts its effect by adsorbing inhibitory concentrations of BAP.

Effect of various additives on shoot bud induction

Silver nitrate

It was used as ethylene antagonist. In the cut explants ethylene production takes place which some times results in the death of explants. Silver Nitrate was used in elongation but total death was observed at 100 ppm.

Polyvinyl pyrrolidon (PVP)

It was used as phenol scavenger at 20 and 100 ppm level. Cultures at 20 ppm concentration were healthy and 60% explants showed shoot bud induction where as at 100 ppm explants resulted in poor growth with no shoot bud production.

Amino Acids

Aspargine, glutamine and tyrosine were tried at 0.5 and 1.0 mM levels. Aspargine was found to have some positive effect at 1mM level but glutamine was more potent than aspargine in the induction of shoots buds. Tyrosine failed to elicit any response in both the concentration. For our future experiments we plan to use PVP (20 ppm) and glutamine (0.5 mM) for studying shoot bud induction in cotyledonary explants.

Effect of combined cytokinin

It is seen that all the combination of cytokinins (BAP and 2 iP) give shoot buds but in some cases the buds clutter around the proximal end of the cotyledons. The combination of BAP / 2 iP, at ppm ratios of (1 : 4) show promising response in early days of culture (first week) but observations after one and a half month shows that the ratio of 2:3 and 3:2 are more promising in the sense that explants in these culture medium have rough surface throughout with the induction of buds at the tip.

It is possible that if the cotyledons are cut into pieces, obviously, not cutting the rough or meristematic regions, may help to obtain number of shoot bud from individual protuberances.

Effect of age of the seed and season on shoot bud induction

Fresh seeds germinate very well (> 90%) in 1% agar; germination percentage declines with the time of storage of the seeds. Cotyledons obtained from fresh seeds exhibit better results for shoot bud induction. Preliminary results from cultures done during June, July

and August gave better results and the responsiveness of explants decreased in other months of the year.

Effect of seed germination period on shoot bud induction

In order to study the effect of germination period on shoot bud induction, seeds after incubating for various lengths of time were taken out for excision of cotyledons and incubated in LP media containing 22 μM BAP. It was observed that elongation of cotyledons increased, swelling decreased and overall mortality decreased with the germination period. Cotyledons obtained from seeds germinated for less than 3 days had a low survival rate whereas those from seeds germinated for more than 4 days elongated so much that shoot buds could not appear (general experience is that for shoot bud induction explants with less elongation and more swelling are preferred). It was found that seeds germinated for three (72 h) to four days (96 h) yielded the most responsive cotyledons.

Elongation of shoot buds

Shoot buds thus obtained on explants were transferred to LP (half strength) medium containing 3% sucrose. They were kept for 2-3 weeks in this medium. Three to four subcultures in this medium were required for elongation of almost all the potential buds. At the end of every passage individual buds were cut with a piece of explants and transferred. By the end of 3-4 subcultures shoots elongated to a size of 1 - 1.5 cm and were suitable for transferring into rooting media.

Rooting

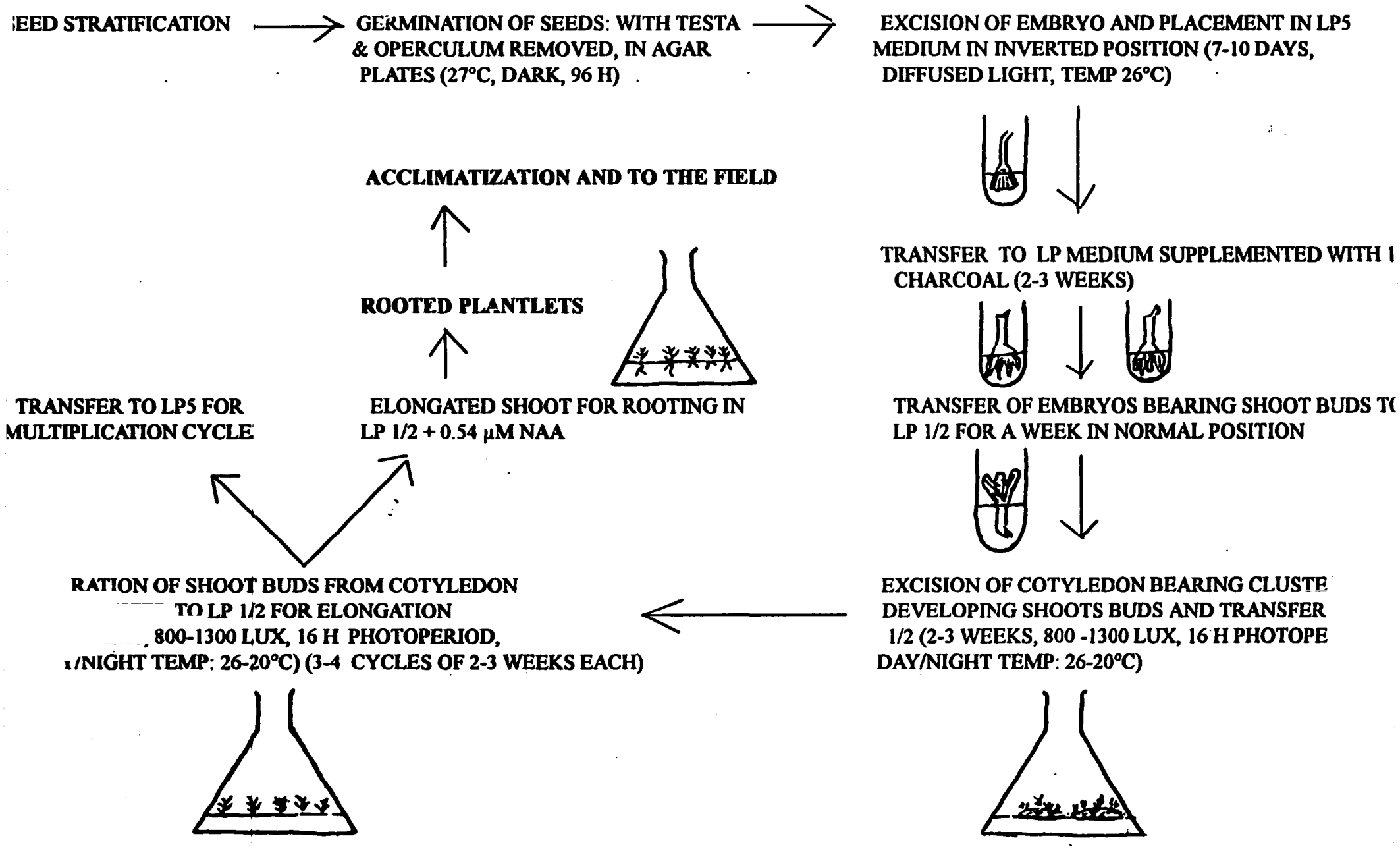
Same LP1/2 medium was used for rooting containing 0.54 μM NAA. Shoot buds started rooting within 2-3 weeks. But the percentage of rooting was rather low (30-40).

A DETAILED DESCRIPTION OF INVERTED EMBRYO TECHNIQUE

Inverted Embryo Technique (IET) in tissue culture is a most useful technique for the propagation of pine (Agrawal et al. 1991 and Shakya et al. 1992). Using this technique, about 200-300 shoots per embryo in case of *Pinus roxburghii* and 110 - 130 shoots in *Pinus wallichiana* can be produced. The IET comprises of following major steps:

Step	Activities
1	Removal of seed testa & operculum and seed germination on agar plates
2	Excision of 5 day old embryo and placing in LP medium in inverted position for 10 days in diffused light
3	Transfer of embryo to LP medium supplemented with 1 % charcoal (2-3 weeks)
4	Transfer of embryos bearing shoot buds to LP 1/2 for a week in normal position
5	Excision of cotyledon bearing cluster of developing shoot buds and transfer to LP 1/2 (2-3 weeks)
6	Separation of shoot buds from cotyledon and transfer to LP 1/2 for elongation (2-3 weeks)
7	Transfer of elongated shoots for rooting in LP 1/2 + 0.5 µM NAA

INVERTED EMBRYO TECHNIQUE



REGENERATION OF PINUS ROXBURGHII PLANTLETS USING INVERTED EMBRYO TECHNIQUE

ABSTRACT

Embryos obtained from mature seeds of *Pinus roxburghii* germinated for 96 hr were incubated in three orientations - vertical, horizontal and inverted, in modified Quoirin and Lepoivre media (LP) containing 22 μM N-6 benzyl amino purine (BAP) at 26°C under diffused light for 1, 3, 5, 7, 10, 14 and 21 days. These embryos were then transferred to LP medium containing 1% activated charcoal devoid of any hormone. After 21 days in charcoal, embryos were transferred to LP 1/2 for a week for greening. Individual cotyledons were dissected out and incubated individually in LP 1/2 for 2-3 weeks. The explants containing bunch of shoots were further dissected in smaller bunches and incubated in LP 1/2 for 2 to 3 weeks. This cycle of dissection followed by transfer was continued till individual buds were separated and further incubated in LP 1/2 for 3 weeks and transferred to fresh medium till shoots elongated to a height of 1 to 1.5 cm. Elongated shoots were transferred to LP 1/2 medium supplemented with 0.54 μM NAA for rooting. Rooting (30-40%) was achieved within three weeks in this medium.

Results obtained from studies suggested that (a) inverted orientation of embryo is most suitable for shoot proliferation (b) incubation in LP5 for 7 to 10 days is optimal for shoot bud induction. (c) charcoal is essential for shoot proliferation (d) about 200-300 shoots per embryo can be obtained in a period of 4 months.

MATERIALS AND METHODS

Collection of seeds and excision of primary explant

Mature *Pinus roxburghii* seeds, obtained through the courtesy of the Forest Development Project, Hattishar, Kathmandu, were washed in liquid detergent for 15 min and rinsed thoroughly in running water. Seeds were stratified at 7° C for 24 h and then sterilized in

20% Chlorox (1.05% NaOC1) for 15 minutes followed by three sterile rinses. The testa and the operculum of the sterilized seeds were removed aseptically. Seeds thus obtained were incubated in dark on agar plates (1% (w/v) for 96 h at 27°C for germination (Fig. 12 A). On the fifth day of germination (after 96 hr) the green embryos were excised from seeds.

Shoot bud induction

Excised embryos were carefully implanted in inverted position (Fig. 12 B) with the cotyledon portion LP medium (Aitken-Christie and Thorpe 1984) containing 22 μM 6-benzyl amino purine (BAP) in 0.8% agar. After 10 days of incubation embryos were reimplanted in inverted position in LP medium containing activated charcoal (1% w/v) and incubated for three weeks. Cultures were maintained at 26°C ± 2 under diffuse light (16 hr/day).

Shoot multiplication and elongation

After treatment with charcoal differentiating embryos were implanted in normal position with hypocotyl in half strength LP (LP 1/2) medium containing full strength sucrose. After a week, individual cotylendons and hypocotyl were dissected and incubated in LP 1/2. After three weeks cotyledons bearing shoot buds were transversely dissected in four to five smaller pieces; care was taken not to cut along the sites having obvious formation of shoot buds. These pieces as well as hypocotyl containing the apical dome were further incubated in LP 1/2 for another three weeks. This cycle of dissecting the explant, followed by transfer, was continued until individual buds are separated (Fig. 12 H). Shoot buds were elongated in LP 1/2 to an average height of 1-1.5 cm. by transferring the cultures every three weeks.

For shoot multiplication and elongation cultures were maintained under 16 h light (800 - 1,300 lux); day and night temperatures being 26° and 20° C, respectively.

Fig. 12: Regeneration of *Pinus roxburghii* from inverted embryo technique. A : Seed germination on agar plate; B : 5-day old inverted embryo in LP + 22μM BAP medium; C: 4-week old inverted embryo in LP medium + 1% activated charcoal; D: 4-week old embryo removed from LP+1% charcoal medium; E: Initiation of shoot buds at the tip of cotyledon; F : Multiple shoot bud formation on a portion of cotyledon; G: Formation of axillary bud during the 4-5 week old culture in LP+1% activated charcoal; H: Placement of shoot bearing cotyledon pieces on LP (1/2 strength) medium for elongation; I: Elongated shoots in rooting media (LP 1/2 strength + 0.54μM NAA); J: Rooted plantlet; AB : Axillary buds.

Rooting of the shoots

For rooting shoots were implanted in LP 1/2 containing 0.54 μM NAA (Fig. 12 I). Culture conditions were the same as given for shoot multiplication.

RESULTS AND DISCUSSION

Pretreatment of *P. roxburghii* seeds

Seeds were stratified before germination because this step increased the percentage of germination.

Effect of BAP and Charcoal on shoot bud induction

In BAP containing shoot induction media the inverted embryos (embryos that are implanted in an inverted fashion) turned dark green in five days. Cotyledons and hypocotyl sections swell significantly by the tenth day, but were devoid of shoot buds. When the BAP treated embryos were transferred to 1% charcoal containing LP, dramatic morphological changes occured with in three weeks (Fig. 12 C & D). Following are the chronology of morphological changes when BAP treated embryo was transferred to charcoal containing medium - 1. elongation and swelling of cotyledons and hypocotyl segments, 2. appearance of shoot buds first on cotyledon tips (Fig. 12 E) and then along the length of cotyledons (Fig. 12 F), 3. appearance of axillary buds (Fig. 12 G) and 4. formation of multiple buds in apical dome.

Inverted embryos incubated with BAP for one day did not give shoots at all. Those incubated for three to five days developed shoots at the cotyledon tips only. Embryos incubated for seven to ten days gave the best response in terms of number of shoots. Incubation for longer periods did not appreciably increase number of shoots. This clearly indicated that a minimum three day BAP Shock for the induction of shoot buds was necessary, as hads been shown by the studies done on radiata pine (Biondi and Thorpe 1982). Positive effect of charcoal on shoot bud proliferation as described here was

consistent with our work on *P. roxburghii* cotyledons and the work on *P. radiata* by Aitken-Christie *et al.* (Aitken *et al.* 1988) but in contrast to the work in *P. strobus* by Kaul (Kaul 1985)

Effect of light and role of hypocotyl

In IET after BAP treatment the embryo was inserted in the medium in inverted manner with hypocotyl end sticking out above the medium. In order to find what role this phypocotyl end does play in IET, two kinds of experiments were carried out. In one set of experiment, incubation was carried out in dark during charcoal treatment. It was very interesting to observe that embroys grown in dark produced shoot but these could not proliferate as in normal IET. It was also noticed that dark grown embryos turned yellow and eventually died. In second set of experiment hypocotyl end of embryo was cut out and remaining embryo was implanted in inverted manner in the medium. In embryos without hypocotyl end, shoot induction was drastically inhibited. These investigation suggested that light plays a very important role in shoot induction and hypocotyl end discharges an important function in shoot proliferation by trapping light. The protein estimation showed that the hypocotylless embryo possessed more protein that intact embryo. This suggests that the protein in hypocotylless embryo was not used in any physiological activity. The SDS-PAGE showed that proteins of high molecular weight appeared and of low molecular weight disappeared in hypocotylless embryo.

Normal vs Inverted Orientation

In IET, embryo was implanted in inverted orientation, both in BAP and charcoal treatments. The requirement of inverted orientation for BAP treatment had been proved earlier, but such a need for charcoal treatment has not been as yet demonstrated. When incubation in normal orientation was carried out, enlargement of cotyledons was observed as in inverted orientation but shoot induction was much suppressed. Interestingly, some of cotyledons in normal position grew larger and part of them even came out of the medium. These cotyledons inserted back bending down in the medium and showed the shoot induction. Therefore, it can be concluded that inverted orientation is needed for shoot induction in charcoal treatment.

Effect of agar volume and its concentration

Experiment using vessels of different shape (test tubes, jars, conical flask etc.) indicated that shoot induction depended upon the area of the medium and its depth. Small diameter of the tube with larger depth of the medium showed more efficient shoot induction in charcoal treatment. A general phenomenon that had been noticed during charcoal treatment in IET is that a good portion of embryo comes out of the medium because of resistance of the medium to the penetration of cotyledons inside it. Therefore, in anticipation that a softer agar will resolve this problem to some extent, experiments with 0.7% agar were carried out. In 0.7% agar, embryos could show higher shoot induction because due to lower resistance of the medium cotyledons spread deeper in the medium and allowed a greater surface area for shoot induction. Moreover SDS-PAGE showed induction of new proteins in embryos cultured in 0.7% agar as compared to 0.8%.

Shoot multiplication and elongation

Transfer of the differentiating embryos to LP 1/2 for a week before its eventual dissection into cotyledon was done in order to increase the explant survival rate; embryos turned green from pale green during this step. The cycle of dissection and transfer yields minimum of 200 shoots (Fig. 12 H) per embryo in a period of about four months. (We were able to get a minimum of 15 shoots per cotyledon, 12 shoots from axillary buds and 10 shoots per apical dome resulting into 15 x 12 + 12 + 10 = 202 shoots per embryo). Selection of light intensity between 800-1,300 lux for shoot multiplication and elongation was based on our previous work with cotyledonary explant where this range of light was found essential for shoot induction.

Literature revealed the production of three different types of adventitious shoots during the shoot elongation stage - waxy, wet and translucent shoots (Aitken-Christie and Thorpe 1984). Translucent shoots are also known as "vitrified" shoots and wet shoots lack epicuticular wax. So these two types of shoots did not survive when exposed to lower humid situations and are unfit for rooting stage. It is interesting to note in our work

with P. roxburghii that all the shoots regenerated belong to waxy types with 100 % survival.

Rooting of shoots

For rooting shoots (1.0 - 1.5 cm.) were planted in LP 1/2 containing 0.54 µM NAA (Fig. 12 I). Rooting began within two weeks of incubation (Fig. 12 J).

Comparison with Aitken - Christie's method (Aitken - Christie *et al.* 1988)

The inverted embryo technique for pine regeneration was first reported by Aitken - Christie (Aitken-Christie *et al.* 1988). This method involves (a) induction of meristematic tissue from embryo, (b) shoot formation, multiplication and elongation and (c) finally root formation (embryo → meristemoids → shoots → roots → plantlets). Embryos from fresh non-germinating *P. radiata* seeds were cultured in LP medium containing BAP for a period of three months or more resulting in the formation of meristematic tissue which further gave shoots when transferred to LP medium containing charcoal (3 weeks) and then without charcoal. This method is highly productive; it can allegedly produce 260,000 shoots per seed in 2.5 years (Aitken-Christie *et al.* 1988). The present method described by us did not involve meristemoid formation and is less efficient (200 shoots per seed in 4 months). Since our method did not require prolong treatment with BAP, it was rather reasonable to assume that pine clones obtained using this method were true-to-type, a highly important trait required in propagation of forest trees with a maturity period of over 50 years. Our attempts to induce the formation of meristematic tissues using embryos from fresh, non-germinating *P. roxburghii* seeds had so far failed.

REGENERATION OF PINUS WALLICHIANA PLANTLETS USING INVERTED EMBRYO TECHNIQUE

Abstract

Embryos excised from seeds of *Pinus wallichiana*, germinated for seven days, were incubated in inverted orientation for 10 days in modified Quoirin and Lepoivre (LP) medium containing 44 µm N6-benzyl aminopurine (BAP). For shoot initiation BAP-treated embryos were further incubated in inverted orientation for 21 days in LP containing 1% activated charcoal. Differentiating embryos were then transferred in normal orientation to half LP for seven days for greening. Individual cotyledons were dissected and incubated individually in half LP for 2-3 weeks. The explants containing bunches of shoots were further dissected into smaller bunches and incubated in half LP for 2-3 weeks. This cycle of dissection followed by transfer was continued until individual buds were separated and grown to a height of 1-2.5 cm. All incubations were maintained under 16 h light (800-1300 lux). Day and night temperatures were 26°C and 21°C respectively. This method yields 40-50 shoots per embryo as compared to 200-300 shoots per embryo for *P. roxburghii*.

MATERIAL AND METHODS

Collection of seeds and excision of primary explant

Seeds of *P. wallichiana*, collected from Daman, Makawanpur District, Central Development Region, were stored at 7°C. Seeds were dewinged and sterilized by stirring with 20% bleach and a drop of Tween-20 (detergent) for 20 min. They were then washed in running water thoroughly for about half an hour. Washed seeds were scarified at their micropylar ends and kept in a small amount of distilled water at 7°C for 24 hours. The seeds were again sterilized with 20% bleach for 15 minutes and rinsed 3-4 times with distilled water. Their testa and operculum were removed aseptically and the embryos were placed on agar plates (1% agar) in the dark at 26°C for between five and ten days for germination. After germination the embryos were excised again aseptically. Keeping the

seeds at 7°C for periods of more than 24 hours did not improve germination. It fell to below 50 per cent.

Shoot-bud induction and proliferation

Excised embryos were implanted in inverted orientation (Fig 13 A) with the cotyledon portion in the LP medium containing various concentrations (0-88 μm) of BAP in 0.8% agar for 10, 14 and 21 days. BAP - treated embryos were reimplanted in inverted orientation in LP medium containing activated charcoal (1% w/v) (Fig 13 B) and incubated for 21 days. All cultures were maintained at 26°C and 21°C day and night temperatures respectively.

Shoot multiplication and elongation

After treatment with charcoal, differentiating embryos were implanted in normal orientation with their hypocotyl in half LP (half strength LP medium containing full strength or 3% sucrose) (Fig 13 C). After a week, individual cotyledons and hypocotyl were dissected and incubated in half LP (Fig 13 D). After a further three weeks cotyledons bearing shoot buds were separated out carefully and, if necessary, were transversely dissected into a few pieces; care was taken not to cut along the sites that had a mass of shoot buds. Unlike *P. roxburghii*, the initial hypocotyl region just below the axillary part of an embryo of *P. wallichiana* is also responsive to shoot bud proliferation. The responsive parts of the hypocotyl were also dissected out. These pieces as well as the hypocotyl containing the apical dome were further incubated in half LP for another three weeks (Fig 13 E). This cycle of dissecting the explant followed by transfer was continued until individual buds were separated. Shoot buds were grown in half LP to an average height of 1-2.5 cm by transferring the cultures every three weeks (Fig 13 F). All cultures were maintained under 16 hours light (800-1,300 lux); day and night temperatures being 26°C and 21°C respectively.

Fig. 13: Regeneration of *Pinus wallichiana* from inverted embryo technique. A: I[illegible] medium containing BAP; B: Inverted embryo in LP + 1% activated charcoal; C: Embryo in Normal orientation; D: Dissected cotyledons and hypocotyls in half LP; E: Shoot buds growing in half LP; F: Shoots developing roots in half LP.

RESULTS AND DISCUSSION

BAP shock was essential for shoot bud induction. Experiments involving various concentrations (0-88 µm) indicated that *P. wallichiana* requires higher concentration of BAP (44µm) compared to P. roxburghii (BAP 22 µm). Time course experiments showed that the minimum duration of BAP shock for *P. wallichiana* embryos was 10 days. Charcoal was necessary for shoot initiation and proliferation. Like *P. roxburghii*, *P. wallichiana* embryos were devoid of shoot buds unless they were incubated in LP medium containing 1% charcoal. In BAP-containing shoot induction media the inverted embryos turned dark green in five days. When the BAP-treated embryos were transferred to LP medium containing 1% charcoal, dramatic morphological changes occurred within three weeks. The chronology of changes was :

(1) Elongation and swelling of cotyledons and hypocotyl segments
(2) Appearance of shoot buds first on cotyledon tips and then along the length of cotyledons.
(3) Appearance of axillary buds.
(4) Formation of multiple buds on apical dome.
(5) Appearance of shoot buds along the length of the hypocotyl.

The transfer of the differentiating embryos to half LP for a week before eventual dissection into cotyledon and hypocotyl along with apical dome was performed in order to increase the explant survival rate; embryos turned from pale to dark green during this phase. The cycle of dissection and transfer yielded a minimum of 40 shoots per embryo in a period of about four months, and sometimes more with an increase of the time period. There was minimum of ten shoots per cotyledon, eight shoots from axillary buds, twelve shoots from the apical dome and ten shoots from the hypocotyl resulting in forty shoots per embryo.

MANPOWER TRAINING

Following persons were trained in micropropagation of Pine :

1.	Sahira Shakya	2.	Shyam K. Agrawal
3.	Jhalendra N. Shrestha	4.	Ganesh K. Agrawal
5.	Arvind K. Keshari	6.	Ram L. Singh
7.	Bina Sthapit	8.	Binay K. Yadav

SEMINARS ATTENDED

(1) Dr. V. P. Agrawal participated in the "International Workshop on Application of Biotechnology in Forestry and Horticulture" organized by National Science Foundation, USA from Jan 14-17, 1988.

(2) Dr. V. P. Agrawal attended the "Biotechnology Conference" organized by USAID from April 17-21, 1988, Washington, USA.

(3) Dr. M. Ranjit participated in the "Third International Plant Biotechnology Conference" held in Nairobi, Kenya from Jan 8-12, 1989.

(4) Mr. D. R. Bastola participated in the "International Symposium on Application of Biotechnological Methods and Recent Accomplishments of Economic Value in Asia" held in Bangkok, 5 - 9 November, 1989.

(5) Dr. V. P. Agrawal participated in the symposium "Molecular and Genetic Approaches to Plant Stress".

REFERENCES

1. Agrawal VP, Sahira Shakya S, Bastola DR and Joshee N (1991) Inverted embryo technique for *in vitro* propagation of Himalayan pines- *Pinus roxburghii and P. wallichiana*. Biotechnology Letters. 1: 14-19.

2. Aitken J, Horgan KJ and Thorpe TA (1981) Influence of explant selection on the shoot - forming capacity of juvenile tissue of *Pinus radiata*. Can J. Forestry Res. 11 : 112-17.

3. Aitken-Christie J and Thorpe TA (1984) Clonal propagation : Gymnosperms. In "Cell Culture and Somatic Cell Genetics of Plants" (I.K. Vasil, ed.), Vol. 1, pp. 82-95, Academic Press.

4. Aitken-Christie J, Singh AP and Davies H (1988) Multiplication of meristematic tissue : A new tissue culture system for radiata pine. In: Genetic Manipulation of Woody Plants (Eds. I. W. Hanover and D.E. Keathley), pp. 413-432. Plenum Press. N.Y.

5. Bajaj YPS (Ed.) (1986) Biotechnology in Agriculture and Forestry. Trees I. Springer-Verlag. N.Y.

6. Bhatnagar SP, Singh M N and Kapur N (1983) Preliminary investigations on organ differentiation in tissue cultures of *Cedrus deodara* and *Pinus roxburghii*. Ind. J. Expt. Biol. 21 : 524-526.

7. Biondi S and Thorpe TA (1982) Clonal propagation of forest three species. In : "Proceedings of the COSTED Symposium on Tissue Culture of Economically Important Plants". (Ed. A N Rao) pp. 197-204, COSTED and Asian Network of Biological Science, Singapore.

8. Biondi S and Thorpe TA (1982) Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot. Gaz. 143: 20-25.

9. Bonga JM (1977) Application of tissue culture in forestry, In "Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture" (Eds. J Reinert and YPS Bajaj), pp. 93-108, Springer - Verlag.

10. Bonga JM (1974) In vitro culture of microsporophylls and megagametophyte tissue of *Pinus*. In Vitro 9: 270-277.

11. Bonga JM (1981) Haploid tissue culture and cytology of conifers. In "Collogue International Sur La Culture *In Vitro* Des Essences Forestieres", pp. 283-293. AFOCEL, Nangis.

12. Bonga JM and Durzan DJ (1982) "Tissue Culture in Forestry" Nijhoff, The Haque.

13. Bormann CH and Jasson E (1980) Organogenesis in cultureed *Pinus sylvestris* tissue. Z. Pflanzenphysiol. 96: 1-6.

14. Cambell RA and Durzan DJ (1975) Induction of multiple buds and needles in tissue cultures in Picea glauca. Can J. Bot. 53, 1652-1657.

15. Cohen MA (1975) Vegetative propagation of P strobus by needle fascicles. Oc. Inter, Plant Prop. Soc 25, 413-419.

16. Cotter HVT, Adhikari MR and Raj JBH (1987) *Cronartium himalayense*, causal agent of Chir pine stem rust, discovered in Nepal. Plant Disease. 71: 761.

17. Dallimore W and Jackson AB (1966) A handbook of Coniferae and Ginkgoaceae. Edward Arnold Publishers, London.

18. David A (1982) Tissue Culture in Forestry (Eds. JM Bonga, DJ durzan) Martinus Nijhoff / Dr. Junk Publishers. The Haque pp. 72-108.

19. David A and David H (1977) Manifestations de diverses potentialites organogenes d'organes ou de fragments d'organes de Pin maritime (*Pinus pinaster* Sol) en cultiv in vitro. C.R. Acad. Sci., Paris 284, 627-630.

20. David A, David H, Eaye M and Isemukali K (1979) The cultivation in vitro and the micropropagation of the maritime pine (*Pinus pinaster* Sol.) in : "Micropropagation d'Arbes Forestieres", AFOCEL etudes et recherches, No. 12, 6/79, pp. 33-40. AFOCEL, Nangis.

21. Durzan DJ (1980) Plant Cell Culture: Results and Perspectives (Eds. F. Sala, B. Baris, R.Cella). Elsevier / North-Holland Biomedical Press. Amsterdum. pp 283-288.

22. Durzan DJ and Champbell RA (1974) Prospects for the production of improved stock of forest trees by cell and tissue culture. Can. J. Forestry Res. 4, 151-174.

23. Evans DA, Sharp WR and Flick CE (1981) Plant regeneration from cell cultures, In "Horticultural Review" (J. Hanick, ed.), vol. 3, pp. 214-314. The Avi Publishing Co.,

24. Girouard RM (1971) Vegetative propagation of *Pinus* by means of needle fascicles -a literature review. nform. Rpt. Environ., Can For. Ser. Quebec.

25. Greenwood MS ( ) Meristems in vitro by hypocotyl section from dormant *Pinus lambertiana* embryos. Can J. Bot. 43: 173-175.

26. Gresshoff PM and Doy CH (1972) Development and differentiation of haploid *Lycopersicum esculentum* (tomato). Planta 107: 161-170.

27. Horgan KJ (1982) The tissue culture of forest trees. Proc. Int. Symp. Natl. Acad. Sci., 9th, pp. 105-120

28. Horgan KJ and Aitken J (1981) Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. Physiol. Plant. 53: 170-175.

29. Howland AK and Howland P (1984) A dictionary of the common forest and farm plants of Nepal. Forest Research and Information Center, Dept. of Forest, Kathmandu.

30. Jalaska S, Koleveska-Pletikapic B and Cidakovic M (1981) Bud regeneration in *Pinus nigra* embryo and seedling tissue culture. In "Cologue International Sur La Culture in vitro Des Essences Forestieres", pp. 159-166. AFOCEL, Nangis.

31. Joshee NB and Agrawal VP (1990) Commercial potential of Inverted Embryo Technique for In Vitro Propagation of Pinus roxburghii. In "Agricell Report : A plant tissue culture newsletter" 14 : 28.

32. Kaul K (1985) Factors influencing in vitro micropropagation of White Pine. In : Genetic Manipulation of Woody Plants. 1988. I.W. Hanover and D.E. Keathley, Eds.). Basic Life Sciences Vol. 44, pp. 473, Plenum Press. N.Y.

33. Minocha SC (1980) Callus and adventitious shoot formation in excised embryos of white pine (Pinus strobus). Can J. Bot. 58, 366-370.

34. Mott RL (1978) Tissue culture propagation of conifers. In : "Propagation of Higher Plants through Tissue Culture - A Bridge between Research and Application" (K.W. Hughes, R. Henke and M. Constantin, Eds.), pp. 125-133. Tech. Inf. Cent., U.S. Dept. of energy, Springfield, Virginia.

35. Mott RL (1981) Trees. In : "Cloning Agricultural Plants via *In Vitro* Techniques" (B.V. Conger, ed.), pp. 217-254. CRC Press, Boca Raton, Florida.

36. Mott RL and Amerson HV (1981) A tissue culture for the clonal production of loblolly pine plantlets. N.C. Agric. Res. Serv. Tech. Bull, 271: 1-14.

37. Mott RL, Amerson HV (1981) Tissue Culture plantlets from *Pinus monticola* embryonic materials. For. Sci. 27(2) 299-304.

38. Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol. 15: 473-479.

39. Puri GS (1969) Indian Forest Ecology, Vol I, Oxford Book and Stationary Co. New Delhi.

40. Quoirin M and Lepoivre P (1977) Etudes de miliex adaptes aux cultures *in vitro* de prunus. Acta Bortica. 437-442.

41. Reily KJ and Washer J (1977) Vegetative propagation of radiata pine by tissue culture : plantlet formation from embryonic tissue. New Zealand J. Forestry Sci. 7, 199-206.

42. Schenk RU and Hilderbrandt AC (1972) Medium and techniques for induction and growth and monocotyledons plant cell culture. Can. J. Bot. 50: 199-204.

43. Shakya S, Joshee N and Agrawal VP (1992) Commercial potential of inverted embryo technique for *in vitro* propagation of *Pinus wallichiana*. Ban Ko Jankari, 3: 29-31.

44. Sommer HE and Brown CL (1974) Plantlet formation in pine tissue culture. Am. J. Bot. 61: 11.

45. Sommer HE, Brown CL and Kormanik PP (1975) Differentiation of plantlets in long-leaf pine (*Pinus palustris* Mill.) tissue cultured *in vitro*. Bot. Gaz. 136: 196-200.

46. Sommer HE and Brown CL (1979) Application of tissue culture to forest tree improvement. In : "Plant Cell and Tissue Culture : Principles and Applications" (Eds. WR Sharp, PO Larsen, EP Paddock and V Raghvan), pp. 451-491. Ohio State Univ. Press, Colmbus.

47. Stainton JDA (1972) Forests of Nepal. John Murry, London.

48. Thorpe TA and Biondi S (1983) Conifers. In "Handbook of Plant Cell Culture" (Eds DA Evans, WR Sharp, PV Ammirato and Y Yamada) Vol. 2 Macmillan, New York.

49. Tominga Y and Oga K (1970) Cytological studies on the calli *of Pinus densiflora in vitro*. Hiroshima Jap. Agr. Coll. Bull. 4, 8-10.

50. Uphof JC Th (1968) Dictionary of Economic Plants, Verlag von J. Cramer.

51. Weier T, Stocking CR, Barbour MG and Rost TL (1982). Botany : An Introduction to Plant Biology. Sixth edition, pp. 609, John Wiley and Sons.

52. Winton L (1978) Morphogenesis in clonal propagation of woody plants. In "Frontiers of Plant Tissue Culture" (Ed. TA Thorpe), pp. 419-426. Univ. of Calgary Press, Calgary.