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CLONAL PROPAGATION OF PINE TREE PROJECT

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WINROCK INTERNATIONAL INSTITUTE FOR AGRICULTURAL DEVELOPMENT

AND

THE RESEARCH LABORATORY FOR AGRICULTURAL BIOTECHNOLOGY AND BIOCHEMISTRY, SANEPA KATHMANDU, NEPAL

PREPARED BY:

Dr. John De Boer
Dr. V.P. Agrawal
Mr. Janak Shrestha

USAID OFFICE OF SCIENCE ADVISOR
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ACKNOWLEDGMENT

All staff of the CPPT project have helped in the preparation of this report. Dr. V. P. Agrawal, Principal Investigator, of this project had primary responsibility for updating the research results, editing and completing the report.

Thanks also to ARPP/WI Chief of Party, Dr. A. John De Boer for overall project management and also proof reading of the report.

Special credit goes to Mr. Janak Shrestha, Administrative Assistant of WI/PVBP project for word processing and production of the report.

B

I. INTRODUCTION

Every year in the monsoon season, Nepal has to face many problems through flooding. Erosion of topsoil makes the lands unusable and causes low intensity of cultivation and low productivity of the land. Therefore, reforestation in Nepal is most important and superior clones adaptable to poor soil and harsh climate in the mountains of Nepal must be used for reforestation.

This project has as its goal to define and develop tissue culture technology that would allow mass propagation of superior pine seedlings suitable to the climate of Nepal. The overall aim of this project is to start a tree tissue culture laboratory for clonal propagation of superior forest trees native to the Indian subcontinent

The total grant to carry out this research project is estimated at US $ 121,996.00 for the period of this grant (August 31, 1987 through August 30, 1991) for salaries, honorariums, equipment, training, travel, per diem, materials, supplies and other direct costs. The project activities during Aug. 31, 1987 through February, 1989 are described in the following chapters.

II. PROJECT AIMS AND SPECIFIC OBJECTIVES

In most countries of the world, forests are important both ecologically and economically. Forest products play a significant role in international trade. Due to mismanagement of forest resources in several developing countries, serious deforestation has resulted. In Nepal, desertification due to deforestation has posed serious problems of erosion of topsoil and water losses in irrigation. Every year the monsoon causes serious soil erosion in Nepal by causing heavier flooding not only in Nepal, but also in its neighboring 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 problem for the India sub-continent as a whole. In order to fight against desertification, adequate measures should be taken for mass propagation of forest trees indigenous to the Indian subcontinent and especially to Nepal.

In Nepal, with the help of international organizations, campaigns have already been launched for reforestation and soil conservation. To date, however, efforts in tissue culture research of forest trees have yet to be initiated. After reviewing this situation, the CPPT project has been established as a research project. This project aims 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 <u>Pinus roxburghii and Pinus</u>

wallichiana. The overall aim of this project is to start a tree tissue culture laboratory for clonal propagation of superior forest trees native to the Indian subcontinent. This project 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. If the task to generate plantlets from callus cultures of pine trees, a goal not realized as yet, is accomplished then the protoplast culture techniques can be used to obtain improved genotypes, which might provide greater disease resistance or suitability for growth in a particular soil or climate.

Findings of this project should be of interest to the research community in the area of tissue culture. These findings would have the potential for use in mass propagation of forest trees and could initiate a plethora of research activities in tissue culture of pine trees and other conifers in less developed countries. Hopefully, the united research efforts in cooperation among these research groups would strengthen afforestation programs worldwide.

This research project is particularly relevant to the needs of Nepal, which is confronted by serious deforestation problems. This project will certainly enhance the capability of Nepalese scientists to do innovative research in tissue culture of forest trees by helping train the appropriate man-power and by increasing their own level of self confidence.

Finally, the objectives of this project are compatible with the policy of AID to stimulate new and innovative research on problems that confront developing countries.

III. FINANCE AND BUDGET

The following is the estimated budget for CPPT project including local cost items. Revision was made to allocate some over expenses of the original budget as shown below:

(a) BUDGET LINE ITEM IN DETAIL

COST ELEMENT IN U. S. $	ORIGINAL BUDGET	REVISED BUDGET
Salaries and wages	18,820.00	16,320.00
Equipment	35,504.00	62,100.00
Materials and supplies	20,000.00	13,000.00
Travel and Per diem	25,000.00	10,000.00
Other direct cost	4,800.00	2,704.00
Total direct cost	104,124.00	104,124.00
VI/Administrative fee	17,842.00	17,842.00
Grand total	121,966.00	121,966.00

(b) EXPENDITURES

The total budget of this project is US $ 121,966.00 and since the project was initiated in August 1987 through February, 1989, CPPT project has spent US $ 105,169.65 including local and Headquarters expenses, or almost 86.13% of the total funds.

A summary of expenses is shown in the following table:

Table No. 1

Expenditures incurred as of February 29, 1989 are : ($):

EXPENDITURE LINE ITEMS	ESTIMATED BUDGET	TOTAL EXPENSES	BALANCE	REMAINING PERCENTAGE
1. SALARIES & WAGES	16320.00	3284.14	13035.86	79.88%
2. EQUIPMENT	62100.00	65069.84	-2969.84	-4.78%
3. MATERIALS & SUPPLIES	13000.00	13819.44	-819.44	-6.30%
4. TRAVEL & PER DIEM	10000.00	4950.03	5049.97	50.50%
5. OTHER DIRECT COST	2704.00	3915.67	-1211.67	-44.81%
TOTAL DIRECT COST	104124.00	91039.12	13084.88	12.57%
6. WI/MANAGEMENT FEE	17842.00	14130.49	3711.51	20.80%
GRAND TOTAL	121966.00	105169.61	16796.39	13.77%

Above statement shows that there are over expenses on some line items. These over expenses were due to heavy custom duty paid on the equipment line which were imported from WI/Headquarters USA.

IV. INTERNATIONAL SCIENTIFIC COLLABORATION

Professor Dr. Don Durzan, Department of Pomology, University of California, Davis is the advisor to this project. Dr. Ralph Mott, North Carolina State University, Dr. Harry Sommer, University of Georgia, and Dr. S.S.Bhojwani, University of Delhi are the collaborators. The Principal Investigator has been continuing correspondence with these scientists to exchange ideas, to assess the potential scientific research on existing project and to coordinate with the problems on research findings.

V. PROJECT ADMINISTRATION

This "Pine Tree Project" is under the administration of Winrock International. The WI supervisor Dr. A. John De Boer has been constantly monitoring the grant with close supervision and direction to the Administrative Assistant to administer the whole grant. The institutional authority to this grant is invested in Dr. A. John De Boer or S.S. Bal, the WI Chief of Party in Kathmandu and Deputy Chief of Party, respectively under the Agricultural Research and Production Project.

VI. PERSONNEL (Local)

On recommendation from Principal Investigator of this project, a research assistant, research associate and a lab boy for the project have been appointed on a temporary basis to assist the investigator in research work. The staff appointment work for CPPT project was completed by January 1, 1989, according to grant approved salary scale. The following staff are working in CPPT lab.

CPPT MEMBER STAFF

1. Dr. V. P. Agrawal, Principal Investigator
2. Dr. Mukunda Ranjit, Co-Investigator
3. Dr. Nirmal Joshee, Research Associate
4. Mr. Dhundy Bastola, Research Assistant
5. Mr. Man B. Tamang, Lab boy

VII. COMMODITIES PROCUREMENT & INVENTORY

Following equipments and chemicals were procured under this project and issued to CPPT lab. Equipments which were ordered from Winrock International Headquarters has arrived and issued to lab and experiment work has been conducted with these facilities.

A. WI/HQ EQUIPMENT PROCUREMENT

ITEM	PARTICULARS	QUANTITY
1.	AIR CONDITIONER MODEL #A1109EH 5C	1
2.	REFRIGERATOR MODEL #KNT16ASE, 16 CU.FT. CAP	1
3.	MICRO WAVE OVEN MODEL KMK76 V	1
4.	COMPAQ DESKPRO, 640K (2) 360K 51/4" DRIVES	2
5.	MONOCROME MONITOR	2
6.	COMPAQ GRAPHICS+ CARD	2
7.	8087 MATH CO-PROCESSOR CHIPS	2 ,,
8.	AST/0 MINI CARD CLOCK ACL/SER/PARA	2 ,,
9.	IBM PC-DOS 3.2 (5.25" FORMAT)	2 ,,
10.	COVER SHEET FOR CPU MONITOR, KEY BOARD	2
11.	EPSON FX 286E PRINTER WITH TRACTOR	1
12.	EPSON FX 86E PRINTER WITH TRACTOR	1
13.	PARALLEL PRINTER CABLE - IBM	2
14.	EPSON FX86E RIBBONS	10
15.	EPSON FX- 86E RIBBONS	10
16.	14 7/8X11 INCH PIN FEED PAPER	1 box
17.	DISKETTE HEAD CLEANING KIT	2
18.	DSDDL 5.25" DOSKS	2 BOX
19.	WORDSTAR PROFESSIONAL 4.0	1
20.	LOTUS 1-2-3	1
21.	PORTABLE ALTERNATOR (GENERATOR)5000WATTS	1
22.	4 WAY HEAVY DUTY OUTDOOR CORD 25 FT.	25 FT.
23.	HEAVY DUTY YELLOW EXTENSION CORD # 7468-K-14	25 FT.
24.	SHAKER SUPER MIXER CAT # 8294-F15	1 EA
25.	STIRS-HOT PLATE, SP 13420 CAT # 8614-R14	1 EA
26.	IDENTI-PLUGS, 21-26MM PKG 400 CAT #9242-F25	2 EA
27.	SP0508, PUMP FILTER UNIT CAT #4617-B10	1 EA
28.	2004020 BX100 FILTER 0.02 UN, CAT #4618-B10	1 BOX
29.	STIR BAR 3/8 X 11/2 INCH, CAT #8608-C08	3 EA
30.	NANOPURE ULTRAPURE WATER SYSTEM CAT #3986-21	1 EA
31	BRINKMAN MICRO CENTRIFUGE CAT # 3514-4A	1 EA
32.	MICRO-CENTRIFUGES FOR ABOVE PK/100	1 CS
33.	MICROSCOPE, STEREO BOX J 3903-00	1 EA
34.	TRANSFORMER J 1578-05	1 EA
35.	LAB CART/RAIL SS J 9363-00	1 EA

Contd...\...

ITEM	PARTICULARS	QUANTITY
36.	RACK, WHT, 25MM TUBES J 6737-80 5 CASE	40 EA
37.	RACK, WTH, 16MM TUBES J 6737-60	40 EA
38.	RACK, WHT, 13MM TUBES J 6737-50	40 EA
39.	MACROPIPETT 2-10ML J 7859-40	2 EA
40.	HEATER 1 - BLOK J 3129-02	1 EA
41.	HEATER BLOK 20 HOLES J 3132-00	1 EA
42.	HEATER BLOK 12 HOLES J 3133-00	1 EA
43.	HEATER BLOK 6 HOLES J 3135-00	1 EA
44.	THERMOMETER -20-15-C J 8128-98	1 EA
45.	WATER BATH J 1603-10	1 EA
46.	TIPS, DISP 2-10 ML 200//BG J 7859-42	5 EA
47.	FINN, DGTL 40-200UL J 6247-54	1 EA
48.	FINNTIP 1-200UL, 1000CS J 6247-82	1 CS
49.	OVEN, LAB 1.3 CU FT J 5015-56	1 EA
50.	TRANSFORMER J 1578-05	1 EA
51.	EVAP, MOTOR/STD J 1603-00	1 EA
52.	CONDENSER, STD GLASS ST J 1603-15	1 EA
53.	DIURNAL GROWTH CHAMBER 15.5 CU.FT. INCUBTOE	1 EA
54.	CHARGE FOR 220V, 50HZ CONVERSION	1 EA
55.	PAIRS OF SPARE LIGHTS	3 EA
56.	SPARE PARTS KIT #99998 FOR MODEL 3740/3744	1 EA
57.	BARNSTEAD AUTOCLAVE, MOD #N9060 01 CAT #A9066-2	1 EA
58.	SARTORIOUS BALANCE CAPACITY 0.1GRM.	1 EA
59.	PH METER FOR 230V 50/60HZ, MOD 800 CAT #H3700-9	1 EA
60.	COMBINATION ELECTRODES WITH GEL FILLED	2 EA
61.	BOROCILICATE GLASS CULTURE TUBES CAT #T-1310-13	5 CS
62.	,, ,, ,, WITH SCREW CAT #T-1346-10	3 CS
63.	,, ,, ,, ,, ,, CAT #T-1346-1	1 CS
64.	,, ,, ,, ,, ,, CAT #T-1346-6	1 CS
65.	HANGING PAN BALANCE SEN. 0.05GM CAT #B-1800-37	1 EA
66.	WEIGHTS FOR PAN BALANCE CAT #B-1245-110	1 EA

B. EQUIPMENT LOCALLY PROCURED

S.NO.	PARTICULARS	QUANTITY
1.	pH METER -PORTABLE, WTW-PH 90/ GERMANY	1 SET
2.	COMPUTER TABLE	1 ,,
3.	SUNMICA TOPPED TABLE WITH RACK, SLIDING DOOR	2 ,,
4.	MARBLE TOPPED TABLE FOR MICRO BALANCE	1 ,,
5.	SUNMICA TOPPED TABLE SMALL SIZE	1 ,,
6.	LAMINAR FLOW HOOD VERTICAL	1 ,,

C. WI/HQ CHEMICAL PROCUREMENT

	DESCRIPTIONS	QUANTITY
1.	AGAR A. 7002	5 KG
2.	6-BENZYLAMINOPURINE B.6750	5GX3
3.	KINETIN K. 2751	1 GM
4.	IBA I. 1875	25X3
5.	IAA I 1250	25 GM
6.	NAA N0375	100 GM
7.	MYOINOSITAL 15125	500 ,,
8.	,, 2- IPA D0636	1 ,,
9.	PYRIDOXINE P. 9755	25 ,,
10.	NICOTINIC ACID N 4126	100 ,,
11.	THIAMINE T 4625	100 ,,
12.	TWEEN 80 P 1379	100 ML
13.	TWEEN 80 P 1754	100 ML
14.	2, 4-D D4517	100 GM
15.	GIBERALLINS G 3250	5 ,,
16.	MANDELONITRILE M 7010	25 ML
17.	SEPHADEX G 100- 120	50 GM
18.	CM- SEPHADEX C 25-120	50 ,,
19.	DEAE-SEPHADEX A 25-120	50 ,,
20.	LH-20-100	5 ,,
21.	BLUE DEXTRAN D5751	1 ,
22.	NITROPHENYLE-B-GLUCOPYRANOISIDE N1377	100 ,,
23.	,, ,, N7005	100 ,,
24.	M 4772 MOLECULAR BIOLOGY OF PLANT MANUAL	1 25.
25.	P 9914 PLANT MOLECULAR BIOLOGY VOLUM 188	1
26.	P 6150 PLANT CELL CULTURE	1

D. CHEMICAL LOCALLY PROCURED

	DESCRIPTIONS	QUANTITY
1.	TIN FOIL	4 RLS.
2.	CHLOROX	500.ML.
3.	CHLOROFORM	10 LTR.
4.	HEXANE	10 ,,
5.	METHANOL	10 ,,
6.	SPIRIT	5 ,,
7.	ALUMINIUM TAG	1000 PCS
8.	CHLOROFORM CR	5 LTR
9.	SPIRIT	10 LTR
10.	PLASTIC REGENT BOTTLE	12 PCS
11.	CHLOROX 470 ML	6 BTL

VIII. OVERVIEW OF PROGRESS

The principal objective of this project is to establish a tree tissue culture laboratory for clonal propagation of superior forest trees native to the Indian subcontinent.

Winrock International, as the grantee, bears responsibility to successfully achieve this objective. Although in the original grant proposal submitted by the PI, it was proposed to carry out this research in Chemistry Department, Tribhuvan University. However, this was not possible due to lack of basic necessities for research like water, electricity, work space and accessibility during off hours. Therefore, in consultation with the PI, WI decided, in the best interest of the grant, to start the project in Research Laboratory for Agricultureal Biotechnology and Biochemistry (RLABB) of which the PI is the director. In order to facilitate pine tree research in RLABB, WI executed two MOU's with RLABB (see appendix I and II).

Since then, WI is providing the Principal Investigator all the facilities needed for successful completion of the project; these facilities include procurement, administration, accounting, transportation, telex facilities etc. In keeping with the principal objective of this project to create a tree tissue culture laboratory, WI furnished and equipped the Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB) for this purpose.

Some of the important achievements in this regard are briefly summarised as below:

1. Repair work such as white-washing, electrification, installation of generator and air conditioner have been completed

2. Now RLABB has its own IBM Compaq computer with the UPS system and other accessories.

3. After waiting for a long period equipments and glasswares that had been ordered on November 1, 1987 arrived in October 1988 from the US. Equipment included growth incubator, autoclave, water purification system, single pan balance, pH meter, microwave oven, air conditioner, generator etc...

4. After a long search, Dr. Nirmal Joshee was appointed as Research Associate on January 1, 1989.

5. Construction of growth room which began in Nov. 1988 was completed in March 1989, which is currently under use.

6. Necessary lab equipment, chemicals and glasswares have been procured

7. In addition to the locally built laminar flow hood, another laminar flow hood has been procured and installed in the lab.

8. Furniture and other office supplies have been procured.

9. Principal Investigator Dr. V. P. Agrawal participated in the "International Workshop on Application of Biotechnology in Forestry and Horticulture" sponsored by National Science Foundation, USA from january 14 - 17, 1988. He also attended the "Biotechnology Conference" organized by AID (April 17 - 21, 1988) in Washington, D. C.

10. Co-Investigator Dr. Mukunda Ranjit also participated in the "Third International Plant Biotechnology Conference" held in Nairobi, Kenya from January 8 - 12, 1989. All the travel expenses were supported by CPPT funds.

11. Significant progress regarding tissue culture of pines has been made (see technical progress report by the PI).

12. On insistence from USAID/ Nepal the PI has also started tissue culture work on multipurpose trees like Artocarpus lakoocha, Ficus nemoralis,etc.

13. In last 3 months many important persons have visited the CPPT facilities and indicated their satisfaction with the progress: notables are Director, Mr. David Wilson, USAID/Nepal, Dr. D.S. Athwal, Vice-president, WI; Secretary, HMG/N; Ministry of Agriculture, Mr. S. S. Mahdi, Resident Representative, FAO; etc..

IX. TECHNICAL PROGRESS REPORT FOR CLONAL PROPAGATION OF PINE TREE PROJECT

MARCH 1, 1988 THROUGH FEBRUARY 29, 1989

BY

DR. V. P. AGRAWAL

PRINCIPAL INVESTIGATOR,

WI- CLONAL PROPAGATION OF PINE TREE PROJECT

A. SUMMARY

Factorial experiments using combination of various levels of cytokinin (N^6-Benzylaminopurine,BAP) and auxin (Naphthalene acetic acid, NAA) in MS, LP, GD, and SH media were conducted to determine the best experimental conditions (media and levels of phytohormones) for optimum shoot bud induction in *in vitro* grown cotyledons of *P. roxburghii*. Results from these experiments suggest that shoot buds in *P. roxburghii* cotyledons (*in vitro*) can be efficiently initiated in LP media containing 5 ppm BAP as compared to MS,GD, and SH. Presence of charcoal (1% w/v) in basal LP media enhances shoot proliferation. One batch of elongated shoots of *P. roxburghii* has been processed for root induction. Although LP is the right medium for shoot bud induction in *P. roxburghii in vitro* cotyledons, callus induction is easier in MS containing NAA or 2, 4-D; NAA results in friable and green callus as compared to 2,4-D wherein callus is pale white and compact.

For *P. wallichiana in vitro* explants factorial experiments are yet to be done, but preliminary results suggests that shoot bud induction takes place within 21 days in LP with BAP. For *wallichiana in vivo* explants green and friable callus is induced at cut ends in LP containing 0.1 ppm NAA.

B. FUTURE PROGRAM

Since shoot induction has not worked well consistently in our lab, the effect of age of the seeds, period of germination, level of sucrose , level of amino acids and mixed phytohormones, vitamins and coconut milk in shoot bud induction will be tried. In the next six months, a minimum of 2000 explants each of *Pinus roxburghii* and *Pinus wallichiana* will be processed. At the same time most ideal rooting substrates will be finalized through various experiments.

C. MATERIALS AND METHODS:

1. Explant:

Three methods , differing mainly in sterilization procedure were used to obtain *in vitro* and *in vivo* explants for regeneration studies (see illustrations in Fig I and II and Flow diagrams on pages).

2. Description of Media:

Four kinds of media were tested for tissue culture research in *Pinus roxburghii* and *Pinus wallichiana*. These are MS, SH, GD, & LP. Compositions of these media are given in Table I.

3. Experiments with P. roxburghii

A. Induction of shoot buds:

For shoot bud induction cotyledons obtained following method II were placed on different media containing combinations of various levels of BAP and NAA; altogether 24 such combinations were chosen (factorial experiment) as shown in Table II.

Explants in these experiments were observed for 21 days. Cultures were provided with 16 h day and 8 h night period in the incubator (Forma Scientific).A temperature of 27_oC and 22_oC was maintained in the day and night period, respectively. Light intensity of approximately 2000 lux was used. Appearance of rough surface (protuberances) on explants was assumed as an indicator of shoot bud induction.

B. Shoot Elongation and multiplication:

After initial shoot bud induction, explants were transferred to basal media for elongation. As elongation is an energy demanding process, concentration of sucrose was increased to 4% from 3%. Two sets of experiments were conducted to find out the role of gibberellic acid and activated charcoal in the process of elongation. Gibberellic acid was incorporated in three different concentrations ie: 1,10, 100 ppm into the basal media. Since ultrafiltration system through millipore filters is not available , media containing gibberellic acid

were autoclaved. Buds were kept and incubated in these media for a period of 4 weeks.

In another set of experiments activated charcoal was added to the basal medium at a concentration of 1 %. Buds were incubated for a period of 4 weeks and then transferred to basal media.

Shoot buds thus obtained were cut into small pieces and transferred to hormone free media. This was done to multiply and grow buds at a faster rate.

C. Root Initiation:

Elongated shoots (10-15 mm) were transferred to rooting medium containing IBA (1mg/lit), NAA (0.5mg/lit) and agar (1 % in water). Elongated shoots were exposed to this medium for 7-10 days.

D. Experiments with in vitro plants .

Cotyledons, hypocotyls and plumules obtained from in vivo grown P. roxburghii seedlings were incubated in LP,SH and MS media containing various phytohormones alone or in combination: LP (BAP - 0.5,1,2 and 5 ppm);MS (2,4-D - 2 and 5 ppm) and SH (BAP - 0.5, 1, 2 and 5 ppm alone and with 0.1 ppm NAA)

4. Experiments with *P. wallichiana*

A. *In vitro experiments* :

Cotyledons obtained by methods II and III were incubated in SH and LP containing BAP - 0.5,1,2 and 5 ppm.

B. *In vivo experiments:*

Cotyledons, hypocotyls and plumules from in vivo grown seedlings were incubated in LP medium containing BAP- 0.5,1,2 and 5 ppm alone and in combination with 0.1 ppm NAA.

D. RESULTS AND DISCUSSION

PINUS ROXBURGHII:

Pinus tissue culture can be divided in following phases:-

(i) Shoot bud induction phase (21 days incubation)

(ii) Bud elongation phase (21 days incubation)

(iii) Root induction phase

(iv) Rooting, hardening and transplantation.

In Pinus roxburghii tissue culture success has been achieved in the (i) and (ii) stage and few elongated shoots are in stage (iii).

All the four media which were initially tried gave varied results. In vitro cotyledons placed on different media gave following results after an incubation period of 21 days:-

1. MS medium:- Factorial experiments involving combination of various levels of NAA and BAP demonstrated that (a) callus formation was observed in all treatments containing NAA (0.1 to 1 ppm), (b) excessive elongation and swelling of the explants took place without NAA and (c) no treatment could induce shoot bud formation within 21 days (Table III).

2. SH Medium:- Table IV reveals the pattern of responses of explants to various treatments in SH media. Shoot bud induction was not seen in any of the treatments . Callus formation restricted to the cut ends alone was induced by 0.5 ppm NAA in combination with 0.1 to 5 ppm BAP.

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

4. LP Medium: LP medium yielded most favorable results among all the media tested. Shoot buds were produced in LP medium containing BAP (1.0, 1.25 and 5.0 ppm),thus revealing the role of cytokinin in this process. Shoot buds become evident as small protuberances after 14 days of incubation . They were allowed to stay and grow in the same medium till 21st day. High BAP concentrations (10 ppm) adversely affected shoot bud induction.

Treatments with NAA alone or in combination with BAP resulted in callus. Callus formation was not profuse and was usually restricted to the cut end regions of explants (Table VI).

Results obtained from these experiments indicate clearly that for shoot bud induction in cotyledons of Pinus roxburghii LP medium containing BAP is the most appropriate one. On the other hand most suitable media for callus induction in P. roxburghii cotyledons is MS containing NAA.

These results were observed during the first 21 day incubation period. In some instances, explants that did not respond during this period developed shoot buds in second or even in third 21 day incubation on basal medium with or without charcoal. Therefore results described here should be taken with due consideration.

Results on Elongation Experiments:

After initial shoot bud induction explants were transferred to basal media for elongation. As elongation is an energy demanding process, concentration of sucrose was increased to 4% from 3%. Two sets of experiments were conducted to find out the role of gibberellic acid and activated charcoal.

Effect of Gibberellic acid: Three regimes of gibberellic acid (1,10 and 100 mg/lit) were tried to assess its role in the process of elongation but contrary to established role of gibberellic acid no effect was noticed. It could be because of the loss of gibberellic acid during autoclaving of the media as millipore filter system was not available with us to sterilize the gibberellic acid solution.

Effect of Charcoal: In order to determine the role of charcoal in shoot proliferation, cotyledons with protuberances were incubated for a period of four weeks in basal LP medium containing activated charcoal (1% w/v). Explants placed on media with and without activated charcoal exhibited distinct trends in

relation to increasing BAP concentration used for shoot induction. Media having charcoal resulted in better proliferation of shoot buds when explants had been incubated in relatively higher concentrations (1.25 and 5 ppm) of BAP (Photograph I). On the contrary , charcoal inhibited the growth of explants that had been incubated with BAP concentration 1 ppm or less. However, explants from 1 ppm BAP when inoculated in the medium devoid of activated charcoal showed better results than those in medium with charcoal (Photograph II).

Shoot buds thus obtained were cut into small pieces and transferred to basal LP media for elongation and multiplication (Photograph III).

<u>In vivo</u> results (<u>P. roxburghii</u>)

i) With SH containing BAP neither shoot bud nor callus induction was observed.

ii) In MS with 2,4-D (2 to 5 ppm) white and compact callus was seen in three weeks (Photograph IV).

iii) In basal LP callus was observed whereas in LP containing BAP no noticeable change was found in 21 days.

PINUS WALLICHIANA

In vitro:

i) In LP all concentrations (0.5 to 5 ppm) of BAP tested induced shoot bud formation within 21 days.

ii) In SH with 1 and 2 ppm BAP callus was obtained. Higher BAP did not elicit any response.

In vivo

i) In LP with 0.1 ppm NAA green and friable callus was seen only at cut ends in a period of three weeks.

E. A GENERAL ACCOUNT OF VARIOUS EXPERIMENTS DONE TO DATE :

The first factorial experiment was initiated on July 4, 1988, using NAA (0.1, 0.5 and 1.0 ppm) and BAP (0.1, 0.5.0 and 10 ppm) in LP medium. But in this experiment no results could be obtained because of severe contamination.

The next experiment was initiated on August 24, 1988 by seed germination. Seeds were sterilized in 20% chlorox for 15 min. After cold treatment for 16 hrs. at 7°and removing seed coat from the seeds, they were placed on 2% agar with water in a petri-dish for germination at 27°n dark. After 5 days of incubation cotyledons were excised and used as explant. These cotyledons were transferred to LP media (factorial design). Explants were subcultured at regular intervals and are being maintained. First subculture which was done on Sept.9 , was on LP basal media containing 1% activated charcoal , 4% sucrose and 0.8% agar. After this, inoculations of P. roxburghii cotyledons

were done on different media at regular intervals. Explants that yielded shoot buds were transferred after a total incubation period of 21 days to basal media with activated charcoal. Experiments were started on Nov.25 (MS1/2 media), Nov. 28 (MS full), Dec. 9 (SH media), Dec.11 (GD media), Dec.25 (MS1/2) and Dec. 26 (MS media). All these experiments were done on factorial design basis.

Experiments which were done in 1988 were observed at regular intervals. Whatever explants that responded to BAP treatments within 21 days were transferred to basal LP media with charcoal. In response to certain treatments calli were also obtained which are being maintained.

By the end of 1988 superiority of LP media was established over other culture media. So in 1989 all the experiments were done in this medium. At the same time seeds of both Pinus spp. were also grown in pots in the green house. Seed sowing was done during 1st week of January 1989. For germination both the species take 20-25 days. With the help of these seedlings some experiments were carried out taking in vivo explants at different growth intervals.

To date, 21 experiments have been done on Pinus roxburghii and Pinus wallichiana. A brief summary of various cultures including their history has been given in table VII.

TABLE I

COMPOSITION OF VARIOUS MEDIA USED FOR PINE RESEARCH

Major	MS mg/lit	LP mg/lit	SH mg/lit	GD 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				
$FeSO_4.7\ H_2O$	27.8	30	15	30
$Na_2\ EDTA.2H_2O$	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
$ZnSO_4.7\ H_2O$	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
$CoCl_2.6\ H_2O$	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	1000	1000	1000
Sucrose	30,000	30,000	30,00	20,000
Agar	1 %	1%	1%	1%
pH	5.7-5.8	5.7-5.8	5.7-5.8	5.7-5.8

Table II

FACTORIAL DESIGN USED FOR SHOOT INDUCTION IN PINE

NAA	BAP—>	0	.5	1	1.25	5	10
0		0,0	0,.5	0,1	0,1.25	0,5	0,10
0.1		.1,0	.1,.5	.1,1	.1,1.25	.1,5	.1,10
0.5		.5,0	.5,.5	.5,1	.5,1.25	.5,5	.5,10
1		1,0	1,.5	1,1	1,1.25	1,5	1,10

Concentrations in mg/lit.

TABLE III

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN MS MEDIA

BAP ->>

NAA	0	0.5	1	1.25	5	10
0	ONLY ELONGATION & SWELLING					
0.1	CALLUSING AT CUT ENDS					
0.5	CALLUSING ALL ALONG THE EXPLANT SURFACE					
1.0	CALLUSING ALL ALONG THE EXPLANT SURFACE					

TABLE IV

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN SH MEDIA

	0	0.5	1	1.25	5	10
0	ONLY ELONGATION & SWELLING					
0.1	CALLUS	SWELLING				
0.5	ELONGATION	CALLUS				ELONGATION & SWELLING
1.0		NO RESPONSE			ELONGATION	

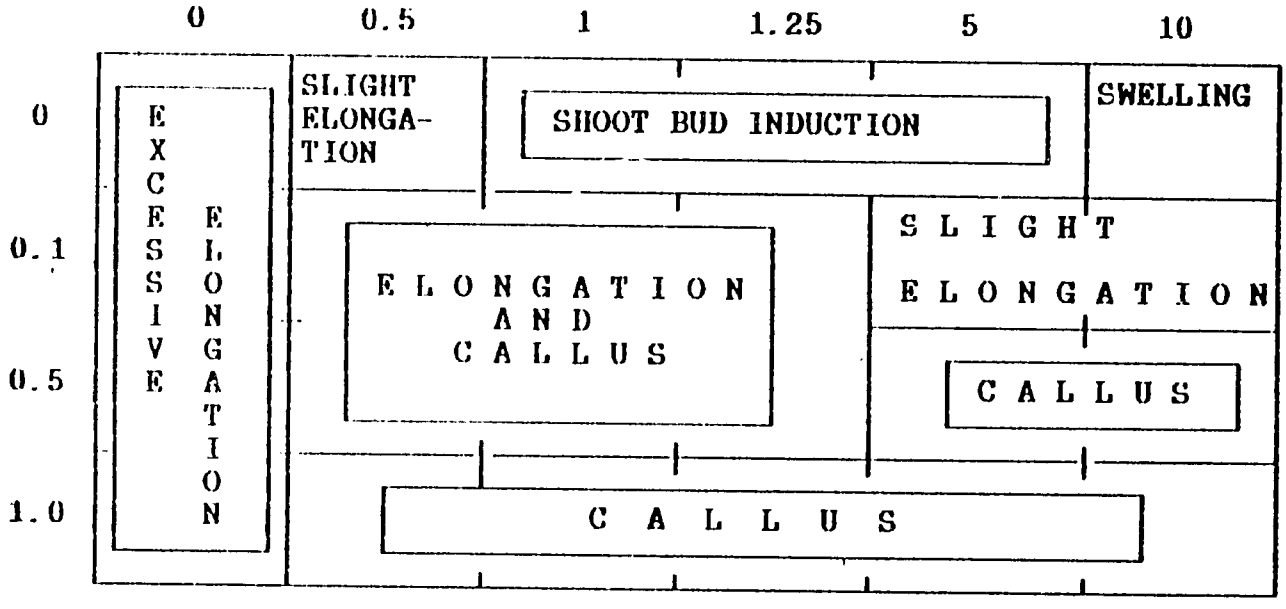
TABLE V

FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN GD MEDIA

	0	0.5	1	1.25	5	10
0	ELONGATION & SLIGHT SWELLING					
0.1	ELONGATION & SWELLING					
0.5	ELONGATION					
1.0	ELONGATION					

TABLE VI

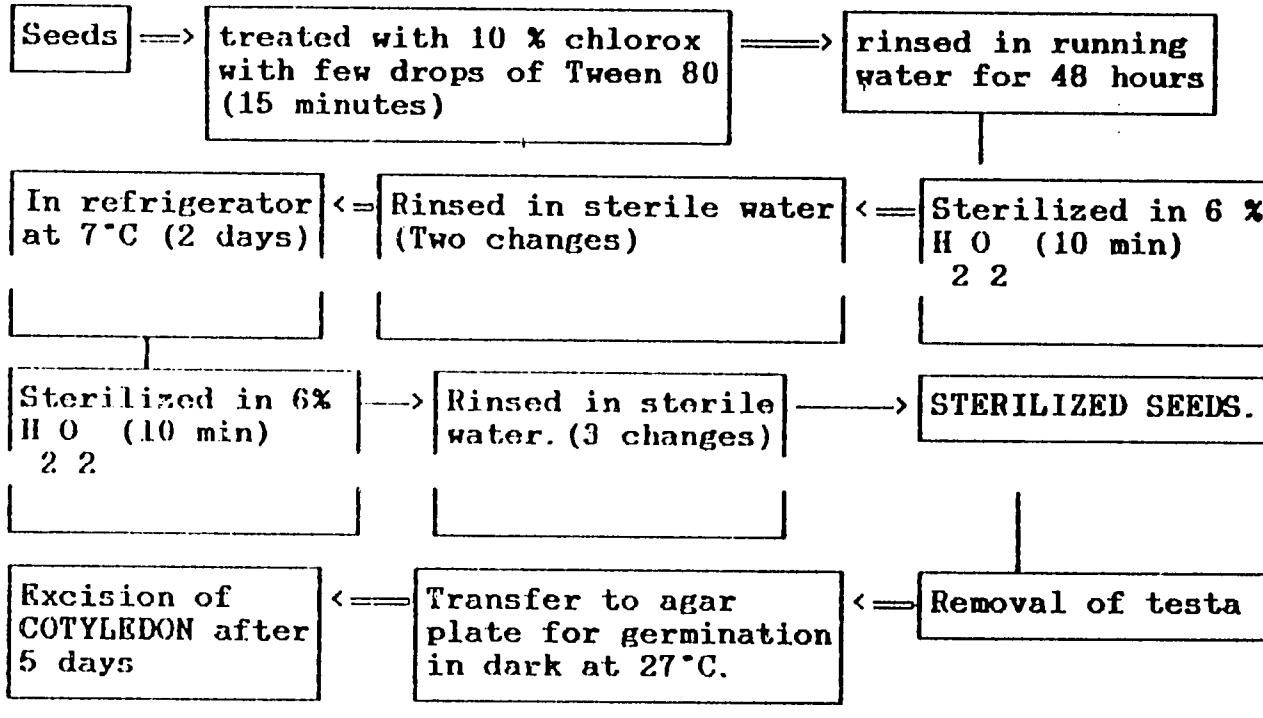
FACTORIAL EXPERIMENTS OF P. ROXBURGHII COTYLEDONS IN LP MEDIA



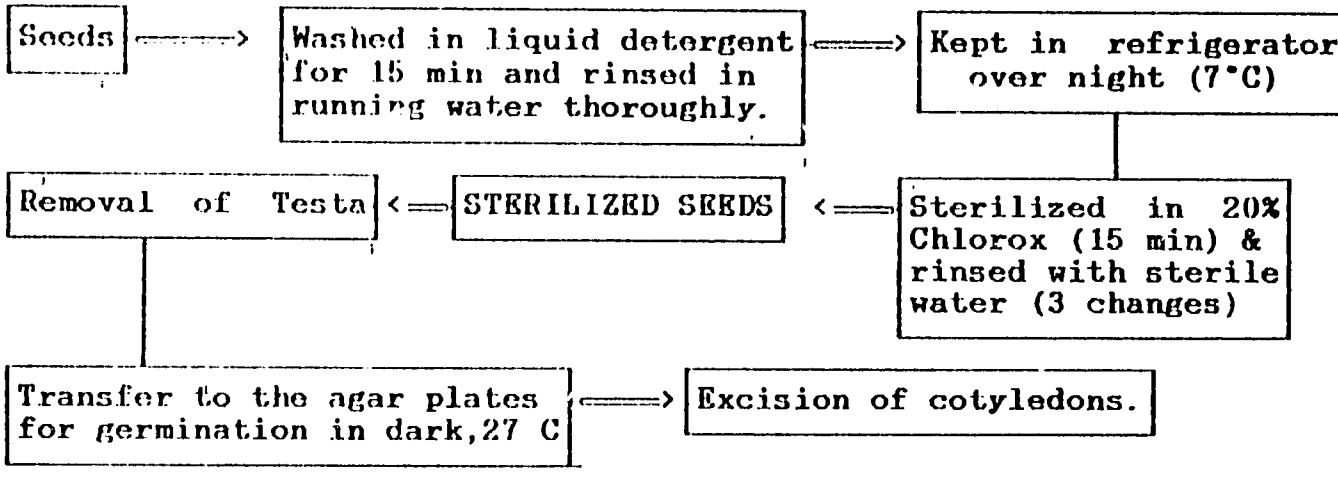
F. FLOW CHART FOR VARIOUS METHOD USED FOR EXPLANT PREPARATION

a) In vitro

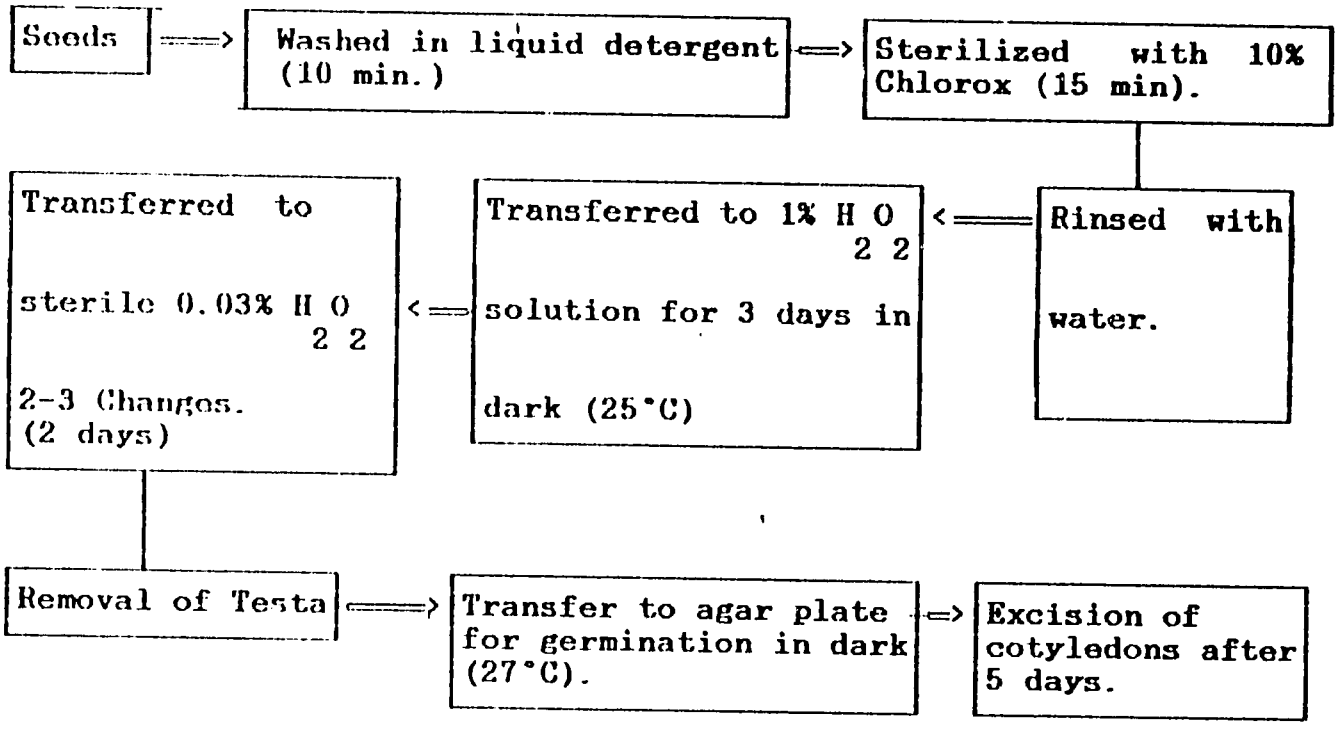
Method I:



Method II:



Method III:



(This method does not include cold treatment step.)

b) In vivo

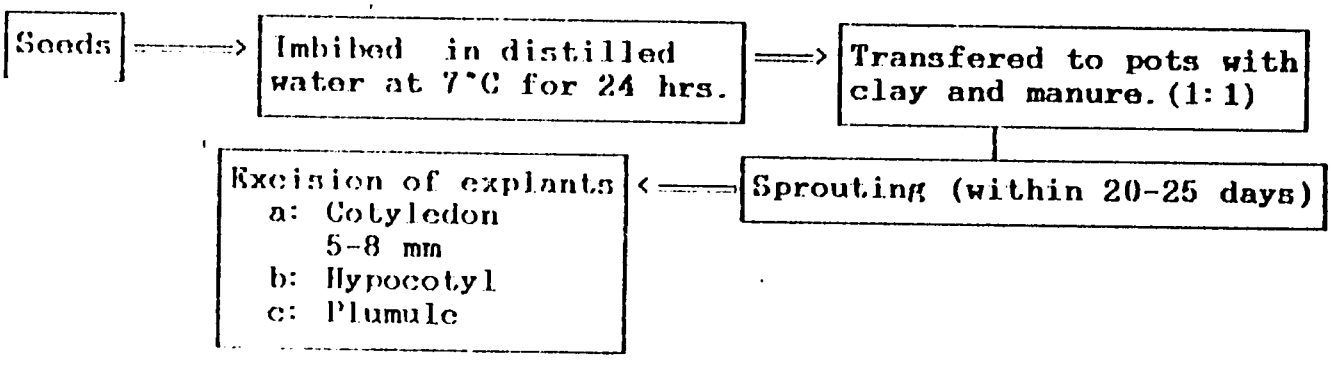


TABLE VII

STATUS REPORT OF VARIOUS EXPERIMENTS DONE ON PINUS

NO.	DATE OF EXPT.	EXPLANT	MEDIA	EXPLANTS AT PRESENT	STAGE	EXPLANT/METHOD
1	Aug.24'88	P.rox, in vitro	LP	19,(2F,2T)	Elg,SB	II
2	Nov.25'88	P.rox, in vitro	MS1/2	3(1F)	SB	II
3	Nov.28'88	P.rox, in vitro	MS	6(4T)	SB	II
4	Dec.09'88	P.rox, in vitro	SH	5(4T)	SB	II
5	Dec.11'88	P.rox, in vitro	GD	2(1F,1T)	No response	II
6	Dec.25'88	P.rox, in vitro	MS1/2	2(2T)	SB	II
7	Dec.26'88	P.rox, in vitro	MS	4(4T)	2SB,2Ca.	II
8	Jan.03'89	P.rox,in vitro	LP	5(5T)	Ca., NC	II
9	Jan.29'89	P.walli,in vitro	SH	4(2T)	SB	II
10	Feb.01'89	P.rox, in vitro	LP	30(10T)	Ca.,SB	II
11	Feb.01'89	P.rox,in vitro	SH	44(15T)	Ca.,SB	II
12	Feb.02'89	P.walli.in vitro	SH	144(35T)	Ca.	II
13	Feb.17'89	P.rox,in vivo	SH	30(15T)	No response	(b)
14	Feb.21'89	P.walli,in vivo	LP	35(22T)	Ca.	(b)
15	Feb.23'89	P.rox,in vivo	LP	5(5T)	Ca.	(b)
16	Feb.23'89	P.rox, in vivo	MS	5(5T)	Ca.	(b)
17	Feb.25'89	P.walli,in vitro	LP	34(12T)	SB,Calli	III
18	Mar.07'89	P.rox,in vivo	LP	48(24T)	Ca,Elg	(b)
19	Mar.12'89	P.walli,in vivo	LP	59(23T)	Elg,Swell,Ca.	(b)
20	Mar.13'89	P.rox,In vitro	LP	204(75T)	Rough & Swell	III
21	Mar.16'89	P.rox,inverted E.	LP	10(10T)	Ca,Swell	III
22	Mar.16'89	P.rox,rooting	Agar +IBA +NAA	13(13T)	Ca. at base	
23	Mar.22'89	P.walli,in vitro	LP	109(45T)		III

Total No.of explant = 700

F= Flask; T= Test Tube
EL= Elongation; SB= Shoot Bud; Cot= Cotyledon
Ca.= Callus; Swell= Swelling; Rough= Rough Surface
P.rox= Pinus roxburghii; P.walli= Pinus wallichiana

FIGURE NO. 1

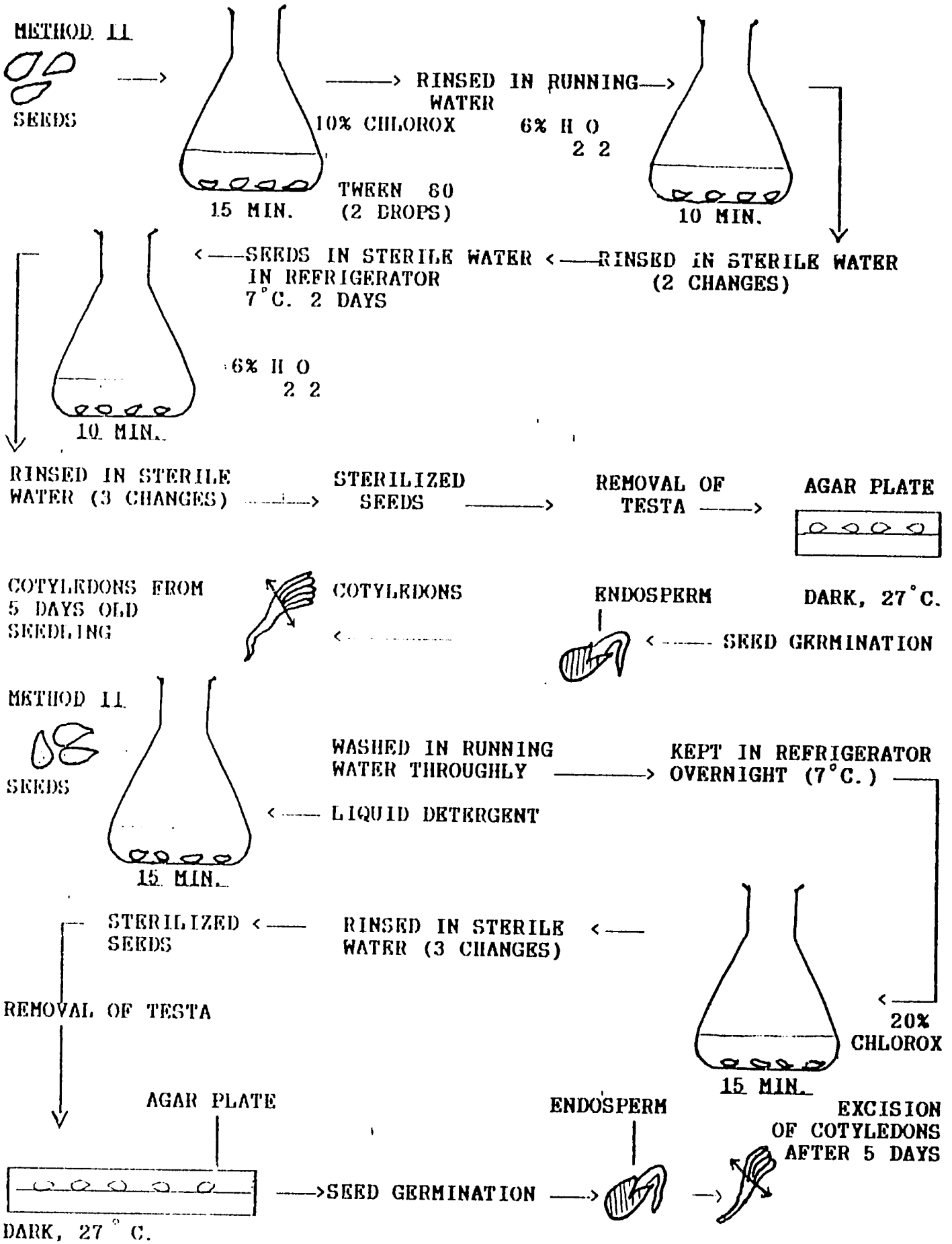
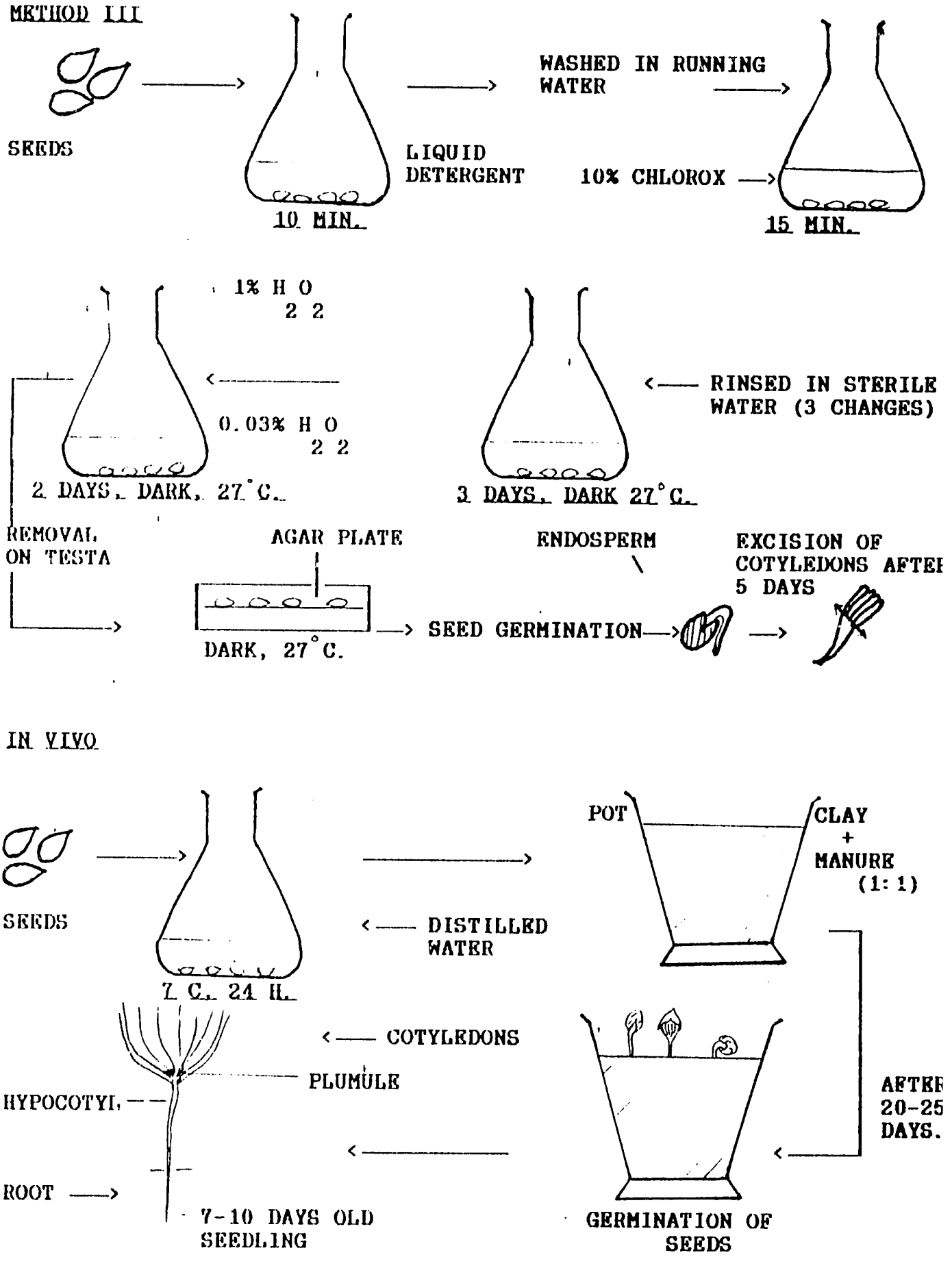

METHOD II
SEEDS
10% CHLOROX
TWEEN 80
(2 DROPS)
15 MIN.
RINSED IN RUNNING WATER
6% H O
2 2
10 MIN.
RINSED IN STERILE WATER
(2 CHANGES)
SEEDS IN STERILE WATER
IN REFRIGERATOR
7°C. 2 DAYS
6% H O
2 2
10 MIN.
RINSED IN STERILE
WATER (3 CHANGES)
STERILIZED
SEEDS
REMOVAL OF
TESTA
AGAR PLATE
COTYLEDONS FROM
5 DAYS OLD
SEEDLING
COTYLEDONS
ENDOSPERM
DARK, 27°C.
SEED GERMINATION
METHOD II
SEEDS
WASHED IN RUNNING
WATER THROUGHLY
KEPT IN REFRIGERATOR
OVERNIGHT (7°C.)
LIQUID DETERGENT
15 MIN.
STERILIZED
SEEDS
RINSED IN STERILE
WATER (3 CHANGES)
20%
CHLOROX
15 MIN.
REMOVAL OF TESTA
AGAR PLATE
ENDOSPERM
EXCISION
OF COTYLEDONS
AFTER 5 DAYS
SEED GERMINATION
DARK, 27° C.

FIGURE 2


METHOD III
SEEDS
LIQUID DETERGENT
10 MIN.
WASHED IN RUNNING WATER
10% CHLOROX
15 MIN.
1% H O 2 2
0.03% H O 2 2
2 DAYS, DARK, 27°C.
RINSED IN STERILE WATER (3 CHANGES)
3 DAYS, DARK 27°C.
REMOVAL ON TESTA
AGAR PLATE
DARK, 27°C.
SEED GERMINATION
ENDOSPERM
EXCISION OF COTYLEDONS AFTER 5 DAYS
IN VIVO
SEEDS
7 C, 24 H.
DISTILLED WATER
POT
CLAY + MANURE (1:1)
COTYLEDONS
PLUMULE
HYPOCOTYL
ROOT
7-10 DAYS OLD SEEDLING
AFTER 20-25 DAYS.
GERMINATION OF SEEDS

INTERNATIONAL TRAVEL

1. Trip to US and France by PI

The principal investigator undertook a trip to the U.S. in April 1988. He attended the conference on "Strengthening Collaboration in Biotechnology: International Agricultural Research and the Private Sector" organized by AID at the Rosslyn West Park Hotel, Arlington, from April 17-21, 1988. This gave the PI opportunity to meet several scientists working in government , university and private sectors in various areas of plant biotechnology and representatives of quite a few private organizations.

During this trip he flew to Raleigh, NC to meet Dr. Ralph Mott, a collaborator of the CPPT project. There he could meet Dr. Emerson who works with Dr. Mott and has considerable experience in tissue culture of pines. He also spent two days with Dr. P.E. Kolattukudy, Director, Biotechnology Center, Ohio State University visiting various facilities and meeting scientists there. He has made some very useful contacts there. In the final leg of his U.S. trip he visited Dr. S.C. Minocha, Chairman, Department of Botany and Plant Pathology, University of New Hamphshire and very useful discussions on Agrobacterium mediated DNA transfer in pines. On the return journey, he also visited Dr. J. M. Bove of University of Bordeaux, Bordeaux to talk about the possibility of using monoclonal antibody methods for detection of greening disease in citrus.

2. Trip to Nairobi by Co-Investigator

The Co-Investigator left kathmandu for Nairobi on January 4, 1989. He participated in the "Third International Plant Biotechnology Conference" held in Nairobi, Kenya from January 8-12, 1989.

PHOTOGRAPH. I

SHOOT BUD INDUCTION ON LP MEDIUM FROM EXPLANT OF *P. roxburghii*

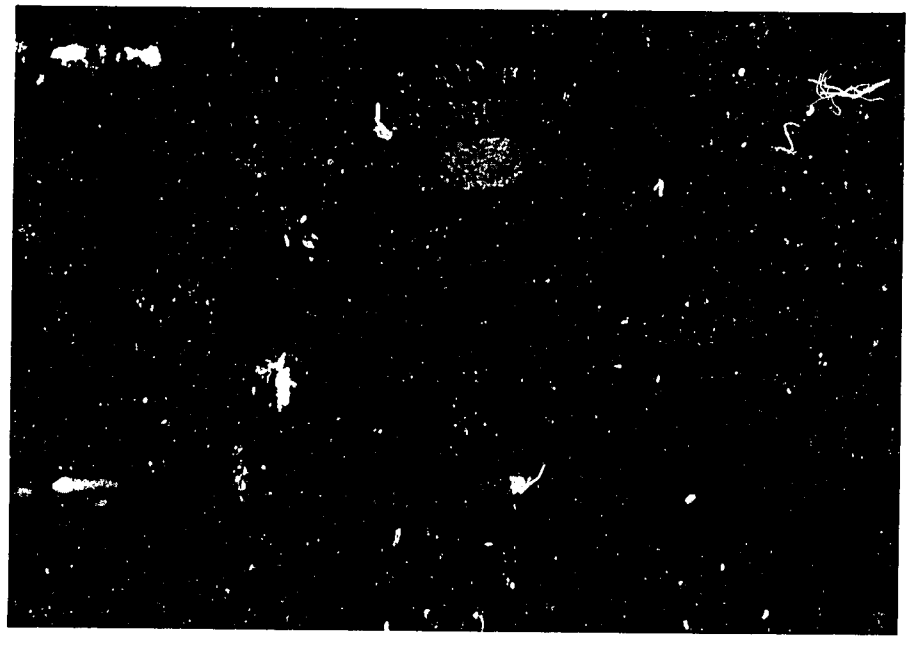
PHTOGRAPH II

EFFECT OF CHARCOAL (1%) ON *P. roxburghii*

a. EFFECT ON BUDS RAISED ON LP - 1.25 ppm BAP.

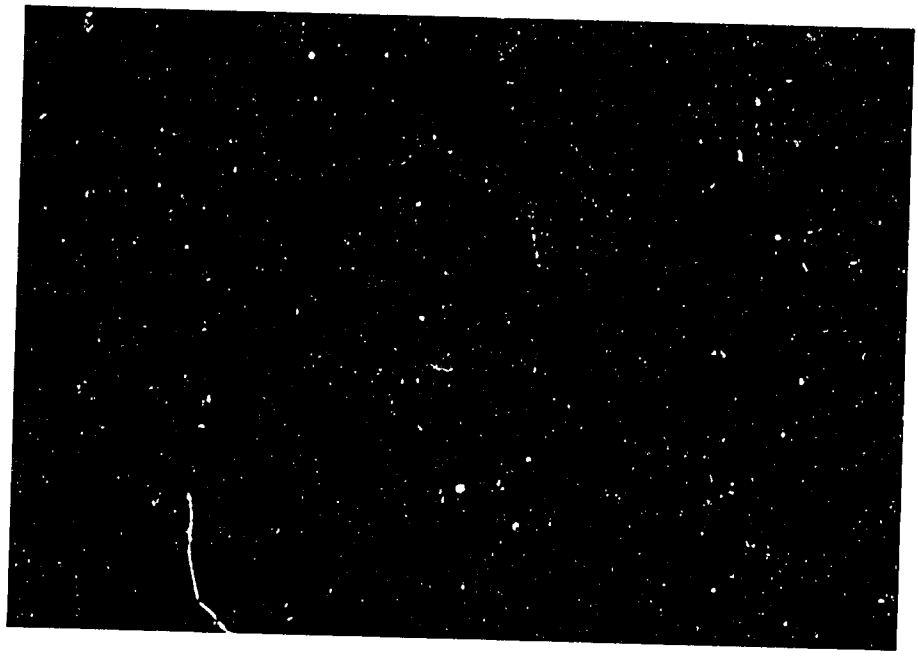
b. EFFECT ON BUDS RAISED ON LP - 1.00 ppm BAP

PHOTOGRAPH III



P. roxburghii SHOOT BUDS IN ELONGATION MEDIUM (LP BASED

PHOTOGRAPH IV



CALLUS INDUCTION IN P.roxburghii COTYLEDON & IN LP 8ppm 2,4-D.

ANNEX I. MEMORANDUM OF UNDERSTANDING 1.

BETWEEN

DR. A. JOHN DEBOER

WINROCK INTERNATIONAL INSTITUTE FOR AGRICULTURAL DEVELOPMENT

AND

DR. VISHWANATH P. AGRAWAL

PRINCIPAL INVESTIGATOR

WI- CLONAL PROPAGATION OF PINE TREE PROJECT

AND

DIRECTOR

RESEARCH LABORATORY FOR AGRICULTURAL

BIOTECHNOLOGY AND BIOCHEMISTRY

SANEPA, KATHMANDU, NEPAL

This is the first memorandum of understanding covering implementation of the Winrock International grant from USAID (Grant No. DPE-5542-G- SS- 7058-00 project No. 936-5542) for "Clonal Propagation of Pine Trees (**Pinus roxburghii and P. Wallichiana)** by methods of tissue culture in Nepal (CPPT Project). This grant is made to Winrock International to provide assistance and support of a research program aimed at the establishment of tree tissue culture laboratory for clonal propagation of superior pine trees and fodder trees, as more fully described in Attachment 1 of this grant entitled "Schedule" and attachment 2 entitled "Program Description". The Memorandum of Understanding resulted from discussions held between Dr.A. John DeBoer, Winrock International, Dr. Vishwanath P. Agrawal, Principal Investigator, WI-CPPT Project, Dr. Mukunda Ranjit, Co-investigator, WI-CPPT project and Krishna Khanal, Program Specialist, Winrock International, on October 19, 1987 at WI-Bakhundole office.

This Memorandum of Understanding will be effective from Oct 1st, 1987 and the Scientific work plan will take place in accordance with the date of this memorandum of understanding. The major revision to the initial grant proposal are thus limited under the budget summary, honorariums, shipment and supplies, vehicle operating cost, international travel and per diems.

Whereas this project aims to define the nutritional and hormonal requirements of plantlets (explant ->shoot ->root ->plantlet) from explants of seeds, parts of seedling - cotyledons, hypocotyles and

epicotyles- and young shoot tips of Pinus Roxburghii and Pinus Wallichiana and eventually for other fodder tree species. The overall aim of this project is to start a tree tissue culture laboratory for clonal propagation of superior forest trees native to the Indian Subcontinent.

The two parties mentioned above have reached agreement on the following points:

Article 1

Winrock International will administer the whole grant including local and overseas procurement, salaries, local and international travel and per diems, training, consultants and reporting. WI will provide, subject to personnel and budgetary limitation, and as may be mutually agreed upon:

A. In order to achieve the objectives set out in the research program, WI will help establish a tissue culture facility in the "Research Laboratory for Agricultural Biotechnology and Biochemistry" (RLABB) located in Sanepa Indrayani, Lalitpur.

B. A full time Research Associate to support research efforts of CPPT project in selected activities, based at tissue culture laboratory for a period of 3 years on an annual renewal basis.

C. A full time Research Assistant to assist the research efforts of the project including filing, record keeping, administering the laboratory procedures, for a period of 3

years starting from Oct 1, 1987 on an annual renewal basis.

D. WI will arrange to pay electricity bills for the laboratory where the PI will install a separate electric sub-meter.

E. WI will use CPPT Project fund for white washing the laboratory areas of approximately 9000 sq. ft.

F. WI will use funds from this revised budget for travel cost required for procurement , custom clearance at Birgunj customs office and equipment, materials and supplies.

G. WI will revise the original budget estimate for all line items and the balance will be used for procurement including a locally made Laminar flow station, stavols, volt guards, furnitures and laboratory supplies. Please refer to the attached revised budget which gives more detail.

Article II

A. Research findings, as a result of the joint cooperative work of the three parties, will be published in the public interest as mutually agreed upon:

B. Research finding published by either party will give due credit to other parties contribution, and at the same time, the conclusions and interpretations reported will be the sole responsibility of the party publishing the findings.

Article III

Honorarium for the research will be as given below:

A. Principal Investigator will be paid a monthly honorarium at the rate of Rupees 1095.00 for a period of 4 years starting from Oct. 1st, 1987.

B. Co-investigator will be paid a monthly honorarium at the rate of Rupees 1095.00 for a period of 4 years starting from Oct. 1st, 1987. Salaries for other research and support staff will be as given below:

C. Research Associate will be paid a monthly salary at the rate of Rupees 5475.00 for a period of 3 years starting from October 1, 1988.

D. Research Assistant will be paid a monthly salary at the rate of Rupees 1323.00 for a period of 4 years starting from Oct. 1st, 1987.

E. Laboratory aide at the rate of US $20. a month for a period of 46 months, to keep the lab equipment clean.

F. All personnel mentioned A-D above will be entitled to a 5% increase in their honorarium on salary and are not entitled to other benefits as per WI - personnel policy in Nepal.

Article IV

A. All the equipment installed in this Tissue culture laboratory and the furnitures will be handed over to an appropriate unit relevant to such research work by the end of the project. WI will retain the title to all equipment purchased under the CPPT program, until such time as it is transferred to an appropriate unit. In

accordance with the spirit of the provision of the grant document (page 45, d-1 &2), WI shall keep the equipment in RLABB as long as they are needed for research in tissue culture.

B. WI will transfer the title to remaining expandable items, furniture, computer etc. to an appropriate unit at the end of the CPPT Project.

C. Institutional authority will be invested with Dr. A. John De Boer or Mr. S. S. Bal, Winrock International.

D. This memorandum of Understanding may be supplemented by work plans submitted with the original project proposal, which describes more specifically the activities to be carried out under this cooperative research program and which set forth the envisaged contributions of each party as well as any such revision in budget, timing of work etc. that may be needed for timely execution of this project.

This memorandum of Understanding shall come into force upon the date of Oct. 1, 1987 and shall remain in force for a period of 4 years or until terminated by either party by giving the other party six months prior notice in writing.

Signed_____
Dr. A. John DeBoer
Winrock International

Signed_____
Dr. V. P. Agrawal
Principal Investigator
CPPT Project

ANNEX II. MEMORANDUM OF UNDERSTANDING NO. 2

BETWEEN

DR. A. JOHN DE BOER

WINROCK INTERNATIONAL

INSTITUTE FOR AGRICULTURAL DEVELOPMENT, (WI)

KATHMANDU, NEPAL

AND

DR. VISHWANATH PRASAD AGRAWAL

PRINCIPAL INVESTIGATOR

CLONAL PROPAGATION OF PINE TREE PROJECT

DIRECTOR

RESEARCH LABORATORY FOR AGRICULTURAL

BIOTECHNOLOGY AND BIOCHEMISTRY

KATHMANDU, NEPAL.

This is the second memorandum of understanding covering implementation of the Winrock International Grant from USAID/ Office of the Science Advisor for Clonal Propagation of Pine Tree Project (CPPT Project). This second memorandum of understanding is needed due to lack of required budget for shipment clearance, custom duties, transportation cost and other expenses made to procure the Project equipment. This MOU is needed to make line item transfers to the original budget.

The original budget is attached as Table 1.

The parties mentioned above have reached agreement on the following points:

Article I

Winrock International has estimated the anticipated cost overruns under the line item No.II for Equipment as stated in the following paragraph:

Line item II.Equipment:

	Original budget	Expenditure to date	Difference
1.Equipment	$ 35504.00	$ 36590.00	$ 1086.00
2.Clearing and transportation			$ 2000.00
3.Taxes at the rate of 60% in average of US $ 36,000.00			US $ 21,600.00
Total expenditure			US $ 24,686.00
4.Estimated additional equipment purchase over life of project			US $ 1,910.00
Total add on			US $ 26,596.00
New total under this item (Equipment)			US $ 62,100.00

In order to provide additional amount of US $ 26,596.00 as shown above, revision will be made to the following line items and transferred to item No. II.

Line item III. Materials and supplies 1. (chemicals)

Most of the chemicals are ordered from US along with the equipment, so US $ 7000.00 will be transferred to the revised budget from the line item III.

Line item IV.Travel and Per diem

1+2 International travel and Per diem (PI)

Since the Principal Investigator has already attended a seminar in the US, he will require little further travel over life of the project. Therefore, the amount of US US $ 10,000.00 will be transferred to line item II. (Equipment)

IV. Advisor (Dr. D. Durzan) Travel.

The consultation cost for Dr. Durzan US $ 5000.00 will be transferred to the revised budget for the present period and a request to USAID will be made for Dr. Durzan's travel and Per diem cost.

Line item I. Salaries and wages

Research Associate's salary was proposed from the beginning of this project but up to now this post has not been filled so that US $ 2,500.00 will be transferred to line item II.(Equipment)

Line item V. Other direct cost:

We propose to transfer $ 2096.00 from line item V. to line item II. (Equipment).

The remaining amount from this revised budget will be utilized under the line items II. and III. which is equipment and materials supplies. The reflected amount is US $ 6844.00 The line item No. II.(Equipment) will cover US $ 3000.00 and the remaining to the line item No. III.(Materials and supplied)

The original budget is attached as Table 1 and the Revised Budget is attached as Table 2.

This memorandum of understanding is signed in Kathmandu on September_____ ,1988.

Signed in _____,in original

Date_____

_____

Dr. A. John De Boer

Winrock International

Institute of Agricultural Development

_____

Dr. V. P. Agrawal

Principal Investigator

CPPT.Project,Direct , RLABB.

Witness_____

Krishna Khanal

Administrative Specialist

Table 1.

ORIGINAL CPPT BUDGET

GRANT BUDGET IN CONTRACT DATED AUGUST 31, 1987

COST ELEMENT	AMOUNT
I. Salaries and wages	US $ 18,820.00
II. Equipment	US $ 35,504.00
III. Materials & supplies	US $ 20,000.00
IV. Travel and Per diem	US $ 25,000.00
V. Other direct cost	US $ 4,800.00
VI. WI/Administrative fee	US $ 17,842.00
Grand total	US $ 121,966.00

PINE TREE GRANT BUDGET STATUS

August 16, 1988 FOV through July 1988.

Table 2.

Proposed Budget for Grant Agreement Amendment

Line item	New budget
I. Salaries and wages	US $ 16,320.00
II. Equipment	US $ 62,100.00
III. Materials and supplies	US $ 13,000.00
IV. Travel and Per diem	US $ 10,000.00
V. Other direct cost	US $ 2,704.00
Total direct cost	US $ 104,124.00
VI. WI/ Administrative fee	US $ 17,842.00
Grand total	US $ 121,966.00

Notes on new budget:

The original estimated cost under the line item No.II.Equipment, was US $35,504.00. Due to lack of required budget for shipment clearance, custom duties, transportation cost and a little over expenses made to procure the project equipment, this revision is needed and transferred the amount as stated in the following paragraph.

Original Budget	Spent	Difference
US $ 35,504.00	US $ 36,590.00	US $ 1086.00
Clearing and transportation		US $ 2000.00
Taxes @ 60% of 36,000.00		US $ 21,600.00
Total estimate over expenditure		US $ 24,686.00
Estimate additional equipment purchases		US $ 1,910.00
Total to add on		US $ 26,596.00
New total		US $ 62,100.00

(a) Transfer $ 15,000.00 from Travel and Per diem.

(b) Transfer $ 7,000.00 from line item III.Materials and supplies, US $ 2,500.00 will be transferred from Salaries under the line item No.I.

(c) Transfer US $ 2096.00 from Other direct cost line item No V.