

PN-ABB-10!

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

DATE:

8/20/88

MEMORANDUM

TO: AID/PFC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 6. 26 2
PR Semi-annual #1

Attachment

PI-ABB-101
57543

CLONAL PROPAGATION OF PINE TREE PROJECT

SEMI-ANNUAL REPORT NO. 1

September 1, 1987 through February 29, 1988.

WINROCK INTERNATIONAL INSTITUTE FOR AGRICULTURE DEVELOPMENT
AND
THE RESEARCH LABORATORY FOR AGRICULTURAL BIOTECHNOLOGY AND
BIOCHEMISTRY,
SANEPA KATHMANDU, NEPAL

USAID OFFICE OF SCIENCE ADVISOR
GRANT NO. DPE-5542-G-SS-7058-00

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1. INTRODUCTION

The "Clonal Propagation of Pine Tree Project" (CPPT) is funded through a grant by the United States Agency for International Development, Program in Science and Technology Cooperation to the Winrock International Institute for Agricultural Development, Morrilton, Arkansas, USA. The grant is administered by the Winrock International Office in Kathmandu, Nepal. The purpose of the grant is to provide assistance to support a research program aimed at the establishment of a tree tissue culture laboratory for "clonal propagation of superior pine trees (Pinus roxburghii and P. wallichiana) and fodder trees by methods of tissue culture" in Nepal, under the USAID Grant No. DEP-5542-G-SS-7058-00. Professor Don J. Durzan, Department of Pomology, University of California, Davis will serve as an advisor to this project.

In Nepal, land degradation due to deforestation has also posed serious problems of erosion of topsoil and water losses in irrigation. Each and every year the monsoon causes serious soil erosion in Nepal by causing heavier flooding. This problem is not only in Nepal but also in its neighbouring countries of India and Bangladesh. Every year in the monsoon season these countries face many problems through flooding. Erosion of topsoil makes the lands unuseable 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 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). This grant provides financing for salaries, honorariums, equipment, training, travel, per diem, materials, supplies and other direct costs. The project activities during the first six months of this project 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 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 problem for the India sub-continent as a

whole. In order to fight against desertification, adequate measures should be taken to propagate on a mass scale the forest trees indigenous to the Indian subcontinent and especially to Nepal. In Nepal, with the help of international organisations, 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 Pinus roxburghii and Pinus 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 would try 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 controlled-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. 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 seedset, 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(7). The potential of plant tissue culture techniques as a means of mass propagation of superior forest trees has been recognized for several years (3-15).

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(15).

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 banksiana (grey pine) from hypocotyl as explant (16), plantlet regeneration in P. palustris (long leaf pine) from embryo (17), plantlet regeneration in P. Pinaster from cotyledons (18), root formation in P. Lambertiana from hypocotyles (19), regeneration in P. strobus from embryos and needle folicles (20, 21), plantlet and embryo regeneration in P. elliotii (slash pine) (22), regeneration in P. mugo from seedling (23), plantlet and embryo regeneration in P. taeda (loblolly pine) from juvenile explants (22, 24), plantlet regeneration in P. radiata from several types of juvenile explants(25,26,27), (one of the best example showing the success of tissue culture research in pine), plantlet regeneration in P. sylvestris from cotyledons and shoot tips (28), and bud regeneration in P. nigra from embryos and shoot tips (29) 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 (14,30). Induction of callus, but not its morphogenesis into plantlets, has been shown in P. strobus (20), P. Lambertiana (19), P. resinosa (31), P. mugo and P. nigra (32), and P. densiflora (33).

IV. TECHNICAL WORK PLAN

In order to achieve the goals above the technical work plan includes the following steps:

1. Selection of explants.
2. Sterilization and dissection of explants.
3. Initiation and multiplication of adventitious shoots.
4. Shoot elongation and remultiplication.
5. Root initiation (14, 48)
6. Root formation and growing on to plantlets.
7. Hardening of plantlets.
8. Generation of plantlets from young shoots.

V. SPECIFIC PROJECT ACTIVITIES

To achieve the goal and purpose of this project, the tasks to be accomplished include the following supporting functions:

1. establishment of laboratory at Saepa Indrayani in the premises of Research Laboratory for Agricultural

Biotechnology and Biochemistry with the capacity to conduct appropriate studies.

2. construction of an environmental room at project site.
3. furnishing the research lab with all equipment needed for the research work.
4. procurement of locally available equipment, glassware and chemicals.
5. procurement of required equipment from overseas.

Some significant progress is summarized below

1. Research laboratory has been established.
2. Repair works, such as white-washing and electrification have been completed.
3. Position of Research Assistant has been filled.
4. Construction of an environmental room is in progress.
5. Chemicals and glassware have been supplied.
6. Construction of laminar flow hood is in progress.
7. Furniture and other office supplies have been procured.
8. Procurement order for equipment has been placed with Winrock International headquarters and has been dispatched.
9. Principal Investigator Dr. V. P. Agrawal got an opportunity to participate in the "International Workshop on Application of Biotechnology in Forestry and Horticulture" sponsored by National Science Foundation, USA from January 14 to 17, 1988.

VI. MEMORANDUM OF UNDERSTANDING

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

SANEGA, 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 DePoer, 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 CPTF 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. PI 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, staveis, 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 ELAEB as long as they are needed for research in tissue culture.

- B. WI will transfer the title to remaining expendible 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. Pal, 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 DeFoer
Winrock International

Signed _____
Dr. V. P. Agrawal
Principal Investigator
CPPT Project

VII. FINANCE AND BUDGET

The following is the estimated budget for CPPT project including local cost items:

BUDGET LINE ITEM IN DETAIL

COST ELEMENT	US \$
Salaries and wages	18,820.00
Equipment	35,504.00
Materials and supplies	20,000.00
Travel and Per diem	25,000.00
Other direct cost	4,800.00
Administrative management fee	17,842.00
Grand total	\$ 121,966.00

Expenditures incurred as of February 29, 1988 are : (\$):

Expenditures line item	Local	WI-HQ's	Total
A. Salaries and wages	694.24	-	694.24
B. Equipment	7,067.52	8,504.29	15,571.81
C. Materials and supplies	9,439.94	2,361.65	11,791.59
D. Travel and Per diem	1,217.92	-	1,217.92
E. Other direct cost	172.43	677.24	849.67
Total direct cost	18,582.05	11,543.18	30,125.23
WI management fee	-	4,910.67	4,910.67
Total expenditure	18,582.05	16,453.85	35,035.90

VIII. CONSTRUCTION AND OTHER FACILITIES

Winrock International has designed an environmental room for the lab and it is under construction at the Research Laboratory For Agricultural Biotechnology And Biochemistry, Sanega. White washing and electrification work is completed and furniture and other materials were provided by Winrock International for office establishment. Estimated cost is shown below.

1. Laminar flow station	\$ 2000.00
2. Furniture	\$ 4000.00
3. Stationery	\$ 800.00
4. White wash and fittings	\$ 3500.00
5. Environmental room	\$ 5000.00
6. Electric supply for lab	\$ 405.00
7. Glassware	\$ 3000.00
8. Chemicals	\$ 1000.00
Total	<hr/> \$19,705.00

IX. OTHER FACILITIES AND REPAIR WORKS

Winrock International has been supporting the researchers by providing facilities to carry out their research activities at their office and laboratory. Dr. V.P. Agrawal has provided laboratory space and WI has furnished the rooms.

Regarding repair of facilities, WI provided financial and technical support to repair the rooms with white washing work.

X. 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 Somer, University of Georgia, and Dr. S.S. Phojwani, 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.

XI. 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 Specialist to administer the whole grant. The institutional authority to this grant is invested in Dr. A. John De Boer or S.S. Pal, the WI Chief of Party in Kathmandu and Deputy Chief of Party, respectively under the Agricultural Research and Production Project.

PERSONNEL (Local)

On recommendation from Principal Investigator of this project, a research assistant for the project has been appointed on a temporary basis to assist the investigator in research work. The staff appointment work for CPPP project was completed by December 4, 1988, according to grant approved salary scale.

LOGISTIC SUPPORT

Winrock International has been contributing all the facilities required to support the project in order to provide strong support in the research activities. The WI accountants have been handling all the accounting for the project. In addition to this, WI has been providing communications support services as well.

COMODITY PROCUREMENT AND INVENTORY

Following commodities were locally procured and provided to the project.

(Equipments local procurement)

Item No.	Descriptions	Quantity
1.	Wooden standard tables	6 pcs
2.	Wooden racks	5 "
3.	Wooden chairs	4 "
4.	Lab stools	4 "
5.	Wash basin	2 "
6.	Kerosene heater	1 set
7.	Kerosene jerry can	1 can
8.	Laminar flow station	1 set
9.	Filing cabinet	1 pcs
10.	Steel skeleton rack	5 "
11.	Micro balance	1 set

(Glasswares locally procured)

Item No.	Description	Quantity
1.	Volumetric flask 1000.ml	2 pcs
2.	Measuring cylinder 1000...	2 "
3.	Conical flask 50...	10 "
4.	Volumetric flask 50...	10 "
5.	Measuring cylinder 50...	10 "
6.	Beaker 50...	10 "
7.	Measuring pipette 1...	10 "
8.	" " 2...	10 "
9.	" " 5...	10 "
10.	" " 10...	10 "
11.	" " 25...	10 "
12.	Culture tubes 6"-1"	1000 "
13.	Graduated pipette 1...	10 "
14.	Conical flask 1000...	200 "
15.	" " 250...	50 "
16.	Petri dishes	100 pair
17.	Aluminum foils	3 pkt
18.	Marker pen	12 pcs
19.	Parafilm	1 pkt
20.	Graduated pipette 10.ml	10 pcs
21.	" " 5...	10 "
22.	" " 1...	10 "
23.	Burets 50...	5 "
24.	" 100...	2 "
25.	Beaker 100...	10 "
26.	Conical flask 100...	10 "
27.	Volumetric flask 100...	10 "
28.	Measuring cylinder 100...	5 "
29.	Conical flask 250...	10 "
30.	Volumetric flask 250...	5 "
31.	Measuring cylinder 250...	5 "
32.	Beaker 250...	5 "
33.	Conical flask 500...	5 "
34.	Beaker 500...	5 "
35.	Volumetric flask 500...	5 "
36.	Measuring cylinder 500...	5 "
37.	Conical flask 1000...	5 "
38.	Beaker 1000...	5 "
39.	U. V. lamp	2 set

CHEMICALS LOCALLY PROCURED

Item No.	Description	Quantity
1.	Calcium nitrate 500. gm.	4 pkt
2.	Ferrous sulfate 500. "	2 "
3.	Magnesium sulfate 500. "	1 "
4.	Boric acid 500. "	1 "
5.	Zinc sulfate 500. "	1 "
6.	Potassium iodine 500. "	2 "
7.	Copper sulfate 500. "	1 "
8.	Sodium molybdate 500. "	1 "
9.	Cobalt chloride 500. "	1 "
10.	Potassium sulfate 500. "	2 "
11.	Hydrochloric acid 500. "	4 pkt
12.	Sulfuric acid 500. "	4 "
13.	Sodium chloride 500. "	4 "
14.	Potassium hydroxide 500. "	4 "
15.	Chloroform 100. "	3 bot
16.	Ethyl alcohol 500. "	20 "
17.	Spirit 5. ltr	1 "
18.	EDTA sodium salt 100. gm	1 "
19.	Sucrose 500. "	20 pkt
20.	Magnesium sulfate 500. "	4 "
21.	Potassium hydrogen 500. "	1 "
22.	Potassium nitrate 500. "	1 "
23.	Ammonium nitrate 500. "	1 "
24.	Calcium chloride 500. "	1 "
25.	Ammonium sulfate 500. "	1 "
26.	Boric acid 500. "	1 "
27.	Magnesium sulfate 500. "	1 "
28.	Zinc sulfate 500. "	1 "
29.	Copper sulfate 500. "	1 "
30.	Cobalt chloride 500. "	1 "
31.	Potassium iodide 250. "	2 "
32.	Ferrous sulfate 500. "	1 "
33.	EDTA sodium salt 100. "	3 "
34.	EDTA Naz salt 100. "	1 "
35.	Potassium nitrate 500. "	10 "
36.	Magnesium sulfate 500. "	4 "
37.	Ammonium phosphate 500. "	3 "
38.	Calcium chloride 500. "	4 "
39.	Aluminium sulfate 500. "	2 "
40.	Potassium chloride 500. "	2 "
41.	Sodium phosphate 500. "	2 "
42.	Ammonium nitrate 500. "	10 "
43.	Potassium phosphate 500. "	2 "
44.	n-Propanal 500. "	18 bot
45.	Isopropanal alcohol 2.5 ltr	4 "
46.	Pexane 500. ml	20 "
47.	Methanol 2.5 ltr.	4 "
48.	Acetone 2.5 "	4 "
49.	Ethanol alcohol 450. ml	20 "

EQUIPMENT WITH PROCUREMENT

Following equipments has being ordered from Winrock International Headquarter and has been dispatched.

Item	Particulars	Quantity
1.	Autoclave model 750 sterilizer 220 V	1
2.	Richard Autoclave glove	12 pr
3.	Electronic Analytical Balance model 2100	1
4.	pH meter for 220 V 50/60Hz " 800	1
5.	Combination Electrodes with Gel filled	2
6.	Polysilicate glass culture tubes	5 case
7.	Polysilicate glass culture tube with screw	3 "
8.	Polysilicate glass culture tube with "	1 "
9.	Polysilicate glass culture tube with screw	1 "
10.	Double hanging pan balance with weights	1
11.	Biorad growth chamber 15.5 cf	1
12.	Vortex Shaker for 220 V	1
13.	Stirrer hot plate	1
14.	Culture tube plate 25mm	2 pkg
15.	Hand pump filter unit	1
16.	Exhaust filter	1 box
17.	Stirring bar 2/PM 1/2"	3
18.	Water Distillation apparatus Aqua-4200	1
19.	Micro Centrifuge TC-14 for 220 V	12000
20.	Microscope (dual power)	1
21.	Transformer	1
22.	Stainless steel centrifuge	1
23.	Autoclavable polypropylene test tube rack 25mm	10 case
24.	" " " " 16mm	5 "
25.	" " " " 13mm	5 "
26.	Cherpette micropipettors	2
27.	Single block heater	1
28.	Block (12x12mm tubes)	1
29.	" (15x16mm tubes)	1
30.	" (25mm tubes)	1
31.	Reagent Thermometer	1
32.	Peter-Path	1
33.	Disposable Line	5
34.	Digital finpipette	1
35.	Disposable tips for 4 above	1 case
36.	Double wall laboratory oven for 230 VAC	1
37.	Transformer for 220 VAC	1
38.	Hotter & stand for 230 VAC	1
39.	Standard glassware set	1
40.	Air conditioner 220 V. 50Hz	1
41.	Refrigerator for 220 V. 50Hz	1
42.	Microwave oven for 220 V. 50Hz	1
43.	IBM-XT Model 287 or compatible (640K RAM)	2
44.	Appropriate Monochrome monitor 120 Volt	2
45.	Pericles Graphics 1 Card	2
46.	8087 Math Co-processor chip	2
47.	AST/O Mini card- clock CBI/SFP/PAPA	2

48.	IBM PC-DOS 3.2 (5.25" FORMAT)	2
49.	Cover Sheet for CPU/Monitor, Keyboard	2
50.	Epson FX 1000 Printer with tractor 120 V	1
51.	Epson FX 86E printer with tractor 120 V	1
52.	IBM parallel printer cable	2
53.	FX-1000 Ribbons	10
54.	FX-86E Ribbons	2
55.	14 7/8x11 inch pin feed paper	1 box
56.	Dickette head cleaner Kit	2
57.	ESDP 5.25" Disks	2 box
58.	Wordstar professional 4.0	1
59.	Lotus 1-2-3	1
60.	Heavy duty portable alternator 5000 watts	1
61.	4 Day heavy duty cut door cord 25 ft	25 ft
62.	Heavy Duty Yellow 12 AWG Extension cord	25 ft

CHEMICAL SUPPLY PROCUREMENT

	Descriptions	Quantity
1.	Agar A. 7002	5 kg
2.	6-Benzylaminopurine B.6750	5gX3
3.	Kinetin E. 2751	1 gm
4.	IPA I. 1875	25X3
5.	IAA I 1250	25 gm
6.	NAA I0375	100 gm
7.	Myoinositol 15125	500 "
8.	" 2- IPA D0636	1 "
9.	Pyridoxine P. 9755	25 "
10.	Nicotinic Acid M 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 P4117	100 gm
15.	Cilofallin C 2250	5 "
16.	Kandolorifate N 7010	25 ml
17.	Sephadex C 100-120	50 gm
18.	" Sephadex C 25-120	50 "
19.	DEAE-Sephadex A 25-120	50 "
20.	IB-20-100	5 "
21.	Blue Fortran B5751	1 "
22.	Nitrophenyl-B-Glucopyranoside N1377	100 "
23.	" " " N7005	100 "
24.	P 4772 Molecular Biology of Plant manual	1 25.
25.	P 9914 plant Molecular Biology Volum 188	1
26.	P 6150 Plant cell culture	1

XII. TECHNICAL PROGRESS REPORT ON
CLONAL PROPAGATION OF PINE TREE PROJECT

AUGUST 31, 1987 THROUGH FEBRUARY 29, 1988

BY

DR. V. P. AGRAWAL

PRINCIPAL INVESTIGATOR,

WI- CLONAL PROPAGATION OF PINE TREE PROJECT

Following is the progress report from the CPPT Principal Investigator, Dr. V.P.Agrawal:

Project Purpose

Throughout history, humans have mistreated forests by extensively clearing forest cover for settlement and forest-products without giving proper attention to plantation of forest trees in proportional numbers. For small countries like Nepal, where forest trees are the primary source of fuel, the rate of deforestation is a serious problem. Deforestation in Nepal has not only caused acute shortage of fuel wood but it has also threatened with extinction several tree species. Many families in the mountains of Nepal spend days searching for fuel wood and hence become victims of mismanagement of our forest resources.

Use of chemicals and fertilizers in forestry is not economically feasible. Therefore, genetically superior clones adaptable to poor soil and harsh climate in mountains must be used for reforestation. It is easy to imagine that millions of good seedlings will be required during the next few years for successful afforestation in Nepal. This project aims to develop the tissue culture technology that would allow mass propagation of superior pine seedlings, more suitable to the climate of Nepal.

TECHNICAL WORK PLAN

In order to define the nutritional and hormonal requirements of steps involved in regeneration of plantlets from explants of seeds, parts of seedlings (cotyledons, hypocotyls and epicotyls)

and young shoot tips of the pine species, we have prepared the following work plan:

Explants:

a) Seed:

To be collected from pine cones or from the Forestry Dept. Seeds of different maturity to be used.

b) Seedlings:

i) Seedlings grown from seeds in green house,

ii) Seedlings from aseptic culture:

The seedlings can be obtained aseptically by incubating (25 c) the seeds on a moist filter paper in a petri dish.

Seeds can also be obtained by culturing a surface sterilized seed, without its seed coat in 4% agar at 25 c or by culturing the embryo in modified MS and LP media.

c) Primary and secondary shoots:

These shoots can be obtained from base of pine trees of different age groups.

They can also be obtained by hedging pine plants.

Culture Media:

Though the basic nutritional requirements of cultured plant cells are very similar to those normally utilized by whole plants, various culture media will be tried to fulfill the requirement of the cells in culture.

Media that will be tried for tissue culture of pines are as follows:

1. Burschige and Skeeg (BS)
2. Carberg et.al, (BS)
3. Schenk & Hilderbrandt and Modified by Peilly & Washer (SH)
4. Crosshoff and Doy and modified by Horgan and Aitken (GD)
5. Quoirin and Lepoivre and modified by Aitken-Christie (LP)
7. Campbell and Purzan (CD)
8. Berberger et al and modified by Eorwann (MCF)

Various growth regulators that will be used are:

Cytokinin:

- a) 6-Benzylamino purine (BAP)
- b) 2-isopentenyl-adenine (2iP)

Auxin:

- a) Indole-3-acetic acid (IAA)
- b) Naphthalene acetic acid (NAA)
- c) 2,4-dichlorophenoxy acetic acid (2,4-D)
- d) Indole-3-butyric acid (IBA)

TECHNICAL REPORT

ABSTRACT

Preliminary experiments on embryo cultures of Pinus roxburghii and Pinus wallichiana are described. Plantlets of Pinus roxburghii and Pinus wallichiana could be obtained from seeds or embryos cultured in MS & LP medium for 10 days. Attempts at regeneration of the explants from germinated seeds have been successful in obtaining adventitious buds and/or callus like tissue.

INTRODUCTION

Pinus roxburghii and Pinus wallichiana are the two native and most abundant pine trees of the Himalayan region . In order to meet the growing demand of pine seedlings in this region, large numbers of seedlings of improved variety will be needed for afforestation .One of the new techniques that shows promise is tissue culture and its use in forestry should be considered.

In this present work, we have tried to define the nutritional and hormonal requirements of steps involved in regeneration of plantlets from explants of seeds and parts of seedling-cotyledons, hypocotyles and epicotyl .

MATERIALS AND METHODS

The seeds of Pinus roxburghii and Pinus wallichiana were obtained from Forest Development Project, Pattishar. They were kept at 7⁰ c in a refrigerator for about three months, before using them. The wings of the seeds were mechanically removed and about 100 of them were placed in a 250 ml beaker containing about

200 ml. water. The seeds that sank were removed and put in a sterilized glass bottle with a lid. Surface sterilization of these seed was conducted in the following manner.

HEALTHY SEEDS---> Treated with 10% solution ---> Rinsed in running
of Sodium Hypochlorite with water for 48 hrs.
few drops of Tween 80 for
15 minutes (100 ml.)



Placed in a refrigerator <--- Rinsed twice <--- Sterilized again in
for two days at 7⁰ c. in sterile 6% Hydrogen peroxide
water. for 10 minutes(30 ml)



Sterilized in 6% Hydrogen ---> Rinsed in sterile -->-STERILIZED
peroxide for 10 minutes. water thrice. SEEDS
(30 ml.)

Asceptic germination of seeds with seed coat intact:

About 25 sterilized seeds were taken and placed in a sterilized petri dish containing two sheets of filter paper. The petri dish was sealed with parafilm and placed in dark at 27⁰c.

Removal of seed coat:

To remove the seed coat the sterilized seed was placed between the arms of a forcep and pressed gently making sure that the pressure applied just ruptured the seed coat and did not crush the entire seed. After the seed coat had been removed, the seed covered with brownish membrane was obtained. These seeds were taken for further experiments.

Germination on agar of seeds without seed coat

The seed without seed coat was placed on a petri dish containing 4% agar. The petri dish sealed with parafilm was placed in dark at 27⁰c. The seeds germinated and developed into seedlings after 7 to 10 days. These seedlings were used as explants for regeneration experiments.

Germination of excised embryos in MS & LP medium containing no growth hormones:

The seed without its seed coat was placed in a sterile petridish. The brownish membrane and the operculum were removed with the help of sterile blade and forcep. An incision was made along the ridge of the seed to obtain embryo which was removed from the concave bed within the female gametophyte. It was then placed in the MS & LP medium containing no growth hormones at 25 c; light intensity was 500 lux for 16 hours/day.

Nutrient Media:

For germination of excised embryos, media developed by Murashige and Skoog (MS) with some modification, (MS) and Quoirin and Lepoivre and modified by Aitken-Christie (LP) have been used.

	(MS)	(LP)
Major	mg/lit	mg/lit
KNO ₃	1900	1,800
MgSO ₄ .7H ₂ O	370	360
CaCl ₂ .2H ₂ O	440	-
NH ₄ NO ₃	1650	400
KH ₂ PO ₄	170	270
Ca(NO ₃) ₂ .4H ₂ O	-	1200

Minor:	(MS) mg/lit	(LP) mg/lit
FeSO ₄ ·7H ₂ O	27.8	30
Na ₂ EDTA·2H ₂ O	37.3	40
MnSO ₄	15.6	1
H ₃ BO ₃	6.2	6.2
ZnSO ₄ ·H ₂ O	8.6	8.6
KI	0.83	0.08
CuSO ₄ ·5H ₂ O		
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25
CoCl ₂ ·6H ₂ O	0.025	0.025

Vitamines:

Thiamine HCl	0.5	0.4
Nicotinic acid	0.05	
Pyridoxine.HCl	0.5	
Nye-inositol	100	1,000
Sucrose	30,000	30,000

Attempts on regeneration:

Parts of seedlings (cotyledons) obtained from germination of seeds without seed coats were placed on MS medium with different concentration of growth hormones.

RESULTS AND DISCUSSION:

The cold pretreatment of the seeds was necessary as untreated fresh seeds did not respond well.

The seeds with or without seed coat germinated and developed into seedlings after 7 to 10 days which were used as explants for regeneration experiment.

Both kinds of excised embryos that were placed in the MS &

LP medium showed development into plantlets within 4-5 days at 25⁰ c and light intensity of 500 lux for 16 hours per day. It was noticed that a slightly damaged cotyledon did not significantly affect the development process. However, embryo with damaged radicle would not develop to root stage.

In order to determine the regeneration potential of the explants taken from seed-grown seedlings, a factorial experiment using different ratio of auxin and cytokinin in MS medium was carried out. The results obtained so far indicated that when either hypocotyls or cotyledons or combination of both were incubated on agar in MS media containing varying concentrations of EAP (0 - 2 mg/lit) and EAA (0 - 2 mg/lit), callus formation was noticed in every case within 2 weeks. When explants were incubated on 20 μ M BAP for 2-3 weeks, instead of callus formation, adventitious buds were observed

OVERVIEW OF PROGRESS:

After the grant agreement was finalised between AID and WI, procurement of chemicals, glassware and furniture and recruitment of research assistant was started in October 1987. Unfortunately the whole process was delayed by a month because of temporary confusion as to the place where the tissue culture laboratory could be set up. On Nov 1, 1987 an order for equipment to be imported from U.S. was sent to WI-Headquarters.

Chemicals and glassware that were available locally were procured without delay. An order for laminar air flow hood was placed to be built locally. A research assistant was hired

effective November 1, 1987. The job to paint and furnish the research laboratory was also undertaken in November and finished within a month.

Appointment of a post-doctoral fellow was postponed for a later date subject to the arrival of equipment from U.S. In December preliminary experiments on pine regeneration (seed germination, embryo isolation etc) were started with initial success. In the same month researchers became capable of growing plantlet from embryo in the test tube for both pine species, an important breakthrough, considering the past failure in doing so as reported by scientists working in this area.

In January 1988, the PI undertook a two-week trip to Delhi for consultation on pine research as well as for attending a symposium on Tissue Culture (see the report on International Travel for detail). Work on pine regeneration has been restricted by non-availability of proper equipment. During the period of January to February 1988, the investigators were able to get callus type material from explants like cotyledons, hypocotyls, etc. In retrospect it turns out that tissues alleged to be callus might not have been regular callus but adventitious buds. The PI learned about this possibility from Folph Nett when he visited him in Raleigh during his two-week trip to the U.S. in April) (to be reported upon in next Semi-Annual Report). Since then the research on pine regeneration has a new direction and approach. The researchers are confident of success in pine regeneration. Their work will be expedited and facilitated once the equipment

arrives from the U.S.

Researchers have already received quite a few top-ranking books on tissue culture. They are getting two leading journals - In vitro and Plant Physiology. Once full facilities are available in the laboratory, a postdoctoral fellow will be appointed; this will give a big boost to the research program.

The research team has been fortunate to have Vinrock International as the grantee because they are doing their best in administering the grant.

INTERNATIONAL TRAVEL

TRIP TO INDIA

In order to consult with Dr. S. S. Phojwani, the collaborator of CPPT project, the Principal Investigator visited Delhi from January 3 to 17, 1988. During this visit he learnt monoclonal antibody techniques in the Biochemistry Department, All India Institute of Medical Sciences. The PI had been invited for this training by Professor S. P. Sharma, Chairperson of the Department. This trip became highly valuable because the PI got an opportunity to participate in an "International Workshop on Applications of Biotechnology in Forestry and Horticulture," sponsored by National Science Foundation USA. This meeting allowed him to meet Dr. Don Durzan, advisor and Dr. Polih Pitt, the collaborator of the CPPT Project. On request from Professor S. C. Maheshwari, the PI gave a lecture in his laboratory on Plant Fatty Acid Biosyntheses. Overall, this trip was a successful one.

FUTURE PROGRAM:

The task of elucidating and developing tissue culture technology for regeneration of pine seedlings is progressing well. In the immediate future, growing pine seedlings in green house will be started so that clean stock material is available for research. Following the work done by Ralph Mott, researchers here will try to graft mature pine branches with young plants so that young shoots from mature trees can be obtained in the green house. Researchers will try to learn different parameters regarding normal plantation of pine trees on a certain piece of land allocated for pine research (Community Forest Department will be approached for help in this direction).

It is generally felt that the research team must learn the hardening process of tissue culture-produced seedlings. To this effect, attempts will be directed towards rooting in green house of the very young shoots obtained by severely hedging young pine seedlings. The rooted shoots (seedlings) will be planted outside in the land allocated for research forming the basis for learning about hardening process.

REFERENCES

1. Unasylva (1985) 37 No. 147, pp. 7-16, FAO, Rome.
2. Keier, T., Stoecking, C.P., Farlour, M. C and Post T. L. (1982) Botany, An Introduction to Plant Biology. Sixth edition, p. 60". John Wiley and Sons.
3. Durzan, D. J. and Campbell, P. A. (1974) Prospects for the production of improved stock of forest trees by cell and tissue culture. Can. J. Forestry Res. 4, 151-174.
4. Durzan P. J. and Campbell, P. A. (1974) Prospects for the Introduction of traits in forest trees by cell and tissue culture. New Zealand J. Forestry Sci. 4, 261-266.
5. Panga, J. V. (1977) Application of tissue culture in forestry. In "Applied and Fundamental Aspects of plant cell, tissue and Organ Culture" (J. Reinert and Y. P. S. Bajaj, eds.), pp. 93-108, Springer-Verlag.
6. Vinton, L. (1978) Morphogenesis in clonal propagation of woody plants. In "Frontiers of Plant Tissue Culture" (T. A. Thorpe, ed.), pp. pp. 419-426. Univ. of Calgary Press, Calgary.
7. Kott, P. L. (1978) Tissue culture propagation of conifers. In "Propagation of Higher Plants through Tissue Culture - A Bridge between Research and Application" (E. F. Hughes, R. Henke and E. Constantin, eds.), pp. 125-133. Tech. Inf. Cent., U.S. Dept. of Energy, Springfield, Virginia.
8. Somers, H. G. and Freen, C. L. (1979) Application of tissue culture to forest tree improvement. In "Plant Cell and Tissue Culture: Principles and Applications" (E. R. Shan, P. O. Larsen, E. P. Federick and V. Raghvan, eds.), pp. 451-491. Ohio State Univ. Press, Columbus.
9. Kott, P. L. (1981) Trees. In "Cloning Agricultural Plants Via in Vitro Techniques" (R. V. Conger, ed.), pp. 217-254. CRC Press, Boca Raton, Florida.
10. Panga, J. V. and Durzan, D. J. (1982) "Tissue Culture in Forestry" Nijhoff, The Hague.
11. Biondi, S. and Thorpe, t. A. (1982) Clonal propagation of forest tree species. In "Proceeding of the COSTED Symposium on Tissue Culture of Economically Important Plants". (A. P. Rao, ed.) pp. 197-204, COSTED and Asian Network of Biological Science, Singapore.
12. Horgan, K. J. (1982). The tissue culture of forest trees. Proc. Int. Symp. Natl. Acad. Sci., 9th, pp. 105-120
13. Thorpe, T. A. and Biondi, S. (1983). Conifers. In "Handbook

- of Plant Cell Culture" (D. A. Evans, W. R. Sharp, P.V. Ammirato and Y. Yanada, eds.) Vol. 2 Macmillan, New York.
14. Aitken-Christie, J. and Thorpe, T. A. (1984) Clonal propagation: Gymnosperms. In "Cell Culture and Somatic Cell Genetics of Plants" (L. K. Vasil, ed.), Vol.1, pp. 82-95. Academic Press.
 15. Evans, D. A., Sharp, W. R., and Flick, C. E. (1981) Plant regeneration from cell cultures, In "Horticultural Review" (J. Hanick, ed.), vol.3, pp. 214-314. The Avi Publishing Co., Westport, Connecticut.
 16. Campbell, P.A. and Purzan, D. J. (1975) Induction of multiple buds and needles in tissue cultures in Picea glauca. Can J. Bot. 53, 1652-1657.
 17. Sommer, H. G., Brown, C. I. and Kornanik, P. P. (1975) Differentiation of plantlets in loblolly pine (Pinus taeda Mill.) tissue cultured in vitro. Bot. Gaz. 136, 196-200.
 18. 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 culture in vitro. C. R. Acad. Sci., Paris 284, 627-630.
 19. Greenwood, F. S. meristems in vitro by hypocotyl section from dormant Pinus taeda embryos. Can J. Bot. 43, 173-175.
 20. Binocha, S. C. (1980) Callus and adventitious shoot formation in excised embryos of white pine (Pinus strobus). Can J. Bot. 58, 366-370.
 21. Cohen, H. A. (1975) Vegetative propagation of P. strobus by needle fascicles. cc. Inter. plant Prop. Soc 25 413-419.
 22. Sommer, P. E. and Brown, C. I. (1974) Plantlet formation in pine tissue culture. Am J. Bot. 61, 11.
 23. Girouard, E. M. (1971) Vegetative propagation of Pinus by means of needle fascicles -a literature review. Inform. Rpt. Environ., Can For. Ser. Quebec.
 24. Mott, P. L. and Amerson, H. V. (1981) A tissue culture for the clonal production of loblolly pine plantlets. N. C. Agric. Res. Serv., Tech. Bull. 270, 1-14.
 25. Peily, K.J. and Fischer, J. (1977) Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue. New Zealand J. Forestry Sci. 7, 199-206.
 26. Aitken, J. , Morgan, K.L. and Thorpe, T. A. (1981) Influence of explant selection on the shoot - forming capacity of juvenile tissue of Pinus radiata. Can J. Forestry Res. 11,

112-117.

27. Horgan, K. J. and Aitken, J. (1981) Reliable plantlet formation from embryos and seedling shoot tips radiata pine. ysiol. plant. 53, 170-175.
28. Polmann, C. H. and Jasson, E. (1980) Organogenesis in culture Pinus sylvestris tissue. Z. pflanzenphysiol. 96, 1-6.
29. Jalaska, S., Poljarecka-Pletikovic, P. and Cidakovic, B. (1981) End regeneration in Pinus nigra embryo and seedling tissue culture. In "Coloqum International Sur La Culture in vitro Des Essences Forestieres", pp. 159-166. AFCCFL, Nanais.
30. David, A., David, P., Faye, M., and Isenokali, K. (1979) The cultivation in vitro and the micropropagation d'Arbes Forestieres", AFCCFL. etudes et recherches, No.12, 6879, pp.33-40. AFCCFL, Nanais.
31. Ponca, J.P. (1974) In vitro culture of microsporephylls and megagametophyte tissue of Pinus. In Vitro 9, 270-277.
32. Ponca, J.P. (1981) Paploid tissue culture and cytology of conifers. In "Coloqum International Sur La Culture In Vitro Des Essences Forestieres", pp. 283-292. AFCCFL, Nanais.
33. Tominga, Y. and Goo, K. (1970) Cytological studies on the calli of Pinus densiflora in vitro. Pfuroshima Jap. Agr. coll. Full. 4, 8-10.
34. Dallimore, B. and Jackson, A.P. (1966) a handbook of Coniferae and Cycadaceae. Edward Arnold Publishers, London.
35. Stainton, J.D.A. (1972) Forests of Nepal. John Hurry, London.
36. Uphof, J.C.Th. (1969) Dictionary of Economic Plants. Verlag von J. Cramer.
37. Chopra, P.P., Chopra, I.C. and Varma, P.S. (1965) Supplement to Glossary of Medicinal Plants. Publications and Information Directorate, New Delhi.
38. Howland, A.E and Howland, P. (1984) A dictionary of the common forest and farm plants of Nepal. Forest Research and Information Center, Dept. of Forest, Kathmandu.
39. Puri, G.S. (1969) Indian forest Ecology. Vol I, Oxford Book and Stationary Co., New Delhi.
40. Murashige, T. and Skoog, F. (1962) a revised medium for rapid growth and bicassays with tobacco tissue cultures. Physiol. Plant. 15, 473-479.
41. Carborg, O.J., Miller, F.A. and Gjime, K. (1968) Nutrient requirements of suspension cultures of soyabean root cells. Exp.Cell Res. 50, 150-158.

42. Schenk, P.H. and Hilderbrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204
43. Gresshoff, P.P. and Doy, C.H. (1972) Development and differentiation of haploid Lycopersicon esculentum (tomato). Planta 107, 161-170.
44. Quoirin, H. and Lepoivre, P. (1977) Etudes de milieux adaptes aux cultures in vitro de prunus. Acta. Hortica. 437-442.
45. Pomeroy, J.A., Varnell, P.J. and Taber, C.A. (1970) Culture of apical meristems and embryonic shoot of Picea abies. Approaches and Techniques. USIA-TECF. 1970, 1046.
46. Pojar, C.H. (1981) In vitro regeneration potential of the conifer phylloclad. In "Symposium on Clonal Forestry", Research Notes 22, Bupala, 43-56.
47. Berman, C.B. and Jansen, F. (1981) Regeneration of plants from the conifer leaf with special reference to Picea abies and Pinus sylvestris. In "Colloque international sur la culture in vitro Des Essences Forestieres", pp. 41-53. AFCEEI.Nangis.
48. Throppe, T. A. and Patel, K.P. (1984) Clonal propagation: Adventitious buds. In "Cell culture and somatic cell genetics of plants" (I.K.Vasil, Ed) Vol.1, pp 49-60. Academic Press.
49. David, A. (1987) In vitro Propagation of gymnosperms. In "Tissue Culture in Forestry" (J.H. Fonga and D.J. Durjan, eds.), pp 72-108, Pijhoff, The Hague.
50. Sagawa, Y. and Fujinaki, J.T. (1984) Clonal propagation: Orchids. In "Cell Culture and Somatic Cell Genetics of Plants" (I.K. Vasil, ed.), pp. 61-67. Academic Press, Inc.

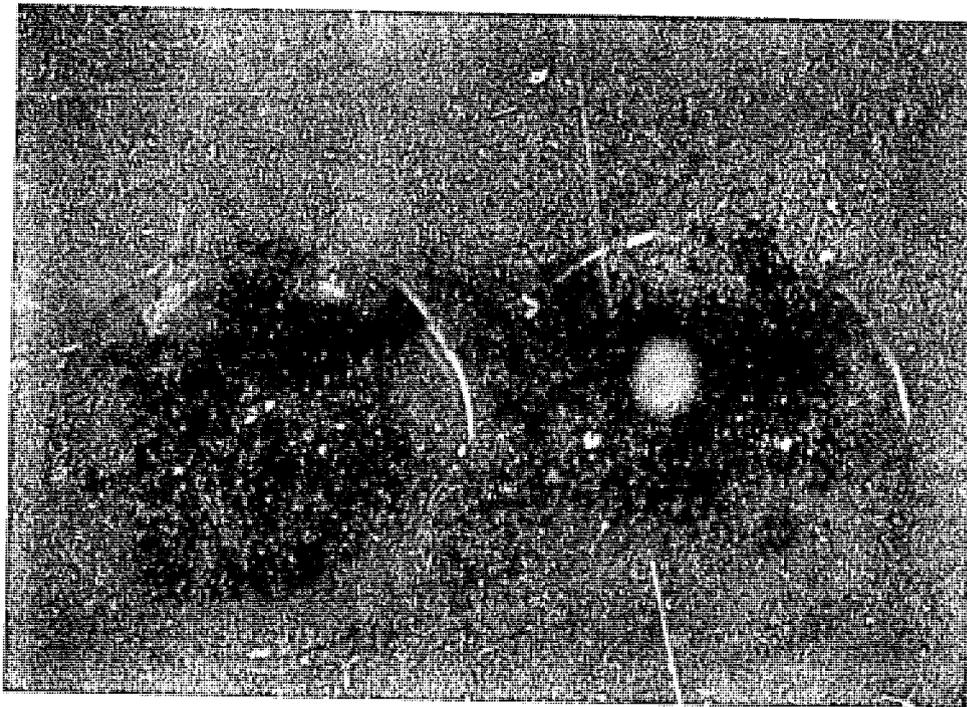


PHOTO 1: BIGGER SEEDS: P. ROXBURGHII
SMALLER SEEDS: P. WALLICHIANA



PHOTO 2: SEED OF P. ROXBURGHII WITHOUT ITS SEED COAT

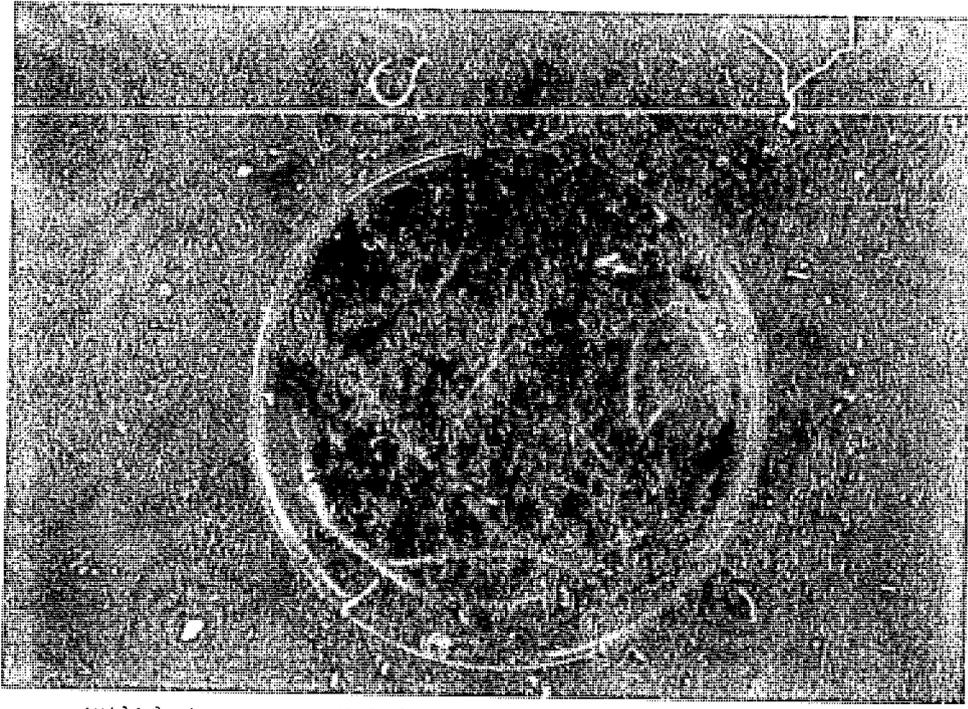


PHOTO 3: SEED OF *P. WALLICHIANA* WITHOUT ITS SEED COAT, DEVELOPED TO SEEDLINGS.



PHOTO 4: ONE DAY EMBRYO PLANTED IN H.S. MEDIA

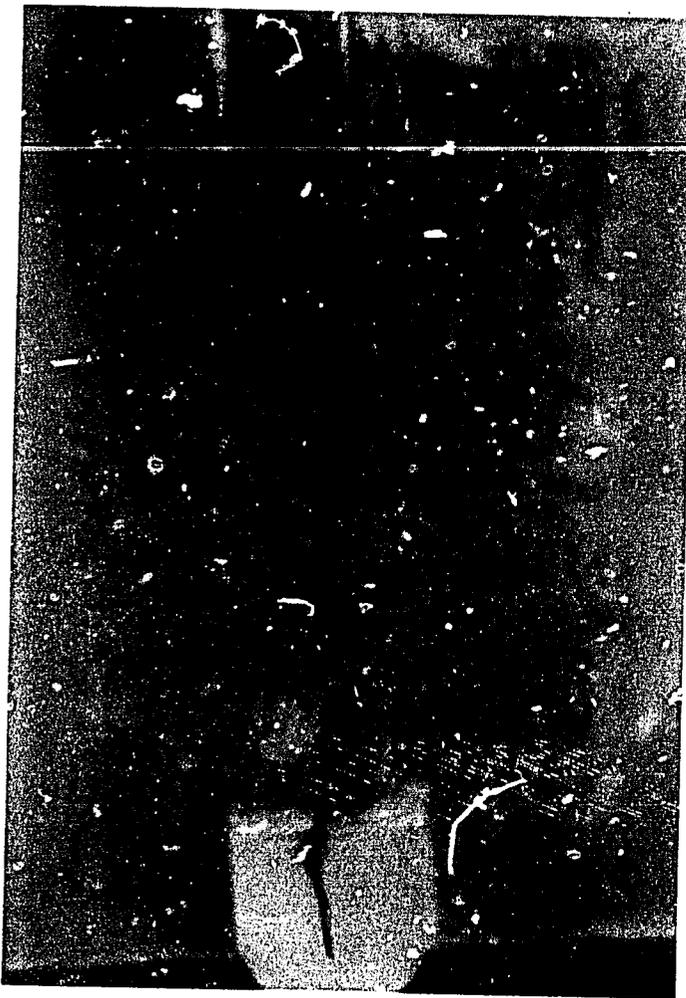


PHOTO 5:
PINUS WALLICHIANA GROWN FROM
EMBRYO IN M. S. MEDIA

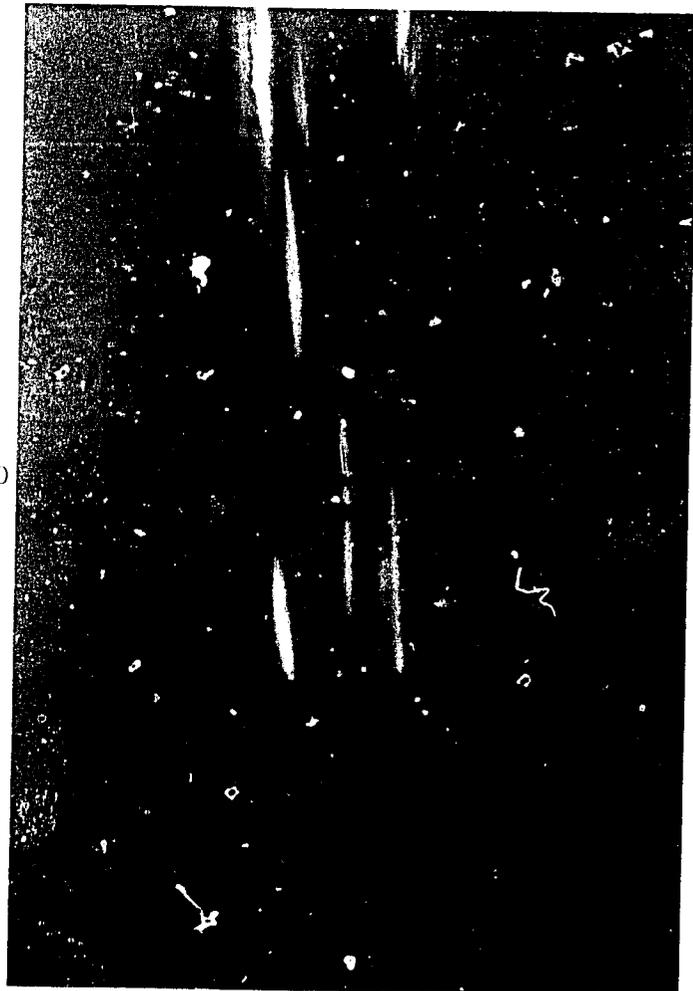


PHOTO 6:
PLANTLET OBTAINED FROM EMBRYO
CULTURE OF P ROXBURGHII



PHOTO 7: THE SHOOT CULTURE OF P. ROXBURGHII