PN-ABL-112
ISN 16230

ANNUAL REPORT

Covering Period: August 31, 1989 to August 30, 1990

Submitted to the Office of The Science Advisor U.S. Agency for International Development.

Tittle: Gene Expression of Thermotolerance and Injury Resistance in the Underused Trees of Chile: Prosopis chilensis and Araucaria araucana.

Principal Investigator: Dr. Liliana Cardemil
Grantee Institution: Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

Collaborator: Dr. Joseph E. Varner
Dr. David Ho
Institution : Department of Biology, Washington University, St. Louis, Missouri, U.S.A.

Project Number: 7.068 Office of the Science Advisor
Project # : 936-5542
Grant # : DPE-5542-G-SS-8073-00
Obligation # : 8361137

A.I.D. Grant Officer: Dr. Mike Benge

Project Duration: August 31, 1988 to August 30, 1991

REQUEST FOR ADVANCE OR REIMBURSEMENT

(See instructions on back)

Budget, No. 80-R0183

1. TYPE OF PAYMENT REQUESTED
a. "X" one, or both boxes: ☐ ADVANCE ☐ REIMBURSEMENT
b. "X" the applicable box: ☐ FINAL ☐ PARTIAL

2. BASIS OF REQUEST: ☐ CASH ☐ ACCRUAL

3. FEDERAL SPONSORING AGENCY AND ORGANIZATIONAL ELEMENT TO WHICH THIS REPORT IS SUBMITTED
Agency for International Development

4. FEDERAL GRANT OR OTHER IDENTIFYING NUMBER ASSIGNED BY FEDERAL AGENCY
DPE-5542-G-SS-8073-00

5. PARTIAL PAYMENT REQUEST NUMBER FOR THIS REQUEST

6. EMPLOYER IDENTIFICATION NUMBER

7. RECIPIENT'S ACCOUNT NUMBER OR IDENTIFYING NUMBER

8. PERIOD COVERED BY THIS REQUEST
FROM (month, day, year): March 1st, 1990
TO (month, day, year): August 30, 1990.

9. RECIPIENT ORGANIZATION
Universidad de Chile, Fac. de Ciencias
Name: Banco de Chile, Dep. del Ext. Sec. Contabilidad Acc. Nº 51
Number and Street: Casilla 151-D
City, State and ZIP Code: Santiago, Chile

10. PAYEE (Where check is to be sent is different than item 9)
Name:
Number and Street:
City, State and ZIP Code:

11. COMPUTATION OF AMOUNT OF REIMBURSEMENTS/ADVANCES REQUESTED

PROGRAMS/FUNCTIONS/ACTIVITIES ▶	(a) Salaries and Travel	(b) Equipment and Other Costs	(c) Reagents and Supplies	TOTAL
a. Total program outlays to date (As of date)	$ 39,661	$ 69,690	$ 15,864	$125,215
b. Less: Cumulative program income	30,572	71,194	20,044	121,810
c. Net program outlays (Line a minus line b)	9,090	- 1,504	- 4,180	3,405
d. Estimated net cash outlays for advance period	5,000	0,000	5,000	10,000
e. Total (Sum of lines c & d)	14,089	0,000	820	13,405
f. Non-Federal share of amount on line e				
g. Federal share of amount on line e				
h. Federal payments previously requested				
i. Federal share now requested (Line g minus line h)				
j. Advances required by month, when requested by Federal grantor agency for use in making prescheduled advances — 1st month				
2nd month				
3rd month				

12. ALTERNATE COMPUTATION FOR ADVANCES ONLY

a. Estimated Federal cash outlays that will be made during period covered by the advance	$
b. Less: Estimated balance of Federal cash on hand as of beginning of advance period	
c. Amount requested (Line a minus line b)	$ 10,000

13. CERTIFICATION

I certify that to the best of my knowledge and belief the data above are correct and that all outlays were made in accordance with the grant conditions or other agreement and that payment is due and has not been previously requested.

SIGNATURE OF AUTHORIZED CERTIFYING OFFICIAL: [signature]

DATE REQUEST SUBMITTED:

TYPED OR PRINTED NAME AND TITLE
Profesor Camilo Quezada Bouey
Dean of Facultad de Ciencias, U. de Chile

TELEPHONE (AREA CODE, NUMBER, EXTENSION)

This space for agency use

2

PAYMENT - PERIODIC ADVANCE (NOVEMBER 1985)

(This provision is applicable when (1) the grantee has an acceptable accounting system (2) the grantee has the ability to maintain procedures that will minimize the time elapsing between the transfer of funds and the disbursement thereof, and (3) the grantee's financial management system meets the standards for fund control and accountability required under the standard provision of this agreement entitled "Accounting, Audit, and Records".)

(a) AID funds shall not be commingled with other grantee owned or controlled funds. The grantee shall deposit all AID cash advances in a separate bank account and shall make all disbursements for goods and services from this account.

(b) Each quarter, after the initial cash advance, the grantee shall submit to the AID Controller, identified in the schedule, voucher SF 1034 (original) and SF 1034-A (three copies), entitled "Public Voucher for Purchases and Services Other Than Personal", copies of which are attached.

(c) Each voucher shall be identified by the appropriate grant number and shall be accompanied by an original and three copies of a report in the following format:

FEDERAL CASH ADVANCE STATUS REPORT
(Report Control No. W-245)

A. Period covered by this report:

FROM (Month, day, year) March 1st, 1990
TO (Month, day, year) August 30, 1990

Period covered by the next report:
FROM (Month, day, year) August 31, 1990
TO (Month, day, year) February 28, 1991

B. Cash Advance Use and Needs:

1. Cash advance on hand at the beginning of this reporting period ..... $ 12,136

2. U.S. Treasury check advance(s) received during this reporting period..... $ 14,175

3. Interest earned on cash advance during this reporting period..... $ 0,000

4. GROSS cash advance available during this reporting period (Lines 1, 2, & 3).....$ 26,311

5. LESS, interest remitted to AID during this reporting period..... $ 0,000

6. NET cash advance available during this reporting period (Line 4 minus Line 5)..... $ 26,311

7. Total disbursements during this reporting period, including subadvances (see footnote 1)..... $ 22,906

8. Amount of cash advances available at the end of this reporting period (Line 6 minus Line 7)..... $ 3,406

9. Projected disbursements, including subadvances, for the next reporting period (see footnote 2)..... $ 13,406

10. Additional cash advance requested for the next reporting period (Line 9 minus Line 8)..... $ 10,000

11. Total interest earned on cash advance from the start of the grant to the end of this reporting period, but not remitted to AID ..... $ 0,000

12. Total cash advances to subgrantees, if any, as of the end of this reporting period..... $ 0,000

FOOTNOTES:

1. The grantee shall submit a cumulative detailed report of disbursements by BUDGET line item quarterly.

2. The grantee shall attach a Summary, by BUDGET line item, of its projected disbursements for the next reporting period.

C. Certification:

The undersigned hereby certifies: (1) that the amount in paragraph B.9 above represents the best estimate of funds needed for the disbursements to be incurred over the period described, (2) that appropriate refund or credit to the grant will be made in the event of disallowance in accordance with the terms of the grant, (3) that appropriate refund or

credit to the grant will be made in the event funds are not expended, and (4) that any interest accrued on the funds made available herein will be refunded to AID.

DATE _____

BY Juan Carrasco Contreras

TITLE Jefe de Ofina de Importaciones y Donaciones, Facultad de Ciencias.

(END OF STANDARD PROVISION)

Disbursements of Budget Grant # DPE-5542-6-SS-8073-00

Cummulative Detailed Report

From: March 1st, 1990
To : August 30, 1990

	US$
Salaries	3,031
Equipment	0,000
Travel	8,196
Reagents	10,174
Other Costs	1,505
TOTAL	22,906

TOTAL DISBURSEMENTS OF BUDGET

CUMMULATIVE REPORT UP TO AUGUST 30, 1990 (First and second year)

	From August 30, 1988 to May 5, 1989 US$	From May 5, 1989 To August 30, 1989 US$	From August 31, 1989 To February 28, 1990 US$	From March 1st, 1990 To August 30, 1990 US$	TOTAL US$
SALARIES	1,440	2,424	5,958	3,031	12,853
EQUIPMENT	45,755	1,431	2,655	0,000	49,841
TRAVEL	5,874	1,794	1,852	8,196	17,718
REAGENTS	4,190	4,174	1,506	10,174	20,044
OTHER COSTS	1,230	12,473	6,146	1,505	21,354
TOTAL ...	58,491	22,296	18,117	22,906	121,810

PRELIMINARY REMARKS

This report corresponds to six months of work of our project, from the period March 1st to August 30 of the present year. It corresponds to the end of the second year of the above grantee. For this reason and following the guidelines of the National Research Council we are including a summary of the work achieved during this second year of research as well a description of the highlights achievements during the past semester.

A manuscript entitled **"Expression of heat shock proteins in seeds and seedlings during growth of Araucaria araucana as a response to thermal stress"**, from the authors Claudio Goycoolea and Liliana Cardemil is included. It is being submitted to the Journal of Plant Physiology and Biochemistry.

A letter from Journal of Experimental Botany is enclosed with the acceptance for publication of our previous manuscript **"Expression of cell wall proteins in seeds and during early seedling growth of Araucaria araucana is a response to wound stress and developmentally regulated"** from Liliana Cardemil and Alejandro Riquelme. A letter from Dr. Maurice Fried of the National Research Council is also enclosed where is stated that

8

our research and previous reports have been found of entire satisfaction.

We have spent so far the amount of US$22,906 in the second year of the project.

We are requesting US$10,000 for the starting period September 1st, 1990 to February 28, 1991.

9

EXECUTIVE SUMMARY

Summary of the Research performed from August 31, 1989 to August 30, 1990 (second year of the AID project).

Western blot analyses of the heat shock proteins synthesized by seedlings of Prosopis chilensis, using antibodies raised against conjugated ubiquitin showed several bands of proteins which cross react with the antibody. These proteins bands may corresponds to proteins which are targeted by ubiquitin to be degraded during heat stress. Western analysis using the antibody raised against free ubiquitin, showed the presence of a low molecular weight protein band, about 9 KDa, which corresponds to the molecular weight of ubiquitin. This protein is not expressed at 35° and 40°C but it is expressed at 45° and 50°C. Monoclonal antibodies raised against the 70 KDa hsp and used for Western analyses showed that in Prosopis chilensis there are two protein bands which cross react with the antibody. The proteins have molecular weights of 62 and 71 KDa. The expression of these two proteins increases in embryo axes subjected to 40°, 45° and 50°C when the seeds are germinated at 35°C and increases at 35°, 40°,

10

45° and 50°C when the seeds are germinated at 25°C.

Field research was started in natural population of Prosopis chilensis during the past semester. One location is at Quilapilún Station of Peldehue (Central Chile) and the other is at La Serena in the gorge of San Carlos (Norte Chico). Maximum and minimum temperatures, humidity, evaporation and radiation were registered for two days of January and two days of February, 1990. Temperatures were also registered in July for San Carlos. The maximum temperatures dropped in 1° and 2°C from January to February at Quilapilún. In San Carlos, the temperature did not dropped during this period. However, between January and July of 1990 dropped 37°C being the maximum temperatures in January 36°C and -1°C in July. Phenological studies started in these natural populations of Prosopis. Samples of leaves were taken at different hours of the day to screen the hsp present in these organs using Western analyses to detect the presence of ubiquitin and 70 KDa protein with their respective antibodies.

Two cell wall proteins of Araucaria araucana have been purified by isochromatofocusing. They have a molecular weight of 60 and 100 KDa. Both protein are rich in glycine and serine. A third protein of 155 KDa have been purified by preparative SDS electrophoresis and is also rich in glycine and serine. The two

aminoacids comprises about 40% of the aminoacids of these proteins. These three proteins are glycosylated with galactose, glucose, xylose and mannose and have peroxidase activity.

The amino terminal sequence have been obtained for the proteins 60 and 100 KDa. The amino terminal sequence is identical in these two proteins suggesting these proteins are genetically related.

Prosopis chilensis cell wall proteins have been analyzed by SDS polyacrylamide electrophoresis and cationic neutral electrophoresis of the native proteins during development from 12 to 36 hours of seedling growth and under wounding and non-wounding conditions. Seedlings of 12 hours of growth show a better response to injury with a higher expression of cell wall proteins than seedlings of 36 hours of growth. Hypocotyls are the organs showing a better response to damage and a higher increase of expression of hydroxyproline-rich proteins. Western analyses of the wall proteins with antibodies raised against soybean seed coat extensin demonstrated the presence of three new proteins in cotyledons which were wounded 24 hours before the analyses.

Part of this research has been performed in collaboration with Professors Joseph Varner and David Ho from Washington University, St. Louis, Missouri. For this purpose Liliana

Cardemil, the principal investigator, visited their laboratories at Washington University two times during the past two years. In 1989, Alejandro Riquelme also spent 2 1/2 months in Professor Varner's Lab. Professor Varner and Professor Ho are planning to visit Chile in 1991.

13

SECTION I

Note on Publications:

The enclose manuscript entitled **"Expression of heat shock proteins in seed and during seedlings growth of Araucaria araucana as a response to thermal stress"** from the authors Claudio Goycoolea and Liliana Cardemil is being submitted to the Journal of Plant Physiology and Biochemistry.

A letter of acceptance from The Journal of Experimental Botany of our previous manuscript entitled **"Expression of cell wall proteins in seeds and during early seedling growth of Araucaria araucana is a response to wound stress and developmentally regulated"** from Liliana Cardemil and Alejandro Riquelme is also enclosed.

A. Research Objectives:

The specific objective of this project is to investigate the expression of genes responsible for the synthesis of specific plant proteins that appear to be present under, and protect against damage from temperature stress and injury stress.

The research is performed with two native trees of Chile:

14

Prosopis chilensis and *Araucaria araucana*. Both trees are well adapted to their harsh environment. They are living under conditions considered to be extreme for most plant systems.

Therefore, these two species of trees will allow us to test the hypothesis that: "Native trees are better adapted to their harsh environment because they show a higher degree of expression of those genes encoding proteins related to thermotolerance and injury resistance". The higher degree of expression may be due either to regulatory mechanisms of gene function or to the number of copies of genes involved.

The specific objective of this research has been accomplished with the following research activities which can be considered as specific research goals.

1. To study the response of embryo and reserve tissues (mainly cotyledons in *Prosopis chilensis* and megagametophyte and seedling hypocotyl of *Araucaria araucana* seedlings) to temperature stress and to injury stress through synthesis of temperature shock proteins (cold and heat shock) and accumulation of HRGPs.

2. To investigate the presence of the genes responsible for the synthesis of temperature shock proteins and HRGPs and the number of copies of these genes using southern blot hybridization

15

techniques with the heterologous probes available.

3. To investigate the control and regulation of the synthesis of these proteins, by quantitative determination of the mRNA using northern blot hybridization with the DNA probes available.

4. To characterize the temperature shock proteins and HRGPs by chemical analysis and immunological methods to compare them with the known proteins of the other groups of plants.

5. To develop histoimmunological methods as a mean to elucidate the cellular locations of these proteins and studying the possible relationship between HPRGs and lignification.

6. To develop an "in vitro" culture system of embryo cell protoplasts and synchronized dividing cells in Prosopis chilensis and Araucaria araucana to study cell wall assembly and regeneration in presence of prolylhydroxylase inhibitors such as the α,α'-dipyridyl and 3,4-dehydro-D,L-proline.

7. To survey in the natural populations of Prosopis chilensis and Araucaria araucana, the degree of expression of the genes involved in temperature shock proteins and HRGPs as a means to select individual trees which have significantly increased amounts of these proteins. Therefore, they should be eventually better protected against temperature stress, wounding and/or

pathogens.

8. To develop methods for *in vitro* culture an vegetative propagation of the selected individual trees, evaluating the stability of the selected phenotypes (high degree of expression for heat shock proteins and HRGPs) and selected genes.

From all the activities mentioned above we have begun to study the expression of heat shock proteins (hsp) in natural populations of *Prosopis chilensis*. During these studies we are expecting to select the resistant individuals to propagate them through *in vitro* culture or vegetative propagation.

All of the other activities are in progress.

B. Research Accomplishments

Because we sent a completed detailed report of our research for the period from August 31st 1989 to February 28, 1990 (Report #3) which corresponds to the first semester of the second year of the project, we are reporting here in detail only the activities and results obtained in the period of March 1st to August 30 of this present year. The Executive Summary, however, summarizes the complete second year of research.

17

Temperature Stress Experiments

We have performed a Western blot analysis with proteins extracted from seedlings of Prosopis chilensis subjected to temperature stress, using a monoclonal antibody against the 72 KDa protein purified from HeLa cells. This antibody is commercially available in Amersham Chem. Co.

Two groups of seedlings were subjected to this analysis. One groups were seedlings coming from seeds germinated and grown to 25°C. They were stressed, 48 hours after germination, for 2 hours at 30°, 35°, 40°, 45° and 50°. The other group were seedlings coming from seeds germinated and grown at 35°C. They were stressed, 48 hours after germination, for 2 hours at 40°, 45° and 50°C.

After heat shock, the proteins were extracted from the seedlings and run in SDS-polyacrylamide electroforesis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes were incubated in the 1st antibody (against the 72 KDa protein) in a dilution of 1/1000 and then in a second antibody alkaline phosphatase conjugated in a dilution of 1/30000.

The results show that two bands of proteins cross react with the antibody. These proteins are 71 and 69 KDa and both of them

are present in seedlings subjected at 25°C.

However the expression of these proteins is different in seedlings grown at 25°C from seedlings grown at 35°C. In the first group the expression of the proteins increases considerably at 35°C as compared with the expression at 25° and 30°C and expressed still higher at 40°, 45° and 50°C (Fig. 1A). In the second group the expression of the proteins at 35°C is about the same in magnitud to the expression shown at 25° and 30° of the first group (Fig. 1 A and B). The expression of these two proteins further increases at 40°, 45° and 50°C, Figures 1 B.

Heat Shock Expression and Field Research

The measurements of the environmental parameters of temperature and relative humidity in the natural population of Prosopis chilensis located at Quilapilun Station in Peldehue (Central Chile) have been expanded to other locations of the IV Region of Chile, see Figure 2.

The location of studies is the gorge of San Carlos at the interior of Elqui Valley, La Serena (Figure 3). The climate of this geographic area has been described as "Mediterranean Arid"

and is characterized with short precipitations during Winter. Table I shows the maximum and minimum temperatures and relative humidities for the period January to July of the present year. Table 2 shows the precipitation registered for the Winter period of 1990 (June and July 1990).

In this station of gorge of San Carlos, three growth parameters of Prosopis chilensis have been measured in 5 selected individuals from the Eastern exposition and 5 selected from the Western exposition. The Eastern exposition of the gorge receive less radiation due to the morning mist. The growth parameters considered in this study are: lenght of the principal axis (Fig. 4A); total number of leaves (Fig. 4B); number of leaflets per leaf (Fig. 4C).

Our results demonstrated that there are not significant differences of growth between individual located on the Eastern exposition compared with those located on the Western exposition during the period measured from April to September 1990.

Injury Stress Experiments

Two cell wall proteins from Araucaria araucana purified as described in our previous report #3, have been analyzed for sugar

-20

composition (since they are glycosilated) and for NH_2 terminal sequence to see how much genetically related they are.

The amino terminal sequence was performed in collaboration with Professors Joseph E. Varner and David Ho, from Washington University, St. Louis, Missouri and with Monsanto Laboratories, Monsanto Company at St. Louis.

For the sugar analysis the proteins identified as B_2 of 100 KDa and B_3 of 60 KDa were electrotransferred to an Inmobilon membrane (Polyvinylidene Difluoride Membrane). The membrane was stained with Coomassie Blue and the protein bands were cut from the membrane. Each strip of membrane was hydrolyzed for 1 hour at 121°C with 2N trifluoroacetic acid (TFA). After hydrolysis the sugars were analyzed by high pressure liqued chromatography (HPLC) using a Carbo Pak PA column and 4.25 mM NaOH, pH 11.5 as buffer. The detector was a dionex pulsed amperometer with a gold electrode.

Table 3 shows the results of these analyses. There are similar amounts of xylose and galactose for both proteins. The B_3 protein, however, has a higher content in glucose and less amount of mannose

The amino terminal sequence of the two proteins were also performed by electrotransferring the proteins to Inmobilon

21

membranes in amounts of 10 µg of protein in a surface of 2 x 10 mm of the membrane. The bands were visualized with Coomassie Blue.

The protein bands were cut from the membrane and the piece of membrane containing the protein was sequenced in an automated liquid phase sequencer using a phenylthiohydantoins derivation of the NH_2 terminal residue of each protein. This is a modified procedure of Edman's degradation (G. Allen, 1989).

Table 4 shows the sequences for B_2 and B_3 proteins. Both proteins have the same sequence and therefore they seem to be genetically related.

The principal investigator of this project spent three months at Dr. Joseph E. Varner's Laboratory performing the sequencing and sugar analyses of the cell wall proteins.

During this period professor Liliana Cardemil performed a "tissue print Northern" analysis with the <u>Araucaria</u> <u>araucana</u> seedlings of 7 and 15 days after germination. For this analysis she used an antisense RNA probe for cell wall extensin from carrot roots. The probe was prepared transcribing a PUC plasmid containing the complementary DNA sequence to the cDNA for carrot extensin. The DNA sequence has also a T_3 promotor that allows to make an <u>in</u> <u>vitro</u> transcription of the DNA using RNA polymerase

22

reaction (Sambrook et al., 1990). The transcription was performed in presence of (^{35}S)UTP as precursor of the RNA synthesis.

Different parts of the seedlings were printed on Inmobilon membranes and hybridized as for a Northern blot analysis. The image of the tissues printed on the membrane were visualized by exposure of the membrane after hybridization to a X-ray film. A protocol with the hybridization procedure is included. Figure 5 shows a "tissue print Northern" obtained from cross sections of seedlings organs of A. araucana. In the prints the vascular bundles of the cotyledons, wounded areas of the megagametophyte, resin ducts of the hypocotyls were visualized with the radioactive probe demonstrating that these are the tissues where the cells seem to be engaged in mRNA synthesis for extensin.

C) Scientific Impact of Collaboration

Professor Joseph E. Varner and Professor David Ho from Department of Biology, Washington University, St. Louis, Missouri have been collaborating in this project since the starting date, August 31, 1988. The have provided a space in their Laboratory for Dr. Liliana Cardemil (the principal investigator of the project) and for one of her students, Alejandro Riquelme. Professor Cardemil visited Dr. Ho and Dr. Varner's Lab. two

22

times during the last two years for three months each. Alejandro Riquelme visited Dr. Varner's Lab for three months in 1989.

Professor Varner's have provided antibodies and probes for extensin proteins, as well for proline and glycine rich proteins of the plant cell wall.

Professor Ho'has provided a probe for the hsp 70 KDa through a collaboration with Monsanto Company.

Similar collaboration between Monsanto Laboratories and Professor Varner allowed Alejandro Riquelme to obtain the NH_2 terminal sequence of two cell wall proteins of Araucana araucana.

Both, Drs. Varner and Ho have provided protocols, and have advised with critical evaluation our results and manuscripts for publications. Both professors are planning to visit Chile in 1991, for a National Meeting of the Sociedad de Botánica de Chile. They will participate in a Symposium on Stress Physiology and will teach a short graduate course at the Facultad de Ciencias, Universidad de Chile.

Recently during this last year, professor Francisco Squeo from Universidad de La Serena has begun to collaborate in the projet, screening the response to heat stress of Prosopis chilensis at San Carlos location.

D) Description of Project Impact

So far we have not used the project results as it was proposed in the original project, the more resistant individuals of both species of trees will be used to perform vegetative and *in vitro* propagation of them.

At the present, we are planning to get next coming Summer the screening data of heat shock resistant individuals in the natural population of *Prosopis chilensis*. From the data we will select and propagate the most resistant ones.

E) Strengthening of Developing Country Institutions

All the equipment we have purchased through AID project is in use in our laboratory. A list of the purchased equipment and a complete descripcion of them has been reported in our previous reports. The equipment is available for all the investigators of our Facultad de Ciencias.

Five students have been trained in this project. One of them, Claudio Goycoolea obtained his Master Degree in December 1989.

25

F) Future work

We hope to complete most of the work that is in progress during the next coming year which is the last year of the project. At that time we hope to have a clear idea of the physiological and molecular responses of both trees to the stress conditions (heat shock and wounding) of their natural environment.

26

SECTION II

A. Managerial Issues

During the past semester we have started a collaboration with Professor Francisco Squeo from Universidad de La Serena with the idea to screen populations of Prosopis chilensis that are exposed to higher temperatures of that present in locations near Santiago. Professor Squeo is a plant ecophysiologist with a great experience on field research and statistics. He and his student will take samples of leaves, buds and branches of Prosopis trees during the hours of the days and during the seasons. The different organs will be analyzed in Professor's Cardemil laboratory for expression of heat shock proteins.

We are trying to have our time schedule on time. However, due that our project started six month later (see report #1 and 2) we would like very much that AID consider to give us six more extra months to pursue the research of the project before to submit the final report.

B. Budget

During the past semester Liliana Cardemil travelled to the

27

Department of Biology, Washington University, St. Louis, Missouri, to work in the laboratories of Professors Varner and Ho. She stayed in St. Louis for three months performing research on heat shock proteins and cell wall proteins. During her visit she bought reagents and supplies for her research and for the Laboratory of the Universidad de Chile. Due to the fact that prices have increased in U.S.A. from the time the project budget was sumitted she spent in reagents twice the amount estimated for the past semester.

The amount of US$1504 in the items other costs corresponds to douane expenses for the reagents imported from U.S.A.

However, the amount spent in salaries was less. Therefore we are taken funds from the item salaries to cover the excedent in reagents and other costs.

C. Special Concern

There is not special concern.

D. Collaboration, Travel, Training and Publication

Professor Cardemil, the principal investigator of the

28

project will go to La Serena to perform Field Research with Professor Francisco Squeo. A student from Francisco Squeo, Miss Nancy Olivares will go with them to screen the behaviour of Prosopis chilensis trees to the temperatures present during the day. Samples of leaves, buds and branches will be taken to screen the presence of heat shock proteins in these organs of the plants using the specific antibodies for hsp. From these activities we expect to select the most resistant individuals to heat stress and those showing a higher expression of hsp.

Miss Nancy Olivares from Universidad de La Serena will be trained in Dr. Cardemil's Laboratory in methods of biochemistry and molecular biology related to hsp.

Field research will be accomplished during the Summer, performing research trips twice a month.

Professor Cardemil and one of her students will travel to the Department of Biology, Washington University, at St. Louis, Missouri to work at Professor David Ho's Lab. to perform Northern hybridization analysis for mRNA quantification of the hsp.

Five students have been trained in the project. They are graduate students enrolled in a Master Program. Four of the students have research assistanship paid by AID grant. The 5th student, José Gregório Rodríguez, from Venezuela has been awarded

with a fellowship from the Red Latinoamericana de Botánica (RLB) (Latinamerican Botany Network).

We are including in this report a letter of acceptance by the Journal of Experimental Botany of a previous manuscript on Cell Wall Proteins of Araucaria araucana. We are also including a second manuscript on heat shock proteins of Araucaria araucana which has been submitted to Plant Physiology and Biochemistry. The work has been presented in scientific meetings in Chile, Brazil and U.S.A.

E. Request for A.I.D. of BOSTID Action

We would like very much that AID will request BOSTID to help us to report our results in International Conferences through organization of Symposia with participation of other investigators from the Third World Countries.

We would like to show in those Conferences our research as a model of scientific achievements that could be used in Plant Conservation and Plant Management. Also as an example that sofisticated research can be performed in non-developed countries.

30

LEGENDS OF FIGURES

Figure 1. Western blot analysis of Prosopis chilensis heat shock proteins, using a monoclonal antibody raised against 72 KDa protein from HeLa cells as a primary antibody. The secondary antibody was a mouse anti IgG conjugated to alkaline phosphatase.

A - Analysis of the proteins extracted from seedlings of 48 hours of inhibition and exposed for 2 hours at temperature of 30°, 35°, 40°, 45° and 50°C. In this case the seedlings originated from seeds germinated at 35°C.

B - Analysis of the proteins extracted from seedlings of 48 hours of imbibition and exposed for 2 hours at 35°, 40°, 45° and 50°C. In this case the seedlings originated from seeds germinated at 25°C.

After extraction, the proteins were run in a SDS-polyacrylamide gel electrophoresis, electrofransferred to nitrocellulose membranes and incubated with the primary and secondary antibodies.

Figure 2. Map of Chile showing in detail the IV Region of Chile, La Serena.

Figure 3. Map of gorge of San Carlos at the interior of Elqui Valley. The cross (x) shows the exact location of the *Prosopis chilensis* population.

Figure 4. Phenological studies of *Prosopis chilensis* from the population of gorge San Carlos. Five individuals from Eastern exposition (-o-) and five individuals from Western exposition (--Δ--) were randomly selected and the length of the principal axe (A), the number of leaves (B) and the number of leaflets/leaf (C) were measured from May to September of the present year.

Figure 5. Tissue print Northern of cross sections of megagametophytes enclosing the cotyledons and cross sections of hypocotyls of 10 and 20 days old seedlings of *Araucaria araucana*. The cross sections were printed by pressuring them on nylon membranes. The membranes were hybridized with a probe containing the genetic information for the carrot extensin (cell wall protein). The genetic information is preceeding by a T_3 promoter that allows to make *in vitro* transcription and to prepare an

32

antisense (^{35}S)RNA probe using a DNA dependent RNA polymerase reaction. (A) shows the print from 20 days old seedling. (B) are the prints from 10 days old seedling. Arrows show the wounded areas.

M; megagametophyte
h; hypocotyl

Figure 1

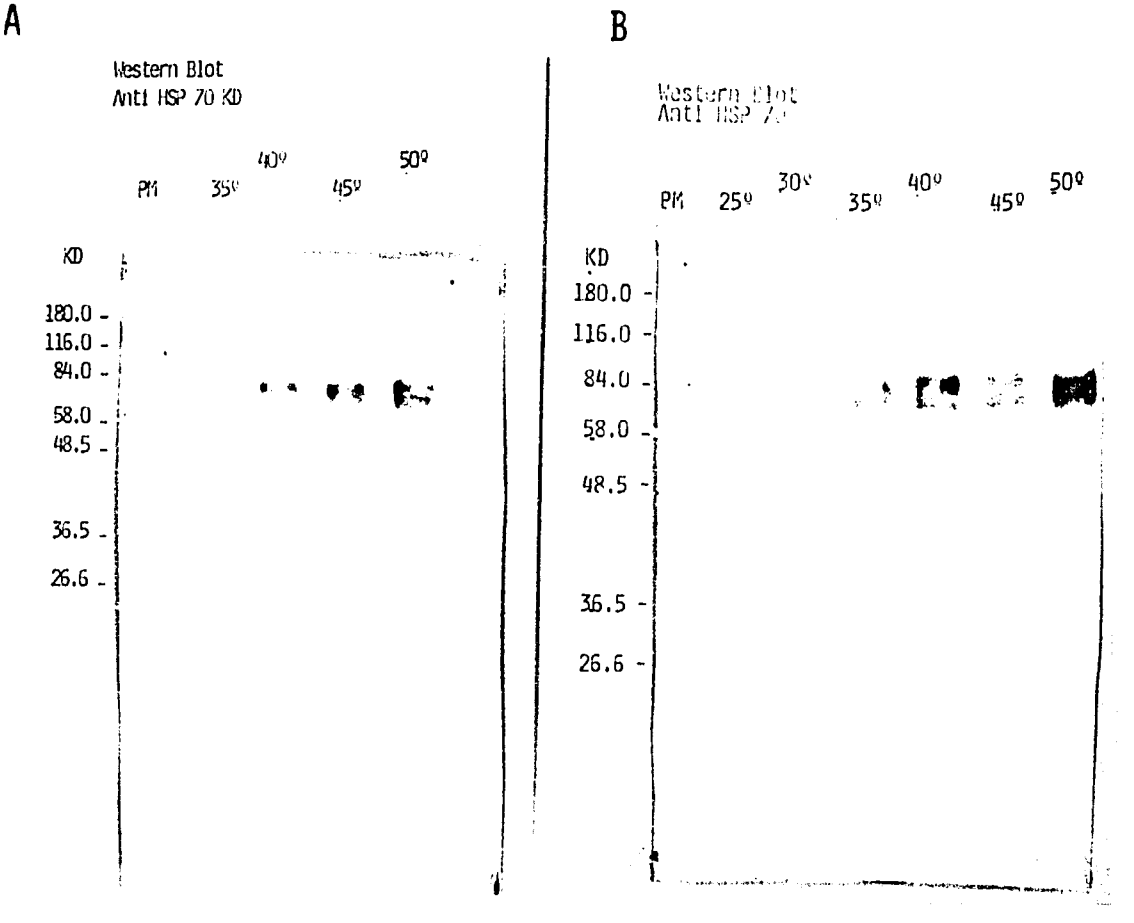
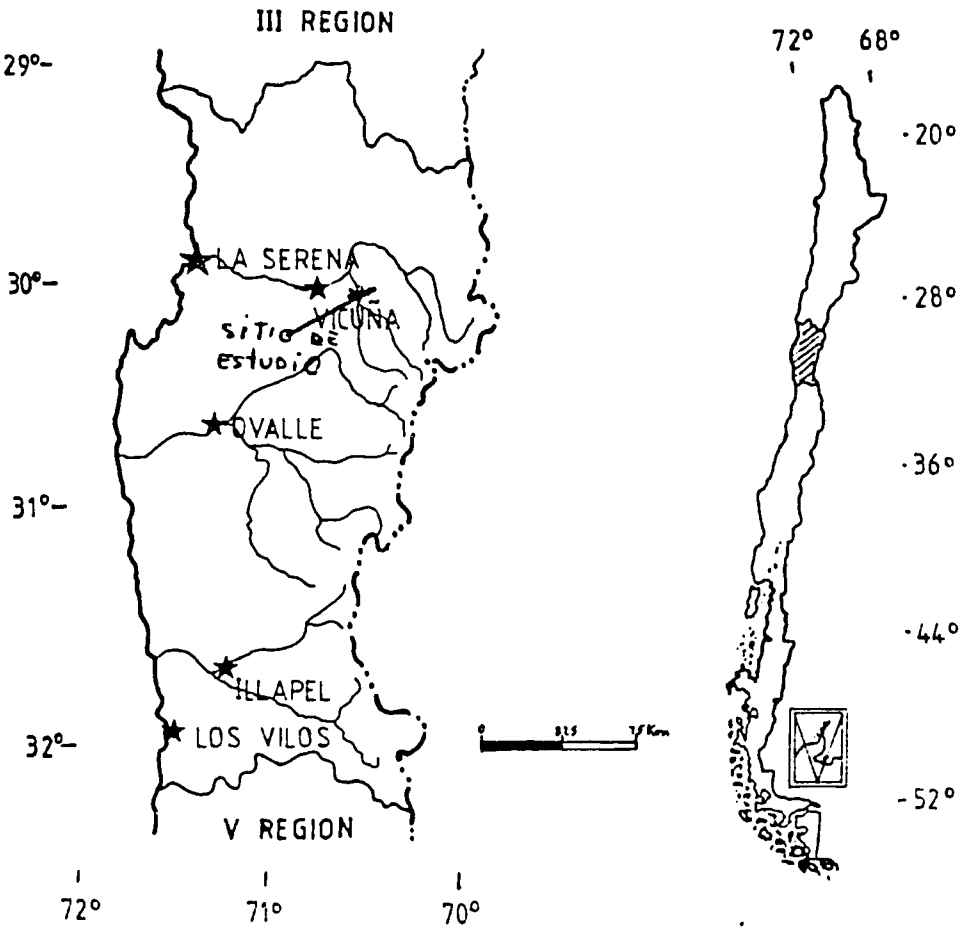

A
Western Blot
Anti HSP 70 KD
PM
35°
40°
45°
50°
KD
180.0
116.0
84.0
58.0
48.5
36.5
26.6
B
Western Blot
PM
25°
30°
35°
40°
45°
50°
KD
180.0
116.0
84.0
58.0
48.5
36.5
26.6

Figure 2


III REGION
29°
30°
31°
32°
LA SERENA
VICUÑA
SITIO DE ESTUDIO
OVALLE
ILLAPEL
LOS VILOS
V REGION
72°
71°
70°
72°
68°
20°
28°
36°
44°
52°

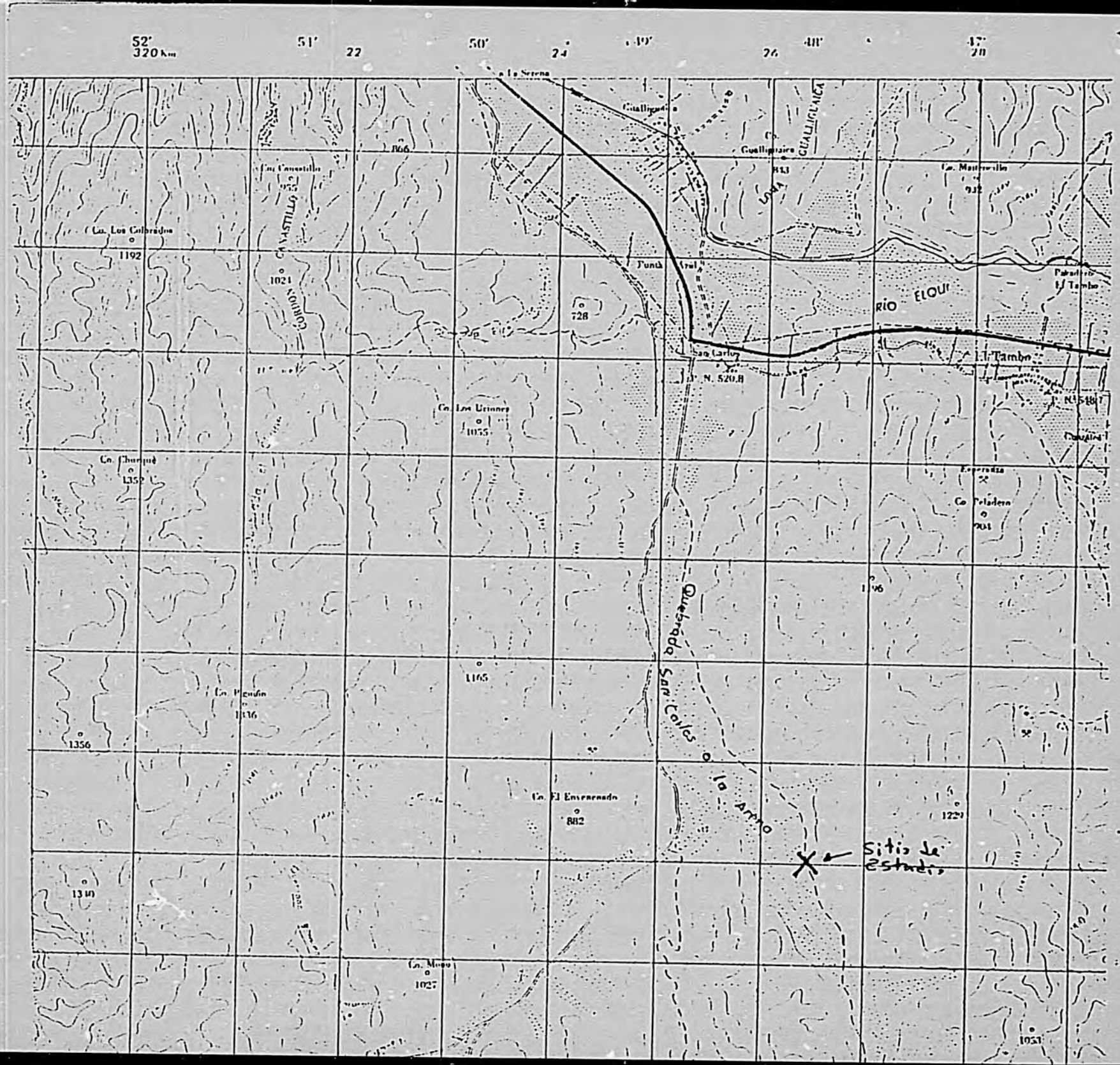

52'
320 km
51'
22
50'
24
49'
26
48'
47'
28
La Serena
Guallipuaire
GUALLIGUAICA
Guallipuaire
833
Ca. Mantrovilla
932
Ca. Los Colorados
1192
Los Castillos
CASTILLO
CONDON
1021
866
Punta Azul
728
LOMA
RIO ELQUI
Paladeros
El Tambo
San Carlos
N. 520.8
El Tambo
Ca. Los Urmeros
1055
Ca. Chuquí
1352
Esperanza
Ca. Peladero
901
1196
Quebrada San Carlos o la Arena
1165
Ca. Pingüin
1336
1356
Ca. El Enseñado
882
1221
Sitio de estudio
1310
Ca. Mono
1027
1053

Figure 3

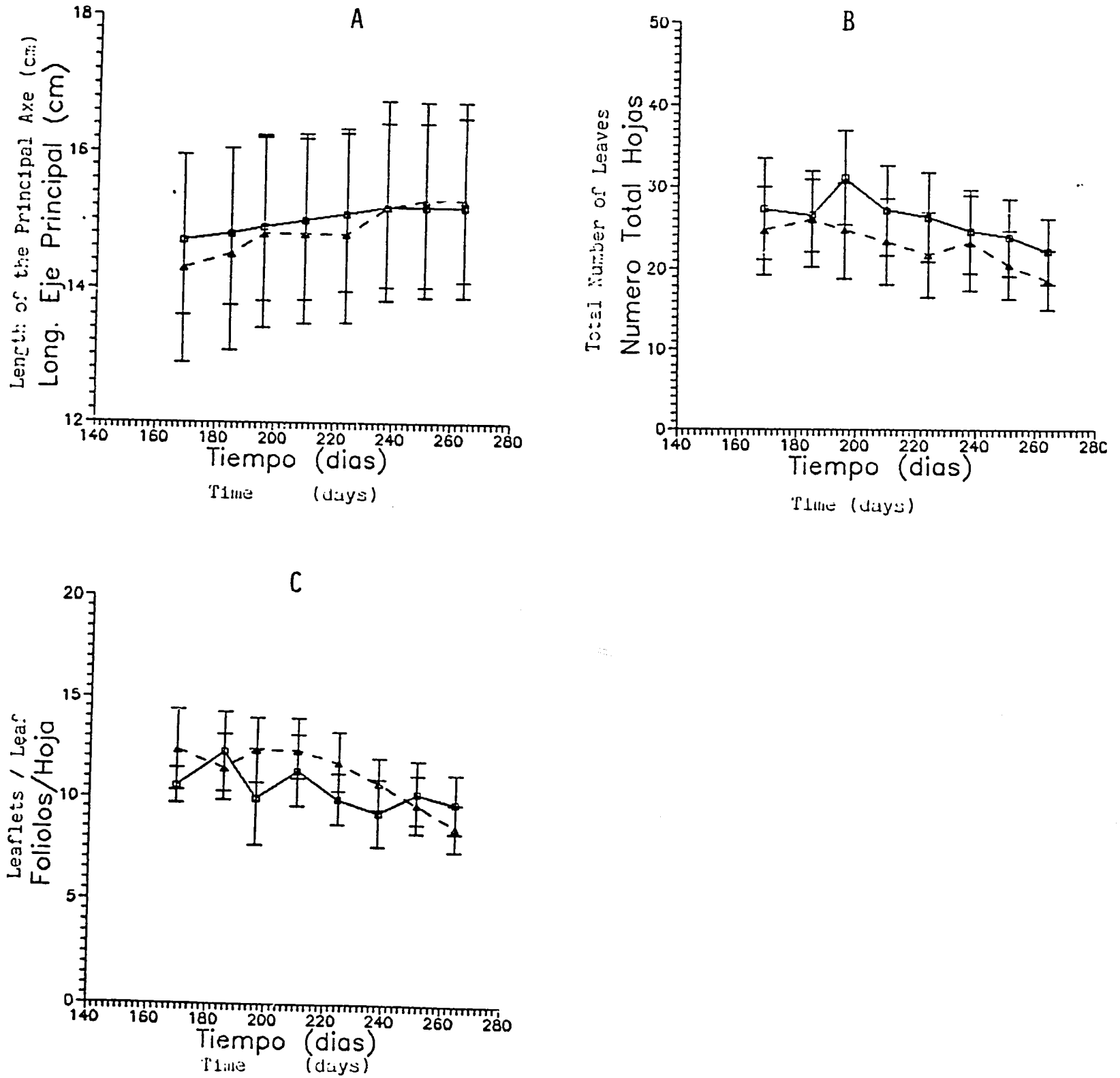
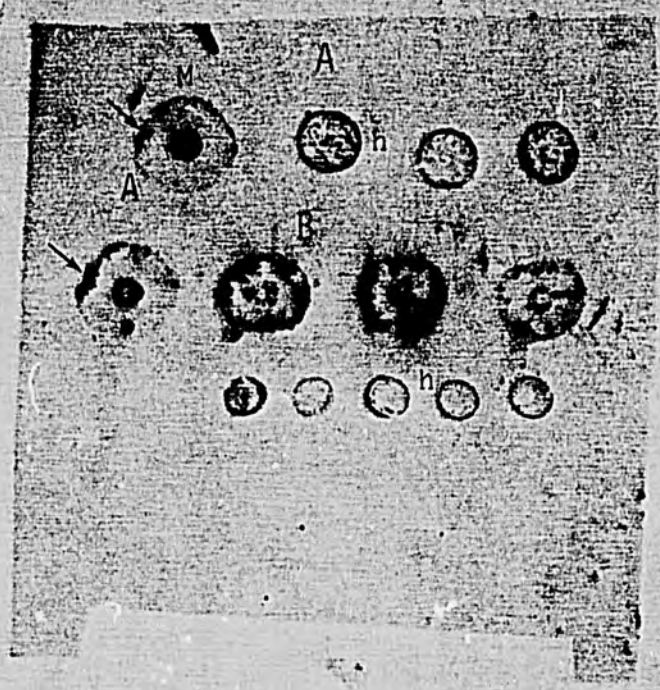

A
Length of the Principal Axe (cm)
Long. Eje Principal (cm)
18
16
14
12
140 160 180 200 220 240 260 280
Tiempo (dias)
Time (days)
B
Total Number of Leaves
Numero Total Hojas
50
40
30
20
10
0
140 160 180 200 220 240 260 280
Tiempo (dias)
Time (days)
C
Leaflets / Leaf
Foliolos/Hoja
20
15
10
5
0
140 160 180 200 220 240 260 280
Tiempo (dias)
Time (days)

Figure 4

Figure 5



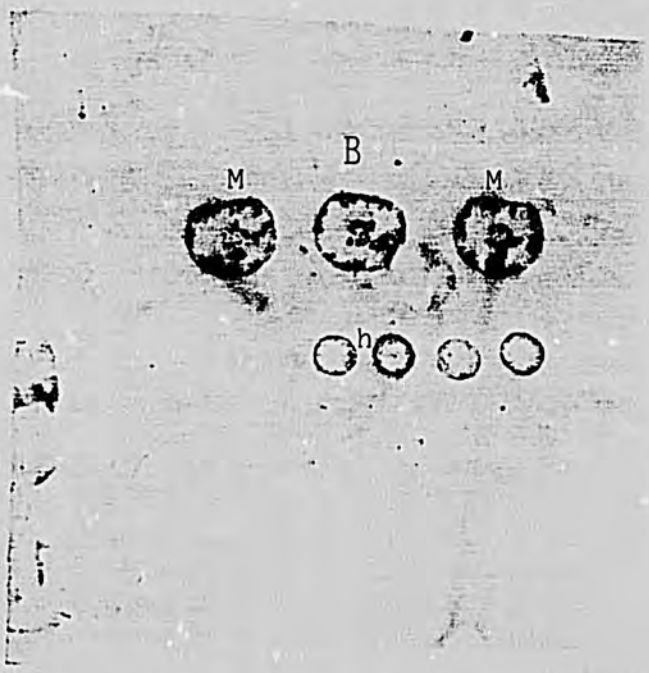


Table 1. Daily average of maximal and minimal temperatures and relative humidities per month and during the first semester of 1990 in the Experimental Station of Vicuña, gorge of San Carlos (30° 02' S - 70° 39' W, 750 m over the sea).

Month		Temperature		Relative Humidity	
		Maximun	Minimun	Maximun	Minimun
January	1990	30.2	12.0	94.4	21.8
February	1990	29.5	11.0	95.5	22.0
March	1990	28.5	9.8	95.1	20.0
April	1990	24.2	6.5	95.7	24.2
May	1990	23.8	3.8	85.1	15.9
June	1990	23.5	4.3	76.5	14.9
July	1990	20.0	2.7	90.0	24.2

Table 2. Millimiters of precipitation during the first semester of 1990 registered in the Experimental Station of Vicuña, gorge of San Carlos (30° 02' S - 70° 39' W, 750 m) over the sea).

Fecha		mm	Total Accumulated (mm)
June	4	0.7	0.7
June	5	0.7	1.4
July	6	5.6	7.0
July	7	45.4	52.4
July	8	7.5	59.9

Table 3. Sugar composition analysis for two cell wall proteins of *Araucaria araucana*. Proteins B_2 of molecular weight of 100 kDa and Protein B_3 of molecular weight of 60 kDa.

Sugar	B_2 ng /ug protein	B_3 ng /ug protein
Galactose	76.80	72.40
Glucose	19.60	42.80
Xylose	1.02	1.40
Mannose	4.36	1.88

Table 4. Sequence analyses of two cell wall proteins of <u>araucaria</u> <u>araucana</u> Protein B_{100} of a molecular weigth of 100 kDa and protein B_{60} of a molecular weight of 60 kDa.

Designated sequence B_{100}	Designated sequence B_{60}
H_2N....AMTXEQVQSSLEPXI	H_2N....AMTXEQVQSSLEPXISXLXA

A = alanine
M = methionine
T = threonine
X = non designated
E = glutamic acid
Q = glutamine
V = valine
S = serine
L = leucine
P = proline
I = isoleucine

Running head: Heat shock proteins in *Araucaria araucana*

Title: Expression of heat shock proteins in seeds and during seedlings growth of *Araucaria araucana* as a response to thermal stress.

Author's names : Claudio Goycoolea[2] and Liliana Cardemil[3].

Institution address : Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

Telephone: 56 (2) 2712865, extensión 221.

Footnotes: 1. Present address: Rengo 31, Concepción, Chile.

2. Liliana Cardemil, for editorial correspondence and to whom reprint requests are to be sent.

ENGLISH ABSTRACT

The purpose of this work was to investigate how seeds and seedlings of Araucaria araucana respond to high temperatures. Embryo axis, cotyledons and megagametophyte, tissues of Araucaria araucana seeds, were subjected to temperatures of 28°, 32°, 36°,40°, 44°C and 48°C for several hours and the viability of the tissues after exposure, was determined with the colorimetric test of the triphenyl tetrazolium chloride, or by measuring the electrolyte leakage. The most sensitive tissues are the cotyledons which die after three hours of treatment at 44° and 48°C. The most resistant is the megagametophyte which loses viability only after 7 hours at 44°C. Embryo axis tissues of seedlings are more sensitive to high temperature treatments. They lose viability after 30 minutes of treatment at 44°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins synthesized by root tips of seedlings at 32°,36°,40° and 44°C in the presence of [^{35}S] methionine and followed by fluorography, shows that the optimum synthesis and accumulation of the heat shock proteins occurs at 36°C. At least eight proteins are synthesized under heat shock among which is a protein 70 KDa. Kinetic experiments performed to determine how soon after heat shock the proteins appear, demonstrated that the new proteins are present 30 minutes after the embryo axes have been exposed to 36°C. Thirty six degrees is also the optimum temperature for acquisition of thermotolerance by Araucaria araucana seedlings.

FRENCH ABSTRACT (To be provided)

ABREVIATIONS: SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. hsp, heat shock proteins. TM, treatment medium. KDa, kilo dalton. TTC, triphenyl tetrazolium chloride. TCA, trichloro acetic acid.

INTRODUCTION

Plants are subjected to harsh environmental conditions such as, frost, water stress, mineral toxicity, nutrient starvation, extreme temperatures and wounding caused by predators, insects and pathogens. However, plants are adapted to adverse conditions through structural, physiological and biochemical adjustments.

As an example of these adjustments, plants respond to exposure to high temperatures by changing the pattern of protein synthesis, most often with a decrease of the proteins normally synthesized and appearance of new proteins **(Schlesinger et al., 1982, Cooper and Ho, 1983 and Meyer and; Chartier, 1983)**. These new proteins synthesized under high temperatures are denominated heat shock proteins, **(Kimpel and Key, 1985)**. The plant heat shock proteins (hsp) reported so far have been classified as hsp of high molecular weight, between 90 and 120 KDa, hsp of medium molecular weight, between 40-89 KDa and hsp of low molecular weight between 15-39 KDa, **(Cooper and Ho, 1983; Cooper et al., 1984)**. These low molecular weight proteins seem to be peculiar to the plant kingdom **(Key et al., 1981)**.

The physiological role of the hsp is still under discussion and not understood, but they seem to protect the organism since there is a high correlation between the

appearance of the proteins and the acquisition of thermotolerance **(Yamamori et al., 1978; Ashburner and Bonner, 1979; Barnett et. al., 1980; Lin et al, 1984; Schlesinger, 1985).**

Araucaria araucana (Mol.) Koch is a conifer specie, endemic to austral South America. Araucaria araucana grows in high mountain forests between latitudes of 37° 30' and 40° 3' S, from 900 to 1700 m altitude, in the oriental and occidental slopes of the Andes and in the Nahuelbuta Cordillera of the chilean cost between latitudes of 37° 2' and 38° 4' S.

It has been suggested that Araucaria araucana has a high degree of physiological adaptations to grow in these areas, constituing relic forests originated in the tertiary times **(Montaldo, 1974).**

The weather, climate and soil of volcanic origin of Araucaria forests, as well as biological factors such as animals and humans make difficult the natural regeneration of this conifer. Seeds are shed and germinate at the end of Summer and seedlings grow on the volcanic soil which reaches temperatures of 50°C and higher at noon of hot Summer days.

The purpose of this work was to investigate how seeds and seedlings of Araucaria araucana respond to high temperatures and to study the pattern of heat shock proteins synthesized under thermal stress, since the genus **Araucaria** has been reported to have a tropical origin **(Montaldo, 1974).**

MATERIAL AND METHODS

Seeds material and germination conditions. Araucaria araucana (Mol.) Koch seeds were collected in the forest reserve of Malalcahuello (latitude 37.5° S, Chile) between the months of March and April of 1986, 1987 and 1988. The seeds were stored in plastic bags at 4°C until used. For the experiments seeds without testa were sterilized in a 10% sodium hypochlorite solution for 5 minutes and then washed thoroughly in distilled water. After sterilization the seeds were germinated in wet vermiculite. The germination temperature was 28°C.

Determination of viability of tissues of quiescent seed subjected to different temperatures. Sterilized seeds without testa, were divided in groups of 50 seeds each and each seed was divided in two halves. Each group of 100 half seeds was subjected to different temperatures going from 28°C (control experiment) to 32°, 36°, 40°, 44° and 48° and with times of exposure of 1, 2, 3, 5, 7 and 9 hours. At the end of treatment the half seeds were dissected into embryo, megagametophyte and cotyledons and viability was determined by measuring the tissue area that changed from colorless to red after staining for 2 hours in darkness with 0.5% of triphenyl tetrazolium chloride (TTC).

Alternatively, the viability was determined after the

temperature treatment by measuring the conductance of electrolytes leaked from the tissue into deionized water (leachate). The conductance of a leachate of dead seeds, killed in boiling water for 30 minutes, was considered 100%. The per cent of leaching was expressed as conductance of leachates after heating x 100 / final conductance after killing. The mean leaching was plotted against the temperature tested, from where the LT_{50} (temperature at which the conductance became 50% of the conductance after killing) was calculated.

Determination of growth of the embryo axis of seedlings subjected to different temperatures. Seedlings of 120 hours of imbibition without megagametophyte were presoaked for 3 hours at 28°C in sterilized vermiculite watered with a solution containing the Murashige-Skoog saline components **(Murashige and Skoog,1962)** and supplemented with 15% sucrose and 50 ug/ml of chloramphenicol (treatment medium=TM). After this pretreatment the length of embryo axes were measured in millimeters and then soaked in the same medium for 1,2 and 3 hours at 28°, 32°, 36°, 40° and 44°C. During the treatment, the medium was aerated constantly with an aquarium pump.

The damage caused by the high temperature treatment was measured by the growth recovery of the embryo axes at 28°C in 48 hours.

Induction of thermotolerance in embryo axes by exposure to sublethal temperatures. Five groups of 15 embryo axes each, coming from seedlings of 120 hours of imbibition were pretreated for 3 hours at 28°C by soaking them in wet vermiculite watered with the saline TM described above. After the pretreatment the lengths of the embryo axes were measured and changed to flasks containing a fresh liquid medium. One group was maintained to 28°C as the control. Another 2nd, 3rd, 4th and 5th groups were subjected to 28°, 32°, 36°, 40° and 44°C respectively for 30 and 120 minutes in separate experiments. Each group was then subjected to 44°C (lethal temperature) for 2 hours. After treatment at 44°C, the embryo axes were changed to solid medium and kept at 28°C for 48 hours. After this time the recovery of growth was determined by measuring the length of the axes.

Incorporation of [^{35}S] methionine. Seedlings of 120 hours of imbibition with radicles between 1.5-2.0 cm. long, were dissected to separate the embryo axes from the megagametophyte. The embryo axes were further cut into pieces of 1cm.

Five groups containing pieces of 15 embryo axes each, were incubated for three hours in liquid TM under continuous aeration. After this time the group 1, 2, 3, 4 and 5 were subjected to 28°, 32°, 36°, 40° and 44°C respectively for 2 hours in aerated liquid TM.

[^{35}S] methionine with a specific radioactivity of

3 X 10^{-8} Bq/mmol (from ICN Radiochemicals) was added to the media, up to obtain a concentration of radioactivity of 10.8 X 10^{-10} uBq/ml. The [^{35}S] methionine was added to the final 30 minutes of two hours of temperature treatment in order to determine the proteins synthesized under heat shock, or, added at the beginning of the two hours of temperature exposure in order to determine the accumulation of proteins synthesized during this time. For kinetics experiments the [^{35}S] methionine of the same specific radioactivity mentioned above, was added to the medium containing the pieces of embryo axes. The [^{35}S] methionine was added for 30 minutes at the end of the period of exposure at 36°C (30 minutes, 1, 2, 3, 4 or 5 hours).

Extraction of proteins labelled with [^{35}S] methionine. The extraction of proteins was performed by the method described by Cooper and Ho (1983) using the buffer of Laemmli (1970) made up of 0.06 M Tris HCl, pH 6.8 and containing 2% of sodium dodecylsulfate (SDS), 10% glycerol and 5% of mercaptoethanol. The pieces of tissue were homogenized with a glass homogenizer and maintained for 2 minutes in water bath at 100°C; the homogenate was then centrifuged in an Eppendorf microfuge for 3 minutes. The incorporation of [^{35}S] methionine was determined in the proteins of the supernatant. For this, the proteins of the extract were precipitated with trichloro acetic acid (TCA) as it has been described by Mans and Novelli (1960), taking

aliquots of 10 ul and placed on a disk of filter paper Whatmman 3MM 1.4 cm diameter. The paper was dried for 20 seconds . Each paper disk was soaked for 60 min. in 10% TCA containing 0.1M cold methionine. The disks were washed with 10% TCA at room temperature for 15 minutes followed by a second wash with 5% TCA at 90°C for 30 minutes. After the TCA washes, the disks were passed through an ethanol ether: solution (1:1) for 30 minutes at 37°C, washed in pure ether for 15 minutes and dried at room temperature. The dried paper disks were placed into scintillation fluid of: toluene and Triton X-100 in a ratio of 2:1 containing 4g/l of 2,5-Diphenyloxazole, (PPO) and 50 mg/l of 1,4-bis (5 Phenyl-2-oxazolyl)-benzene; 2,2'-p-Phenylene bis (5-phenyloxazole) (POPOP). The radioactivity was measured in a Phillips liquid scintillation counter.

Quantification of proteins of the tissue extracts was performed by Bradford's method **(Bradford, 1976)** after precipitation of the proteins with 10 volumes of cold acetone and resuspension in 0.01M Tris. A radioactivity of 3000 cpm/ug of protein was considered as a good incorporation.

Protein electrophoresis. The proteins labelled with [^{35}S] methionine were separated by electrophoresis in SDS-polyacrylamide gels, by the method described by Laemmli (1970). The running gel was 10% polyacrylamide containing 0.1% SDS, pH 8.8. The stacking gel was 3.5% polyacrylamide

containing 0.1% SDS, pH 6.8. Each channel of the gel was loaded with 80 ug of proteins with a total radioactivity of 180,000-300,000 cpm. The electrophoresis was run for 7 hours at 180 V, using the Laemmli's buffer (1970). The proteins bands were visualized on the gel with 0.25% coomasie R 250 in 50% methanol and 10% acetic acid. The gel was destained with 10% acetic acid and 50% methanol for 4 hours at room temperature.

Fluorography. In order to detect the synthesized proteins during the thermal stress, the protein bands were visualized by the radioactivity of the [^{35}S] methionine incorporated in the proteins. For this, after electrophoresis, the gel was twice washed with 10% glacial acetic acid and 40% ethanol for 30 minutes each wash. Afterwards, the gel was submerged in solution of 0.4% PPO, 20% ethanol, 30% xylol and 50% glacial acetic acid for 3 hours. The PPO was precipitated into the gel with distilled water for 2.5 hours. After precipitation of the PPO, the gel was dried under vacuum in between two sheets of Saran Wrap. The dried gel was exposed to a Kodak X Omat film at -80°C for four days.

RESULTS

Determination of viability of seeds and seedlings after thermal stress. The three tissues of quiescent seeds of Araucaria araucana, embryo axes, cotyledons and megagametophytes were subjected to temperatures of 28°C (control experiment) and higher, 32°, 36°, 40°, 44° and 48°C for different periods of time, between 1-8 hours. These three tissues are affected differently in their viability by exposure to high temperatures. Figure 1 shows that the embryo axes lose significantly their viability compared with the control experiment, after 5 hours of treatment to 44°-48°C ($p < 0.005$, probability given by Student's test). The cotyledons are more sensitive to thermal stress, losing 20% viability after exposure at 48°C for the period of 3 hours. The megagametophyte tissue is the most resistant. Its viability is only affected 17% (compared with the control samples) after 7 hours of treatment at 44°C, and 30% after exposure at 48°C for the same period of time.

Seedlings 120 hours after imbibition, with a root length between 1.5-2.0 cm, were subjected to temperatures of 28°C and 32°, 36°, 40° and 44°C for periods of 30 and 60 minutes. After the temperature exposures, the seedlings were returned for 48 hours to 28°C and the length of the roots was measured again.

Figure 2 A shows the growth of seedling embryo axes expressed as percent of growth of the control samples. An exposure of 30 minutes at 44°C reduces the growth of the embryo axes to 16%. However, the embryo axes show 80% of control growth when exposed for 30 minutes at 32°, 36° and 40°C.

A thermal treatment for 60 min at 36°C decreases the seedling growth to 58% of the control and to 48% when the temperature is 40°C. Seedlings subjected for 60 min to 44°C do not recover growth, **(fig. 2 B).**

These experiments, to study thermal resistance, allowed us to determine the lethal and sublethal temperatures for quiescent seeds and seedlings of Araucaria araucana . The lethal temperature is therefore, 44°C and the sublethal temperatures are 32°, 36° and 40°C.

Protein pattern of embryo axes after 2 hours of heat shock. The protein pattern present in embryo axes of Araucaria araucana at 28°C and under treatment of 2 hours of heat shock, was studied incubating the tissue in the presence of [^{35}S] methionine as precursor of protein synthesis, followed of SDS-PAGE of the extracted proteins and fluorography of the radioactive gel.

Two kinds of experiments were performed to discriminate between protein accumulation and protein synthesis during heat shock. The accumulation of proteins was studied by incubation the tissue in the presence of [^{35}S] methionine during 2 hours

of exposure at the appropiated temperature (28°, 32°, 36°, 40° and 44°C). The synthesis of proteins was studied by incubation of the tissue in the presence of [^{35}S] methionine during the last 30 minutes of the 2 hours of exposure at the appropiate temperatures. Figure 3 is a fluorography of a SDS-PAGE of the proteins accumulated in two hours of heat shock. The proteins were analyzed and compared with those present in embryo axes grown at 28°C. Seven new bands of protein appear in the embryo axes under heat stress, which are not detectable in the control samples. The seven new bands of proteins have molecular weights of 126, 106, 89, 77, 44, 24 and 19 KDa. These new proteins present under heat shock have been denominated heat shock proteins (hsp), **(Kimpel and Key, 1985)**. The seven bands of proteins appear at 32°C and increase in intensity at 36°C. However, two of these proteins, the ones of 126 and 24 KDa are not present at 40°C. There are also some other proteins present at 28°C which increase considerably in intensity at temperatures of 32°, 36° and 40°C Figure 3 also shows that most of the accumulated proteins decline at 44°C.

Figure 4 is a fluorogram of the proteins synthesized during heat shock since the [^{35}S] methionine was incorporated in the last 30 minutes of the 2 hours of thermal exposure. It shows that the same 7 bands of proteins accumulated in two hours of treatment are also synthesized in the last 30 minutes of thermal stress. Because in this fluorogram the bands are

seen better resolved, it is possible to detected one additional band synthesized under stress. This band has a molecular weight of 17 KDa. Furthermore, there are about 9 other proteins present at 28°C whose synthesis increases considerably at 32° and 36°C. The hsp 126, 106 and 17 KDa are no longer synthesized at 44°C. Indeed, at 44°C the synthesis of proteins declines. Table 1 is a summary of the proteins synthesized under heat shock, showing an estimation of the intensity of radioactivity incorporated per band. From these results we conclude that the optimum temperature for synthesis and accumulation of heat shock proteins is 36°C.

Kinetic of appearance of the hsp in embryo axes subjected to 36°C. Since 36°C is the optimum temperature for synthesis and accumulation of hsp, a kinetic experiment of appearance of the proteins bands at 36°C was run, with the purpose to determine how soon proteins appear in the tissue after the beginning of a thermal treatment. for this purpose the tissue of embryo axes was incubated for different periods of time (from 30 minutes to 5 hours) in the presence of [^{35}S] methionine added for 30 minutes at the end of two hours of thermal treatment. Figure 5 is a fluorogram showing the results of this experiment. Six of the eight hsp, are detected after 30 minutes of heat shock at 36°C (106, 89,77, 24, 19 and 17 KDa proteins). After 1 hour, the bands 126 and 44 KDa are also present. Analysis of the intensity of the bands shows that maximum incorporation of

radioactivity per band occurs from 1 to 2 hours after heat shock initiation. After 3 hours of heat shock the proteins 77 and 17 KDa are not longer present. After 5 hours of treatment the proteins 126 is not present and most of the intensity of the protein bands decline, **(tab. 2)**.

Acquisition of thermotolerance by the embryo axes subjected to sublethal temperatures. Because 36°C is the optimum temperature for heat shock protein synthesis and accumulation, the question to ask is whether or not 36°C is the optimum temperature for acquisition of thermotolerance by the embryo axes of Araucaria araucana. Figure 6 A shows the results of induction of thermotolerance in embryo axes after incubation for 30 minutes at sublethal temperatures of 32°, 36° and 40°C. Only the embryo axes exposed at 36°C are able to recover 45% growth after treatment for 2 hours at the lethal temperature of 44°C. However, the recovery of growth after 120 minutes exposure at sublethal temperatures, followed of a 44°C heat shock for 2 hours, was 52% of the control group when the sublethal temperature was 32°C, and 84% when the sublethal temperature was 36°C. The embryo axes subjected for 2 hours to 40°C, prior to exposure at 44°C, recover growth in only 24%, **(fig. 6 B)**. This growth is still less than the growth recovery of embryo axes subjected to 28°C before heat shock for 2 hours at 44°C.

Therefore, 36°C is also the best sublethal temperature to induce thermotolerance in embryo axes of *Araucaria araucana*.

DISCUSSION

The three main tissues of Araucaria araucana seeds respond differently to high temperatures. The cotyledons are the most sensitive to thermal stress while the megagametophyte, is the most resistant tissue of the seed to high temperature exposure.

The loss of viability of the cotyledons could be due to experimental conditions since they are never exposed to external environments. Indeed, the cotyledons remain inside of the megagametophyte during germination and seedling growth, because they are the haustorial organs of the system and transport sugars and amino acids from the megagametophyte to the embryo, **(Lozada and Cardemil, 1983)**.

This relationship between cotyledons and megagametophyte, is perhaps related to another physiological role that the megagametophyte seems to have, besides being the reserve tissue of the seed. That role is to serve as a protective and isolating organ, for the embryo and cotyledons, **(Cardemil and Reinero, 1982; Cardemil and Varner, 1985; Lozada and Cardemil, 1984)**. Therefore, it is important that the megagametophyte, as protective tissue, could tolerate thermal stress. The higher thermotolerance of this tissue may be the consequence of less water content and more dry starch material packed in amyloplasts in the cells of the megagametophyte, **(Cardemil and Reinero, 1982; Reinero el al. 1983)**.

The Araucaria araucana seedlings are sensitive to high temperature exposure, may be due to the high water content of the growing tissues after germination. The seedlings used in these experiments have been imbibed for 120 hours. At this age the seedlings are greening and most of the reserve stored in the embryo has been consumed with a considerably increase in fresh weight **(Cardemil and Reinero, 1982; Reinero et al. 1983 and Cardemil and Varner, 1985).**

The experiments of thermotolerance performed with seeds and seedlings of Araucaria araucana allowed us to define the lethal (44°C) and sublethal temperatures (32°, 36° and 40°C) for the system. This distinction between lethal and sublethal temperatures is very important for Araucaria araucana seedlings as in any other living system because organisms acquire thermotolerance after exposure to sublethal conditions and therefore, can resist lethal temperatures, **(Lin el al., 1984; Cardemil, 1985).** Furthermore the acquisition of thermotolerance by organisms is correlated with the presence of heat shock proteins.

The main characteristics of the hsp can be summarized:

1. The heat shock proteins are synthesized under thermal stress, condition that most often decreases synthesis of normal proteins.

2. The hsp are synthesized at sublethal temperatures and when the organism acquires thermotolerance.
3. The hsp are transient because they disappear under continuous heat shock or when the organism is put back to normal temperature.
4. There are hsp of high, medium and low molecular weight, these last ones being peculiar to the plant kingdom.

In seedlings of Araucaria araucana eight hsp were detected and they fit the hsp characteristics mentioned above.

Among the hsp of Araucaria araucana there is a prominent protein of 89 KDa. A protein of identical molecular weight has been reported as hsp in chicken embryos by Schlesinger et al., 1982 and it seems to be related to glycolysis; possible it is a phosphatase or a phosphotransferase, **(Schlesinger, 1985).**

There are two heat shock proteins in Araucaria araucana seedlings which are good candidates to be considered the equivalent of the universal hsp 70 KDa. These are the proteins 80 and 69 KDa. Both proteins are present at 28°C and both increase considerably under thermal stress. Therefore, strictly speaking the 80 and 69 KDa proteins are not at all hsp, since the expression of these proteins would depend of constitutive genes whose magnitude of expression would be regulated by thermal conditions.

There is another protein of 77 KDa expressed only under heat shock and no present at 28°C. The protein has a maximum accumulation at 40°C, but its synthesis seems to be low and uniform in seedlings treated at 32°, 36° and 40°C. Therefore, this protein may not correspond to the 70 KDa protein. However, with the methods used in this work is not possible to identify the hsp 70 KDa. Antibodies raised against a 70 KDa protein from other organism may help to identify this protein of Araucaria araucana, since it has been reported that there is a high degree of conservation in the sequence of the 70 KDa protein as well as in the gene coding for this protein **(Ingolia and Craig, 1982; Rochester et al., 1986)**. This is the case for corn, where two genes for the protein have been cloned and sequenced. Both genes have a 68% homology between the predicted sequence of amino acids with the amino acid sequence of the 70 KDa protein of Drosophila **(Rochester et al., 1986)**.

There are in embryo axes of Araucaria araucana two other proteins of high molecular weights, one of 126 and other of 106 KDa. In corn, proteins of molecular weight higher than 89 KDa, are not present, **(Cooper and Ho, 1983)**. However, in tobacco protoplasts the expression of hsp 120 and 100 KDa with isoelectric points of 5 and 7 has been reported **(Meyer and Chartier, 1983)**.

In Araucaria araucana three hsp of low molecular weighs are present. In the literature similar proteins have been

considered peculiar to the plant kingdom **(Meyer and Chartier, 1983; Cooper and Ho, 1983; Cooper et al., 1984 and Kimpel and Key, 1985).**

Ubiquitin, a conserved and universal low molecular weight peptide of 8 KDa has been reported to be a heat shock protein, since it increases 6 folds under thermal stress. **(Bond and Schlensinger, 1985; Vierstra, 1987).** Under the SDS-PAGE conditions used for protein analyses of Araucaria araucana, the ubiquitin can not be detected, but, the peptide seems to be expressed in Araucaria araucana seedlings under heat shock. Indeed, Northern analysis of total RNA using a heterologous probe of chicken ubiquitin shows an increases of 6 fold of ubiquitin mRNA in the embryo axes when this tissue is exposed to 36°C (data not shown).

The hsp of embryo axes of Araucaria araucana have their maximum expression at 36°C. Therefore, 36°C is the optimum temperature for accumulation and synthesis of hsp. A kinetic experiment for hsp appearance, shows that the expression of most of the new proteins occurs after 30 minutes of heat shock. Very possibly, these proteins are expressed before 30 minutes, at a level that is not easy to detect by electrophoresis.

It is also important to point out that the synthesis of normal proteins in embryo axes of Araucaria araucana continues under thermal stress and does not decline, as seems to be the case for tobacco or soybean plants where normal protein

synthesis stops under heat conditions. Therefore, the embryo axes of Araucaria araucana respond to thermal stress with synthesis and accumulation of new proteins and increased rate of synthesis of many other proteins already present at 28°C.

Being 36°C the best temperature for hsp expression, the obvious question to ask was if 36°C is also the best temperature for acquisition of thermotolerance. Indeed the experiment of thermotolerance induction by sublethal temperatures in embryo axes of Araucaria araucana demonstrated that 36°C is also the optimum temperature for induction of tolerance to resist lethal temperatures.

AKNOWLEDGEMENTS

We thank Dr. Joseph Varner, Department of Biology, Washington University, St. Louis, MO 63130, U.S.A. for critical comments on the manuscript. This work was part of Claudio Goycoolea's Master Degree Dissertation. The technical assistance of Angélica Vega and Ximena Oñate from Departamento de Biología, Facultad de Ciencias, Universidad de Chile is acknowledged. This research was supported by AID grant #DPE-5542-G-SS-8073-00, FONDECYT 0160/88 and CONICYT/NSF #84-201.

REFERENCES

Ashburner M. and Bonner J.J., 1979.- The induction of gene activity in Drosophila by heat shock. **Cell, 17,** 241-254.

Barnett T., Altschuller M. McDaniel, C.N. and Mascarenhas, J.P., 1980.- Heat Shock induced proteins in plant cells. **Dev. Gen., 1,** 331-340.

Bond U. and Schlesinger M.J., 1985.- Ubiquitin is a heat shock protein in chicken embryo fibroblasts. **Mol. Cell Biol., 5,** 949-956

Bradford M.M., 1976.- A rapid and sensitive method for the quantitation for microgram quantities of protein utilizing the principle of protein dye binding. **Anal. Biochem.,** 72, 248-254.

Cardemil L. and Reinero A., 1982- Changes of Araucaria araucana seed reserves during germination and early seedling growth. **Can. J. of Bot., 60,** 1629-1638.

Cardemil L. and Varner J.E., 1984.- Starch degradation metabolism towards sucrose synthesis in germinating Araucaria araucana seeds. **Plant Physiol., 76,** 1047-1054.

Cardemil L. 1985.- Inducción de termotolerancia en plantas. **Medio Ambiente, 7,** 3-14.

Cooper P. and Ho D.T., 1983.- Heat shock proteins in maize. **Plant Physiol. 71,** 215-222.

Cooper P., Ho D.T., and Hauptmann R.M., 1984.- Tissue specifity of heat shock response in maize. **Plant Physiol., 75,** 431-441.

Ingolia T.D. and Craig E.A., 1982.- Four small Drosophila heat shock proteins are related to each other and to mammalian crystallin. **Proc. Nat. Acad. of Sci., U.S.A., 79,** 2360-2364.

Key J.L., Lin C.Y. and Chen Y.M., 1981.- Heat shock proteins of higher plants. **Proc. Nat. Acad. of Sci., U.S.A., 78,** 3526-3560.

Kimpel J.A. and Key J.L., 1985.- Heat shock in plants. **TIBS, 9,** 353-357.

Laemmli U., 1970.- Clevage of structural proteins during assembly of the head of bacteriophage T_4. **Nature, 227,** 680-685.

Lin Ch-Y., Roberts J.K. and Key, J.L., 1984.- Acquisition of thermotolerance in soybean seedlings. **Plant Physiol. 74,** 152-160.

Lozada R. and Cardemil L., 1983.- Mechanism of sucrose transport by seedling cotyledons of Araucaria araucana (Mol.) Koch. **Plant Physiol., 72,** S-70.

Mans R. and Novelli G.D., 1960.- A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. **Biochem. Biophys. Res. Commun., 3,** 540-548.

Meyer Y. and Chartier Y., 1983.- Long-lived and short-lived heat shock proteins in tobacco mesophyl protoplasts. **Plant Physiol., 72,** 26-32.

Montaldo P.R., 1974.- La bio - ecología de Araucaria araucana (Mol.) Koch. **Boletín Instituto Forestal Latino Americano de Investigación y Capacitación Biológica, 45,** 3-55.

Murashige T. and Skoog F., 1962.- A revised medium for rapid growth and bioassays with tobacco tissue culture. **Plant Physiol., 15,** 473-497.

Reinero A., Balboa O. and Cardemil L., 1983.- Characterization of the amylolytic activity of Araucaria araucana (Mol.) Koch germinating seeds. **Plant and Cell Physiol., 24,** 182-190.

Rochester D.E., Winer J.A. and Shah D.M., 1986.- The structure and expression of maize genes encoding the mayor heat chock protein, hsp 70 Kda. **EMBO, 5** 451-458.

Schlesinger M., Aliperti G. and Kelley, P.M., 1992.- The response of cells to heat shock. **TIBS, 7,** 222-225.

Schlesinger M., 1985.- Stress response in avian cells. In Changes in eukaryotics gene expression in response to environmental stress. Atkinson B.G. and Walden D.B. ed., **Academic Press Inc.,** 183-195.

Vierstra R.D., 1987.- Ubiquitin, a Key component in the degradation of plant proteins. **Physiol. Plant., 70,** 103-106.

Yamamory T., Ito K., Makamura Y. and Yura T., 1978. Transcient regulation of protein synthesis in Echerichia coli upon shift-up of growing temperature. **J. of Bacteriol., 134,** 1133-1140.

Table I. **Protein synthesis and relative intensity of the radioactive protein bands of embryo axes subjected to thermal stress.** The molecular weights (MW) and the relative intensities of the radioactive bands of the synthesized proteins in embryo axes upon thermal stress are shown and compared with the control at 28°C, as seen in **fig. 4.**

MW	28°C control	32°C	36°C	40°C	44°C
126*	-	-	+	+	-
106*	-	-	+	+	-
101	+	+	+++	++	-
98	+	++	+	+	+
94	+	++	+++	++	+
89*	-	++	++++	+++	+
84	+	+	+++	++	-
80	+	+++	++++	+++	+
77*	-	+	++	+	+
69	+	+	+++	++	+
44*	-	+	++	+	+
33	+	+++	+++	+++	+++
24*	-	+++	++	+	+
20	+	+++	++	+	+
19*	-	++	+	+	+
17*	-	++	++	+	-

* Molecular weights of the hsp not present at 28°C.
\+ Relative intensities of the radioactive bands. The intensity is compared among the bands of the same protein.

Table II. **Kinetic experiment of protein synthesis by embryo subjected to 36°C and relative intensity of the radioactive proteins bands.** The molecular weights (MW) and the relative intensity of the protein bands detected in the kinetic experiment of protein synthesis are shown for embryo axes when exposed to 36°C for different periods of time (30 minutes to 5 hours), as seen in **fig.5.**

MW	28°C control.	30min.	1 hour	2 hour	3 hour	5 hour
126*	-	-	+	+	+	-
106*	-	+	++	++	++	++
101	+	+++	+++	+++	+++	+++
98	+	+	+++	+++	+++	+++
94	+	+++	++++	+++	+++	+++
89*	-	+	++	++	++	+
84	+	++	+++	++	++	+
80	+	+++	++++	++++	++++	++++
77*	-	++	+++	++	-	-
69	+	+	++	++	+++	+
44*	-	+	+++	+++	++	++
33	+	++	+++	++	++	++
24*	-	+	++	++	++	+
20	+	++	+++	+++	+++	+++
119*	-	++	+++	++	+	+
17*	-	++	+++	++	-	-

* Molecular weights of the proteins bands (hsp)not present at 28°C
\+ Relative intensities of the radioactive proteins bands. The intensity is compared among the bands of the same protein.

FIGURES

Figure.1 **Viability of the different tissues of quiescent seeds of** ***Araucaria araucana***. The results are shown at the minimum period of time (3h for cotyledons , 5h for embryo axes and 7h for megagametophyte) at which there is a significant decrease of viability by exposure to high temperatures.

The control experiment was performed at 28°C and it is shown as white bars, the experiments were performed at 32°, 36°,40° and 48°C and are shown as dotted bars.

The viability (LT_{50}) was measured by the colorimetric method of TTC or by determination of electrolyte leakage.

The standard deviation is given for each bar.

Figure 2. Growth recovery of embryo axes of ***Araucaria araucana***. After 30 minutes of thermal exposure in A or after 60 minutes of thermal exposure in B. The recovery is given as per cent of growth of the control embryo axes maintained at 28°C. The stress temperatures were 32°, 36°, 40° and 44°C. After the

thermal treatment the axes were placed at 28°C for 48 hours and the length of the embryo axes were measured. The standard deviation is given for each bar.

Figure 3. **Fluorogram of the proteins accumulated in embryo axes under heat stress.** Proteins accumulation correspond to 2 hours of incubation at different temperatures in the presence of 10.8 X 10^{-10} uBq/ml of [^{35}S] methionine used as precursor of protein synthesis. Each experimental group was of six embryo axes. After incubation, the proteins were extracted from the tissue and analyzed by SDS-PAGE followed by fluorography of the gel. The complete arrows point to those new proteins (hsp) not present in the control embryo axes incubated at 28°C . The arrow heads point to those bands which increase in radioactive intensity upon thermal exposure.

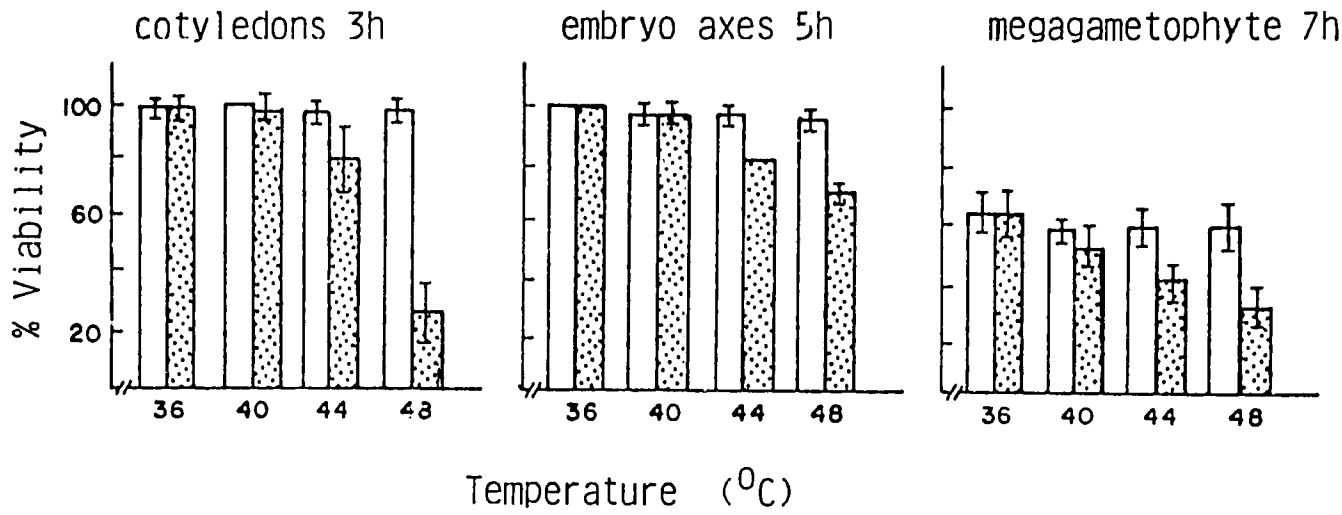
Figure 4. **Fluorogram of the proteins synthesized in embryo axes under heat stress.** The synthesis of proteins correspond to the last 30 minutes of 2 hours of incubation at different temperatures in the presence of 10.8 X 10^{-10} uBq/ml of [^{35}S] methionine used as precursor of protein synthesis. Each experimental

group was of six embryo axes. After incubation the proteins were extracted from the tissue and analyzed by SDS-PAGE,followed by fluorography of the gel. The complete arrows point the bands of new proteins not present in the control embryo axes treated at 28°C (hsp). The arrow heads point to those bands which increase in radioactive intensity upon thermal exposure.

Figure 5. **Kinetics of protein synthesis in embryo axes.** The embryo axes were subjected at 36°C for different periods of time (from 30 minutes to 5 hours). Groups of 6 embryo axes were incubated at 36°C in the presence of 10.8×10^{-10} uCi/ml of [^{35}S] methionine which was added to the medium in the last 30 minutes of the period of thermal treatment. After the exposure to 36°C the proteins were extracted from the tissue and analyzed SDS-PAGE followed by fluorography of the gel. The complete arrows show the new proteins bands not present in embryo axes incubated at 28°C. The arrow heads show those bands of proteins which increase in radioactive intensity upon thermal treatment.

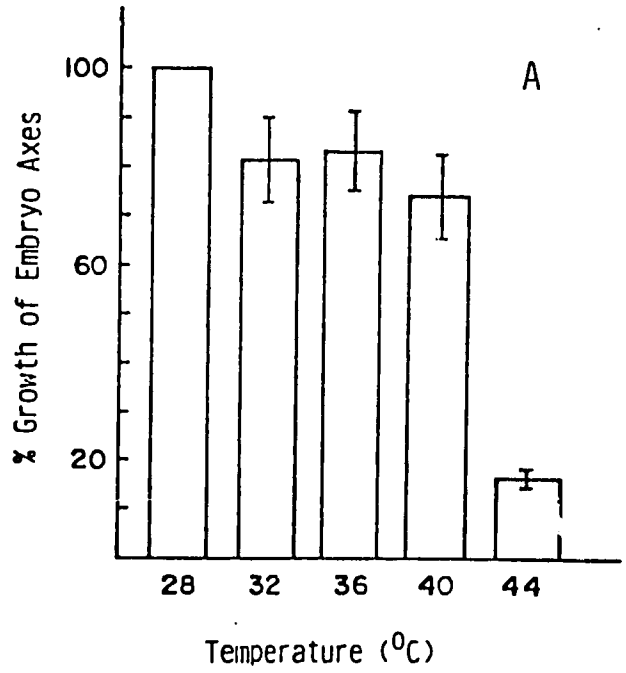
Figure 6. **Thermotolerance induction in embryo axes of a *Araucaria araucana* by exposure of the tissues to sublethal temperatures.** The sublethal conditions were temperatures of 32°, 36° and 40°C for 30 minutes in A and for 120 minutes in B. After the treatment to the sublethal temperatures, the embryo axes were subjected to 2 hours of heat shock at the lethal temperature of 44°C. The acquisition of thermotolerance was detected by the per cent of growth recovery of the axes when further incubated for a period of 48 hours at 28°C after the lethal temperature treatment.

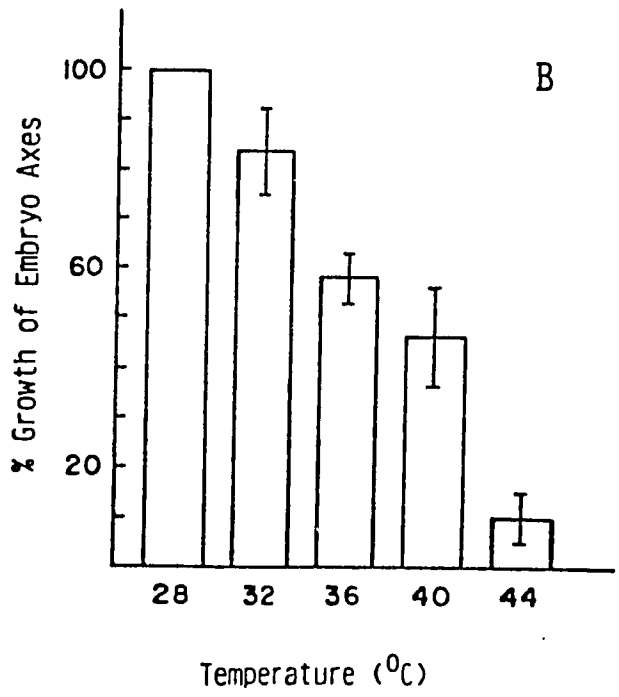
Figure 1

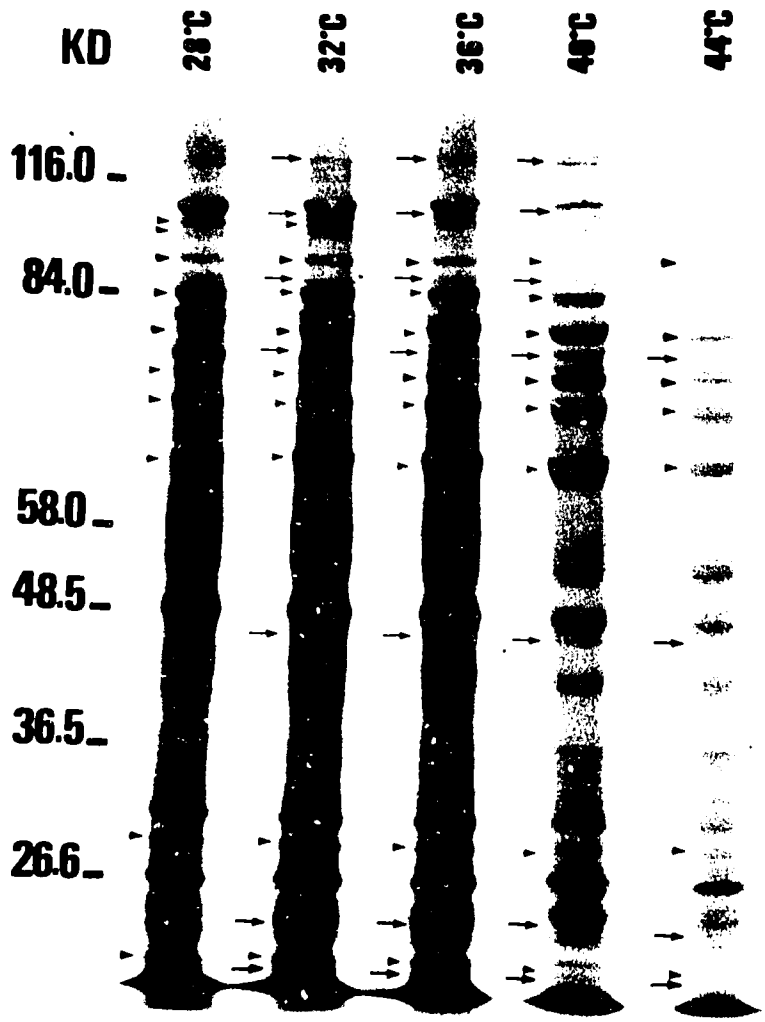

cotyledons 3h
embryo axes 5h
megagametophyte 7h
% Viability
100
60
20
36
40
44
48
Temperature (°C)

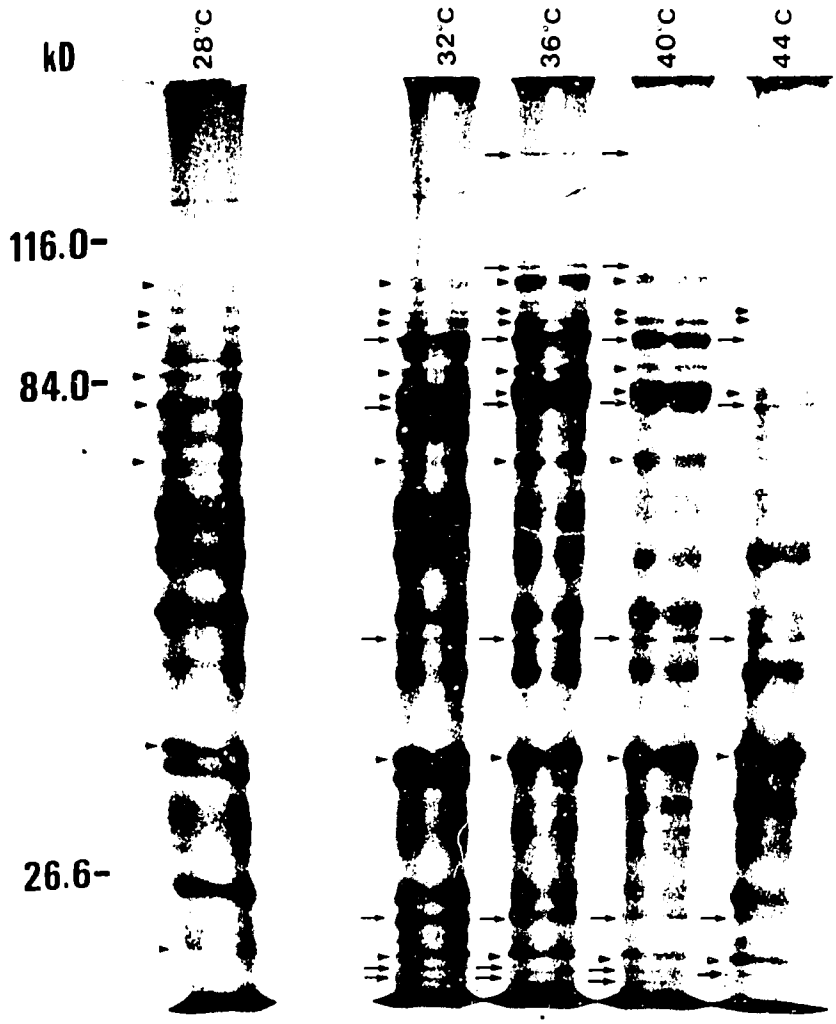
78

Figure 2


A
100
60
20
% Growth of Embryo Axes
28
32
36
40
44
Temperature (°C)


B
100
60
20
% Growth of Embryo Axes
28
32
36
40
44
Temperature (°C)


KD
28°C
32°C
36°C
40°C
44°C
116.0
84.0
58.0
48.5
36.5
26.6


kD
28°C
32°C
36°C
40°C
44 C
116.0-
84.0-
26.6-

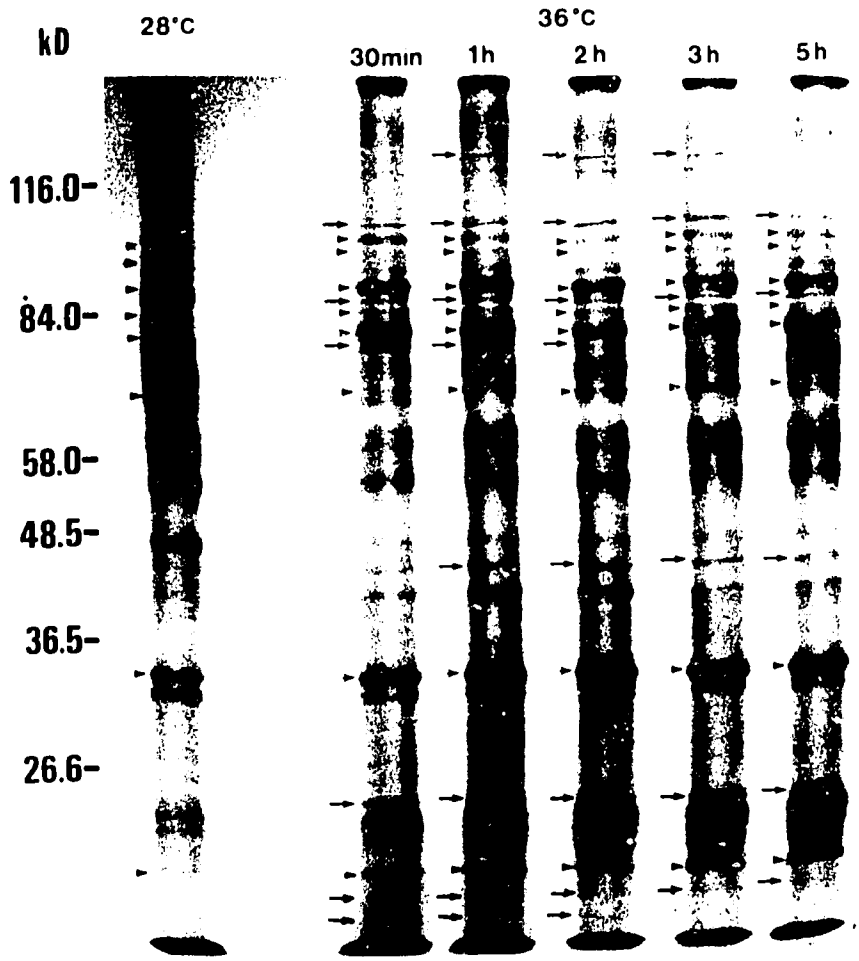
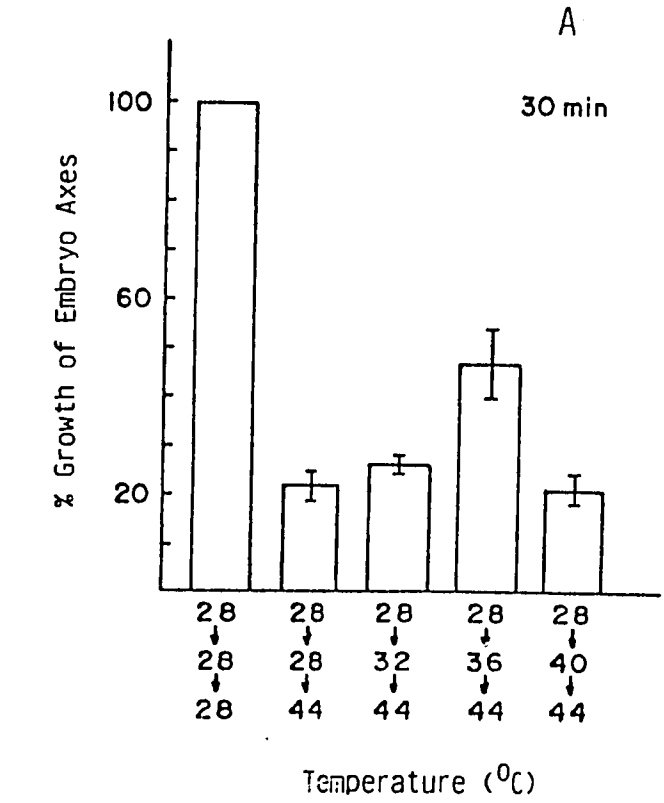
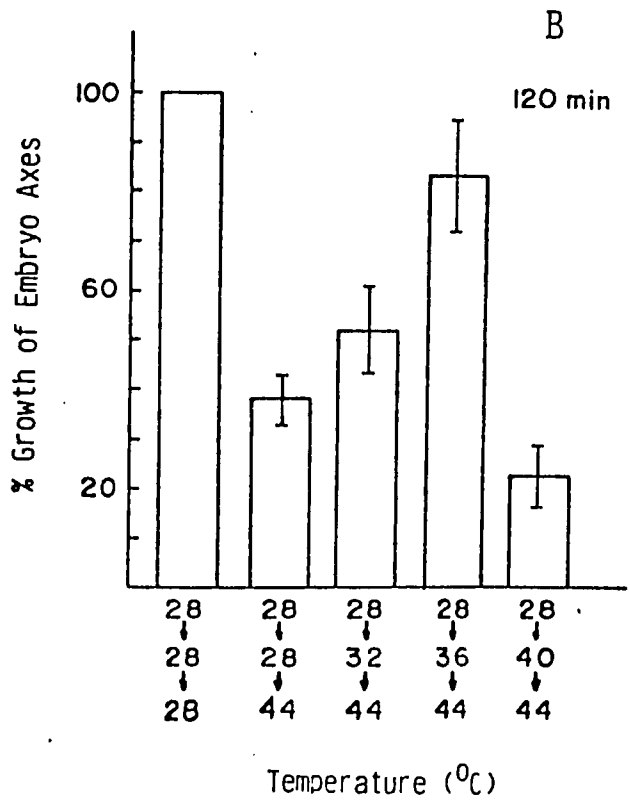

kD
28°C
36°C
30min
1h
2h
3h
5h
116.0-
84.0-
58.0-
48.5-
36.5-
26.6-

Figure 6


A
30 min
100
60
20
% Growth of Embryo Axes
28 28 28 28 28
28 28 32 36 40
28 44 44 44 44
Temperature (°C)


B
120 min
100
60
20
% Growth of Embryo Axes
28 28 28 28 28
28 28 32 36 40
28 44 44 44 44
Temperature (°C)

NATIONAL RESEARCH COUNCIL
OFFICE OF INTERNATIONAL AFFAIRS
BOARD ON SCIENCE AND TECHNOLOGY FOR INTERNATIONAL DEVELOPMENT

2101 CONSTITUTION AVENUE
WASHINGTON, D.C. 20418 USA
TELEPHONE 202/334-2633

TELEX 353001 BOSTID WSH
Cable Address NARECO
Fax No. 202/334-2660

August 28, 1990

Dr. Liliana Cardemil, Professor
Casilla 653
Departamento de Biologia
Facultad de Ciencias
Universidad de Chile
Santiago - Chile

Dear Professor Cardemil,

This is to acknowledge receipt of your last annual report. I read this report and your previous annual report with a great deal of interest. I want to congratulate you on the progress you have made in identifying the heat shock proteins in the two species with which you are working. Both species have a real potential in areas of extreme temperature and possibly in the case of Araucaria, to low water potentials.

It would appear that the data is worthy of publication and I was pleased to see that you have reported your results at a symposium. I have been in touch with Dr. Varner who has informed me that a manuscript describing the responses of Araucaria araucana to wounding has been accepted for publication and that you have another manuscript ready for submission. He also suggests that you have enough data for two more manuscripts. We, at BOSTID put a very high priority on publication of new meaningful data such as indicated in your report and if we can be of any assistance please don't hesitate to call upon us. Since your results are of broad interest we hope that at least some of your publications will be in internationally recognized referied journals. We can help and of course I am sure that Dr. Varner would also be pleased to help. I would appreciate receiving a copy of your above mentioned publication.

You may recall that in the original proposal it was pointed out that the knowledge of the expression of the genes for specific plant proteins produced during stress will allow selection of individual trees having a higher expression of the genes coding for the temperature shock proteins. Do you feel you have reached a stage where this might be considered? How do you visualize such a procedure would be carried out and are you in touch with any breeders in this regard?

I look forward to a dialog and your next report.

Sincerely yours,

Maurice Fried
Program Manager Biot/Pl

The Board on Science and Technology for International Development (BOSTID) conducts programs with and for developing countries on behalf of the National Research Council through its Office of International Affairs which serves the international interests of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine

- 84

From J. Varner's Lab.

Tissue Printing to Detect Proteins and RNA in Plant Tissue

Introduction

When a freshly cut section of an organ is pressed gently on a nitrocellulose, nylon or PVDF membrane the contents of the cut cells are transferred to the membrane.

Tissue Print Western

Section 1. Tissue print on nitrocellulose paper.

Nitrocellulose paper is usually used for tissue print Western. For localization of cellular proteins, nitrocellulose paper can be used without any pretreatment. For localization of plant cell wall proteins, nitrocellulose paper should be soaked in 0.2 M $CaCl_2$ for 30 min, then air-dried before use.

1. Put 6 layers of Whatman No. 1 paper on a plastic plate. On the Whatman paper put one sheet of smooth-surface paper (xerox paper is fine), then lay nitrocellulose paper on it.

2. Use double-edge razor blade to cut 1 mm thick section by free hand. Gently blot the surface of the section on Kimwipes or wash the section in distilled H_2O for a few seconds then gently blot it on Kimwipes. Carefully transfer the section on the nitrocellulose paper by forceps. Do not move the section after it is transferred on the paper. Put 4 layers of Kimwipes on the section, press the section gently and evenly for 15 -20 sec by finger. Remove Kimwipes and section carefully by forceps. Keep the section or cut thin section from mirror-side for anatomy comparison. The section can be stained by toluidine blue for observation of anatomy.

3. Repeat step 2 for the next print. Many prints can be done on the same nitrocellulose paper. Air-dry the paper.

Section 2. Detection of Proteins by Specific Antibody.

1. Wash the paper in washing buffer I for 15 min.

2. Incubate the paper in blocking buffer for 3 hr.

3. Incubate the paper in primary antibody (diluted in blocking buffer) for 2 hr.

4. Wash 4 times with washing buffer I for 10 min each.

5. Incubate in secondary antibody (alkaline phosphatase-conjugated; diluted in blocking buffer) for 1 hr.

6. Wash with washing buffer I for 10 min.

7. Wash 2 times with washing buffer II for 15 min each.

8. Wash with washing buffer I for 10 min.

9. Equilibrate with AP buffer for 10 min.

10. Color development. For 10 ml AP buffer, add 66 ul NBT and 33 ul BCIP, mixed. Put the paper in AP buffer containing substrates. Purple color appears in the place where the interest protein is localized. Develop color until it is satisfactory. Generally the color development will be finished within 10 min. If longer color development is needed, keep the reaction in the dark.

11. Stop the reaction in washing buffer III for 5 min. Wash in distilled H_2O for 5 min.

12. Air-dry the paper.

Section 3. Observation and Recording of Protein Localization on Nitrocellulose Paper.

Observation and recording is generally carried out under a dissecting microscope. By using in succession transmitted light and incident light, the tissue localization of the protein can be seen. The physical print can easily be seen under incident light. To analyze the protein localization on tissue print, it's better to compare the anatomy of the section.

For black and white print, Kodak Technical Pan 2415 can be used. For color slides, use Kodak Ektachrome 160.

Materials and Solutions:

--Nitrocellulose paper (Schleicher & Schuell)

--Whatman No. 1 paper

--Plastic plate

--Smooth surface paper

--Double-edge razor blade

--Kimwipes

--Forceps

--Dishes

--Primary antibody

--Secondary antibody (alkaline phosphatase-conjugated)

--Washing buffer I

-0.1 M Tris-HCl (pH 8.0)

-0.05% Sodium azide

-0.3% Tween-20

--Washing buffer II

-0.1 M Tris-HCl (pH 8.0)

-0.05% Sodium azide

-0.3% Tween-20

-0.05% SDS

--Washing buffer III

-10 mM Tris-HCl (pH 8.0)

-1 mM EDTA

--Blocking buffer

-0.1 M Tris-HCl (pH 8.0)

-0.05% Sodium azide

-0.25% BSA

-0.25% gelatin

-0.3% Tween-20

--AP buffer

-0.1 M Tris-HCl (pH 9.5)

-0.1 M NaCl

-5 mM $MgCl_2$

-Alkaline phosphatase substrates

-NBT and BCIP (Promega)

Tissue Print Northern

Section 1. Tissue Print on Nitrocellulose or Nylon Membrane.

Both nitrocellulose paper and nylon membrane can be used for tissue print without any pretreatment. Generally nylon membrane is preferred because of its easy handling and sometimes higher resolution.

1. Put 6 layers of Whatman No. 1 paper on a plastic plate. On the Whatman paper, put one sheet of smooth-surface paper, then lay nitrocellulose or nylon membrane on it.

2. Use double-edge razor blade to cut 1 mm-thick section by free-hand. If the surface of the section is too juicy, gently blot it on Kimwipes, then carefully transfer the section on the membrane by forceps. Do not move the section after it is transferred on the membrane. Put 4 layers of Kimwipes on the section, press the section gently and evenly for 15-20 sec by finger. Remove Kimwipes and section carefully by forceps. Keep the section or cut thin section from mirror side for anatomy comparison. The section can be stained by toluidine blue for observation of anatomy.

3. Repeat step 2 for the ext print. Many prints can be done on the same membrane. So this is a wonderful tool to screen for organ and tissue specific gene expression in different plants and different developmental stages at the same time on the same membrane. The results can be observed in 3 days!

4. Bake at 80°C under vacuum for 2 hr.

Section 2. Preparation of Radiolabelled Probes.

Either ^{32}P or ^{35}S labeled DNA and RNA probes can be used for hybridization. Higher resolution is obtained by using ^{35}S-labeled probe. The synthesis of radiolabeled probes can be done according to standard method.

Section 3. Detection of mRNA by ^{35}S-RNA Probe.

1. Wash the membrane in washing solution I at 65°C for 4 hr.

2. Prehybridization in hybridization solution at 68°C for 2 hr.

3. Hybridization with ^{35}S-RNA probes at 1×10^7 cpm/ml at 68°C for 20 hr.

4. Wash the membrane in washing solution II at 42°C three times for 20 min each.

5. Wash the membrane in washing solution III at 65°C twice for 30 min each.

6.Expose to x-ray film or T-max 400 film. Generally T-max 400 film gives better resolution. The exposure time depends on the intensity of signal. Generally it takes 4 hrs to 4 days.

7. X-ray film is developed in x-ray film processor. T-max 400 film is developed as follows:

11 min in Kodak T-max developer

30 sec in stop bath

5 min in Kodak rapid fixer

5 min in H_2O

Section 4. Data Analysis.

The mRNA localization on the tissue print is observed under dissect microscopy by comparison with the anatomy of the section. Kodak Technical Pan 2415 can be used for recording results.

Materials and Solutions:

--Nylon (Schleicher and Schuell Nytran
Amersham Hybond N
Bio-Rad Zeta-probe membrane)

--^{35}S-UTP (1mCi/ml) (NEN)

--washing solution I

--0.2 x SSC

-- 1% SDS

--hybridization solution

--2 x SSC

--1% SDS

--5 x Denhardt's solution

--0.1 mg/ml SSD

--10 mM DTT

--washing solution II

-- 2 x SSC

--0.1% SDS

--10 mM DTT

--washing solution III

--0.2 x SSC

--0.1% SDS

-- 1 mM DTT

--T-max-400 film

--Kodak T-max developer

--Kodak stop bath

--Kodak rapid fixer

tissue print Western

9:00 am wash

9:15 am block

12:15 pm 1st AB

2:15 pm wash

3:00 pm 2nd AB

4:00 pm wash, color development

tissue print Northern

9:30 am tissue print

11::00 am wash

3:00 pm prehybridization

5:00 pm hybridization

free time 11:00 am ------> 2:00 pm