MANAGEMENT REPORT

Covering period : August, 31, 1990 to February 28, 1991

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

Title : 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.

Collaborators : Dr. Joseph E. Varner
Dr. David Ho

Institution : Department of Biology, Washington University, St. Louis, M.O. 63130, U.S.A.

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

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

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

Preliminary Remarks

This report correspond to six months of research of our project from the period August 31, 1990 to February 28, 1991. The report has been written according to the guidelines of the National Research Council. This is a Management Report, following the Annual Report which covered the period February 28 - August 30, 1990. We are enclosing here copies of galley proofs of two publications with results obtained under this project. This publications are: "Expression of Cell Wall Proteins in Seeds and During Early Seedling Growth of Araucaria araucana is a Response to Wound Stress and Developmentaly Regulated" from the authors Liliana Cardemil and Alejandro Riquelme. These galley proofs have been published already in the Journal of Experimental Botany (we have not received prints yet of the publication). The other is "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. It will soon be published in the Journal of Plant Physiology and Biochemistry.

We have spent so far US $ 9,527.77 from the last report. We estimate that our expenses will be about US $ 10,000 for this coming semester.

Finally, this report is being sent to A.I.D., two months later, due to familiar problems. My husband is very sick. He recently had a heart stroke.

1) Scientific Summary:

Tissue print analyses have been perfomed with cotyledons, hypocotyls and roots of Prosopis chilensis. The tissues have been wounded by cuts perfomed with razor blades and printed on nitrocellulose membranes 24 hours after the wounding. Intact tissues have been also printed and used as control. The nitrocellulose membranes were incubated with rabbit antibodies (first antibody) raised against extensin from carrot and from soybean seed coats. After incubation with the antibody the membranes were soaked with a goat secondary antibody raised against rabbit IgG and conjugated to alkaline phosphatase.

The prints demonstrated that the vascular bunddles of cotyledons, hypocotyls and the root necks are the tissues and organs of the plant showing a higher cross reactivity with extensin antibodies.

Respect to field research with Prosopis chilensis in Vicuña (gorge of Sn. Carlos) has allows to obtain data so far on :

a) Phenological studies not only in Prosopis chilensis but also in other species present in the same area of study.

b) Daily time course of photosynthesis and transpiration.

c) Foliar Carbon Isotope Analysis for CO_2 assimilation during thermal stress.

d) Daily presure-volumen measurements in branches and leaf petioles.

e) Anatomical studies of the connecting points bethween branches and petioles.

f) Western analysis of heat shock proteins synthesized in leaves collected in the morning, noon, afternoon and evening.

2) Scientific Issues:

We need urgently to develop a method to label DNA and RNA probes with non radioactive nucleotides precursors. Because of this need, the principal investigator of the project Professor L. Cardemil, visited for one month Dr. Elena del Campillo's. Laboratory in the Department of Plant Biology, University of California, Berkeley with the idea to perform in situ hybridization using "the ingenious kit" method to label a RNA probe containing genetic information for extensin . We are hoping to develop this method also in Chile.

3) Managerial Issues :

In our last annual report we have stated that we need extra time to full accomplish the main goals of our original proposal.

For this reason we would like very much to have one extra year of research. This extra time is needed because actually our project begun six months later from the starting date August 31 1988, due to the fact that AID sent to us the agreements papers by slow mail. In the extra year of research we will perform field research and we will evaluate and select the resistant individuals.

Respect to our expenses, we have spent less in salaries and more in reagents. Enclosed is a detailed report of Disbursement of Budget for the reporting period.

4) **Special Concerns :** No special concerns.

5) **Collaborations, Travel, Training and Publications:**

The National Research Council, Office of International Affairs, Board on Science and Technology for International Development, through the PSTC/BOSTID program, invited Dr. Liliana Cardemil to participate in the Fourth Conference of the International Plant Biotechnology Network (IPBNet Conference). The Conference took place in San José, Costa Rica from the 13-18 of January, 1991. A day after the Conference all the PSTC grantees of AID met together with Dr. Jill Conley, Program Officer of the PSTC/BOSTID, in order to present the research progress of our projects. All of us have enjoyable presentations and discussions.

At the IPBNet Conference, Dr. Cardemil gave a 20 minutes talk presentation entitled: "Gene Expression to Thermal Stress in Two Native Trees of Chile: Araucaria araucana y Prosopis chilensis" from the authors Liliana Cardemil, Claudio Goycoolea and Consuelo Medina. Dr. Cardemil also presented a poster entitled: "Response to Wound Stress by Seedlings of Araucaria araucana and Prosopis chilensis" from the authors Liliana Cardemil, Alejandro Riquelme, José Gregorio Rodríguez. Copies of the abstracts of these two presentations are enclosed.

We are also enclosing copies of the two publications, one published in the Journal of Experiemental Botany and in the Plant Physiology and Biochemistry (see Special Remarks).

6) Request for A.I.D. or BOSTID Actions:

In spite of the presentations in the IPBNet Conference, I still would like very much to have other similar meetings to be able to meet people related to our work and discuss our results with them.

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

Cummulative Detailed Report

From :
To :

	US$
Salaries	3,457
Equipment	----
Travel	1,315
Reagents (supplies)	4,152
Other Costs (Bibliography and equipment repair)	637
TOTAL	9,561

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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$	From August 31, 1990 To February 28, 1991 US$	TOTAL US$
SALARIES	1,440	2,424	5,958	3,031	3,457	16,310
EQUIPMENT	45,755	1,431	2,655	0,000	-----	49,841
TRAVEL	5,874	1,794	1,852	8,196	1,315	19,033
REAGENTS	4,190	4,174	1,506	10,174	4,152	24,196
OTHER COSTS	1,230	12,473	6,146	1,505	637	21,991
TOTAL...	58,489	22,296	18,117	22,906	9,561	131,371

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REQUEST FOR ADVANCE OR REIMBURSEMENT

(See instructions on back)

Approved by Office of Management and Budget, No. 80-R0183

PAGE OF PAGES

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): August 31, 1990
TO (month, day, year): February 28, 1991.

9. RECIPIENT ORGANIZATION
Universidad de Chile, Fac. de Ciencias
Name: Banco de Chile, Dep. del Ext. Sec. Contabilidad Acc. # 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 & Travel	(b) Equipment & Other Costs	(c) Reagents & Supplies	TOTAL
a. Total program outlays to date (As of date)	$ 68,350	$ 66,470	$ 15,000	$ 149,820
b. Less: Cumulative program income	35,343	71,831	24,196	131,371
c. Net program outlays (Line a minus line b)	33,007	-5,361	9,196	18,449
d. Estimated net cash outlays for advance period	4,000	0,000	4,000	8,000
e. Total (Sum of lines c & d)	37,007	-5,361	13,000	18,646
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	$ 8,000
b. Less: Estimated balance of Federal cash on hand as of beginning of advance period	
c. Amount requested (Line a minus line b)	$

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: Carlos Michaelis

DATE REQUEST SUBMITTED: May 3, 1991

TYPED OR PRINTED NAME AND TITLE: Dr. Carlos Andrade Plaza, Dean, Facultad de Ciencias, Universidad de Chile

TELEPHONE (AREA CODE, NUMBER, EXTENSION): [illegible] Ext. 271

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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) August 31, 1990
TO (Month, day, year) February 28, 1991

Period covered by the next report:
FROM (Month, day, year) March 1, 1990
TO (Month, day, year) August 30, 1990

B. Cash Advance Use and Needs:

1. Cash advance on hand at the beginning of this reporting period ..... $ 28,010

2. U.S. Treasury check advance(s) received during this reporting period..... $ 24,605

3. Interest earned on cash advance during this reporting period..... $ 90,000

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4. GROSS cash advance available during this reporting period (Lines 1, 2, & 3).....$ 28,010

5. LESS, interest remitted to AID during this reporting period..... $ 00,000

6. NET cash advance available during this reporting period (Line 4 minus Line 5)..... $ 28,010

7. Total disbursements during this reporting period, including subadvances (see footnote 1)..... $ 9,561

8. Amount of cash advances available at the end of this reporting period (Line 6 minus Line 7)..... $ 18,646

9. Projected disbursements, including subadvances, for the next reporting period (see footnote 2)..... $ 8,000

10. Additional cash advance requested for the next reporting period (Line 9 minus Line 8)..... $ 0,000

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

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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 May 3, 1991.

BY Juan Carrasco Contreras

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

(END OF STANDARD PROVISION)

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Journal of Experimental Botany, Vol. 42, No. 236, pp. 000–000, March 1991

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Expression of Cell Wall Proteins in Seeds and During Early Seedling Growth of *Araucaria araucana* is a Response to Wound Stress and is Developmentally Regulated

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Received 22 May 1990

ABSTRACT

Araucaria araucana seeds and seedlings respond to wounding after 48 h with a 3- to 4-fold increase of hydroxyproline-rich glycoproteins (HRGP) in the cell walls of the embryo and with a 15-fold increase in the cell walls of the megagametophyte. The megagametophyte walls accumulate six times more hydroxyproline per μg of cell wall protein than the embryo in this wound response. Tissue immunoprints of different parts of seeds and seedlings obtained with polyclonal antibodies raised against HRGP from carrot roots or soybean seed coats indicate that the response is due to an increase in a protein similar to the ones seen in carrot roots or soybean seed coats. Western blots of embryo and megagametophyte cell wall proteins subjected to SDS-PAGE show three bands that cross-react with these antibodies. In a native cationic gel system followed by Western blot analysis, only two bands react with these antibodies. Expression of such proteins in *Araucaria araucana* seeds seems to be developmentally regulated and tissue specific, since they are present mainly in the megagametophyte and the root cap of the embryo.

Key words: *Araucaria araucana*, seeds, seedlings, cell walls, hydroxyproline-rich glycoproteins.

INTRODUCTION

Araucaria araucana is a South American conifer restricted to high mountain areas in southern Chile and Argentina. *Araucaria araucana* constitutes relict forests from tertiary times (Montaldo, 1974). We assume that there is a great degree of physiological adaptation of *Araucaria araucana* to live in these harsh isolated areas covered by volcanic soil.

It is interesting then to investigate, in species of native trees like *Araucaria* sp., genes whose expression protect the organisms when subjected to environmental stress. Such protection is accomplished through the synthesis of specific proteins.

One class of such protective proteins is the hydroxyproline-rich glycoproteins (HRGPs) of the plant cell wall, also called extensins by Lamport. These were characterized by Lamport (1965, 1969) at Michigan State University and by Stuart and Varner (1980) and Cassab, Nieto-Sotelo, Cooper, Van Holst, and Varner (1985), at Washington University, St. Louis. The HRGPs seem to be a family of proteins, as has been reported by Smith, Muldoon, and Lamport (1984) and Varner and Lin (1989).

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In addition, there are other cell wall proteins, such as a glycine-rich protein present in oat coleoptiles, the seed coats of soybean, petunia cell walls, pumpkin seed coats, and the protoxylem of french bean as reported by Cassab and Varner (1986). A threonine-rich glycoprotein has been found in corn suspension cell cultures by Kieliszewski and Lamport (1987) and in corn pericarp by Hood, Shen, and Varner (1988).

The present work investigates similar proteins in the cell wall of a conifer. It provides the initial characterization of the proteins present in *Araucaria araucana* cell walls from embryo and megagametophyte tissues of seeds and seedlings.

MATERIALS AND METHODS

Plant material

Cell walls were prepared and purified as described by Stuart and Varner (1980) and Cassab *et al.* (1985) from wounded and non-wounded megagametophyte and embryonic tissues of germinating seeds of *Araucaria araucana*.

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Cell wall proteins were extracted from purified walls with 0.1652 M $CaCl_2$ and 0.1652 mM $Na_2S_2O_3$ solution.

0.2 M $CaCl_2$
2 mM $Na_2S_2O_3$

Analytical methods

The hydroxyproline content was quantified by the method of Drozdz after hydrolysis of the walls with 6 N HCl at 121 °C for 12 h under nitrogen (Drozdz, Kucharaz, and Szyja, 1976). Proteins were quantified by the method of Bradford (Bradford, 1976). Arabinogalactan proteins were identified and quantified by a single radial diffusion test in agarose gels containing β-glucosyl Yariv reagent (Van Holst and Clarke, 1985).

Gel electrophoresis and Western blot analyses

could say 5 M Tris 2 M glycine chemist

SDS–PAGE of proteins was performed in 30%:8%, acrylamide:*bis* acrylamide to make a 7% polyacrylamide gel and run for 6 h at 25 mA in 0.165025 M Tris 0.165192 M glycine buffer, pH 6·8, containing 0·1% SDS, following Laemmli's method (Laemmli, 1970). Cationic-neutral gel electrophoresis of native proteins was performed as reported by Thomas and Hodes (1981), using 40%:2%, acrylamide:*bis* acrylamide to make a 7% polyacrylamide gel and run overnight at 15 mA in ~~0.1651~~ M histidine, ~~0.16502~~ M MOPS buffer (3-[*N*-Morpholino] propanesulphonic acid) pH 6·8 as modified by Cassab and Varner (1987). After gel electrophoresis half of the gel was stained for proteins with silver nitrate stain. The other half was subjected to Western blot analysis by transferring the proteins to nitrocellulose paper overnight at 130 mA using either the SDS–Tris-glycine buffer or the histidine–MOPS buffer depending on the type of electrophoresis. The nitrocellulose membrane was incubated with a primary polyclonal antibody raised against either glycosylated or deglycosylated HRGPs from carrot roots used at a dilution of 1/500 or from soybean seed coats used at a dilution of 1/1500. These antibodies were kindly given to us by Gladys Cassab and Joseph E. Varner from Washington University, Department of Biology, St. Louis, MO. The nitrocellulose membranes were then incubated with a goat secondary antibody at a dilution of 1/20 000, raised against the rabbit IgG and conjugated to alkaline phosphatase. The membrane was stained with nitroblue tetrazolium reagent to detect the reaction product of alkaline phosphatase using 5 bromo-4 chloro-3 indoyl phosphate as substrate (Cassab and Varner, 1987).

histidine 7 MOPS

Tissue immunoprints

Tissue immunoprints were performed as described by Cassab and Varner (1987) with sections of quiescent seeds, sections of seeds imbibed for 48, 72, 96, and 110 h; sections of wounded seeds and sections of root tips from germinating seeds.

Three types of wounding were performed on the seeds. The first was made accidentally when the seed coats were removed prior to seed imbibition, i.e. a lengthwise cut with a razor blade that also slightly cut the surface of the megagametophyte. This type of wounding is shown in Fig. 1F. The second type was performed intentionally with a razor blade by removing small pieces of the megagametophyte surface, making shallow depressions in it, see Fig. 3B. The third type was intentionally performed with a razor blade by cross-sectioning the seed half-way through and completing the cross-section immediately before the print, see Fig. 3D. The wounding of the megagametophytes was performed 24–48 h before the impression of the seed cross-sections on nitrocellulose membranes.

To make the prints, the tissue was pressed for 20 s against dry nitrocellulose paper impregnated with a 0.1652 M $CaCl_2$, 0.1650 mM $Na_2S_2O_3$ solution. The wet area of the paper left by the sections was air-dried and the nitrocellulose membrane was incubated with the primary antibody raised against HRGP of soybean seed coats used at a dilution of 1/1500. In only one tissue immunoprint the membrane was incubated with a primary polyclonal antibody raised against a 12 amino acid synthetic peptide; VEATNSVTEDHY, a sequence not present in carrot root or soybean seed coat extensins but present internally in the derived amino acid sequence of carrot root in a cDNA clone which codes a 33 kD for proline-rich protein (p33) (Chen and Varner, 1985). The genetic information for this protein is also expressed after wounding. The antibody against the synthetic polypeptide was kindly provided by Mary L. Tierney and it was used at a dilution of 1/75 (Tierney, Wiechert, and Pluymers, 1988). [redacted]

After incubation with the primary antibody the tissue prints membranes were treated in the same manner as the Western blots.

RESULTS

Protein and hydroxyproline contents of cell walls of wounded and non-wounded tissues

The amount of protein and the hydroxyproline content were quantified in embryonic and megagametophytic tissues of seeds imbibed for 48 h under wounded and non-wounded conditions. Table 1 shows that hydroxyproline content in both tissues, either expressed as μg of hydroxyproline in 100 μg of cell wall protein or as μg of hydroxyproline per gram of fresh weight (gfw), increases after 48 h of wounding. The level of hydroxyproline is 3 to 4 times higher in embryonic tissue and 15 times higher in the megagametophytic tissue under wounded conditions compared with non-wounded conditions.

Protein and hydroxyproline contents of cell walls of germinating seeds and growing seedlings

The hydroxyproline content of the whole seed cell walls also increases during germination and early seedling

TABLE 1. *Hydroxyproline content of $CaCl_2$ cell wall extracts under wounded and non-wounded conditions*

The figures are the average of four experiments with their standard deviations.

Condition	Embryo		Megagametophyte	
	μg Hyp/100 μg c.w.p	μg Hyp/gfw	μg Hyp/100 μg c.w.p	μg Hyp/gfw
Non-wounded	0·6 ± 0·3	2·0 ± 0·5	1·2 ± 0·7	1·7 ± 0·4
Wounded	3·2 ± 0·8	6·5 ± 1·2	18·0 ± 2·3	25·0 ± 2·165

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growth. The hydroxyproline increases from the quiescent seed to 110 h after the start of imbibition and decreases beyond this time (Table 2). The amount of hydroxyproline is 20-fold higher at 110 h of imbibition than in the quiescent seeds.

Tissue immunoprints

The above results are corroborated by the tissue immunoprints shown in Fig. 1A, B, C, D and Fig. 2A where the tissue sections show an increasing cross-reactivity with the antibody raised against the wall protein of soybean coats, from quiescent seeds to seeds imbibed for 110 h. Figure 1E is a diagram of the seed of *Araucaria araucana* where the megagametophyte, embryos and cotyledons are seen.

The tissue immunoprints of Fig. 1B and D also reveal that the root tip and the megagametophyte tissue surrounding the root tip are the ones that show higher cross-reactivity with the antibodies raised against the wall protein of soybean seed coats. The cross-reactivity of the root tip and surrounding tissue increases with the age of the tissue from 48 to 110 h of imbibition. Figure 1C shows a higher cross-reactivity of the wounded area of the megagametophyte that was cut with a razor blade when the seed coats were removed prior to imbibition. A diagram of the wounding cut is shown on Fig. 1F.

Figure 2B shows a magnified area of the megagametophyte section after 110 h of imbibition observed in Fig. 2A. In this micrograph it is possible to see that the cross-reactivity is given mainly by walls surrounding the large hexagonal cells of the megagametophyte.

In Fig. 3A small pieces of the megagametophyte surface of seeds of 48 h of imbibition were removed 24 h prior to performing the cross-sectioning and printing of the seed on nitrocellulose membrane. A diagram of the wounding cut is shown on Fig. 3B. The membrane was incubated with polyclonal antibodies raised against HRGP from soybean seed coats. In Fig. 3C seeds of 48 h of imbibition were cut with a razor blade half-way through: after 24 h the cut was continued through the seed and then the sections were printed on a nitrocellulose membrane. A diagram of the wounding cut is shown in Fig. 3D. Next, the membrane was incubated with polyclonal antibodies raised against the synthetic polypeptide derived from the p33 cDNA coding sequence as reported by Chen and Varner (1985) and by Tierney *et al.* (1985). In both Fig. 3A and 3C there are two control sections, one of them was completely sectioned for 48 h and is

TABLE 2. *Hydroxyproline content of $CaCl_2$ cell wall extracts of the whole seed during the imbibition time*

The figures are the average of four experiments with their four standard deviations.

Time (hours after imbibition)	μg Hyp/100 μg c.w.p.	μg Hyp/gfw
0	0·3 ± 0·05	0·5 ± 0·1
48	1·0 ± 0·20	2·0 ± 0·8
72	3·1 ± 0·30	6·4 ± 0·5
110	6·3 ± 0·80	9·1 ± 1·0
160	4·2 ± 1·20	5·2 ± 0·1659

1.9

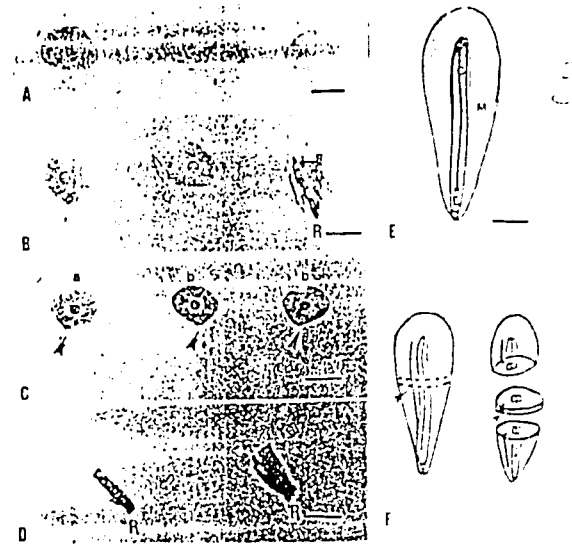

FIG. 1. Tissue immunoprints of cross-sections of seeds and longitudinal sections of root tips of seedlings of *Araucaria araucana*. (A) Cross-sections of quiescent seeds. Scale bar = 1.1650 cm. (B) Cross-sections of seeds and longitudinal sections of a root tip of a seed embryo, imbibed for 48 h. Scale bar = 1.1650 cm. (C) Cross-sections of seeds imbibed for 72 h in *a*, and imbibed for 96 hours in *b*. Arrows show a small wounded area by making a cut with a razor blade. Scale bar = 1.1650 cm. (D) Longitudinal sections of root tips of seedlings after 96 h of imbibition. Scale bar = 1.1650 cm. (E) Diagram of a longitudinal section of a seed of *Araucaria araucana*. M, megagametophyte; E, embryo, C, cotyledons. Scale bar = 1.1655 cm. (F) Diagram of the seed showing the lengthwise cut performed accidentally on the megagametophyte with a razor blade when the seed coats were removed. Arrows show the wounded area of the tissue. Dashed lines show the cross section performed to make the print pointed with the arrows in Fig. 1C. The antibody used in all prints of Fig. 1 was raised against soybean seed coat extensin. R, stands for roots.

(A) 1 c
(B) 1 cm
(C) 1 cm
(D) 1 cm
(E) 1

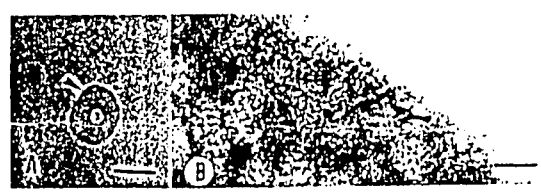

FIG. 2. Tissue immunoprint of a megagametophyte cross-section after 110 h of imbibition, it is shown in (A) at the actual size. The arrow in (A) shows the area that has been magnified in (B), where the cell walls can be observed as the cell structure giving the strongest cross-reactivity with the extensin antibody from soybean seed coats. Scale bar in (A) = 1.1650 cm, in (B) = 390 nm.

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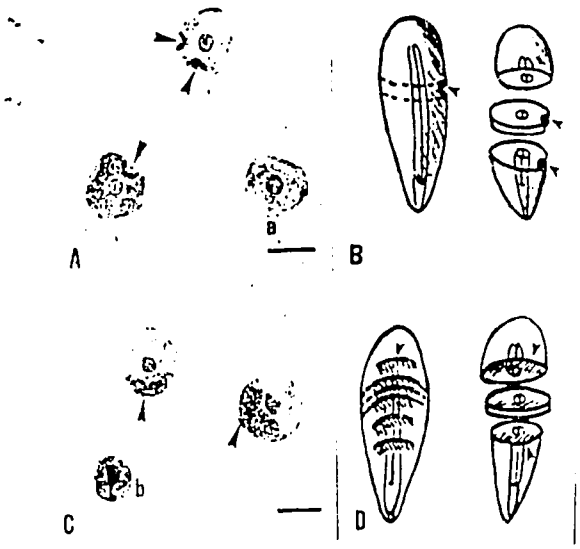

FIG. 3. Tissue immunoprints of partially wounded seeds of 48 h of imbibition. (A) Areas of cross-sections of seeds which were wounded 24 h before the printing, by removing small pieces of the surface of the megagametophyte. The wounded areas show a stronger cross reactivity with the soybean seed coat extensin antibody (arrows). Scale bar = 1/1650 cm. (B) Diagram showing the type of wounding cut performed in (A). Arrows point the wounded area of the megagametophyte. The dashed lines correspond to a cross-section of the seed to make a print. (C) Areas of cross-sections of seeds which were wounded 24 h before the printing by cutting the seeds half-way through. The nitrocellulose paper was incubated with antibodies raised against the 12 amino acid synthetic polypeptide derived from the coding sequence of p33. The wounded areas show a stronger cross-reactivity with the antibody (arrows). Scale bar = 1/1650 cm. (D) Diagram showing the type of wounding cut performed in (C). Arrows point the wounded areas of the megagametophyte. The dashed lines correspond to a cross section of the seed performed to make a print. a, non-wounded section. b, complete wounded section.

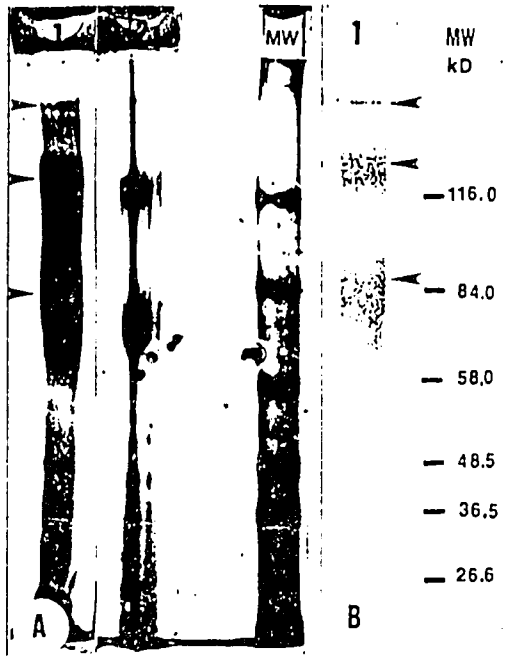

FIG. 4. SDS–polyacrylamide gel electrophoresis of cell wall proteins from embryo and megagametophyte tissues. (A) Gel stained with silver nitrate. (B) Western blot of the same electrophoresis using anti HRGP raised against glycosylated extensin from carrot root tissue. Arrows show the corresponding bands of (A) and (B). Molecular weight markers (MS) are shown at the margin of (A) and (B). In (B) the molecular weight markers are shown with the molecular weight values expressed in kD. Lane 1 of (A) and (B) are proteins from megagametophyte extracted with $Na_2S_2O_5$ and $CaCl_2$. Lane 2 of (A) are proteins from the embryo extracted with $Na_2S_2O_5$ and $CaCl_2$.

marked as section *b* and the other was only cut completely just before the printing and is marked as section *a*.

SDS–polyacrylamide gel electrophoresis and Western blot analysis of the cell wall proteins

Figure 4A shows a silver stained SDS–PAGE gel of the proteins extracted from the cell walls of embryo and megagametophyte tissues. Figure 4B is a Western blot performed with the same samples. The gel shows that multiple proteins can be extracted from the cell walls of these tissues with a solution of $CaCl_2$ and $Na_2S_2O_5$. However, only three protein bands cross-react with the antibodies raised against HRGP of carrot cell walls (Fig. 4B). The bands also cross-react with the antibodies raised against HRGP of soybean seed coats. The estimated molecular weights of these proteins are 187, 134, and 81 kD.

Cationic neutral gel electrophoresis and Western blot analysis of the cell wall proteins

Due to the basic nature of the HRGP, these proteins run towards the cathode (-) in polyacrylamide gels at a near neutral pH of 6·8. To see if these cell wall proteins of *Araucaria araucana* which cross-react with HRGP antibodies are basic proteins when run in SDS–PAGE Western analysis, we performed a cationic neutral gel electrophoresis of the native cell wall proteins, followed by Western blot analysis under cationic conditions.

In native cationic gel electrophoresis systems only two proteins penetrate the gel (Fig. 5A). Both cross-react with the antibodies raised against either the glycosylated or deglycosylated HRGP from carrot. Figure 5B shows the cross-reactivity of *Araucaria araucana* cell wall proteins with the antibodies raised against glycosylated carrot HRGP. These bands also cross-react with the antibodies raised against HRGP of soybean seed coats.

DISCUSSION

From our studies it is clear that the cell walls of different tissues of *Araucaria araucana* seeds and seedlings contain

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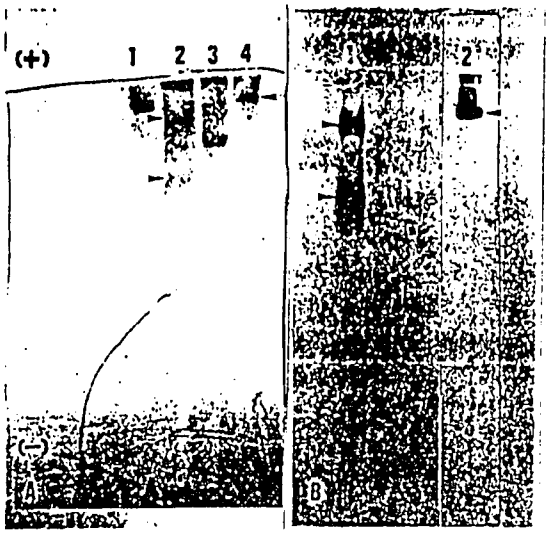

FIG. 5. Cationic neutral gel electrophoresis of cell walls of *Araucaria araucana* extracted with $Na_2S_2O_5$ and $CaCl_2$ 48 h after wounding. (A) Cationic neutral gel stained with silver nitrate stain. Lane 1: 0.1653 μg of glycosylated HRGP from carrot root. Lane 2: 20 μg of *Araucaria* cell wall proteins. Lane 3: 1.1650 μg of deglycosylated HRGP from carrot root. Lane 4: 1.1650 μg of glycosylated HRGP from carrot root. (B) Western blot of cationic neutral gel electrophoresis incubated with polyclonal antibodies raised against glycosylated HRGP of carrot root. Lane 1: 20 μg of *Araucaria* cell wall proteins. Lane 2: 1 μg of glycosylated HRGP from carrot root. Arrows show the corresponding bands of (A) and (B).

proteins that can be extracted by buffers of high ionic strength. The proteins extracted from the cell walls of *A. araucana* in both tissues make up about 2·5% of the total dry weight of the walls. However, after extraction there are still proteins left in the cell walls as demonstrated by the hydroxyproline released on hydrolysis of the residual walls after protein extraction. These are probably proteins insolubilized in the cell walls (data not shown).

The presence of hydroxyproline as a component of the proteins of the cell wall suggests that these proteins could be similar to those which have been called HRGPs or extensins. Extensins as well as the arabinogalactan proteins are localized in the extracellular matrix of higher plants (Lamport, 1965; Varner and Lin, 1987).

The proteins extracted from the cell walls of *Araucaria araucana* are not arabinogalactans because arabinogalactan proteins are washed out from the extracellular matrix during the purification of the walls, and because the extracted proteins of the walls of *Araucaria araucana* tissues give a negative reaction with the β-glucosyl Yariv reagent (data not shown). Furthermore, the proteins show cross-reactivity with antibodies prepared against glycosylated or deglycosylated HRGPs from cell walls of carrot roots.

As in the case of other HRGPs reported (Stuart and Varner, 1980; Chen and Varner, 1985; Showalter, Bell, Cramer, Bailey, Varner, and Lamb, 1985) the amount of these proteins increases greatly after wounding. The megagametophyte tissue shows 6 times higher cell wall protein levels in response to the injury than the embryo. This was an unexpected physiological response for this tissue since the megagametophyte is haploid while the embryo is diploid (Cardemil and Jordan, 1982). Indeed, previous work performed by Cardemil and Reinero (1982) and Cardemil and Varner (1984) demonstrated that the level of starch hydrolytic enzymes is much lower in the megagametophyte as compared with the embryo. It might well be that the megagametophyte responds better to wounding because it may have a protective role for the embryo during germination and for the cotyledons during seedling growth since this tissue encloses the cotyledons during the development of the seedling (Cardemil and Reinero, 1982).

The tissue immunoprints also show that the cells which have been damaged respond to stress by increasing the levels of these HRGPs. It is apparent that two different classes of cell wall proteins are expressed in wounded tissue of *A. araucana*, since the wounded tissue prints strongly cross-react in those wounded areas with polyclonal antibodies raised against extensin of soybean seed coats and carrot roots and against the synthetic polypeptide contained in p33 genetic information.

It is evident that these proteins are part of the cell wall since they are present in the saline extraction of purified walls and because high magnification of tissue immunoprints reveals that the cell wall is the cellular compartment showing a higher cross-reactivity with the antibodies.

The HRGPs of *A. araucana* walls are tissue-specific, because there is more accumulation of these proteins in the root tip and in the megagametophyte tissue surrounding the root tip.

Quantification of the cell wall proteins of the whole seed as well as the tissue prints, demonstrated that the cell wall proteins which contain hydroxyproline are developmentally expressed during germination and early seedling growth of *A. araucana* seeds. That the extensins are developmentally regulated and tissue specific has been already demonstrated during development of angiosperms by Cassab *et al.* (1985) and Hood *et al.* (1988). Our results show for the first time for the gymnosperm plant, *A. araucana*, that the cell wall proteins are also developmentally expressed during germination and early seedling growth.

SDS–polyacrylamide gel electrophoresis of the proteins extracted from the cell walls detect many bands of proteins. However, only three of them are good candidates to be considered as possible extensins because these three protein bands cross-react with the antibodies raised against carrot root extensins.

The broad pattern of migration in 7% polyacrylamide

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gels containing 0·1% SDS suggests that these proteins could by glycoproteins.

The estimated molecular weights of these proteins are high. With this method, however, the molecular weights of glycoproteins are overestimated because of their larger hydrodynamic radii (Van Holst and Varner, 1984; Cassab *et al.*, 1985).

The HRGPs reported so far are basic proteins because of the high content of lysine. The basic nature of these proteins makes them penetrate, in their native form, into polyacrylamide gels under cationic conditions (Cassab and Varner, 1987; Hodes, 1981). Two of the three proteins of *Araucaria araucana* which cross-react with extensin antibodies run in gels under cationic neutral conditions.

The observed accumulation of these proteins in plant cell walls during development, wounding and infection suggests a role in plant protection and plant defence (Showalter *et al.*, 1985). Therefore, the presence of these proteins in the cell walls of the embryo and megagametophyte of *Araucaria araucana* seeds may be related to conservation and propagation of this endangered species in its harsh natural environment. Seeds and seedlings of *Araucaria araucana* are difficult to propagate and grow under environmental conditions other than those of the restricted geographic area of Chile where *Araucaria* forests still exist, because the climate, soil, and biological factors including fire, animals (though mainly birds which eat the seed and propagate it) and humans make the natural regeneration and propagation of the seed difficult. How these proteins may function in protection and defence is unknown but they may cross-link to form a chemical/mechanical barrier and/or provide sites of lignin and carbohydrate deposition during the assembly of new cell walls or during wall repair (Whitmore, 1978; Varner and Lin, 1989).

Purification of these proteins for amino acid analysis and sequencing is underway.

ACKNOWLEDGEMENTS

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Expression of heat shock proteins in seeds and during seedling growth of *Araucaria araucana* as a response to thermal stress

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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 *A. araucana* seeds were subjected to temperatures of 28, 32, 36, 40, 44 and 48°C for several hours and the viability of the tissues after exposure was determined with the colorimetric test of the triphenyltetrazolium chloride or by measuring the electrolyte leakage. The most sensitive tissues are the cotyledons which die after 3 h of treatment at 44 and 48°C. The most resistant is the megagametophyte which loses viability only after 7 h at 44°C. Embryo axis of seedlings more sensitive to high temperature treatments. They lose viability after 30 min of treatment at 44°C. Sodium dodecylsulfate-polyacrylamide gel electrophoresis 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. Eight proteins are synthesized under heat shock among which is a protein 70 kDa. Kinetic experiments demonstrated that the new proteins are present 30 min after the embryo axes have been exposed to 36°C. Thirty six degrees is also the optimum temperature for acquisition of thermotolerance by *A. araucana* seedlings.

Additional key words – Megagametophyte, heat shock, lethal temperature, thermotolerance, sublethal temperature.

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Résumé. *Le but du travail est de rechercher comment les graines et les plantules d'*Araucaria araucana *réagissent aux températures élevées. Les tissus des axes embryonnaires, des cotylédons et des megagamétophytes de graines d'*A. araucana *ont été soumis à des températures de 28, 32, 36, 40, 44 et 48°C pendant plusieurs heures et la viabilité des tissus après le traitement a été déterminée par le test au chlorure de triphénltétrazolium ou par la mesure des fuites d'électrolytes. Les tissus les plus sensibles sont les cotylédons, qui meurent après 3 h de traitement à 44 et 48°C. Le plus résistant est le mégagamétophyte, dont la viabilité disparaît après 7 h à 44°C. L'axe embryonnaire est le plus sensible aux traitements par des températures élevées, il perd sa viabilité après un traitement de 30 min à 44°C. L'électrophorèse sur gel de polyacrylamide-dodécyl sulfate de sodium des protéines synthétisées par les extrémités des racines de plantules à 32, 36, 40 et 44°C en présence de méthionine[^{35}S] et révélées par fluorographie, montre que la synthèse optimale et l'accumulation des protéines de choc thermique se produit à 36°C. Huit protéines sont synthétisées*

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durant le stress thermique, parmi lesquelles se trouve une protéine de 70 kDa. Des expériences de cinétique indiquent que les nouvelles protéines sont présentes 30 min après l'exposition des axes embryonnaires à 36°C. La température de 36°C est aussi la température optimale pour acquisition de l thermotolérance par les plantules d'A. araucana. Mots clés additionnels : mégagamétophyte, choc thermique, temperature létale, thermotolérance, temperature sublétale.

Abbreviations. SDS-PAGE, sodium dodecysulfate-polyacrylamide gel electrophoresis; hsp, heat shock proteins. TM, treatment medium. TTC, triphenyltetrazolium chloride, TCA, trichloroacetic 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 responds 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; 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 mass, between 90 and 120 kDa, hsp of medium molecular mass, between 40-89 kDa and hsp of low molecular mass between 15-39 kDa (Cooper and Ho, 1983; Cooper *et al.*, 1984). These low relative molecular mass 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.*; Schlesinger, 1985).

Araucaria araucana is a conifer species, endemic to austral South America. *A. araucana* grows in high mountain forests between latitudes of 37° 30' and 40° 3' S, from 900 to 1 700 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 *A. araucana* has a high degree of physiological adaptation to grow in these areas, constituting relic forests originated in the tertiary times (Montaldo, 1974).

The weather, climate and soil of volcanic origin of *A. araucana* 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 on hot summer days.

The purpose of this work was to investigate how seeds and seedlings of *A. 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).

MATERIALS AND METHODS

Seeds material and germination conditions. *Araucaria araucana* (Mol.) Koch seeds were colected 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 min 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°C and with times of exposure of , 2, 3, 5, 7 and 9 h. 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 h 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,

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killed in boiling water for 30 min, was considered 100%. The per cent of leaching was expressed as conductance of leachates after heating × 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 h of imbibition without megagametophyte were presoaked for 3 h 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 μg ml^{-1} 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 h 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 h. Induction of thermotolerance in embryo axes by exposure to sublethal temperatures. Five groups of 15 embryo axes each, coming from seedlings of 120 h of imbibition were pretreated for 3 h 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 min in separate experiments. Each group was then subjected to 44°C (lethal temperature) for 2 h. After treatment at 44°C, the embryo axes were changed to solid medium and kept at 28°C for 48 h. After this time the recovery of growth was determined by measuring the length of the axes.

Incorporation of [^{35}S]methionine. Seedlings of 120 h 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 1 cm. 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 h in aerated liquid TM. [^{35}S]methionine with a specific radioactivity of 3.42 MBq mmol^{-1} (from ICN Radiochemicals) was added to the media, to obtain a concentration of radioactivity of 1.48 MBq ml^{-1}. The [^{35}S]methionine was added to the final 30 min of 2 h of temperature treatment in order to determine the proteins synthesized under heat shock, or added at the beginning of the 2 h 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 min at the end of the period of exposure at 36°C (30 min, 1, 2, 3, 4 or 5 h).

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-HI (pH 6.8) and containing 2% of sodium dodecylsulfate (SDS), 10% glycerol and 5% mercaptoethanol. The pieces of tissue were homogenized with a glass homogenizer and maintained for 2 min in water bath at 100°C, the homogenate was then centrifuged in an Eppendorf microfuge for 3 min. The incorporation of [^{35}S]methionine was determined in the proteins of the supernatant. For this, the proteins of the extract were precipitated with trichloroacetic acid (TCA) as it has been described by Mans and Novelli (1960), taking aliquots of 10 μl and placed on a disk of filter paper Whatmman 3MM 1.4 cm in diameter. The paper was dried for 20 s. Each paper disk was soaked for 60 min in 10% TCA containing 0.1 M cold methionine. The disks were washed with 10% TCA at room temperature for 15 min followed by a second wash with 5% TCA at 90°C for 30 min. After the TCA washes, the disks were passed through an ethanol ether solution (1:1, v/v) for 30 min at 37°C, washed in pure ether for 15 min 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 4 g l^{-1} of 2,5-diphenyloxazole, (PPO) and 50 mg l^{-1} 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 0.01 M Tris. A radioactivity of 50 Bq μg^{-1} 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 μg of proteins with a total radioactivity of 2,500-5,000 Bq. The electrophoresis was run for 7 h 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

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with 10% acetic acid and 50% methanol for 4 h 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% acetic and 40% ethanol for 30 min each wash. Afterwards, the gel was submerged in solution of 0.4% PPO, 20% ethanol, 30% xylol and 50% acetic acid for 3 h. The PPO was precipitated into the gel with distilled water for 2.5 h. 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

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 h. These three tissues are affected differently in their viability by exposure to high temperatures. Figure 1 shows that the embryo axes significantly lose their viability compared with the control experiment, after 5 h 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 h. The megagametophyte tissue is the most resistant. Its viability is only affected 17% (compared with the control samples) after 7 h of treatment at 44°C, and 30% after exposure at 48°C for the same period of time.

Seedlings 120 h after imbibition, with a root length between 1.5-.0 cm, were subjected to temperatures of 28, 32, 36, 40 and 44°C for periods of 30 and 60 min. After the temperature exposures, the seedlings were returned for 48 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 min 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 min at 32, 36 and 4p°C.

A thermal treatment for 60 min at 36°C decreaes 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 *A. araucana*. The lethal temperature is therefore 44°C and the sublethal temperatures are 32, 36 and 40°C.

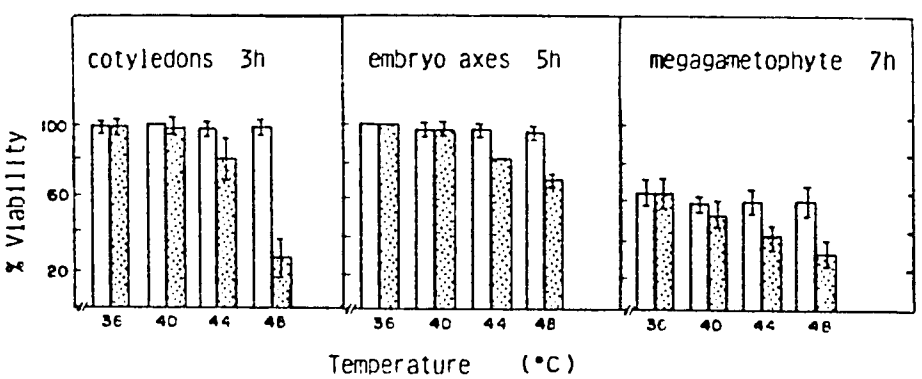

Figure 1. *Viability of the different tissues of quiescent seeds of* Araucaria araucana. The results are shown at the minimum period of time (3 h for cotyledons, 5 h for embryo axes and 7 h for megagametophyte) at which there is a significant decrease of viability by exposure to high temperatures. The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

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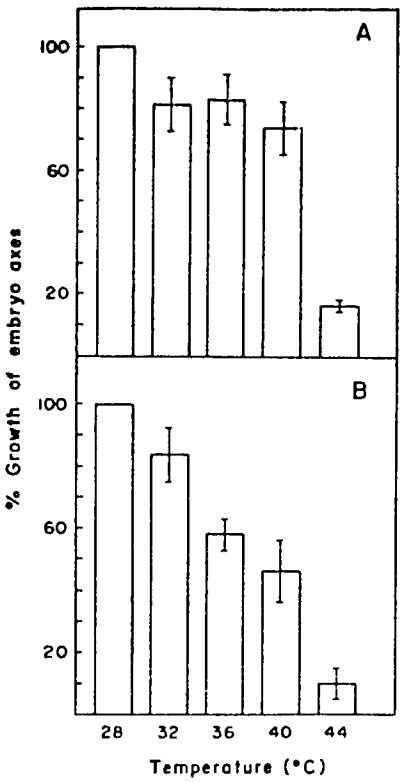

Figure 2. *Growth recovery of embryo axes of* Araucaria araucana. After 30 min of thermal exposure in A or after 60 min of thermal exposure in B. The recovery is given as per cent of growth of the control embryo axes maintained at 28°C. The stress temperatures were 32, 36, 40 and 44°C. After the thermal treatment the axes were placed at 28°C for 48 h and the length of the embryo axes were measured. The standard deviation is given for each bar.

Protein pattern of embryo axes after 2 h of heat shock

The protein pattern present in embryo axes of *A. araucana* at 28°C and under treatment of 2 h 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 of the tissue in the presence [^{35}S]methionine during 2 h 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 min of the 2 h of exposure at the appropiate temperatures. Figure 3 is a fluorography of a SDS-PAGE

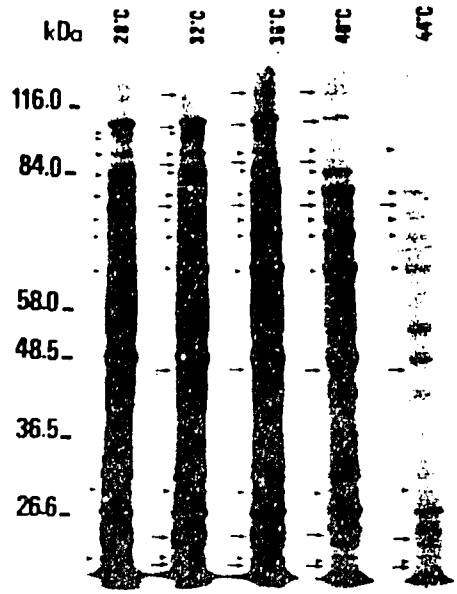

Figure 3. *Fluorogram of the proteins accumulated in embryo axes under heat stress.* Proteins accumulation correspond to 2 h of incubation at different temperatures in the presence 1.48 MBq ml^{-1} of [^{35}S]methionine used as precursor of protein synthesis. Each experimental group was of six embryo 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.

of the proteins accumulated in 2 h 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 relative molecular masses of 126, 106, 89, 77, 44, 24 and 19 kDa. These new proteins present under heat shock have been denominated heat

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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 those 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 temperatures of 32, 36 and 40°C. Figure 3 also shows that most the accumulated proteins decline at 44°C.

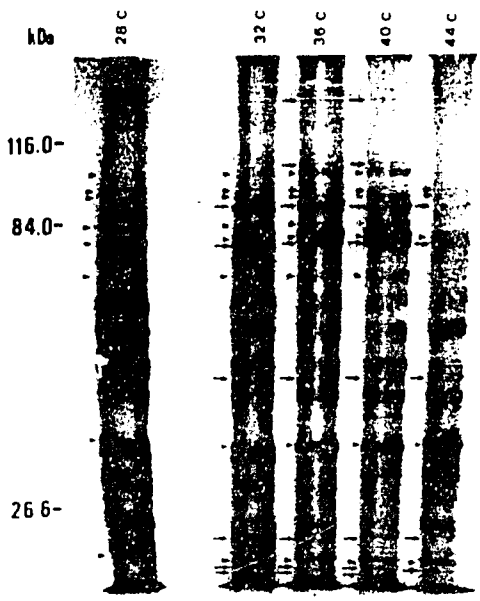

Figure 4. *Fluorogram of the proteins synthesized in embryo axes under heat stress.* The synthesis of proteins correspond to the last 30 min of 2 h of incubation at different temperatures in the presence of 1.48 MBq ml^{-1} of [^{35}S]methionine used as precursor of protein synthesis. Each experimental 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 4 is a fluorogram of the proteins synthesized during heat shock, since the [^{35}S]methionine was incorporated in the last 30 min of the 2 h of thermal exposure. It shows that the same 7 bands of proteins accumulated in 2 h of treatment are also synthesized in the last 30 min of thermal stress. Because in this fluorogram the bands are seen better resolved, it is possible to detect one additional band synthesized under stress. This band has a molecular mass 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.

Table 1. *Protein synthesis and relative intensity of the radioactive protein bands of embryo axes subjected to thermal stress.* The molecular mass 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 figure 4. +, relative intensities of the radioactive bands. The intensity is compared among the bands of the same protein.

Molecular Mass (kDa)	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 mass of the proteins bands (hsp) not present at 28°C.

Kinetic of appearance of the hsp in embryo axes

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 min to 5 h) in the presence of [^{35}S]methionine added for 30 min at the end of

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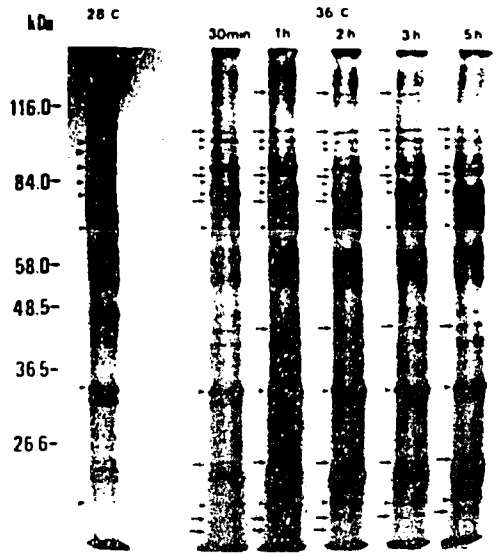

Figure 5. *Kinetics of proteins synthesis in embryo axes.* The embryo axes were subjected at 36°C for different periods of time (from 30 min to 5 h). Groups of 6 embryo axes were incubated at 36°C in the presence of 1.48 MBq ml^{-1} of [^{35}S]methionine which was added to the medium in the last 30 min 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.

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 min of heat shock at 36°C (106, 89, 77, 2, 19 and 17 kDa proteins). After 1 h, 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 h after heat shock initiation. After 3 h of heat shock the proteins 77 and 17 kDa are not longer present. After 5 h 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

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 *A. araucana*. Figure 6A shows the result of induction of thermotolerance in embryo axes after incubation for 30 min 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 h at the lethal temperature of 44°C. However, the recovery of growth after 120 min exposure at sublethal temperatures, followed of a 44°C heat shock for 2 h, 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 h to 40°C, prior to exposure at 44°C, recover growth in only 24% (*fig.* 6A). This growth is still less than the growth recovery of embryo axes subjected to 28°C before heat shock for 2 h at 44°C.

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

Table 2. *Kinetics experiment of proteins synthesis by embryo subjected to* 36°C and relative intensity of the radioactive proteins bands. The molecular mass 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 min to 5 h), as seen in figure 5. +, relative intensities of the radioactive proteins bands. The intensity is compared among the bands of the same protein.

Molecular Mass (*kDa*)	Control	30 min	1h	2 h	3 h	5 h
126*	–	–	+	+	+	–
106*	–	+	+ +	+ +	+ +	+ +
10	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
98	+	+	+ + +	+ + + +	+ + + +	+ + + +
94	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
89*	–	+	+ +	+ +	+ +	+
84	+	+ +	+ + +	+ +	+ +	+
80	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
77*	–	+ +	+ + +	+ +	–	–
69	+	+	+ +	+ +	+ +	+
44*	–	+	+ + +	+ + +	+ +	+ +
33	+	+ +	+ + +	+ +	+ +	+ +
24*	–	+	+ +	+ +	+ +	+
20	+	+ +	+ + +	+ + +	+ + + +	+ + + +
19*	–	+ +	+ + +	+ +	+	+
17*	–	+ +	+ + +	+ +	–	–

* Molecular mass of the proteins bands (hsp) not present at 28°C

DISCUSSION

The three main tissues of *Araucaria araucana* seeds respond differently to high temperatures. The

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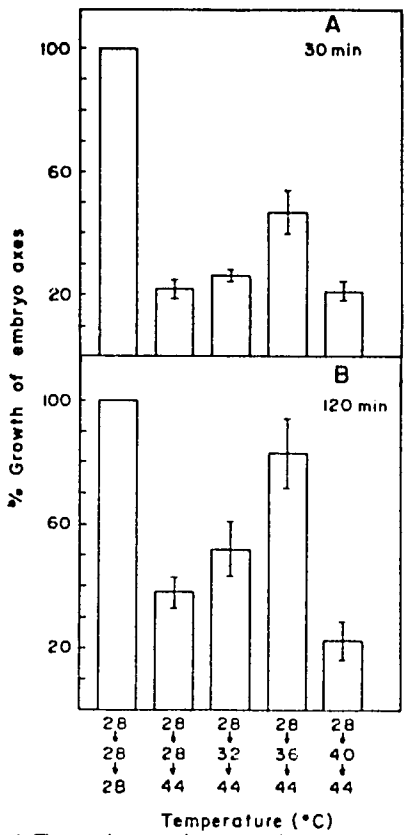

Figure 6. *Thermotolerance induction in embryo axes of* Araucana araucana *by exposure of the tissues to sublethal temperatures.* The sublethal conditions were temperatures of 32, 36 and 40°C for 30 min in A and for 120 min in B. After the treatment to the sublethal temperatures, the embryo axes were subjected to 2 h 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 h at 28°C after the lethal temperature treatment.

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 the megametophyte 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 *et al.*, 1983).

The *A. 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 h. At this age the seedlings are greening and most of the reserve stored in the embryo has been consumed with a considerable increase in fresh weight (Cardemil and Reinero, 1982; Reinero *et al.*, 1983; Cardemil and Varner, 1985).

The experiments of thermotolerance performed with seeds and seedlings of *A. 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 *A. araucana* seedlings as in any other living system because organisms acquire thermotolerance after exposure to sublethal conditions and therefore can resist lethal temperatures, (Lin *et 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: (*a*) The heat shock proteins are synthesized under thermal stress, condition that most often decreases synthesis of normal proteins. (*b*) The hsp are synthesized at sublethal temperatures and when the organism acquires thermotolerance. (*c*) The hsp are transient because they disappear under continuous heat shock or when the organism is put back to normal temperature. (*d*) There are hsp of high, medium and low molecular masses, these last ones being peculiar to the plant kingdom.

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In seedlings of *A. araucana* eight hsp were detected and they fit the hsp characteristics mentioned above. Among the hsp of *A. araucana* there is a prominent protein of 89 kDa. A protein of identical molecular mass has been reported as hsp in chicken embryos by Schlesingger *et al.* (1982) and it seems to be related to glycolysis; it has been suggested that it could be a phosphatase or a phosphtransferase (Schlesinger, 1985).

There are two heat shock proteins in *A. araucana* seedlings which are good condidates 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 not 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 it 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 *A. 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 *A. araucana* two other proteins of high molecular mass, one of 126 and other of 106 kDa. In corn, proteins of molecular mass 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 *A. araucana* three hsp of low molecular masses 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; Kimpel and Key, 1985).

Ubiquitin, a conserved and universal low molecular mass peptide of 8 kDa has been reported to be a heat shock protein, since it increases 6-fold under thermal stress (Bond and Schlensinger, 1985; Vierstra, 1987). Under the SDS-PAGE conditions used for protein analyses of *A. araucana* the ubiquitin can not be detected, but the peptide seems to be expressed in *A. 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 *A. 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 mn of heat shock. Very possibly, these proteins are expressed before 30 min, at a level that is not easy to detect by electrophoresis.

It is also important to point out that the systhesis of normal proteins in embryo axes of *A. 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 *A. 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 *A. araucana* demonstrated that 36°C is also the optimum temperature for induction of tolerance to resist lethal temperatures.

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IN VITRO MULTIPLICATION OF PINEAPPLE (ANANAS COMOSUS L MERR.) USING AXILLARY BUDS FROM THE CROWN.

Veronica Brooms. National Agricultural Research Institute, Mon Repos, East Coast Demerara, GUYANA.

Studies were conducted to develop a system for the rapid *in vitro* multiplication of pineapple shoots. Dormant axillary buds, 4-6mm in diameter, excised from the stem region of the crowns of the pineapple cultivar Montserrat were used.

The results show that buds which were initiated in liquid or on agar-solified MS medium supplemented with various concentrations of NAA, BAP and/or GA3 could be induced to form multiple shoots. At bud burst, i.e. approximately 8 weeks after initiation, buds were transferred to liquid medium for shoot multiplication. Apical meristems, in contrast, began shoot multiplication earlier than the axillary buds.

Generally, shoots were produced at the rate of 8-10 every three weeks. Some cultures which used shoots older than six weeks produced up to five shoots per explant weekly. During shoot multiplication, cultures were maintained either non-shaking or with continuous aeration. Shoots remained green during culture and were transferred to agar-solidified medium prior to weaning. Shoots were rooted either during *in vitro* culture or at the weaning stage.

GENE EXPRESSION TO THERMAL STRESS IN TWO NATIVE TREES OF CHILE: ARAUCARIA ARAUCANA AND PROSOPIS CHILENSIS.

Liliana Cardemil, Claudio Goycoolea and Consuelo Medina. Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

Araucaria araucana constitutes temperated relic forests of high mountains in the austral region of South America with differences of temperatures between day and night and between seasons of 20°C. *Prosopis chilensis* is a leguminous tree of the arid and semi arid from northern and central Chile. In these regions the temperature reaches over 60°C at noon of Summer days. By performing heat shock experiments, we have investigated how seeds and seedlings of these trees respond to high temperature and how they acquire thermotolerance when exposed to sublethal temperatures.

Indeed, the main purpose of this research has been to test the hypothesis that "Native trees are adapted to their natural environment because they have a high degree of expression of those resistant genes to stand harsh environmental conditions. The high degree of expression could be due to a high number of genes conferring resistance or due to regulatory mechanisms of gene function".

SDS-Polyacrylamide gel electrophoresis of the synthetized proteins under thermal stress and in the presence of ^{35}S-methionine, Western blot analyses using polyclonal antibodies against heat shock proteins (hsp) and Northern hybridization analyses using probes for the 70 kD hsp and for the ubiquitin have allow us to test the above hypothesis as well to select for high resistant individuals in the natural populations of these trees.

AID Grant #DPE 5542-G-SS-8073-00 and FONDECYT 0160/88.

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24 **RESPONSE TO WOUND STRESS BY SEEDLINGS OF ARAUCARIA ARAUCANA AND PROSOPIS CHILENSIS.**

Liliana Cardemil, Alejandro Riquelme and José Gregorio Rodríguez. Departamento de Biología Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

Tissues of seedlings of *Araucaria araucana* and *Prosopis chilensis* respond to wounding by increasing from 10 - 20 folds the basal level of cell wall proteins.
Tissue immunoprints of the different organs present in seeds and seedlings of *A. araucana* and *P. chilensis* using polyclonal antibodies raised against hydroxyproline-rich glycoproteins (HRGP's, extensins) present in cell walls of carrot roots or soybean cell walls.
The tissue immunoprints also reveal that in *A. araucana*, the megagametophyte, root tips and resin canals of the cotyledons, are the tissues which show higher expression of extensin-like cell wall proteins. In *P. chilensis*, the vascular bundles of the cotyledons and of the hypo and epicotyls are the tissues showing a higher expression for these proteins.
SDS-polyacrylamide gel electrophoresis of the cell wall proteins followed by Western blot analysis reveals that are 3 cell wall proteins *A. araucana* and 4 in the case of *P. chilensis*, immunocross react with carrot and soybean extensin antibodies. Some of these proteins can run in their native forms under conditions of cationic neutral gel electrophoresis indicating a basic nature of these proteins.
In *A. araucana* two of the cell wall proteins have been purified and further characterized for amino acid composition and for NH_2 terminal sequences. Two proteins have peroxidase activity.
AID Grant BDPF 5542-G-SS-8073-00 and FONDECYT 0160/88.

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