ANNUAL REPORT


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

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PRELIMINARY REMARKS

This report corresponds to the third year of research of the above grantee, from the period August 31, 1990 to August 30, 1991.

The project has been extended for six additional months under the request from the principal investigator to the PSTC/AID Program, due to the fact that the project begun six months after the starting date. Therefore, the final report of the project will be handle in to AID after February 28, 1992. Copy from the AID letter adressed to Dr. Cardemil is enclosed with permission to extend the project.

Reprints of two publications with the results of the research performed under this grant are enclosed. A new manuscript has been recently submitted.

We have spent the amount of US $ 8,509 in the last semester and US $ 18,070 in the third year of research. We are planning to spend US $ 9,940 in the next six months.
SECTION I

Note on Publications:

Reprints of two publications are enclosed. One of them is "Expression of heat shock proteins in seeds and during seedling growth of *Araucaria araucana* as a response to thermal stress" from the authors Claudio Goycoolea and Liliana Cardemil. It was published in the Plant Physiology and Biochemistry 29: 213-222. The other is "Expression of cell wall proteins in seeds and during seedling growth of *Araucaria araucana* is a response to wound stress and developmentally regulated" from the authors Liliana Cardemil and Alejandro Riquelme. It was published in the Journal of Experimental Botany 342: 415-421.

A new manuscript entitled: "*Prosopis chilensis* is a plant highly tolerant to heat shock" from the authors Consuelo Medina and Liliana Cardemil, has been recently submitted to AMBIO journal. A copy of this manuscript is 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 has been performed with two native trees of Chile: *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 have allowed us to test the hypothesis that: "Native trees are better adapted to their harsh environment because they show a higher 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*) to temperature stress and to injury stress through synthesis of temperature shock proteins (heat shock) and accumulation and synthesis of cell wall proteins (extensin, HRGPs).

2. To investigate the presence of the genes responsible for the synthesis of temperature shock proteins and of the HRGPs and the number of copies of these genes using Southern blot hybridization techniques with 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 the HRGPs by chemical analysis and immunological methods to compare them with known proteins of other groups of plants.
5. To develop histoimmunological methods as a mean to elucidate the cellular locations of these proteins and to study the possible relationship between HRGPs 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 $\alpha,\alpha'$-dipyridyl and the 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 mean 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 and 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 are studying the expression of heat shock proteins (hsp) in natural populations of Prosopis chilensis. We are also studying the expression of cell wall proteins in Prosopis chilensis as a response to wound stress. From these studies we have concluded already that the expression of the cell wall proteins is tissue specific and developmentally regulated. The characterization of
cell wall proteins in Prosopis chilensis constitutes José Gregorio Rodríguez’s dissertation. All of the other objectives are still in progress.

B. Research Accomplishments

Heat shock responses in Prosopis chilensis.

We have pursued our observations and field research in the population of Prosopis chilensis located in the Gorge of San Carlos at the interior of Vicuña (30°02′S-70°44′W, 730 m over the sea).

The climate data have been provided by the Experimental Station of Vicuña of the Instituto Nacional de Investigaciones Agropecuarias (INIA), Tables 1, 2, 3 and 4.

The climate of the studied area has been described as a mediterranean arid climate because of the higher interannual variability of precipitations being the months of June and July with higher probability of rainfall.

The minimum temperature registered last year was -3°C in June and the maximum temperature registered was 38°C in January, 1991.

From May 1990 to April 1991, we performed 25 field trips to the Gorge of San Carlos. The material collected, mainly leaves, is being screened at the laboratory to detect expression of heat shock proteins (hsp).

The foliar temperatures were registered in five individuals (performing two replicates) using thin thermocouples of copper-constantan which were placed on the abaxial face of the
folios. The temperature of the canopies was registered with thermocouples suspended 20 cm over the leaves. The microclimate was registered with soil thermometers and with surface and air thermocouples placed between -7 cm to 100 cm.

Determinations of water potentials (xylem) of the stems and petioles were performed with a nitrogen-pressure bomb. The water potential of the folioles was registered by psicometer chambers connected to a microvoltimeter of Dew Point.

For the pressure-volumen plots, branches from 3 to 5 individuals were cut under water and kept in plastic bags for 24 hours in dark to determine in the Lab the relation between water potential and water content.

The foliar samples for hsp analysis were taken from the same five individuals which foliar temperatures were registered. The leaves removed from the trees were placed immediately on dry ice to be analyzed in the Lab for hsp expression.

Results:

Phenology: Between May 19, 1990 to February 28, 1991 the principal axis, the stem of the tree, increase 0.46 cm in altitud (var. = 0.05), Figure 1. The number of leaves per node (Figure 2) and the number of folioles per leaf (Figure 3) decrease to a minimum number in October. In November there is a fast recovery of them. The minimum values of, foliar area (Figure 4) and foliar area per node (Figure 5) also occur in October and start to increase in November (Figure 6). However, this increment in the area of the folioles during November does not compensate
the previous loss of foliar area and of foliar area per node. At this time, new leaves begin to appear reaching maximum values of leaf areas per node from December to January. The area of the folioles also reaches a maximum during these months.

**Water potential:** The water potential of the xylem of branches decrease at noon time in 20 bars from the middle of the Spring to the Summer, Figure 7. The daily course of the water potential in the stem and in the folioles shows minimum values around midday,. Figures 8, 9, and 10. In all of them the mid-day values are between -70 and -90 bars, while the minimum water potentials of the branches are between -35 and -50 bars during the same days. Interesting, the water potentials of the folioles show a second decrease during the sunset hours of those days, reaching values between -60 and -75 bars. This second decrease does not occur in the branches. The maximum values for both water potentials (folioles and branches) become equal during the sunrise hours with values of -30 bars. Figure 11 shows a daily time course of the water potential of the branch and leaf (petiole) xylems. The average water potential of the petioles were 20 bars higher than those shown in the branches during the afternoon hours. In both cases the water potentials decrease at noon but only in the case of the leaves there is a second decrease during the sunset hours.

These results suggest the presence of an active mechanism to keep the water potentials less negatives in the leaves respect to the branches. Such mechanism could be inhibited in the dark or by lower temperatures. This inhibition is under investigation in the field and in the Lab.

**Foliar temperatures:** The foliar temperatures follow the
same fluctuations as the air temperature in the canopy. The maximum temperature values become over 30°C and take place between noon and 4 PM. The minimum temperatures are from 7°C to 14°C and take place from 4 to 7 AM (Figures 12, 13 and 14). Close to midday, the foliar temperatures are lower in 2°C compared with those of the air.

Microclimate: During the day hours of February, the maximum temperatures registered in the soil-air phase were 55°C at the soil level (Figure 15 c) and always higher than in the air taken at 10 cm (Figure 15 a) and at 50 cm (Figure 15 b). The temperatures beneath the soil surface were also higher than those of the air, 43°C at -2 cm (Figure 15 d), 35°C at -5 cm and 32°C at -7 cm (Figure 15 f).

Other results: We are in the process of performing research and obtaining other results based on the observations and on the field studies.

a.- Time course of expression of heat shock proteins.
b.- Anatomical studies of the connecting tissues branch-petiole.
c.- Pressure-volumen plots in petioles and branches.
d.- Carbon isotopes analysis of leaves (performed at the University of Hawaii).
e.- Time course of photosynthesis and transpiration.
f.- Fenological studies of the reproductive phase.
Wound Stress Responses in *Prosopis chilensis*.

Three different classes of tissues of seedlings of *P. chilensis* were subjected to wound stress with cuts performed with razor blades: cotyledons, hypocotyls and root tips. Seedling hypocotyls of 24 hours after the start of imbibition were the organ showing a higher response to wounding with an increase in cell wall protein expression 24 hours after the damage was performed. The increase in the expression was detected by quantification of the protein content in the walls and by tissue immunoprints using polyclonal antibodies originated against extensin from soybean seed coats or against extensin from carrot roots.

The expression of the extensin protein in normal tissue cell walls of seedlings (non damaged) can also be detected by tissue immunoprints because the protein is developmentally expressed. In the three organs, the tissues which show a higher expression of the extensin protein are the vascular bundles (Figure 16 A and B).

The cell wall proteins were analyzed by SDS-polyacrylamide gel electrophoresis and by cationic neutral gel electrophoresis of the native proteins, followed by Western analysis using the same antibodies as for the tissue immunoprints. The results of the analysis showed that the pattern of the proteins present in the cell walls of cotyledons changes after wounding. Four proteins are present in the wounded tissue (not present in the unwounded tissue) and cross react with the antibody (Figure 17 A and B).
Furthermore, *Prosopis chilensis* has an exceptional response to wound stress. Seedlings of 24 hours of imbibition differentiate secondary roots when the main root is wounded, Figure 18.

We are trying to localize those cells of the vascular bundles with a higher expression for extensin like proteins. For this, José Gregorio Rodríguez, a master degree student from Venezuela is performing histoimmunohistochemistry for electron microscopy.

In the previous Management Report, we have said that we need urgently to develop a method to label DNA and RNA probes with non radioactive nucleotide precursors.

For this, Professor Cardemil, visited for one month Dr. Elena del Campillo's Laboratory in order to perform in situ hybridization using "the ingenious kit" method to label a RNA probe containing genetic information for extensin.

However, due to the fact that we had the extensin gene in the original vector pDC5A1 from Varner's Lab (Chen & Varner, 1985) we needed to subclone the extensin gene into pBluescript II KS (+/-) to be able to get the RNA probe for histochemical localization of the mRNA for this protein.

The pBluescript II KS (+/-) is a 2961 base pair phagemid derived from pCU19. The KS designation indicates the polylinker is oriented such that the Lac Z transcription proceeds from KpnI to SacI. The polylinker of the plasmid is flanked by the T3 and T7 promoters. Therefore, any insertion in the many restriction sites of the polylinker region can be copy from either one strand using the T3 RNA polymerase or the T7 RNA polymerase. The
pBluescript II KS (+/-) phagemid has the ampicillin resistant gene for antibiotic selection and the Lac Z which provides an α-complementation for blue/white color selection of recombinant phagemids.

Since the extensin gene has four XbaI restriction sites and one PstI site as can be seen in Figure 19, from Chen and Varner, 1985, we decided to cut the pDC5Al with EcoRI and PstI to generate a fragment of 3447 bases which contains the encoding region of the gen. The fragment was next inserted in the pBluescript II KS (+/-) that was also digested with EcoRI and PstI. The extensin fragment was then ligated in to the EcoRI-PstI ends of the phagemid in such a way that the PstI end of the extensin which is the 5'end became oriented towards the T₇ promoter and the EcoRI end, which is the 3'end, became oriented towards the T₃ promoter. Because the RNA polymerase reaction always occurs from the 5'to the 3'end, the polymerase that has to be used for transcriptional copy is the T₇ RNA polymerase.

For the ligation reaction, the extensin fragment of 3447 bases and the pBluescript fragment, were purified preparatively from the agarose gel. The purification of the fragments was performed by electroblotting the DNA bands on S&S NA45 DEAE membranes followed by elution of the DNA from the membranes with NET high salt buffer, at 4°C overnight. The ligase reaction was performed overnight according to standard protocols (Sambrook et al., 1989) and the DNA product of ligation was purified with phenol-chloroform. After purification the DNA (pBluescript II KS (+/-) containing the extensin fragment) was used to transform competent bacterias (Sambrook et al., 1989). The transformants
were selected on LB agarose media containing ampicillin and suplemented with 20 ul of X gal-IPTG (isopropylthio-β-D-galactoside) in formamide for color selection of the transformants. The DNA of the blue colonies was analyzed for restriction fragments. This analysis finally confirm the subcloning of the extensin information in the phagemid.

References.


Figures Legends.

Figure 1. Growth in length of the principal axis of the plant during a period of 12 months, from May 1990 to April 1991.

Figure 2. Variation in the number of leaves per node in a period of 12 months, May 1990 to April 1991.

Figure 3. Variation in the number of folioles per leaf in a period of 12 months, May 1990 to April 1991.

Figure 4. Variation in the foliar area during 12 months, from May 1990 to April 1991.

Figure 5. Variation in the foliar area per node, from May 1990 to April 1991.

Figure 6. Variation in the area of the foliole, from May 1990 to April 1991.

Figure 7. Variation in the water potential of the branch xylem, from October 1990 to March 1991.

Figure 8. Daily time course of the water potential of the folioles and of the branches, registered November 1-2, 1990.
\[\text{o, branches.}\]
\[\text{--m--, folioles.}\]

Figure 9. Daily time course of the water potential of the folioles and of the branches, registered December 26-27, 1990.
\[\text{o, branches.}\]
\[\text{--m--, folioles.}\]

Figure 10. Daily time course of the water potential of the folioles and of the branches, registered January 29-30, 1991.
\[\text{o, branches.}\]
\[\text{--m--, folioles.}\]

Figure 11. Daily time course of the xylem potential of the petiole and of the branch, registered February 18, 1991.
\[\text{o, branches.}\]
\[\text{--m--, petioles.}\]
Figure 12. Temperature variation in the leaves and in the air in a period of 24 hours, registered November 1-2, 1990.
--.--, air temperature.
_0_, leaf temperature.

Figure 13. Temperature variation in the leaves and in the air in a period of 24 hours, registered December 26-27, 1990.
__-_ _, air temperature.
__o_, leaf temperature.

Figure 14. Temperature variation in the leaves and in the air in a period of 24 hours, registered January 29-30, 1991.
___- _, air temperature.
__o_, leaf temperature.

Figure 15. Microclimate variation. Variation of temperature in the air-soil gradient in a period of 24 hours of a Summer day, February 1991.

a. 10 cm over the soil.
b. 50 cm over the soil.
c. soil temperature.
d. 2 cm beneath the soil surface.
e. 5 cm beneath the soil surface.
f. 7 cm beneath the soil surface.

Figure 16. Tissue immunoprints of a cotyledon transversal section of Prosopis chilensis seedlings of 60 hours of age. The transversal section of cotyledons were pressed for 30 seconds on a nitrocellulose membrane. The membrane was incubated with rabbit polyclonal antibodies raised against extensin of soybean seed coats and next incubated with a goat anti-IgG conjugated with alkaline phosphatase.

In A: Tissue immunoprint of a cotyledon transversal section. HV1 is a principal vascular bundle and HV2 are the secondary vascular bundles.
In B: Hand-free transversal section stained with methylene blue. HV1 is a principal vascular bundle and HV2 are the secondary vascular bundles.
Figure 17. Gel electrophoresis of the native cell wall proteins of seedling embryo axis of *Prosopis chilensis*.  
In A: The gel was stained with silver nitrate.  
In B: Western analysis of the electrophoresis shown in A. The proteins of the gel were electrotransferred to a nitrocellulose membrane and incubated with a first rabbit antibody raised against extensin from soybean seed coats and then incubated with a second antibody, a goat anti-IgG conjugated with alkaline phosphatase. The arrows point the equivalent bands of proteins in A and B.

Figure 18. Wound effect produced with razor blade cuts on seedling hypocotyls of *Prosopis chilensis*, of 24 hours of imbibition. One day after wounding, the radicle of the seedlings differentiate secondary roots as a response to the wound stress.  
D: Damaged seedlings  
ND: Non-damaged (control) seedlings  
The arrows point the induced secondary roots. The damaged seedlings are almost 2.5 longer than the control seedlings.

Figure 19. Restriction map and sequence analysis of the gene encoded in the pDC5A1. Note the four XbaI sites and one PstI site flanked by the two EcoRI sites of the genetic information proline-rich, Chen & Varner, 1985.
C. Scientific Impact and Collaboration

Professors Joseph Varner and David Ho from the Department of Biology, Washington University, St Louis, Missouri will visit Chile in October 15, 1991 to discuss part of the project with Professor Cardemil. Both Professors are consultants in this project. They will advise on the project and will revise manuscripts for publications.

D. Description of the Project Impact

So far we are still hoping that during this Summer (December, January and February) to get the physiological data from the field research and to be able to know how the Prosopis trees are responding in their natural environment to temperature and to wound stress. We are working very hard on that.

However, it has been difficult to perform the molecular analysis due to the fact that the plant extracts from adult trees are contaminated with resins and oils. So we are in the process of purifying the plant extracts.

Because the field research with Prosopis has become extremely interesting due to the fact that Prosopis chilensis seems to be highly resistant to heat shock, Professor Francisco Squeo, who is collaborator in this project and Professor Cardemil, are in the process of submitting other proposal to the National Geographic Society on field research with this fascinating tree.

Another similar project in Phaseolus vulgaris has been already submitted to the BID (Banco Interamericano para el
Desarrollo). This project is proposing to select *Phaseolus* cultivars which are resistant to heat shock and to water stress and particularly, other colleagues from Universidad de Chile will be training in Dr. Cardemil's Laboratory with methods and expertises acquired and improved with the A.I.D. project.

**E. Strengthening of Developing Country Institutions**

Another degree dissertation, with research performed under this project, is ready to be evaluated by a scientific committee at the Universidad de Santiago de Chile. The student is Alejandro Riquelme, a candidate to Biochemist Degree who performed a wonderfull research on the characterization of two cell wall proteins expressed in *Araucaria araucana* seedlings under injury stress. He will get now the Degree in Biochemistry and the results obtained during this research will allow him to get a Master Degree in Science very soon.

A copy of the cover and abstract of Alejandro Riquelme's dissertation is enclosed. Part of his results has been already published in the Journal of Experimental Botany (a print of the article is included). We are planning to get two other publications out of his work.

Four other students are still being trained under this grant.

Professors Varner and Ho will stay in Chile for 15 days when coming to visit Dr. Cardemil's Lab. During this period, they will teach a graduate course in the Facultad de Ciencias for 15
students from Master Degree and Ph.D., programs. Two of these students will be from Perú which is one of the countries in South America which most needs to develop plant science. The money to bring these two students from Perú was kindly given to us by the Red Latinoamericana de Botánica (RLB).

Professors Varner and Ho will also participate in a Symposium on Plant Stress Physiology which will be held in the VIII Reunión Nacional de Botánica of la Sociedad de Botánica de Chile.

F. Future Work

In the six months still left, we are hoping to complete the screening of *Prosopis chilensis* to select thermotolerant and injury resistance individuals. We are also expecting to publish the phenological data collected for *Prosopis chilensis* in the Gorge of San Carlos, IV Región.
SECTION II

A. Managerial Issues

In our last Management Report we said that we needed urgently to develop a method to label DNA and RNA probes with non radioactive nucleotides. In order to learn the Ingenious Kit Method, Professor Cardemil visited Dr, Elena Del Campillo at the Department of Plant Biology, Berkeley, University of California. However, because Professor Cardemil had to return to Chile due to her husband suffered a heart stroke, she could only subclone the extensin gene in the Bluescript phagemid. We are planning to try and get the method in a near future.

B. Budget

During this 3rd year we have spent US $ 18,070. From this amount, US $ 8,509 is the money spent in the second semester.

Most of the expenses have been in travel and field research. The items reagents and salaries make up the rest. See additional pages for details in expenses. We are planning to spend US $ 9,940 in the next coming semester.
C. Special Concerns

We are still concerned about the time schedule. We hope to be able to complete most of the objectives proposed.

D. Collaboration, Travel, Training and Publication

The collaboration of Dr. Cardemil's Lab. with Professors Varner and Ho has been very productive. We would like very much to continue this collaboration with them in future investigations.

The collaboration with Professor Francisco Squeo from Universidad de La Serena has been also very important to develop the field research on *Prosopis* and both groups are planning to continue this collaborative research on *Prosopis* sp. in the near future.

The data obtained in field research will be presented in an abstract in the VIII Reunión Nacional de Botánica in Santiago, October 16-19, 1991 from the authors: Francisco Squeo, Nancy Olivares and Liliana Cardemil (copy of the abstract is enclosed).

Professor Cardemil is planning to travel to Washington University in St. Louis, Mo and to Berkeley, University of California in the next semester.
Respect to publications, a new manuscript have been submitted to AMBIO, entitled: "Prospis chilensis is a Plant Highly Tolerant to Heat Shock" from the authors Consuelo Medina and Liliana Cardemil.

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

We requested last year to BOSTID to help us to report our results in International Conferences. Indeed The National Research Council, Office of the International Affairs, Board on Science and Technology for International Development (PSTC/BOSTID), invited the principal investigator of the project to participate in the Fourth Conference of the International Plant Biotechnology Network hold in San José Costa Rica. We had two abstract in this conference and a day after the conference all the PSTC grantees made enjoyable presentations of their projects. Dr. Jill Conley was the organizer. She also came to visit Chile last August to evaluate the progress of the A.I.D./PSTC grant research. Dr. Jill Conley is apparently organizing another similar conference for all the A.I.D. grantees working on plants. We are expecting to attend the Conference.
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Figure 16

[A] E

HV1

HV2

[B]

HV1
Fig. 2. Restriction map of pDC5A1 insert, containing a carrot extensin gene. Restriction endonucleases are abbreviated as follows: R: EcoRI, P: PstI and X: XhoI. Relative positions of the long and short transcripts of pDC5A1, and homologous region of cDNA clone pDC5 to pDC5A1 are indicated. Subclones are diagrammed showing the region of pDC5A1 insert they contain along with the vector which was used in the construct.

Fig. 3. DNA sequence of pDC5A1. The inserted DNA of pDC5A1 was subcloned into M13 phage vectors and sequenced according to methods described by Barnes et al. (1983). Only the first 2230 bp of pDC5A1 insert (4 kb) was sequenced. The sites of the two RNA transcripts, an 18-bp direct repeat, the intron splicing sites and the polyadenylation sites are indicated. Sequences similar to the CAAT box and TATA box and polyadenylation signal sequences were underlined with **. The predicted amino acid sequence along with the putative signal peptide cleavage site is also shown.
RESPUESTA A ESTRES MECANICO EN SEMILLAS DE Araucaria araucana (MOL.) KOCH.

Tesis presentada a la Universidad de Santiago en cumplimiento parcial a los requisitos para optar al grado de Licenciado en Bioquímica

por

ALEJANDRO ANDRES RIQUELME ESCOBAR

Prof. Patrocinante: Mg. Francisca Masardo.
Prof. Tutor: Dra. Liliana Cardemil O.

CHILE
RESUMEN

En la naturaleza las plantas están sujetas frecuentemente a sufrir daños por heridas causados por insectos, animales herbívoros, hongos y otros agentes patógenos. Se ha visto que las plantas responden a dicho estrés con la expresión de algunas proteínas específicas.

Semillas de Araucaria araucana responden al daño causado por heridas (ya sea después de 24 ó 72 horas) con un aumento de 1.5 veces los niveles de proteínas asociadas a la pared celular. Una clase de estas proteínas son las peroxidasas cuya actividad es incrementada de 2 a 50 veces, en un tiempo de 24 a 72 horas después de provocado el daño.

Analisis por SDS-PAGE de las proteínas presente en la pared celular de semillas enteras de A. araucana, reveló la presencia de múltiples proteínas. Las dos proteínas mayoritarias encontradas en las paredes celulares y que aumentan significativamente cuando las semillas son dañadas poseen un peso molecular de 83 y 145 kD.

Estas dos proteínas fueron positiva al test de marcación con dansilhidrazina estableciéndose que se trataban de glicoproteínas. A pH neutro ambas proteínas tienen carga positiva lo que les permite ser analizadas por electroforesis de proteínas nativas en geles
cationicos-neutros. Por su naturaleza catiónica pudieron ser analizadas por cromatoenfoque con gradiente de pH de 11-8.5 determinándos que ambas proteínas poseen un punto isoelectrico de 10.5 ± 0.3.

Centrifugación con gradiente de densidad de CsCl permitió establecer que estas proteínas tienen una densidad boyante de 1.333 g ml⁻¹. Esta densidad no es propia de enzimas de pared más que de glicoproteínas ricas en hidroxiprolina. Proteínas de pared celular con esta densidad han sido descritas con actividad enzimática de peroxidasa. Por esta razón, se quiso detectar actividad peroxidásica en estas proteínas en geles nativos con o-fenilenediamina, como sustrato, encontrándose reacción positiva para ambas proteínas.

Se determinó que las isoperoxidasas de pI 10.5 presente en la pared celular de semillas de *A. araucana* poseen un pH óptimo de 5 para la oxidación de o-PND y tiene un Km app. de 13.6 mM para peróxido de hidrógeno y un Km app. de 3.4 mM para o-fenilenediamina, con una V máx de 525 M o-PNDox.min⁻¹.g⁻¹.

Impresiones de tejido de semillas dañadas en membranas de nitrocelulosa y reveladas con el test de actividad de peroxididasas mostraron que en las regiones heridas incrementa de esta enzima. Además se observó que la expresión de estas peroxididasas en la pared de semillas *A. araucana* están reguladas por el desarrollo y son tejido específico.
Nuestros resultados sugieren que las peroxidasas de pared celular de 83 y 145 kD, son isoformas de una misma proteína y pueden jugar un papel importante en las semillas de *Araucaria araucana* como una respuesta defensiva en tejido dañado.
ABSTRACT

In nature, plants are subjected frequently to suffer damage by wounding caused by insects bites, herbivore animals, fungi and other pathogens. Previous reports suggest that plants respond to the wound stress with expression of specific proteins.

*Araucaria araucana* seeds respond from 24 to 72 hours after wounding with a 1.5 fold increase of the cell wall proteins. One class of the cell wall proteins are the peroxidases which increase from 2 to 50 folds between 24 and 72 hours after of wounding.

Analysis by SDS-PAGE of the cell wall proteins from *A. araucana* seeds, shows many bands of proteins. The two major cell wall proteins that increase upon wounding, have an estimated molecular weights of 83 and 145 kD.

These proteins exhibit positive reaction to the fluorescent labeling with dansylhydrazine and therefore were identified as glycoproteins.

Both proteins are positively charged at neutral pH and could be analyzed by native cationic gel electrophoresis. Because of this cationic nature, the proteins could also be analyzed by chromatofocusing with a pH gradient from 11 to 8.5, these proteins have a pI of 10.5 ± 0.3.

Density gradient centrifugation in CsCl determine a buoyant density of 1.333 g·ml⁻¹ for these
proteins, suggesting that these proteins are enzymes present in the plant cell wall of *A.araucana* seeds, rather than hydroxyproline rich proteins. Proteins with a similar buoyant density have been described as peroxidases. Detection of peroxidase activity in natives gels using o-phenylenediamine and hydrogen peroxide, as substrates, demonstrate than both proteins are peroxidases.

The cell wall peroxidases of pI 10.5 of *A.araucana* seeds, have a optimum pH of 5 for activity tested with o-phenylenediamine, as substrate. Both have a Km app. of 13.6 mM for hydrogen peroxide and a Km app. of 3.4 mM for o-phenylenediamine with a V máx of 525 M of o-phenylenediamine oxidado · min⁻¹ · g⁻¹.

Tissue prints performed in nitrocellulose membranes of wounded seeds show a higher peroxidase activity on the wounded area. Expression of these peroxidases in *A.araucana* seeds seems to be developmentally regulated and tissue specific.

Our results suggest that the cell wall peroxidases of 145 and 83 kD are isoforms of the same protein and may play an important role in *A.araucana*, as a defensive mechanism to the damage caused by wounding.
Trabajo presentado a la VIII Reunión Nacional de Botánica de Chile, 16-19 de Octubre de 1991.


Las poblaciones de Prosopis chilensis de la zona semiárida de la IV Región habitan en un ambiente con alta demanda evapotranspirativa. Esta situación sólo se revierte durante cortas temporadas en los años lluviosos. Adicionalmente, como producto de la camanchaca, existen diferencias marcadas en la HR del aire durante un ciclo de 24 hrs. Dado que los individuos nunca quedan completamente sin hojas, esta especie debería poseer un mecanismo eficiente en el control del potencial hídrico foliar.

La población de P. chilensis estudiada se ubica en la Quebrada San Carlos (Valle del Elqui, 39°02'S, 70°49'O, 520 msnm). Se registraron durante 24 horas, cada dos meses y por un año: el potencial xilemático de rama (PXR) y peciolo foliar (PXP), y el potencial hídrico foliar (PHF). PXR y PXP fueron medidos con una bomba de Scholander, y PHF con una cámara psicrométrica. Para el estudio anatómico de la interfase rama-peciolo, se utilizó la técnica de tinción safranina-verde rápido.

Los valores de potencial menos negativos y más semejantes fueron registrados entre las 5:00 y 7:00 hrs. y los más negativos pasado el medio día. En promedio, PHF presentó una mayor rango de variación (ca., -22 a -48 bar), en comparación con PXR (ca., -21 a -49 bar) y PXP (ca., -17 a -39 bar). El PXR fue siempre ≤ el PXP en todas las mediciones. El PHF fue siempre ≤ PXR y PXP. El estudio anatómico evidencia una discontinuidad en el tejido conductor entre rama y peciolo foliar. Estos resultados permiten postular que P. chilensis es capaz de mantener activamente un PXP ≤ PXR. Una disminución del potencial osmótico en el tejido xilemático del peciolo (aumento de azúcares), permitiría explicar los mayores valores de PXP.

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PHENOLOGICAL AND ECOPHYSIOLOGICAL PATTERNS OF *Prosopis chilensis* FROM NORTH-CENTRAL CHILE

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*Prosopis chilensis* has been extensively and intensively used for firewood and their fruits used to feed domestic animals in the last 500 years. In the present century, increasingly man pressure reduced old big "algarrobales" to small populations. In north-central Chile, algarrobos are found in areas with a high demand of evapotranspiration. This condition is reverted only during short seasons in rainy years and during short daily periods with fog ("camanchaca").

Since June 1990 we are studying the phenological and ecophysiological patterns of *P. chilensis* in a population of Quebrada San Carlos (30°02'S, 70°49'W, 520 masl).

*P. chilensis* is a partial deciduous tree. The total minimum leaf area was found at mid spring (15%). New leaves appeared at the end of spring, and reproductive processes occurred in summer, ending with the fall of fruits at the end of summer. No seedlings were seen in the study site.

Leaf water potential (LWP) showed a greater variability (ca., -22 to -88 bar) than the branch (BXP) (ca., -21 to -49 bar) and leaf petiole xilematic potential (LXP) (ca., -17 to -39 bar). The BXP was always ≤ LXP. The LWP was always ≤ BXP and LXP. The most negative values were recorded after midday. Anatomical studies show a discontinuity of the conductive tissue between the branch and the leaf petiole. These results indicate that *P. chilensis* is able to actively maintain a LXP ≥ BXP. A decrease of the osmotic potential in the petiole xilematic tissue may account for the highest values of LXP.

At midday, with maximum air temperature prevails (ca., 36°C), leaf temperature of *P. chilensis* was always lower, up to 5°C difference. We propose that active maintenance of leaf turgor and leaf cooling are tightly related processes. Next we will study the ecological and ecophysiological factors limiting the germination of seeds and the establishment of seedlings in the field.

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Abstract.

Seeds of *Prosopis chilensis* germinate 100% in 24 hours at temperatures from 25° to 35°C. At higher temperatures the germination slows down. At 50°C, seeds do not germinate. After germination at 25°C, the optimal temperature for seedling growth is 35°C and the seedlings do not grow at a temperature of 50°C. However, when germination is at 35°C, the optimal temperature for seedling growth becomes 40°C and some seedlings grow at the lethal temperature of 50°C, suggesting that thermotolerance is induced during seed germination at 35°C. Further thermotolerance can be induced in seedlings germinated at 35°C and grown at 35°C for 48 hours, if the seedlings are exposed for two hours at 40°C. Under these conditions, seedlings increase the growth rate at 45°C and at 50°C. Fluorography of SDS-polyacrylamide gel electrophoresis of the proteins synthesized and accumulated during two hours at temperatures of 35°, 40°, 45° and 50°C and in the presence of [35S]methionine, reveals the expression of eleven new proteins. Most of the proteins present at 35°C also increase in expression. The most prominent proteins accumulated during stress are of molecular masses of 100, 80, 71, 69 and 60 kDa, they are strongly expressed at the lethal temperature of 50°C. We have concluded that *Prosopis chilensis* is a plant with a thermotolerance higher in about 5°C compared with other plants.
Introduction.

The greenhouse effect due to the increase of CO₂ content in the atmosphere along with the rapid deforestation of earth, are present problems. They have created the need for a rapid search of living organisms, among them plants, with high adaptability to the present environmental conditions. These adapted plants can grow and propagate under harsh conditions such as high or low temperature, drought stress, high salinity, high level of chemicals, etc. These plants could be also considered as gene banks. Therefore, the overall planet's environmental problems have urged scientists to look for genetic variability among native plants, which have not been selected by men and which can tolerate stress conditions. The scientists have also looked for methods to propagate and conserve these plant species.

For many years botanists have thought that Prosopis species are some of these native resistant plants to environmental stress, because they are present in arid areas, subjected to extreme temperatures and growing on salty soils, (1, 2).

P. chilensis is a leguminous tree with a high degree of genetic variability among the individuals of natural populations, due to its high embreeding by cross pollination.
and to its wide geographic distribution. Natural populations of *Prosopis chilensis*, forming small monospecific forests, are in the arid and semi-arid regions in Chile. The species has a geographic distribution from Copiapó at the North (27°S), to Santiago at the South (33°S), and from the Central Valley (West) to 2,400 m altitude in the Cordillera de los Andes (East). In these regions daily temperatures go from 6°C in the night to 50°C in the afternoon of Summer days.

In spite that *P. chilensis* has been considered a resistant plant to heat stress, there is not evaluation so far of the physiological and molecular responses of these trees under this stress. No such evaluation has been reported for any other *Prosopis* species!

For the past three years, we have been working on the physiological and molecular responses of the seedlings of *P. chilensis* to thermal stress. The general purpose of the work has been to evaluate the hypothesis that "native plants are well adapted to their natural environmental conditions" because they have a higher expression of the genes that protect plants against harsh conditions.

On the other hand is well known that plants respond to high temperatures as all living organisms do by the synthesis of a new set of proteins which are the heat shock proteins (hsp). Several genes coding for hsp have been cloned (3), and their encoding sequences seem to be very
conserved (4, 5).

The present work shows the results of seed germination and seedling growth of *P. chilensis* when subjected to temperatures going from 25° to 50°C. The acquisition of thermotolerance and the pattern of proteins synthesized under heat shock, are also reported. The results also demonstrate that *Prosopis chilensis* indeed can be considered a plant highly resistant to heat stress.
Materials and Methods

Source of material. Seeds of *Prosopis chilensis* (Mol) Koch were collected in Peldehue located 35 Km East from Santiago and at 33°S latitud, in the years of 1988 and 1989.

Seed germination. Seeds not infected by Bruchids were selected for germination. For germination, the seeds were scarified for 15 minutes in concentrated sulfuric acid and rinsed for 30 minutes with abundant running water. After the rinse, the seeds were soaked for one hour in deionized water and transfer to germination trays having wet vermiculite. The trays containing the seeds and covered with Saran Wrap were placed in growth chambers under wet saturating conditions at different temperatures. Groups of 100 seeds in duplicate for each temperature treatment were germinated at 25°, 30°, 35°, 40°, 45° and 50°C. After 24 and 48 hours of imbibition, the germination response was checked by protrusion of the root through the seed coat. The number of seeds germinated was counted.

Determination of lethal and sublethal temperatures for seedling growth. For these experiments two kind of seedlings imbibed during 48 hours were used. One kind were seedlings germinated at at 25°C. The others were seedlings germinated at 35°C. All the seedlings were presoaked for three hours at 25°C.
and 35°C respectively, in sterilized wet vermiculite. After this pretreatment the lengths of the embryo axes were measured and placed in groups of 50 seedlings per tray on fresh steril wet vermiculite. Seedlings germinated at 25°C were grown in chambers at 25°, 30°, 35°, 40°, 45° and 50°C, and seedlings germinated at 35°C were grown at 35°, 40°, 45°, and 50°C. After 24 and 48 hours the length of the axis was measured again. During all the period of temperature treatment the chambers were maintained under wet saturating conditions.

Induction of thermotolerance. Groups of 50 seedlings each, 48 hours after germination and coming from seeds germinated at 35°C, were pretreated for two hours in wet vermiculite at 35°C. After the pretreatment, the lengths of the embryo axes were measured. Then, the seedlings were changed to fresh vermiculite and subjected for two hours to the sublethal temperatures of 35° and 40°C, prior to incubation for growth at the sublethal temperature of 45°C and the lethal temperature of 50°C. Two other groups of seedling were subjected directly from 35° to 50°C during 24 hours, without pretreatment of 40°C. After the high temperature treatment the lengths of the axes were measured and the seedlings were returned to grow at 35°C. In this experiment, the control seedlings were maintained at 35°C during all the period of the experiment.
Incorporation of \([{}^{35}\text{S}}\)methionine. Seedlings grown for 48 hours after germination at 35°C, were cut in pieces of 1 cm. Four groups containing pieces of 15 embryos each, were placed in trays with steril vermiculite and incubated for two hours at 35°C in water saturated chambers. After this period the pieces of each group were washed in running water and soaked in aerated solution containing the saline components of the Murashige and Skoog's media \((6)\) and subjected for two hours at 35°, 40°, 45° and 50°C. \([{}^{35}\text{S}}\)methionine with specific radioactivity of \(3.5 \times 10^{-7} \text{MBq mmol}^{-1}\) (from ICN Radiochemicals) was added to the water at the beginning of the two hours of treatment up to obtain a concentration of radioactivity of \(1.5 \text{MBq ml}^{-1}\). The labelled proteins were those accumulated during the two hours of incubation in the presence of \([{}^{35}\text{S}}\)methionine.

Extraction of proteins. Proteins labelled with \([{}^{35}\text{S}}\)methionine were extracted by homogenizing the tissues in the buffer of \(\text{Laemmli}(7)\). The homogenate was kept two minutes in a water bath at 100°C. The incorporation of \([{}^{35}\text{S}}\)methionine was determined in the proteins of the extracts which were precipitated with trichloroacetic acid (TCA) as it has been described by Mans and Novelli \((8)\). The radioactivity was measured in a Beckman liquid scintillation spectrophotometer. Quantification of the proteins in the extracts was performed.
by Bradford's method (9), after precipitation of the proteins with 10 volumes of cold acetone and resuspension of the precipitate in 0.01M Tris. A radioactivity of 80 Bq ug⁻¹ was considered a good incorporation.

Protein electroforesis. The proteins labelled with [³⁵S]methionine were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) by the method described by Laemmli. 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 2500-5000 Bq. The electrophoresis was run for seven hours at 180 V, using Tris-glycine buffer. 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 50% methanol and 10% acetic acid for four hours at room temperature.

Fluorography. In order to detect the synthesized and accumulated proteins during the thermal stress, the protein bands were visualized by the radioactivity of the [³⁵S]methionine incorporated in the proteins. For this, after electrophoresis, the gel was twice washed with 10% acetic acid and 40% ethanol for 30 minutes each wash. Afterwards, the gel was submerged in solution of 0.4% 2,5-Diphenyloxazole (PPO), 20% ethanol, 30% xylol and 50% acetic acid for three hours.
The PPO was precipitated into the gel with distilled water for two and a half hours. After precipitation of the PPO, the gel was dried under vacuum in between two sheets of Saram Wrap. The dried gel was exposed to Kodak X Omat film at -80°C for four days.
Results.

Seeds of *Prosopis chilensis* germinated at 25° and 30°C did not show significant differences in percent or rate of germination respect to the seeds germinated at 35°C. In all these temperatures, the seeds germinate in about 100% in 24 hours, (Fig. 1). At 40°C the germination rate decrease to 60% in 24 hours. However, after 48 hours, 90% of the seeds germinate. At 45°C less than 10% of the seeds germinate in 24 hours and about 20% are germinated after 48 hours of imbibition. None seeds germinate at 50°C.

The growth of the seedlings of *Prosopis chilensis* was determined by measuring the lengths of the embryo axes at different temperatures, going from 25° to 50°C, 24 and 48 hours after germination at 25°C (Fig. 2). In all the temperature treatments, the total growth of the seedlings at 48 hours is almost three folds of the growth after 24 hours of germination. The optimal temperature of seedling growth is 35°C. At this temperature the lengths of the seedlings are about three folds of the lengths at 25°C. At 50°C there is not growth.

When seeds are germinated at 35°C and the seedlings are grown at temperatures over 35°C, the optimal temperature for growth of the seedlings is now 40°C, suggesting an induction of thermotolerance during germination at 35°C.
(Fig. 3 B). At 40°C, the growth of the embryo axes become 40% larger than the growth achieved by the seedlings germinated and grown at 35°C and three folds the length achieved by seedlings germinated at 25°C and grown at 40°C for the same period of time (Figs. 3 A and 3 B). After germination at 35°C, some of the seedlings can grow now at 50°C.

Because the optimal temperature of seedling growth is displaced from 35°C to 40°C when the seeds are germinated at 35°C, the question to ask is if the thermotolerance acquired during germination, could increase further by exposure of the seedlings at the sublethal temperature of 40°C prior to a treatment at 45°C and to a treatment at the lethal temperature of 50°C. Two independent experiments were performed where the lengths of the embryo axes were measured after a treatment for two hours at the optimal temperature of growth of 40°C, followed by an exposure for 24 hours to the sublethal temperature of 45°C or to the lethal temperature of 50°C. The growth of the seedlings under this treatment was compared with the growth of seedlings going directly from 35°C to the thermal treatment of 45°C and 50°C (Fig. 4). The results show that the seedlings treated at 45°C after exposure for two hours at 40°C have a growth 38% higher than the seedlings exposed at 45°C directly from 35°C. The
seedlings treated at 50°C after exposure for two hours at 40°C have a growth 16% higher than the seedlings exposed directly from 35°C to 50°C, with a p < 0.01 as shown by Student Test. Therefore, a further acquisition of thermotolerance is achieved by seedlings of Prosopis chilensis when these are subjected to a treatment for two hours at the sublethal temperature of 40°C prior to a treatment at 45°C or at 50°C.

The pattern of the synthesized and accumulated proteins during two hours of incubation of embryo axes at 35°C, 40°C, 45°C and 50°C in the presence of [15S]methionine, as precursor of protein synthesis was studied (Fig. 5). The results of the fluorography of the SDS-PAGE analysis of the extracted proteins, reveals that eleven new proteins are expressed in the seedling axes treated at temperatures over 35°C. The molecular masses of these proteins are: 150, 108, 100, 88, 82, 60, 52, 36, 31, 20 and 18 kDa. These new proteins expressed under heat shock have been denominated heat shock proteins (hsp), by Kimpel and Key (10). Nine out of the eleven new bands appear at 40°C, being the most prominent of the proteins the one of 60 kDa. The protein of 88 kDa is only present at 45°C and 50°C. In the seedlings there are many other proteins which being present at 35°C, increase further in expression at 40°C and 45°C. The three most prominent of them are the bands of 80, 71 and 69 kDa. At 50°C the
accumulation of proteins decreases considerably. However, there are five proteins, of molecular masses of 100, 80, 71, 69 and 60 kDa which are strongly accumulated at 50°C. The proteins of 100 and 60 kDa are not expressed at 35°C, while the proteins of 80, 71 and 69 kDa increase in expression under heat shock (Tabla 1). From these results, it is conclusive that the optimal temperature for protein accumulation in the embryo axes of P. chilensis occurs at 45°C.
Discussion

The seeds of *Prosopis chilensis* seem to be very resistant to germinate at high temperatures. One hundred percent of the seeds germinate in 24 hours at 35°C, and 90% of the seeds germinate in 48 hours at 40°C, temperatures which are considered extreme temperatures for germination in most plants (11). As an example, in seeds of *Araucaria araucana*, a southamerican conifer, germination is reduced in a 60% at 40°C. From 35°C to 40°C are considered sublethal temperatures for growth of seedlings in *A. araucana*, being 35°C the optimal temperature for thermotolerance induction and expression of the hsp (12, 13). The results on germination of seeds of *P. chilensis* also demonstrate that the population of seeds is heterogeneous. Few seeds are able to germinate at 45°C. This is a lethal temperature for most seeds of the plant kingdom.

During germination at 35°C, the seeds of *Prosopis chilensis* acquire thermotolerance, because the optimal temperature for growth becomes 40°C instead of 35°C and some seedlings can grow now at the lethal temperature of 50°C. The induction of thermotolerance during germination at 35°C has to be investigated further to know the physiological and molecular mechanisms for this protection.
Our results also demonstrate that the thermotolerance can be improved further in seedlings with exposure for two hours at 40°C prior to treatment during 24 hours at 45°C and 50°C. In other seedlings the exposure to the lethal temperature can not go beyond two to three hours after the treatments at the sublethal temperatures which induce the thermotolerance (14). In the case of the seedlings of *P. chilensis* they actually grow during the 24 hour treatment at the lethal temperature of 50°C.

The induction of thermotolerance in *Prosopis chilensis* as well as in other plants correlates with the appearance of heat shock proteins. In seedlings of *Prosopis chilensis* derived from seeds germinated at 35°C, ten new proteins can be detected at the sublethal temperatures of 40° and 45°C, with an optimal protein expression through accumulation at 45°C.

The hsp characteristics of all living organisms can be summarized: a) The hsp 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 back to normal temperature. d) There are hsp of high, medium and low molecular masses, these last ones being peculiar to the plant kingdom (15).
In general we can conclude that the hsp of *P. chilensis* fit the characteristics of these proteins above mentioned.

Among the hsp of *P. chilensis* there are two prominent bands of 60 and 69 kDa. The protein of 69 kDa is expressed constitutively while the one of 60 kDa is expressed from 40°C to 50°C with maximum accumulation at 45°C and 50°C.

Two other less prominent bands but also strongly accumulated during heat shock have molecular masses of 90, 80 and 71 kDa. From these the protein of 90 kDa is the only one expressed under heat shock.

Heat shock proteins of 71, 69 and 60 kDa have been reported to be related proteins and function as chaperon proteins (16, 17). In *P. chilensis* unlike other organisms the 71 and 69 kDa proteins are expressed constitutively. The 60 kDa protein also seem to be related in this plant to the 69 and 71 kDa proteins because monoclonal antibodies raised against a 72 kDa hsp of HeLa cells cross react with these two bands (data not shown). This cross reactivity is not surprising because it has been reported a high degree of conservation in the amino acid sequence of these proteins as well as in the encoding region of their respective genes (18).

In corn, two genes which have been cloned for the 70 kDa protein having 60% homology between the predicted sequence of amino acids and the amino acid sequence of of the 70 kDa
protein of *Drosophila* (19).

In *Prosopis chilensis* as in the case of *Araucaria araucana* there are hsp of molecular masses larger than 116 kDa. In corn the larger molecular mass protein is one of 89 kDa (20). However, in tobacco protoplasts hsp of 120 and 100 kDa with isoelectric points of five and seven have been reported (21).

In our analysis we can detect only two proteins expressed under heat stress of 20 and 18 kDa. The low molecular mass proteins have been reported to be present only in the plant kingdom (22). Recent work (23), demonstrates that the low molecular mass proteins are also related having common amino acid sequences in two domains of the proteins. The roles of these low molecular mass hsp are unknown, although they could play a role on membrane compartmentation and stabilization.

The optimal temperature for expression and accumulation for most of the hsp in *P. chilensis* as well as for those which increase considerably under heat shock, is 45°C. It is also important to point out that the accumulation of normal proteins does not decline in heat stressed seedlings of *P. chilensis*. This is also true for accumulation and synthesis of proteins in seedlings of *A. araucana*, other native tree of South America, while in most of
the cultivated plants the synthesis and the accumulation of normal proteins decline under heat shock (24, 25). All of our results suggest that *Prosopis chilensis* is a plant with higher thermotolerance. Indeed, we have estimated that the thermotolerance of seeds and seedlings of *P. chilensis* is from 5° to 10°C higher than in seeds and seedlings of *A. araucana*. 
References:


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Notes: Key words: Heat shock, thermotolerance, lethal temperature, sublethal temperature.
Color Photographs.

1. Natural population of *Prosopis chileensis* in the Gorge of San Carlos, at the interior of Vicuña, IV Región of Chile (20°02'S; 70°44'W and 730 m over the sea level).

2. Legume fruit of *Prosopis chilensis*.

Photos: Francisco Siqueo.
Figure Legends.

Figure 1. Percent of seed germination of *Prosopis chilensis*, at different temperatures. Seeds were germinated at different temperatures, going from 25° to 50°C. White bars are the % of germination after 24 hours of imbibition. The dotted bars are the % of germination after 48 hours of imbibition. Standard deviations are shown for each experiment.

Figure 2. Growth of seedlings of *Prosopis chilensis* at different temperatures. Growth of seedlings axes was measured in mm, 24 hours after germination (white bars) and 48 hours after germination (dotted bars). Standard deviations are shown for each experiment.
Figure 3. Comparative % of growth between seedlings treated at different temperatures for 24 hours after germination at 25° and at 35°C. In A, groups of seedlings coming from seeds germinated at 25°C were grown at temperatures of 25°, 30°, 35°, 40°, 45° and 50°C for 24 hours. In B, groups of seedlings coming from seeds germinated at 35°C were grown at 35°, 40°, 45° and 50°C for 24 hours. In A and B, the axis lengths were measured in mm after the 24 hours of treatment at higher temperatures. Standard deviations are shown for each experiment.

* 100% was considered the length of seedlings grown at 35°C.

Figure 4. Thermotolerance induction in seedlings coming from seeds germinated at 35°C. Groups of seedlings grown for 48 hours at 35°C were exposed for two hours at 40°C before treatment for 24 hours at 45° and 50°C. The length of the axes were measured in mm after 24 hours of treatment at the sublethal and lethal temperatures of 45° and 50°C, respectively. In this experiment the control seedlings were those directly exposed from 35° to 45° and 50°C for 24 hours. Standard deviations are shown for each experiment. * is the Significant Difference by Student Test, p< 0.01.
Figure 5. Fluorogram of the proteins accumulated in the seedling axis during two hours of heat stress. Proteins accumulation correspond to two hours of incubation at different temperatures in the presence of 1.5 MBq ml$^{-1}$ of [$^{35}$S]methionine used as precursor of protein synthesis. Each experimental group was of segments of 15 seedling 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 axes incubated at 35°C. The arrow heads point to those bands which increase in radioactive intensity upon thermal exposure.
Figure 1

The graph shows the percentage of germination (% Germination) at different temperatures (°C). The temperatures tested were 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. The x-axis represents the temperature, and the y-axis represents the percentage of germination. The data indicates a significant decrease in germination at 45°C compared to other temperatures.
Figure 2

Growth (mm)

Temperature (°C)

25 30 35 40 45 50
Figure 4

Temperature (°C)

Growth (mm)

2 hrs

35 40 35 40

45 45 50 50

*
Figure 5

TEMPERATURE (°C)

MM (KDa)

35  40  45  50

116.0

84.0

58.0

48.5

36.5

26.6
Table I. Protein accumulation and relative intensity of the radioactive protein bands of seedling axes subjected to thermal stress. The molecular masses and the relative intensities of the radioactive bands of the accumulated proteins (hsp) in seedling axes upon thermal stress are shown and compared with the control at 35°C, as seen in Figure 5. The three most prominent proteins which accumulation increase from 35° to 50°C, are also included.

* Molecular mass of the hsp not present at 35°C.
+ Relative intensities of the radioactive bands.

The intensity is compared among the bands of the same protein.

<table>
<thead>
<tr>
<th>Molecular Mass (kDa)</th>
<th>Control</th>
<th>40°C</th>
<th>45°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>150*</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>108*</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>100*</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>88*</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>82*</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>80</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>71</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>69</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>60*</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>52*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>36*</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>31*</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>20*</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>18*</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>
BUDGET
## REQUEST FOR ADVANCE OR REIMBURSEMENT

(See instructions on back)

**Agency for International Development**

**E. EMPLOYER IDENTIFICATION NUMBER**

**F. REGIMENT ORGANIZATION**

Universidad de Chile, Facultad de Ciencias

Banco de Chile, Dep. del Exterior

Sección Contabilidad

Acc. # 51

Casilla 151-D Santiago, Chile

### COMPUTATION OF AMOUNT OF REIMBURSEMENTS/ADVANCES REQUESTED

**PROGRAMS/FUNCTIONS/ACTIVITIES**

<table>
<thead>
<tr>
<th></th>
<th>(a) Salaries &amp; Travel</th>
<th>(b) Equipment &amp; Other Costs</th>
<th>(c) Reagents &amp; Supplies</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Total program outlays to date</td>
<td>$68,350</td>
<td>$66,470</td>
<td>$15,000</td>
<td>$149,820</td>
</tr>
<tr>
<td>b. Less: Cumulative program income</td>
<td>39,678</td>
<td>72,696</td>
<td>27,504</td>
<td>139,878</td>
</tr>
<tr>
<td>c. Net program outlays (Line a minus line b)</td>
<td>28,672</td>
<td>-6,226</td>
<td>-12,504</td>
<td>9,942</td>
</tr>
<tr>
<td>d. Estimated net cash outlays for advance period</td>
<td>6,775</td>
<td>1,000</td>
<td>2,167</td>
<td>9,942</td>
</tr>
<tr>
<td>e. Total (Sum of lines a &amp; d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f. Non-Federal share of amount on line e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g. Federal share of amount on line e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h. Federal payments previously requested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Federal share now requested (Line g minus line h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>j. Advances required by month, when request ed by Federal grantor agency for use in making prescheduled advances</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st month</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd month</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd month</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### ALTERNATE COMPUTATION FOR ADVANCES ONLY

a. Estimated Federal cash outlays that will be made during period covered by the advance | $9,942 |

b. Less: Estimated balance of Federal cash on hand as of beginning of advance period | |

c. Amount requested (Line a minus line b) | $ |

### 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 Ardade Plaza, Dean

Facultad de Ciencias, Universidad de Chile

(56-2) 271-2865 Ext. 221
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, 1991
TO (Month, day, year) August 30th, 1991

Period covered by the next report:
FROM (Month, day, year) August 31, 1991
TO (Month, day, year) February 28, 1992

B. Cash Advance Use and Needs:

1. Cash advance on hand at the beginning of this reporting period $ 18,451

2. U.S. Treasury check advance(s) received during this reporting period $ 00,000

3. Interest earned on cash advance during this reporting period $ 00,000
<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>GROSS cash advance available during this reporting period (Lines 1, 2, &amp; 3)</td>
<td>$18,451</td>
</tr>
<tr>
<td>5</td>
<td>LESS Interest remitted to AID during this reporting period</td>
<td>$00,000</td>
</tr>
<tr>
<td>6</td>
<td>NET cash advance available during this reporting period (Line 4 minus Line 5)</td>
<td>$18,451</td>
</tr>
<tr>
<td>7</td>
<td>Total disbursements during this reporting period, including subadvances</td>
<td>$8,509</td>
</tr>
<tr>
<td>8</td>
<td>Amount of cash advances available at the end of this reporting period</td>
<td>$9,942</td>
</tr>
<tr>
<td>9</td>
<td>Projected disbursements, including subadvances, for the next reporting period</td>
<td>$00,000</td>
</tr>
<tr>
<td>10</td>
<td>Additional cash advance requested for the next reporting period</td>
<td>$00,000</td>
</tr>
<tr>
<td>11</td>
<td>Total interest earned on cash advance from the start of the grant to the end of this reporting period</td>
<td>$00,000</td>
</tr>
<tr>
<td>12</td>
<td>Total cash advances to subgrantees, if any, as of the end of this reporting period</td>
<td>$00,000</td>
</tr>
</tbody>
</table>

**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 September 28, 1991

TITLE Jefe de Oficina de Aportaciones y Donaciones, Facultad de Ciencias, Universidad de Chile

By Juan Carlos Hidalgo

(END OF STANDARD PROVISION)
Disbursements of Budget

Cumulative Detailed Report

From: March 1st, 1991
To: August 30, 1991

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
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<tr>
<td>Salaries</td>
<td>$2,222</td>
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<tr>
<td>Equipment</td>
<td>$0,000</td>
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<tr>
<td>Travel</td>
<td>$2,115</td>
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<tr>
<td>Reagents</td>
<td>$3,308</td>
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<tr>
<td>Other Costs</td>
<td>$864</td>
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<tr>
<td>TOTAL</td>
<td>$8,509</td>
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</tbody>
</table>
TOTAL DISBURSEMENTS OF BUDGET
CUMULATIVE REPORT UP TO AUGUST 30, 1991 (First to third year)

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SALARIES</td>
<td>1,440</td>
<td>2,424</td>
<td>5,958</td>
<td>3,031</td>
<td>3,457</td>
<td>2,222</td>
<td>18,532</td>
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<tr>
<td>EQUIPMENT</td>
<td>45,755</td>
<td>1,431</td>
<td>2,655</td>
<td>0,000</td>
<td>-----</td>
<td>0,000</td>
<td>49,841</td>
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<tr>
<td>TRAVEL</td>
<td>5,874</td>
<td>1,794</td>
<td>1,852</td>
<td>8,196</td>
<td>1,315</td>
<td>2,115</td>
<td>21,146</td>
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<tr>
<td>REAGENTS</td>
<td>4,190</td>
<td>4,174</td>
<td>1,506</td>
<td>10,174</td>
<td>4,152</td>
<td>3,308</td>
<td>27,504</td>
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<tr>
<td>OTHER COSTS</td>
<td>1,230</td>
<td>12,473</td>
<td>6,146</td>
<td>1,505</td>
<td>637</td>
<td>864</td>
<td>22,855</td>
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<tr>
<td>TOTAL...</td>
<td>58,489</td>
<td>22,296</td>
<td>18,117</td>
<td>22,906</td>
<td>9,561</td>
<td>8,509</td>
<td>139,878</td>
</tr>
</tbody>
</table>

US$