1 PN-ABL-695 7.068
78222

FINAL REPORT

Covering Period: August 31,1988 to February 29, 1992.

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

Title: Gene Expression of the 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, MO 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: 8561137

AID Grant Officer: Dr. Mike Benge

Project Duration: August 31, 1988 to February 29, 1992.

REC'D IN R&D/R cc: CDIE

JUL 21 1992

2. TABLE OF CONTENTS

	PG.
EXECUTIVE SUMMARY	3
RESEARCH OBJECTIVES	6
METHODS AND RESULTS	8
Heat shock stress	9
In *Araucaria araucana*	9
In *Prosopis chilensis*	11
Wound stress	15
In *Araucaria araucana*	15
In *Prosopis chilensis*	19
Specific protocols	21
IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER	22
PROJECT ACTIVITIES/OUTPUTS	25
A. Meetings	25
B. Publications	28
PROJECT PRODUCTIVITY	29
FUTURE WORK	30
LITERATURE CITED	31
FIGURE LEGENDS	36

3. EXECUTIVE SUMMARY.

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".**

We have studied the expression of two classes of resistant genes: those related to heat shock and those related to wound stress.

The hypothesis has been tested in two trees of Chile, Araucaria araucana and Prosopis chilensis. Araucaria araucana constitutes temperate relic forests of high mountains in the austral region of South America. Prosopis chilensis is a leguminous tree of the arid and semi arid regions of northern and central Chile. In these regions exist differences of temperatures between day and night and between seasons of about 20°C. The temperature most often reaches between 50° to 60°C at noon and afternoon of warm Summer days.

In both plants the fruits and seeds are susceptible of injuries because they are dispersed and predated by birds, insects and mammals.

On thermal stress we have performed heat shock experiments investigating how seeds and seedlings of these trees respond to high temperature. We have also studied the magnitude of acquisition of thermotolerance when the seedlings

are exposed to sublethal temperatures. In both species, we have been able to define the lethal and sublethal temperatures and the temperature of thermotolerance acquisition: 36°C in A. araucana and 40°C in P. chilensis.

The expression of heat shock genes was evaluated by study and analysis of the proteins which are the final product of the expression, the heat shock proteins (hsp); also by investigating the synthesis of mRNA for hsp.

In the first case we have incubated pieces of plant tissue in the presence of [^{35}S]-methionine which is a precursor of protein synthesis; the proteins have been analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blot analysis using polyclonal and monoclonal antibodies raised against two classes of hsp, ubiquitin and the 70 kDa protein. In the case of the mRNA we have used probes containing the genetic information for the same two classes of hsp. With these approaches we have found that in both species there is a high expression of hsp. The physiological and molecular experiments have allowed to demonstrate that Prosopis chilensis has a thermotolerance higher in 5°C compared with Araucaria araucana and corn plants.

Respect to wound stress, we have investigated the expression of cell wall protein genes under wound stress and normal conditions. Araucaria araucana seeds and seedlings respond to wounding with a high expression of cell wall proteins. Tissue immunoprints and tissue print Northern to

detect the *in situ* expression of extensin proteins and of its mRNA respectivelly, demonstrated that the megagametophyte, wounded areas, and vascular bunddles are the tissues and cells with higher expression of extensin and extensin mRNA. In *Araucaria araucana* two major proteins of the cell walls which greatly increase after wound stress have been purified, characterized and identified as peroxidases. The peroxidases have similar amino acid composition and an identical amino terminal sequence. However, the proteins are different in the sugar components, in susceptibility to degradation when heated in 5M urea and in expression during seed germination. These results suggest that these proteins could be forms of the same class of peroxidase. In *Prosopis chilensis*, Western blot analysis of the cell wall proteins performed with antibodies raised against carrot extensin, demonstrated that four new proteins are expressed when the cotyledons are wounded. Tissue immunoprints reveal that the vascular bundles and the epidermis are the tissues showing higher immunocross reactivity with the antibodies. The constitutive expression of the proteins during development and the premature emergence of the secondary roots when the root is wounded favor the idea that wound stress advances the developmental clock in *Prosopis chilensis* seedlings.

4. RESEARCH OBJECTIVES:

The original and specific objective of this project was 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 has 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 the 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 cotyledos 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 α,α'-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.

5. METHODS AND RESULTS.

During the past three years, the Plant Physiological Genetic Laboratory from the Facultad de Ciencias, Universidad de Chile, directed by the principal investigator of this proposal, Dr. Liliana Cardemil, has studied the physiological and molecular responses to heat shock and to wound stress of two chilean plants, Araucaria araucana and Prosopis chilensis.

Araucaria araucana constitutes temperate relic forests of high mountains in the austral region of South America where exists differences of temperatures between day and night and between seasons of about 20°C. Araucaria araucana also grows on volcano soil with surface temperatures reaching 50°C at noon of warm Summer days. On this soil, at the end of the Summer, the seeds are shed, germinate and the seedlings have to grow (Montaldo, 1974). Prosopis chilensis is a leguminous tree of the arid and semi arid region of northern and central Chile. In theses regions the temperature reaches

over 60°C at noon and afternoon of Summer days (Felker et al, 1982 and 1983).

In both plants the fruits and seeds are susceptible of injuries because they are dispersed and predated by birds, insects and mammals. In Araucaria araucana the seeds are dispersed by parrots (choroy) and in Prosopis chilesis by guanacos, goats and wild rabbits that in most cases eat the fruits and seeds (Montaldo, 1974; Felker et al. 1982 and 1983)

A summary of the results of these three years of research is described below. For more details see the enclosed reprints and prepared manuscripts for publication.

Heat shock stress.

In Araucaria araucana: In this Sci-Funded Work from A.I.D./PSTC Program, Claudio Goycoolea, who was a graduate student of our Facultad de Ciencias, Universidad de Chile, performed his Master Degree dissertation on heat shock stress in Araucaria araucana. The dissertation is entitled: "Las Proteinas de Estrés Térmico Juegan un Rol Fisiológico en la Adquisición de Tolerancia a las Altas Temperaturas en Plántulas de Araucaria araucana (Mol.) Koch" (The Heat Shock Proteins Play a Physiological Rol in The Acquisition of Thermotolerance in Seedlings of Araucaria araucana (Mol.) (Koch.). He was able to establish that seedlings of this specie synthezises 8 hsp when subjected for two hours to temperatures over 32°C in presence

of [^{35}S]-methionine used as precursor of protein synthesis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the proteins extracted from the seedlings demonstrated that the temperature for maximal expression of the hsp is 36°C. The temperature of 36°C is also the optimal temperature for acquisition of thermotolerance. Kinetics experiments of appearance of the hsp showed that 30 minutes after heat shock at 36°C starts, the proteins are expressed, (Goycoolea and Cardemil, 1991).

Claudio Goycoolea was able to detect and quantify in this system, the expression of mRNA for two hsp. The laboratory has probes for the hsp of 70 kDa and for ubiquitin. The detection of mRNA for these proteins was performed by analysis Northern according to Sambrook et al. (1989), in three tissues of A. araucana seedlings: embryo axis, cotyledons and megagametophyte. This last tissue is the reserve tissue of the seed and remains in the seedlings up to a very late stage of development (Cardemil and Reinero, 1982); Cardemil et al., 1990).

The probe for the hsp of 70 kDa, was kindly given to us by Dr. Dean Rochester, from Monsanto Chemical Co., St. Louis, MO (Rochester et al., 1986). With this probe was possible to detect the presence of two populations of mRNA when the tissues were subjected to 36°C. At temperatures less than 36°C the mRNA for this protein is little expressed, Figure 1.

The other hsp probe encloses the genetic information for ubiquitin which is a small heat shock peptide. This probe

was a gift from Dr. Milton Schlesinger, from Washington University, St. Louis, MO. (Bond and Schlesinger, 1985). The probe detects a prominent mRNA band when the tissues are subjected to temperatures of 28°, 32° and 36°C being the expression of mRNA at 36°C six folds the amount of mRNA present at 28°C, Figure 2.

The Northern blot hybridization analyses demonstrated that the embryo axis was the tissue showing a higher expression of mRNA for these two proteins, Figures 1 and 2.

The Master Degree Dissertation of Claudio Goycoolea was approved with Maximal Distinction in December 27, 1989. Part of this dissertation was published in the journal Plant Physiology and Biochemistry (Goycoolea and Cardemil, 1991). Enclosed is a photocopy of the abstract of Claudio Goycoolea's dissertation and a reprint of the publication.

In *Prosopis chilensis*: The initial experiments in *P. chilensis* were performed to evaluate the physiological responses of seeds and seedlings to heat shock (Felker et al., 1982 and 1983). We have found that the seeds of *Prosopis chilensis* can germinate 100% in 24 hours when incubated at temperatures of 25° to 35°C. At temperatures of 40°C the germination rate decreases to 90% after 48 hours. At 45°C, 20% of the seeds germinate in 48 hours and at 50°C there is not germination. In the seedlings germinated at 25°C the optimal growth of the embryo axis occurs at 35°C . At 40°C the growth is reduced to 45% and at 45°C is reduced to 5%. At 50°C there is not growth, therefore, 50°C is considered the lethal

temperature. However, seedlings of P. chilensis germinated at 35°C have the maximal growth at the temperature of 40°C and some seedlings can grow at 50°C, suggesting that germination at 35°C induces thermotolerance. Seedlings germinated and grown at 35°C can acquire further thermotolerance if the seedlings are subjected to 40°C for 2 hours before exposure at 45° or 50°C. Under these conditions the seedlings can grow 10% more than the controls directly exposed from 35°C to 45° or 50°C. The growth difference found between controls and seedlings subjected to heat shock, was statically significant as shown by Student's Test with $p < 0.01$.

Analysis of the proteins synthesized and accumulated during the thermal stress by SDS-PAGE in presence of [^{35}S]-methionine, allowed to detect the expression of 11 proteins not present at 35°C. From these hsp, those of 103 and of 58 kDa are the most prominent. Most other proteins highly increase their expression under heat shock like the proteins of 80, 71 and 69 kDa which have a maximal accumulation at 50°C. The temperature for maximal synthesis and accumulation of hsp is 45°C for seedlings of Prosopis chilensis germinated at 35°C (see enclosed manuscript from Medina and Cardemil).

In Prosopis chilensis, we have performed Western analysis of the hsp expressed in the seedlings when subjected to temperatures of 35°-50°C. For these analyses, we used polyclonal antibodies raised against ubiquitin, a generous gift from Dr. Aaron Ciechanover from the Medical School, Washington University, St. Louis, MO, and a mouse monoclonal

antibody raised against the hsp 72 kDa from HeLa cells (Amersham Chem. Co.).

The antibodies generated against ubiquitin are of two classes: one raised against free ubiquitin and other against conjugated ubiquitin. The results are shown in Figure 3A and 3B. In this Figure is observed that the antibody raised against free ubiquitin immunocross reacts with a protein band of 9 kDa expressed in seedlings subjected for two hours at 45° and 50°C; Figure 3A. The antibody raised against conjugated ubiquitin identifies many protein bands which expression increases slightly with heat shock from 35°-50°C. This results suggests the presence of ubiquitin-bound proteins. Probably, these proteins have lost their normal configuration under heat shock with the subsequent protein misfunction. Therefore, these proteins are tagged by ubiquitin for protease degradation (Vierstra 1987), Figure 3B. The monoclonal antibody raised against the hsp 72 kDa from HeLa cells gives immunocross reactivity with two bands of proteins constitutive expressed in seedlings of Prosopis chilensis with molecular masses of 69 and 71 kDa. Proteins of similar molecular masses have been informed to be chaperonins playing a protective role during stress by folding proteins into the normal configuration (Vierling, 1989; Rothman, 1989; Gatenby et al., 1990), Figure 4, A and B, shows the results of these analyses for seedlings germinated at 25°C (A) or at 35°C (B) when subjected to increasing temperatures with a maximal expression for ubiquitin to 50°C.

From the physiological and molecular experiments, we have concluded that the thermotolerance of Prosopis chilensis is 5°-10°C higher than in other plants, (Medina and Cardemil, manuscript, submitted).

Field research in the Quebrada de Sn. Carlos, Vicuña, IV Región has been performed in collaboration with Professor Francisco Squeo from the Universidad de La Serena. The research has investigated the physiological and metabolic reposes of Prosopis chilensis to fluctuations of temperature between day and night and between seasons. The temperature can reach up to 45°C at 4.00 PM from December to February. Early in the morning and before 4.00 PM, the temperatures of the leaves are 2°-3°C lower than the surrounding air temperature. At 16°C the leaves temperature becomes equal to that of the air. At this time the stomata closes.

Analysis of leaf samples taken at different hours of the day allowed to study daily course expression of the hsp by Western analysis, using the available antibodies. The proteins have their maximal expression between 12.00-4.00 PM in Summer days (December, 1991 to January, 1992). At this time the binding of ubiquitin for other proteins also increases.

For field research see manuscript from Squeo et al., in preparation (enclosed).

We have been able to select two ecotypes using the antibodies raised against the 70 kDa and against ubiquitin. One of these is from Caldera (27°S latitude, III Región) and the other from Quebrada Sn. Carlos (30°S latitude, IV Región).

Several individuals from Caldera shows significantly higher expression of these two hsp, compared with the average individual trees from Sn. Carlos. Therefore, the selection have been preformed by marking the trees and taking seeds from those trees of Caldera which show a higher hsp expression and from those of Sn. Carlos showing a lower expression of these proteins. The higher expression of protein 70 kDa and ubiquitin is correlated with a higher *in vitro* growth rate of buds taken from these individuals when exposed to 45° and 50°C. This work is in progress and constitutes the dissertation project of Héctor Sánchez.

Wound Stress.

In *Araucaria araucana*: We have found that wounds performed intentionally on tissues of seedlings of *A. araucana* (by making cuts on the tissues using razor blades), increase the expression of cell wall proteins from 24 to 72 hours after wounding. In cell walls of wounded embryo tissue, there is a 5 folds increase in cell walls proteins compared with normal tissue. The expression is still higher in the megagametophyte tissue with a 20 fold increase in cell wall proteins respect to the control megagametophyte (non wounded). The cell wall proteins also increase with the developmental stage of the seedling, having a maximum expression at the age of 110 hours of imbibition.

The structural cell wall proteins are characterized by a high content in the uncommon amino acid hydroxyproline or

in glycine (Lamport, 1965; Esquerre-Tugaye and Lamport, 1979; Cassab and Varner, 1986; Condit and Meagher, 1986). The proteins are also glycosilated (Varner and Lin, 1989). The hydroxiproline-rich glycoproteins (HRGP) have been denominated extensins and these are the proteins expressed considerably more during wound stress.

Our Laboratory has polyclonal antibodies raised against, carrot or soybean seed coat extensins (Cassab and Varner, 1987) and the extensin probe, the pDC5A1 containing the genetic information for carrot extensin which is a genomic clone, (Chen and Varner, 1986). These antibodies and the probe were available to us thanks to our project's collaborator Dr. Joseph E. Varner, Department of Biology, Washington University, St. Louis, Missouri. With the antibodies, we have been able to perform tissue immunoprints of the seedlings of A. araucana (see enclosed reprint from Cardemil and Riquelme, 1991) and with the pCD5A1 probe we have performed a tissue print Northern what is similar to an in situ mRNA hybridization, Figure 5, (Ye and Varner, 1991). The tissue immunoprints and the tissue print Northern, revealed that the megagametophyte, root tips, resin chanels and wounded areas are the tissues and cells showing higher expression for extensin protein and extensin mRNA (Cardemil and Riquelme, 1991 and Figure 5). For the tissue print Northern we needed to subclone the extensin gene into pBluescript II KS (+/-) vector to be able to get a RNA probe for histochemical localization of the extensin mRNA (see **Specific Protocols** section).

Alejandro Riquelme, who obtained his Bachelor Degree in Biochemistry (Universidad de Santiago de Chile), purified three cell wall proteins from the seedlings using these antibodies. These proteins were analyzed by SDS-polyacrylamide gels electrophoresis (SDS-PAGE) or by electrophoresis of the native proteins in cationic neutral gels. The cationic neutral gel electrophoresis analysis could be done with these proteins because they have an isoelectric point (pI) of 10-11 and therefore, penetrate the cationic gels. The molecular masses of these proteins are 187 kDa (B1), 145 kDa (B2) and 83 kDa (B3). The proteins immunocross react with antibodies raised against carrot or soybean seed coat, extensins. The results of the preliminary characterization of the cell wall proteins of A. araucana originated the first publication entitled: " Expression of Cell Wall Proteins in Seeds and During Early Seedling Growth of Araucaria araucana is a Response to Wound Stress and Developmentally Regulated", Journal of Experimental Botany Vol. 42: 415-421, from the authors Liliana Cardemil and Alejandro Riquelme (reprint is enclosed, also the dissertation summary).

A further characterization of the proteins of 145 and 83 kDa using o-Phenylenediamine (o-PDA) to visualize the proteins bands on native gels, demonstrated that these proteins are peroxidases. Kinetic studies of these peroxidases defined a Km app. of 13.6 mM for H_2O_2 and 3.4 mM for o-PDA. The Vmax was, for both peroxidases, 525 µmol o-PDA oxidized min^{-1} mg^{-1}. The peroxidases have a pI of 10.5 and an optimal pH of 5. An amino

acid composition analysis was performed for each enzyme showing that the proteins are rich in glycine and serine. The glycine constitutes between 26-32% of total amino acid content of the proteins (Table I). The peroxidases are glycosilated as shown by staining, on gels, the proteins bands with periodic acid-dansylhydrazine after SDS-PAGE (Estep and Miller, 1986). Sugar composition analysis was performed by HPLC showing that the peroxidases are glycosilated with galactose, glucose, xylose and mannose, being the galactose the most abundant residue, Table II, (see enclosed manuscript from Riquelme and Cardemil, accepted in Phytochemistry). Analysis of the NH_2-terminal sequence was performed for both peroxidases, by transferring the proteins from gels to Immobilon-P membranes (polyvinylidene difluoride membranes, Nakagawa and Fukuda, 1989). On the membranes, the proteins bands were identified and stained with coomassie blue. After staining, the proteins bands were cut out from the membrane and sequenced on the surface of the Immobilon-P piece by the Edman's degradation method of the phenylthiohydantoine derivatives of the amino acid residues and using a liquid phase automated sequencer (Spiess, 1986). These sequences are shown on the Table III and were obtained thanks to collaboration of Monsanto Chem. Co., St. Louis, MO, U.S.A. On Table III is also possible to see that both peroxidases have identical NH_2-terminal sequences. The sequences are different from those of horseradish and turnip peroxidases, except for three amino acid residues which are very much conserved in their positions: the threonine

at position # 3, the proline at position # 4 and the serine at the position # 10 (Mazza and Welinder, 1980).

However, the two peroxidases are differently expressed during seed germination and they also show a different susceptibility to degradation when the proteins are heated in the presence of 5 M urea. These results suggest that both cell wall proteins could be forms of the same class of peroxidase .

All the above research on cell wall proteins of Araucaria araucana constituted Alejandro Riquelme's Dissertation who obtained his Degree in Biochemistry from the Universidad de Santiago de Chile in September, 1991 with a grade of excellent.

In Prosopis chilensis: The research on wound stress responses of Prosopis chilensis seedlings constitutes the Master Degree's Dissertation of José Gregorio Rodríguez, who will get his degree in august or september of this year. Three tissues of P. chilensis seedlings were subjected to wound stress by performing cuts on the tissue surface with razor blades: cotyledons, hypocotyls and root tips. The hypocotyls of seedlings of 24 hours of imbibition were the tissue with greater response to wound stress showing an increase in cell wall protein expression 24 hours after wounding. The increase in expression was detected by quantification of the cell wall proteins and by tissue immunoprints using extensin antibodies. Tissue immunoprints of cotyledons and histoimmunochemical localization of the protein in the cotyledonary tissue,

revealed that a greater expression of cell proteins exists in the vascular bundles of these organs (Figure 6A, 6B and 6C). SDS-PAGE and cationic neutral gel electrophoresis of the native proteins followed by Western blot analysis using polyclonal antibodies raised against carrot and soybean seed coats identifies four bands of proteins which immunocross react with the antibodies. These proteins are not present in the control tissue, Figure 7A and 7B.

Amino acid analysis of these proteins reveals that three of the four proteins, those of molecular masses of 180, 126 and 107 kDa have a very similar composition. The 4th protein of 63 kDa differs significantly from the other three, Table IV. Furthermore, NH_2terminal sequence analysis of the proteins, Table V, confirms the similarity between the proteins of 180, 126 and 107 kDa, while the 63 kDa protein shows similar NH_2terminal sequence with proline-rich proteins from soybean.

<u>Prosopis</u> <u>chilensis</u> has exceptional responses to wound stress. Seedlings of 24 hours of imbibition differentiate secondary roots when the root is damaged, Figure 8. Immunohistochemical localization of the cell wall proteins in the area of the cortical tissues of the roots, naturally wounded by the emergence of secondary roots, reveals a very high expression of the cell wall proteins in these areas, Figure 9, A and B.

The constitutive expression of the cell wall proteins for specific tissues and the premature emergence of secondary roots when the tissue is wounded, favor the hypothesis that

wound stress advances the developmental clock in Prosopis chilensis seedlings (Rodriguez and Cardemil, 1991).

Specific Protocols (prepared and used on this research).

1. Subcloning the Extensin Gene in Bluescript Phagemid. The pBluescript II KS (+/-) is a 2961 base pair phagemid derived from pUC19. The KS designation indicates the polylinker is oriented such that the Lac Z transcription proceeds from KpnI restriction site to SacI. The polylinker of the plasmid is flanked by the T_3 and T_7 promoters. Therefore, any insertion in the restriction sites of the polylinker region can be copied from either one strand using the T_3 RNA polymerase or the T_7 polymerase. The pBleuscript 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 genes has four XbaI restriction sites and one PstI site (Chen and Varner, 1986) we decided to cut the pDC5A1 probe 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 pBleuscript 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 T_7 promotor and the EcoRI end, which is the 3'end became

oriented towards the T_3. promoter. Because the RNA polymerase reaction always occurs from the 5'to the 3'end, the polymerase to be used for transcription is the T_7 RNA polymerase. For the ligation reaction the extensin fragment of 3447 bases and the pBluescript fragment, were preparative purified from the agarose gel. The purification was performed by electroblotting of the DNA bands on S&S NA45 DEAE membrane 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 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 bacteria (Sambrook et al., 1989).

The transformants were selected on LB agarose media containing ampicillin and supplemented with 20 µl of X gal-IPTG (isopropylthio-ß-D-galactoside) in formamide for color selection of the transformants. The DNA of the blue colony was analyzed for restriction fragments (Sambrook et al., 1989).

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER:

The development of the project has been the opportunity to open a new area of research in our country, that is Stress Plant Physiology. Being Chile a long country, there is a high latitudinal diversity in climate and geography and, therefore, extreme environmental conditions, from the

North (a desert) to the South (the Antarctic) and from the East (the Andes) to the West (the Pacific).

The methodological approach of our research on temperature stress and heat shock proteins, is being applied by other research groups: Dr. Miren Alberdi (Instituto de Botánica, Universidad Austral de Chile) working on plant cold acclimation and by Dr. Luis Corcuera (Facultad de Ciencias, Universidad de Chile) working on plant cold tolerance and acclimation with plant of the Antarctic.

Our research on wound stress and expression of cell wall proteins has been also used as a model research for plant defense against pathogens and insect attack by Dr. Luis Corcuera. whose laboratory is also working on plant-aphids interactions, and by Dr. Hermann Niemeyer (Facultad de Ciencias, Universidad de Chile) on cereal-insect interaction.

The tissue immunoprints and tissue print Northern methods have been learned from us and used by the Plant Biochemistry group in the Facultad de Ciencias Quimicas y Farmacéuticas de la Universidad de Chile.

Two students working on this project have obtained their degrees : Claudio Goycoolea (Master Degree) and Alejandro Riquelme (Degree in Biochemistry, equivalent to a Master Degree). Two other students are in progress to get their degrees: José Gregorio Rodriguez, who will get his Master Degree very soon and Héctor Sánchez who is doing field research and simulation experiments to induce heat shock on two ecotypes of Prosopis chilensis under conditions mimicked from La Serena

and Caldera from where the ecotypes have been selected. It is very important to point out that José Gregorio Rodríguez is from Venezuela and Héctor Sánchez is from Perú. Both of them will go back to their countries after they get the degree. In their countries, either they will continue their research on these subjects or they will applied the methodology and technologies learned and used on their own future projects. Furthermore, we have to consider the indirect impact of this project on Venezuela and Perú by receiving high trained people, who, hopefully, will improve Plant Physiology in their respective countries.

The equipment bought with the grant: a lyophilizer, scintillation spectrophotometer, autoclave , deep freezer and growth chambers, have been available to all other laboratories from our Facultad de Ciencias and to people from other universities of Chile.

Professor Varner came two times during the project to visit Chile (1988 and 1991). He worked in the Lab., evaluated the research progress, gave conferences and taught a graduate class on "Plant Stress Physiology". His generous help, advice and expertise were a great benefit not only for our Lab. but also for the country.

Professor David Ho came once to Chile (1991). He was evaluating the research going, teaching with Dr. Varner the graduate class on "Plant Stress Physiology". He was also great help for the project and the students enjoyed very much his teaching.

7. PROJECT ACTIVITIES/OUTPUTS:

A. Meetings

a) International Meetings:

1.- Goycoolea, Claudio, Cardemil, Liliana 1988 : Expression of heat shock proteins (hsp) in tissues of seeds and seedlings of Araucaria araucana (Mol.) Koch.
-Annual Meeting of the American Society of Plant Physiology
-Plant Physiology suppl. 86: 143.

2.- Medina, Consuelo and Cardemil, Liliana, 1989. Changes in the pattern of protein synthesis of Prosopis chilensis induced by high temperatures.
-Annual Meeting of the American Society of Plant Physiology
-Plant Physiology suppl. 89: 202.

3.- Cardemil, Liliana, Goycoolea, Claudio and Medina, Consuelo, 1991. Gene expression to thermal stress in two native tress of Chile: Araucaria araucana and Prosopis chilensis.
-IV Conference of the International Plant Biotechnology Network (IPB Net). San José, Costa Rica.
-Abstract Book p. 3:6.

4.- Cardemil, Liliana, Riquelme, Alejandro and Rodriguez, José G., 1991. Response to wound stress by seedlings of Araucaria araucana and Prosopis chilensis.
-IV Conference of the International Plant Biotechnology Network (IPB Net). San José, Costa Rica.
-Abstract Book p. 30: 24.

b) National Meetings:

1.- Cardemil, Liliana y Riquelme, Alejandro, 1988. Respuesta fisiológica al estrés de heridas en semillas de Araucaria araucana.
-XII Reunión Anual de la Sociedad de Bioquímica de Chile.
-Libro de Resúmenes de la Reunión. p. 21.

2.- Goycoolea, Claudio y Cardemil, Liliana, 1988. Respuesta fisiológica al tratamiento térmico de los tejidos de semillas y plántulas de Araucaria araucana (Mol.) Koch.
-Congreso: XII Reunión Anual de la Sociedad de Bioquímica de
Chile.
-Libro de Resúmenes de la Reunión. p. 22.

3.- Medina, Consuelo y Cardemil, Liliana, 1988. Germinación y crecimiento de plántulas de Prosopis chilensis en altas temperaturas y su correlación con el patrón de síntesis proteica.
-VII Reunión Nacional de Botánica. 31 agosto al 3 septiembre. Valparaíso.
-Libro de Resúmenes de la Reunión. p. 125.

4.- Rodríguez, José G. y Cardemil, Liliana, 1990. Glicoproteínas ricas en hidroxiprolina de pared celular de Prosopis chilensis.
-XXXIII Reunión Anual de la Sociedad de Biología de Chile. 21-23 noviembre. Punta de tralca.
-Arch. Biol. Med. Exp. Vol. 23 p.R. 234: 100.

5.- Riquelme, Alejandro (Trabajo dirigido por Liliana Cardemil), 1990. Caracterización de dos proteínas catiónicas presentes en paredes celulares en semillas de Araucaria araucana (Mol.) Koch.
-XXXIII Reunión anual Sociedad de Biología de Chile. 21-23 noviembre. Punta de Tralca.
-Arch. Biol. Med. Exp. Vol. 23 p.R. 234: 101.

6.- Riquelme, Alejandro y Cardemil, Liliana, 1991. Caracterización de dos glicoproteinas de pared celular de semillas de Araucaria araucana que responden al estrés mecánico.
-VIII Reunión Nacional de Botánica, Sociedad de Botánica de Chile. 16-19 de octubre. CENIT, Universidad de Santiago de Chile, Santiago.
-Libro de Resúmenes de la Reunión, p. 147.

7. -Rodríguez, José G. y Cardemil, Liliana, 1991. Expresión de las proteínas de pared celular en Prosopis chilensis en respuesta al estrés producido por heridas.
-VIII Reunión Nacional de Botánica, Sociedad de Botánica de Chile. 16-19 de octubre. CENIT, Universidad de Santiago de Chile, Santiago.
-Libro de Resúmenes de la Reunión, p. 77.

8.- Rodríguez, José G. y Cardemil, Liliana, 1991. Expresión de las proteínas de pared celular en *Prosopis chilensis* en respuesta al estrés de heridas.
-XXXIV Reunión Anual de la Sociedad de Biología de Chile. 27 al 10 de noviembre. Hotel Termas de Puyehue, Puyehue.
-Arch. Biol. y Med. Exp. Vol. 24 p.R 194: 339

c) International Symposia.

1.- Cardemil Liliana; Goycoolea, Claudio and Medina Consuelo. ecial Interest Symposium "Physiology of Land Plants of the Pacific Basin: Plant physiological responses to stress conditions in ecosystem of the Pacific Basin".
-Pacific Science Association. VI Inter Congress. Valparaíso-Viña del Mar. 7 - 10 agosto.
-Abstracts Book p.11

2.- Cardemil, Liliana, 1989. Simposio sobre "Genoma y eventos fisiológicos en células vegetales: Expresión de genes de resistencia al estrés térmico en dos árboles nativos de Chile".
-XXXII Reunión Anual de la Sociedad de Biología de Chile y IV Congreso de la Sociedad Iberoamericana de Biología Celular. 21 - 25 noviembre. Viña del Mar.
-Arch. Biol. y Med. Exp. Vol. 22: R 224

3.- Cardemil, Liliana, 1989. Simposio sobre "Diferenciación celular en Planta. Las proteínas de la pared celular y su posible rol en el crecimiento y diferenciación de la célula vegetal".
-XXXII Reunión Anual de la Sociedad de Biología de Chile y IV Congreso de la Sociedad Iberoamericana de Biología Celular. 21-25 noviembre. Viña del Mar.
-Arch. Biol. y Med. Exp. Vol. 22: R 199

4.- Liliana Cardemil (organizer), 1991. Simposio sobre "Respuestas Fisiológicas y Moleculares de las Plantas a Condiciones de Estrés".
-VIII Reunión Nacional de Botánica de la Sociedad de Botánica de Chile. 16-19 de octubre. CENIT, Universidad de Santiago de Chile, Santiago.
-Libro de Resúmenes de la Reunión p. 22

d) National Symposia

1.- Goycoolea, Claudio; Riquelme, Alejandro y Cardemil, Liliana, 1988. Simposio sobre "Cultivo de Tejido enMicropropagación de Plantas de Aplicaciones Biotecnológicas. Selección para Alta Expresión de Genes de Resistencia alEstrés Térmico y de Heridas en Plantas".
-VII Reunión Nacional de Botánica, 31 de agosto al 3 de septiembre, Valparaíso.
-Libro de Resúmenes de la Reunión p. 25

2.- Cardemil Liliana, 1990. Simposio sobre "Interrelaciones Metabólicas Entre La Planta y su Entorno: Respuestas de las plantas a condiciones de estrés".
-XIV Reunión Anual Sociedad de Bioquímica de Chile. Termas de Catillo. 30 de agosto al 1 de septiembre.
-Libro de resúmenes de la Reunión p. R 365

B. Publications

1.- Cardemil, Liliana y Riquelme, Alejandro, 1991. Expression of cell wall proteins in seeds of *Araucaria araucana* and early seedling growth is a response to wound stress and developmentally regulated. Journal of Experimental Botany. 42: 415-421.

2.- Goycoolea, Claudio and Cardemil, Liliana, 1991. Expression of heat shock proteins in seeds and during seedling growth of *Araucaria araucana* as a response to thermal stress. Plant Physiology and Biochemistry. 29: 213-222.

3.- Riquelme, Alejandro and Cardemil, Liliana, 1992. Two major proteins present in the cell walls of seed and seedlings of *Araucaria araucana* are peroxidases. Phytochemistry, in press.

4.- Medina, Consuelo y Cardemil, Liliana, 1992. *Prosopis chilensis* is a plant highly tolerant to heat shock.
Submitted to Plant Cell and Environment.

5.- Squeo, Francisco; Cardemil, Liliana and Olivares, Nancy, 1992. Water potential components in the branch system of *Prosopis chilenis* (Mol.) (Mimosaceae): A new regulation model? In preparation.

8. PROJECT PRODUCTIVITY

The project accomplish most of proposed goals. The objectives 6, 7 and 8 of our original proposal are goals that need to be worked further. Respect to these, I need to point out that in the field we have selected two Prosopis chilensis ecotypes: one in Quebrada Sn Carlos,Vicuña, IV Región and other in Caldera III Región. A third one in Peldehue near Santiago, V Región, is under study. These 3 regions are located in a latitudinal gradient from parallel 27°S to parallel 33°S. At the present, we are checking for the behavior of the seeds during germination of the selected ecotypes and performing experiments on thermotolerance acquisition to be sure that the selection really means more tolerance to heat shock. It is important to emphasize that this part of the project is in progress because constitutes the research project of Héctor Sánchez who is in the master program of the Facultad de Ciencias.

We feel that the project has been very productive. We have already three publications in prestigious international journals, one paper submitted and another in preparation. Two dissertations have been completed during this period. A third one will be done very soon and a fourth is in progress.

9. FUTURE WORK

The project will continue in two other projects: One is on: "Caracterización de ecotipos de Prosopis chilensis resistentes y sensibles a estrés térmico y estrés de heridas en una gradiente latitudinal de 3ra a 5ta región" (Characterization of resistant and susceptible ecotypes of Prosopis chilensis in a latitudinal gradient from 3th to 5th region). Part of this project is already funded by CONICYT in Chile, part of it will be submitted to National Geographic Society.

We are preparing other project, molecular oriented proposing to clone the heat shock and injury resistant genes of these species. This will be submitted to Agency for International Development in the PSTC program.

10. LITERATURE CITED.

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

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

Cardemil, L; E Salas and M Godoy, 1984. Comparative studies of the karyotypes of South American species of Araucaria. J. of Heredity. 75: 121-125.

Cardemil, L and J E Varner, 1984. Starch degradation metabolism towards sucrose synthesis in germinating Araucaria araucana seeds. Plant Physiol. 77: 532-535.

Cassab, G I and J E Varner, 1986. A new protein in petunia. Nature. 323: 110.

Cassab, G I and J E Varner, 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J. Cell Biol. 105: 2581-2588.

Chen, J and J E Varner, 1986. An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin. The EMBO Journal. 4: 2145-2151.

Condit, C M and Meagher, R B, 1986. A gene encoding a novel glycine-rich structural protein in petunia. Nature: 323, 178-181.

Esquerre-Tugaye, M T and D T A Lamport, 1979. Cell surface in plant-microorganism interactions. I. A structural investigation of cell wall hydroxyproline-rich glycoproteins which accumulate in fungus-infected plants. Plant Physiol. 64: 314-319.

Estep, T N and T J Miller, 1986. Optimization of erythrocyte membrane glycoprotein fluorescent labeling with dansylhydrazine after polyacrylamide gel electrophoresis. Anal. Biochem. 157: 100-105.

Felker, P; P R Clark, P Nash, J F Osborn and G H Cannell, 1982. Screening Prosopis (mesquite) for cold tolerance. Forest. Sci. 28: 556-562.

Felker, P; G H Cannell, P R Clark, J F Osborn and P Nash. 1983. Biomass production of Prosopis species (mesquite), Leucaena, and other leguminous trees grown under heat/drought stress. Forest. Sci. 29: 592-606.

Gatenby, A A; Viitanen P V and Lorimer G H, 1990. Chaperonin assisted polypeptide folding and assembly: implications for the production of functional proteins in bacteria. Trends in Biotech. 5: 354-357.

Goycoolea, C and L Cardemil, 1991. Expression of heat shock proteins in seeds and during seedling growth of Araucaria araucana. Plant Physiol. Biochem. 29: 213-222.

Lamport, D T A, 1965. The protein component of primary cell walls. Advances in Botany Research. 2: 151-218.

Mazza, G and K G Welinder, 1980 Covalent structure of turnip peroxidase. Biochim. Biophys. Acta. 322, 218-223.

Medina, C and L Cardemil, 1992. Prosopis chilensis is a plant highly tolerant to heat shock. Submitted to Plant Cell and Environment.

Montaldo, P R, 1974. La bio-ecologia de Araucaria araucana (Mol.) Koch. Instituto Forestal Latino-Americana de Investigación y Capacitación. Boletin 46-48.

Nakagawa, S and T Fukuda, 1989. Direct amino acid analysis of proteins electroblotted into polyvinylidene difuoride membrane from sodium dodecyl sulfate-polyacrylamide gel. Anal. Biochem. 181: 75-58.

Riquelme, A and L Cardemil, 1992. Two major cell wall proteins of seeds and seedlings of Araucaria araucana are peroxidases. Phytochemistry, accepted.

Rochester, D E; J A Winer and D M Shah, 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp 70 kDa. The EMBO Journal 5: 451-458..

Rodriguez, J G and L Cardemil, 1991. Expresión de las proteinas de pared celular de Prosopis chilensis en respuesta al estrés producido por heridas. Arch. Biol. y Med. Exp. 24: R 194.

Rothman, J E, 1989. Polypeptide chain binding proteins: Catalyst of protein folding and related processes in cells. Cell. 59: 591-601.

Sambrook, J; E F Fritsch and T. Maniatis, 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, N. Y.

Spiess, J, 1986. Strategies of biochemical characterization of hormonal peptides. In Advanced Methods in Protein Microsequence Analysis. Wittmann-Liebold, B, J Salnikow and V A Erdmann, ed. Springer-Verlag.

Varner, J E and L.-S. Lin, 1989. Plant cell wall architecture. Cell. 56: 231-239.

Vierling, E, 1991. The roles of heat shock proteins in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42: 579-620.

Vierstra, R D, 1987. Ubiquitin, a key component in the degradation of plant proteins. Physiol. Plantarum 70: 103-106.

Ye, Z-H and J E Varner, 1991. Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell 3: 23-37.

Figure Legends.

Figure 1.- X-ray film of Northern blot hybridization of total RNA extracted from cotyledons (C) and embryo axes (E) of Araucaria araucana seedlings exposed for 2 hours at different temperatures (28°, 32° and 36°C). The hybridization was performed with a probe containing the cDNA information for ubiquitin from chicken embryo. "Cont" is a positive control where 3 µg of maize RNA extracted from plants subjected to 42°C, was run. The 25S and 18S mark the points where the ribosomal RNA for the large and small subunits run in the gel. These points were used for size estimation of RNA. The two channels marked as "C 28°C*" were overloaded with 3 times of RNA amount present in the extracts of the cotyledons exposed at 28°C and those marked as E 28°C* were loaded with 5 times the amount of RNA present in the embryo axes exposed at 28°C, thus to estimate how much more mRNA for ubiquitin was present in the tissue exposed at 36°C.

Figure 2.- X-ray film of Northern blot hybridization of total RNA extracted from megagametophyte (M) and cotyledons (C) of Araucaria araucana seedlings exposed for two hours at different temperatures (28°, 32° and 36°C). The hybridization was performed with a probe containing the cDNA information for hsp 70 kDa for maize. "Cont" is a channel for a positive control where was loaded 3 µg of maize RNA extracted from plants subjected at 42°C. 25S and 18S mark the points where the ribosomal RNA for large and small subunits run in the gel, and was used for size estimation of RNA.

Figure 3.- Western blot analysis of the proteins extracted from Prosopis chilensis seedlings, using polyclonal antibodies raised against free ubiquitin (**A**) or against conjugated ubiquitin (**B**). The seedlings were grown from seeds germinated at 35°C and then subjected to heat stress at temperatures of 35°, 40°, 45° and 50°C, for two hours. The proteins were extracted and run in SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were incubated with the first antibody (IgG anti free ubiquitin or IgG anti conjugated ubiquitin) and with the secondary antibody conjugated to alkaline phosphatase.

Figure 4.- Western blot analysis of Prosopis chilensis heat shock proteins, using a monoclonal antibody raised against 72 kDa protein from HeLa cells as primary antibody. the secondary antibody was a mouse anti IgG conjugated to alkaline phosphatase.
A.- Analysis of the proteins extracted from seedlings of 48 hours of imbibition and exposed for two hours at temperatures of 30°, 35°,40°, 45° and 50°C.
B.- Analysis of the proteins extracted from seedlings of 48 hours of imbibition and exposed for 2 hours at temperatures of 35°, 40°, 45° and 50°C. In this case the seedlings were from seeds germinated at 35°C. After extraction the proteins were run in SDS-PAGE, electrotransferred to nitrocellulose paper and incubated with the primary and secondary antibodies.

Figure 5.- Tissue print Northern of cross sections of hypocotyls and of megagametophytes enclosing the cotyledons, of 10 and 20 days seedlings of Araucaria araucana. The tissues were printed by pressuring the cross sections on nylon membranes for 20 seconds. The membranes were hybridized with a probe containing the genetic information for the carrot extensin. The genetic information is preceding by a T_3 promoter that allows to make in vitro transcription and to prepare an antisense [^{35}S]RNA probe using a DNA dependent RNA polymerase reaction. **A** shows the prints from seedlings of 20 days. **B** shows the prints from seedlings of 10 days.

Figure 6.- Immunocytolocalization of extensin protein in Cotyledon (**A**) and in Hypocotyl (**B**).
A. 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 then incubated with a goat anti-IgG conjugated with alkaline phosphatase.
HV1 is a principal vascular bundle and HV2 are the secondary vascular bundles.
B. Cross section of hypocotyl of 60 hours of age which was paraplast embedded, sectioned and incubated with the same antibodies used in **A**.
HV are the vascular bundles.
Ep, means epidermis.

Figure 7.- 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 8.- Wound effect produced with razor blade cuts on seedling roots of Prosopis chilensis, of 24 hours after germination. One day after wounding, the radicle of the seedlings differentiates 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 9.- Immunocytolocalization of extensin during development of secondary roots as a response to wound stress when seedling radicles has been injured with razor blade cuts, as shown in Figure 8.
A is a longitudinal section of the radicle showing the beginning of a secondary root development. **B** is a cross section of a radicle with a secondary root full developed.
The arrows point the root cortical tissue that seems to be wounded by the emergency of the secondary root.

Table I. Sugar composition of cell wall peroxidases.

Sugar	83 kDa protein	145 kDa protein	Horseradish peroxidase *
	µg sugar/mg protein		
D-galactose	72.4 ± 2.9 (61.1%)	76.8 ± 1.6 (75.1%)	0.0 (0.0%)
D-glucose	42.8 ± 2.0 (36.1%)	19.6 ± 3.6 (19.2%)	5.7 (3.8%)
D-xylose	1.4 ± 0.3 (1.2%)	1.3 ± 0.5 (1.3%)	12.8 (8.5%)
D-mannose	2.0 ± 0.6 (1.6%)	4.5 ± 0.9 (4.4%)	48.2 (32.1%)
L-arabinose	0.0 (0.0%)	0.0 (0.0%)	2.9 (1.9%)
D-N-Acetyl glucosamine	N.D.	N.D.	66.5 (44.3%)
Carbohydrate in the protein	12	10	15

The sugar composition was obtained after hidrolysis of the protein bands electroeluted from polyacrylamide gels after SDS-PAGE. HPLC was performed in Knauer HPLC type 364 equipped with a CARBOPACK PA1 column using 5.25 mM NaOH, pH 11.5, as solvent. The gas chromatograph was provided with a gold electrode present in the Dionex pulsed amperometric detector.

N.D. not determined

* from Clarke and Shannon, 1976 (22).

± s.d.

TABLE II

AMINO ACID COMPOSITION OF CELL WALL PEROXIDASES OF *ARAUCARIA ARAUCANA*.

% MOL

AMINO ACIDS	PEROXIDASE 83 kDa	PEROXIDASE 145 kDa	HORSERADISH PEROXIDASE 40 kDa
LYS	5.37 (23)	4.80 (42)	1.80 (4)
HIS	2.93 (12)	1.70 (14)	1.00 (2)
ARG	2.26 (8)	4.03 (29)	6.90 (13)
ASX	4.87 (23)	4.42 (42)	17.40 (45)
GLX	8.60 (36)	6.87 (60)	6.50 (15)
THR	2.50 (13)	4.22 (45)	6.50 (19)
SER	15.65 (92)	18.40 (223)	7.30 (24)
GLY	32.81 (270)	35.32 (598)	5.60 (25)
ALA	10.23 (71)	6.70 (96)	8.10 (31)
PRO	4.92 (26)	5.91 (65)	5.50 (16)
CYS	N.D.	N.D.	1.00 (3)
MET	N.D.	N.D.	0.90 (2)
VAL	3.97 (21)	1.50 (16)	5.80 (17)
ILE	1.90 (9)	1.72 (17)	4.20 (11)
LEU	3.19 (15)	3.65 (35)	12.30 (32)
TYR	0.00	0.00	1.40 (3)
PHE	0.75 (3)	0.83 (2)	7.70 (16)
TRP	N.D.	N.D.	N.D.

In parentheses, number of residues per molecule.

TABLE III

NH2 TERMINAL SEQUENCES OF CELL WALL PEROXIDASES OF
ARAUCARIA ARAUCANA

Peroxidase		3	4							10						
145 kDa	Ala	Met	Thr	Pro	Glu	Gln	Val	Gln	Ser	Ser	Leu	Glu	Pro	Tyr	Ileu	
Peroxidase		3	4							10						
83 kDa	Ala	Met	Thr	Pro	Glu	Gln	Val	Gln	Ser	Ser	Leu	Glu	Pro	Tyr	Ileu	Ser
	Tyr	Leu	Thr	Ala												
Horseradish		3	4							10						
Peroxidase	Glx	Leu	Thr	Pro	Thr	Phe	Tyr	Asp	Asn	Ser	Cys	Pro	Asn	Val	Ser	Asn
	Ileu	Val	Arg	Asp												
Turnip ?		3								10						
Peroxidase	Glx	Leu	Thr	Asn	Phe	Tyr	Tyr	Ser	Thr	Ser	Cys	Pro	Asn	Leu	Leu	Ser
	Thr	Val	Lys	Ser												

TABLE IV

Amino acid composition of the cell wall proteins present in the cotyledons of *Prosopis chilensis*. The composition is compared with proteins encoded by genes cloned from soybean.

Mol %

Amino Acid	*Prosopis chilensis*				Soybean	
	180 kDa	126 kDa	107 kDa	63 kDa	SbPRP1	ENOD2
Hyp	n.d.	n.d.	n.d.	n.d.		
Pro	6.7	7.8	8.2	14.7	35.9	44.4
Asx	11.5	11.7	10.5	8.9	1.2	1.2
Thr	2.3	2.9	2.9	3.1	0.4	0.4
Ser	6.8	7.1	6.9	3.5	2.3	0.4
Glx	23.8	21.3	18.6	18.7	3.9	22.0
Gly	6.2	5.7	4.8	3.3	0.4	0.4
Ala	5.0	5.4	4.6	3.8	1.6	0.0
Val	4.7	5.5	6.4	12.3	15.2	2.1
Cys	0.8	0.7	0.1	0.2	0.0	0.0
Met	0.0	0.0	2.0	0.0	0.0	0.0
Ile	4.4	3.5	3.0	1.8	2.7	0.4
Leu	7.4	6.4	5.1	2.5	3.1	1.2
Tyr	2.6	3.4	5.7	8.5	14.5	7.9
Phe	2.8	2.6	2.0	1.3	0.0	0.0
His	0.9	2.4	4.6	2.1	0.0	9.5
Lys	4.8	6.2	8.5	13.5	17.6	9.5
Arg	9.3	7.4	6.2	2.2	0.4	0.4
Trp	n.d.	n.d.	n.d.	n.d.	0.0	0.0

n.d. No determined

TABLE V

NH_2 -terminal sequence of the cell wall proteins present in cotyledons of *Prosopis chilensis*. The sequences are compared with homologous sequences coded by genes from soybean

PROSOPIS CHILENSIS:

Protein of 180 kDa

X-X-Gly-X-Asn-Glu-Ala-Leu-Gln-Glu-Ile-Met-X-Gln-Ile-Gly-

Protein of 126 kDa

X-X-Gln-X-Gln-Ala-Leu-Lys-X-Ile-Met-X-X-Glu-Gly-GLy-Ser-Leu-(Leu)-X

Protein of 107 kDa

X-X-Gln-Ile-Gln-Ala-Leu-Lys-X-Ile-Met-Arg-Arg-X-Gly

Protein of 63 kDa

Asn-Tyr-Tyr-Gln-Pro-Hyp-Thr-Tyr-Lys- Pro-Pro-Glu-Lys-Pro-X

SOYBEAN CLONES:

Sb PRP 1: Pro-Pro-Ile-Tyr-Lys
Pro-Pro-Val-Tyr-Lys

Pro-Pro-Val-Glu-Lys
Pro-Pro-Ile-Glu-Lys

ENOD 2: Pro-Pro-His-Glu-Lys

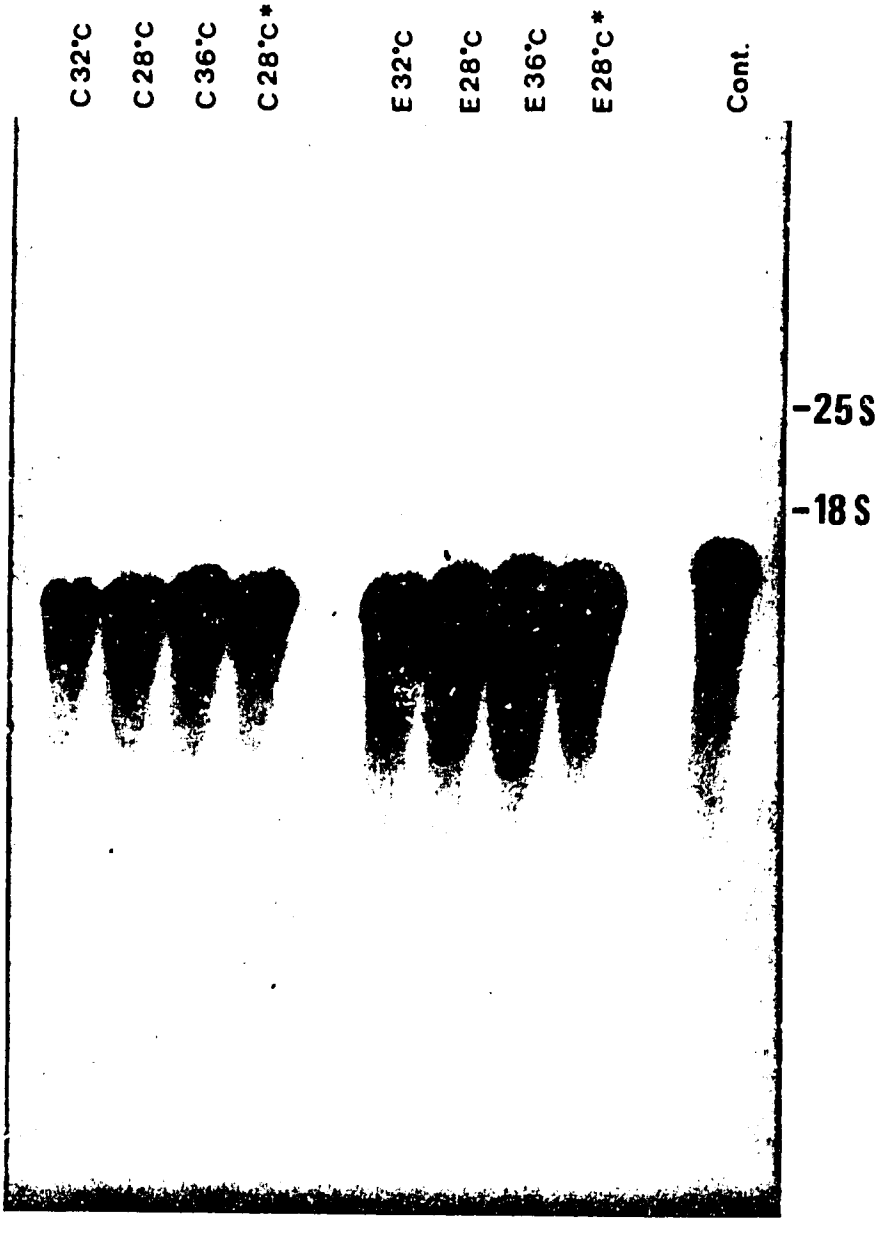


Figure 1

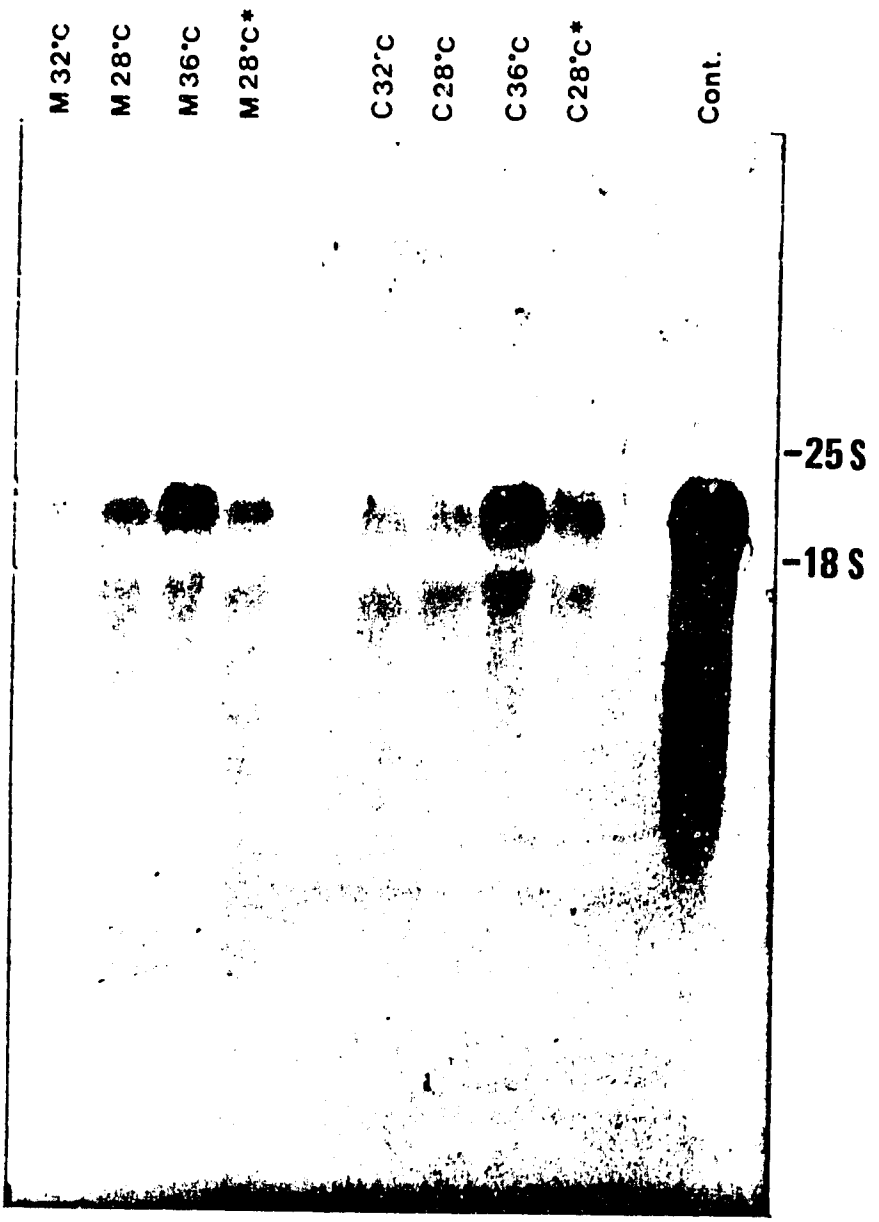


Figure 2

45

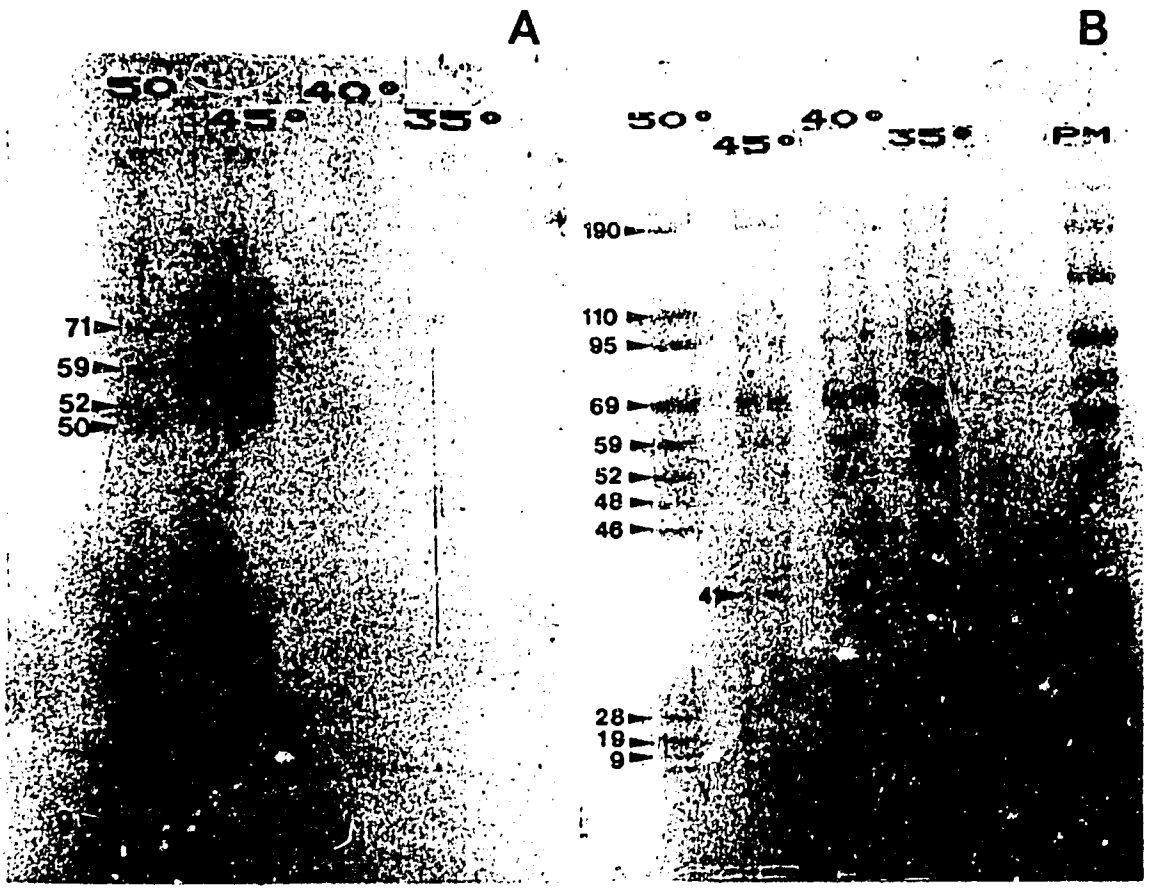

A
B
50°
40°
45°
35°
50°
40°
45°
35°
PM
190
110
95
69
59
52
48
46
41
28
19
9
71
59
52
50

Figure 3

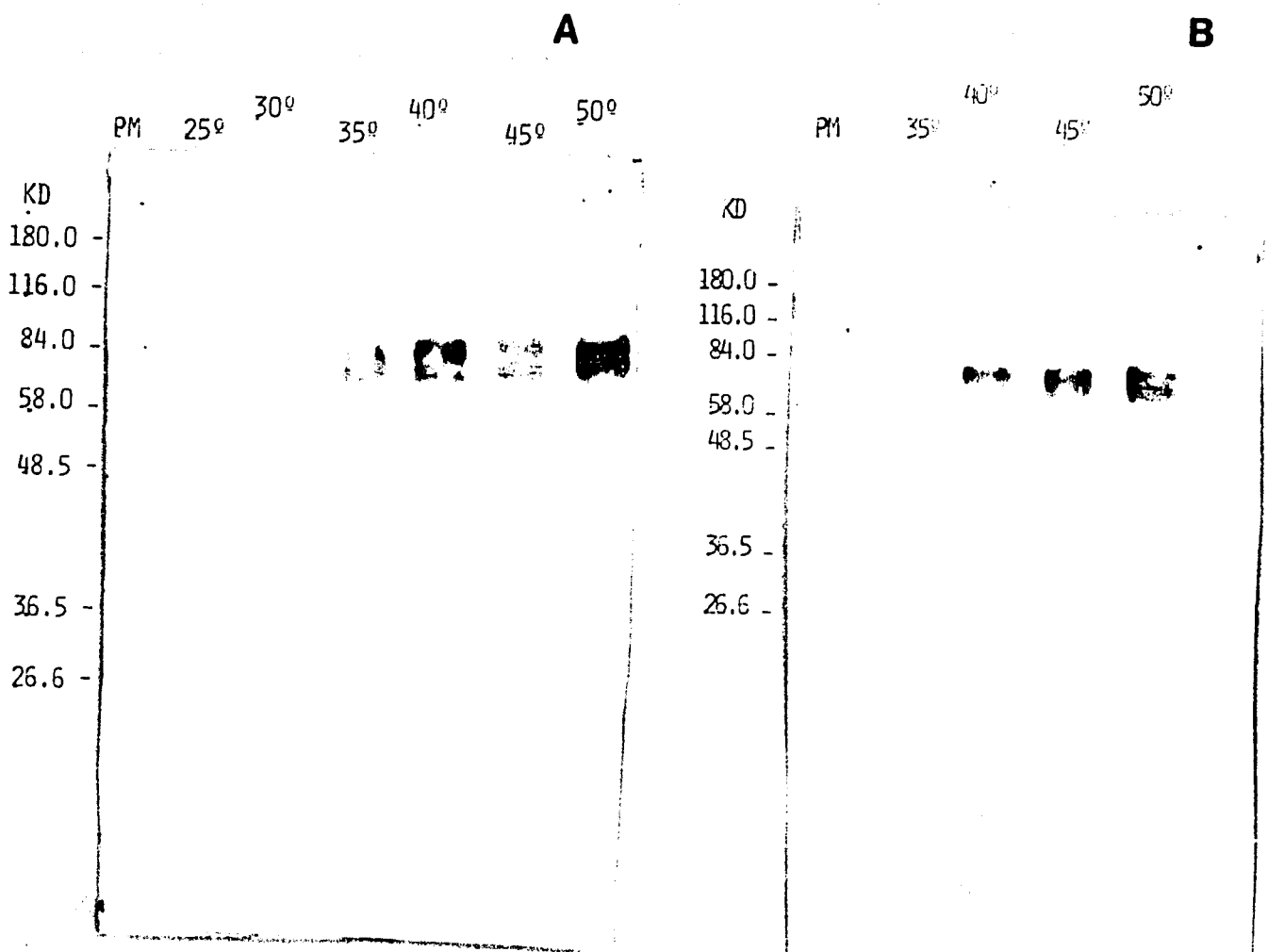
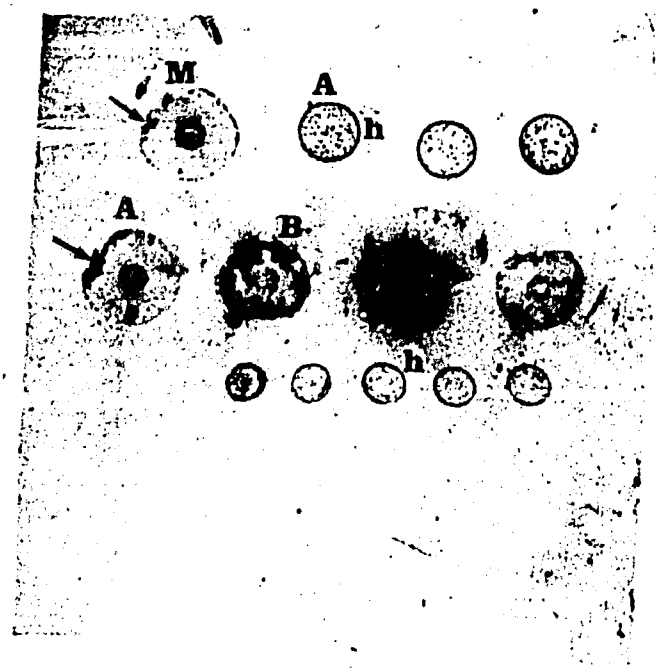

A
PM
25º
30º
35º
40º
45º
50º
KD
180.0
116.0
84.0
58.0
48.5
36.5
26.6
B
PM
35º
40º
45º
50º
KD
180.0
116.0
84.0
58.0
48.5
36.5
26.6

Figure 4



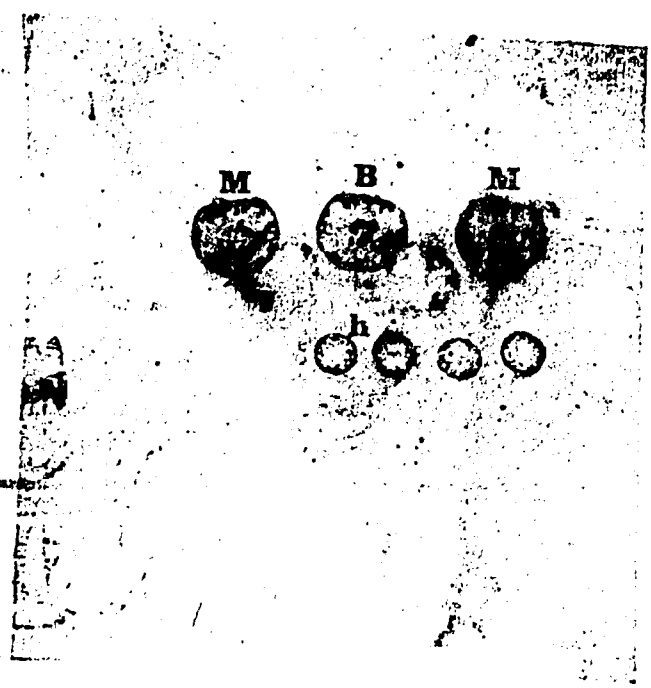


Figure 5

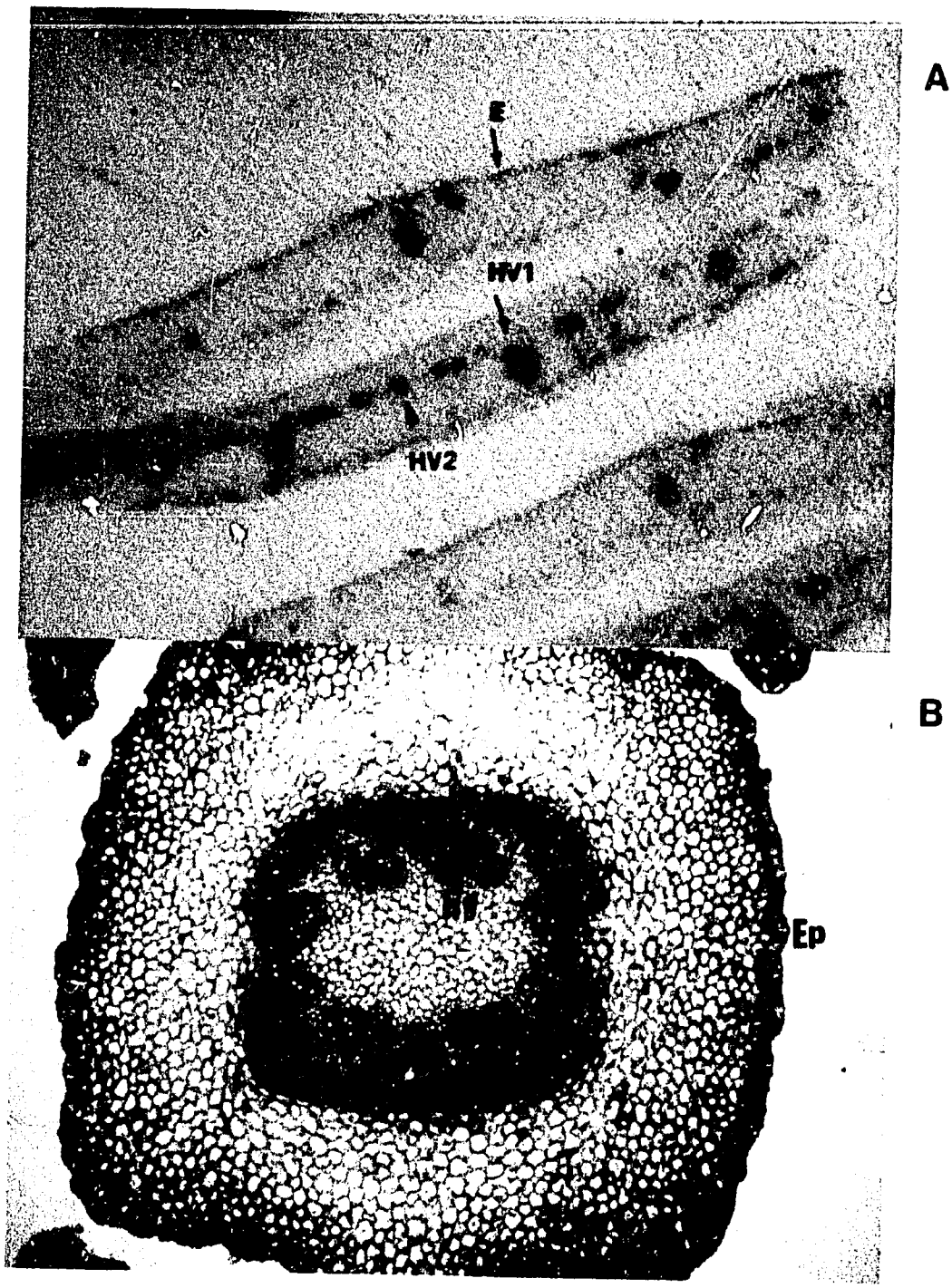


Figure 6

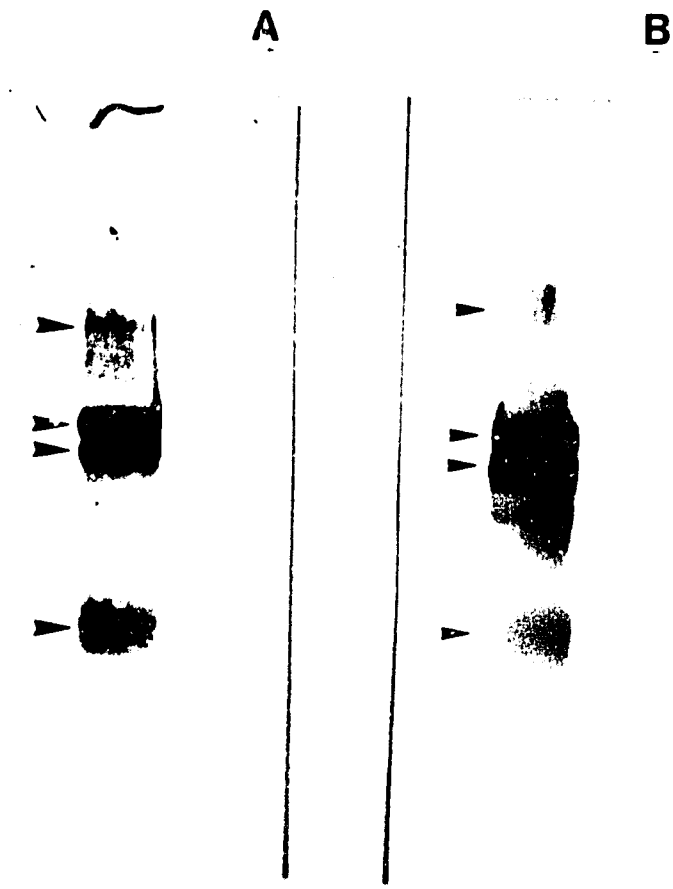


Figure 7

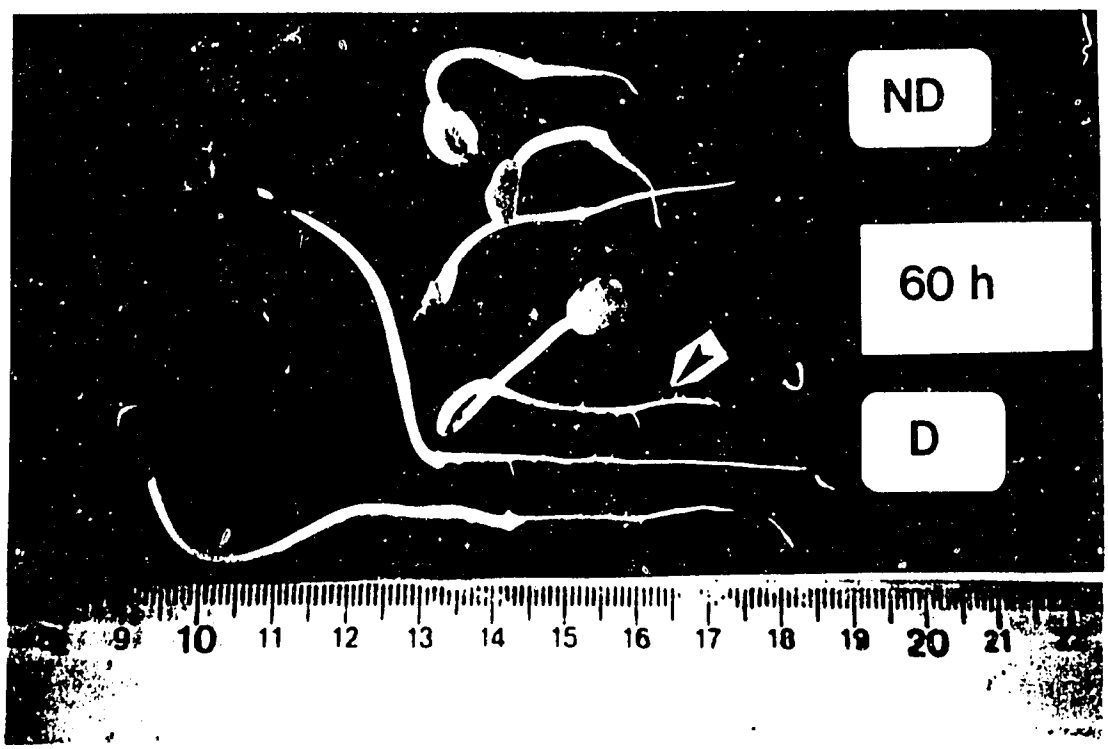


Figure 8

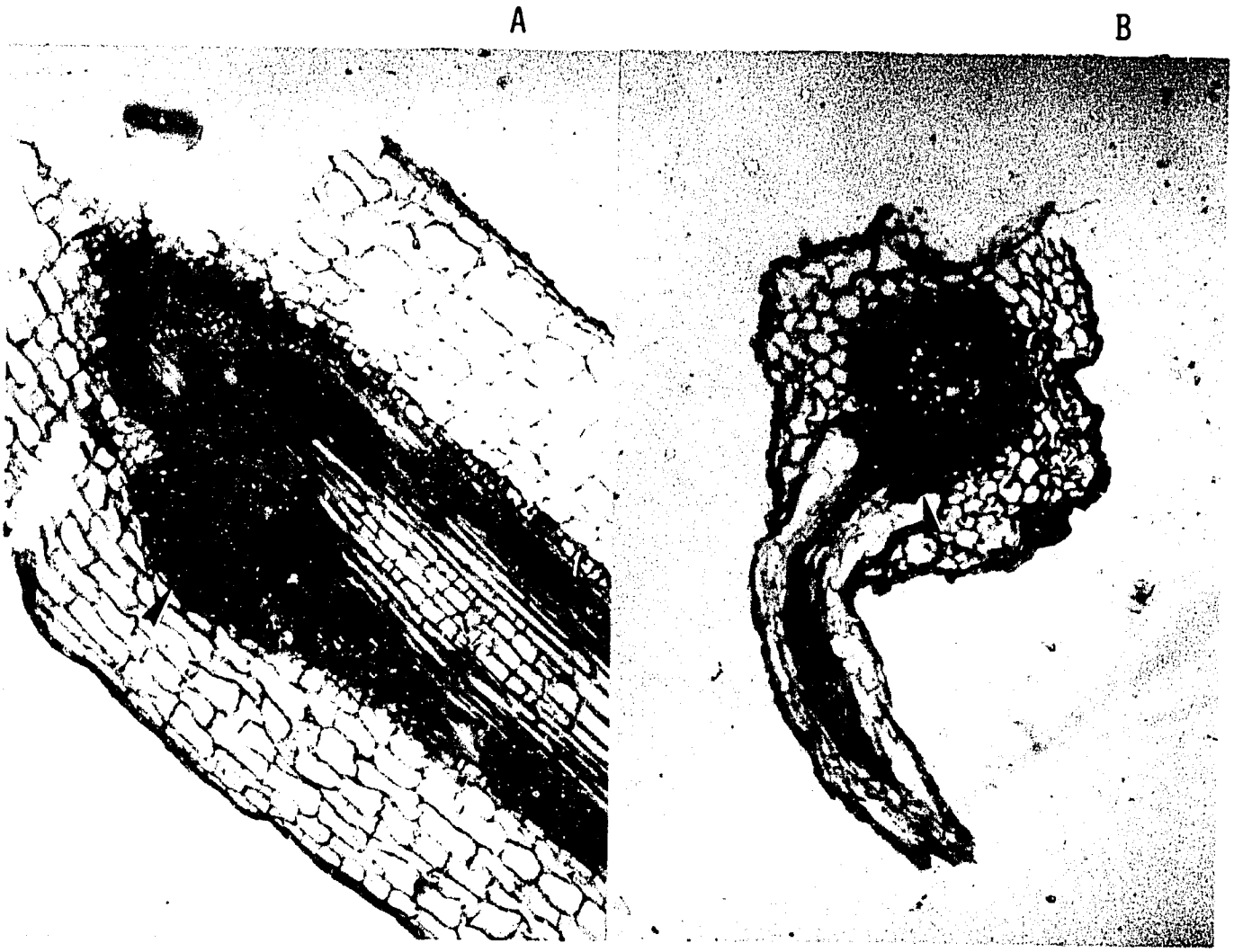


Figure 9

PUBLICATIONS
AND
PUBLICATIONS IN PREPARATION

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 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 1.5-2.0 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 40°C.

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

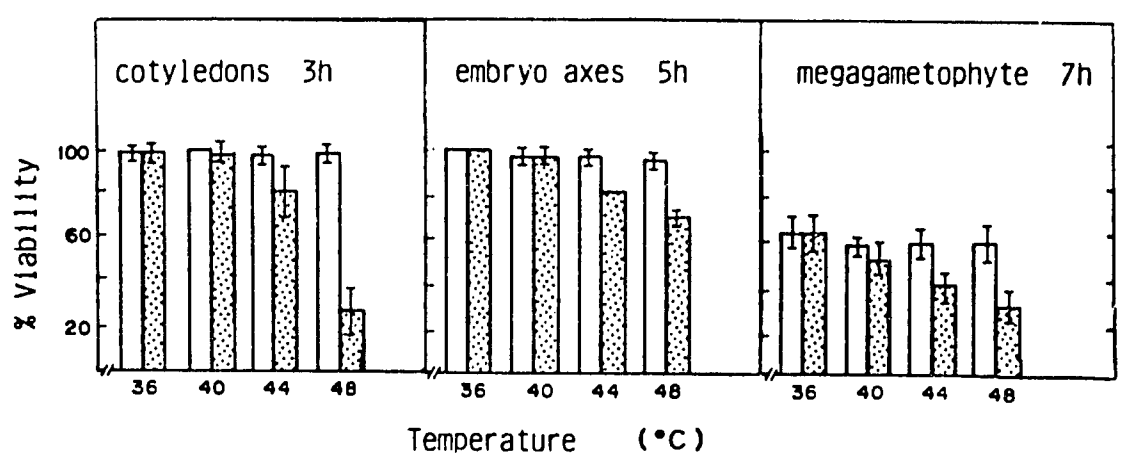


Figure 1. *Viability of the different tissues of quiescent seeds of* Araucaria araucana. The results are shown at the minimum period of time (3 h for cotyledons, 5 h for embryo axes and 7 h for megagametophyte) at which there is a significant decrease of viability by exposure to high temperatures. The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

54

chloride (TTC). Alternatively, the viability was determined after the temperature treatment by measuring the conductance of electrolytes leaked from the tissue into deionized water (leachate). The conductance of a leachate of dead seeds, killed in boiling water for 30 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-HCl (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

-55-

*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 l'acquisition de la thermotolérance par les plantules d'*A. araucana. Mots clés additionnels : mégagamétophyte, choc thermique, température létale, thermotolérance, température 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 respond to exposure to high temperatures by changing the pattern of protein synthesis, most often with a decrease of the proteins normally synthesized and appearance of new proteins (Schlesinger *et al.*, 1982; Cooper and Ho, 1983; 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.*, 1984; 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 of 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 collected in the forest reserve of Malalcahuello (latitude 37.5° S, Chile) between the months of March and April of 1986, 1987 and 1988. The seeds were stored in plastic bags at 4°C until used. For the experiments seeds without testa were sterilized in a 10% sodium hypochlorite solution for 5 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 1, 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

Plant Physiol. Biochem., 1991, **29** (3), 213-222.

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

Claudio Goycoolea and Liliana Cardemil

Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

(Received for publication October 8, 1990; accepted January 7, 1991)

Goycoolea, C. and Cardemil, L. 1991. Expression of heat shock proteins in seeds and during seedling growth of *Araucaria araucana* as a response to thermal stress – Plant Physiol. Biochem. 29 : 213-222.

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.

C. Goycoolea, Rengo 31, Concepción, Chile; L. Cardemil (reprint requests), Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

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 mégagamétophytes de graines d'*A. aracana *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 triphényltétrazolium 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*

51

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 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.

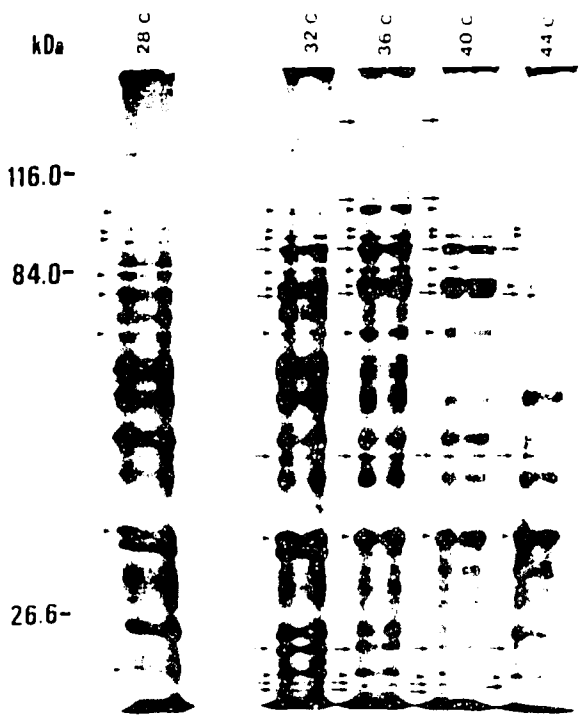


Figure 4. *Fluorogram of the proteins synthesized in embryo axes under heat stress.* The synthesis of proteins correspond to the last 30 min of 2 h of incubation at different temperatures in the presence of 1.48 MBq ml^{-1} of [^{35}S]methionine used as precursor of protein synthesis. Each experimental 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[a]	–	–	+	+	–
106[a]	–	–	+	+	–
101	+	+	+ + +	+ +	–
98	+	+ +	+	+	+
94	+	+ +	+	+ +	+
89[a]	–	+ +	+ + + +	+ + +	+
84	+	+	+ + +	+ +	–
80	+	+ + +	+ + + +	+ + +	+
77[a]	–	+	+ + +	+	+
69	+	+	+ + +	+ +	+
44	–	+	+ +	+	+
33	+	+ + +	+ + +	+ + +	+ + +
24[a]	–	+ + +	+ +	+	+
20	+	+ + +	+ +	+	+
19[a]	–	+ +	+	+	+
17[a]	–	+ +	+ +	+	–

[a] 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

58

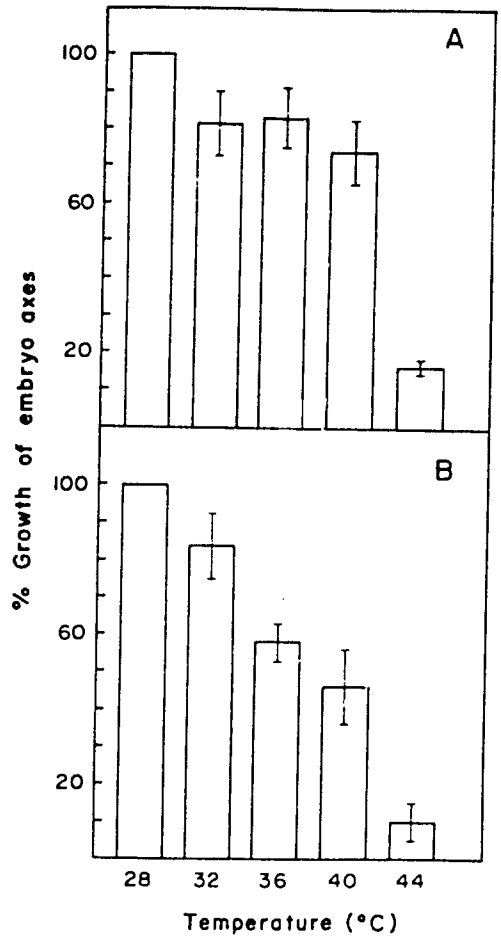


Figure 2. *Growth recovery of embryo axes of* Araucaria araucana. After 30 min of thermal exposure in A or after 60 min of thermal exposure in B. The recovery is given as per cent of growth of the control embryo axes maintained at 28°C. The stress temperatures were 32, 36, 40 and 44°C. After the thermal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

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.

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

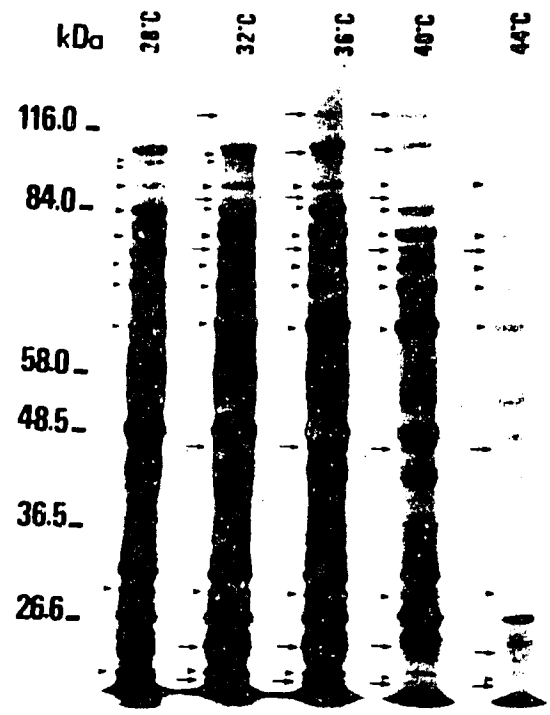


Figure 3. *Fluorogram of the proteins accumulated in embryo axes under heat stress.* Proteins accumulation correspond to 2 h of incubation at different temperatures in the presence 1.48 MBq ml^{-1} of [^{35}S]methionine used as precursor of protein synthesis. Each experimental group was of six embryo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

59

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

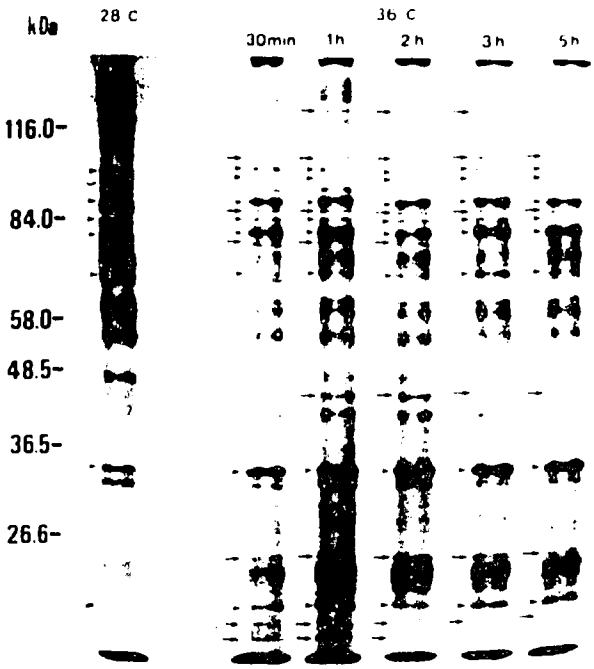


Figure 5. *Kinetics of proteins synthesis in embryo axes.* The embryo axes were subjected at 36°C for different periods of time (from 30 min to 5 h). Groups of 6 embryo axes were incubated at 36°C in the presence of 1.48 MBq ml^{-1} of [^{35}S]methionine which was added to the medium in the last 30 min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

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[a]	−	−	+	+	+	−
106[a]	−	+	+ +	+ +	+ +	+ +
101	+	+ + +	+ + +	+ + +	+ + +	+ + +
98	+	+	+ + +	+ + +	+ + +	+ + +
94	+	+ + +	+ + + +	+ + +	+ + +	+ + +
89[a]	−	+	+ +	+ +	+ +	+
84	+	+ +	+ + +	+ +	+ +	+
80	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
77[a]	−	+ +	+ + +	+ +	−	−
69	+	+	+ +	+ +	+ + +	+
44[a]	−	+	+ + +	+ + +	+ +	+ +
33	+	+ +	+ + +	+ +	+ +	+ +
24[a]	−	+	+ +	+ +	+ +	+
20	+	+ +	+ + +	+ + +	+ + +	+ + +
19[a]	−	+ +	+ + +	+ +	+	+
17[a]	−	+ +	+ + +	+ +	−	−

[a] Molecular mass of the proteins bands (hsp) not present at 28°C.

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.* 6B). 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.*

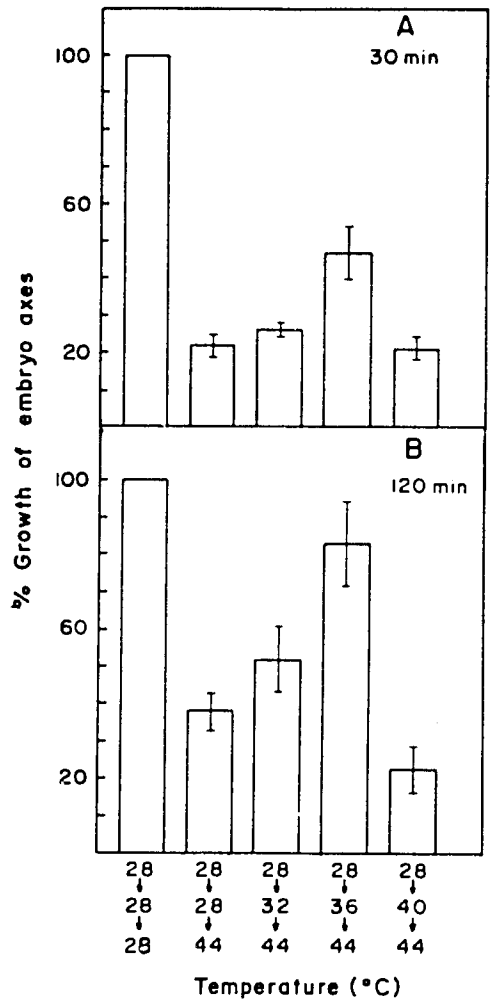


Figure 6. *Thermotolerance induction in embryo axes of* Araucaria araucana *by exposure of the tissues to sublethal temperatures.* The sublethal conditions were temperatures of 32, 36 and 40°C for 30 min in A and for 120 min in B. After the treatment to the sublethal temperatures, the embryo axes were 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.

DISCUSSION

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

The loss of viability of the cotyledons could be due to experimental conditions since they are never exposed to external environments. Indeed, the cotyledons remain inside 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.

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 Schlesinger *et al.* (1982) and it seems to be related to glycolysis: it has been suggested that it could be a phosphatase or a phosphotransferase (Schlesinger, 1985).

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

There is another protein of 77 kDa expressed only under heat shock and 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, Nortnern 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 min 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 synthesis 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.

Acknowledgements. We thank Dr. Joseph Varner, Department of Biology, Washington University, St Louis, U.S.A., for critical comments on the manuscript. This work was part of Claudio Goycoolea's Master Degree Dissertation. The technical assistance of Angélica Vega and Ximena Oñate from Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, is acknowledged. This research was supported by grant #7.068, Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development; FONDECYT 0160/88;

-16

CONICYT NSF #84-201 and DTI # B 2806-8923 from Universidad de Chile.

REFERENCES

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

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

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

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

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

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

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

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

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

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

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

Kimpel J. A. and Key J. L., 1985. – Heat shock in plants. *Trends Biochem. Sci.*, **9**, 353-357.

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

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

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

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

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

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

Murashige T. and Skoog F., 1962. – A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiol.*, **15**, 473-97.

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

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

Schlesinger M., Aliperti G. and Kelley, P. M., 1982. – The response of cells to heat shock. *Trends Biochem. Sci.*, **7**, 222-225.

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

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

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

Journal of Experimental Botany, Vol. 42, No. 236, pp. 415–421, March 1991

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

LILIANA CARDEMIL[1] and ALEJANDRO RIQUELME

Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

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). 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.

[1] To whom correspondence should be addressed.

Abbreviations: HRGP, hydroxyproline-rich glycoprotein; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; c.w.p., cell wall protein; MOPS, 3-[*N*-morpholino] propane sulphonic acid.

© Oxford University Press 1991

64

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 megagametophytic and embryonic tissues of germinating seeds of *Araucaria araucana*.

Cell wall proteins were extracted from purified walls with 0·2 M $CaCl_2$ and 2·0 mM $Na_2S_2O_5$ solution.

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

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 0·025 M Tris 0·192 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 0·1 M histidine, 0·02 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).

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 0·2 M $CaCl_2$, 2·0 mM $Na_2S_2O_5$ 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).

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 2 to 4 times higher in embryonic tissue and 15 times higher in the megagametophytic tissue under wounded conditions compared with non-wounded conditions.

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	0·8 ± 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 ± 2·6

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 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 in 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 in 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

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 ± 0·9

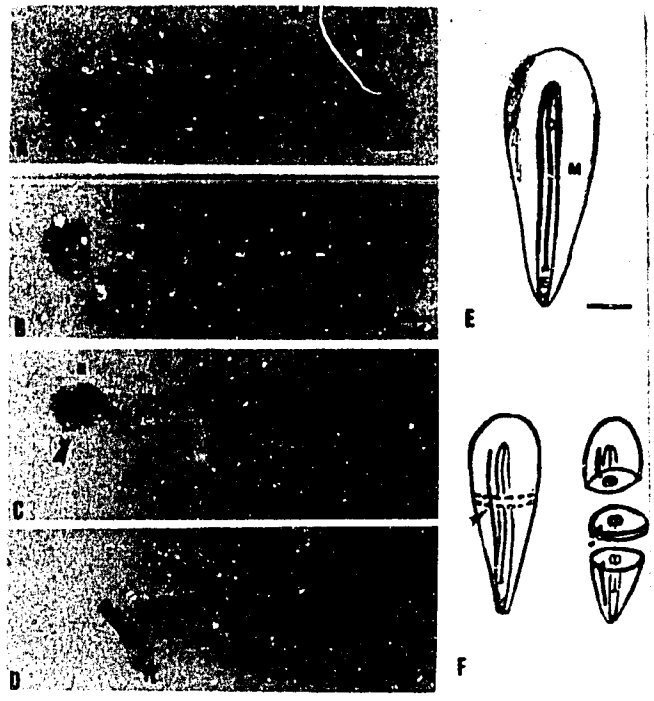


FIG. 1. Tissue immunoprints of cross-sections of seeds and longitudinal sections of root tips of seedlings of *Araucaria araucana*. (A) Cross-sections of quiescent seeds. Scale bar = 1·0 cm. (B) Cross-sections of seeds and longitudinal sections of a root tip of a seed embryo, imbibed for 48 h. Scale bar = 1·0 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 = 1·0 cm. (D) Longitudinal sections of root tips of seedlings after 96 h of imbibition. Scale bar = 1·0 cm. (E) Diagram of a longitudinal section of a seed of *Araucaria araucana*. M, megagametophyte; E, embryo; C, cotyledons. Scale bar = 1·5 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.

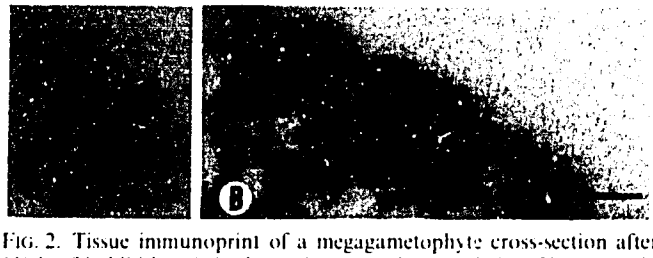


FIG. 2. Tissue immunoprint of a megagametophyte cross-section after 110 h of imbibition; it is shown in (A) at the actual size. The arrow in (A) shows the area that has been magnified in (B), where the cell walls can be observed as the cell structure giving the strongest cross-reactivity with the extensin antibody from soybean seed coats. Scale bar in (A) = 1·0 cm; in (B) = 300 nm.

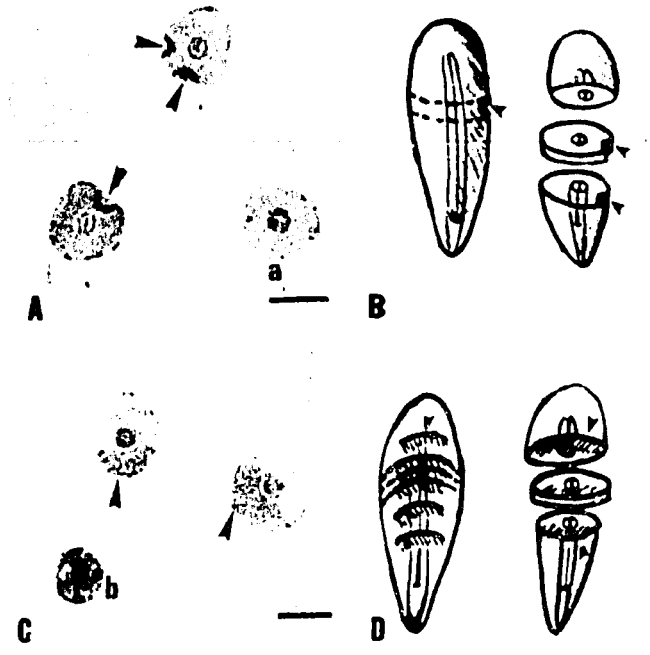


FIG. 3. Tissue immunoprints of partially wounded seeds of 48 h of imbibition. (A) Areas of cross-sections of seeds which were wounded 24 h before the printing, by removing small pieces of the surface of the megagametophyte. The wounded areas show a stronger cross-reactivity with the soybean seed coat extensin antibody (arrows). Scale bar = 1·0 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 = 1·0 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.

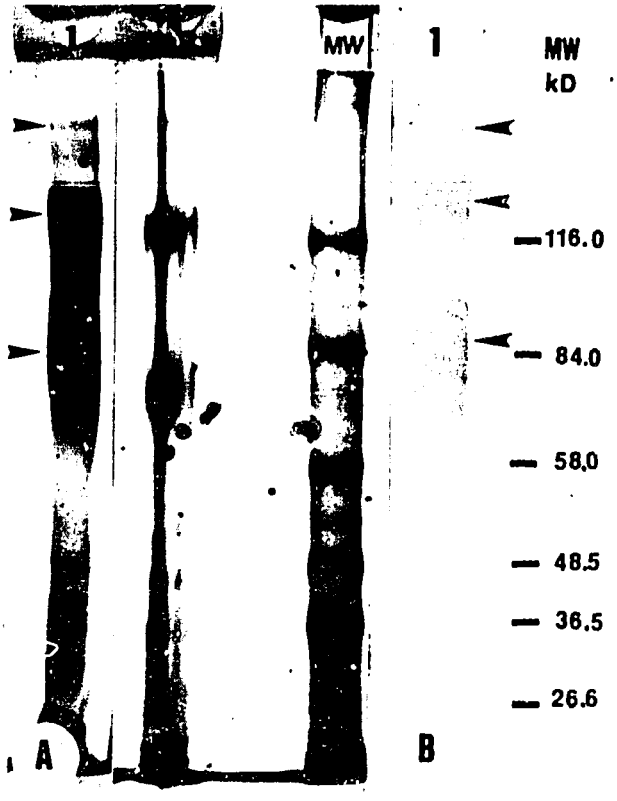


FIG. 4. SDS polyacrylamide gel electrophoresis of cell wall proteins from embryo and megagametophyte tissues. (A) Gel stained with silver nitrate. (B) Western blot of the same electrophoresis using anti HRGP raised against glycosylated extensin from carrot root tissue. Arrows show the corresponding bands 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 $CaCl_2$.

by Chen and Varner (1985) and by Tierney *et al.* (1988). In both Fig. 3A and 3C there are two control sections: one of them was completely sectioned for 48 h and is 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.

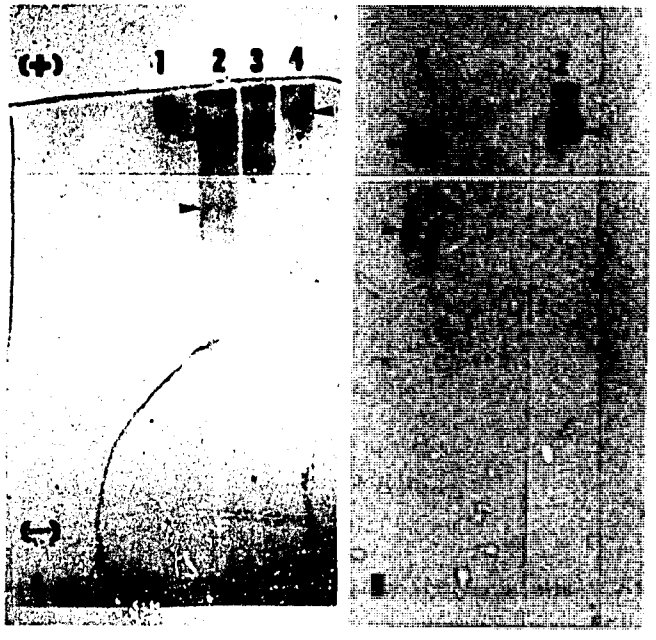


FIG. 5. Cationic neutral gel electrophoresis of cell walls of *Araucaria araucana* extracted with $Na_2S_2O_5$ and $CaCl_2$ 48 h after wounding. (A) Cationic neutral gel stained with silver nitrate stain. Lane 1: 0·3 μg of glycosylated HRGP from carrot root. Lane 2: 20 μg of *Araucaria* cell wall proteins. Lane 3: 1·0 μg of deglycosylated HRGP from carrot root. Lane 4: 1·0 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: 1·0 μg of glycosylated HRGP from carrot root. Arrows show the corresponding bands of (A) and (B).

DISCUSSION

From our studies it is clear that the cell walls of different tissues of *Araucaria araucana* seeds and seedlings contain 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, 1989).

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 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; Thomas and 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

We thank Dr Joseph E. Varner and Dr Rafael Pont Lezica from the Department of Biology, Washington University, St. Louis, MO, for extensive revision of this manuscript, and Dr Gladys Cassab and Dr Mary Tierney for their generous supply of antibodies. We also gratefully acknowledge the following supporting grants—FONDECYT 0160/88, CONICYT/NSF 84-201, AID grant DEP-5542-G-SS-8073-00.

LITERATURE CITED

BRADFORD, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, **72**, 248–54.

CARDEMIL, L., and JORDAN, M., 1982. Light and electron microscopy study of an *in vitro* cultured female gametophyte of *Araucaria araucana* (Mol.) Koch. *Zeitschrift für Pflanzenphysiology*, **107**, 329–38.

— and REINERO, A., 1982. Changes in *Araucaria araucana* seed reserves during germination and early seedling growth. *Canadian Journal of Botany*, **60**, 1629–38.

— and VARNER, J. E., 1984. Starch degradation metabolism towards sucrose synthesis in germinating *Araucaria araucana* seeds. *Plant Physiology*, **76**, 1047–54.

CASSAB, G., NIETO-SOTELO, J., COOPER, J. B., VAN HOLST, G. J., and VARNER, J. E., 1985. A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. *Plant Physiology*, **77**, 532–5.

— and VARNER, J. E., 1986. A new protein in petunia. *Nature*, **323**, 110.

— — — 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. *Journal of Cell Biology*, **105**, 2581–8.

CHEN, J., and VARNER, J. E., 1985. Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33 kDa protein (wound response). *Proceedings of The National Academy of Sciences, USA*, **82**, 4399–4403.

DROZDZ, M., KUCHARAZ, E., and SZYJA, J., 1976. A colorimetric micromethod for determination of hydroxyproline in blood serum. *Zeitschrift für Medical Labortechnik*, **17**, 163–71.

HOOD, E., SHEN, Q. X., and VARNER, J. E., 1988. A developmentally regulated hydroxyproline-rich glycoprotein in maize pericarp cell walls. *Plant Physiology*, **87**, 132–42.

KIELISZEWSKI, M., and LAMPORT, D. T. A., 1987. Purification and partial characterization of a hydroxyproline-rich glycoprotein in a graminaceous monocot *Zea mays*. Ibid. **85**, 823–7.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature*, **227**, 680–5.

LAMPORT, D. T. A., 1965. The protein component of primary cell walls. *Advances in Botany Research*, **2**, 151–218.

— 1969. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymatic degradation of primary cell walls. *Biochemistry*, **8**, 1155–63.

MONTALDO, P., 1974. La bio-ecologia de *Araucaria araucana* (Mol.) Koch. *Institute Forestal Latinoamericano de Investigación y Capacitación. Boletin*, **46–48**, 3–5.

SHOWALTER, A. M., BELL, J. N., CRAMER, C. L., BAILEY, J. A., VARNER, J. E., and LAMB, C. L., 1985. Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. *Proceedings of The National Academy of Sciences*, **82**, 6551–5.

SMITH, J. J., MULDOON, E. P., and LAMPORT, D. T. A., 1984. Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. *Phytochemistry*, **23**, 1233–9.

STUART, D. A., and VARNER, J. E., 1980. Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. *Plant Physiology*, **66**, 787–92.

THOMAS, J. M., and HODES, M. E., 1981. A new discontinuous buffer system for electrophoresis of cationic proteins at near-neutral pH. *Analytical Biochemistry*, **118**, 194–6.

TIERNEY, J. L., WIECHERT, J. H., and PLUYMERS, D., 1988. Analysis of the expression of extensin and p33-related cell

wall proteins in carrot and soybean. *Molecular and General Genetics*, **211**, 393–9.

VAN HOLST, G. J., and CLARKE, A. E., 1985. Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Analytical Biochemistry*, **148**, 446–50.

——— and VARNER, J. E., 1984. Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. *Plant Physiology*, **74**, 247–51.

VARNER, J. E., and LIN, L.-S., 1989. Plant cell wall architecture. *Cell*, **56**, 231–9.

WHITMORE, F. W., 1978. Lignin–protein complex catalysed by perioxidase. *Plant Science Letters*, **13**, 241–5.

(1) Title: Two major proteins present in the cell walls of seeds and seedlings of Araucaria araucana are peroxidases.

(2) Authors: Alejandro Riquelme and Liliana Cardemil*.
Departamento de Biología, Facultad de Ciencias, Universidad de Chile. Casilla 653, Santiago, Chile.
Phone: (562) 271-2865.
FAX: (562) 271-2983.

(3) Key Words Index- Araucaria araucana, cationic neutral gel, conifer, embryo, megagametophyte, o-Phenylenediamine, danzylhydrazine, horseradish peroxidase.

* To whom correspondence should be addressed.

Abbreviations: o-PDA, o-Phenylenediamine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Vmax, maximun velocity; Km app., apparent Michaelis constant; kDa, kilodalton; MOPS, 3-[N-morpholino]propanesulfonic acid; CAPS, 3-[cyclohexilamino]-1-propane-sulfonic acid; Bis-Tris, bis[2Hydroxyethylimino-tris[hidroxymethyl]methane; pI, isoelectric point; EU, enzyme unit; MM, molecular mass.

Fax from Dr Terence A.Smith
Executive Editor,
Phytochemistry,
Research Station,
Long Ashton,
Bristol BS18 9AF,
England

Tel. 0275 85 4317

Fax No. 0275 39 4007

To:- Dr L. Cardemil,
Departamento de Biologia
Facultad de Ciencias
Universidad de Chile
Casilla 653
Santiago, Chile

Fax No.
56 2 271 2983

Number of pages to follow:- 0

Date 9/5/92

Dear Cardemil,

Ms 128 Two peroxidases from cell walls of Araucaria araucana

Thank you for your fax of 29 April.

It seems that you have not received our letter of 26 February in which we sent the referee's report on the above paper. Since this letter appears to have been lost in the mail, I am trying to expedite the processing of your manuscript and I have now edited this and am sending it to you by air mail for revision. Please check my corrections, and rewrite your manuscript according to the instructions written on the title page. Retype only when needed for clarity, and return to me in Bristol, together with the original pages.
Please follow the Instructions to Authors given in the January copy of Phytochemistry.

We look forward to hearing from you.
Yours sincerely,

Terence A. Smith

12

(4) Abstract

Two major proteins present in the cell walls of the embryo and megagametophyte tissues of Araucaria araucana seeds were partially purified, characterized and identified as peroxidases. These two proteins have molecular masses (MM) of 83 and 145 kDa, a pI of 10.5, an optimal pH of 5.0 and a buoyant density of 1.333 $g \cdot ml^{-1}$. Kinetic studies of these peroxidases define a Km app. of 13.6 mM for H_2O_2 and 3.4 mM for o-Phenylenediamine (o-PDA). The Vmax is 525 µmol o-PDA oxidized $min^{-1} \cdot mg^{-1}$. After oxidation with periodic acid the peroxidases react on gels with dansylhydrazine demostrating that the proteins are glycosylated. The sugar components linked to the proteins are galactose, glucose, xylose and mannose. Galactose comprises more than 60% of the sugar residues in both peroxidases. The other sugar contents show significative quantitative differences between the peroxidases. Expression of the peroxidases increases upon wounding of the seed tissues. The two proteins were differently expressed during seed germination. They showed also a different susceptibility to degradation when the proteins are heated in the presence of 5M urea.

(5) Introduction

In plant cell walls there are structural proteins like the hydroxyproline-rich (1), glycine-rich (2) and threonine-rich (3), proteins and enzymes which catalyze important reactions related to cell wall metabolism. Among the enzymes there are peroxidases (E.C. 1.11.1.7) and different glycosidases such as ß-1,4-D-glucanase (E.C. 3.2.1.4, celulase), pectinases and hemicellulases, (4, 2). Most of the cell wall enzymes are involved in plant protection against pathogens and wounding (4, 5) and they also seem to have a role in cell elongation control (6). Peroxidases are the most studied plant enzymes due to their abundance in plant tissues. In cell walls, peroxidases can be found soluble or linked by ionic or covalent bonds to other components of cell walls (7). The presence of peroxidases is induced during cell wall repairing (8). Therefore, the peroxidase role could be to repair the cell wall originating an impermeable barrier to water at the site of injury by mean of aliphatic and aromatic compounds (9). Peroxidases also catalize cross-linking reactions between macromolecules such as those involved in lignine biosynthesis (10), hemicellulose and ferulic acid (11), and cross linking of proteins by isodityrosine (12) or in lysine residues that have been deaminated by oxidation (2).

We have already reported the pattern of proteins present in the cell walls of embryo and megagametophyte

14

tissues of Araucaria araucana seeds and seedlings during early growth (13). Araucaria araucana is a conifer tree whose seeds will express oxidative pathways leading to lignification during germination and early development . The seeds are also propagated by birds. These birds many times do not eat the the seeds but they wound them with their beaks. Therefore, the tissues of the seeds need to repair the injury before or during germination. In these two processes, lignification and repairing, the cell wall peroxidases may be involved.

The purpose of this work was to find out whether there are peroxidases among the cell wall proteins of Araucaria araucana seeds. Two major cell wall proteins were identified as peroxidases.

Results and Discussion

Cell wall protein pattern.

The protein pattern present of cell walls of Araucaria araucana seeds was analyzed by SDS-PAGE (Fig.1). The arrows point the two predominant proteins expressed 24 and 72 hours after seed imbibition in wound and non-wounded seeds. The proteins had MM of 145 and 83 kDa. After wounding there was a considerably increase in expression of the same two proteins. This increase in expression was correlated with other physiological events such as the protrusion of the root tips, an increment in the diameter of the hypocotyl (probably a consequence of the ethylene production caused by wounding) and an increment in the total weight of the seed. The weight change may be caused by an increase in the water content of the tissues or by the increase of other proteins not present in the cell wall of non wounded seeds, (14, 15).

Cationic neutral electrophoresis of these proteins demonstrated that they have a basic nature at a gel pH 6.8. The proteins could run on cationic neutral gels and be analyzed by electrophoresis of their native forms due to their basic nature (2, 16, 17).

Purification and separation of the two majors cell wall proteins.

The proteins were partially purified by chromatofocusing using a column made of the polybuffer exchanger PBE 118. The chromatofocusing was run in a pH gradient from 11 to 8.5 (Fig. 2). The two proteins elute together in fractions 8 to 15 at a pI of 10.5. In the insert, a photograph of a SDS-PAGE analysis corroborates the degree of purification. The specific activity and the yield percent of this purification was also determined (Table 1).

A separation of the two proteins was attempted by isopycnic centrifugation in a CsCl gradient (Fig. 3). Both proteins came together in the fractions 28 to 35. They have a buoyant density of 1.333 $g \cdot ml^{-1}$. This result suggested that these proteins are not structural proteins, namely extensin-like, because these proteins have a density of 1.44 $g\ ml^{-1}$ (6, 16, 17). The high MM of the proteins also rules out they could be arabinogalactans (18). Furthermore, the ß-glucosyl Yariv antigen (19) has demonstrated that the arabinogalactans are not present in the cell wall extracts of <u>Araucaria</u> <u>araucana</u> (13).

Identification of the two major proteins as peroxidases.

The peroxidases are enzymes frequently present in the plant cell wall of woody plants. Identification of these proteins as peroxidases was performed after cationic neutral electrophoresis of the native proteins incubating the gel with o-PDA and H_2O_2 (Fig, 4). The two major proteins gave a positive peroxidase reaction, as it does the horseradish peroxidase run on the gel as a positive control.

Kinetic characterization of the peroxidases.

A saturation curve with increasing concentration of o-PDA at a constant concentration of 20 mM H_2O_2 was obtained together with the derived graph of Lineweaver Burk for o-PDA (Fig. 5 A and B). The effect of the increment of H_2O_2 in the velocity of the reaction at a constant concentration of o-PDA of 5.5 mM with the correspondig derived graph of Lineweaver-Burk were also obtained (Fig. 5 C and D). These results give a Km app. for o-PDA of 3.4 mM and Vmax of 525 µmol of o-PDA oxidized $min^{-1} \cdot mg^{-1}$ with a Km app. for H_2O_2 of 13.6 mM. The kinetic constant for the cell wall peroxidases of A. araucana seeds in the o-PDA oxidation suggests that the binding of H_2O_2 to the active site is the rate limiting step because a four fold higher concentration of H_2O_2 than of o-PDA is needed to get 1/2 of the Vmax. The two peroxidases studied

were inhibited at high concentration of H_2O_2 (over 64 mM). Other peroxidases like those isolated from cell walls of lupines are inhibited with concentration of 0.75 mM of H_2O_2 and peroxidases of tomato cell culture are inhibited with a concentration of 0.1 mM (20). It is known that the efficiency of peroxidase oxidation is affected by the type of substrate used (21). Therefore, the two cell wall peroxidases of A. araucana seeds could have a different oxidation efficiency with their natural biological substrates present in the cell wall, which are unknown.

Optimal enzyme pH.

The pH profile studied for these peroxidases showed and optimal pH of 5.0 (Fig.6). At this pH the activity of the enzyme is 0.059 µmol of o-PDA oxidized·min^{-1}. The 1/2 of the maximal activity occurs at pH of 4.6 and 5.6. This optimal pH might suggest that these enzymes are not the responsible for the synthesis of molecular bonds which probably contributes to decrease the cell wall plasticity or they are not present or not active in the walls of those cells which elongates (6).

Characterization of the peroxidases as glycoproteins.

The wide migration pattern of these proteins in the SDS-PAGE, suggested that these peroxidases are glycoproteins.

The glycoprotein nature of these enzymes was demonstrated incubating the gel after SDS-PAGE with periodic acid and dansylhydrazine. Both proteins gave positive fluorescence bands with these reagents (Fig. 7).

Identification of the sugar residues.

The sugar residues linked to the proteins were identified by HPLC after hydrolysis of the proteins electroeluted from the gel after SDS-PAGE, (Table 2). The sugar components were compared with those present in a horseradish peroxidase (22). In both cell wall peroxidases, galactose was the predominant sugar making more than 60% of the sugar residues in both peroxidases. The glucose was the second most common sugar in the A. araucana peroxidases (36% in that of 83 kDa and a 19% in that of 145 kDa). D-N-acetyl glucosamine is the major sugar in horseradish peroxidase (44.3%) being mannose the second abundant sugar residue in the horseradish enzyme (32.1%). Arabinose and fucose were not found in A. araucana peroxidases but they are present in the horseradish with a 2% of arabinose and 9.4% of fucose.

Expression of peroxidases during development.

The expression of the enzymes during seed germination, from quiescent seeds to 96 hours of imbibition,

was quantified by scannography of a SDS-PAGE of the proteins (Fig. 8). The lanes of the gel were loaded with the amount of proteins contained in 100 mg of dried weight of cell walls. After 72 hours of seed imbibition, the 145 kDa peroxidase decreased to 27% of the original level in the quiescent seeds, while the 83 kDa peroxidase increased 20% after 24 hours of seed imbibition and remained stable up to 96 hours. The maximun peroxidase activity in the cell walls was observed after 48 hours of imbibition. At this time the seeds have already germinated (23). The increase in peroxidase activity at this time could be related to the protective role of this enzyme (cell wall repair) since Araucaria araucana seeds are propagated by birds as was mentioned in the introduction.

Conclusions

The two cell wall peroxidases studied can be analyzed in their native forms by electrophoresis run under cationic neutral conditions, they have identical pI and density. They are glycoproteins having similar sugar components. However, the differential expression of the peroxidases during development suggests that these proteins could be isoforms of the same class of peroxidase. This is supported by quantitative differences in the sugar residues present in the peroxidases and because extraction of these proteins in the presence of protease inhibitors always gives the same two forms of peroxidases. This rules out the hypothesis that the 145 kDa protein could originate the 83 kDa form by degradation. Furthermore, the 83 kDa peroxidase is resistant to heat in the presence of 5M urea while the 145 kDa degrades after 5 minutes of boiling in this reagent. Further analyses like amino acid composition, N-terminal sequence and peptide maps could confirm the idea that the two cell wall peroxidases present in seeds and seedlings of <u>Araucaria</u> <u>araucana</u> are related proteins.

(6) Experimental

Biological material. Uncoated seeds of Araucaria araucana (Mol.) Koch were sterilized in a 5% solution of commercial leach for 15 minutes at room temperature. The seeds were germinated in dark at 20°C. For the experiments three groups of seeds were transversally sectioned half a way of the seed with a razor blade (13), and three groups of intact seeds were used as control.

Purification of cell walls and proteins extraction. The cell walls were purified and extracted for proteins after purification with 0.2M $CaCl_2$ in 2mM $Na_2S_2O_5$ from the wounded and non wounded seeds using the method described (16, 17, 13). After extraction, the proteins were precipitated with 5 volumes of acetone and the protein pellet separated by centrifugation at 15,000 g. The proteins were resuspended in 0.1M Tris HCl, pH 6.8. The salt free solution containing the proteins was obtained by ultrafiltration using an Amicon membrane PM-10. The proteins were quantified by the method of Bradford (24).

Electrophoretic analyses. The cell wall proteins were analyzed by SDS-PAGE, in a 7% gel as has been described (13). A SDS-PAGE in the presence of urea, at a concentration of 5M in the concentration gel and 8M in the separation gel, was performed to evaluate if the major proteins of the wall

could have two or more peptides. Before loading the gel, the samples were heated in the presence of 5M urea at 90°C for 5, 10 and 20 minutes. Cationic neutral gel electrophoresis analysis of the native proteins were performed as described by Thomas & Hodes, (25). In all types of electrophoresis, the protein bands were visualized with silver stain reagent using the method of Nielsen and Brown (26) or 0.25% coomassie blue R-250 in 50% methanol and 10% acetic acid.

Protein purification by chromatofocusing. A glass column of 18 cm long and 1 cm of diameter was packed with a Polybuffer Exchanger (PBE 118) from Pharmacia to perform an isofocusing chromatography. The column was equilibrated with 0.025M triethylamine, pH 11. Fractions of 1 ml were collected. Aliquots of each fraction were used for protein determination by the Bradford's method and for peroxidase activity as described below.

Isopycnic centrifugation on CsCl gradient. Three g of CsCl were disolved in 3 ml of deionized water and placed in a centrifuge tube. On top of the CsCl a solution of 500 µl of cell wall protein extract containing 500 µg of protein was layered. Deionized water was added to make a volumen of 5 ml. The tubes with the samples were centrifuged in a Beckmann L5-50 centrifuge at 45,000 rpm for 72 hours at 4°C. After centrifugation the tube bottoms were pinched off to get fractions of 6 drops each. Five µl from each 5 fractions

were used to measure the refraction index using a Bausch & Lomb refractometer. The amount of proteins was determined in the fractions collected.

Identification of protein bands by peroxidase activity. Peroxidase activity was detected in native proteins run on cationic neutral gel electrophoresis using a 0.1% solution of o-PDA and 0.012% H_2O_2 as substrates. The buffer was 0.1M sodium citrate, pH 5.0 (27). After visualization of the bands the reaction was stopped using successive washes with deionized water. The gels were dried under vacuum in a gel drier.

Peroxidase assay. The peroxidase activity was detected using 5 µl of each fraction and 1 ml of 0.1% o-PDA and 0.012% H_2O_2 in 0.1M sodium citrate, pH 5.0. After 10 minutes of incubation at 25°C the reaction was stopped by addition of 10 µl of ß-mercaptoethanol to the reaction media. The absorbance was measured at 450 nm in a Schimadzu UV-240 spectrophorometer. One enzyme unit (EU) was defined as the amount of enzyme which oxidizes 1 µmol of o-PDA·min^{-1}.

Standard curve to quantify the oxidized o-Phenylendiamine. Known quantities of o-PDA in the range of 10-100 µg were added to one ml of the assay mix made up 32 mM H_2O_2, excess of horseradish peroxidase (Sigma type VI) and 0.1M sodium citrate, pH 5.0. The reaction mix was incubated at 20°C until the absorbance reached a maximun and remained stable at

450 nm. At this point is assumed that all the substrate is converted into product. In the standard curve the absorbance at 450 nm was plotted against the concentration of oxidized o-PDA.

Determination of the optimal pH of the cell wall peroxidases. The reaction mix used was 5.5 mM o-PDA and 4 mM H_2O_2 made up in the corresponding buffer to which 0.42 µg of proteins was added. For the pH range between 3-5 the buffer used was 0.1M sodium citrate; for pH 6 was 0.6 M Bis-Tris; for pH 7 was 0.7M MOPS; for pH 8-9 was 0.7M Tris-Base and for the pH range 10-11 was 0.5 M CAPS. The reaction was monitored for 10 minutes at 20°C. The activity of the enzyme for the o-PDA oxidation was calculated from the plot A_{450} v/s time and obtained in the Shimadzu UV-240 spectrophotometer and was expressed in µmol of o-PDA oxidized·min^{-1}.

Kinetics studies. Initial velocity as function of the H_2O_2 concentration. The standard conditions of the reactions were: 0.42 µg of purified proteins added to 1 ml of a mix containing 5.5 mM o-PDA and from 1 to 64 mM H_2O_2 in 0.1M sodium citrate, pH 5 incubated at 20°C. The initial velocity was obtained as described before. The kinetic parameters were calculated taking the average values of the Lineaweavwr-Burk, Hanes and Eadie-Hofstee plots.

Initial velocity as function of the o-PDA. The standard conditions of the reaction were: 0.42 µg of purified proteins

added to 1 ml of a mix containing 20 mM H_2O_2 and from 2.75 to 27.5 mM of o-PDA in 0.1M sodium citrate, pH 5 incubated at 20°C. The initial velocity was obtained as described above. The kinetic parameters were calculated taking the average values of the Lineaweavwr-Burk, Hanes and Eadie-Hofstee plots.

Identification of the peroxidases as glycoproteins. The glycosilation of the peroxidases was detected by the reaction periodic acid /dansylhydrazine (28), on gels after SDS-PAGE or electrophoresis of the native proteins under cationic neutral conditions. The protein bands were visualized by transillumination with UV light.

Identification of sugar residues present in the glycoproteins. To obtain 0.5 mg of each peroxidase, the enzymes were electroeluted from gels after SDS-PAGE. The proteins were hydrolyzed with 300 µl of 2N TFA heating for 1 hour at 121°C. The TFA was evaporated under vacuum and the product of the hydrolysis was resuspended in deionized water. The sugar analysis was performed in a Knauer HPLC type 364 equipped with a column of CARBOPACK PA1 (Dionex Corporation, USA) and using 5.25 mM NaOH as solvent. Sugars were detected with a gold electrode present on the Dionex pulsed amperometric detector.

Determination of the peroxidase expression during development. Cell wall proteins of quiescent seeds and from seeds embibed for 24, 48, 72 and 96 hours were extracted and analyzed by SDS-PAGE loading the gel with the amount of

proteins present in the 100 mg of dried cell walls. After electrophoresis, the gel was stained with silver reagent and vacuum dried in a gel dryer. Densitometry of the proteins bands was performed in a scanner Geniscan GS-4500 and using the Scannedit computarized program.

(7) Acknowledgements

We thank Dr. Joseph Varner, Department of Biology, Washington University, St. Louis, Missouri for advice and encouragement during the development of this research and Dr. Luis Corcuera, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, for critical reading of the manuscript. We are grateful of Angélica Vega and Ximena Oñate for technical assistance. This research was supported by grants: # 7,068, Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development; CONICYT/NSF 84-201; FONDECYT 0160/88 and DTI # B 2806-8923 from Universidad de Chile.

Figure Legends

Figure 1. Analysis by SDS-PAGE of the cell wall proteins present in seeds embibed for 24 hours in A and for 72 hours in B.

In A and B: Lane 1, loaded with cell wall proteins of non wounded seeds. Lane 2, loaded with cell wall proteins of wounded seeds.

Each lane was loaded with amount of proteins present in 250 mg of dried walls.

Figure 2. Chromatofocusing profile of the cell wall proteins after elution from the PBE 118 column. The column was loaded with 600 µg of crude extract of cell wall proteins.

_____ amount of proteins present in each fraction.

----- peroxidase activity

--o-- pH.

▬▬ indicates the fraction where the two proteins elute.

The insert is a SDS-PAGE of the proteins present in fractions 10-15 (lane 2) and compared with those present in the crude extract (lane 1).

Figure 3. Isopycnic centrifugation in CsCl gradient of the cell wall proteins.

___ Proteins elution profile.

--- Density gradient

▬▬ Fractions where the two proteins are found.

The centrifugation tube was loaded with 500 µg of crude extract of cell wall proteins.

Figure 4. Peroxidase activity after cationic neutral gel electrophoresis of the cell wall proteins. The electrophoretic gel was incubated with o-PDA and H_2O_2. The bands giving positive reactions are pointed by arrows in lane 2. In lane 1 was loaded horseradish peroxidase as positive control.

Figure 5. Kinetics parameters of the wall peroxidases.

A. Saturation curve of o-PDA. The amount of proteins used in each assay was 0.4 µg in 20 mM H_2O_2. The temperature of incubation was 20°C.

B. Lineweaver-Burk plot of **A.**

C. Saturation curve of H_2O_2. The amount of protein used in each assay was 0.4 µg in presence of 1 mg ml of o-PDA. The temperature of incubation was 20°C.

D. Lineweaver-Burk plot of **C.**

Figure 6. pH profile of cell wall peroxidases. The activity is expressed in µmol of o-PDA oxidized· min^{-1}.

91

Figure 7. Identification of the cell wall peroxidases as glycoproteins. The proteins were run in a SDS-PAGE and the gel was stained with periodic acid/dansylhydrazine.

Lane 1. Cell wall peroxidases.

Lane 2. Ovoalbumine run as positive control.

* Shows positive reaction for the two peroxidases.

Figure 8. Expression of cell wall peroxidases after 96 hours of seed imbibition. The plot is a densitometric analysis of the protein bands run on SDS-PAGE. The wells of the gel were loaded with the proteins present in 100 mg of dried walls of quiescent seeds (0 hours) and of seeds embibed for 24, 48, 72 and 96 hours.

___●___ Expression of the 145 kDa peroxidase.

---o--- Expression of the 83 kDa peroxidase.

References

1. Lamport, D.T.A. and Catt, J.W. (1981) in Encyclopedia of Plant Physiology (New Series) Vol. 13 B (Loewus, F.A. and Tanner, W. eds) pp. 133-165. Springer, Berlin.
2. Cassab, G.I. and Varner, J.E. (1988) Cell Wall Proteins. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 321.
3. Kieliszewki, M. J. and Lamport, D.T.A. (1987) Plant Physiol. 85, 823.
4. Mc Neil, M., Darwill, A. G., Fry, S.C. and Albersheim, P. (1984) Ann. Rev. Biochem. 53, 625.
5. Varner, J.E. and Lin. L.-Sh. (1989) Cell. 56, 231.
6. Cooper, J.B. and Varner, J.E. (1984) Plant Physiol. 76, 414.
7. Ros Barceló, A., Pedreño, M.A., Muñoz, R. and Sabater, F. (1988) Physiol. Plantarum. 73, 238
8. Davies, E. (1987) in Biochemistry of Plants (Davies, D.D., ed.) Vol. 12, pp. 243-264. Academic Press, New York.
9. Espelie, K.E., Franceshi, V.R. (1986) Plant Physiol. 81, 487.
10. Whitmore, F.W. (1978) Plant Sci. Lett. 13, 241.
11. Fry, S.C. (1983) Planta. 157, 111.
12. Fry, S.C. (1982) Biochem. J. 204, 449.
13. Cardemil, L. and Riquelme, A. (1991) J.Exp. Bot. 42, 415.
14. Bacic, A. and Delmer, D. (1981) Planta. 152, 346.

15. Hendry, G. A. F. and Jones, O.T.G. (1984) New Phytol. 96, 153.

16. Stuart, D. A. and Varner, J. E. (1980) Plant Physiol. 66, 787.

17. Cassab, G.I., Nieto-Sotelo, J., Cooper, J.B. Van Holst, G. J. and Varner, J.E. (1985). Plant Physiol. 77, 532.

18. Van Holst, G. J., Klis, F., Bouman, F. and Stegwee, D. (1980) Planta. 149, 209.

19. Fincher, G.B., Stone, B.A. and Clarke, A.E. (1983) Ann. Rev. Plant Physiol. 34, 47.

20. Everdeen, D.S., Kiefer, S., Willard, J.J., Muldoon, E.P., Dey, P.M., Li, X.B. and Lamport, D.T.A. (1988) Plant Physiol. 87, 616.

21. Chibbar, R.N. and van Huystee, R.B. (1984) Plant Physiol. 75, 956.

22. Clarke, J. and Shannon, L. M. (1976) Biochim. Biophys. Acta. 427, 428.

23. Cardemil, L. and Reinero, A. (1982) Can. J. Bot. 60, 1629.

24. Bradford, M.M. (1976) Anal. Biochem. 72, 248.

25. Thomas, J.M. and Hodes, M. E. (1981) Anal. Biochem. 118, 194.

26. Nielsen, B.L. and Brown, L.R. (1984) Anal. Biochem. 141, 311.

27. Cassab, G.I., Lin, J.J., Lin, L.S. and Varner, J.E. (1988) Plant Physiol. 88, 522.

28. Estep, T.N. and Miller, T.J. (1986) Anal. Biochem. 157, 100.

94

Table 1. Partial purification by chromatofocusing in PBE 118 of the cell wall peroxidases.

					% Yield	
Step	Volumen ml	Protein mg	Units of Activity [a]	Specific Activity [b]	Protein	EU
Crude Extract	50	0.576	92.8	161.1	100	100
PBE-118	3	0.078	27.4	351.2	14	30

The PBE 118 column was loaded with 600 ul of cell wall extract after equilibration with 0.25 mM of triethylamine at pH 11. In the fractions collected the amount of protein and the peroxidase activity was determined.

a. EU, enzyme units: µmol o-PDA oxidized·min^{-1}

b. Specific activity: µmol o-PDA oxidized· min^{-1} mg^{-1} of protein.

Table 2. Sugar composition of cell wall peroxidases.

Sugar	83 kDa protein	145 kDa protein	Horseradish * peroxidase
	µg sugar/mg protein		
D-galactose	72.4 ± 2.9 (61.1%)	76.8 ± 1.6 (75.1%)	0.0 (0.0%)
D-glucose	42.8 ± 2.0 (36.1%)	19.6 ± 3.6 (19.2%)	5.7 (3.8%)
D-xylose	1.4 ± 0.3 (1.2%)	1.3 ± 0.5 (1.3%)	12.8 (8.5%)
D-mannose	2.0 ± 0.6 (1.6%)	4.5 ± 0.9 (4.4%)	48.2 (32.1%)
L-arabinose	0.0 (0.0%)	0.0 (0.0%)	2.9 (1.9%)
D-N-Acetyl glucosamine	N.D.	N.D.	66.5 (44.3%)
Carbohydrate in the protein	12	10	15

The sugar composition was obtained after hidrolysis of the protein bands electroeluted from polyacrylamide gels after SDS-PAGE. HPLC was performed in Knauer HPLC type 364 equipped with a CARBOPACK PA1 column using 5.25 mM NaOH, pH 11.5, as solvent. The gas chromatograph was provided with a gold electrode present in the Dionex pulsed amperometric detector.

N.D. not determined

* from Clarke and Shannon, 1976 (22).

± s.d.

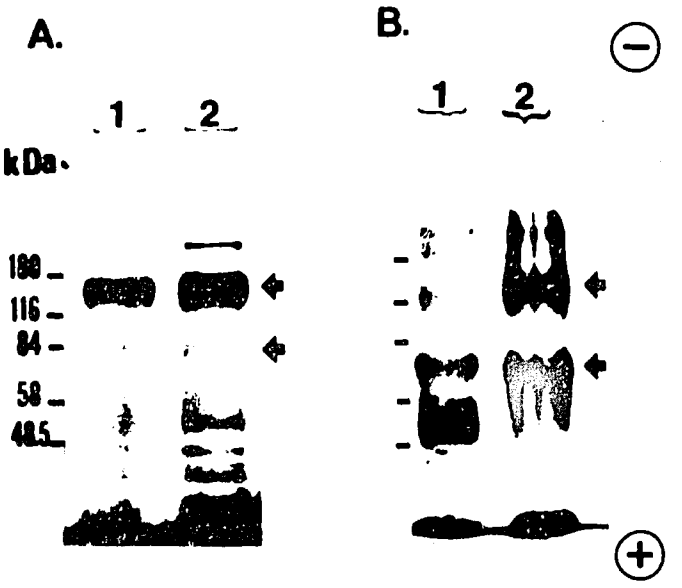


Figure 1

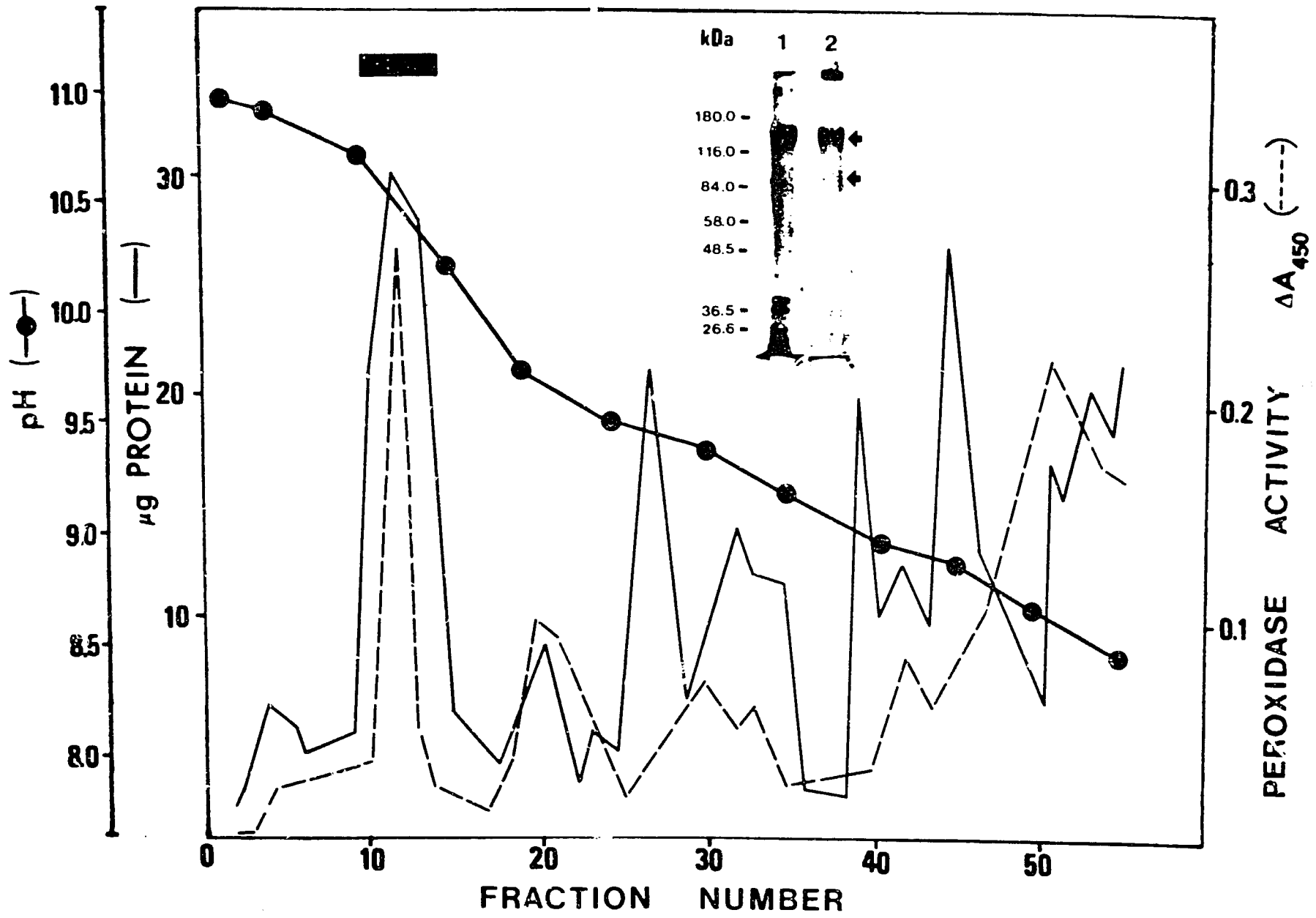

kDa
1
2
180.0
116.0
84.0
58.0
48.5
36.5
26.6
11.0
10.5
10.0
9.5
9.0
8.5
8.0
pH
30
20
10
µg PROTEIN
0.3
0.2
0.1
PEROXIDASE ACTIVITY
ΔA450
0
10
20
30
40
50
FRACTION NUMBER

Figure 2

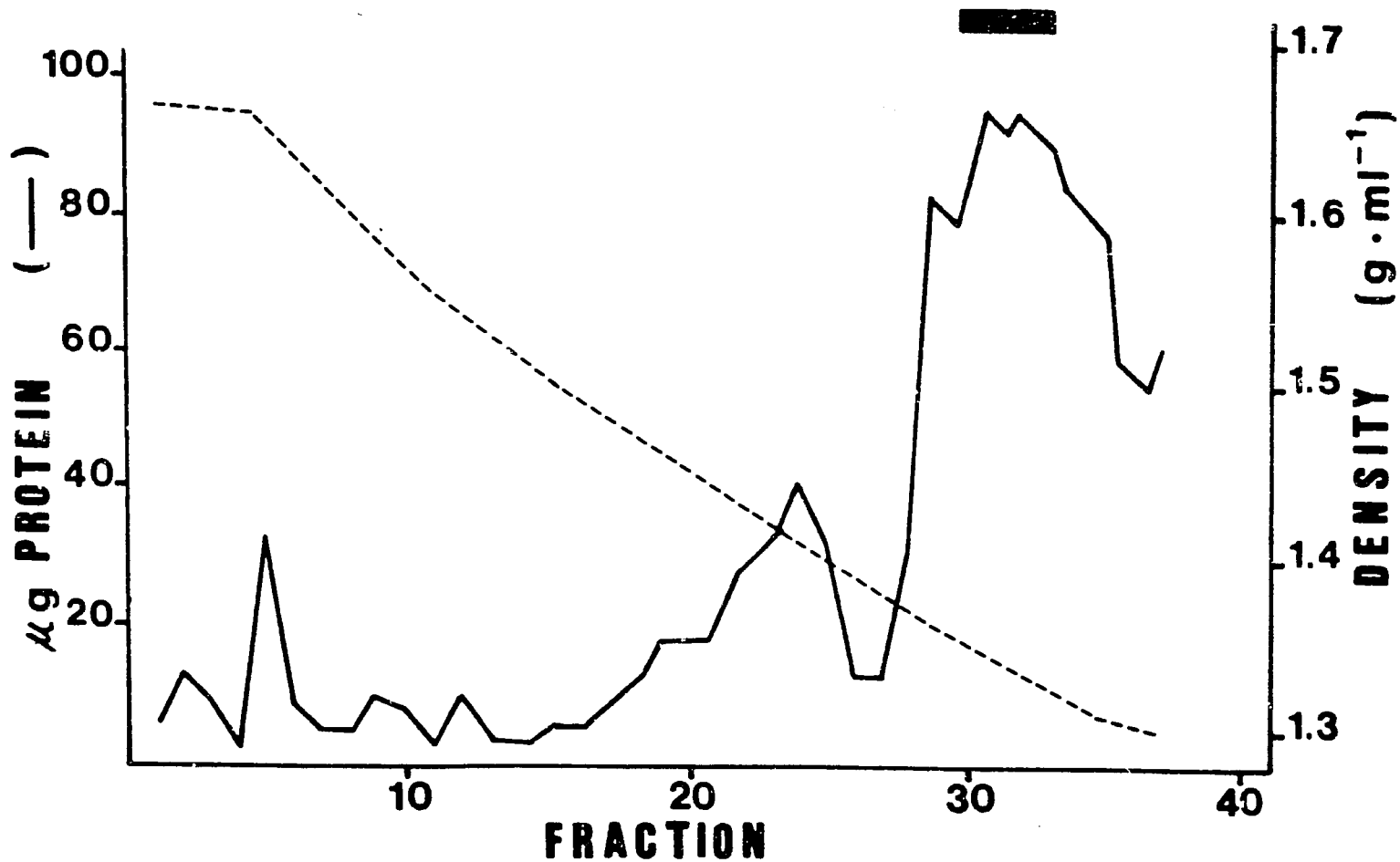

100
80
60
40
20
μg PROTEIN (——)
1.7
1.6
1.5
1.4
1.3
DENSITY (g·ml⁻¹)
10
20
30
40
FRACTION

Figure 3

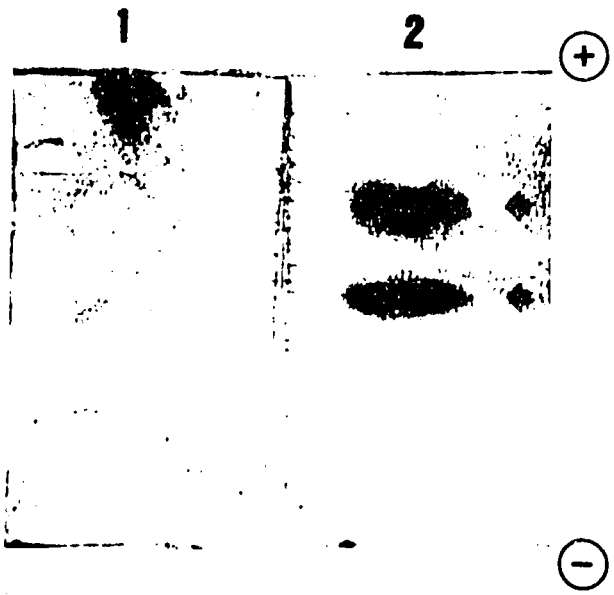


Figure 4

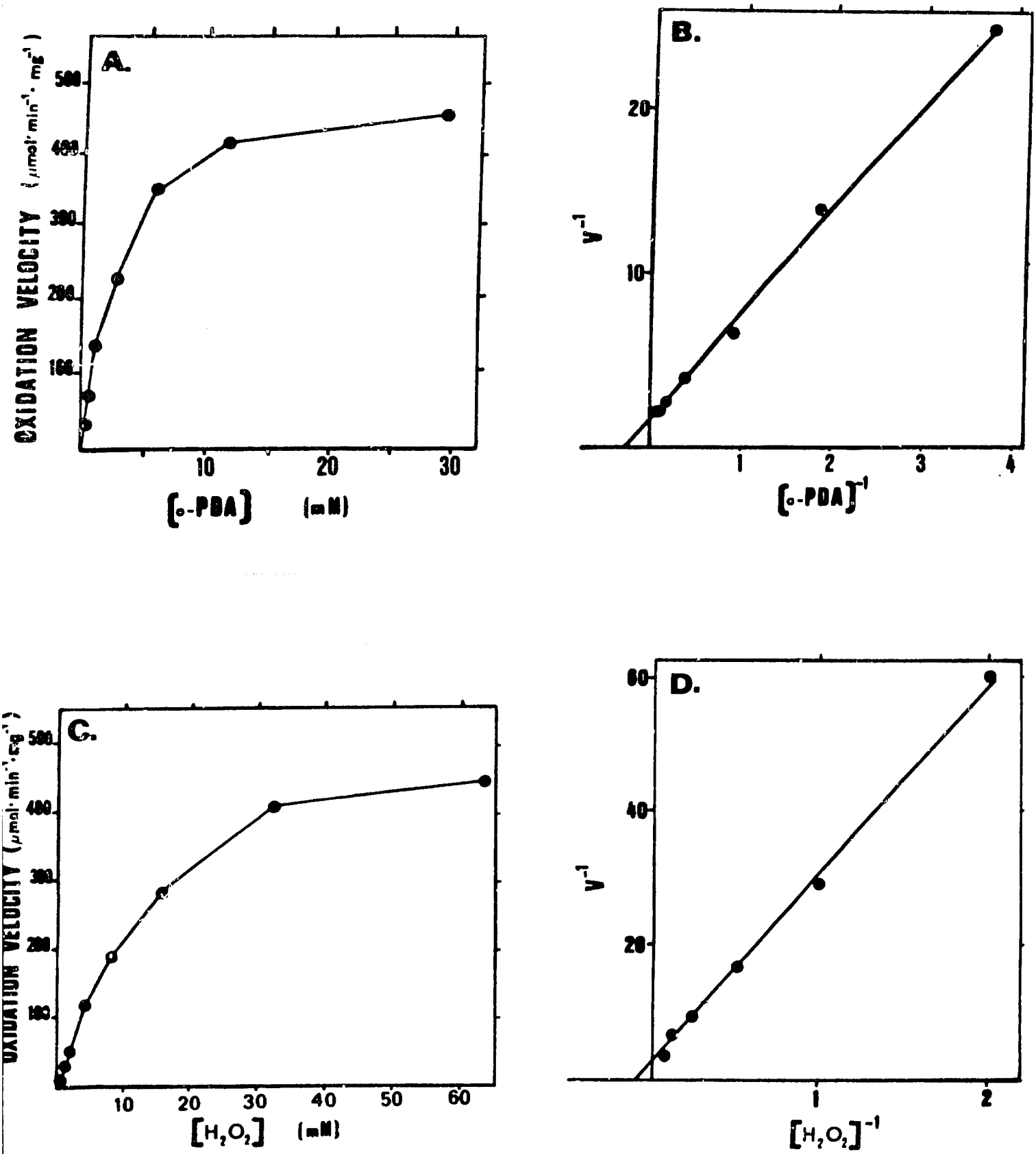

A.
OXIDATION VELOCITY (μmol·min⁻¹·mg⁻¹)
500
400
300
200
100
10
20
30
[o-PDA] (mM)
B.
20
10
V⁻¹
1
2
3
4
[o-PDA]⁻¹
C.
OXIDATION VELOCITY (μmol·min⁻¹·mg⁻¹)
500
400
300
200
100
10
20
30
40
50
60
[H₂O₂] (mM)
D.
60
40
20
V⁻¹
1
2
[H₂O₂]⁻¹

Figure 5

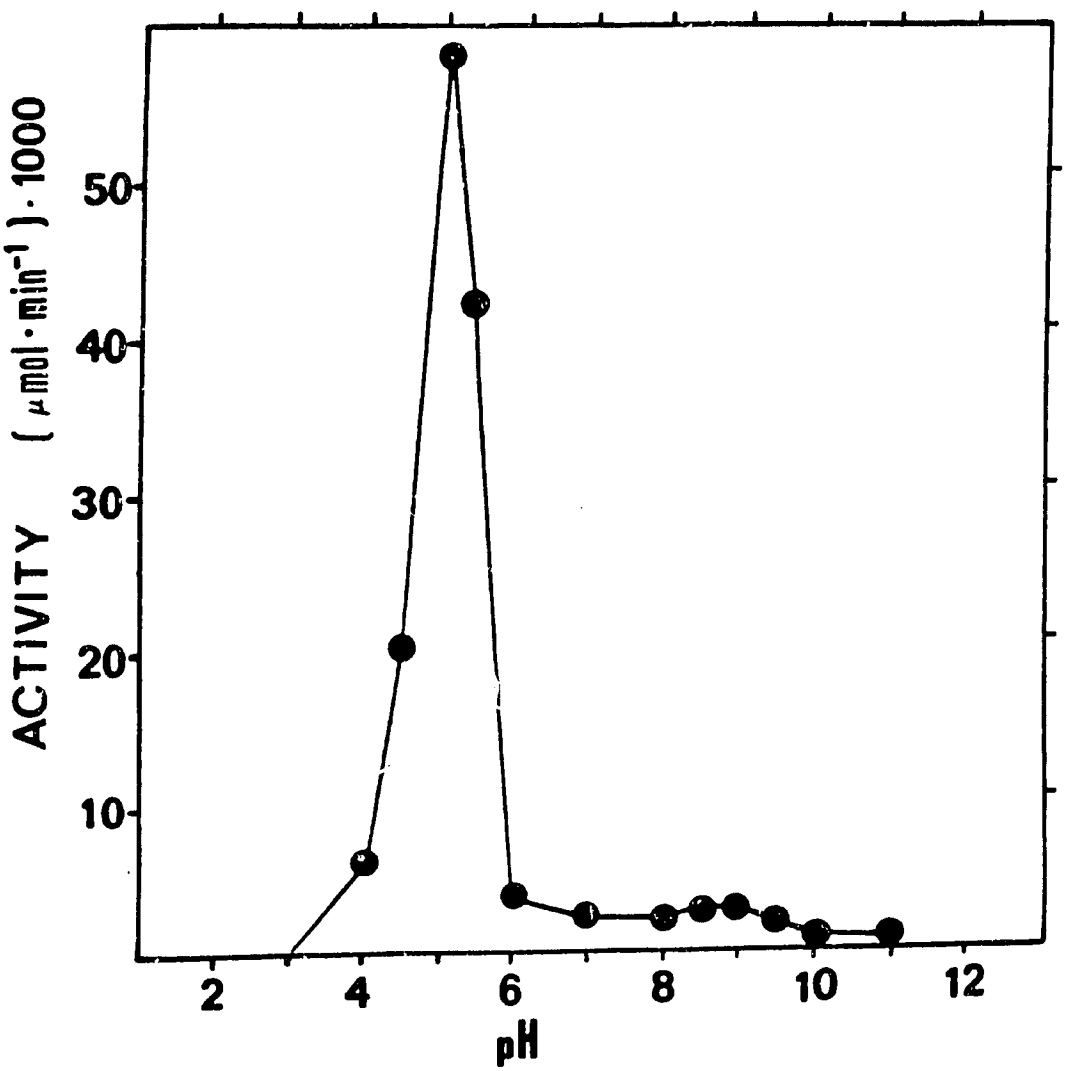


Figure 6

102

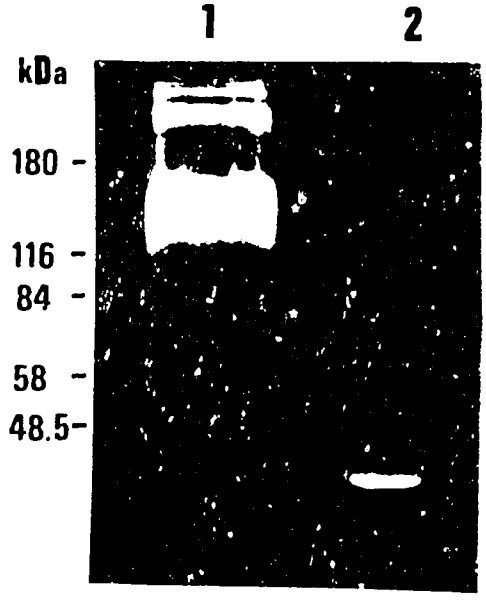


Figure 7

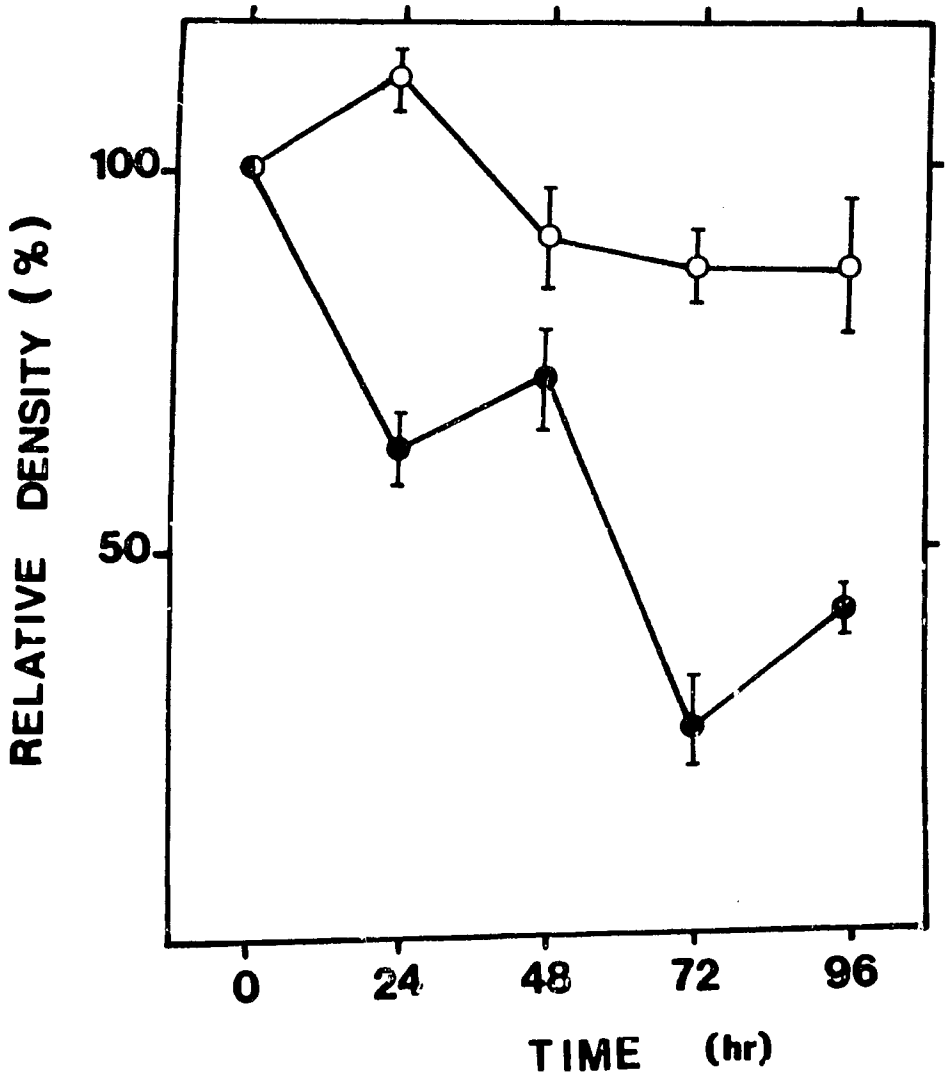


Figure 8

(i) **Title:** *Prosopis chilensis* is a plant highly tolerant to heat shock.

Running title: Thermotolerance in *Prosopis chilensis*

(ii) **Author's names:** Consuelo Medina[1]
Liliana Cardemil[2]

(iii) **Institution address:** Departamento de Biología,
Facultad de Ciencias,
Universidad de Chile, Casilla 653,
Santiago, Chile.
Telephone: (56-2) 2712865, extension 221.
FAX: (56-2) 2712983.

(iv) **Footnotes:**

1. Present address: CINVESTAD, Apartado Postal 629, Irapuato, Guanajuato, México.

2. Liliana Cardemil, Casilla 653, Santiago, Chile, to whom proofs and offprints requests are to be sent.

105

Abstract.

At temperatures from 25 to 35°C, 100 % of *Prosopis chilensis* seeds germinate within 24 hours. At higher temperatures the germination rate is reduced; 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 50°C, suggesting that thermotolerance is induced during seed germination at 35°C. Further thermotolerance can be induced in seedlings germinated at 35°C, by exposing them to 40°C for two hours. Under these conditions, seedlings increase their 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 in the presence of [^{35}S]methionine, reveals the expression of eleven proteins not detectable at 35°C. Most of the proteins present at 35°C also increase in expression. The temperature for maximal expression of these proteins is 45°C. We conclude that *Prosopis chilensis* has a thermotolerance about 5°C higher than other plants.

106

Key-word index

Heat shock, thermotolerance, lethal temperature, sublethal temperature, protein synthesis.

Introduction.

The greenhouse effect due to the increase of CO_2 content in the atmosphere, along with the rapid deforestation of the earth, are current problems. They have created the need for a rapid search for living organisms, among them plants, with high adaptability to present environmental conditions. These adaptable plants could be also considered as natural gene banks. Therefore, the overall planet's global environmental problems have urged scientists to look for genetic variability among native plants which can tolerate stress conditions and for methods to propagate and conserve these plant species.

For many years botanists have considered that Prosopis species as good examples of native plants resistant to environmental stress, because they are present in arid areas, subject to extreme temperatures and grow on salty soils, (Felker, Clark, Nash, Osborn & Cannell, 1982; 1983). P. chilensis is a leguminous tree with a high degree of genetic variability in natural populations, due to its high outbreeding by cross pollination and to its wide geographic distribution (Hunziker, Naranjo, Palacios & Poggio, 1977). Natural populations of Prosopis chilensis, forming small monospecific forests, are found

in the arid and semi arid regions in Chile. The species has a geographic distribution from Copiapó in the North (27°S), to Santiago in the South (33°S), and from the Central Valley (West) to 2,400 m altitude in the Andes (East). In these regions daily temperatures range from 6°C at night to 50°C in the afternoon of Summer days (Hunziker et al., 1977; Balboa, Parraguez & Arce, 1991).

Although P. chilensis has been considered a plant resistant to heat stress up to now there has been no evaluation of the physiological and molecular responses of Prosopis species to this type of stress. For the past three years, we have been studying the physiological and molecular responses of the seedlings of P. chilensis to thermal stress. It is well known that plants respond to high temperatures as do all living organisms, by the synthesis of a new set of proteins which are the heat shock proteins, hsp (Lin, Roberts & Key, 1984).

This study presents the results of seed germination and seedling growth of P. chilensis when subjected to temperatures from 25° to 50°C. The acquisition of thermotolerance and the pattern of proteins synthesized under heat shock, are also reported.

Materials and Methods.

Source of material. Seeds of Prosopis chilensis (Mol.) Koch were collected in Peldehue 35 Km East of Santiago at 33°S latitude, 1988 and 1989.

Seed germination. Seeds not infected by bruchid beetles were selected for germination. Seeds were first scarified for 15 minutes in concentrated sulfuric acid and rinsed for 30 minutes with abundant running water. After the rinse, seeds were soaked for one hour in deionized water and transferred to germination trays containing vermiculite moistened with tap water. The trays containing the seeds were covered with plastic wrap and placed in growth chambers under high humidity 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 percentage was determined using the criterion of protrusion of roots 2 mm long through the seed coat.

Determination of lethal and sublethal temperatures for seedling growth. For these experiments two groups of seedlings imbibed for 48 hours were used: seedlings germinated at 25°C, and seedlings germinated at 35°C. All the seedlings were presoaked for three hours at 25° and 35°C respectively, in

sterilized vermiculite moistened with tap water. After this pretreatment the lengths of the embryo axes were measured and groups of 50 seedlings per tray were placed on fresh wet sterile 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. The length of the axis was measured again after 24 and 48 hours. The chambers were mantained under high humidity during the temperature treatment.

Induction of thermotolerance. Groups of 50 seedlings each, 48 hours after germination at 35°C, were presoaked as described above for two hours at 35°C, after which the lengths of the embryo axes were measured. The seedlings were then placed in wet fresh vermiculite and subjected for two hours to 35° or 40°C, prior to incubation for 24 hours at 45°C or 50°C. Two other groups of seedling were subjected to 50°C for 24 hours, without pretreatment of 40°C. After the high temperature treatment the lengths of the axes were measured. Control seedlings were maintained at 35°C for the whole experimental period.

Incorporation of [^{35}S]methionine. Seedlings grown for 48 hours after germination at 35°C, were cut in 1 cm sections. Four groups containing pieces of 15 embryos each, were placed in trays with sterile moistened vermiculite and incubated for two hours at 35°C in chambers. After this period the sections of each group

were washed in running water and soaked in an aerated solution containing the mineral nutrients of Murashige and Skoog's media (Murashige & Skoog, 1962) and subjected for two hours at 35°, 40°, 45° and 50°C. [^{35}S]methionine with specific radioactivity of $3.5 \cdot 10^{-7}$ MBq $mmol^{-1}$ (from ICN Radiochemicals) was added to the water at the beginnig of the two hours of treatment to obtain a concentration of 1.5 MBq ml^{-1}. The labelled proteins were those accumulated during the two hours of incubation in the presence of [^{35}S]methionine.

Extraction of proteins. Proteins labelled with [^{35}S]methionine were extracted by homogenizing the tissues in the buffer of Laemmli (Laemmli, 1970). The homogenate was kept for two minutes in a water bath at 100°C. The incorporation of [^{35}S]methionine was determined in 10 μl aliquots of the protein extracts placed on a disk of Whatmman 3 MM filter paper 1.4 cm in diameter. The disk was soaked for 60 min in 10% TCA containing 0.1 M cold methionine, then the radioctivity measured on the disk as described by Mans and Novelli (1960) and Goycoolea and Cardemil (1991), in a Beckman liquid scintillation spectrophotometer.

Quantification of the proteins in the extracts was performed by Bradford's method (Bradford, 1976), 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 μg^{-1} was considered a good incorporation.

Protein electrophoresis. The proteins labelled with [^{35}S]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 either the same amount of radioctivity or the same amount of total protein (about 30 μg per channel). 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% Coomassie Blue 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. To detect the proteins synthesized and accumulated during thermal stress, protein bands were visualized by the radioactivity of the [^{35}S]methionine incorporated in the proteins. For this purpose, after electrophoresis, gels were washed twice with 10% acetic acid and 40% ethanol for 30 minutes each time. Afterwards, the gel was submerged in a 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 gels with distilled water for 2 1/2 hours, then the gels were dried under vacuum in between two sheets of cellophane. The dried gel was exposed to Kodak X Omat film at - 80°C for 4 days. Densitometry of the proteins bands was performed in a Geniscan

CS-4500 scanner, using the Scannedit computer program. The intensities of the radioactive bands were recorded as peaks and the integrated areas of the peaks were compared among the bands of the same protein expressed at different temperatures, but not among proteins of different molecular masses. For a particular protein, one + sign was asigned to the peak integrated area of the band showing minimal expression, and 2 or more + for multiples of this area.

Results.

At temperatures of 25°, 30° and 35°C, 90 to 100 % of the seeds of Prosopis chilensis germinate in 24 hours (Fig. 1). At 40°C the germination rate decreases 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. No seeds germinated at 50°C.

The growth of the seedlings of Prosopis chilensis was determined by measuring the lengths of the embryo axes at temperatures, from 25° to 50°C, 24 and 48 hours after germination at 25°C (Fig. 2). In all the temperature treatments, the total length of the seedlings at 48 hours is almost three times the length after 24 hours. The temperature for maximal seedling growth is 35°C. At this temperature the lengths of the seedlings are about three times greater than at 25°C. At 50°C there is no growth.

When seeds are germinated at 35°C and the seedlings are grown at temperatures of 35°C or more, the temperature for maximal 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 is 40% greater than that achieved by seedlings germinated and grown at

35°C and three times the length of 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 seedlings can now grow at 50°C (Fig. 3 B).

Because the temperature of maximun seedling growth is displaced from 35° to 40°C when seeds are germinated at 35°C, the question may be asked whether the thermotolerance acquired during germination could be increased further by exposure of the seedlings to the sublethal temperature of 40°C prior to a treatment at 45°C and 50°C. To answer this question the lengths of the embryo axes were measured after a treatment for two hours at the temperature of growth of 40°C, followed by an exposure for 24 hours to the temperature of 45°C or 50°C. The growth of the seedlings under these treatments was compared with the growth of seedlings going directly from 35°C to the thermal treatment of 45° and 50°C (Fig. 4). The results show that the seedlings treated at 45°C after exposure for two hours at 40°C grow 38% more 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% greater than the seedlings exposed directly from 35° to 50°C ($p < 0.01$ for each pair by Student's Test). Therefore, a further acquisition of thermotolerance is achieved by seedlings of <u>Prosopis chilensis</u> when these are subjected to a treatment for two hours at 40°C prior to a treatment at 45° or at 50°C.

116

The pattern of the proteins synthesized and accumulated during two hours of incubation of embryo axes at 35°, 40°, 45° and 50°C in the presence of [^{35}S]methionine was studied (Fig. 5). The results of the fluorography of the SDS-PAGE analysis of the extracted proteins reveal that eleven proteins not detectable at 35°C are expressed in the seedling axes treated at temperatures over 35°C. The molecular masses of these proteins are 165, 108, 103, 95, 83, 60, 58, 50, 36, 31 and 24 kDa. Proteins expressed under heat shock have been called heat shock proteins, hsp (Kimpel & Key, 1985). Ten out of the eleven bands appear at 40°C, the most prominent of the proteins being the one of 58 kDa. The protein of 95 kDa is only present at 45° and 50°C. In the seedlings there are many other proteins which, being present at 35°C, have greater expression at 40° and 45°C. The three most prominent are the bands of 80, 71 and 69 kDa. At 50°C the accumulation of proteins decreases considerably. However, there are five proteins, with molecular masses of 103, 80, 71, 69 and 58 kDa which are strongly accumulated at 50°C. Of these, the proteins of 103 and 58 kDa are not expressed at 35°C, while the proteins of 80, 71 and 69 kDa increase in expression under heat shock (Table 1). From these results, it is concluded that the temperature for maximal protein accumulation in the embryo axes of P. chilensis is 45°C.

Discussion.

The seeds of Prosopis chilensis seem to be very able to germinate at high temperatures. One hundred percent of the seeds germinate in 24 hours at 35°C, and 90% germinate in 48 hours at 40°C, temperatures which are considered extreme for germination in most plants (Bewley & Black, 1982). As an example, in seeds of Araucaria araucana, a South American conifer, germination is reduced by 60% at 40°C . From 35° to 40°C are considered sublethal temperatures for growth of seedlings in A. araucana, 35°C being the temperature for maximal induction of thermotolerance and maximal expression of the hsp (Cardemil & Reinero, 1982; Goycoolea & Cardemil, 1991). The results of germination of seeds of P. chilensis also demonstrate that the population of seeds is heterogeneous, since a few seeds have a slower germination rate at 25° and 30°C and only a few germinate at 45°C. This is a lethal temperature for most seeds of P. chilensis and other plant species.

During germination at 35°C, the seeds of Prosopis chilensis acquire thermotolerance, because the temperature for maximal growth becomes 40° instead of 35°C and some seedlings can now grow at the temperature of 50°C. The induction of thermotolerance during germination at 35°C has to be investigated further to understand the physiological and molecular mechanisms for this protection.

118

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° and 50°C. In other species the exposure to the temperature of 50°C cannot go beyond two to three hours after the treatments at the sublethal temperatures which induce the thermotolerance (Lin, Roberts & Key, 1984). Seedlings of P. chilensis actually grow during the 24 hour treatment at 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, eleven proteins not detectable at 35°C are observed at the sublethal temperatures of 40° and 45°C, with a maximal protein accumulation at 45°C.

These proteins are not the result of expression caused by other stress conditions such as mechanical or wound stress because they are not expressed in control seedlings. Additionally, antibodies raised against proteins that are expressed under wound stress do not cross react with the proteins expressed under heat shock (unpublished results).

The hsp characteristics of living organisms may be summarized: a) The hsp are synthesized under high temperature stress, a 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 returned to normal temperature. d) There are hsp of high medium and low molecular masses, the last being peculiar to the plant kingdom (Cardemil, 1985).

In general we may conclude that the hsp of Prosopis chilensis fit the characteristics of these proteins mentioned above.

Among the hsp of Prosopis chilensis there are two prominent bands of 69 and 58 kDa. The protein of 69 KDa is expressed constitutively while the one of 58 kDa is expressed from 40° to 50°C with maximun accumulation at 45° and 50°C.

Three other bands, less prominent but also strongly accumulated during heat shock, have molecular masses of 103, 80 and 71 kDa. Of these the protein of 103 kDa is the only one not detectable at 35°C.

Heat shock proteins of 71, 69 and 60 kDa have been reported to be related proteins which function as chaperone proteins (Rothman, 1989; Gatenby, Viitanen & Lorimer, 1990). In P. chilensis, as in other organims, the 71 and 69 kDa proteins are expressed constitutively. In this species, the 58 kDa protein also seems to be related to the 71 and 69 kDa proteins because monoclonal antibodies raised against a 72 kDa hsp of HeLa cells cross react with those three bands (data not shown). This cross reactivity is not surprising, since a high degree of conservation in the amino acid sequence of these

proteins as well as in the encoding region of their respective genes has been reported (Rochester, Wiener & Sha, 1986; Vierstra, 1987).

In Prosopis chilensis as in the case of Araucaria araucana there are hsp of molecular masses larger than 116 kDa (Goycoolea & Cardemil, 1991). In corn the largest molecular mass protein is of 89 kDa (Cooper & Ho, 1983), while in tobacco protoplasts hsp of 120 and 100 kDa have been reported (Meyer & Chartier, 1983).

In our analysis we detected one low molecular mass protein (24 kDa) expressed under heat stress. The low molecular mass proteins have been reported to be present only in the plant kingdom (Nieto-Sotelo, Vierling & Ho, 1990). The roles of these low molecular mass hsp are unknown, although they could play a role in membrane compartmentation and stabilization (Vierling, 1990).

The temperature for maximal 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, another native tree of South America, while in most the cultivated plants the synthesis and the accumulation of normal proteins decline under heat shock (Key,

Lin & Chen, 1981; Cooper, Hauptmann & Ho, 1984). All our results suggest that Prosopis chilensis is a plant with high 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 Aaraucaria araucana (Goycoolea & Cardemil, 1991).

Acknowledgements. The technical assistance of Angélica Vega and Ximena Oñate from the Department of Biology, Facultad de Ciencias, Universidad de Chile is acknowledged. This research was supported by the grant N° 7.068, Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development; FONDECYT 0160/88; CONICYT/NSF N° 84-201 and DTI N° B 2806-8923.

References:

Balboa, O., Parraguez, J.M. & Arce, P. (1991) Phenology studies of Prosopis species growing in Chile. Annals of 2nd International Meeting of Prosopis juliflora, Recife, Pernambuco, Prazil, 1986). FAO (ed.). 259-267.

Bewley, J.D. & Black, M. (1982) Physiology and Biochemistry of Seeds in Relation to Germination. Springer-Verlag, Berlin, Heilderberg, New York. Vol. 2, p. 168-314.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation for microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry. 72, 248-254.

Cardemil, L. & Reinero, A. 1982. Changes in Araucaria araucana seed reserves during germination and early seedling growth. Canadian Journal of Botany. 60, 1629-1638.

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

Cooper, P. & Ho, D. T. (1983) Heat shock proteins in maize. Plant Physiology. 71, 215-222.

Cooper, P. Ho, T.D. & Hauptmann, R.M. (1984) Tissue specificity of heat shock proteins in maize. Plant Physiology. 75, 431-441.

Felker, P. Clark ,P.R. Nash, P., Osborn, J.F. & Cannell G.H. (1982) Screening Prosopis (mesquite) for cold tolerance. Forestry Science. 28, 556-562.

Felker, P. Cannell, G.H. Clark, P.R. Osborn, J.F. & Nash, P. (1983). Biomass production of Prosopis species (mesquite), Leucaena, and other leguminous trees grown under heat/drought stress. Forestry Science. 29, 592-606.

Gatenby, A.A. Viitanen, P.V. & Lorimer, G.H. (1990) Chaperonin assisted polypeptide folding and assembly: Implications for the production of functional proteins in bacteria. Trends in Biotechnology. 5, 354-357.

Goycoolea, C. & Cardemil, L. (1991) Expression of heat shock proteins in seeds and during early seedling growth

of Araucaria araucana as a response to thermal stress. Plant Physiolgy and Biochemistry. 29, 213-222.

Hunziker, J.H., Naranjo, C.A., Palacios, R.A. & Poggio, L. (1977). Chromosomal cytology and hybridization. In Mesquite, its biology in two desert ecosystems. Simpson, B.B. (ed.). Dowden, Hutchinson & Ross, Stroudsburg. 56-59.

Key, J. L. Lin, C. Y. & Chen, Y. M. (1981) Heat shock proteins of higher plants. Proceedings of The National Academy of Science, USA. 78, 3526-3530.

Kimpel, J.A. & Key, J.L. (1985) Heat shock in plants. Trends Biochemical Sciences. 9, 353-357.

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

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

Lin, Ch-Y., Roberts, J.K. & Key, J.L. (1984) Acquisition of thermotolerance in soybean seedlings. Plant Physiology. 74, 152-160.

Mans, R. & Novelli, G.D. (1960) A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochemistry Biophysics Research Communication. 3, 540-548.

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

Nieto-Sotelo, J. Vierling, E. & Ho, T-H. D. (1990) Cloning, sequence analysis and expression of a cDNA encoding plastid-localized heat shock protein in maize. Plant Physiology. 93, 1321-1328.

Rochester, D.E. Winer, J.A. & Shah, D.M. (1986) The structure and expression of maize genes encoding the major heat shock protein, hsp 70 KDa. EMBO J. 5, 451-458.

126

Rothman, J.E. (1989) Polypeptide chain binding proteins: Catalysts of protein folding and related processes in cells. Cell. 59, 591-601.

Vierling, E. (1990) Heat shock proteins: function and expression. In Stress Responses in Plants: Adaptation mechanisms, Alscher R. (ed.). Alan R. Liss, Inc. N.Y. 357-354.

Vierstra, R.D. (1987) Ubiquitin, a key component in the degradation of plant proteins. Physiologia Plantarum. 70, 103-106.

Figure Legends.

Figure 1. Percentage of seed germination of *Prosopis chilensis* at different temperatures. Seeds were germinated at temperatures from 25° to 50°C. White bars represent the germination percentage after 24 hours of imbibition. The dotted bars are the germination percentage 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 seedling 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. **Compararison of growth of seedlings treated at different temperatures for 24 hours after germination at 25° and at 35°C.** In **A**, groups of seedlings 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 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. The average length of seedlings grown at 35°C was considered as 100 %.

Figure 4. **Thermotolerance induction in seedlings 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 lengths 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 transferred 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.** Protein accumulation corresponding 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 consisted 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 proteins (hsp) not detectable in the control axes incubated at 35°C. The arrow heads point to those prominent bands which increase in radioactive intensity upon exposure to high temperatures. M. wt. are the molecular weight standards run in the same gel: α2- Macroglobulin (180 kDa); ß-Galactosidase (116 kDa); Fructose-6-Phosphate Kinase (84 kDA); Pyruvate Kinase (58 kDa); Fumarase 48.5 kDA); Lactic Dehydrogenase (36.5 kDa); Triosephosphate Isomerase (26.6 kDa).

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

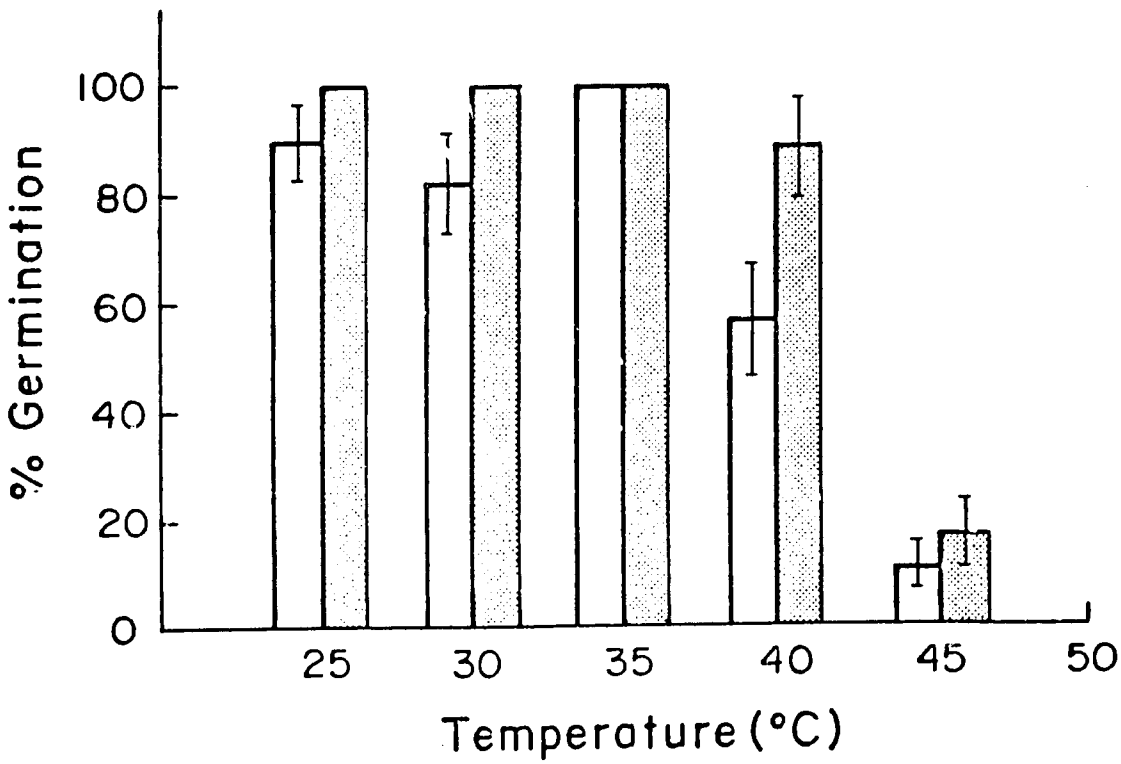
*** Molecular mass of the hsp not present at 35° C.**

+ Relative intensities of the radioactive bands.

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

Molecular Mass (kDa)	Control	40°	45°	50°
165*	-	+	++	-
108*	-	+	+	-
103*	-	+	++	++
95*	-	-	+	++
83*	-	++	+++	+
80	+	++	+++	+++
71	+	++	+++	+++
69	+	++	+++	++++
60*	-	+	++	++
58*	-	+	+++	++++
50*	-	+	+	-
36*	-	+	++	-
31*	-	+	++	-
24*	-	++	+++	+

Figure 1



132

Figure 2

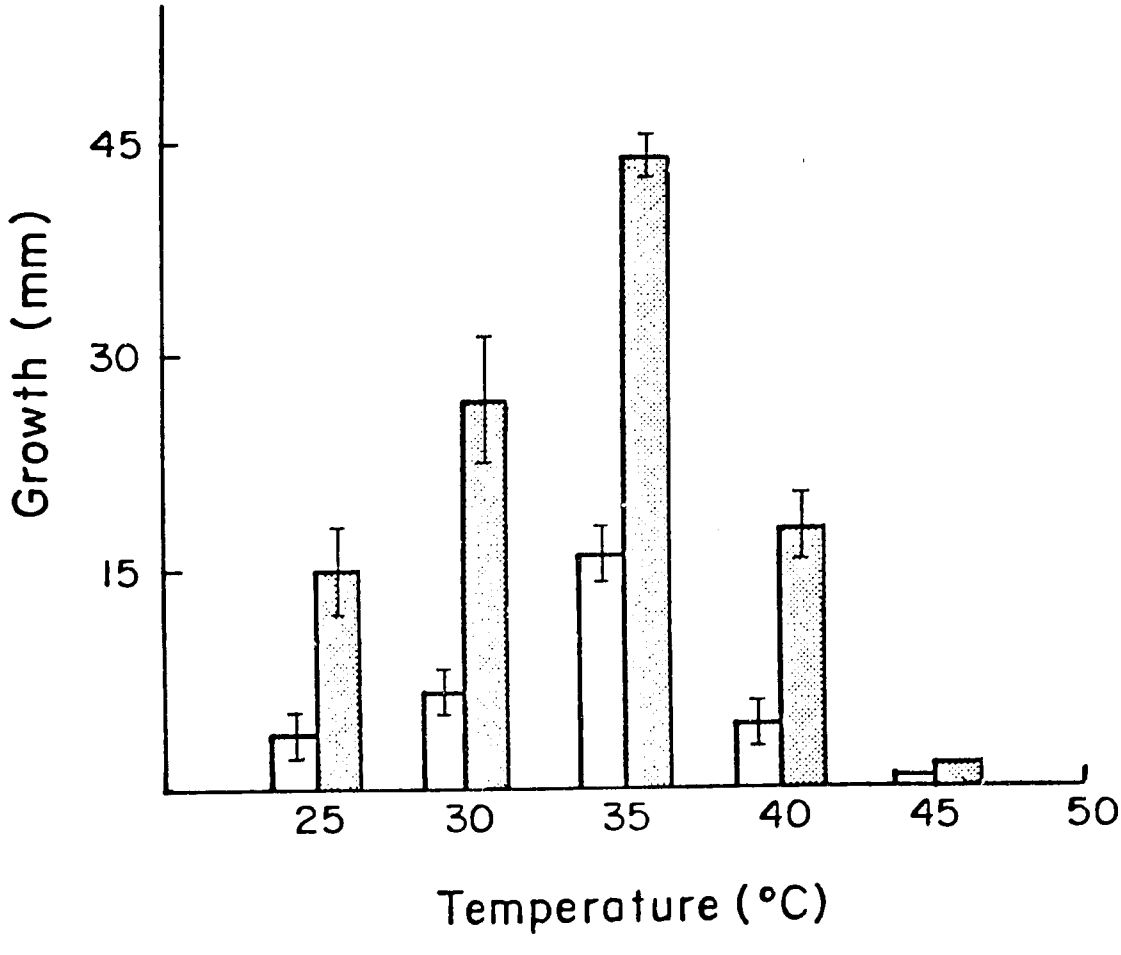
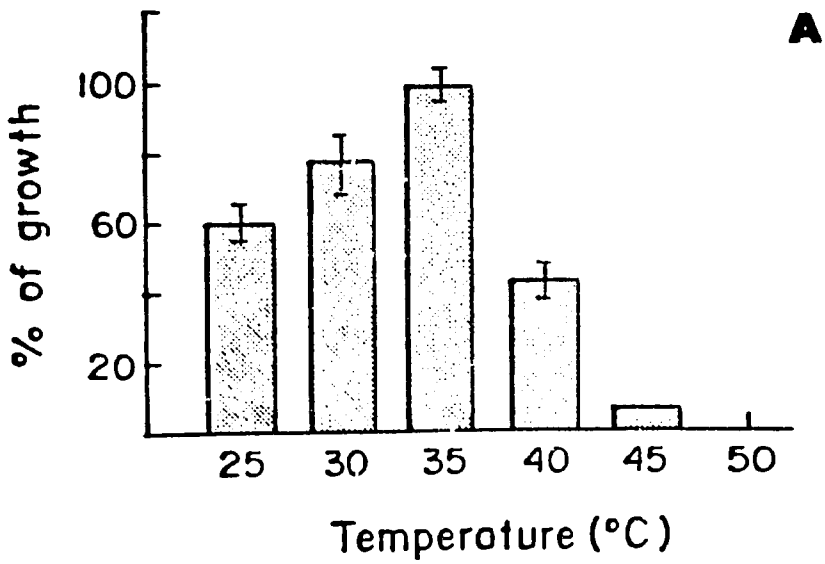


Figure 3


A
% of growth
100
60
20
25
30
35
40
45
50
Temperature (°C)

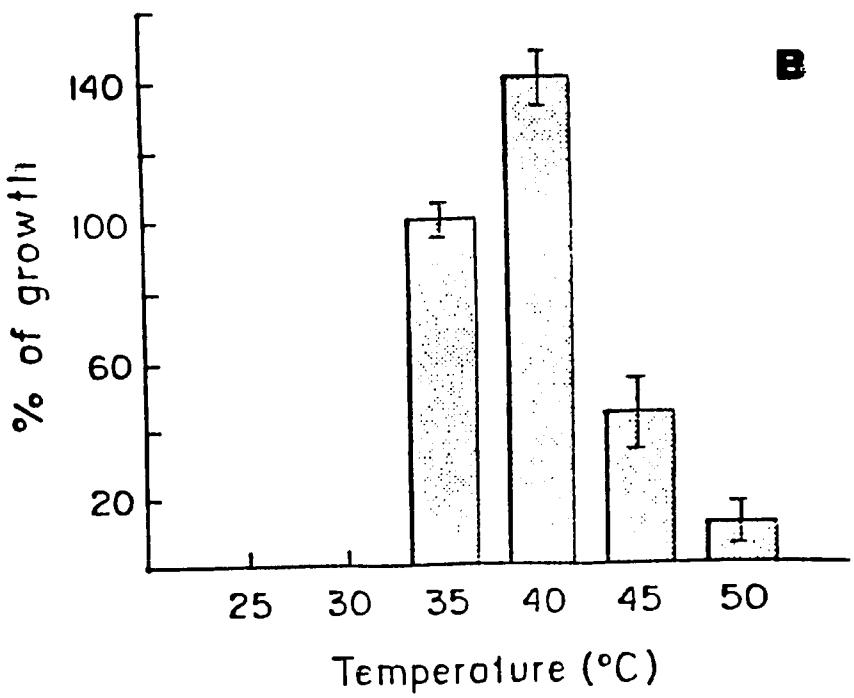

B
% of growth
140
100
60
20
25
30
35
40
45
50
Temperature (°C)

Figure 4

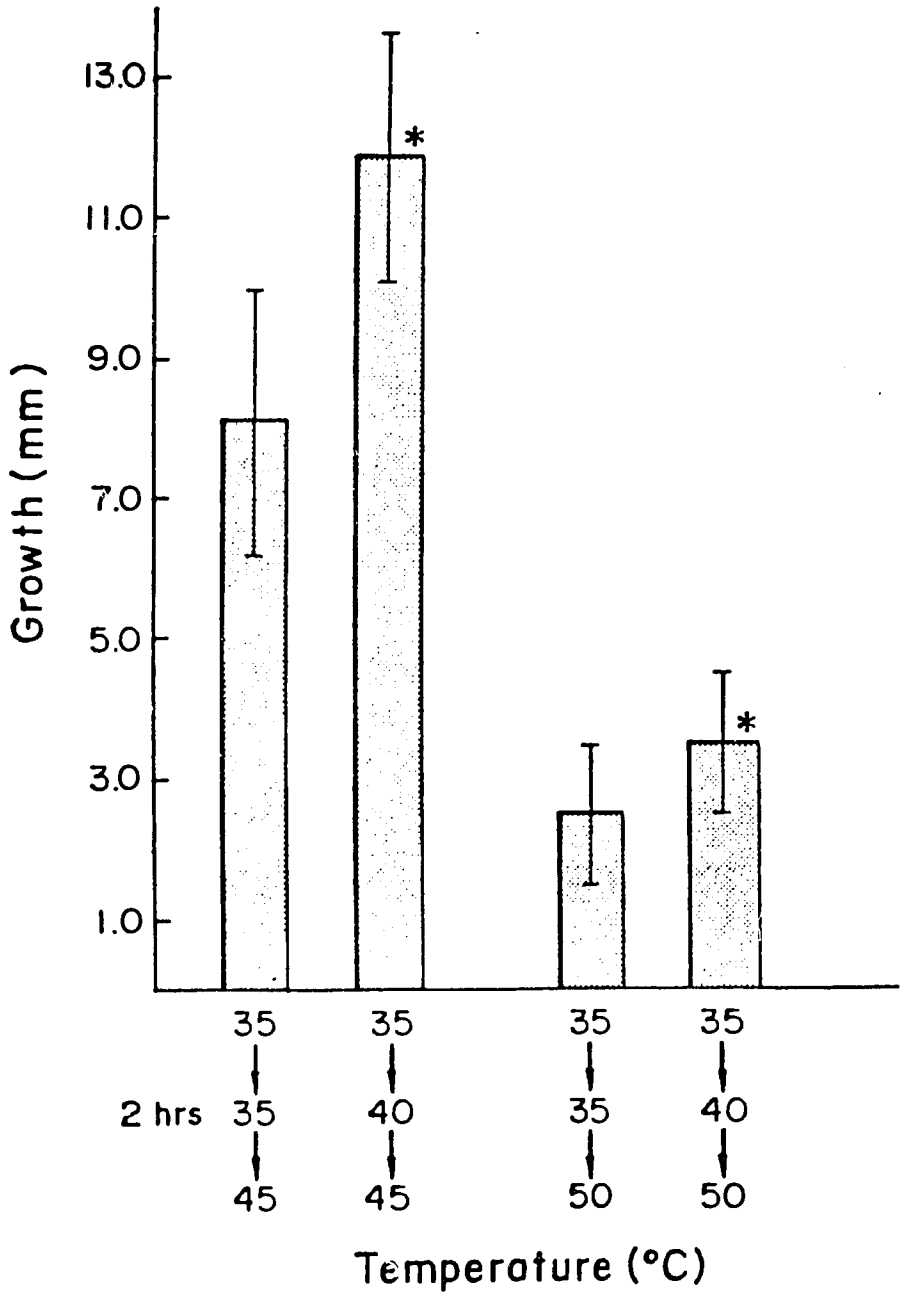
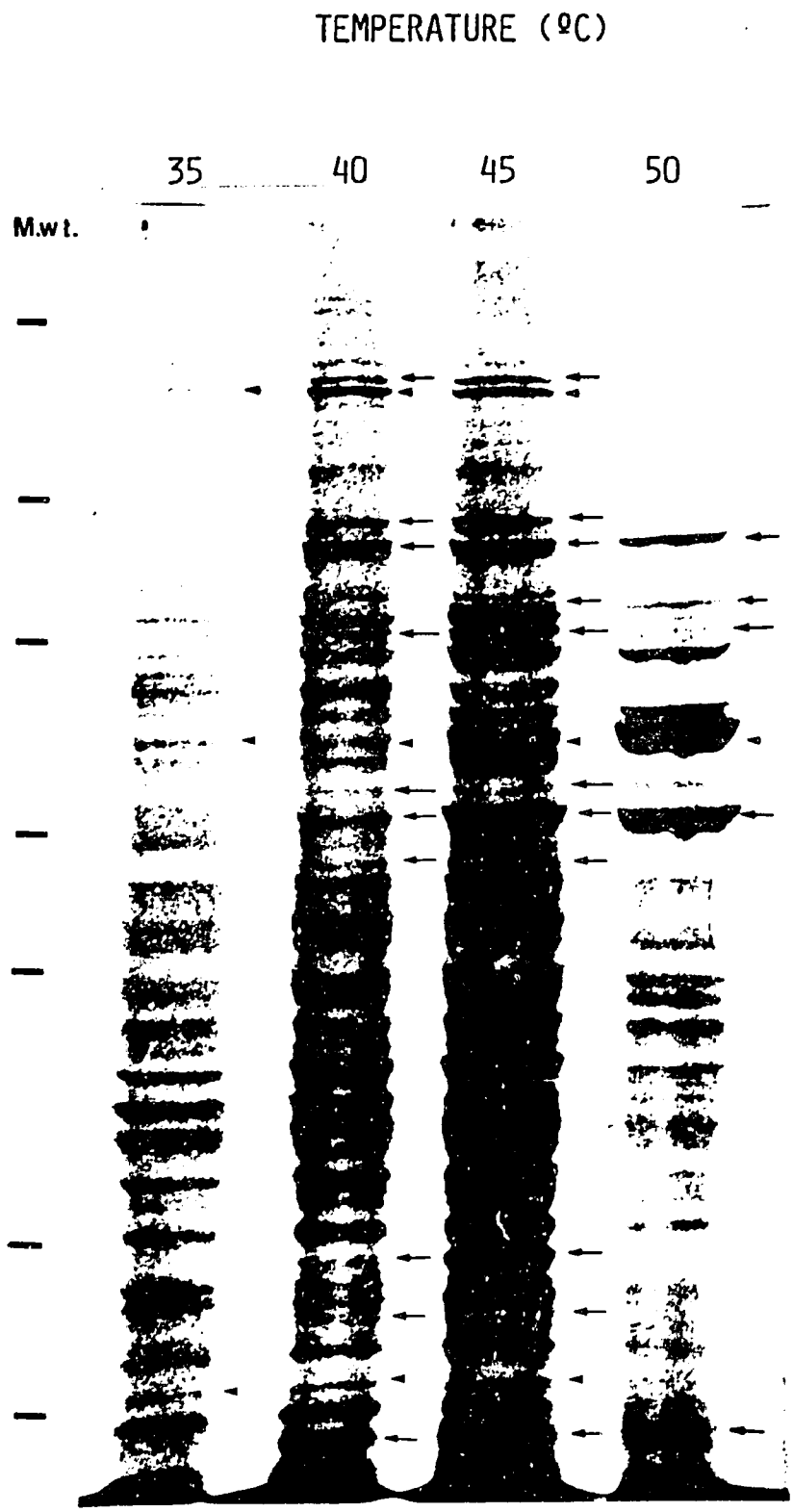

13.0
11.0
9.0
7.0
5.0
3.0
1.0
Growth (mm)
*
*
35
35
35
35
2 hrs
35
40
35
40
45
45
50
50
Temperature (°C)

Figure 5



Water potential components in the branch system of *Prosopis chilensis* (Mol.) (Mimosaceae): A new regulation model?

Francisco A. Squeo (1), **Liliana Cardemil** (2), and **Nancy Olivares** (1)

(1) Departamento de Biología, Facultad de Ciencias, Universidad de La Serena. Casilla 599. La Serena. Chile.

(2) Departamento de Biología, Facultad de Ciencias, Universidad de Chile. Casilla 653. Santiago. Chile.

* This is a contribution of the Program of Arid Zone Studies of Universidad de La Serena, performed in collaboration with Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Please send editorial correspondence and proofs to:

Dr. Francisco A. Squeo
Departamento de Biología
Facultad de Ciencias
Universidad de La Serena
Casilla 599 - La Serena
Chile
FAX: 56 (51) 211473
Telephone: 56 (51) 226080 anexo 401

137

Summary.

The contributions of the turgor pressure and the osmotic potential on the water potential of branch and leaf petiole xylem in Prosopis chilensis were studied in a Northen-central Chile population at 30°S. Leaf water potential (LWP) showed a greater variability (ca., -2.2 to -8.8 MPa) than the branch xylem potential (BXP) (ca., -2.1 to -4.9 MPa) and leaf petiole xylem potential (LPXP) (ca., -1.7 to -3.9 MPa) in daily courses for all seasons. The BXP was always ≤ LPXP. The LWP was always ≤ BXP and LPXP, except for some days at early hours. The most negative values were recorded after midday. Osmotic potential in leaf petiole was 2.4 MPa lower than in the branch, compensating the water potential gradient in the branch systems.

Anatomical studies show a discontinuity of the conductive tissue (apoplasmic route) between the branch and the leaf petiole. The branch-leaf petiole connection has undifferentiated vessel elements with protoplasm (simplasmic route). This data was corroborated with the specific hydraulic conductance (Cs) values. Branch and leaf petiole Cs was always >> branch-leaf petiole connection. The selective advantages of this system are discussed.

Key words: Water potential - Turgor pressure - Osmotic potential - Specific conductance - Xylem - Arid environment - Chile.

The conduction of water in the branches of all higher plants takes place in the lumina of dead xylem elements (apoplasmic route). Protoplasma into vessel elements could only interfere with water flow (Mauseth 1988). The structure of the vascular system differs markedly among groups of plants species (i.e., ring-porous species, diffuse-porous species, conifers) and consequently differs in the potential velocities of water movement (Zimmermann and Brown 1971). Now then, the water potential gradiente from soil to air via the plant, constitutes the driving force for water movement, from high to low water potential (Fitter and Hay 1981). Under certain circumstances, measured gradient in water potential in high trees are not consistent with these theorical gradients. To explain this, it has been suggested that tree leaves are capable of absorbing water from their surfaces, thereby raising leaf water potential (Connor et al. 1977; Suzuki 19..).

The tree species with habit in arid ecosystems of the world are exposed to severe water stress during several times in the year. It has been postulated that mechanisms to avoid cavitation in xylem cells under water stress, are related to, for example, lower tracheid and vessel diameters, change in their relative proportion, margo-torus system and other pit membrane characteristics (Zimmermann and Brown 1971; Salisbury and Ross 1978; Fitter and Hay 1981; Mauseth 1988).

For apoplasmic water, the dominant component is usually the hydrostatic pressure (pressure potential), contrary the osmotic

potential is usually negligible. The apoplasmic value of osmotic potential is usually higher than -0.1 MPa and is often higher than -0.02 MPa (Koide et al. 1991), though it can be much lower in halophytes (Ritchie and Hinckley 1975; Turner 1981).

The main objective of this paper is to study the relationships between the components of the water potential in the branch system of Prosopis chilensis, a chilean desert tree species. This study is part of a major project related to evaluate the resistance of P. chilensis to extreme environmental conditions.

Methods

Study Site

This study was conducted in Quebrada San Carlos (30°02'S, 70°49'W, 520 m elevation), within Observatorio Interamericano El Tololo area, 50 km west to La Serena in the Elqui Valley. The climate is arid mediterranean with a light maritime influence. Climatological records from the Vicuña (30°02'S-70°44'W, 730 m altitude, 8 km west to study site) show a mean annual precipitation of 94.9 mm (C.V.= 65.8%, range 4.9 - 256.5 mm) for the last 47 years. June (21.6%), july (28.8%) and august (19.7%) are the most rainy months and making 70% of total annual precipitation. Temperature and humidity varies daily and seasonaly. Mean maximum monthly temperature varies between 29.0°C (january) and 19.0°C (july) and mean minimum between 12.3°C (january) and 5.3°C (june). Mean maximum monthly relative

140

humidity varies between 89% (may) and 84% (september), and minimum 25% (april) and 37% (june). Total annual tray evaporation is 2000 mm, and the mean monthly varies between 280 mm (january) and 72 mm (june).

The vegetation in study area is an open desert forest longest dominated by Prosopis chilensis Mol., with low height (< 4 m). The most important shrubs species are Encelia canescens Lam., Gutierrezia recinosa (H. et A.) Blake, Ephedra chilensis K.Presl. and Adesmia pedecillata H. et A. In rainy years, annual plant species are abundant. The site has been exposed to low grazing by goats and cattle, and the tree have been cut for firewood over the present century.

Water Potential Measurement

Xylem potentials were determinated seasonaly in 24 h daily courses during one year, from spring 1990 to summer 1991. Xylem potential in brach and leaf petiole were measured with a pressure chamber without phloem tissue (Scholander et al. 1965; Ritchie and Hinckley 1975; Turner 1981; 1987; Koide et al. 1991). Leaf water potential and, brach and leaf petiole xylem osmotic potential were measured with psychrometric chambers (C-52 chamber, Wescor Corp., Logan, Utha).

Hydraulic conductance

The branch was cut and a hose-system was installed below the water level. Specific hydraulic conductance (Cs) of branch, petiole and branch-petiole system were determinated for: Cs= (Q* A) / dP, where: Q= flux rate (m^3 s^{-1}), A= transversal section

area of transversal of xylem (m^2), dP= pressure difference (MPa m^{-1}) (.....).

Branch-leaf petiole anatomy.

Transversal and longitudinal section of branchs were obtained. The section were dehydrated, paraplast embeded and stained with safranina-fast green (Cardemil et al. 1990).

Results

Water potential.

Leaf water potential (LWP) showed a greater variability than the xylem potential of branch (BXP) and leaf petiole (LPXP) in a 24 h daily course (Fig.1). The most negative values were recorded after midday. The typical water potential gradient (i.e., BXP > LPXP > LWP) was found apparently modified in *P. chilensis*. BXP was always ≤ LPX. The LWP was always ≤ BXP and LPXP except early in the morning (some days).

The pressure chamber method assume that the osmotic potential in the xylem sap is close to zero. But, the xylem pressure differences between the branch and the leaf petiole could only be explained by the osmotic potential compensation if there exists an anatomical discontinuity of the conductive system between this two xylem tissues.

Simultaneous measurements of osmotic and pressure potentials in branch and leaf petiole xylem tissues were obtained from 14:00 to 16:00 during december 1991 (Table 1). This data showed that water potential gradient was consistent

147

with the classical model (i.e., branch > leaf petiole water potential), and that there exists osmotic potential differences between the xylem sap of branch and leaf petiole. Osmotic and water potential in the leaf petiole were significatively lower than in the branch (2.40 and 2.12 MPa of difference, respectively). The pressure potential mean in the branch was 0.28 MPa lower than in the leaf petiole. However, statistic difference was not found during this day period.

Hydraulic conductance.

In table 2 are shown the specific hydraulic conductances (Cs) for the branch, the leaf petiole and the branch-leaf petiole system. The branch showed the higher values in Cs ($3.46*10^{-6}$ m^2/s MPa), 4.4 fold higher than the leaf petiole Cs. The branch-leaf petiole system Cs was 32 fold lower than the leaf petiole Cs. The three Cs mean values were statisticaly differents. This low specific conductance (i.e., high resistence) indicates that the route of the xylem tissue between the branch and the leaf petiole are very close for tension water flow.

Branch-leaf petiole anatomy.

Anatomical studies confirmed a discontinuity of the conductive tissue between the branch and the leaf petiole. Apoplasmic route of the branch and of the leaf petiole xylem tissues were closed by undifferentiated xylem cells with citoplasm (simplasmic route). In agreement with the specific conductivity differences, the diameters of the vessel elements

in the branch were significative higher than in the leaf petiole (branch: X= 52.9 µm, s.d.= 14.6 µm, n= 16; leaf petiole: X= 33.2 µm, s.d.= 13.7 µm, n= 16; t= 3.94, P < 0.001, t-test). Teorical conductance in a Prosopis chilensis branch vessel will be 6.5 fold high than the leaf petiole vessel. Additionaly, in death xylem cells of branch and leaf petiole exist starch granules.

Discussion

Our results indicate that the xylem of the branch-leaf petiole system in Prosopis chilensis posses a simplasmic route between two apoplasmic routes. It has not been documented so far in other plant species. This system may explain an osmotic potential control over water potential in the leaf petiole.

An apparent evidence for a possible reverse tranpiration are shown in Fig. 1 (between 8:00 and 9:00 hrs), similarity to the proposed for Prosopis tamarugo and some other species (Connor et al. 1977; Suzuki 19..). Additionaly, the osmotic potential in leaf petiole may be lower than -5 MPa (or less), increasing the internal reverse water potential gradient. In the distribution range of the P. chilensis, the fog is frequent in the first morning hours (i.e., relative humidity close to 100%). We propose that if reverse transpiration occurs, it may be important for recovery of the possible leaf petiole cavitations.

The physical compromise between high water flux rate and lower cavitation risk in Prosopis chilensis xylem may be resolved by the branch-leaf petiole xylem system. The branch

shows high water conductivity and high diameter in their vessel elements compared with those of the leaf petiole, and a lower cavitation risk by simplasmic discontinuity in the apoplasmic route.

In the semi-desert of North-central Chile, Prosopis chilensis is a partial deciduous tree. The total minimum leaf area is found at mid spring (15%). New leaves appeared at the end of the spring, and the reproductive processes occurred in summer, ending with abscission of fruits at the end of the summer (Squeo et al. 1991). For mediterranean central Chile (30°S), Arce and Balboa (1991) shows the same phenological pattern, and that the branch xylem tension is lower than -3.0 MPa during this period. For the same month (october), the branch xylem tension is at least 2.0 MPa more negative in the semi-desert population than the mediterranean population. Besides, P. chilensis is capable of obtaining subsoil water. However, the periods of highest demand for evapotranspiration are when new leaves appear and reproductive processes occur. Branch cavitation and finally branch death caused by severe water stress are clearly more expensive for a tree species than in the other perennial species. This suggests that branch-leaf petiole xylem system could help to understand and partially explain the desert range distribution of P. chilensis.

The selective advantage of this system might be: a) to prevent that cavitation occurs in the branch xylem tissue due to an indirect transmission of the water column tension caused by

the fast transpiration rate, b) if cavitation occurs in leaf petiole, this may be reversed in the morning when leaf water potential is higher than the leaf petiole water potential and sometime lower than the air water potential, and c) leaf is able to maintain actively the turgor.

This type of mechanism in other Prosopis species and other woody desert plant species should be further investigated.

Aknowledgements. We thank Dr. R. Williams (director) and E. Figueroa from Observatorio Interamericano El Tololo for kindly permit given to use Quebrada San Carlos as the study site. We also thank Dr. ..... (director) and M.I. Rojas from Subestación Experimental de Vicuña from Instituto de Investigaciones Agropecuarias (INIA) to make available unpublished climatic data. This research was supported by the grant #7.068, Program in Science and Technology Cooperation, Office of the Science Advisor U.S. Agency for International Development and FONDECYT 92-1140 granted to Liliana Cardemil.

References

Arce P, Balboa O. (1991) Seasonality in rooting of Prosopis chilensis cuttings and in-vitro micropropagation. For Ecol. Manage 40:163-173.

Cardemil L, Lozada R, Cortés M (1990) Sucrose uptake and anatomical studies ... J. Plant Physiol & Biochem xx:xx-xx

Connor DJ, Legge NJ, Turner NC (1977), Water relations of Mountain ash (Eucalyptus regnans F. Muell.) forest. Austr J Plant Physiol 4: 753-762.

Fitter AH, Hay RKM (1981) Environmental Physiology of Plants. Academic Press, London.

Koide RT, Robichaux RH, Morse SR, Smith CM (1991) Plant water status, hydraulic resistance and capacitance. In: Pearcy RW, Ehleringer J, Mooney HA, Rundel PW (eds) Plant Physiological Ecology, Chapman and Hall, London, pp 161-183.

Mauseth JD (1988) Plant anatomy. The Benjamin/Cummings Publ. Comp. Inc., Menlo Park, California.

Ritchie GA, Hinckley TM (1975) The pressure chamber as an instrument for ecological research. Adv. Ecol. Res. 9: 165-254.

Scholander PF, Hammel HT, Bradstreet ED, Hemmingsen EA (1965) Sap pressure in vascular plants. Science 148: 339-346.

Squeo FA., Olivares N, Cardemil L (1991) Phenological and ecophysiological patterns of Prosopis chilensis from North-central Chile. International Workshop Desertification: A Landscape-ecosystem perspective, La Serena, Chile pp 36.

Suzuki F 1969. Absorción foliar de humedad atmosférica en tamarugo P. tamarugo Phil. Boletin Técnico, Facultad de Agronomía, Universidad de Chile. 30: 1-23

Turner NC (1981) Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58: 339-366.

Turner NC (1987) The use of the pressure chamber in studies of plant water status. Proc. Int. Conf. Measurement Soil Plant Water Status 2: 13-24.

Zimmermann MH, Brown CL (1971) Tree: Structure and Function. Springer-Verlag, New York.

Table 1. Water potential and their component in branch and leaf petiole of *Prosopis chilensis*. The data were obtained from 14:00 to 16:00 hr during december 1991. Standard desviations are shown in parentheses. n= 12 in all cases.

	Water potential (MPa)		
	Osmotic	Pressure	Total
Leaf petiole	-8.48 (1.17)	-2.47 (0.41)	-10.94 (0.99)
Branch	-6.07 (1.57)	-2.75 (0.36)	-8.83 (1.38)
Branch minus Leaf petiole	2.40 (1.52)	-0.28 (0.37)	2.12 (1.43)

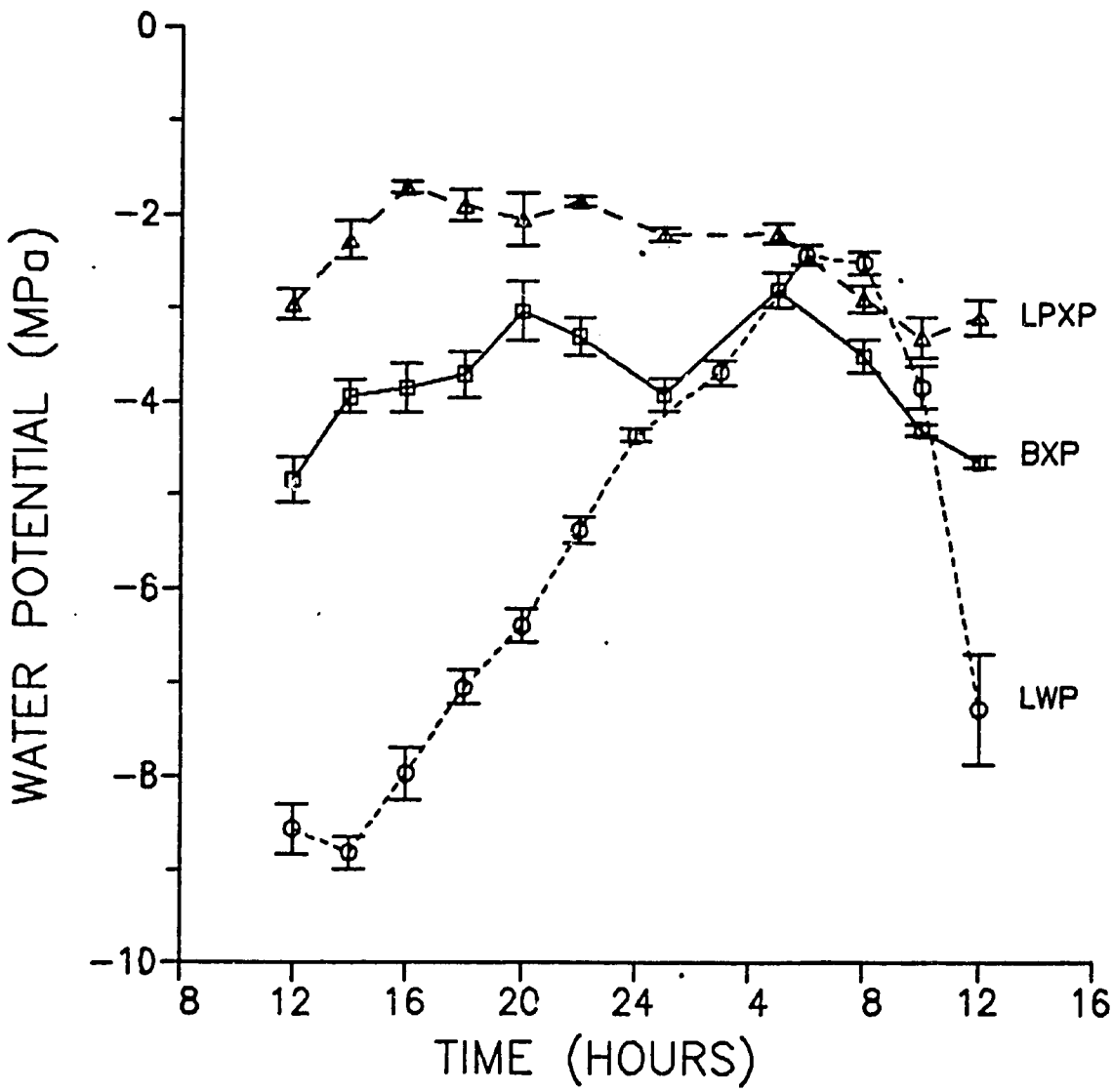
t-test: Branch versus leaf petiole (osmotic potential: t= 4.06, P < 0.001; pressure potential: t= 1.72, P > 0.05; water potential: t=4.14, P < 0.001).

Table 2. Specific hydraulic conductance in the branch, leaf petiole and branch-leaf petiole systems. n = 4 in all cases.

	Mean (m^2/s MPa)	Variance
Leaf petiole	$7.84 * 10^{-7}$	$2.3 * 10^{-14}$
Branch	$3.46 * 10^{-6}$	$9.1 * 10^{-13}$
Branch-Leaf petiole system	$2.42 * 10^{-6}$	$1.4 * 10^{-16}$

t-test: Leaf petiole versus Branch (t= 4.64, P < 0.001), Leaf petiole versus Branch-Leaf petiole system (t= 8.72, P < 0.001), Branch versus Branch-Leaf petiole system (t= 6.24, P < 0.001).

Fig. 1. Typical daily course of branch (BXP, square) and leaf petiole xylem potential (LPXP, triangle), and leaf water potential (LWP, circle) in Prosopis chilensis. Data correspond to may 30-31 of 1991. Each mean shows one standard error bar.



CONGRESS
AND
SYMPOSIUM

UNIVERSIDAD DE VALPARAISO
FACULTAD DE MEDICINA
ESCUELA DE QUIMICA Y FARMACIA

PROGRAMA Y RESUMENES

VII REUNION NACIONAL DE BOTANICA

31 DE AGOSTO
3 DE SEPTIEMBRE
1988
VALPARAISO

SECCION BOTANICA
SOCIEDAD DE BIOLOGIA DE CHILE

157

SELECCION PARA ALTA EXPRESION DE GENES DE RESISTENCIA AL ESTRES TERMICO Y DE HERIDAS EN PLANTAS. (Biotechnological applications in agriculture and in conservation of native plants. Selection for a high degree of gene expression of thermotolerance and resistance to wounding in plants). Goycoolea, C., Riquelme, A. y Cardemil, L. Dep. de Biología, Facultad de Ciencias, U. de Chile. (Proyecto FONDECYT 0160/88).

Las plantas como todos los organismos vivos, responden a situaciones de estrés con síntesis de nuevas proteínas que parecen estar relacionadas a la protección del organismo cuando es expuesto a condiciones extremas del medio ambiente. Las nuevas proteínas detectadas son el producto final de la expresión de genes que las codifican.

Técnicas de Biología Molecualr y de Biología Celular, permiten facilmente detectar y cuantificar la expresión de estos genes, ya sea, a través del uso de sondas radioactivas, obtenidas por la integración de la información génica, contenida en un cDNA, en un plásmido que ha sido clonado en *E. coli*, lo que permite detectar genes y mensajeros, o ya sea, a través del uso de anticuerpos policlonales que permiten detectar y cuantificar las proteínas.

La respuesta fisiológica y la expresión génica para termotolerancia por semillas y plántulas de *Araucaria araucana* ha sido investigada por fluorografía de proteinas sintetizadas durante estrés térmico, en presencia de ^{35}S-metionina y sometidas a electroforesis SDS-poliacrilamida. Los estudios de proteínas han sido completados con detección de mRNAs para las proteínas de estrés térmico: de 70 Kd y de ubiquitina.

Anticuerpos policlonales, nos han permitido la detección de proteínas de pared celular que se sintetizan en condiciones de heridas cortantes en el tejido vegetal. La síntesis de las proteínas ha sido detectada por electrotransferencia Western y por inmunoimpresión en nitrocelulosa de tejidos vegetales sometidos a cortes. Se discute el uso de estas técnicas para selección de plantas resistentes.

GERMINACION Y CRECIMIENTO DE LAS PLANTULAS DE PROSOPIS CHILENSIS EN ALTAS TEMPERATURAS Y SU CORRELACION CON EL PATRON DE SINTESIS PROTEICA. (Germination and seedling growth of Prosopis chilensis and their correlation with the pattern of protein synthesis). Medina, C. y Cardemil L. Depto. de Biol., Fac. de Ciencias, Universidad de Chile.

Semillas de Prosopis chilensis provenientes de la población de Chacabuco fueron escarificadas y puestas a temperaturas de 25°, 30°, 35° , 40°, 45° y 50° C., encontrándose el óptimo a 35° C. El crecimiento radicular fue también óptimo a esta temperatura decayendo drásticamente a temperaturas superiores y prácticamente deteniéndose a 45° C. Plántulas germinadas en condiciones óptimas logran crecer a 45° C., lo que se incrementa si previamente se someten a un tratamiento de 2 horas a 40° C.

Es conocido que, en una variada gama de organismos incluyendo los vegetales, la elevación de temperatura a niveles supraóptimos induce cambios en el patrón de síntesis de proteínas y éste se estudió en *Prosopis chilensis*.

Apices radiculares fueron incubados en presencia de un precursor de proteínas radiactivo (Met-S^{35}) a diferentes temperaturas. La fluorografía de electroforesis SDS muestra aparición de nuevas proteínas a temperaturas superiores a 35° C., alguna de las cuales podría tener importancia en latolerancia de *Prosopis chilensis* a altas temperaturas. (Proyecto FONDECYT 0160/88).

April 1989
Volume 89
Number 4
ISSN-0032-0889

Supplement to

PLANT PHYSIOLOGY

Published monthly by the American Society of Plant Physiologists

150

1206

EFFECT OF THE TIMING OF COLD STORAGE ON COLD HARDINESS OF INTERIOR DOUGLAS-FIR SEEDLINGS

Karen E. Burr & Richard W. Tinus U.S. Forest Service, Rocky Mtn. Forest and Range Exp. Stn., Flagstaff, AZ 86001

Cold storage of dormant tree seedlings is an essential tool in the successful production and establishment of forest planting stock. Thus, to maintain stock quality, we must know the impact of storage on seedling cold hardiness. Actively growing container seedlings were acclimated in growth chambers as follows; 4 wks SD20/N15 (10-hr day), 16 wks SD10/N3. At weekly intervals, cold hardiness was measured by electrolyte leakage from needles, and seedlings were transferred to 1°C dark storage for 4 wks. Cold hardiness was reassessed following storage. The relation between cold hardiness and growth chamber acclimation time was sigmoidal, with a maximum stable hardiness of -32°C (LT50) reached in 14 weeks. Cold hardening continued in storage regardless of when during acclimation seedlings were stored. Thus, cold hardening was restarted in storage after stabilizing under growth chamber conditions. In addition, immediate reacclimation (2°C/wk) of deacclimating seedlings (LD22/N22, 14-hr day) was possible in storage.

1207

COORDINATION OF C AND N METABOLISM OF BEAN PLANTS GROWN UNDER DIFFERENT ROOT TEMPERATURES

Eugene Koshkin and Nicolai Treliakov, Dept. of Plant Physiol., Agr. Academy, 127550, Moscow, USSR.

The effect of root temperature on C and N metabolism of non-nodulated bean plants was determined for root temperatures of 12, 22 and 32°C. Shoot temperature was maintained at 22 °C with a 16 hr photoperiod (400 µmol m^{-2} s^{-1} PAR). Gas exchange measurements in the light and dark were conducted separately for the intact roots and shoots at 3-4 day intervals throughout the experimental period (80 days). Photosynthesis, respiration (on a whole plant basis) and seed yield were approximately 2-fold greater at root temperatures of 22 °C than at 12 °C, and intermediate to values at 32 °C. Over the experimental period, true growth efficiency (0.78-0.81), photosynthetic efficiency (1.40-1.70 g C per g seed), true respiratory C loss (total minus maintenance respiration) on a seed yield (0.28-0.32 g C per g) and protein yield (6.63-7.22 g C per g N) basis remained fairly constant regardless of root temperature. These data suggest that long term adaptation of the plants to growth conditions causes compensatory changes in photosynthesis, respiration, and C and N metabolism which are coordinated for optimal efficiency.

1208

CHARACTERIZATION OF HEAT INJURY IN GRAPES USING NMR METHODS

Majeed Abass, & C.B. Rajashekar Hort. Dept. Kansas State Univ., Manhattan, KS 66506

Transverse relaxation times (T_2) of tissue water (^1H) in leaves and suspension-cell cultures of grape hybrids (Vitis spp, cv Veeblanc and Ken-1) were measured at various temperatures. A sharp decrease in T_2 for the major fraction of tissue water was observed in association with heat injury in both leaves and suspension-cell cultures. The changes in T_2 as a result of heat injury were irreversible as indicated by a temperature dependent hysteresis of T_2 in samples subjected to heat injury. Results on T_2 using paramagnetic probe (Mn^{2+}) suggest that the plasma membrane is irreversibly damaged at the injury temperature resulting in a loss of cell compartmentalization. T_2 for the major fraction of tissue water was significantly smaller in heat-killed tissues than in live tissues. T_2 for the major fraction of tissue water in live leaves and cell cultures, doped with Mn^{2+} (0.01M) was 52.8 ms and 87 ms and decreased to 5.5 ms and 14.3 ms respectively after heat-killing.

1209

CHANGES IN THE PATTERN OF PROTEIN SYNTHESIS OF PROSOPIS CHILENSIS INDUCED BY HIGH TEMPERATURES.

Consuelo Medina & Liliana Cardemil Dep. de Biol., Facultad de Ciencias, U. de Chile, Santiago, Chile.

Seeds of Prosopis chilensis, a leguminous tree from semi-arid regions of Central Chile, were germinated at temperatures of 25-30-35-40-45 and 50°C. Germination was 100% between 25 and 40°C, being faster at 35°C. The best temperature for root growth was also 35°C. There was not germination at 50°C. However, seedlings coming from seeds germinated at 35°C were capable of growing at higher temperatures of 45 and 50°C. Pattern of protein synthesis was followed in roots incubated with ^{35}S-methionine at increasing temperatures between 35 and 50°C. SDS-PAGE of the proteins followed by fluorography shows that at temperatures above 35°C, new protein bands appear while others become thicker. Most of the protein bands have decreased at 50°C., with the exception of the new bands. A band of 70 KD, that is present at 35°C, is more prominent at 50°C. These proteins may have an important role in the thermotolerance of Prosopis chilensis to stressing temperatures. AID Grant # DPE 5548-G-SS-8073-00 & FONDECYT 0160/88.

1210

UNICONAZOL-INDUCED AMELIORATION OF LOW TEMPERATURE DAMAGE IN CUCUMBER SEEDLINGS IN RELATION TO ANTIOXIDANT ACTIVITY

Abha Upadhyaya, Tim D. Davis, R.H. Walser, A.B. Galbraith, & N. Sankhla, Department of Agronomy and Horticulture, Brigham Young University, Provo, UT 84602

Cucumber seedlings were treated with 0 or 100 µg of soil-applied uniconazol (UNI), a gibberellin biosynthesis inhibitor, and then exposed to 22 or -1C for 8 h one week later. Following exposure to -1C, electrolyte leakage from leaf tissue of treated plants was about 33% that of the controls indicating that UNI reduced low temperature damage. Following low temperature exposure, the content of malondialdehyde was about 25% less in treated seedlings indicating that UNI decreased low temperature-induced lipid peroxidation. Uniconazol-induced low temperature tolerance was accompanied by increased levels or activities of various antioxidant systems including glutathione, peroxidase, and catalase. These results are consistent with the hypothesis that triazole-induced stress tolerance is due, at least in part, to increased antioxidant activity which reduces stress-related oxidative damage to membranes.

1211

SURVIVAL STRATEGIES OF PLANTS DURING WATER STRESS

R. Scheuermann, T. Stuhlfauth, D. Sültemeyer, H. Fock Universität D-6750 Kaiserslautern, FB Biologie

Fluorescence and gas exchange of bean, maize, sunflower and wooly foxglove were simultaneously measured as described by Stuhlfauth et al. (Plant Physiol. 86, 246-250, 1988) at 250 µmol quanta/m^2/s. Under severe water stress conditions about 40 % of the photochemical energy was converted to heat at PS II. This is interpreted as a protective mechanism against photoinhibitory damage when net CO_2 uptake is reduced by about 70 %. After $^{14}CO_2$ gas exchange, only in bean a homogenous distribution of radioactivity over the leaf was observed. In all other plants we found a patchy distribution of regions with either an intensive or a reduced gas exchange. We conclude that CO_2-recycling (photorespiration and reassimilation) behind closed stomata also contributes to energy dissipation under severe stress conditions.

PACIFIC SCIENCE ASSOCIATION

VI INTER CONGRESS

VALPARAISO - VIÑA DEL MAR, CHILE, AUGUST 7-10, 1989

THE PACIFIC, BRIDGE OR BARRIER?

ABSTRACTS

ORGANIZED BY
THE INSTITUTE OF INTERNATIONAL STUDIES
OF THE UNIVERSITY OF CHILE
VENUE
FEDERICO SANTA MARIA UNIVERSITY-VALPARAISO
SPONSORED BY
COUNCIL OF RECTORS OF CHILEAN UNIVERSITIES
INFORMATION: CONDELL 249 SANTIAGO 9 CHILE P.O. BOX 14187 SUC. 21
SANTIAGO, CHILE. CABLES INTERACADEMIC.
TELEX 443024 INTERC TELEPHONES 2740139 2740850

CHANGES IN THE PATTERN OF PROTEIN SYNTHESIS IN TWO TREES OF CHILE, ARAUCARIA ARAUCANA AND PROSOPIS CHILENSIS, INDUCED BY HIGH TEMPERATURE.

Cardemil, L.; Goycoolea, C. and Medina, C. Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Araucaria araucana constitutes temperated relictual forests in the high mountains of Chile Austral, with 20ºC differences between days and nights and between seasons. Prosopis chilensis instead, is a leguminous, nitrogen-fixing tree of the arid and semi arid regions of Chile with temperatures reaching 60ºC or higher, in Summer time. We have investigated how seeds and seedlings of these two plant trees, respond to high temperature exposure, performing heat shock experiments, and how seeds and seedlings acquire thermotolerance when exposed to sublethal temperatures. Indeed, the main idea of this research has been to test the hypothesis that "Native plants are adapted to their natural environments because they show a higher degree of expression of those genes encoding proteins related to plant protection during environmental harsh conditions". The higher degree of expression may be due either to regulatory mechanisms of gene function or to the number of copies of the genes involved. SDS-gel electrophoresis of proteins synthesized during heat shock experiments and Northern hybridization analysis to detect the mRNA of these proteins, using probes for heat shock genes, allow to evaluate this hypothesis. Proyecto FONDECYT 0160/88 and AID grant # DPE-5542-G-SS-8073-00. Universidad de Chile Proyecto de Desarrollo # 178501-4.

159

ISSN 0004-0533

ARCHIVOS DE BIOLOGIA Y MEDICINA EXPERIMENTALES

VOL. 22 NOVIEMBRE 1989 Nº 3

SOCIEDAD IBEROAMERICANA DE BIOLOGIA CELULAR
4º Congreso

SOCIEDAD DE BIOLOGIA DE CHILE
32ª Reunión Anual

SOCIEDAD DE GENETICA DE CHILE
22ª Reunión Anual

SOCIEDAD DE BIOQUIMICA DE CHILE
13ª Reunión Anual

SOCIEDAD DE BIOLOGIA CELULAR DE CHILE
3ª Reunión Anual

SOCIEDAD CHILENA DE REPRODUCCION Y DESARROLLO
2ª Reunión Anual

Viña del Mar
21 al 25 de noviembre de 1989

Resúmenes de
Conferencias, Simposios y Comunicaciones

Sociedad de Biología de Chile

160

PEPTIDOS OPIOIDES Y ACTIVIDAD CITOLITICA NK. (Opioid peptides and cytolytic NK activity). Javier Duente Piccardo. Departamento de Bioquímica y Biología Molecular. Facultad de Ciencias Químicas y Farmacéuticas. Universidad de Chile.

La interrelación entre el sistema inmune y el sistema nervioso se basa en la directa inervación del parenquima de tejidos tales como timo, bazo, ganglios linfáticos, médula ósea y otros tejidos linfoides. Además la presencia en las células inmuno competentes de receptores para diversas hormonas y neurotransmisores, como también la propiedad de éstas células de producir múltiples señales, en muchos casos compartidas con el sistema neuroendocrino, han establecido claramente una vasta inter-comunicación que ha permitido avanzar en el entendimiento de diversas situaciones fisiológicas y patológicas.

Esta comunicación bidireccional ha sido estudiada a muchos niveles y, en el presente caso, nuestro interés se ha centrado en la actividad citolítica mediada por células NK y su modulación por péptidos opioides.

Hemos estudiado la regulación ejercida sobre esta actividad por los péptidos, metionina-encefalina, leucina-encefalina y β-endorfina, tratando de correlacionar la estimulación observada con la existencia y propiedad de los receptores para péptidos opioides tanto en muestras normales como patológicas. Es un hecho establecido que los péptidos opioides influyen en diversas funciones del sistema inmune, como la síntesis de anticuerpos, proliferación de linfocitos y citolisis NK. Sin embargo, las características de los receptores involucrados y el mecanismo general de acción de estos péptidos en el sistema inmune no han sido hasta ahora aclarados.

Proyecto Fondecyt 88-0874

INTERACCIONES ENTRE SISTEMA INMUNE Y NEUROENDOCRINO (Inmune - Neuroendocrine Network). Sepúlveda Cecilia Departamento de Medicina Hospital Clínico Universidad de Chile.

Aunque el sistema inmune posee sus propios mecanismos de regulación, actualmente está bien establecida la inmunorregulación neuroendocrina. Esta tiene su base en una compleja red de interacciones bidireccional entre sistema inmune, sistema neuroendocrino, y estructuras autonómicas.

Las linfoquinas, monoquinas y Ac producidos durante la respuesta inmune afectan mecanismos neuroendocrinos. El sistema neuroendocrino puede o no responder emitiendo señales reguladoras que limiten su expansión excesiva, previniendo enfermedades autoinmunes y linfoproliferativas.

La sobreestimulación del sistema neuroendocrinopuede ser uno de los factores mediadores de la inmunosupresión que se observa en la fase aguda de enfermedades infecciosas, shock séptico, trauma, quemaduras, cáncer, y otras condiciones patológicas.

La inmunomodulación puede contribuir a restablecer la función inmune en estos individuos.

Proyecto Fondecyt 88-0874

SIMPOSIO GENOMA Y EVENTOS FISIOLOGICOS EN CELULAS VEGETALES

Coordinador: *Jorge Sans*

SIMPOSIO
GENOMA Y EVENTOS FISIOLOGICOS EN CELULAS VEGETALES (Genome and physiological events in vegetal cells).
Sans, J. (Coordinador).

Durante la vida celular ocurren una infinidad de eventos fisiológicos, que de una u otra manera, dependen de la expresión de genes en momentos, muy particulares.

Se han utilizado diversas estrategias experimentales en células vegetales mantenidas en un tejido in toto, para tratar de vincular eventos celulares a sectores del genoma. Una de estas estrategias ha sido la producción de células multinucleadas con núcleos homo y heteroploides para asociar eventos propios del ciclo celular con la presencia, en estos núcleos, de determinados cromosomas. Otra metodología utilizada con este mismo fin, ha sido el incorporar al DNA nucleótidos que bajo determinadas condiciones pueden alterar la expresión del genoma. De esta manera, la bromosustitución del genoma e irradiación posterior con luz de 313 nm., por una parte y la incorporación al DNA de un análogo de la citidina (5-azacitidina) que provocan hipometilación del DNA, por otra parte, han permitido asociar algunos eventos a sectores del genoma que replican en momentos particulares del periodo S.

Por último, mediante experimentos en que se utiliza el estrés térmico, se ha podido detectar sectores del genoma que confieren propiedades de termo tolerancia en semillas de árboles autóctonos chilenos.

En este Simposio se analizan estas estrategias y los logros con ellas alcanzados como así las perspectivas futuras de nuevos abordajes metodológicos en este tipo de estudios.

LA INDUCCION DE CELULAS MULTINUCLEADAS Y SU EMPLEO EN EL ESTUDIO DE EVENTOS DEL CICLO PROLIFERATIVO (The induction of multinucleated cells and its usefulness in the study of events in the proliferative cycle).
Giménez-Martín, G. Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006-Madrid, España.

La inducción experimental de células homocarióticas poliploides 8n en Allium cepa L. puede llevarse a cabo por inhibición de dos citocinesis sucesivas. Así se pueden formar células binucleadas (4n-4n), trinucleadas (2n-4n-2n) o tetranucleadas (2n-2n-2n-2n), dependiendo de la proximidad de los grupos cromosómicos en metafase y de su inclusión o no en un único núcleo al cursar la telofase.

Si simultáneamente se emplea un agente multipolarizante, las células 8n pueden distribuir su genoma en varios núcleos de ploidías muy diferentes y decididamente aneuploides en su composición génica.

Tanto las primeras, homocarióticas, como las segundas, heteroploides y aneuploides, se han utilizado para determinar el grado de autonomía de cada núcleo en la replicación y culminación de las etapas interfásicas y mitóticas, así como el papel de ciertos cromosomas (como el cromosoma NOR) en dicha autonomía nuclear, proporcionando una metodología útil para la disección cromosómica del ciclo celular.

GENOMA Y REGULACION DE EVENTOS DEL CICLO CELULAR (Genome and regulation of events the cell cycle).

De la Torre, C., Sans, J., Merqudich, D., Galanti, N., Gonzalez-Fernández, A., Giménez-Abián, M. y Allen, P.
Centro de Investigaciones Biológicas, CSIC, Madrid, España y Departamento Biología Celular y Genética, Facultad de Medicina, Universidad de Chile.

La replicación secuencial del genoma permite alterar la funcionalidad de segmentos de éste por bromación durante fracciones del período S y posterior irradiación, en anoxia, de las células con su DNA parcialmente bromado.

Esta metodología ha permitido, en meristemos de la raíz de *Allium cepa L.*, determinar el momento de replicación de secuencias del genoma relacionados con cada uno de los cuatro puntos de transición (TPs) donde se requiere síntesis de proteínas para la progresión de la célula a etapas más avanzadas de su ciclo celular. La replicación de las secuencias implicadas en los dos TPs para síntesis de proteínas situadas en el temprano y tardío G1 están en el temprano y tardío S respectivamente. Las secuencias implicadas en el TP situado en G2 lo hacen en el centro del S, mientras que secuencias dispersas por todo el genoma parecen ser responsables del TP situado en profase.

METILACION DEL DNA Y EVENTOS RELACIONADOS CON DIFERENCIACION Y DIVISION CELULAR. (DNA methylation and events retaled to cell differentiation and cell division).

Sans, J., Leyton, C. and Merqudich, D. Departamento Biología Celular y Genética, Facultad de Medicina, Universidad de Chile.

Se ha postulado que la metilación de citocina en secuencias dinucleotídicas C-G del DNA, sería un mecanismo mediante el cual se controlaría la expresión génica. Se ha comprobado, en una gran variedad de células, que la administración de 5-azacitidina (5-AzaC), análogo de la citidina, y poderoso agente hipometilante del DNA, produce activación génica.

En nuestro laboratorio, con el fin de asociar, desde un punto de vista morfológico, sectores del genoma con eventos celulares involucrados con el proceso de división o diferenciación celular hemos usado como estrategia experimental el efecto hipometilante del DNA DE 5-AzaC en raíces de *Allium cepa L.*

Nuestros resultados muestran que la administración de 5-AzaC provoca : a) un aumento significativo en la elongación celular que ocurre en la zona de diferenciación de la raíz, b) una aceleración de la reorganización nucleolar que se lleva a cabo al final de la mitosis y c) una alteración en el grado de condensación de la cromatina durante la mitosis. Los dos últimos eventos son particularmente evidentes cuando el análogo se administra durante la primera mitad del período replicativo.

Estos resultados sugerirían que los eventos estudiados estarían controlados por genes cuya expresión se regularía por el grado de metilación del DNA. (Proyecto FONDECYT 89/812).

EXPRESION DE GENES DE RESISTENCIA AL ESTRES TERMICO EN DOS ARBOLES NATIVOS DE CHILE, *Araucaria araucana* Y *Prosopis chilensis*. (Gene expression of resistance to heat shock in two native trees of Chile, *Araucaria araucana* and *Prosopis chilensis*).

Cardemil, L. Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Araucaria araucana constituye bosques relictuales templados de alta montaña en la Región Austral de Chile, donde existen diferencias de temperatura entre día y noche y entre estaciones de 20ºC. *Prosopis chilensis* en cambio, es una leguminosa, fijadora de nitrógeno de la región árida y semi árida del Norte y Centro de Chile. En estas regiones la temperatura llega a mas de 60ºC en los días de verano.

Hemos investigado como las semillas y plántulas de estos dos árboles responden a una exposición a altas temperatura, a través de experimentos de estrés térmico y como las semillas y plántulas adquieren termotolerancia, cuando son expuestas a temperaturas subletales.

En verdad, el principal objetivo de esta investigación es probar la hipótesis de que "Arboles nativos están adaptados a su medio natural porque ellos muestran un alto grado de expresión de los genes de resistencia para soportar condiciones extremas ambientales. El alto grado de expresión puede deberse ya sea a mecanismos de regulación de la función del gen, o a un mayor número de copia de los genes de resistencia".

Electroforesis en poliacrilamida SDS, de las proteínas sintetizadas durante el estrés de temperatura y en presencia de ^{35}S-metionina, como también hibridización Norte usando dos sondas de proteínas de estrés térmico, nos ha permitido evaluar esta hipótesis. PROYECTO FONDECYT, 0160/89; AID GRANT# DPE-5542-G-SS-8073-00; PROYECTO de Desarrollo, Universidad de Chile # 178501-4.

ISSN 0004-0533

ARCHIVOS DE BIOLOGIA Y MEDICINA EXPERIMENTALES

VOL. 23 NOVIEMBRE 1990 Nº 3

33a. REUNION ANUAL

SOCIEDAD DE BIOLOGIA DE CHILE
Y SOCIEDADES AFILIADAS

Sociedad Chilena de Biología de la Reproducción y Desarrollo
Sociedad de Biología Celular de Chile
Sociedad de Bioquímica de Chile
Sociedad de Botánica de Chile
Sociedad Chilena de Ciencias Fisiológicas
Sociedad de Farmacología de Chile
Sociedad de Genética de Chile

RESUMENES DE
CONFERENCIAS, SIMPOSIOS Y COMUNICACIONES

21 - 23 de noviembre de 1990
Punta de Tralca, Chile

Sociedad de Biología de Chile

163

100

GLICOPROTEINAS RICAS EN HIDROXIPROLINA DE PARED CELULAR DE PROSOPIS CHILENSIS (Cell wall Hydroxiproline-Rich Glycoproteins from P. chilensis). Rodríguez, J.G., Cardemil, L. Laboratorio de Fisiología y Genética Molecular Vegetal, Facultad de Ciencias, Universidad de Chile.

Uno de los aspectos a considerar en la adaptación fisiológica del algarrobo (Prosopis chilensis) es el aumento de la síntesis de glicoproteínas ricas en hidroxiprolina (HRGP's) de pared celular, como respuesta al daño mecánico.

Las inmunoimpresiones realizadas con tejidos dañados y no dañados han revelado una reacción positiva cruzada del anticuerpo antiextensina de testa de poroto de soya, con una posible HRGP de P. chilensis, encontrándose una mayor respuesta en hipocótilos de plántulas de 24 horas de edad, 24 horas después del daño. Los hipocótilos dañados dieron mayor inmunoreacción cruzada con el anticuerpo que los no dañados.

El contenido de hidroxiprolina por microgramo de proteína confirmó que existe una mayor riqueza de HGRP's en todos los tejidos dañados. La respuesta al daño es máxima en cotiledones de semillas de 36 horas de edad, 12 horas después del daño mecánico.

Las electroforesis aniónicas y catiónicas, seguidas del análisis western, permitieron detectar diferencias en el patrón de bandas de proteínas entre cotiledones dañados y no dañados. En el tejido dañado el anticuerpo antiextensina de testa de poroto de soya reacciona con tres bandas de proteínas.

Este último resultado ha permitido sugerir, que aparentemente, existen proteínas tipo extensina o que existe más de una forma para esta proteína en respuesta al daño mecánico.

PROYECTO FONDECYT 0160/88; AID Grant DPE-5542-G-SS-8073-00

101

CARACTERIZACION DE DOS PROTEINAS CATIONICAS PRESENTES EN PAREDES CELULARES DE SEMILLAS DE ARAUCARIA ARAUCANA (MOL.) KOCH. (Characterization of two cell wall cationic proteins in A. araucana seeds). Riquelme, A. Departamento de Biología, Facultad de Ciencias, Universidad de Chile. Patrocinado por Dra. L. Cardemil.

Paredes de embrión y megagametofito de Araucaria araucana fueron aisladas, purificadas y posteriormente sus proteínas extraídas con $CaCl_2$. Electroforesis en geles SDS-poliacrilamida de las proteínas asociadas a la pared celular de ambos tejidos de la semilla, revelaron la presencia de varias proteínas de las cuales las dos proteínas más prominentes fueron purificadas por cromatografía de isoenfoque, teniendo ambas un punto isoeléctrico de 10.5 ± 0.3. Las proteínas purificadas poseen un peso molecular de 65 y 100 KD.

Además estas proteínas dieron positiva la reacción de marcación con dansilhidrazina, estableciéndose que se trataban de glicoproteínas. Un posterior análisis de los azúcares componentes de estas glicoproteínas, reveló que ambas están glicosiladas con Galactosa, Glucosa y con una menor proporción de Xilosa y Manosa.

Análisis de la composición de aminoácidos muestra que ambas son ricas en Serina y Glicina, constituyendo estos aminoácidos casi un 50% de las proteínas. Análisis de secuencia amino terminal demostró que ambas proteínas poseen una secuencia idéntica, lo cual podría indicar que posiblemente son isoformas de una misma especie proteica.

Debido a que las peroxidasas son proteínas comúnmente presentes en las paredes celulares de plantas, especialmente en especies leñosas, como es A. araucana, se quiso detectar actividad de peroxidasa de estas proteínas en geles nativos con o-fenilenediamina como sustrato, encontrándose reacción positiva para ambas proteínas.

Financiado por Proyecto Fondecyt 0160/88; AID GRANT DPE-5542-G-SS-8073-00.

102

DISTRIBUCION DE CAROTENOS EN SIETE CEPAS DE DUNALIELLA SALINA RECOLECTADAS EN EL SALAR DE ATACAMA (Distribution of Dunaliella salina collected at Atacama saltern). Erazo S. y Viertel M. Instituto de Química, Facultad de Ciencias Básicas y Matemáticas, Universidad Católica de Valparaíso. (Patrocinio: P. Domínguez).

La Dunaliella salina (Chlorophyceae) es una microalga halotolerante encontrada en la poza la Rinconada en Antofagasta, produce una mezcla de carotenos: el todo trans-α-caroteno (A), el todo trans-β-caroteno (B) y el 9-cis-β-caroteno (C) en proporciones variables. Esto nos ha motivado ha efectuar una prospección de fitoplancton en diferentes lagunas salinas del Norte de Chile, con el objeto de seleccionar cepas que sean productoras de los isómeros (B) y (C) en forma mayoritaria.

En el presente trabajo se informan los resultados obtenidos con muestras recolectadas, en tres zonas diferentes, del salar de Atacama. Estas muestras fueron propagadas con un medio de cultivo de agua de mar enriquecida de Provosoli (PES), más una cantidad adicional de cloruro de sodio (15 a 20%) a temperatura de 23 ± 4°C y fotoperiodo 12:12, sin aireación. En estas muestras se analizó la distribución relativa de los isómeros (A), (B) y (C) y la relación xantofilas/carotenos mediante cromatografía HPLC y también se evaluó el contenido de carotenos totales. De los resultados obtenidos se estableció que en muestras recolectadas en la misma zona se encuentran cepas con contenidos de caroteno totales y distribución de los isómeros significativamente diferentes. Existen cepas en las cuales el isómero que predomina es (C) con un 62% relativo, valor superior al obtenido por D. chilena de la Rinconada (CONC-001) y por D. bardawil. De los tres isómeros (C) es el más liposoluble y puede acumularse en el hígado sin los problemas observados para el isómero (B).

Agradecimiento: Al PNUD CHI-87/009. Al Dr. Oscar Parra y Sra. Ana Silvia Cifuentes, Depto. Botánica, Fac. Ciencias Biológicas y Recursos Naturales de la U. de Concepción. Fondecyt 89/0823 que han aportado las cepas.

103

CURVAS DE CRECIMIENTO Y CAROTENOGENESIS EN CEPAS NATIVAS DE *Dunaliella salina* TEODORESCO. (Growth curves and carotenogenesis in native strains of *Dunaliella salina* Teodoresco)

Cifuentes, A.S., Contreras, R., Parra, O., Bellarosa, V. y González, M.

Departamento de Botánica, Facultad de Ciencias Biológicas y de Recursos Naturales, Universidad de Concepción. (Proyecto PNUD CHI 87/009, FONDECYT 89/0823).

Dunaliella es una microalga que se caracteriza por su notable capacidad para producir betacaroteno, pigmento utilizado a nivel mundial en la industria alimentaria, para reemplazar colorantes sintéticos. La aplicación de métodos biotecnológicos ha permitido en Australia, Israel, Estados Unidos y China, optimizar la producción de este pigmento bajo condiciones controladas de cultivo.

En Chile, *Dunaliella* crece naturalmente en los ambientes hipersalinos de la zona norte. Su tolerancia a altas salinidades, temperaturas e irradiación le permite vivir en estos ambientes libre de competidores.

Los objetivos de este estudio pretenden caracterizar y comparar el crecimiento y rendimiento de carotenos, de 8 cepas de *Dunaliella salina* recolectadas en la II Región. Las cepas aisladas y mantenidas en cultivos unialgales, se hicieron crecer en frascos Erlenmeyer conteniendo medio Erschreiber, más una cantidad adicional de 12.5% de NaCl, a una intensidad de flujo fotónico continuo de 150 uE m^{-2} s^{-1} y a 25 ± 4°C, sin aireación.

La cepa CONC-001 (Laguna La Rinconada, Antofagasta) es la que presenta la más alta tasa de crecimiento (k=0.22 div/día), y el menor rendimiento de carotenos (ca. 7.40 ug/l, en fase estacionaria temprana). Por el contrario, la cepa CONC-007 (Salar de Atacama) presenta la tasa de crecimiento más baja (k=0.14 div/día) y produce la mayor cantidad de carotenos (ca. 20.600 ug/l), con una relación carotenos/clorofila de 6:1. Las otras cepas provenientes del Salar de Atacama (cepas CONC-003, 004, 005, 008 y 009) también presentan rendimientos satisfactorios de carotenos, por unidad de volumen.

Estos resultados, aparte de dar a conocer cual o cuales podrían ser las cepas a utilizar en un proyecto de cultivo industrial, también podrían servir de base para un programa de mejoramiento por selección continuada.

ARCHIVOS DE BIOLOGIA Y MEDICINA EXPERIMENTALES

VOL. 23 DICIEMBRE 1990 Nº 4

HUMAN, WHEN?

CYSTIC FIBROSIS

TRANSCRIPTION PROMOTERS FROM THIOBACILLUS

PROGESTERONE BLUNTS OVIDUCTAL RESPONSE TO ESTROGEN

MODELLING OF SELF-EVALUATION OF DRIVE AND MOOD

Abstracts

V Annual Meeting Sociedad Chilena de Ciencias Fisiológicas
XII Annual Meeting Sociedad de Farmacología de Chile
III Annual Meeting Sociedad Chilena de Reproducción y Desarrollo
IV Annual Meeting Sociedad de Biología Celular de Chile
XIV Annual Meeting Sociedad de Bioquímica de Chile
II Biophysics Meeting American Southern Cone

Cumulative Indexes

Sociedad de Biología de Chile

TALLER INTERRELACIONES METABOLICAS ENTRE LAS PLANTAS Y SU ENTORNO

Coordinadora: Dra. Luz María Pérez

RESPUESTAS DE LAS PLANTAS A CONDICIONES DE ESTRES. (Plant responses to stress conditions) Cardemil, L. Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Las plantas, al igual que todos los organismos vivos, responden a condiciones de estrés con síntesis de nuevas proteínas. Entre éstas debemos mencionar a las proteínas universales de estrés térmico de 70 kDa y a la ubiquitina. Esto es lo que ocurre en dos plantas chilenas *Prosopis chilensis* y *Araucaria araucana*, donde los niveles de mRNA para ambas proteínas aumentan en forma significativa, con síntesis *de novo* de la proteína de 70 kDa y aumento de ubiquitina. Ambas proteínas protegen a la planta otorgándole termotolerancia al calor. La ubiquitina marca a las proteínas que han perdido su conformación debido a la condición de estrés térmico, permitiendo que sean reconocidas por proteasas y degradadas. Por lo tanto, libera a la planta de moléculas incapaces de cumplir su función, quedando en las células sólo las proteínas que tienen la estructura terciaria o cuaternaria adecuada para su funcionalidad.

En ningún caso, las plantas sometidas a estrés térmico sufren de una degradación masiva de proteínas. Tal degradación es un proceso extremadamente bien regulado en las plantas. Cualquier señal de estrés del medio ambiente que pueda significar peligro de sobrevivencia para la planta, induce la síntesis de un péptido pequeño que es inhibidor de proteasas. La presencia de tal inhibidor se debe a síntesis *de novo* de la molécula, provocada por señales químicas de metabolitos secundarios volátiles como es el ácido jasmónico.

RESPUESTAS METABOLICAS DE LA CEBADA AL ESTRES HIDRICO Y BAJAS TEMPERATURAS. (Metabolic responses of barley to water and cold stress). Corcuera, L.J., Zúñiga, G.E. y Alberdi, M. Departamento de Biología, Facultad de Ciencias, Universidad de Chile; Instituto de Botánica, Universidad Austral de Chile, Valdivia.

La escasez de agua y las bajas temperaturas son algunos de los factores ambientales más limitantes para el crecimiento de las plantas en el mundo. A pesar de su importancia, los mecanismos moleculares de resistencia a la sequía y al frío son aún poco comprendidos. En este trabajo se pretende discutir algunos de los cambios metabólicos que ocurren en las plántulas de cebada (*Hordeum vulgare*) sometidas a estos dos factores limitantes.

Plántulas bajo estrés hídrico acumularon prolina, glicil-betaína, glucosa y lípidos polares. Plantas tratadas a baja temperatura, en cambio, no acumularon prolina ni betaína, pero sí lípidos polares. La proporción del ácido linolénico en varias fracciones de lípidos polares varió significativamente. Este y otros cambios metabólicos observados parecen tener importancia en la adaptación al estrés. Sin embargo, plantas sometidas a estrés hídrico se hicieron susceptibles al ataque de áfidos. Glicil-betaína podría ser el factor susceptibilizante, pues aumentó la reproducción de los insectos alimentados con una dieta artificial.

Financiado por FONDECYT 1126-89 y 0897-88/DIC-UACH

DEGRADACION DE LIGNINA POR HONGOS Y BACTERIAS (Lignin degradation by fungi and bacteria). González,B. Laboratorio de Bioquímica. Facultad de Ciencias Biológicas. P. Universidad Católica de Chile. Casilla 114-D. Santiago, Chile.

La celulosa, la hemicelulosa y la lignina son los componentes poliméricos principales de las estructuras vegetales. La lignina está formada por monómeros del tipo fenilpropanoico unidos por distintos tipos de enlace. Por ser insoluble y estereoirregular es difícilmente atacada por insectos y microorganismos, lo que le confiere a esta macromolécula un rol protector de la célula vegetal. A pesar de ello, la lignina es degradada biológicamente, proceso en el que ciertos grupos de hongos juegan un rol fundamental.

Evidencia bioquímica obtenida recientemente indica que los hongos ligninolíticos poseen un conjunto de enzimas extracelulares capaces de atacar diversas preparaciones de lignina. Por su parte, las bacterias filamentosas degradan preferentemente lignocelulosas de plantas anuales, produciendo intermediarios del tipo ligninoso. Finalmente, existen varios grupos de bacterias capaces de utilizar subestructuras de la lignina como única fuente de carbono y energía. Entre estas subestructuras están las del tipo ariglicerol-β-ariléter que contienen el enlace β-O-4, el más abundante de la lignina. Estos compuestos de bajo peso molecular son liberados por la acción de los hongos en ambientes en los que está ocurriendo la ligninolisis. De esta manera, bacterias y hongos actuarían conjuntamente en la transformación completa de lignina a CO_2.

INTERACCION PLANTA-FITOPATOGENO: UN MODELO DE DEFENSA DEL TEJIDO VEGETAL. (Plant-phytopathogen interaction: the plant tissue defense model). Pérez, L. M. Dep. Bioq. Biol. Molec., Fac. Cs. Quím. Farm., Univ. de Chile.

Los tejidos vegetales se encuentran expuestos al ataque de fitopatógenos. Para defenderse de esta agresión, poseen la información génica que les permite sintetizar entre otros, a metabolitos secundarios, de bajo peso molecular, llamados fitoalexinas.

La especificidad para el ataque de un patógeno parece estar dada por factores de reconocimiento que involucran la presencia de glicoproteínas, tanto en la superficie del patógeno como de la planta.

La secreción de enzimas hidrolíticas le permiten al patógeno disolver la pared celular vegetal, pero a su vez permiten generar fragmentos de pared celular que actúan como inductores de la respuesta defensiva de la planta.

El conjunto de factores involucrados en el reconocimiento del fitopatógeno y la planta, junto con los mecanismos defensivos que ésta induce, se plantean en un modelo que puede ser resuelto experimentalmente. El modelo es aplicable al mejoramiento genético de plantas sensibles al ataque por patógenos.

Financiado por FONDECYT (157/88) y DTI (B 2950-8923).

Abstracts

of the

FOURTH CONFERENCE OF THE

INTERNATIONAL PLANT BIOTECHNOLOGY NETWORK (IPBNet)

Biotechnology for Tropical Crop Improvement in Latin America

San Jose, Costa Rica
January 14 – 18, 1991

Sponsored by:
Tissue Culture for Crops Project (TCCP)
United States Agency for International Development

Co–sponsored by:
El Centro Agronomico Tropical de Investigacion y Ensenanza (CATIE)

IN VITRO MULTIPLICATION OF PINEAPPLE (ANANAS COMOSUS L ME 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 9 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 fiv 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 Cardenil, 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 perfoming 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 synthezised 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 AUPE 5542-G-SS-8073-00 and FONDECYT 0160/88.

24

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

Liliana Cardenil, Alejandro Riquelme and José Gregorio Rodriguez. 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 #DPE 5542-G-SS-8073-00 and FONDECYT 0160/88.

VIII Reunión Nacional de Botánica

16 al 19 de Octubre de 1991

CENTRO DE EVENTOS INTERNACIONALES
UNIVERSIDAD DE SANTIAGO DE CHILE
AV. LIBERTADOR B. O'HIGGINS 3363

SOCIEDAD BOTANICA DE CHILE
PRESENTA LA
NION NACIONAL DE BOTANICA

de Paneles y Presentaciones Libres.
Las Dunas chilenas como hábitat
humano, florístico y fitosociológico.
Respuestas fisiológicas y moleculares
de las plantas a condiciones de estrés.
La conservación y manejo del
Chile.

Chile
Santiago de Chile
Chile

Catolica de Chile). Regeneracion *in vitro* y respuestas morfogenéticas en algunas especies de importancia económica en Chile.

11:30 : HOPP, E., SALADRIGAS, M.V., CRAMER, P. y HUZZINI, A. (Instituto de Biologia Molecular (CICV) INTA Castelar, Argentina). Expresion de genes de resistencia a virosis en protoplastos de papa.

12:00 : DURZAN, D. (Environmental Horticulture, University of California, Davis, CA 95616 8587). Recent developement in forest biotechnology.

12:30 : LITZ, R.E. (University of Florida, Tropical Research and Education Center 18905 SW280 Street Homestead, Florida 33031, U.S.A.). Fruit biotechnology.

SIMPOSIO III

RESPUESTAS FISIOLOGICAS Y MOLECULARES DE LAS PLANTAS A CONDICIONES DE ESTRES

Sala : Enrique Froemel
Moderador : CARDEMIL, L.

14:30 : PEREZ, L.M. (Facultad de Ciencias Quimicas y Farmacéuticas, Universidad de Chile). Las plantas superiores usan diferentes estrategias para evitar el ataque por fitopatogenos.

15:00 : GOLDSTEIN, G. (Department of Botany, University of Hawaii, Honolulu, HI96822). Respuestas de las plantas con distintos fotosistemas al deficit hidrico.

15:30 : DIETRICH, S.M.C., CORDEIRO NETO, F. (Departamento de Micologia, Universidade Federal de Pernambuco e Instituto de Botanica, San Paulo, Brasil). Induccion de fitoalexinas por hongos de la superficie foliar de Rubiaceae tropicales silvestres.

16:00 PANELES II

17:00 : PARLICH, E. (Inst. Pflanzenphysiol. Hrch Buff Ring 64, 6300 GieBen.). Red metabólica homeostática y sensibilidad al estrés en plantas.

17:30 : HO, T.H.D. (Department of Biology, Washington University, St. Louis, Missouri 63130 U.S.A.). Proteinas inducidas por estrés hidrico/acido

EXPRESION DE LAS PROTEINAS DE PARED CELULAR DE PROSOPIS CHILENSIS EN RESPUESTA AL ESTRES PRODUCIDO POR HERIDAS (Expression of cell wall proteins in Prosopis chilensis as a response to wound stress). Rodríguez, J. y Cardemil, L. Laboratorio de Fisiología y Genética Molecular vegetal. Facultad de Ciencias , Universidad de Chile.

En el presente trabajo se estudia la expresión de las proteínas de pared celular de Prosopis chilensis ("algarrobo chileno") en respuesta al estrés producido por heridas. La hipótesis fundamental es que la expresión de estas proteínas no es específica a este tipo de estrés, y que corresponde a la activación de genes cuya expresión es propia de algún tejido no dañado en un determinado estadio de la diferenciación.

Esta hipótesis se ha evaluado por análisis electroforético (geles catiónicos y aniónicos) e inmunológico (Western) de las proteínas de pared, expresadas durante las primeras etapas del desarrollo y en respuesta al estrés de heridas.

Las inmunoimpresiones, así como una inmunocitoquímica fina, realizadas con tejidos dañados y no dañados durante el desarrollo temprano, han revelado una reacción positiva cruzada con el anticuerpo policlonal anti-extensina de testa de poroto de soya. Las mismas evidencias indican que las células xilemáticas y las epidérmicas son las que reaccionan más intensamente con el anticuerpo y que existe una respuesta al estrés producido por heridas caracterizada por una intensificación de la reacción alrededor de la zona dañada.

La expresión constitutiva y tejido específica de estas proteínas, así como la emergencia prematura de raíces secundarias en respuesta a las heridas son evidencias de que la expresión en respuesta a este tipo de daño podría ser equivalente a un adelantamiento del reloj biológico del desarrollo.

Financiado por proyecto Fondecyt 0150/88; AID GRANT DPE -5542-G-SS-8073-00. Beca de Magister otorgada por la Red Latinoamericana de Botánica.

172

CARACTERIZACION DE DOS GLICOPROTEINAS DE PARED CELULAR DE SEMILLAS DE ARAUCARIA ARAUCANA (MOL.) KOCH QUE RESPONDEN AL ESTRES MECANICO. (Characterization of two cell wall glycoproteins of A.araucana seeds that respond to wounding stress). Ricardo, A y Cardemil, L. Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Las plantas responden al daño por heridas incrementando la síntesis de proteínas que ayudarían a reparar y curar la injuria. El presente trabajo investiga proteínas similares en la pared celular de A.araucana. SDS-PAGE de las proteínas extraídas con $CaCl_2$ desde las paredes celulares purificadas de semillas dañadas y no dañadas, revelaron la presencia particular de dos proteínas predominantes que se acumulan en la pared en presencia de daño. Estas dos proteínas poseen un peso molecular de 83 y 145 kD y ambas tienen un punto isoeléctrico de 10.5 ± 0.3. Además estas proteínas son glicosiladas, debido que dió positiva la reacción de marcación con dasilhidrazina. Un posterior análisis de las azúcares presentes en dichas glicoproteínas, reveló que ambas estan glicosilados con Galactosa, Glucosa y en menor proporción Xilosa y Manosa. Debido a que las peroxidasas son proteínas comunmente presente en las paredes celulares, se quiso dectectar dicha actividad en estas proteínas, encontrandose reacción positiva para ambas proteínas. Además se vió que poseen un pH óptimo de 5 para la oxidación de o-fenilenediamina (o-PND) y con una V máx de 525 M o-PND oxidado·min^{-1}·g^{-1}, Km app. de 13.6 mM para H_2O_2 y Km app. de 3.4 mM para o-PND. Análisis de la composición de aminoácidos muestra que ambas peroxidasas son ricas en Serina y Glicina. Análisis de secuencia amino terminal demostró que ambas poseen una secuencia idéntica.

Financiado por Proyecto Fondecyt 0160/88; AID GRANT DPE-5542-G-SS-8073-00.

MECANISMO DE CONTROL DEL POTENCIAL HIDRICO FOLIAR EN *Prosopis chilensis*: **UNA HIPOTESIS.** (Control mechanism of leaf water potential in *Prosopis chilensis*: a hypothesis). Squeo, F.A. (1), Olivares, N. (1) y Cardemil, L. (2). (1) Depto. Biología y Química, Fac. Ciencias, Universidad de La Serena. (2) Depto. Biología, Fac. Ciencias, Universidad de Chile.

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, 30°02'S, 70° 42'O, 520 msm). Se registraron durante 24 horas, cada los 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 -88 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 fué 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.

Proyecto financiado por AID (USA).

INTERNATIONAL WORKSHOP

DESERTIFICATION: A LANDSCAPE-ECOSYSTEM PERSPECTIVE

TALLER INTERNACIONAL

DESERTIFICACION: UNA PERSPECTIVA PAISAJISTICO-ECOSISTEMICA

AREA ACADEMICA DE DESARROLLO PRIORITARIO DE ZONAS ARIDAS

UNIVERSIDAD DE LA SERENA
LA SERENA, CHILE
OCTOBER, 1991

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

Francisco A. Squeo (1), **Nancy Olivares** (1) and **Liliana Cardemil** (2). (1) Departamento de Biología y Química, Facultad de Ciencias, Universidad de La Serena, Casilla 599, La Serena, Chile. Phone: 56(51)226080 ext. 401, FAX: 56(51)211473. (2) Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile. Phone: 56(2)2712865, FAX: 56(2)2712983.

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 of 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 stablishment of seedlings in the field.

This work has been funded by AID-5542-G-SS-8073-00 (USA).

ISSN 0004 0533

ARCHIVOS DE BIOLOGIA Y MEDICINA EXPERIMENTALES

VOL. 24 NOVIEMBRE 1991 Nº 2

34a. REUNION ANUAL

SOCIEDAD DE BIOLOGIA DE CHILE
Y SOCIEDADES AFILIADAS

Sociedad Chilena de Biología de la Reproducción y Desarrollo
Sociedad de Biología Celular de Chile
Sociedad de Bioquímica de Chile
Sociedad de Botánica de Chile
Sociedad Chilena de Ciencias Fisiológicas
Sociedad de Farmacología de Chile
Sociedad de Genética de Chile

RESUMENES DE
CONFERENCIAS, SIMPOSIOS Y COMUNICACIONES

27 - 30 de noviembre de 1991
Puyehue, Chile

Sociedad de Biología de Chile

339

EXPRESION DE LAS PROTEINAS DE PARED CELULAR DE PROSOPIS CHILENSIS EN RESPUESTA AL ESTRES PRODUCIDO POR HERIDAS. (Expression of cell wall proteins in Prosopis chilensis as a response to wound stress). Rodríguez, C. y Cardemil, L. Laboratorio de Fisiología y Genética Molecular Vegetal. Facultad de Ciencias, Universidad de Chile.

En el presente trabajo se estudia la expresión de las proteínas de pared celular de Prosopis chilensis ("algarrobo chileno") en respuesta al estrés producido por heridas. La hipótesis fundamental es que la expresión de estas proteínas no es específica a este tipo de estrés, y que corresponde a la activación de genes cuya expresión es propia de algún tejido no dañado en un determinado estadio de la diferenciación.

Esta hipótesis se ha evaluado por analisis electroforético (geles catiónicos y aniónicos) e inmunológico (Western) de las proteínas de pared, expresadas durante las primeras etapas del desarrollo y en respuesta al estrés de heridas. Las inmunoimpresiones, así como una inmunocitoquímica fina, realizadas con tejidos dañados y no dañados durante el desarrollo temprano, han revelado una reacción positiva cruzada con el anticuerpo policlonal anti-extensina de testa de poroto de soya. Las mismas evidencias indican que las células xilemáticas y las epidérmicas son las que reaccionan más intensamente con el anticuerpo y que existe una respuesta al estrés producido por heridas caracterizada por una intensificación de la reacción alrededor de la zona dañada.

La expresión constitutiva y tejido específica de estas proteínas, así como la emergencia prematura de raíces secundarias en respuesta a las heridas son evidencias de que la expresión en respuesta a este tipo de daño podría ser equivalente a un adelantamiento del reloj biológico del desarrollo.

Financiado por proyecto Fondecyt Otorgado AID GRANT DPE-5542-G-SS-8073-00. Beca de Magister y financiamiento otorgados por la Red Latinoamericana de Botánica.

340

ESTUDIO QUIMICO BASADO EN FLAVONOIDES DE Sophora linearifolia GRISEBACH (FABACEAE). (Chemical study based on flavonoids of Sophora linearifolia Grisebach (Fabaceae)). Donoso, C., Becerra, J. y Silva, M. Departamento de Botánica, Universidad de Concepción Casilla 2407-10, Concepción.

En Argentina se conocen sólo 2 especies de Sophora, S. rhynchocarpa, arbusto que habita en los valles húmedos de Tucumán y Salta a 1200-1300 m de altura, su nombre vulgar es "ovejero" y parece bastante raro. S. linearifolia, subarbusto plateado con hermosas flores, endémico de las sierras Córdoba y San Luis.

De este último Sophora hemos estudiado sus flavonoides para realizar luego un estudio quimiotaxonómico con Sophora de Nueva Zelanda, Chile, Archipiélago Juan Fernández y Hawaii.

Se aisló un total de 4 flavonoides del tipo isoflavona (Fig. 1), Flavonol (Fig. 2) y Flavonas (Fig. 2) los cuales fueron aislados, identificados y analizados por técnicas cromatográficas y espectroscópicas (U.V., RMN de protones), coloración a los vapores de amoniaco y valores de Rf.

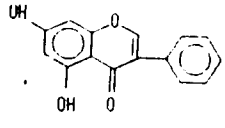


Fig. 1

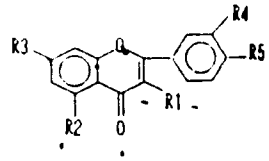


Fig. 2

	C 1	C 2	C 3
R 1	O-galactosa	H	H
R 2	H	OH	OH
R 3	O-galactosa	H	OH
R 4	OH	OH	H
R 5	OH	OH	OH

Agradecimientos. Laboratorio de química de productos naturales de la Universidad de Concepción

341

APROXIMACION CITOGENETICA A LA TAXONOMIA DE Amaryllidaceae CHILENAS (Cityogenetic approach to chilean Amaryllidaceae taxonomy). Palma-Rojas C. Dpto. Biología, Fac. Cs., U. de La Serena.

La nomenclatura de los generos de Amaryllidaceae en Chile ha sido desde sus primeras descripciones muy confusa. Ravenna (1979) reconoce para Chile nueve géneros, señalando que las especies adscritas a Hippeastrum por Baker (1888), pertenecen a los generos Rhodophiala Presl., Phycella Lindl. y Rhodolirium Phil. Sobre este aspecto, Philippi (1885), Hunziker (1985), y Marticorena (1985) coinciden en reconocer a Rhodophiala, pero difieren en cuanto a la validez para Chile de Hippeastrum, Phycella y Rhodolirium. A objeto de contrastar parcialmente algunas de las hipótesis sobre la taxonomía de Amaryllidaceae en Chile, se describen y comparan los cariotipos de Phycella scarlatina (Psc.), Phycella ignea (Psc.), Rhodolirium montanum (Rmo.), Rhodophiala phycelloides (Rpt.), Rhodophiala bagnoldii (Rba.) y la especie cultivada Hippeastrum sp.

Los cromosomas se obtuvieron por aplastado de raicillas pretratadas con actinotótico y teñidas con Feulgen. En fotomicrografías los cromosomas se midieron, recortaron y ordenaron, y las mediciones se presentan en gráficos de dispersión.

Rpt. y Rba. poseen un mismo cariotipo 2n=18. Psc. y Rmo. presentan un cariotipo similar 2n=16, morfológicamente distinto del compartido por las especies de Rhodophiala, pero similar al cariotipo 2n=32 encontrado para Psc. que sería una especie alotetraploide. Hsp. tiene un cariotipo 2n=44 similar al descrito para el genero (n=11) por Naranjo (1975), pero muy diferente de aquellos encontrados para las especies 2n=18, 2n=16 y 2n=32.

Estos resultados concuerdan con distinciones taxonómicas previas que reconocen la validez para Chile de Rhodophiala Presl. (2n=18; n=9) y Phycella Lindl. (2n=16-32, n=8), pero no apoyan dentro del conjunto de especies consideradas la existencia de Rhodolirium Phil. e Hippeastrum Herb.

Financiado por proyecto DIULS 120-2-66.

342

UN NUEVO GENERO EN AEGLIDAE (CRUST.:DECAP.: ANOM.)? (A new genus in Aeglidae (Crust.: Decap.:Anom.)?). Jara, C.G. Instituto de Zoología, Fac. Ciencias, U. Austral de Chile.

Proponer un género nuevo para acomodar una especie ya incluida en otro, desestabiliza la clasificación. Empero, la clasificación resulta de la interpretación de atributos biológicos. Consecuentemente, la clasificación está siempre sujeta a revisión.

Aeglidae comprende 1 gén. fósil (Haumuriaegla, 1 sp.) y 1 gén. actual (Aegla, 40 spp.). Comparten suturas del caparazón que no son homólogas a las del resto de los Anomuros. En Haumuriaegla éstas son incipientes. En Aegla el sistema de suturas es complejo, el lóbulo epibranquial separado del pterygostomio y el telson típicamente dímero.

Se examinaron caparazones de A. papudo, A. rostrata, y Haumuriaegla glaessneri (molde). El material se sonificó (5 min.), se bañó en NaOH 0,5M (85oC, 2 min.), se enjuagó y secó. Se fotografió con estereomicroscopio WILD M8 y equipo MPS 45/51 Photoautomat (DF).

A. papudo tiene órbitas estrechas y profundas, borde orbital lateroventral expandido, lóbulo epibranquial no separado del pterygostomio y telson monómero. Tales atributos justificarían la segregación de A. papudo en un taxón hermano de Aegla. El telson monómero de A. alacalufi sería homoplásico.

* Financiado por Proy. FONDECYT 81-0900 y Proy. S-81-4 DID UACh.

DISSERTATIONS

"LAS PROTEINAS DE ESTRES TERMICO JUEGAN UN ROL FISIOLOGICO EN LA ADQUISICION DE TOLERANCIA A ALTAS TEMPERATURAS EN PLANTULAS DE *Araucaria araucana* (MOL)KOCH."

Tesis
presentada a la
Universidad de Chile
en cumplimiento parcial a los requisitos
para optar al grado de
Magister en Ciencias Biológicas
Mención en Botánica

por

CLAUDIO ALBERTO GOYCOOLEA PRADO

Prof. Patrocinante: Dra. Liliana Cardemil O.

SANTIAGO. Diciembre, 1989
CHILE

150

FACULTAD DE CIENCIAS
UNIVERSIDAD DE CHILE

INFORME DE APROBACION

TESIS DE MAGISTER

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magister presentada por el candidato

CLAUDIO ALBERTO GOYCOOLEA PRADO

ha sido aprobada por la comisión Informante de Tesis como requisito de tesis para el grado de Magister en Ciencias Biológicas con Mención en Botánica, en el Examen de Defensa de Tesis.

Patrocinante de Tesis

Dra. Liliana Cardemil O. .....

Comisión Informante de Tesis

Dr. Luis Corcuera. .....

Dra. Mirén Alberdi. .....

Dr. Carlos Jerez. .....

Dr. Luis Meza. .....

RESUMEN

El objetivo de esta tesis fue el investigar la respuesta fisiológica que presentan semillas y plántulas de Araucaria araucana frente al estrés provocado por calor, orientada a la hipótesis que: "Las proteínas de estrés térmico (PETs) juegan un rol fisiológico en la adquisición de tolerancia a altas temperaturas en plántulas de Araucaria araucana". A. araucana constituye bosques relictuales, desde el Terciario, donde existen diferencias de temperaturas de 20°C entre día y noche, y entre invierno y verano. Las semillas caen, germinan, y las plántulas crecen y se establecen en el suelo volcánico de estos bosques, donde la temperatura alcanza hasta 50°C a medio día en verano.

Se midió la viabilidad de semillas quiescentes de Araucaria araucana por el test del cloruro de trifenil tetrazolio cuando fueron sometidas a 28°C (control) y a 32, 36, 40, 44 y 48°C, distinguiendo tres partes de éstas; eje embrionario, cotiledones y megagametofito. Se determinó que los ejes embrionarios pierden viabilidad después de 5 horas de estar expuestos a temperaturas de 44°C. Los cotiledones demostraron ser un tejido más sensible al calor, ya que pierden viabilidad después de 3 horas de exposición a 44°C. El megagametofito, en cambio, fue el tejido más resistente,

ya que pierde su viabilidad después de 7 horas de exposición a 44°C. La pérdida de viabilidad de los cotiledones podría, sin embargo, ser una condición artificial, porque los cotiledones están protegidos y aislados del medio ambiente por el tejido megagametofítico.

La tolerancia de las plántulas al estrés térmico fue determinada por la capacidad de recuperar su crecimiento a 28°C después de una exposición por 2 horas a temperaturas de 32, 36, 40 y 44°C. Plántulas expuestas a 44°C no recuperaron el crecimiento una vez vueltas a 28°C. Por lo tanto 44°C fue considerada la temperatura letal. Plántulas expuestas a 32, 36 y 40°C por 2 horas, recuperaron su crecimiento una vez vueltas a 28°C y por lo tanto estas temperaturas fueron consideradas temperaturas subletales. La temperatura subletal que permitió una mejor recuperación del crecimiento a 28°C fue la temperatura de 36°C.

Tratamiento a temperaturas subletales indujo en ejes embrionarios una tolerancia para resistir una exposición de 2 horas a 44°C (temperatura letal), siendo la temperatura óptima para la adquisición de termotolerancia 36°C, siempre que se otorgue por más de 30 min.

Se estudió el patrón de proteínas sintetizadas durante el estrés, mediante marcación de las proteínas usando ^{35}S-Metionina en el medio de incubación donde ejes embrionarios fueron sometidos al tratamiento térmico. Después que las proteínas fueron extraídas del tejido, se analizaron por

electroforesis en geles de poliacrilamida-SDS seguida de fluorografía del gel y exposición de éste en un película de Rayos X. Se encontraron 8 nuevas bandas de proteínas no presentes a 28°C. Además, 7 bandas de proteínas mostraron un aumento considerable en su expresión durante el estrés térmico.

Las 8 proteínas sintetizadas solo durante el estrés térmico fueron de un peso molecular aparente de 126, 106, 89, 77, 44, 24, 19 y 17 kD de peso molecular, mientras que aquéllas que aumentaron su expresión durante el estrés térmico fueron de 101, 98, 94, 84, 80, 69 y 20 kD de peso molecular. La temperatura óptima tanto para la síntesis de las nuevas PETs como para las que aumentaron su expresión fue de 36°C.

La cinética realizada a 36°C demuestra que la expresión de la mayoría de las PETs de ejes embrionarios ocurre a los 30 min de iniciado el tratamiento a la temperatura subletal, perdurando la mayoría de ellas después de 5 horas de exposición del tejido a esta temperatura.

Análisis de hibridación "Northern", de los RNA mensajeros para dos de las PETs, usando sondas de cDNA para ubiquitina y proteína 70 kD, evidenciaron que el mayor acúmulo de RNA mensajero ocurre a 36°C, detectando una población de RNA mensajero para ubiquitina y dos para proteína de 70 kD.

El RNA mensajero para ubiquitina estubo presente a

28°C pero aumenta considerablemente durante la exposición del tejido embrionario a 36°C, llegando a ser entre 4 y 6 veces más que a 28°C en ejes embrionarios. Los RNAs mensajeros para la proteína 70 kD fueron muy poco expresados a 28 y 32°C pero la expresión de mensajeros se gatilla a 36°C.

Se concluye que las plántulas de Araucaria araucana respondieron bien al estrés térmico, con la síntesis de nuevas proteínas, aumento de la expresión de proteínas y de los mRNA para las PETs ubiquitina y la proteína 70 kD.

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 Massardo.

Prof. Tutor : Dra. Liliana Cardemil O.

SANTIAGO. Septiembre, 1991.
CHILE

RESPUESTA A ESTRES MECANICO DE SEMILLAS DE
Araucaria araucana (MOL.) KOCH.

ALEJANDRO ANDRES RIQUELME ESCOBAR

Este trabajo de graduación fue preparado bajo la dirección del profesor tutor Dra. Liliana Cardemil Oliva del Departamento de Biología de la Facultad de Ciencias de la Universidad de Chile y ha sido aprobado por los miembros de la comisión calificadora del candidato: Srta. Maria A. Valenzuela, Srta. Francisca Massardo y Sr. Gustavo Zuñiga.

Profesor tutor

Profesor Patrocinante

Director

RESUMEN

En la naturaleza las plantas están expuestas frecuentemente a sufrir daños por heridas causados por insectos, animales herviboros, hongos y otros agentes patógenos. Se ha visto que las plantas responden a dicho estrés con la expresión de algunas proteínas específicas (Lamport, 1980; Davies, 1987; Lagrimini y Rothstein, 1987; Showalter y Varner, 1989).

Las 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 de los niveles de proteínas asociadas a la pared celular. Una clase de estas proteínas son las peroxidasas, cuya actividad fue incrementada de 2 a 50 veces, en un tiempo de 24 a 72 horas después de provocado el daño.

El análisis por SDS-PAGE de las proteínas presentes 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 una masa molecular de 83 y 145 kD.

Estas dos proteínas fueron positivas el ensayo de marcación con dansilhidrazina estableciéndose que se trataba de glicoproteínas. A pH neutro ambas proteínas tienen carga positiva, lo que les permite ser analizadas por

electroforesis de proteínas nativas en geles catiónicos-neutros. Por su naturaleza catiónica pudieron ser analizadas por cromatoenfoque con gradiente de pH de 11-8,5 determinándo que ambas proteínas poseen un punto isoeléctrico de 10,5 ± 0,3.

La centrifugación en gradiente de densidad de CsCl permitió establecer que estas proteínas tienen una densidad boyante de 1,333 $g \cdot ml^{-1}$. Esta densidad no es común de enzimas de pared y tampoco de glicoproteínas ricas en hidroxiprolina. Proteínas de pared celular con esta densidad se han descrito con actividad enzimática de peroxidasa (Liu, 1975). 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 presentes en la pared celular de semillas de A.araucana poseen un pH óptimo de 5 para la oxidación de o-PND y tiene un valor de Km app. de 13,6 mM para peróxido de hidrógeno y una Km app. de 3,4 mM para o-fenilenediamina, con una V máx de 0,525 M o-PNDox$\cdot min^{-1} \cdot mg^{-1}$.

Las impresiones de tejido de semillas dañadas en membranas de nitrocelulosa y reveladas con el ensayo de actividad de peroxidasas mostraron que en las regiones heridas hay un incremento de esta enzima. Además, se observó que la expresión de estas peroxidasas en la pared de semillas A.araucana está regulada por el desarrollo y es

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.