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PHYSIOLOGICAL AND MOLECULAR RESPONSES OF *PROSOPIS CHILENSIS* UNDER FIELD AND SIMULATION CONDITIONS

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Key Word Index—*Prosopis chilensis*; Mimosaceae; algarrobo; high temperature; heat shock proteins; ubiquitin.

Abstract—The diurnal courses of CO_2 assimilation and stomatal conductance in spring were determined in trees of *Prosopis chilensis* growing in Quebrada San Carlos, Vicuña, 4th Region, Chile. These two parameters along with proline content, and the expression of free and conjugated ubiquitin and the heat shock protein (HSP) M_r 70 K were determined at the same time intervals in the leaves of six-month-old plants acclimated in a growth chamber with similar conditions to those of Quebrada San Carlos. The expression of the HSPs was studied by Western and immuno dot blot analyses using monoclonal and polyclonal antibodies generated against these types of proteins. The CO_2 assimilation of northwest-facing trees and plants under simulated conditions showed a bimodal response with maxima at 12.00 and 18.00 hr, when the temperature was 25° and the relative humidities were 35 and 33%, respectively; the minimum was around 15.00 hr with a temperature of 35° and a relative humidity of 32%. The low CO_2 assimilation at 35° was not correlated with stomatal closure, but rather with a nyctinastic movement of leaf and foliole petioles, which greatly decreased the foliar area. After 18.00 hr, CO_2 assimilation declined to become zero or negative at 21.00 hr. Proline, the expression of M_r 70 K and conjugated ubiquitin also reached a maximum at 35°. The expression of free ubiquitin showed the reverse pattern being high at night, early in the morning and late in the evening. The physiological implications of these responses to temperature stress are discussed.

INTRODUCTION

Under natural and cultivated conditions, plants are subjected to different types of environmental stress which act simultaneously e.g. high temperature and drought, or high temperature and UV radiation. It has been demonstrated that temperatures over 40° greatly affect the photosynthesis and respiration of plants [1, 2]. There is evidence that photosynthesis is more sensitive than respiration to thermal stress, photosystem II being the most affected [3]. However, the limits of thermotolerance vary with the level of plant acclimation, with the genotype of the individual plant or with plant species. This indicates that plants are able to tolerate environmental stress by means of morphological, physiological and molecular strategies. Examples of these are: to decrease leaf solar radiation by leaf folding, or to increase compatible solutes, which seems to protect the plants against heat shock or water stress [4, 5] and/or to increase the expression of specific proteins which protect the plant during stress [6, 7].

Prosopis chilensis (Mol.) Stuntz is a leguminous tree from the Mimosaceae, which is found in arid and semi-arid zones of northern and central Chile. In these zones, the daily temperature fluctuates between 8° and 50° during summer [8]. We have previously shown that *P. chilensis* is highly tolerant to heat shock. Seedlings acquire thermotolerance when germination takes place at 35°, showing a displacement of the optimal growth temperature from 35° to 40°, and they are able to grow at the lethal temperature of 50°. Seedlings of *P. chilensis* also express high levels of heat shock proteins (HSPs), and the synthesis of other proteins does not decline under heat shock [9]. In the present work, we have investigated the physiological and molecular responses of young plants of *P. chilensis* during daylight hours in spring in Quebrada San Carlos, Vicuña, a semi-arid region located at 30°0.2′ S, 70°49′ W and at 520 m elevation, and in simulated conditions with temperatures and humidities similar to those found at the natural site. Under these conditions there are simultaneous changes in temperature, radiation and water availability.

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RESULTS

Figure 1 shows a daily course of the foliar temperatures of *P. chilensis* during spring, under natural and

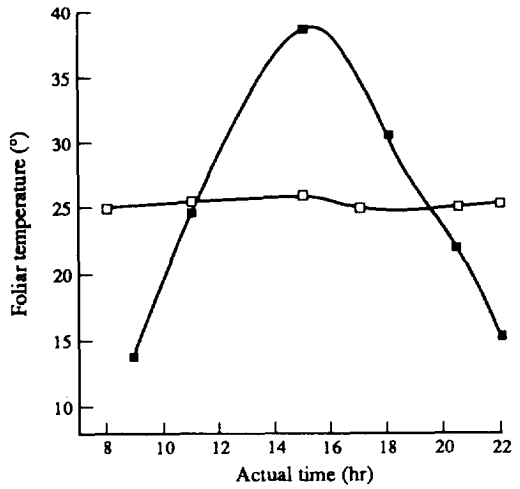

Fig. 1. Foliar temperatures of *P. chilensis* plants during the day: —■— experimental plants; —□—, control plants.

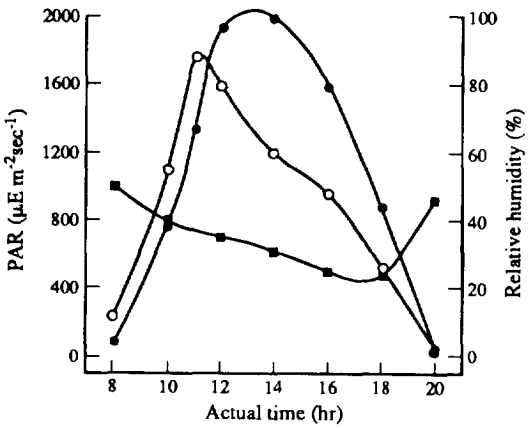

Fig. 2. Daily course of RH (—■—) and PAR under natural conditions in SE-facing trees (—○—) and NW-facing trees (—●—) of *P. chilensis*.

experimental conditions. The foliar temperatures of the experimental and control plants are also shown. A daily course of the photosynthetic active radiation (PAR) for northwest (NW)- and southeast (SE)-facing trees and the relative humidities (RHs) found in Quebrada San Carlos in springtime are shown in Fig. 2.

Carbon dioxide assimilation by SE-facing trees began earlier in the morning (1 μmol m^{-2} sec^{-1} at 8.00 hr) than CO_2 assimilation of NW-facing trees, which began 1 hr later (Fig. 3A). At that time the PAR was higher in the SE than in the NW. However, in both groups of trees the foliar temperature was 13°, the water potential − 0.8 MPa and the RH 50%. The maximum rate of assimilation recorded for the SE-facing trees (8.55 μmol m^{-2} sec^{-1}) took place at 10.00 hr (foliar temp. 18°, water potential − 1.1 MPa and RH 40%). After that time, the assimilation decreased steadily to became negative at 16.00 hr, showing a minimum of − 3 μmol m^{-2} sec^{-1} at 20.00 hr.

Carbon dioxide assimilation of NW-facing trees showed a bimodal response, increasing from a negative CO_2 assimilation of − 0.25 μmol m^{-2} sec^{-1} at 8.00 hr (foliar temp. 13° and RH 50%) to 8.5 μmol m^{-2} sec^{-1} at noon (foliar temp. 30°, water potential − 1.5 MPa and RH 35%). Carbon dioxide assimilation decreased to 2 μmol m^{-2} sec^{-1} at 16.00 hr (foliar temp. 36°, water potential − 2.5 MPa and RH 25%) and then rose to become 5 μmol m^{-2} sec^{-1} at 18.00 hr (foliar temp. 30°, water potential − 1.9 MPa and RH 28%). After 18 hr, CO_2 assimilation declined and again became negative (− 0.5 μmol m^{-2} sec^{-1}) at 20.00 hr (foliar temp. 23° and RH 50%) (Fig. 3A.). At 21.00 hr the water potential was − 1.0 MPa.

Carbon dioxide assimilation under simulated conditions similar to those found in San Carlos for the NW-facing trees also showed a bimodal response (Fig. 3B). However, the rate of assimilation was considerably less than that of trees in Quebrada San Carlos, (from 20 to

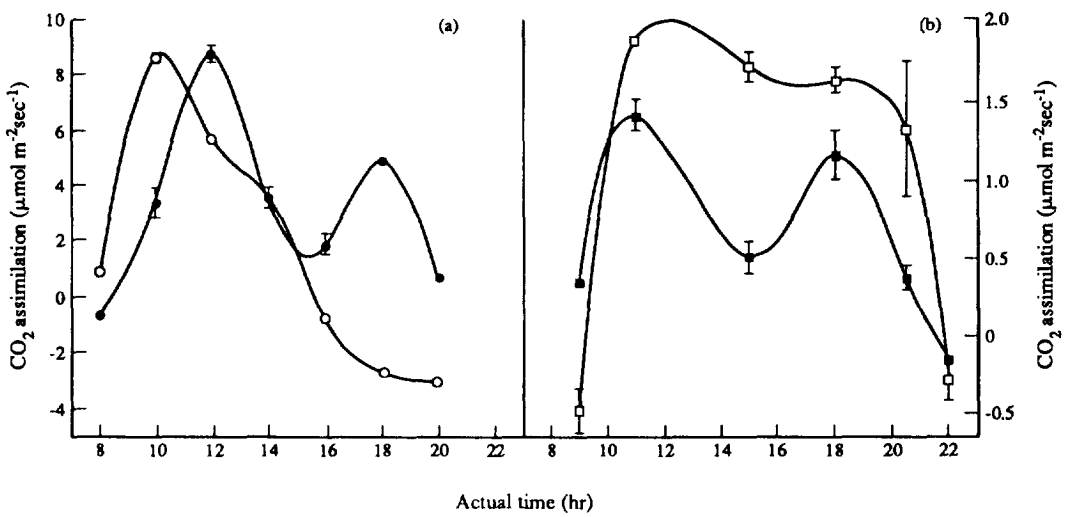

Fig. 3. Diurnal course of CO_2 assimilation in *P. chilensis* plants under natural (a) and simulated conditions (b): —○—, SE-facing trees; —●—, NW-facing trees; —□—, control plants; —■—, experimental plants. Standard deviations correspond to three independent measurements.

25% of the rate of assimilation found in the NW-facing trees). In control plants kept at a constant temp. of 25° and 60% RH, CO_2 assimilation increased at 11.00 hr and was maintained at the same level during daylight hours; it then decreased after 19.00 hr, becoming negative at 21.00 hr (Fig. 3B).

The values for stomatal conductance demonstrated that in both natural and experimental conditions, as well as in control plants (Fig. 4), the stomas remained open during the light period, including the hours of maximum temperature and minimum RH. The stomas closed at the beginning of the dark period, i.e. around 20.00 hr (water potential − 1.5 MPa) in the wild plants and around 23.00 hr in the control plants (water potential − 0.5 MPa). The stomas were closed during the dark period.

Under variable and constant temperatures, the proline concentration in leaves increased with temperature to reach a maximum at 14.00 hr and then decreased to the initial levels at 23.00 hr (Fig. 5). These results showed that the proline levels were always higher in the experimental plants than in the control plants.

In experimental and control plants, the expression of the ubiquitin as conjugated ubiquitin (Fig. 6A) was low early in the morning. It increased as the foliar temperature increased and then decreased in the evening, reaching a minimum expression after 20.00 hr. In the controls, the expression of conjugated ubiquitin was always lower than in the experimental plants, (Fig. 6A). The expression of free ubiquitin showed the reverse pattern to that of conjugated ubiquitin, for both experimental and control plants (Fig. 6B). In experimental plants, the level of free ubiquitin was high in the morning and decreased to 20% of the initial level at 14.00 hr. It started to increase after 14.00 hr, reaching a maximal level at 21.00 hr. In the control plants, the level of expression was also at a minimum at 14.00 hr, increasing after that to reach a maximum level at 21.00 hr. At 14.00 hr, the level of conjugated ubiquitin in the control plants was four times the level found in the experimental plants (Fig. 6A).

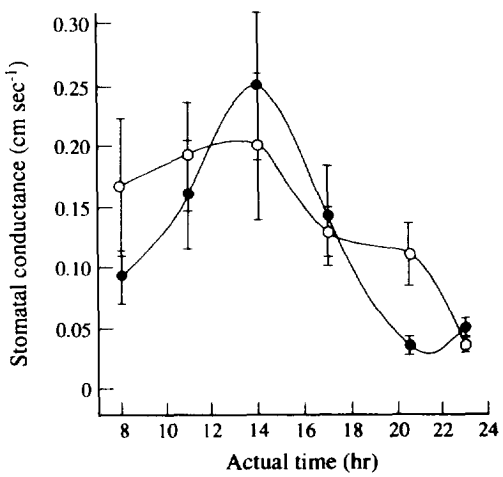

Fig. 4. Stomatal conductance of leaves of *P. chilensis* during the day: —●—, experimental plants; —○—, control plants. Standard deviations correspond to three independent measurements.

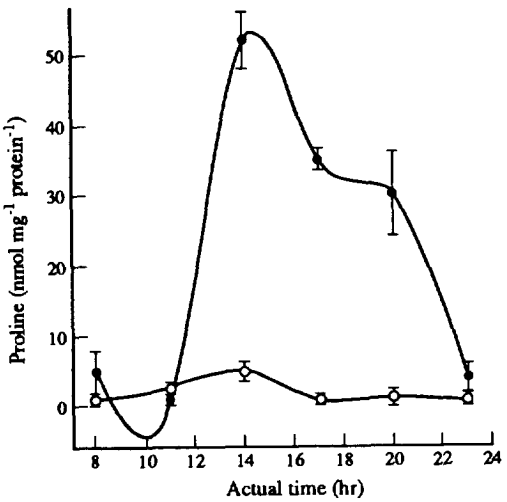

Fig. 5. Proline levels of *P. chilensis* plants during the day:—●—, experimental plants; —○—, control plants. Standard deviations correspond to three independent determinations.

Western blots of free and conjugated ubiquitin corroborated the results obtained with the immuno dot blot analyses. The results are shown for the experimental plants (Fig. 7). Two bands of proteins cross-reacted with the antibody raised against free ubiquitin (Fig. 7A). These were the monomer of M_r 8 K and the dimer of M_r 16 K. Both bands were well expressed at 8.00 hr, fainter at 11.00 and 15.00 hr and stronger at 17.00 hr. The antibody raised against conjugated ubiquitin cross-reacted with several protein bands. All of them seem to have a high M_r since their mass is the protein plus, probably, the mass of several ubiquitin molecules linked to it. The pattern of protein bands is also smeared due to several degradative forms of each protein. These conjugated ubiquitin–protein bands were evident from 11.00 to 21.00 hr, with a higher expression of the ubiquitin-conjugated proteins between 15.00 and 17.00 hr (Fig. 7B).

Western blot analyses for the HSP M_r 70 K family of proteins showed that two proteins of M_r 71 K and 69 K cross-reacted with the monoclonal antibody raised against the M_r 72 K HSP from HeLa cells (Fig. 8). The analysis was performed with 72 hr-old seedlings. In seedling of *P. chilensis*, an increase of expression of these two HSPs was also observed with increase in the temperature treatment from 25° to 50°.

The daily course of expression of the *P. chilensis* HSPs, which were similar to the HSP M_r 72 K from HeLa cells, demonstrated that these HSPs increased in experimental plants with the rise of the foliar temperature, decreased at 17.00 hr when the foliar temp. was 26°, and remained low during the evening and overnight (Fig. 9). In control plants, the expression of these HSPs increased slightly at 11.00 hr, but decreased faster after this time, although the foliar temperature was almost constant at 26° (Fig. 9).

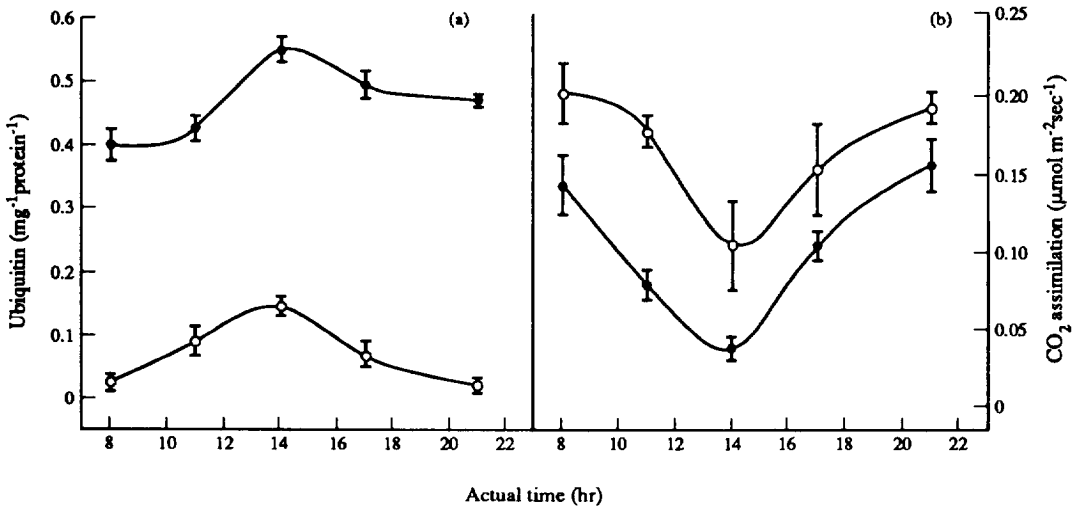

Fig. 6. Levels of conjugated ubiquitin (a) and free ubiquitin (b) in *P. chilensis* plants during the day: —●—, experimental plants; —o—, control plants. Standard deviations correspond to three independent determinations.

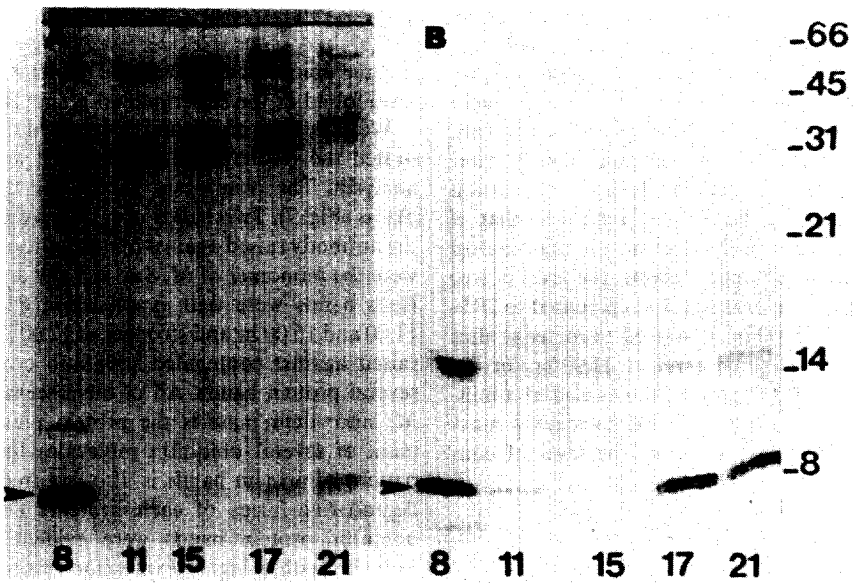

Fig. 7. Western blot analyses of proteins present in experimental plants of *P. chilensis*. Analyses to detect the hours of expression of the free and conjugated ubiquitin were performed as described in the Experimental. The hour of the day appears at the bottom of the photographs and the standard M_rs used in the right margin. **A**, membrane incubated with the antibody raised against conjugated ubiquitin; **B**, membrane incubated with the antibody raised against free ubiquitin. The arrows indicate the protein bands identified as free ubiquitin. The dimer of ubiquitin is seen over alongside the M_r 14 K standard.

DISCUSSION

The results of this study have shown that the rate of CO_2 assimilation is a good indicator of stress in *P. chilensis*. Net photosynthesis is clearly limited at the time of highest temperature and lowest RH. It is well known that the rate of CO_2 assimilation is determined by light intensity, temperature, RH and stomatal resistance [1, 3]. As a consequence, CO_2 assimilation in *P. chilensis* declined at the time of highest temperature and lowest RH and showed a bimodal response during the day in the case of the NW-facing trees and in plants incubated under those conditions. However, in the SE-facing trees, CO_2 assimilation decreased irreversibly after reaching its highest level at 10.00 hr. This probably occurs because the PAR was higher earlier in the SE-than in the NW-facing trees and therefore the maximum PAR also takes place earlier in the SE-facing trees than in the NW-facing

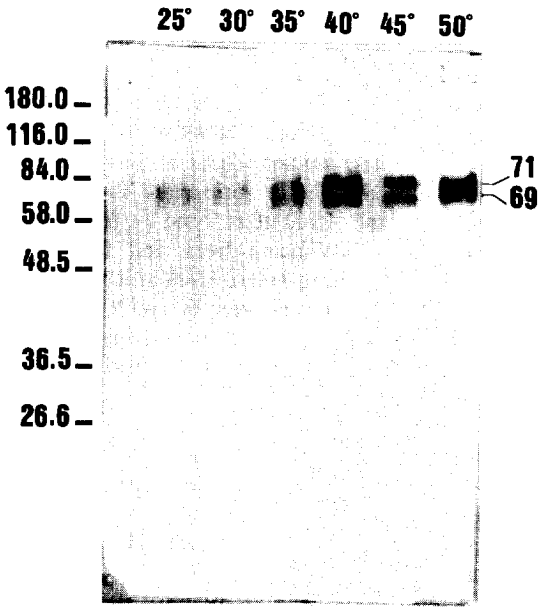

Fig. 8. Western blot analysis of proteins present in seedlings of *P. chilensis* after heat shock treatments for 2 hr at 30°, 35°, 40°, 45° and 50°. The control seedlings were maintained at 25°. Analysis to detect the expression of HSP M_r 70 K was performed as described in Experimental. The temperatures are indicated for each lane at the top of the photograph. M_r markers are shown in the left margin. The M_r for the two cross-reacting proteins appear in the right margin.

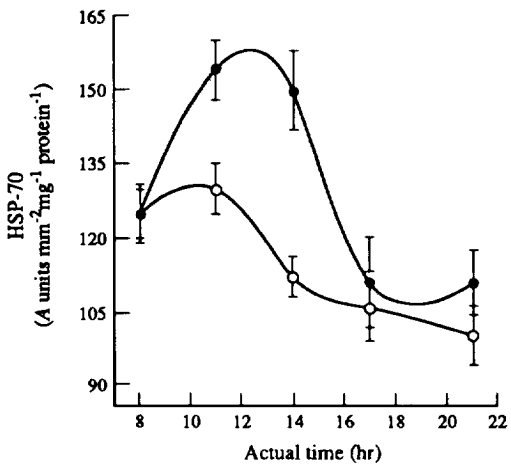

Fig. 9. Levels of M_r 70 K HSPs in *P. chilensis* plants during the day: —●—, experimental plants; —○—, control plants. Standard deviations correspond to three independent determinations.

trees (10.00 hr vs 13.00 hr) with lower foliar temperature and higher RH than those of NW-facing trees at the time of maximum PAR in this orientation. In SE-facing trees, CO_2 assimilation dropped irreversibly after 10.00 hr, probably because the PAR declined when foliar temperatures were still high in those trees.

The bimodal pattern of CO_2 assimilation observed for NW-facing trees occurred under simulated conditions as well. In control plants, CO_2 assimilation was maintained without variation during the light period, because the plants were subjected to optimum conditions of temperature and RH. In all cases, under natural and experimental conditions and in the control plants, there was no CO_2 assimilation during the night. The negative values found for CO_2 assimilation at night were probably due to respiration.

Prosopis chilensis is a legume which belongs to the Mimosaceae. Consequently, one of the strategies of this plant to avoid solar radiation (high light intensity) and high temperature is to activate the petiole pulvinus, causing a nyctinastic movement of the leaves (pinna) and folioles (pinnule) [10, 11]. After the motor cells of the pulvinus have been activated, the reorientation of the folioles takes place reducing the surface of the leaves to near zero when exposed to high solar radiation. Furthermore, in the new foliole position the stomas are hidden, but not closed and therefore continue to transpire and refresh the plant. All these anatomical and physiological responses of *P. chilensis* take place with a very low energy cost to the plant [1]. The nyctinastic movements of the folioles and the stomas' responses have also been shown to occur in *P. chilensis* at Quebrada San Carlos at almost the same hour of the day (unpublished results).

The diurnal levels of synthesis or accumulation of proline were as expected for the experimental plants, i.e. becoming high at the hour of maximum stress. Proline has been considered as a compatible solute whose synthesis or accumulation is induced by heat, water or salt stress [12] and it probably serves to protect the plants under these conditions [13]. Therefore, the increase in proline levels is a good indication that the experimental plants were stressed at the time of maximum temperature and low RH.

Our results demonstrate that this amino acid also increases in the control plants (although to lower levels). The increase in proline in the control plants might be due to the presence of light and the high rate of CO_2 assimilation during the day, since both conditions have been reported to influence proline biosynthesis [12, 14, 15]. We cannot discard, however, the possibility that the fluctuations in proline levels in the control plants could also be caused by a biological clock which might be maintained in these plants by the photoperiod even after 10 days of acclimation to the uniform conditions of 50% RH and 25°.

The expression levels of conjugated ubiquitin also confirmed that the experimental plants were stressed at the time of maximum temperature and minimum RH. Ubiquitin has been described as a HSP [16], which tags the proteins altered by stress, allowing them to be further degraded by proteases [17]. To perform its role, ubiquitin conjugates to the denatured proteins to permit further recognition of the protein–ubiquitin complex by proteases [17–19]. Therefore, ubiquitin can be found in the organisms in its free or conjugated form. The use of an antibody, specifically raised against the unusual pept-

ide linkage between α- and ε-amino groups of proteins and the terminal glycine carboxyl group of the ubiquitin, allowed us to determine the time of day (11.00 to 16.00 hr) when ubiquitin conjugated to proteins with abnormal conformation in *P. chilensis*. Conversely, the use of an antibody raised against free ubiquitin allowed us to quantify the levels of unlinked ubiquitin. Therefore, the levels of free ubiquitin found in *P. chilensis* might indicate that the plant is not under stress overnight, early in the morning or late in the evening. The lower levels found during stress hours might be due to the fact that free ubiquitin is linked to denatured proteins, thus increasing the levels of the conjugated ubiquitin. Pulse-chase experiments need to be performed in order to determine the relationship between free- and conjugated-ubiquitin levels.

The HSP M_r 70 K has been described as a family of proteins which are expressed under several types of stress [20]. Western blot analysis demonstrated that the monoclonal antibody raised against the HSP M_r 72 K of HeLa cells, cross-reacted with two bands of proteins of *P. chilensis*. Both proteins seem to be constitutive HSPs and have M_rs of 69 K and 71 K, respectively. Their expression increased following heat shock treatment when the temperature was raised from 25° to 50°. Since both proteins are constitutive HSPs, the expression level of this family of HSPs is always above zero, as we have found in daily time course expression studies. The expression of the HSPs M_r 69 K and 71 K of *P. chilensis*, showed that the levels of these proteins increased for a shorter period of time at the hours of stress and with less prominent fluctuations than those found for ubiquitin and conjugated ubiquitin. We know that HSPs M_r 69 K and 71 K are moderately expressed in *P. chilensis* seedlings when exposed to a temperature of 35° and that the expression increases considerably from 40° to 50°. Therefore, the moderate levels of expression of these proteins at 35° might be due to the fact that 35° is not the temperature for maximum expression of these two HSPs in *P. chilensis*.

The M_r 70 K family of proteins has been reported to act as chaperones playing a protective role during stress. They fold proteins into their normal configuration when the proteins become unfolded by the stress conditions [20–22]. Our results show that the expression of HSPs M_r 69 K and 71 K is higher in the experimental plants and extends 2 hr beyond the declining hours of expression for the control plants. The M_r 70 K HSPs have been described as transient proteins since their expression declines if the temperature returns to normal or if the heat shock stress continues [23, 24]. It is possible then that 4 hr of expression of these proteins in the experimental plants was long enough for the chaperones to accomplish their protective role in *P. chilensis*.

The smaller increase of the M_r 69 K and 71 K HSPs and conjugated ubiquitin during the daylight hours in the control plants would also be explained by a biological clock, imposed on these plants by the photoperiod regimen and CO_2 assimilation.

EXPERIMENTAL

Source of material. Six trees of *P. chilensis* of similar size (*ca* 50 years old) in Quebrada San Carlos, Vicuña, 4th Region of Chile (30°0.2′S, 70°49′W, 520 m elevation) were used for these studies. Three of them were facing NW and three the SE. In spring, the PAR fluctuates between 100 and 2100 $\mu E\,m^{-2}\,sec^{-1}$ from 8.00 to 15.00 hr in the NW-facing trees and from 100 to 1800 $\mu E\,m^{-2}\,sec^{-1}$ from 8.00 to 15.00 hr in the SE-facing trees. The RH fluctuates between 60 and 25% from 6.00 to 18.00 hr. Samples for analyses and readings were taken at 8.00, 10.00, 12.00, 14.00, 16.00, 18.00 and 20.00 hr.

For simulation experiments, seeds of *P. chilensis* were collected in Quebrada San Carlos, in 1991 and 1992, and germinated at 25° as described in ref. [9]. Seedlings and young plants were grown in a greenhouse at 25°. Three-month-old plants were acclimatized for 10 days in a growth chamber (Heraeus vötsch HPS 2000) under conditions simulating those of the NW-facing trees found in San Carlos during spring, except for the max. light intensity, which was 25% of that found under natural conditions (550 $\mu E\,m^{-2}\,sec^{-1}$) and is not enough for the plant to achieve photosynthetic saturation [25].

Light conditions in the growth chamber. Light conditions were programmed for a photoperiod of 11 hr of light and 13 hr of darkness and temp. and RH cycles similar to those found for the NW-facing trees. The light period began at 8.00 hr and the dark period at 20.00 hr. The light intensity was regulated by varying the height of mobile panels of fluorescent light tubes. The minimum light intensity was 100 $\mu E\,m^{-2}\,sec^{-1}$ at 8.00 hr, increasing to 300 $\mu E\,m^{-2}\,sec^{-1}$ at 11.00 hr and reaching a max. of 550 $\mu E\,m^{-2}\,sec^{-1}$ at 14.00 hr, decreasing after this time to reach the initial intensity at 19.00 hr.

Temp. and humidity conditions. In line with natural conditions, the temp. in the chamber was raised *ca* 4° hr^{-1}, from 6° at 6.00 hr with a RH of 60% to reach a max. of 35° in the afternoon (14.00–15.00 hr) with an RH of 25%. The temp. was lowered after this time by 4° hr^{-1} to reach the minimum of 6° at night (22.00 hr), with an RH of 50%. After 22.00 hr the conditions were kept uniform, at 6° and an RH of 60%. Control experiments were run under the same conditions, except for the temp. and RH, which were kept uniform at 25° and 60%, respectively.

Samples for analyses and readings in the chamber were taken at 8.30, 11.00. 15.00, 17.00, 20.00 and 23.00 hr. The foliar temp. was measured with thin thermocouples of copper constantan.

Heat shock experiments. These were performed with seedlings grown at 25° for 72 hr after germination. Groups of 20 seedlings were exposed for 2 hr at 30°, 35°, 40°, 45° and 50°. After the treatments, the seedlings were frozen in dry ice and processed for protein extraction as described in ref. [9].

Physiological determinations. The net CO_2 assimilation was measured in the field in the leaves of adult trees and under simulated conditions in 6-month-old plants using

an IR gas analyser with an open system for air flow (IRGA, Analytical Development Co. Ltd, type 225/2/SS/B) over 4 consecutive days. For this, the leaves were placed in a Plexiglas chamber. Under simulated conditions, the humidity of the chamber was controlled by passing the air flow through a humidifier system, with the RH going from 60 to 25%. The CO_2 concn was kept between 340 and 370 ppm.

Stomatal conductance measurements were performed with a transient automatic porometer (MK3 Delta-T). In experiments performed under simulated conditions, the porometer was calibrated for the chamber conditions found when the samples were taken for analysis. The total water potential and its components were measured in 6–8 cm terminal shoots with a pressure pump. The water potential was between -0.8 and -2.8 MPa from 6.00 to 15 hr.

Proline determination. Proline was quantified only under simulated conditions. It was determined by the same method as in ref. [26]. The proline concn was determined with a calibration curve, using proline (Sigma) as standard. Total proteins were extracted from the tissue by grinding fresh leaves in 5 ml 1.5 mM Tris containing 2% Triton X-100 and 0.1% mercaptoethanol, pH 7.5. Total proteins were quantified as in ref. [27]. A calibration curve was obtained using commercial BSA as standard.

Electrophoretic analyses. These were performed on SDS-PAGE gels, by the same method as in ref. [28], using a 10% gel. Each gel lane was loaded with 30 μg protein. The proteins were run for 7 hr at 180 V, using Tris–glycine buffer.

Western blot analyses. For western blot analyses, the proteins were electro-transferred overnight from the PAGE gels to nitrocellulose membranes at 180 V [29]. After several washes, the membranes were incubated with polyclonal antibodies raised against free ubiquitin, or conjugated ubiquitin, both from chicken embryos [19], in a dilution for free ubiquitin antibody of 1:200 and for conjugated ubiquitin antibody of 1:300, or with a mouse monoclonal antibody, raised against the HSP M_r 72 K from HeLa cells in a dilution of 1:1000. After incubation with the first antibody, the membranes were incubated with a goat anti-IgG secondary antibody conjugated to alkaline phosphatase in a dilution of 1:30 000. To test the specificity of the antibodies, controls such as membranes incubated with preimmune serum or without the first antibody or second antibody, were run. Immuno dot blot analyses were performed with proteins extracted from plants under simulated conditions, using a Bio Rad Chamber (Bio Dot model), loading all wells of the chamber with the same amount of proteins (12.5 or 20 μg). After this, the membranes were incubated with the first and second antibody as described for Western blot analysis. Immuno dot blots with serial dilutions of commercial ubiquitin were run for calibration curves. Densitometry of the dots was performed in a Gelscan CS-4500 scanner using the Scannedit Computer Program Gray 256. The absorbances of the dots were recorded as peaks, and the integrated areas of the peaks were compared with the absorbances of the calibration curves. The results were expressed in μg of ubiquitin μg^{-1} of protein or μg of conjugated ubiquitin μg^{-1} of protein. In the case of the M_r 70 K HSPs, the results were expressed in A units into $mm^2\ \mu g^{-1}$ of protein due to the absence of a M_r 70 K HSP standard by which to run a calibration curve.

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