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CHANGES IN FREE AMINO ACID CONTENT AND FROST RESISTANCE IN *NOTHOFAGUS DOMBEYI* LEAVES

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Key Word Index—*Nothofagus dombeyi*; Fagaceae; cold resistance; proline accumulation.

Abstract—The annual course of frost resistance and free proline content was studied in leaves at different stages of development of a woody species (*Nothofagus dombeyi*) from Southern Chile. The freezing resistance reached a minimum in late spring or summer and a maximum in the autumn–winter period. Adult and juvenile trees showed a similar degree of resistance; meanwhile, cold resistance was maximum at the seedling stage. Free proline levels and frost resistance in leaves changed throughout the seasonal cycle, increasing in winter and decreasing in summer. Artificial hardening caused changes in amino acid content of leaves; while valine, proline, lysine, histidine, serine and alanine increased upon hardening, aspartic acid, glutamic acid and arginine decreased. The nature of cold-induced metabolic adjustments is discussed as well as its ecological significance.

INTRODUCTION

The factors and mechanisms involved in freezing tolerance in plants are not well-understood. Apparently the accumulation of specific low molecular weight metabolites and macromolecules seems to be associated with development of frost hardiness [1, 2]. This parallel has been found when species or varieties differing in hardiness are compared [3] and when solutes are fed artificially to potentially cold resistant plants [1].

The actual concentrations of free amino acids in plants reflect the steady state between protein synthesis, proteolysis and transport processes to and from the organs involved. These processes are dependent on physiological characteristics, developmental stages and environmental factors. Based on existing data, it seems that the accumulation of certain amino acids is frequently observed in plants subjected to environmental stresses and is sometimes associated with cold hardiness [4, 5].

In this work, we report investigations concerning free amino acid concentration changes, the hardening process and seasonal cycle of resistance to freezing at different stages of development of *Nothofagus dombeyi* (Mirb.) Oerst. (coigüe), a woody evergreen plant species from the Chilean southern rain forest.

RESULTS AND DISCUSSION

Freezing resistance of leaves of *N. dombeyi* reached a maximum in the autumn–winter period (Fig. 1B). From a comparison of seasonal cycle of frost resistance with annual temperature fluctuations in Valdivia (Chile), it is apparent that a relatively close relationship exists between the cycle of frost tolerance and the environmental temperatures (Fig. 1A). Changes in freezing resistance in the leaves were observed at various developmental stages. A small, but consistent, difference in frost resistance between adult and juvenile stages and seedlings was observed, giving LT_{50} values (lethal temperatures for 50% foliar damage) of −5.5°, −6.0° and −7.5°, respectively (Figs 1B and 2). It should be mentioned that the maximum frost resistance at the seedling stage is not a common feature in woody plant species [6].

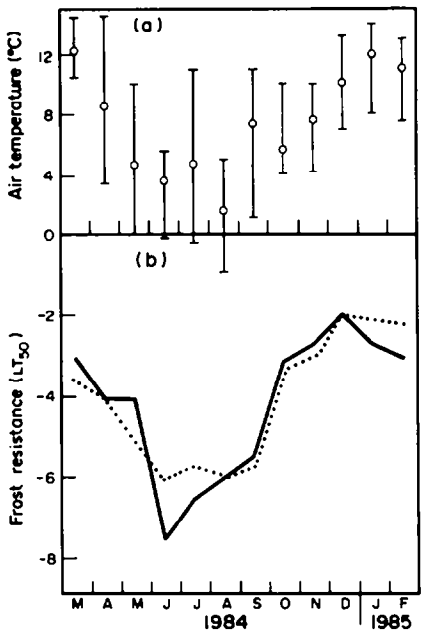

Fig. 1. Seasonal changes in: A, average of air temperature minimum (O) and the oscillation of the temperature 2 weeks before sampling. B, Frost resistance in *N. dombeyi* leaves from juvenile plants (.....) and seedlings (——).

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The low level of frost resistance found in *N. dombeyi* seems to be a common feature of plants from the southern hemisphere [6–8]. Our results would support the hypothesis that the species from the southern hemisphere show a lower freezing tolerance than comparable species from the northern hemisphere, mainly due to the mild oceanic winter conditions prevailing in the southern hemisphere [9, 10].

The seasonal variations of frost hardiness and soluble proline in leaves of adult trees are shown in Fig. 2. The analysis revealed a gradual increase of proline content from summer to winter months. A four- to five-fold increase was reached during the June–September period. As in many other cold resistant plants [4, 5], the accumulation of free proline seems to be associated with increased frost resistance. Nevertheless, the accumulation of free proline as a consequence of frost resistance in cold tolerant plants remains uncertain.

Changes in the levels of free proline in leaves can be also experimentally induced. Juvenile plants were subjected to artificial hardening for various periods of time. Figure 3 shows the time course of the accumulation of proline in leaves in cold-treated plants at 0°. After 24 hr of treatment, proline content reached a steady state level, that was eight-fold higher than controls kept at room temperature. In every experiment performed, there was a consistent lag of about 8 hr from the beginning of the cold-treatment until proline started accumulating. This property, that allows proline levels to change quickly in response to an abrupt temperature change, was also observed in juvenile plants and seedlings kept in the natural habitat. Figure 3 also shows that the rapid increase in free proline coincides closely with the maximum change in frost hardiness.

Nothofagus dombeyi can grow in areas without vegetation formed mainly by volcanic scoria or by deforestation, both unfavourable places with large thermic fluctuations [11, 12]. Thus, seedlings and juvenile trees are subjected to frost danger even during warmer seasons. In response to these abrupt low temperature changes, seedlings and juvenile plants could quickly accumulate proline while the frost resistance capacity increases. This feature could explain the pioneer character of this species as a consequence of their excellent capacity for adaptation [11].

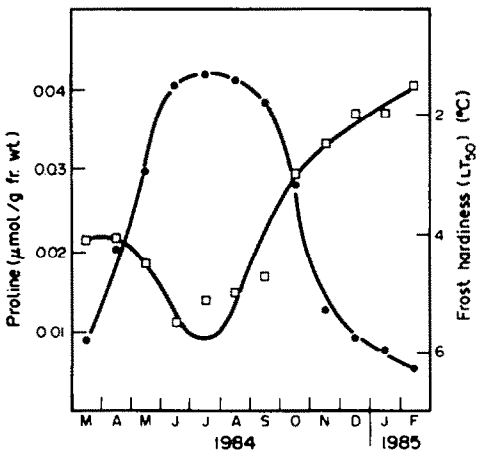

Fig. 2. Seasonal course of frost hardiness (□) and levels of free proline (●) in leaves of *N. dombeyi* adult trees.

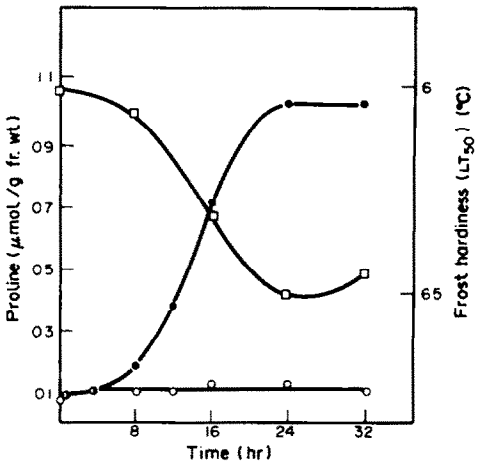

Fig. 3. Time course of frost hardiness (□) and free proline accumulation (●) in leaves of *N. dombeyi*. Juvenile plants were exposed at 0° for various periods of time. The frost hardiness was determined after 0, 8, 16, 24 and 32 hr of cold treatment. Unhardened plants kept at room temperature (○) served as references.

Twigs of unhardened adult trees were also exposed to cold for 24 hr and the frost hardiness as well as free proline were determined (Fig. 4). It is observed that proline accumulation is temperature-dependent. Free proline in leaves increased up to three-fold in cold-treated samples. Experimental data would suggest that free proline can accumulate at an increased level in response to an increased stress. The net increase in free proline was correlated with the increase of frost hardiness. However, under a temperature regime lower than −2°, frost resistance did not parallel the increasing accumulation of proline. Our experiment did not allow us to rule out a cold-induced increase of proline from a water-stress induced accumulation that could occur as a secondary

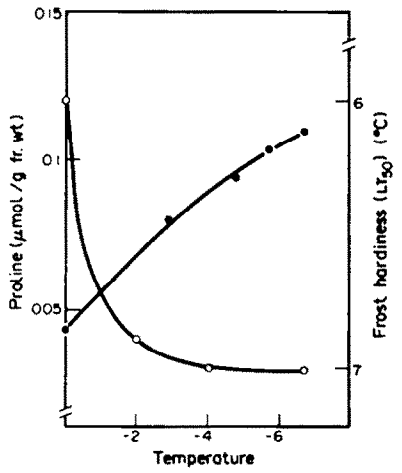

Fig. 4. Variations of free proline contents (●) and frost hardiness (□) in adult *N. dombeyi* leaves. Twigs of unhardened trees were subjected to increasing cold stress for 24 hr. After each cold treatment leaves of free proline and frost hardiness were determined as in the Experimental.

effect due to the efflux of cellular water towards intercellular spaces [1].

As shown in Fig. 5, free amino acid analyses were performed in leaves of unhardened and artificially hardened (24 hr, 0°) juvenile plants. After cold treatment, an eight- to ten-fold increase was observed in valine and proline levels. Meanwhile, alanine, serine, histidine and lysine increased to a lower extent (three- to five-fold). On the other hand, arginine, glutamic acid and aspartic acid declined between 25 and 60% in the same experimental conditions. The remainder of the amino acids analysed were found either in trace amounts or did not reveal significant differences after cold treatment. Methionine, glutamine and asparagine were not analysed.

The cold induced lowering of free glutamic acid could be related to some extent to the high increase of free proline and valine. Glutamate, through the glutamate pathway [13], is a direct precursor of proline and also participates as the amino group donor during transamination of 2-oxoisovaleric acid, a direct precursor of valine [14]. Concerning the decline in arginine, a stimulatory effect has been reported [15] on the conversion of arginine, via ornithine to proline as a consequence of the concomitant water-stress of cold hardening. On the other hand, certain plant species during the fall accumulate organic nitrogen in the form of asparagine [1]. In that case, aspartic acid would be involved as a direct precursor of asparagine [16]. However, it is unknown whether the cold-induced lowering of aspartic acid is related to the increased synthesis of asparagine.

The exact mechanism which triggers the increase of specific amino acids during seasonal or experimentally induced hardening, is not clear. Nevertheless, as far as proline is concerned, there are numerous factors involved in its accumulation. Specifically, the increase in free proline levels appears to occur with accompanying effects on tissue water balance [17], that causes, among other effects, inhibition of proline oxidation [18], decreased protein synthesis [19] as well as stimulation of proline synthesis from glutamate and arginine [15, 18]. Each of these factors might contribute to proline accumulation. To the best of our knowledge the specific accumulation of valine found in *N. dombeyi*, has not been reported before in any other plant species under environmental stress. The positive correlation found between the seasonal cycle of frost resistance and cold hardiness and changes in the levels of certain specific amino acids in leaves of *N. dombeyi*, provides additional support for a direct involvement of free amino acids in the development of hardiness.

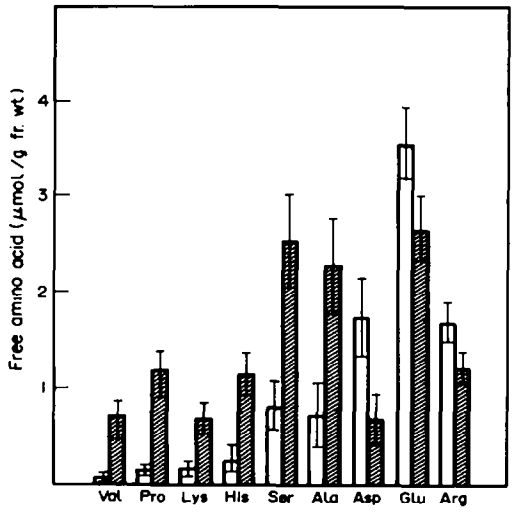

Fig. 5. Changes of free amino acid contents in leaves of juvenile trees of *N. dombeyi*. Individuals were exposed 24 hr at 0°. Each column is the mean ± standard deviation of triplicate samples of: (▨) cold-treated trees or (□) controls kept at room temperature. LT_{50} values were measured at the end of cold treatment, being −6.5° and −5.9° for cold-treated samples and controls respectively.

EXPERIMENTAL

Materials. One-month-old *Nothofagus dombeyi* seedlings, juvenile individuals (1-year-old) and mature trees (25-year-old) were provided by the Botanical Garden of Universidad Austral de Chile, Valdivia, located at 39° 48′ S. Seedlings or twigs (10–15 cm in length) of juvenile or mature trees with leaves of equivalent ages were collected from March 1984 through February 1985, and taken to the laboratory in insulated containers. The seasonal cycle of frost hardiness, experimental cold hardening, as well as free amino acid levels, were determined in leaves.

Determination of frost hardiness. The plant material (10 repetitions per trial) was placed in polyethylene bags and kept in controlled low temp. regimes for 190 min as previously described [8]. After treatment, the samples were thawed in the air at 1°. The lower parts of the shoots were kept in water for 7 days and then freezing injury (browning) was measured [20, 21]. According to Levitt [1] the temp. producing damage in *ca* half of the leaf was used as a measure of frost hardiness (LT_{50}). The results are given as the average for each month.

Cold treatment. Unhardened juvenile plants of *N. dombeyi* were subjected for various periods of time at 0°. Also, twigs of mature trees kept in polyethylene bags were hardened at −3°, −5° or −7° for 24 hr.

Method of proline determination. Leaves were frozen in liquid N_2 and then crushed and homogenized in water, using a Sorvall Omni-mixer at full speed for 30 sec. The homogenate was extracted twice with H_2O at 80° for 20 min. The combined extract was passed through a cation exchanger as previously described [22] and total amino acids eluted with 0.5 N NH_3. Eluates were brought to dryness using a rotary evaporator. Samples for amino acid analysis were prepared in 0.1 M sodium citrate buffer pH 2.2 and separated and quantified, using an Amino Acid Analyzer. Proline was also determined by the acid ninhydrin method of Singh *et al.* [23].

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