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Effects of low temperatures on the first development stages and protein composition of two genotypes of *Lupinus albus* L

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Summary — Some characteristics related to low temperature effects were studied in 2 white lupin genotypes: a spring one (Lutop) and a winter one (LA99). These characteristics concerned seed imbibition and germination and protein metabolism in young plants. Low temperatures influenced protein metabolism: they stimulated synthesis of some proteins, suppressed synthesis of others and had no apparent effect on some others. The spring genotype showed more important alterations in the young, growing organs (root tips, leaves, shoot apices) whereas, in the winter form, alterations were more important in fully developed leaves. The most hydrophilic proteins seemed to be the most concerned by these alterations.

protein analysis / CLHP / genetic expression

Résumé — Effets des basses températures sur les premiers stades de développement et la composition protéique de 2 génotypes de *Lupinus albus* L. *L'étude de deux génotypes du Lupin blanc, l'un «de printemps» (Lutop), l'autre «d'hiver» (LA99) a permis de mettre en évidence certains effets des basses températures sur l'imbibition des graines, leur germination et le métabolisme protéique des jeunes plants. À basse température, l'inhibition et la germination ne diffèrent pas de façon importante pour les deux variétés (fig 1 et 2). Toutefois, une faible imbibition de la germination (de l'ordre de 5%) se manifeste pour la variété Lutop placée à 5 °C dès le début de l'apport d'eau. L'analyse des protéines indique que l'action du froid se traduit par une activation, une inhibition ou n'a aucun effet, sur la synthèse de différents polypeptides. Pour la variété de printemps, ces variations sont nettement plus marquées au niveau des organes jeunes en croissance (racines (fig 3 et 4), feuilles (fig 6), apex caulinaires (fig 5)). Au contraire, pour la forme d'hiver, elles sont plus importantes au niveau des feuilles complètement formées (fig 6). Les protéines les plus hydrophiles semblent plus affectées par ces modifications. Les réponses des deux génotypes de lupin blanc à l'action des basses températures sur la composition en protéines indiquent donc des modifications du métabolisme des protéines. Ces modifications dépendent de la variété et de chaque organe étudié. De plus, elles traduisent une résistance (ou une adaptabilité) relativement importante du génotype de printemps à l'action du froid.*

analyse des protéines / CLHP / expression génétique

INTRODUCTION

Crop plants are submitted to numerous environmental stresses (salinity, drought, extreme temperatures...). These constraints are significant limiting factors in agricultural productivity, and can cause decreased yields or plant death. But plants possess a high degree of metabolic plasticity. So, chilling sensitive species become more resistant to freezing temperatures when submitted first to moderate stress conditions (cold hardening or cold acclimation).

Cold acclimation is a complex response involving physical and biochemical changes. Variations in protein and fatty acid composition of membranes, as in free sugar and soluble protein content are known to occur in plant cells exposed to low temperatures (Steponkus, 1981; Graham and Patterson, 1982; Guy, 1990). Photosynthesis is greatly affected (Berry and Bjorkman, 1980; Levitt, 1980; Berry and Raison, 1981; Steponkus, 1981; Carter and Brenner, 1985; Herner, 1986; Markhart, 1986). But the molecular basis for cold hardening is not yet understood and the cause and effect relationships remain to be established.

An important component in the adaptation of plants to low temperatures is change in the syn-

Abbreviations : mE, micro-Einstein; 2-ME, 2-mercaptoethanol; TFA, trifluoroacetic acid; ACN, acetonitrile.

thesis of proteins (Sarhan and D'Aoust, 1975; Fowler *et al,* 1977; Kacperska-Palacz *et al,* 1977; Chen *et al,* 1983; Cloutier, 1983; Guy, 1990). Some specific mRNA accumulated at low temperature while others decreased (Meza-Basso *et al,* 1986; Gilmour *et al,* 1988; Mohapatra *et al,* 1988, 1989; Wu *et al,* 1988). Thus, cold hardening might involve change in gene expression (Weiser, 1970; Berry and Raison, 1981; Sarhan and Chevrier, 1985; Mohapatra *et al,* 1989). Cold acclimation of some perennial plants may require both transcriptional activation of genes and the synthesis of new proteins (Weiser, 1970; Voinikov *et al,* 1989; Lin *et al,* 1990). In order to understand the molecular basis of plant adaptation to low temperature, it is important to know if the protein-synthesizing possibilities of the cells change following a genotypic adaptation. However, very little is known about the nature of cold regulated genes. The stress protein synthesis in plant under the influence of low temperature has been poorly studied. No information exists about their identity and their function.

In the present study, we compared protein composition of roots and leaves from two varieties of white lupin, a spring one (Lutop), and a winter type (LA99). This last cultivar, collected in the USSR (Georgia) was studied for cold resistance at the "INRA Station d'Amélioration des Plantes Fourragères de Lusignan, France" (Papineau, 1987). As other winter ecotypes from central Italy, this lupin presented a better resistance to low temperatures than the spring varieties. So, LA99 was selected for the present study as a potentially resistant cultivar and Lutop, the spring variety, as a presumed sensitive one. The aim of this work was to detect protein(s) which could be used as marker(s) for cold resistance. It was also to improve our understanding of the mechanisms of gene expression involved in metabolic response to low temperatures for the sweet white lupin, a species which could be an important crop for protein-rich seed production in European countries.

MATERIALS AND METHODS

Seeds of the two varieties (Lutop and LA99) of *Lupinus albus* L, came from INRA (Station de Lusignan, France, which was associated with this work).

Cultures

Seeds of homogeneous weight (approximately 320 mg per seed for Lutop and 350 mg for LA99) were surface sterilized in a solution of Ca hypochloride (90 g/l; 30 min) and washed in sterile distilled water. Imbibition was obtained by placing the seeds (55 per sample) for 12 h in sterile water and then in Petri dishes on wet filter paper, in the dark, until germination occurred. Samples were subjected either to 25 °C (control) or to 5 °C for different time periods. They were harvested, weighed and stored at –80 °C. Results were expressed as percentages of the weight of the dry seeds.

Seedlings of similar size (24 h imbibition and 40 h in Petri dishes at 25 °C) were grown in growth chambers (24°/18 °C, day/night; 14 h/10 h, light/dark; 54.4 $mE.m^{-2}.s^{-1}$), in sterile distilled-water moistened vermiculite. Plants with 3–4 leaves were divided into two groups: one was maintained under the same growing conditions, the second was transferred to 5 °C (20.4 $mE/m^2/s$). Leaves 1 and 4 (development order) and stem apex (apical bud) were harvested after different exposition times at 5° or 25 °C. Samples were fixed and stored at –80 °C.

For root studies, 3 homogeneous seed samples (50 seeds each) were imbibed and exposed at 25 °C for 4 days in Petri dishes. Then, a control was maintained under the same conditions and samples were submitted to 5 °C for 10, 24 and 48 h, and to 0 °C for 2, 10 and 24 h in total darkness.

HPLC

Proteins were extracted in 0.05 M Tris–HCl buffer, pH 7.5, containing 8 M urea and 5% 2-ME. Polypeptides were separated on a Brownlee RP 300 column (4.6 x 250 mm) with an acetonitrile gradient, in 80 min (solvent A = 0.1% TFA in water: acetonitrile, 88: 12, v/v, solvent B = 0.1% TFA in water: acetonitrile, 20 : 80, v/v).Temperature was 60 °C and flow rate 1 ml/min. Detection was with UV at 280 nm (LKB apparatus). These methods were developed by Burnouf and Bietz (1984) for the study of the reserve proteins of wheat endosperm. The extraction medium as well as the reserve phase wide pore (300 Å) silica gel column used for the polypeptides separation are very powerful (Buehler *et al,* 1989). About 100 µg of proteins were injected for each radicle sample and 200 µg for the shoot apice and the leaves.

RESULTS

Imbibition and germination

At 25 °C, full imbibition of the seeds of both lupin varieties was practically reached within 24 h (fig 1). Increase of seed fresh weight was 150% after 40 h. Imbibition was more rapid for LA99 samples than for those of Lutop during the first hours of water absorption. But this difference disappeared after about 20 h.

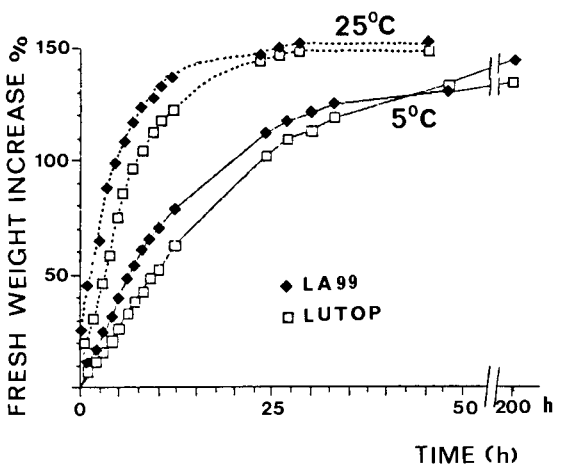

Fig 1. Imbibition of Lutop and LA99 seeds at 25 °C and 5 °C. The increasing fresh weight of each sample (55 seeds) was expressed as percentages of the weight of the dry seeds (initial dry weight = 0%).

Imbibition proceeded more slowly at low temperature: 20 to 25 h were needed to obtain 100% increase of fresh weight at 5 °C, compared to only 5–7 h at 25 °C. 150% increase in fresh weight was obtained only after 220 h at 5 °C (fig 1).

Germination (radicle emergence through the teguments) was obtained at 25 °C after 24 h imbibition followed by 24 h in Petri dishes, on water saturated filter paper. At 5 °C, 168 h in Petri dishes were needed for germination, after imbibition. Initial growth was very slow at 5 °C (fig 2).

Modifications of protein composition

Roots

Plants were grown at 25 °C during 4 days, then at 5° or at 0 °C for various time periods in darkness. Protein compositions of the two controls (Lutop and LA99, at 25 °C) were similar but they could be differentiated quantitatively (peaks 10, 11, 25, fig 4). At low temperature some polypeptides were synthesized and some others disappeared.

Effects of low temperature (5 °C) differed greatly between genotypes. In LA99, these effects (fig 3) were revealed essentially by a decrease of peaks 1, 2 after 24 h and an increase of peaks 10, 11 after 48 h. On the contrary in Lutop, synthesis was evident for polypeptides 23, 24, 25 after only 10 h exposure to 5 °C. Later (after 24 and 48 h), the relative importance of peak 25 persisted, but increased for peak 3, 4 and decreased for 1, 2 (as in LA99).

After exposure to 0 °C, (fig 4), differences between the two varieties were confirmed. Protein composition of LA99 was slightly modified only after 10 h: 3 and 4 peaks were higher than for controls, but 1 and 2 peaks were lower. After 24 h, an important increase of peak 25 and a decrease of peak 3 and 4 was noted. For Lutop, the polypeptide composition varied greatly and rapidly. After 2 h at 0 °C, many peak heights had increased: 3, 4, 6, 7, 23, 24, 25, 32 but this increase was attenuated for peaks 10, 11. After

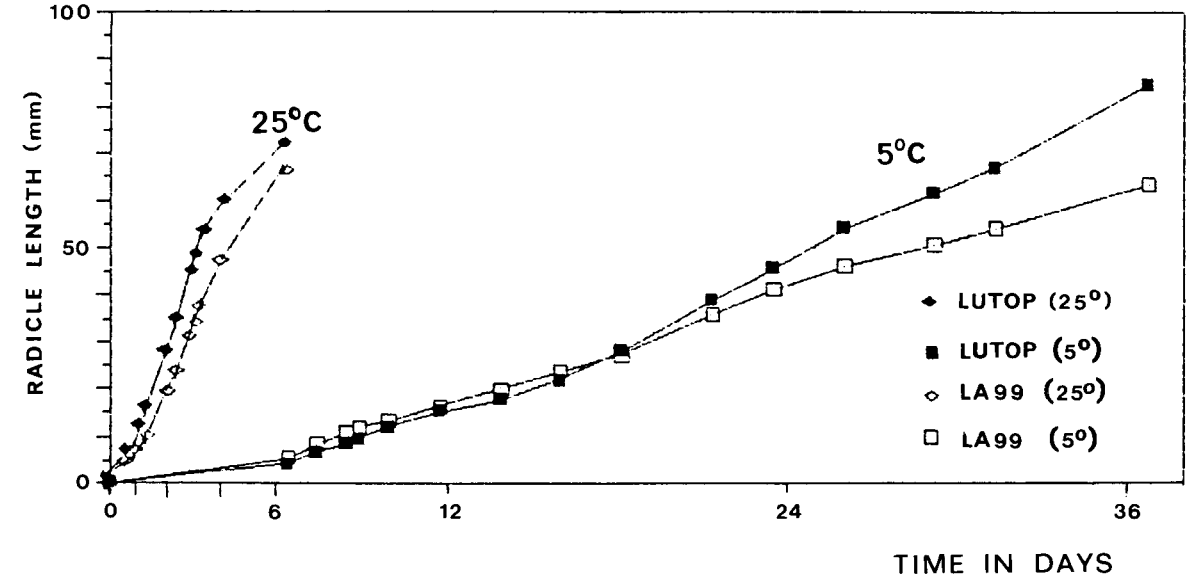

Fig 2. Growth of Lutop and LA99 seedlings at 25 °C and 5 °C. Growth was expressed as increasing length of the radicles (55 seeds).

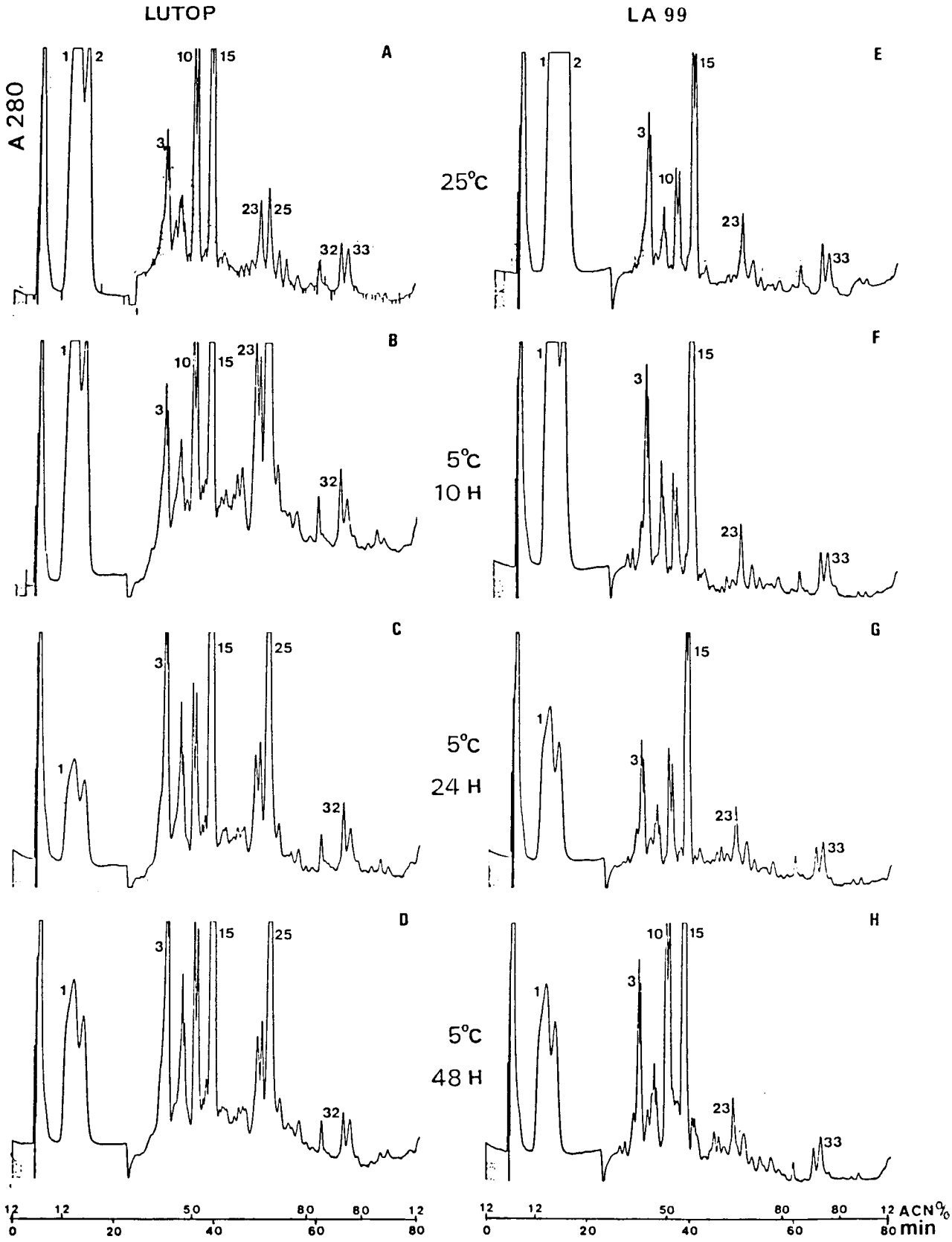

Fig 3. Analysis of root apex proteins of Lutop and LA99 by high performance liquid chromatography (HPLC). Lutop: A: control, 4 days at 25 °C, B, C, D: 4 days at 25 °C followed by 10, 24 or 48 h at 5 °C, respectively. LA99: E: control, 25 °C, F, G, H: 5 °C: same conditions as for Lutop. Absorbance at 280 nm.

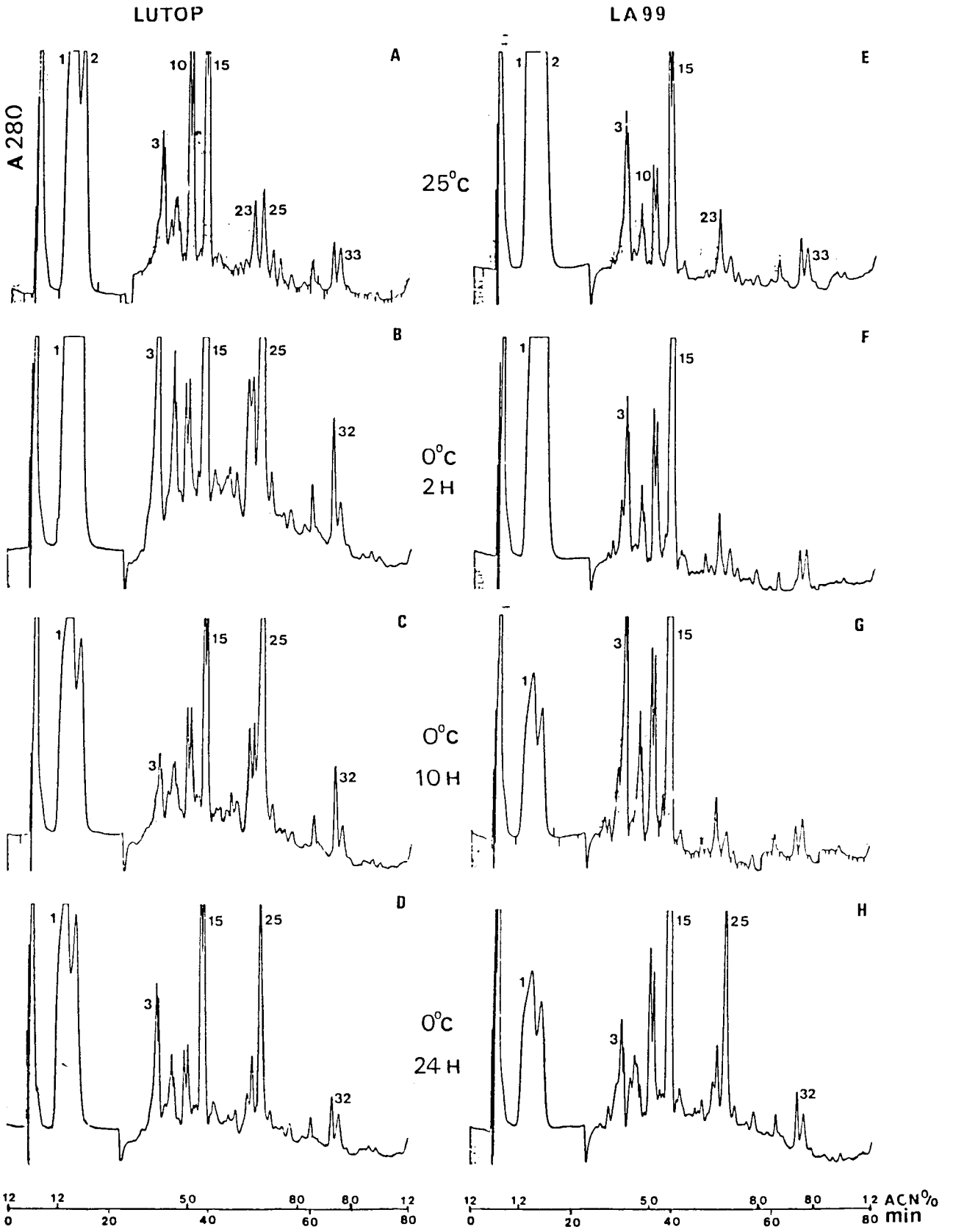

Fig 4. Analysis of root apex proteins of Lutop (A, B, C, D) and LA99 (E, F, G, H) by HPLC. Lutop: A: control: 4 days at 25 °C; B, C, D: 4 days at 25 °C followed by 10, 24, or 48 h at 0 °C. LA99: E: control, 25 °C; F, G, H, 0 °C. Absorbance at 280 nm.

24 h, this increase of peak 25 was maintained, but surface areas of peaks 10, 11 decreased. At this stage, the chromatographic profiles of Lutop and of LA99 were relatively similar, the main difference concerning peaks 10, 11.

Shoot apices

Controls for apex extracts from Lutop and from LA99 gave comparable chromatograms (fig 5). After 24 h at 5 °C, some peak areas (7, 8, 9) showed an important increase for Lutop. But alterations were limited for LA99, the most important one concerned peak 17 which was very slight in controls. After 14 days at 5 °C, chromatograms from the two genotypes were more similar, but some differences persisted, essentially for the hydrophilic polypeptides (peaks 3 to 9 and peak 16).

Leaf 4 (fig 6a, b, e, f)

The polypeptide compositions were very similar in both genotype controls. However, higher relative concentration of the proteins eluted in peaks 1 and 2 of the Lutop variety could be noted. After

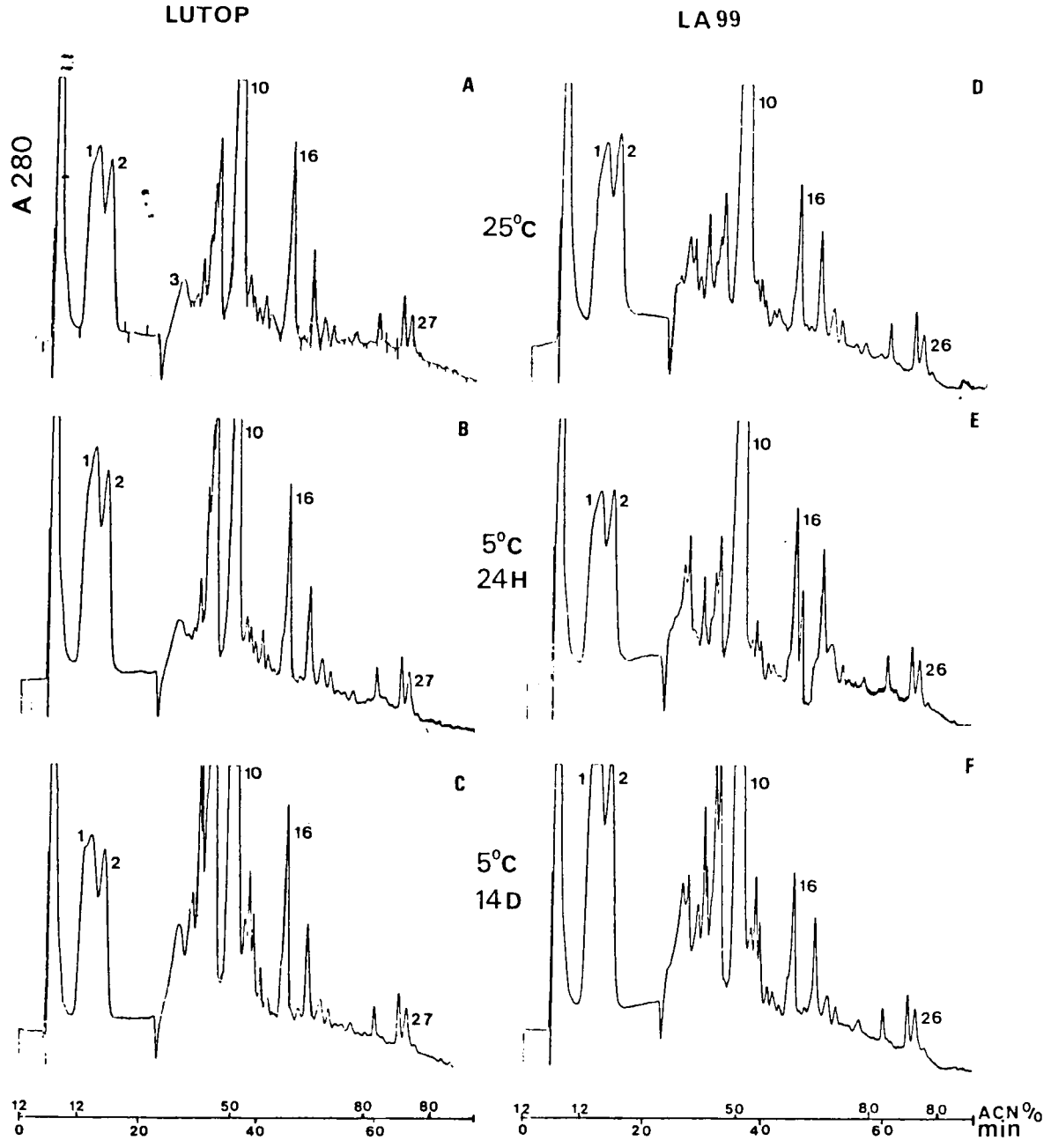

Fig 5. Analysis of shoot apex proteins of Lutop (A, B, C) and LA99 (D, E, F) by HPLC. Samples were grown at 25 °C during 14 days and exposed to 5 °C for 24 h or for 14 days. Absorbance at 280 nm.

14 days at 5 °C, the chromatogram for LA99 extract was not greatly modified (peaks 1, 2 decreased, 7 increased) but for Lutop, peaks 8 and 9 increased.

Leaf 1 (fig 6c, d, g, h)

The protein compositions of the controls were once more very similar in both lupins. Surprisingly, modifications of polypeptide compositions by low temperatures (14 days at 5 °C) were inverted compared to those described for the other organs. Indeed, for this leaf, the oldest one, the most important alterations were obtained for LA99 extracts. These alterations concerned peaks 5 to 9 (relatively hydrophilic polypeptides) and 19, 20 (more hydrophobic). For Lutop samples, peaks 4 to 6 (hydrophilic) were the most affected ones. The appearance of a new peak (31), missing in controls, should also be noted.

DISCUSSION

Imbibition and germination were studied only to obtain some morphological data indicating the effects of low temperatures on the lupin seeds development.

Imbibition of white lupin seeds (cultivar Kalina) has been previously studied (Le Deunff *et al,* 1989). Water uptake was different for soaked seeds or for seeds imbibed on a moistened support, as for the different parts of the seed (teguments, cotyledons, axis), the axis absorbed more rapidly. Moreover, the rate of water uptake was higher when the different seed compartments were hydrated separately. Compared to the present results obtained for Lutop and LA99 genotypes, water penetration in the "Kalina" seeds proceeded apparently more rapidly (at 20–25 °C).

For Lutop and LA99 genotypes, seed imbibition differed under normal growing temperature (25 °C) and under low temperature conditions (5 °C). For the soybean, the highest low temperature sensitivity appeared during seed imbibition and germination was reduced when imbibition proceeded at 12–14 °C (Bramlage *et al,* 1978; Hobbs and Obendorf, 1978). For the lupin, germination percentage was reduced for the Lutop variety at low temperature: 5% of the seeds did not germinate at 5 °C. Seed exposure at 5 °C resulted in an increase of the duration (4 times) needed for germination. Seeds from truly chilling sensitive plants do not germinate below 10 °C. Seeds from chilling resistant species are able to germinate under the same conditions, but germination rate may be slow and germination percentage may decrease (Herner, 1986). Moreover, the plasma membrane reorganization that occurred during water uptake by the seeds during imbibition is delayed by low temperatures (Bramlage *et al,* 1978). More injury (electrolyte leakage, necrosis at the radicle tip, damage to the root cortex) could occur if imbibition is accomplished under low temperatures (Bramlage *et al,* 1978). It is also well known that low soil temperatures decreased highly the water absorption by herbaceous plants (Chen *et al,* 1983). Thus, owing to their different imbibition and germination behaviours, even the more sensitive genotype of white lupin studied here possesses only a relative sensitivity to the low (5 °C) temperature applied, compared to other plants such as soybean.

At the protein composition level, exposure of plants to low temperatures has been shown to induce alterations in protein metabolism (Guy *et al,* 1985; Meza-Basso *et al,* 1986; Mohapatra *et al,* 1987a, b, 1988). The development of cold hardening is also correlated to changes in the levels of translatable mRNA (Meza-Basso *et al,* 1986; Mohapatra *et al,* 1987a; Gilmour *et al,* 1988). It was proposed that low temperature could induce the expression of some genes (Schaffer and Fisher, 1988). The proteins synthesized appear to be specific to cold induced stress. Some are related to photosynthesis. Indeed, quantitative increases in the levels of RuBP carboxylase and fructose BP phosphatase appear to enable plants to maintain a sufficient photosynthetic rate at low temperature (Berry and Raison, 1981). Cold treatment also induced an increased synthesis of several membrane proteins (Yoshida and Uemura, 1984; Yoshida, 1984; Mohapatra *et al,* 1988).

Alterations of the protein composition of the two lupin genotypes were recorded for roots, shoot apices and leaves. Some proteins were synthesized (cold induced proteins), whereas some others disappeared (cold repressed proteins). These alterations of protein metabolism were obtained at 5 °C, a temperature generally considered as related to conditions of cold-hardening (Guy, 1990). At 0 °C, stronger damage is often observed, but for the two lupins studied, protein composition of the roots was not dramatically modified during the first 24 h of cold exposure. Under these conditions, alterations in

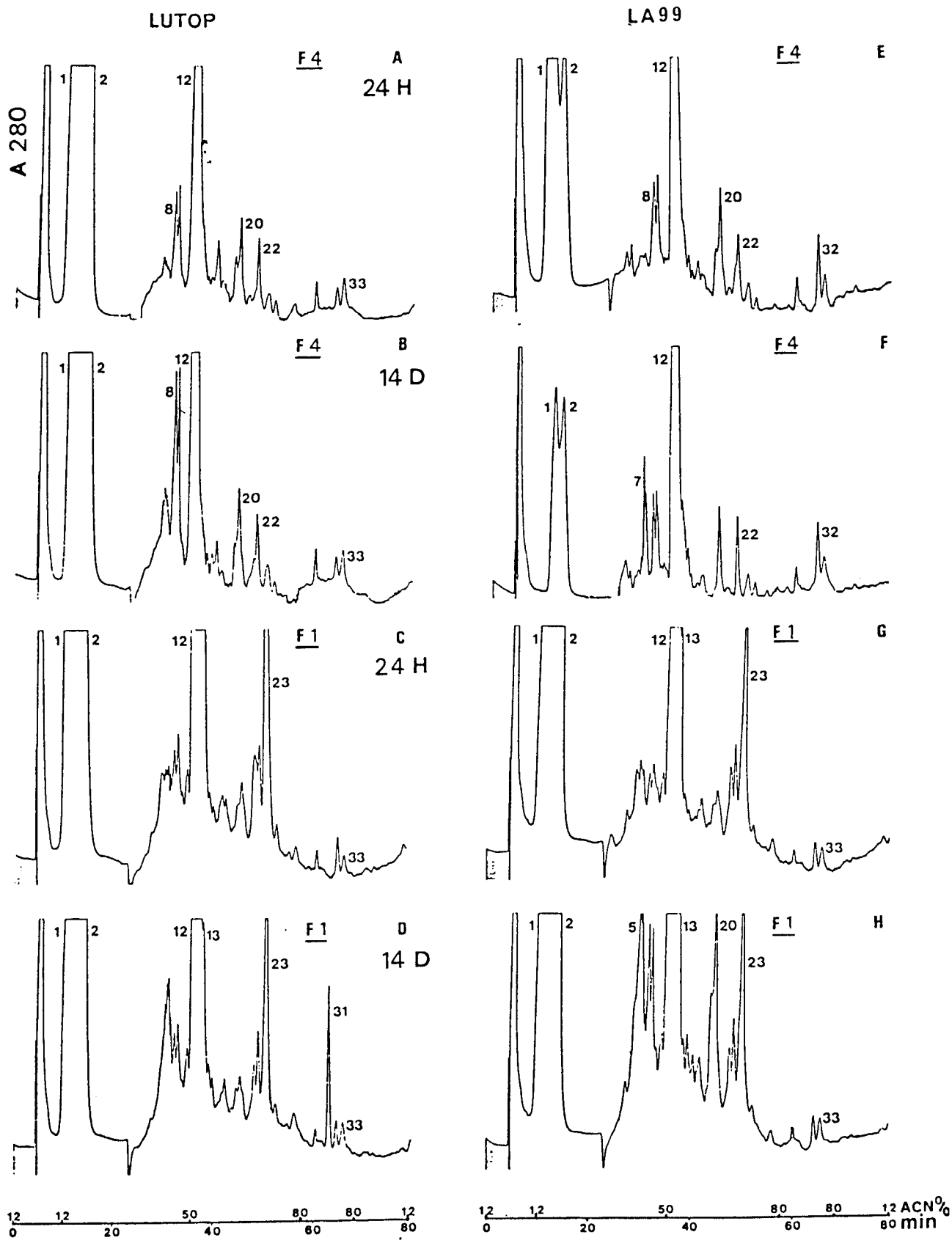

Fig 6. Analysis of leaf 4 (F4) and leaf 1 (F1) proteins of Lutop (A, B, C, D) and LA99) (E, F, G, H) by HPLC. Plants were grown at 25 °C during 14 days and exposed to 5 °C for 24 h or for 14 days. Absorbance at 280 nm.

chromatographic profiles appeared more rapidly and were more important for the spring variety. These results confirm that roots have great capacities to acclimate to chilling temperature (Markhart, 1986). They also show that it is probably more the sensitivity of the plant to cold which induces metabolic adaptation rather than its resistance. The results indicate other features: roots, shoot apices and young leaves (*ie* young growing tissues) present the same type of response in protein pattern alterations during cold exposure, *ie* a more important reaction in the spring variety. On the contrary, older leaves show greater alterations in the winter genotype. Thus, for the white lupin, metabolic responses of the plants to low temperature are highly dependant on organ type and on the physiological state of a given organ.

The results reported in this study indicate that the spring variety Lutop of the white lupin is more sensitive to the low temperatures. When these conditions were applied to the plant, its protein composition presented more modifications than that of the winter variety LA99. The greatest resistance of this last type is reflected by its greater metabolic stability. The modifications of protein composition that appeared for Lutop probably indicate part of the metabolic adaptations that could be implicated in the chilling acclimation processes of this variety.

It should also be noted that HPLC is a very convenient method for investigating very small alterations in protein metabolism that usually require the use of labelled compounds. This technique could be used to identify proteins specific of cold hardening or cold resistance. Studies are in progress in our laboratory to isolate such markers.

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