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Effect of drought and rewatering on the metabolism of *Lupinus albus* organs

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Summary

Alterations in the metabolism of *Lupinus albus* organs that result from and subsequently follow a period of severe water deficit (WD) are described. By means of ^{13}C-nuclear magnetic resonance (NMR), changes in the major metabolites were monitored in several plant organs (leaflets and petiole, roots, stem stele and cortex). During the stress, most of the leaves were lost and the stem functioned as a storage repository of sugars (glucose and sucrose) and amino acids (asparagine and proline). Upon rewatering, lupin plants rapidly re-established the relative water content (RWC) and produced new leaves. However, at the metabolic level, the events seem to be more complex, since proline (a stress related metabolite) disappeared rapidly while sugars and asparagine reached the initial pattern more slowly, particularly in the stem.

Introduction

Lupinus albus L. is an important grain legume crop (Petterson, 1998) that is able to recover from severe water deficit (Pinheiro et al., 2001). During the progression of water deficit (WD), the several tissues behave quite distinctly in the amount and rate of water loss. In contrast to the stem stele, which loses only a small amount of water during the stress period, the water content of mature leaves is rapidly and strongly affected by the water shortage (Rodrigues et al., 1995; Pinheiro et al., 2001).

Abbreviations: DAW, days after withholding water; NMR, nuclear magnetic resonance; RW, rewatering; RWC, relative water content; WD, water deficit

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Metabolites, as the end products of the cellular processes, can express the ultimate responses of biological systems to environmental changes (Fiehn, 2002). When subjected to water stress, leaves are known to accumulate organic solutes of several types. The accumulation of such compounds, often called osmolytes, is regarded as one of the mechanisms by which plants tolerate WD, mediate osmotic adjustments, and protect subcellular structures (Hare et al., 1998; Tabaeizadeh, 1998; van Heerden and Krüger, 2002). The role played by osmotic adjustment in WD tolerance is under debate, since some of the data obtained through engineered plants are contradictory (e.g. Nanjo et al., 1999 and Mani et al., 2002), and an increased tolerance is often accompanied by a decrease in growth (Hare et al., 1998; Smirnoff, 1998; Serraj and Sinclair, 2002). However, in *L. albus*, leaf osmotic adjustment does not seem to play an important role in WD survival (Rodrigues et al., 1995). In fact, the plant readily loses the mature leaves, with the stem emerging as a central organ in WD tolerance (Rodrigues et al., 1995; Pinheiro et al., 2001). Since WD affects virtually every aspect of plant metabolism (Zhu, 2002), it is essential to characterise the metabolic alterations induced in the individual organs in order to achieve a more integrated understanding of the whole plant system.

Nuclear magnetic resonance (NMR) spectroscopy can be used for the analysis of the composition, or metabolic profiling, of plant tissues, which allows one to identify and quantify the most abundant mobile metabolites and to determine how concentrations change as a result of environmental disturbances (Bligny and Douce, 2001; Ratcliffe and Shachar-Hill, 2001). By using NMR we analysed the metabolic profiles of *L. albus* organs during WD and rewatering. These analyses may disclose tissue-specific responses, which are important in understanding the metabolic strategy needed to overcome WD in lupin plants.

Material and methods

Plant material

L. albus L. plants (cv. Rio Maior) were cultivated either on a 1:1:1 soil, peat, sand mixture or on washed siliceous fine sand under controlled light (290–320 μmol m^{-2} s^{-1} PAR), photoperiod (12 h), temperature (19/25°C, night/day) and relative humidity (65–70%). Sand was used instead of a soil mixture in order to allow for an adequate collection of roots. Watering was done every other day with a nutritive solution (9 mM KNO_3, 3 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.5 mM KH_2PO_4) and micronutrients (added according to Johnson et al., 1957).

Twenty-four days after sowing, WD was induced by withholding watering. This caused a natural and slow induction of WD. The plants were collected at days 0 and 11 from the imposing of the stress. Rewatering was resumed at day 11 and a recovery sample was collected 2 d later. Sample collection took place 3–5 h after the beginning of the photoperiod. Leaves were separated into leaflets and petiole, and the stem was separated into vascular (stele) and cortical (cortex) tissues. The stem was cut at both the cotyledon level and the shoot/root junction. A small vertical incision was produced at the first of the extremities and the cortex was peeled off of the stele. The separation occurred at the cambium level so that the stele included both the parenchymatous pith tissue and the vascular tissue. Samples of roots and of the very young leaves were also collected.

Characterisation of the plant water status

The leaf water potential was measured with a Sholander pressure chamber (PMS instrument Co, Corvallis, Oregon, USA) at predawn (Ψpd) and the relative water contents (RWC) for leaflets and petiole, stem cortex and stele, and root were determined according to Rodrigues et al. (1995).

NMR analysis of the tissue extracts

In a preliminary study we compared the extraction capacity of the perchloric acid extraction method (Gout et al., 2000) with that of a hot water extraction as described below. We found no significant differences between the two methods in the levels of the several metabolites analysed. Hence, for simplicity we selected the hot water extraction method.

Frozen tissue samples (3–10 g) were dropped into boiling water and boiled for 5 min. Boiled material was ground in a mortar. The slush was centrifuged, the recovered supernatant lyophilised, and the residue resuspended in 4 ml of an aqueous solution containing D_2O (5.8 M), Na_2EDTA (2.5 mM) and NaN_3 (2.5 mM). Spectra were obtained at 75.47 MHz on a Bruker AMX300 spectrometer using a 10 mm diameter broadband probe head with the following parameters: number of scans = 1500; recycle time = 2.9 s; sweep width = 22.7 kHz; and pulse width = 11 μs corresponding to a flip angle of 45°. Proton decoupling was applied during the

acquisition only. The temperature of the probe head was kept at 300 K. Data were processed with 4 Hz exponential line broadening. Quantification of metabolites was carried out by ^{13}C-NMR using dioxan as an internal concentration standard. Well defined peaks for each of the analysed metabolites were integrated using the spectrometer software. Fully relaxed spectra were obtained using 31.4 s for the repetition delay and were then used to calculate correction factors for the areas of relevant peaks. Chemical shifts are expressed in ppm relative to dioxan at 67.0 ppm. For each tissue analysed, triplicate spectra were performed. Resonances due to glucose, sucrose, fructose, asparagine, proline, citric, and malic acids were identified by their chemical shifts.

Results

Severe WD with marked wilting can be observed in plants at 11 DAW (Fig. 1). The youngest leaves were green but with folded leaflets, whereas most of the mature leaves were chlorotic or had been detached. Lupin plants are able to recover from severe WD with Ψpd values 4 to 5 times more negative than those of the control (Fig. 1). We observed recoveries from even more negative Ψpd (−2.4 MPa; data not shown). The imposed WD affected the RWC in a tissue-specific way (Table 1). The stem stele showed a very low decrease in its RWC (circa 5%), whereas the leaflets and the fleshy root were very sensitive to WD, losing 35% and 40% of their water, respectively.

Using ^{13}C-NMR, we detected the major metabolites of *L. albus* organs and followed the changes in their concentrations that were due to WD and occurred during the subsequent plant recovery that came with rewatering. Glucose, fructose, sucrose, malate, citrate, asparagine, and proline were detected, but mannitol, pinitol, and glycine-betaine, which are common stress related osmolytes in other plant species, were not. Fig. 2 represents a stem cortex ^{13}C-NMR spectrum of a WD *L. albus*.

The sugar levels in the several organs after WD and rewatering are shown in Fig. 3. Fructose was present at very low concentrations in control and WD plants, while sucrose and glucose concentrations decreased with stress in leaves and roots, but increased in the stem. Regarding the relative contribution of the two stem components (cortex and stele), 78% of sucrose and 91% of glucose were found in the stele (Table 2). After rewatering, a tendency was observed in all organs to re-establish the initial sugar values, showing an increase in leaflets and roots and a decrease in the stem and petioles. This process was slower in the stele,

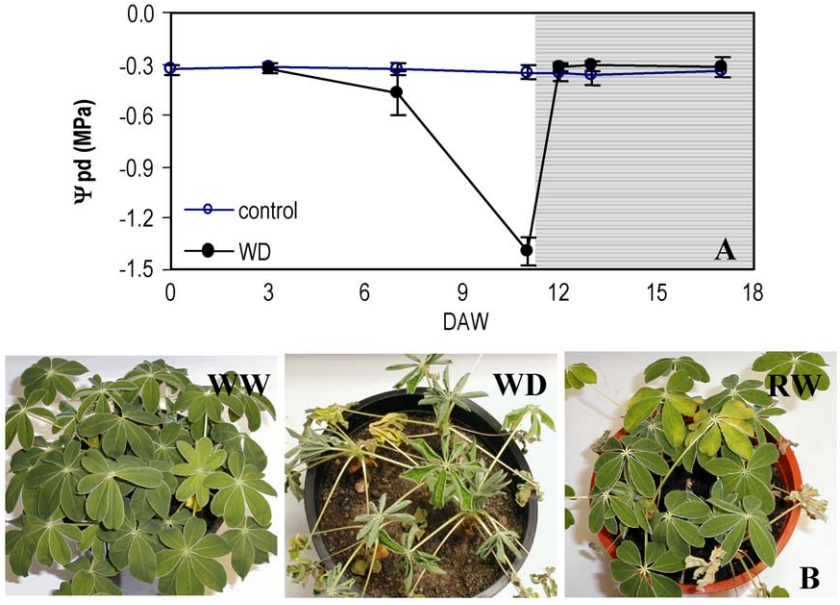

Figure 1. (A) Leaf water potential (ψ_{pd}) of *L. albus* plants, well watered (○), submitted to drought (●) for 3, 7 and 11 d and subsequently rewatered for 1, 2 and 6 d (shaded area). The water potential was determined by Sholander pressure chamber measurements on the 5th or 6th leaf before the beginning of illumination period. Data are the means ±sd of three independent experiments. (B) Effect of water availability on the *L. albus*. WW—plants well watered for the whole growing period (35 d from sowing); WD—plants well watered for the initial 24 d from sowing and to which water was withhold for the following 11 d; RW—plants subjected to WD and subsequently rewatered for 2 d.

where 6 d were necessary for full sugar recovery (data not shown).

In respect to organic acids (Fig. 4), malate concentration decreased with WD in all tissues, most markedly in the roots and the leaves. Rewatering led to a malate increase in all of the organs except in the petioles and cortex. In control plants, citrate was only detected in the leaflets, young leaves, and, more intensely, in the roots. During WD, it almost disappeared in the leaflets and roots (Fig. 4B). Upon rewatering, there was a tendency for an increase in citrate in young leaves and roots.

Examining the amino acids (Fig. 5), we found that asparagine was at a very high level in young leaves of control plants. During WD it greatly decreased in young leaves and roots but tremendously increased in the stem, particularly in the stele. Similar to what was observed for sugars, the stem stele accumulated most of the total amino acids present in the stem (Table 2). After 2 d of rewatering, asparagine was maintained at a high level in the stem tissues but recovered to its initial values in young leaves and roots. Even 6 d after rewatering, asparagine levels remained high in the stem, particularly in the cortex (data not shown). In control plants, proline was only evident in the leaflets and only at a very low level. However, when the stress was applied, proline was detected in all tissues. It was in the stem stele where proline increased the most and in the root where it increased the least (Fig. 5B). Upon rewatering, proline rapidly disappeared from all tissues, including the leaflets.

Table 1. Relative water content (RWC) of *L. albus* leaf, stem and root tissues from well watered plants (WW), plants subjected to water deficit for 11 days (WD) and 2 days rewatered plants (RW)

		DAW	WW	WD	RW
Leaf	Blade	0[a]	90±3	—	—
		11	89±2	57±4	—
		13[b]	88±3	—	87±3
	Petiole	0[a]	88±3	—	—
		11	87±2	71±1	—
		13[b]	86±2	—	83±2
Stem	Stele	0[a]	89±2	—	—
		11	87±2	83±2	—
		13[b]	88±1	—	89±4
	Cortex	0[a]	74±3	—	—
		11	72±2	58±2	—
		13[b]	73±2	—	74±2
Root	Fleshy zone	0[a]	97±4	—	—
		11	99±3	58±4	—
		13[b]	97±2	—	94±3

Data are the means±sd.
[a]Plants 24 days from sowing.
[b]2 d of rewatering.

Discussion

The plant responses to WD include both deleterious effects and adaptive changes (Chaves et al., 2002; Zhu, 2002) that occur in a complex framework of organ interactions through metabolic exchanges and signalling events. By studying the stress reactions of a single organ, as is done in most WD studies, it is difficult to unravel the plant strategy

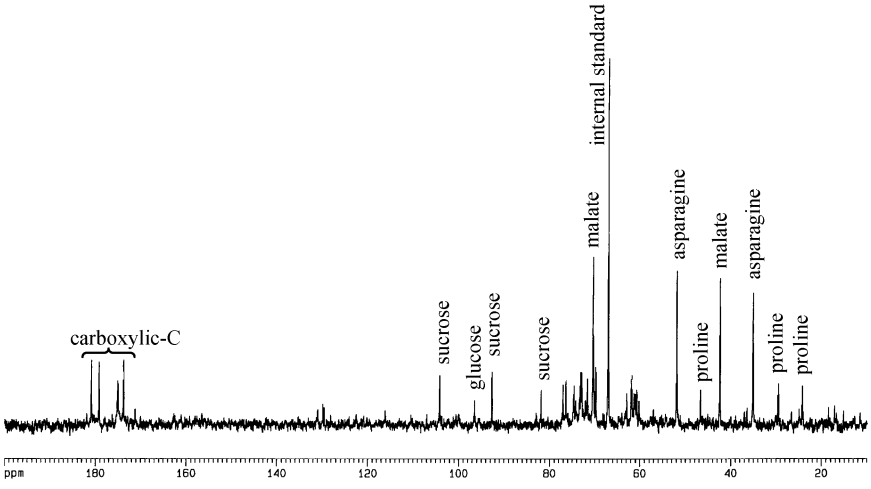

Figure 2. Representative ^{13}C-NMR spectrum of *L. albus* stem cortex of plants subjected to drought for 11 days. The main compounds, sucrose, glucose, malate, asparagine and proline, are identified by means of the chemical shifts. Only the most representative peaks are marked. The internal standard dioxane allows the quantification of the several metabolites.

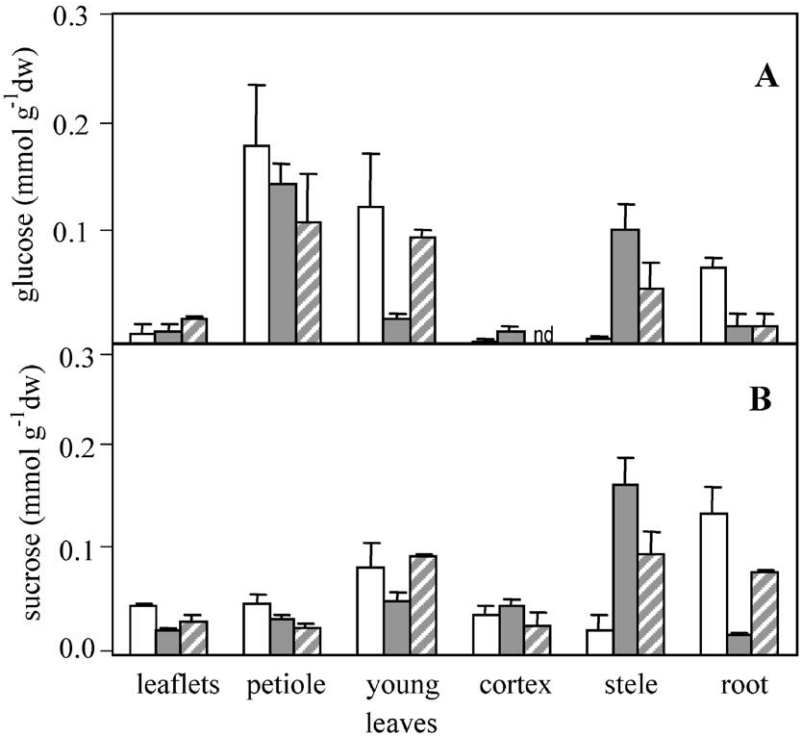

Figure 3. Glucose (A) and sucrose (B) concentrations of *L. albus* leaves (leaflets, petiole and young leaves), stems (cortex and stele) and roots (fleshy zone) in plants under well watered conditions (empty bars), subjected to severe water deficit (11 d; filled bars) and after 2 d rewatering (partly filled bars). Data are the means±sd of three independent determinations; nd—not detected.

Table 2. Relative storage capacity of the two *L. albus* stem components (cortex and stele) for sugars and amino acids in control (WW) and water stressed (WD) plants

		Cortex		Stele	
		μmol per stem cortex	%[a]	μmol per stem stele	%[a]
Glucose	WW	0.06±0.03	4	1.4±0.03	96
	WD	0.6±0.17	9	5.8±1.02	91
Sucrose	WW	1.2±0.24	63	0.7±0.03	37
	WD	2.6±0.35	22	9.1±1.12	78
Asparagine	WW	0.4±0.09	100	nd	0
	WD	11.6±1.48	31	24.9±3.51	69
Proline	WW	nd	0	nd	0
	WD	2.6±0.48	20	10.3±0.99	80

[a]Percentage accumulated in the stem component in relation to the whole stem.nd—not detected. The data are expressed as μmol of metabolite accumulated per stem component of an average plant and as the % relative to the total amount accumulated in the whole stem. The dry weight of the stem of an average plant is 0.06 g (±0.005) for the control and 0.11 g (±0.013) for WD plant. On a dry weight basis, stele and cortex weights are equivalent. WD plants had been deprived of water for 11 days.

used to cope with the stress. We have been investigating the changes produced by WD in several lupin organs in an attempt to understand the whole-plant responses to this stress.

Malate, which is often considered to increase under stress conditions in plants that display osmotic adjustment (Büssis and Heineke, 1998; Patonnier et al., 1999; Gebre and Tschaplisnki, 2002), decreased markedly in all lupin organs, especially in the leaflets and root. This may be related to the fact that in *L. albus* osmotic adjustment is not a relevant mechanism of WD tolerance, at least not in leaves (Rodrigues et al., 1995) and roots (Dracup et al., 1998). So, the decrease in leaf malate level may indicate that WD affected the photosynthetic apparatus, since malate as an alternative end product of glycolysis is considered a good indicator of the photosynthetic

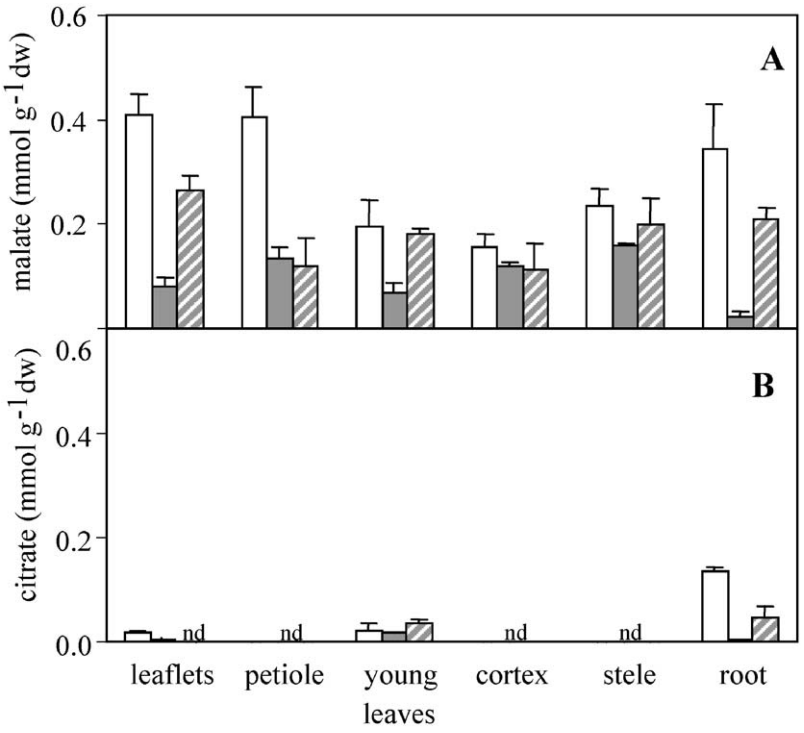

Figure 4. Malate (A) and citrate (B) concentrations of *L. albus* leaves (leaflets, petiole and young leaves), stems (cortex and stele) and roots (fleshy zone) in plants under well watered conditions (empty bars), subjected to severe water deficit (11 d; filled bars) and after 2 d rewatering (partly filled bars). Data are the means±sd of three independent determinations; nd—not detected.

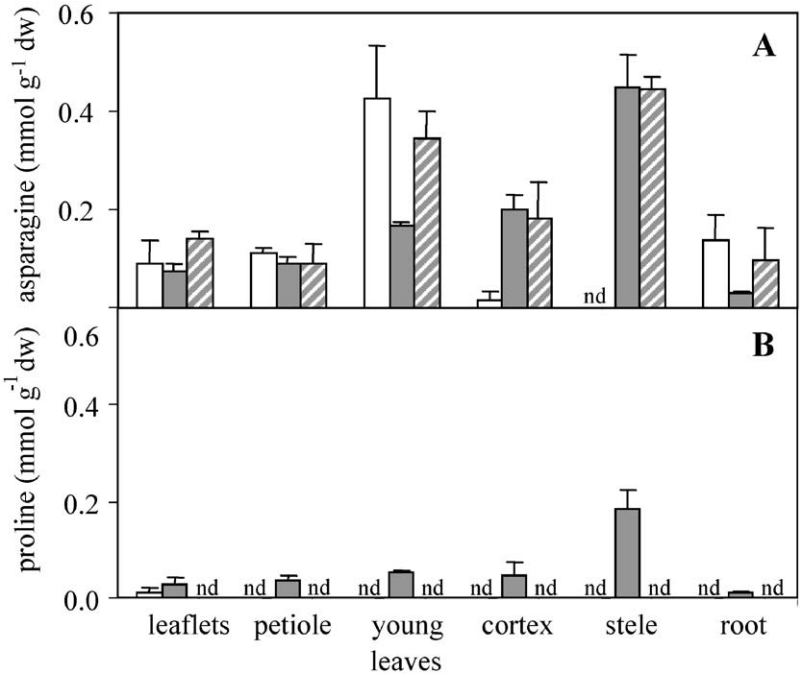

Figure 5. Asparagine (A) and proline (B) concentrations of *L. albus* leaves (leaflets, petiole and young leaves), stems (cortex and stele) and roots (fleshy zone) in plants under well watered conditions (empty bars), subjected to severe water deficit (11 d; filled bars) and after 2 d rewatering (partly filled bars). Data are the means±sd of three independent determinations; nd—not detected.

capacity (Lance and Rustin, 1984; Patonnier et al., 1999). The recovery of malate concentrations upon rewatering suggests that the photosynthetic capacity was only temporarily affected during WD, confirming the remarkable resistance of the lupin photosynthetic apparatus to dehydration, especially in young leaves (Chaves et al., 2002). On the other hand, since malate is closely coupled to nitrate uptake (Foyer et al., 2003), the malate decrease in the root further suggests that nitrogen metabolism may also have been affected.

Considering the changes that occurred in the other metabolites upon WD, the several organs can be associated in two distinct groups: leaves and roots (which do not accumulate sugars and asparagine) and stem tissues (which show the opposite pattern). Interestingly, similar groups are formed when the distinction is based upon the ability of the organ to retain water during the WD stress. This suggests a possible relationship between the metabolite changes and the hydration status of the tissues. These metabolic alterations reinforce the idea that the stem is an essential structure for WD survival serving as a temporary storage organ, as evidenced by dry weight accumulation (Rodrigues et al., 1995) and nitrogen content (Pinheiro et al., 2001). Although Rodrigues et al. (1995) suggested that under WD the remobilised biomass was distributed to the shoots and the roots, we can only confirm a storage role for the stem. Furthermore, it should be emphasised that of the two stem components it is the stele, with a higher ability to retain water, that exhibits the largest storage capacity and that accounts for 70–80% of the sugars and 75–90% of the amino acids accumulated in the stem.

Sugars, in addition to their storage functions, are considered to have an important role in controlling cellular metabolism. In lupin, changes in leaf sucrose were found to precede the accumulation in the stem, not only of sugars, but also of nitrogen and sulphur (Pinheiro et al., 2001). Our present results indicate that asparagine and proline are significant contributors to such nitrogen accumulation. It is important to note that the expression of the asparagine synthetase gene was shown to be dependent on the sugar status of the cell (Lam et al., 1994; Chevalier et al., 1996). Thus, sucrose seems to play a key role in the integration of plant growth and seems to be part of a wider mechanism for balancing carbon acquisition and allocation within and between organs (Farrar et al., 2000; Foyer et al., 2003).

Proline and asparagine behaved quite differently from each other, particularly in the stem and during the recovery period. In fact, asparagine levels were still elevated 2 d after rewatering, whereas proline was rapidly remobilised. The presence of asparagine in excess is considered to be a good marker of protein degradation under stress (Genix et al., 1990; Brouquisse et al., 1992). However, transport events can also be one reason for the maintenance upon rewatering of high asparagine levels in the stem. During rewatering, asparagine rapidly increased in the roots where it is intensely synthesised and from where it could be transported to the actively growing organs (Smith and Gallon, 1993; Lam et al., 1994; Pate et al., 1998).

It is noteworthy that the other amino acid, proline (actually an imino acid), does not follow the asparagine trends during WD and rewatering, and instead appears to be strictly correlated to stress. Proline is rapidly metabolised during the stress relief, possibly as a substrate for the TCA cycle (van Heerden and Krüger, 2002). Hare et al. (1998) discussed the conflicting evidence about proline, which is simultaneously a signal of stress-induced injury and a reliable measure of stress tolerance. This behaviour could explain why proline was simultaneously accumulated in the *L. albus* leaflets (programmed to be shed) and in the stem (functioning as a storage and survival organ).

As a general conclusion we want to stress the importance of NMR metabolite fingerprinting in the characterisation of several organs during the *L. albus* reaction to WD and during the subsequent recovery period. The stem has a central role in the plant strategy to cope with WD, and displays metabolic alterations that markedly differ from those of other survival organs, such as the root and the young leaves.

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References

Bligny R, Douce R. NMR and plant metabolism. Curr Opin Plant Biol 2001;4:191–6.

Brouquisse R, James F, Pradet A, Raymond P. Asparagine metabolism and nitrogen distribution during protein degradation in sugar-starved maize root tips. Planta 1992;188:384–95.

Büssis D, Heineke D. Acclimation of potato plants to polyethylene glycol-induced water deficit II. Contents and subcellular distribution of organic solutes. J Exp Bot 1998;49:1361–70.

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CP, Osorio ML, Carvalho I, Faria T, Pinheiro C. How plants cope with water stress in the field. Photosynthesis and growth. Ann Bot 2002;89:907–16.

Chevalier C, Bourgeois E, Just D, Raymond P. Metabolic regulation of asparagine synthetase gene expression in maize (*Zea mays* L.) root tips. Plant J 1996;9:1–11.

Dracup M, Turner NC, Tang C, Reader M, Palta J. Responses to abiotic stresses. In: Gladstones JS, Atkins C, Hamblin J, editors. Lupin as Crop Plants. Biology, Production and Utilization. Wallingford: CAB International; 1998. p. 227–62.

Farrar J, Pollock C, Gallanger J. Sucrose and the integration of metabolism in vascular plants. Plant Sci 2000;154:1–11.

Fiehn O. Metabolomics- the link between genotypes and phenotypes. Plant Mol Biol 2002;48:155–71.

Foyer CH, Parry M, Noctor G. Markers and signals associated with nitrogen assimilation in higher plants. J Exp Bot 2003;54:585–93.

Gebre GM, Tschaplisnki TJ. Solute accumulation of chestnut oak and dogwood leaves in response to throughfall manipulation of an upland oak forest. Tree Physiol 2002;22:251–60.

Genix P, Bligny R, Martin JB, Douce R. Transient accumulation of asparagine in sycamore cells after a long period of sucrose starvation. Plant Physiol 1990;94:717–22.

Gout E, Aubert S, Bligny R, Rébeillé F, Nonomura AR, Benson AA, Douce R. Metabolism of methanol in plant cells. Carbon-13 nuclear magnetic resonance studies. Plant Physiol 2000;123:287–96.

Hare PD, Cress WA, Van Staden J. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Env 1998;21:535–53.

Johnson CM, Stout PR, Boyer TC, Carlton AB. Comparative chlorine requirements of different plant species. Plant Soil 1957;8:337–53.

Lam HM, Peng S, Coruzzi G. Metabolic control of asparagine synthetase gene expression in *Arabidopsis thaliana*. Plant Physiol 1994;106:1347–57.

Lance C, Rustin P. The central role of malate in plant metabolism. Phisiol Vég 1984;22:625–41.

Mani S, Van de Cotte B, Van Montagu M, Verbruggen N. Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. Plant Physiol 2002;128:73–83.

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K. Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett 1999;461:205–10.

Pate JS, Emery RJN, Atkins CA. Transport physiology and partitioning. In: Gladstones JS, Atkins C, Hamblin J, editors. Lupin as Crop Plants. Biology, Production and Utilization. Wallingford: CAB International; 1998. p. 181–226.

Patonnier M, Peltier J, Marigo G. Drought-induced increase in xylem malate and mannitol concentrations and closure of *Fraxinus excelsior* L. stomata. J Exp Bot 1999;50:1223–31.

Petterson DS. Composition and food uses of lupins. In: Gladstones JS, Atkins C, Hamblin J, editors. Lupin as Crop Plants. Biology, Production and Utilization. Wallingford: CAB International; 1998. p. 353–84.

Pinheiro C, Chaves MM, Ricardo CP. Alterations in carbon and nitrogen metabolism induced by water deficit in the stems and leaves of *Lupinus albus* L. J Exp Bot 2001;52:1063–70.

Ratcliffe RG, Shachar-Hill Y. Probing plant metabolism with NMR. Ann Rev Plant Physiol Plant Mol Biol 2001;52:499–526.

Rodrigues ML, Pacheco CMA, Chaves MM. Soil-plant water relations, root distribution and biomass partitioning in *Lupinus albus* L. under drought conditions. J Exp Bot 1995;46:947–56.

Serraj R, Sinclair TR. Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant Cell Env 2002;25:333–41.

Smirnoff N. Plant resistance to environmental stress. Curr Opin Biotechnol 1998;9:214–9.

Smith RJ, Gallon JR. Nitrogen fixation. In: Lea PJ, Leegood RC, editors. Plant biochemistry and molecular biology. England: Wiley; 1993. p. 129–53.

Tabaeizadeh Z. Drought-induced responses in plant cells. Int Rev Cytol 1998;182:193–247.

Van Heerden PD, Krüger GHJ. Separately and simultaneously induced dark chilling and drought stress effects on photosynthesis, proline accumulation and antioxidant metabolism in soybean. J Plant Physiol 2002;159:1077–86.

Zhu JK. Salt and drought stress signal transduction in plants. Ann Rev Plant Biol 2002;53:247–73.