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RESEARCH PAPER

Sugar metabolism in developing lupin seeds is affected by a short-term water deficit

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Abstract

A short-term water deficit (WD) imposed during the pre-storage phase of lupin seed development [15–22 d after anthesis (DAA)] accelerated seed maturation and led to smaller and lighter seeds. During seed development, neutral invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13) have a central role in carbohydrate metabolism. Neutral invertase is predominant during early seed development (up to 40 DAA) and sucrose synthase during the growing and storage phase (40–70 DAA). The contribution of acid invertase is marginal. WD decreased sucrose synthase activity by 2-fold and neutral invertase activity by 5–6-fold. These changes were linked to a large decrease in sucrose (~60%) and an increase of the hexose:sucrose ratio. Rewatering restored sucrose synthase activity to control levels while neutral invertase activity remained depressed (30–60%). A transient accumulation of starch observed in control seeds was abolished by WD. Despite the several metabolic changes the final seed composition was largely unaltered by WD except for ~60% increase in stachyose and raffinose (raffinose family oligosaccharides). This increase in raffinose family oligosaccharides appears as the WD imprinting on mature seeds.

Key words: Galactinol synthase, invertase, *Lupinus albus*, rewatering, seed development, storage compounds, sucrose synthase, sugars, water deficit.

Introduction

Lupinus albus L. is an important grain legume crop (Petterson, 1998) that is often subjected to water deficit (WD) during late spring. A transient water shortage during vegetative development can cause large physiological and metabolic alterations (Rodrigues *et al*., 1995; Pinheiro *et al*., 2001, 2004), but usually does not reduce seed yield appreciably (Dracup *et al*., 1998). However, if WD occurs after flower anthesis, seed yield is greatly affected in *L. albus* (Huyghe, 1997; Dracup *et al*., 1998). Previous studies have been largely centred on the composition and nutritional value of the fully developed seeds (Huyghe, 1997; Petterson, 1998), and little is known about the effect WD exerts on seed composition and metabolism during seed development.

Sucrose and related sugars are key metabolites and important forms of carbon translocation in the plant that provide backbones for the synthesis of seed storage compounds. WD greatly affects photosynthesis and growth, which results in a decrease in sugar availability and alterations in relative sink strength. Therefore, sucrose-related metabolism must have a central role in the events triggered by WD as well as the enzymes responsible for the initial metabolization of sucrose (e.g. sucrose-phosphate synthase, sucrose synthase, and acid and neutral invertases). Sucrose is also involved in the synthesis of galactosyl-sucrose oligosaccharides, known as the raffinose family oligosaccharides (RFO), which are important carbohydrate reserves considered to have an additional role in the desiccation tolerance of seeds (Keller and Pharr, 1996). Galactinol synthase is considered a key enzyme in this pathway (Keller and Pharr, 1996).

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The aim of this work was to study *L. albus* seed development and the effect exerted by a transient WD on seed metabolism and composition. A transient stress, imposed during the pre-storage phase, is a common event during this stage of plant development under natural conditions and will restrict the availability of the photo-assimilates to the forming sink. It is the chain of events associated with this stress, namely those aspects of sucrose metabolism expected to have some impact on the reserve accumulation pattern and mature seed composition, which is of interest.

Materials and methods

Plant material

Lupinus albus L. (cv. Rio Maior) seeds were sown in sand/soil/peat (1:1:1 by vol.) mixture and the plants grown under greenhouse conditions from December to July, with temperatures ranging from 15 °C to 35 °C and relative humidity from 40% to 80%. Four assays were performed between 1998 and 2002. A similar trend of responses was observed in each of the experiments. The data shown in this work refer to the last one and the most complete of those experiments. The time of flower pollination was considered as day zero [0 DAA (days after anthesis)].

WD was imposed by suppressing watering at 15 DAA, until pre-dawn leaf water potential (Ψ_{pd}) reached −1.0 MPa (~7 d), after which, the plants were watered regularly. Control plants were kept well watered (WW) throughout the experiment. Ψ_{pd} was measured in the most recently fully expanded leaves using a Scholander pressure chamber (PMS Instrument Co., Corvallis, OR, USA).

The effect of WD on photosynthesis was analysed at the end of the stress imposition period. Stomatal conductance and net photosynthetic rate were measured with an IRGA (Li-Cor 6400; Li-Cor Inc., Nebraska, USA) between 10.00 h and 12.00 h with constant irradiation (1000 μmol m^{-2} s^{-1} PAR). Leaf area was measured with a LI-3000 A (Li-Cor Inc.).

Seeds were collected at 10, 20, 30, 40, 55, 70, 85, 100, and 120 DAA, between 14.00 h and 15.30 h, weighed, frozen in liquid nitrogen (for quantification of carbohydrates, enzymatic activities, and soluble protein content) or dried at 80 °C [for determination of seed dry weight (DW), total lipid and C, N, H, and S content]. Seed water content (%) was calculated as: (FW–DW)×100/FW.

Element analysis

Element analysis (C, N, S, and H) of homogenized dried samples was performed by combustion on a Vario EL, CNHS Elemental Analyzer (Elementar Americas Inc., New Jersey, USA).

Total lipid quantification

A minimum of 0.5 g DW was used for total lipid determination by a gravimetric method, involving the extraction with petroleum ether (50–75 °C) in a Soxtec System HT 1043 extraction unit (Tecator AB, Höganäs, Sweden).

Enzymatic activities and soluble protein content

Seed proteins were extracted twice (2.0 ml g^{-1} FW) with sodium phosphate buffer (50 mM, pH 7.0) containing 1 mM $MgCl_2$, 1 mM EDTA (ethylenedinitrilotetraacetic acid), 1 mM DTT (dithiothreitol), 1% Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, no. 1836153), 10% (w/v) NaCl, and 2% PVP 40000 (w/v). The extract was centrifuged at 15 000 *g*, at 4 °C for 15 min. The combined supernatants were desalted in PD-10 columns (Amersham BioSciences) equilibrated in the same solution without NaCl, and used for quantification of soluble protein content (Bradford, 1976) and enzymatic determinations.

Sucrose synthase (SS) (EC 2.4.1.13), acid invertase (INV_A), and neutral invertase (INV_N) (EC 3.2.1.26) activities were measured as described by Pinheiro *et al*. (2001). Sucrose-phosphate synthase (SPS) (EC 2.4.1.14) activity was measured according to Wardlaw and Willenbrink (1994) but with the addition of 10 mM G-6-P as an activator (Copeland, 1990). Determination of galactinol synthase activity (GS) (EC 2.4.1.123) was adapted from Peterbauer *et al*. (2001), by quantifying galactinol (α-gal[1→1]myo-inositol) formed with α-galactosidase (Boehringer Mannheim/R-Biopharm no. EO 428167) using the modification of Hatterscheid and Willinbrink (1991) and galactinol as a standard. This enzyme is able to hydrolyse α-galactosides, such as galactinol (Keller and Pharr, 1996). Blanks without UDP-galactose and/or myo-inositol) were used to correct for interference of other α-galactosides.

Carbohydrate analysis

Seed samples were extracted with 80% (v/v) ethanol (1 ml per 0.2 g sample FW) at 80 °C for 20 min. The ethanol extracts were freeze-dried and the pellets solubilized with water. After extract clean-up through a Waters Sep-Pak columns (C18, Accell Plus QMA and Accell Plus CM), the sugars (glucose, fructose, galactose, sucrose, raffinose, stachyose, and verbascose) were quantified by HLPC (precolumn Shodex SH-G and column Shodex SH 1011) in a Merck Hitachi system (AS-2000 auto sampler; D-2500 chromato-integrator; T-6300 column thermostat; L-6000 pump). The separations were performed at 75 °C with a flow rate of 1 ml min^{-1} using 0.01 N H_2SO_4. The retention times for the sugars analysed were: glucose, 13.49 min; galactose, 14.29 min; fructose, 14.97 min; sucrose, 13.92 min; raffinose, 8.40 min; stachyose, 7.29 min; verbascose, 6.90 min. Myo-inositol retention time was 15.52, but cannot be detected after clean-up through Waters Sep-Pak columns.

The ethanol-insoluble fraction (insoluble carbohydrates) was freeze-dried, resuspended in two volumes of H_2O and autoclaved at 120 °C for 3 h. The supernatants were used to quantify starch and non-starch polysaccharides. Starch was enzymatically quantified according to the modification of Hatterscheid and Willenbrink (1991) with kit no. EO 207748 (Boehringer Mannheim/R-Biopharm). For the quantification of non-starch polysaccharides the supernatants were precipitated with absolute ethanol, and the resulting pellets subjected to acid hydrolysis with 72% H_2SO_4 for 1 h at 30 °C, and then with 1 M H_2SO_4 for 1 h at 120 °C, following Al-Kaisey and Wilkie (1992). After adjusting to neutral pH with a saturated sodium bicarbonate solution, samples were freeze-dried and resuspended in H_2O. The monosaccharides in solution were quantified by HLPC as described above.

Statistical analysis

All the presented data are averages affected by standard deviation. The significance levels were calculated with the program STATISTICA® version 5.0 (StatSoft Inc., Oklahoma, USA). Data referring to the same DAA that differ significantly, between control and WD samples, are labelled with * (P <0.05), ** (P <0.01) or *** (P <0.001).

Results

Seed development

During lupin seed development the following four stages can be identified: very early development (until 10–20

DAA); early development (20–30 DAA); growing and storage phase (>30–70 DAA); yellowing and drying (>70 DAA). The drying process was completed at 90–100 DAA (Fig. 1).

The imposition of WD in the pre-storage phase (15–22 DAA) decreased the number of seeds per pod (data not shown) and altered seed development, since seeds from stressed plants exhibited an accelerated drying rate and were smaller at the mature stage (Fig. 1). On average, seed FW was decreased by 40% and seed DW was decreased by 30% (Fig. 1A, B). During the period of WD imposition, seed water content was not significantly affected (Fig. 1C), but leaf water status was affected, as indicated by the leaf water potential at predawn (Table 1). By the end of the stress imposition period, stomatal conductance was severely reduced (by 80%), while the specific leaf area was reduced by 45% and the net photosynthetic rate by 30% (Table 2).

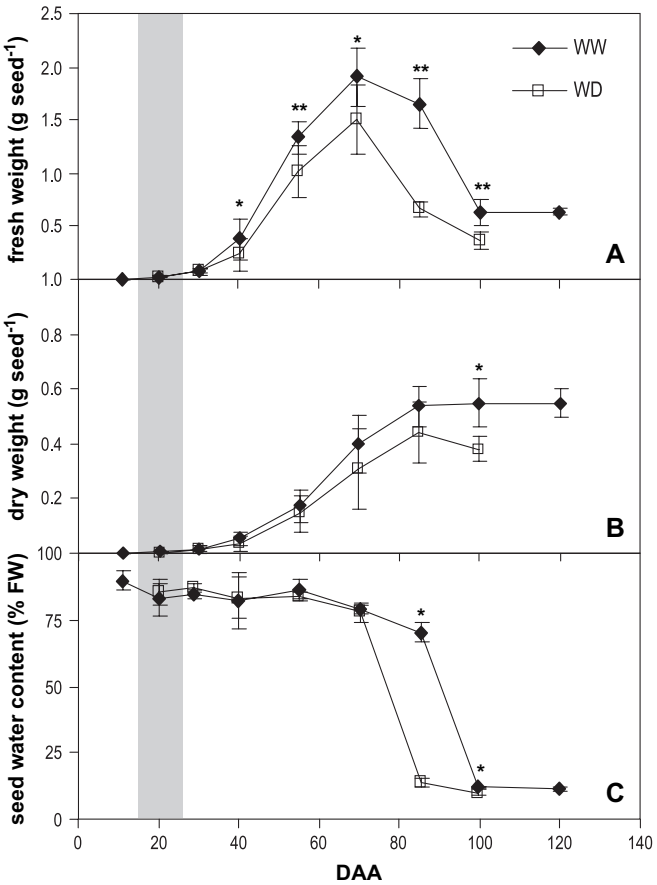

Fig. 1. *Lupinus albus* seed development of well-watered (WW) and water-stressed (WD) plants. The stress was imposed between 15 DAA and 22 DAA (shaded area). (A) Seed fresh weight; (B) seed dry weight; (C) seed water content. Data are the means ±standard deviation of at least five samples. Significant differences between control and WD are labelled with * (P <0.05) or ** (P <0.01). Cotyledon formation was observed after 20 DAA, and the seed coat was filled by 30 DAA (data not shown).

Reserve accumulation

During normal seed development, C and H content remained relatively unchanged during the whole period. S was always below the detection limit (data not shown), while N content increased from 40 DAA onwards, reaching about 35 mg seed^{-1} at 100 DAA (Fig. 2B). Soluble protein followed the same trend (Fig. 2A) and at 100 DAA was around 50 mg seed^{-1}. Lipids had markedly increased (about 6-fold) from 40 DAA until seed maturity (Fig. 2C). A transient accumulation of starch was observed between 30 and 70 DAA (reaching 20 μmol glucose equivalents seed^{-1}) in seeds of well-watered plants, but after 85 DAA it was barely detected (Fig. 2D). Non-starch polysaccharides were only detected from 30 DAA onwards (Fig. 2E) and were, essentially, galactose based (galactans). A sharp increase in non-starch polysaccharide content was observed in actively growing seeds between 40 and 55 DAA, the mature seeds containing approximately 30 μmol galactose equivalents seed^{-1}.

When considering soluble sugars, hexoses were detected at 10 DAA and decreased after 70 DAA (Fig. 3). Galactose content was constitutively very low. Sucrose was only detected at 20 DAA and started to be accumulated after 40 DAA (Fig. 3D). The highest hexose-to-sucrose ratio was detected in pre-storage seeds (10, 20, and 30 DAA). In addition to sucrose, the oligosaccharides raffinose and stachyose were also detected, but only from 55 DAA (Fig. 3E, F).

Table 1. *Predawn leaf water potential (Ψ_{pd}) of* Lupinus albus *plants in well-watered conditions (WW) and under water deficit (WD)*

The water potential was determined by Scholander pressure chamber measurements on the most recently expanded leaves before the beginning of the illumination period. Data are the means ±standard deviation of five measurements. Significantly different points between control and WD samples are labelled with * (P <0.05).

DAA	Ψ_{pd} (MPa)	
	WW	WD
15	−0.30±0.03	–
19	−0.29±0.03	−0.55±0.10*
22	−0.34±0.04	−1.03±0.06*

Table 2. *Stomatal conductance (*g*), net photosynthesis (*A*), and specific leaf area (*SLA*) at the end of the stress imposition period of* Lupinus albus *plants in well-watered conditions (WW) and under water deficit (WD)*

Data are the means ±standard deviation of six measurements. Significantly different points between control and WD samples are labelled with * (P <0.05), ** (P <0.01), or *** (P <0.001).

	g (mmol H_2O m^{-2} s^{-1})	*A* (μmol CO_2 m^{-2} s^{-1})	*SLA* (m^2 kg^{-1})
WW	73.4±6.2	6.90±1.07	12.7±1.9
WD	13.5±5.1***	4.90±0.03*	6.9±1.0**

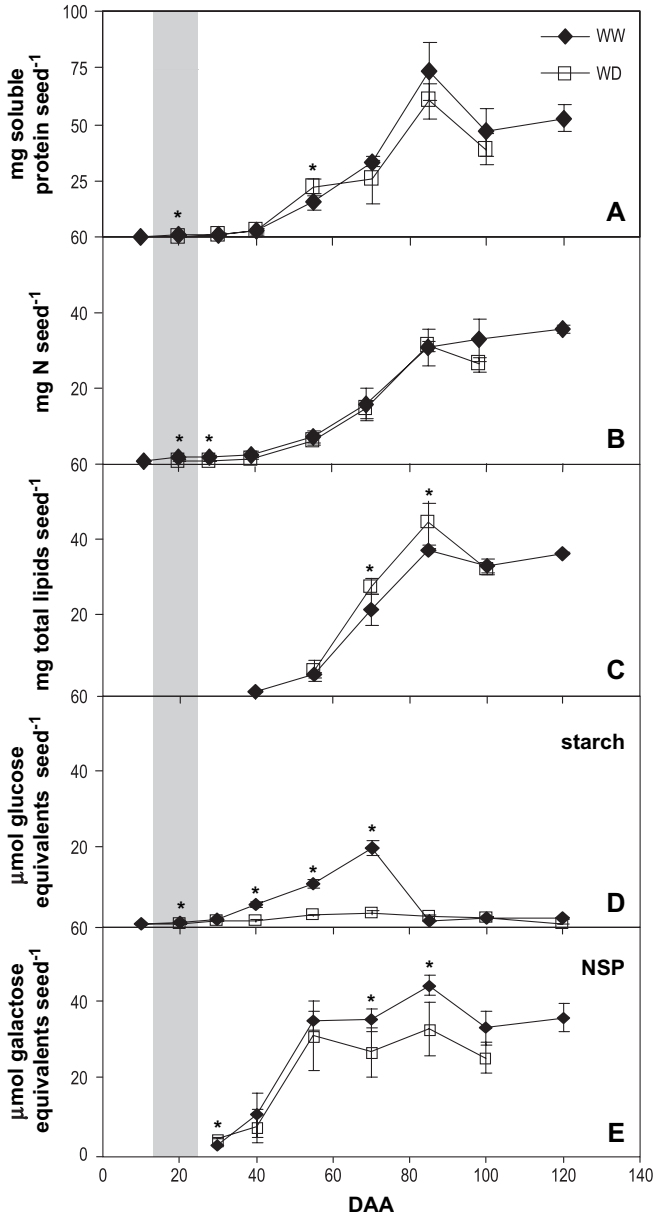

Fig. 2. Accumulation of soluble proteins (A), total nitrogen (B), total lipids (C), starch (D), and galactan-based non-starch polysaccharides (E) during *Lupinus albus* seed development under well-watered conditions (WW) and when subjected to a transient post-anthesis water deficit (WD). The stress was imposed between 15 DAA and 22 DAA (shaded area). Data are the means ±standard deviation of at least four samples. Significant differences between control and WD are labelled with * (P <0.05).

Effects of WD on the seed metabolites became noticeable during the stress imposition period. A decrease in the order of 50–60% was observed in the contents of N (from 1.48 to 0.70 mg seed^{-1}), soluble protein (from 0.73 to 0.27 mg seed^{-1}), starch (from 0.20 to 0.10 µmol seed^{-1}), and sucrose (from 2.8 to 1.0 µmol seed^{-1}) (Figs 2A, B, D, 3D). During the subsequent rewatering starch was greatly reduced, galactans were transiently decreased, and total lipids transiently increased (Fig. 2C–E). However, in spite of these transient changes, no significant differences were observed for these compounds in the mature seeds (Fig. 2). Glucose increased at 30 DAA and 40 DAA, but not fructose or galactose. As a result of WD, the hexose-to-sucrose ratio was greatly increased. During the stress imposition period (20 DAA) it changed from 1.6 to 3.6 and immediately after rewatering (30 DAA) from 1.3 to 6.3. This increase in the hexose to sucrose ratio is the result of a combination of the increase in glucose and the marked decrease in sucrose. In fact, the sucrose accumulation peak observed at 30 DAA in control seeds was abolished by WD (Fig. 3D, inset). Concerning raffinose and stachyose, their accumulation was advanced and much more intense (more than two to three times) in seeds of WD plants. Whereas in control seeds a sharp increase occurred between 85 DAA and 100 DAA, in WD seeds such an event occurred between 70 DAA and 85 DAA. As a result of WD, the sucrose-to-raffinose-to-stachyose ratio was altered in the mature seeds from 1.0:0.29:0.34 to 1.0:0.50:1.04.

Enzymatic activities

The activities of the sucrose metabolizing enzymes (INV_N, INV_A, SS, and SPS) and GS were studied during seed development, both under control and transient WD conditions (Fig. 4). Considering that the lupin seeds store protein, the enzyme activities are represented per seed. However, since the protein started to be accumulated only after 40 DAA, specific activities could be considered before that date (see insets in Fig. 4).

INV_A activity was constitutively low (Fig. 4A). By contrast, the other enzymes displayed a high total activity during the growing and accumulation phase (Fig. 4). Until 40 DAA INV_N activity was higher than SS and SPS activities, but afterwards SS dramatically increased and at 70 DAA was about 10-fold higher than INV_N and SPS. This increase occurred simultaneously with an intense accumulation of reserve compounds (N, soluble protein, galactans, lipids, and starch; Fig. 2). GS, which is considered to be involved in RFO synthesis, also markedly increased in activity after 40 DAA, but RFO accumulation was only initiated after the activity of the enzyme had reached its maximum (Figs 3, 4).

Expressing the data as specific activity reinforces the association of SS activity with reserve accumulation, even when seeds are actively accumulating protein (Fig. 4C, inset). No such association was found between GS activity and RFO accumulation. Specific activities also allow the association of INV_N with the cotyledon formation stage (20–30 DAA; Fig. 4B, inset) and high sucrose concentration (Fig. 3D, inset).

WD led to the decrease in the total activities of INV_N, SS, SPS, and GS during the stress imposition period, but while INV_N activity decreased to 20% of control activities, the other enzymatic activities were reduced by 50% (Fig. 4). However, if specific activities are considered, then only

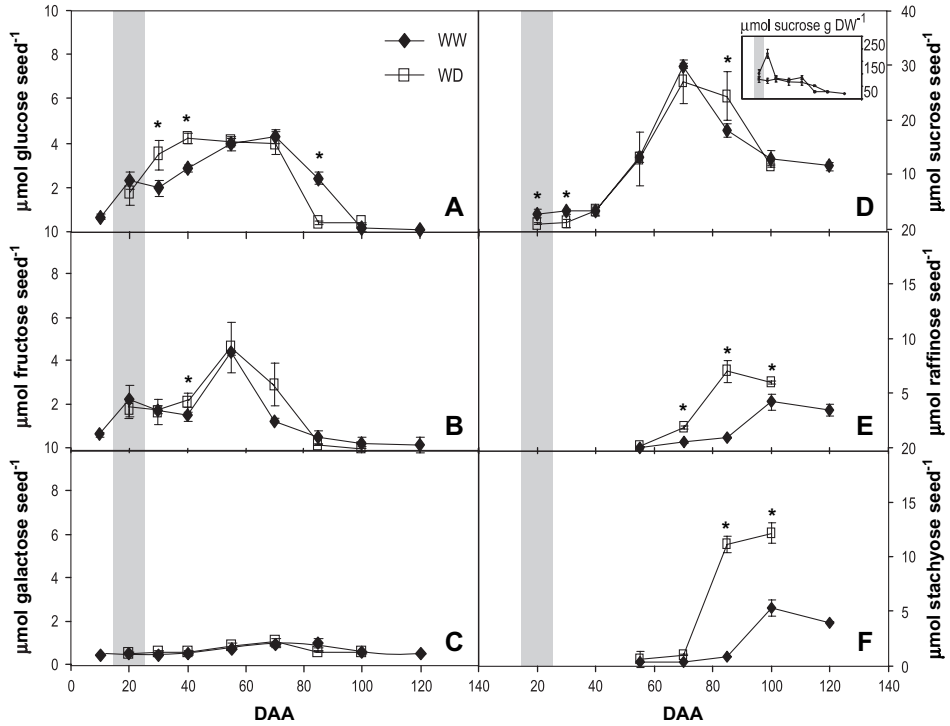

Fig. 3. Soluble sugars levels during *Lupinus albus* seed development under well-watered conditions (WW) and when subjected to a transient post-anthesis water deficit (WD). The stress was imposed between 15 DAA and 22 DAA (shaded area). (A) Glucose; (B) fructose; (C) galactose; (D) sucrose; (E) raffinose; (F) stachyose. Inset represents sucrose data expressed per gram of dry weight. Data are the means ±standard deviation of at least four samples. Significant differences between control and WD are labelled with * (P <0.05).

INV_N was negatively affected by WD and the other enzymes increased by 2–3-fold (Fig. 4, insets). This was concomitant with a two to three times reduction in seed soluble protein (Fig. 2A).

After rewatering, SS and SPS total activities were similar to those of the control. However, seed desiccation occurred earlier in stressed seeds (Fig. 1C) as well as the decrease in SS and SPS activities. By contrast, total INV_N activity was permanently affected, remaining 40–60% lower than in the control seeds (Fig. 4B). GS activity was affected similarly to INV_N but not so intensely (Fig. 4E). This lowering in GS activity was opposed to the RFO accumulation pattern (Figs 2, 3).

Discussion

The reduction in seed yield due to WD is a consequence of restricted photosynthesis and accelerated leaf senescence that disturb the primary source of assimilates. Seed survival under such conditions appears to be linked to the ability of the water-stressed plants temporarily to accumulate assimilates in the shoots that are later diverted to the pods (Chaves *et al*., 2002). The shortening in the seed-filling period due to WD is associated with important metabolic modifications that occur not only during WD imposition but are also prolonged into the rewatering period.

Sucrose metabolism is pivotal in seed development and is particularly susceptible to WD. The 3-fold reduction in this sugar in 20 DAA and 30 DAA seeds, as a result of stress, might reflect a lower availability at source due to lower photosynthesis and blockage of transport into the seeds. A higher rate of sucrose hydrolysis is unlikely because INV_A activity is residual and INV_N and SS activities are reduced by WD. Such a decrease in sucrose is an important signal for the adjustment of the seed developmental programme to WD, in agreement with the regulatory functions attributed to sugars (Gibson, 2005; Weber *et al*., 2005). An example is the modulation of the gene expression of several SS, SPS, and INV isoforms by sugars (Koch, 1996; Weber *et al*., 1998). A shift in the hexose-to-sucrose ratio is also known to promote a switch in development and metabolism in legume seeds, from cell division to expansion and reserve accumulation (Weber *et al*., 2005). The prolonged high hexose-to-sucrose ratio triggered in lupin by WD thereby alters the timing of such a switch.

It was proposed that the control of the hexose-to-sucrose ratio in legume seeds is dependent on the activities of INV_A in the early developing phase and SS during the storage phase (Weber *et al*., 2005). In lupin seeds, INV_N rather than INV_A is associated with early development. As far as is known this is the first time that such a predominance of INV_N in developing seeds is shown. However, a high ratio

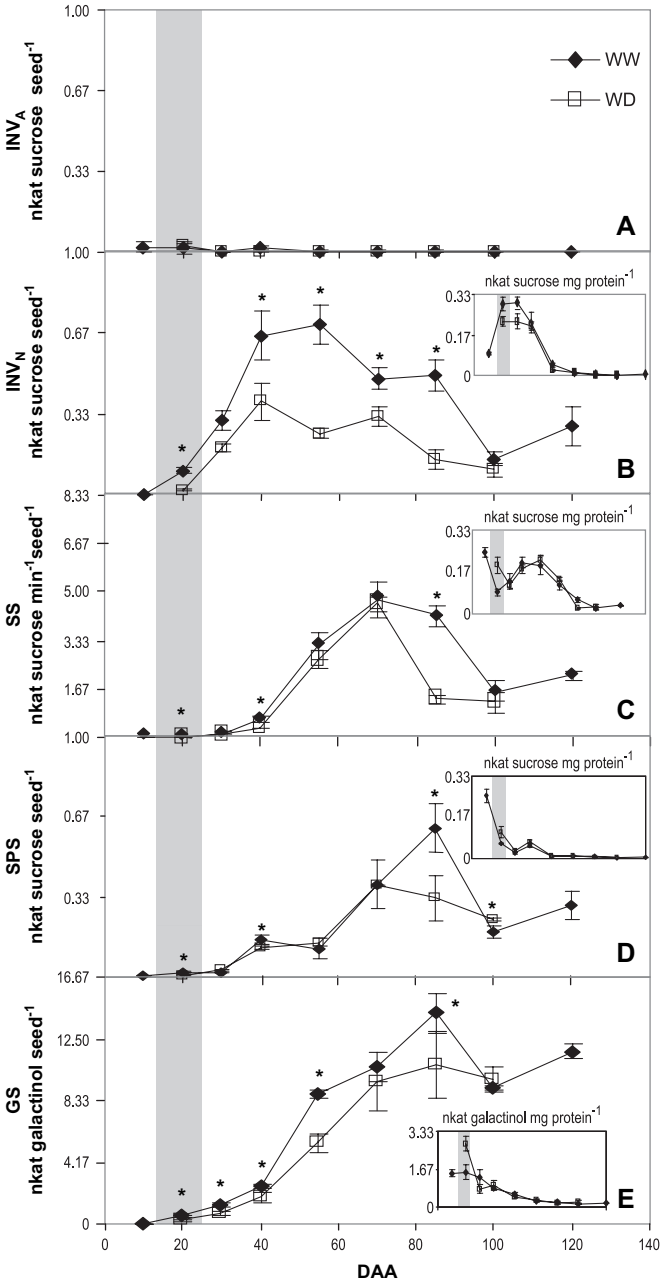

Fig. 4. Enzymatic activities of acid invertase (A), neutral invertase (B), sucrose synthase (C), sucrose phosphate synthase (D), and galactinol synthase (E) during *Lupinus albus* seed development under well-watered conditions (WW) and when subjected to a transient post-anthesis water deficit (WD). The stress was imposed between 15 DAA and 22 DAA (shaded area). Insets represent data expressed per milligram of protein (specific activity). Data are the means ±standard deviation of at least four samples. Significant differences between control and WD are labelled with * (P <0.05).

of INV_N to INV_A activities in mature seeds of several legumes has been observed (Cooper and Greenshields, 1961; Pridham and Walter, 1964; Silva *et al*., 1988). It is not clear why INV_N should be so preponderant in lupin seeds. INV_N and INV_A are distinct enzymes with a distinct pH optimum, kinetic characteristics, and amino acid sequences (Sturm, 1999), and appear to regulate sucrose metabolism under distinct conditions. INV_A activity is often related to sucrose hydrolysis in tissues with a high demand of hexoses (Ricardo and ap Rees, 1970; Sturm, 1999), while INV_N is associated with the need for sucrose accumulation (Ricardo and ap Rees, 1970; Ricardo, 1974). It is interesting that in lupin seeds the stage of cotyledon formation (20–30 DAA) is associated with the highest INV_N specific activity and with a peak of sucrose concentration, both of which are strongly affected by WD.

While INV_N is permanently affected by WD, SS is not. This enzyme is considered to have a key role in seed storage by controlling sink activity (Weber *et al*., 1998). SS activity is decreased by WD during the stress imposition period, but not during the growing phase, when its function is crucial (Weber *et al*., 2005). This lack of sensitivity of SS to WD during the growing phase can be connected to the small effect of WD on reserve accumulation (starch and RFO excepted). Unlike other legumes, mature lupin seeds store galactans rather than starch (Al-Kaisey and Wilkie, 1992; Petterson, 1998), but nonetheless transiently accumulate starch. The parallelism between SS activity and starch content reported for pea and faba bean seeds (Weber *et al*., 1998) is also observed in control lupin seeds but does not exist in WD seeds. Since the major polyssacharides stored in lupin seed are the galactans, the transient starch accumulation indicates a metabolic intermediary function for this polysaccharide, which at low photoassimilate availability is abolished. A transient starch accumulation dependent on photoassimilate availability has been also described during tomato fruit development (N'tchobo *et al*., 1999). Galactans are considered to have a dual function, acting as a reserve and as a constraint to cotyledon expansion (Buckeridge *et al*., 2000). With the acquisition by the cell wall of a polysaccharide reserve function (acquired during evolution on legume seeds), a balance of the carbon exchange between wall (galactans) and intracellular carbon (starch) metabolism must be achieved (Buckeridge *et al*., 2000). This is evident during germination of fenugreek seeds where galactomannan remobilization is accompanied by a transient starch accumulation, which functions as a temporary storage of otherwise osmotically active sugars (Bewley *et al*., 1993). It can be admitted that during seed formation an inverse mechanism is active, the starch pool being dependent on the availability of sucrose.

The other important class of lupin seed carbohydrates is RFO. These are quantitatively more affected by WD than any other storage compounds and their accumulation pattern is also altered. This alteration is probably related to the earlier onset of seed desiccation, since RFO start to be accumulated at the onset of the drying stage (Saini and Lymbery, 1983; Frias *et al*., 1996). RFO are important carbon reserves readily available on germination, and are also considered stress metabolites during cold and WD tolerance (Keller and Pharr, 1996; Górecki *et al*., 1997;

Peterbauer and Richter, 2001). Desiccation tolerance associated with higher RFO content has been described for *Brassica campestris* (Sinniah *et al*., 1998), *Vicia faba* (Lahuta *et al*., 2000), and *Acer* spp. seeds (Pukacka and Wójkiewicz, 2002). The importance to the seed of this increased tolerance and how it is achieved still requires clarification. Although a good relationship is observed between RFO accumulation and the activity of GS, the enzyme considered to regulate RFO synthesis (Keller and Pharr, 1996) in control lupin seeds, the same does not apply to WD seeds. This research, like that of Peterbauer *et al*. (2001), calls into question the key role of GS on RFO metabolism and the necessity for RFO biosynthesis to be elucidated further. This is an important field of research, considering that possible advantages to the plant conferred by RFO accumulation could have nutritive implications. Indeed, RFO are considered to be a major cause of flatulence in animals and humans (Petterson, 1998).

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