Sugarbeet seed priming: effects of priming conditions on germination, solubilization of 11-S globulin and accumulation of LEA proteins

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Abstract

The relationship between initial mobilization of 11-S globulin storage protein (leading to solubilization of its B-subunit) and germination performance after priming was studied in sugarbeet (*Beta vulgaris* L.) seeds. Priming was conducted for 2 d either in water (hydropriming) or in –2.0 MPa polyethylene glycol-8000 (osmopriming), at various temperatures ranging from 5 to 40°C and oxygen concentrations in the atmosphere ranging from 0 to 21%. For both types of pre-treatments, the range of temperatures and the concentrations of oxygen which were effective in priming were very similar to those which allowed solubilization of the B-subunit of 11-S globulin, supporting the robustness of this protein marker for optimization of sugarbeet seed priming. Furthermore, the temperature and oxygen dependence of priming efficiency closely paralleled that for germination of the untreated seeds, reinforcing the finding that the beneficial effect of priming corresponded to the advancement of germination *sensu stricto* (i.e. phase II of the germination process). For priming times longer than 2 d, particularly for osmopriming, there was a very dramatic decrease in germination of the treated seeds. For instance, following a 14 d osmopriming at 25°C as much as 60% of the pre-treated seed population failed to germinate when transferred to water. This loss in germination performance quite closely paralleled degradation of LEA (late embryogenesis abundant) proteins, notably a heat-stable seed-specific protein of about 60 kDa and a seed-specific biotinylated LEA protein.

Keywords: seed priming, 11-S globulins, LEA proteins, biotinylated proteins, sugarbeet, *Beta vulgaris*

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Introduction

Seed priming (pre-sowing imbibition treatment) is widely used to enhance seed performance with respect to rate and uniformity of germination (Heydecker *et al.*, 1973; Hegarty, 1978; Heydecker, 1978; for reviews see Bradford, 1986; Parera and Cantliffe, 1994; Taylor *et al.*, 1998). These treatments are based upon controlled hydration of the seeds; during priming, seeds are brought up to, and eventually held at, the end of phase II of water uptake of the germination process without entering the initial growth phase characterized by radicle protrusion through the seed coat (Bray *et al.*, 1989; Bray, 1995). Since most embryos of seeds are desiccation tolerant up to this developmental stage (Kermode, 1997), the advancement of the seeds in the germination process during priming can be arrested by drying.

Several methods have been described to prime seeds. For osmopriming, controlled water uptake by the seeds is achieved by the use of an inert osmoticum such as polyethylene glycol (PEG) (see Bradford, 1986, and references therein). There are other priming techniques in which controlled hydration of the seeds is effected with a water-absorbing carrier as in solid matrix priming (Taylor *et al.*, 1988), with pure water as in hydropriming (Tarquis and Bradford, 1992; C. Job *et al.*, 1997) or with only water vapour as in drum priming (Rowse, 1996). A major difficulty encountered in seed priming, particularly with the hydropriming and drum priming techniques, is to control seed imbibition to a level permitting pre-germinative processes to proceed without reaching the stage of radicle emergence where seeds become desiccation intolerant. The consequence of drying back intolerant primed seeds for storage purposes can be a total loss of the treated batch.

Optimization of priming treatments rests mainly on conducting germination assays, which can only yield *a posteriori* indications of the priming conditions

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(e.g. duration, water potential and availability, temperature, oxygen availability). Thus, the characterization of early germination and/or priming markers that are easy to detect would allow continuous monitoring of the priming process and to facilitate optimization of priming treatments. Besides these practical aspects, an identification of pre-germination markers would also be useful to unravel the key molecular and biochemical processes involved in germination. Of special interest in this context are the recent studies pointing out the importance of DNA repair on embryo rehydration for successful germination (Boubriak *et al.*, 1997), as well as that of cell-cycle-related processes (DNA replication, β-tubulin accumulation) and of weakening of the endosperm cap (endo-β-mannanase accumulation) in control of tomato seed germination (Bino *et al.*, 1992; De Castro *et al.*, 1995; Still *et al.*, 1997; Toorop *et al.*, 1998; Özbingöl *et al.*, 1999). Recent work also demonstrated a substantial increase of root-tip nuclei with DNA contents of 4C and above in sugarbeet seeds which had been submitted to a pre-sowing imbibition treatment called seed advancing (Redfearn and Osborne, 1997).

We reported the occurrence of an initial mobilization of the 11-S globulin storage protein during priming of sugarbeet seeds (C. Job *et al.*, 1997; D. Job *et al.*, 1997; Chareyre *et al.*, 1998). The 11-S globulins, which are stored during seed maturation in protein bodies, are composed of two polypeptides, an acidic A-chain ($M_r \approx 30{,}000$) and a basic B-chain ($M_r \approx 20{,}000$) that are linked by a disulphide bond; they are known to be insoluble in low ionic strength extraction buffers (Lawrence *et al.*, 1990; Shewry *et al.*, 1995). Yet the basic B-subunit becomes the most abundant polypeptide in soluble protein extracts from germinated and primed seeds, being very easily detected by ELISA from whole seed extracts (C. Job *et al.*, 1997; Chareyre *et al.*, 1998). This solubilization is a result of an endoproteolytic attack on the A-chain, which liberates a complex composed of the B-subunit and a peptide of the A-chain (C. Job *et al.*, 1997; D. Job *et al.*, 1997).

In this work the existence of a positive correlation between extent of B-subunit solubilization and the advancement of germination by priming was assessed further. Toward this goal the effects of priming conditions (temperature, oxygen tension, duration of treatment) on the soluble protein patterns in sugarbeet seeds were examined by using two priming protocols, hydropriming and osmopriming. Mature embryos of various species contain a highly abundant set of hydrophilic heat-stable proteins referred to as LEA (late embryogenesis abundant) proteins, which are presumed to play a role in conferring desiccation tolerance during late stages of embryo development (Dure, 1993; Kermode, 1997; Russouw *et al.*, 1997; Walters *et al.*, 1997). Since these proteins can be degraded very rapidly during germination (Dure, 1993), we also analysed the behaviour of members of the heat-stable protein fraction during sugarbeet seed priming.

Materials and methods

Seed material

Sugarbeet (*Beta vulgaris* L.) seeds were provided by KWS (Einbeck, Germany). They were primed as follows:

(1) Hydropriming pre-treatment. This pre-treatment included the three following steps (C. Job *et al.*, 1997): (a) a washing step of the seeds [4 hours in milli-Q water (Millipore) at 20°C] to remove germination inhibitors from the seed coats (Battle and Whittington, 1969; Morris *et al.*, 1984), followed by re-drying in air at approx. 20°C for 12 h to the initial moisture content (8.45% on a dry weight basis); (b) a controlled hydration of the washed seeds at various temperatures ranging from 5 to 36°C (± 1°C) for various durations up to 5 d; and (c) a dehydration of the treated seeds to the initial moisture content as described above. Controlled hydration was achieved by placing 2.5 g of seeds (on a fresh weight basis) on filter paper (0.82 g) moistened with a calculated amount of milli-Q water (0.81 ml) in plastic tubes (h = 7 cm; d = 3 cm) sealed with airtight closures. Then, the tubes containing the seeds were slowly agitated in a chamber at the desired temperature in the dark. Following incubation, seeds were weighed to calculate their water uptake (under these conditions seed fresh weight increased by 30 ± 1%) and then were re-dried at room temperature as described above. None of the seeds germinated by 5 d of incubation.

(2) Osmotic pre-treatment. Seeds were placed on a layer of cotton wool imbibed with a solution of PEG-8000 at –2.0 MPa for various durations up to 14 d at 25°C, or for 2 and 7 d at various temperatures ranging from 5 to 35°C (± 0.5°C). The concentration of the PEG solution was calculated for each temperature according to Michel and Kaufmann (1973). Osmotic pre-treatment in atmospheres containing 0 (pure nitrogen) to 100% oxygen was performed at 25°C for 2 d according to the procedure developed by Côme and Tissaoui (1968), as for germination assays (see below). Following incubation, treated seeds were rinsed and dried to their original moisture content as described by Özbingöl *et al.* (1998) for tomato seeds.

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Germination assays

Germination assays in air were carried out on batches of 100 seeds placed in 9 cm Petri dishes (25 seeds per dish, four replicates) on a layer of cotton wool moistened with distilled water. Assays were carried out in darkness over a range of constant temperatures between 5 and 40°C (± 0.5°C).

Studies on the effects of oxygen concentration on seed germination were performed according to Côme and Tissaoui (1968). Gas mixtures containing from 0 to 21% oxygen were obtained by mixing pure nitrogen and compressed air. The gaseous atmospheres were passed continuously through germination chambers at a constant flow rate (4 l h^{-1}). Each assay was performed with two germination chambers (replicates) containing 50 seeds placed on a layer of cotton wool moistened with distilled water. Germination assays were conducted in darkness and counts were made daily up to 10 d. The results presented are the means of germination percentages obtained in two or four replicates ± standard deviation (SD). Germination performance of the seeds is expressed as the percentages obtained after 10 d (G_{10}), and as the time taken for 50% of the seeds sown to germinate (t_{50}). t_{50} was estimated graphically from the cumulative germination curves.

Preparation of the protein extracts

One hundred seeds were ground with two stainless steel balls (d = 12 mm) in a stainless steel cylinder (10 ml) using a grinder (Retsch) operating at full speed for 1 min. Eight millilitres of extraction buffer E, consisting of 50 mM HEPES, pH 8.0, and 1 mM EDTA, were added to the powder. This suspension was stirred at 5°C for 15 min and was then centrifuged (Sigma type 3K30) at 9000 *g* for 10 min at 5°C. Four millilitres of the supernatant were withdrawn and subjected to a second, clarifying, centrifugation (35,000 *g*, 10 min, 5°C). This fraction corresponded to the soluble protein extract. A heat-stable protein fraction was obtained by incubating an aliquot of the soluble protein extract at 90°C for 10 min, chilling on ice, and then centrifuging (35,000 *g*, 10 min, 5°C).

Protein analyses

Protein concentrations in the various extracts were measured according to Bradford (1976). Bovine serum albumin was used as a standard.

SDS-PAGE of the protein extracts was carried out according to Laemmli (1970), using a Mighty Small II SE250 electrophoresis cell (Hoefer Scientific Instruments, San Francisco). Samples were mixed with load buffer [10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 2.5% (v/v) SDS, 50 mM dithiothreitiol (DTT) and 0.01% (w/v) bromophenol blue], heated to 100°C for 5 min and loaded onto gels (15% homogeneous polyacrylamide gels). Electrophoresis was conducted at a constant current intensity of 30 mA for 90 min at 25°C. Proteins were revealed by staining with Coomassie blue R250 (Serva).

Antibodies

Primary rabbit antibodies directed against the B-subunit of 11-S sugarbeet globulin were used as described previously (C. Job *et al.*, 1997). SDS-PAGE analyses revealed the presence of a doublet of proteins of about 60 kDa in the heat-stable protein fraction from control sugarbeet seeds (see Fig. 7A). The most abundant of these two proteins, referred to as HS60 (for heat-stable protein of about 60 kDa apparent molecular mass), was gel purified and used to raise primary antibodies in a guinea pig as described previously (C. Job *et al.*, 1997). Analyses of the heat-stable protein fraction from various tissues and organs of sugarbeet (leaves, roots, mature seeds, germinated seeds) by Western blotting using streptavidin conjugated to horseradish peroxidase as a specific reagent for biotin (Duval *et al.*, 1994b) disclosed the existence of a seed-specific biotinylated protein of about 60 kDa (see Fig. 7B). This protein, referred to as SBP (for seed biotinylated protein), was purified to homogeneity by affinity chromatography using a monomeric avidin column (Duval *et al.*, 1994b) and then used to raise primary antibodies in a guinea pig.

Western blot and ELISA analyses

Western blot analyses were carried out as described by Duval *et al.* (1994b). Briefly, following SDS-PAGE, proteins were transferred from the polyacrylamide gel on to nitrocellulose (Gelman Sciences) using a semi-dry electroblotter (Towbin *et al.*, 1979). Blots were rinsed twice for 5 min in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl (TBS), then incubated for 1 h at 25°C in TBS containing 1% (v/v) "Blocking Solution" (Boehringer Mannheim). After incubation for 1 h with appropriate primary antibodies (dilution 1:10,000 in TBS containing 0.5% Blocking Solution), blots were washed twice for 10 min in TBS containing 0.1% Tween 20 and twice for 10 min in TBS containing 0.5% Blocking Solution, then reacted for 30 min with appropriate secondary antibodies conjugated to horseradish peroxidase (Sigma; dilution 1:10,000 in TBS containing 0.5% Blocking Solution). After four

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washing steps of the blots as described above, proteins were detected using the BM chemiluminescence kit from Boehringer Mannheim according to the instruction booklet "BM Chemiluminescence Blotting Substrate (POD)" from Boehringer Mannheim. Detection of biotinylated proteins from the protein blots followed the same protocol, except that proteins were specifically labelled and revealed using streptavidin conjugated to horseradish peroxidase as a specific reagent for biotin (Duval *et al.*, 1994b).

The contents of 11-S globulin B-subunit, HS60 protein and SBP per seed were determined by ELISA as described previously (Duval *et al.*, 1994b; C. Job *et al.*, 1997). Serial dilutions of soluble or heat-stable protein extracts were loaded into wells of microtitre plates. Wells were then washed with PBS (10 mM sodium phosphate, 150 mM NaCl) containing 0.05% Tween 20, incubated with appropriate primary antibodies (dilution 1:5000 in PBS containing 0.05% Tween 20), washed as above, and then incubated with appropriate secondary antibodies [peroxidase-labelled goat anti-rabbit or goat anti-guinea-pig antibodies (Sigma); dilution 1:5000 in PBS containing 0.05% Tween 20] and peroxidase substrates [1.8 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Aldrich), and 10^{-3}% (w/w) H_2O_2 (Sigma) in 0.1 M citrate–phosphate buffer, pH 4.0]. Green colour development was monitored at 405 nm using a microplate reader (EL340 from Bio-Tek Instruments). Curve fitting and statistical data analyses were carried out with the KaleidaGraph™ program (Abelbeck Software). The contents of 11-S globulin B-subunit, HS60 protein and SBP per seed were calculated by linear regression analysis from the slope (± SD) of the linear part of the plots A_{405} vs. quantity of seeds in the extracts.

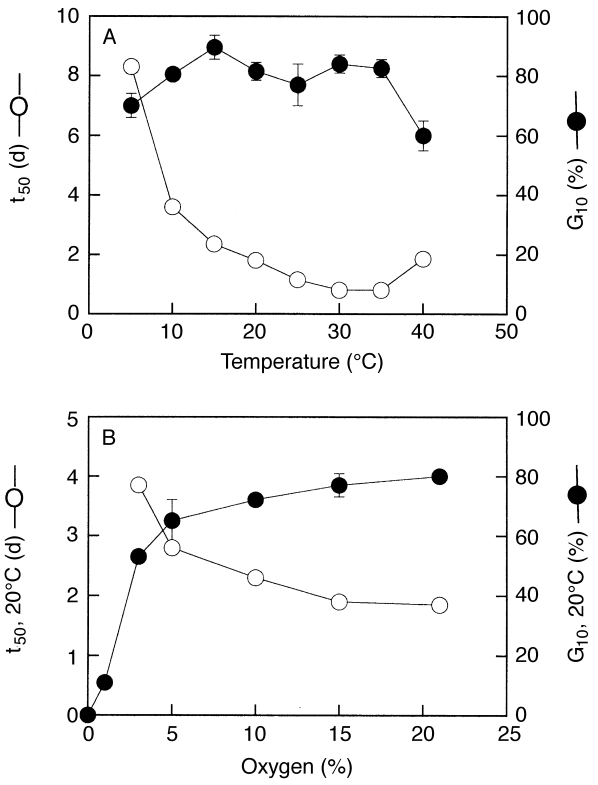

Figure 1. Effects of temperature (A) and oxygen concentration in the atmosphere (at 20°C) (B) on the germination percentages obtained after 10 d (G_{10}, ●) and the time to obtain 50% germination (t_{50}, ○). The values correspond to the means of four measurements ± SD for the germination percentages.

Amino acid sequencing of sugarbeet HS60

Following digestion of the gel-purified HS60 protein by endoproteinase Lys-C (Sigma) (100 pmol HS60 protein, 0.4 µg enzyme in 350 µl of 50 mM Tris–HCl, pH 8.6, buffer containing 0.03% SDS; 18 h incubation at 37°C), the reaction mixture was injected on to a DEAE C18 HPLC column (2.1 mm diameter), and the peptides were eluted at 0.2 ml min^{-1} with an acetonitrile gradient (2–45% in 35 min) in 0.1% trifluoroacetic acid. Peptides contained in fractions with a symmetrical peak form were directly sequenced. Amino acid sequencing was performed by Dr Jacques D'Alayer (Institut Pasteur, Paris, France) by automated Edman degradation of the peptides in a PE Applied Biosystem sequencer.

Results and discussion

Germination characteristics of the control untreated sugarbeet seeds

The germination characteristics of the sugarbeet seed lot used throughout the present study are shown in Fig. 1. The seeds germinated at temperatures ranging from 5 to 40°C (Fig. 1A). However, the germination percentages obtained after 10 d (G_{10}) were the highest (80–90%) between 5 and 35°C. Only about 60% of the seeds were able to germinate at 40°C. The optimal temperature of this seed lot was in the range 25–35°C, as shown by the lowest t_{50}. At lower temperatures, t_{50} increased and reached more than 8 d at 5°C.

Germination was very sensitive to oxygen deprivation (Fig. 1B). The lower the oxygen concentration below 21% (air), the smaller was the germination percentage obtained after 10 d at 20°C and the longer was the time to obtain 50% germination. Germination was strongly reduced in atmospheres containing less than 5% oxygen. No seed

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germinated in complete anoxia, and only about 10% of the seeds were capable of germinating within 10 d at 20°C in 1% oxygen.

Influence of priming conditions on priming efficiency and solubilization of the B-subunit of 11-S globulin

Sugarbeet seeds pre-treated for 2 d at 25°C in air, according to either of the two priming protocols (hydro- or osmopriming) described under Materials and Methods, germinated much faster than the untreated control seeds (Table 1). The beneficial effect of priming was most evident when pre-treated seeds were germinated under stressful conditions, in this case 5°C.

For both hydro- and osmopriming, priming efficiency was strongly dependent on the pre-treatment temperature (Fig. 2A). Thus, priming was rather inefficient when the pre-treatment was carried out at 5°C, as inferred from t_{50} measurements. Then, priming efficiency strongly increased upon increasing the pre-treatment temperature, up to an optimal pre-treatment temperature of about 25°C for both treatments. Above this optimum, there was a slight but significant loss in germination performance of the primed seeds.

The influence of oxygen concentration in the atmosphere during priming was assessed by using the osmoconditioning technique (Fig. 3). Pre-treatment with –2.0 MPa PEG for 2 d at 25°C was totally ineffective in complete anoxia. However, seeds tolerated such a treatment and later germinated in air similar to the control ones. To markedly improve subsequent germination, the osmotic pre-treatment required at least 3% oxygen in the atmosphere, and maximal enhancement was obtained in air. Under the temperature and oxygen ranges investigated, germination percentages of primed seeds obtained after 10 d (G_{10}) of subsequent germination in air at 10°C remained nearly constant [(88.2 ± 3)% for the osmoprimed seeds, and (97.6 ± 2)% for the hydroprimed seeds] (data not shown).

In summary, the range of temperatures (Fig. 2A) and the concentrations of oxygen (Fig. 3) that are effective in priming are nearly the same as those which allow germination to occur (Fig. 1). These results confirm recent findings obtained with tomato seeds (Özbingöl *et al.*, 1998) that priming probably corresponds to the advancement of germination *sensu stricto* (phase II of the germination process) as defined by Côme (1980/81) and Côme and Thévenot (1982).

Having different seed populations issued from the same seed lot but characterized by different germination abilities (i.e. the control seeds and the seeds primed to different extents), we assessed the

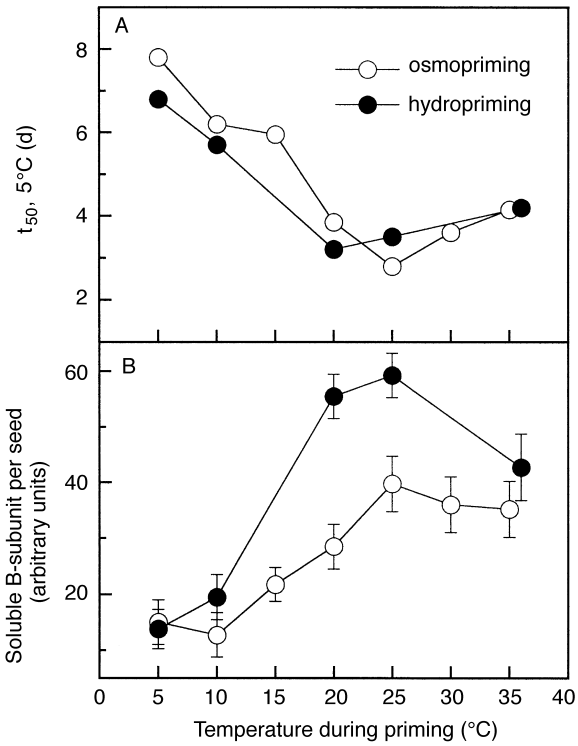

Figure 2. Effects of temperature during hydropriming (●) and osmopriming (○) on the germination characteristics of primed seeds and on the extent of solubilization of the B-subunit of 11-S globulin during priming. Hydropriming and osmopriming were carried out for 2 d in air, and seeds were germinated in water, in air at 5°C. (A) Times to obtain 50% germination (t_{50}) at 5°C. (B) Soluble B-subunit of 11-S globulin contents per seed ± SD at the end of the priming treatments. The values for unprimed seeds were 8.3 d for t_{50} and 11 ± 6 arbitrary units for soluble B-subunit content of dry ungerminated seeds.

Table 1. Germination characteristics of the hydroprimed and osmoprimed sugarbeet seeds. Hydropriming and osmopriming were carried out for 2 d in air and at 25°C, as described in Materials and Methods. Germination performance is expressed as the time to obtain 50% germination upon subsequent germination of the treated seeds at 5°C (t_{50}) and by the germination percentages recorded after 10 d upon subsequent germination of the treated seeds at 10°C (G_{10}). For comparison, germination performance of the control untreated seeds at the same temperatures is also shown

Seed sample	t_{50}, 5°C (d)	G_{10}, 10°C (%)
Control (untreated)	8.3 ± 1.3	80.5 ± 2.0
Osmoprimed	3.4 ± 0.5	89 ± 3
Hydroprimed	2.5 ± 0.5	98 ± 1

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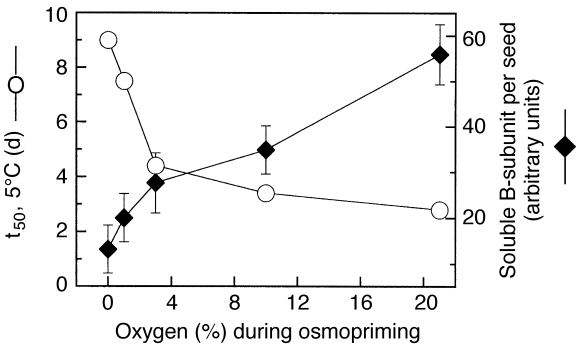

Figure 3. Effects of oxygen concentration of the atmosphere during osmopriming on the germination characteristics of primed seeds and on the extent of solubilization of the B-subunit of 11-S globulin during priming. Osmopriming was carried out for 2 d at 25°C in the indicated oxygen concentration range, and seeds were then germinated in water, in air at 5°C. (○) Times to obtain 50% germination (t_{50}) at 5°C; (◆) soluble B-subunit of 11-S globulin contents per seed ± SD at the end of the priming treatments. The values for unprimed seeds germinated in air were 8.3 d for t_{50} and 11 ± 6 arbitrary units for soluble B-subunit content of dry ungerminated seeds.

existence of a positive correlation between content of soluble 11-S globulin B-subunit per seed and priming efficiency by SDS-PAGE analyses of soluble seed protein extracts in the presence of the disulphide reductant DTT. In agreement with previous results (C. Job *et al.*, 1997; Chareyre *et al.*, 1998), these analyses disclosed an accumulation of the B-subunit of 11-S globulin during priming (Figs 4A and 4B; compare lanes 1 and 3). Furthermore, these data were consistent with the conclusion that solubilization during priming of part of the stored B-subunit of 11-S globulin occurs independently of the method of priming (C. Job *et al.*, 1997). Such an accumulation of the soluble B-subunit, as quantitated by ELISA, was strongly dependent on both the pre-treatment temperature (Fig. 2B) and oxygen concentration in the pre-treatment atmosphere (Fig. 3). For both pre-treatments, the soluble B-subunit content per seed closely paralleled priming efficiency (Figs 2 and 3), testifying to the robustness of this protein marker for prediction of sugarbeet seed vigour. Since part of the total B-subunit of 11-S globulin also becomes soluble during early stages of germination (C. Job *et al.*, 1997), these features further reinforce the finding that priming corresponds to the realization of germination *sensu stricto*.

To further characterize the priming conditions with both hydropriming and osmopriming techniques, kinetic analyses were performed by varying the duration of the incubation phase at 25°C in air. Under these conditions the hydropriming pre-treatment could be prolonged up to 5 d since none of the seeds germinated by this time. Yet, increasing the hydropriming treatment to 6 d may cause some of the seeds to germinate. The optimal incubation time was 2–3 d (Fig. 5A); for longer priming treatments, priming efficiency was slightly depressed compared with that observed under optimal conditions, as judged from a decrease in germination percentages obtained after 10 d at 10°C (Fig. 5B).

The influence of longer incubation times upon priming efficiency could be investigated in more detail with the osmopriming technique since none of the seeds germinated by up to 14 d of incubation under standard conditions (i.e. incubation in a PEG solution at –2.0 MPa at 25°C in air). As for

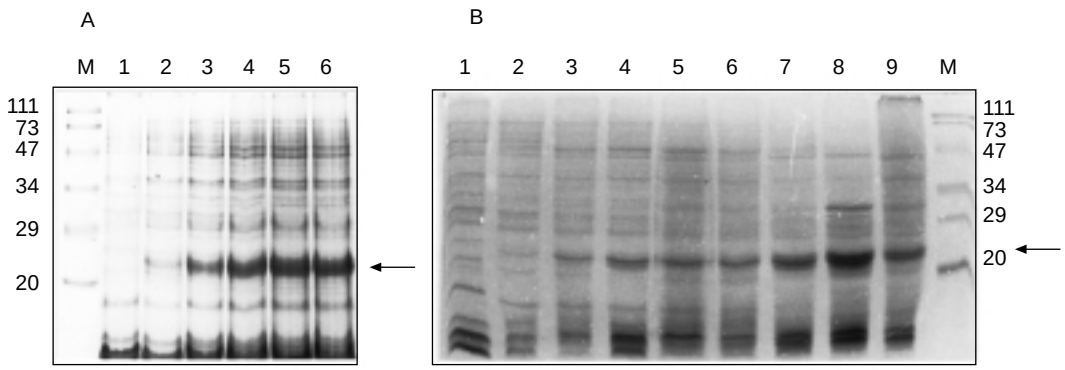

Figure 4. SDS-PAGE profiles after Coomassie-blue staining of soluble protein extracts from untreated and primed sugarbeet seeds. An equal amount of extracts corresponding to 0.10 seed was applied to each lane of 18% homogeneous polyacrylamide gels. The size of molecular mass markers (lane M) is indicated in kDa. The arrows mark the migration of the B-subunit of 11-S globulin. (A) Kinetics of hydropriming at 25°C in air. Lane 1, soluble proteins from control untreated seeds; lanes 2–6, seeds that were submitted to the hydropriming pre-treatment for 1, 2, 3, 4, and 5 d, respectively. (B) Kinetics of osmopriming at 25°C in air. Lane 1, soluble proteins from control untreated seeds; lanes 2–9, seeds that were submitted to the osmopriming pre-treatment for 1, 2, 3, 4, 5, 9, 11, and 14 d, respectively.

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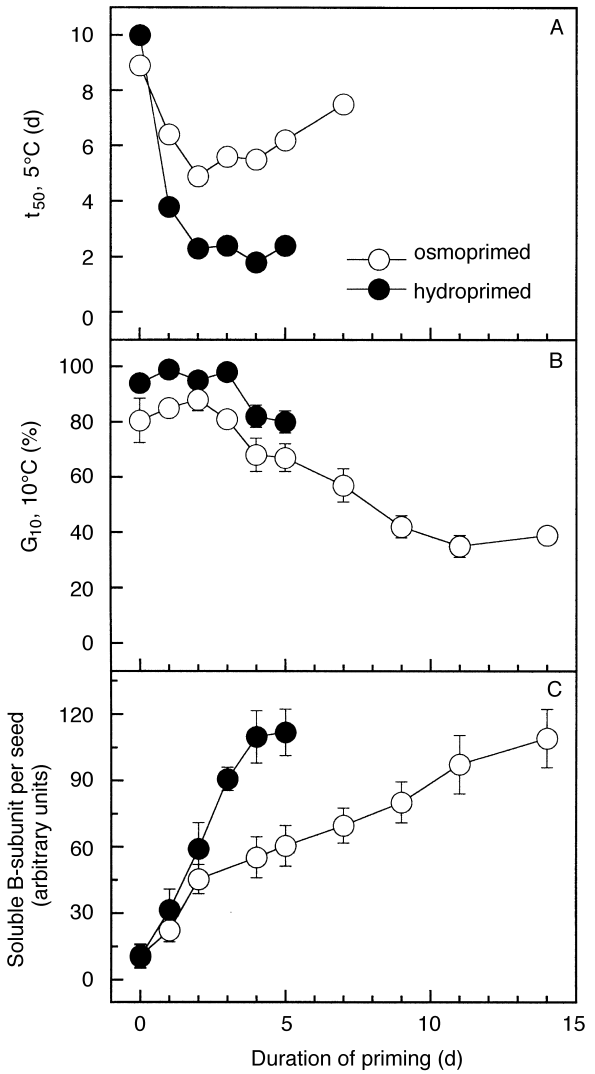

Figure 5. Effects of duration of hydropriming (●) and osmopriming (○) at 25°C in air on germination characteristics of primed seeds and on the extent of solubilization of the B-subunit of 11-S globulin during priming. (A) Times to obtain 50% germination (t_{50}) at 5°C. (B) Germination percentages obtained after 10 d at 10°C (G_{10}) ± SD. (C) Soluble B-subunit contents per seed ± SD.

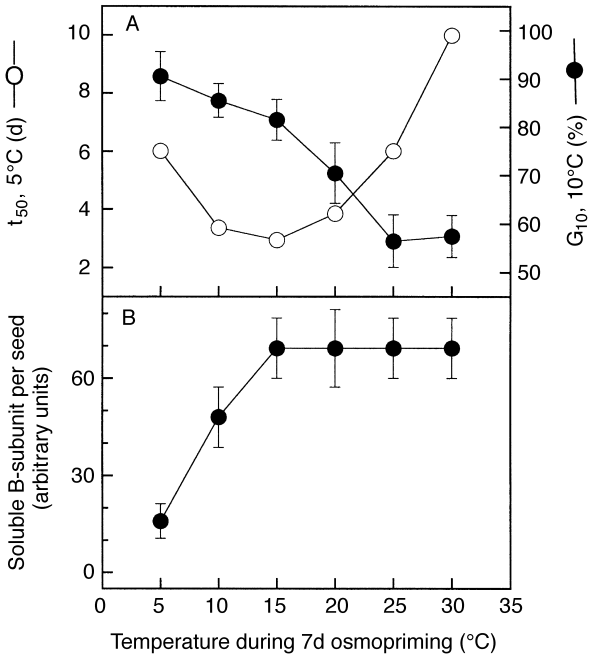

Figure 6. Effects of temperature during a 7 d osmopriming in air on germination characteristics of primed seeds and on the extent of solubilization of the B-subunit of 11-S globulin. (A) Germination performance of the osmoprimed seeds. (○) Times to obtain 50% germination (t_{50}) at 5°C; (●) germination percentages obtained after 10 d at 10°C (G_{10}) ± SD. (B) Soluble B-subunit of 11-S globulin contents per seed ± SD. The values for unprimed seeds germinated in air were 8.3 d for t_{50}, 81 ± 2% for G_{10} and 11 ± 6 arbitrary units for soluble B-subunit content of dry ungerminated seeds.

hydropriming, the optimal priming time was 2 d (Figs 5A and 5B). For longer incubations there was a progressive reduction in both germination rates at 5°C (i.e. t_{50} increased; Fig. 5A) and germination percentages obtained after 10 d at 10°C (i.e. G_{10} decreased; Fig. 5B) with the osmoprimed seeds. For instance, following an osmopriming of 7 d at 25°C, as much as about 40% of the pre-treated seed population failed to germinate when transferred to water at 10°C; when osmopriming was prolonged up to 14 d, this loss concerned about 60% of the seeds (Fig. 5B). We verified that this dramatic decline in germination performance was not due to a decreased tolerance of the seeds toward desiccation as seeds approached the time for radicle protrusion during the osmopriming treatment. Indeed, about 60% of the seeds pre-treated in the same conditions and transferred to water without re-drying failed to germinate (data not shown). The loss in germination ability of the 7 d osmoprimed seeds was strongly dependent on the temperature during the pre-treatment: for example, use of suboptimal temperatures (e.g. below 20°C) during a 7 d osmopriming treatment largely prevented this loss in germination ability and promoted priming of the seeds (Fig. 6A). Although the mechanisms responsible for this loss in germination performance of osmoprimed seeds remain to be elucidated, these experiments illustrate the difficulty in readily optimizing priming of sugarbeet seeds in the absence of related markers. That priming may reduce longevity of seeds was previously reported. This was shown, for example, for tomato (Argerich *et al.*, 1989), lettuce (Tarquis and Bradford, 1992) and pepper (Saracco *et al.*, 1995;

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Bruggink *et al.*, 1999). It should be noted, however, that this reduced longevity was usually revealed by conducting controlled deterioration tests, e.g. following exposure of primed seeds to high temperatures. In marked contrast, in the case of sugarbeet seeds that were osmoprimed at 25°C for periods longer than 3–4 d, the loss in germination ability occurred very rapidly, concomitantly with priming.

SDS-PAGE (Fig. 4) and ELISA (Figs 5C and 6B) analyses showed a progressive accumulation of the B-subunit of 11-S globulin in the soluble protein extracts from the hydro- and osmoprimed seeds; kinetics of accumulation of soluble B-subunit of 11-S globulin were slower during osmopriming than during hydropriming. A comparison of these results with the germination data (Fig. 5) indicated, however, that although such an accumulation is a characteristic feature of priming, it does not allow prediction of reduced seed germination performance after osmopriming for periods longer than 3–4 d at 25°C in air.

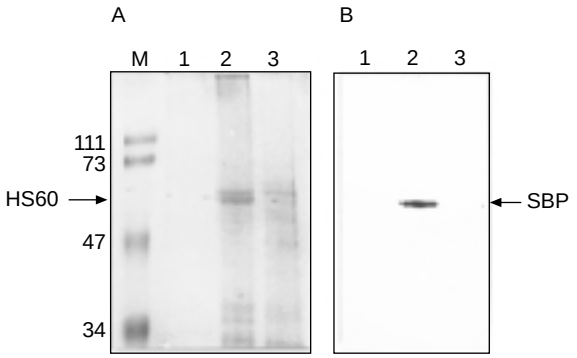

Figure 7. Analysis of heat-soluble proteins by SDS-PAGE. Heat-soluble protein extracts were prepared as described in Materials and Methods from sugarbeet leaves (lane 1), mature seeds (lane 2) and germinated seeds (lane 3), and analysed by SDS-PAGE on a 12% polyacrylamide gel. An equal amount of proteins (1 µg) was loaded on each lane. The size of molecular mass markers (lane M) is indicated in kDa. (A) Staining with Coomassie blue. (B) Western blot analysis of the protein gel shown in (A) probed with streptavidin-peroxidase as a specific reagent for biotin.

Characterization of heat-stable sugarbeet proteins

From the above results, it was of importance to characterize other protein markers for sugarbeet seed priming that would discriminate between the positive effects induced by priming itself and the negative side-effects induced by over-priming on seed performance. To this end, we chose to investigate the class of highly abundant LEA proteins because of their rapid degradation during germination (Dure, 1993). From this temporal expression pattern, a decrease in LEA protein contents during over-priming might be expected. Since one of the distinguishing features of many LEA proteins is their solubility in water after boiling (Dure, 1993; Russouw *et al.*, 1997; Walters *et al.*, 1997), we characterized the heat-soluble protein fraction from untreated and primed sugarbeet seeds by SDS-PAGE.

The heat-soluble protein fraction from untreated sugarbeet seeds contained only a few proteins of which the most prominent corresponded to a doublet of polypeptides of about 60 kDa (Fig. 7A). The smallest and most abundant of these two polypeptides, HS60, was gel purified, digested in the presence of endoproteinase Lys-C, and the resulting peptides purified by HPLC. Some of these peptides were sequenced and the sequences PGIMSSVFGALESTYEHAK, NNILGAIGNVTDAMK and TWETAEAAK were found. Scrutiny of molecular databases revealed strong homologies between two LEA group 3 proteins from *Arabidopsis thaliana* (GenBank accession numbers AAD20140 and BAA11017) and the two first peptides. Also the first of these three peptides showed significant homologies with embryonic protein BP8 from European white birch (GenBank accession number S39475) and embryonic abundant protein of 59 kDa from soybean (GenBank accession number S27757). Finally, the third peptide showed significant homology with cotton LEA D-7 protein (GenBank accession number P13939). The heat-stable protein of about 60 kDa from mature sugarbeet seeds is, therefore, likely to belong to the LEA protein group. In agreement with this finding, this protein disappears after germination (Fig. 7A).

Because of their abundance and conserved hydrophilic structure, LEA proteins have been proposed to impart protection against desiccation damage (Kermode, 1997, and references therein). There exists, however, among the heat-stable proteins found in seeds, a unique seed-specific biotinylated LEA protein that we have documented in pea and called SBP65 (for seed biotinylated protein of 65 kDa apparent molecular mass) (Duval *et al.*, 1994a, b, 1996, 1997). The best understood role of biotin is to serve as an essential cofactor for a small number of housekeeping carboxylases (e.g. acetyl-CoA carboxylase; see Knowles, 1989; Dehaye *et al.*, 1994; Alban *et al.*, 2000). In contrast, SBP65 does not exhibit any biotin-dependent carboxylase activity (Duval *et al.*, 1994a, b). Instead, this protein behaves as a sink for free biotin during late stages of embryo development and is rapidly degraded during germination (Duval *et al.*, 1994b; Dehaye *et al.*, 1997). Putative roles for SBP65 are that this protein constitutes a storage form of biotin necessary for

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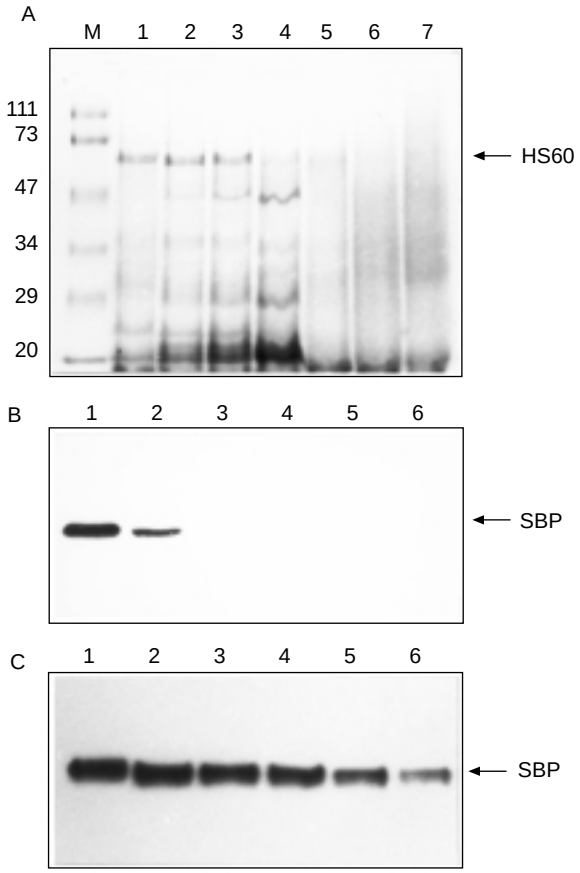

Figure 8. Accumulation pattern of heat-soluble proteins during priming of sugarbeet seeds. Heat-soluble proteins were analysed by SDS-PAGE on an 18% polyacrylamide gel. An equal amount of heat-stable protein extracts, corresponding to 0.3 seed, was loaded on the various lanes. The size of molecular mass markers (lane M) is indicated in kDa. (A) Staining with Coomassie blue. Lane 1, untreated control seeds; lanes 2, 3, and 4, seeds hydroprimed at 25°C in air for 1, 2, and 5 d, respectively. Lanes 5, 6, and 7, seeds osmoprimed at 25°C in air for 2, 5, and 11 d, respectively. (B) Western blot analysis of heat-soluble proteins from control and osmoprimed seeds probed with streptavidin-peroxidase as a specific reagent for biotin. Lane 1, untreated control seeds; lanes 2–6, seeds osmoprimed at 25°C in air for 1, 2, 3, 4, and 5 d, respectively. (C) Western blot analysis of heat-soluble proteins from control and hydroprimed seeds probed with streptavidin-peroxidase as a specific reagent for biotin. Lane 1, untreated control seeds; lanes 2–6, seeds hydroprimed at 25°C in air for 1, 2, 3, 4, and 5 d, respectively.

germination and/or that, by depleting the free biotin pool during late maturation, it may help the embryonic cells to enter into and/or to maintain a quiescent state. The existence of SBP65 does not seem to be restricted to pea; putative homologues have been described in soybean seeds (Neto *et al.*, 1997; Shatters *et al.*, 1997; Hsing *et al.*, 1998), castor bean seeds (Roesler *et al.*, 1996) and carrot somatic embryos (Wurtele and Nikolau, 1992). Figure 7B shows that an equivalent of the pea SBP65 exists in sugarbeet. As for LEA proteins, this protein is resistant to heat denaturation. Also, we verified that it exhibits the same spatial and temporal accumulation patterns as those described for the pea protein. In particular, the protein specifically accumulates in mature seeds (Fig. 7B). Therefore, we refer to this sugarbeet biotinylated protein as SBP (for seed biotinylated protein).

Behaviour of heat-stable proteins (HS60 and SBP) during priming

SDS-PAGE analyses disclosed a very fast degradation of the heat-stable proteins of about 60 kDa during osmopriming (Fig. 8A). Thus, these proteins almost completely disappeared by 2 d of incubation of sugarbeet seeds on –2.0 MPa PEG at 25°C in air. In contrast, very little degradation of these proteins was observed when seeds were pre-treated for 2 d by hydropriming (Fig. 8A). Yet, after 5 d of hydropriming pre-treatment, some degradation was occurring, as evidenced by the disappearance of the 60 kDa bands and the appearance of discrete bands of lower molecular mass (Fig. 8A). The SBP content per seed showed the same pattern of accumulation during priming. Thus, its level decreased at a fast rate during osmopriming (Fig. 8B) and at a much reduced rate during hydropriming (Fig. 8C). A comparison of these results with the germination data in Fig. 5 suggests, therefore, that the disappearance of the HS60 heat-stable proteins and of SBP correlates with loss of germination performance during osmopriming.

To strengthen this apparent correlation, additional measurements of HS60 and SBP contents per seed were performed by ELISA in a range of priming conditions (i.e. by varying duration, temperature, and oxygen concentration of the pre-treatments). These values were plotted against germination percentages of treated seeds obtained after 10 d of subsequent germination in air at 10°C (Fig. 9). From these data it is clear that germination ability during priming was not strongly affected provided that the levels of both types of proteins remained close to their initial maximal contents present in the untreated seeds. In marked contrast, treated seeds containing less than 50% of their original content of HS60 protein and SBP exhibited a very dramatic loss in germination ability. In view of the rapid disappearance of HS60 and SBP during early germination (Fig. 7), one possibility to account for the behaviour of osmoprimed seeds could be that sugarbeet seeds are going too far into phase II

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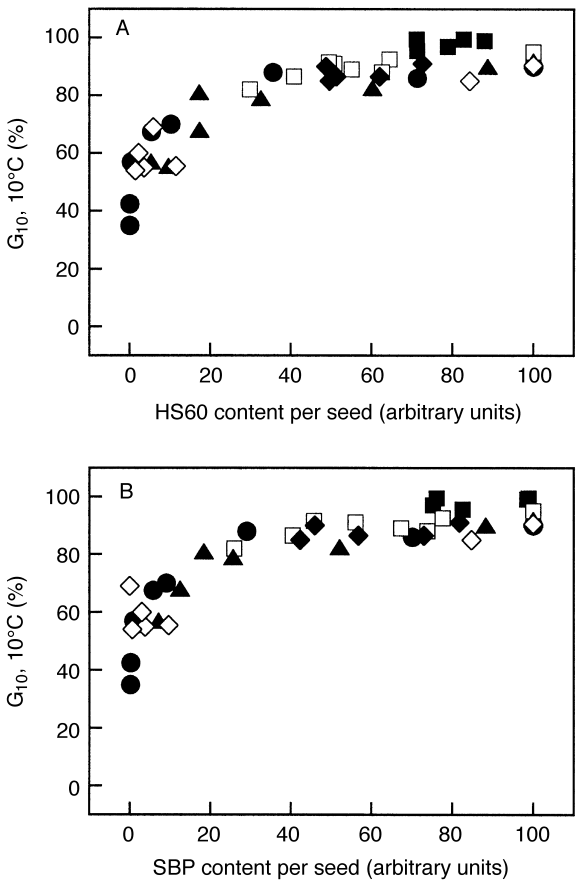

Figure 9. Germination percentages (G_{10}) obtained after 10 d at 10°C with control and primed sugarbeet seed samples vs. HS60 content per seed (A) or SBP content per seed (B). The contents of heat-soluble protein of about 60 kDa (HS60) per seed and of seed-specific biotinylated protein (SBP) were evaluated by ELISA. Symbols: (●) seeds osmoprimed at 25°C in air for different times; (■) seeds hydroprimed during 2 d in air at different temperatures; (□) seeds osmoprimed for 2 d in air at different temperatures; (▲) seeds osmoprimed for 7 d in air at different temperatures; (◆) seeds osmoprimed for 2 d at 25°C at various oxygen concentrations; (◇) seeds osmoprimed for 7 d at 25°C at various oxygen concentrations.

of the germination process during osmopriming and reach a developmental stage where ageing processes are activated if radicle emergence is prevented. Accordingly, both HS60 protein and SBP might be viewed as priming stress proteins. It is worth noting in this context the recent work of Bettey and Finch-Savage (1998) suggesting that accumulation patterns of some stress proteins (e.g. the heat-shock protein HSP17.6) in *Brassica* seeds correlate with seed performance.

Conclusion

The present study conducted with sugarbeet seeds has documented both the beneficial effects of priming treatments to improve germination ability and the difficulties of controlling such treatments. Kinetic experiments revealed that these seeds may lose their germination ability upon prolonged osmopriming treatment and then perform worse than untreated seeds. The mechanism of such a loss of the beneficial effect of priming is presently unknown. Experiments are in progress to investigate whether this osmopriming-induced reduced germination ability was due to an acceleration of general ageing processes leading to an irreversible deterioration of the seeds (see Taylor *et al.*, 1998) or to commitment of the osmoprimed seeds to a dormant state. We found, however, that this loss in germination ability was not due to decreased desiccation tolerance as seeds approached the time for radicle protrusion during priming treatments (see Welbaum *et al.*, 1998).

The present work also illustrates the possibility of predicting seedlot performance from measurements of selected germination markers. The current results indicate that, for a priming treatment of sugarbeet seeds to be the most efficient, there should be an increase in the level of soluble 11-S globulin B-subunit per seed *but* no decrease in that of heat-stable seed-specific proteins such as HS60 protein (a putative member of the LEA class 3 proteins) and SBP (the sugarbeet seed-specific biotinylated protein homologous to pea SBP65). Both these contents can be easily measured from whole seed extracts by ELISA (Duval *et al.*, 1994a, b; C. Job *et al.*, 1997; D. Job *et al.*, 1997; Chareyre *et al.*, 1998).

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References

Alban, C., Job, D. and Douce, R. (2000) Biotin metabolism in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 17–47.

Argerich, C.A., Bradford, K.J. and Tarquis, A.N. (1989) The effects of priming and ageing on resistance to deterioration of tomato seeds. *Journal of Experimental Botany* **40**, 593–598.

Battle, J.P. and Whittington, W.J. (1969) The relation between inhibitory substances and variability in time to

germination of sugar-beet clusters. *Journal of Agricultural Science (Cambridge)* **73**, 337–346.

Bettey, M. and Finch-Savage, W.E. (1998) Stress protein content of mature *Brassica* seeds and their germination performance. *Seed Science Research* **8**, 347–355.

Bino, R.J., De Vries, J.N., Kraak, H.L. and Van Pijlen, J.G. (1992) Flow cytometric determination of nuclear replication stages in tomato seeds during priming and germination. *Annals of Botany* **69**, 231–236.

Boubriak, I., Kargiolaki, H., Lyne, L. and Osborne, D.J. (1997) The requirement for DNA repair in desiccation tolerance of germinating embryos. *Seed Science Research* **7**, 97–105.

Bradford, K.J. (1986) Manipulation of seed water relations via osmotic priming to improve germination under stress conditions. *HortScience* **21**, 1105–1112.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.

Bray, C.M. (1995) Biochemical processes during the osmopriming of seeds. pp. 767–789 *in* Kigel, J.; Galili, G. (Eds) *Seed development and germination*. New York, Marcel Dekker.

Bray, C.M., Davison, P.A., Ashraf, M. and Taylor, R.M. (1989) Biochemical changes during osmopriming of leek seeds. *Annals of Botany* **63**, 185–193.

Bruggink, G.T., Ooms, J.J.J. and van der Toorn, P. (1999) Induction of longevity in primed seeds. *Seed Science Research* **9**, 49–53.

Chareyre, S., Kersulec, A., Job, D. and Job, C. (1998) The use of an ELISA to quantitate the extent of 11-S globulin mobilization in untreated and primed sugar beet seed lots. *Compte Rendus de l'Académie des Sciences, Sciences de la Vie, Paris* **321**, 705–711.

Côme, D. (1980/81) Problems of embryonal dormancy as exemplified by apple embryo. *Israel Journal of Botany* **29**, 145–156.

Côme, D. and Thévenot, C. (1982) Environmental control of embryo dormancy and germination. pp. 271–298 *in* Khan, A.A. (Ed.) *The physiology and biochemistry of seed development, dormancy and germination*. Amsterdam, Elsevier Biomedical Press.

Côme, D. and Tissaoui, T. (1968) Induction d'une dormance embryonnaire secondaire chez le pommier (*Pirus malus* L.) par des atmosphères très appauvries en oxygène. *Compte Rendus de l'Académie des Sciences, Sciences de la Vie, Paris* **266**, 477–479.

De Castro, R.D., Zheng, X.Y., Bergervoet, J.H.W., De Vos, C.H.R. and Bino, R.J. (1995) β-Tubulin accumulation and DNA replication in imbibing tomato seeds. *Plant Physiology* **109**, 499–504.

Dehaye, L., Alban, C., Job, C., Douce, R. and Job, D. (1994) Kinetics of the two forms of acetyl-CoA carboxylase from *Pisum sativum*. Correlation of the substrate specificity of the enzymes and sensitivity towards aryloxyphenoxypropionate herbicides. *European Journal of Biochemistry* **225**, 1113–1123.

Dehaye, L., Duval, M., Viguier, D., Yaxley, J. and Job, D. (1997) Cloning and expression of the pea gene encoding SBP65, a seed-specific biotinylated protein. *Plant Molecular Biology* **35**, 605–621.

Dure, L. III (1993) The LEA proteins of higher plants. pp. 325–335 *in* Verma, D.P.S. (Ed.) *Control of plant gene expression*. Boca Raton, CRC Press.

Duval, M., DeRose, R.T., Job, C., Faucher, D., Douce, R. and Job, D. (1994a) The major biotinyl protein from *Pisum sativum* seeds covalently binds biotin at a novel site. *Plant Molecular Biology* **26**, 265–273.

Duval, M., Job, C., Alban, C., Douce, R. and Job, D. (1994b) Developmental patterns of free and protein-bound biotin during maturation and germination of seeds of *Pisum sativum*. Characterization of a novel seed-specific biotinylated protein. *Biochemical Journal* **299**, 141–150.

Duval, M., Loiseau, J., Dehaye, L., Pépin, R., Le Deunff, Y., Wang, T. and Job, D. (1996) SBP65, a seed-specific biotinylated protein, behaves as a LEA protein in developing pea embryos. *Compte Rendus de l'Académie des Sciences, Sciences de la Vie, Paris* **319**, 585–594.

Duval, M., Dehaye, L., Alban, C., DeRose, R., Douce, R., Job, C. and Job, D. (1997) The role of protein biotinylation in the development and germination of seeds. pp. 33–43 *in* Ellis, R.H.; Black, M.; Murdoch, A.J.; Hong, T.D. (Eds) *Basic and applied aspects of seed biology*. Dordrecht, Kluwer.

Hegarty, T.W. (1978) The physiology of seed hydration and dehydration, and the relation between water stress and the control of germination: a review. *Plant, Cell and Environment* **1**, 101–119.

Heydecker, W. (1978) "Primed" seeds for better crop establishment? *Span* **21**, 12–14.

Heydecker, W., Higgins, J. and Gulliver, R.L. (1973) Accelerated germination by osmotic seed treatment. *Nature* **246**, 42–44.

Hsing, Y.I.C., Tsou, C.H., Hsu, T.F., Chen, Z.Y., Hsieh, K.L., Hsieh, J.S. and Chow, T.Y. (1998) Tissue- and stage-specific expression of a soybean (*Glycine max* L.) seed-maturation, biotinylated protein. *Plant Molecular Biology* **38**, 481–490.

Job, C., Kersulec, A., Ravasio, L., Chareyre, S., Pépin, R. and Job, D. (1997) The solubilization of the basic subunit of sugarbeet seed 11-S globulin during priming and early germination. *Seed Science Research* **7**, 225–243.

Job, D., Kersulec, A. and Job, C. (1997) Globulin protein 11S, usable as a seed impregnation marker during plant seed germination. *International Patent* WO9743418.

Kermode, A.R. (1997) Approaches to elucidate the basis of desiccation-tolerance in seeds. *Seed Science Research* **7**, 75–95.

Knowles, J.R. (1989) The mechanism of biotin-dependent enzymes. *Annual Review of Biochemistry* **58**, 195–221.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lawrence, D.M., Halmer, P. and Bowles, D.J. (1990) Mobilisation of storage reserves during germination and early seedling growth of sugar beet. *Physiologia Plantarum* **78**, 421–429.

Michel, B.E. and Kaufmann, M.R. (1973) The osmotic potential of polyethylene glycol 6000. *Plant Physiology* **51**, 914–916.

Morris, P.C., Grierson, D. and Whittington, W.J. (1984) Endogenous inhibitors and germination of *Beta vulgaris*. *Journal of Experimental Botany* **35**, 994–1002.

Neto, J.B.F., Shatters, R.G. and West, S.H. (1997) Developmental pattern of biotinylated proteins during embryogenesis and maturation of soybean seed. *Seed Science Research* **7**, 377–384.

Özbingöl, N., Corbineau, F. and Côme, D. (1998) Responses of tomato seeds to osmoconditioning as related to temperature and oxygen. *Seed Science Research* **8**, 377–384.

Özbingöl, N., Corbineau, F., Groot, S.P.C., Bino, R.J. and Côme, D. (1999) Activation of the cell cycle in tomato (*Lycopersicon esculentum* Mill.) seeds during osmoconditioning as related to temperature and oxygen. *Annals of Botany* **84**, 245–251.

Parera, C.A. and Cantliffe, D.J. (1994) Presowing seed priming. *Horticultural Reviews* **16**, 109–141.

Redfearn, M. and Osborne, D.J. (1997) Effect of advancement on nucleic acids in sugarbeet (*Beta vulgaris*) seeds. *Seed Science Research* **7**, 261–267.

Roesler, K.R., Savage, L.J., Shintani, D.K., Shorrosh, B.S. and Ohlrogge, J.B. (1996) Co-purification, co-immunoprecipitation, and coordinate expression of acetyl-coenzyme A carboxylase activity, biotin carboxylase, and biotin carboxyl carrier protein of higher plants. *Planta* **198**, 517–525.

Rowse, H.R. (1996) Drum priming – a non-osmotic method of priming seeds. *Seed Science and Technology* **24**, 281–294.

Russouw, P., Farrant, J., Brandt, W. and Lindsley, G.G. (1997) The most prevalent protein in a heat-treated extract of pea (*Pisum sativum*) embryos is an LEA group I protein; its conformation is not affected by exposure to high temperature. *Seed Science Research* **7**, 117–123.

Saracco, F., Bino, R.J., Bergervoet, J.H.W. and Lanteri, S. (1995) Influence of priming-induced nuclear replication activity on storability of pepper (*Capsicum annuum* L.) seed. *Seed Science Research* **5**, 25–29.

Shatters, R.G., Boo, S.P., Neto, J.B.F. and West, S.H. (1997) Identification of biotinylated proteins in soybean [*Glycine max* (L.) Merrill] seeds and their characterization during germination and seedling growth. *Seed Science Research* **7**, 373–376.

Shewry, P.R., Napier, J.A. and Tatham, A.S. (1995) Seed storage proteins: structures and biosynthesis. *Plant Cell* **7**, 945–956.

Still, D.W., Dahal, P. and Bradford, K.J. (1997) A single-seed assay for endo-β-mannanase activity from tomato endosperm and radicle tissues. *Plant Physiology* **113**, 13–20.

Tarquis, A.M. and Bradford, K.J. (1992) Prehydration and priming treatments that advance germination also increase the rate of deterioration of lettuce seeds. *Journal of Experimental Botany* **43**, 307–317.

Taylor, A.G., Klein, D.E. and Whitlow, T.H. (1988) SMP: solid matrix priming. *Scientia Horticulturae* **37**, 1–11.

Taylor, A.G., Allen, P.S., Bennett, M.A., Bradford, K.J., Burris, J.S. and Misra, M.K. (1998) Seed enhancements. *Seed Science Research* **8**, 245–256.

Toorop, P.E., van Aelst, A.C. and Hilhorst, H.W.M. (1998) Endosperm cap weakening and endo-β-mannanase activity during priming of tomato (*Lycopersicon esculentum* cv. Moneymaker) seeds are initiated upon crossing a threshold water potential. *Seed Science Research* **8**, 483–491.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedures and some applications. *Proceedings of the National Academy of Sciences, USA* **76**, 4350–4354.

Walters, C., Ried, J.L. and Walker-Simmons, M.K. (1997) Heat-soluble proteins extracted from wheat embryos have tightly bound sugars and unusual hydration properties. *Seed Science Research* **7**, 125–134.

Welbaum, G.E., Shen, Z., Oluoch, M.O. and Jett, L.W. (1998) The evolution and effects of priming vegetable seeds. *Seed Technology* **20**, 209–235.

Wurtele, E.S. and Nikolau, B.J. (1992) Differential accumulation of biotin enzymes during carrot somatic embryogenesis. *Plant Physiology* **99**, 1699–1703.

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