Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties

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Abstract

Germination and carbohydrate metabolism were studied in fresh developing pea (*Pisum sativum* L., cv Baccara) seeds and after artificial drying at 25°C and various relative humidities (20, 75 and 99% RH) to investigate whether the occurrence of desiccation tolerance was related to sucrose, raffinose and stachyose contents. Seeds became completely tolerant to fast drying at 25°C and 20% RH a few days after the end of reserve accumulation, i.e. when their moisture content dropped to approx. 50% (fresh weight basis). This acquisition of desiccation tolerance was associated with an accumulation of raffinose and stachyose, the latter being more abundant in the embryonic axis than in the cotyledons. The (raffinose+stachyose)/sucrose ratio increased during seed development and reached 1.1 in the axis and 0.2 in the cotyledons just before the onset of desiccation tolerance. When the natural acquisition of desiccation tolerance occurred on the mother plant, artificial drying of isolated seeds induced an increase in oligosaccharide content in the cotyledons. Immature seeds, the moisture content of which was higher than about 60% (fresh weight basis), did not tolerate fast drying (25°C and 20 or 75% RH). Such drying did not result in the synthesis of stachyose and induced an increase in electrolyte leakage, a decrease in the ability of seeds to convert 1-aminocyclopropane 1-carboxylic acid (ACC) to ethylene and an increase in ethane synthesis, thus indicating a deterioration of cell membrane properties and lipid peroxidation. In contrast, immature seeds tolerated drying either in the pods or at 25°C and 99% RH, and such drying induced a decrease in sucrose content, an increase in oligosaccharide content and a (raffinose+stachyose)/sucrose ratio higher than around 1. Soluble sugar contents of dried immature seeds depended on the conditions of dehydration. In cotyledons, the (raffinose+stachyose)/sucrose ratio reached 0.61 when seeds were dried at 25°C and 99% RH, whereas it was as low as 0.15 when drying was performed at 25°C and 20% RH. All the results obtained are consistent with the concept that oligosaccharides may well be involved in the protection of membranes during dehydration.

Keywords: Desiccation tolerance, membrane properties, pea seed, *Pisum sativum*, raffinose, seed dehydration, seed development, stachyose, sucrose

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Abbreviations: ACC, 1-aminocyclopropane 1-carboxylic acid; Ra, raffinose; RFO, raffinose family oligosaccharides; RH, relative humidity; Sta, stachyose; Su, sucrose

Introduction

Seed dehydration, in association with reduced metabolism, is the normal terminal event in the development of orthodox seeds (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999). However, desiccation tolerance in seeds depends on their developmental stage. Immature embryos do not tolerate fast drying during histodifferentiation, but generally acquire this tolerance during reserve deposition or when maximal dry mass is established,

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i.e. prior to maturation drying (Vertucci and Farrrant, 1995). The time of onset of desiccation tolerance during seed development depends on the species. For example, wheat, barley and maize embryos are completely tolerant to fast drying at fairly early stages of reserve accumulation (Bartels *et al*., 1988; Black *et al*., 1996; Brenac *et al*., 1997) while desiccation tolerance of leguminous seeds occurs at later times during development, i.e. at maturation drying (Matthews, 1973; Ellis *et al*., 1987; Sun and Leopold, 1993; Sanhewe and Ellis, 1996; Gorecki *et al*., 1997; Corbineau *et al*., 1998).

The critical moisture content to which immature embryos can be dried without induction of cellular damage also depends on the rate of water loss (Vertucci and Farrant, 1995). Slow drying can initiate desiccation tolerance in immature orthodox seeds (Blackman *et al*., 1992; Sanhewe and Ellis, 1996; Bochicchio *et al*., 1997; Black *et al*., 1999) or in somatic embryos of some orthodox species (Senaratna *et al*., 1989; Compton *et al*., 1992). In contrast, slow drying of recalcitrant seeds results in lower desiccation tolerance than rapid ('flash') drying (Pammenter and Berjak, 1999).

A sequence of irreversible cellular and metabolic damage is associated with dehydration of desiccation-intolerant tissues (Salmen Espindola *et al*., 1994; Vertucci and Farrrant, 1995; Côme and Corbineau, 1996). Critical features of desiccation intolerance are probably the inability to maintain physiological integrity of membranes in the dry state or during reimbibition (Crowe *et al*., 1992; Vertucci and Farrant, 1995) and to limit the occurrence of oxidative processes or accumulation of free radicals, both resulting in cellular deterioration (Leprince *et al*., 1990*b*, 1993).

Acquisition of desiccation tolerance (reviewed by Pammenter and Berjak, 1999) is associated with several cellular and metabolic events leading to the biosynthesis and accumulation of potentially protective molecules such as late-embryogenesis abundant (LEAs) and dehydrin proteins (Kermode, 1997), and sucrose, oligosaccharides or galactosyl cyclitols (Koster and Leopold, 1988; Horbowicz and Obendorf, 1994; Obendorf, 1997), partitioning of amphipathic molecules into membranes (Golovina *et al*., 1998), and induction or activation of antioxidative defence systems (Leprince *et al*., 1993; Vertucci and Farrant, 1995). Sucrose, particularly, has been suggested to be involved in preventing liquid crystalline to gel phase transitions in the membrane lipid bilayer (Crowe *et al*., 1992), and oligosaccharides enhance its protective effect by limiting its crystallization (Koster, 1991).

Accumulation of sucrose and the raffinose family oligosaccharides (RFO), e.g. raffinose and/or stachyose, has been correlated with the development of desiccation tolerance in seeds of maize (Chen and Burris, 1990; Brenac *et al*., 1997), wheat (Black *et al*., 1996, 1999), *Brassica campestris* (Leprince *et al*., 1990*a*), soybean (Blackman *et al*., 1992), lupin (Gorecki *et al*., 1997) and bean (Corbineau *et al*., 1998). On the other hand, a decrease in RFO and an increase in reducing sugars have been associated with loss of desiccation tolerance during germination of various species (Koster and Leopold, 1988; Hoekstra *et al*., 1994; Gorecki *et al*., 1997). However, other reports obtained with imbibed germinating seeds (Lin *et al*., 1998) or isolated immature wheat grains (Black *et al*., 1999) indicate that accumulation of RFO is not closely correlated with the degree of desiccation tolerance.

The aims of the present work, which concerns pea seeds, were (i) to investigate the effects of various conditions of artificial drying of the developing seeds within the pods and out of the pods on their subsequent germination ability, (ii) to study the changes in oligosaccharides and in the (raffinose + stachyose)/sucrose ratio during seed development as related to desiccation tolerance, (iii) to specify the effects of artificial drying on soluble carbohydrate contents and on membrane properties evaluated by electrolyte leakage, ACC conversion to ethylene and ethane production by seeds collected at various stages of their development, and (iv) to determine whether the desiccation damage is related to soluble sugars and the (raffinose + stachyose)/sucrose ratio.

Materials and methods

Plant material and drying conditions

Pea (*Pisum sativum* L., cv Baccara) plants were grown in 1996 and 1997 in experimental fields of FNAMS (Fédération Nationale des Agriculteurs Multiplicateurs de Semences) located in Brain-sur-l'Authion, near Angers, France. Developing pods were harvested at various times from 14 to 50 days after flowering.

For each developmental stage, seeds or excised organs (embryonic axes and cotyledons) were immediately frozen in liquid nitrogen and stored at –30°C until soluble sugar determination. Additional seeds were used for moisture content determinations and germination tests.

Other seed batches were dried at various rates by placing the pods or the isolated seeds at 25°C in ventilated incubators in which the relative humidity (RH) of the air was maintained at approx. 20% in the presence of silica gel, 75% over a saturated solution of NaCl, and 99% over water until the equilibrium moisture content (EMC), approx. 12–13%, 15–16% and 35–36% fresh weight basis, respectively, was reached. All seeds were then transferred to 25°C and 75% RH

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to obtain a final EMC of 15–16% (fresh weight basis). As an example, Table 1 gives the times to reach the different intermediate EMCs in the various desiccation procedures and the final EMC (15–16%) after transfer to 75% RH, for immature seeds collected 31 days after flowering in 1997, i.e. with 63.2% moisture content. Drying of seeds at 25°C and 75% RH corresponded to the usual drying conditions in practice, and was therefore considered as the standard drying condition.

Water content measurement

Whole seeds, embryonic axes or excised cotyledons were oven dried at 105°C for 3 days for determination of water content. Moisture content was calculated on a dry or fresh weight basis. Results are expressed as the mean moisture content obtained from three to five replicates of 10 organs.

Germination tests

After drying the seeds as described above, germination was tested at 20°C for samples of 200 seeds placed in sand moistened with water (10% w/w), in plastic boxes (180 × 120 × 55 mm) as four replicates of 50 seeds. Tolerance to desiccation was evaluated by the mean percentages of germination or of normal seedlings obtained after 8 days, according to the rules of ISTA (1993). A seed was considered tolerant when it germinated and produced a normal seedling, or intolerant when it did not germinate or did not produce a normal seedling.

Determination of soluble sugars

Sugars from 10 embryonic axes (corresponding to 30–75 mg fresh weight) or from cotyledons (50 mg fresh weight) were extracted in 0.8 ml hot (70°C) 80% aqueous ethanol containing 2.5 mg ml^{-1} melezitose as an internal standard, as described by Black *et al.* (1996). After centrifugation and removal of the supernatant, the pellet was resuspended and re-extracted twice, first in 0.5 ml and then in 0.3 ml hot 80% aqueous ethanol. The three supernatants were combined and reduced to dryness in a centrifugal evaporator. The dry extract was dissolved in 50–100 µl distilled water. The solutions of soluble carbohydrates were filtered through a 0.45 mm pore filter (Nalgene) and then analysed by HPLC. Samples (10 µl) were injected and eluted with 80:20 (v/v) acetonitrile/H_2O at a flow rate of 1 ml min^{-1} using a Spectra Physics 8700 pump on a 200 × 4.6 mm Spherisorb-NH_2 column (Thermo Separation Products, Les Ullis, France). The eluents were analysed with a differential refractometer (Spectra Physics 8430) and the peak areas were electronically integrated by a Spectra Physics 4290 integrator. The various sugars were identified by co-elution with standards (Sigma). There was a linear response of the refractive index over the concentration range evaluated so that the amount of each sugar could be calculated. Results are expressed as µg sugars per mg dry matter. They correspond to the means of measurements carried out with three to five extracts ± SD.

Electrolyte leakage measurements

Solute leakage was determined by placing four seeds in 10 ml of distilled water at 20°C and measuring the conductivity of the medium after 24 h of soaking with a conductivity meter (K220 Consort). Results are expressed as percentages of the total leakage from seeds boiled for 15 min in water and are the means of five measurements ± SD.

Measurements of ethylene and ethane production

The conversion of 1-aminocyclopropane 1-carboxylic acid (ACC) to ethylene was measured in seeds placed in 15-ml flasks (four seeds per flask) containing 1 ml ACC solution (1 mM) as described by Salmen Espindola *et al.* (1994). After 24-h incubation in the presence of ACC, flasks were tightly closed with serum caps and placed at 20°C. After 3 h, a 1 ml gas sample was taken from each flask and injected into a gas chromatograph (type 330, Girdel-France) equipped with a flame ionisation detector and an activated alumina column for ethylene and ethane determination. Results are the means of seven measurements ± SD and are expressed as nl ethylene or ethane produced in 1 h per 1 g of dry matter.

Results

Seed development

Results presented here concern seeds collected on plants grown in 1996. Those obtained in 1997 were very similar, but seeds developed a little slower than in 1996.

Figure 1 shows the changes in fresh and dry weights, and in moisture content of whole pea seeds during their development on the mother plants. Seeds were very small at 14 days after flowering, and their moisture content was very high (86.3% fresh weight basis). Their fresh and dry weights increased up to 32 days after flowering. At the end of reserve accumulation, the mean seed dry weight was around 230 mg, and the moisture content was around 55% fresh weight basis (i.e. 1.1 g H_2O g^{-1} dry weight).

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Maturation of seeds, corresponding to the phase of decreasing fresh weight and stabilisation of dry weight, was correlated with a marked increase in water loss. [It should be noted that prior to this phase, although water content (calculated on a fresh mass basis) decreased, the absolute amount of water in the seed actually increased.] During the development of seeds, the moisture content of cotyledons was always lower than that of the embryonic axis (Table 2).

Acquisition of desiccation tolerance and changes in soluble carbohydrates during seed development

Seeds younger than 37 days after flowering, the moisture content of which was higher than about 50% fresh weight basis (i.e. 1.2 g H_2O g^{-1} dry weight), did not completely tolerate the fastest drying condition (25°C and 20% RH) (Fig. 2, curve 1). Though all dried seeds germinated at 32 days after flowering (Fig. 2A), some seedlings were abnormal (Fig. 2B). Tolerance to this drying condition developed fully a few days after seed filling, when the seed moisture content dropped sharply (Fig. 1).

However, the acquisition of tolerance to artificial desiccation during seed development depended on the conditions of drying (Fig. 2). The higher the RH during drying of isolated seeds, i.e. the slower the drying (cf. Table 1), the better immature seeds tolerated dehydration. Thus, many seeds, the moisture content of which was around 60% (FW basis), did not tolerate dehydration at 25°C and 20% RH, whereas tolerance was apparent when such seeds were dried at 25°C and 99% RH. Drying of seeds within the pods at 25°C and 75% RH resulted in a higher percentage of tolerant seeds than drying of detached seeds at every RH. In various cases, dehydrated immature seeds were able to germinate (Fig. 2A) but did not give rise to normal seedlings (Fig. 2B).

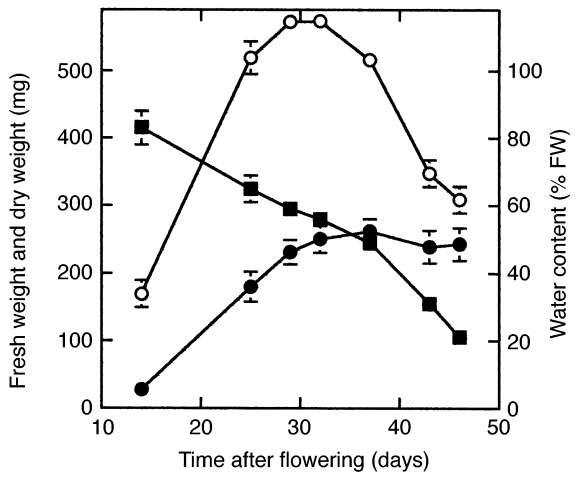

Figure 1. Changes in fresh and dry weights, and in moisture content during pea seed development in 1996. Fresh (○) and dry weights (●) per seed, and moisture content of whole seeds (■). Means of three measurements. Vertical bars correspond to SD. Where no bars are shown, SD is less than the size of the symbols.

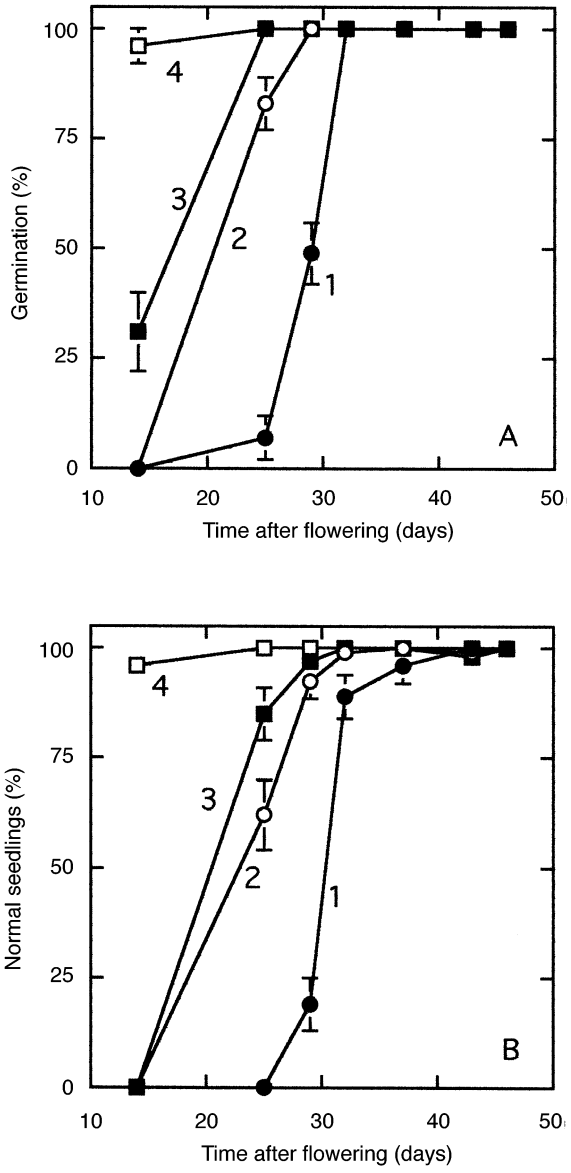

Figure 2. Changes in desiccation tolerance during pea seed development in 1996. Desiccation tolerance was expressed as germination percentages (A) or as percentages of normal seedlings (B) obtained after 8 days at 20°C. Seeds were dried out of the pods at 25°C and 20% (1), 75% (2) or 99% RH (3), or within the pods at 25°C and 75% RH (4). Means of four replicates. Vertical bars correspond to SD. Where no bars are shown, SD is less than the size of the symbols.

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Table 1. Drying parameters for immature seeds harvested 31 days after flowering, at a mean moisture content of 63.2% (fresh weight basis). The times to reach the intermediate equilibrium moisture contents (EMC) of seeds dried under various conditions, as well as the final EMC (15–16%) reached after seed transfer to 25°C and 75% RH, are indicated.

Drying conditions	Intermediate EMC (% FW)	Time (h) to reach		Additional time (h) to reach the final EMC of 15–16% at 75% RH
		15–16% EMC (% FW)	Intermediate EMC	
Isolated seeds				
25°C, 20% RH	12–13	60	117	49
25°C, 75% RH	15–16	117	117	–
25°C, 99% RH	35–36	–	142	92
Within the pods				
25°C, 20% RH	12–13	–	168	49*
25°C, 75% RH	15–16	216	216	–

*Isolated seeds.

Acquisition of desiccation tolerance during seed development on the mother plants was associated with a synthesis of raffinose and stachyose, and a decrease in sucrose content (Fig. 3). Raffinose was more abundant in the embryonic axis (Fig. 3A) than in the cotyledons (Fig. 3B), and stachyose was present only in the axis. The (Ra + Sta)/Su ratio increased in both organs during seed filling and then remained constant (Table 3). It reached 1.09 in the embryonic axis 32 days after flowering and then its value remained around 1 during seed maturation. It was lower in the cotyledons, reaching only 0.12–0.17 at the end of seed development.

Table 2. Changes in water content (% fresh weight) of the embryonic axis and cotyledons during seed development in 1996. Means of three or four measurements ± SD.

Time after flowering (days)	Water content (% FW)	
	Axis	Cotyledons
14	86.3 ± 6.1*	86.3 ± 5.2*
25	67.3 ± 3.2	60.6 ± 2.1
29	63.8 ± 1.6	53.4 ± 1.9
32	62.5 ± 2.1	51.2 ± 2.0
37	52.1 ± 2.3	–
43	37.6 ± 2.1	28.7 ± 1.6
46	14.7 ± 1.6	11.7 ± 1.3

*Whole embryo (too small for isolating axis and cotyledons).

Table 3. Changes in the (Ra + Sta)/Su ratio in the embryonic axis and the cotyledons during seed development in 1996, and after drying out of the pods at 25°C and 75% RH. Ra, raffinose; Sta, stachyose; Su, sucrose. Means of three or four measurements. No measurements were made at 14 days after flowering because seeds were too small for isolation of the axis and cotyledons.

Time after flowering (days)	(Ra + Sta)/Su			
	Before drying		After drying	
	Axis	Cotyledons	Axis	Cotyledons
14	–	–	0.04	0.04
25	0.07	0.01	0.09	0.09
29	0.73	0.09	0.60	0.43
32	1.09	0.14	1.64	0.99
37	1.09	–	1.17	0.95
43	1.06	0.12	1.16	0.63
46	1.01	0.17	0.85	0.53

Effects of artificial drying on soluble carbohydrates and desiccation tolerance

For seeds collected in 1996 (Fig. 4A), drying at 25°C and 75% RH markedly decreased the sucrose content in the axis of very young seeds (25 days after flowering), but had almost no effect on sucrose, raffinose and stachyose contents thereafter, whatever the stage of seed development (cf. Fig. 3A). Consequently, the (Ra + Sta)/Su ratio did not change much in this organ after drying (Table 3). In the cotyledons (Fig. 4B), the sucrose content was decreased in young seeds by the same drying but was not significantly altered when seeds became tolerant to desiccation under such conditions (cf. Fig. 3B). Such drying induced an increase in the raffinose content of the cotyledons and particularly in the synthesis of stachyose, which was absent in the cotyledons of freshly collected seeds (Fig. 4B, to be compared with Fig. 3B). These changes in soluble carbohydrates in the cotyledons resulted in a marked increase in the (Ra + Sta)/Su ratio (Table 3). This ratio reached values around 1 when seeds were collected 32 days after flowering (Table 3) and practically all of them produced normal seedlings after drying (cf. Fig. 2B), i.e. when they were completely tolerant to drying at 25°C and 75% RH. Very similar results were obtained with seeds collected in 1997 and dried in the same conditions (data not shown).

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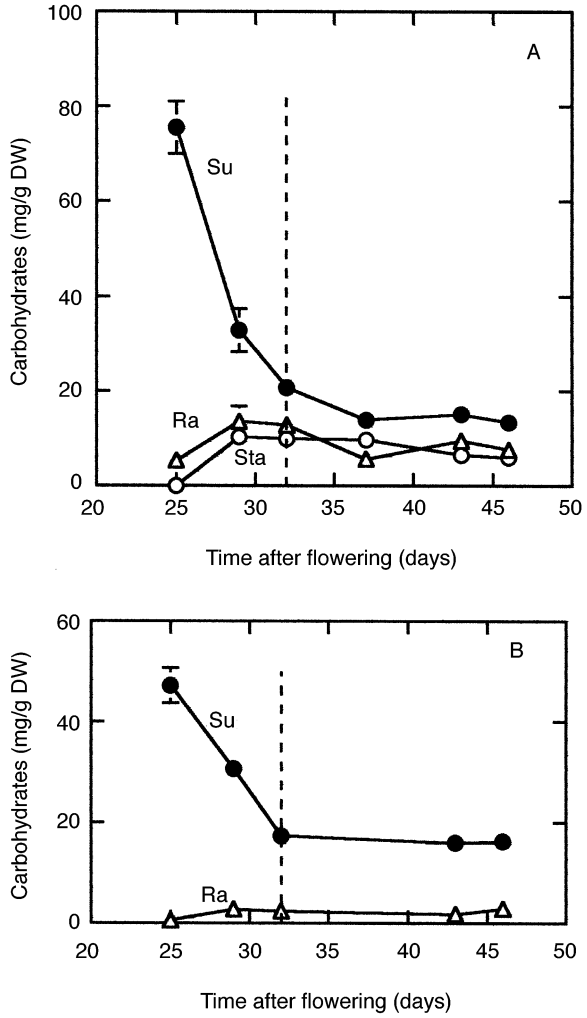

Figure 3. Changes in soluble carbohydrate contents in axes (A) and cotyledons (B) of seeds during their development on the mother plant in 1996. Ra, raffinose; Sta, stachyose; Su, sucrose. Vertical dotted lines indicate the onset of tolerance to drying at 25°C and 75% RH. Means of three or four measurements. Vertical bars correspond to SD. Where no bars are shown, SD is less than the size of the symbols.

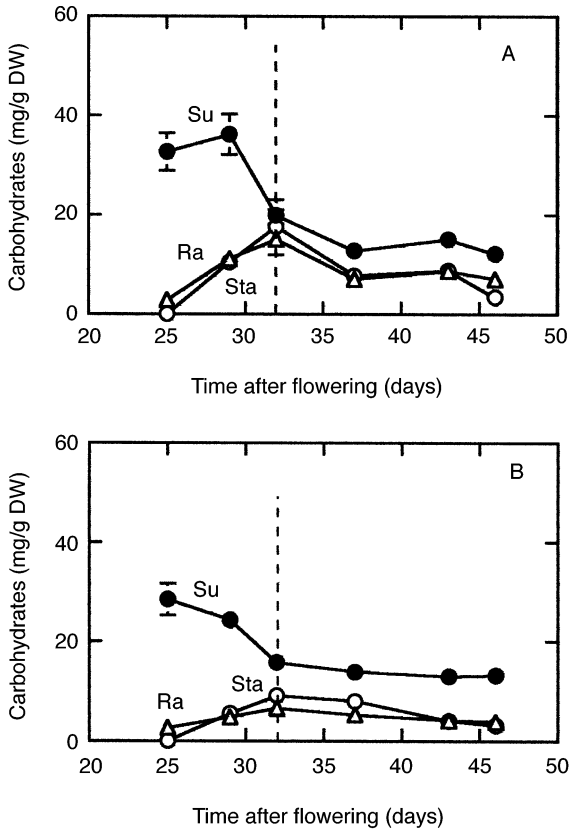

Figure 4. Soluble carbohydrate contents in axes (A) and cotyledons (B) of seeds dried at 25°C and 75% RH at various stages of development in 1996. Ra, raffinose; Sta, stachyose; Su, sucrose. Vertical dotted lines indicate the onset of tolerance to drying at 25°C and 75% RH. Means of three or four measurements. Vertical bars correspond to SD. Where no bars are shown, SD is less than the size of the symbols.

The effects of drying of immature seeds on soluble carbohydrates and tolerance to desiccation depended on the conditions of dehydration. These effects are shown in Table 4 with seeds collected 31 days after flowering in 1997, the mean moisture content of which was 63.2% (fresh weight basis). The axis and the cotyledons of fresh, non-dried seeds contained a high level of sucrose and much less raffinose and stachyose, the latter being absent in the cotyledons. The (Ra + Sta)/Su ratio was therefore low (0.49 in the axis and 0.02 in the cotyledons). Natural drying on the mother plants induced an increase in this ratio (1.22 in the axis and 0.35 in the cotyledons) which was associated with the acquisition of desiccation tolerance (92% normal seedlings). Drying of isolated seeds (i.e. seeds out of the pods), 31 days after flowering, at 25°C and 20% RH or at 25°C and 75% RH had little effect on the (Ra + Sta)/Su ratio and did not result in desiccation tolerance since no or only a few seedlings were normal. In contrast, slower drying (25°C and 99% RH) of isolated seeds induced a marked increase in the (Ra + Sta)/Su ratio (0.56 in the axis and 0.61 in the cotyledons) which was due to a large decrease in the sucrose content in both organs and the synthesis of raffinose and stachyose in the cotyledons. This increase in the (Ra + Sta)/Su ratio corresponded to a partial tolerance of seeds to desiccation (59% normal seedlings). Drying of seeds within the pods at 25°C and 20% RH or at 25°C and 75% RH resulted in a higher increase in the (Ra + Sta)/Su ratio (1.29 and 1.06 in the axis, 0.66 and 1.64 in the cotyledons) and a better tolerance of seeds to desiccation (96–98% normal seedlings).

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Table 4. Effects of drying conditions of immature seeds on sucrose (Su), raffinose (Ra) and stachyose (Sta) contents and the (Ra + Sta)/Su ratio measured in the axis and the cotyledons, and tolerance to desiccation expressed as percentages of normal seedlings obtained at 20°C. Seeds were harvested 31 days after flowering in 1997 with 63.2% mean moisture content (fresh weight basis). Means of three or four measurements ± SD as indicated.

Organ	Drying conditions	Carbohydrates (mg g^{-1} DW)			(Ra + Sta)/Su	Normal seedlings (%)
		Su	Ra	Sta		
Axis	No drying	31.1 ± 0.8	10.9 ± 1.3	4.3 ± 0.8	0.49	–
	Natural drying on plant	12.4 ± 1.1	13.2 ± 3.8	1.9 ± 1.3	1.22	92
	Isolated seeds at					
	25°C, 20% RH	36.5 ± 5.3	8.0 ± 1.7	1.9 ± 0.6	0.27	0
	25°C, 75% RH	15.2 ± 2.1	6.1 ± 1.5	0	0.40	3
	25°C, 99% RH	16.8 ± 1.8	8.3 ± 0.3	1.2 ± 0.3	0.56	59
	In pods at					
	25°C, 20% RH	16.3 ± 3.6	15.7 ± 3.8	5.4 ± 2.1	1.29	96
	25°C, 75% RH	14.4 ± 1.8	12.4 ± 0.8	2.8 ± 1.2	1.06	98
Cotyledons	No drying	31.9 ± 2.1	0.5 ± 0.2	0	0.02	–
	Natural drying on plant	12.7 ± 1.5	4.5 ± 0.9	0	0.35	92
	Isolated seeds at					
	25°C, 20% RH	34.6 ± 6.4	4.1 ± 0.9	1.2 ± 1.1	0.15	0
	25°C, 75% RH	28.9 ± 3.6	5.5 ± 2.0	0	0.19	3
	25°C, 99% RH	13.5 ± 2.4	5.2 ± 1.9	3.0 ± 2.0	0.61	59
	In pods at					
	25°C, 20% RH	10.5 ± 1.5	3.9 ± 0.6	3.1 ± 1.6	0.66	96
	25°C, 75% RH	7.6 ± 1.7	3.7 ± 0.5	8.8 ± 1.5	1.64	98

Effects of artificial drying on membrane properties

Figure 5A summarises the effects of drying at 25°C and 75% RH on electrolyte leakage, and Fig. 5B the ACC conversion to ethylene and ethane production by seeds collected in 1996 at various stages of development. Drying of very young seeds (14 days after flowering) which were not tolerant to desiccation (cf. Fig. 2) induced strong deterioration of cell membranes, indicated by high electrolyte leakage (Fig. 5A), the inability of seeds to convert ACC to ethylene (Fig. 5B) and a high ethane production (Fig. 5B). The deleterious effects of drying on membranes decreased during seed development. From 32 days after flowering, i.e. when seeds became completely tolerant to desiccation at 25°C and 75% RH (cf. Fig. 2), electrolyte leakage was much reduced (Fig. 5A), and seeds did not produce ethane and became able to convert ACC to ethylene (Fig. 5B). It is interesting to notice that after the development of desiccation tolerance the ability of seeds to convert ACC to ethylene increased during their maturation (Fig. 5B), suggesting that this phase of seed development is associated with changes in membrane properties.

Figure 6 shows that in the seeds collected during 1996 and 1997, and dried under the various conditions indicated in Table 4, electrolyte leakage decreased when the (Ra + Sta)/Su ratio in the cotyledons increased. Leakage was less than 20% of total electrolytes within 24 h of soaking at 20°C when this ratio was higher than 0.5.

The effects of drying on the membranes of immature seeds depended on the conditions of dehydration, as shown in Table 5 for seeds collected 31 days after flowering in 1997, the mean moisture content of which was 63.2% (fresh weight basis). After natural drying on the plants, seeds exhibited little electrolyte leakage, were able to convert ACC to ethylene and produced very little ethane. Similar behaviour was observed with seeds dried out of the pods at 25°C and 99% RH or within the pods. In contrast, drying of isolated seeds at 25°C and 20% or 75% RH resulted in considerable electrolyte leakage and ethane production, and an almost complete inability to convert ACC to ethylene.

Discussion and conclusion

As in other leguminous species, such as bean (Sanhewe and Ellis, 1996; Corbineau *et al*., 1998), soybean (Blackman *et al*., 1992; Sun and Leopold, 1993) and lupin (Gorecki *et al*., 1997), detached pea seeds became fully tolerant to rapid drying (25°C and 20% RH) just after the seed-filling phase when their moisture content declined to approx. 50% (fresh weight basis) (Fig. 1). However, development of tolerance to drying at 25°C and 20% RH in the whole

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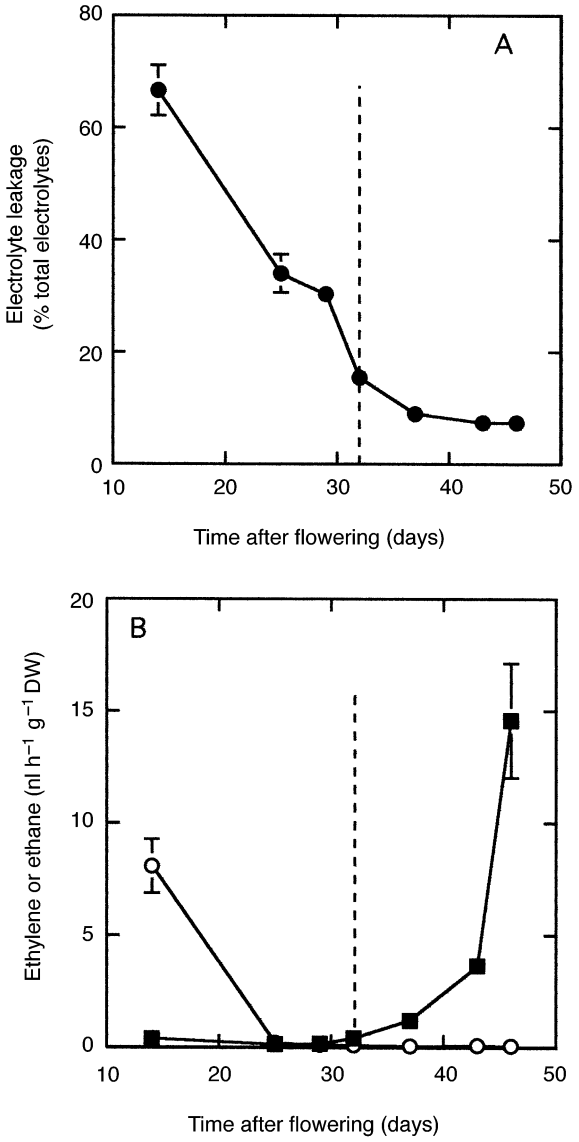

Figure 5. Electrolyte leakage (A), and ethylene (■) and ethane (○) production (B) by seeds dried at 25°C and 75% RH at various stages of development in 1996. Measurements were made at 20°C after 24 h of soaking in water (electrolyte leakage) or in a solution of 1 mM ACC (ethylene and ethane production). Vertical dotted lines indicate the onset of tolerance to drying at 25°C and 75% RH. Means of five or seven measurements. Vertical bars correspond to SD. Where no bars are shown, SD is less than the size of the symbols.

population of detached seeds depended on the year of cultivation. It occurred about 37 days after flowering in 1996 (Fig. 2), when the mean moisture content of the embryonic axis was around 52% (fresh weight basis) (Table 2). In 1997, complete tolerance to the same drying was acquired 10–15 days later (data not shown).

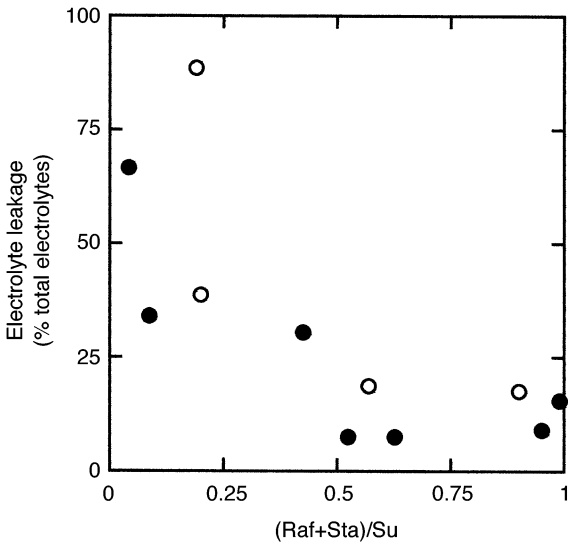

Figure 6. Relationship between electrolyte leakage by seeds and the (Ra + Sta)/Su ratio measured in the cotyledons. Leakage was measured at 20°C after 24 h of soaking in water. Results obtained with seeds collected in 1996 (●) and 1997 (○) and dried under the various conditions indicated in Table 4.

The tolerance of pea seeds to desiccation to a 15–16% (fresh weight basis) final moisture content depended on the conditions of dehydration. As in maize (Bochicchio *et al.*, 1997) and soybean (Adams *et al.*, 1983; Blackman *et al.*, 1992), slow drying of detached seeds or seeds within the pods resulted in a higher percentage of tolerant seeds (Fig. 2). Continued drying to the final low moisture content used in the present work did not allow determination of the critical amount of water loss required for the initiation of tolerance to dehydration. However, Blackman *et al.* (1992) and Black *et al.* (1999) have reported that a slight decrease in water content was enough to induce desiccation tolerance in soybean and wheat embryos.

Loss of viability of immature pea seeds induced by dehydration at 25°C and 20% or 75% RH was associated with cell membrane damage as shown by high electrolyte leakage (Fig. 5A and Table 5) and the inability of seeds to convert ACC to ethylene (Fig. 5B and Table 5). ACC-dependent ethylene production is a good indicator of membrane deterioration, since it is mediated by ACC oxidase, the *in vivo* activity of which depends on membrane integrity or properties (Odawara *et al.*, 1977; Porter *et al.*, 1986). Decreased ACC conversion to ethylene was one of the early indicators of cell membrane damage during dehydration of recalcitrant *Araucaria angustifolia* embryos (Salmen Espindola *et al.*, 1994) or cold temperature exposure of chilling-sensitive tissues (Chen and Patterson, 1985; Corbineau *et al.*, 1999). The

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Table 5. Effects of drying conditions of immature seeds on electrolyte leakage (% total electrolytes), and ethylene and ethane production by seeds. Seeds were harvested 31 days after flowering in 1997 with 63.2% mean moisture content (fresh weight basis). Measurements were made at 20°C after 24 h of soaking in water (electrolyte leakage) or in a solution of 1 mM ACC (ethylene and ethane production). Means of five or six measurements ± SD.

Drying conditions	Electrolyte leakage (%)	Ethylene (nl h^{-1} g^{-1} DW)	Ethane (nl h^{-1} g^{-1} DW)
Natural drying on the plant	17.4 ± 2.7	0.33 ± 0.10	0.07 ± 0.03
Isolated seeds, 25°C and 20% RH	94.1 ± 4.4	0.06 ± 0.03	6.75 ± 0.77
Isolated seeds, 25°C and 75% RH	83.5 ± 4.5	0.19 ± 0.08	10.50 ± 1.40
Isolated seeds, 25°C and 99% RH	16.5 ± 6.6	1.28 ± 0.30	0.09 ± 0.01
In the pods, 25°C and 20% RH	11.2 ± 0.9	1.03 ± 0.25	0.10 ± 0.02
In the pods, 25°C and 75% RH	8.3 ± 0.7	0.40 ± 0.06	0.09 ± 0.02

high ethane production by rapidly dried immature pea seeds (Fig. 5B and Table 5) probably resulted from lipid peroxidation of damaged membranes (Kimmerer and Kozlowski, 1982). On the other hand, the increased ability of seeds to convert ACC to ethylene after the onset of tolerance to dehydration at 25°C and 75% RH (Fig. 5B), which might be a good marker of seed vigour in various species including pea (Gorecki *et al*., 1991; Khan, 1994), suggests that the maturation-drying phase is probably associated with an increase in seed resilience.

Acquisition of desiccation tolerance in pea seeds was associated with a decrease in sucrose content (expressed per g DW) in the embryonic axis and the cotyledons (Fig. 3) and accumulation of raffinose and stachyose in the axis (Fig. 3A). The (raffinose + stachyose)/sucrose ratio in the axis increased during seed development *in planta* and reached values of at least 1 when seeds were tolerant to drying, i.e. 32 days after flowering for seeds collected in 1996 and dried at 25°C and 75% RH (Table 3 and Fig. 2). In quiescent axes, the main soluble sugars were sucrose, raffinose and stachyose, and the value of the oligosaccharides/sucrose ratio remained close to 1 during the late maturation-drying phase of seed development (Table 3). There was no accumulation of stachyose in the cotyledons *in planta* (Fig. 3B), which suggests that desiccation tolerance of pea seeds does not require this oligosaccharide in cotyledons. Accumulation of RFO probably resulted from an increase in galactinol synthase, raffinose synthase and stachyose synthase activities as observed in *Vigna umbellata* (Peterbauer *et al*., 1998), kidney bean (Castillo *et al*., 1990) and soybean (Saravitz *et al*., 1987; Lowell and Kuo, 1989; Castillo *et al*., 1990). Comparison of the timing of soluble carbohydrate accumulation in the axis and the cotyledons during pea seed development on the mother plants suggests different regulatory mechanisms of oligosaccharide biosynthesis in each organ, particularly stachyose synthase activity.

Artificial drying of very young seeds resulted in a decrease in sucrose content in both the axis and the cotyledons (Fig. 4 and Table 4). At the end of seed filling and thereafter, drying had no important effect on RFO content in the embryonic axis (Fig. 4A) and then did not change the (raffinose + stachyose)/sucrose ratio in this organ much (Table 3), while it induced stachyose synthesis (Fig. 4B) and an increase in the (raffinose + stachyose)/sucrose ratio (Table 3) in the cotyledons. These specific effects of drying increased with a decreased rate of dehydration of detached seeds and were particularly marked in seeds dried within the pods (Table 4), suggesting that a rapid initial water loss was unfavourable to the development of enzymatic reactions including those involved in RFO synthesis. Induction of RFO synthesis by slow drying was previously shown by Blackman *et al.* (1992), Bochicchio *et al.* (1997) and Black *et al.* (1999) in other species. Comparison of soluble sugar contents in seeds matured *in planta* with those in seeds dried *ex planta* (Table 4) also emphasises the role of seed detachment from the mother plant as the signal to initiate oligosaccharide synthesis, specifically in the cotyledons. Such an effect of seed abscission has already been suggested in the regulation of raffinose and dehydrin syntheses in wheat embryos (Black *et al*., 1999). In the case of pea, the pod itself might also play a role in RFO synthesis in seeds.

Our results demonstrate that desiccation tolerance of most of the seed population was established in pea as the oligosaccharides/sucrose ratio in the axis increased above a value close to 1 (Table 4), which was consistent with the potential role of sucrose associated with oligosaccharides in protection of membranes from dehydration damage. However, there was no clear correlation between acquisition of desiccation tolerance and the value of this ratio measured in cotyledons of seeds dried under various conditions (Table 4), which cast doubt on a specific requirement for a high oligosaccharides/sucrose ratio in the development of desiccation tolerance. However, the relationship which existed between electrolyte leakage and RFO/sucrose ratio in cotyledons (Fig. 6) suggests that oligosaccharides

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could play a role in seed quality or storability as claimed by Horbowicz and Obendorf (1994).

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