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Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* in relation to the acquisition of tolerance to drying

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Changes in ultrastructural, biochemical and biophysical characteristics of embryonic axes of *Aesculus hippocastanum* during development are related to changing levels of desiccation tolerance. Histodifferentiation was complete 30 days after flowering (DAF) and fruits were shed about 120 DAF. During this period, the dry mass of embryonic axes increased from about 0.5 to 4 mg and the water content decreased from 10.2 to 2.0 g H_2O g^{-1} dry mass (g g^{-1}). In spite of the large changes in water content, water potentials of freshly harvested material declined slightly during development from −0.65 to −2.0 MPa. Tolerance of desiccation increased as embryos matured. If evaluated on the basis of critical water contents for survival, tolerance appeared to increase continuously, maximum tolerance being achieved at 120 DAF when embryos survived water contents as low as 0.30 g g^{-1}. When evaluated from critical water potentials, three distinct levels of desiccation tolerance could be recognized at −1.8 MPa (30–40 DAF), −4 MPa (48–90 DAF) and −12 MPa (100–120 DAF). During development, total dry matter increased while sugar content (g g^{-1} dry mass) and osmotically active material (mmol g^{-1} dry mass) decreased. The subcellular organisation of axes was always typical of metabolically active tissues. Maximum tolerance (−12 MPa) was associated with a reduced amount of monosaccharides and the appearance of water with unusual calorimetric behaviour. Our data are consistent with several of the current hypotheses regarding the mechanisms of desiccation tolerance. Accumulation of dry matter reserves, reduced levels of monosaccharides, presence of dehydrin-like proteins and ability to form glasses appear to be associated with the changes in desiccation tolerance. However, none of these factors allow *A. hippocastanum* embryos to achieve the extreme level of desiccation tolerance typical of orthodox seeds. This may be because *A. hippocastanum* embryos do not reach physiological maturity and remain metabolically active even after they are shed from the parent plant. Also, embryos may acquire incompetent protectants or lack as yet unidentified protective mechanisms.

Abbreviations – DAF, days after flowering; DM, dry mass; DSC, differential scanning calorimetry; g g^{-1}, g water g^{-1} dry mass; PEG, polyethylene glycol (M_r = 8 000); TEM, transmission electron microscopy.

Introduction

Seeds can be divided into two categories based on their ability to tolerate drying and consequently on their storage longevity (Roberts 1973). Mature orthodox seeds can tolerate complete water loss and when dried to water contents less than 0.15 g g^{-1} are metabolically quiescent. Recalcitrant seeds are shed at a high moisture content (water contents vary but are usually greater than 1.0 g g^{-1}) and are sensitive to some degree of drying (to water contents between 0.25 and 1.0 g g^{-1}, depending on species). It has been shown that these seeds are metabolically active and most appear to initiate germination upon abscission (reviewed

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by Berjak et al. 1989). Studies on mature (post-abscised) recalcitrant seeds have shown that there is considerable variation among different species in the extent of dehydration tolerated (reviewed by Vertucci and Farrant 1995). Furthermore, it appears that the apparent desiccation tolerance of a species can be influenced by factors such as the progress towards germination after shedding (reviewed by Berjak et al. 1989), the rate of drying (Pammenter et al. 1991, Berjak et al. 1993; reviewed by Kermode 1990, Vertucci and Farrant 1995) and the temperature to which seeds are exposed (Ellis et al. 1990, 1991, Berjak et al. 1994, Vertucci et al. 1994a). Such results indicate that the phenomenon of seed recalcitrance is complex.

The differences in desiccation tolerance among different seed types arise from differences in their embryological development on the parent plant. Orthodox seeds develop desiccation tolerance during the mid to late stages of development (reviewed by Kermode 1990, 1995, Galau et al. 1991, Vertucci and Farrant 1995). Comparison of the developmental processes occurring in different recalcitrant seeds and orthodox seeds could lead to an understanding of varying sensitivity to water loss (reviewed by Farrant et al. 1993).

The ability of desiccation-tolerant organisms to tolerate severe desiccation has been variously attributed to (1) the accumulation of protectant sugars (reviewed by Hoekstra et al. 1989, Crowe et al. 1992, Horbowicz and Obendorf 1994, Leopold et al. 1994) or the loss of reducing sugars (Leprince et al. 1993, Tetteroo et al. 1994), (2) the ability to express late embryogenesis abundant (LEA) proteins (reviewed by Kermode 1990, 1995, Chandler and Robertson 1994), (3) the ability to limit the degree of vacuolation in the embryonic tissues (Bewley 1979, Kermode 1990, Vertucci and Farrant 1995, Farrant et al. 1997) or (4) the ability to turn off metabolism (Leprince et al. 1995, Farrant et al. 1997; reviewed by Leprince et al. 1993, Vertucci and Farrant 1995). The ability to tolerate water loss has also been related to qualitative differences in the nature of water present in different seed types (Sun et al. 1994; reviewed by Leopold et al. 1994, Vertucci and Farrant 1995) or the inability of seeds to tolerate loss of the different types of water (Pammenter et al. 1991, 1993).

Because of the differing responses of tissues to water loss, it is necessary to consider the interaction of several factors (reviewed by Farrant et al. 1993, Leprince et al. 1993, Vertucci and Farrant 1995). The physical and chemical processes which confer mechanical stability and/or protection should in turn influence the physical properties of subcellular water. Conversely, the ability to alter the physical properties of water might confer tolerance by changing the metabolic status of a seed (Walters et al. 1997; reviewed by Vertucci and Farrant 1995). Perhaps orthodox seeds tolerate drying because they acquire all the necessary factors during development. Recalcitrant seeds have differing degrees of tolerance because of the absence (or malfunctioning) of one or more of these factors.

In the present study, the embryological development of the recalcitrant seeds of *Aesculus hippocastanum* was followed to compare the developmental process of these seeds to those reported for orthodox seeds and some other recalcitrant seeds. Developmental changes in mass, moisture content, ultrastructure, sugar content and the physical properties of water were monitored. The amount of water loss tolerated by embryos of different developmental stages was assessed and was related to these parameters.

Materials and methods

Plant material

Aesculus hippocastanum L. seeds were collected at weekly intervals from the grounds of Colorado State University, Fort Collins, USA during the summers of 1992 through 1995. Trees flowered in mid-May and fruits were shed in mid-September. Embryos were classified into 16 maturity stages based on the colour, texture and water content of the cotyledons. The average days after flowering (DAF) required to achieve a maturity class was calculated from DAF determined each year. Serial sections through wax-embedded whole seeds indicated that histodifferentiation was complete by about 30 DAF.

Mass and water content determination

The testa was removed and axes and cotyledons were separated from each of 10–20 seeds. The fresh mass of individual axes and cotyledons was recorded and the dry mass was measured after tissues were held at 95°C for 36 h. Water content was calculated from the fresh and dry mass and is expressed as g water per g dry mass (g g^{-1}).

Water and osmotic potential determinations

The relationship between water content and water potential was determined for embryonic axes throughout development by constructing water sorption isotherms at high water potentials ($\Psi_w \geq -35$ MPa). Usually, this is done using psychrometric methods, but whole embryonic tissues of *A. hippocastanum* did not equilibrate in the sample chamber of the SC-10 thermocouple psychrometer (Decagon Devices, Pullman, WA, USA) and slowly dried out during measurements. To gain more reliable water potential measurements, axes were soaked in polyethylene glycol (PEG) ($M_r = 8000$) solutions of different concentrations for 16 h (constant mass being achieved with 8–10 h) and then fresh and dry mass were measured. The water potentials of the

PEG solutions were calculated according to equations provided by Michel and Kaufmann (1973) and confirmed by thermocouple psychrometry.

The molality of osmotically active solutes in freshly harvested axes was calculated from the equations

$$\Psi_w = \frac{RT}{\bar{V}} \cdot \ln(x_a) \quad (1)$$

$$m = \frac{x_b}{x_a \cdot \frac{M_a}{1\,000}}, \quad (x_b = 1 - x_a) \quad (2)$$

where R, $\bar{V}$, and M_a are constants of 8.314 J mol^{-1} K^{-1}, 18.334 l mol^{-1}, and 18 g mol^{-1}, respectively, x_a and x_b are the mole fractions of water (water content on a fresh weight basis) and solute, and m is the molality.

Assessment of tolerance to water loss

Excised axes were dried in an air stream as described by Pammenter et al. (1991). The total drying time varied from 30 to 180 min depending on the developmental status of the axes. Young axes, being small, dried more rapidly and required less time to reach low moisture contents. Axes were removed from the airstream at regular intervals and the level of damage was assessed by measuring the extent of electrolyte leakage from the tissue and by growth in culture. To prevent imbibitional damage, variously dried axes were slowly rehydrated on damp blotting paper. Thereafter, the rate of leakage was measured for 5–10 replicates on an individual axis basis. Leakage was monitored at 5-min intervals for 1 h using an ASAC-1000 (Applied Intelligence Systems Inc./Neogen, Lansing, MI, USA) conductivity meter and was corrected by the axis dry mass. The water content at which leakage was judged to have increased markedly is termed the critical water content and was determined as described by Berjak et al. (1993). Each axis was then transferred to embryo culture and was assessed on its ability to expand and produce roots and shoots. Individual axes were surface sterilized for 10 min in 2.5% (v/v) sodium hypochlorite containing 0.2% (w/v) Tween 20, washed in sterile water and transferred aseptically to separate culture vessels. The culture medium was comprised of Murashige and Skoog (1962) basal salts supplemented with 3% (w/v) sucrose and 2 mg l^{-1} benzylaminopurine (BAP) and 2 mg l^{-1} naphthaleneacetic acid (NAA). Mature seeds of *A. hippocastanum* have been reported to be dormant (Tompsett and Pritchard 1993). The effect of plant growth regulators in the culture media varied with maturity of the axes and the harvest year. The addition of growth regulators rarely had an effect on axes harvested before September, but in some years they hastened development in culture of more mature axes by 2–4 weeks.

Ultrastructural studies

The changes which occurred in axis meristem and hypocotyl tissues were examined using transmission electron microscopy (TEM). Tissues were processed using a caffeinated gluteraldehyde-osmium procedure described by Drennan and Berjak (1982). Material was sectioned with a Reichart (Wein, Austria) ultramicrotome and viewed with a Jeol Ltd (Tokyo, Japan) 2000 EX II TEM.

Sugar analyses

The types and quantities of sugars present in embryonic axes were determined using HPLC and colorimetric assays. Sugar extraction followed procedures of Kuo et al. (1988). Freeze-dried tissues (approximately 10–30 axes depending on maturity stage) were ground in a glass microhomogenizer and sugars were extracted in 75% (v/v) ethanol (crude extract). The extract was then heated for 30 min at 75°C, centrifuged at 10000 *g* for 10 min and the resulting supernatant was freeze-dried. Prior to HPLC analysis, the sample was redissolved in water and filtered using a 0.2-μm filter. Sugars were separated by HPLC on a Benson carbohydrate column (Alltech Assoc. Inc., Dearfield, IL, USA) using water as the solvent and detected by refractive index. Sugars were identified by comparison of retention times with standards and quantified by comparison of peak heights with the internal standard (sorbitol). Sugar standards used were arabinose, ribose, xylose, glucose, mannose, galactose, fructose, sucrose, maltose, cellobiose, melezitose, raffinose, stachyose, verbascose, and several sugar alcohols. Retention times varied from 5 to 25 min. Total sugars were also quantified by adding orcinol in concentrated sulfuric acid to the crude sugar extract. After heating them at 80°C for 15 min, samples were cooled and absorbance read at 510 nm (Chaplin and Kennedy 1986). Reducing sugars were quantified by the Somogyi-Nelson alkaline-copper method (Robyt and White 1987). Sugar concentrations in *A. hippocastanum* axes were determined from standard curves constructed using sucrose and glucose, respectively, for the orcinol/sulfuric and Somogyi-Nelson assays. The slopes of standard curves for the orcinol/sulfuric acid assay were 5 and 15% of the value calculated for sucrose when sorbitol and *myo*-inositol, respectively, where used as standards.

Calorimetry

Calorimetric properties of water were measured for individual axes (undried and dried to various levels) using a Perkin Elmer (Norwalk, CT, USA) DSC 7 apparatus calibrated for onset temperature using indium and methylene chloride standards and for energy using indium. From this calibration, the onset tempera-

ture of deionized water was $-0.6°C$ when scanning at $10°C\ min^{-1}$ and the melting enthalpy was 335 J g^{-1} H_2O. Embryonic axes of *A. hippocastanum*, dried to various levels and hermetically sealed in aluminium pans, were cooled from 20°C to $-70°C$ at $10°C\ min^{-1}$ and then rewarmed to 20°C at the same rate. After the DSC measurements, water contents were determined by puncturing the pans (which had been preweighed empty and immediately after the sample was enclosed) and holding the contents at 95°C for 36 h. The temperature of the transition was determined from the onset temperature since peak temperatures are known to be influenced by the thermal mass of the sample. The onset temperature of the transitions was determined, using Perkin Elmer software, from the intersection of the baseline and the tangent to the sharpest part of the main peak. The enthalpy of the transition, in joules, was calculated from the peak area, also using Perkin Elmer software. The moisture content below which freezing and melting transitions were not observed was determined from the x-intercept of the regression line between the water content and the enthalpy of transition, in J g^{-1} dry mass (Vertucci 1990). The slope of that regression line gave the energy associated with the melting of water, in J g^{-1} H_2O. The ratio of water in the sharp peak relative to the broad peak was calculated from the area of the two peaks (Vertucci et al. 1994a).

Results

In the present study, we report changes in the status of water and in ultrastructural and biochemical characteristics of *A. hippocastanum* which occur from the onset of reserve accumulation to its completion when the seed is shed from the parent. During this period, both the fresh and dry mass of the cotyledons increased progressively and the water content decreased progressively (Fig. 1B). Similar trends were observed for axes (Fig. 1A). Although there was considerable variation in axis water content within each harvest year during the early stages of development (Fig. 1A), the average water contents of axes and cotyledons at each harvest date were fairly similar among all study years (1992–1995) and were also similar to other reports of recalcitrant seeds from temperate climates given for the latter stages of maturation (Pritchard 1991, Finch-Savage 1992, Grange and Finch-Savage 1992, Pence 1992, Tompsett and Pritchard 1993, Vertucci et al. 1994a). The increase in dry matter continued until shedding for cotyledons (Fig. 1B) and slowed or stopped a week prior to shedding for axes (Fig. 1A). The continued accumulation of dry matter until shedding was also observed in *Quercus robur* embryos and is the basis for the hypothesis that recalcitrant seeds do not achieve 'physiological maturity' because development is prematurely terminated (Finch-Savage 1992). While axis and cotyledon water contents of embryos harvested from Wakehurst Place, UK (Tompsett and Pritchard 1993) and from Ft. Collins, CO, USA (presented here) were similar (after conversion to common units), embryos from Ft. Collins were significantly smaller. This may be explained by the shorter growing season (by about 25 days) and presumably drier conditions in Ft. Collins. Except that moisture loss prior to shedding was not as extensive as what is observed in many orthodox seeds (see Welbaum and Bradford 1988 for exceptional behaviour in an orthodox seed), the patterns of dry matter accumulation and water loss were similar for *A. hippocastanum* and orthodox seeds.

The relationship between water content and water potential was determined from water potential isotherms drawn for embryonic axes throughout development (Fig. 2). Our data for mature axes are similar to those presented earlier for *A. hippocastanum* (Tompsett and Pritchard 1993). Like developing embryos from other species, water potential increased with maturity for embryos at the same water content. The osmotic potential of axes at harvest, calculated from isotherms and Eqs 1 and 2 (Table 1, 2nd row from bottom), decreased with maturity, perhaps because small molecular mass substances were replaced by larger molecules during dry matter accumulation.

Desiccation damage was suggested by an increase in leakage or a decline in germination when axes were dried below a critical moisture content (Fig. 3, inset).

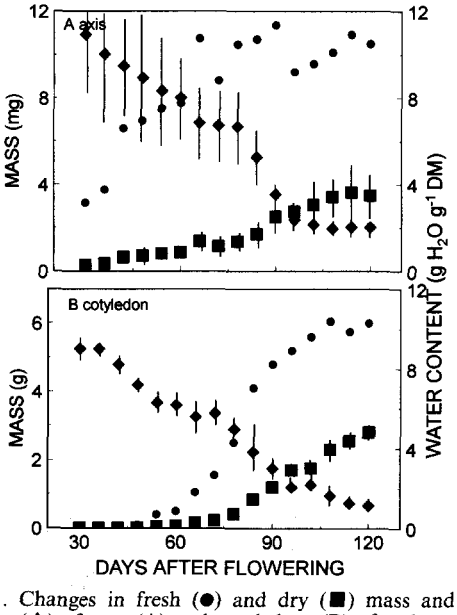


Fig. 1. Changes in fresh (●) and dry (■) mass and water content (◆) of axes (A) and cotyledons (B) of embryos of *A. hippocastanum* from the completion of histodifferentiation (30 DAF) until newly shed (120 DAF). Data points represent averages of 4 harvest years and error bars are the average standard deviation among all years.

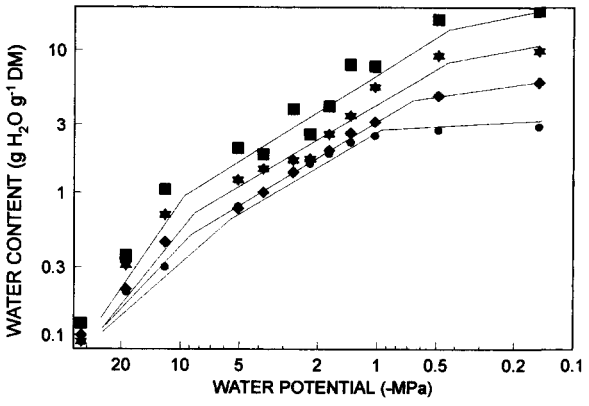


Fig. 2. Water potential isotherms illustrating the relationship between water potential and water content of embryonic axes of *A. hippocastanum* at 30 (■), 60 (✱), 90 (◆) and 120 (●) DAF. Isotherms at other harvest dates show the same trends as those presented.

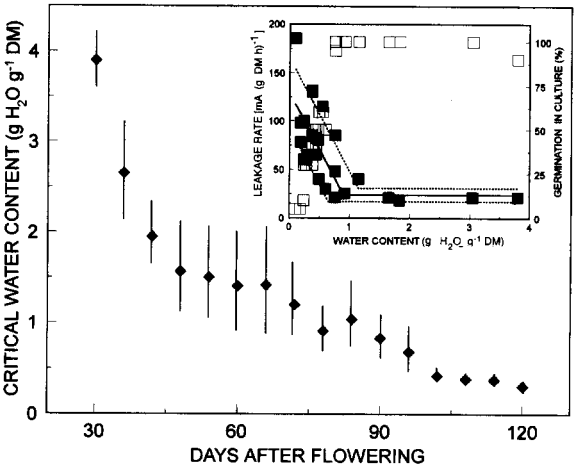


Fig. 3. Assessment of desiccation tolerance of axes of *A. hippocastanum* at different developmental stages. The inset shows the change in leakage rates (■) and germination (shoot production) in culture (□) when axes are dried to different water contents. Data presented in the inset are for 78 DAF but are representative of data collected for all other stages, except 30 DAF where growth of control axes in culture was not achieved. The solid and dashed lines in the inset represent the regression lines and standard error of the estimate, respectively, of the water content vs leakage rate relationship. In the main figure, diamonds (◆) represent the intersection points of the solid lines as drawn in the inset and the ranges are delimited by the intersection points of the dashed lines as drawn in the inset.

There was good correspondence between the critical moisture contents estimated by leakage and growth assays, and critical moisture content ranges were calculated as the moisture contents at which significant changes were observed in either assay. Axes from the most immature stage studied were very sensitive to drying, being damaged at moisture contents below 4.0 g g^{-1} (Fig. 3). The critical water content of axes decreased with development, but even the most mature embryonic axes were damaged at water contents less than 0.3 g g^{-1} (Fig. 3), a moisture level that is high in comparison to orthodox seeds which can usually survive the immediate effects of complete water removal.

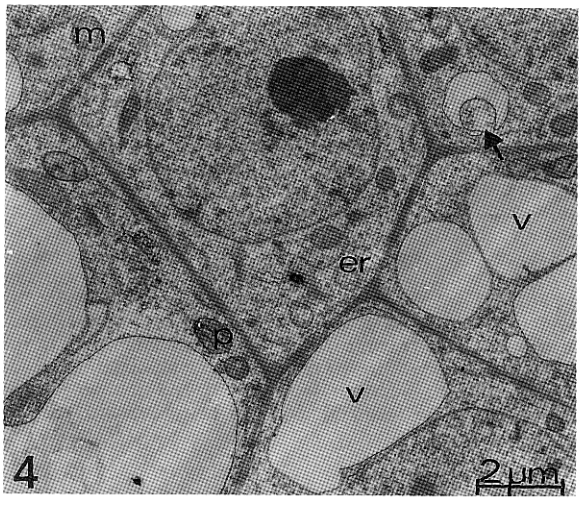
The ultrastructural organisation of hypocotyl tissue of axes harvested at 36 DAF was typical of reserve tissue which had started the growth phase. Cells were vacuolated and there was evidence of new vacuole formation (arrowed, Fig. 4). Plastids had some internal

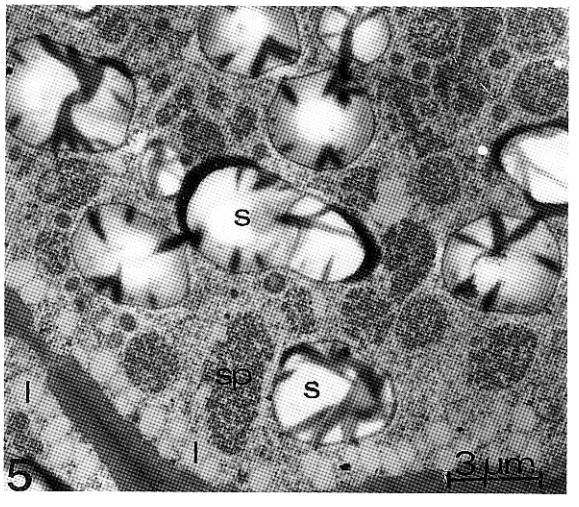
Table 1. Types and quantities of soluble carbohydrates in axes at various stages of development. Total amount of sugar was determined using HPLC analyses of 3 replicate samples and values in parentheses indicate the standard deviation of the mean. Molar quantities were calculated assuming a M_r for unknown sugars of 340. The contribution of sugars to water potential was calculated using Eqs 1 and 2 and isotherms similar to those in Fig. 2.

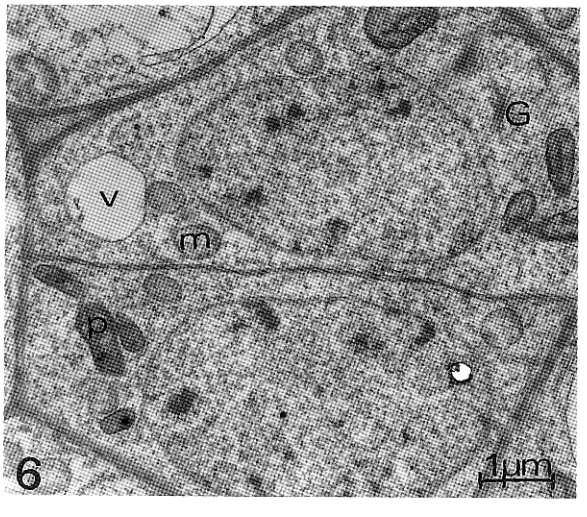
Parameter	Maturity stage (DAF)			
	48	60	96	114
	Soluble carbohydrates content (mg mg^{-1} DM)			
Detected by colorimetric assays				
Total	0.14	0.12	0.13	0.14
Reducing sugars	0.052	0.049	0.003	0.002
Detected by HPLC analysis				
Arabinose	0	0	0.006	0.024
Sucrose	0.082	0.104	0.113	0.116
Glucose	0.034	0.026	0.004	0
myo-Inositol	0.061	0.033	0.009	0
Unknowns	0.117	0.095	0.038	0.026
Total	0.294 (0.044)	0.258 (0.029)	0.170 (0.028)	0.166 (0.010)
	Contribution to water potential			
Sugar concentration (mmol g^{-1} DM)	1.11	0.91	0.53	0.49
Substances contributing to water potential (mmol g^{-1} DM)	2.88	2.48	2.21	1.63
Contribution of soluble carbohydrates to water potential (%)	38.5	36.7	23.9	29.8

membranes. While there was some evidence of endoplasmic reticulum and Golgi bodies, marked endomembrane development normally associated with storage protein synthesis had not yet commenced (Fig. 4). As axes developed, reserves accumulated in the hypocotyl tissue. Vacuoles became progressively filled with electron-dense material. As this process was accompanied by extensive endomembrane development, this material was assumed to be storage protein. Starch accumulated in plastids and by 120 DAF, the hypocotyl tissue was characterised by numerous small protein bodies, large starch deposits and peripheral lipid droplets (Fig. 5).

Radicle meristem cells from axes harvested 36 DAF were typical of hydrated, relatively active tissues. Mitochondria had well developed cristae and, like the hypocotyl tissue of the same stage, plastids contained some internal membranes. The endomembrane system was not well developed (Fig. 6). Meristematic cells remained metabolically active throughout embryo maturation and there was considerable development of the







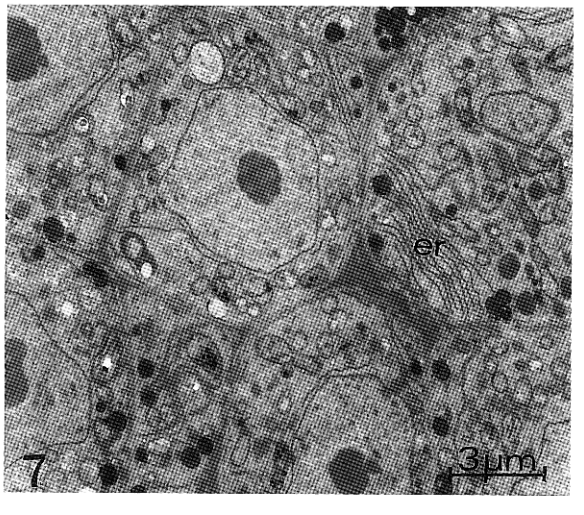


Fig. 4. Subcellular organisation of hypocotyl tissue of axes of *A. hippocastanum* at 36 DAF is typical of embryo tissues which have just completed histodifferentiation and initiated cell growth by vacuolation. er, Endoplasmic reticulum; p, plastid; v, vacuole; m, mitochondron. × 6000.

Fig. 5. Subcellular organisation of hypocotyl tissue of mature, newly shed axes (120 DAF) of *A. hippocastanum*. Considerable amounts of reserves have accumulated in the tissue. Storage proteins (sp) have accumulated in the vacuoles, large starch (s) deposits are evident and lipid bodies (l) are evident at the cell periphery. × 4500.

Fig. 6. Subcellular organisation of radicle meristem cells of axes of *A. hippocastanum* at 36 DAF. The organisation is typical of metabolically active tissue. There are few vacuoles (v) and little endomembrane development. G, Golgi; m, mitochondrion; p, plastid. × 10250.

Fig. 7. Subcellular organisation of radicle meristem cells of mature, newly shed axes (120 DAF) of *A. hippocastanum*. Endoplasmic reticulum profiles (er) are prevalent. × 4600.

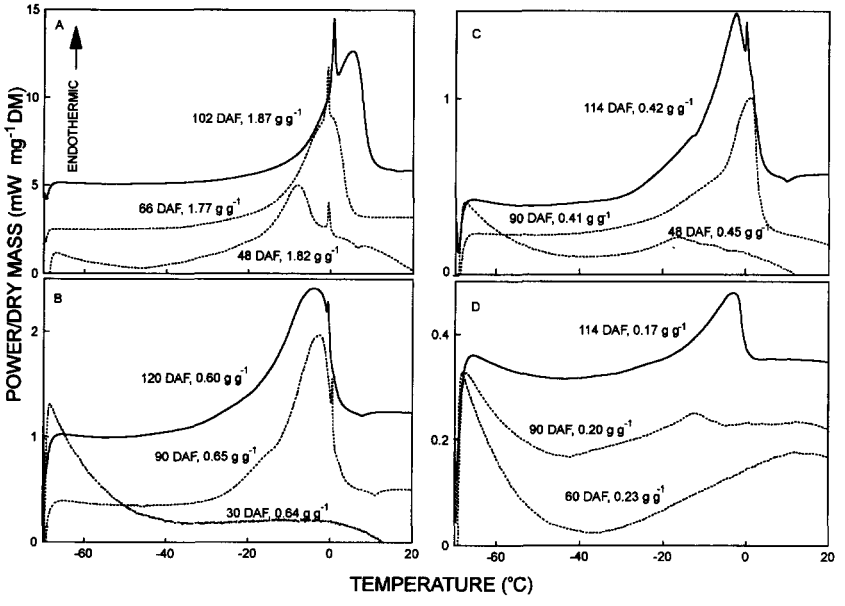


Fig. 8. Representative thermograms of axes from seeds of *A. hippocastanum*. Each panel shows power curves of axes at three different developmental stages (young, intermediate and mature) but at different water contents. A, 1.8 g g^{-1}; B, 0.6 g g^{-1}; C, 0.4 g g^{-1} and D, 0.2 g g^{-1}. Axis size varied with age and so the curves have been corrected by axis dry mass. Peak size is thus a function of the water content and maturity stage.

endomembrane system. Figure 7 shows the subcellular detail typical of radicle meristem cells from newly shed axes (120 DAF). Endoplasmic reticulum profiles were conspicuous and there were numerous mitochondria. Many of the plastids had accumulated darkly staining material. This degree of subcellular organisation suggested ongoing metabolic activity in the radicle meristem tissues.

Three sugars, viz. arabinose, glucose and sucrose, and *myo*-inositol were identified in axes using HPLC separation of soluble carbohydrates (Table 1). In addition, three peaks in HPLC traces (labelled unidentified in Table 1) had retention times that did not correspond with any of the standards used in the study (see Materials and methods). The Somogyi-Nelson colorimetric assay gave slightly higher estimates of reducing sugars (between 0.02 and 0.002 mg mg^{-1} dry mass) than the HPLC measurement which detected only glucose. Total sugar content, quantified using the orcinol/sulfuric acid assay, was considerably less than the HPLC measurement, an expected result if extracts contain sugar alcohols. The unknown carbohydrates, which contribute 40 and 16% of the total carbohydrates in axes 48 and 114 DAF, respectively, may be sugar alcohols, which are fairly common in seeds (Horbowicz and Obendorf 1994), but poorly detected by the orcinol/sulfuric acid method (see Materials and methods).

Qualitative and quantitative changes in sugar amounts were noted during embryo maturation (Table 1). Arabinose and sucrose increased in axes as they matured, the amounts of sucrose being considerably higher than those of arabinose. The amounts of glucose, *myo*-inositol, and the unidentified carbohydrates declined with developmental stage, as did the total level of soluble carbohydrates. This trend of decreasing concentrations of reducing sugar and sugar alcohols during maturation is common among embryos of different species (Horbowicz and Obendorf 1994). The contribution of soluble carbohydrates to the total osmolarity also declined with maturity and this trend has been observed previously in maturing oak embryos (Grange and Finch-Savage 1992). The total soluble carbohydrates constituted about 37 and 27% of the osmotically active components of the dry mass in immature and mature axes (Table 1, last row).

Changes in the freezing and melting behaviour of water in embryonic axes of *A. hippocastanum* were studied using DSC. Figure 8 is a composite of representative warming scans. Based on qualitative characteristics of the thermocurves, the melting behaviour of water can be distinguished into three categories: (1) water that melts as a sharp peak at 0°C, (2) water that melts as a broad peak, the onset temperature decreasing with decreasing water content, and (3) water in which no melting transition is observed. The relative proportion of water exhibiting these behaviours varied with water content and maturity status (Fig. 8).

The physiological significance of the sharp peak is unknown, although it is more prevalent in immature axes (Berjak et al. 1993, Vertucci et al. 1994a,b) and has been linked with freezing injury (Wesley-Smith et al. 1992). The ratio of water in the sharp peak relative to the broad peak in *A. hippocastanum* was comparable to what has been reported previously for both recalcitrant and orthodox seeds (Pammenter et al. 1991, Berjak et al. 1993, Vertucci et al. 1994a,b). Both the sharp and broad peaks diminished in size with decreasing water content [Fig. 8 (note scale changes on ordinates)]. The water content at which no melting transition was observed decreased as seed maturation progressed [no

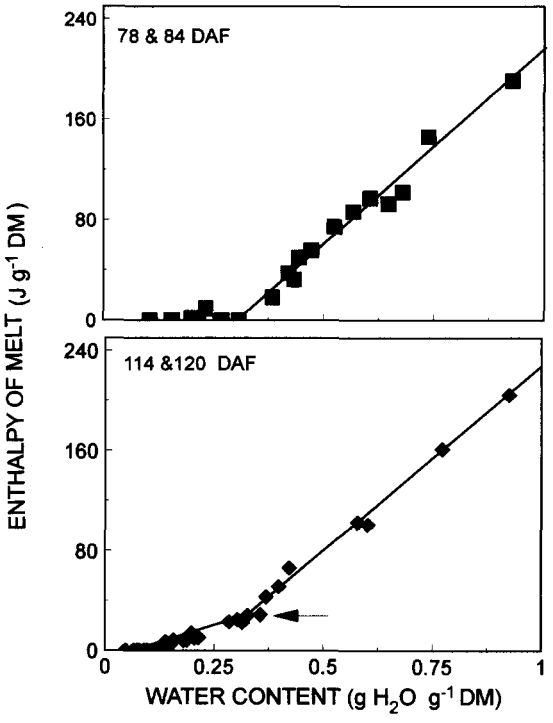


Fig. 9. Relationship between the enthalpy of water melting and water content in axes of *A. hippocastanum*, flash-dried to different water contents. Enthalpy values are calculated by integrating peaks similar to those presented in Fig. 8. Data are combined for 78 and 84 DAF in the upper panel and for 114 and 120 DAF in the lower panel. The ΔH vs water content data given here are representative of data collected for other stages. Note the break in the enthalpy vs water content line (indicated by arrow) of the more mature axes.

transition observed in 30-day axes at 0.64 g g^{-1} (Fig. 8B) while transitions were observed in more mature axes at lower water contents (Fig. 8C,D)].

The enthalpy of the water melting transition (J g^{-1} H_2O) and the moisture content of unfrozen water can be calculated from the slope and x-intercept of the regression line between ΔH (J g^{-1} dry mass) and water content. This treatment revealed a fourth category of water melting behaviour, observed only in axes harvested later than 90 DAF, as a break in the regression curve at water contents of about 0.35 g g^{-1} (Fig. 9). Regression coefficients were greater than 0.97 and 0.93 for lines drawn above and below the break, respectively (an exception to this was a regression coefficient of 0.84 for the regression line below the break point for seeds at 96 DAF). Pure water has a melting transition of 333 J g^{-1} H_2O. The enthalpy of the melt at water contents above the break ranged from 334 for the most immature stages (30–54 DAF combined) to 281 J g^{-1} H_2O for axes at 120 DAF. At water contents below the break (96–120 DAFs only), the melting enthalpy averaged 126 J g^{-1} H_2O. The moisture content of the break (calculated from the intersection of the two regression lines) decreased slightly with increasing maturity from 0.38 g g^{-1} for axes at 96 DAF to 0.30 g g^{-1} for mature axes (Table 2). The water content at which water remained unfrozen (calculated from the x-intercept of the regression lines) decreased with increasing maturity from about 0.36 g g^{-1} for axes at 30–54 DAF to 0.10 g g^{-1} for mature axes (Table 2). The trend of decreasing unfrozen water with maturation has also been observed in orthodox bean seeds and recalcitrant *Zizania palustris* embryos (Vertucci et al. 1994a,b).

The onset temperature of the main transition peak varied with the water content within a maturity class (Fig. 10) and for a given water content among maturity classes (Fig. 10, inset). Water from the less mature embryos generally melted at lower temperatures and this is consistent with a higher osmotic activity in the less mature samples (Table 1).

The water potential of axes (Fig. 11) corresponding to the average water content at harvest (Fig. 1), the critical water content (Fig. 3) and the point at which melting transitions were not observed (Table 2) were determined from isotherms (data similar to those presented in Fig. 2). Water potential determinations fluctuated from −0.65 to −0.90 MPa in axes harvested 30–90 DAF, but then declined abruptly to about −2.0 MPa in more mature axes (Fig. 11A). The critical Ψ_w for desiccation damage was about −1.9 MPa for very

Table 2. Ratio of water in sharp and broad peaks and the moisture contents at which water with various calorimetric properties are observed. The x-intercepts for lines with slopes of ca 300 and 100 J g^{-1} H_2O represent the water contents at which water becomes 'unfreezable' for axes harvested 30–90 DAF and 96–120 DAF, respectively.

DAF	Ratio of water in sharp and broad peaks	Water content		
		x-Intercept for the line with a slope of 300 J g^{-1}	Intersection between lines with slopes of 100 and 300 J g^{-1}	x-Intercept for the line with a slope of 100 J g^{-1}
30–54	0.032	0.41	None	None
60–84	0.030	0.34	None	None
90	0.028	0.23	None	None
96	0.028		0.38	0.13
102	0.028		0.33	0.12
108	0.028		0.33	0.12
114	0.027		0.35	0.11
120	0.026		0.30	0.10

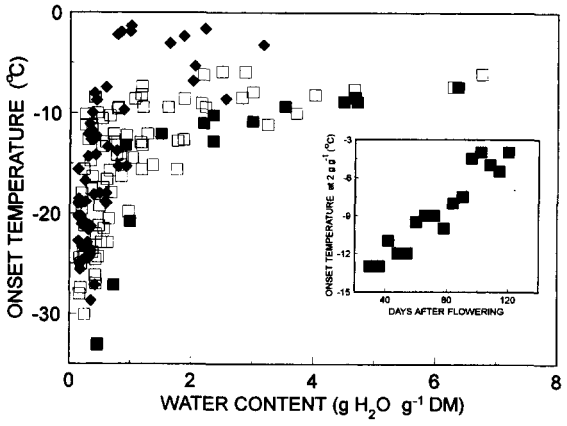


Fig. 10. Onset temperature of the broad peak in the melting transitions of axes of *A. hippocastanum* at different stages of development: (■) immature axes (30–54 DAF); (□) axes of intermediate developmental status (stages 60–90); (◆) most mature axes (96–120 DAF). The inset shows onset temperature of axes at different stages of development, which had been dried to a moisture content of 2.0 g g^{-1}.

immature axes (< 40 DAF), but then decreased to about −4 MPa and remained constant through 90 DAF. The critical Ψ_w decreased further to about −12 MPa at about 102 DAF (Fig. 11B). Water melting transitions were observed in immature seeds (< 96 DAF) at $\Psi_w > -19$ MPa and in more mature seeds (≥ 96 DAF) at $\Psi_w < -38$ MPa (Fig. 11C). At 96 DAF, the Ψ_w at which melting transitions with $\Delta H \approx 300$ J g^{-1} H_2O were no longer observed increased from −19 to −12 MPa (Fig. 11C), and this was coincident with the appearance of water melting with $\Delta H \approx 126$ J g^{-1} H_2O (Table 2).

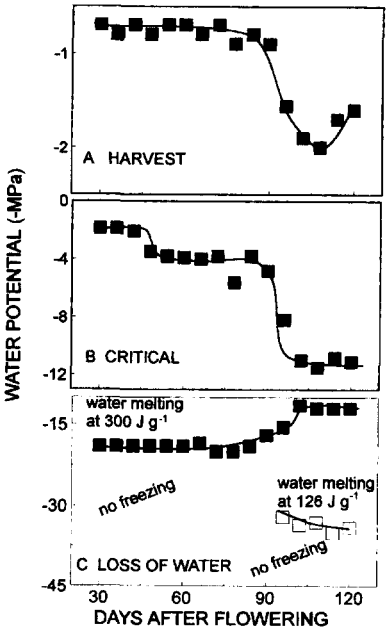


Fig. 11. Water potentials of embryonic axes of *A. hippocastanum* at harvest (A), at which damage by desiccation was observed (B), and where water melting transitions at the enthalpy indicated were not observed (C) as a function of embryological development. Water potentials were determined from the average water contents given in Figs 1 and 3 (A and B, respectively) and Table 2 (C) and from isotherms similar to those in Fig. 2.

Discussion

Newly shed embryos of *A. hippocastanum* have a high moisture content and are killed at relatively high water contents and water potentials (Table 1, Fig. 10). In this regard the seeds of this species conform to the general definition of recalcitrance. Ultrastructural studies reported here are also consistent with those reported for other recalcitrant seeds, in that axis cells of *A. hippocastanum*, particularly in the radicle meristem, had a subcellular organisation typical of hydrated, metabolically active tissue (Berjak et al. 1989, 1993, Farrant et al. 1992, 1997). A reduction in respiratory activity occurs in orthodox seeds just after dry matter accumulation is completed but prior to a severe reduction in water potential (Fischer et al. 1988, Farrant et al. 1997). This reduction in metabolic activity was not observed in *A. hippocastanum*, possibly because the post-abscission processes that mark physiological maturity (i.e. Galau et al. 1991, Demir and Ellis 1992) do not occur in this species. It is not possible to discern from the studies presented here whether high metabolic activity is a cause (no switch to shut off metabolism) or an effect (high water contents lead to high levels of metabolic activity) of recalcitrance (Finch-Savage 1992; reviewed by Leprince et al. 1993, Vertucci and Farrant 1995).

The ultrastructural and physiological changes observed during the early stages of development of *A. hippocastanum* embryos are typical of development of most seeds. Water relations during development appear to be typical of seed development in general (Fischer et al. 1988, Welbaum and Bradford 1988, Saab and Obendorf 1989; reviewed by Bradford 1994). Major differences between *A. hippocastanum* and orthodox seed behaviour appear to be manifested during the very last stages of development.

As with most seeds, embryos of *A. hippocastanum* became increasingly tolerant of drying with development (reviewed by Kermode 1990, 1995, Galau et al. 1991, Leprince et al. 1993, Vertucci and Farrant 1995). When desiccation tolerance was evaluated from critical water content measurements (Fig. 3), the acquisition of tolerance appeared to be a rather continuous process. A similar observation was made by Sun and Leopold (1993) using an orthodox seed, and they concluded that desiccation tolerance was quantitative. However, when evaluated on the basis of Ψ_w (Fig. 11B), the change in desiccation tolerance occurred in three distinct phases:

the critical Ψ_w decreased between 36 and 48 DAF from -1.8 to -4 MPa and between 90 and 102 DAF from -4 to -12 MPa. These discrete changes in desiccation tolerance suggest that tolerance is acquired via thresholds.

The abrupt changes in desiccation tolerance, as measured by critical Ψ_ws, allow us to define critical stages in *A. hippocastanum* development, and to determine corresponding ultrastructural, biochemical and biophysical features. During the period between 48 and 90 DAF, embryos maintain tolerance to $\Psi_w \approx -4$ MPa (Fig. 11B). This period is marked by rapid dry matter accumulation into vacuoles and plastids (Figs 1A, 4, 5). The constant Ψ_w, in spite of reduced vacuolar volume, suggests that there is not a quantitative relationship between vacuole size and desiccation tolerance (reviewed by Bewley 1979, Kermode 1990, Vertucci and Farrant 1995). However, the abrupt decrease in Ψ_w at 96 DAF may indicate a maximum allowable vacuole space for tolerance at -12 MPa.

The developmental stage which marks an abrupt change in tolerance from $\Psi_w \approx -4$ to -12 MPa coincides with a shift in sugar composition (Table 1). Total sugar concentration in axes harvested after 96 DAF declined to $\leq 65\%$ of levels found in less mature embryos. Associated with this change is a marked decrease in reducing sugars, *myo*-inositol and three unknown carbohydrates. Low levels of reducing sugars have been correlated with desiccation tolerance (Tetteroo et al. 1994; reviewed by Leprince et al. 1993) and may be effective in the increase of desiccation tolerance observed in *A. hippocastanum* embryos. In spite of the complete removal of glucose, embryos harvested after 96 DAF did not achieve the level of desiccation tolerance observed in orthodox seeds. Thus, complete removal of reducing sugars may be important for achieving a certain level of desiccation tolerance but is not sufficient to achieve complete tolerance.

Sucrose has been suggested as an important protectant of macromolecular structure when water is removed (reviewed by Hoekstra et al. 1989, Crowe et al. 1992, Leopold et al. 1994). Sucrose content increases slightly with development of *A. hippocastanum* embryos (Table 1), but the amounts at all stages of development are not significantly different from those reported for desiccation-tolerant organisms (reviewed by Vertucci and Farrant 1995). Sucrose in combination with oligosaccharides has also been suggested as important in desiccation tolerance of seeds (reviewed by Horbowicz and Obendorf 1994, Leopold et al. 1994). We could not detect oligosaccharides in axes of *A. hippocastanum* at any stage of development and perhaps this deficit is responsible for the incomplete desiccation tolerance of the species. While raffinose may be a limiting factor to achieving full desiccation tolerance in *A. hippocastanum* embryos, it is present in appropriate concentrations in other recalcitrant species (reviewed by Berjak et al. 1989, Still et al. 1994, Vertucci and Farrant 1995), indicating that it is not always limiting.

Also associated with the abrupt change in the desiccation tolerance to about -12 MPa (after 96 DAF) is the detection of water that melts with enthalpies of 126 J g^{-1} H_2O (Table 2, Figs 9 and 11C). This unusually low enthalpy of melting transitions for water is indicative of the formation of an aqueous state that is sufficiently viscous to alter the freezing properties of water but not sufficiently viscous to prevent the freezing altogether. Water that melts with low enthalpies has been detected in mature orthodox tissues (Vertucci 1990, Vertucci et al. 1994b) and also in recalcitrant embryos from temperate species [*Z. palustris* (Vertucci et al. 1994a) and *A. hippocastanum* (presented here)], but not in recalcitrant embryos from tropical areas (Pammenter et al. 1991, Berjak et al. 1993). As with recalcitrant *Z. palustris* embryos, the water content limit at which water melting transitions were observed was less in *A. hippocastanum* embryos (ca 0.12 g g^{-1}) in comparison to orthodox tissues (ca 0.24 g g^{-1}), indicating that at water contents between 0.1 and 0.3 g g^{-1}, water mobility is more restricted in orthodox embryos compared to recalcitrant ones. This finding is consistent with the hypothesis introduced by Burke (1986) and forwarded by Leopold et al. (1994) that the formation of an aqueous glass (amorphous state with extreme viscosity) is associated with protection during desiccation, if the idea of glass formation is extended to include formation of other, less viscous states as well. From this discussion, one may suggest that the intracellular component(s) responsible for viscous states is(are) lacking in immature embryos, and accumulate as embryos mature to various degrees depending on the level of recalcitrance.

The formation of viscous amorphous states (i.e. syrups, rubbers and glasses) is often attributed to sugar composition with sucrose being the most important component because it readily forms glasses (e.g. Slade and Levine 1991). Oligosaccharides (which do not form glasses) are also considered important because they inhibit sucrose crystallisation at water contents near the glass transition (reviewed by Leopold et al. 1994). Neither sucrose nor raffinose are likely to be responsible for the semi-viscous state that we detected via low melting enthalpies in more mature embryos (Fig. 9B, Table 2) since the amount of sucrose did not change during maturation and raffinose was absent altogether (Table 1). Alternatively, we have suggested that LEA-like proteins, in combination with sugars, may be important in the formation of viscous states observed in orthodox seeds (Walters et al. 1997). These proteins are present in mature *A. hippocastanum* (Finch-Savage et al. 1994, Farrant et al. 1996) and in *Z. palustris* (Still et al. 1994), but a correlation between time of expression and water behaviour is not possible here as the developmental stage at which these proteins arise is not known.

Conclusions

There are a number of challenges to overcome if an organism is to be totally tolerant of desiccation. Mature, orthodox seeds meet these challenges during development while recalcitrant seeds do not acquire a complete set of tolerance mechanisms. Because we assume that there are many components required to achieve full tolerance, studies of tolerance mechanisms are necessarily studies of limiting factors. Limiting factors may be dependent on species or growing conditions and efforts to determine them are confounded by (1) the lack of clear quantitation of desiccation tolerances or damage, (2) an inability to distinguish between developmental processes that lead to desiccation tolerance and those that are purely a consequence of embryogenesis, and (3) the possibility of a false negative relationship when cells acquire some mechanisms, but not sufficient ones to tolerate the initial insults of desiccation. We have monitored changes in numerous characteristics during the development of embryonic axes from *A. hippocastanum* and have identified those that do and do not correspond to changes in the level of desiccation tolerance. However, we cannot completely eliminate the above confounding factors from our study.

We observed discrete changes in desiccation tolerance in *A. hippocastanum*. Embryonic axes from this species ultimately achieve tolerance to -12 MPa, demonstrating that they acquire some potent protectants. These coincide with elements of dry matter accumulation (perhaps a minimum vacuolar volume and depletion of reducing sugars) and the appearance of a semi-viscous state. *A. hippocastanum* axes do not attain the extreme level of tolerance that orthodox seeds acquire during the final stages of embryogenesis. The amount (mg mg^{-1} dry mass) of sucrose, a putative protectant of desiccation, was not substantially different from the amount found in orthodox seeds, suggesting that sucrose content is not the limiting factor in achieving profound desiccation tolerance. Unlike many orthodox seeds, *A. hippocastanum* axes did not accumulate oligosaccharides, completely fill vacuoles, experience a decrease in metabolic activity or achieve a sufficiently viscous state to prevent water freezing transitions at water contents less than 0.2 g g^{-1}. These features, which may be interrelated, are perhaps the basis for recalcitrance in this species.

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References

Berjak, P., Farrant, J. M. and Pammenter, N. W. 1989. The basis of recalcitrant seed behaviour: Cell biology of the homoiohydrous seed condition. – *In* Recent Advances in the Development and Germination of Seeds (R. B. Taylorson, ed.), pp. 89–108. Plenum Press, New York, NY. ISBN 0-306-43521-7.

Berjak, P., Vertucci, C. W. and Pammenter, N. W. 1993. Desiccation-sensitive (recalcitrant) seeds: Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in Camellia sinensis. – Seed Sci. Res. 3: 155–166.

Berjak, P., Bradford, K. J., Kovach, D. A. and Pammenter, N. W. 1994. Differential effects of temperature on ultrastructural responses to dehydration in seeds of *Zizania palustris*. – Seed Sci. Res. 4: 111–122.

Bewley, J. D. 1979. Physiological aspects of desiccation tolerance. – Annu. Rev. Plant Physiol. 30: 195–238.

Bradford, K. J. 1994. Water stress and the water relations of seed development: A critical review. – Crop Sci. 34: 1–10.

Burke, M. J. 1986. The glassy state and survival of anhydrous biological systems. – *In* Membranes, Metabolism and Dry Organisms (A. C. Leopold, ed.), pp. 358–363. Cornell University Press, Ithaca, NY. ISBN 0-8014-1979-4.

Chandler, P. M and Robertson, M. 1994. Gene expression regulated by abscisic acid and its relation to stress tolerance. – Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 113–141.

Chaplin, M. F. and Kennedy, J. F. 1986. Carbohydrate Analysis: A Practical Approach. – IRL Press, Washington, DC. pp. 228. ISBN 0-947946-44-6.

Crowe, J. H., Hoekstra, F. A. and Crowe, L. M. 1992. Anhydrobiosis. – Annu. Rev. Physiol. 54: 579–599.

Demir, I. and Ellis, R. H. 1992. Changes in seed quality during seed development and maturation in tomato. – Seed Sci. Res. 2: 81–87.

Drennan, P. M. and Berjak, P. 1982. Degeneration of the salt glands accompanying foliar maturation in *Avicennia marina* (Forsskal) Vierh. – New Phytol. 90: 165–176.

Ellis, R. H., Hong, T. D. and Roberts, E. H. 1990. An intermediate category of seed storage behaviour. I. Coffee. – J. Exp. Bot. 41: 1167–1174.

Ellis, R. H., Hong, T. D. and Roberts, E. H. 1991. Effect of storage temperature and moisture on the germination of papaya seeds. – Seed Sci. Res. 1: 69–72.

Farrant, J. M., Pammenter, N. W. and Berjak, P. 1992. Development of the recalcitrant (homoiohydrous) seeds of *Avicennia marina*: Ultrastructural and biochemical events associated with development from histodifferentiation to maturation. – Ann. Bot. 70: 75–86.

Farrant, J. M., Pammenter, N. W. and Berjak, P. 1993. Seed development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. – Seed Sci. Res. 3: 1–13.

Farrant, J. M., Pammenter, N. W., Berjak, P., Farnsworth, E. J. and Vertucci, C. W. 1996. Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may be related to habitat. – Seed Sci. Res. 6: 175–182.

Farrant, J. M., Pammenter, N. W., Berjak, P. and Walters, C. 1997. Subcellular organisation and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. – Seed Sci. Res. 7: 135–144.

Finch-Savage, W. E. 1992. Seed development in the recalcitrant species *Quercus robur* L.: Germinability and desiccation tolerance. – Seed Sci. Res. 2: 17–22.

Finch-Savage, W. E., Pramanik, S. K. and Bewley, J. D. 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. – Planta 193: 478–485.

Fischer, W., Bergfeld, R., Plachy, C., Schäfer, R. and Schopfer, P. 1988. Accumulation of storage materials, precocious germination and development of desiccation tolerance during seed maturation in mustard (*Sinapis alba* L.). – Bot. Acta 101: 344–354.

Galau, G. A, Jakobsen, K. S. and Hughes, D. W. 1991. The controls of late dicot embryogenesis and early germination. – Physiol. Plant. 81: 280–288.
Grange, R. I. and Finch-Savage, W. E. 1992. Embryo water status and development of the recalcitrant species *Quercus robur* L.: Determination of water relation parameters and pressure-volume analysis. – J. Exp. Bot. 43: 657–662.
Hoekstra, F. A., Crowe, J. H. and Crowe, L. M. 1989. Membrane behaviour in drought and its physiological significance. – *In* Recent Advances in the Development and Germination of Seeds (R. B. Taylorson, ed.), pp. 71–88. Plenum Press, New York, NY. ISBN 0-306-43521-7.
Horbowicz, M. and Obendorf, R. L. 1994. Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols – review and survey. – Seed Sci. Res. 4: 385–406.
Kermode, A. R. 1990. Regulatory mechanisms involved in the transition from seed development to germination. – CRC Crit. Rev. Plant Sci. 9: 155–195.
Kermode, A. R. 1995. Regulatory mechanisms in the transition from seed development to germination: Interactions between the embryo and the seed environment. – *In* Seed Development and Germination (J. Kigel and G. Galili, eds), pp. 273–331. Marcel Dekker, New York, NY. ISBN 0-8247-9229-7.
Kuo, T. M., van Middlesworth, J. F. and Wolf, W. J. 1988. Content of raffinose oligosaccharides and sucrose in various plant seeds. – J. Agric. Food Chem. 36: 32–36.
Leopold, A. C., Sun, W. Q. and Bernal-Lugo, I. 1994. The glassy state in seeds: Analysis and function. – Seed Sci. Res. 4: 267–274.
Leprince, O., Hendry, G. A. F. and McKersie, B. D. 1993. The mechanisms of desiccation tolerance in developing seeds. – Seed Sci. Res. 3: 231–246.
Leprince, O., Vertucci, C. W., Hendry, G. A. F. and Atherton, N. M. 1995. The expression of desiccation-induced damage in orthodox seeds is a function of oxygen and temperature. – Physiol. Plant. 94: 233–240.
Michel, B. E. and Kaufmann, M. R. 1973. The osmotic potential of polyethylene glycol 6000. – Plant Physiol. 51: 914–916.
Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. – Physiol. Plant. 15: 473–497.
Pammenter, N. W., Vertucci, C. W. and Berjak, P. 1991. Homeohydrous (recalcitrant) seeds: Dehydration, the state of water and viability characteristics in *Landophia kirkii*. – Plant Physiol. 96: 1093–1098.
Pammenter, N. W., Vertucci, C. W. and Berjak, P. 1993. Responses to dehydration in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. – *In* Basic and Applied Aspects of Seed Biology (D. Côme and F. Corbineau, eds), pp. 867–872. Fourth International Workshop on Seeds, ASFIS, Paris. ISBN 2-9507351-4-2.
Pence, V. C. 1992. Desiccation and the survival of *Aesculus*, *Castanea*, and *Quercus* embryo axes through cryopreservation. – Cryobiology 29: 391–399.
Pritchard, H. W. 1991. Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. – Ann. Bot. 67: 43–49.
Roberts, E. H. 1973. Predicting the storage life of seeds. – Seed Sci. Technol. 1: 499–514.
Robyt, J. F. and White, F. J. 1987. Biochemical Techniques: Theory and Practice. – Brooks Cole, Monterey, CA, pp. 407. ISBN 0-534-07944-X.
Saab, I. N. and Obendorf, R. L. 1989. Soybean seed water relations during in situ and in vitro growth and maturation. – Plant Physiol. 89: 610–616.
Slade, L. and Levine, H. 1991. A food polymer science approach to structure-property relationships in aqueous food systems: Nonequilibrium behavior of carbohydrate-water systems. – *In* Water Relationships in Food (H. Levine and L. Slade, eds), pp. 29–101. Plenum Press, New York, NY. ISBN 0-3034-3936-0.
Still, D. W., Kovach, D. A. and Bradford, K. J. 1994. Development of desiccation tolerance during embryogenesis in rice (*Oryza sativa*) and wild rice (*Zizania palustris*): Dehydrin expression, abscisic acid content, and sucrose accumulation. – Plant Physiol. 104: 431–438.
Sun, W. Q. and Leopold, A. C. 1993. Acquisition of desiccation tolerance in soybeans. – Physiol. Plant. 87: 403–409.
Sun, W. Q., Irving, T. C. and Leopold, A. C. 1994. The role of sugar, vitrification and membrane phase transition in seed desiccation tolerance. – Physiol. Plant. 90: 621–628.
Tetteroo, F. A. A., Bomal, C., Hoekstra, F. and Karssen, C. M. 1994. Effect of abscisic acid and slow drying on soluble carbohydrate content in developing embryoids of carrot (*Daucus carota* L.) and alfalfa (*Medicago sativa* L.). – Seed Sci. Res. 4: 203–210.
Tompsett, P. B. and Pritchard, H. W. 1993. Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. – Ann. Bot. 71: 107–116.
Vertucci, C. W. 1990. Calorimetric studies of the state of water in seed tissues. – Biophys. J. 58: 1463–1471.
Vertucci, C. W. and Farrant, J. M. 1995. Acquisition and loss of desiccation tolerance. – *In* Seed Development and Germination (J. Kigel and G. Galili, eds), pp. 237–271. Marcel Dekker, New York, NY. ISBN 0-8247-9229-7.
Vertucci, C. W., Crane, J., Porter, R. A. and Oelke, F. A. 1994a. Physical properties of water in *Zizania* embryos in relation to maturity status, moisture content and temperature. – Seed Sci. Res. 4: 211–224.
Vertucci, C. W., Farrant, J. M. and Crane, J. 1994b. The status of and requirement for water in developing bean embryos. – Plant Physiol. 105 (Suppl.): 164.
Walters, C., Ried, J. L. and Walker-Simmons, M. K. 1997. Hydration characteristics of the heat soluble protein fraction from mature wheat embryos. – Seed Sci. Res. 7: 125–134.
Welbaum, G. E. and Bradford, K. J. 1988. Water relations of seed development and germination in muskmelon (*Cucumis melo* L.). I. Water relations of seed and fruit development. – Plant Physiol. 86: 406–411.
Wesley-Smith, J., Vertucci, C. W., Berjak, P., Pammenter, N. W. and Crane, J. 1992. Cryopreservation of recalcitrant axes of *Camellia sinensis* in relation to dehydration, freezing rate and thermal properties of water. – J. Plant Physiol. 140: 596–604.

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