AoB PLANTS http://aobplants.oxfordjournals.org/

Open access – Research article

Vacuolar status and water relations in embryonic axes of recalcitrant *Aesculus hippocastanum* seeds during stratification and early germination

Natalie V. Obroucheva*, Snezhana V. Lityagina, Galina V. Novikova and Irina A. Sin'kevich

Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya str. 35, Moscow 127276, Russia

Received: 10 May 2011; **Returned for revision:** 8 August 2011; **Accepted:** 26 March 2012; **Published:** 10 April 2012

Citation details: Obroucheva NV, Lityagina SV, Novikova GV, Sin'kevich IA. 2012. Vacuolar status and water relations in embryonic axes of recalcitrant *Aesculus hippocastanum* seeds during stratification and early germination. *AoB PLANTS* **2012**: pls008; doi:10.1093/aobpla/pls008

Abstract

Backgrounds and aims In tropical recalcitrant seeds, their rapid transition from shedding to germination at high hydration level is of physiological interest but difficult to study because of the time constraint. In recalcitrant horse chestnut seeds produced in central Russia, this transition is much longer and extends through dormancy and dormancy release. This extended time period permits studies of the water relations in embryonic axes during the long recalcitrant period in terms of vacuolar status and water transport.

Methodology Horse chestnut (*Aesculus hippocastanum*) seeds sampled in Moscow were stratified in cold wet sand for 4 months. Vacuole presence and development in embryonic axes were examined by vital staining, light and electron microscopy. Aquaporins and vacuolar H^+-ATPase were identified immunochemically. Water channel operation was tested by water inflow rate. Vacuolar acid invertase was estimated in terms of activity and electrophoretic properties.

Principal results Throughout the long recalcitrant period after seed shedding, cells of embryonic axes maintained active vacuoles and a high water content. Preservation of enzyme machinery in vacuoles was evident from retention of invertase activity, substrate specificity, molecular mass and subunit composition. Plasmalemma and tonoplast aquaporins and the E subunit of vacuolar H^+-ATPase were also present. In non-dormant seeds prior to growth initiation, vacuoles enlarged at first in hypocotyls, and then in radicles, with their biogenesis being similar. Vacuolation was accompanied by increasing invertase activity, leading to sugar accumulation and active osmotic functioning. After growth initiation, vacuole enlargement was favoured by enhanced water inflow through water channels formed by aquaporins.

Conclusions Maintenance of high water content and desiccation sensitivity, as well as preservation of active vacuoles in embryonic axes after shedding, can be considered a specific feature of recalcitrant seeds, overlooked when studying tropical recalcitrants due to the short duration. The retained physiological activity of vacuoles allows them to function rapidly as dormancy is lost and when external conditions permit. Cell vacuolation precedes cell elongation in both hypocotyl and radicle, and provides impetus for rapid germination.

* Corresponding author's e-mail address: obroucheva@ippras.ru; n.obroucheva@mail.ru

Introduction

Modern-day seed biology recognizes two types of seeds, namely orthodox and recalcitrant seeds. These differ in their tolerance to desiccation during maturation. The term 'orthodox seeds' embraces maturing seeds, which tolerate desiccation without damage and remain viable at water contents as low as 8–10 %, on a fresh weight basis. In contrast, recalcitrant seeds are damaged by water loss and die quickly if water levels decline to 40–65 % (Steadman *et al*. 1996). Thus, they are desiccation sensitive (Berjak and Pammenter 2008). In other words, recalcitrant seeds undergo little or no maturation drying and remain desiccation sensitive during maturation and after shedding. Such a large difference in response to dehydration has attracted research interest for at least 30 years (Pammenter and Berjak 1999; Kermode and Finch-Savage 2002). Recently, it was found that desiccation-induced damage was caused by a dehydration-driven burst of reactive oxygen species in embryonic axes accompanied by the ineffective development of antioxidant systems (Pukacka and Ratajczak 2006; Cheng and Song 2008; Roach *et al.* 2008, 2010).

Seeds of tropical and subtropical forest species are typically recalcitrant, and have very short intervals between shedding and germination. This makes the study of recalcitrance difficult. A high water content (40–65 %) was recorded in embryonic axes of 12 such species while the water content in cotyledons was 10 % lower than this (Steadman *et al.* 1996). Water potentials in four seed species were found to be high and within the range −1.5 to −2.5 MPa (Walters 2000). A high water content can indicate the presence of vacuoles; however, cell vacuolation in embryonic axes has not been adequately studied until now. In mature seeds of tropical *Araucaria angustifolia* (woody gymnosperm) and *Landolpha kirkii* (woody dicot), the radicles were found to contain no vacuoles or vacuoles comprising not more than 3 % of cell cross-sectional area, respectively (Farrant *et al*. 1989). In cotyledons of *Scadoxus membranaceus* (tropical grass), vacuoles occupied ∼25 % of cell cross-sectional area. In *Avicenna marina*, a tropical mangrove, small vacuoles were found in radicle meristem (Motete *et al*. 1997) with large vacuoles only in cotyledons (Farrant *et al.* 1997). In no case were vacuoles studied in embryonic axes after seed shedding and the time leading up to germination.

Recalcitrant seeds are distinguished by high water content in their embryonic axes. We aimed at prolonging the period between shedding and germination of recalcitrant seeds in order to characterize their water status. Earlier attempts to prolong mature seed lifespan by long-term storage under conditions preventing water loss have resulted in seed deterioration (Farrant *et al.* 1989; Motete *et al*. 1997). We therefore studied recalcitrant seeds of horse chestnut (*Aesculus hippocastanum)* trees growing in central Russia. These were chosen because the period between shedding and germination is long. Horse chestnut originated in the Balkan mountains and gradually spread over much of Europe after the last ice age. The spread north from Greece to Scotland was accompanied by a reduction in seed size and water content, and faster seed maturation (Daws *et al.* 2004). In horse chestnut growing to the north of southern England, seeds do not germinate after shedding in autumn because of low temperatures. Instead, such seeds enter dormancy and need a short cold stratification to enable germination (Pritchard *et al*. 1999). In contrast, in central Russia, shedding of horse chestnut seeds takes place after almost 5 months of seed development. These seeds are mature and excised embryonic axes can germinate in 3 days under optimum laboratory conditions. Mature seeds possess a thick dense wax coating, a fully differentiated embryonic axis consisting of radicle, hypocotyl and plumule, and large massive cotyledons rich in starch. Intact shed seeds respond to cold winter temperatures by a long deep dormancy and need a cold wet stratification of 16–18 weeks to germinate. Dormancy is due mainly to a long coat-imposed dormancy and can be relieved by cytokinin application (Oboucheva and Antipova 2000; Oboucheva and Lityagina 2007). Weak embryo dormancy lasts for 6–7 weeks and is characterized by a slower growth rate of excised embryonic axes. This is probably the effect of preformed abscisic acid (ABA) and can be relieved by fusicoccin, an activator of plasmalemma proton ATPase (Antipova *et al*. 2003; Oboucheva and Antipova 2004).

Previous investigations of water status in mature horse chestnut seeds are limited to seeds collected in Colorado, USA. These data reveal 65 % water content and −1.9 MPa water potential in embryonic axes (Farrant and Walters 1998; Walters 2000) or 60 % water content in embryonic axes and 55 % water content in cotyledons (Farrant *et al.* 1997). The vacuoles in embryonic axes were found to be almost disappeared by full maturation. In hypocotyls, Farrant and Walters (1998) noted an absence of vacuoles and the presence of protein bodies, but the latter lacked membranes and had amorphous content associated with precipitated saponins. Thus, only weak vacuolation was evident in radicle meristems. In contrast, mature freshly shed seeds collected in Kiev (Ukraine) (Musatenko *et al.* 1997) possess large vacuoles in embryonic axes.

In seeds from central Russia, water content in embryonic axes is maintained at 63–65 % during the entire stratification period and must increase to 73–74 % for germination (Obroucheva and Antipova 2004). The maintenance of water content in tissues at the same high level for a prolonged period makes horse chestnut seeds ideal for the study of recalcitrance in terms of vacuole preservation. An additional simplifying advantage is that growth initiation in embryonic axes of germinating horse chestnut seeds occurs only by cell elongation, with no contribution by cell division (Obroucheva 1999; Obroucheva and Antipova 2003). The hypogeal germination of horse chestnut seeds begins by elongation of hypocotyl cells, which results in radicle protrusion. Cell elongation in radicle cells commences only after radicle protrusion. The embryonic axis grows by cell elongation up to a length of 3 cm. For this reason, the growth initiation in horse chestnut seeds may be considered a model of seed germination as cell elongation is the primary and obligatory germination event (Obroucheva 1999). The vacuolar status of cells in embryonic axes and further cell vacuolation in elongating cells are of primary importance, because the water entering vacuoles produces additional pressure on cell walls, thus favouring elongation.

Vacuolar status in embryonic axes includes the ability to regulate water inflow. To enter a cell and vacuole, water penetrates the membranes by diffusion and by transport through water channels (Tyerman *et al.* 1999; Javot and Maurel 2002; Maurel *et al.* 2008). To clarify these possibilities, we identified plasmalemma and tonoplast aquaporins (i.e. water channel proteins) and evaluated their participation in water inflow. The presence of aquaporins and their activity are known to be closely related to cell elongation in growing plant organs (Obroucheva and Sin'kevich 2010). This study establishes the vacuolar status of recalcitrant seeds in terms of vacuole preservation, enlargement and activity in relation to water inflow in embryonic axes. Vacuolar status is shown to be closely related to the maintenance of high water content during a long stratification time and to cell elongation at early germination.

Materials and methods

Freshly shed horse chestnut (*A. hippocastanum*) seeds were collected from the Main Botanical Garden (Moscow, Russia) in October 2007–2009. They were stratified at 4 °C in wet sand for 4 months to break dormancy. During the course of stratification, seeds were regularly examined for radicle emergence under optimum water supply and temperature (27 °C) in the dark. In this way, we were able to distinguish between deep dormancy, dormancy release and non-dormant state. Initiation of germination, i.e. radicle emergence, was evaluated as the appearance of a 1-mm-long radicle tip. During stratification, excised embryonic axes were fixed for microscopical examination and also stored at −20 °C for later biochemical analysis.

Water content was routinely measured in embryonic axes by weighing before and after drying. Vital staining of vacuoles in longitudinal sections of freshly excised embryonic axes was performed with 0.01 % Neutral Red (Baryckina *et al.* 2004). Sections 60 μm thick were cut with an HM-650V vibratome and examined under an Axio Imager D1 microscope (Carl Zeiss, Oberhofen, Germany). For light microscopy, embryonic axes were fixed according to Birch-Girschfeld with a Zenker fixative modified for vacuole preservation. The fixative consisted of 3 g of $HgCl_2$, 2.5 g of $K_2Cr_2O_7$ and 1 g of Na_2SO_4 per 100 mL of H_2O. Just prior to fixation, 5 mL of 10 % aqueous formaldehyde were added. After 24 h fixation, axes were washed in running water and transferred to I_2+KI solution to remove residual $HgCl_2$. The material was then dehydrated and embedded in paraffin. Longitudinal sections were stained with Procion Bright Blue RS and Procion Bright Red 2BS dyes according to Ivanov and Litinskaya (Ivanov 1987). The size and number of cells in radicles and hypocotyls were measured under a light microscope in the third longitudinal rows of the outer cortex.

To characterize membrane proteins, microsomal fractions from embryonic axes were used. The axes were first excised from intact seeds during stratification and then either immediately frozen or cultivated to radicle emergence in water in Petri dishes in the dark at 27 °C. Frozen axes weighing 15 g (300 embryonic axes from dormant seeds or 150 protruding axes) were homogenized at 4 °C in extraction buffer comprising 300 mM sucrose, 100 mM Tris-HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid, 5 mM potassium meta-bisulphite, 5 mM dithiothreitol (DTT), 5 mM phenylmethylsulphonyl fluoride, 0.6 % polyvinylpyrollidone in the ratio of 1 : 20. The microsomal fraction was sedimented by centrifugation for 1 h at 100 000 *g*, and the pellet resuspended in the same medium. Prior to electrophoresis, microsomal preparations were transferred to two-fold buffer, containing 0.125 M Tris-HCl (pH 6.8), 4 % sodium dodecyl sulphate (SDS), 20 % glycerol, 200 mM DTT and 0.02 % bromophenol blue. Proteins were then separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis with a Midget Electrophoresis Unit 2050 (LKB, Bromma, Sweden) according to Laemmli (1970). Gels were stained with Coomassie R-250. Molecular masses were determined with protein markers (10–225 kDa) from Promega (Madison, WI, USA).

For western blot analysis, electrophoretically separated proteins were transferred onto nitrocellulose membranes (Sigma, St Louis, MO, USA) using a Multiphor Electrophoresis Unit (LKB, Sweden). The membrane was washed three times for 5 min with a buffer containing 10 mM phosphate buffer (pH 7.4), 2.7 mM KCl, 137 mM NaCl, 0.05 % Tween 20 (PBS-T) and blocked with 5 % non-fat dry milk in PBS-T.

To identify plasmalemma (PIP) and tonoplast (TIP) aquaporins as well as vacuolar H^+-ATPase, nitrocellulose membranes were treated with appropriate antibodies. Nitrocellulose blots were treated with anti-PIP1;1, anti-PIP2;2 and anti-TIP2 antibodies kindly provided by Professor C. Maurel (École supérieure agronomique, Montpellier, France). Antibodies against TIP3;1 were produced in our institute, and the antibodies against subunit E of plant vacuolar H^+-ATPase from *Arabidopsis thaliana* were purchased from Antisera (Vannas, Sweden). Protein bands were visualized with secondary antibodies coupled with horseradish peroxidase (Promega).

The open or closed state of water channels in the membranes was assessed by a method previously developed for growing roots (Barrouclough *et al.* 2000; Javot and Maurel 2002). This involves measuring water absorption, its inhibition by mercuric chloride and subsequent restoration by certain reductants such as DTT. Water uptake by control embryonic axes was measured as an increase in fresh weight. Other embryonic axes were weighed and transferred to 0.5 mM mercuric chloride for 30 min, then weighed again to evaluate the inhibition of water inflow by Hg^{2+} ions. The rinsed embryonic axes were then incubated in 10 mM DTT to eliminate the mercury-induced inhibition of water uptake and weighed again.

Vacuolar acid invertase activity was measured after protein extraction with 0.01 M phosphate buffer, pH 6.5, followed by centrifugation and subsequent supernatant dialysis against the same buffer for 24 h. The reaction was carried out in 0.01 M phosphate-citrate buffer, pH 5.5, with the substrates (50 mM sucrose or raffinose) at 30 °C for 40 min. Fructose content was estimated by colour reaction with resorcinol at 520 nm with a Genesys 10uv spectrophotometer (Thermo, Madison, WI, USA). To characterize the molecular properties of acid vacuolar invertase, native electrophoresis in 7 % polyacrylamide gels (Davis 1964) was used with subsequent invertase identification. Protein separation was carried out for 2.5 h at 20 mA, the gel was washed in 0.01 M phosphate-citrate buffer, pH 5.5, for 10 min at 35 °C in darkness and transferred to fresh buffer containing 0.6 M sucrose for an hour at 37 °C in darkness. The invertase reaction was terminated by boiling in 4 % NaOH containing 0.2 % 2,3,5-triphenyltetrazolium chloride. The red colour produced by fructose formed indicated the location of invertase protein within the gel.

Total protein was estimated with a Sigma BCA protein assay. The colour reaction was measured at 620 nm with a Genesys 10uv spectrophotometer (Thermo) with bovine serum albumin as the standard.

Results

Characterization of mature horse chestnut seeds after shedding

Under the conditions of central Russia, mature horse chestnut seeds are shed in October in a dormant state. Cold wet stratification needs to continue for at least 4 months for germination to take place. During stratification, deep dormancy is gradually removed, as can be seen from the acceleration of the rate of radicle emergence and increasing germination percentage when tested under optimum conditions (Fig. 1). During stratification, we distinguished deep dormancy (freshly shed seeds, curve 0 weeks), dormancy release (curves 4 and 12 weeks) and complete loss of dormancy (curve 16 weeks). At the end of stratification, non-dormant seeds were able to germinate within 1–2 days. Throughout stratification, the water content of the embryonic axes remained at 63.5–65.0 % on a fresh weight basis (Fig. 2). Seeds remained recalcitrant, i.e. sensitive to desiccation, and died if embryonic axes were dehydrated to 58 % water content. In imbibing non-dormant seeds, the water content in embryonic axes had increased to 74–75 % at radicle emergence. Such an increase in embryonic axis hydration is necessary for germination to begin as any retardation of this hydration prevents radicle emergence (Oboucheva and Antipova 2000). The preservation of desiccation sensitivity in parallel with the maintenance of a high water content during seed stratification is evidence of a prolonged recalcitrant state.

In mature freshly shed seeds, each embryonic axis consists of a radicle (1–2 mm) and hypocotyl (3–7 mm) below a small plumula. Both cell size and cell number remained unchanged at dormancy with hypocotyl cells somewhat longer than typical meristematic cells making up the radicle (Fig. 3, curve 1). The hypocotyl may be considered a storage organ since it contains many large starch grains. In radicle cells, starch grains are small and scarce.

Vacuoles in embryonic axes of dormant seeds

A high water content found in embryonic axes from shedding to the end of dormancy (Fig. 2) indirectly indicates the presence of vacuolated cells. Striking evidence of vacuole preservation in embryonic axial cells was obtained by vital staining with Neutral Red, which

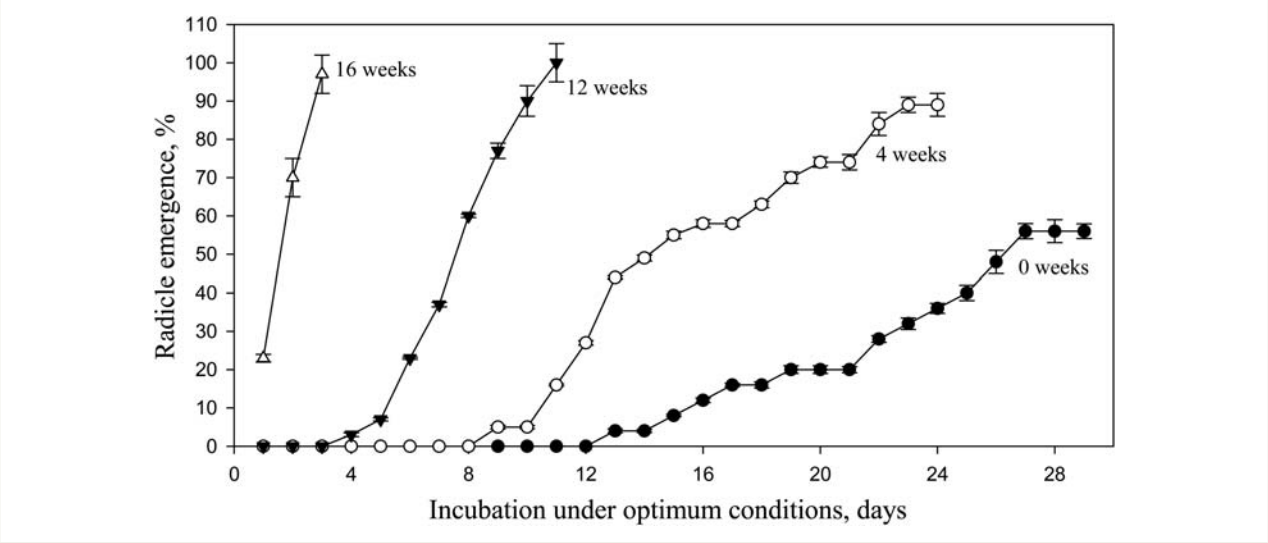

Fig. 1 Dormancy release in imbibing horse chestnut seeds subjected to cold wet stratification. Data represent the germination rate of seeds incubated under optimum conditions after 0, 4, 9, 12 and 16 weeks of stratification. Each sample consisted of 200 seeds. Data are shown as the means and standard errors.

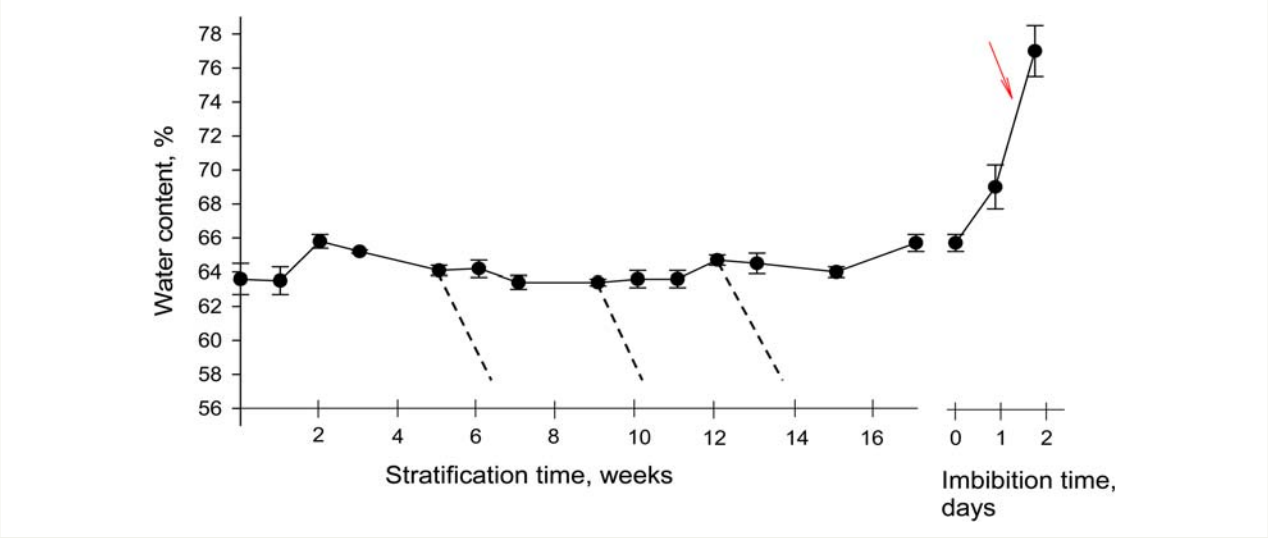

Fig. 2 Water content in embryonic axes of horse chestnut seeds during seed stratification and imbibition. Water content is expressed as % fresh weight. Dashed lines indicate water loss during desiccation. These seeds did not survive after desiccation. The measurements at each date were performed in triplicate, with 10 embryonic axes per sample, and are represented as the means and standard errors.

produces a crimson colour at acidic pHs typical of vacuolar contents (Fig. 4). The cells of the outer cortex in both radicle and hypocotyl contained vacuoles preserved throughout stratification. Colour was absent from cap cells, innermost cortical cells and hypocotyl epidermis, indicating an absence of vacuoles in these tissues. The presence of vacuoles in hypocotyl cells was confirmed by electron microscopy. Figure 5A demonstrates numerous small vacuoles typical of hypocotyl cortical cells.

Vacuoles at growth initiation in embryonic axes of non-dormant seeds

Radicle cells did not elongate at radicle protrusion (Fig. 3, curve 2). Radicle tips are instead pushed out by the

elongating hypocotyl. Radicles retained a meristematic pattern and contained only small vacuoles at this time (Fig. 5B). Radicle protrusion results from cell elongation starting from the upper end of the hypocotyl and gradually spreading downward (Fig. 3, curve 2). For radicle protrusion, a doubling of length in the upper part of the hypocotyl operates as the driving force. These cells were 30 μm long on average with the uppermost cells elongating to a shorter size.

All cells of the outer cortex along the hypocotyl were found to be vacuolated. Even at the base of the hypocotyl (third millimetre of the embryonic axis), cortical non-elongating cells contain vacuoles, which are greater in size (Fig. 5C) than in hypocotyls of non-protruded embryonic axes (Fig. 5A). No small vacuoles were observed. Vacuoles appear to increase in size by fusion and subsequent dilation. This is in line with previous observations in oat root cells of vacuoles forming by the enlargement and fusion of numerous small vacuoles, and the disappearance of the membranes situated between the fusing adjacent vacuoles (Herman *et al.* 1994).

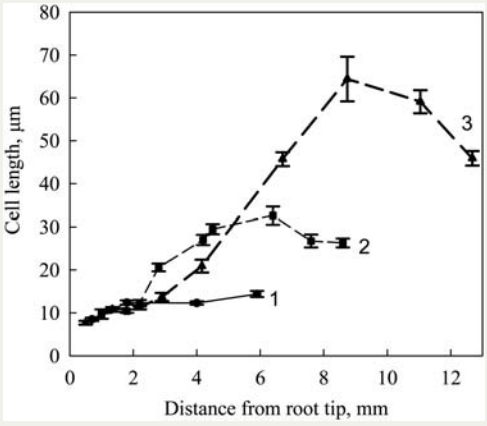

Fig. 3 Length of cortical cells in embryonic axes of horse chestnut seeds as a function of distance from the root tip. Data represent average cell lengths per field of vision measured consecutively along the cortical cell row in three embryonic axes. 1, dormant seed; 2, non-dormant seed at radicle protrusion; 3, seed after radicle protrusion. The embryonic axes were 15 mm long. Data are shown as the means and standard errors.

Vacuoles in growing embryonic axes

Figure 3 (curve 3) shows the cell length increase in 15-mm-long embryonic axes that were continuing to grow immediately after radicle emergence. The radicle still did not start to extend, and its apical cells remain meristematic, with small vacuoles. However, cortical cells in the upper part of the first millimetre of the radicle are more vacuolated. This process is clearly seen in radicle cells of the second millimetre (Fig. 5D). As in the hypocotyl, vacuolation in the radicle precedes the initiation of cell elongation.

Vacuolation in hypocotyl cells continued, with increasing cell hydration (Fig. 2). Cells in the central region elongated more actively than the upper cells while the lowest hypocotyl cells only started to extend. Vacuoles in hypocotyl basal cells (third millimetre of the axis) (Fig. 5E) were similar to those in the third millimetre of the hypocotyl at

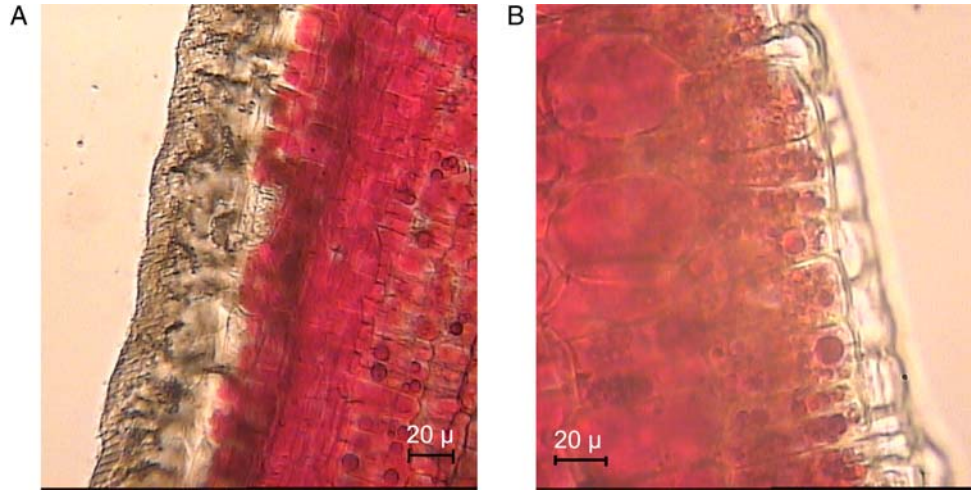

Fig. 4 Neutral Red-stained longitudinal sections of the radicle (A) and hypocotyl (B) in horse chestnut seeds. Vacuoles are visualized in the outer cortex of the radicle and hypocotyl of living embryonic axes. The radicle cap (A) and hypocotyl epidermis (B) remained colourless.

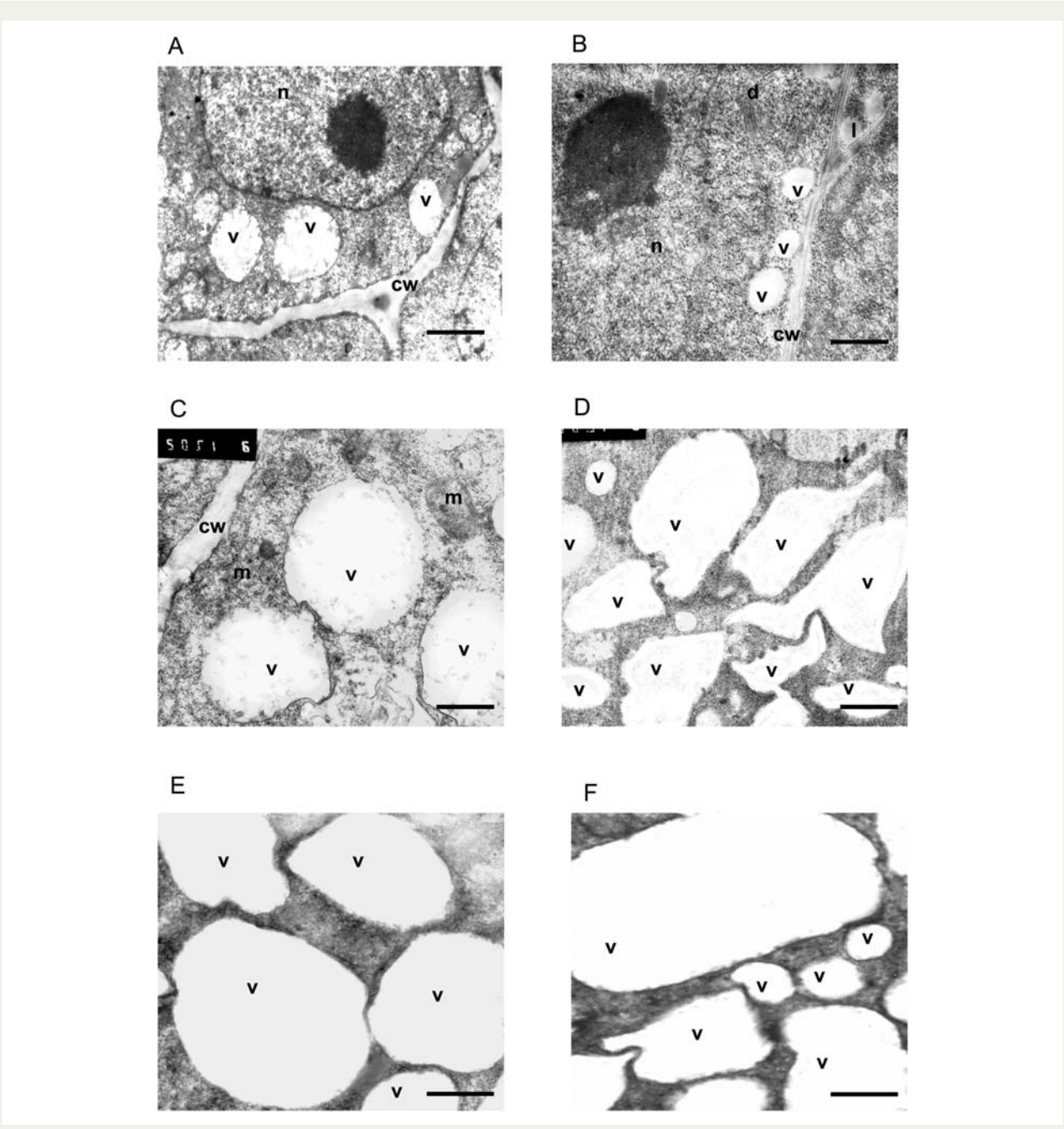

Fig. 5 Electron micrographs of hypocotyl (A, C, E, F) and radicle (B, D) cells in embryonic axes of horse chestnut seeds. (A) Hypocotyl in a dormant seed; (B) radicle (first millimetre) in the protruding embryonic axis; (C) hypocotyl (third millimetre) in protruding embryonic axis; (D) radicle (second millimetre) in growing embryonic axis; (E) hypocotyl (third millimetre) in growing embryonic axis; (F) hypocotyl (fifth millimetre) in growing embryonic axis. Bar: 0.5 μm; cw, cell wall; d, dictyosome; l, lipid droplet; m, mitochondrion; n, nucleus; v, vacuole.

the time of radicle emergence (Fig. 5C). The middle cells (fifth millimetre) possessed numerous large vacuoles (Fig. 5F) with thin cytoplasmic bands between them. The most elongated cells of such hypocotyls were characterized by large vacuoles of various sizes but no central vacuole had yet formed. These cells were still far below the final size of fully elongated cells (150 μm on average) and their vacuolation remained incomplete.

Membrane proteins in embryonic axes

To characterize the water inflow to embryonic axes needed to maintain their high water content (Fig. 2),

we investigated whether functional water channels formed by PIPs and TIPs are present in cells during the recalcitrant period. No data on aquaporins in recalcitrant seeds are currently available. To identify aquaporins in microsomal fractions from embryonic axes, we used antibodies to two widespread plant plasmalemma aquaporins (PIP1 and PIP2) and to two tonoplast aquaporins [TIP2 (previously called γ-TIP) and TIP3;1 (previously called α-TIP)], characteristic of plant vacuoles (Karlsson *et al.* 2000). The latter is a marker for tonoplasts in protein bodies (Johnson *et al.* 1989; Hunter *et al.* 2007) that are restored to vacuoles at germination of orthodox dicot seeds. Figure 6 shows that membranes of embryonic axes in horse chestnut seeds contain similar PIPs during dormancy (0 weeks), dormancy release (8–13 weeks of stratification), dormancy loss (16–18 weeks) (Fig. 6A and C) and at radicle emergence (Fig. 6B and D). PIP1 was represented by a 25-kDa protein; PIP2 was found as a 29-kDa protein. In the same microsomal fractions, we examined the presence of TIP3;1 and TIP2 in the tonoplast (Fig. 7). The TIP3;1 monomers have molecular masses of 27-kDa (Fig. 7A and B). In the case of TIP2, monomers were of 29 kDa (Fig. 7C and D). Molecular masses of aquaporin monomers correspond to those in other plants (Maurel *et al.* 2008). These TIPs were already present in shed seeds and did not change substantially by the time to radicle emergence, i.e. growth initiation. Therefore, tonoplast aquaporins, like plasmalemma aquaporins, were previously synthesized and present in readiness throughout the long recalcitrant period.

We also followed the presence of TIP along the embryonic axis in non-dormant seeds (Fig. 8) prior to and after radicle emergence. The length of the radicles remained 2 mm (see Fig. 2), and they exhibited identical patterns

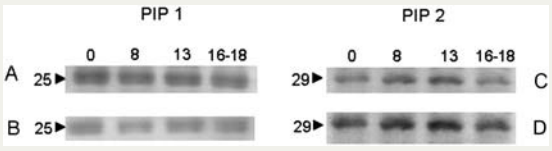

Fig. 6 Identification of plasmalemma aquaporins PIP1 and PIP2 by immunoblotting of microsomal fractions from horse chestnut embryonic axes. (A and C) Embryonic axes excised from intact seeds during stratification. (B and D) Embryonic axes excised from stratified seeds and incubated *in vitro* under optimum conditions up to radicle emergence. The numbers indicate the stratification weeks at which the seeds were sampled. Protein loading was normalized to equivalent microsomal protein. The arrows indicate the molecular masses of aquaporins in kilodaltons.

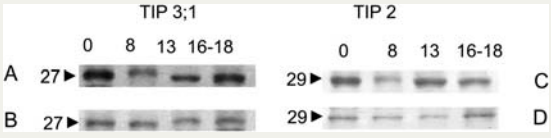

Fig. 7 Identification of tonoplast aquaporins TIP3;1 and TIP2 by immunoblotting of microsomal fractions isolated from horse chestnut embryonic axes. (A and C) Embryonic axes excised from intact seeds during stratification. (B and D) Embryonic axes excised from stratified seeds and incubated *in vitro* under optimum conditions up to radicle emergence. The numbers indicate the stratification weeks at which the seeds were sampled. Protein loading was normalized to equivalent microsomal protein. The arrows indicate the molecular masses of aquaporins in kilodaltons.

of distribution for both TIPs. Hypocotyls were 3–8 mm long prior to radicle emergence, then extended by 2 mm at radicle emergence, thereby pushing the radicle tip through the seed coat, and subsequently elongated. The same TIPs were present in hypocotyl cells preparing for elongation, in cells starting to elongate and in actively elongating cells.

TIP3;1 is usually considered a tonoplast marker in protein bodies of maturing orthodox seeds (Johnson *et al.* 1989; Hunter *et al.* 2007) and is present in preserved vacuoles of horse chestnut embryonic axes. It seems reasonable to classify it not just as a marker of protein bodies, but rather as a tonoplast marker in maturing vacuoles, independent of their further transformation or non-transformation to protein bodies.

We have also identified the vacuolar protein, H^+-ATPase. Prior to and at radicle protrusion, two isoforms of the E subunit of this enzyme (combining V_1 and V_0 complexes) were identified, having molecular masses of 31 and 29 kDa (Fig. 9). In growing embryonic axes, only one isoform with a molecular mass of 29 kDa was revealed. The results were similar in both the radicle and hypocotyl, which suggested an identical pattern of vacuole biogenesis in embryonic organs.

Water channel operation in embryonic axes

The presence of aquaporins in the membranes does not necessarily mean the operation of water channels. This is because they may be closed (inactive) or in an open (active) state. To distinguish between these possibilities, specific tests were performed. Figure 10A and B shows that Hg^{2+} ions inhibited water uptake by growing embryonic axes, and that DTT restored water absorption. These data demonstrated the presence of open water channels and confirmed the similarity of their operation with

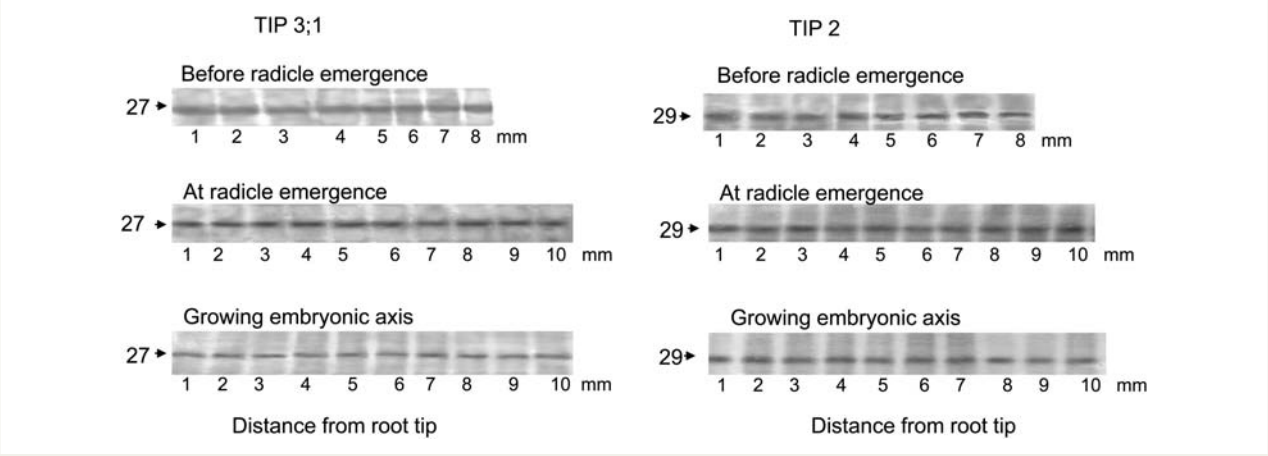

Fig. 8 Identification of tonoplast aquaporins TIP3;1 and TIP2 by immunoblotting of microsomal fractions isolated from 1-mm segments along embryonic axes from non-dormant horse chestnut seeds. Data included immunoblots prior to and at radicle emergence, and in apical 10-mm segments of 15- to 20-mm-long growing embryonic axes. Protein loading was normalized to equivalent microsomal protein. The arrows indicate the molecular masses of aquaporins in kilodaltons.

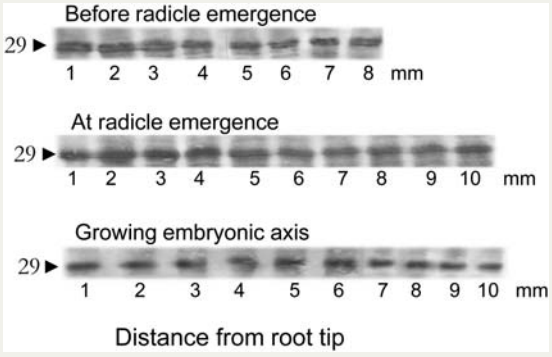

Fig. 9 Identification of vacuolar H^+-ATPase by immunoblotting of microsomal fractions isolated from 1-mm segments along embryonic axes from non-dormant horse chestnut seeds. Data included immunoblots prior to and at radicle emergence, and in apical 10-mm segments of 15- to 20-mm-long growing embryonic axes. Protein loading was normalized to equivalent microsomal protein. The arrows indicate the molecular masses of aquaporins in kilodaltons.

water channels in growing roots (Javot and Maurel 2002; Wan *et al.* 2004; Maurel *et al.* 2008; Obroucheva and Sin'kevich 2010). However, no such effect was shown with recently protruded embryonic axes, even at longer exposures to mercury ions (Fig. 10C). The inhibition of water uptake was weak and no recovery by DTT occurred. Therefore, at radicle protrusion, embryonic axes have closed water channels, and water enters cells only by diffusion through membranes.

Vacuolar enzyme (acid invertase) in embryonic axes

To characterize the vacuolar status in embryonic axes, it was necessary to check whether vacuoles retain the activity of enzymes such as invertase. Acid vacuolar invertase participates in the breakdown of sucrose, the content of which in embryonic axes of horse chestnut seeds amounts to 150 mg g^{-1} dry weight (Obroucheva *et al.* 2006). The dominating reserve carbohydrate, starch, is utilized in embryonic axes only after early germination. Sucrose appears to be a main readily metabolized storage compound. Acid vacuolar invertase (Fig. 11) was active in embryonic axes excised from mature freshly shed dormant seeds and later during the recalcitrant period. Its activity remained at the same level to the end of stratification. This enzyme is capable of hydrolysing not only sucrose, but also raffinose, an oligosaccharide which is present only in small amounts in the axes (4 mg g^{-1} dry weight) (Obroucheva *et al.* 2006). The enzyme activities towards both substrates (curves A1 and B1) were preserved in embryonic axes for 4 months after shedding. Native electrophoresis revealed the molecular mass of invertase (Fig. 12A) to be high (500–550 kDa). Denaturing electrophoresis (Fig. 12B) showed that it comprises 63- and 65-kDa subunits, and appears to be a multimer protein consisting of eight subunits. The molecular masses of these subunits are comparable with other plants (Obenland *et al.* 1993; Lee and Sturm 1996), but the extent of subunit aggregation is much higher. These data show that the properties of vacuolar invertase protein are preserved

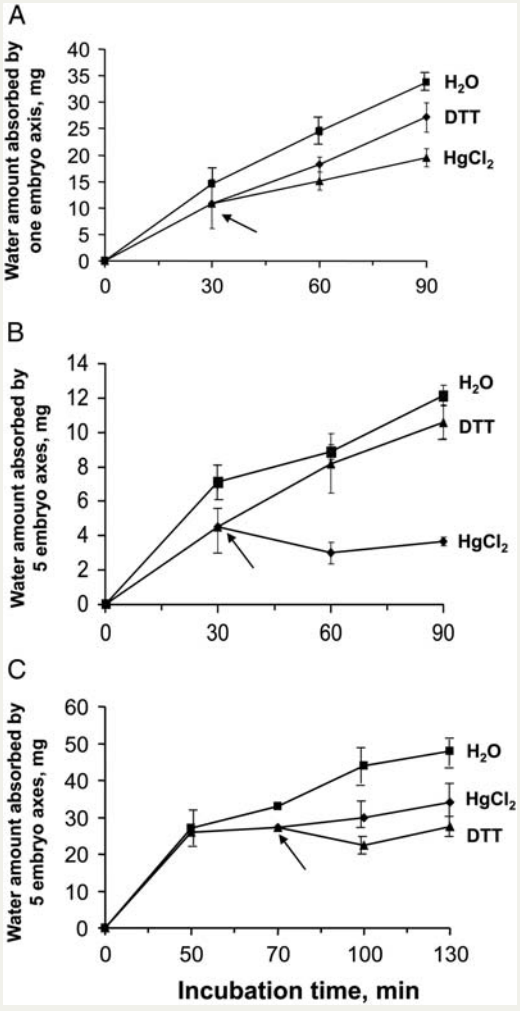

Fig. 10 Water uptake by excised embryonic axes from non-dormant horse chestnut seeds as a test for water channel operation. (A) Embryonic axes of 3–5 cm; (B) embryonic axes of 1.5–2.0 cm; (C) embryonic axes of 1.0–1.1 cm (at radicle emergence). The upper curve shows water uptake by control axes. Arrows indicate the transfer of embryonic axes from mercury chloride solution to DTT solution. Each sample consisted of 5–6 axes. The experiments were repeated six times. Data are shown as the means and standard errors.

during the long recalcitrant period and that the enzyme retains its potential activity. We measured the enzyme activity towards both substrates in embryonic axes from imbibing seeds (Fig. 11B and C). The activity of acid invertase increased in imbibing axes to the time of growth initiation. This rise did not depend on seed stratification time and imbibition time, i.e. embryonic axes retained and enhanced the capacity to efficiently hydrolyse both sucrose (Fig. 11B) and raffinose (Fig. 11C) prior to radicle emergence. These data confirm that vacuoles preformed in embryonic axes of horse chestnut seeds are capable of preserving and maintaining their functional activity in a prolonged recalcitrant state.

Discussion

Seed recalcitrance is most widespread in tropical rain forests characterized by humid air, moist soil and high temperatures. High humidity prevents water loss and desiccation of maturing seeds. These are shed when well hydrated and can immediately germinate in wet litter and soil. Similar seed behaviour is typical of plants in subtropical regions, as the shedding and germination coincide with the warm rainy season. When seed production in 886 tree and shrub species belonging to 93 families was studied (Tweedle *et al.* 2003), the proportion of species producing recalcitrant seeds was found to decrease as the habitat became more arid. Because of a very short interval between shedding and germination, no attention was paid to the specific physiological features of recalcitrant seeds at this time. The present paper addresses this shortcoming by following the fate of vacuoles in embryonic axes of a recalcitrant seed, horse chestnut. This species was chosen because it is capable of preserving recalcitrance and maintaining a high water content for a long period after seed shedding. This specific feature of horse chestnut seeds is an adaptation to the climatic conditions of central Russia. It prevents the rapid seed germination typical of recalcitrant seeds and replaces it with a state of deep dormancy and a slow release following low-temperature stratification. During this long period, seeds remain desiccation sensitive and maintain high hydration levels (Fig. 2). This makes these seeds ideal for studying the physiology of recalcitrance and the role of vacuoles.

High hydration of embryonic axes (Fig. 2) indicates the preservation of vacuoles. This phenomenon, impossible in orthodox seeds, was demonstrated by vital staining of embryonic axes (Fig. 4) as well as by electron microscopy (Fig. 5A). The question arises as to whether or not these vacuoles remain physiologically active during the recalcitrant period. The properties of the vacuolar enzyme, acid invertase, showed that this enzyme is able to hydrolyse sucrose and raffinose during the entire recalcitrant period (Fig. 11A) and beyond (Fig. 11B and C), i.e. it preserved its activity due to retention of molecular mass and subunit composition (Fig. 12). Therefore, these vacuoles preserve the enzyme machinery ready to operate under conditions favourable for germination.

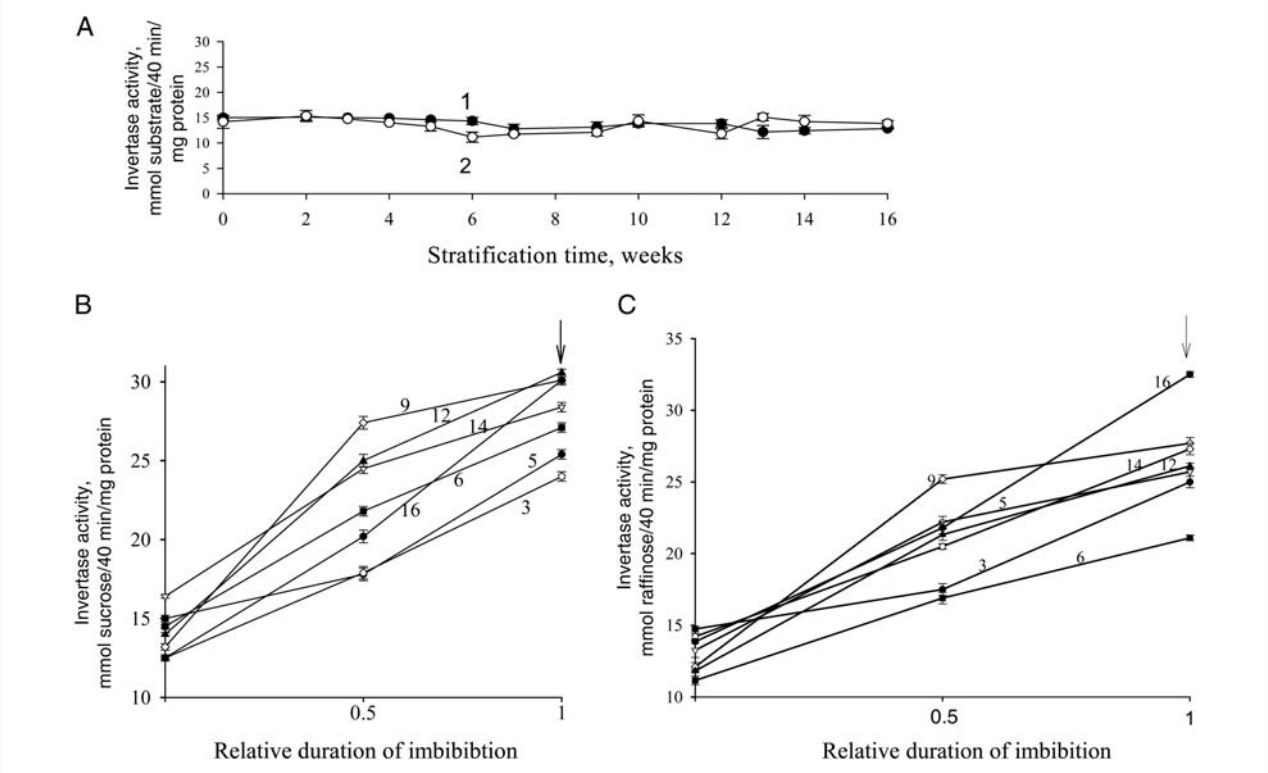

Fig. 11 Activity of acid vacuolar invertase in embryonic axes of horse chestnut seeds during stratification (A) and of seeds imbibing under optimum conditions up to radicle emergence (B and C). A1 and B with sucrose as a substrate; A2 and C with raffinose as a substrate. Numbers on curves indicate the weeks of stratification at which seeds were transferred for imbibition. The abscissa shows the relative duration of imbibition. Arrows indicate radicle protrusion. Data are shown as the means and standard errors.

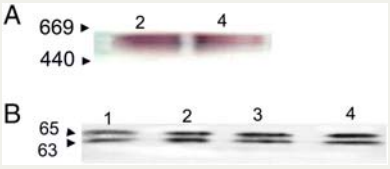

Fig. 12 Molecular mass and subunit composition of acid vacuolar invertase from embryonic axes of horse chestnut seeds. (A) Electrophoregrams of the enzyme protein after native electrophoresis. (B) Electrophoregrams of invertase subunits after denaturing electrophoresis. 1, dormancy; 2, dormancy release; 3, non-dormant seeds; 4, radicle emergence.

For recalcitrant seeds, water relations appear to be a key aspect of their behaviour. The maintenance of high hydration and vacuoles that operate as osmotic compartments was indicated by the water transport and water channel experiments. In addition to continuous water diffusion through membranes, water channels allow for rapid osmotic-driven water flow not only into cells but also from cell to cell (transcellular water transport) (Tyerman *et al.* 1999; Maurel *et al.* 2008). This accelerates water transport. The aquaporins in imbibing and germinating seeds were analysed in orthodox arabidopsis seeds by Willigen *et al.* (2006), but were studied not in embryonic axes but in intact seeds. In horse chestnut we found water channel proteins in the membranes of both plasmalemma and tonoplast (Figs 6 and 7) during the long recalcitrant period, and showed that they were preserved in embryonic axes up to growth initiation (Fig. 7B and D). In non-dormant horse chestnut seeds, water channels appeared to remain closed at radicle emergence (Fig. 10C). They take part in water inflow only after radicle emergence, i.e. in actively elongating cells (Fig. 10A and B), their opening presumably being a result of phosphorylation (Maurel *et al.* 2008). This conclusion corresponds to the observations made on intact arabidopsis (Willigen *et al.* 2006) and pea seeds (Veselova and Veselovsky 2006). After growth initiation, in elongating cells, water transport occurs not only by diffusion, but through open water channels in

the membranes too. Active cell vacuolation is accompanied by the commencement of osmotic-driven accelerated water inflow through the water channels.

In horse chestnuts, the vacuoles were not transformed to protein bodies at seed maturation and did not function as reserve protein deposits as often seen in orthodox dicot seeds. Furthermore, no restoration of protein bodies to vacuoles was observed. Vacuoles in horse chestnut axes remained unconverted, and preserved their contents, the enzyme machinery and tonoplast aquaporins (Fig. 7). Therefore, these vacuoles retain their physiological potential during the recalcitrant period; their functional readiness being realized in the rapid commencement of cell elongation in non-dormant recalcitrant seeds.

Being an obligatory pre-germinative event, cell vacuolation in non-dormant imbibing horse chestnut seeds develops rapidly, starting from pre-existing and functionally ready vacuoles. As an active osmotic compartment, these vacuoles take up water and enlarge before cell elongation begins in the hypocotyl and root. This additional amount of water raises the turgor pressure needed to drive cell elongation (Oboucheva 1999). Such activity depends directly on the accumulation of osmolytes such as sugars (Oboucheva *et al.* 2006) following a rise in invertase activity. For comparison, in orthodox arabidopsis seeds, invertase genes are expressed much later, after radicle emergence (Mitsuhashi *et al.* 2004). Thereafter, vacuoles are seen to enlarge by fusion and subsequent dilation, and vacuolation follows the pattern typical for growing cells. It should be emphasized that the opening of water channels in embryonic axes after radicle protrusion greatly favours cell vacuolation by facilitating accelerated osmotically driven water inflow.

The physiological strategy of mature recalcitrant seeds is not to lose water below a relatively high fatal value and to be able to germinate at once. A key component of this strategy is the maintenance of a constant high water content by minimizing water evaporation through a wax-covered dense seed coat, maintenance of an osmotic potential of approximately −3 MPa generated by sucrose and potassium ions in the embryonic axis (Oboucheva 1999; Oboucheva and Antipova 1999), and maintaining 63–65 % water content (Fig. 2), which that is sufficient for metabolic activity. Metabolism is suppressed during stratification by cold temperatures. After dormancy release, if both temperature and water supply are within a permissible range, cells immediately begin the pre-germinative preparation for growth. It is their advantage over orthodox seeds because germination of recalcitrant seeds is not delayed by the need for imbibition and metabolic activation.

The second strategic advantage of recalcitrant seeds is the preservation of intact functionally active vacuoles. In contrast, in orthodox seeds, protein bodies must first be restored to vacuoles. In horse chestnut seeds, vacuolar readiness in embryonic cells enables rapid germination. A further advantage of recalcitrant seeds is an early import of sucrose from cotyledons to the imbibing embryonic axis (Oboucheva *et al.* 2006, 2009). For example, after 9-week-long stratification, the sucrose content increased in the axes on the fourth day, whereas the radicle first emerged on the tenth day. Sucrose import begins in recalcitrant seeds prior to growth initiation, whereas in orthodox seeds sucrose translocation from the cotyledons commences much later, after growth initiation. Early transport in recalcitrant seeds is possible because of the high hydration level maintained in vascular tissues. Owing to rapid delivery of sucrose and increasing activities of two acid invertases located in cell walls and vacuoles (Lityagina 2010), fructose and glucose, which are of central importance for various metabolic pathways, are accumulated. Increasing activity of vacuolar invertase is mostly the outcome of new enzyme synthesis based on long-lived mRNA (Oboucheva and Lityagina 2009). The activities of both invertases contribute to a 20 % increase in osmotic pressure in embryonic axes just prior to radicle emergence, thus favouring commencement of cell elongation (Oboucheva *et al.* 2006).

Taken together, these physiological features of recalcitrant seeds explain their readiness for germination and subsequent successful development. Here we have probed some of the physiological features determining the recalcitrant habit. We identified the maintenance of high water content, preservation of potentiated vacuoles, early import of sucrose to the embryonic axis and rapid initiation of cell elongation as the outstanding functional features contributing to the ability of recalcitrant seeds to germinate promptly.

Conclusions and forward look

We show that cells of embryonic axes in mature recalcitrant horse chestnut seeds are characterized by a prolonged recalcitrant state associated with the maintenance of a high water content and preservation of vacuoles after seed shedding. These vacuoles appear to be physiologically active because membrane proteins, e.g. aquaporins, and activity of invertase enzyme are preserved. These vacuoles are therefore ready to function in imbibing seeds to support rapid onset of the cell elongation that results in germination. Future work to demonstrate the extent of the applicability of our finding to other recalcitrant species will give further

insight into a seed survival strategy that contrasts markedly with that of orthodox seeds, in which drying at maturation is essential for survival.

Sources of funding

We acknowledge financial support from the Russian Federation for Basic Research (grants no. 08-04-004160 and 11-04-01139) and the Program for Molecular and Cell Biology of the Presidium of Russian Academy of Sciences.

Contributions by the authors

The overall contributions by the authors were similar.

Acknowledgements

The authors are very grateful to Professor Christophe Maurel for his gift of aquaporin antibodies.

Conflict of interest statement

None declared.

References

Antipova OV, **Bartova LM**, **Kalashnikova TS**, **Obroucheva NV**, **Voblikova VD**, **Muromtsev GS**. **2003**. Fusicoccin-induced cell elongation and endogenous fusicoccin-like ligands in germinating seeds. *Plant Physiology and Biochemistry* **41**: 157–164.

Barrouclough D, **Peterson C**, **Steudle E**. **2000**. Radial hydraulic conductivity along developing onion roots. *Journal of Experimental Botany* **51**: 547–557.

Baryckina RP, **Veselova TD**, **Devyatov AG**, **Djalilova KK**, **Il'ina GM**, **Chubatova NV**. **2004**. *Handbook on botanical microtechnique: bases and methods*. Moscow: Nauka.

Berjak P, **Pammenter NW**. **2008**. From *Avicennia* to *Zizania:* seed recalcitrance in perspective. *Annals of Botany* **101**: 213–228.

Cheng H-Y, **Song S-Q**. **2008**. Possible involvement of reactive oxygen species scavenging enzymes in desiccation sensitivity of *Antiaris toxicaria* seeds and axes. *Journal of Integrative Plant Biology* **50**: 1549–1556.

Davis BJ. **1964**. Description of discontinuous buffer system for non-denaturing gels and disc electrophoresis. *Annals of the New York Academy of Sciences* **209**: 373–381.

Daws MI, **Lydall E**, **Chmielarz P**, **Leprince O**, **Matthews S**, **Thanos CA**, **Pritchard HW**. **2004**. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. *New Phytologist* **162**: 157–166.

Farrant JM, **Walters C**. **1998**. Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* in relation to the acquisition of tolerance to drying. *Physiologia Plantarum* **104**: 513–524.

Farrant JM, **Pammenter NW**, **Berjak P**. **1989**. Germination-associated events and the desiccation sensitivity of recalcitrant seeds—a study on three unrelated species. *Planta* **178**: 189–198.

Farrant JM, **Pammenter NW**, **Berjak P**, **Walters C**. **1997**. Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. *Seed Science Research* **7**: 135–144.

Herman EM, **Li X**, **Su RT**, **Larsen P**, **Hsu H**, **Sze H**. **1994**. Vacuolar-type H^+-ATPases are associated with the endoplasmic reticulum and provacuoles of root tip cells. *Plant Physiology* **106**: 1313–1324.

Hunter PR, **Craddock CP**, **Benedetto SD**, **Roberts LM**, **Frigerio L**. **2007**. Fluorescent reporter proteins for the tonoplast and the vacuolar lumen identify a single vacuolar compartment in arabidopsis cells. *Plant Physiology* **145**: 1371–1382.

Ivanov VB. **1987**. *Reactive dyes in biology*. London: Harwood Academic Publishers.

Javot H, **Maurel C**. **2002**. The role of aquaporins in root water uptake. *Annals of Botany* **90**: 301–313.

Johnson KD, **Herman EM**, **Chrispeels MJ**. **1989**. An abundant, highly conserved tonoplast protein in seeds. *Plant Physiology* **91**: 1006–1013.

Karlsson M, **Johansson I**, **Bush M**, **McCann M**, **Maurel C**, **Larsson C**, **Kjellbom P**. **2000**. An abundant TIP expressed in mature highly vacuolated cells. *Plant Journal* **21**: 83–90.

Kermode AR, **Finch-Savage WE**. **2002**. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. In: Black M, Pritchard HW, eds. *Desiccation and survival in plants: drying without dying*. Wallingford: CABI Publishing, 149–184.

Laemmli UR. **1970**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lee H, **Sturm A**. **1996**. Purification and characterization of neutral and alkaline invertase from carrot. *Plant Physiology* **112**: 1513–1522.

Lityagina SV. **2010**. *Physiological traits of seed recalcitrance exemplified by horse chestnuts*. PhD Thesis, Institute of Plant Physiology, Moscow, Russia.

Maurel C, **Verdoucq L**, **Luu DT**, **Santoni VP**. **2008**. Plant aquaporins. Membrane channels with multiple integrated functions. *Annual Review of Plant Biology* **59**: 595–624.

Mitsuhashi W, **Sasaki S**, **Kanazawa A**, **Yang Y-Y**, **Kamiya Y**, **Toyomasu T**. **2004**. Differential expression of acid invertase genes during seed germination in *Arabidopsis thaliana*. *Bioscience, Biotechnology and Biochemistry* **68**: 602–608.

Motete N, **Pammenter NW**, **Berjak P**, **Frederic JC**. **1997**. Response of the recalcitrant seeds of *Avicennia marina* to hydrated storage: events occurring at the root primordia. *Seed Science Research* **7**: 169–178.

Musatenko LI, **Generalova VM**, **Martyn GG**. **1997**. About physiology of *Aesculus hippocastanum* L. seeds. *Ukrainskii Botanicheskii Zhurnal (Ukranian Botanical Journal)* **54**: 86–91.

Obenland DM, **Simmen U**, **Boller T**, **Wiemken A**. **1993**. Purification and characterization of three soluble invertases from barley (*Hordeum vulgare* L.) leaves. *Plant Physiology* **101**: 1331–1339.

Obroucheva NV. **1999**. *Seed germination: a guide to the early stages*. Leiden: Buckhuys.

Obroucheva NV, **Antipova OV**. **1999**. Common physiological mechanisms prepare seeds with different dormancy types for germination. *Russian Journal of Plant Physiology* **46**: 363–368.

Obroucheva NV, **Antipova OV**. **2000**. The distinct controlling of dormancy release and germination commencement in seeds. In: Viemont J-D, Crabbe J, eds. *Dormancy in plants: from whole plant behaviour to cellular control*. Willingford: CABI Publishing, 35–46.

Obroucheva NV, **Antipova OV**. **2003**. Germination of horse chestnut seeds—cell growth and hormonal regulation. *Seed Technology* **25**: 128–139.

Obroucheva NV, **Antipova OV**. **2004**. Role of water in the transition of recalcitrant seeds from dormancy to germination. *Russian Journal of Plant Physiology* **51**: 942–951.

Obroucheva NV, **Lityagina SV**. **2007**. Dormancy release and germination in recalcitrant *Aesculus hippocastanum* seeds. *Dendrobiology* **57**: 27–33.

Obroucheva NV, **Lityagina SV**. **2009**. Acid vacuolar invertase in dormant and germinating horse chestnut seeds. *Russian Journal of Developmental Biology* **40**: 339–344.

Obroucheva NV, **Sin'kevich IA**. **2010**. Aquaporins and cell growth. *Russian Journal of Plant Physiology* **57**: 153–165.

Obroucheva NV, **Lityagina SV**, **Richter A**. **2006**. Dynamics of carbohydrates in the embryo axes of horse chestnut seeds during their transition from dormancy to germination. *Russian Journal of Plant Physiology* **53**: 768–778.

Obroucheva NV, **Lityagina SV**, **Richter A**. **2009**. Initiation of source–sink relations during horse chestnut germination. *Acta Horticulturae* **835**: 137–141.

Pammenter NW, **Berjak P**. **1999**. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9**: 13–37.

Pritchard H, **Steadman K**, **Nash J**, **Jones C**. **1999**. Kinetics of dormancy release and the high temperature germination response in *Aesculus hippocastanum* seeds. *Journal of Experimental Botany* **50**: 1507–1514.

Pukacka S, **Ratajczak E**. **2006**. Antioxidative response of ascorbate-glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. *Journal of Plant Physiology* **163**: 1259–1266.

Roach T, **Ivanova M**, **Beckett RP**, **Minibayeva FV**, **Green I**, **Pritchard HW**, **Kranner I**. **2008**. An oxidative burst of superoxide in embryonic axes of recalcitrant sweet chestnut seeds as induced by excision and desiccation. *Physiologia Plantarum* **133**: 131–139.

Roach T, **Beckett RP**, **Minibayeva F**, **Colville LE**, **Whitaker C**, **Chen H**, **Bailly C**, **Kranner I**. **2010**. Extracellular superoxide production, viability and redox poise in response to desiccation in recalcitrant *Castanea sativa* seeds. *Plant, Cell and Environment* **33**: 59–75.

Steadman KJ, **Pritchard HW**, **Dey PM**. **1996**. Tissue-specific soluble sugars in seeds as indicators of storage category. *Annals of Botany* **77**: 667–674.

Tweedle JC, **Dickie JB**, **Baskin CC**, **Baskin JM**. **2003**. Ecological aspects of seed desiccation sensitivity. *Journal of Ecology* **91**: 294–304.

Tyerman SD, **Bohnert HJ**, **Maurel C**, **Steudle E**, **Smith JAC**. **1999**. Plant aquaporins: their molecular biology, biophysics and significance for plant water relation. *Journal of Experimental Botany* **50**: 1055–1071.

Veselova TV, **Veselovsky VA**. **2006**. Possible involvement of aquaporins in water uptake by pea seeds of different quality. *Russian Journal of Plant Physiology* **53**: 96–101.

Walters C. **2000**. Levels of recalcitrance in seeds. *Revista Brasileira de Fisiologia Vegetal* **12**: 7–21.

Wan X, **Steudle E**, **Hartung W**. **2004**. Gating of water channels (aquaporins) in cortical cells of young corn roots by mechanical stimuli (pressure pulses): effects of ABA and $HgCl_2$. *Journal of Experimental Botany* **55**: 411–422.

Willigen VC, **Postaire O**, **Tournair-Roux C**, **Boursiac Y**, **Maurel C**. **2006**. Expression and inhibition of aquaporins in germinating arabidopsis seeds. *Plant and Cell Physiology* **47**: 1241–1250.