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Storage compounds, ABA and fumarase in *Fagus sylvatica* embryos during stratification

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Abstract: Understanding the mechanisms of seed dormancy is essential for planning optimal seed storage and for breaking the dormancy of stored seeds. Our experiments focused on three aspects of characterization of breaking the dormancy of *Fagus sylvatica* seeds during moist low temperature stratification. 1) We used the changes of ABA content in embryos during the whole process of stratification as the marker of the depth of dormancy: the decrease in the depth of dormancy (according standard germination tests) was directly proportional to the drop in endogenous ABA content in both cotyledons and embryonic axes. 2) Fumarase activity (the second marker of dormancy) increased gradually during stratification. 3) The histochemical visualisation of storage compounds as the third marker of dormancy documented the changes in storage protein deposition during stratification. The proteinaceous content of storage vacuoles disappeared; the vacuoles diminished, petered out or fused, creating central vacuoles. This process commenced with imbibition of the seeds and it was more pronounced in the cells in the external parts of the cotyledons. No changes in the size and location of starch grains or calcium oxalate crystals linked with stratification were detected.

Additional key words: embryonic axis, *Fagus sylvatica,* seed dormancy, storage proteins

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Introduction

Seed dormancy is an important adaptive mechanism, necessary for plants to survive severe seasonal variation. The temporary inability of mature seeds to germinate prevents germination and seedling development during periods with unfavourable conditions. Dormancy is determined genetically but it can be regulated by environmental factors. The techniques for breaking the dormancy of beechnuts have been improved using a variety of treatments (chemical or physical) (Gendreau and Corbineau 2009; Kolářová et al. 2010). Many substances controlling seed viability and dormancy are associated with germination capacity. Phenolic compounds, alpha-tocopherol, sterols, ascorbic acid, glutathione and soluble proteins have all been measured in stored beechnuts and found to be associated with germination, whilst a negative correlation has been found with reactive oxygen species (Pukacka and Ratajczak 2014).

Hormonal regulation of dormancy has been shown to be especially linked to the balance between abscisic acid (ABA) and gibberellins (GA). These hormones play essential and antagonistic roles in dormancy and germination (Rodríguez-Gacio et al.

2009; Graeber et al. 2012). The main role of ABA in seed dormancy is in the regulation of seed formation, the deposition of storage reserves, the prevention of germination, the acquisition of desiccation tolerance and the induction of primary dormancy (Kermode 2005). The role of ABA was confirmed by the experiments of Nicolás et al. (1996) in which ABA treatment induced the return of seed dormancy previously eliminated by low temperature. The opposite effect is achieved by the application of GAs, which can break seed dormancy and induce germination. An increase in endogenous GAs has been interrelated to the dormancy-breaking process (Fernández et al. 1997). Calvo et al. (2004) suggest cross-talk regulation by several phytohormones in the transition from seed dormancy to germination. Kucera et al. (2005) summarize the phytohormone effects: GA releases dormancy, promotes germination and counteracts ABA effects. Ethylene and brassinosteroids promote seed germination and also counteract the effects of ABA. Both ABA and GA may be involved in the regulation of protein metabolism during dormancy (Nicolás et al. 1996). ABA has been found mostly to down-regulate proteins, whereas GA up-regulates them (Pawłowski 2010).

According to Shen and Odén (2000), one indicator of the depth of dormancy is fumarase activity since this is a key enzyme of mitochondrial respiration. An increase in fumarase activity correlates also with higher degradation of storage lipids before the start of germination (Shen and Odén 1999).

Fagus seeds do not contain an endosperm; instead, the nutrients are stored in the cotyledons. The distribution or redistribution of the main reserves (starch, proteins, oils, calcium oxalate) during breaking the dormancy plays an important role in the process of germination. Seed cells usually contain several different storage organelles including dynamic protein vacuoles in which storage proteins are deposited during seed development. Storage proteins can be transported from vacuoles through the cell (Ibl and Stoger 2012). Partial mobilization of storage proteins starts with the uptake of water by dry seeds (Bewley et al. 2013). Calcium oxalate (CaOx) crystals are usually not regarded as the seed storage compounds; however the major demonstrated function of CaOx crystals is the bulk calcium regulation (Franceschi and Nakata 2005). CaOx thus provide a reservoir of calcium that can be used during germination. Crystal deposition can occur in the vacuoles of idioblasts or alternatively in storage parenchyma cells (Webb 1999). CaOx is present frequently in many plant species; in beech seeds calcium-rich crystals have been reported by Collada et al. (1993). The major mobilization of reserves within storage tissues commences after protrusion of the radicle (Bewley et al. 2013).

We were interested in the changes that occur in beech embryos during moist stratification and dormancy breaking with the aim to assign the criteria of the stratification efficiency.

Our experiments were conducted in three steps:

1. We characterized the process of beechnut breaking the dormancy in terms of the changes in endogenous ABA level in embryos during stratification.
2. We determined changes in fumarase activity in the embryonic axes during stratification to assess the depth of dormancy in beechnuts.
3. We described the changes in the distribution of storage proteins, starch grains and CaOx in beechnuts during stratification on the histological level.

Methods

The seeds of common beech (*Fagus sylvatica* L.) used in all experiments were obtained from the Forestry and Game Management Research Institute – Research Station Kunovice, Czech Republic. The changes in endogenous ABA levels in embryonic axis and in cotyledons were conducted using lots P1, P2 and P3 (harvested in 2010 in Poland). The changes of ABA content and fumarase activity in embryonic axes as well as all histological experiments were conducted using lot B7 and verified using lot S12 (both harvested in 2011 in Moravia, Czech Republic). Dormant seeds were analysed just after harvesting; moist stratification (without any growing medium) at a low temperature (3°C) was applied for 13–15 weeks (Bezděčková et al. 2014). The seeds after stratification were not dormant, according to germination tests conducted at the Research Station Kunovice.
Seed material was collected every week during stratification at the Research Station Kunovice. Selected 100 seeds per 1 sample were frozen and stored in plastic bags at –80°C while awaiting analysis. Accurate number of seeds was prepared for every analysis. All manipulations were done using this frozen material under low temperature – in boxes filled with ice.

ABA analysis

Accurately weighted plant material (around 0.1 g FW) was ground to powder in liquid nitrogen and extracted overnight by 3 ml (3 times) of modified Bieleski solution (methanol : water : formic acid, 75 : 20 : 5). For monitoring of every step of analysis the internal standard (deuterated ABA – Olchemim, Czech Republic) was added to grounding process. The extract was centrifuged and purified by using solid phase extraction (SPE) method. For that, SPE column C18 (Phenomenex, USA) was used. The el-

uate from C18 column was partly evaporated by the rotary vacuum evaporator (RVE – Buchi Labortechnik, Switzerland) to end volume of approx. 3 ml. The pH was then adjusted by 1 ml of 1 M formic acid (Sigma-Aldrich, Germany). The adjusted eluate was applied on the MCX SPE column (Waters, France), cleaned by 2 ml of 1 M formic acid, eluted by 5 ml of 100% methanol (Sigma-Aldrich, Germany) and dried on the rotary vacuum concentrator (Christ, Germany). Dried samples were diluted in 15% acetonitrile:water v/v (Sigma-Aldrich, Germany) solution. Filtrates were injected to HPLC equipped with UV-detector at 270 nm (Agilent, USA), and precleaned on C-18 column (Phenomenex, USA) with gradient elution and fractionated on a fraction collector (Gilson 203B, Middleton, WI, U.S.A.). Fraction at time 23.05 min was collected for 1 min and dried. After drying collected fraction was derivatized by 0.3 ml diazomethane for 15 min, dried, dissolved in 10 μl of acetone (Sigma-Aldrich, Germany). 8 μl of redissolved sample were injected into GC-MS/MS (Thermo, USA) and analyzed on column DB-5MS (Agilent, USA) with detection by Ion trap in MS/MS scan mode (MS1: full scan 50–300 amu; ABA (Sigma-Aldrich, Germany):precursor 190.2 amu, product full scan 65–200 amu; MS2 labeled ABA (Olchemim, Czech Republic): precursor 194.2 amu, product full scan 70–200 amu). The method is described in detail by Žďárská et al. (2013).

Fumarase activity

We adapted the methods of Hatch (1978) and Shen and Odén (2000) based on the measurement of the increase in absorbance due to NADPH formation in the NADP malate dehydrogenase-coupled assay of fumarate hydratase (fumarase, L-malate hydrolyase, EC 4.2.1.2.). The optimization of the enzymatic reaction was realized in two steps: 1) the timing of the reaction using porcine fumarase (Sigma-Aldrich, Germany) and the optimum for measurement was assessed to be 340 nm in the 12th minute of reaction; 2) the effect of fumarase concentration on absorbance. The linearity of absorbance was assessed between 0.01 and 0.25 IU of fumarase.

Weighted embryo axes were ground with the ice-cold extraction buffer containing 50 mM HEPES buffer (pH 7.6), 2 mM dithiotreitol (both Duchefa, The Netherlands) and 0.2% (v/v) Triton X-100 (Sigma-Aldrich, Germany) using a mixer mill MM301 Retsch (Haan, Germany) for 2 min at 30Hz. The homogenates were centrifuged and the supernatants were filtered using Alltech centrifuge filters (regenerated cellulose 0.2 μm) (Grace Davison Discovery Sciences, Deerfield, IL). A quantity of 150 μl of filtered extract was added to a reaction mixture (25 mM HEPES buffer, ca 0.2 IU malate dehydrogenase (NADP malic enzyme, EC 1.1.1.40., E.coli, Sigma-Aldrich), 0.4 mM NADP+, 4 mM $MgCl_2$ (both Sigma-Aldrich, Germany), 5 mM KH_2PO_4 (Lachner, Czech Republic)). Reaction was started by adding fumarate (final concentration 10 mM; sodium fumarate dibasic, Sigma-Aldrich) up to a total volume 1 ml. Absorbance was measured at 340 nm using a Helios β spectrophotometer (Thermo Spectronic, Cambridge, UK) after 3, 6, 9 and 12 minutes of reaction in a 50 mm cuvette. The changes in absorbance were proportional to the activity of fumarase in the sample. Each sample point consisted of 6 replicates, each containing 5 embryonic axes (around 0.05 g FW).

Anatomical and histological study

Anatomical and histochemical analyses were conducted on longitudinal paraffin sections of seed parts (approx. 5 mm), which contained embryonic axes and the adjoining portion of the cotyledons. Material was fixed and embedded into paraffin according to Svobodová et al. (1999). Longitudinal sections (12 μm) were prepared using rotary microtome. Histochemical proof of proteins was done using protein-specific dyes: 0.5% Ponceau xylidine in 2% acetic acid (Gutmann et al. 1996), 0.1% amido black 10B (Schaffner and Weissmann 1973) and 2.5% Commassie brilliant blue (CBB) R250 (Bradford 1976), both in methanol: acetic acid: water 45 : 10 : 45 v/v/v solution. In order to locate starch grains, the sections were stained using IIK (Lugol solution) (Sass 1958). Crystals were detected using polarized light. CaOx was determined by a chemical test (HCl dissolved the crystals, in contrast to CH_3COOH) according to Yasue (1969).

Histological observations were made using a Jenaval microscope (Zeiss, Germany) equipped with a Nikon DS-5M digital camera and processed using the Nis-Elements AR 3.0 (Laboratory Imaging, Prague, Czech Republic) computer image analysis system.

Statistical analyses

At least three independent sample preparations and measurements were carried out for every step of our experimental work. Mean values and their standard deviations obtained in each experiment (with three replicates) are shown in the figures. They were obtained using these equations:

Both ABA content and fumarase activity were measured in the linear response ($y = \alpha \times x + b$) range. The constant b was tested using the Student's t tests for significance.

Results

Endogenous level of ABA

We compared the ABA content in cotyledons and in the axes of dormant and non-dormant embryos. The lower ABA level – under 100 pmol/g of fresh weight was found in cotyledons, in contrast to approximately 1000–1500 pmol/g of fresh weight in the embryonic axes of dormant embryos. A continual decrease in the level of ABA over the period of stratification was observed in the axes as well as in the cotyledons (Fig. 1). A drop of more than 50% was recorded in the axes and cotyledons of the non-dormant embryos from 3 lots (P1, P2, P3) of beechnuts (after 13 weeks of stratification). The proportional changes in ABA content in the axes were comparable to that in cotyledons, so all subsequent measurements were conducted using embryonic axes only. We confirmed our results using embryonic axes derived from lots B7 (Fig. 2) and S12 (non-published results).

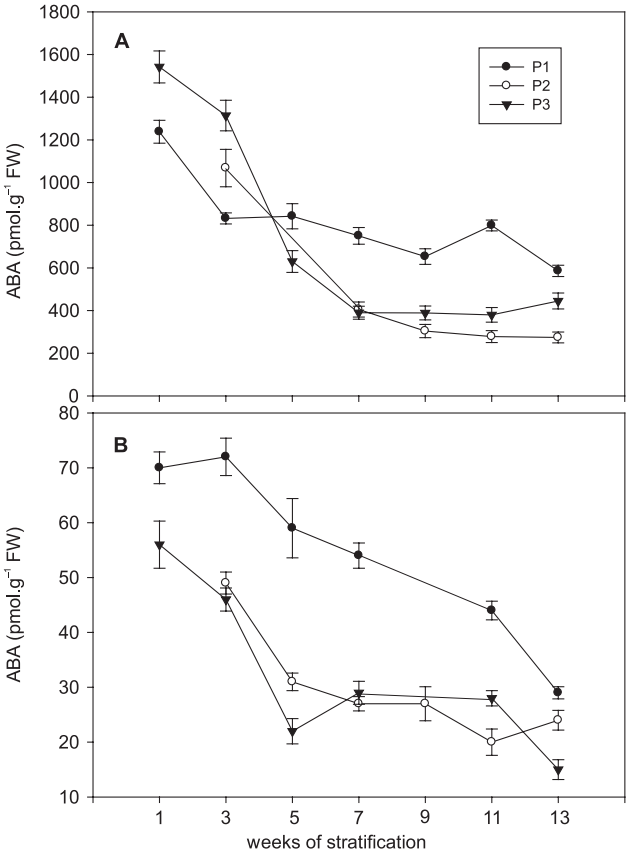

Fig. 1. Changes in ABA content in embryonic axes and cotyledons from 3 lots of beech seeds during stratification
A – ABA content in ebryonic axes (pmolg^{-1} of fresh weight)
B – ABA content in cotyledons (pmolg^{-1} of fresh weight)
P1, P2, P3 – lots of beech seeds harvested in 2010; experiments performed in 2011–2012

Fumarase activity

The changes of fumarase activity were estimated in embryonic axes derived from B7 lot of seeds. In contrast to ABA content, the fumarase activity in embryonic axes increased gradually during stratification. The enzyme activity in dormant embryos was approximately one half of that in non-dormant embryos (Fig. 2). The values of fumarase activity were assessed under 1 unit/g of fresh weight of dormant embryonic axes at the start of stratification. During the whole process of stratification fumarase activity increased to approx. 2 units/g of fresh weight of embryonic axes derived from non-dormant embryos. Similar results were obtained for S12 lot of seeds (non-published data).

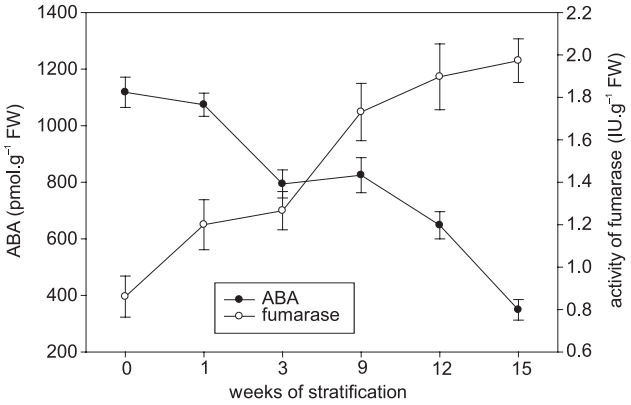

Fig. 2. Changes in ABA content and fumarase activity in embryonic axes from beech seeds (lot B7) during stratification
• – ABA content in embryonic axes (pmolg^{-1} of fresh weight)
° – Fumarase activity in embryonic axes (IUg^{-1} of fresh weight)
B7 – lot of beech seeds harvested in 2011; experiments performed in 2012–13

Storage compounds distribution

We focused our attention to starch grains, storage proteins and crystals of CaOx that we found in cotyledons as well as in embryonic axes of all embryos.

A large number of starch grains were present in cotyledons and embryonic axes of both dormant and non-dormant embryos. The largest starch grains were located in the parts of cotyledons clinging to the embryo axes. The smallest ones occurred near the vascular tissues. However, no relationship between the size, number or distribution of starch grains and the depth of dormancy was found (Fig. 3a, b).

We observed abundant druses and prismatic crystals of CaOx in vacuoles of the cotyledons of dormant embryos as well as in embryos after stratification (Fig. 3c, d). Small druses were rarely present also in embryonic axes. The amount and distribution of these crystals were not influenced by stratification.

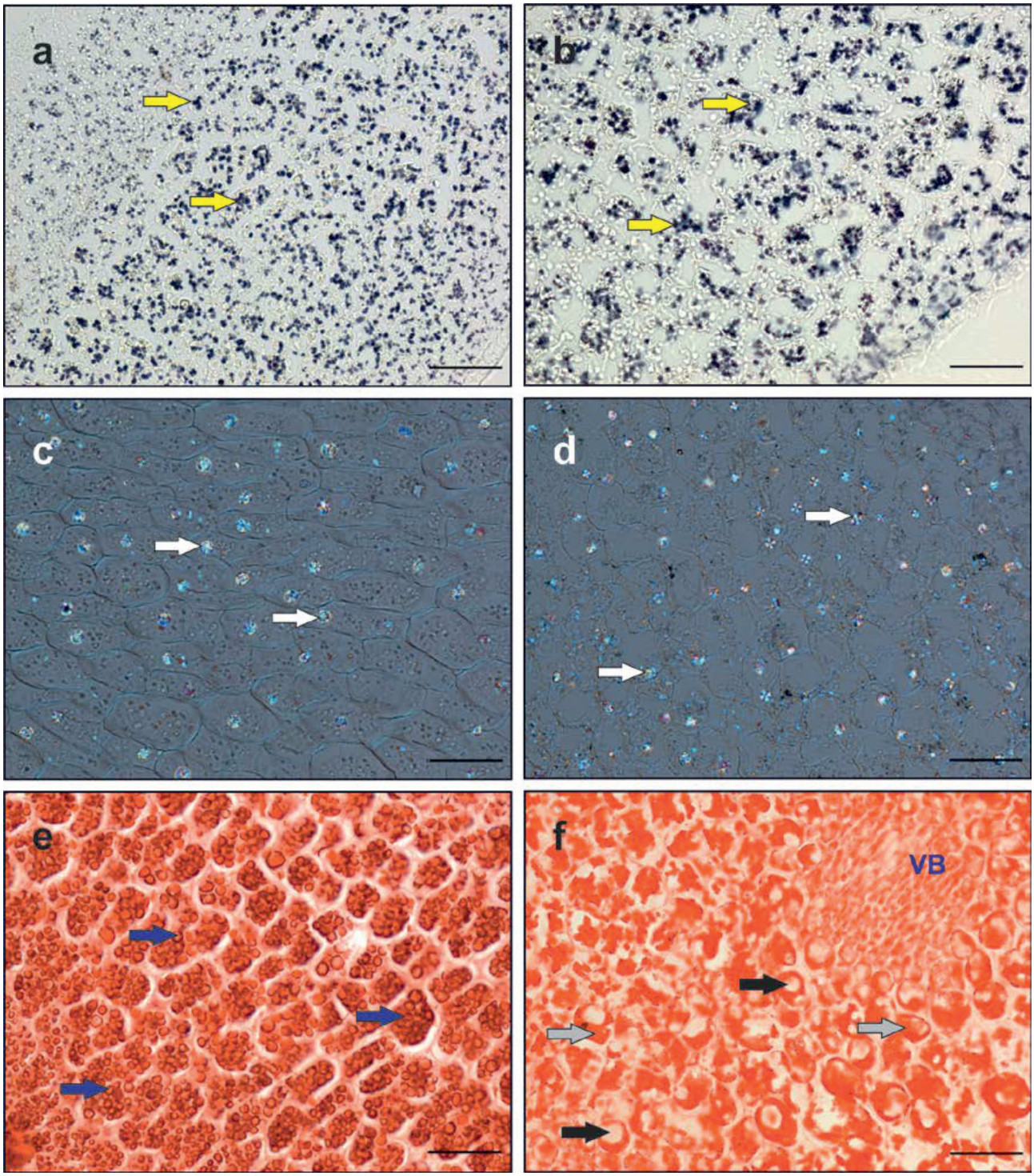

Fig. 3. Storage compounds in central part of cotyledons from dormant (left) and non-dormant (right) embryos (lot B7)
1st row – starch grains (stained using Lugol solution) in cotyledon of dormant (a) and non-dormant embryo (b).
2nd row – CaOx crystals (in polarized light) in cotyledon of dormant (c) and non-dormant embryo (d).
3rd row – storage proteins (stained using Ponceau xylidine) in dormant (e) and non-dormant embryo (f).
Starch grains – yellow arrows; CaOx crystals – white arrows; protein storage vacuoles (PSVs) – blue arrows; central vacuole – grey arrows; VB – vascular bundle.
Bars represent 50 μm.

The most remarkable reserves in beech embryos were proteins. We determined the proteinaceous content of vacuoles during the whole process of stratification using three independent protein-specific dyes: Ponceau xylidine (Fig. 3), amido black 10B and CCB R250 (non-published data). We detected plenty of protein storage vacuoles (PSVs) of different sizes entirely filling the cells of the cotyledons of

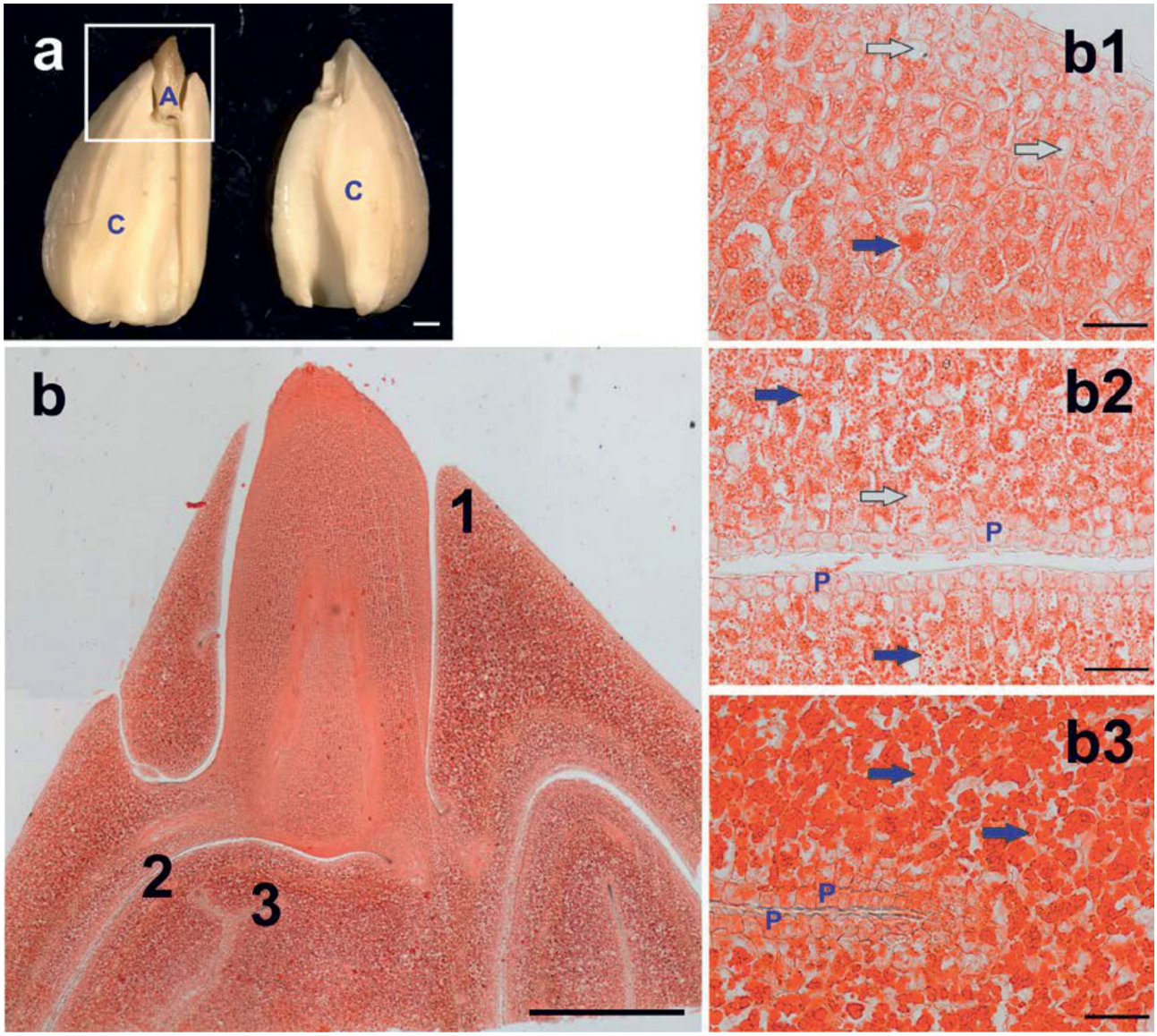

Fig. 4. Storage proteins in cotyledons of beech embryos (lot B7) after the first week of stratification
a – embryo consisting of cotyledons (C) and embryonic axis (A); bar represents 1 mm.
b – anatomy of embryonic axis and adjoining part of cotyledons, stained using Ponceau xylidine; bar represents 1 mm.
In the details:
b1 – PSVs confined to the cortical cytoplasm by central vacuoles in the cotyledonary cells located close to the radicle; proteins present in PSVs and in the cytoplasm.
b2 – PSVs with the rest of the storage proteins located in the cells of the exterior part of the cotyledons.
b3 – PSVs replete with storage proteins fill the cells in the interior of the embryo.
Protein storage vacuoles (PSVs) – blue arrow; central vacuoles – grey arrow; P – protodermis.
Bars represent 50 μm.

dormant embryos (Fig. 3e; Ponceau xylidine dying). Small PSVs were also distinguishable in embryo axes, predominantly in the region close to the apical meristem. Fundamental histological changes appeared in cotyledons after the first week of stratification, i.e. shortly after imbibition (Fig. 4). In the external parts of the cotyledons, which were exposed to the moistened surface of the seed, storage vacuoles diminished or disappeared, and eventually even the protein content disappeared (Fig. 4b1, b2). Below the apical meristems of the embryo axes, in the interior of the embryo, PSVs and their content remained almost unchanged (Fig. 4b3). At the end of stratification, the storage proteins in the vacuoles had mostly been utilized (Fig. 3f).

Discussion

ABA was established as key in maintaining seed dormancy (Matilla and Matilla-Vázquez 2008). It corresponded with the efficiency of stratification (re-

viewed by Kermode 2005). According to our results ABA level decreased continually in cotyledons as well as in embryonic axes during stratification and the drop of more than 50% is linked with the successful breaking the dormancy. In beech seeds the decrease in the level of ABA correlated with ABA degradation during the dormancy breaking and with the inability to synthesize ABA (Le Page-Degivry et al. 1997). High ABA level in embryos inhibits the transition to germination (Rodríguez-Gacio et al. 2009). We confirmed the correlation between endogenous ABA level and depth of dormancy in all lots of beech seeds used in our experiments. The gradual changes of ABA content in embryogenic axes show clearly the progress in dormancy breaking during stratification. According to large differences between the ABA levels in embryonic axes derived from embryos at the start and the end of stratification the dormant embryos can be distinguished from non-dormant ones. The changes in ABA content thus can be used as good indicator of depth of dormancy.

We tried to verify the possibility of routine measurement of the changes of fumarase activity as the second marker of stratification efficiency. Fumarase activity was measured as the indicator of depth of dormancy by Shen and Odén (2002) for *Picea, Pinus, Betula* and *Fagus*; their experiments were not often repeated and their methods are so far not generally used for dormancy characterization. Our results correspond well with that of Shen and Odén (2002). They showed that fumarase activity in non-dormant beech seeds was about twice that in dormant seeds. Shen and Odén (2000) recommended using a correlation between mean germination time and fumarase activity for identifying changes in seed vigour during storage. According to our results, the gradual changes in fumarase activity in embryonic axes may be a suitable indicator of the depth of dormancy. We can recommend using of fumarase activity method to indicate the efficiency of stratification as well as the measurement of ABA content in embryos.

Starch grains were present in cotyledons and in embryonic axes of dormant as well as non-dormant embryos. We did not find any indication of starch exploitation during dormancy breaking. This observation corresponded with the finding that hydrolysis of starch reserves in the cotyledons commences after germination (Bewley et al. 2013). The degradation of storage reserves including starch (and proteins) can contribute to the embryo growth potential (Rosental et al. 2014).

CaOx crystals were present widely in cotyledons and seldom in embryonic axes of both dormant and non-dormant embryos. We did not detect any differences in localization and quantity of CaOx crystals during stratification. We can suppose that the demand for Ca necessary for germination will be make later after breaking the dormancy; together with other storage compounds. Druse crystals of CaOx have been reported in some protein bodies of jojoba and *Eucalyptus* seeds (Buttrose and Lott 1978); Collada et al. (1993) showed druse-like calcium-rich crystals with a proteinaceous matrix in the protein bodies of beech seeds. Nevertheless CaOx as the storage compound is not utilized during stratification.

The most expressive changes induced by moist stratification we detected in the localization and exhausting of storage proteins both in cotyledons and in embryonic axes. In the cotyledons of beech seeds, the majority of storage proteins correspond to 11S legumin-type and 7S vicilin-type globulins (Collada et al. 1993). They are accumulated in PSVs rather than in protein bodies (Ibl and Stoger 2012). The first changes of PSVs were observed during the first week of stratification and they probably correlate with the increase of water content in embryos after imbibition. Proteinaceous content gradually disappeared and PSVs diminished. Afterwards, during stratification proteins were detected in the cytoplasm, while small storage vacuoles fused to form central vacuoles. Following Pawłowski (2010), we suggest that the storage proteins were hydrolysed to provide amino acids for new protein synthesis after seed imbibition. A high level of protein synthesis was observed in beech seeds as early as within 48 h of imbibition by Szczotka et al. (2003). Our observations are complementary to the results of Pawłowski (2007). He undertook a proteomic analysis of beech seeds during seed dormancy breaking and germination. His results showed that most of the changes in protein expression were observed at the end of stratification and in the germinated seeds. Our experiments support his conclusions at the histological level.

We can conclude that both the decrease in endogenous ABA and the increase in fumarase activity in embryonic axes can be used as indicators of the depth of dormancy in beechnuts. The main storage compounds (starch, proteins and CaOx) were found to be present in beech embryos during stratification. No differences in size and location of starch grains and/or CaOx crystals linked with stratification were detected. The storage proteins localized in vacuoles were exhausted during the process of stratification; the first histological changes were evident after imbibition. Three tested parameters bring three different directions of dormancy breaking characterization. The ABA level correlates directly with the depth of dormancy (that determine next seeds development); fumarase activity increase represents the release of inhibition and the start of the processes towards germination; the changes in storage proteins distribution and utilization depend on water uptake during the start of stratification that induce protein hydrolysis yielding transportable amino acids essential for germination.

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