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ORIGINAL RESEARCH

# Ascorbate and glutathione metabolism during development and desiccation of beech (*Fagus sylvatica* L.) seeds

**Stanislawa Pukacka · Ewelina Ratajczak**

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**Abstract** The ascorbate-glutathione system was studied during development and maturation of beech (*Fagus sylvatica* L.) seeds, the classification of which in the orthodox category is controversial. This study revealed an increase in glutathione content after acquisition of desiccation tolerance, which was more intensive in embryonic axes than in cotyledons. During seed maturation, the redox status of glutathione markedly changed toward the more reducing state, especially in cotyledons. Ascorbic acid content decreased during maturation, mostly in cotyledons. Activities of the enzymes of the ascorbate-glutathione cycle—ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2)—were markedly higher in embryonic axes than in cotyledons throughout the study period. In the course of seed maturation, the activities of these enzymes decreased. Importance of the ascorbate-glutathione cycle in desiccation tolerance of beech seeds was discussed in relation to results for typical orthodox and recalcitrant seeds of other broadleaved species.

**Keywords** Ascorbic acid · Ascorbate peroxidase · Dehydroascorbate reductase · Glutathione reductase · Monodehydroascorbate reductase · Redox balance

S. Pukacka (✉) · E. Ratajczak
Seed Biochemistry Lab, Institute of Dendrology,
Polish Academy of Sciences, 62-035 Kórnik, Poland
e-mail: spukacka@man.poznan.pl

## Introduction

Seed development can be divided into three stages: embryogenesis, seed filling (cell enlargement and reserve deposition) and maturation. Seed development starts with a zygotic embryo and ends with a mature seed that is capable of germination. During seed development, metabolic activity and mitochondrial respiration rate are high. These high rates suggest that a significant amount of reactive oxygen species (ROS) can be generated at this time. The level of ROS is strictly controlled by antioxidant mechanisms, which prevent the undesirable, destructive effects of their activity. However, ROS also perform important roles as signaling molecules regulating plant and seed growth and development as well as in coordinating responses to abiotic and biotic stress (Desikan et al. 2005; Bailly et al. 2008; Oracz et al. 2009). The ascorbate system may play a central role in embryogenesis and seed filling (Arrigoni et al. 1992; De Gara et al. 2003; De Tullio and Arrigoni 2003). Ascorbic acid (ASA) may affect the progression of the cell cycle (Noctor and Foyer 1998; Potters et al. 2002). ASA may also influence cell growth by modulating the expression of genes involved in hormonal signalling pathways (Pastori et al. 2003). In developing seeds, ASA modulates the synthesis of plant hormones such as ethylene, GA and ABA (Rodriguez-Gacio and Matilla 2001; Peng and Harberd 2002, Nambara and Marion-Poll 2003). In this way, ASA indirectly affects seed growth. Most studies of the role of ROS in seed development have focused on how seeds acquire tolerance to desiccation. During development of orthodox (desiccation-tolerant) seeds, acquisition of desiccation tolerance occurs during seed feeling and is associated with various biochemical events such as specific protein synthesis, oligosaccharide accumulation and activation of antioxidant defense systems

(Vertucci and Farrant 1995; Kermode and Finch Savage 2002). In orthodox seeds, ROS production during maturation drying and upon rehydration is under control of the antioxidant defense system. In contrast, ROS overproduction and an insufficient antioxidative system are considered to be the main cause of cellular damages during desiccation of recalcitrant (desiccation-sensitive) seeds (Pukacka and Ratajczak 2006, 2007a).

The ascorbate-glutathione (A-G) cycle is an important pathway of indirect or direct ROS scavenging in cells (Noctor and Foyer 1998; Foyer and Noctor 2003). Earlier research confirmed its role in plant defense reactions to many abiotic stresses including desiccation (Allen et al. 1997; Foyer et al. 1997). Ascorbate peroxidase (APX) is an important enzyme participating in cell detoxification due to its presence in all cell compartments and its high affinity for hydrogen peroxide ($H_2O_2$). The electron donor is ASA, the pool of which is regenerated by monodehydroascorbate reductase (MR) and dehydroascorbate reductase (DHAR) with the participation of reduced glutathione (GSH) and NADPH. GSH is oxidized to GSSG and reduced back to GSH by glutathione reductase (GR). Besides participating in the A-G cycle, ASA can react directly with hydroxyl radicals, superoxide anion radicals and singlet oxygen (Noctor and Foyer 1998). ASA is able also to reduce the oxidized form of α-tocopherol, an important antioxidant associated with cell membranes (Sattler et al. 2004).

Tommasi et al. (1999) showed differences in ASA content and ascorbate-related enzymes (such as APX, MR and DHAR) between orthodox seeds (*Pinus pinea* L., *Vicia faba* L. and *Avena sativa* L.) and recalcitrant seeds (*Ginkgo biloba* L., *Aesculus hippocastanum* L., *Quercus cerris* L. and *Cycas revoluta* Thunb.) before and after dehydration. In their study, recalcitrant seeds were characterized by high ascorbate content and high activity of enzymes of the ascorbate system compared with orthodox seeds, in which ASA content and APX activity were significantly diminished or totally disappeared during seed maturation and desiccation. No such regularity was found in the case of orthodox and recalcitrant seeds of Norway maple (*Acer platanoides* L.) and sycamore (*A. pseudoplatanus* L.) (Pukacka and Ratajczak 2007a).

On the other hand, the placement of *Ginkgo biloba* and *Cycas revoluta* seeds in the recalcitrant category is rather doubtful (Dickie and Pritchard 2002).

Glutathione is the major antioxidant and redox buffer in plant cells (Schafer and Buettner 2001). In response to oxidative stress, GSH is converted to GSSG, which changes the redox status of the cell to be a more oxidizing status. Kranner and Grill (1993) observed accumulation of GSSG in pea seeds in the dry state. Kranner et al. (2006) supposed that glutathione half-cell reduction potential is an important marker of lethal changes caused by oxidative stress in plant cells including seed cells during aging and desiccation caused by oxidative stress. Pukacka and Ratajczak (2007a) found that during development of Norway maple (orthodox) and sycamore (recalcitrant) seeds, the level of glutathione reflected the acquisition of desiccation tolerance, and in mature orthodox seeds it was nearly twice or three times as high as in recalcitrant seeds, in embryonic axes and cotyledons, respectively. The glutathione redox status was also involved in desiccation tolerance of orthodox seeds (Pukacka and Ratajczak 2007a). During maturation, this status was in a more reduced state in orthodox seeds than in recalcitrant ones.

Beech (*Fagus sylvatica* L.) is one of the most important broadleaved species in European forestry. Beech seeds were acknowledged as intermediate by Bonner (1990), Gosling (1991), León-Lobos and Ellis (2002) and Pukacka et al. (2003) because they have greater sensitivity to drying and storage conditions than do typical orthodox seeds of other species (Pukacka and Wojkiewicz 2003; Pukacka and Ratajczak 2007b). On the other hand, Poulsen (1993) and Poulsen and Knudsen (1999) classified them as orthodox. Recent data (Kalemba et al. 2009) on the acquisition of desiccation tolerance in beech seeds during their maturation (mainly in relation to LEA protein accumulation with additional parameters such as mass maturity, germination capacity, ABA level and the ratio of heat-stable proteins accompanying this process) indicated a typical orthodox behavior of these seeds.

The aims of the present study were to investigate the activity of ascorbate and glutathione metabolism in beech seeds during seed development, maturation and desiccation and to determine the differences and similarities of beech seeds to orthodox and recalcitrant seeds of two closely related broadleaved tree species of the temperate zone: Norway maple and sycamore.

## Materials and methods

The seeds were collected 11–19 weeks after flowering (WAF) from one single tree growing in the Kórnik Arboretum in western Poland, during the 2006 growing season. Seeds were collected in 1- or 2-week intervals, beginning at 11 WAF, when the embryonic axes and cotyledons were morphologically completely formed as solid tissue and they could be separate for analyses. After harvest, the seeds were immediately transported to the laboratory. At each harvest, the moisture contents (MC) were determined using three samples of 10 cotyledons and 50 embryonic axes that were dried at 100°C for 24 h. Suitable samples of embryonic axes and cotyledons were weighed and then stored at −80°C until use for another analyses.

### Tolerance to desiccation of beech seeds during development

Acquisition of desiccation tolerance was determined by testing seed germination after previous desiccation to approximately 10% of water content. Results were confirmed by testing ionic conductance as described in Kalemba et al. (2009).

### Ascorbate assays

ASA and DHA content were measured using the procedure described by Kampfenkel et al. (1995). The assay is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by ascorbic acid in acidic solution. $Fe^{2+}$ forms complexes with bipyridyl, yielding a pink color with the absorbance peak at 525 nm. Each measurement was made using three samples of 50 embryonic axes or 10 cotyledons. The samples were homogenized in cold 6% (w/v) TCA. The homogenate was centrifuged for 10 min at 12,000*g* and the supernatant was assayed. The data presented are the means and standard deviations of two measurements with three different extracts.

### Glutathione assays

Glutathione in the reduced (GSH) and oxidized (GSSG) forms were determined according to Smith (1985). Samples of 50 embryonic axes or 10 cotyledons were homogenized in 5% (w/v) sulfosalicylic acid on ice and centrifuged at 10,000*g* for 20 min. A 1 ml aliquot of the supernatant was removed and neutralized by adding 1.5 ml of 0.5 M K-phosphate buffer (pH 7.5). This sample was used for the determination of total glutathione (GSH + GSSG). Another 1 ml of neutralized supernatant was pretreated with 0.2 ml of 2-vinylpyridine for 1.5 h at 25°C to mask GSH and allow determination of GSSG alone. Both samples were extracted twice with 5 ml of diethylether. The reaction mixture contained 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.5) with 5 mM EDTA, 0.2 ml of 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH, 0.1 ml (1 unit) of glutathione reductase type III (Sigma–Aldrich, Poland) and 0.1 ml of seed extract. The change in absorbance at 412 nm was followed at 25°C. A standard curve was prepared by using the GSH (Sigma–Aldrich) standard. Results are expressed as means (± SD) of two measurements conducted with three different extracts.

### Enzyme extractions and assays

All extraction procedures were conducted at 4°C. Samples of 50 embryonic axes or 10 cotyledons each were ground in liquid nitrogen and homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 20% polyvinylpolypyrrolidone (PVPP) for 10 min. Homogenates were filtered through two layers of cheesecloth and centrifuged at 4°C at 15,000*g* for 20 min. The supernatant was desalted on Sephadex G25 (Sigma–Aldrich) columns according to Helmerhorst and Stokes (1980).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by following the decrease in absorbance at 290 nm owing to ASA oxidation for 5–10 min, according to Nakano and Asada (1981). The reaction mixture contained 1 ml of 0.68 mM ASA and 0.1 mM EDTA in 0.1 M phosphate buffer (pH 7.0), 1 ml of 4 mM $H_2O_2$ and 50–100 µl of the enzyme extract. APX activity was expressed as nanokat (i.e. nmol ASA $min^{-1}$) per mg protein.

Monodehydroascorbate reductase (MR, EC 1.6.5.4) activity was tested according to Zhang and Kirkham (1996). The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 0.1 mM NADH, 2.5 mM ASA, four units of ASA oxidase (Sigma–Aldrich) and 50 µl of enzyme extract. NADH oxidation was monitored at 340 nm. MR activity was expressed as nanokat (i.e. nmol NADH $min^{-1}$) per mg protein.

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured according to Arrigoni et al. (1992). The reaction mixture contained 0.9 ml of 0.05 M potassium phosphate buffer (pH 6.3), 100 µl of 13.5 mM dehydroascorbate (DHA), 100 µl of 13.5 mM reduced glutathione (GSH) and 100–200 µl of enzyme extract. The DHAR activity was monitored by following the formation of ascorbate at 265 nm for 5 min. Enzyme activity was expressed as nanokat (i.e. nmol ASA $min^{-1}$) per mg protein.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined according to Esterbauer and Grill (1978), by following the rate of NADPH oxidation at 340 nm. The assay mixture contained 0.5 mM NADPH, 10 mM oxidized glutathione (GSSG), 10 mM EDTA, 0.1 M phosphate buffer (pH 7.8), and 50–100 µl of the enzyme extract. GR activity was expressed as nanokat (i.e. nmol NADPH $min^{-1}$) per mg protein.

The protein content of crude enzyme extracts was estimated according to Bradford (1976), using BSA as a standard.

### Statistical analysis

Data are presented as means ± standard deviation of three or six replicates. The statistical differences between terms of seed development for embryonic axes and cotyledons were tested using an analysis of variance (ANOVA) and LSD test ($P < 0.05$) for multiple comparisons.

## Results

Beech seeds were collected starting 11 weeks after flowering (WAF). At that time, the embryos were large enough to allow isolation of embryonic axes from cotyledons so they could be analyzed separately. Seed development was observed until 18 WAF, when seeds were fully mature, and during the next week they started to be shed. On the last date of collection, the seeds were gathered under the tree and then desiccated in the laboratory, at room temperature and 45–50% relative humidity, until they reached 10% moisture content (MC). During development, starting from 14 WAF, substantial differences in water loss between embryonic axes and cotyledons were observed (Fig. 1). The MC of embryonic axes was markedly higher than that of cotyledons. Desiccation tolerance tests (germination capacity and electrolyte leakage after drying freshly harvested seeds to 10% MC) showed that beech seeds became tolerant to desiccation at 16 WAF (Fig. 1). At that time, in seeds previously desiccated to ca 10% MC, electrolyte leakage decreased to a low level. Simultaneously, germination capacity increased to 86%. In embryonic axes and cotyledons, the ASA content decreased during seed development and maturation. The decrease was strongest in cotyledons at 14 WAF. The total pool of ASA was higher in embryonic axes than in cotyledons throughout the maturation period (Fig. 2). GSH content of embryonic axes, after a clear decrease at 16 WAF, started to increase in the following weeks of maturation and desiccation (Fig. 3). A significant change in redox status, represented by the GSH:GSSG ratio, toward a more reducing state (from 1.7 to 2.9) was observed during seed maturation and desiccation. As in the case of ASA, cotyledon GSH content

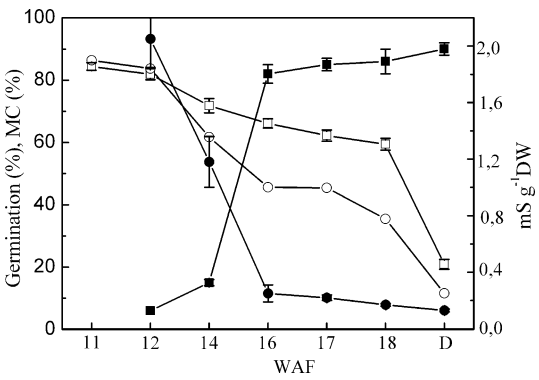


**Fig. 1** Changes in moisture content (MC) in embryonic axes (*open square*) and cotyledons (*open circle*), germination capacity (*filled square*) and electrolyte leakage (*filled circle*) of beech (*Fagus sylvatica*) seeds during development. Germination capacity and electrolyte leakage were determined after previous desiccation of seeds to ca 10% MC. Data are means of three replicates ±SD

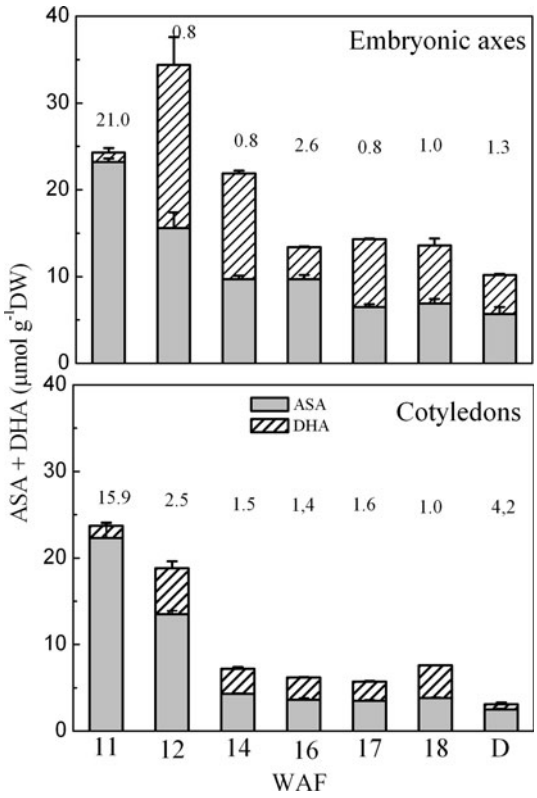


**Fig. 2** Changes in ascorbate (ASA) and dehydroascorbate (DHA) contents and ASA:DHA ratio in embryonic axes and cotyledons of *F. sylvatica* seeds during development and desiccation. Data are means of six replicates ±SD

decreased until 16 WAF, and during seed maturation it slightly increased. From 14 WAF until the time of shedding and desiccation, the level of GSSG strongly decreased and the redox status markedly changed toward a more reducing state (Fig. 3). In cotyledons, a large decrease in ASA and GSH took place at 14 WAF, when the differences between water contents of embryonic axes and of cotyledons became significant (Fig. 1). The patterns of APX, DHAR, MR and GR activities were similar in embryonic axes and cotyledons (Fig. 4). The activities of all these enzymes were highest in embryonic axes, at the beginning of the study period. Later, enzyme activities decreased, and this decrease was stronger in cotyledons. In embryonic axes, enzyme activities were markedly higher than in cotyledons, and from 16 WAF until desiccation they remained at a stable level. After desiccation of seeds, no increase in the activities of enzymes of the A-G cycle was observed. The content of soluble protein, expressed as mg $g^{-1}$ DW (Fig. 5), of embryonic axes, was significantly higher than that of cotyledons during most of the developmental period. Between 12 and 14 WAF it decreased drastically and remained at a stable level for the next 4 weeks and then diminished significantly before shedding and after desiccation of seeds.

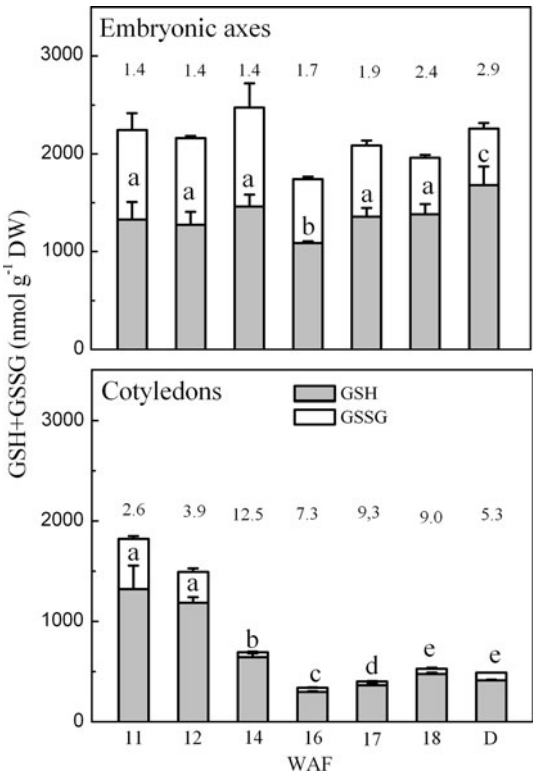


**Fig. 3** Changes in the contents of reduced (GSH) and oxidized (GSSG) forms of glutathione and GSH:GSSG ratio in embryonic axes and cotyledons of *F. sylvatica* seeds during development and desiccation. Data are means of six replicates ±SD. *Bars* of GSH labeled with the same letter do not differ significantly

## Discussion

The study period encompassed part of embryogenesis, seed filling, maturation and desiccation. Developmental changes in the A-G system were observed in embryonic axes and cotyledons until seeds were fully mature and after desiccation. Moisture content measured separately in embryonic axes and cotyledons showed that in cotyledons the water level was significantly lower than in embryonic axes starting from 14 WAF, i.e. during maturation and drying. The decrease in MC at 14 WAF in cotyledons was greater than in embryonic axes (from 83.7 to 61.7% and from 81.9 to 71.8%, respectively). Such a large decrease in MC could affect the metabolism and cause the remarkable decline in ASA and GSH content of cotyledons at 14 WAF. Maximal ASA content and ASA:DHA ratio were recorded at 11 WAF (21.0 for embryonic axes and 15.9 for cotyledons). High values of ASA contents and ASA:DHA ratio are characteristic for periods of active cell divisions (Arrigoni et al. 1992). The high activities of APX and the enzymes of ASA recycling, especially MR, indicate that embryogenesis in beech seed embryonic axes lasted until 11 WAF. In embryonic axes, a marked decrease in MR activity was accompanied by a temporary increase in DHA level, which was observed at 12 WAF. The two processes are correlated (Arrigoni 1994). Actively dividing cells use large amounts of ASA (Liso et al. 1984), so a high level of MR activity is needed in actively dividing cells (Paciolla et al. 2001;

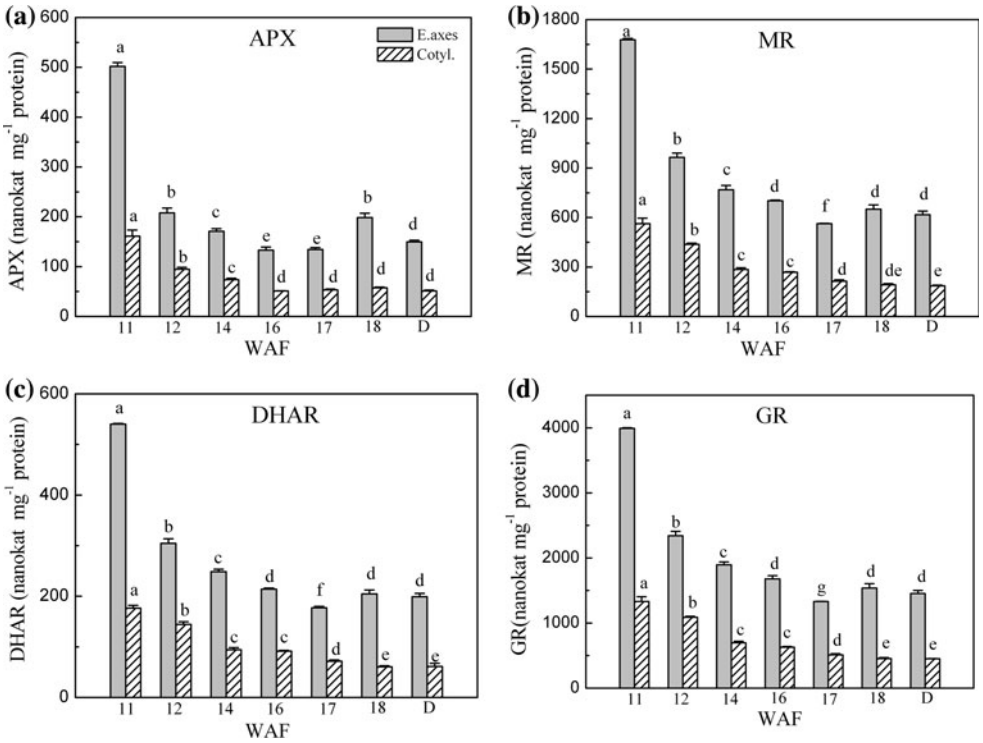


**Fig. 4** Changes in the activities of ascorbate peroxidase (APX) (a), monodehydroascorbate reductase (MR) (b), dehydroascorbate reductase (DHAR) (c) and glutathione reductase (GR) (d) in embryonic axes and cotyledons of *F. sylvatica* seeds during development and desiccation. Data are means of six replicates ± SD. Bars labeled with the same letter do not differ significantly

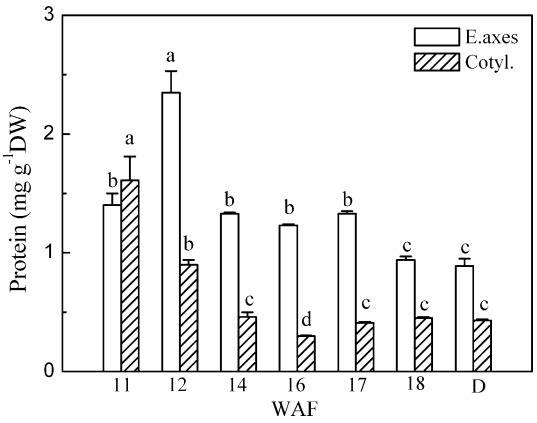


**Fig. 5** The protein content in crude enzymatic extracts from embryonic axes and cotyledons of *F. sylvatica* seeds during development and desiccation. Data are means of six replicates ±SD. Bars labeled with the same letter do not differ significantly

Potters et al. 2002). In cotyledons, this was less conspicuous: the decrease in activity of A-G cycle enzymes between 11 and 12 WAF was not as high as in the case of embryonic axes. In cotyledons, embryogenesis probably ended before 11 WAF. In cotyledons, from 11 WAF to the maturation and drying, activity of A-G cycle enzymes gradually decreased and was significantly lower than in embryonic axes. Similar results were obtained in research on ascorbate metabolism during development of orthodox Norway maple seeds (Pukacka and Ratajczak 2007a), however, in that species, during seed desiccation a marked increase of the activity of enzymes of A-G cycle was observed (Pukacka and Ratajczak 2007a). In beech seeds, activities of the enzymes of the A-G cycle in embryonic axes were almost three times higher than in cotyledons. Such large differences between embryonic axes and cotyledons were not observed in Norway maple seeds.

The pattern of changes in ASA content of beech seed embryonic axes and cotyledons was similar to that of Norway maple. In seeds of the both species after desiccation, a lower content of ASA (5–10 μmol $g^{-1}$ DW) was retained.

Changes in glutathione content of beech embryonic axes were similar to those in Norway maple, but its absolute content at the end of maturation was markedly lower in beech. Clear differences between those species were observed in cotyledons. In beech seeds cotyledons, glutathione content substantially declined during seed maturation, and before desiccation (18 WAF) it was about fourfold lower than during the corresponding developmental phase of Norway maple seed cotyledons. In desiccated seeds, these differences were maintained. Although the glutathione redox status, represented by the GSH:GSSG ratio, in beech seed cotyledons during seed maturation and desiccation was higher than in the same organs of Norway maple (9.0 vs. 4.4), beech seed cotyledon cells probably had a lower redox potential than those of Norway maple because the glutathione redox potential depends on the ratio of GSH to GSSG and on the absolute GSH content of the cell (Schafer and Buettner 2001). This may be an important reason for the greater sensitivity of beech seeds to drying and storage as compared to Norway maple seeds. Glutathione redox potential is an important factor in the maintenance of seed viability after desiccation and during storage (Kranner and Birtić 2005).

In conclusion, our research showed that A-G cycle metabolism in beech seeds during their development, maturation and desiccation proceeds similarly as in the typically orthodox Norway maple seeds. However, the potency of the antioxidant system of A-G cycle in beech seeds is lower in some points: first, a weak reaction of APX, MR, DHAR and GR to desiccation stress and second, a lower concentration of glutathione in embryonic axes and cotyledons than observed in Norway maple seeds. On the other hand, beech seeds indicate some beneficial features that help them to maintain viability during desiccation specifically, the higher activity of enzymes of A-G cycle in embryonic axes than in cotyledons and higher level of glutathione in these organs. This quality suggests that embryonic axes of beech seeds are preferentially protected against oxidative stress during desiccation.

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