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Research article

Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds

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Abstract

The behaviour of the *Ginkgo biloba* L. seeds was studied during storage at 4 and 25 °C. When stored at 25 °C, all the seeds died in 6 months. Cold temperatures preserved seed tissue viability for 1 year but did not preserve their capability to germinate, since such capability decreased after 6 months. A significant increase in lipid peroxidation occurred in the seed both in the embryo and in the endosperm. During storage a progressive deterioration of the endosperm tissues was evident. The two major water soluble antioxidants, ascorbate (ASC) and glutathione (GSH), showed different behaviour in the two conditions of storage and in the two main structures of the seed, the embryo and the endosperm. The ASC content of embryos and endosperms remained quite unchanged in the first 9 months at 4 °C, then increased. At 25 °C a significant decrease in the ASC content in the embryos was evident, whereas it remained more stable in the endosperm. The GSH pool decreased at both storage temperatures in the embryos. As far as the ASC–GSH redox enzymes are concerned, their activities decreased with storage, but changes appeared to be time-dependent more than temperature-dependent, with the exception of the endosperm ascorbate free radical (AFR) reductase (EC 1.6.5.4), the activity of which rapidly decreased at 25 °C. Therefore overall the antioxidant enzymes were scarcely regulated and unable to counteract oxidative stress occurring during the long-term storage.

Keywords: Antioxidants; Ascorbate; *Ginkgo biloba*; Glutathione; Recalcitrant seeds; Seed storage

1. Introduction

A number of different plant species, both of tropical and temperate origin, produce seeds considered as recalcitrant because, differently from the orthodox seeds, they are shed from the mother plant with a high moisture content and are desiccation-sensitive. They generally directly pass from development to germination, even if in some cases a dormant phase occurs [4,9,32]. There are many types of recalcitrant seeds with different desiccation tolerance; moreover some species produce seeds with a behaviour intermediate between orthodox and recalcitrant [4]. For few recalcitrant seeds, there is consistent literature on some aspects of seed development [15], on the basic physiology and response to desiccation [11,17,28,35,38], as well as ecology and evolution [29,31,39]. However many questions are still open, for example concerning the lifespan of seeds of many species, long term-storage and the processes occurring during loss of viability [30], also because there are a wide range of differences in the post harvest responses of recalcitrant seeds. Some data report that many recalcitrant seeds, particularly those of tropical origins, are also chilling sensitive and cannot be stored at temperatures below 15 °C. Their storage lifespan is quite short varying from 2 weeks to some months [8,28]. Recalcitrant seeds of temperate origin, like *Aesculus hippocastanum* or *Quercus robur,* seem to have a storage lifespan of some months or 2–3 years, respectively [8]. During short term storage, embryonic axes of recalcitrant seeds undergo to ultrastructural changes similar to those occurring during orthodox seed germination, among which increase in cell size, extensive vacuolization, consumption of reserves and development of mitochondria.

Abbreviations: AFR, ascorbate free radical; ASC, ascorbate; DHA, dehydroascorbate; GLDH, galactono-γ-lactone dehydrogenase; GSH, glutathione; GSSG, oxidized glutathione; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

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Such changes imply an additional water requirement and for this reason recalcitrant seeds are exposed to a progressive water depletion during storage [28]. It is well known that the antioxidant systems play a pivotal role in limiting damage during water stress in vegetative tissues [27,33] and during orthodox seed development and germination [2,3,36] by removing the reactive oxygen species (ROS) generated in these conditions. Some data relate the loss of the germination capability of *Shorea robusta* recalcitrant seeds to a drop in the antioxidant system efficiency [7]. Moreover, the decrease in specific activities of antioxidant enzymes seems to be directly associated with loss of viability in *Quercus robur* where the maintenance in the ascorbate pool alone was unable to prevent or delay peroxidative damage induced by desiccation [20]. The origin of this damage in drying recalcitrant seeds has been attributed to the formation of ROS in conjunction with a decline in protection afforded by antioxidants [6,17–20]. Some authors recently reported that the antioxidant activity, at least the lipid soluble component, varies during seed storage, but it was not related to seed viability in some Australian species [26]. The presence of antioxidant systems have been also reported in *Ginkgo biloba* L. seeds which, diversely from orthodox seeds, contain large amount of ascorbate (ASC), a certain amount of dehydroascorbate (DHA), the oxidized form of the ASC, and the enzymes of the ASC metabolism [35,37]. No information is available about glutathione (GSH) metabolism in *G. biloba* seeds and antioxidant behaviour during seed storage. The morphology of *G. biloba* seeds, such as the well developed embryo contained in an haploid endosperm from which it is easy to separate it, makes them particularly suitable as a "model system" for seed physiology studies. Consistently, *G. biloba* seeds, have already been used as a model for demonstrating that the desiccation rate is a key factor for desiccation tolerance in seeds [25]. For their dimensions, tropical origin, elevated water content, and desiccation sensitivity *G. biloba* seeds seem to be recalcitrant [25]; however, this question is still under debate because some authors consider them as orthodox ones [1]. Not much information is available about lifespan and viability in different storage conditions of these seeds.

The goal of this work was to obtain information about the lifespan of the *G. biloba* seeds, to verify the possibility of their conservation in middle and long term and to investigate the putative relation between seed viability, germinability and antioxidant metabolism. The content and the redox state of ASC and GSH as well as the enzymes involved in the metabolism of these redox pairs were studied during storage of *G. biloba* seeds at different temperatures.

2. Results

2.1. Seed viability and germinability

Among the freshly collected *G. biloba* seeds, only 80% contained an embryo. The viability of the seeds during storage at 4 and 25 °C was expressed as a percentage of the embryos showing respiratory capability (Table 1). At the beginning of storage 100% of the embryos were viable (i.e. positive to formazans test, see Section 4); after 3 months the percentage of viable embryos delete had decreased both at 4 and 25 °C. After 6 months the number of viable embryos had decreased up to 53% at 25 °C, while at 4 °C only up to 90%. All the embryos from the seeds stored at 25 °C were no longer viable after 9 months, whereas at 4 °C embryos viability of 87% was maintained for 12 months.

Table 1
Ginkgo biloba seed viability during storage at 4 and 25 °C
Seed viability was measured with TTC test on isolated embryos. The results are given as the mean value of 10 experiments ± S.D. Values with different letters indicate differences statistically significant according to the Student's *t*-test ($P < 0.05$); nd = not determined

Storage (months)	Viable embryos (%)	
	4 °C	25°C
0	100 a	100 a
3	97 ± 3 a/b	93 ± 4 b
6	92 ± 2 b	53 ± 10 d
9	90 ± 5 b/c	0
12	87 ± 4 c	nd

Table 2 reports data on germinability, and on water content of the embryos and the endosperms from the *G. biloba* seeds stored at the two different temperatures. At the beginning of storage, 100% of the seeds equipped with embryos were able to germinate. The germinability of the seeds lowered to 99%, 86%, 13% and 7% after 3, 6, 9 and 12 months of storage at 4 °C, respectively (Table 2). When the seeds were stored at 25 °C their germinability decreased much more rapidly; after 6 months more than 50% of the seeds did not germinate, and after 9 months none of the seeds germinated. In the freshly harvested seeds the moisture content of the embryos was 67%; whereas that of the endosperms was significantly lower (36%). The water content decreased to a value of 52% in the embryos and of 28% in the endosperm after 12 months of storage at 4 °C. In the seeds stored at 25 °C for 6 months, the water content reached values similar to those of seeds maintained at 4 °C for 12 months (Table 2).

Fig. 1 shows cell viability in embryos and endosperm from freshly collected seeds (Fig. 1A, E), after 6 months at 4 °C (Fig. 1B, F) and 25 °C (Fig. 1C, G) and after 1 year at 4 °C (Fig. 1D, H). The embryo tissues did not show visible damage symptoms due to storage (Fig. 1D), even if the cotyledons became thinner at 25 °C (Fig. 1C) than at 4 °C (Fig. 1B). On the other hand, the endosperm tissues showed a progressive deterioration; dead cells appeared after 6 months of cold sto-

Table 2
Germinability and water content of seeds of *Ginkgo biloba* during storage at 4 and 25 °C. The results are given as the mean value of 10 experiments ± S.D.; nd = not determined

Storage (months)	Germinability (%)		Water content (%)			
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
			Embryos		Endosperms	
0	100	100	67 ± 0.7	67 ± 0.7	36 ± 1.6	36 ± 1.6
3	99 ± 1	80 ± 2	66 ± 0.6	60 ± 1.1	35 ± 1.2	33 ± 2.1
6	86 ± 2	46 ± 5	64 ± 0.6	51 ± 0.8	30 ± 1.1	27 ± 1.2
9	13 ± 3	0	63 ± 1.3	nd	28 ± 1.3	nd
12	7 ± 2	0	52 ± 0.9	nd	28 ± 0.8	nd

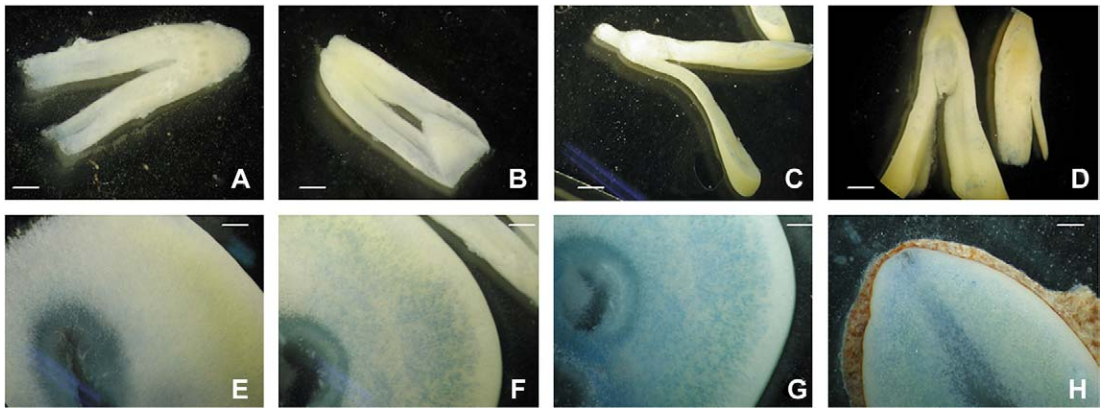

Fig. 1. Cell viability in *Ginkgo biloba* seed tissues.
1A: embryo from after shedding seed; 1B: embryo after 6 months of storage at 4 °C; 1C: embryo after 6 months of storage at 25 °C; 1D: embryos after 1 year of storage at 4 °C; 1E: endosperm from after shedding seed; 1F: endosperm after 6 months of storage at 4 °C; 1G: endosperm after 6 months of storage at 25 °C; 1H: endosperm after 1 year of storage at 4 °C. The blue coloured cells are dead. Bar = 1 mm.

rage (Fig. 1F) and their number increased after 1 year (Fig. 1H). In the seeds stored at 25 °C wide necrotic regions were already evident after 6 months (Fig. 1G).

2.2. Analyses of redox balance during storage

In order to understand whether the decrease in germinability was due to the onset of oxidative damage in the seed tissues, analyses of parameters indicative of oxidative stress and of antioxidant defences were performed throughout the period in which *G. biloba* seeds were still able to germinate (until 6 months for the seeds stored at 25 °C and 12 months for those maintained at 4 °C).

Fig. 2 shows data concerning lipid peroxidation in embryos and endosperms during storage. A significant increase in lipid peroxidation occurred in the seeds stored at 4 °C for 9 months both in the embryo and in the endosperm. However, at 25 °C a remarkable increase in lipid peroxidation was already evident after 3 months and further increased after 6 months.

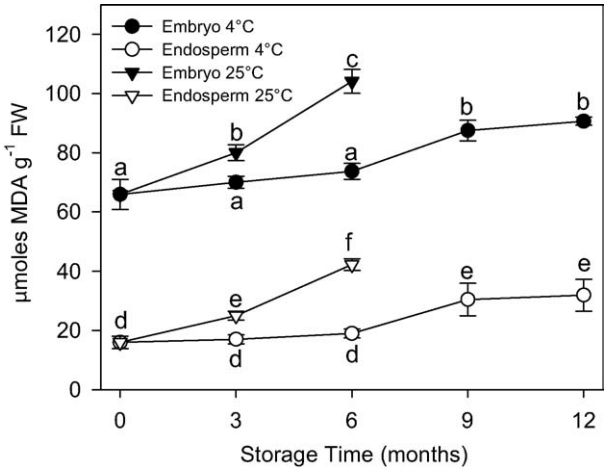

Fig. 2. Lipid peroxidation in the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C.
The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

Fig. 3 shows the ASC content in the embryos and in the endosperms during the storage period. Both embryos and endosperms were characterised by an ASC pool, reduced (ASC) plus oxidized (DHA) forms, in which the reduced form was prevalent. At 4 °C, the ASC content of embryos and endosperms remained quite unchanged in the first 9 months, then increased at 12 months of storage. On the contrary, at 25 °C a significant decrease in the ASC content of the embryos was evident after 3 months. At 6 months of storage, the embryos contained almost 50% less ascorbate than the embryos from freshly collected seeds. In the endosperm of the seeds stored at 25 °C, an increment in the ASC pool was evident after 6 months. The ASC/DHA ratio was 4.4 and 5.8, respectively, in the embryo and in the endosperm of the freshly collected seeds and increased to 14.7 and 7.9 in the embryos and in the endosperms, respectively, during 12 months of storage at 4 °C. On the contrary, no significant differences in the ASC redox state were evident in embryos and endosperms of the seeds maintained at 25 °C.

The total GSH content was about 20-fold higher in the embryos than in the endosperms (Fig. 4). In the embryos maintained at 4 °C the GSH content decreased progressively and such a decrease was earlier at 25 °C. In the endosperms GSH remained constant with a significant decrease occurring only after 12 months of the 4 °C storage (Fig. 4). Also for this redox pair, the reduced form was prevalent in the two main tissues of the seed. During cold storage, the GSH/GSSG ratio fell progressively from a value of 18.3 to a value of 10.3 in the embryos and from 33.25 to 16.15 in the endosperms after 1 year. At 25 °C the glutathione redox state only decreased in the embryos.

2.3. Ascorbate and glutathione related enzymes

ASC peroxidase activity behaved differently in embryos and endosperms (Fig. 5). The activity of the enzyme was higher in the embryos of the freshly harvested seeds and decreased progressively during cold storage. During storage at 25 °C the behaviour of the ASC peroxidase in the embryos was quite

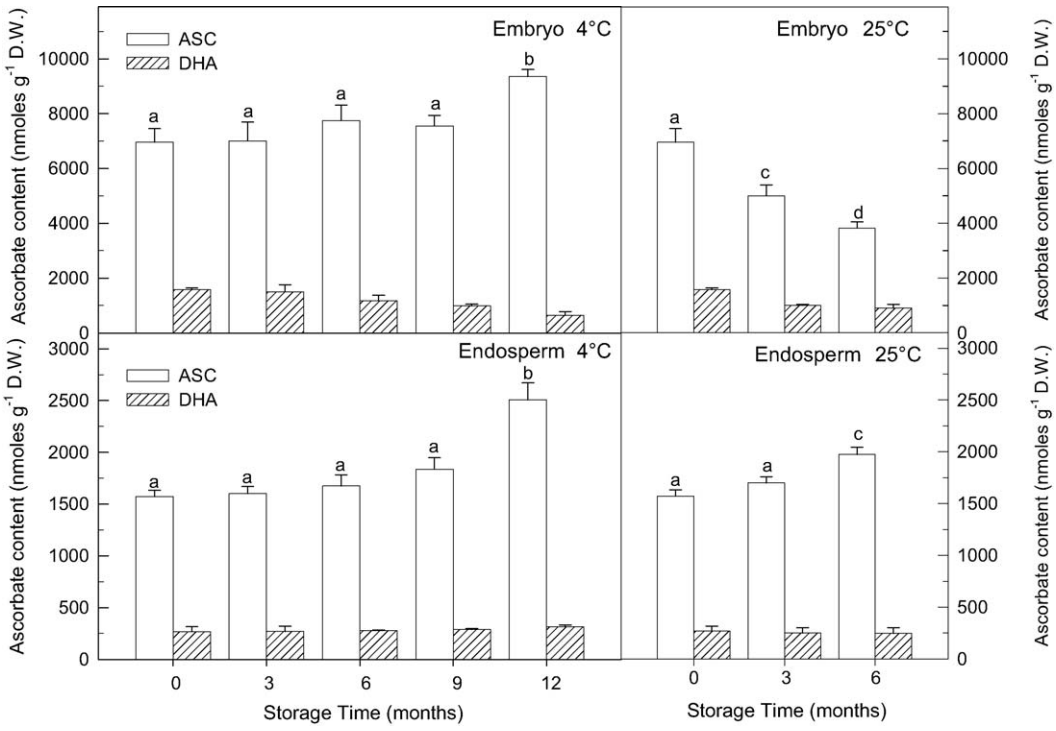

Fig. 3. Ascorbate and dehydroascorbate content in the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

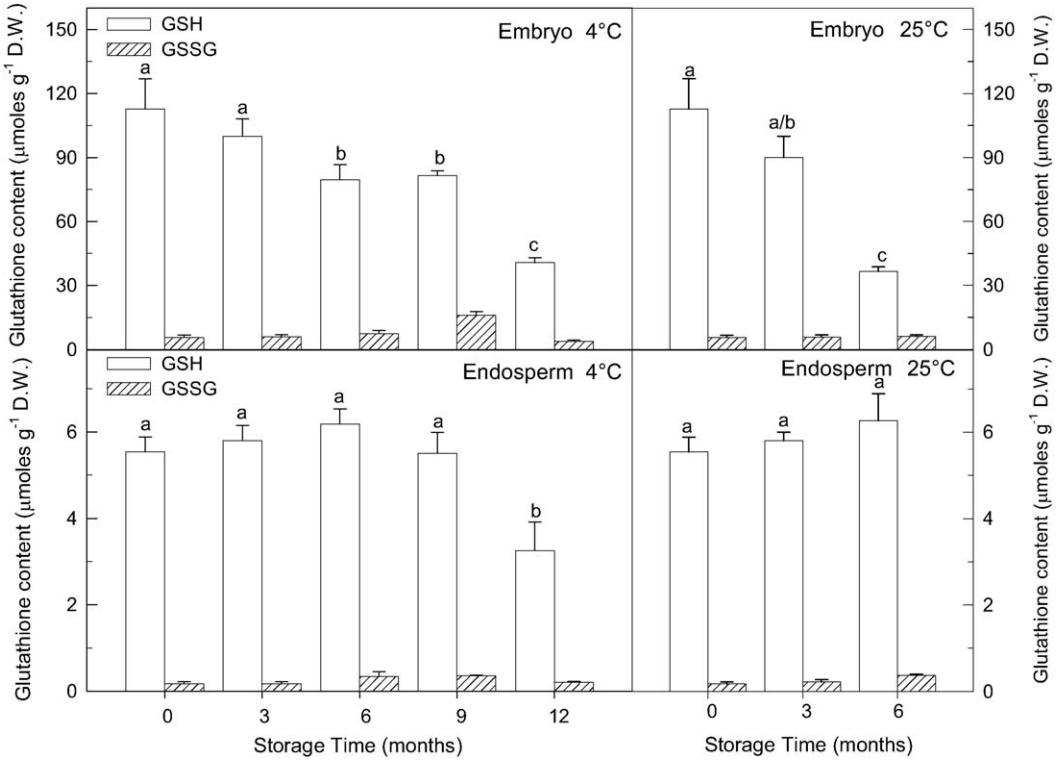

Fig. 4. Reduced and oxidized glutathione content in the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

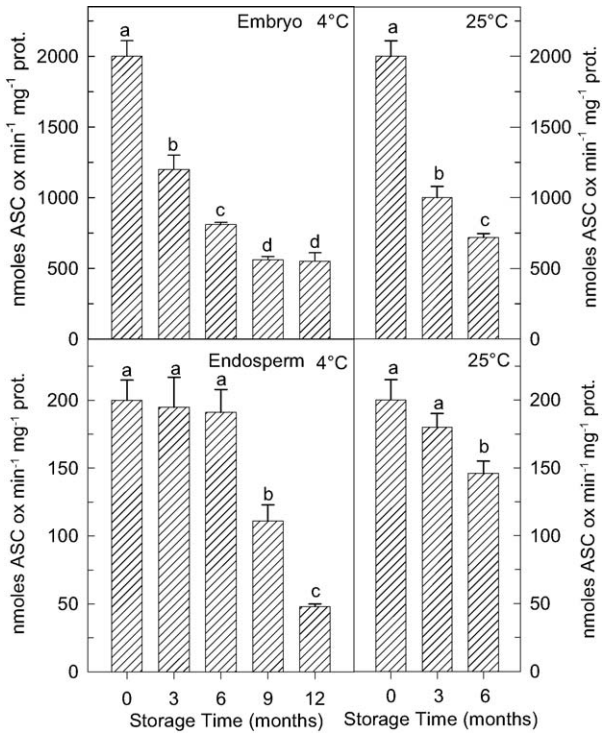

Fig. 5. Ascorbate peroxidase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

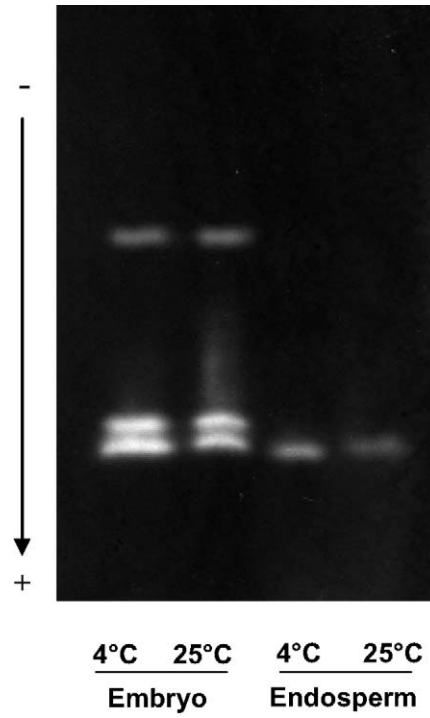

Fig. 6. Native PAGE of ascorbate peroxidase of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C for 6 months. 600 μg of protein was loaded per lane.

similar to that observed at 4 °C. In the endosperms ASC peroxidase activity was one order of magnitude lower in comparison with the embryos and decreased only after 9 and 12 months of cold storage. At 25 °C the decrease in ASC peroxidase activity was more rapid. These data were confirmed by native-PAGE (Fig. 6). The embryos showed three different proteins with ASC peroxidase activity with similar intensity after 6 months of storage at both temperatures. On the contrary, the endosperms had a single protein with ASC peroxidase activity that was less intense when the seeds were stored at 25 °C. The electrophoretic pattern of ASC peroxidase of embryos and endosperms in the freshly collected seeds is the same to that observed at 6 months of storage at 4 °C (data not shown).

In order to have more information on the global hydrogen peroxide detoxification capability of the different seed tissues during conservation, catalase was also analysed (Fig. 7). The activity of this enzyme remained more stable during storage: at 4 °C a significant decrease only occurred after 12 months both in the embryos and in the endosperms, while at 25 °C it remained unchanged in the embryos and only slightly decreased in the endosperms after 6 months. These results were confirmed by native-PAGE that also showed the presence of a single isoenzyme with the same migration rate in both embryos and endosperms (data not shown).

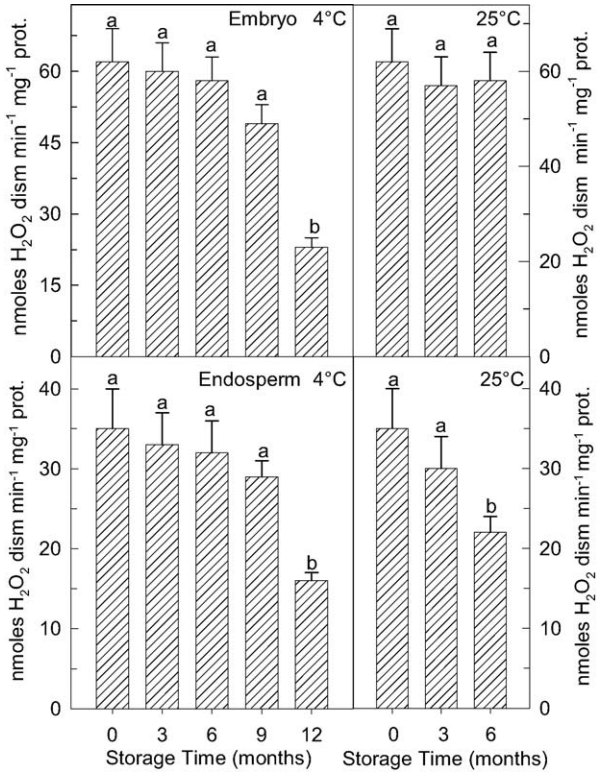

Fig. 7. Catalase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

The activities of the enzymes of ASC-GSH recycling, AFR reductase, DHA reductase and GSSG reductase were also analysed. At 4 °C AFR reductase did not show significant changes in the embryos and in the endosperms until 6 and 9 months of storage, respectively; after which it decreased. During storage at 25 °C its activity was unchanged in the embryos, whereas a remarkable decrease occurred in the endosperms after 3 months of storage (Fig. 8). DHA reductase and GSSG reductase behaved similarly to AFR reductase, but with a more pronounced decrease in the endosperm of the seeds maintained at 4 °C, and a less remarkable decrease in the same tissue of the seeds conserved at 25 °C (Figs. 9 and 10). The native-PAGE showed the presence of many proteins with DHA-reductase activity both in the embryos and in the endosperms. The pattern of DHA reductase in the freshly collected seeds is the same of that found at 6 months of cold storage both in the embryos and in the endosperms (data not shown). After 6 months of storage, the pattern of the DHA reducing proteins in the embryos remained similar at both temperatures, whereas in the endosperms of the seeds stored at 25 °C one protein with DHA reductase activity disappeared (Fig. 11).

The activity of the galactone-γ-lactone dehydrogenase (GLDH), the last enzyme in the ascorbate biosynthetic pathway, decreased in the endosperms after 9 and 12 months of cold storage, while it progressively increased in the embryos. No significant changes appeared when the seeds were stored at 25 °C (Fig. 12).

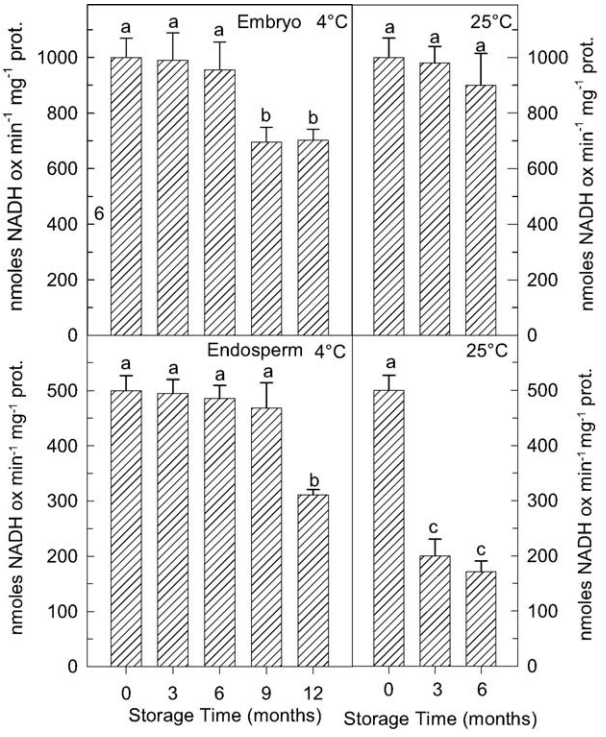

Fig. 8. Ascorbate free radical reductase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

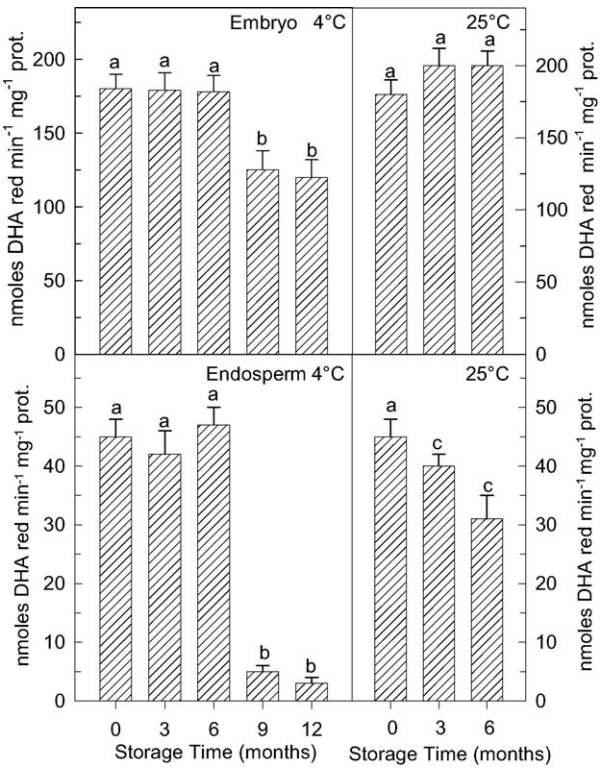

Fig. 9. Dehydroascorbate reductase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

3. Discussion

Although *G. biloba,* one of the oldest extant seed-bearing species, has been the subject of many studies from its first description, attributed to Linnaeus in the remote 1771, many aspects of its reproductive biology are still not completely known.

For long time it was believed as a "Prephanerogame" or an "oviparous" plant because, according to many authors in the past, the length of time from ovule initiation to seed germination was 12–14 months with fertilization taking place either immediately before or closely following abscission from the parent tree [16]. A recent study reports that the phenology of this species is less primitive than previously believed: Holt and Rotwell [21] report that the seeds removed directly from the mother plant contain completely developed embryos. Data here reported agree with these observations, because the seeds with embryos show 100% germinability after collection from the mother plant (Table 2). The lack of the embryos in 20% of the freshly harvested seeds is due to the development of the seed starting with pollination and not with the fertilization process, as it has been reported to occur in *G. biloba* to a certain extent [16]. Results here reported indicate that *G. biloba* seeds lose their viability in about 6 months when they are maintained at 25 °C (Table 1). Therefore, they remain viable for a much more prolonged time in comparison with

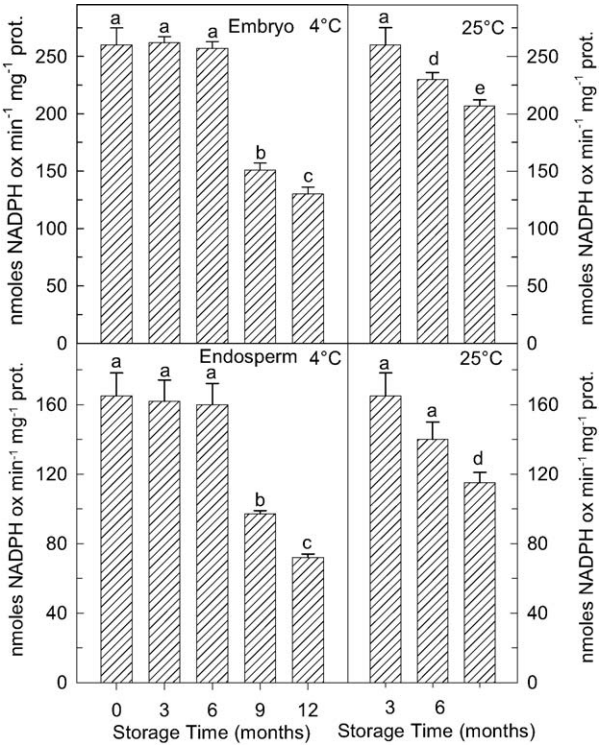

Fig. 10. Glutathione reductase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

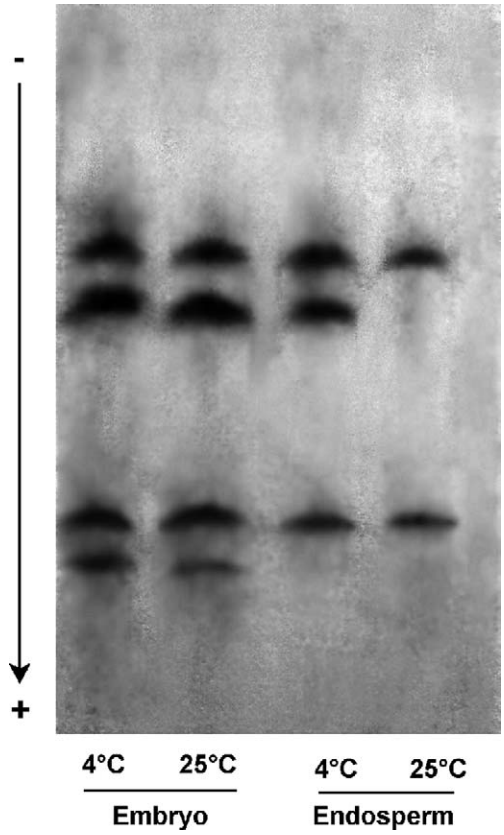

Fig. 11. Native PAGE of DHA reductase of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C for 6 months. 300 μg of protein was loaded per lane.

other recalcitrant species such as *Trichilia degeana,* a tropical species, the seeds of which lose their viability after 8 days at 25 °C [14], but comparable to that reported for temperate species like *Aesculus hippocastanum* [8]. The *G. biloba* seeds are not chilling sensitive, as expected for a temperate species. Cold storage preserves tissue viability for many months (Table 1), but only partly their germinability, that falls significantly after 6 months (Table 2). As far as the desiccation sensitivity is concerned, under the same level of water content (about 60%) remarkable differences in the germinability are evident both in seeds maintained at 4 and 25 °C. Indeed the water content of the seeds also seems not to be correlated with seed germinability. The *G. biloba* seeds do not show, at least in these experimental conditions, high dehydration sensitivity, in agreement with the authors which insert this species in an intermediate class of recalcitrance [25]. Time of storage seems to be more critical than temperature for the maintenance of the germinability. However, cold conservation remarkably prevents the oxidative damage that has been reported to occurring during storage [20,22,23]. Indeed, the level of lipid peroxidation is higher at 25 °C than at 4 °C both in the embryos and in the endosperm (Fig. 2). The increment in lipid peroxidation seems to be correlated to a decrease of germinability of the seeds. It is interesting to notice that the two major soluble antioxidants involved in the protective mechanisms against stress, ASC and GSH, show different behaviour during storage and differences are also evident between the two main structures of the seed, the embryo and the endosperm.

Few data are present in the literature on GSH metabolism in recalcitrant seeds. Hendry et al. [20] found that the GSH content was below detectable levels in the *Quercus* seeds. However, recalcitrant seeds of *G. biloba* contain an amount of GSH higher than that detected in orthodox germinating seeds [36] and similar to that observed in vegetative tissues also for its redox state [12,36]. A decrease in the total GSH content, in its redox state and in the GSSG reductase activity occurs during storage (Figs. 4 and 10) and this is accompanied to the loss of germinability, thus suggesting that the alteration of glutathione metabolism occurring under the storage conditions could be involved in the germinability decrease.

On the contrary, changes in ASC content are not strictly related to the seed germinability. Indeed the total ASC content (ASC + DHA), remarkably higher in the embryos than in the endosperms (4.6-fold), and its redox state, similar in the two parts of the seed, remained unchanged after 1 year of storage at 4 °C and decrease only in the embryos maintained at 25 °C (Fig. 3).

A different behaviour in the antioxidant content between various structures of the seed has often been pointed out. Particularly, Hendry et al. [20] report in the *Quercus* species that several of the major protective mechanisms against the activated oxygen species operate in different ways in the embryos and in the cotyledons. In *G. biloba* seeds the endosperm, a haploid tissue, plays a food storage role similar to the cotyledons in the *Quercus* genus.

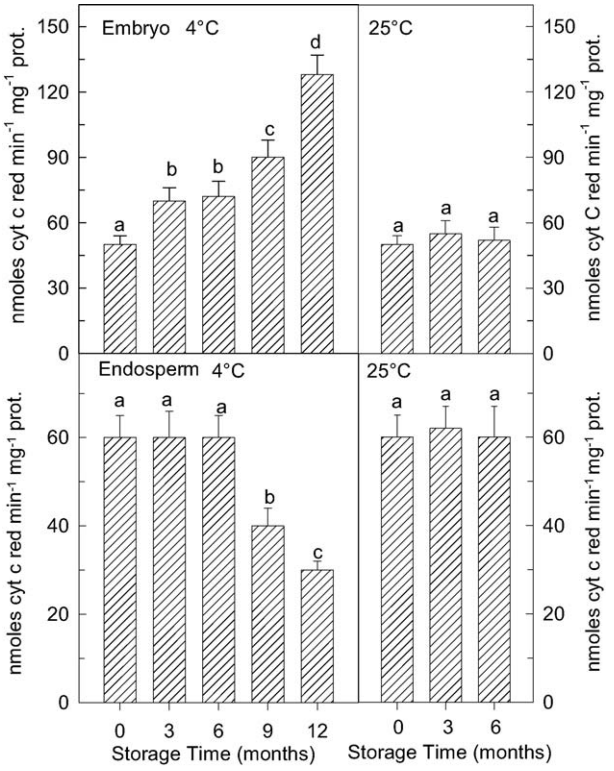

Fig. 12. Galactono-γ-lactone dehydrogenase activity of the embryos and endosperms of *Ginkgo biloba* seeds stored at 4 and 25 °C. The results are given as the mean value of six experiments ± S.D. Values with different letters indicate differences among endosperms or embryos statistically significant according to the Student's *t*-test ($P < 0.05$).

The ASC peroxidase activity decreased during storage (Fig. 5), even if, in particular in the embryos, its activity remains at higher levels than in germinating orthodox seeds [36]. The electrophoretic analyses shows the presence of a single protein in the endosperm (haploid tissue) and of three bands in the embryos (diploid tissues) and this supports the differences in the specific total activity found in the two seed structures. Catalase activity seems to be less affected by storage than ASC peroxidase. The behaviour of ASC peroxidase and catalase indicates a reduced ability of the endosperm, respect to the embryo, to eliminate toxic products of the oxidative metabolism. Cold storage seems to prevent partially the decrease in the two ascorbate recycling enzymes, AFR and DHA reductase, mainly in the endosperm tissues. In addition, at 25 °C, the decrease in the DHA reductase activity is accompanied by a change in the electrophoretic pattern. Indeed after 6 months of conservation, one of the DHA reducing proteins disappears. A decrease in the activity of the ascorbate recycling enzymes is also induced by other treatments affecting the water content such as flash-drying or osmotic stress [37]. On the contrary, in the immature *Vicia faba* embryo axes, which are desiccation insensitive, a remarkable increase in the activities of DHA reductase and AFR reductase has been reported during the desiccation occurring during seed development [2] and after water or osmoticum stress [37]. A transient increase in the ASC recycling enzymes, in particular in DHA reductase has also been reported during dehydration of wheat kernels [12]. The AFR reductase and the DHA reductase, named as ASC recycling enzymes, are able to maintain ascorbate in the reduced form and to prevent the DHA accumulation. These enzymes are differently regulated in orthodox and recalcitrant seeds: the orthodox seeds are able to enhance and to modulate the activity of these enzymes in different conditions, while recalcitrant seeds can not modulate them. The inability of *G. biloba* seeds to increase the activities of these enzymes during aging and desiccation could be one of the causes of the damages occurring during storage. It must be noted that the enzymes of the reduction of the ASC and GSH oxidized forms not only contribute to maintain these two redox pair in the reduced form, but also contribute to supply oxidized pyridine nucleotides for other metabolic pathways. Changes in the pyridine nucleotide redox state could also contribute to the impairment of *G. biloba* seed metabolism.

In *G. biloba* seeds, the maintenance of the ASC pool during storage is due to the ASC ex novo synthesis at the expense of the endosperm reserves, more than to the ASC recycling enzymes. It is possible that during conservation the metabolism of reserve carbohydrates generates intermediates for the ASC biosynthetic pathway, thus promoting the maintenance or even an increase in the ASC levels. Consistently, GLDH, the last enzyme in ASC biosynthesis, increases in the embryos stored at 4 °C and remains unchanged in the endosperms, until 6 months of storage both at 4 and 25 °C.

In conclusion, storage at 4 °C preserves tissue viability, but only in part seed germinability. Moreover, embryos seem more equipped with antioxidant systems than endosperms. However, seed tissues are not able to counteract the damage occurring during storage.

4. Methods

4.1. Plant materials

The *G. biloba* seeds used in the experiments were obtained from a commercial source (Florsilva Ansaloni, Bologna, Italy) from plants cultivated in a single locality of the North of Italy, collected at the end of October, deprived of sarcotesta and transferred to the laboratory few days after the harvesting. In order to avoid variations due to dimensions of the seed and development status of the embryos [10,11], in all the experiments homogeneous lots of seeds ($23 \pm 1{,}5 \times 15 \pm 1{,}5$ mm, 1.2 ± 0.2 g), with embryos completely developed (7–9 mm long) were used. The seeds were stored in the dark at 4 and 25 °C in thermostated rooms, in juta bags for 12 months. The analyses described below were carried out at the beginning of the storage and after 3, 6, 9 and 12 months of storage.

4.2. Water content

Moisture content was estimated gravimetrically by drying 100 mg of embryos or endosperms for 20 min at 105 °C with a infra-red drier (Mettler LP 16-M). All measures were expressed on a fresh weight basis.

4.3. Seed viability

The viability of the seeds was evaluated measuring the respiratory capability of isolated embryos according to Lester and Smith [24], opportunely modified. Lots of four isolated embryos were incubated for 1 hour in 5 ml of a solution of 1% (w/v) 2,3,5 triphenyl tetrazolium choride (TTC) in phosphate buffer 0.05 M, pH 7.3. The development of red colour was considered as positive test. The coloured formazans formed were extracted in 80% (v/v) acetone. The homogenate was centrifuged at 18,000 × *g* and the absorbance of the supernatant nm was evaluated at 495. A standard curve was prepared by using TTC reduced with sodium hydrosulfite.

Cell viability in the seed tissues was analysed by using trypan blue staining [13]. Briefly, whole embryos and endosperm cross sections were incubated with a 0.4% (w/v) Trypan Blue solution for 10 min. Stained sections were washed with water, observed and photographed using a Leica DMLS stereo microscopy with tungsten lighting.

4.4. Germination capability

The germination capability of the seeds was estimated as a percentage of germination on batches of 40 seeds. The seeds, deprived of the sclerotesta, were maintained in Petri dishes on moist Whatman 3M paper for 20 days in the dark at 22 ± 2 °C, 65% relative humidity, in a thermostated room. Germination was scored as production of a radicle > 3 mm and was assessed daily. At the end of 20 days, not germinated seeds were cut and examined to verify the presence of an embryo. The percentage of germination was calculated only on the seeds equipped with embryo.

4.5. Lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA), a product of lipid peroxidation, content determined by the thiobarbituric acid reaction as described by Zhang and Kirkam [40]. 0.3 g of embryos or endosperms were homogenized with 4 ml of 0.1% (v/v) trichloracetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 10 min. To 1 ml aliquot of the supernatant, 4 ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid was added. The mixture was heated at 95 °C for 30 min and than quickly cooled in an ice bath. After the tube was centrifuged at 10,000 × *g* for 10 min, the absorbance at 532 nm was read. The value for the non specific absorption at 600 nm was subtracted from the 532 reading. The concentration of MDA was calculated using an extinction coefficient of 155 $nM^{-1}cm^{-1}$ [40].

4.6. Enzyme assays

Five to ten gram of homogeneous embryos completely developed (7–9 mm long) or endosperms collected from the seeds were homogenised in a mortar at 4 °C with a medium containing 0.3 M mannitol, 1 mM EDTA, 50 mM Tris–HCl pH 7.8, 0.1% (w/v) bovine serum albumin and 0.05% (w/v) cysteine in a 1/6 ratio (w/v). The cytosolic and mitochondrial fractions were obtained in accordance with the procedure reported by Tommasi et al. [36]. The activity of ASC peroxidase (EC 1.11.1.11), was tested in accordance with Tommasi et al. [35]. Since no ascorbic acid was added to the grinding medium, only the cytosolic component of ASC peroxidase was detected [34]. The activities of dehydroascorbate (DHA) reductase (EC 1.8.5.1), ascorbate free radical (AFR) reductase (EC 1.6.5.4), oxidized glutathione (GSSG) reductase (EC 1.6.4.2), galactono-γ-lactone dehydrogenase (GLDH) (EC 1.3.2.3) were tested according to Tommasi et al. [36].

Protein measurement was performed according to Bradford [5] using bovine serum albumin as a standard.

Native-PAGE analyses of ASC peroxidase and DHA reductase were performed on the cytosolic fraction in accordance with Tommasi et al. [36].

4.7. Extraction and analysis of ascorbate and glutathione

Embryos or endosperms (1 g) collected as above reported were homogenised with 8 volumes of cold 5% (w/v) metaphosphoric acid. The homogenate was centrifuged at 18,000 × *g* for 15 min at 4 °C and the supernatant was collected for analyses. The ASC and GSH pool were assayed according to de Pinto et al. [13].

4.8. Statistics

The reported values are the average of 10 replicates ± S.D. for the experiments concerning seed viability, germination tests and water content and six replicates ± S.D. in the other cases. Different letters indicate values obtained for the same structure (endosperm or embryos) the difference of which was statistically significant according to Student's *t*-test ($P < 0.05$).

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References

[1] R.R. Alexander, *Ginkgo biloba* L, in: C.S. Schopmeyer (Ed.), Seed woody plants in the United States, Agriculture Handbook no 450, Washington DC. Forest Service, USDA, USGPO, Washington, 1974, pp. 429–430.

[2] O. Arrigoni, L. De Gara, F. Tommasi, R. Liso, Changes in the ascorbate system during seed development of *Vicia faba* L, Plant Physiol. 99 (1992) 235–238.

[3] B. Bailly, Active oxygen species and antioxidants in seed biology, Seed Sci. Res. 14 (2004) 93–107.

[4] P. Berjak, N.W. Pammenter, Recalcitrance is not an all-or-nothing situation, Seed Sci. Res. 4 (1994) 263–264.

[5] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[6] K.S.K. Chaitanya, S.C. Naithani, Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn. f, New Phytol. 126 (1994) 623–627.
[7] K.S.K. Chaitanya, R. Naithani, S.C. Naithani, Ascorbic acid metabolism in ageing recalcitrant sal (*Shorea robusta* Gaertn. f.) seeds, Indian J. Exp. Bot. 38 (2000) 1031–1035.
[8] H.F. Chin, E.H. Roberts, Recalcitrant Crop Seeds, Tropical Press, SDN BDH, Kuala Lampur, Malaysia, 1980.
[9] H.F. Chin, Y.L. Hor, M.B. Mohd Lassim, Identification of recalcitrant seeds, Seed Sci. and Technol. 12 (1984) 429–436.
[10] M.I. Daws, C.S. Gaméné, S. Glidewell, H.W. Pritchard, Seed mass variation potentially masks a critical water content in recalcitrant seeds, Seed Sci. Res. 14 (2004) 185–195.
[11] M.I. Daws, E. Lydall, P. Chmierlaz, O. Leprince, S. Matthews, C.A. Thanos, H.W. Pritchard, Developmental heat sum influences recalcitrant seed traits in A*esculus hippocastanum* across Europe, New Phytol. 162 (2004) 157–166.
[12] L. De Gara, M.C. de Pinto, V.M.C. Moliterni, M.G. D'Egidio, Redox regulation and storage processes during maturation in kernels of *Triticum durum*, J. Exp. Bot. 54 (2003) 249–258.
[13] M.C. de Pinto, D. Francis, L. De Gara, The redox state of the ascorbate-dehydroascorbate pair as a specific sensor of cell division in tobacco BY-2 cells, Protoplasma 209 (1999) 90–97.
[14] P. Drew, N.W. Pammenter, P. Berjak, "Sub- imbibed" storage is not an option for extending longevity of recalcitrant seeds of the tropical species *Trichilia dregeana* Sond, Seed Sci. Res. 10 (2000) 355–363.
[15] J.M. Farrant, N.W. Pammenter, P. Berjak, Seed development in relation to desiccation tolerance: a comparison between desiccation sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation tolerant types, Seed Sci. Res. 3 (1993) 1–13.
[16] M. Favre-Duchartre, *Ginkgo biloba*, an oviparous plant, Phytomorphology 8 (1958) 377–390.
[17] W.E. Finch-Savage, P.S. Blake, H.A. Clay, Desiccation stress in recalcitrant *Quercus robur* L. seeds results in lipid peroxidation and increased synthesis of jasmonate and abscisic acid, J. Exp. Bot. 47 (1996) 661–667.
[18] V. Greggains, W.E. Finch-Savage, N.M. Atherton, P. Berjak, Viability loss and free radical processes during desiccation of recalcitrant *Avicennia marina* seeds, Seed Sci. Res. 11 (2001) 235–242.
[19] G.A.F. Hendry, Oxygen free radical processes and seed longevity, Seed Sci. Res. 3 (1993) 141–153.
[20] G.A.F. Hendry, W.E. Finch-Savage, P.C. Thorpe, N.M. Atherton, S.M. Buckland, K.A. Nilsson, W.E. Seel, Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L, New Phytol. 122 (1992) 273–279.
[21] B.F. Holt, G.W. Rothwell, Is *Ginkgo biloba* (Ginkgoaceae) really an oviparous plant?, Am. J. Bot. 84 (1997) 870–872.
[22] O. Leprince, N.M. Atherton, R. Deltour, G.A.F. Hendry, The involvement of respiration free radical processes during loss of desiccation tolerance in germinating *Zea mays* L. an electron paramagnetic resonance study, Plant Physiol. 104 (1994) 1333–1339.
[23] O. Leprince, J. Buitink, F.A. Hoekstra, Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity, J. Exp. Bot. 50 (1999) 1515–1524.
[24] R. Lester, A.L. Smith, The mode of reduction of tetrazolium salt by beef hearth mitochondria; role of coenzyme Q and other lipids, Biochim. Biophys. Acta 47 (1961) 475–496.
[25] Y.H. Liang, W.Q. Sun, Rate of dehydration and cumulative desiccation stress interated to modulate desiccation tolerance of recalcitrant Cocoa and Ginkgo embryonic tissues, Plant Physiol. 128 (2002) 1323–1331.
[26] D.J. Merritt, T. Senaratna, D.H. Touchell, K.W. Dixon, K. Sivasithamparam, Seed ageing of four Western Australian species in relation to storage environment and seed antioxidant acitivity, Seed Sci. Res. 13 (2003) 155–165.
[27] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 9 (2002) 405–409.
[28] N.W. Pammenter, P. Berjak, A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms, Seed Sci. Res. 9 (1999) 13–37.
[29] N.W. Pammenter, P. Berjak, Evolutionary and ecological aspects of recalcitrant seed biology, Seed Sci. Res. 10 (2000) 1301–1306.
[30] N.W. Pammenter, P. Berjak, J.M. Farrant, M.T. Smith, G. Ross, Why do stored hydrated recalcitrant seeds die?, Seed Sci. Res. 4 (1994) 187–191.
[31] H.W. Pritchard, M.I. Daws, B.J. Fletcher, C.S. Gamene, H.P. Msanga, W. Omondi, Ecological correlates of seed desiccation tolerance in tropical African dryland trees, Am. J. Bot. 91 (2004) 863–870.
[32] E.H. Roberts, Predicting the storage life of seeds, Seed Sci. and Technol. 1 (1973) 499–514.
[33] N. Smirnoff, Plant resistance to environmental stress, Current Opinion in Biotecnology 9 (1998) 214–219.
[34] S. Shigeoka, T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta, K. Yoshimura, Regulation and function of ascorbate peroxidase isoenzymes, J. Exp. Bot. 53 (2002) 1305–1319.
[35] F. Tommasi, C. Paciolla, O. Arrigoni, The ascorbate system in recalcitrant and orthodox seeds, Physiol. Plant. 105 (1999) 193–198.
[36] F. Tommasi, C. Paciolla, M.C. de Pinto, L. De Gara, A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds, J. Exp. Bot. 52 (2001) 1647–1654.
[37] F. Tommasi, C. Paciolla, M.C. de Pinto, L. De Gara, Relationships between water levels and ascorbate peroxidase – ascorbate recycling enzymes in recalcitrant and orthodox seeds, in: M. Acosta, J.N. Rodriguez-Lopez, M.A. Pedreno (Eds.), Plant Peroxidases: Biochemistry and Physiology, Univesidad de Murcia, Murcia, 2003, pp. 209–213.
[38] P.B. Tompsett, H.W. Pritchard, Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* seeds, Ann. Bot. (Lond.) 71 (1993) 107–116.
[39] I. von Teichman, A.E. van Wyk, Structural aspects and trends in the evolution of recalcitrant seeds in dicotyledons, Seed Sci. Res. 4 (1994) 225–239.
[40] F. Zhang, M.B. Kirkham, Antioxidant responses to drought in sunflower and sorghum seedlings, New Phytol. 132 (1996) 361–373.