PHYSIOLOGIA PLANTARUM 105: 193–198. 1999
Printed in Ireland—all rights reserved

ISSN 0031-9317

The ascorbate system in recalcitrant and orthodox seeds

Franca Tommasi*, C. Paciolla and O. Arrigoni

Istituto Botanico, via E. Orabona 4, 70125 Bari, Italy
**Corresponding author: e-mail tommasi@botanica.uniba.it*

Received 26 May 1998; in final form 19 October 1998

Recalcitrant seeds of *Ginkgo biloba* L., *Quercus cerris* L., *Aesculus hippocastanum* L. and *Cycas revoluta* Thunb. are shed by the plant at a high moisture content, contain a large amount of ascorbic acid (ASA) and maintain high ascorbate (ASC) peroxidase (EC 1.11.1.11) activity. Three proteins showing ASC peroxidase activity are present in *G. biloba* seeds. Conversely, dry orthodox seeds (*Vicia faba* L., *Avena sativa* L., *Pinus pinea* L.) are completely devoid of ASA and ASC peroxidase. Experimentally induced rapid variations of the water level in both recalcitrant and orthodox seeds do not affect the ASC peroxidase; slow dehydration affects the ASC peroxidase activity moderately in recalcitrant seeds, but provokes a complete loss of germinability. Another peculiar difference between orthodox and recalcitrant seeds concerns the ascorbate recycling enzymes, ascorbate free radical (AFR) reductase (EC 1.6.5.4) and dehydroascorbate (DHA) reductase (EC 1.8.5.1). The DHA reduction capability is low in recalcitrant seeds, but is high in the orthodox ones. In contrast, AFR reductase activity is high in recalcitrant seeds and low in the orthodox ones. Data reported here concerning the ASC system appear to contribute to better understanding the recalcitrance. The presence of three different proteins showing ASC peroxidase activity in the archaic seed-bearing plant *G. biloba* and its involvement in the spermatophyte evolution is discussed.

Introduction

'Orthodox' seeds undergo a definite desiccation prior to shedding at low moisture content (Roberts 1973, von Teichman and van Wyk 1994). Dry orthodox seeds maintain their viability for long periods and are able to germinate even after many years (Roberts 1973). Marked changes in ascorbate (ASC) metabolism occur during the development of orthodox seeds. In the early stage (stage I, according to Müntz 1982), the 'orthodox' *Vicia faba* seed shows the highest ascorbic acid (ASA) content and a very high ASC peroxidase activity. During the desiccation phase, ASA gradually decreases and finally completely disappears (Arrigoni et al. 1992). With water loss the ASC peroxidase activity also progressively decreases and is undetectable in the dry seed (Arrigoni et al. 1992, Tommasi et al. 1995). Consequently, the seeds at the end of their development are devoid of both ASA and ASC peroxidase (De Gara et al. 1987, Klapheck et al. 1990, Tommasi and De Gara 1990, Arrigoni et al. 1992, Tommasi et al. 1995, De Gara et al. 1997, Ushimaru et al. 1997). Dry seeds contain a moderate amount of dehydroascorbic acid (DHA) and proteins able to catalyze the reduction of dehydroascorbate to ASC in the presence of glutathione (Arrigoni et al. 1992, Tommasi et al. 1995, De Gara et al. 1997).

By contrast, the seeds referred to as 'recalcitrant' (Roberts 1973) lack the desiccation phase. They are shed from the mother plant at a high moisture content, remain metabolically very active (Roberts 1973, Berjak and Pammenter 1994, Pammenter et al. 1994, von Teichman and van Wyk 1994) and generally do not have a typical quiescent stage, passing directly from development to germination. Recalcitrant seeds are characterized by short storage life (Roberts 1973) and this represents a significant problem in the maintenance of genetic seed banks for long-term conservation.

At the present time there are important studies available concerning anatomic characters of recalcitrant seeds like ovule structure (von Teichman and van Wyk 1994), the size of the seed (Chin et al. 1984), the site and nature of storage reserve (Farrant et al. 1992, 1993). Less data are available about the physiological and molecular aspects of recalcitrance (Finch-Savage et al. 1992, Hendry et al. 1992, Berjak and Pammenter 1994, Finch-Savage et al. 1994, 1996). Re-

Abbreviations – ASA: ascorbic acid; ASC: ascorbate; AFR: ascorbate free radical; DHA: dehydroascorbate; FDEBD: fully developed *Vicia faba* embryo axes just before the beginning of the desiccation period; GSH: reduced glutathione.

cently it has been reported that the loss of viability of seeds could be related to a drop in the antioxidants (Hendry et al. 1992). Since the ASC system could have an important role in this context, the aim of this work was to ascertain: (1) if recalcitrant seeds maintain ASA and ASC peroxidase when they are shed; (2) if the ASC system always remains fully operating; (3) if experimentally rapid desiccation affects the ASC system.

Materials and methods

Plant material

In the experiments whole embryos or embryo axes from recalcitrant or orthodox seeds were used depending on the localization of seed-stored food. In seeds with food reserves outside the embryo (primary or secondary endosperm), the whole embryo was analyzed. On the contrary, in seeds with stored metabolites accumulated in the cotyledons, only the embryo axes were collected. In particular, whole embryos of *Ginkgo biloba* L., *Cycas revoluta* Thunb. (recalcitrant seeds), *Pinus pinea* L. and *Avena sativa* L. (orthodox seeds) were used, while only embryo axes of *Quercus cerris* L., *Aesculus hippocastanum* L. (recalcitrant), *Vicia faba* L. (orthodox) were considered. The seeds were collected or purchased, but in every case the collection data were carefully checked to avoid the comparison of different developmental stages. The seeds of *Cycas revoluta* were stored at 5°C for 24 weeks to complete embryo maturation as described in Dehgan and Schutzman (1989). All recalcitrant seeds were wet-stored at 4°C, as described in Berjak et al. (1992) in order to maintain their moisture level. The viability of the seeds was estimated as a percentage of germination (Berjak et al. 1992); water content and dry weights were estimated using a Mettler infra-red drier LP 16-M and expressed on a fresh weight basis.

Flash-drying treatments were carried out for 1 h according to the procedure of Berjak et al. (1992), utilising *G. biloba* embryos and *V. faba* embryo axes collected from developing seeds at the end of seed reserve accumulation (45 days after anthesis).

Enzyme assays

Batches of 0.3 g of embryos or embryo axes were homogenized in a mortar at 4°C in 1:4 (w/v) ratio with a medium containing 0.3 *M* mannitol, 1 m*M* EDTA, 50 m*M* Tris-HCl, pH 7.8, 0.1% (w/v) bovine serum albumin (BSA) and 0.05% (w/v) cysteine. The cytosolic fraction was obtained according to the procedure reported by Arrigoni et al. (1997). The activities of cytosolic ASC peroxidase (EC 1.11.1.11), dehydroascorbate (DHA) reductase (EC 1.8.5.1) and ascorbate free radical (AFR) reductase (EC 1.6.5.4) were tested according to Arrigoni et al. (1992). The term ASA was referred to the assays carried out at acid pH values; the term ASC was referred to the condition in which the acid was mostly in the dissociated form.

Since no ASA was added to the grinding medium only the so-called cytosolic component of ASC peroxidase was detected (Amako et al. 1994). Proteins were determined according to Bradford (1976) with BSA as standard.

Native-PAGE analysis of ASC peroxidase and DHA-reducing proteins

Embryos were ground as previously described, the supernatant at 30000 *g* was used for electrophoretic analysis.

Native-PAGE was performed on the cytosolic fraction using a stacking gel containing 4.3% acrylamide and a separating gel containing 7.3% acrylamide with a running buffer composed of 4 m*M* Tris-HCl, pH 8.3, and 38 m*M* glycine.

After non-denaturing electrophoresis, the gels were incubated for 15 min at room temperature under agitation in 0.1 *M* Na-phosphate buffer, pH 6.2, containing appropriate substrates for the enzymatic reactions to occur: 4 m*M* ASA and 4 m*M* H_2O_2 for ASC peroxidase; 4 m*M* GSH and 2 m*M* DHA for DHA-reducing proteins. The gels were then washed with distilled water and stained with a solution of 0.125 *M* HCl containing 0.1% (w/v) ferricyanide and 0.1% (w/v) ferrichloride. ASC peroxidase was located as an achromatic band on a Prussian blue background, as a result of the reaction between ferrichloride and ferrocyanide, the latter having been produced by the reduction of ferricyanide with unreacted ASA; in contrast, DHA-reducing proteins were observed as dark blue bands on a light blue background.

For the elution of ASC peroxidase isozymes from the gel, a lane was cut and dyed as described above to identify the position of each band. The areas containing ASC peroxidase isozymes were electroeluted with GE 200 Sixpac Gel Eluter (Hoefer Scientific Instruments, San Francisco, CA, USA) according to the procedure recently reported by De Gara et al. (1997).

The apparent K_m for ascorbate of each eluted isozyme was determined according to Allison and Purich (1979).

Ascorbic acid content assay

Embryos (0.2–0.4 g) were homogenized in 5% metaphosphoric acid. The homogenate was centrifuged at 10000 *g* and the supernatant was then used. The ASA and DHA content were determined according to Arrigoni et al. (1992).

Results

All seeds tested were able to germinate; low values of germinability occurred in *C. revoluta* and *G. biloba* because some seeds lacked embryos. It is well known that in these 'gymnosperms', and particularly in *Cycas*, the seed can develop without fertilization (Dehgan and Schutzman 1989).

G. biloba embryos had the highest water content (67%), while *C. revoluta* showed the lowest water content (25%) among the recalcitrant seeds examined. The orthodox embryos tested showed a water content of 5–6% (Table 1).

The embryos of recalcitrant seeds were found to contain ASA; the highest value was observed in *G. biloba* embryos (6.3 μmol g^{-1} dry weight), the lowest value was measured in

Table 1. Germinability of recalcitrant and orthodox seeds and water content of embryos or embryo axes. The results are given as the mean value of three experiments ± SD.

Species	Germination (%)	Water content (%)
Recalcitrant seeds		
G. biloba	80 ± 9	67 ± 3
Q. cerris	90 ± 6	45 ± 5
A. hippocastanum	90 ± 4	48 ± 3
C. revoluta	50 ± 3	25 ± 3
Orthodox seeds		
P. pinea	98 ± 4	5 ± 2
V. faba	98 ± 1	6 ± 2
A. sativa	98 ± 4	6 ± 3

C. revoluta (0.65 μmol g^{-1} dry weight). Orthodox seeds lacked ASA (Fig. 1).

DHA was present in all the embryos examined; however, DHA content was higher in the recalcitrant seeds than in the orthodox (i.e. 2.27 μmol g^{-1} dry weight in *G. biloba*, 1.04 in *C. revoluta*, compared with 0.52 μmol g^{-1} dry weight in *A. sativa*) (Fig. 1).

Table 2 shows that the DHA reductase activity of the recalcitrant seeds was 90–140 nmol DHA reduced mg^{-1} protein min^{-1}, while in the orthodox embryos it was 200–500 nmol DHA reduced mg^{-1} protein min^{-1}. Two DHA-reducing proteins were detectable by native-PAGE in *G. biloba* and *Q. cerris*, only one in *A. hippocastanum* and *C. revoluta*. In *P. pinea* and *A. sativa* several DHA-reducing proteins were evident, eight and five, respectively, while four bands were present in *V. faba* (Fig. 2).

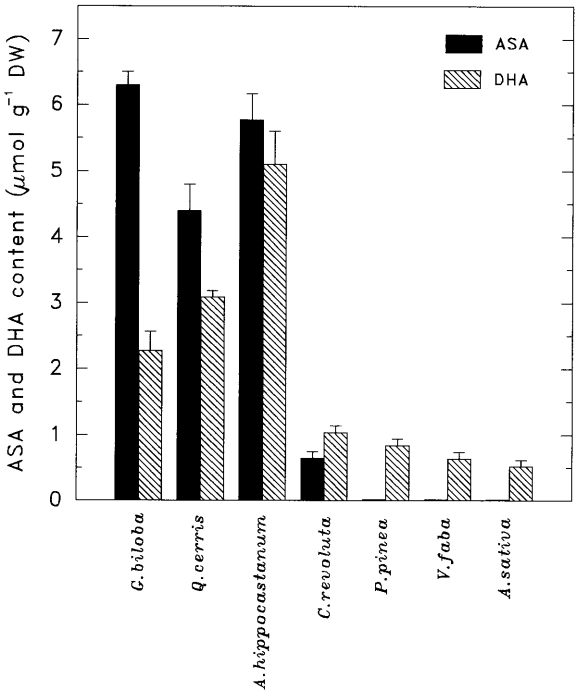

Fig. 1. Ascorbic acid (ASA) and dehydroascorbic (DHA) acid content in the embryos of *Ginkgo biloba*, *Quercus cerris*, *Aesculus hippocastanum*, *Cycas revoluta* (recalcitrant seeds), *Pinus pinea*, *Vicia faba* and *Avena sativa* (orthodox seeds). The values are the means ± SD of three experiments.

The activity of AFR reductase, the enzyme that reconverts the AFR (or monodehydroascorbate radical, i.e. MDAR) to ASC by using NAD(P)H as an electron donor, was very high in all recalcitrant seeds tested in comparison with orthodox seeds (Table 2). The highest value was measured in *Q. cerris* (1100 nmol NADH oxidized mg^{-1} protein min^{-1}); while orthodox seeds of *P. pinea*, *V. faba* and *A. sativa* gave values of 134, 180, 310 nmol NADH oxidized mg^{-1} protein min^{-1}, respectively. It is necessary to point out that, as has been previously demonstrated (De Gara et al. 1997), the activity of the enzyme rose during germination and in the seedlings of *Poales* reached a value of approximately 800 nmol NADH oxidized mg^{-1} protein min^{-1}.

ASC peroxidase was undetectable in orthodox dry seeds, but was very active in the embryos of recalcitrant seeds. *G. biloba* embryos showed the maximum value (2000 nmol ASC oxidized mg^{-1} protein min^{-1}) and *Q. cerris* the minimum (350 nmol ASC oxidized mg^{-1} protein min^{-1}) (Table 2). Native-PAGE gave three clearly distinct ASC peroxidase bands for *G. biloba*, whereas only one band of activity was present in *Q. cerris*, *A. hippocastanum* and *C. revoluta* embryos (Fig. 3). No bands were detectable in dry orthodox seeds. In order to characterize the *G. biloba* proteins showing ASC peroxidase activity on the gel, they were electroeluted according to the procedure described in De Gara et al. (1997). Although in the electroeluted fluid other proteins were present together with ascorbate peroxidase and consequently the enzyme was only partially purified, some kinetic characteristics were analyzed. The *G. biloba* protein band with the intermediate migration rate showed the highest affinity for ASC (apparent K_m 18 μ*M*) whereas the other two bands, with low and fast migration rates, had K_m for ASC of 22 and 28 μ*M*, respectively.

Since the ASC peroxidase disappears together with ascorbic acid during the long physiological desiccation phase in the orthodox seed, we studied whether rapid desiccation by means of flash-drying process affected the ASC peroxidase activity and the ASA content of recalcitrant *G. biloba* embryos and of fully developed orthodox *V. faba* embryo axes just before the beginning of the desiccation period.

Table 3 shows that as the water content rapidly decreased to 32%, the ASC peroxidase activity of *G. biloba* embryos was unchanged. No changes appeared in the native-PAGE results and also the embryo viability was not compromised (data not shown). Only a moderate drop in the ASA content (27%) occurred. The same rapid dehydration was applied to fully developed *Vicia faba* embryo axes just before the beginning of the desiccation period (FDEBD) (Table 4). At this stage the water content of the embryo axes was about 75%, the ASA content 10 μmol g^{-1} dry weight and the ASC peroxidase activity was 350 nmol mg^{-1} protein min^{-1}. After flash-drying treatment, the water content dropped to 20%, but the ASC peroxidase activity and ASA content were quite unchanged.

Table 2. ASC peroxidase, DHA reductase and AFR reductase activities in embryos or embryo axes of recalcitrant and orthodox seeds. The results are given as the mean value of three experiments ± SD. Activities are expressed as nmol substrate metabolized mg^{-1} protein min^{-1}.

Species	ASC peroxidase	DHA reductase	AFR reductase
Recalcitrant seeds			
G. biloba	2 000 ± 90	107 ± 18	817 ± 22
Q. cerris	350 ± 45	90 ± 20	1 100 ± 75
A. hippocastanum	400 ± 86	100 ± 20	1 000 ± 90
C. revoluta	500 ± 20	140 ± 20	781 ± 30
Orthodox seeds			
P. pinea	0	200 ± 20	134 ± 11
V. faba	0	500 ± 43	180 ± 15
A. sativa	0	340 ± 29	310 ± 24

Slow dehydration of *G. biloba* whole seeds (3 weeks at 25°C) induced a drop in the embryo water content to 40%; the ASC peroxidase activity decreases 28%, but the enzyme did not disappear and a total loss of germinability occurred. Conversely, in the orthodox seeds slow drying induces the quiescence of the seed, as it is well documented (Kermode 1990).

The effects of rapid hydration on ASA-ASC peroxidase are reported in Table 4. When dry orthodox seeds of *V. faba* containing no more than 7% water were hydrated until their water content reached 20%, both ASA and ASC peroxidase remained undetectable. They appeared together some hours later in the germination when ASA biosynthesis started.

Discussion

Data reported in the present paper show that large differences in the ascorbate system do exist between recalcitrant and orthodox seeds. Recalcitrant seeds contain ASA and have a high ASC peroxidase activity, while orthodox seeds are devoid of both ASA and ASC peroxidase. The maintenance of the ASC peroxidase activity of recalcitrant seeds is very important because they never reach the quiescent phase that most dry seeds of the advanced plants do. They keep a high water content, active metabolism and inevitably produce hydrogen peroxide. ASC peroxidase, the enzyme that co-operates with catalase in eliminating the toxic hydrogen peroxide, is therefore essential. Recalcitrant seeds must maintain a functional ASC biosynthetic pathway because it is well known that ASC and ASC peroxidase activity are in vivo tightly related (De Tullio et al. 1994). ASC is not only the substrate of the enzyme, but it also controls the expression of the enzyme (De Gara et al. 1997). On the contrary, orthodox seeds need neither ASC nor ASC peroxidase, because they are dry with a low oxidative metabolic activity and consequently a poor hydrogen peroxide production (Mayer and Poljakoff-Mayber 1982, Puntarulo et al. 1988).

The electrophoretic pattern of ASC peroxidase of *G. biloba* embryos shows the presence of three distinct bands having kinetic characteristics similar to those found in *Poales* (De Gara et al. 1997). This is an unexpected finding because *G. biloba* is an archaic seed-bearing plant, considered the most ancient living tree (Major 1967) or a 'living fossil'. In the majority of angiosperms a single cytosolic

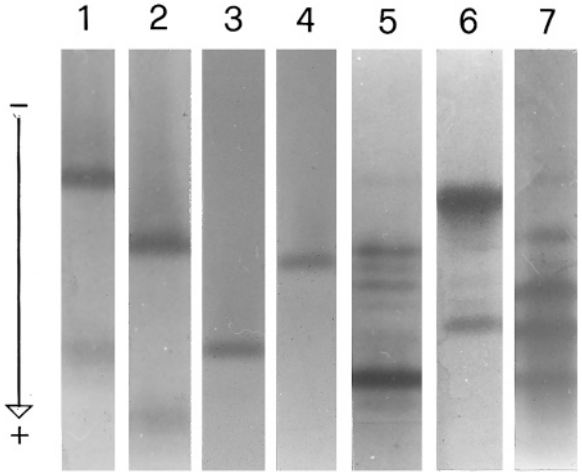

Fig. 2. Native-PAGE of DHA-reducing proteins of *Ginkgo biloba* (1), *Quercus cerris* (2), *Aesculus hippocastanum* (3), *Cycas revoluta* (4), *Pinus pinea* (5), *Vicia faba* (6) and *Avena sativa* (7). 200 μg of protein was loaded per lane.

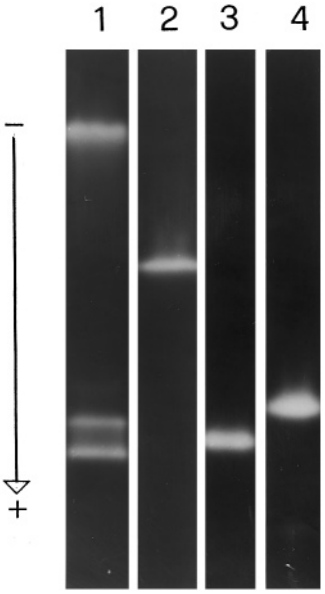

Fig. 3. Native-PAGE of ASC peroxidase of *Ginkgo biloba* (1), *Quercus cerris* (2), *Aesculus hippocastanum* (3) and *Cycas revoluta* (4). 250 μg of protein was loaded per lane.

Table 3. Effect of rapid dehydration on ASC peroxidase activity, and ASA content in *Ginkgo biloba* embryos. The results are given as the mean value of three experiments ± SD. ASC peroxidase activity is expressed in units; 1 unit = 1 nmol ASC oxidized mg^{-1} protein min^{-1}.

Treatment	Water content (%)	ASC peroxidase	ASA content (nmol g^{-1} DW)
After shedding seeds	67 ± 3	2 089 ± 140	6 200 ± 250
Flash-dried embryos	32 ± 4	2 100 ± 150	4 500 ± 300
Slow-dried embryos	40 ± 5	1 500 ± 200	3 300 ± 200

Table 4. Effect of moisture content variation on ASC peroxidase activity and ASA content in *V. faba* embryo axes. Values are means of three experiments ± SD. ASC peroxidase activity is expressed in units; 1 unit = 1 nmol ASC oxidized mg^{-1} protein min^{-1}.

Treatment	Water content (%)	ASC peroxidase	ASA content (nmol g^{-1} DW)
FDEBD	75 ± 5	350 ± 15	10 000 ± 580
Flash-dried seeds	20 ± 4	311 ± 20	9 500 ± 200
Dry seeds	6 ± 2	0	0
Hydrated seeds (1 h)	20 ± 3	0	0
Germinating seeds (24 h)	80 ± 10	160 ± 10	800 ± 65

ASC peroxidase isozyme is usually detectable; only in maize and in other advanced *Liliopsida* (*Poales*) are three distinct isozymes present (De Gara et al. 1993, Paciolla et al. 1996).

Recently, Van Breusegem and co-workers reported the presence of three different genes encoding the ASC peroxidase isozymes in maize (Van Breusegem et al. 1995) and it has been suggested that the presence of three isozymes and of three different genes represents an important evolutive acquisition by the *Poales* (De Gara et al. 1993, Arrigoni 1994, Paciolla et al. 1996). Data reported here, even if indirectly, seem to suggest that probably three genes for ASC peroxidase were already present in the archaic *Ginkgo biloba*. This could indicate that during plant evolution ASC peroxidase gene expression occurred in a different way rather than the acquisition of a new gene.

Recalcitrant seeds show a higher DHA content and a lower DHA reduction capability than dry orthodox seeds that contain a moderate amount of DHA and, frequently, a population of detectable DHA-reducing proteins. The presence of high DHA reductase activity in the dry orthodox seed is essential at the beginning of germination because it is the only way to provide the embryo cells with ASC. In fact, the ex novo ASC biosynthesis from sugars starts after 6–20 h of germination (De Gara et al. 1997). When the ex novo ASC biosynthesis is fully functional, the majority of DHA-reducing proteins disappear and DHA reduction plays a secondary role in the cell ASC pool. The DHA reductase remains at a low level in the plant unless stress conditions occur (Arrigoni 1994, Foyer et al. 1995, Smirnoff 1996) or the ASC biosynthesis is experimentally inhibited by lycorine (De Gara et al. 1994, De Tullio et al. 1998). In the recalcitrant seeds the ASC biosynthesis through galactono dehydrogenase is always functional (F. Tommasi, unpublished data) and DHA reduction plays a secondary role in the maintenance of the ASC pool of the cell.

AFR reductase, the other enzyme of the 'ascorbate recycling' process, is higher in recalcitrant seeds than in orthodox ones. This agrees with data previously reported showing that when cell metabolism activity is high (i.e. recalcitrant seeds), the AFR reductase activity is very high, while when metabolic activity is low (i.e. orthodox seeds), the AFR reductase activity is also low (Arrigoni et al. 1981, Tommasi et al. 1990, Arrigoni 1994, De Gara et al. 1997).

The last point that we can briefly comment on concerns the behavior of ASC peroxidase during the dehydration and rehydration processes. A rapid decrease in water content did not substantially affect ASC peroxidase activity in either orthodox or recalcitrant seeds. However, dramatic differences exist between recalcitrant and orthodox seeds. In orthodox seeds slow physiological dehydration induces the disappearance of the ASA-ASC peroxidase system, seed quiescence and transition from development to germination (Kermode 1990). In recalcitrant seeds slow drying causes the complete loss of germinability; under these conditions, differing from that reported by Hendry (Hendry et al. 1992), only moderate changes occur in the ASA-ASC peroxidase system. Recalcitrant seeds seem to lack the molecular mechanism that controls the switching-off of the ASA-ASC peroxidase system. At present we do not have any clear explanation; however, some experimental data suggested that abscisic acid could be involved in this process (Black 1992).

Acknowledgements – This work was supported by M.U.R.S.T. grants.

References

Allison RD, Purich DL (1979) Practical considerations in design of initial velocity enzyme rate assays. Methods Enzymol 63: 3–22

Amako K, Chen GX, Asada K (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for chloroplastic and cytosolic isoenzymes of ascorbate peroxidase in plants. Plant Cell Physiol 35: 497–504

Arrigoni O (1994) Ascorbate system in plant development. J Bioenerg Biomembr 26: 407–419

Arrigoni O, Dipierro S, Borraccino G (1981) Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. FEBS Lett 125: 242–244

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

Arrigoni O, Calabrese G, De Gara L, Bitonti MB, Liso R (1997) Correlation between changes in cell ascorbate and growth of *Lupinus albus* seedlings. J Plant Physiol 150: 302–308

Berjak P, Pammenter NW (1994) Recalcitrance is not an all-or-nothing situation. Seed Sci Res 4: 263–264
Berjak P, Pammenter NW, Vertucci C (1992) Homoiohydrous (recalcitrant) seeds: Developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. Planta 186: 249–261
Black M (1992) Involvement of ABA in the physiology of developing and mature seeds. In: WJ Davies, HJ Jones (eds) Abscisic Acid: Physiology and Biochemistry, Bio Scientific Publishers, London, pp 99–124. ISBN 0-7923-2985-6
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
Chin HF, Hor YL, Mohd Lassim MB (1984) Identification of recalcitrant seeds. Seed Sci Technol 12: 429–436
De Gara L, Tommasi F, Liso R, Arrigoni O (1987) Il sistema dell'acido ascorbico in *Vicia faba* L. Boll Soc Ital Biol Sper 63: 551–558
De Gara L, De Tullio M, Paciolla C, Liso R, Arrigoni O (1993) Cytosolic ascorbate peroxidase in angiosperms and different expression of its isoforms in maize embryos during germination. In: Welinder KG, Rasmussen SK, Penel C, Greppin H (eds) Plant Peroxidases, Biochemistry and Physiology. University of Geneva, Geneva, pp 251–255. ISBN 2-88164-006-0
De Gara L, Paciolla C, Tommasi F, Liso R, Arrigoni O (1994) In vivo inhibition of galactono-γ-lactone conversion to ascorbate by lycorine. J Plant Physiol 144: 649–653
De Gara L, de Pinto MC, Arrigoni O (1997) Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. Physiol Plant 100: 894–900
Dehgan B, Schutzman B (1989) Embryo development and germination of *Cycas* seeds. J Am Soc Hortic Sci 114: 125–129
De Tullio M, De Gara L, Paciolla C, Arrigoni O (1994) Correlation between ascorbate biosynthesis and ascorbate peroxidase activity. J Biol Res 70: 63–68
De Tullio M, De Gara L, Paciolla C, Arrigoni O (1998) Dehydroascorbate-reducing proteins in maize are induced by the ascorbate biosynthesis inhibitor lycorine. Plant Physiol Biochem 36: 440–443
Farrant JM, Pammenter NW, Berjak P (1992) Development of the recalcitrant (homoiohydrous) seed of *Avicennia marina*: Anatomical, ultrastructural and biochemical events associated with development from histodifferentiation to maturation. Ann Bot 70: 75–86
Farrant JM, Pammenter NW, Berjak P (1993) Seed development in relation to desiccation tolerance: A comparison between desiccation sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation tolerant types. Seed Sci Res 3: 1–13
Finch-Savage WE, Clay HA, Blake PS, Browning G (1992) Seed development in the recalcitrant species *Quercus robur* L.: Water status and endogenous abscisic acid levels. J Exp Bot 43: 671–679
Finch-Savage WE, Pramanik SK, Bewley JD (1994) The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. Planta 193: 478–485
Finch-Savage WE, Blake PS, Clay HA (1996) Desiccation stress in recalcitrant *Quercus robur* L. seeds results in lipid peroxidation and increased synthesis of jasmonate and abscisic acid. J Exp Bot 47: 661–667
Foyer C, Souriau N, Perret S, Lelandais M, Kunert KJ, Pruvost C, Jouanin L (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinibition in poplar trees. Plant Physiol 109: 1047–1057
Hendry GAF, Finch-Savage WE, Thorpe PC, Atherton NM, Buckland SM, Nilsson KA, Seel WE (1992) Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. New Phytol 122: 273–279
Kermode AR (1990) Regulatory mechanisms involved in the transition from seed development to germination. Crit Rev Plant Sci 9: 155–195
Klapheck S, Zimmer I, Cosse H (1990) Scavenging of hydrogen peroxide in the endosperm of *Ricinus communis* by ascorbate peroxidase. Plant Cell Physiol 31: 1005–1013
Major RT (1967) The *Ginkgo*, the most ancient living tree. Science 157: 1270–1273
Mayer AM, Poljakoff-Mayber A (1982) Metabolism of germinating seeds. In: PF Wareing, AY Galston (eds) The Germination of the Seeds. Pergamon Press, New York, NY, pp 85–139. ISBN 0-08028853-7
Müntz K (1982) Seed development. Encycl Plant Physiol 14: 505–558
Paciolla C, De Gara L, De Tullio MC, Arrigoni O (1996) Distribution of cytosolic ascorbate peroxidase in angiosperms. Giorn Bot Ital 130: 729–737
Pammenter NW, Berjak P, Farrant JM, Smith MT, Ross G (1994) Why do stored hydrated recalcitrant seeds die? Seed Sci Res 4: 187–191
Puntarulo S, Sanchez RA, Boveris A (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol 86: 626–630
Roberts EH (1973) Predicting the storage life of seeds. Seed Sci Technol 1: 499–514
Smirnoff N (1996) The function and metabolism of ascorbic acid in plants. Ann Bot 78: 661–669
Tommasi F, De Gara L (1990) Correlazione tra presenza di acido ascorbico e comparsa dell'attività ascorbico perossidasica in embrioni di *Avena sativa* L. Boll Soc Ital Biol Sper 66: 357–364
Tommasi F, De Gara L, Liso R, Arrigoni O (1990) The ascorbic acid system in *Cuscuta reflexa* Roxb. J Plant Physiol 135: 766–768
Tommasi F, Paciolla C, D'Elia F, Arrigoni O (1995) Changes of ascorbate peroxidase and dehydroascorbate reductase activities during seed development of *Vicia faba* L. J Biol Res 71: 341–346
Ushimaru T, Maki Y, Sano S, Koshiba K, Asada K, Tsuji H (1997) Induction of enzymes involved in the ascorbate-dependent antioxidative system, namely ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase, after exposure to air of rice (*Oryza sativa*) seedlings germinated under water. Plant Cell Physiol 38: 541–549
Van Breusegem F, Villaroel R, Van Montagu M, Inzé D (1995) Ascorbate peroxidase cDNA from maize. Plant Physiol 107: 649–650
von Teichman I, van Wyk AE (1994) Structural aspects and trends in the evolution of recalcitrant seeds in dicotyledons. Seed Sci Res 4: 225–239

Edited by C. H. Foyer