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A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds

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Abstract

The ascorbate and glutathione systems have been studied during the first stages of germination in orthodox seeds of the gymnosperm *Pinus pinea* L. (pine). The results indicate that remarkable changes in the content and redox balance of these metabolites occur in both the embryo and endosperm; even if with different patterns for the two redox pairs. Dry seeds are devoid of the ascorbate reduced form (ASC) and contain only dehydroascorbic acid (DHA). By contrast, glutathione is present both in the reduced (GSH) and in the oxidized (GSSG) forms. During imbibition the increase in ASC seems to be mainly caused by the reactivation of its biosynthesis. On the other hand, the GSH rise occurring during the first 24 h seems to be largely due to GSSG reduction, even if GSH biosynthesis is still active in the seeds. The enzymes of the ascorbate–glutathione cycle also change during germination, but in different ways. ASC peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2) activities progressively rise both in the embryo and in endosperm. These changes are probably required for counteracting production of reactive oxygen species caused by recovery of oxidative metabolism. The two enzymes involved in the ascorbate recycling, ascorbate free radical (AFR) reductase (EC 1.6.5.4) and DHA reductase (EC 1.8.5.1), show different behaviour: the DHA reductase activity decreases, while that of AFR reductase remains unchanged. The relationship between ascorbate and glutathione metabolism and their relevance in the germination of orthodox seeds are also discussed.

Key words: Ascorbate metabolism, glutathione metabolism, pine, *Pinus pinea*, imbibition, seed germination, ROS protection.

Introduction

Germination is a crucial step in the plant life cycle. In particular, in the seeds reaching maturity in a highly dehydrated state, the orthodox seeds (Roberts, 1973), it is characterized by dramatic changes in metabolism which allow the degradation of stored macromolecules and the recovery of the biosynthetic processes necessary for efficient germination. Upon imbibition, the quiescent dry seeds rapidly resume oxygen uptake and oxidative phosphorylation, processes required for supporting the high energy cost of germination (Mayer and Poljakoff-Mayber, 1982; Hourmant and Pradet, 1981). Oxidative phosphorylation and the mobilization of food storage generate reactive oxygen species (ROS), which, if they are not rapidly removed, cause considerable structural and functional damage in cells. Moreover, during the lag required for the gradual transition of the membrane phospholipid components from the gel phase (achieved during maturation drying) to the normal hydrated liquid-crystalline state (a transition that also involves mitochondrial membranes), ROS production as phosphorylation by-products could be higher than in other physiological phases (Crowe and Crowe, 1992). Indeed, the enzymes and metabolites responsible for ROS scavenging are of particular importance for the success of germination. However, it has been widely reported that orthodox seeds

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Abbreviations: AFR, ascorbate free radical; ASC, ascorbate; BSO, L-buthionine (S,R) sulphoximine; DHA, dehydroascorbate; GLDH, galactono-γ-lactone dehydrogenase; GR, glutathione reductase; GSH, glutathione-reduced form; GSSG, glutathione disulphide; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

are completely devoid of ascorbate (ASC) and ASC peroxidase, two key factors in ROS detoxification (Asada, 1997), they contain only moderate amounts of dehydroascorbate (DHA), and have ascorbate free radical (AFR) reductase and DHA reductase, enzymes catalysing the reduction of the two products of ASC oxidation into ASC (Klapheck *et al*., 1990; Arrigoni *et al*., 1992; De Gara *et al*., 1997; Ushimaru *et al*., 1997; Tommasi *et al*., 1999). The other ROS scavenger enzymes, superoxide dismutase and catalase, even if present, have very low activities in dry orthodox seeds of angiosperms (Gidrol *et al*., 1994; Puntarulo *et al*., 1988). A rapid rise in ASC content and ASC peroxidase activity during the first stages of germination is a common strategy developed in orthodox seeds of herbaceous plants to cope with an increased level of ROS (Cakmak *et al*., 1993; De Gara *et al*., 1997).

Besides ASC, another redox pair involved in ROS detoxification is glutathione (GSH) (Foyer *et al*., 1991). ASC and GSH are part of a cycle of reactions, the ascorbate–glutathione cycle, in which ASC peroxidase is directly involved in H_2O_2 removal (Noctor and Foyer, 1998) and AFR reductase, DHA reductase and glutathione reductase (GR), contribute to maintaining a high level of ASC and to avoiding the accumulation of DHA, a product of ASC oxidation that can negatively interfere with plant growth (Arrigoni, 1994; Cordoba-Pedregosa *et al*., 1996; De Gara and Tommasi, 1999; de Pinto *et al*., 1999).

Compared with other tissues of higher plants, dry seeds contain higher amount of glutathione (Klapheck, 1988). Changes in glutathione content and GR activity during germination of some herbaceous plants have also been reported (Fahey *et al*., 1980; Kranner and Grill, 1993).

In this paper the pattern of events involving the ASC and GSH systems during the first stages of germination in the orthodox seeds of the gymnosperm *Pinus pinea* L. (pine) have been studied, with the aim of obtaining an integrated picture of the behaviour of the two main antioxidant metabolites during the first stages of seed germination of an arboreous plant, both in the embryo and in the primary endosperm, a typical haploid storage tissue that in the gymnosperm is physically and genetically distinguished from the embryo.

Materials and methods

Plant material

Seeds of *Pinus pinea* purchased from commercial sources (Florsilva, Anzaloni, Bologna, Italy) were deprived of their lignified tegument and sown in Petri dishes on moist Whatman 3M paper at 22 °C in the dark for 24–48 h. Embryos and endosperms collected from dry and germinating seeds at the times indicated in each experiment were utilized in the experiments. Dry weights (DW) were measured in accordance with Tommasi *et al*. (Tommasi *et al*., 1999).

Enzyme assays

5–10 g of embryos or endosperms was homogenized 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 fraction and the mitochondria were obtained in accordance with the procedure reported previously (De Gara *et al*., 1997). The activity of ASC peroxidase (EC 1.11.1.11), was tested in accordance with Tommasi *et al*. (Tommasi *et al*., 1999). Since no ascorbic acid was added to the grinding medium, only the cytosolic component of ASC peroxidase was detected because the plastidial ones were completely inactivated (Amako *et al*., 1994). The activities of DHA reductase (EC 1.8.5.1) and AFR reductase (EC 1.6.5.4) were tested (de Pinto *et al*., 2000). Glutathione reductase (GR) (EC 1.6.4.2) was measured as reported previously (Foster and Hess, 1982). The activity of galactono-γ-lactone dehydrogenase (GLDH) (EC 1.3.2.3) was assayed on the mitochondria washed fraction, utilizing cytochrome *c* as an electron acceptor for galactono-γ-lactone (Ôba *et al*., 1995), with minor modifications (de Pinto *et al*., 2000). Proteins were determined in accordance with Bradford (Bradford, 1976).

Native-PAGE analysis of ASC peroxidase and DHA reducing proteins

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 mM TRIS-HCl, pH 8.3, and 38 mM glycine. In each lane 300–600 μg of total proteins was loaded. 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 mM ascorbic acid and 4 mM H_2O_2 for ASC peroxidase; 4 mM GSH and 2 mM 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) potassium ferricyanide and 0.1% (w/v) ferric chloride. ASC peroxidase was located as an achromatic band on a Prussian blue background, as a result of the reaction between ferric chloride and potassium ferrocyanide, the latter having been produced by the reduction of potassium ferricyanide with unreacted ascorbic acid; by contrast, DHA-reducing proteins were observed as dark blue bands on a light blue background.

Extraction and analysis of ascorbate and glutathione

Embryos or endosperms (1 g) were homogenized in 5% metaphosphoric acid. The homogenate was centrifuged at 18 000 *g* and the supernatant was then used. The ASC and DHA content were determined (Kampfenkel *et al*., 1995) with modifications (de Pinto *et al*., 1999). The glutathione pool was assayed according to previously described methods (Griffith, 1985; Smith, 1985) as reported by Zhang and Kirkham (Zhang and Kirkham, 1996) utilizing 0.4 ml aliquots of supernatant neutralized with 0.6 ml of 0.5 M phosphate buffer (pH 7.5). For GSSG assay, the GSH was masked by adding 20 μl of 2-vinylpyridine to the neutralized supernatant, whereas 20 μl of water was added in the aliquots utilized for the total glutathione pool (GSH + GSSG) assay. Tubes were mixed until an emulsion was formed. Glutathione content was in 1 ml of reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5′ dithiobis(2-nitrobenzoic acid), and 0.1 ml

of sample obtained as described above. The reaction was started by adding 3 units of GR and was monitored by measuring the change in absorbance at 412 nm for 1 min. GSH was estimated as the difference between the amount of total glutathione and that of GSSG. A standard curve for GSH in the range of 0–30 μmol ml^{-1} was prepared.

In some experiments germinating seeds were treated with 1 mM L-buthionine (S,R) sulphoximine (BSO), a specific inhibitor of GSH biosynthesis (Griffith and Meister, 1979).

Statistics

All the experiments were repeated at least six times; values reported are the mean of six experiments ±SD.

Results

During germination of *Pinus pinea* remarkable changes in the content and redox balance of ascorbate occurred both in the embryos and in the endosperm. Dry seeds were completely devoid of the reduced form of vitamin C, ASC, whereas both embryos and endosperms contained its oxidized form, DHA (Fig. 1).

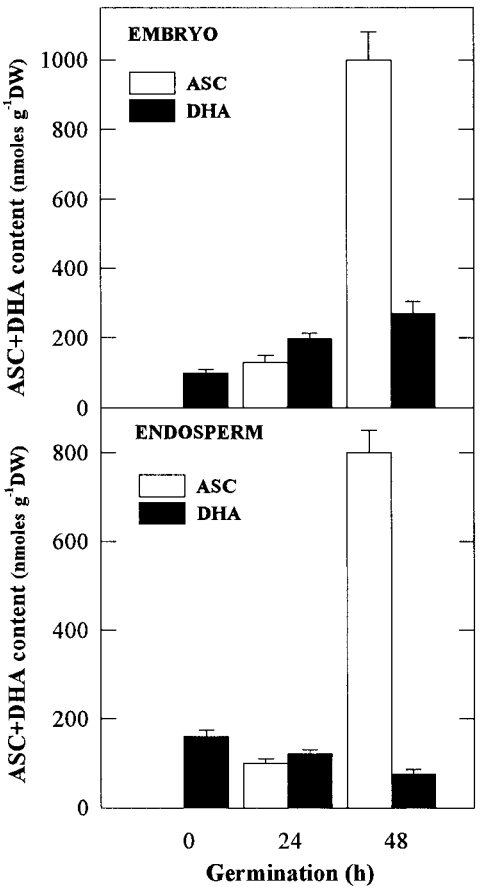

Fig. 1. Ascorbate and dehydroascorbate content during the first stages of germination in embryo and endosperm of *Pinus pinea*. The results are given as the mean value of six experiments ±SD.

When the seeds, deprived of the lignified tegument, were sown on moist filter paper, a rapid imbibition started. After 24 h the ascorbate pool (ASC plus DHA) increased by approximately 3 and 1.4 times in the embryos and endosperm, respectively, even if seeds did not show evident germination signs, such as radicle protrusion. A further rise in the ascorbate pool occurred in the following hours both in embryos and endosperms (Fig. 1).

The activity of GLDH, the enzyme that synthesizes ASC by oxidizing its last precursor, also increased during germination (Fig. 2). During the first 24 h, the GLDH rose more rapidly in the endosperm than in the embryo. However, in this reserve tissue a steady-state situation seemed to be reached at the end of the first day of germination, since no significant additional increase occurred in the following hours. On the other hand, in the embryos GLDH progressively showed further increases throughout the analysed period.

During germination changes in the glutathione pool also occurred (Fig. 3). Dry seeds contained a certain amount of glutathione. However, the redox balance of this pair was quite different in the two analysed parts, since dry embryos contained a similar amount of GSH and GSSG, whereas the endosperm of dry seeds had a GSH content more than 3 times higher than that of GSSG. The increase in the total glutathione pool which occurred during the 24 h of seed imbibition, both in embryos and in endosperms, was only transient, since, at 48 h, the glutathione (GSH + GSSG) contents were lower than in the dry seeds (Figs 3, 4). In spite of this decrease, glutathione biosynthesis occurred throughout the analysed period both in embryos and endosperms, since

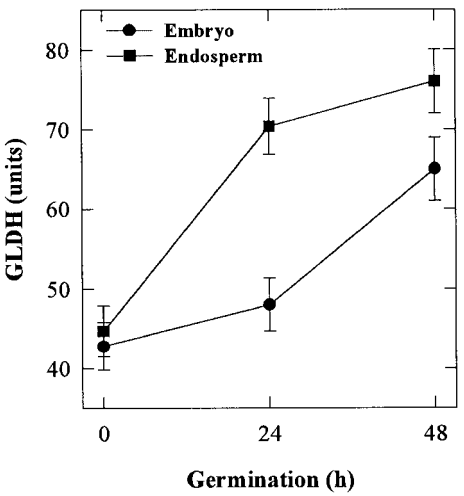

Fig. 2. Galactono-γ-lactone dehydrogenase activity during the first 48 h of germination in embryo and endosperm of *P. pinea*. The results are given as the mean value of six experiments ±SD. 1 unit = 1 nmol cytocrome *c* reduced mg^{-1} protein min^{-1}.

treatment with BSO, an inhibitor of glutathione biosynthesis, affected its content (Fig. 4). Interestingly, the increase in GSH observed after 24 h was accompanied by a strong decrease in GSSG content (Fig. 3).

The enzymes of the ascorbate–glutathione cycle also changed their activities during germination (Fig. 5). ASC peroxidase was completely absent in dry seeds, but gradually increased its activity during the analysed period both in embryos and in endosperms. The presence of different ASC peroxidase isoenzymes and the timing of their appearance during germination was tested by native PAGE analysis. The electrophoretic pattern of the ASC peroxidase shows one band with ASC peroxidase activity detectable after 24 h of germination both in the embryo and in the endosperm. This band became more intense during germination, thus confirming the increase in ASC peroxidase activity, but no extra bands appeared in the following hours (data not shown).

The two ASC recycling enzymes, DHA and AFR reductase, behaved differently. The DHA reductase activity progressively decreased during germination both in the embryos and in the endosperm. The electrophoretic pattern of the embryo's DHA reducing proteins showed the presence of several bands in the dry seeds. During germination some proteins sharing DHA reducing activity reduced their intensity or disappeared. The protein with the highest migration rate maintained the same activity (Fig. 6). Moreover, the AFR reductase activity was the same in the endosperm, and only slightly increased in the embryos (Fig. 5).

As far as the GR activity is concerned, its activity increased progressively both in the embryo and in the endosperm (Fig. 5).

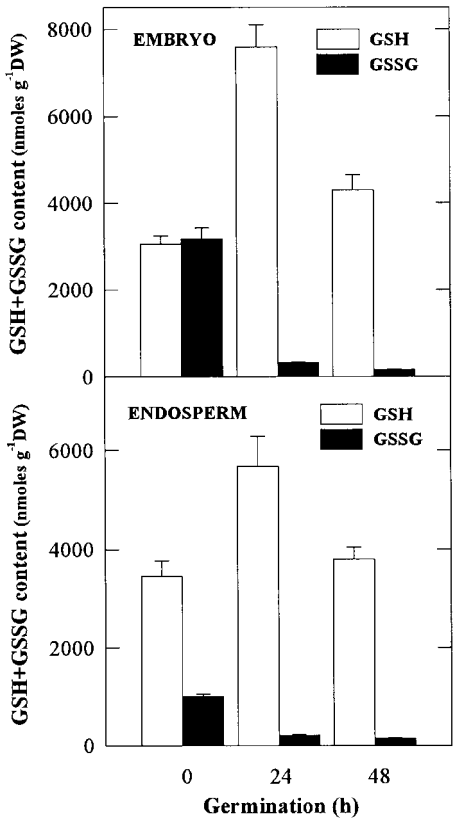

Fig. 3. Glutathione content during the first 48 h of germination in embryo and endosperm of *P. pinea*. The results are given as the mean value of six experiments ±SD.

Discussion

In the early stages of germination of *Pinus pinea* seeds the restoration of the oxidative metabolism is accompanied by changes in the redox state and metabolism of ascorbate and glutathione, two redox pairs playing a key role in ROS scavenging.

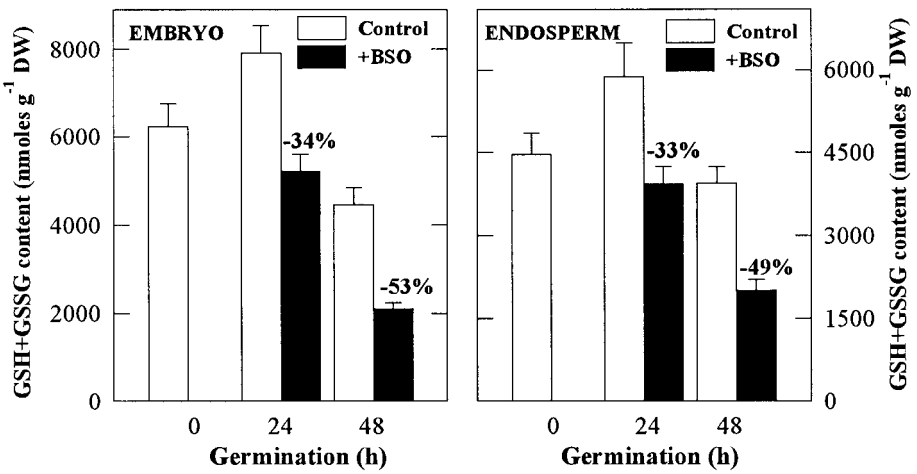

Fig. 4. Effects of L-buthionine(S,R) sulphoximine (BSO) on the biosynthesis of glutathione pool in *Pinus pinea* seeds during 48 h of germination. The results are given as the mean value of six experiments ±SD.

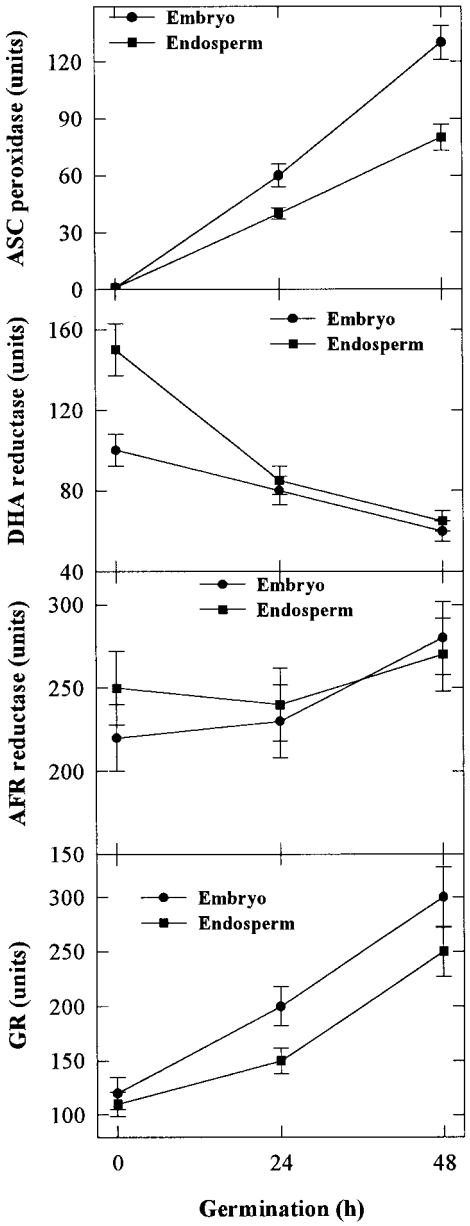

Fig. 5. Ascorbate peroxidase, dehydroascorbate reductase, ascorbate free radical reductase, and glutathione reductase activities during the first 48 h of germination in *P. pinea* seeds (embryo and endosperm). The results are given as the mean value of six experiments ±SD. Activities are expressed in units; 1 unit = 1 nmol substrate metabolized mg^{-1} protein min^{-1}.

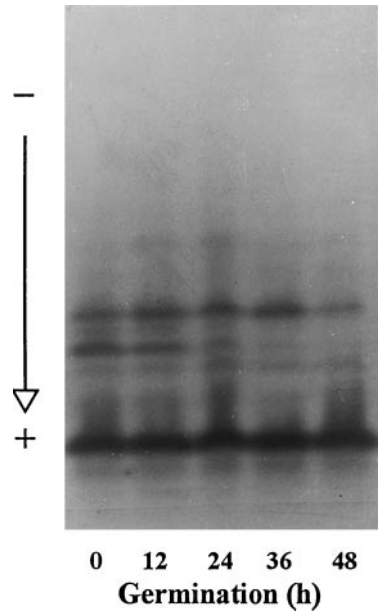

Fig. 6. Native PAGE of DHA-reducing proteins of *P. pinea* embryos in dry seeds and during germination. 300 μg of protein was loaded per lane.

The absence of ASC in dry seeds and its progressive increase during germination is not a peculiarity of *P. pinea*, since the same behaviour has been observed in some angiosperm seeds. According to data reported here, the increase in ASC and the change in the ASC/DHA ratio occurring during germination are probably due to *ex novo* ASC biosynthesis rather than the ASC recycling capability. GLDH, the last enzyme in the ASC biosynthetic pathway (Wheeler *et al.*, 1998), is present both in embryos and in endosperm of dry and germinating seeds, and its activity progressively increases during germination (Figs 2, 5). The existence of active ASC biosynthesis in the embryos is not surprising, since it has been widely reported that both cell division and cell expansion require ASC (Liso *et al.*, 1984; Innocenti *et al.*, 1990; Kerk and Feldman, 1995; De Gara *et al.*, 1996; Arrigoni *et al.*, 1997; De Tullio *et al.*, 1999); and these two processes are fundamental for seedling development. The fact that the endosperm, a peculiar haploid tissue with a temporary role of supplying reserves to the embryos, is also autonomous in ASC biosynthesis suggests that ASC could also be involved, directly or by maintaining the opportune cellular redox balance, in the processes responsible for rendering the reserve substances available for the germinating embryos. In this reserve tissue, GLDH activity reaches the steady-state level after 24 h, whereas in the embryos its activity seems to have a trend of continuous increase, even after 48 h of analysis. The different behaviour of this enzyme fully agrees with the different fate of the two structures (depletion of storage substances and death for the endosperm, generation of a new organism for the embryo).

Proteins able to reduce DHA are also present both in embryos and endosperm, and have a particularly high activity in dry seeds. The presence of many proteins with DHA-reducing capability in the embryos of *P. pinea* collected from dry seeds agrees with what occurs in *Triticum durum* L. and *Vicia faba* L. as well as the disappearance of part of these proteins during germination (Tommasi *et al.*, 1995; De Gara *et al.*, 1997). As previously suggested, some of the DHA reducing proteins

active only in the early stages of germination could play a key role in ASC provision until the ASC biosynthetic capability is adequately restored. Moreover, some of the proteins with DHA reducing capability, that are present in dry seeds and disappear during germination, could also have a different physiological function. The capability of DHA reducing proteins to work as protease inhibitors (Trümper *et al*., 1994), suggests that some of these proteins could contribute to regulate the hydrolysis of reserve proteins.

The other enzyme responsible for ASC recycling, AFR reductase, does not significantly change in the first 48 h of germination. Exclusively in the embryos a slight increase is detectable at the end of the analysed period. Since the only source of AFR is ASC oxidation (Borraccino *et al*., 1982; Arrigoni, 1994), the presence of AFR reductase in dry seeds also substantiates precocious ASC generation during the very early stages of germination through DHA reduction or GL conversion into ASC.

The absence of ASC peroxidase activity in dry *P. pinea* seeds confirms that this character is typical of orthodox seeds since orthodox angiosperm seeds also lack ASC peroxidase (De Gara *et al*., 1997, 2000; Ushimaru *et al*., 1997).

In contrast to ascorbate, the glutathione pool is present in dry seeds both in the reduced and oxidized forms. Moreover, it only transiently increases during germination and after 48 h of germination the total glutathione (GSH + GSSG) content is lower than that in dry seeds (Fig. 4). The high level of glutathione in dry *P. pinea* seeds confirms reports for several herbaceous higher plants (Klapheck, 1988; Kranner and Grill, 1993). The treatment of BSO, a specific inhibitor of glutathione biosynthesis, shows that GSH synthesis starts during the first 24 h of germination both in the embryos and in the endosperm (Fig. 4). However, the data from this study suggest that the GSSG reduction plays a relevant part in the GSH rise occurring during the first 24 h (Fig. 3). This is particularly evident in the embryos, where GSSG decreases from a value higher than 50% of the total glutathione pool in the dry seed to a value lower than 4% after 24 h of germination (Fig. 3). The presence of an amount of GSSG in dry embryos higher than in germinating seed could contribute to prevent the germination process, since it has been suggested that in dormant wheat embryos GSSG blocks protein synthesis (Fahey *et al*., 1980). Moreover, a reversible inhibition of protein synthesis, dependent on the glutathione redox state, has also been described in mosses exposed to drought and subsequent dehydration (Dhindsa, 1987, 1991). In animal cells, a molecular mechanism for the GSSG-dependent inhibition of protein synthesis has been suggested. GSSG seems to activate a protein kinase responsible for the inactivation of initiation factor 2 by phosphorylating its α-subunit (Kranner and Grill, 1996, and references therein). The reduction of GSSG, occurring as soon as germination starts, could have the function of removing the block of protein synthesis, besides generating molecules with antioxidant properties.

In contrast to what has been reported for pea seedlings (Kranner and Grill, 1993), GR activity increases during germination both in embryos and in endosperm. It is interesting to note that an increase in the availability of NADPH, the electron donor of the enzyme, also occurs during seed germination since, in this process, the pathway responsible for NADPH generation in non-green tissues, the pentose phosphate cycle, is promptly activated (Mayer and Poljakoff-Mayber, 1982). The rise in GR activity also indicates an increase in the utilization of GSH during germination. This utilization probably involves processes other than the ascorbate–glutathione cycle, since DHA reductase and GR, the activities of which are tightly correlated in the ascorbate–glutathione cycle, change in opposite ways during germination. This is not surprising, since glutathione, besides being involved in the protection against oxidative stress both by contributing ASC regeneration and directly scavenging organic and hydrogen peroxides by means of GSH peroxidase, is also involved in sulphur metabolism (Noctor *et al*., 1998; De Kok and Stulen, 1993).

The fact that in dry seeds the main physiological redox pairs are shifted throughout the oxidized state could represent a strategy that, with cellular dehydration, contributes to maintaining a low level of metabolic activity. Interestingly, the oxidative status is more pronounced in the embryos than in the endosperm.

The GSH/GSSG ratio, besides affecting protein synthesis, seems to regulate enzyme activity by interfering with the redox state of proteic cysteine residues (De Kok and Stulen, 1993; Noctor and Foyer, 1998). Moreover, recent reports suggest that the ASC/DHA redox pair works as a redox sensor able to block or delay cell division on the basis of its redox state (de Pinto *et al*., 1999). In addition, it is known that DHA inhibits the activity of the enzymes regulated by the thioredoxin–thioredoxin system (Morell *et al*., 1997). Data presented here also suggest that the redox state of the ascorbate and glutathione pools and the level of the activities of the enzymes involved in their redox reactions are finely regulated according to the plant developmental stages. Further studies are in progress to evaluate the relevance of the redox state of ASC and GSH systems in seed maturation and in the viability of orthodox seeds.

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