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Dehydroascorbate-reducing proteins in maize are induced by the ascorbate biosynthesis inhibitor lycorine

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Abstract – Dehydroascorbate (DHA) reductase (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) has been generally considered a specific enzyme of the ascorbate-glutathione cycle. However, at least four distinct proteins can catalyze *in vitro* both glutathione-dependent DHA reduction and other reactions mainly related to thiol-disulphide exchange. These data have raised questions both on the existence of specific DHA reductase and the actual physiological role of DHA-reducing proteins (DRP). We have observed characteristic electrophoretic patterns of DRP in dark-germinating embryos of different plant species. Marked differences were observed not only in the number, but also in the migration rate of DRP under non-denaturing conditions. In order to evaluate the actual contribution of DRP activity to ascorbate (ASC) regeneration under conditions limiting ASC biosynthesis, *Z. mays* germinating embryos excised from endosperm were either incubated in distilled water or treated with the alkaloid lycorine, an inhibitor of ASC biosynthesis. In parallel with the decrease in ASC content, a strong enhancement in DRP activity occurred. The increase in DRP activity was prevented by cycloheximide, and thus seems to be due to *de novo* protein synthesis. The possible involvement of DRP in avoiding DHA accumulation under adverse environmental conditions is discussed. © Elsevier, Paris

Ascorbate regeneration / dehydroascorbate / lycorine / *Zea mays*

AFR, ascorbate free radical (monodehydroascorbate) / APX, ascorbate peroxidase (EC 1.11.1.11) / ASC, ascorbate / CHI, cycloheximide / DHA, dehydroascorbate / DRP, dehydroascorbate-reducing proteins / FW, fresh weight / GL, L-galactono-γ-lactone / GSH, glutathione / GSSG, glutathione disulphide / PAGE, polyacrylamide gel electrophoresis

1. INTRODUCTION

A large number of early reports dealt with the presence in plant and animal tissues of DHA reductase (EC 1.8.5.1), the enzyme catalysing the two-electron reduction of DHA to ASC using GSH as the electron donor [e.g. 12, 41]. The interest of several researchers in the activity of this enzyme resided in the fact that DHA reductase virtually links both the ASC and the GSH systems together, in the sequence of detoxification activities often referred to as the ascorbate-glutathione cycle [26]. In this series of reactions, ASC utilization produces AFR that can either be enzymatically reduced back to ASC or undergo a disproportionation reaction yielding both ASC and DHA. Further analysis of DHA reductase activity, however, indicated a small contribution by this enzyme to ASC regeneration, as compared to AFR reduction [6, 15].

It has been reported that different proteins such as glutaredoxin (thioltransferase), protein disulphide isomerase, thioredoxin (*m* and *f*) and Kunitz-type trypsin inhibitor can also have DHA reductase activity in vitro [1, 35, 37, 39]. DHA reduction performed by these proteins (all sharing in their sequence a tetrapeptide domain with a redox-active dicysteine site) could however be a mere side reaction with no physiological value. In the light of these observations, the existence of a specific DHA reductase has been questioned [32] at least in chloroplasts, where non-enzymatic ASC regeneration is likely to be highly efficient due both to the direct electron transport from photosystem I to AFR, and to the GSH-dependent DHA reduction occurring at alkaline pH in the stroma. Some recent reports on specific DHA reductases in animal [34, 40] and plant tissues [23, 29] have appeared. The presence of the enzyme has also been demonstrated in plant mitochondria and peroxisomes [27]. This necessitates

a definite assessment of the effectiveness of enzymatic DHA reduction in different organisms and cell compartments.

A physiological role for enzymatic DHA reduction has been observed in our laboratory at the onset of seed germination. It has been demonstrated that dry (orthodox) seeds are devoid of ASC, but still have a small DHA pool [7]. New ASC biosynthesis starts only after 10-20 h of germination [18]. At this stage, highly active protein-mediated DHA reduction seems to be the only ASC source the cell can rely on to start ASC-requiring metabolic reactions. A progressive decrease both in the specific activity and in the number of DRP occurs when ASC biosynthesis is progressively restored [2, 20].

Another more general possible function of DRP is suggested by an increasing number of reports dealing with the toxic effect of DHA on cell metabolism [9, 24, 25, 32, 36, 42, 43]. In the light of these reports, enzymatic DHA reduction could represent a mechanism allowing plant cells to avoid DHA accumulation, rather than a means to regenerate ASC.

The simultaneous presence of several distinct proteins all potentially performing DHA reduction and the possible physiological implications of this multiplicity are currently under investigation in our laboratory. We present here observations on the distribution of DRP in different plant materials, the presence of these proteins during germination of maize embryos and their behaviour under conditions limiting ascorbate biosynthesis.

2. RESULTS

2.1. Presence of distinct DRP patterns in different plant materials

The native-PAGE method set up in our laboratory makes possible the direct observation of DRP activity [19, 21]. By using this technique, we have been able to detect marked differences in the soluble fraction between the electrophoretic patterns of several plant species analysed. *Figure 1* shows the results obtained with the dicots *Capsicum annuum*, *Spinacia oleracea*, *Sinapis alba* and the monocot *Allium porrum*. A number of protein bands was observed in the germinating embryos of different species, ranging from 1 (*C. annuum*, *figure 1*) to 6 (*Zea mays*, *figure 2*). Both the number and the migration rate of DRP bands observed for each plant species were constant and reproducible.

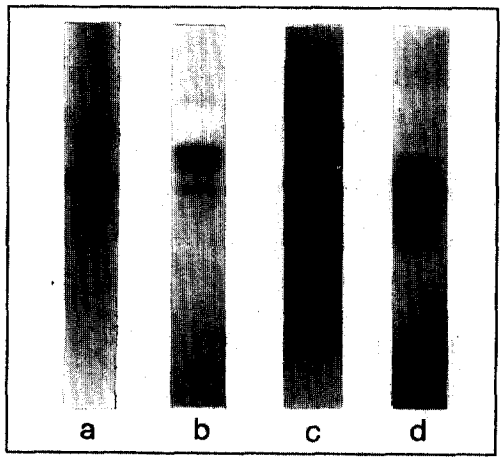

Figure 1. Native-PAGE and DRP activity staining in *Capsicum annuum* (a), *Spinacia oleracea* (b), *Allium porrum* (c) and *Sinapis alba* (d). Several attempts were made for each plant species, in order to find the protein concentration allowing the best resolution of DRP bands. The amount of total protein loaded for each lane ranged from 350 to 500 µg. Bromophenol blue was used as an internal standard to evaluate the relative migration rate of the protein bands.

The time-course of DRP activity in *Z. mays* embryos during germination was also analysed (*figure 2*). Dry embryos showed very high activity, which remained constant also in seedlings after 72 h of germination (*figure 2*, lane d). At this stage, much less activity

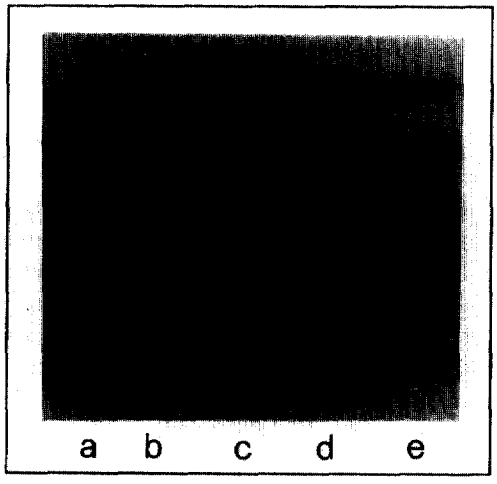

Figure 2. Dehydroascorbate-reducing proteins in maize during germination. Embryos were excised at different stages of caryopses germination: (a) dry seeds; (b) 24 h; (c) 48 h; (d) 72 h (coleoptile); (e) 72 h (root). The same amount of total proteins (600 µg) was loaded in each lane.

could be detected in the root (*figure 2*, lane e) as compared to the coleoptile.

2.2. Ascorbate regeneration under conditions limiting its biosynthesis

In ASC-synthesizing organisms, ASC content depends on four different parameters: (a) rate of ASC synthesis, (b) its utilization in ASC-requiring metabolic activities, (c) regeneration from its oxidized forms AFR and DHA, and (d) breakdown and degradation into oxalate via diketogulonic acid. Little information regarding the fourth point is presently available. In order to evaluate the actual contribution of DRP to ASC regeneration, we investigated the effect of lycorine on ASC content and DRP activity in maize. Lycorine was used since this alkaloid proved to be a strong inhibitor of GL dehydrogenase (EC 1.3.2.3), the last enzyme of ASC biosynthetic pathway, both in vivo [3, 4, 16, 19] and in vitro [8].

Embryos were used after 48 h of germination because at this stage both ASC biosynthesis and APX (indicative of ASC utilization) are fully active in maize [18]. The effect of different treatments on ASC content in maize embryos excised from endosperm is shown in *figure 3*. At time 0 (immediately after excision, *figure 3* A) ASC content exceeded 2 000 nmoles per gram fresh weight. After 3 h of incubation (*figure 3* B), ASC consumption led to a marked decrease in ASC content. Lycorine treatment caused a further 14 % decrease in ASC content, whereas administration of 1 mM GL, the last precursor of ASC biosynthesis, induced a 35 % increase, keeping ASC content at levels comparable to time 0. Incubation in lycorine plus GL gave values comparable to water. After 5 h of incubation (*figure 3* C) ASC content was also lower in lycorine-treated samples (–26 %) than in water-incubated ones. The progressive decrease observed in ASC content of water-incubated samples is not surprising, since embryos excised from their storage tissues are not supplied with adequate ASC precursors; in fact administration of exogenous GL promptly induced ASC synthesis, both when this precursor was added immediately after excision (*figure 3* B and C) and after 5 h of incubation in water (*figure 3* D). The latter result indicates that neither the viability nor the biosynthetic capability were affected during incubation. An appreciable increase was also induced by GL plus lycorine after 5 h simultaneous administration (*figure 3* C). However, when embryos were pre-incubated in lycorine (*figure 3* D), the GL conversion into ASC was virtually blocked, thus suggesting a protective effect of GL on GL dehydrogenase. The same treatments causing marked differences in ASC content apparently did not affect DHA content (*figure 4*). However, a lower DHA level could be observed at time 0. We hypothesize that embryo excision induced a wounding effect resulting in ASC decrease and DHA increase.

Lycorine treatment of maize embryos affected the activity of the enzymes of the ASC system in different ways (*table I*). An increase in protein-mediated DHA reduction was marked after 3 h of lycorine incubation

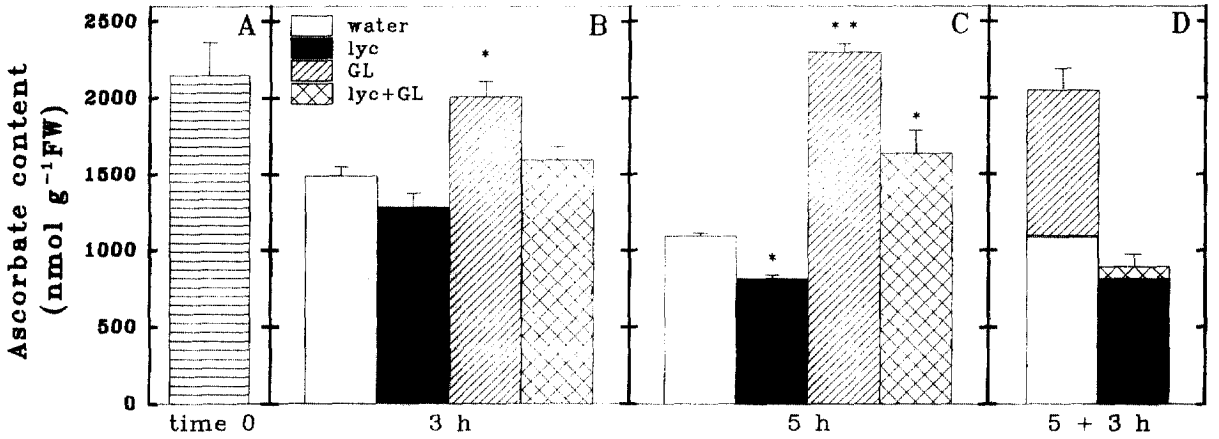

Figure 3. Ascorbate content in maize embryos. After 48 h of germination, embryos were excised and ascorbate measured at time 0 (**A**), after 3 h (**B**) and 5 h (**C**) of incubation in distilled water, 100 µM lycorine (lyc), 1 mM L-galactono- γ-lactone (GL), 100 µM lycorine plus 1 mM L-galactono-γ-lactone (lyc + GL). **D**, Embryos treated 5 h in water were further incubated 3 h in GL; embryos treated 5 h in lycorine were further incubated 3 h in GL plus lycorine. Mean value of 3 replicates + SE. Asterisks indicate significant differences in the Student's *t*-test between control (water) and treatment. (*) $P < 0.05$; (**) $P < 0.01$.

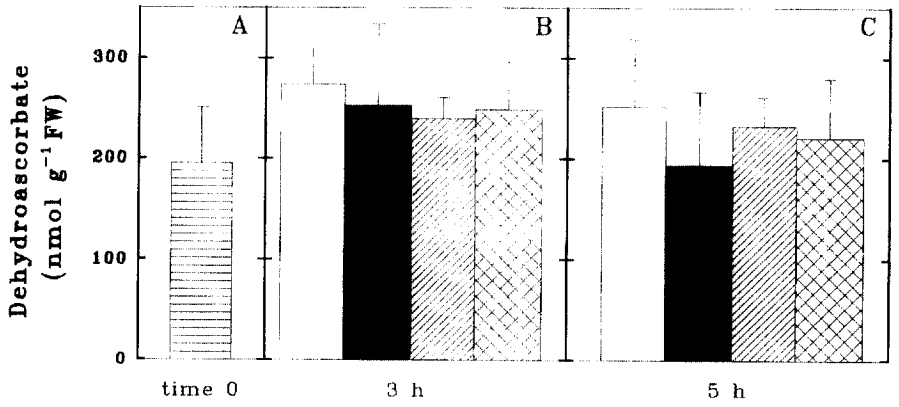

Figure 4. Dehydroascorbate content in maize embryos. After 48 h of germination, embryos were excised and dehydroascorbate measured at time 0 (**A**), after 3 h (**B**) and 5 h (**C**) of incubation. Abbreviations and symbols as in *figure 3*.

Table I. Effect of lycorine on enzyme activities in maize embryos immediately after excision (time 0) and after 3 and 5 h of incubation. Mean of 3 replicates ± S.E. [a, b] different letters indicate significant differences in the Student's *t*-test ($P< 0.05$).

Treatment	Total DRP activity (nmol DHA reduced·min^{-1}·mg^{-1} prot)	AFR reductase (nmol NADH oxidized·min^{-1}·mg^{-1}prot)	GSSG reductase (nmol NADPH oxidized·min^{-1}·mg^{-1} prot)	ASC peroxidase (nmol ASC oxidized·min^{-1}·mg^{-1} prot)
Time 0	73 ± 15	230 ± 21	75 ± 12	160 ± 7
3 h distilled water	47 ± 4 [a]	201 ± 6	58 ± 2	175 ± 4
3 h 100 µM lycorine	87 ± 7 [b]	198 ± 12	46 ± 5	139 ± 20
5 h distilled water	76 ± 12 [a]	221 ± 66	89 ± 23	160 ± 16
5 h 100 µM lycorine	130 ± 19 [b]	212 ± 59	87 ± 25	138 ± 19

(+ 85 % in comparison to water). Lycorine did not induce any significant change in either AFR reductase or glutathione reductase activity. Longer incubation (5 h) showed an increase in DRP activity in both water- and lycorine-treated samples. In this case, however, lycorine treatment also induced a 70 % further increase in DRP activity. There was still no change in AFR reductase, whereas both treatments induced a similar increase in GSSG reductase. Treatment with lycorine caused only slight changes in the activity of APX, an enzyme that usually consumes a large part of the whole ASC pool in plant cells.

Interestingly, the assayed enzymes of the ASC-GSH pathway showed different behaviour in response to wounding (excision). The activities of DRP and GSSG reductase were higher in embryos at time 0. On the other hand, during the period of our analysis, AFR reductase and APX activities did not show significant differences between time 0 and later treatments.

Our native-PAGE assay allowed us to further characterize the effect of lycorine on maize embryo DRP. *Figure 5* A shows the electrophoretic patterns obtained by incubating maize embryos for 5 h in 100 µM lycorine. A remarkable increase in the activity of DRP was observed. The effect of lycorine on DRP activity was not specific for maize embryos since a similar effect was also observed in *Pisum sativum* cotyledonless embryos (*figure 5* B), *Lupinus albus* roots and hypocotyls and *Triticum durum* embryos (data not shown).

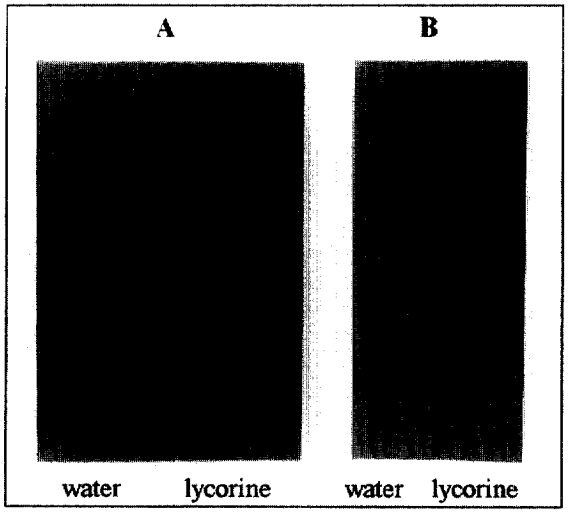

Figure 5. Electrophoretic determination of dehydroascorbate reducing protein activity in maize (**A**) and pea (**B**) embryos. Materials were incubated 5 h either in water or in 100 µM lycorine. For each lane 800 µg of total proteins were loaded.

Data reported in *table II* show that cycloheximide prevented the rise in DRP activity induced by lycorine.

Table II. Effect of cycloheximide on dehydroascorbate reducing protein and ascorbate free radical reductase activities in maize embryos. Mean of three replicates ± S.E. n.d.: not determined.

Treatment	Total DRP activity (nmol DHA red·min^{-1}·mg^{-1} prot)	AFR reductase (nmol NADH ox·min^{-1}·mg^{-1} prot)
Distilled water	48 ± 7	194 ± 8
CHI 100 μg ml^{-1}	10 ± 2	218 ± 20
CHI 100 μg ml^{-1} + 100 μM lyc	16 ± 5	n.d.

The elicitation of DRP activity seems therefore to be largely due to de novo synthesis. Lycorine did not affect DRP activity in vitro (*table III*).

Table III. Lycorine effect on the activity of dehydroascorbate-reducing proteins in cell-free maize extract. Mean of 3 replicates ± S.E.

Assay conditions	Total DRP activity (nmol DHA reduced·min^{-1}·mg^{-1} prot)
Without lycorine	47 ± 5
50 μM lycorine	46 ± 4

3. DISCUSSION

Large amounts of ASC are utilized in several metabolic reactions including the biosynthesis of hydroxyproline containing proteins [5, 17], ASC peroxidase-mediated scavenging of hydrogen peroxide [2] and safe dissipation of excess light energy via the xanthophyll cycle [22]. These activities all lead to the production of AFR, that is either reduced to ASC by means of AFR reductase, or undergoes a spontaneous disproportionation reaction yielding both ASC and DHA [2, 10].

Plant cells can rely on three enzyme-regulated ASC sources: biosynthesis, regeneration via AFR reductase and regeneration via DRP. Control of degradation and breakdown of the oxidized forms AFR and DHA could be a possible fourth mechanism regulating ASC content. Very little information is presently available about the mechanisms and enzymes modulating ASC catabolism [30], although such regulation is very likely to occur.

Recent observations on the capability of several proteins to catalyze DHA reduction (see introduction) and our results confirming the multiplicity of DRP (*figure 1*) led us to study DRP contribution to ASC regeneration under conditions limiting its biosynthesis.

We observed a decrease in ASC content in excised embryos, and an even more severe ASC loss after lycorine treatment. The decrease observed in ASC content could be either due to impaired ASC biosynthesis, or to increased ASC breakdown. To our knowledge, no data about a lycorine-mediated induction of ASC breakdown are presently available. On the other hand, we have a lot of data on the effect of lycorine on ASC synthesis. On the basis of these observations, we speculate that the contribution of AFR and DHA recycling to the maintenance of an adequate ASC pool is negligible, since recycling mechanisms are apparently not able to keep pace with ASC consumption.

Although apparently not effective in keeping ASC in the reduced form, the activities of AFR reductase and of DRP could have an important role in avoiding DHA accumulation, since DHA itself proved to have a possible disruptive effect on cell membranes and to inhibit the activity of several enzymes (see introduction). We tried to investigate whether DHA content could be correlated with lycorine-mediated induction of DRP activity. In our experiments, DHA content was not significantly affected by treatments imposed (*figure 4*). ASC/DHA ratios ranged between 4 and 5.4 in both water-treated and lycorine-treated samples. However, DHA quantitation is very troublesome due to its well documented instability [11, 28, 31]. Moreover, according to some authors DHA could form condensation products with amino groups of proteins [23, 43]. In this case, some DHA could be precipitated with proteins during the extraction. An actual role for DHA in DRP activation cannot therefore be totally excluded. It is noticeable that the highly productive maize hybrid used for our experiments showed efficient DHA reduction not only in the very early germination stage, as previously observed in other species [2, 20; F. Tommasi, pers. comm.], but also in seedlings. Moreover, when DRP activity was compared in maize pure lines and the corresponding first generation hybrid, higher DHA recycling capability was detected in the latter [De Gara et al., unpublished]. These results suggest a possible correlation between DRP activity and plant productivity.

We observed that DRP activity can be experimentally induced by lycorine administration. Treatment with lycorine apparently lowers ASC content below a threshold value that seemingly activates DHA recy-

cling. This is consistent with the observation that an increase in DRP activity also occurred in water-incubated samples in parallel with ASC decrease (*figure 3*; *table I*). Our data, however, cannot exclude a possible alternative mechanism in which lycorine directly induces DHA recycling mediated by DRP. Further studies presently underway in our laboratory will hopefully elucidate this point. Addition of lycorine to the cell-free system, however, did not induce any change in DRP activity (*table III*). This seems to suggest a complex mechanism requiring cell integrity for its activation.

Lycorine-induced DRP activation seems to be due to de novo protein synthesis, whereas AFR reductase is not affected by treatment with cycloheximide (*table II*). Our data also seem to suggest a high DRP turnover (although only pulse-chase experiments could give conclusive evidence on this point). This would indicate a fast modulable response in DRP activity, whereas AFR reductase would not be as readily inducible. We believe that the presence of several different DRP could be linked to the necessity to rapidly remove toxic DHA in all ASC-utilizing sites, where DHA is inevitably formed.

Several indications suggest a role for DRP in some critical stages of plant life, such as germination [2, 20] or response to environmental stress conditions [14, 33, 38, 44]. The number of DRP observed and the apparent plasticity of their activation make further DRP investigation worthwhile. We have observed that lycorine is a useful tool for the elicitation of DRP activity. Our next goal will be the identification and characterization of the proteins involved in this response.

4. METHODS

4.1. Plant materials. Seeds of *Pisum sativum* L., *Sinapis alba* L., *Capsicum annuum* L., *Allium porrum* L., *Spinacia oleracea* L. and caryopses of *Zea mays* L. cv Sele were sown in glass Petri dishes on moist filter paper at 23 °C. After 48 h in the dark, germinating embryos were excised and used for analysis. Maize and pea embryos were incubated in 100 mL of the indicated incubation medium, in Petri dishes at 25 °C under mild agitation. Materials were homogenized in an ice cold porcelain mortar with a grinding medium composed of 50 mM Tris-HCl (pH 7.8), 0.3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.05 % (w/v) cysteine, and the homogenate centrifuged 15 min at 20 000 x *g*. The supernatant was used for both spectrophotometric and electrophoretic determinations. Ascorbate and dehydroascorbate were measured according to Kampfenkel et al. [28].

4.2. Electrophoresis. Native-PAGE was performed according to De Gara et al. [20], using 14 x 24 cm slab gels. After completion of the electrophoretic run, gels were incubated under agitation for 20 min in 0.1 M phosphate buffer (pH 6.2) containing 4 mM GSH and 2 mM DHA. Gels were then washed with distilled water and specifically stained for DHA reductase activity by incubation for 15 min in a 0.125 N HCl solution containing 0.1 % (w/v) ferrichloride and 0.1 % (w/v) ferricyanide, their reaction with ASC yielding a coloured product. Proteins presenting DHA reductase activity were observed as dark blue bands on a light blue background, the latter due to the non-enzymatic ASC formation occurring in the reaction between DHA and GSH.

4.3. Enzyme assay. Spectrophotometric determination of DHA reductase activity was performed as reported by Arrigoni et al. [7]. The reaction mixture contained 0.1 M phosphate buffer (pH 6.2) and 2 mM GSH. The reaction was started upon addition of 1 mM DHA. The rate of non-enzymatic DHA reduction was subtracted. AFR reductase and APX activities were assayed according to Arrigoni et al. [7]. DRP and AFR reductase assays in the presence of the protein synthesis inhibitor cycloheximide were performed after incubation of excised maize embryos (48 h of germination) for 3 h in water (control), in 100 $\mu g \cdot mL^{-1}$ cycloheximide and in cycloheximide plus 100 µM lycorine. Glutathione reductase was measured as indicated in Osswald et al. [33]. Protein measurement was performed according to Bradford [13].

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REFERENCES

[1] Ahn B.Y., Moss B., Glutaredoxin homologue encoded by vaccinia virus is a virion-associated enzyme with thioltransferase and dehydroascorbate reductase activities, Proc. Natl. Acad. Sci. USA 89 (1992) 7060-7065.

[2] Arrigoni O., Ascorbate system and plant development, J. Bioenerg. Biomemb. 26 (1994) 407-419.

[3] Arrigoni O., Arrigoni-Liso R., Calabrese G., Lycorine as an inhibitor of ascorbic acid biosynthesis, Nature 256 (1975) 513-514.

[4] Arrigoni O., Arrigoni-Liso R., Calabrese G., Ascorbic acid as a factor controlling the development of cyanide insensitive respiration, Science 194 (1976) 332-333.

[5] Arrigoni O., Arrigoni-Liso R., Calabrese G., Ascorbic acid requirement for biosynthesis of hydroxyproline-containing proteins in plants, FEBS Lett. 82 (1977) 135-137.

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

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

[8] Arrigoni O., De Gara L., Paciolla C., Evidente A., de Pinto M.C., Liso R., Lycorine: a powerful inhibitor of L-galactono-γ-lactone dehydrogenase activity, J. Plant Physiol. 150 (1997) 362-364.

[9] Bianchi J., Rose R.C., Dehydroascorbic acid and cell membranes: possible disruptive effects, Toxicology 40 (1987) 75-82.

[10] Bielski B.H.J., Chemistry of ascorbic acid radicals, in: Seib P.A., Tolbert B.M. (eds.), Ascorbic acid: chemistry, metabolism and uses, Advances in chemistry series 200, American chemical society, 1981, pp. 81-93.

[11] Bode A.M., Cunningham L., Rose R.C., Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography, Clin. Chem. 36 (1990) 1807-1809.

[12] Borsook H., Davenport H.W., Jeffreys C.E.P., Warner C., The oxidation of ascorbic acid and its reduction in vitro and in vivo, J. Biol. Chem. 117 (1937) 237-279.

[13] Bradford M.M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[14] Castillo F.J., Greppin H., Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure, Environ. Exp. Bot. 28 (1988) 231-238.

[15] Coassin M., Tomasi A., Vannini V., Ursini F., Enzymatic recycling of oxidized ascorbate in pig heart: one-electron vs. two-electron pathway, Arch. Biochem. Biophys. 290 (1991) 456-462.

[16] Córdoba-Pedregosa C., González-Reyes J.A., Cañadillas M.S., Navas P., Córdoba F., Role of apoplastic and cell-wall peroxidases on the stimulation of root elongation by ascorbate, Plant Physiol. 112 (1996) 1119-1125.

[17] De Gara L., Tommasi F., Liso R., Arrigoni O., Ascorbic acid utilization by prolyl hydroxylase in vivo, Phytochemistry 30 (1991) 1397-1399.

[18] De Gara L., De Tullio M., Paciolla C., Liso R., Arrigoni O., Cytosolic ascorbate peroxidase in Angiosperms and the different expression of its isoforms in maize embryos during germination, in: Welinder K.G., Rasmussen S.K., Penel C., Greppin H. (eds.), Plant peroxidases, biochemistry and physiology, University of Geneva Press, Geneva, 1993, pp. 251-255.

[19] De Gara L., Paciolla C., Tommasi F., Liso R., Arrigoni O., In vivo inhibition of galactono-γ-lactone conversion to ascorbate by lycorine, J. Plant Physiol. 144 (1994) 649-653.

[20] De Gara L., de Pinto M.C., Arrigoni O., Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination, Physiol. Plant. 100 (1997) 894-900.

[21] De Tullio M.C., Paciolla C., de Pinto M.C., De Gara L., The multiplicity of enzymatic DHA reduction: a new purification procedure for a DHA reducing protein from potato tubers, J. Biol. Res. - Boll. Soc. It. Biol. Sper. 71 (1995) 163-170.

[22] Demmig-Adams B., Adams III W.W., Photoprotection and other responses of plants to high light stress, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1992) 599-626.

[23] Every D., Enzymatic method to determine dehydroascorbic acid in biological samples and in bread dough at various stages of mixing, Anal. Biochem. 242 (1996) 234-239.

[24] Fiorani M., De Sanctis R., Saltarelli R., Stocchi V., Hexokinase inactivation induced by ascorbic acid/Fe(II) in rabbit erythrocyte is independent of glutathione-reductive processes and appears to be mediated by dehydroascorbic acid, Arch. Biochem. Biophys. 342 (1997) 191-196.

[25] Forti G., Studi sulla fisiologia dell'acido ascorbico XXIII: Azione inibente dell'acido deidroascorbico sulla fosforilazione ossidativa di mitocondri vegetali, Rend. Acc. Naz. Lincei 24 (1958) 70-77.

[26] Foyer C.H., Lelandais M., Kunert K.J., Photooxidative stress in plants, Physiol. Plant. 92 (1994) 696-717.

[27] Jiménez A., Hernández J.A., del Río L.A., Sevilla F., Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves, Plant Physiol. 114 (1997) 275-284.

[28] Kampfenkel K., Van Montagu M., Inzé D., Extraction and determination of ascorbate and dehydroascorbate from plant tissue, Anal. Biochem. 225 (1995) 165-167.

[29] Kato Y., Urano J., Maki Y., Ushimaru T., Purification and characterization of dehydroascorbate reductase from rice, Plant Cell Physiol. 38 (1997) 173-178.

[30] Keates S.E., Loewus F.A., Franceschi V.R., Oxalate biosynthesis from analogs of ascorbic acid in the pathogenic fungus *Sclerotinia sclerotiorum*, Plant Physiol. 114 Suppl. (1997) 48.

[31] Lykkesfeldt J., Loft S., Poulsen H.E., Determination of ascorbic acid and dehydroascorbic acid in plasma by high-performance liquid chromatography with coulometric detection - are they reliable biomarkers of oxidative stress? Anal. Biochem. 229 (1995) 329-335.

[32] Morell S., Follmann H., De Tullio M., Häberlein I., Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants, FEBS Lett. 414 (1997) 567-570.

[33] Osswald W.F., Kraus R., Hippeli S., Benz B., Volpert R., Elstner E.F., Comparison of the enzymatic activities of dehydroascorbic acid reductase, glutathione reductase, catalase, peroxidase and superoxide dismutase of healthy and damaged spruce needles (*Picea abies* (L.) Karst.), J. Plant Physiol. 139 (1992) 742-748.

[34] Paolicchi A., Pezzini A., Saviozzi M., Piaggi S., Andreuccetti M., Chieli E., Mavaldi G., Casini A.F., Localization of a GSH-dependent dehydroascorbate reductase in rat tissues and subcellular fractions, Arch. Biochem. Biophys. 333 (1996) 489-495.

[35] Park J.B., Levine M., Purification, cloning and expression of dehydroascorbic acid-reducing activity from human neutrophils: identification as glutaredoxin, Biochem. J. 315 (1996) 931-938.

[36] Rose R.C., Choi J.L., Bode A.M., Short term effects of oxidized ascorbic acid on bovine corneal endothelium and human placenta, Life Sci. 50 (1992) 1543-1549.
[37] Trümper S., Follmann H., Häberlein I., A novel dehydroascorbate reductase from spinach chloroplasts homologous to plant trypsin inhibitor, FEBS Lett. 352 (1994) 159-162.
[38] Van Camp W., Capiau K., Van Montagu M., Inzé D., Slooten L., Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts, Plant Physiol. 112 (1996) 1703-1714.
[39] Wells W.W., Xu D.P., Yang Y., Rocque P.A., Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity, J. Biol. Chem. 265 (1990) 15361-15364.
[40] Xu D.P., Washburn M.P., Sun G.P., Wells W.W., Purification and characterization of a glutathione dependent dehydroascorbate reductase from human erythrocytes, Biochem. Biophys. Res. Commun. 221 (1996) 117-121.
[41] Yamaguchi M., Joslyn M.A., Investigation of ascorbic acid dehydrogenase of pea (*Pisum sativum*) and its distribution in the developing plant, Plant Physiol. 26 (1951) 757-772.
[42] Yano M., Hayashi T., Namiki M., Formation of free-radical products by the reaction of dehydroascorbic acid with amino acids, J. Agricol. Food Chem. 24 (1976) 815-819.
[43] Yin D., Lipofuscin-like fluorophores can result from reactions between oxidized ascorbic acid and glutamine. Carbonyl-protein cross-linking may represent a common reaction in oxygen radical and glycosylation-related ageing processes, Mech. Ageing Dev. 62 (1992) 35-46.
[44] Zhang J., Kirkham M.B., Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants, Plant Sci. 113 (1996) 139-147.