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Ascorbate and glutathione metabolism in embryo axes and cotyledons of germinating lupine seeds

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Abstract

Changes in ascorbate and glutathione contents and the activities and isoenzyme patterns of enzymes of the ascorbate-glutathione cycle were investigated in embryo axes and cotyledons of germinating lupine (*Lupinus luteus* L.) seeds. Ascorbate content was not significantly affected over the initial 12 h of imbibition in embryo axes, but afterwards increased, with the most rapid accumulation coinciding with radicle emergence. A somewhat similar trend was observed for glutathione with significant increase in embryo axes shortly before radicle protrusion followed by decline in the next hours. In cotyledons the ascorbate pool rose gradually during germination but the amount of glutathione showed fluctuations during a whole germination period. The activity of ascorbate peroxidase (APX) rose progressively in embryo axes, while activities of dehydroascorbate reductase (DHAR) and glutathione reductase (GR) showed transient increase during germination. New isoforms of APX and GR were synthesized, suggesting that they play a relevant role during germination. All analyzed enzymes were already present in dry seeds which allowed them to be active immediately after imbibition.

Additional key words: antioxidative enzymes, *Lupinus luteus* L., radicle emergence.

Introduction

The reactivation of metabolism during germination may provide an important source of reactive oxygen species (ROS), so the enzymes and metabolites responsible for ROS scavenging are of particular importance for the success of germination (Bailly 2004). The antioxidant defence system would involve low molecular mass antioxidants such as reduced glutathione or ascorbate and enzymes of the ascorbate-glutathione cycle. It has been widely reported that dry orthodox seeds are completely devoid of ascorbate and ascorbate peroxidase but contain only moderate amounts of dehydroascorbate (De Gara *et al*. 1997, Tommasi *et al*. 2001). A rapid increase in ascorbate (AsA) content and ascorbate peroxidase (APX, EC 1.11.1.11) activity during the first stages of germination was confirmed for some orthodox seeds of herbaceous and arboreous plants (De Gara *et al*. 1997, Tommasi *et al*. 2001). Glutathione, another redox pair involved in ROS detoxification, also increases during early seed imbibition (Kranner and Grill 1993, De Gara *et al*. 1997, Tommasi *et al*. 2001). The major function of glutathione in protection against oxidative stress is the re-reduction of ascorbate in the ascorbate-glutathione cycle. ROS production during germination should also be regarded as an active, beneficial biological reaction that is connected with high germination capacity and vigorous seedling development (Schopfer *et al*. 2001). ROS, and particularly H_2O_2, may induce expression of many genes, intervene in the cell-wall modification required for elongation of the radicle, and protect the embryo against pathogens (Bailly 2004).

Considering the increasing evidence on the role of ascorbate-glutathione cycle in antioxidative responses to various physiological processes, it was of particular interest to investigate if this pathway plays a role in lupine seed germination. Some aspects of ascorbate-glutathione cycle responses were investigated in embryos and endosperm of germinating recalcitrant seeds. Since a comprehensive investigation in orthodox seeds in both embryo axes and cotyledons has not been published so far, the present work focuses on changes in ascorbate

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Abbreviations: APX - ascorbate peroxidase; AsA - reduced ascorbate; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DTNB - 5,5'-dithiobis(2-nitrobenzoic acid); DTT - dithiothreitol; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; NMR - nuclear magnetic resonance; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; TCA - trichloroacetic acid

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and glutathione content and related enzyme activities in two analysed organs. The aim of this study was to determine whether the organs with different physiological function show differences in development and behaviour of antioxidative mechanism during seed germination.

Materials and methods

Plants: Lupine (*Lupinus luteus* L. cv. Juno) seeds were sown in Petri dishes containing filter paper wetted with water and incubated at 25 °C in darkness up to 48 h. Embryos collected from dry seeds or those germinating at the times indicated in each experiment were separated into cotyledons and embryonic axes and used either immediately or frozen in liquid nitrogen. A seed was considered germinated when the radicle pierced the seed coat. Emergence of the radicle occurred at 23 to 25 h of incubation so among seeds incubated for 24 h we distinguished two populations: seeds just before radicle protrusion (labelled as 24 h) and immediately after radicle protrusion (labelled as 24' h).

Glutathione assay: Non-protein thiols were extracted by homogenizing 0.3 g of axes or cotyledons in 3 cm^3 of 0.1 M HCl, 1 g polyvinylpyrrolidone (PVP) as in Costa *et al*. (2002). After centrifugation at 10 000 *g* for 10 min, the supernatants were used for analysis. Total glutathione (GSH plus GSSG) was determined in the homogenates by spectrophotometry at 412 nm, using yeast-GR, DTNB and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG.

Ascorbate and dehydroascorbate determination: AsA and DHA were determined according to Kampfenkel (1995). Axes or cotyledons (1 g) were homogenized in ice-cold 6 % (m/v) trichloroacetic acid (TCA), and centrifuged at 20 000 *g*. For the concentration quoted, half of a sample was assayed for total ascorbate (AsA plus DHA), and the other half was assayed for reduced ascorbate (AsA) only. DHA concentration was calculated from difference between the total and reduced ascorbate. This assay is based on the reduction of Fe^{3+} by AsA, followed by complex formation between Fe^{2+} and bipyridil, that absorbs at 525 nm. A standard curve of AsA or DHA was used for calibration.

Protein extraction: Extracts for determination of APX (EC 1.1.1.11) and GR (EC 1.6.4.2.) were prepared from 1 g of of liquid-nitrogen-frozen samples homogenized in 5 cm^3 of extraction buffer, containing 50 mM phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 4 % PVP. For measuring APX activity, the tissue was separately ground with homogenizing medium containing 1 mM ascorbate in additions to other ingredients. The homogenates were centrifuged at 14 000 *g* for 20 min and the supernatants were used for spectrophotometric and electrophoretic assays. Extracts for determination of DHAR (EC 1.8.5.1) were prepared from 1 g of samples homogenized in 4 cm^3 of extraction buffer containing 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM DTT and 4 % PVP. The homogenates were centrifuged at 14 000 *g* for 20 min. Protein measurement was performed according to Bradford (1976), using bovine serum albumin as standard.

Enzyme assays: APX and GR activities were determined as described by Garnczarska (2005). For APX activity the hydrogen peroxide dependent oxidation of AsA was followed by a decrease in the absorbance at 290 nm (coefficient of absorbance 2.8 mM^{-1} cm^{-1}). GR activity was measured by following the increase in absorbance at 412 nm due to 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reduction by glutathione reduced form (GSH) generated from glutathione oxidized form (GSSG). DHA reductase activity assay was performed according to Hossain and Asada (1984) by monitoring the glutathione-dependent production of ascorbate at 265 nm.

Native-PAGE of APX, GR and DHA-reducing proteins was performed as described in Tommasi *et al*. (2001). APX isozymes were detected in gels as reported in Mittler and Zilinskas (1993). GR activity was detected according to Rao *et al*. (1996). For DHA reductase activity gels were stained as described in Tommasi *et al*. (2001).

Statistical analysis: All determinations were performed in at least three replicates in 3 independent experiments. SD was calculated and is shown in the figures. The significance of differences between dry and germinating seeds was analysed using Student's *t*-test.

Results

A low ascorbate content was observed in dry seeds both in axes and cotyledons (Fig. 1*A*,*B*). In an ascorbate pool AsA was prevalent and DHA constituted 8 to 27 % in axes and 5 to 16 % in cotyledons of the total ascorbate pool, respectively. In axes, the increase in total ascorbate content occurred shortly before the radicle protruded the seed coat and remained relatively constant in the next hours. The AsA/DHA ratio increased from 3 in axes from dry seeds to 11 in axes immediately after radicle emergence. In cotyledons, total ascorbate pool increased

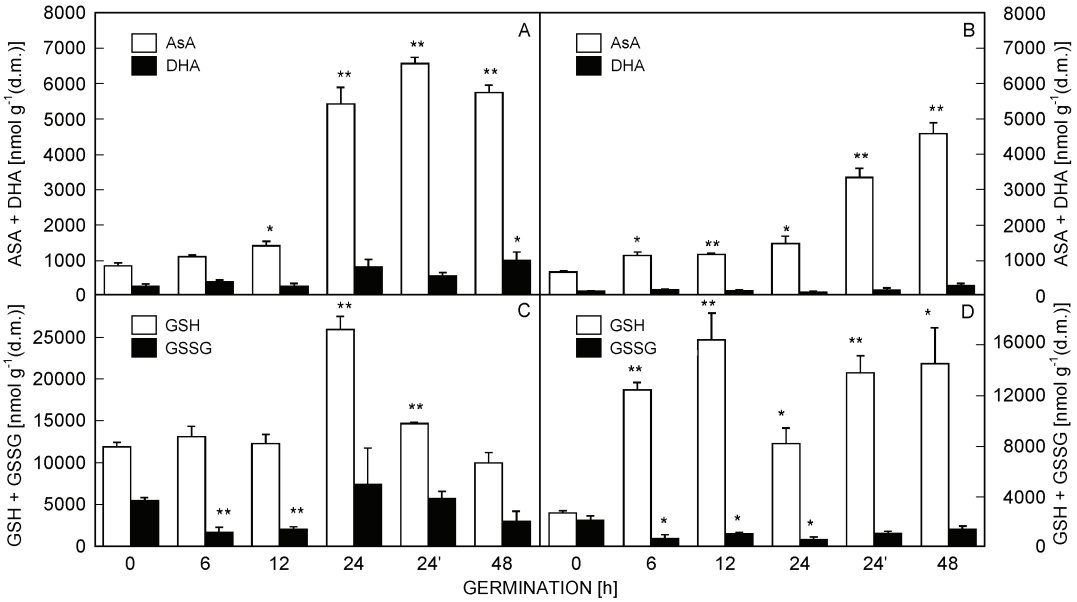

Fig. 1. Ascorbate content in embryo axes (*A*) and cotyledons (*B*) and glutathione content in embryo axes (*C*) and cotyledons (*D*) of germinating lupine seeds. Emergence of the radicle occurred at 23 to 25 h of incubation so among seeds incubated for 24 h we distinguished two populations: seeds just before radicle protrusion (labelled as 24 h) and immediately after radicle protrusion (labelled as 24' h). Means ± SD of three independent experiments, * - $P < 0.01$, ** - $P < 0.001$.

during germination reaching three times higher concentration after 48 h than after 6 h of germination. In dry cotyledons AsA/DHA ratio was 5 and increased to 22 in cotyledons immediately after radicle protrusion.

During germination changes in the glutathione pool were observed both in axes and cotyledons. However, the redox balance of this pair was quite different in the two analysed parts of dry seeds. Cotyledons isolated from dry seeds contained a similar amount of GSH and GSSG (Fig. 1*D*), whereas dry embryo axes had a GSH content more than 2 times higher than that of GSSG (Fig. 1*C*). In axes the glutathione pool remained relatively constant during imbibition but increased before the onset of germination and decreased afterwards. In cotyledons fluctuations in the glutathione content were observed and the GSH/GSSG ratio declined during germination from 18 at 6 h to 10 at 48 h.

APX activity was very low in dry seeds, but gradually increased during germination in embryo axes, while in cotyledons no significant changes were noticed during the analysed period (Fig. 2*A*). The embryo axes showed six proteins with APX activity after 6 and 12 h of germination similar as in dry axes (Fig. 3). One new isoform APX-6, was noticed in axes germinating for 24 and 48 h. The increase in APX activity observed in axes germinating for 24 and 48 h was associated with APX-6, since other isoforms were not detectable on the gel. On the contrary, cotyledons had four APX isoforms but two isoforms (APX-2 and APX-3) disappeared in cotyledons germinating for 48 h.

DHAR activity increased in the first hours of germination both in embryo axes and cotyledons and

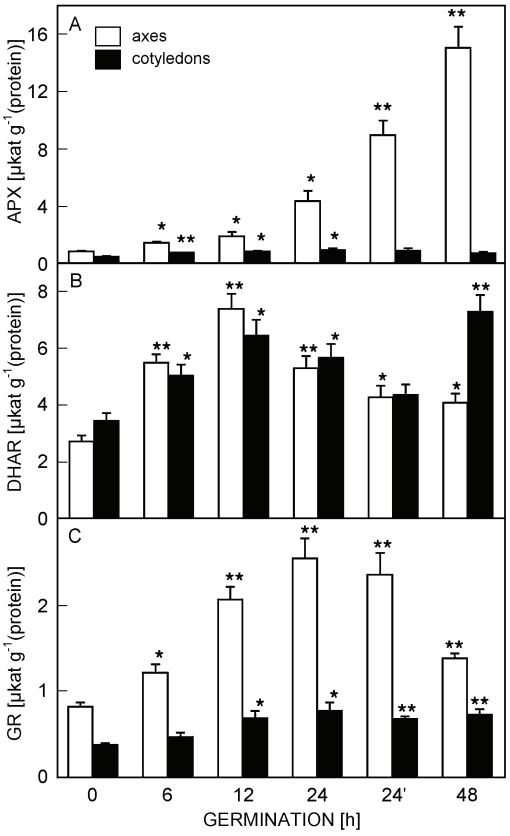

Fig. 2. Activities of APX (*A*), DHAR (*B*) and GR (*C*) in embryo axes and cotyledons of germinating lupine seeds. Means ± SD of three independent experiments, * - $P < 0.01$, ** - $P < 0.001$.

dropped thereafter (Fig. 2*B*). The electrophoretic pattern of the embryo's and cotyledon's DHA reducing proteins showed the presence of two bands (Fig. 3). A fast-migrating band sharing DHA reducing activity disappeared in axes germinating for 48 h.

GR activity showed a significant increase in embryo axes shortly before the radicle protruded the seed coat and decreased on subsequent 24 h. In cotyledons GR activity showed the increase after 12 h of germination and remained unaltered in the next hours (Fig. 2*C*). GR was represented by four isoforms both in embryo axes and cotyledons (Fig. 3). However, in axes germinating for 48 h GR-1 and GR-3 isoforms were not detected and new GR-5 isoform with the highest electrophoretic mobility appeared on the gel.

Discussion

Our previous research showed that germination of lupine seeds was accompanied by increase in contents of free radicals in lupine embryonic axes immediately after radicle protruded through the seed coat (Garnczarska *et al*. 2005). Apart from free radicals generation increased H_2O_2 concentration was observed in embryo axes before radicle emergence.

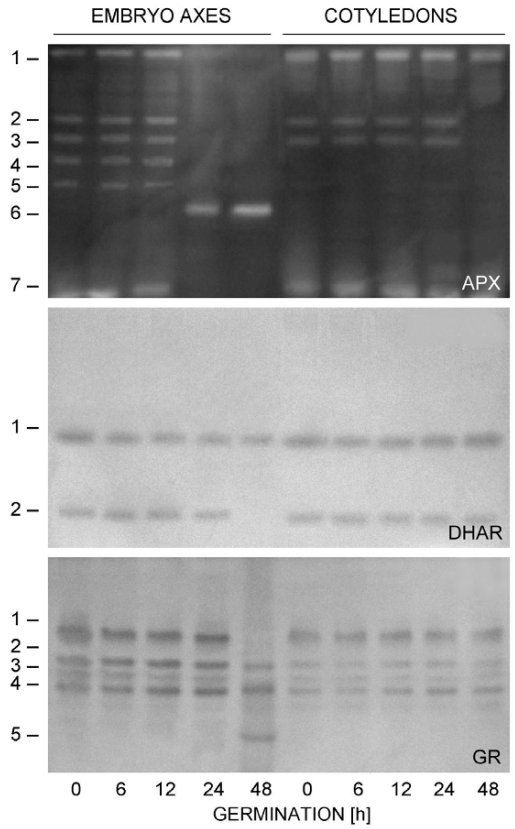

Fig. 3. Isoenzyme patterns of APX, DHAR and GR in embryo axes and cotyledons of germinating lupine seeds. The different isoforms are numbered from cathode to anode. For APX 100 μg proteins were loaded per each well, for DHAR and GR 300 μg. Similar results were obtained in at least four independent experiments.

Ascorbate-glutathione cycle was shown to be involved in the regulation of H_2O_2 content in plant tissues (Noctor and Foyer 1998). Therefore, in the present study we investigated the modulations of the content of ascorbate and glutathione and related enzyme activities in both embryonic axes and cotyledons of germinating lupine seeds. Dry lupine seeds contained a low amount of AsA (Fig. 1*A*,*B*) although, the absence of AsA has been observed in wheat (De Gara *et al*. 1997), maize (De Gara *et al*. 2000) and *Pinus pinea* (Tommasi *et al*. 2001). During seed desiccation AsA gradually decrease and dry seed are completely devoid of it (De Gara *et al*. 1997). However, NMR studies of temporal and spatial water uptake and distribution in germinating lupine seeds showed the existence of areas with elevated local moisture content and free water in dry seeds (Garnczarska *et al*. 2007) and that may partially explain the presence of AsA in dry lupine seeds. Leubner-Metzger (2005) showed that in air-dry tobacco seeds local elevation in moisture content was enough to permit de novo β-1,3-glucanase expression.

In most examined seeds, a strong correlation has been demonstrated between AsA availability and APX activity. According to De Tullio and Arrigoni (2003) the capability of rapidly re-starting both ascorbate biosynthesis and APX activity could be crucial to ensure seed germinability, although it cannot be ruled out that they are the consequence, rather than the cause, of seed vigour. In lupine embryo axes the increase in APX activity during germination (Fig. 2*A*) occurred in parallel with the rise in ascorbate content (Fig. 1*A*). As APX activity in embryo axes increased during germination it is reasonable to conclude that this enzyme utilized ascorbate to efficiently remove H_2O_2 produced by embryos. The increase of APX activity in embryo axes was due to the expression of a new APX-6 isoform (Fig. 3). Presented data refer to electrophoretic patterns of APX and other enzymes isolated from dry seeds and seeds germinating for 6, 12, 24 and 48 h (Fig. 3). For the technical reason variant 24' h is omitted but for all tested enzymes from this variant molecular forms in embryo axes and cotyledons were the same as for variant 24 h. Our results suggest that the response timing of APX isoforms against oxidative stress is not the same for all isoforms. The disappearance of some isoforms during germination suggest that these could play a key role in the early stages of germination until emergence of the radicle but new form synthesized in the following hours could have a different physiological function. APX activity increases under H_2O_2-over-producing conditions, such as those induced by biotic and abiotic stresses (Foyer *et al*. 1994,

Mandhania *et al*. 2006). The DHA-reduction capability of the embryonic axes and cotyledons was high in the first hours of lupine germination but then decreased. DHA reduction is postulated to have a role in rendering available a small, but significant, ascorbic acid supply in the very early stages of germination before the recovery of ascorbic acid biosynthetic activity (De Tullio and Arrigoni 2003). The relatively high DHAR activity during early germination (Fig. 2*B*) could protect the ascorbate pool from being lost by oxidation.

The glutathione was present in dry lupine seeds both in the reduced and oxidized forms (Fig. 1*C*,*D*). The high content of glutathione in dry lupine seeds confirms reports for several higher plants (Kranner and Grill 1993, Tommasi *et al*. 2001). When seeds start to germinate, high GSSG content must be rapidly reduced in order to allow protein synthesis (Kranner and Grill 1993). On the basis of dry mass, much more glutathione was present in lupine embryo axes than in cotyledons. In embryonic axes total glutathione pool increased before the onset of germination and decreased afterwards (Fig. 1*C*). The decline in the level of GSH may be attributed to decrease in GR activity (Fig. 2*C*) connected with disappearance of two GR isoforms (Fig. 3). However, GR showed transient increase in embryonic axes shortly before radicle protrusion. Another reason for an overall reduction in the endogenous content of GSH might be its utilization as a reducing substrate in the synthesis of ascorbate. In cotyledons the transient increase in GSH was also observed (Fig. 1*D*). Probably GSSG reduction plays a relevant role in the GSH rise in cotyledons occurring during the first 12 h of germination. The reduction of GSSG occurring as soon as germination starts allows the generation of molecules with antioxidant properties. However, ascorbate and glutathione might play a wider role than the sole scavenging of ROS, through control of the cellular redox balance (Tommasi *et al*. 2001) or protein synthesis (Kranner and Grill 1993).

Conclusions: Correlating changes in low molecular mass antioxidants and antioxidative enzymes with ROS production and kinetic of germination indicate a marked trend for a role of ascorbate-glutathione cycle in antioxidative responses during lupine seeds germination. The differential responses of embryo axes and cotyledons may be attributed to varied level of ROS generation in two functionally distinct organs of the seed. The observed changes appear to be closely related to metabolic and developmental processes associated with preparation for germination. Embryonic axes are better protected against the destructive effects of ROS since they are characterized by higher concentrations of low molecular mass antioxidants and a higher activity of related enzymes than cotyledons. The increase in enzymes activity maintains ascorbate and glutathione turnover and activation of the hydrogen peroxide scavenging ascorbate-glutathione cycle.

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Jones, J.B.: **Tomato Plant Culture. In the Field, Greenhouse, and Home Garden.** - CRC Press, Taylor and Francis Group, Boca Raton - London - New York 2008. 399 pp. ISBN 0-8493-7395-6.

Modern vegetable production uses incessantly improving growing technologies based on the latest results of science and research. Due to these effective methods applied in modern vegetable culture, it is possible to gradually increase the intensity, quality and rentability of production; however, implementation of new growing technologies to production systems requires high professional potential of growers, which presupposes more profound theoretical knowledge of the issue.

The book "Tomato Plant Culture" consists of eight chapters including six appendixes, references and index. The text is supplemented with numerous black and white photographs, well-ordered tables and graphs; a CD-ROM with colour photographs is enclosed to better illustrate tomato culture issues. The text offers many Internet links to provide more details about the topic.

Chapter I is an introduction to the issue, chapter II presents botanical and physiological characteristics of tomatoes, chapter III describes seed and seedling production, paying a special attention to tomato grafting, which is gaining more importance in modern tomato production. Chapter IV deals with fruit characteristics from different aspects (physical characteristics, chemical composition, quality, packaging, storage, *etc*.), chapter V gives details on tomato nutrition; individual nutrients and their physiological importance for tomato plant are described there. Following two chapters present details on tomato culture technologies, both in field and greenhouse conditions. Chapter VI focuses on field production and describes mainly the conventional system of commercial tomato production; however, the attention is paid also to ecological and home garden production. Chapter VII, concerning greenhouse tomato production, not only handles the optimization of growing environment but also the modern ways of greenhouse hydroponic growing. The last chapter offers to reader an insight to the most significant diseases and pests including their characteristics and ways for prophylaxis against their harmful effect; the author outlines the ways of chemical control and other methods of integrated pest management. Furthermore, there is a section devoted to weed control.

This book brings both theoretical facts and descriptions of special technological procedures of tomato plant culture. It can be thus a valuable source of information for professional tomato growers and advanced home gardeners as well as for research workers who specialize in this particular plant species and search for information leading to deeper knowledge of biological nature of tomato production. The work is also noteworthy for its high didactical quality; it might be certainly useful as a studying literature in the Vegetable production studies.

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