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Reduced mitochondrial and ascorbate–glutathione activity after artificial ageing in soybean seed

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ABSTRACT

The effect of artificial ageing on the relationship between mitochondrial activities and the antioxidant system was studied in soybean seeds (*Glycine max* L. cv. Zhongdou No. 27). Ageing seeds for 18 d and 41 d at 40 °C reduced germination from 99% to 52% and 0%, respectively. In comparison to the control, malondialdehyde content and leachate conductivity in aged seeds increased and were associated with membrane damage. Transmission electron microscopy and Percoll density gradient centrifugation showed that aged seeds mainly contained poorly developed mitochondria in which respiration and marker enzymes activities were significantly reduced. Heavy mitochondria isolated from the interface of the 21% and 40% Percoll were analyzed. Mitochondrial antioxidant enzymes activities including superoxide dismutase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase were significantly reduced in aged seeds. A decrease in total ascorbic acid (ASC) and glutathione (GSH) content as well as the reduced/oxidized ratio of ASC and GSH in mitochondria with prolonged ageing showed that artificial ageing reduced ASC–GSH cycle activity. These results suggested an elevated reactive oxygen species (ROS) level in the aged seeds, which was confirmed by measurements of superoxide radical and hydrogen peroxide levels. We conclude that mitochondrial dysfunction in artificially aged seeds is due to retarded mitochondrial and ASC-GSH cycle activity and elevated ROS accumulation.

Introduction

Seed ageing leads to seed deterioration. In agricultural production, aged seeds lead to commercial and genetic losses. Two common storage methods to limit seed deterioration are to reduce the moisture content and storage temperature (Walters et al., 2004; Lu et al., 2005). Even under optimal storage conditions, however, seeds suffer a variety of biochemical and metabolic alterations, including lipid peroxidation, enzyme inactivation, and disruption of cellular membranes (Rajjou et al., 2008; Sveinsdóttir et al., 2009; Kaewnaree et al., 2011; Hu et al., 2012). Understanding the mechanisms of seed ageing will lead to new methods for seed conservation and longevity.

Superoxide radical ($O2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are reduce seed vigour (Bailly et al., 2008) and the accumulation of reactive oxygen species (ROS) is the main contributor to seed deterioration (Bailly et al., 2008; Rajjou et al., 2008; Oracz et al., 2009; Bailly and Kranner, 2011; Bellani et al., 2012; Hu et al., 2012; Yao et al., 2012). The antioxidative system prevents ROS accumulation in seed ageing (Bailly et al., 2002; Pukacka and Ratajczak, 2005, 2006; Yao et al., 2012) and several studies have revealed a close relationship between seed deterioration and a reduction in the activity of various antioxidative systems in soybean (Sung, 1996), cotton (Goel et al., 2003), sunflower (Kibinza et al., 2006), pea (Yao et al., 2012), and elm (Hu et al., 2012) seeds.

The mitochondrion is the major site for production and scavenging of ROS during seed germination (Bewley, 1997; Møller, 2001; Howell et al., 2006; Navrot et al., 2007) and mitochondrial

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbic acid; ASH, Reduced ascorbate; ATP, adenosine triphosphate; BSA, bovine serum albumin; COX, cytochrome *c* oxidase; DHA, dehydroascorbate (oxidized ascorbate); DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; RCR, respiration control rate; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, tricarboxylic acid; TEM, transmission electron microscopy; TES, N-Tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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biogenesis and restoration provide energy compounds and intermediates for cellular biosynthesis (Logan et al., 2001; Howell et al., 2006; Macherel et al., 2007; Taylor et al., 2010; Carrie et al., 2013). Mitochondria are also important in stress tolerance (Stupnikova et al., 2006; Roschzttardtz et al., 2009; Smiri and Chaoui, 2009). However, the role of the mitochondria in seed ageing and ROS accumulation has not been thoroughly described.

In this work, we analyzed the physiological deterioration of artificially aged seed by studying mitochondrial ultra-structure, enzymatic activity, respiration, the antioxidative enzymatic defense system, and cellular ROS accumulation.

Materials and methods

Plant material and treatment

Soybean (*Glycine max* L. cv. Zhongdou No. 27) seeds were obtained from the National Genebank of China. The seed germination percentage was 99% and moisture content was 12.6%; storage was at −20 °C. Seeds were artificially aged by sealing them in an air-tight aluminium foil bag for 0, 18 d, 41 d at 40 °C. After treatment, the seeds were stored at −20 °C. Prior to each experiment, the frozen seeds were transferred to 25 °C for 2 d in an aluminium foil bag. For germination analysis, seeds were incubated for 7 d in an artificial climate incubator at 25 °C in the dark as described by the International Seed Testing Association (ISTA, 1996). Germination percentage was measured on the 7th day after imbibition. To measure seedling dry weight, seedlings without cotyledons were dried at 103 °C for 5 h.

Electric conductivity

Ten whole dry seeds were soaked in 25 mL Milli-Q water (Millipore, Milford, MA, USA) at 25 °C. The electric conductivity of the seed leachate was determined at regular intervals with an electrical conductivity metre (Delta 326, METTLER-TOLEDO, Switzerland). Absolute conductivity was measured after treating the seeds with boiling water for 30 min. Results were presented as relative electric conductivity, i.e. electric conductivity at different soaking times relative to the absolute conductivity.

Lipid peroxidation

Axes collected from 24 h-imbibed soybean seeds were ground at 4 °C. Lipid peroxidation was determined as the concentration of malondialdehyde (MDA) according to Heath and Packer (1968) and Hendry et al. (1992). This assay was based on thiobarbituric acid.

Transmission electron microscopy

Axes collected from 24 h-imbibed soybean seeds were cut into slices and fixed immediately with 2.5% (*v*/*v*) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). Each sample was dehydrated with a graded ethanol series and embedded in Spurr resin. Ultra-thin sections were prepared with a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained with uranyl acetate followed by lead citrate prior to observation. The sections were observed and photographed with a Jeol JEM-1230 transmission electron microscope (TEM, Kyoto, Japan).

Mitochondrial isolation

Axes collected from 24 h-imbibed soybean seeds were ground at 4 °C in extraction buffer (0.3 M sucrose, 5 mM tetrasodium pyrophosphate, 10 mM KH_2PO_4, pH 7.5, 2 mM EDTA, 1% [*w*/*v*] PVP40, 1% [*w*/*v*] bovine serum albumin (BSA), 5 mM cysteine, and 1 mM DTT). The homogenate was centrifuged for 5 min at 2000 × *g* and the resulting supernatant was centrifuged for 10 min at 20,000 × *g*. The pellet was resuspended in wash buffer (0.3 M sucrose, 1 mM EGTA, and 10 mM MOPS/KOH, pH 7.2) and spun at low speed (2000 × *g*) for 15 min followed by high-speed centrifugation (20,000 × *g*) for 15 min. The pellet was re-suspended in a small volume of wash buffer [0.3 M mannitol, 0.5% (w/v) BSA and 10 mM N-Tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES; pH 7.5)] and loaded on a Percoll step gradient (from bottom to top: 1 volume 40% and 1 volume 21% Percoll in wash buffer). The gradient was centrifuged at 40,000 × *g* for 1 h. The mitochondrial fraction located at the interface between the 40% and 21% layers was aspirated and washed 3 times in wash buffer before respiratory activity measurements.

Respiratory activity measurements in isolated mitochondria

All oxygen consumption measurements were performed using a Clark-type oxygen electrode (Chlorolab2, Hansatech Instrument, UK) at 25 °C. Mitochondria equivalent to 100 μg mitochondrial protein were suspended in 1 mL respiration buffer (0.3 M sucrose, 5 mM KH_2PO_4, 10 mM TES, 10 mM NaCl, 2 mM $MgSO_4$, and 0.1% [*w*/*v*] BSA, pH 6.8). Succinate (10 mM), NADH (1 mM), and ADP (0.8 mM) were added as required (Millar et al., 2001).

Cytochrome *c* oxidase (COX) (EC 1.9.3.1) activity in isolated mitochondria was measured as a decrease in absorbance at 550 nm (ε_{550} = 13.5 mM^{-1} cm^{-1}) and 25 °C due to cytochrome *c* oxidation as described by Neuburger, 1985. Mitochondrial malate dehydrogenase (MDH) (EC 1.1.1.37) activity was determined by monitoring the increase in absorbance at 340 nm (ε_{340} = 6.2 mM^{-1} cm^{-1}) and 25 °C due to NADH production as described by Glatthaar et al. (1974).

Antioxidative enzyme activities in isolated mitochondria

All antioxidative enzymes were extracted from purified mitochondria isolated from soybean embryonic axes after 24 h imbibition. All extraction procedures were carried out at 0 to 4 °C. Measurements were performed as follows:

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by monitoring inhibition of the photochemical reduction of nitro blue tetrazolium at 560 nm and 25 °C as described by Beyer and Fridovich (1987). Ascorbate peroxidase (APX) (EC 1.11.1.7) activity was measured as the decrease in absorbance of ASC at 290 nm (ε_{290} = 2.8 mM^{-1} cm^{-1}) and 25 °C due to oxidation by H_2O_2 as described by Nakano and Asada (1981). Glutathione reductase (GR) (EC 1.6.4.2) activity was determined by the decrease in absorbance at 340 nm (ε_{340} = 6.2 mM^{-1} cm^{-1}) and 25 °C due to NADPH oxidation, as described by Madamanchi and Alscher (1991). Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was measured according to Dalton et al. (1993) by monitoring the increase in absorbance at 265 nm (ε_{265} = 14 mM^{-1} cm^{-1}) and 25 °C caused by DHA formation. Monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) activity was assayed as described by Arrigoni et al. (1992) by monitoring the decrease in absorbance at 340 nm (ε_{340} = 6.2 mM^{-1} cm^{-1}) and 25 °C due to NADH oxidation.

Determination of ASC and glutathione (GSH) in isolated mitochondria

ASC and GSH in purified mitochondria were extracted with ice-cold 5% sulfosalicylic acid. After vortex-mixing for about 5 min, it was centrifuged at 20,000 × *g* for 20 min at 4 °C, and the content of ASC and GSH in the supernatant was measured. Reduced ascorbate (ASH) and dehydroascorbic acid (DHA) were determined as described by Law et al. (1983). Reduced glutathione (GSH) and