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RESEARCH PAPER

Phytic acid prevents oxidative stress in seeds: evidence from a maize (*Zea mays* L.) *low phytic acid* mutant

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Abstract

A maize mutant defective in the synthesis of phytic acid during seed maturation was used as a tool to study the consequences of the lack of this important reserve substance on seed survival. Data on germinability, free iron level, free radical relative abundance, protein carbonylation level, damage to DNA, degree of lipid peroxidation, α- and γ-tocopherol amount and antioxidant capacity were recorded on seeds of maize B73 and of an isogenic *low phytic acid* mutant (*lpa1-241*), either unaged or incubated for 7 d in accelerated ageing conditions (46 °C and 100% relative humidity). The *lpa1-241* mutant, compared to wild type (wt), showed a lower germination capacity, which decreased further after accelerated ageing. Whole *lpa1-241* mutant kernels contained about 50% more free or weakly bound iron than wt ones and showed a higher content of free radicals, mainly concentrated in embryos; in addition, upon accelerated ageing, *lpa1-241* seed proteins were more carbonylated and DNA was more damaged, whereas lipids did not appear to be more peroxidated, but the γ-tocopherol content was decreased by about 50%. These findings can be interpreted in terms of previously reported but never proven antioxidant activity of phytic acid through iron complexation. Therefore, a novel role in plant seed physiology can be assigned to phytic acid, that is, protection against oxidative stress during the seed's life span. As in maize kernels the greater part of phytic acid (and thus of metal ions) is concentrated in the embryo, its antioxidant action may be of particular relevance in this crop.

Key words: Antioxidant, low phytic acid mutant, oxidative stress, seed.

Introduction

Oxidative stress is a pervasive and ubiquitous environment-related problem that plant cells must cope with. Because of photosynthetic activity, it is particularly frequent in the green parts of the plant, but also affects seeds, usually in the last phase of maturation when seed tissues undergo dehydration which is often accompanied by oxidative stress (Bailly, 2004). The expression of specific gene products such as late embryogenesis abundant (*lea*) proteins, mostly induced by abscisic acid (ABA), is probably an effective means to prevent major damage to cell macromolecules and structures. However, both in this phase and during seed storage, the occurrence of reactive oxygen species (ROS) and other free radicals may lead to devastating consequences on seed cells. For example, damage and degradation of DNA and RNA with impaired transcription and translation may occur, together with the alteration of cell membranes' permeability and carbohydrates reserve accumulation (McDonald and Nelson, 1986; Wilson and McDonald, 1986; Smith and Berjak, 1995; McDonald, 1998, 1999). The presence in seed tissues of ascorbate, glutathione, carotenoids, tocopherols, polyphenols, and other molecules endowed with antioxidant properties can provide protection against reactive oxygen species (Shi *et al.*, 2003*a*; Sattler *et al.*, 2004; Ogawa, 2005; Howitt and Pogson, 2006;

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Kranner *et al.*, 2006; Maeda and Della Penna, 2007; Pourcel *et al.*, 2007) and this is consistent with the progressive decrease observed in antioxidant reserves during seed storage and ageing (Pinzino *et al.*, 1999; Galleschi *et al.*, 2002; Calucci *et al.*, 2004). However, so far, no other mechanism has definitely been proven to play a major role in protecting embryo viability.

Myo-inositol 1,2,3,4,5,6-hexakisphosphate, commonly called phytic acid, is the primary storage form of phosphorus in seeds, representing 50% to over 80% of total phosphorus in mature seeds and accounting for one to several per cent of the dry weight (Lott, 1984; Lin *et al.*, 2005). In maize (*Zea mays* L.), the majority of the kernel phytic acid, 88% or 95% according to O'Dell *et al.* (1972) and Lin *et al.* (2005), respectively, is found in the embryo, with the remainder in the aleurone layer. In seed tissues, as a polyanion at physiological pH, phytic acid is an effective chelator of positively charged cations of important macro- and micro-nutrients including K, Mg, Ca Fe, Zn, and Mn, forming phytate (also called phytin), which is sequestered in specialized vacuoles termed protein bodies or protein storage vacuoles (Lott, 1984; Wada and Lott, 1997; Raboy, 2002). Phosphorus and mineral cation reserves deposited in the phytate molecule are essential for germination and for the growth and development of seedlings (Lott, 1984). However, the same is not true for phytic acid itself. Several viable low phytic acid (*lpa*) mutant plants were isolated, which produced seeds with a substantial decrease in phytic acid phosphorus, but not in total phosphorus and mineral element content (Raboy, 2000). Moreover, phytic acid, due to its ability to chelate metal cations and, therefore, to reduce their bioavailability in the digestive apparatus, has long been regarded as an anti-nutrient for monogastric animals (Zhou and Erdman, 1995; Lönnerdal, 2003).

On the other hand, phytic acid, by virtue of its ability to chelate iron, is a potent inhibitor of the iron-driven formation of reactive oxygen species (Graf *et al.*, 1984) and of lipid peroxidation *in vitro* (Graf *et al.*, 1987; Graf and Eaton, 1990; Empson *et al.*, 1991). In particular, it ensures the removal of Fe^{2+}, which alone has been shown to cause the production of reactive oxygen species and lipid peroxidation by oxidation to Fe^{3+}, which is relatively inert even in the presence of oxygen and polyunsaturated lipids. The prevention of these oxidative events, ordinarily catalysed by free and weakly bound iron (Burkitt and Gilbert, 1990, 1991), may well be an important antioxidant function of phytate within plant seeds as hypothesized 20 years ago by Graf *et al.* (1987; Graf and Eaton, 1990) and may contribute towards explaining why seeds belonging to many plant species are viable for a long time, in spite of the fact they contain a potentially dangerous mixture of iron, oxygen, and unsaturated fatty acids. However, so far, no experimental evidence of such an *in vivo* role of phytic acid has been reported.

The *lpa* mutants cited above, which have been isolated mainly in order to avoid the antinutrient function of phytic acid in edible seeds (Raboy, 2002; Lönnerdal, 2003), provide an ideal tool for dissecting phytic acid synthesis pathways in plants and understanding its functions in seeds. A few papers on *lpa* mutations in maize have appeared in the literature in recent years (Larson and Raboy, 1999; Raboy *et al.*, 2000; Raboy, 2000, 2002; Pilu *et al.*, 2003; Lin *et al.*, 2005). A study on the effects of the *lpa1-1* mutation on the concentration and distribution of phytic acid and other mineral nutrients in maize kernels and its parts indicated that the embryo scutellum was the major site for phytic acid deposition and storage (Lin *et al.*, 2005). Moreover, the *lpa1-1* mutation had an impact on globoid formation. The whole kernel concentrations of P, K, Mg, Fe, Zn, and Mn were comparable or higher in *lpa1-1* seeds than in wt seeds, while Ca was lower. In particular, the increase in Fe level was the greatest, with an approximately 1/3 increase in whole grains and embryos (Lin *et al.*, 2005).

The maize *lpa1-241* mutation we isolated in 2001 and showed to be allelic to Raboy's *lpa1-1* mutation (Pilu *et al.*, 2003) causes, compared to wt, a 90% reduction of phytic acid phosphorus and a corresponding 10-fold increase in inorganic phosphorus level without affecting the total amount of phosphorus stored in the kernel. In the same work, we demonstrated that, in the maturing seeds of the *lpa-241* mutant, the expression of the gene coding *myo*-inositol-3-phosphate synthase (MIPS), the first and key enzyme of the pathway leading to phytic acid, is severely affected, as it is in another maize *lpa1*-type mutant (Shukla *et al.*, 2004). In addition, our *lpa1-241* mutant was shown to display a few negative pleiotropic effects, the most relevant of which is a decrease in germination capacity and rate (Pilu *et al.*, 2005). These latter results prompted an investigation into the possible correlation between the *lpa1-241* mutation and seed deterioration which might be attibuted to the occurrence of oxidative processes during maturation and/or storage. The focus would be on the chelating properties and, consequently, on the potential antioxidant function of phytate in seeds.

With this aim, in this paper the changes in maize kernel due to the lack of phytic acid induced by the *lpa1-241* mutation were investigated from different points of view. In particular, various analytical methods were used to verify the content of long-lived organic free radicals in seed embryo and endosperm, the production of H_2O_2 in the scutellum, the amount of paramagnetic metal cations, such as Mn^{2+} and Fe^{3+} in seed tissues, and the seed content of weakly bound or free or chelated iron. Moreover, the amounts of tocopherols and glutathione and the level of antioxidant activity present in the seed were assayed and compared. Finally, damage to seed macromolecules was looked for, such as that shown by protein carbonylation, lipid peroxidation, and the amount of DNA apurinic/apirimidinic sites. The possible effects of lack of phytic acid in enhancing ageing-related damage was investigated by carrying out the planned measurements on both wt and *lpa1-241* mutant seeds subjected to accelerated ageing (Delouche and Baskin, 1973).

The discussion also takes into account the possible role of the phytic acid precursor, *myo*-inositol, involved in the synthesis of many other plant metabolites such as polyols,

cell wall components and phosphoinositides, and the precursor of an important second messenger such as IP_3 (inositol 1,4,5-trisphosphate) (Loewus and Murthy, 2000; Xiong *et al.*, 2002).

Materials and methods

Chemicals

Unless specified, all chemicals were from Sigma, while the kit used for DNA analyses was from Biovision® (Switzerland).

Plant material

Seeds of B73 wt and *lpa1-241* maize (*Zea mays* L.) were collected from plants grown in the field during the 2006 or 2008 growing season and several fractions were subjected to the accelerated ageing procedure described farther on.

For most analyses, seeds were ground in a mixer mill type MM 200 (Retsch GmbH & Co. KG, Germany) equipped with stainless steel vessels cooled with liquid nitrogen. For EPR analyses, kernels were also separated into embryo and endosperm, which were ground following the same procedure. The ground materials (i.e. whole kernel flour, embryo, and endosperm) were stored at –20 °C before analyses.

Measurement of seed water content

The water content of maize seeds, undesiccated or previously subjected to desiccation treatment, was measured gravimetrically upon drying in an oven according to ISTA (1985) rules, using at least 50 seeds/sample.

Seed desiccation

Seven replicates of 50 wt or *lpa1-241* maize seeds harvested in 2008 were individually weighed and then put to dry with silica gel in a desiccator (6.0 l capacity) at 25 °C. About 2 kg of silica gel were placed at the bottom of the desiccator and covered by a wire mesh. The seed lots were folded into nylon netting and placed on top of the wire mesh in the desiccator. Seeds were removed after 11 d, and water losses were measured by reweighing them as precisely as possible. Six lots/sample were then used to evaluate germination capacity, immediately, or after accelerated ageing treatment (see below), while one lot was used to measure seed water content.

Accelerated ageing

Accelerated ageing was performed by treating the wt and *lpa1-241* maize seeds at 46 °C and 100% relative humidity according to Delouche and Baskin (1973). Seeds were suspended over distilled water on a plastic mesh tray within closed plastic boxes, which were maintained at 46 °C in a growth chamber for 7 d. After such treatment, seeds were rapidly air dried until they reached their original moisture content.

Germination tests

Germination tests were performed on wt and *lpa1-241* maize seeds, either aged or not. Four replicates of 100 seeds from each lot were uniformly spread in Petri dishes with filter paper placed on the top and on the bottom and imbibed with distilled water. Germination was carried out at 26 °C in the dark for 72 h. A seed was considered germinated when the primary root was at least 1 mm long. Results were expressed as germination capacity, defined as the percentage of completely germinated seeds after a 72 h incubation (Bewley and Black, 1994).

Iron extraction and detection

Extraction of total iron: Flour (300 mg) prepared from maize kernels was mixed thoroughly with 1.5 ml of ultra-pure nitric acid and the sample was digested for 2 h at room temperature. A fraction of the digested sample (0.6 ml) was evaporated to dryness in a mineralizer at 100 °C. The residue was processed with desferrioxamine (DFO) for HPLC analysis as described farther on.

Differential extraction of iron at different HCl concentrations: Flour samples (5 g samples, five replicates for each experimental material) were mixed with 15 ml of HCl at different concentrations (0.01, 0.03, 0.1, 0.4 N) in a test tube and shaken overnight at 4 °C. After a 15 min centrifugation at 12 000 rpm, the pellets containing the unextracted iron were discarded and supernatants were put in a mineralizer at 100 °C until completely evaporated. The residues were dissolved in ultra pure nitric acid, dried again in the mineralizer, and processed with DFO as described below.

HPLC analysis: Iron was determined by HPLC analysis as described by Tesoro *et al.* (2005). This method is based on the use of DFO, a potent iron chelator with little affinity for other metal ions. Briefly, the dried samples prepared as above described were dissolved in 1.0 ml of 20 mM DFO freshly prepared in 10 mM TRIS-HCl buffer (pH 5), incubated at room temperature in the dark for 3 h, and then centrifuged for 5 min at 10 000 rpm. Twenty µl of the clear supernatant were finally injected into the HPLC system (Kontron Instrument 420 system, equipped with a C18 column Zorbax ODS column 250×4.6 mm, 5 µm, Agilent Technologies). Data from three replicas per sample were collected in two independent experiments.

EPR measurements

EPR measurements were performed on powder samples (~100 mg) lightly packed into EPR quartz tubes using a Varian E112 X-band spectrometer interfaced to a PC by means of a homemade data acquisition system (Ambrosetti and Ricci, 1991; Pinzino and Forte, 1992) and equipped with a standard cavity. Scan ranges of 100 Gauss and 8000 Gauss were used. For the 8000 Gauss range the microwave power was 10 mW and the modulation amplitude 4 Gauss.

The 100 Gauss range was used to look closely at the free radical signal with 0.25 mW microwave power and 4 Gauss modulation amplitude. Free radical concentrations were obtained by double integration of the first derivative signals and comparison with the Varian Strong Pitch standard. Three replicates were analysed for each material.

Histological detection of H_2O_2 in embryo scutellum

For light microscopy studies, unaged or aged wt and *lpa1-241* mature dry seeds were soaked in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (1 mg ml^{-1} DAB, pH 3.8 with NaOH) for 18 h and subsequently fixed for 24 h in freshly prepared 3:1 100% ethanol:glacial acetic acid at 4 °C. The fixed material was placed in 70% ethanol and stored at 4 °C until processed. After dehydration in an ethanol series and embedding in Paraplast Plus (Ted Pella, Inc. and Pelco International, Redding, CA), sections were cut at 15 μm and serially arranged on slides. H_2O_2 was revealed by DAB staining as brown/black coloration as described by Murgia *et al.* (2004).

Myo-*inositol detection*

Myo-inositol was extracted from 100 mg flour samples (four replicates for each experimental material) by the method described in Shi *et al.* (2003*b*), and quantified by GC-MS analysis as described in March *et al.* (2001).

Analysis of protein carbonylation level

This analysis was carried out following Lenz *et al.* (1989): proteins were extracted from 100 mg of seed flour (four replicates for each experimental material) using 1 ml of 50 mM TRIS-HCl buffer (pH 6.8) containing 3% SDS and 5% β-mercaptoethanol. Then samples were vortexed for 10 min, put in a shaker for 2 h, centrifuged for 5 min at 12 000 rpm, placed at 100 °C for 5 min, and finally stored at –20 °C until analysed. For the analyses, 300 μl of sample was evaporated to dryness using a Speedvac centrifuge (Sevylor). 500 μl of 20 mM dinitrophenyl hydrazine (DNPH) dissolved in 2 M HCl was added to the residue and the sample was incubated for 60 min at room temperature (vortexing every 10 min) before adding 500 μl of 20% TCA and centrifuging at 12 000 rpm for 3 min. The supernatant was discarded and the pellet was washed three times with a 1:1 v/v ethanol/ethyl acetate solution, resuspended in 800 μl of 6 M guanidine HCl and 200 μl of phosphate buffer (pH 6.0), incubated for 15 min at 37 °C, and centrifuged at 12 000 rpm for 3 min. The supernatant absorbance at 370 nm was finally measured and the amount of carbonyl groups was calculated using a molar absorption coefficient of 22 000 M^{-1} cm^{-1} (Levine and Lehrman, 1984; Lenz *et al.*, 1989).

Analysis of the amount of apurinic/apyrimidinic sites in DNA

A kit obtained from Biovision®, Switzerland (www.biovision.com), was used to measure apurinc/apyrimidinic sites in DNA of both wt and *lpa1-241* maize seeds, unaged or artificially aged (three replicates for each material). DNA was extracted as follows: 700 μl of the kit extraction buffer was added to 100 mg of maize seed flour and the sample was incubated for 10 min in a water bath at 65 °C. Then 200 μl of 5 M potassium acetate was added and, after swirling, samples were put on ice for 5 min. After centrifuging for 10 min at 13 000 rpm, the supernatant was transferred in a new test tube and the same volume of chloroform:phenol 1:1 v/v was added. After centrifugation, 1 vol. of iso-propanol was added to the aqueous phase of each sample and, upon a further centrifugation, the supernatant was discarded and the pellet was washed twice with 80% ethanol. The final pellet was dried and then suspended in 20–50 μl of extraction buffer. Lastly, 0.5–1 μl of RNAse A was added, and samples were stored at –20 °C until spectrophotometric analysis at 450 nm and 650 nm was performed.

Analysis of TBARS amount in lipids

The TBARS test is based on malondialdehyde (MDA) production during the oxidation of polyunsaturated fatty acids. The reaction between MDA and thiobarbituric acid (TBA) yields a reddish colour, which corresponds to an absorbance maximum at 532 nm. The basic protocol used or adapted in numerous studies dealing with lipid peroxidation (Heath and Packer, 1968) was chosen. wt and *lpa1-241* unaged or artificially aged seeds were homogenized extensively in liquid nitrogen with a mortar and pestle. The homogenized tissue powder (0.2 g) was suspended in 5 ml of 0.1% TCA on ice, and centrifuged at 10 000 rpm for 10 min. To 1 ml of supernatant, 2 ml of 20% TCA and 0.025 ml of 0.5% TBA were added and the mixture was incubated at 95 °C for 30 min, cooled on ice, and centrifuged at 10 000 rpm for 10 min before reading absorbance at 532 nm subsequent to subtraction of non-specific absorption at 600 nm. The MDA concentration was calculated using its extinction coefficient 155 mM^{-1} cm^{-1}. Four replicates were analysed for each experimental material.

Quantification of tocopherols

The analysis of α- and γ-tocopherol was performed using a procedure described by Weber (1987) and by Kurilich and Juvic (1999), with some modifications. Six ml of ethanol in 0.1% butylated hydroxytoluene (BHT) were added to 600 mg of flour prepared from aged and unaged wt or *lpa1-241* seeds (four replicates for each material). After a 5 min incubation in an 85 °C water bath, samples were saponified by a 10 min treatment with 150 μl of 80% KOH and vortexed for 10 min, 3 ml of distilled water was added and they were placed on ice for 3 min. Three ml of hexane were added and samples were vortexed and centrifuged for 10 min at 1200 *g*. The upper layer was placed into another test tube and the pellet was re-extracted using two ml of hexane and recentrifuged. Supernatants were added to the hexane fraction with 3 ml of distilled water. Upon a last centrifugation, the hexane fraction of each sample was dried down

in an evaporating centrifuge (Speedvac, Sevylor) and the residue was reconstituted in 200 µl of acetonitrile:methanol:methylene chloride (45:20:35 by vol.) prior to injection into the RP-HPLC system (Kontron Instrument 420 system) equipped with a C18 column (Zorbax ODS column 250×4.6 mm, 5 µ, Agilent Technologies). The mobile phase consisted of acetonitrile:methanol (80:20 v/v). The flow rate was 1.3 ml min^{-1} at room temperature. Absorbance was measured at 210 nm and α- and γ-tocopherols were identified in the chromatograms and quantified by comparison with the respective standards.

Quantification of glutathione

The method described by De Pinto *et al.* (1999) was used to assay the amount of GSH (reduced glutathione) and GSSG (oxidized glutathione). Briefly, flours were extracted with 2 vol. of cold 5% metaphosphoric acid at 4 °C in a porcelain mortar. The homogenate was centrifuged at 20 000 *g* for 15 min at 4 °C and the supernatant neutralized with 0.5 M phosphate buffer (pH 7.5). The glutathione pool was assayed according to Zhang and Kirkham (1996). For GSSG assay, the GSH presence was masked by adding 20 µl of 2-vinylpyridine (of water in the sample utilized for total glutathione pool assay). The extracts (three replicates for each experimental material) were then added to a reaction mixture containing DTNB (5,5′-dithiobis-2-nitrobenzoic acid). The sulphydryl group of GSH reacts with DTNB producing the yellow-coloured compound TNB (5-thio-2-nitrobenzoic acid). The mixed disulphide GSTNB concomitantly produced is then reduced by glutathione reductase so as to recycle GSH and produce more TNB, whose final amount, proportional to the initial amount of GSH in the sample, is measured spectrophotometrically at 405 nm.

DPPH test

By means of the widely used 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, following the procedure described by Brand-Williams *et al.* (1995), it is possible to measure the ARP (anti-radical power) of extracts prepared from any biological material. Five hundred mg of the flours prepared from the seed samples (unaged and aged wt or *lpa1-241*) to be investigated (four replicates for each sample) were extracted with 1 ml of a water:methanol 1:2 v/v solution by shaking vigorously for 1 h at 4 °C. Upon centrifugation at 10 000 rpm for 5 min, different amounts (10–100 µl) of the supernatants were added to 900 µl of a water/methanol 1:1 v/v deep purple solution of DPPH. This stable free radical reacts with antioxidants and its consequent colour loss measurable at 515 nm correlates to antioxidant content. Each reaction mixture was incubated at room temperature in the dark and was allowed to reach a steady-state before reading its absorbance. Residual DPPH values were plotted against the extract volumes so as to calculate by interpolation the amount of extract required to consume 50% of the initial DPPH amount. The reciprocal of this figure corresponds to the ARP value.

Statistical analysis

In order to check the significance of the differences between the mean values registered for wt and *lpa* experimental materials, aged and unaged, the results of all experiments (exception made for the histological detection of H_2O_2) were statistically evaluated by applying the Student *t* test.

Results

Germination tests

In the experiments carried out in 2007, unaged *lpa1-241* seeds showed a lower germination capacity than wt seeds (72±15% versus 98±2%) After accelerated ageing, the germination capacity was further decreased for both wt (89±10%) and *lpa1-241* (45±14%) seeds, the loss being much higher for mutant (about 38%) than for wild type seeds (about 9%). Student's *t* test showed that these differences were significant at $P < 0.05$. In order to verify whether the higher moisture content recorded in *lpa1-241* with respect to wt seeds (11.2±0.1% versus 10.7±0.1%) might be partially or totally responsible for the higher germination capacity loss registered in *lpa1-241*, a further experiment was performed using a fraction of the 2008 harvest: wt and *lpa1-241* seeds were dried in a desiccator for 11 d, after which their initial moisture contents (11±0.1% and 11.4±0.1%, respectively) had dropped to 10.7±0.1% and 10.8±0.1%, respectively. Finally, the accelerated ageing test was performed on half of the desiccated seeds and the germination capacity of both the unaged and the aged desiccated seeds was measured. Although the water content of *lpa1-241* seeds were so close (0.1% difference) to that of wt seeds, *lpa1-241* was confirmed to have a much lower germination capacity than wt seeds either unaged (78±6% versus 97±5%) or after accelerated ageing treatment (52±9% vs. 88±8%). The Student *t* test showed that both differences are significant at $P < 0.01$. Germination capacity losses recorded in unaged (–19%) and aged (–41%) *lpa1-241* mutant seeds with respect to wt seeds were nonetheless slightly lower than those recorded in the 2007 experiment (–26% and –49% in the case of unaged and aged seeds, respectively) carried out on undessicated seeds with a 0.5% higher water content in *lpa1-241* with respect to wt seeds. This might indicate that the lower seed water content in wt could have resulted in slower ageing, thus contributing, in a small part, to account for the observed differences between the germination capacities of wt and *lpa1-241* seeds.

EPR analyses

Electron Paramagnetic Resonance (EPR) spectra were recorded at room temperature on powder samples of whole kernel, embryo, and endosperm of both wt and *lpa1-241* maize, either subjected to accelerated ageing or not; representative spectra are shown in Figs 1 and 2. Wide scan spectra of embryo samples (Fig. 1) showed the presence of

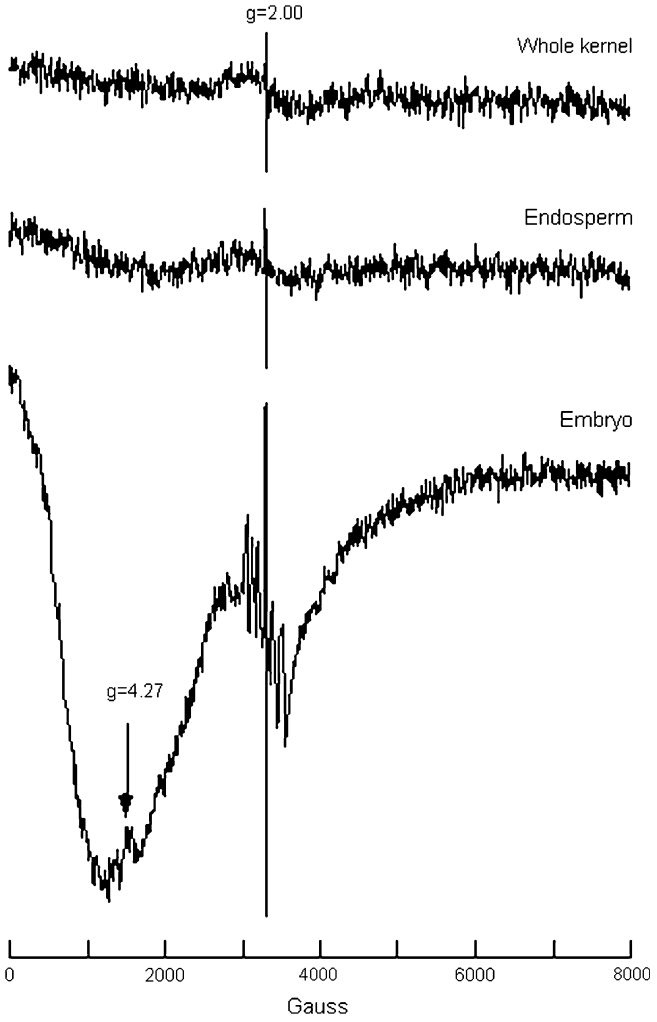

Fig. 1. Examples of wide scan EPR spectra of embryo, endosperm and whole kernel samples.

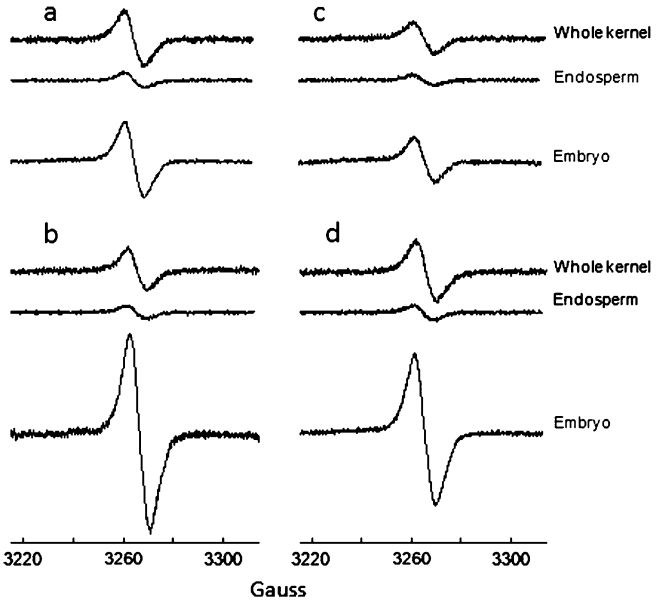

Fig. 2. Examples of EPR spectra of organic radicals in embryo, endosperm and whole kernel of wt (a), *lpa1-241* (b), artificially aged wt (c), and artificially aged *lpa1-241* (d) samples.

four distinct features: a low field component with g=4.27 and three separate components with $g \approx 2.0$; such spectra are common in plant-derived products (Conger and Randolph, 1968; Evans and Windle, 1970; Hepburn *et al.*, 1986; Reichenauer and Goodman, 2003). The signal at $g = 4.27$ is commonly assigned to mononuclear Fe(III) complexes (Griffith, 1964; Kedzie *et al.*, 1965; Aasa, 1970), while the broad resonance with $g \approx 2.0$ is associated with clusters of Fe(III) and/or Mn(II) ions (Reid *et al.*, 1968; Rodrigues-Filho *et al.*, 2005), and the sextet centred at $g \approx 2.0$ is due to isolated Mn(II) ions (Conger and Randolph, 1968; Evans and Windle, 1970; Bharti *et al.*, 1978; Singh and Bharti, 1985; Hepburn *et al.*, 1986; Reichenauer and Goodman, 2003; Rodrigues-Filho *et al.*, 2005), whereas the sharp signal at $g \approx 2.00$ is ascribable to organic free radicals. An analogous Mn(II) signal has been assigned to mononuclear manganese–phytate complexes in wheat flour (Rodrigues-Filho *et al.*, 2005); however, in this work no differences could be found in the spectrum of wt and *lpa1-241* mutant seeds to allow this signal to be unequivocally assigned to mononuclear manganese–phytate complexes in the maize embryo. The sharp signal at $g \approx 2.00$, more closely inspected by performing small scan range EPR spectra (Fig. 2), was a single line without any fine structure characterized by a g value of 2.0045 ± 0.0003 and a peak-to-peak line width of 8–9 Gauss. This signal is commonly found in plant materials (Conger and Randolph, 1968; Evans and Windle, 1970; Buchvarov and Gantcheff, 1984; Priestley *et al.*, 1985; Hepburn *et al.*, 1986; Atherton *et al.*, 1993; Bertolini *et al.*, 2001; Szöcs, 2002; Reichenauer and Goodman, 2003) and has been assigned to carbon-centred organic free radicals on a conjugated structure with oxygen-containing functional groups (Fischer, 1965; Atherton *et al.*, 1993), such as that of a quinone involved in an electron transport chain, a simple phenolic secondary metabolite, or a more complex polyphenol.

The signals at $g \approx 2.0$ due to clusters of Fe(III) and/or Mn(II) ions and organic radicals were also present in the whole kernel and endosperm spectra (Figs 1, 2) but with much lower intensity, whereas the other signals were not distinct enough to stand out clearly from the background noise, reflecting the much lower concentration of metal cations in maize endosperm compared with the embryo (O'Dell *et al.*, 1972; Bityutskii *et al.*, 1999; Lin *et al.*, 2005). No significant differences were found between paramagnetic ions EPR signals of corresponding materials prepared from wt and *lpa1-241* seeds, even after the accelerated ageing of seeds (data not shown). By contrast, different free radical contents were found for the different samples (Table 1). First of all, embryos showed one order of magnitude higher concentrations than the corresponding endosperms. In unaged samples, a significantly higher radical concentration (almost 2-fold) was found in the *lpa1-241* embryo sample compared with the wt one, whereas no significant difference was observed for endosperm and whole kernel samples. For seeds subjected to accelerated ageing, no further increase, or rather a moderate decrease of free radical concentration was

observed upon ageing in both wt and *lpa1-241* seeds. However, *lpa1-241* materials still showed a higher free radical content than the corresponding wt ones.

Histological analysis of H_2O_2 production

In order to evaluate the production of H_2O_2 qualitatively, a histological analysis of the scutellum of unaged or artificially aged wt and *lpa1-241* maize seeds was performed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining as described by Murgia *et al.* (2004). The visual inspection of the images obtained from longitudinal sections of mature kernels stained with DAB clearly revealed a more intense staining, corresponding to a higher H_2O_2 concentration, in aged than in unaged seeds, as well as in *lpa1-241* with respect to wt seeds either aged or not (Fig. 3).

Analysis of myo*-inositol seed content*

A comparative analysis of *myo*-inositol amount in both wt and *lpa1-241* unaged seeds carried out by Gas Chromathography coupled to Mass Spectrometry (GC–MS) showed a decrease of about 40% in the metabolite amount in *lpa1-241* seeds (122±8 versus 72±6 μg *myo*-inositol g^{-1} extracted flour). The Student *t* test showed that the observed difference is significant at $P < 0.01$.

Table 1. Free radical concentration in unaged or artificially aged whole kernels and kernel fractions of wt or *lpa1-241* maize.

Sample	Free radical concentration (spins g^{-1})		
	Whole kernel	Embryo	Endosperm
wt	$(12\pm1)\times10^{15}$	$(23\pm5)\times10^{15}$	$(3.0\pm0.2)\times10^{15}$
lpa1-241	$(10\pm1)\times10^{15}$	$(43\pm5)\times10^{15}$	$(3.1\pm0.2)\times10^{15}$
Aged wt	$(10\pm1)\times10^{15}$	$(15\pm3)\times10^{15}$	$(2.0\pm0.2)\times10^{15}$
Aged *lpa1-241*	$(15\pm2)\times10^{15}$	$(39\pm3)\times10^{15}$	$(3.1\pm0.3)\times10^{15}$

Free iron cations content

The content of iron in maize seeds was measured in both wt and *lpa1-241* kernels by extracting flour samples with HCl solutions at different concentrations. Solutions at low HCl (0.01–0.03 N) concentration should extract only or mainly free iron and organic iron not complexed with phytic acid, whereas both non-phytic and phytic iron should be extracted by a more concentrated HCl solution (0.4 N) (Chauhan and Mahjan, 1988; Rakhi and Khetarpaul, 1995; Duhan *et al.*, 2002; Engle-Stone *et al.*, 2005). Results, shown in Table 2, indicate that the total amount of iron extractable with 0.4 N HCl from wt and *lpa1-241* seed flours is not significantly different. Conversely, a much higher amount of iron could be extracted from *lpa1-241* compared with wt flour using lower HCl concentrations. In particular, when HCl 0.03 N was used, about 16% and 8% of the total iron was extracted from *lpa1-241* and wt maize flour, respectively, indicating that a lower amount of phytic acid accumulated in seed is correlated to a higher level of iron easily extractable from flour.

Analysis of damage to protein, DNA and lipids

Data on the damage to protein and to DNA reported in Table 3 showed that, as far as unaged seeds are concerned, no significant differences between wt and *lpa1-241* were evident in protein carbonylation rate, while the number of DNA apurinic/apyrimidinic sites was under the detection threshold

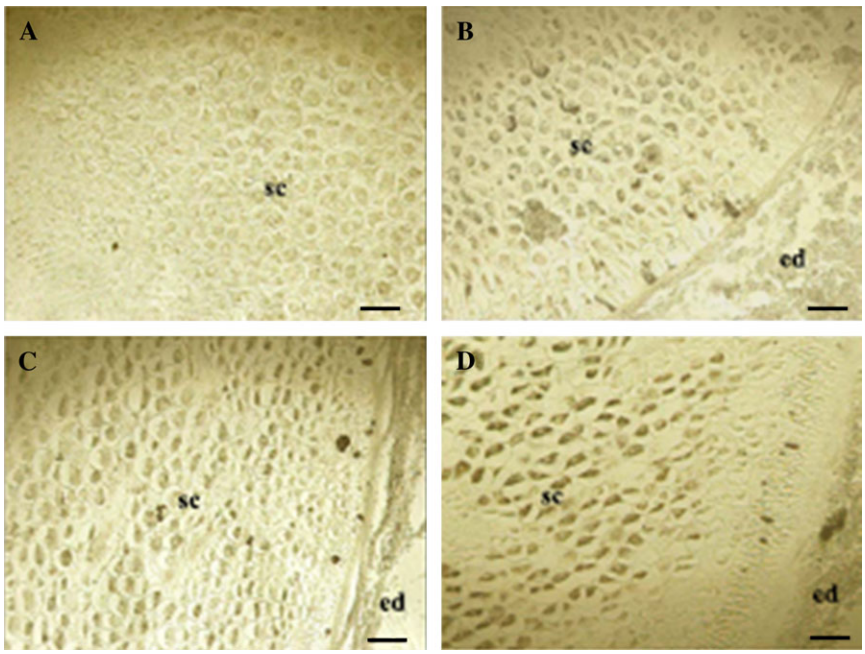

Fig. 3. DAB-staining of H_2O_2: longitudinal section of mature unaged wt (A), artificially aged wt (B), unaged *lpa1-241* (C), and artificially aged *lpa1-241* (D) kernels stained with a DAB solution (ed, endosperm; sc, scutellum). Bar: 50 μm.

in both cases. Conversely, in artificially aged seeds, the *lpa1-241* mutation led to a 3-fold increase in protein carbonylation level compared with wt, as well as about a 5-fold increase in the amount of apurinic-apyrimidinic sites in DNA. Concerning the damage to lipids, as judged by the amount of TBARS (thiobarbituric acid reactive substances) present in seed extracts, no significant differences were found between wt and *lpa1-241* seeds, both artificially aged and unaged.

Analysis of tocopherols

Table 4 shows that a significant difference was found in the amount of γ-tocopherol which, compared to wt, in unaged and artificially aged seeds of *lpa1-241* was about 20% and 45% lower, respectively. In the case of α-tocopherol, no significant difference was found.

Analysis of glutathione

The results of glutathione analysis shown in Table 5 revealed that unaged *lpa1-241* seeds contained a higher level of total glutathione, but a 50% lower ratio GSH/GSSG than the corresponding unaged wt seeds. Upon ageing, a drop in the amounts of total and reduced glutathione and an increase of oxidized glutathione was observed in both samples. However, this trend was much more marked in *lpa1-241* than in the wt aged sample: compared to unaged samples, the GSH/GSSG ratio decreased 8-fold in wt and as much as about 96-fold in *lpa*.

DPPH test

Measurement of ARP (anti radical power) by means of the DPPH test showed (Table 6) that unaged *lpa1-241* seeds have higher ARP than the corresponding wt seeds (about 1.5-fold), whilst the accelerated ageing treatment caused a statistically significant decrease (of about 40%) of the

Table 2. Iron concentration in extracts from flours of unaged wt or *lpa1-241* maize seeds upon extraction with HCl at different concentrations

Sample	μg iron g^{-1} flour[a]			
	HCl 0.01 N	HCl 0.03 N	HCl 0.1 N	HCl 0.4 N
wt	2.0±0.1	7.4±0.4	31±3	94±9
lpa1-241	5.1±0.1*	15.2±0.4*	46±12	89±12

[a] * Value significantly different from that of wt by Student *t* test at $P < 0.05$.

Table 3. Extent of damage to macromolecules in flour of unaged and artificially aged wt or *lpa1-241* maize seeds

Sample	Protein[a] (nmoles carbonylic groups 100 mg^{-1} flour)	DNA[a] (apurinic-apyrimidinic sites mg^{-1} flour)	Lipids TBARS (nmoles g^{-1} flour)
wt	33±7	Under detectable threshold	38±6
lpa1-241	43±9	Under detectable threshold	40±10
Aged wt	42±11	970±60	43±9
Aged *lpa1-241*	124±14**	4400±800**	42±5

[a] ** Value significantly different (Student *t* test) from that of aged wt as well as from those of unaged wt and *lpa1-241* samples at $P < 0.01$.

Table 4. Content of α- and γ-tocopherols in unaged or artificially aged wt and *lpa1-241* maize seeds

Sample	μg α-tocopherol g^{-1} flour	μg γ-tocopherol g^{-1} flour[a]
wt	4.40±0.80	12.70±1.45
lpa1-241	4.0±0.67	10.00±0.55*
Aged wt	3.76±1.07	12.38±0.36
Aged *lpa1-241*	4.16±2.06	6.94±1.69**[b]

[a] *,** Values significantly different from that of the corresponding wt sample by Student *t* test at $P < 0.05$ and $P < 0.01$, respectively.
[b] Value significantly different from that of the corresponding unaged sample by Student *t* test at $P < 0.05$.

Table 5. GSH and GSSG concentration values registered in flour of unaged and artificially aged wt or *lpa1-241* maize seeds

Sample	nmoles GSH g^{-1} flour[a]	nmoles GSSG g^{-1} flour[a]	nmoles total glutathione g^{-1} flour	GSH/GSSG
wt	100.9±12.6	8.1±2.3	109	12.4
lpa1-241	135.2±26.6	20.1±4.0*	155	6.7
Aged wt	32.7±7.4	22.4±1.6[b]	56	1.5
Aged *lpa1-241*	3.7±1.0**	50.2±2.b[b]	54	0.07

[a] *,** Value significantly different from that of the corresponding wt sample by Student's *t* test at $P < 0.05$ and $P < 0.01$, respectively.
[b] Value significantly different from that of the corresponding unaged sample by Student's *t* test at $P < 0.01$.

Table 6. ARP values determined in flour of unaged and artificially aged wt or *lpa1-241* maize seeds

Sample	DPPH test: ARP mg^{-1} flour[a]
wt	5.3±0.91
lpa1-241	8.0±0.56*
Aged wt	5.2±0.94
Aged *lpa1-241*	4.9±0.82[b]

[a] * Value significantly different from that of the corresponding wt sample by Student's *t* test at $P < 0.05$.
[b] Value significantly different from that of the corresponding unaged sample by Student *t* test at $P < 0.01$.

ARP values registered in the corresponding unaged material only in the case of *lpa1-241* maize.

Discussion

As observed in a previous work (Pilu *et al.*, 2005), the most evident negative pleiotropic effect caused by the *lpa1-241* mutation is a decrease in germination capacity. Moreover, the results obtained in the present work show that, compared to the control, the germination capacity loss of *lpa1-241* seeds tends to rise further following seed ageing.

That the viability of plant seeds is severely and primarily influenced by the degree of oxidative stress to which the embryo is exposed during maturation and storage is not a new concept (Wilson and McDonald, 1986). However, apart from a few partially successful attempts to show that antioxidant molecules (carotenoids, tocopherols, polyphenols, etc) stored in seed tissues may constitute defensive tools against oxidative stress, so far, no other small molecule or mechanism has definitely been proven to play a major role in protecting embryo viability. Taking into account its ability to remove Fe^{2+}, which alone has been shown to cause the production of reactive oxygen species in the Fenton reaction, by oxidation to Fe^{3+}, phytic acid is indeed a good candidate for protecting the embryo from oxidative processes. That phytic acid might have a potential role as an antioxidant was hypothesized earlier (Graf *et al.*, 1987; Graf and Eaton, 1990; Empson *et al.*, 1991), while the idea that it might be important for the maintenance of the long-term viability of seeds was put forward by Raboy *et al.* (2000) and also suggested by Dorsch *et al.* (2003). Nevertheless, to the best of our knowledge, no experimental evidence was reported in the literature to prove this hypothesis.

In the present work, the availability of *lpa* mutant maize allowed us to collect results clearly supporting the function of phytic acid in the prevention of oxidative stress in seeds.

A qualitative indication was achieved by means of the DAB histological test in which a higher production of hydrogen peroxide was found in the embryo scutellum of aged compared to unaged seeds, as well as in *lpa1-241* seeds with respect to wt ones (Fig. 3).

Quantitative information regarding long-lived radical concentration in seed tissues of both wt and *lpa* maize, either unaged or subjected to accelerated ageing, were provided by EPR spectroscopy (Table 2). A higher radical concentration was found in the embryo compared to the corresponding endosperm of all samples, confirming the expected higher exposure to oxidative events of embryo compared to endosperm. This is probably due to the presence, reported by various authors using different techniques (O'Dell *et al.*, 1972; Bityuskii *et al.*, 1999; Lin *et al.*, 2005), of a higher amount of metals in the embryo, where phytic acid is also more concentrated. Moreover, a higher accumulation of free radicals was observed in *lpa* mutant embryos than in wt ones, whereas only insignificant differences were found for endosperm tissues. On the other hand, no further significant increase, or even some decrease, in radical concentration of whole kernel and kernel fractions of both wt and *lpa* maize was observed upon accelerated ageing. Following seed ageing, the amount of free radicals remains more than 2-fold higher in *lpa* than in wt maize embryos.

However, damage to protein and DNA macromolecules in maize seeds induced by the *lpa* mutation become evident only after accelerated ageing, as indicated by the results obtained on the degree of protein carbonylation and the amount of apurinic/apirimidinic sites in DNA (Table 3), whereas no change in the degree of lipid peroxidation was found between *lpa1-241* and wt seeds, even upon accelerated ageing. This finding may depend on the remarkable amount of carotenoids and, most of all, tocopherols present in the lipid fraction of maize seeds (Kurilich and Juvic, 1999) which might be particularly competent and efficient in preventing the ROS-induced damage to membrane lipids during both maturation and ageing.

There are no evident direct metabolic links between the pathways of phytic acid and those of 'classical' antioxidants such as tocopherols, ascorbate and glutathione. However, it is possible that the increased oxidative stress (shown by the DAB test and EPR measurements) that *lpa* seeds undergo during maturation may stimulate the synthesis and accumulation in dry seeds of a larger amount of water-soluble antioxidants. The higher level of total glutathione (Table 5) and of anti-radical power measurable by the DPPH test (Table 6) in unaged *lpa1-241* as compared to unaged wt seeds appears to be consistent with this hypothesis. It may also be possible that, in mature dry seeds, during ageing, such increased oxidative stress 'consumes' more antioxidants in *lpa* than in wt seeds. Our findings indicate this is indeed the case for reduced glutathione (Table 5) and for the lipidic antioxidant γ-tocopherol (Table 4). Moreover, the level of the total antioxidant activity measured by the DPPH test appears to decrease by only 2% in wt seeds during ageing, while, strikingly, it dropped by about 40% in *lpa* seeds (Table 6). These results, showing more oxidative damage in DNA and protein of *lpa1-241* than of wt seeds only following artificial ageing (Table 3), can thus be accounted for by the hypothesis that the combined action of antioxidant enzymes and compounds succeeds in counteracting the oxidative stress and the related damage to cell macromolecules during seed maturation of both wt and *lpa1-241*, but fail to do so during ageing in *lpa1-241* seeds.

Overall, these data indicate that the *lpa1-241* mutation isolated in maize is correlated to a high degree of oxidative stress that seeds must cope with during maturation and ageing and that eventually results in loss of embryo viability. However, *lpa* seeds of other species accumulating seed phytic acid (and iron) mainly in tissues different from the embryo, or lacking tocopherols, or endowed with other kinds or quantities of antioxidant compounds, might show different behaviour. In order to clarify this point, studies are underway on *lpa* mutants of other plant species such as common bean.

These findings may also explain some previously obtained results regarding the much lower seedling emergence

reported (Meis *et al.*, 2003) for *lpa* mutant lines of soybean grown under subtropical environmental conditions, that is high temperature and humidity, at which high levels of free iron cations may be more damaging to the seeds. On the basis of our findings, the presence and level of phytate may be, through the mechanism of prevention of ROS formation, among the factors influencing to some extent seed recalcitrance (see review by Berjak and Pammenter, 2003). Appropriate and specific investigations are, however, required to clarify this point.

As to the mechanism linking the *lpa1-241* mutation to the generation of oxidative stress in maize, the following hypotheses can be made.

(i) Acting as signal transducer molecules, phytic acid may be able to induce an appropriate response to oxidative stress such as the synthesis of enzymatic systems able to detoxify reactive oxygen species during maturation. So, in *lpa* mutants its shortage might decrease the embryo's survival chances. It has indeed been reported (Lemtiri-Chlieh *et al.*, 2003) that phytic acid is directly involved in signal transduction events in guard cells, playing a role in the processes by which the drought-stress hormone abscisic acid (ABA) induces stomatal closure, conserving water and ensuring plant survival. An alternative possibility is that the missing signal molecule may not be phytic acid itself, but *myo*-inositol 1,4,5-triphosphate (IP_3), a well-known second messenger that would also be able to induce adaptive responses to oxidative stress. In fact, as in *lpa1-241* maturing seeds the MIPS expression level is severely diminished (Pilu *et al.*, 2003, 2005), *myo*-inositol-3-phosphate and thus also the level of *myo*-inositol (the precursor of IP_3) might be very low. However, it is ascertained here that the amount of *myo*-inositol in *lpa1-241* seeds is only about 40% lower than that found in wt seeds. Taking into account that signal molecules are not required in substrate amounts, it seems unlikely that such a decrease may in turn lead to an IP_3 shortage sufficient to prevent the adaptive response from being induced in embryo tissues.

(ii) The very relevant increase (about 10-fold) in free phosphate level found in *lpa1-241* mutant seeds (Pilu *et al.*, 2003) might perturb phosphorus homeostasis during seed maturation and interfere with any phosphorylation cascade involved, once again, in signal transduction.

(iii) The increase in Fenton reaction caused by the low phytic acid level and by the consequent decrease in iron chelating ability might be the direct and main factor generating ROS, further increasing the oxidative stress to which the *lpa-241* embryo is exposed during maturation. Indeed, in wt maturing seeds most iron cations are transported mainly to embryo tissues where they are bound to the negative phosphate residues of phytic acid. If, in spite of the paucity of phytic acid in *lpa* mutant, cations are nonetheless transported to embryo tissue, most of them might accumulate as free cations or be weakly bound to organic molecules. Indeed, we have verified that, in *lpa* kernels, iron is partially accumulated in a form easily extractable by a mild acid treatment (Table 2). As stated above, in an aerobic cell environment, iron cations, if not linked to chelating compounds or sequestered by other molecules (for instance phytoferritin), can give rise to reactive oxygen species. These in turn, as by now widely observed, are the most common and widespread species able to damage cell molecules and structures and to lead to cell senescence and to apoptosis phenomena.

Therefore, this third hypothesis seems to be the most likely, even if the signalling role and the maintenance of the phosphate homeostasis in maturing seed as a further 'shelter factor' cannot yet be excluded.

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