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Research article

Relationship between ureidic/amidic metabolism and antioxidant enzymatic activities in legume seedlings

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ABSTRACT

Ureides are nitrogenous compounds with a special function in some legume under nitrogen fixing conditions, the ureidic legumes. In this group, ureides are the predominant nitrogen transport molecule from nodules to the upper part, whereas amidic legumes use amides as nitrogen transport compounds. In this study, the ureide levels have been analysed in seedlings from four ureidic and four amidic legume plants. It has been found that the differentiation among ureide and amide plants already exists in seedlings during early seedling development, with high levels of ureide and allantoinase activity in cotyledons and embryonic axes from ureide plants. Since ureides have been implicated in the response of plant to several stress, total hydrosoluble antioxidant capacity and the levels of several antioxidant activities have been determined and compared among these two legume groups. The total antioxidant capacity did not follow any differential pattern in cotyledons or embryonic axes for the analysed plants. The levels of superoxide dismutase, guaiacol peroxidase and ascorbate peroxidase in both embryonic axes and cotyledons are statistical different between amide and ureide seedlings, whereas the catalase activity was similar among these groups of plants. We discuss than amides and ureides could follow different strategies to protect against oxidation.

1. Introduction

Seeds represent a crucial stage in the life cycle of higher plants. Seed germination and postgerminative growth are heterotrophic phases in the sense that seedlings completely depend on seed reserves. At the end of these periods, its nutrient reserves became depleted and the seedling must achieve photoautotrophism. The amount of reserves in the embryo are very small and are quickly consumed and, therefore, the additional growth depends on the supply from cotyledons (Bewley, 1997). Protein mobilization and metabolization has been analysed during germination and seedling development (Shutov et al., 2003). Protein mobilization starts much earlier in the embryonic axis than in the cotyledons, where mobilization only begins after axes protein reserves are depleted (Tiedemann et al., 2000; Lambert et al., 2016). The mobilization of phosphorous in germinating seeds and seedlings has received little attention. In addition to nitrogen, the developing axes require high amounts of phosphorus for the synthesis of nucleic acids, among other molecules. The nucleic acid content in cotyledons might be considered as a nitrogen and phosphorous storage during these phases. Nucleases and ribonucleases activities, which catalyze the hydrolysis of the phosphodiester linkage present in nucleic acids releasing nucleotides, have been determined in axes and cotyledons from French bean during postgerminative growth (Lambert et al., 2016). The first step in the degradation of the nucleotides released by nuclease activities is the removal of phosphate group catalysed by a phosphatase. In French bean, a phosphatase activity with high affinity for nucleotides is induced as well during this phase of seedling development (Cabello-Díaz et al., 2012, 2015). A final compound used as nitrogen transport by seedlings can be the ureides, which are derived from the purine moieties present in purinic nucleotides. All the above metabolism could

Abbreviations: ALNase, allantoinase activity; APX, ascorbate peroxidase; DAI, days after imbibition; GPX, guaiacol peroxidase; SOD, superoxide dismutase; ROS, Reactive Oxygen Species; Pv, *Phaseolus vulgaris*; Vr, *Vigna radiata*; GM, *Glycine max*; Vu, *Vigna unguiculata*; Lc, *Lens culinaris*; Ps, *Pisum sativum*; Ca, *Cicer arietinum*; Ls, *Lathyrus sativus*

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result in the accumulation of ureides and induction of its metabolism described in French bean during postgerminative growth (Quiles et al., 2009).

The ureides have been mostly studied as the predominant nitrogen transport molecules in the xylem of nitrogen fixing tropical legumes (reviewed by Todd et al., 2006). Legumes can fix atmospheric nitrogen through their root nodules and the fixed nitrogen can be exported to the aerial parts in the form of amides, glutamine and asparagine (amidic species), whereas others export the nitrogen from the nodules as ureides, allantoin and allantoate (ureidic species). The N:C ratio in ureides is higher than in amides and, therefore, they are efficient nitrogen transport molecules due to the high rate of nitrogen:carbon (1:1). The advantage of ureides as nitrogen-transporter can be exploited, as well, during other processes that require high mobilization as the seedling development described above, or during the leaf senescence. Accumulation of ureides in association with nucleic acid degradation have been described in dark-induced leaf senescence in French bean (Lambert et al., 2017).

A role of Reactive Oxygen Species (ROS) in reserve mobilization from cotyledons to axes during postgerminative growth has been proposed by promoting oxidative modifications of stored protein (Verma et al., 2015). Thus, the addition of external hydrogen peroxide leads to enhanced rates of storage protein mobilization (Puntarulo et al., 1988). ROS have a dual nature acting as signaling and as toxic molecules. As signaling molecules, ROS interact with phytohormones resulting in signaling networks that control seed germination (Wojtyla et al., 2016). Therefore, seed germination requires a regulated and controlled generation of ROS, which has been named as the oxidative window for germination (Bailly et al., 2008). Among ROS, hydrogen peroxide is a good signaling molecule due to its stability and lower reactivity in comparison with the other ROS molecules (Quan et al., 2008). Regarding the second nature of ROS, as toxic molecules, they are formed as toxic byproducts of photosynthesis, cellular respiration, and other metabolic reactions in mitochondria, chloroplasts, peroxisomes and apoplast (Liu and He, 2017). The high rate of respiration caused by a high rate of cell division in the growing axes, and the high nutrient mobilization from cotyledons leads to the increased production of ROS (Verma et al., 2015).

Biotic and abiotic stress are the main cause of crop yield loss for the major crops worldwide (Cramer et al., 2011). The concentration of cellular ROS increases when plants are challenged by both type of stresses (Saxena et al., 2016). ROS stress can cause oxidative damage to proteins, lipids, nucleic acids or pigments (Moller et al., 2007). Damaging effect of ROS can be alleviated by enzymatic and nonenzymatic mechanisms. Enzymatic mechanisms include several antioxidant enzymes such as peroxidases, catalase or superoxide dismutase, whereas the non-enzymatic mechanisms involve the action of antioxidant species such as glutathione, ascorbate or vitamin E (Foyer and Noctor, 2009).

Recent studies suggest a function for ureides in response to abiotic stress. Most of these studies have been performed in Arabidopsis, in which an increase in the amount of ureides have been described under drought (Watanabe et al., 2014; Irani and Todd, 2016), dark (Brychkova et al., 2008) and salt stresses (Irani and Todd, 2016) and cadmium stress (Nourimand and Todd, 2017). In French bean plants, an increase in ureides have been described under drought stress (Alamillo et al., 2010; Coleto et al., 2014). Furthermore, the addition of exogenous allantoin altered the ROS levels in Arabidopsis dark-treated leaf disks (Brychkova et al., 2008), as well as in seedlings treated with salt (Irani and Todd, 2018). The accumulation of ureides in plants under abiotic stress might suggest a possible role for ureides protecting plants against the effects of reactive oxygen species, directly acting as ROS protector or mediating the activation of ABA signaling pathways (Watanabe et al., 2014).

Consequently, we hypothesized that ureide metabolism is related to the antioxidant activities in legume seedlings. To contrast this, the antioxidant activities and ureide metabolism of four ureidic and four amidic legumes have been investigated during early seedling development. Our results suggest that ureidic and amidic plants can use different strategies to cope with the oxidative stress involved in seedlings development.

2. Material and methods

2.1. Seeds and seed germination

Seeds from four ureidic legumes (French bean (*Phaseolus vulgaris* L. cv. Great Northern), mung bean (*Vigna radiata*), soybean (*Glycine max*), cowpea (*Vigna unguiculata*)) and four amidic legumes (lentil (*Lens culinaris*), garden pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and grass pea (*Lathyrus sativus*)) were purchased in local markets. Seeds were surface-sterilized in ethanol 80% (30 s) and 0.2% (w/v) sodium hypochlorite (10 min) and then washed thoroughly with sterile distilled water. Soaked seeds were allowed to germinate in Petri dishes (120 mm diameter) with wet paper in sterile conditions. The moisture was maintained by regularly adding solutions to the dishes. Germination was carry out in a growth chamber under a long-day photoperiod (16 h light, 8 h dark) and 70% relative humidity at 26 - 21 °C (day - night temperatures).

2.2. Preparation of crude extract

Plant material was ground on liquid nitrogen and stored as a fine powder at −80 °C. All the following procedures were carried out at 0–4 °C. Plant powder was homogenized in an appropriated container by adding 4 ml of extraction buffer per gram of tissue. The extraction buffer was 50 mM Tris-HCl (pH 7.8), 1 mM $MnSO_4$ and 0.1% (w/v) sodium deoxycholate. After 15 min of incubation, the resulting homogenate was centrifuged at 12000 g for 10 min. The supernatant was used as crude extract for activity determinations, soluble protein, total antioxidant capacity and total ureides.

2.3. Enzymatic activities

2.3.1. Allantoinase activity (ALNase)

Allantoinase activity (ALNase) was determined following the production of allantoate as indicated by Raso et al. (2007a). The reaction mixture consisted of 50 mM Tris–HCl (pH 7.8), 1 mM $MnSO_4$, 12 mM allantoin and appropriate amount of crude extract. The reaction was carried out at 35 °C and aliquots were taken at several time points (up to 1 h) to determine allantoate concentration.

2.3.2. Superoxide dismutase activity (SOD)

Superoxide dismutase activity (SOD) was determined as the ability of inhibit the photochemical reduction of nitroblue tetrazolium as described by (Giannopolitis and Ries, 1977). The reaction mixture was composed of 50 mM potassium phosphate (pH 7.3), 13 mM of L-methionine, 0.1 mM of EDTA, 0.06 mg/ml of nitroblue tetrazolium, 4 μM of riboflavin and the adequate amount of crude extract. Absorbance was measured at 560 nm after incubation for 20 min at room temperature with 30 W fluorescent bulb. One unit of SOD activity was defined as the amount of enzyme required to inhibit NBT photoreduction by 50%.

2.3.3. Guaiacol peroxidase activity (GPX)

Guaiacol peroxidase activity (GPX) was determined as described by Castillo et al. (1984) with minor changes. The continuous assay was performed for 2 min at 30 °C in a spectrophotometer Jasco V-630. The reaction mixture consisted of 100 mM phosphate buffer (pH 5.8), 30 mM of guaiacol, 11 mM of hydrogen peroxide and the adequate amount of crude extract. Absorbance increase was measured at 470 nm and enzyme activity was calculated using an extinction coefficient of 26.6 $mM^{-1}\,cm^{-1}$.

2.3.4. Ascorbate peroxidase activity (APX)

Ascorbate peroxidase activity (APX) was determined following the method described by Chen and Asada (1989) with little modifications. The reaction mixture consisted of PBS (pH 7), 0.5 mM of ascorbate, 0.5 mM of peroxide and the adequate amount of crude extract. The decrease in A290 was recorded and an extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for calculations.

2.3.5. Catalase activity (CAT)

Catalase activity (CAT) was obtained according to the method described by Lu et al. (2013) with little modifications. Reaction mixture consisted of PBS (pH 7.0), 15 mM of hydrogen peroxide and the adequate amount of crude extract. These mixtures were incubated at 25 °C. The decrease of absorbance at 240 nm for 3 min was recorded and an extinction coefficient of 0.0265 mM^{-1} cm^{-1} was used for calculations.

2.4. Analytic determinations

Soluble protein concentrations were determined by the Bradford (1976) method with bovine serum albumin as a protein standard.

The concentration of ureides (allantoin and allantoate) was determined by the colorimetric analysis of glyoxylate derivatives as described by Piedras et al. (1998). In this method, allantoin and allantoate are determined after its chemical transformation to glyoxylate. The values of total ureides in crude extracts indicated in the paper are the sum of both allantoin and allantoate.

Total hydrosoluble antioxidant capacity was established by the method previously described by Prieto et al. (1999). Briefly, 0.1 ml of extract was added to 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and the mix was incubated at 95 °C for 90 min. After cooling at room temperature, the absorbance was measured at 695 nm against a blank. Antioxidant capacity was expressed in mg of ascorbate equivalent g^{-1} FW using a calibration curve of ascorbate.

The concentration of H_2O_2 in tissues was determined by the enzymatic-catalyzed oxidation of Amplex-Red. Briefly, powder samples (0.1 g) was homogenized in an ice bath with 0.4 ml of a solution consisted of 3% (w/v) activated charcoal and 0.1 M HCl. The mixture was centrifuged at 12000 g for 40 min and 4 °C. Appropriate amount of supernatant was added to 0.2 M sodium phosphate (pH 7.3), 0.1 mM Amplex-Red and 0.2 U ml^{-1} of horseradish peroxidase. After 15 min at room temperature, the absorbance was measured at 569 nm and the amount of H_2O_2 was calculated based on a standard curve.

2.5. Statistical analysis

Firstly, to test the difference in ureides content and allantoinase activity between day 1 and day 4 in the same species, Student's *t*-tests were performed. Secondly, three different sets of general linear models were built to check differences between plant species and between ureidic and amidic legumes. Four different response variables were used in the first set of the models: total ureides in the axis, total ureides in the cotyledon, ALNasa in the axis and ALNasa in the cotyledon (Figs. 1 and 2, panels B and D). In these models, the variables 'Day' (Day 1, and Day 4), 'Family' (ureidic and amidic) and the interaction 'Day*Family' were included as explanatory variables, whereas the variable 'Species' was added as a random factor in order to account that the samples were obtained from different species. In the second and third sets, the response variables were the antioxidants (Fig. 3), the superoxide dismutase (Fig. 4), the guaicol peroxidase (Fig. 5), the ascorbate peroxidase (Fig. 6), and the catalase (Fig. 7) activities measured separately in the axis and in the cotyledon. The variable 'Species' was the unique explanatory variable included in the second set of the models (panels A and C), whereas in the third set the 'Family' was include as an explanatory variable and the 'Species' was treated as a random variable (panels B and D). In all models, post hoc tests within the mixed analysis was developed to check for differences among the level of categorical variables and to explore the interactions. The assumptions of normality and homogeneity of the residuals were confirmed in all models. The entire statistical analysis was performed using InfoStat software.

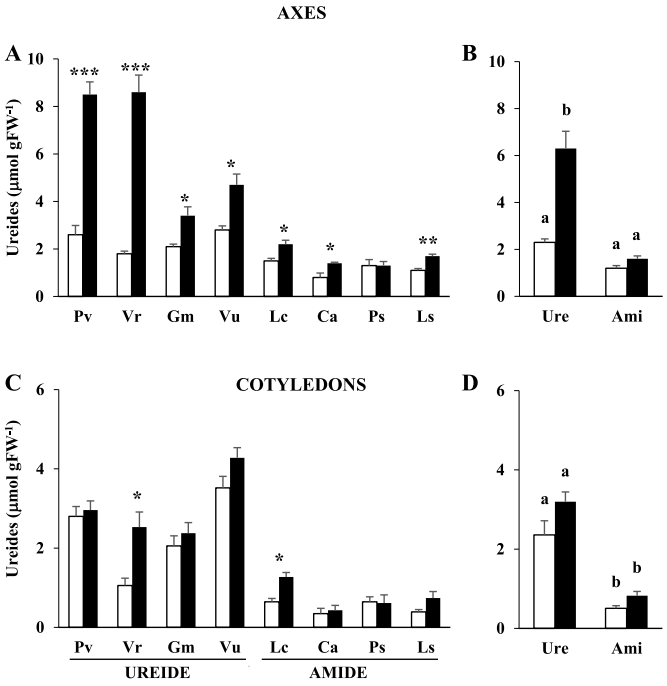

Fig. 1. Ureide concentration in seedlings from four ureidic and four amidic legumes. **A.** Crude extracts were obtained from embryonic axes (**A**) and cotyledons (**C**) at 1 (white bars) and 4 days (black bars) after start of imbibition and the levels of ureides were determined and normalised against fresh weight. Results are the mean ± SE of three independent replicates. Student's *t*-test was performed to compare the difference between 1 and 4 DAI in each species. Asterisks indicate significant difference at $P < 0.05$, $P < 0.005$ or $P < 0.0005$. The mean values ± SE of ureide concentration for the four ureidic (Ure) and amidic (Ami) legumes at both times (1 and 4 DAI) in embryonic axes (**B**) and cotyledons (**D**) were represented. General lineal model test was performed and different letters indicates statistical differences among the four means according to the pot hoc tests developed within the model procedure (Supplementary Table 1). Each species is abbreviated as follow: *Phaseolus vulgaris* (Pv), *Vigna radiata* (Vr), *Glicine max* (Gm), *Vigna unguiculata* (Vu), *Lens culinaris* (Lc), *Pisum sativum* (Ps), *Cicer arietinum* (Ca) and *Lathyrus sativus* (Ls).

3. Results

3.1. Ureide content in legume seedlings during germination

Total ureide content was determined in the embryonic axes and cotyledons of non-germinated (1 DAI, white bars) and germinated (4 DAI, black bars) seedlings from several ureidic and amidic legumes (Fig. 1). The concentration of ureides in embryonic axes of all the ureidic plants increased significantly after germination (Fig. 1A). In the amidic legumes, a moderate increase along development was observed in all the species with the exception of *P. sativum* (Fig. 1A). To test if there was a common pattern for each group of legumes, and statistical analysis was performed comparing the ureide concentration in all the amidic and ureidic legumes at both times (Fig. 1B). At 1 DAI, not statistical differences were found in ureide concentration in embryonic axes between both groups, but a clear statistical difference was observed at 4 DAI between amidic and ureidic plants since only in ureidic legumes the concentration of ureides increased (Fig. 1B).

Total ureide was also determined in cotyledons from the seedlings analysed above (Fig. 1C). Among all the species studied, the

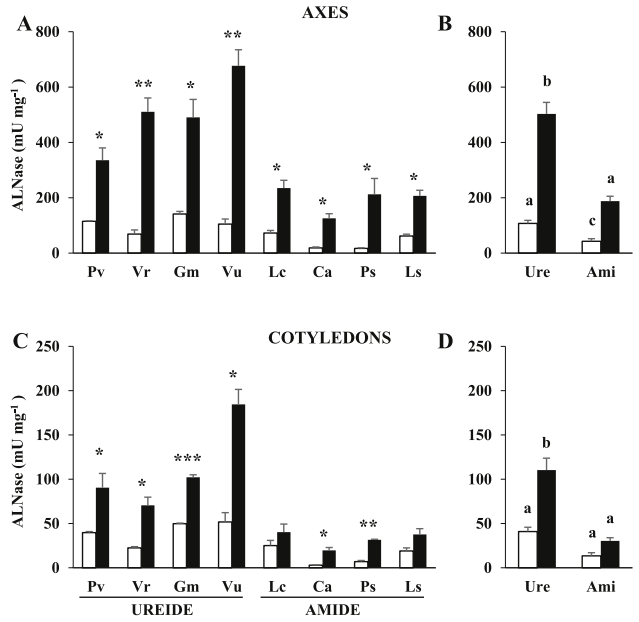

Fig. 2. Allantoinase activity in seedlings from four ureidic and four amidic legumes. Specific allantoinase activity was determined in the same crude extracts from embryonic axes (**A**) and cotyledons (**C**) indicated in Fig. 1. Results are the mean ± SE of three independent replicates. Student's *t*-test was performed to compare the difference between 1 and 4 DAI in each species. Asterisks indicate significant difference at $P < 0.05$, $P < 0.005$ or $P < 0.0005$. The mean values ± SE of allantoinase activity for the four ureidic (Ure) and amidic (Ami) legumes at both times (1 and 4 DAI) in embryonic axes (**B**) and cotyledons (**D**) were represented. General lineal model test was performed and different letters indicates statistical differences among the four means according to the pot hoc tests developed within the model procedure (Supplementary Table 1). The abbreviated name correspond to the specie indicated in Fig. 1.

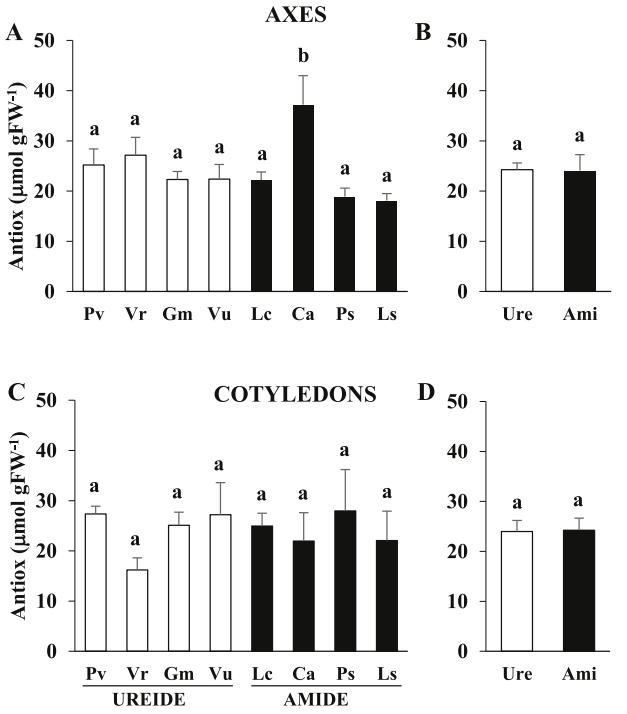

Fig. 3. Total antioxidant capacity in seedlings from ureidic (white bars) and amidic (black bars) legumes. Total antioxidant was determined in the crude extracts obtained from embryonic axes (**A**) and cotyledons (**C**) from seedlings at 4 DAI. The abbreviated name correspond to the specie indicated in Fig. 1. Results are the mean ± SE of three independent replicates. General lineal model test was performed and different letters indicates statistical differences among the eight species according to the pot hoc tests developed within the model procedure (Supplementary Table 2). The mean values for total antioxidant in axes (**B**) and cotyledons (**D**) for all the ureidic (Ure) and amidic (Ami) was calculated and different letters indicated statistical differences among the mean values.

concentration of ureides increased in cotyledons along germination at an statistically significant level only in *V. radiata* (ureidic legume) and *L. culinaris* (amidic legume) and not changes were observed between both periods in the remaining species (Fig. 1C). When ureidic and amidic groups were compared, significant differences were observed in the ureide content in cotyledons among both groups in both days analysed, with more ureides in cotyledons from ureidic legumes (Fig. 1D). In cotyledons, no increase in ureide concentration was observed in these groups with the germination (Fig. 1D).

3.2. Allantoinase activity in legume seedlings during germination

Allantoinase activity was determined in the same crude extracts from axes and cotyledons (Fig. 2). In embryonic axes, the allantoinase activity increased significantly after germination in all the species (Fig. 2A). When both types of legumes were grouped and compared, the allantoinase activity was higher after germination in both groups, with higher value in ureidic seedlings than in amidic ones at both times (Fig. 2B).

In cotyledons, the allantoinase activity increased after germination in all the species, although the increase was not statistical significant in *L. culinaris* and *L. sativus*, both amidic legumes (Fig. 2C). When the activity was compared between both groups at both times, a significantly different allantoinase activity was obtained in cotyledons from ureidic seedlings at 4 DAI compared with amidic ones (Fig. 2D). Allantoinase activity at 4 DAI was higher in axes than in cotyledons for the eight species analysed (Fig. 2 A, C).

3.3. Total hydrosoluble antioxidant capacity in legume seedlings

The total hydrosoluble antioxidant activity was determined in the crude extracts obtained from the eight seedling legumes (Fig. 3). The analysis was performed at 4 DAI because clear differences in ureide content and allantoinase activity have been found during early seedling development. The total antioxidant capacity was very similar in embryonic axes from all the species analysed with the exception of *Cicer arietinum*, which showed a higher capacity than the others (Fig. 3A). Statistical differences were not found when total antioxidant capacity in embryonic axes was compared between both groups (Fig. 3B). The total antioxidant capacity values in cotyledons were very similar to those from embryonic axes (Fig. 3A, C). The antioxidant activity in cotyledons did not show statistical differences when the eight species were compared individually (Fig. 3C) or when they were grouped as amidic and ureidic plants (Fig. 3D).

3.4. Antioxidant enzyme activities in legume seedlings

Several antioxidant enzymatic activities were determined in crude extracts from embryonic axes and cotyledons from seedlings at 4 DAI. Superoxide dismutase (SOD) activity in embryonic axes of *V. radiata*, *G. max* and *V. unguiculata* was slightly higher than in *P. vulgaris*, while it was very similar among amidic plants (Fig. 4A). SOD activity was higher in embryonic axes of ureidic than in amidic legumes (Fig. 4B). In the case of cotyledons, the SOD activity of *P. vulgaris* and *V. unguiculata* was higher than in the rest of species and the lowest SOD activity was found in *C. arietinum* (Fig. 4C). When compared as groups, statistical differences were obtained between amidic and ureidic legumes (Fig. 4D).

The guaiacol peroxidase (GPX) activity in embryonic axes from

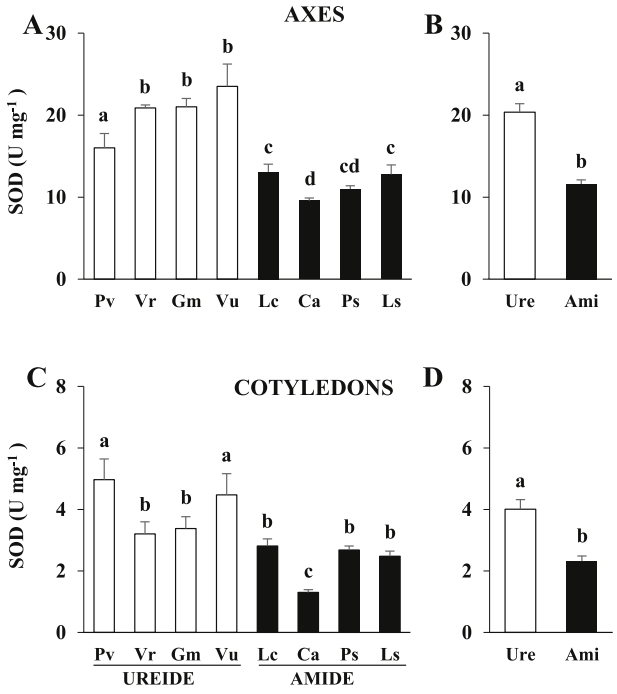

Fig. 4. Superoxide dismutase activity in seedlings from ureidic (white bars) and amidic (black bars) legumes. Specific activity of SOD was determined in crude extracts obtained from embryonic axes (**A**) and cotyledons (**C**) of seedlings at 4 DAI. The abbreviated name correspond to the specie indicated in Fig. 1. General lineal model test was performed and different letters indicates statistical differences among the eight species according to the pot hoc tests developed within the model procedure (Supplementary Table 2). The mean values ± SE of SOD activity for the four ureidic (Ure) and amidic (Ami) legumes in embryonic axes (**B**) and cotyledons (**D**) were represented.

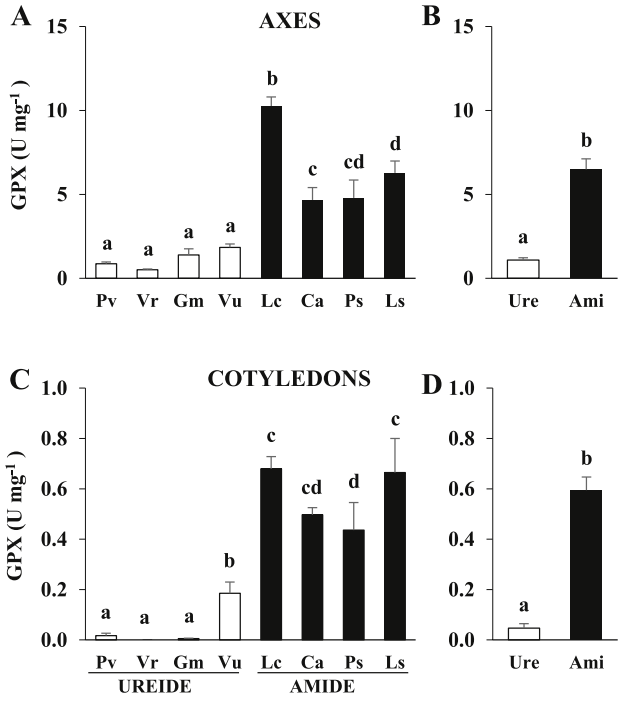

Fig. 5. Guaiacol peroxidase activity in seedlings from several legumes. The same as Fig. 4 but for guaiacol peroxidase.

ureidic legumes was very similar among species (Fig. 5A). This activity for amidic plants was higher than for the ureidic ones, and the highest activity was obtained for embryonic axes of *L. culinaris* (Fig. 5A). A

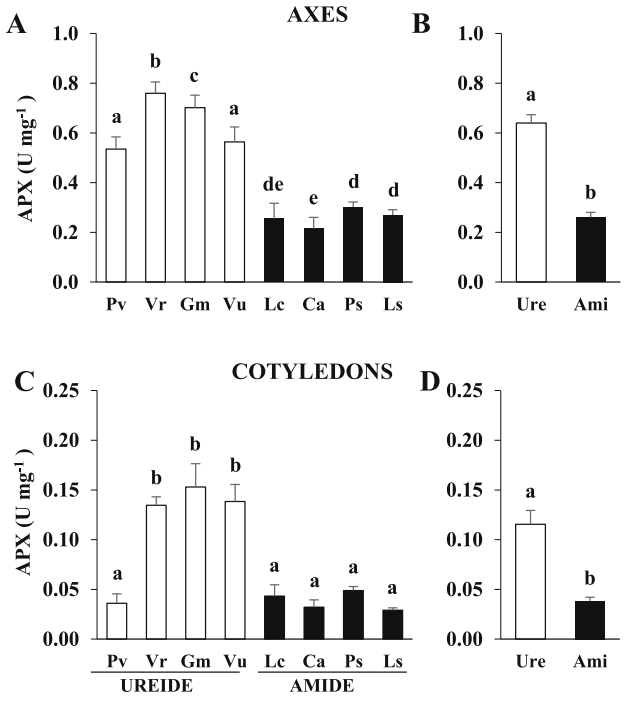

Fig. 6. Ascorbate peroxidase activity in seedlings from several legumes. The same as Fig. 4 but for ascorbate peroxidase.

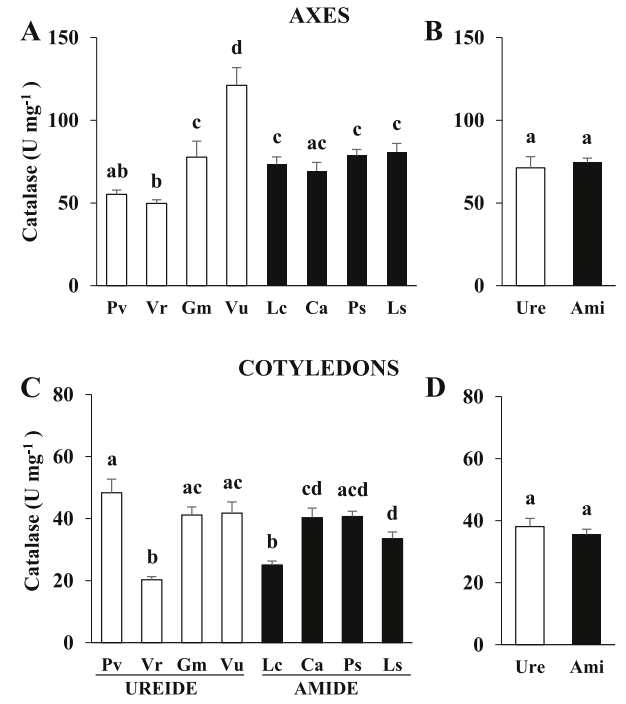

Fig. 7. Catalase activity in seedlings from several legumes. The same as Fig. 4 but for catalase.

clear difference was obtained when the values were compared between ureidic and amidic groups (Fig. 5B). A similar pattern was observed in crude extracts from cotyledons, with GPX activity being lower in the ureidic species than in the amidic ones (Fig. 5C). Among the ureidic legumes, GPX was higher in *V. unguiculata* than in the other three species (Fig. 5C). A clear statistical difference between the two groups was obtained (Fig. 5 D).

Ascorbate peroxidase (APX) activity was higher in axes from the ureidic seedlings than in the amidic species (Fig. 6A). In axes from

ureidic plants, the highest activity was observed in *V. radiata*, followed by *G. max*. The activity in *P. vulgaris* and *V. unguiculata* was very similar between them, and lower than in the other ureidic plants (Fig. 6A). A clear and statistically significant difference between amidic and ureidic groups was also found (Fig. 6B). APX activity in cotyledons was very similar en *V. radiata*, *G. max* and *V. unguiculata* and statistically different from the other five species including the ureidic *P. vulgaris*, which showed similar activity to the amidic legumes (Fig. 6C). Besides the low activity in cotyledons of *P. vulgaris*, statistical difference was obtained between the two legume groups (Fig. 6D).

The highest catalase (CAT) activity was observed in embryonic axes from *V. unguiculata* and the lowest one in those from *P. vulgaris* and *V. radiata*, all of them ureidic legumes (Fig. 7A). In the other five species the activity was very similar among them (Fig. 7A). The absence of a pattern highlighted the lack of variation in catalase activity when compared ureidic and amidic seedlings (Fig. 7B). The catalase activity in cotyledons was variable in the eight species studied without a clear pattern (Fig. 7C and D).

It is interesting to note that specific values for all the enzymatic activities analysed were generally higher in axes than in cotyledons for the eight species studied, with the exception of catalase activity in *P. vulgaris* which showed similar values in axes and in cotyledons.

To summarize, when the level of antioxidant activities in ureidic and amide legumes was compared, SOD and APX clearly showed differences between both groups, being these activities higher in ureidic than in amidic plants. On the contrary, GPX was lower in ureidic than in amides seedlings, whereas catalase activity did not show significant differences between both groups of legumes.

4. Discusion

Legumes are important crops with great agronomic and ecologic interest, since they can get most of the nitrogen for development thanks to symbiotic association with nitrogen-fixing bacteria. Legumes develop nodules to carry out the nitrogen fixation process. The nitrogen fixed in nodules can be transported to the upper part of the plants as amides (amidic legumes) or ureides (ureidic legumes) (review by Zrenner et al., 2006). In nodulated ureidic legumes, the ureides are synthesized via the purine oxidation pathway in the nodule. Interestingly, the novo synthesis of purine starts from an amide, the glutamine, and therefore, could be considered a specialized pathway. The use of ureides confers a major advantage as N-transport molecules due to fact that N:C ratio is higher for ureides than for amides, transporting nitrogen to aerial parts at a minimal carbon cost. The use of ureides in nitrate feeding legumes is reduced, and the concentration of ureides was seven-fold higher in the leaves of nodulated than non nodulated leaves of *P. vulgaris* (Raso et al., 2007b; Diaz-Leal et al., 2012). Traditionally, ureides have been studied because of this role in the transport of fixed nitrogen, but ureide metabolism is common to all plants as purine recovery mechanism. In the last years, the metabolism and role of ureides has been studied in no legume plant as Arabidopsis, where the abundance relative of ureides in leaves (Werner et al., 2008) is lower than in soybean (Duran and Todd, 2012) or French bean (Diaz-Leal et al., 2012).

Beyond transport of fixed nitrogen, ureides can be also important in other conditions that require nutrient mobilization. The transport of ureides from cotyledons to the developing seedling has been suggested in French bean (Quiles et al., 2009) and soybean (Duran and Todd, 2012) during postgerminative growth. In this paper we show that although ureides are higher in cotyledons and axes of ureidic plants, they are also present in all amide species analysed. In the embryonic axes of ureidic and amidic legumes differences in ureide concentration was found at 4 DAI, being the amount similar among amidic and ureidic legumes at 1 DAI (Fig. 1). This is, the concentration of ureides only increases in axes from ureidic seedlings. In cotyledons, the differences were at both times, being much higher in ureidic than in amidic plants, but without an increase in concentration along postgerminative development in any group. Likewise, allantoinase activity was clearly different among both groups of legumes. The changes in allantoinase activity has been postulated as the responsible for the ureide levels in French bean during development (Diaz-Leal et al., 2012). These data suggest that the differentiation among ureide and amide metabolism is not only true in adult plant when legumes are fixing nitrogen biologically, but it is also present in other process and developing stages with high nutrient mobilization. In French bean, the involvement of ureides in nutrient mobilization has also been suggested during cotyledons and leaves senescence in association with an increase in the degradation of nucleic acids that could be the origin for the ureides (Lambert et al., 2016, 2017).

A potential role as ROS scavengers during plant stress response has also been proposed for ureides (Brychkova et al., 2008; Watanabe et al., 2014; Takagi et al., 2016). It has been shown that externally added allantoin alters the levels of ROS as well as the transcription of several genes encoding antioxidant activities (Watanabe et al., 2014; Irani and Todd, 2018). In addition, a protective effect of ureides has been proposed for osmotic (Watanabe et al., 2014), dark (Brychkova et al., 2008) and salt stress (Irani and Todd, 2018). Furthermore, ureides may also participate in stress responses regulating the basal levels of stress associated hormones (Watanabe et al., 2014; Takagi et al., 2016). The possible relation between ureides and ROS in other physiological process has not been determined. ROS are generated during germination and seedling development, and different functions have been proposed recently for these compounds (Oracz and Karpinski, 2016; Wojtyla et al., 2016). ROS quenching in *Vigna radiata* resulted in reduced rates of germination establishing the importance of ROS in germination and seedling development (Verma et al., 2015). The diminution in ROS concentration affects protein reserve mobilization in cotyledons, and it has been postulated that high levels of ROS can cause oxidative modifications acting as signals to mobilize reserves to the growing axis (Verma et al., 2015). The clear differences in ureide and allantoinase activity between both groups of legumes, and the relationship between ureides and oxidative stress prompted us to investigate the antioxidant capacities and activities of these legumes. Not differences were observed for the total antioxidant capacities of crude extracts. Nevertheless, some clear differences among ureide and amide legumes have been found in several antioxidant enzymatic activities.

All the antioxidant activities assayed were much higher in axes than in cotyledons with the exception of catalase, whose values were only slightly higher in axes. At the stage of four days after imbibition, the embryonic axes are high growing tissues and therefore have an elevated rate of respiration that could result in increased production of ROS (Verma et al., 2015). Catalase is more suited for gross removal and control of high peroxide levels and less for maintaining delicate control (Verma et al., 2015). Although the antioxidant activities showed some differences among all the species analysed, it is noteworthy that SOD, APX and GPX presented different patterns in ureidic and amidic legumes. In the ureide biosynthetic pathway, there are two enzymes, xanthine oxidase and uricase, that produce considerable amounts of ROS. Therefore, the production of ureides in the ureidic seedlings, probably used as nutrient mobilization, could result in a different ROS pattern. The hydrogen peroxide in embryonic axes of the eight species analysed was very similar (data not shown). The pathway of ureide synthesis is compartmentalized among several organelles. The ROS-generating xanthine oxidoreductase and urate oxidase have been demonstrated to be present in peroxisomes (Corpas et al., 1997). Catalase is one of the most important antioxidant enzymes and is also predominately localized in peroxisomes. This enzymatic activity is similar in ureidic and amidic legumes, but this does not exclude that part of the hydrogen peroxide could leak out of peroxisome and induce other enzymatic activities in other compartments. New functions have been proposed for peroxisomes as the source of signal molecules involved in the functional interrelation with other subcellular compartments (Corpas et al., 2019). The silencing of one of these ROS-generating

enzyme, the XDH, reveals the role of this enzyme in several processes (Brychkova et al., 2008; Han et al., 2018; Soltabayeva et al., 2018).

APX and GPX presented different patterns in ureidic and amidic legumes. Ascorbate peroxidase belongs to the family of heme-containing peroxidases that catalyse the H2O2 -dependent oxidation of a wide range of organic molecules (Pandey et al., 2017). Ascorbate peroxidase is reported to contribute to hydrogen peroxide detoxification being an efficient regulator of ROS (Pandey et al., 2017). Guaiacol peroxidase correspond to the peroxidase that can use guaiacol as artificial phenolic substrate, property associated with class III peroxidases (Mika et al., 2010). Class III peroxidases are bifunctional enzymes which can either generate or detoxify hydrogen peroxide. Guaiacol peroxidase activity was significantly higher in embryonic axes and cotyledons from amidic legumes than in those from ureidic, whereas ascorbate peroxidase was the opposite. Therefore a possible complementation between both activities in order to reduce the peroxide levels during seedlings development can be deduced.

Our results suggest that ureides and amides follow different strategies to regulate the level of ROS. Whether this differentiation is related with the presence of different levels of ureides or to other factors needs further investigation, but the fact that ureides have been implicated as antioxidant compounds indicated that it can be plausible that ureides complemente/participate in the control of ROS during the early seedling development in ureidic legumes.

Author contribution

FAQ, GG-V and PP conceived the research plan and designed the experiments. FAQ performed the experiments. PP was the primary author involved in writing the original draft of the paper. FAQ and JG-C performed the statistical analyses. GG-V and MP contributed to review and editing of the paper, providing helpful comments and discussions. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.016.

References

Alamillo, J.M., Diaz-Leal, J.L., Sanchez-Moran, M.V., Pineda, M., 2010. Molecular analysis of ureide accumulation under drought stress in Phaseolus vulgaris L. Plant Cell Environ. 33, 1828–1837.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bailly, C., El-Maarouf-Bouteau, H., Corbineau, F., 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. C R Biol 331, 806–814.

Bewley, J.D., 1997. Seed germination and dormancy. Plant Cell 9, 1055–1066.

Brychkova, G., Alikulov, Z., Fluhr, R., Sagi, M., 2008. A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the Atxdh1 *Arabidopsis* mutant. Plant J. 54, 496–509.

Cabello-Díaz, J.M., Quiles, F.A., Lambert, R., Pineda, M., Piedras, P., 2012. Identification of a novel phosphatase with high affinity for nucleotides monophosphate from common bean (*Phaseolus vulgaris*). Plant Physiol. Biochem. 53, 54–60.

Cabello-Diaz, J.M., Galvez-Valdivieso, G., Caballo, C., Lambert, R., Quiles, F.A., Pineda, M., Piedras, P., 2015. Identification and characterization of a gene encoding for a nucleotidase from *Phaseolus vulgaris*. J. Plant Physiol. 185, 44–51.

Castillo, F.J., Celardin, F., Greppin, H., 1984. Peroxidase assay in plants. Interference by ascorbic acid and endogenous inhibitors in sedun and pelargonium enzyme extracts. Plant Physiol. 2, 69–75.

Chen, G.X., Asada, K., 1989. Ascorbate peroxidase in tea leaves. Ocurrence of 2 isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol. 30, 987–998.

Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M., Shinozaki, K., 2011. Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol. 11, 163.

Coleto, I., Pineda, M., Rodino, A.P., De Ron, A.M., Alamillo, J.M., 2014. Comparison of inhibition of N2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. Ann. Bot. 113, 1071–1092.

Corpas, F.J., de la Colina, C., Sanchez-Rasero, F., del Río, L.A., 1997. A role for leaf peroxisomes in the catabolism of purines. J. Plant Physiol. 151, 246–250.

Corpas, F.J., del Rio, L.A., Palma, J.M., 2019. Plant peroxisomes at the crossroad of NO and H_2O_2 metabolism. J. Integr. Plant Biol. https://doi.org/10.1111/jipb.12772.

Diaz-Leal, J.L., Gálvez-Valdivieso, G., Fernandez, J., Pineda, M., Alamillo, J.M., 2012. Developmental effects on ureide levels are mediated by tissue-specific regulation of allantoinase in *Phaseolus vulgaris* L. J. Exp. Bot. 63, 4095–4106.

Duran, V., Todd, C.D., 2012. Four allantoinase genes are expressed in nitrogen-fixing soybean. Plant Physiol. Biochem. 54, 149–155.

Foyer, C.H., Noctor, G., 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid. Redox Signal 11, 861–905.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases. I. Ocurrence in higher plants. Plant Physiol. 59, 309–314.

Han, R.C., Rasheed, A., Wang, Y.P., Wu, Z.F., Tang, S.Q., Pan, X.H., Shi, Q.H., Wu, Z.M., 2018. Silencing of OsXDH reveals the role o purine metabolism in dark tolerance in rice seedlings. J. Integr. Agric. 17, 1736–1744.

Irani, S., Todd, C.D., 2016. Ureide metabolism under abiotic stress in *Arabidopsis thaliana*. J. Plant Physiol. 199, 87–95.

Irani, S., Todd, C.D., 2018. Exogenous allantoin increases Arabidopsis seedlings tolerance to NaCl stress and regulates expression of oxidative stress response genes. J. Plant Physiol. 221, 43–50.

Lambert, R., Cabello-Diaz, J.M., Quiles, F.A., Piedras, P., 2016. Identification of nucleases related to nutrient mobilization in senescing cotyledons from French bean. Acta Physiol. Plant. 38, 11.

Lambert, R., Quiles, F.A., Gálvez-Valdivieso, G., Piedras, P., 2017. Nucleases activities during French bean leaf aging and dark-induced senescence. J. Plant Physiol. 218, 235–242.

Liu, Y., He, C., 2017. A review of redox signaling and the control of MAP kinase pathway in plants. Redox Biology 11 192-2014.

Lu, J., Li, X.N., Yang, Y.L., Jia, L.Y., You, J., Wang, W.R., 2013. Effect of hydrogen peroxide on seedling growth and antioxidants in two wheat cultivars. Biol. Plant. 57, 487–494.

Mika, A., Boenisch, M.J., Hopff, D., Luthje, S., 2010. Membrane-bound guaiacol peroxidases from maize (*Zea mays* L.) roots are regulated by methyl jasmonate, salicylic acid, and pathogen elicitors. J. Exp. Bot. 61, 831–841.

Moller, I.M., Jensen, P.E., Hansson, A., 2007. Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. 58, 459–481.

Nourimand, M., Todd, C.D., 2017. Allantoin increases cadmium tolerance in Arabidopsis via activation of antioxidant mechanisms. Plant Cell Physiol. 57, 2485–2496.

Oracz, K., Karpinski, S., 2016. Phytohormones signaling pathways and ROS involvement in seed germination. Front. Plant Sci. 7, 864.

Pandey, S., Fartyal, D., Agarwal, A., Shukla, T., James, D., Kaul, T., Negi, Y.K., Arora, S., Reddy, M.K., 2017. Abiotic stress tolerance in plants: myriad roles of ascorbate peroxidase. Front. Plant Sci. 8, 581.

Piedras, P., Aguilar, M., Pineda, M., 1998. Uptake and metabolism of allantoin and allantoate by cells of *Chlamydomonas reinhardtii* (Chlorophyceae). Eur. J. Phycol. 33, 57–64.

Prieto, P., Pineda, M., Aguilar, M., 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of phosphomolybdenum complex. Specific application of the determination of vitamin E. Anal. Biochem. 269, 337–341.

Puntarulo, S., Sanchez, R.A., Boveris, A., 1988. Hydrogen-peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol. 86, 626–630.

Quan, L.J., Zhang, B., Shi, W.W., Li, H.Y., 2008. Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. J. Integr. Plant Biol. 50, 2–18.

Quiles, F.A., Raso, M.J., Pineda, M., Piedras, P., 2009. Ureide metabolism during seedling development in French bean (*Phaseolus vulgaris*). Physiol. Plantarum 135, 19–28.

Raso, M.J., Pineda, M., Piedras, P., 2007a. Tissue abundance and characterisation of two purified proteins with allantoinase activity from French bean (*Phaseolus vulgaris*). Physiol. Plantarum 131, 355–366.

Raso, M.J., Muñoz, A., Pineda, M., Piedras, P., 2007b. Biochemical characterisation of an allantoate-degrading enzyme from French bean (*Phaseolus vulgaris*): the requirement of phenylhydrazine. Planta 226, 1333–1342.

Saxena, I., Srikanth, S., Chen, Z., 2016. Cross talk between H_2O_2 and interacting signal molecules under plant stress response. Front. Plant Sci. 7, 570.

Shutov, A.D., Baumlein, H., Blattner, F.R., Muntz, K., 2003. Storage and mobilization as antagonistic functional constraints on seed storage globulin evolution. J. Exp. Bot. 54, 1645–1654.

Soltabayeva, A., Srivastava, S., Kurmanbayeva, A., Bekturova, A., Fluhr, R., Sagi, M., 2018. Early senescence in older leaves of low nitrate-grown Atxdh1 uncovers a role for purine catabolism in N supply. Plant Physiol. 178, 1027–1044.

Takagi, H., Ishiga, Y., Watenabe, S., Konishi, T., Egusa, M., Akiyoshi, N., Matsuura, T., Mori, I.C., Hirayama, T., Kaminaka, H., Shimada, H., Sakamoto, A., 2016. Allantoin, a stress-related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. J. Exp. Bot. 67, 2519–2532.

Tiedemann, J., Neubohn, B., Muntz, K., 2000. Different functions of vicilin and legumin are reflected in the histopattern of globulin mobilization during germination of vetch (*Vicia sativa* L.). Planta 211, 1–12.

Todd, C.D., Tipton, P.A., Blevins, D.G., Piedras, P., Pineda, M., Polacco, J.C., 2006. Update on ureide degradation in legumes. J. Exp. Bot. 57, 5–12.

Verma, G., Mishra, S.B., Sangwan, N., Sharma, S., 2015. Reactive oxygen species mediate axis cotyledon signaling to induce reserve mobilization during germination and seedling establishment in *Vigna radiata*. J. Plant Physiol. 184, 79–88.
Watanabe, S., Matsumoto, M., Hakomori, Y., Takagi, H., Shimada, H., Sakamoto, A., 2014. The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. Plant Cell Environ. 37, 1022–1036.
Werner, A.K., Sparkes, I.A., Romeis, T., Witte, C.P., 2008. Identification, biochemical characterization, and subcellular localization of allantoate amidohydrolases from Arabidopsis and soybean. Plant Physiol. 146, 418–430.
Wojtyla, L., Lechowska, K., Kubala, S., Garnczarska, M., 2016. Different modes of hydrogen peroxide action during seed germination. Front. Plant Sci. 7, 66.
Zrenner, R., Stitt, M., Sonnewald, U., Boldt, R., 2006. Pyrimidine and purine biosynthesis and degradation in plants. Annu. Rev. Plant Biol. 57, 805–836.