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ORIGINAL ARTICLE

Aluminum inhibits root growth and induces hydrogen peroxide accumulation in *Plantago algarbiensis* and *P. almogravensis* seedlings

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Abstract We have evaluated the impact of aluminum (Al) on germination, relative root growth, Al accumulation in roots tips, H_2O_2 levels, plasma membrane integrity, pigment levels, protein content, and the activities of superoxide dismutase (SOD) and catalase (CAT) in seedlings of the endangered Portuguese species *Plantago algarbiensis* and *Plantago almogravensis*. We found that up to 400 μM Al had no impact on the germination percentage in either species but inhibited root growth in a concentration-dependent manner (more severely in *P. algarbiensis*). Al accumulation in the root tips of both species was concentration dependent up to 200 μM but declined thereafter despite the absence of membrane damage. We observed a concentration-dependent induction of SOD activity but no change in CAT activity resulting in the accumulation of H_2O_2 (a known growth inhibitor), although its impact in *P. almogravensis* may be partially ameliorated by the accumulation of carotenoid pigments. Our data suggest an association between Al uptake, H_2O_2 production, and the inhibition of root growth during early seedling development in *P. algarbiensis* and *P. almogravensis*, although the latter is more tolerant towards higher concentrations of the metal.

Keywords Al uptake · Antioxidant enzymes · Hydrogen peroxide · Metal toxicity · Plasma membrane integrity · Root growth inhibition

Abbreviations

CAT	Catalase
FW	Fresh weight
MGT	Mean germination time
NBT	Nitroblue tetrazolium
ROS	Reactive oxygen species
SOD	Superoxide dismutase

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Introduction

Germination is a complex process involving the coordination of physiological, biochemical, and molecular mechanisms that are particularly susceptible to adverse environmental factors (Bewley 1997). Although the impact of metal toxicity on the growth of vegetative tissues such as shoots and roots has been well documented, there have been few studies focusing on metal toxicity and germination (Kranner and Colville 2011). Physiological metal tolerance strategies in plants depend on age and the nature of the metal ions, but metal-tolerant species are thought to deploy their adaptive mechanisms throughout development, particularly during germination when the young plant is most vulnerable (Lefèvre et al. 2009).

Aluminum (Al) is a major metal component in soils and is solubilized as phytotoxic ions (predominantly Al^{3+}) which inhibit plant growth under low-pH conditions (Tahara et al. 2008). Both germination and seedling growth can be inhibited if the medium surrounding the seed is contaminated with Al (Marciano et al. 2010; Gui et al. 2011). Germination and root growth tests have been used to evaluate the effect of Al in several plant species, particularly crops (Marciano et al. 2010; Zhang et al. 2010). Furthermore, Ezaki et al. (2007, 2008) used germination and relative root growth tests to measure Al tolerance in 49 wild species and in panels of *Arabidopsis thaliana* mutants.

Plantago almogravensis Franco is an endemic species that grows along the southwest coast of Portugal in acidic, podzolic soils naturally enriched in bioavailable Al and iron (Pimentel et al. 1996; Buurman and Jongmans 2005; Serrano

et al. 2011). This species is considered an Al hyperaccumulator plant based on the results of field testing (Branquinho et al. 2007; Serrano et al. 2011). *Plantago algarbiensis* Samp. is an endemic species from the West-Central Algarve region that occurs in clay-rich soils preferably downstream from small springs or clearings containing acidophilic brushes. Given that both species are currently endangered, micropropagation protocols were established for these species (Gonçalves et al. 2009) in order to provide enough plant material for experiments, allowing us to confirm their ability to grow normally in low-pH medium (Martins et al. 2011). Also, we observed that micropropagated shoots and plantlets of both species accumulate considerable amounts of Al and are moderately tolerant to this metal (Martins et al. 2013a, b, c). These two wild plants may conserve Al resistance genetic information, and therefore, will be useful sources to understand the Al resistance mechanisms and to discover and isolate tolerant genes that can be applied in breeding strategies for crops growing in acidic soils. In addition, due to the endangered nature of these species, the knowledge of their physiological and biochemical response to Al is important for the ecological understanding and for the definition of conservation strategies. The aim of this work was to evaluate the effect of Al on germination and seedling development in *P. algarbiensis* and *P. almogravensis* to gain insight into their Al toxicity and tolerance mechanisms during a stage when the young plants are most vulnerable. Although the requirements for germination in both species have been studied (Martins et al. 2012) the behavior of germinating seeds in the presence of Al has not been investigated.

Materials and methods

Plant material

P. algarbiensis and *P. almogravensis* seeds were collected in June 2010 from wild plants growing among natural populations in Algoz (Algarve Region, Portugal) and Vila Nova de Milfontes (Portugal), respectively. Several plants from both species were selected to ensure the seeds were genetically diverse. The seeds were stored in hermetic glass jars inside paper bags and were kept in the dark under laboratory conditions at 25±2 °C for 2 months.

Seed germination and seedling growth

Seeds were sown in 9-cm glass Petri dishes on 1 mM $CaCl_2$ solution (pH 4.5) solidified with 0.5 % (*w*/*v*) bactoagar (Difco, USA) supplemented with 0, 100, 200, or 400 μM $AlCl_3$, corresponding to 0, 78, 158, or 317 μM Al^{3+}, respectively, as estimated by Geochem-EZ (Shaff et al. 2010). Seeds were incubated under optimal germination conditions (15±2 °C, 16-h photoperiod, 69 μmol m^{-2} s^{-1}) as previously described (Martins et al. 2012). The seeds were monitored every 2 days over 30 days and germination was scored on the basis of visible radical protrusion.

For each treatment, we recorded the final germination percentage (in percent) and the mean germination time (MGT), which was determined using the formula $MGT=\Sigma DN / \Sigma N$ (Ellis and Roberts 1981), where *D* is the number of days from sowing and *N* is the number of seeds that have germinated by day *D*. Root length was measured for each treatment after 30 days of germination and relative root growth was estimated by calculating the root lengths of Al-treated seedlings as a percentage of the root length of untreated seedlings (Ezaki et al. 2007).

Aluminum uptake

The content was determined by hematoxylin staining (Polle et al. 1978). Roots were washed in distilled water for 30 min at room temperature, stained in 0.2 % (*w*/*v*) hematoxylin solution containing 0.02 % (*w*/*v*) KIO_3 for 30 min and washed again as above. Stained roots were photographed using a Moticam 350 (Motic, China) under a stereomicroscope (Olympus SZ40, Japan). For quantitation, 5-mm root tips were excised and soaked in 1 M HCl for 1 h to release the hematoxylin, and the concentration was determined by measuring absorbance at 490 nm.

H_2O_2 content

The H_2O_2 content of the seedlings was determined as described by Loreto and Velikova (2001). Fresh plant material (100 mg) was homogenized in 1 ml 0.1 % (*w*/*v*) trichloroacetic acid at 4 °C and centrifuged at 12,000×*g* for 15 min. We then mixed 0.2 ml of the supernatant with 0.2 ml 10 mM sodium phosphate buffer (pH 7.0) and 0.4 ml 1 M KI. The reaction was developed for 30 min in darkness and the H_2O_2 content was determined by measuring absorbance at 390 nm against a set of H_2O_2 standards, and subtracting a blank sample lacking the plant extract. The results were expressed as micromole per gram fresh weight (FW).

Plasma membrane integrity

Plasma membrane integrity was evaluated using Evans blue as described by Baker and Mock (1994). Intact roots were stained with 0.25 % (*w*/*v*) Evans blue solution for 10 min at room temperature, washed three times with distilled water for 10 min and photographed under a stereomicroscope as above. For quantitation, 5-mm root tips were excised and soaked in *N*,*N*-dimethylformamide for 1 h at room temperature to release the stain, and the concentration was determined by measuring absorbance at 600 nm.

Photosynthetic pigments

Photosynthetic pigments were extracted with 100 % acetone from fresh plant material (25 mg) and quantified by measuring the absorbance at 661.6, 644.8, and 470 nm (Lichtenthaler 1987).

SOD and CAT activities and soluble protein levels

Superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) activities were evaluated in extracts from seedlings after 30 days of germination. Fresh tissue (100 mg) was ground in a pre-chilled mortar containing 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid, 1 % (*w*/*v*) polyvinylpolypyrrolidone, and 2.5 mM dithiothreitol. The homogenate was centrifuged at 20,000×*g* for 10 min at 4 °C and the supernatant was used for subsequent enzyme assays. The specific activity for both enzymes was expressed as enzyme units per milligram of protein, based on the measurement of total soluble protein levels according to the method of Bradford (1976) using bovine serum albumin as standard.

SOD activity was measured by the reduction of nitroblue tetrazolium chloride (NBT) as described by Beauchamp and Fridovich (1971). The reaction mixture comprised 50 mM sodium phosphate buffer (pH 7.8), 10 mM methionine, 0.075 mM NBT, 0.2 mM riboflavin and 20 μl of the enzyme extract. The reaction was monitored by measuring absorbance at 560 nm for 6 min. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50 %.

CAT activity was measured by the degradation of H_2O_2 as described by Aebi (1983). The reaction mixture comprised of 50 mM sodium phosphate buffer (pH 7.0), 40 mM H_2O_2 and 20 μl of the enzyme extract. The reaction was monitored by measuring absorbance at 240 nm (ε=39.4 mM^{-1} cm^{-1}). One unit of CAT was defined as the amount of enzyme required to degrade 1 μmol H_2O_2 per min.

Data analysis

Final germination percentages, MGT, and relative root growth values were based on data from ten replicates of ten seeds or seedlings, whereas other values were determined from five replicates of plant material randomly collected from different Petri dishes. The values obtained were expressed as means±standard errors. The impact of different treatments was tested by one-way analyses of variance using the SPSS statistical package for Windows (release 19.0, SPSS Inc., California). We used SigmaPlot version 11.0 (Systat Software, San Jose, CA, USA) for data presentation. Significant differences between means were identified using Duncan's New Multiple Range Test or Dunnett's test. Final germination percentages were arcsine transformed prior to statistical analysis.

Results

Effect of Al on seed germination and seedling growth

To investigate the effect of Al on germination of *P. algarbiensis* and *P. almogravensis* seeds, the final germination percentages, and the mean germination time (MGT) were measured (Table 1). We observed that the final germination percentages and MGT values were not significantly affected ($p \geq 0.05$) by the Al loading in both species (Table 1). As shown in the Fig. 1a, there were no visible signs of toxicity such as chlorotic shoots and stubby, brittle, and brown roots in seedlings of both *Plantago* species exposed to different concentrations of Al even at the highest concentration. The quantitative impact of Al loading was investigated by measuring root growth in Al-treated seedlings relative to root growth in untreated controls. In *P. almogravensis* seedlings, there was no difference ($p \geq 0.05$) in relative root growth at 100 μM Al, a negligible ($p \geq 0.05$) reduction at 200 μM Al and even at 400 μM Al the relative root growth was still ~60 % (Fig. 2). In *P. algarbiensis*, there was a slight reduction in relative root growth at 100 μM Al, and the values fell ($p < 0.05$) to approximately 63 and 41 % at 200 and 400 μM Al, respectively.

Al accumulation in root tips

To monitor the accumulation and distribution of Al ions in the two species, we incubated seedling roots with the Al-specific dye hematoxylin. The intensity of hematoxylin staining in the root tips of both species increased in concert with Al loading (Fig. 1b) and the corresponding quantitative data showed that Al accumulates in the roots in a concentration-dependent manner in both species when exposed to media containing up to 200 μM Al (Table 2). However, in both species

Table 1 Effect of aluminum on the final germination percentage and mean germination time (MGT) of *P. algarbiensis* and *P. almogravensis* seeds

Al (μM)	Germination (%)	MGT (days)
P. algarbiensis		
0	98.00±1.33 a	5.35±0.20 a
100	100.00±0.00 a	5.26±0.24 a
200	96.00±2.21 a	5.53±0.20 a
400	99.00±1.00 a	5.50±0.50 a
P. almogravensis		
0	97.00±2.13 a	4.49±0.31 a
100	98.00±1.33 a	4.35±0.32 a
200	98.00±1.33 a	4.28±0.27 a
400	96.00±2.67 a	4.72±0.37 a

Values are expressed as the mean±SE (n=10). For each species, mean values followed by the same letter are not significantly different at $p \geq 0.05$, according to Duncan's test

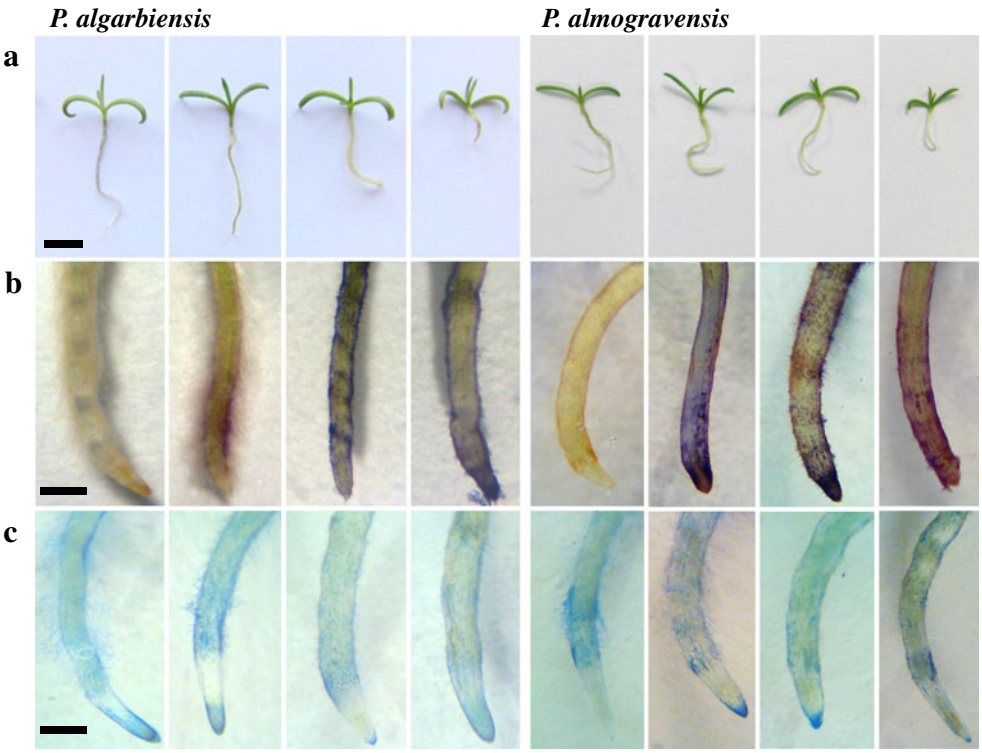

Fig. 1 **a** Effect of aluminum on *P. algarbiensis* and *P. almogravensis* seedling growth (*bar*=1 cm). **b** Aluminum accumulation in roots tips revealed by hematoxylin staining (*bar*=1 mm). **c** Evaluation of plasma membrane integrity in the roots tips using Evans blue (*bar*=1 mm). Panels from *left* to *right* show seedlings grown in medium containing 0, 100, 200, and 400 μM Al respectively, for 30 days. Images represent at least three seedlings or roots

there was a significant reduction in hematoxylin staining ($p<0.05$) between 200 and 400 μM Al (Table 2).

Effect of Al on the H_2O_2 content

We investigated the H_2O_2 content of Al-treated seedlings compared to untreated controls, and observed a significant increase ($p<0.05$) in both species at the highest Al concentration and also at 200 μM in *P. algarbiensis* (Fig. 3a).

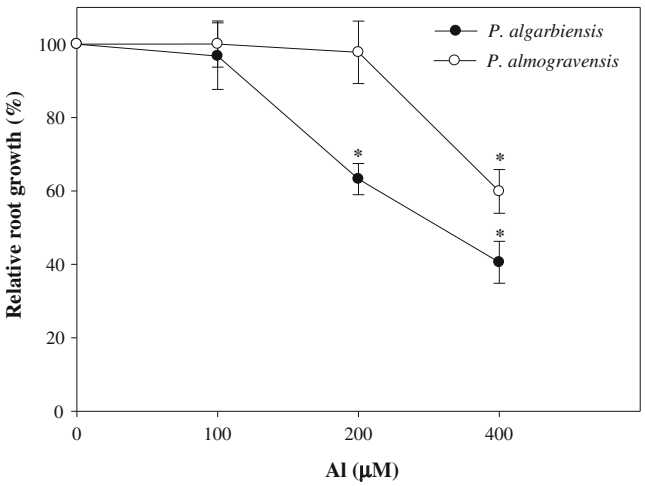

Fig. 2 Effect of aluminum on relative root growth in *P. algarbiensis* and *P. almogravensis* seedlings. Values are expressed as the mean±SE ($n=10$). *$p<0.05$, significant difference from control (Dunnett's test)

Effect of Al on the plasma membrane integrity, photosynthetic pigments, and soluble protein levels

To evaluate the oxidative damage caused by Al loading, the plasma membrane integrity, photosynthetic pigments, and soluble protein contents were measured. The integrity of the plasma membrane (measured as Evans blue dye uptake) of roots tips was maintained ($p\geq0.05$) in both species under Al treatment (Fig. 1c; Table 2). We observed no significant differences ($p\geq0.05$) in the total chlorophyll content when we compared Al-treated seedlings and controls, but a

Table 2 Quantitative determination of hematoxylin staining and the uptake of Evans blue in *P. algarbiensis* and *P. almogravensis* root tips in the presence of aluminum

Al (μM)	Hematoxylin (*A* 490 nm)	Evans blue (*A* 600 nm)
P. algarbiensis		
0	0.013±0.001 c	0.060±0.006 a
100	0.021±0.001 bc	0.061±0.005 a
200	0.045±0.005 a	0.059±0.011 a
400	0.026±0.003 b	0.064±0.008 a
P. almogravensis		
0	0.008±0.001 c	0.061±0.003 a
100	0.040±0.004 a	0.057±0.003 a
200	0.036±0.005 a	0.056±0.007 a
400	0.021±0.004 b	0.062±0.005 a

Values are expressed as the mean±SE ($n=5$). For each species, mean values followed by different letters are significantly different at $p<0.05$, according to Duncan's test

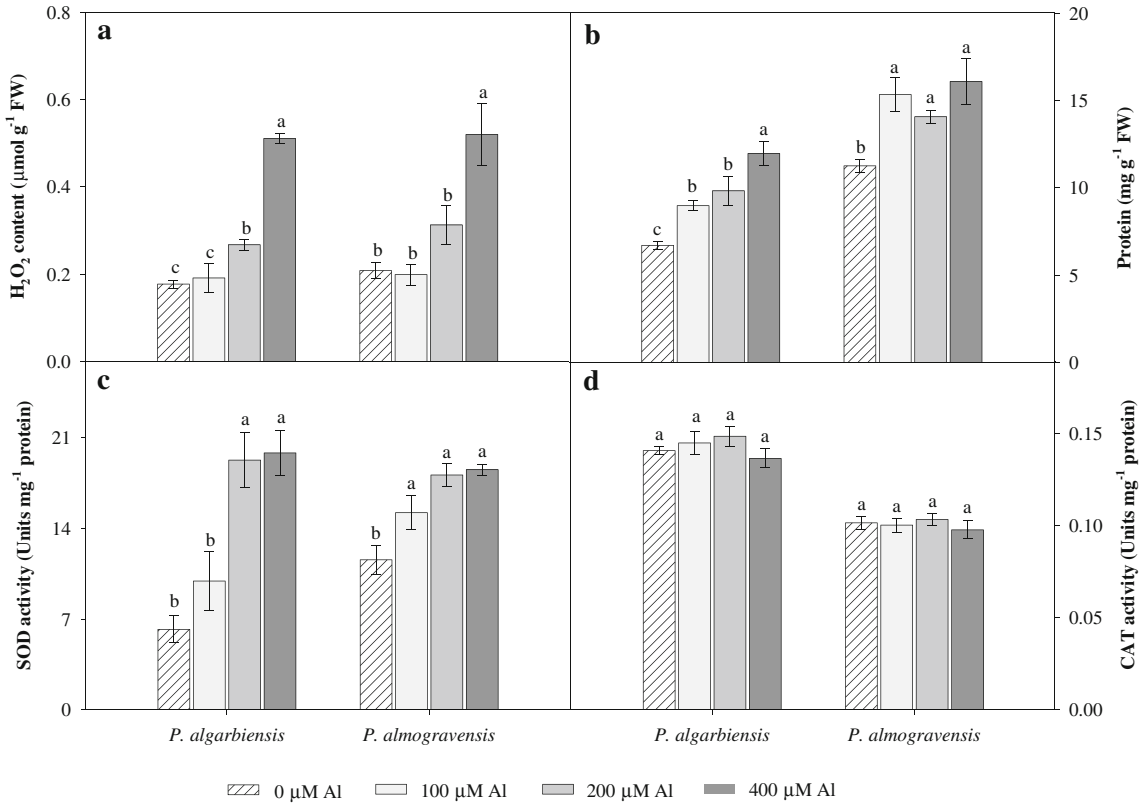

Fig. 3 Effect of aluminum on **a** H_2O_2 content, **b** protein content, **c** SOD activity, and **d** CAT activity in *P. algarbiensis* and *P. almogravensis* seedlings. Values are expressed as the mean±SE (n=5). For each species, mean values are significantly different at p<0.05, according to Duncan's test

significant (p<0.05) increase in the carotenoid content was observed in Al-treated *P. almogravensis* seedlings (Table 3). The protein content in seedlings of both species was higher (p<0.05) in response to Al loading (Fig. 3b).

Effect of Al on SOD and CAT activities

To investigate if the antioxidant system efficiently protects *P. algarbiensis* and *P. almogravensis* seedlings from Al-induced oxidative stress we measured the SOD and CAT activities (Fig. 3c, d). In *P. almogravensis* seedlings, there was a significant increase in SOD activity (p<0.05) at all three Al concentrations, whereas in *P. algarbiensis* the significant increase was observed at the two highest concentrations (Fig. 3c). Conversely, Al had no impact (p≥0.05) on CAT activity in either species (Fig. 3d).

Discussion

Germination assays are often used to evaluate the toxicity of exogenous compounds including metals such as Al (Labra et al. 2006; Ezaki et al. 2008). Therefore, we investigated the effect of Al on germination of *P. algarbiensis* and *P. almogravensis* seeds. We found no significant differences in either species when we compared the final germination percentages and MGT values between the Al-treated seeds and controls (Table 1). This reflects the behavior of other Al-tolerant plants, which have also been shown to germinate

Table 3 Effect of aluminum on the pigment content of *P. algarbiensis* and *P. almogravensis* seedlings

Al (μM)	Total chlorophyll (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)
P. algarbiensis		
0	0.51±0.04 a	0.18±0.01 a
100	0.50±0.02 a	0.15±0.01 a
200	0.54±0.04 a	0.18±0.01 a
400	0.52±0.03 a	0.17±0.00 a
P. almogravensis		
0	0.58±0.02 a	0.17±0.00 b
100	0.57±0.03 a	0.20±0.00 a
200	0.56±0.03 a	0.21±0.01 a
400	0.58±0.01 a	0.21±0.01 a

Values are expressed as the mean±SE (n=5). For each species, mean values followed by different letters are significantly different at p<0.05, according to Duncan's test

successfully despite high concentrations of Al ions in the environment (Ezaki et al. 2008).

Seedling growth is more sensitive to metal toxicity than germination (Kranner and Colville 2011) and seedling root growth is the most relevant indicator because roots are generally the first point of contact between plants and toxic metals in the environment (Lamhamdi et al. 2011). In this study, there were no overt signs of toxicity in seedlings of both *Plantago* species exposed to Al (Fig. 1a). However, Al loading inhibited the relative root growth of both species in a concentration-dependent manner, but more severely in *P. algarbiensis* (Fig. 2). In a previous work, we observed that Al at 400 μM induced root growth inhibition in *P. algarbiensis* in vitro-grown plantlets but did not affect root growth in *P. almogravensis* (Martins et al. 2013c).

The extent of Al accumulation in root cells can be easily evaluated through hematoxylin staining that has been widely used to study Al tolerance in several plant species (Polle et al. 1978; Marciano et al. 2010). Our results showed that Al accumulates in the root tips of both species in a concentration-dependent manner up to 200 μM Al (Fig. 1b; Table 2). This suggests that both *Plantago* species lack effective aluminum exclusion mechanisms and corroborates the Al accumulation capacity already observed in these species (Branquinho et al. 2007; Serrano et al. 2011; Martins et al. 2013a, b, c). However, in both species there was a reduction in hematoxylin staining between 200 and 400 μM Al (Table 2), which could reflect either the induction of Al exclusion mechanisms under high-loading conditions or the consequences of root apex damage caused by metal toxicity.

Excess Al is thought to cause oxidative damage to cells by triggering the production of reactive oxygen species (ROS) that interact with nucleic acids in the embryo as well as stored proteins and lipids in the seed (Pereira et al. 2010). These processes can interfere with germination and seedling development, and also generate potentially harmful mutations (Kranner and Colville 2011). Oxidative damage triggered by Al is primarily thought to result from the production of H_2O_2 (Yamamoto et al. 2003). The increased H_2O_2 content observed in seedlings of *P. algarbiensis* and *P. almogravensis* grown at the highest Al concentrations (Fig. 3a) seems to be associated with the inhibition of relative root growth (Fig. 2). We also observed Al-induced oxidative damage in *P. algarbiensis* roots in later stages of development (Martins et al. 2013d). Our results are consistent with previous studies where the H_2O_2 accumulation increase in plants undergoing Al toxicity (Pereira et al. 2010; Xu et al. 2011) and the resulting oxidative stress may be responsible for the inhibition of root growth (Zhang et al. 2010; Xu et al. 2011).

Membrane lipids and proteins are especially vulnerable to ROS, therefore damaged membranes are considered to be reliable indicators of cell death caused by oxidative stress in plants (Halliwell and Gutteridge 1993). We investigated the potential link between Al loading, the inhibition of root growth, ROS production, and cell death by measuring plasma membrane integrity. The azo dye Evans blue is not taken up by living cells because it cannot cross functional lipid membranes, but it can leak through ruptured membranes and is therefore a sensitive indicator of membrane integrity and cell death (Xu et al. 2011; Zelinová et al. 2011). No differences in dye uptake were observed between Al-treated roots and controls suggesting that the plasma membrane was not damaged to a significant extent by the increased production of ROS in either species (Fig. 1c; Table 2). These findings suggest that ROS production does not inhibit root growth by damaging membranes, although it is possible that other targets including nucleic acids and cytosolic proteins may be involved (Gill and Tuteja 2010).

Although several studies have shown that photosynthetic pigment levels decline in plants exposed to Al (Yadav and Mohanpuria 2009; Pereira et al. 2010), our results showed no significant differences in the total chlorophyll content when we compared Al-treated plants and controls of either species (Table 3) suggesting that Al does not interfere with early photosynthetic activity. In contrast, the carotenoid contents were higher in Al-treated *P. almogravensis* seedlings (Table 3) indicating that the production of antioxidant pigments may be induced to prevent oxidative damage (Larson 1988).

The soluble protein content of plants is an important indicator of metabolic changes which responds to a wide variety of stresses (Singh and Tewari 2003) and predicts the physiological status of seedlings (Nataraj and Parmar 2008). It is likely that the increased protein content observed in the seedlings of both species in response to Al (Fig. 3b) reflects the de novo synthesis of stress-induced proteins that mediate the adaptive physiological mechanisms of metal tolerance (Verma and Dubey 2003). Al may also be detoxified through the formation of complexes with cytosolic Al-binding proteins (Basu et al. 1999).

The oxidative damage caused by ROS can be prevented by the induction of both enzymatic and non-enzymatic antioxidant systems in plants (Apel and Hirt 2004). These systems are thought to play a substantial role during the germination and development of seedlings exposed to metals (Kranner and Colville 2011), although the precise roles of antioxidant enzymes in Al tolerance have not been investigated in detail. We therefore studied the activities of two pivotal antioxidant enzymes, namely superoxide dismutase (SOD) and catalase (CAT). SOD represents the first step in the detoxifying process, catalyzing the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2, whereas CAT converts H_2O_2 into harmless oxygen and water. The complementary activities of SOD and CAT are therefore necessary to mitigate oxidative stress caused by superoxides (Benavides et al. 2005). In our study, the increased H_2O_2 generation observed

in *P. algarbiensis* and *P. almogravensis* seedlings exposed to Al (Fig. 3a) reflects a parallel increase in SOD activity (Fig. 3c) but the maintenance of CAT activity at normal levels (Fig. 3d).

Our results indicate that both species may be able to tolerate the presence of Al and germinate in the acidic Al-rich soils colonized by them. However, the Al induced H_2O_2 accumulation and root growth inhibition observed in both species during the initial seedling development, indicate that high Al amounts (≥ 400 μM) may compromise plants establishment in their natural habitats. Moreover, *P. almogravensis* demonstrated to be more tolerant in this early stage than *P. algarbiensis*, which is in agreement with our previous results (Martins et al. 2013c, d) and its predominance in geochemical islands rich in Al and their inexistence outside these areas with the surrounding vegetation, reflecting an obligate metallophyte behavior (Serrano et al. 2011). The concentration-dependent inhibition of root growth we observed was associated with a quantitative increase in H_2O_2 levels resulting from the induction of SOD with no compensatory increase in the level of CAT activity. However, the higher levels of H_2O_2 were not sufficient to induce membrane damage suggesting that the inhibition of root growth reflects the impact of oxidative stress on intracellular targets, such as cytosolic proteins and nucleic acids, although this issue should be clarified in future works.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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