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Metabolism and aluminum accumulation in *Plantago almogravensis* and *P. algarbiensis* in response to low pH and aluminum stress

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Abstract

We investigated the impact of low pH and aluminum on the metabolism and capacity for Al accumulation in shoots of the plantain species *Plantago algarbiensis* and *P. almogravensis*. We found that increasing the concentration of Al in the medium increased accumulation of it in the shoots of both plants (although more in *P. almogravensis* than in *P. algarbiensis*). The presence of Al in the medium induced proline and saccharide synthesis in *P. almogravensis* without affecting lipid peroxidation, but increased proline synthesis and lipid peroxidation in *P. algarbiensis* without affecting the saccharide content. Lipid peroxidation in *P. algarbiensis* was also enhanced at pH 4.0. The activity of antioxidant enzymes was increased as a response to low pH and Al in both species. Our data indicate that both species can accumulate high levels of Al but they have different sensitivities to low pH and/or the presence of Al in the growth medium.

Additional key words: antioxidant enzymes, lipid peroxidation, proline, saccharides, tolerance.

Introduction

Aluminum (Al) is one of the most abundant elements in the earth's crust and at pH values below 5.5 becomes soluble in a phytotoxic form (predominantly Al^{3+}) that inhibits plant growth (Kochian *et al*. 2004, Xu *et al*. 2010). Anthropogenic activity is leading to the progressive acidification of environments, and acidic soils that currently account for ~30 % of the global ice-free land area are gradually expanding (Von Uexküll and Mutert 1995). Low pH stress (proton toxicity) and Al toxicity are therefore already becoming important constraints to crop productivity in several countries.

Some plant species have evolved strategies to reduce the impact of Al toxicity and can therefore grow well in acidic Al-rich soils and accumulate high content of Al in their shoots (Kochian *et al*. 2004). Plants may be classified as Al hyperaccumulators if at least 1 mg(Al) g^{-1}(d.m.) can be stored in the shoots (Foy 1984, Baker and Brooks 1989). Wild plants are often more tolerant than crops towards environmental stresses (Mittova *et al*. 2004) and this includes low pH stress and excess Al (Ezaki *et al*. 2008, Olivares *et al*. 2009). Therefore, wild plants may be useful sources of Al/low pH tolerance genes (Ezaki *et al*. 2008).

In this context, we investigated the ability of two *Plantago* species (plantains) to grow under low pH and presence of Al as a first step towards determining the mechanisms of stress tolerance. *Plantago almogravensis* is an endemic plantain species that grows along the southwest coast of Portugal. It grows in podzolic soils with high content of iron and Al, and is considered as Al hyperaccumulator (Buurman and Jongmans 2002, Branquinho *et al*. 2007). *Plantago algarbiensis* is an endemic plantain species from the West-Central Algarve region. It grows in clay-rich soils preferably downstream

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Abbreviations: APX - ascorbate peroxidase, CAT - catalase, d.m. - dry mass, f.m. - fresh mass, GPX - guaiacol peroxidase, MDA - malondialdehyde, MS - Murashige and Skoog medium, NBT - nitroblue tetrazolium, SOD - superoxide dismutase, TBA - thiobarbituric acid, TCA - trichloroacetic acid.

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from small springs or clearings containing acidophilic brushes, and its response to Al is unknown. Since both species belongs to endangered ones, we established *in vitro* cultures (Gonçalves *et al*. 2009) and have recently demonstrated that they can grow in medium with low pH values (Martins *et al*. 2011). *In vitro* cultures have previously been used to select Al-tolerant species and to understand the tolerance mechanisms (Barnabas *et al*. 2000, Wen *et al*. 2009, Tabaldi *et al.* 2011). We therefore set out to evaluate the capacity of each species to accumulate Al, and the effects of low pH and Al^{3+} on *in vitro* shoots in terms of: *1*) the degree of membrane damage through lipid peroxidation, *2*) proline and saccharide accumulations, and *3*) the activity of antioxidant enzymes.

Materials and methods

Plantago algarbiensis Samp. shoots (~6 cm in length) and *Plantago almogravensis* Franco shoots (~3 cm in length) were separated from *in vitro* cultures proliferating in Murashige and Skoog (1962, MS) medium containing 0.5 mg dm^{-3} zeatin (*P. algarbiensis*) or 0.2 mg dm^{-3} 6-benzyladenine (*P. almogravensis*) as described by Gonçalves *et al*. (2009). The cultures were maintained at 25 ± 2 ºC and a 16-h photoperiod (cool white fluorescent lamps, irradiance of 69 μmol m^{-2} s^{-1}). Low pH stress was applied by transferring shoots to ¼ MS liquid medium with the pH adjusted to 4.00 (stress) or 5.75 (control). Al stress was applied by transferring shoots to ¼ MS liquid medium containing 100, 200, or 400 μM $AlCl_3$ which dissociates to form Al^{3+} ions when the pH is adjusted to 4.00. Shoots were inoculated individually in test tubes (32 × 200 mm) containing 20 cm^3 of liquid medium on filter paper bridges and incubated under the conditions described above.

Shoots were cultured for 7 d under stress or control conditions and then oven dried at 65 ºC until they reached a constant dry mass (d.m.). The dried material was ground, burned to ash in a muffle furnace at 500 ºC, and acid digested. The Al content was then determined according to the aluminon method described by Kerven *et al*. (1989) using a commercial kit and a *DR 2800* spectrophotometer (both provided by *Hach LANGE*, Düsseldorf, Germany). The accuracy of the results was verified against reference beech leaves *BCR-100*.

The lipid peroxidation was measured by determining the amount of malondialdehyde (MDA) available to react with 2-thiobarbituric acid (TBA) (Hodges *et al*. 1999). Fresh plant material was homogenized with 0.1 % (m/v) trichloroacetic acid (TCA) and centrifuged at 10 000 *g* for 5 min. The supernatant was added to either 20 % (m/v) TCA (-TBA solution) or 0.5 % (m/v) TBA in 20 % (m/v) TCA (+TBA solution) and incubated at 95 ºC for 30 min. The reaction was quenched on ice and the samples were centrifuged at 3 000 *g* for 10 min. The absorbance of the supernatant was measured at 532, 600, and 440 nm and MDA equivalents were calculated as described by Hodges *et al*. (1999).

Harvested shoots were exhaustively extracted with 80 % (v/v) ethanol. The content of free proline was determined using the ninhydrin method (Troll and Lindsley 1955) as modified by Magné and Larher (1992). The extract was added to 1 % ninhydrin (m/v, in 60 % acetic acid) and the mixture was boiled for 1 h. The resulting chromogen was extracted with toluene and the free proline was quantified at 520 nm against a set of proline standards (0 - 750 μM). The saccharide content was determined using the anthrone method (Yemm and Willis 1954). The sample was mixed with 75 % (v/v) sulfuric acid containing 0.01 M anthrone reagent and was boiled until the reaction was complete. The resulting 5-(hydroxymethyl)furfural/anthrone complex was quantified at 578 nm against a set of glucose standards (0 to 500 μM) and the results were expressed as glucose equivalents.

Superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (GPX; EC 1.11.1.7), and ascorbate peroxidase (APX; EC 1.11.1.11) activities were evaluated after shoots were cultured for 1, 2, and 7 d. Fresh shoot tissue (100 mg) was homogenized with a pre-chilled mortar and pestle in 50 mM sodium phosphate buffer (pH 7) supplemented with 0.1 mM ethylenediaminetetraacetic acid, 1 % (m/v) polyvinylpolypyrrolidone and 2.5 mM dithiothreitol (and 5 mM ascorbate in the case of APX). The homogenate was centrifuged at 20 000 *g* at 4 ºC for 10 min and the supernatant was used for subsequent enzymes assays. The specific enzyme activity for all enzymes was expressed as enzyme units per mg of protein based on the measurement of total soluble protein content according to the method of Bradford (1976) using bovine serum albumin as a standard.

SOD activity was determined by the reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The photo-reduction of NBT was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50 %. CAT activity was determined by the degradation of H_2O_2 using the method of Aebi (1983). The H_2O_2 decomposition was monitored 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. GPX activity was measured by the modification of guaiacol as described by Egley *et al*. (1983). The tetraguaiacol formation was measured at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). One unit of GPX was defined as the amount of enzyme required to produce 1 μmol of

tetraguaiacol per min. APX activity was determined by the oxidation of ascorbate as described by Nakano and Asada (1981). The H_2O_2-dependent oxidation of ascorbate was monitored at 290 nm ($\varepsilon = 2.8$ mM^{-1} cm^{-1}). One unit of APX was defined as the amount of enzyme required to degrade 1 μmol of ascorbate per min.

Five shoots were used in all the experiments and the values obtained were expressed as means ± standard errors. We carried out a one-way analysis of variance (*ANOVA*) to assess treatment differences using the *SPSS* statistical package for Windows (*v. 15.0*; *SPSS Inc.*, Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test.

Results

Neither species accumulated detectable levels of Al in the shoots when cultivated on control MS medium without Al. However, both species accumulated large amounts of Al [1.0 - 4.5 mg g^{-1}(d.m.)] when cultivated in MS medium supplemented with Al and there was a positive correlation between the accumulation of Al *in planta* and the concentration of Al^{3+} in the medium (Fig. 1). *P. almogravensis* shoots showed a tendency to accumulate more Al than *P. algarbiensis* shoots, although this only became statistically significant ($P < 0.05$) in the medium containing 200 μM Al. The biomass increment after 7 d of culture in all the tested media was negligible in both *Plantago* species and, thus, it was not possible to evaluate the effect of low pH and Al on shoot growth.

The lipid peroxidation (expressed as the content of MDA) in *P. algarbiensis* shoots was higher ($P < 0.05$) in the low pH medium with or without Al than in the control medium at pH 5.75 (Table 1). The MDA content was also affected by Al. Shoots cultivated in medium containing 100 μM Al showed lower lipid peroxidation than those cultivated in the absence of Al, but more severe lipid peroxidation occurred when the amount of Al in the medium further increased. In contrast, neither low pH stress nor the presence of Al had a significant ($P \geq 0.05$) impact on lipid peroxidation in *P. almogravensis* (Table 1).

In comparison to shoots cultured in the low pH medium lacking Al, there was a significant increase ($P < 0.05$) in the proline content of *P. algarbiensis* shoots cultured in medium containing 100 and 400 μM Al but no significant change ($P \geq 0.05$) in saccharide content regardless the Al concentration (Table 1). In contrast, *P. almogravensis* shoots cultured in medium containing 200 and 400 μM Al contained higher content ($P < 0.05$) of both proline and saccharides compared to shoots

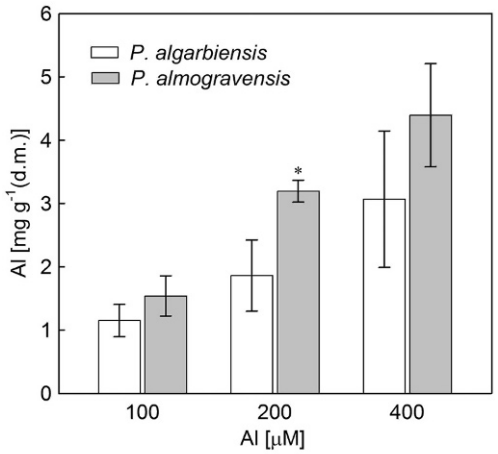

Fig. 1. Al accumulation in *P. algarbiensis* and *P. almogravensis* shoots after 7 d of culture in medium containing different concentrations of $AlCl_3$ (100, 200, and 400 μM). Means ± SE ($n = 5$). * indicates a significant difference between species ($P < 0.05$).

Table 1. Effect of low pH and Al on the content of MDA, proline, and saccharides in *P. algarbiensis* and *P. almogravensis* shoots after 7 d in culture. Values are expressed as the mean ± SE ($n = 5$). Mean values with common letters are not significantly different at $P \geq 0.05$, according to Duncan's test. Saccharide content is expressed as μmol of glucose equivalents.

Species	pH	Al [μM]	MDA [nmol g^{-1}(f.m.)]	Proline [μmol g^{-1}(f.m.)]	Saccharides [μmol g^{-1}(f.m.)]
P. algarbiensis	5.75	0	22.06 ± 2.72c	0.43 ± 0.03ab	107.18 ± 20.83a
	4.00	0	76.48 ± 4.36a	0.31 ± 0.04b	91.29 ± 9.76a
	4.00	100	55.13 ± 7.27b	0.58 ± 0.07a	117.13 ± 14.60a
	4.00	200	73.06 ± 7.25ab	0.46 ± 0.03ab	114.75 ± 7.99a
	4.00	400	82.55 ± 6.22a	0.48 ± 0.06a	113.33 ± 10.02a
P. almogravensis	5.75	0	48.50 ± 3.25a	0.38 ± 0.09b	39.33 ± 6.36b
	4.00	0	42.65 ± 7.05a	0.51 ± 0.06b	52.68 ± 1.98b
	4.00	100	42.60 ± 6.55a	0.44 ± 0.06b	48.75 ± 4.12b
	4.00	200	54.76 ± 2.76a	0.80 ± 0.08a	80.47 ± 6.53a
	4.00	400	53.13 ± 3.63a	0.87 ± 0.10a	79.51 ± 1.78a

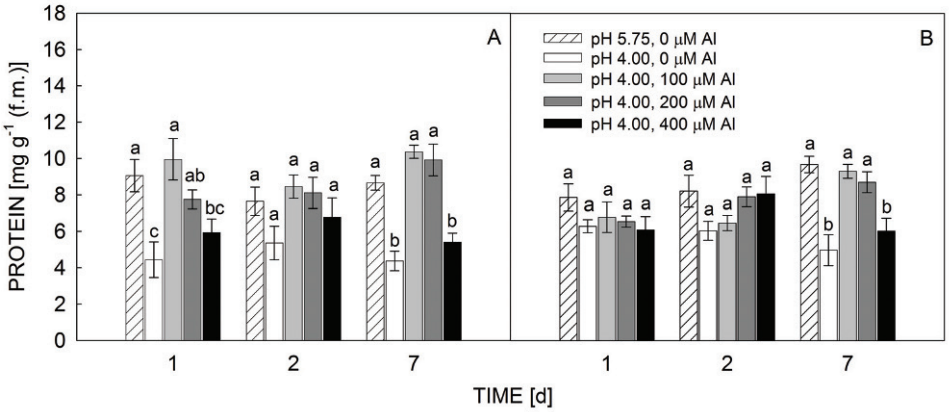

Fig. 2. Effect of low pH and Al on the protein content of *P. algarbiensis* (*A*) and *P. almogravensis* (*B*) shoots after 1, 2, and 7 d of culture. Means ± SE ($n = 5$). Mean values followed by the same letter are not significantly different at $P \geq 0.05$.

cultivated in the absence of Al (Table 1).

We observed a significant reduction ($P < 0.05$) in the protein content of *P. algarbiensis* shoots grown for 1 and 7 d in low pH medium either without Al or containing the highest test concentration, 400 μM Al (Fig. 2*A*). The same pattern was observed after 7 d in *P. almogravensis* shoots (Fig. 2*B*).

We also observed significant changes in the activities of antioxidant enzymes in both species in response to low pH and Al stress (Fig. 3). After cultivation for 1 or 2 d in the low pH medium with or without Al, *P. algarbiensis* shoots generally showed a three-fold increase in SOD activity compared to shoots growing at pH 5.75 (Fig. 3*A*), although after 2 d in medium containing 400 μM Al the SOD activity was no higher ($P \geq 0.05$) than in the control. There was also a significant increase ($P < 0.05$) in SOD activity in *P. almogravensis* shoots after cultivation for 1 d in low-pH medium without Al or containing 200 μM Al (Fig. 3*B*). SOD activity also increased ($P < 0.05$) after cultivation for 7 d in low-pH medium lacking Al.

We observed a two-fold increase in CAT activity when *P. algarbiensis* shoots were cultivated for 2 d in the low pH medium with or without Al (Fig. 3*C*). Similar results were observed for *P. almogravensis* shoots, although in this species the CAT activity did not increase in shoots cultivated in the low pH medium supplemented with 400 μM Al until 7 d of cultivation had passed (Fig. 3*D*).

We also observed a two-fold increase in APX activity in *P. algarbiensis* shoots cultivated for 2 d in low pH media containing 100 and 200 μM Al, and after 7 d in all the low pH media regardless of Al content (Fig. 3*E*). In *P. almogravensis*, a significant ($P < 0.05$) increase in APX activity occurred only after 7 d and only in the low pH media with no Al and 100 μM Al (Fig. 3*F*).

GPX activity in *P. algarbiensis* shoots remained low for the first few days of cultivation but showed a significant ($P < 0.05$) increase after 7 d in the low-pH medium without Al or in the presence of the highest Al concentration (Fig. 3*G*). In *P. almogravensis* shoots, there was a significant increase ($P < 0.05$) in GPX activity after 7 d of cultivation in medium containing the lowest Al concentration (Fig. 3*H*).

Discussion

We studied the low pH and Al stress responses in two protected wild plantain species, *P. algarbiensis* and *P. almogravensis* using *in vitro* shoot cultures. We found that both species were able to accumulate large amounts of Al without visible toxicity symptoms in agreement with previous observations of Al accumulation in field-grown *P. almogravensis* plants (Branquinho *et al*. 2007).

One of the main symptoms of Al toxicity in plants is lipid peroxidation. The damaged membranes release MDA which is therefore considered a good stress indicator (Liu *et al*. 2008, Xu *et al*. 2012). We observed a striking difference in the content of MDA induced by low pH and Al stress when the two *Plantago* species were compared (Table 1). There was no significant change in MDA content when *P. almogravensis* shoots were exposed to low pH with or without Al suggesting this species was protected from oxidative stress and the resulting membrane damage. In contrast, there was a significant increase in MDA content when *P. algarbiensis* shoots were exposed to low pH with or without Al suggesting this species is susceptible to this stress. In a previous work, we also observed an increase in the content of lipid peroxidation in shoots of *P. algarbiensis* grown for 6 weeks in medium with low pH (Martins *et al.* 2011). There was less membrane damage in *P. algarbiensis* at the lowest Al concentration

that has also been reported in other species (Andersson and Brunet 1993, Kinraide 1993). The protective mechanism is unclear, but the trivalent Al^{3+} ion may stabilize the membrane by reducing the surface negative charge reducing the interaction with protons and therefore reducing exosmosis (Kinraide 1993, Liu *et al*. 2003).

Saccharides and proline are scavengers of reactive oxygen species helping to prevent the oxidative damage that results from many forms of abiotic stress (Smirnoff 1998). In accordance with our previous results (Martins *et al.* 2011) we observed that the proline and saccharide content was not affected by the reduction of medium pH

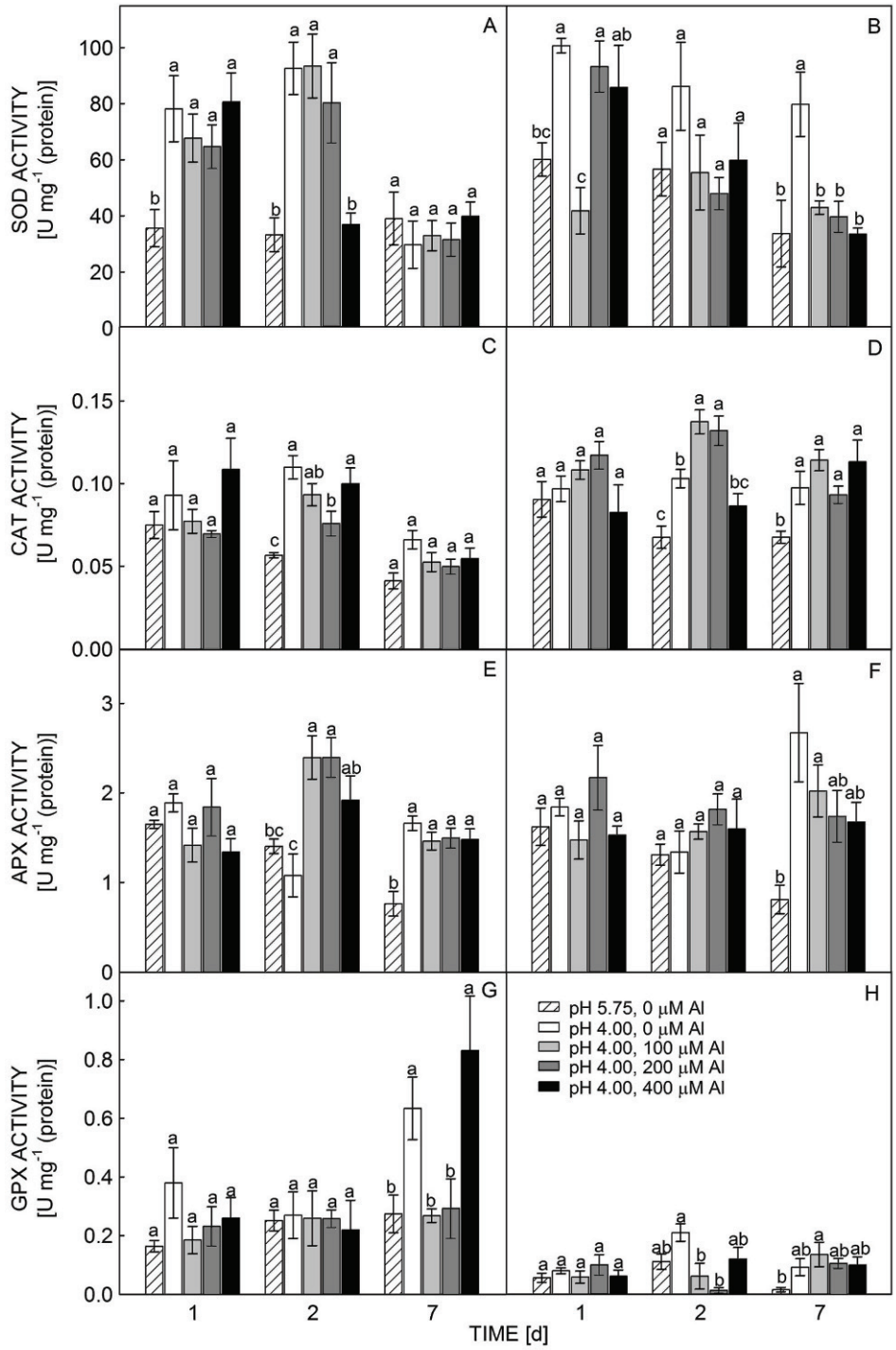

Fig. 3. Effect of low pH and Al on the SOD (*A*, *B*), CAT (*C*, *D*), APX (*E*, *F*), and GPX (*G*, *H*) activities of *P. algarbiensis* (*left*) and *P. almogravensis* (*right*) shoots, after 1, 2, and 7 d of culture. Means ± SE (n = 5). Mean values followed by the same letter are not significantly different at $P \geq 0.05$.

in both *Plantago* species. Nevertheless, the proline content of *P. algarbiensis* shoots increased in the low pH medium containing 100 and 400 µM Al compared to the same without Al, whereas the proline content of *P. almogravensis* shoots increased when the shoots were cultured in media with the highest Al concentrations. These results corroborated the role of proline on the protection of plants against Al stress (Khan *et al*. 2000, Yadav and Mohanpuria 2009, Giannakoula *et al*. 2010). The saccharide content of *P. almogravensis* shoots exposed to the two highest Al concentrations was higher than that of shoots cultivated in the low pH medium without Al (Table 1). The modulation of saccharide metabolism in response to Al stress has been reported previously (Khan *et al*. 2000, Giannakoula *et al*. 2010).

Al toxicity can also induce changes in protein synthesis (Delhaize and Ryan 1995) and protein degradation by proteolytic enzymes (Basu *et al*. 1994). We observed a significant reduction in total protein content in the shoots of both species in consequence of low pH with or without Al (Fig. 2).

The oxidative stress caused by Al ions induces a well characterized response pathway that includes the production of antioxidant enzymes (Shamsi *et al.* 2008, Xu *et al.* 2012). However, little is known about the induction of antioxidants in response to low pH. Our data suggest that SOD, CAT, APX, and GPX are induced by both Al and low pH. The most significant induction of all four enzymes occurs when shoots growing in the low pH medium are compared to those growing at pH 5.75. Again, it appears that the presence of both Al^{3+} and H^{+} reduces the overall stress suggesting that each ion alleviates the toxicity of the other to a certain extent.

We conclude that both *Plantago* species can accumulate high content of Al, but that there are differences in terms of sensitivity to low pH and/or Al and in the specific responses. In *P. algarbiensis*, the high content of proline and antioxidant enzyme activity induced by stress were not sufficient to suppress oxidative membrane damage whereas no damage was detected in *P. almogravensis* suggesting this is the more stress-tolerant of the two species.

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