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ORIGINAL PAPER

The influence of low pH on in vitro growth and biochemical parameters of *Plantago almogravensis* and *P. algarbiensis*

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Abstract The effects of low medium pH (4.50, 5.00 and 5.75) on in vitro growth and on several biochemical parameters (lipid peroxidation, proline and carbohydrate content, antioxidant enzymes activities and total soluble protein) of *Plantago almogravensis* and *P. algarbiensis* micropropagated shoots were investigated. Overall, it was observed that medium pH did not affect in vitro proliferation and rooting. Interestingly, cultures of both species modify the initial pH value to the same final value. Results have shown that the lowest pH tested induced an increase in the level of lipid peroxidation in roots of both species and in shoots of *P. algarbiensis*, indicating plasma membrane damage. An accumulation of carbohydrates was observed in roots of *P. almogravensis* cultured in pH 4.50 and 5.00. It was observed a slight response of the enzymatic system to medium pH, particularly in *P. almogravensis*. Based on the results obtained we can conclude that *Plantago* species are apt to grow in vitro in medium with pH values much lower than the usually used in tissue culture, which is in agreement with the fact that both species colonize acid soils.

Keywords Antioxidant enzymes · Carbohydrates · Endemic species · In vitro propagation · Lipid peroxidation · Proline

Abbreviation

Al	Aluminum
APX	Ascorbate peroxidase
BA	6-benzyladenine
CAT	Catalase
FW	Fresh weight
GPX	Guaiacol peroxidase
IAA	Indole-3-acetic acid
MDA	Malondialdehyde
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
Pro	Proline
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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Introduction

Low pH (high H^+ activity) may directly inhibit plant growth and development (Kidd and Proctor 2001; Pavlovkin et al. 2009), probably by adverse effects at the root plasmalemma level. Additionally, low pH can affect plant growth indirectly by elevated levels of Aluminum (Al) and Manganese, as well as by limited mineral nutrient uptake and translocation. Among these complex factors, the combined Al^{3+} and H^+ stresses are the major causes of poor plant growth. However, there is growing evidence suggesting that these two stresses differ in their inhibition of plant growth (Bose et al. 2010) and that resistance to Al and to protons is controlled by separate mechanisms (Lazof and Holland 1999; Kidd and Proctor 2001). Hence, it is necessary to evaluate the effects of low pH separately from the combination of low pH and Al to better understand H^+ toxicity.

Acid soils are found throughout the world and the atmospheric inputs of natural nitric and sulphuric acids, anthropogenic pollutants, and certain fertilization practices are

increasing the area affected by acidity (Marschner 1995). Species differ in their physiological tolerance to acidity and examples of tolerance to low pH in crops are numerous (Llugany et al. 1995; Osaki et al. 1997; Fageria et al. 2009). However, reduced information is known regarding H^+ tolerance in wild species. *Plantago almogravensis* Franco and *Plantago algarbiensis* Samp. are endemic species from the Portuguese Southwest coast and the Western-centre of the Algarve region (Portugal), respectively, that are in risk of global extinction. *Plantago almogravensis* colonizes a sandy eroded podzol-like soil, enriched in iron and Al (Buurman and Jongmans 2002; Branquinho et al. 2007), and *P. algarbiensis* occurs in clay-rich soils and prefers areas that are located downstream from small spring or clearings of low acidophilic brushes (ICN 2007). Branquinho et al. (2007) showed with field plants that *P. almogravensis* is an Al hyperaccumulator species, however its tolerance to Al and H^+ stress remains unclear.

The micropropagation protocol for both *Plantago* species utilizing the medium pH usually used in in vitro culture (pH = 5.75) was recently published by our group (Gonçalves et al. 2009). Most factors affecting in vitro growth are similar to those limiting the growth in vivo (Hew and Yong 1997), thus the effect of low medium pH on in vitro growth of both *Plantago* species will be useful to understand their tolerance to low pH, without the interference of indirect factors. Cell and/or tissue culture have been extensively used to evaluate the abiotic stress tolerance of many species, since responses are relatively fast, the generation times are short, and the environment is controlled (Cui et al. 2010; Lokhande et al. 2010; Xu et al. 2011).

Besides affecting growth, environmental adverse conditions can induce physiological and biochemical effects on plants (Çiçek and Çakirlar 2008). Therefore, the aim of this work was to investigate the ability of *P. algarbiensis* and *P. almogravensis* micropropagated shoots to grow in vitro in low pH conditions, as well as to elucidate the impact of this condition on the degree of membrane damage through lipid peroxidation level, proline (Pro) and carbohydrates content, and antioxidant enzyme activities.

Materials and methods

Plant material

The cultures of *P. algarbiensis* and *P. almogravensis* were initiated in vitro as described earlier (Gonçalves et al. 2009), and were proliferated and maintained on MS medium (Murashige and Shoog 1962) with 0.2 mg l^{-1} 6-benzyladenine (BA) (pH 5.75), at an interval of 6 weeks, under a 16 h photoperiod (cool white fluorescent lamps at 69 μmol m^{-2} s^{-1}) and a temperature of 25 ± 2°C.

Influence of medium pH on shoot proliferation and rooting

To investigate the effect of medium pH on proliferation, *P. algarbiensis* and *P. almogravensis* shoots (about 5 and 3 cm of size, respectively) were inoculated in MS medium supplemented with 0.2 mg l^{-1} BA. Five shoots were cultured per 250 ml Erlenmeyer flask with 50 ml of culture medium, capped with aluminum foil.

For root induction, shoots of *P. algarbiensis* and *P. almogravensis* with identical size (about 7 and 6 cm of size, respectively) were inoculated in ½MS medium (half-strength macronutrients) supplemented with 0.5 mg l^{-1} indole-3-acetic acid (IAA). Five shoots were cultured per 500 ml Erlenmeyer flask with 80 ml of culture medium, capped with aluminum foil.

All the media were supplemented with 2% (w/v) sucrose and solidified with 0.5% (w/v) gelrite. The pH was adjusted to 4.50, 5.00 or 5.75 before autoclaving, at 121°C and 1.1 kg cm^{-2} for 20 min. The post-autoclave pH values were determined after medium cooling until 40°C.

The cultures were grown in the conditions described above. After 6 weeks, the shoot proliferation was assessed by the proliferation frequency, the number of shoots and the length of the longest shoot, and the rooting was evaluated in terms of rooting frequency, root number and the longest root length. The pH of the culture media was measured at the end proliferation or rooting phases after harvesting the cultures. Media were liquefied and cooled until 40°C before pH measurements. As control, the pH values in the culture media maintained during 6 weeks in the same incubation conditions but without cultures were also measured.

Effect of medium pH on biochemical parameters

The level of lipid peroxidation, Pro and carbohydrates content, and antioxidant enzyme activities were analyzed in samples of shoots and roots randomly collected from different Erlenmeyers within each medium.

Determination of lipid peroxidation

The lipid peroxidation was estimated by determining the malondialdehyde (MDA) content according to the thiobarbituric acid-reactive-substances method (Hodges et al. 1999). Fresh tissue was ground in 0.1% (w/v) trichloroacetic acid (TCA) using mortar and pestle and then centrifuged at 10,000*g* for 5 min. After centrifugation, the supernatant was mixed to either 20% (w/v) TCA (−TBA solution) or 0.5% (w/v) thiobarbituric acid (TBA) prepared in 20% (w/v) TCA (+TBA solution). The mixture was heated at 95°C for 30 min and then quickly cooled on ice. After centrifugation

at 3,000*g* for 10 min, the concentration of MDA was calculated from the absorbance at 532, 600 and 440 nm using the extinction coefficient of 157 $mM^{-1}cm^{-1}$ and expressed as nmol MDA g^{-1} fresh weight (FW).

Proline and carbohydrate estimations

The shoots and roots were repeatedly extracted with 80% (v/v) ethanol, and heated at 80°C for 30 min in each extraction for free Pro and carbohydrates determination.

The free Pro accumulation was determined using the acid-ninhydrin reagent method (Troll and Lindsley 1955) modified by Magné and Larher (1992). The extract was reacted with 1% (w/v) ninhydrin reagent, in 60% (v/v) acetic acid, for 1 h at 100°C and the reaction was terminated in an ice bath. The mixture was extracted with toluene and the chromophore optical density was measured at 520 nm. The content of Pro was determined from a standard curve in the range of 0–750 µM.

The carbohydrates content was determined based on the anthrone method (Dreywood 1946; Yemm and Willis 1954). The extract was reacted with 75% (v/v) sulfuric acid and 0.01 M anthrone reagent for 15 min at 100°C. After cooled at room temperature the absorbance was read at 578 nm. Glucose solutions at different concentration (0–500 µM) were used as standards.

Enzyme assays and soluble protein

The activities of the superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX; EC 1.11.1.7) were evaluated in extracts from shoots and roots after 6 weeks of culture. Fresh tissue (100 mg) was ground in a prechilled mortar using a homogenization medium consisting of 50 mM sodium phosphate (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpolypyrrolidone and 2.5 mM dithiothreitol. For APX the homogenizing solution contained 5 mM ascorbate. The homogenate was centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was used for enzyme activities and protein content assays. The SOD activity was assayed using the method of Beauchamp and Fridovich (1971) by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 10 mM methionine, 0.075 mM NBT, 0.2 mM riboflavin and 20 µl enzyme extract. One unit of SOD was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm in the presence of riboflavin in the light during 6 min. The CAT activity was determined by the method described by Aebi (1983). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 40 mM H_2O_2 and 20 µl enzyme extract. The CAT activity was measured by following the decrease in absorbance at 240 nm ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$) due H_2O_2 decomposition. One unit of CAT was defined as the amount of enzyme which breaks down 1 µmol H_2O_2 per min. The activity of APX was determined as described previously (Nakano and Asada 1981). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.5 mM H_2O_2 and 20 µl enzyme extract. The oxidation of ascorbic acid in the reaction mixture was measured using the rate of decrease in absorbance at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of APX was defined as the amount of enzyme which breaks down 1 µmol of ascorbate per min. The GPX activity was defined as outlined by Egley et al. (1983) through monitoring the increase in absorbance at 470 nm due to tetraguaiacol formation ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 8 mM guaiacol, 8 mM H_2O_2 and 20 µl enzyme extract. One unit of GPX was defined as the amount of enzyme to produce 1 µmol tetraguaiacol per min. These assays were conducted in a total volume of 1 ml at 25 ± 2°C. The specific enzyme activity for all enzymes was expressed as unit mg^{-1} protein. Detection of total soluble protein was determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard.

Statistical analysis

To evaluate the influence of medium pH on in vitro proliferation and rooting 3 repetitions with 10 shoots each (two flasks) were performed. To investigate the effect of medium pH on the biochemical parameters plantlets (shoots and roots separately) were analysed in all the experiments and the values obtained were expressed as the mean ± standard error of five replicates. The results were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 15.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test.

Results and discussion

Influence of medium pH on shoot proliferation and rooting

The pH of the culture medium is an important factor for the in vitro shoot and root formation and healthy culture growth. Some plants can tolerate a broader pH range, while in others pH tolerance is limited. Proliferation frequencies

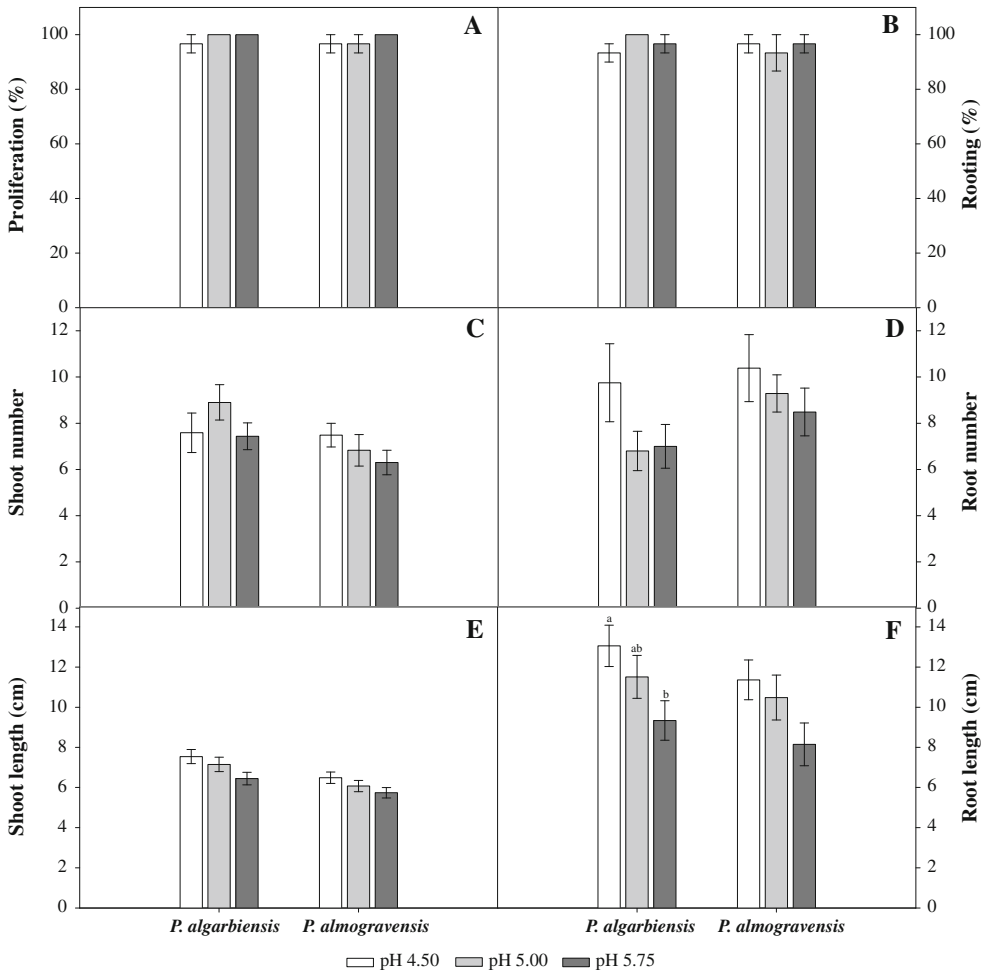

Fig. 1 Effect of medium pH (4.50, 5.00 and 5.75) on proliferation and rooting of *P. algarbiensis* and *P. almogravensis* shoots. Values are expressed as the mean ± SE (n = 3). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed

of both *Plantago* species were very high, near 100%, in all the medium pH tested (Fig. 1a), and no significant differences ($P \geq 0.05$) were observed in the mean number of shoots (values from 7.43 ± 0.58 to 8.90 ± 0.77 in *P. algarbiensis* and 6.30 ± 0.53 to 7.48 ± 0.51 in *P. almogravensis*) and shoot length (6.44 ± 0.31 to 7.54 ± 0.35 cm in *P. algarbiensis* and 5.74 ± 0.26 to 6.49 ± 0.29 cm in *P. almogravensis*) (Fig. 1c, e). Plantlets developed normally in all the medium pH tested, without visually noticed damages caused by low pH (Fig. 2). Xu et al. (2008) in *Malus zumi* also observed that shoot growth was not significantly affected within a broad range (5.0–7.0) of initial medium pH. In contrast, Naik et al. (2010) observed that shoot regeneration of *Bacopa monnieri* was significantly affected by medium pH, obtaining the best results under pH 4.5.

Although proton toxicity usually causes severe inhibition of root growth in various plant species (Kidd and Proctor 2001; Koyama et al. 2001; Sawaki et al. 2009), in this work high rooting frequencies were attained for both species regardless the pH of the rooting medium ($P \geq 0.05$) (Fig. 1b). Also the root number, in both species (Fig. 1d) and root length, in *P. almogravensis* (Fig. 1f), were not affected by pH levels ($P \geq 0.05$). In concordance with our results, Geneve and Heuser (1982) reported that pH ranging from 3.0 to 7.0 did not affect rooting of *Vigna radiana*. Furthermore Bennet et al. (2003) observed that the pH of the medium did not affect the number of roots produced by *Eucalyptus globules* shoots. Curiously, in *P. algarbiensis* roots obtained at pH 4.50 were longer than at pH 5.75 ($P < 0.05$) (Fig. 1f). Nutrient bioavailability often decreases at low pH, thus the increase in root surface area could be considered a strategy for enhancing nutrient acquisition (Marschner 1995).

Changes in medium pH can occur during culture, depending on the initial pH, medium composition and plant species (Schuch et al. 2010). Thus, in this work the pH was monitored after autoclaving and at the end of the subculture period. As expected post-autoclave pH values were lower (0.20–0.75 units) than the pre-autoclave in all the media tested (Table 1). Furthermore, in most media without plantlets the pH decreased to a more acid condition over the 6 week period (Table 1). This acidification was previously observed and can be caused by dehydration of media

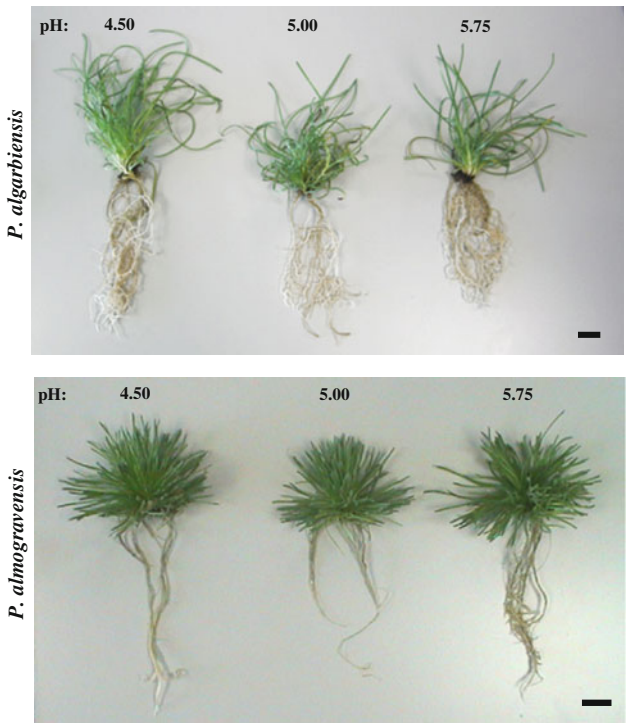

Fig. 2 *P. algarbiensis* and *P. almogravensis* plantlets (bar = 2 cm) at the end of the rooting phase in medium with different pH values (4.50, 5.00 and 5.75)

and/or by the precipitation of medium components such as mineral nutrients (Skirvin et al. 1986; Leifert et al. 1992; Shibli et al. 1999).

Independently of the initial pH, after 6 weeks of proliferation or rooting, there were not significant differences ($P \geq 0.05$) regarding the final pH value, for both species (Table 1). The shift of a wide range of initial pH to the same final pH value has been reported for other plant species (Skirvin et al. 1986; Minocha 1987). It is tempting to suggest that plant material has an active role in establishing an optimum pH environment. The direction and extent of the pH change might also have been influenced by the plant's in vivo optimum pH.

When cultures were inoculated the pH of the media significantly decreased ($P < 0.05$) (Table 1) after 6 weeks, with the exception of *P. almogravensis* during proliferation. Moreover, the lowest pH values were observed in the rooting media for both species ($P < 0.05$). These results can be due to differences in the composition of the proliferation and rooting media but also to the root biomass in the medium. In fact, soil acidification by roots has been demonstrated for many plant species (Marschner et al. 1986; Hauter and Mengel 1988). Under in vivo conditions, plant roots not only respond to pH changes in the soil, but also contribute to the modulation of ambient soil by a variety of mechanisms including: differential uptake of NO_3^- and NH_4^+, release of H^+ and/or OH^-, changes in the uptake and release of CO_2, and release of various organic acids and organic compounds (Minocha 1987; Yan et al. 1992).

Influence of medium pH on biochemical status

It has been suggested that the excess of H^+ competes with other cations for root absorption sites, interfering with ion transport and uptake, and causes root membranes to become leaky in sensitive plants (Foy 1992). According to Koyama et al. (2001) the exposure to low pH damages the plasma membrane of root tip cells irreversibly within a short time. Furthermore, changes in biochemical parameters would occur before any visible symptom of toxicity appears (Lin et al. 2007), and conclusions based on these parameters may be more reliable than morphological observations in revealing H^+ toxicity. Thus, in this work, although in vitro proliferation and rooting were not affected by medium pH, its effect on lipid peroxidation was investigated. Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids, has been utilized as

Table 1 Changes in the pH of proliferation and rooting media after autoclaving and after 6 weeks, with or without *P. algarbiensis* and *P. almogravensis* plantlets

Medium	Original pH	Post-autoclave pH	pH after 6 weeks		
			Without plantlets	*P. algarbiensis*	*P. almogravensis*
Proliferation	4.50	4.20 ± 0.01 b	4.11 ± 0.02 b	3.65 ± 0.10* c	4.60 ± 0.07* a
	5.00	4.52 ± 0.01 ab	4.39 ± 0.04 b	3.72 ± 0.13* c	4.68 ± 0.07* a
	5.75	4.97 ± 0.02 a	4.74 ± 0.01 a	3.50 ± 0.03* b	4.72 ± 0.18* a
Rooting	4.50	4.26 ± 0.02 a	4.08 ± 0.01 b	3.15 ± 0.05 d	3.46 ± 0.09 c
	5.00	4.74 ± 0.02 a	4.35 ± 0.06 b	2.92 ± 0.08 d	3.62 ± 0.09 c
	5.75	5.09 ± 0.01 a	4.85 ± 0.02 b	3.15 ± 0.10 d	3.72 ± 0.10 c

Values are expressed as the mean ± SE (n = 3). In each row, mean values followed by different letters are significantly different at $P < 0.05$, according to Duncan's test

* Significant difference between proliferation and rooting media for each original pH ($P < 0.05$)

Table 2 Contents of MDA, Pro and carbohydrates in *P. algarbiensis* and *P. almogravensis* plantlets (shoot and root) cultured in media with different pH

pH	MDA (nmol g^{-1} FW)		Pro (μmol g^{-1} FW)		Carbohydrates (μmol g^{-1} FW)	
	Shoot	Root	Shoot	Root	Shoot	Root
P. algarbiensis						
4.50	70.52 ± 8.11 a	40.60 ± 3.50 a	0.50 ± 0.09 a	0.20 ± 0.03 a	91.85 ± 9.73 a	285.90 ± 11.46 a
5.00	26.97 ± 4.01 b	29.12 ± 4.12 b	0.57 ± 0.07 a	0.16 ± 0.04 a	100.83 ± 5.54 a	269.08 ± 35.61 a
5.75	26.05 ± 5.68 b	30.90 ± 2.08 b	0.54 ± 0.10 a	0.21 ± 0.04 a	87.90 ± 14.39 a	240.90 ± 62.45 a
P. almogravensis						
4.50	59.51 ± 8.96 a	47.10 ± 4.11 a	0.20 ± 0.01 a	0.16 ± 0.03 b	33.25 ± 3.93 a	168.22 ± 27.15 a
5.00	48.32 ± 9.06 a	31.21 ± 2.99 b	0.33 ± 0.08 a	0.26 ± 0.04 ab	57.09 ± 12.20 a	190.57 ± 14.72 a
5.75	42.12 ± 4.22 a	29.76 ± 3.02 b	0.35 ± 0.06 a	0.39 ± 0.10 a	42.19 ± 5.54 a	86.30 ± 22.73 b

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

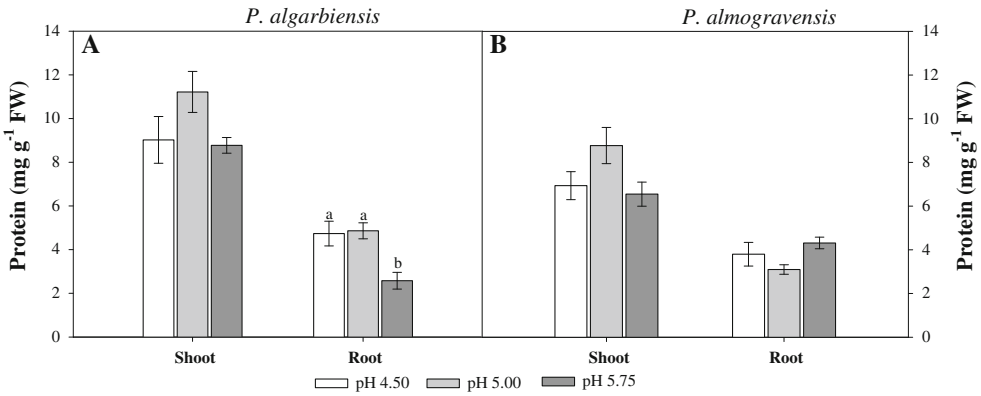

Fig. 3 Effect of medium pH (4.50, 5.00 and 5.75) on protein content in shoots and roots of *P. algarbiensis* (**a**) and *P. almogravensis* (**b**). Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed

a biomarker for lipid peroxidation (Sivanesan et al. 2011). The MDA content was significantly higher ($P < 0.05$) in shoots of *P. algarbiensis* and in roots of both species cultured at pH 4.50 in comparison with shoots cultured at the remaining pH (Table 2). This indicates membrane damage stress and was analytically detectable before growth differences appeared.

The accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress conditions (Chen et al. 2007). In addition to the role of compatible solutes in cell osmotic adjustment they can also function as reactive oxygen species (ROS) scavengers (Hong et al. 2000). Therefore, the enhanced content of these solutes may probably ameliorate the tolerance through improving oxidative status. Although the accumulation of Pro and carbohydrates in response to abiotic stress has been well documented (Ghnaya et al. 2010; Lokhande et al. 2010; Somboonwatthanaku et al. 2010), few studies related to H^+ stress conditions were reported.

A significant increase ($P < 0.05$) in Pro content was observed in *P. almogravensis* roots cultured at pH 5.75, while the roots grown at pH 4.50 and 5.00 showed a significant ($P < 0.05$) increase in carbohydrates content (Table 2). The Pro and carbohydrate contents in *P. algarbiensis* shoots and roots remained unchanged after grown under different pH values (Table 2).

In *P. almogravensis* no significant differences ($P \geq 0.05$) were observed in protein content between all the pH values assayed (Fig. 3b), while in *P. algarbiensis* that content was significantly higher ($P < 0.05$) in roots developed at pH 4.50 and 5.00 (Fig. 3a). Soluble protein content is an important indicator of changes in metabolism, and it is known to respond to a wide variety of stresses (Singh and Tewari 2003). A higher content of soluble protein has been reported in several plant species under adverse growth conditions (Ashraf and Harris 2004; He and Huang 2007).

One of the plant responses to environmental stresses is the rapid and increased generation of ROS (Yang et al. 2010). These free radicals have an important role in the metabolism and development of aerobic organisms; however, their uncontrolled production leads to oxidative stress (Abbasi et al. 2011). Different plants develop different protection mechanisms to eliminate ROS and prevent oxidative damage (Yang et al. 2010). Antioxidant enzymes

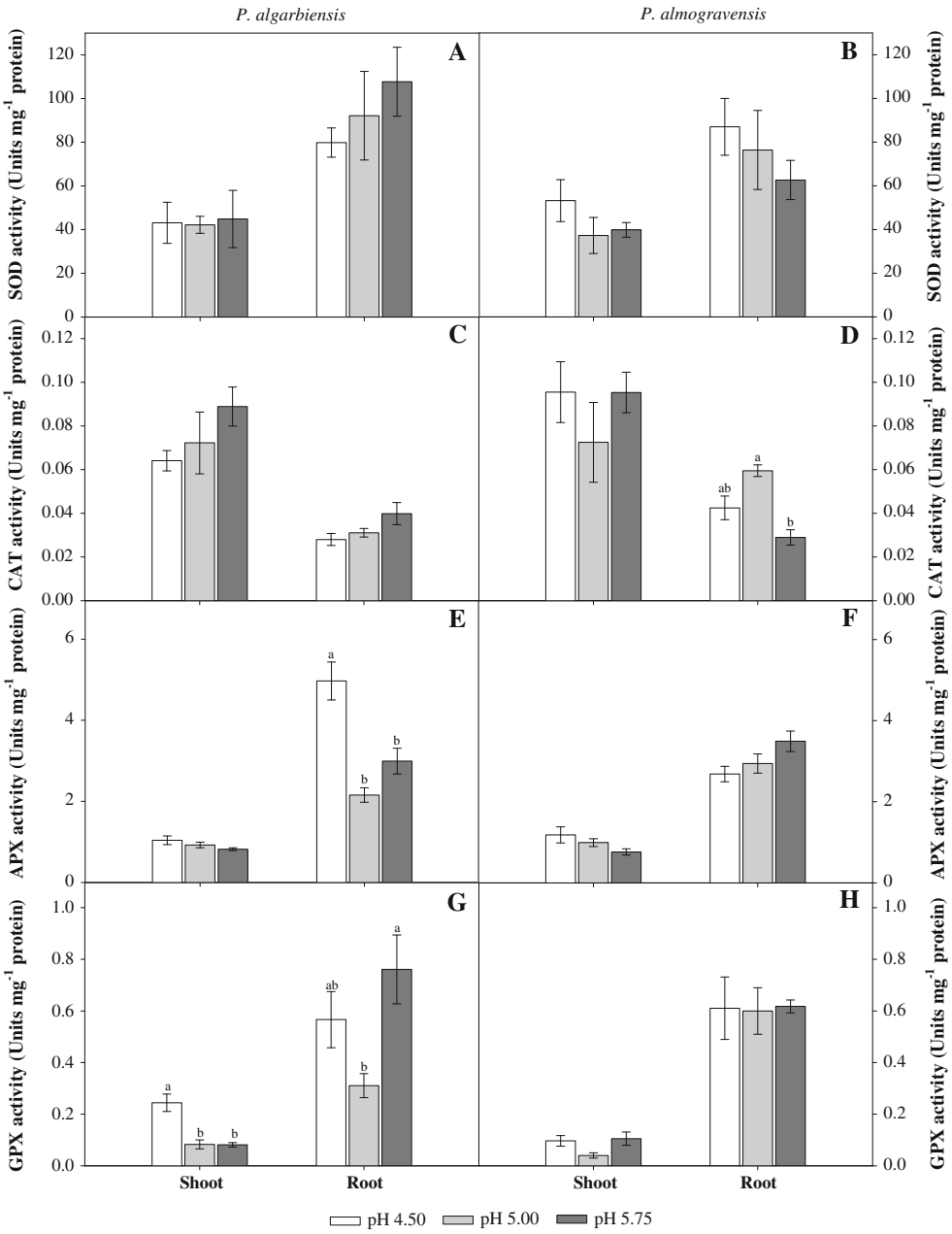

Fig. 4 Effect of medium pH (4.50, 5.00 and 5.75) on SOD (**a**, **b**), CAT (**c**, **d**), APX (**e**, **f**) and GPX (**g**, **h**) activities in shoots and roots of *P. algarbiensis* and *P. almogravensis*. Values are expressed as the mean ± SE (n = 5). For each species, mean values followed by different letters are significantly different at $P < 0.05$ and the absence of letters indicates that no significant differences were observed

like SOD, CAT, APX and other enzymes are efficiently involved in ROS scavenging and can act as one of the main tolerance mechanisms against oxidative stress in plants (Lokhande et al. 2010). It is known that pH affects nutrient uptake as well as enzymatic and hormonal activities in plants (Bhatia and Ashwath 2005). However, reports about the response of antioxidant system for plants subjected to H^+ stress are scarce.

To investigate the antioxidant response of *P. algarbiensis* and *P. almogravensis* shoots and roots to medium pH the activities of SOD, CAT, APX and GPX were measured (Fig. 4). No significant changes ($P \geq 0.05$) in SOD activity were observed in shoots and roots of both *Plantago* species cultured in medium with different pH values (Fig. 4a, b). The CAT activity was not affected ($P \geq 0.05$) by the pH of the medium in *P. algarbiensis* (Fig. 4c), while a significant activation ($P < 0.05$) of this enzyme was observed in roots of *P. almogravensis* developed under pH 5.00 as compared with pH 5.75 (Fig. 4d). In *P. algarbiensis* there was a significant increase ($P < 0.05$) in APX and GPX activity in roots and shoots, respectively, grown at pH 4.50 (Fig. 4e, g). The APX and GPX activity of *P. almogravensis* shoots and roots was not affected by medium pH (Fig. 4f, h).

The level of antioxidative response depends on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Reddy

et al. 2004). Our results demonstrate that after 6 weeks of culture SOD activity was unaffected by the pH of the culture medium in both *Plantago* species but minor changes were detected in the activity of the other enzymes (Fig. 4). This is quite acceptable because superoxide anions could also be mitigated through non-enzymatic pathways (Costa et al. 2010).

The general conclusion of this investigation is that medium pH did not affect in vitro proliferation and rooting of micropropagated shoots of *P. almogravensis* and *P. algarbiensis*. Nevertheless, plasma membrane damage was observed in both species at the lowest pH (pH 4.50), which is controversial because at the end of the rooting period pH values were similar in all the media, regardless the initial value. Although this remains unexplained and should be clarified in future works, from these results it seems evident that both *Plantago* species are apt to grow in vitro in medium with pH values much lower than the usually used in tissue culture (pH 5.70–5.80). This is further reinforced by the fact that medium pH even decreases to a more acid condition during rooting and is in agreement with the fact that both species colonize acid soils.

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