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Nutrient deficiency and hypoxia as constraints to *Panicum coloratum* growth in alkaline soils

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Abstract

Alkaline and saline–alkaline soils impose severe restrictions on plant growth. *Panicum coloratum* var. *coloratum* is a perennial C4 forage grass widely used in tropical and subtropical environments. Published information on its responses to alkaline soil conditions is scarce. The objectives of this study were (i) to characterize the effects of alkaline substrates on germination and initial growth in this species, (ii) to assess the influence of high pH in combination with reduced availability of either nutrients or oxygen and salinity, on plant growth and (iii) to evaluate some physiological traits potentially responsible for growth restrictions under alkaline soil conditions. Trials were conducted in a greenhouse. Germination and early plant survival were not affected by alkalinity. To isolate the effects of high pH, reduced nutrient and oxygen availability on growth, plants were grown either in neutral or alkaline soil, in hydroponics, in neutralized alkaline soil (with or without supplementary fertilization), or were flooded to induce hypoxia. Alkalinity effects on growth in hydroponics were milder than in soil. Growth in alkaline soil with nutrient supplement was still significantly lower (by 40%) than in neutral soil. Both alkalinity and hypoxia reduced growth non-synergistically. These results show that studies of plant response to alkaline substrates carried out in aerated nutrient solutions can only partially address the complexity of this stress. Photosynthesis and PSII activity were among the physiological mechanisms negatively affected by alkalinity and may be partially responsible for the growth limitations observed in *P. coloratum* under alkaline conditions.

Keywords: *Panicum coloratum* cv *coloratum*, alkaline soil, hydroponics, nutrient deficiency, JIP analysis, chlorophyll fluorescence

Introduction

Saline, alkaline and saline–alkaline soils are regarded as problem soils for agricultural purposes. Estimates suggest that, globally, there are about 412 million ha affected by salinity and 618 million ha by sodicity, although these figures do not differentiate areas where both types of soil conditions overlap (FAO and ITPS, 2015). Alkaline soils occur most frequently in steppe, dry steppe and semidesert zones, which periodically suffer from water deficits, with major areas in Ukraine, the Russian Federation, Kazakhstan, Hungary, Bulgaria, Romania, China, USA, Canada, South Africa, Argentina and Australia (Lyubimova *et al.*, 2009). In saline soils (also termed Solonchaks), electrical conductivity (EC) of the saturation extract is >4 mmhos cm^{-1} at 25°C and exchangeable sodium percentage (ESP) is <15, whereas in saline–alkaline soils, ESP is >15 and the pH of the saturation extract is above 8·5. Soda–saline soils, a subgroup of the latter soils, are characterized by high sodium carbonates in the soil solution (Vorob'eva and Pankova, 2008). Non-saline–alkaline soils (Solonetz) have saturation extract EC <4 mmhos cm^{-1} but ESP > 15. Both saline and saline–alkaline soils have high EC in the saturation extract, a condition associated with salinity stress.

Agricultural productivity of alkaline soils is restricted by their agrophysical, physiological and hydrological properties. Agrophysical properties are related to the high swelling capability of soil colloids, which deform during shrinking, leading to low porosity and oxygen deficiency (Lyubimova *et al.*, 2009).

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Physiological factors are associated with high concentrations of water-soluble salts and nutrition imbalance, with micronutrient deficiencies (Fe and Zn) being a typical consequence of alkaline soil pH (Marschner, 1995). Hydrological factors refer to the low water permeability of these soils; alkali soils are less permeable to water than non-alkali soils. Another adverse feature of these soils is a high content of unavailable moisture. These soils, which are marginal for agriculture, support forage species and cattle production activities.

Most studies on the response of plants to alkaline stress have been performed on plants grown in nutrient solutions (Yang *et al.*, 2008, 2012; Li *et al.*, 2009; Zhang and Mu, 2009; Guo *et al.*, 2010; Cao *et al.*, 2012; Javid *et al.*, 2012; Gong *et al.*, 2013; Babuin *et al.*, 2014; Hu *et al.*, 2015) and, exceptionally, in soil (Ahmad *et al.*, 2014). However, nutrient solutions do not reproduce all the limitations of alkaline soils; studies performed in hydroponics, with plants supplied with high nutrient concentrations, have only addressed the high pH feature of alkaline soils.

Panicum coloratum var. *coloratum* is a perennial C4 grass native to eastern Africa, which has been introduced as a forage species to tropical and subtropical areas worldwide. It is known to tolerate drought, flooding and waterlogging soil conditions, as well as mild salinity stress (Cook *et al.*, 2005). It also tolerates alkaline soil conditions (Lloyd, 2007). There is, however, limited published information to support this claim. A plethora of studies have addressed tolerance to salinity stress in many plant species (Munns and Tester, 2008), including this grass species (Taleisnik *et al.*, 1998; Pittaro *et al.*, 2015). However, although alkaline stress is more complex and damaging to plants than salinity, and saline–alkaline stress is even more damaging (Yang *et al.*, 2008; Guo *et al.*, 2010; Javid *et al.*, 2012; Xu *et al.*, 2012), alkalinity effects on plants have not been extensively explored.

The objectives of this study were (i) to characterize the effect of alkaline substrates on *P. coloratum* germination, initial establishment and later stages of vegetative plant growth; (ii) to assess separately the influence of high pH, in combination with reduced nutrient and oxygen availability, on the restriction of *P. coloratum* growth in those substrates; and (iii) to evaluate some physiological traits potentially responsible for growth restrictions under these conditions.

Restricted uptake of Zn and Fe by plant roots in alkaline soils can lead to deficiencies of these elements. Given the close positive correlations often observed between the rate of net photosynthesis and mineral nutrient content of leaves (Marschner, 1995), photosynthetic carbon fixation and photosystem II (PSII) activity were characterized in these plants to gain insight into the separate effects of pH and nutrient stress. Measuring the quantum efficiency of light utilization by PSII can contribute to the identification of non-stomatal causes for reduced carbon fixation under stress (Gururani *et al.*, 2015). Here, we assessed the functioning of PSII in response to alkaline stress conditions. Zn deficiency may produce decreases in antioxidant enzyme activity, specifically Cu/Zn-SOD (Yu *et al.*, 1998; Cakmak, 2000), and consequently generate oxidative stress (Sharma *et al.*, 2004), which may contribute to the negative effect of alkaline soils on plant growth. Therefore, Cu/Zn-SOD concentration, oxidative stress expression and some antioxidant activities were compared in plants grown at neutral and alkaline pH.

The analyses performed in this work constitute an initial approach to discriminating the effects of the various components of saline–alkaline stress on *P. coloratum* growth and to identify the physiological bases of the responses that limit the success of this species in soils affected by these conditions.

Material and methods

Experimental environment, plant material and soil

Panicum coloratum (L.) var *coloratum* (Klein) seeds were obtained from Oscar Pemán SA, a local seed company. Trials were performed in a naturally illuminated greenhouse. Air temperature and incident photosynthetically active radiation (PAR) in the greenhouse were measured and recorded every hour with a digital datalogger (Cavadevices SA™ Buenos Aires, Argentina). Supplemental illumination was provided with Powerstar HQI-T 400W/D (OSRAM) lamps set to a 16-h photoperiod. Mean air temperature was 27·7 ± 0·2°C and PAR was 342 ± 9·6 μmol m^{-2} s^{-1}.

Neutral and alkaline soils were collected from Alto Alegre, Córdoba Province, Argentina (32°24′S, 62°49′W). Neutral pH soil samples (pH 7·3) were obtained from alfalfa plots, and alkaline soil (pH 10·1) was obtained from bare spots where only *Distichlis spicata* was found. This is a characteristic halophytic grass (Glenn *et al.*, 1999 and references therein, see also http://www.sussex.ac.uk/affiliates/halophytes/index.php?content=plantView&id=4772). The results of the soil analysis are given in Table 1. The alkaline soil exhibited a dark clayish organic film on its surface; it is non-saline–alkaline type, since ESP > 15 and EC < 4.

Effect of alkalinity on germination and early plant survival

Alkalinity effects on seed germination were assessed in both Petri dishes and soil. In the first case, seeds were

Table 1 Chemical characterization of soils from Alto Alegre, province of Córdoba, Argentina, used in this study.

	Alfalfa plot (S32°24′28·2″; W62°49′6·3″)	**Bare spots with *D. spicata* (S32°25′9·4″; W62°49′25·7″)**
Organic matter (g kg^{-1})	2·38	0·78
Organic carbon (g kg^{-1})	1·38	0·45
Total nitrogen (g kg^{-1})	0·19	0·08
Extractable P (Bray)	13·70	50·76
Nitrate (mg kg^{-1})	27·36	39·83
Calcium carbonate*	-	xx
pH	7·27	10·16
EC of the saturation extract (mmho/cm)	0·079	0·626
Exchangeable cations (me/100 g)		
Calcium	9·34	10·16
Magnesium	3·02	1·10
Sodium	1·37	13·22
Potassium	2·92	3·09
Exchangeable Na (g kg^{-1})	7·38	68·32
S from sulphate (μg g^{-1})	11·85	39·87

-: non-detectable, x: low, xx: high.

*The calcium carbonate level is indicated with symbols.

placed on filter paper moistened either with distilled water or with a pH 9 solution (9 mM $NaHCO_3$ and 1 mM Na_2CO_3) and kept at 28°C in a temperature-regulated oven. One hundred seeds were placed per dish, using three replicates per treatment. Germinated seeds were counted daily, and the filter papers were moistened with distilled water if needed.

Soil was mixed with vermiculite (2:1 on a volume basis) to improve agrophysical and hydraulic properties. Pots were filled with mixtures of neutral and alkaline soil:vermiculite (two pots per type of soil) and irrigated to field capacity; 150 seeds were sown in each pot. Pots were covered with a plastic film until initial plant emergence. Emerged seedlings were initially counted daily for 9 d and then weekly until day 48.

Effect of alkalinity and salinity on plant growth

Growth in natural soil, pH 7 or 10

For growth Trial 1, the soil:vermiculite mixture was used to fill 450-cm^3 black plastic pots, 5·5 cm diameter. Treatments included saline and non-saline conditions of each soil type. To obtain homogeneously salinized soil, pots were filled by thirds; each layer was irrigated with 100 mM NaCl adjusted to either pH 7 or 9·9 (in this case, using the carbonate and bicarbonate solution mentioned above) and allowed to drain before adding the next layer. The same procedure was used for each layer. Non-saline pots were filled following the same procedure, but layers were irrigated with tap water.

Plants for this trial were obtained from seeds of *P. coloratum* (L.) var *coloratum* sown in vermiculite. Seedlings at the two-leaf stage were transplanted to the pots before the addition of the last soil layer, which was carefully laid to prevent root damage. The EC of the drainage solution was measured the following morning; it was 0·3–0·9 dS m^{-1} in non-salinized treatments and 10·7–11·1 dS m^{-1} in salinized treatments. The surface of each pot was covered with sand to prevent excessive evaporation. All pots were weighed at that point and subsequently every 2 d, and consumed water was added. There were 12 replicates per treatment. Seedling death was observed during the first week after transplanting, mainly in the alkaline treatments, and individuals were replaced. The trial was harvested after 53 d, and fresh and dry weight of shoots and roots was measured.

Isolating pH effects: growth in hydroponics

The aim of Trial 2 was to assess pH effects separately from other characteristics that distinguish alkaline from neutral pH soils. Growth was evaluated in plants cultivated in an inert substrate (perlite and washed river sand, 2:1 on a volume basis). Seeds were sown in 10 cm × 10 cm × 33 cm plastic pots containing the inert substrate, and emerged plants were thinned to one plant per pot. Pots were irrigated with half-strength Hoagland solution (Hoagland and Arnon, 1950) pH 7, or adjusted to pH 9 with 9 mM $NaHCO_3$ and 1 mM Na_2CO_3. A preliminary trial performed at pH 7, 8 and 9 showed differences between pH 9 and the other conditions; therefore, subsequent trials were performed only at pH 7 and 9. To mimic saline–alkaline conditions, the effect of salinity in combination with high pH was also evaluated. One month after sowing, pots were gradually salinized to 100 or 200 mM NaCl by adding salinized nutrient solution. These concentrations were used to provide a level of salinity comparable to that found in soils with EC = 10 dSm^{-1} in the drainage solution, considering that drainage solutions are obtained when soils are above field capacity, and actual EC would increase with decreasing soil water content. Plants were irrigated every 2–3 d with 200–250 mL nutrient solution to ensure complete rinsing of the void volume and prevent salt accumulation. The EC and pH in the

drainage solution were monitored after each irrigation. Plants were harvested 51 and 97 d after sowing, i.e. 39 and 76 d from the first salinization. There were 10 replicates per pH/salinity combination.

Isolating pH effects: growth in neutralized alkaline soil

In another approach to restrict soil effects to pH differences and to evaluate whether fertilization helped to overcome the growth constraints imposed by high pH, plants were grown in alkaline soil or neutralized alkaline soil, with or without the addition of nutritive solution (Trial 3).

The alkaline soil:vermiculite mixture mentioned earlier was used to fill PVC pots (5·5 cm diameter × 33 cm height). To reduce soil pH, HCl 100 mM was applied by drip irrigation, until pots started to drain. At this point, the substrate in all pots (both acid-treated and controls) was extensively rinsed with distilled water, and pH and EC were monitored in the drainage solution. The procedure was repeated until the drainage solution from acid-treated pots stabilized close to pH 7.

Plants for this trial were obtained by vegetative propagation of four types of *P. coloratum* (L.) var *coloratum* plants: plants obtained from seeds (PK), and plants from *P. coloratum* clones P3, P4 and P15, selected for stress tolerance (Pittaro *et al.*, 2015). Tillers bearing two or three leaves were transplanted to alkaline or neutralized soil. The pots containing the two types of soil (pH ca. 7 and 9·5) were in turn divided into two groups: one that was drip-irrigated with distilled water and the other with half-strength Hoagland solution.

Tillers were counted once a week. Shoot fresh weight was determined at harvest (37 d after transplantation) and samples were subsequently dried at 70°C for 4 d to obtain dry weight.

Effect of hypoxia and Zn deficiency on plant growth

Low porosity and oxygen deficiency are a characteristic of Solonetz soils (Lyubimova *et al.*, 2009). To induce oxygen deficiency, plants were cultured in waterlogged conditions (Trial 4). Seeds were sown in 0·7-L pots filled with perlite and washed river sand (2:1, v:v), which in turn were placed in 6-L trays. Upon emergence, seedlings were thinned to 15 per pot. Twelve days after sowing, pots were divided into two groups and irrigated with half-strength Hoagland solution pH 7 or 9 (obtained as explained above). Half of the pots in each group were waterlogged with the nutrient solution, and the other half was kept at field capacity. There were six pots per treatment. Plants were harvested after 28 d, and height, number of tillers, and fresh and dry weight were measured.

To determine whether Zn deficiency could reproduce the effects of the alkaline growth medium, a set of plants was grown under the above conditions, but including a Zn-deficient treatment at pH 7 (Trial 5).

Carbon fixation, chlorophyll fluorescence and JIP test parameters

Net CO_2 assimilation rate (A), stomatal conductance (g_s) and internal CO_2 (C_i) were measured, 2 d before harvest, on the youngest fully expanded leaves using a portable photosynthesis system (LICOR 6400, Lincoln, NA, USA). The internal LED light source was set at saturating incident photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1}; the cuvette temperature was fixed at 25°C and the CO_2 flow at 500 μmol m^{-2} s^{-1}.

Measurements of chlorophyll a fluorescence transients were taken using a Pocket PEA (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK), on the same type of leaves as above (Hansatech, 2006). The leaves were previously darkened for 25 min to achieve the complete oxidation of the primary electron carriers (F0); then, they were exposed to 3500 mmol photons m^{-2} s^{-1} (637 nm peak wavelength) during 3 s and recorded at 685 nm. Electron transport activity of PSII was assessed by calculating the JIP test parameters according to Strasser and Tsimilli-Michael (2004). (An explanation of the JIP parameters is given in Table S1 in the Supporting Information.)

Root anatomy

Root sections obtained at 3 cm from the apex were fixed in FAA (formaldehyde: 95% ethanol: 37% glacial acetic acid: water, 50:5:10:35) for several days and subsequently transferred to 70% ethanol. Cross-sections were stained with berberine hemisulphate and aniline blue according to Brundrett *et al.* (1988) and observed under a fluorescence microscope (Nikon Eclipse Cs1 Spectral Confocal Microscope, Nikon Corporation, Tokyo, Japan).

Ion and mineral concentration

Samples for ion or mineral determination were obtained from mature leaf blades, at harvest time, in all the trials, and various determinations were performed. For ions, tissues were suspended in 0·1 N HNO_3 and ions in the suspension medium were determined by HPLC (Shimadzu Prominence Modular HPLC, Kyoto, Japan). Samples were subsequently dried and weighed and ion concentrations were calculated on a dry weight (DW) basis.

To determine Fe and Zn in plant samples, dry matter was ground and digested according to Malavolta *et al.* (1997) and concentrations of these elements were quantified by atomic absorption spectrometry (PinAAcle 900H Atomic Absorption Spectrometer, Perkin Elmer, Waltham, Mass, USA). The same elements in soils were determined after extraction with Mehlich 3 (Mehlich, 1984). Total nitrogen in leaf tissues was determined by the Kjeldahl method and phosphorus by spectrophotometry, following Malavolta *et al.* (1997).

Oxidative damage and antioxidant activity

Superoxide presence in root tips was determined with nitro blue tetrazolium (NBT), following Bustos *et al.* (2008). Oxidative damage in leaves was evaluated by malondialdehyde (MDA) concentration, measured in alcohol extracts according to Heath and Parker (1968).

Total non-enzymatic antioxidant activity was determined in alcohol extracts of frozen leaf blade segments, following Benzie and Strain (1996).

Superoxide dismutase (SOD) activity was determined according to Beauchamp and Fridovich (1971). Frozen leaf samples (100 mg fresh weight) were ground to a fine powder in liquid nitrogen and homogenized in 50 mM potassium phosphate buffer (pH 7·5), containing 1 mM EDTA and 1% PVPP (polyvinylpolypyrrolidone). Homogenates were centrifuged at 16 000***g*** for 25 min at 4°C and the supernatant was used to determine protein concentration (Bradford, 1976). Total superoxide dismutase (SOD) activity was assayed spectrophotometrically at A_{560} by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of the photochemical reduction of NBT, and SOD specific activity was expressed as units per mg protein in the sample. Catalase (CAT) activity was determined at room temperature by measuring the decrease in A_{240} after adding 5 mM H_2O_2 to samples (Gallego *et al.*, 1996).

Results

Effect of alkalinity on germination and early plant survival

Germination in Petri dishes was completed within 5–6 d after seed sowing; nearly 50% of the seeds germinated, and the process was not affected by the pH of the medium (Supporting Information: Figure S1). In soil, germination was somewhat slower, but also reached statistically similar levels in both types of soil. Seedling survival in this trial was checked up to 48 d after sowing, and it was not affected by soil pH until day 30. On day 48, the last sampling date, a decrease in survival at pH 10 was observed.

Influence of high substrate pH, in combination with salinity, reduced nutrient and oxygen availability, on *P. coloratum* growth

After nearly two months of growth in pots with soil (Trial 1), shoot dry weight of *P. coloratum* was severely affected (about 85%) by alkaline pH (Figure 1a). Growth in the combination of saline and alkaline stress was similar to that in the non-salinized alkaline control. Similar results (data not shown) were obtained in a previous, longer trial, in which soil had not been mixed with vermiculite: significant negative effects of alkaline pH and no further growth reductions were observed in the combination treatments as compared to the alkaline control. These results indicate that alkaline stress in soil has much more negative effects on growth than (this level) salinity stress. The similar growth trends obtained in soil and soil-vermiculite mixtures indicate that physiological features of alkaline soils exert a negative impact on growth, independently of agrophysical and hydrological features. Consequently, growth in nutrient solutions of different pH was expected to replicate the effects of soil pH on plant growth.

In hydroponics (Trial 2), under non-saline conditions there was, as could be predicted, a significant negative effect of pH 10 on growth (Figure 1b), but it was much lower (about 40%) than that observed in soil. However, in these plants, salinity did exert a statistically significant negative effect on growth. In this case, however, the effects of the salinity treatments were similar at both pH levels, indicating that under optimal nutrient supply, as in a hydroponics setting, pH effects were buffered, and negative salinity effects were disclosed. This tendency was maintained in a second harvest at 91 d (data not shown); in that harvest, however, plants grown at the highest salinity level of salinity were smaller than those at 100 mM NaCl. Similar results had been obtained in a previous trial harvested 34 d after sowing (data not shown).

To further separate pH and nutritive effects from other features of alkaline soil, plants were grown in natural and neutralized alkaline soil (Trial 3), with or without supplementary fertilization. Neutralized soil was extensively washed to eliminate any remnants of the acid used for this process, and the non-neutralized soil was subject to the same rinsing scheme. Potential mineral deficiencies may have resulted from such extensive rinsing; therefore, to overcome this condition, half of the pots were irrigated with half-strength nutrient solution (Hoagland and Arnon, 1950) and

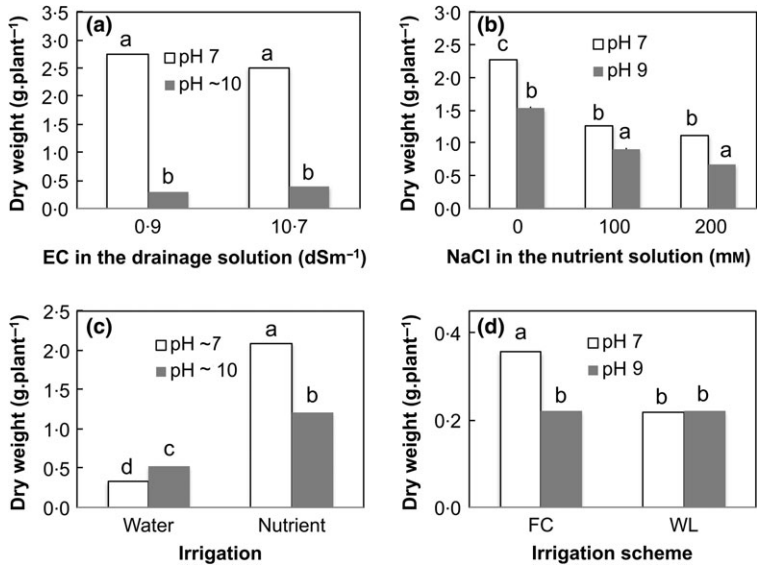

Figure 1 Shoot dry weight of *Panicum coloratum* plants grown under alkaline, saline and waterlogged conditions. (a) Plants grown for 53 d in pots with soil pH 7 or ~10, irrigated with 0 or 100 mM NaCl (Trial 1). (b) Plants grown for 51 d in nutrient solution pH 7 or 10 and salinized with 0, 100 or 200 mM NaCl (Trial 2). Results are means ± s.e. of 7–11 plants. (c) Plants grown for 37 d in pots containing soil pH ~10 or neutralized to pH 7, irrigated with water or nutrient solution (Trial 3). Results are means of 23 plants. (d) Plants grown for 28 d in nutrient solution pH 7 or 9·5, under field capacity (FC) or waterlogged (WL) conditions. Results are means of 21 plants per treatment. In all panels, different letters indicate significant differences at $P < 0{\cdot}05$ (Fisher's test).

Table 2 Chemical characterization of alkaline and neutralized soils, irrigated with water or nutrient solution.

	Alkaline		Neutralized	
	+ Water	+ Nutrients	+ Water	+ Nutrients
Organic matter (g kg^{-1})	1·17	0·45	0·37	0·48
Organic carbon (g kg^{-1})	0·68	0·26	0·21	0·28
Total nitrogen (g kg^{-1})	0·07	0·03	0·02	0·03
Extractable P (Bray)	14·12	12·15	44·15	36·21
Nitrate (mg kg^{-1})	49·53	92·37	7·43	23·15
Calcium carbonate*	xx	xx	xx	xx
pH	9·21	9·34	6·48	6·52
EC of the saturation extract (dS m^{-1})	1·56	1·90	4·49	2·88
Exchangeable cations (me/100 g)				
Calcium	5·30	6·10	7·10	8·40
Magnesium	1·50	1·10	1·80	1·70
Sodium	7·30	8·21	2·27	0·69
Potassium	1·94	1·98	1·76	1·91
Exchangeable Na (g kg^{-1})	45·50	47·20	16·84	5·24
S from sulphate (μg g^{-1})	55·00	44·14	1·77	2·26
Fe (mg kg^{-1})	18·00	19·60	30·60	23·10
Zn (mg kg^{-1})	5·9	6·0	5·7	5·7

-: non-detectable, x: low, xx: high.

*The calcium carbonate level is indicated with symbols.

the other half was irrigated with distilled water. Soil analyses are shown in Table 2. The results indicate that available P was higher in neutralized soil, whereas nitrate, despite the extensive rinsing, was not completely washed from alkaline soil. This may be a consequence of the reduced water permeability of this

soil. Neutralized soil had lower nitrate content, increasing after fertilization. Exchangeable Na was reduced in neutralized soil, but Zn and Fe remained at similar levels in alkaline and neutralized soil.

As anticipated, growth in nutrient-supplemented pots was significantly higher than in non-supplemented pots, confirming that the washed substrate was deficient in nutrients (Figure 1c). Growth in alkaline soil with nutrient supplement was still significantly lower (by 40%) than in neutral soil with the same supplement, in agreement with the results from the hydroponics treatment.

Poor aeration and particle dispersion are other features of alkaline soils. In the previous trial, it was observed that even neutralized alkaline soil had better drainage than alkaline soil. Hence, it was presumed that alkaline soil with poor drainage was oxygen deficient, a condition normally associated with waterlogging. Therefore, to separate effects of oxygen deficiency from those of high pH, plants were grown on an inert substrate (the mixture of sand and perlite mentioned earlier) saturated or not with nutrient solution (Figure 1d). Both high pH and waterlogging significantly decreased the growth of these plants, but there were no synergistic effects, indicating that negative effects of alkaline soils on growth could have been due to both high pH and oxygen deficiencies.

Roots growing in alkaline soil were expected to have a higher degree of aerenchyma development as a consequence of the reduced aeration typical of those soils. Roots of plants growing in neutral soil already showed a high degree of aerenchyma development (Supporting Information, Figure S2) as previously reported for this species (Imaz *et al.*, 2013), but root anatomy was similar in both substrates (Figure S2). Since plants exhibited lower growth in a waterlogged substrate than in an aerated one, the presence of aerenchyma was not effectively contributing to overcome the negative incidence of waterlogging. A slightly brighter endodermis was observed in roots from plants grown in alkaline substrate, suggesting it may be more suberized than in control plants, but the functional relevance of this observation was not explored.

Carbon fixation, stomatal conductance and PSII electron transport activity

Stomatal conductance and carbon fixation were reduced by high pH in fertilized plants, and even further reduced by nutrient deficiency, regardless of the pH (Figure 2). Growth in neutralized soil with or without nutrient supplement (Figure 1c) was positively related to stomatal conductance and carbon fixation (Figure 2), indicating that alkaline pH had a negative effect on these variables, despite nutrient supplement. The correlation between growth and carbon fixation was $y = 0{\cdot}23x - 0{\cdot}47$, R^2 0·99.

The effects of substrate pH on electron transport components were analysed in plants grown with nutrient supplement (Figure 3). The decrease in Fv/Fm indicates that the quantum efficiency of PSII photosynthesis was reduced. All parameters related to the reaction centres (RC) increased, which may simply indicate that RCs were affected, as shown by the decrease in Fv/Fm. The greatest effect of the alkaline substrate was in the light absorption component per RC (ABS/RC), and alkalinity also induced a greater degree of energy dissipation as latent heat (DIo/RC), which is expectable given the observed decrease in Fv/Fm.

Nutrient concentration in plants grown in neutral and alkaline substrate

Availability of Zn and Fe is normally reduced in alkaline soils (Marschner, 1995), and this in turn may lead to deficiencies of these elements in the plants. While plants grown in alkaline soil were markedly deficient in Zn, those grown in hydroponics also had reduced Zn concentration in leaves, but not below the critical level (Marschner, 1993) (Figure 4). Growth in a Zn-deprived medium was negatively affected, but to a much lower degree than by the alkaline medium (data not shown), indicating that Zn deficiency is only one aspect of the growth restrictions imposed by alkaline growth solutions. Fe concentrations did not differ

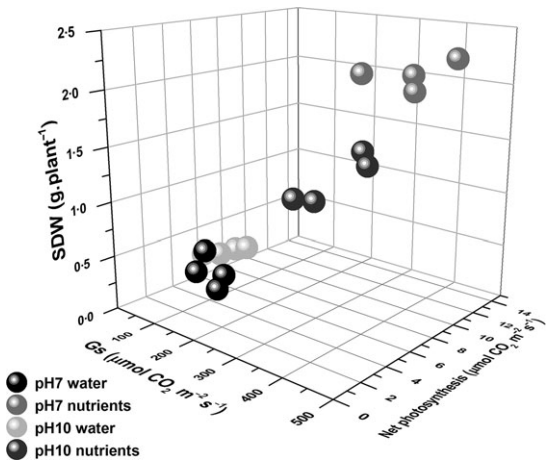

Figure 2 Growth, carbon fixation and stomatal conductance in four genotypes of *P. coloratum* plants grown for 37 d in pots containing soil pH ~10 or neutralized to pH 7 and irrigated with water or nutrient solution. Carbon fixation results are means of five plants per genotype. There were no differences among genotypes in the response to the treatments.

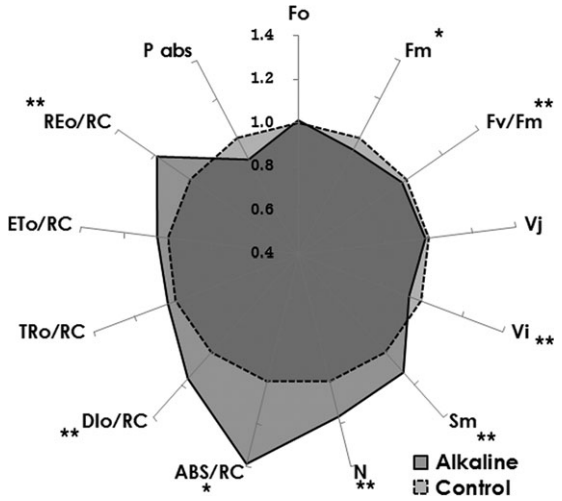

Figure 3 Radar plot showing the differences in JIP test parameters between *P. coloratum* plants growing for 37 d in soil pH ~10 or neutralized to pH 7 and irrigated with nutrient solution. The values represent the means of 20 plants. All JIP test parameters were normalized to pH 7 values. * and ** indicate significant differences at $P < 0{\cdot}01$ and $P < 0{\cdot}0001$, respectively (Fisher's test), between treatments.

between plants grown at high or neutral pH, either in soil or hydroponics; however, since total and active Fe were not distinguished in these assays, it was not possible to determine whether Fe deficiencies occurred despite similar Fe concentrations.

As with Fe, leaf blade K and Mg concentrations were not affected by soil pH, and, as expected, they were higher in plants irrigated with nutrient solution (Supporting Information: Figure S3). Leaf blade N and P concentrations did not differ between plants grown in alkaline or neutralized soil supplemented with nutrient solution. Ca concentration in leaf blades was lower in plants grown in alkaline soil, but was similar in plants irrigated with water or nutrient solution. In plants grown in hydroponics, there was no significant effect of pH on ion accumulation, and the effects of salinity were variable (results not shown).

Oxidative stress and antioxidant activity

Root tips of plants grown at pH 9 showed intensive blue colour (Figure 5a), indicating the presence of apoplastic superoxide (Rodríguez and Taleisnik, 2012). Yet MDA concentration, indicating oxidative damage to membranes, was not statistically higher in leaves from plants grown at pH 9 than at 7, except when plants were waterlogged (Figure 5b). Total non-enzymatic antioxidant activity showed similar responses in those plants (Figure 5d). In plants grown in soil, this activity was very low when plants were fertilized, but it was higher in nutrient-poor substrate, especially under alkaline conditions (Figure 5c).

Enzymatic antioxidant activity (SOD and CAT) was not affected by soil pH, and specifically, in SOD, Cu–Zn isoforms did not react to this condition (data not shown).

Discussion

Effect of alkalinity on germination and early plant survival

Germination in this experiment was not affected by the alkaline substrate. This finding supports the results obtained for several other crops: sunflower (Liu *et al.*, 2010), *Lathyrus quinquenervius* (Zhang and Mu, 2009), *Medicago ruthenica* (Guan *et al.*, 2009) and sorghum

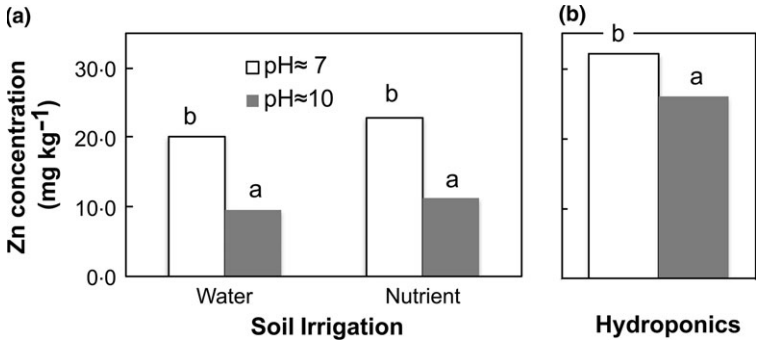

Figure 4 Zn concentration on a dry weight basis (mg kg^{-1}) in *P. coloratum* leaf tissues. (a) Plants grown for 37 d in pots containing soil pH ~10 or neutralized to pH 7, irrigated with water or nutrient solution (Trial 3). (b) Plants grown for 51 d in nutrient solution pH 7 or 10. Results are means of 5–9 plants (Trial 2). Different letters indicate significant differences at $P < 0{\cdot}05$ (Fisher's test).

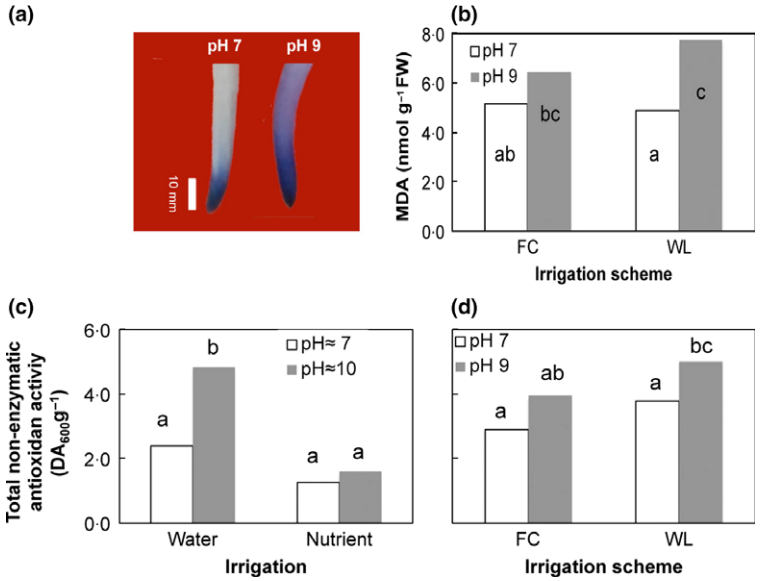

Figure 5 Oxidative stress and total non-enzymatic antioxidant activity in *P. coloratum* plants grown at pH 7 or 9. (a) Superoxide production (as shown by NBT staining) in root tips from plants grown for 28 d in nutrient solution pH 7 or 9, under field capacity (FC). (b) Malondialdehyde (MDA) concentration in leaves from plants grown for 28 d in nutrient solution pH 7 or 9, under field capacity (FC) or waterlogged (WL) conditions. (c) Total non-enzymatic antioxidant activity in leaves from plants grown for 37 d in pots containing soil pH ~10 or neutralized to pH 7, irrigated with water or nutrient solution (Trial 3). Results are means of 20–23 plants. (d) Total non-enzymatic antioxidant activity in leaves from plants in panel B. Results are means of nine plants per treatment. In all panels, different letters indicate significant differences at $P < 0{\cdot}05$ (Fisher's test).

(Zhao *et al.*, 2014), where seed germination was not affected by high pH, even in the presence of low salinity levels. Nevertheless, in the present trial, the substrate in the pots was kept at field capacity, whereas under field conditions this is unlikely, and fine-textured, alkaline or poorly drained soils may exhibit surface crusts that affect seedling emergence, as reported for lupins (White, 1990). Therefore, while these results indicate that *P. coloratum* is tolerant of high pH at germination, under field conditions this may be influenced by other characteristics associated with alkaline soils. Germination assessment under controlled conditions can only predict the response to a specific component of the complex alkaline stress syndrome, in this case, high pH.

Seedling survival, measured up to 40 d after sowing, was also not affected by high pH, as shown previously for alfalfa (Peng *et al.*, 2008) and the alkaline-tolerant grass *Aneurolepidium chinense* (Shi and Wang, 2005) after short-term exposure to high pH. A longer evaluation period may have revealed decreased survival under these conditions. (This is suggested by the results in Figure S1 (Supporting Information), with a declining tendency observed on the last sampling date.)

Separate effects of high pH, low fertility and hypoxia on plant growth in alkaline substrates

The next stage of this work was to characterize vegetative plant growth under alkaline conditions and to evaluate how growth is influenced by some components of this stress: high pH, low fertility and hypoxia. The first two are related to physiological soil features and the third to agrophysical and hydrological features. To distinguish how these characteristics influence growth under alkaline conditions, results of growth trials using alkaline soil, neutralized alkaline soil (with or without added fertilizer) and hydroponics were compared. High pH in soil, soil-vermiculite mixtures and hydroponics, all resulted in growth reductions compared to neutral pH, suggesting that alkaline substrates exert a negative impact on *P. coloratum* growth, independently of agrophysical and hydrological features. Taken together, these results challenge the common notion that *P. coloratum* is tolerant to alkaline stress, at least at this stage of development. The development of stress-adaptive mechanisms may support tolerance at later developmental stages, or contribute to its persistence in the sward, aspects that require field-scale investigations.

Regardless of substrate pH, low nutrient availability severely limited *P. coloratum* growth in our trials, consistent with previous evidence for its positive response to fertilization (Squires and Myers, 1970; Carr, 2014). Under conditions of sufficient nutrient supply (Figure 1b, d), growth was still negatively affected by substrate pH. Major nutritional constraints of sodic soils include toxicity by Na and B, and deficiencies of Zn, Fe and P, and, to a lesser extent, Ca, K and Mg. In turn, Fe deficiencies can lead to Mn toxicity (Marschner, 1995). Alkalinity *per se* is not usually a direct constraint and generally influences pH-dependent factors such as nutrient availability (Adcock *et al.*, 2007). These authors reported that in south-eastern Australia the major chemical constraints for agricultural productivity associated with alkaline soils include low P and N availability, micronutrient (Zn, Cu, Mn, Fe) deficiencies and/or toxicities (e.g. excessive B, sodicity, salinity). While the concentration of most macronutrients was not affected by substrate pH (see Supporting Information: Figure S3), since plants grown at pH 10 were smaller, total macronutrient uptake was lower; therefore, growth restrictions associated with limited macronutrient uptake cannot be precluded. Macronutrient limitations have been claimed to be responsible for the negative effect of alkalinity on *Brassica juncea* (Javid *et al.*, 2012). Nitrogen is the main growth-limiting factor for most non-legume crops growing in alkaline soils, since more than 90% of the soil nitrogen is bound to organic matter and only becomes available after mineralization (Marschner, 1995). This means that in short-term trials, such as in the current study, growth relies mostly on available nitrate as the N source. As a result of extensive rinsing, neutralized soil was particularly deficient in nitrate (compare nitrate in Tables 1 and 2), which was partially replaced when pots were irrigated with nutrient solution. Nitrogen availability could not have been the cause of reduced growth, since growth was highest in neutralized soil with the addition of nutrients, and this soil had only about 25% of the nitrate content found in fertilized alkaline soil.

Micronutrient deficiencies, specifically Zn deficiency, are common in plants grown in alkaline soils (Rashid and Ryan, 2004). Accordingly, in these trials, Zn concentration was significantly lower in plants grown in alkaline substrate. On account of the fundamental role of Zn in protein metabolism, structural and functional integrity of biomembranes, photosynthetic C metabolism and IAA metabolism, Zn deficiency can cause significant inhibitions of plant growth and development (Cakmak, 2000) and may be partially responsible for the growth inhibition observed at high pH, even in nutrient-supplemented plants.

Vegetative growth is negatively affected by salinity in *P. coloratum* with adequate nutrient supply, as verified in experiments carried out in hydroponics by Pittaro *et al.* (2015), and also observed in the present study. Nutrient availability can affect the response to salinity, an example being that of *Triadica sebifera* plants in fertilized media showing more tolerance to saline conditions (Yang *et al.*, 2015). Accordingly, plants in soil should have expressed more sensitivity to salinity, since soil is poorer in nutrients than Hoagland solution. Yet, plant growth in soil was not affected by a level of salinity similar to the one in hydroponics, suggesting that the soil itself exerted negative effects on growth, masking the effects of salinity. This was most notably observed in plants grown in alkaline soil, where the negative effect of the substrate was very strong and no further negative effects of salinity were recorded. By contrast, in hydroponics, the salinity–alkalinity combination had more negative effects than either stress alone. Similar results have repeatedly been observed in plants grown in hydroponics (Shi and Sheng, 2005; Yang *et al.*, 2012; Wang *et al.*, 2015), but the physiological causes for the synergy remain to be investigated. These results highlight again that caution should be exerted when interpreting results to alkaline stress obtained only in hydroponic setups, as these only partially mimic the effects of alkaline soil.

Both high pH and waterlogging significantly reduced growth of these plants (Figure 1d), despite the reported flooding tolerance of *P. coloratum* (Imaz *et al.*, 2015), but there were no synergistic effects, indicating that negative effects of alkaline soils on growth could have been due to both high pH and oxygen deficiencies. Deficient aeration might have prevented the incidence of further negative effects of high pH, which may require normal oxygen pressure to be expressed. Alkaline soils exert a significantly negative effect on growth resulting from high pH and ion-exchange features that contribute to nutrient imbalance, along with deficient aeration and reduced hydraulic conductance. Experiments in hydroponics, using an aerated substrate and high nutrient concentrations, only mimic high pH. This may help to explain why effects of alkaline pH in hydroponics were always less remarkable than in soil, even when the soil was mixed with vermiculite to increase hydraulic conductivity and aeration.

Physiological causes potentially responsible for growth reductions in alkaline substrates

Carbon fixation, stomatal conductance and electron transport activity

CO_2 assimilation was positively correlated with stomatal closure, and the decrease in both variables was

positively related to growth impairment in plants grown in alkaline substrate (Figure 2). Measuring the quantum efficiency of light utilization by PSII can contribute to identifying non-stomatal causes for reduced carbon fixation under stress. Despite the extensive use of chlorophyll fluorescence kinetics for monitoring the photosynthetic machinery of plants under diverse stressful environmental situations, very few studies have addressed the impact of soil alkalinity on the functionality of the PSII electron transport activity. The photosynthetic performance index (PI_{ABS}) integrates parameters that contribute to overall photosynthesis (Strasser *et al.*, 2000); this index decreased in plants grown at high pH (Figure 3), in accordance with the alteration in photosynthesis observed under those conditions (Figure 2). The maximum quantum efficiency of PSII (Fv/Fm; Figure 3) was significantly reduced in plants grown under alkaline conditions in comparison with those grown in neutral pH soil. This parameter was linearly related to the quantum efficiency for CO_2 assimilation in leaves of C4 plants (Baker, 1993). The decrease in Fv/Fm may indicate that RCs were negatively affected and this may explain why all parameters related to RCs increased in plants grown at high pH (as shown on the left side of Figure 3). The reduction found in the variable fluorescence emission in the first step may have been responsible for the increment of REo/RC, suggesting that there was a significant impact on the donor side of PSI (Gururani *et al.*, 2015). These results highlight that alkaline was stress affected both stomatal and non-stomatal components of photosynthesis.

Oxidative stress and antioxidant activity

Root tips of *P. coloratum* plants grown under alkaline conditions showed increased superoxide production (Figure 5a). The plasma membrane-bound superoxide-generating NADPH oxidase complex is mainly responsible for apoplastic superoxide production in roots (Bustos *et al.*, 2008). This complex is a key player in the ROS signalling network that participates in growth and development regulation as well as in the responses to multiple stress conditions (Suzuki *et al.*, 2011). Increased activity of this complex may induce antioxidant activity. Yet, neither non-enzymatic nor the enzymatic antioxidants measured changed much in plants grown in alkaline substrate. Nevertheless, since these measurements were performed after plant exposure to alkaline conditions for at least 28 d, it may also be possible that initial responses leading to oxidative stress may have already been mitigated by the induction of antioxidant activity, which, in turn, may have led to a new steady state with low oxidative stress and antioxidant activity. Accordingly, induction of gene expression for antioxidants other than thioredoxins was not mentioned in *Lotus* plants exposed to alkaline stress for 21 d (Babuin *et al.*, 2014), supporting our interpretation. Disclosing the relationship between alkalinity and oxidative stress control would probably require sequenced samplings.

Conclusions

Germination and early plant survival were not the main limiting stages for reduced success of *P. coloratum* var. *coloratum* in alkaline soil. Observed growth limitations under alkaline conditions challenge the common notion that *P. coloratum* is tolerant to alkaline stress. Stomatal and non-stomatal conditioners of photosynthetic carbon fixation are in part responsible for the growth reductions imposed by alkaline substrates. Both oxygen and nutrient deficiencies, which characterize alkaline soils, contributed to growth restrictions under these conditions. As several features of alkaline soils contribute to the limitations these substrates exert on the growth of this species, individual amendments of any one of them would only have a limited effect on growth. The use of tolerant species may be the most reasonable approach for the productive inclusion of alkaline areas. Studies of plant response to alkaline substrates carried out in aerated nutrient solutions, with high nutrient concentrations, can only partially address the complexity of this stress.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Panicum coloratum* cv Klein seed germination and seedling survival in neutral and alkaline substrate. (A). Cumulative germination percentage in Petri dishes containing several layers of filter paper dampened with either distilled water (pH 7) or a pH 9 solution ($NaHCO_3$ 9 mM: Na_2CO_3 1 mM). Dishes were incubated at 28°C. Results are means ± s.e. of three dishes per treatment, containing 100 seeds each. (B). Cumulative germination percentage in pots containing soil pH 7·27 or pH 10·06, mixed with vermiculite (2:1, soil to vermiculite, on a volume basis). Results are means ± SE of two pots per type of soil, containing 150 seeds each. (C). Seedling survival in the above pots.

Figure S2. Cross sections obtained at 3 cm from the apex of roots of *P. coloratum* plants grown for 53 days in pots with soil pH 7 (A) or ~10 (B). Sections were stained with berberine hemisulphate and aniline, and observed under UV.

Figure S3. Ion concentration in leaf tissues of plants grown in pots containing soil pH ~10 or neutralized to pH 7, irrigated with nutrient solution for 37 days (Trial 3). No significant differences were detected in ion concentration in either type of soil, except for Ca.

Table S1. Formulae and glossary of terms used for the JIP test (modified from Strasser *et al.*, 2000; Hansatech, 2006 and Gururani *et al.*, 2015).

