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Salinity induced anatomical and morphological changes in *Chloris gayana* Kunth roots

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Key words: abiotic stress, tolerance, vascular tissue.

ABSTRACT: *Chloris gayana* Kunth is a grass species valuable as forage which was introduced into Argentina to be used as pasture in saline soils of subtropical and warm-temperate zones, given its good adaptability to drought, salinity and mild freezing. However, its tolerance varies according to the cultivar. In tetraploid cultivars, important reductions in yield have been observed. Here, a study of the variations produced on the root and stem system by salinity at different NaCl concentrations (0, 150 y 250 mM) was performed in the Boma cultivar, with the aim of determining the anatomical and morphological alterations produced by the salt excess. Plants cultivated with the highest level of salinity showed, in the whole, significant differences in the measured variables.

A diminution in absolute values of the variables and a major reduction in vascular tissue dimensions were observed, which suggests that the lack of tolerance to salt stress could be related to a deficient adaptation to absorb and transport water and nutrients from the roots.

Introduction

Environmental factors influence both shoot and root growth. In the first case, they affect both bud activation and growth rate (Bell, 1986; Huber, 1997). Likewise lateral roots quantity, development and function are affected in the radical system (Glinski and Lipiec, 1990; Hauser *et al.*, 1995; Lynch, 1995). As consequence, plant architecture is modified and so, its capacity to explore and acquire resources from the medium (Dong and de Kroon, 1994).

Among abiotic stresses, drought and salinity cause a reduction in hydraulic conductivity in plants (Peyrano *et al.*, 1997; Steudle, 2000). The stress imposed by salts excess is an important restriction for the productive use of lands (Sanderson *et al.*, 1997) as it reduces plant growth and productivity at a soil conductivity over 4.5 dS/m (50 mM) (Muscolo *et al.*, 2003).

High salt content, especially chloride and sodium sulphates, affects plant growth by modifying their morphological, anatomical (Kalaji and Pietkiewicz, 1993; Huang and Redmann, 1995) and physiological traits (Muscolo *et al.*, 2003). Such growth impairment is due to osmotic effects and ionic imbalances affecting plant metabolism (Greenway and Munns, 1980).

It is known that under stressful conditions, root system biomass decreases, however, detailed studies about morphological and anatomical changes of roots challenged to different saline conditions are scarce (Córdoba *et al.*, 2001).

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Salinity reduces root length and diameter (Neumann, 1995). Anatomically, it affects cell division and expansion processes (Kurth *et al.*, 1986; Zidan *et al.*, 1990), reduces the size of apical meristems, cortex and vascular cylinder. Additionally, stimulates exodermis and endodermis suberization (Reinhardt and Rost, 1995; Sanderson *et al.*, 1997; Ramos *et al.*, 2004) or the occurrence of atypical structures such as rhizodermis with phi-thickenings. (Degenhardt and Gimmler, 2000). The most common anatomical response to salinity is related to cell wall modifications. In cotton, an accelerated deposition of suberin in cells of the Casparian strip was observed (Wilson and Peterson, 1983; Reinhardt and Rost, 1995). Zhong and Lauchli (1993) found a rise in uronic acid along with lower cellulose content per unit of dry matter in cell walls of primary roots of cotton as effect of elevated salts levels. These alterations could be the cause of the change observed in the relative proportions of root tissues (Ramos *et al.*, 2004), which modify the shape of the organ and its function (Hauser *et al.*, 1995). At a molecular level it is known that several genes are expressed upon salt exposure and a number of proteins involved in salt-tolerance have been identified (Bohnert and Jensen, 1996).

Chloris gayana Kunth is a grass species valuable as forage which was introduced to Argentina to be used as pasture in saline soils of subtropical and warm-temperate zones (Burkart, 1969) given its good adaptability to drought, salinity and mild freezing (Bogdan, 1969; Duke, 1978). One of the known mechanisms of this species to cope with salinity is the presence of bi-cellular salt glands in its leaves which build up and excrete the sodium excess (Amarasinghe and Watson, 1989; Liphshitz and Waisel, 1974).

Due to the biological and agronomical importance of *C. gayana*, the study of its mechanisms of tolerance is of special importance (Kobayashi *et al.*, 2007). In this case, it exist a broad variability in function of cultivars. Significant reductions in the tetraploid cultivars productivities have been observed (Taleisnik *et al.*, 1997; de Luca *et al.*, 2001). Reduced yielding in these cultivars under salinity is manifested as a higher number of senescent leaves, a lower leaf area expansion and a minor number of stolons per plant (de Luca *et al.*, 2001).

Plant roots provide an ideal experimental system to investigate the effects of salinity on growth and other parameters given that, a) growth is restricted to a definite region, the millimetres immediately above the apical meristem, followed by a non-growing zone consisting of mature elongated cells located just some centimetres above the tip (Ishikawa and Evans, 1995);

b) root cells can be directly exposed to different NaCl concentrations by changing the root medium (Hilal *et al.*, 1998). For these reasons, we have chosen this system to deepen the knowledge of the responses of this halophyte growing under salt stress. In addition, there is almost no study addressing this kind of studies from a multivariate analysis perspective which allows correlating and discriminating the relevance of each variable involved.

Anatomical changes could compromise plant ability to conduct water and nutrients in high salinity. Ortega *et al.* (2006) observed a diminution of leaf protoxylem diameter in Rhodes grass leaves. Applying the law of Hagen-Poiseuille which relates water flux to the forth power of the xylem conduits radius, it can be deduced that a small variation in this value would imply a big increase in hydraulic resistance (Lewis and Bosse, 1995). A reduction in root hydraulic conductivity was observed by Peyrano *et al.* (1997) in tomato under salinity. Thus, we hypothesized that a reduction in vascular tissue dimensions more than any reduction in other tissue, would limit growth under salt stress conditions.

Thus, the aims of this work were determining, in tetraploid *C. gayana* roots, the morphological and anatomical alterations produced by salt excess (as NaCl) in the medium and whether they were correlated.

Materials and Methods

Plant material

Assays were carried out at the Juan Donnet Experimental Field of the Agronomic Sciences College of the Universidad Nacional del Litoral (Province of Santa Fe, Argentina). Seeds of the tetraploid cultivar "Boma" were germinated in a growing chamber at 26°C. After 48 h, plantlets were transferred to 4 L pots filled with perlite-vermiculite (3:1). Plants were watered daily with a half-strength Hoagland solution (Hoagland and Arnon, 1950). For plants subjected to experimental salinization, this solution was supplemented with NaCl in two final concentrations. Thus, three treatments were defined as: no NaCl added (control), 150mM NaCl (low NaCl concentration) and 250 mM NaCl (high NaCl concentration). Salinization began 5 days after seedling emergence watering with a 50mM NaCl supplement. From that time, daily 50mM increments were performed until the desired final NaCl concentration was reached. Salt concentration in the pots was monitored by conductivity measurement in the leaked excess solution.

After 40 days of treatments beginning, plants were harvested for morphological and anatomical studies. Roots were thoroughly washed and immediately immersed in FAA fixative solution (ethanol 50% [v/v], glacial acetic acid 5% [v/v], and formol 10% [v/v]) for 48 h; then they were stored in 70% (v/v) ethanol until use.

Segments from the absorption zone (1.5 cm from the tip) were sampled to perform permanent histological preparations (Berlin and Miksche, 1976). Shoots were oven-dried for 72 h at 90°C to obtain dry matter. After taking samples for morphological and anatomical studies, roots were oven-dried in the same manner.

Morphological parameters

Direct and derivative morphological variables were determined: shoot and root dry mass, total dry mass, shoot/root ratio, total root length according to Tennant (1975) and specific root length as the ratio between total root length and root dry mass.

Anatomical parameters

Digital images of the histological preparations were acquired using the Image-Pro Plus 7.0 Software Development Kit (SDK, © Media Cybernetics v. 6, Silver Spring CO., EEUU, 2000). In these, total area of each root cross-section, and areas corresponding to cortical parenchyma and vascular cylinder were measured. From these data, the ratio between vascular cylinder and cortical parenchyma areas was calculated.

Statistical analysis

An experimental design of completely randomized plots was run using between 9-12 plants per treatment. Four roots per plant were sampled for anatomical studies.

Data were analyzed through ANOVA using the Fisher least significant difference (LSD) test for comparisons of means (Sokal and Rohlf, 1971) using a 5% level of significance. The adjustment of errors to normal distribution was verified through the Shapiro-Wilks test and homoscedasticity was verified through the Levene test.

Given that all variables were measured on the same experimental units, multivariate analysis was applied in order to obtain an appropriate picture of the correlation and relative “weight” of each variable on the final results.

Thus, Principal Components Analysis, Biplot and Minimum Spanning Tree of the parameters were run to get a summary of the effects of salinity on the variables, as a whole.

Principal Components Analysis reduces the multivariate space to only two variables allowing, through these new variables (principal component 1 and 2), to discriminate those which influence the most the statistical results. It is a simplified way to explain the total variability of the set of samples.

Minimum Spanning Tree was plotted in the Biplot to show Principal Component Analysis or dimension reduction accuracy. Lines joining the experimental units

TABLE 1.

Effects of NaCl treatments on several growth parameters. Results are mean \pm SD; *n* was 9-12 per group. Different letters indicate significant differences ($P < 0.05$). Last row indicates the least significant difference (LSD) value determined for each parameter.

Treatment	Shoot dry mass (g)	Root dry mass (g)	Total dry mass (g)	Shoot/root ratio	Total root length (m)	Specific root length (m.g ⁻¹)
no NaCl added	0.46 \pm 0.15 a	0.14 \pm 0.05 a	0.60 \pm 0.19 a	3.27 \pm 0.58 a	8.18 \pm 2.01 a	60.74 \pm 14.7 a
Low NaCl concentration	0.30 \pm 0.13 b	0.10 \pm 0.04 b	0.40 \pm 0.17 b	3.04 \pm 0.60 a	5.96 \pm 1.80 b	64.29 \pm 16.4 a
High NaCl concentration	0.31 \pm 0.12 b	0.07 \pm 0.03 b	0.38 \pm 0.15 b	4.32 \pm 0.88 b	4.75 \pm 1.15 b	71.31 \pm 18.7 a
LSD	0.12	0.04	0.15	0.64	1.47	0.67

without crossing each other in the Biplot indicate a good dimension reduction, therefore a good explanation of the multivariate reality. Cofenetic correlation coefficient quantifies how good the dimension reduction through Principal Component Analysis was.

Statistical analyses were run using the InfoStat Professional (v. p2, Group InfoStat, 2006) software program.

Results

Morphological parameters

C. gayana 'Boma' biomass accumulation was reduced by salinity as seen from the significant lower values of shoot dry mass, root dry mass and total dry mass in low NaCl concentration and high NaCl concentra-

tion respect to no NaCl added (Table 1).

Total root length was notably reduced upon exposure to salt but, as with shoot dry mass, root dry mass and total dry mass did not show significant differences between low NaCl concentration and high NaCl concentration, despite the lower average value in high NaCl concentration.

When the effects were expressed as percentage of control (no NaCl added) shoot dry mass, root dry mass and total dry mass displayed diminishing values as salinity level increased but without statistical significance (Table 2). On the other side, shoot/root ratio decreased in low NaCl concentration but rose to about a significant 32% in high NaCl concentration reflecting the higher diminution of root dry mass against shoot dry mass upon the highest salinity.

On respect of specific root length, there were no

TABLE 2.

Changes induced by salinity in morphological parameters values. Results are expressed as percentages of control plants. Different letters indicate significant differences ($P < 0.05$; $n = 9-12$)

Treatment	shoot dry mass	root dry mass	total dry mass	shot/root ratio	total length	specific length
Low NaCl concentration	-34.78 a	-28.57 a	-33.33 a	-7.03 a	-27.14 a	+5.84 a
High NaCl concentration	-32.61 a	-50.00 a	-36.67 a	+32.11 b	-41.93 a	+17.40 a

TABLE 3.

Salinity-induced effects cross-sectional total root area, vascular cylinder; cortical parenchyma and vascular cylinder/cortical parenchyma ratio. Results are means \pm SD; n was 9-12 per group. Different letters indicate significant differences ($P < 0.05$). Last row indicates the least significant difference (LSD) value determined for each parameter.

Treatment	Total root area (mm ²)	Vascular cylinder (mm ²)	Cortical parenchyma (mm ²)	Vascular cylinder/cortical parenchyma
No NaCl added	0.59 \pm 0.17 a	0.10 \pm 0.03 a	0.42 \pm 0.14 a	0.25 \pm 0.06 a
Low NaCl concentration	0.53 \pm 0.14 b	0.08 \pm 0.02 b	0.39 \pm 0.11 ab	0.20 \pm 0.06 b
High NaCl concentration	0.46 \pm 0.16 b	0.06 \pm 0.02 c	0.34 \pm 0.14 b	0.18 \pm 0.05 b
LSD	0.07	0.01	0.06	0.03

significant differences between treatments; although a mild increase could be observed as salinity level increased (Table 2).

Anatomical parameters

Root cross-sectional area, sampled on about 1.5 cm from root apex, decreased as salt concentration increased (Table 3, Fig. 1). Although in absolute figures salt treatments were equivalent on this parameter, significant differences were detected between the relative percentages of diminution on respect to no NaCl added.

On the contrary, cross-sectional area of the vascular cylinder showed significant differences between all treatments (Table 3) decreasing from control to high NaCl concentration.

On regard to the parenchyma, only the highest salt level was significantly different to control. Low NaCl concentration was not different to either of the other treatments.

The vascular cylinder/cortical parenchyma ratio was affected by salinity as both levels of NaCl were different from no NaCl added (control plants). However, despite of the decreasing tendency, no differences were detected between salinity levels in absolute values (Table 3). In percentage values, there was a reduction in the vascular cylinder/cortical parenchyma ratio of a 20% and 28% for low NaCl concentration and high NaCl concentration respectively, reflecting the greater reduction for vascular cylinder compared to cortical parenchyma in the same treatments, (Table 4). This would indicate that the reduction in vascular cylinder and cortical parenchyma cross-sectional area are equivalent and proportional as salinity increases, at least at the sensibility of the experimental system of this study.

Multivariate analysis

A high cofenetic correlation coefficient (0.99) indicated a good projection quality of the observations in the plane of the selected components. The Principal Components explained about 89.4% of the total variation. Principal Component 1 explained a 74.6% of total variation and discriminated plants according to the assayed salinity levels. As it can be seen on Figure 2, control plants tended to cluster to the right, low NaCl concentration ones to the middle and high NaCl concentration to the left of the graph.

Considering Principal Component 1, there was no prevalence of any variable: all of them clustered around an eigenvalue of 2.5. Shoot dry mass and total length

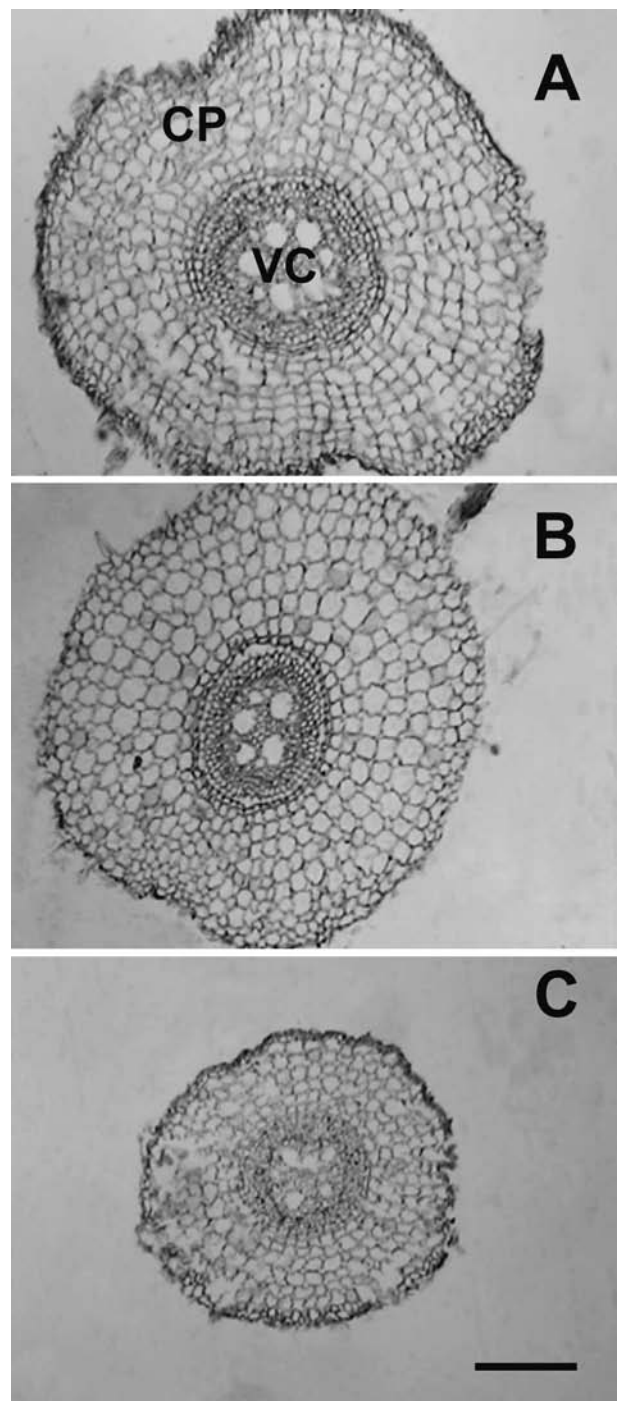


FIGURE 1. Effects of salinity on anatomical parameters. Representative cross-sections of permanent microscopic preparations of *Chloris gayana* roots. A: control roots; B: root under 150 mM NaCl (low NaCl concentration); C: root under 250 mM NaCl (high NaCl concentration). VC: vascular cylinder; CP: cortical parenchyma. Bar represents 200 μ m.

were highly correlated. On the other hand, the same situation was observed between total area and cortical parenchyma (Fig. 2). However, no correlation was detected between the former two and these later ones.

Considering Principal Component 2, shoot dry mass and total area are the variables which weighted the most, however as this Principal Component did not discriminate observations between treatments, and the variability explained is just about 16%, it was not considered for the analysis.

Discussion

According to their susceptibility to soil salt concentration, plants can be classified as halophytes or glycophytes (Hester *et al.*, 2001). The former are capable of absorbing and accumulating high salt levels in tissues through ion inclusion in vacuoles, synthesis of osmotic compounds (Gorham *et al.*, 1985) which protect against dehydration (Shinozaki and Yamaguchi-Shinozaki, 1999) and the existence of excretory glands

TABLE 4.

Changes induced by salinity in anatomical parameters values. Results are expressed as percentages of control plants. Different letters show significant differences ($P < 0.05$; $n = 9-12$).

Treatment	Total root area	Vascular cylinder	Cortical parenchyma	Vascular cylinder/cortical parenchyma
Low NaCl concentration	-10.17 a	-20.00 a	-7.14 a	-20.00 a
High NaCl concentration	-22.03 b	-40.00 b	-19.05 b	-28.00 a

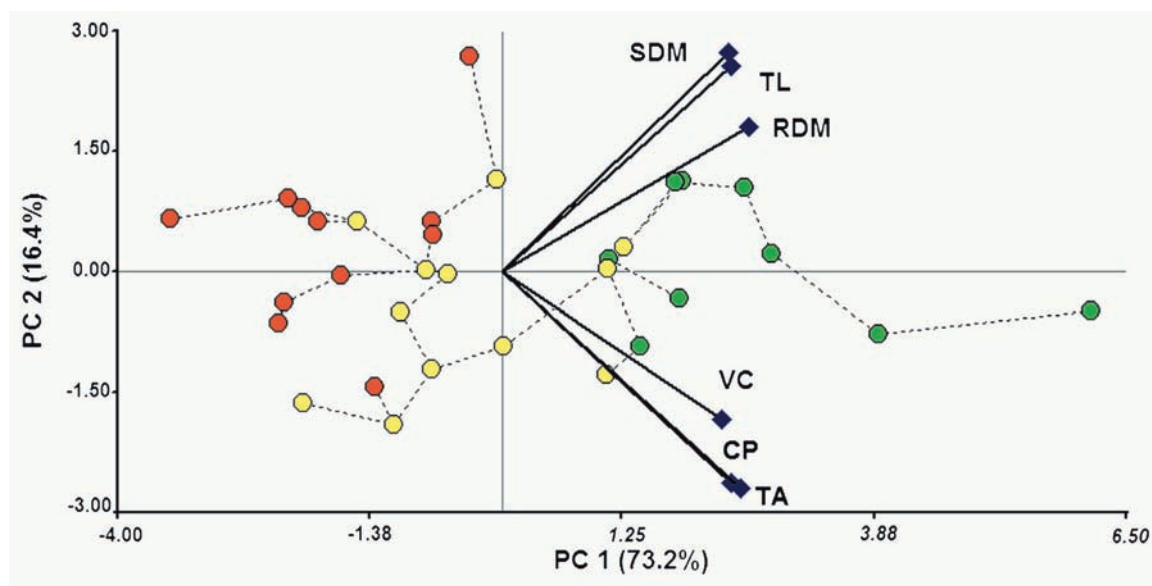


FIGURE 2. Principal Components (PC1 and PC2), Biplot graph (variables and observations) and Minimum Spanning Tree of the parameters (broken line between circles). Green circles: no NaCl added plants, yellow: low NaCl concentration plants, red: high NaCl concentration plants. SDM: shot dry mass, TL: total root length, RDM: root dry mass, VC: vascular cylinder area of cross-sectional root, CP: cortical parenchyma area of cross-sectional root, TA: total area of cross-sectional root.

located on the leaf surface (Luna *et al.*, 2002; Kobayashi *et al.*, 2007). On the contrary, glycophytes can not avoid ion entrance to tissues and experience the adverse effects of poisonous ions accumulation (Greenway and Munns, 1980). Only a few can avoid ion intake into tissues by osmotic adjustment, but in both kind of plants, the highest level of salinity tolerated is very low (Yeo, 1983). A gradient of salinity tolerance exists between both plant categories and the morpho-physiologic response depends on the particular species (Alshmmmary *et al.*, 2004).

As it has been seen in this work *C. gayana* biomass accumulation was significantly affected by salinity. Although growth continues even at high salt concentrations, shoot dry mass was significantly reduced (Table 1). Same response was observed by Muscolo *et al.* (2003) in kikuyu grass (*Pennisetum clandestinum* Hochst).

In coincidence with other studies in which root biomass rarely overcome shoot biomass (Agren and Ingestad, 1987; Baxter *et al.*, 1994; Canham *et al.*, 1996; Bolinder *et al.*, 1997; Ryser *et al.*, 1997; McConnaughay and Coleman, 1998), shoot biomass always over passed root biomass, in absolute figures (Table 1). The significantly higher shoot/root ratio at the highest salt level indicated that the accumulated dry mass in roots was more affected than the one in the shoot (Table 2). This reduction in shoot/root ratio would reflect that the mechanisms controlling shoot growth are less affected by salinity than those which control root growth. This pose an interesting trait to study whether this factor is one of the determinants of the higher sensibility to saline stress reported for *C. gayana* tetraploid cultivars (Taleisnik *et al.*, 1997; de Luca *et al.*, 2001; Luna *et al.*, 2002).

On the contrary to tetraploid cultivars, diploid ones possess a major tolerance conferred by resistance mechanisms (Guggenheim and Waisel, 1977; Russell, 1976).

Ortega and Taleisnik (2003) observed a diminution in elongation leaf rates of *C. gayana*, partially compensated with a longer elongation time. Similar effects were observed by Beemster y Masle (1996) in wheat growing in soils with high resistance to penetration. Total length decreased sensibly as medium salinity increased as seen before (Neumann, 1995). However, specific length was not reduced. In percentage, there was a tendency to a higher specific length in high NaCl concentration plants. This would show certain adaptability in this tetraploid cultivar (probably a longer elongation time) which would not be enough to compensate the negative effects of the saline environment. This adaptation (higher specific length) was also described by Miller

(1986), who pointed out that in higher plants exist mechanisms to cope with different stressful situations, namely root system slendering and expansion, changes in shoot/root ratio and an additional development of adventitial roots. Similar observations were done by Zwieniecki and Newton (1995), in plant roots growing under mechanical stress.

As some morphological parameters were negatively affected by salinity, some anatomical variables were diminished as well. Cortical parenchyma and vascular cylinder were reduced (Table 3), an effect that was observed in cotton (Reinhardt and Rost, 1995), perennial forages (Sanderson *et al.*, 1997) and *Pappophorum philippianum* Parodi (Ramos *et al.*, 2004). However, in our case the reduction was greater in the transport tissue (Table 4) supporting our hypothesis about the relevance of this effect on the ability of the plant to conduct water and nutrients in high salinity. This effect was previously observed in sorghum (Baum *et al.*, 2000) and rhodesgrass (Ortega *et al.*, 2006) leaves. In the latter, the leaf protoxylem diameter was a 65% lower in salinized plants being a probable limiting factor to hydraulic conductivity and therefore to tissue expansion. Xylem elements diameter and characteristics of the terminal cell walls are the main determinants of xylem hydraulic resistance (Tyree and Sperry, 1989; Davis *et al.*, 1999; Hacke and Sperry, 2001). Peyrano *et al.* (1997) observed a reduction in root hydraulic conductivity in tomato under salinity. However, Principal Component Analysis revealed that anatomical and morphological parameters were not correlated (Fig. 2). In fact, on the contrary to our expectations, the significant reduction in vascular cylinder did not have the same effect either on shoot nor root development (shoot dry mass and total root length). The positive correlation between the latter variables was some higher than the one between shoot dry mass and root dry mass (Fig. 2). The apparent "privilege" of shoot dry mass over root dry mass indicated by the higher shoot/root ratio would make soil exploration difficult for salinized plants.

It must be noted that anatomical observations were performed on the absorption zone which is just a small part of the entire root system. The obtained results may not reflect the salt-induced disturbances at organ level.

In conclusion, *C. gayana* Kunth tetraploid cultivar Boma showed a salinity-induced reduction in almost all morphological and anatomical variables recorded in this study. The lower shoot/root ratio would indicate a higher sensibility of root growth machinery against saline stress conditions. The increase in specific root length would not be enough to compensate the susceptibility of this cultivar to salinity.

In salinity, vascular cylinder/cortical parenchyma ratio was reduced, evidencing a low tolerance of this tetraploid cultivar to saline environments due to a lower capacity to conduct water and nutrients (vascular tissues were more affected than any other ones). Despite of this, significant differences were not detected in either assayed salinity levels on regard of this variable. Therefore, the existence of tolerance mechanisms to high salinity could not be ruled out.

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