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Title

Interspecific hybridization improves the performance of *Lotus* spp. under saline stress

Short running title

Improving saline tolerance of *Lotus* spp.

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Highlights

- Accumulation of ions associated to salt conditions differs between tissues and *Lotus* spp. genotypes.

- Accumulation of anthocyanins in stems of salt treated *Lotus* spp. plants is associated to Cl$^-$ levels.

- Interspecific hybridation could provide a tool to obtain cultivars with a high potential to use at saline environments.

Abstract

Salinity is one of the most frequent limiting conditions in pasture production for grazing livestock. Legumes, such as *Lotus* spp. with high forage quality and capable of adapting to different environments, improves pasture performance in restrictive areas. In order to determine potential cultivars with better forage traits, the current study assess the response to salt stress of *L. tenuis*, *L. corniculatus* and a novel *L. tenuis* x *L. corniculatus* accession. For this purpose, chlorophyll fluorescence, biomass production, ion accumulation and anthocyanins and proanthocyanidins levels have been evaluated in control and salt-treated plants PSII activity was affected by salt in *L. tenuis*, but not in *L. corniculatus* or hybrid plants. Analyzed accessions showed similar values of biomass, Na$^+$ and K$^+$ levels after salt treatment. Increasing Cl$^-$ concentrations were observed in all accessions. However, hybrid plants accumulate Cl$^-$ in stems at higher levels than their parental. At the same time, the levels of anthocyanins considerably increased in *L. tenuis* x *L. corniculatus* stems. Chloride and anthocyanin accumulation in stems could explain the best performance of hybrid plants after a long saline treatment. Finally, as

proanthocyanidins levels were no affected by salt, *L. tenuis* x *L. corniculatus* plants maintained adequate levels to be used as ruminant feed. In conclusion, these results suggest that hybrid plants have a high potential to be used as forage on salt-affected lands. High Cl⁻ and anthocyanins accumulation in *Lotus* spp. stems seems to be a trait associated to salinity tolerance, with the possibility of being used in legume breeding programs.

Abbreviations

NPQ, non photochemical fluorescence quenching; PA, proanthocyanidins; PSII, photosystem II; q_P, photochemical fluorescence quenching coefficients; ROS, reactive oxygen species; ϕ_{PSII}, quantum efficiency of PSII in light.

Keywords

LEGUMES, SALINITY, FORAGE, INTERSPECIFIC HYBRIDIZATION, ANTHOCYANINS

1. Introduction

*Lotus tenui*s Waldst. et Kit. and *L. corniculatus* L. are legume species that have been acknowledged worldwide for their high nutritious value as forage [1,2]. Although both species are phylogenetically close [3], they differ in their adaptability to restrictive environments. While *L. tenui*s become naturalized in the Flooding Pampa [2], characterized by halomorphic soils and periodic exposure to waterlogging [4,5], commercial cultivars of *L. corniculatus* display less relative tolerance to restrictive environments and are intended for soils with a better agronomical aptitude. In spite of this, the advantage of *L. corniculatus* cultivars lies in their higher yield potential and their moderate levels of proanthocyanidins (PA), which strongly affect their nutritional value [6,7]. Moderate levels of these metabolites in ruminant feeding prevent bloating and make plant protein utilization more efficient [7].

In forage breeding it is of great interest to obtain legume cultivars with a similar or better adaptability to restrictive environments than *L. tenuis,* along with moderate levels of PA as *L. corniculatus*. Interspecific hybridization has been previously used to obtain a *L. tenuis x L. corniculatus* hybrid with improved agronomic traits such as adequate foliar PA levels [8]. Beyond the hybrid vigor and growth differences, heterozygote plants generally display higher levels of tolerance to biotic and abiotic stresses than parental ones [9,10]. Based on this statement, the hypothesis of this work is that gene arrangement in *L. tenuis* x *L. corniculatus* hybrid could give rise to plants with greater tolerance to the saline conditions associated to marginal soils for agriculture.

Excess of NaCl in soil causes hyperosmotic stress and specific ion effects to most plants [11,12]. Salinity may cause nutrient deficiencies or imbalances due to the competition of Na⁺ and Cl⁻ with nutrients such as K⁺, Ca²⁺ and NO₃⁻ [13,14]. As a result, above- and below-ground

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biomass allocation may be affected by salt accumulation in plant tissues [15,16]. Several mechanisms have been described as the basis of salinity tolerance at the cellular and whole plant levels [16,17]. Among them, restriction of Na^+ and Cl^- accumulation in young leaves by using older leaves as sinks is thought to be important for salinity tolerance in glycophytes [16,18,19]. Vacuolar compartmentation of Na^+ and Cl^- at the intracellular level to avoid toxic concentrations within the cytoplasm has also been related to increased salt tolerance [16,20].

It has also been described that salinity and other abiotic stresses, like cold and drought, lead to the accumulation of anthocyanins and other flavonoids [21]. Furthermore, a role as ROS scavengers has been suggested for these colorful compounds in plant abiotic stress tolerance [22,23]. The species that belong to the *Lotus* genus show differences in PA and anthocyanins accumulation; indeed, the shoots of *L. corniculatus* accumulate higher levels of both flavonoids than those in *L. tenuis*. Its hybrid offspring shows intermediate levels of them [8]. Since PA also have antioxidant properties [24], they could play a role in stress tolerance. However, the relation between PA levels and salt stress responses has not been profoundly evaluated in species of the genus *Lotus*. In the present work ion accumulation and changes in PA and anthocyanins have been determined in different tissues of *Lotus* spp. with the objective to compare salt reponses of the *L. tenuis* x *L. corniculatus* hybrid plants with its parental. The interspecific hybridization between *L. tenuis* and *L. corniculatus* provides us with a tool to correlate PA and anthocyanins levels with tolerance to salinity, or to other biotic and abiotic stresses.

2. Materials and Methods

2.1. Plant material and growth conditions

Plant genotypes used in this work was a parental *L. tenuis* plant (from a commercial variety adapted to saline, alkaline and floodable soils from the Salado River Basin area, Argentine), a parental *L. corniculatus* plant (from wild population of saline areas at Devesa de El Saler, Spain) and *L. tenuis* x *L. corniculatus* hybrid plants obtained by the cross-pollination of both diploid species [8]. The manual collection of the parental material originating in Spain was carried out during summer 2009, in a similar manner to the procedure used for the collection of the seeds of *L. tenuis* in Argentina. The sexual cross for obtaining the interspecific hybrid and the clonal multiplication by nodal cuttings, were carried out following the usual methodology described previously [8,25]. Cuttings were transferred to a 30-cm^3 pot that contained a mixture of washed sand-perlite (1:1 V:V), which was irrigated throughout the experiment with 0.5 x Hoagland's nutrient solution [28]. Plants were grown in a chamber under controlled conditions using a 16/8 h photoperiod at 24 °C / 21 °C ± 2 °C (day / night) and at 55 / 65 ± 5 % relative humidity. Light intensity (250 μmol m^{-2} s^{-1}) was provided by Grolux fluorescent lamps (F-40

W). Treatments were carried out on 10-day-old plants for 21 or 35 days, duration of experiments were 31 and 45 days respectively.

2.2. Experimental design

The experiments followed a completely randomized design. All the accessions were evaluated under two conditions: i) Control, irrigated with nutrient solution without salt; ii) Saline, irrigated with nutrient solution plus 150 mM of NaCl. In order to avoid any osmotic shock due to saline treatment, plants initially received 50 mM NaCl and the concentration was then step-wise increased for 1 week (acclimation) until the final 150 mM concentration was reached [29]. There were four pots per treatment (each plant per pot was considered as one biological repetition, n = 4). We conducted three independent experiments in order to determine all parameters studies. PSII activity and biomass production were measured in all experiments to ensure reproducibility.

2.3. Chlorophyll fluorescence measurements

Photosystem II (PSII) activity was estimated by non invasive fluorescence measurements before collecting the plant material. Measurements were taken in a growth chamber on intact leaves with a portable fluorometer (PAM 2000, Heinz Walz, Effeltrich, Germany). Leaves were pre-darkened for 20 min before starting the experiment, and were then excited with a weak measuring beam to obtain minimum dark fluorescence yield (F_o). A 1.3-second saturating pulse of white light was then given to determine the maximum fluorescence yield when all the PSII reaction centers were closed in the dark (F_m). The fluorescence-measuring light was removed for 5 min prior to beginning irradiance.

The maximum fluorescence in actinic light (F'_m) was obtained by giving a 1.3-second saturating pulse. The steady-state components of fluorescence were assumed to be nearly complete after 10 min at the lowest intensities or 7 min at all the other photon flux densities. Therefore, a saturating pulse of white light was given after a 10- or 7-minute period following each stepwise, and by increasing the photon flux density to determine F'_m.

The fluence utilized during the fluorescence induction kinetics was about 1200 µmol m^{-2} s^{-1}. Twenty pulses of saturating white light were applied at 60-second intervals during actinic illumination to determine F'_m. The minimum light fluorescence yield (F'_o) values were determined during a brief interruption of actinic illumination in the presence of far-red illumination, which should preferentially excite PSI [30].

The quantum efficiency of PSII in light was determined as $\phi_{PSII} = (F'_m - F_t)/F'_m$, where F_t estimates the fluorescence yield during illumination. Non photochemical fluorescence

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quenching was determined according to the Stern–Volmer equation: $NPQ = (F_m/F'_m)-1$. The photochemical fluorescence quenching coefficient, q_p, was calculated from $q_p = (F_t-F'_o)/(F'_m-F'_o)$. The reduction state of the quinone pool was calculated from $1-q_p$. The electron transport rate was estimated from the *in situ* photon flux density and the quantum efficiency of PSII.

2.4. Biomass measurement

Tissues from harvested plants were divided into four groups: new and old leaves, stems and roots; and their dry matter was determined after drying at 60 °C until constant weight. The weight of shoots was calculated as the addition of the weight of new and old leaves and stems, and the total dry weight considering all tissues.

2.5. Analytical determinations

A 10-mg aliquot of dried material was used to estimate the Na^+ and K^+ concentrations by standard flame photometry [31]. Chloride was determined by a thiocyanate-Hg-based colorimetric reaction [32]. For this purpose, 12.5 mg of powdered dry plant material were extracted in 0.5 ml of a solution that contained H_2O_2 (30 %) : concentrated HNO_3 : isoamyl alcohol : H_2O at 1 : 1 : 0.08 : 7.9 (V : V), incubated at room temperature for 15 min, diluted to 5 ml with Milli-Q water, and vigorously agitated by vortexing. Then 1.5 ml of the extraction mixture were centrifuged (10 000 rpm, 5 min) and the supernatant was transferred to another Eppendorf tube. The colorimetric reaction solution contained polyethylene glycol dodecyl ether–water (Brij 35®, 4 %): mercuric thiocyanate (4.17 g l^{-1} methanol) : $(NO_3)_3Fe$ (202 g l^{-1} Milli-Q water, plus 21 ml of concentrated HNO_3) : Milli-Q water at 0.05 : 15 : 15 : 70 (V : V). One milliliter of this reaction was added to 320 μl of the supernatant (control treatment). With the saline treatments, 50 μl of the supernatant were previously diluted to 320 μl with the extraction solution. Sample absorbance was determined at 450 nm by a spectrophotometer (Hitachi U-1100) and was interpolated into a KCl calibration curve (0, 5, 10, 15 and 20 ppm) to calculate the Cl^- concentration.

Anthocyanins were extracted from 100 mg of frozen ground matter with 3 ml of 0.1 % HCl : methanol solution for 60 min at room temperature. Then 0.75 ml of water and 2 ml of chloroform were added to 1 ml of extract. Finally, anthocyanins and PA were extracted and quantified as described [8] .

2.6. Statistical analysis

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Data were subjected to a two-way analysis of variance with the following factors: Lotus accession (three levels: *L. corniculatus*, *L. tenuis* and hybrids plants) and Salinity (two levels: 0 and 150 mM NaCl). The Shapiro-Wilk test to determinate normality and Levene`s test to assess the equality of variances were used. Duncan`s test was used for multiple comparisons ($P <$ 0.05). For the non parametric data, the Kruskal-Wallis *H* test, followed by a paired comparison for the Lotus x Salinity factor, were used. Analysis were performed with the Infostat software tool [33].

3. Results

3.1. *Lotus* spp. accessions respond phenotypically different to saline stress

Reduced growth was observed in the genotpyes of *Lotus* spp. studied after 21 days of exposure to 150 mM NaCl. However, after 35 days of salt treatment symptoms were clearly different between genotypes (Fig. 1). In both parental, the saline treatment induced senescence in the basal leaves of shoots. Moreover, salt induced senescence progressed differently in each species: *L. tenuis* leaves showed earlier senescence from the basal to the apical leaves, and finally to the shoot apex, leaves became necrotic and remained on the stem (Fig 1A), whereas *L. corniculatus* leaves remained green and were detached from the stem in a posterior phase (Fig. 1C).In contrast, all the evaluated hybrid plants did not show any symptoms of senescence after 35 days of saline treatment (Fig. 1B). The differential accumulation of ions in the leaves would trigger their fall-up process, which is noteworthy in *L. tenuis*. This trait would act as a complement to other plant stress response mechanisms, which would also have different relevancy for each vegetal accession.

Chlorophyll fluorescence measurements were taken to estimate PSII activity after 21 days of treatment. Under salt stress, hybrid plants showed a higher maximum fluorescence yield *F*v/*F*m value than control non stressed plants (Table I). However, no significative differences were observed as consequence of salt treatment in both parental (Table 1). Differences were observed among accession only under saline conditions; the *L. tenuis* plants had a lower F*v*/F*m* than the *L. corniculatus* or hybrids plants (Table I). The photochemical fluorescence quenching coefficients (q$_P$) were similar in the control and salt exposed plants. Similar q$_p$ values were also observed among plant accessions under both conditions. Lower q$_P$ values were observed only in the *L. corniculatus* control plants (Table 1). Saline stress decreased the quantum efficiency of PSII in light (ϕ_{PSII}) and increased non photochemical fluorescence quenching (NPQ) in the *L. tenuis* plants. On the contrary, the *L. corniculatus* or the hybrid plants subjected to saline stress showed similar ϕPSII and NPQ to the control plants (Table 1).

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Finally, evaluation of the dry weight from different *Lotus* spp. plant tissues after 21 days exposure to salt was carried out. Under the control conditions, hybrid plants exhibited a dry weight higher than *L. corniculatus* and similar to *L. tenuis*. The main differences came principally from the stems and, in a smaller proportion, from the roots of hybrid plants (Appendix S1). Under saline treatment, all the plants produced between 45-55 % less total dry matter compared with the control condition (Appendix S1). Stems (60-74 % reduction) and old leaves (40-56 % reduction) were the tissues more affected by saline stress. The roots of the *L. corniculatus* and *L. tenuis* accessions were not significantly affected. However, roots of the hybrid plants under the salt condition had a light reduction of dry weight.

3.2. Ion accumulation in the saline treatment differs between tissues and accessions

Na^+, K^+ and Cl^- concentrations were determined in the different tissues harvested from *Lotus* spp. in both the control and salt-exposed plants. Because of saline treatment, Na^+ and Cl^- levels increased in all tissues from different accessions evaluated (Table 2). Meanwhile, the K^+ concentration were lower in aerial tissues from salt-treated plants. However, the saline treatment affected K^+ levels only in the roots of *L. tenuis* plants (Table 2).

Under control conditions, Na^+ levels were less than 5 mg g^{-1} of DW, only stem samples were slightly higher (Table 2). Under salt stress, the old leaves generally had a higher Na^+ concentration than any other tissue; the Na^+ levels in both stems and roots were markedly lower than in leaves (Table 2). The differences observed among genotypes as a result of salt stress in Na^+ levels were observed mostly in the young leaves. The new leaves of the saline-treated hybrid plants showed a similar Na^+ concentration to its *L. corniculatus* parental; whereas *L. tenuis* had lower Na^+ levels (Table 2). In roots of hybrid plants under saline conditions, the level of this ion was higher than *L. corniculatus* ones, but similar to *L. tenuis* samples (Table 2).

The K^+ concentration under the control conditions was variable in both tissues and accessions (Table 2). Higher K^+ levels were observed in leaves, being higher in old leaves (Table 2). Roots showed a lower concentration of K^+ among tissues, less than a half than the old leaves (Table 2). However, when plants were subjected to salt stress, the old leaves tissue was markedly affected (reduction of around 70%) and showed lower K^+ values in relation with other tissues. After salt stress, the K^+ levels in stem tissue were higher than in the other tissues. In general, *L. corniculatus* had higher K^+ levels in the aerial tissues of the salt-treated plants than those from *L. tenuis* and the *L. tenuis* x *L. corniculatus* plants under similar conditions (Table 2). Under the control and saline conditions, the *L. corniculatus* roots had lower K^+ levels than those of other accessions (Table 2).

The tissues of the control plants had a low Cl^- concentration (under 6 mg g^{-1} of DW), roots and stems had a slightly lower Cl^- concentration than leaves (Table 2). Under stress, new

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leaves and roots showed similar Cl^- concentrations; old leaves and stems also showed similar but higher Cl^- concentrations (Table 2). Chloride accumulation under stress was 2.2- to 3.4-fold greater in the old than in the new leaves (Table 2). In particular, the samples taken from the *L. tenuis* plants under saline stress showed the larger difference between the young and mature leaves (Table 2). Similar Cl^- levels were also observed in the new leaves of the different plant accessions subjected to salt treatment. However, the Cl^- levels in the old leaves were higher in *L. tenuis* than in the *L. corniculatus* or hybrid plants. The most outstanding results were observed for the Cl^- concentrations in stems, where the level of this anion markedly increased due the saline treatment (Table 2).

Under salt stress, plants were exposed to equal amounts of Na^+ and Cl^-. However, the accumulation of these ions did not maintain this equality as showed at the Na^+ meq g^{-1} DW / Cl^- meq g^{-1} DW biplot (Fig. 2). Well-differentiated groups were observed in the Na^+ / Cl^- biplot depending on the tissue (Fig. 2). On the one hand, the Na^+ concentration was higher in the old than in the new leaves, but stems and roots showed lower concentrations (Table 2). On the other hand, the Cl^- concentration was higher in stems and the old leaves than in the new leaves and roots (Table 2). In general, new leaves accumulated more sodium than chloride; on the contrary, stems accumulated more chloride than sodium (Table 2). In old leaves and roots of stressed plants, the Na^+ and Cl^- concentrations were proportionally similar (Fig. 2).

Among *Lotus* spp. accessions, differences in Na^+ / Cl^- ratio were observed mostly in stems and at a lower extension in old leaves (Fig. 2). *L. tenuis* differentiates of the others accession due to Cl^- accumulation (Table 2) that lead to a lower Na^+ / Cl^- ratio in old leaves (Fig. 2). The Na^+ / Cl^- biplot grouped stems as the most particular tissue for ions accumulation in the NaCl-treated plants (Fig. 2). Higher Cl^- levels in the stems of the treated *L. tenuis* x *L. corniculatus* plants than in their parental were observed (Table 2). In turn, the *L. tenuis* stems showed higher levels than *L. corniculatus* (Table 2). The Na^+ / Cl^- ratio was lower in the hybrid plants than in *L. tenuis*, and both accessions gave lower ratios than *L. corniculatus* (Fig. 2).

3.3. Anthocyanins, but not PA, modified their accumulation under salt stress

Anthocyanins were measured spectrophotometrically in extracts of stems from *Lotus* spp. plants. However, no anthocyanins were detected in leaves or roots. In the absence of salt, the levels of anthocyanins were higher in stems of *L. corniculatus* than of *L. tenuis*. The *L. tenuis* x *L. corniculatus* plants showed intermediate levels. Despite these differences, under salt stress the hybrid accessions increased the anthocyanins concentration until the levels observed in *L. corniculatus*. It was indeed outstanding that *L. tenuis* x *L. corniculatus* increased their

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anthocyanin levels by around 3-fold in the salinized plants. However, *L. corniculatus* increased only 1.7-fold. The *L. tenuis* plants showed a similar increase in anthocyanins to the interspecific hybrid, but its levels were still lower than other accessions.

The PA levels were quantified on both leaves and stems. In general, the PA concentration was much higher in *L. corniculatus* than in the *L. tenuis* samples, while the *L. tenuis* x *L. corniculatus* samples showed intermediate values between both parental (Appendix S2). The PA levels in the young leaves were not affected by saline stress. In the mature leaves of *L. corniculatus* or the hybrid plants, the PA levels remained unaffected. However in the old leaves of *L. tenuis,* a reduction (62 %) in the PA levels was observed. Finally, in stems, saline stress led to lower PA levels of the *L. corniculatus* and *L. tenuis* x *L. corniculatus* accessions (66 % and 58 %, respectively), but no differences were detected in the stems of the *L. tenuis* plants (Appendix S2).

4. Discussion

In the last few decades, the proportion of cultivated superficies in agronomical areas from South America (i.e. Pampa Grasslands) has increased. Nevertheless, the increase in soils used for agriculture has forced cattle production to be displaced to more restrictive soils like saline-affected areas [34,35]. At the same time, soil and water salinization in many agricultural areas has resulted in the "biosaline agriculture" concept, in which grazing livestock has been paid much attention as a means to exploit such areas [36]. However, biosaline agriculture has to take into account that plant growth and, therefore, forage production are affected by saline stress to a greater or lesser extent. For these reasons, obtaining cultivars with improved quality and performance under saline conditions is interesting in plant breeding [37]. In the present work the performance under the saline conditions of two forage legumes of the *Lotus* genus (*L. tenuis* and *L. corniculatus*) and its hybrid progeny is evaluated.

L. tenuis and *L. corniculatus* are considered salt-tolerant glycophytes, displaying *L. tenuis* a higher relative tolerance to saline conditions than the commercial cultivars of *L. corniculatus* [38,39]. However, some authors criticize that the adaptability of *L. corniculatus* to constrained environments has been largely affected in commercial cultivars because breeding programs focus on dry matter production and persistence under non restrictive conditions [2]. For this reason, a wild *L. corniculatus* accession grown in extremely alkaline-saline areas of Spain was selected in a previous work [8]. Given its adaptation to a restrictive environment, a better tolerance to abiotic stress in the wild *L. corniculatus* accession than those of the commercial cultivars was expected.

PSII activity, estimated by chlorophyll fluorescence measurements, was altered in the *L. tenuis* plants after 21 days of treatment with 150 mM of NaCl, but not in the *L. corniculatus* and

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L. tenuis x *L. corniculatus* accessions (Table 1). The ϕ_{PSII} parameter used as an indicator of overall photosynthesis [40]. decreased in the *L. tenuis* plants under salt stress (Table 1) suggesting that this specied could lose carbon fixation efficiency. Higher NPQ values were observed in the salt-exposed *L. tenuis* plants compared with the control plants (Table 1). An anticipatory induction of NPQ has been described as a plant adaptive response to dissipate excess excitation energy as heat. This photoprotection mechanism helps to minimize ROS production, and consequently protects from oxidative stress [41,42]. It is worth mentioning that three major processes determine NPQ: energy-dependent quenching (qE), state-transition quenching (qT), and photohinibitory quenching (qI); the last process is associated with the quenching caused by photoinhibition [42]. Higher NPQ values, due to the qI component, could also indicate damage to PSII. It has been postulated that saline stress affects Rubisco activity by reducing CO_2 fixation and, hence, inducing ROS generation [43]. The increased NPQ values observed under the saline conditions (Table 1) suggest that salt induces ROS generation in the *L. tenuis* plants as either a photoprotection mechanism or by indicating photodamage. Finally, even if the maximum fluorescence yield (*F*v/*F*m) was not significantly affected, a tendency to diminish through the effect of salt was observed in *L. tenuis* (Table 1).

Although shoot biomass production under salinity has been used as a single trait to obtain tolerant plants in traditional breeding programs, its success has been limited because other traits associated with salt tolerance were left aside [44]. The evaluated *Lotus* spp. accessions showed a similar shoot biomass under saline conditions (Appendix S1). Nevertheless, this does not mean that they respond similarly to salt, since other parameters as PSII activity or ion accumulation differ amog accessions, especially under long-term salt exposure. It is noteworthy that plants respond to saline stress in two phases: i) the osmotic phase and ii) the ion-specific phase. In the osmotic phase, salt outside roots causes osmotic effect and reduced water uptake by plants and, consequently, a lower growth rate. Depending on the plant species, the osmotic phase of saline stress occurs on the first days or in the first weeks to salt exposure, and its effect is comparable to drought stress. Probably the first phase of saline stress equally affected all the evaluated accessions, and resulted in a similarly reduced total biomass (Appendix S1). To ameliorate tolerance to the osmotic stress brought about by salt, some plants increase compatible osmolytes, such as proline, sucrose or glycine betaine [44]. Although proline concentration rises in *L. corniculatus*, *L. tenuis* and *L. japonicus* in response to salt conditions, this parameter has not been correlated with saline tolerance in these species [27,39,45]. Indeed metabolite profiling done on *L. creticus*, *L. tenuis* and *L. corniculatus* subjected to saline stress has demonstrated a similar metabolic response among species, regardless of its saline tolerance [39]. The ion-specific phase occurs when ions accumulate at toxic concentrations [16]. In fact the effect of the ion-specific phase on the *L. tenuis* plants became more evident after a longer period (35 days under saline treatment), when the majority

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of the *L. tenuis* plants showed senescence symptoms that progressed from the basal to the apical leaves, and finally to the shoot apex, only the secondary shoots remained alive (Fig. 1A). The *L. corniculatus* plants were less affected, although a large proportion of the old leaves was detached (Fig. 1C). Interestingly, *L. tenuis* x *L. corniculatus* plants did not showed senescence symptoms after long period of salt treatment (Fig. 1B). Such evidence allows us to assume that in *Lotus* spp., the major differences in saline tolerance occur in the ion-specific phase.

Since the *L. corniculatus* genotype used herein came from an extremely saline environment, and it is possible that its high tolerance to salt stress was due to developed anatomical adaptations to grow under these constrained conditions and *L. tenuis* has also been considered a tolerant glycophite [39]; the genetic pool displayed by both *Lotus spp.* are invaluable to improve salt stress tolerance in the genus. In fact, it has been previously demonstrated that the *L. tenuis* x *L. corniculatus* hybrid plants outperform the traits of their parental and present improved nutritional characteristics to forage utilization [8]. Interestingly under the saline conditions, the hybrid plants also seemed to overcome their parental because no senescense symptoms were observed after the long-term treatment (Fig. 1B).

The improved performance of the hybrids plants under the saline conditions could be partially explained by a differential accumulation of Na^+ and Cl^- ions at the ion-specific phase. In this sense, sensitive glycophyte plants are more affected since they are unable to exclude ions and accumulate them up to toxicity, specially in old leaves due to the transpiration stream [16].

Salt tolerance in legumes is strongly associated with Cl^- exclusion [15,46]. This is also valid for the *Lotus* genus, including the sensitive genotypes that accumulate higher levels of Cl^- [38,47]. However, these previous studies evaluated Cl^- accumulation in shoots, with no differentiation between tissues.The results of this work suggest that the *Lotus* spp. plants differentially regulated Cl^- accumulation among shoot tissues (Table 2 and Appendix S3). All the *Lotus* spp. accessions subjected to saline conditions had a higher Cl^- concentration in stems than in leaves, and maintained lower concentrations in new than in old leaves (Table 2 and Appendix S3). Interestingly, the *L. tenuis* x *L. corniculatus* plants accumulated a higher Cl^- concentration in stems than their parental (Table 2 and Fig. 2). *L. tenuis* plants accumulted intermediate Cl^- concentration in stems. However, despite the differences in concentration, the total level of this anion was similar among *L. tenuis* and hybrid plants (Appendix S3). Although concentration and total level of Cl^- were similar in new leaves of salt-treated plants, *L. tenuis* accumulated higher Cl^- levels in old leaves (Table 2 and Appendix S3). No significant differences were observed for the Na^+ and Cl^- concentrations in the roots of the salt-exposed plants (Table 2). However, the *L. tenuis* x *L. corniculatus* plants displayed a tendency to accumulate slightly higher concentrations of both ions (Table 2). This accession gave a heavier root weight under saline conditions than its parental (Appendix S1). Furthermore, when considering both weight and the ion concentration, the final quantity of both ions retained in the roots of the hybrid

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plants were 6.5 for Na^+ and 12.6 mg for Cl^-, while these amounts were smaller in *L. tenuis* (4.7 and 8.0 for Na^+ and Cl^-, respectively) and *L. corniculatus* (2.4 and 4.6 for Na^+ and Cl^-, respectively) (Appendix S3).

Tissue accumulation of ions, seems to be a differential response among *L. corniculatus* and *L. tenuis* plants when exposed to salt. On one hand, *L. corniculatus* displayed low accumulation of Cl^- and Na^+ in all tissues (Appendix S3) what could suppose a more efficient "exclusion" mechanism. Ion "exclusion", defined as "the ability of plants to prevent root uptake of Cl^- from the soil and subsequent transport in the xylem to the shoot from shoots" has also been described as saline tolerance adaptation in many plant species [16,44,46]. On the other hand, in *L. tenuis,* which is also a tolerant glycophite, the accumulation of both ions, and particularly Cl^-, was high in stems (Appendix S3), indicating that location of Cl^- in this tissue helps to maintain lower levels in leaves. . The performance of hybrid plants exposed to salt stress could be due to the fact that hybrid plants share responses from both parental. In this sense, Na^+ total levels in new leaves were similar between *L. corniculatus* and hybrids, but higher than in *L. tenuis*. At the same time, Cl^- total levels in old leaves and Na^+ in stems were similar between *L. corniculatus* and hybrid, but lower than in *L. tenuis*. On the other hand, Cl^- levels in stems of hybrid plants were similar to *L. tenuis* but higher than in *L. corniculatus* stems (Table 2 and Appendix S3).

Other response similar to *L. corniculatus* plants (a relative more tolerant parental) is the concordance between the induction of anthocyanin biosynthesis (Table 3) and Cl^- levels in stems (Table 2). In fact, *L. tenuis* x *L. corniculatus* plants showed a marked increase in the anthocyanin concentration, with similar levels to *L. corniculatus* stems (Table 3). Although the anthocyanin levels of *L. tenuis* rise due to saline stress, the concentration of this metabolite remains much lower than in other accessions (Table 3), and agrees with its relatively poorer performance under salt stress.

Antocyanins and other phenolic compounds acts as scavenging molecules to alleviate oxidative stress [23,49]. Induction of anthocyanin biosynthesis has been reported under saline conditions [21]. Some authors have suggested a relation between salt tolerance and anthocyanin levels due to their protecting effect against the oxidative damage produced by excess ROS [22]. These observations suggest that the accumulation of anthocyanins could play a role in saline tolerance mechanisms from *Lotus* spp. The evaluation of anthocyanin levels under salt stress in *Lotus* spp. has not been reported to date. However, since anthocyanin-related genes are induced by cold in *L. japonicus* [50], more studies should be carried out to evaluate the role of anthocyanin in the abiotic stress responses in this genus.

Similarly to anthocyanins, PA have been functionally described as potent antioxidant molecules [24]. The PA levels also vary among accessions, although its main accumulation occurs in leaves (Appendix S2). However, the PA concentration was not affected by saline treatment in the leaves of the PA-rich accessions (Appendix S2). Unlike anthocyanins, the PA

concentration in stems was lowered by salinity in the *L. corniculatus* and hybrid plants (Appendix S2). Since transcriptional shift regulation has been described between PA and anthocyanin metabolic pathways [51], the repression of PA biosynthesis to favor the anthocyanin flux would lower PA contents and increase anthocyanins levels in stems. This would suggest that anthocyanins more than PA are involved in salt stress tolerance.

In conclusion, the *L. tenuis* x *L. corniculatus* plants displayed the best performance under saline stress than their parental. This could be explained, at least in part, by the combination of responses from both parental displayed by the hybrid under salt stress. Moreover, the obtained results suggest that salt-induced anthocyanin accumulation in stems may play a role in saline tolerance, indicating that studies on anthocyanin metabolism and its regulation under stress conditions are needed. The new gene assortments obtained by the interspecific cross between *L. tenuis* and *L. corniculatus* has led to the generation of novel genotypes with a high potential to be used as forage on salt-affected lands.

Author contributions

FJE and CJA performed the saline assay and the plant material harvest; FJE took the chlorophyll fluorescence measurements, and ran the ion quantification and determination of the anthocyanins and proanthocyanins levels; FJE and CJA ran the statistical analyses; FJE, CJA, PCS and OAR wrote the paper; FJE, PCS and OAR conceived and designed the research. All the authors read and approved the manuscript.

Acknowledgments

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Figure legends

Figure 1. The *Lotus* spp. accessions cultivated with the control (with no NaCl added) and saline (150 mM of NaCl). Treatments were applied to 10-day-old plants for 35 days. **A.** *Lotus tenuis*; **B.** *L. tenuis* x *L. corniculatus*; **C.** *L. corniculatus*.

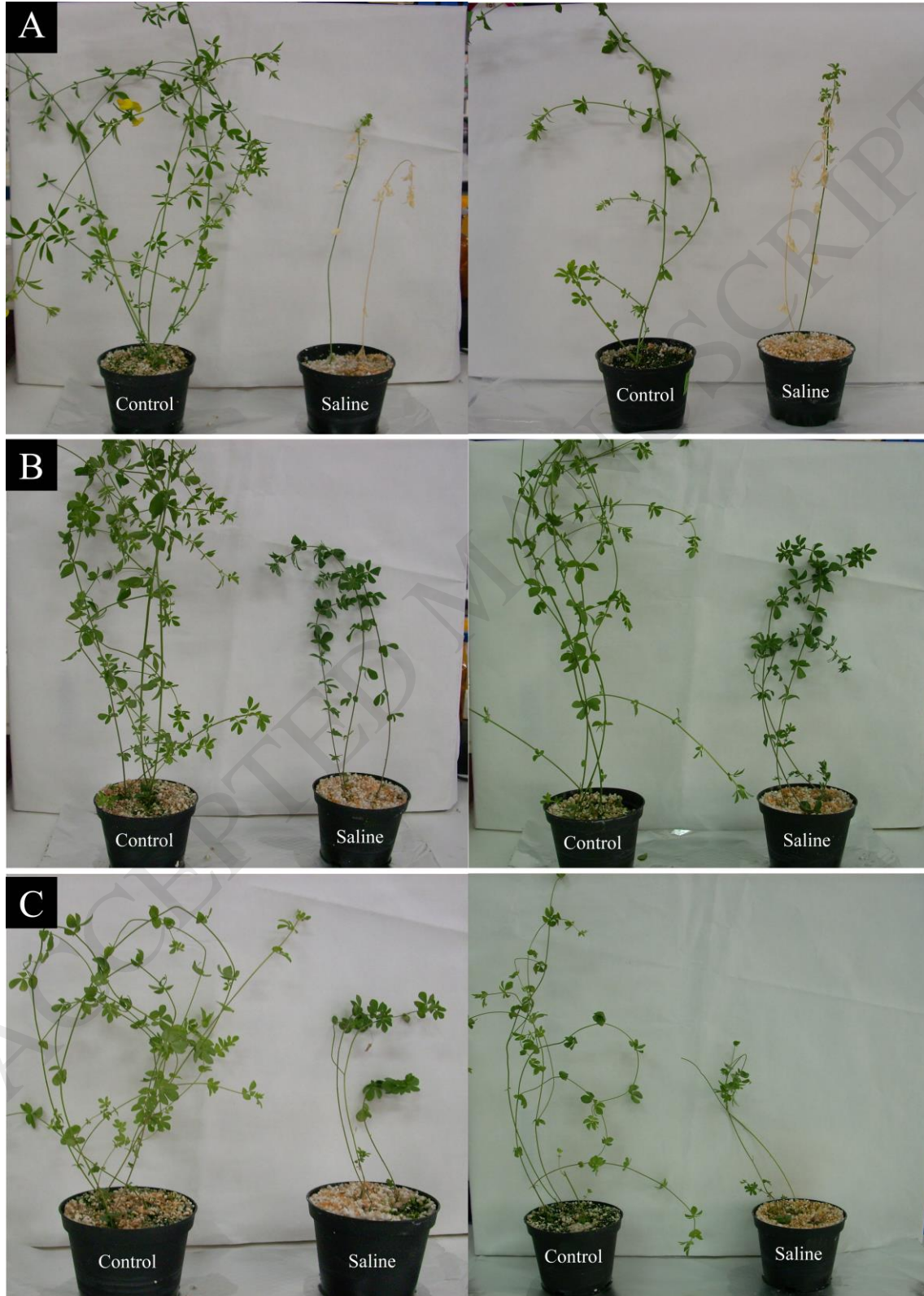**Figure 2.** The Na^+ / Cl^- biplot from the saline-treated plants. The means of meq Cl^- g^{-1} of DW $\pm$ SD were shown according to the means of meq Na^+ g^{-1} of DW $\pm$ SD. Square symbols are the means of the new leaves, diamonds of the old leaves, circles of stems and triangles of roots. The plus symbol indicates the mean of each tissue.
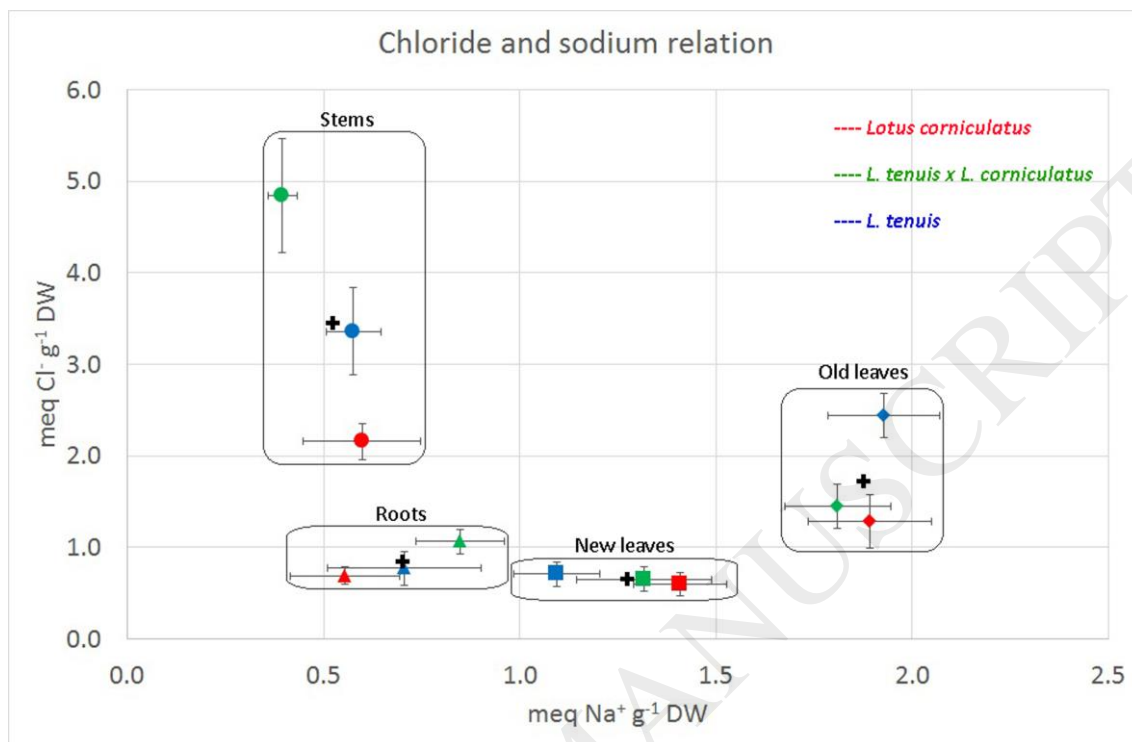
Table 1. Fluorescence parameters measured in the *Lotus* spp. plants with the control and saline treatments. Treatments were applied to 10-day-old plants for 21 days. Values are means (n = 6) ± SD. Within each number block, the means with a similar letter do not differ significantly ($P <$ 0.05), based on Duncan`s test. Fv/Fm, maximum fluorescence yield; q_P, photochemical fluorescence quenching; ϕ_{PSII}, quantum efficiency of PSII in light; NPQ, nonphotochemical fluorescence quenching.

Fluorescence parameter	Accession	Control			Saline			Control vs. Saline (P-value)
Fv / Fm	*L. corniculatus*	0.728	±	0.061 a	0.765	±	0.036 b	0.1422
	Lt x Lc	0.724	±	0.051 a	0.766	±	0.037 b	0.0065
	L. tenuis	0.730	±	0.060 a	0.696	±	0.056 a	0.2546
q_P	*L. corniculatus*	0.550	±	0.060 a	0.566	±	0.052 a	0.6245
	Lt x Lc	0.649	±	0.052 b	0.622	±	0.054 a	0.4036
	L. tenuis	0.682	±	0.068 b	0.574	±	0.122 a	0.0883
ϕ_{PSII}	*L. corniculatus*	0.226	±	0.065 a	0.221	±	0.052 a	0.9011
	Lt x Lc	0.277	±	0.032 a	0.253	±	0.032 a	0.2245
	L. tenuis	0.367	±	0.074 b	0.270	±	0.067 a	0.0374
NPQ	*L. corniculatus*	0.628	±	0.110 b	0.749	±	0.490 a	0.6187
	Lt x Lc	0.537	±	0.085 b	0.635	±	0.292 a	0.5029
	L. tenuis	0.397	±	0.057 a	1.392	±	0.208 b	0.0005

Table 2. The Na^+, K^+ and Cl^- concentrations of the different tissues from the control and saline-treated *Lotus* spp. plants. Treatments were applied to 10-day-old plants for 21 days. Values are means (n = 4) ± SD, followed by Rank values when non parametric analyses were performed. Within each number the block means with a similar letter do not differ significantly ($P < 0.05$), based on Duncan`s test or the Kruskal-Wallis *H* test followed by a paired comparison.

Sodium concentration

Tissue	Sample	Control (mg Na^+ g^{-1} DW)		Saline (mg Na^+ g^{-1} DW)		Control vs. Saline (*P*-value)
New leaves	*L. corniculatus*	4.205	± 1.651 c	32.371	± 2.726 e	<0.0001
	Lt x Lc	2.403	± 0.337 ab	30.258	± 3.961 e	0.0008
	L. tenuis	2.269	± 0.511 a	25.163	± 2.536 d	0.0004
Old leaves	*L. corniculatus*	5.077	± 1.272 c	43.492	± 3.614 f	<0.0001
	Lt x Lc	4.147	± 0.274 c	41.612	± 3.104 f	0.0002
	L. tenuis	3.875	± 0.831 bc	44.293	± 3.283 f	0.0002
Stems	*L. corniculatus*	8.774	± 1.345 e	13.740	± 3.466 ab	0.0369
	Lt x Lc	6.589	± 0.986 d	9.093	± 0.854 a	0.0086
	L. tenuis	9.333	± 1.584 e	13.248	± 1.594 ab	0.0131
Roots	*L. corniculatus*	2.179	± 0.444 a	12.727	± 3.212 ab	0.0074
	Lt x Lc	2.533	± 0.648 ab	19.489	± 2.618 c	0.0011
	L. tenuis	3.748	± 0.537 abc	16.214	± 4.484 bc	0.0117

Tissue	Control (mg Na^+ g^{-1} DW)		Saline (mg Na^+ g^{-1} DW)		Control vs. Saline (*P*-value)
New leaves	2.960	± 1.300 a	29.260	± 4.250 c	<0.0001
Old leaves	4.370	± 0.970 b	43.130	± 3.240 d	<0.0001
Stems	8.230	± 1.720 c	12.030	± 2.980 a	0.0009
Roots	2.820	± 0.860 a	16.140	± 4.300 b	<0.0001

Potassium concentration

Tissue	Sample	Control (mg K^+ g^{-1} DW)	Saline (mg K^+ g^{-1} DW)	Control vs. Saline

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		Control		Saline		Control vs. Saline (*P*-value)
New leaves	*L. corniculatus*	58.006 ±	5.359 e	32.540 ±	1.497 e	0.0001
	Lt x Lc	52.088 ±	3.646 de	16.412 ±	1.811 b	<0.0001
	L. tenuis	48.952 ±	5.418 d	18.817 ±	1.625 bc	<0.0001
Old leaves	*L. corniculatus*	68.144 ±	5.641 fg	23.805 ±	3.662 d	<0.0001
	Lt x Lc	71.479 ±	8.782 g	11.009 ±	2.724 a	<0.0001
	L. tenuis	60.296 ±	6.540 ef	11.248 ±	1.317 a	0.0007
Stems	*L. corniculatus*	47.758 ±	4.618 d	30.563 ±	4.628 e	0.0019
	Lt x Lc	34.000 ±	5.917 c	24.512 ±	3.228 d	0.0305
	L. tenuis	46.388 ±	6.478 d	24.112 ±	3.267 d	0.0009
Roots	*L. corniculatus*	15.440 ±	4.016 a	14.225 ±	3.189 ab	0.6526
	Lt x Lc	25.350 ±	6.324 b	23.170 ±	1.576 cd	0.5515
	L. tenuis	37.125 ±	6.723 c	24.808 ±	5.070 d	0.0264

Tissue	Control (mg K^+ g^{-1} DW)		Saline (mg K^+ g^{-1} DW)		Control vs. Saline (*P*-value)
New leaves	53.020 ±	5.900 c	22.590 ±	7.570 b	<0.0001
Old leaves	66.640 ±	8.080 d	15.350 ±	6.720 a	<0.0001
Stems	42.720 ±	8.280 b	26.400 ±	4.590 c	<0.0001
Roots	25.970 ±	10.650 a	20.730 ±	5.840 b	0.1493

Chloride concentration

Tissue	Sample	Control (mg Cl^- g^{-1} DW)		Saline (mg Cl^- g^{-1} DW)		Control vs. Saline (*P*-value)
New leaves	*L. corniculatus*	5.581 ±	1.039 def	21.028 ±	4.478 a	0.0067
	Lt x Lc	4.125 ±	0.268 abcde	23.163 ±	4.884 ab	0.0044
	L. tenuis	5.357 ±	1.213 def	25.086 ±	4.768 ab	0.0040
Old leaves	*L. corniculatus*	5.816 ±	2.408 ef	45.445 ±	10.301 c	0.0049
	Lt x Lc	5.111 ±	1.307 cdef	51.415 ±	8.591 c	0.0018
	L. tenuis	6.673 ±	2.228 f	86.437 ±	8.643 d	<0.0001
Stems	*L. corniculatus*	5.575 ±	0.737 def	76.510 ±	7.036 d	0.0010

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							P-value
	Lt x Lc	3.711	±	1.074 abcd	171.695	± 22.148 f	0.0008
	L. tenuis	4.918	±	0.818 bcdef	119.153	± 16.791 e	0.0053
	L. corniculatus	2.467	±	0.557 a	24.461	± 3.352 ab	0.0003
Roots	*Lt x Lc*	3.226	±	0.434 abc	37.649	± 4.881 bc	0.0006
	L. tenuis	2.967	±	1.066 ab	27.299	± 6.594 ab	0.0009

Tissue	Control (mg Cl$^-$ g^{-1} DW)	Saline (mg Cl$^-$ g^{-1} DW)		Control vs. Saline (P-value)
New leaves	5.020 ± 1.080 b	23.090 ± 4.600	9.1 a	<0.0001
Old leaves	5.870 ± 1.960 c	61.100 ± 20.640	31.3 b	<0.0001
Stems	4.730 ± 1.140 a	122.450 ± 43.320	41.3 b	<0.0001
Roots	2.890 ± 0.740 a	29.800 ± 7.510	16.3 a	<0.0001

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Table 3. The anthocyanins (cyanidin-3-O-glucoside eq.) levels in the stems of *Lotus* spp. Treatments were applied to 10-day-old plants for 21 days. Values are means (n = 4) ± SD, followed by Rank values when non parametric analyses were performed. Within each number the block means with a similar letter do not differ significantly ($P < 0.05$), based on the Kruskal-Wallis *H* test followed by a paired comparison.

Accession	Control (mmol anthocyanins g^{-1} FW)			Saline (mmol anthocyanins g^{-1} FW)			Control vs. Saline (*P*-value)
L. corniculatus	0.2006	± 0.0303	30.5 c	0.3435	± 0.0683	22.1 b	0.0058
Lt x Lc	0.1176	± 0.0245	16.9 b	0.3439	± 0.1564	19.7 b	<0,0001
L. tenuis	0.0162	± 0.0051	3.9 a	0.0485	± 0.0080	5.3 a	0.0225