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Lotus tenuis seedlings subjected to drought or waterlogging in a saline sodic soil

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ABSTRACT

The growth response of *Lotus tenuis* seedlings to drought and waterlogging in a saline-sodic soil, the mobilization of Na+ in tissue, and the association with native AM fungi and *Rhizobium* bacteria were studied by means of two experiments under controlled conditions.

In the first experiment, we tested the effect of the duration of waterlogging on seedlings very early after sowing, when they had the two cotyledons totally expanded and, in the second experiment, we tested the effect of water stress on seedlings of different growth stages, at a wide range of water availability in the soil, from deficit to water excess *L. tenuis* seedlings were able to deal with the intensity of water stress at very early growth stages. They established symbiotic associations with AM fungi and *Rhizobium* bacteria and regulated Na+ concentration in plant tissue to prevent leaf injury. The seedling strategy consisted in decreasing the shoot:root ratio under water deficit and increasing the shoot:root ratio under water excess.

Lotus seedlings are highly tolerant to the combination of water and salt stress from early stages in their development and early plant establishment in adverse soil conditions is an important ability of *L. tenuis* seedlings to grow and survive longer within a grassland ecosystem.

1. Introduction

Species of the genus *Lotus* are increasingly used in pastures over the world because of their plasticity and productivity in a wide range of soils (Blumenthal and McGraw, 1999). *Lotus tenuis* Waldst. & Kit. is a widely spread leguminous plant which grows naturally in the low grasslands of the flooding Pampas of Argentina. These grasslands are subject to periodic droughts or floods, and their soils show extreme values of salinity and sodicity (García and Mendoza, 2008). During the rainy season (autumn, winter and early spring) flooding is a common phenomenon in these lowlands and *L. tenuis* can be flooded for up to six months (Escudero and Mendoza, 2005). In contrast, in late spring and summer, *L. tenuis* is frequently exposed to periods of high temperatures and dry conditions, alternating with wet spells. These rainfall episodes, coupled with high evaporation rates and the existence of a shallow, saline and water table, enhance the salinization process of the top soil (Lavado et al., 1992). The combination of these edaphic stress conditions represents a particular situation to study the mechanisms involved in the spread of *L. tenuis* in the lowlands of the flooding Pampas of Argentina.

L. tenuis adult plants are considered tolerant to water excess (Vignolio et al., 1999; Mendoza et al., 2005; Striker et al., 2005; Teakle et al., 2006). Greenhouse experiments have shown that 2-3-month-old plants can tolerate more than one month of waterlogging or drought growing in saline-sodic soils (Mendoza et al., 2005; García et al., 2008). Previous studies have shown that *L. tenuis* plants can grow, become nodulated by *Rhizobium* and colonized by arbuscular mycorrhizal (AM) fungi under deficit or excess of water in the soil by using a different strategy to invest their available resources (Mendoza et al., 2005; García et al., 2008). Recently, it has been reported that *L. tenuis* seedlings with three pentafoliate leaves (14 days after germination) tolerated a 12-day submergence period and were able to grow more vigorously after the period of submergence (Striker et al., 2012). It is important to note that the artificial fertilized substrate used for seedlings growth in that study was better in terms of fertility than the saline-sodic conditions commonly present in the flooding Pampas.

One of the critical periods of a forage plant to grow in field conditions is the first weeks after germination early in the growing season. Accordingly, the N in the soil may be sufficient to grow, but P may be deficient because of its low mobility and their limited access of the roots in young plants (Krigel, 1967). In this sense, the

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Abbreviations: AM, arbuscular mycorrhizal; NW, control treatment; W, water excess treatment; 2C, two cotyledons totally expanded growing stage; 3L, three leaves totally expanded growing stage; MC, root length colonized by AM fungi; AC, root length containing arbuscules; VC, root length containing vesicles.

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ability of a species to establish early symbiotic associations with soil microorganisms like AM fungi or *Rhizobium* bacteria is important to improve its access to available nutrients in the soil, and to alleviate the effects of water stress or saline-sodic conditions. Surprisingly, little information is available regarding the tolerance of *Lotus* seedlings subject to water stress and saline-sodic conditions, and the possible role of soil microorganisms in water stress tolerance and plant nutrition during the early stages of growth. These aspects are important to understand the mechanisms involved in the establishment of seedlings in stressed environments and to find out the keys to the wide spread of *Lotus* species in several areas of the world.

The purpose of the present work was to study the ability of *L. tenuis* seedlings to tolerate the stress conditions commonly present in the lowlands of the flooding Pampas of Argentina. We planned two experiments to study the growth response of *Lotus* seedlings to waterlogging or drought in a saline-sodic soil, the mobilization of Na^+ in tissue and the early association with native AM fungi and *Rhizobium* bacteria which may facilitate the establishment of *Lotus* in the grassland community. In the first experiment, we tested the effect of the duration of waterlogging on seedlings very early after sowing, when they had the two cotyledons totally expanded, and in the second experiment we tested the effect of water stress on seedlings of different growth stages, at a wide range of water availability in the soil, from deficit to water excess, as it is supposed to occur at field conditions.

2. Materials and methods

2.1. Soil collection and experimental set up

Soil samples were collected from the top 10 cm layer of a natural grassland located in San Vicente, Buenos Aires Province, Argentina. The soil was classified as Typic Natraqualf; its physical–chemical characteristics were: pH, 8.7; electrical conductivity, 5.1 dS m^{-1}; exchangeable sodium percentage, 56%; available P, 13.5 $mg.kg^{-1}$ (Bray I); total C, 1.2%; and total N, 0.11% (further details in García and Mendoza, 2007). The soil was air-dried and sieved through a 2-mm-mesh screen. Soil moisture contents at saturation, field capacity and permanent wilting point were 40% (0 kPa), 36% (2 kPa) and 12.5% w/w (1500 kPa), respectively. Two separate experiments were conducted under controlled conditions.

2.1.1. Experiment 1: The effect of the duration of waterlogging

The effect of the duration of waterlogging was examined on seedling response in Experiment 1. Twenty-five closed bottom trays of 750 ml were filled with 0.65 kg of dry soil. Pre-germinated seeds of *L. tenuis* cv. Chaja were sown in each tray, in which the soil surface was covered with a 2-mm layer of sterilized sand to minimize water evaporation. The trays were placed in a greenhouse under a mean day temperature of 30 ± 4 °C, a mean night temperature of 19 ± 3 °C, mean humidity of 65–75% and a photoperiod length of 10–12 h during the experimental period. The soil in the trays was daily irrigated with deionized water to reach 80% of field capacity (29% of soil water content).

Five days after sowing, 50% of the seedlings of each tray showed the two cotyledons totally expanded. At this growing stage, one group of five trays was harvested (initial seedlings), another group of trays was maintained at 80% of field capacity (control treatment, NW), and a third group of trays was waterlogged, keeping the cotyledons above the water layer for 7 days (water excess treatment, W). Twelve days after sowing, the seedlings of the control (NW) and water excess (W) treatments were harvested. Seedlings of another group of five trays of the control and water excess treatments continued growing for an additional period of 7 days (14 days of soil water treatment), and 19 days after sowing the seedlings were harvested. Eighty seedlings by tray were harvested on each date and treatment.

Shoot and root dry weight, shoot length and Na^+ concentration were measured in plant tissue as described below. Mycorrhizal root colonization and root nodulation were determined as described below.

Plant and fungal variables were analyzed through two-way ANOVA with duration of treatment and water treatment as the first and second factors respectively. Mean separation was performed by the Tukey test. The normality and homogeneity of variances were previously verified. Non-normal distributed data were appropriately transformed for comparing treatment means. Statgraphic 5.0 software was used for statistical analyses.

2.1.2. Experiment 2: Water stress, from deficit to water excess

This experiment was conducted to determine the effect of water stress on the response of seedlings of different ages at a wide range of water availability in the soil, from deficit to water excess. Thirty-six closed bottom trays of 750 ml were filled with 0.65 kg of dry soil. Pre-germinated seeds of *L. tenuis* cv. Chaja were superficially sown in each tray. The soil surface of each tray was covered with 2 mm of sterilized sand to minimize water evaporation. Before each treatment, the trays were irrigated with deionized water until reaching 25% of water content in the soil (70% of field capacity). This soil water content was selected in relation to the data previously collected in a field study conducted in the sampling area, where the annual mean moisture content was 25% (García and Mendoza, 2008).

The trays were separated into two groups according to the growing stage of the seedlings. One group of trays was classified as growing stage 2C six days after sowing. This growing stage was reached when 50% of the seedlings showed the two cotyledons totally expanded. A second group of trays was classified as growing stage 3L 28 days after sowing. This growing stage was reached when 50% of the seedlings showed the first three leaves totally expanded. Then, 2C and 3L seedlings were subjected to a range of soil water content from deficit to excess of water. The gradient of soil water content was determined as 15% (water deficit), 20%, 25%, 30%, 35% and 40% (water excess), which corresponded to soil suction matrix of 94 kPa, 33 kPa, 12 kPa, 2 kPa, 1 kPa and 0 kPa, respectively. The trays were maintained at the indicated range of water content by daily watering to constant weight. Three replicates per growing stage and soil water content were grown in a greenhouse at environmental conditions similar to those mentioned for experiment 1. All the trays were harvested 45 days after sowing and 80 seedlings in each tray were harvested.

Shoot and root dry weight and Na^+ concentration were measured in plant tissue as described below. The cotyledons were separated from stems and the dry masses of the cotyledons were determined. Shoot length was measured and root length was determined by the line intercept method (Giovannetti and Mosse, 1980). Mycorrhizal root colonization and root nodulation were determined as described below.

2.2. Plant yield and analytical determinations in tissue

For both experiments, plant biomass was separated in shoots and roots after harvesting. Shoots and roots were divided into two portions; one was oven-dried at 70 °C for 48 h to determine the dry weight and the concentration of Na^+ in tissue, and the other portion of fresh roots was used to measure AM colonization morphology and number of *Rhizobium* nodules. Dry shoots and roots were digested separately in a nitric-perchloric acid mixture (3:2)

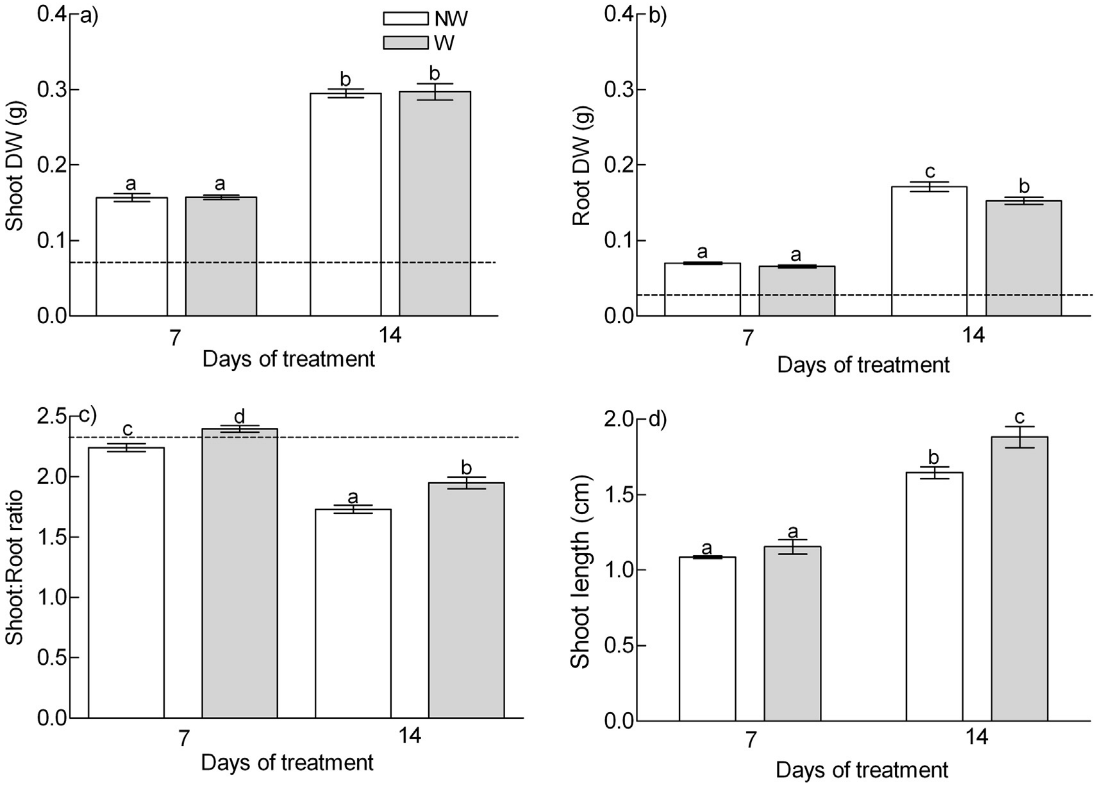

Fig. 1. Biomass of *Lotus tenuis* seedlings subjected to 7 and 14 days of soil water excess or control conditions. (a) Shoot dry weight, (b) root dry weight, (c) shoot:root ratio and (d) shoot length (Experiment 1). The dashed line represents the initial values of each variable. Different letters indicate significant differences ($P < 0.05$) between mean values of 5 replications ± standard error according to the Tukey test. The initial values of each variable are not included in the analyses. NW: control treatment, W: water excess treatment.

Table 1
Results of two-way ANOVA (*F* and *P* values) for the effects of the duration of treatment (DT), water treatment (WT) and the interaction between DT and WT on plant and fungal variables.

Variable	Duration of treatment (DT)		Water treatment (WT)		DT × WT	
	F	*P*	*F*	*P*	*F*	*P*
Seedling growth						
Shoot DW	663.57	<0.0001	0.07	0.7976	0.01	0.9203
Root DW	926.80	<0.0001	13.36	0.0021	5.55	0.0316
Shoot:Root ratio	290.06	<0.0001	44.33	<0.0001	1.21	0.2871
Shoot length	316.00	<0.0001	17.68	0.0007	5.27	0.0356
AM colonization						
MC index	244.23	<0.0001	39.02	<0.0001	10.79	0.0047
AC index	138.97	<0.0001	4.29	0.0549	0.19	0.6725
VC index	35.88	<0.0001	11.11	0.0042	0.09	0.7661
EP	16.78	0.0000	10.02	0.0060	0.01	0.9331
Rhizobium nodules						
Number of nodules	51.55	<0.0001	101.93	<0.0001	36.6	<0.0001
Na^+ in tissue						
Shoot Na^+ concentration	6.19	0.0243	20.17	0.0004	8.89	0.0088
Root Na^+ concentration	27.75	0.0001	18.84	0.0005	4.39	0.0524
Allocation Na^+ to roots	87.27	<0.0001	4.03	0.0618	0.88	0.3634

Table 2
AM fungi variables and *Rhizobium* nodules in roots of *Lotus tenuis* seedlings subjected to 7 and 14 days of soil water excess or control conditions (Experiment 1). Different letters in each row indicate a significant difference ($P < 0.05$) between mean values of five replications ± standard error according to the Tukey test. VC index was sqr transformed for this analysis. The initial values of each variable are not included in the analyses.

Variable	Initial seedlings (5 days)	Period of treatment			
		7 days		14 days	
		Control seedlings	Water excess	Control seedlings	Water excess
AM fungal variables					
MC index	0.00	0.09 ± 0.01 a	0.12 ± 0.01 a	0.22 ± 0.01 b	0.31 ± 0.02 c
AC index	0.00	0.06 ± 0.01 a	0.07 ± 0.01 a	0.17 ± 0.01 b	0.19 ± 0.01 b
VC index	0.000	0.000 ± 0.000 a	0.003 ± 0.001 ab	0.005 ± 0.000 bc	0.011 ± 0.004 c
Entry point (per mm root)	0.00	5.34 ± 0.17 b	4.59 ± 0.14 ab	4.38 ± 0.42 a	3.67 ± 0.35 a
Rhizobium nodules					
Nodules (per g fresh root)	0.00	9.28 ± 2.71 a	18.70 ± 2.49 a	11.91 ± 2.97 a	49.49 ± 3.71 b

Table 3
Statistical comparison of two equations (Eqs. (1) and (2)) fitting the response of *Lotus tenuis* seedlings (2C and 3L treatments) to a range of soil water content 45 days after sowing (Experiment 2).

Variable	Source of variation	Sums of square	Degree of freedom	Variance	Variance ratio *(F)*
Shoot DW	Eq. (1)	0.05731	4	0.01433	
	Residual Eq. (1)	0.01006	8	0.00127	11.28^{**}
	Eq. (2)	0.06443	5	0.01289	
	Residual Eq. (2)	0.00294	7	0.00042	30.70^{***}
	Improv. Eq. (2) vs. Eq. (1)	0.00712	1	0.00712	16.96^{**}
Root DW	Eq. (1)	0.00479	4	0.001197	
	Residual Eq. (1)	0.00456	8	0.00057	2.10 ns
	Eq. (2)	0.007581	5	0.001516	
	Residual Eq. (2)	0.001771	7	0.000253	5.99^{*}
	Improv. Eq. (2) vs. Eq. (1)	0.001873	1	0.001873	7.40^{*}
Shoot:root ratio	Eq. (1)	0.37670	4	0.09417	
	Residual Eq. (1)	0.02025	8	0.00253	37.21^{***}
	Eq. (2)	0.39528	5	0.07906	
	Residual Eq. (2)	0.01858	7	0.00265	29.79^{***}
	Improv. Eq. (2) vs. Eq. (1)	0.00167	1	0.00167	0.63 ns
Root length	Eq. (1)	147.83	4	33.96	
	Residual Eq. (1)	60.69	8	7.59	4.47 ns
	Eq. (2)	192.52	5	38.50	
	Residual Eq. (2)	15.80	7	2.26	17.04^{***}
	Improv. Eq. (2) vs. Eq. (1)	44.69	1	44.69	19.80^{***}

ns = $P > 0.05$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

to determine Na^+ by standard flame photometry (Benton Jones and Wernon, 1990).

2.2.1. Plant growth

In experiment 2, the growth response of the seedlings to the range of water content in the soil (15–40%) was described by a polynomial equation:

$$Y = a + bx + cx^2 + dx^3 \tag{1}$$

where a, b, c and d are coefficients, x is the soil water content (%) and Y is the dry weight (g) at the end of the experiment. A polynomial equation was used by Colwell and Goedert (1988) to measure the relative effectiveness of one fertilizer relative to a standard fertilizer by the estimation of the substitution rate. The substitution rate indicates the amount of one fertilizer that may be substituted for the other, to supply the same amount of nutrient to achieve the same yield. Similarly, in the present experiment, we estimated the substitution rates by assuming that the growth response of 2C and 3L seedlings differed because of differences in the available water content in the soil.

The response of 2C and 3L treatments was described together by adding an extra coefficient (k) in Eq. (1):

$$Y = a + b(xk) + c(xk)^2 + d(xk)^3 \tag{2}$$

where the coefficient k represents the substitution rate. The coefficient k can be interpreted as the amount of water that may be substituted for some other amount to supply the same amount of water in the soil to achieve the same yield. A value of k equal 1 is the substitution rate for the 2C seedlings (standard treatment) and a value of k different from 1 is the substitution rate for the 3L seedling (alternative treatment). One of the advantages of using Eq. (2) to fit the data is that the two fitting curves differed only by the coefficient k, which represents the substitution rate, since the other coefficients are the same for the two curves.

The simplex method of Nelder and Mead (1965) was used to find the values of the coefficients which minimized the residual sum of squares of the values of dry weight yield observed. The statistical difference between the fitting curves of both groups of seedlings was only justified when there was a significant improvement ($P < 0.05$) in the residual sum of squares of deviation by adding the extra coefficient k in Eq. (2). The changes in shoot length, cotylendons dry weight, mycorrhizal colonization (MC) and arbuscular colonization (AC) indexes, entry point number, and nodules in roots with water content in the soil were described by a polynomial equation for 2C and 3L seedlings separately.

2.2.2. AM colonization and root nodulation

Mycorrhizal root colonization in experiment 1 and 2 was measured by Mc Gonigle et al. (1990). The number of entry points was determined following Amijee et al. (1989). *Rhizobium* nodules were counted in whole fresh root systems with a binocular stereomicroscope (×7.5).

3. Results

3.1. Experiment 1: The effect of the duration of waterlogging

Shoot biomass did not differ between waterlogged (W) and non-waterlogged (NW) seedlings after 7 and 14 days of treatment

Table 4
Values of the coefficients of Eq. (2) to describe the effect of a range of water content in the soil on the growth of *Lotus tenuis* seedlings (2C and 3L treatments) after 45 days of sowing and the confident limits (95%) of coefficient k (Experiment 2).

Variables	*a*	*b*	*c*	*d*	*k*			R^2
					Value	Maximum	Minimum	
Shoot DW	−0.5207	0.1051	−0.00389	4.88×10^{-5}	0.8592	0.9447	0.7645	0.948
Root DW	0.1307	0.01024	−0.0003203	3.42×10^{-6}	0.5501	0.9311	0.3301	0.631
Shoot:Root ratio	−0.6267	0.2589	−0.009149	1.10×10^{-4}	0.9890	1.0540	0.9845	0.952
Root length	−385.582	69.003	−0.2944	4.03×10^{-3}	0.9089	0.9729	0.8439	0.873

(Fig. 1a and Table 1). Five days after sowing, the shoots of waterlogged and non-waterlogged seedlings grew 2.6 and 5.0 times in the next 7 and 14 days respectively. Root biomass was not affected by waterlogging 7 days of treatment, but decreased approximately 10% compared with non-waterlogged seedlings after 14 days (Fig. 1b and Table 1). The shoot:root ratio increased in waterlogged seedlings after 7 and 14 days of treatment compared to control seedlings (Fig. 1c and Table 1). Shoot length did not change after 7 days of treatment, but increased after 14 days of waterlogging compared to control seedlings (Fig. 1d and Table 1).

Five days after sowing, the roots of initial seedlings were not colonized by AM fungi or nodulated by *Rhizobium* bacteria, but after 7 days of treatment, (i.e. 12 days after sowing), both waterlogged and non-waterlogged seedlings were colonized by AM fungi near 10% of the root length (MC index) (Tables 1 and 2). After 14 days of treatment, the MC index increased to 0.31 in waterlogged and to 0.22 in non-waterlogged seedlings (Table 2), whereas the arbuscular colonization (AC index) did not differ between waterlogged and non-waterlogged seedlings (Tables 1 and 2). The length of the roots colonized by vesicles (VC index) ranged from 0 to 1.1% of the total root length. The VC index increased from 0 to 0.003 and from 0.005 to 0.011 after 7 and 14 days of waterlogging, respectively (Table 2). The number of entry points in roots decreased after 7 days of waterlogging, but did not change after 14 days of treatment (Tables 1 and 2). *Rhizobium* nodules significantly increased after 14 days of waterlogging (Tables 1 and 2).

The concentration of Na^+ in shoot and root tissue was not affected by 7 days of waterlogging, but increased in waterlogged seedlings compared to non-waterlogged seedlings after 14 days (Fig. 2a and b and Table 1). Allocation of Na^+ to roots did not differ between waterlogged and non-waterlogged seedlings, but increased with the plant age (Fig. 2c and Table 1).

3.2. Experiment 2: Water stress, from deficit to water excess

The growth response of *L. tenuis* seedlings to a wide range of water availability in the soil (15–40%) was adequately described by Eq. (2) (Fig. 3; Tables 3 and 4). The shape of the fitted curve showed two different phases with an inflexion point near 25% of water content in the soil, which was the initial water content before the seedlings were subjected to a range of soil water content (Fig. 3). In the first phase of water deficit of 15–20%, shoot biomass decreased exponentially with decreasing soil water content (Fig. 3a). At water contents near 25%, shoot biomass showed little changes (Fig. 3a). Then, after the flattening of the curve at 25–30% of water content, shoot biomass increased exponentially with increasing soil water content (Fig. 3a). Both 2C and 3L seedlings showed a similar response at the range of water content from 15 to 40% but 2C seedlings showed a higher shoot biomass at both deficit (15–20%) and excess of water in the soil (35–40%) than 3L seedlings (Fig. 3a). At 25–30% of soil water content, both groups of seedlings approached similar shoot biomasses. Table 3 shows that for shoot dry weight, fitting the data with Eq. (2) resulted in a statistically significant decrease in the residual sum of squares of deviations compared to Eq. (1), so two curves were used to describe the changes in shoot dry weight at the range of soil water content used. Root biomass responded similarly to shoot biomass, but the changes to the range of soil water content were less marked, especially for 2C seedlings, which showed little variation along the availability of water in the soil (Fig. 3b). The shoot:root ratio of 2C and 3L seedlings increased with increasing soil water content and showed no statistical differences between the fitting curve of both groups of seedlings. Eq. (2) did not statistically improve the fit over Eq. (1) (Table 3), so one fitting curve for both groups of seedling was used to describe the changes in the shoot:root ratio at the range of soil water content used (Fig. 3c). The dry biomass of cotyledons decreased under water excess and deficit in both groups of seedlings (Fig. 3d). The shoot length of 2C seedlings showed an increase at high levels of soil water content, but showed little differences in 3L seedlings at the range of water content used (Fig. 3e). The root length of both groups of seedlings responded similarly to shoot dry weight to the range of water content used. The roots of the 2C seedlings were longer at deficit or excess of water in the soil than the roots of 3L seedlings (Fig. 3f).

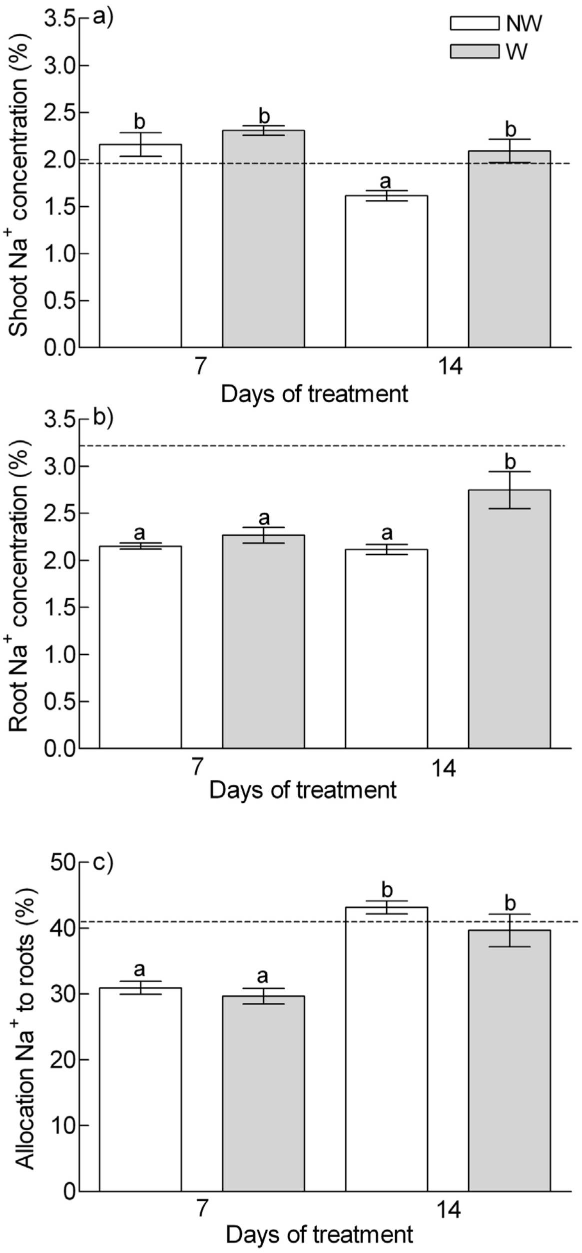

Fig. 2. Concentration of Na^+ in shoots (a), roots (b) and the allocation to roots (c) in *Lotus tenuis* seedlings subjected to 7 and 14 days of soil water excess or control conditions (Experiment 1). The dashed line represents the initial values of each variable. Different letters indicate significant differences ($P < 0.05$) between mean values of 5 replications ± standard error according to the Tukey test. Root Na^+ concentration was log transformed for this analysis. The initial values of each variable are not included in the analyses. NW: control treatment, W: water excess treatment.

The AM colonization indexes (MC and AC) in roots of both groups of seedlings increased with increasing water content in the soil and there was a cross-over between the fitted curves of 2C and 3L seedlings (Fig. 4a and b). At water deficit (15–20%), the two indexes were higher for 3L than for 2C seedlings, but at

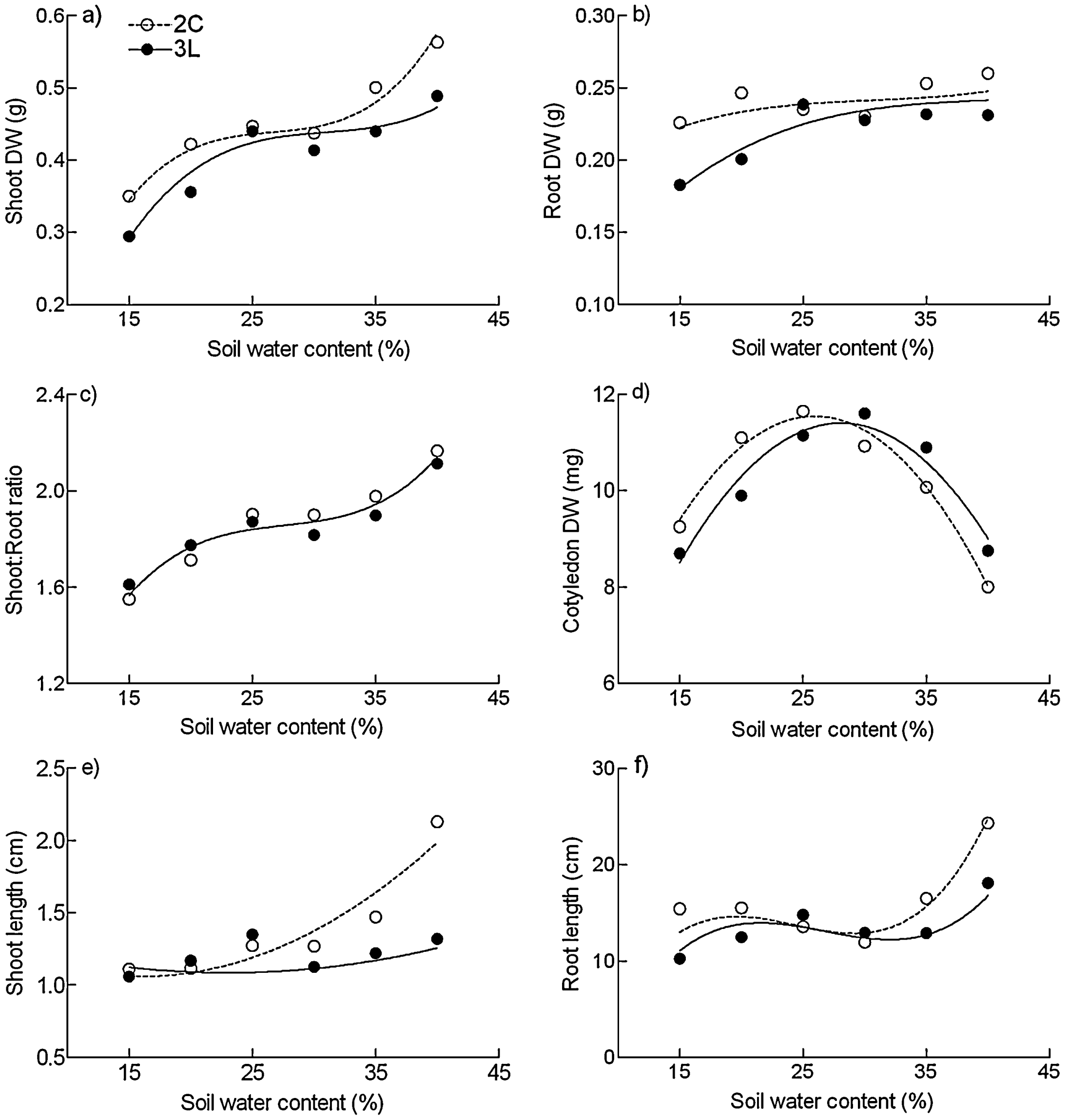

Fig. 3. Curves fitted by Eq. (2) to describe the effect of a range of soil water content from 15 to 40%, on the *Lotus tenuis* seedling biomass at different ages (2C and 3L treatments) 45 days after sowing. (a) Shoot dry weight, (b) root dry weight, (c) shoot:root ratio, (d) cotyledon dry weight, (e) shoot length and (f) root length (Experiment 2). Each point represents the mean of three replicates per growing stage and soil water content. 2C growing stage: two cotyledons totally expanded. 3L growing stage: three leaves totally expanded.

water excess (35–40%) the indexes were higher for 2C than for 3L seedlings (Fig. 4a, b). Only near 6-7% of the root length was colonized by vesicles. The VC index increased exponentially from near no-colonization at water deficit (15%) in roots of both groups of seedlings to a maximum value of 0.07 for 2C seedlings and 0.06 for 3L seedlings in 40% of soil water content. Entry point number decreased in roots of 3L seedlings and increased in roots of 2C seedlings with increasing soil water content (Fig. 4c). The number of nodules in 2C seedlings was higher at water excess, whereas that in 3L seedlings showed little differences along the wide range of soil water content used (Fig. 4d).

The concentration of Na^+ in shoot tissue showed a similar but less marked pattern of shoot dry weight for 2C and 3L seedlings (Fig. 5a). Na^+ concentration was higher at deficit or excess of water in the soil for 2C seedlings than for 3L seedlings. Two curves fitted by Eq. (2) were used to describe the pattern of Na^+ concentration in shoots of 2C and 3L seedlings. Na^+ concentration in roots consistently increased with increasing soil water content for both groups of seedlings and one curve fitted by Eq. (2) was used to describe this pattern (Fig. 5b). The shoot:root Na^+ concentration ratio decreased when the water in the soil increased. This ratio was higher at deficit for 2C than for 3L seedlings and approached a minimum value at water excess for both groups of seedlings (Fig. 5c).

4. Discussion

Our results indicate that *L. tenuis* seedlings were able to deal with the intensity of water stress at very early stages of growth, establish symbiotic associations with AM fungi and *Rhizobium* bacteria and regulate the Na^+ concentration in tissue in a saline-sodic soil.

The response of *L. tenuis* seedlings under water excess in saline-sodic conditions involved an increase in shoot growth and length and a decrease in root biomass, which increased the shoot:root ratio. The increase in the shoot:root ratio in *Lotus* waterlogged seedlings is the response usually described in flooding-tolerant plants (Mendoza et al., 2005; García et al., 2008), as a strategy to facilitate transpiration rates (Blom and Voesenek, 1996;

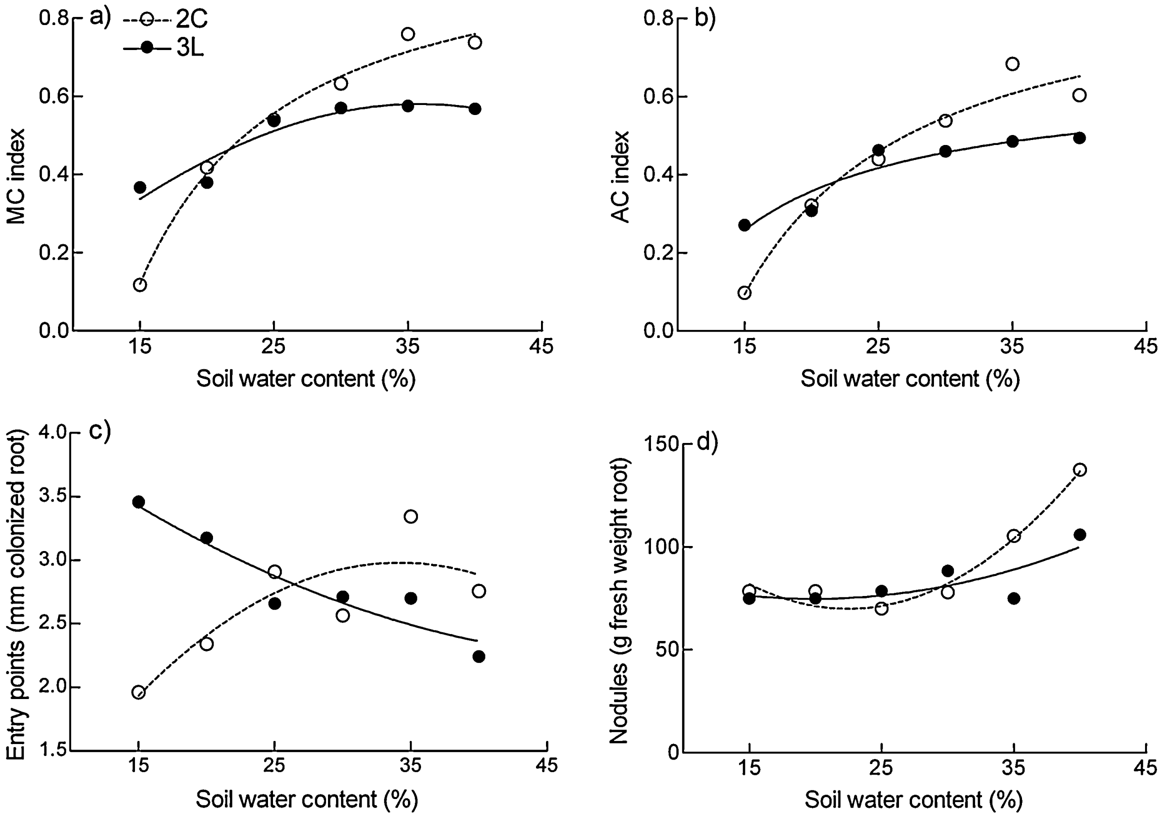

Fig. 4. Curves fitted by Eq. (2) to describe the effect of a range of soil water content from 15 to 40% on AM fungi variables and *Rhizobium* nodules in roots of *Lotus tenuis* seedlings at different ages (2C and 3L treatments) 45 days after sowing. (a) MC index, (b) AC index, (c) number of entry points and (d) *Rhizobium* nodules (Experiment 2). Each point represents the mean of three replicates per growing stage and soil water content. 2C growing stage: two cotyledons totally expanded. 3L growing stage: three leaves totally expanded.

Vartapetian and Jackson, 1997; Colmer and Voesenek, 2009). Water excess also changes the soil chemical properties in saline-sodic soil conditions by decreasing soil pH, reducing salinity and increasing nutrient availability (Rubio et al., 1997; Mendoza et al., 2005; García et al., 2008). In the present study, shoot growth and elongation may have been satisfied by the change in soil nutrient availability and the capability to mobilize carbohydrate reserves from the cotyledons.

In general, the tolerance of *L. tenuis* seedlings to water excess observed in the present study is partly consistent with previous results reported by Striker et al. (2012). However, the previous study differs from the present experiments in several aspects: the substrate used for seedling growth was better in terms of fertility compared to the present experiment, where a saline-sodic soil in which *L. tenuis* spontaneously grows at the field was used. Then in the previous study, *L. tenuis* seedlings grew in an environment without nutritional limitations and it could increase the tolerance of the seedlings to stress conditions. Other point to note is the stage of seedling growth when water stress treatment is applied, in the present study the water treatment was applied when the seedlings showed the two cotyledons totally expanded (5 days after sowing) and in the previous study when the seedlings showed three pentafoliate leaves (14 days after sowing). Another difference between the two studies is the water layer above the soil surface used. Striker et al. (2012) investigated the strategies in seedlings of *L. tenuis* subjected to submergence and the subsequent recovery after this stress. In our study, we analyzed the tolerance of *Lotus* seedlings under water stress (water deficit and excess) in natural soil conditions, the development of relationships with symbiotic microorganisms and plant nutritional aspects. All of these different aspects between the two studies may change the tolerance of the seedlings to water stress. As far as we know, this is the first study which involves plant growth variables, symbiotic associations and Na$^+$ concentrations in early stages of *L. tenuis* in a saline-sodic soil. *Lotus* seedlings also showed tolerance to dry conditions. They decrease both shoot and root growth under water deficit but the decrease of shoot was higher than of root resulting in the decline of the shoot:root ratio. The decrease in the shoot:root ratio is one of the main plant responses to water deficit (Chaves et al., 2002; García et al., 2008; Jaleel et al., 2009; Li et al., 2011). The seedling response is a strategy to reduce the foliar area exposed to the atmosphere for transpiration and to enhance water use efficiency, maintaining a sufficient water supply to sustain seedling growth (Chaves et al., 2002; Jaleel et al., 2009; Rivas et al., 2013). Another point to highlight is that seedlings showed a decrease in the cotyledon biomass under the range of water excess and deficit studied. Unlike *Lotus* adult plants, which grow at the expense of the reserves of the crown and roots, carbohydrate reserves in seedlings are centered on cotyledons. Hence, the decrease in biomass of cotyledons suggests that carbohydrate reserves were used to support seedling growth. These results are in agreement with previous observations by Mujica and Rumi (1998) and Striker et al. (2012).

In the present study, 2C seedlings produced more biomass than 3L seedlings under deficit and excess of water content in the soil. The other point to consider is that 2C seedlings had a longer adaptation period to the stress conditions than 3L seedlings, because 2C were subjected to stress conditions in an earlier stage of growth than 3L. We propose that the differences observed in biomass between 2C and 3L seedlings under a wide range of soil water content are due to changes in metabolic processes that lead to

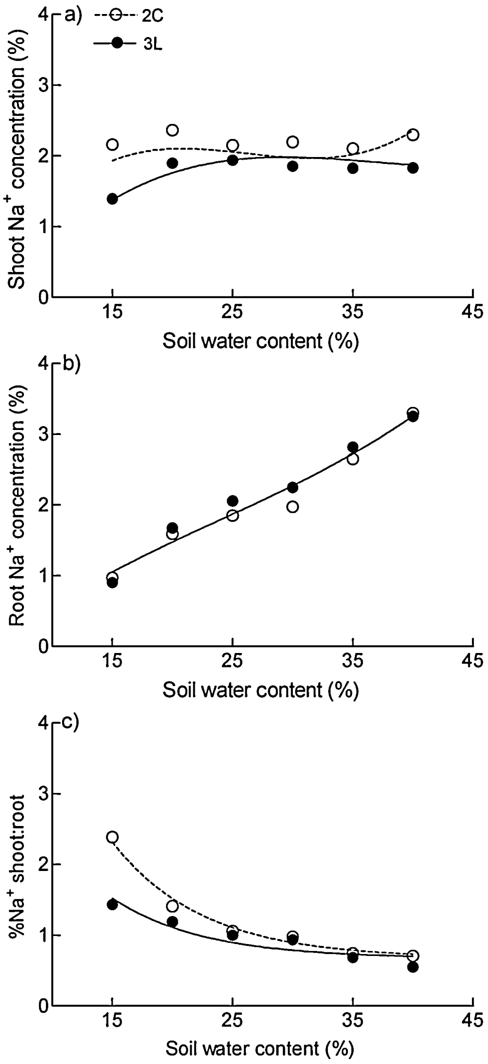

Fig. 5. Curves fitted by Eq. (2) to describe the effect of a range of soil water content from 15 to 40% on Na^+ concentration in *Lotus tenuis* seedlings at different ages (2C and 3L treatments) 45 days after sowing. (a) Shoot Na^+ concentration, (b) root Na^+ concentration and (c) Na^+ shoot:root ratio (Experiment 2). Each point represents the mean of three replicates per growing stage and soil water content. 2C growing stage: two cotyledons totally expanded. 3L growing stage: three leaves totally expanded.

a decrease in the daily growth rate of 3L seedlings. In turn, this decrease would be due to the fact that 3L seedlings use the energy reserves for the maintenance of the foliar biomass to avoid the damage of photosynthetic and root tissues. After the first exposure to environmental disturbance, plants acquire greater tolerance to stress from biochemical changes and/or epigenetic alterations (Bruce et al., 2007), and thus have the ability to respond faster and more vigorously to a recurring stress event (Walter et al., 2011). In this sense, our results indicate that *Lotus* seedlings are highly tolerant to the combination of water and salt stress from very early stages in their development. This tolerance may improve the performance of *Lotus* adult plants against recurring stress events in the grassland.

Results of this study show that *Lotus* seedlings are colonized by the native AM fungal community between 5 and 12 days after sowing. These results are in agreement with those reported by Read et al. (1976), in which *L. corniculatus* seedlings were AM-colonized at cotyledon and 1–4 leaf stages under field conditions. The early association with AM fungi and *Rhizobium* bacteria is a key factor to improve not only plant nutrition but also the water balance of seedlings, such that AM fungi improve the resistance of plants to water stress and saline conditions (Ruiz-Lozano and Azcón, 2000; Kumar et al., 2010; Birhane et al., 2012).

AM colonization and nodulation in 2C and 3L seedling roots increased with increasing soil water content, but the increase was less marked in 3L seedlings. Miller and Sharitz (2000) reported that once mycorrhizal colonization was established, subsequent flooding did not affect future colonization in the submerged root. This could explain the little differences in AM colonization and nodulation in 3L seedlings under water excess compared to 2C seedlings, and this could also be proposed as an explanation to drought conditions.

In the present work, we observed a high proportion of roots colonized by arbuscules and a low colonization of vesicles under different soil water content in saline-sodic conditions. Arbuscules constitute the primary sites of nutrients and C exchange between the symbionts (Smith and Read, 2008). The high proportion of roots colonized by arbuscules suggests that *Lotus* seedlings and AM fungi may establish a functional symbiosis independently of the intensity of soil water stress. It is expected that actively growing seedlings present a greater proportion of their roots colonized by structures of nutrient exchange between the symbionts than storage structures, like vesicles, independently of the soil water content.

In the current experiment, the concentration of Na^+ in shoot tissue showed little changes at the range of water content in the soil studied, but that in roots markedly increased with increasing water content. Salinity and osmotic pressure are much higher under soil water deficit than at either field capacity or water excess soils (García et al., 2008), and it would also contribute to decreasing plant growth at water deficit (Munns, 2002). The primary mechanism of salinity tolerance is centered on the control of Na^+ uptake by the roots and the subsequent distribution of Na^+ in plant tissue to minimize the amount of Na^+ transported to shoots (Plett and Møller, 2010). We propose that in the water excess treatments of the present study (soil water content higher than 25%), there is a mechanism whereby an increase in Na^+ concentration in shoots of *L. tenuis* seedlings is prevented by a reduction in Na^+ uptake by roots from the soil solution and a subsequent reduction of Na^+ transport from roots to shoots through an increase in the amount of Na^+ accumulated by the root system, which prevents the effect on seedling growth. Under the water deficit treatments (soil water content lower than 25%), the roots of *L. tenuis* seedlings were subjected to higher salinity values in the soil and the Na^+ concentration in shoots became higher than that in roots, decreasing the amount of Na^+ accumulated by the root system and increasing that transported to the shoot. Therefore, our results indicate that *L. tenuis* seedlings have a regulatory mechanism that allows tolerating high concentrations of Na^+ in shoots without causing leaf injury under water deficit.

In temperate latitudes, natural grasslands are dominated by perennial grasses associated with a decline or absence of native or naturalized legumes because of land use, soil fertility and/or selective grazing (Muir et al., 2011). The ability of a forage legume plant to tolerate adverse soil conditions imposed by the environment during the first week after germination is a key factor to compete for nutrient resources with neighbor plants of the grassland. Our results indicate that *Lotus* seedlings cope better with water stress and salinity when imposed earlier in plant development. Hence, early plant establishment in adverse soil conditions is an important ability of *L. tenuis* seedlings to grow and survive longer within the grassland and probably contributes to the wide spread of *Lotus* species in areas where the low fertility and/or water stress conditions negatively affect the persistence of other forage legumes.

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