


# Physiological and anatomical traits associated with tolerance to long-term partial submergence stress in the *Lotus* genus: responses of forage species, a model and an interspecific hybrid

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## Abstract

Cattle production based on natural pastures is often subject to flooding periods, which affect plant performance and as a result, forage production. Although most forage legumes are not tolerant to flooding, *Lotus* spp. are outstanding alternatives, since species, such as *L. tenuis* (Lt) and *L. corniculatus* (LcT), have high forage quality and are adaptable to different environments. We recently obtained a *L. tenuis* × *L. corniculatus* hybrid (LtxLc) with potential new cultivar traits, although its tolerance to flooding stress has not yet been evaluated. In the present study, the performance of LtxLc, its parental diploid accessions, the model legume *L. japonicus* and tetraploid LcT were evaluated under 55 days of partial submergence stress and a 35-day recovery period. Physiological, morphological and anatomical traits were analysed, showing that tolerance to partial submergence was positively associated with aerenchyma and adventitious root formation and relative growth rates. Overall, Lt and LtxLc showed the best responses under stress and during the recovery period. Nevertheless, the higher forage value of LtxLc makes it recommendable for use in environments affected by flooding. Our results could be used as breeding criteria for the generation of new cultivars tolerant to partial submergence stress.

## KEYWORDS

aerenchyma, flooding pampa, forage, interspecific hybridization, legume, waterlogging

## 1 | INTRODUCTION

Flooding periods are natural disturbances that affect plant growth in different regions around the world (Rosenzweig, Tubiello, Goldberg, Mills, & Bloomfield, 2002). The frequency and intensity of flooding are expected to increase over the next decades due to global climate change, affecting crop yield and forage production (Arnell & Liu, 2001; Hirabayashi et al., 2013).

Flooding rapidly reduces O<sub>2</sub> levels in the rhizosphere, thereby altering aerobic ATP production in roots and submerged organs, as well as plant nutrient uptake (Armstrong, 1980; Ponnampuram,

1984). Moreover, flooding causes reduction in water absorption, stomatal closure, changes in hormonal balance and accumulation of toxic compounds, all of which worsen plant performance (Bailey-Serres & Voesenek, 2008).

Plant responses to flooding stress involve different anatomical and morphological changes, such as aerenchyma formation and development of adventitious roots (Colmer & Voesenek, 2009; McDonald, Galwey, & Colmer, 2002). These changes favour acclimation to the new environmental condition and constitute the tolerance traits to be evaluated in plant breeding programmes to obtain improved cultivars for flood-prone areas (Malik, Islam, & Colmer,

2011; Vignolio, Fernández, & Maceira, 1999). Plant responses depend on the duration and magnitude of the stress period, which can be classified as waterlogging, partial submergence or complete submergence. In particular, partial submergence is defined as flooding of the entire root system and part of the aboveground organs (Sasidharan et al., 2017) and is the most common stress situation occurring in the Flooding Pampa in Argentina.

The Flooding Pampa (9 million hectares) is the most important cattle-rearing area in Argentina. It undergoes periods of excessively wet conditions almost annually during the spring season (Paruelo, 1990). The negative effect of flooding periods is increased by the presence of soils with low permeability and the shallow depth of the water table (Lavado, Taleisnik, Grunberg, & Santa María, 2008; Soriano et al., 1991). Moreover, poor nutrient availability and saline, alkaline edaphic conditions severely limit crops and most common forages, making the natural grasslands the primary forage source for cattle production (Soriano et al., 1991). There are few native legumes in these natural grasslands, so forage quality needs to be improved by introducing other legumes (Lüscher, Mueller-Harvey, Soussana, Rees, & Peyraud, 2014; Zemenchik, Albrecht, & Shaver, 2002). One remarkable alternative is *L. tenuis* Waldst et Kit. (narrowleaf trefoil), which is adapted to floodable saline soils (Striker, Insausti, Grimoldi, Ploschuk, & Vasellati, 2005; Teakle, Amtmann, Real, & Colmer, 2010) and could be used to improve the forage quality produced by natural grasslands (Antonelli et al., 2016; Blumenthal & McGraw, 1999; Escaray et al., 2012).

*Lotus tenuis* produces fodder of quality comparable to that of other forage legumes such as *Medicago* spp. and *Trifolium* spp. However, it contains a lower level of foliar proanthocyanidins (PA) compared to other species of *Lotus* genus. A moderate level of PA (also called condensed tannins) is another important forage quality trait, due to their contribution for ruminant diet, avoiding cattle bloat, improving protein fraction assimilation and reducing intestinal parasites (McNabb, Waghorn, Peters, & Barry, 1996; Min, Barry, Attwood, & McNabb, 2003). In addition, PA reduce enteric fermentation, decreasing greenhouse gases production (mostly methane) (Patra & Saxena, 2010; Tavendale et al., 2005). Within the *Lotus* genus, a species which accumulates PA at foliar level is *L. corniculatus* L. (birdsfoot trefoil), phylogenetically close to *L. tenuis*. Nevertheless, attempts to implant *L. corniculatus* commercial cultivars (all tetraploid populations) in the lowlands of the Flooding Pampa have failed due to its more demanding edaphic requirements and lower tolerance to flooding.

Recently, a wild diploid *L. corniculatus* accession was crossed with *L. tenuis*, producing highly fertile interspecific hybrids with adequate foliar PA levels inherited from the first accession (Escaray et al., 2014). Therefore, we also hypothesized that the interspecific hybrid would acquire flooding tolerance traits from *L. tenuis*, incorporating a new forage material harbouring adequate PA levels in flood-prone areas of the Flooding Pampas in Argentina. Thus, plant responses to partial submergence were evaluated in the interspecific hybrid and its parental accession along with the responses of a commercial tetraploid *L. corniculatus* cultivar and the model legume

*L. japonicus* Regel. Our results could be used as breeding criteria for the generation of new tolerant cultivars, enabling forage production under flooding conditions to be increased.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and treatments

Seeds of the commercial cultivar of *L. corniculatus* cv. "San Gabriel" (LcT), diploid accession of *L. corniculatus* cv. "Charlii" (LcD), naturalized accession of *L. tenuis* (Lt), F1 population of *L. corniculatus* × *L. tenuis* (LtxLc) (Escaray et al., 2014) and *L. japonicus* MG-20 ecotype (Lj) were used to perform assays. Seeds were scarified with sulphuric acid (100%) for 3 min, washed 10 times with sterile distilled water and sown in Petri dishes containing water/agar (0.8%). They were incubated for 7 days in a growth chamber with a 16/8-hour day/night cycle at 24/21 ± 2°C and 55/65 ± 5% relative humidity. Light (at an intensity of 250 μmol m<sup>-2</sup> s<sup>-1</sup>) was provided by Gro-lux F40 W fluorescent lamps. Seedlings were transferred to 4-L pots containing sand-soil (1:1), placed in a naturally lit greenhouse and irrigated with water from a rainwater harvesting system. Temperature and irradiance were recorded throughout the experiment. Mean temperature was 23.3 ± 5.2°C, and mean maximum irradiance per day was 1150 ± 225 μmol m<sup>-2</sup> s<sup>-1</sup>. The soil used in the substrate (phosphorus: 6.3 ppm; pH: 6.28; electric conductivity: 0.54 dS; organic matter: 8.8%; total N: 0.25%) was obtained from the top 20 cm of a flooding land horizon (location: 35°37'47" S, 57°59'50" W, Chascomús, Province of Buenos Aires, Argentina). Five-month-old plants were used for all treatments.

Control and partial submergence (flooding) treatments were initiated during spring (October 2012) for all accessions, in the season during which waterlogging frequently occurs in the Pampa Grasslands (Paruelo, 1990). The flooding treatment was performed by obstructing drainage and adding rainwater until the water level was 6 cm above the substrate surface. The control treatment was performed by cultivating plants with periodical irrigation and free drainage, with substrate humidity maintained at not less than 80% field capacity.

A first harvest was done at the beginning of the experiment (*initial*), using 10 plants per accession. The *flooding* period lasted for 55 days, after which a second harvest was done (*flooding harvest*) using 14 plants per treatment and accession. Then, partial submergence was eliminated, and the plants which had undergone flooding treatment were irrigated in the same way as the control in free drainage pots during a 35-day *recovery* period. A final harvest was carried out at this time for both treatments (*recovery harvest*), using 10 plants per treatment and accession.

### 2.2 | Determination of growth parameters

Plant biomass was separated into leaves, stems and roots at each harvest, and dry weight was measured after oven-drying for 72 hr at 70°C in all cases. The number of adventitious roots was also

recorded. Only for the *flooding* harvest, the diameter of three primary stems per plant was measured 3 cm above the crown with a calliper, in 14 plants per treatment and accession.

The biomass data obtained were used to calculate the relative growth rate (RGR) for the *flooding* period (from the *initial* harvest to the *flooding* harvest) and for the *recovery* period (from the *flooding* harvest to the *recovery* harvest). The RGR was calculated using the equation proposed by Malik et al. (2011):

$$\text{RGR} = (\text{LN } DW_2 - \text{LN } DW_1) / (t_2 - t_1) \quad (1)$$

where  $DW_1$  is dry weight (g) for the first harvest considered;  $DW_2$  is dry weight (g) for the second harvest considered;  $t_2$  and  $t_1$  are the plant growth times (d) for both harvests; and LN is natural logarithm. For RGR estimations, daily growth rates are assumed to be constant throughout the period considered.

Shoots/roots (S/R) and leaves/stems (L/S) ratio were also calculated for all measurements.

### 2.3 | Gas exchange measurements

Net photosynthetic rate ( $A_{sat}$ ) and stomatal conductance ( $g_s$ ) were measured one day before the *flooding* harvest on the fifth fully expanded leaf from apex, using a TPS-2 (Portable Photosynthesis, MA, USA). Measurements were performed using a LED light unit at saturating irradiance intensity ( $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) on four plants per treatment and accession.

### 2.4 | Chlorophyll fluorescence transient analysis

Non-invasive OJIP tests (Strasser & Srivastava, 1995) were performed on the third fully expanded leaf on the day of the *flooding* harvest using a Pocket PEA Chlorophyll Fluorimeter (Hansatech Instruments, UK). Leaves were dark adapted for 20 min before analysis and then exposed for 3 s to light at an intensity of  $3500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Data were processed by PEA Plus software (Hansatech Instruments, UK) and Windows Excel (Microsoft, WA, USA). The maximum quantum yield of primary photochemistry ( $F_v/F_m$ ) was calculated, and the absorption of light energy (ABS), the trapping of excitation energy (TR) and the conversion of excitation energy to electron transport (ET) were analysed through the multiparametric expression performance index (Plabs; Strasser, Srivastava, & Tsimilli-Michael, 2000). Measurements were performed on six plants per treatment and accession.

### 2.5 | Electrolyte leakage measurements

Electrolyte leakage was measured according to Lee, Lur, and Chu (1993) with modifications, at the *flooding* harvest. Briefly, three leaflets from the fifth fully expanded leaf of three different stems per plant were incubated in 15 mL of deionized water, and the initial conductivity ( $C_0$ ) was measured using a combined pH meter/conductometer (HI 255, Hanna Instrument). Then, the leaves were shaken in a gyratory shaker at room temperature for 24 hr, and conductivity

was measured again ( $C_1$ ). Finally, the samples were autoclaved for 20 min, and the conductivity was determined ( $C_{max}$ ). The relative electrolyte leakage was calculated as:

$$\text{EL} = (C_1 - C_0) / (C_{max} - C_0) \times 100 \quad (2)$$

### 2.6 | Root anatomy

At the end of the *flooding* period, root aerenchyma formation was measured using optical techniques following Striker et al. (2005). Briefly, young lateral root samples were taken at 2 cm from the tip, carefully washed and then preserved in formalin acetic alcohol (FAA) until dehydration with increasing ethanol concentrations, for paraffin embedding. Thin sections ( $10 \mu\text{m}$ ) were cut, stained with safranin and Fast Green, mounted in Canada balsam and photographed in a Nikon Eclipse E600 microscope (Nikon Instruments, NY, USA). Microscopy was performed on different root sections of five plants per treatment and accession. The aerenchyma proportion in each root was calculated as the total aerenchyma lacunae area with respect to the total root area of each cross section. The values were expressed in percentage. All the micrographs were analysed using Image-Pro Plus software (v4.1, Media Cybernetics, Bethesda, MD, USA).

### 2.7 | Experimental design and statistical analysis

A completely randomized experimental design was used in all cases. Two-way ANOVA was performed in all analyses, except for the analysis of the number of adventitious roots within the *flooding* treatment, where a one-way ANOVA was performed. The assumptions of variance homogeneity and normality were tested for all variables. When a significant interaction was detected between accession and treatment, or significance was found for the main effects, the analysis was followed by Duncan's test ( $p < 0.05$ ). All the statistical analyses were performed using InfoStat software (Universidad Nacional de Córdoba, Argentina) (Di Rienzo et al., 2011).

## 3 | RESULTS

### 3.1 | Biomass response

Under the *flooding* treatment, all the assayed accessions had lower accumulated biomass than their controls (Figure 1). Lt was the least affected accession after the *flooding* period, with 28% and 48% less shoot and root biomass than the control, respectively. In contrast, LcT was the most affected accession, with 63% and 88% less shoot and root biomass, respectively (Figure 1). For LtxLc, LcD and Lj accessions, biomass was in between the values recorded for the aforementioned accessions. In particular, for shoot biomass accumulation, LtxLc reached  $8.99 \pm 3.06$  g per plant after the *flooding* treatment, just behind Lt ( $13.95 \pm 3.21$  g) (Figure 1).

The shoot, root and total relative growth rates (RGR) were calculated for each accession at the end of the *flooding* period (Table 1).

All stressed accessions had lower RGR than their controls, although in different proportions. Shoot RGR (sRGR) was least affected in Lt and LtxLc (20% and 29%, respectively), while it was most affected in LcD, LcT and Lj (52, 78 and 86%, respectively). Root RGR (rRGR) of LcT was extremely low during the flooding treatment, and showed a negative rate value ( $-0.009 \pm 0.011 \text{ g g}^{-1} \text{ day}^{-1}$ ), indicating root biomass loss due to stress treatment. Total RGR (tRGR) in Lt and LtxLc was 25 and 30% lower, respectively, during the stress condition, than in their controls, while in LcD, Lj and LcT it was 55, 86 and 93% lower, respectively. It is worth mentioning that the LtxLc accession had the highest sRGR in the flooding and control treatments ( $0.048 \pm 0.003$  and  $0.034 \pm 0.007 \text{ g g}^{-1} \text{ day}^{-1}$ , respectively) (Table 1).

The effect of the flooding treatment on shoot and root biomass was also evaluated at the end of the *recovery* period (in the *recovery harvest*) (Figure 2). The accessions subjected to the stress treatment that accumulated the highest shoot biomass after the *recovery* period were Lt and LtxLc ( $20.65 \pm 2.45$  and  $18.53 \pm 2.99 \text{ g}$  per plant, respectively), with values that were 15% and 25% lower than their controls. Both *L. corniculatus* accessions accumulated significantly lower biomass under the stress situation ( $10.46 \pm 3.06$  and  $8.98 \pm 2.12 \text{ g}$  per plant for LcT and LcD, respectively). Lastly, the Lj subjected to the stress treatment presented the lowest shoot biomass accumulation after the *recovery* period ( $3.84 \pm 1.20 \text{ g}$  per plant) (Figure 2a).

With regard to root biomass accumulation in flooded plants, no significant difference was observed in LtxLc compared to control

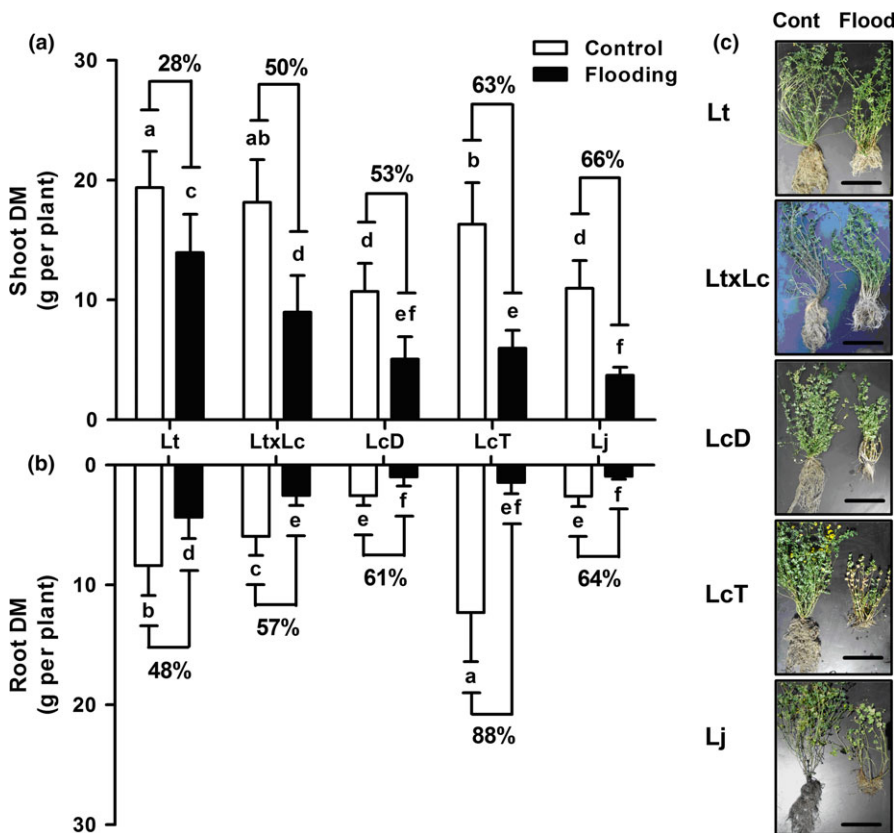
plants after the *recovery* period (Figure 2b). However, in the others accessions, there was significantly lower root biomass accumulation under stress. In particular, the most affected accession was LcT, with 80% root biomass reduction compared to its control, followed by LcD, Lj and Lt (73%, 55% and 37%, respectively).

Moreover, shoot, root and total relative growth rates (RGR) were calculated for each accession at the end of the *recovery* period (Table 2). The sRGR and the tRGR of the plants subjected to the stress treatment were different for each accession, with sRGR being higher for LtxLc and LcD under stress, compared to controls.

Lastly, regarding rRGR, no difference was found between treatments in each accession, although their values differed (Table 2). In particular, the highest rate was measured for LcD, followed by LtxLc, LcT and Lt. As for the sRGR parameter, a negative rRGR was calculated for Lj, showing root biomass loss for this accession in the *recovery* period due to stress treatment.

### 3.2 | Biomass ratios and adventitious roots generation

Shoot/root (S/R) and leaf/stem (L/S) biomass ratios were calculated at the end of the *flooding* period (Table 3). Both accessions of *L. corniculatus* (LcD and LcT) showed higher S/R ratio under stress, compared to controls, although this increase was highest in the latter (44% and 250%, respectively). For the other three accessions, S/R ratio values did not change between treatments ( $p > 0.05$ ). Similar results were

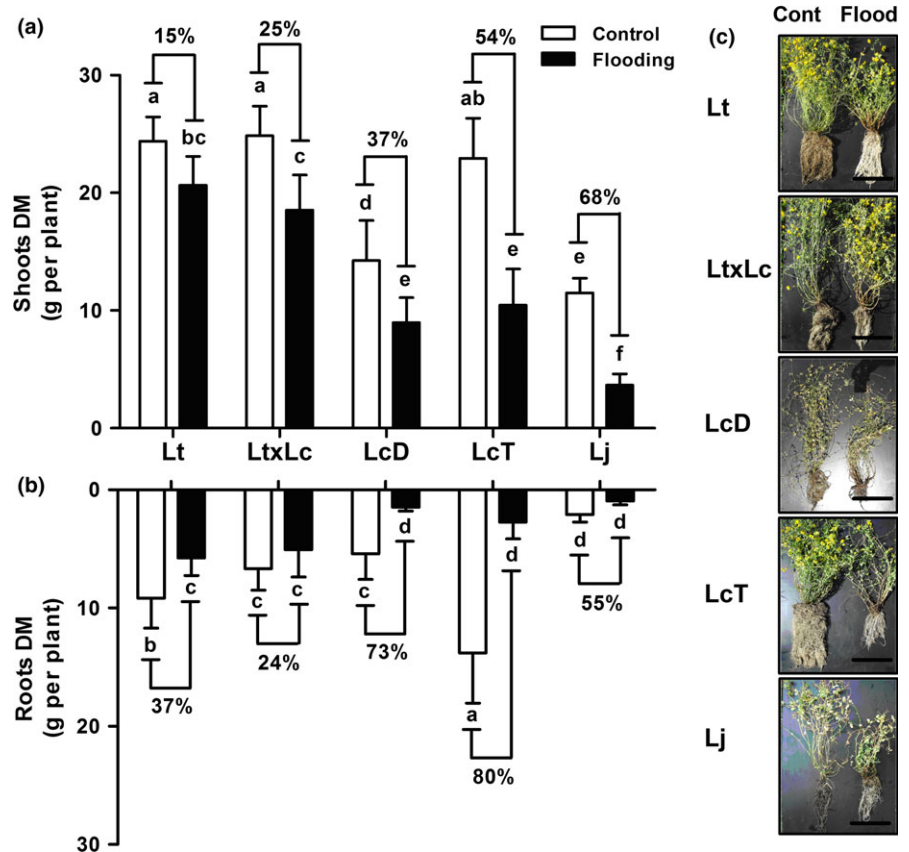


**FIGURE 1** Biomass production during flooding stress. Effect of partial submergence on plant biomass production. Lt: *Lotus tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. a and b: shoot and root dry biomass, respectively. Percentages show the reduction in biomass between stressed plants and their controls. Columns without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 14 \pm \text{SD}$ ). c: representative images of different plants during the *flooding harvest*. Black bars: 25 cm

**TABLE 1** Effect of partial submergence on relative growth rates (RGR,  $\text{g g}^{-1} \text{day}^{-1}$ )

Treatment		RGR Shoot		RGR Root		RGR Total	
(Acc × Flood)		Mean ± SD		Mean ± SD		Mean ± SD	
Lt	Ctrl	0.030 ± 0.003	c	0.033 ± 0.006	b	0.031 ± 0.003	bc
	Flood	0.024 ± 0.004	d	0.020 ± 0.007	d	0.023 ± 0.004	e
LtxLc	Ctrl	0.048 ± 0.003	a	0.044 ± 0.005	a	0.047 ± 0.003	a
	Flood	0.034 ± 0.007	b	0.028 ± 0.006	bc	0.033 ± 0.007	b
LcD	Ctrl	0.029 ± 0.005	c	0.025 ± 0.006	cd	0.028 ± 0.004	cd
	Flood	0.014 ± 0.007	e	0.004 ± 0.014	e	0.013 ± 0.008	f
LcT	Ctrl	0.023 ± 0.004	d	0.032 ± 0.006	b	0.027 ± 0.004	d
	Flood	0.005 ± 0.005	f	-0.009 ± 0.011	f	0.002 ± 0.006	g
Lj	Ctrl	0.022 ± 0.004	d	0.023 ± 0.007	cd	0.023 ± 0.004	e
	Flood	0.003 ± 0.003	f	0.004 ± 0.005	e	0.003 ± 0.003	g

Lt: *L. tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. For each variable, means without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 14 \pm \text{SD}$ ).



**FIGURE 2** Biomass production after the recovery period. Effect of the recovery period on plant biomass production. Lt: *Lotus tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. a and b: shoot and root dry biomass, respectively. Percentages show the reduction in biomass between stressed plants and their controls. Columns without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 10 \pm \text{SD}$ ). c: representative images of different plants during the recovery harvest. Black bars: 25 cm

observed for the L/S ratio, which for LcD and LcT were 37% and 57% lower (respectively) than their controls.

No adventitious root was found in plants under the control treatment at the end of the flooding period. However, differences among accessions were observed for this parameter under stress at this time point. In particular, Lt had the highest number of adventitious roots ( $207 \pm 67$ , Table 3), followed by LcT and LtxLc accessions ( $114.29 \pm 67.78$  and  $95.64 \pm 49.54$  roots per plant, respectively).

The lowest values for this parameter were observed for LcD and Lj (Table 3).

### 3.3 | Photosynthesis and electrolyte leakage

Net photosynthesis rate under saturating irradiance ( $A_{\text{sat}}$ ) was differentially affected by the flooding treatment in the evaluated accessions (Figure 3a). While  $A_{\text{sat}}$  values were not affected under stress

Treatment		RGR Shoot		RGR Root		RGR Total	
(Acc × Flood)		Mean ± SD		Mean ± SD		Mean ± SD	
Lt	Ctrl	0.006 ± 0.002	de	0.001 ± 0.008	b	0.005 ± 0.003	cd
	Flood	0.011 ± 0.003	bcd	0.007 ± 0.007		0.010 ± 0.003	bc
LtxLc	Ctrl	0.009 ± 0.003	cd	0.002 ± 0.008	ab	0.008 ± 0.003	c
	Flood	0.020 ± 0.005	a	0.017 ± 0.013		0.020 ± 0.006	a
LcD	Ctrl	0.008 ± 0.008	d	0.020 ± 0.013	a	0.011 ± 0.006	bc
	Flood	0.016 ± 0.007	ab	0.010 ± 0.007		0.015 ± 0.006	ab
LcT	Ctrl	0.009 ± 0.004	cd	0.002 ± 0.009	ab	0.007 ± 0.004	c
	Flood	0.015 ± 0.009	bc	0.014 ± 0.017		0.015 ± 0.010	ab
Lj	Ctrl	0.001 ± 0.003	ef	-0.007 ± 0.009	c	0.000 ± 0.004	de
	Flood	-0.001 ± 0.007	f	-0.001 ± 0.009		-0.001 ± 0.007	e

Lt: *L. tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. For each variable, means without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 10 \pm SD$ ). No interaction between factors was observed for RGR roots, so significant differences among means of each accession are shown.

**TABLE 2** Effect of the recovery period on relative growth rates (RGR,  $g\ g^{-1}\ day^{-1}$ )

Treatment		Shoot/Root		Leaves/Stems		Adventitious Roots	
(Acc × Flood)		Mean ± SD		Mean ± SD		Mean ± SD	
Lt	Ctrl	2.49 ± 0.83	ef	0.46 ± 0.10	def		
	Flood	3.50 ± 0.97	cde	0.52 ± 0.14	cde	206.6 ± 67.44	a
LtxLc	Ctrl	3.20 ± 0.80	de	0.65 ± 0.14	c		
	Flood	3.57 ± 0.84	cde	0.58 ± 0.12	cd	95.6 ± 49.54	b
LcD	Ctrl	4.67 ± 2.44	bc	1.05 ± 0.38	a		
	Flood	6.75 ± 3.30	a	0.66 ± 0.25	c	53.9 ± 36.05	c
LcT	Ctrl	1.42 ± 0.42	f	0.84 ± 0.12	b		
	Flood	4.97 ± 1.80	b	0.36 ± 0.17	f	114.3 ± 67.78	b
Lj	Ctrl	4.61 ± 1.63	bc	0.41 ± 0.06	ef		
	Flood	4.19 ± 0.95	bcd	0.36 ± 0.12	f	56.2 ± 12.07	c

Lt: *L. tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. For each variable, means without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 14 \pm SD$ ).

**TABLE 3** Effect of partial submergence on biomass-associated variables (S/R and L/S) and adventitious root formation

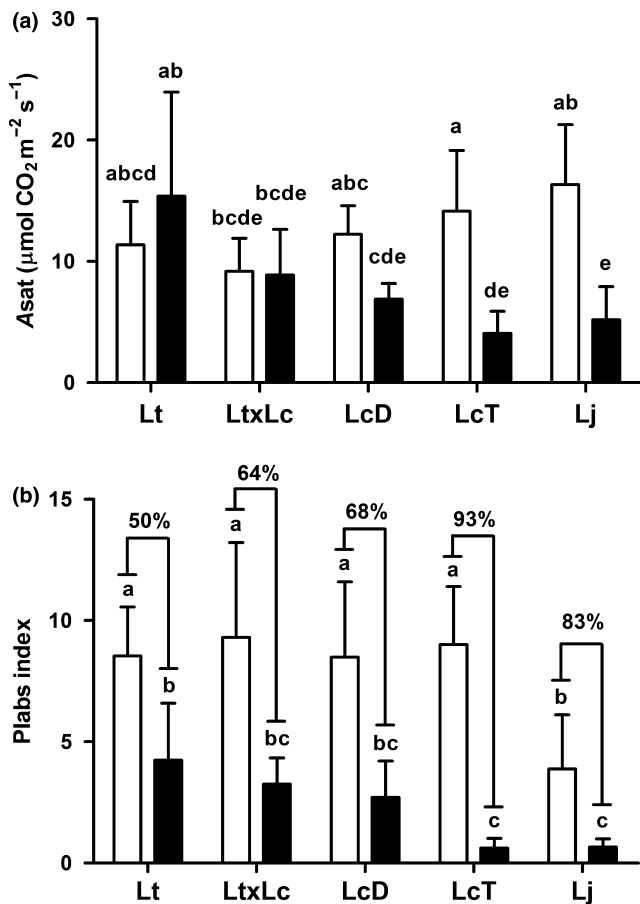
in Lt and LtxLc plants, the values for LcT and Lj were 68 and 71% lower, respectively, than controls. A similar pattern was observed for the  $g_s$  data, although no significant difference was observed in this case (Table S2).

In addition, chlorophyll fluorescence was evaluated and an OJIP analysis was performed (Figure 3B, Table S2). While no change was observed between treatments for the maximum quantum yield of primary PSII photochemistry (Fv/Fm, Table S2), the performance index (Plabs) was differentially lower for each accession under stress (Figure 3B). Regarding this parameter, Lt and LtxLc were the accessions least affected by the flooding treatment (50% and 64% lower, respectively), while LcD, LcT and Lj were the most affected (68%, 93% and 83%, respectively), compared to controls.

Moreover, electrolyte leakage was measured as a stress indicator and as an estimation of membrane integrity after flooding (Table S2). Differences between treatments were only observed for LcT and Lj, which had higher values under stress, while no change was recorded for the rest of the accessions.

### 3.4 | Root anatomy and shoots diameter

A higher proportion of aerenchymatous tissue was observed in root cross sections under the flooding treatment in Lt, LtxLc and LcD (34.9%, 32.0% and 31.3%, respectively) (Figure 4a and c). Moreover, under control conditions, differences in the proportion of aerenchyma were also observed among accessions. In this case, LtxLc and Lt showed the highest aerenchyma percentage per root cross section



**FIGURE 3** Photosynthetic response under flooding. Effect of partial submergence on net photosynthetic rate under saturating irradiance (Asat; a) and performance index (Plabs; b). Lt: *Lotus tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. Percentages show the reduction in the evaluated parameters between stressed plants and their controls. Columns without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 4 \pm \text{SD}$ )

(16.5% and 10.6%, respectively), while LcD, LcT and Lj showed the lowest (3.1%, 2.8% and 2.1%, respectively).

In addition, an increase in stem diameter was observed under the stress treatment for all the evaluated accessions (Figure 4b). The greatest increases were observed for LtxLc, LcD and Lt (143%, 141% and 137% compared to controls, respectively), while the lowest increases were observed for LcT and Lj (60% and 59% compared to controls, respectively).

## 4 | DISCUSSION

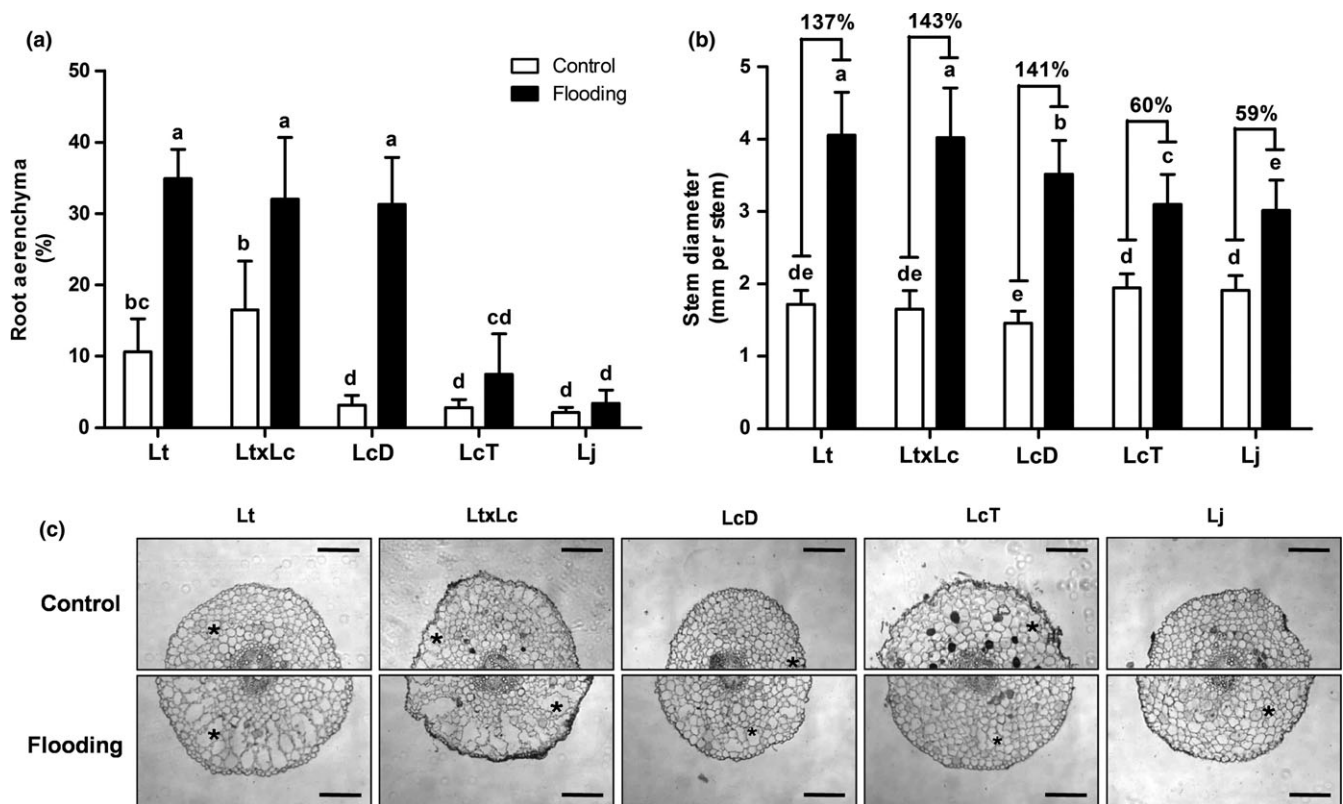
Obtaining new forage legumes adapted to constraint soils is highly relevant to cattle production in marginal areas (León, Striker, Insausti, & Perelman, 2007; Striker et al., 2005). In particular, flooding affects forage legume establishment and thereby forage production (Radović, Sokolović, & Marković, 2009; Striker & Colmer, 2016). In this

sense, *Lotus* species are a highly valued alternative for improving the nutritional quality of pastures in flood-prone environments (Antonelli et al., 2016; Escaray et al., 2012; Radović et al., 2009). However, differences have been reported in the ability of different *Lotus* species to adapt to flooding conditions (Escaray et al., 2012; Vignolio, Fernández, & Maceira, 2002). *L. tenuis* is one of the legume forage species most tolerant to the restrictive conditions of the Flooding Pampa (Striker et al., 2005). Although it has been able to become naturalized in this wide region (Kade, Pagani, & Mendoza, 2003; Montes, 1980), several researchers suggested that its content of PA is below the optimum levels for ruminants feeding (Escaray et al., 2014; Li, Tanner, & Larkin, 1996). The higher level of PA in the interspecific hybrid compared to *L. tenuis* described by Escaray et al. (2014) could increase the quality of the forage produced for feeding ruminants. Moreover, in vitro ruminal methane production tests have found a decrease in enteric methane production (approximately 15%) using forage from the interspecific hybrid with respect to *L. tenuis* (unpublished own data). Therefore, obtaining materials with optimum forage characteristics regarding their nutritional value would also be relevant for the mitigation of greenhouse gas production from cattle areas. In the current study, we evaluated the morphological, physiological and anatomical responses of different *Lotus* species and the LtxLc interspecific hybrid under partial submergence stress, with focus on the response of the accessions with adequate PA content for feeding cattle.

### 4.1 | *Lotus* species responses to flooding stress

Different authors have classified the tolerance of plant species to situations of environmental constraint by considering the reduction in plant dry mass (Cramer, Urano, Delrot, Pezzotti, & Shinozaki, 2011; Sairam, Rao, & Srivastava, 2002). Using the same criteria, our results showed that Lt and LtxLc were the most relatively tolerant accessions, while Lj and LcT the most relatively sensitive (Figure 1, Table S1), with LcD tolerance in between. Other authors have reported similar responses for Lt and LcT to flooding stress, with weight reduction being less marked in Lt than in LcT when subjected to a similar flooding treatment (Striker et al., 2005; Vignolio et al., 2002). However, LcT and Lj have also been classified as more tolerant accessions than other plant genera (Real, Warden, Sandral, & Colmer, 2008; Rocha et al., 2010), although the experimental conditions used in these cases differ from the ones used in the current study. In particular, plant responses to flooding stress depend on the duration of the stress treatment and on the nature of the substrate used in the experiments. In this regard, the potential toxicity of some ions (such as  $\text{Fe}^{2+}$  or  $\text{Mn}^{2+}$ ) can only be present if natural soils are used as a substrate (Shabala, 2011; Zeng, Shabala, Zhou, Zhang, & Shabala, 2013).

Although the reduction in dry weight was greater for LcD than for Lt and LtxLc, it was smaller than the reduction observed for the other accession of the same species (LcT) (Figure 1). It is worth noting that LcD was obtained from a restrictive environment characterized by highly saline, floodable soils (Escaray et al., 2014), so its



**FIGURE 4** Aerenchyma formation in root cross sections and stem diameter under flooding. Effect of partial submergence on aerenchyma formation in roots (a) and primary stem diameters (b). Lt: *L. tenuis*; LtxLc: interspecific hybrid *L. tenuis* × *L. corniculatus* diploid; LcD: *L. corniculatus* diploid; LcT: *L. corniculatus* tetraploid and Lj: *L. japonicus*. Columns without common letters differ significantly (Duncan test,  $p < 0.05$ ;  $n = 5 \pm SD$ , a;  $n = 14 \pm SD$ , b). c: Representative images of root cross sections. Asterisks indicate the presence of aerenchyma lacunae. Black bars: 150  $\mu\text{m}$

better response might be related to a potential adaptation of its germplasm to this stress situation.

The LtxLc accession showed a smaller reduction in dry mass due to flooding than the rest of the evaluated accessions (except Lt) (Figure 1). The similar responses of the hybrid and Lt to the stress condition suggest the heritability of some tolerance traits that participate in the flooding response. In this regard, some agronomic characteristics have already been observed to be heritable by interspecific hybridization in the *Lotus* genus (Castillo et al., 2012; Escaray et al., 2014).

Another important agronomic trait in forage legume species is the L/S ratio. In particular, in alfalfa breeding programmes, the L/S ratio is strongly correlated to digestibility and forage intake by livestock (Kephart, Buxton, & Hill, 1990; Lemaire & Allirand, 1993). Moreover, it has also been suggested that the L/S ratio in the *Lotus* genus is an important selection trait for improving condensed tannin content in forage (Häring, Suter, Amrhein, & Lüscher, 2007). Of all the accessions evaluated, LcD had the highest L/S ratio both in control and stress treatments (Table 3), followed by LtxLc. Annicchiarico (2015) showed that L/S has a high heritability in alfalfa, and our results seem to support this observation in *Lotus*.

During partial submergence, the roots are the main organs affected by stress (Colmer & Voesenek, 2009; Smethurst & Shabala, 2003). In the present study, roots were more affected than shoots in

all the accessions evaluated except for Lj (Figure 1). These observations were also reflected by the S/R ratio (Table 3). A higher S/R ratio is usually associated with a higher relative partition of photosynthates to shoots (Mendoza, Escudero, & García, 2005; Rubio, Casasola, & Lavado, 1995). However, under our stress conditions, changes in S/R may also be related to the decomposition of the root system due to partial submergence, which was particularly observed for the LcT accession, with a negative root RGR during the flooding period (Table 1).

Long-term flooding stress promotes adventitious root generation in different plant species (Colmer & Voesenek, 2009; Voesenek & Bailey-Serres, 2015), and a positive correlation has been suggested between long-term flooding tolerance and adventitious root formation (Real et al., 2008; Song, Shi, Gao, Fan, & Wang, 2011). Our results showed that adventitious roots were only present in the stress treatment plants and mainly in the most tolerant accession (Lt) (Table 3). It is worth noting that LcT and LtxLc presented similar values for adventitious roots (Table 3), although the roots observed in the first accession were smaller than those observed in the second (Figure 1c).

Differences in RGR also suggested differences in flooding tolerance among accessions. In particular, tRGR has been used for studying plant growth under waterlogging stress in different species (Alamri, Barrett-Lennard, Teakle, & Colmer, 2013; Imaz, Giménez,



Grimoldi, & Striker, 2015), including *Lotus* spp. (Teakle et al., 2010). In the current study, LtxLc had the highest tRGR value during the *flooding* period (Table 2), although it presented lower biomass accumulation than Lt under the same treatment. This could be explained by its lower dry mass at the beginning of the experiment. Our data suggest that LtxLc grows more slowly initially, in autumn/winter months, but faster than the rest of the accessions during the spring *flooding* period.

#### 4.2 | Carbon fixation, chlorophyll fluorescence and electrolyte leakage under flooding

Waterlogging stress alters carbon fixation in plants, reducing the net photosynthetic rate (*Asat*) in sensitive species (Ashraf, 2003) but not in some tolerant ones (Insausti, Grimoldi, Chaneton, & Vasellati, 2001). Our results showed that *Asat* did not change for Lt and LtxLc at the end of the stress treatment (55 days) while it was considerably lower for LcT and Lj under the same condition (Figure 3a), compared to controls. These data are consistent with the differences observed in the biomass accumulation of each accession during the *flooding* period (Figure 1). Similar results were also reported for *L. corniculatus* by Striker et al. (2005), who observed that net photosynthetic rate dropped more than 50% in partially submerged plants (6 cm water depth) with respect to controls, while stressed *L. tenuis* plants were less affected. They concluded that *L. tenuis* was more tolerant to prolonged soil *flooding* than *L. corniculatus* due to the maintenance of carbon fixation rates and biomass production under these conditions.

Lower *Asat* values could be partially explained through the impairment of PSII. Alterations in the Plabs parameter were consistent with the alterations observed for *Asat* values in the different accessions (Figure 3b). However, the maximum quantum efficiency of PSII (*Fv/Fm*) was not strongly modified due to waterlogging. The *Fv/Fm* has been used to characterize physiological state in different plant species under abiotic stress conditions, although its reliability depends strongly on the severity of the stress applied in each case (Silvestre, de Sousa Araújo, Vaz Patto, & Marques da Silva, 2014; Strauss, Krüger, Strasser, & Van Heerden, 2006; Van Heerden, Strasser, & Krüger, 2004). Our results are in agreement with previous reports indicating that the Plabs parameter is more sensitive when assessing moderate abiotic stress conditions in plants (Oukarroum, El Madidi, Schansker, & Strasser, 2007; Wang et al., 2012). Furthermore, it has also been used in the detection of tolerant phenotypes in legume species, such as soybean (Krüger et al., 2014) and *Lathyrus* spp. (Silvestre et al., 2014) under abiotic stresses.

On the other hand, stressful conditions cause inefficient functioning of the membranes, which can be estimated through electrolyte leakage (Bajji, Kinet, & Lutts, 2002; Koyro, Hussain, Huchzermeyer, & Khan, 2013). LcT and Lj had the highest in electrolyte leakage during waterlogging, compared to controls, which would indicate a lower extent of tolerance of these two accessions to this stress condition (Table S2).

#### 4.3 | Aerenchyma formation during flooding

Oxygen transport from shoots through the tissues is essential for maintaining root oxygenation during a *flooding* period (Armstrong, 1980; Colmer, 2003). In consequence, low resistance to gas movement due to the formation of aerenchyma (porosity) has been associated with a tolerance trait in different plant species (Armstrong, 1980; Colmer & Voesenek, 2009). Striker and Colmer (2016) showed that different forage legumes with low root porosity (below 10%-12%) under *flooding* presented high biomass reductions due to stress. In line with these results, our data showed that the accessions with the highest capacity of aerenchyma formation, Lt and LtxLc, showed the lowest biomass reduction and the highest tRGR during the *flooding* period (Table 1, Figure 4). Similar results have been observed for *L. tenuis* under comparable conditions (Striker et al., 2005; Teakle, Real, & Colmer, 2006). On the contrary, Lj was the accession with the lowest aerenchyma formation and lowest biomass accumulation under stress. Our results are in agreement with Striker, Casas, Manzur, Ploschuk, and Casal (2014), who concluded that there was no correlation between aerenchyma formation and shoot biomass production in this species. It would not be able to form enough aerenchymatous tissue in *flooding* conditions, further highlighting the importance of this trait in the tolerance to long-term partial submergence in the *Lotus* genus.

In addition, because partial submergence also affects shoot bases, morphological changes in the shoot were analysed. The formation of a white spongy secondary tissue was observed (Figure 1c), which could be related to secondary aerenchyma formation (Yamauchi, Shimamura, Nakazono, & Mochizuki, 2013). In order to quantify this observation, the diameter of the submerged stems was measured, and our results showed that the most tolerant accessions presented the highest increases in stem diameters (Figure 4b). Data suggest that this tissue could be related to the availability of root oxygenation under *flooding* in the *Lotus* genus, in line with other authors who have described the relevance of these secondary tissues in other plants (Teakle, Armstrong, Barrett-Lennard, & Colmer, 2011; Yamauchi et al., 2013). Similar observations have been found in the legume *Melilotus siculus* (Teakle et al., 2011; Verboven et al., 2012).

#### 4.4 | Recovery period

Plant responses during stress period are not always correlated to their responses during a recovery period (Parad, Zarafshar, Striker, & Sattarian, 2013; Striker, 2012). This is a matter of relevance for forage production, because waterlogging periods usually precede the moment of highest cattle-rearing demand on pastures. However, only 20% of the studies conducted on forage legumes have evaluated this issue (Striker & Colmer, 2016). In our work, the plants remained in the recovery period for 5 weeks, trying to emulate a field situation in which the forage resource can then be grazed without being detrimental to the survival of the plants. It has been demonstrated that *L. tenuis* plants with a high proportion of aerenchymatous tissue are severely affected during a post-*flooding*-early grazing due to roots collapse by cattle trampling (Striker,

Insausti, Grimoldi, & Leon, 2006). Our results showed that Lt and LtxLc, which presented the best response under flooding, also performed best during the recovery period (Figure 2). In line with our data, similar results were observed in *L. tenuis* plants after long-term flooding stress and a 90-day recovery period (Vignolio, Maceira, & Fernández, 1994).

Regarding the performance of *L. japonicus*, its biomass accumulation and RGR in the recovery period were the lowest when compared with the other accessions (Table 2). In particular, negative RGR values were recorded, indicating leaves and root tissue lost due to stress without the capacity to recover. To the best of our knowledge, no previous study addresses long-term flooding stress on the model legume *L. japonicus* or its performance after a recovery period.

## 5 | CONCLUDING REMARKS

This study evaluated the responses to partial submergence in five *Lotus* accessions during long-term stress and after a recovery period. Our results showed that *L. tenuis* and the interspecific hybrid LtxLc presented the best response parameters to flooding stress, compared to *L. corniculatus* accessions and *L. japonicus*. Of the three accessions containing PA in the leaves (LcT, LcD and LtxLc), the interspecific hybrid LtxLc was the most tolerant under stress. As a whole, a connection between key traits for flooding stress tolerance (such as aerenchymatous tissue development, relative growth rates and development of adventitious roots) and performance under this environmental constraint was observed. In particular, we suggest the LtxLc accession as a potential material with better forage quality and similar tolerance to floodable soils than *L. tenuis*. To conclude, the interspecific hybridization in the *Lotus* genus could be used as a tool for generating new varieties intended to improve the productivity of cattle production in restrictive areas such as the Flooding Pampa in Argentina.

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## REFERENCES

- Alamri, S. A., Barrett-Lennard, E. G., Teakle, N. L., & Colmer, T. D. (2013). Improvement of salt and waterlogging tolerance in wheat: Comparative physiology of *Hordeum marinum*-*Triticum aestivum* amphiploids with their *H. marinum* and wheat parents. *Functional Plant Biology*, 40(11), 1168–1178. <https://doi.org/10.1071/FP12385>
- Annicchiarico, P. (2015). Alfalfa forage yield and leaf/stem ratio: Narrow-sense heritability, genetic correlation, and parent selection procedures. *Euphytica*, 205(2), 409–420. <https://doi.org/10.1007/s10681-015-1399-y>
- Antonelli, C. J., Calzadilla, P. I., Escaray, F. J., Babuin, M. F., Campestre, M. P., Rocco, R., ... Llamas, M. E. (2016). Lotus spp: Biotechnological strategies to improve the bioeconomy of lowlands in the Salado River Basin (Argentina). *AGROFOR International Journal*, 1(2), 43–53.
- Armstrong, W. (1980). Aeration in higher plants. *Advances in Botanical Research*, 7, 225–332. [https://doi.org/10.1016/S0065-2296\(08\)60089-0](https://doi.org/10.1016/S0065-2296(08)60089-0)
- Arnell, N. W., & Liu, C. (2001). Hydrology and water resources. In J. J. McCarthy, O. F. Canziani, N. A. Leary, D. J. Dokken & K. S. White (Eds.), *Climate change 2001: impacts, adaptation, and vulnerability. Contribution of working group II to the third assessment report of the Intergovernmental Panel on Climate Change (IPCC)* (191–234). Cambridge, UK: Cambridge University Press.
- Ashraf, M. (2003). Relationships between leaf gas exchange characteristics and growth of differently adapted populations of Blue panicgrass (*Panicum antidotale* Retz.) under salinity or waterlogging. *Plant Science*, 165(1), 69–75. [https://doi.org/10.1016/S0168-9452\(03\)00128-6](https://doi.org/10.1016/S0168-9452(03)00128-6)
- Bailey-Serres, J., & Voesenek, L. (2008). Flooding stress: Acclimations and genetic diversity. *Annual Review of Plant Biology*, 59, 313–339. <https://doi.org/10.1146/annurev.arplant.59.032607.092752>
- Bajji, M., Kinet, J.-M., & Lutts, S. (2002). The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regulation*, 36(1), 61–70. <https://doi.org/10.1023/A:1014732714549>
- Blumenthal, M. J., & McGraw, R. L. (1999). Lotus adaptation, use, and management. In P. R. Beuselinck (Ed.), *Trefoil: The science and technology of Lotus* (pp. 97–119). Madison, Wisconsin: CSSA Special Publication Number 28.
- Castillo, A., Rebuffo, M., Rizza, M. D., Folle, G., Santiñaque, F., Borsani, O., & Monza, J. (2012). Generation and Characterization of Interspecific Hybrids of *Lotus uliginosus* × *Lotus corniculatus*. *Crop Science*, 52(4), 1572–1582. <https://doi.org/10.2135/cropsci2011.07.0374>
- Colmer, T. D. (2003). Long-distance transport of gases in plants: A perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell & Environment*, 26(1), 17–36. <https://doi.org/10.1046/j.1365-3040.2003.00846.x>
- Colmer, T. D., & Voesenek, L. (2009). Flooding tolerance: Suites of plant traits in variable environments. *Functional Plant Biology*, 36(8), 665–681. <https://doi.org/10.1071/FP09144>
- Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biology*, 11(1), 163. <https://doi.org/10.1186/1471-2229-11-163>
- Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M., & Robledo, C. W. (2011). InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. Retrieved from <http://www.infostat.com.ar>
- Escaray, F. J., Menéndez, A. B., Gárriz, A., Pieckenstein, F. L., Estrella, M. J., Castagno, L. N., ... Ruiz, O. A. (2012). Ecological and agronomic importance of the plant genus *Lotus*. Its application in grassland sustainability and the amelioration of constrained and contaminated soils. *Plant Science*, 182, 121–133. <https://doi.org/10.1016/j.plantsci.2011.03.016>
- Escaray, F. J., Passeri, V., Babuin, M. F., Marco, F., Carrasco, P., Damiani, F., ... Ruiz, O. A. (2014). *Lotus tenuis* × *L. corniculatus* interspecific hybridization as a means to breed bloat-safe pastures and gain insight

- into the genetic control of proanthocyanidin biosynthesis in legumes. *BMC Plant Biology*, 14(1), 14–40. <https://doi.org/10.1186/1471-2229-14-40>
- Häring, D. A., Suter, D., Amrhein, N., & Lüscher, A. (2007). Biomass allocation is an important determinant of the tannin concentration in growing plants. *Annals of Botany*, 99(1), 111–120. <https://doi.org/10.1093/aob/mcl227>
- Hirabayashi, Y., Mahendran, R., Koirala, S., Konoshima, L., Yamazaki, D., Watanabe, S., ... Kanae, S. (2013). Global flood risk under climate change. *Nature Climate Change*, 3(9), 816–821. <https://doi.org/10.1038/nclimate1911>
- Imaz, J. A., Giménez, D. O., Grimoldi, A. A., & Striker, G. G. (2015). Ability to recover overrides the negative effects of flooding on growth of tropical grasses *Chloris gayana* and *Panicum coloratum*. *Crop and Pasture Science*, 66(1), 100–106.
- Insausti, P., Grimoldi, A. A., Chaneton, E. J., & Vasellati, V. (2001). Flooding induces a suite of adaptive plastic responses in the grass *Paspalum dilatatum*. *New Phytologist*, 152(2), 291–299. [https://doi.org/10.1111/j.0028-646X.2001.257\\_1.x](https://doi.org/10.1111/j.0028-646X.2001.257_1.x)
- Kade, M., Pagani, E. A., & Mendoza, R. E. (2003). A morphological study of populations of *Lotus glaber* Mill. (Fabaceae). *Agronomie*, 23, 203–207. <https://doi.org/10.1051/agro>
- Kephart, K. D., Buxton, D. R., & Hill, R. R. (1990). Digestibility and cell-wall components of alfalfa following selection for divergent herbage lignin concentration. *Crop Science*, 30(1), 207–212. <https://doi.org/10.2135/cropsci1990.0011183X003000010045x>
- Koyro, H.-W., Hussain, T., Huchzermeyer, B., & Khan, M. A. (2013). Photosynthetic and growth responses of a perennial halophytic grass *Panicum turgidum* to increasing NaCl concentrations. *Environmental and Experimental Botany*, 91, 22–29. <https://doi.org/10.1016/j.envexpbot.2013.02.007>
- Krüger, G. H. J., De Villiers, M. F., Strauss, A. J., De Beer, M., Van Heerden, P. D. R., Maldonado, R., & Strasser, R. J. (2014). Inhibition of photosystem II activities in soybean (*Glycine max*) genotypes differing in chilling sensitivity. *South African Journal of Botany*, 95, 85–96. <https://doi.org/10.1016/j.sajb.2014.07.010>
- Lavado, R., Taleisnik, E., Grunberg, K., & Santa María, G. (2008). Visión sintética de la distribución y magnitud de los suelos afectados por salinidad en la Argentina. La Salinización de Suelos En La Argentina: Su Impacto En La Producción Agropecuaria. EDUCC (Editorial Universidad Católica de Córdoba), Córdoba. ISBN, 978–987.
- Lee, T., Lur, H., & Chu, C. (1993). Role of abscisic acid in chilling tolerance of rice (*Oryza sativa* L.) seedlings. I. Endogenous abscisic acid levels. *Plant, Cell & Environment*, 16(5), 481–490. <https://doi.org/10.1111/j.1365-3040.1993.tb00895.x>
- Lemaire, G., & Allirand, J. M. (1993). Relation entre croissance et qualité de la luzerne: Interaction génotype-mode d'exploitation. *Fourrages*, 134, 183–198.
- León, R. J. C., Striker, G. G., Insausti, P., & Perelman, S. B. (2007). Río de la Plata grasslands and *Lotus tenuis*. *Lotus Newsletter*, 37, 67–68.
- Li, Y.-G., Tanner, G., & Larkin, P. (1996). The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture*, 70, 89–101. [https://doi.org/10.1002/\(ISSN\)1097-0010](https://doi.org/10.1002/(ISSN)1097-0010)
- Lüscher, A., Mueller-Harvey, I., Soussana, J.-F., Rees, R. M., & Peyraud, J.-L. (2014). Potential of legume-based grassland–livestock systems in Europe: A review. *Grass and Forage Science*, 69(2), 206–228. <https://doi.org/10.1111/gfs.12124>
- Malik, A. I., Islam, A., & Colmer, T. D. (2011). Transfer of the barrier to radial oxygen loss in roots of *Hordeum marinum* to wheat (*Triticum aestivum*): Evaluation of four *H. marinum*–wheat amphiploids. *New Phytologist*, 190(2), 499–508. <https://doi.org/10.1111/j.1469-8137.2010.03519.x>
- McDonald, M. P., Galwey, N. W., & Colmer, T. D. (2002). Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wetland and dryland grass species. *Plant, Cell & Environment*, 25(3), 441–451. <https://doi.org/10.1046/j.0016-8025.2001.00817.x>
- McNabb, W. C., Waghorn, G. C., Peters, J. S., & Barry, T. N. (1996). The effect of condensed tannins in *Lotus pedunculatus* on the solubilization and degradation of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39; Rubisco) protein in the rumen and the sites of Rubisco digestion. *British Journal of Nutrition*, 76, 535–549. <https://doi.org/10.1079/BJN19960061>
- Mendoza, R., Escudero, V., & García, I. (2005). Plant growth, nutrient acquisition and mycorrhizal symbioses of a waterlogging tolerant legume (*Lotus glaber* Mill.) in a saline-sodic soil. *Plant and Soil*, 275(1–2), 305–315. <https://doi.org/10.1007/s11004-005-2501-3>
- Min, B. R., Barry, T. N., Attwood, G. T., & McNabb, W. C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. *Animal Feed Science and Technology*, 106(1–4), 3–19. [https://doi.org/10.1016/S0377-8401\(03\)00041-5](https://doi.org/10.1016/S0377-8401(03)00041-5)
- Montes, L. (1980). Narrowleaf trefoil naturalized in low-land fields in Buenos Aires province (Argentina). *Lotus Newsletter*, 11, 9–10.
- Oukarroum, A., El Madidi, S., Schansker, G., & Strasser, R. J. (2007). Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll a fluorescence OLKJIP under drought stress and re-watering. *Environmental and Experimental Botany*, 60(3), 438–446. <https://doi.org/10.1016/j.envexpbot.2007.01.002>
- Parad, G. A., Zarafshar, M., Striker, G. G., & Sattarian, A. (2013). Some physiological and morphological responses of *Pyrus boissieriana* to flooding. *Trees*, 27(5), 1387–1393. <https://doi.org/10.1007/s00468-013-0886-9>
- Paruelo, J. M. (1990). Caracterización de las inundaciones en la Depresión del Salado (Buenos Aires, Argentina): Dinámica de la capa freática. *Turrialba (IICA)*, 40(1), 5–11.
- Patra, A. K., & Saxena, J. (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry*, 71(11–12), 1198–1222. <http://www.ncbi.nlm.nih.gov/pubmed/20570294>
- Ponnamperuma, F. N. (1984). Effects of flooding on soils. In T. T. Kozłowski (Ed.), *Flooding and plant growth* (pp. 9–45). New York: Academic Press. <https://doi.org/10.1016/B978-0-12-424120-6.50007-9>
- Radović, J., Sokolović, D., & Marković, J. (2009). Alfalfa—most important perennial forage legume in animal husbandry. *Biotechnology in Animal Husbandry*, 25(5–6–1), 465–475. <https://doi.org/10.2298/BAH0906465R>
- Real, D., Warden, J., Sandral, G. A., & Colmer, T. D. (2008). Waterlogging tolerance and recovery of 10 *Lotus* species. *Australian Journal of Experimental Agriculture*, 48(4), 480–487. <https://doi.org/10.1071/ea07110>
- Rocha, M., Licausi, F., Araujo, W. L., Nunes-Nesi, A., Sodek, L., Fernie, A. R., & van Dongen, J. T. (2010). Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. *Plant Physiology*, 152(3), 1501–1513. <https://doi.org/10.1104/pp.109.150045>
- Rosenzweig, C., Tubiello, F. N., Goldberg, R., Mills, E., & Bloomfield, J. (2002). Increased crop damage in the US from excess precipitation under climate change. *Global Environmental Change*, 12(3), 197–202. [https://doi.org/10.1016/S0959-3780\(02\)00008-0](https://doi.org/10.1016/S0959-3780(02)00008-0)
- Rubio, G., Casasola, G., & Lavado, R. S. (1995). Adaptations and biomass production of two grasses in response to waterlogging and soil nutrient enrichment. *Oecologia*, 102(1), 102–105. <https://doi.org/10.1007/BF00333316>
- Sairam, R. K., Rao, K. V., & Srivastava, G. C. (2002). Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. *Plant Science*, 163(5), 1037–1046. [https://doi.org/10.1016/S0168-9452\(02\)00278-9](https://doi.org/10.1016/S0168-9452(02)00278-9)

- Sasidharan, R., Bailey-Serres, J., Ashikari, M., Atwell, B. J., Colmer, T. D., Fagerstedt, K., ... Hill, R. D. (2017). Community recommendations on terminology and procedures used in flooding and low oxygen stress research. *New Phytologist*, 214(4), 1403–1407. <https://doi.org/10.1111/nph.14519>
- Shabala, S. (2011). Physiological and cellular aspects of phytotoxicity tolerance in plants: The role of membrane transporters and implications for crop breeding for waterlogging tolerance. *New Phytologist*, 190(2), 289–298. <https://doi.org/10.1111/j.1469-8137.2010.03575.x>
- Silvestre, S., de Sousa Araújo, S., Vaz Patto, M. C., & Marques da Silva, J. (2014). Performance index: An expeditious tool to screen for improved drought resistance in the Lathyrus genus. *Journal of Integrative Plant Biology*, 56(7), 610–621. <https://doi.org/10.1111/jipb.12186>
- Smethurst, C. F., & Shabala, S. (2003). Screening methods for waterlogging tolerance in lucerne: Comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. *Functional Plant Biology*, 30(3), 335–343. <https://doi.org/10.1071/FP02192>
- Song, J., Shi, G., Gao, B., Fan, H., & Wang, B. (2011). Waterlogging and salinity effects on two *Suaeda salsa* populations. *Physiologia Plantarum*, 141(4), 343–351. <https://doi.org/10.1111/j.1399-3054.2011.01445.x>
- Soriano, A., León, R. J. C., Sala, O. E., Lavado, R. S., Deregibus, V. A., Cauhépé, M. A., ... Lemcoff, J. H. (1991). Río de la Plata Grasslands. In R. T. Coupland (Ed.), *Ecosystems of the world 8A. Natural grasslands. Introduction and Western Hemisphere* (pp. 367–407). Amsterdam: Elsevier.
- Strasser, R. J., & Srivastava, A. (1995). Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. *Photochemistry and Photobiology*, 61(1), 32–42. <https://doi.org/10.1111/j.1751-1097.1995.tb09240.x>
- Strasser, R. J., Srivastava, A., & Tsimilli-Michael, M. (2000). The fluorescence transient as a tool to characterize and screen photosynthetic samples. In M. Yunus, U. Pathre & P. Mohanty (Eds.), *Probing photosynthesis: Mechanisms, regulation and adaptation* (pp. 445–483). London, UK: Taylor and Francis.
- Strauss, A. J., Krüger, G. H. J., Strasser, R. J., & Van Heerden, P. D. R. (2006). Ranking of dark chilling tolerance in soybean genotypes probed by the chlorophyll a fluorescence transient OJIP. *Environmental and Experimental Botany*, 56(2), 147–157. <https://doi.org/10.1016/j.envexpbot.2005.01.011>
- Striker, G. G. (2012). Time is on our side: The importance of considering a recovery period when assessing flooding tolerance in plants. *Ecological Research*, 27(5), 983–987. <https://doi.org/10.1007/s11284-012-0978-9>
- Striker, G. G., Casas, C., Manzur, M. E., Ploschuk, R. A., & Casal, J. J. (2014). Phenomic networks reveal largely independent root and shoot adjustment in waterlogged plants of *Lotus japonicus*. *Plant, Cell & Environment*, 37(10), 2278–2293.
- Striker, G. G., & Colmer, T. D. (2016). Flooding tolerance of forage legumes. *Journal of Experimental Botany*, 68(8), 1851–1872. [erw239](https://doi.org/10.1093/jxb/erw239)
- Striker, G. G., Insausti, P., Grimoldi, A. A., & Leon, R. J. C. (2006). Root strength and trampling tolerance in the grass *Paspalum dilatatum* and the dicot *Lotus glaber* in flooded soil. *Functional Ecology*, 20(1), 4–10. <https://doi.org/10.1111/j.1365-2435.2006.01075.x>
- Striker, G. G., Insausti, P., Grimoldi, A. A., Ploschuk, E. L., & Vasellati, V. (2005). Physiological and anatomical basis of differential tolerance to soil flooding of *Lotus corniculatus* L. and *Lotus glaber* Mill. *Plant and Soil*, 276(1–2), 301–311. <https://doi.org/10.1007/s11104-005-5084-0>
- Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., & Sivakumaran, S. (2005). Methane production from in vitro rumen incubations with and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology*, 123–124, 403–419. <https://doi.org/10.1016/j.anifeeds.2005.04.037>
- Teakle, N. L., Amtmann, A., Real, D., & Colmer, T. D. (2010). *Lotus tenuis* tolerates combined salinity and waterlogging: Maintaining O<sub>2</sub> transport to roots and expression of an NHX1-like gene contribute to regulation of Na<sup>+</sup> transport. *Physiologia Plantarum*, 139(4), 358–374.
- Teakle, N. L., Armstrong, J., Barrett-Lennard, E. G., & Colmer, T. D. (2011). Aerenchymatous phellem in hypocotyl and roots enables O<sub>2</sub> transport in *Melilotus siculus*. *New Phytologist*, 190(2), 340–350. <https://doi.org/10.1111/j.1469-8137.2011.03655.x>
- Teakle, N. L., Real, D., & Colmer, T. D. (2006). Growth and ion relations in response to combined salinity and waterlogging in the perennial forage legumes *Lotus corniculatus* and *Lotus tenuis*. *Plant and Soil*, 289, 369–383. <https://doi.org/10.1007/s11104-006-9146-8>
- Van Heerden, P. D. R., Strasser, R. J., & Krüger, G. H. J. (2004). Reduction of dark chilling stress in N<sub>2</sub>-fixing soybean by nitrate as indicated by chlorophyll a fluorescence kinetics. *Physiologia Plantarum*, 121(2), 239–249. <https://doi.org/10.1111/j.0031-9317.2004.0312.x>
- Verboven, P., Pedersen, O., Herremans, E., Ho, Q. T., Nicolai, B. M., Colmer, T. D., & Teakle, N. L. (2012). Root aeration via aerenchymatous phellem: Three-dimensional micro-imaging and radial O<sub>2</sub> profiles in *Melilotus siculus*. *New Phytologist*, 193(2), 420–431. <https://doi.org/10.1111/j.1469-8137.2011.03934.x>
- Vignolio, O. R., Fernández, O. N., & Maceira, N. O. (1999). Flooding tolerance in five populations of *Lotus glaber* Mill. (Syn. *Lotus tenuis* Waldst. et Kit.). *Australian Journal of Agricultural Research*, 50(4), 555–559. <https://doi.org/10.1071/A98112>
- Vignolio, O. R., Fernández, O. N., & Maceira, N. O. (2002). Biomass allocation to vegetative and reproductive organs in *Lotus glaber* and *L. corniculatus* (Fabaceae). *Australian Journal of Botany*, 50(1), 75–82. <https://doi.org/10.1071/BT01012>
- Vignolio, O. R., Maceira, N. O., & Fernández, O. N. (1994). Efectos del anegamiento en invierno y verano sobre el crecimiento y la supervivencia de *Lotus tenuis* y *Lotus corniculatus*. *Ecología Austral*, 4, 19–28.
- Voesenek, L. A. C. J., & Bailey-Serres, J. (2015). Flood adaptive traits and processes: An overview. *New Phytologist*, 206(1), 57–73. <https://doi.org/10.1111/nph.13209>
- Wang, Z. X., Chen, L., Ai, J., Qin, H. Y., Liu, Y. X., Xu, P. L., ... Zhang, Q. T. (2012). Photosynthesis and activity of photosystem II in response to drought stress in Amur Grape (*Vitis amurensis* Rupr.). *Photosynthetica*, 50(2), 189–196. <https://doi.org/10.1007/s11099-012-0023-9>
- Yamauchi, T., Shimamura, S., Nakazono, M., & Mochizuki, T. (2013). Aerenchyma formation in crop species: A review. *Field Crops Research*, 152, 8–16. <https://doi.org/10.1016/j.fcr.2012.12.008>
- Zemenchik, R. A., Albrecht, K. A., & Shaver, R. D. (2002). Improved nutritive value of Kura clover–and birdsfoot trefoil–grass mixtures compared with grass monocultures. *Agronomy Journal*, 94(5), 1131–1138. <https://doi.org/10.2134/agronj2002.1131>
- Zeng, F., Shabala, L., Zhou, M., Zhang, G., & Shabala, S. (2013). Barley responses to combined waterlogging and salinity stress: Separating effects of oxygen deprivation and elemental toxicity. *Frontiers in Plant Science*, 4, 313. <https://doi.org/10.3389/fpls.2013.00313>

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