REGULAR ARTICLE

Increasing defoliation frequency constrains regrowth of the forage legume *Lotus tenuis* under flooding. The role of crown reserves

Gustavo G. Striker · Milena E. Manzur ·
Agustín A. Grimoldi

Abstract Repeated defoliation and flooding trigger opposite plant morphologies, prostrated and erect ones, respectively; while both induce the consumption of carbohydrate reserves to sustain plant recovery. This study is aimed at evaluating the effects of the combination of defoliation frequency and flooding on plant regrowth and levels of crown reserves of *Lotus tenuis* Waldst. & Kit., a forage legume of increasing importance in grazing areas prone to soil flooding. Adult plants of *L. tenuis* were subjected to 40 days of flooding at a water depth of 4 cm in combination with increasing defoliation frequencies by clipping shoot mass above water level. The following plant responses were assessed: tissue porosity, plant height, biomass of the different organs, and utilization of water-soluble carbohydrates (WSCs) and starch in the crown. Flooding consistently increased plant height independently of the defoliation frequency. This response was associated with a preferential location of shoot biomass above water level and a reduction in root biomass accumulation. As a result, a second defoliation in the middle of the flooding period was more intense among plants that are taller due to

flooding. These plants lost *ca.* 90% of their leaf biomass vs. *ca.* 50% among non-flooded plants. The continuous de-submergence shoot response of frequently defoliated plants was attained in accordance to a decrease of their crown reserves. Consequently, these plants registered only 27.8% of WSCs and 9.1% of starch concentrations with respect to controls. Under such stressful conditions, plants showed a marked reduction in their regrowth as evidenced by the lowest biomass in all plant compartments: shoot, crowns and roots. Increasing defoliation frequency negatively affects the tolerance of the forage legume *L. tenuis* to flooding stress. Our results reveal a trade-off between the common increase in plant height to emerge from water and the amount of shoot removed to tolerate defoliation. When both factors are combined and defoliation persists, plant regrowth would be constrained by the reduction of crown reserves.

Keywords Crown reserves · Defoliation frequency · Flooding · Leaf removal · *Lotus tenuis* · Plant height

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G. G. Striker (✉) · M. E. Manzur · A. A. Grimoldi
IFEVA-CONICET, Facultad de Agronomía,
Universidad de Buenos Aires,
Avenida San Martín 4453,
CPA 1417 DSE Buenos Aires, Argentina
e-mail: striker@ifeva.edu.ar

Introduction

The ability of plants to shift their morphology according to the environment provides a better chance to improve its performance under changing growth conditions (Sultan 2000). However, in flood-prone grasslands ecosystems, there is a conflict in the expression of plant morphology due to the combination

of defoliation by grazing and flooding. This is because defoliation promotes prostrated plant growth while flooding induces exactly the opposite: erect plant forms (Oesterheld and McNaughton 1991). Defoliated plants are of low stature and concentrate their biomass near the soil surface (Briske and Richards 1995; McNaughton 1983; Zheng et al. 2010). Both features help them to ameliorate subsequent defoliation events (Briske and Richards 1995; Lemaire and Chapman 1996; McNaughton 1983). On the contrary, flooded plants are taller due to promoted plant height, thus showing a higher leaf proportion above water, facilitating oxygen capture for root aeration through the aerenchymatic tissue (Bailey-Serres and Voesenek 2008; Colmer 2003; Laan et al. 1990; Striker et al. 2005; Visser and Voesenek 2004). It has been stated that such conflicting morphological responses in plant height can depress plant regrowth by affecting proportions of submerged/emergent shoots (Striker et al. 2008). The results obtained in grass species, which have a high dependence on current assimilation for regrowth (Lattanzi et al. 2005; Schnyder and de Visser 1999), support this idea as a result of a higher proportion of submerged shoots after defoliation (Hayball and Pearce 2004; Oesterheld and McNaughton 1991; Striker et al. 2008). However, in legumes with a higher capacity of using crown reserves during regrowth, the only available report showed that regrowth was slightly affected in Lotus tenuis when plants were defoliated once before flooding (Striker et al. 2008). In this work we study the effects of defoliation frequency under flooding conditions on the regrowth of Lotus tenuis Waldst. & Kit., a forage legume of increasing importance in areas prone to soil flooding.

Studies on the effects of defoliation frequency in legume species were mostly done on Medicago sativa (alfalfa). They showed that organic reserves are critical for sustaining shoot regrowth (Avice et al. 1997; Meuriot et al. 2005; Teixeira et al. 2007). In particular, carbon reserves (water-soluble carbohydrates and starch) are known to support the respiration of residual plant organs during early regrowth (Avice et al. 1996). It has been shown that the contribution of reserves for plant regrowth is dependent on the remnant leaf biomass after defoliation (Meuriot et al. 2004). Intense and/or frequent defoliations (i.e. lower cutting height and short time for recovery, respectively) determine a lower remnant leaf biomass and, in

consequence, a higher dependence on reserves for sustaining regrowth (Avice et al. 1996; Sinclair et al. 2007). In addition, the capacity of using reserves allowing for shoot regrowth after defoliation was reported to differ among legumes (Li et al. 1996; Smith 1962). In this respect, it was suggested that Lotus species are less dependent on crown reserves for regrowth than other forage legumes (i.e. alfalfa, Kallenbach et al. 2001; Smith 1962). In this paper, we explore whether L. tenuis can intensively use reserves during regrowth when it is subjected to extreme carbon starvation caused by successive defoliations under flooding conditions.

Studies on how anoxia by flooding affects the reserve levels in flood-tolerant species have shown that some plants are capable of sustaining their basic metabolism by consuming reserve carbohydrates (Dixon et al. 2006; Laan and Blom 1990; Schlüter and Crawford 2001). In flood-sensitive alfalfa, authors frequently noted the accumulation of water-soluble carbohydrates (hereafter WSCs) and minor changes on the starch concentration of the crowns due to the lack of growth and sugar demand under waterlogged conditions (Barta 1988; Castonguay et al. 1993). In contrast, in flood-tolerant L. tenuis, which is able to maintain carbon fixation and plant growth during long-term flooding (Striker et al. 2005; Teakle et al. 2006), we found a high consumption of reserves that ensured plant survival when aerial photosynthesis was impeded by completely submerging the plants for 30 days (Manzur et al. 2009). Considering that both flooding and defoliation can trigger the consumption of reserves and that this consumption is related to the level of stress suffered by plants (Gibbs and Greenway 2003), we now predict that progressive stressful conditions imposed by repeated defoliation on plants that are taller due to flooding could determine a depletion of crown reserves. The understanding and quantification of the reserves status in crowns and how reserves are affected by such stress can be useful in defining best management practices in order to enhance the forage yield and the persistence of the legume L. tenuis under field conditions.

In this work we test the hypothesis that legume regrowth under flooding conditions depends on the frequency of defoliation. We predict a lower plant regrowth under a higher defoliation frequency as a result of higher leaf removal on plants that are taller

due to flooding, which would also present lower crown reserves. We focused on the role of crown carbohydrates (WSCs and starch) in dealing with higher leaf removal due to increasing plant height as the result of flooding. For this purpose, adult plants of *L. tenuis* were subjected to 40 days of flooding at a water depth of 4 cm in combination with one or two defoliation events (days 0 and 20) that consisted in clipping the shoot mass above water level. The following plant responses were assessed: tissue porosity, height, biomass of the different organs and the utilization of crown carbohydrates. As far as we know, this is the first study to address the effects of defoliation frequency on the performance of flooded plants, and the first to reveal the causes of the depressed regrowth of a flood-tolerant legume species when defoliation occurs at the same time as the flooding.

Materials and methods

Plant material

Lotus tenuis Waldst. & Kit. (syn. *Lotus glaber* Mill.) is a warm-season perennial legume naturalized in the Flooding Pampa grasslands, a region subjected to periodic flooding events (Soriano 1991). Its growing season ranges from spring to autumn. It usually presents a well-developed crown and taproot with extensive lateral root branching. Leaves are pentafoliate with two of the leaflets at the petiole base resembling stipules. This species is able to alter its growth habit in response to environmental conditions: plants grow prostrated (plagiotropic orientation of their shoots) under grazing, and become erect by shifting their shoots to an orthotropic position under flooding conditions (Striker et al. 2008) and closed canopies (Striker et al. 2010).

Experimental details

Seeds of *L. tenuis* (cv. Pampa INTA) were germinated in an incubator (25°C) in polystyrene boxes containing absorbent white paper saturated with distilled water. After 2–3 days, germinated seeds were transplanted to 2 L plastic pots (three seedlings per pot) filled with sand and topsoil (1:1) from a lowland grassland of the Flooding Pampa (3.3% organic carbon; for further details, see Soriano 1991) and transferred to a glasshouse at the Faculty of Agronomy at University of Buenos Aires. Seedlings were subsequently thinned to one plant per pot and grown for 6 months at 22 ± 6 °C and midday photosynthetic photon flux density (PPFD) of 1400 ± 40 μmol m^{-2} s^{-1}.

Flooding and defoliation treatments

Six-month-old plants of *L. tenuis* (biomass: 1.8 ± 0.1 g plant^{-1}, height: 4.6 ± 0.2 cm; see Day 0 in Figs. 1 and 3) were subjected to flooding and defoliation treatments following a fully randomised design (2×3: non–flooding and flooding×non–defoliation, one defoliation and two defoliations) with six replicates.
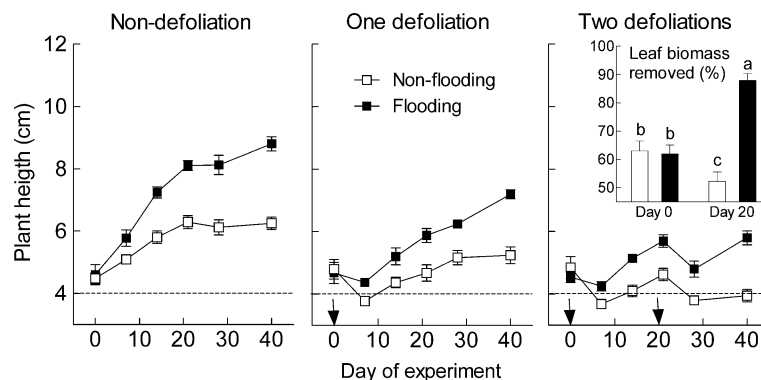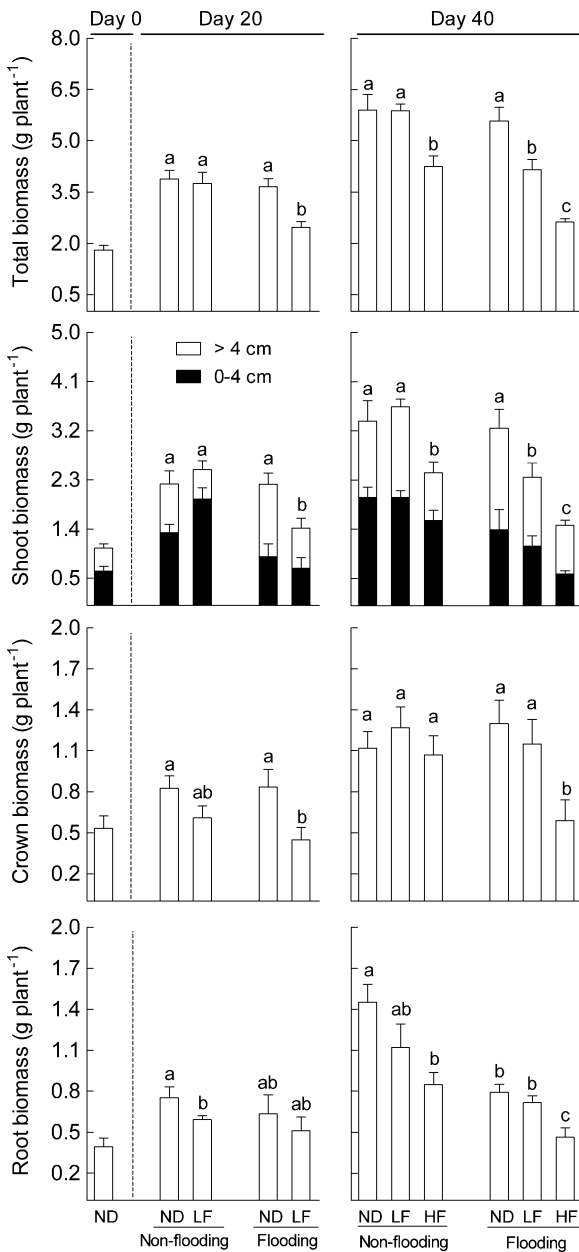

Fig. 1 Plant height of *Lotus tenuis* subjected to a combination of flooding and defoliation treatments. Arrows indicate each defoliation event. Dashed lines indicate the flooding level. The inset graph shows the proportion of leaf biomass removed by clipping (with respect to total leaf biomass per plant) at days 0 and 20 under non-flooding and flooding conditions. Different letters indicate significant differences ($P<0.05$) between treatments (date and flooding). Values are means±SE of six replicates

Fig. 2 Biomass of *Lotus tenuis* plants subjected to a combination of flooding and defoliation treatments. Harvests were carried out at the beginning (day 0), at the middle (day 20) and at the end of the experiment (day 40). Biomass removed by defoliation at each opportunity was added to the corresponding shoot fraction of the upper layer. Abbreviations: ND, non-defoliation; LF, low defoliation frequency (cutting only at day 0); HF, high defoliation frequency (cutting at days 0 and 20). Different letters indicate significant differences ($P<0.05$) among treatments within each date based on Tukey's test. Values are means±SE of six replicates

maintaining a water level of 4 cm above the soil surface in accordance to the duration and intensity of a typical flooding in these grasslands. Non-flooded pots were watered daily to field capacity and allowed to drain freely. It should be noted that six additional plants per treatment were used in the destructive sampling done at the middle of the flooding period (carbohydrates in plant crowns and biomass determinations). The experiment
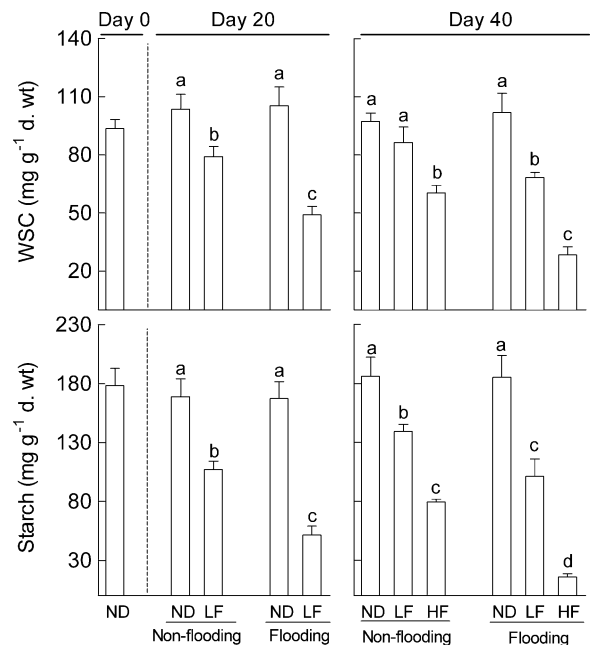

Fig. 3 Water-soluble carbohydrates (WSCs) and starch concentration in crowns of *Lotus tenuis* plants subjected to a combination of flooding and defoliation treatments. Measurements were carried out at the beginning (day 0), at the middle (day 20) and at the end of the experiment (day 40). Abbreviations: ND, non-defoliation; LF, low defoliation frequency (cutting only at day 0); HF, high defoliation frequency (cutting at days 0 and 20). Different letters indicate significant differences ($P<0.05$) among treatments within each date based on Tukey's test. Values are means±SE of six replicates

Defoliation was simulated by clipping plant biomass above 4 cm height. Biomass removals were made once (day 0) in plants subjected to a low defoliation frequency (referred to here as "LF") and twice (days 0 and 20) in plants subjected to a high defoliation frequency (referred to here as "HF"). A defoliation interval of 20 days can be considered representative of frequently defoliated plants of *L. tenuis* in these grasslands (*sensu* Agnusdei and Mazzanti 2001). The flooding treatment was applied for 40 days by

ran during early spring, when the air vapour pressure deficit in the glasshouse was on average 1.9 KPa. Soil anoxia by flooding was characterized by measuring the oxygen diffusion rate (ODR) at a soil depth of 5 cm with platinum microelectrodes.

Tissue porosity

Fractional porosity of roots and shoots were measured at the end of the experiment (day 40) using the pycnometer method (Jensen et al. 1969), based on the weight increase that occurs when air spaces of plant tissues (i.e. intercellular air spaces and aerenchyma) are replaced by water after maceration. The porosity in aerial (shoot) and submerged tissues (roots) allows us to compare the capacity for internal aeration from shoot to root among flooded and non-flooded plants (see reviews by Colmer and Voesenek 2009, and Visser and Voesenek 2004). This variable was only measured in plants grown under flooding and non-flooding conditions because it was demonstrated that defoliation does not alter the capacity to generate aerenchyma in this species (see Striker et al. 2008).

Plant height and shoot number

Plant height was quantified every week during the course of experiment. This variable provides information about plant morphology induced by treatments (prostrate under defoliation *vs.* erect under flooding) and the consequences on plant regrowth when plants that are taller due to flooding are defoliated and a higher shoot mass removal is expected. The number of shoots per plant was measured three times: at the beginning of the treatments (day 0), in the middle of experiment to coincide with the second defoliation (day 20), and at the end of the experiment (day 40).

Biomass responses

Plant harvests were carried out at the beginning of the treatments (day 0) in randomly chosen individuals ($n=6$), at day 20 to coincide with the second defoliation and at the end of the experiment (day 40). Plant biomass was separated into shoot, crown and root. Shoot biomass was harvested in two layers: 0–4 cm and >4 cm, in accordance with the defoliation height and flooding water level. The biomass removed by defoliation at each opportunity was weighed, and later included in the shoot fraction of the corresponding plants. The fraction of shoot biomass above 4 cm with respect to the total shoot biomass was calculated to evaluate the preferential placement of the aerial biomass induced by treatments. In addition, the quotient between clipped leaf biomass (g plant^{-1}) and the total leaf biomass (g plant^{-1}) was calculated to determine the proportion of leaves removed by defoliation under flooding and non-flooding conditions at days 0 and 20. Harvested material was weighed after oven drying for 72 h at 70°C.

Carbohydrates analyses

Crown reserves were examined to study their role in plant regrowth after stressful conditions imposed by repeated defoliation in flooded soil. Crown samples (2 cm of the transition zone between shoots and roots that functions as a storage reserve tissue for plant sprout) were used for the analyses. A minimal quantity of 300 mg (d. wt.) of dried sample was milled to determine WSCs with anthrone reagent. The flour was extracted in 80% ethanol (v/v) for 1 h, stirred and then centrifuged for 5 minutes. The supernatant was filtered twice and diluted with 25 mL distilled water and heated to 60°C for 1 h. All supernatants were made up to 100 mL with 80% ethanol and aliquots mixed with anthrone reagent (200 mg anthrone in 75% v/v H_2SO_4), left to stand for 30 min, shaken and heated for 10 min in an 80°C water bath (Yemm and Willis 1954). Absorbance was recorded at 620 nm and the total soluble carbohydrate concentration was calculated based on a glucose calibration curve. The remaining residue was used for starch determination. The ethanol-extracted residue was dried under vacuum at 4°C. Water (500 µL) was added to each tube, and the tubes were heated in a boiling water bath for 10 min to gelatinise starch. The resulting homogenate was incubated with 0.7 ml of a solution containing 18 units.ml^{-1} of amyloglucosidase in an acetate buffer at a pH 5.1 and a temperature of 37°C overnight. The amount of glucose released was measured using the anthrone method as described above (Yemm and Willis 1954). WSCs and starch concentration in plant crowns (mg g^{-1} d. wt.) were determined at the beginning (day 0), the middle (day 20) and the end of the experiment (day 40).

Statistical analysis

The variations in plant height during the experiment were evaluated by two-way repeated measures ANOVA with defoliation and flooding as the main between-subject factors and time as the within-subject factor (Von Ende 1993). In addition, orthogonal contrasts were done to compare plant height between flooded and non-flooded plants within each date and defoliation level. Biomass, shoot number per plant, WSCs and starch in plant crowns were analysed through two-way ANOVAs for each date of harvest (days 20 and 40). When significant interactions were detected, a subsequent Tukey test was applied to determine the effects of the treatments. Normality and the homogeneity of variances were previously verified. The variables involving proportions (i.e. tissue porosity and proportion of leaves removed) were arcsine $\sqrt{x}$ transformed previous analyses. Statistical analyses were performed using the STATISTICA package for Windows (StatSoft, Tulsa, OK, USA). All results are presented as non-transformed means of six replicates ± standard error.

Results

Flooding effects on soil aeration

Oxygen diffusion rate (ODR) under non-flooding treatments remained between 72.4 and 82.3×10^{-8} g cm^{-2} min^{-1}, indicating well-oxygenated soil conditions. In flooded soil, ODR decreased rapidly from 78.3 ± 3.7 to $4.5\pm1.1\times10^{-8}$ g cm^{-2} min^{-1} during the first 4 days, and remained near zero until the end of the experiment, indicating anaerobic soil conditions.

Plant height and leaf tissue removal

Initial plant height was similar among plants assigned to the different treatments ($P=0.73$). Accordingly, the proportion of leaf biomass removed by defoliation at day 0 was the same among plants assigned to non-flooded and flooded treatments ($62\pm3.5\%$; $P=0.64$; inset of Fig. 1). During the course of the experiment, the plant height varied depending on the combination of flooding and defoliation treatments (rmANOVA, flooding×defoliation×time: $P<0.05$; Fig. 1). Increasing defoliation frequency reduced plant height that

was particularly noticeable under non-flooded conditions, while flooding increased plant height (flooding×defoliation: $P<0.01$). Therefore, for the same frequency of defoliation, flooded plants were always taller than non–flooded ones ($P<0.01$ in all cases; see Fig. 1). Importantly, this incremented plant height under flooding conditions led to a higher proportion of shoot biomass located above the water level, even in the plants that had been defoliated (51.7–59.7%; Fig. 2 and Table 1). As a result, the second defoliation (at day 20) provoked a much higher leaf removal on flooded plants than in non-flooded ones ($88.9\pm2.5\%$ vs. $51.1\pm3.2\%$ of leaves removed, respectively; $P<0.001$; see inset in Fig. 1). For that reason, flooded plants started with much less remnant leaf biomass (only 10%) than the non-flooded plants to sustain plant recovery from the second defoliation event.

Biomass responses

In non-flooded soil, a single initial defoliation (LF; Fig. 2) did not alter plant biomass accumulation in comparison to non-defoliated plants. However, high defoliation frequency (HF) negatively affected plant growth by reducing the final plant biomass by 27.9% with respect to non-defoliated or once defoliated (LF) plants (Fig. 2; Table 1). Such lower biomass was the result of lower shoot and root biomass accumulation (Fig. 2). Conversely, in flooded soil, the initial defoliation was enough to provoke a negative effect on biomass accumulation rapidly evident at day 20 of the experiment (see F×D in Table 1; Fig. 2). At this time, total biomass was 22.3% lower as a result of a reduced shoot and crown biomass than under all other treatments (see left panel in Fig. 2; Table 1). During the following 20 days, once defoliated (LF; Fig. 2) plants continued growing under flooding conditions without further depressing their growth, thus allowing them to maintain small differences in biomass with respect to non-flooded plants (25.6 %, see the right panel in Fig. 2).

A second defoliation under flooding conditions intensified the negative effect on plant growth (see F×D in Table 1; Fig. 2). Thus, flooded plants subjected to high defoliation frequency (HF) registered the lowest final biomass accumulation: 56.6% less than non-defoliated plants ($P<0.05$; Fig. 2). This response was a product of lower shoot, crown and root biomass with a minimal growth rate of each

Table 1 Results of two-way ANOVA for the effects of flooding and defoliation on different variables for *Lotus tenuis* plants

Variable	Main effects		Interaction	MS Error
	Flooding	Defoliation	F×D	
Crown reserves				
Day 20				
Water soluble carbohydrates	1.21 ns	14.83 **	4.12 *	8.87
Starch	2.29 ns	21.90 ***	4.02 *	7.10
Day 40				
Water soluble carbohydrates	10.74 **	48.65 ***	5.59 **	5.63
Starch	9.68 **	53.22 ***	3.87 *	8.62
Biomass responses				
Day 20				
Total	5.82 *	2.10 ns	4.48 *	0.30
Shoot	4.38 *	0.42 ns	4.54 *	0.26
Crown	0.15 ns	8.83 ***	0.23 ns	0.05
Root	0.09 ns	0.92 ns	0.66 ns	0.08
Shoot fraction above 4 cm	28.26 ***	11.85 ***	5.08 *	0.06
Day 40				
Total	24.27 ***	28.65 ***	3.36 *	0.63
Shoot	29.59 ***	29.38 ***	4.70 *	0.23
Crown	1.2 ns	3.95 *	2.23 ns	0.14
Root	28.2 ***	10.02 **	1.96 ns	0.07
Shoot fraction above 4 cm	4.21 *	0.01 ns	0.37 ns	0.06

F-values and the Mean Squares of the Errors (MS Error) of the ANOVAs are presented. Degrees of freedom for each source of variation are: 1 (Flooding), 2 (Defoliation), 2 (F×D) and 30 (Error). Significant differences: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, $P>0.05$

compartment throughout the experiment (cf. day 0 vs. day 40 in Fig. 2). These responses were entirely in line with that which could be expected for plants with a minimal proportion of remnant leaves (inset in Fig. 1) and diminished reserve levels in crowns (Fig. 3). It should be noted that in absence of defoliation, flooding did not provoke significant reductions in final plant biomass although the biomass allocation did change, favouring shoot over root growth as a common response to flooding (Fig. 2).

Shoot number

At the beginning of the experiment, plants assigned to the different treatments presented similar shoot numbers ($P>0.72$; Table 2). After 20 days of the experiment, the initial defoliation increased shoot

Table 2 Shoot number of *Lotus tenuis* plants subjected to a combination of flooding and defoliation treatments at the beginning (day 0), at the middle (day 20) and at the end of the experiment (day 40). Abbreviations: ND, non-defoliation; LF, low defoliation frequency (cutting only at day 0); HF, high defoliation frequency (cutting at days 0 and 20). Different letters indicate significant differences ($P<0.05$) among treatments within each date based on Tukey's test. Values are means ±SE of six replicates

Shoot number (plant⁻¹)	Non-flooding			Flooding		
	ND	LF	HF	ND	LF	HF
Day 0	31.4±1.9 a	32.9±2.2 a	32.0±2.1 a	32.3±2.2 a	32.1±1.8 a	32.9±1.8 a
Day 20	37.6±2.2 b	44.9±1.9 a	45.7±2.1 a	35.6±2.3 b	34.9±2.0 b	34.7±1.7 b
Day 40	42.9±2.1 c	49.9±2.7 b	55.2±1.9 a	41.6±1.9 c	38.6±2.1 c	28.5±3.0 d

number by 20.4% in plants grown under non-flooded conditions; while flooding, as a single factor or in combination with defoliation, did not modify this parameter (F×D interaction: $P<0.05$; see Table 2). When this parameter was evaluated at the end of the experiment, plants subjected to a high defoliation frequency (HF; Table 2) showed a significant increase in the shoot number of non-flooded plants (28.6% higher than non-defoliated plants; $P=0.035$), a response that was not registered under flooding conditions (F×D interaction: $P<0.05$; Table 2). As a result, in the most stressful scenario (a high defoliation frequency in flooded soil), the lowest number of shoots per plant was recorded. Remarkably, such stressed plants attained a final number of shoots that did not differ from that registered on day 0 ($P=0.24$).

Water soluble carbohydrates and starch in plant crowns

In non-flooded soil, the initial defoliation determined a slight reduction in WSCs and starch concentration in plant crowns already evident at day 20 of the experiment. On this date, defoliated plants registered decreased concentrations of WSCs (23.4% lower) and starch (35.5% lower) than non-defoliated ones. Forty days after defoliation, plants only subjected to initial defoliation (LF; Fig. 3) recovered the concentration levels of WSCs but not for starch. In plants subjected to a second defoliation (HF; Fig. 3), the consumption of reserves persisted in a way that final concentrations of WSCs and starch were 37.8% and 57.3% lower (respectively) than in non-defoliated plants (Fig. 3; Table 1). Importantly, after each defoliation event, plants progressively consumed their starch reserves, but these never recovered their initial levels.

In flooded soil, the use of reserves by defoliated plants was much more intense than in non-flooded soil (see D×F in Table 1; Fig. 3). At day 20, defoliated plants grown in flooded soil showed 52.7% and 69.1% lower concentrations of WSCs and starch than non-defoliated ones. In addition, flooded plants subjected to a second defoliation (HF; Fig. 3) showed a major depletion of reserves in crowns as revealed by the low values registered for WSCs and starch in the final measurement. Consequently, plants subjected to a high defoliation frequency showed 72.2% lower WSC concentration and 90.9% lower starch concentrations than non-defoliated ones

(respectively; Fig. 3; Table 1). It should be noted that flooding without defoliation did not change the concentration of either WSCs or starch at any measurement date as could be expected for flood-tolerant species (Fig. 3).

Plant tissue porosity

As expected, root porosity was lower under non-flooding conditions (13.4 %) and higher under flooding at the end of the experiment (31.8%; t_{10}: 5.41, $P<0.001$). Constitutive shoot porosity in non-flooded plants was high (e.g. 23.9 %) and was further increased by 70% in plants grown under flooding conditions (40.9%; t_{10}: 3.79, $P<0.01$).

Discussion

Regrowth of Lotus tenuis as affected by repeated defoliation under flooding conditions

Research indicates a trade-off between the increment of plant height in order to emerge from water and the amount of shoot removed in order to minimize the subsequent effects of defoliation. When plants are flooded, there is an increment in plant height that exposes more leaves above the water (see also Colmer and Voesenek 2009 and references therein; Striker et al. 2005; 2008). Conversely, when plants are defoliated under non-flooding conditions, a lower proportion of leaves are located above the cutting level as a result of decreased plant height and the number of shoots per plant rises. Both of these are typical responses associated with grazing (Briske and Richards 1995; Lemaire and Chapman 1996; McNaughton 1983; Zheng et al. 2010). When both of these factors were combined in the forage legume Lotus tenuis, the flood-induced plant morphology prevailed and the de-submergence of shoots continued at the expense of the consumption of crown reserves and without an increase in plant shoot number (Figs. 1 and 3 and Table 2). Hence, as flooded plants were taller, most of their leaves were removed by defoliation in a second cutting (inset Fig. 1). As a consequence, when the defoliation frequency increased, plant regrowth under flooding conditions was highly limited in terms of both biomass accumulation (Fig. 2) as well as shoot sprouting (Table 2). As

far as we know, this is the first work demonstrating this trade-off in a legume species re-growing from crown reserves. This idea was previously stated only for grass species, which regrow mainly from current assimilation (see Hayball and Pearce 2004 for *Bolboschoenus caldwellii*; Merril and Colberg 2003 for *Deschampsia caespitose*; Oesterheld and McNaughton 1991 for *Cenchrus ciliaris* and *Panicum coloratum*; and Striker et al. 2008 for *Paspalum dilatatum*).

At all defoliation treatments, the continuous emergence of shoots from water in flooded soil allows plants to restore contact of leaves with atmospheric oxygen (Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009; Grimoldi et al. 1999; Laan et al. 1990; Striker et al. 2005), allowing the capture and transport of oxygen from shoots to roots of increased porosity (i.e. higher aerenchyma tissue) through passive diffusion (Colmer 2003). This de-submergence shoot response, which is relevant in determining the flooding tolerance of once defoliated plants (see also Striker et al. 2008), negatively determined the outcome of plants subjected to a higher defoliation frequency. In this situation, photosynthesis at the plant level seems to be a major constraint for plant regrowth under flooding conditions, as plants presented only a minimal residual (fully submerged) leaf biomass (10% d. wt basis) after the second defoliation event. In addition, it is known that the regrowth from defoliation of *L. tenuis* is mainly produced by the sprouting of basal axillary buds of the old stems (Smith 1962). Therefore, plants with only a little amount of (mostly submerged) leaf mass regrowing from buds on basal positions appeared unable to rapidly capture the carbon dioxide and oxygen they required to sustain vigorous photosynthesis and aerobic metabolism after cutting (Kozlowski and Pallardy 1984). Here, an intense use of reserves (discussed in the next section) appears to be the main carbon source to start a self-sustained process of leaf area expansion and shoot de-submergence when considering that the alternative source of energy, the underwater photosynthesis, seems not to be highly important in this case (but see Mommer and Visser 2005). In addition, the lower residual leaf biomass of plants subjected to a high defoliation frequency, and the accelerated yellowing of submerged leaves of this species (probably mediated by ethylene; Jackson 2008) registered by Manzur et al. (2009) support this idea. In contrast, in grass species lacking of reserve organs (i.e. crowns like *L. tenuis*) it is possible that underwater photosynthesis would play a major role facilitating plant regrowth. In this respect, a work on six wetland species revealed that values for underwater photosynthesis of a species having large amounts of reserves (*Acorus calamus*; Schlüter and Crawford 2001) were three times lower than among other species (Colmer and Pedersen 2008).

It should be noted that *L. tenuis* responds differentially to complete submersion if the immersion is due to greater water depth (i.e. higher flooding intensity; Manzur et al. 2009) or defoliation (as we have presented here). Plants completely submerged as a result of greater water depth adopt a non-elongating quiescent strategy (Manzur et al. 2009), while plants submerged due to defoliation show a submergence-escape response (as we have presented here) similar to the strategy of plants growing under partial submergence conditions (Manzur et al. 2009). On that note, there are two mutually non-excluding reasons, which may help to explain this differential response: (*i*) the environmental cues and available energy needed in order to sustain shoot elongation and (*ii*) hormonal changes that would occur from combined defoliation and flooding. First, the environment is expected to be more stressful for plant growth in deep water (i.e. submerged plants at a water depth of 20 cm) than in shallow water (i.e. submerged plants due to defoliation at a water depth of 4 cm). This is because at deeper water positions, the availability of light (and to a lesser extent dissolved oxygen) is much lower (Jackson and Ram 2003). It could be expected that the plant's emergence from water through shoot elongation would be more likely in shallow water (Colmer and Voesenek 2009). Second, defoliation could increase the levels of bioactive gibberellins (see Morvan-Bertrand et al. 2001) which, in conjunction with a decline in endogenous abscisic acid due to ethylene accumulation in submerged tissues (Chen et al. 2010; Jackson 2008; Visser and Voesenek 2004), would promote shoots elongation. This issue deserves further research as it can reveal the mechanisms by which the submergence-escape response prevails in flooded plants that are defoliated due to grazing.

Crown reserves and plant regrowth under defoliation and flooding conditions

Under a low defoliation frequency in non-flooding conditions, *L. tenuis* was able to fully recover its

biomass production until the end of the experiment by using WSCs and starch stored in its crowns (Figs. 2 and 3), a finding that concurs with previous works (Kallenbach et al. 2001; Smith 1962; Striker et al. 2008). This result indicates that carbon utilization from crown reserves allowed plants to compensate for leaf removal as seen in *Medicago sativa* cv. Lodi under infrequent cutting (45 days between defoliations; Avice et al. 1997). In contrast, under a high defoliation frequency, plants were not able to maintain their biomass accumulation in spite of the persistent use of their crown reserves. Here, the slightly higher remnant leaf biomass of plants after the second defoliation (inset Fig. 1) was not enough to compensate for two events of shoot removal during a period of 40 days. It is also possible that the second defoliation event, done on plants that were more prostrated (Fig. 1) and with a higher proportion of shoot mass below the cutting height, provoked the removal of young leaves (in upper layers of the canopy), while the remnant leaf biomass was mainly composed of older leaves with less photosynthetic potential for regrowth (see Boller and Nösberger 1985; Lemaire et al. 2009). Therefore, plant biomass accumulation was slightly decreased at a high defoliation frequency, even under non-flooding conditions (Fig. 2). In all cases, it should be noticed that once defoliated and twice defoliated plants did not fully recover their starch level either (i.e. rest periods of 40 days and 20 days, respectively). These results are in line with the known characteristics of *Lotus*, which maintains relatively low levels of carbohydrate reserves when subjected to grazing during the growing season until the fall storage takes place (Li et al. 1996; Smith 1962).

The response of flooded plants to repeated leaf removal (i.e. high defoliation frequency) through cycles of submergence/de-submergence involved intense stress. In this case, the gain of plant biomass was minimal (cf. day 0 vs. day 40 in Fig. 2), and upward shoot elongation seemed mainly aimed at survival. Our results suggest that the energy used by plants to sustain respiration and shoot elongation under this treatment was drawn from WSC consumption and starch breakdown in the crowns (Fig. 3), as also reported for tap roots in submerged (non-defoliated) plants of the weed *Rumex crispus* (Laan and Blom 1990) and culms of submerged (non-defoliated) plants of the crop *Oryza sativa*

(Ram et al. 2002). These responses were not previously reported for a forage legume able to tolerate long-term flooding like *L. tenuis*. Results also showed that extreme carbon starvation attributable to repeated defoliation under flooding led plants to use more reserves than expected due to the addition of each of the factors (Fig. 3; Table 1). Interestingly, it should be pointed out that flooding per se did not compel plants to use reserves, indicating that this factor did not create major stress for this flood-tolerant species as corroborated by the plant biomass and the shoot number responses (Fig. 2 and Table 2; see also Striker et al. 2005, 2008). Therefore, repeated defoliation appears to provoke carbon starvation by submerging the plants (Fig. 1) and compelling them to consume WSCs and starch from their crowns as revealed by the drop of these reserves after each defoliation event (Fig. 3). The capacity of *L. tenuis* to use stored reserves in extreme situations (when carbon gain through photosynthesis is negligible) was recently reported by Manzur et al. (2009) for plants surviving 30 days of complete submergence. In addition to carbon reserves, it is known that nitrogen reserves also play an important role in facilitating regrowth in legumes (Avice et al. 1996; Meuriot et al. 2004). So, the dynamics of nitrogen reserves under the combination of defoliation and flooding is an interesting step for future research.

Implications for grazing management of *Lotus tenuis* in flood-prone areas

The results obtained in this experiment provide significant information in terms of the ecophysiology and management of this forage legume species. The optimal grazing strategies will depend on the water regime of the specific environment in which *L. tenuis* is introduced as part of the forage resources. In areas that rarely flood, this species seems to be able to tolerate frequent defoliation reasonably well by decreasing its stature and increasing its shoot number, two classical responses to grazing. In contrast, in lowland areas in which plants periodically deal with flooding conditions, our results suggest that defoliation should be limited during flooding events and a longer recovery time should be considered to allow plants to build back up their reserves. Otherwise, the biomass production and vigour of plants descend if

subject to repeated defoliation during flooding and/or immediately after the water subsides. Thus, grazing management should consider the reported results as the described short-term effects of high defoliation frequency under flooding conditions would also have long-term effects on both the forage yield and on the perenniality of *L. tenuis* in the grasslands.

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