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RESEARCH PAPER

Different strategies of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* to deal with complete submergence at seedling stage

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Keywords

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ABSTRACT

Two main strategies allow plants to deal with submergence: (i) escape from below water by means of shoot elongation, or (ii) remaining quiescent under the water until water subsides and then resume growth. We investigated these strategies in seedlings of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* subjected to control and submergence for 12 days, with a subsequent 30-day recovery period. All three species survived submergence but used different strategies. Submerged seedlings of *L. japonicus* exhibited an escape strategy (emerging from water) as a result of preferential carbon allocation towards shoot mass and lengthening, in detriment to root growth. In contrast, seedlings of *L. corniculatus* and *L. tenuis* became quiescent, with no biomass accumulation, no new unfolding of leaves and no shoot elongation. Upon de-submergence, seedlings of *L. japonicus* had the lowest recovery growth (a biomass and shoot height 58% and 40% less than controls, respectively), *L. corniculatus* was intermediate and *L. tenuis* showed the greatest recovery growth. Previously submerged seedlings of *L. tenuis* did not differ from their controls, either in final shoot biomass or shoot height. Thus, for the studied species, quiescence appears to be an adequate strategy for tolerance of short-term (*i.e.*, 12 days) complete submergence, being consistent with field observations of *L. tenuis* colonisation of flood-prone environments.

INTRODUCTION

Complete submergence is one of the most stressful scenarios that plants can confront in environments prone to soil flooding (Blom 1999; Mommer & Visser 2005; Colmer & Voesenek 2009). In addition to oxygen deprivation at root level, which occurs during water excess in soil, plants subjected to complete submergence conditions are restricted in obtaining enough oxygen for tissue aeration, even though some species can be partially supplied through underwater photosynthesis (Vashisht *et al.* 2011). Hence, aerobic metabolism for energy production shifts to the much less efficient anaerobic/fermentative pathways (Gibbs & Greenway 2003; Voesenek *et al.* 2006; Kulichikhin *et al.* 2009). Moreover, depending on turbidity of the water, light reduction can constrain carbon gain by photosynthesis (Sand-Jensen 1989; Colmer & Pedersen 2008). Therefore, complete submergence can cause a drastic energy and carbohydrate crisis that can threaten plant survival (Voesenek *et al.* 2006; Colmer & Voesenek 2009).

Some plants cope with complete submergence using one of two main strategies recognised in plant submergence responses (*sensu* Bailey-Serres & Voesenek 2008, 2010). The first is an escape strategy (LOES, low-oxygen escape syndrome) and the second is a 'sit-and-wait' strategy (LOQS, low-oxygen quiescence syndrome) (Bailey-Serres & Voesenek 2008, 2010; Hattori *et al.* 2010). The LOES involves shoot elongation in order to restore leaf contact with the atmosphere, while the LOQS is based on maintaining steady energy conservation without shoot elongation (Bailey-Serres & Voesenek 2008). It has been postulated that LOES offers plants a better chance to survive under shallow, long-term flooding (>1 week), where shoot de-submergence is generally plausible. In contrast, LOQS is more likely to be adopted by species coping with deep, short-term flooding (<1 week), where shoot emergence seems to represent a higher cost in energy and might compromise eventual recovery when the water recedes (Colmer & Voesenek 2009).

Both escape and quiescence strategies have been studied mostly in the crop *Oryza sativa* (Setter & Laureles 1996; Fukao *et al.* 2006; Hattori *et al.* 2009; Bailey-Serres & Voesenek 2010; and references therein) and some species of the genus *Rumex* (Voesenek *et al.* 1990; Chen *et al.* 2009, 2011). In rice, both strategies were associated with interspecific differences among cultivars. For the *Rumex* species, Pierik *et al.* (2009) showed that *R. palustris* displays submergence escape while *R. acetosa* invokes quiescence. Within the genus *Lotus*, which includes important forage species used in flood-prone soils (Dear *et al.* 2003; Striker *et al.* 2005, 2008), there is only a single study showing the ability of adult *L. tenuis* plants to shift between escaping from water or adopting a non-elongating quiescent strategy depending on the water depth. Manzur *et al.* (2009) demonstrated that *L. tenuis* has the flexibility to elongate its shoot more vigorously (LOES) when partially submerged or to 'sit-and-wait' (LOQS) when completely submerged, at the expense of its crown reserves. However, information on the use of these strategies at the seedling stage is

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scarce and mainly limited to rice cultivars (Setter & Laureles 1996). Seedlings possess fewer reserves in comparison to adult plants, but are more likely to be completely submerged during a flood due to their small size and height. The present study compared the responses of seedlings of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* to complete submergence, specifically with regard to escape or quiescence strategies. In addition, recovery of each species 1 month after the water subsided was also determined, as this attribute is related to seedling recruitment in natural environments (Lenssen *et al.* 2004). As far as we know, this is the first study to address tolerance responses to complete submergence in seedlings of legume species with potential forage use in prone-flood areas.

MATERIAL AND METHODS

Plant material and experimental details

Lotus japonicus (Regel) Larsen is a waterlogging-tolerant species (Rocha *et al.* 2010) that inhabits a broad range of environments, which include sporadic events of water excess (Pajuelo & Stougaard 2005). *Lotus corniculatus* L. and *L. tenuis* Waldst. & Kit. are also flooding-tolerant species but they inhabit lowland areas regularly subjected to floods of variable magnitudes (water depth and duration) (Dear *et al.* 2003; Striker *et al.* 2005).

Seeds of *L. japonicus* (cv. Miyakojima MG-20), *L. corniculatus* (cv. San Gabriel) and *L. tenuis* (cv. Chajá) 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 0.3-l plastic pots (two or three seedlings per pot) filled with sand and topsoil (1:1) from lowland grassland of the Flooding Pampa of Argentina (3.3% organic carbon) and transferred to a glasshouse at the Faculty of Agronomy at the University of Buenos Aires. In order to avoid nutrient limitation for seedling growth, pots were irrigated twice with 50 ml half-strength modified Hoagland solution (additionally enriched with 0.23 μM H_3BO_3, 0.71 μM $MnCl_2$, 5 μM $ZnSO_4$, 0.6 nM $(NH_4)_6Mo_7O_{24}$, 1.6 nM $CuSO_4$) before the treatments. Seedlings of each species with three pentafoliate leaves (approximately 14 days after germination) were subjected to control and submergence conditions for 12 days following a fully randomised design with ten replicates. The experimental units (true replicates) were pots with two or three seedlings each. Submergence was achieved by fully covering the seedlings (2.4 ± 0.1 cm height, see day 0 in Fig. 1) with clear water to a height of 4 cm above the soil. The photosynthetic photon flux density (PPFD) at the top of the submerged seedlings was 360 ± 29 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (LI-192 Underwater Quantum Sensor; Li-Cor Inc., Lincoln, NE, USA), a light environment that allows underwater photosynthesis of C3 species (Colmer & Pedersen 2008). The dissolved oxygen of submergence water ranged between 0.6 and 0.9 mg O_2 l^{-1} (equipment LT Lutron DO-5510, Taipei, Taiwan), indicating the hypoxic environment caused by this treatment. Control pots were watered daily to field capacity. After submergence, a growing period of 30 days under well-watered conditions was allowed to study the recovery capacity of seedlings of each species. During the experiment, the glasshouse temperature was controlled to remain between 18.5 and 25.3 °C.

The variables measured included shoot height (equivalent to seedling height at the same age because of the orthotropic growth of the seedlings), number of unfolded leaves and biomass of the shoot and root portions. For the shoot, cotyledons were separated from leaves and stems. In all cases, the material was weighed after oven drying for 72 h at 80 °C. Variables were determined at the beginning (day 0), at the

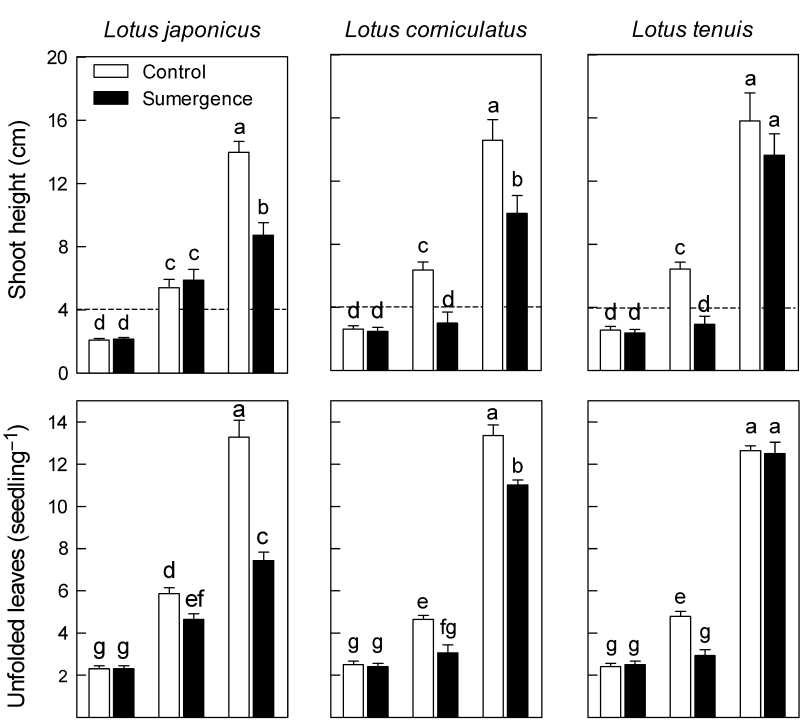

Fig. 1. Shoot height (upper panel) and number of unfolded leaves (lower panel) of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* subjected to control (C) and submergence (S) conditions. Submergence period lasted 12 days (D12). Recovery period lasted 30 days (D42). Dashed lines (upper panel) indicate the water level of the submergence treatment. Different letters indicate significant differences (P < 0.05) among bars. Values are means ± SE of 10 replicates.

end of submergence (day 12) and at the end of the recovery period (day 42).

Statistical analysis

Treatment effects on shoot height, number of unfolded leaves and biomass were evaluated using two-way ANOVAS, with 'submergence' and 'species' as the main factors. When significant interactions were detected, a subsequent Tukey test was done to determine the treatment effects. The analyses were carried out separately for the submergence and the recovery period. In addition, orthogonal contrasts were performed to compare data between day 0 and day 12 within species. The relationships between shoot and root biomass of seedlings of each species and treatment combination were studied through linear regression using Ln-transformed data (see Poorter & Lewis 1986; Megonigal & Day 1992). Additionally, slope tests were done in order to compare these relationships between treatments within species (Zar 1984). An increase in the slope of this relation between treatments allowed us to deduce a preferential active mass allocation toward shoots typical of the low-oxygen escape syndrome (LOES). In contrast, we concluded that a lack of variation in the slope of such a relationship together with passive reductions in seedling growth was indicative of the quiescence syndrome (LOQS). The normality and homoscedasticity of data were checked to ensure compliance with ANOVA assumptions. Statistical analyses were done using the STATISTICA package for Windows (StatSoft, Tulsa, OK, USA). All results are presented as means of ten replicates ± standard error (SE).

RESULTS AND DISCUSSION

Differences in strategies of seedlings of three *Lotus* species to deal with complete submergence

Our results showed that the *Lotus* species responded differently to complete submergence in terms of seedling morphology (Fig. 1) and biomass accumulation (see interaction submergence × species in Table 1, Fig. 2). In this respect, two opposing strategies were identified during complete submergence: LOES in *L. japonicus* and LOQS in *L. corniculatus* and *L. tenuis* (*sensu* Bailey-Serres & Voesenek 2008). Submerged seedlings of *L. japonicus* were able to maintain their shoot height as well as control plants and their leaves continued to unfold underwater (Fig. 1), as typical responses of the 'escape' syndrome. Shoot height maintenance during submergence appeared to be driven by preferential biomass allocation towards shoots without adventitious root formation (a common response to water excess; see Jackson & Armstrong 1999; Colmer & Voesenek 2009). Such a response leads to a higher slope of the shoot–root relationship of the submerged seedlings (Fig. 3; Table 2). As a result, young leaves of this species were able to emerge above the water to complete their escape strategy (Fig. 1). The energy used for seedling growth – before leaves surpassed the water level – does not appear to be related to cotyledon reserves, as cotyledon biomass was even higher during complete submergence than under control conditions (Fig. 2). Here, the underwater photosynthesis of submerged leaves is suggested to be the main source of substrate, allowing seedling growth in the first days of submergence (see Mommer *et al.* 2004; Mommer & Visser 2005). The relatively high amount of light intercepted by submerged seedlings supports this suggestion as the measured values (>350 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were much higher than the minimum required (180 $\mu mol \cdot m^{-2} \cdot s^{-1}$) to sustain considerable underwater photosynthesis in species possessing C3 photosynthetic carbon metabolism (for rice: Panda *et al.* 2006; Das *et al.* 2009; for non-crop wetland species: Colmer & Pedersen 2008). After leaves emerged above the water level, the seedlings would then have been able to conduct aerial photosynthesis as well as access atmospheric oxygen (Kozlowski & Pallardy 1984; Striker *et al.* 2005; Hattori *et al.* 2010); aerial photosynthesis being higher than rates underwater (*e.g.*, rice, Pedersen *et al.* 2009). The LOES strategy adopted by seedlings of *L. japonicus*, subjected to 12 days of submergence could be associated with the characteristics of the flooding regime of their natural habitats, in which sporadic events of water excess (Pajuelo & Stougaard 2005) would generate shallow but not deep submergence conditions.

In contrast to the response of *L. japonicus*, submerged seedlings of *L. corniculatus* and *L. tenuis* had the same number of unfolded leaves as on day 0 and these remained below water level during the entire 12 days of submergence (Fig. 1). These two species did not increase their shoot and root biomass when submerged ($P > 0.15$ in all cases; compare day 0 and day 12 in Fig. 2) and the relationships between shoot

Table 1. Results of two-way ANOVAS for the effects of 'submergence' and 'species' on biomass of *Lotus* seedlings.

	main effects		interaction	
biomass responses	submergence	species	sub × species	MS error
after submergence (day 12)				
total	322.68***	9.00**	18.38***	4.74
aerial	197.98***	0.97^{ns}	2.96^{ns}	2.62
cotyledons	14.04**	7.14*	23.78***	0.04
roots	228.90***	59.36***	29.83***	1.05
after recovery (day 42)				
total	178.00***	23.83***	10.59**	86.29
aerial	149.29***	11.68**	11.45**	46.87
roots	100.65***	28.45***	4.10*	20.15

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 (Submergence), 2 (Species), 2 (Submergence × Species) and 54 (Error). Significant differences: *P < 0.05; **P < 0.01; ***P < 0.001; ^{ns}P > 0.05.

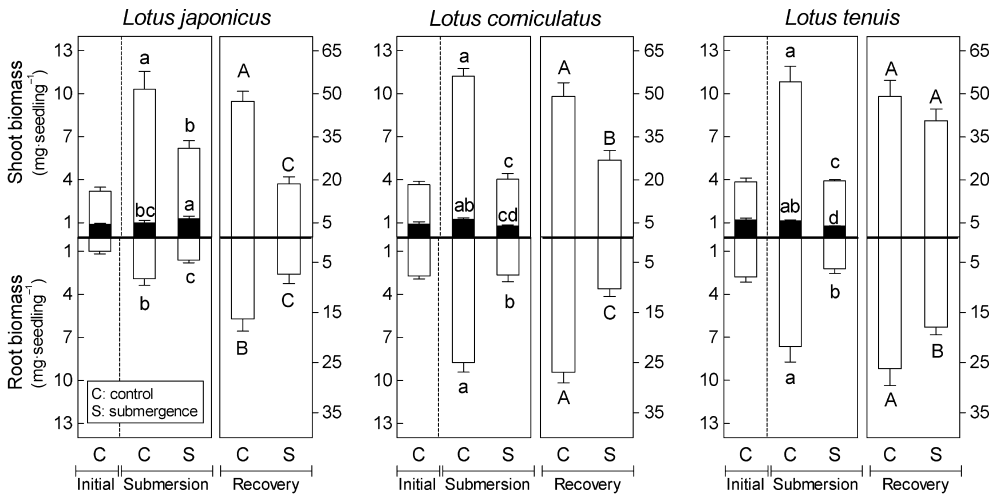

Fig. 2. Shoot and root dry mass of seedlings of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* subjected to control (C) and submergence (S) conditions. Black bars within shoot compartment indicate cotyledon biomass. Graphs of each species are split into two panels: left panels show biomass at day 0 (initial) and at day 12 (submergence) while right panels show biomass at day 42 (recovery). Note the different scales between left and right panels. Lowercase letters allow comparison of treatments and species within the submergence period, while uppercase letters allow comparison within the recovery period. Different letters indicate significant differences (P < 0.05) based on Tukey's test. Values are means ± SE of 10 replicates.

and root biomass did not change between treatments (Fig. 3; Table 2). Considering these responses, the proposal of quiescence (LOQS) for *L. corniculatus* and *L. tenuis* seedlings due to submergence becomes apparent. The LOQS developed by these species is in line with what would be expected when one considers that they usually inhabit lowland areas (Dear *et al.* 2003; Striker *et al.* 2005), in which deep submergence can be a regular situation. Also, this finding concurs with a quiescent behaviour already described by Manzur *et al.* (2009) for adult plants of *L. tenuis* surviving 30 days during complete submergence by utilising their crown reserves. As submerged seedlings, both *L. corniculatus* and *L. tenuis* showed reduc-

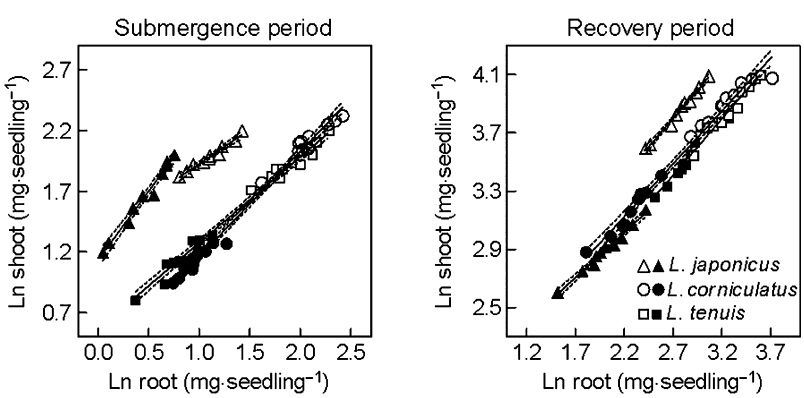

Fig. 3. Relationship between Ln of shoot *versus* Ln of root biomass of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* seedlings subjected to control and submergence conditions. Submergence period lasted 12 days. Recovery period lasted 30 days. Open symbols: control treatment, closed symbols: submergence treatment. Note that when slopes and intercepts between the fitted regressions did not differ, the data were pooled and a single regression line for species is presented for brevity and clarity.

Table 2. Linear regression analysis of the relationship between Ln of shoot mass and Ln of root mass for seedlings of the *Lotus* species evaluated.

species	treatment	slope	intercept	r^2
submergence period				
Lotus japonicus	control	0.68 (0.038)	0.94 (0.055)	0.93
Lotus japonicus	submergence	1.17 (0.062)	1.21 (0.081)	0.91
Lotus corniculatus	control and submergence	0.76 (0.049)	0.29 (0.047)	0.89
Lotus tenuis	control and submergence	0.72 (0.034)	0.43 (0.027)	0.92
recovery period				
Lotus japonicus	control	0.77 (0.057)	1.70 (0.055)	0.94
Lotus japonicus	submergence	0.67 (0.051)	1.31 (0.061)	0.93
Lotus corniculatus	control and submergence	0.72 (0.023)	1.48 (0.065)	0.98
Lotus tenuis	control and submergence	0.77 (0.033)	1.33 (0.104)	0.86

Equations with significant parameters (P < 0.05) are shown. When differences between slopes were not detected, data within species were pooled to obtain a single linear regression. Values in brackets are SE (n = 10).

tions in cotyledon biomass (Fig. 2; note the difference from *L. japonicus*), which suggests that reserves were consumed to maintain a basal metabolism typical of the quiescence response during submergence. In this respect, when cotyledons were removed from *L. tenuis* seedlings, growth reduction was substantial (Mujica & Rumi 1998), which further demonstrates the importance of cotyledon reserves in providing carbohydrates for seedling growth.

Recovery from submergence by seedlings of three *Lotus* species

During the recovery period, the three species responded in the opposite manner to that expected from their performance during submergence. This highlights the importance of considering a recovery period for evaluating plant responses after the application of stress conditions (Malik *et al.* 2002; Striker 2008). *Lotus japonicus*, the species that continued to grow during complete submergence, showed the lowest recovery capacity, as evaluated in terms of biomass accumulation and shoot height (Figs 1 and 2). By the end of the experiment, previously submerged seedlings of this species registered the lowest shoot biomass among the three *Lotus* species examined (Fig. 2; Table 1). The reduced accumulation of shoot biomass was in line with the low number of photosynthesising leaves present at the end of the experiment on de-submerged seedlings, as compared with controls (Fig. 1). Indeed, this species had the lowest final number of unfolded leaves among the three species analysed, as a consequence of the abscission of basal leaves after its de-submergence. In this respect, it is possible that the re-exposure to ambient O_2 levels after de-submergence provoked increased production of reactive oxygen species (ROS) in leaf tissues (Kawano *et al.* 2002; Blokhina *et al.* 2003), which in turn caused the observed leaf abscission (Sakamoto *et al.* 2008). On the other hand, root growth was relatively less affected during recovery, indicating that shoots and roots have different capacities for recovery after submergence (Fig. 2; cf. responses of wheat upon drainage following waterlogging, Malik *et al.* 2002). As a result, the relationship between the shoot and root biomass decreased to a level similar to that recorded for the previously submerged seedlings of *L. corniculatus* and *L. tenuis* (Fig. 3; Table 2). From a practical point of view, the poor recovery of *L. japonicus* could constrain its recruitment in field situations, as seedlings might be out-competed by flood-tolerant or facultative wetland macrophytes (*e.g.*, see Lenssen *et al.* 2004).

Seedlings of *L. corniculatus* presented an intermediate recovery capacity after submergence, as revealed by the higher shoot biomass accumulation and shoot height increase during the recovery period, in comparison with *L. japonicus* (Figs 1 and 2). These results shed light on the moderate tolerance to flooding (or reduced pO_2) of *L. corniculatus* at the seedling stage, a process that has only previously been examined in more mature and adult plants to date (James & Crawford 1998; Striker *et al.* 2005; Teakle *et al.* 2006). Seedlings of *L. corniculatus* seemed to be less tolerant than older plants (see Striker *et al.* 2005), suggesting that seedling performance during/after flooding could be a constraint for the recruitment of this species in periodically flooded habitats. On the other hand, *L. tenuis* had the highest recovery capacity among the three species tested. After the quiescence exhibited during submergence, seedlings of this species grew at a high rate following de-submergence, and attained a similar shoot height and biomass to the controls by the end of experiment (Figs 1 and 2; Table 1). This high recovery capacity of previously submerged seedlings of *L. tenuis* was probably related to their ability to maintain a number of leaves similar to controls, without noticeable leaf abscission (cf. *Lotus* species at day 42 in Fig. 1). This high capacity for recovery in *L. tenuis* is in line with that reported for adult plants (Vignolio *et al.* 1999; Striker *et al.* 2005, 2008; Manzur *et al.* 2009) and probably gives its seedlings a better chance for recruitment in periodically flooded environments. Moreover, the inclusion of a flooding-sensitive species of *Lotus* would be of interest in future experiments in order to broaden the spectrum of responses recorded.

CONCLUSIONS

The genus *Lotus* includes species that have different strategies to deal with submergence at the seedling stage. *Lotus japonicus* continues growing during submergence and invests resources in escaping above the water, whereas *L. corniculatus* and *L. tenuis* adopt a quiescent strategy without noticeable growth when under water. However, the apparently better performance of *L. japonicus* during submergence does not correspond with a high seedling growth rate during the recovery period. Instead, the two species that adopt a 'sit-and-wait strategy', *L. corniculatus* and *L. tenuis*, were able to grow more vigorously after the period of submergence. These findings demonstrate that performance during submergence alone is not conclusive for assessments of seedling submergence tolerance – post-submergence recovery also needs to be assessed. So, in terms of growth, the cost–benefit relation for promoting shoot elongation (LOES) or remaining quiescent (LOQS) needs to be carefully evaluated in experiments that do not include a recovery period. On the basis of our results, we can conclude that the quiescence strategy appears to be a better strategy than shoot elongation for sustaining high seedling recovery after a 12-day submergence, and *L. tenuis* is thus the most promising species for soils prone to flooding that lead to submergence for periods up to 12 days. Future experiments that evaluate the performance of seedlings under different submergence regimes (see also Chen *et al.* 2011) would contribute to a better understanding of the costs and benefits associated with the use of each of the two main strategies for submergence tolerance in plants.

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