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REVIEW PAPER

Flooding tolerance of forage legumes

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Abstract

We review waterlogging and submergence tolerances of forage (pasture) legumes. Growth reductions from waterlogging in perennial species ranged from >50% for *Medicago sativa* and *Trifolium pratense* to <25% for *Lotus corniculatus*, *L. tenuis*, and *T. fragiferum*. For annual species, waterlogging reduced *Medicago truncatula* by ~50%, whereas *Melilotus siculus* and *T. michelianum* were not reduced. Tolerant species have higher root porosity (gas-filled volume in tissues) owing to aerenchyma formation. Plant dry mass (waterlogged relative to control) had a positive (hyperbolic) relationship to root porosity across eight species. Metabolism in hypoxic roots was influenced by internal aeration. Sugars accumulate in *M. sativa* due to growth inhibition from limited respiration and low energy in roots of low porosity (i.e. 4.5%). In contrast, *L. corniculatus*, with higher root porosity (i.e. 17.2%) and O_2 supply allowing respiration, maintained growth better and sugars did not accumulate. Tolerant legumes form nodules, and internal O_2 diffusion along roots can sustain metabolism, including N_2 fixation, in submerged nodules. Shoot physiology depends on species tolerance. In *M. sativa*, photosynthesis soon declines and in the longer term (>10 d) leaves suffer chlorophyll degradation, damage, and N, P, and K deficiencies. In tolerant *L. corniculatus* and *L. tenuis*, photosynthesis is maintained longer, shoot N is less affected, and shoot P can even increase during waterlogging. Species also differ in tolerance of partial and complete shoot submergence. Gaps in knowledge include anoxia tolerance of roots, N_2 fixation during field waterlogging, and identification of traits conferring the ability to recover after water subsides.

Key words: Aerenchyma, N_2 fixation under hypoxia, nitrogen deficiency, photosynthesis and stress, plant submergence stress, root hypoxia, root porosity, waterlogging tolerance.

Introduction

Soil flooding affects >1700 Mha of land worldwide every year (Voesenek and Sasidharan, 2013), including grasslands and pastures where forage legumes are grown for livestock. The frequency and severity of floods are expected to increase in some regions during the next decades due to climate change (Arnell and Liu, 2001; Hirabayashi *et al.*, 2013). The global use of forage legumes is wide, and the diversity of species used as forage is important for different climates and farming systems (Phelam *et al.*, 2015). The animal feed information repository by FAO lists 153 species of legume used for forage (see the complete list at http://www.feedipedia.org/content/feeds?category=13594); however, a small number of these are recognized as being of wide commercial importance. In terms of land area or production, the use of forage legumes is poorly documented, but estimates are available for certain species in some regions (Phelam *et al.*, 2015). Graham and Vance (2003) reported that forage legumes in monocultures cover ~20 Mha of land, with annual production of 605×10^6

Mt, while more recently Bouton (2013) estimated a larger area of ~35 Mha worldwide just for *Medicago sativa*. Nevertheless, as many forage legumes (e.g. *Trifolium* spp. and *Lotus* spp.) are grown in mixtures with grasses, the use of forage legumes is far greater than that only in monocultures; grazing lands cover ~3300 Mha (Asner *et al.*, 2004). In Table 1 we summarize some key environmental conditions (i.e. soil conditions, precipitation range), waterlogging tolerances, sown areas, and biomass production for the main seven perennial and 10 annual forage legumes in world agriculture, plus *Melilotus siculus* which is a waterlogging- (and salinity) tolerant annual legume species that could be used for forage (Nichols *et al.*, 2008*a*; Rogers *et al.*, 2011; Teakle *et al.*, 2012; Striker *et al.*, 2015).

Soil flooding, also called waterlogging, results in severe hypoxia or even anoxia in roots, due to the rapid consumption of O_2 and the slow diffusion in water impeding entry of O_2 into waterlogged soils (Armstrong, 1979). The shift from aerobic respiration to the low ATP-yielding fermentation leads to an 'energy crisis' (Gibbs and Greenway, 2003), which reduces root growth and functioning for water and nutrient uptake (Jackson and Drew, 1984; Colmer and Greenway, 2011). As a result, the shoots of poorly adapted species with roots in excess water typically suffer severe growth reductions, or eventually die (Armstrong, 1979; Jackson and Drew, 1984; Colmer and Voesenek, 2009). When shoots become completely submerged during deeper floods, the additional adverse effects directly on shoots are reduced sunlight and decreased CO_2 entry to the leaves for photosynthesis (P_n) and night-time hypoxia (among other factors; Voesenek *et al.*, 2006). Here, we review the tolerance to waterlogging of the main forage legumes and examine physiological mechanisms contributing to tolerance (or explaining sensitivity) to root zone O_2 deficiency. Responses to partial and complete shoot submergence are also reviewed for the few forage legume species evaluated.

Main forage legumes used in world agriculture and diversity in waterlogging tolerance

Forage legumes contribute to the nitrogen (N) economy of grazing lands and cropping systems (e.g. cereal–pasture rotations) due to the symbiosis with rhizobia (N_2-fixing bacteria). Forage legumes increase herbage production and quality for grazing livestock, especially when grown mixed with grasses in areas with no or low N fertilizer inputs (Marten *et al.*, 1989; Frame *et al.*, 1998; Phelam *et al.*, 2015). The addition of legumes with higher leaf protein than grasses to an otherwise grass-dominated diet of ruminants increases the digestibility and metabolizable energy of the feed and better meets the protein requirements of the animals (Rochon *et al.*, 2004). Of the forage legumes listed by the FAO (see web-link above), we focused our analyses on the main perennial (seven species) and annual (10 species) forage legumes used, plus *M. siculus* as a very tolerant annual species (Table 1).

Medicago sativa is the most widely used forage legume, but has poor growth in soils with poor drainage and prone to waterlogging (Table 1). *Trifolium pratense* and *T. repens* can perform well in a variety of soils, and both are often sown with grasses (Kirwan *et al.*, 2007; Finn *et al.*, 2013). *Trifolium fragiferum* can also grow in a variety of soil textures and wide pH range (Table 1). *Lotus corniculatus* and *L. tenuis* are used much less than *M. sativa* and the above three *Trifolium* spp. (Phelam *et al.*, 2015; Table 1), but are important in South America and are (inter)sown in grasslands in less intensive systems often dealing with stressful soils (e.g. waterlogged, saline, or alkaline) (Table 1; Escaray *et al.*, 2012). *Lotus pedunculatus* is used less than the above two *Lotus* spp. and it can perform in loamy to clayey (not sandy) soils but needs >1000 mm of annual precipitation to behave as a perennial (Table 1).

Annual forage legumes commonly used in various parts of the world are *Melilotus albus*, *Medicago polymorpha*, *T. subterraneum*, and *T. michelianum*. The other annual species listed in Table 1 are utilized much less. Annual species are grown where perennial species cannot persist (e.g. summer drought) and/or where these suit the farming system. *Melilotus albus* and *T. subterraneum* can grow in a variety of soil textures, but with preferences, respectively, for neutral and acidic soils, and both are used across relatively wide amplitudes of annual rainfall (Table 1). *Medicago polymorpha* and *T. michelianum* grow well in alkaline soils, providing forage legume options for these soil types. The reported yields for annual forage species show high variability, largely reflecting the variability in rainfall across areas where these are grown (Table 1).

The waterlogging tolerance classifications for the species, provided in Table 1, are based on our analysis of the effects of waterlogging on whole-plant dry mass (percentage of controls) using data from the literature (Fig. 1; see Supplementary text at *JXB* online for details on data compilation and analyses). We classified the species as 'sensitive', 'intermediate', 'tolerant', or 'very tolerant' to waterlogging according to their positions in a ranking of tolerance for perennials and annuals, separately. Among perennials, the ranking from sensitive to tolerant was: *M. sativa*, *T. pratense, T. repens*, *L. pedunculatus*, *L. corniculatus*, *T. fragiferum*, and *L. tenuis*. Among annuals, the ranking from sensitive to very tolerant was: *M. truncatula*, *M. polymorpha*, *T. subterraneum*, *T. michelianum*, and *M. siculus*. The outcomes from our analysis (Fig. 1) of data across 49 studies (Supplementary Table S1) mostly agreed with the previous reputations for each species from individual studies. Data are from studies where 64% were performed in pots (n=120), 33% in nutrient solution (n=62), and 3% in field conditions (n=6). The perennial and annual species had the same median duration of treatment of 21 d (perennials: Q_1: 14 d and Q_3: 30 d; annuals: Q_1: 19 d and Q_3: 28 d), but the most tolerant perennial species, *Lotus* spp., were in several studies subjected to longer durations of waterlogging (Supplementary Fig. S1). Assessment of the waterlogging tolerance when comparing results from experiments which used pots containing soil, potting mix, or other substrates with experiments in nutrient solutions,

Table 1. *Main perennial and annual forage legumes used in world agriculture, and* Melilotus siculus *(a very waterlogging-tolerant species which could be used as a forage legume, see text)*

For each species, the centre of origin, soil texture, soil pH, waterlogging (WL) tolerance, range of precipitation, sown area, current areas of distribution/use, and dry mass yield are presented.

Species	Common name	Centre of origin	Soil texture, soil pH[a] and waterlogging tolerance[b]	Precipitation range (mm, annual)	Sown area (Mha)	Current areas of distribution/use	DM yield[c] (t/ha)	References
Perennial species								
Medicago sativa	Lucerne, alfalfa	Mediterranean basin and Southwest Asia	Sandy to clayey pH 6.5–7.5 WL sensitive	600–1200	35 Total (13 USA)	Europe, Australia, North and South America, and East Asia	Up to 20	1, 2, 3
Trifolium pratense	Red clover	Mediterranean basin	Loam, silt loams and clay pH 6–7 WL sensitive	310–1920	6 USA 9 NZ	New Zealand, Europe, North and South America, and Australia	4–18	3, 4
Trifolium repens	White clover	Eastern Mediterranean and Asia Minor	Loam, clay (rarely sandy) pH 5.5–7 Intermediate WL tolerance	>700	5 USA	New Zealand, North America, China, Australia, and South America	9–12	3, 5, 6
Trifolium fragiferum	Strawberry clover	Eastern Mediterranean and Southern Asia Minor	Sandy, peats and clayey pH 5.5–9 WL tolerant	750–1500	0.04 Australia	Western USA, New Zealand, and Southern Australia	3–6	7, 8
Lotus corniculatus	Birdsfoot trefoil	Mediterranean basin, parts of Asia, and Northern Africa	Sandy to clayey pH >6.2 WL tolerant	600– 1500	N/A[d]	Europe, New Zealand, North and South America, China, and India	6–14	9, 10, 11
Lotus tenuis	Narrow-leaf birdsfoot trefoil		Loam, clay (rarely sandy) pH 5.5–8 WL tolerant	440–1160	N/A	Argentina and Southern Australia	5–9	10, 12, 34
Lotus pedunculatus (syn. *L. uliginosus*)	Greater lotus, big trefoil		Loam, clay (not sandy) pH 5.5–8.2 WL tolerant	>1000 (to behave as perennial)		Western Europe, North Africa, New Zealand, South-East Australia, USA, and Uruguay	Up to 12	10, 12, 13
Annual species								
Melilotus albus[e]	White sweetclover, white melilot	Eastern Mediterranean and Northwest Asia	Sandy to clayey pH 6.5–7.5 WL sensitive[f]	90–1600	N/A	Central Europe, Eastern Mediterranean, Northwest Asia, USA, Australia, and Argentina	Up to 7–8	14, 15
Trifolium subterraneum (subspecies: *yanninicum, subterraneum, brachycalycinum*)	Subterranean clover	Southern Europe, North Africa, and Southern England	Sandy to clayey pH 4–6.5 Intermediate WL tolerance	350–1200	N/A	Southern Australia, New Zealand[g]	3–12	16, 17
Trifolium michelianum	Balansa clover	Mediterranean basin	Sandy to clayey pH 4.5–8.0 Very WL tolerant	350–750	N/A	Southern Europe from Spain to Turkey, Southern and Eastern Australia	5–8	18
Medicago polymorpha	Burr medic, bur clover	Western and Central Asia and Mediterranean basin	Loam, clay loam and clay pH 4.7–8 Intermediate WL tolerance	100–800	N/A	Mediterranean countries, Australia, Chile, South Africa, and USA	1.5–5	19, 20, 21, 37

Table 1. *Continued*

Species	Common name	Centre of origin	Soil texture, soil pH[a] and waterlogging tolerance[b]	Precipitation range (mm, annual)	Sown area (Mha)	Current areas of distribution/use	DM yield[c] (t/ha)	References
Melilotus siculus	Messina	Mediterranean basin and Western Asia	Sandy loam, loam, and clay pH 5.5–8.5 Very WL tolerant[h]	>400	N/A	New option for Southern Australia	1.3–8.7	22, 39,40, 41
Medicago truncatula	Barrel medic	Mediterranean basin	Sandy to clayey pH 6–8 WL sensitive	250–600	N/A	Mediterranean Basin, West Asia, and Southern Australia	Up to 5	23, 24, 25
Trifolium alexandrinum	Egyptian clover, berseem clover	Syria	Loamy to clay pH 6.5–8 WL sensitive[f]	550–750	N/A	Egypt, Northern India, USA and Southern Europe	Up to 8	26, 38
Trifolium tomentosum	Cottonball clover, woolly clover	Northern Africa, Southern Europe, and Western Asia	Sandy to loamy pH >6.5 WL tolerant[f]	>300	N/A	South-West Asia and Southern Australia	N/A	27, 28, 29
Trifolium resupinatum	Persian clover	Central and Southern Europe, Mediterranean basin, and Southwest Asia	Clay loams to heavy clay pH 5.5–8 WL tolerant[f]	>500	N/A	Iran, Afghanistan, and Northern Pakistan[i]	Up to 5	33
Ornithopus spp. (*O. compressus*, *O. pinnatus*, *O. sativus*)	Serradella	Mediterranean basin	Sandy to loam pH 3.5–6.5 WL sensitive[f]	>325	N/A	Mediterranean countries, Southern Europe, Morocco, and Algeria	3–5	30, 31, 39
Biserrula pelecinus	Biserrula	Mediterranean basin	Sandy (and duplex type[j]) pH 4.5–7.5 WL sensitive[f]	325–700	N/A	Mediterranean Europe, North Africa, West Asia, and Western Australia	2.5–10	32, 35, 36

[a] Soil pH range for adequate plant performance.
[b] Waterlogging tolerance based on our analysis presented in Fig. 1 and associated text.
[c] Dry mass yield when in monoculture.
[d] N/A = not available. For this case, however, data for *Lotus* genus but mostly for *L. corniculatus*: 1.85 Mha in South America, 1.39 Mha in USA, and 1.38 Mha in Europe.
[e] Annual but can behave as biennial.
[f] Waterlogging tolerance reputations are preliminary as only one or two studies are available.
[g] To a lesser extent South America, southern Europe, north-west and south-east USA, and South Africa.
[h] Studies conducted in nutrient solution and some unpublished field trials (see reference 41), so needs confirmation in controlled experiments with pots of waterlogged soil.
[i] Minor forage/fodder in southern Europe, southern Australia, and USA.
[j] Duplex soils have variable texture, with coarse sand to clay loam top-soils and light to heavy clay subsoils.

References: 1 Radovic *et al*. (2009); 2 Bouton (2013); 3 Marten *et al.* (1989); 4 Satell *et al.* (1998); 5 Jones and Kersten (1992); 6 http://www.fao.org/ag/agp/agpc/doc/gbase/data/Pf000350.htm; 7 Smetham (1973); 8 http://www.fao.org/ag/AGP/AGPC/doc/gbase/data/pf000500.htm; 9 Jones and Turkington (1986); 10 Díaz *et al*. (2005); 11 Bullard and Crawford (1995); 12 Dear *et al.* (2003); 13 http://www.fao.org/ag/AGP/AGPC/doc/Gbase/data/pf000345.htm; 14 http://www.fao.org/ag/AGP/AGPC/doc/Gbase/data/pf000488.htm; 15 Sparrow *et al.* (1993); 16 http://www.fao.org/ag/agp/AGPC/doc/Gbase/data/pf000351.htm; 17 Puckeridge and French (1983); 18 http://www.fao.org/ag/AGP/AGPC/doc/Gbase/data/pf000413.htm; 19 Hannachi *et al.* (1998); 20 Del Pozo *et al.* (2001); 21 http://www.cabi.org/isc/datasheet/33031; 22 Marañon *et al.* (1989); 23 http://ucanr.org/sites/asi/db/covercrops.cfm?crop_id=4; 24 http://www.fao.org/ag/agp/AGPC/doc/Gbase/data/meditrunc.htm; 25 Anonymous (1972); 26 http://www.fao.org/ag/agp/AGPC/doc/Gbase/data/pf000414.htm; 27 Zohary and Heller (1984); 28 Gibberd *et al.* (1999); 29 Gibberd and Cocks (1997); 30 https://www.daf.qld.gov.au/plants/field-crops-and-pastures/pastures/serradella; 31 Loi *et al.* (2000); 32 Bennet *et al.* (2001); 33 http://www.fao.org/ag/agp/AGPC/doc/Gbase/data/pf000415.htm; 34 http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=7416; 35 http://www.fao.org/ag/agp/agpc/doc/Gbase/data/pf000467.htm; 36 Loi *et al.* (1995); 37 Bullitta *et al.* (1994); 38 Hannaway and Larson (2004); 39 Nichols *et al.* (2007); 40 Nichols *et al.* (2008*a*; for low yield in harsh environment); 41 P. Nichols personal communication (high yield of selected accessions in recent field trials).

where data for both types of growth media (solid substrates and solutions) were available for six species, showed that only in the case of *L. tenuis* was the apparent waterlogging tolerance slightly higher in nutrient solution (see comments on this species below) while for the other five species (*L. pedunculatus*, *L. corniculatus*, *T. fragiferum*, *T. subterraneum*, and *T. michelianum*), tolerance did not differ between experiments in soil/substrates and in nutrient solutions (Supplementary Table S2).

Among the perennial forage legumes, *M. sativa* and *T. pratense* were in line with their reputations as waterlogging sensitive (e.g. for *M. sativa*, Barta, 1988*a*, *b*, *c*; Barta and Sulc, 2002; Smethurst *et al.*, 2005), as when waterlogged/O_2-deficient, *M. sativa* attained median whole-plant dry mass of only 40% of controls (Q_1: 30% and Q_3: 58%) and *T. pratense* was 39% (Q_1: 29% and Q_3: 45%); both these species had lower median values than the other five perennial species (Fig. 1; medians <50% of controls: $P<0.05$, Wilcoxon signed rank tests). *Trifolium repens* attained 65% of controls (Q_1: 50% and Q_3: 72%) and *L. pedunculatus* achieved 64% of controls. For *L. pedunculatus*, however, the data were of wide variability (Q_1: 54% and Q_3: 92%; the median did not differ from 75% but was <100% of controls; $P=0.023$); so balancing our analysis and based on its reputation which included side-by-side comparisons with other *Lotus* species (Justin and Armstrong, 1987; Shiferaw *et al.*, 1992; Real *et al.*, 2008), we consider *L. pedunculatus* as tolerant. However, the most waterlogging-tolerant perennial species are *L. corniculatus*, *T. fragiferum*, and *L. tenuis* with, respectively, median values of dry mass relative to controls of 77% (Q_1: 66% and Q_3: 88%), 79% (Q_1: 74% and Q_3: 93%), and 81% (Q_1: 52% and Q_3: 89%) when in waterlogged/O_2-deficient conditions (Fig. 1; medians did not differ from 85% of controls: $P>0.12$, but were <100%: $P<0.05$). High variability in growth responses was observed for *L. tenuis*, which presumably reflects genotypic differences (15 accessions/cultivars), and the ranges in waterlogging duration (14–120 d; median of 29 d), water depths from the soil surface to 6 cm above the surface, temperatures from 19 °C to 30 °C, and some studies using moderately saline soils (e.g. Vignolio *et al.*, 1999; Mendoza *et al.*, 2005; García and Mendoza, 2014; Supplementary Table S1).

Annual forage legumes also showed large variation in tolerance to waterlogging (Fig. 1). *Medicago truncatula* was most sensitive to waterlogging, with a median dry mass of 53% of the control (Q_1: 42% and Q_3: 56%; median <60%: $P<0.031$). *Medicago polymorpha* attained 72% (Q_1: 51% and Q_3: 89%) and *T. subterraneum* was 74% (Q_1: 59% and Q_3: 83%) of their controls (Fig. 1, medians did not differ from 75%: $P>0.38$, but were <85%: $P<0.5$). For *T. subterraneum*, three subspecies were analysed (*yanninicum, subterraneum* and *brachycalycinum*) and found to be similarly tolerant ($P=0.56$; (Supplementary Fig. S2), so these were pooled for this species in Fig. 1, although *yanninicum* is considered to be more tolerant than the other two subspecies (Francis and Devitt, 1969; Devitt and Francis, 1972). Importantly, *T. michaelianum* at 98% (Q_1: 91% and Q_3: 107%) and *M. siculus* at 101% (Q_1: 91% and Q_3: 104%) of their controls are both very tolerant to waterlogging (medians did not differ from controls; $P=0.93$ and $P=0.84$, respectively). Although the data on *M. siculus* are from nutrient solution experiments (Supplementary Table S1), its high waterlogging tolerance was evident in field trials (P. Nichols, pers. comm.). In the cases of *M. albus*, *Ornithopus sativus*, *T. alexandrinus*, *T. resupinatum* and *T. tomentosum*, the waterlogging tolerances specified in Table 1 are based on limited data (one or two studies), so these are preliminary conclusions and need confirmation.

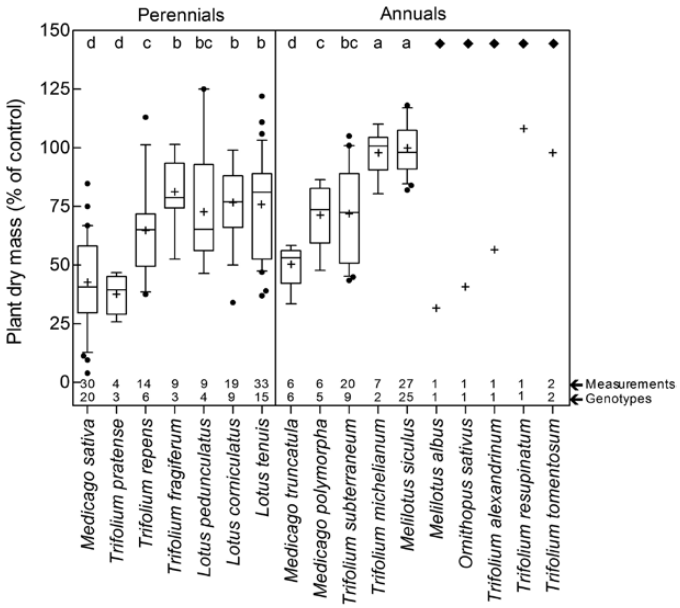

Fig. 1. The effect of waterlogging or O_2 deficiency in the root zone on whole-plant dry mass (% of drained or aerated controls) of the main perennial and annual forage legumes used in world agriculture (and *Melilotus siculus*). Boxes are 50% of the observations (numbers of measurements and genotypes per species are indicated along the *x*-axis) with the median shown as the horizontal line within each box, mean as '+' within each box, and bars extending from each box are 10 and 90 percentiles; outliers are shown as •. Data are from plants in pots ($n=122$), nutrient solution ($n=62$), and field conditions ($n=6$) reported in 49 peer-reviewed articles (data values, key experimental conditions, and references are given in Supplementary Table S1). Waterlogging or low O_2 treatment durations (days) for experiments with the various species were (ranges with median in parentheses): *Medicago sativa*, 5–42(18); *Trifolium* pratense, 14–21(17); *T. repens*, 6–30(17); *T. fragiferum*, 6–28(20); *Lotus pedunculatus*, 14–60(38.5); *L. corniculatus*, 14–60(30); *L. tenuis*, 14–120(29); *Medicago truncatula*, 16–60(16); *Medicago polymorpha*, 16–18(16); *T. subterraneum*, 10–35(21); *T. michaelianum*, 10–35(21); *Melilotus siculus*, 21–28(28); *M. albus*, 20; *T. resupinatum*, 10; *T. tomentosum*, 7–35(21); *Ornithopus sativus*, 21. Median duration of waterlogging or low O_2 treatment was 21 d for both perennials (Q_1: 14; Q_3: 30) and annuals (Q_1: 19; Q_3: 28). Waterlogging depths ranged from the soil/substrate surface to 7.5 cm above the surface (soil pots and field; nutrient solutions were roots and at most the shoot base only) and in all cases without complete shoot submergence. Letters denote dry mass of plants in treatments for different ranges as a percentage of controls based on Wilcoxon signed rank tests where a: median did not differ from 100%; b, 75%<median<99%; c, 60%<median<74%; and d, median<60%. For *T. subterraneum*, we pooled the three subspecies (*yanninicum*, *subterraneum*, and *brachycalycinum*) as these were not significantly different (Supplementary Fig. S2). Black diamonds indicate species excluded from analysis due to too few data (in those cases $n=1$ or 2 so only the mean is shown).

Some additional information comes from the separation of plant dry mass into shoot and root components, which was

possible only for some of the data (Supplementary Fig. S3). For the sensitive perennial species *M. sativa*, root dry mass was more adversely affected than shoot dry mass when subjected to soil waterlogging (n=29) or stagnant deoxygenated nutrient solution (n=1) (Mann–Whitney test P=0.0117), with medians relative to controls of 30% (Q_1: 22% and Q_3: 45%) and 45.5% (Q_1: 30% and Q_3: 68%), respectively. As a result, the root to shoot ratio dropped by 36% from 0.85 (controls) to 0.54 (waterlogged/O_2 deficient) (P<0.05; Supplementary Fig. S4). For *T. repens* and the tolerant perennials *L. pedunculatus*, *L. corniculatus*, and *L. tenuis*, no differential reductions of shoot and root dry mass were detected, whereas *T. fragiferum* (also considered tolerant) had a lower root (median 64%, Q_1: 63% and Q_3: 81%) than shoot (median 82%, Q_1: 75% and Q_3: 96%) dry mass (Mann–Whitney test, P=0.10) (Supplementary Fig. S3). For the annual species, data availability allowed us to analyse only root and shoot components for *T. subterraneum*, *T. michelianum*, and *M. siculus* (Supplementary Fig. S3). In *T. subterraneum*, waterlogging did not differentially affect root and shoot dry mass (P=0.91), while in *T. michelianum* and *M. siculus*, roots appeared less affected than shoots (Mann–Whitney test: P=0.017 and P=0.0043, respectively) (Supplementary Fig. S3), but these differences were slight and did not significantly affect the root to shoot ratio (Supplementary Fig. S4).

The responses of growth to waterlogging or O_2 deficiency presented in Fig. 1 are data at the end of waterlogging. Recovery after the water recedes is also of importance, but has often been overlooked in research on waterlogging tolerance (Striker, 2012). For forage legumes, 20% of the studies on waterlogging tolerance of perennial species have included a recovery period, whereas recovery following waterlogging has not been considered in any of the studies of annual species (Supplementary Table S1). Amongst the studies of perennial species, an impressive 37% of those on *M. sativa* included assessments of plant recovery after waterlogging. In the cases where recovery was considered, however, only final dry mass is typically reported, whereas physiological measurements after waterlogging are generally lacking. For perennial forage legumes, the recovery ability is important to consider as this would not only influence forage production but also plant persistence within the pasture. For annual species, the ability to recover from waterlogging would impact on feed supply and, importantly, also on seed production for self-regeneration of the pasture.

To illustrate the importance of recovery, in *M. sativa* it was shown that waterlogging duration impacts negatively on the subsequent recovery; for plants cut to 1 cm above the soil at the flowering stage and allowed to grow for 5 d, and then waterlogged for 3, 5, or 7 d, recoveries were 83, 66, and 38% in terms of whole-plant dry mass relative to the controls when assessed 19 d after the waterlogging ended (Barta, 1980). The growth stage when waterlogging stress occurs is also important; as an example for *M. sativa*, 14 d of waterlogging reduced shoot and root dry masses (relative to controls) to 43% and 22% for early seedling (1–2 trifoliate leaves), 60% and 27% for early vegetative (3–6 trifoliate leaves), and 81% and 36% for mid-to-late vegetative (5–11 branches on main stem) stages, when measured at 36 d after waterlogging (Teusch and Sulc, 1987). Finally, soil temperature during waterlogging proved to be important in determining tolerance and subsequent recovery of *M. sativa* (Thompson and Fick, 1981). Injury occurs sooner with increasing temperatures, evident as a lack of shoot growth (i.e. relative growth rate was zero) for 21 d upon drainage following 14 d of waterlogging at 16 ºC, 10 d at 21 ºC, 8 d at 27 ºC, and 6 d at 32 ºC (5-week-old plants; Thompson and Fick, 1981). These examples demonstrate that various factors, such as duration, growth stage, and environmental conditions during (and after) waterlogging, should be considered in future research examining the recovery ability after waterlogging of forage legumes.

Summary of diversity in waterlogging tolerance of forage legumes

Forage legume species differ markedly in waterlogging tolerance. Growth reductions are >50% for the sensitive perennial species *M. sativa* and *T. pratense*, whereas reductions are <25% (median) for the more tolerant *L. pedunculatus*, *L. corniculatus*, *T. fragiferum*, and *L. tenuis*. Annual forage legume species also show variable waterlogging tolerances, and *T. michelianum* and *M. siculus* are both very tolerant of root zone O_2 deficiency. Plant recovery after waterlogging has been rarely examined, with the exception of *M. sativa*. In the next sections, we consider the causes of damage from waterlogging to sensitive species and various physiological mechanisms in the tolerant species of forage legumes.

Physiological mechanisms in roots contributing to waterlogging tolerance

Aerenchyma formation and gas-filled porosity in roots

One of the major traits conferring tolerance to waterlogging is the formation of aerenchyma in roots (Armstrong, 1979), and plant species differ markedly in root aerenchyma (91 species; Justin and Armstrong, 1987). Aerenchyma formation increases tissue porosity (gas volume per unit tissue volume; Armstrong, 1979), and largely determines O_2 movement within roots (Armstrong, 1979; Colmer, 2003). Aerenchyma can also develop in the cortex of nodules, which connects to that in the roots (see the section 'Nodulation and N_2 fixation').

The constitutive porosity of roots (i.e. porosity in drained or aerated conditions) varies among forage legumes from 2.1% (*M. sativa*) to 14.5% (*L. tenuis*) and even 18% (*M. siculus*) (Fig. 2). Constitutive porosity of roots is determined by the pattern of cell packing in the cortex; a cubic arrangement has greater tissue porosity than a hexagonal arrangement of cells (Justin and Armstrong, 1987) in combination with any lacunae (gas-filled channels) formed in some roots even when in aerated conditions. In forage species with low constitutive porosity of roots (<3%), the arrangement of cells in the cortex is hexagonal, such as for roots of *M. sativa* (cross-sections in Zook *et al.*, 1986; Arrese-Igor *et al.*, 1993) and eight *Trifolium* species/accessions (not any of the major forage species) mostly sensitive to waterlogging (Table 2 in Gibberd *et*

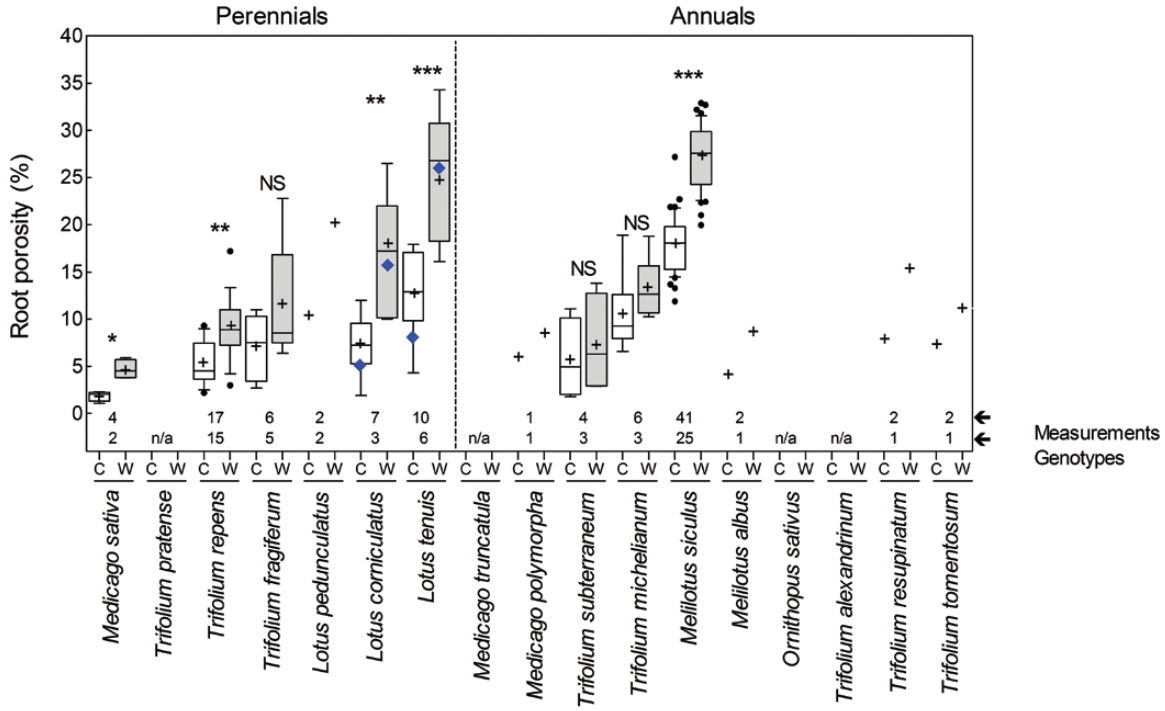

Fig. 2. Root porosity (%, v/v) of plants in aerated solution or drained soil (white boxes; C = control) and O_2-deficient solution or waterlogged soil (grey boxes; W = waterlogged) for the main perennial and annual forage legumes used in world agriculture (and *Melilotus siculus*). Boxes are 50% of the observations (numbers of measurements and genotypes per species are indicated above the *x*-axis; n/a, not available) with the median shown as a horizontal line within the box, mean as '+', and bars extending from each box are 10 and 90 percentiles; outliers are shown as •. Data are from plants in pots (n=40) and nutrient solution (n=63) reported in 22 articles (data values, key experimental conditions, root type, and references are given in Supplementary Table S3). Median duration of waterlogging or low O_2 treatment was 28 d for perennials (Q_1: 21; Q_3: 30) and 21 d for annuals (Q_1: 21; Q_3: 28). Asterisks indicate significant differences between treatments based on Mann–Whitney tests in species with at least three replicates to fulfil the requirement of the analysis (*P<0.05; **P<0.01; ***P<0.0001; NS: P>0.05). For *Trifolium subterraneum*, two values were for subspecies *brachycalycinum*, one for *yanninicum,* and one for *subterraneum* (Supplementary Table S3). Diamonds indicate root aerenchyma values in addition to the root porosity data for *Lotus corniculatus* and *L. tenuis* (only porosity values were used in the statistical analysis and not aerenchyma values). (This figure is available in colour at *JXB* online.)

al., 2001). In contrast, species with higher constitutive porosity (e.g. >5%) commonly have cubic (or mixed) cell packing in the root cortex, such as in *L. pedunculatus* (Justin and Armstrong, 1987; James and Crawford, 1998), *L. corniculatus*, *L. tenuis* (Justin and Armstrong, 1987; Striker *et al.*, 2005; Mendoza *et al.*, 2005; García *et al.*, 2008), *T. repens* (Rogers and West, 1983), *T. subterraneum* (Aschi-Smiti *et al.*, 2003), and *M. siculus* (Teakle *et al.*, 2011 for the primary cortex).

Mechanisms of aerenchyma formation upon hypoxia and associated signalling in roots have been reviewed (plants generally, Evans, 2004; Steffens and Sauter, 2014; grain crops, Yamauchi *et al.*, 2013), but studies of aerenchyma in forage legume species are relatively few. The cross-sections available for roots of some forage legume species (references in Supplementary Table S3) indicate that aerenchyma in the primary cortex is mainly of the lysigenous type (with some notable exceptions, e.g. *M. siculus* has schizogenous-type aerenchyma). Lysigenous aerenchyma results from programmed cell death (PCD) triggered by ethylene (Sasidharan and Voesenek, 2015; see Visser and Bögemann, 2006 for the exception of *Juncus effusus*). Recent work highlights the role of reactive oxygen species (ROS) as part of PCD in aerenchyma formation in wheat roots (Xu *et al.*, 2013; Yamauchi *et al.*, 2014). No studies have been conducted on ethylene as the trigger for aerenchyma formation in roots of forage legumes, but we do not expect the mechanism to differ substantially from that already described more generally for plants.

Among the perennial forage legume species, the waterlogging-sensitive *M. sativa* has only 2.1% constitutive porosity in the tap root, which during hypoxia increases only to 4.5% (Fig. 2); despite this doubling in root porosity, the final gas-filled volume is still too low to provide adequate longitudinal O_2 diffusion for tissue aeration (Justin and Armstrong, 1987; Fig. 2; Supplementary Table S3). Zook *et al.* (1986) documented for *M. sativa* roots with secondary growth also some gas-filled spaces within the stele and small lacunae in the outer cortex, whereas Teutsch and Sulc (1997) did not observe aerenchyma formation in tap roots (0.5 cm below the crown) of 33- to 36-day-old *M. sativa* (secondary growth already evident) after 14 d of waterlogging. In contrast, the more tolerant *L. corniculatus* and *L. tenuis* have, respectively, median constitutive root porosities of 7.3% and 12.8%, and these increased to 17.2% and 26.5% when in waterlogged soil (n=7) or stagnant nutrient solution (n=9) (Fig. 2); these levels of porosity typically result in substantially improved internal aeration of roots (Armstrong, 1979). For the other perennial forage species measured for this trait when in low O_2 conditions, *L. pedunculatus* root porosity was 20.3% (n=2), that of *T. repens* was 8.5%, and that of *T. fragiferum* was 8.9%. Data are not available for root porosity of the sensitive *T. pratense* (Fig. 2).

Annual species of forage legumes also show variability for constitutive root porosity and median porosity induced by root zone O_2 deficiency (Fig. 2). *Trifolium subterraneum* and *T. michelianum* have roots with constitutive porosities of 4.9% and 9.3%, respectively, with a trend for porosity to increase to 6.3% and 12.7% (both not statistically significant) under O_2-deficient conditions (Fig. 2). *Melilotus siculus*, a very waterlogging-tolerant species, has a median root porosity of 18% when in aerated conditions and 27.6% when in stagnant deoxygenated solution (Fig. 2). *Melilotus siculus* develops an aerenchymatous phellem (i.e. secondary aerenchyma, see next paragraph) along the upper portions of the tap root and the base of many lateral roots, in addition to aerenchyma in the root primary cortex (Teakle *et al.*, 2011); the above whole-root porosity values are the net result of both the primary and secondary aerenchyma and all root tissues. The porosity of the aerenchymatous phellem tissue alone is 45–55% (Verboven *et al.*, 2012). For the other annual species listed in Table 1, root porosity values have only been measured in one or two studies and this prevented a statistical analysis of this trait, but for the four species measured the values were 4.1–7.9% in aerated conditions and 8.5–15.3% in stagnant conditions (Fig. 2; Supplementary Table S3); the highest of these values was for *T. resupinatum* in O_2-deficient conditions.

Secondary aerenchyma can develop in some species in the hypocotyl, tap root, and lateral roots. This secondary growth differentiates centrifugally from a phellogen, and so the gas-filled channels in this secondary tissue are called secondary aerenchyma (Jackson and Armstrong, 1999; Yamauchi *et al.*, 2013). Most studies on secondary aerenchyma have been carried out on the grain legume *Glycine max* (e.g. Shimamura et al., 2002, 2003, 2010) and on the tropical semi-aquatic leguminous tree *Sesbania rostrata* (e.g. Saraswati *et al.*, 1992; Shiba and Daimon, 2003). In *S. rostrata*, phellem formation can be triggered within 36 h of waterlogging (15-day-old plants; Shiba and Daimon, 2003). The functional importance of phellem for root aeration was demonstrated by vaseline application to the hypocotyl of *G. max* to prevent O_2 entry which caused rapid inhibition of root growth in waterlogged conditions (Shimamura *et al.*, 2003). Amongst the forage legumes, *L. pedunculatus* (James and Sprent, 1999) and *M. siculus* (Teakle *et al.*, 2011, 2012, 2014; Verboven *et al.*, 2012; Striker *et al.*, 2015) have been reported to form this porous secondary tissue.

Secondary aerenchyma of *M. siculus* has been studied using non-nodulated plants in nutrient solutions, and the 'aerenchymatous phellem' is a white spongy tissue that forms externally from a phellogen (Teakle *et al.*, 2011). The aerenchymatous phellem develops in hypocotyl, tap root, and basal portions of lateral and adventitious roots, and enables hypocotyl-to-root O_2 diffusion as demonstrated experimentally by: (i) removing a 1 cm ring of phellem just below the hypocotyl–shoot junction (interrupting the 'phellem continuity' below the water level); and (ii) increasing the water depth above the phellem collar over the hypocotyl; which in both cases dramatically reduced the O_2 within the roots (Teakle *et al.*, 2011). The microstructure of the phellem tissue in roots was examined using micro-computed tridimensional tomography, and this revealed the extensive network of interconnected gas-filled spaces in this tissue of high porosity (45–55%), which allowed relatively high internal O_2 partial pressures in *M. siculus* roots of 18–20 kPa near the root–hypocotyl junction and up to 8–9 kPa at 9 cm below this junction (Verboven *et al.*, 2012). The porosity of the main root was high in all *M. siculus* accessions tested; constitutive porosity in aerated nutrient solution was 13.3–14.4% and increased to 19–24.5% for plants in stagnant deoxygenated nutrient solution (22 accessions, Rogers *et al.*, 2011; 15 accessions, Striker *et al.*, 2015). It remains to be examined whether some other forage legumes, in addition to *M. siculus* and *L. pedunculatus*, have an ability to form secondary aerenchyma.

Tolerance to waterlogging related to enhanced root aeration can benefit from a barrier to radial O_2 loss (ROL) in the outer tissues of roots, in addition to aerenchyma formation (Armstrong, 1979; Colmer, 2003). A barrier to ROL in basal root zones can enhance longitudinal O_2 diffusion in the aerenchyma towards the root apex, by reducing losses to the rhizosphere, and this feature could also impede entry of potential 'phytotoxins' which increase in anoxic and chemically reduced soils (Armstrong, 1979; Colmer, 2003). In forage legumes, the anatomical/histological features in roots that might form a barrier to ROL have not been examined, but patterns of ROL along roots have been measured for only a couple of species: *L. tenuis* and *L. corniculatus* (Teakle *et al.*, 2010). ROL patterns along roots of *L. tenuis* indicated a 'partial' barrier to ROL, as O_2 flux from lateral roots of stagnant-treated plants (4 weeks old) did not differ significantly for positions at 10–50 mm from the root tip, whereas in *L. corniculatus* ROL increased progressively from the root tip to the more basal positions, indicating that these roots lacked, or had only a weak barrier to ROL (Teakle *et al.*, 2010). The partial barrier to ROL in roots of *L. tenuis* is much weaker than the barrier found in many wetland plants, such as rice (Armstrong, 1979; Colmer, 2003).

ROL measurements were also conducted for roots of *T. tomentosum* (waterlogging tolerant) and *T. glomeratum* (waterlogging sensitive) (Gibberd *et al.*, 1999) and for *M. siculus* (Teakle *et al.*, 2011), to test the functionality of aerenchyma by measurements near the root tips, rather than for assessments of ROL patterns along roots. *Trifolium tomentosum* with root porosity of 11.2% after 21 d in O_2-deficient nutrient solution (0.03–0.06 mM O_2), and transferred into an O_2-free root medium with shoots in air, showed a 20-fold higher ROL from the lateral roots (10 mm behind tip) and a 5-fold higher ROL from the primary root (60 mm below the root–shoot junction) than *T. glomeratum* with root porosity of 6.1%, suggesting that greater porosity and enhanced internal O_2 supply in roots contributed to the higher waterlogging tolerance of *T. tomentosum* (Gibberd *et al.*, 1999). For lateral roots of *M. siculus* in an O_2-free medium with shoots initially in air, ROL near the tips was relatively high, and remained high as the water depth was progressively raised until water was above the hypocotyl phellem, at which time ROL decreased markedly; thus, air-exposed phellem is an entry point for atmospheric O_2 to reach the root tips (Teakle *et al.*, 2011).

The relationship between plant dry mass (as a percentage of the control) and root porosity in waterlogged conditions was explored across the various forage legume species (excluding those having <4 replicates, thereby not meeting the criteria when analysed separately for Fig. 1 and/or Fig. 2; however, see Supplementary Fig. S5 which also includes the data for the five additional species with low replicates). A hyperbola fitted best the positive relationship ($P<0.01$; $r^2=0.61$) between median root porosity (i.e. when waterlogged/O_2 deficient) and median plant dry mass, and highlighted that root porosity below 10–12% is associated with dramatic reductions in plant dry mass in waterlogged/O_2-deficient conditions (Fig. 3). This correlation across eight species supports the hypothesis that root porosity is a trait contributing to waterlogging tolerance in forage legumes, as seen more widely in other plant species (Justin and Armstrong, 1987).

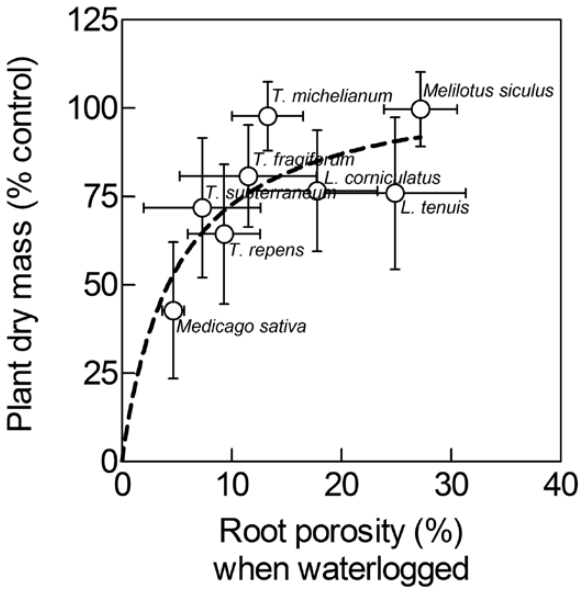

Fig. 3. Relationship between whole dry mass of plants in waterlogged soil or O_2-deficient root zone treatments as a percentage of drained or aerated controls, and root porosity under waterlogging or O_2-deficient conditions of eight species of forage legumes. Species were included only when data of at least four values for root porosity were available (Fig. 2; genotypes for each species are given in Supplementary Table S1 for whole-plant dry mass and in Supplementary Table S3 for root porosity). The fitted relationship is a saturation binding curve (hyperbola) of the type $y=[(B_{max}\times x)/(K_d+x)]$; where it is assumed that dry mass as a percentage of controls ('y') is zero (i.e. no plant survival, so zero dry mass) when root porosity is zero (note, zero porosity is for convenience only as root tissues always have some porosity, but this can be as low as 0.5–1%; Justin and Armstrong, 1987), and dry mass increases to a maximum plateau value B_{max}. Fitted values were B_{max}=108.5% and K_d=4.93% (R^2=0.61). Values shown for each species are means ±SD and were taken from Figs 1 and 2, and Supplementary Tables S1 and S2. Additional data points for five species with only one or two replicates are shown in Supplementary Fig. S5.

Root metabolic responses to hypoxia in a waterlogging-sensitive and a waterlogging-tolerant forage legume

Metabolism in hypoxic roots has been compared for waterlogging-sensitive *M. sativa* and waterlogging-tolerant *L. corniculatus* (Barta, 1984, 1986, 1988*a*, *b*, *c*). Crude extracts from root samples from field-waterlogged plants (1 cm water depth for 12 d) with shoots intact had increased alcohol dehydrogenase (ADH) activities of 4.8-fold in *M. sativa* and 6.8-fold in *L. corniculatus*, with respect to drained controls (Barta, 1984). Although the *in vitro* ADH activity was ~7.5-fold higher for *L. corniculatus* than for *M. sativa*, ethanol was not detected in roots of *L. corniculatus* but was present in roots of *M. sativa* (Barta, 1984); *in vitro* ADH activity and *in vivo* ethanol production can differ for several reasons (Gibbs and Greenway, 2003), and ethanol produced in 'anoxic cores' of tissue zones can also diffuse and be metabolized in aerobic tissues (Gibbs and Greenway, 2003). Tissue ethanol concentrations would only represent a small fraction of the ethanol produced, as ethanol would diffuse to the root medium and some can also enter the transpiration stream and move to the shoots (Barta, 1984). In a pot experiment with shoots enclosed in chambers, *M. sativa* in waterlogged vermiculite emitted as much as 10 µmol ethanol per day per plant, whereas no ethanol was detected from shoots of *L. corniculatus*, even up to 15 d of waterlogging (Barta, 1984). The capacity of roots of both species to produce ethanol was demonstrated for roots washed from vermiculite and incubated in sterile water with antibiotics (O_2 in medium not specified) (Barta, 1984). Although Barta (1984) speculated that ethanol might contribute to shoot damage in *M. sativa*, he also noted that the levels were relatively low and that an earlier 'ethanol toxicity hypothesis' had been refuted by others (e.g. as summarized in Chan and Burton, 1992); indeed glycolysis linked to ethanol production provides valuable ATP in anoxic cells (Greenway and Gibbs, 2003). We suggest that the greater ethanol production in roots of *M. sativa* than in *L. corniculatus* could relate to the low porosity and thus poor internal aeration of *M. sativa* (Fig. 2) and that the leaf yellowing in *M. sativa* could result from other causes such as nutrient deficiency (see the section 'Consequences for shoots of waterlogging and root O_2 deficiency') related to poor root functioning in waterlogged soils.

Effects of hypoxic (2–4% O_2 at 28 °C) nutrient solution on *M. sativa* and *L. corniculatus* root tissue ATP/ADP and injury (solute leakage) were assessed for 4- to 5-week-old plants (Barta, 1986). Unfortunately, anoxia was not included as a treatment and the critical oxygen pressure for respiration (COP_R) is not available for these species. The ATP/ADP ratio of hypoxic roots declined during 4 d from 1.78 to 0.95 in *M. sativa* and from 1.87 to 1.47 in *L. corniculatus*; maintenance of a higher ATP/ADP ratio in *L. corniculatus* could result from higher root porosity (Fig. 2) and thus internal O_2 supply for respiration in a greater proportion of the root system than in *M. sativa*. The solute leakage assay involved transferring plants into deionized water, but nevertheless showed that *M. sativa* roots from the hypoxic treatment lost more solutes (58% of K^+, 90% of sugars, 78% of amino-N) than did those of *L. corniculatus*, indicating greater membrane damage (and given these massive losses, probably also death of some or many cells) in roots of *M. sativa*.

An adequate supply of carbohydrates is required for respiration in the tissues that receive O_2 and for fermentation in anoxic tissues, in both cases to produce ATP to maintain

cell viability and functions (Saglio and Pradet, 1980; Webb and Armstrong, 1983; Bailey-Serres and Voesenek, 2008). Hypoxia resulted for *M. sativa* in increased sucrose (from 124 μmol g^{-1} DW to 403 μmol g^{-1} DW) in roots, whereas in *L. corniculatus* sucrose was similar in control (55.5 μmol g^{-1} DW) and hypoxic (62.4 μmol g^{-1} DW) roots (Barta, 1988*a*). Similarly, and with a different cultivar of *M. sativa*, root sucrose concentration was up to 7.5-fold higher at 7 d of waterlogging than in controls (Castonguay *et al.*, 1993). The increased sugars in roots of *M. sativa* could reflect a lower consumption due to inhibition of growth by waterlogging (Barta, 1986, 1988*a*; Barta and Sulc, 2002; Smethurst *et al.*, 2005, among others; Supplementary Fig. S2) and limited respiration due to poor internal aeration in the roots of low porosity, in comparison with *L. corniculatus* with roots of higher porosity (Fig. 2). Indeed, sucrose in roots of two cultivars of *M. sativa* waterlogged for 14 d in a field increased from 196 μmol g^{-1} DW to 388 μmol g^{-1} DW (Barta, 1988*b*) and glucose-6-phosphate increased from 493 nmol g^{-1} DW to 1510 nmol g^{-1} DW, but the ATP/ADP ratio decreased from 3.5 to 0.88 and ATP concentration declined from 171 nmol ATP g^{-1} DW to 40 nmol ATP g^{-1} DW (Barta, 1988*c*); supporting that ATP production was inhibited despite the high substrate availability in this waterlogging-sensitive species with little capacity to form aerenchyma (Arrese-Igor *et al.*, 1993; Fig. 2).

Ethanol production, ADH activity, and waterlogging tolerance were explored in *T. repens* for three populations collected from rarely or frequently flooded sites; 6-month-old plants were waterlogged to 1 cm above the substrate for 5 d (Chan and Burton, 1992). The main findings were that *in vitro* ADH activity in extracts from 2–3 cm root tips was positively correlated with ethanol production (r^2=0.29; excised roots, 24 h in anoxia with 0.25 mM glucose) and with waterlogging tolerance (r^2=0.55; waterlogged/control relative growth rate during 5 d treatment) (Chan and Burton, 1992). *In vitro* ADH activity of root extracts was 25% higher and *in vivo* ethanol production by excised roots in anoxia was 45% higher for plants of the frequently flooded compared with rarely flooded populations (Chan and Burton, 1992). Pyruvate decarboxylase (PDC) activity, which can limit the rate of ethanol formation (Gibbs and Greenway, 2003), was not measured. Other characteristics, in addition to ethanol production rates, should also be evaluated to gain a full picture of traits contributing to waterlogging tolerance of these populations.

Metabolic acclimation to hypoxia, and tolerance of anoxia, has been studied for roots of *T. subterraneum* (cv. Park). Roots of 30-day-old plants exposed to 15 d of hypoxia (1.5% O_2 in nutrient solution) showed increased *in vitro* activities of sucrose synthase (SuSy) and of ADH [as well as lactate dehydrogenase (LDH)], and ethanol formation by excised root segments incubated in anoxia (with 100 mM sucrose) (Aschi-Smiti *et al.*, 2003). Moreover, the adenylate energy charge (AEC) in anoxia was 0.6 for segments from hypoxia-pre-treated roots but 0.4 for anoxia-shocked (i.e. aerated-to-anoxia) root segments (Aschi-Smiti *et al.*, 2003). Increased ethanol formation typically also involves, in addition to increased ADH activity, increased PDC activity, and these inductions can occur at various levels from transcription to post-translational modifications (Gibbs and Greenway, 2003). Indeed the abundances of PDC and ADH transcripts both increased markedly in hypoxic roots of *L. japonicus* and declined again upon reaeration (Rocha *et al.*, 2010). Unfortunately, information on anoxia tolerance appears to be lacking for *M. sativa* and the tolerant *Lotus* spp.; future research should assess anoxia tolerance in the various commercially relevant forage legume species.

The studies above, with the exception of the couple of experiments with excised tissues of *T. subterraneum* (Aschi-Smiti *et al.*, 2003) and *T. repens* (Chan and Burton, 1992) in anoxia, were all for hypoxic roots. ATP production in anoxia (via glycolysis linked to fermentation) can be substantially lower than in hypoxia (with some oxidative phosphorylation), of course depending on the O_2 concentration in the various cells. Anoxia tolerance is presumably determined both by an ability to produce some ATP without O_2, and by how that limited amount of ATP is used or directed to processes essential for survival (Gibbs and Greenway, 2003; Atwell *et al.*, 2015). Energy is required for cellular maintenance, but, during the 'energy crisis' in anoxia, membrane integrity can deteriorate so that cellular compartmentation is compromised (Gibbs and Greenway, 2003). Root tips can only survive anoxia for a relatively short duration; as examples, 15 h for root tips of 5-day-old seedlings of *T. subterraneum* (Aschi-Smiti *et al.*, 2003) and 24 h for root tips of *Triticum aestivum* seedlings (15–30 h hypoxic pre-treatment, Waters *et al.*, 1991). The anoxia tolerances of roots of most forage legume species remain to be determined.

Summary of effects of O_2 deficiency on root porosity and metabolism

Root porosity is variable among forage legume species, and the more tolerant species generally have higher constitutive porosity and are able to increase porosity under O_2 deficiency by formation of aerenchyma in the root cortex. There is a positive (hyperbolic) relationship between plant dry mass (relative to controls) and root porosity in waterlogged or O_2-deficient conditions. Some forage legumes (*M. siculus* and *L. pedunculatus*) can form secondary aerenchyma from phellogen in the hypocotyl, tap root, and the basal parts of lateral roots. The aerenchymatous phellem connects to the aerenchyma in the primary cortex and so O_2 reaches the tips of lateral roots. Aerenchyma also forms in the cortex of nodules (see the section 'Nodulation and N_2 fixation'). The presence/induction of barriers to ROL in roots of forage legumes has scarcely been investigated. Root metabolism under hypoxia is differentially affected in sensitive and tolerant species. In the waterlogging-sensitive *M. sativa* with low root porosity and so little internal O_2 supply, sugars accumulated in tissues, probably due to growth inhibition resulting from limited respiration and low energy. In the more tolerant *L. corniculatus* with higher root porosity and thus O_2 supply which allows respiration for energy to better maintain growth, sugars did not accumulate. Eventually, and earlier in sensitive than in tolerant species, cell membranes can be damaged during hypoxia, as evidenced by leakage of solutes (e.g.

Table 2. *Nitrogen (N) concentration in shoots (S) or leaves (L) and in roots of plants in waterlogged or O_2-deficient root zone treatments (as a percentage of drained or aerated controls) for* Medicago sativa *(a waterlogging-sensitive species) and* Lotus tenuis*,* L. corniculatus*, and* L. pedunculatus *(three waterlogging-tolerant species)*

Shoot and root dry mass (DM) when waterlogged or O_2-deficient (as a percentage of controls) for these studies with shoot nutrient data are also presented. The treatment duration, culture system, and rhizobia inoculation for each study, and the reference, are given. A scatter plot of shoot growth (% of control) and shoot N (% of control) is shown in Supplementary Fig. S4.

Species and tissue	Shoot DM (% of controls)	N concentration (% of DM)[a] in shoot (S) or leaves (L)			Root DM (% of controls)	N concentration (% of DM)[a] in roots			Treatment duration and culture system	Inoculation with rhizobia and/or N supply	Reference
		C	WL	% of control		C	WL	% of control			
Waterlogging sensitive[b]											
Medicago sativa (S)	89%	2.4	1.6	67%	52%	1.25	1.26	101%	60 d (1 kPa O_2) in perlite with nutrient solution in pots	Inoculated (1 ml of suspension of *Rhizobium meliloti*) prior to WL. Plants watered with N-free solution	Arrese-Igor *et al.* (1993)
Medicago sativa (S)	81%	40[a]	28[a]	70%	74%	86[a]	82[a]	96%	5 d (2–4% O_2) in nutrient solution in pots	Inoculated with *Rhizobium meliloti*. 0.5 mM NH_4NO_3 in nutrient solution	Barta (1988*c*)
Medicago sativa (L)	78%	3.9	1.8	46%	34%	2.8	3.2	114%	14 d water at soil surface in pots with Turface as substrate	Not inoculated. Half-strength Hoagland solution (105 ppm N)	Castonguay *et al.* (1993)
Medicago sativa (L)	46%	5.5	2.3	42%	30%				20 d in sand:perlite mixture plus nutrient solution in pots	Not inoculated. Half-strength Hoagland solution (105 ppm N)	Smethurst *et al.* (2005)[c]
Medicago sativa (S)	40%	2.6	1.7	52%	n/a[e]				10 d water at soil surface (field experiment)	n/a (potentially in soil but nodulation not mentioned). N in soil is not provided	Rogers (1974)[d]
Medicago sativa (S)	37%	3	2.1	70%	n/a[e]				14 d water at 3 cm above soil level in pots	Inoculated. Infertile soil Haploxerult (yellow podzolic) as substrate with no N addition; initial N not provided	Shiferaw *et al.* (1992)
Medicago sativa (S)					n/a	35	33	95%	14 d water saturated soil (field experiment)	Inoculated with *Rhizobium meliloti*. Fertilizer applied at the rate of 11 kg N ha^{-1} at seeding	Barta (1988*b*)
Waterlogging tolerant[b]											
Lotus tenuis (S)	113%	1.2	0.9	77%	64%	1.6	1.4	83%	35 d water at 1 cm above soil level in pots	Inoculation not mentioned, but rhizobia in soil as nodules formed. Natraqualf soil as substrate with 0.11% of N.	Garcia *et al.* (2008)

Table 2. *Continued*

Species and tissue	Shoot DM (% of controls)	N concentration (% of DM)[a] in shoot (S) or leaves (L)			Root DM (% of controls)	N concentration (% of DM)[a] in roots			Treatment duration and culture system	Inoculation with rhizobia and/or N supply	Reference
		C	WL	% of control		C	WL	% of control			
Lotus tenuis (S)	71%	1.9	2.3	124%	56%	2.2	2.7	121%	40 d water at 1 cm above soil level in pots	Inoculation not mentioned, but rhizobia in soil as nodules formed. Natraqualf soil as substrate with 0.26% of N.	Mendoza *et al.* (2005)
Lotus corniculatus (S)	82%	50[a]	32.5[1]	65%	93%	118[a]	101[a]	86%	5 d (2–4% O_2) in nutrient solution in pots	Inoculated with *Rhizobium meliloti*. 0.5 mM NH_4NO_3 in nutrient solution	Barta (1988c)
Lotus corniculatus (S)	50%	2.6	1.9	73%	n/a[e]				14 d water at 3 cm above soil level in pots	Inoculated. Infertile soil Haploxerult (yellow podzolic) as substrate with no N addition; initial N not provided	Shiferaw *et al.* (1992)
Lotus pedunculatus (S)	67%	2.8	2.8	101%	n/a[e]					Inoculated. Infertile soil Haploxerult (yellow podzolic) as substrate with no N addition; initial N not provided	Shiferaw *et al.* (1992)
Lotus pedunculatus (S)	62%	2.1	2.2	101%	74%				20 d water at 1 cm above soil level in pots	Inoculated: 5 ml of suspension (10^7 ml of rhizobia). N-free solution (Crone nutrient solution + Hoagland A-Z micronutrients).	Allan *et al.* (2001)
Lotus corniculatus (S)	103%	2.7	2.9	110%	82%				60 d in pots with vermiculite and placed in tank with water flushed with N_2 (0.094 mol m^{-3} O_2)	Inoculated with *Rhizobium loti* strain DUS341at sowing. N_2 flushed in water (no addition of mineral N informed)	James and Crawford (1998)
Lotus pedunculatus (S)	140%	2.6	2.6	100%	111%						James and Crawford (1998)

[a] With the exception of Barta (1988*b*, *c*) where units are amino-N (μmol alanine equivalents g^{-1} DM).
[b] Tolerance to waterlogging for the different species as defined in Fig. 1 and listed in Table 1; these species were chosen as representatives of sensitive and tolerant species for which data on tissue N concentrations were available.
[c] Values are the average of four genotypes (Aurora, Hunter River, Sequel HR, and SARDI L153).
[d] Values are the average of five genotypes (Hunter River, Du Puits, Lahontan, Siro Peruvian, and P10).
[e] n/a = not available.

K^+, sugars, and amino-N). So far, knowledge on metabolic root responses of forage legumes to low O_2 is limited, and experiments have largely focused on hypoxia, so the anoxia tolerance of roots remains to be elucidated.

Nodulation and N_2 fixation

Waterlogging adversely affects root growth, alters root morphology, and impacts on root metabolism. The altered roots, and possible effects on rhizobia of anoxia and low soil redox, could disturb establishment and/or functioning of the plant–rhizobia symbiosis and thus impact on the N nutrition and growth of legumes in waterlogged soils. More generally for plants, O_2 deficiency inhibits nutrient uptake by roots and can result in deficiencies in the shoots, particularly for N in the case of non-legumes (e.g. *T. aestivum*; Herzog *et al.*, 2016) and as summarized for forage legumes in Table 2.

Establishment of the symbiosis involves signalling between the host root and rhizobia, with root 'infection' via root hairs (Jones *et al.*, 2007) and/or other sites (Sprent *et al.*, 2013). Nodules 'house' the rhizobia and provide the low O_2 microenvironment needed for the enzyme nitrogenase, to enable N_2 fixation with the host and also providing the substrate for respiration to produce the required energy. O_2 within the infected zone is regulated via a diffusion barrier in the nodule cortex and the O_2 which enters is bound by leghaemoglobin which then delivers this O_2 throughout the infected tissue for respiration (reviewed by Minchin *et al.*, 2008).

The nodulation process during flooding has been studied for the flood-tolerant tropical legume *Sesbania rostrata*. Root hair growth is reduced by waterlogging and then infection by rhizobia occurs at the branch point of lateral roots, with nodule formation at the base of lateral roots, and nodules also form on the stem at positions of adventitious root initials even above the water (Capoen *et al.*, 2010). In *Sesbania exasperata*, and several other wetland species, nodules form at the base of adventitious roots that emerge from the stem (James *et al.*, 2001) and at sites with root primordia along the stem (Boivin *et al.*, 1997).

Lotus pedunculatus formed nodules on the stem when the shoot was partially submerged for 30 d; these nodules were most abundant where adventitious roots had emerged (James and Sprent, 1999) and are rather different from the 'aerial' stem nodules on *S. rostrata* (which contain chloroplasts and are photosynthetically active; James *et al.*, 1998), and nodules also formed on these roots (James and Sprent, 1999). In this study of *L. pedunculatus*, seeds germinated under 1–2 mm of water (N-free nutrient solution) on the surface of vermiculite, and the water was raised to 1 cm above the hypocotyl as the seedlings grew, so that nodulation occurred under waterlogged conditions. Root hairs were present and nodules had formed via root hair infection on lateral roots as well as at the base of lateral roots of 3-week-old seedlings. After 60 d, growth, nodule fresh weight per plant, and nitrogenase activity (acetylene reduction by undetached nodules) did not differ in the two treatments. The development of lenticels and extensive aerenchyma for internal aeration of root tissues and nodules would have enabled gas exchange by nodules on the submerged roots of *L. pedunculatus* (James and Sprent, 1999).

Nodules formed and functioned when 1-week-old *T. repens* was inoculated with rhizobia and waterlogged for the next 9 weeks (Pugh *et al.*, 1995). Interestingly, however, when plants were raised for 9 weeks in drained soil and then waterlogged, the nitrogenase activity (acetylene reduction by undetached nodules) was inhibited for at least 7 d (experiment terminated at 7 d; Pugh *et al.*, 1995). Indeed, acclimation of root systems to soil waterlogging can take several days, as time is needed for aerenchyma to form in existing roots and nodules (and for the emergence of new, higher porosity, adventitious roots), before significant N_2 fixation can resume (e.g. *G. max*; Thomas *et al.*, 2005). By contrast, acclimation by nodules to changes in external O_2 by modulation of the gas diffusion barrier within nodules can occur within hours (Minchin *et al.*, 2008; Roberts *et al.*, 2010) and changes in radial permeability can sustain O_2 entry for nodule respiration in hypoxic conditions (e.g. 2.5–5% O_2 for nodulated root crown of *G. max*; Dakora and Atkins, 1990*a*). Below the external critical O_2 pressure (COP) for nodule (and root) respiration (dependent on tissue diffusion pathlength, porosity, and O_2 consumption rates), formation of aerenchyma and longitudinal O_2 diffusion from the shoot into and along the roots to the nodules becomes necessary for metabolism to be sustained, or for nodule functioning to recover from low O_2. Root aerenchyma which connects to the porous outer cortex of nodules, and reduced resistance across the nodule gas diffusion barrier, would enable O_2 supply to the infected zone where leghaemoglobin protects the O_2-sensitive nitrogenase but enables O_2 use for respiration (Minchin *et al.*, 2008).

N_2 fixation by waterlogging-sensitive forage legume species has been studied on relatively few occasions. Exposure of nodulated, 1-month-old *M. sativa* to 1 kPa O_2 in the root zone for 60 d resulted in increased gas-filled porosity in the outer cortex of nodules, and N_2 fixed per unit nodule dry mass was approximately equal to that of controls at 21 kPa O_2, even though bacteroid density had declined (Arrese-Igor *et al.*, 1993). This result showed that even nodules of this waterlogging-sensitive species can function with an O_2-deficient root zone, but since nodule mass was ~50% less than for the control plants by 60 d of treatment the shoot N concentration had declined (Arrese-Igor *et al.*, 1993). In contrast, when 70-day-old *L. corniculatus* was placed in an N_2-flushed nutrient solution (0.094 mM O_2), this resulted in apparent senescence of some nodules, although whole-plant N_2 fixation and growth were not affected during 60 d (James and Crawford, 1998). Functioning of roots and nodules in a hypoxic root environment, however, could be different from that in anoxic soil, and particularly for species with relatively low root porosity which would limit internal O_2 movement to nodules. Interestingly, field observations even of waterlogging-tolerant *L. pedunculatus* revealed that almost all of the nodule biomass was on adventitious roots on the soil surface or aerial roots in the surface litter and vegetation, so that these nodules were

not in direct contact with the underlying anoxic soil (Allan *et al.*, 2001).

Few studies have considered nodulation by forage legumes in waterlogged field situations. Nodulation ratings of 19 species of annual legumes after 7 weeks of soil flooding at a mildly saline site in southern Australia showed that most species had 'effective nodulation', including, for example, the waterlogging-sensitive *M. albus* (Nichols *et al.*, 2008*a*). Nodulation ratings were also conducted for 13 species of perennial legumes at the same site, but for some species on the very few surviving plants; nevertheless, nodules were present on the waterlogging-sensitive *M. sativa* (Nichols *et al.*, 2008*b*). *Medicago sativa* was also well nodulated after 11 weeks of waterlogging in a potting mix, being similar in nodulation score to the more tolerant *L. tenuis*, *L. corniculatus*, and *L. pedunculatus*, even though growth of *M. sativa* was severely inhibited (Real *et al.*, 2008). The field observations of nodulation of forage legumes are useful, but it is unknown whether the nodules formed prior to and/or during the waterlogging; clearly the nodules had persisted but the N_2 fixation capacities were not assessed. N_2 fixation estimates based on stable isotope measurements of foliage of 27 species at three field sites prone to transient waterlogging in southern Australia (waterlogging depth and duration not documented) indicated that many forage legumes had functional symbioses at these sites (Dear *et al.*, 2003). For example, *T. subterraneum* (subsp. *brachycalycinum* at 62% and *yanninicum* at 55%) contained proportions of fixed N similar to those found in other studies of these plants which had not targeted waterlogging-prone sites (Dear *et al.*, 2003, and references therein). Additional studies of priority annual forage legume species in regenerated stands, so that plant regeneration and rhizobia persistence and symbiosis formation are monitored across seasons, and with durations and depths of soil waterlogging recorded, are needed to determine whether N_2 fixation capacity does (or does not) become limiting for forage production in some waterlogged situations.

Little information is available for post-waterlogging recovery of nodules of forage legumes. Nodules of *T. repens* showed enhanced nitrogenase activity (acetylene reduction by undetached nodules) upon drainage after 9 weeks of soil waterlogging, relative to continuously drained controls (Pugh *et al.*, 1995). Nodule operation after return from low O_2 conditions to air, with the morphological changes (e.g. increased porosity of nodule outer cortex) to enhance O_2 entry, would rely on the internal diffusion barrier to regulate O_2 entry (e.g. *G. max*; Atkins *et al.*, 1993). These responses of *T. repens* and *G. max* contrast markedly with the loss of nitrogenase activity of nodules of *Vigna unguiculata* when moved from 1% or 2.5% O_2 back to air (Dakora and Atkins, 1990*b*). Inhibition of nitrogenase activity also occurred for the submerged root nodules of *Neptunia plena* (a semi-aquatic species) when transferred from non-aerated nutrient solution directly to humidified air, but, when O_2 was increased in 5% O_2 steps to 21% O_2 (over a 60 min period), nitrogenase activity was then maintained as nodular diffusion resistance could then adjust and prevent excessive O_2 entry into the infected zone (James *et al.*, 1992). Additional assessments are needed for forage legume species in conditions of fluctuating waterlogging, as species can differ in nodulation both during waterlogging and following soil drainage; for example, some species of *Lotus* showed increased nodulation during a recovery phase, but others had a low recovery of nodulation post-waterlogging (Real *et al.*, 2008).

In summary, waterlogging-tolerant forage legumes appear to maintain N_2 fixation as nodule formation proceeds, and internal O_2 diffusion along roots and into nodules can sustain metabolism in submerged nodules. Increased porosity of the outer cortex of nodules which connects to the root aerenchyma, together with reduction of the gas diffusion barrier within nodules, enables O_2 supply to the infected zone where leghaemoglobin protects the O_2-sensitive nitrogenase and enables O_2 use for respiration. Nodules can also form on adventitious roots, surface and aerial roots, and the stem, at least of some species. Data are lacking for N_2 fixation in flooded situations for some key species of interest, such as *M. siculus*. Few data are available on N_2 fixation by forage legumes in waterlogged field situations.

Consequences for shoots of waterlogging and root O_2 deficiency

The above-mentioned effects of waterlogging on roots have consequences for the shoots, the forage production of the pasture (Fig. 1). In this section we consider the effects of waterlogging on net photosynthesis (P_n), tissue sugars, and shoot nutrient status.

Net photosynthesis and tissue sugar concentrations

Waterlogging can adversely impact on P_n of forage legumes, with quicker and greater declines for a waterlogging-sensitive as compared with a waterlogging-tolerant species (Castonguay *et al.*, 1993; Smethurst and Shabala, 2003; Irving *et al.*, 2007 for *M. sativa*; Striker *et al.*, 2005, 2007 for *L. tenuis*). Short- and longer term effects on P_n of root zone O_2 deficiency are discussed in the following paragraphs.

In the sensitive *M. sativa*, P_n showed progressive declines, commencing at 2 d of waterlogging, and reduced to only 16–18% of controls at 14 d of waterlogging (cv. Apica; Castonguay *et al.*, 1993). In a second study, initial declines in P_n occurred in parallel with reductions in stomatal conductance [2–4 d; the intercellular CO_2 concentration (C_i) was not presented] with additional reductions in P_n to 40% of controls in the longer term (11–16 d) associated with damage to the photosynthetic apparatus (cvs L153 and Sequel HR; Smethurst and Shabala, 2003). Reductions in stomatal conductance within the first days of waterlogging might be the result of a reduced root hydraulic conductivity (Bramley and Tyerman, 2010), which can result in shoot wilting (*M. sativa* wilted in Smethurst and Shabala, 2003). Leaf water potential was on average 44% more negative for *M. sativa* at 2–6 d of waterlogging (at 20 °C; Castonguay *et al.*, 1993). Declines in stomatal conductance of *M. sativa* were correlated with increased leaf abscisic acid (ABA) concentration,

but without increases in the roots (Castonguay *et al.*, 1993), which supported the notion that increased ABA synthesis in, or reduced export from, leaves of plants with hypoxic roots (cf. Jackson and Hall, 1987) is responsible for the increased leaf ABA, rather than root-derived ABA as classically for drought (Zhang and Davies, 1989; Goicoechea *et al.*, 1997 for *M. sativa*). However, at 3–5 d of waterlogging, *M. sativa* shoots had increased soluble sugars (41% in Barta, 1987; 18.5% in Barta, 1988*c*) and starch (126% in Barta, 1988*c*; 3- to 4-fold in Catonguay *et al.*, 1993); so, negative feedback from carbohydrate accumulation could reduce P_n (as in wheat during waterlogging; Herzog *et al.*, 2016). C_i has rarely been reported together with stomatal conductance and P_n (the one exception only gave C_i at 15 d of waterlogging—it had decreased; Smethurst *et al.*, 2005). So, although stomatal limitation of C_i could reduce P_n by *M. sativa* during waterlogging, since sugars and starch increased, the hypothesis of negative feedback also seems likely. Measurements of the effect of waterlogging on P_n should report C_i values in order to aid interpretations.

Reduced P_n during longer term waterlogging might be associated with reductions in chlorophyll and damage to the photosynthetic apparatus (Smethurst and Shabala, 2003; Smethurst *et al.*, 2005; Irving *et al.*, 2007). The concentration of total chlorophyll decreased by 18–22%, with Chl *a* being more affected than Chl *b*, in two *M. sativa* cultivars after 9–11 d of waterlogging, and total chlorophyll continued to decrease to ~60% of controls at 16 d (Smethurst and Shabala, 2003). Damage to the photosynthetic apparatus in leaves of *M. sativa* (cvs Aurora, Hunter River, L153, and Sequel HR) after 15 d of waterlogging was evident as the maximal quantum efficiency of PSII (F_v/F_m) declined to 0.75, ETR (electron transport rate) had decreased by 25%, and NPQ (non-photochemical quenching) increased by 42% (Smethurst and Shabala, 2003; Smethurst *et al.*, 2005). So, during longer term waterlogging, decreases in chlorophyll (e.g. possibly as a result of N deficiency; see subsection 'Mineral nutrition') or damage to components of the photosynthetic apparatus possibly from ROS when excess energy is not dissipated (i.e. energy not used in photochemistry; Maxwell and Johnson, 2000; Shabala, 2011) could reduce P_n in *M. sativa*.

In contrast to *M. sativa* for which P_n declines within 2–4 d upon waterlogging, in *L. corniculatus* and *L. tenuis* P_n was maintained for 18 d and 25 d, respectively, when waterlogged [water 6 cm above soil, 21 ºC, and average vapour pressure deficit in air (VPD_{air}) 1.82 kPa] (Striker *et al.*, 2005). In both of these *Lotus* species, following these initial periods without effects, stomatal conductance then declined by 45–55% and C_i was reduced by 26–28%, which coincided with decreases in P_n of 30–40% at 40 d of waterlogging, but leaf water potential was not affected (Striker *et al.*, 2005). Plant water relations and P_n responses are also dependent on evaporative demand, as shown for *L. tenuis* which developed strong leaf wilting in shoots when waterlogged during summer (29 ºC and average VPD_{air}: 3.28 kPa); by 15 d almost complete stomatal closure had occurred along with a 70% reduction in leaf water potential, and, although survival was not compromised and P_n recovered within 1 week upon drainage, dry mass per plant was 54% of controls at 30 d of recovery (Striker *et al.*, 2007).

In *M. sativa*, sugars accumulate in leaves within 2–4 d of root hypoxia (2–4% O_2 in solution) as the root system stops growing and is no longer a strong sink for sugars (Barta, 1986); $^{14}CO_2$ labelling of photoassimilates showed that translocation to the roots was only 35–40% of the control values at 2, 4, or 6 d of root hypoxia (Barta, 1987). Nevertheless, even with this reduced translocation of sugars from the shoots to roots, root sugar concentrations were 2.8- to 3.2-fold higher than in the controls; as discussed in an earlier subsection of this review ('Root metabolic responses to hypoxia in a waterlogging-sensitive and a waterlogging-tolerant forage legume'), inadequate root aeration impeding respiration and growth would have contributed to the reduced sugar consumption in roots. Experiments with shade treatments [photosynthetic photon flux density (PPFD) 60–75 µmol m^{-2} s^{-1} versus >300 µmol m^{-2} s^{-1} in Barta, 1987; 150–200 µmol m^{-2} s^{-1} versus 950–1 000 µmol m^{-2} s^{-1} in Barta and Sulc, 2002] in combination with root hypoxia support the notion of greatly reduced demand for sugars in *M. sativa* when waterlogged; shading reduced shoot growth (44–52% of controls) and sugar concentrations in shoots (59% of controls) and aerobic roots (56–62% of controls), but in hypoxic roots sugars still increased even when the plants were in shade (115% of controls at 3 d waterlogging in Barta, 1987; 155% of controls at 6 d waterlogging in Barta and Sulc, 2002).

In waterlogging-tolerant *L. tenuis*, in contrast to *M. sativa*, sugar concentrations in the crown (i.e. tissues at the shoot base where reserves are stored) were reduced to 74% of controls when 6-month-old plants were waterlogged for 30 d (0.5 cm above soil; Manzur *et al.*, 2009). Waterlogging itself did not compel plants to use starch (Manzur *et al.*, 2009) but, when soil waterlogging (4 cm above soil) was combined with defoliation (e.g. 90% leaf removal to simulate grazing) at 20 d of waterlogging, the sugar concentration in the remaining tissues (shoot base and main root, G. Striker, pers. comm.) was reduced to 32% of that of controls and the starch concentration in the crown had declined to 30% of that of controls, which presumably facilitated shoot re-growth during the next 20 d with the waterlogging continued (Striker *et al.*, 2011). The use of carbohydrates under waterlogging/shallow flooding (not complete submergence) in *L. tenuis* can be associated with enhanced shoot elongation (Manzur *et al.*, 2009) which results in a higher proportion of leaves above water (Striker *et al.*, 2005) to maximize shoot atmospheric contact and P_n in air. The role of starch reserves in waterlogging tolerance could be further investigated using *L. japonicus* as a model species. *Lotus japonicus* has some waterlogging tolerance (dry mass was 68% of control after 21 d of waterlogging), but with interesting variability to be explored (Rocha *et al.*, 2010; Striker *et al.*, 2014), and genetic resources of wild accessions, recombinant inbred line (RIL) populations, and mutants (see genetic resources available for *L. japonicus* at https://www.legumebase.brc.miyazaki-u.ac.jp/lotus/top/top.jsp;jsessionid=6480FEE5DFB43B42FC6F75C3CD5C30D4). For example, mutants for starch synthesis and degradation (Vriet *et al.*, 2010, 2014) should be useful to investigate the role of starch

reserves in tolerance to waterlogging and submergence, and plant recovery after water recedes.

Mineral nutrition

Data are available for tissue concentrations of N, P, and K as affected by low O_2 in the root zone (soil waterlogging or hypoxia in nutrient solution) for the waterlogging-sensitive *M. sativa* and waterlogging-tolerant *L. corniculatus*, *L. pedunculatus*, and *L. tenuis* (Arrese-Igor *et al.*, 1993; Castonguay *et al.*, 1993; James and Crawford, 1998; Smethurst *et al.*, 2005; Mendoza *et al.*, 2005; García *et al.*, 2008), and we have summarized the findings for N in roots and shoots in Table 2 and Supplementary Fig. S6. The reductions in shoot N, P, and K concentrations in *M. sativa*, but not in the three *Lotus* species, presumably reflect the impaired root functionality in nutrient uptake and/or transport to the shoots as a result of inadequate internal aeration of the low porosity roots of *M. sativa*, compared with the *Lotus* species with higher root porosity (see the section 'Physiological mechanisms in roots contributing to waterlogging tolerance').

In *M. sativa*, the median shoot N concentration was reduced by waterlogging or O_2 deficiency to 59% of controls (Q_1: 45%; Q_3: 69%; n=6; Wilcoxon test P<0.05), similar to the effect on shoot dry mass which was 62% of that of controls (Q_1: 39%; Q_3: 82%; n=6). Despite waterlogging impacting negatively on shoot N concentration in *M. sativa* which potentially might contribute to reductions in P_n as a longer term effect (subsection 'Net photosynthesis and tissue sugar concentrations'), there was no correlation between shoot N and dry mass (Supplementary Fig. S6). In contrast to the reductions in shoot N, the N concentrations in roots were 98% of those of controls (Q_1: 94%; Q_3: 110%; n=4; Wilcoxon test P=0.9) while root dry mass was only 43% of that of controls (Q_1: 31%; Q_3: 68%; n=4) (Table 2). For the three *Lotus* spp., we pooled the values, and the median shoot N concentration of plants in waterlogging/hypoxic treatments was 100% of that of controls (Q_1: 74%; Q_3: 107%; n=8), while shoot dry mass was 76% of that of controls (Q_1: 63%; Q_3: 110%; n=6), which indicates that the 24% reduction in shoot growth was not due to altered shoot N concentration (Table 2; Supplementary Fig. S6). In the roots of these three *Lotus* species, tissue N concentrations were 86% of those of controls.

The tissue concentrations of P and K in shoots of *M. sativa* were reduced by low O_2 in the root zone in three studies: (i) from 0.76% to 0.52% (w/w) in dry mass (P) and 2.75% to 1.65% (K) in 84-day-old plants in aerobic or hypoxic (1 kPa O_2) nutrient solutions for the final 60 d (Arrese-Igor *et al.*, 1993); (ii) from 7.2 mg g^{-1} DW to 3.8 mg g^{-1} DW (P) and 23 mg g^{-1} DW to 13 mg g^{-1} DW (K) in 42-day-old plants in an organic-free substrate ('turface'—porous calcined clay media) supplied with half-strength Hoagland solution and waterlogged to the substrate surface for the final 14 d (Castonguay *et al.*, 1993); and (iii) from 0.43% to 0.23% (P) and 3.40% to 2.16% (K) in 60-day-old plants in waterlogged pots (sand:perlite 2:1 irrigated with half-strength Hoagland solution) for the final 20 d (Smethurst *et al.*, 2005). In contrast, in *L. tenuis*, the concentration of P in shoots was higher under low O_2 in the root zone than in drained soil in two pot studies: (i) 0.13% (control) versus 0.25% (waterlogged) in 49-day-old plants exposed to 40 d of waterlogging (Mendoza *et al.*, 2005); and (ii) 0.058% (control) versus 0.073% (waterlogged) in 75-day-old plants after 35 d of waterlogging (García *et al.*, 2008). The larger older plants (8.9 g versus 2.35 g per plant in controls), along with 30% lower P availability in the second waterlogged soil, might contribute to the lower shoot P concentration in the second study (soil pH was 8.7 in both). The higher concentration of P in shoots of *L. tenuis* when waterlogged might be the combined result of some reduction in plant growth (6% in García *et al.*, 2008; 28% in Mendoza *et al.*, 2005) and higher P availability in waterlogged soils (Kirk *et al.*, 1990) which in these studies had increased by 17% (García *et al.*, 2008) and 38% (Mendoza *et al.*, 2005). The concentrations of several nutrients in leaves of *T. subterraneum* were assessed at 21 d of waterlogging in a loam-based soil mixture in pots; leaf tissue concentrations (average across subspecies *yanninicum, subterraneum*, and *brachycalycinum*) were reduced by 16% for N, 31% for Ca, 18% for K, and 28% for Mg, but increased by 30% for P, 2.4-fold for Fe, and 23% for Mn (up to 123 ppm dry matter when waterlogged) (Devitt and Francis, 1972).

Shoot concentrations of Mn, an element that becomes reduced and increases in solubility to potentially toxic concentrations in some waterlogged soils, have been assessed for *M. sativa* and some *Lotus* species (Shiferaw *et al.*, 1992). Fe has not, to our knowledge, been assessed for these species. Microelement toxicities could be of concern in acid soils, but *M. sativa* already grows poorly on such soils (Dear *et al.*, 2003) and *Lotus* species are usually sown in more alkaline rather than acid soils (Escaray *et al.*, 2012), so these species might not be subjected to high concentrations of these microelements. Nevertheless, leaf Mn concentration did not change in *M. sativa* (146 ppm in dry matter in both conditions) or in two accessions of *L. pedunculatus* (averages were 103 ppm and 180 ppm in dry matter for control and waterlogged treatments, respectively) but increased by 5.3-fold in *L. corniculatus* (from 80 ppm to 424 ppm in dry matter) at 14 d of waterlogging where soil redox potential dropped from +350 mV to −307 mV (pH 6.14); so, leaf Mn concentration was not related to waterlogging tolerance (Shiferaw *et al.*, 1992). The critical tissue concentration for Mn toxicity in these legumes is not available, but in sweet clover the value is 500–700 ppm in dry matter (Morris and Pierre, 1949), which is higher than the levels in *L. corniculatus* when waterlogged in the study by Shiferaw *et al.* (1992).

Summary of consequences for shoots of O_2 deficiency in roots

Waterlogging effects on shoot physiology depend on species tolerance. In the sensitive *M. sativa*, P_n declines within 2–4 d of waterlogging, and in the longer term (>10 d) leaves suffer chlorophyll degradation, damage to the photosynthetic apparatus (e.g. PSII), and shoot N, P, and K deficiencies. In the more tolerant *L. corniculatus* and/or *L. tenuis*, the negative effects on P_n occur later, concentrations of N in shoots are maintained (or little affected), or can even increase in the case

of P, and plant growth is much less affected. Fe^{2+} and Mn^{2+} toxicity in shoot tissues seems unlikely for species grown in neutral to alkaline soils, but cannot be ruled out for some species and waterlogging situations in acidic soils.

Submergence tolerance in forage legumes

Complete submergence imposes a more severe stress for plants than soil waterlogging alone (Colmer and Voesenek, 2009). In addition to low O_2 in the root zone, when the shoot is fully submerged, the tissue O_2 is typically low at night due to respiratory consumption and slow inwards diffusion, but O_2 is relatively high during the light period when some P_n occurs (e.g. rice; Winkel *et al.*, 2013). Rates of P_n when leaves are under water are, however, much lower than when the leaves are in air, as when submerged the entry of CO_2 into the leaves is impeded and light can be low (Mommer and Visser, 2005; Colmer *et al.*, 2011). The greatly reduced P_n under water can threaten plant survival as a result of 'carbohydrate starvation' owing to sugar consumption exceeding production, so that the 'energy crisis' associated with low O_2 in tissues can be further compounded by depletion of sugar substrates in completely submerged plants (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009).

The 'energy crisis' that occurs when tissues become anoxic, such as would occur each night to a portion of the root system of completely submerged plants (e.g. rice; Waters *et al.*, 1989), is a consequence of the inhibition of ATP production via oxidative phosphorylation. Cells can switch to fermentation, so that glycolysis linked to ethanol formation: (i) provides substrate-level phosphorylation of ADP to ATP; and (ii) recycles NADH to NAD^+ (Gibbs and Greenway, 2003). The ATP production in anoxia, however, is only 5.5–12% of that in aerobic tissues, and the resulting low ATP availability can lead to cellular damage and eventually death (Gibbs and Greenway, 2003). Sugars, including those mobilized from starch, are used to sustain glycolysis/fermentation, but these are not adequately replenished by the low rates of P_n during the daylight hours when submerged. So, as the duration of submergence progresses, exhaustion of carbohydrates might result in low energy and death of cells, tissues, and eventually organs (Bailey-Serres and Voesenek, 2008).

Two main syndromes have been recognized for plant adaptation to submergence; an escape strategy (LOES, low O_2 escape syndrome) and a sit-and-wait strategy (LOQS, low O_2 quiescence syndrome) (Bailey-Serres and Voesenek, 2008; Hattori *et al.*, 2010). The LOES involves shoot elongation in order to restore leaf contact with the atmosphere (beneficial for prolonged, 'shallow' submergence), while the LOQS is based on energy conservation (e.g. no shoot elongation) to prolong survival during submergence and enable resumption of growth after the water recedes (beneficial for short duration and/or 'deep' submergence) (Bailey-Serres and Voesenek, 2008). The LOQS strategy, where underwater shoot extension and carbohydrate metabolism are repressed, is conferred in rice by the *SUB1A* gene of the polygenic *Submergence1* (*Sub1*) locus which results in a lack of responsiveness to ethylene of shoot extension (Fukao *et al.*, 2006; Bailey-Serres and Voesenek, 2008).

In forage legumes, shoot extension responses have been evaluated for some species of the *Lotus* genus (Manzur *et al.*, 2009; Striker *et al.*, 2012). Fourteen-day-old seedlings of 2.4 cm height of *L. japonicus*, *L. corniculatus*, and *L. tenuis* were exposed to submergence for 12 d (4 cm water above soil), and a subsequent recovery period of 30 d in de-submerged and drained conditions. All three species survived submergence but exhibited different responses of shoot extension. Submerged *L. japonicus* exhibited an 'escape strategy' and shoots emerged above the water, whereas *L. corniculatus* and *L. tenuis* both displayed a 'sit-and-wait strategy' with no shoot extension, no new unfolding of leaves, and no dry mass accumulation. Upon de-submergence and soil drainage, *L. japonicus* had the lowest recovery growth of shoots, with dry mass of 42% of the control at 30 d after the excess water was removed, *L. corniculatus* was intermediate, and *L. tenuis* showed the greatest recovery growth, with final shoot dry mass equal to the control (Striker *et al.*, 2012).

Interestingly, the shoot extension response of *L. tenuis* has the ability, depending on the water depth, to shift between the two strategies of 'sit-and-wait' (complete submergence) or 'escape' (partial submergence) (Manzur *et al.*, 2009). Under 30 d of partial submergence, 6-month-old plants of *L. tenuis* ceased root growth but showed shoot lengthening by 31% compared with drained controls, so that the shoot proportion above the water increased. These plants with shoots emergent above the water also developed greater shoot and root tissue porosity compared with completely submerged plants (shoot porosity, 40.9% versus 27.1%; root porosity, 32.5% versus 21.6%). In contrast, completely submerged plants (water 8–10 cm above plants) displayed a 'sit-and-wait strategy', with no shoot elongation, no production of new leaves, and dry mass slightly lower than at the beginning of the experiment. The survival of completely submerged plants is attributed to consumption of sugars and starch reserves from crowns (concentrations 50–75% less than in partially submerged and control plants) (Manzur *et al.*, 2009), presumably with some small supplementation also from underwater P_n as plants were submerged in clear water and bubbles were observed on the leaves (G. Striker, pers. obs.).

A leaf feature which might enhance submergence tolerance is a hydrophobic surface that results in a thin layer of gas on the leaves (Colmer and Pedersen, 2008; Colmer *et al.*, 2011; Pedersen and Colmer, 2012). The ability to retain a 'gas film' on leaves when under water is dependent on the hydrophobicity of the cuticle, and a gas film improves the entry of CO_2 for underwater P_n when in light and of O_2 from floodwaters when in darkness (Pedersen *et al.*, 2009; Verboven *et al.*, 2014). Further, root aeration is also improved as: (i) underwater P_n produces O_2, some of which diffuses into and along the shoot and root aerenchyma; and (ii) during the night the enhanced O_2 entry from the floodwater into the leaves also increases the amount of O_2 that reaches the roots (e.g. rice; Pedersen *et al.*, 2009; Winkel *et al.*, 2014). Thus, leaf gas films can improve both tissue O_2 and sugar status of completely submerged plants (Pedersen *et al.*, 2009).

Submergence tolerance in forage legumes in relation to the presence/absence of leaf gas films has been examined only in *M. siculus* (in non-saline and moderately saline water; Teakle *et al.*, 2014). The main findings were that plants with gas films on leaves, which persisted only for 3 d on both leaf sides, were able to survive 7 d of complete submergence in water with up to 50 mM NaCl, but were unable to resume growth after submergence at 100 mM NaCl (Teakle *et al.*, 2014). The presence of leaf gas films reduced the Na^+ and Cl^- ingress into leaves when submerged in saline water, and leaves with gas films better maintained chlorophyll and rates of underwater P_n after 3 d of complete submergence (Teakle *et al.*, 2014). So, leaf gas films can benefit *M. siculus* exposed to short-term (non-saline and moderately saline) submergence (Teakle *et al.*, 2014).

In submerged *M. siculus*, no shoot extension response was evident, but leaves did re-orientate to be vertical (not simply 'buoyancy'; Teakle *et al.*, 2014; T. Colmer, pers. obs.), and such 'hyponastic growth' near the base of petioles has been studied in detail for *Rumex palustris* (Cox *et al.*, 2003). In the case of *M. siculus*, the more vertical leaves might in shallow submergence again place lamina in air contact, or at least nearer the water surface where light might be higher for P_n. Increased ethylene in tissues probably triggers this hyponastic growth, as demonstrated for petioles of *R. palustris* (Heydarian *et al.*, 2010). Ethylene also promotes shoot elongation in species with an 'escape response' (Voesenek *et al.*, 2006). Responses of forage legumes to ethylene have not been studied, with the exception of *T. fragiferum* (cv. Salina) treated with ethephon (ethylene treatments would be preferred), for which stolons became erect (hyponasty) with subsequent rapid elongation (Hansen and Bendixen, 1974).

In summary, *L. tenuis* and *M. siculus* show tolerance of complete submergence, but the molecular mechanisms underpinning their submergence tolerance remain to be elucidated. The use of *L. japonicus* as a model for identifying the genetic basis of submergence tolerance of *Lotus* spp. looks promising and, despite the fact that a single accession (MG-20) displayed a LOES when submerged (Striker *et al.*, 2012), wide variability was found when screening 94 RILs for waterlogging tolerance (Striker *et al.*, 2014), so additional variability also might be expected for submergence tolerance. Potential orthologues of the *Sub1A* gene in rice (conferring LOQS; Xu *et al.*, 1996) should be searched for in the sequenced genome of *L. japonicus*, which then could assist identification of related genes in *L. tenuis*. The present physiological understanding of *L. tenuis* and *M. siculus* also makes these species well placed for future studies using '-omics' tools to unravel the molecular basis of their submergence tolerance.

Conclusions and future research

Forage legumes (annual and perennial species) differ markedly in waterlogging tolerance. Tolerant species (e.g. *Lotus tenuis* and *Melilotus siculus*) generally have higher constitutive porosity in roots and are able to increase root porosity under O_2 deficiency by formation of aerenchyma. There is a positive (hyperbolic) relationship between whole-plant dry mass (as a percentage of control) and root porosity when in waterlogged/O_2-deficient conditions. Higher root porosity improves the internal O_2 supply for respiration and thus the energy status of roots, enabling growth and functioning, including N_2 fixation. Aerenchyma in roots and the outer cortex of nodules, and increased permeability of the gas diffusion barrier within nodules, enables O_2 supply to nodules of submerged roots. In sensitive species, using *Medicago sativa* as an example, P_n declines within a few days of waterlogging and decreases further with longer duration waterlogging as leaves suffer chlorophyll degradation, N deficiency, and damage; yet since growth is severely inhibited, demand for sugars is markedly reduced and tissue sugar concentrations can increase. Such effects are absent, or greatly delayed, in the more tolerant species (e.g. *L. tenuis*). Fe^{2+} and Mn^{2+} toxicities are unlikely for species grown in neutral to alkaline soils. Tolerance of forage legumes to complete submergence is much less studied than soil waterlogging. *Lotus tenuis* responds with shoot elongation ('escape') during partial submergence but does not elongate ('sit-and-wait') when completely submerged. Leaf gas films can contribute to submergence tolerance, as demonstrated for *M. siculus*. The diversity in waterlogging (and submergence) tolerance amongst forage legumes, for both annual and perennial species, provides at least some options for forage production in flood-prone areas.

We suggest six priority areas requiring further study to improve knowledge of flooding tolerance in forage legumes which are of importance to world agriculture: (i) metabolic responses and anoxia tolerance in roots; (ii) N_2 fixation and productivity as affected by soil waterlogging (including in field situations with regenerated annual pastures); (iii) evaluations of ability to recover after water subsides and identification of traits conferring recovery ability; (iv) improved understanding of the responses of P_n as related to possible stomatal and non-stomatal limitations, and especially including data on C_i and tissue sugars; (v) identification of molecular mechanisms defining tolerance to submergence, including the role of ethylene signalling; and (vi) evaluations of within-species variation for tolerance and key contributing traits (and recovery ability), and their genetic regulation, knowledge which could underpin future breeding programmes aimed at improvement of waterlogging and/or submergence tolerance of some priority (widely used) species. Future studies should include increased focus on nodulated plants, in addition to the use of mineral N-fed plants, so that both the N_2-fixing symbiosis and plant tolerance *per se* are evaluated.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Waterlogging and root zone O_2 deficiency treatment durations of experiments performed on the main perennial and annual forage legumes used in world agriculture.

Figure S2. The effect of waterlogging or O_2 deficiency in the root zone on whole-plant dry mass of *Trifolium subterraneum* subspecies '*yanninicum*', '*subterraneum*', and '*brachycalycinum*'.

Figure S3. The effect of waterlogging or O_2 deficiency in the root zone on dry mass of shoots and roots of the main perennial and annual forage legumes used in world agriculture.

Figure S4. The effect of waterlogging or O_2 deficiency in the root zone on the root to shoot ratio of the main perennial and annual forage legumes used in world agriculture.

Figure S5. Relationship between whole-plant dry mass in waterlogged or O_2-deficient root zone conditions and root porosity of 13 forage legume species.

Figure S6. (a) Shoot dry mass in waterlogged or O_2-deficient root zone treatments plotted against shoot nitrogen (N) concentration. (b) Box plots of shoot N concentration in waterlogged or O_2-deficient treatments for *Medicago sativa* and three *Lotus* species.

Table S1. List of the perennial and annual forage legume species, cultivars, waterlogging or hypoxic treatment duration, growth conditions, shoot dry mass, root dry mass, inclusion (or not) of a recovery period, and references for data used in Fig. 1.

Table S2. Comparison of waterlogging tolerance results from experiments in pots with soil or other substrates and in nutrient solutions for six of the perennial and annual forage legume species shown in Fig. 1.

Table S3. Summary table showing the perennial and annual forage legume species, cultivars, waterlogging or hypoxic treatment duration, growth conditions, root type, porosity in drained or aerated conditions, porosity in waterlogging or hypoxic (O_2 deficient) conditions, and references of data used in Fig. 2.

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