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The contrasting influence of short-term hypoxia on the hydraulic properties of cells and roots of wheat and lupin

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Abstract. Little is known about water flow across intact root cells and roots in response to hypoxia. Responses may be rapid if regulated by aquaporin activity, but only if water crosses membranes. We measured the transport properties of roots and cortical cells of three important crop species in response to hypoxia (0.05 mol O_2 m^{-3}): wheat (*Triticum aestivum* L.), narrow-leafed lupin (*Lupinus angustifolius* L.) and yellow lupin (*Lupinus luteus* L.). Hypoxia influenced solute transport within minutes of exposure as indicated by increases in root pressure (P_r) and decreases in turgor pressure (P_c), but these effects were only significant in lupins. Re-aeration returned P_r to original levels in yellow lupin, but in narrow-leafed lupin, P_r declined to zero or lower values without recovery even when re-aerated. Hypoxia inhibited hydraulic conductivity of root cortical cells (Lp_c) in all three species, but only inhibited hydraulic conductivity of roots (Lp_r) in wheat, indicating different pathways for radial water flow across lupin and wheat roots. The inhibition of Lp_r of wheat depended on the length of the root, and inhibition of Lp_c in the endodermis could account for the changes in Lp_r. During re-aeration, aquaporin activity increased in wheat roots causing an overshoot in Lp_r. The results of this study demonstrate that the roots of these species not only vary in hydraulic properties but also vary in their sensitivity to the same external O_2 concentration.

Additional keywords: hydraulic conductivity, oxygen deficiency, pressure probe, root pressure, turgor pressure.

Introduction

Research investigating the tolerance of different species to waterlogging has predominantly focussed on the long-term effects on growth (days to weeks), yet many physiological processes are affected within much shorter time frames. Waterlogging leads to a depletion of oxygen in the rhizosphere and whilst this depletion can happen quickly, no plant experiences anoxia (zero O_2) immediately upon its root system becoming submerged (Blackwell 1983). Examining responses that occur when a plant senses the changing environment will help reveal mechanisms involved in acclimation to O_2 deficiency. For example, pre-exposure to hypoxia (low O_2) increases the tolerance of a tissue to subsequent anoxia through improved cytosolic pH regulation and respiration, higher rates of alcoholic fermentation and changes in protein production and gene expression (Saglio *et al*. 1988; Waters *et al*. 1991; Xia and Roberts 1996; Dennis *et al*. 2000; Kato-Noguchi 2000). Pre-exposure to hypoxia is also required to stimulate development of lysigenous aerenchyma (Drew *et al*. 2000).

Waterlogging and low O_2 in the rhizosphere tend to reduce the hydraulic conductivity of roots (Lp_r), but responses vary between species and type of treatment imposed (see review in Bramley *et al*. 2007*a*). Root death or anatomical changes may reduce Lp_r during long-term exposure to O_2 deficiency by creating physical barriers to water flow. Anatomical changes that influence Lp_r are usually associated with water and nutrient deficiencies and have rarely been characterised for waterlogging (Enstone and Peterson 2005). Even less is known about responses in Lp_r to short-term exposure to O_2 deficiency, but Lp_r can change rapidly in response to transpiration and other abiotic perturbations (Steudle 2001). Rapid changes in Lp_r due to O_2 deficiency may be caused, indirectly or directly, by reduced respiration rates or pH-induced gating of aquaporins (Tournaire-Roux *et al*. 2003). However, these effects require bulk water flow to cross membranes somewhere along its flow-path between the root surface and vascular tissue. If water flows entirely through the apoplast (cell walls and intercellular spaces), then aquaporins will have little influence on Lp_r.

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This study examined the effects of short-term (0.5 h) hypoxia on water flow in wheat (*Triticum aestivum* L.), narrow-leafed lupin (*Lupinus angustifolius* L.) and yellow lupin (*Lupinus luteus* L.) roots when all three species were exposed to the same external oxygen concentration (0.05 mol O_2 m^{-3}). Wheat and lupins are important crops grown in winter in southern Australia. When sown on duplex soils, where a sandy/loam soil overlies clay, these crops often experience transient waterlogging during periods of high rainfall (Belford *et al.* 1992). Wheat is thought to tolerate waterlogging better than lupins, although direct comparisons under the same field conditions have not been undertaken (Dracup *et al.* 1992). In a glasshouse experiment comparing root growth in root chambers flooded for 1 week, wheat roots had superior survival and recovery than narrow-leafed lupin or yellow lupin (Bramley 2006). Roots of both lupin species died when submerged and only yellow lupin grew new roots when the chambers were drained. In a study on whole plants, Davies *et al.* (2000*a*) found that yellow lupin was more tolerant to waterlogging than narrow-leafed lupin due to properties of their root system, as indicated by cross-grafting roots and shoots, although it is not yet known what properties confer that response.

The effect of O_2 deficiency on water flow in lupin roots has not been previously investigated, but Zhang and Tyerman (1991) found that 0.5 h of hypoxia reduced the hydraulic conductivity of root cortical cells (Lp_c) of wheat by 85%. Water uptake in wheat roots occurs predominantly in a region close to the root tip and under ambient conditions, radial water flow is influenced by aquaporins, probably located in the endodermis (Bramley *et al.* 2009). Therefore, hypoxia may inhibit Lp_r of wheat roots and the extent of the inhibition should be related to the inhibition at the cell level. In comparison, radial water flow in lupin roots occurs predominantly through the apoplast, despite significant aquaporin activity in cortical cells (Bramley *et al.* 2009). Any inhibitory effects of O_2 deficiency on aquaporin activity should therefore have less influence on Lp_r, unless O_2 deficiency causes water flow to switch to the cell-to-cell pathway.

Water flow was measured across individual roots before, during and after a mild hypoxic treatment. Because wheat roots resume growth after waterlogging (Bramley 2006), water uptake must also resume and therefore, Lp_r should recover when wheat roots have sufficient oxygen. To determine whether effects on Lp_r were due to transport across cell membranes, the effects of hypoxia on Lp_c of root cortical cells were also measured. In comparison to the majority of studies that simulate waterlogging or O_2 deficient conditions by imposing sudden anoxia (zero oxygen), 0.05 mol O_2 m^{-3} is a mild hypoxic treatment. However, it is similar to the concentration that reduced Lp_c of wheat root cells (Zhang and Tyerman 1991) through closure of aquaporins (Zhang and Tyerman 1999). This O_2 concentration can also induce changes in aquaporin expression within 0.5 h of treatment (Klok *et al.* 2002).

Materials and methods

Plant material

Seeds of wheat (*Triticum aestivum* L. cv. Kulin), narrow-leafed lupin (*Lupinus angustifolius* L. cv. Merrit) and yellow lupin (*L. luteus* L. cv. Wodjil) were germinated and grown as described by Bramley *et al.* (2007*b*). Plants were grown in individual sand-filled pots that allowed access to the roots without injury. Roots were carefully washed from the sand 10–14 days after sowing. The taproot of lupin or the longest seminal root of wheat was excised below emerging lateral roots as lateral roots prevented the root sealing to the root pressure probe. Roots were 70–180 mm long for measurements with the root pressure probe and roots were excised 80 mm from the tip for measurements on intact cells. Root diameters in the region punctured with the cell pressure probe were 0.61 ± 0.02 mm for wheat, 0.89 ± 0.05 mm for yellow lupin and 1.2 ± 0.20 mm for narrow-leafed lupin.

Roots had the same anatomy as those reported in Bramley *et al.* (2009), with none of the species developing an exodermis and the endodermis matured closer to the root tip in wheat than in either lupin species (Fig. 1).

Hydraulic conductivity of roots (Lp_r)

The excised end of the root was connected to a root pressure probe (Steudle 1993) via a seal made from silicon impression material (Exaflex, Halas Dental Supplies, Adelaide, SA, Australia). The root was supported inside a glass tube (5 mm internal diameter), which was connected to a reservoir (maintained at 25°C by a water bath) of the same nutrient solution used to water the plants, supplemented with 5 mM glucose. Glucose was included as a surrogate carbohydrate (Gibbs *et al.* 1998*a*) supply because measurements on excised roots took several hours. The solution circulated past the root at a rate of 10–15 mm s^{-1}. The nutrient solution was bubbled with air or a combination of N_2 plus air (hypoxic treatment), through an air-stone, whilst a Rank oxygen electrode continuously monitored the O_2 concentration just before the solution passed the root. When the nutrient solution was bubbled with N_2 plus air, it took 120–600 s for the O_2 concentration to decrease to 0.05 mol O_2 m^{-3}. The circulation system was totally sealed apart from where the root entered the glass tube.

Measurements of Lp_r commenced when root pressure (P_r) was stable, which took a minimum of 2 h depending on the individual root. Water flow through the root was induced by applying a series of successive step changes in root pressure by rotating the rod inside the probe (Bramley *et al.* 2007*b*). Pressure was clamped for 60–120 s to ensure that pressures and flows were measured under steady-state conditions (Bramley *et al.* 2007*b*). The hydraulic conductance of the root (L_r) was calculated from the slope of the linear regression of volume flow rate against applied pressure. L_r was normalised by the surface area of the root to give Lp_r. Lp_r was measured on the same root three times; during perfusion with aerated solution (0.25 mol O_2 m^{-3}), after 0.5 h of hypoxia (0.05 mol O_2 m^{-3}) and after 1 h of re-aeration (0.25 mol O_2 m^{-3}).

The seal connecting the root to the pressure probe was tested at the end of the measurements to ensure that it had not restricted water flow during the experiment (Steudle 1993).

Water relations of root cortical cells

Effects of hypoxia on cell water relations were determined using a cell pressure probe mounted on a micromanipulator with 1-μm increments (MX1, Narishige, Tokyo, Japan). The root segment was secured inside a small perspex chamber, as described by

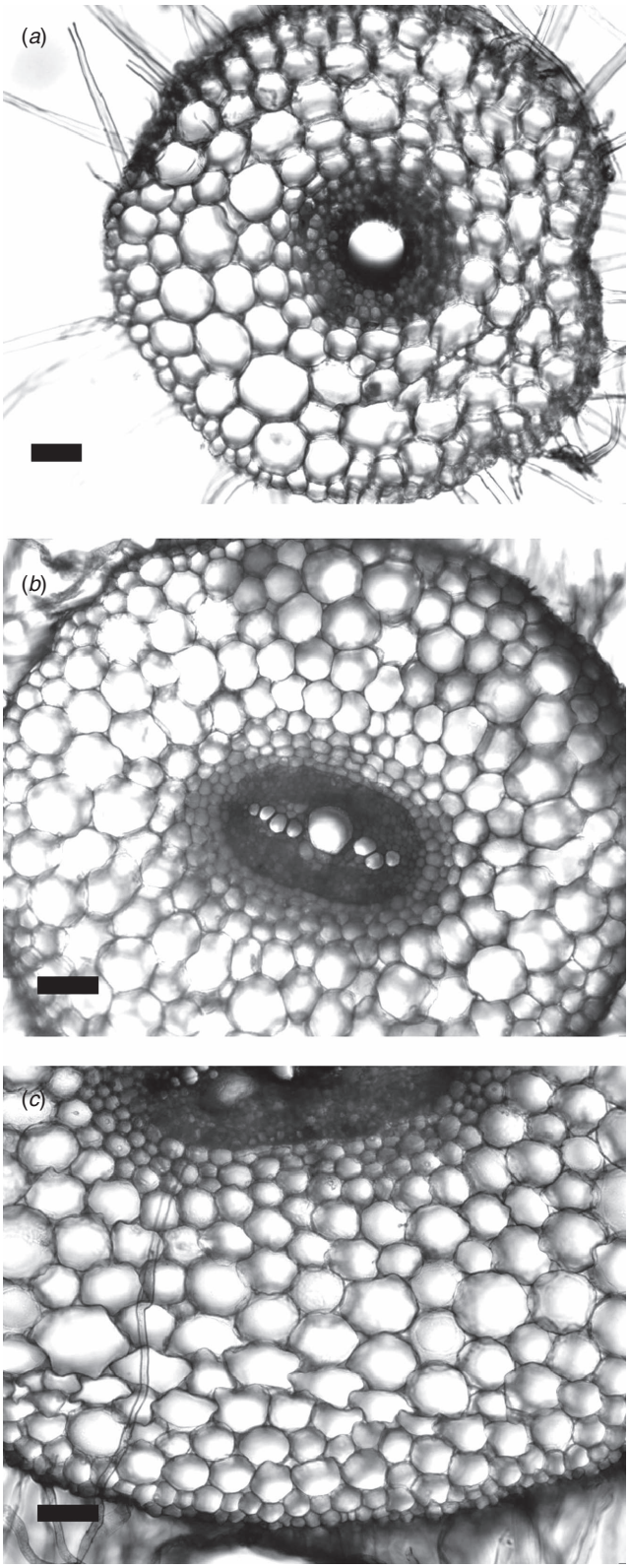

Fig. 1. Freehand cross-sections of (*a*) wheat, (*b*) yellow lupin and (*c*) narrow-leafed lupin roots, 10 cm from the root tip. Examples are typical of 14-day-old roots grown in sand. Sections were stained for 5 min with 0.05% toludine blue. Bars = 50 µm.

Zhang and Tyerman (1991), and positioned on the stage of a microscope. A transparent cover was sealed to the top of the chamber with vacuum grease so that the circulatory system was entirely closed, apart from a 5-mm opening for entry of the microcapillary of the pressure probe. The same nutrient solution as that used in measurements of Lp_r was circulated through the chamber at a rate of 420 mL h^{-1} using a peristaltic pump (Exatech, Melbourne, Vic, Australia). The O_2 concentration of the nutrient solution was adjusted and monitored as described above.

Cortical cells were punctured by the microcapillary of the pressure probe in the second to sixth cell layer from the root surface (20–200 µm depth from root surface), 30–50 mm from the root tip. The methodology for measuring the water relations of cortical cells and the size of the cells were reported in Bramley *et al*. (2009). Turgor pressure (P_c), half-time of the rate of water exchange ($T_{1/2}$) and volumetric elastic modulus (ε) were measured and used to calculate the hydraulic conductivity of cells (Lp_c). Where water flow across membranes was rapid ($T_{1/2} < 1$ s), ε was corrected for the curvilinear relationship between volume and pressure change (Steudle *et al*. 1980). Because of the difficulty in maintaining constant turgor pressure and an unblocked probe capillary for long periods of time, measurements were conducted on roots within 0.2–0.5 h, after bathing roots for 0.5 h with aerated solution (0.25 mol O_2 m^{-3}) or hypoxic solution (0.05 mol O_2 m^{-3}).

Statistical analyses

All data were analysed with SPSS ver. 11.0 (SPSS, Chicago, IL, USA) and GraphPad Prism ver. 5.01 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA compared the initial root pressure (P_r) between species. To test whether hypoxia significantly changed P_r of each species, P_r before and during hypoxia were compared in a paired *t*-test. Repeated-measures ANOVA tested the effects of the O_2 treatments on Lp_r with Bonferroni post-test. Two-way ANOVA with Bonferroni post-tests compared the effects of O_2 treatment on the water transport parameters of cells.

Results

Root pressure

When connected to the root pressure probe, wheat roots generated significantly higher P_r than either species of lupin ($P < 0.0001$; Table 1). Roots responded to reduced O_2 concentration by increased P_r (Table 1, Fig. 2). P_r of both lupin species significantly increased in response to hypoxia ($P = 0.001$ for narrow-leafed lupin and $P = 0.021$ for yellow lupin), but the response in wheat was not statistically significant ($P = 0.295$). P_r of yellow lupin began to increase soon after the commencement of bubbling with N_2 plus air when the O_2 concentration of the solution was between 0.06 and 0.07 mol O_2 m^{-3}, and then returned to pre-hypoxia values when roots were re-aerated (Fig. 2*b*). In comparison, P_r of narrow-leafed lupin took 161 (±32) s at 0.05 mol O_2 m^{-3} before increasing, but the increase in P_r was only transient, declining during the hypoxic treatment (Fig. 2*c*). Re-aeration did not arrest the decline in 6 of the 11 narrow-leafed lupin roots, but in those roots, P_r continued to decline to zero without subsequent recovery (Fig. 2*c*).

Table 1. Root pressure (P_r) of roots bathed with aerated solution (0.25 mol O_2 m^{-3}) and the maximum increase in P_r (ΔP_r) when bathed with hypoxic solution (0.05 mol O_2 m^{-3})

P_r was measured with the root pressure probe. Values are means ± s.e.m. and values in brackets = n. Different superscript letters represent significant difference between the species ($P < 0.05$)

	P_r (MPa × 10^{-3})	ΔP_r (MPa × 10^{-3})
Wheat	96 ± 6 (10)^{a}	5 ± 6 (10)^{a}
Yellow lupin	52 ± 7 (9)^{b}	14 ± 4 (9)^{ab}
Narrow-leafed lupin	40 ± 5 (11)^{b}	22 ± 4 (11)^{b}

Hydraulic conductivity of roots

Lp_r of aerated wheat roots was almost twice that of both lupin species (Fig. 3; $P = 0.008$). Aeration treatment significantly affected Lp_r of wheat roots ($P = 0.0007$; Fig. 3), but did not significantly affect Lp_r of either lupin species when roots with stable root pressure at the end of the experiment were analysed ($P = 0.294$ for narrow-leafed lupin and 0.331 for yellow lupin; Fig. 3). The effect of O_2 treatment on Lp_r was not analysed in lupin roots where P_r decreased to zero without recovery, as this 'leakiness' creates artificially high water flows. For wheat roots, hypoxia reduced Lp_r by 2–45%, but due to this variability, the effect was not significant ($P > 0.05$; Fig. 3). However, after re-aeration, Lp_r was on average 1.4 and 1.6 times greater than Lp_r before and during hypoxia, respectively ($P < 0.05$; Fig. 3). Because Lp_r of individual wheat roots is inversely related to root length (Bramley *et al*. 2009), the relative change in L_r ($Lp_r \times A$) due to O_2 treatment (normalised to the value before hypoxia) was examined in relation to root length (Fig. 4). The relative change in L_r due to hypoxia tended to be greater in shorter roots (Fig. 4*a*), but was independent of root length after re-aeration (Fig. 4*a*).

Because changes in root anatomy could be discounted during such short-term aeration treatments, changes in Lp_r of wheat roots may have been mediated by varying aquaporin activity. Aquaporin activity was tested before hypoxia and after re-aeration using mercuric chloride to inhibit water flow across cell membranes, the assumption being that if more aquaporins were active there would be greater inhibition of water flow. The influence of aquaporins on Lp_r was not tested during hypoxic treatment as the combined treatment of mercury and hypoxia was toxic to roots, causing loss of root pressure without recovery. Wheat roots were incubated for 0.5 h with 50 μM $HgCl_2$ before hypoxia or after 0.5 h hypoxia plus 1 h re-aeration. Aquaporin activity was also tested in lupin roots, but only for pre-hypoxia treatment as Lp_r was not significantly affected by O_2 treatments.

Mercuric chloride inhibited water flow of wheat roots more after re-aeration (60%) than before hypoxia (40%), reducing Lp_r to the same level (Fig. 5*a*). In comparison, mercury did not significantly affect Lp_r of either lupin species ($P > 0.05$; Fig. 5*b*).

Cell water relations

Cortical cells of wheat had significantly higher turgor pressure than cells of either lupin species (Table 2; $P < 0.001$). Hypoxia decreased the turgor pressure of yellow lupin cells by 0.12 MPa ($P < 0.001$; Table 2), but did not affect turgor pressure of cells in wheat roots ($P > 0.05$; Table 2). Cells of narrow-leafed lupin roots bathed with hypoxic solution were less stable than cells in aerated roots (Fig. 6). Cells often lost turgor pressure almost immediately after puncturing and the consistency of the cell sap appeared

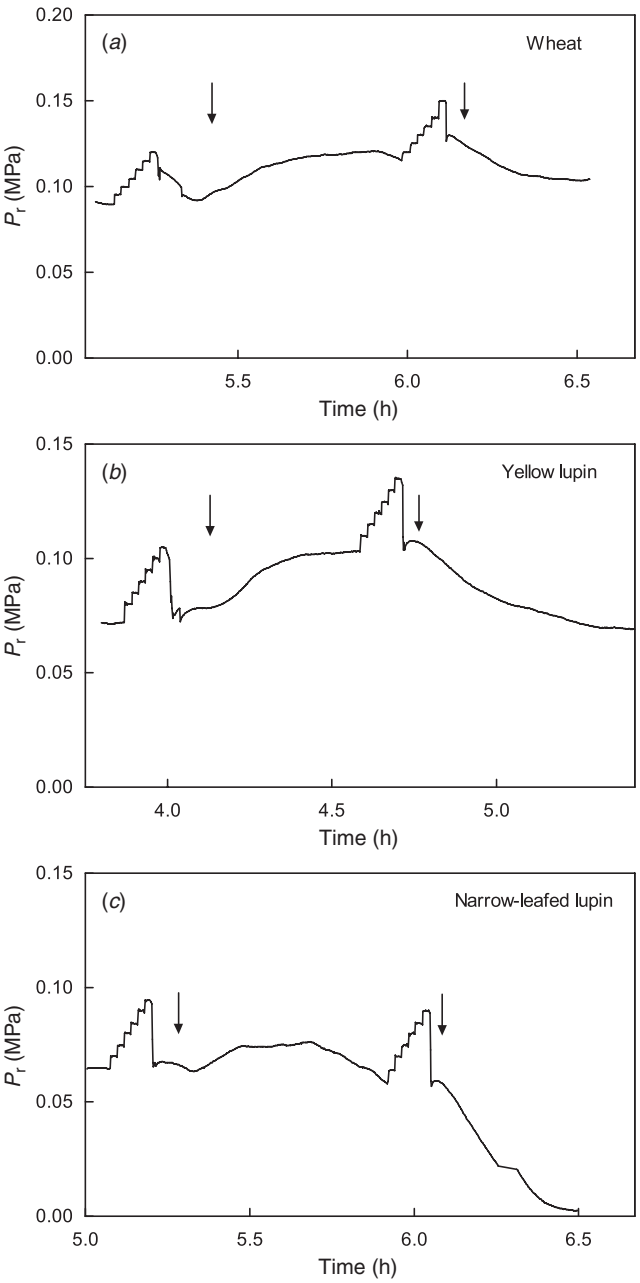

Fig. 2. Examples of the response in root pressure (P_r) when individual roots of (*a*) wheat, (*b*) yellow lupin and (*c*) narrow-leafed lupin were bathed with hypoxic solution (0.05 mol O_2 m^{-3}) and then re-aerated (0.25 mol O_2 m^{-3}). Arrows indicate the switch from aerated to hypoxic solution and then back to aerated solution. The steps in pressure are pressure-clamps for measuring Lp_r. The example shown for narrow-leafed lupin (*c*) was a typical response for six roots, with the remaining roots ($n = 5$) behaving similar to yellow lupin (*b*).

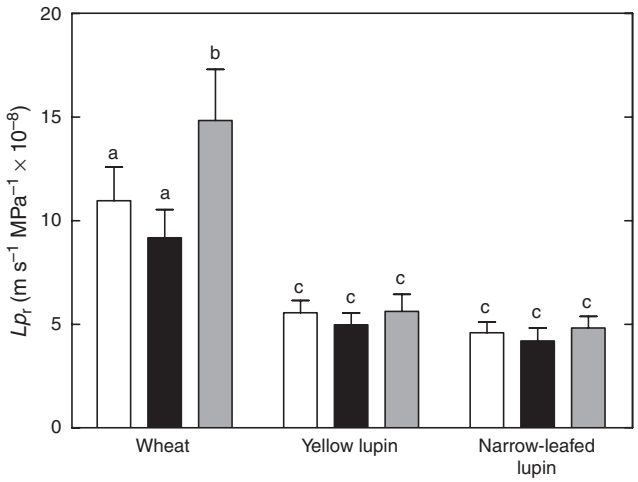

Fig. 3. The effect of O_2 treatment on the hydraulic conductivity (Lp_r) of individual roots. Lp_r was measured on the same root when bathed with aerated solution (0.25 mol O_2 m^{-3}, white bars), then after 0.5 h with hypoxic solution (0.05 mol O_2 m^{-3}, black bars) and then after 1 h of re-aeration (0.25 mol O_2 m^{-3}, grey bars). Values of Lp_r were only considered for those roots with stable root pressure at the end of the measurements. Error bars represent + s.e.m.; $n = 5$–11. Significant differences due to treatment and between species ($P < 0.05$) are marked by different letters.

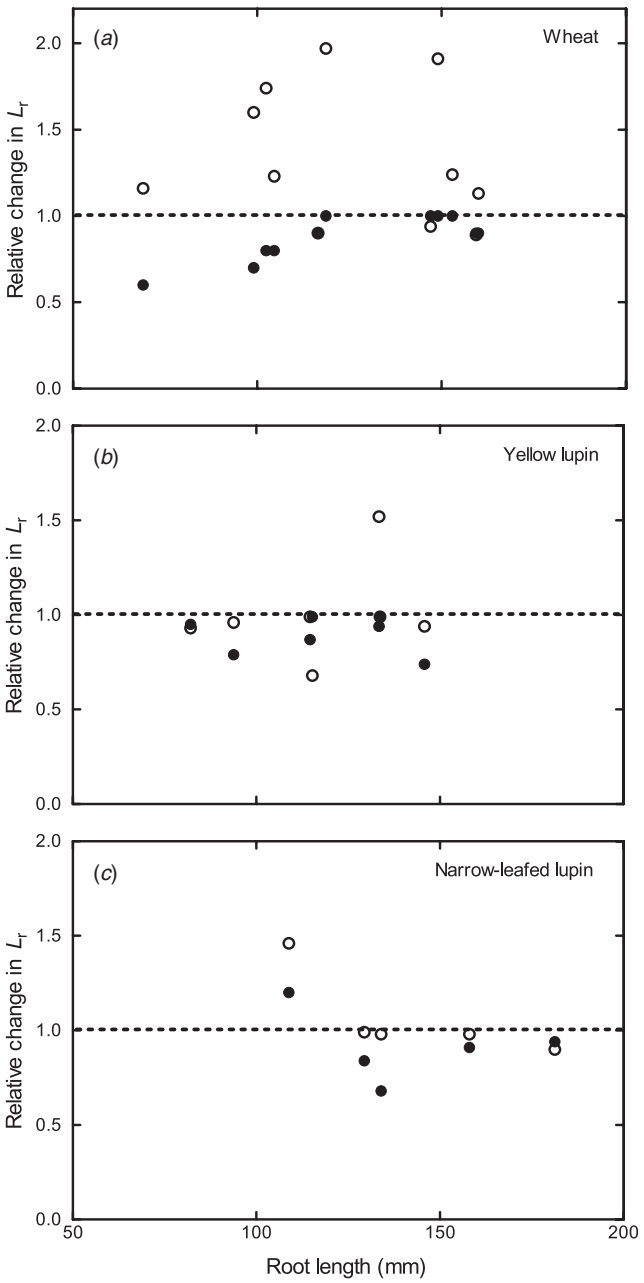

Fig. 4. The relationship between relative change in hydraulic conductance (L_r) and length of root connected to the root pressure probe. Changes in L_r due to hypoxia (closed circles) and then re-aeration (open circles) were normalised to the values before hypoxia treatment. Values above or below the dotted line denote increases or decreases in L_r, respectively. Values are shown only for those roots with stable root pressure at the end of the measurements.

to be altered by hypoxia, causing frequent blockages of the microcapillary.

Lp_c of cortical cells in aerated roots was not significantly different between the species ($P = 0.333$), but was significantly reduced by hypoxia ($P < 0.0001$; Table 2). Lp_c of hypoxic treated cells was lower than aerated cells, by an average of 46% for wheat, 63% for yellow lupin and 74% for narrow-leafed lupin (Table 2). Bramley *et al*. (2009) demonstrated that Lp_c of cells in aerated wheat and lupin roots tends to increase with depth from the root surface, but the effect of hypoxia on Lp_c was independent of cell location within the cortex.

Lp_c influences the rate of water flow across the membrane and hence influences $T_{1/2}$. $T_{1/2}$ for cells in roots bathed in aerated solution ranged between 0.3 and 1.3 s, and was not significantly different between the species ($P = 0.678$; Table 2). For all species, mean $T_{1/2}$ of cells from hypoxic roots was significantly greater than cells from aerated roots ($P < 0.0001$; Table 2; Fig. 6). Increase in $T_{1/2}$ due to hypoxia was greater for narrow-leafed lupin root cells than for the other species (Table 2), but only five narrow-leafed lupin cells were stable for sufficient time to obtain measurements. Mean $T_{1/2}$ of wheat and yellow lupin root cells doubled in response to hypoxia (Table 2), however, the effect on individual cells was variable as $T_{1/2}$ ranged between 0.6–3.0 s and 0.7–4.6 s, respectively.

The elastic modulus of cortical cells from aerated roots ranged from 3.8 to 9.0 MPa for wheat and 1.8 to 16.7 MPa for lupins, and the average was not significantly different between the species ($P = 0.705$; Table 2). The variability in ε was due to the dependence of ε on cell volume (Fig. 7). Hypoxia had no effect on ε of wheat or yellow lupin ($P > 0.05$; Table 2, Fig. 7). However, hypoxia apparently reduced ε of narrow-leafed lupin cells, so that there was no longer a relationship between cell volume and ε ($P = 0.0005$, Fig. 7). The rate of water exchange across the cell is dependent on ε and therefore, a lower ε would result in an increase in $T_{1/2}$. However, the apparent reduction in ε may be an artefact of the measurement process since cells with a lower ε may have been the only ones that were measurable due to greater membrane instability during hypoxia. When the pressure probe microcapillary is pushed against the cell wall just before puncturing the cell, it could cause a surge in turgor pressure,

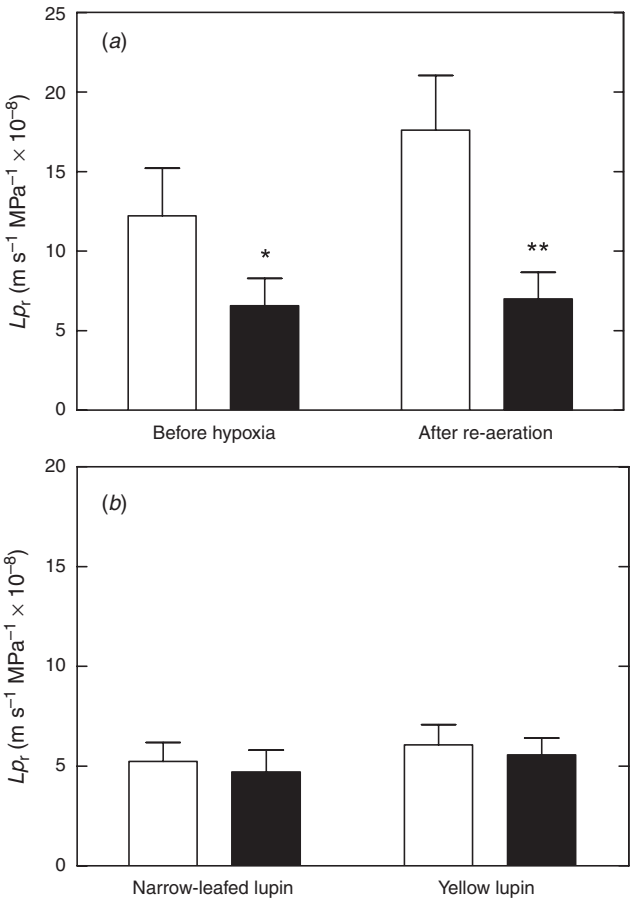

Fig. 5. The hydraulic conductivity (Lp_r) of roots before (white bars) or after (black bars) treatment with 50 μM $HgCl_2$. The mercury treatment was applied to roots of (*a*) wheat before hypoxia or after re-aeration and (*b*) both lupin species before hypoxia. Error bars represent + s.e.m.; $n = 4–5$. Significant differences due to $HgCl_2$ treatment are indicated by * ($P < 0.05$) and ** ($P < 0.01$).

disrupting cells with unstable membranes. Cells with a lower ε would buffer against sudden changes in turgor pressure.

To test aquaporin activity, incubating roots with mercury and low O_2 caused membranes to become leaky when punctured with the cell pressure probe; therefore, Lp_c with $HgCl_2$ during hypoxia was not measured.

Discussion

Short-term hypoxia rapidly affected transport properties in the roots of wheat and both lupin species. Root pressure changed within minutes of bathing roots with hypoxic solution, particularly lupin roots. This is a rapid response in P_r and demonstrates how sensitive roots are to their changing environment and especially how sensitive solute transport is to oxygen deficiency. The reported effects of O_2 deficiency or waterlogging on water transport in roots are diverse, ranging from zero effect to more than 70% inhibition (reviewed in Bramley *et al*. 2007*a*). The source of this variability relates not only to the different species, but also to the nature of the treatment imposed. There have been few direct comparisons between species treated with the same external low O_2. In this study, hypoxia affected different components of water transport in the three species, which is related to their contrasting hydraulic properties (Bramley *et al*. 2009) and may relate to their tolerance to waterlogging. In addition, measurements during re-aeration identified an important regulation of water transport in wheat roots, which may be beneficial for recovery in growth and the transport of water and nutrients to the shoot when flooded soils have drained. The results of this short-term study are consistent with earlier speculations (Dracup *et al*. 1992) based on anecdotal field observations that wheat roots are more tolerant of waterlogging than the roots of lupins. Lupin roots are more sensitive to low O_2 than wheat roots, especially narrow-leafed lupin roots that become leaky.

Solute transport

The accumulation of solutes in the xylem creates an osmotic gradient that draws water into the xylem generating P_r (Steudle 1993). Solutes are able to accumulate in the apoplast of the stele because Casparian bands, located in the endodermis of all three species (Bramley *et al*. 2009), prevent backflow out of the stele (Steudle *et al*. 1993). When aerated, P_r of wheat was similar to a range of other crop species (Steudle and Jeschke 1983; Steudle and Brinckmann 1989; Azaizeh and Steudle 1991; Miyamoto *et al*. 2001; Lee *et al*. 2004). In comparison, lupins only generated low P_r when connected to the root pressure probe, similar to woody species and *Lotus japonicus* (Regel) K. Larsen (Hallgren *et al*. 1994; Steudle and Meshcheryakov 1996; Henzler *et al*. 1999). The difference in P_r between wheat and lupins may be related to greater active transport of ions or organic solutes in wheat roots or a lower effective reflection coefficient in lupin roots. Whilst increases in concentration of certain solutes in the

Table 2. Water relation parameters of root cortical cells bathed with aerated (0.25 mol O_2 m^{-3}) or hypoxic (0.05 mol O_2 m^{-3}) solution
Measurements of cell turgor pressure (P_c), half-time of the rate of water exchange ($T_{1/2}$) and elastic modulus (ε) were made with the cell pressure probe and used to calculate cell hydraulic conductivity (Lp_c). Values are means ± s.e.m. and values in brackets = n. Significant difference due to aeration treatment for each species is indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$)

	$[O_2]$ (mol O_2 m^{-3})	P_c (MPa)	$T_{1/2}$ (s)	ε (MPa)	Lp_c (m s^{-1} MPa^{-1} × 10^{-6})
Wheat	0.25	0.55 ± 0.02 (18)	0.8 ± 0.1 (10)	7.5 ± 0.5 (10)	1.3 ± 0.2 (10)
	0.05	0.60 ± 0.01 (12)	1.6 ± 0.3* (10)	7.7 ± 0.6 (10)	0.7 ± 0.1** (10)
Yellow lupin	0.25	0.39 ± 0.01 (12)	0.7 ± 0.1 (12)	7.9 ± 1.0 (12)	1.6 ± 0.2 (12)
	0.05	0.27 ± 0.01*** (22)	1.9 ± 0.4*** (12)	9.6 ± 1.2 (12)	0.6 ± 0.1*** (12)
Narrow-leafed lupin	0.25	0.41 ± 0.01 (17)	0.6 ± 0.1 (12)	11.0 ± 1.2 (12)	1.4 ± 0.2 (12)
	0.05	0.39 ± 0.04 (7)	4.3 ± 0.7*** (5)	5.0 ± 0.4*** (5)	0.4 ± 0.1*** (5)

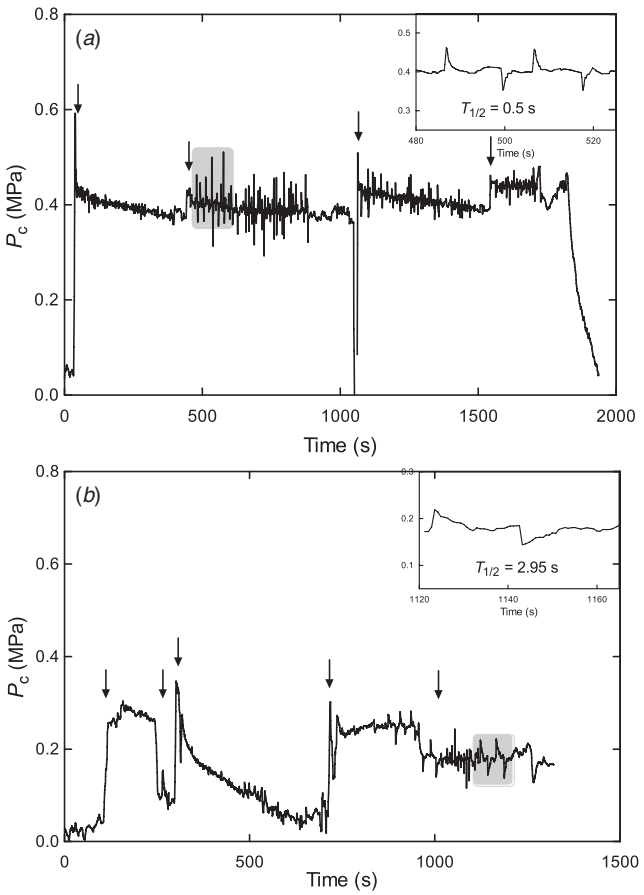

Fig. 6. Examples of turgor pressure (P_c) traces recorded with the cell pressure probe, for cells in narrow-leafed lupin roots bathed with aerated solution (0.25 mol O_2 m^{-3}; *a*) or hypoxic solution (0.05 mol O_2 m^{-3}; *b*). Arrows indicate where new cells were stabbed by the microcapillary of the probe. The highlighted regions are expanded in the figure insets to show the differences in $T_{1/2}$ of pressure relaxations due to aeration treatment.

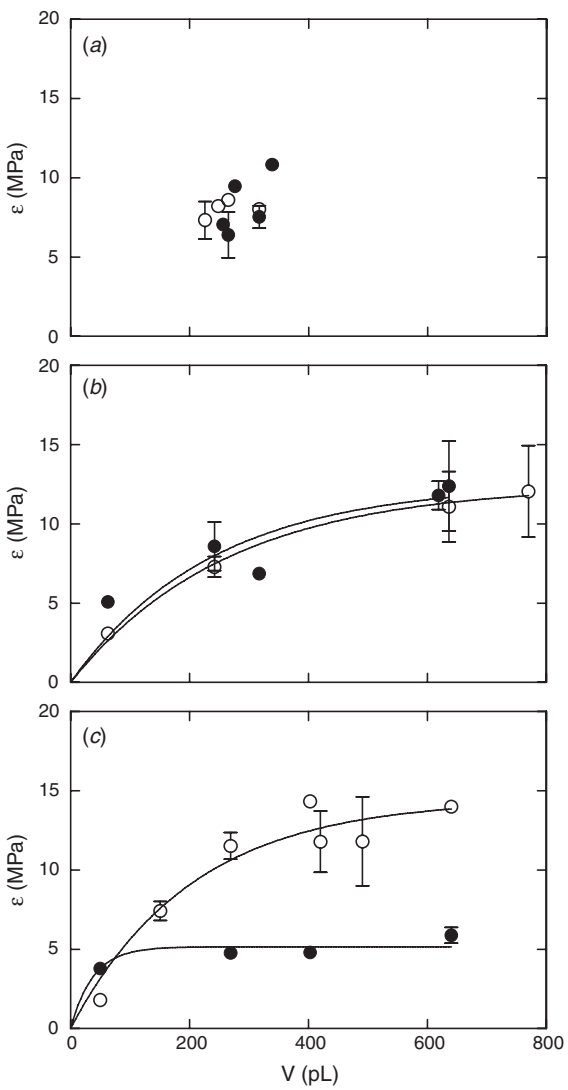

Fig. 7. Relationship between volumetric elastic modulus (ε) and cell volume (*V*) of cortical cells in roots of (*a*) wheat, (*b*) yellow lupin and (*c*) narrow-leafed lupin bathed with aerated solution (open circles) or hypoxic solution (closed circles). Regression analysis was not relevant for wheat because of the small range of cell sizes. Error bars represent ± s.e.m.; $n = 5–12$.

xylem during flooding have been found earlier (Jackson *et al*. 1996), changes in P_r in response to oxygen deficiency have only previously been reported for *Zea mays* (L.) (Birner and Steudle 1993; Gibbs *et al*. 1998*a*). However, the response depended on the oxygen concentration because P_r of hypoxic *Z. mays* roots gradually recovered when re-aerated (Birner and Steudle 1993), but not roots treated for 5 h with anoxia (Gibbs *et al*. 1998*a*).

The effect of hypoxia on P_r was related to root diameter with the greatest response in narrow-leafed lupin. Root diameter is an important factor in comparisons of waterlogging tolerance as greater diffusive distances from the external medium to the root centre could lead to greater internal oxygen deficiency. Therefore, the oxygen deficiency in the stele of narrow-leafed lupin could be twice as severe as wheat, assuming similar rates of respiration. Using microelectrodes, radial profiles of the O_2 partial pressure across roots have shown that the stele may be close to anoxic even with appreciable amounts of O_2 in the cortex (Armstrong and Beckett 1987; Armstrong *et al*. 1994).

The response in P_r is likely to be due to leakage of ions and/or carbohydrates rather than an effect on energy-dependent processes because P_r began to change immediately upon O_2 decreasing in the bathing medium of yellow lupin and within a few minutes of the lowered O_2 for narrow-leafed lupin. Whilst the effects of hypoxia on energy-dependent solute transport to hypoxia are not slow (Trought and Drew 1980; Buwalda *et al.* 1988; Thomson *et al.* 1989), the response has not been shown to occur so rapidly. In comparison, patch clamping techniques have shown that reversible leakage of ions due to membrane depolarisation/hyperpolarisation in response to changes in aeration can occur within seconds, as has been observed for cortical cells of wheat roots (Zhang and Tyerman 1997). The process in yellow lupin roots appears to be reversible as P_r returned to original levels during re-aeration. However, the subsequent loss of P_r in narrow-leafed lupin indicates that either the endodermis became leaky or solutes leaked from the

root tip. Root tips are usually the most sensitive region of roots to O_2 deficiency (Drew 1997) and when waterlogged, narrow-leafed lupin roots die first at the tip (Bramley 2006).

Changes in turgor pressure provide evidence that solute leakage occurred from cortical cells of lupin roots, but not wheat, in response to hypoxia. Turgor pressure decreased indicating a loss of osmotica. Leakage from epidermal cells may also occur, but cells were physically too small to measure with the pressure probe. Unlike the effect on P_r, which was related to root diameter, cortical cells measured with the cell pressure probe were within the same cell layers and depth from the root surface for all species so the distance for O_2 diffusion would have been the same. However, the instability of narrow-leafed lupin cortical cells suggests that this species is particularly sensitive to hypoxia.

Cell and root hydraulics

The influence of O_2 deficiency on Lp_c has only been previously measured in wheat and *Z. mays* (Zhang and Tyerman 1991; Tyerman *et al*. 1992; Zhang and Tyerman 1999). In those studies, the impact on wheat (60–85%) may have been slightly greater than the present study (46%) because cells were closer to the root tip (10–20 mm) and exposed to a slightly lower O_2 concentration (0.04 mol O_2 m^{-3}). Hypoxia dramatically reduced Lp_c in all species, with the effect being greater in lupins than wheat. It is unlikely that the greater reduction of Lp_c in lupins was due to greater internal oxygen deficiency due to thicker roots because Lp_c was measured within the same range of depth from the root surface in all species. In addition, recent evidence by Armstrong *et al*. (2009) indicates that respiration rates may not decline in the cortex with increasing depth into the root even when oxygen concentrations decline. Instead, the hypoxia results are analogous to those of mercury inhibition of aerated roots (Bramley *et al*. 2009), indicating greater inhibition of aquaporin activity in lupins than wheat. Closure of aquaporins in membranes decreases the hydraulic conductivity of membranes and hence increases the half-time of the rate of water exchange ($T_{1/2}$) across the cell. Cytoplasmic pH, Ca^{2+} and respiration, processes that are affected by hypoxia, can regulate cell water permeability through aquaporin gating and expression (Gerbeau *et al*. 2002; Tournaire-Roux *et al*. 2003; Alleva *et al*. 2006; Verdoucq *et al*. 2008). However, although 0.05 mol O_2 m^{-3} can induce changes in aquaporin expression within 0.5 h of treatment (Klok *et al*. 2002), those aquaporins that are highly expressed in roots and facilitate water transport across membranes (e.g. PIP2s, tables 2 and 5 of Bramley *et al*. 2007*a*) tend to be downregulated after a few hours of hypoxia. Given the short-term exposure of wheat and lupin roots to hypoxia, it seems more probable that Lp_c was inhibited through a closure of aquaporins, rather than a reduction in their expression. What mechanism closed aquaporins is not known, but 0.5 h of zero O_2 reduced cytoplasmic pH of *Arabidopsis thaliana* (L.) roots to 7.1 and *Z. mays* root tips to 6.9 (Xia and Roberts 1996; Tournaire-Roux *et al.* 2003). These values of pH are within the range of pH that can decrease the osmotic water permeability of plasma membranes by half due to aquaporin closure (Alleva *et al*. 2006; Verdoucq *et al*. 2008).

Despite the large reductions in Lp_c in lupin roots due to hypoxia, the insignificant reduction in Lp_r indicates that water flow across the cortex occurs predominantly through the apoplastic space. If flow occurred by the cell-to-cell pathway in the cortex, then Lp_r would be proportionally reduced. This is consistent with Bramley *et al*. (2009) where Lp_r of narrow-leafed lupin and yellow lupin was not affected by mercury treatment despite dramatic reductions in Lp_c. This is also consistent with a smaller inhibition in Lp_r compared with Lp_c for *Z. mays* (Tyerman

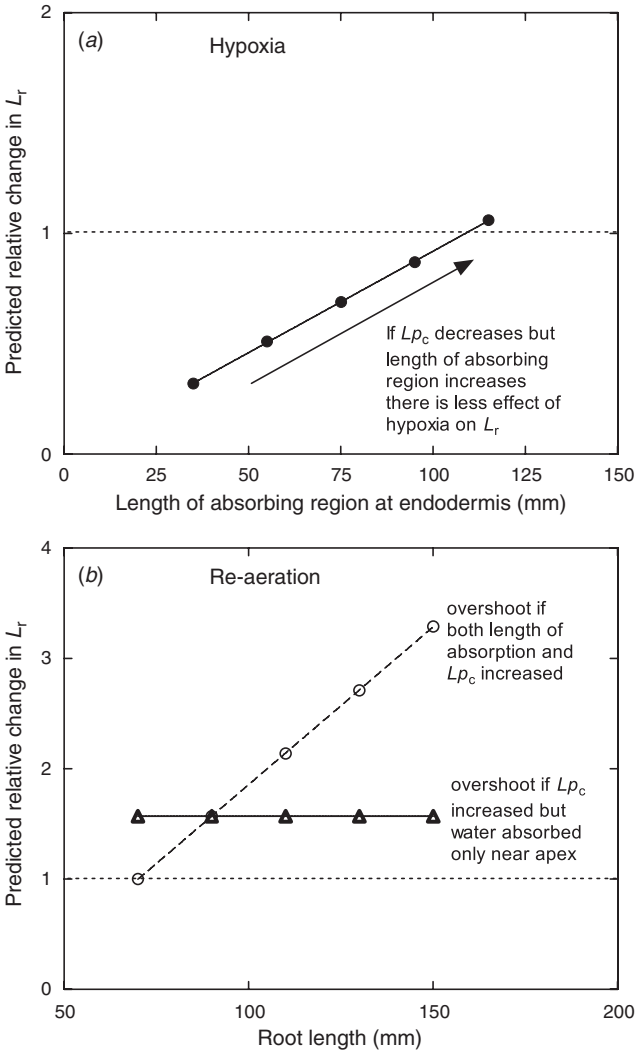

Fig. 8. Predicted relative change (normalised to the value before treatment) in hydraulic conductance (L_r) of wheat roots calculated using Lp_c and a model describing the root as a series of concentric cylinders (Bramley *et al*. 2009). The results show the effect on L_r when Lp_c and length of absorption at the endodermis changes. During hypoxia (*a*), if a root extends the length of effective water transport across the endodermis, L_r can be maintained even if Lp_c is low. This would account for the observation that roots that were longer showed less effect of hypoxia on L_r (Fig. 4*a*). During re-aeration (*b*), L_r would increase with measured length of root (open circles) if Lp_c returned to the original values and the length of the absorbing region had increased. Alternatively, L_r is constant with measured root length (open triangles) if only a small region of the root near the apex absorbs water. For there to be an overshoot in L_r similar to the measured values in Fig. 4*a*, Lp_c in the endodermis either increased by the same proportion as L_r (1.6-fold) or a larger region of the root contributed to flow than that before hypoxia treatment.

et al. 1992), another species believed to have predominantly apoplastic flow (Steudle and Frensch 1989), although Gibbs *et al*. (1998*b*) argue that the symplast is important. Measurements by Armstrong *et al*. (2009) indicate that the critical oxygen pressure (COP) at the root surface is a function of respiratory decline in the stele and that roots with larger stelar diameters should tend to have higher COPs, confirming original predictions by Berry and Norris (1949). Narrow-leafed lupin may therefore experience greater respiratory decline in the stele in comparison to the other species, when exposed to the same external oxygen concentration. However, this does not change our conclusion that bulk water flow across lupin roots bypasses cells without crossing membranes.

Using a model of a root comprising of concentric cylinders in conjunction with measurements of Lp_c of each cortical cell layer and L_r, Bramley *et al*. (2009) identified which regions of the root contribute to L_r. In aerated wheat roots, L_r is constant with root length because water uptake preferentially occurs within 40 mm of the root tip and is probably controlled by the endodermis (Bramley *et al*. 2009). Changes in L_r due to aeration treatment can be predicted by altering Lp_c, the length of the absorbing region and the contribution of cortical cell layers. Applying the model here shows that increasing the length of the endodermis that transports water via aquaporins reduces the inhibition by hypoxia on root hydraulic conductance (Fig. 8). Despite hypoxia inhibiting Lp_c, increasing the length of the absorbing region creates more parallel pathways for flow, allowing longer roots to maintain their hydraulic conductance.

During re-aeration, water transport recovered in wheat roots, but with an overshoot. Overshoots in Lp_r have also been observed during recovery from water deficit in *Agave deserti* Engelm., nutrient deprivation in wheat, hypoxia in *Z. mays*, and low temperature in *Cucurbita ficifolia* Bouché (Carvajal *et al*. 1996; Gibbs *et al*. 1998*b*; North *et al*. 2004; Lee *et al*. 2005), but the reason for this phenomenon has not been investigated. In this study, a greater reduction in Lp_r by mercury after re-aeration, than before hypoxia, indicated that the overshoot in Lp_r of wheat roots was due to greater aquaporin activity. Therefore, more aquaporins were gated open or more active aquaporins were embedded in plasma-membranes during re-aeration. Furthermore, Lp_r was greater in wheat roots during re-aeration, irrespective of root length. If longer roots were absorbing water over a longer length than shorter roots, then L_r would increase with length (Fig. 8). Either Lp_c returned to its original values but the length of the absorbing region was slightly longer (55 mm predicted by the model) than before hypoxic treatment, or additional pathways were opened (Fig. 8). If Lp_c increased 1.6-fold during re-aeration, but only in the endodermis (35 mm, not including 5 mm apex), then L_r would be 60% greater than the value before hypoxia (Fig. 8). It would be difficult to measure such an increase in Lp_c without automating the cell pressure probe, as half-times can be <1 s under ambient conditions. However, with care and a fine resolution micro-manipulator (Bramley *et al*. 2009), it may be possible to detect changes in Lp_c in different regions of the endodermis.

The physiological significance for the effect of re-aeration could be related to the recovery of root growth when waterlogging has subsided. The rate of extension of previously waterlogged wheat roots during the recovery period was greater than control roots (Bramley 2006). An increased rate of water uptake into the cells in the zones of elongation, mediated by aquaporins, may increase the rate of root extension. In addition, an increase in the rate of water uptake after waterlogging would be needed to restore the water balance and transport of nutrients to the shoot. In comparison to wheat, lupin roots have inferior survival during waterlogging and recovery is dependent on the growth of new roots originating near the base of the stem (Bramley 2006). The hydraulics of lupin roots depends on changes in anatomy and morphology, but appears to have little ability to regulate water flow in the short term, under ambient or hypoxic conditions.

Our study focussed on the rapid responses to hypoxia using excised roots to examine their hydraulic properties. Water transport through roots is only part of the multifaceted soil–plant–atmosphere continuum that has many regulatory controls. Further research is required to link root hydraulic properties to those of shoots and functioning of whole plants. For example, although root systems of wheat and lupins are generally more sensitive to waterlogging than shoots (Trought and Drew 1980; Davies *et al*. 2000*b*), sensitivity is not solely dependent on the root system. In longer-term studies, waterlogging decreased stomatal conductance and transpiration in narrow-leafed lupin and yellow lupin, but despite closure of stomata shoot water potential of narrow-leafed lupin also declined (Davies *et al*. 2000*c*). Waterlogging also reduced leaf gas exchange in wheat plants, with a greater inhibition in a more sensitive genotype that also experienced decreased shoot water potential (Huang *et al*. 1994). Reductions in leaf gas exchange are believed to be caused by insufficient supply of water from the roots (Cannell and Jackson 1981), although correlations between the two processes are not clear.

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