CSIRO PUBLISHING

Functional Plant Biology, 2015, **42**, 9–17
http://dx.doi.org/10.1071/FP14078

Radial oxygen loss and physical barriers in relation to root tissue age in species with different types of aerenchyma

Milena E. Manzur[A,B], *Agustín A. Grimoldi*[A], *Pedro Insausti*[A] *and Gustavo G. Striker*[A]

[A]IFEVA, CONICET/Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, C1417DSE Buenos Aires, Argentina.
[B]Corresponding author. Email: mmanzur@ifeva.edu.ar

Abstract. Plant root aeration relies on aerenchyma and barrier formation in outer cortex influencing the radial oxygen loss (ROL) from roots towards the rhizosphere. Plant species display large variation in strategies for both responses. We investigated the impacts of root-zone hypoxia on aerenchyma formation and development of ROL apoplastic barriers in the outer cortex as a function of root tissue age using three lowland grassland species, each with alternative aerenchyma structure. All species increased root aerenchyma and continued with root elongation after imposing hypoxia. However, ROL barrier development differed: (i) *Rumex crispus* L. displayed only 'partial' barrier to ROL evidenced at older tissue ages, (ii) *Cyperus eragrostis* Lam. initiated a 'tighter' barrier to ROL following exposure to hypoxia in tissues older than 3 days, and (iii) *Paspalidium geminatum* (Forssk.) Stapf demonstrated highly effective inhibition of ROL under aerated and hypoxic conditions at all tissue ages related to constitutive 'tight' apoplastic barriers in outer cortex. Thus, hypoxic conditions affected root elongation and 'tightness' of apoplastic barriers depending on species. The physiological implications of the different ROL responses among species in relation to the differential formation of barriers are discussed.

Additional keywords: apoplastic barriers, *Cyperus eragrostis*, root-zone hypoxia, *Rumex crispus*, *Paspalidium geminatum*, tissue age.

Received 9 March 2014, accepted 2 July 2014, published online 28 August 2014

Introduction

Floods can be extremely damaging to plants, and can affect crops and natural ecosystems (Bailey-Serres and Voesenek 2008). The main effect of flooding is O_2 deprivation at root level as a result of a drastic reduction of the inward flux of atmospheric O_2 into the soil (Armstrong 1979). Such lack of O_2 can limit root growth; thereby, it constrains soil exploration (Armstrong 1979). However, flood tolerant species can adjust their morphology, anatomy and physiology at root level for maintaining root aeration (Armstrong 1979; Colmer and Voesenek 2009).

Plant responses to flooding depend on the intensity and duration of water excess (Colmer and Voesenek 2009; Striker *et al*. 2012). The main traits contributing to flooding tolerance at the morphological level are the formation of adventitious roots and at the anatomical level, generation and/or increase of root aerenchyma (Justin and Armstrong 1987; Seago *et al*. 2005; Colmer and Voesenek 2009). Aerenchyma forms a low resistance pathway that facilitates gaseous exchange between the atmosphere and the submerged organs, and it is an essential feature for plant growth in O_2 deficient soils (Armstrong 1979; Colmer and Voesenek 2009; Abiko *et al*. 2012). Flood-tolerant species have different types of aerenchyma resembling different shapes, which were classified according to the spatial arrangement of gas-filled lacunae in the cortex (related to the packing of the cells) and some characteristics of the exodermal cell layers like cell wall thickening (Justin and Armstrong 1987; Visser *et al*. 2000; Seago *et al*. 2005). Here, we studied the aerenchyma formation of three species with alternative aerenchyma structure: *Rumex crispus* L. ('honeycomb aerenchyma', named rumex type), *Cyperus eragrostis* Lam. ('spiderweb aerenchyma', named cyperaceous type) and *Paspalidium geminatum* (Forssk.) Stapf ('bicycle wheel aerenchyma', named graminaceous type).

A variable amount of O_2 transported through aerenchyma is consumed (i.e. via root respiration) or is lost along the root in its way to the apex (Armstrong 1971). In contrast, the oxygen can also be lost in a radial direction towards the rhizosphere, which is referred to as radial oxygen loss (ROL). In this respect, flood tolerant species can diminish ROL by having a constitutive apoplastic physical barrier against oxygen loss in the exodermis or by inducing a physical barrier under hypoxic conditions (Colmer *et al*. 1998; Visser *et al*. 2000; Abiko *et al*. 2012). The selected species have different characteristics in the exodermal layers – thin cell walls in outer cortex for *R. crispus*, and differential degree of thicker cell walls with presence of sclerenchyma in *C. eragrostis* and *P. geminatum* (Striker *et al*. 2007) – so that they could show weaker, intermediate or stronger barriers against ROL. We addressed this issue by comparing these species regarding the presence/absence of apoplastic barriers in the outer cortex and their responses in ROL.

It is known that suberin and lignin deposition (as components of apoplastic barriers) are related to root tissue age (Rittinger *et al*. 1987; Enstone and Peterson 2005), and that hypoxic conditions can modify root elongation rate (Armstrong 1971). Therefore, if roots growing under hypoxia are compared with roots growing in aerated soils, tissue age and the related appearance of apoplastic barriers could be different for a given distance behind the root apex (Enstone and Peterson 2005). To date, the approach for evaluating oxygen loss from roots has followed a 'classical' spatial pattern in which ROL is always presented at different fixed distances from the root tip (e.g. Visser *et al*. 2000; Abiko *et al*. 2012); with one recent exception (see Kotula *et al*. 2014). Although there is no doubt about the physiological significance of this approach, when ROL and root elongation are both reduced as a result of O_2 deficiency, confusing effects render it difficult to conclude if the reduction of O_2 loss is a direct effect of the treatment, or if it is caused by high suberin and/or lignin deposition typical of older tissues. In this paper, we analyse the effects of ROL as a function of root tissue age in order to disentangle the direct effects of the hypoxic treatment provoked by anaerobiosis from those associated to the age of the evaluated tissue by examining the presence of apoplastic barriers in the outer cortex. This evaluation was performed by comparing three flood tolerant species each with a different type of aerenchyma, which coexist in grasslands prone to flooding.

Materials and methods

Plant material and growth conditions

Three flood tolerant terrestrial species – *Rumex crispus* L., *Cyperus eragrostis* Lam. and *Paspalidium geminatum* (Forssk.) Stapf – were selected for possessing different types of root aerenchyma (Grimoldi *et al*. 2005; Striker *et al*. 2007). Dicotyledonous *R. crispus* exhibits aerenchyma all over the cortex with a honeycomb-like appearance and uniseriate epidermis; *C. eragrostis* and *P. geminatum* display aerenchyma lacunae in tangential and radial arrangement, resembling a spider web and a bicycle wheel respectively (Justin and Armstrong 1987; Seago *et al*. 2005). The latter two species also present a multiseriate ring of densely packed cells in the outer cortex and uniseriate epidermis (Striker *et al*. 2007). Thirty individual plants of each species were collected from lowland areas of a grassland prone to soil flooding, located in the Department of Pila, Province of Buenos Aires, Argentina (36°30′S, 58°30′W). Each plant was transplanted into plastic pots (4 L), filled with sand and topsoil (1 : 1 v/v) from the grassland (3% organic carbon), and transferred to a glasshouse at the School of Agriculture, University of Buenos Aires for an acclimatisation period of three months. Glasshouse temperature was maintained at 22 ± 6°C and photosynthetic photon flux density (PPFD) was 1400 ± 40 µmol m^{-2} s^{-1}.

Experimental setup and treatments

Similar-sized plants were selected, roots were washed free of soil, and transferred to hydroponic culture (floating support in plastic containers of 30 L), containing 1/4 Hoagland III nutrient solution, enriched with 0.23 µM H_3BO_3, 0.71 µM $MnCl_2$, 5 nM $ZnSO_4$, 0.6 nM $(NH_4)_6$ Mo_7O_{24} and 1.6 nM $CuSO_4$. The nutrient solution in tanks was continuously bubbled with air using aquarium air pumps. After 1 week in hydroponics conditions, the plants did not show stress symptoms due to the changes in growing media. Then, two treatments were imposed on acclimated plants for 8 days: (i) aerated – nutrient solution supplied with constant air bubbling (oxygen concentration: 0.247 moles O_2 m^{-3}); or (ii) hypoxic – nutrient solution first bubbled with N_2 for 10 min to obtain hypoxic conditions for 12 h, to avoid anoxic shock, and then replaced with de-oxygenated agar nutrient solution (0.05% w/v dissolved agar added to the nutrient solution; final oxygen concentration: 0.016 moles O_2 m^{-3}). Agar addition prevented convective movements in the nutrient solution, helping to simulate the decrease of O_2 and the accumulation of plant-generated ethylene (Wiengweera *et al*. 1997) as it occurs in waterlogged soils. Oxygen concentration and temperature (20 ± 6°C) of the hydroponics growth chambers were monitored daily using a dissolved oxygen meter (Lutron, DO-5510, Taipei, Taiwan). All adventitious roots used for measurements were formed during under hydroponic conditions.

Root elongation rate

New adventitious roots of both treatments for each species were marked (2–4 roots per plant) in a non-destructive way, separating target roots from the rest with a thin thread. We measured roots of 5–8 plants per treatment. Target adventitious roots were photographed daily during 8 days, and afterwards the increase in root length was measured on digital images by using Image Tool 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA). The results were plotted as root length (millimetres), as a function of time (days), and then their relationship were fitted through linear regressions. The slope of the relationship between root length and time was used to estimate root elongation rate for each combination of treatment and species. Root elongation rate was used for determining the root tissue age at each position where aerenchyma, and ROL were quantified and apoplastic barriers were identified (see below). This approach allowed us not only to compare the aerenchyma, ROL and physical barriers generation at the measured positions along the root ('classical' approach) but also, for the first time, to separate the effects of root tissue age on such parameters. All results were analysed taking into account both age and position along the root, but special focus was given to root tissue age, as described below.

Radial oxygen loss (ROL) measurements

ROL measurements were performed on adventitious roots developed under each one of the treatments (1–3 roots per plant, and 5–8 plants per treatment). To do this, intact plants were transferred to plastic boxes with hypoxic nutrient solution (i.e. the same solution described above) plus bubbling with N_2 for 30 min to purge out all O_2. Shoots were in contact with atmospheric air (except shoots bases that were in contact with hypoxic solution) and one of the adventitious roots was carefully positioned horizontally through guides into the box, while the rest of the root system was isolated inside the box with septum (as in work by Armstrong *et al*. 2000). Oxygen concentrations at the root surface and at 100 µm of distance (in vertical position)

in the solution were measured by using Clark-type O_2 microelectrodes of 25 µm tip diameter (OX25, Unisense, Aarhus, Denmark). Measurements were taken at 0.5, 1, 2, 3, 4 and 5 cm behind the root apex. After preliminary tests, consistent and stable values were obtained when all positions along the root were measured within timeframes of 20–25 min, after a root acclimation period of 30 min in the anoxic medium. Accurate positioning (and the moving) of microelectrodes was achieved by using a micromanipulator (MM33, Unisense), and viewing it through binocular lens. The microelectrode was connected to a high-sensitivity picoammeter (PA2000, Unisense) with outputs logged on a computer using an A/D converter (ADC 216, Unisense). ROL from the root towards the rhizosphere was calculated according to Henriksen *et al*. (1992), using oxygen concentration measurements at two radial positions:

$$\mathrm{ROL} = 2\pi D = \frac{c2 - c1}{\ln\left(\frac{r2}{r1}\right)}, \qquad (1)$$

where D is the diffusion coefficient of oxygen in the rooting medium (in water at 23°C: 2.27×10^{-9} m^2 s^{-1}, although this coefficient is expected to be lower in agar medium), $c1$ is the oxygen concentration on the root surface (mol m^{-3}) calculated from the partial pressure, $c2$ is the oxygen concentration at 100 µm distance from the root surface, $r2$ is the radial distance from the given distance (100 µm) to the centre of the root (m) and $r1$ is the root radius (m). Root diameters were measured on the same roots from digital images, using Image Tool 3.0 software. All measurements were at 23°C. PPFD received by shoots during measurements was ~350 µmol m^{-2} s^{-1}.

Root aerenchyma determination

Aerenchyma formation was studied on the same roots as ROL measurements in order to describe and determine relationships among variables. For this, roots were stored in 70% alcohol for further histological analyses. Root pieces ~0.5 cm long were obtained from each position (see '*ROL measurements*' above), embedded in paraffin, and then cut into 15–20 µm sections using a rotating microtome with a steel blade. Then, root cross-sections were transferred to glass slides and digital photographs were taken with a camera (Nikon E8700, Tokyo, Japan) connected to an optical microscope (Zeiss Axioplan, Zeiss, Oberkochen, Germany). Aerenchyma percentage was obtained as the proportion of area occupied by aerenchyma lacunae over the total area of root cross-section (as in work by Striker *et al*. 2007) by using ImageTool 3.0 software.

Detection of apoplastic barriers

Apoplastic barriers identification was performed on the same cross-sections examined for aerenchyma. Cross-sections of roots were analysed by UV light (excitation at 395 nm, Zeiss UV filter set, 50–200 ms of exposition time) to reveal by autofluorescence the presence of lignin, aromatic suberin and other phenolics (Brundrett *et al*. 1991; Schreiber and Franke 2011) as components of the apoplastic barriers to radial oxygen loss (Kotula *et al*. 2009*a*, 2009*b*). Digital images of all cross-sections and ages (resolution: 600 dpi) were recorded with a digital camera (Nikon E8700).

Statistical analyses

A linear regression was set for root length as function of days of experiment for each species and treatment. Slope tests were done with a confidence level of 95%. Adjusted r^2, P-values and F-tests are stated within the figures. For each species, root aerenchyma (%) and radial oxygen loss (ROL) were analysed using two-sample t-test to examine the effect of 'treatment' between points in a same range of tissue age (i.e. average values overlapping ± 10% of tissue age between treatments). The same dataset was also evaluated through paired t-test to examine the effects of 'tissue age' within the same treatment. Comparisons of means in all cases were done with a confidence level of 95%. In addition, classical two-way ANOVAs were performed on aerenchyma and ROL dataset to evaluate 'treatment' and 'position' effect for each species. Means were subsequently compared with Tukey's tests ($P < 0.05$). Normality and homogeneity of variances were previously verified in order to satisfy ANOVA assumptions. The variables involving proportions were arcsine $\sqrt{x}$ transformed before analyses. Statistical analyses were performed using InfoStat 2010 package for Windows (University of Córdoba, Córdoba, Argentina). All results are presented as non-transformed means ± s.e.

Results

Root elongation rate

All species continued adventitious root elongation after low O_2 hypoxic treatment was imposed (Fig. 1*a–c*), in accordance to their reputation for being flood tolerant. However, root elongation rate differed between treatments depending on the species. This parameter was reduced by 61% as a result of hypoxic treatment in *R. crispus* (slope test: F-value$_{1,59}$: 43.93; $P < 0.0001$; Fig. 1*a*). In contrast, for *C. eragrostis* and *P. geminatum*, root elongation rate was not affected by root-zone oxygen deficiency as indicated by the similar slopes of the fitted equations for each treatment (slope tests: $P > 0.9$ and $P > 0.1$ respectively; Fig. 1*b*, *c*). These root elongation rates for each combination of species and treatment were used to estimate the root tissue age at each position along roots where ROL, aerenchyma and barrier formation were measured and analysed.

ROL profiles as a function of root tissue age

Different responses in ROL across tissue age were exhibited by the studied species, as a result of their differential responses of aerenchyma generation and apoplastic barrier formation in the outer cortex. (i) *R. crispus*: relatively high ROL under both treatments at all root tissue ages, (ii) *C. eragrostis*: high ROL in very young root tissues, and low ROL in older root tissues, but ROL further decreased under hypoxic conditions, and (iii) *P. geminatum*: very low ROL under both treatments at all root tissue ages. In particular, *R. crispus* presented higher ROL values when root tissues were young (and near root apex, Fig. 2*d–f*), then ROL slightly decreased as root tissue became older (and distant from apex, Fig. 2*d–f*) without significant differences between treatments ($P > 0.05$). This pattern suggests the presence of a 'partial barrier' to ROL in this species (Fig. 2*a*). Cyperaceous *C. eragrostis* displayed marked

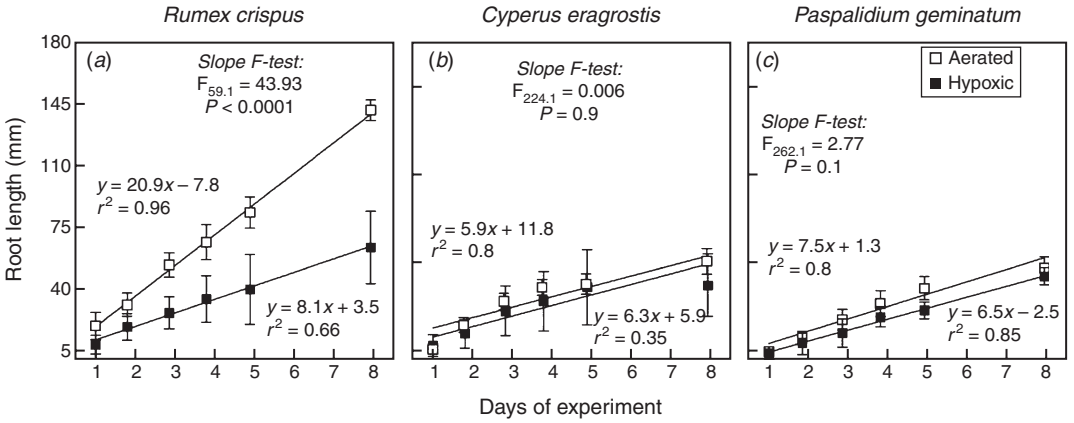

Fig. 1. Adventitious root length (mm) as a function of days of experiment of (*a*) *Rumex crispus*, (*b*) *Cyperus eragrostis*, and (*c*) *Paspalidium geminatum* grown in nutrient solution under aerated (0.247 moles O_2 m^{-3}) and hypoxic conditions (0.016 moles O_2 m^{-3}) for 8 days. Each point represents the mean ± s.e. of 5–8 plants. Adjusted equations through lineal regression for each treatment, *F*-tests and *P*-values between treatments are presented.

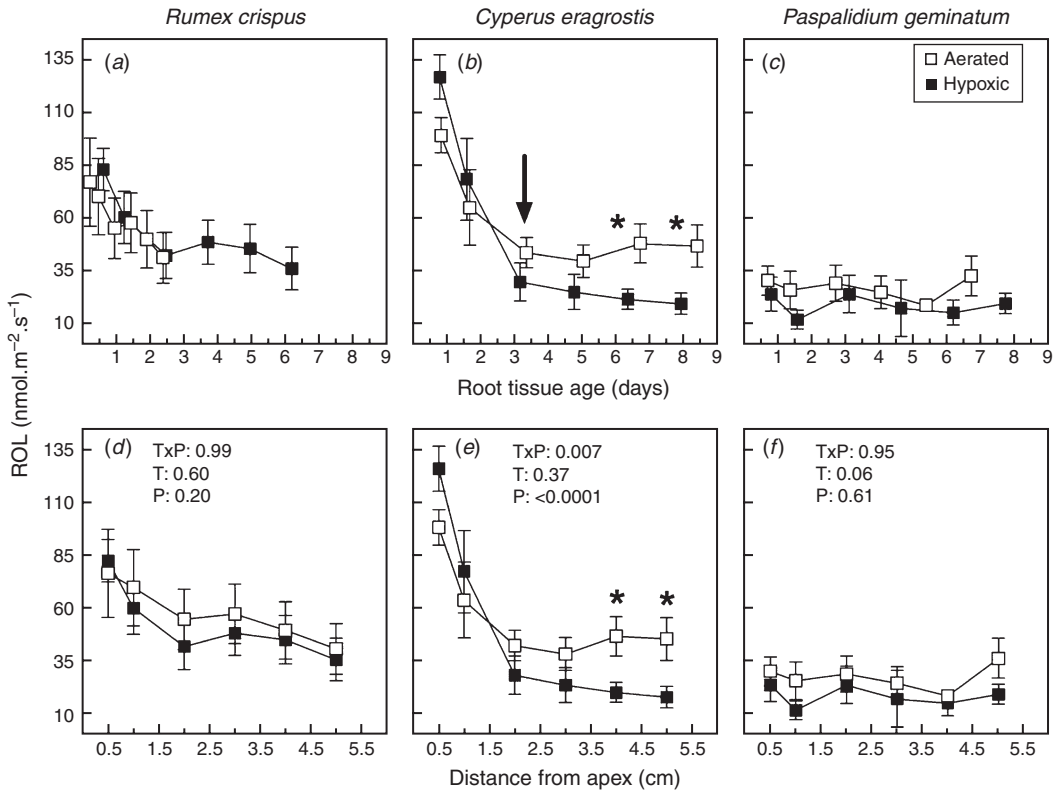

Fig. 2. Radial oxygen loss (ROL) as a function of root tissue age (*a–c*) or distance from apex (*d–f*) of *Rumex crispus* (*a*, *d*), *Cyperus eragrostis* (*b*, *e*) and *Paspalidium geminatum* (*c*, *f*) grown in nutrient solution under aerated (0.247 moles O_2 m^{-3}) and hypoxic conditions (0.016 moles O_2 m^{-3}). Each point represents the mean ± s.e. of 5–8 plants. Arrow in (*b*) indicates, for *C. eragrostis*, root tissue age when ROL starts to be lower for roots growing under root zone O_2 hypoxia than in aerated conditions. Significant differences ($P < 0.05$) between treatments for each root tissue age or position are indicated: *. *P*-values of ANOVAs analyses are shown in the graphs of ROL vs distance from apex (*d–f*) where T is treatment and P is position. For further statistical details, see 'Materials and methods'.

differences in ROL profile according to root tissue age: very high ROL towards the hypoxic external environment when tissues were young (<2 days old) followed by a dramatic drop of ROL (5-fold lower) in older tissues (>2–3 days old; $P < 0.05$). In this species, ROL values in older tissues were further reduced when roots grew under hypoxic conditions (the process started

at day 3 as indicated by the arrow in Fig. 2*b*, *e*; and the differences started to be significant in tissues older than 5 days old; $P < 0.05$), evidencing the presence of an 'induced barrier' to ROL provoked by root zone O_2 deficiency. This analysis, where ROL is plotted as a function of root tissue age allows us to ascribe the hypoxic treatment as responsible for the induction to the barrier against ROL, as there were no possible confusing ontogenetic effects. Graminaceous *P. geminatum* showed a flat ROL pattern as a function of root tissue age where no differences were evident between treatments (i.e. low ROL values irrespective of root tissue age and treatment; $P > 0.05$; Fig. 2*c*, *f*). This suggests the presence of a constitutive 'tight barrier' to ROL in this species.

Root aerenchyma and apoplastic barriers as a function of root tissue age

As expected for flood tolerant plants, all species contained aerenchyma in their roots even under aerated control conditions (i.e. constitutive aerenchyma; see Fig. 3*a–f*). However, this parameter was variable among species, treatments and age of the root tissues. In this respect, *R. crispus* showed typical expansigenous aerenchyma (Seago *et al*. 2005) in a shape resembling a honeycomb structure (Fig. 4, left panels). This species displayed high percentage of constitutive aerenchyma (>30%; Fig. 3*a*, *d*), even in very young root tissues, which was not increased when root tissues became older ($P = 0.87$). Under hypoxic conditions, aerenchyma lacunae of this species expanded into bigger-sized lacunae at all estimated root tissue ages (Fig. 4 compare *a* with *b*, *g* with *h* and m with *n*), thus, showing a 1.8-fold increase in the amount of aerenchyma with respect to roots of plants that grew in aerated conditions ($P < 0.05$; Fig. 3*a*, *d*). In this species, apoplastic barrier in the outer cortex cells was undetectable in cross-sections exposed to UV light (Fig. 5).

Cyperaceous *C. eragrostis* exhibited a spiderweb-like appearance for the aerenchyma arrangement (Fig. 4, middle panels) with 15% of constitutive aerenchyma for the youngest root tissues (1 day old; Fig. 3*b*). As root tissues became older (from 2 to 4 days old) aerenchyma proportion increased to $34 \pm 5\%$ under aerated conditions, and it was further increased to $51 \pm 4\%$ (Fig. 3*e*; $P < 0.05$) under hypoxic conditions as a product of cell lysis in root cortex, typical of the tangential-lysigenous aerenchyma formation process (in Fig. 4 compare *c* with *d*, i with *j* and *o* with *p*). Apoplastic barrier in outer cortex cells of very young tissues was weak irrespective of the

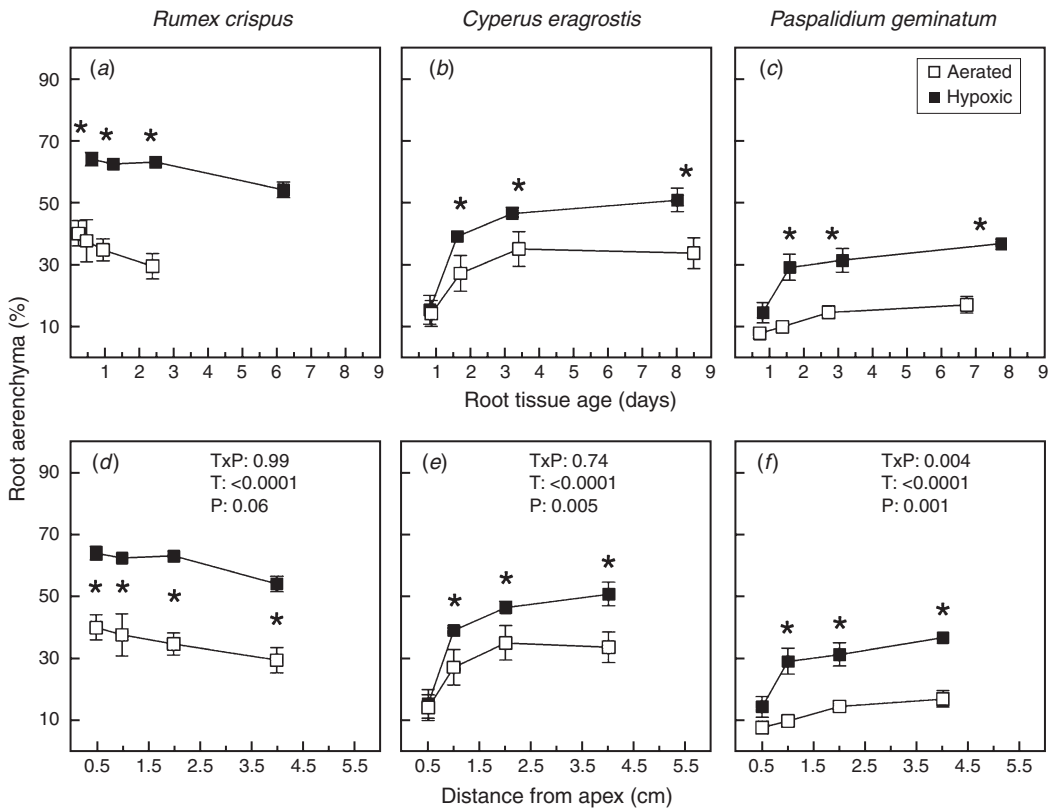

Fig. 3. Root aerenchyma (%) as a function of root tissue age (*a–c*) or distance from apex (*d–f*) of *Rumex crispus* (*a*, *d*), *Cyperus eragrostis* (*b*, *e*) and *Paspalidium geminatum* (*c*, *f*) grown in nutrient solution under aerated (0.247 moles O_2 m^{-3}) and hypoxic conditions (0.016 moles O_2 m^{-3}). Each point represents the mean ± s.e. 5–8 plants. Significant differences ($P < 0.05$) between treatments for each root tissue age or position are indicated: *. Note that root aerenchyma values at oldest tissue age under aerated conditions in (*a*) were not statistically evaluated as there were no comparable values under hypoxic conditions. *P*-values of ANOVAs analyses are shown in graphs of root aerenchyma vs distance from apex (*d–f*) where T is treatment and P is position. For further statistical details, see 'Materials and methods'.

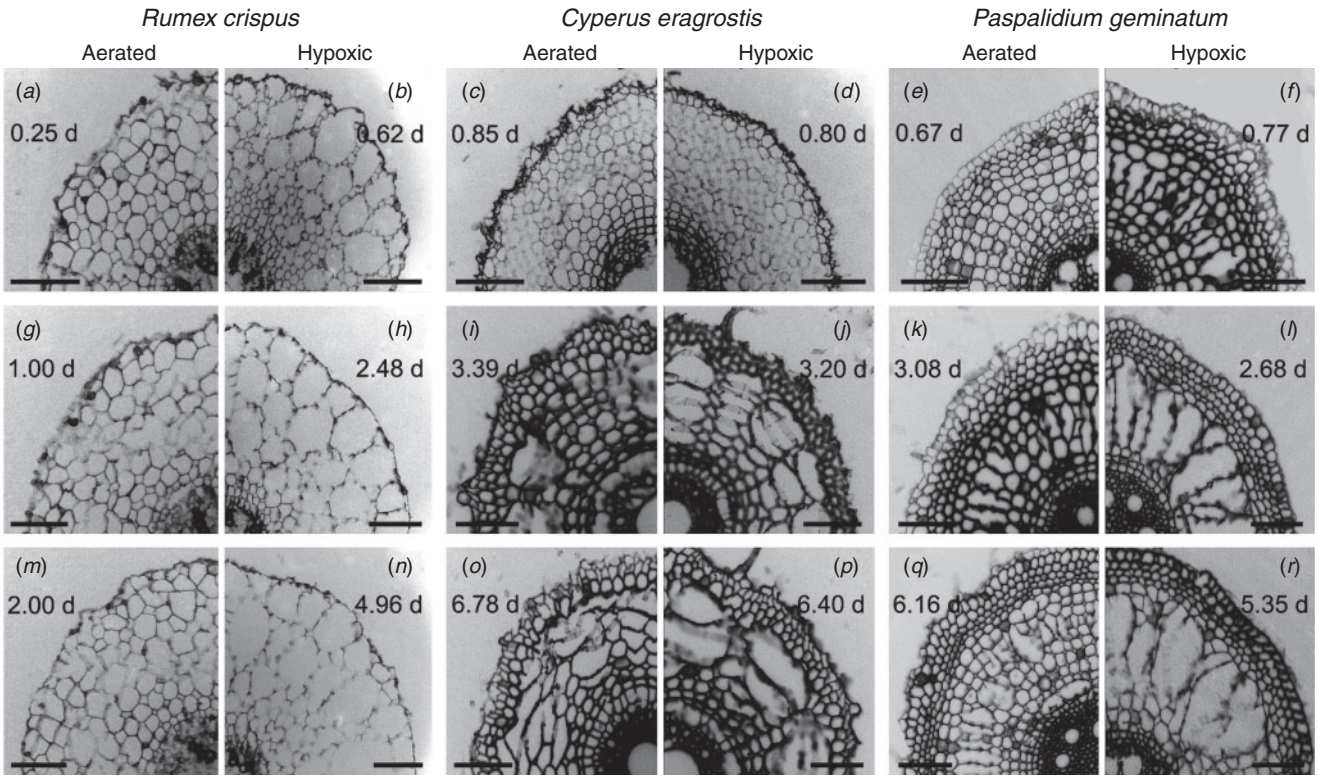

Fig. 4. Root aerenchyma development of *Rumex crispus*, *Cyperus eragrostis* and *Paspalidium geminatum* grown under aerated (0.247 moles O_2 m^{-3}) and hypoxic conditions (0.016 moles O_2 m^{-3}). Samples were taken at three positions from root apex: 0.5 cm (*a–f*), 2 cm (*g–l*) and 4 cm (*m–r*). Numbers within each photomicrography indicate the corresponding root tissue age (days). Scale bars represent 100 µm.

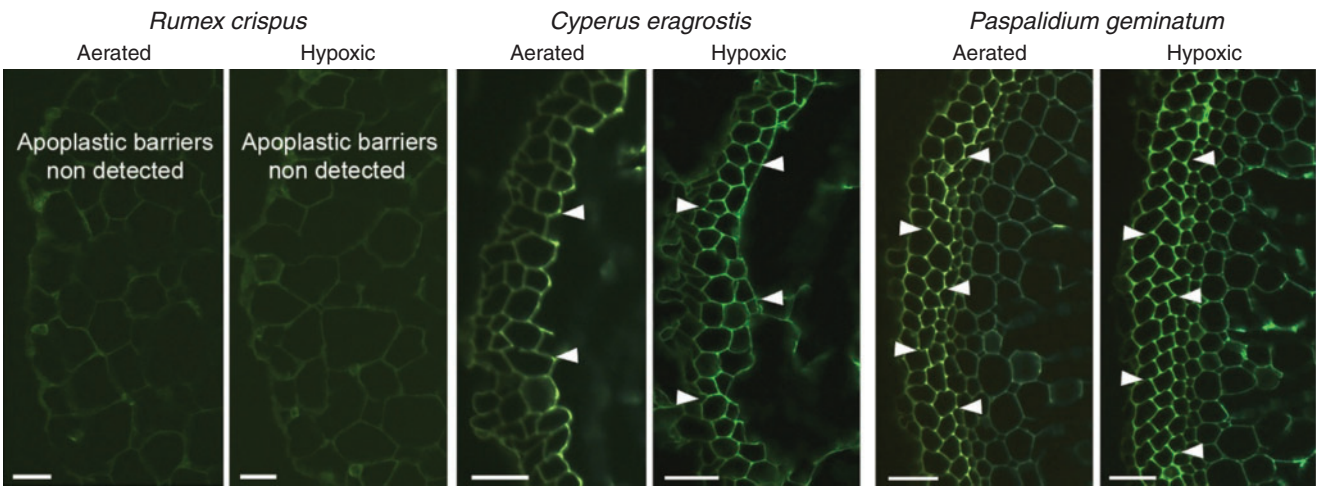

Fig. 5. Magnified sections of hypodermis/outer root cortex of *Rumex crispus*, *Cyperus eragrostis* and *Paspalidium geminatum* at 2 cm from root apex (*g–l* from Fig. 4) where ROL responses started to differ between treatments in *C. eragrostis* (see Fig. 2). Plants were grown in nutrient solution under aerated (0.247 moles O_2 m^{-3}) and hypoxic conditions (0.016 moles O_2 m^{-3}) for 8 days. White arrows indicate presence of apoplastic barriers as revealed by autofluorescence when cross-sections were exposed to UV light (excitation at 395 nm, Zeiss UV filter set, 50–200 ms of exposition time). Scale bars represent 20 µm.

treatment. However, as root tissues became older (>2 days old), apoplastic barrier started being evident, particularly under hypoxic conditions (Fig. 5), which suggested the development of a more 'tight' barrier induced by root zone hypoxia ($P < 0.05$). This was in accordance with the decline in ROL values at such root tissue age (compare Fig. 2*b*, *e* and Fig. 5). Such 'tight' apoplastic barrier was evident in 3 days old tissues (and older) (Fig. 5, middle panel).

Graminaceous *P. geminatum* (aerenchyma lacunae radially arranged in a shape resembling a bicycle wheel; Fig. 4 right panels) showed a relatively low value of aerenchyma in young root tissues (8.1–10.3%; but still significant for oxygen transport; see Armstrong 1979; Colmer 2003*b*) with respect to the other species, and with significant increases as root tissues became older when in aerated conditions ($P < 0.05$; ~17%, Fig. 3*c*, *f*). Under hypoxic treatment, more than 1.5-day-old tissues showed evidence of strong lysogenic processes (see progression in Fig. 4*f*–*l*–*r*), which quickly resulted in higher root aerenchyma amounts (>30%; $P < 0.05$; Fig. 3*c*, *f*). In this species, a 'tight' apoplastic barrier in the outer cortex was constitutively present at all evaluated root tissue ages without apparent differences between treatments (Fig. 5 right panel). So, in this species, the 'tight' physical barrier against ROL was also in accordance with the very low ROL from these roots under both treatments.

Discussion

Our results showed that the presence or induction of physical barriers in the outer cortex restrict the ROL under root zone O_2 deficiency (Colmer *et al*. 1998; Colmer 2003*a* for rice; Abiko *et al*. 2012 for teosinte). This was showed by comparing the responses of three species with alternative type of aerenchyma. ROL followed different patterns according to each species: (i) relatively high oxygen lost to rhizosphere irrespective of tissue age (rumex aerenchyma type of *R. crispus*); (ii) reduction of oxygen lost to rhizosphere – further induced by hypoxia – with induction of a 'tight' barrier in outer cortex in tissues older than 3–5 days (cyperaceous aerenchyma type of *C. eragrostis*); (iii) very low oxygen lost to rhizosphere, with constitutive 'tight' apoplastic barrier in the outer cortex (graminaceous aerenchyma type of *P. geminatum*). Our approach including root tissue age as a novel dimension of analyses, allowed concluding for *C. eragrostis* that the stronger apoplastic barrier induced by root zone O_2 deficiency (Fig. 5) can be certainly ascribed to an active response to hypoxia, not being an ontogenetic response.

The oxygen lost from roots growing in hypoxic soil can differ depending on the distance from apex, growth conditions and species (Armstrong 1971; Colmer *et al*. 1998; Visser *et al*. 2000; Shiono *et al*. 2011). In this work, we have shown that under hypoxic conditions, oxygen was lost from roots in a different magnitude, also according to root tissue age (this contribution) and species. The oxygen was lost from young tissues to old tissues (from high to low quantity) in *R. crispus* and *C. eragrostis* (Figs 2*a*–*d*, *b*–*e*), whereas in *P. geminatum* ROL was low all along the root and homogeneous irrespective of tissue age (Fig. 2*c*–*f*). For *R. crispus*, a similar ROL profile was reported in the closely related *R. palustris*, with more than 40% porosity (Visser *et al*. 2000). In our study, the high aerenchyma content and high ROL near the root apex in plants of *R. crispus* under hypoxia suggest that oxygen effectively reached the apex. Thus, the observed reductions in root elongation rate in this species might be related with other factors like ethylene entrapment and its accumulation in root tissues as seen in *R. palustris* and *R. thyrsiflorus* (Visser *et al*. 1997). Under hypoxic conditions, a low root elongation rate (eventually forming a more shallow and aggregated root system) and the relatively high cost of oxygen from roots seems to be beneficial in maintaining the rhizosphere oxidised in order to avoid the presence of potential toxic ions such as Fe^{2+}, S^{2-} and Mn^{2+} (this last idea formerly suggested by Pedersen *et al*. 2004; Armstrong and Armstrong 2005; Cheng *et al*. 2012).

In *C. eragrostis*, an additional response was the decrease of ROL (barrier induction) at older tissue ages while the plant maintained high ROL in very young root apical tissues. This might reflect a capacity to preserve an oxidised zone around the apex – sensitive to toxic ions and crucial for allowing cell division and enlargement – while reducing the release of oxygen towards the root base, thereby improving the longitudinal transport of oxygen (Armstrong 1971; Colmer 2003*b*). This plastic hypoxic-induced barrier was reported in other flood tolerant species as *Caltha palustris* (Visser *et al*. 2000), *Phragmites australis* and *Glyceria maxima* (Soukup *et al*. 2007), but this is the first time that it is informed for a cyperaceous root type like that of *C. eragrostis*. The lack of variation in ROL responses of *P. geminatum*, which increased root aerenchyma but maintained very low ROL when in hypoxic conditions, is likely to be related to maximising the conservation of oxygen inside the root as well as to impede the entry of toxic ions (see further discussion below), both of these responses were reported even under submergence in very tolerant rice genotypes (Waters *et al*. 1989).

The opposite patterns of ROL for each species corresponded to the presence and 'strength' of apoplastic barriers in relation to root tissue age (compare Figs 2 and 5). In this respect, *R. crispus* showed a 'partial' barrier, which was not detected under both treatments and root tissue ages with our methodological approach (Fig. 5, left panel). This response described in *R. crispus* was also observed in other flood tolerant species like *Acanthus ilicifolius* and *Avicennia marina*, with high aerenchyma content and high ROL; being the latter related to very weak physical barriers in exodermal cells (Pi *et al*. 2009). In contrast, *C. eragrostis* and *P. geminatum*, both with 'tight' ROL barriers, had apoplastic barriers in their outer cortex, and in *C. eragrostis* a more 'tight' barrier was induced by hypoxia (Fig. 5, middle panel). However, our results should be carefully interpreted as Shiono *et al*. (2011) have shown that barrier induction, as measured in functional assays for patterns of ROL, can occur even before histochemically detectable changes in apoplastic barrier presence as viewed by light microscopy. Induction of 'tighter' barriers presumably due to extra suberin and/or lignin deposition in root exodermis by oxygen deprivation was reported for rice (Kotula *et al*. 2009*a*, 2009*b*; Shiono *et al*. 2011), maize (Enstone and Peterson 2005), *Hordeum marinum* (Garthwaite *et al*. 2003), teosinte (Abiko *et al*. 2012) and *C. eragrostis* in this study. This plastic response, presumably with an extra cost due to extra suberin and lignin deposition, might account for the benefit of better adjustment of root growth under changing water soil conditions. So, this ability would be part of the mechanisms developed by this species for inhabiting different environments as suggested by its wide distribution in grasslands from lowlands to uplands (Soriano 1991) as it was also suggested for rice (Colmer 2003*a*).

Reports on flood tolerant species with constitutive apoplastic barriers in root outer cortex/exodermis like *P. geminatum* in our experiment (Fig. 5) are available: *Carex acuta* (Visser *et al*.

2000), *Echinocloa crusgalli* and *Schoenoplectus validus* (McDonald *et al*. 2002). The benefits associated with constitutively physical barriers are clear as these roots not only minimise the loss of oxygen towards rhizosphere but they would also act as apoplastic barriers impeding the entry of reduced toxic ions such as Fe^{2+}, S^{2-} and Mn^{2+} into roots (these ions increase under reductive soil conditions, e.g. Pedersen *et al.* 2004; Armstrong and Armstrong 2005; Cheng *et al*. 2012) and also organic acids with proved detrimental effects on root extension rate (Kotula *et al*. 2014). In addition, the presence of apoplastic barriers in the outer cortex might reduce the uptake of some nutrients, like Na^+ and Cl^- (Ranathunge *et al*. 2011), but not others like NO_3^- (Rubinigg *et al*. 2002). However, the constitutive formation of ROL barriers, as those observed in *P. geminatum*, would have costs associated to their generation and maintenance, which might be reflected in its lower value of absolute root elongation rate if compared with that of *R. crispus* (compare Fig. 1*a* with Fig. 1*c*). Concurring with this idea, similar differences were reported by Visser *et al*. (2000) for absolute values of root growth between *Carex acuta* (species similar to *P. geminatum* in ROL responses and constitutive barriers) and *R. palustris*.

In conclusion, we found that roots of *P. geminatum* and *C. eragrostis* have (or are able to form) a 'tight' apoplastic barrier against ROL, whereas *R. crispus* has a 'partial' apoplastic barrier to ROL. The presence of such apoplastic barriers in both graminoid species seems aimed at avoiding the intrusion of potential toxic elements from the reduced rhizosphere. The functionality of having a high ROL from roots, as it was the case of the dicot *R. crispus*, is likely to be a way to maintain the rhizosphere oxidised to avoiding the presence of reduced elements potentially toxic for the plant. The ecological consequences of both strategies at root level presumably to deal with ionic toxicity as a result of the reduction of soil redox potential due to flooding should be analysed by scaling up to the responses at plant level in the context of their grassland natural habitat.

Acknowledgements

We specially thank Professor Timothy D Colmer (University of Western, Australia) and two anonymous reviewers for their critical review of the manuscript. We thank Gabriela Zarlavsky for anatomical work and helpful comments from Professor Amy Austin (University of Buenos Aires, Argentina) on an earlier version of the text. This research was supported by grants from the National Scientific and Technical Research Council of Argentina (CONICET – PIP 0087/2012) and University of Buenos Aires (UBA 01/K108). Milena E Manzur was supported by a postdoctoral fellowship from CONICET (Argentina).

References

Abiko T, Kotula L, Shiono K, Malik AI, Colmer TD, Nakazono M (2012) Enhanced formation of aerenchyma and induction of a barrier to radial oxygen loss in adventitious roots of *Zea nicaraguensis* contribute to its waterlogging tolerance as compared with maize (*Zea mays* ssp. *mays*). *Plant, Cell & Environment* **35**, 1618–1630. doi:10.1111/j.1365-3040.2012.02513.x

Armstrong W (1971) Radial oxygen losses from intact rice roots as affected by distance from the apex, respiration and waterlogging. *Physiologia Plantarum* **25**, 192–197. doi:10.1111/j.1399-3054.1971.tb01427.x

Armstrong W (1979) Aeration in higher plants. *Advances in Botanical Research* **7**, 225–332. doi:10.1016/S0065-2296(08)60089-0

Armstrong J, Armstrong W (2005) Rice: sulfide-induced barriers to root radial oxygen loss, Fe^{2+} and water uptake, and lateral root emergence. *Annals of Botany* **96**, 625–638. doi:10.1093/aob/mci215

Armstrong W, Cousins D, Armstrong J, Turner DW, Beckett PM (2000) Oxygen distribution in wetland plant roots and permeability barriers to gas-exchange with the rhizosphere: a microelectrode and modeling study with *Phragmites australis*. *Annals of Botany* **86**, 687–703. doi:10.1006/anbo.2000.1236

Bailey-Serres J, Voesenek LACJ (2008) Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* **59**, 313–339. doi:10.1146/annurev.arplant.59.032607.092752

Brundrett MC, Kendrick B, Peterson CA (1991) Efficient lipid staining in plant material with Sudan red 7B or Fluorol yellow 088 in polyethylene glycol-glycerol. *Biotechnic & Histochemistry* **66**, 111–116. doi:10.3109/10520299109110562

Cheng H, Chen D, Fung-Yee Tam N, Chen GZ, Li SY, Ye ZH (2012) Interactions among Fe^{2+}, S^{2-}, and Zn^{2+} tolerance, root anatomy, and radial oxygen loss in mangrove plants. *Journal of Experimental Botany* **63**, 2619–2630. doi:10.1093/jxb/err440

Colmer TD (2003*a*) Aerenchyma and an inducible barrier to radial oxygen loss facilitate root aeration in upland, paddy and deepwater rice (*Oryza sativa* L.). *Annals of Botany* **91**, 301–309. doi:10.1093/aob/mcf114

Colmer TD (2003*b*) Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell & Environment* **26**, 17–36. doi:10.1046/j.1365-3040.2003.00846.x

Colmer TD, Voesenek LACJ (2009) Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology* **36**, 665–681. doi:10.1071/FP09144

Colmer TD, Gibberd MR, Wiengweera A, Tinh TK (1998) The barrier to radial oxygen loss from roots of rice (*Oryza sativa* L.) is induced by growth in stagnant solution. *Journal of Experimental Botany* **49**, 1431–1436. doi:10.1093/jxb/49.325.1431

Enstone DE, Peterson CA (2005) Suberin lamella development in maize seedling roots grown in aerated and stagnant conditions. *Plant, Cell & Environment* **28**, 444–455. doi:10.1111/j.1365-3040.2005.01286.x

Garthwaite AJ, von Bothmer R, Colmer TD (2003) Diversity in root aeration traits associated with waterlogging tolerance in the genus *Hordeum*. *Functional Plant Biology* **30**, 875–889. doi:10.1071/FP03058

Grimoldi AA, Insausti P, Vasellati V, Striker GG (2005) Constitutive and plastic root traits and their role in differential tolerance to soil flooding among co-existing species of lowland grassland. *International Journal of Plant Sciences* **166**, 805–813. doi:10.1086/431805

Henriksen GH, Raman DR, Walker LP, Spanswick RM (1992) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes: II. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. *Plant Physiology* **99**, 734–747. doi:10.1104/pp.99.2.734

Justin SHFW, Armstrong W (1987) The anatomical characteristics of roots and plant responses to soil flooding. *New Phytologist* **106**, 465–495. doi:10.1111/j.1469-8137.1987.tb00153.x

Kotula L, Ranathunge K, Schreiber L, Steudle E (2009*a*) Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution. *Journal of Experimental Botany* **60**, 2155–2167. doi:10.1093/jxb/erp089

Kotula L, Ranathunge K, Steudle E (2009*b*) Apoplastic barriers effectively block oxygen permeability across outer cell layers of rice roots under deoxygenated conditions: roles of apoplastic pores and of respiration. *New Phytologist* **184**, 909–917. doi:10.1111/j.1469-8137.2009.03021.x

Kotula L, Colmer TD, Nakazono M (2014) Effects of organic acids on the formation of the barrier to radial oxygen loss in roots of *Hordeum marinum*. *Functional Plant Biology* **41**, 187–202. doi:10.1071/FP13178

McDonald MP, Galwey NW, Colmer TD (2002) Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wet- and dry-land grass species. *Plant, Cell & Environment* **25**, 441–451. doi:10.1046/j.0016-8025.2001.00817.x

Pedersen O, Binzer T, Borum J (2004) Sulphide intrusion in eelgrass (*Zostera marina* L.). *Plant, Cell & Environment* **27**, 595–602. doi:10.1111/j.1365-3040.2004.01173.x

Pi N, Tam NFY, Wu Y, Wong MH (2009) Root anatomy and spatial pattern of radial oxygen loss of eight true mangrove species. *Aquatic Botany* **90**, 222–230. doi:10.1016/j.aquabot.2008.10.002

Ranathunge K, Lin J, Steudle E, Schreiber L (2011) Stagnant deoxygenated growth enhances root suberization and lignifications, but differentially affects water and NaCl permeabilities in rice (*Oryza sativa* L.) roots. *Plant, Cell & Environment* **34**, 1223–1240. doi:10.1111/j.1365-3040.2011.02318.x

Rittinger PA, Biggs AR, Peirson DR (1987) Histochemistry of lignin and suberin deposition in boundary layers formed after wounding in various plant species and organs. *Canadian Journal of Botany* **65**, 1886–1892. doi:10.1139/b87-258

Rubinigg M, Stulen I, Elzenga JTM, Colmer TD (2002) Spatial patterns of radial oxygen loss and nitrate net flux along adventitious roots of rice raised in aerated or stagnant solution. *Functional Plant Biology* **29**, 1475–1481. doi:10.1071/FP02081

Schreiber L, Franke R (2011) 'Endodermis and exodermis in roots.' (John Wiley & Sons Ltd: Chichester, UK)

Seago JL, Jr, Marsh LC, Stevens KJ, Soukup A, Votrubová O, Enstone DE (2005) A re-examination of the root cortex in wetland flowering plants with respect to aerenchyma. *Annals of Botany* **96**, 565–579. doi:10.1093/aob/mci211

Shiono K, Ogawa S, Yamazaki S, Isoda H, Fujimura T, Nakazono M, Colmer TD (2011) Contrasting dynamics of radial O_2 loss barrier induction and aerenchyma formation in rice roots of two lengths. *Annals of Botany* **107**, 89–99. doi:10.1093/aob/mcq221

Soriano A (1991) Río de la Plata Grasslands. In 'Ecosystems of the world 8A. Natural grasslands. Introduction and western hemisphere'. (Ed RT Coupland) pp. 367–407. (Elsevier: Amsterdam, The Netherlands)

Soukup A, Armstrong W, Schreiber L, Franke R, Votrubová O (2007) Apoplastic barriers to radial oxygen loss and solute penetration: a chemical and functional comparison of the exodermis of two wetland species, *Phragmites australis* and *Glyceria maxima*. *New Phytologist* **173**, 264–278. doi:10.1111/j.1469-8137.2006.01907.x

Striker GG, Insausti P, Grimoldi AA, Vega AS (2007) Trade-off between root porosity and mechanical strength in species with different types of aerenchyma. *Plant, Cell & Environment* **30**, 580–589. doi:10.1111/j.1365-3040.2007.01639.x

Striker GG, Izaguirre RF, Manzur ME, Grimoldi AA (2012) Different strategies of *Lotus japonicus, L. corniculatus* and *L. tenuis* to deal with complete submergence at seedling stage. *Plant Biology* **14**, 50–55.

Visser EJW, Nabben RHM, Blom CWPM, Voesenek LACJ (1997) Growth of primary lateral roots and adventitious roots during conditions of hypoxia and high ethylene concentrations. *Plant, Cell & Environment* **20**, 647–653. doi:10.1111/j.1365-3040.1997.00097.x

Visser EJW, Colmer TD, Blom CWPM, Voesenek LACJ (2000) Changes in growth, porosity and radial oxygen loss from adventitious roots of selected mono- and dicotyledonous wetland species with contrasting types of aerenchyma. *Plant, Cell & Environment* **23**, 1237–1245. doi:10.1046/j.1365-3040.2000.00628.x

Waters I, Armstrong W, Thompson J, Setter TL, Adkins S, Gibbs J, Greenway H (1989) Diurnal changes in radial oxygen loss and ethanol metabolism in roots of submerged and non-submerged rice seedlings. *New Phytologist* **113**, 439–451. doi:10.1111/j.1469-8137.1989.tb00355.x

Wiengweera A, Greenway H, Thomson C (1997) The use of agar nutrient solution to simulate lack of convection in waterlogged soils. *Annals of Botany* **80**, 115–123. doi:10.1006/anbo.1996.0405