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Peralta Ogorek, Lucas León; Pellegrini, Elisa; Pedersen, Ole

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Novel functions of the root barrier to radial oxygen loss – radial diffusion resistance to H₂ and water vapour

Lucas León Peralta Ogorek, Elisa Pellegrini and Ole Pedersen

The Freshwater Biological Laboratory, Department of Biology, University of Copenhagen, Universitetsparken 4, 3rd Floor, Copenhagen 2100, Denmark

Authors for correspondence:
Lucas León Peralta Ogorek
Email: lucas.ogorek@bio.ku.dk

Ole Pedersen
Email: opedersen@bio.ku.dk

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Summary

- The root barrier to radial O₂ loss (ROL) is a trait enabling waterlogging tolerance of plants. The ROL barrier restricts O₂ diffusion to the anoxic soil so that O₂ is retained inside root tissues.
- We hypothesised that the ROL barrier can also arrest radial diffusion of other gases (H₂ and water vapour) in rice roots with a barrier to ROL. We used O₂ and H₂ microsensors to measure ROL and permeability of rice roots, and gravimetric measurements to assess the influence of the ROL barrier on radial water loss (RWL).
- The ROL barrier greatly restricted radial diffusion of O₂ as well as H₂. At 60 kPa pO₂, we found no radial diffusion of O₂ across the barrier, and for H₂ the barrier reduced radial diffusion by 73%. Similarly, RWL was reduced by 93% in roots with a ROL barrier.
- Our study showed that the root barrier to ROL not only completely blocks radial O₂ diffusion under steep concentration gradients but is also a diffusive barrier to H₂ and to water vapour. The strong correlation between ROL and RWL presents a case in which simple measurements of RWL can be used to predict ROL in screening studies with a focus on waterlogging tolerance.

Introduction

The root barrier to radial O₂ loss (ROL) is a trait enabling waterlogging tolerance of several wetland plants, including rice (Colmer, 2003b). The ROL barrier restricts O₂ diffusion to the anoxic soil so that O₂ is retained in the root, and during waterlogging roots with a ROL barrier can obtain greater maximum length compared with roots without this trait (Colmer & Voesenek, 2009). However, the role of the ROL barrier in restricting radial diffusion of other gases was unknown and therefore the present study evaluates its importance for tissue water retention and bench-measurements of RWL can be used to predict ROL in screening studies with a focus on waterlogging tolerance.

Plants need water to sustain growth but too much or too little can result in abiotic stress. The ongoing climate changes produce more extreme weather events with severe droughts and floods (IPCC, 2019). Drought limits the growth and reproductive capacity of cereal crops, ultimately impacting their yield (Barnabas et al., 2008). By contrast, too much water results in soil waterlogging that leads to soil anoxia because gas diffusion in water is much slower compared with air; moreover, O₂ is rapidly consumed both chemically and by microorganisms (Ponnamperuma, 1972; Armstrong et al., 1991). As O₂ becomes limited, the soil microbes turn from aerobic to anaerobic metabolism and the soil redox potential decreases resulting in production of reduced iron or sulphur, which are known as potent plant phytoxins (Laanbroek, 1990; Armstrong et al., 1991).

Wetland plants possess a number of root traits enabling growth in waterlogged soils. Due to the slow diffusion in water, the gaseous hormone ethylene accumulates in the root tissues triggering the production of adventitious roots of high gas-filled porosity (aerenchyma) (Colmer & Voesenek, 2009). The aerenchyma enhances the transport of gases within the root while reducing the tissue respiratory demand (Armstrong, 1979; Zhu et al., 2010). Aerenchyma can extend from the roots to the stem, allowing longitudinal diffusion of O₂ from the aerial to the waterlogged parts of the plant (Colmer & Voesenek, 2009). Another trait is the surface area to volume ratio (SA : V); roots are larger in diameter when grown in stagnant, deoxygenated conditions (mimicking waterlogging) compared with aerated conditions (mimicking drained soils) (Ranathunge et al., 2011a; Yamauchi et al., 2019). Thicker roots reduce the SA : V ratio so that the relative area where O₂ can be lost to the rhizosphere is decreased, helping to conserve O₂ inside the root (Pedersen et al., 2020). While aerenchyma and thicker roots both facilitate internal aeration, the barrier to ROL is needed to further sustain growth in waterlogged soils.

The barrier to ROL improves the internal O₂ status of the root by restricting ROL. The ROL barrier can be constitutive or induced during waterlogging (Visser et al., 2000) and starts being formed 20–40 mm behind the tip (for examples on constitutive barriers in the genera of Urochloa or Echinochloa, see Jiménez et al. (2019) and Ejiri & Shiono (2019), and for inducible barriers in rice or Hordeum marinum, see Colmer (2003a) and
Garthwaite et al. (2003)). Some species possess a ‘tight’ barrier with low ROL in the basal zones, whereas others with ‘weak’ barriers show comparatively higher ROL in the basal part of the root, resulting in reduced O₂ supply to the growing tip (Colmer, 2003b). Low and nontoxic concentrations of organic acids (Colmer et al., 2019) and reduced Fe (Mongon et al., 2014), all of which are produced by anaerobic soil bacteria, can induce the formation of the ROL barrier in roots of rice. Moreover, exposing rice roots that already had formed a barrier to ROL to sulphides further reduced ROL (Armstrong & Armstrong, 2005).

Suberin and lignin are thought to be the active components of the barrier to ROL and these are both deposited in the apoplast on the cell walls of the root exodermis. Roots with a barrier to ROL possess higher amounts of suberin in the exodermis (Ranathunge et al., 2011b). Lignin content is also higher in roots grown under stagnant, deoxygenated conditions (Kotula et al., 2009) but does not always contribute to reducing ROL (De Simone et al., 2003; Nishiuichi et al., 2012). A strong apoplastic barrier could possibly restrict radial water uptake, particularly during transpiration in which the majority of the radial water uptake occurs via the apoplastic pathway (Steudle, 1994).

Studies on the influence of the barrier on root hydraulic conductivity are, however, not conclusive. Roots of neither rice (Ranathunge et al., 2011a) nor H. marinum (Garthwaite et al., 2006) with a ROL barrier showed any significant reduction in hydraulic conductivity compared with roots without. By contrast, when exposed to sulphones, roots of rice suffered a reduction in water uptake of 9–25% compared with controls, but factors other than the barrier such as xylem blockage, stunted root growth and reduced number of laterals could also have resulted in the observed reduction in water uptake (Armstrong & Armstrong, 2005). Similarly, Iris germanica, which can develop a multi-erated exodermis, showed a reduction in hydraulic conductivity of c. 50% in roots with several layers of suberin compared with roots with only few layers (Meyer et al., 2011). Finally, radial water movements have been measured in roots with or without an exodermis and it was found that radial water movements were greatly reduced in roots with an exodermis. However, this study did not test if the exodermis had formed a barrier to ROL (Taleisnik et al., 1999).

H₂ serves as an important signalling molecule in many responses of plants. Applied exogenously, H₂ stimulates adventitious root formation (Zhu & Liao, 2016), root elongation, seed germination and seedling growth, and postharvest freshness of fruits (Li et al., 2018). In addition, treatment with H₂-rich water increases tolerance to environmental stresses (Li et al., 2018). Moreover, H₂ is produced in soil by the N₂-fixing enzyme nitrogenase in legume nodules (Dong et al., 2003). The presence of H₂ changes the soil microbial composition, favouring the development of H₂-oxidising bacteria that promotes growth of Arabidopsis (Maimaiti et al., 2007) or cereals such as wheat (Angus et al., 2015) and barley (Dong et al., 2003). For these reasons, identification of resistance components (such as the barrier ROL) to radial diffusion of H₂ in roots is a research priority.

We tested the hypothesis that ‘the root barrier to ROL, in addition to O₂, also restricts radial diffusion of other gases such as H₂ and water vapour in rice roots without or with a barrier’. We used two contrasting pO₂ to test if the barrier to ROL can block radial diffusion regardless of the gradient. We also used two contrasting pH₂ to investigate the capacity of the barrier to block a nonpolar gas, H₂, which is smaller than O₂ and diffuses faster. Moreover, we assessed the capacity of the barrier to restrict loss of water vapour (a polar gas). Artificial roots (silicone tubing) were used to compare the radial diffusion of the three gases through a material with known diffusion characteristics. Finally, we correlated ROL with RWL, hypothesising a strong positive correlation.

Materials and Methods

The following sections conceptually describe the experimental procedures (see Supporting Information Methods S1 for details).

Plant material

Seeds of Oryza sativa (cultivar IR42, a moderate drought and submergence tolerant rice cultivar; Ponnamperuma, 1979), were germinated and transferred to aerated nutrient solution for 7 d. The seedlings were then grown in aerated hydroponics until 21 d old. A group of plants was maintained in aerated nutrient solution and others were transferred to stagnant, deoxygenated nutrient solution to simulate waterlogging (Wiengweera et al., 1997). Roots of stagnant plants were used for measurements after 7–10 d. Adventitious roots (10–14 cm) of 28–31-d-old plants without (aerated) or with a tight (stagnant) barrier to radial O₂ loss (ROL) were used in side-by-side comparisons in all experiments; these roots were growing at 17–32 mm d⁻¹ (see Methods S1). For some experiments, plants were grown for 45–55 d in aerated nutrient solution to induce a weak barrier to ROL. Artificial roots (silicone tubing) were used to compare gas intrusion and radial water loss (RWL) through a material with known diffusion characteristics.

Radial O₂ or H₂ intrusion

This experiment aimed at assessing rates of O₂ or H₂ intrusion (from external solution and into the root cortex) at two contrasting external gas pressures (pO₂ 20.6 and c. 60 kPa or pH₂ c. 6.2 and c. 31.3 kPa) for roots without or with a tight barrier to ROL. Root segments (corresponding to a position 30–80 mm behind the tip) without the tip and sealed in both cut ends with lanoline were fixed on a metal mesh. The microsensor (O₂ or H₂) was positioned midway along the segment, inserted 180–250 μm into the cortex after which the root segment was submerged in the medium. Measurements continued until a quasi-steady state was achieved (c. 50 min) at which point the bulk water was replaced with the higher pO₂ (or pH₂). The bulk water was monitored using an O₂ mini-oeptode (or a H₂ microsensor). H₂ intrusion rates were compared using two-way ANOVA. For O₂, there was no detectable intrusion into segments with a tight barrier.
Root respiration rates

Root respiration rates (O_2 consumption based on the approach by Winkel et al., 2013) were measured at 25 or 5°C to account for the O_2 consumed during the O_2 intrusion experiments. Respiration was measured using an O_2 minioptode inserted into glass vials with a root segment in a constant temperature bath and the rate was expressed per gram of fresh mass. Respiration was only measured on roots without an ROL barrier as the barrier restricts O_2 consumption from the medium, underestimating tissue respiration (Jiménez et al., 2020).

Radial root profiles of O_2 or H_2

Radial profiles of pO_2 or pH_2 were taken to verify that the measured gas pressures were independent of the exact microsensor position in the cortex. The radial profiles followed the approach described in Colmer et al. (2020). The root segments and gas pressures used for profiling were prepared similarly to those used for O_2 or H_2 intrusion. The radial profiles were taken starting within the diffusive boundary layer (DBL), penetrating the surface of the root and into cortex, stele, and finishing once inside the cortex again. The segments were exposed to relevant gas pressures for 15–20 min before the profiles were taken.

Radial water loss

We tested whether the barrier to ROL, root tip or lateral roots influenced RWL by conducting gravimetric measurements. We used root segments without, with a weak or with a tight barrier to ROL, without or with the root tip, and without or with root laterals. The cut end(s) were sealed with Vaseline and placed in a closed chamber of a balance with silica gel to maintain a constantly dry atmosphere. The loss in mass as the water evaporated was recorded every minute for 1 h. The segments were then dried to calculate the cumulated water loss (% total water content). RWL was calculated from the cumulated water loss and root surface area and normalised based on root diameter (see Fig. S1a,b for non-normalised data). Statistical comparisons were conducted using the RWL rates at the time point at which 15% of the tissue water had evaporated using a one-way ANOVA.

Radial O_2 loss (ROL)

We hypothesised a strong positive correlation between RWL and ROL and therefore we measured rates of ROL using a microoptode on intact plants without, a weak or with a tight barrier. ROL was measured using the slope of O_2 concentrations within the DBL (HenrikSEN et al., 1990; Colmer et al., 2020). The ROL measurements were taken 40–60 mm behind the root tip on the exact same plant from which the root segments for RWL measurements were taken. The obtained ROL values were adjusted to root diameter (see Fig. S1a) to normalise data to roots of 1.00 mm in diameter (see Fig. S1b for non-normalised data and Fig. S1c for example DBL profile).

Visualisation of O_2 leakage from roots

A qualitative assessment of ROL was conducted using methylene blue staining. Methylene blue is a colourless redox indicator in its reduced form and turns blue when oxidised. Plants with or without a tight barrier had all but one or two roots trimmed off and roots were subsequently submerged 1–2 cm below the root–shoot junction in a methylene blue solution prepared as described by Yamauchi et al. (2019).

Visualisation of the apoplastic barrier

A qualitative assay was conducted to visualise the permeability of the apoplastic barrier using the approach described in Soukup et al. (2002). Periodic acid acts as tracer travelling the apoplast and can be detected using Schiff’s reagent, resulting in a purple coloration. Root segments without or with a tight barrier were sealed on the cut ends using lanoline and incubated in periodic acid. Segments were then washed and incubated in a reducing solution and rinsed in deionised (DI) water. Cross-sections were taken at a position 40 mm behind the root tip and stained using Schiff’s reagent.

Apparent permeance (P_a) to O_2, H_2 and water vapour

To compare the capacity of the barrier to restrict radial diffusion of O_2, H_2 and water vapour, we calculated the apparent permeance (P_a, m s^-1) following the equation described in Lendzian (2006):

\[ P_a = \frac{F}{Ax\Delta C} \]

where F (mol s^-1) is the intrusion rate of O_2 or H_2 or loss rate of water, A (m^2) is surface area of the root segments and \( \Delta C \) (mol m^{-3}) is the concentration gradient. For O_2 and H_2, the concentration gradient was calculated as the maximum concentration difference between tissue and bulk. For water, \( \Delta C \) was calculated as the difference in relative humidity (100%) of the tissue and the mean external atmosphere (20%).

Statistical analyses

GraphPad PRISM software (v.8.3.1) was used for statistical analyses. The maximum concentrations at quasi-steady state for O_2 and intrusion rates for H_2 were tested using two-way ANOVA followed by a Tukey test. For both O_2 and H_2, the data were log-transformed to meet the ANOVA assumptions (normality and homoscedasticity). The effect of the ROL barrier on RWL was evaluated using one-way ANOVA followed by a Tukey test on log-transformed data to meet the assumptions (normality and homoscedasticity). Artificial roots were excluded from all statistical analysis. Finally a linear regression model was used to test the...
relationship between RWL and ROL data; the data were transformed using the z-score to meet normality and homoscedasticity assumptions. For all figures, nontransformed data are shown.

Results

Radial O₂ intrusion

The permeability to O₂ of the barrier to ROL was evaluated using steep gradients in O₂ between the external medium and the tissue. We found that the barrier essentially prevented radial diffusion of O₂ regardless of the gradient applied. An O₂ microsensor was used to measure ρO₂ inside the cortex of root segments (Fig. 1a). With an external water ρO₂ of 20.6 kPa, cortex ρO₂ in segments without a barrier initially declined slightly due to tissue consumption until a diffusion equilibrium was established (Fig. 1b). When the bulk water was replaced with 60 kPa, the intrusion of O₂ into the cortex increased steeply during the first minutes before a new quasi-steady state was reached (Fig. 1b). Root cortex ρO₂ never matched the external ρO₂ (Fig. 1c) as some O₂ was consumed by respiration when diffusing from the medium to the cortex (see below).

Root segments with a tight barrier showed contrasting behaviour compared with roots without a barrier. The initial decline in ρO₂ continued until the cortex reached anoxia (detection limit < 0.02 kPa). The tissue consumed O₂, but the barrier essentially prevented resupply from the external medium (Fig. 1b,c). After changing the bulk water to 60 kPa, the cortex O₂ status remained unaffected and below the detection limit. However, the tissue O₂ consumption could mask an insignificant O₂ intrusion if O₂ was consumed at a rate matching the diffusional flux across the barrier to ROL. We therefore carried out measurements on segments with a tight barrier now at 5°C when the respiration of root segments was reduced by 81% (Fig. S2a) corresponding to a Q₁₀ for root respiration of 2.27. However at 5°C, there was also no detectable intrusion of O₂ (Fig. S2b).

O₂ intrusion into artificial roots made from silicone rubber were conducted to compare the intrusion rates. The ρO₂ inside the lumen of the artificial root segments showed no change when submerged into bulk water with 20.6 kPa, as the lumen already was at atmospheric equilibrium. Upon change in bulk water to 60 kPa, the O₂ increased rapidly to c. 60 kPa; this contrasted with that of root segments without a barrier as there was no O₂ consumption by the silicone rubber (Fig. 1b,c).

The barrier to ROL effectively prevented any intrusion of O₂ and therefore it was not possible to estimate intrusion rates. Instead, the maximum ρO₂ achieved at quasi-steady state was used to compare roots (Fig. 1c). Roots without a barrier had a maximum ρO₂ 54-fold higher than that of roots with a tight barrier at 20.6 kPa. At 60 kPa, roots without a barrier had 1000-fold higher ρO₂ compared with roots with a tight barrier. Compared with roots without a barrier, the artificial roots achieved the highest ρO₂ at quasi-steady state, being 3.1-fold and 1.8-fold higher with external ρO₂ at 20.6 and 60 kPa, respectively.

Radial O₂ profiles at 20.6 kPa were taken of root segments without or with a tight barrier to ROL. The ρO₂ in the DBL (~200 to 0 µm) declined slightly (Fig. 1d,e) indicating that the root segments were consuming O₂. Inside segments without a barrier, ρO₂ declined steeply across the outer cell layers (Fig. 1d); at c. 120 µm inside the root, ρO₂ remained relatively constant until the tip of the microsensor encountered the stele where the ρO₂ dropped to roughly half of that found in the cortex (Fig. 1d). Inside the stele, ρO₂ remained constant until the microsensor reached the cortex on the other side where the ρO₂ again increased (Fig. 1d). The radial O₂ profiles taken at 60 kPa (Fig. 1e) were similar to those at 20.6 kPa, except that the tissue ρO₂ was greatly elevated, but the decline in ρO₂ inside the stele was still present due to reduced diffusion (tissue of low porosity) and enhanced O₂ consumption.

For root segments with a tight barrier to ROL, the radial O₂ profiles were profoundly different. First, the initial decline in ρO₂ within the DBL was much smaller, indicating that the net O₂ consumption of these segments was lower compared with segments without a barrier. Once the microsensor penetrated the outer cell layers, the ρO₂ declined and remained below the detection limit in the cortex as well as in the stele (Fig. 1d); the tissues also remained below the detection limit even with external ρO₂ at 60 kPa (Fig. 1e).

Radial H₂ intrusion

The permeability of the barrier to ROL was also evaluated using steep pH₂ gradients (c. 6.2 and c. 31.3 kPa). For O₂, we found that the barrier substantially reduced radial diffusion of H₂ even though the H₂ molecule was much smaller than O₂ and diffused faster. At both external gas pressures, pH₂ inside the cortex of segments without a barrier increased steeply shortly after exposing the segment to H₂. The intrusion gradually slowed until a quasi-steady state was reached after c. 25 min (Fig. 2a). However, intrusion of H₂ into segments with a tight barrier followed a different pattern. Although there was intrusion, the barrier clearly restricted radial H₂ diffusion and it took longer (c. 50 min) to reach the quasi-steady state; this response was similar at both external pH₂.

H₂ intrusion rates were calculated for root segments without or with a tight barrier and were significantly different (Fig. 2b). Segments without a barrier had intrusion rates 3.7-fold and 3.3-fold higher compared with segments with a tight barrier at pH₂ 6.3 and 31.4 kPa externally, respectively. The barrier therefore restricted (but did not completely block) radial diffusion of H₂ into the cortex. Interestingly, the intrusion rates of roots without a barrier were 1.3-fold higher compared with artificial roots for both external pH₂.

We also obtained radial tissue profiles of pH₂ of root segments without or with a tight barrier (Fig. 2c,d). However, the pH₂ continued to change across the root until in equilibrium with the external pH₂, after which the profile would be flat. Therefore, we started measuring at the time point when 50% of equilibrium had been achieved. The profiles showed a clear decline in H₂ both across the DBL and the first few outer cell layers and then again across the cortex and stele (Fig. 2c). As for O₂, H₂ profiles...
were similar at the elevated bulk water pH2 only differing in absolute concentration levels (Fig. 2d).

Radial water loss

The capacity of the ROL barrier to restrict water loss was assessed measuring the evaporation from root segments into a dry atmosphere. Similar to O2 and H2, the barrier significantly restricted RWL. Moreover, the strong influence of the barrier on RWL was directly visible to the naked eye. After 1 h in a dry atmosphere, root segments without a barrier had shrivelled, whereas segments with a tight barrier had not changed in structure (Fig. 3a).

Quantitative measurements of the rate of desiccation also revealed vast effects of the barrier to ROL. Cumulated water loss showed that roots without a barrier had lost almost 80% of the total water content after 35 min, and 15% were already lost after 2 min (Fig. 3b). Cumulated water loss followed a saturating function as the evaporation of water slowed down as the tissue gradually desiccated. By contrast, root segments with a tight barrier showed a more linear response with only a brief initial
enhanced evaporation before entering a relatively linear phase (Fig. 3b). After 1 h, segments with a tight barrier had lost only 18% of the tissue water. Root segments with a weak barrier showed an intermediate response with high variability but followed a linear desiccation pattern similar to segments with a tight barrier (Fig. 3b).

Rates of RWL also differed significantly between root segments without or with a tight barrier as these rates are calculated based on cumulated water loss. For the three barrier types, the initial fast evaporation from the outer cell layers resulted in elevated RWL with the initial rate being three-fold higher for roots without a barrier compared with roots with a tight barrier (Fig. 3c). Segments without a barrier showed three phases of desiccation: (1) an elevated initial phase, (2) a phase that lasted for the next 15–20 min, and (3) a phase in which RWL declined to values below 470 µmol H₂O m⁻² s⁻¹. Shortly after 40 min, the RWL of root segments without a barrier matched the RWL rates of segments with a tight barrier. By contrast, segments with a tight barrier only showed two phases with, again, an elevated initial phase and a second phase with RWL of 300 µmol H₂O m⁻² s⁻¹.

Root segments with a weak barrier showed high initial RWL but soon followed the same pattern of root segments with a tight barrier. However, RWL was consistently higher even after 1 h (595 µmol H₂O m⁻² s⁻¹), compared with those without a barrier. Finally, RWL of artificial roots were constant at 2.8 µmol H₂O m⁻² s⁻¹ as these continued to contain liquid water inside the lumen throughout the measurements (Fig. 3c), maintaining a constant gradient.

RWL is driven by gradients in water between the tissue surface and the surrounding atmosphere and rates of RWL therefore changed with time. To facilitate comparison between the barrier types, we extracted the RWL for the time point when the root segments had lost 15% of the water content. At this time, RWL rates were 14-fold higher for root segments without a barrier compared with those with a tight barrier (Fig. 3d) showing the substantial effect of the barrier in also restricting water loss. RWL rates of roots with a weak barrier were 33% lower than roots without a barrier but 4.5-fold higher than roots with a tight barrier.

Relationship between ROL and RWL

We hypothesised that rates of ROL and rates of RWL would be positively correlated even if O₂ is a nonpolar gas and H₂O is polar. ROL was measured on positions representative of the segments used for RWL. ROL and RWL were plotted against each other for root segments without or with a tight barrier to ROL (Fig. 3e). Indeed, the relationship between ROL and

Fig. 2 H₂ intrusion in roots without or with a tight barrier to radial O₂ loss (ROL) in rice (Oryza sativa). The experimental setup of H₂ intrusion measurements followed the same protocol used for O₂ intrusion measurements. Panel (a) shows an example time trace of H₂ intrusion into segments without or with a tight barrier to ROL at two external pH₂ (c. 6.2 and c. 31.3 kPa); examples from artificial roots of silicone rubber are shown for comparison. Assessment of intrusion rates (b) for roots without or with a tight barrier were conducted at the point at which c. 50% quasi-steady state was achieved and is shown as box–whisker plots (mean, ±; median, horizontal line; 50% quartiles, box; minimum and maximum values, whiskers). Number of replicates are n = 6 or 7 without a barrier, n = 6 with a tight barrier and n = 4 for artificial roots; level of replication is one root per plant. Comparisons between root types were tested using Tukey’s test (***, P < 0.001; ****, P < 0.0001); statistical comparisons did not include artificial roots. Examples of radial H₂ tissue profiles from root segments without or with a tight barrier to ROL with an external pH₂ of c. 6.2 and c. 31.3 kPa are shown in (c) and (d), respectively, obtained at c. 50% of quasi-steady state. Distance ‘0’ indicates root surface with negative distance showing bulk water H₂ and positive distance showing tissue H₂. Number of replicates are n = 5 for roots without a barrier and n = 3 for roots with a tight barrier; level of replication is one root segment per plant. For all experiments, an additional H₂ microsensor was used to monitor the bulk water H₂.
RWL was positive and strongly significant ($r^2 = 0.89$, $P < 0.0001$).

**O₂** leakage into anoxic medium and visualisation of the apoplastic barrier

Methylene blue was used to qualitatively assess the patterns of ROL of intact roots without or with a tight barrier to ROL. Roots grown in aerated nutrient solution stained blue after 1 h 45 min confirming that no barrier had formed (Fig. 4a); the blue halo enveloping the root was due to molecular O₂ leaking into the anoxic medium. By contrast, roots formed in stagnant, deoxygenated solution only showed leakage of O₂ close to the root tip (Fig. 4b). These results validated our approach using root segments taken 30 mm behind the root tip, as these would not have formed a barrier when grown in aerated solution or clearly have formed a barrier when grown in stagnant, deoxygenated nutrient solution.

Moreover, periodic acid was used to visualise an apoplastic barrier in roots grown in aerated or stagnant nutrient solution. Root segments from aerated solution were highly permeable; the entire cross-section stained purple (Fig. 4c). However, roots from
stagnant nutrient solution had formed a strong apoplastic barrier; the diffusion of periodic acid was completely blocked within the first 50–75 µm from the root surface (Fig. 4d). The outer cell layers stained purple, but the apoplastic barrier prevented the periodic acid from reaching the cortex, further confirming our experimental approach.

Apparent permeance ($P_a$) of O$_2$, H$_2$ and water vapour

To enable a direct comparison of the diffusive resistance of the root ROL barrier to O$_2$, H$_2$ and water vapour, we calculated the apparent permeance ($P_a$, Table 1). The $P_a$ for O$_2$ and H$_2$ of root segments without a ROL barrier was similar (3.66 and 2.63 m s$^{-1}$ × 10$^{-5}$, respectively), whereas $P_a$ was substantially higher for water vapour (475 m s$^{-1}$ × 10$^{-5}$), being 180-fold and 130-fold higher than H$_2$ and O$_2$, respectively. For roots with a tight barrier, it was not possible to calculate the $P_a$ for O$_2$ as the intrusion of O$_2$ was below the detection limit (Fig. S2b). While the $P_a$ for water vapour in roots with a tight barrier (30 m s$^{-1}$ × 10$^{-5}$) was reduced by 94% compared with roots without a barrier, it was still 35-fold higher compared with H$_2$ (0.86 m s$^{-1}$ × 10$^{-5}$).

Discussion

We found that the barrier to ROL in roots of rice greatly restricted radial diffusion of both O$_2$ and H$_2$ although H$_2$ is 59% smaller in covalent radius and has an 2.1-fold faster diffusion compared with O$_2$ (Zhang & Cloud, 2006). Importantly, radial diffusion of water was also greatly restricted by the barrier, as demonstrated by the slow loss of tissue water and implying that the properties of the barrier restricted diffusion of nonpolar (O$_2$ and H$_2$) as well as of polar (H$_2$O) gases.

Table 1 Apparent permeance, $P_a$ (m s$^{-1}$ × 10$^{-5}$), for O$_2$, H$_2$ and water vapour without or with a tight barrier to radial O$_2$ loss (ROL) in rice (Oryza sativa).

<table>
<thead>
<tr>
<th>Type of barrier</th>
<th>O$_2$</th>
<th>H$_2$</th>
<th>Water vapour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without barrier</td>
<td>3.66</td>
<td>2.63</td>
<td>475</td>
</tr>
<tr>
<td>Tight barrier</td>
<td>na</td>
<td>0.858</td>
<td>30.1</td>
</tr>
<tr>
<td>Artificial root</td>
<td>6.93</td>
<td>1.08</td>
<td>6.93</td>
</tr>
</tbody>
</table>

Rice was grown in aerated solution (without barrier) for 21 d after which half the plants were transferred to stagnant, deoxygenated nutrient solution (without a barrier). Measurements were taken 7–10 d after transferring the plants (n = 4–6).

The ROL barrier restricts radial diffusion of O$_2$ and H$_2$

Measurements based on segments showed that the barrier virtually blocked all intrusion of O$_2$ at pO$_2$ 20.6 and 60 kPa (Fig. 1c). After reducing tissue respiration by 81%, there was no detectable intrusion of O$_2$ (Fig. S2a,b), but the remaining respiration could still mask some intrusion. Consequently, we suggest that the ROL barrier could allow for a negligible intrusion of O$_2$, which is consumed in respiration within the first few cell layers, preventing O$_2$ to accumulate in the cortex. Indeed, ROL measurements showed some O$_2$ loss, although small (Fig. 3e).

We also found that the barrier restricted radial diffusion of H$_2$ but not to the same extent as for O$_2$. Given enough time, root segments without a barrier as well as segments with a tight barrier would reach the same pH$_2$ inside and outside the cortex.
The radial H₂ profiles (Fig. 2c,d) were overall similar to those of O₂ (Fig. 1d,e) showing a flat plateau in the cortex and another one, but with lower values, in the stele. For H₂, the lower values of the stele is likely caused by the lower diffusivity of stelar tissue (lower gas-filled porosity (Pedersen et al., 2020)). For O₂, the decline within the stele is caused by lower diffusivity and also by the higher O₂ demand of the stelar tissue. The radial concentration profiles obtained for O₂ on these root segments match those of intact roots from barley (Kotula et al., 2015), maize (Darwent et al., 2003), chickpea (Colmer et al., 2020), pea (Armstrong et al., 2009), and some wild wetland plants (Herzog & Pedersen, 2014), all showing higher O₂ status in the cortex compared with the stele.

The findings related to H₂ suggest that H₂ could be used to gauge the tightness of the barrier to ROL, as it behaves similar to O₂ but with much higher mobility due to its smaller molecular size and resulting in faster diffusion. Moreover, the fact that the barrier restricts the movement of such a small molecule indicates that it could also restrict the movement of bigger molecules such as H₂S or volatile organic acids as suggested by Colmer (2003b).

Finally, H₂ in plants has lately become a growing research area due to its important properties. For example, H₂ in plants treated with H₂-rich water has been shown to enhance the tolerance to stresses such as salt (Xu et al., 2013; Wu et al., 2020), metal toxicity (Wu et al., 2015), drought or low temperatures (Jin et al., 2013). It is therefore important to better understand the diffusive properties of H₂ in plant tissues and potential barriers that restrict diffusion, such as the barrier to ROL.

The ROL barrier restricts RWL

We assessed the tightness of the barrier to water vapour by measuring desiccation of root segments without or with a barrier to ROL. A similar approach using root segments has previously been used to demonstrate the importance of the exodermis as a barrier to RWL comparing species with (maize, onion and sunflower) or without an exodermis (pea, bean and wheat); the study found that the exodermis restricted water loss (Taleisnik et al., 1999). The present study is distinguished from the former using three phases of desiccation, whereas segments with a barrier only showed two (Fig. 3c). We speculate that the initial phase present for both root types (showing high rates of RWL) is primarily caused by mass flow in the apoplast externally to the exodermis. As desiccation progresses, root shrivelling occurs and the structural deformation of the segments imposed additional diffusional restrictions. This does not occur in segments with a weak or a tight barrier and they therefore preserve their natural shape.

In conclusion, the root barrier to ROL present a substantial barrier also to water. This is further revealed by comparing the apparent permeance (Pₐ) for water to other plant tissues. The Pₐ of rice roots in the present study is only 1.7-fold higher than that of a bell pepper, *Capsicum annuum* (Lendzian & Kerstiens, 1991), 2.4-fold higher than that of a conifer needle, *Abies alba* (Lendzian et al., 1986) but 58-fold higher than the median of the leaf cuticles of 60 species (Riederer & Schreiber, 2001). By contrast, the Pₐ of the rice root is only 0.3% of the leaf of *Potamogeton lucens* (Schönherr, 1976); living completely submerged, there is no cuticle to restrict desiccation in this aquatic plant.

Finally, when water becomes limiting, roots can shrink and lose contact with the soil potentially further restricting water uptake (Carminati et al., 2009). Even extensive transpiration can cause shrinking (Carminati et al., 2017), but water uptake can be restored by closing the root–soil gaps (Faiz & Weatherley, 1982). Consequently, loss of root–soil contact can be delayed if the barrier prevents shrinking (see Fig. 3a).

The relationship between ROL and RWL

The strong relationship between ROL and RWL suggests that RWL can possibly be used as a proxy for ROL in screening root phenotypes. ROL measurements require specialised equipment and are typically ‘destructive’, as the entire plant is used when applying the traditional root-sleeving electrodes (Armstrong, 1979) or O₂ microsensors (Manzur et al., 2015). By contrast, RWL can be assessed on a number of individual root segments and requires only an analytical balance connected up to a computer (Taleisnik et al., 1999).

In the present study, we provide a model to predict ROL based on RWL. We suggest to further expand such relationship using a
range of rice genotypes, species in the genus of *Oryza*, wetland species (e.g. *Phragmites australis*) and species of *Echinochloa* and *Urochloa*. A multispecies approach would capture potential species-specific differences in the chemical composition of the ROL barrier and provide a range in root dimensions. We have normalised ROL and RWL data to SA : V to correct for the higher tendency for both O₂ and water to be lost from thin roots compared with thick roots. It is possible that this correction is insufficient when comparing roots that differ several fold in root diameter. Therefore, a multispecies approach is likely to allow for understanding of the drivers of radial diffusion of O₂ and water and also a better normalisation of data.

**Radial O₂ intrusion as a proxy for ROL**

The present study introduced a new experimental approach to characterise the strength of diffusion barriers in roots. Rather than assessing radial loss rates of the target gas (e.g. O₂), we measured intrusion of the target gas from the exterior bulk medium into the root cortex using detached root segments. This approach has at least three advantages over the conventional methods using intact plants for ROL measurements obtained by either root-sleeving electrodes (Armstrong, 1979) or O₂ microsensors (Manzur et al., 2015): (1) The magnitude of ROL depends on the strength of the barrier and the internal O₂ status of the cortex (Armstrong et al., 2000). The O₂ status is higher closer to the source, that is the root–shoot junction. The further away from the source, the lower the O₂ status as O₂ might have been lost via ROL or consumed in respiration (Darwent et al., 2003). Using detached segments with sealed cut ends eliminates the problem of longitudinal internal gradients of O₂. The segments enabled characterisation of diffusion while manipulating the concentration gradients of O₂ or other gases. (2) Using root segments conserves experimental material, making such measurements possible with limited numbers of individual plants. (3) Working with gases other than O₂ (such as H₂) is not feasible using conventional approaches as such gases would first have to accumulate inside the roots before any loss rates from the tissues to the exterior could be detected.

However, there are some considerations to account for when using this approach. When working with biologically active gases (e.g. O₂), consumption or production of the gas should be reduced to obtain representative intrusion rates. In addition, emerging laterals could act as intrusion points (windows) bypassing the barrier to ROL (Armstrong & Armstrong, 2001, 2005). Finally, tissue porosity must be accounted for; if the tissue is dense, gas intrusion will be slowed by diffusional resistance in addition to a possible barrier to ROL.

**Conclusion**

The barrier to ROL in roots of rice can be considered as a ‘Jack of all trades’ as the trait restricts radial diffusion of O₂, H₂ and also water. As the barrier is able to slow the diffusion of these gases, it could as well restrict the intrusion of gaseous sulphide (H₂S) as has been previously suggested (Armstrong & Armstrong, 2005; Soukup et al., 2007; Nishiuchi et al., 2012). Although the barrier to ROL was first described as a root trait conferring flood tolerance, the results of the present study related to RWL suggest that the barrier could also play a potential role in dry soils. We speculate that the barrier to ROL could restrict water loss in dry top soils, if soil desiccation follows a period of waterlogging when the barrier to ROL would have been formed. It would not be the first time that a well described trait serves multiple purposes; aerenchyma formation is another example, as it improves survival of roots during flooding as well as under drought stress (Zhu et al., 2010; Yamauchi et al., 2020). Finally, the strong positive relationship between ROL and RWL presents an exciting case in which simple measurements of RWL potentially can be used to predict ROL in screening studies with the focus on waterlogging tolerance.

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**Author contributions**

LLPO and OP planned and designed the research. LLPO performed all experiments and analysed data. EP performed ROL experiments and subsequent data analysis. LLPO and OP wrote the manuscript. EP wrote and provided input for RWL and ROL sections. LLPO, EP and OP all provided comments to the final version of the manuscript.

**ORCID**

Ole Pedersen https://orcid.org/0000-0002-0827-946X
Elisa Pellegrini https://orcid.org/0000-0002-6972-9540
Lucas León Peralta Ogorek https://orcid.org/0000-0002-9178-7050

**References**


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Theoretical relationship between root diameter and surface area to volume ratio (SA : V) of a cylinder, uncorrected RWL–ROL data, and example ROL profile using microoptodes.

**Fig. S2** Respiration rates of roots without a barrier to ROL and O2 intrusion in roots with a tight barrier to radial O2 loss (ROL) in rice (*Oryza sativa*).

**Fig. S3** Radial water loss in root segments without or with tip, without or with laterals, and without or with a tight barrier to radial O2 loss (ROL) in rice (*Oryza sativa*).

**Methods S1** Detailed Materials and Methods.

**Notes S1** RWL results of root segments with tip and laterals.

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