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Lateral roots, in addition to adventitious roots, form a barrier to radial oxygen loss in *Zea nicaraguensis* and a chromosome segment introgression line in maize

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Introduction

Changes in climate during the past 50 yr have resulted in an increase in flood events (Pedersen *et al.*, 2017). Periods of intense precipitation can lead to soil flooding (waterlogging), a condition that may also result from slow drainage or poor irrigation management (Sasidharan *et al.*, 2017). During waterlogging, soil anoxia can occur as the gas spaces within the soil are replaced by water and plant roots and soil microorganisms consume O₂ in respiration but the slow molecular diffusion of O₂ in water, compared with in air, restricts replenishment of O₂ from the atmosphere (Ponnamperuma, 1972). The capacity for internal aeration of roots is an important trait for plant growth in flooded soils. This paper provides a comparative analysis of *Zea mays* ssp. *mays* (maize), *Zea nicaraguensis* (a wild relative of maize, which has high waterlogging tolerance; Itlis & Benz, 2000; Mano & Omori, 2007) and a unique chromosome segment introgression line (IL) of the wild relative *Z. nicaraguensis* into maize, to improve knowledge of internal aeration and radial O₂ loss (ROL) of lateral roots.

Plants typically respond to waterlogging by producing new adventitious roots with aerenchyma and many wetland plants form a root barrier to radial O₂ loss (ROL), but it was not known if this was also the case for lateral roots.

We tested the hypothesis that lateral roots arising from adventitious roots can form a ROL barrier, using root-sleeving electrodes and O₂ microsensors to assess ROL of *Zea nicaraguensis*, the maize (*Zea mays* ssp. *mays*) introgression line with a locus for ROL barrier formation (introgression line (IL) #468) from *Z. nicaraguensis* and a maize inbred line (Mi29).

Lateral roots of *Z. nicaraguensis* and IL #468 both formed a ROL barrier under stagnant, deoxygenated conditions, whereas Mi29 did not. Lateral roots of *Z. nicaraguensis* had higher tissue O₂ status than for IL #468 and Mi29. The ROL barrier was visible as suberin in the root hypodermis/exodermis. Modelling showed that laterals roots can grow to a maximum length of 74 mm with a ROL barrier, but only to 33 mm without a barrier.

Presence of a ROL barrier in lateral roots requires reconsideration of the role of these roots as sites of O₂ loss, which for some species now appears to be less than hitherto thought.

Summary

- Plants typically respond to waterlogging by producing new adventitious roots with aerenchyma and many wetland plants form a root barrier to radial O₂ loss (ROL), but it was not known if this was also the case for lateral roots.
- We tested the hypothesis that lateral roots arising from adventitious roots can form a ROL barrier, using root-sleeving electrodes and O₂ microsensors to assess ROL of *Zea nicaraguensis*, the maize (*Zea mays* ssp. *mays*) introgression line with a locus for ROL barrier formation (introgression line (IL) #468) from *Z. nicaraguensis* and a maize inbred line (Mi29).
- Lateral roots of *Z. nicaraguensis* and IL #468 both formed a ROL barrier under stagnant, deoxygenated conditions, whereas Mi29 did not. Lateral roots of *Z. nicaraguensis* had higher tissue O₂ status than for IL #468 and Mi29. The ROL barrier was visible as suberin in the root hypodermis/exodermis. Modelling showed that laterals roots can grow to a maximum length of 74 mm with a ROL barrier, but only to 33 mm without a barrier.
- Presence of a ROL barrier in lateral roots requires reconsideration of the role of these roots as sites of O₂ loss, which for some species now appears to be less than hitherto thought.
Interestingly, soil phytoxins can trigger the formation of a ROL barrier (Armstrong & Armstrong, 1999; Kotula et al., 2014) and for roots of rice this can occur at subtoxic concentrations (Colmer et al., 2019). For low-molecular-weight carboxylic acids, the root barrier to ROL was shown to be induced in Hordeum marinum (Kotula et al., 2014) as well as in rice (Armstrong & Armstrong, 2001; Colmer et al., 2019). Suberin is a likely component of the ROL barrier as it is deposited in the walls of root hypodermis/exodermis cells (Armstrong et al., 2000; Soukup et al., 2007; Garthwaite et al., 2008; Kotula et al., 2009). Indeed, we found that many suberin biosynthesis-related genes were upregulated within the outer parts of rice roots during ROL barrier formation, assessed by transcriptome analyses of laser-microdissected tissues (Shiono et al., 2014). Moreover, histochemical staining revealed that suberin lamellae were formed at the hypodermis/exodermis of ROL barrier-induced roots in rice (Kotula et al., 2009; Ranathunge et al., 2011) and Phragmites australis (Armstrong et al., 2000; Soukup et al., 2007). Thus, suberin lamellae formation at the root hypodermis/exodermis has been associated with ROL barrier formation. However, suberin lamellae were also observed at the hypodermis/exodermis of adventitious roots in Zea mays ssp. mays (maize, which does not form a ROL barrier) as well as in Z. nicaraguensis, which can form a ROL barrier (Abiko et al., 2012; Watanabe et al., 2017). These observations showed that the root ROL barrier formation is not always distinguished by histochemical staining of suberin, and thus the permeability of suberin lamellae to O2 may depend on the suberin monomer composition and/or the structure (Watanabe et al., 2017).

The ROL barrier has so far mainly been studied for the main axis of adventitious roots, with few studies considering whether thin lateral roots can also form a ROL barrier. Thin lateral roots are of high surface area to volume ratio, and so ROL would significantly reduce the longitudinal O2 diffusion along these roots. In the wetland plants P. australis and rice, the lateral roots appeared to be major sites of soil oxygenation (Armstrong, 1970) and there were no indications that these laterals possessed a barrier to ROL (Armstrong et al., 1996). A more recent study on rice, however, showed that ROL from laterals declined substantially as a response to application of sulphide but the lateral roots still showed substantial ROL (Armstrong & Armstrong, 2005). We have recently observed for the wetland plant Z. nicaraguensis that lateral roots can grow up to several cm in length; such lengths of lateral roots in waterlogged, anoxic soils might not be possible in the absence of a ROL barrier unless these roots had formed substantial aerenchyma (Armstrong & Beckett, 1987).

A set of Z. nicaraguensis chromosome segment introgression lines (ILs) in maize (inbred line Mi29) was produced by Mano & Omori (2013). The ILs were used to identify the chromosomal region involved in controlling the ROL barrier formation in the main axis of adventitious roots, and the locus for the root ROL barrier formation was located on the short-arm of chromosome 3 of Z. nicaraguensis (Watanabe et al., 2017). IL #468 contains the short-arm of chromosome 3 and can form a ROL barrier in adventitious roots (Watanabe et al., 2017). Using Z. nicaraguensis, Mi29 and IL #468, in the present study, we assessed ROL from the main axis of adventitious roots and also for the fine lateral roots by root-sleeving electrodes and O2 microsensors in order to address the overall question related to whether lateral roots can also form a barrier to ROL. We hypothesised that not only thick adventitious roots, but also the long and thin lateral roots of Z. nicaraguensis, possess a barrier to ROL to restrain O2 loss, despite the large surface area to volume of these roots. Furthermore, we hypothesised that the maize introgression line possessing the locus for a ROL barrier (IL #468) forms a barrier to ROL in adventitious roots when grown under experimental conditions mimicking soil waterlogging and that the barrier, if present, is also expressed in lateral roots. The present evaluation of lateral root ROL is of importance for understanding wetland root ecophysiology, as in some wetland species lateral roots are regarded as a major site of ROL (Sorrell et al., 2000).

Materials and Methods

Plant material

Maize (Zea mays ssp. mays) inbred line Mi29 was provided by the Kyushu Okinawa Agricultural Research Centre, NARO, Japan, and Z. nicaraguensis (CIMMYT 13451), a wild relative of maize, was provided by the International Maize and Wheat Improvement Centre (CIMMYT), Mexico. Moreover, IL #468 possessing a segment of chromosome 3, which includes a locus for ROL barrier formation, as well as segments of chromosomes 9 and 10 of Z. nicaraguensis in the genetic background of Mi29 (Watanabe et al., 2017), was used. Waterlogging tolerance in IL #468 is intermediate between those in Z. nicaraguensis and Mi29 (M. Nakazono et al., unpublished).

Growth conditions

Seeds of Mi29, Z. nicaraguensis and IL #468 were surface-sterilised with 10% (v/v) hydrogen peroxide for 2 min (Naredo et al., 1998), rinsed with deionised (DI) water and placed at the upper end within a roll of wet filter paper. The rolled papers were transferred into 2 l grey plastic pots with 200 ml DI water, with the base of the rolled papers in the water, and incubated in a growth chamber (LH-411SP; Nippon Medical & Chemical Instruments Co. Ltd, Osaka, Japan) for 4 d at 28°C (the same growth chamber and temperature was used for the plant cultures; to be described later). On the 5th day, the seedlings were exposed to light for 1 d (PAR of 250–300 µmol m⁻² s⁻¹; 14 h:10 h, light:dark). After the shoots had grown to a height of at least 3 cm, seedlings were transplanted to 51 grey pots (250 mm height x 190 mm length x 120 mm width; four plants per pot) containing 4.5 l half-strength nutrient solution. The composition of the nutrient solution at full strength was 1.0 mM NH₄NO₃, 0.50 mM NaH₂PO₄, 0.30 mM K₂SO₄, 0.30 mM CaCl₂, 0.60 mM MgCl₂, 0.045 mM Fe-EDTA, and 50 µM H₂B₄O₇, 9.0 µM MnSO₄, 0.30 µM CuSO₄, 0.70 µM ZnSO₄, 0.10 µM Na₂MoO₄ (Mae & Ohira, 1981) with 5.0 mM 2-ethanesulfonic acid (MES) buffer and adjusted to pH 5.5 using KOH (Abiko et al., 2000).
The microprofiling of O₂ consisted of three different experiments (aerated as well as stagnant, deoxygenated) were renewed every 7 d. Control plants of *Z. nicaraguensis* were grown in aerated nutrient solution; the solutions (aerated as well as stagnant, deoxygenated) were renewed every 7 d.

For microsensor profiling of O₂, evaluation of ROL barrier formation, methylene blue staining and histochemical staining of suberin, we used adventitious roots (3rd nodal roots), emerging after transfer to stagnant, deoxygenated solution; the plants were 21-d-old to 25-d-old when these roots were severed. These had 33–55 lateral roots in *Z. nicaraguensis* and 15–21 lateral roots in Mi29 and the length of the lateral roots differed slightly so that laterals were c. 40 mm in *Z. nicaraguensis* and c. 30 mm in Mi29; we chose roots with the largest number of laterals.

O₂ microprofiles in the diffusive boundary layer and in root tissues

The measurements of radial O₂ fluxes in the diffusive boundary layer and associated internal tissue profiles followed the approach of Colmer *et al.* (2020). An intact plant was placed in a glass chamber (250 × 60 × 62 mm), hereafter referred to as a ‘flume’, into which deoxygenated nutrient solution flowed with a mean flow velocity of 0.63 mm s⁻¹ (see Supporting information Fig. S1 for a diagram of the experimental set-up). The target root was mounted onto a series of 10 vertically orientated 1.2 mm glass plates, with the root held by four stainless-steel rings attached to four of the glass plates. Measurements were taken on 52–109 mm length (median = 82 mm, n = 20) adventitious roots and 5–25 mm length (median = 13 mm, n = 13) lateral roots. The microprofiling of O₂ consisted of three different experiments: (1) assessment of the radial O₂ fluxes in the diffusive boundary layer, and the O₂ status of cortex; (2) radial O₂ profiles across tissues (exodermis, cortex and stele) of adventitious and lateral roots; and (3) visualisation of O₂ distribution within the main axis of an adventitious root at the point where a lateral root had emerged.

(1) For measurements of O₂ in the diffusive boundary layer and outer root tissues, a 25-µm tip diameter microsensor (OX25, Unisense A/S) was mounted on a micromanipulator (MM33-2, Unisense A/S), which was fitted on a motorised stage (1D Motorised MicroProfiling System, Unisense A/S) connected to a picoamperemeter (OxyMeter; Unisense A/S, Denmark) and the sensor signals were collected with data acquisition software (SENSORTRACE SUITE v.2.3.100; Unisense A/S). Measurements were taken for intervals of 25 µm for the tip of the microsensor, starting 500 µm away from the root surface and at ending c. 500 µm into the root tissue. Measurements were not taken for aerated Mi29 or IL #468, as both are already known not to form a barrier to ROL when in aerated medium (Abiko *et al.*, 2012; Watanabe *et al.*, 2017).

(2) Additional high-resolution tissue measurements of O₂ were obtained with a 10-µm tip diameter microsensor (OX10; Unisense A/S). O₂ profiles were taken with a spatial resolution of 10 µm starting c. 100 µm away from the root surface and penetrating one side of the cortex, the whole stele and into the opposite side of the cortex before exiting the root tissue on the other side of the root.

(3) Finally, we mapped the tissue O₂ distribution in a complex network of lateral roots emerging from the main axis of an adventitious root. The purpose was to describe internal tissue O₂ gradients within a position where a lateral root penetrated the cortex of the main axis. These detailed O₂ profiles were also taken with a spatial resolution of 10 µm starting c. 100 µm away from the root surface and penetrating one side of the cortex, the whole stele and into the opposite side of the cortex before exiting the root tissue on the other side of the root for the main axis as well as for the lateral root.

Bulk medium pO₂ in the flume was measured c. 5 mm away from the root using an O₂ optode (OP–MR, Unisense A/S) connected to an optode meter (MicroOptode Meter, Unisense). All measurements were taken at 27°C.

Radial O₂ loss from roots measured by root-sleeving electrodes

Plants were transferred to an acrylic chamber with the roots immersed in deoxygenated solution containing 0.1% (w/v) agar, 5.0 mM KCl and 0.50 mM CaSO₄ (Colmer *et al.*, 1998). Each plant was mounted to ensure that the root–shoot junction was kept at 30 mm below the surface of the deoxygenated medium and with the shoot in air. The measurements were taken in the light at 28°C in the growth chamber with conditions the same as during plant growth. A cylindrical platinum electrode (inner diameter 1.0 mm, height 5.0 mm) was placed around a selected lateral root (c. 30 mm in length) of 80–120 mm long adventitious root. ROL was measured at distances of 5, 10 and 20 mm behind the root tip, according to Watanabe *et al.* (2017).

Respiration rates of root tissues

Respiration was measured as O₂ consumption by excised root segments and followed the procedure described in Colmer *et al.* (2019) using a MicroResp system (Unisense A/S, Aarhus, Denmark). Two 10 mm long root segments were excised from 65–90 mm long adventitious roots immediately below the youngest lateral root and inserted into a 4-ml glass vial containing fresh incubation medium (see the ‘Growth conditions’ subsection). Each vial was fitted with a stir bar and stirring rate was set at 600 rpm using the stirrer controller unit (MR2-St-Co, Unisense A/S) and O₂ was measured in each vial using an O₂ optode (OP–MR; Unisense A/S) every 10 to 15 min during which the external O₂ pO₂ decreased from c. 21 kPa to 18 kPa. Wicks with incubation medium but without root tissues served as blanks. O₂...
consumption rates were calculated as the difference in O₂ concentration (µmol O₂ l⁻¹) between two time points; any background O₂ consumption of the medium was subtracted before multiplying by the vial volume (l). Finally, the rate was divided by the fresh mass (FM, g) of the tissue.

Visualisation of radial O₂ loss from roots by methylene blue staining

Methylene blue can be used for qualitative assessments of spatial ROL profiles from roots (Armstrong & Armstrong, 1988) because the reduced form is colourless, whereas the oxidised form shows blue colour. Methylene blue was at a concentration of 13 mg l⁻¹ in a deoxygenated solution containing 0.1% (w/v) agar, also containing sodium dithionite (Na₂S₂O₄) at 130 mg l⁻¹. One adventitious root with a length of 80–120 mm was selected, and all other roots were trimmed off immediately before use. The plant was transferred to the methylene blue solution in an acrylic tank (c. 25°C under white light) and held with the root–shoot junction positioned at 30 mm below the surface of the solution. After 15–20 min, the staining patterns of methylene blue around the lateral roots were evaluated.

Root aerenchyma and suberin staining

Root cross-sections were prepared from lateral roots at a length of 20–30 mm. Root segments were sampled at distances of 2.5–7.5, 7.5–12.5 or 17.5–22.5 mm behind the tip of a lateral root, and embedded in 4% (w/v) agar. Segments of 80-µm thickness were cut using a vibrating microtome (VT 1200S; Leica Biosystems Nussloch GmbH, Nussloch, Germany). Adherent agar was removed in a clearing solution (2 g ml⁻¹ chloral hydrate in 25% (v/v) glycerol) for 1 h at 70°C. After clearing, the cross-sections were washed several times with warm DI water.

The root cross-sections were mounted in water and viewed with bright field illumination using a light microscope (BX60; Olympus, Tokyo, Japan) fitted with a charged coupled device (CCD) camera (DP70; Olympus). Areas occupied by aerenchyma were measured using IMAGEJ software (v.1.46r; National Institutes of Health, Bethesda, MD, USA). The percentage of aerenchyma compared with the entire root-cross-sectional area was calculated.

Suberin lamellae in the cross-sections were detected by staining with 0.01% (w/v) Fluorol Yellow 088 (Sigma-Aldrich) in polyethylene glycol 400 (PEG-400) for 1 h in darkness at room temperature. Excess stain was washed away by several rinses with warm DI water. Suberin lamellae were detected with a CCD camera (DFC310FX; Leica) as yellow fluorescence upon excitation with ultraviolet (UV) light under a fluorescence microscope (DM500B; Leica).

Data analysis

Calculation of O₂ fluxes measured by O₂ microsensors used the equation from Henriksen et al. (1992) that describes the molecular diffusion of solutes (ions or dissolved gases) from, or into, a cylinder in aqueous solution. For O₂:

\[ J_{O_2} = \frac{D_{O_2} [O_2]_{200} - [O_2]_0}{r_0 \log (r_{200}/r_0)} \]

where \( J_{O_2} \) (mol m⁻² s⁻¹) is the O₂ flux, \( D_{O_2} \) is the diffusion coefficient of O₂ at 27°C (2.2 × 10⁻⁹ m² s⁻¹; Ferrell & Himmelblau, 1967), \( r_0 \) (m) is the radius of the root, \( r_{200} \) is the radius of the root +200 µm, \([O_2]_0\) is the O₂ concentration (mol m⁻³) at the root surface and \([O_2]_{200}\) is the O₂ concentration (mol m⁻³) 200 µm away from the root surface, but still within the diffusive boundary layer of both adventitious roots and thin laterals. The O₂ concentrations at the two positions within the diffusive boundary layer were derived from linear regression of the O₂ from profiling.

GraphPad PRISM 8.0 software was used for statistical analyses and graphing of data. Figure and table captions provide the details on numbers of replicates, the various statistical tests used as well as significance levels.

Results

Root tissue O₂ status

Tissue O₂ status of the cortex of adventitious roots and their fine laterals was assessed with O₂ microsensors for roots in a deoxygenated, slowly flowing medium within the flume chamber (see Fig. S1), that is root O₂ status was reliant on internal movement of O₂ from the shoot in air and into and along the roots. Radial microprofiling showed that O₂ of the root medium was low (Z. nicaraguensis, Fig. 1a,b) but in some cases O₂ increased towards the root surface (Mi29, Fig. 1c,d) for adventitious roots as well as for the fine laterals. The increase in O₂ for some of the roots was caused by radial O₂ loss (ROL); see the ‘Radial O₂ loss and ROL barrier formation’ subsection.

Roots of Z. nicaraguensis grown in stagnant, deoxygenated root medium before transfer into the flume, showed significantly higher tissue O₂ status than Mi29 (also grown in stagnant, deoxygenated root medium) both for adventitious roots and laterals (Fig. 1a–d). For adventitious roots, mean cortex pO₂ was 14.0 kPa in Z. nicaraguensis but only 5.5 kPa in Mi29; lateral roots, mean cortex pO₂ was 10.4 kPa in Z. nicaraguensis and 4.9 kPa in Mi29 (Fig. 1a–d). The substantially higher tissue O₂ status was caused by a root barrier to ROL formed by Z. nicaraguensis when in stagnant, deoxygenated solution, but not by Mi29.

Zea nicaraguensis, Mi29 and IL #468 were all grown in deoxygenated, stagnant solution, a condition known to stimulate a root barrier to ROL in Z. nicaraguensis (Abiko et al., 2012). For comparison, we also included Z. nicaraguensis grown in aerated medium, a condition in which a root barrier to ROL is not induced (Abiko et al., 2012). In addition to our main focus on cortex tissue O₂ status near the base of the root where numerous laterals had emerged, we also took O₂ measurements of cortex tissue near the root apex where laterals had not yet been formed (see Fig. S2 for an example of a radial O₂ profile obtained 10 mm behind the root tip in Z. nicaraguensis grown in stagnant,
Fig. 1 Examples of O\textsubscript{2} profiles in the diffusive boundary layer (negative distances) and 300 µm into the root tissues (positive distances) of *Zea nicaraguensis* and maize inbred line Mi29 (a–d), example of an adventitious root with laterals and the two O\textsubscript{2} sensors (e) and summary of root tissue O\textsubscript{2} status (f). The profiles were taken with a Clark-type O\textsubscript{2} microsensor with tip diameter of 10 µm (tip indicated with * in (e)) and medium O\textsubscript{2} was monitored with an O\textsubscript{2} optode with a tip diameter of 500 µm (tip indicated with ** in (e)). Bar, 5 mm. Mean root cortex pO\textsubscript{2} are shown in (f) for the main apex or base of the target adventitious root as well as for the cortex of 5–25 mm long lateral roots of *Z. nicaraguensis* (roots in aerated or deoxygenated medium), Mi29 or introgression line #468 (IL #468) (both with roots in deoxygenated medium). The box–whisker plot shows mean (+), median (horizontal line), 50% of the observations (box) and minimum or maximum values (error bars); n = 3 or 4 (replicates represent individual plants from which one adventitious root was used). For panel (f), the results of a two-way ANOVA testing species/treatment and root type/position are shown in the panel. The plants were grown with the roots in deoxygenated medium and measurements were on intact 21-d-old to 24-d-old plants with the roots immersed in hypoxic (2–5 µmol O\textsubscript{2} l\textsuperscript{-1}) and slowly flowing nutrient solution and with the shoot in air; see Supporting Information Fig. S1 for experimental diagram. Measurements were taken in dim light (<5 µmol photons m\textsuperscript{-2} s\textsuperscript{-1}) and at 27°C (O\textsubscript{2} solubility = 249 µmol O\textsubscript{2} l\textsuperscript{-1} corresponding to pO\textsubscript{2} of 20.6 kPa). ns, not significant.
deoxygenated solution). A two-way analysis of variance (ANOVA) showed highly significant effects of ‘genotype and growth conditions’ and also significant effects of the ‘position of measurements’, but with no significant interaction (Fig. 1). However, cortex tissue O₂ status of IL #468 did not differ from that of *Z. nicaraguensis* grown aerated or Mi29 grown in stagnant, deoxygenated solution; the lack of difference was present both in adventitious roots (base and root tip) as well as in lateral roots (Fig. 1f). This finding indicated that IL #468 either: (1) did not form a root barrier to ROL; or (2) if it did form a ROL barrier, this did not result in higher cortex tissue O₂ status.

**Respiration**

Respiration was measured as O₂ uptake of 10-mm long root segments excised from adventitious roots immediately below the youngest emerging lateral root. These respiration measurements of root tissues showed no significant differences in root respiration rates among *Z. nicaraguensis*, Mi29 or IL #468 when grown in aerated solution (Table 1). For practical reasons, it is not possible to measure respiration as O₂ uptake by root segments with a barrier to ROL, as the barrier would greatly restrict O₂ uptake from the solution.

**Radial O₂ loss and ROL barrier formation**

The relatively long lateral roots of *Z. nicaraguensis* and the high O₂ status of the cortex of the lateral roots (section above) indicated that the lateral roots of *Z. nicaraguensis* were also likely to possess a ROL barrier in order to maintain O₂ inside the root. Therefore, we used a cylindrical root-sleeving electrode to measure ROL from intact lateral roots in an O₂-free medium (Fig. 2a). These measurements showed that lateral roots of *Z. nicaraguensis* grown in aerated nutrient solution did not form a barrier to ROL; ROL was high at around 175 nmol m⁻² s⁻¹ along the entire length of the c. 30 mm long lateral roots (Fig. 2b). In stark contrast, lateral roots of *Z. nicaraguensis* formed under stagnant, deoxygenated conditions had lower ROL (c. 50 nmol m⁻² s⁻¹) near the base and also at 10 mm behind the root tip, whereas ROL peaked near the root tip (c. 350 nmol m⁻² s⁻¹), a pattern typical of roots with a barrier to ROL (Colmer, 2003). Importantly, lateral roots of IL #468 grown in stagnant, deoxygenated solution showed a very similar ROL pattern to that of *Z. nicaraguensis*; these lateral roots of IL #468 had formed a barrier to ROL (Fig. 2e), which was not the case for Mi29 grown in stagnant, deoxygenated conditions (Fig. 2d).

The novel finding of an inducible barrier to ROL in the lateral roots of *Z. nicaraguensis* and IL #468 prompted us to confirm the findings using O₂ microsensors (Fig. 3a). The principles of these experiments utilise the fact that exchange of O₂ between the root and the external medium takes place via molecular diffusion across a diffusive boundary layer that envelops all tissues. ROL from roots results in high O₂ on the tissue surface and a linearly declining gradient towards the surrounding root medium when still inside the diffusive boundary layer (Fig. 3b). Under aerated conditions, there is no ROL barrier formed and O₂ is high on the root surface (67 µmol O₂ l⁻¹) and the concentration inside the diffusive boundary layer declines linearly to around 20 µmol O₂ l⁻¹ 250 µm away from the root surface; the O₂ status of the slowly, flowing root medium was around 2–5 µmol O₂ l⁻¹. By contrast, for roots with a barrier to ROL, there was still a gradient in the diffusive boundary layer but in the opposite direction (Fig. 3c) when the external medium contained low amounts of O₂, as the cell layers external to the root ROL barrier consumed some O₂ from the medium. When grown in stagnant, deoxygenated solution, a barrier to ROL was formed by these roots and, in the specific case shown in Fig. 3(c), this leads to O₂ uptake by the outer root cells so that O₂ is highest 250 µm away from the root surface (around 3 µmol O₂ l⁻¹) and lowest on the root surface (below 0.5 µmol O₂ l⁻¹).

We used the O₂ microsensors to measure ROL based on analyses of any O₂ gradient in the diffusive boundary layer. ROL measurements indicated that a barrier to ROL was present in the basal regions of adventitious roots of *Z. nicaraguensis* and IL #468 when grown in stagnant, deoxygenated solution, as these roots all showed O₂ uptake from the ‘deoxygenated’ (i.e. 2–5 µmol O₂ l⁻¹) slowly flowing medium (Fig. 3d). By contrast, the base of adventitious roots of *Z. nicaraguensis* grown in aerated solution and from Mi29 with roots in stagnant, deoxygenated solution both showed substantial loss of O₂ to the surrounding root medium (Fig. 3d). Similarly, for the lateral roots, those of *Z. nicaraguensis* and IL #468 each had a barrier to ROL when these roots were formed in stagnant, deoxygenated solution, whereas the lateral roots of *Z. nicaraguensis* formed in aerated solution, or of Mi29 grown in stagnant, deoxygenated solution, showed no sign of ROL barrier formation (Fig. 3e). Employing contrasting experimental procedures, we have shown that the lateral roots of *Z. nicaraguensis* and IL #468 each form a barrier to ROL when grown in stagnant, deoxygenated nutrient solution.

**Visualisation of the locations of ROL in roots**

The locations of ROL along lateral roots in O₂-deficient media were visualised by staining with methylene blue. Root parts where ROL occurs (without a ROL barrier) would appear blue (i.e. show the presence of O₂), whereas root parts with a ROL

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**Table 1** Root respiration rates of *Zea nicaraguensis*, maize inbred line Mi29 and an introgression line (IL #468) all grown in aerated nutrient solution.

<table>
<thead>
<tr>
<th></th>
<th>Mean (nmol O₂ g⁻¹ FM s⁻¹)</th>
<th>SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zea nicaraguensis</em></td>
<td>5.93</td>
<td>0.18</td>
<td>2</td>
</tr>
<tr>
<td>Mi29</td>
<td>4.92</td>
<td>0.40</td>
<td>4</td>
</tr>
<tr>
<td>IL #468</td>
<td>4.38</td>
<td>0.51</td>
<td>4</td>
</tr>
</tbody>
</table>

Measurements were taken on 10 mm long adventitious root segments from 21-d-old to 24-d-old plants excised immediately below the youngest lateral root; measurements were at 27°C. Replicates represent individual plants from which one adventitious root was severed. The mean rates between Mi29 and IL #468 were not significantly different (Mann–Whitney test, P > 0.05).
barrier would lack or only show slight blue coloration from methylene blue staining. In *Z. nicaraguensis* grown in aerated conditions or Mi29 grown in stagnant, deoxygenated conditions, the entire surface of lateral roots (Fig. 4a,c) were stained blue. By contrast, in *Z. nicaraguensis* and IL #468 grown in stagnant, deoxygenated solution, only the apical parts strongly stained blue, while the rest of the lateral root lacked or was only weakly stained (Fig. 4b,d). These results confirmed that a barrier to ROL is formed in lateral roots of *Z. nicaraguensis* and IL #468, but not in Mi29 grown under stagnant, deoxygenated conditions or in *Z. nicaraguensis* grown in aerated nutrient solution.

**Suberin staining and aerenchyma formation**

In adventitious roots, suberin accumulation has previously been observed in the hypodermis/exodermis of *Z. nicaraguensis*, Mi29 and IL #468 (Watanabe *et al.*, 2017). Similarly, in the lateral roots, suberin was deposited in the hypodermis/exodermis at 5, 10 and 20 mm from the root tips in *Z. nicaraguensis* and IL #468 (Fig. 5b,f,j,d,h,l) and at 10 and 20 mm from the root tips in Mi29 (Fig. 5c,g,k) under stagnant, deoxygenated conditions. In *Z. nicaraguensis* grown under aerated conditions, suberin deposition was also observed at 10 and 20 mm from the root tips of lateral roots (Fig. 5a,e,i).

In *Z. nicaraguensis* grown under aerated conditions, aerenchyma formation in lateral roots was 2.9%, 6.8% and 12.3% at positions 5, 10 and 20 mm, respectively, behind the root tip (Fig. 6). Under stagnant, deoxygenated conditions, the aerenchyma ratios increased to 9.8% at 10 mm and 15.8% at 20 mm (Fig. 6), suggesting that aerenchyma is constitutively formed under aerated conditions and further induced under stagnant, deoxygenated conditions in lateral roots (this study) as well as adventitious roots (Abiko *et al.*, 2012) in *Z. nicaraguensis*. In lateral roots of Mi29 and IL #468, the aerenchyma ratios were similar to each other under stagnant, deoxygenated conditions (Fig. 6), but the aerenchyma ratios of these two genotypes were lower than those of *Z. nicaraguensis* at positions 10 and 20 mm behind the root tips.

**Discussion**

Our study demonstrated that not only the main axis of adventitious roots, but also their lateral roots, can form a barrier to ROL. This observation is novel and improves knowledge of wetland plant ecophysiology, for which previous understanding was that formation of a barrier to ROL is limited to the main axis of adventitious roots and where the fine laterals are seen as sources of substantial ROL limiting maximum root length (Armstrong, 1970; Sorrell *et al.*, 2000). Moreover, we have also shown that our maize IL #468, with a locus for a ROL barrier formation in adventitious roots (Watanabe *et al.*, 2017) forms a ROL barrier also in the lateral roots when grown in stagnant, deoxygenated conditions. Our
Radial O2 loss (ROL) from lateral roots of *Zea nicaraguensis*, maize inbred line Mi29 or introgression line #468 (IL #468). Rates of ROL were measured using a customised Clark-type microsensor with a tip diameter of 10 or 25 µm (a, microsensor indicated by *), and ROL was calculated, using the equation of Henriksen et al. (1992), from O2 measurements in the diffusive boundary layer (b, c) on intact 21-d-old to 24-d-old plants with the roots immersed in hypoxic nutrient solution (2–5 µmol O2 l−1) and with the shoot in air. Rates of ROL (d, e) are shown in a box–whisker plot with medians (horizontal line), 50% of the observations (box) and minimum or maximum values (error bars); n = 3 or 4 (replicates represent individual plants from which one adventitious root was used). In (d) and (e), positive values indicate O2 flux from the roots (i.e. ROL), whereas negative values indicate O2 flux into the roots (i.e. O2 consumption). Different letters indicate significant differences (P < 0.05); Mann–Whitney test. Measurements were taken in dim light (<5 µmol photons m−2 s−1) and at 27 °C (O2 solubility = 249 µmol O2 l−1 corresponding to pO2 of 20.6 kPa) with O2 concentration in the external medium at 2–5 µmol O2 l−1. Bar, 1 mm.

Findings are timely, as ongoing climate change has already resulted in an exponential increase in flood events in recent years (Pedersen et al., 2017) resulting in soil flooding and damage of poorly adapted crops. Below, we discuss the implications of a barrier to ROL in lateral roots, focusing on tissue O2 status.

We used three contrasting approaches to determine the presence or absence of a barrier to ROL in both adventitious roots and their fine laterals. Methylene blue staining is a qualitative approach suitable for visualising sites of ROL along the root (Armstrong & Armstrong, 1988). Mi29 did not form a barrier to ROL in lateral roots and the roots stained blue along the entire root length (Fig. 4). By contrast, and when grown in stagnant, deoxygenated solution, both *Z. nicaraguensis* and IL #468 formed a barrier in the laterals; ROL was only detected from the root tips as blue stains (Fig. 4). We then applied root-sleeving electrodes to quantify ROL from fine laterals. A semiquantitative approach involving naked platinum wires has previously been used to demonstrate how application of sulphide can reduce ROL from lateral roots (Armstrong & Armstrong, 2005), but in our study we succeeded in quantifying ROL at three positions along 30-mm long lateral roots by using root-sleeving electrodes (Fig. 2). The measurements showed that a ROL barrier formed in lateral roots of *Z. nicaraguensis* and IL #468, and the rates of ROL in the position with a barrier were similar to those observed for the main axis of adventitious roots of various rice varieties (Colmer, 2003), *Hordeum marinus* (Malik et al., 2009) and some roots of *Phragmites australis* (Armstrong & Armstrong, 2001), ranging from 20–70 nmol O2 m−2 s−1 (Fig. 2). A shortcoming of the root-sleeving electrodes, however, is the lack of ability to detect O2 consumption from the root in a scenario in which the rhizosphere contains trace amounts of O2 leaked from tips of neighbouring roots lacking a barrier to ROL (Armstrong, 1970).

Indeed, our measurements of O2 gradients in the diffusive boundary layer of adventitious and lateral roots revealed different situations of either ROL or radial O2 consumption. Radial O2 consumption was clearly present in adventitious roots of *Z. nicaraguensis* and IL #468 and also in laterals of *Z. nicaraguensis*, while different positions for laterals of IL #468 showed either a weak radial O2 consumption or a weak radial O2 loss when grown in stagnant, deoxygenated medium (Fig. 3).
The inference of radial O₂ consumption is that tissue O₂ status is not minimal on the root surface, but rather within a cell layer below the root surface. This has previously been shown for maize using microsensor profiling in which the root tissue O₂ minimum of the outer cell layers appeared c. 100 µm below the root surface (Fig. 7 in Darwent et al., 2003). The implications of a local O₂ minimum below the root surface is that cells positioned between an O₂ minimum within the tissues and the root surface can rely on an external O₂ supply, whereas cells between the minimum and the cortex obtain O₂ from the porous cortex tissue, which is usually high in O₂ (Figs 1, S2 and Armstrong et al., 1993; Darwent et al., 2003; Kotula et al., 2015).

We found that the lateral root portion emerging from the surface of the adventitious roots formed a ROL barrier, whereas the lateral root portion embedded inside the cortex of the parent adventitious roots formed no ROL barrier (Fig. S3). The fact that there is no ROL barrier formation in this portion of the lateral roots indicated a high permeability of O₂ at the outer cell layers of the lateral root tissues within the parent root, resulting in high flux of O₂ from the cortex of the adventitious root into the tissues of lateral roots. In stark contrast, the lateral root tissue, which is immediately outside the adventitious root, had formed a ROL barrier (Figs 3, 4). After the emergence of the lateral root from the surface of the adventitious root, the outer cell layers of the lateral root of Z. nicaraguensis may sense a signal(s) for ROL barrier formation from the external medium. As one example, low concentrations of low-molecular-weight monocarboxylic acids were recently shown to induce the ROL barrier in the main axis of adventitious roots of rice (Colmer et al., 2019); lateral roots were not examined in this previous work on rice.

In Z. nicaraguensis lateral roots, the amount of aerenchyma in the stagnant, deoxygenated conditions was 1.3-fold and 1.4-fold that under aerated conditions, at 10 and 20 mm behind the root tip, respectively (Fig. 6). Moreover, mean root cortex pO₂ in lateral roots were about two-fold higher under stagnant, deoxygenated conditions than under aerated conditions (Fig. 1f). These results suggested that, in addition to the aerenchyma development, ROL barrier formation contributed to keeping higher O₂ status in the cortex of the lateral roots by restricting O₂ leakage to the rhizosphere under stagnant, deoxygenated conditions. By contrast, mean cortex pO₂ in lateral roots under stagnant, deoxygenated conditions were similar in Mi29 and IL #468 (Fig. 1f), implying that ROL barrier formation in IL #468 lateral roots did not result in higher O₂ status in the cortex. As shown in Fig. 6, the aerenchyma areas at 10 and 20 mm from the tips of lateral roots grown under stagnant, deoxygenated conditions were 1.9-fold to 2.0-fold greater in Z. nicaraguensis than in Mi29 and IL #468, whereas these areas were of comparable sizes in Mi29 and IL #468. Both aerenchyma formation and ROL barrier formation can act together to enhance internal O₂ movement and thus tissue O₂ status in roots (Armstrong, 1979).

The functional implications of a ROL barrier in lateral roots are that O₂ is conserved inside these roots and thus the laterals can grow further into anoxic soils (see Armstrong (1979) as discussed for the main axes of roots). The surface area to volume ratio of the lateral roots is 7.8-fold larger than for the main axis of adventitious roots.
roots (caption in Fig. S3). Hence, these thin laterals are substantially more prone to ROL than the main axes of the adventitious roots. Internal O2 was higher in the lateral roots of *Z. nicaraguensis* (with a ROL barrier) than in Mi29 (without a ROL barrier), but for IL #468 (with a ROL barrier) the internal O2 level did not differ from Mi29 (albeit showing a greater range of values than Mi29). Direct comparisons of the present measurements of internal O2 are complicated by these being taken on roots that would have differed in the numbers of laterals (and thus O2 sink) and in the distance of the measured lateral from the root–shoot junction, so that ROL barrier strength was not the only influence on internal O2 concentration. A further quantitative analysis shows that a lateral root with a ROL barrier and mean root aerenchyma area of 10% (Fig. 6), emerging at a distance of 50 mm from the base of the main axis of an adventitious root, could grow to maximum length of 74 mm, which corresponds well with the maximum length of lateral roots observed in *Z. nicaraguensis* (80 mm). In stark contrast, the maximum length would only be 33 mm without a barrier (see Methods S1 for details on modelling). Another implication of the present discovery of lateral roots possessing a ROL barrier is that, for some species, laterals are perhaps not such a vast source of ROL as previously thought more broadly for wetland plants (Armstrong, 1970; Sorrell *et al.*, 2000). Our study

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**Fig. 5** Histochemical staining of suberin in lateral roots of *Zea* (*Z.* nicaraguensis), maize inbred line Mi29 or introgression line #468 (IL #468). *Z. nicaraguensis* was grown under both aerated (a, e, i) and stagnant, deoxygenated conditions (b, f, j), and Mi29 (c, g, k) and IL #468 (d, h, l) were grown under stagnant, deoxygenated conditions. Lateral roots of 20–30 mm in length emerging from 80–120 mm long adventitious roots were selected from 21-d-old plants. Cross-sections of 80-μm thickness were made at 5 (i–l), 10 (e–h) and 20 (a–d) mm from the root tips using a vibrating microtome. Cross-sections were stained by 0.01% Fluorol Yellow 088. The suberin staining was detected as yellow–green fluorescence under UV illumination. Bars, 200 μm.

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**Fig. 6** Aerenchyma of lateral roots of *Zea* (*Z.* nicaraguensis), maize inbred line Mi29 and introgression line #468 (IL #468) at 5, 10 and 20 mm from the root tips. *Z. nicaraguensis* was grown under both aerated and stagnant, deoxygenated conditions and Mi29 and IL #468 were grown under stagnant, deoxygenated conditions. Lateral roots of 20–30 mm in length emerging from 80–120 mm long adventitious roots were selected from 21-d-old plants. Bars show means ± SE (*n* = 3; replicates represent individual plants from which three lateral roots were severed). The results of a two-way ANOVA testing the combinations of genotype/treatment and position in the root is shown inside the panel.
shows that O₂ does indeed leak from the apices of the laterals but that the barrier is relatively tight, only a few mm behind the tip. The tight barrier along most of the lateral root length would greatly reduce the overall ROL from intact root systems. Finally, the root ROL barrier has also been proposed to protect against soil phytotoxins. In some waterlogged soils, anaerobic mineralisation by bacteria can produce carboxylic acids (Armstrong & Armstrong, 1999), resulting in concentrations that can reduce root respiration (Colmer et al., 2019) and stunt root growth (Armstrong & Armstrong, 1999; Kotula et al., 2014). However, experimental evidence of the ROL barrier acting as a barrier also against phytotoxin intrusion is currently lacking.

In conclusion, we have shown that Z. nicaraguensis forms a barrier to ROL not only in the main axis of adventitious roots, but also in the lateral roots when grown in stagnant, deoxygenated solution. The Z. nicaraguensis locus for ROL barrier formation introgressed into maize, which has previously been shown to result in ROL barrier formation of the basal parts of the main axis of adventitious roots when grown in stagnant, deoxygenated solution (Watanabe et al., 2017), also results in ROL barrier formation in the lateral roots of IL #468. This 1st affirmation of lateral roots being able to form a barrier to ROL has significant implications for our understanding of laterals as sites of O₂ loss and possible entry points of soil phytotoxins for plants in waterlogged soils.

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Author contributions

OP, TDC, HT and MN planned and designed the research. OP, YN, HY, YT, HT, AHF, FO, YM, TDC and MN performed experiments and analysed data. OP, TDC, HT and MN wrote the manuscript. OP and MN contributed equally to this work.

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References


Supporting Information

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

**Fig. S1** Diagram of the experimental set-up used for O₂ profiling.

**Fig. S2** Root tissue O₂ profile of an adventitious root of *Zea nicaraguensis* taken 10 mm behind the root tip.

**Fig. S3** Visualisation of O₂ distribution within the main axis of an adventitious root at the point where a lateral root had emerged.

**Methods S1** Modelling of maximum length of lateral roots.

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