Anatomical and physiological responses of roots and rhizomes in *Oryza longistaminata* to soil water gradients

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Abstract

Background and aims:

Roots and rhizomes are critical for the adaptation of clonal plants to soil water gradients. *Oryza longistaminata*, a rhizomatous wild rice, is of particular interest for perennial rice breeding due to its resilience under abiotic stress conditions. While root responses to soil flooding are well-studied, rhizome responses to water gradients remain underexplored. We hypothesize that physiological integration of *Oryza longistaminata* mitigates heterogeneous water deficit stress through interconnected rhizomes, and both roots and rhizomes respond to contrasting water conditions.

Methods:

We investigated the physiological integration between mother plants and ramets, measuring key photosynthetic parameters (photosynthetic and transpiration rate, and stomatal conductance) using an Infrared Gas Analyzer. Moreover, root and rhizome responses to three water regimes (flooding, well-watered, and water deficit) were examined by measuring radial water loss and apparent permeance to O$_2$, along with histochemical and anatomical characterization.

Key results:

Our experiment highlights the role of physiological integration via interconnected rhizomes in mitigating water deficit stress. Severing rhizome connections from mother plants or ramets exposed to water deficit conditions led to significant decreases in key photosynthetic parameters, underscoring the importance of rhizome connections in bidirectional stress mitigation. Additionally, *O. longistaminata* rhizomes exhibited constitutive suberized and lignified apoplastic barriers, while such barriers were induced in roots under water stress. Anatomically, both rhizomes and roots respond similarly to water gradients, showing thinner diameters under water deficit conditions and larger diameters under flooding conditions.

Conclusion:

Our findings indicate that physiological integration through interconnected rhizomes helps alleviate water deficit stress when either the mother plant or the ramet is experiencing water deficit, while the counterpart is in control conditions. Moreover, *O. longistaminata* can adapt to various soil water regimes by regulating anatomical and physiological traits of roots and rhizomes.

Keywords: Apparent permeance to O$_2$; Cortex to stele ratio; Drought; Flooding; Large gas spaces; Number of vascular bundles; Perennial rice; Physiological integration; Radial water loss; Red rice; Root porosity; Tissue diameter
INTRODUCTION

The water regime largely determines productivity and adoption of rice, where lowland or upland rice varieties are selected and cultivated based on their outstanding performance under specific conditions of flooding or drained soils, respectively. Perennial rice, developed by hybridization of annual domesticated Asian rice (*Oryza sativa*) and its rhizomatous perennial African relative, *O. longistaminata*, has recently appeared as a sustainable yet productive rice alternative with great potential for cultivation in extensive areas allowing minimum intervention without the need to replant the crop every season (Zhang et al., 2023). Although some efforts have been conducted to improve flooding or water deficit tolerance of perennial rice through breeding (Hu et al., 2003; Soto-Gómez and Pérez-Rodríguez, 2022; Dossou-Yovo et al., 2024), little is known about the concurrent root and rhizome mechanisms of adaptation to different water conditions.

Perennial rice develops a robust and extensive root system along with rhizomes containing axillary buds capable of developing roots, secondary rhizomes, or ramets. In contrast to the well-studied anatomical and morphological responses of rice roots to flooding or water deficit, information regarding the rhizome responses to such conditions is limited. In roots, the formation of aerenchyma (large gas-filled spaces) increases in response to flooding or water deficit, facilitating O$_2$ diffusion in the former (Yamauchi and Nakazono, 2022) or reducing the metabolic costs for resource exploration in the latter (Zhu et al., 2010; Lynch et al., 2014). Moreover, aerenchyma formation has been shown to increase radial water loss (RWL) in roots of rice under dry conditions (Song et al., 2023). In flooded soils, the root diameter is often thicker, allowing more spaces to form aerenchyma (Yamauchi et al., 2013), whereas roots can be thinner under water deficit conditions (Azhiri-Sigari et al., 2000), thus increasing the capacity to explore for water. With thinner root diameters, longer roots can be formed with the same investment in resources, and also increasing the surface area in contact with the soil (Comas et al., 2013).

Rhizomes of *O. longistaminata* are characterized by several nodes and internodes, with a dense cortex structure, aerenchyma spaces, a pith cavity, and vascular bundles (He et al., 2014; Bessho-Uehara et al., 2018). Well-developed aerenchyma spaces are also common in rhizomes from other wetland plants including *Phragmites australis* (Armstrong et al., 1999), *Glyceria maxima* (Braendle and Crawford, 1987), *Typha domingensis* (Duarte et al., 2021), and *Paspalum wrightii* (Fabbri et al., 2005). Similar to roots, the aerenchyma spaces in rhizomes can also increase in response to flooding conditions as shown for *Sporobolus virginicus* (Naidoo and Naidoo, 1992), *Carex rostrata* (Fagerstedt, 1992), *Paspalum wrightii* (Fabbri et al., 2005), and *Phragmites australis* (Danin and Naenny, 2008). However, information about the rhizome plasticity of *O. longistaminata* in response to varying water stress conditions is lacking.

Outer apoplastic barriers, composed of deposits of suberin and lignin in root cell walls of the exodermis or sclerenchyma, are commonly formed in response to flooding or water deficit conditions (Peralta Ogorek et al., 2024a). These outer apoplastic barriers restrict radial O$_2$ loss (ROL) from roots to the rhizosphere, enhancing longitudinal O$_2$ diffusion towards the root tip of plants grown in flooded soils (Jiménez et al., 2021b). Interestingly, these outer apoplastic barriers also decrease radial water loss (RWL) in rice roots (Song et al., 2023; Peralta Ogorek et al., 2024b). A barrier to impede ROL...
has been characterized in roots of *O. longistaminata* when grown in stagnant conditions (Tong et al., 2023). However, information of such barriers in rhizomes is not available, but the lignification of rhizomes of *Oryza coarctata* (Rajakani et al., 2022) suggests that the permeability to O2 or water in these tissues can be limited.

Rhizomes not only have the role of storing energy and facilitating vegetative reproduction, but also serve to exchange water, nutrients, and signaling molecules between mother plants and ramets (Guo et al., 2021). Therefore, rhizomes can play an important role in plant adaptation to abiotic stress conditions. The clonal integration between a stressed mother plant and a ramet with sufficient resources (or vice versa), via the rhizome, can efficiently mitigate heterogeneous water stress or nutrient deficit (Kroon et al., 1996; Touchette et al., 2012; Tian et al., 2023). In the rhizomatous invasive grass, *Imperata cylindrica*, the translocation of photosynthates between mother plants and ramets enhances its invasive behavior (Estrada et al., 2020). Moreover, nitrogen translocation in the clonal plant, *Carex flacca*, between mother plants and ramets is similar with the pattern of water transport (Kroon et al., 1998). Furthermore, the clonal integration in *Carpobrotus edulis* alleviates drought stress to the damage of photochemical activity in ramets via interconnected rhizomes, and as a consequence, to increase their growth (Lechuga-Lago et al., 2016).

Understanding the concurrent plasticity of both root and rhizome traits of perennial rice in response to different water conditions (i.e., flooding, well-watered, water deficit) will serve as a basis for developing cultivars adapted to such conditions. We hypothesized that clonal integration helps alleviate water deficit stress within the clone, and that both roots and rhizomes can respond to soil water gradients. We therefore studied the physiological integration via interconnected rhizomes when only the mother plant or ramet was grown under water deficit conditions. We measured the photosynthetic parameters (photosynthetic and transpiration rate, and stomatal conductance) of *O. longistaminata* before and after severing the interconnected rhizomes when mother plants or ramets were exposed to water deficit stress. We found that the physiological integration of *O. longistaminata* via interconnected rhizomes mitigates the water deficit stress when the mother plant or the ramet is under water deficit conditions. We grew *O. longistaminata* under flooding, well-watered or water deficit conditions and measured RWL and the apparent permeance to O2. We conducted histochemical staining and used an apoplastic tracer to visualize outer apoplastic barriers, and characterized the anatomy of roots and rhizomes under varying water stress conditions. We found that rhizomes have constitutive barriers impeding water movement and O2 diffusion while these barriers are inducible in roots when grown under flooding, well-watered, or water deficit conditions.

**MATERIALS AND METHODS**

*Physiological integration via the interconnected rhizomes*

In order to investigate the physiological integration via interconnected rhizomes in perennial wild rice, we designed an experiment to measure photosynthetic rate, transpiration rate and stomatal conductance before and after severing the sympodial rhizomes when mother plant or ramet had been exposed to severe water deficit and its counterpart is in control conditions. Six rhizomes were collected from the growth container and were propagated in non-aerated full strength nutrient solutions (see details for the nutrient solutions below). Further, plants composed of one mother plant...
and one ramet, and interconnected via rhizomes, were grown separately in two independent 3-L pots. All roots, developed from either the mother plant or the ramet, were contained within their respective pots. After the ramet had developed three leaves (ca. eight weeks after rhizomes collection), either the ramet or the mother plant was exposed to severe water deficit by supplementing the non-aerated nutrient solution with 20% (w/v) PEG6000. Meanwhile, the respective counterpart ramet or mother plant continued to grow in a full-strength non-aerated nutrient solution without PEG6000. Treatments lasted for 2 weeks, and each treatment had 3 replicates. Photosynthetic and transpiration rates, and stomatal conductance were measured in the first fully expanded leaf of either mother or ramet for each combination of exposed-unexposed plants to PEG6000, using an Infrared gas analyzer system LI-6800 (LI-COR Biosciences Inc., Lincoln, NE, USA). Measurements were collected at a photosynthetically active radiation of 800 µmol photons m⁻² s⁻¹, leaf temperature of 28 °C, CO₂ concentration of 450ppm and relative humidity of 70-80%. The measurements were taken between 11:00 to 14:00. Measurements were collected in both mother and ramet plants 10 min before the connecting tissue was severed and 1.5 hours after cutting in 30 min intervals.

Root and rhizome responses to water gradients

Fifteen rhizomes of *Oryza longistaminata* were collected from 12-months old plants grown in an 80-L container filled with potting mix soil. Rhizomes were washed with DI water and transplanted into pots filled with full strength aerated nutrient solution during 2 weeks to allow propagation. The nutrient solution composition (in mM) was 1.5 CaSO₄·2H₂O, 7.5 MES (buffer), 0.4 MgSO₄·7H₂O, 3.75 KNO₃, 0.625 NH₄NO₃, 0.1 Na₂O₃Si·5H₂O, 0.05 Fe-EDTA, and 1.0 micronutrients. Micronutrients (in mM): 0.05 KCl, 0.025 H₃BO₃, 0.002 MnSO₄·H₂O, 0.002 ZnSO₄·7H₂O, 0.0005 CuSO₄·5H₂O, 0.0005 Na₂MoO₄·2H₂O, and 0.001 NiSO₄·7H₂O (pH adjusted to 6.5 using 2 M KOH). After two weeks of propagation, the 15 seedlings that emerged from rhizomes were carefully transplanted into 0.8-L rhizoboxes filled with sand culture (supplementary data Figure S1). The rhizoboxes were irrigated using a dripping system flowing a 20% strength nutrient solution and the seedlings were maintained in this condition for an extra month of establishment.

After one month of establishment (one and a half months after rhizome collection), plants were randomly selected and three water treatments were conducted: i) flooding, ii) well-watered and iii) water deficit. For flooding treatment, rhizoboxes were inserted into a plastic tank and filled with a 0.1% (w/v) starch solution until reaching a water table of 3 cm above the sand surface. The application of starch solution accelerates microbial growth and thereby the consumption of O₂ in the porewater (Mano and Omori, 2013). For well-watered treatment, rhizoboxes were irrigated using the dripping system, whereas for water deficit treatment, the dripping system was removed and the rhizoboxes were put into a big plate with DI water and the water depth was 3 cm from the bottom. Due to the capillary force among sand granules in water deficit treatment, the plants can keep relatively low water availability. Rhizoboxes were organized in a completely randomized design with three treatments (flooding, well-watered and water deficit) × 5 replications. Treatments lasted for 3 weeks. During those three weeks, the plants were irrigated daily with 20% strength nutrient solution for well-watered treatment. For flooding treatment, 20% strength nutrient solution was added weekly to the plastic tank to compensate for the consumed water and nutrient by plants, and finally for water deficit treatment, 20% strength nutrient solution was added weekly to the big plate to compensate for the consumed water and nutrient by plants.
Anatomical analyses
We evaluated the anatomical changes of roots and rhizomes of plants grown in the three different water treatments. Cross-sections from roots and from 1st, 2nd, and 3rd rhizome internodes were prepared for anatomical analyses. Roots of 100 to 110 mm long were harvested from the plants and cut at positions of 5 mm, 20 mm, 40 mm, 60 mm and 80 mm behind the apex using a sharp razor blade. Root segments were imbedded in 5% (w/v) agar and cross-sections were made using a vibrating microtome (Leica VT1200S, Leica Biosystems). Root cross-sections were stained with 1.5% (w/v) Toluidine Blue O to increase contrast and were visualized under a bright field microscope (Olympus, BX60, Olympus Optional CO., LTD Tokyo, Japan). Total root porosity (aerenchyma and small intercellular air spaces), root diameter, number of cell files, cortex to stele ratio (CSR) in root cross-sections were calculated by using ImageJ2 software (National Institutes of Health, Bethesda, USA). Small intercellular air spaces were measured as described in Justin and Armstrong (1987) for cells with a cubic geometry. For rhizomes, cross-sections were made from 1.2, 2.4, 3.6, 4.8, and 6.0 mm behind the node at the 1st, 2nd, and 3rd rhizome internodes. Rhizome diameter, large gas spaces (i.e., pith cavity and aerenchyma), and number of vascular bundles were measured.

Radial water loss
The radial water loss (RWL) from roots and rhizomes of plants grown in flooding, well-watered and water deficit conditions was measured following the protocol described in Song et al. (2023). For roots, fresh samples were collected from rhizoboxes and rinsed carefully with DI water to remove sand granules from the root surface. About 150–200 mg fresh mass of root segments were prepared from intact roots (100–120 mm of length) by removing lateral roots and the most apical 40 mm in the developing part of the root. The cut ends of the root segments were sealed with Vaseline and placed on a metal mesh into a 5-digits balance (Mettler Toledo Analytical Balance ME54). A filter bag with silica gel granules was located inside the balance chamber for maintaining a relative humidity of 18-28% and the measurements were conducted at room temperature of 22-24 °C (HOBO UX100-011 Temperature and RH data logger, Onset). The loss in mass during desiccation was recorded automatically every 30 s for 1 h using the software Balancelink V4.1.3. Changes in root diameter were recorded every 5 min by time-lapse pictures using a USB camera (Dino-Eye Eyepiece Camera) connected with the software Dino-Capture 2.0. For rhizomes, about 150–200 mg fresh mass were collected from 2nd internodes, the cut ends were sealed with Vaseline and the loss in mass was automatically recorded every 1 min for 3 h, and the rest of process followed as previous described in roots.

Cumulative water loss (% of total water content) and RWL (mmol H₂O m⁻² s⁻¹) were calculated based on total tissue water content and surface area, respectively. For roots, data of cumulative water loss and RWL were fitted by using a two-phase decay function, except for plants grown in flooding or water deficit treatments, where a 6th order polynomial curve showed the best fit. For rhizomes, a one-phase decay function was used to fit all cumulative water loss data from three treatments and a 6th order polynomial curve was used to fit the RWL data. The process of fitting data was conducted in order to identify the time at which 15% of cumulated water is lost in roots, while in rhizome we used the time at which 2% of cumulated water is lost (see Figure 4A to C; supplementary data Figure S4).

Apparent permeance to O₂
In addition to RWL, we aimed to quantitatively assess the capacity of roots and rhizomes to restrict radial O₂ loss (ROL) when grown in flooding, well-watered and water deficit conditions. For this purpose, we measured the O₂ intrusion into tissues using a O₂ Clark-type microsensor (OX-25, Unisense A/S, Denmark) as described elsewhere (Peralta Ogorek et al., 2021). Roots of c. 100-140 mm long were collected, shortened to 15 mm to 20 mm segments corresponding to positions at 35
mm to 50 mm behind the root tip, and their cut ends were sealed with lanolin, and fixed on a metal mesh inside an aquarium. The O$_2$ microsensor was positioned midway along the segment, inserted 125 – 175 µm into the cortex before being submerged in an O$_2$ saturated medium. For rhizomes, both apex and 2nd internode regions were measured. Rhizome segments of 20 to 25 mm were collected from rhizoboxes, sealed with lanoline in cut ends and then fixed on a metal mesh inside an aquarium. The O$_2$ microsensor was positioned midway along the segment, inserted 1200 – 1500 µm into the pith cavity before being submerged in O$_2$ saturated medium. Two contrasting external gas pressures (pO$_2$ 20.6 and c. 60 kPa) in the medium were used for both roots and rhizomes O$_2$ intrusion and the O$_2$ concentrations inside tissues were recorded using software Logger (SensorTrace Suite v.3.2, Unisense A/S, Denmark) (see Figure 4D to F).

**Histochemical analyses**

Roots (100-140 mm long) and rhizomes (c. 100 mm long) were taken from plants grown in the three different water treatments. Cross-sections at 40 mm behind the root tip and from the 2nd rhizome internode were obtained using a vibrating microtome (Leica VT1200S, Leica Biosystems). Lignification of cells were evident as red coloration of cross-sections stained with 5% (w/v) phloroglucinol and 20% (w/v) HCl as described in Vallet et al. (1996). Cell suberization was evident as green-yellowish fluorescence in cross-sections stained with a 0.01% (w/v) Fluorol Yellow 088 solution dissolved in polyethylene glycol 400 for 1 hour as described elsewhere (Brundrett et al., 1991). The stained cross-sections were mounted in a glass slide with 70% (w/v) glycerol and observed in a bright field microscope (Olympus, BX60, Olympus Optional CO., LTD Tokyo, Japan) for lignin or an epifluorescence microscope under UV light (Nikon ECLIPSE Ci, Excitation filter Ex 365/10, Dichroic mirror DM-400, Barrier filter BA-400, camera Nikon DS-Fi3) for suberin.

**Apoplastic tracer**

The permeability of roots and rhizomes to solutes was assessed following the intrusion of periodic acid into the roots as described in Soukup et al. (2007). Briefly, root segments of 100-110 mm length were harvested, the apical 40 mm was removed and the cut ends were sealed with lanolin. Root segments were incubated in 0.1% (w/v) periodic acid for 1 h, followed by incubations in a reducing solution (1 g of potassium iodide and 1 g of sodium thiosulfate dissolved in 50 ml of DI water and acidified with 1 ml of 1 M hydrochloric acid) for 1 h at room temperature. After storing segments in DI water overnight at 4 °C, the segments were embedded in 5% (w/v) agar for up to 3 days and 100 μm thick cross-sections were made using a vibrating microtome (Leica VT1200S, Leica Biosystems). Cross-sections were stained with Schiff’s reagent for 3–5 min and periodic acid penetration was visualized as violet colorations (i.e., aldehydes generated after the lipid peroxidation caused by periodic acid) across the examined roots under a bright field microscope (Olympus, BX60, Olympus Optional CO., LTD Tokyo, Japan). For rhizomes, segments of 100-120 mm length were harvested and the cut ends were sealed with lanolin. Rhizome internode segments were incubated in 0.1% (w/v) periodic acid for 2 h, followed by incubations in a reducing solution (see above) for 2 h at room temperature. After storing segments in DI water overnight at 4 °C, the segments were vertically mounted on the sample plate and ca. 100 µm thick cross-sections were prepared using a vibrating microtome (Leica VT1200S, Leica Biosystems). Cross-sections were stained with Schiff’s reagent for 5–10 min and periodic acid penetration was visualized as violet coloration (see above) across the examined rhizomes under a bright field microscope (Olympus, BX60, Olympus Optional CO., LTD Tokyo, Japan).

**Statistical analyses**

GraphPad Prism software (v.9.0.0) was used for statistical analyses. For the physiological integration, differences between treatments were analyzed by using repeated measures ANOVA followed by Šídák’s test. For the anatomical analyses, RWL, and the apparent permeance to O$_2$, differences between treatments were evaluated by using two-way ANOVA followed by Tukey’s test. All data
satisfied the assumption of normality (Shapiro-Wilk’s test) and homoscedasticity (Bartlett’s test) without requiring data transformation. Figure legends provide details on the tests used and the significance levels.

RESULTS

Physiological integration via interconnected rhizomes

The ability of some rhizomatous plants to alleviate stress in parts of a clone experiencing water deficit by using other parts with ample water access has been demonstrated (Kroon et al., 1996; Roiloa and Retuerto, 2007; Touchette et al., 2012; Lechuga-Lago et al., 2016). However, for *O. longistaminata*, such physiological integration via rhizomes had not previously been established. Using key photosynthetic parameters that are particularly sensitive to water supply, we investigated the physiological integration of interconnected mother plants and ramets via rhizomes, when either of these was under water deficit conditions and the other had ample water supply (Figure 1A and B). We did so by cutting the sympodial rhizome while measuring, e.g., photosynthesis (A), transpiration (E) and stomatal conductance (g
d). The photosynthetic and transpiration rates, as well as the stomatal conductance of either mother plants or ramets were similar when these were interconnected via the rhizomes, irrespective of whether one of them was under stress conditions (Figure 1). After the rhizomes connecting mother plants and ramets were cut, photosynthetic, transpiration and stomatal conductance significantly decreased in ramets (Figure 1C, E and G) and mother plants (Figure 1D, F and H) exposed to stress conditions, whereas these parameters remained stable in counterparts grown in control conditions.

These findings suggest *i)* that clonal integration is significant at the physiological level, and *ii)* that the integration is bidirectional, i.e., water can be supplied in both directions depending on the demand. Consequently, we expanded our approach to examine the anatomical responses of roots and rhizomes not only to water deficit but also to excess water resulting in flooding. This helped us understand how roots and rhizomes interact in adapting the belowground organs to gradients in soil water.

Root and rhizome anatomical traits in response to water gradients

Anatomical traits involved in gas diffusion, water transport, or resource optimization are known to respond significantly to gradients in soil water (Yamauchi et al., 2021). We therefore evaluated anatomical changes at several positions behind the root tip (i.e., 5, 20, 40, 60, 80 mm) and at the 1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} internodes for rhizomes, in response to the three water regimes used. Root porosity (i.e. aerenchyma and small intercellular air spaces) was higher at basal regions and significantly decreased towards the root apex for all plants, independently of the water regimes (Figure 2A and B). However, the mean root porosity from well-watered conditions (8.7%) increased under flooding (17%) and water deficit conditions (21%). Notably, roots under water deficit had 2.0-fold higher porosity at 5 mm behind the root tip than that of roots from flooding or well-watered conditions. At 80 mm behind the root tip, the root porosity responded even more with 13% under well-watered conditions to 33% and 34% under flooding or water deficit conditions, respectively (Figure 2B). The root diameter also responded to soil water content, with roots being thinner under water deficit conditions (0.74 mm) compared to 1.00 mm and 1.31 mm from well-watered and flooding conditions, respectively (Figure 2C). Similarly, roots under water deficit conditions had a lower number of cell files, averaging 10, compared with 14 and 15 under well-watered and flooding conditions, respectively (Figure 2D).
Finally, the cortex to stele ratio (17.2) was significantly higher for roots under flooding conditions in comparison with well-watered or water deficit conditions (9.6 vs. 9.1) (Figure 2E).

We found that rhizomes also responded to soil water gradients. Larger internode diameters (4.67 mm) were observed in plants grown under flooding compared with well-watered or water deficit conditions (3.30 vs. 3.20 mm) (Figure 3A and B). The diameter and large gas spaces (i.e. pith cavity and aerenchyma) of the 1st, 2nd, and 3rd rhizome internode of plants grown under flooding were significantly higher than those from well-watered or water deficit conditions (supplementary data Figure S3). Longitudinally and under well-water conditions, the large gas spaces significantly increased from 10.9% at the 1st to 20.5% at the 3rd internode (Figure 3C; supplementary data Figure S3). However, the large gas spaces also increased in response to flooding (36.1%) compared with well-watered or water deficit conditions (15.4 vs. 17.3%) (Figure 3C). On the other hand, the mean number of vascular bundles was 54 and 53 in rhizomes from flooding and in well-watered conditions respectively, whereas it was 58 in rhizomes under soil water deficit (Figure 3D). In contrast, the various anatomical traits along different longitudinal positions within the same rhizome internode (0 to 6 mm behind the node) exhibited no discernible response to different water treatments (supplementary data Figure S3).

Permeability of roots and rhizomes to water and O2

The apoplastic barrier in the outer part of the root can restrict solute flow as well as gas diffusion (Ranathunge et al., 2011; Peralta Ogorek et al., 2021). Using RWL as a diagnostic tool, we found that adventitious roots of O. longistaminata formed a tight outer apoplastic barrier under flooding, whereas only a weak barrier was present under well-watered or water deficit conditions. After 1 h of desiccation, roots growing in well-watered conditions reached 80% of the cumulative water loss and only 40% and 60% under flooding and water deficit conditions, respectively (Figure 4A). RWL from roots of plants grown in well-watered conditions was 4.1-fold higher compared to that of roots from flooding conditions. Similarly, RWL was 2.9-fold higher in roots from water deficit compared to those from flooding conditions (Figure 4G). In comparison, we found no differences in RWL from rhizome internodes or rhizome apexes for plants grown under the three soil water regimes (Figure 4G). Furthermore, after 3 h of desiccation, the cumulative water loss reached 10-15% in rhizome internodes grown under the three treatments, but 20-25% in rhizome apexes (Figure 4B and C).

After moving roots from air equilibrium to high O2, the cortical O2 concentration increased over time in roots from both well-watered and water deficit conditions, whereas it remained below detection limit under flooding conditions (Figure 4D). In addition, the increase in O2 concentration in roots grown in water deficit conditions was much faster than that of roots grown in well-watered conditions (Figure 4D). The apparent permeance to O2 in roots from well-watered and water deficit conditions were significantly higher than that of roots from flooding conditions (Figure 4H). For flooding conditions, the apparent permeance to O2 was $1.83 \times 10^{-8}$ m sec$^{-1}$, whereas it increased 30- to 63.0-fold for plants under well-watered or water deficit conditions, respectively. For rhizome internodes and apexes from flooding, well-watered or water deficit conditions, the O2 intrusion decreased over time after moving roots to high O2 (Figure 4E) or was below the detection limit (Figure 4F), indicating that tissue O2 consumption exceeded O2 intrusion (Figure 4H).
Root apoplastic barriers

Cell wall components involved in apoplastic barrier formation such as lignin can be visualized using histochemical staining (Liu and Kreszies, 2023). Root and rhizome cross-sections were therefore stained with phloroglucinol-HCl, which produce a red coloration in the presence of lignin (Vallet et al., 1996). We found substantial lignification of sclerenchyma and exodermal cell walls regardless of soil water regime, but roots from flooding or water deficit conditions showed stronger colorations (Figure 5A). Suberin is also an important cell wall component in apoplastic barriers (Vishwanath et al., 2015), and suberization of exodermal cells, indicated by green-yellowish fluorescence, was evident in all roots and to similar extent regardless of water regime (Figure 5A). Interestingly, there were several cells in the sclerenchyma and exodermis without lignin or suberin (i.e., “windows”), particularly in roots from well-watered or water deficit conditions, while these windows were significantly fewer in roots from flooding (supplementary data Figure S2). The solute permeability across the outer part of the root, investigated by penetration of periodic acid and evident as purple colorations, indicated that periodic acid penetrated the outer root cells and reached inner tissues (cortex) in roots from well-watered or water deficit conditions, whereas the penetration stopped at the exodermis in roots from flooding (Figure 5A).

In rhizomes, lignification was evident at epidermal and sub-epidermal cells of rhizomes from all three water regimes, but with higher coloration in rhizomes from water deficit conditions (Figure 5B). Moreover, suberization of epidermal and sub-epidermal cells was evident for rhizomes regardless of soil water conditions (Figure 5B). Finally, penetration of the apoplastic tracer (periodic acid) stopped at the cortical cells of rhizomes independently of the water treatment, indicating that rhizomes of O. longistaminata form a constitutive apoplastic barrier in their rhizomes (Figure 5B).

DISCUSSION

Cultivation of perennial rice is a sustainable and productive option in many rice growing areas, but very little is known about the mechanisms of adaptation to different water gradients of rhizome-bearing rice. This study documents significant acclimations in roots and rhizomes of the perennial rhizomatous O. longistaminata in response to flooding, well-watered and water deficit conditions. The development of barriers to impede radial O₂ loss (ROL) and radial water loss (RWL) is constitutive on rhizomes, while this trait is modulated in roots in response to water conditions. We also provide compelling evidence for the physiological integration alleviating water deficit stress of mother or ramet plants when one is short of water. Below, we discuss these findings with focus on the roots and rhizomes anatomical changes, and how these traits contribute to the physiological integration under water deficit stress.

The physiological integration of O. longistaminata mitigates heterogeneous water deficit stress via the interconnected rhizomes

To justify in-depth investigation of rhizome and root responses to gradient in soil water, we first designed an experiment to examine if there is significant physiological integration within clones of O. longistaminata. We measured key photosynthetic parameters (i.e. photosynthetic and transpiration rate, and stomatal conductance) in the mother plant and ramet before and after cutting the
interconnected rhizomes (Figure 1). When either the mother plant or the ramet was exposed to severe water deficit, cutting the interconnected rhizome led to significant declines in the photosynthetic parameters of the mother plant or ramet that was exposed to stress. However, the counterpart under control conditions remained unchanged (Figure 1C to H). These results suggest an exchange of water, nutrients, or molecular signals through the horizontal rhizomes. Physiological integration is one of the key traits associated with clonal growth, which allows resource sharing between interconnected ramets within a clonal system (Portela et al., 2021). Through the support given by the mother plant to the ramet, clonal plants can explore new soil water resources during water deficit. Once the ramet reaches new water resources, the ramet can in turn support the mother plant remaining under water deficit stress. Therefore, physiological integration can greatly enhance the ability to avoid drought in *O. longistaminata*. Notably, the ramet in the present study exhibited a swifter response to water deficit than the mother plant (Figure 1), possibly due to its greater reliance on the mother plant’s water status. This dependence likely renders the ramet more sensitive to sudden water deficit conditions than the mother plant. Our results suggest that the physiological integration of *O. longistaminata* is bidirectional via the interconnected rhizomes, making the rhizome key in mitigating heterogeneous water deficit stress.

**Constitutive barriers developed in rhizomes, while this trait is modulated in roots in response to water gradients**

The finding that physiological integration within a clone of *O. longistaminata* is significant merits a more thorough investigation of the underlying reasons. We found that the rhizome exhibited a constitutive barrier to radial O$_2$ loss (ROL), with depositions of lignin and suberin observed at the epidermis and sub-epidermis in rhizomes grown under flooding, well-watered, or water deficit conditions (Figure 4H and Figure 5B). In addition, the movements of the apoplastic tracer were blocked at epidermis and sub-epidermis, indicating that rhizomes were radially impermeable to solutes. The constitutive barrier also significantly restricted radial water loss (RWL) in rhizomes as shown under flooding, well-watered, or water deficit conditions (Figure 4G); this would have possible functional implications in a dry soil. The constitutive barrier in the outer part of the rhizome was further demonstrated by the low apparent permeance to O$_2$ under all three conditions of soil water availability (Figure 4H). In addition to the cell wall depositions, the dense cortical cells (Figure 3A) in rhizomes could also contribute to the low apparent permeance to O$_2$ as O$_2$ consumption can be high and diffusivity low in bulky tissues (Jiménez et al., 2024). A previous study has shown that a suberized and lignified constitutive barrier formed in adventitious roots of wild rice, *O. glumaepatula*, where it restricted ROL under aerated conditions (an experimental approach to simulate a drained soil) (Ejiri et al., 2020). However, our study is the first to report the formation of constitutive barriers in rhizomes of *O. longistaminata*.

The roots of *O. longistaminata* had significantly higher RWL and apparent permeance to O$_2$ in well-watered or water deficit conditions than that under flooding conditions (Figure 4G and H). The higher RWL and apparent permeance to O$_2$ (i.e., indicating a weak barrier) could be due to leaks in the apoplastic barriers, influenced by more lateral roots developing and the presence of ‘windows’ of non-suberized or non-lignified cells in conditions of well-watered or water deficit (supplementary data Figure S2). The formation of a window would provide a radial diffusive pathway for water and O$_2$. The formation of window sites have been reported in roots of several wetland plant species, such as cultivated rice (Justin and Armstrong, 1987), *Phragmites australis* (Armstrong et al., 2000), *Hordeum*
marinum (Garthwaite et al., 2008), in which the outer apoplastic barriers can be induced and in roots of *Urochloa humidicola* with constitutive barriers to ROL (Jiménez et al., 2021a). In a previous study, where roots of *O. longistaminata* induced outer apoplastic barriers when grown in stagnant, deoxygenated nutrient solutions (an experimental approach mimicking soil flooding, (Wiengweera et al., 1997)), the RWL was 2 mmol m$^{-2}$ s$^{-1}$ (Tong et al., 2023) compared with 1.8 mmol m$^{-2}$ s$^{-1}$ in the present study with roots in flooded sand. Moreover, the apoplastic tracer was blocked at the outer part of roots grown in flooding conditions, while the tracer penetrated into the cortex in roots from well-watered or water deficit conditions (Figure 5A). These results show that roots of *O. longistaminata* can induce a strong outer apoplastic barrier under flooding conditions. It might be logical to assume that the formation of more windows in the outer apoplastic barrier of roots from water-deficit conditions, would facilitate water uptake, in contrast to a completely sealed barrier with very few windows in flooding conditions preventing ROL to the rhizosphere.

**Key anatomical traits of roots and rhizomes respond significantly to soil water gradients**

It is well-known that plants can acclimate to soil water gradients via plasticity in root anatomical traits (e.g., Yamauchi et al. (2021); Yamauchi et al. (2024)). In the present study, we found that root porosity (aerenchyma plus minor intercellular gas spaces) was higher (21.0%) under water deficit, compared with flooding (17.0%) or well-watered (8.7%) conditions (Figure 2B). This finding suggests that *O. longistaminata* could reduce energy costs on root growth through programmed cell death for adaptation to water deficit conditions (Lynch, 2018). Moreover, thinner roots with lower number of cell files in the cortex were formed under water deficit conditions compared with flooding or well-watered conditions (Figure 2C and D). This respond results in higher surface area to volume ratio facilitating the relative contact area to the soil, and at the same time it reduces the distance for water and nutrients movement from the rhizosphere to the stele. The cortex to stele ratio (CSR) represents the balance between cortex and stele proportions in roots, and a high CSR is beneficial for aerenchyma formation under flooding conditions (due to more cortex tissues), whereas a low CSR facilitates water uptake under drought conditions (Yamauchi et al., 2024). Here, we found that the CSR in roots from water deficit conditions was similar to roots from well-watered conditions (Figure 2E). This reduction in CSR was related to a reduced number of cell files (Figure 2D) rather than changes in root diameters (Figure 2C). In contrast, the CSR of flooded roots was higher than that of roots grown in well-watered or water deficit conditions, which may be associated with a higher number of cell files (Figure 2D) and thicker root diameters (Figure 2C). In summary, the roots of *O. longistaminata* exhibit significant acclimations to water deficit conditions by increasing root porosity, decreasing root diameter, and reducing the number of cell files, to lower metabolic costs and enhance water uptake. In contrast, under flooding conditions, thicker roots of high porosity facilitate oxygen diffusion from the basal to the apical regions.

Interestingly, in contrast to the constitutive nature of the outer apoplastic barriers, the anatomical features of rhizomes of *O. longistaminata* respond to soil water gradients. The rhizome internodes had significantly lower tissue diameter and lower areas of large gas spaces in water deficit or well-watered conditions in comparison with flooding conditions (Figure 3B and C). This suggests that rhizomes might have lower metabolic costs under water deficit or well-watered conditions compared to flooding conditions. Additionally, the lower area of large gas spaces could facilitate cortical carbohydrate storage for recovery after drought stress. Moreover, thicker rhizomes in flooded conditions provides more space for the formation of cortical aerenchyma (Braendle and Crawford,
1987) with the pith cavity and cortical aerenchyma in rhizomes serving as an important aeration pathway under waterlogged conditions (Armstrong et al., 1992). On the other hand, the increase in the number of vascular bundles in rhizomes under water deficit demonstrates that rhizomes regulate the formation of vascular bundles to enhance water transport (Figure 3D). Finally, the constitutive barriers in rhizomes can effectively reduce water and O₂ loss thereby improving longitudinal fluxes to other tissues (Figure 4G and H; Figure 5B).

Roots and rhizomes share many strategies for acclimating to water gradients, but they also differ in some aspects. Roots and rhizomes both had thinner tissues under water deficit and thicker tissue diameters in flooding conditions (Figure 2C and Figure 3B). We propose, that *O. longistaminata* increases water uptake and transport under water deficit conditions by investing energy into tissue expansion to form a larger stele in roots (i.e., lower CSR, see Figure 2E) and by forming more vascular bundles in the rhizomes (see Figure 3D). However, under water deficit, the higher root porosity but much lower areas of large gas spaces in rhizomes, show that roots and rhizomes have different strategies under water deficit conditions, i.e., roots form more porosity to save resources, while rhizomes might require more cortical cells for carbohydrate storage for recovery after the stress (Figure 2B and Figure 3C).

**CONCLUSION**

We demonstrated that bi-directional physiological integration is significant, as indicated by the rapid changes in key photosynthetic parameters when the horizontal communication between mother plants and ramets was interrupted. We also showed that roots of *O. longistaminata* form a strong outer apoplastic barrier under flooded conditions, while the barrier is weak under well-watered or water deficit conditions. The rhizomes had constitutive barriers in their outer parts, as indicated by low RWL, low apparent permeance to O₂, histochemical staining of lignin and suberin, and limited radial movements of the apoplastic tracer. Anatomically, both roots and rhizomes were thinner under water deficit, while they were thicker during flooding. Compared with flooding, roots had a lower cortex to stele ratio in water deficit conditions, and rhizomes had a higher number of vascular bundles. Interestingly, root porosity increased under flooding or water deficit conditions, while large gas spaces in rhizomes decreased under well-watered or water deficit conditions. We therefore propose that *O. longistaminata* acclimates to soil water deficits via inducing an outer apoplastic barrier in the roots to restrict radial water loss, decreasing root diameter, and forming windows to improve the water uptake. The rhizomes acclimate by increasing the number of vascular bundles to enhance water transport capability, and reducing rhizome diameter to decrease growth and metabolic costs.
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Contributions by authors
ZS, CL and OP conceptualized and designed the study. ZS collected data. ZS, CL, JdlCJ and OP analyzed and interpreted data. ZS, JdlCJ and OP drafted the paper and all authors provided input to the draft and approved the final submission.

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Literature cited


Figure legends

Figure 1
Response of key photosynthetic parameters to interconnected rhizome cutting. A (left panel), cutting the interconnected rhizome when the ramet is exposed to water deficit and mother plant is in control solutions. B (right panel), cutting the interconnected rhizome when the mother plant is exposed to water deficit and the ramet is in control solutions. C and D, photosynthetic rate. E and F, transpiration rate. G and H, stomatal conductance. Mother plant or ramet had been exposed to severe water deficit for two weeks, and key photosynthetic parameters were measured before and after cutting the interconnected rhizome. Black arrows indicate the time that the interconnected rhizomes were severed. Data are means ± SE, n = 3. The statistical comparisons were conducted with repeated measures ANOVA (P < 0.01, see supplementary data Table S1) followed by Šidák’s test (**, P ≤ 0.05; ****, P ≤ 0.0001).

Figure 2
Root cross-sections of O. longistaminata grown in flooding, well-watered or water deficit conditions (A). Root porosity (B), root diameter (C), number of cell files (D) and cortex to stele ratio (E). Measurements were collected longitudinally at 5, 20, 40, 60, and 80 mm behind the root tip. In B, root porosity refers to both aerenchyma and small intercellular air spaces. Data are means ± SE, n= 5. The statistical comparisons were conducted using two-way ANOVA (P < 0.01, see supplementary data Table S2) followed by Tukey’s test (different letters indicate significant difference, P < 0.05), and all data passed Shapiro-Wilk’s normality test. In panel B, P_T = 0.0026; P_P = 0.0001; P_TxP = 0.0489. In panel C, P_T = 0.0001; P_P = 0.0014; P_TxP = 0.8465. In panel D, P_T = 0.0024; P_P = 0.0403; P_TxP = 0.6600. In panel E, P_T = 0.0001; P_P = 0.0191; P_TxP = 0.8690. T, treatment; P, position; T×P, treatment and position interaction. Scale bar = 200 µm.

Figure 3
Rhizome cross-sections of O. longistaminata grown in flooding, well-watered or water deficit conditions (A). Rhizome diameter (B), large gas spaces (C) and number of vascular bundles (D). Measurements were collected longitudinally at the 1st, 2nd, and 3rd rhizome internodes. Refer to supplementary data Figure S3 for the longitudinal variation in these traits along different internodes. In C, the large gas spaces refer to both cortical aerenchyma and the pith cavity. Data are means ± SE, n= 3. The statistical comparisons were conducted with two-way ANOVA (P < 0.01, see supplementary data Table S3) followed by Tukey’s test (different letters indicate significant difference, P < 0.05), and all data passed Shapiro-Wilk’s normality test. In panel B, P_T = 0.0054; P_P = 0.0007; P_TxP = 0.6208. In panel C, P_T = 0.0036; P_P = 0.0008; P_TxP = 0.2165. In panel D, P_T = 0.0110; P_P = 0.6611; P_TxP = 0.5312. T, treatment; P, position; T×P, treatment and position interaction. Scale bar = 1 mm. PC = pith cavity, Ae = aerenchyma.

Figure 4
The radial water loss (RWL) and the apparent permeance to O\textsubscript{2} of roots and rhizomes in O. longistaminata grown in flooding, well-watered or water deficit conditions. Cumulative water loss (A-C). Time trace of O\textsubscript{2} intrusion (D-F), RWL (G) and apparent permeance to O\textsubscript{2} (H). RWL values for roots were extracted at the time point at which 15% cumulative water loss had occurred (dashed
line in A and Figure S4). For rhizome internodes and apexes, RWL values were extracted at the time at which 2\% cumulative water loss had occurred (dashed line in B, C and Figure S4). For apparent permeance to O\(_2\), root segments of 15-20 mm, corresponding to positions at 35 to 50 mm behind the root tip, and rhizomes of 20 to 25 mm long from the 2\(^{nd}\) internode or the apex were used. The statistical comparisons were conducted using two-way ANOVA (see supplementary data Table S4 and Table S5, \(P < 0.01\)) followed by Tukey’s test (different letters indicate significant difference, \(P < 0.05\)), and all data passed Shapiro-Wilk’s normality test. Data are means ± SE, \(n=5\). Mean, +; median, horizontal line; 2\(^{nd}\) and 3\(^{rd}\) quartiles, box; minimum and maximum values, whisker. b.d. = below detection limit (i.e., \(pO_2 < 0.02\) kPa).

**Figure 5**
Patterns of lignification, suberization and permeability to apoplastic tracer of roots (A) and rhizomes (B) of *O. longistaminata* grown in flooding, well-watered or water deficit conditions. Root cross-sections were taken at 40 mm behind the root tip and rhizome cross-sections at the 2\(^{nd}\) internode. Black arrow heads point at lignified exodermal and sclerenchymal cells in roots, and at epidermal and sub-epidermal cells of rhizomes. Yellow arrow heads point at suberized exodermal cells in roots, and at epidermal and sub-epidermal cells in rhizomes. EP = epidermis, EX = exodermis, SCL = sclerenchyma, CO = cortex, Sub-EP = sub-epidermis. Scale bar = 100 µm.
Figure 1

A. Normal nutrient solution vs. 20% PEG6000 nutrient solution

B. 23% PEG6000 nutrient solution vs. normal nutrient solution

C. Photosynthesis rate (µmol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Photosynthesis rate

D. Photosynthesis rate (µmol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Photosynthesis rate

E. Transpiration rate (mol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Transpiration rate

F. Transpiration rate (mol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Transpiration rate

G. Stomatal conductance (mol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Stomatal conductance

H. Stomatal conductance (mol m⁻² s⁻¹)
   - Mother plant vs. Ramet
   - Time (min) vs. Stomatal conductance
Figure 3

A

3rd internode

Flooding | Well-watered | Water deficit

2nd internode

1st internode

B

C

D

 Rhizome diameter (mm)  Large gas spaces (%)  Number of vascular bundles

Flooding  Well-watered  Water deficit
Figure 4

A. Root
Cumulative water loss (% of total)

B. Rhizome internode
Cumulative water loss (% of total)

C. Rhizome apex
Cumulative water loss (% of total)

D. Bulk water
O₂_solute (μmol·liter⁻¹)

E. Flooding
O₂_solute (μmol·liter⁻¹)

F. Well-watered
O₂_solute (μmol·liter⁻¹)

G. Water deficit
O₂_solute (μmol·liter⁻¹)

H. Flooding
Apparent permeance to O₂ (m sec⁻¹)

Root
Rhizome internode
Rhizome apex

a, b, ab, abc, abcd

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Figure 5

A. Benchmark, Flooding, Well-watered, Water deficit

Root:
- Lignin
- Suberin
- Apoplastic tracer

B. Root:
- Lignin
- Suberin
- Apoplastic tracer, Suberin

Chiome: