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Water retention capacity for roots with and without the barrier to ROL

A quantitative report on the significance of the root barrier to radial oxygen loss for water retention under drought simulation

Picture: Elisa Pellegrini. University of Copenhagen.

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Abstract

Increasing floods and droughts events has major impact on plants performance. Too much or too little water effects adventitious roots that both causes abiotic stress. Flooded soils are anoxic and roots of rice, develops a barrier consisting of a suberized exodermis to prevent radial oxygen loss (ROL) to the soil. The root exodermis has also been suggested to lower water loss to soils with low water content, but no study has previously investigated how the barrier to ROL can prevent radial water loss under drought conditions. This study attempts assessing the importance of the barrier to ROL for the retention of water under simulated drought conditions. The Michalis-Menten equation worked well (r^2 = 0.99) to describe the initial 30 minutes of desiccation. V_{max} as a parameter was estimated for three root types; roots without a barrier to ROL, roots with a weak barrier and roots with a tight barrier. There was a significant difference between the water loss of roots without the barrier and roots with either a tight barrier or a weak barrier. Roots without the barrier had around 2.5 times faster water loss compared to roots with a barrier. There was no significant difference between roots with a tight barrier and roots with a weak barrier. The lack of difference was also reflected in the time for 95% loss of the total water pool; roots with a tight barrier showed around 22 h compared to roots with a weak barrier of 19 h. Roots without the barrier had approx. 5 h for 95 % desiccation. The plants were grown in hydroponics in either aerated or deoxygenated conditions, to obtain adventitious roots without the barrier to ROL or roots with a barrier. Using an apoplastic tracer on roots cross-sections showed which roots that had developed the barrier or not. The barrier as a specific root trait have previously only been propitious in terms of flooded conditions. This study clearly indicates that this root traits also holds the ability to retain water inside the roots.

Contents

Introduction

1.Increasing of floods and drought events due to climate change

An increasing number of drought and flood events is observed on a global scale. An increase in floods has been especially observed in Asia (*Figure 1*) with 325 flood events in the year 2000, compared to the very few flood events previously. America had around 225 flood incidents in 2000 compared to 15-25 floods in 1960. The warning is not only related to the number of flood events but also to the increase of the sea level.

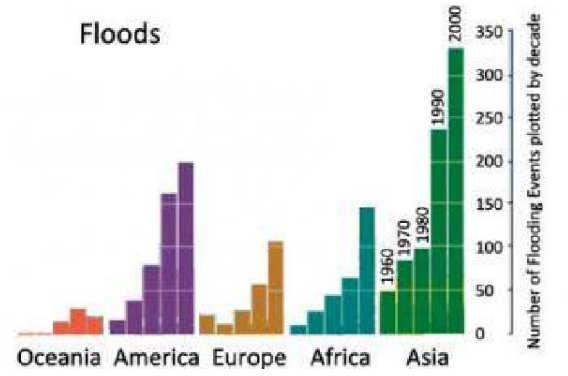


Figure 1: Number of flood events recorded from the International Disaster Database of the University of Louvain, Belgium. The floods can be rainfall, broken rivers, coastal floating or snow melting. *Millennium Ecosystem Assessment map.*

The Second Assessment report of IPCC (Intergovernmental Panel on Climate Change) reported that global sea-level rise is accelerating. Global sea-level is rising around 3-4 mm/year (Watson, White et al. 2015), and over the next 100 years it is expected that sea-level will rise between 10-90 cm (IPCC, 2001). Global sea-level rise will increase the costal flood events (Vitousek, Barnard et al. 2017) and change flood magnitude. This would have an important impact on the coastal population and ecosystems globally (Bijlsma, Ehler et al. 1996) The expected damage will lead to the loss of 22 % of wetlands in 2080 (Nicholls, Hoozemans et al. 1999).

Parallel to the increase in flood events along shorelines, drought is expected to dramatically affect only some places, e.g. the Mediterranean basin (Ipcc 2001).

Figure 2 shows different scenarios of meteorological drought frequency in Europe. RCP.4.5 assumes the “best scenario” of low CO_2 emission and small temperature rise while RCP.8.5 as the “worst scenario” represents the extreme scenario with higher CO_2 emission and temperature rise. Both scenarios show an increase in the drought frequency across Europe but mostly in southern Europe. Spain has an increases of drought events from 0.4 up to 0.7 per 10 years, following the highest emission (RCP.8.5) scenario. Only few areas of northern Europe show a decrease in future droughts events for the scenario RCP.8.5.

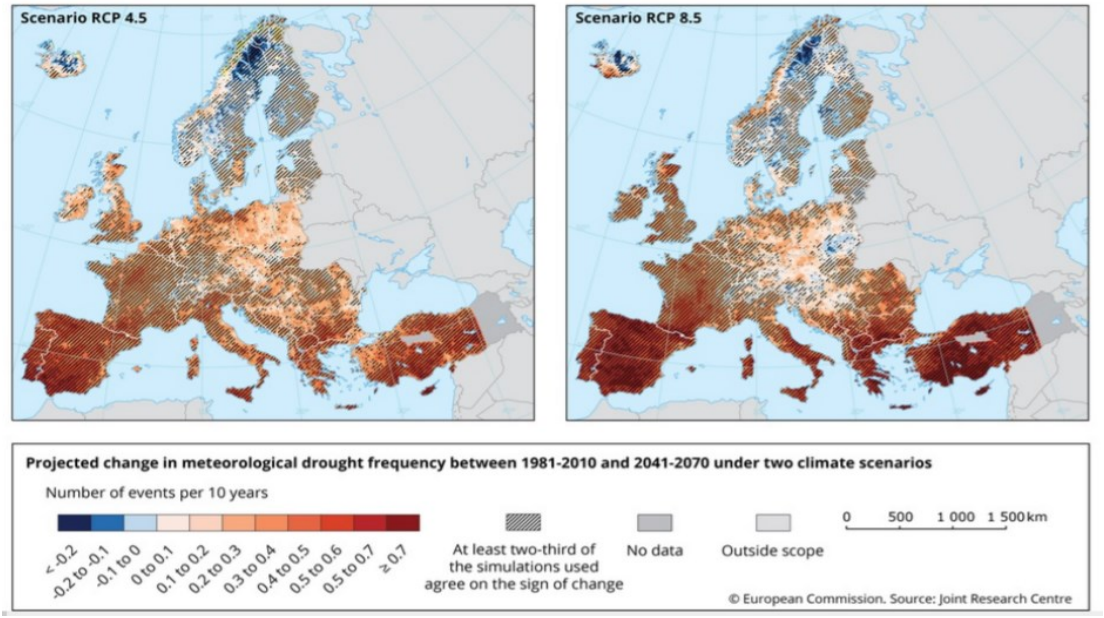


Figure 2: **Changes in meteorological drought frequency projected and compared between two time-intervals for two different future emission scenarios in Europe.** The time-interval is between 1982-2010 (present times) and 2041-2070 (mid-21st century). The scenario RCP.4.5 is for the end of the 21st century and is assumed to have CO_2 of around 650 ppm and an increase of temperature of 1.8 ^{O}C. For scenario RCP.8.5, the CO_2 emission is about 1370 ppm and a temperature rise of 4 C^{O}. A darker red colour indicates higher frequency of the drought events per 10 years. Sources: European commission. Joint Research Centre. Emission scenario data: (Spinoni, Vogt et al. 2018)

Prolonged drought events can cause severe damage. Agricultural is often the most damaged factor. The crops that are loss in a drought season could be around 1/2-1/3 exceeding the average yield. Other damages are soil degradation and erosion because of severe water evaporation. Vegetation damage and wildlife loss is also a consequence with prolonged drought (Maybank, Bonsai et al. 1995)

Being a warning scenario, the aim of the present thesis is to focus on plant traits against flooding and drought, and on root traits that significantly contribute to stress tolerance.

2. Flooding implications on roots

2.1 Flood stress in plants

Flood stress is excess of water from e.g. heavy rainfall, overflow by rivers, tides, wrong irrigation practice leading to waterlogged soils or even submergence (Whitfield, 2012). This results in a decrease of availability of soil O_2. In fact, O_2 diffusion in water is approx. 10,000 slower than in air (Armstrong and Drew 2002) and the slow diffusion is insufficient to replenish O_2 that is being consumed by roots and soil microorganisms; hence, waterlogged soils turn anoxic soon after flooding (Ponnamperuma 1972). O_2 can decrease by 60 % in an hour and almost 95 % in a day compared to air equilibrium (Smith et al. 2010, (Ponnamperuma 1972). Plants need O_2 as terminal electron acceptor in the oxidative phosphorylation process to obtain energy in the form of ATP, but most plants can acclimate to short-term hypoxia (below 50 mmol/m^3) or anoxia. If the soil is anoxic, some species uses carbohydrates reserves (Cronk and Fennessy 2016) and fermentative pathways (i.e. anaerobic carbohydrate catabolism) that allow the pyruvate to be converted into ethanol or lactate and to provide some ATP. Fermentative pathways can contribute to 3-35 % of the energy used in the aerobic cells (Colmer and Flowers 2008) and are essential to overcome the temporary lack of O_2 due to submergence.

Nutrient uptake is also reduced in sensitive species under hypoxia and anoxia. When waterlogged, shoots continue to grow while roots may down-regulate the growth. This may cause an imbalance in root: shoot ratio, shoots nutrient demand and root nutrient uptake capacity (Elzenga and van Veen 2010).
When flooding is due to tidal water as e.g. rising sea-level, salinity represents an additional stress that plants must overcome. Salinity can cause ion-specific stresses where excess Na^+ can alter intracellular K^+ fluxes and cause osmotic stress (Zhu 2002). Traits against salinity are e.g. glandular cells in leaves of many halophytes to excrete salt (Breckle 2002) or the production of osmolytes (Flowers and Colmer 2008). In fact, salinity tolerance is achieved by controlling distribution and uptake of anions and cations and it is commonly addressed using vacuoles for storing osmo-compatible solutes (Jones and Gorham 2002).

2.2 Root traits against flooding

Wetland plants have a suite of root traits that enable them to thrive in waterlogged soil (Yamauchi, Colmer et al. 2018).

Many flood-tolerant plants develop adventitious roots during flood stress. Adventitious roots can form in air or underground and are usually short-lived (Pederson 1989). Adventitious roots have higher porosity due to aerenchyma formation (Lorbiecke and Sauter 1999). Moreover, adventitious roots have much lower SA: V (Surface-to volume area) compared to seminal roots and can develop a barrier to radial O_2 loss (ROL) enhancing internal O_2 diffusion (Colmer 2003, Yamauchi, Abe et al. 2019)

2.2.1 Aerenchyma formation

Plants need a reliable internal aeration system to enable diffusion of O_2 from shoots to roots when experiencing anoxic conditions in the surrounding soil. Inside the cortex, interconnected gas-filled spaces called aerenchyma are developed in adventitious roots of many wetland plants (Armstrong 1980) Roots of wheat *(Triticum aestivum)* and maize (*Zea mays*) have a small percentage of aerenchyma in aerobic conditions (constitutive) that increase under hypoxic stress (inducible) (Abiko, Kotula et al. 2012). The sum of all gas-filled spaces in roots are also referred to as root porosity.

Aerenchyma is a great advantage for cell respiration i.e. better at obtaining O_2 because molecular O_2 moves 10,000-fold faster in the gas-filled aerenchyma compared to the liquid cell sap (Colmer 2003).

There are two main types of aerenchyma: lysigenous aerenchyma (found in *Oryza sativa*) and schizogenous aerenchyma. Lysigenous aerenchyma is formed due to programmed cell death whereas schizogenous aerenchyma is formed due to separation of some cortical cells (Voesenek, Colmer et al. 2006). Aerenchyma can be inducible (i.e. occurring only when waterlogged) or constitutively formed (Colmer and Voesenek 2009).

The inducible lysigenous aerenchyma is triggered by the plant hormone ethylene. Ethylene production rises at low O_2 levels in soil, thus with tissue hypoxia. A chain reaction happens in the plasma membrane which involve ROS species (O_2^- and H_2O_2). Superoxide and peroxide induce the programmed cell death (Drew, He et al. 2000) and hereafter the formation of aerenchyma (Yamauchi, Shiono et al. 2015, Yamauchi, Fukazawa et al. 2017, Yamauchi, Yoshioka et al. 2017).

2.2.2 Low SA:V

Low surface-area to volume ratio (i.e. thicker roots/higher root porosity) is besides aerenchyma, an important trait for the plant internal aeration. Root porosity is defined in many studies as the gas-filled volume per unit of root volume (Justin and Armstrong 1987). Root porosity is associated with the thickness of the adventitious roots i.e. more aerenchyma in thicker roots (Colmer 2003). In O_2

deprived conditions, root porosities are higher in wetland plants compared to terrestrial plants (Visser, Bögemann et al. 2000). A study conducted by Yamauchi et al. (2019) highlighted the importance of this trait. The cortex to stele ratio and the aerenchyma to cortex ratio were larger in rice roots compared to maize and wheat that typically are non-wetland species (Yamauchi, Abe et al. 2019). A large cortex to stele ratio also showed that without the aerenchyma (cuboidal cell arrangement instead as the root porosity), the apical part of the root leaked radial O_2 in higher amount than thin roots. This indicates that lower SA:V in roots has an increase in higher internal aeration and O_2 diffusion (Yamauchi, Abe et al. 2019).

2.2.3 Barrier to radial O_2 loss

Another important trait is the barrier to radial O_2 loss (ROL). The barrier prevents losses of O_2 into the rhizosphere (Colmer 2003) enhancing root internal aeration. Alongside aerenchyma, the ROL barrier stimulates the longitudinal diffusion of O_2 towards the root tip (i.e. apex) and thereby enables root elongation into anaerobic soil. The barrier is mostly tighter at the basal parts of the roots and patchy or absent in younger roots, e.g. in common reed (*Phragmites australis*). Some O_2 is, however, lost into the rhizosphere through the apex and through the lateral roots (Jackson and Armstrong 1999, Colmer 2003). In species with an inducible barrier to ROL, the loss is significantly reduced when the barrier is present and this was proved for many species like *Hordeum marinum* (Kotula et al 2017), the genus of *Echinocloa* (Ejiri and Shiono 2019) and rice in upland, paddy and deep-water rice (*Oryza sativa)* (Rao, Johnson et al. 2007*).* A tight barrier to ROL is inducible when plants are grown in stagnant, deoxygenated conditions (Colmer 2002). Species with morphological similarities can show large differences in the 'tightness' of the barrier, even within the same genus (Inoue and Tsuchiya 2008). Aerenchyma and an inducible barrier to ROL facilitate root aeration in rice (*Oryza sativa* L.) (Colmer 2003). Another example of an inducible barrier to ROL is seen in a wild relative to maize. Cultivated in stagnant conditions, *Zea nicaraguensis* showed a clear decline in ROL compared to maize, showing the presence of a barrier (Abiko, Kotula et al. 2012).

3.Drought implications on roots

3.1 Drought stress in plants

Drought stress is considered for the plants to be a moderate loss of water which leads to malfunction of physiological processes (Jaleel, Manivannan et al. 2009). It affects cell turgor pressure that is responsible for the cell structure. When the cell expansion or turgor pressure is reduced it causes wilting of leaves and growth arrest (Smith, Coupland et al. 2010).

Nutrient uptake is greatly reduced under drought. Limited soil interstitial water reduce microbial activity and further inorganic nutrient uptake by plants (Borken and Matzner 2009). Root nutrient uptake is reduced due to decreased rates of water movement and ion diffusion in dry soil (Hagan, Haise et al. 1967).

The sensing of water deficit in plants seems to be due to the 'hormone' abscisic acid (ABA). ABA controls different mechanisms, like stomatal closure, but is possibly also involved in drought stress signalling (Smith, Coupland et al. 2010).

Cell respiration could be also affected by drought, but it is still not clear if the respiration rate is negatively or positively affected. Some authors showed an increase in respiration rate or no changes under drought stress (Upchurch, Peterson et al. 1955, Shearman, Eastin et al. 1972) Other authors reported opposite results (Brix 1962, Brown and Thomas 1980). A data comparison was done by Flexas et al. (2005). They collected several different studies focusing on relative water content as the reference parameter for 14 different species. They found that the relationship between respiration and relative water content was a trend of biphasic response, (e.g. a response that has two phases). The first

initial tendency showed a decrease in respiration attributed to a decrease in energy demand, for plant growth. At severe water deficit a second trend showed an increase in respiration attributed to an enhanced osmo-production and in general to a drought-stress defence response (Flexas, Galmes et al. 2005).

3.2 Root traits against drought

Drought conditions also contributes to different stresses in plants and the consequent development of acclimation traits. For instance, cactus has a short root system to get advantage of the moisture in the shallower ground during the rare rainfalls. Eeucalyptus on the contrary has a very deep root system, to reach the very low water table (Pierret, Maeght et al. 2016). Some species don't possess leaves but have green stems designated for carbon fixation. The stems may have lower SA: V compared to leaves. Lower surface area: volume (SA: V) reduce transpiration (Smith, Coupland et al. 2010).

Aerenchyma in the root cortical in maize can increase drought tolerance in the plant. Under water stress, maize plants with high aerenchyma in the root cortical had greater root length and 30 % more shoot biomass. Relative water content was a 10 % greater. One possible reason could be that when cortical tissue cells are being converted into aerenchyma (gas spaces) via programmed cell death, the amount of root tissue per volume of root is reduced and thereby water and energy requirements decline (Zhu, Brown et al. 2010)

4. Composition of the root ROL barrier

The ROL barrier is composed by suberin and lignin depositions in the root hypodermis/exodermis (Ejiri and Shiono 2019). Suberin is a hydrophobic macromolecule, a polyester containing long fatty-chain acids (suberin acids) and glycerol. The core back-bone is glycerol units linked in α, ω-diacids connections. These can either be in an organized or disorganized manner (Pollard, Beisson et al. 2008).

Lignin is a hydrophobic polyphenolic polymer existing as a matrix in the cell wall surrounding the polysaccharide components of the membrane (Whetten and Sederoff 1995, Funaoka 2003). Lignin is built by lignin monomers (i.e. monolignols) that holds three different alcohols (Barros, Serk et al. 2015).
Suberin lamellae are commonly found in the endodermis (the tissue surrounding the vascular cylinder in roots) of most plants (Pollard, Beisson et al. 2008) or can be found in the exodermis (*Figure 3*). Suberin lamellae consist of biopolymers of suberin and small amounts of lignin (Bernards 2002, Enstone, Peterson et al. 2002).

Adventitious roots, which can develop a barrier to ROL, are composed of different layers with the epidermis, hypodermis/exodermis and sclerenchyma (i.e. dead cells which cell wall contains lignin) forming the outer layers (*Figure 3)*. The exodermis is not a constant structure, the formation is under strong environmental influence (PERUMALLA, PETERSON et al. 1990) Moving closer to root stele, cortex/mesodermis and endodermis are distinguishable. Phloem and xylem are positioned in the center of the root (Atkinson, Rasmussen et al. 2014). In rice, the outer part of the roots consists of four layers; *rhizodermis, exodermis, sclerenchyma and one layer of cortical cells* (Ranathunge, Steudle et al. 2003). The barrier to ROL is known to be the suberized exodermis and/or lignified sclerenchyma (Kotula and Steudle 2009).

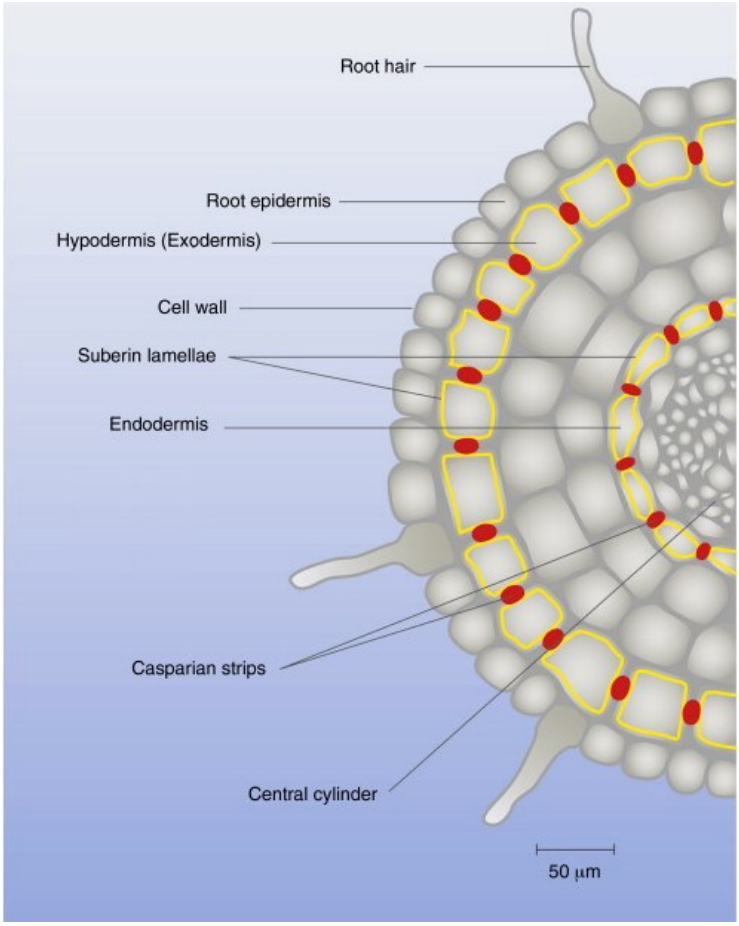


Figure 3: The figure illustrates a diagram of a dicotyledonous root in its first developmental state. It is showing the suberized endodermis and hypodermis (exodermis). Both shown with the Casparian strips/bands (red dots) in the radial cell wall. The suberin lamellae (shown in yellow) is deposited onto the inner surface of the primary cell wall (shown in grey). Trends in Plant Science. (Schreiber 2010)

Genes that controls the biosynthesis of suberin in the ROL barrier are more up-regulated than those controlling the synthesis of lignin (Soukup, Armstrong et al. 2007, Ejiri and Shiono 2019). This has been show in *Zea nicaraguensis* where adventitious roots showed suberized exodermis in the basal parts but no lignified epidermis (Watanabe, Takahashi et al. 2017). Lignin could not contribute to the formation of the barrier but acts like a mechanical support and a general plant defence against abiotic stress such as extreme temperature, flooding and high winds (Whetten and Sederoff 1995, Ejiri and Shiono 2019).

Some wild species of *Echinochloa* form a constitutive barrier to ROL whereas *E. oryzicola* form an inducible barrier to ROL, like rice. All the types showed suberized exodermis in stagnant, deoxygenated conditions and 97 % of the cells developed suberin lamellae (Ejiri and Shiono 2019).

In rice, exodermis deposition of Casparian bands (i.e. diffusion barrier that direct nutrient and water) was observed in plants grown in stagnant, deoxygenated conditions close to the apex but none in plants grown in aerated conditions. Suberin lamellae were also absent in aerated conditions 20 mm from the apex. Stagnant, deoxygenated conditions induced stronger lignified sclerenchyma 60 mm from the apex compared to aerated conditions (Kotula, Ranathunge et al. 2009). Suberized cell walls were noticed in the exodermal hypodermis of two wetland grasses, *Glyceria maxima* and *Phragmites australis*. *P. australis* showed a multi-layer exodermis and a scherenchymous ring, whereas *G.*

maxima had a two-layer exodermis (Soukup, Armstrong et al. 2007). *G. maxima* had suberin lamellae with denser deposition of suberin in the hypodermal layers (Soukup, Armstrong et al. 2007).

5. Signalling for the ROL barrier formation

Plants can sense O_2 shortage in their environment (Licausi, Kosmacz et al. 2011). The main mechanism in sensing O_2 is the regulation of the N-end rule of protein degradation (Gibbs, Lee et al. 2011). However, the barrier to ROL is not induced either by shortage of O_2 or low plant hormone ethylene. The same was the case with elevated concentration of CO_2 (Colmer, Cox et al. 2006). Though ethylene is shown to enhance aerenchyma formation, it is implied that the trait of aerenchyma and barrier to ROL is differently regulated (Armstrong 1971).

The barrier to ROL may be triggered at sub-toxic concentration of organic acids, sulphides and Fe^{2+} (Armstrong and Armstrong 2005, Kotula, Colmer et al. 2014, Mongon, Konnerup et al. 2014).
The consequences of waterlogged soils are not only relevant to plants but also to soil microorganisms. Under anoxia, soil microorganisms produce toxic compounds such as reduced iron, sulphides and low molecular carboxylic acids (Ponnamperuma 1984). Colmer et al. (2019) demonstrated that organic acids trigger the ROL barrier in rice at sub-toxic concentrations (0.04 mM). Four organic acids were compared (butyric, acetic, hexanoic and propionic acid) and effects on ROL were measured. Under influence of the organic acids, the ROL decreased from ~250 nmol O_2 m^{-2} s^{-1} to 5-10 nmol O_2 m^{-2} s^{-1} 20 mm from the apex (i.e. the tip of the root) in all treatments. This is evident for the barrier formation. Roots without the barrier shows a steady loss of radial O_2. The study also tested for elevated amounts of transcript for suberin and lignin (both are histological components of the barrier in the exodermis) but found no significant evidence for higher amounts with sub-toxic treatments suggesting the presence of other molecules limiting gases diffusion (e.g. waxes).

Heavy metals pollution is a concerning problem with an increasing frequency of lead (Pb), zinc (Zn) and copper (Cu) in the estuarine systems (Mills 1985). For three different mangroves species*; A. corniculatum, B. gymnorrhiza* and *A. marina* a treatment with Pb, Zn and Cu decreased the ROL proportional to the increase of metal concentration suggesting that other elements can trigger the ROL barrier (Liu, Tam et al. 2009).

6. ROL barrier against phytotoxins

The barrier for ROL may also act as a barrier against phytotoxins (Armstrong and Armstrong 2005). From previous paragraph Colmer et al., (2019) showed that the barrier of ROL was developed upon sub-toxic concentration of organic acids, suggesting that the barrier may act as a protective barrier against harmful toxins.

There is no barrier (i.e. higher amount of ROL) at the growing root tip (apex). This may help to re-oxidise the reduced compounds in the rhizosphere protecting the sensitive growing root tip .The same potential strategy is observed in mangroves trees to avoid reduced compounds and metals uptake (Armstrong 1980, Pedersen, Binzer et al. 2004)

7. Research question and hypothesis

The ROL barrier serves to enable longitudinal O_2 diffusion from shoots to roots under waterlogged conditions. However, direct observations pointed out possible additional roles and functions of the ROL barrier related to soil phytotoxin exclusion.

The aim of this project is to further investigate additional roles of the ROL barrier other than the well-know one that restricts radial O_2 loss.

This project focuses on the role of the ROL barrier against tissue dehydration as the roots would experience it in dry soil. Supplementary to the well-known function of the barrier to reduce radial O_2 loss, it could also potentially reduce radial water loss. The hypothesis for this study is that *adventitious roots with a barrier to ROL will lose water at a slower rate compared to roots without a barrier to ROL.*

The project tests the hypothesis by growing rice of the genotype IR45 in hydroponics. This facilitates a controlled environment with easy access to adventitious root sampling of the same age. The plants can be grown in stagnant, deoxygenated or aerated conditions to produce roots with or without a ROL barrier. Radial water loss will be assessed in evaporation experiments for root segments in dry air. To visualize the barrier, a qualitative analysis of root sections will be applied using the periodic acid as an apoplastic tracer. Additionally, methylene blue staining will be used to confirm the presence or absence of a barrier to ROL.

Materials and Method

8.1 Seed germination and hydroponic culture

About 24 seeds of the rice genotype IR42 were imbibed in 0.5 mM $CaSO_4$ for 3 h and transferred to a Petri dish with wet blotting paper. To help starting the germination, the Petri dish was wrapped with aluminium foil and kept at 30 °C for 3-4 days until germination. This was done to simulate the seed being in the soil for dark conditions. The seedlings were then transferred to the hydroponic culture using a 50% strength nutrient solution (see information S1). Seedlings were placed on a floating mesh in a 3 L black bucket with light conditions at about >200 PAR (12 h light and 12 h darkness) and temperature of 30/28 °C day/night. Aerated conditions of the nutrient solution were achieved by gently purging the solution with atmospheric air. When the seedlings were about 5-7 cm in height (equivalent to approximately 7- days old), the nutrient solution was replaced using a 100% strength nutrient solution. After about 1-2 weeks, half of the seedlings were transferred to stagnant, deoxygenated conditions. 3 to 4 plants (pseudo replicates) were positioned in each bucket forming one replicate with 3 replicates for each treatment; aerated or stagnant, deoxygenated. Plants of the stagnant treatment were conditioned imposing a hypoxic pre-treatment (bubbling the nutrient solution with N_2 for 2-3 min) the day before the transfer. The stagnant nutrient solution was of the same composition as the aerated nutrient solution but with the addition of 0.1% agar.

8.2 Measurements of evaporation of tissue moisture from root segments

Measurements of evaporation of tissue moisture were based on mass records. Root tissue samples were exposed to dry air for 30 minutes and root tissue mass was recorded every second using a lab balance (*Mettler Toledo ME54*) connected to the software *LabX direct balance 2.4* with measurements taken at 25 °C.

The relative humidity inside the weight chamber of the balance was about 18-22% (HOBO RH logger, Onset) and it was kept low using about 12 g of silica gel desiccant grains held in paper filters hanging from the top of the closed balance chamber. Silica gel grains were replaced prior to each measurement. About 10-14 roots (200-300 mg of root tissue) were collected for each replicate bucket and kept wet until the start of the recordings. First, the most apical thirty mm were discarding and then a 25-60 mm root segment was prepared from each root; lateral roots were removed, and root segment were kept wet. Vaseline was used to seal both cut ends of the root segments before these were quickly positioned on a metallic mesh. The mesh ensured maximum exposure of root surfaces to air. The mesh was placed on the balance and the recording was immediately initiated with automatic recording for 30 minutes. Subsequently, roots were collected into a piece of aluminium foil and dried 2-3 days at 50 °C. The dry mass was then recorded.

In total 12 replicates were conducted with 4 replicates respectively to each group (roots without the barrier, tight barrier and weak barrier). *These results were kindly provided by Elisa Pellegrini, Freshwater Biological Laboratory, University of Copenhagen.*

8.3 Root surface area

ImageJ was used to measure the radius of cross-sections of IR42 with and without the barrier. Around 24 replicates of radius measurements were done for the root with the barrier and the root without the barrier. Knowing the cylinder surface area formula, the length and the number of root segments, water loss data were calculated based on root surface.

Data as how many roots per replicate and the length of the roots was provided by Elisa Pellegrini, Freshwater Biological Laboratory, University of Copenhagen.

8.4 Michael Menten model fitting and statistics

The Michael Menten model was applied to the data. Michael Menten model follows this equation:

$$v = \frac{d[P]}{dt} = \frac{V_{max} * [S]}{K_M + [S]}$$

The function is commonly applied to enzymatic processes. The reaction rate v for the P(product) of enzymes binding to the S (substrate). The curve will reach the saturation point (Vmax) where there are no more binding sites on the substrate. Km is the S of when $2/V_{max}$ is reached.

V_{max} was estimated for the 12 replicates. A one-way Anova with a Tukey post hoc test was applied to compare the mean of each group (without barrier, with barrier and partial barrier) and determine if there is a significance differences for radial water loss between each group. Prior to Anova, the normality and homoscedastic of data were verified with Shapito's test and Bartlett's test respectively. A one-tailed t-test tested if any significance difference would be between roots that has a weak barrier over roots that had a tight barrier.

Using software *Graphpad Prism 8.4.2* one could calculate the three root types desiccation time of 95 %. This was done by setting the V_{max} as 100 % for total desiccation. 95 % could be read in the program.

8.5 Qualitative test: Permeability test with periodic acid and methylene blue staining

For the periodic acid permeability test, single roots were sealed with vaseline at the cut end and incubated in a 0.1 % w/v solution of HIO4 (=periodic acid 0.1% aqueous solution) for 60 min. The excess of acid was rinsed off with water 3 times. Roots were then treated with a reducing solution (Information S1), (Pearse, 1968) and incubated overnight in DI water.
Cross sections were hand made. Schiff's solution was used to stain the cross-sections for 10 min. Cross sections were then washed 3 times with DI water and afterwards mounted in glycerol (70% v/v or pure). Using bright field optics, the indictive purple staining could be observed. (Soukup et al. 2002, 2006, 2014; Pecková et al. 2016; Watanabe et al. 2017).

For the methylene blue staining 0,1 % agar was prepared and purged with N_2 for 2 hours to remove O_2. Methylene blue (0.03 mM) and sodium dithionite (0.3 mM $Na_2S_2O_4$) was added and mixed with N_2 purging to dissolved. The solution was syphoned into a glass chamber where the plants where positioned. After 45 minutes the staining was recorded using a camera.
Pictures and data was kindly provided by Lucas León Peralta Ogorek, Freshwater Biological Laboratory, University of Copenhagen.

Results

9. The barrier to ROL enhances tissue water retention

The overall purpose with this study was to test if the barrier to ROL could prevent tissue dehydration for the roots of the rice. Retention of tissue moisture was measured as cumulated water loss in a dry atmosphere from roots segments over time. The cumulated water loss (*Figure 4)* resembles a saturation curve, i.e. the water loss is initially greatest at the beginning and then declines with time, due to tissue desiccation and the resulting decline in moisture gradient. *Figure 4* shows the accumulated water loss for three treatments of the root segments; roots that had no barrier to ROL, roots that possessed a weak barrier and roots that had a tight barrier to ROL.

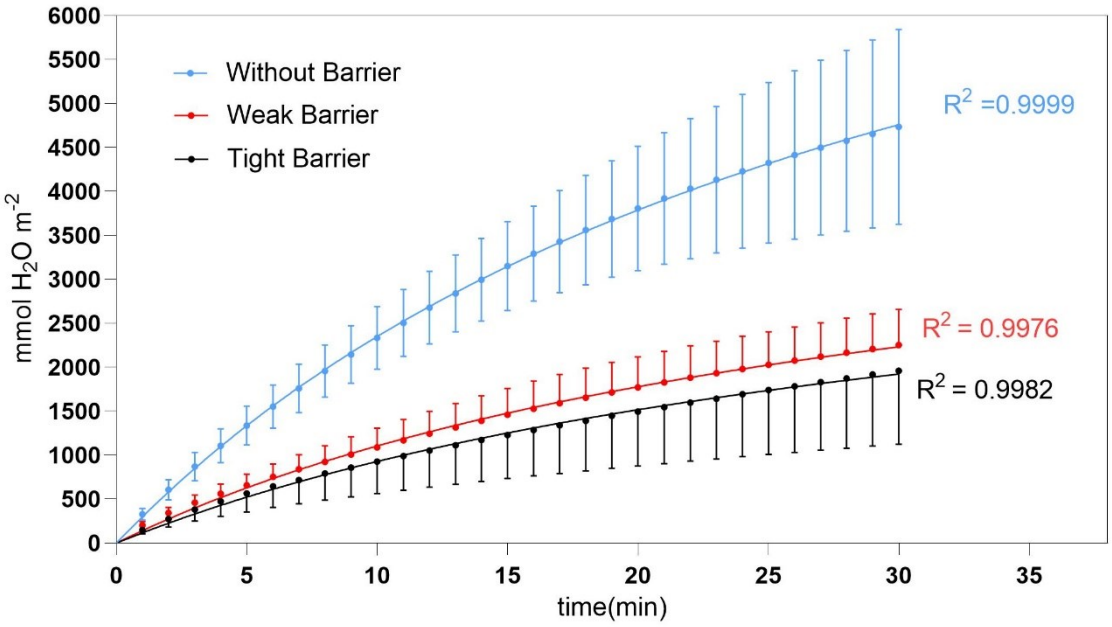


Figure 4: Cumulated water loss from roots without a barrier to ROL, a weak barrier or a tight barrier*. Tissue dehydration was recorded for 30 minutes. Each data point represents the mean of 4 replicates; error bars show the standard deviation. R^2 represents the non-linear regression fit of the Michalis-Menten model.*

Roots that had developed a barrier to ROL had lost around 2000 mmol H_2O m^{-2} water loss after 30 minutes compared to roots without the barrier that lost up to 5000 mmol H_2O m^{-2} at the end of the experiment. Hence, roots with a tight barrier had lost 58 % less water compared to the roots without the barrier. If roots with a weak barrier is compared to roots without the barrier, we also observe less water loss from roots with a barrier. Here the roots with a weak the barrier had lost around 52 % less water compared to roots without the barrier. The difference is only around 3 % less water loss between roots with a weak and a strong barrier (*Figure 4, black and red*). This difference of 3 % is not significant. A performed one-tailed t-test with a p- value of 0.33 for a significance-level of 95 % rejected that there was any significant difference between the roots with a weak barrier and roots with a tight barrier.

The Michaelis-Menten model showed the best fit for the data presented in *Figure 4*. First a linear model was initially applied to evaluate the data. The r^2 showed a good match of 0,98 (*Figure S1).* Nevertheless, since the relationship is obviously non-linear, a non-linear model was applied. The Michalis-Menten model shows a great fit with a r^2 of 0.999, 0.997 or 0.998 for roots without a barrier, a weak barrier or a tight barrier, respectively.

Table 1. Estimated values of V_{max} and Km from the Michalis-Menten model.

Root type:	V_{max} (mmol H_2O m^{-2})	Km (min)
Without Barrier	9767	31.56
Tight Barrier	4139	34.57
Weak Barrier	4564	31.32

For the three root types Km and V_{max} was isolated from Michalis-Menten model (*see table 1).*

The V_{max} constant was considered the best parameter to apply to the Anova-test since it seemed to respond to the treatment, where Km did not. Km indicates the time when half of V_{max} is reached which seemed to occur around the same time for all three root types.

Table 2 shows the estimated times for when the three root types would reach 95 % desiccation. Roots with a tight barrier takes approx. 22 hours to reach 95 % desiccation. That is around 4.5-times higher desiccation time, compared to roots without the barrier. The difference between roots with a weak barrier to roots with the tight barrier only differs with around 3 hours.

Table 2. Estimates the time for 95 % desiccation for the three root types; roots without barrier, tight barrier, weak barrier. V_{max} as 100 % desiccation for the roots

Root type:	Desiccation 95 % time (min)
Without Barrier	312 (approx. 5 h)
Tight Barrier	1341.4 (approx. 22 h)
Weak barrier	1148.6 (approx. 19 h)

Figure 5 shows the obtained results of the mean V_{max} for the three root types.

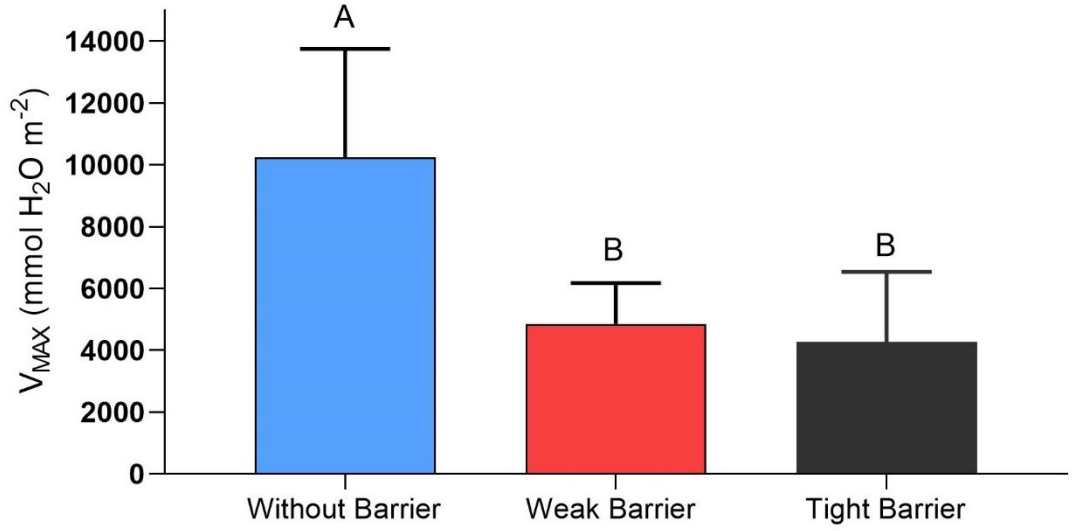


Figure 5. Mean V_{max} values for roots without a barrier to ROL, a weak barrier or a tight barrier. *The bar graph illustrates the different mean V_{max} values from the Michalis-Menten model for three root types roots without barrier, roots with weak barrier and roots with a tight barrier. The V_{max} values are in mmol H_2O m^{-2}. A one-way Anova with a Tukey post-hoc test was conducted. Different letters indicate a significant difference while same letter indicate no significance difference. Means ± SD, n=4.*

The roots without a barrier show the greatest mean value of V_{max} of around 10000 mmol H_2O m^{-2}, while the roots with tight or weak barriers shows the similarity with a V_{max} of 4500 mmol H_2O m^{-2}.

For the mean values of V_{max}, a one-way Anova with a Tukey post-hoc test was applied to test if there were any significant differences in the water loss between each root type. The test-showed a significant difference between roots without the barrier and roots with a tight barrier at a 95 % significance-level with a p-value of 0.02. A significant difference was likewise observed in V_{max} between roots without a barrier and roots with a weak barrier, with a p-value of 0.0362. Roots with a tight barrier compared to roots with a weak barrier showed no significant differences with a p-value of 0.9396. This shows that roots that hold some sort of a barrier to ROL losses significantly less water compared to roots without the barrier.

Table 3: Results from Tukey's test showing the combinations of treatments, p-value and if the difference is significant.

Tukey's multiple comparisons test (Combination)	Adjusted P Value	Significant
Without Barrier vs. Tight Barrier	0.0214	Yes
Without Barrier vs. Weak Barrier	0.0362	Yes
Weak Barrier vs. Tight Barrier	0.9396	No

Figure 6 illustrates the rate loss of water with time. The loss of water is initially high, then it declines with time and reached a quasi-steady state. Roots without a barrier have higher loss rates with 4-5 mmol H_2O m^{-2} s^{-1} during the first minutes compared to the rate of around 2 mmol H_2O m^{-2} s^{-1} for roots with a tight or weak barrier. This means that roots without the barrier have approx. 2.5 times higher water loss compared to roots with a tight or weak barrier. Even after 30 minutes, roots without a barrier still had higher loss rates compared to roots with some sort of a barrier.

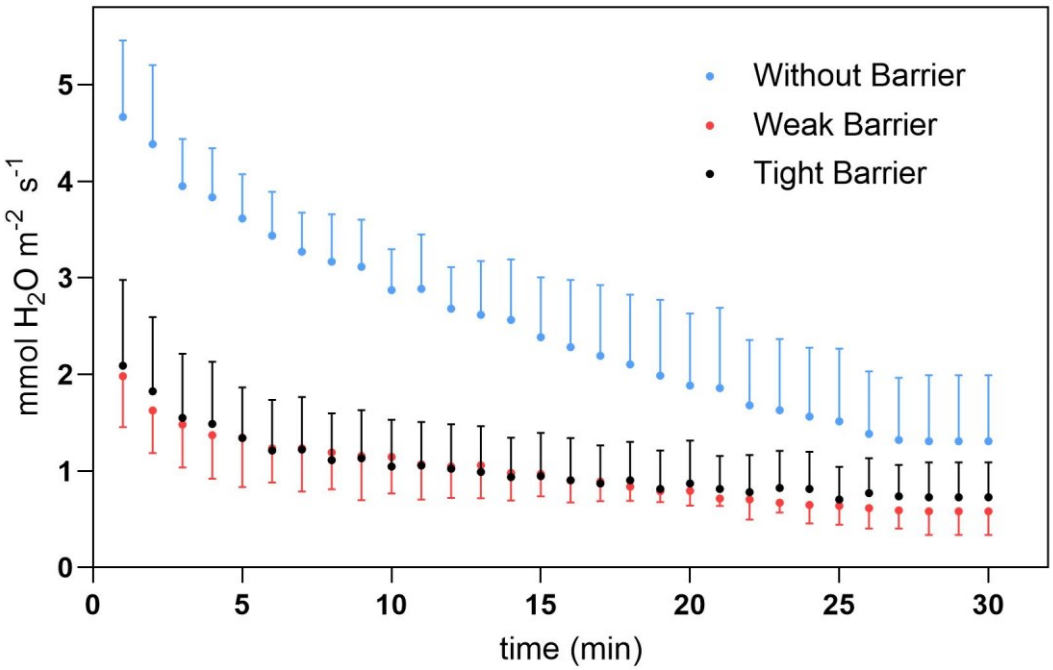


Figure 6. Radial water loss rate for roots without a barrier to ROL, a weak barrier or a tight barrier: *The graph presents the rate of radial water loss as mmol H_2O m^{-2} s^{-1} for the time interval of 0-30 minutes.*

Another way to illustrates tissue dehydration is shown in *figure 7:*

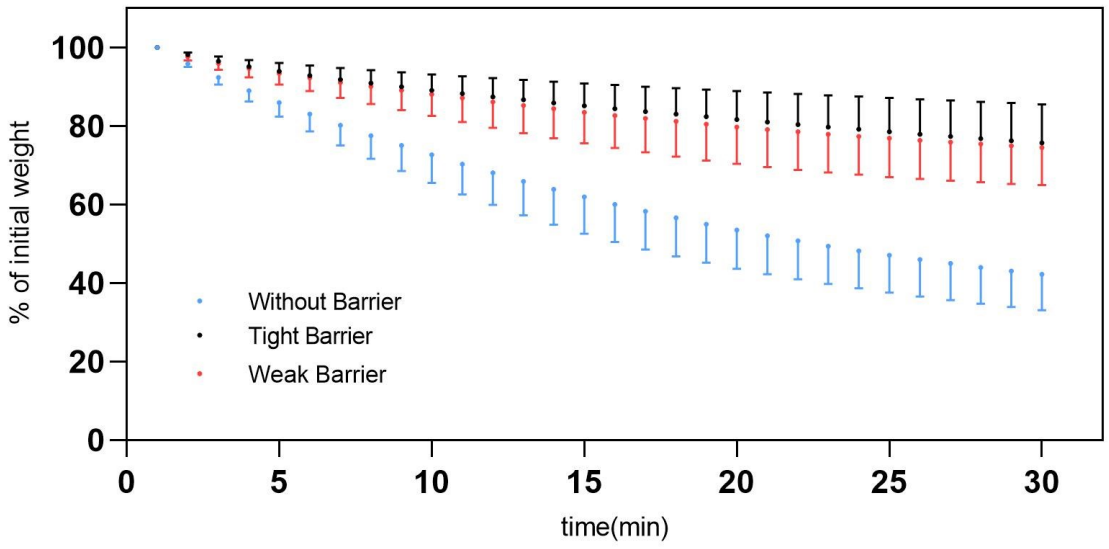


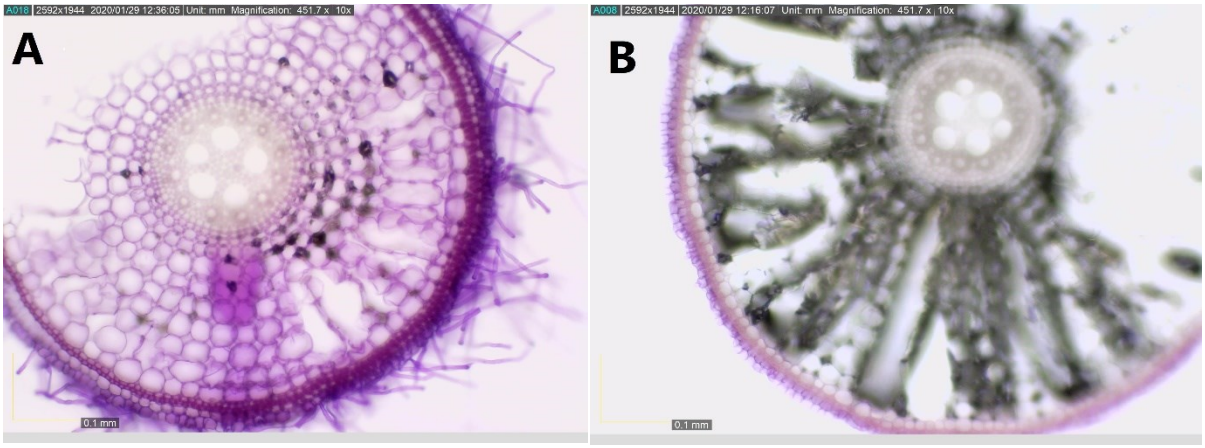
Figure 7. Percentages of initial weight for the three root types; without the barrier, a tight barrier and a weak barrier*. This graph presents the general weight loss in percentages, for the three root types in the time interval of 0-30 minutes. Means ± SD*

The figure presents the weight in % for the total mass of the roots for each root type. After around 30 minutes roots without the barrier has lost 58 % weight in water, while roots with a barrier after 30 minutes had lost 24-25 % weight in water.

10. Presence of the barrier to ROL

Periodic acid staining was conducted to confirm the presence of an apoplastic barrier. *Figure 8* shows two cross-sections of roots without a barrier or with a tight barrier.

Figure 8. Cross-section of rice root with or without a barrier. *The two cross-sections show a root without a barrier (A) or with a barrier (B) in the exodermis. The purple colour indicates the presence of the periodic acid tracer. Pictures kindly provided by Lucas León Peralta Ogorek. Freshwater Biological Laboratory, University of Copenhagen.*



The periodic acid functions as an apoplastic tracer infiltrating the apoplastic pathway between the cells; this path may also be the pathway that the water molecule follow during evaporation (*see Discussion*). A root without the barrier to ROL will appear with an overall coloured midsection because the apoplastic tracer can infiltrate (Figure 8, A). If the root possesses a barrier, the suberin and possibly lignin synthesized between the cell walls, act as a barrier against the apoplastic tracer leaving the whole-mid centre colourless (Figure 8, B).

The periodic acid as a permeability test shows only the presence or absence of a barrier but not the tightness of this trait or possible changes along the whole root (patchy barrier). *Figure 9* presents the radial O_2 loss through the root via. methylene blue staining.

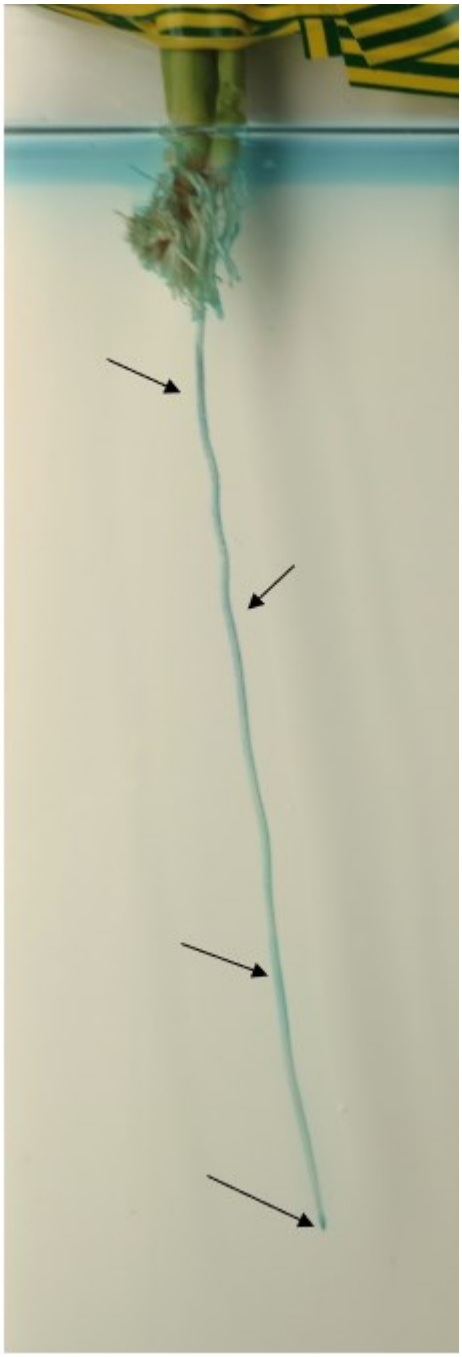


Figure 9. Radial oxygen loss from an 18.5 cm long root of rice. *The figure visualizes radial oxygen by means of the methylene staining method. Reduced methylene is colourless while when oxidised it is blue. The darker the blue the more O_2 is leaking from the root. Reduced methylene can be observed in the mid top of the root and in the lower part of the root or at the apex (i.e. tip). Picture kindly provided by Lucas León Peralta Ogorek***. *Freshwater Biological Laboratory, University of Copenhagen.*

The picture has been modified with a warmer background colour in order to highlight the colour differentiation on the root. Methylene blue oxidises in the presence of molecular O_2 turning into a blue colour. It remains colourless when no O_2 is present. The root has dark blue spots in the top from cuttings of laterals that leaks some O_2. This root demonstrates a root without the barrier because the presents of blue downward the root. of Towards the apex, the root shows some different colour gradient that are visible as patches of clear blue to a paler tone (pointing arrows). At the apex, where a barrier never develops, one can observe it's very dark blue staining due to leakage of O_2 (see last arrow). Around some mm from the apex a very pale colour occurs demonstrating a tighter barrier to ROL.

Discussion

This study hypothesized that roots with a barrier to ROL would lose water at a lower rate than roots without a barrier. The result of the conducted experiments strongly supports this hypothesis. It was shown that there is a difference in water loss for roots without the barrier that lost significant more water compared to the roots with a barrier to ROL (*Table 3*). Accordingly, roots without a barrier had 2.5 times higher water loss rates compared to roots with the barrier *(Figure 6).* The water loss measured in this experiment was assessed for the first 30 minutes of desiccation. A video *(Video S1*) show footages equivalent for *1 hour* (speeded up to last 1 min) but around 10 – 20 minutes into the footage, a noticeable reduction of the size of the roots can be observed, particularly of roots without a barrier. Using the Michalis-Menten model from the results section in *Figure 4*, one could estimate the time for the root types to lose 95 % of their total water pool (*Table 2*). It would take approx. 5 h for roots without the barrier to lose 95 % of tissue water. For roots with tight barrier it would take 22 h and for roots with a weak barrier it would approx. take 19 h. These times strongly suggest that a barrier to ROL prolongs the total desiccation time compared to root without the barrier.

The Michaelis-Menten function was originally developed to describe the initial reaction rate for enzymes binding with substrate. The curve of Michalis-Menten showed a great fit ($r^2 = 0.99$) for tissue dehydration, because of the high initial rate of water loss that reaches a saturation point, showing a *physical process*. If this was a classic general *biological* phenomenon, one would expect a greater deviation and fluctuation in the data from this curve, which was not the case in the present study since evaporation is driven by gradient in water vapour, i.e. an entirely physical process. Any experimental noise would have been reflected in the r^2. V_{max} showed the most favourable fit for the parameter for the Tuckey test, whereas K_m did not differ between the three root types.

11. Water retention in roots with exodermis

Only one previous study has attempted to determine the influence of a well-developed root exodermis on tissue water retention (Taleisnik, Peyrano et al. 1999). The study was conducted on different species that either develop an exodermis in the roots as exemplified by maize (*Zea mays* L.) or species without an exodermis such as pea *(Pisum sativum L.).* There may be a relationship between the exodermis and the barrier to ROL. The exodermis shares some important similarities with the barrier to ROL. These important similarities are, with various degree in compositions, Casparian bands and suberin lamellae (Enstone, Peterson et al. 2002). Especially suberin as a molecule has been shown to be the major component in the barrier to ROL (Ejiri and Shiono 2019). But also, suberin lamellae is not formed in every root or every cell (Enstone, Peterson et al. 2002). The exodermis and the barrier to ROL formation are both influenced by environmental cues such as drought, flooding, sub-toxic levels of soil phytotoxins and low temperatures etc. (Enstone, Peterson et al. 2002, Kotula, Colmer et al. 2014). Suberin is hydrophobic macromolecule containing of long-fatty suberin acids and glycerol as the backbone (Pollard, Beisson et al. 2008). Suberin lamellae as a consequence of suberin's high hydrophobicity, should have a major impact on the water flow in the roots (Ma and Peterson 2003). In summary, all of the above indicate that the barrier to ROL in the exodermis, may not be the same, but share these important similarities that makes it an interesting comparison.
The study of Taleisnik, Peyrano et al. (1999) showed that after 20 min when the water content was approx. 75%, the retention of water had become significantly lower compared to roots with an exodermis (Taleisnik, Peyrano et al. 1999). One can compare *Figure 7* from this study with results from Taleisnik, Peyrano et al. (1999) that presents water loss kinetics (*Figure S2). It* is shown that roots without an exodermis have a much higher tissue dehydration. At 30 minutes the roots had lost 60-65 % water from their total initial weight compared to roots with exodermis, which had lost only approx. 35 % water loss (Taleisnik, Peyrano et al. 1999). *A*fter 30 minutes, roots without a barrier had lost 58 % water from their initial weight and roots with a barrier (tight and weak) had lost around 24-25 % water *(Figure 7).* Both figures show a difference in water retention for different types of roots. Though the roots from this study all had an exodermis for the three root types but differed in having a

barrier or not. The conditions for the air exposure are different between the experiments. In the study of Taleisnik, Peyrano et al. (1999) the roots were placed on the lab bench in room conditions to be periodically weighed. This would result in more experimental noise compared to present study's experiments where the roots were placed in a controlled enviroment of the weight chamber with a set humidity of 18-22 % and silica gel grains to maintain the humidity, and weight mesurements every 1 minute. The consistent intervals of weighting and the more controlled relative humidity is clearly demonstrated when comparing *Figures 7* and *S2*.

12. Experimental limitations

One limitation of the present experiments is to know with certainties where exactly the formation of the barrier is located along the length of the root. It has been shown that water retention is higher in basal (i.e. more developed exodermis) than the apical (i.e. less developed exodermis) parts of the root in several species (Taleisnik, Peyrano et al. 1999). Usually the barrier is not formed around 2-4 cm from the apex (Yamauchi, Colmer et al. 2018). In this study, around 2.5 - 3 cm of the root apex was discarded assuming that the remaining part of the root had the barrier to ROL (when grown in stagnant deoxygenated conditions). However, the remianing part may still have tissue without the barrier to ROL left. This could underestimate the results of the barrier to ROL influence on the water retention, because water from root tissue could evaporate from "windows" without the barrier. The methylene blue staining method showed these windows, when the barrier was abscent (see *Figure 9 pointing arrows*). This would affect especially V_{max}. Km would likely not show any differences because the parameter did not respond to the treatment in the first place.

13. Determination the presence of the barrier

To reveal the presence of the barrier, periodic acid as an apoplastic tracer was used. Apoplastic tracers are used to analyse the root permeability by binding to certain proteins or components of the root cells walls (Pecková, Tylová et al. 2016). For this study, Schiff's reagent reacts with cleaved polysaccharides that is detectable in the cell wall (Soukup, Votrubová et al. 2002). The periodic acid is thought to be blocked in the apoplastic pathway in the exodermis that contains suberin and Casparian bands (Kotula and Steudle 2009). From *Figures 8.B and 8.A*, the periodic acid method clearly shows the presence of a barrier compared to a root section without the barrier. The presence of the barrier is shown as a non-coloured cortex i.e. where suberin and possible lignin blocked Schiff's reagent. The roots with no barrier appeared with a coloured cortex because no suberin blocked the tracer. This method can be used to visualize if a barrier is presents or not, though it does not identify the tightness or the gradient of the barrier, i.e. this method remains qualitative. Additionally, methylene blue staining can be used to test for the tightness and gradient of the barrier by colour gradients through the root *(Figure 9)* of oxidised blue (O_2 leaking) and colourless (no O_2 leak).

14.Tissue dehydration through the apoplastic pathway

The greater reduction in size of the water loss from roots without the barrier, raised the question how water exists as vapor inside the root tissue. Ion and gasses travels through the apoplastic pathway of the exodermis (i.e. also through the epidermis and cortex) blocked by the casparian bands in the endodermis. After the ions have been taken up through the apoplastic pathway they enter the symplastic pathway through the plasmalemma into the stele (Sattelmacher 2001, Enstone, Peterson et al. 2002). For the barrier to ROL it is known that the barrier is developed in a suberized exodermis (Kotula and Steudle 2009) and the Casparian bands have been shown to be established in the exodermis (Kotula, Ranathunge et al. 2009). Because of these modifications of the apoplast (i.e. cell wall) it suggests that O_2 gas must use the apoplastic pathway when leaking into anoxic soils. Nutrients are also observed to be obtained through the exodermis and redistributed across the endodermis into the stele (Enstone, Peterson et al. 2002). But when it comes to water it is a more passive process for the uptake regulated by; an osmotic component, the plants transpiration (i.e. plant

tissue dehydration over the surface) and the roots hydraulic resistance (Steudle and Peterson 1998). At high transpiration the apoplastic pathway is primarily used where the hydraulic resistance is low allowing rapid water flow. When the transpiration is low (e.g. stress factors as drought, salinity, nutrient deprivation – or night time) the apoplastic pathway is less used and the water flow is restricted and directed through the symplastic pathway (Steudle 1994, Steudle and Peterson 1998). Data has shown that suberin functions to reduce water permeability (Peterson and Enstone 1996). The modification of the exodermis with suberin indicates that the exodermis must function as apoplastic regulator for water retention. It has also been suggested that when it comes to hydrostatic pressure, water flow is predominantly through the apoplastic pathway (Steudle 1994). In the study of Taleisnik, Peyrano et al (1999) with their investigation of water loss as a diffusional process, the authors concluded that this process must also have an apoplastic component (Taleisnik, Peyrano et al. 1999).

15. Outlook for further research

For further research more and different species are required to test if the barrier to ROL also shows same significant resistance to radial water loss as observed with rice in the present study. Also testing on species that has constitutive barriers to see if a barrier developed regardless of environmental clues is less or more effective for water retention compared to an inducible barrier. The present study showed no significant difference in water loss between weak barriers and tight barriers (*Table 3*). Therefore, further research is needed to test if greater root tightness for O_2 is correlated with better water retention. Another interesting aspect would be to see if the formation of the barrier could happen as a respond to drought conditions. It was shown that under prolonged drought simulation sorghum nodal roots had significantly thicker exodermis compared to the control, though no significant differences in water loss was observed (Taleisnik, Peyrano et al. 1999).

For future implementation, if extreme weather events such as floods and droughts would increase on a global scale it would be beneficial though introgression to introduce traits such as the barrier to ROL to increase crops' overall tolerance to climate changes.

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Supporting information

Information S1:

Formula of growth solution for rice in hydroponics aerated conditions:

1 pot = 3,5 L (around 4-5 plants)

0.904 g $CaSO_4$ * $2H_2O$ 1.708 g MES (Buffer) 1.4 ml $MgSO_4$ *$7H_2O$ 13.125 ml KNO_3 2.1875 ml NH_4NO_3 1.4 ml KH_2PO_4 1.4 ml Na_2O_3Si * $9H_2O$ 3.5 ml Fe-EDTA 3.5 ml Micronutrients.

Micronutrients: Prepared in one solution in g/L:
KCL: 3.725
H_3BO_3 : 1.545
$MnSO_4$ *H_2O: 0.338
$ZnSO_4$ *$7H_2O$: 0.575
$CuSO_4$*$5H_2O$: 0.12485
Na_2MoO_4*$2H_2O$: 0.12095
$NiSO_4$*$7H_2O$: 0.2808

Figure S1: Linear- fit

The linear fit for the water loss data. N = 4. Error bars = SD. Regression for a linear-fit.

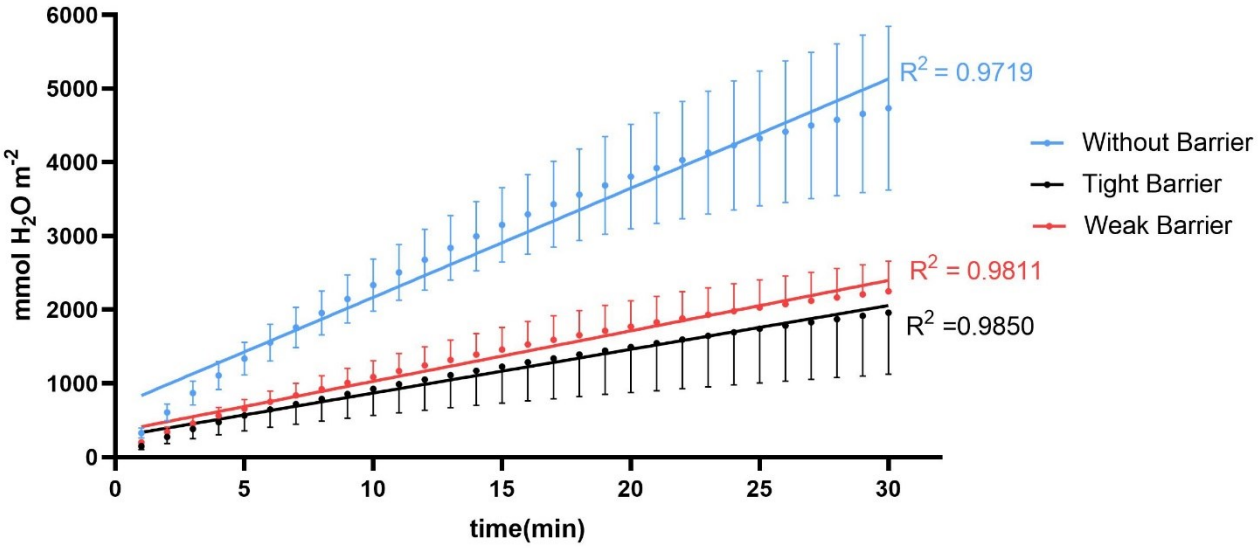

Figure S1. Linear fit of data. N = 4. Error bars = SD. Regression for a linear-fit.

Video S1

Observed differences in tissue dehydration for roots with and without the barrier to ROL

https://youtu.be/-H6L5O-yU-g (Video should remain private).

Figure S2:

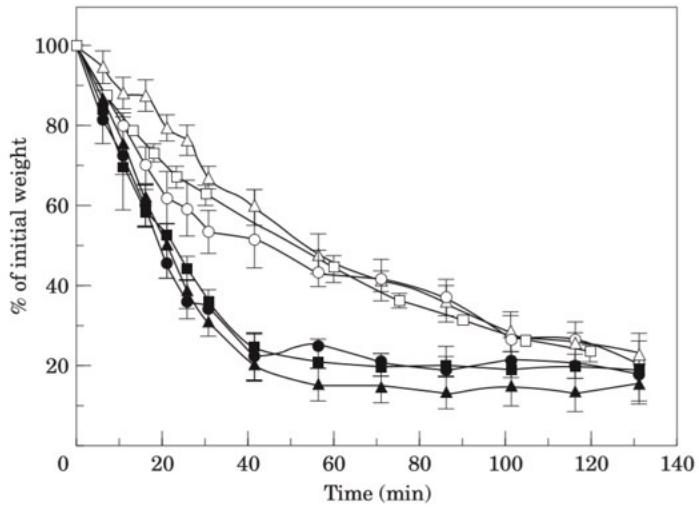

Figure S2: *Water loss kinetics in basal (100-140 mm from root apex) root segments from roots without exodermis (black, circle, square and triangle) and roots with exodermis (white circle, square, triangle).*

Figure obtained from the study of Taleisnik, Peyrano et al. (1999) side 23.