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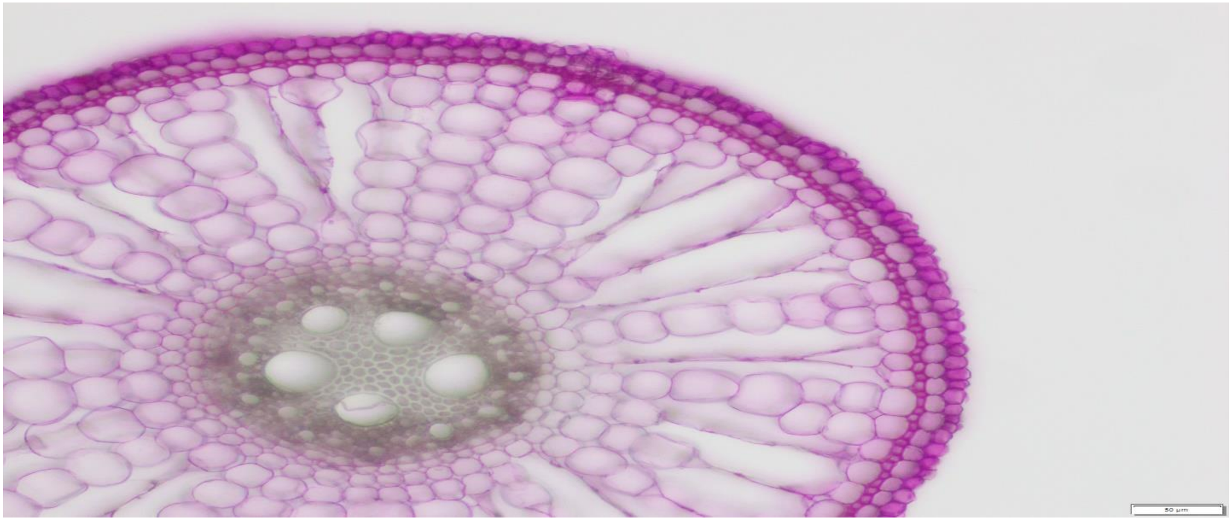

Bachelor Thesis

Effect of osmotic stress on formation of ROL-barrier in roots of rice

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Abstract

Global warming is expected to increase drought stress in many rice producing areas, warranting the search for drought stress tolerance traits. A trait normally considered important for flood tolerance, the formation of a barrier to radial oxygen loss (ROL), has recently been shown to reduce root water loss under dry conditions. The aim of the study was to investigate if roots of rice (*Oryza sativa* X O. *glaberrima)* induced a ROL-barrier when subject to osmotic stress. NERICA-1 rice was grown hydroponically in aerated (control), stagnant deoxygenated and 10% Polyethylene glycol (PEG) containing nutrient solutions. Stagnant nutrient solution was used to mimic waterlogged, known to induce barrier formation and PEG to induce osmotic stress mimicking a drought situation. Plant roots of all treatments were subject to measurements of root water loss (RWL) rates, shoot & root dry mass, SPAD Measurements on days 1 to 16, and root cross sectioning with staining for apoplastic barrier on day 0, 2, 4, and 8. RWL rates were lowest in roots grown in stagnant nutrient solution but not in PEG and aerated solutions. The presence of a ROL-barrier in stagnant nutrient solution was confirmed by root cross-section staining's. SPAD measurements show stagnant nutrient solution being the most visibly stressed, with PEG treated plants not showing chlorophyll degradation. NERICA-1 roots grown in PEG and aerated (control) nutrient solution had RWL rates which were insignificantly different. However, pictures of root-cross sections show the presence of a weak-barrier in PEG nutrient solution on days 2, 4 and 8. The highest root dry mass was found in plants treated with PEG, indicating a possible drought stress response in NERICA-1 toward osmotic stress. The results support the hypothesis that a ROL-barrier is induced in stagnant, deoxygenated nutrient solution (mimicking waterlogged conditions), while the hypothesis that osmotic stress induces a similarly strong ROL-barrier in the roots of rice was not supported.

Introduction

Why is rice important?

Rice is an important crop everywhere, especially in LEDCs (Less economically developed countries) where it can amount up to 19% of the total crop areas (Maclean et al., 2013). Up to 94% of total rice area is located in LEDCs, cultivated by existing millions of rice farms (Maclean et al., 2013). Compared to other major crops such as wheat and maize, rice is not as commonly used for animal feed and instead consumed by humans. In 2009 human rice consumption made up 78% of total rice production (Maclean et al., 2013). During the same year 159 million ha. rice was harvested equalling maize production, making rice one of the worlds most harvested crops. In 2009 rice provided 13% per capita protein, ranking rice high in protein compared to other cereals. The protein content can have a ranging value of 4,3%-18,2% protein. Variation in protein content can be due to different environmental factors such as flooding, drought, soil fertility, temperature and solar radiation (Maclean et al., 2013). Rice also provided for 19% global human per capita energy. There are two main species of rice that are central to human nutrition, *Oryza sativa* and *O. glaberrima*, O. *sativa* grown worldwide and O. *glaberrima* grown in West Africa (Maclean et al., 2013).

In sub-Saharan Africa (West Africa) rainfed rice makes up more than 80% of the total rice area, with large areas often struggling to cope with drought stress (Kijoji et al., 2014). Six million hectares (60%) of the continents rice growing is located in West Africa (Niang et al., 2017), making it the most important rice production region in Africa that has a total of 9 million ha. rice fields (Wopereis et al., 2013). There are several challenges with growing rice in West Africa. High temperatures can cause heat or cold induced spikelet sterility which in turn gives a lower yield. The harsh climatic conditions affect the soil in several ways. Weathering can change the amount of nutrients in the soil, the ability to cation exchange capacity (CEC) and the pH of the soil. Humid forest rice soils are therefore often weathered and acidic with macronutrient deficiencies. Meanwhile semi-arid soils are less weathered and have higher CEC and pH but suffer due to salinity and alkalinity problems (Niang et al., 2017). Rice is grown mostly in rainfed and irrigated lowlands and upland rice fields. Irrigated rice is normally grown in paddy fields with irrigation, which enables the farmer to cultivate more than one crop annually. In rainfed lowland fields, rice is grown at a slope similarly to upland rice. These varying soil and geographical conditions make it hard to have a steady yield. Rice yield is also affected by the means which the crop is produced. Means of production can vary largely farmer to farmer especially with the lack of monetary resources available to the farmers in these regions (Niang et al., 2017). This causes there to be a big difference in crop establishment date and methods as observed by (Niang et al., 2017) as well as tillage methods, choice of rice, water management, use of fertilizer (timing and amount) and pesticides/insecticides (Niang et al., 2017). Taking all these issues into account, drought still poses one of the largest threats to the rice industry (Wopereis et al., 2013).

Abiotic stress

Drought

Drought can be defined as a lack of water relative to normal conditions (Sheffield & Wood, 2011). With an increasing population and a limited water supply, it is expected that drought (which is already a big threat to the worlds food security) will continue to increase (Farooq et al., 2009). Drought can cause severe stress on plants because of its damaging effects on the plant itself but also the soil in which the plant is grown in. Due to a more varying climate, plants will often face different stresses during a shorter time span. It is therefore hard to predict the plants response and capability to survive in a changing climate (Farooq et al., 2009). This is an important area of research as there currently are no affordable technologies that can solve the issues crop production faces during drought. Hence many researchers opting to find new sorts of crop plants which can tolerate drought. To do this it Is important to know about the physiological mechanisms of the plant as drought can have an effect both morphologically and molecularly. The effects can also be measured at all phenological stages when water loss is taking place (Farooq et al., 2009). Drought has an impact on germination, plant growth, and yield of the plants. When affected by drought plants have reduced plant development and growth which leads to less flowers developed and a decrease in grain filling (Farooq et al., 2009). Almost all plants that experience water deficit first response is to close their stomata to prevent the loss of water by transpiration (Mansfield & Atkinson, 1990). If the stomata closes it could lead to a decrease in leaf turgor and water potential (Ludlow & Muchow, 1990). Drought-induced stomatal closure that limits CO_2 uptake by the leaves can cause limitations to photosynthesis and lead to increased photo-damage (Cornic & Massacci, 1996). A decrease in turgor pressure results in impaired cell growth (Taiz & Zeiger, 2006). Reduction in photosynthesis is also limited by premature leaf senescence and decrease in leaf expansion as a result of drought (Farooq et al., 2009). Drought can lead to limited nutrient uptake, as water is usually used by the roots to uptake nutrients and transport it further via the shoot (Garg, 2003). The restraints of drought are not only damaging to the plants but also to those who farm them.

In (Wopereis et al., 2013) a table was made displaying sub-Saharan African farmers perceptions of climate related abiotic stresses in rice environments across 18 different countries. The average percentage of areas affected by drought when experienced was 37% and average percentage yield caused by drought when experienced was 29%. The table indicates the percentages for each rice production environment, irrigated, upland, rainfed lowland and other. Drought reaches more than 20% for each (percentage affected, yield affected) environment. A total of 30% sub-Saharan farmers have perceived drought as a major problem across all rice environments. (Wopereis et al., 2013) In 2008 it was estimated that 10% of farmers struggled with drought that affected 37% of their rice area and led to a 29% yield loss (Wopereis et al., 2013). Later in the report (Wopereis et al., 2013) conclude that drought and flooding are the two most important climate related stresses.

Flooding

Alongside drought, flooding is one of the major abiotic stresses. According to (FAO, 2021) floods are the second (next to drought) most damaging natural disaster. From 2006 till 2016, two-thirds of the total damage and loss of crops was caused by floods (Conforti et al., 2017), costing several countries billions of USD. Estimating a total of 19% production loss. In the (FAO, 2021) report it is also stated that floods happened on an average of 30 times a year during the 1970s. The average for the 2000s was 180 and in 2006 a record number of 246 floods were counted. Flooding was estimated by 25% of sub-Saharan farmers to be a major issue, 5% of farmers experienced flooding that affected up to 37% of the rice area damaging 27% of their yield in 2008 (Wopereis et al., 2013). Soil waterlogging is defined as when the soil is in an excess of water that limits gas diffusion (Nishiuchi et al., 2012). Waterlogging is often a result of flooding, with potential for complete submergence of crops. When waterlogging occurs, an imbalance forms due to the slow diffusions of gasses in water and oxygen being consumed by organisms and plant roots. When the oxygen in the soil is used up it can be detrimental to the roots of the plants as ion uptake and transport becomes more difficult (Visser, 2003). Flooding can also decrease the soil redox potential, leading to major changes in the soil profile. The decreased ability for gas exchange will cause root hypoxia, increased CO_2 levels in the root zone as well as anoxia of the submerged area (Colmer et al., 2018). These factors all result in effects on the ability of shoot and root growth, nutrient intake, and metabolism in the roots. Microorganisms in the soil use NO_3^- to respire when O_2 has been depleted. NO_3^- is then reduced to NH_4^+, afterwards manganese oxides are used by the microorganism, which increase the concentration of manganese in the water to a point where they are toxic for the plants (Colmer et al., 2018). After the water recedes, pollutants are left in the soil and can cause water contamination. Soil erosion is another side effect of floods, that happen due to accumulation of silt in the soil (Conforti et al., 2017). Not all plants are equally tolerant to shoot submergence and soil waterlogging, different strategies are applied by plants to survive abiotic stresses (Colmer et al., 2018).

Plant adaptation to abiotic stress

Drought

Plants have made several adaptations to drought stress. To avoid drought stress, some plants have attained a shorter life cycle and can reproduce before the dry season (Farooq et al., 2009). Plants have also attained mechanism that help reduce water loss, this can be by stomatal control of transpiration and the ability to maintain water uptake through a larger/longer root system. Root properties such as length, biomass and depth help improve the yield. Roots are key during drought, as only the root can uptake water from the soil, often the plant will limit the number of leaves to avoid using more water. A deep thick root is then favoured for the plant as it will be able to reach water deep in the soil (Farooq et al., 2009). O. *glaberrima* has shown phenotypic traits suitable for hot and dry areas. These traits are such as leaf thickness that correlate with efficiency of transpiration and drought tolerance (Atwell et al., 2014). During germination drought can cause a shortened hypocotyl length, while shoot and root weight can be reduced. Normally to combat drought it is seen in plants that roots become thicker (Farooq et al., 2009). Modern commercial rice have been modified by artificial selection over many years, hereby the differences between commercial and wild rice types can vary quite a bit. Plants can vary from leaf structure, morphology, and physiology to canopy architecture, as well as fast growth rates of shoot biomass needed in the short-wet seasons in areas such as Australia and Africa. Traits in wild rice may be relevant to explore and could be found beneficial to domesticated rice (Atwell et al., 2014). In a study done by (Atwell et al., 2014) it was found that cultivars with bigger leaf area had better transpiration efficiency during drought, compared to plants with more slender leaves. Large leaves with more vascular tissue are considered characteristic of rice species more tolerant to drought. In the study it was also shown that species that are more tolerant to drought have increased mesophyll conductance, this helps increase CO2 uptake and water movement in the plant. This is much like the plant increasing the cell wall to stop the loss of water, both drought resistance strategies known to be used by plants (Atwell et al., 2014). Strategies that help increase drought tolerance have a high chance of not being present in fertilised rice that are grown in well irrigated fields. (Atwell et al., 2014) highlighting the importance of possible traits found in O. *glaberrima*. This report is relevant as it looks at NERICA-1 rice which is a cross between O. *sativa* and O. *glaberrima*, two of most widely grown rice, however O. *glaberrima* is mostly grown in West Africa and O. *sativa* in Asia. Crossing these species will hopefully allow for a rice that can grow in both dry and wet regions.

Flooding

Plants have evolved several responses to reduce the impact of stress from oxygen depletion in soil and water. These responses can be both morphological and biochemical (Visser, 2003). Such as internal oxygen transport, which can avoid anaerobic conditions inside the plant or the development of aerenchyma. Plants that can tolerate flooding tend to either have traits that can be categorized as escape or quiescence. Escape strategy is summarized in (Colmer et al., 2018) as increase in growth rate in petioles and stems (shoot organs) which enable the plant to reach above the water. Furthermore the plants develop aerenchyma to facilitate internal diffusion of gas. (Colmer et al., 2018) summarizes quiescent strategy as conservation of carbohydrates and energy and an increase in molecular components that support root and shoot organs in low O_2 conditions. Rice is unique for a major crop in its ability to be flood tolerant during seed germination and establishment (Ismail et al., 2012). However rice seedlings are limited to coleoptile development, as shoots and root do not develop in anaerobic conditions (Ismail et al., 2012). The Coleoptiles exist of pre-formed cells which can increase their length every hour to reach O_2 enabling growth of the shoot and root (Colmer et al., 2018). The growth of the coleoptile is induced by ethylene enabling them stretch towards the surface of the water. A clear example of a response to abiotic stress in the rice plant (Colmer et al., 2018). Species that are tolerant to waterlogging often form larger adventitious root systems, than species that are not tolerant. These roots are able to grow into anoxic flooded soil (Colmer et al., 2018). To stop the plant tissue from becoming anoxic, the plant increases the amount of aerenchyma in the roots, however this is not immediate (Justin & Armstrong, 1987). Aerenchyma are channels of cells that allow for gas to pass through easily (Visser, 2003). It allows for transport of O_2 to parts of the plant that are submerged (Colmer et al., 2018). However it is not common for all species to have as much aerenchyma, as it is more seen in wetland species than dryland species, with some not capable of forming aerenchyma (Colmer et al., 2018). Aerenchyma is made in the cortex of the root and can be developed in two main ways. The first is lysigeny which is formed by the collapse of cortical cells that then form gas-filled spaces. The cell collapse is a result of programmed cell death. The second is schizogeny formed when cells spread in a radial direction, leaving gas-filled spaces between cells (Colmer et al., 2018). To make aerenchyma more effective it is possible to form gas tight barriers in the epidermis or exodermis of the roots to prevent loss of oxygen (Colmer, 2003). The Reduced oxygen loss (ROL) barrier inhibits oxygen loss but also intake, aerenchyma therefore has to supply oxygen by longitudinal diffusion (Colmer, 2003). This tight barrier has been found in wetland species, but as found in (Colmer, 2003) the barrier can be induced by growth in stagnant deoxygenated mediums. Roots with a tight ROL barrier are still affected by O_2 loss in the root tip and laterals. However, the ROL in the root tip can stop toxins from affecting the apex and hereby not only stop the loss of O_2 (Colmer et al., 2018). The ROL barrier is also know to reduce root water loss (Peralta Ogorek et al., 2021). I therefore hypothesize that barriers to ROL are induced when rice is stressed by drought.

Does drought induce ROL-barrier in Nerica-1?

An ROL-barrier either be endodermal suberin, that blocks apoplastic movement of water and solutes into the stele, or Exodermal suberin that stops apoplastic transport on the root surface (Baxter et al., 2009). In (Colmer et al., 1998) four rice genotypes were grown in aerated and stagnant nutrient solutions, growth in stagnant solution showed an barrier that reduced oxygen loss (ROL-barrier). Colmer et al. (1998) suggests that the barrier not only reduces ROL but also stops toxic substances formed in the waterlogged soil from entering the roots. During a study done by (Henry et al., 2012) it was found that drought caused the diameter of the stele to increase as well as an increase in suberization of the endodermis but not the exodermis. The article also mentioned that suberization of the outer part of the root (OPR) is possibly done to keep water from going out of the root (Henry et al., 2012). The endodermis and stele are mentioned to have a similar effect (Henry et al., 2012). The ROL-barrier was found to restrict O_2, H_2 and water in (Peralta Ogorek et al., 2021), where O. *sativa* had been stressed in stagnant deoxygenated nutrient solution, to simulate similar conditions to waterlogging. The article suggest that the ROL-barrier could restrict water loss when in dry soils. Similarly in Kjær (2020) an O. *sativa* X *O. glaberrima* species was found to have tight ROL barriers when grown under stagnant conditions. To test the claim that the ROL-barrier could restrict RWL, I hypothesize that Nerica-1 rice roots will develop an ROL-barrier when exposed to drought-like conditions posed by adding PEG to plants growing in nutrient solution. The formation of a ROL barrier was assessed by measuring root water loss, and staining root cross sections for the formation of an endodermis. In addition to PEG, rice was also grown in aerated nutrient solution (control) and in stagnant nutrient solution. Growing the rice in a stagnant nutrient solution we expect to see an ROL-barrier developed as found in several rice when waterlogged (Colmer, 2003; Colmer et al., 2018; Peralta Ogorek et al., 2021)

Materials & Methods

Germinating Seeds

Seeds of O. *glaberrima x sativa 'NERICA-1'* were imbibed in aerated 0.5 mM CaSO4 solution for two hours, covered in aluminum foil to keep light out. After 2 hours the seeds were placed on 0.5 mM CaS04 moistened tissue paper in aluminum covered petri dishes. The seeds were then left to germinate for 2-3 days in the glasshouse. The temperature in the glasshouse ranged from a max of 41.51 °C, minimum of 16.28 °C and had an average of 28.27 °C (6 am till 6 pm). From 6 pm till 6 am the max was 30.20 °C, minimum 15.99 °C with an average of 22.35 °C. After germination, the seeds were transferred to a mesh floating on 25% concentrated aerated nutrient solution (composition seen below) in a 3.6 L pot. A week later the seedlings were transferred to a 100% concentrated aerated nutrient solution in pots of 3.6 L. The composition of 100% aerated nutrient solution consists of (mmol L^{-1}): CaSO4; 1.5, MES; 2.5, MgSO4; 0.4, KNO3; 3.75, NH4NO3; 0.625, KH2PO4; 0.2, Na2O3Si; 0.1, Fe-EDTA; 0.05, Micronutrients; 1. Each pot with space for 8 seedlings, put in place with foam and Parafilm for the roots not to grow into the foam keeping them in place. The nutrient solution was pH adjusted with 1 M KOH to ca. pH 6. Hereafter the solution was optimally changed weekly (sometimes biweekly). Pots were topped up with deionized (DI) water to compensate for transpiration. The rice was kept in a glass house with varying temperatures as mentioned before. Each pot was aerated and had additional lights set on a timer allowing the rice 10 hours of darkness.

After reaching an age of 4 weeks the rice plants had developed enough roots for measurements to be taken. The plants were divided into four 3.6 L pots per treatments (Control, Stagnant and PEG), each containing 4 plants. Control pots were kept in aerated 100% nutrient solution. Nutrient solutions made stagnant with 0.1 % agar mimicked an anoxic, waterlogged soil (Wiengweera, 1997). Rice is known to induce a ROL-barrier under these conditions (Colmer, 2003). Plants for the stagnant solution had been given hypoxic pre-treatment the day before to avoid anoxic shock. Hypoxic pre-treatment consisted of bubbling with N2 gas for 2-3 minutes and no further aeration. On the day of treatment, "stagnant" plants were moved to a 100% nutrient solution made stagnant with 0.1% agar and deoxygenated by bubbling with N2 for 2 hours. Two leftover pots were kept as a Day '0' pot. Due to time constraints the Day 0 pot allowed for measurements of rice plants in aerated nutrient solution the following day.

PEG Concentration

Polyethylene glycol (PEG) 6000 was used to induce a "synthetic" drought in the rice pots. E.g., Dos Santos et al. (2018) determined the water potential threshold of rice drought responses using PEG 6000. PEG 6000 is useful due to It being a high molecular weight polymer, that is insoluble to plants but highly soluble in water (Dos Santos et al., 2018). PEG is neither tox nor has any saline effects (Dos Santos et al., 2018). An equation (Michel, 1983) allows for the calculation of the solution water potential at various PEG 6000 concentrations, which reads: $\psi w = -(1.18 \times 10^{-2})C - (1.18 \times 10^{-4})C^2 + (2.67 \times 10^{-4})CT + 8.39 \times 10^{-7})C^2T$. In the equation C = concentration of PEG6000 (g L^{-1}), T = temperature (^{o}C), ψw = water potential of nutrient solution (bar). The equation was then calculated using a concentration of 10%; C = 100 and T = 30°C and for 20%; C = 200 and T= 300 °C. Resulting in -0.13 mPA for 10% PEG and -0.44 mPA for 20% PEG. According to (Dos Santos et al., 2018) rice experiences drought at -0.045 mPa. To help determine which concentration would have the better affect to measure, a pilot test was made with two pots of rice with 10% and 20% PEG 6000. After 1-2 days the rice in the pot with PEG 20% wilted and the 10% rice pot looked healthy (Fig. 1S) with the most notable sign of stress in 10% PEG shown by the accumulation of brown roots. From the pilot test it was then decided that 10% PEG 6000 would be used for the actual experiment. The PEG treatment consisted of 100% nutrient solution mixed with 10% PEG-6000 (w/v, i.e 360 g per pot). The PEG is solid, so it was slowly mixed with the nutrient solution and stirred till the solid was dissolved. These 4 pots were kept aerated.

Visualization of ROL barrier by root staining

Two to three mature roots were cut in lengths 3-6 cm from rice grown in stagnant, aerated and PEG nutrient solutions. About one cm was cut off from the root tip and lateral roots were removed to best ability. Roots were blotted with a paper towel. The ends of the root were then sealed with Vaseline or lanoline. Hereafter they are incubated in 0.1% w/v periodic acid (H3IO6) for one hour. After the roots are carefully washed with DI water and incubated in a reducing solution (1 g KI, 1 g

$Na_2S_2O_3*5H_2O$ dissolved in 50 ml DI water acidified with 1 ml 1 M HCL) for one hour (Shiono et al., 2014). An 5% agar solution is made by adding 25 g agar to 500 ml DI water in a 1 L bottle. The bottle is then heated using an autoclave. The Agar solution is then poured into a petri dish where the roots are added. When the solution has cooled down and hardened the petri dish is stored in a refrigerator. To prepare the cross-sections for the microscope, a 1 cm piece of agar with the root was cut and glued to a sample plate. The sample plate was assembled onto a vibrating microtome, which was set to cut 1.50 mm cross sections. After being cut the cross sections were collected with a pipette and stained with Schiff's reagent. Schiff's reagent is used to stain the periodic acid the roots previously were incubated in (Shiono et al., 2014). Schiff's reagent produces a bright purple color when reacting with periodic acid and is applied to the roots for 5-10 minutes before being washed off with DI. After the cross sections are carefully placed on a glass-slide using a brush covered in glycerol. The cross-sections were then looked at in a microscope at 4x,10x and 20x magnification. A program linked to a camera on the microscope was used on the computer to take pictures of the cross-sections.

Radial Water Loss

Radial water loss was measured on four 8 cm roots from each pot, with each pot serving as a replicate. The roots were cut into 5 cm lengths to ensure a mature part of the root was being measured and that if present the ROL-barrier had formed. The roots were then dried with paper towels. Vaseline was applied to the ends of the roots and put on a metal mesh in a 5-digit scale to measure RWL. The measurements were taken at room temperature (20-22°C) and silica gel was kept inside the scale to keep humidity below 30%. The scale was linked to a computer where measurements were taken every minute for 60 minutes. The root diameter was measured for each root before being dried using a digital caliper. The diameter was then used to calculate the surface area of the root. Radial water loss is then calculated by the change in weight between each measurement divided by surface area of the sample (Kjær, 2020). After the measurements had been taken on the digital scale the roots were further dried in an oven at 60°C for 3 days. The roots were then measured to find the dry biomass and for further calculations of the total water content and cumulative water loss.

The time point at which the root reached at cumulative water loss of 40% was found and used to determine a corresponding rate of radial water loss (Kjær, 2020) which will be presented in the results section.

SPAD

Spad was measured using a SPAD-502plus Konica Minolta chlorophyll meter. A leaf was marked with a red marker on each 4 replicates for each treatment (Aerated, Stagnant, PEG). To measure SPAD the youngest fully developed leaf was marked and then measured at the beginning, middle and end of the leaf and then averaged. Measurements were taken on day 1 (beginning of experiment), 2, 4, 6, 8, and 16. The point of the SPAD measurement was to show physiological stress on the plant, such as discolouring which would show on the chlorophyll meter.

Dry Mass

On day 16, after the final SPAD measurements were taken, each replicate consisting of four rice plants were separated into root and shoot. Roots & shoots were cut from one another and put into brown paper bags marked with the treatment and replicate number Ex. C1 (Control replicate one). The bags were then left to dry for 2-3 days in a heat-oven at 60 °C. After drying the weight each replicate was measured (both shoot & root).

Statistics

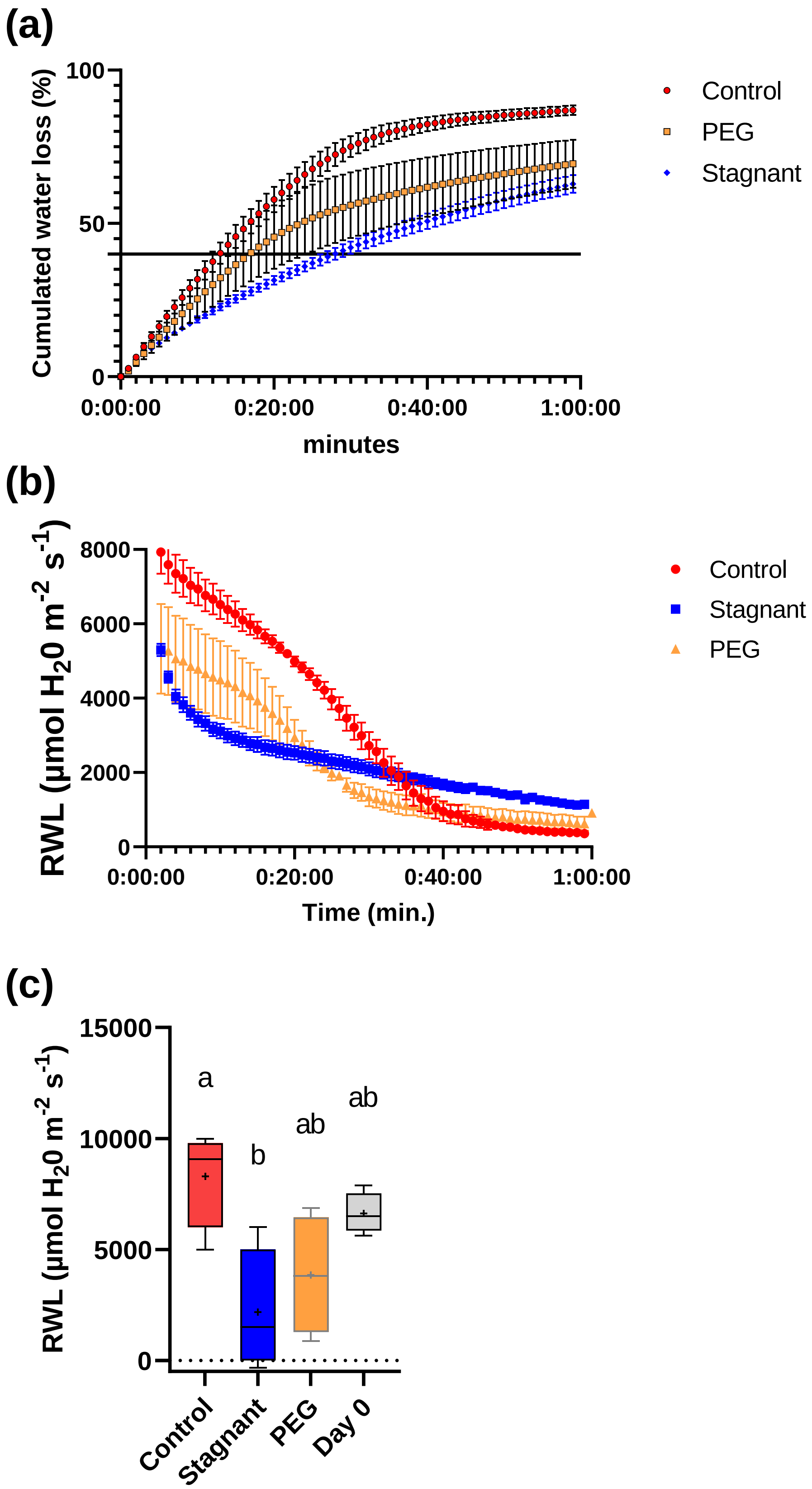
GraphPad prism (v9.1.0) was used for statistical analysis. To test the effect of the ROL-barrier on RWL a one-way ANOVA was used. A one-way ANOVA was also used to evaluate the effect of growing rice in PEG, Stagnant and aerated nutrient solutions, on root, shoot biomass and root diameter. Spad measurements were analysed using a two-way repeated measure ANOVA, since SPAD measurements were performed on the same leaf throughout the experiment. Tukey's and Bennett's multiple comparisons test were used as post hoc test to see significance difference between variables.

Results

To test for ROL-barrier formation in NERICA-1 grown in aerated, stagnant and under osmotic stress (10% PEG) I measured root water loss, SPAD, shoot and root biomass and performed staining of root cross sections for an apoplastic barrier. In this section data will be presented and the results summarized.

ROL-barrier induced by stagnant nutrient solution growing conditions.

Water loss of root segments was quantified to assess the exodermal barrier strength. Fig 1 (a) shows the cumulative water loss (%) of root segments over 1 hour of desiccation. Roots of plants grown in stagnant nutrient solution lost 62.8% water while PEG lost 69.5% water. Aerated (control) roots lost 87% water after 60 minutes. In fig 1 (b), the corresponding root water loss rates (calculated as µmol H_2O m^{-2} S^{-1}) are plotted. A similar trend is seen as in fig 1 (a), Stagnant has lost least water (1142.5 µmol H_2O m^{-2} S^{-1}) followed by PEG (912.2 µmol H_2O m^{-2} S^{-1}) and the aerated control (355.2 µmol H_2O m^{-2} S^{-1}) is lowest by the end of the hour. Aerated day 0 (Day 0) which was included to show the state of the roots before starting the experiment had a mean RWL of 6625 (µmol H_20 m^{-2} s^{-1}). The rest of the variables were measured 5 days later. Aerated day 5 (Control) has the highest RWL with a mean of 8291 (µmol H_20 m^{-2} s^{-1}). To compare RWL rates between treatments, I identified the RWL rate (consulting Fig. 1b) at which the root segments reached 40% cumulated water loss (time point identified in Fig. 1a). These RWL rates where then corrected for root diameter (see materials and methods section) and are shown in Fig. 1 (c). This graph shows the values of RWL when CWL is at 40%. A 73.8% significant difference was found between Stagnant and aerated day 5 (control) RWL rates (Tukey multiple comparisons test, $P<0.05$)-hence suggesting the existence of a barrier to Root water loss (RWL) in rice grown in stagnant nutrient solution. There was no significant difference between PEG and control RWL rates. This indicates that PEG treatment had not induced a strong ROL barrier formation by day 5. PEG and Stagnant both have lower RWL with a mean of 3844 µmol H_2O m^{-2} S^{-1}) and 2167 µmol H_2O m^{-2} S^{-1}) respectively, compared to aerated (control) nutrient solution that has a mean of 8292 µmol H_2O m^{-2} S^{-1}). However, due to PEG being insignificant it is not possible to assume why PEG has a low RWL like Stagnant.



(a)
Cumulated water loss (%)
100
50
0
0:00:00
0:20:00
0:40:00
1:00:00
minutes
Control
PEG
Stagnant
(b)
RWL (µmol H20 m-2 s-1)
8000
6000
4000
2000
0
0:00:00
0:20:00
0:40:00
1:00:00
Time (min.)
Control
Stagnant
PEG
(c)
RWL (µmol H20 m-2 s-1)
15000
10000
5000
0
a
b
ab
ab
Control
Stagnant
PEG
Day 0

Figure 1. Root water loss from in NERICA-1 roots grown in aerated, stagnant and PEG nutrient solutions.

(a) Cumulative water loss (%) with time of 5 cm root segments incubated at <35% relative humidity on a 5-digit balance taking one measurement per minute. A line is plotted at y=40, to represent time point at which RWL rates were compared. Symbols indicate +/- S.E.M (n=4). “Control” refers to aerated nutrient solution, “stagnant” refers to stagnant nutrient solution and “PEG” refers to osmotic stress (10% PEG)-nutrient solution. **(b)** Radial Water Loss (µmol H_2O m^{-2} S^{-1}) calculated using total lateral surface (m^2), water lost every measurement (g) and time (60 seconds) for roots grown under aerated, stagnant, and osmotic stress (10% PEG)-nutrient solution conditions. Symbols indicate +/- S.E.M (n=4). **(C)** RWL rates corrected for root diameter, showing the values for RWL found at 40% CWL. One-way ANOVA Post Hoc Tukey’s multiple comparisons test showed significance between stagnant and aerated day 5 (Control) ($P<0.05$, n=4). No significant difference was found in RWL between roots grown with 10% PEG nutrient solution and the aerated day 5 control. Aerated day 0 (Day 0) shows the RWL for the rice plants the day the treatments were added. Lines indicate median, +; means, whiskers; Min to Max, Box; 25^{th}-75^{th} percentile (n=4).

Root staining

Root cross sections stained with Schiff’s reagent after incubating roots with an apoplastic tracer indicate if a barrier was present or not in roots grown with aerated (control), Stagnant and PEG nutrient solutions. Through day 0 till day 4 there is no sign of an ROL-barrier in the roots of the Control, this was expected as no stress was induced on it (Fig. 2). However, on day 8 it is possible to see that the aerenchyma of control roots is very white and no reagent has gone inside the root. It should be noted that the pictures shown in figure 6 does only represent single examples. In some instances, the Control root showed at day 8 that the root had been stained, although on all samples taken the root cross-section was not fully stained as on the previous days. I.e., some weak barrier formation appeared to take place from day 4-8 even in control plants. For stagnant it is visible that the barrier has already formed by day 2. This trend continues for day 4 and 8 as the cross section remains unstained. The centre of the cross section does appear slightly red for day 4 and 8, but this could be due to longitudinal diffusion of the periodic acid if roots were not properly sealed during incubation. PEG showed varying results. Day 2 there were both samples that were completely stained indicating no barrier formation. However, some cross sections as shown in Fig. 2, show that the dye did not stain the whole cross-section. The same trend is shown in both Day 4 and 8, some PEG samples were completely stained, and other samples remained slightly stained. With such different examples for PEG, it is hard to deduce whether the plant has developed an ROL-barrier or if there have been problems during the staining.

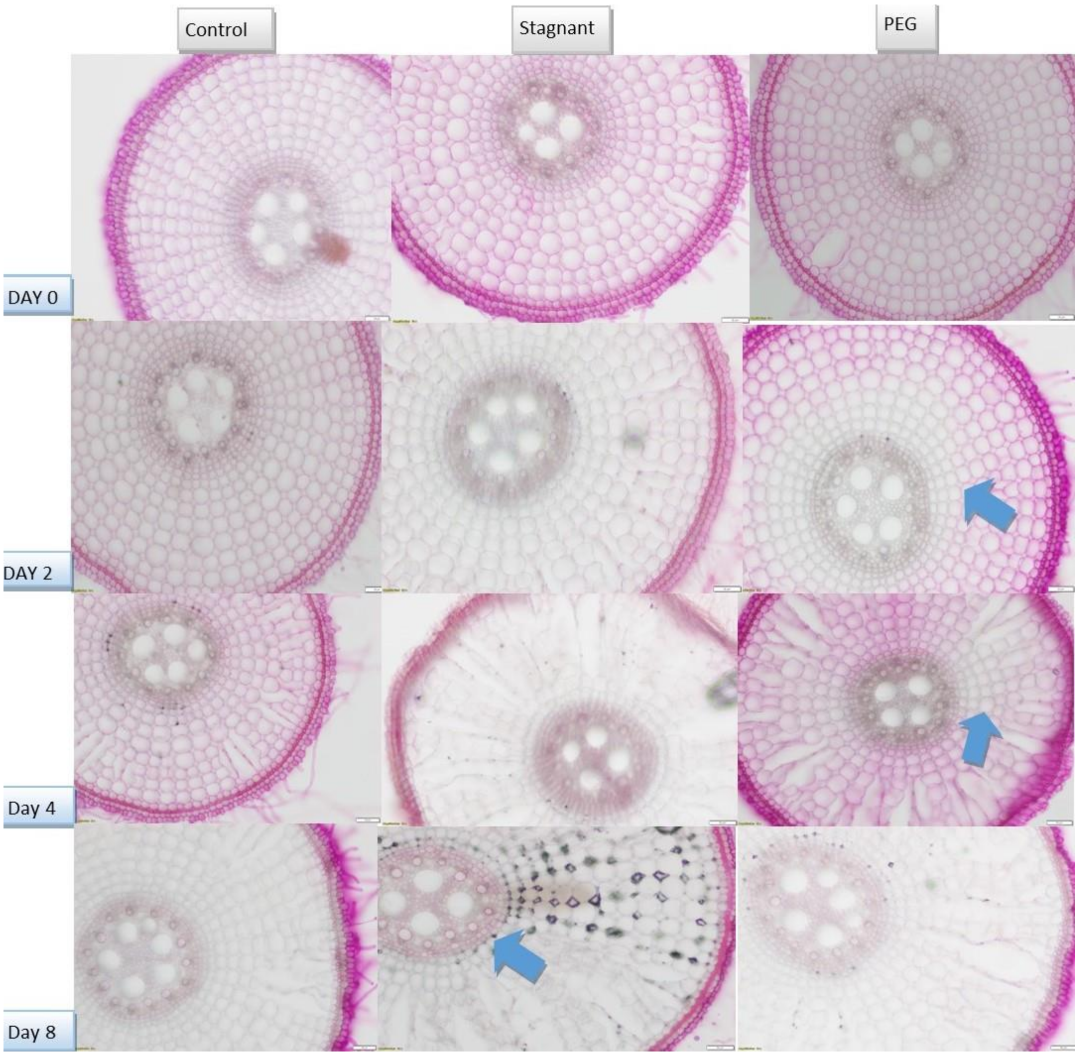


Figure 2. Stained root cross-section

Cross-sections of the root stained with Schiff's reagent (purple color). Each Column shows roots grown in aerated (control), Stagnant and PEG nutrient solutions. All pictures at a magnification of 50 µm.

Root Diameter

Root diameter of excised adventitious roots (5 cm long) was measured prior to RWL measurements. In (Fig. 3) a bar graph of the average root diameter for each treatment is shown. Stagnant has the thickest roots with a mean of about 1 mm. Aerated (control) stagnant solution has a mean of 0.9 mm and PEG nutrient solution 0.8 mm. However, the difference between the three variables is small and could be considered insignificant.

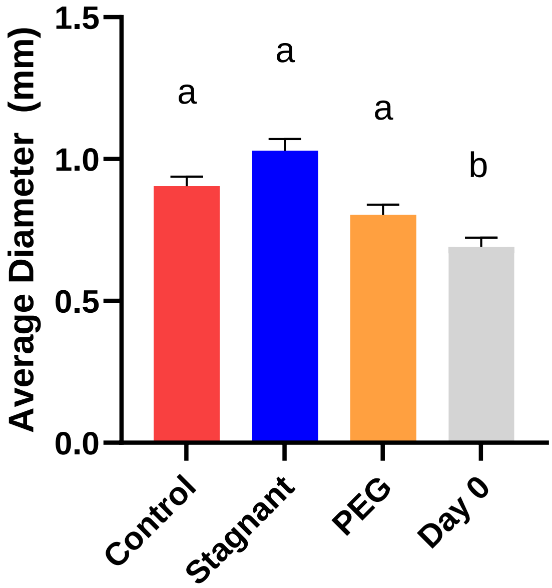


Figure 3. Average mean Root Diameter (mm) of O. *glaberrima* for aerated, stagnant and PEG nutrient solutions.

Root diameter of NERICA-1 plants grown in aerated (control), stagnant, PEG nutrient solutions. The results were analysed using Tukey's multiple comparisons test. Tukey's test showed a significant difference between root diameter of NERICA-1 rice grown in DAY 0 and PEG ($P<0.05$), Stagnant ($P<0.05$) and Control ($P<0.01$) nutrient solutions. Bars indicate mean +/- S.E.M, Letters indicate significance (n=4).

Dry Biomass shows increased root growth when stressed with PEG nutrient solution.

After 16 days of treatment, plants were harvested for roots and shoots DM determinations. In Fig. 4 (a) the shoot dry mass is shown. Overall NERICA-1 grown in aerated (control) nutrient solution had the highest shoot biomass (almost 8 g). NERICA-1 grown under osmotic stress (10% PEG) nutrient solution had a shoot biomass of ca. 7 g and NERICA-1 grown in stagnant nutrient solution ca. 5 g. A One-way ANOVA analysis shows that there is a significant difference between shoot biomass of NERICA-1 grown in aerated (control) and stagnant but no difference between shoot biomass of plant growth PEG and control nutrient solution. This could be expected as the rice is not under drought stress. Root dry mass is shown in Fig. 4 (b), where plants grown with PEG nutrient solution has a median of circa 5 g. The median weight of dry root biomass for both Control and Stagnant lie just above 2 g.

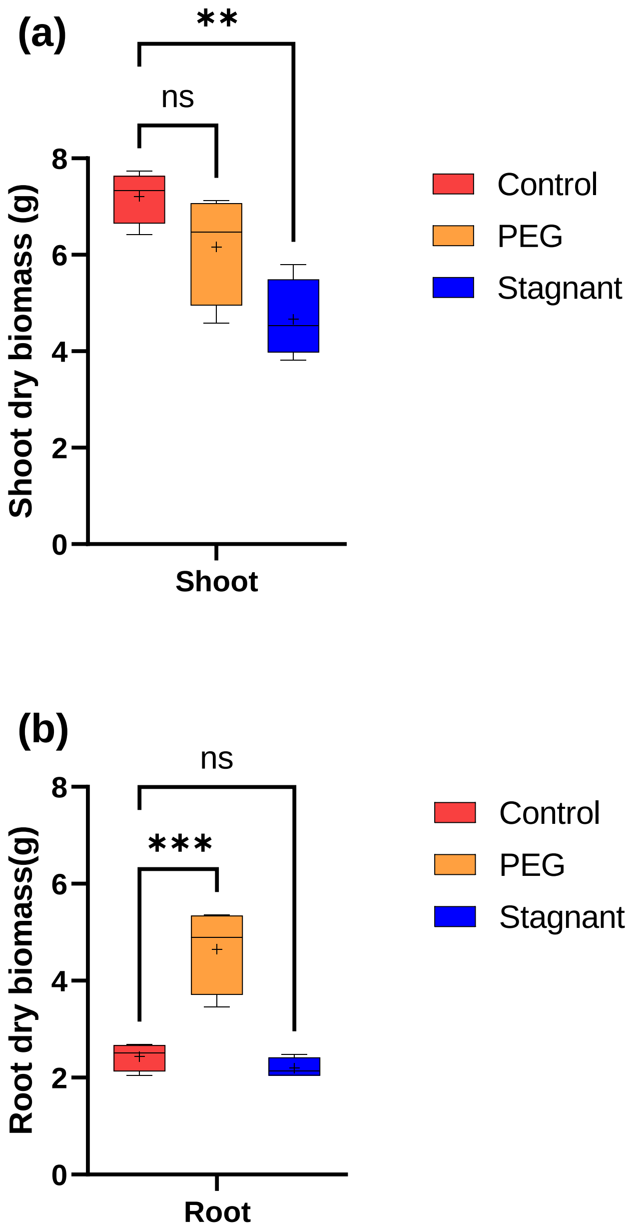


Figure 4. Dry Biomass of Root (g) and Shoot (g) of Nerica-1.

NERICA-1 plants grown under Aerated (control), PEG and stagnant conditions shoot (a) and root (b) dry mass. The results in Fig. 4 (a) were analysed using Dunnett's multiple comparisons test and showed significant difference ($P<0.01$) between stagnant and aerated nutrient solution. In Fig. 4 (b) the same analysis showed significant difference ($P<0.001$) between PEG and aerated nutrient solution. The Asterisk * shows significant difference determined by Dunnett's multiple comparisons test. The asterisk indicates the level of significance of the result. *: $\alpha = 0.05$, **: $\alpha=0.01$ and ***: $\alpha= 0.001$. Lines indicate median, +; means, whiskers; Min to Max, Box; 25^{th}-75^{th} percentile (n=4).

SPAD

Spad was measured from Day 1 to Day 16 in order to achieve data that would indicate if rice plants showed physiological. Aerated (control) showed an increase in chlorophyll until day 8 whereafter it remains steady to about 40 units. PEG followed a similar trend with a final value of 38 units after 16 days of treatments. In contrast the values for NERICA-1 plants grown in stagnant nutrient solution decrease after day 8 to a SPAD value about 20 units. The graph shows that NERICA-1 rice grown in stagnant nutrient solution is visibly more stressed than in Aerated (control) and PEG nutrient solutions. A two-way ANOVA showed significant difference between the date and treatment ($P<0.0001$). SPAD measurements are therefore affected by the treatments and time passed by as seen in (Fig. 5).

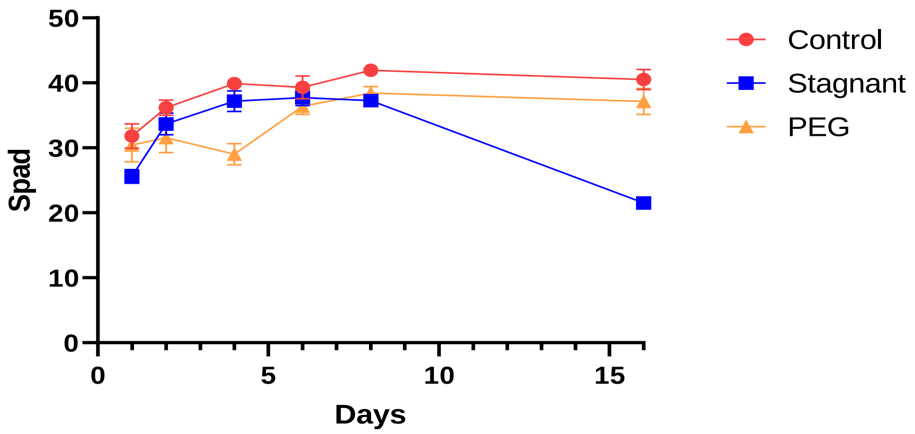


Figure 5. Spad Measurements over 16-day period on NERICA-1 leaves.

NERICA-1 plants grown in Aerated (Control), Stagnant, and PEG nutrient solution conditions were measured with a chlorophyll meter to sample SPAD measurements over a 16-day period. Using Tukey's multiple comparisons test a significant difference was found between several variables. On Day 4 a significant difference was found between SPAD of NERICA-1 grown in Control and PEG nutrient solutions ($P<0.05$). Day 4 also showed significant difference ($P<0.05$) between SPAD of NERICA-1 grown in PEG and stagnant nutrient solutions. Day 8 shows a significant difference ($P<0.05$) between SPAD of NERICA-1 grown in aerated (control) and stagnant nutrient solutions. For day 16 significant differences ($P<0.01$) were found in SPAD of NERICA-1 grown in aerated (control) and Stagnant nutrient solutions and then in stagnant and PEG nutrient solutions. Symbols indicate means +/- S.E.M (n=4).

Discussion

This project investigated the formation of an apoplastic barrier in the root exodermis (also termed ROL-barrier) in NERICA-1 rice plants grown under osmotic stress (10% PEG), in stagnant deoxygenated agar and in aerated nutrient solution. It was found that a ROL-barrier in the root of Nerica-1 rice restricted water loss when grown in stagnant, deoxygenated agar, indicating a strong ROL barrier formation under these conditions. However, RWL was not significantly lower in plants subject to PEG than in aerated controls, thereby not suggesting that drought induced a strong ROL-barrier formation in NERICA-1 rice. Nerica-1 Rice suffering from drought induced by PEG had a larger root biomass than aerated controls, suggesting increased root growth as possible plant acclimation to osmotic stress. Spad was found to be affected by time and treatment, rice grown in stagnant nutrient solution became visibly more stressed. These findings will be discussed in relation to previous studies in the following sections.

In past studies, roots with and without exodermis from different species with varying exodermal development were grown with frequent and non-frequent irrigation (to simulate drought) and tested for water loss (Taleisnik, 1999). These results showed that the exodermis restricted water loss (Taleisnik, 1999). In the present study stagnant nutrient solutions (Fig. 1c) also showed a barrier to ROL was only present when plants were grown in stagnant nutrient solution. In another study assessing cumulated water loss it was found that after an hour roots without a barrier had lost 80% tissue water and roots with a barrier has lost 18% (Peralta Ogorek et al., 2021). In this study it was found that roots grown in aerated (control) nutrient solution had lost 87% tissue water after an hour, and stagnant had lost 63% (Fig. 1a). In Peralta Ogorek et al. (2021) it was found that RWL rates were 14-fold higher in roots without a barrier than roots with a tight barrier. In the present study, (Fig. 1c) showed Control's (no barrier) rate of RWL only four times higher than Stagnant (barrier present). The large difference between stagnant and aerated (control) nutrient solution RWL rates as seen in (Fig. 1a) could be due to several factors. Short roots are less mature, and it is therefore possible that the barrier has not formed yet. It is also possible that that the age of the plant influenced the barrier and that if the plant had been one or two weeks older the barrier would have been more present in roots grown in aerated (control) nutrient solution. It was visible that roots grown in stagnant nutrient solution were thicker. A thicker root could be more favourable for the formation of a ROL-barrier or its ability to store water. Roots cut from the plant were of different lengths to start with. This variation could also affect formation of the ROL-barrier and as the roots were measured at varying stages of development. Another study found a decrease in RWL rates of 77% when growing O. *glaberrima* X O. *sativa* in stagnant conditions compared to aerated conditions (Kjær, 2020). ROL-barrier formation in stagnantly grown plants was supported by root cross section pictures (discussed in detail below). These results confirm that NERICA-1 is capable of forming a strong ROL-barrier.

It can be speculated whether PEG induces a response limiting RWL, as after an hour roots grown in PEG nutrient solution lost 69% tissue water compared to 63% lost tissue water in stagnant nutrient solution. While not statistically different, PEG's rate of RWL was two times higher than stagnant (Fig. 1c), compared to 4 times higher in roots grown under aerated conditions. When looking at (Fig. 1a,b) PEG follows a similar CWL and RWL curve as Stagnant. In future studies, it would be interesting to look more detailed at the presence of Casparian band and suberin in the hypodermal walls, as a study done by (Perumalla et al., 1990) found a correlation suggesting exodermis that have a Casparian band and have been suberized protect roots from RWL.

Looking at the pictures of the root cross section, there was evidence for and against ROL-barrier being present in PEG. In (Fig. 2) on Day 0 all root cross sections are visibly stained. On day 2 stagnant, is seen having a clear barrier already. Kjær (2020) also finds a clear cross-section in a O. *sativa* X O. *glaberrima* species grown in stagnant nutrient solution. For PEG on day 2 there is a sign of a barrier, as some of the periodic acid had diffused into the cross-section however it is not fully stained, leading to speculation if the root has indeed developed a barrier to ROL as hypothesized. For PEG Day 3, 4 and 8 show similar results. On day 3 it is only a small part of the root section that remains clear, whereas on day 4 the cross section is almost fully stained and on day 8 the cross-section is clear. The pictures displayed in (Fig. 2) show a variety of results for PEG, as for both day 3 and 4 cross sections were found that were fully stained and some that were not. The varied results seen in the cross-sections resembles that of the varying results seen in the RWL rates in (Fig. 1b). For different replicates on the same days, variations of periodic acid diffusion are seen, sometimes the barrier seems to be present and at other time not at all. RWL rates for roots grown in PEG are similar to that of stagnant but contain a lot more variation. This variation is an overall trend for data relating to roots grown in PEG nutrient solution, and it is therefore only possible to speculate on what responses are seen. More consistent results could have been found if there had been time to optimize the use of the vibrating microtome. The cross sections were not always easily attainable and often cut in ways that made it difficult to get a clear picture in the microscope. However, roots are naturally different which makes it hard to get consistently similar results. It is possible that while the roots were incubated in periodic acid, it diffused longitudinally into the cross-section where not properly sealed as seen in (Fig. 2) Stagnant day 8. In this case the root cortex is clear but the endodermis, pericycle and vascular tissue appears stained.

Rice species grown in waterlogged conditions tend to develop thicker adventitious roots (Colmer, 2003; Colmer et al., 2018). The data suits (Fig. 3) that shows roots grown in stagnant nutrient solution have a mean diameter of 1 mm compared to aerated (control) nutrient solution that has a mean diameter of 0.9 mm and PEG nutrient solution has a mean diameter of 0.8 mm. It was clear when collecting roots that Stagnant had many thicker roots than PEG and aerated (control) nutrient solutions.

In (Fig. 4) rice grown in PEG has by far the highest dry root biomass (ca. 5 g) compared to Stagnant (ca. 2 g) and Control (ca. 3 g). According to (Dos Santos et al., 2018) drought causes a decrease in accumulation of roots and shoots, although data from this study shows otherwise. It is possible that a larger root system is a survival strategy used specifically by Nerica-1 to resist drought. In a past study, it was found that root diameter was not correlated to the rate of RWL (Taleisnik, 1999).

Spad was measured to see if the different treatments would have any visible effects on the plant. Each variable showed significant results for Spad measurements, showing a clear effect of time and treatment on the plants. However, conclusions able to be drawn from Spad are limited. Measuring Spad using a chlorophyll meter resulted in some day-to-day variation, even when sampling identical leaves throughout the experiment. In future studies, leaves could be harvested, and leaf chlorophyll determined using a spectrophotometer to deduce variation. Throughout the 16-day period Stagnant showed the most signs of leaf discoloration (Fig. 5) show a big decrease in Spad between day 8 and 16. Control and PEG follow similar trends, PEG varying more in the beginning but at the end of the 16th day PEG lies only 3 spad units under the Control. It was expected that PEG would show more signs of stress, due to results observed in a pilot test, where 20% PEG after few days disabled plant growth and killed the rice plant. The use of 10% PEG was also validated by calculating the resulting water potential (see M&M) in the nutrient solution at 10% PEG; and relating that to the water potential threshold at which rice experiences drought. It would therefore be beneficial when conducting further research about Nerica-1 to stress it at a higher percentage of PEG. It would also have been preferred to measure water potential in the solution (e.g., using a potentiometer or psychrometer), especially following some days of treatment, rather than relying on calculations.

Conclusion

The hypothesis for an ROL-barrier induced in NERICA-1 when simulating waterlogged conditions in stagnant, deoxygenated nutrient solution is accepted. Meanwhile, the hypothesis that an RWL-barrier is formed in NERICA-1 when simulating drought induced by PEG was not supported, based on RWL rates and the overall interpretation of root cross sections. However, it would be worthwhile to conduct more studies, possibly testing NERICA-1 with higher percentages of PEG, as the root biomass increased and shoot biomass was not affected at 10% PEG. Nerica-1 remains worthwhile to study, since it combines O. *sativa* and O. *glaberrima* traits into a good solution for African farmers who due to the varying climate are struggling to produce a big enough yield.

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Supplementary Materials

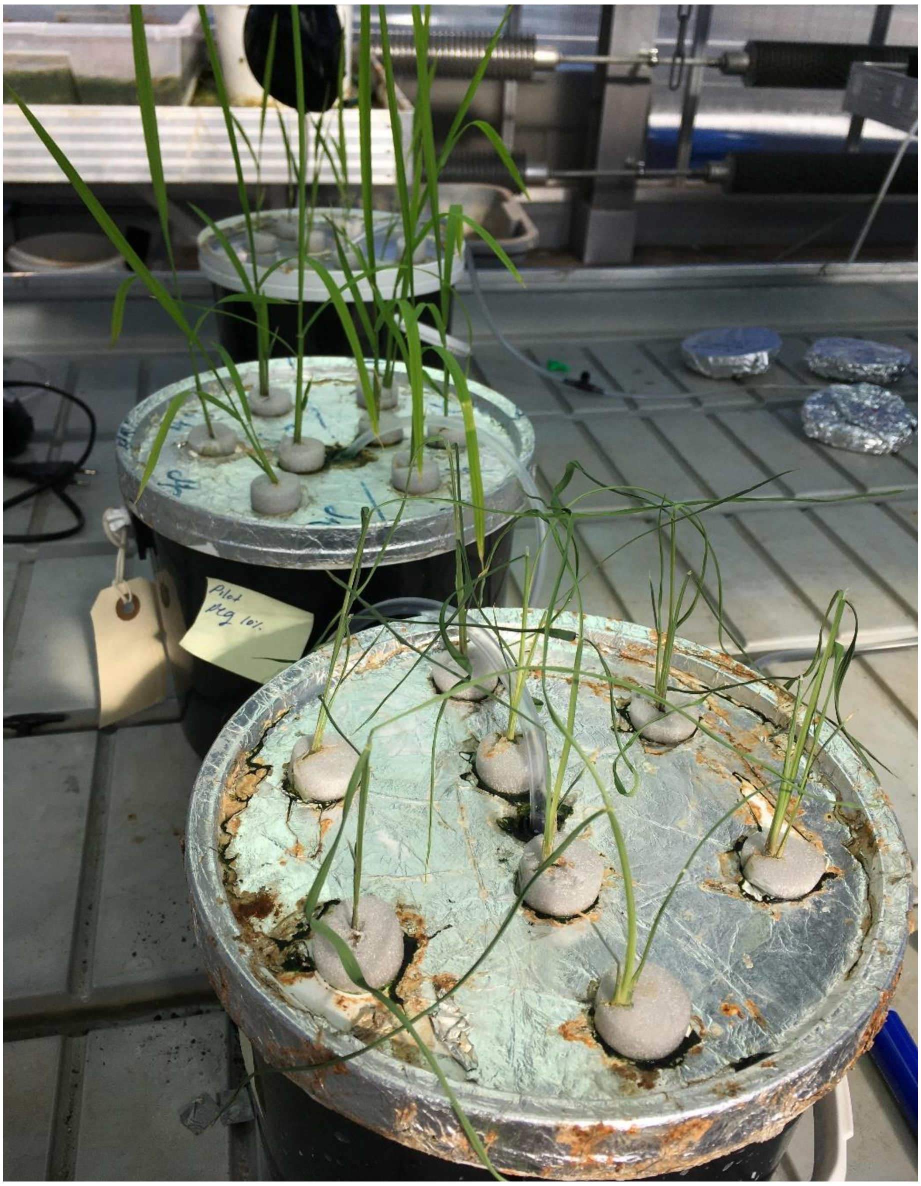

Figure 1. NERICA-1 rice in 20% PEG after 1-2 days (pot nearest)