

Physiological analysis of anaerobic response
mechanisms in Poaceae crops
(イネ科作物の低酸素応答の生理学的解析)

田丸 翔太郎
(2024)

概要

洪水は、作物生産において干ばつに次ぐ非生物学的ストレスである。洪水は冠水、部分冠水、湛水に分類されるが、畑作物はそもそも湛水環境にも適応ができず、生育および収量に重大な負の影響を受ける。土壌と根の低酸素状態を起点とするストレスへに対応するためには、湛水下での作物の通気能を制御するメカニズムを解明することが重要であるが、酸素の実計測を絡めた理解は、作物学の分野ではあまり進んでいない。そこで、本研究では、低酸素に耐性の植物種と感受性の植物種間に存在する通気能の差を、酸素計測により解明することを目的とした。まず、低酸素耐性種であるイネ (*Oryza sativa*) において、酸素要求度の高い根の部位である中心柱を高い割合で持つ品種 Sensho を含む 4 品種の間で根の酸素消費・供給特性の品種間差異を解析した。その結果、仮説としては根の形態的特性と相関関係にあると想定されていた根における酸素消費速度および酸素供給速度は、葉面積/根乾物重比との間にそれぞれ正の相関が認められた。イネが根の好気呼吸に必要とする酸素供給の点からは、根の酸素供給速度/酸素消費速度比は、供試品種内で 49%~109%であることが認められた。本研究はこれまでに酸素の輸送の面から議論されていた通気制御について、呼吸の要素を加えて議論をしている点が新規的であり、根の呼吸に必要な酸素量を供給する仕組みについて掘り下げるうえで地上部特性の影響を加味する重要性を強調した。

一方で、過去に報告されているトウモロコシおよびエンバクの根の酸素消費速度および酸素供給速度から根の酸素供給速度/酸素消費速度比を算出すると、それぞれ 8%および 0%となり、イネと低酸素に感受性を示す種の通気能の間には大きな差があることが示唆された。そこで、イネと低酸素感受性種の間をカバーする通気能を有する遺伝資源を探索するために、根への低酸素ストレスが葉におよぼす影響と個体の乾物重、根の特性の關係に着目し、4 種の雑穀類のキビ (*Panicum miliaceum*)、シコクビエ (*Eleusine coracana*)、ヒエ (*Echinochloa utilis*)、およびハトムギ (*Coix lacryma-jobi* var. *ma-yuen*) について低酸素応

答を比較・検討した。その結果、キビおよびシコクビエでは、ストレス処理期間が進むにつれて葉のクロロフィル蛍光 F_v/F_m および SPAD 値が低下したが、ヒエおよびハトムギでは同様の傾向は認められなかったことから、これらの種は低酸素ストレスの影響を回避していることが示唆された。その回避機作において、地上部乾物重の低酸素区/通気区と総根長の低酸素区/通気区には強い正の相関が認められ、根の伸長を低酸素下で維持することが乾物生産の維持に重要であることが示唆された。ハトムギとヒエの根は、土壌低酸素条件下において、恒常的に高い根の通気組織/切片断面積比、皮層/中心柱断面積を示し、これらの特性が低酸素環境への順化を早めることが示唆された。さらに、ハトムギとヒエでは主根長/側根長比の増加が認められたため、これらの形質の特徴が低酸素下での効率的な酸素分配を介して総根長の伸長に寄与していると推察された。

ハトムギは F_v/F_m および SPAD 値を低酸素区で維持していたことから、葉への酸化ストレスを回避していたと推察されるが、地上部乾物重は低酸素条件下で有意に減少していた。このことは、ハトムギの通気能はイネやヒエと低酸素感受性種の間をカバーするものである可能性を示唆した。このことから、ハトムギと低酸素感受性種の間での差を酸素計測で定量化することは、畑作物の耐湿性向上に必要な通気能についての具体的な提案につながる考えた。そこで、低酸素感受性種であるソルガムとハトムギを供試して、湛水下での植物体内通気経路の差異を、微小酸素電極を用いた葉から根端までの酸素プロファイルの比較により明らかにした。その結果、根と茎の接合部で種間の酸素分圧に異なる傾向が認められ、特に根の基部でハトムギとソルガムの間には有意差が認められた。これらの結果から、ハトムギとソルガムの湛水下での酸素拡散経路の間には根の基部付近を起点として差異が生じると結論づけた。ハトムギの根の基部では、根の皮層から表皮への酸素拡散がソルガムと比較してより制限されていることが明らかになり、このことはハトムギの根の基部での ROL バリアの存在を示唆した。また、ハトムギの根の皮層/中心柱比がソルガムと比較して高いことは、皮層から中心柱への効率的な酸素拡散に寄与すると考えられた。以上より、本研究は

低酸素感受性種と低酸素耐性種の間にある湛水下での通気能とその制御機作の差異について有用な知見を提供し、畑イネ科作物の耐湿性を向上するための作物デザインの発展に寄与するものである。

Summary

Flooding is the second largest abiotic stress in crop production after drought. Flooding is categorized into submergence, partial submergence, or waterlogging, but field crops are not adapted to even in waterlogged environments, and growth and yield are severely negatively affected. To cope with stresses originating from hypoxia in soil and roots, it is important to understand the mechanisms that control the plant internal aeration capacity of crops under waterlogging, but the crop science has not made much progress in understanding the mechanisms involving actual measurements of oxygen. Therefore, the objective of this study was to elucidate the oxygen-based differences in aeration capacity between hypoxia-tolerant and hypoxia-sensitive species. First, interspecific differences in root oxygen consumption and supply characteristics were analyzed among four hypoxia-tolerant rice (*Oryza sativa*) cultivars, including the cultivar Sensho, which has a high proportion of the root stele with high oxygen demand. The results showed that oxygen consumption and oxygen transportation rates in roots, which were hypothesized to be correlated with root morphological characteristics, were positively correlated with leaf area/root dry weight ratio, respectively. In terms of oxygen transportation required by rice for root aerobic respiration, the oxygen transportation rate/oxygen consumption rate ratio in roots was found to be 49%~109% within the tested cultivars. This study is novel in that it discusses aeration control, which has previously been discussed in terms of oxygen transport, by adding a respiration component, and emphasizes the importance of taking into account the influence of above-ground characteristics to delve into the mechanism of supplying the amount of oxygen required for root respiration.

On the other hand, root oxygen transportation rate/oxygen consumption rate ratios calculated from previously reported value of maize and oat, respectively, were 8% and 0%, suggesting a significant difference between the aeration capacities of rice and hypoxia-sensitive species. Therefore, in order to

search for genetic resources with aeration capacities that cover the gap between rice and hypoxia-sensitive species, we focused on the relationship between the effects of hypoxia stress on roots on leaves, individual dry matter weight and root characteristics of four millet species, common millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*), Japanese barnyard millet (*Echinochloa utilis*) and job's tears (*Coix lacryma-jobi* var. *ma-yuen*) were compared and investigated for hypoxia response. The results showed that leaf chlorophyll fluorescence F_v/F_m and SPAD values decreased with increasing duration of stress treatment in common millet and finger millet, but no similar trend was observed in Japanese barnyard millet and job's tears, suggesting that these species avoid the effects of hypoxic stress. In the avoidance mechanism, a strong positive correlation was observed between hypoxia/aeration of shoot dry weight and hypoxia/aeration of total root length, suggesting that maintaining root elongation under hypoxia is important for maintaining dry matter production. Job's tears and Japanese barnyard millet roots exhibited permanently high aerenchyma/ cross-sectional area ratios and cortex/stele cross-sectional area under hypoxia, suggesting that these characteristics accelerate acclimatization to hypoxic conditions. Furthermore, an increase in the main root length/lateral root length ratio was observed in job's tears and Japanese barnyard millet, suggesting that these traits contribute to total root length elongation via efficient oxygen partitioning under hypoxia.

Job's tears maintained F_v/F_m and SPAD values under hypoxia, suggesting that it avoided oxidative stress to the leaves, but shoot dry weight was significantly reduced under hypoxic conditions. This suggested that the aeration capacity of job's tears may cover between rice or Japanese barnyard millet and hypoxia-sensitive species. Therefore, we considered that quantifying the difference in aeration between job's tears and hypoxia-sensitive species using oxygen measurements would lead to specific suggestions on the aeration capacity required to improve the waterlogging tolerance of field crops. Therefore, the hypoxia-sensitive species sorghum and job's tears were tested to determine the

differences in oxygen diffusion pathways in plants under waterlogging by comparing oxygen profiles from leaves to root tips using micro oxygen electrodes. The results showed a different trend in oxygen partial pressure between species at the root-stem junction, with significant differences between job's tears and sorghum, particularly at the root base. From these results, it was concluded that differences occur between the oxygen diffusion pathways of job's tears and sorghum under waterlogging, starting near the root base. Oxygen diffusion from the root cortex to the epidermis was found to be more restricted at the root base of job's tears compared to sorghum, suggesting the presence of a ROL barrier at the root base of job's tears. The higher root cortex/stele area ratio of job's tears compared to sorghum may contribute to efficient oxygen diffusion from the cortex to the stele. In conclusion, this study provides useful insights into the differences in aeration capacity and control mechanisms under waterlogging between hypoxia-sensitive and hypoxia-tolerant species and contributes to the development of crop design to improve waterlogging tolerance in field Poaceae crops.

Part of this dissertation has been published in:

1. S. Tamaru, S. Yabuta, K. Goto, P. Khanthavong, J-I. Sakagami. Oxygen consumption and transportation in rice roots are highly dependent on leaf area and root dry weight ratio. *Rhizosphere* 25: 100644. **2023**.
2. S. Tamaru, K. Goto, J-I. Sakagami. Spatial O₂ Profile in *Coix lacryma-jobi* and *Sorghum bicolor* along the Gas Diffusion Pathway under Waterlogging Conditions. *Plants* 13(1):3 **2024**.

Acknowledgments

First of all, the author pays to Dr. Jun-Ichi Sakagami, my utmost gratitude for the seven years that his patiently and warmly nurtured my immaturity, not only in terms of research but also in terms of humanity, in a way that best suited me. The author also pays to the co-supervisors, Dr. Zheng Shao-Hui and Dr. Makoto Ikenaga, my gratitude for their patience, always showed me best advice warmly and constructive way to carry out this research. Thanks to Dr. Shin Yabuta, Dr. Yoshihiro Nakao and Dr. Phanthasin Khanthavong, as seniors in the laboratory, they have shown me their attitude toward research and their way of life, and they have always helped me when I was in trouble. Thanks to Keita Goto, as colleague, he always went ahead of the field and gave me great motivation, and not only that, the role he played as co-researcher was also very significant. Also, thanks to Emmanuel Odama, as colleague, showed his attitude toward research as a veteran and was also my English mentor. Thanks to Naoya Katsuhama, as younger, always inspired me, and that helped me advance.

In addition, author thank everyone at the Tropical Crop Science laboratory of Kagoshima University and neighbors for all their help. And thanks to the research in this field and the societies that have helped me, these have always shown and inspired high quality research.

Finally, special credit to my family and partner for their warmth encourage and support in my doctoral studies.

Kagoshima, February 2024

Shotaro Tamaru

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CHAPTER 1

General Introduction

1. Background

Poaceae, one of the largest families of flowering plants, is crucial for human economic activity and the composition of natural plant communities (Tzvelev, 1989). Major cereals form the basis of caloric intake as staple foods, while minor cereals provide beneficial health effects to the diet (Wang et al., 2023). Among three major cereals—rice, wheat, and maize—waterlogging stress has emerged as a primary limiting factor in wheat (Collaku & Harrison, 2002) and maize (Mano & Nakazono, 2021). Even in minor cereals, many crop species have been reported to be sensitive to waterlogging (Kono et al., 1987), and this poses a threat not only to direct human food, but also to indirect human food production activities, such as forage. Therefore, there is a growing need to better understand the adaptation mechanisms of adaptive poaceae species.

Flooding is the second most damaging abiotic stress to agriculture (Kaur et al., 2020). Flood is categorized into completely submerge, partial submerge, waterlogging (Sasidharan et al., 2017). Submergence is a serious environmental stress that even threatens the production of rice which adapt to wetland well (Bailey-Serres et al., 2019), to begin with, almost field crops cannot endure the stress from partial submerge or even waterlogging, which limits their growth and yield. Waterlogging can occur anywhere due to heavy rainfall, so estimated 10–12 % of the agricultural area is affected by waterlogging or severe soil drainage constraints globally (Kaur et al., 2020). Furthermore, flood modelling based on the RCP4.5 scenario estimates a 26.4% increase in flood risk by 2050 in the USA (Wing et al., 2022). This means that the damage caused by waterlogging associated with flooding is potentially going to affect crops more than ever as a result of climate change.

When breaking down the stress of waterlogging conditions, the first thing that oxygen movement is impeded under waterlogged soil conditions, causing hypoxic conditions for plant roots

(Colmer et al., 2010). Such soil hypoxia causes root hypoxia. Oxygen is crucial for various root functions, including nutrient absorption, root elongation, and root maintenance (Veen, 1981), this means that root hypoxia limits these root function. If soil and root hypoxia continue for a long time, the balance of nutrient supply in the plant body can be disrupted, and ROS can begin to affect not only the roots but also the leaves (Luan et al., 2018). This can be exacerbated by the accumulation of reduced minerals (Laanbroek, 1990) and soil phytotoxins (Casolo et al., 2023), depending on the type of soil. Wetland plant species can continue to grow in such situations because they can continuously supply oxygen to their roots (Armstrong, 1980). The oxygen sent to the roots not only maintains root respiration but also protects the roots from reducing conditions by oxidizing the vicinity of the roots (Koop-Jakobsen et al., 2018; Larsen et al., 2015; Tian et al., 2015). Comparisons of plants in wetlands have indicated that their ability to maintain respiration depends not on controlling the amount of respiration, but on being able to supply the oxygen needed for respiration (Striker, 2023). Thus, the stress caused by soil becoming hypoxic diversifies as spatiotemporal elements expand, but it is clear that the ability to supply oxygen to the roots is important as a starting point.

Under hypoxic soil conditions such as waterlogged soil, oxygen diffuses more efficiently from the atmosphere to the leaf, stem, root, and soil, and plants adapted to wetlands can develop increased intercellular space, creating gas pathways that provide the necessary oxygen to sustain root activity (Armstrong, 1980). Although wheat and maize can induce lysigenous aerenchyma in response to hypoxia, they cannot develop constitutive aerenchyma or an induced barrier to radial oxygen loss (ROL), a trait found in rice. Consequently, intraspecific variation in waterlogging tolerance among major crops, such as wheat, is limited. Therefore, understanding adaptation strategies for waterlogging within a broad gene pool has become increasingly important (Oyanagi, 2011). Researchers have made substantial progress in identifying genes that could enhance waterlogging tolerance using *Hordeum marinum* (Malik et al., 2011), which can be crossed with wheat, and *Zea nicaraguensis* (Gong et al.,

2019), which can be crossed with maize. However, the transgenic introduction of the ROL barrier alone did not significantly enhance waterlogging tolerance in maize varieties (Pedersen, Nakayama, et al., 2021). Additionally, varieties with introduced constitutive aerenchyma formation ability exhibited a 29% increase in shoot dry matter production under waterlogged conditions compared with the parental variety; however, this increase was still 29% lower than the shoot dry matter production of *Z. nicaraguensis*, with the difference increasing in waterlogged soils (Gong et al., 2019). Presently, no practical waterlogging-tolerant field crop varieties have been successfully developed. This highlights the need for incorporating a range of waterlogging-tolerant crop strategies into the development of waterlogging-sensitive crops to effectively mitigate flood damage.

2. Problem Statement

Because waterlogging can occur on any agricultural land and is difficult to deal with after it has occurred, developments in agricultural practices and crops, respectively, are proposed. In addition, waterlogging will continue in situations after finishing submergence, so waterlogging susceptible crops will not be able to adapt to higher flooding e.g. submergence. Waterlogging stress is a complex phenomenon, but because it originates from hypoxia in the rhizosphere and root tissue, the focus has been on plant internal aeration capacity. Because of the history in crop science, which has tended to focus on quantitative comparisons of aerenchyma for selection of waterlogging tolerant varieties, there is a significant lack of knowledge of plant internal aeration involving oxygen measurements compared to wetland plants treated in the field of ecology. Considering that no practical waterlogging tolerant varieties have been developed for field crops in the long history of research, we need to go back once again to the mechanisms that control the plant internal aeration. In addition, this research needs to be done with millets, which have not been focused on to cover the tolerance gap between rice, a current model crop, and susceptible crops. Therefore, this study will provide fundamental knowledge on the extent to which aeration capacity needs to be improved to enhance waterlogging tolerance in field

poaceae crops.

3. Novelties

This study offers the following novelties:

- i Changes in hypoxic stress over time and hypoxic response of roots in millets.
- ii Quantitative differences in oxygen basis between poaceae crops tolerant to hypoxic stress and those sensitive to hypoxic stress

4. General Objectives

This study aims:

- i To identify intra- and interspecific variation in hypoxia response of poaceae crops.
- ii To quantify the differences in oxygen permeability between hypoxia-tolerant and hypoxia-sensitive species in the poaceae crops.

5. Research Scope

This research was focus on the root traits related to oxygen transport and respiration. Less injured root are needed for these analysis. Therefore, these three researches were carried out under hydroponic experiment as below:

1. Oxygen consumption and transportation in rice roots: comparison between upland rice and lowland rice
2. Inter-and intra-specific variation in hypoxic response of four millets: *Panicum miliaceum*, *Eleusine coracana*, *Echinochloa utilis* and *Coix lacryma-jobi* var. *ma-yuen*
3. Comparison of plant internal oxygen pathway between *Coix lacryma-jobi* and *Sorghum bicolor*

CHAPTER 2

Oxygen consumption and transportation in rice roots: comparison between upland rice and lowland rice

II-1 Introduction

A larger proportion of root respiration is devoted to ion uptake than to growth or maintenance (T. Nakamura & Nakamura, 2016; Veen, 1981). The root stele contains xylem vessels; it is important for nutrient and water uptake (Petricka et al., 2012), so root stele respiration is higher than that of the root cortex (Aguilar et al., 2003; Colmer et al., 2020). When external O₂ and internal aeration are insufficient, hypoxia and anoxia in stellar cells restrict the uptake of essential ions (Colmer & Greenway, 2011; Kotula et al., 2015; Shabala et al., 2014). Therefore, the narrower root stele area might reduce the root oxygen consumption and increase their tolerance to hypoxia (Armstrong & Beckett, 1987; Cardoso et al., 2013; McDonald et al., 2002; Sundgren et al., 2018). It is known that species adapted to wetlands have a low root oxygen consumption; indeed, it was lower in rice than in maize, and even lower in plant species that live in salt marsh areas where oxygen is very limited (Maricle & Lee, 2007). Therefore, a low root oxygen consumption is also considered important for plants to adapt to oxygen-limited environments.

In contrast to the stele, the cortex can supply oxygen through the aerenchyma and intercellular spaces (Yoshida & Eguchi, 1994). In addition, aerenchyma formation is considered to decrease the oxygen consumption as the root respiration in maize lines with high aerenchyma levels was lower than in those with lower aerenchyma levels (Zhu et al., 2010). In rice, maize, and wheat, root cortex expansion was observed in stagnant solutions, and the cortex to stele ratio (CSR) increased accordingly (Yamauchi, Abe, et al., 2019). CSR provides a good reflection of plant adaptations to soil water conditions, and wetland species are reported to have a high CSR (Yamauchi, Pedersen, et al., 2021).

Thus, the root anatomical morphology may play an important role in oxygen consumption and transportation to the roots under hypoxia, but there is still no quantitative knowledge linking them to the root oxygen consumption and transportation.

Rice is a crop adapted to paddy fields, but it is also grown in aerobic environments like upland fields. Rice varieties with enhanced tolerance to dryland conditions and varieties for aerobic rice system suitable for areas with limited water resources are being developed (Bouman, 2001; Lafitte et al., 2002) and both varieties have been found to grow well in waterlogged conditions (Colmer, 2003a; Suralta & Yamauchi, 2008). In aerobic rice varieties, the xylem area is larger, and it is considered to contribute to water uptake (Phule et al., 2019). In other words, rice varieties with diverse root internal structures may exist due to various development, and the mechanisms by which they adapt to waterlogging may be diverse. This study aimed to investigate which traits affect the root oxygen consumption and transportation ability of rice varieties with different root anatomical traits.

II-2 Materials and Methods

2.1. Experiment A: Plant materials and growth conditions

Three lowland rice varieties (Inpari29, Inpari30, and Koshihikari) and three upland rice varieties (BlackGora, LAC23, and Sensho) were used in this experiment. Upland and lowland rice varieties were used to compare the genotypic variations in responses to waterlogging. According to Colmer (2003), further insight into the diversity of root aeration traits in upland rice would be gained by studies on genotypes taken from well-drained environments, especially in well-drained soils. Therefore, BlackGora, LAC23, and Sensho were used as upland rice varieties. BlackGora is a moderately drought-tolerant variety (Comas et al., 2013). LAC23 also has drought-tolerant characteristics (Lamo et al., 2007). Sensho is a traditional upland rice of japonica that has a high water use efficiency (Matsuo et al., 2010). Koshihikari is a typical Japanese rice variety, while Inpari29 and Inpari30 are indica

varieties that have been introduced with the *Sub1A* gene, which is useful for flash-flood tolerance. The experiment was conducted at 28°C, and the light–dark cycle (photosynthetic active radiation of light: 410 $\mu\text{mol m}^{-2}\text{s}^{-1}$) was set to 12 h using an LED light (SMD-50W, W-LITE). Seeds were disinfected with Benomyl (Benlate wettable powder, Sumitomo Chemical Co.). After being disinfected for 12 h, the seeds were placed into Petri dishes, and tap water was used to provide moisture to filter papers on Petri dishes. When the length of the seminal root reached approximately 1 mm after soaking, the seedlings were transplanted to a container by randomized block design. Plastic nets were floated in six plastic containers with a volume of 0.9 L, and 10 seedlings per variety were transplanted onto each. A hydroponic solution (Kyowa Corporation) was diluted 500 times with tap water and adjusted to pH 5.6. This solution contained 80 mg L^{-1} N, 76 mg L^{-1} P, 188 mg L^{-1} K, and minor elements (Tada et al., 2014).

Immediately after transplanting, two air pump (e-AIR 1000SB GEX.Co.) were used to aerate three containers, and the container with increased dissolved oxygen concentration was designated as aerated. The three containers (length 140 mm, width 92 mm, height 76 mm) grown without bubbling were designated as stagnant. The hydroponic solution was changed every three days after transplanting. Nine days after transplanting, three plants with moderate height were taken from each container. The average of these three plants were taken as the representative values for each container(n=3).

2.2 Experiment B: Plant materials and growth conditions

Two lowland rice varieties (IR42 and Koshihikari) and two upland rice varieties (BlackGora and Sensho) were used in this experiment. IR42 was added instead of the indica variety of Inpari29. This variety was confirmed to be high yield on irrigated lowlands (Katsura et al., 2016). Seedlings were raised in a growth chamber (Nippon Medical and Chemical Instruments Co., Ltd) at 25°C, and the light–dark cycle was set to 12 h (PAR:200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ condition). The seeds were disinfected with

Benomyl (Benlate wettable powder, Sumitomo Chemical Co.). After being disinfected for 12 h, the seeds were sown on plastic plates that were floated in a plastic container with a volume of 4 L. A high concentration of hydroponic solution (Kyowa Corporation) was diluted 500 times with tap water and adjust to pH 5.1–5.4.

The hydroponic solution was changed every two days until sampling. Fifteen plants (Sensho and Koshihikari: 16 days after sowing, BlackGora and IR42: 18 days after sowing) of each variety with four leaves and medium height were selected and used for testing. Each plant was transplanted into a 100-mL flask and acclimated in a growth chamber for one day. The PAR, day/night period, and temperature inside the growth chamber were the same as during the seedling growth period, and the relative humidity was set at 70%. After acclimatization, five plants of each variety were sampled for dry weight measurement, as described below, but these data of this timing were not used in this study. Based on the plant height of the remaining 10 plants, they were divided into two treatments(n=5). Individuals grown in a 0.1% nutrient agar solution with an increased dissolved oxygen concentration after bubbling for 2 h were designated as preaerated. Meanwhile, individuals grown in a 0.1% agar nutrient solution whose initial dissolved oxygen was adjusted to under 0.9 mg L⁻¹ by nitrogen gas flushing were designated as deoxygenated. In Experiment B, the aim of these operation was to reduce the source of inter-treatment differences as close as possible to differences in dissolved oxygen concentration alone. In addition, agar solutions are more suitable than water in reproducing the gas composition of waterlogged soil because there is less convection (Wiengweera et al., 1997). After seven days of growth on each flask, the 5 plants per each treatment were sampled, and the measured parameters were described later (Sensho and Koshihikari: 23 days after sowing, BlackGora and IR42: 25 days after sowing). After finishing each measurement, the shoots and roots were oven-dried at 80°C for 72 h and weighed using an electronic balance.

2.4. Anatomical analysis of root cross sections

Experiment A, seminal roots were taken from each sampled plant. In Experiment B, three nodal roots were taken from each plant. A newer short thick root (length range, 3.5–8.3 cm), long thick root (length range, 10.1–16.0 cm), and long thin root (length range, 11.5–25.7 cm) were selected from each plant in experiment B. The average value of these different roots was considered to be a representative of the roots of that plant. Root sections were made at half of these root lengths \pm 0.5 cm in both experiments. The areas of each root tissue were measured using an ImageJ software (US National Institutes of Health), and the ratio of each root cortex area and stele area was calculated using a cross-sectional area. The average area of three nodal root types in Experiment B was determined.

2.5. Experiment B: Measurement of oxygen consumption

Root oxygen consumption was measured using the method described by Suralta and Yamauchi (2008). Before root oxygen consumption measurements, the stomatal conductance of the youngest expanded leaf was measured using a porometer (AP4, Delta-T Devices, UK) from 9:00 am to 10:30 am in a growth chamber. Therefore, plants were moved to laboratory (air temperature: 25°C); their roots were lightly washed with distilled water. Then, 100 mL of 0.1% agar solution was prepared with a measuring cylinder, poured into a 100-mL flask, and the plant roots were inserted into the solution. At this time, the roots were completely immersed in the solution, and the shoot part was fixed with a sponge to come out of the flask. This was shaken in laboratory condition (PAR:10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ condition, 25°C) using a rotary shaker (R-20 TAITEC) at a speed of 95–100 r min^{-1} . After 3 h, the dissolved oxygen was measured using a dissolved oxygen meter (D-210D, HORIBA, Ltd.) while being stirred by a magnetic stirrer. Changes in dissolved oxygen were determined in a flask without plants to adjust the O_2 measurements. The shoot part was removed at the time of solution exchange, and the dissolved oxygen was measured 3 h later using the same process. Great care was taken to

ensure that the submerged cut surface was not exposed to air during stirring. Leaf area was measured using an automatic area meter (AAM-9, Hayashi Denko Co., Ltd., Japan). The root oxygen transportation rate was calculated by subtracting the root oxygen consumption rate without the shoot from the root oxygen consumption rate with the shoot. In this study, the following value was calculated to determine how well oxygen transportation compensates for root oxygen consumption.

Compensation of oxygen consumption by oxygen transport

$$= (\text{Oxygen transport rate} / \text{Root oxygen consumption rate}) \times 100 \quad (1)$$

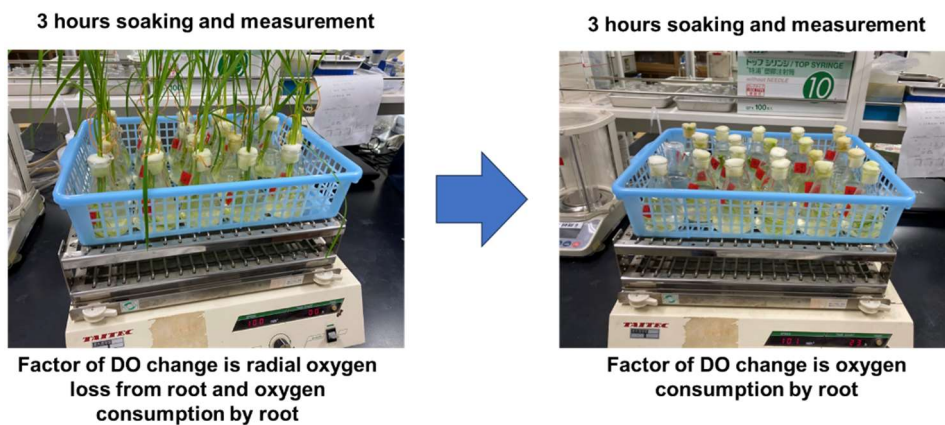


Fig.2-1. The photos during oxygen consumption measurement in Experiment B

2.6. Experiment B: Porosity measurements

Porosity measurements was based on Jensen et al., (1969) and removing air was conducting by vacuum pump. The whole root was measured to assess the porosity. First, the roots were cut to 2–3 cm, wrapped in a paper (Kimwipe, NIPPON PAPER CRECIA Co., Ltd.), put in a 15-mL tube, and then centrifuged at 2000 rpm for 2 min using a low-speed centrifuge (LC-100, TOMY SEIKO Co., Ltd). The fresh weight was measured by removing the water (w1). A 25-mL pycnometer was filled with distilled water up to the marked line and weighed (w2). Little water was drained from the pycnometer, the roots were inserted into it, distilled water was added up to the marked line, and the weight was measured (w3). Then, the roots were degassed and depressurized with distilled water in a vacuum desiccator until the pressure reached –80 kPa, and the roots were left to stand for 10 min. This process

was repeated three times for a total of 30 min. The air in the root voids was replaced with distilled water. If bubbles were still released from the roots after 30 min of degassing, decompression was repeated. After degassing, water was added up to the marked line, and the weight was measured (w4).

Porosity was calculated using the following formula.

$$\text{Porosity(\%)} = \{(w4 - w3)/(w1 + w2 - w)\} \times 100 \quad (2)$$

2.6. Statistical analysis

Correlation analysis and multiple regression analysis were also performed using Excel. The mean values were compared using SPSS statistics Version 26 (IBM software). One-way analysis of variance was performed between the varieties, and comparisons between the varieties were conducted using Tukey HSD. In addition, T-test was used to compare the data between the treatments for each variety. $p < 0.05$ was considered statistically significant to all analysis.

II-3 Results

3.1. Cortex to stele ratio (CSR) in the Experiment A

The CSR of seminal roots are shown in Fig. 2-2. Treatment effects were observed in the CSR of BlackGora, Inpari29 LAC23, and Sensho, and there was a significant difference between the aerated and stagnant conditions (Fig. 2-2). Among the six varieties, the average CSR ranged from 2.98 (Sensho) to 5.09 (Inpari29) in aerated conditions and 3.86 (Sensho) to 7.89 (Inpari29) in stagnant conditions. Statistical analysis showed that BlackGora, Inpari29, Inpari30, and Koshihikari had a significantly higher CSR than Sensho in aerated conditions, and BlackGora and Inpari29 had a significantly higher CSR than Sensho in stagnant conditions.

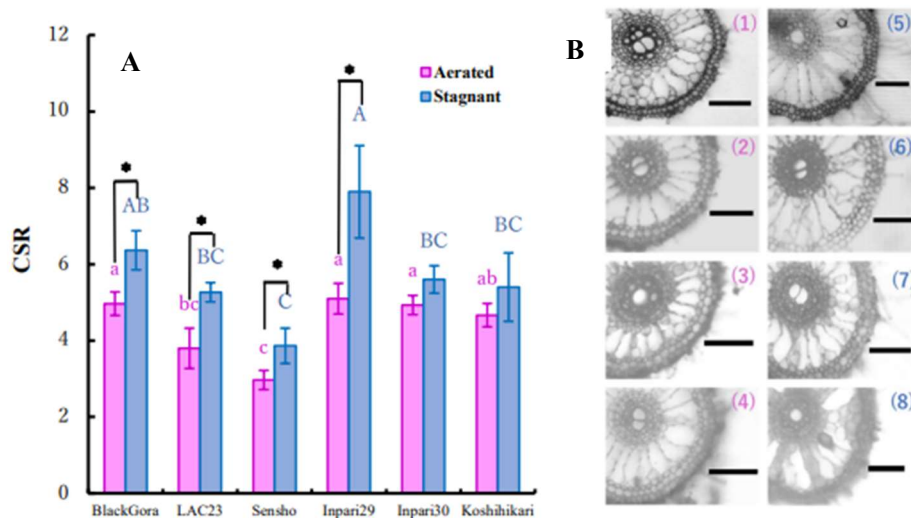


Fig. 2-2. Cortex to stele ratio (CSR) in seminal roots (A) and root section picture of varieties with significant inter-treatment difference (B) on Experiment A (n = 3). Different letters indicate a significant difference at $P < 0.05$ among varieties in each treatment according to Tukey HSD. Significant differences between aerated and stagnant conditions at $P < 0.05$ are denoted by *. Error bars indicate standard deviations (S.D.). Letters on pictures mean BlackGora (1: Aerated, 5: Stagnant), LAC23(2: Aerated, 6: Stagnant), Sensho (3: Aerated, 7: Stagnant), Inpari29(4: Aerated, 8: Stagnant). Bars in pictures measure 100 μm .

3.2. Plant growth in Experiment B

Significant effects from different initial oxygen concentrations were not observed in the growth parameters, except for the shoot dry weight of Koshihikari (Table 2-1). The shoot dry weight was significantly higher in both treatments with BlackGora and lower in both treatments with IR42 and Koshihikari. The root dry weight was also the highest in BlackGora and lowest in Koshihikari. Leaf area and shoot dry weight showed the same tendency. The leaf area/root dry weight (LA/RDW) was significantly lower in BlackGora and IR42 than in Sensho and Koshihikari, and it was higher in Sensho than in Koshihikari.

Table 2-1. Shoot and root dry weight, leaf area of each variety under pre-aerated or deoxygenated 0.1% agar solution on experiment B.(n=5)

Variety	Shoot dry weight (mg)			Root dry weight (mg)			Leaf area(cm ²)			Leaf area/Root dry weight(cm ² /g)		
	P	D	t-test	P	D	t-test	P	D	t-test	P	D	t-test
BlackGora	387.5 a	400.8 A	n.s	127.4 a	131.7 A	n.s	63.4 a	63.3 A	n.s	496.7 c	482.8 C	n.s
Sensho	239.0 b	269.2 B	n.s	44.3 c	51.7 BC	n.s	42.7 b	47.5 B	n.s	971.7 a	929.5 A	n.s
IR42	174.9 c	175.7 C	n.s	57.9 b	61.6 B	n.s	26.2 c	29.1 C	n.s	452.2 c	470.5 C	n.s
Koshihikari	171.8 c	190.8 C	*	35.1 c	41.7 C	n.s	26.4 c	27.2 C	n.s	767.1 b	661.6 B	n.s

Significant differences between P(Pre-aerated) and D(Deoxygenated) at $P < 0.05$ are denoted by *. Different letters indicate a significant difference at $P < 0.05$ according to Tukey HSD.

3.3. Root anatomical traits in Experiment B

In preaerated (P) conditions, there were no significant differences among the four varieties. In deoxygenated (D) conditions, the root porosity of BlackGora(14.8%), IR42(14.9%) and Koshihikari(14.9%) was significantly higher than that of Sensho(5.6%), respectively. Image analysis results are shown in Fig. 2-4. Significant treatment effects were observed in the cortex and CSR of Koshihikari. Root section areas were significantly larger in BlackGora (P: 4.6 mm², D: 4.6 mm²) than in other varieties (smallest in Koshihikari; P: 1.8 mm², D: 2.1 mm²). The stele was significantly larger in BlackGora (P: 0.32 mm², D: 0.28 mm²) and Sensho (P: 0.22 mm², D: 0.23 mm²) than in Koshihikari (P: 0.11 mm², D: 0.11 mm²). The cortex was significantly larger in BlackGora (P: 3.5 mm², D: 3.5 mm²) than in other varieties (lowest in Koshihikari; P: 1.3 mm², D: 1.6 mm²). The CSR was significantly lower in Sensho (P: 7.0, D: 7.2) than in other varieties (BlackGora, P: 11.1, D: 12.1; IR42, P: 12.7, D: 12.7; Koshihikari, P: 11.9, D: 13.1).

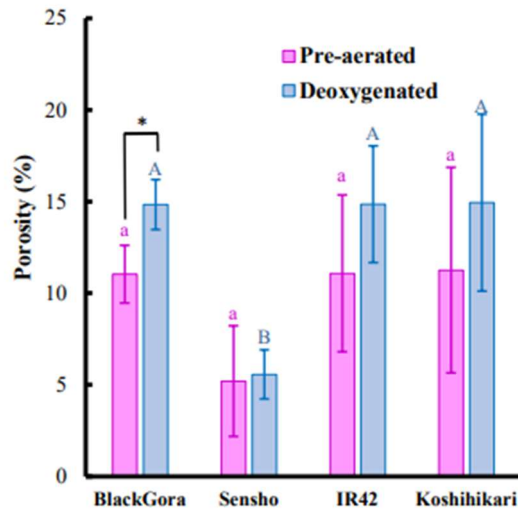


Fig. 2-3. Porosity of all roots from each variety in Experiment B (n = 3). Significant differences between pre-aerated and deoxygenated conditions at $P < 0.05$ are denoted by *. Different letters indicate a significant difference at $P < 0.05$ according to Tukey HSD. Error bars indicate standard deviations (S.D.).

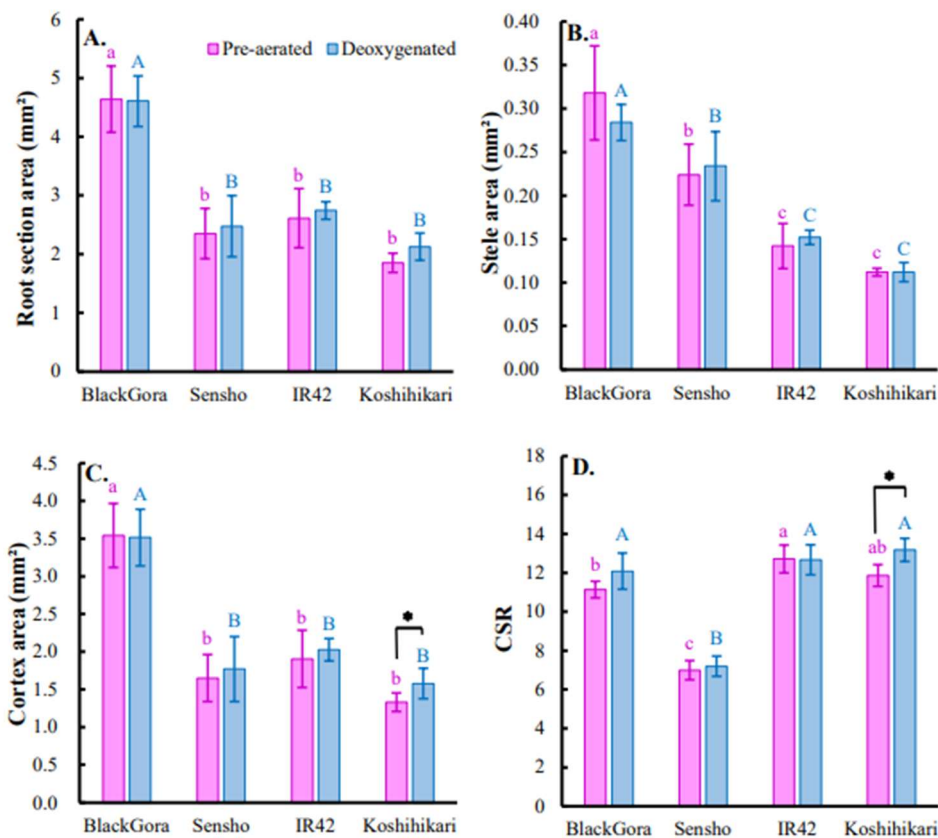


Fig. 2-4. Average of root anatomical traits from three different nodal root types (n = 5). A. indicates Root section area, B. indicates stele area, C. indicates Cortex area, D. indicates Cortex to stele ratio (CSR), respectively. Significant differences between pre-aerated and deoxygenated conditions at $P < 0.05$ are denoted by *.

P<0.05 are denoted by *. Different letters indicate a significant difference at P < 0.05 according to Tukey HSD. Error bars indicate standard deviations (S.D.).

3.4. Root oxygen consumption in Experiment B

The results of root oxygen consumption per root dry weight are shown in Table 2-2. Significant treatment effects were observed in the compensation of oxygen consumption by oxygen transportation of Koshihikari and Sensho. Root oxygen consumption was lowest in BlackGora (P: 0.79 mg h⁻¹ g⁻¹, D: 0.82 mg h⁻¹ g⁻¹) and highest in Sensho (P: 2.69 mg h⁻¹ g⁻¹, D: 2.44 mg h⁻¹ g⁻¹). Root oxygen transportation was lowest in BlackGora (P: 0.51 mg h⁻¹ g⁻¹, D: 0.40 mg h⁻¹ g⁻¹) and highest in Sensho (P: 1.36 mg h⁻¹ g⁻¹, D: 1.63 mg h⁻¹ g⁻¹). The compensation of consumption by transportation was highest in IR42 (P: 96.1%, D: 108.6%).

Table 2-2. Root oxygen consumption and transportation in Experiment B (n = 5).

Variety	Oxygen consumption rate (mg g ⁻¹ h ⁻¹)			Oxygen transportation rate (mg g ⁻¹ h ⁻¹)			Compensation of oxygen consumption by oxygen transportation (%)		
	P	D	t-test	P	D	t-test	P	D	t-test
BlackGora	0.79	0.82	c B n.s	0.51	0.40	b B n.s	63.1	49.1	b B n.s
Sensho	2.69	2.44	a A n.s	1.36	1.63	a A n.s	51.0	66.6	b B *
IR42	1.24	1.17	b B n.s	1.20	1.26	a A n.s	96.1	108.6	a A n.s
Koshihikari	2.29	2.19	a A n.s	1.18	1.42	a A n.s	51.3	65.0	b B *

Significant differences between P (pre-aerated) and D (deoxygenated) at P<0.05 are denoted by *. Different letters indicate a significant difference at P < 0.05 according to Tukey HSD.

II-4. Discussion

The Experiment A showed that there was a significant increase in CSR in stagnant conditions compared to that in aerated conditions for BlackGora, Inpari29, LAC23, and Sensho (Fig.2-2). These results were similar to previous research in which CSR increments in stagnant deoxygenated agar conditions were observed in cereal crops (Yamauchi, Abe, et al., 2019), so this response was also considered to be related to improve oxygen diffusion in root. Looking at genotypic differences, three

varieties except Inpari29 are varieties that are operated as upland rice. One of the factors affecting the amount of oxygen transported from the ground to the root tips is root length (Armstrong et al., 1982; Armstrong et al., 1983). It is therefore speculated that differences in rooting between upland and lowland rice are related to differences in CSR responses. Especially, BlackGora is proved as deeper rooting variety (Islam et al., 2021).

The Experiment B showed that deoxygenated conditions did not result in any negative effects on biomass production in different varieties (Table 2-1). However, considering that root porosity in Deoxygenated only increased in BlackGora (Fig. 2-3) and that the increase in CSR was also lower (Fig. 2-2) than result in Experiment A (Fig. 2-2) and report by Yamauchi et al (Yamauchi, Abe, et al., 2019). It means the effect of the treatment in Experiment B was considered lower than in conventional methods and in waterlogged soil trials. In a pot experiment, Sensho was confirmed to be comparatively sensitive to waterlogging in rice, as oxygen was lost more readily from the root basal area (Colmer, 2003a). However, field experiments reported that Sensho performed well in both waterlogged and aerobic fields (Nguyen et al., 2015). Although plant species with higher CSR are considered to be more waterlogging adapted (Yamauchi, Abe, et al., 2019; Yamauchi, Pedersen, et al., 2021), the waterlogging adaptability demonstrated by Sensho in previous study and the root morphology results obtained in this study, taken as a whole, suggest that high adaptability of rice to waterlogging.

The difference in absolute CSR value between adventitious and seminal roots is due to the fact that the rate of increase in stele area is less than the rate of increase in root sectional area in rice when comparing seminal and adventitious roots (table 2-3.). This trend may differ between species. Sundgren et al., (2018) report data for wheat seminal roots and adventitious roots, but when Seminal root/Adventitious root was calculated, the ratio was higher in stele area than in the ratio of root sectional area (table 2-3.). This means that depending on the plant species, cortex formation is promoted, or stele formation is promoted. These differences in the process of primary root tissue

development may be linked to the relationship between the moisture conditions of the plant habitat and the internal morphology of the roots.

Although this study was conducted under the assumption that root oxygen consumption and transportation characteristics are related to the internal morphology of the root, no correlation was found between them. There are two possibilities: one is that differences in root internal morphology among rice varieties are not large enough to be associated with oxygen transportation and consumption characteristics; the other possibility is that the root internal morphology plays an important role in the efficiency of oxygen diffusion within the root, but other traits are more influential on the amount of oxygen delivered and consumed. Indicators of root internal morphology, such as fractional root porosity and CSR, were found to correlate well with the aquatic environment to which plants are adapted (Justin & Armstrong, 1987; Yamauchi et al., 2021). However, Leaf area/Root dry weight (LA/RDW) was found to have the greatest impact on root oxygen consumption and transportation (Fig. 2-5). Therefore, the importance of these root internal morphologies in terms of oxygen transportation may require the elucidation of local oxygen distribution in the roots using micro oxygen electrodes (Armstrong et al., 2000, 2019; Colmer et al., 2020; Lin et al., 2021).

A trait that has not been examined in the present study but that affects oxygen consumption in roots is the formation of the ROL barrier. The ROL barrier inhibits oxygen absorption in areas where it is formed (Pedersen, Nakayama, et al., 2021). Of the four varieties used in the study, Sensho has been reported to have a low capacity to form ROL barriers in previous studies (Colmer, 2003a). Maricle & Lee (2007) also reported an interspecific comparison of oxygen uptake and found that when respiration is not inhibited, most tested wetland species had lower oxygen uptake when compared to Maize, which does not form ROL barriers. Based on these results, if the other varieties tested in this study had higher ability of formation the ROL barrier than Sensho, this may have affected the oxygen consumption results of the respective rice varieties. However, even if this is discounted, the positive correlation

between root oxygen consumption and LA/RDW observed in this study was very strong in present study (Fig.2-5). Relationship between LA/RDW and root oxygen consumption was similar trends in Sensho and Koshihikari, so we believe that it is not due to the specific variety selection.

In terms of oxygen production and uptake through photosynthesis and transpiration, leaf area is more appropriate than dry weight in discussing the relationship to oxygen supply, and LA/RDW was adopted instead of shoot dry weight/root dry weight (S/R ratio) in this study. LA/RDW and S/R ratio showed a very strong positive correlation ($r = 0.96$ Fig. 2-6). Many wetland-adapted species have an increased S/R ratio to reduce the oxygen sink under hypoxia and to allow better access to oxygen from the shoot (Jung et al., 2009; Miller & Zedler, 2003). Actually, root oxygen consumption was positively correlated with the S/R ratio (Nakamura et al., 2010). In addition, plants with higher S/R ratio from an increasing assimilation of NH_4^+ to the shoot was thought to supply oxygen to the roots more efficiently than plants with lower S/R ratios where NO_3^- is the main source of utilization (Nakamura & Noguchi, 2020). The interpretation that LA/RDW was influenced by oxygen transportation in the root as a result it was a strongly positive correlation with root oxygen consumption is possible. But then the oxygen consumption rate of IR42 roots with high Compensation of oxygen consumption by oxygen transportation (CCT) should be much higher without following LA/RDW, we interpreted that LA/RDW influenced oxygen transportation and root oxygen consumption. It is still unclear why there is such a strong relationship between root oxygen consumption and LA/RDW. According to Veen (1981) and Nakamura and Nakamura (2016), a larger proportion of root oxygen consumption was used for ion uptake than for root growth and maintenance. For traits related to root ion uptake, diurnal variations in transpiration were shown to be related to diurnal variations in root respiration (Bekku et al., 2011). Therefore, we theorized that the changes in balance between the number of leaves, which are the sites of transpiration, and roots, which are the sites of nutrient absorption, affected the root oxygen consumption. In the stele, ion accumulation, like energy-dependent K^+ accumulation in the

xylem parenchyma cells and transportation, use energy, and their activities were restricted when the stele becomes hypoxic (Kotula et al., 2015; Shabala et al., 2014). Therefore, plants with high LA/RDW may need to achieve a higher ion uptake by transpiration with fewer ion uptake parts than plants with lower LA/RDW, it increases the oxygen consumption in high LA/RDW plant.

In this study, there was a significant increase in the compensation of consumption by transportation in deoxygenated conditions of Sensho and Koshihikari, as their root oxygen consumption was higher (Table 2-2). CSR increments due to cortex expansion were thought to contribute to the increase in the compensation of consumption by transportation in Koshihikari (Fig. 2-3). However, Sensho did not show any anatomical root changes. The mechanism how Sensho increased the compensation of consumption by transportation in deoxygenated conditions is unclear, and further verification is required. In addition, IR42 had the highest compensation of oxygen consumption by oxygen transportation, but the reason was unclear in this study. Therefore, multiple regression analysis was performed with root oxygen transportation rate as an objective variable; this showed that the contributions of leaf stomatal conductance and shoot dry weight were second only to LA/RDW, but the coefficient of determination (R^2) was only 0.65 to oxygen transportation (Table 2-4). Plants transport oxygen from the atmosphere, and oxygen is produced by photosynthesis, so vapor pressure deficit, stomatal conductance, and photosynthetic rate are correlated with radial oxygen loss (Sorrell & Brix, 2003; Q. Wang et al., 2019). Although not measured in this study, when considering the pathways of oxygen transportation from the leaves to the roots, morphological characteristics, such as aerenchyma and intercellular gas spaces, also have an important influence on the amount of gas being transported (Evans & Carvalho-Evans, 2021). Based on multiple regression coefficients, shoot dry weight had a negative effect on oxygen transportation in rice. Variations in air space within the shoot and root–shoot junction affect the amount of transported gas as resistance even in aquatic plants (Aulakh et al., 2000; Rich et al., 2013), which may cause oxygen losses as the shoot gets larger even

similar LA/RDW plant. In future studies, a detailed organ oxygen concentration profiling from the shoot to the root is needed to sort out the characteristics that affect the amount of gas being transported, the oxygen concentration in that gas, and the way oxygen diffuses in the root to clarify the mechanism that can efficiently supply oxygen to the roots. In addition, crops susceptible to waterlogging tend to close their stomata when their stress progressed on waterlogging (Goto et al., 2022; Herrera, 2013; Khanthavong et al., 2022). Therefore, to enhance the waterlogging tolerance of such crops, it will be necessary to study both root and shoot characteristics with respect to their impact on oxygen transport using species with diverse tolerance strengths.

Table 2-3. Adventitious root/Seminal root of root section area and stele area in present research and previous research

	Variety	Adventitious root/Seminal root	
		Root section area	Stele area
Rice	BlackGora	30.0	19.0
	Sensho	22.0	14.6
	Koshiihikari	20.9	11.9
Wheat (Table1, Sundgren et al., 2018)	Bjarne	2.1	2.4
	NK93602	1.5	1.9
	Zebra	1.8	2.0
	Naxos	1.9	2.3
	Quarna	1.9	2.3
	T9040	1.9	2.3

Adventitious root/Seminal root in wheat were calculated from data in Table 1 of Sundgren et al., (2018)

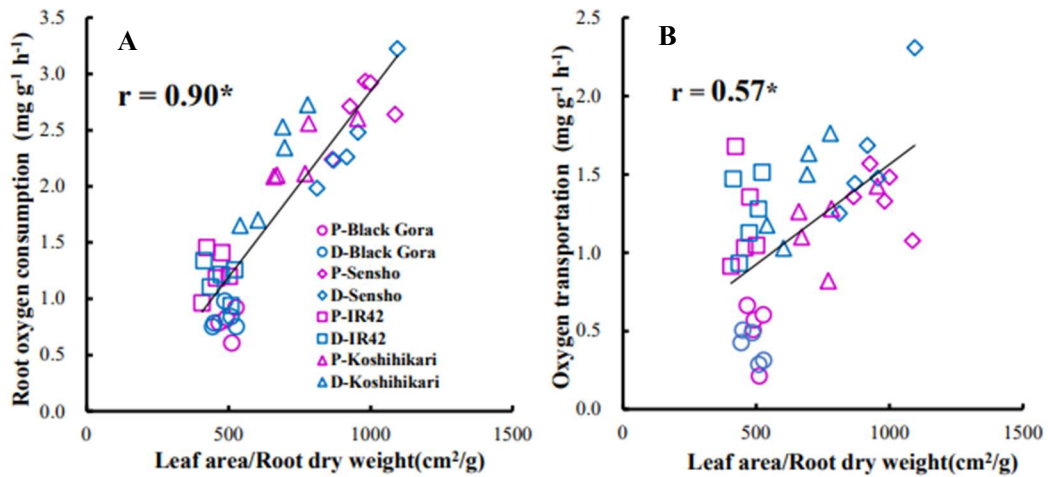


Fig. 2-5. Relationships between leaf area/root dry weight and root oxygen consumption (A) and transportation (B). Significant correlation coefficients defined by Pearson at $P < 0.05$ are denoted by *.

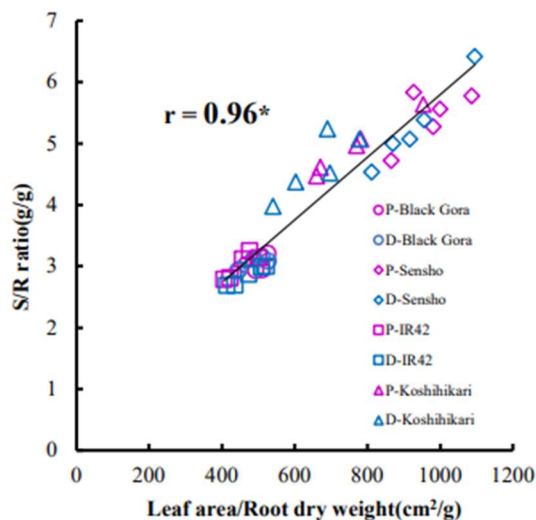


Fig. 2-6. Relationships between leaf area/root dry weight and shoot dry weight/root dry weight (S/R ratio). Significant correlation coefficients defined by Pearson at $P < 0.05$ are denoted by *.

Table 2-4. T-value and p-value in coefficients of multiple regression analysis with oxygen Transportation as an objective variable ($n = 40$).

	Unstandardized coefficient	S.E.	t value	p value
Intercept	0.734	0.311	2.358	0.024
Leaf area/Root dry weight	0.001	0.000	5.167	0.000
Shoot dry weight	-0.003	0.001	-4.958	0.000
Stomatal conductance	0.001	0.000	1.899	0.066

Adjusted determination factor (R^2) = 0.650

II-5. Conclusion

This is the first study to investigate the regulation of root oxygen consumption and transportation in rice, a crop with strong waterlogging adaptability, using cultivars with different internal root morphologies. Although the effect of treatment in Experiment B is considered weaker than that of the conventional method and Experiment A, the increase in CSR of Koshihikari and an increase in porosity in all varieties, except Sensho, was observed, as like indicated anaerobic response by a previous study. LA/RDW was strongly positively correlated with root oxygen consumption and

positively correlated with oxygen transportation, indicating the strong influence of these characteristics. However, the resulting compensation of oxygen consumption by oxygen transportation was highest for IR42, indicating that this variety supplied oxygen to the roots most efficiently. The reason for this and the fact that Sensho improved the compensation of oxygen consumption by oxygen transportation without changes in root morphology could not be determined, but multiple regression analysis with root oxygen transportation rate as an objective variable showed that the contributions of stomatal conductance and shoot dry weight were second only to LA/RDW, suggesting that gas exchange and oxygen pathway in shoot may be involved in enhancing the oxygen concentration in transported gas.

CHAPTER 3

Inter-and intra-specific variation in hypoxic response of four millets: *Panicum miliaceum*, *Eleusine coracana*, *Echinochloa utilis* and *Coix lacryma-jobi* var. *ma-yuen*

III-1. Introduction

Millet species are diverse genetic resources with high adaptability to environmental stresses (Bandyopadhyay et al., 2017). Hypoxia response in these species has been observed, with tolerant species such as Japanese barnyard millet demonstrating the ability to maintain more root hairs, which contribute to nutrient absorption (Matsuura et al., 2022). These findings emphasize the importance of conserving and evaluating genetic resources while implementing breeding strategies to adapt agriculture to climate change challenges. Recent comparisons of Poaceae grasses at various soil moisture levels (Yamauchi, Pedersen, et al., 2021) have demonstrated the significance of achieving an optimal root cortex-to-stele area ratio (CSR) for soil moisture management. Evaluating plants from a diverse gene pool can provide valuable insights for crop development, considering changing climate conditions. Despite limited research on waterlogging tolerance in Poaceae crops, including minor crops, there have been some comparative studies (Galamay et al., 1991; Kono et al., 1987, 1988; Yamauchi et al., 1987). Notably, Job's tears exhibit a high waterlogging tolerance level (Kono et al., 1987), with genetic similarities to those of sorghum and maize (Guo et al., 2020; Zhai et al., 2023). This indicates that they might be a better model crop for studying waterlogging tolerance than rice. These crops are produced on a considerably smaller scale than major crops, which has historically limited research on their hypoxia response. However, current research has developed methods to induce hypoxic stress at the laboratory level using deoxygenated agar hydroponic solutions, effectively mimicking waterlogged soil conditions (Wiengweera et al., 1997). This approach has facilitated in-depth investigations into root responses. Additionally, the availability of handheld devices that can capture leaf conditions has enabled early-stage stress assessment and stress level measurement in terms

of leaf physiology and changes in dry matter production. Moreover, the use of root analysis software has simplified the analysis of complex root system elements. Leveraging these advancements, herein, we explored the hypoxic responses of four Poaceae crops with respect to leaf physiology and root morphology using an experimental system based on a stagnant agar solution.

III-2. Material and methods

2.1. Growth and sampling

Following earlier studies of dry matter weights on control and waterlogged plots (Kono et al., 1987, 1988), we investigated common millet (*Panicum miliaceum*) and finger millet (*Eleusine coracana*) as waterlogging-sensitive species and Japanese barnyard millet (*Echinochloa utilis*) and Job's tears (*Coix lacryma-jobi* var. *ma-yuen*) as waterlogging-tolerant species. A total of 18 varieties were examined, five each variety for common millet, finger millet, and Japanese barnyard millet, and three varieties for job's tears (Table 3-1). Tests were conducted independently for each species with the following common protocols.

Twenty-five to thirty seeds of each variety were sterilized with 0.5% (v/v) sodium hypochlorite for 30 min, followed by thorough rinsing with distilled water. The seeds were germinated in a sponge using tap water within a growth chamber, with the air temperature maintained at 25°C and a 14-h light period with photosynthetically active radiation (PAR) at 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$, followed by a 10-h dark period. From seedling growth to the sampling stage, the nutrient solution at full strength comprised 1.0 mmol NH_4NO_3 , 0.50 mmol NaH_2PO_4 , 0.30 mmol K_2SO_4 , 0.30 mmol CaCl_2 , 0.60 mmol MgCl_2 , 90 μmol Fe-EDTA, 50 μmol H_3BO_3 , 9.0 μmol MnSO_4 , 0.30 μmol CuSO_4 , 0.70 μmol ZnSO_4 , and 0.10 μmol Na_2MoO_4 . Room temperature was maintained at 25°C throughout the 14-h light period, with PAR ranging from 204 to 350 $\mu\text{mol m}^{-2}\text{s}^{-1}$. This temperature setting was also applied during the 10-h dark period, from seedling growth to the sampling stage. Following germination, each seedling was transplanted into its designated 17-L pot corresponding to its variety and placed in an aerated half-

strength nutrient solution until it had grown to 4–5 leaves. Acclimation to a 0.1% agar hydroponic solution was conducted on seedlings with 4–5 leaves. The seedlings of each variety were transferred to four 17-L pots, with two plants per variety, totaling 10 plants per pot. Following 6 days of growth in the full-strength aerated 0.1% solution, two different conditions of the nutrient solution were introduced. The plant ages at the start of the treatment were as follows, Common millet; 26 days after sowing (DAS), Finger millet; 30 DAS, job`s tears; 31DAS, Japanese barnyard millet; 31 DAS. Two pots received continued treatment of aerated full-strength nutrient solution (aerated conditions), whereas the other two pots were treated with a deoxygenated solution (dissolved oxygen, <1.0 mg L⁻¹) by flushing them with nitrogen gas, creating deoxygenated stagnant conditions. The pH of the solution was adjusted to 5.8, and the solution was renewed every 7–10 days. To prevent iron deficiency in the young seedlings under aerated conditions, FeSO₄ was added to the aerated nutrient solution at a final concentration of 2–5 µM L⁻¹ daily, from each initial day of rewatering and continuing for 5 days (Kulichikhin et al., 2014). Two types of measurements were conducted every 2 days from the beginning of the treatment to assess the impact of rhizosphere hypoxia on leaf function. First, the chlorophyll content of the leaves was measured three times on the middle part of the newest fully expanded leaves using a soil plant analysis development (SPAD) meter (SPAD-502 plus, Konica Minolta, Japan). The average value obtained from these three measurements represents the SPAD value for each sample. Second, a chlorophyll fluorescence meter (AquaPen AP100-P, Photon Systems Instruments, Czech Republic) was used to measure the maximum quantum yield of photosystem II on the same leaves. These fluorescence measurements were taken 5–8 h after the lights were turned off, with vertical exposure to excitation light facilitated by leaf clips (Sone et al., 2012). After 16 days of growth from treatment initiation, the seedlings were sampled and used for subsequent measurements.

Table 3-1. Information of tested varieties in this research

Specie	Variety	Origin	JP number	
Common millet	INAZAIRAI 3	Nagano, Japan	49760	
	MINERI	Sri Lanka	177482	
	SHINANO 1	Nagano, Japan	77160	
	TOBI	Mongolia	4021	
	YUSUHARA KEI 1	Kochi, Japan	36235	
Finger millet	IE929	Africa	25336	
	MURUWA	Nepal	99911	
	PURNA	India	25326	
	WHITE RAGI	India	25337	
	YUKIJIRUSHI KEI	Japan	25310	
	Japanese barnyard millet	KINSHU	China	3684
		MURASAKI	Iwate, Japan	3649
NANBU MOCHIMUCHI		Iwate, Japan	251036	
RIKUU 3		Akita, Japan	3611	
WHITE PANICK		Japan	162385	
Job's tears	HATOHKARI	Iwate, Japan	146894	
	OKAYAMA ZAIRAI	Okayama, Japan	204	
	RIOGRANDE DE SUL	Brazil	207	

2.2. Measurements of shoot morphological parameters

During sampling, the number of stems on each plant was counted, and the plant was dissected into leaves, stems, and roots. The roots were preserved in 50% ethanol and stored in a low-temperature room at 4°C for further analysis. Following excision, the leaves were rapidly passed through an automatic area meter (AAM-9, Hayashi Denko Co., Ltd., Japan) to measure the leaf area. Subsequently, the leaves and stems were oven-dried at 80°C for at least 72 h, brought to room temperature in a desiccator to prevent moisture absorption, and weighed using an electronic balance.

2.3. Measurements of root morphological parameters

Before root scanning, the root number of each sample was counted. Root samples were scanned at 300 dpi (EPSON GT-X830, Epson American Inc., USA), and the scanned images were analyzed using an image analysis system (WinRHIZO, Regent Instruments Inc., Canada) to determine the total length and surface area of the roots. Root diameters were classified into 0.1-mm intervals for each measurement. During root image analysis, we focused on the maximum lateral root diameter for each species. Consequently, the main and lateral roots were classified as follows: common millet lateral root < 0.5 mm, finger millet lateral root < 0.6 mm, Japanese barnyard millet lateral root < 0.6 mm, and Job's tear lateral root < 0.9 mm. The main and lateral root lengths were subsequently calculated.

2.4. Measurements of root anatomical parameters

Root anatomical traits were measured on adventitious roots, focusing on roots with lengths close to the average length of the roots. The range of root lengths tested varied depending on the species and treatment as follows: common millet (aerated 210–310 mm, stagnant 110–200 mm), finger millet (aerated 250–530 mm, stagnant 80–140 mm), Japanese barnyard millet (aerated 150–300 mm, stagnant 110–220 mm), and Job's tears (aerated 300–570 mm, stagnant 260–360 mm). Root segments were carefully cut approximately 20–30 mm from the root apex, and root sections were manually prepared using a razor blade. The areas of each root tissue, including the whole cross-sectional area, epidermis area, cortex area, stele area, and total aerenchyma area, were manually measured using ImageJ software (National Institutes of Health, America). The aerenchyma-to-whole area ratio (AWR) was calculated by dividing the total aerenchyma area by the whole cross-sectional area, whereas the CSR was calculated by dividing the cortex area by the stele area.

2.5. Statistical analysis

Microsoft Excel was used to conduct t-tests and correlation analyses. The *aov* function in the standard package of R software (v.4.2.2) was used to conduct a two-way analysis of variance (ANOVA). The *prcomp* function in the Stats package of R software (v.4.2.2) was used to conduct principal component analysis on stagnant/aerated averages of each parameter.

III-3 Results

3.1. Growth of the four Poaceae crops under aerated or stagnant conditions

During the treatment period, the maximum quantum yield (F_v/F_m) under aerated conditions for each species remained consistently around 0.78 (Fig. 3-1). However, under stagnant conditions, Japanese barnyard millet and Job's tears displayed a similar trend to the aerated condition, whereas common millet and finger millet exhibited significant decreases compared with the aerated condition (Fig. 3-1). Notably, common millet exhibited significant reductions in F_v/F_m from 2 days after

treatment initiation to throughout the remaining treatment period, while finger millet showed significant reductions from 6 days after treatment initiation to throughout the remaining treatment period (Fig. 3-1).

In summary, common millet exhibited an earlier and more pronounced reduction in Fv/Fm (ranging 0.43–0.66), whereas finger millet exhibited a milder reduction (ranging 0.71–0.77) (Fig. 3-1). The SPAD value, which measures chlorophyll content, followed a similar pattern, decreasing significantly under stagnant conditions for both common millet (from 10 days after treatment initiation to throughout the remaining treatment period, except at 14 days) and finger millet (at 14 and 16 days after treatment initiation; Fig. 3-1).

The average shoot dry weight of three millet species was approximately 1.0 g under aerated conditions (common millet: 1.1 g; finger millet: 9.4 g; Japanese barnyard millet: 1.0 g), whereas in Job's tears, a nearly fourfold difference in plant size was observed at 4.3 g. For each species, two-way ANOVA was performed for between-varieties, between-treatment, and interaction effects. At the variety level, significant differences were found for common millet, finger millet, and Japanese barnyard millet. Significant reductions in shoot dry weight were observed under stagnant conditions for common millet, finger millet, and Job's tears. The range of stagnant/aerated ratio (S/A) of shoot dry weight was as follows: common millet (0.28–0.61), finger millet (0.42–0.68), Japanese barnyard millet (0.85–1.20), and Job's tears (0.69–0.82). There was no significant interaction between variety and treatment in any species (Fig. 3-2).

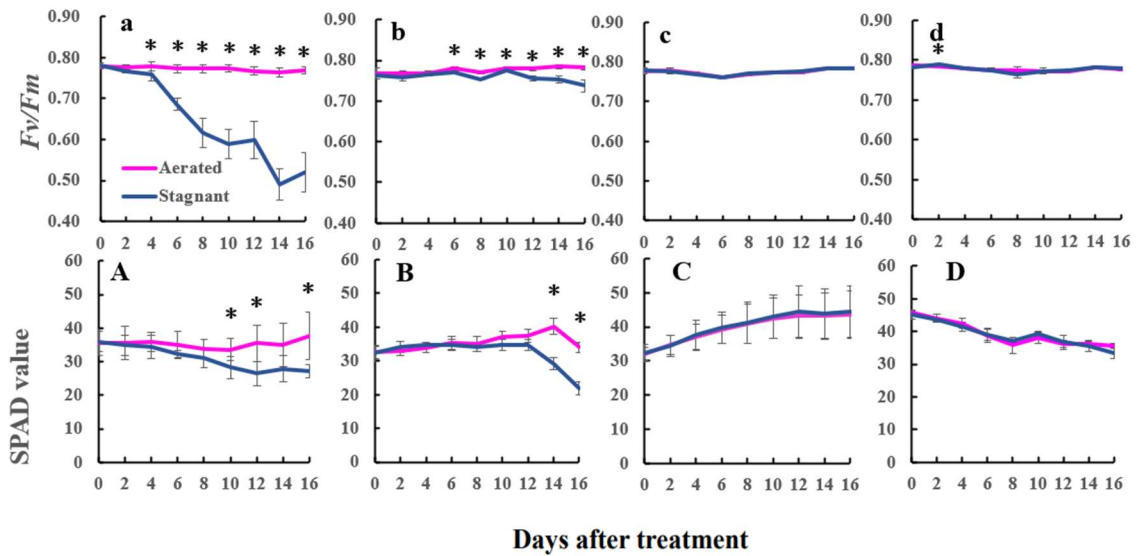


Fig. 3-1. Maximum quantum yield (F_v/F_m) and SPAD value of the four Poaceae crops. (a, A) common millet, (b, B) finger millet, (c, C) Japanese barnyard millet, and (d, D) Job's tears under aerated and deoxygenated stagnant conditions. The lines represent the averages of each species calculated from the averages of each variety as replications ($n = 5$, except $n = 3$ for Job's tears). Data were collected from the newest fully expanded leaf on each measurement day. Significant differences between aerated and deoxygenated stagnant conditions at $p < 0.05$ were tested using the Student's t-test and are denoted by *. Error bars indicate standard deviations (SD).

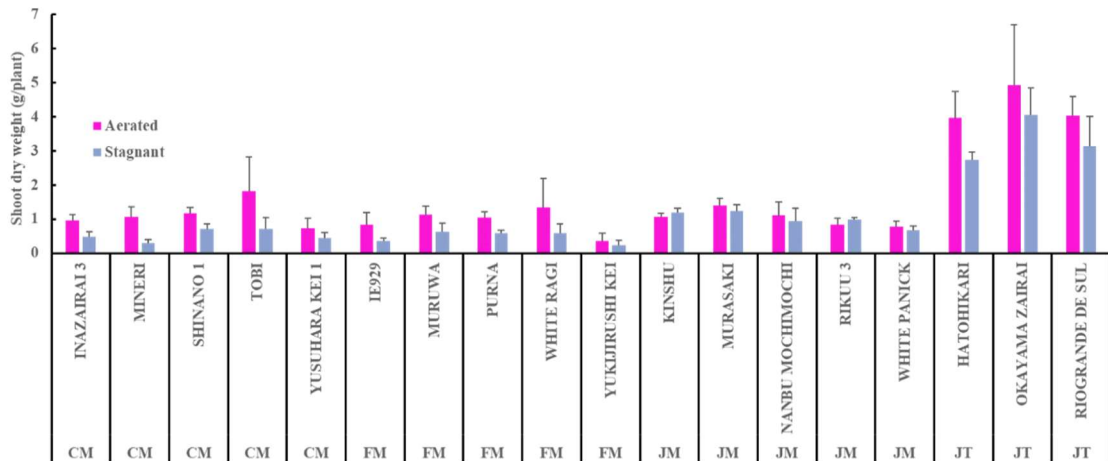


Fig. 3-2. Shoot dry weight of the four Poaceae crops. (CM = common millet, FM = finger millet, JM = Japanese barnyard millet, and JT = Job's tears) under aerated and deoxygenated stagnant conditions. The bars represent the average of each variety, with $n = 3$ for CM and FM and $n = 4$ for JM and JT. Data were collected from plants treated for 16 days under aerated or deoxygenated stagnant solutions.

3.2. Root morphology of the four Poaceae crops under aerated or stagnant conditions

Significant reductions in total root length were observed in common millet and finger millet under stagnant conditions (Table 3-2). Moreover, these species' main and lateral root lengths were significantly reduced under stagnant conditions (Table 3-2). In contrast, Japanese barnyard millet exhibited a significant increase in total and lateral root lengths under stagnant conditions (Table 3-2). Considering the S/A (common millet: 0.28–0.56, finger millet: 0.12–0.31, Japanese barnyard millet: 1.03–1.30, Job's tears: 0.75–1.08) and absolute values, common millet and finger millet could not maintain total root length as effectively as Japanese barnyard millet and Job's tears (Table 3-2). Under stagnant conditions, a significant increase in root number was observed for finger millet, Japanese barnyard millet, and Job's tears. Furthermore, the root number of finger millet exhibited significant differences among varieties, although there was no significant interaction between variety and treatment (Table 3-2).

Given that oxygen diffusion to the lateral root is believed to occur through the main root under hypoxic rhizosphere conditions, we examined the length ratio between the lateral and main roots under aerated and stagnant conditions. Additionally, we assessed the single root length in terms of the oxygen diffusion distance, calculated by dividing the main root length by the main root number. The lateral/main root length ratio under stagnant conditions significantly decreased in common millet but significantly increased in Japanese barnyard millet and Job's tears (Table 3-3). Significant varietal differences and interactions were observed in common millet and Japanese barnyard millet (Table 3-3). Furthermore, the main root length to root number ratio under stagnant conditions decreased significantly in all species (Table 3-3). Significant varietal differences were also observed in common millet and Japanese barnyard millet, but no significant interaction between variety and treatment was observed (Table 3-3).

Table 3-2. Root morphological traits of each species (n = 3 for common millet and finger millet; n = 4 for Japanese barnyard millet and Job's tears) grown under aerated and deoxygenated stagnant conditions for 16 days.

Species	Variety	Total root length (cm)						Lateral root length (cm)						Root number			
		Aerated			Stagnant			Aerated			Stagnant			Aerated	Stagnant		
		Ave.	S.D.	n.s.	Ave.	S.D.	n.s.	Ave.	S.D.	n.s.	Ave.	S.D.	n.s.	Ave.	S.D.		
Common millet	INAZAIRAI 3	6625.3	1106.5	2832.4	1110.9	1027.3	207.8	527.3	180.6	5598.0	910.6	2305.1	935.0	26.0	5.6	23.3	3.1
	MINERI	7681.0	3195.4	2171.1	434.6	1456.7	524.6	454.3	113.1	6224.4	2682.7	1716.8	327.8	37.3	2.1	24.7	5.5
	SHIMANO 1	6521.0	677.8	3657.1	766.3	1323.2	236.5	713.9	280.4	5197.9	441.6	2943.2	490.7	23.3	4.2	30.0	7.2
Finger millet	TOBI	8789.8	5024.6	2677.4	1066.1	1302.6	776.0	484.0	198.3	7487.1	4250.0	2193.4	891.3	27.3	10.0	23.7	7.6
	YUSUHARA KEI 1	6027.6	2887.1	2661.8	1123.0	782.3	518.0	520.9	202.8	5245.3	2383.6	2141.0	938.5	26.3	4.2	25.0	5.6
	Variety difference	n.s.								n.s.							n.s.
Treatment difference	p < 0.001																n.s.
Interaction	n.s.																n.s.
Japanese barnyard millet	IE929	8301.7	4458.1	1020.0	366.6	1251.2	686.1	119.9	51.6	7050.5	3773.0	900.1	316.6	13.7	2.9	14.0	1.0
	MURUWA	9135.8	4887.1	1984.8	572.4	1348.1	513.3	306.3	128.3	7787.7	4380.8	1678.6	445.0	21.3	2.9	30.0	3.0
	PURNA	6058.7	988.3	1892.1	435.6	1070.3	181.0	298.7	20.2	4988.4	828.2	1593.4	420.8	21.7	4.0	29.0	4.0
Job's tears	WHITE RAGI	8263.9	4630.9	1451.2	426.2	1326.6	837.0	230.5	72.5	6937.3	3794.2	1220.7	353.8	16.3	6.4	23.3	2.1
	YUKIJURUSHI KEI	2755.9	1517.5	614.2	476.4	395.4	317.1	100.7	87.3	2360.6	1200.7	513.5	389.2	11.7	5.0	16.3	9.5
	Variety difference	n.s.								n.s.							p < 0.001
Treatment difference	p < 0.001																p = 0.004
Interaction	n.s.																n.s.
Japanese barnyard millet	KINSHU	4434.4	681.8	4580.7	462.2	579.3	139.0	498.6	70.1	3855.1	549.3	4082.1	421.6	20.8	2.6	28.0	2.3
	MURASAKI	3381.8	590.5	3969.1	449.2	441.8	71.0	425.9	24.5	2940.0	525.0	3543.2	431.8	24.8	3.0	28.3	1.0
	NANBU MOCHIMOCHI	3456.2	1256.3	4258.4	1793.8	536.9	154.5	480.4	172.5	2919.2	1103.1	3778.0	1629.1	27.0	3.7	31.0	8.4
Job's tears	RIKUU 3	3188.3	1101.5	4138.9	360.7	363.4	129.3	473.3	35.5	2824.8	979.4	3665.6	325.7	20.0	1.4	30.5	0.6
	WHITE PANICK	3490.6	983.2	4221.1	549.6	493.6	109.0	428.5	57.5	2997.0	880.4	3792.6	501.1	26.8	3.9	29.3	1.7
	Variety difference	n.s.								n.s.							n.s.
Treatment difference	p = 0.037																p < 0.001
Interaction	n.s.																n.s.
Job's tears	HATOHIKARI	16383.6	2966.2	12242.1	722.7	1425.1	222.4	954.8	110.6	14958.5	2768.6	11287.3	615.1	19.5	4.4	30.5	4.0
	OKAYAMA ZAIRAI	13744.1	6354.5	12267.6	2243.9	1231.9	532.5	1056.8	138.1	12512.2	5822.4	11210.8	2180.4	22.3	3.8	28.8	2.8
	RIOGRANDE DE SUL	12375.1	3895.2	13337.3	2561.3	1272.1	268.6	1037.8	245.2	11103.0	3627.2	12299.4	2413.1	22.5	1.7	27.0	4.8
Job's tears	Variety difference	n.s.								n.s.							n.s.
	Treatment difference	n.s.															p < 0.001
	Interaction	n.s.															n.s.

SD denotes standard deviation. Nonsignificant differences are indicated by "n.s." Common millet and finger millet have a sample size of n = 3, whereas Japanese barnyard millet and Job's tears have a sample size of n = 4.

Table 3-3. Lateral root length to main root length ratio and main root length to root number ratio of each species (n = 3 for common millet and finger millet; n = 4 for Japanese barnyard millet and Job's tears) grown under aerated and deoxygenated stagnant conditions for 16 days.

Specie	Variety	Lateral / Main root length ratio (cm / cm)				Main root length/root number (cm/root)			
		Aerated		Stagnant		Aerated		Stagnant	
		Ave.	S.D.	Ave.	S.D.	Ave.	S.D.	Ave.	S.D.
Common millet	INAZAIRAI 3	5.49	0.43	4.29	0.51	39.6	2.7	22.2	5.3
	MINERI	4.22	0.42	3.82	0.42	38.6	12.1	18.6	3.0
	SHINANO 1	3.98	0.42	4.36	0.97	59.0	19.0	23.5	4.9
	TOBI	5.92	0.52	4.70	1.10	44.3	15.5	20.1	4.4
	YUSUHARA KEI 1	7.35	1.53	4.13	0.68	28.6	15.1	20.3	4.5
	Variety difference	p = 0.005				p = 0.047			
	Treatment difference	p < 0.001				p < 0.001			
	Interaction	p = 0.01				n.s.			
Finger millet	IE929	5.65	0.25	7.76	1.34	87.6	28.7	8.5	3.2
	MURUWA	5.48	1.20	5.72	0.93	61.8	17.3	10.5	5.4
	PURNA	4.68	0.42	5.30	1.15	49.5	3.0	10.4	1.8
	WHITE RAGI	5.65	1.06	5.33	0.19	75.4	31.3	9.8	2.4
	YUKIJIRUSHI KEI	7.35	2.84	5.56	1.06	33.5	19.3	5.5	1.8
	Variety difference	n.s.				n.s.			
	Treatment difference	n.s.				p < 0.001			
	Interaction	n.s.				n.s.			
Japanese barnyard millet	KINSHU	6.83	1.06	8.25	0.91	28.5	9.3	17.9	3.1
	MURASAKI	6.64	0.48	8.31	0.78	18.1	4.0	15.1	1.0
	NANBU MOCHIMUCHI	5.30	0.76	7.72	1.12	19.6	3.2	15.4	4.1
	RIKUU 3	7.84	1.09	7.74	0.15	18.2	6.8	15.5	1.0
	WHITE PANICK	6.03	0.74	8.88	0.76	18.5	2.9	14.6	1.6
	Variety difference	p = 0.045				p = 0.024			
	Treatment difference	p < 0.001				p = 0.002			
	Interaction	p = 0.014				n.s.			
Job's tears	HATOHKARI	10.49	0.95	11.88	0.71	76.8	23.5	31.4	2.4
	OKAYAMA ZAIRAI	10.05	0.46	10.67	1.85	57.6	31.0	37.0	5.9
	RIOGRANDE DE SUL	8.53	1.32	12.01	1.88	57.0	13.4	39.7	12.5
	Variety difference	n.s.				n.s.			
	Treatment difference	p = 0.003				p = 0.001			
	Interaction	n.s.				n.s.			

SD denotes standard deviation. Nonsignificant differences are indicated by “n.s.” Common millet and finger millet have a sample size of n = 3, whereas Japanese barnyard millet and Job's tears have a sample size of n = 4.

3.3. Root anatomy of the four Poaceae crops under aerated or stagnant conditions

Significant increases were observed in the cross-sectional area of the whole root in all species under stagnant conditions (Table 3-4). Furthermore, significant differences between varieties were observed in finger millet and Japanese barnyard millet. A significant interaction between variety and treatment was also observed in common millet (Table 3-4).

Significant increases in AWR were observed in common millet, finger millet, and Japanese

barnyard millet under stagnant conditions (Table 3-4). Additionally, significant differences between varieties were observed in these species. Furthermore, significant interactions between variety and treatment were observed in finger millet and Japanese barnyard millet (Table 3-4).

Significant increases in CSR were observed in common millet, finger millet, and Japanese barnyard millet under stagnant conditions (Table 3-4). Moreover, Job's tears showed an increasing trend in CSR under stagnant conditions (Table 3-4). Significant differences between varieties were observed in common millet and Japanese barnyard millet, with a significant interaction between variety and treatment observed in common millet (Table 3-4). Images of root sections for one variety per species under both aerated and stagnant conditions are shown in Fig. 3-3.

Table 3-4. Root cross-section parameters of each species (n = 3 for common millet and finger millet; n = 4 for Japanese barnyard millet and Job's tears) grown under aerated and deoxygenated stagnant conditions for 16 days.

Specie	Variety	Whole root cross-section area (mm ²)				Aerenchyma to Whole area Ratio (mm ² /mm ²)				Cortex area to Stele area Ratio (mm ² /mm ²)				
		Aerated		Stagnant		Aerated		Stagnant		Aerated		Stagnant		
		Ave.	S.D.	Ave.	S.D.	Ave.	S.D.	Ave.	S.D.	Ave.	S.D.	Ave.	S.D.	
Common millet	INAZAIRAI 3	2.3	0.3	3.9	1.0	0.044	0.048	0.253	0.038	2.4	0.4	5.6	1.2	
	MINERI	2.5	0.6	3.4	1.2	0.181	0.096	0.367	0.041	7.2	1.4	13.2	1.4	
	SHINANO 1	2.1	0.8	4.9	0.8	0.015	0.018	0.304	0.084	2.1	0.5	5.5	0.9	
	TOBI	0.9	0.1	4.8	1.2	0.016	0.028	0.322	0.034	3.1	0.4	6.2	1.4	
	YUSUHARA KEI 1	1.7	0.4	3.9	0.4	0.013	0.023	0.341	0.032	2.4	0.3	5.0	0.3	
	Variety difference	n.s.				p = 0.002				p < 0.001				
	Treatment difference	p < 0.001				p < 0.001				p < 0.001				
	Interaction	p = 0.029				n.s.				p = 0.034				
Finger millet	IE929	3.1	0.2	7.7	2.2	0.016	0.020	0.285	0.024	4.4	0.1	6.0	0.8	
	MURUWA	3.9	0.6	7.5	2.7	0.007	0.007	0.306	0.027	4.4	0.9	6.5	1.3	
	PURNA	4.5	0.4	7.2	1.3	0.006	0.002	0.216	0.042	3.9	0.4	5.4	1.2	
	WHITE RAGI	4.2	1.9	8.8	2.9	0.005	0.009	0.166	0.047	4.3	0.1	5.7	0.4	
	YUKIJIRUSHI KEI	2.6	1.4	3.6	1.8	0.001	0.002	0.274	0.064	3.7	0.6	4.4	0.8	
	Variety difference	p = 0.035				p = 0.001				n.s.				
	Treatment difference	p < 0.001				p < 0.001				p < 0.001				
	Interaction	n.s.				p = 0.007				n.s.				
Japanese barnyard millet	KINSHU	1.9	0.7	4.7	0.7	0.072	0.088	0.218	0.066	6.9	0.8	11.8	2.4	
	MURASAKI	2.8	1.2	5.5	1.1	0.128	0.044	0.270	0.055	6.7	0.8	12.5	0.7	
	NANBU MOCHIMOCHI	5.5	3.2	9.9	3.1	0.136	0.089	0.325	0.065	7.1	1.3	12.3	1.0	
	RIKUU 3	2.0	1.1	6.8	3.5	0.162	0.029	0.349	0.053	5.1	0.6	12.8	1.8	
	WHITE PANICK	4.4	0.8	7.0	1.2	0.004	0.006	0.240	0.102	5.8	0.4	8.2	1.8	
	Variety difference	p = 0.001				p = 0.001				p = 0.001				
	Treatment difference	p < 0.001				p < 0.001				p < 0.001				
	Interaction	n.s.				p = 0.007				n.s.				
Job's tears	HATOHKARI	12.4	3.3	28.2	5.6	0.171	0.065	0.156	0.085	10.8	2.3	13.0	2.2	
	OKAYAMA ZAIRAI	18.4	4.7	27.1	8.0	0.206	0.151	0.199	0.097	11.7	2.5	13.6	1.5	
	RIOGRANDE DE SUL	19.3	5.2	27.0	13.0	0.211	0.052	0.218	0.056	11.9	2.7	13.6	2.5	
		Variety difference	n.s.				n.s.				n.s.			
		Treatment difference	p = 0.002				n.s.				n.s.			
	Interaction	n.s.				n.s.				n.s.				

SD denotes standard deviation. Nonsignificant differences are indicated by "n.s." Common millet and finger millet have a sample size of n = 3, whereas Japanese barnyard millet and Job's tears have a sample size of n = 4.

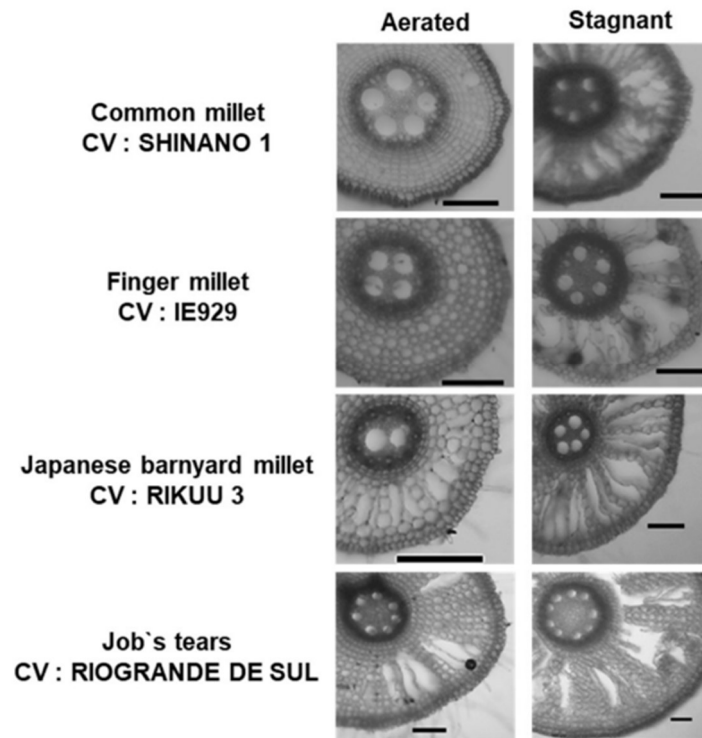


Fig. 3-3. Root section images of each species. Scale bar = 500 μ m.

III-4 Discussion

In this study, we initially investigated the differences in shoot hypoxia responses between waterlogging-tolerant and waterlogging-sensitive species. Subsequently, we explored possible causes of the shoot hypoxia response from morphological and anatomical perspectives, focusing on the root hypoxia response. Finally, we focused on shorter main root and longer lateral root development under hypoxia in tolerant species, aiming to discuss their implications to adapt to soil hypoxia.

4.1. Shoot response to hypoxia

Our experiment revealed different shoot responses to hypoxia between waterlogging-tolerant and waterlogging-sensitive species (Figs. 3-1 and 3-2). The results showed that waterlogging-tolerant species exhibited no leaf damage under stress, whereas waterlogging-sensitive species exhibited a decrease in their maximum quantum yield (F_v/F_m), followed by a decrease in their SPAD value. Goto et al. (2022) found that waterlogging in *Capsicum annuum* caused similar changes in leaf function. This suggests that chlorophyll degradation is caused by a decline in carotenoid levels, which typically

protects chlorophyll molecules from photo-oxidative damage caused by excessive light absorption and an accumulation of reactive oxygen species (ROS). Hypoxic stress has been found to elevate ROS levels in wheat roots (Biemelt et al., 2000) and barley roots and leaves (Luan et al., 2018). Our study indicates that the leaf dysfunction induced by hypoxia, including the initial reduction in Fv/Fm and subsequent chlorophyll degradation, contributes to the dynamic loss of dry weight in common millet and finger millet.

4.2. Maintaining total root length under hypoxic conditions is important to maintain plant shoot production activity

Roots are the first plant part to encounter soil environmental stresses, explaining the dynamic changes observed in this study. Interestingly, although no significant intertreatment differences were observed in the shoot characteristics of waterlogging-tolerant species, certain root characteristics exhibited significant differences between treatments. Specifically, total root length was significantly reduced in common millet and finger millet but not in Job's tears under stagnant conditions; conversely, it significantly increased in Japanese barnyard millet (Table 3-2). The results revealed a positive relationship between the S/A values for aboveground dry matter weight and total root length, with a correlation coefficient (r) of 0.86 (Fig. 3-5).

Related studies comparing wet and non-wet plant species have shown that increased root porosity allows for deeper root elongation under waterlogging conditions (Justin & Armstrong, 1987). Additionally, at the intraspecific level within the Poaceae family, *Urochloa* spp., which has longer roots, can maintain more dry matter under hypoxic conditions (Jiménez et al., 2021). Instead of focusing solely on absolute root length as a measure of adaptability to hypoxic soils, our study introduces a novel approach by considering differences in absolute plant size between species and establishing a relationship between root length maintenance and shoot dry matter production using

intertreatment ratios. Although the shoot dry weight response exhibits a relatively continuous distribution within a close range (sensitive species: 0.26–0.68, coefficient of variation 0.23; tolerant species: 0.69–1.20, CV 0.18), the total root length response is more distinctly differentiated between tolerant and sensitive species (sensitive species: 0.12–0.56, CV 0.42; tolerant species: 0.74–1.30, CV 0.16). One plausible explanation for this difference in performance between tolerant and sensitive species regarding their ability to maintain more stable roots under hypoxic conditions is the variations in root anatomical traits that influence internal aeration. Aerenchyma serves as a diffusion pathway for oxygen, and the formation of lysigenous aerenchyma is attributed to programmed cell death (Jackson & Armstrong, 1999). This process reduces the number of living cells, consequently decreasing oxygen consumption and oxygen diffusion resistance (Zhu et al., 2010). It also facilitates efficient oxygen transport by forming thicker roots, thereby reducing oxygen diffusion resistance and substantially enlarging the cortex area. The cortex area is particularly aerated and has a lower oxygen demand than the stele area (Yamauchi, Abe, et al., 2019). Our study revealed that roots under hypoxic conditions showed increased thickness in all species (Table 3-4). Furthermore, AWR (an indicator of the degree of aerenchyma formation) and CSR were higher in the tolerant species than in the sensitive ones, even under aerated conditions. This suggests that maintaining a consistently high level of root aeration ability during acclimatization to hypoxia reduces hypoxic stress, emphasizing the importance of research into the development of constitutive high-aeration roots by introducing constitutive aerenchyma formation and higher CSR into the roots of waterlogging-sensitive species (Yamauchi, Tanaka, et al., 2019).

4.3. Shorter main root axis and longer lateral root development in waterlogging-tolerant species under hypoxic conditions

Japanese barnyard millet has been shown to maintain nutrient uptake under hypoxic conditions (Matsuura et al., 2022). The ability of tolerant species to perform better than sensitive species is related

to the maintenance of or increase in total root length, as observed in the two tolerant species in this study. However, based on our visual observations, even tolerant species seem to form shorter roots under hypoxic conditions. Therefore, we calculated the center of gravity of a single root length by dividing the main root length by the root number. The results demonstrated that the main root length/root number ratio was significantly lower in all species under stagnant conditions than under aerated conditions, although the extent of change varied between tolerant and sensitive species (Table 3-2, Fig. 3-2). This finding is consistent with a previous report indicating that the maximum root lengths are shorter under stagnant conditions than under aerated conditions in some wild *Echinochloa* accessions (Ejiri & Shiono, 2019). It has been well established in pea roots that root length is positively correlated with oxygen diffusion resistance, where the amount of oxygen transported to the root tip decreases with increasing root length (Armstrong et al., 1983). Even in the wetland plant species *Z. nicaraguensis*, differences in oxygen concentration have been observed between the root base and root tips (Pedersen, Nakayama, et al., 2021); this suggests that the applicability of this rule varies in tolerant species compared to sensitive ones. In other words, it is not surprising that even tolerant species form shorter roots under hypoxic conditions, depending on their ability to access oxygen more efficiently at the root tips under hypoxic conditions.

Lateral root formation was suppressed in common millet and finger millet, consistent with observations in other sensitive species (Jiménez et al., 2021; Loades et al., 2013). Conversely, lateral root formation was increased in both tolerant species (Table 3-3). Specifically, Japanese barnyard millet exhibited a distinct increase in lateral root length, while a decrease in the main root length in Job's tears contributed to the increased lateral/main root length ratio. Although lateral roots are considered oxygen leakage points, similar to the main root apex, their formation is essential for nutrient absorption. Therefore, maintaining lateral roots is crucial even under hypoxic conditions. Moreover, recent evidence of ROL barrier formation in the lateral roots of *Z. nicaraguensis* and its

introggression lines indicates that lateral roots may not be as significant a source of oxygen leakage as previously believed (Pedersen, Nakayama, et al., 2021). If oxygen leakage is also suppressed in the lateral roots of Japanese barnyard millet and Job’s tears, as tested in this study, it is likely that this phenomenon, combined with their high aeration, has been sufficient to sustain lateral roots.

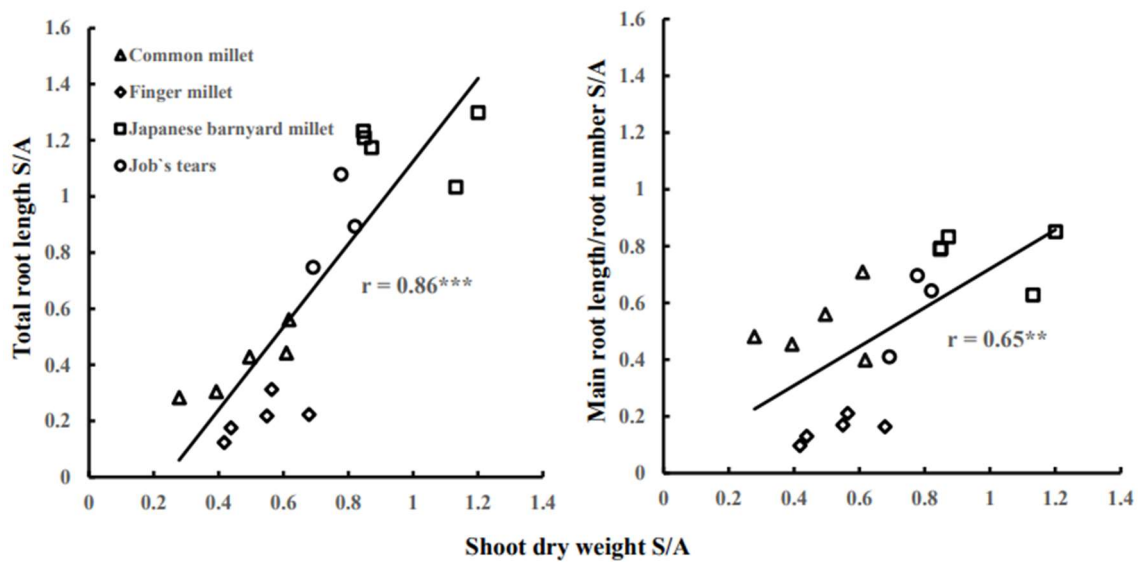


Fig. 3-5. Scatter plot depicting the hypoxia response (under stagnant/aerated conditions) of shoot dry weight and root length among the four Poaceae crops (n = 18). The value of “r” represents the correlation coefficient. Significant correlation coefficients defined by Pearson at $p < 0.001$ and $p < 0.01$ are denoted by “***” and “**”, respectively.

III-5. Conclusion

In this study, we investigated the hypoxia responses of four Poaceae crops with varying levels of waterlogging adaptability. On an interspecific scale, we found that maintaining the total root length is crucial for preserving shoot dry weight and leaf functionality in poaceous root systems. Principal component analysis also revealed species-specific challenges, such as root number responsiveness in common millet and LA/RA responsiveness in finger millet. The roots of Japanese barnyard millet and Job’s tears exhibited a high oxygen diffusivity constitutively in by aerenchyma formation and

higher root cortex area to stele area compared to common millet and finger millet. Moreover, they showed shortening the main root axis while developing lateral roots. Consequently, Japanese barnyard millet and Job's tears offer valuable insights for root development to adapt to hypoxic condition, potentially improving the waterlogging tolerance of terrestrial crops.

CHAPTER 4

Comparison of plant internal oxygen pathway between *Coix lacryma-jobi* and *Sorghum bicolor*

IV-1. Introduction

The efficient supply of oxygen to the roots involves considering the characteristics of the above-ground parts as an oxygen supply source and the roots as a sink (Armstrong, 1980; Tamaru et al., 2023). A meta-analysis integrating 129 studies on tissue oxygen concentrations in plants demonstrated longitudinal oxygen diffusion from the leaves to the roots (Herzog et al., 2023). Additionally, evidence from noninvasive methods using N-isotopes showed leaf-to-root gas diffusion in partially submerged rice plants (Yin et al., 2021). Thus, the oxygen transport among the root, stem, and leaf organs is becoming clearer, but still only a limited number of these tissue oxygen dynamics have been elucidated in a single study. In particular, studies of oxygen dynamics in oxygen diffusion pathways under waterlogging conditions are limited, and tissue oxygen concentration profiles remain at the forefront. Concerning the significant contribution of the above-ground parts as an oxygen source, photosynthetically derived oxygen accumulates in the above-ground portions of submerged plants and serves as an oxygen source to the roots (Koch et al., 2022; Pedersen et al., 2006; Sand-Jensen et al., 2005). However, it remains unknown whether the oxygen transport properties of the leaves and stems contribute to interspecific differences in oxygen concentrations in waterlogged plants in which the above-ground parts are exposed to an atmosphere with a lower gas diffusion resistance than water. Although some studies suggest that differences in the above-ground parts' porosity can affect gas diffusion to roots (Arikado et al., 1990; Evans & Carvalho-Evans, 2021), no tissue oxygen data have been reported to discuss the extent of this effect under waterlogged conditions. Therefore, this study aims to elucidate the differences in the oxygen diffusion gradient from shoots to roots between hypoxia-tolerant and -sensitive species under waterlogging conditions. This investigation could offer valuable insights for enhancing plant internal aeration in the design of waterlogging-tolerant varieties.

Micro-oxygen electrodes are a powerful tool to address this issue, combining high resolution, rapid response, and signal stability (Pedersen et al., 2020; Revsbech, 1989). By ascertaining the distance at which the sensor is inserted by the manipulator, it is possible to measure the tissue oxygen concentration by matching the position of the tissue with the oxygen concentration (Aguilar et al., 2003; Colmer et al., 2020; Lin et al., 2021). In this study, we test job's tears (*Coix lacryma-jobi* var. ma-yuen; cv. Riogrande de sul) as a waterlogging-tolerant species and sorghum (*Sorghum bicolor*; cv. High grain sorghum) as a waterlogging-sensitive species. Our endeavor is noteworthy as it introduces these two plant species, Job's tears and sorghum, which have not been previously examined, to the existing collection of 112 species studied for tissue oxygen concentrations (Herzog et al., 2023). Tissue oxygen measurements have never been performed on these two species, but based on the stability of growth and transpiration under waterlogging in these two species (Kono et al., 1987, 1988), we hypothesize that tissue oxygen levels will be higher at least in the root tip of Job's tears than in that of sorghum. It is worth highlighting that Job's tears are considered the most closely related crop to sorghum (H. Liu et al., 2020). The waterlogging-tolerant and humidity-induced disease-resistant traits observed in the *Coix* family, to which Job's tears belong, are anticipated to contribute to the improvement of resistance breeding in maize and sorghum (Guo et al., 2020; Zhai et al., 2023).

IV-2. Materials and Methods

2.1. Plant material and sample preparation

Seeds from each species were sterilized using a 0.5% sodium hypochlorite solution for 30 min, followed by rinsing with distilled water. The sterilized seeds were then placed in Petri dishes with distilled water and incubated for three days at 31 °C. The germinated seeds were subsequently transferred to a 10 L tank filled with a hydroponic solution (Hyponica, Kyowa, Japan; contents were as shown in Tada et al., (2014); pH was adjusted to 5.5-5.7 after dilution) diluted 500 times and floated for seven days at 27 °C. Light and dark cycles were set at 12 h each, with a photosynthetic active

radiation of $>400 \mu\text{mol m}^{-2} \text{s}^{-1}$ on the leaves during the daytime. The light source was a white light-emitting diode (SMD-50W, Gentos, Japan). Following this, the plants underwent a 12-day hypoxic acclimation process. Each of the three plants was grown in a 0.1% nutrient agar solution that was flushed with N_2 gas until the dissolved oxygen concentration was lower than 1.0 mg/L. This 0.1% agar solution is a useful material to mimic the gas convection process under waterlogged soils (Wiengweera et al., 1997). The nutrient and atmospheric conditions were the same as those after germination. The 0.1% nutrient agar solution was renewed once on the 7th day after treatment. Plants on the 13th day of hypoxia acclimation were used for the subsequent O_2 measurement.

2.2. O_2 micro profiles in in root and shoot tissues

To profile the oxygen status in root tissue (30 mm from the root tip and 30 mm from the root base), a microsensor with a 25 μm tip diameter (OX25, Unisense A/S, Denmark) was utilized. For measurements in shoot tissue (root shoot junction, stem, and leaf in the atmosphere), a microsensor with a 100- μm tip diameter (OX100, Unisense A/S, Denmark) was employed. Each sensor was connected to a picoampere meter (UniAmp Single Channel; Unisense A/S, Denmark) and polarized overnight one day prior to each measurement. An intact plant was secured in a 2 L tank filled with a 0.1% agar solution. For each measurement, the stems were submerged in the agar solution for 1 to 2 cm. A light emitting diode, serving as the light source, was installed for each measurement, providing photosynthetic active radiation on the leaf tip ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$), leaf base (200 to $210 \mu\text{mol m}^{-2} \text{s}^{-1}$), and stem (120 to $150 \mu\text{mol m}^{-2} \text{s}^{-1}$). The sensor calibrated following the method by Jiménez et al. (2021) was mounted on a micromanipulator (MP-2, Narishige Group, Japan). For submerged parts (30 mm from root tip, 30 mm from root base, root shoot junction), the sensor was carefully inserted into the agar solution, with the sensor tip monitored by an endoscope until it reached the tissue surface. For the aerial parts (stem and leaf), the sensor was carefully positioned to reach the tissue surface, guided by visual observation. Measurements were taken at intervals of 125 μm for over 30 sec, and

sensor signals were collected with data acquisition software (SENSORTRACE SUITE v.3.4.400; Unisense A/S, Denmark). The dissolved oxygen concentration in the agar solution was measured before the measurements, and if it exceeded 1.0 mg/L, N₂ gas was flushed out of the plant to maintain the concentration below 1.0 mg/L. Measurements on the root were taken at 27.5°C, while those on the shoot were taken at 26.0°C. Since the units of the oxygen concentration values obtained were in μmol/L, they were converted to kPa using the following equation.

$$pO_2 = 100 \frac{10^{-6}[O_2]}{K(T)}$$

where pO₂ is the partial oxygen pressure. [O₂] is the measured value (μmol/L) using the micro-oxygen sensor. K(T) is Henry's constant at temperature T. In this study, 0.00128 mol/(kg·bar) (27.5 °C) and 0.00123 mol/(kg·bar) (26.0 °C) were used as the Henry's constant values to calculate the partial pressure of the oxygen dissolved in water. To convert bar to kPa, we multiplied the values by 100.

2.3. Root dissection and measurements of root anatomical traits

Following the O₂ measurements, the roots that were measured were immediately cut from the shoot and stored at 4 °C. Cross-sections of the roots were obtained at 30 mm from the tip, at the halfway point between the two O₂ measurements, 30 mm from the root base, and within 10 mm from the root base. These sections were prepared manually using a razor blade and were immediately placed on glass slides. Each section was photographed using a microscope (MP38T, As one, Japan), equipped with a microscope camera (PCM500, As one, Japan). The outlines of the tissues (the whole area of the cross-section, the internal area of the epidermis, and the stele area) in the cross-sectional images were traced by freehand selection, and their areas were quantified using the ImageJ software (v.1.53e; National Institutes of Health, USA). The root cross-section cortex area to stele area ratio (CSR) was calculated by dividing the cortex area (calculated by subtracting the stele area from the internal area of the epidermis) by the stele area.

2.4. Statistical analysis

The Shapiro–Wilk normality test, F-test, Student's t-test, and Welch's t-test were conducted using EZR software (v.1.61), which was programmed by Kanda (2013) and is based on R software (v.4.2.2). Some datasets on root section area and root CSR of position between job's tears and sorghum included unequal variance. Therefore, Welch's t-test was used for these parameters instead of the Student's t-test.

IV-3. Results

3. 1. The pO₂ profile in root and shoot

The pO₂ in the root was continuously measured in each sample until a steep O₂ decrease was observed in the root stele. The highest peak of pO₂ (9.3 kPa in the root tip of Job's tears; 3.5 kPa in the root tip of sorghum) was found in the root cortex layer, while in the stele, pO₂ (6.4 kPa in the root tip of Job's tears; 1.5 kPa in the root tip of sorghum) decreased in each root (Figure 4-1a, b, f, g, respectively). The lowest pO₂ values were collected as stele pO₂ for statistical comparison. Additionally, Job's tears showed a steep pO₂ decrease across the root cortex to the root epidermis (41% lower in the epidermis relative to the cortex layer) at 30 mm from the root base (Figure 4-1b). Even at the root base, sorghum pO₂ in the cortex and stele was lower than that of Job's tears (62.4% lower in the cortex and 76.5% lower in the stele; Figure 4-1b, g, respectively). Therefore, we conducted a pO₂ investigation of the above-ground parts using plants of the same age.

The results of the root shoot junction profile showed that the highest pO₂ peaks (13.4 kPa in Job's tears; 8.4 kPa in sorghum) were present in more internal tissues than on the tissue surface (7.0 kPa in Job's tears and 4.9 kPa in Sorghum; Figure 4-1c, h, respectively). The highest peak of pO₂ in the root shoot junction tended to be higher in Job's tears than in sorghum (Figure 4-1c, h). However, the maximum pO₂ in the root shoot junction of sorghum was 14.7 kPa, which was not inferior when compared to that of Job's tears. The highest pO₂ values of the shoot and leaves were clearly higher

than those of the agar-submerged parts (Figure 4-1d, e, i, j, respectively). The gap in pO_2 between the species was quite narrow on the leaf and stem exposed to the atmosphere (11.0 % lower in the stem and 19% lower in the leaf) compared to the root.

The highest peak of pO_2 in the root cortical layer and root shoot junction was used for statistical comparison as the pO_2 in the intercellular space. To exclude direct atmospheric involvement, the highest peaks of pO_2 of the leaf and stem inside the point where the pO_2 decreased were used for statistical comparison as the intercellular space.

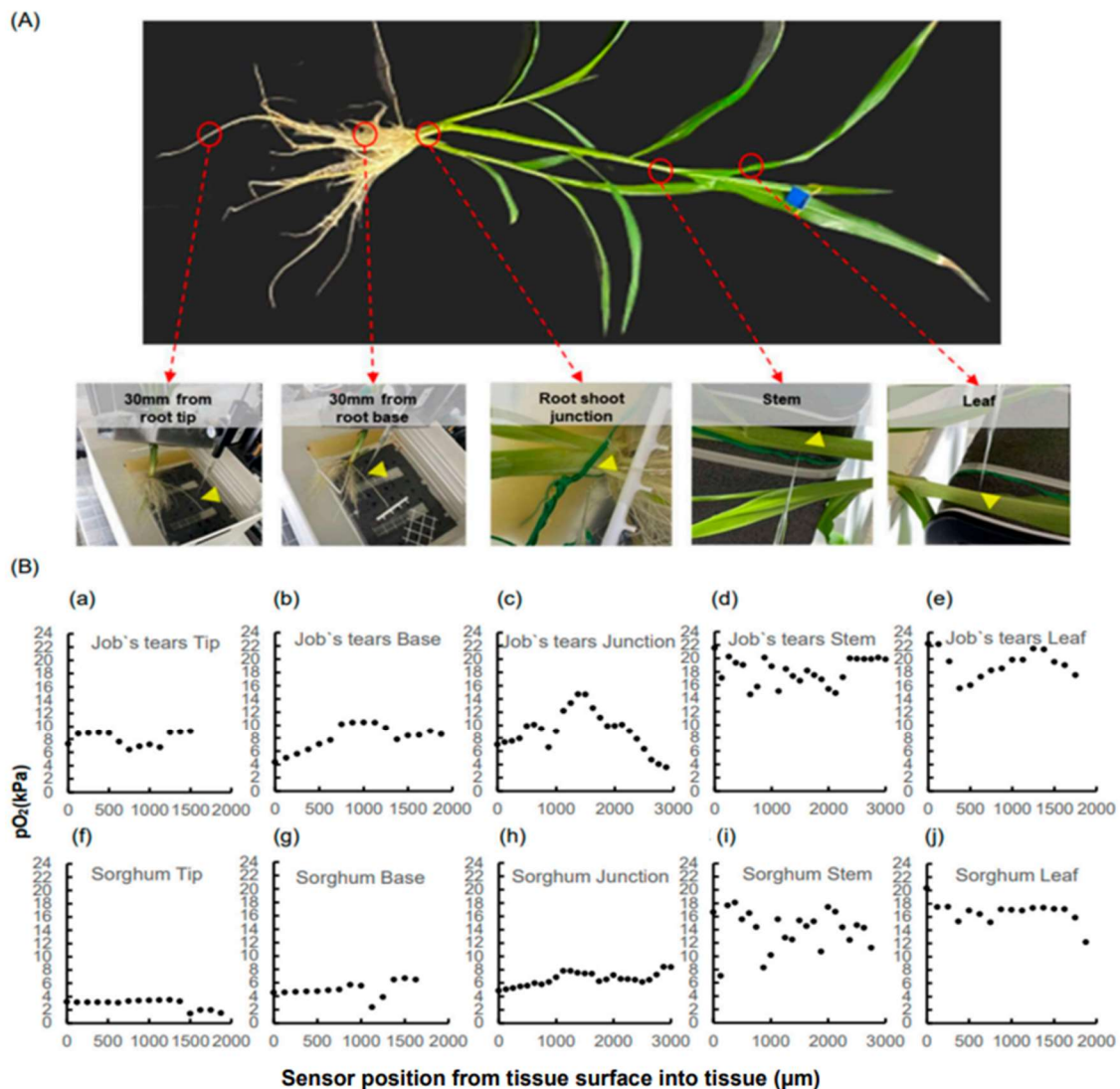


Fig. 4-1. Example pictures of data collection locations (A) and data (B) illustrating pO_2 profiles within the root structures of Job's tears (a–e) and sorghum (f–j). The positive distances were measured at the root tip (a, f), root base (b, g), root shoot junction (c, h), stem (d, i), and leaf (e, j). The yellow arrows in the pictures indicate the points where the sensor was inserted. These points represent the median of the data collected >30 s. In this context, “tip” refers to precisely 30 mm from the root tip, “base” indicates precisely 30 mm from the root base, and “junction” means precisely within 10 mm from the root base on the root shoot junction. Profiles on the leaf were conducted in the youngest expanded leaf. Profiles on the stem were conducted near the auricle of the youngest expanded leaf.

2.2. Statistical comparison for the pO_2 of inter-cellular space, root stele, gradient from cortex to epidermis

The pO_2 levels in the intercellular space tended to decrease in the following order: from the root

shoot junction to the root base, and root tip for both species compared to the stem and leaf exposed to the atmosphere (Figure 4-2A). The intercellular pO₂ of sorghum was significantly lower than that of Job's tears at the root base and near the tips, by 6.1 kPa and 4.0 kPa, respectively (Figure 4-2A). In terms of stele pO₂, it was significantly lower, at 83%, at the root tip of sorghum and 68% lower at the root base, compared to the corresponding positions in Job's tears (Figure 4-2C). To evaluate the ability to retain oxygen by preventing its diffusion from the root cortex to the epidermis, we calculated the pO₂ difference between the intercellular space and the lowest pO₂ around the root epidermis (within 250 μm). The statistical comparisons of these differences revealed that Job's tears had a significantly higher pO₂ difference near the root base than sorghum (Figure 4-2C).

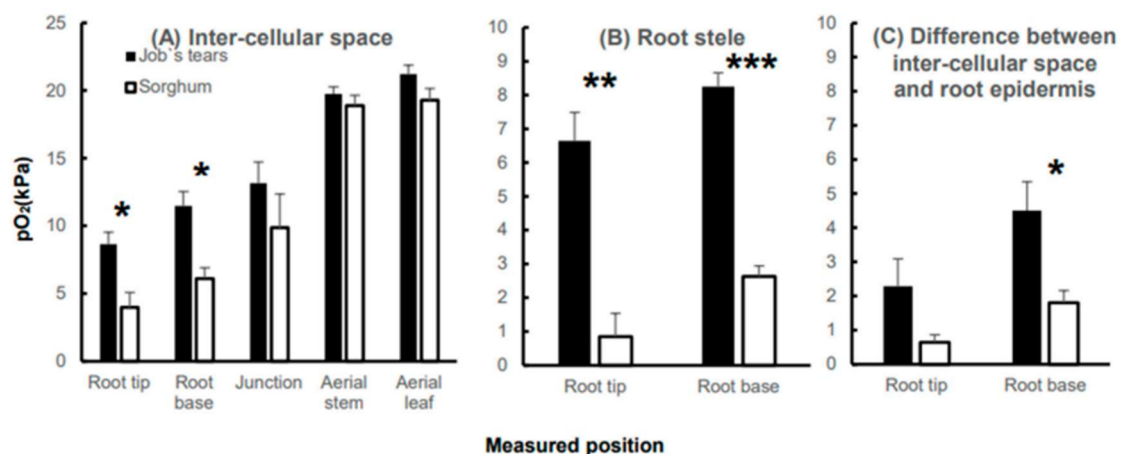


Fig. 4-2. Statistical comparisons of the pO₂ levels in different tissues of Job's tears and sorghum. (A) depicts the pO₂ in the intercellular space. (B) illustrates the pO₂ in the root stele. (C) presents the pO₂ difference between the intercellular space and the root epidermis. The bars represent the standard error (S.E.). Significant differences in pO₂ between Job's tears and sorghum were assessed using a Student's *t*-test and denoted by asterisks: * 0.01 ≤ *p* < 0.05; ** 0.001 ≤ *p* < 0.01; and *** *p* < 0.001.

2.3. Root cross-section area and cortex area to stele area ratio

To explore the plant factors that influenced root pO₂, we examined the pertinent characteristics through the dissection of the roots whose O₂ level was profiled. The root aerenchyma was well identified in both species, especially from 30 mm from the root base to near the midpoint of the root, but was rarely identified within 10 mm of the base (Figure 4-3A). The root section area tended to be

higher in Job's tears than in sorghum within 10 mm from the root base ($p = 0.09$), with a significant difference observed at 30 mm from the root base (Figure 4-3B). Additionally, the root CSR was significantly higher in Job's tears compared to sorghum at every sampled point (Figure 4-3C).

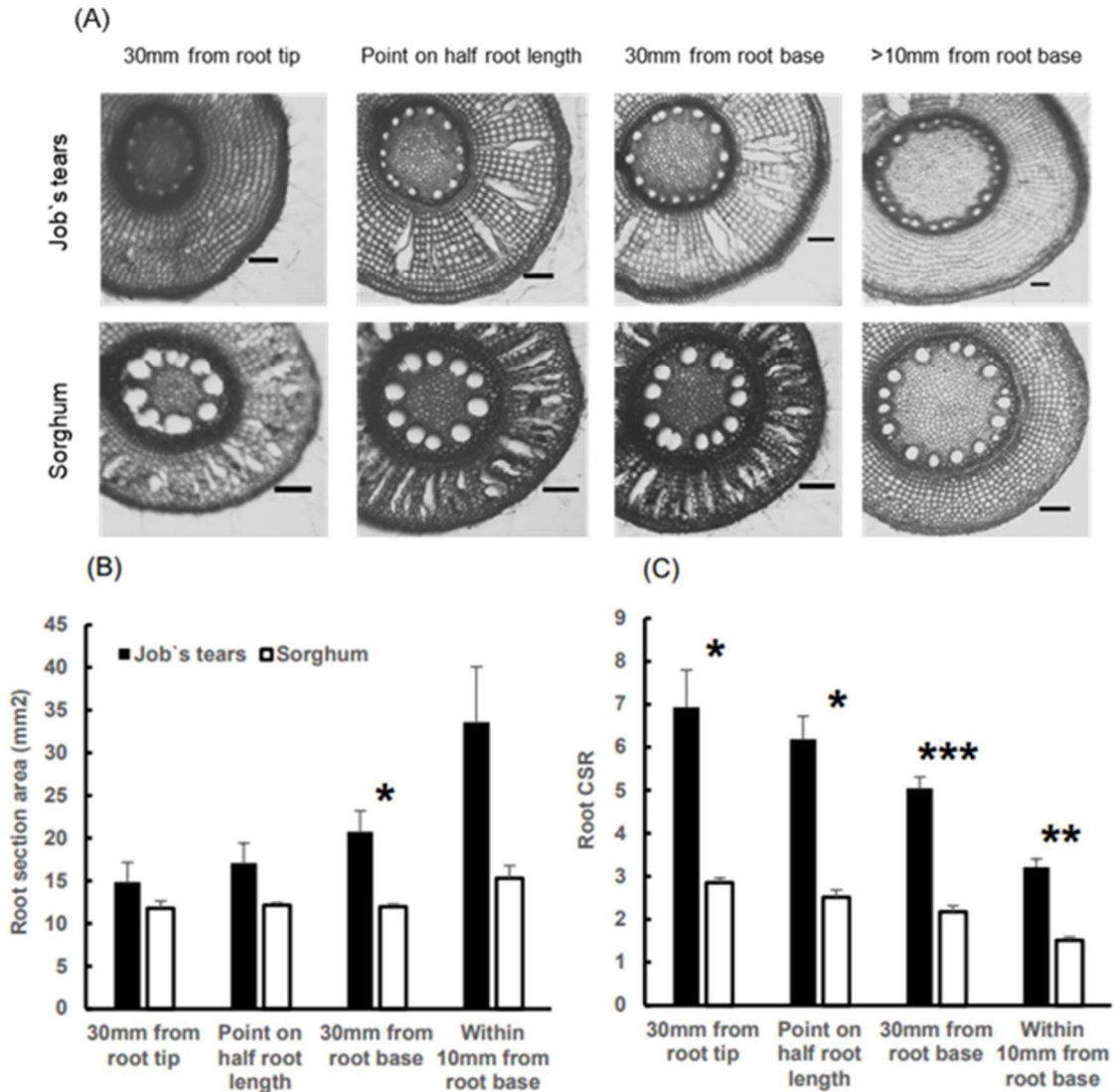


Fig. 4-3. Examples of root cross-section (A) and statistical comparisons of the root cross-section area (B) and the root cortex area to the stele area ratio (C) in the O₂-profiled roots of Job's tears and sorghum. The black bars in A represent 500 μm. The bars in B and C indicate the standard error (S.E.). The significance between Job's tears and sorghum was assessed using Welch's *t*-test and denoted by asterisks: * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$; and *** $p < 0.001$.

IV-3. Discussion

In this study, we analyzed the tissue oxygen profiles from the leaves and stems to root tips for two plant species that differ in waterlogging tolerance. Initially, we explored the interspecific differences in pO_2 values in the leaves and stems exposed to the atmosphere. When inserting the sensor into the stem, we observed points of extreme decrease in pO_2 within the inner part of the stem (Figure 4-1d, j), possibly due to gas diffusion blocking the effect of the cuticle (Møller & Sand-Jensen, 2008). There was no significant difference between species in the value of the high peak, assumed to indicate a supply of oxygen to the surrounding tissues, and it was not significantly higher than the pO_2 exposed to the atmosphere (Figure 4-2A). Contrary to observations in submerged plants (Koch et al., 2022; Pedersen et al., 2006; Sand-Jensen et al., 2005), the high oxygen accumulation and its contribution to roots were not observed under the waterlogged conditions in this study. As discussed by Sou et al., the oxygen produced by photosynthesis did not contribute to the aeration of adventitious roots in the water (Sou et al., 2020). Additionally, the comparison of tissue oxygen concentrations in light and dark conditions revealed that the differences in the oxygen concentrations in the leaves, clear in submerged and partially submerged conditions, were no longer significant in waterlogging and drained conditions (Pellegrini et al., 2017). In summary, photosynthetically produced oxygen from leaves and stems in the atmosphere contributes harder to the roots under waterlogged conditions than under submergence. However, these studies, including our current study, were conducted indoors, and a more rigorous investigation of whether oxygen levels of tissues exposed to the atmosphere affect the submerged portion of the plant will need to be further tested in the field in the future to account for the effects of intense light and atmospheric conditions, as was conducted in field trials on submerged rice plants (Winkel et al., 2013).

In this study, stem submergence in the agar solution mimicking waterlogged soil was 1–2 cm, yet the oxygen partial pressure decreased by 6.6 kPa (S.E. 1.9) in Job's tears and 9.0 kPa (S.E. 1.7) in

sorghum as the oxygen diffused from the stem into the root shoot junction (Figure 4-2A). Although interspecific differences at the root shoot junction were not significant, the differences were more pronounced than in the atmospheric stem and leaf (Figure 4-2A). The tested samples used in this study formed little aerenchyma within 10 mm of the root base, but interestingly, oxygen was transported at the root sites beyond that point. Although oxygen diffusion resistance is expected to be higher than in tissues with well-formed aerenchyma, oxygen transport through intercellular spaces is considered to occur even in such tissues (Yoshida & Eguchi, 1994). Considering the pO_2 of the aerial stem at 100%, 44% of the oxygen was diffused to the cortex near the root tip and 33% to the stele of Job's tears, while in sorghum, only 21% of the oxygen reached the cortex near the root tip and a mere 4% the stele (Figure 4-2A,B). Insufficient oxygen reaching the root tips, the central metabolic site of the root, implies severe limitations to root activity (Veen, 1981; Wiengweera & Greenway, 2004). Root stele hypoxia and anoxia severely restrict the loading of essential ions to the xylem (Colmer & Greenway, 2011; Kotula et al., 2015; Shabala et al., 2014). When plants encounter hypoxia due to waterlogging, they adapt by switching metabolic systems early in the stress period, but morphological changes are essential for adaptation to long-term stress (Drew, 1997). In susceptible field crops, like wheat, roots that develop after encountering hypoxic stress are more adapted to hypoxia than roots that develop before the stress encounter (Yamauchi et al., 2014). In our study, sorghum exposed to 12 days of hypoxia exhibited a fundamental difference in oxygen transportation compared to Job's tears. This underscores the need to address weaknesses in plant tissues exhibiting differences in oxygen concentration in field crops such as sorghum. Consequently, we will continue our study of root properties affecting oxygen diffusion longitudinally and radially.

The pO_2 difference between the two species increased significantly as the oxygen diffused from the root shoot junction to the root base (Fig. 4-2A). Oxygen retainability at the root base of Job's tears was markedly higher than that of sorghum (Fig. 4-2C), suggesting the presence of a ROL barrier-like

function in job's tears roots. A similar pattern of tissue oxygen concentrations with a decrease in epidermal oxygen concentrations in the cortical layer has been reported in *Glyceria maxima*, which suppresses oxygen leakage from the roots (Soukup et al., 2007). The ROL barrier serves to prevent oxygen loss from the root base, enabling extended oxygen transport (Colmer, 2003b). The ROL barrier has never been tested in Job's tears. The ROL barrier has been termed the outer apoplastic barrier in a recent study and has multiple functions (Peralta Ogorek et al., 2023). Future studies should examine the extent to which Job's tears has any of these functions. Moreover, the advantage of thicker and higher CSR roots in oxygen transport, as noted by Yamauchi et al., (Yamauchi, Abe, et al., 2019), is consistent with the characteristics of job's tear roots in this study (Fig. 4-3B, C). The differences in longitudinal oxygen diffusion, influenced by high aeration and oxygen retention, contribute to variations in pO₂ in the intercellular space at the root tips. Additionally, the formation of a larger cortical layer is crucial for facilitating sufficient oxygen diffusion to the stele (Armstrong & Beckett, 1987). The CSR of Job's tears exceeded that of sorghum from the root base to the root tips, resulting in a radial difference in oxygen penetration into the stele. The chromosomal region controlling the inducible ROL barrier has already been identified (Watanabe et al., 2017), and there is a possibility of further QTL studies into the constitutive ROL barrier since the discovery of the wild rice that forms it (Ejiri et al., 2020). In contrast, CSR regulation is a more recently discovered phenomenon than the ROL barrier, and its genetic domain has not yet been fully elucidated (Pedersen, Sauter, et al., 2021; Yamauchi, Pedersen, et al., 2021).

Utilizing the spatial measurements of tissue oxygen partial pressure, this study unveiled where differences in oxygen permeability occur in closely related species, sorghum and Job's tears, under hypoxic agar-mimicked waterlogged soil conditions. However, this does not imply the absence of tolerant species with clearer differences in oxygen transport capacity at the root shoot junction. For instance, compared to previous studies, Nicaragua teosinte (14.0 kPa) exhibited a higher oxygen

partial pressure at the root base (Pedersen, Nakayama, et al., 2021) than the root base of Job's tears (11.5 kPa) in this study. It is conceivable that the above-ground aerating capacity is similar or even higher in such plants. In addition, although the present study compared stress-acclimated plants, waterlogging-sensitive crops do not have constitutive aerenchyma, a trait present in adapted species (Gong et al., 2019; Matsuura et al., 2022), and therefore, tissue oxygen differences may be even greater at the early stages of stress. How hypoxia is sensed and morphological adaptation occurs are still matters of debate for hypoxic-sensitive plants (Armstrong et al., 2019), and therefore, a follow-up comparison from the early stages of stress to stress acclimation should be investigated in future studies. At present, certain traits beneficial for adaptation to waterlogged environments are associated with trade-offs in adaptation to drought environments (Striker et al., 2007; Yamauchi, Noshita, et al., 2021). Therefore, traits with low adaptive costs that provide sufficient oxygen permeability for adaptation to target flooding environments need to be incorporated into field crops. The proper evaluation of such characteristics requires future research linking oxygen levels to traits in various crops.

IV-5. Conclusion

From the longitudinal and radial comparisons of oxygen partial pressure from the leaves to the root tips, notable differences in the pO_2 gradient emerged, particularly at the root shoot junction and root base longitudinally, as well as in the root cortex and root stele radially between Job's tears (cv. Riogrande de sul) and sorghum (cv. High-grain sorghum). The oxygen retainability of the root and thicker and higher CSR roots contributed to longitudinal oxygen diffusion in Job's tears. The high CSR allowed for the higher radial oxygen diffusion to the stele of Job's tears.

CHAPTER 5

General discussion

Present research has included 3 trials which aimed to clarify the question “How cereal crop transport the sufficient oxygen to root tip under waterlogging”. All trials were conducted on hypoxic agar solution to mimic gas conviction under waterlogged soil. Therefore, detailed analysis of the physiological and morphological parameters to the roots were common to each of the studies. Characteristics related to the plant internal aeration are a central topic in this study entitled Physiological Analysis of Hypoxic Responses. This chapter summarizes some of the characteristics that have been focused on in previous waterlogging stress studies with the results of this study, and discusses the mechanisms that provide sufficient oxygen to the roots and adapt to waterlogged environments. In addition, plants are expected to be exposed to severe flooding in some seasons, followed by long droughts, as proposed at the ISPLORE conference in Bamberg, Germany (2022), and there is a growing focus on studying the impact of increased flood and drought tolerance on crops, not only individually but also in tandem (Fagerstedt et al., 2023). Therefore, for each item, the minimum thresholds required for waterlogging adaptation and future research to understand them are discussed.

Root aerenchyma under waterlogging

Aerenchyma is air space which connect from leaf to root (Arikado et al., 1990; Kludze et al., 1993; Yin et al., 2021). This is most important component for plant internal aeration. However, there was no trend in either study for oxygen diffusion to increase as the proportion of root occupied by aerenchyma increased. Referring to studies dealing with 91 plant species, root elongation under waterlogging has a steep positive relationship in the low porosity range, but the relationship becomes

more moderate as porosity increases (Justin & Armstrong, 1987). In Chapters 3 and 4 in present study, we compared cereals with clear differences in waterlogging tolerance, but when we compared plants exposed to hypoxia, we found no clear interspecific differences in the percentage of aerenchyma formation. In addition, In Chapter 2, interestingly, the rice variety Sensho had about half the root porosity of the other varieties, but showed an oxygen transportation to root comparable to the other rice varieties. However, in both studies, the aerenchyma formation exhibited by the waterlogging tolerant species under the aeration hydroponic solution was higher than that of the waterlogging sensitive species. This would indicate, in other words, that aerenchyma is a necessary but not sufficient condition for adequate aeration to the roots. The formation of aerenchyma creates a tradeoff that compensates adaptation costs under continuous waterlogging (Pan et al., 2022) but leads to reduced nutrient water absorption under variable moisture conditions (Yamauchi, Noshita, et al., 2021). Aerenchyma formation also reduces the physical strength of the roots of non-wetland plants (Striker et al., 2007). The amount of plant internal aeration under waterlogging is difficult because it involves not only the aerenchyma but also several other characteristics described below. However, meta-analysis should be used to elucidate the minimum quantitative threshold of aerenchyma required for adaptation to waterlogging as future perspective. And the important thing that this study shows about aerenchyma is that poaceae crops adapted to waterlogging form constitutive aerenchyma, so that such threshold can be accessed quickly depending on the oxygen situation around the roots.

Root cortex and stele under waterlogging

The cortical layer and the stele of the root have different oxygen demand (Aguilar et al., 2003; Colmer et al., 2020). This was also evident from the oxygen profiles of the roots in Chapter 4. Cell density (Herzog et al., 2023) and suberization of endodermis (Lin et al., 2021) are considered to be responsible for this difference in oxygen partial pressure between tissues. The process of nutrient absorption associated with transpiration may also be involved in the variation in oxygen demand of

the stele, since the ratio of aboveground to belowground parts strongly influence oxygen consumption from Chapter 2 (Tamaru et al., 2023).

Root cortex and stele area ratio (CSR) is a quantitative characteristic whose importance to waterlogging adaptation has been recognized in recent studies (Pedersen, Sauter, et al., 2021; Yamauchi, Abe, et al., 2019). In this study, we accumulated knowledge about this root characteristic in each trial. When comparing between plant species of similar age, higher CSRs in resistant species and lower CSRs in susceptible species were common in Chapters 2 and 3, strongly supporting previous studies. Rice varieties in Chapter 1 also showed higher CSRs compared to common millet, finger millet, and sorghum. On the other hand, there was considerable variation in CSR within the highly tolerant species, which, again, indicates that root aeration is a result of multiple trait control. Such inter-specific variation in CSR within resistant species has been reported in recent years, particularly in rice (Ejiri & Shiono, 2023; Tong et al., 2023). The root CSR variation was also observed among plants of different ages within the same species e.g. rice (cv Black Gora, Koshihikari, Sensho in chapter2) and job's tears (cv Riogrande de sul in chapters3 and 4).

While the large root cortex layer ensures root aeration for wetland plants, there is a tradeoff in adaptation to drought because water is consumed in the cortex layer while it is transported from the root epidermis to the stele. In fact, plants in arid areas have a CSR below 4 (Yamauchi et al., 2021). Therefore, designing the roots of such plants as CSR over 10 like wetland plants risks inducing a trade-off. To increase adaptability to waterlogged environments while retaining adaptability to aerobic environments, it is important to focus on low-land plants with low CSRs, such as *Oryza grumaepatula* (CSR 6.9, Ejiri & Shiono, 2023) and *Oryza granulate* (CSR 4.7 (Tong et al., 2023), to identify the minimum CSRs to guarantee aeration and to elucidate the mechanism of how sufficient oxygen diffusion is ensured with such roots.

Oxygen retainability under waterlogging

The ROL barrier is a barrier that not only allows long-distance oxygen transport at the roots, but also protects the roots from toxic substances such as reduced minerals and soil phytotoxin (Colmer, 2003b). Because of this multifunctionality, the latest research (Peralta et al., 2023) proposes to call this barrier “Outer apoplastic barriers”, and this name will be used henceforth. There are interspecific differences in the composition and barrier performance of the outer apoplastic barrier, even among the wetland plants that strongly form it (Soukup et al., 2007). Therefore, characterization is done to confirm barrier properties and composition as well as oxygen measurements. In this study, we found that oxygen diffusion outward from the root is inhibited at the root base of job’s tears in Chapter 4. However, future studies are needed to characterize outer apoplastic barrier of job’s tears.

Outer apoplastic barrier trade-offs are complex and unexplored because it involves not only the degree of root suberization but also selective transport by transporters (Kamiya et al., 2015; Peralta Ogorek et al., 2023). However, it is also being found to have an effect on root water retention and is being recognized as an important factor in adaptation to diverse environmental stresses (Peralta Ogorek et al., 2021). Therefore, the impact of introducing outer apoplastic barriers to field crops is unknown but will be a requirement for designing crops that maintain root oxygen permeability while maintaining adaptability to resilient environment.

Root architecture under waterlogging

In the treatment of hypoxia to the roots in Chapters 3 and 4, even the tolerant species showed a response that resulted in shorter apparent root length. Detailed root length analysis in Chapter 3 revealed that this is a root length control that maintains total root length under hypoxia, a response that form longer lateral roots and shorter main roots. Nevertheless, we cannot fully elucidate the functional implications of significant lateral root development under hypoxic conditions. For instance, lateral root development is important in maintaining nutrient and water absorption under drought (Kano et

al., 2011) and high osmotic conditions (Toyofuku et al., 2015). Attempts have been made to leverage lateral root development for breeding purposes (Hasegawa et al., 2022; Kawai et al., 2022). Thus, understanding the significance of lateral root development under hypoxic conditions is essential. Although the lack of actual measurements of oxygen data in this study limits definitive conclusions, we inferred three hypotheses regarding the function of lateral root development by combining previous findings shown in following sentences. The first hypothesis proposes that similar to responses observed under other stresses, more roots need to form under hypoxic conditions to maintain optimal nutrient absorption efficiency. Studies investigating root oxygen availability and oxygen consumption, including those focusing on wetland plants, have shown that most plants cannot supply 100% of the required oxygen relative to their oxygen consumption (Maricle & Lee, 2007; Suralta & Yamauchi, 2008; Tamaru et al., 2023). Even in wetland plant species, root activity is maintained under hypoxic conditions, with some root tissue engaging in anaerobic metabolism. Consistently, in our study, we observed that in Japanese barnyard millet, an increase in total root length resulted in an increase in lateral root length. Furthermore, those plants that maintained their roots exhibited a higher shoot dry weight S/A. The second hypothesis proposes that one aspect of root system formation involves reducing the distance between each root tip of the main and lateral roots and the oxygen source. Allocating more energy to the lateral roots while shortening the length of each main root could cause the tips of both lateral and main roots to be positioned closer to the root shoot junction, depending on the location of lateral root formation. To investigate this hypothesis, comprehensive studies that collect oxygen data at various root tips, including the number and position of lateral root formations, are required. The third hypothesis proposes that promoting lateral root formation and shortening the main root, thereby concentrating lateral roots in shallow layers, can enhance soil aeration and nutrient absorption. Shallow rooting is advantageous for wetland adaptation (Fan et al., 2017; Mano & Omori, 2007). This adaptation involves changes in root angle (Eysholdt-Derzso & Sauter, 2017; Haque et al.,

2012; Yamauchi et al., 1987) and the development of soil surface roots extending into flooded water (Gomathi et al., 2015; Hanzawa et al., 2013; Miyashita & Shiono, 2023) to escape hypoxic soil conditions. Our focus on main root shortening and the promotion of lateral root formation may contribute to the development of shallow rooting. The dense distribution of lateral roots in shallow layers increases the likelihood of close contact between roots. Studies visualizing oxygen leakage from roots in the soil (Frederiksen & Glud, 2006; Koop-Jakobsen et al., 2018; Koop-Jakobsen & Wenzhöfer, 2015) have suggested that root oxygen release likely has an impact within approximately 3 mm from the roots. Research has demonstrated that planting rice and soybean roots close to each other can alleviate the waterlogging stress for soybeans in terms of gas exchange and survival rates (Iijima et al., 2022). This implies oxygen transfer between roots with varying oxygen availability. Additionally, it is hypothesized that roots support each other through ROL based on differences in ROL barrier formation between roots of different ages within individual plants (Ejiri et al., 2021). The development of lateral roots, known as oxygen leakage sites, within a more limited range than under aerobic conditions may contribute to creating an oxygen network. This network could serve as a strategy to support the survival of diverse roots within and outside the root system under hypoxic conditions. If there is an external aeration pathway as well as internal aeration of single roots, which was the focus of this study, it should be a characteristic that widens the gap between waterlogging tolerant and susceptible species and should be clarified in the future.

Sufficient oxygen supply to maintain root activity under waterlogging

Oyanagi questions the history of research linking quantitative comparisons of aerenchyma to waterlogging tolerance among wheat varieties (Oyanagi, 2011). While quantitative comparisons of wetland plants and field crops are often made between them in terms of characteristics (e.g. Aerenchyma; Justin & Armstrong, 1987 CSR; Yamauchi, Pedersen, et al., 2021 outer apoplastic barrier; McDonald et al., 2002 Root distribution; Khanthavong et al., 2021) related to waterlogging

tolerance, there has been little oxygen based discussion of that is a product of them. This is probably due to the fact that the main interest of researchers who measure oxygen in plants is wetland plants (Herzog et al., 2023; Pedersen et al., 2020). In this study, we attempted to use two oxygen measurement methods to provide an oxygen-based discussion of the differences in oxygen transport capacity across waterlogging tolerant and sensitive species.

First, in Chapter 2, compensation of O₂ consumption by O₂ transport (CCT) was calculated from the difference in dissolved oxygen concentration in the root soaked hydroponic solution when the roots and aboveground parts were intact and when they were excised. This value was used to estimate what percentage of oxygen the plant was able to provide to its roots to compensate for aerobic respiration. Among rice varieties, the CCT values ranged considerably from 49.1% - 108.6 %, but there were clear differences when compared to data (maize: 8.3%, oat: 0% calculated from fig.2 and fig.3 in Maricle & Lee, 2007) for land crops measured by the same method. In other words, because the oxygen permeability of rice, the model crop in the waterlogging tolerance study, differs significantly from that of field crops, a root design to fill this gap could create tradeoffs to the existing field environment. Therefore, we compared millets that have not been studied for their hypoxia response in Chapter 3 and selected job's tears, which has adaptations that can be said to be closer between terrestrial and wetland, avoiding hypoxia-induced stress on leaves but decreasing shoot dry weight by 18% - 31%.

In chapter4, we conducted tissue O₂ sensing by micro needle sensor. Unlike the method described above, this method can reveal local oxygen concentrations, so how does oxygen diffuse through the plant? How are the O₂ status differences, especially near the root tips where the demand for oxygen is high due to root activity? This was a powerful tool that helped us to answer questions such as. The interspecific differences in oxygen partial pressure (job's tears inter-cellular space: 8.6kPa, Stele: 6.6kPa; Sorghum inter-cellular space: 3.9kPa, Stele: 0.8kPa) near root tip revealed by these results are likely to be the differences in oxygen permeability that need to be filled in crop waterlogging tolerance

breeding. While this study mainly aimed to provide knowledge that could serve as a basis for application in enhancing waterlogging tolerance of field crops, methane gas emitted from paddy crops such as rice is also considered a problem, and attempts are being made to mitigate this by enhancing the oxidative capacity of the roots (Jiménez & Pedersen, 2023). More than 30 years after the development of the stable micro oxygen electrode (Revsbech, 1989), knowledge of tissue O₂ in plants still lags far behind that of mammalian studies (Herzog et al., 2023), and cell-level oxygen sensing is still a frontier, especially in non-model plants (Dalle Carbonare et al., 2023). In addition, there has been a remarkable introduction of technologies that allow nondestructive measurement of oxygen, such as planar optodes (Shiono et al., 2022), which allow environmental information to be obtained in a flat plane, and the application of positron emission tomography, which is used in cancer research, to plant science (Herzog et al., 2023; Mincke et al., 2021; Schmidt et al., 2018). Research on linking oxygen and crops will become more and more standard in order to create crops that are adaptable to flood environments.

CHAPTER 6

General Conclusions

Hypoxia condition induced by 0.1 % agar solution to mimic gas conviction under waterlogged soil served detailed poaceae root analysis. Rice, Japanese barnyard millet, and job`s tears avoided hypoxic stress by forming a constitutive aerenchyma and higher CSR root compared to common millet, finger millet, and sorghum. Japanese barnyard millet and job`s tears were considered to distribute oxygen well by changing the distribution of root length under hypoxia, forming shorter main roots and longer lateral roots. Finally, a comparison was made between the plant internal aeration of job`s tears, a moderate hypoxia tolerant species, and sorghum, a susceptible species, and oxygen-based suggestions for differences that should be filled in order for the susceptible species to become more tolerant.

This study provides knowledge of significance to the plant anaerobiosis research field in that it reveals the hypoxic response of rice as well as non-model crops such as job`s tears and Japanese barnyard millet, and furthermore, it reveals differences in the oxygen diffusion pathway between job`s tears and sorghum.

CHAPTER 7

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