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CHANGES IN MORPHOLOGICAL TRAITS UNDER HYPOXIC CONDITIONS IN BROCCOLI CULTIVARS WITH DIFFERENT WATERLOGGING TOLERANCE

Ryo HARA, Yutaka JITSUYAMA*, Takashi SUZUKI
Graduate School of Agriculture, Hokkaido University, Japan

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ABSTRACT

In our previous study, sensitive broccoli cultivars exhibited adverse effects on head yield after just 3 days of waterlogging. Mature plants of sensitive cultivars show severely reduced water content under waterlogging stress. This phenomenon suggests a strong correlation between impaired water uptake under waterlogged conditions and broccoli tolerance to such conditions. In this study, the relationship between these factors was assessed by focusing on the root system, using a primarily morphological approach in three characteristic cultivars, and comparing changes in biomass, water content, root length, internal root structure, and hydraulic conductivity under hypoxic conditions (AIR−). Hypoxia caused by hydroponic cultivation without aeration reduced shoot biomass and substantially increased the shoot-to-root dry weight ratio and leaf area-to-root dry weight ratio in the sensitive cultivars ‘Sawayutaka’ and ‘First Star’. The interaction between aeration and cultivar affected internal root structure but, not root length, radial barriers to oxygen loss, or lignin and suberin deposition. Hypoxia reduced xylem area in the roots of the sensitive cultivars but not in the tolerant ‘Shigemori’. Moreover, the tolerant cultivar maintained high hydraulic conductivity under hypoxic conditions. These results suggest that maintaining root xylem size and hydraulic conductivity, even under hypoxic conditions, may enhance broccoli tolerance to waterlogging.

Key words: aeration, hydraulic conductivity, hypoxic response, root system, root histology

INTRODUCTION

The frequency and severity of floods worldwide have recently increased, severely affecting agriculture (Bailey-Serres et al. 2012). Therefore, breeding of waterlogging-resistant cultivars and research on phenotypes or genes protecting against waterlogging have intensified, with particular emphasis on valuable food crops (Mano et al. 2005; Bailey-Serres et al. 2012; Jitsuyama 2015; Van Nguyen et al. 2017; Pedersen et al. 2021). Especially in Japan, rainfall is expected to increase in midsummer; making it necessary to develop mitigation strategies to prevent waterlogging damage. Broccoli (*Brassica oleracea* var. *italica*) is particularly sensitive to flooding (Tsukazawa et al. 2009; Higashio et al. 2012; Hara et al. 2023). In 2016, Hokkaido was hit by three typhoons, which caused severe damage to broccoli crops in Ebetsu, one of the main broccoli production centers in Hokkaido (Takada, unpublished data). Floods have also affected broccoli crops overseas, in places such as Queensland, Australia, in 2013–2014 (ALIC 2017) and Zhejiang China, in 2015 (ALIC 2019).

Flooding is a common cause of hypoxic or anoxic conditions during plant growth. Soil flooding and waterlogging initially affect roots, which respond to hypoxia by altering their structure, gene expression, metabolism, and internal morphology, including the formation of aerenchyma and barriers

*Corresponding author
e-mail: y-jitsu@agr.hokudai.ac.jp

to radial oxygen loss and improved hydraulic conductivity (Abiko et al. 2012; Sauter 2013; Voesenek & Bailey-Serres 2015; Jitsuyama 2017). Major changes in root systems under hypoxic conditions include alterations in root lateral arrangement and slant (Daniel & Hartman 2024), root diameter (Yamauchi et al. 2014), ratio of cross-sectional area between cortex and stele (Yamauchi et al. 2019), and xylem size (Pang et al. 2004). A large number of hypoxia-regulated genes in plants encode enzymes involved in anaerobic metabolism (Daniel & Hartman 2024), such as alcohol dehydrogenase and lactate dehydrogenase (Kato-Noguchi et al. 1999), and these genes are commonly (though not always) regulated by ethylene (Loreti et al. 2016). Under hypoxic conditions, ethylene-responsive factor genes participate in the transcription of hypoxia-responsive genes (Mustroph et al. 2010) and regulate root behavior induced by polar auxin transport (Eysholdt-Derzsó & Sauter 2017).

Under hypoxic conditions, plants alter gene expression and undergo metabolic adaptations while allocating certain resources to maintain photosynthetic performance, such as efficient gas exchange and ease of water uptake. Aerenchyma formed in adventitious roots (Abiko et al. 2012) and cortical cell arrangement (Yamauchi et al. 2019) may positively affect gas exchange under hypoxic conditions. Induction of a barrier to radial oxygen loss (ROL) in adventitious roots also results in more efficient oxygen transport from the shoot base to the root tip (Abiko et al. 2012). Root hydraulic conductivity, which affects the ease of water uptake, increased under hypoxic conditions in waterlogging-tolerant soybean cultivars (Jitsuyama 2017).

Previous studies on broccoli tolerance to waterlogging have focused on the effects of soil waterlogging on biomass and yield (Hara et al. 2023) and on proteomic analyses (Lin et al. 2015). This study analyzed morphological changes in broccoli roots in response to hypoxia. This study aimed to investigate the complex effects of hypoxia on the phenotypes of three characteristic broccoli cultivars during the initial growth phase.

MATERIALS AND METHODS

Plant materials and hydroponic conditions

The experiment was conducted in spring (Experiment 1: March 20 – April 23) and autumn (Experiment 2: October 1 – November 10) of 2021 in a glass greenhouse at the Experimental Farm of the Field Science Centre for the Northern Biosphere, Hokkaido University, Sapporo, Hokkaido, Japan (43°04′13″ N, 141°20′22″ E). Three broccoli cultivars were tested: ‘Shigemori’ (Vilmorin Mikado, Chiba, Japan), ‘Sawayutaka’ (Sakata Seed, Kanagawa, Japan), and ‘First Star’ (Asahi Agria, Tokyo, Japan). According to the results of the previous study (Hara et al. 2023), ‘Shigemori’ was used in this experiment as a waterlogging-tolerant cultivar, whereas ‘Sawayutaka’ and ‘First Star’ were used as waterlogging-sensitive cultivars.

From late March to mid-April, seedlings were germinated and grown to three true leaves in 128-cell trays filled with potting soil (nursery soil, Takii, Kyoto, Japan) with a base fertilizer containing 320 $mg \cdot L^{-1}$ N, 210 $mg \cdot L^{-1}$ P_2O_5, and 300 $mg \cdot L^{-1}$ K_2O. Broccoli seedlings were then transplanted to a hydroponic system equipped with 1/2000a Wagner pots (2-550-52, AS ONE Corporation). A 5-mm-thick circular polystyrene board with five or seven holes was placed in each Wagner pot filled with a hydroponic nutrient solution (diluted 500 times with Hyponica solution, Kyowa, Osaka, Japan, containing 80 $mg \cdot L^{-1}$ nitrate, 76 $mg \cdot L^{-1}$ phosphate, and 178 $mg \cdot L^{-1}$ potassium). Five plants for Experiment 1 and seven plants for Experiment 2 were then planted in each pot, with one plant serving as a replicate. Hydroponic nutrient solution was added daily during aeration (Fig. 1). Experiment 2 was designed to analyze root hydraulic conductivity under the same hydroponic conditions.

Aeration treatments and environmental measurements

Two treatments were used to simulate different oxygen conditions in the rhizosphere: AIR− without air supply to the nutrient solution by a pump, and

AIR+ (control) with aeration of the nutrient solution by means of 1.5 $L \cdot min^{-1}$ air supply by a pump (GEX E-Air 4000WB, JEX, China). Treatments began after plants were transplanted into the hydroponic system, and the treatment period was 8 days. Hydroponic solutions were monitored by measuring air temperature, oxygen concentration, temperature, pH, and electrical conductivity (EC). Oxygen concentration, water temperature, pH, and EC were measured daily for each treatment between 9:00 and 10:00 AM. The mean greenhouse air temperature during Experiments 1 and 2was 23.3 °C. The temperature of the hydroponic nutrient solution was maintained at 21–22 °C in both treatments.

Morphological and histological analysis of shoot and root biomass

Shoot and root biomass was measured in fresh material and after drying at 80 °C for 72 h using a forced-air dryer. The water content of the shoots was determined based on the difference between the fresh samples and the dry biomass. Before drying for weighing, the roots were scanned with an Epson Expression 1640XL scanner (Seiko Epson, Tokyo, Japan), and their morphological characteristics were recorded as image data. Root length was analyzed for each diameter (classification in 0.1 mm increments over a range of 0–1 mm) using WinRHIZO software (Regent Instruments, QC, Canada). Root diameter ranges were classified based on the significance of the treatment-by-cultivar interaction.

Root lengths of the different treatments were compared using classifications: 0.0–0.1 for fine roots, 0.1–0.5 for medium roots, and 0.5–0.9 mm for thick roots. Root elongation from the initial state was also analyzed before treatment. The leaf area of each plant and biomass traits were simultaneously evaluated using WinRHIZO. The shoot-to-root ratio, based on dry weight, and the leaf area-to-root weight ratio were calculated. The shoot-to-root ratio, based on dry weight, and the leaf area-to-root weight ratio, expressing leaf area per unit of root dry weight, were calculated. Root length was the sum of all root lengths, and root weight was the dry weight of all root biomass per plant.

Newly developed roots that emerged near the root base during the aeration treatment were selected for analysis, and cross-sectional root anatomy was observed. After scanning, three roots (categorized as thick) were randomly selected and collected from each plant, each approximately 5 cm long and emerging near the root base. The collected roots were immediately immersed in 50% ethanol and refrigerated at 4 °C until observation.

Root cross-sections of 35-μm thickness were prepared using a microslicer (DKT-1000, Dosaka EM, Kyoto, Japan) from approximately 1 cm from the base of each root. These sections were subsequently stained for lignin with 1% (w/v) phloroglucinol in 25% HCl (phloroglucinol–HCl reaction) (Jensen 1962) and for suberin with 0.01% (w/v) Fluorol Yellow 088 solution (Brundrett et al. 1991) to assess the integrity of the ROL barrier. Additionally, the Casparian strip was examined to distinguish cortex from stele. All images were obtained with an optical microscope (ECLIPSE Ti, Nikon, Tokyo, Japan), and suberin was particularly visualized as yellow-greenish fluorescence excited by ultraviolet light with a cubic filter (DAPI, Nikon). The areas of cortex, stele, and xylem, as well as their ratios (cortex-to-stele and xylem-to-stele), were calculated using image integration software (NIS-Elements BR, Nikon, Tokyo, Japan).

Spatial patterns of radial oxygen loss (ROL) from the root system in an oxygen-free medium

The formation of a barrier to ROL in root systems was evaluated using methylene blue staining in a deoxygenated medium. Methylene blue, a redox indicator dye, enables the quantitative assessment of spatial ROL profiles from root systems (Watanabe et al. 2017). The reduced form of methylene blue is colorless, whereas the oxidized form is blue. Methylene blue was added at 13 $mg \cdot L^{-1}$ to deoxygenated medium containing 0.1% (w/v) agar and sodium dithionite ($Na_2S_2O_4$) was added at 1.3 $mg \cdot L^{-1}$ to reduce the methylene blue to a colorless solution. The seedlings described above were then treated in AIR− or AIR+ for 8 days, transferred to methylene blue medium in an acrylic box, and maintained with

the root–shoot junction 30 mm below the surface of the solution. The box was kept at room temperature (~25 °C) and illuminated from above and below with white light-emitting diodes. After one hour, methylene blue staining patterns were observed and recorded around the stems and roots of five plants from each sample.

Root hydraulic conductivity

Samples from Experiment 2 were used to analyze root hydraulic conductivity (Lp_r), which was measured in a pressure chamber according to Jitsuyama (2017) using a specially modified pressure chamber (Pump-up chamber; PMS Instrument Company, OR, USA). Initially, hypocotyls of broccoli plants, which had been cut just below the cotyledons, were tightly fixed to the top lid of the pressure chamber, and the roots were immersed in pure water in a vinyl bag inside the chamber (Fig. 1). The internal pressure of the chamber was gradually increased by 0.1 MPa to 0.4 MPa using an air compressor (AM02-04N; Fujiwara Sangyo, Hyogo, Japan), and the sap exuding from the hypocotyl was captured using a piece of pre-weighed cotton wool at 60 s intervals. The wool was then weighed on an electric balance (VIC212; ACCULAB Sartorius AG, Göttingen, Germany) at each pressure. Additionally, the total root length and root surface area were analyzed using WinRHIZO. Lp_r was calculated from the slope of the time–sap flow curve, and the values were expressed as root hydraulic conductivity per root surface area and pressure ($\mu L \cdot m^{-2} \cdot s^{-1} \cdot MPa^{-1}$).

Statistical analysis

A split-plot experimental design was implemented with five replicates (or seven replicates for root hydraulic conductivity). Main plots and subplots were defined based on two hypoxia treatments (AIR+ and AIR−) and three cultivars (‘Shigemori’, ‘Sawayutaka’, and ‘First Star’). Analysis of variance (ANOVA) significance was calculated as described by Little and Hills (1978). Other statistical analyses, including Student’s *t*-test and Tukey–Kramer test, were performed using Statcel4, a Microsoft Excel 2019 add-in (OMS Publishing, Saitama, Japan).

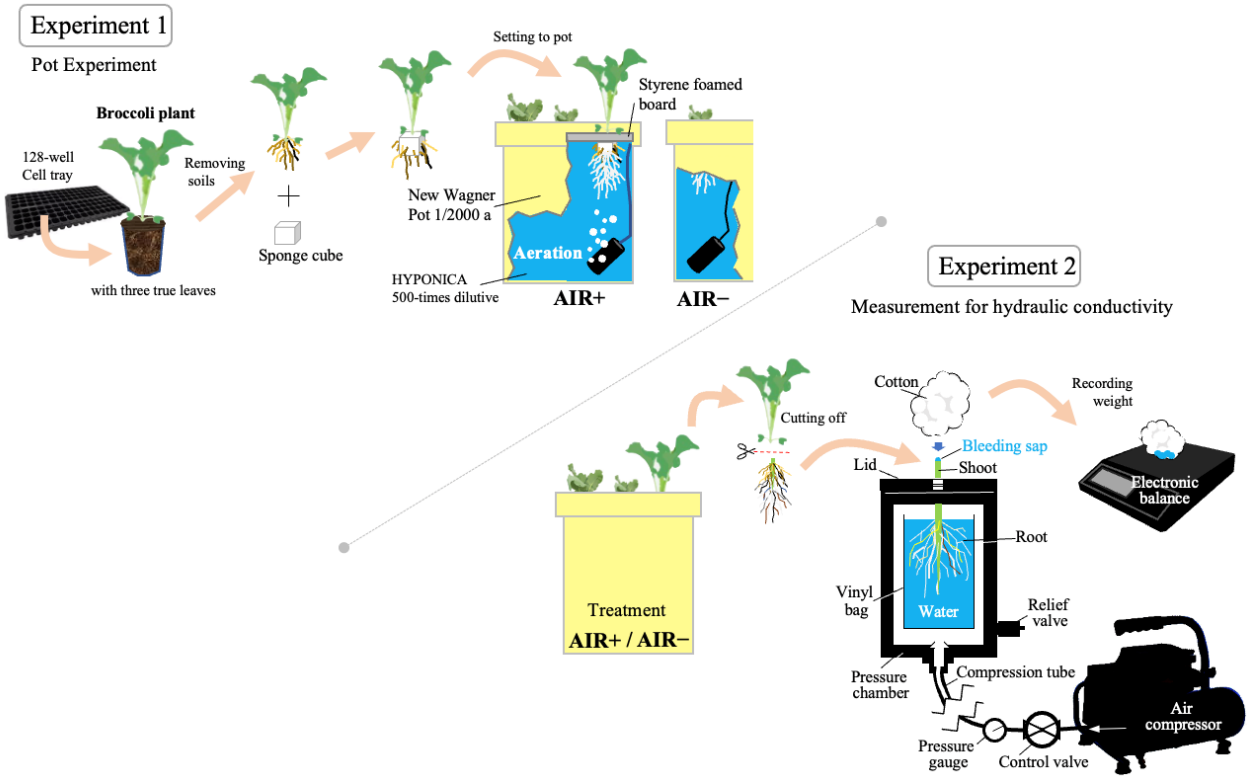

Figure 1. Experimental diagram analyzing the effect of hypoxia on broccoli seedlings

RESULTS

Influence of hypoxia on the characteristics of the medium

Rhizosphere hypoxia treatment drastically altered the dissolved oxygen concentration of the solution. In AIR+, the oxygen concentration was 8.44 mg·L^{-1} and in AIR− 3.42 mg·L^{-1} ($p < 0.05$), decreasing to approximately 1.6 mg·L^{-1} in AIR−. Electrical conductivity (EC) in AIR− was higher than in AIR+ (1.39 mS·cm^{-1} vs. 1.37 mS·cm^{-1}, $p < 0.05$), and the difference occurred after day 6 of treatment. The pH of AIR− was lower than that of AIR+ during the experiment (6.74 vs. 7.58, $p < 0.05$).

Plant biomass

Shoot and root dry weight, shoot water content, root dry weight, shoot-to-root ratio, and leaf area-to-root ratio were compared between the two aeration treatments and the three cultivars (Table 1). Aeration increased shoot water content and root dry weight, while decreasing stem dry weight and shoot-to-root dry weight ratio ($p < 0.001$). The ratio of leaf area to root dry weight was not affected. Plant appearance was also monitored visually, indicating that the AIR− treatment induced shoot wilting symptoms from mid-morning to noon (Fig. 2). Cultivar affected only stem dry weight ($p < 0.05$), with 'Shigemori' having the strongest effect.

The interaction between aeration and cultivar was statistically significant for stem dry weight (DW), shoot-to-root DW ratio, and leaf area-to-root DW ratio (Fig. 3). Stem DW of 'Shigemori' ($p < 0.05$) and 'Sawayutaka' ($p < 0.001$) increased more in AIR− than in AIR+. The increased magnitude of the ratio resulting from the AIR− treatment varied among cultivars. The shoot-to-root ratio of 'Sawayutaka' and 'First Star' increased more drastically than that of 'Shigemori', and the leaf area-to-root ratios of 'Sawayutaka' and 'First Star' were significantly increased, but not in 'Shigemori' (Fig. 3).

Root length

Total root length per plant and root length at three thickness levels were compared between the two aeration treatments and the three cultivars (Table 2). Aeration drastically increased total root length and root length at each thickness level ($p < 0.001$). In the case of thick root length, the effect of cultivar was significant ($p < 0.05$), but no significant differences between cultivars were found. Treatment–cultivar interactions were observed for fine roots (0–0.1 mm, $p < 0.01$) and thick roots (0.5–0.9 mm, $p < 0.05$). The decrease in root length resulting from the AIR treatment was nearly identical for all cultivars, except for fine roots, where an elongation was observed for 'Shigemori' (Fig. 4).

Radial oxygen loss (ROL) evaluation

The pattern of oxygen leakage from lower stems and roots, i.e., ROL, was observed at the whole root level in methylene blue agar media and compared between the two aeration treatments and the three cultivars (Fig. 5). Blue-stained stems were detected in all samples (Fig. 5B–G). Root tips and junctions of the main and lateral roots of the root system, as well as the whole root surfaces, were stained in both aeration treatments and in all cultivars (Fig. 5H–N).

Table 1. Effect of aeration and cultivar on water content, stem and root dry weight (DW), shoot-to-root dry weight (DW) ratio, and leaf area-to-root dry weight (DW) ratio of broccoli seedlings under hypoxic conditions

Source		Shoot water content (%)	Stem DW (mg)	Root DW (mg)	Shoot-to-root DW ratio	Leaf area-to-root DW ratio
Aeration (A)	AIR+	91.0 ± 0.2 a	99.7 ± 3.4 b	75.7 ± 2.4 a	11.3 ± 0.3 b	2.7 ± 0.1
	AIR−	87.1 ± 0.3 b	117.4 ± 4.1 a	38.9 ± 1.8 b	17.6 ± 0.6 a	3.0 ± 1.8
Cultivar (C)	'Shigemori'	88.8 ± 0.8	120.0 ± 3.6 a	60.0 ± 5.4	14.2 ± 0.7	2.9 ± 0.1
	'Sawayutaka'	88.9 ± 0.6	108.5 ± 6.3 ab	53.2 ± 7.0	15.1 ± 1.6	2.9 ± 0.1
	'First Star'	89.6 ± 0.6	97.1 ± 3.1 b	58.7 ± 7.2	14.0 ± 1.1	2.7 ± 0.1
ANOVA	df	MS	MS	MS	MS	MS
A	1	111.6***	2347.2**	10163.4***	292.9***	0.6 ns
C	2	1.9*	1308.7***	130.2 ns	3.3 ns	0.1 ns
A × C	2	1.0 ns	520.2*	142.1 ns	19.9**	1.0***

***, **, * indicate significance at the 0.1%, 1%, and 5% levels, respectively, ns – not significant; Each value represents the mean (± standard error – SE); Different letters within each column indicate significant differences at the 5% level according to the Student's *t*-test (Aeration: n = 15) and Tukey–Kramer test (Cultivar: n = 10); ANOVA was performed using a split-plot design (n = 5); DW – dry weight; control – AIR+; hypoxia treatment – AIR−; df – degree of freedom; MS – mean square

Table 2. Effect of aeration on total root length and root length with three diameter ranks of broccoli seedlings under hypoxic conditions

Source		Total root length (cm)	Fine root length (cm)	Medium root length (cm)	Thick root length (cm)
	Before aeration	971.3 ± 60.6 c	331.4 ± 22.0 c	571.7 ± 34.1 c	45.6 ± 4.3 c
Aeration (A)	AIR+	3350.0 ± 84.0 a	745.0 ± 19.2 a	2041.4 ± 49.1 a	456.8 ± 24.7 a
	AIR−	1730.3 ± 47.9 b	397.5 ± 14.3 b	1103.6 ± 34.6 b	188.4 ± 4.4 b
Cultivar (C)	'Shigemori'	2075.2 ± 330.7	516.0 ± 69.1	1258.2 ± 190.9	243.5 ± 60.6
	'Sawayutaka'	1991.3 ± 279.2	494.5 ± 51.0	1239.8 ± 181.3	202.9 ± 41.6
	'First Star'	1985.1 ± 330.5	463.5 ± 51.4	1218.7 ± 188.3	244.4 ± 55.6
ANOVA	df	MS	MS	MS	MS
A	2	22144549.2***	740661***	8306662***	653826***
C	2	37935 ns	10452 †	5872 ns	8418*
A × C	4	119451 ns	16938**	38080 ns	8532*

***, **, *, † indicate significance at the 0.1%, 1%, 5%, and 10% levels, respectively, ns – not significant; Each value represents the mean (± standard error – SE); Different letters within each column indicate significant differences at the 5% level according to Tukey–Kramer test (Aeration: n = 15, Cultivar: n = 15); ANOVA was performed using a split-plot design (n = 5); control – AIR+; hypoxia treatment – AIR−; df – degree of freedom; MS – mean square

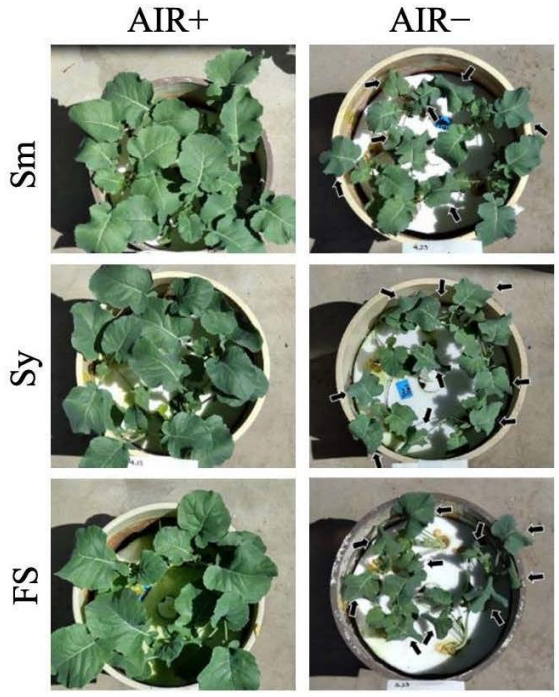

Figure 2. Effect of aeration on three broccoli seedlings grown hydroponically under hypoxic conditions
Arrows indicate wilted leaves; Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'

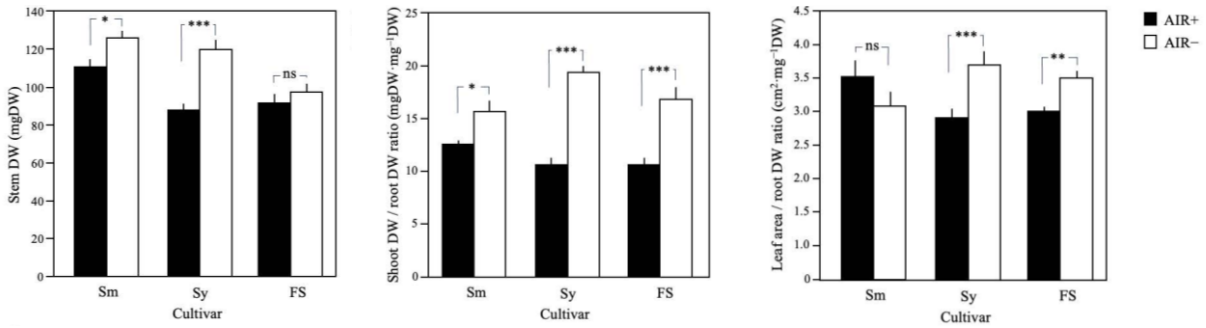

Figure 3. Effect of aeration on stem dry weight (DW), shoot-to-root DW ratio, and leaf area-to-root DW ratio in three broccoli cultivars under hypoxic conditions
***, **, * indicate significant differences between the two treatments at the 0.1%, 1%, and 5% levels, respectively, according to the *t*-test (n = 5); ns – not significant; Vertical bars indicate standard errors; Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'

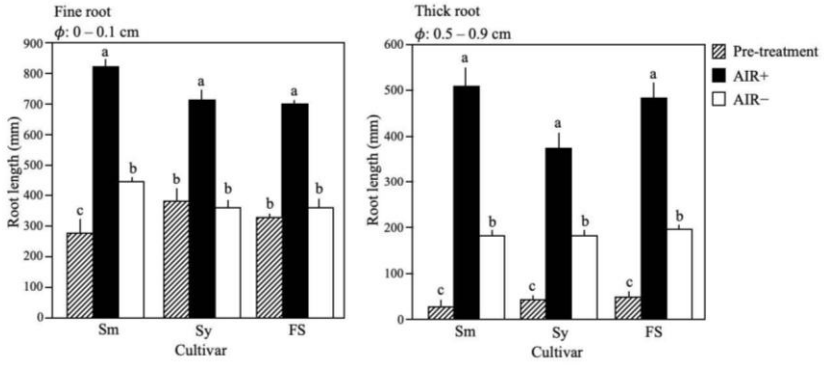

Figure 4. Effect of aeration on the length of roots of different thicknesses in three broccoli cultivars under hypoxic conditions
Different letters within each bar indicate significant differences at the 5% level according to the Tukey–Kramer test (n = 5); Vertical bars indicate standard errors; Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'

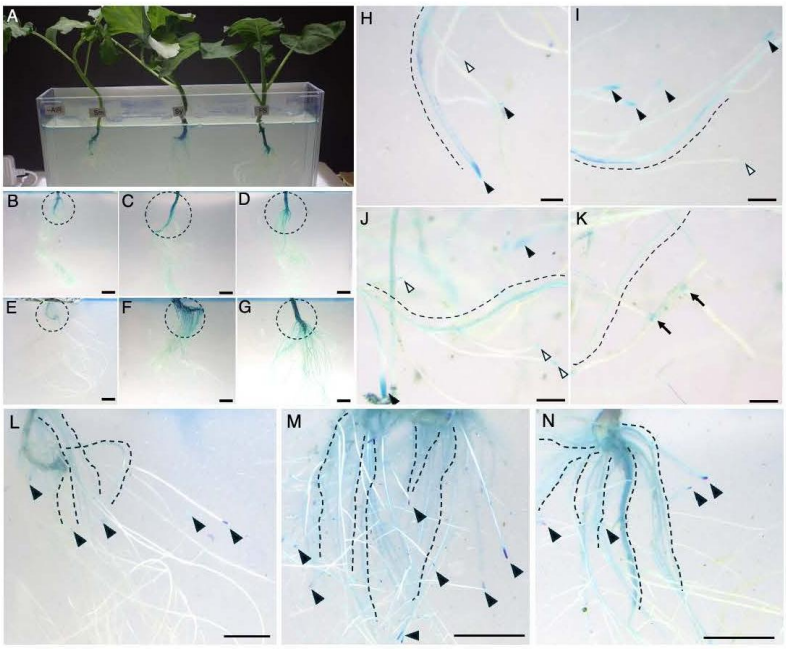

Figure 5. Oxygen leakage pattern from stems and roots visualized using methylene blue in three broccoli cultivars after two aeration treatments (AIR+, AIR−): colorimetric methylene blue method in a transparent box (A), root profiles in AIR+ (B–D) and in AIR− (E–G), stems strongly stained blue (circles with dotted lines), scale bars indicate 1 cm, locations of oxygen loss through root surfaces of 'Shigemori' (H & J), 'First Star' (I), and 'Sawayutaka' (K); In AIR+ scale bars indicate 1 mm, locations of oxygen loss through root surfaces of 'Shigemori' (L), 'Sawayutaka' (M), and 'First Star' (N); In AIR− scale bars indicate 1 cm, main root apices (closed arrowheads), lateral root apices (open arrowheads), junctions (arrows), and whole roots (dot lines) were stained blue
There are no barriers to radial oxygen loss (ROL); Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'; control – AIR+; hypoxia treatment – AIR−

Internal root structure

In the thick root category, the total cross-sectional areas of the root, cortex, and xylem, the cortex-to-stele ratio, and the xylem-to-stele ratio were affected by aeration treatments and cultivar (Table 3). ANOVA indicated that aeration increased xylem area and xylem-to-stele ratio, while cortex area decreased. The total root cross-sectional area and the cortex-to-stele ratio were nonsignificantly lower. Cultivars affected the total cross-sectional areas of the root, cortex, and xylem. The cortex-to-stele and xylem-to-stele ratios did not differ between cultivars.

Interactions between aeration and cultivar were significant for total root, cortex, and xylem cross-sectional areas, but not for cortex-to-stele and xylem-to-stele ratios (Fig. 6). Root response characteristics were verified based on cross-sectional images (Fig. 7), which indicated that the total root and cortex cross-sectional areas of 'Shigemori' were larger in AIR− than in AIR+. Furthermore, only the xylem area of 'Shigemori' did not decrease in AIR−.The observation of lignin and suberin deposition in the internal root structure (Fig. 8), constituting the ROL barrier, did not show any clear relationships with the factors studied.

Root hydraulic conductivity

Experiment 2 provided information on root length and surface and hydraulic conductivity (Lp_r) depending on the aeration treatment and cultivar (Table 4). Aeration increased total root length ($p < 0.001$), root surface area ($p < 0.001$), and hydraulic conductivity ($p < 0.05$), but the interaction of aeration and cultivar was not significantly different. The highest conductivity was observed in 'Shigemori'. The effect of aeration was not detected in 'Shigemori' and 'Sawayutaka', while in 'First Star' aeration increased this characteristic (Fig. 9).

Table 3. Effect of aeration on root cross-sectional area, cortex-to-stele ratio, and xylem-to-stele ratio in broccoli seedlings under hypoxic conditions

Source		Total root cross-sectional area (μm^2)	Cortex area (μm^2)	Xylem area (μm^2)	Cortex-to-stele ratio	Xylem-to-stele ratio
Aeration (A)	AIR+	127126 ± 5122.2 b	78918 ± 3976 b	3476 ± 195 a	2.75 ± 0.1	0.12 ± 0.01 a
	AIR−	159668 ± 5684.0 a	105056 ± 11263 a	2545 ± 323 b	3.52 ± 0.2	0.08 ± 0.01 b
Cultivar (C)	'Shigemori'	181847 ± 8888.5 a	120215 ± 13877 a	3784 ± 302 a	3.27 ± 0.2	0.11 ± 0.01
	'Sawayutaka'	124847 ± 7825.0 b	77240 ± 5554 b	2575 ± 283 b	2.98 ± 0.24	0.10 ± 0.01
	'First Star'	123495 ± 7604.3 b	78506 ± 5523 b	2673 ± 364 ab	3.15 ± 0.24	0.10 ± 0.01
ANOVA	df	MS	MS	MS	MS	MS
A	1	7942283828**	5123629701**	6509790 †	4.419 ns	0.0108**
C	2	11092776890**	5980112959***	4509618**	0.218 ns	0.0003 ns
A × C	2	698716501**	372896954**	2743963*	0.165 ns	0.0002 ns

***, **, * indicate significance at the 0.1%, 1%, and 5% levels, respectively, ns – not significant; Each value represents the mean (± standard error – SE); Different letters within each column indicate significant differences at the 5% level according to the Student's *t*-test (Aeration: n = 15) and Tukey–Kramer test (Cultivar: n = 10); ANOVA was performed using a split-plot design (n = 5); control– AIR+; hypoxia treatment – AIR−; df – degree of freedom; MS – mean square

Table 4. Effect of aeration on total root length, root surface area, and root hydraulic conductivity (Lp_r) of broccoli seedlings under hypoxic conditions

Source		Total root length (cm)	Root surface area (cm^2)	Lp_r ($\mu L \cdot m^{-2} \cdot s^{-1} \cdot MPa^{-1}$)
Aeration (A)	AIR+	1920 ± 74 a	208.7 ± 8.9 a	108.2 ± 8.6 a
	AIR−	839 ± 58 b	82.5 ± 6.5 b	79.8 ± 8.9 b
Cultivar (C)	'Shigemori'	1188 ± 156	122.4 ± 17.2	125.0 ± 11.0 a
	'Sawayutaka'	1321 ± 156	137.5 ± 18.6	92.1 ± 8.6 b
	'First Star'	1629 ± 177	176.8 ± 21	64.9 ± 7.9 b
ANOVA	df	MS	MS	MS
A	1	12277228***	167271.9***	8473.1*
C	2	716072***	11040.1***	12688.5***
A × C	2	6091 ns	180.4 ns	623.1 ns

*** and * indicate significance at the 0.1% and 5% levels, respectively, ns – not significant; Each value represents the mean (± standard error – SE); Different letters within each column indicate significant differences at the 5% level according to the *t*-test (Aeration: n = 21) and Tukey–Kramer test (Cultivar: n = 14); ANOVA was performed using a split-plot design (n = 7); control – AIR+; hypoxia treatment – AIR−; df – degree of freedom; MS – mean square

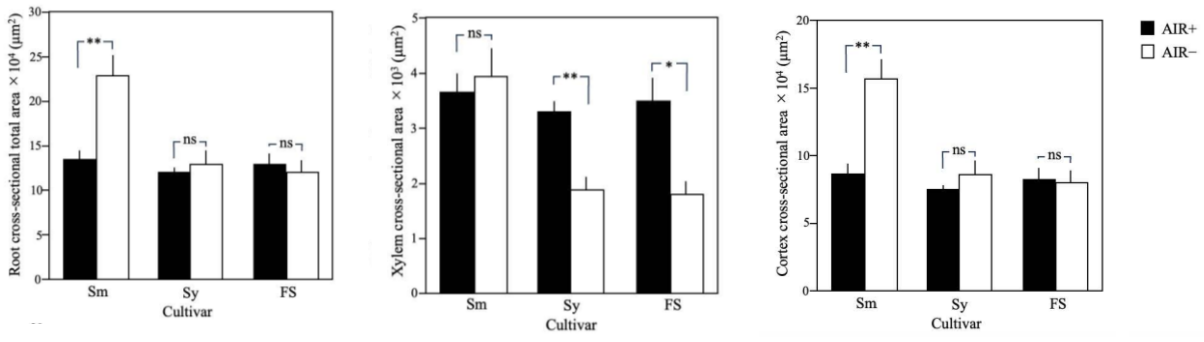

Figure 6. Effect of aeration on the total cross-sectional areas of root, xylem, and cortex of broccoli seedlings under hypoxic conditions
**, * indicate significant differences between the two treatments at the 1% and 5% levels, respectively, according to the *t*-test (n = 5), ns – not significant; Vertical bars indicate standard errors; Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'

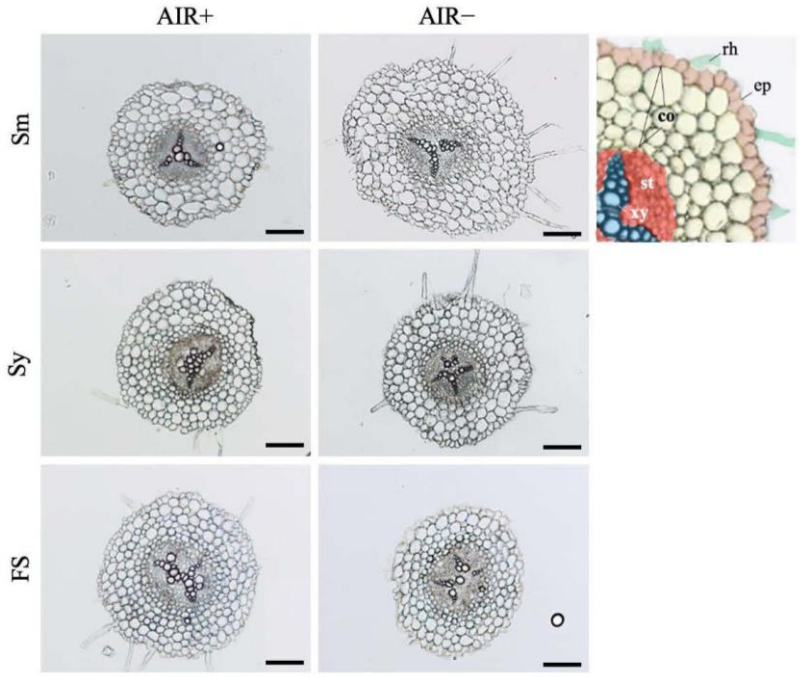

Figure 7. Microscopic images of broccoli root cross-sections
Scale bars indicate 100 µm; Sm – 'Shigemori', Sy – 'Sawayutaka', FS – 'First Star', co – cortex, ep – epidermis, rh – root hair, st – stele, xy – xylem

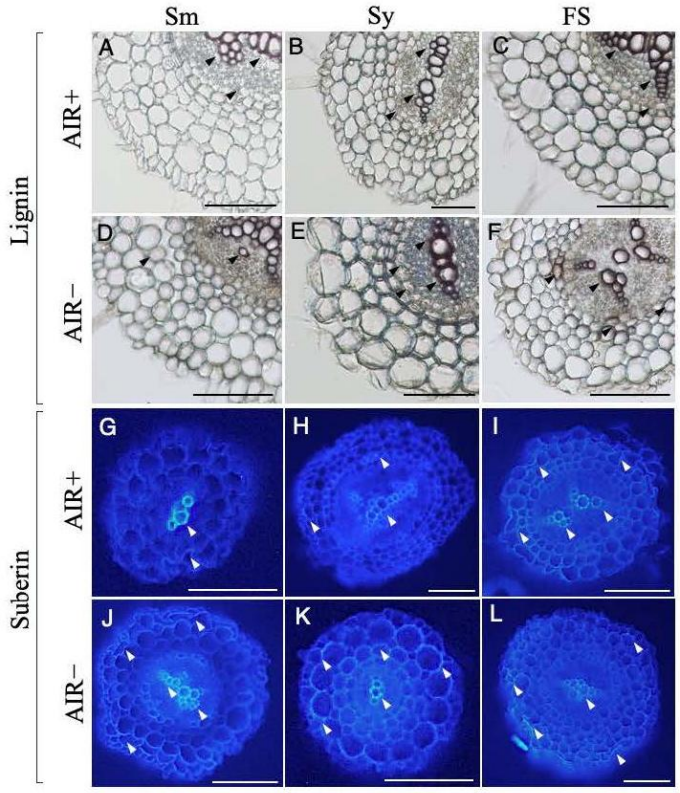

Figure 8. Effect of seedling aeration under hypoxic conditions, visible on root cross-sections, and the occurrence of lignin (A–F) and suberin (G–L), in two types of aeration treatments – AIR+ (A–C, G–I) and AIR− (D–F, J–L) of three broccoli cultivars – 'Shigemori' – Sm (A, D, G, and J), 'Sawayutaka' – Sy (B, E, H, and K), and 'First Star' – FS (C, F, I, and L)
Lignin (closed arrowheads) – red staining after phloroglucinol–HCl reaction; Suberin (open arrowheads) – yellow-greenish fluorescence (Fluorol Yellow 088); Blue fluorescence – autofluorescence; Scale bars indicate 1 mm; control – AIR+; hypoxia treatment – AIR−

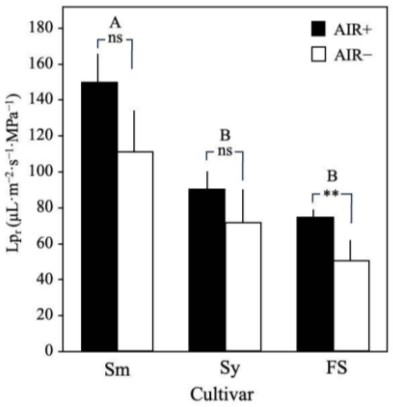

Figure 9. Effect of aeration on root hydraulic conductivity (Lp_r) in three broccoli cultivars
** indicate significant differences between the two treatments at the 1% levels, according to the *t*-test (n = 7); ns – not significant; Different capital letters within each cultivar indicate significant differences at the 5% level according to the Tukey–Kramer test (n = 7); Vertical bars indicate standard errors; Sm – 'Shigemori'; Sy – 'Sawayutaka'; FS – 'First Star'

DISCUSSION

Differences in environmental parameters between treatments

The aim of this study was to subject the rhizosphere of broccoli seedlings under waterlogging conditions to different oxygen concentration. In contrast to AIR−, where oxygen concentration gradually decreased, oxygen concentration in AIR+ was maintained at a constant level of 8–9 $mg \cdot L^{-1}$. The rate of oxygen diffusion in water is substantially slower than in air (Ponnamperuma 1972). The results of the current study suggest that in AIR−, oxygen was continuously used for plant respiration, and the supply from air was insufficient to meet this consumption level; therefore, definite differences in oxygen concentration were observed between the two aeration treatments.

There was no statistically significant difference in water temperature between the two aeration treatments. However, the electrical conductivity (EC) was considerably affected by aeration. The EC of the solution in AIR− was higher than that in the AIR+ treatment. Nutrient uptake by plant roots is reduced in hypoxic environments (Ma et al. 2005). The differences in EC between the treatments could be due to the fact that plants in AIR+ absorbed more nutrients than those in AIR−. In contrast, the pH of the solution in the AIR− treatment was lower than in AIR+, which could be attributed to the increase in relative CO_2 concentration in AIR− compared to that in AIR+.

Differences in plant morphology between treatments

Shoot biomass decreases under hypoxic stress in crops sensitive to waterlogging, such as soybean, wheat, and maize (Jitsuyama 2017; Yamauchi et al. 2019). Broccoli shoot biomass also decreased substantially under hypoxic conditions during the AIR− treatment. The effect of hypoxia included wilting symptoms during the day; however, this was mitigated in 'Shigemori' compared to the sensitive cultivars. When water emission through transpiration surpasses water uptake by roots, the plant wilts due to absolute water deficit. 'Shigemori' may have an excellent ability to maintain both water emission and uptake.

A strong interaction was found between treatment and cultivar in terms of shoot-to-root ratio. The development of the plant root system ceases under hypoxic conditions (Gliński & Lipiec 1990), and under water stress conditions, plants lose their leaves due to water loss through transpiration (Begg & Turner 1976). The shoot-to-root ratio of all cultivars tended to increase under hypoxic conditions; however, 'Shigemori' showed a smaller increase than the other two cultivars. This indicates that the biomass balance between shoot and root was relatively stable in 'Shigemori', even under hypoxic conditions.

The leaf area-to-root DW ratio reflects the proportion of transpiration water loss to water absorption. It indicates the morphological balance between the capacity for transpiration and the ability of roots to take up and transport water (Meinzer & Grantz 1990). A strong interaction was found between treatment and cultivar. In the present study, the leaf area-to-root DW ratio of 'Shigemori' was similar under both aeration conditions but increased under hypoxic conditions in the other two sensitive cultivars. 'Shigemori' improved plant morphology by maintaining a constant shoot-to-root balance.

Difference in root length between treatments

Broccoli root length was clearly inhibited by hypoxic conditions (Table 3), as observed in previous studies on general plants and other crops, tomatoes, and soybeans (Gliński & Lipiec 1990; Jitsuyama 2015; Ide et al. 2022). However, no interaction between treatment and cultivar was detected for total root length, and cultivar-specific characteristics of the root length response were unclear. In tolerant soybean cultivars, thick roots are particularly elongated, even under hypoxic conditions (Jitsuyama 2015). The present study examined root length with three root diameter categories and the interaction between treatment and cultivar. The interaction between treatment and cultivar was detected in fine and thick roots. Root elongation was particularly evident in fine roots. No root elongation was detected before AIR− treatment in the two sensitive cultivars. However, 'Shigemori' roots were substantially elongated. This suggests that the effect of hypoxia, which hinders root elongation, was suppressed in the root of 'Shigemori'. Specific responses to hypoxia were then assessed by examining the anatomy of roots newly formed under hypoxic conditions.

Differences in internal root structure between treatments

Previous studies have reported the formation of adventitious root aerenchyma tissue (Armstrong 1979) and lignification of outer cell layers as a root response to hypoxic conditions, which may suggest the existence of a barrier to ROL (Colmer 2003). However, in the present study, these morphological changes could not be detected in plants under hypoxic conditions.

Hypoxia is known to affect stem growth, resulting in increased stem thickness (Kawase 1981). Hypertrophic stem lenticels and secondary stem aerenchyma facilitate oxygen transport to roots in flooded soil (Shimamura et al. 2010). In the present study, hypoxia led to slightly greater (1.18-fold, $p < 0.01$; Table 2) stem weight compared with AIR+, although it did not affect adventitious aerenchyma and lignification of the outer cell layers in the root tissue. When broccoli was completely submerged (AIR− treatment in this experiment), oxygen leakage from the plant surface appeared to occur prominently in the stem, regardless of cultivars or root zone oxygen concentration. In the AIR+ and AIR− treatments, there was oxygen leakage from different parts of the roots of all cultivars. Regarding ROL, no cultivar specificity was observed in response to the oxygen environment in the root zone. This was also reflected in the randomness of lignin and suberin deposits of (Ejiri et al. 2021), which are known to be the main components of the ROL barrier. Although each substance was usually detected in vessels, detection in the exodermis and Casparian strip showed no consistency in root location, thickness, cultivar, or oxygen treatment, with only slight differences in suberin deposition, even in the roots of 'Shigemori' in the AIR− treatment. In paddy rice, stagnant hydroponic treatment and the presence of abscisic acid influence suberin deposition in the exodermis and the formation of a ROL barrier (Shiono et al. 2022). Broccoli did not produce developmental adventitious aerenchyma in this study, although lignin and suberin deposition were observed at different locations, regardless of cultivar. It was inferred that modification of the ROL barrier as an adaptation strategy to low-oxygen environments in flood-sensitive plants has not yet been developed. Therefore, the adaptability of broccoli to hypoxia may be lower than that of other waterlogging-tolerant plants, such as paddy rice.

The intercellular spaces in the cortex may facilitate gas exchange within the tissue under hypoxic conditions, and the cortical cell arrangement may contribute to waterlogging tolerance (Kawase 1981; Yamauchi et al. 2019). The cortical cell arrangement of broccoli roots was similar to the hexagonal pattern observed in wheat (which offers less intercellular space) but different from the cubic pattern observed in rice (which provides more intercellular space). These results suggest that broccoli is a plant sensitive to waterlogging.

The total root cross-sectional area, corresponding to root thickness, tended to increase under hypoxic conditions. This may be attributed to the growth of cortical tissue, particularly in 'Shigemori'. Under hypoxic conditions, the root thickness of wheat, maize, and soybean increased, and the root cortex provided a site for gas space formation (Yamauchi et al. 2014, 2019). Furthermore, the magnitude of ROL is lower in thicker roots (Pedersen et al. 2021). Therefore, the thick roots of broccoli observed in the present study under hypoxic conditions may play a role in preventing severe ROL, and the response to root thickening may vary among cultivars.

An interaction between treatment and cultivar was found for another characteristic of root cross-sectional area, namely xylem area. In the two sensitive cultivars, xylem area decreased significantly under hypoxic conditions, whereas this was not observed in the tolerant 'Shigemori' (Fig. 6). This suggests that 'Shigemori' may retain the original size of the xylem tissues as the cortex thickens due to root thickening. Changes in root internal structure under hypoxic conditions were previously been confirmed by observations of root cross-sectional images, which showed an increased cortex-to-stele ratio (Yamauchi et al. 2019) and a decreased xylem area (Pang et al. 2004). While these trends were observed in the present study, the preservation of xylem size under hypoxic conditions may be more important than other internal morphological changes in broccoli plants subjected to waterlogging. In broccoli, the deposition of lignin and suberin on the cell wall is not necessarily a critical factor for improved waterlogging tolerance.

Differences in hydraulic conductivity between treatments

For Lp_r, which indicates hydraulic conductivity per unit root area, the interaction between treatment and cultivar was not statistically significant. However, conductivity decreased by 20% in each cultivar grown in AIR−. Root hydraulic conductivity is influenced by environmental stresses in the rhizosphere, such as osmotic or salinity stress (Meng & Fricke 2017; Vaziriyeganeh et al. 2018). Hypoxic environments also affect root hydraulic conductivity in soybean cultivars (Jitsuyama 2017). Conductivity is dependent on the amount or activity of aquaporins, proteins that form water channels, and this activity is known to be reduced by hypoxia (Tournaire-Roux et al. 2003; Bramley et al. 2007). Aquaporins account for 80% of broccoli root conductivity (López-Berenguer et al. 2006). Therefore, differences in root hydraulic conductivity between cultivars and aeration treatments in the current study may be related to the amount or activity of aquaporins.

Although no interaction between treatment and cultivar could be detected in Lp_r, the absolute conductivity value differed significantly among cultivars. The conductivity of 'Shigemori' was higher than that of other cultivars and approximately twice that of 'First Star', which exhibited the lowest conductivity among the cultivars.

Root hydraulic conductivity plays a key role in water transport within the plant body (Hirasawa & Ishihara 1991). Biomass reduction due to waterlogging in mature 'Shigemori' plants was lower than in other cultivars (Hara et al. 2023). Therefore, the primary conductivity of 'Shigemori' roots may have been higher than in other cultivars, resulting in a higher water absorption capacity of the whole roots, even after exposure to hypoxic conditions. The Lp_r threshold range used to distinguish tolerant and sensitive plants was set at 60–70 $\mu L \cdot m^{-2} \cdot s^{-1} \cdot MPa^{-1}$ in this study. This conductivity threshold can preserve the leaf area-to-root DW ratio and maintain high photosynthetic activity (Katsuhara et al. 2003; Hanba et al. 2004). However, the present study did not identify a specific factor leading to variation in root hydraulic conductivity, highlighting the need

for further research on the three-dimensional root structure, water channel dynamics, and hypoxic metabolites such as acetaldehyde and ethanol using other characteristic broccoli cultivars (Higashio et al. 2012).

CONCLUSION

This study focused on the response of broccoli seedlings to waterlogging conditions, with particular emphasis on roots. Roots under these conditions were shorter and thicker, mainly due to the increase in cortical tissue. The response to hypoxia also included shoot–root imbalance, a reduction in xylem tissue volume, and low root hydraulic conductivity. This was particularly true for the cultivar more sensitive to waterlogging. Aeration partially mitigated these negative consequences of hypoxia. The study results provide further knowledge about the waterlogging tolerance of broccoli and may inform breeding programs.

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Conflicts of interest
The authors declare that they have no competing interests.

Data availability
The data supporting the findings of this study are available from the corresponding authors on reasonable request.

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