Original research article

Formation and extension of lysigenous aerenchyma in seminal root cortex of spring wheat (*Triticum aestivum* cv. Bobwhite line SH 98 26) seedlings under different strengths of waterlogging

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Abstract: Aerenchyma promotes gas exchange between shoots and roots that supports plant to survive under waterlogged conditions. To understand the process of aerenchyma formation under waterlogged conditions, we developed a method for creating hypoxic pot-culture conditions using different water depths, and used this system to examine the effects of hypoxia on seedling growth and the anatomy of the seminal roots of spring wheat (*Triticum aestivum* cv. Bobwhite line SH 98 26). After 72 h of waterlogging, the redox potentials of a well-drained control and treatments with a water depth 15 cm below (T-15) and 3 cm above (T+3) the soil surface were +426, +357, and +292 mV, respectively. The root growth of the seedlings was reduced in T+3 plants while the shoot growth did not change significantly during 72 h waterlogging. Root anatomy study showed that wheat formed no aerenchyma under our control condition, but formed aerenchyma in the root cortex in response to hypoxia in T-15 and T+3 conditions. The aerenchyma was initially formed at 2 to 5 cm behind the root tip after 72 h in T-15 and 48 h in T+3. The aerenchyma in T+3 plants then extended by an additional 5 cm towards root base during the next 24 h. Evans blue staining indicated that wheat aerenchyma was lysigenous which resulted from degradation of cortical cells. Thus, the combination of the plant material and the pot-culture method can be used for a basic tool with which to analyse the molecular and physiological mechanisms of lysigenous aerenchyma formation in wheat.

Keywords: aerenchyma, hypoxia, root cortex, seminal roots, waterlogging, wheat (*Triticum aestivum* L.)

Introduction

Waterlogging is a major agricultural constraint that limits crop growth and remains to be solved. This is also true when grain crops such as wheat are cultivated in upland fields that have been converted from paddy fields, which is a current agricultural strategy to mitigate the problem of overproduction of rice in Japan. It has been estimated that approximately 20% of Japan's wheat-producing areas are affected by waterlogging (Oyanagi 2008). Waterlogging reduces the concentration of oxygen (O_2) in the rhizosphere; this initially affects the flooded roots of the plants, but the aerial parts of the plant sense the resulting root stress through a systemic signaling mechanism (Bradford and Yang 1981, Jackson et al. 1996).

Terrestrial plants have evolved a variety of mechanisms to overcome the growth limiting effects of oxygen deprivation under waterlogged conditions. These adaptive mechanisms include aerenchyma formation in the roots, tolerance of the soil toxicity and the emergence of adventitious roots (Mano and Omori 2007). Aerenchyma tissue, which comprises a high proportion of gas-filled spaces, provides plants with an alternative strategy for obtaining O_2 (Drew et al. 2000). Therefore, a plant's capacity to form aerenchyma is an important factor that affects its tolerance of waterlogging or flooding (Arikado and Adachi 1955, Jat et al. 1975, Burdick 1989, Armstrong et al. 1991, Bacanamwo and Purcell 1999, McDonald et al. 2001). In general, aerenchyma is categorized to two types by the developmental

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process: lysigeny, which is caused by the selective death and degradation of cortical cells, and schizogeny, in which cells separate at the middle lamellae during development (Evans 2003). Lysigenous aerenchyma that forms under hypoxia has been well documented in the roots of many monocotyledons and dicotyledons (Justin and Armstrong 1987).

In wheat, the seedling stage begins with the appearance of the first leaf and ends with the emergence of the first tiller (Bauer et al. 1983). Up to six seminal roots and three leaves support the plant at this stage. Root survival is essential during the early seedling stage to prevent seedling loss caused by waterlogging. Seminal roots of 10-d-old wheat seedlings grown on flooded sand had developed 25 to 30% gas space in the cortex, compared with 6 to 8% in roots with sufficient O_2 supply (Erdmann and Wiedenroth 1988). Similarly, Konings (1982) demonstrated that seminal roots of maize seedlings grown for 4 d in a non-aerated nutrient solution had a cavity area amounting to 13% of the root cortex, compared with 3% in plants under well-aerated solution. However, the capacity to form aerenchyma along seminal roots at a very young seedling stage of wheat under soil hypoxic conditions remained to be elucidated. Also, little is known about the process of aerenchyma formation, i.e. necessary time to form, its position within the root, and the direction in which the aerenchyma extends, etc., in response to different intensities of hypoxia.

In this study, we investigated the elapsed time before aerenchyma forms in wheat seminal roots under soil hypoxia conditions, T-15 (water level 15 cm below the soil surface) and T+3 (water level 3 cm above the soil surface), comparing with the well drained control. We also obtained information on the possible position where lysigenous aerenchyma formation begins and the direction of its extension.

Materials and Methods

Plant materials and growth conditions

Spring wheat (*Triticum aestivum* L.) cv. Bobwhite line SH 98 26 was used for all experiments. Seeds were germinated on moist filter paper in petri dishes in a dark growth cabinet at 23°C. Three healthy germinated seeds were sowed at a 3-cm depth in each of 27 soil-filled pots (Fujiwara Scientific Co. LTD., Tokyo, Japan; 1/5000a deep type Wagner pot, height 30 cm x inner diameter 15.9 cm). The wheat plants were grown in a greenhouse maintained at a temperature of approximately 20°C day/night, with natural light at the National Institute of Crop Science (36°0'N, 140°1'E) in 2009. The plant growing periods for three replications were (i) July 24 to August 1, (ii) October 28 to November 5, and (iii) November 21 to November 29.

Pots used for the control plants (the well-drained treatment) had a total of eight 0.6-cm-diameter holes drilled in the bottom. The bottom of each pot was filled with a 3-cm layer of coarse perlite. The pots contained two types of granular soils: the first (bottom) layer above the perlite was 2.6 kg of fertilized granular soil (Kureha Corporation, Tokyo, Japan; pH 6.5, 1.0 g N, 2.6 g P_2O_5, 1.6 g K_2O in each pot) and second (top) layer was 1.8 kg of volcanic soil (Green Tech Company Limited, Tochigi, Japan; pH 6.1). Thus, the soil distribution in the pot as appeared from the bottom are; 3 cm perlite, 14 cm granular soil and 10 cm volcanic soil. The pots were watered to a depth of 14 cm from the bottom by means of daily immersion in a container of water, and were rotated within the greenhouse every second day to minimize the effect of differences in growing conditions at different positions.

Water treatments were imposed when the seedlings were 5 d old and their primary leaves had fully expanded. The three treatments were: (i) the control, with a well-drained soil watered daily to a depth of 12 cm from the bottom and then allowed to drain; (ii) the T-15 treatment, in which the water level was maintained at a 15 cm depth below the soil surface, and (iii) the T+3 treatment, in which the water level was maintained at 3 cm above the soil surface. The water treatments began at around 14:00 to 15:00 pm.

Measurement of physicochemical parameters of the growth conditions and seedling growth

The soil water contents were measured using a HydroSense CD620 display and CS620 sensor with a 12-cm probe (Campbell Scientific Australia Pty. Ltd., Thuringowa Central, Australia) in the top 12 cm of the soil. The O_2 concentrations were recorded at a 14 cm depth in the soil using an OXY-10 O_2-sensor (PreSens Precision Sensing GmbH, Regensburg, Germany). The soil's redox potentials were recorded using an Eh meter (PRN-41, Fujiwara Scientific Co.LTD., Tokyo, Japan) with a reference electrode (Type 4400 DKK-TOA Corporation, Tokyo, Japan) and Pt electrodes (Type EP-201, Fujiwara Scientific Co.LTD., Tokyo, Japan) that were set at a 14-cm depth in the soil during the experiment period. The relative changes in chlorophyll concentration were measured for the same four plants in each treatment, 4.5 cm from the base of first emerged leaves, using a SPAD 502 chlorophyll meter (Konica Minolta, Osaka, Japan).

Three pots in each treatment (i.e., nine plants) were harvested at 0, 24, 48, and 72 h after treatment initiation. One plant from each pot (three plants from

three pots in each treatment) was harvested to determine plant height, the longest root length and root dry mass. The roots were oven-dried for 2 d at 80°C and the dry weights were measured. The other two plants from each pot (six plants from the three pots in each treatment) were used for root anatomy. Within 72 h of waterlogging, the root lengths were less than 24 cm; as a result, no roots had reached the bottoms of the pots. Three of the six plants that showed no injury during root preparation were selected, and used them for tissue sections. Data were analysed by calculating means, standard errors and one-way analysis of variance (ANOVA). Significant differences were stated where $P < 0.05$.

Seminal root anatomy

For each treatment, three primary seminal roots from three individual plants at each sampling time were evaluated to look for the presence of aerenchyma. After a gentle washing, we segmented the roots at 0, 3, and 5 cm beyond the root base (i.e., the root–shoot junction) and at intervals of 5 cm starting 2.0 cm behind the root tip. The root segments were then embedded in 5% agar. Transverse cross-sections of each root segment (four to six sections per segment), with each section 100 to 400 μm thick, were obtained using a D.S.K. Microslicer (DTK-1000, Dosaka EM Co. Ltd., Kyoto, Japan). The sections were examined and photographed with a light microscope equipped with a camera (BX51 microscope and DP72 camera, Olympus Co. Ltd., Tokyo, Japan) at ×10.

We classified the root sections into aerenchymatous or non-aerenchymatous by shape and size of the cortical cells framed in by cell wall: in aerenchymatous root, there is a cell disturbed with rough wall and edged by black shade in some cases. The compartment size often expands to about 2 or 3 normal cell volumes; in non-aerenchymatous root, cortical cells has normal and round like shape with smooth wall.

Evaluation of cortical cell death using Evans blue staining

We evaluated cell death by means of Evans blue staining, as described by Baker and Mock (1994). This non-toxic, water-soluble pigment stains dead cells (Gaff and Okong'O-Ogola 1971, Kanai and Edwards 1973). The semipermeable membranes of living plant cells exclude Evans blue dye, whereas the dye penetrates and stains dead cells. Plants were grown as described in "plant materials and growth conditions" section and harvested at 24h and 48 h after the start of the waterlogging under T+3 condition. For each treatment (24h or 48h), we sampled a total of 9 primary seminal roots from three pots each containing three plants. Roots were first cut into 0.7-cm segments starting 5 cm behind the root tip, and the segments were washed twice for 10 min with distilled water, followed by de-aeration three times for 3 min using a desiccator linked to a vaccum pump (DTC-60, ULVAC Kiko Inc., Miyazaki, Japan) at a pressure of 0.06 MPa. Each root segment was then stained separately in a vial in 1 mL of 1% aqueous Evans blue for 15 min at room temperature on a rotary shaker. The root segments were then washed several times within 15 min using distilled water and directly photographed under a light microscope (SZ2-ILST, Olympus Co. Ltd.). Root sections were then prepared at the midpoint of each segment and viewed under the light microscope, as described above.

Results

Establishment of hypoxic pot conditions

In order to establish hypoxic pot conditions, we attempted to provide different strengths of waterlogging conditions by using different depths of water treatment, 15 cm below (T-15) and 3 cm above (T+3) the soil surface. The waterlogging effects were examined by monitoring the physicochemical parameters in soil, such as water content, redox potential and O_2 concentration during 72 h treatment. Under the control, T-15, and T+3, the soil water contents remained nearly constant within each conditions, those values were 12-14 %, 23-27 % and 74-82 %, respectively (Table 1). The redox potential in the control soil remained roughly constant throughout the experimental period, it was +426 mV at 72 h (Fig. 1). In contrast, the redox potentials in the T-15 and T+3 soils had declined during waterlogging treatment, the values at 72 h were +357, and +292 mV, respectively. The O_2 concentration after 72 h in T-15 soil had decreased by about 46% compared with that in the control soil, whereas that in T+3 soil had decreased by about 74% (Table 1). These results showed that the soil conditions in T-15 and T+3 were becoming hypoxic in order of water depth during waterlogging treatment.

Seedling growth under hypoxic conditions

We measured growth of the seedlings for 72 h to determine any differences between the control condition and the hypoxic (T-15 and T+3) conditions. The length of primary seminal roots was not different between the control and T-15 plants after 72 h of waterlogging, but was reduced by 25% in the T+3 plants (Fig. 2A). The corresponding reduction of seminal root dry mass was 12.5% in T-15 plants and 31% in the T+3 plants (Fig. 2B). For the aerial parts,

the plants did not show significant difference in shoot length (Fig. 2C) and in leaf chlorophyll content (Fig. 2D). These data indicated the adverse effect of waterlogging on plant growth was progressive (i.e., the root growth was restricted with increasing severity of hypoxia).

Table 1. Physicochemical parameters of the soil during waterlogging treatment

Treatment	Water content (%)[a]	O_2 concentration (%)[b]
	24 h of waterlogging	
Control	12 ± 1.0	nd
T-15	23± 1.0	nd
T+3	74± 1.0	nd
	48 h of waterlogging	
Control	14 ± 1.2	nd
T-15	26± 1.2	nd
T+3	81± 1.3	nd
	72 h of waterlogging	
Control	14 ± 1.2	18.7± 0.6
T-15	27± 1.1	10.1± 0.7
T+3	82± 1.8	4.9± 1.1

Control, well-drained; T-15, water level 15 cm below the soil surface; T+3, water level 3 cm above the soil surface
[a] The water contents were recorded after 24, 48 and 72 h of waterlogging at depth of 12 cm in the soil.
[b] O_2 concentrations were recorded after 72 h of waterlogging at depths of 14 cm in the soil.
Values represent the mean ± SE obtained from three independent replicates (pots).

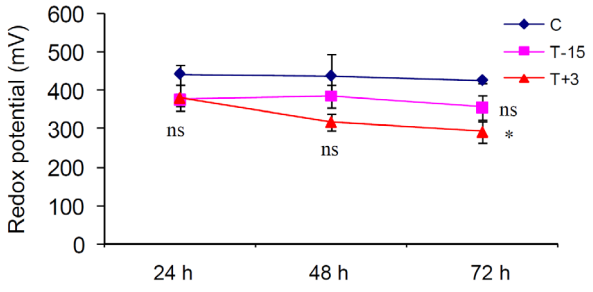

Fig. 1. The redox potentials of the soil during waterlogging treatment in control, T-15 and T+3 conditions. The redox potential was recorded at a 14-cm depth in the soil during the experimental period. Values are the mean ± SE (n=3). * indicate significant difference at the 5% level (analysis of variance).

Formation of lysigenous aerenchyma under hypoxyc conditions

Elapsed time to form aerenchyma was different between waterlogging conditions. Wheat primary seminal roots showed no aerenchyma in our control condition. In T-15, the almost roots showed no aerenchyma until 48 h waterlogging and then after 72 h, they showed conspicuous aerenchyma at 2 to 5 cm behind the tip. In T+3, the roots showed conspicuous aerenchyma after 48 h of waterlogging at 2 to 5 cm behind the tip (Figs. 3 and 4). Therefore, aerenchyma was formed sooner in the deep water treatment. After 72 h waterlogging, the aerenchyma in T+3 plants appeared at 2-10 cm behind the tip (Fig. 3). This result indicated that the aerenchyma might extended longitudinally for an additional 5 cm from tip towards base (root-shoot junction) in the seminal roots during 48 - 72 h waterlogging.

To examine an involvement of cell death during the process of aerechyma formation in the cortex of seminal roots of wheat, the root segments were excised from 5 cm behind the root tip and were stained by Evans blue dye, then the cross-sections were observed. In the results, some cells around the inner cortex had begun to absorb the blue stain within 24 h, indicating the initiation of cell death (Fig. 5A). After 48 h, cells stained with Evans blue were abundant and had extended a considerable volume in the mid cortex, indicating the cell collapse (Fig. 5B). These results indicated the aerenchyma that forms in the cortex of wheat roots was lysigenous, where cell death started after 24 h of waterlogging and continued to 48 h in T+3 condition.

Discussion

Our primary concern was to establish a waterlogging treatment that would provide reproducible effects on young wheat seedlings. So, we examined the physicochemical parameters of the soil and the plant growth under waterlogging treatment. The results showed that the soil conditions in T-15 and T+3 were becoming hypoxic in order of water depth (Table 1 and Fig. 1). In previous pot studies, the redox potential and O_2 concentration also decreased with increasing duration of waterlogging (Malik et al. 2003), although the two parameters were not shown to depend on the soil water content. Then, we measured growth of the wheat seedlings for 72 h to find any difference between the soil conditions. The results showed that only root growth was delayed within 72 h and it was apparent in T+3 plants rather than T-15 plants (Fig. 2). These data showed a good correlation between treatment intensity: i.e., water depth, and the results: i.e., changes in the physicochemical parameters in soil and

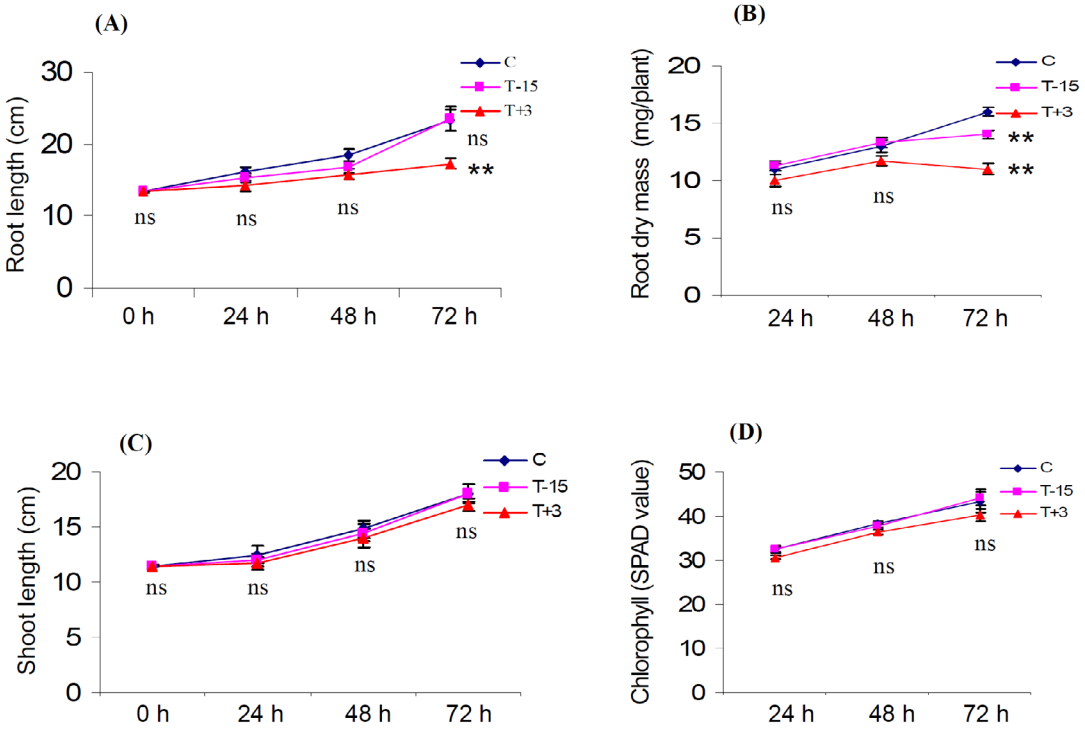

Fig. 2. Effects of waterlogging on the seedlings of wheat cv. Bobwhite under different severities of hypoxia. (A) Root length (seminal roots only), (B) Root dry mass, (C) Shoot height, and (D) SPAD chlorophyll content were determined for the well-drained controls, T-15 plants (water level at 15 cm below the soil surface), and T+3 plants (water level 3 cm above the soil surface) at every 24 h during waterlogging. Experiments were repeated in three replications, where four individual plants in chlorophyll measurements and three in root and shoot measurements were considered. Value represents the mean ± SE (n=3). * indicate significant difference at the 5% level (analysis of variance).

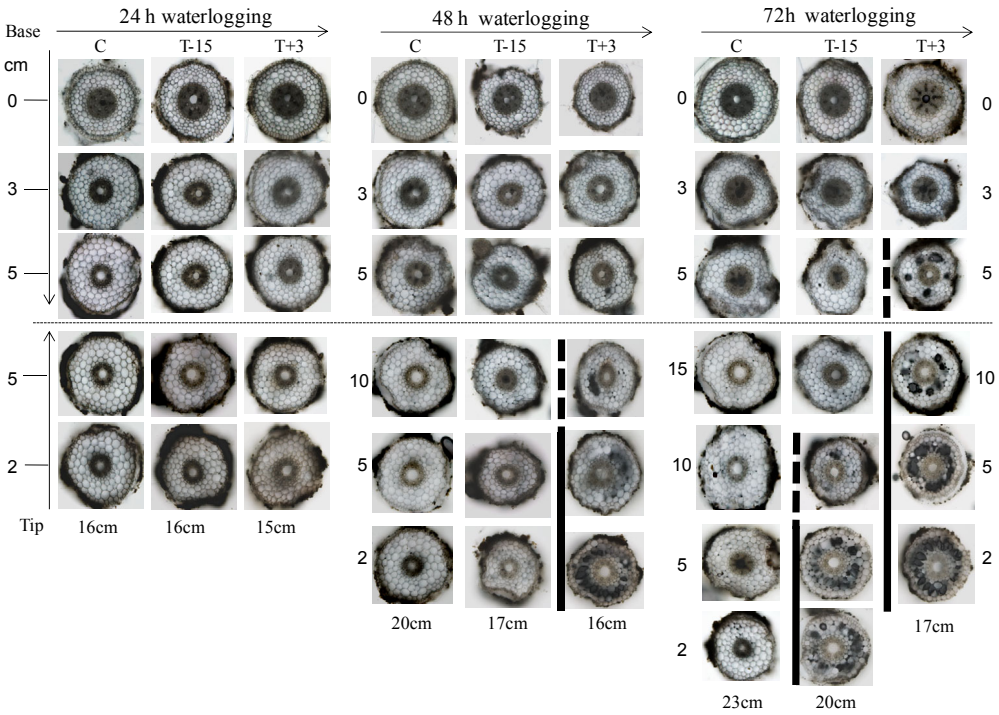

Fig. 3. Cross-sections of primary seminal roots of wheat cv. Bobwhite subjected to different severities of hypoxia after 24, 48, and 72 h, showing the distribution of aerenchyma formation along the roots. Sections were taken at 5 cm distance over 15 cm from 2 cm tip and over 5 cm from 0 cm base (root-shoot junction). n= 9 roots from 9 individual plants were observed. Base, distance measured from the root–shoot junction; Tip, distance measured from the root tip. Numbers below the photographs represent the mean length of the seminal roots. (24 h) Root sections after 24 h of waterlogging. (48 h) Root sections after 48 h of waterlogging. (72 h) Root sections after 72 h of waterlogging. Vertical solid lines indicate completely formed aerenchyma; dashed line indicates the initial stage of aerenchyma formation.

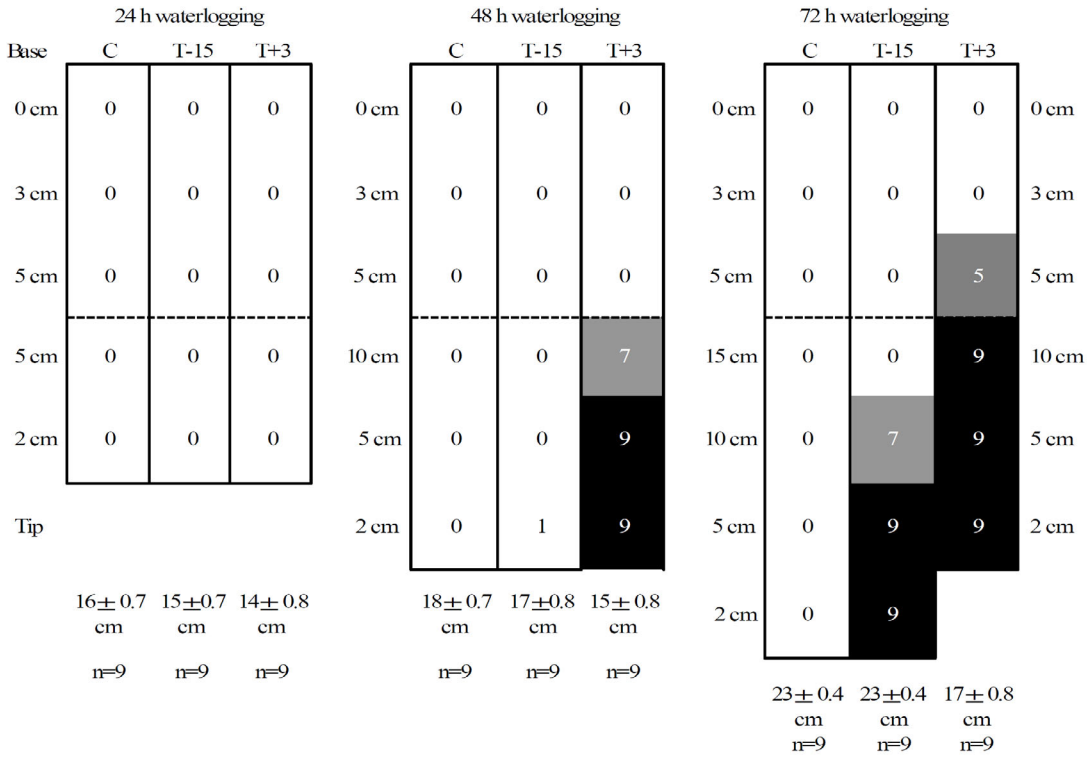

Fig. 4. Numbers of seminal root cross-sections that displayed aerenchyma formation as shown in Fig. 3. n= 9 roots from 9 individual plants were observed. Black and gray color background indicate aerenchyma formation in all nine sections and in more than half number of the nine sections, respectively. Numbers below the figures represent the mean length ± SE of the seminal roots.

the root growth. Previous researches support our results that roots show the effects of waterlogging earlier compared to aerial parts (Trought and Drew 1980, Thomson et al. 1992, Huang et al. 1994) and water depth affected the magnitude of the growth reduction in wheat (Malik et al. 2001, 2002). Thus, the system we used in this study worked as expected, and the low standard errors in the results indicated that the waterlogging treatment in our system has reproducible effects on the plants.

The formation of aerenchyma in seminal and adventitious roots in wheat has mostly been reported for plants grown in solution and in sand culture (Erdmann and Wiedenroth 1988, Thomson et al. 1990, 1992, Huang et al. 1994, 1997, Malik et al. 2003), with few reports for plants grown in soil (Thomson et al. 1992, Malik et al. 2001). Wheat cv. Gamenya were reported to develop more aerenchyma (% of root cross-sectional area) in their adventitious roots when grown in waterlogged soil than in solution culture (Thomson et al. 1992). Since the growth analysis in this study indicated that the waterlogging had significant effects to roots rather than shoots, aerenchyma was expected to be formed in the roots. In our conditions, seminal roots did not form aerenchyma in control, but formed lysigenous aerenchyma until 72 h in T-15 and until 48 h in T+3 at 2 to 5 cm behind the root tip in a reproducible fashion (Figs. 3 and 4). The timing of aerenchyma formation was earlier in T+3 than T-15 as expected in accordance with the severity of hypoxia. To our knowledge, this is the first report to describe the timing of the aerenchyma formation in wheat seminal roots under two different hypoxic conditions and, again, our hypoxia conditions have reproducible effects on the aerenchyma formation as well as plant growth.

The development nature of aerenchyma, such as the formation timing and position, might be similar in part among several crops. First, in respect to timing, aerenchyma can be seen within 48 h after the initiation of hypoxia in the seminal (Fig. 3) and adventitious roots (Malik et al. 2003) in wheat. In maize, aerenchyma formation in the seminal (Gunawardena et al. 2001) and adventitious roots (Drew et al. 1981) is induced by hypoxia within a few days. Second, in respect to the position, aerenchyma is first detected within a few cm from the root tip of wheat (Figs. 3 and 4) (Huang et al. 1994, Malik et al. 2003) and other plants, such as maize (Campbell and Drew 1983), rice (Kawai et al. 1998) and *Sagittaria lancifolia* (Schussler and Longstreth 2000). Cell collapse in seminal roots start at mid cortex in rice (Kawai et al.

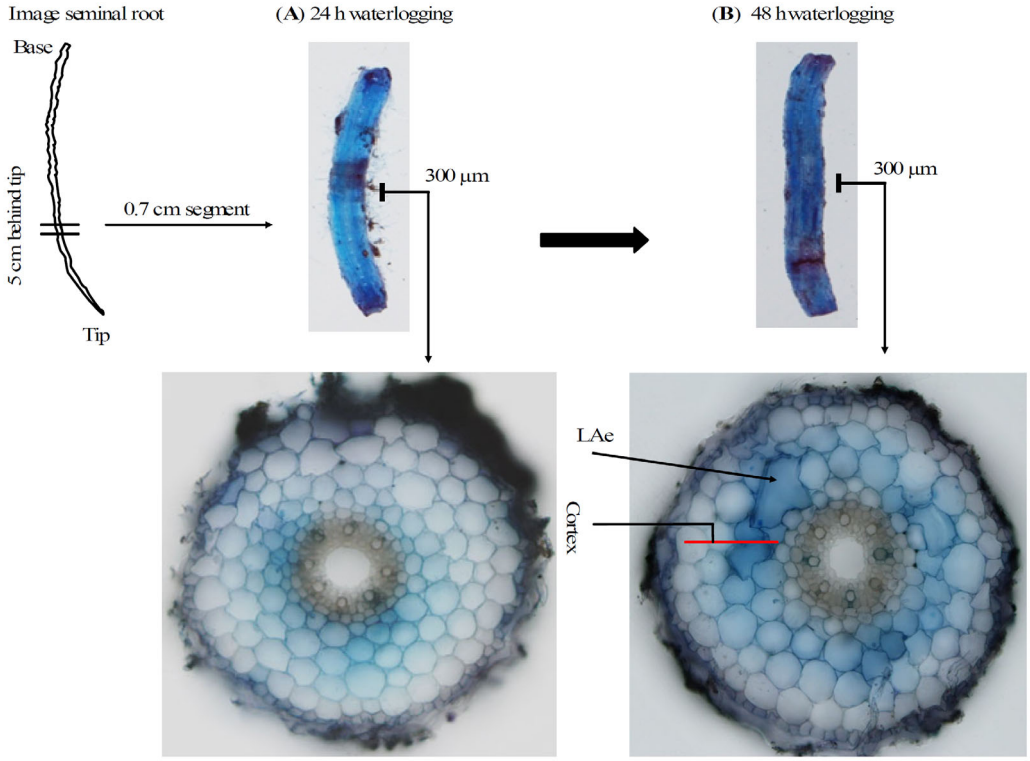

Fig. 5. Evaluation of aerenchyma formation in the cortex of seminal roots of wheat cv. Bobwhite grown under T+3 conditions (a water depth 3 cm above the soil surface). (A) Root section after 24 h of waterlogging, showing the initiation of cell death in the mid cortex. (B) Root section after 48 h of waterlogging, showing the advanced stage of cell death, resulting in the formation of lysigenous aerenchyma (LAe).

1998) and wheat (Fig. 5). Therefore, there would be common physiological events in the underlying process of aerenchyma formation, however, the precise mechanism are not clarified. The initial trigger to lysigenous aerenchyma formation is considered to be due to the stimulation of the plant hormone ethylene in roots (Drew et al. 1979, 1981, 2000). The response of cell collapsing during lysigenous aerenchyma formation is associated with a characteristic of programmed cell death (Kawai et al. 1998; Bouranis et al. 2007). These informations suggest that ethylene stimulation and subsequent programmed cell death would be involved in the physiological mechanisms for aerenchyma formation in wheat seminal roots under hypoxia, though it should be examine experimentally.

In conclusion, our results provide basic information on the aerenchyma formation process in seminal roots of wheat. We detected that the lysigenous aerenchyma formed behind the root tip sooner and to a greater extent with increasing severity of the hypoxic soil conditions. Herewith, our experiment system can be used as a basic tool to analyse the molecular and physiological mechanisms of aerenchyma formation in wheat. The plant material, cv. Bobwhite line SH 98 26, is known to be one of the most useful hosts for producing fertile transgenic wheat (Pellegrineschi et al. 2002). Therefore, we hope the information obtained here will be useful not only for understanding the basic nature of aerenchyma formation, but also for efforts to improve aerenchyma formation, including the creation of transgenic plants by introducing genes thought to be responsible for a high ability to form aerenchyma (Mano and Omori 2009).

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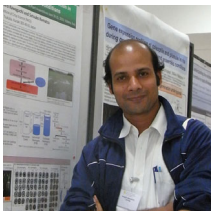
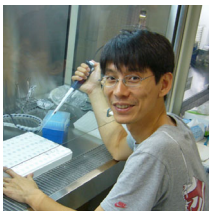


Dr. Md. Emdadul Haque is interested in the relationship between stress-mediated signaling and multiple-stress resistance: mechanisms in crop plants.



Dr. Fumitaka Abe's interest is the gene functions involving in the yield performance of crops. His excellent technique for producing fertile trans-genic plants of wheat is our powerful tool.

Dr. Kentaro Kawaguchi has an interest in the morphological and physiological responses of crops to various environmental stresses, e.g. low-temperature, waterlogging, etc.