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To cite this article: Katsuhiko Shiono, Masato Ejiri, Kana Shimizu & Sumiyo Yamada (2019) Improved waterlogging tolerance of barley (*Hordeum vulgare*) by pretreatment with ethephon, Plant Production Science, 22:2, 285-295, DOI: [10.1080/1343943X.2019.1581579](https://doi.org/10.1080/1343943X.2019.1581579)

To link to this article: <https://doi.org/10.1080/1343943X.2019.1581579>



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Improved waterlogging tolerance of barley (*Hordeum vulgare*) by pretreatment with ethephon

Katsuhiro Shiono , Masato Ejiri, Kana Shimizu and Sumiyo Yamada

Graduate School of Bioscience and Biotechnology, Fukui Prefectural University, Fukui, Japan

ABSTRACT

Root growth into hypoxic or anoxic waterlogged soil relies on internal aeration in plants. The plant hormone ethylene helps adapt to waterlogging by inducing the formation of aerenchyma, which provides a low-resistance pathway for the transport of oxygen from the shoot to the root apex. Waterlogging-susceptible crops including barley start to form aerenchyma after suffering waterlogging stress. But waterlogging can be fatal if aerenchyma formation is not fast enough. Here, we investigated whether pre-treating barley with ethephon, an ethylene-releasing agrochemical, could improve its tolerance to mimicked waterlogging conditions (using stagnant deoxygenated agar nutrient solution). In barley growing in aerated nutrient solution, ethephon treatment enhanced aerenchyma formation at the root tips and induced the development of shorter and shallower roots. Pre-treating barley leaves also delayed waterlogging-caused whitening and increased the percentages of viable root-tips under waterlogging conditions. However, the pretreatment did not noticeably increase fresh weight or shoot length. Further studies are needed to optimize ethephon treatment conditions to improve barley production under waterlogged conditions.

ARTICLE HISTORY

Received 17 July 2018
Revised 25 December 2018
Accepted 7 February 2019

KEYWORDS

Adventitious root; aerenchyma; barley (*Hordeum vulgare*); deoxygenated stagnant agar nutrient solutions; ethylene; hypoxia; waterlogging

CLASSIFICATION

Crop Physiology

Introduction

Plants can suffer from hypoxia or even anoxia when soils become waterlogged. Well-drained soil is porous and normally filled with gas; excess water fills the pores, preventing the entry of atmospheric oxygen, as the diffusivity of oxygen in water is extremely low (Jackson, Fenning, Drew & Saker, 1985). Other problems associated with waterlogging are the accumulation of phytotoxic compounds in the soil (Ernst, 1990; Kreuzwieser, Papadopoulou & Rennenberg, 2004; Lamers, Tomassen & Roelofs, 1998; Ponnamperuma, 1984) and a decline in the availability of some nutrients (Laanbroek, 1990).

Worldwide, waterlogging damages many field crops including soybean, wheat, oats and barley (Bertholdsson, 2013; Setter & Waters, 2003). Barley (*Hordeum vulgare*) is more susceptible to waterlogging stress than other cereals (Setter & Waters, 2003) and thus has been the target of genetic, agronomic and cropping-system approaches to improve its waterlogging tolerance (Bertholdsson, 2013; Mano & Takeda, 2012; Setter & Waters, 2003; Zhang et al., 2016). Similar efforts have targeted maize (Mano & Omori, 2007), soybean (Matsunami, Jung, Oki & Kokubun, 2007) and wheat (Haque, Oyanagi & Kawaguchi, 2012). So far, these efforts have met with only limited success.

The mechanisms by which plants tolerate waterlogging include shifting metabolic pathways, transporting

oxygen internally via aerenchyma (large internal gas spaces that connect the shoot to the roots) and sending out adventitious roots to the soil surface where the oxygen concentration is higher (Bailey-Serres & Voesenek, 2008; Shiono, Takahashi, Colmer & Nakazono, 2008). Ethylene is a plant hormone that induces adaptive responses to waterlogging (Sasidharan & Voesenek, 2015; Shiono et al., 2008; Voesenek & Sasidharan, 2013). Ethylene (i) enhances the expression of genes of fermentation enzymes (Drew, Jackson & Giffard, 1979; Kennedy, Rumpho & Fox, 1992; Sachs, Subbaiah & Saab, 1996), (ii) promotes lysigenous aerenchyma formation (Jackson et al., 1985; Konings, 1982; Rajhi et al., 2011), (iii) induces epidermal cell death of stem internodes of rice at the sites of adventitious root emergence (Mergemann & Sauter, 2000) and (iv) interacts with auxin to initiate the growth of adventitious root (Visser, Cohen, Barendse, Blom & Voesenek, 1996; Visser et al., 1995).

Aerenchyma and newly formed roots are found in not only wetland plants but also waterlogging-susceptible crops (e.g. barley, wheat, maize and soybean) (Shiono et al., 2008; Visser & Voesenek, 2004). However, the latter crops have little or no aerenchyma under well-drained soil or aerated nutrient conditions (Haque et al., 2012; Mano & Omori, 2007). After suffering waterlogging stress, these crops start to form aerenchyma and new

CONTACT Katsuhiro Shiono  shionok@fpu.ac.jp

 Supplemental data for this article can be accessed [here](#).

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roots via ethylene-responsive signalling (Shiono et al., 2008; Visser & Voeselek, 2004), while some wetland plants (e.g. rice and *Zea nicaraguensis*) form aerenchyma constitutively under well-drained soil or aerated nutrient conditions (Kawai, Samarajeewa, Barrero, Nishiguchi & Uchimiya, 1998; Mano et al., 2006; Nishiuchi, Yamauchi, Takahashi, Kotula & Nakazono, 2012; Shiono et al., 2011). Crops require at least a few days to complete formation of aerenchyma and adventitious roots (Haque et al., 2012). Without these changes, only 3 days waterlogging is enough to severely reduce their growth (Malik, Colmer, Lambers, Setter & Schortemeyer, 2002). Thus, we assumed that waterlogging damage would be decreased and growth would be improved if aerenchyma and roots to the soil surface could be induced before the commencement of waterlogging stress.

Ethylene is not easy to use in experimental studies because it is a gas. Instead, researchers often use 1-aminocyclopropanecarboxylic acid (ACC), which is a metabolic intermediate of ethylene and is degraded into ethylene by the activity of ACC oxidase in plants (Roblin & Pérault, 1985; Zhang & Wen, 2010). Recently, pre-treating wheat with ACC before exposing it to waterlogging-like conditions was shown to improve growth, stimulate aerenchyma formation and upregulate the expression of ethanol fermentation enzymes in first seminal roots (Yamauchi et al., 2014). Another ethylene-producing chemical is ethephon. It is widely used in horticultural crops to improve lodging resistance, ripen fruit and stimulates flowering (Gent & McAvoy, 2000). It releases ethylene over a long period as it is chemically degraded (Maynard & Swan, 1963; Peng, Chan, Shih & Yang, 2001; Roblin & Pérault, 1985; Zhang & Wen, 2010). Here, we investigated whether ethephon pretreatment could also improve waterlogging tolerance. Whereas other studies of ACC pretreatment mainly focused on adaptive responses in single roots, we mainly focused on adaptive responses in whole root system under waterlogged conditions.

Materials and methods

Growth conditions

Seeds of barley (*Hordeum vulgare* cv. Morex) were kindly provided by the Genetic Resources Center, National Agriculture and Food Research Organization, Japan (https://www.gene.affrc.go.jp/index_en.php). Barley plants were grown in the same nutrient solution used in earlier studies (Colmer, 2003; Shiono et al., 2011). Plants were grown in a controlled-environment chamber (20-h light at 23°C/4-h dark at 23°C, relative humidity over 50%, photosynthetic photon flux density at 249 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Seeds were sterilized for 30 min in 0.6% (w/v) sodium hypochlorite, washed thoroughly with deionized water, and for imbibition, were placed in Petri dishes containing about 5 mm of deionized water for 1 day in darkness at 23°C. For germination, we used a tank containing aerated, quarter-strength nutrient solution. A stainless mesh was placed in the tank so that its upper surface was at the water level. The mesh pores sized smaller than seeds but large enough for root growth. One day after imbibition, seeds were placed on the mesh and exposed to light. For the ethephon treatment, we prepared 5-liter pots (120 mm \times 180 mm \times 250 mm high) containing aerated full-strength nutrient solution containing 0, 0.1, 1 or 37.5 μM ethephon (Nissan Ethrel®10, Nissan Chemical Industries, Tokyo, Japan). Following Steffens, Kovalev, Gorb and Sauter (2012), we used ethephon treatments of 0, 37.5, 70 and 150 μM . In each pot, a rectangular 2-cm-thick piece of foam was placed on the solution and aluminium foil was placed on the top of the foam to keep the solution dark. In each pot, vertical cuts were made on the six sides of the foam to accommodate stems. Then 6 plants were transferred to each pot, sliding the stems into the cuts. In this way, the root was kept in the dark. Seven days later (13 days after imbibition), plants were transferred to pots containing aerated nutrient solution or stagnant deoxygenated nutrient solution for 7 days. The stagnant solution was prepared by adding 0.1% (w/v) agar to the nutrient solution and boiling the solution to dissolve the agar. The low concentration of agar produced a viscous liquid rather than a gel. By preventing convective movements, the solution mimics the changes in gas composition found in waterlogged soils (e.g. decreased oxygen and increased ethylene) (Wiengweera, Greenway & Thomson, 1997). The solution was poured into the pots and deoxygenated by bubbling N_2 gas through two air stones at a flow rate of about 2.2 L min^{-1} for 15 min per pot. The dissolved oxygen level was confirmed to be less than 1.0 mg L^{-1} by dissolved-oxygen meter (SG6-ELK, Mettler Toledo, Greifensee, Switzerland).

Growth parameters

Plants were harvested before waterlogging treatment (i.e. after pretreatment) and after 7 days of waterlogging treatment under stagnant conditions. Shoot fresh weight (FW), shoot length, leaf age, longest root length and total root number were recorded for each plant. Chlorophyll content of leaves was measured using a Soil Plant Analysis Development (SPAD) meter

(SPAD-502Plus, Konica Minolta, Tokyo, Japan). The chlorophyll content was read at the middle of a leaf. During the 7 days of waterlogging treatment only the 1st leaf (the oldest leaf) was read. After 7 days of waterlogging treatment (i.e. when the seedlings were 20 day-old), all leaves (i.e. 1st, 2nd and 3rd leaves) were read.

Root porosity

Root porosity (P) is a measure of the gas volume in the roots:

$$P = \frac{V_g}{V_r} \times 100$$

where V_g is the gas volume in the roots (including aerenchyma and intercellular space) and V_r is the volume of the root tissue. It is measured by determining root buoyancy before and after vacuum infiltration of water into the gas spaces in the roots (Katayama, 1961; Raskin, 1983; Thomson, Armstrong, Waters & Greenway, 1990).

Roots were cut into 50 mm segments. All of the segments from one plant were combined, gently blotted to remove excess water and weighed (w_1). Three paper clips were used to hold the segments together and act as a sinker. Using an underhook balance (PA213CJP, Ohaus corporation, New Jersey, USA), which weighs objects suspended below the balance, we measured the weight of just the paper clips hanging from a metal hook below the balance in a 2-L beaker of water (w_2) and the weight of the roots held with the same paper clips in the water (w_3). A smaller beaker containing the roots and paper clips in the water was placed in a vacuum desiccator and subjected to three 5-min periods of light vacuum to release the gas in the roots and infiltrate the roots with water. Finally, the paper clips and infiltrated roots were weighed in water (w_4). Following Thomson et al. (1990), the above equation can then be expressed as

$$P = \frac{w_4 - w_3}{w_1 + w_2 - w_3} \times 100$$

Aerenchyma formation

Adventitious roots (8–15 cm length) were cut at the root–shoot junction. Formation of aerenchyma was measured in cross-sections at 15–20 mm from the apex. Parts of the roots were collected and stored in deionized water at 4°C for no more than 4 days before observation. Fresh roots were sliced by hand under a stereomicroscope (Leica S9D, Leica

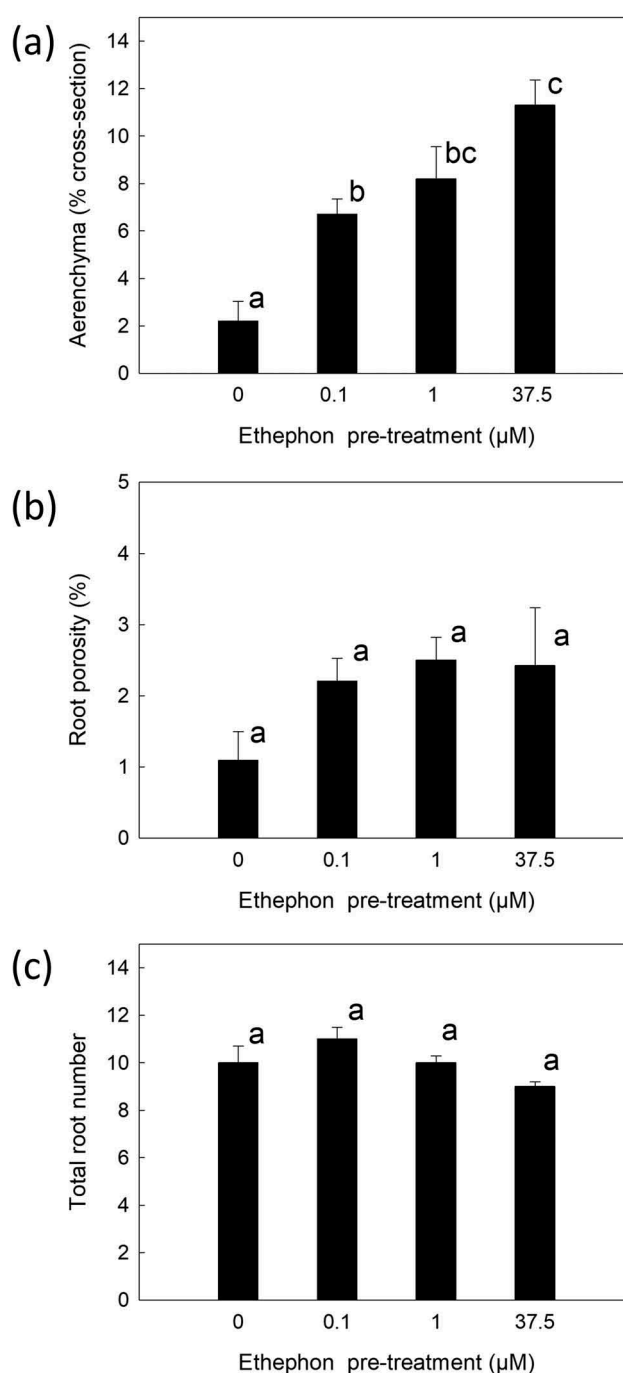


Figure 1. Effect of ethephon pretreatment on aerenchyma formation and root porosity. (a) Aerenchyma formation, expressed as a per cent of the cross-section it occupies, at the 2 cm behind the apex of adventitious roots. Values show means \pm SE. $n = 4$ –7. The lengths of adventitious roots ranged from 9 to 15 cm. (b) Root porosity, total gas volume per volume of whole root system. Means \pm SE. $n = 3$ –5. (c) Total root number. Mean \pm SE. $n = 6$. Different letters denote significant differences among four different concentrations of ethephon pretreatment ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon.



Figure 2. Effect of ethephon pretreatment on root system.

Overview of root system. Scale bar = 5 cm. Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon.

Biosystems, Wetzlar, Germany). The cross-sections were viewed with an Axio Imager A2 microscope and photographed with an AxioCam MRC camera (both Carl Zeiss, Oberkochen, Germany). The proportion of each cross-section occupied by aerenchyma was determined using Image J software (Ver. 1.50i; NIH, Bethesda, USA).

TTC reduction assay

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) is a colourless compound that turns red when reduced by dehydrogenases in living cells. TTC (WAKO, Osaka, Japan) was dissolved in 0.1 M sodium phosphate buffer

(pH 7.0) to a final concentration of 0.6% (w/v). To determine whether a barley root was alive, the root was cut at the root-shoot junction, incubated in TTC solution at 40°C for 30 min, rinsed in deionized water and scanned with a scanner (Colour mode, 400 dpi; EPSON Perfection V800 Photo, SEIKO EPSON, Suwa, Japan). Root tips that turned red were considered to be alive. The percentage of viable root-tips (V) was taken as the per cent of roots that were alive:

$$V = \frac{a}{n} \times 100$$

where a is the number of root-tips turned red by TTC staining and n is the root numbers.

Table 1. Growth of barley seedlings after 7 days ethephon pretreatment (13 day-old-seedlings).

Ethephon pretreatment (μM)	Shoot FW (g)	Shoot length (cm)	Leaf age	Longest roots (cm)	Total root number
0	0.95 ± 0.07^b	27 ± 1^c	2.6 ± 0.0^a	22 ± 1^b	10 ± 0.5^{ab}
0.1	0.99 ± 0.09^b	25 ± 0^{bc}	2.7 ± 0.1^{ab}	21 ± 1^b	11 ± 0.4^b
1	0.74 ± 0.06^a	24 ± 1^b	2.5 ± 0.0^a	13 ± 1^a	10 ± 0.7^{ab}
37.5	0.62 ± 0.06^a	18 ± 0^a	2.8 ± 0.1^b	10 ± 1^a	9 ± 0.3^a

Mean \pm SE. $n = 7$ – 8 . Different superscript letters denote significant differences among concentrations of ethephon treatments ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon.

Statistical analysis

In comparisons of more than three groups, the means were compared with one-way ANOVA and Tukey HSD tests for multiple comparisons at the 5% probability level. The data were analysed with IBM SPSS Statistics version 25 (IBM, New York, USA). Differences in percentage of viable root-tips were compared with Fisher's exact test at the 5% probability level. The data were analysed with R version 3.5.0 (R Core Team, 2018) (R packages: pwr).

Results

Effects of ethephon on morphology and growth

After ethephon pretreatment, aerenchyma formation (% cross-section) was significantly enhanced at 2 cm from the root apex ($P < 0.05$, Figure 1(a)). Ethephon pretreatments at concentrations of 0.1, 1 and 37.5 μM resulted in aerenchyma occupying 6.7%, 8.2% and 11.3% of the cross-sections, respectively. The percentages of aerenchyma were 2.2% without ethephon pretreatment. Thus, pretreatment with ethephon at concentrations of 0.1, 1 and 37.5 μM increased aerenchyma formation 3.0, 3.7 and 5.1 times, respectively. Ethephon pretreatment at concentrations of 70 and 150 μM remarkably reduced root porosities (data not shown), so that we used 37.5 μM as the maximum concentration. Root porosities were not significantly enhanced by ethephon pretreatment, being lower than 3% even with 37.5 μM ethephon ($P > 0.05$, Figure 1(b)).

We observed that plants with 1 or 37.5 μM ethephon pretreatment had shallower root systems than did untreated plants (Figure 2). The lengths of the longest roots with 1 or 37.5 μM ethephon were 10 and 13 cm, respectively (Table 1), significantly shorter than the longest roots (21–22 cm) in plants treated with 0 and 0.1 μM ethephon ($P < 0.05$). However, the ethephon treatments did not significantly affect total root numbers ($P > 0.05$, Table 1). Thus, these shallow root systems were caused by a reduction of root growth. Treatments of 1 and 37.5 μM ethephon also reduced shoot FW ($P < 0.05$, Table 1).

Effect of ethephon on waterlogging tolerance

Without ethephon pretreatment, the first (oldest) leaf was dark green. Its chlorophyll content (SPAD value) remained high during the first 3 days in the stagnant agar solution, declined after 4 days and fell to zero at 6 days as the leaf turned yellowish-white (Figure 3). Pretreatment with ethephon, especially at the higher concentrations, slowed the decline in the SPAD value of the first leaf. Pretreatment with 37.5 μM ethephon significantly increased the SPAD value after 5 and 6 days of stagnant treatment ($P < 0.05$). Thus, ethephon delayed the whitening of the first leaf. At 7 days of stagnant treatment, ethephon reduced the number of yellowish-white leaves (Figure 4(a)). Ethephon also significantly increased the SPAD values in second and third leaves ($P < 0.05$, Figure 4(b)). Ethephon at 37.5 μM remarkably increased the SPAD values in all leaves.

Regarding growth under stagnant conditions, ethephon pretreatment did not noticeably increase the FW of shoots, shoot length, leaf age or total root

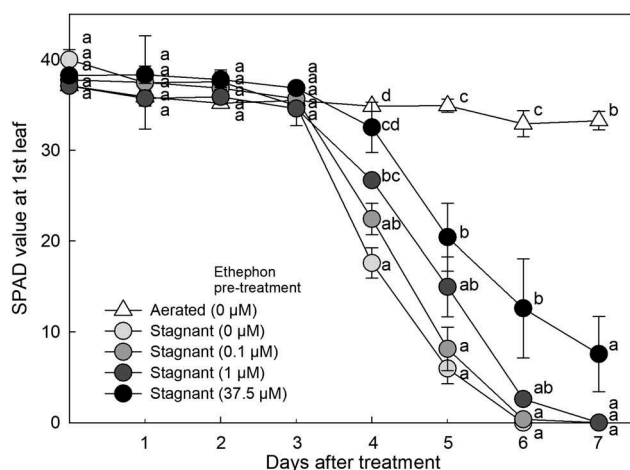


Figure 3. Changes of chlorophyll content (SPAD value) of 1st leaf during waterlogging stress induced by stagnant deoxygenated nutrient solutions.

Means \pm SE. $n = 3$ – 6 . Different letters denote significant differences among different concentrations of ethephon pre-treatment ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution or stagnant deoxygenated nutrient solution for 7 days.

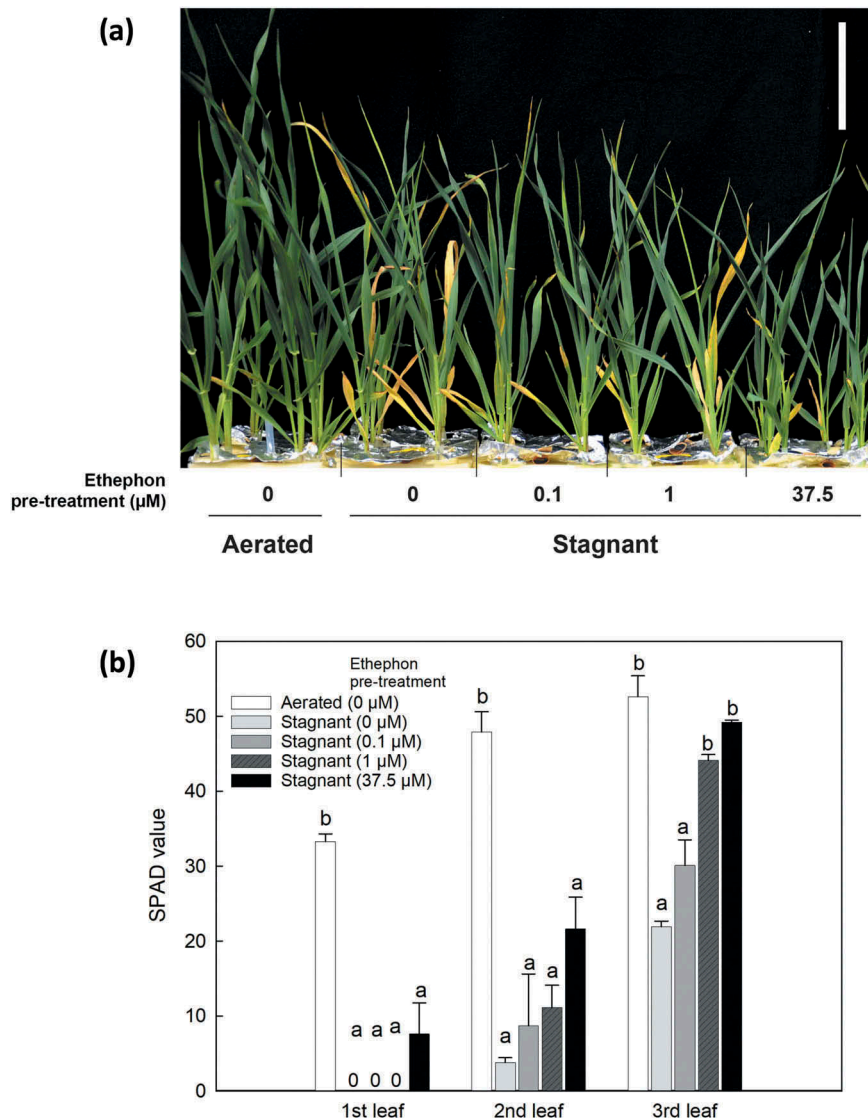


Figure 4. Condition of leaves at 7 days after stagnant treatments.

(a) Overview of leaf colour. Scale bar = 10 cm. (b) SPAD values of 1st, 2nd or 3rd leaves. Means \pm SE. $n = 3$. Different letters denote significant differences among different concentrations of ethephon pre-treatment ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon. Thirteen-day-old seedlings were transferred to pots containing aerated nutrient solution or stagnant deoxygenated nutrient solution for 7 days.

number ($P > 0.05$, Table 2). Without ethephon pre-treatment, shoot FW in stagnant conditions was reduced to 61% of the FW under aerated conditions. The reductions of shoot FW with ethephon pretreatment ranged from 56% to 64%, close to the value of untreated plants grown under stagnant conditions. However, the per cent increase in the growth of the longest root lengths during the treatment period was significantly greater in the 1 μM and 37.5 μM ethephon treatments than in the control ($P < 0.05$, Table 3). This implies that the longest roots kept growing under stagnant conditions.

Root activities under stagnant conditions

After 2 days of stagnant conditions, most of roots treated with 37.5 μM ethephon were still alive, although many inactive roots were observed in the untreated plants (Figure 5). After 7 days of stagnant treatment, most of the root apices of the plants treated with 37.5 μM ethephon were still alive (i.e. they turned red with TTC staining), while many of the roots of the untreated plants were inactive (Figure 6(a)). For roots with lengths of 11–15 cm, the percentage of viable root-tips increased dramatically with increasing ethephon concentration ($P < 0.05$, Figure 6(b)). Few inactive roots were observed

Table 2. Effects of ethephon pretreatment on the growth of barley seedlings after 7 days stagnant treatments (20 day-old-seedlings).

Ethephon pre-treatment (μM)	Aerated	Stagnant	% control (Stagnant/Aerated) [§]
Shoot FW (g)			
0	3.96 \pm 0.67 ^a	2.40 \pm 0.17 ^a	61 \pm 4 ^a
0.1	4.11 \pm 0.79 ^a	2.43 \pm 0.09 ^a	59 \pm 2 ^a
1	3.28 \pm 0.51 ^a	2.09 \pm 0.10 ^a	64 \pm 3 ^a
37.5	3.20 \pm 0.67 ^a	1.78 \pm 0.08 ^a	56 \pm 3 ^a
Shoot length (cm)			
0	36 \pm 1 ^a	32 \pm 1 ^b	90 \pm 3 ^{ab}
0.1	34 \pm 2 ^a	32 \pm 1 ^b	92 \pm 4 ^b
1	33 \pm 2 ^a	31 \pm 1 ^b	93 \pm 2 ^b
37.5	30 \pm 1 ^a	24 \pm 1 ^a	79 \pm 2 ^a
Leaf age			
0	4.8 \pm 0.2 ^a	3.8 \pm 0.2 ^a	79 \pm 4 ^a
0.1	5.0 \pm 0.2 ^a	3.9 \pm 0.0 ^a	78 \pm 1 ^a
1	5.4 \pm 0.3 ^a	3.9 \pm 0.0 ^a	73 \pm 0 ^a
37.5	5.5 \pm 0.2 ^a	3.9 \pm 0.1 ^a	71 \pm 1 ^a
Longest root length (cm)			
0	50 \pm 2 ^b	18 \pm 1 ^a	37 \pm 2 ^a
0.1	47 \pm 3 ^{ab}	18 \pm 2 ^a	38 \pm 3 ^a
1	41 \pm 2 ^{ab}	15 \pm 1 ^a	36 \pm 2 ^a
37.5	36 \pm 3 ^a	13 \pm 1 ^a	36 \pm 2 ^a
Total root number			
0	19 \pm 2 ^a	20 \pm 1 ^b	104 \pm 3 ^b
0.1	19 \pm 2 ^a	20 \pm 2 ^b	106 \pm 11 ^b
1	17 \pm 2 ^a	19 \pm 1 ^b	111 \pm 7 ^b
37.5	19 \pm 2 ^a	13 \pm 1 ^a	69 \pm 3 ^a

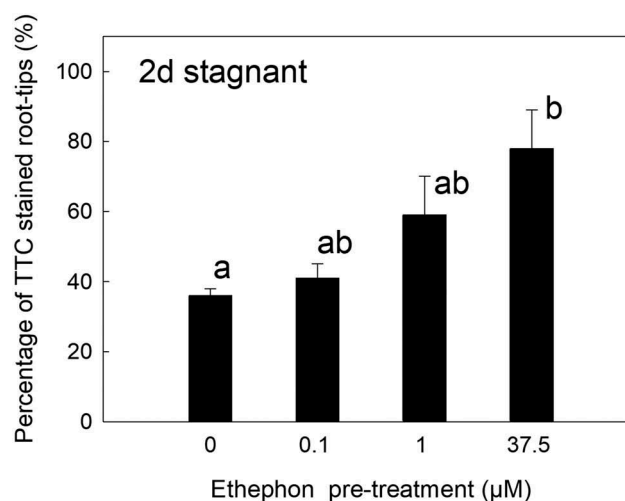
Mean \pm SE. $n = 3-6$. Different superscript letters denote significant differences among concentrations of ethephon treatments ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). [§]Per cent controls were calculated by each growth in aerated or stagnant solution with each concentration of pretreatment. Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon. Thirteen-day-old seedlings were transferred to pots containing stagnant deoxygenated nutrient solution for 7 days.

in the plants treated with 37.5 μM ethephon. Ethephon pretreatment maintained the viability of roots over 11 cm length under stagnant conditions.

Discussion

Ethylene is a key inducer of adaptive responses to waterlogging including aerenchyma formation, development of new adventitious roots and activation of fermentation pathways (Bailey-Serres & Voeselek, 2008; Shiono et al., 2008). Ethephon pretreatment enhanced aerenchyma

development at the root tip before waterlogging, but not at the basal part of root (Figure 1). Additionally, plants pre-treated with ethephon developed a shallow root system and their longest roots were shorter than those of untreated plants (Figure 2, Table 1). Ethylene inhibits root extension (Visser, Nabben, Blom & Voeselek, 1997; Visser & Pierik, 2007). A shallow root system allows plants to grow and survive in waterlogged soil because the infiltration of atmospheric air keeps the oxygen concentration at the soil surface relatively high (Jackson & Drew, 1984; Shiono et al., 2008). For instance, some wetland plants including rice have shallow root systems (Jackson & Drew, 1984; Kawata, Yamazaki, Ishihara, Shibayama & Lai, 1963; Shiono et al., 2008). Wheat cultivars with shallow root system have a higher waterlogging tolerance rather than cultivars with deeper root system (Haque et al., 2012; Oyanagi et al., 2004). Dissolved oxygen levels at depths of

**Figure 5.** Percentage of viable root-tips at 2 days after stagnant treatments.

Viable root tips are indicated by red TTC staining. The number of viable root-tips is expressed as a percentage of the total number of roots. Means \pm SE. $n = 3$. Different letters denote significant differences among four different concentrations of ethephon pre-treatment ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparison). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon. Thirteen-day-old seedlings were transferred to pots containing stagnant deoxygenated nutrient solution for 2 days.

Table 3. Effects of ethephon pretreatment on the growth of barley seedlings.

Ethephon pre-treatment (μM)	% increase (20-day-old/13-day-old) [§]			
	Shoot FW	Shoot length	Leaf age	Longest roots
0	263 \pm 18 ^a	121 \pm 3 ^a	147 \pm 7 ^{ab}	86 \pm 5 ^a
0.1	249 \pm 9 ^a	127 \pm 5 ^a	139 \pm 1 ^b	83 \pm 8 ^a
1	281 \pm 13 ^a	127 \pm 2 ^a	152 \pm 0 ^{ab}	117 \pm 5 ^{ab}
37.5	285 \pm 13 ^a	137 \pm 4 ^a	136 \pm 2 ^a	144 \pm 8 ^b

Mean \pm SE. $n = 3$. Different superscript letters denote significant differences among concentrations of ethephon treatments ($P < 0.05$, one-way ANOVA and then Tukey HSD test for multiple comparisons). Plants were grown in aerated nutrient solution for 6 days, and then transferred to aerated nutrient solution for 7 days in the absence or presence of ethephon. Thirteen-day-old seedlings were transferred to pots containing stagnant deoxygenated nutrient solution for 7 days. [§]Per cent increases were calculated from each value at the commencement day of stagnant treatment (13-day-old seedlings) and the end day of the treatment (20-day-old seedlings).

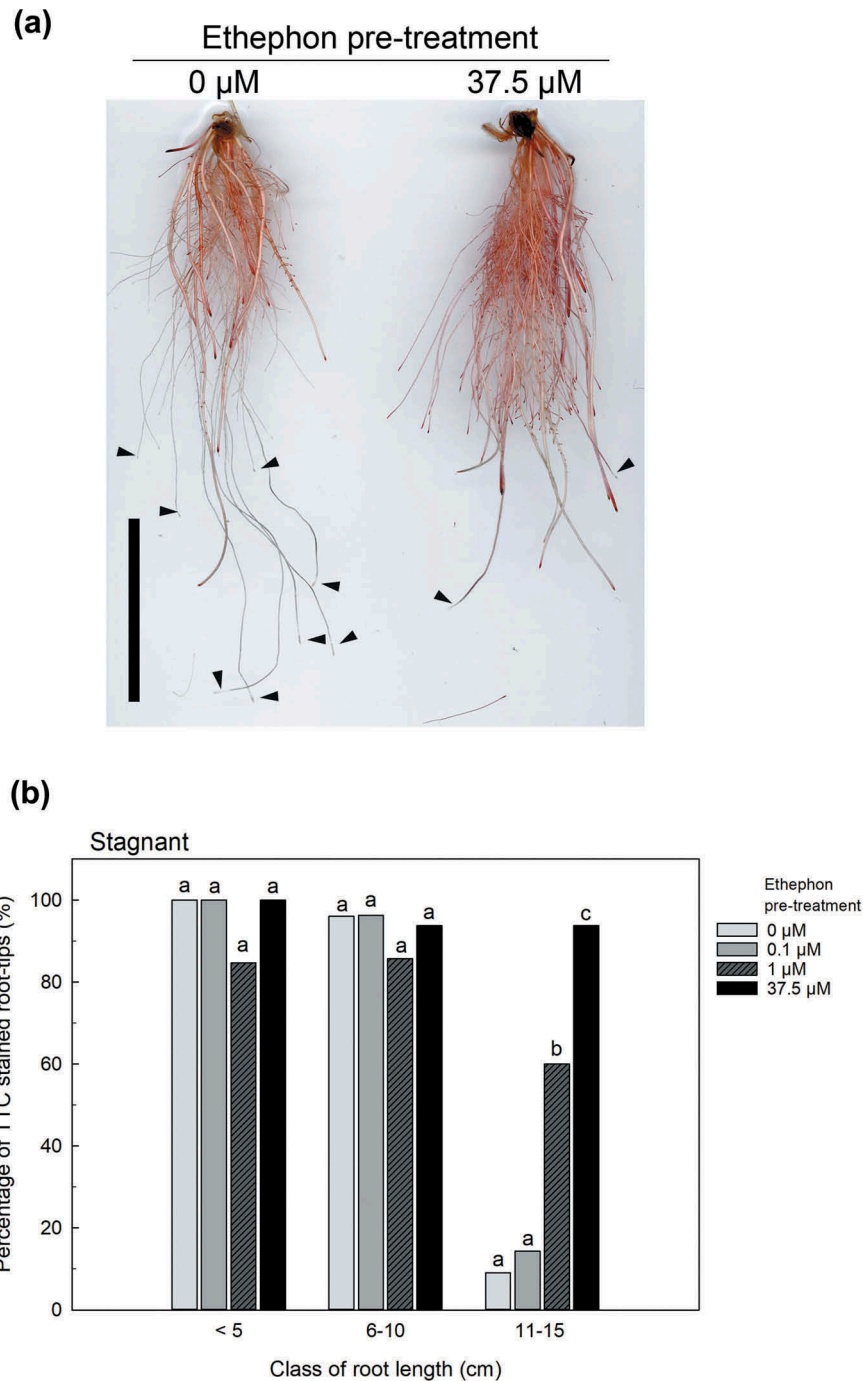


Figure 6. Root-tip activities and percentages of viable root-tips at 7 days after stagnant treatments.

(a) Root-tip activities visualized by TTC staining. Red colour indicated live cells. Arrowhead denotes non-stained root-tips (i.e. inactive root-tips) by TTC staining. Scale bar = 5 cm. (b) Percentage of viable root-tips in each class of root length. The number of viable root-tips is expressed as a percentage of root number in each root-length class. For ethephon pre-treatments of 0, 0.1, 1 and 37.5 μ M, 53, 54, 56 and 39 roots from 3 independent plants, respectively, were used to calculate the percentages of viable roots. Different letters denote significant differences among four different concentrations of ethephon pre-treatment ($P < 0.05$, Fisher's exact test for multiple comparisons).

1, 5 and 15 cm in the stagnant deoxygenated agar solutions after 7 days were 4.9 ± 0.1 , 1.5 ± 0.2 and 0.3 ± 0.0 mg/L (mean \pm SE), respectively. The oxygen concentrations were higher at the surface of stagnant solutions than at the bottom (Supplemental Table 1). Under waterlogged conditions, longer roots (> 10 cm) were inactive

(TTC-negative), but relatively short roots (≤ 10 cm) were still active without ethephon pretreatment (Figure 6(b)). The shallow root system in treated plants may contribute to waterlogging tolerance. Additionally, ethephon pre-treatment enhanced aerenchyma formation (Figure 1(a)). A mathematical simulation (Armstrong, 1979) predicted

that non-aerenchymatous roots would be limited to a depth because of the difficulty of respiration at the root tips, while aerenchymatous roots could go deeper. Enhancement of aerenchyma formation and shallow root system by ethephon pretreatment might maintain the activities of root tips under waterlogged conditions (Figures 4,5), which could explain the delay of leaf chlorosis during waterlogged treatments (Figures 2,3).

Pre-treating wheat with ACC, an ethylene precursor, was also found to improve its waterlogging tolerance (Yamauchi et al., 2014). However, there are a few dissimilarities between the present results and the previous report. In this study, the only effect of ethephon on barley growth was that it increased the length of the longest roots (Table 3). The growth parameters on shoot growth (i.e. Shoot FW and shoot length) were not improved by ethephon pretreatment (Tables 2,3). On the other hand, in wheat, 2-days of pretreatment using 20 μ M ACC, an ethylene precursor, enhanced not only shoot length and shoot dry weight but also the lengths of the longest roots (Yamauchi et al., 2014). Different responses to ACC and ethephon were sometimes observed in other plants and tissues (Lavee & Martin, 1981). The effects of ACC were limited to the treated tissues. Additionally, exogenously applied ACC failed to have prolonged effects (Zhang & Wen, 2010). Ethephon can release ethylene over longer periods. However, it may also cause non-specific responses (Zhang & Wen, 2010). Another difference is that when ethephon is degraded in aqueous solution, it produces toxic acids (e.g. phosphoric acid and hydrochloride) (Biddle et al., 1976; Goudey, Saini & Spencer, 1987).

Although ethephon pretreatment did not significantly increase growth in this study, it prolonged the survival of barley for a few days under waterlogging conditions. Further studies are needed to optimize the period, growth stage and concentrations of ethephon treatment.

Acknowledgments

We thank Prof. Koji Murai for amplifying the seeds of Barley (cv. Morex). We thank Prof. Timothy D. Colmer and Prof. Toshihito Yoshioka for discussion and comments on our experiments.

Author contributions

K.S. designed the study; K.S. M.E., K.S. and S.Y. performed the experiments; K.S. drafted the manuscript. All authors interpreted data, edited the manuscript, and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Fukui prefectural university regional contribution research project participated by local residents and JSPS under Grant [JP16KK0173 and JP17K15211].

ORCID

Katsuhiro Shiono  <http://orcid.org/0000-0001-7897-978X>

References

- Armstrong, W. (1979). Aeration in higher plants. *Advances in Botanical Research*, 7, 225–332.
- Bailey-Serres, J., & Voeseenek, L. A. C. J. (2008). Flooding stress: Acclimations and genetic diversity. *Annual Review of Plant Biology*, 59, 313–339.
- Bertholdsson, N.-O. (2013). Screening for barley waterlogging tolerance in Nordic barley cultivars (*Hordeum vulgare* L.) using chlorophyll fluorescence on hydroponically-grown plants. *Agronomy*, 3, 376–390.
- Biddle, E., Kerfoot, D. G. S., Kho, Y. H., & Russell, K. E. (1976). Kinetic studies of the thermal decomposition of 2-chloroethylphosphonic acid in aqueous solution. *Plant Physiology*, 58, 700–702.
- Colmer, T. D. (2003). Aerenchyma and an inducible barrier to radial oxygen loss facilitate root aeration in upland, paddy and deep-water rice (*Oryza sativa* L.). *Annals of Botany*, 91, 301–309.
- Drew, M. C., Jackson, M. B., & Giffard, S. (1979). Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptive responses to flooding in *Zea mays* L. *Planta*, 147, 83–88.
- Ernst, W. H. O. (1990). Ecophysiology of plants in waterlogged and flooded environments. *Aquatic Botany*, 38, 73–90.
- Gent, M. P. N., & McAvoy, R. J. (2000). Plant growth retardants in ornamental horticulture. In A. S. Basra (Ed.), *Plant growth regulators in agriculture and horticulture: Their role and commercial uses* (pp. 89–130). New York, NY: Food Product Press.
- Goudey, J. S., Saini, H. S., & Spencer, M. S. (1987). Uptake and fate of ethephon ([2-chloroethyl] phosphonic acid) in dormant weed seeds. *Plant Physiology*, 85, 155–157.
- Haque, M. E., Oyanagi, A., & Kawaguchi, K. (2012). Aerenchyma formation in the seminal roots of Japanese wheat cultivars in relation to growth under waterlogged conditions. *Plant Production Science*, 15, 164–173.
- Jackson, M. B., & Drew, M. C. (1984). Effects of flooding on growth and metabolism of herbaceous plants. In T. T. Kozlowski (Ed.), *Flooding and plant growth* (pp. 47–128). New York, NY: Academic Press.
- Jackson, M. B., Fenning, T. M., Drew, M. C., & Saker, L. R. (1985). Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L. by small partial pressures of oxygen. *Planta*, 165, 486–492.
- Katayama, T. (1961). Studies on the intercellular spaces in rice I. *Japanese Journal of Crop Science*, 29, 229–233.
- Kawai, M., Samarajeewa, P. K., Barrero, R. A., Nishiguchi, M., & Uchimiya, H. (1998). Cellular dissection of the degradation

- pattern of cortical cell death during aerenchyma formation of rice roots. *Planta*, *204*, 277–287.
- Kawata, S., Yamazaki, K., Ishihara, K., Shibayama, H., & Lai, K.-L. (1963). Studies on root system formation in rice plants in a paddy. *Japanese Journal of Crop Science*, *32*, 163–180. * In Japanese with English summary.
- Kennedy, R. A., Rumpho, M. E., & Fox, T. C. (1992). Anaerobic metabolism in plants. *Plant Physiology*, *100*, 1–6.
- Konings, H. (1982). Ethylene-promoted formation of aerenchyma in seedling roots of *Zea mays* L. under aerated and non-aerated conditions. *Physiologia Plantarum*, *54*, 119–124.
- Kreuzwieser, J., Papadopoulou, E., & Rennenberg, H. (2004). Interaction of flooding with carbon metabolism of forest trees. *Plant Biology*, *6*, 299–306.
- Laanbroek, H. J. (1990). Bacterial cycling of minerals that affect plant growth in waterlogged soils: A review. *Aquatic Botany*, *38*, 109–125.
- Lamers, L. P. M., Tomassen, H. B. M., & Roelofs, J. G. M. (1998). Sulfate-induced entrophication and phytotoxicity in freshwater wetlands. *Environmental Science and Technology*, *32*, 199–205.
- Lavee, S., & Martin, G. C. (1981). Ethylene evolution following treatment with 1-aminocyclopropane-1-carboxylic acid and ethephon in an *in vitro* olive shoot system in relation to leaf abscission. *Plant Physiology*, *67*, 1204–1207.
- Malik, A. I., Colmer, T. D., Lambers, H., Setter, T. L., & Schortemeyer, M. (2002). Short-term waterlogging has long-term effects on the growth and physiology of wheat. *New Phytologist*, *153*, 225–236.
- Mano, Y., & Omori, F. (2007). Breeding for flooding tolerant maize using “teosinte” as a germplasm resource. *Plant Root*, *1*, 17–21.
- Mano, Y., Omori, F., Takamizo, T., Kindiger, B., Bird, R. M., & Loaisiga, C. H. (2006). Variation for root aerenchyma formation in flooded and non-flooded maize and teosinte seedlings. *Plant and Soil*, *281*, 269–279.
- Mano, Y., & Takeda, K. (2012). Accurate evaluation and verification of varietal ranking for flooding tolerance at the seedling stage in barley (*Hordeum vulgare* L.). *Breeding Science*, *62*, 3–10.
- Matsunami, T., Jung, G.-H., Oki, Y., & Kokubun, M. (2007). Effect of waterlogging during vegetative stage on growth and yield in supermodulating soybean cultivar Sakukei 4. *Plant Production Science*, *10*, 112–121.
- Maynard, J.A., & Swan, J.M. (1963). Organophosphorus compounds. I. 2-chloroalkylphosphonic acids as phosphorylating agents. *Australian Journal of Chemistry*, *16*, 596–608.
- Mergemann, H., & Sauter, M. (2000). Ethylene induces epidermal cell death at the site of adventitious root emergence in rice. *Plant Physiology*, *124*, 609–614.
- Nishiuchi, S., Yamauchi, T., Takahashi, H., Kotula, L., & Nakazono, M. (2012). Mechanisms for coping with submergence and waterlogging in rice. *Rice*, *5*, 2.
- Oyanagi, A., Kiribuchi-Otobe, C., Yanagisawa, T., Miura, S., Kobayashi, H., & Muranaka, S. (2004). Growth and grain yield of wheat experimental lines with deep and shallow root system in wet paddy fields. *Japanese Journal of Crop Science*, *73*, 300–308. * In Japanese with English summary.
- Peng, H.-P., Chan, C.-S., Shih, M.-C., & Yang, S. F. (2001). Signaling events in the hypoxic induction of alcohol dehydrogenase gene in Arabidopsis. *Plant Physiology*, *126*, 742–749.
- Ponnamperuma, F. N. (1984). Effects of flooding on soils. In T. T. Kozłowski (Ed.), *Flooding and plant growth* (pp. 9–45). New York, NY: Academic press.
- Rajhi, I., Yamauchi, T., Takahashi, H., Nishiuchi, S., Shiono, K., Watanabe, R., ... Nakazono, M. (2011). Identification of genes expressed in maize root cortical cells during lysigenous aerenchyma formation using laser microdissection and microarray analyses. *New Phytologist*, *190*, 351–368.
- Raskin, I. (1983). A method for measuring leaf volume, density, thickness, and internal gas volume. *Hortscience*, *18*, 698–699.
- R core Team. (2018). R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Vienna. Retrieved from <https://www.R-project.org/>
- Roblin, G., & Pérault, J.-M. (1985). Effects of ethephon, 1-aminocyclopropane-1-carboxylic acid, and inhibitors of ethylene synthesis on the gravitropically induced movement of *Mimosa pudica* pulvinus. *Plant Physiology*, *77*, 922–925.
- Sachs, M. M., Subbaiah, C. C., & Saab, I. N. (1996). Anaerobic gene expression and flooding tolerance in maize. *Journal of Experimental Botany*, *47*, 1–15.
- Sasidharan, R., & Voesenek, L. A. C. J. (2015). Ethylene-mediated acclimations to flooding stress. *Plant Physiology*, *169*, 3–12.
- Setter, T. L., & Waters, I. (2003). Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant and Soil*, *253*, 1–34.
- Shiono, K., Ogawa, S., Yamazaki, S., Isoda, H., Fujimura, T., Nakazono, M., & Colmer, T. D. (2011). Contrasting dynamics of radial O₂-loss barrier induction and aerenchyma formation in rice roots of two lengths. *Annals of Botany*, *107*, 89–99.
- Shiono, K., Takahashi, H., Colmer, T. D., & Nakazono, M. (2008). Role of ethylene in acclimations to promote oxygen transport in roots of plants in waterlogged soils. *Plant Science*, *175*, 52–58.
- Steffens, B., Kovalev, A., Gorb, S. N., & Sauter, M. (2012). Emerging roots alter epidermal cell fate through mechanical and reactive oxygen species signaling. *The Plant Cell*, *24*, 3296–3306.
- Thomson, C. J., Armstrong, W., Waters, I., & Greenway, H. (1990). Aerenchyma formation and associated oxygen movement in seminal and nodal roots of wheat. *Plant, Cell and Environment*, *13*, 395–403.
- Visser, E. J. W., Cohen, J. D., Barendse, G. W. M., Blom, C. W. P. M., & Voesenek, L. A. C. J. (1996). An ethylene-mediated increase in sensitivity to auxin induces adventitious root formation in flooded *Rumex palustris* Sm. *Plant Physiology*, *112*, 1687–1692.
- Visser, E. J. W., Heijink, C. J., Van Hout, K. J. G. M., Voesenek, L. A. C. J., Barendse, G. W. M., & Blom, C. W. P. M. (1995). Regulatory role of auxin in adventitious root formation in two species of *Rumex*, differing in their sensitivity to waterlogging. *Physiologia Plantarum*, *93*, 116–122.
- Visser, E. J. W., Nabben, R. H. M., Blom, C. W. P. M., & Voesenek, L. A. C. J. (1997). Elongation by primary lateral roots and adventitious roots during conditions of hypoxia and high ethylene concentrations. *Plant, Cell and Environment*, *20*, 647–653.
- Visser, E. J. W., & Pierik, R. (2007). Inhibition of root elongation by ethylene in wetland and non-wetland plant species and the impact of longitudinal ventilation. *Plant, Cell and Environment*, *30*, 31–38.

- Visser, E. J. W., & Voesenek, L. A. C. J. (2004). Acclimation to soil flooding - sensing and signal-transduction. *Plant and Soil*, 254, 197–214.
- Voesenek, L. A. C. J., & Sasidharan, R. (2013). Ethylene - and oxygen signalling - drive plant survival during flooding. *Plant Biology*, 15, 426–435.
- Wiengweera, A., Greenway, H., & Thomson, C. J. (1997). The use of agar nutrient solution to simulate lack of convection in waterlogged soils. *Annals of Botany*, 80, 115–123.
- Yamauchi, T., Watanabe, K., Fukazawa, A., Mori, H., Abe, F., Kawaguchi, K., ... Nakazono, M. (2014). Ethylene and reactive oxygen species are involved in root aerenchyma formation and adaptation of wheat seedlings to oxygen-deficient conditions. *Journal of Experimental Botany*, 65, 261–273.
- Zhang, W., & Wen, C.-K. (2010). Preparation of ethylene gas and comparison of ethylene responses induced by ethylene, ACC, and ethephon. *Plant Physiology and Biochemistry*, 48, 45–53.
- Zhang, X., Zhou, G., Shabala, S., Koutoulis, A., Shabala, L., Johnson, P., ... Zhou, M. (2016). Identification of aerenchyma formation-related QTL in barley that can be effective in breeding for waterlogging tolerance. *Theoretical and Applied Genetics*, 129, 1167–1177.