Phenotypic variation in root development of 162 soybean accessions under hypoxia condition at the seedling stage

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ABSTRACT

Soybean is often damaged by hypoxia caused by waterlogging at the seedling stage. Hypoxia severely inhibits root development and retards plant growth. We aimed to clarify phenotypic variation in root development under hypoxia condition at the seedling stage using diverse soybean accessions. Root development in 162 accessions was evaluated in hydroponic culture. Substantial changes under hypoxia were investigated by means of WinRHIZO analysis before and after the treatment. We found significant phenotypic variation in hypoxia tolerance in root among the 162 accessions. A principal components analysis indicated an association between hypoxia tolerance and the country of origin. We found three new accessions which have a high ability to develop roots under hypoxia (Kokubu 7, Maetsue zairai 90B, and Yahagi). Root development in selected accessions was also evaluated in soil culture. Root development levels in hydroponic and soil culture were significantly correlated. These results will provide important information on waterlogging damage in regions where waterlogging occurs. The three accessions with hypoxia-tolerant roots might be useful for genetic improvement of waterlogging tolerance of modern soybean varieties.

ABBREVIATIONS: ANOVA: analysis of variance; ARD: average root diameter; CARD: change in average root diameter; DAS: days after sowing; IRL: increment in total root length; IRSA: increment in total root surface area; LS: large plants with hypoxia-sensitive roots; LT: large plants with hypoxia-tolerant roots; PC: principal component; PCA: principal components analysis; RD: Wroot dry weight; RL: total root length; RSA: total root surface area; RV: total root volume; SDW: shoot dry weight; SNP: single-nucleotide polymorphism; SS: small plants with hypoxia-sensitive roots; ST: small plants with hypoxia-tolerant roots

Introduction

Waterlogging is a major environmental stress for soybean (Glycine max (L.) Merr.). Soybean is often grown in fields converted from paddy rice or undrained lowlands in parts of monsoon Asia (Kokubun, 2013) such as Japan (Githiri et al., 2006), Viet Nam (Loc et al., 2015), and Indonesia (Ghulamahdi et al., 2016), as well as in other parts of the world, such as the Mississippi Delta of the U.S.A (Linkemer et al., 1998; VanToai et al., 2010) and the lowlands of Brazil’s Rio Grande (Pedó et al., 2015). In such areas, soil easily becomes waterlogged after rainfall. As a result, soybean often faces waterlogging stress. This stress inhibits plant growth, leading to decreased dry weight of roots and shoots (Henshaw et al., 2007a, 2007b; Sallam & Scott, 1987; Shimamura et al., 2003), decreased photosynthesis (Loc et al., 2015; Oosterhuis et al., 1990), decreased nitrogen fixation (Bacanamwo & Purcell, 1999; Sung, 1993), and decreased greenness of leaves (Bacanamwo & Purcell, 1999). Thus, grain yield is severely reduced by waterlogging stress at different growth stages (Linkemer et al., 1998; Oosterhuis et al., 1990; Rhine et al., 2010; Sullivan et al., 2001).

Depletion of oxygen in the rhizosphere has been proposed as the main factor responsible for waterlogging stress. The gas diffusion rate in water is approximately four orders of magnitude slower than that in air (Armstrong, 1980; Armstrong & Drew, 2002). The oxygen remaining in waterlogged soil is quickly consumed by the respiration of plant roots and micro-organisms. If the flooding lasts long enough, soybean develops adventitious roots and aerenchyma that transport oxygen from the shoot to the roots (Shimamura et al., 2003, 2010; Thomas et al., 2005). Roots still suffer from an oxygen deficiency because of the slow transport rate, forcing their tissues to depend...
on the inefficient anaerobic respiration pathway (Drew, 1983; Perata & Alpi, 1993). Under hypoxia, root development and nitrogen fixation are inhibited (Bacanamwo & Purcell, 1999). Thus, oxygen deficiency decreases the plant’s transpiration rate and causes leaf yellowing, probably due to decreased nitrogen uptake (Araki, 2006). Therefore, the ability of soybean to develop roots under hypoxia is an important trait for genetic improvement of hypoxia tolerance.

Soybean is damaged more by waterlogging during the early vegetative stage (at V2) and early reproductive stages (from R1 to R3) (Linkemer et al., 1998). Long spells of rainy weather overlap during the early vegetative stages of soybean in mainland Japan, leading to waterlogging (Githiri et al., 2006). Root growth and morphology under waterlogging and the resulting hypoxia during these early stages have been verified in soil and hydroponic cultivation systems. In soil cultivation, root dry weight (RDW) had decreased significantly by 7 days of waterlogging at the V1 stage (Sallam & Scott, 1987; Valliyodan et al., 2014; Yamane & Iijima, 2016). Although genetic variation in the response to waterlogging at the flowering stage was observed in various soybean cultivars (Rhine et al., 2010; VanToai et al., 1994, 2010), few researchers have screened cultivars by root morphology under waterlogging during the early vegetative stage. In a study using hydroponic culture, Iyodaizu was selected from 92 accessions as a hypoxia-tolerant cultivar on the basis of the ability of its seedlings to develop roots under hypoxia (Sakazono et al., 2014). In addition, Shoku-kei 32 and Toyohomare showed high waterlogging tolerance (Jitsuyama, 2015).

These researches in soybean roots were focused on the phenotype only after hypoxia treatment. Substantial root change during hypoxia treatment was not evaluated. However, early growth in soybean is diverse among cultivars (Vandamme et al., 2016). Thus, the difference of plant size before hypoxia treatment makes evaluation of hypoxia tolerance less clear. To solve this problem, it will be necessary to develop a reliable method that can evaluate substantial root growth under hypoxia by measurement before and after the treatment.

Soybean appears to have originated and been domesticated in China (Hymowitz, 1990). Since its domestication, it has spread widely and is grown around the world. Kaga et al. (2012) showed phenotypic variation in parameters such as seed size and flowering time among accessions distributed around the world. Although waterlogging tolerance during vegetative growth has not been compared among Asian accessions (including Chinese accessions) and those in the United States, their tolerance during reproductive growth is higher than that of American accessions (VanToai et al., 1994, 2010).

A wide diversity of soybean accessions is required in research to identify genes that are potentially responsible for hypoxia tolerance and that can be used as resources in genetic improvement programs. Given the higher tolerance of some Chinese and Asian accessions, they are an important component of any such research program. Japan’s National Institute of Agrobiological Sciences has established a mini core collection that consists of soybeans from around the world, including dozens of Chinese and Asian accessions, and has evaluated them for genetic variations based on single-nucleotide polymorphisms (SNPs) and their expressed phenotypic variations (Kaga et al., 2012).

Our objectives in the present study were (i) to establish an efficient system to identify soybean accessions with substantial hypoxia tolerance in root combined with an analytical method, (ii) to reveal phenotypic variation in root development under hypoxia associated with the country of origin of the accessions, and (iii) to select soybean parents with hypoxia-tolerant roots for use in a breeding program to develop waterlogging-tolerant soybean. To accomplish this, we used 162 accessions, including the mini core collection.

Materials and methods

Experiment 1: evaluation of hypoxia tolerance of soybean in hydroponic culture

We used 162 accessions of soybean (Glycine max (L.) Merr.) in this experiment. Most were obtained from two National Institute of Agrobiological Sciences collections: 79 from the World Soybean Core Collection (https://www.gene.afrgc.go.jp/databases-core_collections_wg_en.php) and 78 from the Japanese Soybean Core Collection (https://www.gene.afrgc.go.jp/databases-core_collections_jg_en.php). Their origin is summarized in Supplementary Tables S1 and S2. An additional five Japanese accessions (Iyodaizu, Tachinagaha, Shoku-kei 32, Toyoharuka, and Toyomusume) were used (Supplementary Table S3). Iyodaizu is tolerant of and Tachinagaha is susceptible to hypoxia at the seedling stage (Sakazono et al., 2014). Shoku-kei 32, which was bred recently in Hokkaido, Japan, has high waterlogging tolerance at the flowering stage, whereas Toyoharuka is sensitive to waterlogging at this stage (Kousaka et al., 2013). Toyomusume is a major cultivar in Hokkaido and is moderately sensitive to waterlogging at the flowering stage (Jitsuyama, 2015). All seeds were multiplied at the Kyushu University Farm, Fukuoka Prefecture, Japan (33°6’N, 130°5’E), in 2013 or 2014 and stored at 4 °C until they were ready for use.

To make the germination rate uniform, six or seven seeds from each accession were placed in a Petri dish at 80% (v/v) relative humidity at 23 °C and left to absorb moisture for...
3 days in a sealed container. The treated seeds were then sown in wet vermiculite (Midorisangyou, Fukuoka, Japan) in plastic pots (51 mm in bottom diameter; 72 mm in top diameter; 116 mm in height) at 23 °C for 2 days. At 2 days after sowing (DAS), representative seedlings for each accession-condition combination (i.e. one for hypoxia and one for normoxia) were transplanted into aerated deionized distilled water and grown for 2 days. At 2 days after sowing (DAS), representative seedlings for each accession-condition combination (i.e. one for hypoxia and one for normoxia) were transplanted into aerated deionized distilled water and grown for 4 days, until 6 DAS, at 23 °C in a growth chamber. An airflow of 1.0 L min⁻¹ was continuously supplied into the water by air pumps (Figure 1(a)). Seedlings were exposed to light with an intensity of 140 μmol m⁻² s⁻¹ of photosynthetically active radiation for 14 h, followed by 10 h of darkness. A total of 20 seedlings (one each from 20 accessions) were grown in an opaque plastic container (386 mm × 256 mm × 135 mm). To ensure that the seedlings remained stably afloat, each was held in place by a silicone stopper inserted in a polystyrene board that was used to cover the container (Figure 1(a) and (b)).

Seedlings at 6 DAS (i.e. the VE vegetative growth stage) were grown for 7 days under one of two conditions: normoxia (control) and hypoxia. They were then harvested at 13 DAS (i.e. the VC vegetative growth stage) (Figure 1(c)). Under normoxia, seedlings were grown in deionized-distilled water aerated by an air pump (dissolved oxygen content >7.0 mg L⁻¹). Under hypoxia, seedlings were grown in stagnant (hypoxic) deionized-distilled water with 0.1% (w/v) agar; the solution was deoxygenated by flushing with nitrogen gas until the desired dissolved oxygen content (<1.0 mg L⁻¹) was obtained (Figure 1(b)). The dissolved oxygen concentration was measured with a DO meter (CM-51, Horiba, Kyoto, Japan) at the start (6 DAS) and end (13 DAS) of the treatment. Each accession was evaluated from three to six plants under control and hypoxia. To evaluate 162 accessions with replications (3-6 replications, single plant per replication in each accession), randomized complete block method was conducted to enhance reliability. To identify accessions that showed substantial growth under hypoxia for 7 days, we measured the root traits at 6 and 13 DAS. Total root length including lateral roots (RL), total root surface area (RSA), total root volume (RV), and average root diameter (ARD) were analyzed using the WinRHIZO software (http://regent.qc.ca/assets/winrhizo_software.html). The increments in total root length (IRL), in total root surface area (IRSA), and in total root volume (IRV), as well as the change in average root diameter (CARD), represented the difference between the initial and final values. Shoot dry weight (SDW) and root dry weight (RDW) of the seedlings were measured after drying at 80 °C for 2 days. The relative values of each root trait were calculated as the ratio of the value under hypoxia to the value under normoxia.

**Experiment 2: evaluation of waterlogging tolerance of soybean in soil**

We selected 11 accessions that showed different responses to hypoxia in experiment 1 (based on the principal components analysis described later in the Methods) for this experiment: 5 from the Japanese Soybean Core Collection...
versus hypoxia) and among accessions. In experiment 1, we performed principal components analysis (PCA) based on the averages of each trait. PCA scores of the principal components (PCs) for each accession were calculated. Accessions with a wide range of different PCA scores for hypoxia were selected for use in experiment 2. Correlations (Pearson’s r) were also calculated between the results in the hydroponic and soil experiments. All analyses were performed in version 13 of the Systat software (https://systatsoftware.com/).

Results

Experiment 1: Growth of the 162 soybean accessions grown under normoxia and hypoxia

Plants grown under hypoxia showed typical symptoms of hypoxia (Table 1; Supplementary Tables S1–S3). All root parameters differed significantly among the accessions and between the two treatments (p < 0.001). IRL, IRSA, and IRV in plants grown under hypoxia from 6 to 13 DAS were significantly lower than the corresponding values under normoxia. The mean IRL under normoxia (234.3 cm) was nearly twice that under hypoxia (126.8 cm). The mean IRSA under normoxia (37.3 cm²) was about 1.5 times that under hypoxia (25.5 cm²). The mean IRV under normoxia (0.47 cm³) was about 15% higher than that under hypoxia (0.41 cm³). On the other hand, CARD was negative under normoxia (i.e. roots decreased in diameter) but positive under hypoxia (a total of 0.115 mm larger than under normoxia).

At 13 DAS, RDW was about 9% lower under hypoxia (51 mg) than under normoxia (57 mg). SDW was about 9% higher under hypoxia (182 mg) than under noroxia (167 mg). We found significant (p < 0.01) accession × treatment interactions for all parameters except SDW.

Statistical analysis

We used a two-way analysis of variance (ANOVA) to compare the average values among treatments (normoxia versus hypoxia) and among accessions. In experiment 1, we performed principal components analysis (PCA) based on the averages of each trait. PCA scores of the principal components (PCs) for each accession were calculated. Accessions with a wide range of different PCA scores for hypoxia were selected for use in experiment 2. Correlations (Pearson’s r) were also calculated between the results in the hydroponic and soil experiments. All analyses were performed in version 13 of the Systat software (https://systatsoftware.com/).

Table 1. Growth and root traits of the 162 soybean accessions grown under normoxia and hypoxia in experiment 1. Mean ± standard deviation (SD) and the minimum and maximum values are provided for the increment of root length (IRL), increment of root surface area (IRSA), increment of root volume (IRV), change in average root diameter (CARD), shoot dry weight (SDW) and root dry weight (RDW), and F values of a two-way ANOVA.

| Trait       | Treatment | Mean ± SD  | Min. | Max.       | F value                |
|-------------|-----------|------------|------|------------|------------------------|
| IRL (cm)    | Normoxia  | 234.3 ± 57.4 | 88.7 | 381.0 | 9.30** 2125.55** 4.18** |
|             | Hypoxia   | 126.8 ± 49.7 | 30.9 | 314.9 | 15.72** 1041.29** 4.04** |
| IRSA (cm²)  | Normoxia  | 37.3 ± 11.1  | 13.3 | 65.0 | 0.045 ± 0.030 0.126 |
|             | Hypoxia   | 25.5 ± 9.3   | 6.5  | 59.2 | 0.070 ± 0.023 0.210 |
| IRV (cm³)   | Normoxia  | 0.47 ± 0.18  | 0.15 | 1.06 | 0.045 ± 0.030 0.126 |
|             | Hypoxia   | 0.41 ± 0.16  | 0.10 | 0.93 | 0.070 ± 0.023 0.210 |
| CARD (mm)   | Normoxia  | −0.070 ± 0.023 | −0.210 | 0.93 | 0.045 ± 0.030 0.126 |
|             | Hypoxia   | 0.045 ± 0.030 | −0.039 | 0.126 | 0.070 ± 0.023 0.210 |
| SDW (mg)    | Normoxia  | 167 ± 65     | 51   | 436  | 55.10** 112.88** 0.92** |
|             | Hypoxia   | 182 ± 66     | 54   | 466  | 55.10** 112.88** 0.92** |
| RDW (mg)    | Normoxia  | 57 ± 17      | 25   | 99   | 24.18** 141.58** 1.42*  |
|             | Hypoxia   | 51 ± 14      | 23   | 90   | 24.18** 141.58** 1.42*  |

Significance: **p < 0.001, *p < 0.01, ns not significant (two-way ANOVA).
Figure 2. Increment in root length (IRL) of (a) 79 accessions from the World Soybean Core Collection under normoxia and hypoxia. Iyodaizu, Shoku-kei 32, Tachinagaha, Toyomusume, and Toyoharuka are also indicated. Values with each bar indicate relative IRL.

(a) World Soybean Core Collection

(b) Japanese Soybean Core Collection
To classify the characteristics of the 162 accessions under normoxia and hypoxia, we performed PCA using the values of IRL, IRSA, IRV, CARD, and RDW, of which accession × treatment interactions are significant ($p < 0.01$) (Table 2). The first two PCs (PC1 and PC2) accounted for 80.9% of the total variance. Their eigenvalues were 6.18 and 1.91, respectively. The eigenvalue of PC3 was just over 1.0 (data not shown), but because of the low additional proportion of variance that it explained (10.9%), we used only the first two PCs in the rest of our analysis.

PC1 accounted for 61.8% of the total variance and was most strongly related to plant size (IRL, IRSA, IRV and RDW) under both normoxia and hypoxia. All these traits showed high positive factor loadings. PC2 accounted for 19.1% of the total variance and was influenced mainly by parameters related to the response of roots to hypoxia (i.e. hypoxia tolerance in root) such as IRL (with a high negative factor loading under hypoxia) and CARD (with a high positive factor loading under hypoxia).

Figure 3 presents scatterplots of PC1 versus PC2 for the three groups of accessions by the country of origin. A large positive score for PC1 represents larger plant size, and a large negative score for PC2 represents higher tolerance to hypoxia. We classified the 162 accessions into four groups based on the two PCs. The SS group represents small plants with hypoxia-sensitive roots, and had negative PC1 and positive PC2 scores (SS: PC1 < 0, PC2 > 0). The LS group represents large plants with hypoxia-sensitive roots, and had positive PC1 and PC2 scores (LS: PC1 > 0, PC2 > 0). The ST group represents small plants with hypoxia-tolerant roots, with negative PC1 and PC2 scores (ST: PC1 < 0, PC2 < 0). The LT group represents large plants with hypoxia-tolerant roots, and had positive PC1 and negative PC2 scores (LT: PC1 > 0, PC2 < 0).

By the country of origin, 15 of 22 (68%) accessions from China and Taiwan had hypoxia-tolerant roots (groups ST and LT), along with 12 of 17 (71%) accessions from Korea (Figure 3(a)). Williams 82, from the U.S.A, belonged to the SS group (Figure 3(a)). 15 of 17 (88%) accessions from South-East Asia and 18 of 22 (82%) accessions from South Asia had small plants (groups SS and ST), and most of them had negative or less positive PC2 scores. (Figure 3(b)). In contrast, 56 of 83 (67%) accessions from Japan were widely distributed in the SS and LS groups (Figure 3(c)). Another 27 (33%) were belonged to the LT and ST group. In particular, Kokubu 7, Maetsue zairai 90B, and Yahagi had large plants with hypoxia-tolerant roots.

### Table 2. Component loadings in the principal components analysis of growth and root traits. Eigenvalues and contributions to the total variance in PC1 and PC2 are provided.

|       | PC1       | PC2       |
|-------|-----------|-----------|
|       | **Normoxia** |          |
| IRL   | 0.819     | 0.160     |
| IRSA  | 0.927     | 0.232     |
| IRV   | 0.929     | 0.256     |
| CARD  | 0.006     | 0.464     |
| RDW   | 0.926     | 0.308     |
|       | **Hypoxia** |          |
| IRL   | 0.678     | −0.668    |
| IRSA  | 0.850     | −0.437    |
| IRV   | 0.916     | −0.179    |
| CARD  | 0.076     | 0.887     |
| RDW   | 0.950     | 0.050     |
| Eigen value | 6.18 | 1.914     |
| Variance (%) | 61.80 | 19.14     |

### Phenotypic variation of IRL in the 162 accessions

Among the 79 accessions from the World Soybean Core Collection, IRL under normoxia ranged from 88.7 cm (Local var. Seputih Raman) to 359.9 cm (Seita) (Figure 2(a); Supplementary Table S1). IRL under hypoxia ranged from 30.9 cm (U 1155-4) to 251.4 cm (M 42). The relative values (i.e. the ratio of the value under hypoxia to the value under normoxia) ranged from 0.214 (Baritou 3 A) to 1.048 (M 652). Higher relative values (all >0.8), which indicate higher hypoxia tolerance in root, were observed in L 2A, U-1741-2-2 No.3, M 44, Heukdaelip, Merapi, Nezumi meta, Col/Pak/1989/lbgr/2326(1), M 42, and M 652. Most other root parameters followed the same pattern as IRL, though with some differences in the variety order.

Among the 78 accessions from the Japanese Soybean Core Collection, IRL under normoxia ranged from 160.6 cm (Gin daizu) to 381.0 cm (Oojiro) (Figure 2(b); Supplementary Table S2). IRL under hypoxia ranged from 42.1 cm (Gin daizu) to 314.9 cm (Kokubu 7). The relative values ranged from 0.216 (Miyagishirome and Abura mame) to 1.246 (Yahagi). Higher relative hypoxia tolerance in root (>0.8) was observed in Yahagi, Kokubu 7, Ban kuro daizu, Daizu, Aoakimame, Kosa mame, Maetsue zairai 90B, and Mochi-daizu.

Among the 5 Japanese accessions, IRL under normoxia ranged from 210.0 cm (Iyodaizu) to 318.8 cm (Tachinagaha) (Supplementary Table S3). IRL under hypoxia ranged from 90.9 cm (Tachinagaha) to 239.9 cm (Iyodaizu). The relative IRL was highest in Iyodaizu (1.142) and lowest in Tachinagaha (0.285). Although Shoku-kei 32 had a higher relative IRL than Toyomusume and Toyoharuka, it did not outperform them in other characteristics and did not have a higher relative IRL than most other LT or ST accessions (Supplementary Table S3).

### Classification of the 162 accessions based on plant size and hypoxia tolerance in root and relation to the country of origin

To classify the characteristics of the 162 accessions under normoxia and hypoxia, we performed PCA using the values of IRL, IRSA, IRV, CARD, and RDW, of which accession × treatment interactions are significant ($p < 0.01$) (Table 2). The first two PCs (PC1 and PC2) accounted for 80.9% of the total variance. Their eigenvalues were 6.18 and 1.91, respectively. The eigenvalue of PC3 was just over 1.0 (data not shown), but because of the low additional proportion of variance that it explained (10.9%), we used only the first two PCs in the rest of our analysis. PC1 accounted for 61.8% of the total variance and was most strongly related to plant size (IRL, IRSA, IRV and RDW) under both normoxia and hypoxia. All these traits showed high positive factor loadings. PC2 accounted for 19.1% of the total variance and was influenced mainly by parameters related to the response of roots to hypoxia (i.e. hypoxia tolerance in root) such as IRL (with a high negative factor loading under hypoxia) and CARD (with a high positive factor loading under hypoxia).
We did not distinguish between lateral and adventitious roots and did not attempt to identify aerenchyma, as these characteristics will be the subject of a future study. Roots of Natto kotsubu (SS) were small under both normoxia and hypoxia (Figure 4(a)). Development of lateral roots was inhibited under hypoxia. Roots of Miyagishirome (LS) were large under normoxia, but the development of lateral roots was severely inhibited under hypoxia (Figure 4(b)). Roots of M 652 (ST) were small under normoxia, but root development showed little inhibition under hypoxia because it formed long lateral roots in the upper part (Figure 4(c)). Roots of Kokubu 7 (LT) were large under normoxia and showed no inhibition of lateral root development under hypoxia (Figure 4(d)).

Experiment 2: Waterlogging tolerance of 11 accessions in control and waterlogged soil

To clarify the hypoxia tolerance in soil cultivation, we selected 11 accessions based on the PCA results (i.e. from accessions with the negative and positive PC2 scores, we selected 11 accessions that covered a wide range of PC1 values) for use in experiment 2. Three accessions from the LS group (Tachinagaha, Miyagishirome, and Komame), two from the SS group (Nattou kotsubu and U 1155-4), two from the ST group (Okjo and M 652), and four from the LT group (Iyodaizu, Maetsue zairai 90B, Kokubu 7 and M 42) were selected by the criteria (PC1 > 0 & PC2 > 1.0, PC1 < 0 & PC2 > 1.0, PC1 < 0 & PC2 < −2.0 and PC1 > 0 & PC2 < −2.0, respectively) set strictly to evaluate the accessions which have distinctly different tolerances to hypoxia.

Table 3 shows the effects of the waterlogging treatment on all traits of these accessions, and the ANOVA results. All traits showed significant (p < 0.001) differences among accessions, significant differences between treatments, and a significant accession × treatment interaction. Plant development except for root diameter was significantly decreased by the seven-day waterlogging treatment. Mean plant sizes under control and waterlogged conditions (respectively) were 843.4 and 326.8 cm in RL, 111.5 and 59.1 cm2 in RSA, 1.19 and 0.87 cm3 in RV, 303 and 271 mg in SDW, and 83 and 57 mg in RDW; that is, all decreased under waterlogging. In contrast, ARD was significantly lower under control conditions than under waterlogging; that is, waterlogging treatment increased root diameter by 40%.

Table 4 shows the RL values of each accession. RL ranged from 613 cm (M 652; ST) to 1177 cm (Maetsue zairai 90B; LT) under control conditions, versus 216 cm (Nattou kotsubu; SS) to 498 cm (Maetsue zairai 90B; LT) under waterlogging. Relative RL, which represents the ratio of RL in the waterlogging treatment to that in the control, ranged from 0.271 (Tachinagaha) to 0.610 (Iyodaizu). The mean

Root morphology of representative accessions from the LS, SS, ST, and LT groups

To characterize root morphology in the four groups, we have illustrated the roots of representative accessions

![Figure 3](image-url)
We discovered three soybean accessions (Kokubu 7, Maetsue zairai 90B, and Yahagi) which have relatively hypoxia-tolerant roots in this study. These materials would be useful as new germplasm resources for the genetic improvement of waterlogging (hypoxia) tolerance of soybean at the seedling stage.

To identify accessions that showed substantial growth during both treatments, we compared plant growth during a seven-day hypoxia treatment with that during a seven-day normoxia treatment in experiment 1 (Figure 2; Supplementary Tables S1-S3). We accounted for differences in growth among the accessions before these treatments began using a modified hydroponic method because early growth of soybean is strongly affected by seed characteristics such as seed weight (Vandamme et al., 2016). The PCA results confirmed that hypoxia tolerance of the roots does not appear to depend on plant size (Table 2).

Correlation between the results in experiment 1 (hydroponics) and experiment 2 (soil)

Among the 11 accessions that were used in both experiments, the values of all traits under normoxia in experiment 1 and control conditions in experiment 2 were significantly positively correlated ($p < 0.001$; Table 5). The same is true of the correlation between the values under hypoxia in experiment 1 and under waterlogging in experiment 2. In addition, we found significant positive correlations between relative RL in experiment 2 and relative IRL in experiment 1 and between relative RSA in experiment 2 and relative IRSA in experiment 1 (Figure 5(a) and (b)). However, relative RV in experiment 2 was not significantly correlated with relative IRV in experiment 1 (Figure 5(c)).

Discussion

We discovered three soybean accessions (Kokubu 7, Maetsue zairai 90B, and Yahagi) which have relatively hypoxia-tolerant roots in this study. These materials would be useful as new germplasm resources for the genetic improvement of waterlogging (hypoxia) tolerance of soybean at the seedling stage.

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Root development, and especially root elongation, was dramatically inhibited and root diameter increased under hypoxia. The mean values decreased relative to the values
under normoxia: IRL by 45%, IRSA by 31%, IRV by 13%, and RDW by 9%. The value of ARD under hypoxia increased by 0.115 mm relative to normoxia. These results agree with those of a previous study, in which hypoxia decreased root elongation and branching and increased root diameter (Sakazono et al., 2014). These symptoms under hypoxia were also observed in other species (Colmer, 2003; Visser et al., 2000; Yamauchi et al., 2014).

Among root traits, RL was inhibited most under both hypoxia and waterlogged conditions (Tables 1 and 3). Therefore, RL is one of the most important indicators of waterlogging tolerance during the early growth of soybean. In addition, RL, RSA, RV, and RDW decreased and ARD increased under waterlogging in experiment 2 (Table 3). Previous studies showed that root traits such as length and dry weight were inhibited in waterlogged soil (Sallam & Scott, 1987; Shimamura et al., 2003; Henshaw et al., 2007a; Yamane & Iijima, 2016). Root length in maize also reflected its waterlogging tolerance under field conditions (Souza et al., 2012). Although waterlogging treatment in experiment 2 inhibited root development more strongly than hypoxia treatment in experiment 1, IRL and relative IRL in experiment 1 were strongly correlated with RL and relative RL in experiment 2, respectively (Table 5; Figure 5(a)). Relative IRSA in experiment 1 also correlated with relative RSA in experiment 2 (Figure 5(b)). This suggests that hypoxia tolerance under hydroponic culture reflected hypoxia tolerance in waterlogged soil at seedling stage. However, relative IRV in experiment 1 was not correlated with relative RV in experiment 2 (Figure 5(c)). The variation in RV was smaller than that in RL in this study. Soybean roots get shorter and thicker under hypoxia and

\[
RV = \pi r^2 \times RL
\]

depends on values of RL more strongly than RSA (RSA = 2\pi r \times RL). Therefore, significant correlation in relative RV would not be detected. RV may not reflect hypoxia tolerance as well as RL and RSA. SDW of plants was greater under hypoxia (hydroponic conditions) than under normoxia (Table 1). Soybean SDW has been reported to show no significant changes (Jitsuyama, 2015) or increased SDW (Sakazono et al., 2014) under short-term hypoxia in hydroponic culture. On the other hand, SDW of plants grown in waterlogged soil was significantly lower than that under control conditions (Table 3). Waterlogging generally leads to decreased shoot growth, accompanied

### Table 3. Growth and root traits of 11 representatives of the 162 soybean accessions grown in control and waterlogged soil (Experiment 2). Mean ± standard deviation (SD), minimum, and maximum values are provided for root length (RL), root surface area (RSA), root volume (RV), average root diameter (ARD), shoot dry weight (SDW) and root dry weight (RDW), and F values of a two-way ANOVA.

| Trait      | Treatment   | Mean ± SD | Min.   | Max.   | F value          |
|------------|-------------|-----------|--------|--------|-----------------|
| Accession  | Treatment   |           |        |        | Accession | Treatment | Accession × Treatment |
| RL (cm)    | Control     | 843.4 ± 172.3 | 613.2  | 1176.5 | 19.80**      |
|            | Waterlogging| 326.8 ± 104.1 | 215.6  | 497.6  | 970.86**     |
| RSA (cm²)  | Control     | 111.5 ± 32.0  | 63.2   | 162.1  | 45.38**      |
|            | Waterlogging| 59.1 ± 18.9   | 36.5   | 95.7   | 611.00**     |
| RV (cm³)   | Control     | 1.19 ± 0.45   | 0.52   | 1.89   | 91.12**      |
|            | Waterlogging| 0.87 ± 0.32   | 0.40   | 1.47   | 181.78**     |
| ARD (mm)   | Control     | 0.42 ± 0.052  | 0.328  | 0.522  | 43.29**      |
|            | Waterlogging| 0.59 ± 0.091  | 0.440  | 0.768  | 694.90**     |
| SDW (mg)   | Control     | 303 ± 94      | 125    | 398    | 144.74**     |
|            | Waterlogging| 271 ± 119     | 135    | 466    | 34.62**      |
| RDW (mg)   | Control     | 83 ± 30       | 39     | 126    | 54.02**      |
|            | Waterlogging| 57 ± 18       | 30     | 87     | 188.66**     |

**Significant at p < 0.001 (two-way ANOVA).

### Table 4. Comparison of root increments in root length (IRL) between normoxia and hypoxia in Experiment 1 and root lengths (RL) between control and waterlogged conditions in Experiment 2. Relative values represent the ratio of the value in hypoxia to the value in normoxia or the value in waterlogged soil to the value in the control. PCA groups are illustrated in Figure 3.

| Accession | PCA group | Normoxia | Hypoxia | Relative value | Control | Waterlogging | Relative value |
|-----------|-----------|----------|---------|----------------|---------|--------------|----------------|
| U 1155-4  | SS        | 134 ± 13.3 | 31 ± 5.2 | 0.231          | 622 ± 73.3 | 242 ± 40.4   | 0.388          |
| Nattou kotsubu | SS     | 162 ± 17.0 | 66 ± 43.6 | 0.409          | 698 ± 59.1 | 216 ± 32.3   | 0.309          |
| Miyagishirome | LS   | 335 ± 69.6 | 72 ± 11.3 | 0.216          | 885 ± 81.9 | 261 ± 75.0   | 0.295          |
| Komame    | LS        | 262 ± 100.3 | 94 ± 13.1 | 0.360          | 934 ± 91.1 | 270 ± 55.2   | 0.289          |
| Tachinaga  | LS        | 319 ± 55.5  | 91 ± 24.4 | 0.285          | 955 ± 172.2 | 259 ± 30.4   | 0.271          |
| M 652     | ST        | 118 ± 13.9  | 124 ± 32.9 | 1.048          | 613 ± 24.6 | 265 ± 66.7   | 0.432          |
| Okjo      | ST        | 165 ± 32.7  | 127 ± 45.5 | 0.771          | 842 ± 85.7 | 265 ± 43.8   | 0.314          |
| Iyodaizu  | LT        | 210 ± 33.1  | 240 ± 57.2 | 1.142          | 732 ± 47.0 | 447 ± 52.2   | 0.610          |
| M 42      | LT        | 258 ± 68.5  | 251 ± 37.1 | 0.973          | 811 ± 95.8 | 413 ± 24.4   | 0.509          |
| Kokubu 7  | LT        | 299 ± 59.6  | 315 ± 39.9 | 1.055          | 1008 ± 100.4 | 461 ± 145.1 | 0.457          |
| Maetsue zairai 90B | LT | 353 ± 51.0 | 299 ± 8.4  | 0.848          | 1177 ± 12.2 | 498 ± 83.2   | 0.423          |
culture systems. Waterlogging stress under soil condition would be more complex for soybean seedling than hypoxia stress in hydroponics because of other factors in addition to hypoxia such as production of toxic substance following soil reduction (Shabala, 2011). For this reason, soybean seedlings, even the accessions with hypoxia-tolerant roots in hydroponics, might be inhibited root development and SDW under waterlogging condition in experiment 2 compared to hypoxia condition in experiment 1.

We found a significant accession × treatment interaction in all aspects of plant growth except SDW (Table 1), which indicates that root development under hypoxia varied widely among the 162 accessions (Figure 3). The PCA classification of the accessions by plant growth and hypoxia tolerance in root into groups SS, LS, ST, and LT (Figure 3) showed some geographical patterns, with most accessions from a given region classified into the same group. Williams 82, from the USA, was classified in SS. There are relatively few landraces in North America compared with Asia (Gizlice et al., 1994, 1996; Hyten et al., 2006). Soybeans from the U.S.A are generally intolerant of waterlogging (VanToai et al., 2010). Although we tested only one American accession, roots of Williams 82 were hypoxia-intolerant at the seedling stage (Figure 3(a)). Hypoxia tolerance was moderate in the Chinese accessions, with a narrow range, based on the PC2 score. Genetic diversity of Chinese and Taiwanese accessions is reported to be wide (Kaga et al., 2012) because Chinese accessions are grown in a wide range of environments. However, we did not find extremely high hypoxia tolerance in root at the seedling stage in the 22 Chinese and Taiwanese accessions we tested (Figure 3(a)). Although many Korean accessions were also classified into the tolerant ST or LT groups, their tolerance was not remarkable compared with some tolerant Japanese accessions.

Accessions from south-eastern and southern Asia were classified mainly into the ST group (Figure 3(b)). Waterlogging after heavy and continuous rain often occurs in these areas (Ahmed et al., 2013), and soybeans are often grown in water-saturated soil in South-East Asia (Ghulamahdi et al., 2016). Nam Vang, a cultivar from Cambodia, showed waterlogging tolerance at the R2 reproductive stage, as indicated by a relatively stable yield (VanToai et al., 2010). This agrees with the present results, which showed that many cultivars from south-eastern and southern Asia had relatively low PC2 score and were able to tolerate hypoxia during the seedling stages. Shoku-kei 32, which was derived from the Indonesian cultivar Wilis, was classified in the LT group. Although it was reported to have high waterlogging tolerance during late growth stages (Kousaka et al., 2013), its PC2 score was less negative than that of highly tolerant accessions such as Kokubu 7 in the present study (Figure 3(c)). To adapt to frequent

### Table 5. Pearson’s correlation coefficient (r) for the relationships between the results of experiment 1 (hydroponics) and experiment 2 (soil). IRL, increment in root length (RL); IRSA, increment in root surface area (RSA); IRV, increment in root volume (RV); SDW, shoot dry weight; RDW, root dry weight.

|                      | IRL  | IRSA | IRV  | SDW | RDW |
|----------------------|------|------|------|-----|-----|
| Control (Experiment 2)| .880**| .920**| .898**| .924**| .872**|
| Hypoxia (Experiment 1)| IRL  | IRSA | IRV  | SDW | RDW |
| Waterlogging (Experiment 2)| RL  | RSA | RV  | SDW | RDW |
| r                    | .966**| .936**| .876**| .961**| .899**|

**Significant at p < 0.001.

Figure 5. Relationships between (a) relative increment in root length (IRL) in experiment 1 and relative root length (RL) in experiment 2, (b) relative increment in root surface area (IRSA) in experiment 1 and relative root surface area (RSA) in experiment 2, and (c) relative increment in root volume (IRV) in experiment 1 and relative root volume (RV) in experiment 2. *p < 0.01; ns, not significant.

by stress symptoms such as yellowing leaves, cracking of the stem, or death (Henshaw et al., 2007b; Sallam & Scott, 1987; Sullivan et al., 2001). In the present study, the adverse effects of waterlogging had already appeared in the shoots after 7 days of waterlogging treatment, accompanied by inhibition of root development in experiment 2. This suggests that shoot phenology may differ between the two
waterlogging, soybean accessions from south-eastern and southern Asia might have been selected for their high hypoxia tolerance at various growth stages. Most of them are semi-wild soybeans, still close to their wild relatives, and have small seeds, a vine-like growth habit, or late flowering. Germplasms of wild soybeans are thought to be useful materials for breeding to enhance stress tolerance, because much of the diversity in cultivated soybeans has been lost during the genetic bottleneck that occurred during domestication (Kaga et al., 2012; Lam et al., 2010). In fact, accessions that still resemble their wild relatives have high tolerance to various stresses; these include Peking, which tolerates pre-germination waterlogging (Muramatsu et al., 2008; Nakajima et al., 2015); JWS156-1, which tolerates salt stress (Hamwieh & Xu, 2008); and PI468917, which has high drought tolerance (Seversike et al., 2014). Sakazono et al. (2014) reported that some wild soybeans also had a high ability to develop roots under waterlogging during early growth stages. Wild soybean, also known as *tsurumame* (*Glycine soja*), produces large amounts of aerenchyma when grown under conditions with excess water (Arikado, 1954). Using genetic data from wild soybeans (Hyten et al., 2006; Lam et al., 2010), it should be possible to select accessions from South-East Asia and South Asia that would be useful for improving the waterlogging tolerance of soybean.

Soybeans from Japan showed wide variation in their characteristics (Figure 3(c)). Three accessions (Kokubu 7, Maetsue zairai 90B, and Yahagi) had large plant size and high hypoxia tolerance in root at the seedling stage. All are Japanese landraces (Kaga et al., 2012). Interestingly, the relative IRLs of Kokubu 7 and Yahagi were >1.0, so their root elongation under hypoxia was vigorous. In addition, Kokubu 7 and Maetsue zairai 90B showed waterlogging tolerance in the soil in experiment 2 (Table 4). The tolerance of these landraces was newly discovered in this study. Iyodaizu, another Japanese landrace, had a medium plant size with high hypoxia-tolerant roots (i.e. was an LT type). Iyodaizu was previously studied because of its high tolerance of hypoxia stress (Sakazono et al., 2014). The result of Iyodaizu in this study was similar with Sakazono et al. (2014). Kokubu 7, Maetsue zairai 90B, and Iyodaizu formed longer roots near the proximal part under waterlogging (data not shown). Root development near the soil surface was observed in waterlogged soil in a wild relative of maize, teosinte (Mano et al., 2009). Hypoxia tolerance in soybean might be related to its ability to develop lateral roots in the upper part of the soil, which is typically better aerated. On the other hand, several Japanese modern elite cultivars (Fukuyutaka, Tamahomare, Tachinagaha, Enrei, Miyagishirome, and Nattou kotsubu) were classified into the susceptible groups (LS or SS; Figure 3(c)). Thus, root development was inhibited in Tachinagaha, Miyagishirome, and Nattou kotsubu under waterlogging (Table 4). These results imply a serious problem for soybean production in Japan, because of the long spell of rainy weather during the time when soybean is sown in fields managed in rotation with paddy rice (Githiri et al., 2006). Many Japanese cultivars were susceptible to hypoxia. The soybean breeding program in Japan has focused mainly on seed quality characteristics such as seed weight and on agricultural characteristics such as flowering time (Zhou et al., 2002). During the breeding process, hypoxia tolerance traits might have been neglected. In addition to Iyodaizu, our results suggest that Kokubu 7, Maetsue zairai 90B, and Yahagi would be useful as new germplasm resources for the genetic improvement of waterlogging tolerance of soybean at the seedling stage.

Genetic analysis can be conducted in further study. Although quantitative trait loci responsible for root development under hypoxia were detected using Iyodaizu and Tachinagaha (Van Nguyen et al., 2017), crucial mechanisms of hypoxia tolerance in soybean were not yet clear. Combining SNPs information of core collections, the method and results of this study would strongly assist to identify genes related to root development under hypoxia. Information on various agro-morphological traits in core collections was accumulated and available to researchers. However, the traits related to waterlogging tolerance including root traits have not been evaluated. The data of this study would supply new information on root morphology for core collections and advance study of waterlogging tolerance combining the existing data. Additionally, evaluation of tolerance at the recovering stage of hypoxia is also necessary to verify whether the accessions with hypoxia tolerant roots improve plant development including shoot after waterlogging and establish cultivation methods to take advantage of developed roots under waterlogging.

**Conclusion**

The present study provides three main results. First, the hydroponic culture method allows more reliable measurement of the relative increment of root growth under hypoxia and normoxia, which correlates well with the values observed in soil cultivation. Second, clear phenotypic variation in root development under hypoxia condition associated with the country of origin of the accessions was found using PCA analysis, which accounted for the effects of plant size. Third, we identified three soybean accessions (Kokubu 7, Maetsue zairai 90B, and Yahagi) that have a high ability to develop roots under hypoxia and show promise as genetic resources. In future research, these accessions will provide insights into the mechanisms of waterlogging adaptation and resources to improve the waterlogging tolerance of modern cultivars at the seedling stage.
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Disclosure statement

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