STUDY ON THE EFFECTS OF QTLS FOR WATERLOGGING TOLERANCE USING NEAR-ISOGENIC LINES OF SOYBEAN (Glycine max (L.) Merr.)

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STUDY ON THE EFFECTS OF QTLS FOR WATERLOGGING TOLERANCE USING NEAR-ISOGENIC LINES OF SOYBEAN (*Glycine max* (L.) Merr.)

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

By

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LIST OF ABBREVIATIONS

AA:	Area of aerenchyma
AF	Aerenchyma formation
Chr.	Chromosome
CSA	Cut surface area
GR:	Germination rate
MAS	Maker assisted selection
NSR:	Normal seedling rate
NIL	Near-isogenic line
POA	Percentage of aerenchyma
QTLs	Quantitative trait loci
RD	Root diameter
RDD	Root diameter development
RL	Root length
RLD	Root length development
RSA	Root surface area
RSAD	Root surface area development
RV	Root volume
RVD	Root volume development
RDW	Root dry weight
SDW	Shoot dry weight
TDW	Total dry weight
WTI	Waterlogging tolerance index

ABSTRACTS

CHAPTER II

THE EFFECTS OF A QTL FOR ROOT DEVELOPMENT UNDER HYPOXIA ON YIELD OF SOYBEAN EXPOSED TO 7-DAY WATERLOGGING AT SEEDLING STAGE

In humid part of the world, waterlogging is a major environmental stress limiting the yield of soybean and its root development is an important indicator of tolerance. Recently, quantitative trait loci (QTLs) for this trait were detected on the chromosome 12 (chr. 12) of soybean through the analyses of recombinant inbred lines (RILs) developed from a cross between a hypoxia-sensitive cultivar, Tachinagaha, and a tolerant landrace, Iyodaizu. In order to get a detailed understanding of these QTLs, NIL-9-4-5, a near-isogenic line (NIL), was selected from Tachinagaha/Iyodaizu BC_6F_3 population through marker assisted selection.

In this chapter, Parents and NIL-9-4-5 was tested for yield performance under hypoxia exposed to 7-day waterlogging at the seedling stage. Plants were grown in pots containing loam soil. From six days after germination, seedlings were grown for 7 days under two treatments: control and waterlogging conditions. Pots were kept in containers where the water level was maintained at 2.0 cm depth from bottom in the control and at 2.0 cm above the soil surface in waterlogged condition.

Results showed that seed yields and root development of Iyodaizu and NIL-9-4-5 were not reduced under waterlogging, whereas those of Tachinagaha were reduced. These results indicate that the tested QTLs are able to maintain or increase yield by stimulating root development under waterlogging.

CHAPTER III

RELATIONSHIP BETWEEN ROOT DEVELOPMENT AND AERENCHYMA FORMATION IN SOYBEAN SEEDLINGS GROWN UNDER HYPOXIC CONDITION

Waterlogging imposes serious hypoxic stress on the roots of most land crops, including soybean. One of the most common anatomical responses of plants to soil hypoxia is the generation of aerenchyma in tissues, which facilitates the transport of oxygen from shoots to roots in order to maintain root growth.

In this chapter, the aerenchyma formation and the relationship with root development were evaluated. The branch root was cross sectioned to calculate the percentage of aerenchyma in root cut surface area. Root length and root surface area were determined through the analyses of scanned root images. Root length development and root surface area development were determined by calculating the value change between before and after the waterlogging treatment.

The obtained results showed that Root length development and root surface area development of Tachinagaha were greatly decreased under hypoxia, while those of Iyodaizu and NIL-9-4-5 tended to increase. Correspondingly, under hypoxia, NIL-9-4-5 exhibited similar aerenchyma percentage to Iyodaizu, which was significantly higher compared to Tachinagaha. These results indicate that in response to waterlogging, the QTLs confer rapid root development through the concomitant formation of aerenchyma.

CHAPTER IV

NEAR-ISOGENIC LINES OF SOYBEAN CONFIRM A QTL FOR SEED WATERLOGGING AT DIFFERENT TEMPERATURES

Climate change is predicted to increase the probability of soil waterlogging due to severe rainfall, causing significant damage to soybean at the germination stage. Germination under waterlogging is also greatly influenced by temperature.

In this chapter, to clarify the variation in germination responses of soybean genotypes to waterlogging at different temperatures, the seeds of soybean genotypes including Iyodaizu and Tachinagaha were treated by soaking for 2 days at four temperatures: 21°C, 23°C, 25°C, 27°C and 29°C.

Differences in the germination rate and normal seedling rate were observed among soybean genotypes after soaking treatments regardless of the temperature. Among the examined genotypes, Iyodaizu was classified as waterlogging tolerant at the germination stage, and Tachinagaha was classified as sensitive. Germination rate of NIL-9-4-5 was nearly the same as that of Iyodaizu and was significantly higher than that of Tachinagaha. These results may indicate that the candidate QTL region for root development under hypoxia at the seedling stage located on Chr.12 contributes to the seed waterlogging tolerance of soybean plants at the germination stage.

CHAPTER I

GENERAL INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is the most important legume and oilseed crop in the world, providing an essential source of protein to human diet, feed for live-stock and as biodiesel and oil for commercial products. It is also used to produce a high protein animal feed. The soy proteins have the highest nutritional value of all the plant proteins for human food, being particularly high in lysine. Over 64 percent of the world's oilseed meal supply and is the major source of oil, made from soybean seed, accounting for about 28 percent of total production (USDA, 2019-2020). Soybean plant is becoming one of the most useful crop for the sustainable development of the major cereal based cropping systems in the world.

Recent years, the rapid rise in the commercial value of soybean in an international market, the total area under soybean cultivation has been increasing from last three decades. Soybean is an important cash crop with a total production of over 336.56 million metric tons in 2019-2020 (USDA data). During this year, the Brazil has been the world's leading producer of soybean representing 37% of the world production, followed by USA with 31%, Argentina with 16%, China with 5%, Paraguay with 3.8%, India with 2.7%, and Canada with 1.8 % (USDA data). Li, Man Wah., et al. (2020) showed that soybean as a major economic crop of high plant oil and protein contents in East Asia and with the advents of modern farming practices and genetic improvements, the yield of soybean has steadily increased since 1961.

In Japan and South Asian countries, soybean is often sown in Spring to early Summer season (Lee et al., 2003), and converted from paddy field (Fig. 1 and Fig. 2), which period occurs with highest frequency of heavy rain is usually common in these months. Therefore, waterlogging is a common limiting factor for soybean growth at seedling stage (Araki, 2012). And breeding varieties with strong waterlogging-tolerance has been desired in countries with much rainfall at early stage. Moreover, soybean cultivars are sensitive to flooding stress and their seed yields are substantially reduced in response to the stress (Githiri et al., 2006). Root development is an important indicator not only for hypoxia tolerance but also for waterlogging in soybean (Nguyen et al., 2017). Under hypoxic experiment in growth chamber, Sakazono (2014) reported that short-term waterlogging inhibited root elongation and root branching in waterlogging susceptible soybean genotypes but did not inhibit those in waterlogging tolerant genotypes during early growth stage. Therefore, maintaining root development, yield and yield components are the most important in waterlogging and hypoxia tolerance of soybean.

Armstrong (2002) described the mechanism of hypoxia stress and its effects on root system of soybean (Fig. 3), under waterlogging condition slow diffusion of oxygen in water causes hypoxia stress at root zone of soybean (Armstrong et al., 2002), micro-organism depletes the remnant oxygen, and the environment becomes hypoxia. It results in decrease respiration (Armstrong et al., 1979) of root and increase fermentation process (Yin et al., 2013), especial, fermentation process included more toxic products like alcohol or acetaldehyde (Brion et al., 2014), these functions restrict root development of soybean under waterlogging condition (Fig. 3).

For mitigating the negative impact of flooding stress, plants use a number of strategies for their survival, mainly escape and quiescence strategies (Zhao et al., 2018). In escape strategy, morphological (aerenchyma development, shoot elongation and adventitious root formation) and anatomical alterations allow the plant to exchange gas between cell and atmosphere (Zhao et al., 2018). Plants including soybean activate various mechanism to cope with flooding stress, in which anatomical and morphological responses to deal with partial submergence imposed by flooding have been paid more attention in numerous of studies (Armstrong, 1979; Kozlowski et a., 1997; Striker et al., 2005; Colmer et al., 2009, Nguyen, L. V. et al., 2020).

The most common anatomical response is the generation of aerenchyma in tissues (Justin et al., 1987; Seago et al., 2005), which facilitates the transport of oxygen from shoots to roots (Colmer, 2003). At morphological level, usual responses to flooding include adventitious rooting and increases in plant height and consequently, in the proportion of biomass above water level (Naidoo et al., 1993; Grimoldi et al., 1999). This also helps to facilitate the oxygenation of submerged tissues through the aerenchyma tissue (Laan et al., 1990; Colmer, 2003). Under field condition, soybean plant formed adventitious root (Thomas et al., 2005), and aerenchyma (Mochizuki et al., 2000; Shimamura et al., 2003) during flooding occurred. Understanding the root development in hypoxia condition will lead to the elucidation of the mechanisms that controlled the formation of root of soybean. In the pass decades, many studies were focused on the development of root of soybean under hypoxia condition. Some reports described that the aerenchyma was formed under hypoxia condition (Yamauchi et al., 2013), and (Sakazono et al., 2014). However, litter is known about relationship between root deveropment and aerenchyma formation in soybean under waterlogging condition.

Near-isogenic lines (NILs) are useful and valuable materials for gene mapping. Backcrossing and maker assisted selection methods are commonly used to create NILs. Backcrossing is a well-known and long-established breeding strategy where a characteristic is introgressed from a donor parent into the genomic background of a recurrent parent (Babu et al., 2004). Marker assisted selection (MAS) is simply understood as the use of genetic markers to follow regions of the genome that encode specific characteristics of a plant (Hospital et al., 1997). Therefore, NILs have been shown to be more suitable for efficient the identification of specific genes.

Seeds of a near-isogenic line (NIL-9-4-5) and parents (Tachinagaha and Iyodaizu) were used as materials in this study. NIL-9-4-5, (100-seed weight 26 gr) derived from Iyodaizu (100-seed weight 13 gr), a hypoxia tolerant variety, in genetic background of Tachinagaha (100-seed weight 36 gr), a intolerance hypoxia, was selected through recurrent backcrossing and MAS at BC_6F_3 (Fig. 4).

Nguyen, V. L. (2017) reported that the QTLs conferring root development including root length development (RLD) and root surface area development (RSAD) under hypoxia conditions at the seedling stage on soybean Chr.12 and developed a NIL population carrying targeted QTLs at BC_6F_3. QTLs for RLD and RSAD (*Qrld-12*, *Qrsad-12*) on Chr.12 are stable across years (Fig. 5).

For evaluating the effects of QTLs for waterlogging tolerance of NIL and parents, these experiments were conducted to (1) test the yield perfomance under hypoxia exposed to 7-day waterlogging at seedling stage; (2) to get a deeper understanding of the candidate region of the QTLs for root development through aerenchyma formation, and (3) to identify the relationship between root development and aerenchyma formation in soybean seedlings grown under waterlogging condition; and (4) to clarify the variation in germination response of soybean genotypes and whether the candidate QTL region for root development is involved in seed waterlogging tolerance.

Fig.1. Soybean field converted from paddy field at Harumachi Farm of Kyushu University.

Fig. 2. Waterlogging damage at the seedling stage of soybean in the field.

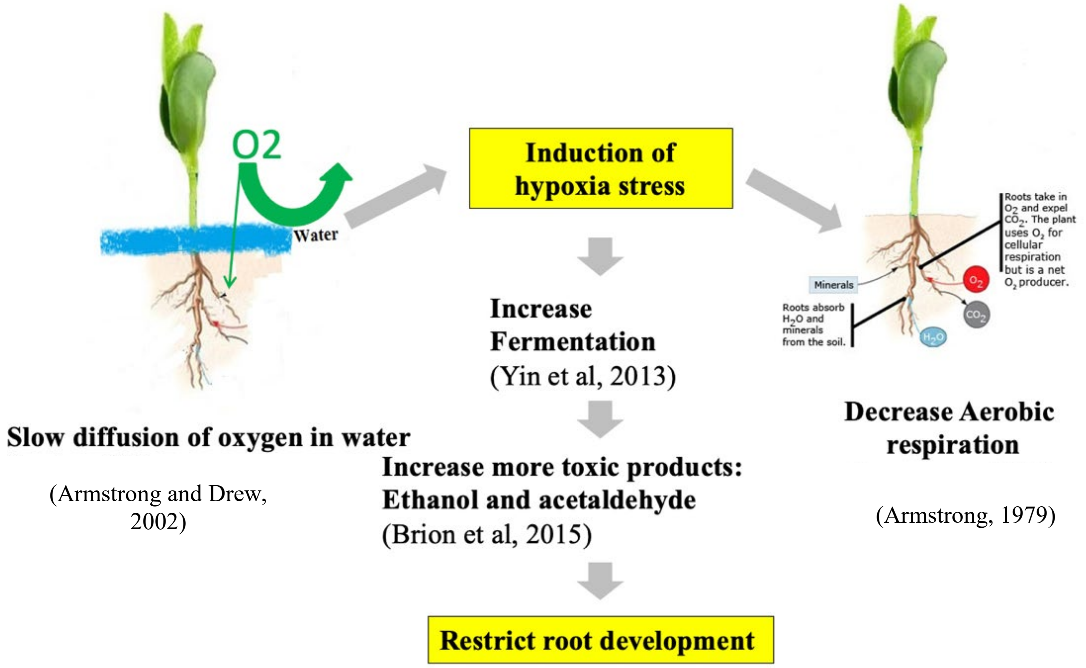


Fig. 3. Mechanism of waterlogging effects on root development of soybean under waterlogging condition.

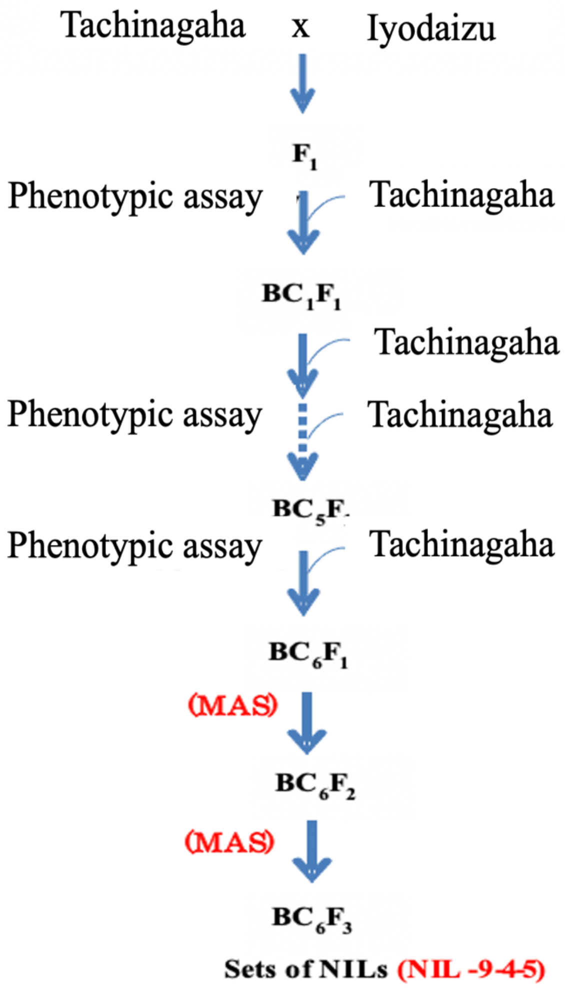
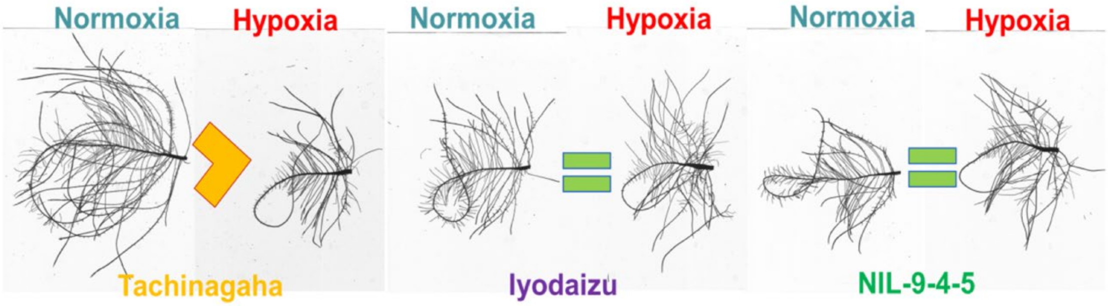
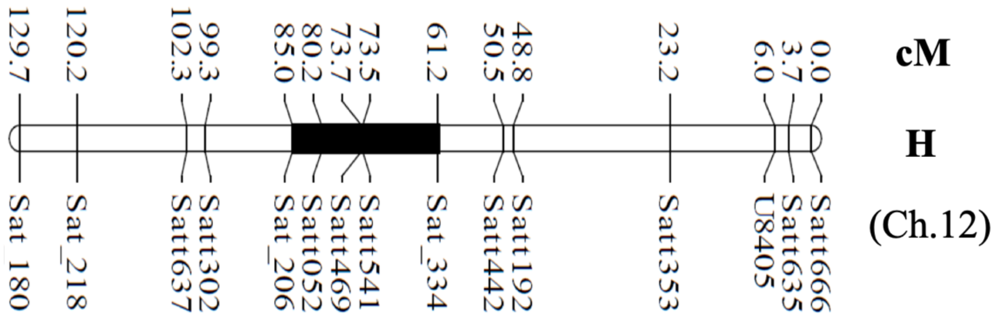


Fig. 4. Diagram showing the material development.

MAS: Marker assisted selection, BC: backcrossing through recurrent parents, NILs: Near-isogenic lines.



Root system of studied genotypes under normoxia and hypoxia



NIL-9-4-5 carrying the target QTLs for root development under hypoxia on Ch.r 12 at linkage group H.

Fig. 5. Information of parents and NIL-9-4-5 at BC_6F_3.

CHAPTER II

THE EFFECTS OF A QTL FOR ROOT DEVELOPMENT UNDER HYPOXIA ON YIELD OF SOYBEAN EXPOSED TO 7-DAY WATERLOGGING AT SEEDLING STAGE

1. Introduction

In humid part of the world, waterlogging is a major environmental factor cause hypoxia stress for root system, and limiting soybean production (Kokubun et al., 2013, Dang et al., 2020). The stress inhibits growth, leading to decreased root growth, nitrogen fixation, leaf chlorophyll content, and photosynthesis activity (Scott et al., 1989; Oosterhuis et al., 1990; Sung, 1993; Linkemer et al., 1998; Bacanamwo et al., 1999, McNamara et al., 1990, Pedó et al., 2015; Suematsu et al., 2017). Therefore, breeding varieties with strong waterlogging-tolerance is a desirable trait in countries with much rainfall (Nguyen et al., 2017).

To contribute for plant growth under waterlogging stress, a common morphological response of plants to low oxygen condition is the production of adventitious roots (Colmer and Voesenek, 2009; Rich et al., 2012). Under waterlogging, adventitious roots are formed/initiated in upland. Maintaining root development under hypoxia is most important factor for accessing the tolarance of soybean, therefore, this chapter was conducted to examine the effects of QTLs for waterlogging tolerance on yield of soybean.

2. Materials and methods

2.1. Plant materials

Parents Iyodaizu and Tachinagaha plus NIL-9-4-5 were used in this study. Iyodaizu is hypoxia-tolerant, and Tachinagaha is hypoxia-susceptible (Sakazono et al., 2014). NIL-9-4-5 carrying the QTL region derived from Iyodaizu in the genetic background of Tachinagaha was selected at BC_6F_3 through recurrent backcrossing and MAS (Fig. 4).

2.2. Experimental design

The waterlogging experiment was conducted in a greenhouse at the Experimental Farm of Kyushu University, Fukuoka, Japan (33°37′N and 130°27′E) (Fig. 6, 7). The experiment was conducted to evaluate the effects of QTLs for root development under hypoxia on root traits and seed yield of soybean exposed to 7 days waterlogging at seedling stage. Eight seeds on Petri dish were incubated at 23°C and 70% RH for 3 days to uniform rate of germination. To select uniform seedlings for next steps, treated seeds were sown in moist vermiculite (Midori Sangyo, Fukuoka, Japan) at 23°C for 2 days. Selected seedling was transplanted into a pot (16 cm diameter and 18 cm height) containing 3 kg of dried paddy soil (56.1% sand, 23.2% silt, and 20.7% clay). Then all pots were putted into ten big trays kept 2 cm water level on its surface bottom. The factorial experiments were arranged in a

Randomized Complete Bock Design with five replications. Three plants of each genotype in a big tray were used as a replication. After germination, seedlings were grown for 7 days under two treatments: drained (control) and waterlogging conditions. Under control condition, water level was maintained at 2.0 cm from bottom of container (Fig. 7). Under waterlogging, water level was maintained at 2.0 cm above soil surface (Fig. 7). The root traits of a plant in each replication were measured before and after treatments. All the remaining pots treated with waterlogging were transferred to new containers to keep water level at 2.0 cm from the bottom like control conditions. Then, all plants were grown under the drained (control) conditions until pod harvesting (Dang, T. T. H. et al., 2020).

2.3. Measurements

The root systems of plants were scanned with an image scanner using the WinRhizo software to measure root morphological traits including root length (RL), root surface area (RSA), and root diameter (RD). RL was calculated on the so-called skeleton images of root system. Roots were classified into ten groups (from x_1 to x_{10}, mm) with unequal width ($x_1 \leqq 0.5$, $0.5 > x_2 \leqq 1.0$, $1.0 > x_3 \leqq 1.5$, $1.5 > x_4 \leqq 2.0$, $2.0 > x_5 \leqq 2.5$, $2.5 > x_6 \leqq 3.0$, $3.0 > x_7 \leqq 3.5$, $3.5 > x_8 \leqq 4.0$, $4.0 > x_9 \leqq 4.5$, $x_{10} > 4.5$) for evaluation of root diameter (RD). RSA was automatically calculated by software based on RD and RL of root in each class. Root development including root length development (RLD), root surface area development (RSAD) and change in root diameter (CARD) were calculated to measure their value changes between before and after treatments in each replication. Waterlogging-tolerant index (WTI) was calculated for each root trait as the ratio of the value under waterlogging to that in control.

To measure seed yield (Y), three components of seed yield of each genotype were assessed: number of pods per plant, number of seeds per pod, and average of seed weight.

2.4. Statistical analysis of phenotypic data

Using the SYSTAT 13 (SYSTAT Software, Inc., Washington, USA) for statistical processing all data in this study. Simple *Pearson* correlation coefficients between traits were calculated using value means. The genotype and treatment effects on the level of each trait were assessed by two-way ANOVA. The *t*-test was used to compare the average values among treatments.

3. Results

3.1. Effects of genotype and waterlogging treatments

Table 1 shows the ANOVA results of the effects of genotypes (G), waterlogging treatments (T), and their interaction (G×T) on the root traits. Genotypes had non-significant effects on all measured traits except root diameter. Treatment had a significant effect on all traits except yield. The interaction had a significant effect on all traits except root diameter and change in average.

3.2. Root development and seed yield of parents and NIL-9-4-5 under waterlogging

Under condition treated with waterlogging, RL, RLD, RSA and RSAD of Tachinagaha were reduced significantly than those of Iyodaizu. Mean of WTI values for RL was 0.4 in Tachinagaha and 1.0 in Iyodaizu (Table 2) while RL of NIL-9-4-5 was 1.0; probably, NIL 9-4-5 maintained root development under waterlogging (Table 2, Fig. 8). RD of all genotypes tended to increase under waterlogging (WTI >1) but not significantly deference in both of Iyodaizu and NIL-9-4-5 (Table 2).

Seed yield, and yield components of Tachinagaha were greatly decreased, but those of Iyodaizu and NIL-9-4-5 showed no significant decrease (Fig. 9). Compared to Tachinagaha, NIL-9-4-5 showed higher root development and seed yield under condition treated with waterlogging (Fig. 8, 9).

3.3. Trait correlations

The relationship between the studied traits under control and waterlogging conditions were calculated by Pearson correlation coefficients (Table 3). All the root traits such as root length (RL), root length development (RLD), root surface area (RSA), and root surface area development (RSAD) were observed under both control and waterlogging treatments using positive correlations. Root diameter (RD) and change avarage root diameter (CARD) showed positive correlation with other root traits under control while they tended to negatively be correlated under waterlogging. The results obtained a strong and highly significant correlation between seed yield (Y) and each of the root traits. However, contrasting responses were found for the correlation between Y and root diameter traits (RD and CARD). RD and CARD showed a positive correlation with Y under controls, while they presented a negative correlation under waterlogging.

4. Discussion

The detected QTLs for a trait are important, but just the first step in molecular breeding program for crop genetic improvement. They should be confirmed in genetic background and different environments. Currently, approaches utilizing near iso-genic lines (NILs) have been effectively used to validate the identified QTLs (Collard and Mackill, 2008). Genes or QTL detection studies in relation to root related abiotic stress using introgression lines or near isogenic lines were conducted in rice (Steele et al., 2006; Uga et al., 2013), and barley (Naz et al., 2012, 2014).

In rice, Uga et al., (2013) reported that positional cloning of QTL underlying DEEPROOTING1 (Dro1) gene; NIL containing Dro1 in IR64 background was developed via marker selection. Shoot biomass, yield and drought stress resistance of Dro1-NIL illustrated significant increase as compared to control genotype IR64 suggesting that the alteration of root system architecture improves yield and drought avoidance in rice plant (Uga et al., 2013).

In this study, NILs was used to confirm the contribution of the region in LG H (Chr. 12) carrying the root development QTLs under waterlogging. The resultant increase in root development of NIL-9-4-5 was most likely inherited from the waterlogging-tolerant parent, Iyodaizu (Table 2). These finding indicates that genes from Iyodaizu could reduce cultivars' waterlogging susceptibility, and that QTLs for root development are strongly involved in it.

Sallam and Scott (1987), showed that the relationship between RL and RSA in soybean plants is consistent. And Sakazono et al., (2014) also reported that this consistency supports the hypothesis that the maintenance of root extension can serve as an indicator of soybean waterlogging tolerance. Under both conditions, the Pearson correlation analysis between traits of root system indicated strong positive relationships among root traits such as RL, RSA, and root development traits included RLD and RSAD.

Sakazono (2014), Suematsu (2017), Nguyen, V. L (2017) and Dang, T. T. H. (2020) showed that RD and CARD have a tendency to increase under waterlogging as compared to controls due to a reduction in root elongation and branching. In this study, RD traits showed a tendency to be negatively correlated with other root traits (Table 3).

The results showed that root development of Tachinagaha was strongly decreased and consequently the seed yield was reduced under waterlogging. Interestingly, experimental results consistently showed that seed yields, yield components and root development of both Iyodaizu and NIL-9-4-5 were not reduced under waterlogging (Fig. 9, 10, 11, 12), these results are most probably related to their ability to maintain root development.

In conclusions, the obtained results showed that RLD and RSAD of Tachinagaha were greatly decreased under condition treated with waterlogging, but those of Iyodaizu and NIL-9-4-5 showed a slight decrease and no decrease, respectively. Interestingly, NIL-9-4-5 also had increased seed yield compared to Tachinagaha, indicating that the tested QTLs can enhance increase yield under waterlogging.

Table 1. Analysis of variance results for the growth and root traits of studied cultivars under control and waterlogging treatment.

Traits	**Analysis of Variance *(F*-Ratio)**		
	Genotype (G)	**Waterlogging (T)**	**GxT**
RL (cm)	3.48^{ns}	33.17^{**}	18.15^{**}
RLD (cm)	2.63^{ns}	31.14^{**}	16.08^{**}
RSA (cm^2)	3.67^{ns}	28.56^{**}	16.27^{**}
RSAD (cm^2)	0.99^{ns}	23.35^{**}	15.32^{**}
RD (mm)	19.32^{**}	16.11^{*}	4.3^{ns}
CARD (mm)	2.56^{ns}	0.95^{ns}	0.87^{ns}
Y (g $plant^{-1}$)	1.13^{ns}	2.98^{ns}	14.33^{**}

RL, root length; RLD, root length development; RSA, root surface area; RSAD, root surface area development; RD, root diameter; CARD, change in average root diameter; Y; seed yield. ns, Not significant at $P > 0.05$; * and ** significant at $P < 0.05$ and $P < 0.01$, respectively.

Table 2. Root traits and waterlogging-tolerance index (WTI) of Tachinagaha, Iyodaizu and NIL-9-4-5.

Traits	**Tachinagaha**			**Iyodaizu**			**NIL-9-4-5**		
	Control	Waterlogging	WTI	Control	Waterlogging	WTI	Control	Waterlogging	WTI
RL (cm)	551±34	245*±19	**0.4**	396±16	392^{ns}±14	**1.0**	445±26	461^{ns} ±29	**1.0**
RLD (cm)	445±29	231*±23	**0.5**	368±11	331*±21	**0.9**	378±34	388^{ns} ±31	**1.0**
RSA (cm^2)	113±11	66*±8	**0.6**	81±6	62*±6	**0.8**	102±6	113^{ns}±8	**1.1**
RSAD (cm^2)	81±12	32*±5	**0.4**	65±7	45*±4	**0.7**	71±3	72^{ns}±6	**1.0**
RD (mm)	0.8±0.2	0.75*±0.02	**1.1**	0.61±0.02	0.63^{ns}±0.02	**1.0**	0.74±0.02	0.71^{ns}±0.04	**1.0**
CARD (mm)	-0.17±0.11	-0.11*±0.05	**0.6**	-0.16±0.09	-0.16 ^{ns}±0.03	**1.0**	-0.24±0.05	-0.21^{ns}±0.06	**1.0**

RL, root length; RLD, root length development; RSA, root surface area; RSAD = root surface area development; RD, root diameter; CARD, change in average root diameter; WTI, waterlogging-tolerance index. ns indicate means are not significantly different within a genotype; * within a genotype are significantly different by *t*-test at $p < 0.05$ (n=5). ns, not significantly different.

Table 3. Phenotypic correlation coefficients among traits under control condition (above diagonal) and waterlogging (below diagonal).

Traits	**RL**	**RLD**	**RSA**	**RSAD**	**RD**	**CARD**	**Y**
RL	-	0.969**	0.916**	0.891**	0.778**	0.612**	0.773**
RLD	0.983**	-	0.964**	0.961**	0.731**	0.551**	0.868**
RSA	0.922**	0.912**	-	0.932**	0.891**	0.688**	0.731**
RSAD	0.916**	0.908**	0.942**	-	0.799**	0.587**	0.678**
RD	-0.899**	-0.911**	-0.839**	-0.801**	-	0.368*	0.544**
CARD	-0.816**	-0.813**	-0.784**	-0.713**	0.768**	-	0.881**
Y	0.881**	0.873**	0.891**	0.861**	-0.801**	-0.601**	-

RL, root length; RLD, root length development; RSA, root surface area; RSAD, root surface area development; RD, root diameter; CARD, change in average root diameter; Y; seed yield. * and ** significant at $P < 0.05$ and $P < 0.01$, respectively.

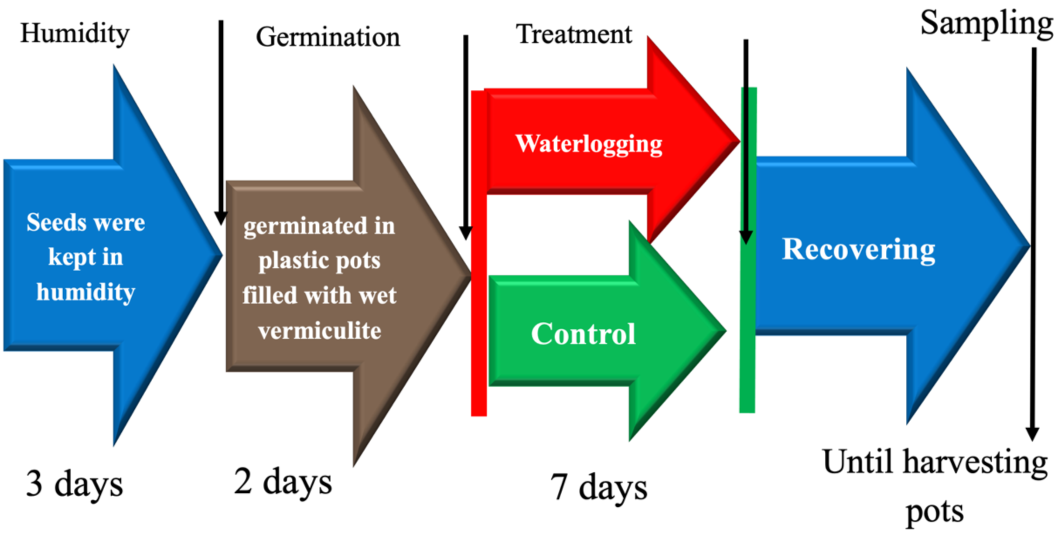


Fig. 6. Experiment designed in Greenhouse.

The waterlogging experiment was conducted in a greenhouse at the Experimental Farm of Kyushu University Fukuoka, Japan (33°37 N and 130°27 E).

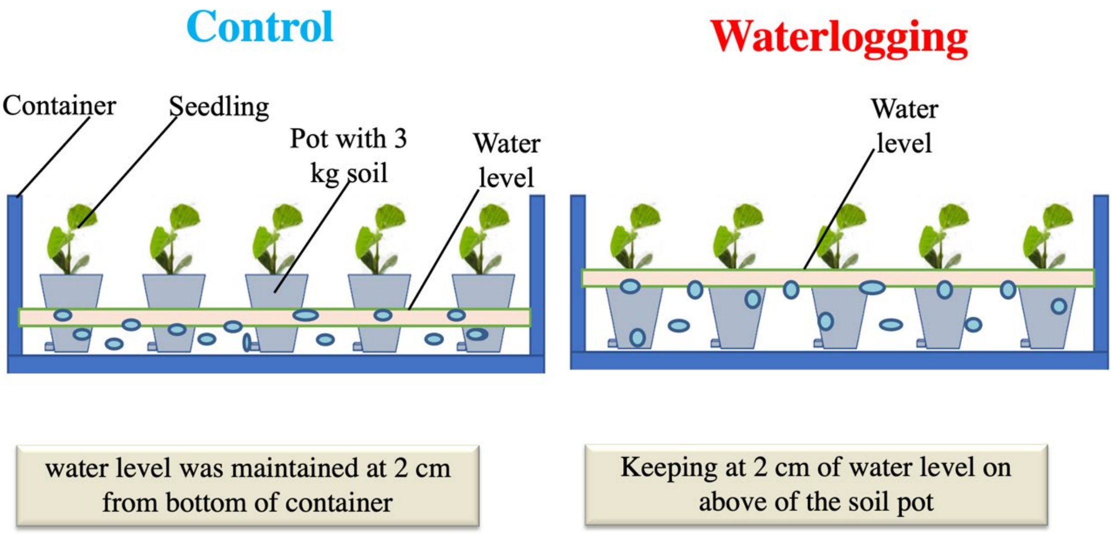


Fig. 7. Plant culture under control and waterlogging conditions

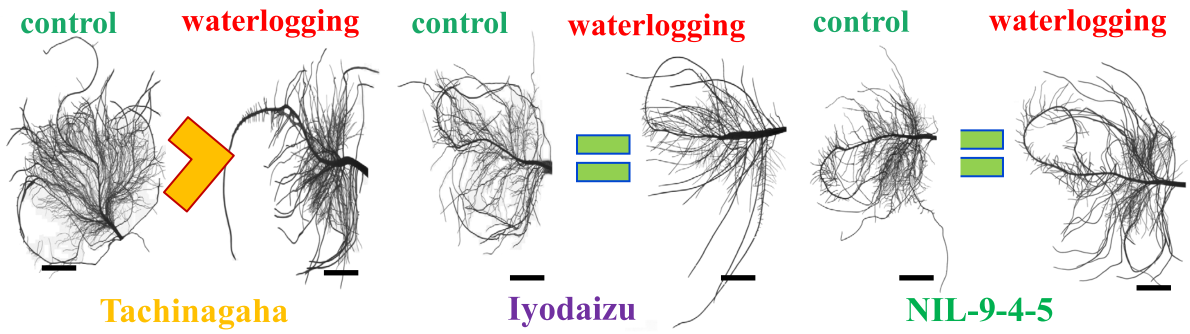


Fig. 8. Root development of parents and NIL 9-4-5 under control and waterlogging conditions in greenhouse. Bar = 4 cm.

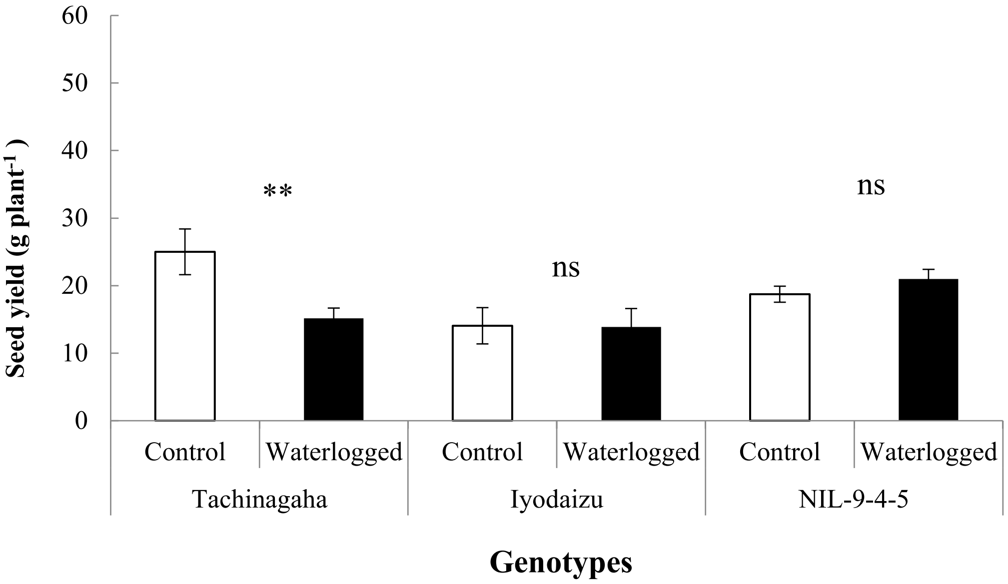


Fig 9. Seed yield per plant of parents and NIL-9-4-5, under control and waterlogging. Vertical bars indicate standard deviations. Bars with asterisk in each genotype indicate significantly different at $P < 0.05$ by *t*-test (n=5). ns, not significantly different.

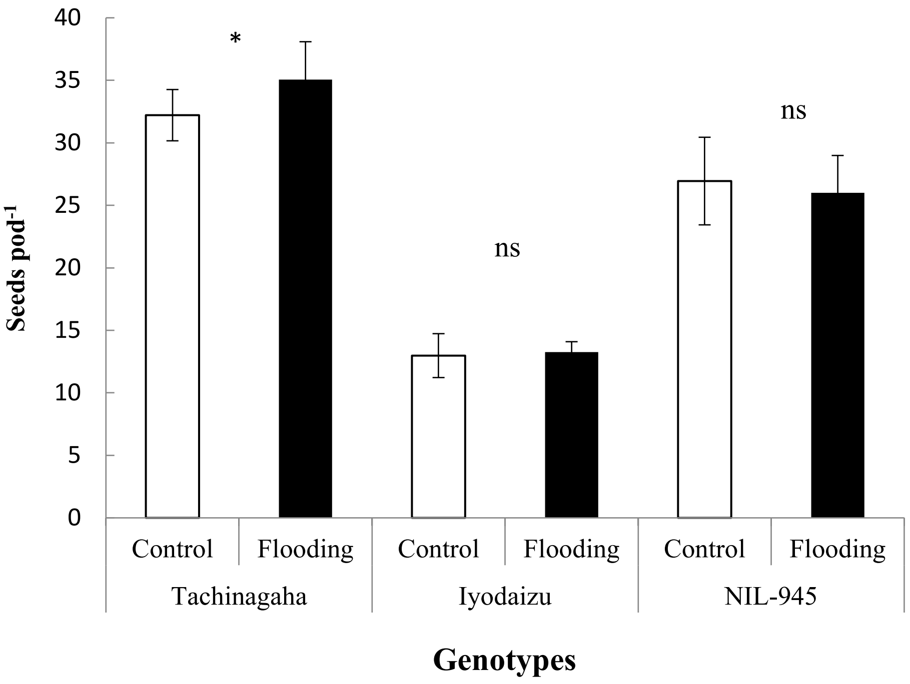


Fig. 10. Number of seed per pod of parents and NIL-9-4-5 under control and waterlogging condition. Vertical bars indicate standard deviations. Bars with * in each genotype indicate significantly different at $P < 0.05$ by *t*-test (n=5). ns, not significantly different.

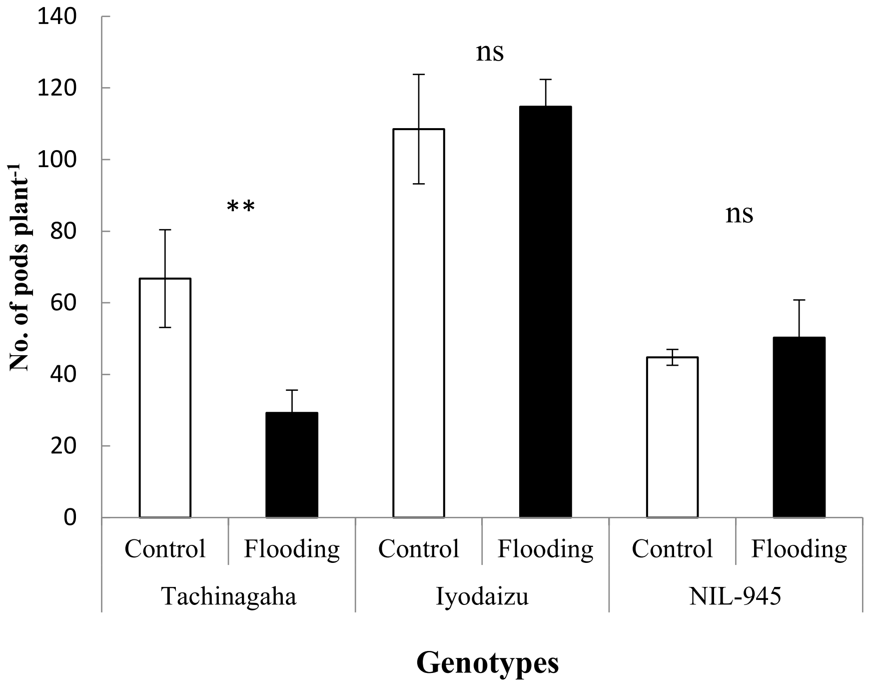


Fig. 11. Number of pods per plant of parents and NIL-9-4-5 under control and waterlogging condition. Vertical bars indicate standard deviations. Bars with ** indicate significantly different at $P < 0.01$ by *t*-test (n=5). ns, not significantly different.

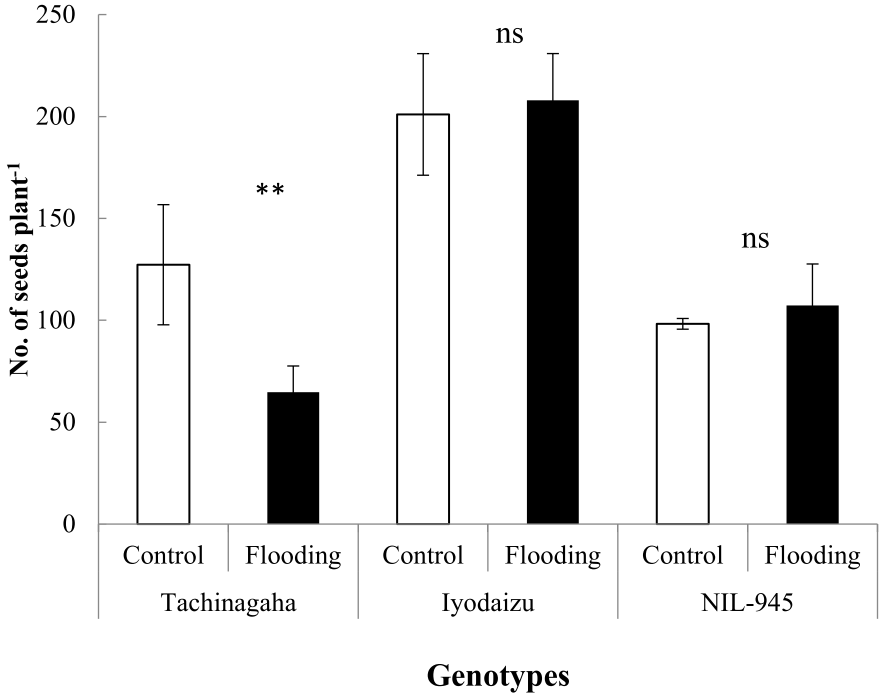


Fig. 12. Number of seed per plant of parents and NIL-9-4-5 under control and waterlogging condition. Vertical bars indicate standard deviations. Bars with ** in each genotype indicate significantly different at $P < 0.01$ by *t*-test (*n*=5). ns, not significantly different.

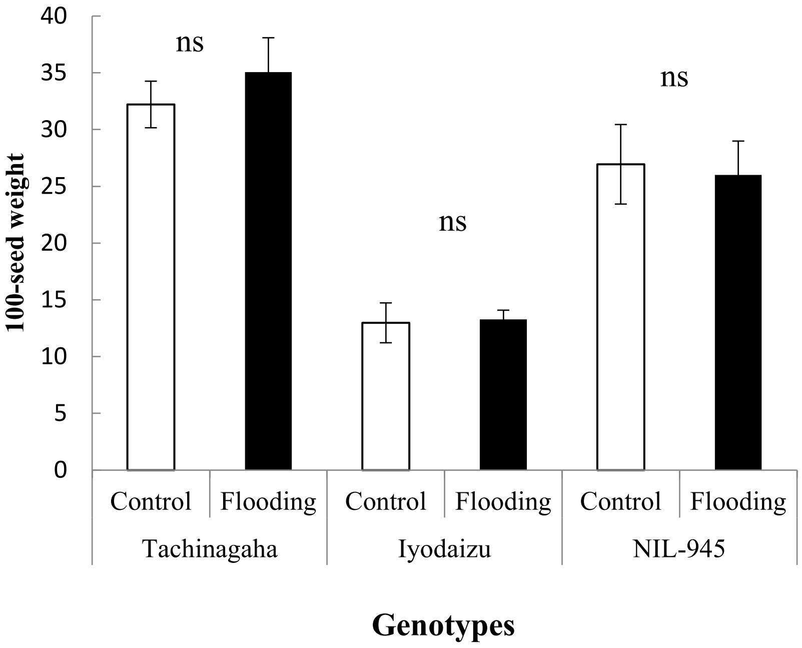


Fig. 13. 100-seed weight of parents and NIL-9-4-5 under control and waterlogging condition. Vertical bars indicate standard deviations. Bars with asterisk ^{ns} in each genotype indicate not significantly different at $P < 0.05$ by *t*-test (n=5).

CHAPTER III

RELATIONSHIP BETWEEN ROOT DEVELOPMENT AND AERENCHYMA FORMATION IN SOYBEAN SEEDLINGS GROWN UNDER HYPOXIC CONDITION

1. Introduction

Soybean (*Glycine max (*L.) Merr.) is the most produced legume in the world. This legume is predominantly consumed in Asia, where it is often grown in fields converted from paddy field or undrained lowland (Kokobun et al., 2013), including Japan (Githiri et al., 2006), Viet Nam (Loc et al., 2015), and Indonesia (Ghulamahdi et al., 2016). In these cases, the soil is easily got waterlogged during rainy season, which causes a rapid deprivation of oxygen in soil (Armstrong, 1979), and the environment becomes hypoxia and even anoxia depending on the duration of waterlogging (Blom and Voesenek, 1996; Bailey-Serres and Voesenek, 2008). However, the most impact of hypoxia varied across soybean, and the plant growth was found to be linearly correlated with the degree of root development (Sakazono et al., 2014; Suematsu et al., 2017). The higher root development seems to be achieved through morphological adaptations, including formations of branching root (Thomas, 2005; Colmer, 2003) and root aerenchyma (Mochizuki et al., 2000; Pires et al., 2002; Shimamura et al., 2003).

Formation of aerenchyma is a major structural adaptation in plants to flood-induced hypoxia (Justin and Armstrong, 1987, Jackson and Armstrong, 1999; Seago et al., 2005, Kacprzyk et al., 2011, and Potocka et al., 2018). It facilitates the transport of oxygen from shoots to roots (Colmer et al., 2003 and Shimamura et al., 2010). Adaptive aerenchyma formation is observed in various crops such as maize, rice, taro, sugarcane and wheat (Jackson et al., 1985, Justin et al., 1991, Mano et al., 2007, Colmer and Voesenek, 2009, Abiko et al., 2012, Yamauchi et al., 2014, Tavares et al., 2019, Imene et al., 2019, Yamauchi et al., 2019, Mohammed et al., 2019 and Abiko et al., 2020).

Some soybean cultivars were found to form aerenchyma to adapt to waterlogging conditions (Parsons et al., 1990, Mochizuki et al., 2000, Pires et al., 2002; Shimamura et al., 2003, Thomas et al., 2005, Yamauchi et al., 2013, Shimamura et al., 2016 and Zhao et al., 2018). The formation of aerenchyma contributes to root growth of soybean under hypoxia in response to waterlogging, Kokubun (2013) and Shimamura (2016) reported that some soybean cultivars were found to form aerenchyma, which transports oxygen from aboveground tissues to root system and the elongation of these cells are enhanced, therefore a secondary aerenchyma with high porosity is produced. And waterlogging results in the rapid formation of aerenchyma of branching root (Thomas, 2005 and Shimamura, 2016). In soybean, some QTLs were found to be involved in waterlogging tolerance (VanToai et al., 2001, Shimamura et al., 2003, Sakazono et al., 2014). Nguyen, V. L. (2017) found that QTLs of waterlogging-tolerant soybean cultivar, Iyodaizu, located on the chr. 12.

In this chapter, root development indices, including RLD and RSAD, were increased by the QTLs. The effects were also stable in a NIL where QTLs were introduced to the genetic background of flooding-susceptible cultivar, Iyodaizu at BC_6F_3, confirming their functions (Nguyen et al., 2017 and Dang et al., 2020). In those cases, the effects of QTLs on the root were evaluated through indirect measurements, such as injury score and tolerance index, instead of direct analysis of the root structures. Therefore, this chapter was conducted (1) for deeper understanding of the candidate region of the QTLs for root development through aerenchyma formation, and (2) to identify the relationship between root development and aerenchyma formation in soybean seedlings grown under waterlogging condition.

2. Materials and methods

2.1. Materials

Iyodaizu, a hypoxia tolerant variety, and Tachinagaha was a hypoxia-susceptible variety and NIL-9-4-5 derived from Iyodaizu in genetic background of Tachinagaha was selected through recurrent backcrossing and MAS; were used is this chapter (Fig. 4).

2.2. Experiment design

The experimental design (Fig. 14, 15) was based on the method of Nguyen (2017). The seeds were kept in humidity oven (environmental condition: 23 ºC temperature and 70 % humidity) for three days. Then seeds were applied with a fungicide (benomyl, ½ of dry seed weight), and then sown in plastic pots filled with wet vermiculite (Midorisangyou, Fukuoka, Japan) to encourage the development of straight radicles. Two days after sowing, the seed coat was removed, and uniform seedlings were transplanted into 1/10 Hoagland solution (normoxia condition). The seedlings were inserted through holes punched in a 7-mm-thick polystyrene board and held upright with plugs of silicone rubber. Each board, holding 20 seedlings, was placed over an opaque plastic container (386 mm × 256 mm × 135 mm), with the roots in 1/10 Hoagland solution continuously aerated at 1.0 L min^{-1} by air pumps. Seedlings were grown in growth chamber (220 µmol m^2 s^{-1} light density 14 hours light/10 hours dark and 23 ºC temperature). Then at 7 days after sowing (DAS), the plants were grown for 7 days under control and waterlogging conditions. Under control condition, seedlings were grown in 1/10 Hoagland solution aerated by two air pumps at opposite ends of the container to maintain a dissolved oxygen (DO) concentration of >7.0 mg L^{-1}. Under waterlogging, seedlings were grown in 0.1% (w/v) agar solution in 1/10 Hoagland solution that was deoxygenated with a continuous flow of nitrogen to maintain a DO concentration of < 1.0 mg L^{-1} (Fig. 15).

2.3. Measurement of root development traits

2.3.1. Root and shoot growth traits

Root traits were measured before and after the treatments, the root systems of individual plants were scanned with an image scanner (Epson Perfection V700/V750 2.08 A, Epson, Long Beach, CA, USA) coupled with WinRhizo system (Regent Instruments, Inc., Quebec City, Canada). The images were analyzed using WinRhizo software to measure root morphological traits including RL, RSA, and root diameter (RD). RL was calculated on the so-called skeleton images of root system. Roots were classified into ten groups (from x_1 to $x_{10,}$ mm) with unequal width ($x_1 \leqq 0.5$, $0.5 > x_2 \leqq 1.0$, $1.0 > x_3 \leqq 1.5$, $1.5 > x_4 \leqq 2.0$, $2.0 > x_5 \leqq 2.5$, $2.5 > x_6 \leqq 3.0$, $3.0 > x_7 \leqq 3.5$, $3.5 > x_8 \leqq 4.0$, $4.0 > x_9 \leqq 4.5$, $x_{10} > 4.5$) for evaluation of RD. RSA was automatically calculated by software based on RD and RL of root in each class. Root development including RLD and RSAD were calculated to measure their changes between before and after treatment.

2.3.2. Aerenchyma measurement

The branch root of parents and NIL-9-4-5 (Fig. 16) were sectioned at 10 - 15 positions (belonging root length of cultivars in the study) into 20 µm thickness by Plant Microtome MTH-1 (Nippon medical & chemical instruments CO., LTD); the first is at the top of branch root tip, distance between two position was 2 cm. The samples were examined under a light microscope (Nikon Eclipse TS2-FL) fitted with a CCD camera (camera head: Nikon DS-F3, control unit: Nikon DS-L4) linked to a computer. The aerenchyma area (AA) was measured using NIS Element D software (Supplementary Fig. S1). The percentage of aerenchyma (POA) in root cut surface area (CSA) was calculated by following formula: POA (%) = AA (μm^2)/CSA (μm^2) x 100.

2.4. Statistical analysis

All statically analyses were done with SYSTAT 13 (SYSTAT software, Inc., Washington, USA). The effect of genotype on the level of each trait was assessed by one-way ANOVA. Correlations (Pearson's *r*) were calculated performed in version 13 of the Systat software (http://systatsoftware.com/). The *t*-test was used to compare the average values among treatments.

3. Results

3.1. Shoot and root growth

Shoot dry weight (SDW), root dry weight (RDW) and total dry weight (TDW) of Tachinagaha, Iyodaizu and NIL-9-4-5 are shown in Fig. 18-20. Different response of shoots and root growth to waterlogging was found in the three cultivars studied. SDW of Iyodaizu was significantly increased by 19 % and NIL-9-4-5 was slightly increased by 11 % in comparison with control, whereas SDW of Tachinagaha was not significantly different under both conditions (Fig. 18). Interestingly, under waterlogging condition, Iyodaizu and NIL-9-4-5 could maintain RDW, while RDW of Tachinagaha was strongly decreased by 31 % compared with control (Fig. 19). The contrast results were observed in TDW of Tachinagaha, Iyodaizu, and NIL-9-4-5; under treatment condition, TDW of Iyodaizu and NIL-9-4-5 were significantly increased 13 %, 8 %, respectively in comparison with control, probably related to SDW in the two lines were slightly increased and RDW could maintain whereas the contrast of direction was found in Tachinagaha, TDW was slightly reduced by 10 % because RDW was strongly decreased (Fig. 20).

3.2. Root morphological traits and root development

The root morphology and root development of the parents and NIL-9-4-5 differed significantly between control and waterlogging condition (Table 4). The contrast results of root morphological traits were detected cultivars studied (Table 4, Fig. 17, 18, 19 and 20). Root length (RL), root surface area (RSA), and root volume (RV) of Tachinagaha were reduced by 40 %, 39 %, and 31 %, respectively in comparison with control condition, whereas those of Iyodaizu and NIL-9-4-5 increased 8 %, 10 %, respectively in RL; 12 %, 7 %, respectively in RSA; and 9 %, 11 %, respectively in RV. RD of Tachinagaha decreased significantly by 14%, while NIL-9-4-5 increased by 17 % and Iyodaizu was not changed (Table 4).

The root traits development includes root length development (RLD), root surface area development (RSAD), and root volume development (RVD) of Tachinagaha were rapid under waterlogging condition. The highest value of reduction was detected with a reduction of 85 % in RSAD, followed by a reduction in RLD (73 %), RVD (76 %), and root diameter development (RDD) (42 %). In contrast RLD, RSAD, and RVD of Iyodaizu and NIL-9-4-5 were increased under waterlogging condition (Table 4, Fig. 17). RDD of Tachinagaha decreased by 42 %, while and NIL 9-4-5 increased by 51 % in comparison with control, but no change in RDD was observed in Iyodaizu under both conditions.

3.3. Effects of genotypes and waterlogging treatments

Result of Table 5 showed that the ANOVA on the effects of genotypes (G), waterlogging treatments (T), and their interaction (GxT) on the root traits. Under the waterlogging condition, the significant effects of G on all measured traits. It was not significant effects of T on RVD, RD, and RDD of soybean plants. GxT interaction significantly affected on all traits.

3.4. Traits correlation

Pearson's correlation coefficients (*r)* were calculated to test the relationship between the studied traits under waterlogging condition (Table 6). The positive correlations among root traits such as RL, RLD, RSA, RSAD, RV, RVD, RD, SDW, RDW, and TDW were observed under both conditions; excluding RDD and SDW showed positive with other root traits under control condition while they tended to be negatively correlated under the waterlogging condition. The obtained results showed that a strong and high significant correlation between RDW and each root traits. However, a contrast direction was found in the correlation between SDW under RL, RLD, RSA, and RSAD. RL, RLD, RSA, and RSAD showed a positive correlation with SDW under control condition while they presented negative under waterlogging condition.

3.5. Aerenchyma formation

The aerenchyma formation in the root of soybean was observed under both conditions. The proportion of aerenchyma area under the control condition of Tachinagaha, Iyodaizu, and NIL-9-4-5 formed from 7 cm, 5 cm, and 6 cm of the root tip, respectively (Fig. 24 A). The total of aerenchyma in the cut surface section was not significantly different between Tachinagaha, Iyodaizu, and NIL-9-4-5 (Fig 21, 22 and 23). The proportion of aerenchyma area ranged from 0 % to 13.21 % depending on the cut position (Fig. 20 A). In contrast, under waterlogging conditions, aerenchyma formation of Tachinagaha, Iyodaizu, and NIL-9-4-5 were significant differences. The aerenchyma area of three cultivars was detected from 0 % to 28 % (Fig. 24 B), depending on cut positions. Tachinagaha was detected of aerenchyma formation from 5 cm of root tips, while Iyodaizu and NIL-9-4-5 formed aerenchyma from 0 cm of root tips (Fig. 24 B). The higher area aerenchyma was found in the middle regions of the branch root.

The aerenchyma area of Tachinagaha was observed not significant difference between control and waterlogging conditions (Fig 23). The results showed that the area of aerenchyma formation from 1.76 % to 8.85 % under control (Fig. 24 A) and 3.01 % to 9.65 % under waterlogging condition (Fig. 24 B). In contrast, Iyodaizu and NIL-9-4-5 tended to increase by 11.7 %, and 7.7 %, (Fig. 24 B), respectively in comparison with control condition. The proportion of the aerenchyma area of Iyodaizu was the highest value 17.76 %, followed by NIL-9-4-5 with 12.9 % and Tachinagaha with the lowest value 4.1 % (Fig. 22).

3.6. Correlation between aerenchyma formation and root length (RL), root length development (RLD), root surface area (RSA), and root surface area development (RSAD)

The values of waterlogging tolerance index (WTI) for RL, RLD, RSA, and RSAD and the area of aerenchyma formation in root of the three cultivars in this study under waterlogging condition were significantly positively correlated ($P < 0.001$, Fig. 25 a-d). The pearson's correlation coefficient *(r)* for the relationships between the results of area of aerenchyma formation in root and WTI for RL, RLD, RSA, and RSAD were significant positive correlations ($r > 0.92$, $P < 0.001$, fig. 25 a), ($r > 0.94$, $P < 0.001$, fig. 25 b), ($r > 0.96$, $P < 0.001$, fig. 25 c), *and* ($r > 0.97$, $P < 0.001$, fig. 25 d), respectively.

4. Discussion

The root system of tolerance cultivars of soybean could maintain the root development under short-term hypoxia stress (Hou et al., 1991, Sakazono et al., 2014). Nguyen (2017) reported that root traits such as RL, RSA, RLD, and RSAD of susceptible cultivar Tachinagaha were strongly decreased under waterlogging condition, while Iyodaizu, a hypoxia tolerance variety maintained all traits under waterlogging condition. In this study, root development was measured during the waterlogging period, which reflected the effects of waterlogging on the root system of the soybean plant. The obtained results showed that root traits development of Tachinagaha was reduced from 42 % to 85%. In contrast, root traits and their development in hypoxia-tolerant cultivars Iyodaizu and NIL-9-4-5 increased under waterlogging conditions compared with those under control (Table 4. Fig. 17). The previous study suggested that RD of soybean were increased under waterlogging condition (Sakazono et al., 2014, Jitsuyama et al., 2015, Prince et al., 2018). This corroborates with our results showing that RD and RDD of Tachinagaha was strongly decreased compared with control, whereas no change in RDD was observed in Iyodaizu and NIL 9-4-5 under both treatments. These results indicated that maintaining root elongation under hypoxia could be an important factor contributing to waterlogging tolerance in soybean.

The relationship between root length, root surface area, root diameter is consistent with the finding of Sallam and Scott (1987), Tagliavini Veto, and Looney (1993), and Wu (2016). This consistency supports the hypothesis that maintains of root extension may serve as an indicator of soybean waterlogging tolerance (Sakazono et al., 2014; Van Nguyen et al., 2017). Root length was strongly correlated with root surface area and root diameter under hypoxia (Heshaw et al., 2007; Wu et al., 2016). In this study, Pearson correlation analysis between traits indicated strong positive relationships among root traits such as RL, RSA, RV, RD, and root development traits included RLD, RSAD, RVD and RDD under both conditions. RD and RDD tended to increase under waterlogging treatment as compared to control due to a reduction in root elongation and branching (Freixes et al., 2002; Wu et al., 2016 and Van Nguyen et al., 2017). Recent results showed that RDD and SDW traits tended to negatively be correlated with other root traits under waterlogging condition (table 6) while TDW and RDW indicated strong positive relationships among root traits.

Sallam and Scott (1987), Araki (2012), Sauter (2013), Voesenek and Bailey-Serres (2015), and Herzog (2016) reported that waterlogging causes hampered both shoot and root growth and affects root growth first. Normally, under waterlogged condition, root growth reduced before shoot growth (Malik et al., 2002, Shimura et al., 2003, and Tareq et al., 2020). Some studies showed that soybean SDW showed insignificant changes or increase SDW under the waterlogging condition and normal condition growth. (Sakazono et al., 2014; Jitsuyama et al., 2015; Van Nguyen et al., 2017; Suematsu et al., 2017). The similar result was obtained in the response of shoot growth to waterlogging. The SDW of waterlogging tolerance cultivar, Iyodaizu, and NIL-9-4-5 were increased under 7 days of prolonged waterlogging, but no change in SDW of susceptible cultivar, Tachinagaha (Fig. 18). However, roots are vital for plant function, which play an important role in the absorption, translocation of water and nutrient, and growth hormones, and especially crop productivity (Garcia, 2015). These results also showed that Iyodaizu and NIL-9-4-5 could maintain RDW under waterlogging conditions, while Tachinagaha was observed strong reduction (Fig. 19); TDW was significantly increased in the two lines because Iyodaizu and NIL-9-4-5 RDW of those increased slightly (Fig. 20), while TDW of Tachinagaha tended to decrease.

Aerenchyma formation is a morphological change that occurs in plants growth under waterlogging or hypoxia condition (Mochizuki et al., 2000, Seago et al., 2005, Y. Mano et al., 2007, Takahashi et al., 2014, Nazemi et al., 2015, and Abiko et al., 2012 and 2020) and it is known to enhance the internal diffusion of atmosphere and photosynthetic oxygen from aerial parts to the flooded roots, allowing the root maintain aerobic respiration (Armstrong et al., 1979; Laan et al., 1990; Colmer et al., 2003, Young et al., 2008). Abiko (2012) reported that under aerated condition root of maize formed negligible aerenchyma, but waterlogging soil increase the aerenchyma to 15%. Mohammed (2019) concluded that root cortical aerenchyma in rice is formed constitutively. Abiko et al., 2020 found that some taro cultivars form aerenchyma under wetland conditions.

In leguminous crops such as soybean, Mochizuki et al., (2000) showed that a correlation between the flooder tolerance of several legume crops, including soybean, and the development of secondary aerenchyma in their hypocotyls, and suggested that the most tolerant crops tended to produce high amount aerenchyma. The ability of root elongation depends on the amount of oxygen that reaches the root tips (Colmer et al., 2003). This is greatly supported by aerenchyma (Justin et al., 1987; Armstrong et al., 2002, Thomas et al., 2005, Yamauchi et al., 2013

Results of this study showed that under waterlogging condition, formation of aerenchyma was found in both waterlogging tolerant (Iyodaizu) and susceptible cultivars (NIL-9-4-5), while insignificant aerenchyma was found in susceptible cultivar, Tachinagaha, degree of aerenchyma formation of NIL-9-4-5 was the same with Iyodaizu and significant higher compared to Tachinagaha (Fig. 21-24). These results indicated the near-isogenic line (NIL 9-4-5) were able to maintain root development such as RLD, RSAD and RDD under waterlogging conditions due to the importance of aerenchyma for increasing oxygen transport to flood tissues (Yamauchi et al., 2013, 2014) These finding indicated that waterlogging tolerance in soybean plants is strongly related to the ability of aerenchyma formation under waterlogging condition (Justin et al., 1987; Armstrong et al., 2002; Colmer et al., 2003). However the benefit of aerenchyma are clear, the mechanisms that underlie the formation of this tissue are not yet fully understood (Yamauchi et al., 2013, Tamang et al., 2014)

In conclusion, the waterlogging condition induces aerenchyma formation to improve the oxygen transfer from shoots to roots and reduces the amount of oxygen of cell consumption. Aerenchyma is formed by programmed cell death and contributes to aerenchyma formation in the root of hypoxia tolerant genotypes. Even though many studies of formation and functions of secondary aerenchyma in root of soybean, but little is known about the relationship between root development and aerenchyma formation under waterlogging. Observations results showed that the root development of hypoxia-tolerant genotypes of soybean through the aerenchyma formation and proved by the results in Fig. 21-24. Moreover, RL, RLD, RSA and RSAD were strongly correlated with aerenchyma formation (Fig. 25). Although, the mechanism for this aspect has been unclear, these data have important implications for the mechanism aerenchyma formation under waterlogging condition. Fine mapping and positional cloning may reveal genes responsible for root development under hypoxia condition and help elucidate the mechanism of waterlogging tolerance in soybean.

Table 4. Root traits mean, standard error (SD), waterlogging tolerance index (WTI) of studied cultivars under flooding and control conditions. (n=5)

Traits	**Tachinagaha**					**Iyodaizu**					**NIL-9-4-5**				
	Control		**Waterlogging**		**WTI**	**Control**		**Waterlogging**		**WTI**	**Control**		**Waterlogging**		**WTI**
	Mean	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**		**Mean**	**SD**	**Mean**	**SD**	
RL (cm)	643.85	45.57	386.07***	16.19	**0.60**	456.12	26.17	492.52*	14.35	**1.08**	475.24	22.16	523.01*	18.89	**1.10**
RLD (cm)	331.36	50.02	87.73***	24.49	**0.26**	236.65	44.46	299.91*	35.76	**1.27**	154.77	22.16	182.15^{ns}	31.77	**1.18**
RSA (cm2)	123.78	5.27	75.00***	6.64	**0.61**	100.53	6.36	113.03*	8.21	**1.12**	110.22	5.22	118.33*	2.13	**1.07**
RSAD (cm2)	63.70	5.62	9.32***	8.74	**0.15**	47.06	6.77	59.26*	7.42	**1.26**	44.60	2.30	49.95*	2.97	**1.12**
RV (cm3)	1.80	0.15	1.23*	0.10	**0.69**	1.29	0.09	1.41*	0.04	**1.09**	1.49	0.14	1.66*	0.06	**1.11**
RVD (cm3)	0.69	0.13	0.11*	0.21	**0.16**	0.34	0.11	0.47*	0.08	**1.40**	0.48	0.13	0.93*	0.40	**1.96**
RD (mm)	0.71	0.02	0.61**	0.03	**0.85**	0.53	0.02	0.56^{ns}	0.04	**1.06**	0.51	0.02	0.60*	0.04	**1.17**
RDD (mm)	0.06	0.05	-0.03*	0.07	**-0.62**	-0.08	0.03	-0.07^{ns}	0.05	**0.84**	0.05	0.02	-0.03*	0.03	**-0.52**

RL, RLD, RSA, RSAD, RV, RVD, RD and RDD denote root length, root length development, root surface area, root surface area development, root volume, root volume development, root diameter, root diameter development, respectively. WTI, waterlogging tolerance index. WTI was then calculated to determine value loss for each cultivar when subjected to waterlogging compared to control as WTI (%) = (Waterlogging value/Control value) *100. Means with *, ** and *** within a genotype are significantly differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ by *t*-test (n=5), respectively.

Table 5. The analysis of variance of the growth and root traits of studied cultivars under control and waterlogging treatments.

Traits	**Analysis of Variance (*F*-Ratio)**		
	Genotype (G)	**Treatment (T)**	**GxT**
RL	16.44^{**}	36.96^{**}	110.38^{**}
RLD	15.55^{**}	19.74^{**}	64.07^{**}
RSA	16.73^{**}	18.75^{**}	82.90^{**}
RSAD	22.94^{**}	30.43^{**}	90.22^{**}
RV	10.20^{**}	5.94^{*}	38.52^{**}
RVD	4.74^{**}	0.01	16.36^{**}
RD	30.82^{**}	0.15	30.17^{**}
RDD	5.18^{**}	0.20	6.19^{**}

*, and ** significance at 0.01, and 0.001 probability levels, respectively. RL: root length, RLD: root length development, RSA: root surface area, RSAD: root surface area development, RV: root volume, RVD: root volume development, RD: root diameter, and RDD: root diameter development. * and ** significant at $P < 0.05$ and $P < 0.01$, respectively.

Table 6. Phenotypic correlation coefficients among trait under control condition (above diagonal) and waterlogging (below diagonal).

Traits	**RL**	**RLD**	**RSA**	**RSAD**	**RV**	**RVD**	**RD**	**RDD**	**SDW**	**RDW**	**TDW**
RL	-	0.871**	0.995**	0.925**	0.919**	0.929**	0.444**	0.394**	0.810**	0.886**	0.802**
RLD	0.901**	-	0.661**	0.976**	0.999**	0.999**	0.883**	0.975**	0.421**	0.534**	0.991**
RSA	0.901**	0.874**	-	0.989**	0.780**	0.960**	0.985**	0.906**	0.963**	0.992**	0.556**
RSAD	0.873**	0.938**	0.965**	-	0.865**	0.865**	0.999**	0.959**	0.962**	0.763**	0.749**
RV	0.925**	0.780**	0.833**	0.772**	-	0.990**	0.877**	0.972**	-0.952**	0.983**	0.578**
RVD	0.790**	0.705**	0.800**	0.739**	0.889**	-	0.876**	0.971**	0.951**	0.984**	0.574**
RD	0.785**	0.611**	0.532**	0.494**	0.776**	0.634**	-	0.965**	0.694**	0.774**	0.893**
RDD	-0.754**	-0.549**	-0.607**	-0.529**	-0.768**	-0.656**	-0.883**	-	0.863**	0.917**	0.754**
SDW	-0.372**	-0.835**	-0.464**	-0.697**	0.422**	0.603**	0.997**	0.706**	-	0.663**	0.954**
RDW	0.738**	0.720**	0.612**	0.426**	0.944**	0.935**	0.477**	0.658**	0.704**	-	0.407**
TDW	0.217**	0.735**	0.315**	0.572**	0.184**	0.159**	0.971**	0.811**	0.913**	0.994**	-

*, and ** significance at 0.01, and 0.001 probability levels, respectively.

RL: root length, RLD: root length development, RSA: root surface area, RSAD: root surface area development, RV: root volume, RVD: root volume development, RD: root diameter, and RDD: root diameter development, SDW: shoot dry weight, RDW: root dry weight, TDW: total dry weight. * and ** significant at $P < 0.05$ and $P < 0.01$, respectively.

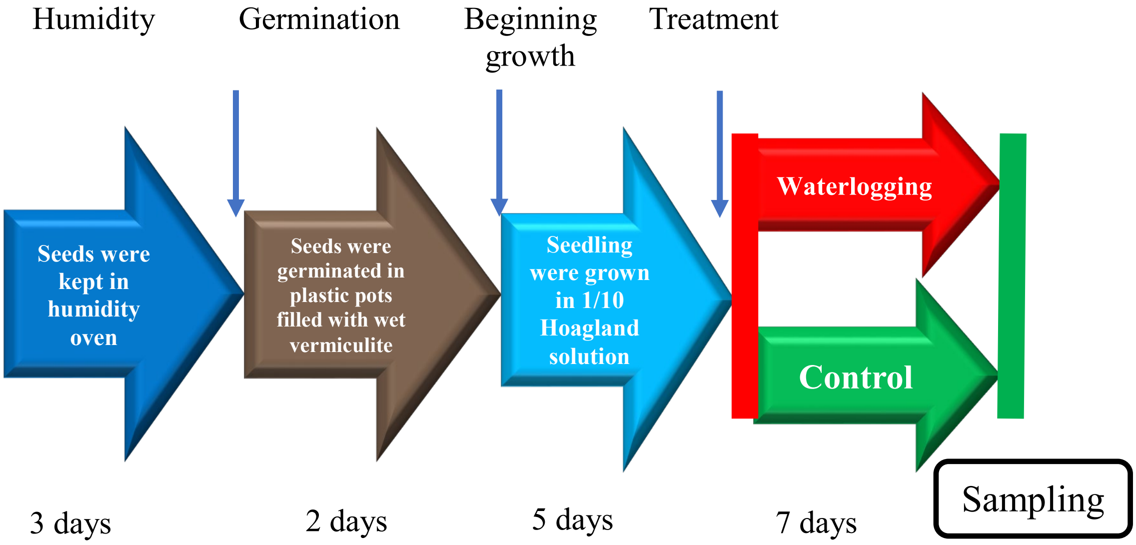


Fig. 14. Experiment design under growth chamber condition.

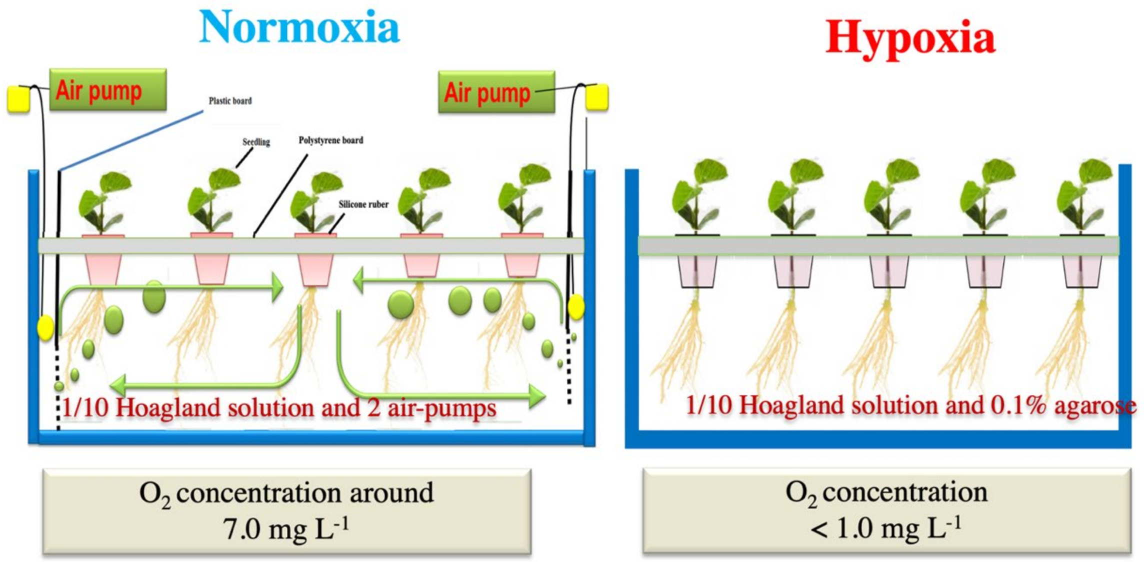


Fig. 15. Plant culture under normoxia (control) and hypoxia conditions

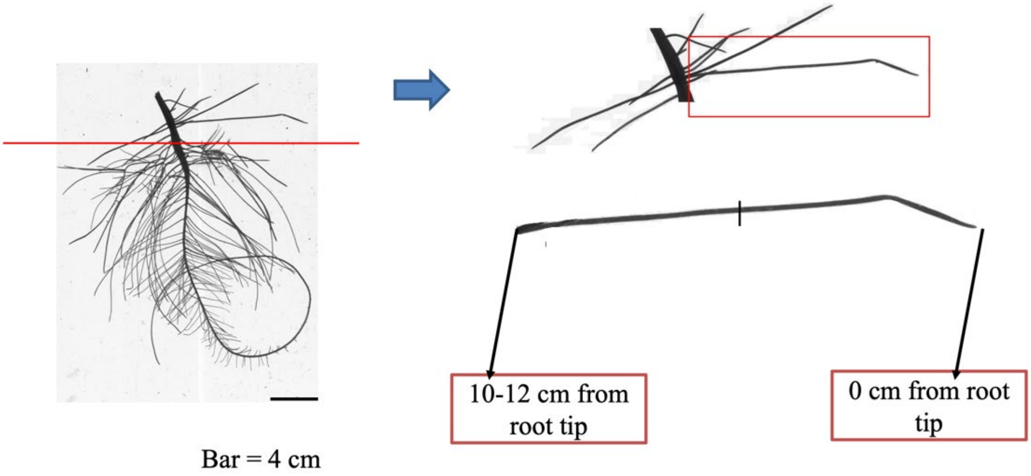


Fig. 16. Chosing branch for cross-section of root

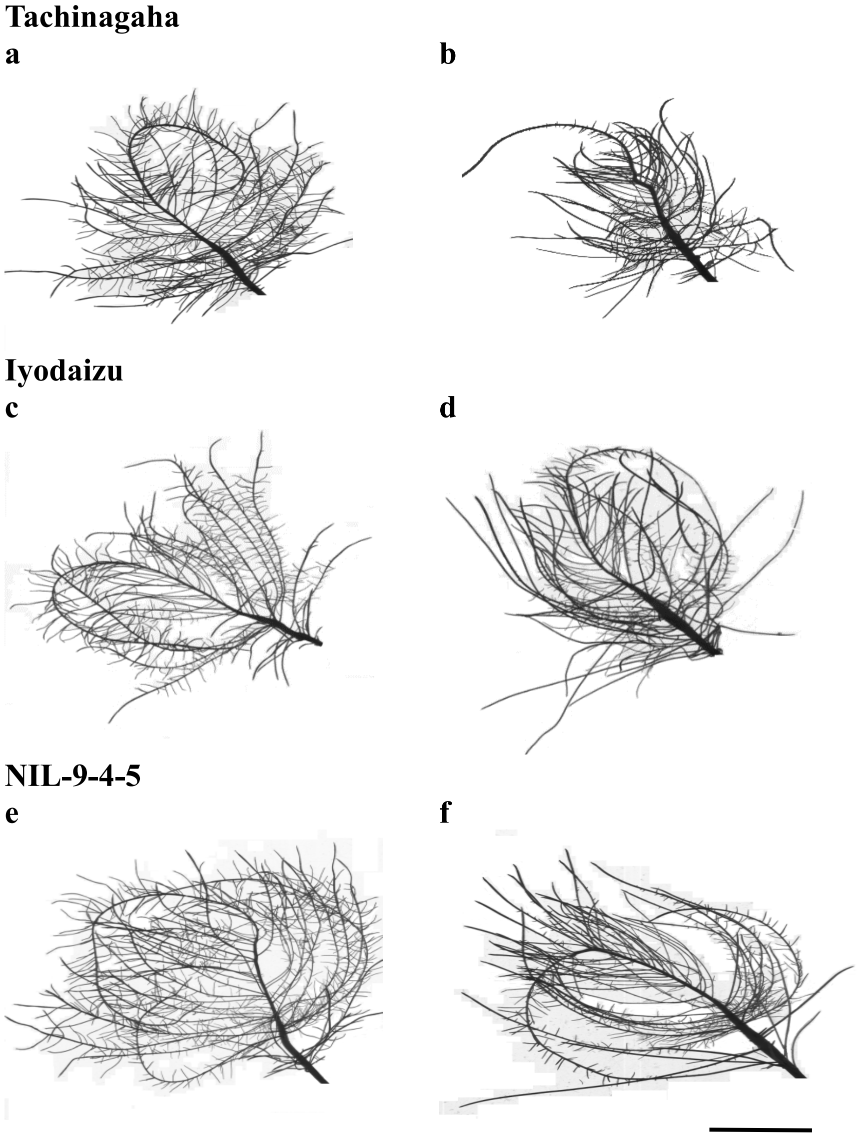


Fig. 17. Root of morphology of parents (Tachinagaha and Iyodaizu) and NIL -9-4-5 under control (a, c, e) and waterlogging (b, d, f) conditions at 13 days after sowing. Bars = 4 cm.

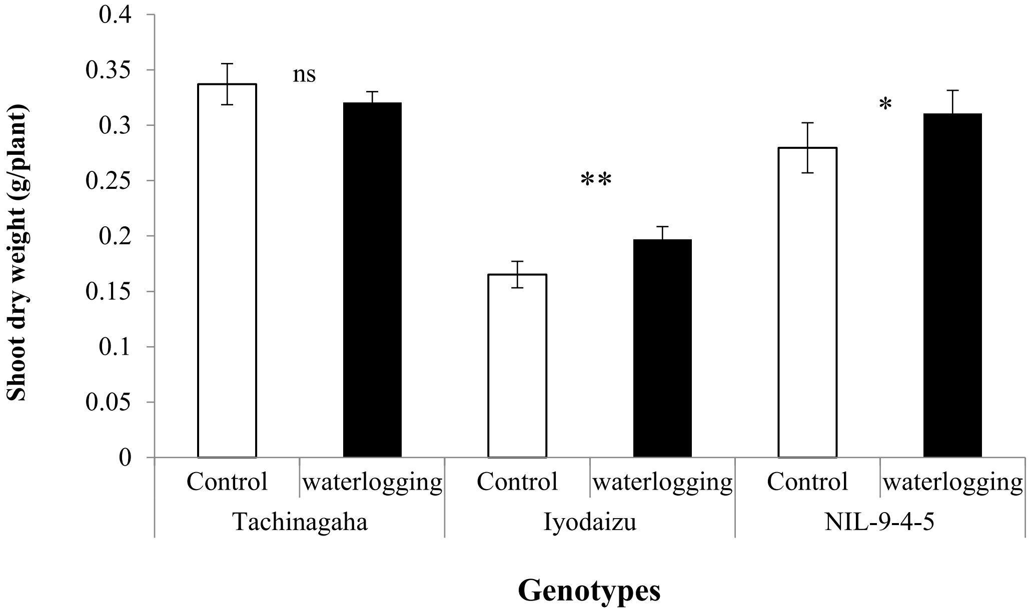


Fig. 18. Shoot dry weight of studied cultivars under control and waterlogging treatments. Vertical bars indicate standard deviations. Bars with asterisk ** and * in each genotype indicate significantly different at $P<0.01$ and $P<0.05$, by *t*-test (n=5), and ns, not significantly different.

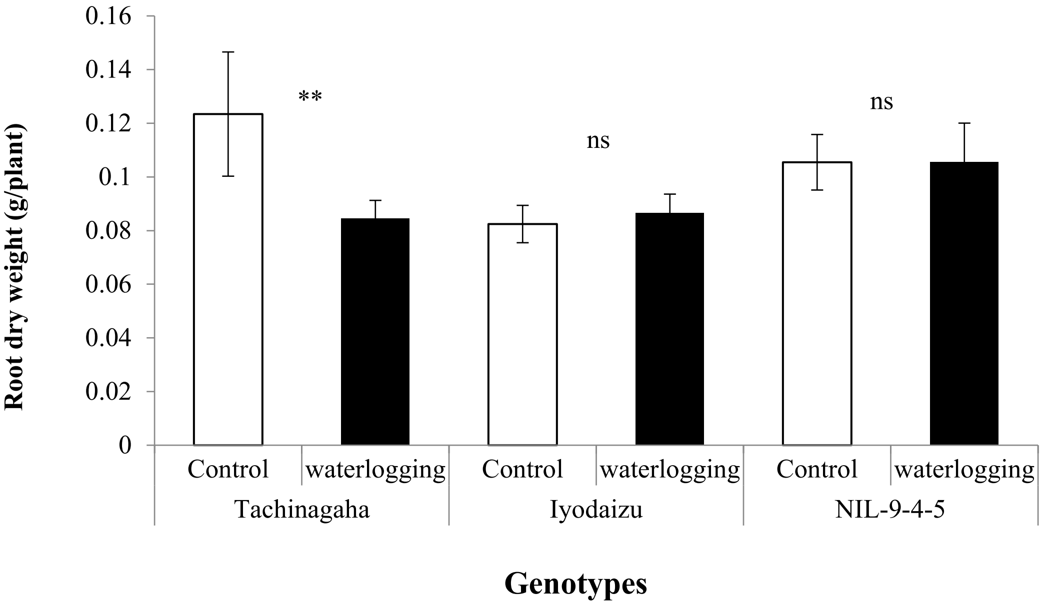


Fig. 19. Root dry weight (RDW) of studied cultivars under control and waterlogging treatments. Vertical bars indicate standard deviations. Bars with asterisk ** in each genotype indicate significantly different at $P<0.01$ by *t*-test (n=5), and ns, not significantly different.

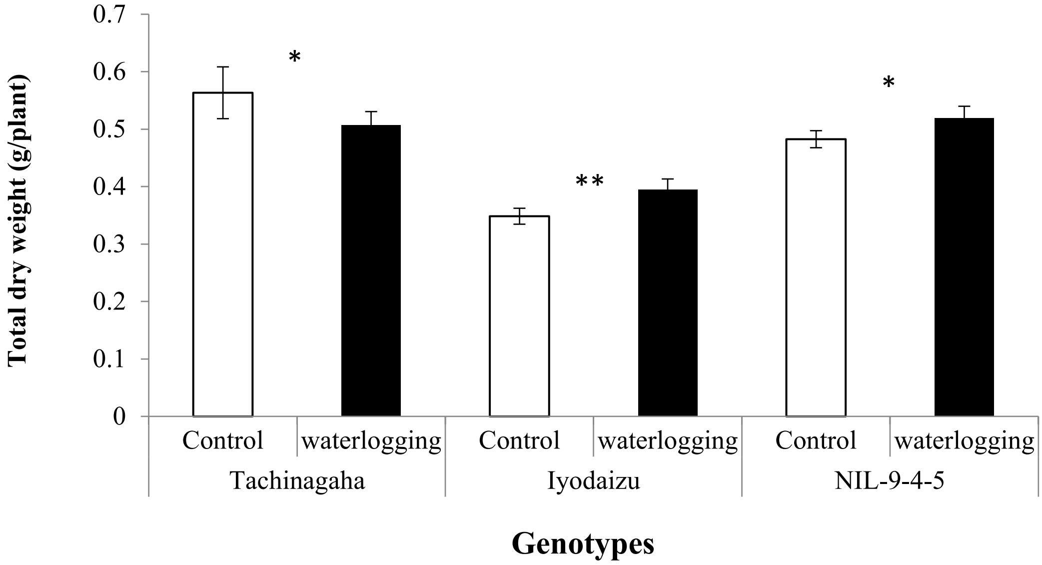


Fig. 20. Total dry weight (TDW) of studied cultivars under control and waterlogging treatments. Vertical bars indicate standard deviations. Bars with * and ** in each genotype indicate significantly different at $P<0.05$ and $P<0.01$ by *t*-test (n=5), respectively.

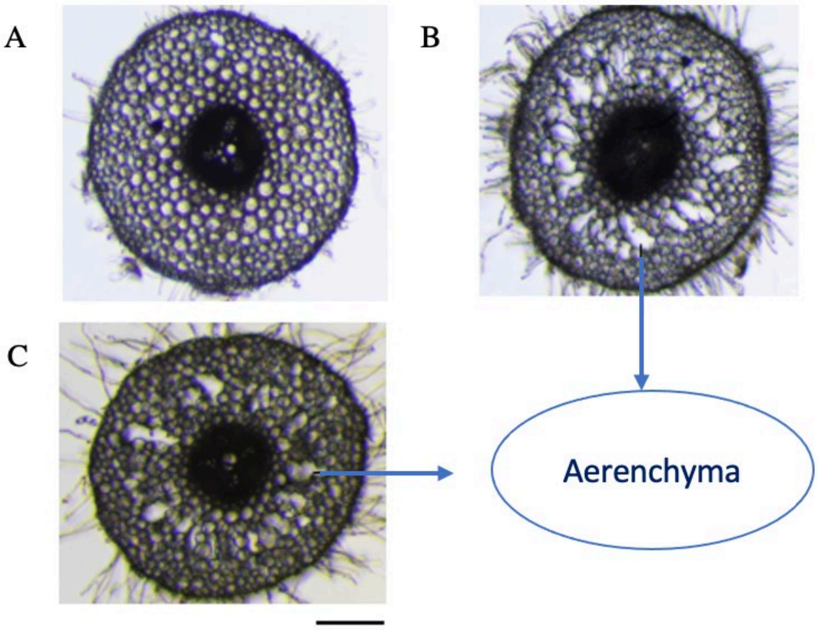


Fig. 21. Cross-section of the branch root tip (10-12 cm) under waterlogging condition of different cultivars: (A) Tachinagaha (B) Iyodaizu, and (C) NIL- 9-4-5. Bars = 200 µm

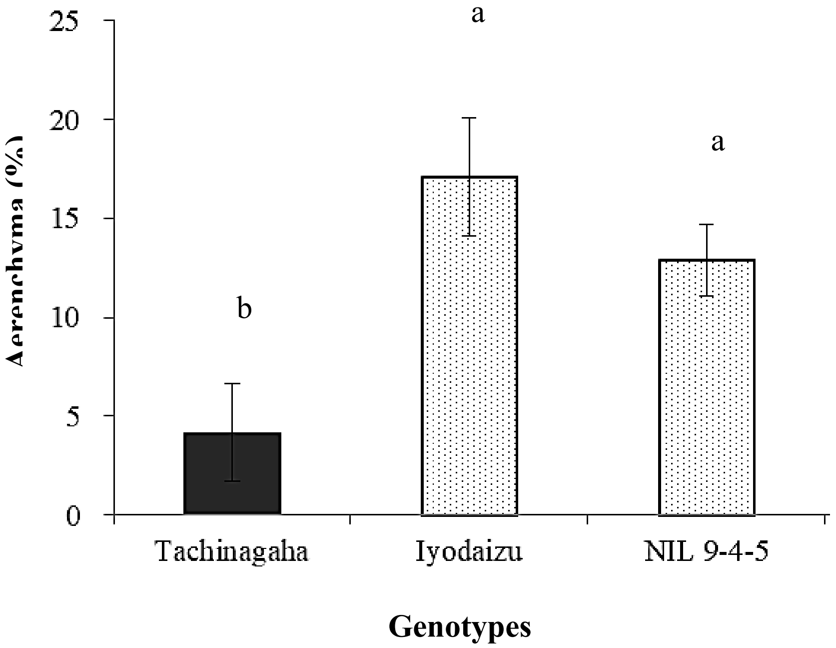


Fig. 22. The percentage of aerenchyma in root of NILs and parents under waterlogging condition. Vartucal bars indicate standard deviations. Bar with the same letter is not significantly different at $P < 0.05$ by *t*-test (n=5).

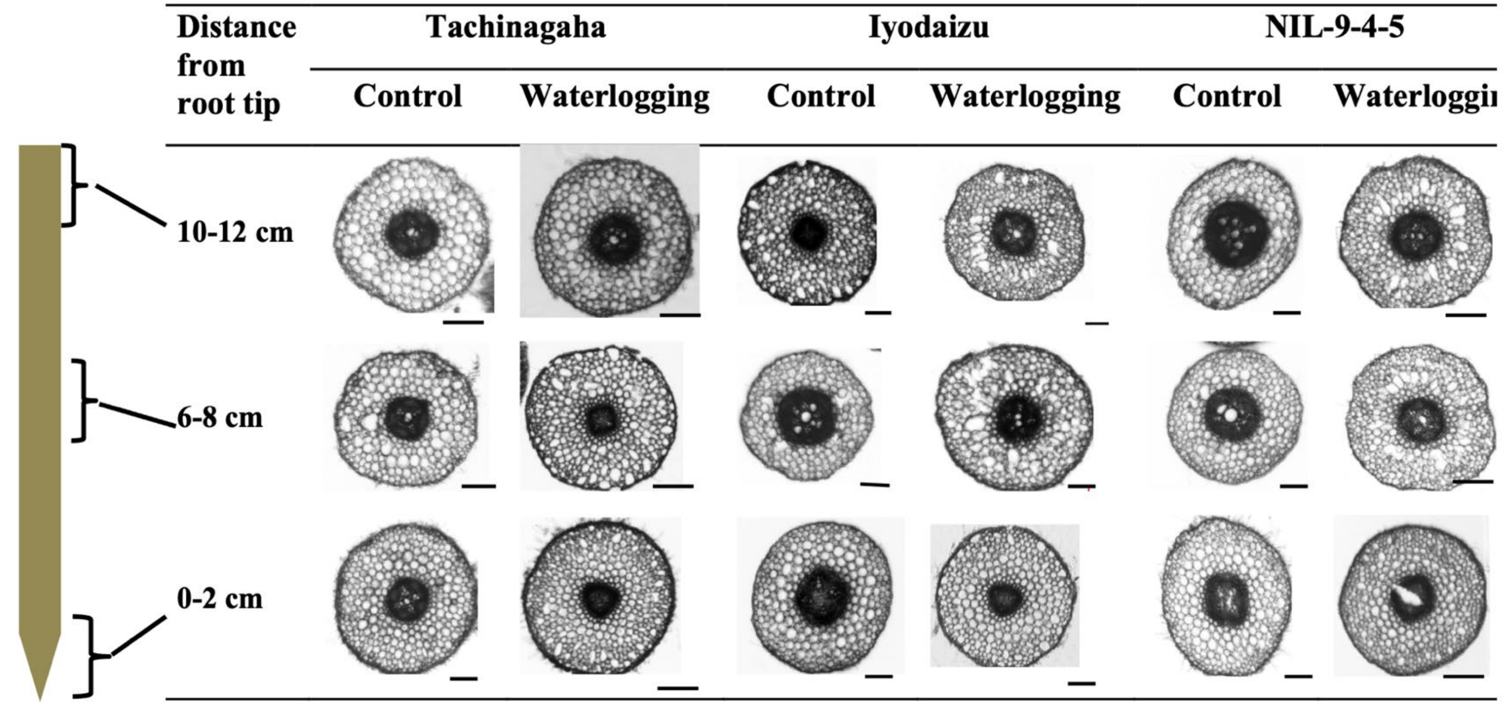
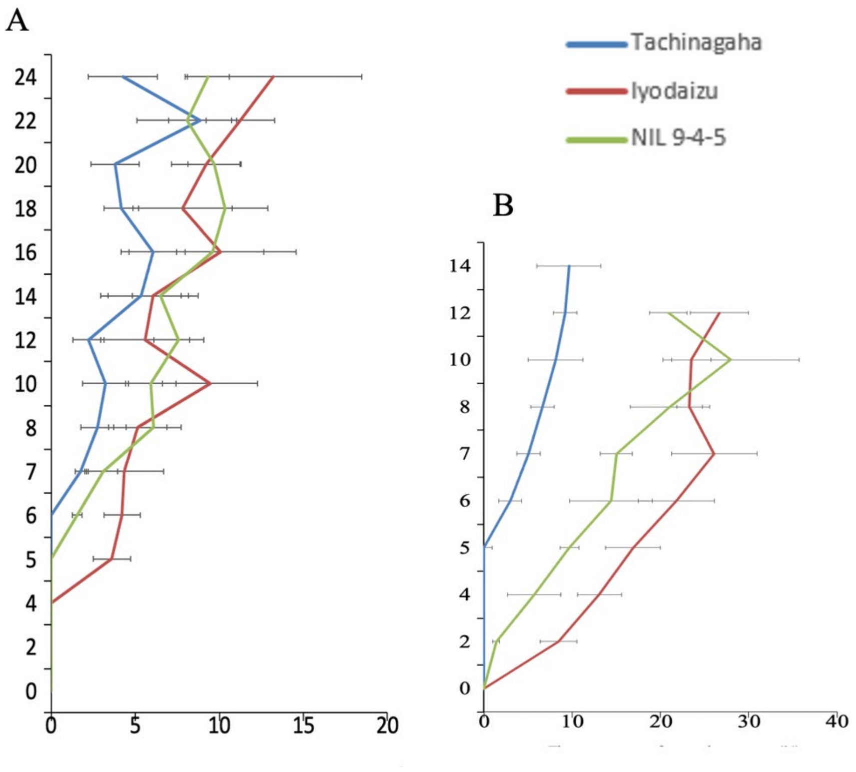


Fig. 23. Cross section at different positions in root of soybean under control and waterlogging conditions. Bars = 100



The percentage of aerenchyma area in root cross section with different positions (%)

Fig. 24. The percentage of aerenchyma area in root cross section with different positions of branch root tip under **A**: control condition and **B:** waterlogging condition. Vertical bars indicate standard deviation. (n=5).

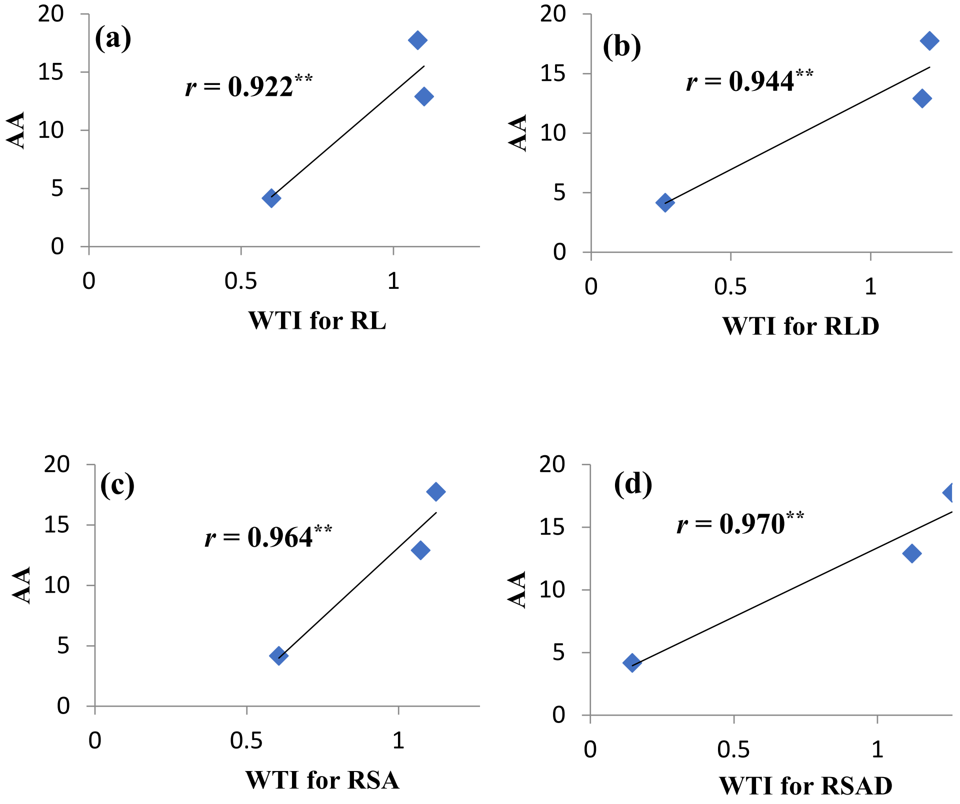
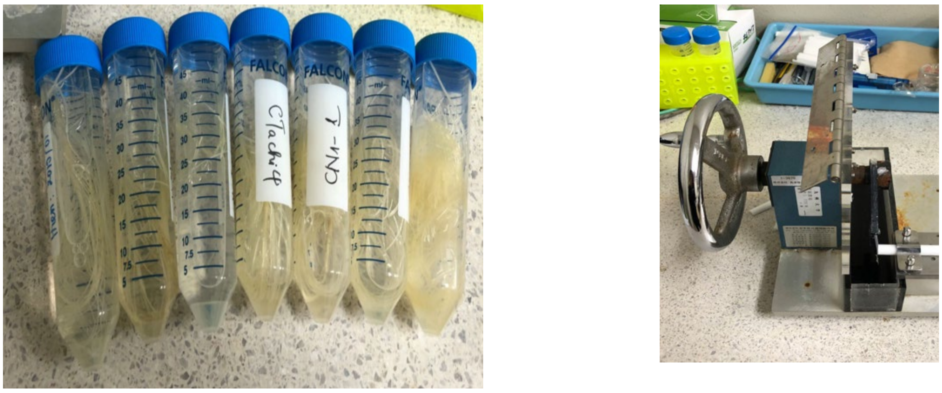
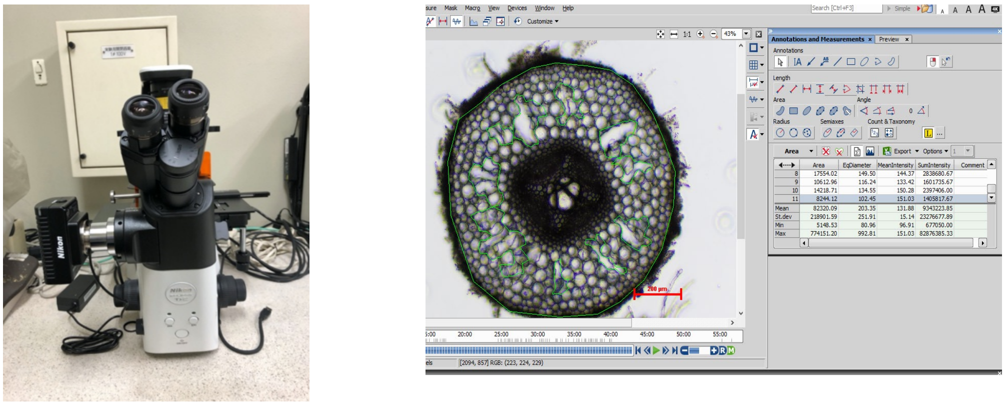


Fig. 25. The relationships between **(a)** area of aerenchyma (AA) and waterlogging tolerant index (WTI) for root length, **(b)** AA and WTI for root length development (RLD), **(c)** AA and WTI for root surface area (RSA), **(d)** AA and WTI for root surface area development (RSAD). **, significant at $P < 0.001$.



A) Sampling was kept in Formalin – Acetic -Alcofo (FAA) solution

B) Using Plant Microtome MTH-1 for cross section branch root into 20 µm thickness



	Area	EqDiameter	MeanIntensity	SumIntensity	Comment
8	17554.02	149.50	144.37	2838680.67	
9	10612.96	116.24	133.42	1601735.67	
10	14218.71	134.55	150.28	2397406.00	
11	8244.12	102.45	151.03	1405817.67	
Mean	82320.09	203.35	131.88	9343223.85	
St.dev	218901.59	251.91	15.14	23276677.89	
Min	5148.53	80.96	96.91	677050.00	
Max	774151.20	992.81	151.03	82876385.33	

C) Light microscope link to a computer

D) Measured aerenchyma by NIS Element D software.

Supplementary Fig. S1. Methodology for keeping sampling, cross-section branch root tip and measurement aerenchyma formation.

CHAPTER IV

NEAR-ISOGENIC LINES OF SOYBEAN CONFIRM A QTL FOR SEED WATERLOGGING AT DIFFERENT TEMPERATURES

1. Introduction

Climate change is regarded as a major factor increasing the probability of soil waterlogging due to severe rainfall. When soil is waterlogged, a hypoxic environment is induced due to the low diffusion of gases in water (Jackson and Colmer, 2005; Licausi and Giuntoli, 2020) and the respiration of organisms (Bailey-Serres and Voesenek, 2008). The low-oxygen atmosphere negatively influences plant growth and productivity. To cope with waterlogging-induced hypoxia, three major strategies (adaptation, escape and quiescence) have evolved in plants; for example, plants exhibit certain root traits that prevent the loss of oxygen from the roots, such as adventitious rooting, root aerenchyma formation and the formation of physical barriers.

Great efforts have been made to understand the effects of waterlogging conditions on the growth and development of various upland crops, such as wheat (*Triticum aestivum*) (Malik et al., 2001), chickpea (*Cicer arietinum*) (Cowie et al., 2013), upland cotton (*Gossypium hirsutum*) (Wang et al., 2017) and maize (*Zea mays*) (Zadi et al., 2003, and Tian et al., 2019). However, few of these studies have addressed the central question of the hypoxia stress response in soybean (*Glycine max*), a crop with poor tolerance to waterlogging.

Soybean is considered the most important legume species to humans and is frequently cultivated from spring to early summer in eastern Asia (Lee et al., 2003). During this period, an increasing occurrence of heavy rains has been reported, causing significant damage to soybean at the germination and seedling stages (Araki et al., 2012; Kokukun et al., 2013). Genetic variation in germination responses to waterlogging was reported in previous studies (Sung, 1993; Sayama et al., 2009; Nanjo et al., 2014), and this response can be greatly affected by temperature (Hou and Thseng, 1991; Wuebker et al., 2001). Warmer temperatures are associated with greater losses during seedling emergence and a complete loss of germination is observed when seeds are soaked for 4 days at 30°C (Hou and Thseng, 1991). Unfortunately, the variation in seed germination responses to waterlogging at different temperatures remains unclear.

Although extensive QTLs for waterlogging tolerance of soybean have been detected, most of these QTLs are related to tolerance at the vegetative stage (Van Toai et al., 2001; Reyna et al., 2003; Cornelious et al., 2005; Githiri et al., 2006; Sayama et al., 2009; Loc, N. V., et al., 2015; Nguyen et al., 2017). Limited information is available about QTLs for waterlogging tolerance in soybean plant in the germination stage. Only five QTLs, *Sft1, Sft2, Sft3*, *Sft4* (Sayama et al., 2009) and *QTN13* (Yu et al., 2019), have been associated with germination and normal seedling rates under seed-soaking stress. Interestingly, one of these QTLs (*Sft1*) is located in a marker interval containing QTLs on chromosome 12, near a candidate QTL region for root development under hypoxia and waterlogging (Nguyen et al., 2017). To obtain a deeper understanding of the effects of candidate QTL regions for root development on waterlogging tolerance of soybean at the germination stage, the seeds of a near-isogenic line (NIL) and their parents were used to confirm the effects of waterlogging at various temperatures on the characteristics of these soybean seeds, including the germination rate and seedling rate.

2. Materials and methods

2.1. Materials

Fifteen soybean genotypes, including seven from Japan (Iyodaizu, Kokubu 7, Komame, Maetsue Zarai 90B, Miyashishirome, Nattou Kotsubu and Tachinagaha), three from India (E C 112828, M42 and M652), two from Nepal (N 2295 and U1155-4), one from Korea Rep. (Okjo), one from China (Peking) and one from the United States (Williams 82), were used in this study (Table 7). In addition, Iyodaizu, Tachinagaha and NIL-9-4-5 were used to confirm the effects of a major QTL for root development under hypoxia on seed waterlogging tolerance in soybean. Among them, NIL-9-4-5 is reported as a near-isogenic line (NIL) that was selected from the Tachinagaha/Iyodaizu BC_6F_3 population through marker-assisted selection (Nguyen et al., 2017).

2.2. Seed waterlogging treatment

Seeds of each genotype were incubated in Petri dishes (diameter: nine cm) at 23 °C and 80% RH in the dark and the number of germinating seeds was counted for three days. Then, three-day-seeds were treated under waterlogging condition at different temperatures: 21, 23, 25, 27 and 29 °C (Fig. 26). Briefly, control seeds were sown in 0.43 l plastic pots (7.2 cm in top diameter; 11.6 cm in height; and 5.1 cm in bottom diameter) filled with humid vermiculite (Midorisangyou, Fukuoka, Japan) for four days, while waterlogging-treated- seeds (10 per temperature treatment per genotype) were subjected to a water soaking treatment in an Erlenmeyer flask containing 120 ml of deionized distilled water for 48 hours. The pots and Erlenmeyer flasks were then placed on trays in growth chambers (220 µmol m^2 s^{-1} light density, 14 h light / 10 h dark) that were set at the target temperatures. Then, the treated seeds were germinated in humid vermiculite for two days as the recovery stage. The experiments were performed under a randomized complete block design with three replications per treatment, and 10 seeds were used per replication (Nguyen, V. L. et al., 2021).

2.3. Measurements

At the end of the seed waterlogging treatment, the appearance of air bubbles at the water surface in the Erlenmeyer flasks containing the seeds of each soybean genotype was recorded. The samples were collected after four days of treatment. Seeds with a radicle longer than 1 cm were recorded as germinating seeds, and seedlings without any damage to the radicle or cotyledon were regarded as normal seedlings. The germination rate (GR) and normal seedling rate (NSR) were calculated with the following formulas:

$$Germiation\ rate\ (\%) = \frac{Number\ of\ germinated\ seeds}{Number\ of\ sowed\ seeds}\ x\ 100$$

$$Normal\ seedling\ rate\ (\%) = \frac{Number\ of\ normal\ seedlings}{Number\ of\ sowed\ seeds}\ x\ 100$$

The relative GR and NSR values at the tested temperatures were calculated as the ratio of the mean value under the control treatment to the mean one under waterlogging.

2.4. Statistical analysis

All statistical analyses were performed with Unistat 6.5. The effect of the genotype on the results for each trait was assessed by two-way ANOVA.

3. Results

3.1. Effects of seed waterlogging stress on GR and NSR

No effects of genotype, temperature or their interactions on GR or NSR were detected under control conditions, while these effects were significant for both variables under waterlogging treatment (Table 8).

Compared to the control, the mean GR under waterlogging stress was reduced from 4 % at 21 °C, to 66 % at 29 °C. The mean NSR was reduced from 18 % at 21 °C, to 82 % at 29 °C (Table 8).

3.2. Variation in seed germination responses to waterlogging stress at different temperatures

The appearance of air bubbles at the water surface in Erlenmeyer flasks showed an increase with increasing temperatures (Fig. 27, 28). Significant genetic variations in NR and NSR responses to temperature under seed waterlogging were found (Fig. 29, Fig. 30). At 21 °C, the GR of the soybean genotypes varied from 73% (Okjo and M42) to 100 % (10/15 varieties), and the NSR varied from 37 % (Williams 82) to 100 % (U 1155-4, Iyodaizu and N 2295). At 23 °C, the GR of the soybean genotypes varied from 50 % (Tachinagaha) to 100 % (U 1155-4 and Iyodaizu), and the NSR varied from 0% (Williams 82) to 100 % (U 1155-4). At 25 °C, the GR of the soybean genotypes varied from 33 % (Tachinagaha) to 100 % (U 1155-4), and the NSR varied from 0 % (Williams 82 and Miyagishirome) to 90 % (U 1155-4). At 27°C, the GR of the soybean genotypes varied from 13 % (Maetsue Zarai 90B and N 2295) to 90 % (U 1155-4), and the NSR varied from 0% (8/15 genotypes including Williams 82, Miyagishirome, Nattou Kotsubu, Komame, Okjo, M42, Kokubu, Maetsue Zarai 90B and N 2295) to 87% (U 1155-4). At 29°C, the GR and NSR showed clear differences among the genotypes. Only five of the 15 genotypes, including U 1155-4, M 652, E

C 112828, Yodaizu, and Peking showed to have the NSR at 29°C. Based on the GR, the genotypes were categorized into three groups, including a tolerant (Peking, U 1155-4, Iyodaizu and M 652), a moderately tolerant (E C 112828, Komame, and Nattou Koshubu) and a sensitive group (others), regardless of the temperature (Fig. 29, 30).

3.3. The seed waterlogging tolerance of soybean is linked to QTLs for root development under hypoxia

NSR of the genotypes under the control treatment was higher than 90% and was not significantly different among all genotypes at different temperatures (Fig. 31, Supplementary Fig. S3). Under waterlogging, a significant reduction was found in GR and NSR of the 3 genotypes associated with increasing temperatures from 21°C to 29°C (Fig. 32, 33, and Supplementary Fig. 4). Among genotypes, Tachinagaha was found to be a waterlogging-sensitive genotype, showing rapid reductions in GR and NSR, while Iyodaizu and NIL-9-4-5 were waterlogging-tolerant compared to controls. GR and NSR of Iyodaizu and NIL-9-4-5 were also significantly greater than those of the recurrent parent Tachinagaha at all temperatures (Fig. 32, 33, and Supplementary Fig. S4).

4. Discussion

Recently reports have described the relationship between waterlogging condition and temperature variation. Here, the effects of a QTL region were confirmed for root development under hypoxia significantly contributes to the seed waterlogging tolerance of soybean plants at the germination stage. Hypoxia has been proposed as the main problem associated with waterlogging because the available oxygen concentration is rapidly decreased due to the slow diffusion of oxygen in water (Armstrong, 1979; Wiengweera et al., 1997; Hossain and Uddin, 2011). Reduction of oxygen partial pressures to 2 kPa and 6 kPa, decreased GR to 0% and 50% respectively, compared to the maximum GR under ambient conditions (Al-Ani et al., 1985). Tian and Arihara (1998) conducted the experiments on the effects of O_2 supplies on GR of 8 soybean genotypes and reported that GR was decreased by 25% to 75% at 5% O_2 compared to 20% O_2. In this study, the GR of soybean under soaking was rapidly decreased by 4% at 21°C to 66% at 29°C (Table 8). These results indicated that hypoxia stress causes a decrease in the GR of soybean under waterlogging.

In this study, the interaction between the waterlogging treatment and temperature was consistent with results of other studies obtained under soaking or soil waterlogging conditions. Previous study has indicated that warmer soil temperatures are related to greater losses in seedling emergence under waterlogging compared with lower temperatures (Fausey and

McDonald, 1985). Under soaking conditions, incubating soybean seeds at 10 or 15°C for up to 8 days prior to germination caused no loss in germination, but germination decreased as the length of the soaking period at 25 and 30°C was increased (Hou and Thseng, 1991). The data obtained in this study showed that the GR was reduced by only 6% at 21°C but was decreased by up to 68% at 29°C (Table 8).

Variation was found among the genotypes included in this study (Fig. 29). Williams 82 and Tachinagaha showed waterlogging-sensitive genotypes exhibiting rapid reductions in GR and NSR regardless of increasing temperatures. Peking, U 1155-4, Iyodaizu and M 652 were better adapted to waterlogging than the other genotypes. Among these genotypes, Peking has been reported as a seed waterlogging-tolerant genotype exhibiting a delay in germination under hypoxia regardless of temperature (Nakajima et al., 2015). Iyodaizu was selected as a tolerant Japanese variety at 25°C (Nanjo et al., 2014). The genotypes showing seed waterlogging tolerance might be useful for the genetic improvement of waterlogging tolerance in modern soybean varieties.

In this study, Iyodaizu was classified in the waterlogging-tolerant group at the germination stage, and Tachinagaha was classified in the sensitive group. In line with our observations, Iyodaizu has been previously reported as a genotype that is tolerant to waterlogging at the germination (Nanjo et al., 2014) and seedling stage (Sakazono et al., 2014; Jitsuyama, 2015; Suematsu et al., 2017; Nguyen et al., 2017), and Tachinagaha has been reported as a moderately sensitive genotype at the germination (Sayama et al., 2009; Nanjo et al., 2014) and seedling stage (Sakazono et al., 2014; Jitsuyama, 2015; Suematsu et al., 2017; Nguyen et al., 2017). These results provide interesting information for exploring the mechanisms involved in the development of adaptations in response to waterlogging in the germination stage. Through the analyses of inbred lines (RILs) developed from a cross between Tachinagaha and Iyodaizu, Nguyen et al. (2017) identified QTLs for root development, including root length development (RLD) and root surface area development (RSAD), on soybean chromosome 12 and developed an NIL-9-4-5 carrying targeted QTLs at BC_6F_2. The QTLs for RLD and RSAD (*Qrld-12*, *Qrsad-12*) on Chr.12 have been shown to be stable across years. The resultant increase in root development in NIL-9-4-5 was most likely inherited from the waterlogging-tolerant parent Iyodaizu. Interestingly, Sayama et al. (2009) also identified a QTL (*Sft1*) for seed flooding tolerance in soybean, which is located near the candidate QTL region mentioned in this study. Therefore, this study was conducted to confirm the existence of QTL effects on seed waterlogging tolerance related to root development under hypoxia using NIL-9-4-5 line. The obtained results suggested that the normal seedling rate of NIL-9-4-5 presented the same trend as that of the donor parent Iyodaizu and was significantly greater than that of the recurrent parent Tachinagaha, indicating that the marker interval may contain a gene for seed waterlogging tolerance in soybean (Fig. 32, 33). These results agreed with those of Nguyen et al. (2017) showing that QTLs for hypoxia tolerance in soybean at the germination and seedling stages are located in the marker interval on Chr. 12.

The important pathway induced under hypoxia is ethanolic fermentation (Liem et al., 2019). By which under the action of pyruvate decarboxylase and alcohol dehydrogenase, carbohydrates convert to alcohol and CO_2 gas (Zabalza et al., 2009). In this study, the formation of gas bubbles indicated that fermentation occurred under seed waterlogging treatment (Fig. 26 - 28). Under waterlogging, the appearance of air bubbles was observed in Erlenmeyer flasks containing the seeds of Iyodaizu and NIL-9-4-5 at 29°C and those of Tachinagaha at 23°C (Supplementary Fig. S2). The mechanisms involved in the hypoxia tolerance of soybean are related to the patterns of alanine aminotransferase (AlaAT) and aldehyde dehydrogenase (ALDH) as previously described (Liem et al., 2019). More specifically, AlaAT plays an important role in regulating the glycolytic flux by preventing the excessive accumulation of pyruvate (Zabalza et al., 2009) while retaining carbon and nitrogen resources within the cell (Rocha et al., 2010). Unlike the production of lactate and ethanol, alanine accumulation does not have detrimental side effects in cells. Another analysis of expression identified aldehyde dehydrogenase, a fermentative enzyme responsible for the metabolization of aldehyde, which is harmful to cells under hypoxia stress (Nakazono et al., 2000; Fukao et al., 2003; Tsuji et al., 2003). These results suggest that by analyzing the expression of genes related to fermentation linked to the seed waterlogging tolerance of soybean evaluated in this study, a more mechanistic understanding of the response to waterlogging stress will be achieved (Nguyen et al., 2021).

Table 7. Characteristics of the studied genotypes

Genotype	**Origin**	**Code at source**	**Seed weight (g/seed)**	**Seed colour**
E C 112828	India	GW80	0.1931	Yellow
Iyodaizu	Japan	Iyo	0.1314	Green
Kokubu 7	Japan	GJ66	0.3332	Yellow
Komame	Japan	GJ32	0.4227	Yellow
M42	India	GJ53	0.1824	Yellow
M652	India	GJ67	0.0688	Black
Maetsue Zarai 90B	Japan	GJ58	0.4997	Yellow
Miyagishirome	Japan	GJ14	0.5141	Yellow
N 2295	Nepal	GW41	0.1973	Brown
Nattou Kotsubu	Japan	GJ13	0.1606	Yellow
Okjo	Rep. Korea	GW18	0.2823	Yellow
Peking	China	GW28	0.1034	Black
Tachinagaha	Japan	Tachi	0.3612	Yellow
U 1155-4	Nepal	1155-4	0.0624	Black
Williams 82	America	GW36	0.2591	Yellow

Table 8. ANOVA and relative values of germination rate and normal seedling rate under control and waterloggingconditions at different temperatures

Traits	***Mean at***					***F-Value***		
	21°C	23°C	25°C	27°C	29°C	Genotype (G)	Temperature (T)	G x T
Control (a)								
Germination rate (%)	98	95	95	95	97	1.54^{Ns}	1.14^{Ns}	1.01^{Ns}
Normal seedling rate (%)	96	94	94	95	96	1.15^{Ns}	1.39^{Ns}	1.37^{Ns}
Seed-waterlogging (b)								
Germination rate (%)	94	80	69	47	31	8.65^{*}	43.75^{*}	66.78^{*}
Normal seedling rate (%)	79	65	48	25	17	11.60^{*}	34.85^{*}	94.77^{*}
Relative value (b/a)								
Germination rate	0.96	0.84	0.72	0.50	0.32			
Normal seedling rate	0.82	0.68	0.50	0.26	0.18			

(**): Significant and (Ns): Not significant at P<0.05

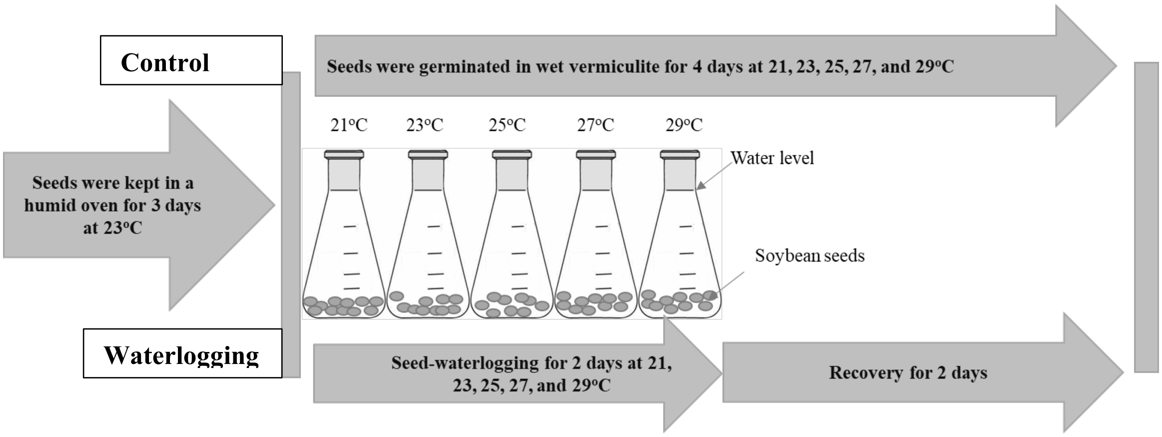


Fig. 26. Experimental schedule and seed-waterlogging treatments

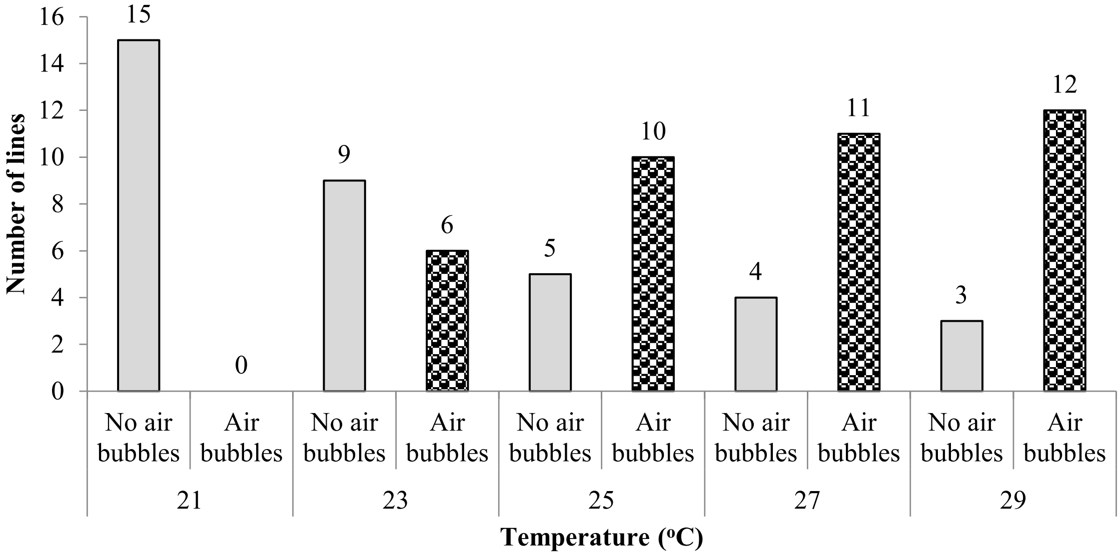


Fig. 27. Frequency distribution of air bubble formation in soybean genotypes after seed-waterlogging treatments at different temperatures

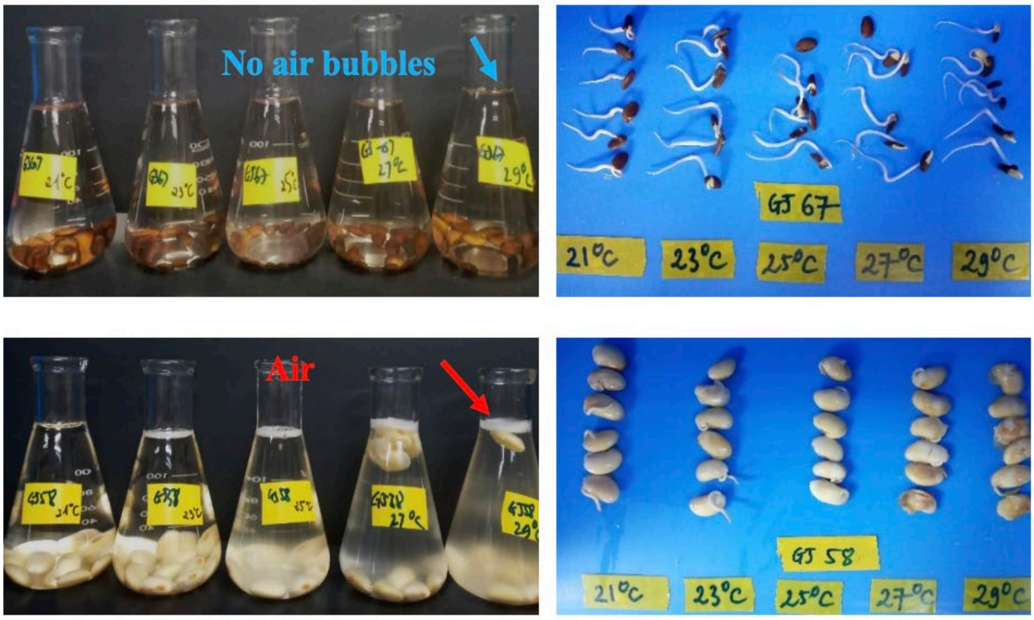


Fig. 28. Air bubble formation after 2 days-waterlogging treatment in Erlenmeyer flasks and germination performance after 2 days recovery of GJ 67 (M652) and GJ 58 (Maetsue Zarai 90B) at different temperatures

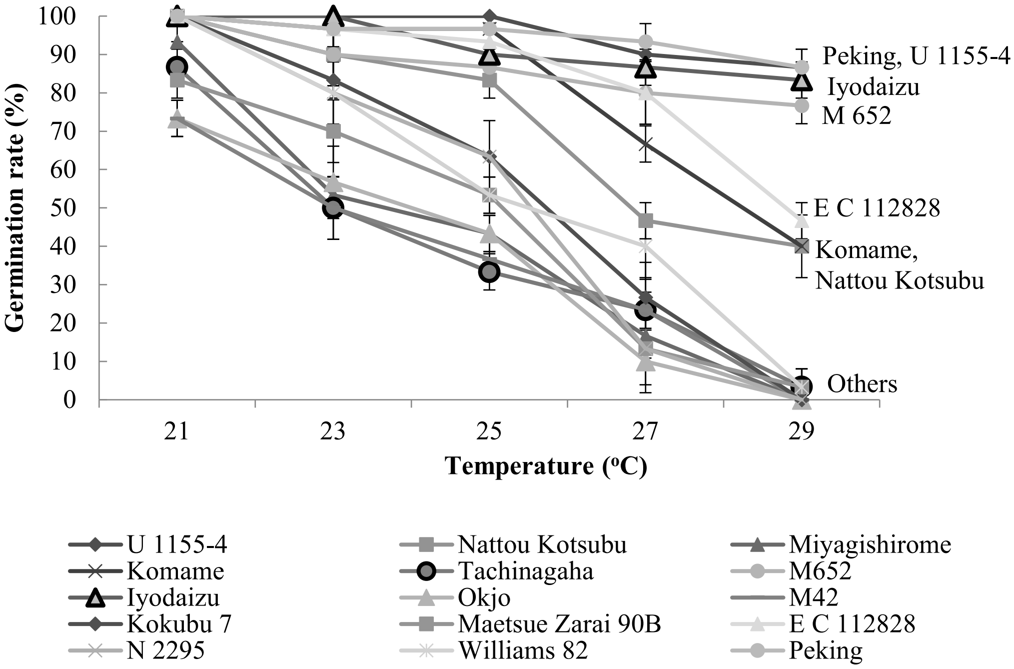


Fig. 29. Variation in the germination rate of soybean genotypes under waterlogging at different temperatures. Vertical bars indicate SEM.

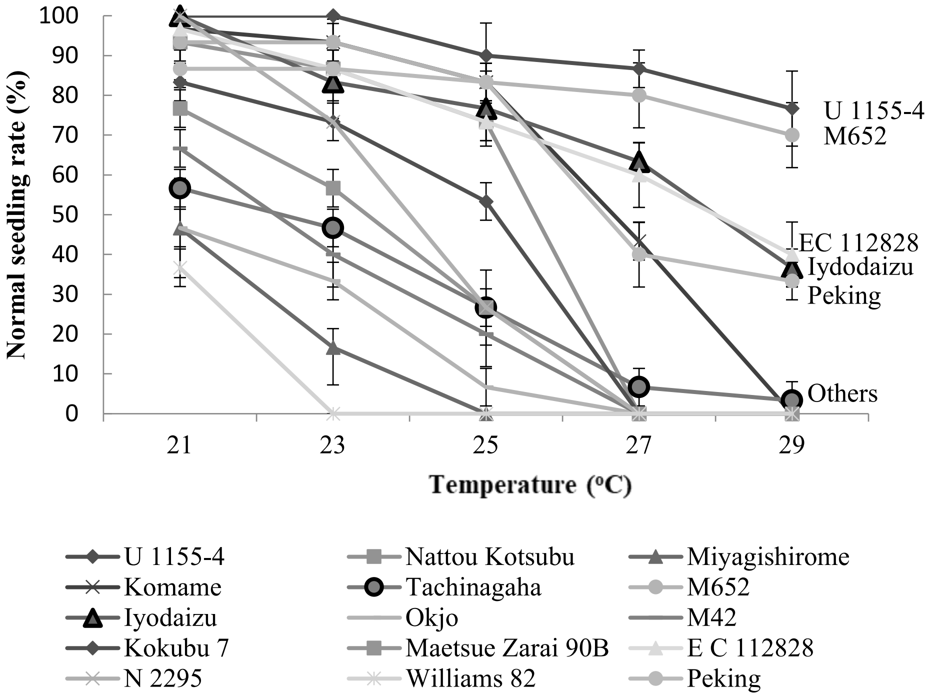


Fig. 30. Variation in the normal seedling rate of soybean genotypes under waterlogging at different temperatures. Vertical bars indicate SEM.

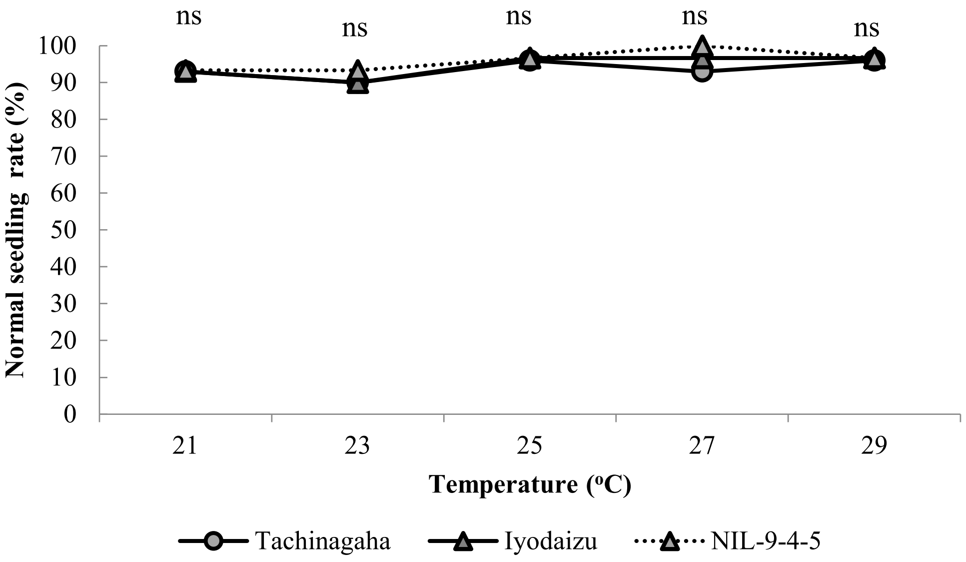


Fig. 31. The normal seedling rate in NIL-9-4-5 and parental genotypes under control conditions at different temperatures. *Ns: Not significant at P<0.05*

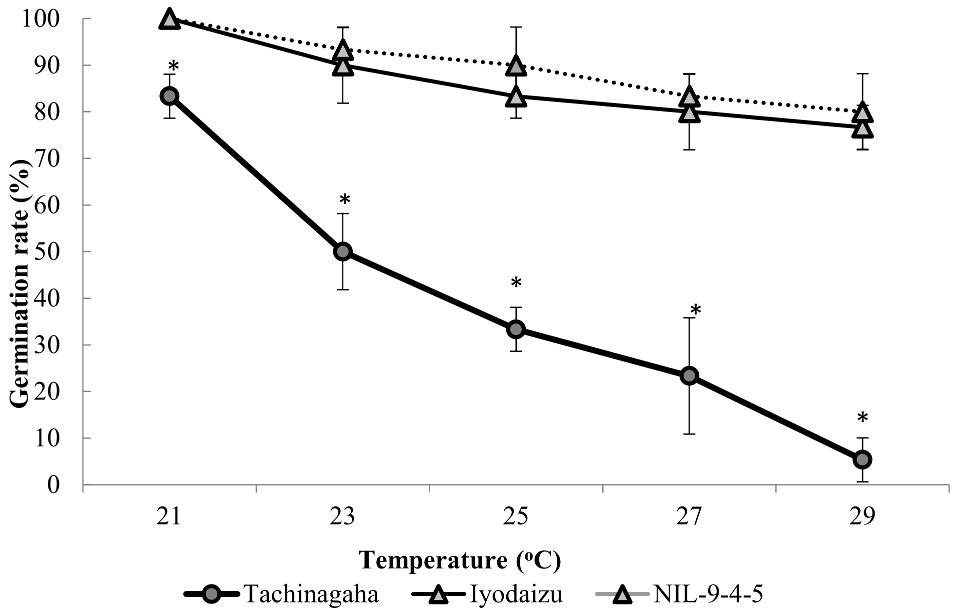


Fig. 32. The germination rate in NIL-9-4-5 and parental genotypes under waterlogging at different temperatures. Vertical bars indicate SEM.**Significant at $P<0.05$.*

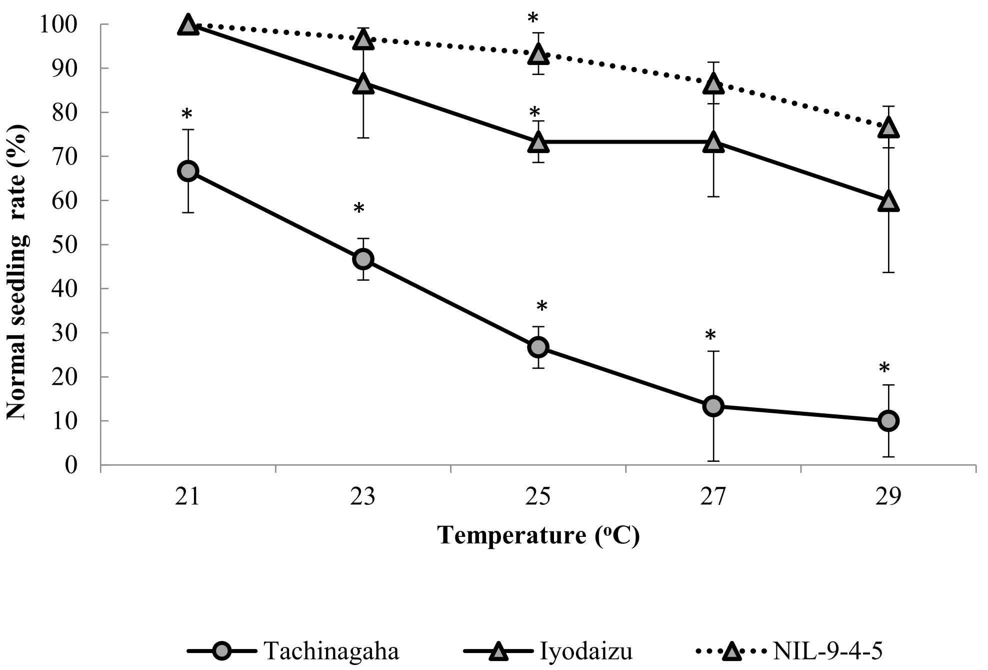
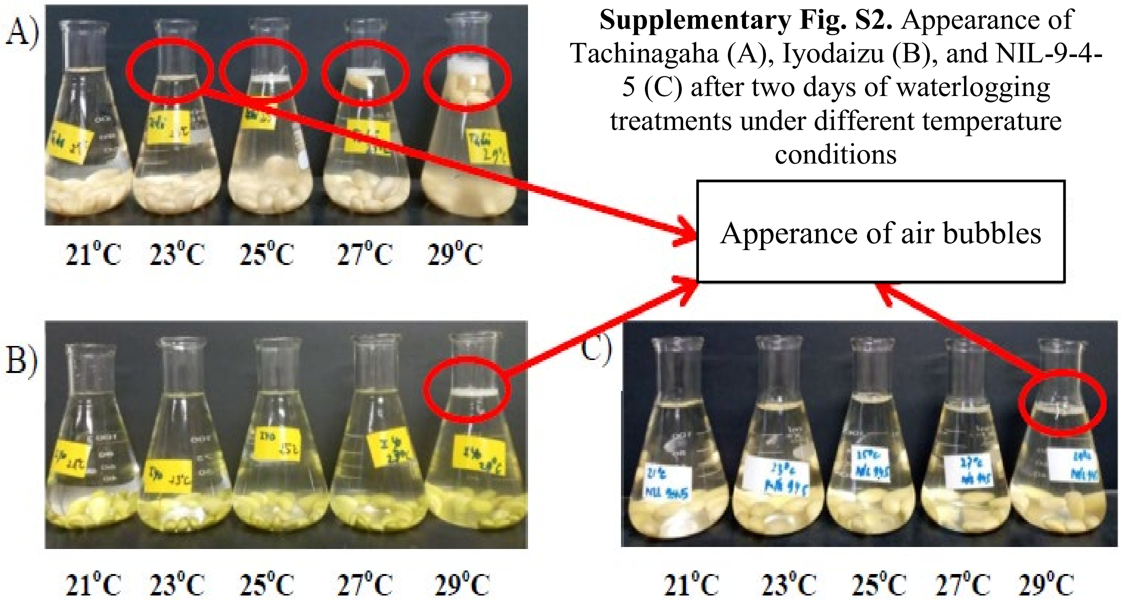
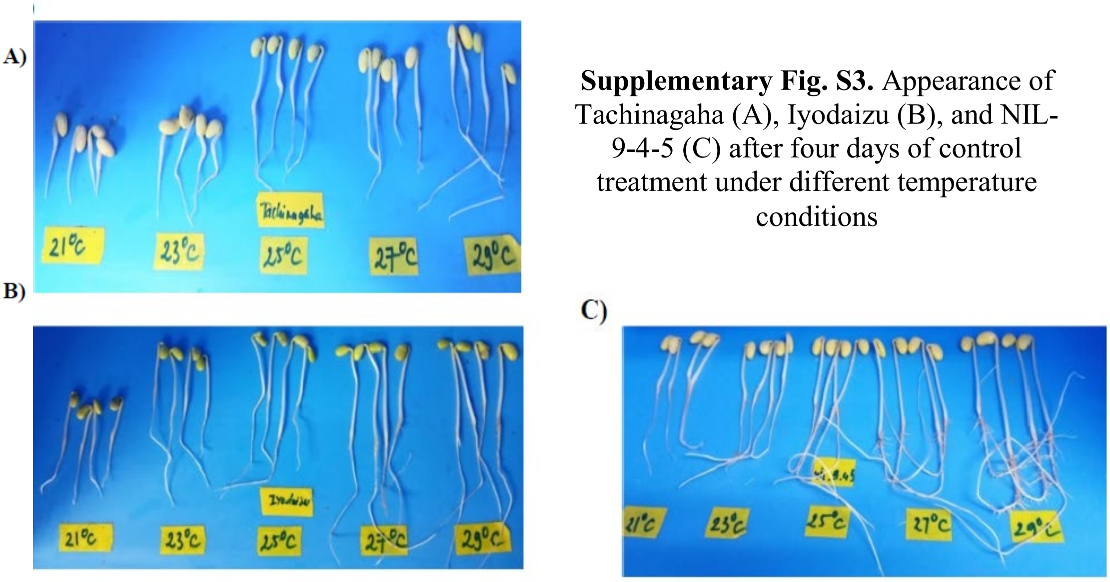


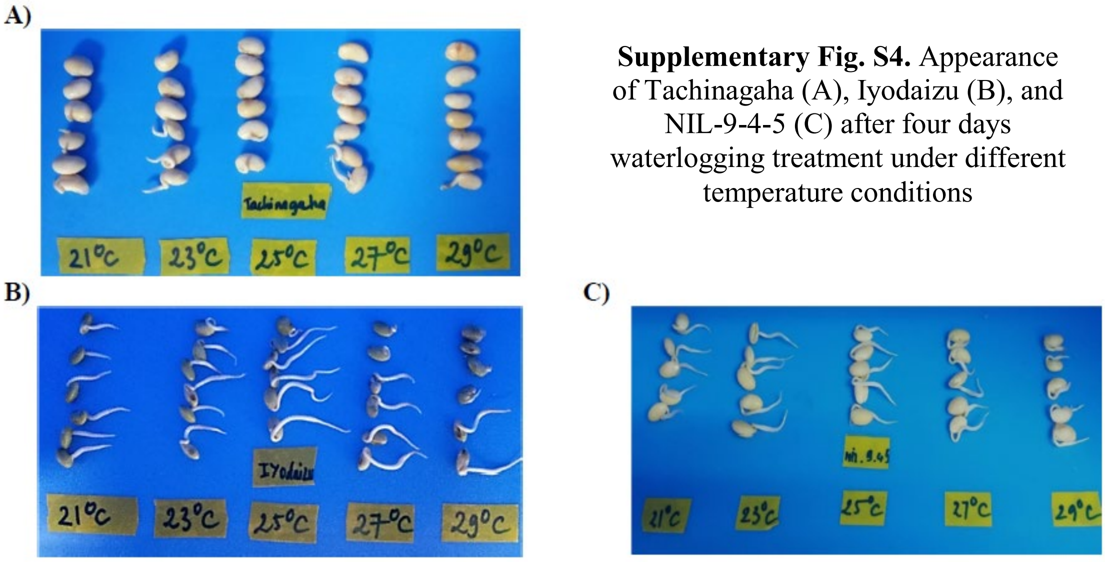
Fig. 33. The normal seedling rate in NIL-9-4-5 and parental genotypes under waterlogging at different temperatures. Vertical bars indicate SEM. **Significant at P<0.05.*



Supplementary Fig. S2. Appearance of Tachinagaha (A), Iyodaizu (B), and NIL-9-4-5 (C) after two days of waterlogging treatments under different temperature conditions



Supplementary Fig. S3. Appearance of Tachinagaha (A), Iyodaizu (B), and NIL-9-4-5 (C) after four days of control treatment under different temperature conditions



Supplementary Fig. S4. Appearance of Tachinagaha (A), Iyodaizu (B), and NIL-9-4-5 (C) after four days waterlogging treatment under different temperature conditions

CHAPTER V

GENERAL DISCUSSION

Climate change is becoming more complicated and unpredictable, it causes waterlogging stress for plant growth. Waterlogging stress includes flooding (hypoxia/waterlogging) and drought stress. Previous studies showed that flooding is a major problem in many areas of the world and most crop species, including soybean, are susceptible to waterlogging (Mochizuki, T. et al., 2000, Nguyen, V. L et al., 2017). Therefore, the development and research on waterlogging tolerance of crops in general and soybean are strongly encouraged.

Soybean is the most important leguminous crop and has a high nutritive value and the cultivation does not require so much labor and soil fertility does not deteriorate, for these reasons it has been cultivated in many countries all over the world (Man, L. W. et al., 2020).

QTL analysis for waterlogging tolerance is usually challenging. However, several types of research have been conducted to detect QTLs for waterlogging tolerance in soybean. QTLs for flooding tolerance of soybean plant at germination stage which were named *Sft1, Sft2, Sft3* and *Sft4* (Sayama et al., 2009). QTLs for waterlogging injury score and tolerance index at vegetative stages were found and mapped on Chrs. 2, 6, 7, 10, 11, 14, and 19

(Githiri et al., 2006), Chrs. 3, 5, 12, 13, and 16 (Cornelious et al., 2005), Chrs. 11 and 13 (Nguyen et al., 2012), and Chr. 12 (Nguyen et al., 2017). QTL associated with better soybean growth and grain yield under waterlogging was identified on Chr. 18 near marker Sat_064 (VanToai et al., 2001). Although many QTL studies of waterlogging tolerance in soybean have been reported, their effects on the overall seed yield of soybean have not yet been validated. Nguyen (2017) reported that QTLs for RLD and RSAD were identified under hypoxia conditions. *Qrld-12* and *Qrsad-12* were found in Satt502 and Satt302 region on Chr.12. A set of NILs was selected at BC_6F_3 generation. Homozygous NIL-9-4-5 showed significantly greater root development than recurrent parent Tachinagaha and equal with donor parent Iyodaizu, indicated that the marker interval may contain the gene for root development QTLs (Nguyen et al., 2017).

For accessing the effects of QTLs for waterlogging tolerance of soybean, the effects of a QTL for yield performance was clarified by subjected soybean plant at 7-day waterlogging at the seedling stage. The seed of each cultivar was sown in a pot containing 3 kg of dried paddy soil (56.1% sand, 23.2% silt, and 20.7% clay). Treatment pots were subjected to watering by maintaining the water level of 2 cm above the soil surface for 7-day waterlogging at the seedling stage. The result showed a strong and highly significant correlation between seed yield and each of the root traits. Seed yields and root development of Iyodaizu and NIL-9-4-5 were not reduced under waterlogging condition, whereas those of Tachinagaha were strongly reduced. These results indicate that tested QTLs are able to maintain or increase yield by stimulating root development under waterlogging.

As mention above, it is strongly suggested that root development is the most important indicator of hypoxia tolerance in soybean. In addition, relationship between root development and aerenchyma formation in soybean seedlings grown under hypoxia condition was investigated. The results showed that under waterlogging conditions, the formation of aerenchyma was found in both waterlogging tolerant Iyodaizu and NIL-9-4-5, while insignificant aerenchyma was found in susceptible cultivar, Tachinagaha. The percentage of aerenchyma formation of NIL-9-4-5 was equivalent to Iyodaizu and significantly higher compared to Tachinagaha. These findings indicated that in response to waterlogging, the QTL confer rapid root development through the concomitant formation of aerenchyma. However, the benefit of aerenchyma is clear, the mechanisms that underlie the formation of this tissue are not yet fully understood (Yamauchi et al., 2013).

Finally, the seed waterlogging was performed using near-isogenic lines of soybean to clarify the variation in germination responses of soybean genotypes to waterlogging at different temperatures. Seeds of each genotype were incubated in Petri dishes at 23 °C and 80% RH in the dark and the number of germinating seeds were counted for three days. Then, two-day-seeds were treated under the waterlogging conditions at different temperatures: 21, 23, 25, 27, and 29 °C. The results showed that the normal seedling rate of NIL-9-4-5 was tendency the same as of the donor parent (Iyodaizu) and was significantly greater than that of the recurrent parent (Tachinagaha), which means that the marker interval may contain a gene for seed waterlogging tolerance in soybean. These results agreed with those of Nguyen et al., 2017, who showed that QTLs for hypoxia tolerance in soybean at the germination and seedling stages are located in the marker interval on Chr. 12.

In conclusion, QTLs for waterlogging tolerance of NIL-9-4-5 and parents could permit an increase of yield and yield components by stimulating root development under waterlogging condition. For responding to waterlogging, the QTLs confer rapid root development through the concomitant formation of aerenchyma in branching root of NIL-9-4-5. The candidate QTLs region (located on Chr. 12) for root development under hypoxia condition has affected on yield, aerenchyma formation and the seed waterlogging tolerance of soybean plants at germination stage. NIL-9-4-5 has been shown to be more suitable for efficient identification of specific genes, and those QTLs on Ch.r.12 are stable across years. Fine mapping and positional cloning may reveal gene(s) responsible for root development under waterlogging conditions and help elucidate the mechanism of waterlogging tolerance in soybean. Effects of the QTLs should be verified under field conditions.

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