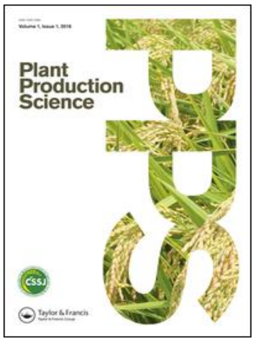

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Nodulation control of crack fertilization technique reduced the growth inhibition of soybean caused by short-term waterlogging at early vegetative stage

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ABSTRACT

Waterlogging is the constraint for soybean growth and yield, because soybean is often cultivated in upland fields converted from paddy in Japan. However, efficient cultivation techniques for alleviating the adverse effects have not been developed. We have proposed the new soybean cultivation technique named crack fertilization which enables yield increase due to enhancing new root growth and N acquisition by increasing nodulation. Waterlogging induces N deficiency due to the suppression of nutrient uptake by the inhibition of root growth and nodule activity. Thus, it is hypothesized that crack fertilization would be effective to alleviate the inhibition of soybean growth and yield. The soybean cultivar of Sachiyutaka was planted in 1/5000 a Wagner pots and root boxes. Two separate waterlogging treatments were imposed to soybean plants at different growth stages, V1 and R4, and crack fertilization was done at V3. After these treatments, soybean plants were sampled at R5 in 2012 and 2013 experiments, respectively. Waterlogging at V1 and R4 inhibited the growth and yield of soybean and nodule growth, and the decreases in physiological parameters of soybean such as photosynthesis, chlorophyll content, and xylem sap exudation rate were observed. The adverse effects of waterlogging at V1 were alleviated by crack fertilization at V3, whereas crack fertilization could not alleviate the adverse effects of waterlogging at R4. Thus, crack fertilization after waterlogging at early vegetative stage would be the cultivation technique that enables to alleviate the adverse effects.

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KEYWORDS
Crack fertilization; *Glycine max* Merr; inoculation; root nodule bacteria; waterlogging

Soybean is widely grown legume in the world and one of the sensitive crops to waterlogging. In Japan, soybean is often cultivated in upland fields converted from paddy (Shimada, 2006), and there are substantial amounts of rain fall in rainy season (from June to mid-July) and the ends of summer (mid-September). Thus, soybean yield in Japan is lower than the global average due to waterlogging.

It is well investigated that even short-term waterlogging severely inhibits root growth of many crops. In addition, the root growth inhibition of the waterlogging-sensitive crops such as wheat (Malik et al., 2002) and grain legumes (Solaiman et al., 2007) does not recover to the control level, even though plants are grown in well-drained condition after waterlogging. The root growth inhibition would induce the reduction of shoot growth due to the decrease in nutrient uptake, especially, nitrogen uptake (Malik et al., 2002; Martínez-Alcántara et al., 2012; Solaiman et al., 2007). Malik et al. (2015) reported that although root growth of grain legumes was inhibited by short-term waterlogging, the tolerant legumes could recover the growth to the control level during recovery periods. Solaiman et al. (2007) also reported similar results in faba bean. These results suggest that the recovery of root growth after waterlogging would be the important trait for the tolerance. Another candidate strategy for the suppression of growth inhibition in soybean caused by waterlogging would be the application of nitrogen fertilizer during recovery periods (Sugimoto & Sato, 1993). However, the application of nitrogen fertilizer is not effective and economic to increase in soybean yield (Barker & Sawyer, 2005; Salvagiotti et al., 2008). In addition, Bacanamwo and Purcell (1999) reported that the decreased N_2 fixation activity under waterlogging was considered to be the main cause of the reduction in soybean growth. Matsunami et al. (2007) reported that the dry matter production of Sakukei 4, which is the supernodulating cultivar derived from a high-yielding Enrei cultivar, significantly decreased by waterlogging, but the yield of Sakukei 4 recovered to similar level to that of Enrei because of its enhanced nodule growth after waterlogging. These results suggest that the maintenance of N_2 fixation activity after waterlogging would be effective to alleviate the reduction in soybean growth and yield. However, there is no attempt to maintain both root development and N

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acquisition after waterlogging using commercial soybean cultivars.

We have proposed the new agronomic concept of nodulation control through 'crack fertilization' (Iijima et al., 2011, 2015). The aim of the technique is that the application of nodule bacteria on biochar that are provided through soil cracks during midterm sub-soiling in the soybean stand *in situ* would enable the continuous nitrogen supply to maturing seeds even after flowering stage (see details in Iijima et al., 2011, 2015). We succeeded to enhance the formation of new roots from the cut surfaces (Iijima et al., 2011) and succeeded to increase soybean yield significantly; up to 1.34 times higher than that of the control by the application of nodule bacteria on biochar. In addition, nitrogen fixation activities in both the field-grown (Iijima et al., 2011) and root box-grown plants (Iijima et al., 2015) were also enhanced. These results lead to the hypothesis that crack fertilization technique would be effective to alleviate the reduction in soybean growth and yield caused by waterlogging by enhancing formation of new roots and by supplying nitrogen due to the maintenance of nodule activity. The aim of the present study is to investigate the alleviative effect of crack fertilization on the reduction of soybean growth and yield caused by waterlogging.

1. Materials and methods

1.1. Plant materials and the treatment with waterlogging and crack fertilization

The pot experiment was conducted at Kinki University in Nara prefecture (latitude 34°40′N, longitude 135°43′E) in two cropping seasons of 2012 and 2013 to evaluate the adverse effects of waterlogging and the alleviative effects of crack fertilization on soybean growth and physiological parameters. In addition, the root box experiment was also conducted in the summer of 2012 to evaluate *in situ* root response to crack fertilization under waterlogging. The soybean cultivar of Sachiyutaka, one of the commercial and recommended cultivar in Nara prefecture, was used in this experiment. The soil used in this study was sandy clay loam with a pH (H_2O) of 5.65, EC of 0.80 ms m^{-1}, total N of 0.03 g kg^{-1}, and total C of 0.46 g kg^{-1}. The soil did not have the experience of soybean cultivation. After the soil was passed through a 2 mm sieve and air dried, powdered synthetic fertilizers ($N:P_2O_5:K_2O$ = 0.2:0.2:0.2 g kg^{-1} soil) were mixed in the soil as basal fertilizers. In addition, magnesium lime of 2.6 g kg^{-1} soil was added to the soil for the adjustment of pH. The value of pH after the adjustment was 6.80. The soil was packed into a 1/5000 a Wagner pot (159 mm diameter, 190 mm tall) with a drain hole, and the soil was filled into a root box (500 × 300 × 20 mm in length, width, and thickness, respectively) at a bulk density of 1.30 g cm^{-3}.

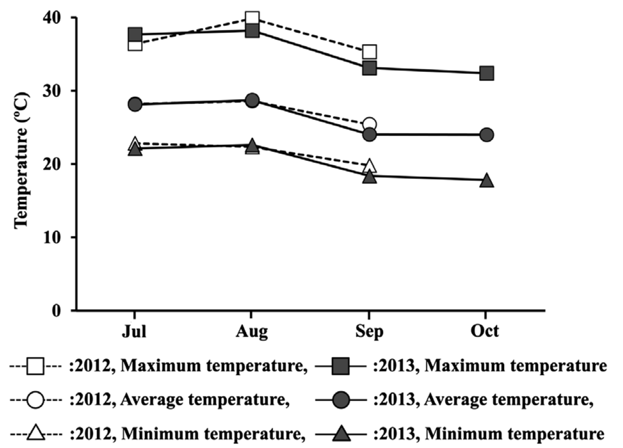

Figure 1. Monthly average, maximum, and minimum temperature during the experimental period in the greenhouse. The temperature in 2012 and 2013 was measured by the sensor for the measurement of temperature in HOBO weather station (Onset Co. Ltd.) and Ondotori (T & D Co. Ltd.), respectively.

Soybean plants were grown and treated with waterlogging and crack fertilization in a greenhouse at Kinki University. The temperature of maximum, minimum, and average in the greenhouse of 2012 and 2013 during the experimental period is shown in Figure 1. The temperature in 2012 and 2013 was measured by the sensor for the measurement of temperature in HOBO weather station (Onset Co. Ltd.) and Ondotori (T & D Co. Ltd.), respectively. We set up four treatments (Control, Crack fertilization (Ck), Waterlogging (WL) and Ck + WL). Ck was defined as crack fertilization. Crack fertilization was conducted at 21 d after sowing (DAS) (V3; soybean stages are according to Fehr et al. (1971)). A crack was made using a shovel (7 cm width and 15 cm depth). The shovel was inserted into soil to make a crack. The crack was made on both sides at 5 cm from a soybean plant. The size of cracks in a pot was 7 cm width and 15 cm depth. Soybean roots in a root box were cut according to Iijima et al., (2011). Charcoals mixed with nodule bacteria were applied to the bottom and along cutting ditch at the rate of 30 kg 10 a^{-1} in each pot and root box, respectively. Experimental protocols for the preparations of charcoals and nodule bacteria were described in detail by Iijima et al. (2015). WL was defined as waterlogging by submerging pots and root boxes up to 5 cm above the soil surface in water pools. In Japan, there are substantial amounts of rainfall at rainy season and there is frequent heavy intensive rainfall at the ends of summer. Thus, two separate waterlogging treatments were imposed to soybean plants at different growth stages, V1 and R4, which were symbolized by WL_{V1} and WL_{R4}, respectively. Ck + WL was defined as the combination with waterlogging and crack fertilization. The soybean plants without treatments were used as a control. Forty-eight pots (4 treatments × 2 different stages of waterlogging × 6 replicates) and 24 root boxes (4 treatments × 1 stages of waterlogging × 6 replicates) in total were used in 2012 experiment. In 2013 experiment, pot experiment (48 pots) was repeated to acquire yield responses to each treatment and additional 10 pots (control and waterlogging × 5 replicates) were prepared for the investigation of the effects of waterlogging at V1 on soybean growth just after waterlogging to check the stress intensities before plant recovery. In total 58 pots were used for 2013 experiment.

In 2012 experiment, pre-germinated seeds were inoculated with commercial soybean nodule bacteria (Konryukin Mame-Zo, National Federation of Agriculture Cooperative Associations, Obihiro, Japan) just before sowing. In the pot experiment, three pre-germinated seeds were directly sown in pots on 17 and 18 July for the waterlogging experiment at V1 and R4, respectively. The germinating seeds were thinned to one plant per pot at 6 DAS. The soybean plants of WL_{V1} and WL_{V1} + Ck were treated with waterlogging for 7 d from 7 DAS. The soybean plants of Ck, WL_{V1} + Ck and WL_{R4} + Ck were treated with crack fertilization at 21 DAS (V3). After the crack fertilization, soybean plants for the experiment of waterlogging at V1 were grown under normal condition until sampling. The soybean plants of WL_{R4} and WL_{R4} + Ck were treated with waterlogging for 14 d from 57 DAS (R4). The sampling was done just after waterlogging. In root box experiments, three pre-germinated seeds were directly sown on 16 July. The germinating seeds were thinned to one plant per pot at 6 DAS. The soybean plants of WL_{V1} and WL_{V1} + Ck at 7 DAS were treated with waterlogging for 7 d. The soybean plants of Ck and WL_{V1} + Ck at 21 DAS were treated with crack fertilization.

In 2013 pot experiment, the pre-germinated seeds which were inoculated with nodule bacteria were directly sown in pots on 8 and 13 July for the experiment of waterlogging at V1 and R4, respectively. The germinating seeds were thinned to one plant per pot at 6 DAS. The soybean plants of WL_{V1} and WL_{V1} + Ck were treated with waterlogging for 7 d from 7 DAS. Five soybean plants from each treatment of control and WL_{V1} were harvested at 14 DAS (V2). Crack fertilization was done at 21 DAS (V3) as with the experiment in 2012. After the crack fertilization, soybean plants for the experiment of waterlogging at V1 were grown under well-drained soil condition until sampling. The soybean plants of WL_{R4} and WL_{R4} + Ck were treated with waterlogging for 7 d from 56 DAS (R4). After waterlogging, these pots were also put in well-drained condition from 63 DAS, and the soybean plants were grown under the condition for 21 d.

Soybean plants were watered everyday. Top dressing ($N:P_2O_5:K_2O$ = 0.1:0.1:0.1 g kg^{-1} soil) was applied at V4 and R2. Insect control was done by spraying MPP (Emulsifiable Concentrate; EC) or Etofenprox against stink bugs and Fenvalerate plus MEP (Wettable Powder) against common cutworms. Pest management was done by spraying thiophanate methyl plus MEP against purpura or crown rot.

1.2. Growth and yield measurements

In 2012 pot experiment, the destructive samplings for the experiment of waterlogging at V1 and R4 were done at 70 DAS (25 September) and 71 DAS (27 September) (R5), respectively. Before the sampling, one leaf of fully expanded uppermost trifoliate leaves was sampled from each pot and frozen with liquid nitrogen for the measurement of chlorophyll content and the activity of antioxidant enzymes. After the leaf sampling, a soybean shoot was cut at 5 cm above the soil surface, and xylem sap was collected for 2 h (from 0900 to 1100) to measure root activity. Thereafter, the rest of the shoot was cut at the surface of the soil, and the shoot dry weight was measured after oven drying at 80 °C for 3 d. In 2012 root box experiment, the root system was sampled at 70 DAS (24 September) according to Iijima and Kono (1991). After the sampling, the pictures of the root system were taken and the photographs were digitized by Adobe photoshop, CS5.0 to check the treatment effects on root system development.

In 2013 pot experiment, five soybean plants of control and WL_{V1} were harvested at 14 DAS (22 July, V2). SPAD value and photosynthetic rate were measured just before the destructive sampling. The rest of soybean plants for reproductive stage sampling started to flower at 33 DAS (10 August), whereas soybean plants of WL_{V1} and WL_{V1} + Ck started to flower at 35 DAS (12 August). Three soybean plants of WL_{V1} and WL_{V1} + Ck started to wilt after flowering time (12 August) for an unknown reason. Thus, six plants from control and Ck and three plants from WL_{V1} and WL_{V1} + Ck were harvested. The soybean plants for the experiment of waterlogging at V1 and R4 were harvested on 30 September and 6 October before ripening (84 DAS), respectively, because the leaves in soybean plants of WL_{V1}, WL_{R4}, and WL_{R4} + Ck turned yellowish. The soybean growth stage would be between R5 and R6 according to the observation of pod development. Thus, in 2013 pot experiment, soybean growth stage at sampling was described as R5. Xylem sap was collected for two hours (from 0900 to 1100) for the analysis of ureide translocation rates by the Young–Conway method (Young and Conway, 1942). After the collection of xylem sap, soybean shoots were cut at the soil surface, and then the root system in pots was washed with tap water. Root nodules were carefully removed from soybean root, and then number and fresh weight of root nodules were measured. Pods were removed from soybean shoots, and the number of pods and seeds were counted. The dry weight of seeds was measured after oven drying at 80 °C for 3 d to estimate the accurate dry matter production of seed at R5. The dry weight of shoots and roots was also measured after oven drying at 80 °C for 3 d.

1.3. Physiological measurements

In 2012 pot experiment, chlorophyll content in soybean plants was measured to evaluate the leaf damage. Since the leaf damage caused by waterlogging is related to the excess accumulation of hydrogen peroxide (H_2O_2) (Blokhina et al., 2001), the activities of ascorbate peroxidase (APX) and catalase (CAT), which are the major scavenging enzymes of H_2O_2 in a leaf, were measured. The leaf (0.1 g) was homogenized with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 7 mM mercaptoethanol, 0.1% (w/v) Triton X-100, 5 mM sodium ascorbate. The part of the homogenate (40 μL) was mixed with 960 μL of ethanol to measure chlorophyll content. The residue of the homogenate was centrifuged at 12,000*g* for 10 min and the supernatant was used for enzyme assays. The chlorophyll content was determined according to Wintermans & De Mots (1965). The activities of APX and CAT were measured according to Yamane et al. (2004).

In 2013 pot experiment, SPAD value and photosynthetic rate were measured as physiological parameters by the SPAD meter (SPAD-502, Konica Minolta) and the portable photosynthetic analyzer (LCpro-SD, ADC BioScience Ltd.), respectively. SPAD value was measured at three points in uppermost trifoliate leaves. Photosynthetic rate was measured using one leaf of uppermost trifoliate leaves. SPAD value and photosynthetic rate of soybean plants treated with waterlogging at V1 were measured after 4, 7, 9, and 10 w of the recovery. In the experiment of waterlogging at R4, SPAD value was measured at 0, 9, and 17 d after the recovery. Photosynthetic rate was measured at 3, 7, and 14 d after the recovery.

1.4. Statistical analysis

F values, probability levels, and standard error of means were indicated in all the parameters. Statistical significance for the experiment of the waterlogging at V1 was tested using Student's *t*-test. Since three plants of WL_{V1} and WL_{V1} + Ck treated with waterlogging at V1 died before sampling in 2013 pot experiment, data in all treatments were calculated using three replicates. In the soybean plants of control and Ck, top three data of six replicates were chosen based on the shoot growth for the calculation of *F* values, probability levels, and standard error of means. One-way analysis of variance (ANOVA) was first applied for statistical evaluation using Excel 2012 for Windows (SSRI Japan, Co. Ltd.). If an ANOVA was significant, *post hoc* analyses were conducted using Dunnett's multiple comparison test, with the level of statistical significance taken as $p < 0.05$ and 0.01.

2. Results

2.1. Effects of waterlogging and crack fertilization in 2012 experiment

Upper side of Table 1 shows the result of shoot dry weight and physiological parameters at R5 in the experiment of waterlogging at V1. Crack fertilization did not affect the shoot dry weight. In addition, the values of physiological parameters were similar to the control. Waterlogging did not induce the decrease in the shoot dry weight, but physiological parameters were severely affected. Chlorophyll content and CAT activity were statistically lower than the control. Xylem sap exudation rate and APX activity decreased, whereas the decreases were not statistically different from the control. On the other hand, the decreases in physiological parameters caused by waterlogging were mitigated by crack fertilization. Xylem sap exudation rate and CAT activity of WL_{V1} + Ck were similar to the control. Although chlorophyll content of WL_{V1} + Ck was statistically lower than the control, the magnitude of the decrease was less pronounced in WL_{V1} + Ck than WL_{V1}.

Lower side of Table 1 shows the result of the shoot dry weight and physiological parameters at R5 (just after waterlogging) in the experiment of waterlogging at R4. The shoot dry weight was not affected by crack fertilization as observed in the experiment of waterlogging at V1. Crack fertilization affected physiological parameters. However, the trend was slightly different from the experiment of waterlogging at V1. Especially, xylem sap exudation rate tended to be lower than the control. Waterlogging at R4 induced the statistically significant decreases in the shoot dry weight and xylem sap exudation rate. Chlorophyll content of WL_{R4} was lower than the control, though the decrease was not statistically different from the control. The activities of APX and CAT of WL_{R4} were not affected by waterlogging. The decrease in the shoot dry weight of WL_{R4} + Ck was suppressed by crack fertilization. However, crack fertilization could not alleviate the adverse effects of waterlogging at R4 on physiological parameters, especially on xylem sap exudation rate and chlorophyll content. These physiological parameters showed the decreased trend than the control.

We investigated root system profiles at R5 using root boxes. Figure 2 shows the digitized root system profiles of soybean. The root system of control well developed (left side and upper), and a lot of roots accumulated in

Table 1. Effects of waterlogging and crack fertilization on shoot growth and physiological parameters of soybean at 70 and 71 DAS (R5) in 2012 pot experiment of waterlogging at V1 (upper) and R4 (lower), respectively.

	Treatment	Shoot dry weight (g)		Xylem sap exudation rate ($g\ h^{-1}$)		Chlorophyll content ($mg\ g^{-1}$ FW)		APX activity (Unit mg^{-1} protein)		CAT activity (Unit mg^{-1} protein)	
V1 stage waterlogging	Control	17.7	(0.69)	0.60	(0.09)	1.35	(0.16)	1.04	(0.03)	0.16	(0.01)
	Ck	17.9	(0.42)	0.70	(0.07)	1.41	(0.14)	1.02	(0.08)	0.18	(0.02)
	WL_{V1}	17.9	(0.22)	0.43	(0.09)	0.41^{**}	(0.15)	0.93	(0.05)	0.10^{**}	(0.01)
	WL_{V1} + Ck	18.3	(0.65)	0.65	(0.09)	0.72^{*}	(0.12)	0.98	(0.16)	0.16	(0.01)
One-way ANOVA	*F* value	0.22		1.90		11.7		0.25		8.86	
	Probability	0.88		0.16		$1.22 \times 10^{-4++}$		0.86		$6.16 \times 10^{-4++}$	
R4 stage waterlogging	Control	18.6	(0.42)	0.38	(0.10)	0.93	(0.13)	2.78	(0.38)	0.10	(0.009)
	Ck	18.0	(0.64)	0.23	(0.06)	1.34	(0.17)	3.48	(0.61)	0.09	(0.012)
	WL_{R4}	15.9^{**}	(0.47)	0.12^{*}	(0.04)	0.76	(0.17)	2.55	(0.23)	0.10	(0.006)
	WL_{R4} + Ck	16.7	(0.71)	0.12^{*}	(0.02)	0.62	(0.15)	2.49	(0.38)	0.08	(0.007)
One-way ANOVA	*F* value	4.57		3.94		4.00		1.55		0.36	
	Probability	0.01^{+}		0.02^{+}		0.02^{+}		0.24		0.78	

Soybean plants were treated with waterlogging at 7 DAS (V1, upper) for 7 d or 56 DAS (R4, lower) for 14 d. Crack fertilization was done at 21 DAS (V3). The data are means of six replicated plants. The values in small parenthesis indicate standard error of mean. When ANOVA was significant, *post hoc* analyses were conducted using Dunnett's multiple comparison test, with the level of statistical difference taken as $P < 0.05$ and $P < 0.01$. The symbols of $^{+}$ and $^{++}$ indicate significant differences at $P < 0.05$ and $P < 0.01$ by ANOVA, respectively. The symbols of * and ** indicate significant differences from control at $P < 0.05$ and $P < 0.01$ by Dunnett's multiple comparison test, respectively.

the bottom of a root box. The treatment with crack fertilization reduced root system (right side and upper). The roots of Ck in the bottom of a root box were slightly less than the control. The treatment with waterlogging at V1 suppressed the development of root system (left side and lower). However, crack fertilization after waterlogging was effective to recover the development of root system (right side and lower).

2.2. *Effects of waterlogging and crack fertilization in 2013 experiment*

In 2013, second season of the experiment, we investigated the impacts of waterlogging at V1 on soybean growth and physiological parameters just after the treatment (Figure 3). Shoot dry weight, SPAD value, and photosynthetic rate of WL_{V1} were similar level to those of the control. However, root growth of WL_{V1} was statistically lower than the control, and the dry weight at V2 was about 50% of the control.

The chlorophyll content of WL_{V1} in 2012 experiment decreased earlier than those of the control and the adverse effects were alleviated by crack fertilization. In 2013 experiment, therefore, we investigated the changes in SPAD value and photosynthetic rate by time course analysis. Left side of Figure 4 shows SPAD values (upper) and photosynthetic rate (lower) in the experiment of waterlogging at V1. SPAD values were similar in all treatments until 7 w after the recovery of waterlogging. After 7 w of the recovery, SPAD value of Ck was maintained at the highest level. SPAD value of WL_{V1} was similar level to the control until 9 w after the recovery. However, the sharp decrease in SPAD value was observed at 10 w after the recovery, whereas the decrease was not statistically different from the control. On the other hand, SPAD value of WL_{V1} + Ck was maintained at similar level to the control during the experimental period. Thus, the decrease in SPAD value tended to be alleviated by crack fertilization as observed in 2012 experiment. Photosynthetic rates of all treatments were similar until 7 w after the recovery. Photosynthetic rate of Ck at 9 w after the recovery was statistically higher than that of the control, but the rate at 10 w after the recovery was similar to the control. The decrease in photosynthetic rate of WL_{V1} was observed at 10 w after the recovery, whereas the decrease was not statistically different from the control as observed in SPAD value. On the other hand, photosynthetic rate of WL_{V1} + Ck was maintained at high level until 10 w after the recovery. Thus, the decreases in photosynthetic rate tended to be alleviated by crack fertilization as well as SPAD value.

Right side of Figure 4 shows SPAD values (upper) and photosynthetic rate (lower) in the experiment of waterlogging at R4. SPAD values just after the recovery were similar to the treatments except for Ck + WL_{R4}. The SPAD

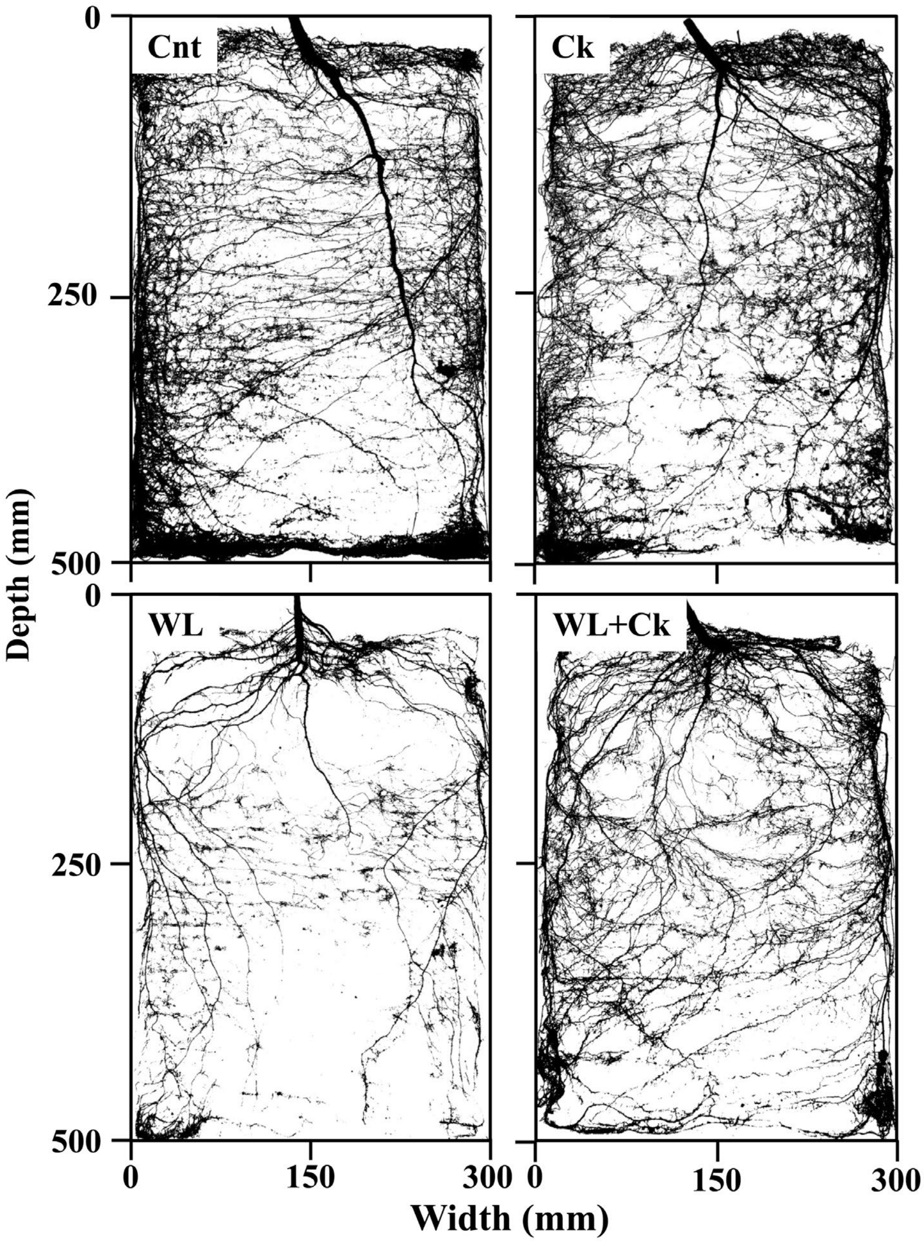

Figure 2. Digitized images of soybean plants in 2012 root box experiment. The root system of control plants (left side, upper) were well watered during the experiment. The root system of Ck (right side, upper) was treated with crack fertilization at 21 DAS. The root system of WL (left side, lower) was treated with waterlogging at 7 DAS. The root system of WL + Ck (right side, lower) was treated with waterlogging at 7 DAS, and then treated with crack fertilization at 21 DAS.

value of Ck + WL_{R4} was statistically lower than the control. SPAD values of Ck after 9 d of the recovery were maintained at high level compared with other treatments. The slight decreases in SPAD values of WL_{R4} and WL_{R4} + Ck were observed from 9 d after the recovery. At 17 d after the recovery, the sharp decrease in SPAD value was observed in WL_{R4}, and the value was 30% of the control. The decrease in SPAD value of WL_{R4} + Ck was also observed, but the rate was less than that of WL_{R4} and the statistical difference was not observed. The decreases in SPAD value of WL_{R4} and WL_{R4} + Ck were not statistically different from the control. Photosynthetic rates of Ck tended to be maintained at the highest level during the measurement period. Photosynthetic rate of WL_{R4} after the recovery showed the decreased trend than the control. On the other hand, the decrease in photosynthetic rate of WL_{R4} + Ck at 3 d after the recovery was suppressed, and the value was 83% of the control. However, the photosynthetic rates of WL_{R4} + Ck after 7 d of the recovery were similar to those of WL_{R4}.

Upper side of Table 2 shows soybean growth, yield, and nodule growth at R5 in the experiment of waterlogging at V1. Shoot dry weight, yield components, and

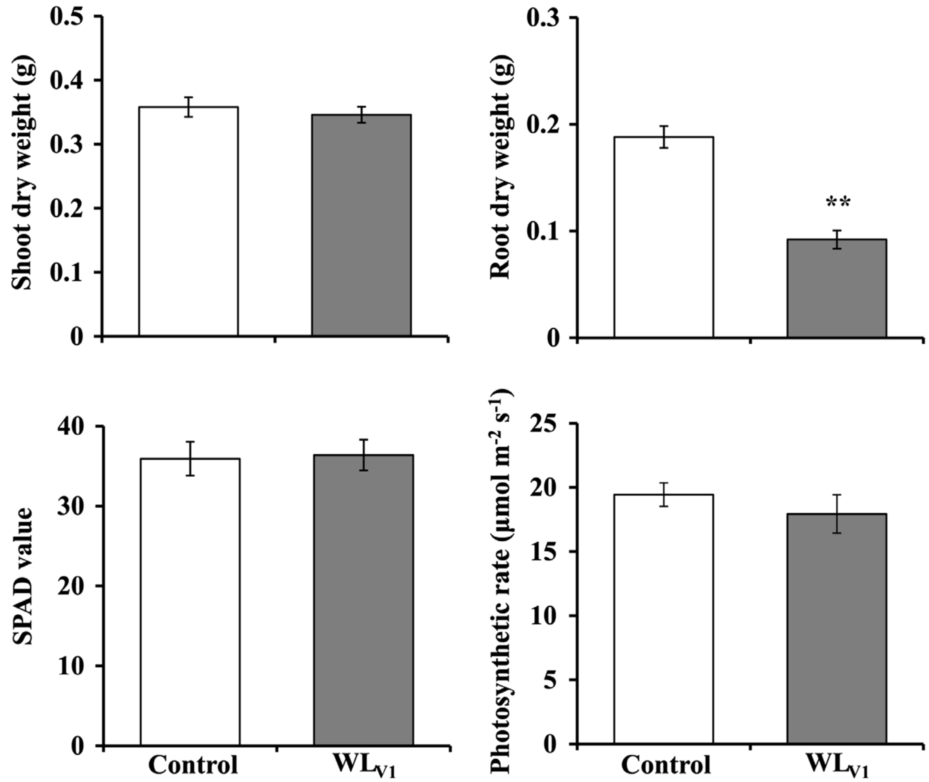

Figure 3. Growth and physiological traits of soybean plants at 14 DAS in 2013 pot experiment. Soybean plants were affected by short-term waterlogging from 7 DAS (V1) for 7 d. The soybean plants were harvested just after the waterlogging (V2, 14 DAS) to check the stress intensity before recovery period. The data are means ± SE ($n = 5$ replicated plants). The symbol of ** indicates significant differences from control at $P < 0.01$ by t-test.

nodule number of Ck showed the increased trend than the control. Especially, crack fertilization could greatly enhance nodule number, whereas the increase was not statistically different from the control. Nodule fresh weight of Ck was similar to that of the control, indicating that small nodules increased by crack fertilization. Waterlogging suppressed the shoot and root growth. Shoot dry weight of WL_{V1} tended to be lower than the control, and the value was 78% of the control. Root dry weight of WL_{V1} was statistically lower than the control and the value was about 50% of the control. Thus, soybean growth did not recover sufficiently even after long recovery period. The numbers of pod and seed of WL_{V1} were similar to the control, whereas seed weight tended to be decreased. Total nodule number of WL_{V1} was similar to that of the control. However, nodule fresh weight of WL_{V1} was statistically lower than the control. In addition, ureide translocation rate of WL_{V1} showed the decreased trend than the control. On the other hand, the inhibitory effects were suppressed by crack fertilization. Shoot dry weight of WL_{V1} + Ck was similar to that of the control. Although root dry weight of WL_{V1} + Ck did not recover to the control level, the magnitude of the decrease was less than that of the WL_{V1}. Seed weight of WL_{V1} + Ck was similar level to the control, and pod and seed number showed the increased trend than the control as observed in Ck. Nodule fresh weight of WL_{V1} + Ck did not fully recover to the level of the control. However, the magnitude of the decrease was lower than WL_{V1}. Ureide translocation rate of Ck was maintained at similar level to the control.

Lower side of Table 2 shows plant growth, yield, and nodule growth at R5 in the experiment of waterlogging at R4. In this experiment, shoot and root dry weight and nodule fresh weight of Ck showed the trend to be enhanced by crack fertilization, though yield components and nodule number were similar to those of the control. Waterlogging at R4 induced statistically significant decreases in all components measured except for pod number. Most nodules of WL_{R4} became black, which is a typical feature of nodule death (data not shown). In addition, ureide translocation rates of WL_{R4} were not detected. Crack fertilization showed the trend to suppress the decrease in shoot and root growth and nodule number. Although nodule number of WL_{R4} + Ck tended to be higher than that of WL_{R4}, most nodules on soybean roots became black and ureide translocation rate were not detected as observed in WL_{R4}. The ameliorative effects on seed number and weight were not observed.

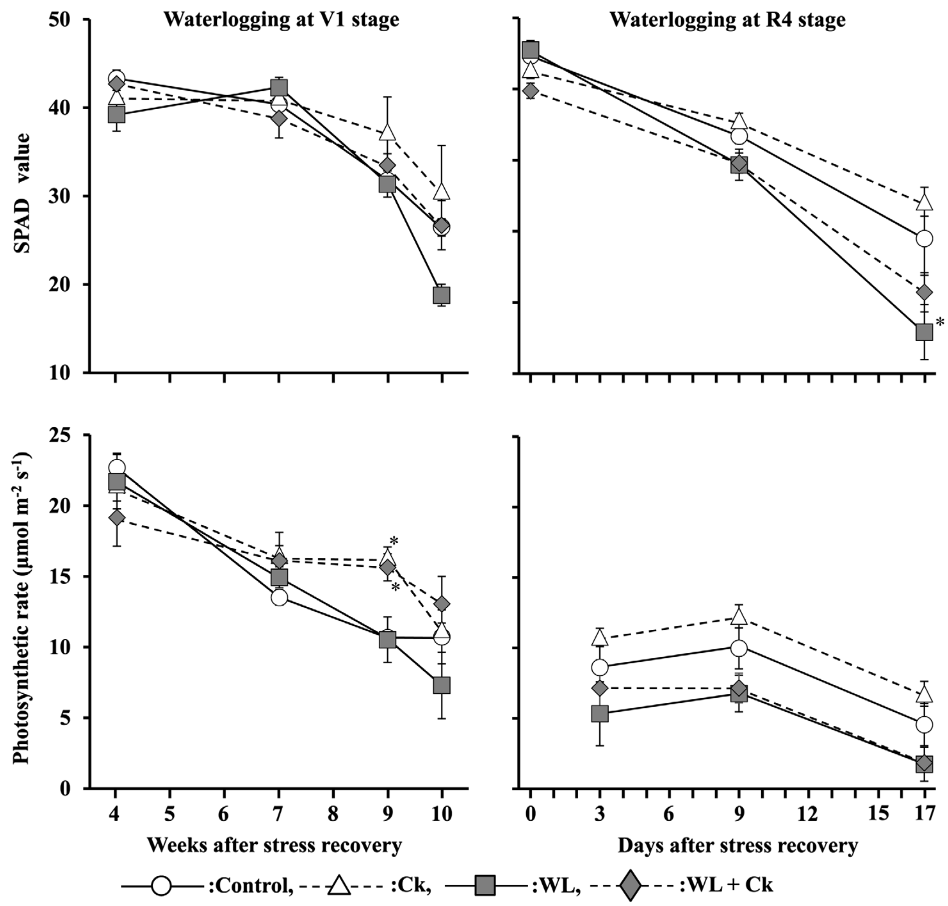

Figure 4. Effects of waterlogging and crack fertilization on SPAD value and photosynthesis in 2013 experiment. Graphs are time course analysis of SPAD value and photosynthesis in the experiment of the treatment with waterlogging at V1 (left side) and at R4 (right side). The data are means ± SE (n = 3 replicated plants for the experiment of waterlogging treatment at V1, n = 6 replicated plants for the experiment of waterlogging treatment at R4). When ANOVA was significant, *post hoc* analyses were conducted using Dunnett's multiple comparison test, with the level of statistical difference taken as $P < 0.05$ and $P < 0.01$. The symbol of * indicates significant differences from control at $P < 0.05$ by Dunnett's multiple comparison test, respectively.

3. Discussion

Waterlogging at early vegetative stage of V1 significantly inhibited the growth of root and nodule (Table 2). Since root growth was significantly suppressed just after waterlogging at V1 (Figure 3 right upper), the inhibitory effects would continue during the recovery period. The continuous inhibitory effects of waterlogging on root growth during the recovery period were observed in wheat (Malik et al., 2002) and grain legumes (Solaiman et al., 2007). In the present study, the root growth inhibition could be due to the impairment of translocation of photosynthetic assimilation products. Photosynthetic rates in waterlogging were maintained to similar level of control at both just after the 7 d treatment (Figure 3 right lower) and 9 w after the recovery (Figure 4 left lower). Shoot dry weight in waterlogging was not significantly different from the control in both 2012 and 2013 experiments. Martínez-Alcántara et al. (2012) reported that the concentration of ^{13}C, which was applied from leaves as $^{13}CO_2$, and carbohydrates such as hexoses, sucrose, and starch in leaves increased compared with the control under waterlogging, whereas roots showed the opposite pattern, suggesting that carbon translocation from leaves to roots was impaired by waterlogging. Thus, the growth inhibition of root and nodule caused by waterlogging could be induced by the impairment of translocation of photosynthetic assimilation products from leaves to roots.

The inhibition of root and nodule growth would induce the early loss of leaf greenness. Chlorophyll content in soybean caused by waterlogging at V1 decreased earlier than the control (Table 1). In addition, time course analysis revealed that the loss of leaf greenness started after more than two months of the recovery (Figure 4 left upper). The early loss of leaf greenness after long-term recovery of waterlogging was also observed in wheat (Araki et al., 2012) and *Prunus* (Amador et al., 2012). It is well documented that waterlogging causes the decrease in leaf nitrogen due to the root growth inhibition and the

Table 2. Effects of waterlogging and crack fertilization on soybean growth at 84 DAS (R5) in 2013 pot experiment.

	Treatment	Shoot dry weight (g)	Root dry weight (g)	Pod number	Seed number	Seed weight (g)	Nodule number	Nodule fresh weight (g)	Ureid translocation rate (mM h^{-1})
V1 stage waterlogging	Control	22.8 (0.05)	18.5 (0.42)	55 (0.85)	102 (2.25)	28.6 (1.37)	134 (15.2)	3.85 (0.32)	0.44 (0.06)
	Ck	26.8 (0.18)	15.2 (2.43)	63 (4.09)	116 (3.06)	31.4 (0.68)	232 (40.1)	3.91 (0.09)	0.56 (0.07)
	WL_{V1}	17.9 (0.58)	9.87* (1.27)	54 (9.21)	102 (19.0)	23.9 (3.89)	138 (45.2)	2.25* (0.37)	0.38 (0.11)
	WL_{V1} + Ck	21.5 (1.46)	11.2 (1.36)	57 (7.51)	112 (12.2)	27.7 (3.02)	121 (14.8)	2.67 (0.13)	0.43 (0.09)
One-way ANOVA	*F* value	5.41	4.02	0.35	0.39	1.34	1.74	7.47	0.78
	Probability	0.03^{+}	0.05	0.79	0.77	0.33	0.24	0.01^{+}	0.54
R4 stage waterlogging	Control	21.0 (1.79)	9.84 (0.90)	43 (0.85)	88 (4.12)	14.8 (0.61)	95 (16.5)	2.54 (0.29)	0.33 (0.14)
	Ck	23.0 (1.06)	10.2 (0.73)	43 (4.09)	87 (3.70)	14.9 (0.60)	89 (14.6)	2.77 (0.19)	0.29 (0.10)
	WL_{R4}	15.7* (1.01)	6.38** (0.52)	38 (9.21)	72** (3.21)	11.7** (0.61)	35** (8.99)	0.66** (0.18)	nd
	WL_{R4} + Ck	17.3 (1.16)	7.33* (0.43)	38 (7.51)	65** (1.92)	10.5** (0.43)	41* (8.04)	0.82** (0.15)	nd
One-way ANOVA	*F* value	6.71	7.74	2.50	11.9	15.51	6.21	28.9	–
	Probability	$2.6 \times 10^{-3++}$	$1.0 \times 10^{-3++}$	0.09	$1.1 \times 10^{-4++}$	$1.9 \times 10^{-5++}$	$3.7 \times 10^{-3++}$	$1.8 \times 10^{-7++}$	–

Soybean plants were treated with waterlogging at 7 DAS (V1) for 7 d or 56 DAS (R4) for 7 d. Crack fertilization was done at 21 DAS (V3). The data in the experiment of waterlogging treatment at V1 and R4 are means of three and six replicated plants, respectively. Seeds were sampled between R5 and R6 according to the observation of pod development. The values in small parenthesis indicate standard error of means. When ANOVA was significant, *post hoc* analyses were conducted using Dunnett's multiple comparison test, with the level of statistical difference taken as $P < 0.05$ and $P < 0.01$. The symbols of $^{+}$ and $^{++}$ indicate significant differences at $P < 0.05$ and $P < 0.01$ by ANOVA, respectively. The symbols of * and ** indicate significant differences from control at $P < 0.05$ and $P < 0.01$ by Dunnett's multiple comparison test, respectively.

inhibition of N_2 fixation activity, leading to the decrease in chlorophyll content (Bacanamwo & Purcell, 1999; Malik et al., 2002; Martínez-Alcántara et al., 2012). In the present study, waterlogging caused the decreases in root growth, xylem exudation rate, nodule fresh weight, and ureide translocation rate (Table 1, 2 and Figure 2 left lower). These results suggest that the inhibition of root growth and nodule activity would be related to the early loss of leaf greenness. Another factor induced the early loss of leaf greenness would be the decrease in CAT activity in leaves. CAT and APX are the key enzymes to detoxify H_2O_2 in leaves (see review in Foyer & Noctor, 2009). However, CAT rather than APX will be responsible for the loss of chlorophyll content. Vanacker et al., (2006) reported that the decrease in chlorophyll content in pea during senescence is concomitant with the decrease in catalase activity, while APX activity remains high. Similar result is obtained from rice under salinity (Yamane et al., 2004). These results suggested that the decrease in CAT activity was related to the early loss of leaf greenness.

To alleviate the adverse effects of waterlogging on soybean growth and yield, the maintenance of root growth (Henshaw et al., 2007; Sauter, 2013; Sakazono et al., 2014), and continuous N acquisition (Sugimoto & Sato, 1993; Bacanamwo & Purcell, 1999) would be essential. In the present study, crack fertilization could alleviate the inhibitory effects of waterlogging at V1 on soybean growth, yield, and nodule growth. The alleviation effects will be due to the maintenance of the root growth and the enhancement of N acquisition after waterlogging. In our previous root box experiment, crack fertilization successfully increased root system development by enhancing the emergency of young root from the cut surfaces (Iijima et al., 2011). In the present study, crack fertilization after waterlogging apparently increased root system development of WL_{V1} + Ck than that of WL_{V1} in both root box and pot experiments (Table 2 and Figure 2 right lower). In addition, xylem sap exudation rate of WL_{V1} + Ck was higher than WL_{V1} (Table 1). Thus, crack fertilization after waterlogging can alleviate the inhibition of the root growth and activity by enhancing the emergence of new roots from the cut surfaces. Crack fertilization would also enable soybean to acquire nitrogen continuously by the alleviation of nodule growth inhibition after waterlogging. Single-year treatment with crack fertilization in field can enhance ureide translocation rate of soybean (Iijima et al., 2011). In the present study, the decrease in nodule fresh weight of Ck + WL_{V1} was suppressed by crack fertilization, and ureide translocation rate of Ck + WL_{V1} was maintained at similar level to that of the control (Table 2). In addition, the decrease in chlorophyll content after long-term recovery was suppressed (Table 1 and Figure 4 left upper). These results suggested that N availability in the soybean treated with crack fertilization after waterlogging was improved due to the alleviation of nodule growth inhibition. Thus, crack fertilization after waterlogging would enable to alleviate the adverse effects on soybean growth and physiological parameters by maintaining root growth and improving N condition.

However, it has been still unclear that the improvement of N condition in soybean plants after waterlogging was achieved by the addition of nodulation (Crack fertilization). In the present study, we used the soil of sandy clay roam without the experience of soybean cultivation. Shibata et al. (1980) reported that the frequency of the symbiosis between nodules and soybean plants was low in the soil without the experience of soybean cultivation, even though nodule bacteria were applied to the seeds before sowing. In the present study, the nodule number of Ck in the experiment of waterlogging at V1 increased (Table 2 upper). However, the nodule number of Ck in the experiment of waterlogging at R4 was similar to the control (Table 2 lower). Thus, the nodulation would be unstable in the soil without the experience of soybean cultivation. In addition, the nodule number of Ck + WL_{V1} was similar to the control, whereas the decrease in the nodule fresh weight was alleviated (Table 2 upper). Thus, the improvement of N condition in soybean plants after waterlogging would be achieved by the alleviation of nodule growth but not by increasing nodulation. The instability of nodulation may be induced by the lack of the flow of photosynthetic products to nodules (Gordon et al., 1999). Further studies are needed to elucidate the mechanisms of the improvement of N condition by crack fertilization after waterlogging. In addition, further field studies are needed to elucidate whether crack fertilization technique is useful to alleviate the adverse effects of waterlogging on soybean growth and yield.

Crack fertilization could not alleviate the adverse effects of waterlogging at R4. Photosynthesis and soybean and nodule growth of Ck were slightly enhanced, though the enhancing effects of crack fertilization on soybean growth would be disappeared by waterlogging. The impacts of waterlogging at reproductive stage on soybean growth, yield, nodule growth, and physiological parameters were greater than that at vegetative stage. Especially, sink organs such as root, seed, and nodule were significantly affected by waterlogging (Table 2). Some studies reported that soybean was more sensitive to waterlogging at reproductive stage than that at the vegetative stage (Griffin & Sazton, 1988; Linkemer et al., 1998; Scott et al., 1989). Since heavy intensive rainfall often occurs in summer of Japan (after flowering time), risks of short-term waterlogging at reproductive stage will increase. Thus, waterlogging at reproductive stage must be avoided, and further studies are needed to suppress the adverse effects of waterlogging at reproductive stage on soybean growth and yield.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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*In Japanese with English abstract.
**In Japanese with English title.
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