Close mixed-planting with paddy rice reduced the flooding stress for upland soybean

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ABSTRACT
Close mixed-planting is a new concept of mixed cropping that allows greater complementary effects under various stress conditions; in this system, oxygen and/or water transfer occurs through the tightly entangled root system of the two species. We aimed to assess whether rice can alleviate flood stress in soybeans via close mixed-planting. A three-year field experiment and one water culture experiment were conducted to compare the crop performance between single and close mixed-planting under short-term flood (or anoxia) stresses. The survival rates, photosynthetic rates, and biomass production were higher in the mixed soybean than in the single soybean plants. Moreover, as the modified land equivalent ratio under flood stress was always >1, rice exhibited greater complementary effect on soybean against short-term flood stress in an upland field converted from paddy. Thus, rice planted as the partner crop of soybean can alleviate short-term anaerobic stress to enhance soybean growth.

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Introduction
The occurrence of floods and droughts is presumed to increase in the near future owing to the drastic climate change. Mixed cropping is a traditional cultivation technique in which different crops are cultivated in the same field to avoid the damage of crops due to floods and droughts (Brooker et al., 2015). In our previous study, a new concept of mixed cropping called ‘close mixed-planting’ has been proposed (Iijima et al., 2016). Close mixed-planting makes the cultivation distance between different species infinitely close, that is, two different crops grow like the main stem and tillers of rice plants (Awala et al., 2016). The crop mixtures will consist of only one single hill, and
they grow together until maturity. In general, the competition between the two species become bigger as the distance between the two species decreases, however, when the distance approaches infinitely small, the complementary effects were highly evident than the competition under stressful conditions, such as those induced by floods (Awala et al., 2019), salinity stress (Nanhapo et al., 2017), and drought (Izumi et al., 2018; Yamane et al., 2018).

By closely entangling the root systems of different crops, mutual exchange of substances among the roots of these crops can be increased, as the radial oxygen loss (ROL) barrier to prevent the leakage of substances from roots is underdeveloped near the young developing portion of roots (Colmer et al., 1998; Yamauchi et al., 2013). Moreover, oxygen (Iijima et al., 2017) and water (Izumi et al., 2018) leaked from immature roots could be easily transferred between the crop species via an entangled root system. In fact, it has been shown previously that when rice and drought-tolerant upland crops (pearl millet and sorghum) were combined, the oxygen released from the roots of rice improved the yield of drought-tolerant upland crops under short-term flood environments (Awala et al., 2016). Under drought conditions, rice survived severe drought stress by receiving the water released from the roots of pearl millet (Izumi et al., 2018). In addition, when ice plants and cowpea were combined for mixed cropping, ice plants enhanced the salinity tolerance of cowpea under severe saline stress conditions, presumably by reducing the salinity level of the rhizosphere soil (Nanhapo et al., 2017).

Previous studies on close mixed-planting examined only the combination of rice and drought-tolerant cereal crops (pearl millet or sorghum), except one study on ice plant and cowpea combination (Nanhapo et al., 2017). Cereal and legume combinations, which are common in crop rotation systems, are preferred for close mixed-planting in terms of field crop production. In the present study, the combination of rice and soybean was tested due to their importance in paddy fields in Japan. Soybean is often cultivated in an upland field converted from paddy, and the field can be temporarily submerged due to the short heavy rain showers that would occur frequently in the future due to climate change. Soybean has been traditionally cultivated in the ridges (narrow foot-pass) in small-scale rice fields in Japan, in particular, rice and soybean can be grown in the same field under paddy and upland conditions. Thus, the present study aimed to assess whether rice can alleviate flood stress in soybean by close mixed-planting.

**Materials and methods**

A three-year field experiment and one water culture experiment were conducted in the Faculty of Agriculture, Kindai University, in the central region of Japan (latitude 34°40’ N, longitude 135°44’ E). Rice (*Oryza sativa*, Nipponbare) and soybean (*Glycine max*, Sachiyutaka) were used as the test plants. The pre-germination procedure is the same as previous report (Iijima et al., 2021, in press). The growth duration; flooding period; maximum, minimum, and average temperatures; relative humidity; solar radiation; and precipitation during each experimental period are summarized in Table 1. Pre-germinated rice seeds were sown in cell trays filled with soil. Soybean was relay-planted 7 days after sowing (DAS) rice. For the planting treatment, single cropping (hereafter referred to as ‘single’) in which only rice or soybean are grown per cell, and close mixed-planting in which both rice and soybean are grown in the same cell (hereinafter referred to as ‘mixed’), were set up. After sowing, the seedlings were watered with tap water, and Hoagland’s solution (Hoagland & Arnon, 1950) was supplied from 7 DAS of rice.

**Field study (experiments 1, 2 and 3)**

Seedlings grown in the glasshouse were transplanted into paddy fields at 28 DAS of rice. Just before transplanting, the seedlings were removed from the cell trays.

| Table 1. Plant growth conditions during the experimental period. Temperature, solar radiation, and rainfall during all growth periods in the water culture experiment and from transplanting to sampling in field experiments. |
|---|---|---|---|
| **Water culture** | **Field** |   |   |
| **2017 (Exp. 4)** | **2015 (Exp. 1)** | **2016 (Exp. 2)** | **2017 (Exp. 3)** |
| **Experimental period** | 16 Oct. – 24 Nov. | 24 Jul. – 7 Sep. | 22 Jun. – 23 Aug. | 31 Jul. – 4 Oct. |
| **Flooding period (DASS)†** | 18–25 | 21–33 | 35–40 | 30–36 |
| **Temperature (°C)** | Mean | 27.5 | 24.6 | 23.0 |
| | Max. | 32.6 | 29.2 | 28.0 |
| | Min. | 23.3 | 21.5 | 18.9 |
| **Solar radiation (MJ m⁻² d⁻¹)** | 12.4 | 19.9 | 14.0 |
| **Rainfall (mm)** | 89.5 | 21.6 | 17.0 |

† DASS, Days after sowing of soybean.
In the field experiment conducted in 2015 (hereafter referred to as Experiment 1), rice was grown for 7 DAS in an environmental control growth room (Iijima et al., 2020a, 2020b) using 25-compartment-type large cell trays (49 mm long × 49 mm wide × 56.5 mm high for each cell) filled with culture soil (Metro-Mix 250, Sun GRO® Horticulture, USA). Thereafter, the seedlings were transferred to the glasshouse and grown for 21 days; following this, the seedlings were transplanted to directly flooded paddy fields (Figure 1). The environmental data in the field and glasshouse were obtained from the Japan Meteorological Agency and Weather Station (HOBO ware Pro, Onset Computer Ltd), respectively.

During the 2016 (Experiment 2) and 2017 (Experiment 3) field experiments, the seedlings were grown in the glasshouse from beginning (sowing of rice) using a 72-compartment-type small cell tray (37 mm long × 37 mm wide × 46 mm height for each cell) filled with different culture soil (small-grained Akadama soil, granular type well-drained Japanese culture soil for gardening), and the seedlings were subsequently transplanted into a well-drained experimental upland field converted from paddy. The environment in the plant growth room used in Experiment 1 was maintained at 28/23°C day/night temperature, with a 14-h photoperiod and 318 ± 2 μmol m⁻² s⁻¹ of photosynthetically active radiation (PAR) at the top canopy level. The glasshouse used in experiment 1, 2 and 3 was equipped with an automatic temperature control unit and artificial light. The artificial light was supplemented by metal halide lamps to adjust the 14-h day length, and temperatures were controlled using a gas heating system and an automatic roof and window opening system (Iijima et al., 2020a). The temperatures in the glasshouse were 38.2, 34.1, and 42.4°C (maximum); 25.1, 23.0, and 19.1°C (minimum); and 30.4, 27.9, and 28.7°C (average), in experiments 1, 2, and 3, respectively. Furthermore, N, P₂O₅, and K₂O were applied to paddy fields as base fertilizers at the rates of 2.0, 3.0, and 2.7 g m⁻², respectively, in experiment 1, and 6.0, 9.0, and 8.0 g m⁻² in both experiments 2 and 3, respectively. Top dressing was not applied during the experimental period, and hand weeding and pest control were conducted as necessary. The row and hill spacings were 35 and 30 cm, respectively, for experiment 1; whereas for experiments 2 and 3, they were 50 and 25 cm, respectively. Both experiments 1 and 2 were conducted with eight replications each, with three individual hills per replication; whereas, experiment 3 was conducted with six replications, with four individual hills per replication. All experiments were conducted using randomized complete block design.

The water levels during flooding treatment were 5.0 ± 0.3, 8.3 ± 0.2, and 6.8 ± 0.9 cm in experiments 1, 2, and 3, respectively. Similarly, water temperatures were 29.5 ± 0.5, 27.8 ± 0.8, and 25.3 ± 0.8°C, respectively, and pH was 6.4-6.8. The Eh of the topsoil layer (7.5 cm below the surface) ranged from +245 to −12, +100 to −45, and +392 to −16 mV during the flooding period in experiments 1, 2, and 3, respectively. The photosynthetic and transpiration rates of soybean were measured at the
center leaf of the trifoliate in the uppermost developed leaves using a photosynthetic analyzer [LC pro-SD (ADC Co-Ltd)]. The measurements were conducted from 9:00 to 12:00, and the photosynthetic photon flux density in the leaf chamber was set to 1,800 μmol m\(^{-2}\) s\(^{-1}\). The youngest, fully expanded leaf was used to determine the survival rates of soybean during the flood treatments, because in most cases, the selected leaf was the last to turn brownish (see the detail in Awala et al., 2016). During sampling, the shoots were oven-dried at 80°C for 72 h before measuring the dry weight and calculating the modified land equivalent ratio (LER). In this study, the concept of LER, which is a common evaluation index for mixed cropping systems (Mead & Willey, 1980), was modified to evaluate midterm soybean growth. Shoot dry weight was used instead of seed yield. The modified LER (mLER) for close mixed-planting of rice and soybean was determined as follows: Total mLER = partial mLER for (rice + soybean) = (DWrm/DWrs) + (DWsm/DWss), where DWrm and DWsm represent the shoot dry weight of rice and soybean as mixed crops, respectively, and DWrs and DWss indicate the respective shoot dry weights of rice and soybean as single crops.

Water culture study (experiment 4)

In the present study, it was difficult to precisely distinguish the dry upland and flood paddy conditions simultaneously in the experimental field. Therefore, a water culture experiment (experiment 4) was carried out to obtain a reference for the growth response of soybean under the control environment in a field. In the water culture experiment, the anaerobic and control conditions can be simultaneously compared using either nitrogen or air as the aeration gases. Anaerobic treatment and continuous aeration treatment can be considered as substitute for field flood treatment and upland field conditions, respectively. Experiment 4 was conducted in the growth room and glass house in the same manner as in experiment 1. The mixed seedlings within each cell tray were transferred to a 5-L container (295 mm × 130 mm × 240 mm) filled with Hoagland solution 7 DAS soybean. The nutrient solution was at quarter-, half-, and full-strength concentrations during the first, second, and third weeks. Hypoxic condition (6 ± 0.2 μM O\(_2\), defined as ‘flood stress,’ was induced by the daily bubbling of the culture solution with N\(_2\) gas flowing at 6 L min\(^{-1}\) for 20 min, whereas air was bubbled continuously for the control treatment. The nutrient water was renewed every 3–4 days, and the pH was adjusted and maintained from 6.0 to 6.5 until the end of the experiment (see Table 1).

**Statistical analysis**

The data obtained were statistically processed using a t-test (Excel statistical ver6.0).

**Results and discussion**

Soybean was subjected to flooding stress for a short period of 6–13 days from the juvenile stage to the early stage of flowering under different conditions of maximum and minimum temperatures and solar radiation over 3 years (Table 1). The photosynthetic and transpiration rates during the recovery period after flooding were higher in mixed crops than in single crops (Table 2). This result was consistent with that of pear millet and sorghum close mixed-planting with rice (Iijima et al., 2016); moreover, it clearly indicated that rice alleviated the damage on the photosynthetic and transpiration rates of soybeans during short-term flooding period. In this study, short-term flood stress was applied in an upland field converted from paddy at Japanese summer climates under high-temperature and humid conditions, and the survival rates of single-planted soybean in 2016 and 2017 were 8% and 29%, respectively (Table 3). In contrast, the survival rates of the mixed-planted soybean were significantly improved in both years, 2.4–4.2 times of the single crop. Shoot biomass

| Water culture | Field |
|---------------|-------|
| 2017 (Exp. 4) | 2016 (Exp. 2) | 2017 (Exp. 3) |
| 5 days after flooding | 9 days after flooding | 3 days after flooding | 8 days after flooding | 10 days after flooding |
| Photosynthetic rates (μmol CO\(_2\) m\(^{-2}\) s\(^{-1}\)) | Control | Single | Mix | ns | - | - | - | - |
| Wet | Single | 16.55 | * | 2.62 | * | 13.11 | * | 5.21 | * | 4.93 | † |
| Mix | 19.76 | - | 5.49 | * | 17.74 | * | 9.02 | * | 6.87 | † |
| Transpiration rates (mmol H\(_2\)O m\(^{-2}\) s\(^{-1}\)) | Control | Single | Mix | ns | - | - | - | - |
| Wet | Single | 4.92 | - | 1.18 | * | 2.96 | * | 1.45 | * | 1.25 | † |
| Mix | 5.83 | - | 2.12 | * | 4.12 | * | 2.44 | * | 1.76 | † |

*, P < 0.05; †, P < 0.1: ns, not significant.
production revealed a significant 3.3-fold increase in soybean biomass in 2016, whereas it was reduced by 50% in rice. No significant difference was observed between the treatments in 2015 and 2017; however, the trend was similar to that observed in 2016. The additive design of mixed cropping (Snaydon, 1991), owing to its increased planting density, generally yields lower production from each species than that obtained with single cropping (Zegada-Lizarazu et al., 2006). The close mixed-planting technique used in this study can be regarded as a modified type of additive design because rice and soybean grow together forming a crop community like a single plant. Therefore, the planting density becomes twice as that of the planting density of single cropping. In contrast, in the water culture experiment, the biomass of soybean did not increase.

A total mLER value of >1 indicates a biomass production advantage for mixed cropping (Table 3). This study involved conducting field cultivation and water culture. Differences in mLER values were observed between the two techniques. These differences can be attributed to the variations in the water environment of the rooting medium. We found that the soybean mixed/single crop ratio was remarkably >0.5, in all field experiments, and the complementary effect was >3 in the case of experiment 2 conducted in 2016. Similarly, as the total mLER was always >1, rice exhibited a strong complementary effect on soybean against short-term flood stress in an upland field converted from a paddy. In contrast, in the water culture experiment (experiment 4), the mLER was approximately 1.6 in both the control and flooded treatments. Well-aerated control treatment in the water culture experiment can be considered as a model system for non-stress control treatment. Therefore, the results suggest that the close mixed-planting of rice and soybean is theoretically possible.

These results demonstrate that rice planted as the partner crop of soybean for close mixed-planting can alleviate short-term anaerobic or flood stresses for soybean growth. Presumably, soybean may receive oxygen released from rice roots into the rhizosphere of closely tangled roots (Iijima et al., 2017), which improves viability and photosynthetic and transpiration rates. A positive antioxidant response was associated with a higher tolerance to flooding stress (Arbona et al., 2008). Oxygen transfer might improve antioxidant enzymatic activity, and this can contribute to the enhancement of physiological activities, such as the rates of photosynthesis and transpiration. Previous findings have demonstrated that close mixed-planting with rice is effective in alleviating short-term flooding stress on the drought-resistant cereal crops of pearl millet and sorghum, which are the main staple foods in semi-arid regions (Awala et al., 2016; Awala et al., 2019). The results of this study demonstrated that the alleviative function can be achieved even in the case of legume crop species such as soybean. Despite careful management of the cultivation of the mixed-seedlings to avoid various stresses before transplanting, one crop of the mixed seedlings tended to have slightly enhanced growth over the other (Figure 1). Unlike a cereal-cereal combination such as rice with pearl millet or sorghum, tiny differences in environmental conditions may result in competition for water and nutrient acquisition due to the tangled root system of crops from two different families (cereal and legume). Optimal environmental characteristics for growth may significantly differ among families of crops. Competition for water and nutrient acquisition can also result from complementarity effects enhanced by the tangled roots of the cereal and legume crop species. These reasons may contribute to the difference in the growth between crops of the mixed-seedlings and control individuals (Figure 1). In this study, field experiments were conducted for three continuous

Table 3. Growth of rice and soybean as influenced by close mixed-planting under root O₂ deficiency stress.

|                      | Water culture 2017 (Exp. 4) | Field |                      |
|----------------------|-----------------------------|-------|----------------------|
|                      | Control | Wet | 2015 (Exp. 1) | 2016 (Exp. 2) | 2017 (Exp. 3) |
| Survival rate (%)    | Soybean | Single | - | - | 62.5 | 8.3 | * | 29.2 | * |
|                      |        | Mix | - | - | 75.0 | 37.5 | 70.8 | * |
| Shoot dry weight (g) | Soybean | Single | 2.23 | * | 2.01 | ns | 4.36 | 2.58 | † | 5.70 | ns |
|                      |        | Mix | 1.86 | * | 1.98 | ns | 6.32 | 8.60 | † | 8.61 | ns |
|                      | Rice   | Single | 0.53 | - | 0.58 | ** | 15.45 | 55.20 | - | 67.95 | - |
|                      |        | Mix | 0.42 | † | 0.35 | ** | 12.20 | 27.19 | - | 57.71 | ns |
| Modified land        | Soybean | 0.83 | 0.98 | 1.45 | 3.33 | 1.51 |
| equivalent ratio     | Rice    | 0.80 | 0.60 | 0.79 | 0.49 | 0.85 |
| Total                | 1.63 | 1.58 | 2.24 | 3.82 | 2.36 |

Soybean was harvested at 38, 55, 58; 32 days after sowing for Exp. 1, 2, 3, and 4, respectively. **, P < 0.01; *, P < 0.05; †, P < 0.1; ns, not significant

†, Shoot dry weight was used instead of seed yield. See text for further explanation.
years and the different growth responses of close mixed-seedlings were recorded. The possibility of differences in climatic and/or environmental factors affecting the growth responses has not been identified at a significant level that can allow for any specific inferences. It was possible to obtain yields from soybean plants that survived the waterlogging treatment. Therefore, more multi-year yield studies need to be conducted to determine the yield performance of mixed-seedlings, as well as the relationship of competition and complementarity among species whose growth was influenced by environmental factors.

It can be said that the versatility of the close mixed-planting technique under short-term flooding stress is further clarified. Similarly, under drought conditions, water was released from soybean roots into the rhizosphere soil by the hydraulic-lift phenomenon (Zegada-Lizarazu & Iijima, 2004), and therefore, water can be supplied to rice from soybean, as observed in the close mixed-planting of pearl millet and rice (Izumi et al., 2018). Since rice (submerged paddy fields) and soybean (upland border roads inside paddy fields) have been grown traditionally in the same field in Japan, it can be expected that cultivation of these crops as a mixture of two species in upland fields converted from paddy can be established as a cultivation technique in the near future during the frequent occurrence of floods and droughts.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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