

# Plant-mediated rhizospheric interactions in rice-water spinach intercropping enhance Si uptake by rice

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## Research Article

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# Abstract

*Aims* Although biodiversity utilization in paddy fields has increased in recent years, silicon (Si) nutrition of rice in intercropping systems is poorly understood. On the basis of our previous study, the present research focused on deciphering the underlying mechanisms involved in rice and water spinach intercropping, which enhance Si uptake by rice.

*Methods* We carried out a series of experiments including field, greenhouse, and incubation experiments to explore the underlying mechanisms.

*Results* The field trials showed that rice-water spinach intercropping with different row ratios can increase Si concentration by 9.8%-52.6% and Si absorption by 34.7%-127.8% in rice leaves at ripening stage compared to rice monoculture. The pot trials further indicated that intercropping can promote rice to increase Si concentration in Si-deficient soil, with an increase of 35.9% in stems and 29.7% in leaves, and intercropping significantly increased soil water-soluble Si content by 168.1% compared to rice monoculture. Further research indicated that there was an interaction between rice roots and water spinach roots under intercropping conditions, which not only induced the up-regulated expression of Si transporter genes in rice roots (*OsLsi1*, *OsLsi2*) and stems (*OsLsi6*), but also stimulated rice roots to secrete more organic acids to increase Si availability in the soil. Moreover, water spinach root exudates helped the mobilization of Si in soil through proton efflux of roots.

*Conclusions* Rice-water spinach intercropping can greatly enhance Si absorption of rice through several beneficial ways, which will have important practical significance for sustainable rice production, especially in Si-deficient soils.

## Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops worldwide. Meanwhile, rice is a typical silicon(Si)-accumulating crop, with an average Si content of up to 10% of shoot dry weight (Yamamoto et al. 2012). The increase of Si content in rice plants can strengthen its rigidity and enhance the defense against abiotic stresses (i.e., heavy metals, salinity, and drought) and biotic stresses (i.e., pests and pathogens), thereby improving the yield and quality of rice (Ma et al. 2001; Mitani et al. 2005; Guntzer et al. 2012). In contrast, the lack of Si will cause rice plants to be soft and with brown spots, which seriously affect the normal growth of rice.

Si uptake by rice is an active process, and monosilicic acid in the soil is efficiently absorbed by rice roots through the transport of carrier proteins (Mitani et al. 2005; Liang et al. 2006). Ma et al. (2001) studied rice root mutants and found that rice absorbs Si through lateral roots rather than root hairs. Further study evidenced that root bases are the main sites for absorbing Si, whereas root tips cannot absorb Si (Yamaji and Ma 2007). Si uptake by rice is regulated by three key transporter genes. *OsLsi1* and *OsLsi2* are mainly expressed in the roots, and any mutation of *OsLsi1* and *OsLsi2* will cause a significant decrease of Si absorption in rice (Ma et al. 2006; Ma et al. 2007). *OsLsi6* is mainly expressed in stems and leaves,

and it is responsible for the upward transport of Si in the xylem. When *OsLsi6* is missing, it will not cause a decrease of Si absorption in rice, but it will affect the distribution of Si in different tissues and organs (Yamaji et al. 2008).

The Si absorbed by rice mainly comes from the soil. Non-crystalline Si is a particularly important component in the soil Si pools, and it is divided into water-soluble Si, active Si (including exchangeable Si and colloidal Si), and amorphous Si, which are of great significance to plant Si nutrition (Liu et al. 2001). In fact, there is a dynamic balance relationship among various forms of Si (Wickramasinghe and Rowell 2006; Sommer et al. 2006), and pH is an important influencing factor in these processes (Ma and Takahashi 2002). Although Si is the second most abundant element in the earth's crust and most soils contain considerable quantities of total Si (Epstein 1994), soluble Si that can be easily absorbed by crops is very limited, usually 3 to 40 mg·L<sup>-1</sup> in soil solution (Meharg and Meharg 2015). Continuous rice cultivation takes away a lot of Si from the soil, which will easily result in a shortage of soluble Si in paddy soil, especially in highly weathered tropical areas. Only relying on the natural release of Si in the soil has been difficult to meet the needs of crops for Si uptake (Tsujiimoto et al. 2014; Marxen et al. 2016). The decline of Si supply in soil will be an important limiting factor for the high and stable yield of rice.

At present, the main specific measures to improve the Si level in paddy fields are Si fertilizer application, including water-soluble high-efficiency Si fertilizer (Wang et al. 2020a) and industrial wastes (iron and steel slag, blast furnace slag, fly ash, etc.) (Makabe-Sasaki et al. 2014; Agostinho et al. 2017). However, these measures all have shortcomings more or less. For example, after water-soluble Si fertilizer is applied to the soil, part of the water-soluble Si will be rapidly fixed by the soil and transformed into ineffective forms, thus reducing utilization efficiency; industrial wastes require a large amount and often have poor effects, and even change soil pH and destroy soil ecological environment. In recent years, the utilization of returning straw, Si-rich manure, and Si-rich biochar in paddy fields have also been proved to be important measures to improve Si bioavailability in soil, but the literature in this area are still limited (Song et al. 2014; Li and Delvaux 2019; Yang et al. 2020).

So, is there another low-input and environmentally-friendly measure to improve the Si level of rice in Si-deficient soils? Numerous studies have demonstrated that reasonable intercropping not only increases farmland biodiversity, improves the productivity and stability of farmland ecosystem, but also significantly improves crop mineral nutrition and increases nutrient utilization efficiency (Vandermeer 1990; Raza et al. 2020). For a long time, studies on intercropping to increase nutrient utilization mainly focused on nitrogen (N) (Fujita et al. 1992; Cong et al. 2015; Rodriguez et al. 2020), phosphorus (P) (Li et al. 2007; Dissanayaka et al. 2015; Messaoudi et al. 2020), and some trace elements such as iron (Fe) and zinc (Zn) (Zuo et al. 2000; Xiong et al. 2013) of upland crops. Reasonable intercropping can reduce the nutrient competition between species due to the difference in roots niche, expand the range of nutrients uptake by roots, and promote the efficient use of soil nutrients (Li et al. 2014; Brooker et al. 2015). Notably, some studies have reported that the improvement in nutrients status under intercropping could be attributed to root interactions and rhizosphere effects (Zuo et al. 2000; Garland et al. 2017). For instance, Li et al. (2016) found that maize root exudates in maize-faba bean intercropping system can

stimulate faba bean to excrete flavonoids (signaling compounds for rhizobia) and up-regulate the expression of a chalcone-flavanone isomerase gene, thereby increasing nodulation and N fixation; Fang et al. (2013) reported that root interactions in maize-soybean intercropping can integrate responses to P status and root behaviors among neighboring plants, and thereby have a positive impact on P nutrition of maize. However, to the best of our knowledge, little information is available on Si nutrition of crop in intercropping system.

In recent years, aquatic crops intercropping (e.g., rice was intercropped with water chestnuts, water spinach, alligator flag, etc.) is attracting scholars' increased interest, which mainly highlighted the advantages of intercropping in increasing crop productivity and reducing pest incidence and even alleviating the accumulation of cadmium in rice (Qin et al. 2013; Liang et al. 2016; Kang et al. 2020; Wang et al. 2020b). Considering that improving soil nutrient use efficiency is an important advantage of intercropping (Zhang and Li 2003; Raza et al. 2019), whether intercropping mode can improve Si nutrition of rice is very worthy of study. Water spinach (*Ipomoea aquatica* Forsk) is an important and common aquatic vegetable in southern China, with shallow root distribution and strong regenerative capacity. Our previous field study initially found that intercropping of four rows of rice and two rows of water spinach significantly increased Si concentration and Si absorption in rice leaves at ripening stage compared to rice monoculture (Ning et al. 2017a), but the intrinsic mechanism in the intercropping system was poorly understood.

In the present study, we hypothesized that there was an interaction between rice roots and water spinach roots under intercropping conditions, which can induce the up-regulated expression of Si transporter genes in rice and stimulate the release of root exudates to increase the Si availability in soil, thereby enhancing Si uptake by rice; meanwhile, water spinach root exudates may play a positive role in the mobilization of Si in soil. Then, a series of trials were carried out to explore the intrinsic mechanism of rice-water spinach intercropping to promote Si uptake of rice, in order to clearly understand a new way of improving Si nutrition of rice.

## Materials And Methods

### Study site

Field trials and greenhouse trials were conducted at the campus farm of South China Agricultural University (113°21'E, 23°9'N), Tianhe District, Guangzhou City, Guangdong Province, China. Incubation trials were conducted in the laboratory inside the campus. This area has a subtropical monsoon climate, and the annual average temperature is 20°C to 22°C and total rainfall is approximately 1720 mm. The season suitable for rice cultivation is from April to November, and usually divided into early season (April to July) and late season (August to November). The soil type in the trial field is yellow-brown soil, and its basic physical and chemical properties are as follows: pH 5.54, 20.28 g·kg<sup>-1</sup> organic matter, 6.45 mg·kg<sup>-1</sup> ammonium N, 4.78 mg·kg<sup>-1</sup> nitrate N, 77.34 mg·kg<sup>-1</sup> available P, 51.26 mg·kg<sup>-1</sup> available K, and 167.88 mg·kg<sup>-1</sup> available Si (citric acid extracting, Acquaye and Tinsley 1965).

## Experimental crops

In all trials of this study, rice variety was Huang-hua-zhan, a traditional high-quality variety provided by the Rice Research Institute, Guangdong Academy of Agricultural Sciences, China, and water spinach with white stems was a local variety in Guangzhou. In the field trial and greenhouse trials, rice seeds and water spinach seeds were sown in a seedling field in advance for seedlings preparation. When rice seedlings grew the fourth leaf, rice seedlings and water spinach seedlings were transplanted simultaneously.

## Field Study

Field trial was conducted in 2014–2015 including four rice planting seasons. There were four treatments with three replicates: (1) rice monoculture, (2) 2:2 intercropping, two rows of rice and two rows of water spinach, (3) 3:2 intercropping, three rows of rice and two rows of water spinach, and (4) 4:2 intercropping, four rows of rice and two rows of water spinach. For planting convenience, the field plot area under rice monoculture and 4:2 intercropping was 48 m<sup>2</sup> (8 m × 6 m), and under 3:2 intercropping and 2:2 intercropping it was 42 m<sup>2</sup> (7 m × 6 m) and 36 m<sup>2</sup> (6 m × 6 m), respectively. The row spacing of rice was 0.2 m and that of water spinach was 0.4 m, and the row distance between rice and water spinach was 0.3 m in intercropping system. Each plot was separated by a 20 cm-high soil dam. In the early stage of each planting season, all plots received 280 kg of urea (total N ≥ 46.4%) hm<sup>-2</sup> and 120 kg of potash (KCl ≥ 60%) hm<sup>-2</sup>.

During the trial, water spinach was harvested in time according to its growth status. In each planting season, water spinach was harvested 3 to 4 times, and the last harvest of water spinach was synchronized with rice. At rice ripening stage, two hills of rice plants in each plot were sampled. Rice plant was cut with a sickle from the ground, and then rapidly divided into stems, leaves, and spikes. Rice stems, leaves, and spikes were baked first at 105°C for 0.5 h, and then baked to constant weight at 80°C. The dried stems, leaves, and spikes were weighed and summed to obtain the above-ground dry biomass of rice. Then, leaves were ground to measure Si concentration. The Si concentration was determined using a spectrophotometer at 650 nm by extracting 0.1 g of plant sample with 3 mL of 50 % NaOH solution at 121°C for 20 min (Dai et al. 2005). The Si absorption was equivalent to Si concentration multiplied by dry weight of leaves.

## Greenhouse Study 1 (Si concentration in rice and various Si contents in soil under pot trial)

A pot trial was conducted using a two-factor random block design with four replicates. The two factors were the amount of Si addition and planting patterns. The amount of Si addition included 4 mmol K<sub>2</sub>SiO<sub>3</sub> (AR) per kilogram of soil (ensure sufficient available Si in the soil) and no K<sub>2</sub>SiO<sub>3</sub> addition. The planting patterns included rice monoculture, water spinach monoculture, and rice-water spinach intercropping. Each pot contained 9 kg of paddy soil (taken from our trial field). The initial concentration of different forms of Si in the soil were as follows: 56.43 mg·kg<sup>-1</sup> water-soluble Si, 225.12 mg·kg<sup>-1</sup> active Si, and 11.57 g·kg<sup>-1</sup> amorphous Si. Rice monoculture and water spinach monoculture were both planted with

four seedlings, and intercropping was planted with two rice seedlings and two water spinach seedlings. Seven days after transplanting, all treatments were applied with 10 g of compound fertilizer (15N-15P-15K) per pot, and Si addition treatments were applied with  $K_2SiO_3$  solution. Water spinach was harvested in time according to its growth situation.

A hill of rice plants in each pot was sampled at ripening stage. Leaves and stems were separated and baked to constant weight, and then ground to measure Si concentration. After rice harvest, soil samples of treatments without Si addition were collected from top to bottom with a 4 cm diameter soil drill. Soil samples from two points in each pot were mixed into a composite sample. The soil samples were air-dried and sieved through a 1 mm mesh to measure pH, water-soluble Si, active Si, and amorphous Si. Soil water-soluble Si was determined using the colorimetry by extracting 10 g of soil sample with 50 mL of  $0.01\text{ mol}\cdot\text{L}^{-1}$   $CaCl_2$  solution at  $25^\circ\text{C}$  for 12 h (Berthelsen et al. 2002). Soil active Si was determined using the colorimetry by extracting 10 g of soil sample with 100 mL of  $0.5\text{ mol}\cdot\text{L}^{-1}$   $CaCl_2$  solution (pH 1.5) at  $25^\circ\text{C}$  for 5 h (Tweneboah et al. 1967). Soil amorphous Si was determined using the colorimetry by boiling 10 g of soil sample with 100 mL of  $0.5\text{ mol}\cdot\text{L}^{-1}$  NaOH solution for 2.5 min (Hashimoto and Jackson 1960).

### **Greenhouse Study 2 (Si concentration in rice under root barriers trial)**

Root barriers trial was carried out to confirm whether the increase of Si concentration in intercropping rice was related to root interactions. The experimental containers were cement ponds with a length  $\times$  width  $\times$  height of 1.2 m  $\times$  0.8 m  $\times$  0.5 m, and each pond was filled with 30 cm deep paddy soil. These cement ponds were arranged with three different root barrier treatments from the middle of the long side (Fig. S1): (I) no barrier, allowing roots and solutions to completely interact; (II) mesh barrier (nylon mesh with 30  $\mu\text{m}$  pores), preventing interspecies root intermingling while permitting solutions exchange; (III) solid barrier (hard plastic board), eliminating root interaction and solutions movement. Each treatment had four replicates. Twelve rice seedlings and twelve water spinach seedlings were transplanted separately on two sides of the cement pond. The row spacing of rice and water spinach were both 20 cm, and they were both 10 cm away from the edge of pond. Thus, rice was positioned in three rows (20 cm, 40 cm, and 60 cm) away from water spinach. Seven days after transplanting, each pond was applied with 60 g of compound fertilizer (15N-15P-15K). Water spinach was harvested in time according to its growth situation. At ripening stage, two hills of rice plants in the middle of each row were sampled. Leaves and stems were separated and baked to constant weight, and then ground to measure Si concentration.

### **Greenhouse Study 3 (quantitative expression of Si transporter genes)**

To compare the expression of Si transporter genes between monoculture rice and intercropping rice, a pot trial was carried out including rice monoculture and rice-water spinach intercropping with three replicates. The planting and management methods were the same as those in Greenhouse Study 1. Rice was sampled at heading stage (peak period of Si uptake). At first, the crops and whole soil column were removed from the pot, and excess soil attached to the roots was washed away with tap water. Then, a

rice plant was selected in each pot and rapidly brought back to the laboratory, and the roots were washed sequentially with tap water and distilled water. The inverted 2 and 3 leaves of each tiller, the stem between the inverted 1 and 2 leaves, and the root located at the 1–3 cm part of the root base were sampled separately and cut into small pieces and then packed into a 10 mL polyethylene tube. Plant samples were rapidly frozen in liquid nitrogen and then placed in a -80°C freezer. The expression levels of *OsLsi1* and *OsLsi2* were measured only for rice roots. The expression level of *OsLsi6* was measured for rice leaves and stems.

Quantitative real-time PCR analysis: Total RNAs were extracted from 0.1 g flash-frozen and powdered leaf/stem/root samples using the TRIzol Reagent (Life Technologies, USA) according to the manufacturer's instructions. The first-strand cDNAs were synthesized from 1 µg of total RNA using a M-MLV Reverse Transcriptase (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara, Japan) with a 7500 Fast Real-Time PCR Sequence Detection System (Applied Biosystems 7500, USA). The thermal profile was 90°C for 30 s, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis were conducted to verify the amplicon specificity. The relative transcript levels were calculated using the double-standard curve method, and the rice housekeeping gene *OsActin* was used as an endogenous control. The gene-specific primers used in this study are listed in Table S1.

#### **Greenhouse Study 4 (organic acids concentration in rice root exudates)**

To compare the organic acids concentration of root exudates between monoculture rice and intercropping rice, a pot trial was carried out including rice monoculture and rice-water spinach intercropping with eight replicates. Each pot contained 5 kg of paddy soil sieved through a 2 mm sieve. Rice monoculture contained two rice seedlings, and intercropping contained a rice seedlings and a water spinach seedlings. Fertilization and management methods were the same as those in Greenhouse Study 1. Sixty days after transplanting, root exudates of monoculture rice and intercropping rice were collected and measured the composition and concentration of organic acids. At first, the crops and whole soil column were removed from the pot, and excess soil attached to the roots was washed away with tap water. Then, crops were rapidly brought back to the laboratory, the roots of the two crops in the same pot were gently separated, and rice roots were washed sequentially with tap water, distilled water, and ultra-pure water. Then, rice roots were placed into a 1 L black hydroponic cup containing 600 mL of 0.5 mmol·L<sup>-1</sup> CaCl<sub>2</sub> solution, and the opening of the hydroponic cup was shaded with a tinfoil. Thereafter, rice shoots were kept under normal light for 5 h. The CaCl<sub>2</sub> solution containing root exudates was passed through a 0.22 µm millipore filter and stored in a 4°C freezer.

Methods of organic acids analysis: Root exudates samples were concentrated 10 times in a rotary evaporator at 40°C and subsequently organic acid concentrations (citric acid, malic acid, fumaric acid, and succinic acid) in root exudates was analyzed using UPLC-MS/MS (zevo-TQD, Waters, USA). The analytical conditions were as follows: chromatographic column: acuity UPLC BEH C18 (1.7 µm, 2.1 mm ×

50 mm); velocity flow:  $0.3 \text{ mL}\cdot\text{min}^{-1}$ ; column temperature:  $30^\circ\text{C}$ ; injection volume:  $10 \mu\text{L}$ ; mobile phase: 0 min, 100% A (0.1% formic acid); 2.5 min, 87.5% A + 12.5% B (acetonitrile with 0.1% formic acid); 3–4 min, 50% A + 50% B; 5–6 min, 100% A.

### **Incubation Study 1 (soil Si availability responds to different organic acids)**

Paddy soil obtained from the trial field was air-dried and sieved through a 1 mm mesh, and then 100 g of soil was packed into a 250 mL polyethylene bottle (15 bottles in total). The initial water-soluble Si, active Si, and amorphous Si contents in the soil were  $25.33 \text{ mg}\cdot\text{kg}^{-1}$ ,  $129.28 \text{ mg}\cdot\text{kg}^{-1}$ , and  $13.84 \text{ g}\cdot\text{kg}^{-1}$ , respectively. In addition, 4 kinds of organic acids (citric acid, malic acid, succinic acid, and fumaric acid, AR) were dissolved separately into a  $0.025 \text{ mol}\cdot\text{L}^{-1}$  solution with distilled water. Here,  $0.025 \text{ mol}\cdot\text{L}^{-1}$  refers to the concentration of citric acid solution to extract soil available Si (Acquaye and Tinsley 1965). Then, each solution was weighed to 100 mL and added into the polyethylene bottle filled with soil, and distilled water was used as a control. Each treatment had three replicates. The bottles were gently shaken and then kept still at room temperature for 7 d. 10 mL of supernatant was taken to measure Si concentration using silicon molybdenum blue colorimetry. Subsequently, the remaining solution in the bottle was discarded, and the soil was spread out and air-dried and sieved through a 1 mm mesh to measure water-soluble Si. Total water-soluble Si in the soil-water system was equal to the sum of Si in the solution and soil water-soluble Si.

### **Greenhouse Study 5 (soil Si availability responds to water spinach root exudates)**

To verify whether water spinach root exudates can increase the Si availability in the soil, 5 kg of paddy soil sieved through a 2 mm sieve was packed into a pot (12 pots in total), and then six pots of rice and six pots of water spinach (both a seedling per pot) were simultaneously cultivated for 30 d to collect the root exudates. The collection method was the same as that in Greenhouse Study 4. 100 g of air-dried and sieved paddy soil (same as that in Incubation Study 1) was packed into a 250 mL polyethylene bottle (12 bottles in total). Then, 100 mL of rice root exudates and 100 mL of water spinach root exudates were added separately into the polyethylene bottle filled with soil. The bottles were gently shaken and then kept still at room temperature for 7 d. 10 mL of supernatant was taken to measure Si concentration. Subsequently, the remaining solution in the bottle was discarded, and the soil was spread out and air-dried and sieved through a 1 mm mesh to measure water-soluble Si, active Si, and amorphous Si. In addition, rice root exudates and water spinach root exudates were measured for the composition and concentration of organic acids, and the methods were the same as that in Greenhouse Study 4.

### **Incubation Study 2 (visualization of rhizosphere acidification)**

Plump rice seeds and water spinach seeds were sterilized with 5%  $\text{H}_2\text{O}_2$  and sown separately in plastic boxes filled with substrate soil. Subsequently, they were cultivated in a light incubator ( $30^\circ\text{C}$ , 85% humidity) for 20 d, and the substrate soil was kept moist during this period. Then, three rice seedlings and three water spinach seedlings were carefully pulled out from the substrate soil and washed with distilled

water to remove the impurities on the roots. Then, the seedlings were transferred separately into hydroponic cups containing 250 mL of Kimura B nutrient solution. These seedlings were placed back in the light incubator (30°C, 85% humidity) for another 30 d, and the nutrient solution was renewed every 3 d during the period.

The difference in rhizosphere acidification between rice seedlings and water spinach seedlings was studied visually by quantifying the proton efflux of roots (Rao et al. 2000). At first, 0.5 g of agar and 0.5 mL of 0.6% bromocresol purple indicator were added into a 50 mL medium containing 50 mg·L<sup>-1</sup> NH<sub>4</sub><sup>+</sup> (1000 mL H<sub>2</sub>O + 183 mg (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>+136 mg CaSO<sub>4</sub>). Subsequently, the mixed medium was placed on an electric stove and stirred and heated to boiling. As soon as the medium cooled to approximately 40°C, its pH was adjusted to 6.0 by adding 0.01 mol·L<sup>-1</sup> HCl or 0.01 mol·L<sup>-1</sup> NaOH. Then, the medium was evenly poured into a petri dish at a depth of approximately 1 cm and gradually solidified. The pre-cultivated rice seedlings and water spinach seedlings were taken out from the hydroponic cups, and the roots were washed with distilled water. After the moisture on the root surface was absorbed using tissue paper, the root was flattened on the agar gel and lightly pressed to embed into the agar gel. After 6 h, we observed the color change of the agar gel around the root, in which yellow means strong acidification, and purple means weak acidification.

## Statistical analysis

The results were presented as mean ± standard deviation. The data of two treatments were statistically analyzed by independent T-test at a 5% significance level. The data of three or more treatments (except Greenhouse Study 2) were statistically analyzed by one-way ANOVA and Duncan's method for multiple comparisons at a 5% significance level. Particularly, the data from Greenhouse Study 2 were statistically analyzed by two-way ANOVA at a 5% significance level. All statistical analyses were performed using SPSS 17.0 and all figures were constructed using Origin 8.0.

## Results

### Effects of intercropping on the above-ground dry biomass, Si concentration, and Si absorption of rice in field study

The above-ground dry biomass of rice was related to the planting seasons, it was obviously higher in early season than in late season, and higher in 2014 than in 2015 (Table 1). Intercropping resulted in a significant increase of the above-ground dry biomass of rice compared to monoculture. Among three intercropping patterns, the above-ground dry biomass of rice generally showed the following order: 2:2 intercropping > 3:2 intercropping > 4:2 intercropping. Among different treatments, the difference in Si concentration in rice leaves showed consistency in four planting seasons, that intercropping resulted in an increase of Si concentration in rice leaves (Table 1). Compared to rice monoculture, 3:2 intercropping and 4:2 intercropping significantly increased the Si concentration in rice leaves by 25.4%-52.6% and 20.3%-30.5%, respectively; but 2:2 intercropping only increased it by 9.8%-15.7%. Si absorption in rice

leaves under three intercropping patterns were all significantly higher than that under monoculture, and the increase rate reached 34.7%-127.8% (Table 1). Among three intercropping patterns, 3:2 intercropping was observed to have the highest Si absorption in rice leaves in the early and late seasons of 2014, however, there was no large difference in Si absorption in rice leaves in 2015.

Table 1

Effects of rice-water spinach intercropping on the above-ground dry biomass, Si concentration, and Si absorption of rice in field study

Planting seasons	Treatments	Above-ground dry biomass of rice (g.hill <sup>-1</sup> )	Si concentration in rice leaves (mg.g <sup>-1</sup> )	Si absorption in rice leaves (mg.hill <sup>-1</sup> )
Early season in 2014	Rice monoculture	56.54 ± 6.08b	39.21 ± 3.64c	333.71 ± 34.81c
	2:2 intercropping	94.24 ± 10.04a	43.06 ± 5.20bc	584.31 ± 61.90b
	3:2 intercropping	87.58 ± 12.15a	59.82 ± 8.46a	760.18 ± 56.58a
	4:2 intercropping	83.25 ± 11.76a	51.16 ± 12.76ab	644.66 ± 52.38b
Late season in 2014	Rice monoculture	49.17 ± 4.47c	50.79 ± 4.11b	288.37 ± 43.54c
	2:2 intercropping	81.64 ± 9.08a	55.94 ± 5.00ab	412.82 ± 32.77b
	3:2 intercropping	75.27 ± 11.39ab	63.69 ± 10.76a	477.70 ± 37.58a
	4:2 intercropping	69.23 ± 9.70b	61.51 ± 7.91a	418.80 ± 62.25b
Early season in 2015	Rice monoculture	39.33 ± 3.37c	46.25 ± 4.43c	281.44 ± 31.62c
	2:2 intercropping	72.90 ± 8.40a	53.45 ± 4.94bc	511.65 ± 60.97ab
	3:2 intercropping	66.89 ± 7.82ab	61.87 ± 8.07a	530.34 ± 40.88a
	4:2 intercropping	60.95 ± 13.10b	55.64 ± 6.23ab	456.74 ± 51.23b
Late season in 2015	Rice monoculture	37.83 ± 5.38b	47.84 ± 4.03c	262.41 ± 38.40b
	2:2 intercropping	56.36 ± 7.49a	55.37 ± 7.10bc	378.16 ± 48.84a
	3:2 intercropping	53.76 ± 10.12a	65.01 ± 9.01a	395.77 ± 41.40a
	4:2 intercropping	47.45 ± 7.79a	59.75 ± 7.56ab	353.51 ± 40.54a

Different small letters indicate significant differences among different treatments at the same planting season ( $p < 0.05$ ).

## Effects of intercropping on Si concentration in rice plants and various Si contents in soil under pot trial

As shown in Fig. 1, exogenous  $K_2SiO_3$  addition could significantly increase Si concentration in rice plants, regardless of monoculture and intercropping. In the case of  $K_2SiO_3$  addition, no significant difference was observed for Si concentration in rice plants between monoculture and intercropping. However, in the case of no  $K_2SiO_3$  addition, compared to rice monoculture, intercropping significantly increased Si concentration in rice plants, where stems increased by 35.9% and leaves increased by 29.7%.

In the case of no  $K_2SiO_3$  addition, soil pH and various forms of Si under different crop planting patterns are shown in Fig. 2. Compared to rice monoculture, water spinach monoculture significantly decreased soil pH by 0.30 unit, and intercropping significantly decreased it by 0.17 unit. Large difference was observed in soil water-soluble Si content among different treatments. Compared to rice monoculture, water spinach monoculture significantly increased soil water-soluble Si content by 302.5%, and intercropping significantly increased it by 168.1%. Soil active Si content among different treatments also showed significant differences, rice monoculture was observed to have the highest soil active Si content, followed by water spinach monoculture, and intercropping had the lowest soil active Si content. In addition, crop planting patterns had no significant effect on soil amorphous Si content in this study.

## Si concentration of rice plants under different root barriers in intercropping system

As shown in Fig. 3, regardless of Si concentration in rice leaves or stems, there is no interaction between root barrier and distance according to a two-way ANOVA. Si concentration in rice leaves and stems under three root barriers generally showed the following order: no barrier > mesh barrier > solid barrier. For rice leaves, this order was significant only at the distance of 20 cm from water spinach; and for rice stems, this order was significant at all distances from water spinach. Furthermore, Si concentration in rice plants under no barrier and mesh barrier gradually declined as the distance from water spinach increased, but this situation was not observed under solid barrier.

## Quantitative expression of Si transporter genes in monoculture rice and intercropping rice

In rice roots, intercropping increased the relative expression of *OsLsi1* and *OsLsi2* by 23.1% and 57.7%, respectively compared to monoculture (Fig. 4a, b). Among them, the expression of *OsLsi2* between monoculture rice and intercropping rice reached a significant difference level ( $p < 0.05$ ). In rice stems, intercropping also increased the relative expression of *OsLsi6* compared to monoculture, with an increase of 81.1%, although the difference did not reach a significant level (Fig. 4c). In rice leaves, there was no significant difference in the relative expression of *OsLsi6* between monoculture and intercropping (Fig. 4d).

## Organic acids in rice root exudates and their effects on soil Si availability under intercropping conditions

It was clearly observed that root exudates of intercropping rice contained significantly higher organic acids concentration (except citric acid) compared to those of monoculture rice (Fig. 5). The concentration of malic acid, succinic acid, and fumaric acid in root exudates of intercropping rice was 2.55, 5.43, and 4.27 times that of monoculture rice, respectively. Furthermore, root exudates of intercropping rice contained relatively high succinic acid concentration. The root exudates of monoculture rice and intercropping rice both contained relatively high citric acid concentration, and its concentration in root exudates of intercropping rice was 1.31 times that of monoculture rice.

After paddy soil was leached with different organic acid solutions, water-soluble Si in the soil-water system changed significantly compared to that with distilled water leaching (Fig. 6). Organic acids treatments, especially citric acid, significantly increased Si concentration in the solution. The Si concentration in the solution with citric acid, succinic acid, malic acid, and fumaric acid leaching was 11.88, 4.03, 3.64, and 2.84 times that with distilled water leaching, respectively (Fig. 6a). Compared to distilled water leaching, organic acids treatments all reduced soil water-soluble Si content (Fig. 6b). The trend of total water-soluble Si in the soil-water system under different treatments was similar to Si concentration in the solution, and citric acid leaching had the highest total water-soluble Si content, which was 3.92 times that with distilled water leaching (Fig. 6c).

### **Effects of water spinach root exudates on soil Si availability in intercropping system**

Compared to rice root exudates leaching, paddy soil leached by water spinach root exudates increased Si concentration in the solution by 62.2% and soil water-soluble Si content by 16.7%, although the differences both did not reach a significant level (Fig. 7a, b). Two root exudates had similar effects on soil active Si and amorphous Si content (Fig. 7c, d). However, organic acids concentration in water spinach root exudates was much lower than that in rice root exudates according to the four detected organic acids (Fig. S3).

According to the Incubation Study 2, rhizosphere acidification of rice seedling and water spinach seedling showed visual difference on the agar gel with bromocresol purple indicator (Fig. 8). It was obviously yellower around water spinach rhizosphere than around rice rhizosphere, and the yellow range around water spinach rhizosphere was wider than that around rice rhizosphere. This indicated that water spinach rhizosphere had stronger acidification ability through proton efflux of roots than rice rhizosphere.

## **Discussion**

### **Intercropping increases Si absorption of rice and soil water-soluble Si content**

In intercropping system, due to the differences in growth characteristics and resource requirements, especially in plant height between species, the combination of two crops with different rows ratio may produce inconsistent effects in some aspects, such as yield and nutrient uptake. In the present study, four-season field trials with three intercropping ratios showed that rice-water spinach intercropping, especially 3:2 intercropping, could increase Si concentration and Si absorption in rice leaves at ripening

stage (Table 1). This was because rice plants absorbed a lot of Si throughout the growth period, while soil water-soluble Si under rice monoculture may be insufficient at the late stage of growth; in contrast, more soil water-soluble Si under intercropping could be absorbed by rice as the Si requirement of water spinach ( $3\text{--}5\text{ mg}\cdot\text{g}^{-1}$  in leaves) was much smaller than that of rice. This can be understood as the niche complementarity enhances resource acquisition (Brooker et al. 2015). Moreover, the above-ground biomass of intercropping rice was obviously greater than that of monoculture rice, which was mainly as a consequence of edge effect (Table 1), enabling the effect of intercropping on Si absorption of rice to be more obvious than Si concentration of rice. It can also be seen that 3:2 intercropping was a better pattern based on comprehensive biomass and Si absorption. As for why 2:2 intercropping exhibited lower Si concentration in rice leaves than 3:2 intercropping and 4:2 intercropping, this can be explained by the dilution effect, wherein 2:2 intercropping obtained the largest individual biomass but no obvious advantage in Si absorption (Table 1).

The results of the pot trial further clarified that rice-water spinach intercropping could increase Si concentration in rice plants in the case of Si deficiency (Fig. 1), which will help rice grow healthily and increase yield. Given that water-soluble Si can be directly absorbed by rice, its increase under intercropping can be considered as an important cause for the increase of Si concentration in rice plants (Fig. 2), correlation analysis showed Si concentration in rice plants and soil water-soluble Si content had a significant positive correlation (Fig. S2), which was consistent with the results from de Camargo et al. (2007). Meanwhile, we think the status of soil water-soluble Si under different treatments was not only affected by crop absorption, the conversion of soil active Si instead of amorphous Si to water-soluble Si was another important reason (Fig. 2). This is because soil amorphous Si is relatively rich and stable in soil Si pools (Majumdar and Prakash 2020), different crop patterns in one planting season are not enough to change soil amorphous Si. In contrast, soil active Si among different treatments may be related to the crop patterns, and it seems to imply that water spinach monoculture and especially intercropping can dissolve soil active Si more easily than rice monoculture. In fact, the changes of various forms of Si in the soil are affected by many factors, such as pH and organic acids, these factors are also the most likely to change in response to crop patterns. In this trial, compared to rice monoculture, water spinach monoculture and rice-water spinach intercropping significantly decreased soil pH by 0.30 unit and 0.17 unit, respectively (Fig. 2), which may be one of the reasons for the change of soil Si. So far, it is sure that rice-water spinach intercropping can enhance Si absorption in rice and Si availability in soil, apart from the difference in Si demand of rice and water spinach, other detailed mechanisms would be explored through the following trials.

### **Interspecific rhizosphere interactions enhance Si uptake by rice**

Does the rhizosphere interactions play a role in Si nutrition of rice under intercropping? The root barriers trial provided an insight into this question. The results indicated that the increase of Si concentration in rice plants was related to the rhizosphere interaction by comparing the Si concentration in rice plants under no barrier and mesh barrier (Fig. 3). Similar results were obtained by Zuo et al. (2000), Li et al. (2001), and Zhao et al. (2020), even though they targeted Fe, P, or N. Obviously, when the roots of two

species come into contact, it will stimulate plants to produce a physiological response, which is often advantageous to the species involved. In our study, the interaction between rice roots and water spinach roots was reflected in promoting rice to absorb Si. Zuo et al. (2000) emphasized that the interactions are likely to be more important in soils with low availability of particular nutrients. In addition, the root barriers trial also evidenced that the silicic acid in water spinach rhizosphere could diffuse into rice rhizosphere by comparing the Si concentration in rice plants under mesh barrier and solid barrier, thereby providing more silicic acid for rice (Fig. 3).

Then, how does the rhizosphere interaction specifically enhance the Si absorption of rice? We first speculated whether it has an effect on the Si absorption inside rice plants. By testing the quantitative expression of Si transporter genes in rice, it was observed that rice-water spinach intercropping can induce the up-regulated expression of Si transporter genes (especially *OsLsi2* in roots) in rice, thereby promoting the uptake and upward transport of Si in rice (Fig. 4). Related studies have also proved that intercropping can induce gene expression. For instance, Xiong et al. (2013) found that maize-peanut intercropping significantly improved the secretion ability of phytosiderophores and the expression of genes related to phytosiderophores synthesis in maize roots compared to maize monoculture. Guo et al. (2014) suggested that maize-peanut intercropping could cause an increase of available Fe in the peanut rhizosphere and the up-regulated expression of Fe uptake genes (*AhFRO1* and *AhYSL1*) in peanut at vegetative stage compared to peanut monoculture. In our study, the more detailed mechanism for the root interaction to induce the expression of Si transporter genes needs to be further studied.

Furthermore, we speculated whether the rhizosphere interaction can increase the Si availability in rice rhizosphere by secreting more organic acids. The results according to Greenhouse Study 4 indicated that rice-water spinach intercropping can induce rice roots to secrete more organic acids, especially succinic acid (Fig. 5). Several studies also indicated that interspecific root interaction can stimulate crops to coordinate rhizosphere activities to enhance plant fitness, resulting in changes in root exudates secretion relative to monoculture (Hess and De Kroon 2007; Li et al. 2010; Zhang et al. 2016). Xiao et al. (2014) found that wheat-faba bean intercropping increased organic acids secretion of wheat roots by 155% at tillering stage, 35.6% at booting stage, and 92.6% at filling stage compared with those in corresponding monoculture. Undoubtedly, the increase of organic acids concentration in rice rhizosphere under intercropping provides a possibility for the mobilization of soil insoluble Si (Barman et al. 1992; Lazo et al. 2017).

Further study showed that organic acids treatments, especially citric acid, significantly increased Si concentration in the solution and total water-soluble Si in the soil-water system compared to distilled water treatment (Fig. 6a, c). Studies have shown that organic acids can mobilize insoluble Si in soil mainly through dissolution, chelation and ion exchange, etc. (Barman et al. 1992; Lazo et al. 2017). As for why citric acid has the strongest ability to mobilize soil Si, an important reason may be that citric acid has three carboxyl groups and is more effective to mobilize soil nutrient than carboxylates with one or two carboxyl groups (Jones et al. 2003). Considering comprehensively that the amount of organic acids in rice root exudates and their ability to mobilize soil Si, we think citric acid and succinic acid are two

more important organic acids for mobilizing soil Si in rice-water spinach intercropping system. Furthermore, compared to distilled water leaching, organic acids treatments all reduced soil water-soluble Si content (Fig. 6b), which showed organic acids can extract the water-soluble Si in soil solid phase into the aqueous solution and facilitate crop Si uptake. This may be because silicic acid has a better affinity towards organic acids compared to water, but further verification is needed.

### **Water spinach root exudates increases soil Si availability in intercropping system**

Some studies have shown that the root exudates of one crop may play an important role in the nutrient acquisition of another crop in intercropping system (Li et al. 2013; Xiong et al. 2013; Latati et al. 2014). For instance, in broad bean-maize intercropping, organic acids secreted by broad bean roots can diffuse into the maize rhizosphere to mobilize insoluble P, thereby improving the P nutrition of maize (Li et al. 2013). Our results according to Greenhouse Study 5 also indicated that water spinach root exudates had the potential ability to increase the soil Si availability compared to rice root exudates (Fig. 7). However, the root exudates collected in a short time may not be enough to show a difference for water-soluble Si, not to mention soil active Si and amorphous Si. Further study found that organic acids concentration in water spinach root exudates was much lower than those in rice root exudates according to the four detected organic acids (Fig. S3), which indicated that the potential of water spinach root exudates to increase the soil Si availability could not be attributed to the effect of organic acids.

Notably, in intercropping system, plant roots can also secrete protons into the rhizosphere to cause rhizosphere acidification, thereby increasing the availability of insoluble nutrients in the rhizosphere soil, mainly for P, Fe, and Zn (Li et al. 2007; Li et al. 2014). By comparing the visualization of rhizosphere acidification of water spinach seedling and rice seedling, we found that water spinach rhizosphere had a stronger acidification ability than rice rhizosphere (Fig. 8). This also gave an explanation for why rice-water spinach intercropping and water spinach monoculture had significantly lower soil pH than rice monoculture in the pot trial (Fig. 2). Soil pH is one of the most important factors for the soil Si availability. In general, soil water-soluble Si will increase as the pH drops within a certain range (Tavakkoli et al. 2011). Therefore, water spinach rhizosphere acidification through proton efflux of roots is considered as a beneficial way to increase the soil Si availability. In addition, the anion-cation balance during the N uptake process and the excretion of organic anions will also drive the secretion of protons in crop roots, which causes rhizosphere acidification (Hinsinger et al. 2003). This means that intercropping rice itself can increase rhizosphere acidification through nutrient competition and root interaction, thereby achieve efficient use of soil Si.

### **Rice-water spinach intercropping advances ecological diversified rice cropping**

The present studies showed that rice-water spinach intercropping can greatly enhance Si absorption of rice through several beneficial ways (Fig. 9), and our previous study also indicated that three intercropping patterns all significantly increased rice yield per unit area and comprehensive yield of the system (Ning et al. 2017b), which shows rice-water spinach intercropping is a feasible eco-agriculture mode. Globally, reducing agro-chemicals is an inevitable trend, however, how to maintain high yields

while reducing agro-chemicals is one problem. It is well known that increasing crop yield, controlling pests, and improving nutrient utilization are important advantages of intercropping (Zhang and Li 2003; Ratnadass et al. 2012). Therefore, intercropping is a promising strategy for the sustainable development of food production based on lesser reliance on chemical fertilizer and pesticide inputs (Zhang et al. 2010; Brooker et al. 2015). In this background, ecological farms are developing rapidly in China and even the world, and diversified rice cropping is also an increasing practice (Liang et al. 2016; Kang et al. 2020; Wang et al. 2020b). Considering the important role of Si for rice, the increase of Si uptake by rice under intercropping can be regarded as an invisible booster within the ecosystem, which advances and escorts ecological diversified rice cropping.

## Conclusions

The present studies showed that rice-water spinach intercropping can greatly enhance Si absorption of rice through several beneficial ways. Besides more water-soluble Si can be absorbed by rice in the intercropping system, as water spinach needs less Si. Interestingly, there was an interaction between rice roots and water spinach roots under intercropping conditions, which can induce the up-regulated expression of Si transporter genes (*OsLsi1*, *OsLsi2* and *OsLsi6*) in rice and stimulate rice roots to secrete more organic acids to increase the Si availability in the soil. Moreover, water spinach root exudates also helped in the mobilization of Si in the soil owing to the proton efflux of roots. This is the first report to explore deeply the Si nutrition of rice under intercropping conditions, which will have important practical significance for sustainable rice production especially in Si-deficient soils. Notably, rice-water spinach intercropping can be a feasible eco-agriculture mode based on the important role of Si for rice, and advance rice biodiversity cultivation.

## Declarations

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**Conflicts of interests** The authors declare no competing interests.

**Author contributions** K.C. supervised the project; C.N., Y.C., and K.C. designed the research; C.N. and R.L. performed most of the experiment; C.N., L.W., T.P., and J.H. analyzed the data; C.N., K.C., and S.L. wrote the paper.

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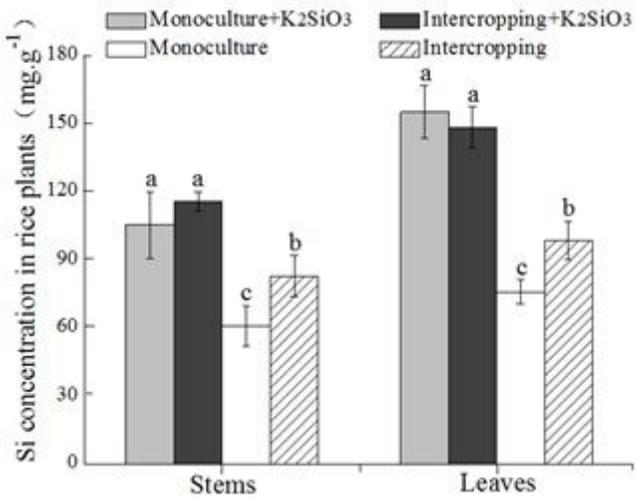
## Tables

**Table 1** Effects of rice-water spinach intercropping on the above-ground dry biomass, Si concentration, and Si absorption of rice in field study

Planting seasons	Treatments	Above-ground dry biomass of rice (g.hill <sup>-1</sup> )	Si concentration in rice leaves (mg.g <sup>-1</sup> )	Si absorption in rice leaves (mg.hill <sup>-1</sup> )
Early season in 2014	Rice monoculture	56.54±6.08b	39.21±3.64c	333.71±34.81c
	2:2 intercropping	94.24±10.04a	43.06±5.20bc	584.31±61.90b
	3:2 intercropping	87.58±12.15a	59.82±8.46a	760.18±56.58a
	4:2 intercropping	83.25±11.76a	51.16±12.76ab	644.66±52.38b
Late season in 2014	Rice monoculture	49.17±4.47c	50.79±4.11b	288.37±43.54c
	2:2 intercropping	81.64±9.08a	55.94±5.00ab	412.82±32.77b
	3:2 intercropping	75.27±11.39ab	63.69±10.76a	477.70±37.58a
	4:2 intercropping	69.23±9.70b	61.51±7.91a	418.80±62.25b
Early season in 2015	Rice monoculture	39.33±3.37c	46.25±4.43c	281.44±31.62c
	2:2 intercropping	72.90±8.40a	53.45±4.94bc	511.65±60.97ab
	3:2 intercropping	66.89±7.82ab	61.87±8.07a	530.34±40.88a
	4:2 intercropping	60.95±13.10b	55.64±6.23ab	456.74±51.23b
Late season in 2015	Rice monoculture	37.83±5.38b	47.84±4.03c	262.41±38.40b
	2:2 intercropping	56.36±7.49a	55.37±7.10bc	378.16±48.84a
	3:2 intercropping	53.76±10.12a	65.01±9.01a	395.77±41.40a
	4:2 intercropping	47.45±7.79a	59.75±7.56ab	353.51±40.54a

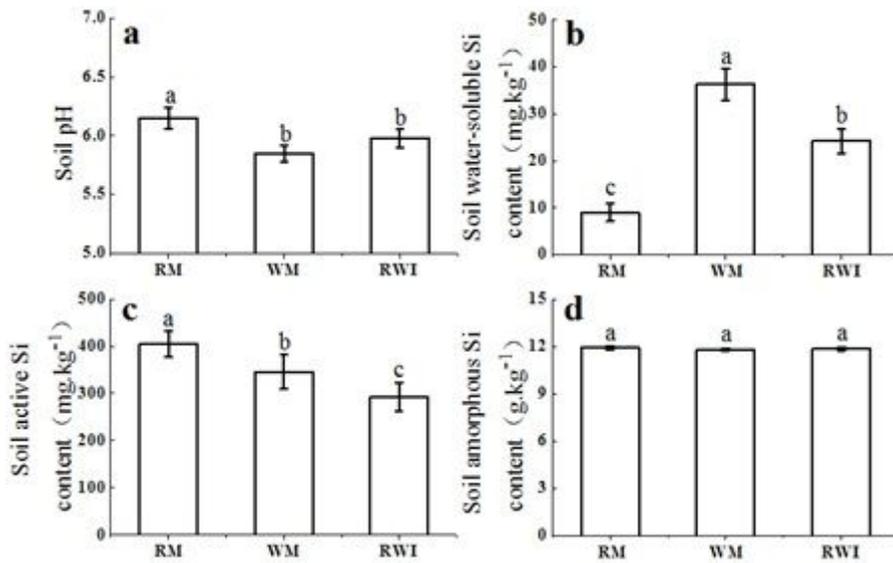
Different small letters indicate significant differences among different treatments at the same planting season ( $p < 0.05$ ).

## Figures



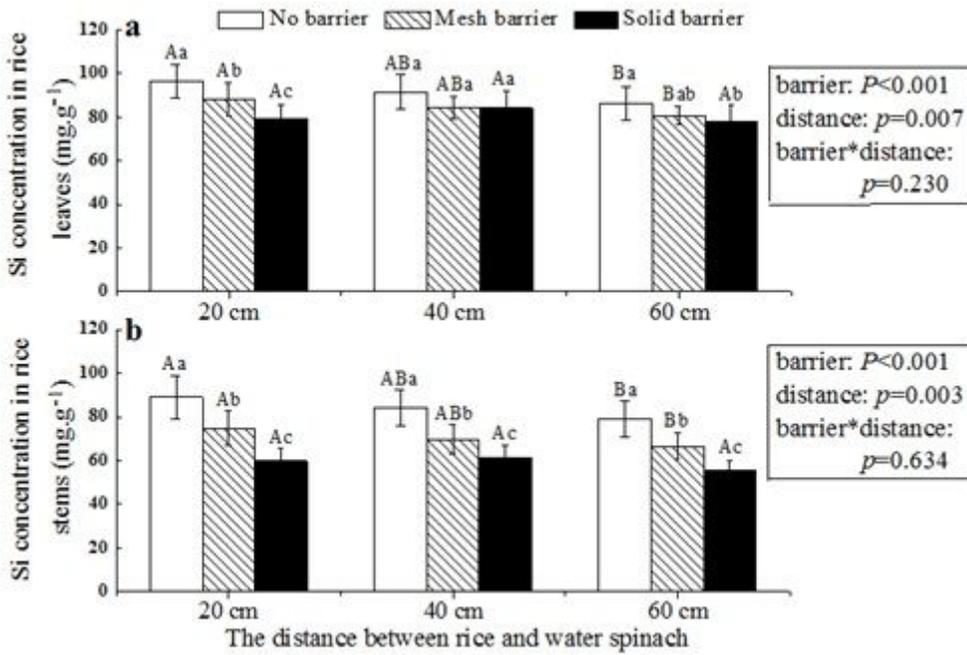
**Figure 1**

Effects of rice-water spinach intercropping with or without K<sub>2</sub>SiO<sub>3</sub> on Si concentration in rice plants in Greenhouse Study 1. Application rate of K<sub>2</sub>SiO<sub>3</sub> was 4 mmol per kilogram of soil. Different small letters indicate significant differences among different treatments in the same rice part ( $p < 0.05$ )



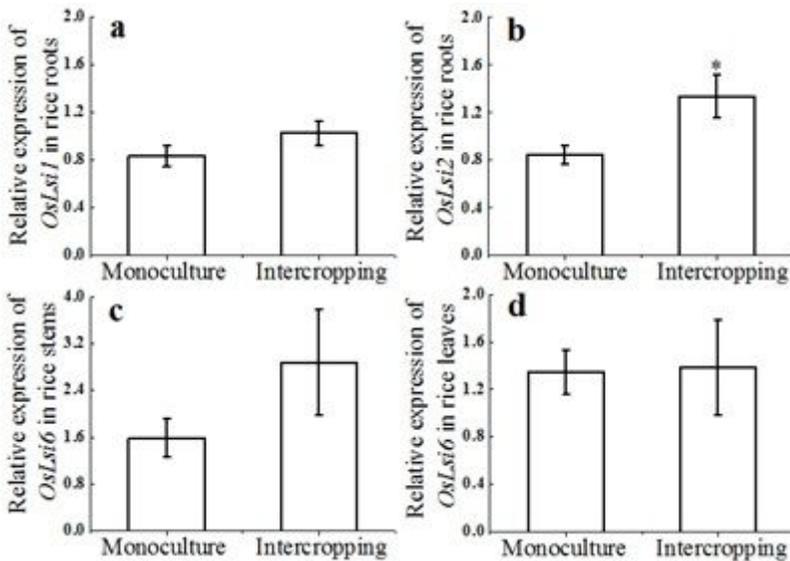
**Figure 2**

Effects of rice-water spinach intercropping on soil pH (a) and various forms of Si (b soil water-soluble Si; c soil active Si; d soil amorphous Si) in Greenhouse Study 1. RM, rice monoculture; WM, water spinach monoculture; RWI, rice-water spinach intercropping. Different letters indicate significant differences among different treatments ( $p < 0.05$ )



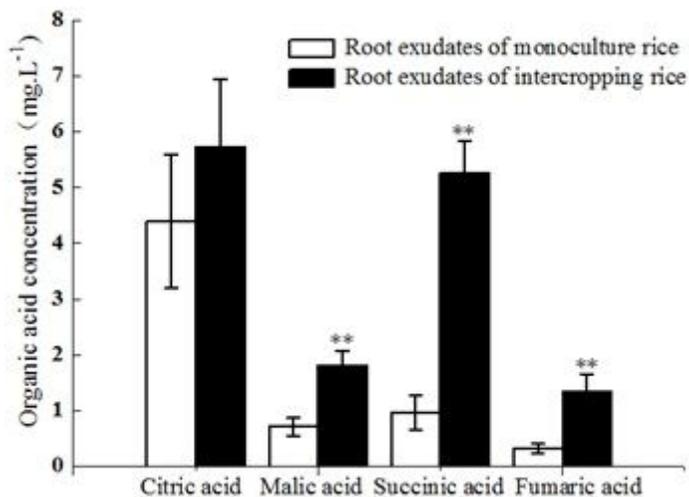
**Figure 3**

Effects of different root barriers between rice and water spinach on Si concentration in rice leaves (a) and stems (b) in Greenhouse Study 2. Three rows of rice were 20 cm, 40 cm, and 60 cm away from water spinach, respectively. Different capital letters indicate significant difference among different distances under the same root barrier ( $p < 0.05$ ). Different small letters indicate significant difference among different root barriers under the same distance ( $p < 0.05$ )



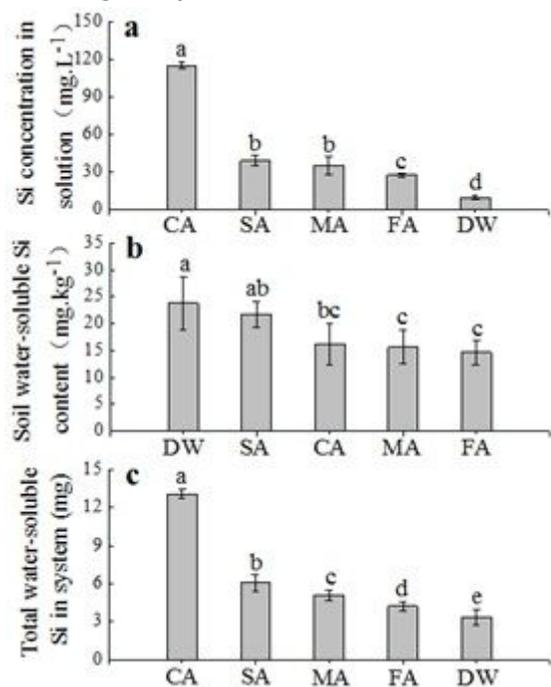
**Figure 4**

Relative expression of Si transporter genes in monoculture rice and intercropping rice in Greenhouse Study 3 (a *OsLsi1* in roots; b *OsLsi2* in roots; c *OsLsi6* in stems; d *OsLsi6* in leaves). Independent T-test was used to determine the significant differences at a 5% (\*) level



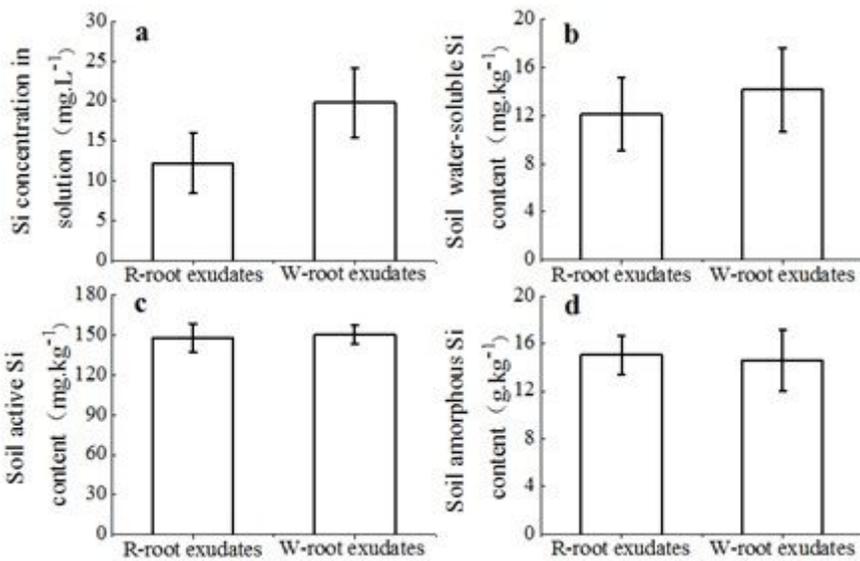
**Figure 5**

Organic acids concentration in root exudates of monoculture rice and intercropping rice in Greenhouse Study 4. Independent T-test was used to determine the significant differences at a 5% (\*) or 1% (\*\*) level, with eight replicates



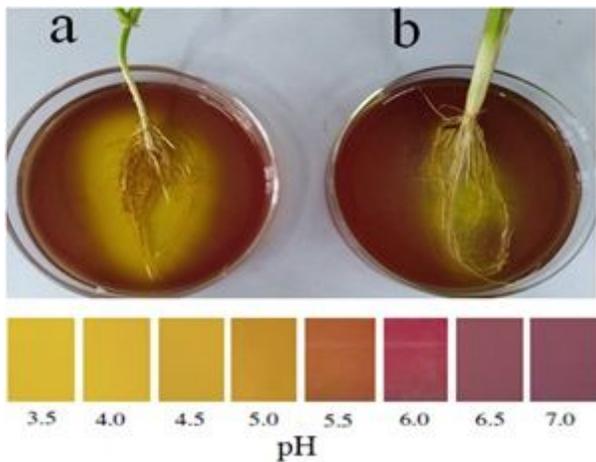
**Figure 6**

Effects of 0.025 mol.L<sup>-1</sup> organic acids solution leaching paddy soil on water-soluble Si in the soil-water system in Incubation Study 1 (a Si in solution; b soil water-soluble Si; c total water-soluble Si). Distilled water (DW) was used as a control. CA, citric acid; SA, succinic acid; MA, malic acid; FA, fumaric acid. Different letters indicate significant differences among different treatments (p<0.05)



**Figure 7**

Effects of root exudates leaching paddy soil on various types of Si in Greenhouse Study 5 (a Si in solution; b soil water-soluble Si; c soil active Si; d soil amorphous Si). R-root exudates, rice root exudates. W-root exudates, water spinach root exudates. All parameters between treatments have no significant difference at a 5% level



**Figure 8**

Visualization of rhizosphere acidification of water spinach seedling (a) and rice seedling (b) in Incubation Study 2. The roots were imbedded in agar gel containing bromocresol purple indicator for 6 h. Yellow means strong acidification, and purple means weak acidification

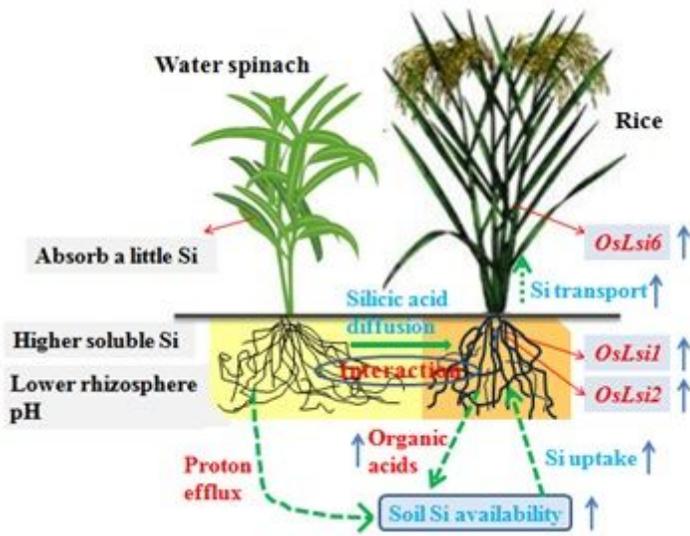


Figure 9

Rice-water spinach intercropping enhances Si absorption of rice through several beneficial ways. On the one hand, silicic acid around water spinach rhizosphere diffuses to rice rhizosphere; on the other hand, rhizospheric interaction induces the up-regulated expression of Si transporter genes in rice and stimulates rice roots to secrete more organic acids to increase the soil Si availability. Moreover, water spinach root exudates help the mobilization of soil Si through the proton efflux of roots

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryInformationforPLSOD2002105.docx](#)