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Rice intercropping with alligator flag (*Thalia dealbata*): A novel model to produce safe cereal grains while remediating cadmium contaminated paddy soil

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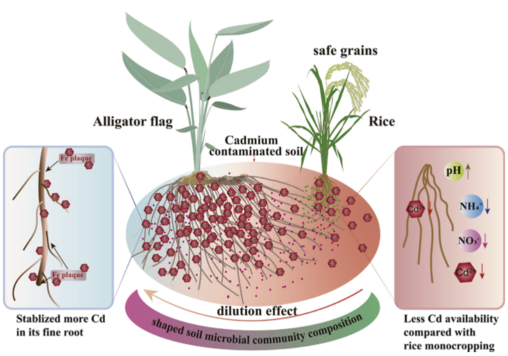
GRAPHICAL ABSTRACT



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ABSTRACT

Phytoremediation has been employed as a cost-effective technique to remove the cadmium (Cd) from soil and water in several ecosystems. However, little is known about whether intercropping the remediating plants with rice (*Oryza sativa*) crop could reduce Cd accumulation in rice grains. We conducted greenhouse pot and concrete pond trials to explore the effects of intercropping alligator flag (*Thalia dealbata*, Marantaceae) on soil Cd remediation, paddy soil and microbial properties, and rice production. Our results suggest that intercropping with alligator flag significantly decreased Cd absorption, transportation, and accumulation from the soil to the rice grains (under 0.2 mg kg^{-1} at a soil Cd content below 2.50 mg kg^{-1}). This decrease was due to the lowered Cd availability and higher soil pH in the rice-alligator flag intercropping system. Although planting alligator flag resulted in the reduction of soil NH_4-N and NO_3-N, Cd content in the rhizosphere was the main factor restricting microbial biomass, species, and community composition. Alligator flag could tolerate higher Cd contamination,

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and accumulate and stabilize more Cd in its tissues than rice. Our study suggests that alligator flag intercropped with rice has potential as a phytostabilization plant to produce rice safely for human consumption in moderately Cd-contaminated soils.

1. Introduction

Heavy metal pollution has become increasingly severe over the past decades. In particular, cadmium (Cd) is one of the most toxic heavy metals to human and ecosystem health. More than 10 million hectares of agricultural land has been polluted with heavy metals, such as Cd, arsenic (As), and lead (Pb), and this figure is increasing annually in China (Lei et al., 2011). This heavy metal pollution occurs mainly due to wastewater irrigation, pesticide application (Sungur et al., 2015), the use of rock phosphate fertilizers, and vehicular and industrial activities (Yu et al., 2006; Rao et al., 2010; Antoniadis et al., 2017; Rizwan et al., 2017). In these contaminated soils, Cd ions of an exchangeable/acid-soluble fraction (Cd^{2+}) are readily absorbed by crops such as cereals, potatoes, vegetables, and fruits (Smolders, 2001; Ingwersen and Streck, 2005). Cd can accumulate throughout the food chain in offal, organs, equine products, shellfish, crustaceans, cocoa, mushrooms, and even some seeds (Smolders, 2001). As it accumulates, Cd poses a significant threat to human health. The long-term intake of Cd can lead to serious health conditions, including renal damage, itai-itai disease, osteoporosis, chondropathy, cancer, and myocardial infarction (Järup and Åkesson, 2009). Cd contamination in the food chain starts with the soil-to-plant transfer of Cd. Thus, Cd absorption by crops could be reduced by lowering the bioavailability of Cd in contaminated soils.

The major methods currently used to lower the bioavailability of Cd are isolation, removal, and stabilization (Martin and Ruby, 2004). Isolation technologies reduce contaminant availability by decreasing the exposed surface area, the contaminant solubility, and/or soil permeability. Removal technologies eliminate metals from contaminated soils. Stabilization technologies reduce the leachability and/or bioavailability of metals in the contaminated soils. More detailed sub-classifications are also reported, including phytoremediation, stabilization and solidification, chemical elution, field management, and combined remediation (Martin and Ruby, 2004; Tang et al., 2016). Phytoremediation, in particular, has been proposed as a cost-efficient method for removing or diluting Cd in the soil (Tang et al., 2016). However, little is known about whether planting the remediating plants alongside rice in paddy fields would reduce the transfer of Cd from the soil to rice grains.

Heavy metal stresses result in oxidative damage to plants by triggering increased production of reactive oxygen species (ROS). Plants possess a series of defensive mechanisms that could protect them from oxidative damage by controlling ROS levels and effects. They regenerate the active form of antioxidants (*i.e.*, enzymatic antioxidants, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT)), and malondialdehyde (MDA) to eradicate or diminish the damage induced by ROS (Alscher et al., 1997). The stable end products of ROS are employed to monitor oxidative stress, because the half-lives of ROS are extremely short. Cd impairs the redox homeostasis of cells and exacerbates the production of ROS, which results in lipid peroxidation, membrane impairment, and enzyme inactivation. This damage eventually affects cell capability (Gill and Tuteja, 2010).

Cd accumulates in the topsoil of agricultural land that has been subjected to sewage sludge application, and contents can be especially high (100–6,000 mg kg^{-1} dry weight) in mining areas (Pereira et al., 2002). Agricultural productivity is limited by toxicity and pollution of heavy metals, especially when the watershed is subjected to irrigation or by discharge of metal-enriched mine drainage (Johnson and Hallberg, 2005). Macrophytes, which possess large biomass both above- and below-ground, can be used to remediate pollutants in constructed wetlands and to produce biomass for bioenergy. However, little to no effort has been made to test whether macrophytes could be intercropped with agricultural crops, especially in paddy fields.

Rice (*Oryza sativa* L.) grows on 24 % of all agricultural land in China and accounts for about 40 % of the overall yield, indicating its significance and popularity as a staple crop in China (Fang et al., 2014). However, its production has been threatened by heavy metal pollution, especially of Cd. To alleviate this pollution, *in-situ* phytoremediation methods are frequently used to lower both the Cd content in rice grains and Cd availability in soils. Alligator flag (*Thalia dealbata*, Marantaceae) is an aquatic plant native to swamps, ponds, and other wetlands in the southern and central United States (Li et al., 2015). Alligator flag has a well-developed fine root system and has been used as a raw material for biochar that can be applied to absorb and remediate heavy metals in soils and wastewater in constructed wetlands (Sohsalam and Sirianuntapiboon, 2008; Cui et al., 2016). However, it is not known whether alligator flag could be intercropped with rice in Cd-contaminated soil to reduce Cd accumulation in rice grains, ultimately to meet the food safety standard of Cd < 0.20 mg kg^{-1} (MHPRC, 2005).

In this study, alligator flag was intercropped with rice in greenhouse pots and concrete ponds in simulated Cd-contaminated soils. Our objectives were to determine: (1) the effects of cropping alligator flag with rice on Cd absorption, transportation, and accumulation from soil to rice grains; (2) the effects of intercropping alligator flag with rice on paddy soil and microbial properties; (3) whether alligator flag has higher tolerance and accumulation of Cd than rice; and (4) to explore the potential mechanisms and develop a sustainable, productive method for rice culture with grains meeting food safety standards in Cd-contaminated soils.

2. Materials and methods

2.1. Paddy soil and experimental plants

Soil was collected from the top layer (0–20 cm) of agricultural land (23°14′22″N, 113°37′57″E) in Guangzhou, Guangdong province, China. The soil had a pH of 6.01, total organic matter content of 27.36 g kg^{-1}, total nitrogen content of 2.14 g kg^{-1}, and total Cd of 0.60–1.50 mg kg^{-1}. According to the USDA textural soil classification, the soil was classified as sandy clay, consisting of medium (36 %) and fine (24 %) sand, silt (5 %), and clay (35 %).

Rice seeds were provided by Guangdong Academy of Agricultural Sciences (Guangzhou, China). Alligator flag seedlings were bought from a garden market in Guangzhou. The rice seeds were sterilized with 2.63 % NaOCl solution for 30 min and rinsed five times with deionized (DI) water. Sterilized rice seeds were soaked in DI water for 24 h at room temperature (23 ~ 25℃), placed in a seed breeding tray (YB-W104, Xian Yubo New Materials Technology Company, Shaanxi, China) padded with moist acid washed sand, and placed in a thermostatic incubator (RXZ-500D, Ningbo Southeast Instrument Company, Zhejiang, China) with a 15 h light period for 12 days at 30℃ under the humidity of 70 % (Farooq et al., 2006). Alligator flag was planted in DI water for one week to dilute and/or remove the potential heavy metals on the root surface. It should be noted that no apparent damaged symptoms on the alligator flag plants was observed during the DI water incubation period, though there was no nutrient element amended.

2.2. Greenhouse experiment

A greenhouse pot experiment was designed to determine the capability of alligator flag to reduce Cd accumulation in rice grains (Fig. 1a). The experiment was conducted in the South China

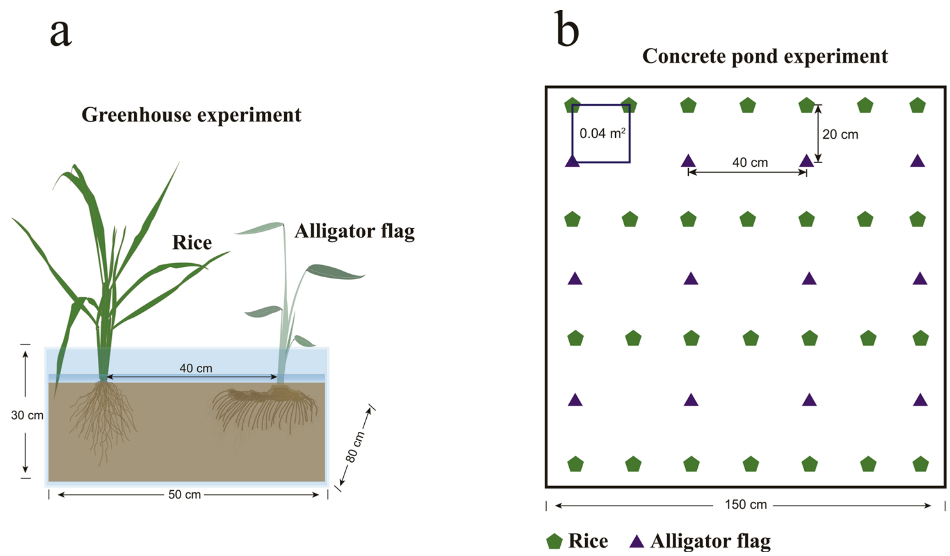

Fig. 1. Schematic diagram of the greenhouse (a) and concrete pond (b) experimental designs.

Agricultural University campus (23°09′30″N, 113°21′29″E). Thirty kg of soil was transferred to each pot (80 cm × 50 cm × 30 cm) and flooded with DI water. Different concentrations of $CdCl_2$ (purity N99 %) solution were added to the pot soils to mimic Cd-contaminated paddy soils. This process resulted in initial soil Cd-contaminated levels of 2.5, 5.0, and 10.0 mg kg^{-1} (the average background soil Cd content was 1.1 mg kg^{-1}). Control (CK) pots just had background soil without any addition of Cd. The pots were then incubated for four months. After incubation, soils were collected from each pot to determine the final Cd concentration. Three treatment systems were set up with five replicates each: (i) rice monoculture (RM, six rice plants per pot) with column and row spacing of 20 cm; (ii) alligator flag monoculture (TM, four alligator flag plants per pot) with column and row spacing of 40 cm; and (iii) rice and alligator flag intercropping (RT, three rice and two alligator flag plants per pot) with column spacing of 40 cm and row spacing of 20 cm for rice and 40 cm for alligator flag (Fig. 1). During the growing period, two PVC pipes with Cd-free (diameter = 6.0 mm) were used to collect non-rhizosphere water and soil samples. Carbamide ($CO(NH_2)_2$) was utilized at the regular levels and timings at each pot to support the normal growth of rice.

2.3. Concrete pond experiment

The concrete pond experiment was designed to further explore the potential mechanisms of Cd reduction in rice grain, plant antioxidant enzyme activity, tissue Cd accumulation, and soil Cd content depletion. A concrete pond system was constructed and partitioned into 16 ponds of dimension 1.5 m × 1.5 m × 0.9 m (Fig. 1b). Nine hundred kg of soil was placed in each pond and flooded with tap water (Cd content < 0.005 mg L^{-1}) using an automatic irrigation system. After 15 days, we created a simulated Cd-contaminated soil by adding $CdCl_2$ (purity N99 %) solution into each pond to obtain initial Cd concentrations of 30, 50, and 120 mg kg^{-1} (Sidhu et al., 2017). Three field alligator flag and rice intercropping plots without any addition of Cd in Guangzhou, where the soil was collected, were set as the control (CK). The simulated Cd-contaminated soil in the concrete ponds was incubated with flooded water for 120 days (from March 10, 2018 to July 8, 2018). After incubation, the same three treatment systems as above (RM with the column spacing of 20 cm and the row spacing of 20 cm, TM with the column spacing of 40 cm and the row spacing of 40 cm, and RT with row spacing of 20 cm for rice and of 40 cm for alligator flag, and the column spacing of 20 cm) were established, with three replicates for each treatment × soil combination. For the RM and TM treatments, the initial soil Cd concentration of 50 mg kg^{-1} was used; for the RT treatment, the initial soil Cd concentrations of 30, 50, and 120 mg kg^{-1} were used. To ensure growth of the rice, a shallow water level of 2–3 cm was maintained until the late tillering stage. From the late tillering stage to the early heading stage, the water level of the field was lowered to ensure that the rice root could penetrate deeply into the soil substrate. During the entire heading period, a 3–5 cm water level was maintained to control weeds. To promote rice survival and yield, the pond soil was kept moist throughout the entire growing season till maturity. The management of fertilization was conducted as that in greenhouse pot trials. Note that the initial soil Cd levels did not include the background soil Cd content (about 1.1 mg kg^{-1}), meaning that the actually initial soil Cd contents were slightly higher.

2.4. Sampling procedures

Two weeks after planting, fresh leaves from both rice and alligator flag plants were sampled from the concrete ponds, washed with tap water and DI water, blotted dry using filter papers, frozen in liquid nitrogen, and stored at −80°C to measure antioxidant enzyme activity. During the growing period, a rice and alligator flag plant were carefully uprooted along with the soil from each treatment, placed in sterile polyethylene bags and transferred to the laboratory for separation of non-rhizosphere and rhizosphere soil. The non-rhizosphere soils were removed by energetically shaking the uprooted rice and alligator flag plants, leaving behind the rhizosphere soils strongly adhering to the roots (Ramakrishna and Sethunathan, 1982; Huang et al., 2016). At maturity, an alligator flag and rice plant including rice grains were removed from the ground from each treatment with the soil intact in their root systems. The plants were washed with tap water and then DI water for five times to remove the residual Cd, and separated into shoot, rhizome (for alligator flag), and fine root. It should be noted that the Cd content in the fine roots might include Cd concentrated in the interior and bound on the surface of the plants' fine roots, because even though the fine roots have been washed with tap water and DI water several times there is still the possibility that the Cd may have combined tightly on the surface of the plants' fine roots (such as plaque fraction) (Zimmer et al., 2011). This tightly combined Cd was considered as the fine root adsorbed or stabilized fraction in order to evaluate the potential of the plants for Cd stabilization. Separated plant tissues samples were oven-dried at 65 °C to constant weight, and smashed to pass through a 0.15 mm mesh before Cd concentration was measured. The plant biomass was collected within a quadrat (0.04 m^2)

between two plants for all three systems in the concrete ponds (Fig. 1b). This sampling approach allowed easy comparison of plant biomass production among the three systems. At the end of the experiment, bulk and rhizosphere soils from the rice and alligator flag were collected using stainless augers and analyzed to determine the plants' ability to remove Cd from the soil.

2.5. Sequential extraction procedure and determination of Cd content

Soil Cd fractions were defined and extracted based on previous methods (Tessier et al., 1979). Briefly, the exchangeable Cd fraction (F1) of the rhizosphere soil was extracted using magnesium chloride solution (1 M $MgCl_2$, pH 7.0) with continuous agitation. The fraction bound to carbonates (F2), the residue from F1, was leached with 1 M NaOAc adjusted to pH 5.0 with acetic acid (HOAc). The fraction bound to iron-manganese oxides (F3), the residues from F2, was extracted with 0.1 M $NH_2OH-HCl$. The fraction bound to organic matter (F4), the residues from F3, was extracted with 30 % H_2O_2 (pH = 2, adjusted with HNO_3) plus 3.2 M NH_4Ac. Finally, the Cd content in the residual soil from F4 was considered as the residual fraction (F5). The dried residual soil from F4 and unextracted soil and plant samples were digested following a microwave-acid method (US EPA method 3052) in a mixture of acid solution consisting of HNO_3 65 %/HF 40 %/HCl 37 %, 9:3:2 (v/v). More specifically, the 0.1 g soil and 0.5 g plant samples were digested in 9 mL of concentrated nitric acid, 3 mL of hydrofluoric acid and 2 mL of hydrochloric acid for 10 min using microwave heating (Multiwave Pro, Anton Paar, Austrian). Properly inert polymeric microwave vessels (Rotor 24HVT50) were used to hold the samples and acids. Sealed vessels were heated in the microwave system to 120 °C for 10 min, maintained for 10 min, and then heated to 180 °C for another 10 min. After maintaining for 40 min at 180 °C, the reactions were completed and the microwave was cooled to 60 °C. After cooling, the samples in the vessel were filtered, centrifuged, decanted, and diluted to 50 mL, and analyzed by flame atomic absorption spectrometry (FLAA) for Cd content. Total Cd content in unextracted soil was considered as the soil total Cd content for which to calculate the recovery ((fractions sum/total Cd) × 100 %). The recovery of Cd was 88 ~ 104 %, which indicates satisfactory quality control (Yu et al., 2016).

2.6. Soil properties analyses and extracellular enzyme assay

Soil pH was measured using a handheld multifunctional pH meter (SanXin721, San Xin, China) with a 1:2.5 soil:water ratio. Total phosphorus (TP) was determined after digestion with perchloric acid using the method described in Turrion et al. (2010). Ammonia (NH_4-N) and nitrate (NO_3-N) were determined with direct colorimetric measurement after extracted with potassium chloride (Dorich and Nelson, 1983). Four enzymes were assayed following the fluorometry method described by Bell et al. (2013). Specifically, β-1,4-glucosidase (BG), β-D-cellobiosidase (cellulose degradation; CB), β-1,4-*N*-acetylglucosaminidase (NAG), and acid phosphatase (ACP) related to carbon, phosphorus and nitrogen cycling were determined.

2.7. Enzyme extraction and assay

Frozen plant tissues were crushed to a fine ash in liquid nitrogen with a pestle and mortar and extracted at a 1:3 ratio (w/v) of fresh weight to extraction buffer (100 mM potassium phosphate buffer [pH 7.5] containing 1 mM EDTA, 3 mM DTT, and 5% [w/v] insoluble PVPP). The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was stored in separate aliquots at −80 °C for later SOD, CAT, and POD analyses. All enzyme extraction and determination were carried out at 4 °C.

The SOD and CAT activity was assayed following the methods described by Azevedo et al. (1998) and modified by Pereira et al. (2002). The POD activity was assayed as described by Ullah et al. (2017). MDA was determined as described by Draper and Hadley (1990), with minor modifications, for plants under a Cd-contaminated system. Fresh leaves (0.5 g) were crushed and mixed thoroughly with 5 mL of 5% v/v trichloroacetic acid (TCA) using a mortar and pestle and centrifuged at 10,000 × g for 10 min. Two mL of a 0.67 % w/v TBA in 10 % v/v TCA solution was added to the 2 mL supernatant in a polypropylene (PP) tube. The tube was placed in boiling water for 30 min and then rapidly cooled on ice. After centrifuged at 10,000 × g for 5 min at 4 ℃, the absorbance of the supernatant was determined at 450, 532, and 600 nm, and the following formula: MDA (μmol/g fresh weight (FW)) = 6.45 (OD532–OD600) − 0.56 OD450 (OD, Optical Density) was applied to calculate the MDA concentration. The lipid peroxidation level was expressed in micromoles of MDA formed per g of leaf tissue (Ullah et al., 2017; Guo et al., 2019).

2.8. Phospholipid fatty acid (PLFA) analysis

PLFA analysis and the calculation of microbial species were conducted following the method described by Bååth and Anderson (2003) and Wei et al. (2017). Potassium phosphate, chloroform, and methanol buffers were used to extract total lipids from the freeze-dried soil samples (8 g) that were sieved (< 2 mm) and completely mixed. A Silica Column (500 mg, Sigma, Germany) was used to fractionate phospholipids from neutral fatty acids and glycolipids. Fractionated samples were dissolved in hexane, and a 7890-gas chromatography outfitted with a Sherlock Microbial Identification System (V. 6.2, MIDI Inc., Newark, DE, USA) was used to identify the phospholipids.

To determine and calculate the content (nmol g^{-1} dry soil) of individual PLFA, the fatty acid 19:00 was added to the fractionated samples before assay as the internal standard. The sum of the total amount of the fatty acids was calculated to identify microbial biomass. The fatty acids contain -anteiso or -iso were summed as the amount of Gram-positive (G+) bacteria; mono-unsaturated, hydroxyl, and cyclopropane fatty acids were summed as Gram-negative (G–) bacteria; compounds with $-COOH$ with $-CH_3$ on the tenth C were counted as Actinomycetes (A); 18:2ω6c and 18:1ω9c were calculated as fungi (F); the sum of G + and G– was calculated as bacteria (B).

2.9. Data analysis

Statistical analysis was conducted with SPSS 25.0 software (IBM Corp., New York, USA). One-way and three-way ANOVA followed by Duncan multiple comparison tests, and paired-sample t-tests were used to compare treatments (α = 0.05). The results are expressed as the mean value ± SE (standard error) of the three replicates. Following Hoffman et al. (2002) and Fernandes et al. (2007), the bioaccumulation factors (BAFs) were calculated as Eq. (1):

$$BAF = \frac{\text{metal content (mg kg}^{-1}\text{ dry weight) in plant tissue}}{\text{metal content (mg kg}^{-1}\text{ dry weight) in soil}} \quad (1)$$

Translocation factors (TFs) were computed as Eq. (2) with the method described by (Ho et al., 2008).

$$TF = \frac{\text{metal content (mg kg}^{-1}\text{ dry weight) in plant aerial part}}{\text{metal content (mg kg}^{-1}\text{ dry weight) in plant root}} \quad (2)$$

The plant tissues include shoots, fine roots, and rhizomes (for alligator flag) and the plant tissue biomass was measured as the total dry weight. The Cd mass was calculated by multiplying the plant tissue Cd content with plant tissue biomass.

3. Results

3.1. Cd content in rice plants and soils in the greenhouse experiment

The Cd content in the rice grains increased significantly for both the

Table 1
Cd contents in plant and soil, and pH in intercropping and monocropping systems under a gradient of Cd contamination in greenhouse pot trials.

Crop species	Cd gradient	Bulk soil Cd		Rhizosphere soil Cd		Shoot Cd		Fine root Cd		Grain or Rhizome Cd		Soil pH	
		Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping
Rice	CK	1.11 ± 0.07d	0.85 ± 0.05d	0.87 ± 0.08d	0.98 ± 0.01d	0.33 ± 0.09b	0.58 ± 0.09d	0.54 ± 0.18c	2.61 ± 0.25d	0.15 ± 0.02b	0.17 ± 0.02c	6.30 ± 0.03a	6.14 ± 0.03a
	Cd2.5	2.98 ± 0.08c	3.25 ± 0.14c	1.78 ± 0.05c	2.14 ± 0.08c	1.14 ± 0.56b	1.82 ± 0.24c	1.40 ± 0.53bc	6.46 ± 0.13c	0.18 ± 0.01b	0.49 ± 0.02b	6.12 ± 0.06b	5.89 ± 0.02b
	Cd5.0	5.26 ± 0.09b	5.30 ± 0.14b	3.80 ± 0.12b	4.46 ± 0.19b	1.18 ± 0.12b	2.73 ± 0.08b	1.56 ± 0.09b	10.68 ± 0.38b	0.24 ± 0.10b	0.70 ± 0.07ab	6.05 ± 0.01b	5.7 ± 0.03c
	Cd10.0	9.88 ± 0.07a	10.2 ± 0.17a	7.73 ± 0.11a	9.35 ± 0.23a	2.17 ± 0.07a	4.36 ± 0.13a	2.76 ± 0.09a	16.84 ± 0.51a	0.65 ± 0.14a	0.82 ± 0.10a	5.92 ± 0.03c	5.21 ± 0.03d
Alligator flag	CK	1.07 ± 0.02d	0.78 ± 0.02d	0.74 ± 0.05d	0.68 ± 0.04d	0.23 ± 0.01c	0.39 ± 0.03c	1.78 ± 0.21d	5.39 ± 0.35d	0.25 ± 0.05c	0.33 ± 0.09c	6.38 ± 0.02a	6.34 ± 0.01a
	Cd2.5	2.79 ± 0.10c	2.24 ± 0.06c	1.40 ± 0.03c	1.02 ± 0.03c	0.49 ± 0.06b	0.61 ± 0.00bc	7.01 ± 0.97c	16.19 ± 1.07c	0.29 ± 0.04c	0.27 ± 0.03c	6.29 ± 0.04a	6.31 ± 0.02ab
	Cd5.0	5.15 ± 0.07b	5.00 ± 0.01b	3.33 ± 0.09b	3.28 ± 0.09b	0.59 ± 0.03b	0.75 ± 0.10b	11.18 ± 0.61b	24.85 ± 1.53b	1.31 ± 0.14b	1.32 ± 0.07b	6.14 ± 0.07b	6.36 ± 0.02b
	Cd10.0	9.53 ± 0.07a	8.30 ± 0.20a	7.45 ± 0.29a	7.33 ± 0.16a	1.25 ± 0.14a	1.44 ± 0.11a	13.79 ± 0.76a	33.15 ± 1.73a	2.41 ± 0.22a	2.44 ± 0.13a	5.89 ± 0.01c	5.94 ± 0.01c

CK indicates the control, Cd2.5 indicates the initial soil Cd concentration of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd concentration of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd concentration of 10.0 mg kg^{-1}. Different lowercase letters in each column indicate the significance among different Cd-contaminated levels (ANOVA, Duncan's multiple range test).

rice and alligator flag intercropping (RT) (ANOVA, $F_{3,11} = 13.87$, $p = 0.002$) and rice monoculture (RM) (ANOVA, $F_{3,11} = 20.18$, $p < 0.001$) systems at higher initial levels of soil Cd (Table 1). However, the Cd content was significantly lower in the rice grains from the RT system than from the RM system at the initial soil Cd levels of 2.5 mg kg^{-1} (paired-sample t-test, t = –7.38, df = 5, $p < 0.001$) and 5.0 mg kg^{-1} (paired-sample t-test, t = –7.77, df = 5, $p < 0.001$). There was no significant difference between the control (CK) and initial soil Cd level of 10.0 mg kg^{-1} treatment. The average Cd content in rice grains from the RT systems was 0.18 mg kg^{-1} in the 2.5 mg kg^{-1} soil Cd treatment, and 0.24 mg kg^{-1} in the 5.0 mg kg^{-1} soil Cd treatment, equivalent to a 63 % and 66 % reduction of Cd in the rice grains in the RT system compared to the RM system.

To estimate the effects of intercropping alligator flag on Cd accumulation in rice, we measured the Cd content in the rice tissues (shoots and fine roots) and soils (bulk and plant rhizosphere soils). In general, the Cd content was lower in the rhizosphere soil than in the bulk soil for all three systems (RM, TM, and RT), and the rice tissue Cd content was lower in the intercropping system than in the monocropping systems (Table 1). Specifically, the Cd content of rice fine roots in the RT system was only 14–20 % of that in the RM system (Table 1). In addition, compared with rice, alligator flag accumulated significantly higher Cd in its fine roots in both the intercropping and monocropping systems across all initial soil Cd levels (Table 1).

The bioaccumulation (BAF) and translocation (TF) factors varied significantly among rice and alligator flag treatments (Table 2). BAFs of rice shoots were significantly lower in the RT than in the RM system at initial soil Cd levels of 5.0 mg kg^{-1} (paired-sample t-test, t = –4.25, df = 5, $p = 0.004$) and 10.0 mg kg^{-1} treatments (paired-sample t-test, t = –3.98, df = 5, $p = 0.003$), while BAFs of rice fine roots were significantly lower in the RT system than in the RM system at all initial soil Cd levels (paired-sample t-test, all $p < 0.01$) (Table 2). It was also significant that BAFs of alligator flag fine roots were higher than those of rice in both the RT system and the monocropping (RM and TM) systems (paired-sample t-test, all $p < 0.01$) (Table 2). In general, the TFs of rice were significantly higher than those of alligator flag both in RT (paired-sample t-test, all $p < 0.05$) and monocropping systems (paired-sample t-test, all $p < 0.01$).

The Cd fraction in plant rhizosphere soils varied significantly between rice and alligator flag across different initial soil Cd levels (Table 3 and, Fig. 2). Generally, although the absolute amount of extractable Cd was increased significantly with the elevation of initial soil Cd levels because of the addition of $CdCl_2$, the relative proportion of the non-extractable Cd fraction was significantly higher increased in the intercropping systems (Fig. 2). Specifically, the net Cd content of each fraction significantly increased with the elevation of initial soil Cd levels (Table 3). The relative proportion of the extractable fractions of Cd (such as F1-F4) was highly increased, but that of the non-extractable fraction of Cd such as F5 was lower, decreased with the elevation of initial soil Cd levels (Fig. 2). It was obvious that the relative proportion of the extractable fractions of Cd in the intercropping systems was less than that in either the rice or alligator flag monocropping systems. In contrast, the relative proportion of the non-extractable fraction of Cd in the intercropping systems was significantly higher than that in the monocropping systems (Fig. 2).

3.2. Cd content in plants and soils in the concrete pond trials

The Cd content in the plant tissues increased at higher levels of initial soil Cd in the concrete ponds (Table 4). For example, the Cd content was 2.76 times greater in the alligator flag fine roots, 1.68 times greater in the rice fine roots, and 1.49 times greater in the alligator flag shoots at 120 mg kg^{-1} initial soil Cd compared to 30 mg kg^{-1} initial soil Cd treatments. Significant differences were found in the shoots of both plants at the initial soil Cd level of 50 mg kg^{-1} (paired-sample t-test, t = –3.14, df = 5, $p < 0.05$): the shoot Cd content was 2.57 times greater

Table 2
Bioaccumulation factor (BAF) and translocation factor (TF) of rice and alligator flag in intercropping and monocropping systems under a gradient of Cd contamination in greenhouse pot trials.

Crop species	Cd gradient	BAF						TF	
		Shoot		Fine root		Grain or Rhizome			
		Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping
Rice	CK	0.40 ± 0.12a	0.59 ± 0.10b	0.67 ± 0.24a	2.65 ± 0.24ab	0.16 ± 0.01a	0.16 ± 0.01b	1.07 ± 0.31a	0.29 ± 0.04a
	Cd2.5	0.66 ± 0.34a	0.84 ± 0.08a	0.80 ± 0.33a	3.03 ± 0.18a	0.10 ± 0.00b	0.23 ± 0.02a	0.91 ± 0.05a	0.36 ± 0.04a
	Cd5.0	0.31 ± 0.03a	0.62 ± 0.04b	0.41 ± 0.03a	2.40 ± 0.15b	0.06 ± 0.03b	0.16 ± 0.01b	0.90 ± 0.09a	0.32 ± 0.01a
	Cd10.0	0.28 ± 0.00a	0.47 ± 0.01b	0.55 ± 0.01a	1.80 ± 0.08c	0.10 ± 0.02b	0.09 ± 0.01c	0.81 ± 0.05a	0.31 ± 0.01a
Alligator flag	CK	0.32 ± 0.03a	0.58 ± 0.08a	2.40 ± 0.16bc	7.93 ± 0.68b	0.34 ± 0.05ab	0.48 ± 0.12a	0.12 ± 0.02a	0.07 ± 0.01a
	Cd2.5	0.35 ± 0.05a	0.60 ± 0.02a	5.04 ± 0.79a	15.98 ± 1.31a	0.21 ± 0.03b	0.26 ± 0.03a	0.07 ± 0.00b	0.04 ± 0.00b
	Cd5.0	0.18 ± 0.01b	0.23 ± 0.04b	3.37 ± 0.22b	7.61 ± 0.66b	0.40 ± 0.05a	0.40 ± 0.02a	0.05 ± 0.00b	0.03 ± 0.00b
	Cd10.0	0.17 ± 0.01b	0.20 ± 0.01b	1.86 ± 0.15c	4.52 ± 0.14c	0.32 ± 0.02ab	0.33 ± 0.02a	0.08 ± 0.01b	0.04 ± 0.00b

CK indicates the control, Cd2.5 indicates the initial soil Cd content of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd content of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd content of 10.0 mg kg^{-1}. Different lowercase letters in each column indicate the significance among different Cd-contaminated levels (ANOVA, Duncan's multiple range test).

in rice than in alligator flag. At the initial soil Cd level of 120 mg kg^{-1}, the Cd content of the alligator flag fine roots was 69 % greater than that in the rice fine root (paired-sample t-test, t = –8.24, df = 5, $p <$ 0.001).

The fine root biomass of alligator flag was significantly larger than that of rice across all initial soil Cd levels (paired-sample t-test, all $p <$ 0.05, Table 4). Specifically, the fine root biomass of alligator flag was 3.33 times greater than that of rice at 30 mg kg^{-1} Cd, 0.91 times greater at 50 mg kg^{-1} Cd, and 1.25 times greater at 120 mg kg^{-1} Cd. The plant biomass of alligator flag decreased at with the elevation of the initial levels of soil Cd. Specifically, the alligator flag shoot biomass decreased by 41 % from 120 mg kg^{-1} soil Cd compared to 30 mg kg^{-1} soil Cd treatment (Table 4). In contrast, the plant biomass of rice first decreased by 24 % and then increased by 43 % at increasing levels of initial soil Cd (Table 4).

The Cd mass was used to evaluate the ability of each plant to remove Cd from the contaminated soils after the growing season. The Cd mass in plant tissue improved with the elevation of initial soil Cd levels (Table 4). For example, the rice shoot Cd mass increased 2.47 times from the initial soil Cd level of 30 mg kg^{-1} to that of 120 mg kg^{-1} treatment. Also, the fine root Cd mass increased 2.92 times in rice and 1.41 times in alligator flag from the initial soil Cd level of 30–120 mg kg^{-1}. Accumulation of Cd mass was greater in the rice shoots and the alligator flag fine roots, while the alligator flag fine roots accumulated more Cd than the rice shoots. For instance, the Cd content in alligator flag fine roots was 0.75 times more than that in rice shoots (Table 4).

The alligator flag monocropping system had the highest total biomass, amount of Cd uptake, and percentage of Cd reduction from the soil (Table 5). The total biomass of alligator flag in the monocropping system was 2.02 times more than that in the rice monocropping system and 2.22 times more than that in the intercropping systems. The amount of Cd uptake in the alligator flag monocropping system was 1.46 times more than that in the rice monocropping system and 4.37 times more than that in the intercropping systems. Cd was reduced by 1.25 % in alligator flag monocropping, 0.51 % in rice monocropping, and 0.23 % in the intercropping system.

3.3. Soil properties and microbial species in different cropping systems under a gradient of Cd contamination

Significant variation in Cd content of bulk soil was found in all treatment systems except for the interaction of plant species and Cd gradient (all $p <$ 0.05) (Table 6). Soil nutrients, such as NH_4-N, NO_3-N, and extracellular enzyme activities varied significantly among different cropping patterns, and lower soil nutrients and extracellular enzyme activities were found in the intercropping system (all $p <$ 0.05), while no significant difference was found between species (all $p >$ 0.05) (Table 6).

Soil microbial biomass and microbial species varied significantly under the interaction of cropping pattern and Cd gradient (Table 7). The highest amount of soil microbial biomass (296.18 ± 17.82 nmol g^{-1} dry soil) was found in the rice monocropping system under the control Cd level, while the lowest amount of microbial biomass (173.20 ± 19.92 nmol g^{-1} dry soil) was found in rice monocropping systems under the initial soil Cd level of 10.0 mg kg^{-1} (Table 7). Soil microbial biomass increased with higher initial soil Cd levels in the intercropping system and the alligator flag monocropping system under the interaction of cropping pattern and Cd gradient, but decreased in the rice monocropping system (Table 7). The microbial community composition indicated by the ratio of different microbial species varied significantly under the interaction of cropping pattern and Cd gradient (all $p <$ 0.05) (Table 7).

3.4. Effect of cadmium stress on antioxidant enzyme activity

In this study, SOD, POD, CAT, and MDA were used to monitor the oxidative stress of rice and alligator flag induced by Cd pollution in the Cd-contaminated soil. Generally, the SOD activity decreased significantly in the two plants at higher initial soil Cd levels. The activity in the two plants also differed significantly (Fig. 3a). That is, the SOD activity in the rice declined by 79 % from the CK to the initial soil Cd level of 30 mg kg^{-1} treatment, then dropped by 46 % from 30 to 50 mg kg^{-1} Cd, and finally increased by 30 % from 50 to 120 mg kg^{-1} Cd treatment. For alligator flag, the SOD activity also declined from 27.73 U g^{-1} FW min^{-1} at CK to 11.19 U g^{-1} FW min^{-1} at the initial soil Cd level of 120 mg kg^{-1}, a decrease in SOD activity of 60 %. The SOD activity of the two species differed significantly (paired-sample t-test, 30 mg kg^{-1}: t = 3.46, df = 4, $p <$ 0.05; 50 mg kg^{-1}: t = 3.00, df = 4, $p =$ 0.04; 120 mg kg^{-1}: t = 3.40, df = 4, $p =$ 0.03) in all the initial soil Cd levels. For example, at the CK and the initial soil Cd level of 50 mg kg^{-1} treatment, the SOD activities were 40 % lower and 134 % greater in alligator flag than those in rice, respectively.

The POD activity was significantly higher in rice than in alligator flag under both the CK and Cd stress (toxicity) conditions (Fig. 3b). For rice, the POD activity increased 77 % from the CK to the initial soil Cd level of 50 mg kg^{-1} treatment, and then dropped by 16 % from the initial soil Cd level of 50–120 mg kg^{-1} treatments. In contrast, the POD activity in alligator flag dropped by 88 % from the CK to the initial soil Cd level of 50 mg kg^{-1} treatment, but then increased by 70 % from the

Table 3
Cd fractions in rhizosphere soil of rice and alligator flag cropping systems under a gradient of Cd contamination in greenhouse pot trials.

Crop species	Cd gradient	F1[a]		F2[b]		F3[c]		F4[d]		F5[e]		Cd_Total[f]	
		Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping	Intercropping	Monocropping
Rice	CK	0.05 ± 0.00d	0.06 ± 0.00d	0.09 ± 0.01c	0.12 ± 0.01c	0.17 ± 0.02c	0.17 ± 0.00c	0.25 ± 0.01d	0.32 ± 0.03c	0.38 ± 0.02d	0.23 ± 0.02d	0.87 ± 0.08d	0.98 ± 0.01d
	Cd2.5	0.29 ± 0.01c	0.37 ± 0.01c	0.19 ± 0.01c	0.28 ± 0.01c	0.33 ± 0.00bc	0.32 ± 0.02c	0.52 ± 0.02c	0.57 ± 0.02c	0.66 ± 0.04c	0.41 ± 0.02c	1.78 ± 0.05c	2.14 ± 0.08c
	Cd5.0	0.62 ± 0.02b	0.78 ± 0.03b	0.46 ± 0.02b	0.55 ± 0.03b	0.57 ± 0.03b	0.70 ± 0.03b	1.03 ± 0.04b	1.26 ± 0.07b	1.40 ± 0.06b	0.90 ± 0.01b	3.80 ± 0.12b	4.46 ± 0.19b
	Cd10.0	0.97 ± 0.07a	1.69 ± 0.09a	0.75 ± 0.06a	1.96 ± 0.11a	1.35 ± 0.15a	1.96 ± 0.11a	2.01 ± 0.10a	1.98 ± 0.14a	2.75 ± 0.13a	1.34 ± 0.03a	7.73 ± 0.11a	9.35 ± 0.23a
Alligator flag	CK	0.04 ± 0.00d	0.04 ± 0.00d	0.07 ± 0.01d	0.08 ± 0.01c	0.14 ± 0.01c	0.13 ± 0.01c	0.18 ± 0.01c	0.29 ± 0.02c	0.26 ± 0.02c	0.20 ± 0.02c	0.74 ± 0.05d	0.68 ± 0.04d
	Cd2.5	0.19 ± 0.01c	0.15 ± 0.01c	0.17 ± 0.01c	0.11 ± 0.00c	0.28 ± 0.01c	0.21 ± 0.01c	0.22 ± 0.01c	0.39 ± 0.02c	0.33 ± 0.03c	0.36 ± 0.02c	1.40 ± 0.03c	1.02 ± 0.03c
	Cd5.0	0.44 ± 0.02b	0.40 ± 0.01b	0.40 ± 0.01b	0.37 ± 0.01b	0.63 ± 0.01b	0.64 ± 0.04b	0.83 ± 0.05b	1.07 ± 0.04b	1.05 ± 0.04b	0.79 ± 0.02b	3.33 ± 0.09b	3.28 ± 0.09b
	Cd10.0	0.98 ± 0.06a	1.00 ± 0.06a	0.94 ± 0.05a	1.75 ± 0.11a	1.57 ± 0.12a	1.75 ± 0.11a	1.63 ± 0.09a	2.45 ± 0.16a	1.95 ± 0.17a	1.51 ± 0.10a	7.45 ± 0.29a	7.33 ± 0.16a

[a] Exchangeable fraction (extracted with 1 M $MgCl_2$).
[b] Bound to the carbonate fraction (extracted with 1 M NaOAc/HOAc).
[c] Iron and manganese oxide bound fraction (extracted with 0.1 M $NH_2OH-HCl$).
[d] Organic-bound metal (extracted with 30 % H_2O_2 plus 3.2 M NH_4Ac).
[e] Residual phase (extracted with HNO_3 65 %/HF 40 %/HCl 37 %, 9:3:2 (v/v)).
[f] The total Cd content calculated by summing Cd fractions (F1-F5). CK indicates the control, Cd2.5 indicates the initial soil Cd content of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd content of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd content of 10.0 mg kg^{-1}. Different lowercase letters in each column indicate the significance among different Cd-contaminated levels (ANOVA, Duncan's multiple range test).

initial soil Cd level of 50–120 mg kg^{-1} treatment. The POD activity of the two plants differed significantly (paired-sample t-test, 30 mg kg^{-1}: t = –7.13, df = 4, p = 0.002; 50 mg kg^{-1}: t = –8.47, df = 4, p = 0.001; 120 mg kg^{-1}: t = –5.46, df = 4, p = 0.005) at all of the initial soil Cd levels. For example, at the CK and the initial soil Cd level of 50 mg kg^{-1} treatment, the POD activities were 1.9 times lower and 43 times lower in alligator flag than in rice, respectively.

The CAT activity varied significantly in the leaves of alligator flag and rice, and the initial Cd level also affected them differently (Fig. 3c). For example, the CAT activity was 47 % lower in alligator flag than in rice at CK, but only 15 % and 26 % less in rice than in alligator flag at the initial soil Cd levels of 30 and 50 mg kg^{-1}. However, at the initial soil Cd level of 120 mg kg^{-1} treatment, the CAT activity was higher in rice than in alligator flag (paired-sample t-test, t = –0.80, df = 4, $p > 0.05$). Generally, the CAT activity decreased at higher Cd levels in alligator flag. In rice, CAT activity decreased from the initial soil Cd level of 30–50 mg kg^{-1} treatment, but then increased from the initial soil Cd level of 50–120 mg kg^{-1} treatment, though these differences were not significant.

In general, the MDA concentration exhibited similar variation to POD activity (Fig. 3b and d). Specifically, MDA concentration decreased significantly in alligator flag from 4.25 μmol g^{-1} FW in the CK to 0.60 μmol g^{-1} FW at the initial soil Cd level of 50 mg kg^{-1} treatment, and then increased to 0.93 μmol g^{-1} FW at 120 mg kg^{-1} Cd treatment. In contrast, the MDA concentration in rice increased 150 % from CK to the initial soil Cd level of 50 mg kg^{-1} treatment, while it decreased significantly from 2.36 to 0.48 μmol g^{-1} FW at the initial soil Cd level of 120 mg kg^{-1} treatment. The two plants differed significantly in MDA concentration at CK and the initial soil Cd level of 50 mg kg^{-1} treatment. The MDA concentration was 3.5 times greater in alligator flag than in rice at CK, while the MDA concentration in alligator flag was only 25 % of that in rice at the initial soil Cd level of 50 mg kg^{-1} treatment.

4. Discussion

4.1. Effects of intercropping alligator flag on the Cd accumulation of rice grains

Cd can be absorbed and transported effectively by rice plants (Shah et al., 2001), although the efficiency of Cd accumulation is affected by Cd availability in the rice rhizosphere soil, soil physicochemical properties, and other factors (Zhao et al., 2010; Yu et al., 2016). In this study, Cd absorption, transportation and accumulation from the soil to the rice grains were all significantly decreased in RT system, primarily due to the reduction of Cd availability in the rice rhizosphere soil by the intercropped alligator flag, because alligator flag exhibited higher fine root biomass and higher Cd content in these fine roots than rice in the RT and monocropping (RM and TM) systems (Table 1 and Fig. 4). The reduction of soil NH_4-N and NO_3-N in the RT system due to the uptake by alligator flag resulted in increased soil pH in the rice rhizosphere soil and a concomitant decrease in Cd availability (Yu et al., 2016). For example, a previous study reported that feeding of NH_4-N nutrient could be a strategy for Cd mobilization in the rhizosphere of sunflowers by decreasing soil pH, while the uptake of NH_4-N and NO_3-N could increase rhizosphere soil pH, which mainly depends on the balance of cations over anions taken up by the roots (Zaccheo et al., 2006). The BAF and TF of rice and alligator flag varied across different cropping patterns and initial soil Cd levels (Table 2), indicating that the BAF and TF may be not the only indicators for judging the potential of plants for phytoremediation. We observed that the presence of alligator flag increased the ratio of the Cd translocated to aerial parts of rice *vs* the Cd in rice roots while decreased the amount of Cd translocation, and the TFs of alligator flag were also increased in the RT system compared to the TM system (Table 2). The TF of plants may vary under different conditions, as suggested by our previous research and others (Zhang et al., 2014; Anning and Akoto, 2018; Wang et al., 2020). For example,

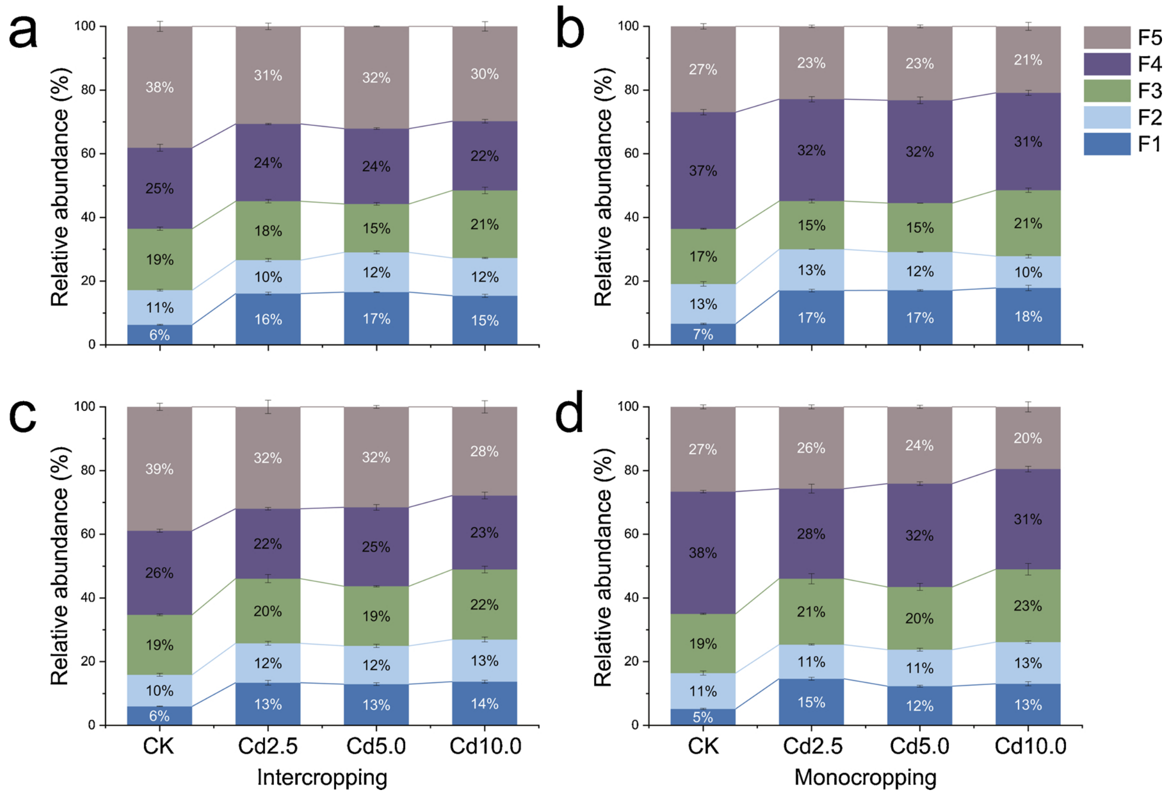

Fig. 2. Relative proportion of the Cd fractions in rice rhizosphere soil under rice-alligator flag intercropping system (a), rice rhizosphere soil under rice monocropping system (b), alligator flag rhizosphere soil under rice–alligator flag intercropping system (c), and alligator flag rhizosphere soil under alligator flag monocropping system (d). F1, exchangeable fraction (extracted with 1 M $MgCl_2$); F2, bound to the carbonate fraction (extracted with 1 M NaOAc/HOAc); F3, iron and manganese oxide-bound fraction (extracted with 0.1 M $NH_2OH-HCl$); F4, organic-bound metal (extracted with 30 % H_2O_2 plus 3.2 M NH_4Ac); F5, residual phase (extracted with HNO_3 65 %/HF 40 %/HCl 37 %, 9:3:2 (v/v)). CK indicates the control, Cd2.5 indicates the initial soil Cd content of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd content of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd content of 10.0 mg kg^{-1}.

Hu et al. (2017) discussed that the transfer of heavy metal in greenhouse soils is affected by the heavy metal concentrations in plants, soil pH, and organic matter (OM) content, and soil pH was observed to positively correlate with the transfer of Cd, As, and Zn. Here, we observed that the Cd content in the rhizosphere soil of rice and alligator flag in the RT system was reduced, and the TFs of rice and alligator flag increased. In contrast, we found that at higher initial Cd contamination levels (from CK to 10 mg kg^{-1} treatment), the TFs of rice and alligator flag decreased (Table 2). These results suggest that soil Cd content negatively correlated with the TF of plants in this study. Similar to the results of (Hu et al., 2017), soil pH was positively correlated with the TFs of Cd. Our results suggest that with the improvement of soil pH in RT systems, the TFs of rice and alligator flag should also rise (Table 2). BAF of fine roots of alligator flag were significantly higher than that of rice (range = 1.86–15.89 in alligator flag planting systems). However, the BAFs of alligator flag shoots were all less than 1.0, indicating that

Table 4
Plant biomass, Cd content, and accumulated Cd mass in plant under a gradient of Cd contamination in concrete pond trials.

Plant	Tissue	Initial soil Cd content (mg kg^{-1})	Cd content (mg kg^{-1})	Biomass[a] (g)	Cd mass (mg)
Rice	Shoot	30	7.29 ± 0.75a	116.33 ± 49.42a	825.08 ± 319.40a
		50	18.37 ± 5.57a	88.50 ± 12.93a	1719.34 ± 628.11a
		120	22.49 ± 0.91a	126.50 ± 24.50a	2866.67 ± 665.51a
	Fine Root	30	38.41 ± 9.24b	11.55 ± 6.48a	327.08 ± 88.27b
		50	87.45 ± 11.95a	12.44 ± 3.74a	951.99 ± 201.71ab
		120	103.04 ± 7.62a	12.78 ± 4.61a	1281.71 ± 377.59a
Alligator flag	Shoot	30	3.28 ± 0.16b	118.33 ± 6.12a	387.34 ± 16.33a
		50	5.15 ± 1.09b	91.00 ± 41.04a	379.57 ± 76.42a
		120	8.16 ± 0.08a	70.00 ± 18.04a	572.62 ± 150.78a
	Fine Root	30	46.40 ± 9.80b	50.05 ± 25.02a	2078.51 ± 770.23b
		50	69.74 ± 6.74b	23.70 ± 2.08a	1674.89 ± 300.01b
		120	174.60 ± 2.73a	28.70 ± 2.51a	5011.30 ± 453.83a
	Rhizome	30	4.71 ± 0.24b	50.39 ± 2.79b	238.00 ± 20.53b
		50	6.47 ± 0.67a	62.08 ± 2.09a	404.48 ± 53.26a
		120	7.92 ± 0.39a	58.90 ± 3.07ab	465.69 ± 25.63a

[a] The biomass for plants is within the 0.04 m^2 quadrat. Different lowercase letters in each column indicate the significance among different Cd-contaminated levels (ANOVA, Duncan's multiple range test).

Table 5
Total biomass, Cd accumulation, and Cd reduction of three cropping systems in concrete pond trials at Cd-contaminated level of 50 mg kg^{-1}.

Tillage Pattern	Total Biomass (g)	Cd accumulation (mg)	Cd reduction (%)
Rice monocropping	7406.44 ± 669.17b	229.18 ± 42.17b	0.51b
Alligator flag monocropping	22347.88 ± 3160.29a	562.92 ± 157.54a	1.25a
Intercropping	6946.59 ± 1491.01b	104.83 ± 11.16b	0.23b

Cd reduction was calculated by comparing to the initial soil Cd content in the Cd-contaminated concrete ponds (50 mg kg^{-1}). Different lowercase letters in each column indicate the significance among different tillage patterns (ANOVA, Duncan's multiple range test).

alligator flag is the optimal phytostabilization plant for Cd. Alligator flag can produce a large root surface area and many root tips (fine roots), Jiang et al. (2011) found that the root surface area of alligator flag reaches 1,488 cm^2, and the number of root tips reaches 12,083 cm^{-2} after 28 days of growth. Our results showed that the biomass of fine roots of alligator flag was significantly higher than that of rice (Table 4). Further, the Cd content in the fine roots of alligator flag was higher than both the background content of Cd in the soil and that of rice roots at the same initial level of Cd contamination (Tables 1 and 4). These results indicate that intercropping alligator flag resulted in the uptake and accumulation of more Cd from the soil to alligator flag than to rice (Fig. 4). In the greenhouse experiment at the initial levels of 2.5 mg kg^{-1} soil Cd, intercropping alligator flag with rice significantly lowered Cd accumulated in the rice grains to a level below the maximum allowable Cd level for food safety standard (< 0.2 mg kg^{-1}) (Zou et al., 2019). Although the Cd accumulation in rice grains was significantly lowered at higher initial soil Cd levels, the reduction of Cd below the standard only occurred when the initial soil Cd level was 2.5 mg kg^{-1} or below.

Table 6
Contents of Cd, nutrients, and extracellular enzyme activities in the studied soil under a gradient of Cd contamination in greenhouse pot trials.

			Cd	Soil nutrient			Extracellular enzyme activity			
			Bulk soil Cd	TP	NH_4-N	NO_3-N	BG	CB	NAG	ACP
Cropping pattern	Mean	Intercropping	6.04 ± 0.32	1.57 ± 0.20	6.53 ± 0.62	1.02 ± 0.27	3.63 ± 0.11	2.58 ± 0.12	4.56 ± 0.40	5.96 ± 0.44
		Rice monocropping	7.32 ± 0.37	1.35 ± 0.24	10.42 ± 0.74	2.17 ± 0.32	4.11 ± 0.13	2.99 ± 0.14	5.30 ± 0.47	6.33 ± 0.52
		Alligator flag monocropping	6.45 ± 0.40	1.57 ± 0.26	9.25 ± 0.93	1.47 ± 0.34	4.22 ± 0.13	2.90 ± 0.18	5.29 ± 0.51	6.60 ± 0.56
	F		3.413	0.369	5.108	3.418	8.091	4.842	1.478	0.630
	P		***0.041***	0.693	***0.010***	***0.041***	***0.001***	***0.012***	0.238	0.537
Species	Mean	Rice	7.27 ± 0.29	1.31 ± 0.19	8.00 ± 0.58	1.55 ± 0.25	3.95 ± 0.10	2.79 ± 0.11	4.68 ± 0.37	5.88 ± 0.40
		Alligator flag	5.66 ± 0.30	1.73 ± 0.19	7.93 ± 0.60	1.29 ± 0.25	3.84 ± 0.10	2.69 ± 0.11	5.18 ± 0.38	6.55 ± 0.42
	F		13.648	2.384	0.010	0.107	2.347	1.833	1.605	1.474
	P		***0.001***	0.129	0.922	0.744	0.132	0.182	0.211	0.231
Gradient of Cd treatment	Mean	CK	3.05 ± 0.43	2.06 ± 0.27	8.25 ± 0.85	1.49 ± 0.36	3.96 ± 0.14	2.81 ± 0.16	4.78 ± 0.54	5.92 ± 0.59
		Cd2.5	4.68 ± 0.40	1.12 ± 0.26	7.78 ± 0.80	1.25 ± 0.34	3.91 ± 0.13	2.85 ± 0.15	4.70 ± 0.51	5.86 ± 0.56
		Cd5.0	7.34 ± 0.43	1.53 ± 0.27	7.58 ± 0.86	1.35 ± 0.36	4.01 ± 0.14	2.88 ± 0.16	4.55 ± 0.54	5.83 ± 0.60
		Cd10.0	10.79 ± 0.41	1.37 ± 0.26	8.26 ± 0.82	1.60 ± 0.35	3.70 ± 0.14	2.43 ± 0.16	5.67 ± 0.52	7.24 ± 0.57
	F		69.209	1.999	0.388	0.362	0.988	1.791	1.321	1.793
	P		***0.000***	0.126	0.762	0.780	0.406	0.161	0.278	0.161
Cropping pattern* Gradient	Intercropping	CK	3.30 ± 0.68	2.45 ± 0.43	7.70 ± 1.34	1.05 ± 0.57	3.54 ± 0.23	2.43 ± 0.26	5.08 ± 0.85	5.92 ± 0.94
		Cd2.5	4.44 ± 0.59	1.14 ± 0.37	6.06 ± 1.16	1.32 ± 0.49	3.60 ± 0.20	2.52 ± 0.22	4.73 ± 0.74	6.23 ± 0.81
		Cd5.0	6.64 ± 0.68	1.18 ± 0.43	7.47 ± 1.34	0.79 ± 0.57	3.91 ± 0.23	2.88 ± 0.26	3.96 ± 0.85	5.37 ± 0.94
		Cd10.0	9.81 ± 0.59	1.52 ± 0.37	5.32 ± 1.16	0.92 ± 0.49	3.48 ± 0.20	2.23 ± 0.22	4.45 ± 0.74	6.31 ± 0.81
	Rice monocropping	CK	2.67 ± 0.74	1.47 ± 0.47	7.20 ± 1.47	1.49 ± 0.62	4.29 ± 0.25	3.06 ± 0.28	4.29 ± 0.94	5.59 ± 1.03
		Cd2.5	5.05 ± 0.74	1.09 ± 0.47	10.31 ± 1.47	1.28 ± 0.62	4.16 ± 0.25	3.13 ± 0.28	4.40 ± 0.94	5.21 ± 1.03
		Cd5.0	8.87 ± 0.68	1.73 ± 0.43	10.57 ± 1.34	2.71 ± 0.57	4.03 ± 0.23	2.80 ± 0.26	5.66 ± 0.85	6.67 ± 0.94
		Cd10.0	12.67 ± 0.83	1.13 ± 0.53	9.16 ± 1.65	3.18 ± 0.70	3.95 ± 0.28	2.58 ± 0.31	6.85 ± 1.05	7.84 ± 1.15
	Alligator flag monocropping	CK	2.94 ± 0.74	1.85 ± 0.47	10.42 ± 1.47	2.36 ± 0.62	4.47 ± 0.25	3.31 ± 0.28	4.67 ± 0.94	6.23 ± 1.03
		Cd2.5	4.78 ± 0.83	1.10 ± 0.53	8.66 ± 1.65	1.06 ± 0.70	4.28 ± 0.28	3.21 ± 0.31	4.95 ± 1.05	5.75 ± 1.15
		Cd5.0	7.21 ± 0.83	2.02 ± 0.53	4.81 ± 1.65	1.10 ± 0.70	4.21 ± 0.28	2.95 ± 0.31	4.61 ± 1.05	5.92 ± 1.15
		Cd10.0	10.86 ± 0.83	1.31 ± 0.53	13.26 ± 1.65	1.36 ± 0.70	3.90 ± 0.28	2.65 ± 0.31	6.94 ± 1.05	8.50 ± 1.15
	F		1.462	1.022	2.562	0.915	0.476	0.633	1.539	1.013
	P		0.211	0.422	***0.031***	0.492	0.823	0.703	0.185	0.428
Species* Gradient	Rice	CK	2.70 ± 0.61	1.43 ± 0.38	7.45 ± 1.20	1.29 ± 0.51	3.98 ± 0.20	2.83 ± 0.23	4.44 ± 0.76	5.67 ± 0.84
		Cd2.5	5.09 ± 0.56	1.13 ± 0.35	9.64 ± 1.10	1.19 ± 0.47	3.97 ± 0.19	2.87 ± 0.21	4.96 ± 0.70	6.06 ± 0.77
		Cd5.0	8.65 ± 0.59	1.51 ± 0.37	8.31 ± 1.16	1.65 ± 0.49	4.07 ± 0.20	2.98 ± 0.22	4.57 ± 0.74	5.47 ± 0.81
		Cd10.0	12.63 ± 0.59	1.16 ± 0.37	6.61 ± 1.16	2.07 ± 0.49	3.78 ± 0.20	2.46 ± 0.22	4.73 ± 0.74	6.31 ± 0.81
	Alligator flag	CK	3.41 ± 0.61	2.68 ± 0.38	9.06 ± 1.20	1.69 ± 0.51	3.94 ± 0.20	2.78 ± 0.23	5.12 ± 0.76	6.16 ± 0.84
		Cd2.5	4.26 ± 0.59	1.10 ± 0.37	5.91 ± 1.16	1.30 ± 0.49	3.85 ± 0.20	2.83 ± 0.22	4.45 ± 0.74	5.65 ± 0.81
		Cd5.0	6.03 ± 0.63	1.55 ± 0.40	6.84 ± 1.26	1.04 ± 0.53	3.96 ± 0.21	2.77 ± 0.24	4.53 ± 0.80	6.19 ± 0.88
		Cd10.0	8.94 ± 0.59	1.57 ± 0.37	9.91 ± 1.16	1.13 ± 0.49	3.62 ± 0.20	2.39 ± 0.22	6.62 ± 0.74	8.17 ± 0.81
	F		5.235	1.621	2.811	0.071	0.024	0.148	2.100	1.406
	P		***0.003***	0.197	***0.049***	0.975	0.995	0.930	0.112	0.252

TP represents total phosphorus, NH_4-N represents Ammonia, NO_3-N represents nitrate; BG represents β-1,4-glucosidase, CB represents β-D-cellobiosidase, NAG represents β-1,4-*N*-acetylglucosaminidase, and ACP represents acid phosphatase. CK indicates the control, Cd2.5 indicates the initial soil Cd content of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd content of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd content of 10.0 mg kg^{-1}.

Table 7
Soil microbial biomass and microbial species mass under a gradient of Cd content in greenhouse trials.

			Microbial species			
			Biomass	G+	G+%	G–
Cropping pattern	Mean	Intercropping	205.07 ± 7.61	54.28 ± 2.24	26.46 ± 0.53	53.81 ± 2.56
		Rice monocropping	225.79 ± 9.09	57.82 ± 2.65	26.17 ± 0.62	63.60 ± 3.03
		Alligator flag monocropping	212.33 ± 9.71	55.01 ± 2.85	25.79 ± 0.67	55.62 ± 3.27
	F		1.145	0.346	0.301	1.944
	P		0.327	0.709	0.741	0.154
Species	Mean	Rice	215.97 ± 7.01	56.16 ± 2.06	26.28 ± 0.49	59.13 ± 2.36
		Alligator flag	208.16 ± 7.25	54.52 ± 2.13	26.16 ± 0.50	54.29 ± 2.44
	F		0.02	0.011	0.023	0.113
	P		0.888	0.916	0.881	0.739
Gradient	Mean	CK	223.14 ± 10.29	54.83 ± 3.02	25.15 ± 0.71	62.81 ± 3.46
		Cd2.5	206.89 ± 9.71	54.68 ± 2.85	26.55 ± 0.67	54.75 ± 3.27
		Cd5.0	211.73 ± 10.37	55.65 ± 3.04	26.11 ± 0.72	55.62 ± 3.49
		Cd10.0	206.50 ± 9.96	56.21 ± 2.92	27.06 ± 0.69	53.67 ± 3.35
	F		1.199	0.03	1.324	2.412
	P		0.32	0.993	0.277	0.078
Cropping pattern* Gradient	Intercropping	CK	193.55 ± 16.27	49.54 ± 4.77	25.67 ± 1.13	52.09 ± 5.47
		Cd2.5	202.59 ± 14.09	52.79 ± 4.14	26.31 ± 0.98	53.61 ± 4.74
		Cd5.0	206.88 ± 16.27	53.46 ± 4.77	25.66 ± 1.13	54.09 ± 5.47
		Cd10.0	217.28 ± 14.09	61.32 ± 4.14	28.18 ± 0.98	55.44 ± 4.74
	Rice monocropping	CK	296.18 ± 17.82	63.69 ± 5.23	22.18 ± 1.23	92.68 ± 5.99
		Cd2.5	211.50 ± 17.82	59.02 ± 5.23	27.87 ± 1.23	55.89 ± 6.00
		Cd5.0	222.26 ± 16.27	61.88 ± 4.77	27.64 ± 1.13	61.29 ± 5.47
		Cd10.0	173.20 ± 19.92	46.66 ± 5.85	27.01 ± 1.38	44.55 ± 6.70
	Alligator flag monocropping	CK	209.30 ± 17.82	56.56 ± 5.23	27.06 ± 1.23	54.39 ± 5.99
		Cd2.5	210.89 ± 19.92	54.12 ± 5.85	25.72 ± 1.38	55.89 ± 6.70
		Cd5.0	210.90 ± 19.92	53.79 ± 5.85	25.49 ± 1.38	53.00 ± 6.70
		Cd10.0	218.22 ± 19.92	55.54 ± 5.85	24.88 ± 1.38	59.22 ± 6.70
	F		3.446	1.71	2.51	4.016
	P		***0.006***	0.139	***0.034***	***0.002***
Species* Gradient	Rice	CK	234.10 ± 14.55	54.37 ± 4.27	24.18 ± 1.01	69.06 ± 4.90
		Cd2.5	213.60 ± 13.37	55.00 ± 3.92	25.82 ± 0.93	57.21 ± 4.50
		Cd5.0	217.01 ± 14.09	60.02 ± 4.14	27.42 ± 0.98	58.56 ± 4.74
		Cd10.0	199.15 ± 14.09	55.26 ± 4.14	27.67 ± 0.98	51.70 ± 4.74
	Alligator flag	CK	212.19 ± 14.55	55.29 ± 4.27	26.11 ± 1.01	56.56 ± 4.89
		Cd2.5	200.19 ± 14.09	54.37 ± 4.14	27.28 ± 0.98	52.28 ± 4.74
		Cd5.0	206.43 ± 15.22	51.28 ± 4.47	24.80 ± 1.05	52.67 ± 5.12
		Cd10.0	213.84 ± 14.09	57.16 ± 4.14	26.45 ± 0.98	55.63 ± 4.74
	F		0.971	0.803	2.887	0.967
	P		0.414	0.498	***0.045***	0.416

	Microbial species							
	G–%	F	F%	A	A%	G+/G–	B/F	A/F
Cropping pattern	26.18 ± 0.37	20.40 ± 1.53	9.81 ± 0.37	25.21 ± 0.93	12.43 ± 0.45	1.02 ± 0.03	5.52 ± 0.23	1.31 ± 0.08
	27.60 ± 0.44	21.86 ± 1.82	8.92 ± 0.44	26.15 ± 1.10	12.51 ± 0.53	0.97 ± 0.03	6.60 ± 0.27	1.62 ± 0.10
	26.16 ± 0.48	18.53 ± 1.96	8.68 ± 0.47	27.51 ± 1.18	13.14 ± 0.57	1.00 ± 0.04	6.24 ± 0.30	1.59 ± 0.11
	1.237	0.109	2.146	0.896	0.265	0.614	4.522	3.515
	0.299	0.897	0.128	0.415	0.768	0.546	***0.016***	***0.037***

(*continued on next page*)

Table 7 (*continued*)

	Microbial species							
	G–%	F	F%	A	A%	G+/G–	B/F	A/F
Species	27.09 ± 0.34	21.50 ± 1.42	9.46 ± 0.34	25.50 ± 0.85	12.41 ± 0.41	0.98 ± 0.03	6.04 ± 0.21	1.46 ± 0.08
	25.97 ± 0.35	19.10 ± 1.46	9.15 ± 0.35	26.53 ± 0.88	12.84 ± 0.43	1.02 ± 0.03	5.89 ± 0.22	1.45 ± 0.07
	1.158	0.228	0.25	0.138	0.069	0.598	0.014	0.028
	0.287	0.635	0.62	0.711	0.794	0.443	0.907	0.868
Gradient	27.62 ± 0.50	23.32 ± 2.08	9.76 ± 0.50	25.62 ± 1.25	12.28 ± 0.61	0.92 ± 0.04	5.84 ± 0.31	1.41 ± 0.11
	26.33 ± 0.48	19.14 ± 1.96	9.13 ± 0.47	25.62 ± 1.18	12.54 ± 0.57	1.02 ± 0.04	6.11 ± 0.30	1.48 ± 0.11
	26.18 ± 0.51	19.66 ± 2.09	9.26 ± 0.50	26.91 ± 1.27	12.87 ± 0.61	1.01 ± 0.04	5.80 ± 0.32	1.44 ± 0.11
	26.01 ± 0.49	19.07 ± 2.01	9.07 ± 0.48	25.91 ± 1.21	12.83 ± 0.59	1.05 ± 0.04	6.12 ± 0.30	1.51 ± 0.11
	2.57	2.133	1.25	0.338	0.665	1.999	0.654	0.689
	0.065	0.108	0.302	0.798	0.578	0.126	0.584	0.563
Cropping pattern* Gradient	26.85 ± 0.80	16.67 ± 3.28	8.53 ± 0.79	26.82 ± 1.98	13.84 ± 0.96	0.96 ± 0.06	6.25 ± 0.50	1.63 ± 0.18
	26.22 ± 0.69	21.36 ± 2.84	10.27 ± 0.68	22.46 ± 1.72	11.38 ± 0.83	1.02 ± 0.05	5.35 ± 0.43	1.17 ± 0.15
	26.13 ± 0.80	21.37 ± 3.28	10.31 ± 0.79	26.00 ± 1.98	12.64 ± 0.96	0.99 ± 0.06	5.06 ± 0.50	1.24 ± 0.18
	25.53 ± 0.69	22.21 ± 2.84	10.12 ± 0.68	25.55 ± 1.72	11.87 ± 0.83	1.11 ± 0.05	5.41 ± 0.43	1.22 ± 0.15
	30.85 ± 0.87	41.31 ± 3.59	13.16 ± 0.86	22.76 ± 2.17	8.79 ± 1.05	0.73 ± 0.07	4.50 ± 0.54	0.83 ± 0.19
	26.41 ± 0.87	16.34 ± 3.59	7.68 ± 0.86	28.16 ± 2.17	13.39 ± 1.05	1.06 ± 0.07	7.19 ± 0.54	1.81 ± 0.19
	27.48 ± 0.80	17.88 ± 3.28	7.98 ± 0.79	27.22 ± 1.98	12.58 ± 0.96	1.02 ± 0.06	6.99 ± 0.50	1.62 ± 0.18
	25.67 ± 0.98	11.89 ± 4.02	6.86 ± 0.97	26.44 ± 2.43	15.28 ± 1.17	1.06 ± 0.08	7.68 ± 0.61	2.23 ± 0.22
	25.92 ± 0.87	18.64 ± 3.59	8.82 ± 0.86	26.09 ± 2.17	12.64 ± 1.05	1.05 ± 0.07	6.36 ± 0.54	1.54 ± 0.19
	26.47 ± 0.98	17.51 ± 4.02	8.29 ± 0.97	29.41 ± 2.43	14.00 ± 1.17	0.97 ± 0.08	6.53 ± 0.61	1.76 ± 0.22
	24.96 ± 0.98	17.99 ± 4.02	8.46 ± 0.97	28.43 ± 2.43	13.59 ± 1.17	1.03 ± 0.08	6.07 ± 0.61	1.65 ± 0.22
	27.31 ± 0.98	19.96 ± 4.02	9.16 ± 0.97	26.11 ± 2.43	12.31 ± 1.17	0.92 ± 0.08	5.98 ± 0.61	1.38 ± 0.22
	2.314	4.863	4.513	1.287	3.28	2.848	2.953	4.331
	0.048	***0.001***	***0.001***	0.281	***0.009***	***0.019***	***0.015***	***0.001***
Species* Gradient	28.63 ± 0.71	27.65 ± 2.94	10.64 ± 0.71	23.54 ± 1.77	11.47 ± 0.86	0.86 ± 0.06	5.51 ± 0.44	1.30 ± 0.16
	26.84 ± 0.65	20.76 ± 2.70	9.41 ± 0.65	26.30 ± 1.63	12.56 ± 0.79	0.96 ± 0.05	6.05 ± 0.41	1.48 ± 0.15
	26.97 ± 0.69	19.82 ± 2.84	9.13 ± 0.68	26.30 ± 1.72	12.33 ± 0.83	1.03 ± 0.05	6.13 ± 0.43	1.40 ± 0.15
	25.92 ± 0.69	17.75 ± 2.84	8.66 ± 0.68	25.90 ± 1.72	13.29 ± 0.83	1.07 ± 0.05	6.47 ± 0.43	1.66 ± 0.15
	26.60 ± 0.71	19.00 ± 2.94	8.88 ± 0.71	27.70 ± 1.77	13.08 ± 0.86	0.99 ± 0.06	6.17 ± 0.44	1.52 ± 0.16
	25.81 ± 0.69	17.52 ± 2.84	8.85 ± 0.68	24.95 ± 1.72	12.51 ± 0.83	1.07 ± 0.05	6.16 ± 0.43	1.47 ± 0.15
	25.38 ± 0.74	19.49 ± 3.07	9.40 ± 0.74	27.53 ± 1.85	13.40 ± 0.90	.99 ± 0.06	5.46 ± 0.46	1.48 ± 0.17
	26.09 ± 0.69	20.39 ± 2.84	9.48 ± 0.68	25.94 ± 1.72	12.38 ± 0.83	1.03 ± 0.05	5.76 ± 0.43	1.36 ± 0.15
	0.709	0.763	0.547	0.998	0.355	2.562	0.514	0.406
	0.552	0.52	0.653	0.402	0.786	0.065	0.674	0.749

G + represents Gram-positive (G+) bacteria; G– represents Gram-negative bacteria; A represents actinomycetes; F represents fungi; B represents bacteria. CK indicates the control, Cd2.5 indicates the initial soil Cd concentration of 2.5 mg kg^{-1}, Cd5.0 indicates the initial soil Cd content of 5.0 mg kg^{-1}, and Cd10.0 indicates the initial soil Cd content of 10.0 mg kg^{-1}.

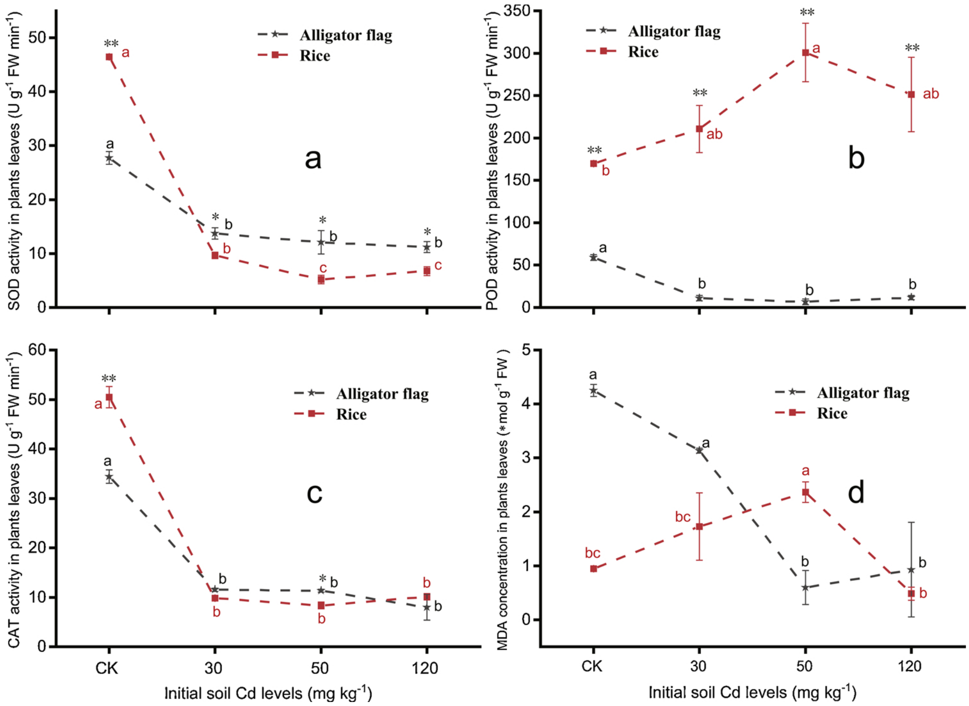

Fig. 3. Antioxidant enzymes: superoxide dismutase (SOD) (a), peroxidase (POD) (b) and catalase (CAT) (c), and malondialdehyde (MDA) (d) in rice and alligator flag leaves after being transplanted in elevated Cd-contaminated soil in concrete ponds for two weeks. Asterisks denote significance between rice and alligator flag plants, levels at $*P < 0.05$ and $**P < 0.01$. Different letters indicate a significant difference between different gradients of initial soil Cd contamination (ANOVA, Duncan's multiple range test).

4.2. Effects of intercropping alligator flag on paddy soil and microbial properties

The partitioning pattern of heavy metals is important to the mobility and toxicity of heavy metals, and the separation fractions and speciation of heavy metals in the contaminated soils or sediments may be more important for hazard evaluation than the total heavy metal contents (Mulligan et al., 2001; Filgueiras et al., 2004). Therefore, the relative proportion of extractable fractions (F1-F4) of Cd was calculated to evaluate the effects of intercropping alligator flag on the availability of Cd. The chemical fractions of extractable Cd are categorized as exchangeable fractions (bioavailable and mobile) and as carbonate, oxide, and organic fractions (fixed and immobile) (Peijnenburg et al., 2007). Extractable fractions (F1-F4) can be varied with properties of the contaminated soil or sediment, such as pH, organic matter (OM) content, clay content, cation exchange capacity (CEC), and available phosphorus (P) content (Xian and Shokohifard, 1989; Pietrzykowski et al., 2014; Lu et al., 2017; Yang et al., 2017). The F1 fraction of Cd (considered as exchangeable Cd) has been reported as negatively correlated with soil pH (Xian and Shokohifard, 1989; Yang et al., 2017). In our study, at higher Cd contamination levels, the F1 fraction rose (from about 5%–17%) in all systems compared with CK. This increase occurred because more $CdCl_2$ was added and induced a lower soil pH (Table 1). The F2 fraction of Cd (carbonate Cd) decreased with lower soil pH (Fig. 2) in the RM system, similar to previous work that reported a positively correlation between F2 and contaminated soil or sediment

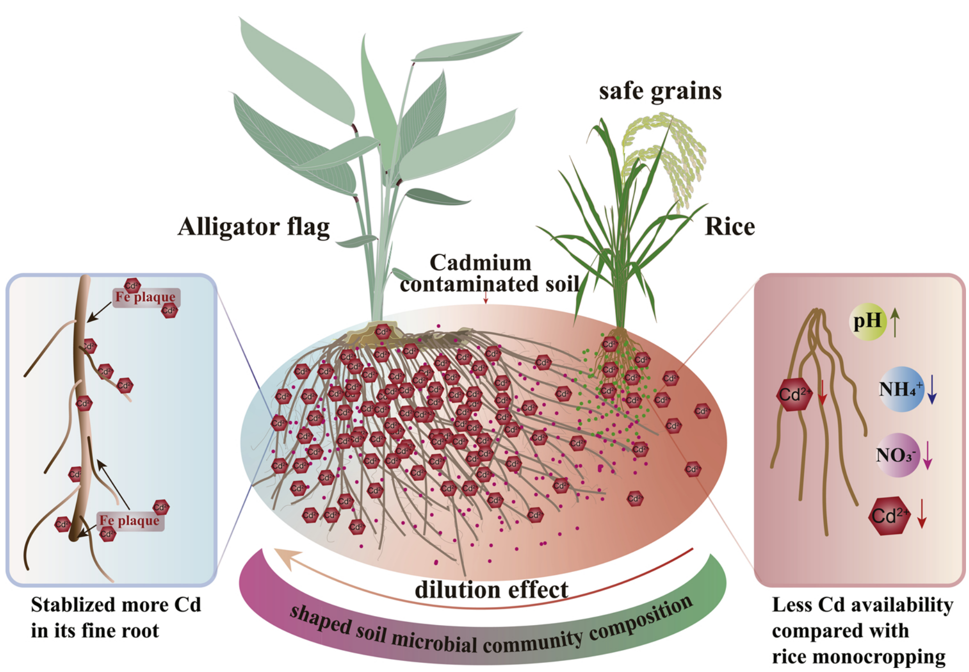

Fig. 4. Potential mechanisms of Cd reduction and remediation in the rice and alligator flag intercropping system. In general, Cd availability decreased as a result of the dilution effect induced by intercropping with alligator flag. Specifically: (1) alligator flag has more fine root mass and could absorb a higher content of Cd in its fine roots than rice; (2) intercropping of alligator flag shaped an inactive environment for Cd, in which soil pH was elevated while NH_4-N and NO_3-N were decreased; (3) more Fe plaque, which could passivate and immobilize Cd, formed on the surface of alligator flag's fine roots; (4) rhizosphere microbial species and community composition varied significantly among different cropping pattern and Cd-contaminated gradients, suggesting that Cd stress impacted microbes, and that as a feedback microbes probably play important roles in Cd immobilization or passivation.

pH (Xian and Shokohifard, 1989). The F3 fraction of Cd (iron and manganese oxide-bound fraction) was higher in alligator flag cropping (TM and RT) systems compared to the RM system, probably because more Fe oxide was induced by planting alligator flag (Jiang et al., 2009, 2011; Liu et al., 2018). The F4 fraction of Cd (organic-bound Cd fraction) decreased at higher contaminated Cd levels (Fig. 2), although an increase of the net amount of F4 fraction was also observed (Table 3). Significant differences were observed between monocropping (either rice or alligator flag) and intercropping systems (Fig. 2). Reports that the addition of compost could alleviate Cd phytotoxicity are attributed primarily to the increase of soil pH and complexation of Cd by the additional organic matter (Liu et al., 2009). In our study, greater plant biomass was found in monocropping (either rice or alligator flag) systems rather than intercropping systems (Table 5). Therefore, more potential organic matter (such as deposits, root exudates, and metabolites) may exist in monocropping systems than in intercropping systems, which may explain why a greater F4 fraction was found in monocropping systems (Fig. 2). In contrast, the lower soil pH may contribute to the lower F4 fraction at higher Cd contamination levels. The relative mobility of the metals, and their redistribution among the different fractions, are more mobile for metals from anthropogenic sources than those from soil parent materials (Chlopecka et al., 1996), which may explain why the relative proportion of the F5 fraction of Cd was lower at higher contaminated Cd levels in this study (because we added anthropogenic Cd).

In this study, a lower Cd content was found in the rhizosphere soil of alligator flag compared to rice at 2.5, 5.0, and even 10.0 mg kg^{-1} Cd, probably because the alligator flag possesses higher fine root biomass and accumulated higher amounts of Cd in its fine roots across the Cd gradient (CK-10.0 mg kg^{-1}) (Tables 1 and 4). In addition, although the TFs of alligator flag were less than that of rice (Table 2), alligator flag exhibited higher above-ground biomass. This higher biomass, combined with the Cd contents in alligator flag shoots and rhizomes gave the higher absolute mass of Cd extracted by alligator flag (2,316 mg per 0.04 m^2 quadrat at 30 mg kg^{-1}, 2,078 mg 0.04 m^{-2} at 50 mg kg^{-1}, and 5,476 mg 0.04 m^{-2} at 120 mg kg^{-1}) compared to that of rice (825 mg per 0.04 m^2 quadrat at 30 mg kg^{-1}, 1,719 mg 0.04 m^{-2} at 50 mg kg^{-1}, and 2,866 mg 0.04 m^{-2} at 120 mg kg^{-1}) (Table 4). Overall, the rhizosphere soil of alligator flag exhibited lower Cd than that of rice mainly because more Cd was extracted and/or stabilized by alligator flag.

As mentioned above, soil NH_4-N and NO_3-N contents were significantly lower in alligator flag cropping systems, mainly ascribed to absorption by alligator flag roots. Our results agree with the previous studies that reported the use of alligator flag in constructed wetlands for the removal of NH_4-N and NO_3-N as well as phosphorus (Jiang et al., 2011; Ying et al., 2011). Although our results suggest that cropping pattern exhibited significant effects on plant rhizosphere soil nutrients and extracellular enzymatic activities, no significant difference was observed for rhizosphere soil microbial species except for the ratios of B:F and A:F (Tables 6 and 7). However, significant variation in microbial species and biomass was found for the interaction of cropping pattern and Cd gradient, though in this case no significant change in soil nutrients or extracellular enzyme activities were observed (Tables 6 and 7). These results may suggest that in alligator flag cropping (TM and RT) systems with elevated Cd contamination, soil Cd content (rather than soil nutrients) is the main factor that constrains microbial species and microbial biomass, although soil nutrients exhibited noticeable effects on microbial community composition. Our results are different from previous studies. For instance, there are some reports that soil pH is the main factor that determines soil microbial diversity and community composition, and higher microbial biomass and higher diversity tend to occur at near-neutral pH soil (Fierer and Jackson, 2006; Lauber et al., 2009; Zhalnina et al., 2015). However, other studies suggest that soil carbon, nitrogen, and nutrients, rather than soil pH, may play more important roles in regulating soil microbial diversity and composition (Campbell et al., 2010; Ramirez et al., 2012; Yu et al., 2019).

4.3. Effects of Cd pollution on plant antioxidant systems

Efficient enzymatic (SOD and CAT) and non-enzymatic antioxidant defense systems exist in plants, which work in concert to control the cascades of uncontrolled oxidation and to protect plant cells from oxidative damage via ROS scavenging (Gill and Tuteja, 2010; Bhaduri and Fulekar, 2012). More antioxidant enzymes are produced to mitigate oxidative stress induced by heavy metal pollution (Rehman et al., 2019). At the high contents treatment of Cd-contaminated soils used in this study, the SOD and CAT activities of alligator flag and rice all decreased as the soil Cd content increased. These results are consistent with those of previous studies, in which the SOD and CAT activities decreased at high soil heavy metal levels due to the heavy metal stress (Devi and Prasad, 1998; He et al., 2014). The POD activity increased in the rice and decreased in the alligator flag at high soil Cd levels. This could be because the rice was more sensitive to Cd that poses a risk to the cytomembrane of rice, causing the POD production for ROS scavenging. If some Cd^{2+} ions were translocated to the aboveground parts of alligator flag, then the leaves of alligator flag would not undergo as much stress as rice. Cd enters plants from the soil and crosses the root barrier through symplasmic or apoplasmic pathways before entering the xylem and being translocated to the shoot (Lux et al., 2010). Importantly, alligator flag differs from rice in its root systems: alligator flag is a macrophyte that possesses more biomass than rice both underground and aboveground, especially in the fine roots, which may contain more vacuoles that capture toxicants (Peng and Gong, 2014). The rhizome is also full of xylogen to protect the Cd transport upward (Lukačová et al., 2013). Also, the decline in POD and CAT activity may be due to the formation of a protein complex with the metals that would change the structure or integrity of the proteins (Mohan and Hosetti, 1997; Hou et al., 2007).

Plants that are under high-level antioxidant stress may produce more MDA, which is the decomposition product of the polyunsaturated fatty acids of biomembranes (Hou et al., 2007). In this study, the Cd stress caused significant increases in the MDA of the rice leaves, indicating that Cd stress could cause injury to the integrity of the cellular membrane and to the cellular components of rice (Shamsi et al., 2008; Nouairi et al., 2009). At Cd levels of 120 mg kg^{-1}, a significant decrease in MDA was observed, indicating that the integrity of the cellular membrane or cellular components may have been damaged or limited by the induced Cd stress. However, the increase in biomass of the rice by 43 % from initial Cd level of 50–120 mg kg^{-1} may suggest that the rice experienced relatively less stress at higher Cd levels. This change may have occurred because rice has extra defensive strategies that were activated at higher Cd stress above a certain threshold (Rascio et al., 2008; Nishizawa et al., 2016). Compared with the rice, alligator flag was less sensitive to Cd stress, and the concentration of MDA was lower in alligator flag, indicating that alligator flag was more tolerant to higher contents of Cd in the soil (Fig. 3).

4.4. Mass of Cd in plants and soil

The TM system extracted a larger mass of Cd from the soil to the plant tissues than the other two cropping systems. The alligator flag was not only better adapted to extract Cd because of its fine roots but also had a larger biomass (Table 4 and Fig. 4). Fe plaque may play an important role in the uptake of metals by aquatic plants, and may form on the root surface of aquatic plants such as alligator flag and rice (Jiang et al., 2009, 2011; Liu et al., 2018). The amount of Fe plaque could be positively correlated with root surface area and the number of root tips (Jiang et al., 2009). In this study, alligator flag exhibited a higher biomass of fine roots, which could represent higher root surface area and more root tips than rice. Hence, more Fe plaque may be generated in the rhizosphere of alligator flag, promoting the absorption of Cd (Fig. 4). The rice likely transferred more Cd to the shoots from the roots than alligator flag because some rice cultivars are potential hyperaccumulators of Cd (Ibaraki et al., 2009; Tezuka et al., 2010) and can produce metal-transporting transmembrane proteins (Tezuka et al., 2010). However, the alligator flag developed a more extensive root system than the rice. In addition, alligator flag is considered a Cd stabilizer and is a candidate for phytostabilization, because it had smaller TFs and larger BAFs (> 2) in the fine roots compared to the rice (Table 2). Therefore, alligator flag could be considered as a potential bio-stabilizer for Cd.

The TM system was the best cropping system to remove Cd from the Cd-contaminated soil because alligator flag produced more biomass than rice and accumulated more Cd in the fine roots. Compared to TM and RM, the RT systems extracted Cd the least efficiently, likely because less biomass was produced in the RT systems than the other two systems due to interspecific competition and potential allelopathy (Olofsdotter, 2001; Mulderij et al., 2006; Jabran, 2017). However, using TM separately could not sustain rice quantity safety (grain yields) due to the occupation of rice growing space in a paddyland totally by the alligator flag, and using RM separately could not realize the safe production of rice (quality safety) due to the Cd-contaminated soils, so our study suggests that RT system is possible to both remediate the Cd-contaminated soils and produce economic yields with two beneficial goals at the same time.

4.5. Limitations

Despite the fact that chloride (Cl) is a ubiquitous and mobile anion found in all natural soils and necessary for plants, the addition of $CdCl_2$ introduced exogenous Cl^- to the soil, which may have had potential impacts on the soil microbes and the experimental plants. Cl is a main ligand for heavy metals, especially Cd (Li et al., 1994). For example, studies reported that in soil, Cl^- is mobile, and its concentration is dependent on the soil porosity, and the Cl^- concentration in soils is another major factor that can determine Cd availability (Babich and Stotzky, 1978; Li et al., 1994; Weggler et al., 2004), and therefore impact soil microbes and plants. As reported by Liu et al. (2010) in their study, cadmium chloride (in $CdCl_2 \cdot 2.5H_2O$ solution) was applied to simulate a gradient of Cd-contaminated soils, and sodium chloride (NaCl) was also added to counterpoise Cl^- in different treatments. However, the addition of NaCl induced extra Na^+, and the elevation of NaCl in soil could be another factor that affects Cd phytoavailability. For instance, Mariem et al. (2014) reported that the addition existence of 200 mM NaCl significantly alleviated Cd toxicity symptoms by reducing Cd uptake through *Sesuvium portulacastrum* (Aizoaceae) in saline soils. Therefore, further efforts are needed to distinguish the relative effects of Cd^{2+} ions and Cl^- anions in $CdCl_2$ simulated soils.

5. Conclusion

In an intercropped rice–alligator flag (RT) system, Cd contents in the harvested rice grains were significantly reduced at low initial soil levels of Cd (2.5–5.0 mg kg^{-1}). This reduction was enough to meet the China food safety standard (< 0.2 mg kg^{-1}) when the initial soil Cd content was below 2.5 mg kg^{-1}. This decrease was achieved because the fine roots of alligator flag take up large amounts of Cd from the rhizosphere soil of the rice, decreasing the Cd uptake by the rice. Alligator flag is likely an optimal phytostabilization plant for Cd remediation. Although significant variation in soil nutrients (NH_4-N and NO_3-N) were found in the alligator flag cropping systems (TM and RT), Cd content was the main factor restricting microbial biomass and community composition. Intercropping alligator flag with rice would therefore make it possible to effectively remediate moderate Cd-contamination farmland with a huge potential of more than 10 million hectares in China, and also to safely produce rice at the same time. Although alligator flag failed to maintain the rice grains meeting China food safety standard in the RT system at higher concentrations of Cd contamination, it still has potential for use as a phytoremediation plant in previously mined area.

Declaration of Competing Interest

The authors declared they had no conflict of interest.

CRediT authorship contribution statement

Jiaxin Wang: Methodology, Formal analysis, Writing - original draft, Investigation, Conceptualization. **Xuening Lu:** Formal analysis, Writing - review & editing. **Jiaen Zhang:** Conceptualization, Supervision, Project administration, Writing - review & editing. **Ying Ouyang:** Supervision, Writing - review & editing. **Guangchang Wei:** Investigation. **Yue Xiong:** Investigation.

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