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Does arbuscular mycorrhizal fungus affect cadmium uptake and chemical forms in rice at different growth stages?

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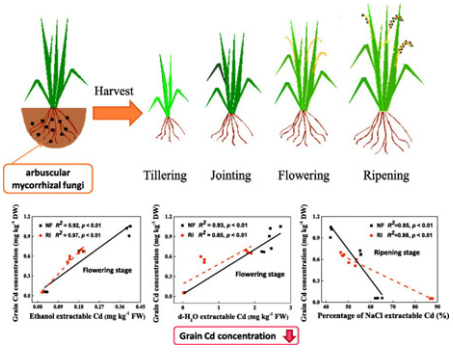
HIGHLIGHTS

- AMF reduced concentrations of ethanol and d-H_2O extractable Cd at flowering stage.
- Ethanol or d-H_2O extractable Cd conc. was positively correlated with grain Cd conc.
- AMF elevated the proportions of NaCl extractable Cd markedly at ripening stage.
- The proportions of NaCl extractable Cd were negatively correlated to grain Cd conc.
- Flowering and ripening stages are critical periods for AMF to limit grain Cd uptake.

GRAPHICAL ABSTRACT



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ABSTRACT

Effects of the arbuscular mycorrhizal fungus (AMF) - *Rhizophagus intraradices* (a mix of root pieces, mycelium pieces and spores) on the temporal variation of Cd uptake and chemical forms in rice at four growth periods (tillering, jointing, flowering, and ripening stages) were investigated in soil added with 0, 2 and 10 mg Cd kg^{-1}. Results showed that the interactions amongst rice growth stages, soil Cd concentrations and mycorrhizal inoculation had significant effects ($P < 0.001$) on root biomass, straw and root Cd concentrations, and straw Cd chemical forms in rice. Root colonization rates fluctuated with growth stages, reaching its peak at jointing stage and then decreasing at flowering and ripening stages. AMF increased the grain yield in rice plant grown in soil added with 10 mg Cd kg^{-1}, whereas no effect was found in soil added with 2 mg Cd kg^{-1}. In soil added with 2 mg Cd kg^{-1}, the concentrations of ethanol and d-H_2O extractable Cd at flowering stage was significantly reduced in mycorrhizal treatments, which subsequently induce less Cd accumulation in grains due to the positive correlations between ethanol or d-H_2O extractable Cd and grain Cd concentrations at flowering stage. In soil added with 10 mg Cd kg^{-1}, AMF significantly elevated the proportions of NaCl extractable Cd at ripening stage which also lead to the reduced grain Cd concentrations, since there was a negative correlation between the percentage of NaCl extractable Cd and grain Cd concentration at this stage. Our study indicated that flowering and ripening stages were important periods for AMF to limit the grain Cd concentrations in rice, when grown in Cd-contaminated soil.

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1. Introduction

Cadmium (Cd) is a toxic heavy metal released into the environment by natural processes and human activities, including disposal of industrial effluents and mining wastes, and agricultural application of sewage sludge or phosphate fertilizer (Gallego et al., 2012). Cadmium pollution in rice paddy fields has been identified in many Asian countries such as India, Thailand, and China (Mori et al., 2016). Rice (*Oryza sativa* L.) is one of the most important crops in the world, especially in Asia. The Cd accumulated in rice grains can enter human body via food chains, leading to various illnesses, including gastroenteritis, renal tubular dysfunction, hypertension, cardiovascular disease, pulmonary emphysema, cancer and osteoporosis (Wagner, 1993). Therefore, Cd contamination in rice has become a major public concern worldwide. The strategy of development for growing rice safely in the presence of Cd contamination may help to counteract the detrimental effects of Cd.

Arbuscular mycorrhizal fungi (AMFs) are widely distributed in the agricultural ecosystem and can form symbiotic associations with most terrestrial plant species (Garg and Chandel, 2011). They play a significant role in the recycling of plant nutrients, maintenance of soil structure, detoxification of noxious chemicals, control of plant pests, and growth regulation of plants and their interactions with the soil environment (Zhang et al., 2006). Most importantly, AM fungi can affect metal uptake by plants from soil and translocation from root to shoot (Chen et al., 2007; Dong et al., 2008; Li et al., 2015). AMF mycelium had high cation exchange capacity and metal sorption capacity and this property was influenced by adaptation to high levels of heavy metals (Takács and Vörös, 2003). The metal non-adapted AM could colonize the contaminated soils and decrease heavy metal uptake by perennial ryegrass (*Lolium perenne*) in a soil artificially contaminated by Cd, Ni and Zn were also demonstrated by Takács and Vörös (2003). Arbuscular mycorrhizal fungi may often lower Cd mobility and toxicity by increasing soil pH (Shen et al., 2006), sequestering Cd inside extra-radical mycelium (Janoušková and Pavlíková, 2010), and binding Cd to glomalin which is an insoluble glycoprotein synthesized and released by AMF (González-Chávez et al., 2004; Wright and Upadhyaya, 1998). In addition, AMF reduced the Cd concentrations markedly in the cell wall and vacuoles fractions of rice which contributed to Cd detoxification (Li et al., 2016). There are a few studies on the effects of AMF on Cd accumulation in plants, e.g., Tomato (*Solanum lycopersicum* L.) (Hashem et al., 2016), Pakchoi (*Brassica chinensis* L.) (Wu et al., 2016) and *Miscanthus* × *giganteus* (Firmin et al., 2015), but only two reports about the role of AMFs in Cd uptake by rice are: (1) Zhang et al. (2005) investigated the role of three species of AMFs (*Glomus versiforme*, *Funneliformis mosseae* and *Rhizophagus diaphanus*) on the uptake of copper (Cu), zinc (Zn), lead (Pb) and Cd by two rice cultivars in co-contaminated soil; (2) our previous study indicated that AMF could reduce Cd uptake by rice exposed to Cd solutions for three days (Li et al., 2016). Nevertheless, the role of AMF on the behavior of Cd in rice during the whole growth stages has not been fully understood.

The phytotoxicity of Cd and capacity of plant Cd tolerance are closely related to the stored forms of Cd and their mobility in plant tissues (Zeng et al., 2011; Yu et al., 2012). Chemical forms of Cd obtained from different extractants, including inorganic, water soluble, pectate and protein-integrated, undissolved phosphate, and oxalate forms, indicate the complex forms and mobility of Cd (Lai, 2015). For example, inorganic form and water-soluble form have a higher migratory capacity and are more toxic to plants (Fu et al., 2011). Therefore, the distribution of chemical forms could be one of the most important heavy metal detoxification mechanisms in plants. It has been reported that AMF might enhance the tolerance to Cd of *Medicago sativa* L. by altering Cd chemical forms in different plant tissues (Wang et al., 2012). Moreover, Su et al. (2014) noted that the percentage of pectate and protein-integrated Cd was negatively and exponentially related to the percentage of Cd in shoots [100 × shoot biomass × $[Cd]_{shoot}$ / (shoot biomass × $[Cd]_{shoot}$ + root biomass × $[Cd]_{root}$)] and Cd translocation factors from roots to shoots of peanut.

It is also worth noting that chemical forms of Cd may change in amount and composition during plant growth periods (Lai and Cai, 2016), and this variation may ultimately influence Cd concentrations in grains. An extended growth period of *Impatiens walleriana* affected the chemical forms of Cd in different plant tissues have been reported by Lai and Cai (2016). For instance, Cd in the stems was mainly in the ethanol and NaCl extracted forms at day 25, but mainly in the ethanol and deionized water extracted forms at day 50 (Lai and Cai, 2016). Therefore, a critical investigation on the chemical forms of Cd and their relationship with Cd accumulation in rice during the whole growth stages will certainly contribute to better understanding of the role of AMF in rice.

To the best of our knowledge, there is a lack of information on how AMF would change the chemical forms over the entire growing period of rice, and effect the Cd accumulation and translocation in grains. Therefore, a long-term pot experiment was conducted to investigate: 1) the effects of AMF [*Rhizophagus intraradices* (*R. intraradices*)] on the growth, Cd uptake and chemical forms in rice at four different growth stages; 2) the relationships between different chemical forms of Cd and grain Cd concentrations; and 3) the critical growth stage in which AMF play a role in rice.

2. Materials and methods

2.1. Plant cultivation

Upland rice (Hanyou 3) seeds were obtained from Henan Academy of Agricultural Sciences, Zhengzhou, PR China. They were sterilized with 10% H_2O_2 for 15 min, and then washed with sterile water. Subsequently, they were germinated on moist filter papers and grown in 20% Hoagland-Arnon nutrient solution (Hoagland and Arnon, 1950). After 1 week, the uniform seedlings of rice (height: 12 cm) were used for the pot trial. Seed germination and plant cultivation were conducted in a temperature-controlled (28/22 °C, day/night) greenhouse with a relative humidity of 85%, under a random block design.

2.2. Soil preparation

Soil was collected from paddy fields at the campus of South China Agricultural University, Guangzhou, PR China. The soil contained 8.1% organic matter, 1.3 g kg^{-1} total N, 1.1 g kg^{-1} total P, and 0.61 g kg^{-1} total K, respectively, with a pH value of 5.8 (Li et al., 2016). The background Cd concentration in soil was 0.18 mg kg^{-1}. After air-drying for 2 weeks, the soil was sieved through a 2-mm mesh and then autoclaved at 121 °C for 120 min, under airtight condition, in order to eliminate the indigenous AMF. Subsequently, the air-dried soil was added with and without 2 (low) or 10 (high) mg Cd kg^{-1} (supplied as $CdCl_2 \cdot 5/2H_2O$). According to the environmental quality standards for soils (GB 15618-1995) in China, the maximum contaminant level for Cd in farmland is 1.0 mg kg^{-1}. The low Cd soil concentration was fixed 2 mg Cd kg^{-1} due to the fact that the Cd contamination is widespread in China, with concentration often exceeded 2 mg kg^{-1}. The treatment of 10 mg Cd kg^{-1} soil used was intended to study the effects of AMF on Cd uptake by rice, under such an extreme condition. The Cd added soil was subjected to three cycles of saturation with deionized water for 3 weeks, mixed every day, and then air-dried for 1 week in the greenhouse, in order to enhance the distribution and stabilization of Cd throughout the entire soil mass (Liu et al., 2015). The final soil Cd concentrations in each treatment were 0.16, 2.09 and 10.05 mg kg^{-1}, respectively, prior to plant cultivation.

2.3. Pot experiment

Two uniform cultivated seedlings (height: 12 cm) were transferred into each pot (15 cm in diameter, 18 cm in height), which contained 1.5 kg soil. Hanyou 3 inoculated with *R. intraradices* was employed due to their preferred performance with enhanced grain yield and less Cd uptake in Cd-contaminated soils (unpublished data). Some of the seedlings were inoculated with 50 g *R. intraradices* inocula obtained from Mycagro Lab (France). The granular inoculum is a mix of root pieces, mycelium pieces and spores. The quality of inoculum is a minimum of 10 infectious propagules (spores, myceliums and roots)/g of inoculum. Non-mycorrhizal controls received an equivalent amount of sterilized inoculum to provide similar conditions. The inoculum was mixed with the 1/3–2/3 depth soil, which is more effective in infecting the roots of seedlings. The pot experiment was conducted in a completely randomized design with a $3 \times 2 \times 12$ factorial scheme with the following variables: three soil Cd concentrations (0, 2 and 10 mg kg^{-1}), two treatments (inoculation without or with *R. intraradices*), and twelve replicates in each treatment, for a total of 72 pots. Soil was maintained at 80% holding capacity during the growing period. Hoagland-Arnon nutrient solution (20%) (Hoagland and Arnon, 1950) was added into each pot every week, in order to maintain an adequate soil nutrient level.

2.4. Sampling

After transplantation, a quarter of plants (three pots for each treatment) were harvested at their tillering stage (day 45), jointing stage (day 75), flowering stage (day 105) and ripening stage (day 145), respectively. They were rinsed thoroughly with deionized water to remove any attached soil/substrate particles before separating into straws and roots. The plants at ripening stage were separated into grains, straws, and roots. After measuring the fresh weight of these tissues, root subsamples were stored in 50% ethanol for mycorrhizal colonization assessment. One part of fresh straws was frozen in liquid N_2 and stored at −80 °C for the analyses of chemical forms.

2.5. Mycorrhizal and total Cd analysis

The cleaned roots stored in 50% ethanol were cut into 1 cm long segments, then rinsed in 10% (w/w) KOH at 90 °C for 1 h, bleached with fresh alkaline H_2O_2 solution (30 mL 10% H_2O_2 + 3 mL NH_4OH + 567 mL deionized water) for 20 min to 1 h, acidified with 1% HCl (1–4 min) and stained with 0.05% Trypan Blue (Phillips and Hayman, 1970; Li et al., 2011). The arbuscular mycorrhizal colonization rates were calculated by the grid-line intersects method under a compound microscope (40×10) (Giovannetti and Mosse, 1980).

Subsamples of grains, straws, and roots were dried at 80 °C to a constant weight for determining dry weight. The dried straws, roots and grains of rice were used to analyze Cd concentrations by an Atomic Absorption Spectrometer (AAS, PinAAcle 900 T, Perkin Elmer, USA), after grinded and digested in an acid mixture of HNO_3:$HClO_4$ (4:1, v/v) at 225 °C.

2.6. Extraction of Cd in different chemical forms

Chemical forms of Cd in rice straws were extracted in the following order: (1) 80% ethanol, for extracting inorganic Cd giving priority to nitrate/nitrite, chloride and aminophenol Cd; (2) deionized water (d-H_2O), for extracting water soluble Cd-organic acid complexes and Cd ($H_2PO_4)_2$; (3) 1 M NaCl, for extracting pectates and protein integrated Cd; (4) 2% acetic acid (HAc), for extracting undissolved cadmium phosphate including Cd_2HPO_4 and $Cd_3(PO_4)_2$ and other Cd-phosphate complexes; (5) 0.6 M HCl, for extracting cadmium oxalate; and (6) the residual (Fu et al., 2011).

Frozen straw tissues (0.5 g) were homogenized in extraction solution with a mortar and a pestle, diluted at a ratio of 1:100 (w/v), and shaken for 22 h at 25 °C. The homogenate was then centrifuged at $5{,}000 \times g$ for 10 min, obtaining the first supernatant solution in a conical beaker. The sedimentation was re-suspended twice in extraction solution, shaken for 2 h at 25 °C, and centrifuged at $5{,}000 \times g$ for 10 min. The supernatants of these three suspensions were then pooled. The residue was extracted with the next extraction solution in the sequence using the same procedure described above. Each pooled solution was evaporated on an electric plate at 70 °C to a constant weight, digested at 145 °C with an acid oxidative mixture of HNO_3:$HClO_4$ (4:1, v/v) and then determined by an Atomic Absorption Spectrometer (AAS, PinAAcle 900 T, Perkin Elmer, USA). To ensure the accuracy of metal determinations, the standard material [GBW07602 (GSV-1)] (obtained from China Standard Materials Research Center, Beijing, PR China) was used. The recovery rates of Cd were within (90 ± 10)%.

2.7. Statistical analysis

All results were tested by three-way ANOVA analysis of variance using the SPSS statistical package and all figures were drawn using the PC-based Origin program. Tukey tests at 5% probability were used for post HOC comparisons to test for differences. Relationships between the chemical forms of Cd and Cd concentrations in rice grains were determined by linear regression analysis (Su et al., 2014).

3. Results

3.1. Root colonization rates by AMF

In this study, growth stages had notable effects ($P < 0.001$) whereas Cd concentrations had no prominent impacts ($P > 0.05$) on root colonization rates by AMF (Table S1). Very low colonization rates [(1 ± 0.5)%] were found in the non-mycorrhizal rice (data not shown). Generally, the colonization rates increased from tillering stage (35.0–38.1%) to jointing stage (62.3–66.5%) and then decreased (40.0–43.0%) at ripening stage (Fig. 1).

3.2. Plant growth

Plant growth stages exerted significant effects ($P < 0.001$) on both straw and root biomass of rice (Table S1). In general, the root biomass of different treatments reached the maximum at ripening stage in soil with or without Cd addition, while the straw biomass tended to increase from tillering to jointing stage and then decreased at ripening stage (Table 1). The mycorrhizal inoculation had a significant effect on straw biomass ($P < 0.001$), but not on root biomass ($P > 0.05$) (Table S1). In soil with or without Cd addition, mycorrhizal inoculation generally had no negative effects on the straw biomass over the entire growth period, but significantly increased it at flowering stage in soil with 10 mg Cd kg^{-1} (Table 1). Root biomass was profoundly enhanced at tillering and jointing stage in soil with 2 mg Cd kg^{-1} (Table 1). As shown in Fig. 2, at ripening stage, the grain yield was reduced from 3.72 to 1.05 g pot^{-1} for non-mycorrhizal rice and from 5.49 to 1.79 g pot^{-1} for mycorrhizal rice, respectively, with the increase of Cd addition from 0 to 10 mg kg^{-1} in soil. Moreover, a positive effect of AMF inoculation was observed in grain yield that increased by 1.48 and 1.70 fold in mycorrhizal treatments, respectively, in soil added with 0 and 10 mg Cd kg^{-1} (Fig. 2).

3.3. Cd uptake by rice

The Cd concentrations in soil, growth stages of rice, AMF, and the interaction amongst them all had marked influences on straw and root Cd concentrations ($P < 0.001$) (Table S1). Concentrations of Cd in straws and roots were enhanced with an increase of soil Cd addition (Fig. 3b

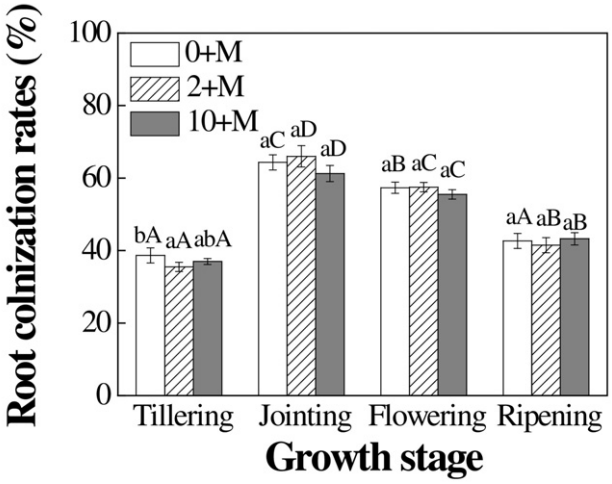

Fig. 1. Root colonization rates (%) of rice inoculated (+M) with *R. intraradices* grown in soil added with 0, 2 or 10 mg Cd kg^{-1} at different growth stages (mean ± S.D., n = 3). Different lowercase letters indicate significant differences under different Cd treatments at same growth stage at the level of $P < 0.05$ (Tukey test). Different uppercase letters indicate significant differences amongst different growth stages with the same Cd addition at the level of $P < 0.05$ (Tukey test).

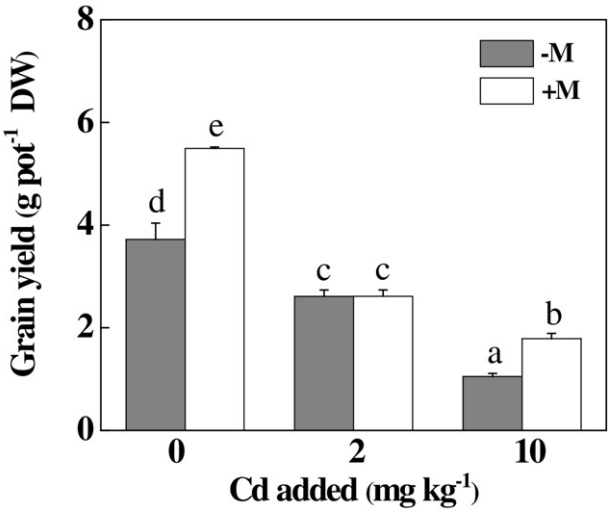

Fig. 2. Grain yield of rice uninoculated (−M) or inoculated (+M) with *R. intraradices* grown in soil added with 0, 2 or 10 mg Cd kg^{-1} (mean ± S.D., n = 3). Different letters indicate significant differences under all the treatments at the level of $P < 0.05$ (Tukey test).

and c). During the whole growth stages, a decreasing trend was observed in root Cd concentrations in high Cd-contaminated soil (10 mg Cd kg^{-1}) (Fig. 3c), while straw Cd concentrations tended to increase with the extension of growth period in Cd-added soil (Fig. 3b). In Cd-contaminated soil, AMF reduced Cd concentrations in straws and roots during the four stages, with an exception in root Cd concentration at tillering stage in soil added with 2 mg Cd kg^{-1} (Fig. 3b and c). This reduction was the most notable in high Cd-contaminated soil (10 mg Cd kg^{-1}), in which the root Cd concentration decreased up to 46% at tillering stage and the straw Cd concentration reduced up to 43% at ripening stage. Furthermore, the concentrations of Cd in the grains of non-mycorrhizal rice were 0.059, 0.69 and 0.99 mg kg^{-1} in soil with 0, 2 and 10 mg Cd kg^{-1}, which were 1.34, 1.26, and 1.46 times higher, respectively, than those of mycorrhizal treatments (Fig. 3a).

3.4. Chemical forms of Cd

Generally, Cd concentrations in soil, growth stages, AMF, and their interactions had significant effects ($P < 0.001$) on both the concentrations and proportions of different chemical forms of Cd in straws (Table S1). Under all treatments, Cd concentrations of different chemical forms increased with the increment of Cd addition (Table 2), which was in consistence with the results of total Cd concentrations in straws (Fig. 3b). In Cd-contaminated soil, NaCl extractable Cd (pectate and protein integrated Cd) were predominant in both non-mycorrhizal and mycorrhizal rice at jointing, flowering and ripening stages, ranging from 32.3% to 55.4% (Fig. 4b and c). The concentrations of NaCl extractable Cd continued to increase significantly with the extension of growth period in soil with 0 and 2 mg Cd kg^{-1}, whereas they increased from jointing to flowering stage and then declined at ripening stage in soil with 10 mg Cd kg^{-1}, both for non-mycorrhizal and mycorrhizal treatments (Table 2). AMF decreased the concentrations of ethanol extractable Cd significantly at flowering stage in soil with 2 mg Cd $kg^{-1}$and at jointing, flowering and ripening stages in soil with 10 mg Cd kg^{-1}. Similarly, the concentrations of d-H_2O extractable Cd at flowering stage in soil with or without Cd addition and at ripening stage in soil with 0 and 10 mg Cd kg^{-1} were significantly reduced in mycorrhizal treatments (Table 2). The concentrations of HAc and HCl extractable Cd and the residues were significantly reduced by AMF at ripening stage in soil with 2 mg Cd kg^{-1}, and at tillering, jointing and ripening stages in soil with 10 mg Cd kg^{-1} (Table 2). The mycorrhizal inoculation increased the proportion of NaCl extractable Cd at flowering stage in soil with or without Cd contamination, while decreased the sum proportion of ethanol and d-H_2O extractable Cd at the same growth stage in soil with 0 and 2 mg Cd kg^{-1}(Fig. 4). In soil added with 10 mg Cd kg^{-1}, the proportion of ethanol extractable Cd was reduced in mycorrhizal treatments at jointing, flowering and ripening stages (Fig. 4c).

Furthermore, significant correlations ($P < 0.01$) were observed between ethanol, d-H_2O, or NaCl extractable Cd and grain Cd

Table 1
Fresh weight of roots and straws (g pot^{-1}) of rice uninoculated (−M) or inoculated (+M) with *R. intraradices* at different growth stages in soil added with 0, 2 or 10 mg Cd kg^{-1} (mean ± S.D., n = 3).

Tissue	Stage	0 − M	0 + M	2 − M	2 + M	10 − M	10 + M
Root	Tillering	4.51 ± 0.35aA	5.32 ± 0.17aA	3.30 ± 0.08aA	4.26 ± 0.21aB	3.32 ± 0.13aA	3.17 ± 0.15aA
	Jointing	6.50 ± 0.18bA	6.71 ± 0.14bA	6.05 ± 0.10bA	6.47 ± 0.18cdB	4.36 ± 0.24bB	3.60 ± 0.04bA
	Flowering	6.96 ± 0.11bcA	7.41 ± 0.24cA	6.15 ± 0.13bA	6.28 ± 0.19bA	4.51 ± 0.40bA	4.71 ± 0.12cA
	Ripening	7.48 ± 0.39cA	7.42 ± 0.33cA	6.41 ± 0.29bA	6.86 ± 0.09cA	4.66 ± 0.30bA	5.10 ± 0.18cA
Straw	Tillering	18.68 ± 1.21aA	22.51 ± 0.83aB	13.98 ± 0.89aA	15.55 ± 0.56aA	14.38 ± 0.63aA	16.45 ± 0.97aA
	Jointing	27.55 ± 1.25bA	29.49 ± 0.66bA	26.55 ± 1.09cA	28.30 ± 0.87cA	22.02 ± 0.53cA	22.87 ± 0.74cA
	Flowering	28.00 ± 1.23bA	28.95 ± 2.17bA	24.43 ± 0.62bcA	24.25 ± 1.00bA	17.57 ± 0.46bA	21.51 ± 1.12bcB
	Ripening	25.70 ± 0.55bA	28.33 ± 0.58bB	22.81 ± 1.03bA	24.05 ± 1.19bA	20.92 ± 1.41cA	20.44 ± 0.62bA

The different lowercase letters represented significant difference at $P < 0.05$ level in roots or straws at different growth stages in non-mycorrhizal or mycorrhizal treatments with the same Cd addition. The different uppercase letters represented significant difference at $P < 0.05$ level in roots or straws between non-mycorrhizal and mycorrhizal treatments with the same Cd addition at same growth stage.

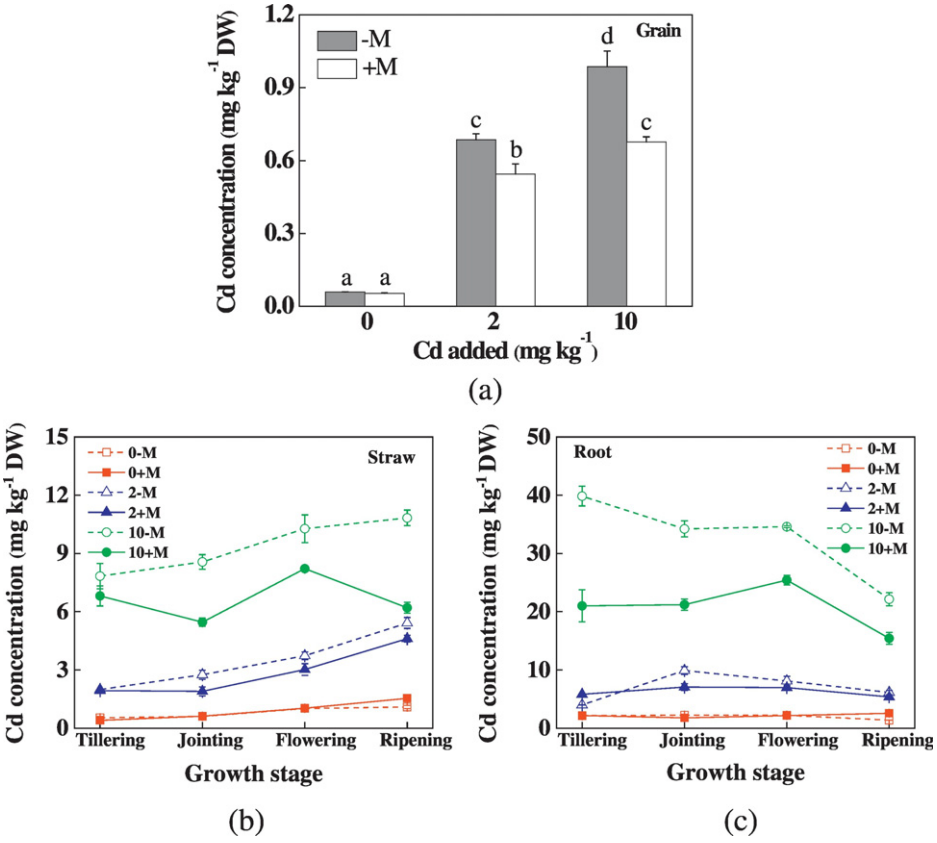

Fig. 3. Grain (a), straw (b) and root (c) Cd concentrations of rice uninoculated (−M) or inoculated (+M) with *R. intraradices* grown in soil added with 0, 2 or 10 mg Cd kg^{-1} (mean ± S.D., n = 3). Different letters indicate significant differences under all the treatments at the level of $P < 0.05$ (Tukey test).

concentrations at different growth stages both in non-mycorrhizal and mycorrhizal treatments (Fig. 5). A high degree of positive linear correlation ($P < 0.01$) was found between ethanol extractable Cd and grain Cd concentration at jointing, flowering and ripening stages (Fig. 5a–c). Similarly, significant correlations ($P < 0.01$) were also noted between d-H_2O extractable Cd and grain Cd concentrations at flowering and ripening stage (Fig. 5d and e). In contrast, the percentage of NaCl extractable Cd showed a negative linear correlation ($P < 0.01$) with grain Cd concentration at ripening stage (Fig. 5f).

4. Discussion

The present study showed that the AMF (*R. intraradices*) was able to colonize the roots of rice under Cd-contaminated conditions, and

Table 2
Concentrations of different chemical forms (mg kg^{-1}) in straws of rice uninoculated (−M) or inoculated (+M) with *R. intraradices* at different growth stages in soil added with 0, 2 or 10 mg Cd kg^{-1}.

Treatment	Growth stage	$F_{ethanol}$ (mg kg^{-1})	$F_{d\text{-}H2O}$ (mg kg^{-1})	F_{NaCl} (mg kg^{-1})	F_{Hac} (mg kg^{-1})	F_{HCl} (mg kg^{-1})	$F_{residue}$ (mg kg^{-1})
0 − M	Jointing	0.025 ± 0.002bA	0.049 ± 0.006aA	0.045 ± 0.008aA	0.036 ± 0.009aA	0.069 ± 0.002cA	0.076 ± 0.007bA
	Flowering	0.035 ± 0.006cA	0.046 ± 0.005aB	0.209 ± 0.008bA	0.031 ± 0.001aB	0.059 ± 0.001bB	0.005 ± 0.0004aA
	Ripening	0.015 ± 0.0003aA	0.085 ± 0.005bB	0.282 ± 0.015cA	0.029 ± 0.001aA	0.029 ± 0.0005aB	0.004 ± 0.0007aA
0 + M	Jointing	0.051 ± 0.00bB	0.051 ± 0.007cA	0.117 ± 0.007aB	0.029 ± 0.005bA	0.080 ± 0.001cB	0.081 ± 0.001cA
	Flowering	0.020 ± 0.001aA	0.024 ± 0.002bA	0.306 ± 0.013bB	0.012 ± 0.001aA	0.035 ± 0.002bA	0.007 ± 0.0004aB
	Ripening	0.025 ± 0.005aA	0.013 ± 0.001aA	0.657 ± 0.021cB	0.036 ± 0.002cB	0.014 ± 0.003aA	0.009 ± 0.0007bB
2 − M	Jointing	0.170 ± 0.017aA	0.208 ± 0.012aA	1.714 ± 0.072aB	0.735 ± 0.038aA	1.033 ± 0.103cB	0.049 ± 0.006cA
	Flowering	0.187 ± 0.018aB	2.228 ± 0.190cB	2.017 ± 0.098bB	0.771 ± 0.027aB	0.163 ± 0.012bA	0.013 ± 0.002aA
	Ripening	0.354 ± 0.018bA	1.002 ± 0.036bA	2.721 ± 0.031cB	0.783 ± 0.090aB	0.056 ± 0.003aB	0.037 ± 0.003bB
2 + M	Jointing	0.160 ± 0.014aA	0.178 ± 0.025aA	1.086 ± 0.081aA	0.832 ± 0.081cA	0.685 ± 0.064cA	0.037 ± 0.008bA
	Flowering	0.140 ± 0.008aA	0.586 ± 0.062bA	1.839 ± 0.043bA	0.585 ± 0.039bA	0.149 ± 0.009bA	0.023 ± 0.001aB
	Ripening	0.438 ± 0.018bB	0.989 ± 0.065cA	2.052 ± 0.094cA	0.434 ± 0.049aA	0.022 ± 0.002aA	0.016 ± 0.003aA
10 − M	Jointing	1.102 ± 0.045bB	1.518 ± 0.185aA	2.520 ± 0.035aB	1.523 ± 0.092bB	1.081 ± 0.044bB	0.068 ± 0.006bB
	Flowering	0.403 ± 0.011aB	2.514 ± 0.142bB	4.454 ± 0.080cB	1.415 ± 0.052bB	0.218 ± 0.006aB	0.018 ± 0.002aB
	Ripening	1.081 ± 0.075bB	2.991 ± 0.117cB	3.960 ± 0.085bB	1.032 ± 0.042aB	0.206 ± 0.005aB	0.061 ± 0.010bB
10 + M	Jointing	0.377 ± 0.018bA	1.494 ± 0.212aA	1.883 ± 0.033aA	1.030 ± 0.119cA	0.654 ± 0.021bA	0.051 ± 0.008bA
	Flowering	0.197 ± 0.016aA	1.846 ± 0.082bA	2.874 ± 0.065cA	0.532 ± 0.007bA	0.073 ± 0.005aA	0.012 ± 0.001aA
	Ripening	0.418 ± 0.018cA	2.011 ± 0.138bA	2.488 ± 0.144bA	0.284 ± 0.015aA	0.056 ± 0.005aA	0.007 ± 0.0008aA

The different lowercase letters represented significant difference at $P < 0.05$ level in roots or straws at different growth stages in non-mycorrhizal or mycorrhizal treatments with the same Cd addition. The different uppercase letters represented significant difference at $P < 0.05$ level in roots or straws between non-mycorrhizal and mycorrhizal treatments with the same Cd addition at same growth stage.

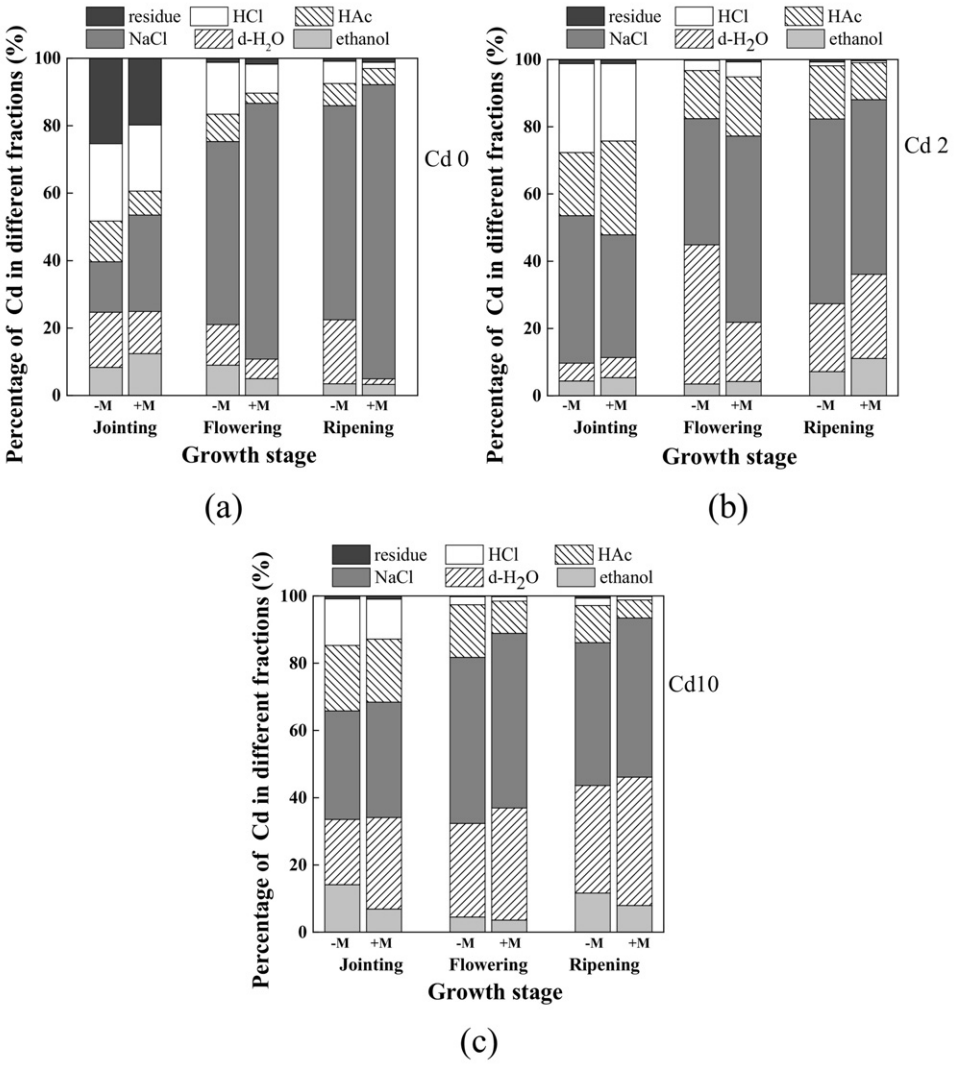

Fig. 4. Percentage of different chemical forms in straws of rice uninoculated (−M) or inoculated (+M) with *R. intraradices* at different growth stages in soil added with (a) 0 mg Cd kg^{-1}, (b) 2 mg Cd kg^{-1}, or (c) 10 mg Cd kg^{-1}.

the increase of Cd addition in soil did not affect root colonization rates ($P > 0.05$) (Table S1). It is consistent with the results of Chen et al. (2004), who found no significant effects of Cd addition in soil (25 and 100 mg kg^{-1}) on the root colonization rates of maize by *Funneliformis mosseae* (Chen et al., 2004). Jiang et al. (2016) also reported that mycorrhizal colonization rates of *Lonicera japonica* by *Glomus versiforme* and *R. intraradices* were hardly affected by Cd addition from 0 to 20 mg Cd kg^{-1}. However, studies showed that high Cd in soil had a strong effect on AMF development and decreased mycorrhizal colonization rates (Shahabivand et al., 2012; Wu et al., 2016). These opposite results demonstrated that different levels of compatibility between host plants and AMFs might occur in Cd-contaminated soil (Zhang et al., 2005). In addition, it was found that the colonization rates fluctuated with growth stages, reaching their peak at jointing stage and then decreasing at flowering and ripening stages (Fig. 1). Wang et al. (2012) also observed a similar trend in alfalfa (*Medicago sativa* L.) that the root colonization rates by *R. intraradices* increased from 17% at day 25 to 69% at day 60 and then decreased to 43% after 80 days, when exposed to 20 mg Cd kg^{-1} in soil. These results were also in accordance with the temporal changes of straw and root biomass which were relatively low at tillering stage and increased dramatically at jointing stage (Table 1). No significant correlations were found between mycorrhizal colonization rates and Cd concentrations in grains, straws or roots (data not shown). This suggests the AMFs constitute an important functional component of the soil-plant system and the mechanisms cannot be explained by root colonization rates simply.

In general, the biomass of rice tissues decreased with increasing Cd addition in soil, which was due to the fact that the toxicity of Cd can greatly inhibit plant growth (Yang et al., 2016a, 2016b). In soil added with 2 mg Cd kg^{-1}, AMF significantly enhanced the root biomass at tillering and jointing stages, but had no effects on straw biomass and grain yield (Table 1 and Fig. 2). The inoculation of AMF significantly promoted straw biomass in soil with 10 mg Cd kg^{-1} addition at flowering stage (Table 1), and also increased grain yield at ripening stage (Fig. 2). Similar results were obtained by Chan et al. (2012), who reported that the grain yield of rice inoculated with *F. mosseae* grown in As-contaminated soil was significantly higher than non-mycorrhizal treatment ($P < 0.05$). The increase in shoot biomass of two upland rice cultivars (91B3 and 277) inoculated with *F. mosseae* when grown in soils contaminated with a mixture of heavy metals (Cu, Zn, Pb and Cd) was also demonstrated (Zhang et al., 2005). All these suggested the important contribution of AMF to plant growth under metal stress. At heavy metal contaminated sites, there is usually a short supply of necessary mineral nutrients (Peter, 2005). It is commonly known that the extensive extraradical hyphal network produced by AMF allows the plants

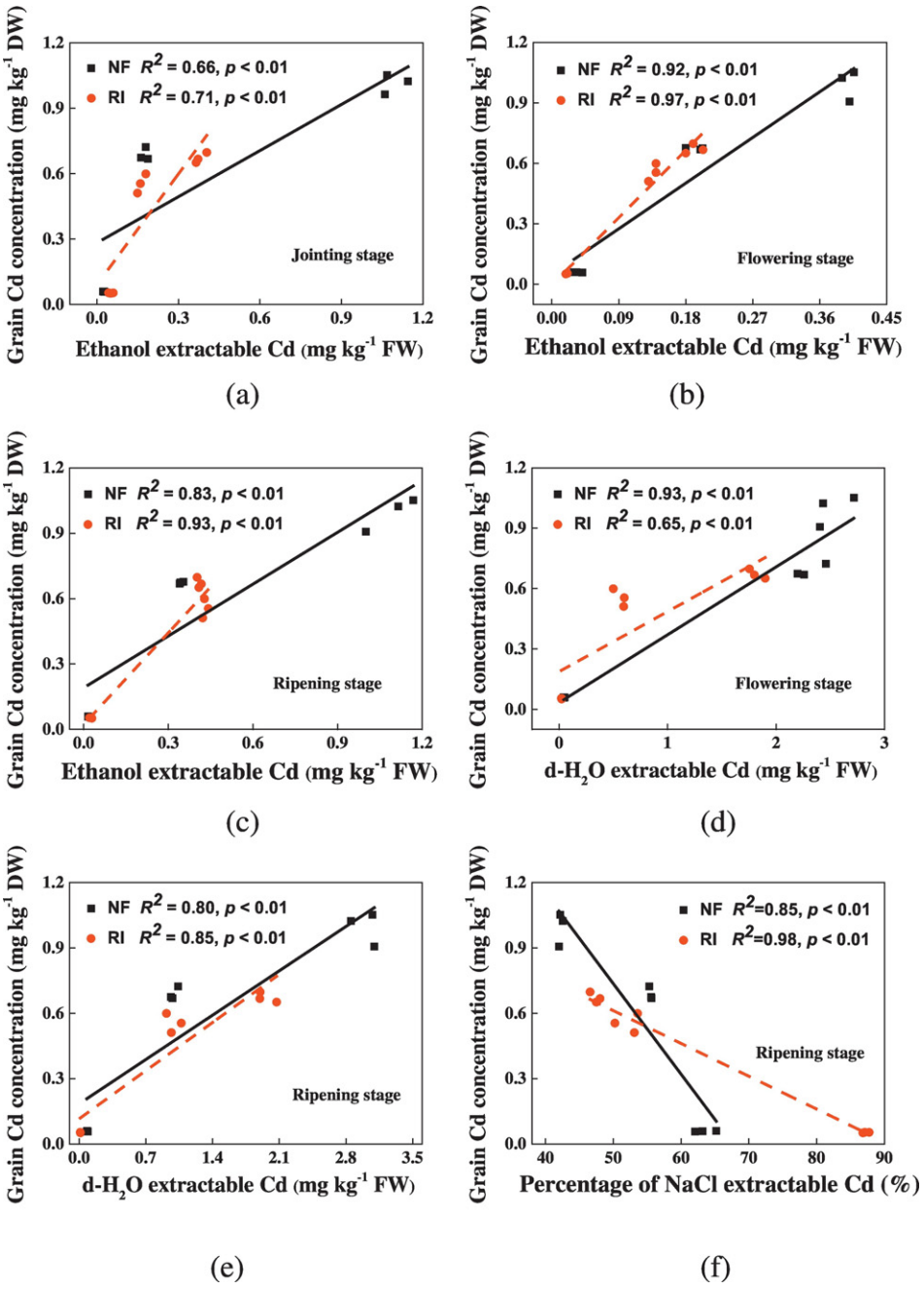

Fig. 5. Relationships between the chemical forms of Cd in straws and grain Cd concentrations of rice uninoculated (NF) or inoculated (RI) with *R. intraradices* at different growth stages.

to access a greater volume of the soil, enhancing plant nutrient (such as phosphorus) absorption and translocation (Garg and Chandel, 2011). Moreover, AMF can modify the architecture and topology of the root system, generally resulting in longer or more branched roots and hence more efficient in nutrient absorption (Garg and Chandel, 2011). Therefore, with the assistance of AMF, rice may obtain more nutrients and higher resistance to Cd.

Cadmium, with high soil-plant mobility, is easily accumulated in rice tissues. In our study, Cd concentrations in different rice tissues decreased following the order of roots > straws > grains (Fig. 3), which suggested a retention of Cd in its translocation from roots to straws, and finally to grains (Wang et al., 2008). This result is consistent with many studies about Cd accumulation in plants (Lehmann and Rebele, 2004; Wang et al., 2008), implying that the translocation of Cd to the aerial part is restricted by internal barriers in order to protect the aboveground part (Ramos et al., 2002). This strategy could be considered as an important tolerance mechanism of rice. Growth stage of rice, AMF, and their interactions all led to significant influences ($P < 0.001$) on straw and root Cd concentrations (Table S1). Li et al. (2012) also noted that rice growth period, AMF and the interaction amongst them had marked effects on shoot As (III) ($P < 0.05$) and As (V) ($P < 0.001$) concentrations and root total As concentrations ($P < 0.001$). The Maximum Contaminant Levels (MCLs) for Cd in rice grains was set at 0.2 mg kg^{-1} in China (Chinese Food Standards Agency, 2005). Although the Cd concentrations of rice grain regardless of AMF inoculation exceeded this standard both in low and high Cd-contaminated soils (2 and 10 mg Cd kg^{-1}), AMF could reduce the grain Cd concentrations significantly (Fig. 3a).

The reduction of Cd concentrations in straws and roots of mycorrhizal rice was more pronounced in soil with 10 mg Cd kg^{-1} (Fig. 3b and c). The increased straw biomass by AMF at flowering stages in high Cd-contaminated soil (10 mg Cd kg^{-1}) may account for the decreased

straw Cd concentrations at the same stages (Table 1 and Fig. 3b), due to the biomass dilution effect (Liu et al., 2011). Similarly, AMF inoculation significantly increased grain yield in high Cd-contaminated soil (10 mg Cd kg^{-1}), leading to the decreased grain Cd concentrations (Figs. 2 and 3a). In soil added with 2 mg Cd kg^{-1}, the decreased Cd concentration in grains by AMF may mainly attribute to the reduced Cd concentrations in straws at jointing, flowering and ripening stages. Glomalin, a glycoprotein exudated by AMF, can strongly and irreversibly sequester metals such as Cu, Cd, and Zn (González-Chávez et al., 2004). In addition, AMF may influence the related heavy metal transporter expressions. Chen et al. (2012) reported that relative mRNA expressions of Lsi1 and Lsi2 related to As transport were notably lower in rice colonized by *R. intraradices*, under different arsenite concentrations. Our previous study demonstrated that AMF could reduce Cd uptake by rice grown in Cd solutions, through altering subcellular distribution and chemical forms of Cd in rice (Li et al., 2016). AMF confer rice with improved ability to enhance rice growth and reduce Cd uptake, which also may be due to the Cd adsorption onto fungal cell walls or extraradical mycelium, Cd chelation by such compounds as siderophores and metallothioneins released by fungi, or Cd sequestration by plant-derived compounds like phytochelatins or phytates (Gaur and Adholeya, 2004).

Straw-to-grain translocation of Cd is also dependent on its chemical forms in rice straws. Different chemical forms of Cd can be isolated with different extracting agents and are associated with the Cd bioavailability in rice (Gao et al., 2013). Cadmium in inorganic form (extracted with 80% ethanol) and water-soluble form (extracted with d-H_2O) possesses a higher migratory capacity and is more damaging to rice cells than pectate- and protein-integrated Cd (extracted with 1 M NaCl), insoluble Cd phosphates (extracted with 2% HAC) and residual Cd (Wang et al., 2008; Zhang et al., 2009). This was verified by high degree of positive correlations between ethanol or d-H_2O extractable Cd in straws and Cd concentration in grains at flowering and ripening stages in our study (Fig. 5b–e). In contrast, Cd integrated with pectates and protein (extracted by 1 M NaCl) was hypothesized to be chelated by some specific polar materials, such as hydroxyl or carboxyl, to form a non-toxic complex, and is less damaged to plants (Fu et al., 2011). In the present study, most of the Cd in rice straws was in the forms of pectate and protein-integrated Cd (extracted by 1 M NaCl) (Fig. 4). This was also found in *Kandelia obovata* (Weng et al., 2012), *Bechmeria nivea* (Wang et al., 2008) and tomato (Zhang et al., 2009), suggesting the resistance of plants to Cd may happen in some cases (Yu et al., 2008; Wu et al., 2005).

At flowering stage, AMF inoculation significantly decreased the concentrations of ethanol and d-H_2O extractable Cd (Table 2), which led to less Cd accumulation in grains grown in Cd-contaminated soil (Fig. 3a). This may due to the positive correlation between ethanol or d-H_2O extractable Cd and grain Cd concentrations (Fig. 5b and d). Moreover, the percentage of NaCl extractable Cd at ripening stage was negatively correlated with grain Cd concentrations (Fig. 5f). At ripening stage, the increment in the proportion of NaCl extractable Cd by AMF in soil added with 0 and 10 mg Cd kg^{-1} (Fig.4a and c) corresponded to the significant decrease of grain Cd concentrations (Fig. 3a). It can be speculated that the decreased Cd concentration in grains by AMF inoculation may be attributed to the changes of the chemical forms of Cd in straws. In soil added with 0 and 2 mg Cd kg^{-1}, AMF played a significant role in decreasing the sum proportions of ethanol and d-H_2O extractable Cd at flowering stage. In high Cd-contaminated soil (10 mg Cd kg^{-1}), AMF reduced the proportions of ethanol extractable Cd at jointing, flowering and ripening stages. Our previous work (Li et al., 2016) also found that AMF might enhance the tolerance to Cd of rice by reducing the proportions of active forms (ethanol and d-H_2O extractable forms) in 0.05–0.1 mM Cd solutions. Plant growth stages, Cd concentrations in soil, and the interaction amongst them exerted profound impacts on biomass, Cd concentrations in rice straws and roots, and the concentrations and proportions of different chemical forms in straws ($P < 0.001$) (Table S1). This implied that Cd uptake, translocation and chemical forms are a dynamic and complicated process, which could not be simply explained by the total Cd concentration detected at a specific time. So far, there is no report regarding to the use of AMF in heavy metal contaminated paddy field. *R. intraradices* has been applied under field conditions for enhancing yield and alleviate heavy metal stress of some plants. Bissonnette et al. (2010) noted that the inoculation of *R. intraradices* decreased Cu and Cd transfer to shoots and increased Cu storage in roots of *S. viminalis* and *Populus* × *generosa* on a heavy metal contaminated site. Thus, *R. intraradices* may be a suitable candidate for employing in contaminated paddy field on the basis of this study, which deserved to further investigation.

5. Conclusion

The interactions amongst AMF, growth stage of rice and Cd concentrations in soil had significant effects on root biomass, straw and root Cd concentrations, and straw Cd chemical forms ($P < 0.001$) in rice. In soil added with 2 mg Cd kg^{-1}, AMF had no effects on grain yield of rice, but significantly reduced Cd concentrations in straws at jointing, flowering and ripening stages. Due to the positive correlation between ethanol or d-H_2O extractable Cd and grain Cd concentrations at flowering and ripening stages, the concentrations of ethanol and d-H_2O extractable Cd at flowering stage reduced by AMF subsequently result in the decrease of Cd concentrations in grains. In soil added with 10 mg Cd kg^{-1}, AMF significantly increased grain yield, reduced straw Cd concentrations at jointing, flowering and ripening stages, increased the proportions of NaCl extractable Cd at ripening stage and finally led to the decreased grain Cd concentrations because of the negative correlation between the percentage of NaCl extractable Cd and grain Cd concentration at ripening stage and grain biomass dilution effect. Moreover, the flowering stage and ripening stage are important periods for reducing the Cd accumulation in grains by AMF. The functional diversity between the different symbioses of AMF strains and plant species under the same condition and related nutrition uptake deserved further studies. Furthermore, there seems to be needed to demonstrate the effectiveness of extensive application of AMF on reducing Cd uptake by rice in the field.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.05.047.

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