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Cadmium uptake and subcellular distribution in rice plants as affected by phosphorus: Soil and hydroponic experiments

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Abstract

High cadmium (Cd) concentrations are a serious environmental problem in various agro-ecosystems and urban areas. Since mobile Cd in the soil can be accumulated in the food chain by plant uptake, remediation techniques to fix Cd in the soil in situ are urgently required, for which the application of phosphorus (P) is auspicious. The effects of P on soil pH, Cd phytoavailability, and Cd distribution with regard to rice plants were examined in a pot experiment using soil contaminated with 82 mg Cd kg^{-1}. A commercial P fertilizer (0-52-34, containing 52% P_2O_5 and 34% K_2O) was applied to the soil to reach P concentrations of 0 (the control), 50, 200, and 1000 mg P kg^{-1} above the baseline P concentration of the soil. P-application increased the soil pH and caused a redistribution of Cd to less mobile fractions. Plant growth was also enhanced by P addition. Plant Cd uptake was only significantly reduced in mature plants receiving a P-application rate of 1000 mg P kg^{-1}. Additionally, a hydroponic experiment was carried out to study the effects of different P concentrations on the subcellular distribution of Cd in rice plants. When a P-application of 1000 mg L^{-1} was applied, the Cd proportions in the cell walls increased by 7% in the roots and 10% in the shoots, while reductions for the other fractions were observed, hinting at the occurrence of a detoxifying effect of P on the rice plant's Cd concentration.

Keywords: P-fertilizer, Cd immobilization, phytoavailability, *Oryza sativa* L., subcellular fractionation

1. Introduction

Cadmium (Cd) is a naturally-occurring heavy metal. However, human activities have raised its natural concentrations through the mining, smelting, and processing of ores (Adriano, 2001). The Mae Sot District of Tak Province, in western Thailand, is an area of former intensive Zn mining, and high Cd contamination of paddy soil has been reported (Simmons *et al*., 2005). High Cd concentrations found in rice grains (*Oryza sativa* L.) grown in this area present potential health and economic problems for consumers and farmers (Simmons *et al*., 2005; Apinan *et al.*, 2009). Consequently, to mitigate the problems of Cd uptake by rice plants, remediation approaches involving Cd immobilization in soil are receiving increased attention.

The aim of *in situ* Cd immobilization in soil is to cause a redistribution of Cd to less mobile soil fractions. The mobile Cd fraction is strongly affected by the pH and surface charge, which can be altered by the addition of phosphorus (P) (Kirkham, 2006). In several studies it was proven that the application of water soluble P compounds can lead to a redistribution of Cd to less mobile fractions and reduce Cd uptake by plants. The Cd immobilizing effect of soluble P compounds is attributed to the formation of insoluble phases or adsorption onto the soil surface, which is mainly influenced by the soil pH (Naidu *et al.*, 1994; Bolan *et al.*, 2003; Chaiyarat *et al*., 2011). The liming effect of the studied P compounds promotes negative charging of soil surfaces, resulting in an increase of the adsorption capacity for Cd (Naidu *et al*., 1994). Additionally, P-fertilizers have a plant growth enhancing effect, increasing the plant's biomass, which can also result in a dilution effect on the Cd within the plant, and thus reduce its Cd concentration. However, these studies mainly focused on the soil's Cd content and not the detoxification mechanisms in the plant, like the modification of the subcellular distribution of Cd in rice. Since cellular sequestration of Cd can greatly affect the level of free Cd in the cells and thus potentially influence the movement of Cd throughout the plant, it is important to study the subcellular distribution of Cd within plants as well. On the basis of computer modeling, Wang et al. (1991) claimed that P additions led to the formation of insoluble Cd-phosphates in plant vacuoles. Yang et al. (1999) confirmed this assumption for the Cd taken up by corn and wheat, as most of the Cd had accumulated in the cell wall and vacuoles as Cd-phosphates. To the best of our knowledge, no information is available on subcellular Cd-P interactions in rice plants. Therefore, the present investigation aimed (1) to study the effects of P-application to Cd contaminated soil on soil properties and the uptake of Cd by rice plants, and (2) to investigate the effects of P-application on the subcellular Cd distribution in hydroponically cultured rice.

2. Materials and Methods

Soil characterization was performed at the Soil Plant and Agricultural Material Testing and Research Unit of Kasetsart University at Kamphaeng Saen. Basic soil physicochemical properties, such as its texture, pH, available P concentration, and organic matter content were determined in accordance with standard procedures (Blume *et al*. 2011) and are presented in Table 1.

Surface soil (0-30 cm) from a rice field having a Cd concentration of 82 mg Cd kg^{-1} was obtained from the Mae Sot region and used in this study to examine the effect of P on the phytoavailability of Cd in a pot experiment. Not only farmers in the Mae Sot region but in whole Asia are forced to grow rice on highly contaminated soils to earn enough money for their living; therefore, this soil was chosen due to its high practical relevance. The experimental site was outdoors at the Kamphaeng Saen campus of the Kasetsart University, Thailand, and protected from rainfall and pests. Every pot was filled with 5 kg dried and crushed soil and then water was added to a height of 2 to 5 cm above soil level, completely submerging the soil. A commercial fertilizer (0-52-34) containing 52% P_2O_5 and 34% K_2O was applied to the submerged soil to achieve 50, 200, and 1000 mg kg^{-1} increases in the P concentration above the inherent concentration of 437 mg P kg^{-1} of the pot experiment soil.

The soil and fertilizer were thoroughly mixed and incubated for 2 weeks. For each variant three replicates were conducted. After incubation, the total P concentration was re-tested to validate the treatment. The addition of P-fertilizer yielded the following increases in the total P: 478 mg kg^{-1} from the addition of 50 mg kg^{-1}, 611 mg kg^{-1} from the addition of 200 mg kg^{-1}, and 1462 mg kg^{-1} from the addition of 1000 mg kg^{-1}. The soil had a low initial available P concentration of 1.8 mg kg^{-1} being the reason for the P application levels of 50, 200, and 1000 mg P kg^{-1}, which increased the available P concentrations to 3.0, 10.7, and 225.4 mg kg^{-1}, respectively. Seeds of the Jasmine rice 105 cultivar

(Khao Dawk Mali 105) were surface sterilized and germinated in deionized (DI)-water for 3 days. After germination the seedlings were transplanted into the pots with a density of 3 plantlets per pot and after 2 weeks of growth the seedlings were thinned to one plant per pot. The pots were arranged in a randomized complete block design and irrigated every day to keep a constant water level of about 2 to 5 cm above soil level. A basic nutrient solution consisting of $NH_4H_2PO_4$ (50 mg L^{-1}), KH_2PO_4 (51 mg L^{-1}), KNO_3 (410 mg L^{-1}), $Ca(NO_3)_2.4H_2O$ (333 mg L^{-1}), $MgSO_4.7H_2O$ (50 mg L^{-1}), Mn-EDTA (1.3 mg L^{-1}), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.3 mg L^{-1}), 2% $ZnSO_4.7H_2O$ (0.26 mg L^{-1}), 2% H_3BO_3 (0.26 mg L^{-1}), $CuSO_4.5H_2O$ (0.3 mg L^{-1}), and Fe-EDTA (4.2 mg L^{-1}) was added twice per week to prevent nutrient deficiency of the plants. The pot experiment started in February 2009, with a duration of day/night of 7.9/16.1 h, an average temperature of 33.8 °C during day and 22.3 °C during the night, and an average humidity of maximum 94.4 and minimum 45.3%. Soil and plant samples were taken at four different stages: shortly after P-application (the background stage) and then after 60 days of plant growth (the vegetative stage), 90 days of plant growth (the panicle formation stage), and 120 days of plant growth (the maturity stage). The plants were harvested by carefully taking them out of the submerged soil to avoid damaging the roots. Then, they were washed thoroughly with tap water to remove the soil prior to being rinsed with DI-water. The plant samples were separated into roots, upper plant parts (stems and leaves), and panicles/grains. After harvesting, the fresh weights were recorded, and the plants were dried to a constant weight at 70 °C. Then, the dry weights were recorded and the plant materials were ground using a stainless steel grinder.

The soil pH and redox potential of the samples were measured at the experimental site at field moisture condition using a portable pH and conductivity meter (model sensION 1, HACH LANGE). For every growth stage, soil subsamples were taken from the tops to the bottoms of the pots and air-dried. After that, they were ground to pass through a 2 mm sieve and stored for further analysis.

The total P in soil samples was determined colorimetrically using a UV/VIS-spectrophotometer (model Heyios α, Thermo Electron Corporation), applying the NaOBr method proposed by Dick and Tabatabai (1977). Microwave-assisted acid digestion was used to analyze the soil samples for their total Cd. In each vessel, 0.5 g soil was placed and 9 mL HCl (37%) and 3 mL HNO_3 (65%) were added and gently swirled to homogenize the solution. After that, the vessels were closed and placed according to the instructions of the microwave (model ETHOS SEL, Milestone). The solutions obtained were analyzed for their Cd concentrations using a flame AAS (model ZEEnit 700 Tech).

Table 1. Physicochemical properties of the soil used for the pot experiment.

pH	SOM[a)]	CEC[b)]	particle size (%)			total P	avail. P[c)]	total Cd
1:1 H_2O	(%)	(meq 100 g^{-1})	clay	silt	sand	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
7.5	2.2	18.66	13.6	47.9	38.5	437	1.8	82

[a)] SOM: soil organic matter; [b)] CEC: cation exchange capacity; [c)] available P concentration.

The Cd fractions in soil were determined by applying BCR methods I and II, proposed by the Standards, Measurements and Testing Programme (Maier and Griepink, 1994). The soil (0.5 g) was placed into a centrifuge tube and suspended in 0.11 M acetic acid (soil/solution ratio of 1:40) to release the exchangeable, water, and acid soluble Cd ions that were bound to the carbonate fraction (F1). The soil solution was shaken for 16 h at 350 rpm at room temperature and then centrifuged for 20 min at 4800 rpm. The supernatant was analyzed directly for Cd using a graphite-furnace AAS (model AAnnalyst 800, Perkin Elmer). Then, 20 mL DI-water was added to wash the residue by manual shaking for 5 min. After that, the centrifuge tubes were centrifuged for 15 min at 4800 rpm to obtain the residue used to extract fraction 2 (F2). In BCR II, the residue of BCR I was suspended in 0.1 M hydroxylammonium chloride (soil/solution ratio of 1:40) at pH 2 (adjusted with 2 M HNO_3) to release the Cd ions that were bound to the iron-manganese oxides, representing the reducible fraction. The same extraction procedure was performed as stated for obtaining the BCR I fraction (Tokalioglu *et al*., 2003). The Cd analysis of the soil samples was validated by incorporating a quality control reference material (Natural Matrix Certified Reference Material Catalog No: CRM027-050, Lot No: HC027) within the experimental program, and the Cd concentration was found to be within the confidence interval.

The dried and ground shoots were digested on a hot plate (80 °C) by adding 8 mL concentrated HNO_3 to 0.5 g of sample. The samples were heated until the copious amounts of NO_2 fumes subsided. After this, the samples were cooled down and 1-2 mL of concentrated reagent-grade H_2O_2 was added. They were returned to the hot plate again to maintain a solution temperature of 180 °C to 200 °C and covered with watch glasses to prevent any loss of the samples. The samples were digested until the denseness of the white fumes had dissipated and only wisps of white vapors were visible. The cooled down samples were filtered using a Whatman No. 41 filter and the volumes were adjusted to the final volumes by adding DI-water. The root samples were digested by adding aqua regia ($HCl:HNO_3$; 3:1) and heated until the copious amounts of NO_2 fumes subsided. These beakers were again covered with watch glasses to prevent sample loss. The cooled samples were filtered and the obtained solutions were measured for their Cd and Zn contents using a flame and graphite-furnace AAS.

The hydroponic pot experiment was conducted after a modified procedure proposed by Makoto *et al*. (1986) at the same experimental site as the pot experiment, outdoors at the Kamphaeng Saen campus of Kasetsart University, Thailand, and protected from rainfall and pests. Seeds of the Jasmine rice 105 cultivar (*Oryza Sativa* L.) were surface-sterilized and germinated in deionized water for 3 days. After germination, the seedlings were transferred to 10 L-containers containing 6 L of a basic nutrient solution consisting of the following: $NH_4H_2PO_4$ (50 mg L^{-1}), KH_2PO_4 (51 mg L^{-1}), KNO_3 (410 mg L^{-1}), $Ca(NO_3)_2.4H_2O$ (333 mg L^{-1}), $MgSO_4.7H_2O$ (50 mg L^{-1}), Mn-EDTA (1.3 mg L^{-1}), $(NH_4)_6Mo7O_{24}.4H_2O$ (0.3 mg L^{-1}), 2% $ZnSO_4.7H_2O$ (0.26 mg L^{-1}), 2% H_3BO_3 (0.26 mg L^{-1}), $CuSO_4.5H_2O$ (0.3 mg L^{-1}), and Fe-EDTA (4.2 mg L^{-1}). Each of the containers was covered with a polystyrol-plate with three evenly spaced holes (for three plants per pot) and arranged in a randomized complete block design. The pH was adjusted to about 5 (with HCl or NaOH) every three days and the nutrient solution was renewed weekly throughout the experiment. After 6 weeks of growth, the plants were transferred into a fresh nutrient solution containing Cd^{2+} (0.3 mg L^{-1}) as $CdNO_3$ and different levels of P (0, 50, 200, and 1000 mg L^{-1}) as KH_2PO_4. The rice plants were harvested after 14 days of treatment. After harvest, the roots were rinsed with deionized water and the fresh roots and shoots were immediately stored at -80 °C.

The roots and upper plant parts were homogenized separately for the subcellular fractionation. The plant samples were ground using a mortar and pestle in a chilled medium containing 0.25 mol L^{-1} sucrose, 50 mmol L^{-1} Tris-HCl (pH 7.5), and 1 mM DL-dithiothreitol. The homogenized plant material was filtrated using a nylon cloth with a mesh size of 240 µm

to obtain the liquid and residue. The residue was washed twice with a grinding medium and then pooled with the first filtrate. The pooled washes were then centrifuged at 300 x g for 30 s. The pellets obtained were combined with the residue from the nylon cloth filtration, which contained mainly cell walls and cell wall debris and thus appointed the cell wall fraction or fraction I. The filtrate from the previous step was centrifuged at 1500 x g for 10 min (root samples: 2500 x g for 20 min) and the pellets obtained were of the chloroplast-shoot/ trophoplast-root containing fraction (i.e., fraction II). The supernatant was then centrifuged at 20 000 x g for 45 min to sediment the cell organelles. The pellets obtained were appointed the organelle fraction or fraction III. The supernatant obtained was considered as the soluble fraction or fraction IV. Fractions I-III were re-suspended in the extraction medium. All steps were performed at 4 °C (Wu *et al*., 2005).

The dried cell wall fraction (fraction I) was digested on a hot plate using aqua regia ($HCl:HNO_3$; 3:1, v:v) on the roots and a mixture of HNO_3 and H_2O_2 on the shoots. The re-suspended fractions II and III, as well as the soluble fraction IV were digested on a hot plate by adding HNO_3 and H_2O_2 at 145 °C prior to analysis. The Cd concentrations in all digests were determined with a graphite-furnace AAS.

The means of the data were calculated along with the standard deviations (SD). Analysis of variance (ANOVA; SPSS 20) for data averaged from independent replicates was performed at a significance level of $p < 0.05$ to test the effects of P-application on the metal contents in plants and soils compared to those of the control treatment. Five replicates were performed for every experiment if not stated otherwise.

3. Results and Discussion

A maximum increase of soil pH of 0.41 pH units was observed during the panicle growth stage and a P addition of 1000 mg kg^{-1}. In the initial growth stage, every increasing level of P-application led to a significant increase in soil pH, whereas in the other growth stages, a significant pH increase only occurred when 1000 mg kg^{-1} of P was added (Table 2). The pH gradually decreased between the initial stage and the vegetative stage.

It is generally accepted that the pH of a soil is one of the most important factors controlling the uptake of heavy metals by plants (Amini *et al*., 2005). The increase in pH after P-application was due to ligand exchange reactions, where surface hydroxyl groups coordinated with a metal cation (e.g., Fe and Al) are replaced by phosphate ions (Bolan *et al*. 2003). The decrease of the pH after the initial stage can be explained by the redox potential of the soil (Table 3). The effect of P addition on the redox potential of the soil was insignificant for every treatment and growth stage (Table 3). However, before rice growth the soil exhibited a positive redox potential, indicating aerobic conditions. For rice propagation, the soil has to be submerged, and under these submerged conditions, the entrained oxygen present in the soil is quickly consumed. The lack of free oxygen causes a reducing environment leading to a reduction of soil pH due to an accumulation of carbon dioxide and the formation of carbonic acid (Sahrawat, 2005).

P-application led to [833]a reduction of the mobile Cd concentration (F1) (Table 4). The Cd concentration in F1 decreased by 1.6 to 16.1% in the initial stage, -1.7 to 8.3% in the vegetative stage, 1.6 to 17.2% in the panicle formation stage, and 2.9 to 11.6% in the maturity stage for P-application levels of 50 to 1000 mg P kg^{-1}. When compared to the control treatment, significant Cd reduction ($p < 0.05$) only occurred when the highest level of P was added (Table 4). As decreases in the F1 fraction occurred, increases in the less mobile F2 fraction of -5.5 to 19.2% in the initial stage, -11.8 to 17.4% in the vegetative stage, -6.3 to 26.1% in the panicle formation stage, and 0.0 to 21.4% in the maturity stage were observed, which were only significant under the highest level of P addition.

Table 2. Soil pH for the different levels of P addition and growth stages.

Phosphate addition	pH values for different growth stages			
(mg kg^{-1})	Initial Stage	Vegetative Stage	Panicle Stage	Maturity Stage
0 (control)	7.51 ± 0.17	7.28 ± 0.27	7.13 ± 0.23	7.41 ± 0.13
50	7.61 ± 0.25*	7.23 ± 0.41	7.15 ± 0.13	7.44 ± 0.30
200	7.63 ± 0.11*	7.21 ± 0.12	7.39 ± 0.21	7.40 ± 0.11
1000	7.63 ± 0.05*	7.55 ± 0.27*	7.54 ± 0.08*	7.52 ± 0.08*

Mean ± SD, n=5; *Significant at $p < 0.05$ (treatment vs. control).

Table 3. Soil redox potential for the different levels of P addition and growth stages.

Phosphate addition	Redox potential for different growth stages			
(mg kg^{-1})	Initial Stage	Vegetative Stage	Panicle Stage	Maturity Stage
0 (control)	129.5 ± 34.47	-85.82 ± 83.99	-172.5 ± 24.61	-128.5 ± 118.2
50	53.54 ± 103.6	-146.3 ± 52.76	-184.7 ± 62.16	-6.760 ± 43.42
200	23.88 ± 119.7	-177.0 ± 54.12	-230.1 ± 8.721	-52.36 ± 130.2
1000	107.1 ± 60.50	-234.2 ± 17.53	-278.5 ± 5.816	-254.8 ± 49.14

Mean ± SD, n=5; *Significant at $p < 0.05$ (treatment vs. control).

As described previously, the pH of the soil increased after the addition of water-soluble KH_2PO_4 due to ligand exchange and fixation reactions. With an increase in pH, the negative surface charge of soil with high clay and/or organic matter content might have increased as well. This enhanced Cd immobilization, as shown by the redistribution of Cd to a less mobile fraction (F2). Another mechanism for the reduction of Cd activity are surface complex formations after P-application. P-anions form a complex with the soil surface featuring the adsorption of Cd-cations onto the adsorbed anions. Precipitation as metal-phosphates and hydroxides has also been proposed to be the main mechanism for the immobilization of metals (Bolan *et al*., 2003). However, the precipitation of Cd as hydroxide compounds ($Cd(OH)_2$) of low solubility was unlikely to occur because of the low pH of the soil, as precipitation starts at a pH between 8.0-9.5. Additionally, the solubility of $Cd_3(PO_4)_2$ was too high to control the activity of Cd in the soil solution (Bolan *et al.*, 2003).

In this study, approximately 75% of the total Cd concentration was extracted during the first extraction step, whereas 25% was extracted during the second extraction step. The almost complete extraction of the total Cd concentration within the first two extraction steps can be explained by the fact that Cd is one of the most mobile heavy metals in soil. Furthermore, about 75% of a soil's Cd can be found in the first two fractions, making Cd such an ecotoxic heavy metal (Ahnstrom and Parker, 1999; Tokalioglu *et al*., 2003). Additionally, the sum of F1 and F2 exceeded the total Cd concentration for some samples. Total Cd was determined using microwave assisted digestion with aqua regia as the reagent. Ahnstrom and Parker (1999) revealed that only 74 to 86% of the total Cd in soil could be extracted using this procedure.

Table 4. Cd concentrations of the different fractions and total Cd concentrations for the four growth stages.

Phosphate addition (mg kg^{-1})	Growth stage	Soil Cd fraction[a]		
		F1	F2	Total Cd
0 (control)	Initial	62 ± 1.1	21 ± 1.2	79 ± 6.7
	Vegetative	60 ± 1.5	19 ± 1.6	83 ± 3.9
	Panicle	64 ± 1.7	17 ± 1.7	86 ± 5.0
	Maturity	69 ± 4.7	22 ± 1.7	79 ± 6.7
50	Initial	61 ± 0.8	20 ± 1.0	72 ± 3.8*
	Vegetative	61 ± 1.9	17 ± 0.8	79 ± 1.6
	Panicle	63 ± 2.2	16 ± 1.1	83 ± 4.4
	Maturity	67 ± 2.6	22 ± 2.4	82 ± 2.7
200	Initial	61 ± 1.0	23 ± 1.0	71 ± 0.6*
	Vegetative	61 ± 2.4	18 ± 1.1	83 ± 4.4
	Panicle	63 ± 2.2	18 ± 1.6	83 ± 3.1
	Maturity	66 ± 2.1	23 ± 1.4	83 ± 1.7
1000	Initial	52 ± 0.9*	26 ± 3.1*	72 ± 2.0*
	Vegetative	55 ± 2.1*	23 ± 2.2*	82 ± 3.7
	Panicle	53 ± 3.7*	23 ± 1.5*	80 ± 1.9*
	Maturity	61 ± 3.3*	28 ± 2.2*	83 ± 1.9

Mean ± SD, n=5; [a] F1: exchangeable, water, and acid soluble Cd fraction; F2: iron-manganese oxides associated Cd fraction; *Significant at $p < 0.05$ (treatment vs. control).

Table 5. Dry matter yields of the rice plants (g pot^{-1}) for the different P-application rates and growth stages.

Phosphate addition (mg kg^{-1})	Growth stage		
	Vegetative	Panicle	Maturity
0 (control)	2.20 ± 0.98	16.12 ± 5.34	18.98 ± 5.40
50	4.24 ± 1.58	25.66 ± 7.20	39.80 ± 9.15*
200	3.56 ± 1.29	33.88 ± 8.14*	55.49 ± 13.36*
1000	7.17 ± 2.28*	40.73 ± 11.20*	-[a)]

Mean ± SD, n=5; [a)] no samples were obtained due to Cd toxicity. *Significant at $p < 0.05$ (treatment vs. control).

To overcome this problem they tried to double-digest the samples, resulting in a slight increase in the recovery to 86 to 91%. However, it is impossible to extract all of the refractory Cd, which can explain the why the sum of F1 and F2 exceeded the total Cd concentration in this study.

The dry matter yield significantly ($p < 0.05$) increased along with the increasing P-application rate, indicating the plant growth enhancing effect of P (Table 5). No rice plants could be harvested for the maturity stage for the highest level of P application (Table 5). This was a result of the high Cd concentration in the soil and at later growth stages plants showed chlorosis, leaf rolls, and stunted growth, all to be known to be signs of Cd toxicity (Benavides *et al*., 2005).

Cd mainly accumulated during the vegetative stage (Table 6), which is consistent with what is reported in the literature (Kashiwagi et al., 2009). The Cd content of the whole rice plants continuously decreased over time for all treatments, and the Cd concentrations at the maturity stage for P-application rates of 50 and 200 mg P kg^{-1} were significantly ($p < 0.05$) lower compared to that of the control treatment. From this, it can be inferred that the translocation of Cd from the roots to the shoots was restrained after P addition — this was likewise found by Li *et al*. (2009). Additionally, this effect can also be explained by the dilution of the plant's Cd, because as the P-application rate increased, so did the plant's biomass. As Cd uptake mainly takes place during the vegetative stage, less Cd was detectable per kilogram of dry mass, which concurs with the findings of Prochnow *et al*. (2001).

The total Cd concentration in the rice plants of the pot experiment generally increased during the vegetative and panicle stages with increasing P addition levels being significant at P-application rates of 200 and 1000 mg P kg^{-1}. However, during the maturity stage, Cd in whole plants significantly decreased (Table 6). Cd accumulation in plant straw and panicles was unaffected by P-application during the vegetative and panicle formation stages, whereas the Cd in straw significantly decreased during the maturity stage. This increase observed during both the vegetative and panicle stages is a result of the augmented Cd complex formations between Cd^{2+} and HPO_4^{2-} and Cd^{2+} and H_2PO^{4-}. Anions like HPO_4^{2-}, H_2PO^{4-}, Cl-, and SO_4^{2-} exist at the outer part of the root cell wall. Being attracted to them, Cd^{2+} adsorbed onto the cell wall, resulting in the increased Cd concentration found in the roots when the anion concentration was increased (Jiang *et al.*, 2007). The reduction of the Cd concentration in whole plants during the maturity stage is a result of (1) a redistribution of soil Cd to less mobile forms, reducing plant uptake, (2) restrained Cd translocation from the roots to shoots, and (3) a dilution effect from increased biomass after P-application. The Joint FAO/WHO Expert Committee on Food Additives (JCEFA) (WHO, 2004) has proposed a maximum level for cadmium in rice grains of 0.4 mg kg^{-1}, which was not exceeded in this study. However, addition of P in form of a commercially available P fertilizer failed to reduce the Cd concentration in the rice grains.

For the subcellular fractionation experiment, the total Cd concentration in the roots exceeded those of the shoots by a factor of 2.1 in the control treatment (0 mg P L^{-1}), which confirmed the findings of He *et al*. (2008) and Li *et al*. (2009). This factor tended to increase with the P-concentration in the nutrient solution and reached 5.0 in the treatment with 1000 mg P L^{-1} (Table 7). Among the subcellular fractions of the control treatment, the order of the fractions was trophoplasts > soluble fraction> cell walls > membranes and organelles for the roots. Following P-application, the relative Cd-proportions among the fractions were redistributed, resulting in increases of 6% for the cell walls and 18% for the soluble fraction and reductions of 15% for the trophoplasts and 9% for the organelles from the highest level of P-addition. In the shoots, the Cd-distribution among the fractions was slightly different: cell walls > soluble fraction > chloroplasts > membranes and organelles (Table 7). This order agrees with comparable findings for lettuce (Ramos *et al*., 2002), barley (Wu *et al*., 2005), and rice (He *et al.*, 2008).

Table 6. Cd concentration (mg Cd kg^{-1} dry weight) in whole plants and rice plant parts for different P-application rates and growth stages.

	Phosphate addition (mg kg^{-1})			
	0 (control)	50	200	1000
	mg Cd kg^{-1}			
Vegetative stage				
Roots	0.317 ± 0.238	1.453 ± 0.649*	0.601 ± 0.706	1.317 ± 0.419*
Straw	0.980 ± 0.242	0.621 ± 0.426	2.465 ± 1.484	1.033 ± 0.169
Total	0.859 ± 0.253	0.791 ± 0.362	1.634 ± 0.526*	1.085 ± 0.062*
Panicle stage				
Roots	0.083 ± 0.054	1.401 ± 0.123*	1.548 ± 0.291*	0.904 ± 0.325*
Straw	0.728 ± 0.200	0.606 ± 0.156	0.857 ± 0.237	0.738 ± 0.539
Panicle	0.555 ± 0.443	0.469 ± 0.367	0.722 ± 0.360	0.577 [a)]
Total	0.661 ± 0.231	0.569 ± 0.281	0.961 ± 0.245	1.036
Maturity stage				
Roots	1.270 ± 0.107	1.036 ± 0.255*	0.682 ± 0.122*	- [b)]
Straw	0.319 ± 0.478	0.013 ± 0.018*	0.023 ± 0.010*	-
Grain	0.252 ± 0.084	0.369 ± 0.148	1.030 ± 0.716	-
Total	0.561 ± 0.413	0.271 ± 0.079*	0.343 ± 0.180*	-

Mean ± SD, n=5; [a)] only one replicate was obtained; b) no samples were obtained due to Cd toxicity. *Significant at $p < 0.05$ (treatment vs. control).

At the highest level of P-addition, the Cd-proportion increased in the cell wall fraction by 10%, while it decreased in the chloroplasts by 3%, cell organelles by 6%, and soluble fraction by 1% (Table 7).

For detoxification, Cd activity must be kept low in the cytosol because Cd preferentially binds to sulphhydryl ligands and thus competes with essential metals like Zn, Ni, and Cu for active functional binding sites at essential cytosolic metabolites (Sanità di Toppi and Gabrielli, 1999). The data in Table 7 indicate that the Cd in rice plants was compartmentalized to regulate the Cd activity in the cytosol. P-addition obviously enhanced the immobilization of Cd in the cell wall fraction and, thereby, alleviated the accumulation of Cd in the other fractions. One reason for the P-induced Cd-deposition could be the formation of immobile Cd-phosphates in the cell wall fraction. This is supported by the increased P-uptake by the rice due to the increased P-concentration in the nutrient solution (Alam, 1983); in addition, direct electron microscopic evidence for the formation of immobile Cd-phosphate deposits has been found in maize cell walls (Jiang *et al.,* 2007). The data in Table 7 also indicate the suppressed transport of Cd from the roots to the shoots resulting from the increased P-concentrations. In conclusion the present study shows that Cd uptake by rice plants from highly contaminated soil could

be significantly reduced through the application of a commercial P-fertilizer. An increase in pH induced by the addition of anions resulted in an increase in the sequestration of Cd to less mobile forms, thereby reducing its phytotoxicity. This study shows that Cd uptake primarily occurs during the vegetative stage, and therefore possible remediation techniques should prevent the uptake of Cd during that stage. Additionally, the present study provides first-time evidence for phosphate's detoxifying effect on rice plants, the result of its increase of Cd-enrichments in the cell walls. Future studies should focus gaining a better plant-level comprehension of Cd immobilization, as understanding the detoxifying mechanisms of the plant is a crucial step towards the development of effective remediation strategies.

Table 7. Subcellular distribution of Cd in roots and shoots (fresh weight) of rice plants at different levels of P-addition; in parentheses, the Cd proportion (%) in each fraction to the total Cd amount in all fractions.

	P concentration [mg L^{-1}]							
Fraction	0	50	200	1000	0	50	200	1000
	Roots				Shoots			
	Cd concentration [µg kg^{-1}]							
Total [a] (= 100%)	1180	1166	953	822	553	443*	436	166*
Cell wall	267±3 (23%)	262±17 (22%)	267±5 (28%)	240±35 (29%)	265±10 (48%)	195±32 * (44%)	213±4 8* (49%)	97±13* (58%)
Trophoplast roots/ chloroplasts shoots	391±13 (33%)	383±14 1 (33%)	266±27 (28%)	145±6 (18%)	73±6 (13%)	63±3 (14%)	49±32 (11%)	16±0.7 (10%)
Membranes and organelles	188±19 (16%)	167±42 (14%)	158±22 (17%)	61±0.8 * (7%)	61±32 (11%)	54±5 (12%)	36±32 (8%)	8±2 (5%)
Soluble	334±26 (28%)	354±60 (30%)	262±3 (27%)	376±18 (46%)	154±34 (28%)	131±27 (30%)	138±7 6 (32%)	45±5* (27%)

Mean ± SD, n=3; a Total: total Cd concentration (sum of all fractions); *Significant at $p < 0.05$ (treatment vs. control).

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