EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/ea/index.html
EnvironmentAsia 10(1) (2017) 63-72
DOI

Alleviation of Cadmium Toxicity in Rice by γ-Polyglutamic Acid Produced by *Bacillus subtilis*

Nuchnapa Kotabin [a, b], Kritchaya Issakul [c], Elke Pawelzik [d], Yasutaka Tahara [e] and Orawan Chunhachart [a, b]

[a] *Bioproducts Science Program, Department of Science, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand*
[b] *Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Chatuchak, Bangkok, 10900, Thailand*
[c] *Department of Environmental Science, School of Energy and Environment, University of Payao, Phayao, 56000, Thailand*
[d] *Section Quality of Plant Products, Department of Crop Sciences, Faculty of Agricultural Sciences, Georg-August-University Goettingen, Goettingen 37075, Germany*
[e] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*

Abstract

The effect of γ-polyglutamic acid (PGA) produced by *Bacillus subtilis* on cadmium (Cd)-damaged rice was investigated in experiments with pots containing soil contaminated with Cd at a concentration of 0-200 mg/kg and PGA at a concentration of 0-500 mg/kg. Although Cd reduced the growth and productivity of rice in comparison to the control, the addition of 500 mg/kg of PGA alleviated the effects of Cd toxicity in rice, resulting in increases in shoot and root lengths, the vigor of the rice and the number of seeds per panicle. Micrographs of ultrathin sections of root cells showed that the addition of 500 mg/kg of PGA decreased Cd accumulation in both the epidermis and the intracellular space of cortex, suggesting that PGA reduced Cd transportation into root cells. Based on these results and the metal-binding characteristics of PGA, it is conceivable that the mechanism by which PGA alleviates Cd toxicity is the extracellular formation of Cd-PGA complexes.

Keywords: *Bacillus subtilis;* γ-polyglutamic acid; cadmium; rice

1. Introduction

Heavy metal contamination in water and soil is a major problem for environmental quality, and cadmium (Cd) is one of the most toxic of the heavy metal pollutants, which are widespread, problematic and difficult to remediate (Grant *et al*., 1998; Simmons *et al*., 2005). Although Cd is a non-essential element, it is readily absorbed by plant roots and then accumulates inside the plant tissues and grains (Wagner, 1993; Grant *et al*., 1998). Therefore, Cd can be transferred to humans via food consumption. For over a decade, some countries have faced Cd contamination problems. For instance, in Mae Sot district in Tak province, Thailand, zinc mining has caused Cd to leak into the neighboring rivers and rice fields, with some reports that local people living in the Cd-contaminated area have high urinary Cd levels (Simmons *et al*., 2003; Swaddiwudhipong *et al*., 2007). The samples collected from sediments of the creeks, paddy soils had high Cd concentrations. Moreover, rice grains (*Oryza sativa* L.) cultivated in the contaminated areas also contained Cd concentrations that exceeded the maximum permissible level established by the Codex Alimentarius Commission of FAO/WHO (0.4 mg/kg) (Swaddiwudhipong *et al*., 2007).

In recent years, the phytoremediation of Cd in agricultural fields by Cd-hyperaccumulator plants has been proposed to be an environmentally friendly method of excluding Cd from Cd-contaminated soil (Khaokaew and Landrot, 2015). Although this technology requires a relatively long time for metal accumulation, critical nutritional conditions and proper soil characteristics, phytoremediation is an economical and practical method (Pulford and Watson, 2003). In addition, several researchers have selected low-Cd rice cultivars that accumulate low levels of Cd in rice grains to replace high-Cd-accumulating cultivars planted in Cd-contaminated areas and reduce human Cd intake (Liu *et al*., 2007a; Sriprachote *et al*., 2012).

For a decade, bioremediation and biosorption using microorganisms has been evaluated as an alternative approach to excluding heavy metals from the environment. Some microbes possess metal mobilization functions, for example, the leaching of metals by oxidation and the production of organic acids to provide protons for metal-complexing organic acid anions. Other microbes can immobilize metals via the secretion of metal binding peptides, the reduction of metals to a lower redox state and the precipitation of metals into sulfide or phosphate form (Gadd, 2000). In general, microbial cell surfaces have several functional groups that can react with metal ions; therefore, the biosorption of Cd by microbial cells has been widely studied (Gadd, 2000; Bai *et al*., 2008).

γ-Polyglutamic acid (PGA), which is an edible, high-molecular-mass (10 to 2,000 kDa) biopolymer that consists of D- and L-glutamic acid polymerized through γ-glutamyl bonds, is produced by *Bacillus* strains as an extracellular viscous material (Shih and Van, 2001; Urushibata *et al*., 2002). PGA has also been reported to be an effective biosorbent for several metals (McLean *et al*., 1990), including Cd (Siao *et al*., 2009), but no studies have proposed the detoxification of Cd in soil and plants using PGA. Thus, we were interested in the bioremediation of Cd-contaminated soil and plants using PGA produced by *Bacillus subtilis* to protect against Cd toxicity. This study describes the effect of PGA on the growth and productivity of Cd-damaged rice.

2. Materials and Methods

2.1 PGA production

PGA was prepared from *Bacillus subtilis* NBRC16449 according to the method described by Ogawa *et al*. (1997). The bacterium was cultured in Luria-Bertani broth with shaking at 120 rpm for 12 h at 30°C. The inoculum was transferred to 100 mL of PGA production medium and then incubated at 30°C with shaking at 120 rpm for 24 h. The bacterial cells were then harvested by centrifugation at 10,000 rpm for 15 min at 4°C. PGA was precipitated using cold ethanol and desalted via dialysis for 12 h at 4°C. Then, the PGA solution was freeze-dried and kept in a desiccator.

2.2 Germination and growth analysis of rice seedlings

The germination, vigor and growth of rice seedlings were evaluated by the modifying method of He *et al*. (2008a). Healthy rice seeds (*Oryza sativa* L. cv. KDML105) were surface sterilized in 10% sodium hypochlorite for 10 min, then rinsed 3 times with deionized water. The seeds were soaked in deionized water for 24 h. Meanwhile, a filter paper was placed on a Petri dish and moistened with 5 mL of Cd (supplied as a $CdCl_2 \cdot 2.5H_2O$ solution) and a PGA mixer solution in a series of different concentrations (Cd: 0, 50, 100, 250, and 500 μM; PGA: 0, 50, and 500 mg/L). Afterward, ten rice seeds were placed on the filter paper and incubated in a growth chamber with 12 h of light and 12 h of darkness at 28ºC±1ºC for 7 days. The growth of the rice seedlings was evaluated using the following procedure. The seeds were soaked in deionized water for 24 h, then wrapped in a wet sheet cloth in the dark until germination. Ten germinated seeds were cultivated on a Petri dish soaked with the mixer solution of Cd and PGA in different concentrations in a growth chamber as described above for 7 days. Root and shoot lengths, the fresh and dry weights of rice seedlings, and the Cd content of seedlings were measured. Three replicates of each treatment were conducted. The germination percentage and vigor index of rice seedlings were calculated according to the formulation described by Vijayalakshmi *et al*. (2003) as follows;

Germination percentage = (Number of seeds germinated / Total number of seeds sown) × 100

Vigor index (seedling length basis) = Germination percentage × (Shoot length + Root length)

Vigor index (dry weight basis) = Germination percentage × Dry weight

2.3 Pot experiment

A pot experiment was carried out by the modifying method of Cheng *et al*. (2009) at the Greenhouse Complex of the Kasetsart University Kamphaeng Saen Campus in Nakhon Pathom, Thailand. The experiment was started from 1 December 2014 until last harvested date on 30 April 2015. The greenhouse used natural light. The experimental soil was excavated from the surface of a paddy field (30 cm depth) and was then air-dried, ground and sieved through a 6.00 mm sieve to remove rock and plant fragments. The properties of the soil were as follows: pH 5.50, organic matter 3.41%, ECe 1.78 dS/m, total nitrogen 0.44%, available P 11.58 mg/kg soil, exchange K 130 mg/kg soil, and Cd content 0.069 mg/kg soil. Plastic pots (25.0 cm diameter × 30.0 cm height) containing 5.0 kg of sieved air-dried topsoil were prepared. The soil was immersed in tap water for 15 days before planting.

The experimental design was carried out using the following procedure. Sieved air-dried topsoil was mixed with Cd and PGA at various concentrations (Cd: 0, 50, 100, and 200 mg/kg; PGA: 0, 50, and 500 mg/kg) resulting in twelve different treatments. Tap water

was added and mixed with soil in the plastic pots until the soil was sludge. Healthy rice seeds were surface sterilized with 10% sodium hypochlorite for 10 min, then rinsed 3 times with deionized water. The rice seeds were soaked in deionized water overnight, then wrapped in a wet sheet cloth and incubated in the dark until germination. Consequently, ten germinated seeds were sown in the plastic pots containing the prepared soil sludge and watered every day. After 7 days, only 5 uniformed healthy seedlings were kept per pot. The water level in all pots was maintained at 2-3 cm above the soil surface until the ripening stage. The experiment was arranged in a randomized complete block design with three replicates. Chemical fertilizers were added to each pot at difference stages, as follows. The formula N:P:K 16:20:0 was added on day 20 after planting (0.4 g/pot), and the formula N:P:K 46:0:0 was added on days 40 and 90 after planting (0.1 g/pot). At maturity stage, the plants and rice grains in each pot were harvested for physical and chemical analysis.

2.4 Analysis of Cd

Cd content in rice seedlings was carried out by the modifying method of He *et al*. (2008a). Rice seedlings were soaked in 20 mM EDTA for 15 min to remove metal ions on the surface, then washed three times with deionized water. The seedlings were dried at 80°C until a constant weight was achieved and ground to a fine powder. The seedling powder was digested with a HNO_3:$HClO_4$ mixture (4:1, v/v) at 120°C for 12 h. The Cd concentration in the seedlings was determined using an atomic absorption spectrometer (Unicam M-Series Solaar, MA, USA). The Cd content in rice grains was analyzed according to the In-house method TE-CH-134 based on AOAC (2012) 999.10. Rice seeds were dried at 80°C until a constant weight was achieved. The rice grains were then removed from the hulk, and the unpolished brown rice grains were ground to a fine powder. The rice powder was digested with 7 mL of HNO_3 and 1 mL of H_2O_2 by microwave digestion (Milestone ETHOS-One, Italy) at 200°C for 45 min. The volume was then adjusted to 25 mL in a volumetric flask. The content of Cd in the rice grains was determined using ICP-MS (Agilent 7500c, Tokyo, Japan).

2.5 Electron microscopy

The anatomy of rice roots was observed via transmission electron microscopy (TEM), according to the modified method described by Doorn *et al*. (2011). After harvesting rice from the pot experiment, 1.0 cm in length was removed from the root tip and the 1.0 cm in root length behind the cutting area was used for further preparation steps. The root samples were prefixed in glutaraldehyde and paraformaldehyde and kept at 4°C. The samples were rinsed with cacodylate buffer for 15-30 min and post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.0). Then, the root samples were rinsed 3 times in phosphate buffer (pH 7.3) for 10-15 min. The samples were dehydrated for 10-15 min each in 30%, 50%, 70%, 80%, 90%, and 95% ethanol solutions and finally dehydrated twice in 100% ethanol for 10-15 min. After that, N-butyl glycidyl ether and 100% ethanol (1:1 v/v) were added, and the samples were soaked for 15 min, followed by the addition of 100% N-butyl glycidyl ether for 15 min and N-butyl with Spurr's resin (1:1 v/v) for 1 h. The root samples were embedded twice in Spurr's resin for 1 h each, and the embedded samples were then heated at 70°C for 8 h. The ultra-sections (60 nm) were prepared using a Leica ultracut microtome (Wetzlar, Germany), and the root sections were then mounted onto copper grids and air-dried before staining in 4% uranyl acetate for 1 h and a lead solution for 10 min. The ultra-structure of the roots was examined using a Jeol TEM-1230 (Tokyo, Japan).

2.6 Statistical analysis

Statistical analysis was performed using the SPSS software package, version 16 (SPSS Inc.; Chicago, IL, USA). The data were analyzed using one-way analysis of variance (ANOVA) at a significance level of 0.05, followed by Tukey's honestly significant difference test.

3. Results and Discussion

3.1 Effect of Cd and PGA on seed germination and seedling growth in the laboratory experiment

Fig. 1 shows the effects of Cd and PGA on the germination and vigor of rice seedlings. The percentage of germination did not differ between the control and the samples treated with 50, 100 and 250 μM Cd, but the addition of 500 μM Cd inhibited germination. Cd also decreased the vigor index in terms of both length and dry weight at concentrations of 250 and 500 μM. In contrast, PGA significantly increased the germination percentage, with a rise in the vigor of the seedlings, particularly at a concentration of 500 μM Cd. Table 1 indicates the effect of Cd and PGA on the growth of the rice seedlings. Cd reduced the root lengths, with decreases of 24%, 48%, 84% and 96% in samples treated with 50, 100, 250 and 500 μM Cd, respectively. The shoot length was gradually reduced by 16%, 24%,

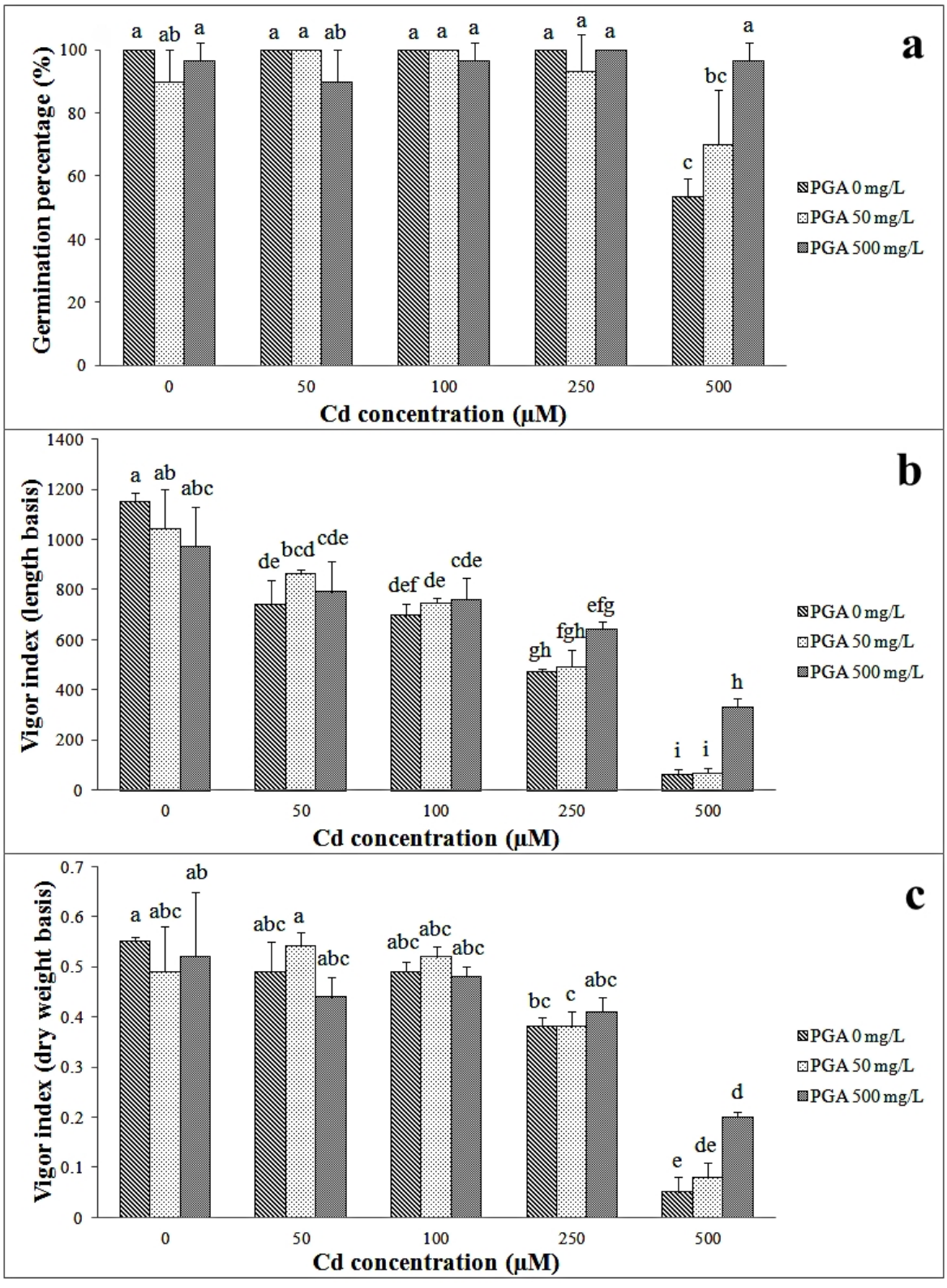

Figure 1. Germination and vigor of rice seedlings

Table 1. Growth and Cd accumulation of rice seedlings

Treatment	Mean ±SD				
	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)	Cd content in rice seedling (mg/kg DW)
Control	5.35 ±0.61 ab	4.17 ±1.65 a	0.0356 ±0.0088 a	0.0072 ±0.0009 a	0.012 ±0.004 c
Cd 0 : PGA 50	5.37 ±0.70 a	4.12 ±1.87 a	0.0331 ±0.0123 ab	0.0068 ±0.0012 ab	0.049 ±0.008 c
Cd 0 : PGA 500	5.48 ±0.76 a	3.85 ±2.05 ab	0.0366 ±0.0132 a	0.0072 ±0.0012 a	0.033 ±0.001 c
Cd 50 : PGA 0	4.52 ±0.50 cd	3.16 ±0.83 bc	0.0303 ±0.0069 abc	0.0061 ±0.0007 bcd	30.387 ±0.618 bc
Cd 50 : PGA 50	4.49 ±0.62 cd	2.49 ±1.09 cd	0.0284 ±0.0085 bcd	0.0058 ±0.0008 cde	35.225 ±0.157 bc
Cd 50 : PGA 500	4.79 ±0.55 bc	2.49 ±0.76 cd	0.0366 ±0.0060 a	0.0064 ±0.0009 bc	36.389 ±1.040 bc
Cd 100 : PGA 0	4.06 ±0.64 de	2.15 ±0.80 de	0.0263 ±0.0062 cde	0.0054 ±0.0009 def	52.875 ±2.453 bc
Cd 100 : PGA 50	4.52 ±0.56 cd	1.96 ±0.82 de	0.0237 ±0.0068 def	0.0055 ±0.0007 def	61.791 ±1.251 b
Cd 100 : PGA 500	4.53 ±0.46 cd	2.77 ±1.14 cd	0.0283 ±0.0066 bcd	0.0053 ±0.0008 ef	51.987 ±0.702 bc
Cd 250 : PGA 0	3.88 ±0.27 e	0.68 ±0.46 fg	0.0183 ±0.0044 fgh	0.0038 ±0.0005 hi	998.85 ±35.324 a
Cd 250 : PGA 50	3.91 ±0.36 e	0.71 ±0.30 fg	0.0199 ±0.0041 efgh	0.0047 ±0.0008 fg	985.77 ±30.652 a
Cd 250 : PGA 500	4.39 ±0.49 cde	1.53 ±0.79 ef	0.0235 ±0.0051 defg	0.0051 ±0.0008 ef	74.89 ±2.571 b
Cd 500 : PGA 0	2.67 ±1.31 f	0.16 ±0.09 g	0.0142 ±0.0055 h	0.0027 ±0.0001 j	3053.76 ±7.774 a
Cd 500 : PGA 50	3.07 ±0.88 f	0.24 ±0.17 g	0.0170 ±0.0042 gh	0.0031 ±0.0008 ij	3209.37 ±44.358 a
Cd 500 : PGA 500	3.90 ±0.28 e	0.74 ±0.54 fg	0.0212 ±0.0028 efg	0.0041 ±0.0008 gh	3109.91 ±160.711 a

* Values in a column with different letters are significantly different at $P<0.05$

28% and 50% after treatments with 50, 100, 250 and 500 μM Cd, respectively. In contrast, PGA recovered both root and shoot lengths when it was added with 250 and 500 μM Cd. The fresh and dry weights were also reduced with increasing Cd concentrations, but the addition of PGA recovered both weight measurements compared to those observed for the Cd treatment. In addition, 500 mg/L of PGA reduced the Cd content in the tissue. These results indicate that PGA can mitigate Cd toxicity in rice seedlings.

Generally, plants exposed to Cd exhibit physiological disorders, for example, reduced enzyme activity related to the assimilation of carbohydrates (He *et al*., 2008a), decreased chlorophyll (Rascio *et al*., 2008), and a drop in production yield (Liu *et al*., 2007b). Although Cd did not significant effect on germination of seedling, it greatly interfered with seedling growth because the activities of amylase were inhibited by Cd resulting in low rate of starch hydrolysis and lack of energy during germination and growth of rice seedling (He *et al*., 2008a).

3.2 Effect of Cd and PGA on the growth of rice in the pot experiment

Table 2 shows the inhibition of the growth of rice by Cd. Cd adversely effected rice growth, resulting in reduced shoot and root lengths, shoot and root fresh weights, and shoot and root dry weights. The shoot length was decreased by 6%, 5% and 12% and the root length was reduced by 15%, 22% and 23% after treatments with 50, 100 and 200 mg/kg Cd, respectively. In contrast, the addition of 500 mg/kg of PGA significantly increased the shoot and root lengths and the shoot and root dry weights. The number of tillers per plant decreased gradually with increasing Cd concentrations. However, the addition of 500 mg/kg of PGA into the treatments with 50 and 100 mg/kg Cd increased the number of tillers significantly. Table 3 indicates the effect of Cd and PGA on the productivity of rice. Cd reduced the number of panicles per plant, but the addition of 500 mg/kg of PGA increased the number of panicles per plant in comparison to the

Table 2. Growth of rice in the pot experiment

Treatment	Mean ±SD					
	Shoot length (cm)	Root length (cm)	Shoot and root fresh weight (g)	Shoot and root dry weight (g)	Increment of dry weight over control (%)	Number of tillers/plant
Control	134.0 ±3.6 abc	35.9 ±8.2 ab	24.1694 ±7.81 bcde	6.7140 ±1.45 abcd		3.5 ±0.8 abcd
Cd 0 : PGA 50	134.9 ±5.4 ab	36.2 ±5.3 ab	27.5024 ±5.12 bc	6.9126 ±1.06 abc	2.96	3.6 ±0.5 abc
Cd 0 : PGA 500	138.7 ±4.3 a	38.4 ±11.6 a	32.0137 ±7.68 ab	7.6178 ±1.77 a	13.47	4.2 ±0.6 a
Cd 50 : PGA 0	125.4 ±7.6 cd	30.4 ±4.0 bc	25.4487 ±4.40 bcd	5.0005 ±0.94 de	-25.52	3.0 ±0.5 cdef
Cd 50 : PGA 50	131.1 ±5.6 abc	30.4 ±7.2 bc	25.9813 ±6.18 bcd	5.1461 ±1.30 cde	-23.35	2.9 ±0.5 cdef
Cd 50 : PGA 500	134.0 ±7.9 abc	37.3 ±5.7 ab	36.1267 ±5.90 a	7.1365 ±0.93 ab	6.30	3.9 ±1.0 ab
Cd 100 : PGA 0	127.6 ±6.2 bc	27.9 ±3.9 c	24.0336 ±3.86 bcd	5.3689 ±0.78 bcde	-20.03	2.8 ±0.6 def
Cd 100 : PGA 50	134.3 ±3.8 abc	31.0 ±7.5 abc	23.6136 ±7.20 cde	5.4385 ±1.48 bcde	-18.99	3.1 ±0.6 cdef
Cd 100 : PGA 500	137.0 ±3.2 a	35.7 ±10.7 ab	28.9580 ±8.94 abc	6.7117 ±1.80 abcd	-0.23	3.3 ±0.5 bcde
Cd 200 : PGA 0	118.3 ±11.9 d	27.5 ±6.4 c	13.2821 ±3.72 f	4.3396 ±1.19 e	-35.36	2.0 ±0.6 g
Cd 200 : PGA 50	131.2 ±5.5 abc	33.4 ±9.4 abc	17.0885 ±3.20 ef	4.5212 ±0.99 e	-32.66	2.4 ±0.6 fg
Cd 200 : PGA 500	135.0 ±5.8 ab	36.0 ±4.1 ab	18.6465 ±8.17 def	4.9805 ±2.24 de	-25.82	2.7 ±0.5 efg

* Values in a column with different letters are significantly different at $P<0.05$

control. In addition, the number of healthy seeds per panicle was reduced after treatment with 200 mg/kg of Cd, but the addition of 500 mg/kg of PGA resulted in an increase in the number of healthy seeds per panicle in comparison to the control. Meanwhile, the number of abnormal seeds drastically increased by 34%, 44% and 279% after treatment with 50, 100 and 200 mg/kg of Cd, respectively, in comparison to the control. However, the addition of 500 mg/kg of PGA to the treatments with 50, 100 and 200 mg/kg of Cd decreased the number of abnormal seeds by 43%, 40% and 25%, respectively. The addition of PGA increased the dry weight in comparison to the control, particularly in the treatments with 50 and 100 mg/kg of Cd and 500 mg/kg of PGA. The increase in dry weight over control for the treatment with 500 mg/kg of PGA with no Cd was also high, implying that PGA could be involved in cell biomass formation. Table 3 also presents the Cd content in unpolished rice grains. Cd accumulated in control grains of rice to a concentration of less than 0.05 mg/kg DW. The increased Cd levels in the experimental soil resulted in significant Cd accumulation in the rice grains, and the application of PGA to the Cd-contaminated soil did not decrease the Cd content in the rice grains. Fig. 2 shows electron-dense deposits in the epidermis and the intracellular space of the cortex of rice roots. The ultrastructure of roots exposed to 200 mg/kg of Cd exhibited electron-dense deposits in both the epidermis (Fig. 2(b)) and the intracellular space of cortex (Fig. 2(e)). The addition of 500 mg/kg of PGA resulted in fewer electron-dense deposits in both the epidermis (Fig. 2(c)) and the intracellular space of cortex (Fig. 2(f)), suggesting that Cd accumulation in roots was reduced by the addition of PGA. Table 4 shows the soil properties before and after the cultivation of rice in the pot experiment. The experimental soil pH was slightly acidic, with low electrical conductivity (ECe), high organic matter and a moderately high cation exchange capacity. After cultivation, the pH changed slightly, while the electrical conductivity, organic matter and total nitrogen were reduced. In contrast, the available phosphorus and exchanged potassium appeared to increase. However, the total nitrogen observed after treatment with 200 mg/kg of Cd and 500 mg/kg of PGA was higher than that observed before planting.

A previous paper showed that the tiller number, the plant height, the leaf area, the dry matter accumulation and the grain yield of rice were significantly reduced when rice was exposed to 100 mg/kg of Cd, which is consistent with our results (Liu *et al*., 2007b). Cd tolerance and Cd accumulation in grains varies among rice species (Arao and Ae, 2003). In general, Cd accumulation in grains is higher in *indica* cultivars than in *japonica* cultivars (Liu *et al*., 2007a). Some specific cultivars among the *indica* rice cultivars accumulate much higher levels of Cd in vegetative tissues and grains (Sriprachote *et al*., 2012). The cultivar used in this study (*Oryza sativa* L. cv. KDML 105) has been reported to accumulate high levels of Cd in grains (Sriprachote *et al*., 2012). Tanaka *et al*. (2007) reported that more than 90% of the Cd that accumulates in rice grains is transported via xylem-phloem transfer. Two possible mechanisms of Cd transport and accumulation in rice have been proposed (Uraguchi *et al*., 2009). One mechanism involves initial Cd accumulation in vegetative tissues

Table 3. Rice yield and Cd content in rice grain

Treatment	Mean ±SD				
	Number of panicles/plant	Number of panicles/pot	Number of healthy seeds/panicle	Number of abnormal seeds/panicle	Cd content in unpolished rice grain (mg/kg)
Control	3.6 ±0.5 ab	16.3 ±0.6 ab	68.4 ±21.3 a	6.2 ±5.1 b	0.047 ±0.020 c
Cd 0 : PGA 50	3.6 ±0.5 ab	18.0 ±1.0 ab	73.6 ±21.4 a	4.8 ±2.6 b	0.062 ±0.025 c
Cd 0 : PGA 500	3.9 ±0.6 a	19.3 ±2.5 a	74.6 ±22.5 a	7.9 ±6.3 b	0.040 ±0.006 c
Cd 50 : PGA 0	3.0 ±0.6 bcd	15.5 ±0.7 ab	64.5 ±20.4 abc	8.3 ±8.3 b	2.137 ±0.182 b
Cd 50 : PGA 50	2.7 ±0.7 cd	13.5 ±0.7 ab	65.9 ±18.9 ab	5.2 ±3.0 b	2.202 ±0.151 b
Cd 50 : PGA 500	3.9 ±0.7 a	19.3 ±3.1 a	68.7 ±17.2 a	4.7 ±3.0 b	2.309 ±0.057 b
Cd 100 : PGA 0	2.9 ±0.7 bcd	14.0 ±2.8 ab	64.1 ±15.8 abc	8.9 ±7.2 b	2.084 ±0.141 b
Cd 100 : PGA 50	3.1 ±0.6 abcd	16.0 ±0.0 ab	67.6 ±17.0 ab	5.8 ±3.5 b	2.317 ±0.089 b
Cd 100 : PGA 500	3.2 ±0.6 abc	16.0 ±1.0 ab	71.6 ±11.8 a	5.3 ±2.9 b	2.058 ±0.062 b
Cd 200 : PGA 0	2.4 ±0.5 cd	16.5 ±3.6 ab	49.9 ±13.2 c	23.5 ±18.6 a	3.889 ±0.139 a
Cd 200 : PGA 50	2.3 ±0.7 d	11.7 ±2.1 b	53.4 ±14.6 bc	18.2 ±9.8 a	3.806 ±0.064 a
Cd 200 : PGA 500	3.6 ±0.8 ab	18.0 ±2.6 ab	61.4 ±21.8 abc	17.7 ±6.3 a	3.576 ±0.226 a

* Values in a column with different letters are significantly different at $P<0.05$

and subsequent mobilization into grains, and the other mechanism posits that Cd absorbed in the roots is transported directly into grains after xylem-phloem transfer. Therefore, Cd can accumulate in all parts of rice, such as roots, leaves, stems, hulk and grains, with varying accumulation levels (Liu *et al*., 2007a).

In addition to rice species and Cd translocation mechanisms, soil properties, such as pH, Cd level and organic matter, also play an important role in Cd translocation and accumulation in rice. The concentration of Cd in the soil solution and the forms of Cd present are important factors that influence Cd availability in the soil, which has a large effect on Cd uptake by plants. Therefore, soil pH is a primary factor that influences the availability of Cd because free Cd is largely released into the soil at acidic pH (Appel and Ma, 2002). Based on our results, the experimental soil pH was slightly acidic. Although the optimum pH for Cd adsorption by PGA is approximately 5.5 (Siao *et al*., 2009), Cd availability to plants is also greater at acidic pH, suggesting that the high concentration of Cd in the soil would exceed the binding capacity of the added PGA. Furthermore, other divalent metal ions such as ferrous, zinc, magnesium and copper are effective competitors and possess some chemical similarities with Cd. Binding of Cd to PGA greatly decreased by the addition of copper ion because of its high electronegativity compared to Cd and other metal ions (Siao *et al*., 2009). Thus, the relatively high Cd content in the rice grains in the present study could be a result of the very high concentration of Cd in the experimental soil, the rice cultivar, the soil pH, other metal competitors in soil and the use of unpolished rice grains for Cd analysis.

Roots accumulate large quantities of Cd, retaining most of the Cd outside of cells. Cd exclusion from protoplasts, which relies on binding to anionic groups of the cell wall, is a defense mechanisms of many plants against metals. The cation exchange sites on root cell walls contain many active ligands, such as carboxyl and hydroxyl groups (He *et al*., 2008b). These ligands can bind to Cd and interfere with Cd transfer across the plasma membrane of roots (Parker *et al*., 1995). As shown in Fig. 2(c) and (f), electron-dense deposits are reduced in both the epidermis and the intracellular space of the cortex of root cells, suggesting that the addition of PGA can reduce Cd accumulation in rice roots. In light of the cation binding characteristics of PGA, the reduction of the effect of Cd toxicity on rice growth by PGA could occur due to the binding of Cd to the carboxyl groups of PGA, which forms insoluble Cd-PGA complexes outside the cell wall (Siao *et al*., 2009).

The phytoremediation of Cd by Cd-hyperaccumulator plants has been carried out using Cd-contaminated soil from the Mae Sot district (Khaokaew and Landrot, 2015). Although this method is cost-effective and practically possible for farmers, the plant species, growth rate, and planting density are still being investigated in field trials (Khaokaew and Landrot, 2015). Meanwhile, Thai rice cultivars with low Cd accumulation in grains have been selected as alternative cultivars with improved genetics (Sriprachote *et al*., 2012). With respect to the microbial remediation of Cd, nearly all reports were performed at the laboratory scale (Siripornadulsil and Siripornadulsil, 2013). In recent years, several reports have proposed integrative strategies for Cd remediation in soil, for

Table 4. Soil property

	pH	ECe (dS/m)	OM (%)	Total N (%)	Available P (mg/kg)	Exchange K (mg/kg)	Cd (mg/kg)
Before planting	5.50	1.78	3.41	0.44	11.58	130.00	0.069
After planting							
Control	6.11	0.65	2.81	0.18	25.65	185.37	ND
Cd 200 : PGA 0	5.51	0.43	2.88	0.17	16.67	206.65	ND
Cd 200 : PGA 500	5.77	0.49	2.94	0.67	19.46	210.88	ND

Note: ND = not detected

example, the use of plant growth-promoting microbes to enhance the growth rate of Cd-hyperaccumulator plants (Mani *et al*., 2015) and the use of symbiosis between leguminous plants and recombinant rhizobia that produce Cd-binding peptides (Ike *et al*., 2007). Therefore, the integration of phytoremediation, the planting of low-Cd grain cultivars and microbial remediation could be the future approach to reducing Cd in both soil and edible plants cultivated in Cd-contaminated areas.

4. Conclusions

This study demonstrated that the addition of PGA to rice cultivated in Cd-contaminated soil alleviates the adverse effects of Cd on rice growth and productivity. The possible mechanism of Cd sequestration by PGA could be the formation of Cd-PGA complexes, which affects Cd mobilization into roots and reduces the available Cd in the soil solution.

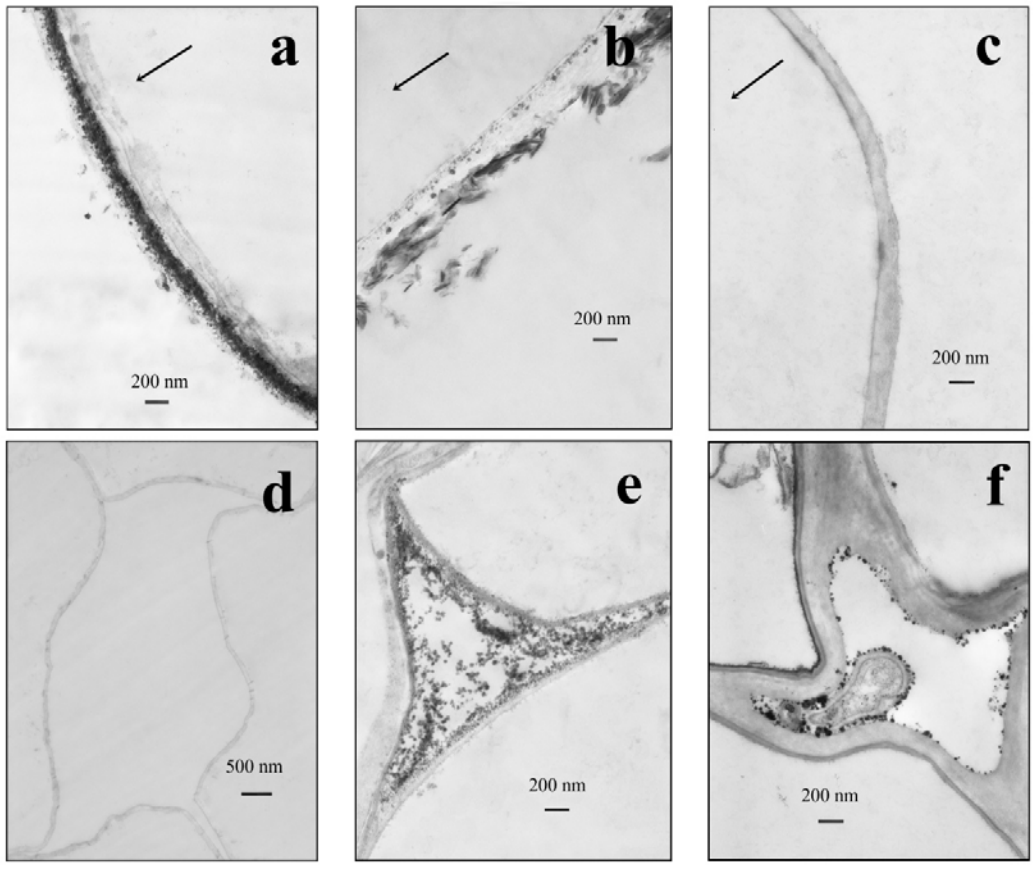

Figure 2. Micrographs of rice root ultrastructure; epidermis of control (a), Cd 200 mg/kg with PGA 0 mg/kg (b), Cd 200 mg/kg with PGA 500 mg/kg (c), intracellular space in cortex of control (d), Cd 200 mg/kg with PGA 0 mg/kg (e) and Cd 200 mg/kg with PGA 500 mg/kg (f)
Note: arrow indicates intracellular of root cell

Acknowledgements

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The authors also thanks to International Cooperation Project under the New Core to Core Program A. Advanced Research Networks on "Establishment of an International Research Core for New Bio-research Fields with Microbes in Tropical Area" Supported by NRCT and JSPS, and Sakon Nakhon Rice Research Center, Thailand.

References

AOAC Official Methods of Analysis. Lead, cadmium, zinc, copper, and iron in foods. Method 999.10. In AOAC. 2012.

Appel C, Ma L. Concentration, pH, and surface charge effects on cadmium and lead sorption in three tropical soils. Journal of Environmental Quality 2002; 31(2): 581-89.

Arao T, Ae N. Genotypic variations in cadmium levels of rice grain. Soil Science and Plant Nutrition 2003; 49(4): 473-79.

Bai HJ, Zhang ZM, Yang GE, Li BZ. Bioremediation of cadmium by growing *Rhodobacter sphaeroides*: kinetic characteristic and mechanism studies. Bioresource Technology 2008; 99(16): 7716-22.

Cheng WD, Yao HG, Zhang HM, Tao XG. Influences of cadmium on grain mineral nutrient contents of two rice genotypes differing in grain cadmium accumulation. Rice Science 2009; 16(2): 151-56.

Doorn WGV, Kirasak K, Sonong A, SrihiranY, Lent JV, Ketsa S. Do plastids in *Dendrobium* cv. Lucky Duan petals function similar to autophagosomes and autolysosomes. Autophagy 2011; 7(6): 584-97.

Gadd GM. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Current Opinion in Biotechnology 2000; 11(3): 271-79.

Grant CA, Buckley WT, Bailey LD, Selles F. Cadmium accumulation in crops. Canadian Journal of Plant Science 1998; 78(1): 1-17.

He JY, Ren YF, Zhu C, Jiang DA. Effects of cadmium stress on seed germination, seedling growth and seed amylase activities in rice (*Oryza sativa*). Rice Science 2008a; 15(4): 319-25.

He JY, Zhu C, Ren YF, Yan YP, Cheng C, Jiang DA, Sun ZX. Uptake, subcellular distribution, and chemical forms of cadmium in wild-type and mutant rice. Pedosphere 2008b; 18(3): 371-77.

Ike A, Sriprang R, Ono H, Murooka Y, Yamashita M. Bioremediation of cadmium contaminated soil using symbiosis between leguminous plant and recombinant rhizobia with the *MTL4* and the *PCS* genes. Chemosphere 2007; 66(9): 1670-76.

Khaokaew S, Landrot G. A field-scale study of cadmium phytoremediation in a contaminated agricultural soil at Mae Sot district, Tak province, Thailand: (1) Determination of Cd-hyperaccumulating plants. Chemosphere 2015; 138: 883-87.

Liu J, Qian M, Cai G, Yang J, Zhu Q. Uptake and translocation of Cd in different rice cultivars and the relation with Cd accumulation in rice grain. Journal of Hazardous Materials 2007a; 143(1-2): 443-47.

Liu J, Cai G, Qian M, Wang D, Xu J, Yang J, Zhu Q. Effect of Cd on the growth, dry matter accumulation and grain yield of different rice cultivars. Journal of the Science of Food and Agriculture 2007b; 87(6): 1088-95.

Mani D, Kumar C, Patel NK. Integrated micro-biochemical approach for phytoremediation of cadmium and zinc contaminated soils. Ecotoxicology and Environmental Safety 2015; 111: 86-95.

McLean RJC, Beauchemin D, Clapham L, Beveridge TJ. Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. Applied and Environmental Microbiology 1990; 56(12): 3671-77.

Ogawa Y, Yamaguchi F, Yuasa K, Tahara Y. Efficient production of γ-polyglutamic acid by *Bacillus subtilis* (natto) in jar fermenters. Bioscience, Biotechnology, and Biochemistry 1997; 61(10): 1684-87.

Parker DR, Chaney RL, Norvell WA. Chemical equilibrium models: applications to plant nutrition research. *In*: Chemistry equilibrium and reaction models (*Eds*: Loeppert RH, Schawb AP, Goldberg S). Soil Science Society of America, Madison, Wisconsin, USA. 1995; 163-200.

Pulford ID, Watson C. Phytoremediation of heavy metal-contaminated land by trees-a review. Environment International 2003; 29(4): 529-40.

Rascio N, Vecchia FD, Rocca NL, Barbato R, Pagliano C, Raviolo M, Gonnelli C, Gabbrielli R. Metal accumulation and damage in rice (*cv*. Vialone nano) seedlings exposed to cadmium. Environmental and Experimental Botany 2008; 62(3): 267-78.

Shih IL, Van YT. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresource Technology 2001; 79(3): 207-25.

Siao FY, Lu JF, Wang JS, Inbaraj BS, Chen BH. *In vitro* binding of heavy metals by an edible biopolymer poly (γ-glutamic acid). Journal of Agricultural and Food Chemistry 2009; 57(2): 777-84.

Simmons RW, Pongsakul P, Chaney RL, Saiyasitpanich D, Klinphoklap S, Nobuntou W. The relative exclusion of zinc and iron from rice grain in relation to rice grain cadmium as compared to soybean: Implications for human health. Plant and Soil 2003; 257(1): 163-70.

Simmons RW, Pongsakul P, Saiyasitpanich D, Klinphoklap S. Elevated levels of cadmium and zinc in paddy soils and elevated levels of cadmium in rice grain downstream of a zinc mineralized area in Thailand: Implications for public health. Environmental Geochemistry and Health 2005; 27 (5-6): 501-11.

Siripornadulsil S, Siripornadulsil W. Cadmium-tolerant bacteria reduce the uptake of cadmium in rice: potential for microbial bioremediation. Ecotoxicology and Environmental Safety 2013; 94: 94-103.

Sriprachote A, Kanyawongha P, Pantuwan G, Ochiai K, Motoh T. Evaluation of Thai rice cultivars with low-grain cadmium. Soil Science and Plant Nutrition 2012; 58(5): 568-72.

Swaddiwudhipong W, Limpatanachote P, Mahasakpan P, Krintratun S, Padungtod C. Cadmium-exposed population in Mae Sot district, Tak province: 1. Prevalence of high urinary cadmium levels in the adults. Journal of the Medical Association of Thailand 2007; 90(1): 143-48.

Tanaka K, Fujimaki S, Fujiwara T, Yoneyama T, Hayashi H. Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oryza sativa* L.). Soil Science and Plant Nutrition 2007; 53(1): 72-77.

Uraguchi S, Mori S, Kuramata M, Kawasaki A, Arao T, Ishikawa S. Root-to-shoot Cd translocation via the xylem is the major process determining shoot and grain cadmium accumulation in rice. Journal of Experimental Botany 2009; 60(9): 2677-88.

Urushibata Y, Tokuyama S, Tahara Y. Difference in transcription levels of *cap* genes for γ-polyglutamic acid production between *Bacillus subtilis* IFO16449 and Marburg 168. Journal of Bioscience and Bioengineering 2002; 93(2): 252-54.

Vijayalakshmi D, Vijayalakshmi C, Djanaguiraman M, Bangarusamy U. Physiological basis of heterotic vigour in rice seeds. The Madras Agricultural Journal 2003; 90(7-9): 545-47.

Wagner GJ. Accumulation of cadmium in crop plants and its consequences to human health. Advances in Agronomy 1993; 51: 173-212.

Received 20 June 2016
Accepted 30 August 2016

Correspondence to
Assistant Professor Dr. Orawan Chunhachart
Department of Science,
Faculty of Liberal Arts and Science,
Kasetsart University Kamphaeng Saen Campus
Nakhon Pathom, 73140,
Thailand
E-mail: faasowc@ku.ac.th