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# Potential of γ-Polyglutamic Acid for Cadmium Toxicity Alleviation in Rice

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***Abstract*****—**Cadmium (II) (Cd) is one of the major toxic elemental pollutants, which is hazardous for humans, animals and plants. γ-Polyglutamic acid (γ-PGA) is an extracellular biopolymer produced by several species of *Bacillus* which has been reported to be an effective biosorbent for metal ions. The effect of γ-PGA on growth of rice grown under laboratory conditions was investigated. Rice seeds were germinated and then grown at 30±1°C on filter paper soaked with Cd solution and γ-PGA for 7 days. The result showed that Cd significantly inhibited the growth of roots, shoots by reducing root, and shoot lengths. Fresh and dry weights also decreased compared with control; however, the addition of 500 $mg{\cdot}L^{-1}$ γ-PGA alleviated rice seedlings from the adverse effects of Cd. The analysis of physiological traits revealed that Cd caused a decrease in the total chlorophyll and soluble protein contents and amylase activities in all treatments. The Cd content in seedling tissues increased for the Cd 250 μM treatment ($P<0.05$) but the addition of 500 $mg{\cdot}L^{-1}$ γ-PGA resulted in a noticeable decrease in Cd ($P<0.05$).

***Keywords*****—**Polyglutamic acid, Cadmium, Rice, *Bacillus subtilis.*

## I. INTRODUCTION

CADMIUM (II) (Cd) is one of the highly toxic, heavy metals and causes a serious threat to plant growth and human health due to its accumulation in edible plant tissues [1], [2]. Thus, Cd can be transferred to animals and humans via the food chain. Agricultural soils of many countries have been contaminated by Cd because of human activities, such as industrial wastes, zinc mining, sewage sludge and phosphate fertilizers [3], [4]. In Mae Sot district, Tak province, Thailand, the contamination of paddy fields and creeks by Cd released from zinc mining has been occurring for more than 20 years. Samples collected from sediments of the creeks, paddy soils, rice grains and soybeans cultivated in the contaminated areas had Cd concentrations exceed the maximum range of permissible levels [4]. Over 80% of rice grain samples collected contain Cd at a concentration exceeding the Codex Committee on Food Additives and Contaminants (CCFAC) Maximum Permissible Level for rice grain of 0.2 mg Cd $kg^{-1}$ and the estimated weekly intake (WI) values ranged from 20 to 82 μg Cd $kg^{-1}$ body weight [5]. Therefore, the people who live in the contaminated area and consume rice grown locally are always exposed to the risk of chronic Cd toxicity. Although the Thai government policies are designed to promote sugarcane cultivation in terms of renewable energy, local farmers are still interested in growing rice in this area because the government rice mortgage program has produced a high market price for rice.

Gamma-polyglutamic acid (γ-PGA) is a naturally occurring anionic polymer, biodegradable, edible, and nontoxic to humans and environments. γ-PGA consists of D- and L-isomers of glutamic acid linked by amide bonds between α-amino group and carboxyl group [6]. In the past few years, it has been interested in various applications such as thickeners, humectants, drug carriers, biological adhesives, foods, cosmetics, medicines, water absorbents, bioflocculants and wastewater treatment [7]. Moreover, it has been reported to be an effective biosorbent for several metal ions; Ni (II), Cu(II), Mn(II), Al(III) and Cr(III) [8], and Cd (II) [9] in water treatment. Thus, it is of interest to consider whether γ-PGA can be used as a Cd biosorbent with plants and to reduce Cd toxicity in rice. This study investigated the effect of Cd and γ-PGA on rice seedlings grown in the laboratory.

## II. MATERIAL AND METHOD

### A. γ-PGA Production

γ-PGA was prepared by *Bacillus subtilis* NBRC16449 according to the method described on [10].

### B. Effect of γ-PGA and Cd on Growth of Rice Seedling

The healthy rice seeds were surface sterilized in 10% sodium hypochlorite for 10 min, then rinsed 4 times with deionized water. Rice seeds were soaked in deionized water for 24 h. A filter paper was placed on a Petri dish (9 cm in diameter) and moistened with 5 mL of Cd (supplied as $CdCl_2{\cdot}2.5H_2O$ solution) and γ-PGA mixer solution in different series concentrations (Cd; 0, 50, 100, 250, 500 μM and γ-PGA; 0, 50, 100, 500, 1,000 $mg{\cdot}L^{-1}$). Ten rice seeds were put on a Petri dish and incubated in the growth chamber with 12 h light and 12 h darkness at 28ºC±1ºC for 7 days. Three replications were conducted in each treatment. Physical parameters of rice seedling were determined.

### C. Chlorophyll Analysis

Chlorophyll content in seedlings was determined by the method described on [11].

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*D. Amylase Activity*

Amylase activities were determined by the method described on [12].

*E. Determination of Cadmium Content*

Rice seedlings were soaked in 20 mM EDTA for 15 min to remove metal ions on surface [13]; then washed with deionized water three times. The seedlings were dried at 80°C until constant weight and ground to fine powder. Seedling powder was digested with $HNO_3$:$HClO_4$ mixture (4:1, v/v) at 120°C for 12 h. The Cd concentration in seedlings was determined using an atomic absorption spectrometer (Unicam M-Series Solaar, USA).

## III. Result

*A. Effect of γ-PGA and Cd on the Growth of Rice Seedlings*

Cd produced a significant decrease in shoot length compared with the control. Shoot length was gradually reduced by 15%, 24%, 27% and 50% in the Cd treatments of 50, 100, 250 and 500 µM, respectively. The root length was decreased by 24%, 48%, 83% and 96% in the Cd treatments of 50, 100, 250 and 500 µM, respectively. Interestingly, the addition of γ-PGA led to an increase in both root and shoot lengths. The addition of 1,000 $mg·L^{-1}$ of γ-PGA significantly increased the shoot length in the Cd treatments with 250 and 500 µM compared with the same Cd treatments with no γ-PGA added. Furthermore, the root length slowly increased when γ-PGA was added. The fresh and dry weights were also found to decrease with increased Cd concentrations. The addition of γ-PGA significantly increased both fresh and dry weights under Cd stress (Table I).

*B. Chlorophyll Content*

The rice seedlings treated with Cd were clearly bleached and subsequent analysis indicated that the chlorophyll content gradually reduced at high concentrations of Cd. The amount of chlorophyll a slowly decreased with an increase in the Cd concentration. In the 250 µM Cd treatment without added γ-PGA, the amounts of chlorophyll a, b and total chlorophyll were significantly reduced compared with the control. Nevertheless, the addition of γ-PGA alleviated the chlorophyll a, b and total chlorophyll contents in the rice tissues, especially at 1,000 $mg·L^{-1}$ γ-PGA which maintained the chlorophyll content at the same level as in the control (Fig. 1).

*C. Amylase Activity*

Total amylase activity in the rice seedlings treated with Cd gradually decreased with an increase in the Cd concentration compared with the control. Cd concentrations of 250 and 500 µM significantly reduced the total amylase and α-amylase activities. However, the β-amylase activity was not significantly different among all treatments. The addition of γ-PGA increased the total amylase, α-amylase and β-amylase contents compared with the Cd treatments without γ-PG (Fig. 1).

*D. Cd Concentration*

An increased Cd concentration in the solution resulted in an increased amount of Cd in the rice tissue samples (Table II). The Cd treatments of 250 and 500 µM without γ-PGA exhibited significantly increased amounts of Cd. After the γ-PGA was added, the Cd content in the rice tissues decreased compared with the treatment with only Cd. In particular, when the Cd solution concentration was 250 µM, the Cd tissue concentration was significantly reduced with the addition of either 500 or 1,000 mg/L γ-PGA. However, at the highest added Cd concentration (500 µM ), γ-PGA at a concentration of 1,000 mg/L was unable to reduce the Cd content in the rice tissues which suggested that this very high concentration of Cd may exceed the adsorption ability of γ-PGA

*E. The Correlation between γ-PGA Concentration and Cd Content in Rice Tissue*

Result of the correlation analyses between γ-PGA concentration used in the experiment (50, 100, 500 and 1,000 $mg·L^{-1}$) and Cd content in rice tissue is shown in Table III. Cd 100 and 250 µM were significantly correlated ($P<0.05$) with negatively correlation coefficient of -.882 and -.893, respectively. It can be generally concluded that the cadmium content in rice tissue tend to be significantly decreased with the increasing of γ-PGA concentration (Table III).

## IV. Discussion

The present study indicated that γ-PGA has the potential to reduce Cd toxicities and decrease the Cd content in rice seedling tissues (Table II) based on the fact that the addition of γ-PGA to Cd-treated seedlings resulted in increased growth (Fig. 1), chlorophyll contents and amylase activities (Fig. 1). Cadmium at high concentrations inhibited the germination of rice consistent with the results from [3], [12]. The inhibition of growth is the first phenotypic evidence in Cd-treated plants, a reduced rates of elongation in the roots and shoots was apparent at low concentrations of Cd. Cd (1 µM) decreased the mitotic index of root tips and they observed noticeable inhibition when the Cd concentration was increased in rice seedling [12]. The effect of Cd was more apparent on roots than shoots because roots are the first point of contact with Cd and the distribution of metals was different in roots and shoots showing that roots had higher quantities of Cd and retained most of the heavy metal in the apoplastic environment outside the cell [14]. In addition, 55-62% of Cd in *Oryza sativa* L. cv Zhonghua 11 was bound onto the root cell wall [3]. A reduction in the chlorophyll content of rice seedlings under Cd stress may be due to an inhibition of the activity of protochlorophyllide reductase, which is an important enzyme for chlorophyll synthesis [15]. Moreover, inhibition at the sulhydryl site of reductase protein can cause a decrease in the density and size of the chloroplast [15]. In addition, Cd induced the production of chlorophyllase, the enzyme that activates the degradation of the chlorophyll [16]. The inhibition of amylase activity resulted in a low rate of starch hydrolysis and insufficient energy supply during seed

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germination and seedling growth [12], [17]. Amylase activities in germinating seed are inhibited by Cd because Cd displaced calcium, which is a cofactor of amylase. The results of displacement are disporting of calcium from α-amylase and β-amylase or changing the steric configurations of α-amylase and β-amylase. The current study showed that the Cd content in rice seedlings sharply increased with an increase in the supplied Cd concentration (Table II) which was in agreement with the previous reports [2]. The addition of γ-PGA reduced the Cd toxicity resulting in a slow increase in the growth rate, amylase activities and the chlorophyll and Cd contents in seedling tissues. Moreover, the correlation between γ-PGA concentration used in the experiment and Cd content in rice tissue was negative correlation. Therefore, increasing of γ-PGA concentration decreased cadmium content in rice tissue.

Because γ-PGA is an anionic polymer of glutamic acid; it has been used as a chelating material as it is capable of binding metal ions via its carboxylic and amide groups [18]. Heavy metal ions bind with γ-PGA in a similar chelation mechanism followed by a conformational change of γ-PGA into an enveloped aggregate precipitate [19]. Plants transport free metal ions by diffusion along the concentration gradient [20] as their results suggested that γ-PGA was bound to Cd outside the cell wall and formed a Cd-PGA complex which had difficulty in penetrating the cell wall. They also noted that the concentration of soluble Cd became low as a result of the formation of the Cd-PGA complexes which affected the Cd in flux or its transport rate by plantlet roots.

TABLE I
EFFECT OF PGA AND CD ON GROWTH OF RICE SEEDLING

| Cd (μM):PGA ($mg \cdot L^{-1}$) | Mean ±SD | | | |
|---|---|---|---|---|
| | Shoot length (cm) | Root length (cm) | Fresh weight (g) | Dry weight (g) |
| Control | 5.35 ±0.61 ab | 4.17 ±1.65 ab | 0.0356 ±0.0088 ab | 0.0072 ±0.0009 ab |
| Cd 0 : PGA 50 | 5.37 ±0.70 ab | 4.20 ±1.87 ab | 0.0331 ±0.0123 abc | 0.0068 ±0.0012 abc |
| Cd 0 : PGA 100 | 5.22 ±0.69 abc | 4.46 ±1.55 a | 0.0352 ±0.0075 ab | 0.0068 ±0.0009 abcd |
| Cd 0 : PGA 500 | 5.48 ±0.76 a | 3.85 ±2.05 ab | 0.0366 ±0.0132 a | 0.0072 ±0.0012 ab |
| Cd 0 : PGA 1,000 | 5.61 ±0.79 a | 4.22 ±1.83 a | 0.0380 ±0.0176 a | 0.0074 ±0.0012 ab |
| Cd 50 : PGA 0 | 4.52 ±0.50 def | 3.16 ±0.83 bc | 0.0303 ±0.0069 abcde | 0.0061 ±0.0007 cdef |
| Cd 50 : PGA 50 | 4.49 ±0.62 defg | 2.49 ±1.09 cdef | 0.0284 ±0.0085 bcdef | 0.0058 ±0.0008 efg |
| Cd 50 : PGA 100 | 4.43 ±0.52 efgh | 2.66 ±1.23 cd | 0.0314 ±0.0072 abcd | 0.0059 ±0.0006 defg |
| Cd 50 : PGA 500 | 4.79 ±0.55 bcde | 2.49 ±0.76 cdef | 0.0366 ±0.0060 a | 0.0064 ±0.0009 bcde |
| Cd 50 : PGA 1,000 | 5.06 ±0.39 abcd | 3.14 ±1.33 bc | 0.0358 ±0.0088 ab | 0.0058 ±0.0011 efg |
| Cd 100 : PGA 0 | 4.06 ±0.64 fgh | 2.15 ±0.80 cdef | 0.0263 ±0.0062 cdefg | 0.0054 ±0.0009 fgh |
| Cd 100 : PGA 50 | 4.52 ±0.56 def | 1.96 ±0.82 def | 0.0237 ±0.0068 defghij | 0.0055 ±0.0007 fgh |
| Cd 100 : PGA 100 | 4.38 ±0.54 efgh | 2.40 ±0.95 cdef | 0.0245 ±0.0075 defghi | 0.0053 ±0.0007 fgh |
| Cd 100 : PGA 500 | 4.53 ±0.46 def | 2.77 ±1.14 cd | 0.0283 ±0.0066 bcdef | 0.0053 ±0.0008 fgh |
| Cd 100 : PGA 1,000 | 4.63 ±0.62 cdef | 2.61 ±1.16 cde | 0.0341 ±0.0059 ab | 0.0059 ±0.0011 defg |
| Cd 250 : PGA 0 | 3.88 ±0.27 h | 0.68 ±0.46 gh | 0.0183 ±0.0044 hijk | 0.0038 ±0.0005 kl |
| Cd 250 : PGA 50 | 3.91 ±0.36 gh | 0.71 ±0.30 gh | 0.0199 ±0.0041 ghijk | 0.0047 ±0.0008 hij |
| Cd 250 : PGA 100 | 3.88 ±0.59 h | 0.75 ±0.41 gh | 0.0202 ±0.0060 ghijk | 0.0043 ±0.0007 ijk |
| Cd 250 : PGA 500 | 4.39 ±0.49 efgh | 1.53 ±0.79 fg | 0.0235 ±0.0051 efghij | 0.0051 ±0.0008 ghi |
| Cd 250 : PGA 1,000 | 4.73 ±0.47 cde | 1.60 ±0.86 ef | 0.0256 ±0.0055 cdefgh | 0.0051 ±0.0008 ghi |
| Cd 500 : PGA 0 | 2.67 ±1.31 i | 0.16 ±0.09 h | 0.0142 ±0.0055 k | 0.0027 ±0.0010 m |
| Cd 500 : PGA 50 | 3.07 ±0.88 i | 0.24 ±0.17 h | 0.0170 ±0.0042 ijk | 0.0031 ±0.0008 lm |
| Cd 500 : PGA 100 | 3.21 ±0.83 i | 0.33 ±0.26 h | 0.0161 ±0.0068 jk | 0.0029 ±0.0009 m |
| Cd 500 : PGA 500 | 3.90 ±0.28 gh | 0.74 ±0.54 gh | 0.0212 ±0.0028 fghijk | 0.0041 ±0.0008 jk |
| Cd 500 : PGA 1,000 | 4.36 ±0.42 efgh | 1.55 ±0.58 fg | 0.0253 ±0.0048 defgh | 0.0047 ±0.0007 hij |

*Values in a column with different letters are significantly different at (*P*<0.05).

TABLE II
CADMIUM CONTENT IN RICE SEEDLING TISSUES

| PGA concentration ($mg \cdot L^{-1}$) | Cd content ($mg \cdot kg^{-1}$ DW) ±SD | | | |
|---|---|---|---|---|
| | Cd 0 μM | Cd 50 μM | Cd 100 μM | Cd 250 μM |
| PGA 0 | 0.012 ±0.004 c | 30.387 ±0.618 b | 52.875 ±2.453 bc | 998.849 ±35.324 b |
| PGA 50 | 0.049 ±0.008 a | 35.225 ±0.157 a | 61.791 ±1.251 a | 985.767 ±30.652 b |
| PGA 100 | 0.022 ±0.004 bc | 36.012 ±0.918 a | 59.472 ±2.736 ab | 1141.471 ±0.066 a |
| PGA 500 | 0.033 ±0.001 ab | 36.389 ±1.040 a | 51.987 ±0.702 c | 74.891 ±2.571 c |
| PGA 1,000 | 0.026 ±0.001 bc | 25.390 ±0.011 c | 40.946 ±0.138 d | 69.521 ±2.987 c |

*Values in a column with different letters are significantly different at (*P*<0.05).

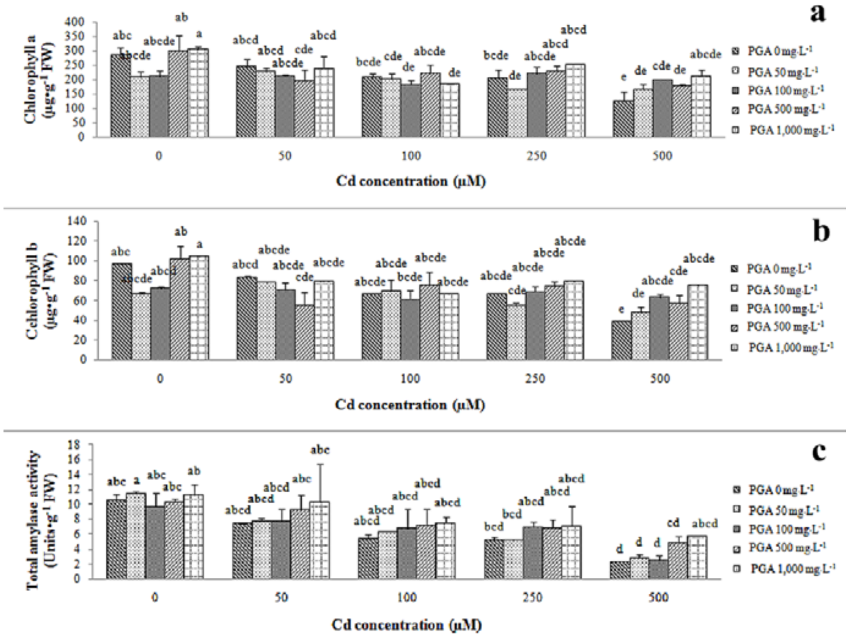


Fig. 1 Effect of PGA and Cd on chlorophyll a and b contents (a and b) and amylase activity (c) (Error lines show standard deviation and bars with different letters are significantly different at ($P$<0.05))

TABLE III
THE CORRELATION BETWEEN PGA CONCENTRATION AND CD CONTENT IN RICE TISSUE

| | Treatments | | | |
|---|---|---|---|---|
| | Cd 0 µM | Cd 50 µM | Cd 100 µM | Cd 250 µM |
| Pearson Correlation | .025 | -.620 | -.882* | -.893* |
| Sig. (2-tailed) | .969 | .264 | .048 | .042 |

*Correlation is significant different at the 0.05 level (2-tailed).

From the results of the current study, the application of γ–PGA was found to be an alternative way for alleviating Cd toxicity. Further study is to investigate effect of PGA on reduction of Cd in rice cultivated in a Cd-contaminated environment.

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