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Hyperaccumulation of Cadmium and DNA Changes in Popular Vegetable, *Brassica chinensis* L.

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ABSTRACT: *Brassica chinensis* L. is a popular vegetable, especially in Asian dishes. For plant growing with inorganic fertilizer, cadmium (Cd) has been one of the highest metal and health-risk factors included. This research aimed to assess the bioaccumulation of Cd by the plant and its genetic changes. The plant was grown in the soil supplemented by Cd at 0, 15, 30, 60 and 120 mg/kg. The accumulations in the roots, stems and leaves, were analyzed using Atomic Absorption Spectrophotometer (AAS), then the Bioconcentration Factor (BCF) and translocation Factor (TF) were calculated. DNA changes were accessed by random Amplified polymorphic DNA (RAPD) with Genomic Template Stability (GTS) tests. The Cd accumulation in the plant parts after 30 days of the treatments ranged from 80.93 to 5053.48 mg/kg, 35.53 to 2439.61 mg/kg, and 21.21 to 2231.02 mg/kg, respectively. The BCF and TF values ranged from 4.54 to 12.66 and 0.70 to 1.67, respectively. From RAPD fingerprints, the GTS values ranged from 51.34 to 80.96%. At the highest concentration of Cd supplemented (120 mg/kg), the DNA resulted in the highest changes (GTS = 51.34%). These results, including BCF and TF values, also indicated that *B. chinensis* is a Cd-hyperaccumulator, therefore, consuming the plants growing in the Cd-polluted area is a health risk

Key words: *Brassica chinensis*, Cd-hyperaccumulator, Bioconcentration factor, Translocation factor, DNA changes

INTRODUCTION

Heavy metal pollution in rivers and soil has become a serious issue of great concern over the last few decades, not only because it can reduce the yield of crops, but also the hazards posed to human health through food chains. Cadmium (Cd) has been used extensively as a material in the agriculture and chemical industry such as inorganic fertilizers, pesticides, paintings, electroplating, etc., and it can be released into the soil and water (Conceição *et al*., 2013; Modaihsh *et al*., 2004; Liu *et al*., 2005). Cadmium accumulation in the food chain could pose a direct threat to human health and can cause diseases (itai disease, cancer), damage to the skeletal system, high blood pressure, adverse cardiovascular effects, enzyme inhibition, DNA damage (Manahan, 2003) and also induces chronic toxicity in animals (Miura *et al*., 2013). The World Health Organization (WHO, 1993) has established a provisional tolerable weekly intake of 7 µg/kg body weight of Cd, whereas the concentration in the soil should be < 37 mg/kg Cd (Ministry of Public Health, Thailand, 1986) and in the leaves of edible plants it should be < 0.2 mg/kg (The Commission of the European Communities, 2006). Thus, Cd effects should be anticipated.

Plants are variously used for the phytoremediation of soil and water pollutions with their capacity in the reduction of toxicity via the physio-biochemical mechanisms. For heavy metal contaminated soil, plants can uptake the metal by the roots and then the translocation to the stems and leaves occurred. Cd contamination in agricultural soils is unlikely to affect plant growth, but Cd is easily transferred to human food chains from the soils. Moreover, an excess of toxic heavy metal ions induces several cellular stress responses and damage to different plant cellular components such as membranes, proteins and DNA

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(Yang *et al*., 2004; Liu *et al*., 2005; 2007a; 2009; Sun *et al*., 2009). Recently, the development of molecular technology has provided suitable tools for DNA analysis in the field of genotoxicology. In many researches, random amplified polymorphic DNA (RAPD) is generally used to effectively indicate the genetic relationships by phylogenetic tree reconstruction (Tanee *et al*., 2012; Kaewdoungdee & Tanee, 2013; Noikotr *et al*., 2013) and species identification (Anuniwat *et al*., 2009). Furthermore, RAPD banding profiles can be scored for genomic template stability (GTS) analysis to detect various types of DNA damage and mutations (point mutation, rearrangement and small deletion or insertion of DNA). The technique has been successfully applied to the study of DNA damage and mutation by heavy metals in plants and animals (Atienzar *et al*., 1999; Liu *et al*., 2005; 2007a; 2009; Duman *et al*., 2014).

Brassica chinensis is one of the popular leafy vegetables in northeastern Thailand, and other Asian countries, especially in China. The knowledge of heavy metal accumulation and DNA damage in the plant species should be clarified for the model of the edible plants. The aims of this study were to determine the contents of Cd in different tissues, *viz.* roots, stems, and leaves, and to detect DNA damage in the plant induced by Cd using RAPD markers.

MATERIALS & METHODS

The tested soil was collected from an agricultural field in Maha Sarakham province, Thailand and determined for Cd concentration before starting the experiments. The collected surface's (depth of 0–30 cm) soil samples were supplemented with five levels of Cd ($CdCl_2 \cdot 2.5H_2O$; Univar, Australia) concentrations: 0 (no supplements), 15, 30, 60, and 120 mg kg^{-1} and 1 kg of the supplemented soils were contained in each plastic pot. Seeds of *B. chinensis* (Chia Tai Seeds Co. Ltd., Thailand) were sown to get five seedlings in each of the pots. Each treatment was replicated three times and arranged in a completely randomized design. To simulate field conditions, the plants were grown under open field conditions with added organic fertilizers. After 30 days (1-30 October 2013) of the experiments, the leaves were collected for DNA analysis. All of the plants and soil were taken for determination of accumulated Cd. The field collected soils (before the experiments) and the collected soil samples from each pot (after the experiments) were dried in an oven at 105°C for 24 h. The plants were dissected into roots, stems, and leaves, and then rinsed thoroughly to get rid of surface materials. After complete drying in an oven (Binder, USA) at 105°C, the dried tissues were ground with a mortar and pestle. A portion (1 g) of each of the dried samples (the soils and plant tissues) was added with 12 ml of $HClO_4$:HNO_3 mixture (1:3) and boiled at 100°C (Tanee *et al*., 2013). This resulted in clear colored solutions which were brought up to 50 ml in a volumetric ûask with deionized-distilled water. The digested samples were analyzed for Cd concentration using the AA 6200 Atomic Absorption Spectrophotometer (Shimadzu, Kyoto, Japan). All the analyses were done in triplicate.

For DNA extraction and RAPD procedures, total genomic DNA was extracted using CTAB method according to Porebski *et al*. (1997). Briefly, each of the leaf samples(50 mg) were finely ground in a 600 µl of warm (65°C) extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (cetyltrimethylammonium bromide)) with a mortar and pestle. The homogenate was transferred to a 1.5-ml microcentrifuge tube then 5 µl of 10 mg/ml RNase A was added and the sample was incubated at 65°C for 30 min. An equal volume of chloroform-isoamyl alcohol mixture (24:1 v/v) was added. The tube was centrifuged at 8,000 ×g for 10 min, then the aqueous phase was transferred to a new tube. Finally, genomic DNA was precipitated by an equal volume of cold (-20°C) 2-propanol for 30 min, then centrifuged. The precipitate was washed with 70% ethanol and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA sample was examined by subjecting it to 0.8% agarose gel electrophoresis in TAE buffer (40 mM tris acetate, 1 mM EDTA, pH 8.0) and visualized using ethidium bromide staining.

In RAPD reaction, amplifications were carried out in 25 µl reactions consisting of GoTaq Green Master Mix (Promega, Madison, USA), 0.5 µM primer (Invitrogen, USA) and 20 ng DNA template. Eighteen RAPD primers were screened and the five primers that successfully amplified clear bands are as follows (52 -32): GCGGCTGGAG (UBC101), GTGACGCCGC (UBC103), GTGCGTCCTC (UBC147), CTGGCGGCTG (UBC155), and CGTGGGCAGG (UBC157). The reaction mixture was denatured at 94°C for 3 min and amplification was performed with the following 35 thermal cycles: denaturation for 30 s at 94°C, annealing for 30 s at 40°C, and extension for 2 min at 72°C, followed by a 7 min final extension at 72°C using a Swift™ Maxi Thermal Cycler (Esco Micro Pte. Ltd., Singapore). The amplified products were separated by 1.2% agarose gel electrophoresis in the TAE buffer and visualized using ethidium bromide staining. The resulting RAPD bands were used to analyze the percentage of genomic template stability (GTS).

All experiments were replicated three times. The means and standard deviations (S.D.) were calculated by Microsoft Office Excel 2010. The means and the S.D. of the Cd in the soil and the plant parts were calculated. Bioconcentration factor (BCF) was calculated as a ratio of Cd concentration in the plant root to the soil, whereas,

translocation factor (TF) was described as a ratio of Cd concentration in the plant shoot to the root (Malik *et al*., 2010). Cd-hyperaccumulating plants were defined, based on the following standards: (1) the accumulating capability (the threshold values of shoots metal concentrations is greater than 100 mg/kg dry weight of shoots), (2) the BCF index is greater than 1.0, sometimes reaching 50-100, and (3) TF index is greater than 1.0, which is used to measure the effectiveness of a plant in the translocation of a metal from the roots to the shoots (Sun *et al*., 2008; 2009; Lorestani *et al*., 2011).

Genomic template stability (GTS) test was calculated by the following equation: GTS (%) = (1-*a*/*n*) × 100; where '*a*' is the number of polymorphic bands detected in each treated sample, and '*n*' is the number of total bands in the control (Liu *et al*., 2007a). The polymorphism observed in RAPD profiles included the disappearance of a normal band and appearance of a new band when compared with the control RAPD profiles. Primers that did not produce changes in RAPD profiles or which were too difficult to score were not used in the calculation.

RESULTS & DISCUSSION

Before the experiments, the field soils contained 14.92±1.18 mg/kg of Cd. The amount and distribution of Cd accumulated in the soil and the *B. chinensis* tissues treated with the different concentrations (0, 15, 30, 60, 120 mg/kg) are shown in Tables 1. The levels of Cd accumulation in soil ranged from 17.82 to 399.06 mg/kg. The Cd accumulation in roots, stems and leaves ranged from 80.93 to 5053, 35.53 to 2439, and 21.21 to 2231 mg/kg, respectively. The distribution of Cd in each type of tissue was found in diminishing order of roots > stems > leaves. The Cd accumulation in all parts of the plant increased with increasing concentrations of Cd spiked in the soils, and there was a positive linear correlation between the root, stem and leaf Cd uptake and Cd concentrations in the soils. The corresponding regression equations can be expressed as:

$$Y_R = 40.396X - 241.93 \quad (R^2 = 0.92) \qquad (1)$$

$$Y_S = 19.946X - 148.09 \quad (R^2 = 0.94) \qquad (2)$$

$$Y_L = 17.679X + 227.18 \quad (R^2 = 0.94) \qquad (3)$$

where Y_R, Y_S and Y_L is Cd concentration in the roots, stems and leaves, respectively, X is the concentration of Cd in soils.

A plant's ability to accumulate metal from soils can be estimated using the BCF and a plant's ability to translocate metal from the roots to the shoots is measured using the TF. From the five different treatments, the BCF values ranged from 4.54 to 12.66, while the TF values ranged from 0.70 to 1.67. The BCF and TF values increased with increasing concentrations of Cd spiked in the soils except for the TF value (0.92) in the treatment that was supplemented with 120 mg/kg of Cd (Table 1).

Cadmium accumulation in edible plants is a serious problem, not only because it could reduce the yield of crops, but also it is a hazard to human health through food chains. *Brassica chinensis* is one the popular vegetables that are used for Asian food, especially Thai and Chinese. From this research, it was observed that the plant can grow in soil that is contaminated with different concentrations of Cd and show high Cd accumulation in both the root and shoot parts (Table 1). The accumulation in the shoots increased with increasing Cd concentrations in the soil (equations 2 and 3). These results are concordant with previous research that shows that the plant can accumulate Cd from the soil (Qin *et al*., 1994; Liu *et al*., 2007b; Yan *et al*., 2009). A Cd-hyperaccumulator should contain Cd in its tissues > 100 mg/kg, whereas the normal level of Cd in most plants is only 0.1 mg/kg. In addition to the total Cd content, both the BCF and the TF indices need to be considered while evaluating the hyperaccumulator. The hyperaccumulating plant should have BCF and TF > 1 (Sun *et al*., 2008; 2009; Lorestani *et al*., 2011). In the present experiment, the concentrations of Cd in both parts of the plants (stems and leaves) ranged from 250 to 2,439 mg/kg and values of the BCF and TF indices ranged from 4.54 to 12.66 and 0.70 to 1.67, respectively. All the values exceeded the critical levels, therefore, *B. chinensis* is defined as a Cd-hyperaccumulator. Plant species that can grow in Cd contaminated soil imply

Table 1. Cadmium accumulation, Bioconcentration Factor (BCF), Translocation Factors (TF) and Genomic template stability (GTS) of *Brassica chinensis* under Cd treatments

Concentration of Cd treated (mg/kg)	Accumulated Cd (mg/kg) in the plant tissues (mean ± SD)			BCF	TF	GTS (%)
	Root	Stem	Leaf			
0 (control)	80.93±10.87	35.53±3.03	21.21±2.29	4.54	0.70	-
15	553.87±25.96	250.43±7.33	426.31±7.73	7.73	1.22	66.44
30	935.28±33.83	322.40±46.61	1,033.24±7.41	6.42	1.45	80.96
60	1,255.82±295.17	699.48±8.59	1,401.96±208.89	6.17	1.67	53.84
120	5,053.48±323.27	2,439.61±305.79	2,231.02±187.27	12.66	0.92	51.34

that it has some tolerant mechanisms on Cd. Although many research studies have documented the Cd tolerance in different plant species such as *Sedum alfredii* (Yang *et al*., 2004), *Solanum nigrum* (Sun *et al*., 2008), and *Biden spilosa* (Sun *et al*., 2009), but the mechanisms of uptake, transport and accumulation of Cd in hyperaccumulating plants are not fully understood yet. Some research results suggested physiological and biochemical effects that Cd can cause a decrease of the root elongation, the height, leaf chlorophyll contents and yields and also increase the concentrations of total non-protein thiols (NPT), the content of free proline and permeability of cell membrane in the shoots (Qin *et al*., 1994; Ren *et al*., 2000; Lin *et al*., 2006; Liu *et al*., 2007b). In addition, from previous researches, BCF and TF values > 1 had been used to evaluate the potential of plant species for phytoextraction while BCF value > 1 and TF value < 1 had been used to evaluate for phytostabilization. The results showed that *B. chinensis* in different treatments had BCF and TF values higher than 1.0 except the TF value in the treatment with 120 mg/kg Cd (Table 1).

The results suggested that *B. chinensis* has characteristics suitable for being used in phytoextraction and phytostabilization of Cd. All of these results support that *B. chinensis* is a Cd-hyperaccumulating plant. Base on the high Cd accumulation in *B. chinensis*, the plants that grow in the contaminated soil are not recommended for food. Because Cd toxicity can pose direct threats to human health via the food chains and it can cause diseases (itai disease, cancer), damage to the skeletal system, adverse cardiovascular effects, enzyme inhibition, and DNA damage on consumer (Manahan, 2003) and its chronotoxicity on animal (Miura *et al*., 2013). Moreover, previous reports have shown that *B. chinensis* can also accumulate other heavy metals such as chromium, lead, zinc, mercury and cupper (Ren & Gao, 2000; Ren *et al*., 2000; Wong *et al*., 2001; Zhang, 2004; Lin *et al*., 2006; Ding *et al*., 2013). However, genotoxic studies in *B. chinensis* exposed to Cd have not been reported. From the RAPD profiles the principal observation was the variations in appearance of new bands and loss of normal bands of the Cd exposed plants when compared with the control non-Cd supplemented plants. The five distinguishing primers produced 69 bands ranging in size from 100 to 3000 bp. The example of RAPD banding patterns are shown in Fig. 1. The RAPD profiles showed substantial differences between the control and the treated plants with apparent changes (disappearance and/or appearance) in the number of DNA bands produced by each primer. In total, 41 RAPD bands occurred due to the loss and/or gain of the amplified fragments in the Cd-treated plants, when compared with the control plants were found. The changes in RAPD profiles are summarized in Table 2. The DNA changes in the plants that were exposed to Cd were expressed as decreases in percentages of GTS that a qualitative measure reflects the changes in the RAPD patterns. The GTS in the different treatments ranged from 51.34% to 80.96% (Table 1). A decrease in GTS values was observed with an increase in Cd concentration, especially in the plants treated with the highest concentration, 120 mg/kg of Cd (Fig. 2). This resulted in the highest DNA changes (GTS = 51.34%), while the lowest DNA change (GTS = 80.96%) was found in the plants treated with 30 mg/kg Cd.

Heavy metal ions induce several cellular stress responses and damage to different plant cellular components such as membranes, proteins and DNA (Yang *et al*., 2004; Liu *et al*., 2005; 2007a; 2009; Sun *et al*., 2009). In the field of genotoxicology, the DNA ûngerprinting assay, especially RAPD profile, was applied to detect the changed DNA. The RAPD assay can be successfully applied to detect genomic DNA changes in plant species induced by several DNA-damaging heavy metals (Liu *et al*., 2007a; 2009; Duman *et al*., 2014). Changes in DNA fingerprint (i.e. band patterns) observed reflect various types of DNA damage and mutations, such as point mutation,

Fig. 1. Random amplified polymorphic DNA (RAPD) proûles of *Brassica chinensis* exposed to different concentrations of Cd, 0 = control, 15, 30, 60, 120 mg/kg, the RAPD profiles from primers UBC130 (A) and UBC157 (B)

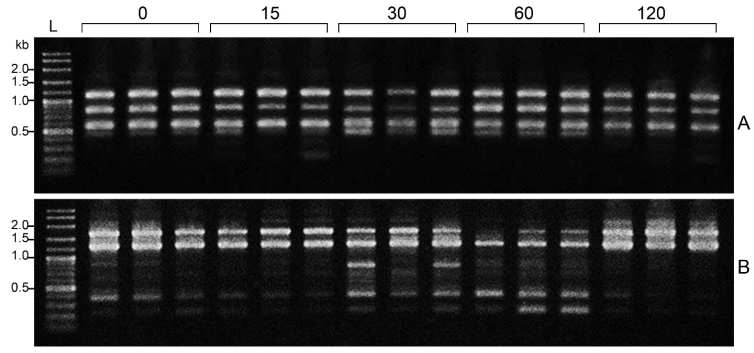

Table 2. Data of random amplified polymorphic DNA (RAPD) profiles including total bands in the control plant and varied bands in the Cd-treated plant when compared with the control plant

Primer	Number of bands in different Cd treatments*								
	0 (control)	15 (mg/kg)		30 (mg/kg)		60 (mg/kg)		120 (mg/kg)	
		a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
UBC101	6	0	2	1	1	1	2	0	1
UBC103	5	2	0	0	0	0	0	2	1
UBC147	7	0	2	1	0	2	3	2	3
UBC155	3	1	1	1	0	1	1	2	0
UBC157	7	0	1	2	0	1	2	0	2
Total	28	3	6	5	1	5	8	6	7
a* + *b		9		6		13		13	

*Symbols: *a*, appearance of new bands; *b*, disappearance of normal bands; *a* + *b*, polymorphic bands.

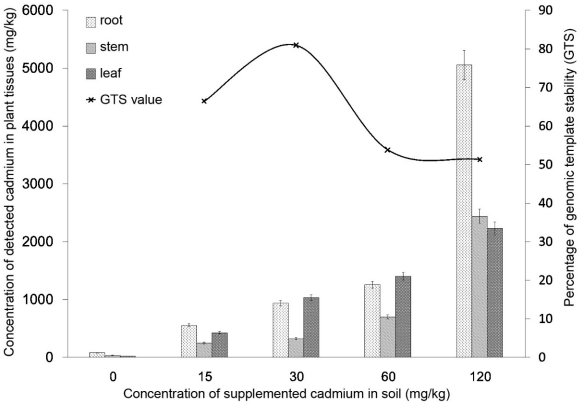

Fig. 2. Comparison of Cd accumulation and GTS values in *Brassica chinensis*

rearrangement and small deletion or insertion of DNA (Liu *et al*., 2007a). Detection of genotoxic effect using RAPD involves the comparison of DNA fingerprints generated from control (unexposed) and treated (exposed) samples. Similarly, in this research, DNA damage induced by Cd exposures for 30 days was reflected by changes in RAPD patterns: appearance of the new amplified fragments and disappearance of bands occurred in the patterns (Fig. 1 and Table 2). Although total bands of DNA fingerprinting showed only 69 characters, these characters are still enough for GTS analysis with 41 characters of DNA changes that compare to the control (untreated). This result supports the proposal that alterations to RAPD profiles due to genotoxic exposure can be regarded as alterations in GTS (Liu *et al*., 2007a). The results indicate that the GTS value in *B. chinensis* was affected by Cd exposure. Moreover, GTS values were shown to enhance with ascending Cd doses (Fig. 2 and Table 1). These results correlated with previous data suggested that Cd could induce DNA changes in plant species (Liu *et al*., 2005; 2007a; 2009; Duman *et al*., 2014). Moreover, this study also suggests that RAPD assay is a very useful tool for ecotoxicology and as a useful biomarker assay for the detection of genotoxic eûects of Cd on plants.

CONCLUSIONS

Cadmium accumulation in plant species should be concerned, not only because of Cd potentially affects the consumers but also causes the genomic damages of *B. chinensis* as shown by RAPD assay. The plant can accumulate Cd at the concentration higher than 100 mg/kg, whereas, the normal level of Cd in most plants is only 0.1 mg/kg. This accumulation level as well as the BCF and TF values indicated that *B. chinensis* is a Cd-hyperaccumulator, therefore, consuming the plants growing in the Cd-polluted area is a health risk. The results of this research should be broadcasted to the public for proper consideration to be taken about consumption of edible plants. Guidance for farmers and others should be provided, especially concerning the instructions for use of pesticides, herbicides, or fertilizers, for food safety in the future.

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