

## Effect of cadmium accumulation on Purple Heart, (*Tradescantia pallida*): An investigation by SEI/ EDX smiling program X-ray images

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### Abstract

This study described a technique for anatomical and ultrastructural investigation of the effects of pH (4.5, 6.0, and 7.5.) on cadmium absorption of Purple Heart (*Tradescantia pallida*) cadmium accumulation in different parts (leaf, stem and root) of the tested plants was determined using chemical method and high vacuum scanning electron microscopy (HV-SEM). We found that, the energy spectrums of the X-rays character emitted from the following elements were carbon (C), oxygen (O), fluorine (F), sodium (Na), magnesium (Mg), aluminum (Al), phosphorus (P), sulfur (S), calcium (Ca), and cadmium (Cd). The percentages of all elements were measured in the following order, C ( $51.830 \pm 6.859$ ) > O ( $43.530 \pm 4.727$ ) > Ca ( $1.496 \pm 2.239$ ) > P ( $1.270 \pm 1.601$ ) > Na ( $0.789 \pm 0.752$ ) > F ( $0.641 \pm 0.804$ ) > Al ( $0.182 \pm 0.447$ ) > S ( $0.090 \pm 0.152$ ) > Mg ( $0.055 \pm 0.043$ ) > Cd ( $0.040 \pm 0.088$ ). The effects of Cd on different pH study indicated that at pH 4.5 the most Cd accumulation was found at lower leaf, lower stem and upper stem of the tested plants, while at pH 7.5 the most Cd accumulation was at upper leaf and tap root of the plants. Results from statistical analysis revealed that there was no significant difference of Cd accumulation at the tap root and lower stem parts between the treatment and control plants ( $p > .05$ ). In contrast, the percentages of Cd at lower and upper leaf, and upper stem parts were significantly different between the treatment and control ( $p < .05$ ). We conclude that, Purple Heart has potential for reduction of Cd in medium. Further study is to use of Purple Heart in reduction of heavy metal contamination in soil and environment.

Key words: Cadmium, Purple Heart, *Tradescantia pallid*, SEI/ EDX Introduction

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Cadmium is a heavy metal that has been ranked 7<sup>th</sup> in the top 20 hazardous substances by ATSDR and classified as human carcinogens by the International Agency for Research on Cancer (IARC, 1997). Contamination of cadmium in the environments results from lead and zinc metallurgies, electroplating industrial, coloring, textile industrial, electronic industrial, rubber and polyvinyl chloride or vinyl (PVC) industrial, fuse production, and utilization of nickel-cadmium batteries (Sanita di toppei & Gabbrielli, 1999). Besides, detection of cadmium also reports in the agricultural areas that have been extensively utilized insecticides, fungicides, and phosphate fertilizers (Prasad, 1995; Sanita di Toppei & Gabbrielli, 1999). In the early of 1990, approximately 22,000 tons of cadmium had been released and mostly entered to water and soil (Liu, 2007).

As mentioned above that cadmium mostly contaminates in surface soil. When being absorbed through plant roots, cadmium is accumulated in both consumable and unconsumed parts of plants in large amounts without any symptom (Monteiro et al., 2008). Presently, contamination of heavy metals such as cadmium in food chains has been increasingly concerned as it is a major route of cadmium accumulation in the tissues, leading to kidney and liver malfunctions, and lung cancer (Yargholi, 2008). Previously, Bunyakitjinda et al. (2008) performed the germination-elongation test to investigate cadmium sensitivity of Purple Heart on its growth. Purple Heart is a trailing perennial plant that has ascending purple stems, and large, pointed, oblong leaves which are fleshy, hairless and rich violet-purple. This plant can reduce heavy metal contamination in soil and environment, so-called phytoremediation. Bunyakitjinda reported that the plants showed either normal growth or higher growth rates than those of the control groups, indicating that cadmium has no inhibitory effects on the growth, and root and stalk elongation. Another factor on cadmium absorption from soil and environment by plants has been documented by Hattori et al (2006). They found that pH has effects on efficiency of cadmium accumulation in the examined plants. Therefore, the objectives of this study were to investigate the effects of cadmium with various pH (4.5, 6.0, and 7.5.) absorption of Purple Heart and to determine cadmium accumulation in different parts of the tested plants using SEI/EDX smiling program X-ray images. The data from this study will be further used as fundamental information for development of a novel technique in reduction of heavy metal contamination in soil and environment by Purple Heart.

## Materials and Methods

### *Plant preparation*

Preparation of Purple Heart was started from selection of the plants with equal size and cultivated them by hydroponic technique in Hoagland' solution for a week before using in the experiments. Investigation of the effects of Cd accumulation on different parts was carried out by growing the plants in freshly prepared Hoagland' solutions containing various CdCl<sub>2</sub> 2.5 H<sub>2</sub>O concentrations at 0 ppm (control group) and 20 ppm with various pH at 4.5, 6.0, and 7.5. After cultivation for 4 weeks, the plants were analyzed elemental accumulation on different parts of plant using SEI and EDX with Smiling program X-ray images.

### *Ultrastructure study and elemental analysis preparation*

The samples were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 h. They were rinsed in 0.1 M phosphate buffer pH 7.2 for 10 min twice. The plant tissues were then dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90% and absolute ethanol), and dried in a critical point dryer machine. Ultrastructure of all samples was investigated using SEI and EDX. Elemental compositions and distribution of all elements was mapped using Smiling program X-ray images. The elemental mapping was showed by means of color coding for each element from each layer.

### *Statistical analysis*

Ten elemental compositions were determined in 5 parts of plants (lower leaf, upper leaf, tap root, lower stem and upper stem) from each treatment. The data were then statistically analyzed using a one-way ANOVA or Kruskal-Wallis test, depending on whether the assumptions of ANOVA were met. The significant difference between treatments was showed in boldface column ( $P < 0.05$ ). The results of multiple pairwise tests are identified by letters followed by the means. Means with the same letters do not differ significantly from each other. For the percentage of Cd in each specimen, Levene's test was first used to test the homogeneity of variances. If variances were homogeneous ( $P < 0.05$ ), a one-way ANOVA and multiple pairwise Duncan tests were conducted. If Levene's test indicated heteroscedasticity ( $P < 0.05$ ), a nonparametric multiple pairwise Duncan tests were performed.

## Results

### **Elemental compositions in different parts of Purple Heart**

Elemental compositions of 5 parts; lower and upper leaf, lower and upper stems and root, from each treatment were analyzed by EDX. The results showed the energy spectrums of the X-rays character emitted from the following elements: carbon (C), oxygen (O), fluorine (F), sodium (Na), magnesium (Mg), aluminum (Al), phosphorus (P), sulfur (S), calcium

(Ca), and cadmium (Cd). The percentages of all elements were found in the following order: C ( $51.830 \pm 6.859$ ) > O ( $43.530 \pm 4.727$ ) > Ca ( $1.496 \pm 2.239$ ) > P ( $1.270 \pm 1.601$ ) > Na ( $0.789 \pm 0.752$ ) > F ( $0.641 \pm 0.804$ ) > Al ( $0.182 \pm 0.447$ ) > S ( $0.090 \pm 0.152$ ) > Mg ( $0.055 \pm 0.043$ ) > Cd ( $0.040 \pm 0.088$ ) (Table 1).

Table 1. Elemental compositions of each treatment analyzed from 5areas.

Area	Treatment	C	O	F	Na	Mg	Al	P	S	Ca	Cd
	pH7.5, 20ppm	56.08 (1.714)	41.49 (1.492)	0.350 (0.416)	0.410 (0.216)	0.076 (0.037)	0.016 (0.020)	0.480 (0.191)	0.080 (0.069)	0.993 (0.083)	0.016 (0.005)
	pH6, 20ppm	49.80 (1.861)	48.25 (2.298)	0.260 (0.336)	0.713 (0.587)	0.667 (0.585)	0.100 (0.147)	0.416 (0.192)	0.026 (0.046)	0.393 (0.270)	0.000 (0.000)
	pH4.5, 20ppm	55.78 (5.015)	41.81 (5.234)	0.723 (0.060)	0.383 (0.365)	0.050 (0.017)	0.056 (0.035)	0.276 (0.172)	0.056 (0.011)	0.830 (0.845)	0.030 (0.010)
	Control	58.29 (3.023)	39.47 (2.921)	0.651 (0.430)	0.468 (0.339)	0.060 (0.050)	0.066 (0.045)	0.371 (0.279)	0.032 (0.018)	0.564 (0.233)	0.001 (0.003)
	pH7.5, 20ppm	56.35 (0.622)	40.82 (0.929)	0.233 (0.404)	1.350 (0.562)	0.106 (0.776)	0.090 (0.108)	0.586 (0.489)	0.463 (0.586)	0.306 (0.100)	0.060 (0.034)
	pH6, 20ppm	57.26 (5.816)	40.72 (5.165)	0.786 (0.138)	0.303 (0.439)	0.036 (0.040)	0.100 (0.045)	0.336 (0.397)	0.016 (0.015)	0.423 (0.304)	0.016 (0.020)
	pH4.5, 20ppm	57.47 (6)	39.72 (3)	0.870 (0)	0.680 (0)	0.066 (0)	0.453 (0)	0.366 (0)	0.006 (0)	0.380 (0)	0.010 (0)

	m	(1.117 )	(2.516 )	0.726 (	0.602 (	0.030 (	0.442 (	0.393 (	0.011 (	0.193 (	0.017 (
	Control	55.36 )4 (2.200	42.27 )2 (1.621	0.647 ) 0.476 (	0.231 ) 0.216 (	0.018 ) 0.016 (	0.097 ) 0.056 (	0.560 ) 0.613 (	0.081 ) 0.110 (	0.706 ) 0.700 (	0.008 ) 0.019 (
	pH7.5, 20ppm	54.32 )0 (1.888	41.82 )6 (0.979	0.420 ) 0.441 (	1.073 ) 0.437 (	0.030 ) 0.042 (	1.630 ) 1.576 (	0.410 ) 0.091 (	0.116 ) 0.101 (	0.113 ) 0.102 (	0.060 ) 0.086 (
	pH6, 20ppm	57.06 )0 (0.200	39.29 )6 (2.268	0.883 ) 0.202 (	0.720 ) 0.346 (	0.040 ) 0.045 (	0.073 ) 0.005 (	0.533 ) 0.510 (	0.066 ) 0.051 (	1.376 ) 2.323 (	0.013 ) 0.023 (
	pH4.5, 20ppm	56.55 )3 (3.486	41.69 )0 (4.091	0.776 ) 0.606 (	0.436 ) 0.080 (	0.020 ) 0.017 (	0.166 ) 0.081 (	0.146 ) 0.142 (	0.033 ) 0.015 (	0.063 ) 0.083 (	0.050 ) 0.045 (
	Control	49.91 )8 (5.749	46.34 )4 (5.925	0.614 ) 0.883 (	1.308 ) 0.956 (	0.060 ) 0.052 (	0.078 ) 0.054 (	0.911 ) 0.360 (	0.105 ) 0.058 (	0.404 ) 0.331 (	0.000 ) 0.000 (
	pH7.5, 20ppm	51.72 )3 (1.761	43.13 )0 (2.565	0.513 ) 0.291 (	1.313 ) 0.387 (	0.060 ) 0.060 (	0.203 ) 0.213 (	1.123 ) 0.165 (	0.230 ) 0.130 (	1.646 ) 0.832 (	0.073 ) 0.020 (
	pH6, 20ppm	52.05 )0 (5.416	44.52 )6 (5.574	1.146 ) 1.330 (	0.793 ) 0.405 (	0.076 ) 0.066 (	0.036 ) 0.030 (	0.656 ) 0.504 (	0.103 ) 0.065 (	0.540 ) 0.634 (	0.086 ) 0.075 (
	pH4.5, 20ppm	48.69 )3 (7.206	42.27 )3 (5.280	2.186 ) 3.144 (	0.743 ) 0.251 (	0.060 ) 0.051 (	0.096 ) 0.126 (	2.390 ) 2.224 (	0.096 ) 0.089 (	3.426 ) 3.892 (	1.200 ) 0.174 (

				(	(	(	(	(	(	(	(
	Contr	45.28	48.71	0.363	1.137	0.078	0.093	2.040	0.033	2.023	0.000
	ol	)3	)3	)	)	)	)	)	)	)	)
		(7.837	(5.220	0.354	1.257	0.056	0.052	1.994	0.030	2.393	0.000
				(	(	(	(	(	(	(	(
	pH7.5,	49.94	42.81	0.270	1.656	0.090	0.340	2.320	0.400	2.470	0.106
	20pp	)6	)3	)	)	)	)	)	)	)	)
	m	(3.740	(1.782	0.275	0.869	0.070	0.289	1.018	0.649	0.269	0.060
				(	(	(	(	(	(	(	(
	pH6	49.95	46.53	0.576	0.690	0.080	0.026	0.626	0.110	1.136	0.070
	,20pp	)6	)0	)	)	)	)	)	)	)	)
	m	(1.545	(0.773	0.282	0.205	0.045	0.020	0.204	0.026	0.255	0.060
				(	(	(	(	(	(	(	(
	pH4.5,	43.55	49.33	0.203	2.046	0.076	0.376	2.533	0.043	1.646	0.113
	20pp	)0	)3	)	)	)	)	)	)	)	)
	m	(4.717	(3.751	0.182	1.506	0.049	0.355	1.451	0.020	1.510	0.080
				(	(	(	(	(	(	(	(
	Contr	46.26	45.31	0.470	1.170	0.073	0.710	2.311	0.722	3.107	0.000
	ol	)6	)0	)	)	)	)	)	)	)	)
		(5.230	(2.052	0.513	1.185	0.040	1.116	2.163	0.045	3.608	0.000
				(	(	(	(	(	(	(	(
Ave	51.83	43.53	0.641(	0.789	0.055	0.182	1.270	0.090	1.496	0.040	
rag	0(6.85	0(4.72	0.804)	(0.75	(0.04	(0.44	(1.60	(0.15	(2.23	(0.08	
e	9)	7)		2)	3)	7)	1)	2)	9)	8)	

### Cadmium accumulation in different parts of the plants and effects of pH on cadmium absorption

From all treatments, the highest cadmium level was detected in the stem following with the tap root while the lowest level of cadmium accumulation was in the leaf (Table 2). This indicated that after being absorbed, some cadmium was remained in the root tissue and some were spread to other parts. This spread cadmium was mostly collected in the stem and small amounts were further transported to the leaf. From the results, it can be concluded that Purple Heart could be used in removal of cadmium from soil and environment.

For the effects of pH on cadmium accumulation, 3 different pH, at pH 4.5, 6.0, and 7.5, were studied. It was found that at pH 4.5 the most Cd accumulation was found at lower leaf, lower stem and upper stem of the tested plants, while at pH 7.5 the most Cd accumulation was at upper leaf and tap root of the plants. The results from statistical analysis revealed that there was no significant difference of Cd accumulation at the tap root and lower stem parts between the treatment and control plants ( $p > .05$ ). In contrast, the percentages of Cd at lower and upper leaf, and upper stem parts were significantly different between the treatment and control ( $p < .05$ ).

Table 2. Average percentage of Cd found in 5 parts of Purple Heart.

Treatment	Lower leaf	Upper leaf	Tap root	Lower stem	Upper stem
pH 7.5, 20 ppm	0.016 <sup>b</sup>	0.060 <sup>a</sup>	0.060	0.073	0.106 <sup>a</sup>
pH 6.0, 20 ppm	0.000 <sup>c</sup>	0.016 <sup>b</sup>	0.013	0.086	0.070 <sup>ab</sup>
pH 4.5, 20 ppm	0.030 <sup>a</sup>	0.010 <sup>b</sup>	0.050	1.200	0.113 <sup>a</sup>
Control group	0.001 <sup>c</sup>	0.008 <sup>b</sup>	0.000	0.000	0.000 <sup>b</sup>

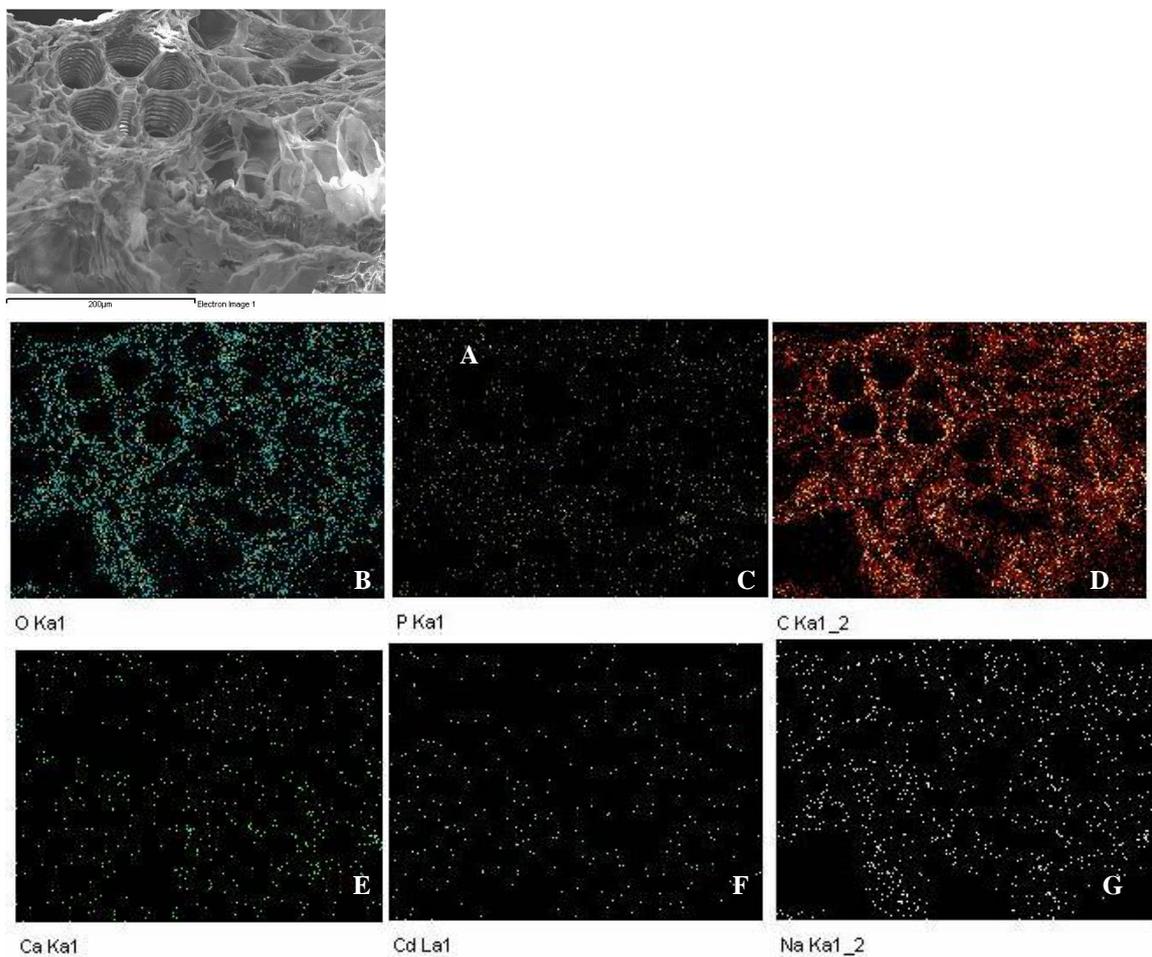
All elements were analyzed for elemental compositions using Smiling program. X-ray images. Distribution of all elements is showed in root (Fig 1.). Carbon, oxygen and calcium, were further investigated as they are elemental composition for formation of  $\text{CaCO}_3$ , which is essential for plant growth. As seen in Fig. 1, C-I, all elements were widely distributed in all areas of tissues. The accumulation of Cd was also observed in all examined part of the plants (Fig. 1 F).

### Statistical analysis of the data

#### *Elemental compositions in different parts of Purple Heart on various pH*

Considering the lower leaf tissue, Cd levels were significantly different between treatments ( $P < 0.05$ ) whilst no statistically difference of other elements observed between treatments. It is clear that difference of Cd accumulation levels among treatments was affecting from the pH in culture medium. In contrast, the percentages of C, O, F, Mg, Al, P, Ca and Cd which were detected in the upper leaf part among 7 treatments showed no significant different ( $P > 0.05$ ) whereas significant difference of Na and S amounts were observed between

treatments ( $P < 0.05$ ), implying that presence of cadmium and pH treatment has effect on accumulation of Na and S in this part of the plant. For the taproot, there was no significant difference of the percentages of C, O, F, Na, Mg, Al, S and Ca observed in all treatments ( $P > 0.05$ ) while significant difference of the P and Cd levels in the tissue were observed ( $P < 0.05$ ), indicating that pH has effect on P and Cd accumulation in this part. Similar to the lower leaf, no significant difference of all examined elements in the lower stem part of all treatment tissues was observed except for Cd. This indicated that pH has influenced on Cd accumulation in the stem. Finally, the percentages of C, O, F, Mg, P and Ca show no significant difference between the upper stem tissues among 7 treatments ( $P > 0.05$ ). However, significant difference of the Na, Al, S and Cd percentages were seen between treatments ( $P < 0.05$ ), suggesting for the effect of pH on Na, Al, S and Cd accumulation at the upper stem. Conclusively, pH has effect on Cd accumulation in all parts of Purple Heart except for the upper leaf.



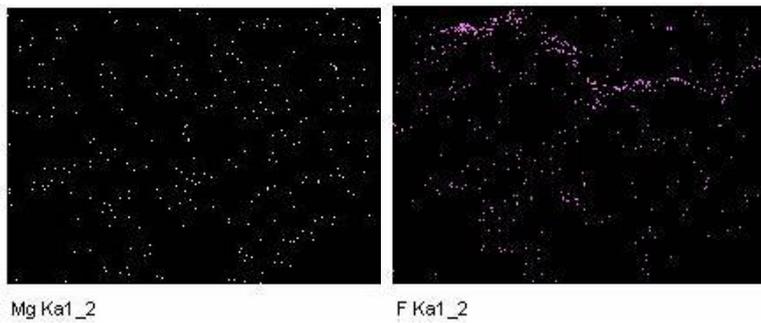


Fig. 1. A: SEM micrographs and the corresponding EDX spectra of elements observed in root tissues, at 4000X. B-I: The energy spectrum of the X-rays character emitted image which mapped the location of the element of oxygen (O), phosphorus (P), Carbon (C), calcium (Ca), cadmium (Cd), sodium (Na), Magnesium (Mg) and Fluoride (F). X-ray images which mapped the location of all elements.

## Discussion

The use of plants in reduction of heavy metal contamination in soil and environment, so-called phytoremediation, has been increasingly interested. Phytoremediation can be processed via several mechanisms depending on the types of plants, which can be concluded as below. Firstly, the metal phytoextraction is observed in the plants that are able to absorb heavy metals through roots and accumulate them in shoots and leaves at high amounts (Jadia & Fulekar, 2009). This type of plants or “hyperaccumulators” can still survive very well under the condition of high cadmium concentration inside them, which is an effective way to remove heavy metals from contamination area. Some plants have ability in accumulating heavy metals 100-1,000 times greater than that was found in the soil from the growing area, or 10-500 times higher than those were detected in normal plants (Hasegawa, 2002). The cadmium accumulating parts of the plants will subsequently be treated by suitable methods (Nandakumar, 1995). Secondary, the phytostabilization is the mechanism found in the plants with root exudation characteristic, in which the plants will release some substances such as organic acids from the roots to form stable complexes with heavy metals. In some plants, they can detoxify the metals accumulating in the roots turning them into stable complexes. This type of plants is useful for removing the toxic substances in soils, silt, and contaminated underground water (Jadia & Fulekar, 2009). Third, the rhizofiltration is found in the plants having a particular type of roots that accumulate toxic substances without further translocation to other parts (Jadia & Fulekar, 2009). The advantage of this method is that the other parts of the plants still can be harvested for further use, while the roots containing the metals will be removed for toxic substance treatment.

From the study in corns, barleys, and cucumbers, it was found that bioaccumulation factor (BAF) or BAF shoot/BAF root in these plants is less than 1, implying that accumulation of cadmium is mostly in the roots (An, 2004). In the opposite of corns, barleys, and cucumbers, sun flower roots (*H. annuus* L.) and Indian mustard (*B. Juncea* (L.) Czern) roots grown by hydroponics can effectively take up  $Cd^{2+}$ ,  $Cu^{2+}$ ,  $Cr^{2+}$ ,  $Ni^{2+}$ ,  $Pb^{2+}$ , and  $Zn^{2+}$  from the solutions (Dushenkov, 1995). A comparison of rhizofiltration characteristic among various plants indicated that Pistia roots performed the most efficient filtration activity in absorbing  $Cd^{2+}$ ,  $Cu^{2+}$ ,  $Cr^{2+}$ ,  $Ni^{2+}$ ,  $Pb^{2+}$ , and  $Zn^{2+}$  from the solution, while duckweed and Water Hyacinth appeared to be the second and third places, respectively (Karkhanis, 2005).

Process of phytoremediation has been found in different mechanisms in several plants. The study in these plants with phytoremediation activity revealed a number of cation exchange sites with active ligands such as carboxyl, hydroxyl, and formylphenyl locating on the cell wall. These sites function to retard transportation of  $Cd^{2+}$  through the membrane (He, 2008). Similar to the report of Nishizono et al (1987), they found 90-70% of the  $Cd^{2+}$  input being trapped within the cell walls of *Athyrium yokoscene* roots.

The other mechanism is that the plants will produce some substances such as phytochelatins (PCs) and organic acids, and storage in cytosols and vacuoles (Grant, 1998; Clemens, 2001). These substances will function as detoxifying agents via forming complexes with Cd metals (chelation) to prevent the ions reacting with sulfhydryl groups of essential enzymes and proteins in the cells, thereby no harmful effects on the cell activity. Additionally, some plants appear to have defense mechanism from Cd accumulation. Observation in barley with 28 genotypes and wheat with 12 genotypes indicated that in tolerant genotypes little Cd ions were transported from roots to straws, and absorbed in very small amounts (Yang, 1995). Likewise, Chardonens et al (1999) found that accumulation of  $Cd^{2+}$  in leaves of tolerant ecotype *Silene vulgaris* is less than that of sensitive ecotype, suggesting that it may result from the detoxification mechanism in preventing transportation of Cd ions into the cell cytosol (Meharg, 1993). From Table 1, carbon and oxygen were the highest percent element (51.830%) and the second highest percent element, respectively (43.530%) found in the examined plants the rest 8 elements were observed in trace amounts (4.64%) throughout 5 parts of Purple Heart. Of which, Cd was the lowest detected level element (about 0.040%). The results shown in Table 2 indicated that purple hearts adsorbed Cd and accumulated it at different parts. It transferred through root into stem and then leaf. This accumulated Cd showed no effect on plant growth (data not shown). When using the medium at various pH (4.5, 6, 7.5), adsorption

of Cd was found in every pH at different levels where the highest level of Cd was seen at pH 4.5. In this study, SEM was used in determination of Cd accumulation, yielding both qualitative and quantitative information. Specially, utilization of smiling program effectively provides information on distribution of Cd including illustration of position of examined elements in the cells.

This study described a technique for anatomical and ultrastructure study of effects on cadmium accumulation in Purple Heart (*Tradescantia pallida*) at various parts (leaf, stems and roots) using chemical method and scanning electron microscopy (SEM). The energy spectrums of the X-rays emitted was found at the following elements: carbon (C), oxygen (O), fluorine (F), sodium (Na), magnesium (Mg), aluminum (Al), phosphorus (P), sulfur (S), calcium (Ca), and cadmium (Cd). The percentages of these elements were detected in the following order: C ( $51.830 \pm 6.859$ ) > O ( $43.530 \pm 4.727$ ) > Ca ( $1.496 \pm 2.239$ ) > P ( $1.270 \pm 1.601$ ) > Na ( $0.789 \pm 0.752$ ) > F ( $0.641 \pm 0.804$ ) > Al ( $0.182 \pm 0.447$ ) > S ( $0.090 \pm 0.152$ ) > Mg ( $0.055 \pm 0.043$ ) > Cd ( $0.040 \pm 0.088$ ). On different pH, at pH 4.5, 6.0, and 7.5, were studied. It was found that at pH 4.5 the most Cd accumulation was found at lower leaf, lower stem and upper stem of the tested plants, while at pH 7.5 the most Ca accumulation was at upper leaf and tap root of the plants. Results from statistical analysis revealed that there was no significant differences of Cd accumulation at the tap root and lower stem parts between the treatment and control plants ( $p > 0.05$ ). In contrast, the percentages of Cd at lower and upper leaf, and upper stem parts were significantly different between the treatment and control ( $p < 0.05$ ). From the results in this study, we conclude that Purple Heart has potential for reduction of Cd amount in medium. The data showed the accumulation of Cd in all parts of plant. Further study is to use of Purple Heart in reduction of heavy metal contamination in soil and environment. Essentially, in this study we have demonstrated the potential of Purple Heart for application in reduction of Cd in soil and environment. The results reported here in are useful for further development of an effective method that do not cause any harm to a living being.

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