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Incidence of Metal Specific Hyper-Accumulation (HMA4) Gene in Wild and Cultivated Plant Species

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Abstract*: Plant hyper-accumulate metals, leading to exceedingly high levels in the plant tissues, with biochemical and possible molecular evidences of this phenomenon. The present work attempts to ascertain the molecular imprint of heavy metal hyper-accumulation in plants using HMA4 gene. Four hyper-accumulating plant species; *Eichhornia crassipes, Ludwigia decurrens, Pistia stratiotes* and *Solanum melongena* were analyzed for heavy metal content using Inductively Coupled Plasma-Mass Spectrometer. The presence of a member gene; HMA4 that expresses heavy metal ATPase for Cadmium (Cd) and Zinc (Zn) within the hyper-accumulation gene-complex HMA complex was analyzed using Polymerase Chain Reaction and Sanger sequencing. The results show that the elemental contents accumulated in the plant species investigated followed the order; Fe > Mn > Na > Zn > Cu > Ni > Cr > Cd > Co, with Se and Pb varying. *Pistia stratiotes* recorded higher concentration of Cd and Cr (1.48 and 16.06ppm) respectively. Other plants range between 0.94 - 0.95 and 6.38 - 9.63 for Cd and Cr respectively. The plant species significantly accumulated higher metal content than permissible levels (ρ = 0.05). However, none of the species showed significant accumulating prowess than the others (ρ = 0.05). Only *Pistia stratiotes* expressed the HMA4 gene; indicating, the gene is probably responsible for the active uptake of Cd and Cr and thus is specific for Cd uptake in *Pistia stratiotes.* The results hold environmental as well as crop improvement potential for the species- *Pistia stratiotes.

***Keywords*: Cadmium, Chromium, Eichhornia crassipes, Gene, HMA4 gene, Hyper-accumulation, Ludwigia decurrens, Pistia stratiotes, Specificity, Solanum melongena.**

1. Introduction

Air and soil pollution is a major problem posing detrimental effect on people and the economy in many parts of the world, limiting agricultural products, also leads to forest decline and numerous harmful substance such as green-house gases. Environmental health impact has been a major problem in many parts of the world. Collecting, transporting and handling coal, mining operations, burning of fossil fuels are major sources of several harmful emissions. These harmful emissions can tremendously contribute to contamination of heavy metal in the soil and atmosphere. However, heavy metals can result from the effect of fertilizers, pesticides, and sewage on the soil. Mineral nutrients used by Plants are obtained from the soil. Plants that grow in soil with elevated level of metals tend to accumulate excess of what is required for its growth. This can affect growth by limiting the plants growth range and also reduce their productivity depending on the plant species. However, certain plant species are capable of growing in excess metalliferous soils. Plant species in these category will hyper-accumulate high amount of metals, which leads to elevated level of metals in plant tissues. Plants ability to remediate heavy metals and accumulate them in its aerial organ has been efficiently used in contaminated soils clean-up. However, not all metal hyper-accumulating plants have high biomass, which is required for a successful and efficient use of plant for remediation. Thus, a very essential inquiry is made to know what is responsible for the ability to accommodate elevated level of heavy metals in certain plants considering the lethal or serious inhibition of growth that might affect a closely related species. [1], grow *Arabidopsis halleri* under elevated levels of Zn. Cross-species transcript profiling was used to identify a set of candidate genes shown to be distinctly expressed in *Arabidopsis halleri* compared to *Arabidopsis thaliana*. Using real time PCR, distinctiveness in transcript level of a subset of these genes was further investigated which shows that 29 genes encode putative metal homeostasis proteins. Prior to this finding, 11 of the 29 genes had been established to involve in metal hyper-accumulation. The other 18 genes show no record of being connected to metal hyper-accumulation.

Four genes (HMA4, ZIP3, ZIP6, and ZIP9) show distinctive high transcript level in *A. halleri*, which may exist as multiple genomic copies. ZIP3, ZIP9, and ZIP6 genes are members of the ZIP metal transporter family and possible genes responsible for roots cytoplasmic metal influx [2], HMA4 on the other hand has been described in *A. thaliana* as a P1B-type heavy metal ATPase and also suggested to play a role in transport of Zn from plants root to shoot [3], [4]. It has also been reported to aid deficiency of Zn and also Cd resistance in yeast, which suggest a role in Cadmium and Zinc transport. The enormous presence of the genes within the genome of *A. halleri* is assumed to be responsible for the distinctive high levels of expression in them,

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supporting the research done by [5] on HMA4 gene. These genes have been shown to have functions directly related to vigorous heavy metals transport in plants. Thus, they can be said to be up-regulated in numerous hyperaccumulating plants such as *Eichhornia crassipes*, *Zea mays*, *Solanum melongena*, *Pistia stratiotes* and so on; as *A. halleri* is one of the model organism used for studying plant biology which conserves metabolic, developmental pathways and genetic material over the course of evolution

Complex heavy metal hyper-accumulator genes have been discovered, but little effort has been made to identify candidate genes responsible for specific heavy metal hyper-accumulation in plant. The outcome of this study is anticipated to provide information on candidate gene responsible for specific heavy metal hyper-accumulation in plants. In advent of the discovery of candidate genes, plants can be genetically modified to hyper-accumulate heavy metals.

The work attempts to ascertain the molecular imprint of heavy metal hyper-accumulation in plants using HMA4 gene.

2. Materials and Methods

A. Plant Materials

Four different plant species (*Pistia stratiotes, Eichhornia crassipes, Solanum melongena,* and *Ludwigia decurrens)* were used for this study.

B. Sample Preparation

Leave samples were collected and placed in a zip-locked bag containing ice-cubes at site of collection and then stored at -80° C in a deep freezer at the laboratory.

C. Study Area

The study areas were majorly locations exposed to different types of pollution. Each plant sample was collected from different notable heavy metal polluted site across Lagos and Ogun state, Nigeria. The brief description of sites and co-ordinates are given in table 1.

D. Determination of Heavy Metals and Other Elements Using Spectrometer

The quantity of heavy metals (Ni, Cu, Pb, Zn, Se, Cd, Cr, Co) and some elements i.e. (Na, Ca, P, Mg and K) was determined in the plant samples. Each fresh screened plant sample is dried at about 105 °C and ground to a grain size of about 0.25 mm. Then the plant sample weighing 1-2 g is placed in an Erlenmeyer flask. 10 mL water and nitric acid of 1:1 (volume/volume) is added to decompose the samples. After decomposition, the sample is then cooled. 5 mL each of H_2O_2, concentrated HCL and H_2O is added before the sample is later heated. After cooling, sample is diluted using distilled H_2O, and then filtered using a 0.45 µm membrane filter. The filtered solution is analyzed using ICP-MS (Inductively Coupled Plasma-Mass Spectrometer, Thermo-Elemental ICP-MS-X Series) at Analytical laboratory IITA Ibadan to determine the presence and concentration of various elements.

E. Extraction of Native DNA

CTAB extraction protocol was used for this research. Dried leaf tissues were grinded into fine powder using GenoGrinder-2000 in the eppendorf tubes. 600 µl of freshly prepared modified CTAB extraction buffer is added and the mixed samples is incubated and placed in water bath at 65°C for 30 minutes while it continues to rock gently. Proper homogenization of the tissue with the extraction buffer was obtained by inverting the tubes regularly. The samples were removed from the water bath and allowed to cool fume hood, sample is gently mixed by tapping and centrifuged at 10000 rpm for 10mins. About 500 µl aqueous phase were transferred into new tubes and 500-600 µl chloroform: isoamyl alcohol (24:1) is added and centrifuge at 10000 rpm for 10 min. The upper aqueous layer is transferred to fresh strip tubes and the chloroform: isoamyl alcohol wash is repeated. Samples were stored in deep freezer at -20℃ for two hours. Centrifuge at 10000 rpm for 20 min. A pellet at the bottom of the tube is formed and the supernatant discarded. 400 µl of 70% ethanol was added. The tubes were gently flapped to let the pellet float for ease of washing, and then centrifuged for 15 min and ethanol discarded by decantation. Pellet is air dried until ethanol evaporates completely. Then 97µl of sterile distilled water and 3µl of RNAse is added to degrade RNA.

F. Determining DNA Quality and Quantity

The quantity and quality of the extracted DNA was determined using a Nano-drop 20002000 spectrophotometer at 260 and 280 mm and the DNA were stored at -20°C. To further substantiate the presence of DNA, 1% of agarose gel containing Ethidium bromide was prepared, the DNA samples were then mixed with loading dye and loaded on the wells of the agarose gel. The gel was run for 25 min at 120 volts. Then, the DNA binds were checked using a UV light Transilluminator.

G. PCR Amplification of DNA

The DNA was subjected to the following cocktail mix and condition for PCR amplification: 10× PCR buffer of 2.5µL, 50 mm $MgCl_2$ of 1.5µL, 5pMol forward primer of 1.0 µL, 5 pMol reverse primer of 1.0 µL, DMSO of 1.0µL, 2.5Mm dNTPs of 2.0, Taq 5 µ/µL of 0.15, 100 ng/µL DNA of 2.0 µL, H_2O of 13.85 µL, all summing up to 25 µL.

HMA4, a P_{1B}type heavy metal ATPase which encodes a protein similar to Zn ATPase. Also shown to aid Zn deficiency and function in Cd resistance in yeast, which suggest a role in

Table 1
Plants, Site description and co-ordinates

S.No.	Plant Name	Site Description	Co-Ordinates (^{0}N, ^{0}E)
1.	*Eichhornia crassipes* (Mart.) Solms	Polluted water body surrounded by heavy metal pollution source such as sites for deposition of several abandoned rusty vehicles, busy over-head bridge with vigorous emission of harmful gases	6.645, 3.380
2.	*Ludwigia decurrens* Walter	Drainage opposite generator house	6.672, 3.162
3.	*Pistia stratiotes* L.	Lake very close to bridge construction site	6.605, 3.229
4.	*Solanum melongena* L.	Site close to a cow dung deposit site, aligned by tricycle park	6.574, 3.258

Cd and Zn transport served as a reference used to design the forward and reverse primers

i) Heavy metal ATPase 4 (HMA4)

HMA4 F (5' TGA TTT ACG TTT TAC AGA AAA TGG C --3')

HMA4 R (5'- GTT CCA GCC CAA ACG GTA GA -3')

The conditions used for PCR were as follows: initial denaturation for 5 min at 94°C; 9 cycles of 15 secs denaturation at 94°C, 20 seconds annealing at 65°C, 30 secs extension at 72°C; 35 cycles of 15 secs denaturation at 94°C, 20 seconds annealing at 55°C, 30 secs extension at 72°C and final extension for 7 min at 72°C.

The amplified products were loaded on a 2.0% agarose gel with a 1kbplus Gene ruler ladder from Thermo Scientific. The gel was run for 45 min at 120 volts. The PCR bands were visualized under a UV light Transilluminator.

3. Results

The elemental contents accumulated in the plant species investigated followed the order; Fe > Mn > Na > Zn > Cu > Ni > Cr > Cd > Co, with Se and Pb varying.

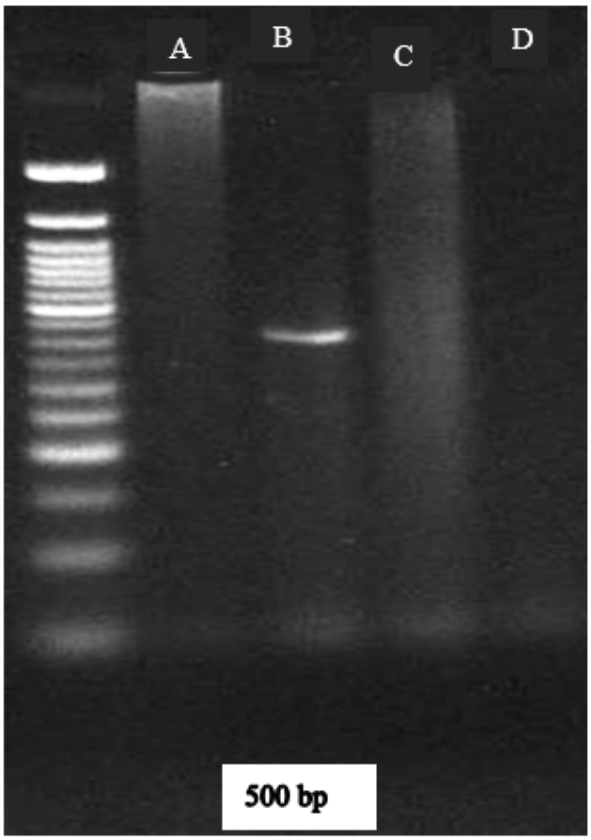

Plate 1: Agarose gel Electrophoresis showing amplification of primer (HMA4 gene) to gDNA of *Pistia stratiotes*
A: *Eichhornia crassipes*; B: *Pistia stratiotes*; C: *Solanum melongena*; D: *Ludwigia decurrens*

Table 2
Spectrometry analysis of heavy metals in plants and its permissible limits

Micro nutrient/heavy metals (ppm	Plants				Limit value
	Eichhornia crassipes	Ludwigia decurrens	Pistia stratiotes	Solanum melongena	(ppm) (system research group 1991)
Na	274.63	32.36	104.42	258.84	150
Mn	795.65	34.44	38.66	665.80	200
Fe	1397.65	204.42	480.66	1092.00	150
Cu	9.42	1.67	1.66	8.20	10
Zn	51.24	12.38	6.12	43.77	50
Pb	1.54	1.55	1.54	1.54	1
Ni	3.00	5.64	3.00	5.62	1.5
Se	1.63	0.84	1.63	0.84	0.02
Co	0.68	0.68	0.49	0.49	0.2
Cd	0.94	0.95	1.48	0.94	0.05
Cr	6.38	9.63	16.06	6.38	1.5
Ca (%)	1.78	0.27	0.28	1.15	1
Mg (%)	0.78	0.02	0.02	0.77	0.2
K (%)	1.84	0.50	0.40	1.83	1.9

Table 3
F-Test analysis for the plant samples based on limit value

Result Details				
Source	***SS***	***df***	***MS***	
Between-treatments	320750.4567	3	106916.8189	$F = 1.17904$
Within-treatments	3627256.0344	40	90681.4009	
Total	**3948006.4912**	**43**		

The *f*-ratio value is 1.17904. The *p*-value is .329821. The result is *not* significant at $p < .05$

Table 4
T-Test analysis for limit values and ecchornia crassipes readings

Permissible limit	*Ecchornia crassipes*	T-value Calculation	*P*-Value
N_1: 11	N_2: 11	s^2_p = 106777.04	.211469
df_1 = 10	df_2 = 10	s^2_{M1} = 9707	
M_1: 51.3	M_2: 231.16	s^2_{M2} = 9707	
SS_1: 58660.03	SS_2: 2076880.77	t = -1.29	
s^2_1 = 5866	s^2_2 = 207688.08		

The *t*-value is -1.29088. The *p*-value is .211469. The result is *not* significant at $p < .05$

Table 5
T-Test analysis for limit values and solanum melongena readings

Permissible limit	*Solanum melongena*	T-value Calculation	*P*-Value
N_1: 11	N_2: 11	s^2_p = 68424.92	.229695
df_1 = 10	df_2 = 10	s^2_{M1} = 6220.45	
M_1: 51.3	M_2: 189.49	s^2_{M2} = 6220.45	
SS_1: 58660.03	SS_2: 1309838.35	t = -1.24	
s^2_1 = 5866	s^2_2 = 130983.84		

The *t*-value is -1.23899. The *p*-value is .229695. The result is *not* significant at $p < .05$

Table 6
T-Test analysis for limit values and pistia stratiotes readings

Permissible limit	*Pistia stratiotes*	T-value Calculation	*P*-Value
N_1: 11	N_2: 11	s^2_p = 13166.08	.866784
df_1 = 10	df_2 = 10	s^2_{M1} = 1196.92	
M_1: 51.3	M_2: 59.61	s^2_{M2} = 1196.92	
SS_1: 58660.03	SS_2: 204661.57	t = -0.17	
s^2_1 = 5866	s^2_2 = 20466.16		

The *t*-value is -0.16992. The *p*-value is .866784. The result is *not* significant at $p < .05$

Table 7
T-Test analysis for limit values and Ludwigia decurrens readings

Permissible limit	*Ludwigia decurrens*	T-value Calculation	*P*-Value
N_1: 11	N_2: 11	s^2_p = 4726.77	.430071
df_1 = 10	df_2 = 10	s^2_{M1} = 429.71	
M_1: 51.3	M_2: 27.69	s^2_{M2} = 429.71	
SS_1: 58660.03	SS_2: 35875.34	$\boldsymbol{t}$ = 0.81	
s^2_1 = 5866	s^2_2 = 3587.53		

The *t*-value is 0.80538. The *p*-value is .430071. The result is *not* significant at $p < .05$

Table 8
Chi-Square analysis of selected heavy metals in plant species based on limit value

	Results					
	Eichhornia crassipes	*Solanum melongena*	*Pistia stratiotes*	*Ludwigia decurrens*	*Permissible limit*	Row Totals
Zn	51 (44.59) [0.92]	44 (39.47) [0.52]	6 (19.74) [9.56]	12 (19.00) [2.58]	50 (40.20) [2.39]	163
Pb	2 (2.46) [0.09]	2 (2.18) [0.01]	2 (1.09) [0.76]	2 (1.05) [0.86]	1 (2.22) [0.67]	9
Co	1 (1.37) [0.10]	1 (1.21) [0.04]	1 (0.61) [0.26]	1 (0.58) [0.30]	1 (1.23) [0.04]	5
Cd	1 (1.64) [0.25]	1 (1.45) [0.14]	2 (0.73) [2.23]	1 (0.70) [0.13]	1 (1.48) [0.16]	6
Cr	6 (10.94) [2.23]	6 (9.69) [1.40]	16 (4.84) [25.70]	10 (4.66) [6.11]	2 (9.87) [6.27]	40
Column Totals	**61**	**54**	**27**	**26**	**55**	**223 (Grand Total)**

The chi-square statistic is 63.7227. The *p*-value is < 0.00001. The result is significant at $p < .05$.

4. Discussion

The large amount of analyzed data derived from environmental trace element research needs a high degree of quality control measure as basis for comparability for the experimental values. The establishment of 'Reference Plant' by the International Commission of Radiological Protection (ICRP) shows the permissible limits for trace elements in plants and can be a useful tool for this comparison.

A. Ascertaining Heavy Metal Hyper-Accumulation in Plant Tissues

Chi-square 5 x 5 contingency analysis on five heavy metals including trace elements (such as Pb, Cd, Cr) required in minute quantity for plant physiological function and permissible limit values for the plant species, shows that there is significant difference between heavy metal and limit values. The plant species significantly accumulated higher metal content than permissible levels (ρ = 0.05), thereby showing the hyper-accumulation property of the plant species.

However, table 2 shows the spectrometry analysis of Plants Heavy Metals and its Permissible Limits. The values of the heavy metal concentration were compared with the limit values. *Eicchornia crassipes* growing on a polluted water body surrounded by heavy metal pollution source such as sites for deposition of several abandoned rusty vehicles, busy over-head bridge with vigorous emission of harmful gases at Ojodu-Berger, Lagos, Nigeria, is characterized by the accumulation of following elements: Na (274.63 ppm), Mn (795.65ppm), Fe (1397.65ppm), Cu (9.42ppm), Zn (51.24ppm), Pb (1.54ppm), Ni (3.00ppm), Se (1.63ppm), Co (0.68ppm), Cd (0.94ppm), Cr (6.38ppm), all appear in higher concentrations except from Copper [Cu] which is lower compared to 'Reference Plant' value. *Ludwigea decurrens* growing in drainage opposite

generator house at Goodness road, Covenant university, Ota, Ogun state, Nigeria, is characterized by the accumulation of following elements: Na (32.36 ppm), Mn (34.44 ppm), Fe (204.42 ppm), Cu (1.67 ppm), Zn (12.38 ppm), Pb (1.55 ppm), Ni (5.64 ppm), Se (0.84 ppm), Co (0.68 ppm), Cd (0.95 ppm), Cr (9.63 ppm), all appear in higher concentrations except from sodium [Na] manganese [Mn], zinc [Zn] and Copper [Cu] which is lower compared to 'Reference Plant' value. *Pistia stratiotes* growing in lake near a bridge construction site at Lagos-Ogun bridge, Aiyetoro road, Ota, Ogun state, is characterized by the accumulation of following elements: Na (104.42 ppm), Mn (38.66 ppm), Fe (480.66 ppm), Cu (1.66 ppm), Zn (6.12 ppm), Pb (1.54 ppm), Ni (3.00 ppm), Se (1.63 ppm), Co (0.49 ppm), Cd (1.48 ppm), Cr (16.06 ppm), all appear in higher concentrations except from sodium [Na] manganese [Mn], zinc [Zn] and Copper [Cu] which is lower compared to 'Reference Plant' value. *Solanum melongena* growing on site close to a cow dung deposit site, aligned by tricycle park at Odo-Eran Bus-stop, Igando, Lagos, Nigeria, is characterized by the accumulation of following elements: Na (258.84 ppm), Mn (665.80 ppm), Fe (1092.00 ppm), Cu (8.20 ppm), Zn (43.77 ppm), Pb (1.54 ppm), Ni (5.62 ppm), Se (0.84 ppm), Co (0.49 ppm), Cd (0.94 ppm), Cr (6.38 ppm), all appear in higher concentrations except from zinc [Zn] and Copper [Cu] which is lower compared to 'Reference Plant' value.

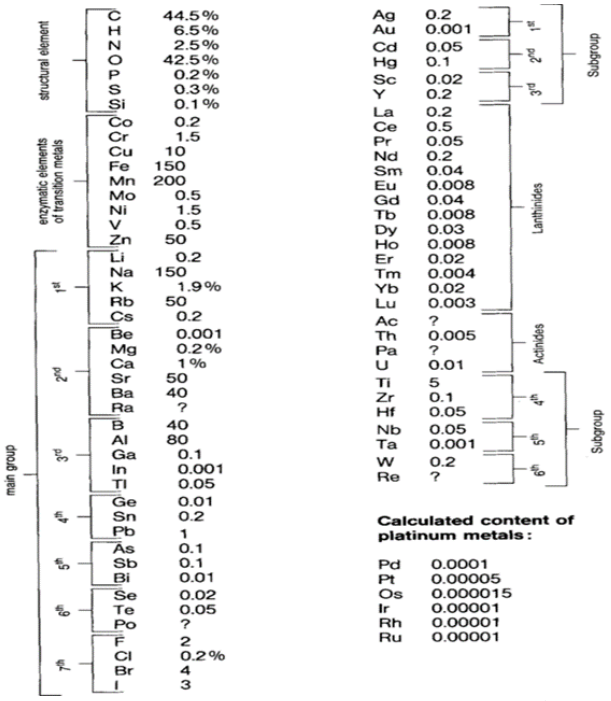

Fig. 1. Element and limit values of 'Reference plant' [mgkg^{-1}/ppm]
Source: [6]

The trace elements in the plant species exceeded most of its permissible limit [i.e. all plant species contain double the value for nickel permissible limit and also the enormous difference in content value of Pb, Cd, Cr between the plants and the given permissible limit], thereby showing that heavy metals are hyper-accumulated and exist in plants tissues.

However, the permissible limits values for leafy vegetables in China (Standard code no GB 2762-2012: 0.30 mg per kg weight for Pb and 0.20 for Cd), Europe (COMMISSION REGULATION (EC) No 1881/2006: 0.30 for Pb and 0.20 for Cd) and United Nations (Codex Standard 193-1995: 0.30 for Pb and 0.20 for Cd), with both of the standard made by these three legislative body looking the same, the values were compared with Pb and Cd content value of the plant species. Results show that Pb ranges between 1.54 – 1.55 ppm in the plant species compared to 0.30 ppm for the standards of leafy vegetables. Cd also has the value range of 0.94 – 1.48ppm compared to 0.20 for the standards of leafy vegetables.

Information about the permissible limits values of Cd and Pb in leafy vegetable in China, Europe and the United Nations have shown leafs of these plants to be inconsumable and distinctively classified them as leafs with high heavy metal content.

B. Variation of Heavy Metal Content in Plants

According to [7], the degree of hyper-accumulation of one or more heavy metals can vary significantly in different species or also in population and ecotypes of the same species. However, F-test analysis carried out to compare mean of the varying heavy metals to the mean of their permissible limits for the plant species reveals that, none of the species showed significant accumulating prowess than the others ($\rho = 0.05$). T-test analysis (table 4 – 8) carried out to compare the value of heavy metals in each plant species with limit values also depicts that none of the species showed significant accumulating prowess than the others ($\rho = 0.05$).

These similar inferences are as a result of inconsistent and varying amount of the different types of heavy metal present in the plants and affinity of plants to different heavy metals.

C. Identifying HMA4 as Heavy Metal Hyper-Accumulating Gene in Plant

Plate1 shows HMA4 gene expressed in *Pistia stratiotes* leaving all other plants un-amplified. Cd and Cr among other heavy metals have higher heavy metal value distinguishing *Pistia stratiotes* from the other plants (Fig. 3 and 4 below).

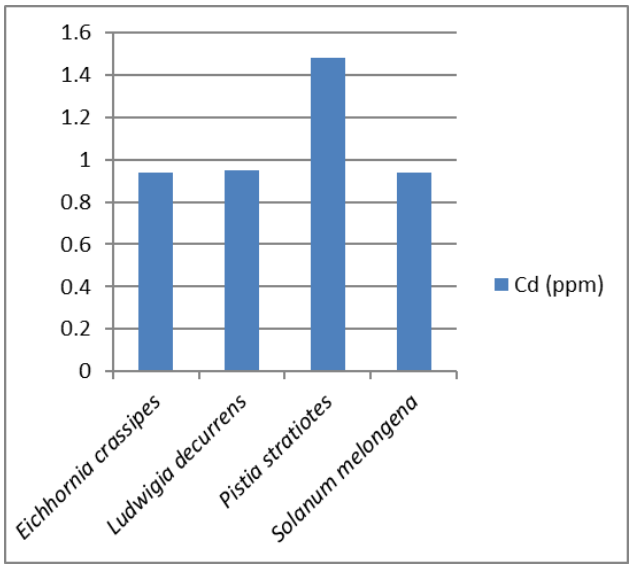

Fig. 2. The different concentration of cadmium (Cd) in the four plants

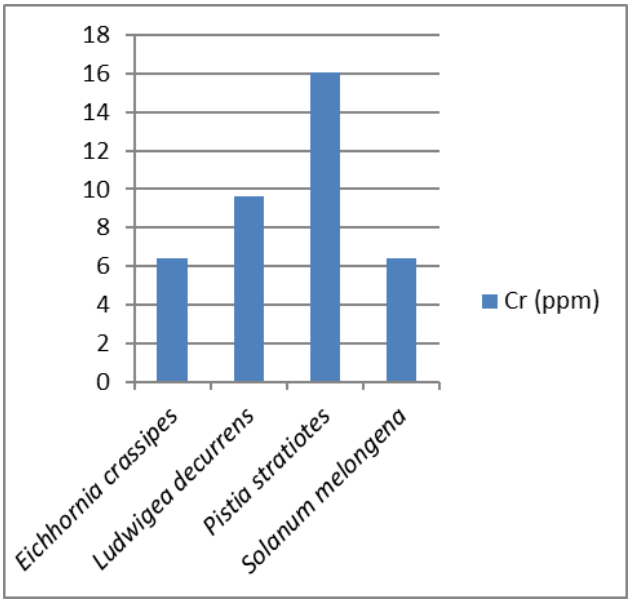

Fig. 3. The different concentration of chromium (Cr) in the four plants

This shows affinity between the primer and the two highly expressed heavy metal (Cd and Cr) in *Pistia stratiotes.* However, HMA4 has been characterized as a P_{1B}-type heavy metal ATPase in *A. thaliana* and has also been suggested to play a role in root to shoot zinc transport (Hussain *et al*., 2004; Verret *et al*., 2004). It has also been reported to aid Zn deficiency and Cd resistance in yeast, suggesting a role in Cadmium and Zinc transport.

This work has confirmed the role of HMA4 for Cd transport in plant and also shows that HMA4 could be responsible for the uptake and transport of Cr in hyper-accumulating plants.

D. Specificity of Hyper-Accumulating Gene in Plants

There is difference in the elevated level of heavy metals with *Eicchornia crassipes* and *Solanum melongena* having more concentration 51.24 and 43.77 ppm respectively. (Fig. 5 below).

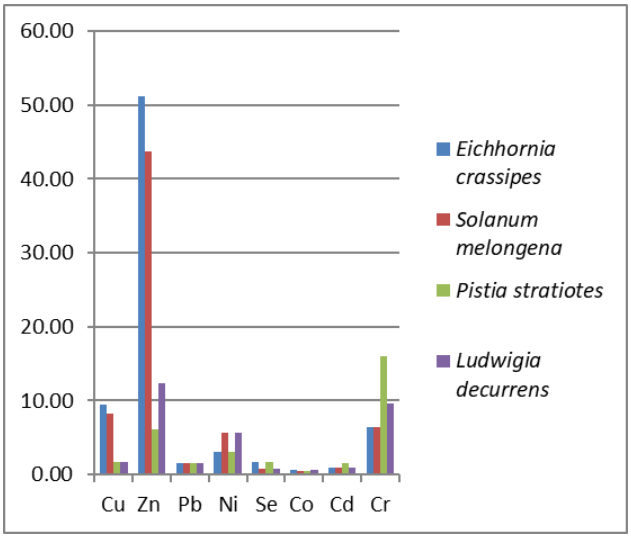

Fig. 4. The different concentration of common heavy metals in the four plants (Element value in ppm)

However, it has been established that HMA4 is specific to elevated concentration of Cd and Cr in *Pistia stratiotes* valued 1.48 and 16.06 ppm respectively. The difference in these values shows that HMA4 gene is specific for elevated level of Cd and Cr in plants, considering Zn in *E. crassipes* and *S. melongena* with higher concentration than Cd and Cr in *Pistia stratiotes*.

5. Conclusion

HMA4 gene has been confirmed to be present in hyper-accumulating plant and also shown to play a major role in heavy metal uptake in plants. It aids in the transport of Cadmium in plant, also suggested to have a role in root to shoot Zinc transport and may be responsible for the uptake and transport of Chromium in the plants.

However, there will be need to identify more plants with such genes and their specificity to heavy metals so as to have a profile for genes that hyper-accumulate. In advent of this exercise, heavy metal genes can be isolated from the plants and used in the improvement of other species for heavy metal clean-up from the environment.

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