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A Preliminary Study on Induction of Phytochelatin in *Mentha piperita* through Cadmium Stress

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Abstract : In this study, it has been proven that *Mentha piperita* can grow in cadmium contaminated soil. It is a fast growing plant, requires minimum attention, and spreads easily. Different concentrations of cadmium were applied to the soil, and the uptake of cadmium was studied using Atomic Absorption Spectroscopy. The plant showed an increase in uptake of cadmium from 5 ppm to 50 ppm in the shoot system; however, there was a decrease in the uptake in 100 ppm concentration. The root system also showed an increased uptake of cadmium from 5 ppm to 100ppm. Identification of Phytochelatin 3 in root tissue sample was carried out by using ESI-MS. Phytochelatin 3 was identified at 753 (m/z). *Menthapiperita* was a metal indicator rather than a hyperaccumulator.
Key words : Heavy metal, Cadmium, *Menth apiperita*, Phytoremediation, Phytochelatin.

1. Introduction

Soil and water contaminated with metals pose a major environmental and human health problem that is still in need of an effective and affordable technological solution. Nonradioactive As, Cd, Cu, Hg, Pb and Zn and radioactive Sr, Cs and U (referred to here as toxic metals) are the most environmentally important metallic pollutants[1,2]. Microbial bioremediation has been somewhat successful for the degradation of certain organic contaminants but is ineffective at addressing the challenge of toxic metal contamination, particularly in soil[3].The phytoremediation of metals is a cost-effective 'green' technology based on the use of metal-accumulating plants to remove toxic metals, including radionuclides, from soil and water[4]. Phytoremediation has recently become a subject of intense public and scientific interest and a topic of many recent reviews. Phytoremediation, in which hyperaccumulators are used to take up huge quantities of toxic metals, has become a hopeful soil remediation technique.

Heavy metals are major environmental pollutants. Among heavy metals, Cd is one of the most perilous elements to plants. Cadmium accumulation in the soil may come from different sources, including air pollution and soil applications of commercial fertilizers, sewage sludge, manure, and lime[5].Human uptake of cadmium takes place mainly through food. Foodstuffs that are rich in cadmium can greatly increase the cadmium concentration in human bodies. Examples are liver, mushrooms, shellfish, mussels, cocoa powder and dried seaweed. The removal of these highly toxic heavy metals can be performed by various biosorption techniques using naturally available biosorbents[6, 7]. The accumulation of Cd in plant tissues may cause a variety of toxicity symptoms ranging from chlorosis, wilting, and growth reduction to cell death[8]. It is, therefore, imperative to develop methods of cleaning up Cd in contaminated soils[9]. Phytoremediation, in which hyperaccumulators are used to take up large quantities of pollutant metals, has become a promising soil remediation technique[10, 11].

Phytoremediation is a natural process that uses various types of green plants to remove, transfer, or stabilize contaminants in soil, sediment, and groundwater[12].

Knowledge of metal-plant interactions is important for the safety of the environment, but also for reducing the risks associated with the introduction of trace metals into the food chain[13]. During the last decade, some studies have been conducted to investigate the mechanisms responsible for enhanced metal uptake and tolerance using natural hyperaccumulators as model plant species[14, 15, & 9]. Plants have evolved some mechanisms to cope with heavy metal stress [14, 17]. It is observed that Glutathione (GSH) plays a central role in protecting plants from environmental stresses, including oxidative stress, xenobiotics, and some heavy metals[18]. Glutathione also serves an additional function in plant responses to heavy metal stress as a precursor of phytochelatins. Phytochelatins (PCs), with the basic structure of (γ- Glu- Cys) n-Gly, where n = 2-11, have been implicated as playing an important role in plant metal tolerance[14]. They are glutathione-derived peptides produced by the transpeptidasephytochelatin synthase. Phytochelatins seems to be an intercellular mechanism for Cd detoxification by shuttling PC-Cd complexes into plant cell vacuoles[19].Although, the induction of PCs *invivo* and activation of PC synthase *invitro* are conferred by a range of metal ions[10]. The best PCS activator tested was Cd followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cations. These metals also induce PC biosynthesis in vivo in plant cell cultures. In in vitro reactions, PC biosynthesis continued until the activating metal ions were chelated either by the PCs formed or by the addition of a metal chelator such as EDTA[20]. However, the only PC complexes identified in vivo were with Cd, Ag, and Cu ions. The objectives of this study were to investigate the induction of phytochelatin in *Mentha piperita* through cadmium stress and to analyze the accumulation PCs, in root and shoot of the plant.

2. Materials and methods

2.1 Chemicals

All chemicals used in the analyses were of the highest purity available. All reagents and solutions were made with Milli-Q Direct 8 purified water. Phytochelatin 3 standard was purchased from AnaSpec, Inc, (Fremont, CA, USA).

2.2 Plant collection and maintenance

Mentha piperita (also known as *M. balsamea Willd*) commonly known as pudhina is a hybrid mint, a cross between water mint and spearmint. It was grown using nodal stem propagation until the stem height reaches to 10 cm. The soil used for the growth of *Mentha piperita* plant was obtained from the nursery. The plant was regularly watered during its growth period, and the soil in which it was grown was obtained from a nursery, free of any metal contamination. It was ensured that the plants were not subjected to synthetic growth regulators like ABA. They regulate Phytochelatin synthases and can hence interfere with experimental results.

2.2 Exposure of plants to cadmium stress

The fraction of the molecular weight of the salt to the atomic weight of the metal gives the amount of salt required to prepare the stock solution.1000ppm cadmium chloride solution was made, and it was then diluted to get the standard solutions of 5, 10, 15, 50 and 100 ppm concentration. The grown plants were exposed to cadmium solutions of various concentrations. The plants which were potted in different pots were exposed to 5, 10, 15, 50 and 100 ppm for ten days. Experiments were carried out in triplicate. All trials were carried out under the same conditions; they were provided with the same mixture of soil (devoid of growth regulators like ABA), watered with equal amounts of water twice a day, had the same exposure to sunlight.

2.3 Analysis of Cadmium content by Atomic Absorption Spectroscopy

After exposure, the amount of cadmium uptake by the roots and shoots were analysed using fully automated double beam Atomic Absorption Spectrophotometer (AAS 4141, ECIL) [21]. The analysis of cadmium uptake by the root and shoot systems were done separately. Metal element contents of the samples were quantified by comparison with the standard solution. One gram of frozen root and shoot tissues of all the concentrations each were ground using a mortar and pestle. The resultant pastes obtained for each concentration were then stirred with 5 ml of sulphuric acid using a glass rod at 4°C for 10 minutes. This was then centrifuged

for 15 minutes at 13000 rpm. The Supernatant from each tube were collected and filtered using Whatman No.1 filter paper to remove suspended particles[22]. The clear solutions were then subjected to Atomic Absorption Spectroscopy. The element contents of the samples were quantified by comparison with the standard solution at appropriate dilution[10].

2.4. Analysis of PC3 synthesis by LC- MS (ESI)

. To identify the presence of phytochelatin 3 in the plants after exposure to various cadmium stresses, the frozen plant tissues were homogenised using mortar and pestle along with quartz sand and 6.3mM of DTPA(Diethylene Triamine Pentaacetic Acid) with 0.1% TFA (Trifluoro Acetic Acid). The mixtures of each concentration were centrifuged at 14,000rpm at 4°C for 10 minutes. Clear supernatant from each tube was collected. The clear solutions of supernatants were analysed for the presence of phytochelatin 3 using Shimadzu LC-MS 2020 with Electro Spray Ionization (ESI)

3. Results and Discussion

3.1. Analysis of Cd contents in plant extracts

The cadmium content in the soil was analysed using AAS. No cadmium content was observed in the soil sample before cadmium exposure. The absorbance of different cadmium concentration is shown in figure 1. The graph shows that there was a linear increase in the absorbance value on the concentration of cadmium.

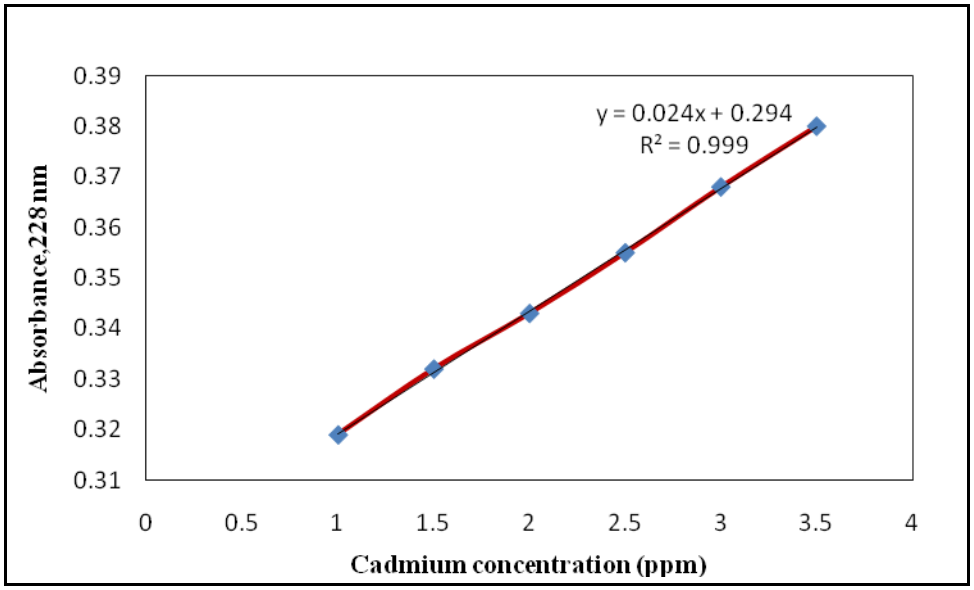

Figure1. Analysis of Cadmium by AAS

The root and shoot tissue extracted samples before and after the cadmium exposure was subjected to AAS analysis. A triplet trial was done to obtain the average uptake of cadmium by the plants and its distribution and transport from the roots to shoot system. From the AAS analysis of shoot tissue extract, it was observed that uptake of cadmium metal was in increment upto 50 ppm. At 100 ppm concentration, there was a marked decrease in the uptake of Cadmium.as shown in fig.2. Whereas, the root tissue extracts had shown increased uptake of cadmium upto 100 ppm as depicted in fig. 3. Unlike the shoot, there was no decrease in the uptake by the roots at 100 ppm.

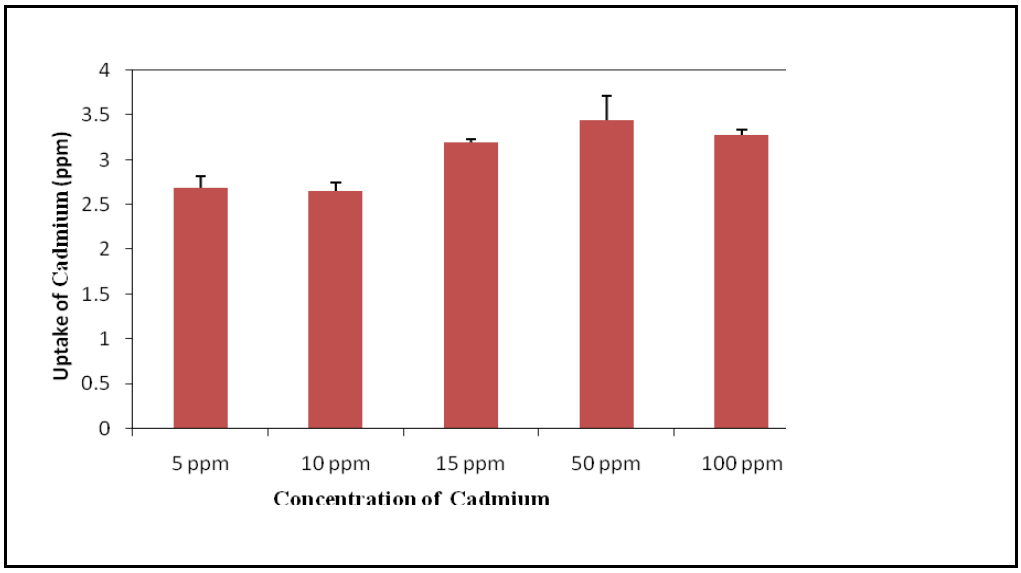

Figure 2.AAS analysis of Cadmium uptake by the shoot system.

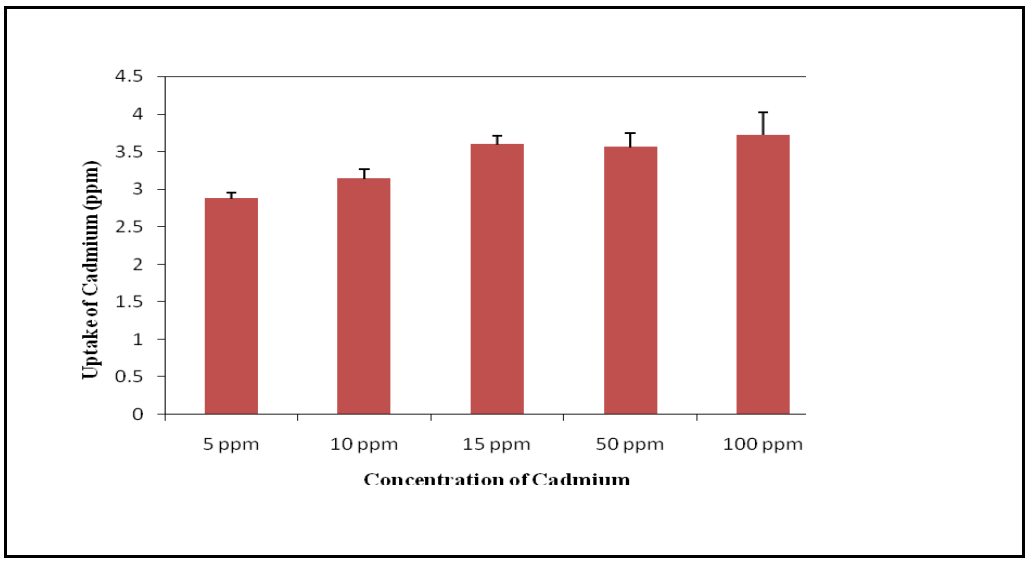

Figure 3. AAS analysis of Cadmium uptake by the root system

The Cd accumulation was lower in shoots than in roots, indicating that a higher proportion of the Cd taken up by plants remained in the roots. Regardless of the different mobility of metals ions in plants than in the above ground tissues[23]. Normally, Cd ions are mainly retained in the roots, and only small amounts are transported to the shoots[24]. This was in agreement with some recent reporton plants such as *Lonicera japonica* Thunb.[9].Such a high metal confinement in theroot tissues may be due to its efficient binding andsequestration to the vacuoles by PCs.

3.2. Chromatographic analysis of Synthetic PC3 and PC3 detection in plant extracts

One very important mechanism for heavy metal detoxification and tolerance in plants is the chelation of the metal ions by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex[25]. PCs have been the most widely studied in plants, particularly about Cd tolerance[26, 27&28].Phytochelatin synthase (PCS) has been shown to be activated only in the presence of heavy metal ions, in particular, Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au and As both in vivo and in- vitro[20]. It has been argued that PCs are involved in the chelation of Cd ions entering the roots. These chelated ions are compartmentalised into vacuoles and could be the cause of the high content of Cd found in roots[29].

The identification of PCs is carried out by comparing the retention times of the signals received for extracts and those obtained for standards. Mass spectrometry with electrospray (ESI-MS) ionisation was applied to support the assumptions framed by the results obtained by LC –MS[30].

Figure 4 and 5 shows the chromatogram obtained from ESI-MS analysis of Phytochelatin 3 standard and root tissue extract of *Mentha piperita*. Two major peaks shown in figure 5 one at753 (m/z) and other at 737(m/z) are observed in the MS data for root tissue extract that has been exposed Cd concentration, and these two peaks were similar to the peaks that were observed in standard PC3 chromatogram, but none of the peaks was observed in the MS data obtained from the control plants (Data not shown). The peak at 737(m/z) represents a trichome, according to the literature. However, the peak at 753 (m/z) of figure 5 is the peak of phytochelatin 3 molecule, in which there has been the removal of a water molecule. The molecular weight of phytochelatin 3 is already known to be 772.8 Daltons[31].

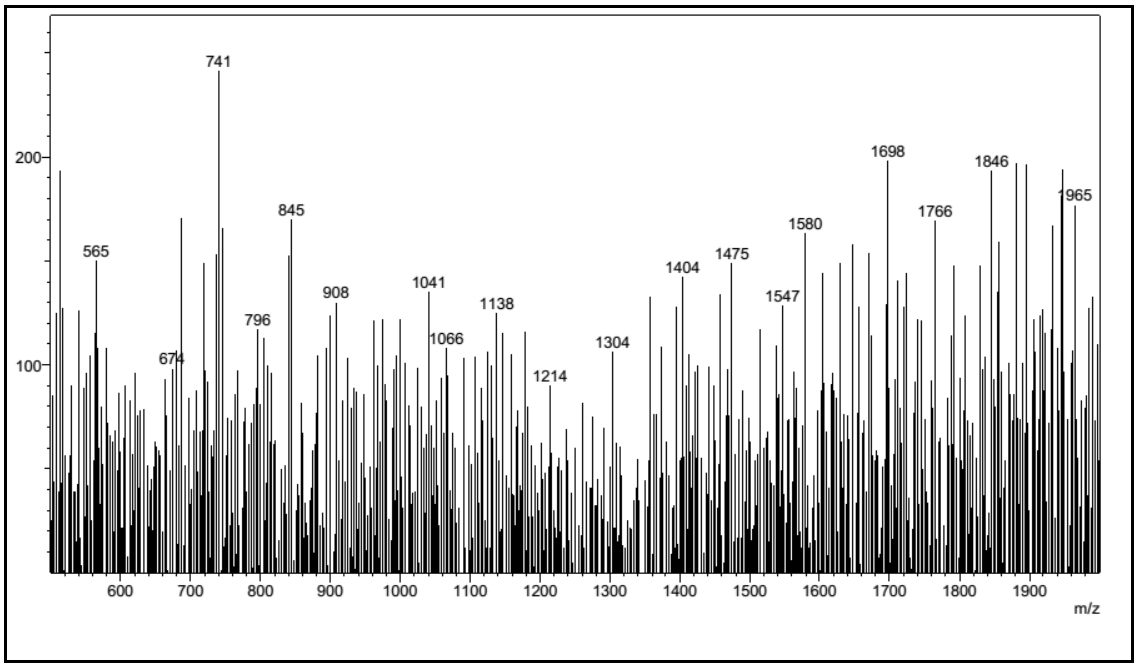

Figure 4. ESI-MS spectra for standard phytochelatin 3

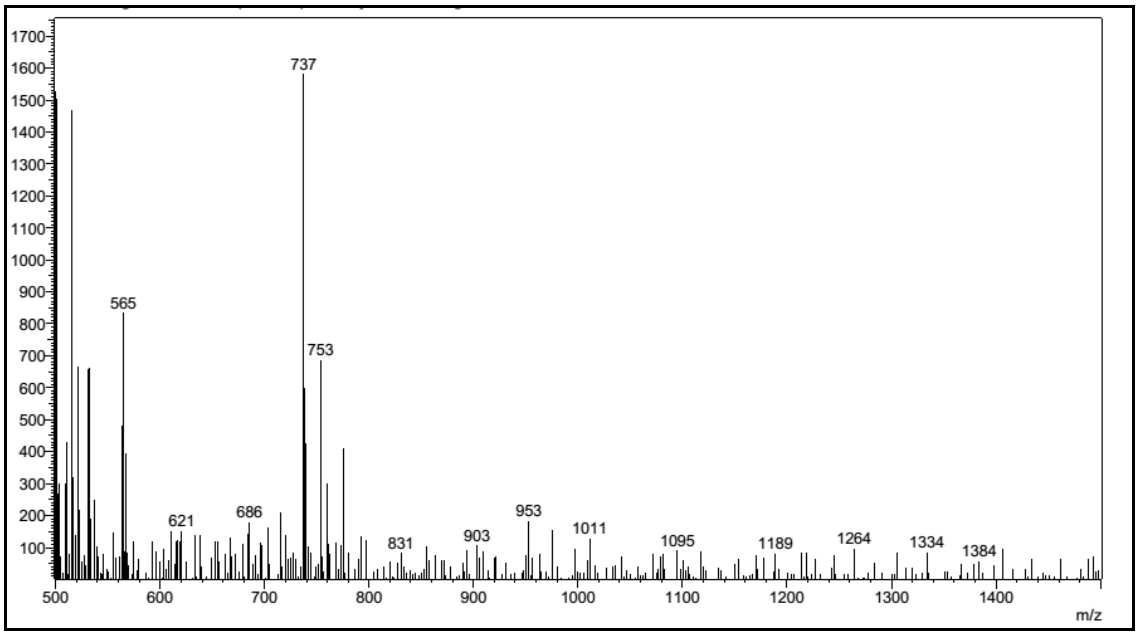

Figure 5. ESI-MS analysis of roots of *Mentha piperita* exposed with cadmium (100 ppm)

Samples from 100ppm of cadmium exposure were also analysed using ESI-MS and similar peaks were observed at 753 (m/z). Hence, the presence of phytochelatin 3 was seen in *Mentha piperita* on exposure to cadmium and its quantity varied directly with concentrations in soil but only partially. The stimulation of PC formation by Cd^{2+} has been reported in roots of various plant species[32, 33]. A possible relationship between Cd-tolerance and Cd-accumulation has been reported at the different family level of plants[34]. PC synthesis in the roots, as seen in our study, may be responsible for increased Cd accumulation in the roots.

4. Conclusion

From the analysis done with the help of AAS, it can be concluded that the distribution of cadmium in the root and shoot system of *Mentha piperita* is almost equal. This implies that there has been the translocation of cadmium from the root to shoot system of plants, and Cd accumulation was significantly enhanced with the increased availability of the metal in the soil. However, the rate of accumulation in shoots decreased at higher concentrations. This shows the production of phytochelatin 3 was observed in *Mentha piperita* root system when exposed to cadmium-contaminated soil but not in shoot system. Hence, it is proven that *Mentha piperita* is one of such plants withstand Cd toxicity and also help in remediation of cadmium metal in the environment by the hyperaccumulation of the metal. Further studies to be carried out to estimate the amount of Phytochelatin3 synthesised by the *Mentha piperita.*

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