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Identification of *Helianthus annuus* Proteins that are Upregulated when Exposed to Heavy Metals

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ABSTRACT

Plants have evolved the ability to extract, transport and use micronutrients from the soil. Many plants utilize this mechanism to accumulate toxic metals from the soil without incurring toxic effects. As a result, phytoremediation is gaining interest as a remediation technology. The study of model plants has shown that plants use a large amount of its genome to deal with stress, including heavy metal uptake and protection from the toxic effects. Although many genes have been identified, knowledge of the basic mechanisms of remediation is limited. Hydroponic and soil based experiments were conducted with two different dwarf sunflowers to identify what proteins were up-regulated as a response to heavy metal exposure. Two-dimensional gels of *Helianthus annuus* have isolated four proteins that were upregulated when the plants were exposed to arsenic or lead. This paper will discuss total metal uptake and translocation of arsenic and lead as well as the proteins that were upregulated as a result of metal sequestration.

Key Words: Phytoremediation, *Helianthus annuus*, proteins, arsenic, lead

INTRODUCTION

Consumption of contaminated drinking water has led to a global epidemic of arsenic poisoning with increased occurrences of cancer and Blackfoot disease in Taiwan, Argentina, Chile, and Bangladesh (Tripathi et al., 2007; Ultra et al., 2007). The toxic forms of As include arsenite (AsO_2^-), which is reactive towards thiol groups and acts on proteins and cofactors (lipoic acid) and arsenate (AsO_4^{3-}), an analogue of phosphate that interferes with phosphorylation. Arsenate is apparently transported by PO_4 transporters and reduced to arsenite in plants by an arsenate reductase (Singh and Ma, 2007).

The most well-known As hyperaccumulators are members of the order of Pteridales: *Pteris vittata* (Chinese brakefern) and *Pityrogramma calomelanos* (silver fern) (Wei and Chen, 2006). Other ferns with the ability to hyperaccumulator As include: *P. ensiformisk, P. umbrosa, P. multifida, P. cretica* and *P. argyraea* (Baldwin and Butcher, 2007; Gonzaga et al., 2006; Wang et al., 2007). Only two studies have documented the ability of dwarf sunflowers, *Helianthus annuus,* to hyperaccumulate As (January et al., 2008; Raab et al., 2005). The hyperaccumulation of Pb has been more widely studied than that of As. The most common Pb hyperaccumulators include *Brassica juncea*, *B. napus* (Marchiol et al., 2004), *Pelagorium spp., Sesbanon drummondi*, *Nicotiana tabacum* (Evangelou et al., 2006), and *H annuus* (Solhi et al., 2005).

There are two classes of peptide families associated that facilitate metal homeostatis and tolerance in plant systems – phytochelatins (PC) and metallothioneins (MT) (Yang et al. 2005). In both cases metal-binding is mediated by thiol groups in the peptide. PCs are synthesized by a PC synthase, which is constitutively present in the cytoplasm of plant cells. PCs are involved in As transport, it appears that this is not the major transportation mode in sunflowers (Raab et al. 2005). A similar observation was made for Cd, which is in roots bound to thiol groups and transported to the vacuole, but GSH and PC appear not to participate in transport to the shoot (Piechalak et al, 2002).

Experiments with two strains of *Helianthus annuus* were conducted under hydroponic conditions as well as in soil. Hydroponic experiments were conducted first to eliminate bioavailability issues that would be present in soil. Each cultivar was exposed to As alone, As in combination with Cd, Cr, and Ni, Pb alone, and Pb with Cd, Cr, and Ni.

MATERIALS and METHODS

Cultivar Source, Preparation, Harvesting and Analysis:

Helianthus annuus was used based on its ability to uptake and translocate heavy metals (Chen and Cutright, 2001). Sundance sunflower (*H. annuus* strain 3508) and Teddy Bear sunflower (*H. annuus* strain 03505) seeds were purchased from the Harris Seed Company (Madison, WI). For hydroponic experiments seeds were initially grown in Rockwool in a greenhouse illuminated with natural light. The average greenhouse temperature was 28 ^{o}C (winter) or 35 ^{o}C (summer) in the day and 20 ^{o}C at night. Seedlings were allowed to grow for 4 wk using a nutrient solution containing 250 mg N (NH_4NO_3), 109 mg P (KH_2PO_4) and 207 mg K (KH_2PO_4) per 1 L distilled water. After the growth period, seedlings of similar size were transferred to troughs in their individual Rockwool compartment to initiate the greenhouse experiment.

Seven sunflowers were utilized per chamber, with two chambers used simultaneously per experiment (one for Sundance and one for Teddy Bear sunflowers). . Both chamber received contaminated solution at a complete recycle rate of 1.59 L h^{-1}. After 17 days of exposure, the plants were harvested for analysis. Half of the biomass was transported on dry ice for protein analysis. The other half of the biomass was dried prior to determining the metal content.

Heavy Metal Source and Determination of Metal Content in Plant Biomass:

The metals were applied as As^{5-} ($Na_2HAsO_4{\cdot}7H_2O$), Pb ($Pb(NO_3)_2$) alone or in combination with Cd^{2+} ($CdSO_4{\cdot}8H_2O$), Cr^{3+} ($CrCl_3{\cdot}6H_2O$), and Ni^{2+} ($NiSO_4{\cdot}6H_2O$). Each compound was added to supply 30 mg of the target element to yield a 30 mg/L water or 30 mg/kg soil concentration.

The roots, leaves, and stems were rinsed with distilled water (to remove any metal-contaminated dust), sectioned and dried in a convection oven at 70 °C for 3 days. Dried tissues were weighed and

milled with mortar and pestle. The crushed tissue sections were then digested according to Zheljazkov and Erickson (1996) and analyzed with flame atomic absorption spectroscopy (Buck 200 AA). The detection limit with the extraction procedure and AA analysis was 0.2 mg/L for each metal. Blind samples and certified reference samples (Supelco) were also analyzed for quality control.

Protein Analyses

Protein Extraction Electrophoresis and in-Gel Tryptic Digestion:

The plant tissues were mashed with mortar and pestle in liquid nitrogen, and 100-200 mg were placed in extraction buffer. The plant extraction solution Focus^{TM} Plant Proteome (G-Bioscience, St. Louis, MO) was used following the manufacturer's instructions, then centrifuged for 20 min at 15,000 x g. Supernatants were collected and the protein concentrations were measured using the Bradford method with albumin as the reference protein solution. One dimensional SDS-PAGE was performed as described (van Keulen et al., 2008). Gels were stained with Coomassie Brilliant Blue.

Two dimensional (2D) gel electrophoresis was performed as described in van Keulen et al. (2008). 2-D SDS-PAGE was performed in NuPAGE Novex 4-12 % Bis-Tris ZOOM gradient Gel (Invitrogen) with IPG wells following manufacturer's instructions. Protein molecular weight marker was applied to the well provided on the gel for calibration of the molecular weight. Gels stained with SimplyBlue, run with an electrophoresis buffer and electrophoresis was performed at 200 V for 35-40 min.

LC-MS/MS:

The digested protein samples were analyzed using an EsquireHCT Brucker Daltonics mass spectrometer equipped with a standard Agilent electrospray (ESI) ion source. Samples were loaded in 10 µl aliquots onto the column as described in van Keulen et al. (2008).

DNA and RNA Methods:

RNA was isolated by modified standard methods. Plant material was ground in liquid nitrogen, and RNA was purified using the Ambion RNA-aqueous mini preparation method. Reverse transcription coupled polymerase chain reaction (RT-PCR) was used to amplify cDNA from total RNA (0.5 µg) using a gene specific antisense primer (see below), and Moloney Murine Leukemia virus reverse transcriptase (Epicentre, Madison, WI) according the manufacturer's protocol. The primers were designed based on homology of the amino acid sequence with TC15508 of chitinase in the Sunflower Gene Index and were, sense: 5' TATCCCACAACATGG*GaATtC*ACTCATC and antisense: 5' TCG*CTcgAG*GACTAGTTTATACTGCCT. The lower case letters stand for mutations introduced to provide restriction enzyme recognition sequences, indicated in italics as *Eco*RI and *Xho*I, resp. The putative thaumatin gene was amplified using a sense primer:

5’ATGACTTGTGCCAAAAACCTTCTACTC and antisense: 5’ ATATACTTAAAGAGTTTATGGACAGAAC designed based on TC15998 in the Sunflower Gene Index. The cDNA (10% of the sample) that was obtained by reverse transcription of RNA using the antisense primer was then subjected to the polymerase chain reaction (PCR) using these gene specific primers and *Taq* polymerase as described (van Keulen et al., 1998). As loading control primers were used to amplify part of the 23S rRNA. Primers were designed to amplify the entire open reading frame and DNA fragments were eluted from agarose gels, purified and ligated into pGEM-T Easy vector (Promega, Madison, WI) for sequence analysis. Plasmids were purified by the alkaline miniprep procedure using the Invitrogen plasmid preparation kit (Invitrogen). The DNA sequence was determined using a Beckman CEQ 8000 Genetic Analysis System.

DNA was purified using the DNeasy plant genomic DNA purification kit using the manufacturer’s instructions (Qiagen, La Jolla, CA). The same primers as described above were used for amplification of genomic DNA, which was cloned into the pGEM–T Easy vector and sequenced.

Antiserum Production:

Antiserum was prepared in rabbits against a synthetic peptide located at the C-end of the sunflower chitinase with sequence: CRFYDKQSGYSDAIK and attached to Keyhole Limpet protein (Sigma-Genosys, The Woodlands, TX). Western Blot analysis was as described (Lopez et al., 2003) using a 1:750 dilution of the antiserum and a 2 h incubation at room temperature. A goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase was used and the signal detected with SuperSignal West Pico (Pierce).

Statistical and Data Analysis:

MINITAB statistical software was used to compare experimental. Statistical significance was determined using Tukey’s Honestly Significant Different comparisons. P-values < 0.05 were considered statistically significant. The translocation factor (TF) is defined as the metal shoot concentration divided by the root concentration. In order for phytoremediation to be considered effective the TF should be greater than one. Selectivity is the preferential uptake of one heavy metal over another.

RESULTS and DISCUSSION

Metal Uptake of as Alone or in Combination with Cd, Cr, Ni:

Analysis of metal distribution indicated that more metals were sequestered in roots than either stems or leaves under the experimental conditions. When As was the only contaminant (Fig 1), the root, leaves and stems concentrations were 1.52, 0.52, and 1.04 mg As g^{-1} biomass, respectively with no statistical difference in sequestration location. When Cd, Cr, and Ni were also present, As concentration

was still highest in the roots at 1.54 mg g^{-1} biomass. There was no difference between the arsenic concentration in the leaves or stems. The presence of the other metals did not appear to alter the As uptake. It is also apparent that uptake of Cr and Cd were significantly greater than Ni or As. These results suggested that the process of accumulating arsenic in *H. annuus* is governed by mechanisms that are distinct from those of other metals.

For soil experiments, Sundance cultivar accumulated 0.96 mg, 0.77 mg, and 1.22 mg As for spiked (no chelator), 0.1 g/kg EDTA, and0.3 g/kg EDTA, respectively. Shoot concentrations for each condition were 999, 1000, and 1776 mg As/kg biomass, thus Sundance achieved hyperaccumulator status.

Similar studies were performed with the Teddy Bear cultivar. The uptake patterns in this strain resembled those of Sundance in that more metals were sequestered in the roots than other tissues. In addition, Cr levels, when compared to other metals, were higher. However, the degree of total metal content in Teddy Bear varied with that of Sundance **(Table 1)**. For instance, the metal uptake was Ni>Cr>Cd>As in Teddy Bear whereas the order in Sundance was Cr>Ni>Cd>As.

Metal uptake of Pb alone or in combination with Cd, Cr and Ni:

Figures 2 and 3 contain the uptake for Sundance and Teddy bear sunflowers, respectively exposed to either Pb alone or in combination with Cd, Cr, and Ni. For both cultivars, when Pb was the only contaminant the concentration was significantly higher in the roots. The root, stem, leaves concentration for Sundance were 3.18, 0.04, and 0.04 mg g^{-1} respectively. With teddy bear the root, stem and leaves contained 0.284, 0.11, and 0.12 mg g^{-1} Pb. When Cd, Cr, and Ni were also present, the majority of Pb was still retained in the roots. However the Pb concentrations in each biomass section was significantly higher. This would indicate that the presence of the other metals decreased the toxic effects of Pb leading to a higher uptake.

Teddy Bear accumulated less Pb than Sundance (Table 2), regardless of whether Pb was the sole contaminant or present with other metals. This was not due to a difference in biomass since there was no statistical difference in biomass weight for the Teddy Bear experiment or with Sundance-mixed metals. In terms of total mg metals, Sundance performed better. In addition, it exhibited a better translocation factor than the Teddy Bear (Table 2). The selectivity with Sundance was Cd>Pb>Cr>>Ni. Selectivity with Teddy Bear cultivar was Cr>Pb>Cd>>Ni.

Protein Analysis:

Protein extracts were size fractionated by 1-D polyacrylamide gel electrophoresis (PAGE-SDS) under denaturing conditions to determine protein distribution in both the control and metal exposed plants (leaves). A comparison between the extracts indicated that protein induction was visible (Figure 4). One protein was clearly present in As-treated plants but was not present in control plants or those treated with Cd, Cr, and Ni (data not shown).

The protein indicated with the arrow was excised, subjected to LC/MS/MS and identified as a type III, family 18 chitinase. This chitinase was truly upregulated in plant leaf samples when all metals including As was present as shown by RT-PCR. Primers were designed to amplify this chitinase, which was detected in the Sunflower Gene Index using the obtained amino acid data from LL/MS/MS.

Figure 5 shows the semi-quantitative RT-PCR that was performed with RNA isolated from leaves of control plants (lanes 1, 2 and 7, 8), leaves from plants treated with all metals except As (lanes 3, 4) and leaves from plants treated with all metals including As (lanes 5, 6). The even-numbered lanes represent 35 cycles of PCR the others 20 cycles. The 1000 basepair (bp) band in lane 6 is the chitinase DNA amplified from the chitinase cDNA. The identity of the DNA was confirmed by DNA sequence analysis. The results demonstrate that the proteomics approach followed by verification via RT-PCR is a valuable approach. In addition, antibodies were generated against a C-terminal peptide of this chitinase. The antiserum showed a high degree of specificity and confirmed the induction of this enzyme by As using Western Blots as described (van Keulen et al., 2008).

A protein dot blot (Figure 6) using several tissues from Sundance plants as source of protein was screened using the anti-chitinase serum. It showed strong signals when leaf proteins of plants exposed to Cd, Cr, Ni and As or As alone were loaded, but no signal when plants were not exposed to metals or to metals without As. Chitinase was also detected in 2-D gels of leaf proteins extracted from plants exposed to Cd, Cr, and Ni plus Pb (spot 1, Figure 7). The mass spec data show 8 peptides covering 21% of the protein sequence. This protein was identical to the one observed after As induction and using 1-D gels. In addition to chitinase, three other proteins appear to be upregulated and are not visible in samples from plants exposed to Cd, Cr, and Ni or no metals. One spot, indicated by number 3 appeared to be a heat-shock protein, belonging to the 70 kDa family with 16 peptides covering 29% of the protein sequence. RT-PCR however failed to confirm this. However, spot 2, which appeared to be a thaumatin-like protein with 6 peptides found to be identical to the database sequence, covering 43% of the sequence. RT-PCR using primers designed to amplify the entire open reading frame, the same approach as described for the chitinase, showed a clear upregulation at the RNA level (Figure 8). Not only was the RNA detected in Cd, Cr, and Ni plus Pb treated samples but also, though at a lower level, in Cd, Cr, and Ni plus As treated samples.

CONCLUSIONS

Both cultivars achieved hyperaccumulator status when As was present as a soil contaminant. Furthermore, the uptake and translocation of As was not impacted by the presence of the other heavy metals. Neither Sundance nor Teddy Bear achieved hyperaccumulator status when Pb was present. However the presence of Cd, Cr, Ni did appear to decrease Pb’s toxicity, leading to a higher Pb uptake for

the mixed metal treatment. Overall, Sundance was more effective at uptaking Pb (1.1 mg) than Teddy Bear (0.75 mg). Sundance also exhibited a significantly higher Pb uptake with the mixed metal condition (2.37 mg versus 0.16 mg with Teddy Bear). Both As and Pb were found to induce proteins that were not present within the controls or with just Cd, Cr and Ni.

Chitinase was first found to be upregulated in the leaves and roots of Sundance sunflowers when exposed to As or As in combination with Cd, Cr, and Ni. Chitinase was not induced in the controls or when As was not present. Teddy bear sunflowers were found to also induce Chitinase when As was present. When experiments were repeated with Pb, four protein spots were upregulated. The fourth spot is believed to be a metal ion binding protein, however this has not been externally verified since there was no match to this protein that has a high degree of identify to an *Arabidopsis* protein, with any known sequence in the Sunflower Gene Index. Two of the proteins were externally verified to be chitinase and thaumatin. These results clearly indicate that chitinase and thaumatin were upregualted in Sundance and Teddy bear sunflowers only when As or Pb was present.

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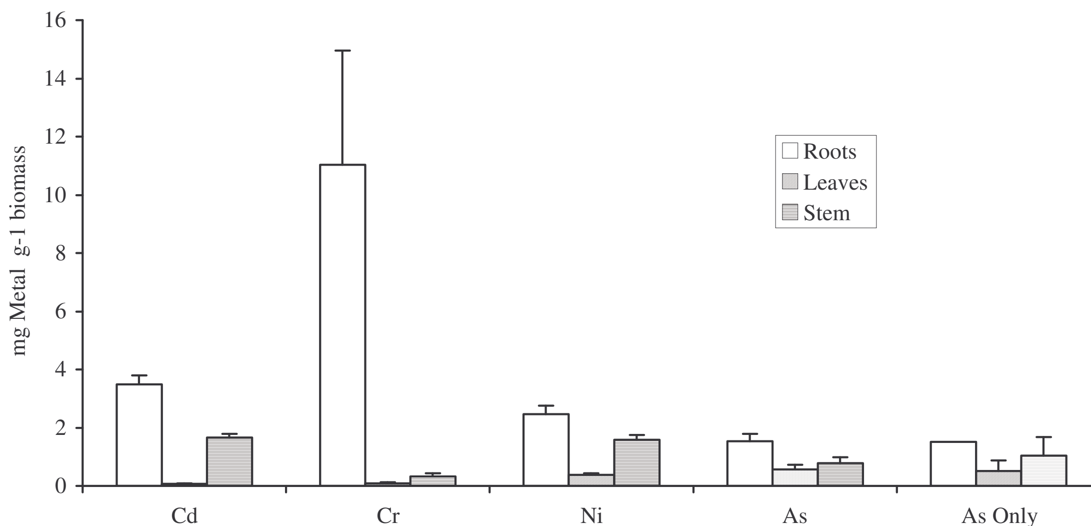


Figure 1 Sundance hydroponic As alone or mixed with Cd, Cr, Ni

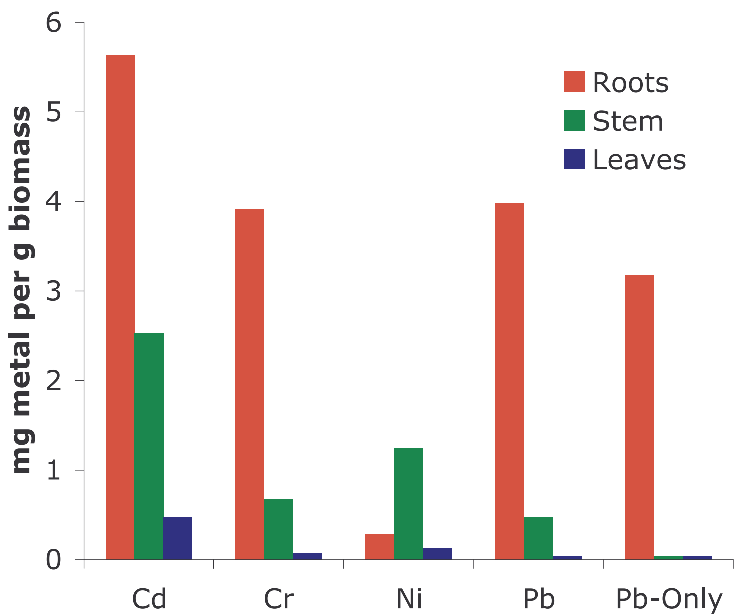


Figure 2 Sundance hydroponic 30 mg/L Pb alone or with Cd, Cr, Ni

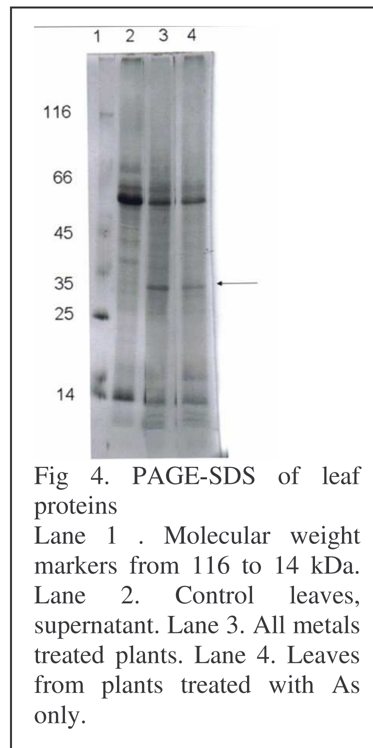


Fig 4. PAGE-SDS of leaf proteins
Lane 1 . Molecular weight markers from 116 to 14 kDa. Lane 2. Control leaves, supernatant. Lane 3. All metals treated plants. Lane 4. Leaves from plants treated with As only.

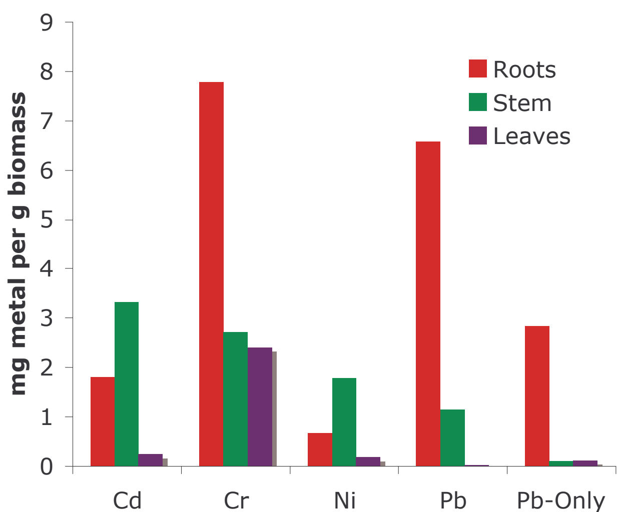


Figure 3 Hydroponic Teddy Bear Pb alone or with Cd, Cr, Ni

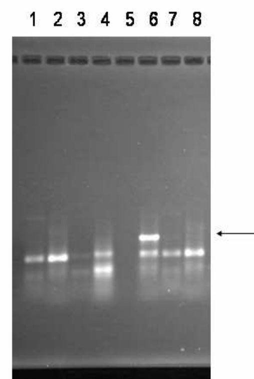


Fig. 5 RT-PCR of Sundance sunflower RNA

Table 1. Total metal uptake, biomass and translocation factors for hydroponic Sundance and Teddy Bear sunflowers in the presence of As.

Cultivar		Total	Metals (mg)				Total
		Biomass g	As	Cd	Cr	Ni	Metal
Sundance	As Cd Ni Cr	2.682	1.95	2.40	3.92	2.55	10.82
	As Only	2.129	1.59				1.59
Teddy Bear	As Cd Ni Cr	4.757	3.34	0.57	3.00	3.59	10.50
			Translocation Factors				
Sundance	As Cd Ni Cr		0.4	0.16	0.02	0.3	
	As Only		0.44				
Teddy Bear	As Cd Ni Cr		0.20	0.13	0.05	0.17	

Translocation factors =shoots (stems and leaves) divided by the metal ion concentration in roots.

Table 2. Total metal uptake, biomass and TFs for hydroponic Sundance and Teddy Bear sunflowers with Pb.

Cultivar		Total	Metals (mg)				Total
		Biomass g	Pb	Cd	Cr	Ni	Metal
Sundance	Pb Cd Ni Cr	1.246	2.37	3.91	2.43	0.42	9.13
	Pb Only	2.21	1.10				1.10
Teddy Bear	Pb Cd Ni Cr	1.198	0.16	0.11	0.41	0.06	0.733
	Pb Only	1.176	0.751				0.751
			Translocation Factors				
Sundance	Pb Cd Ni Cr		0.042	0.52	0.04	0.93	
	Pb Only		0.008				
Teddy Bear	Pb Cd Ni Cr		0.028	0.38	0.32	0.01	
	Pb Only		0.042				

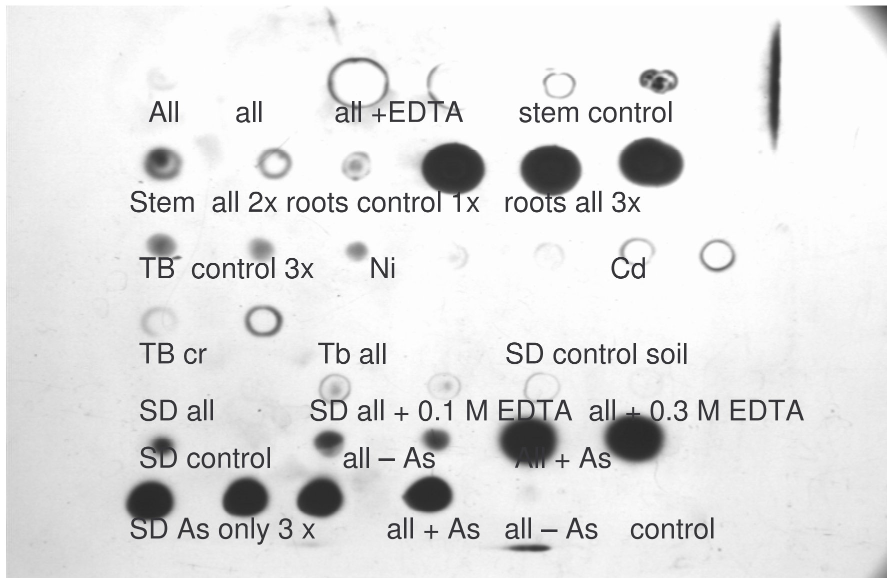
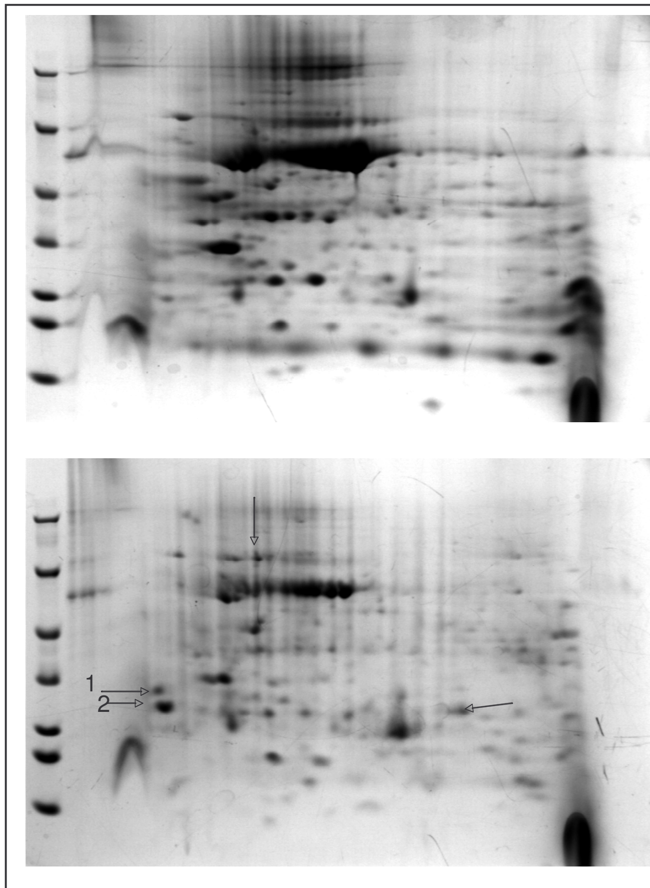


Figure 6. Dot Blot analysis of protein samples in triplicate probed with anti-chitinase serum. Sudance (SD) samples in bottom two rows are hydroponic plants, all others are soil based



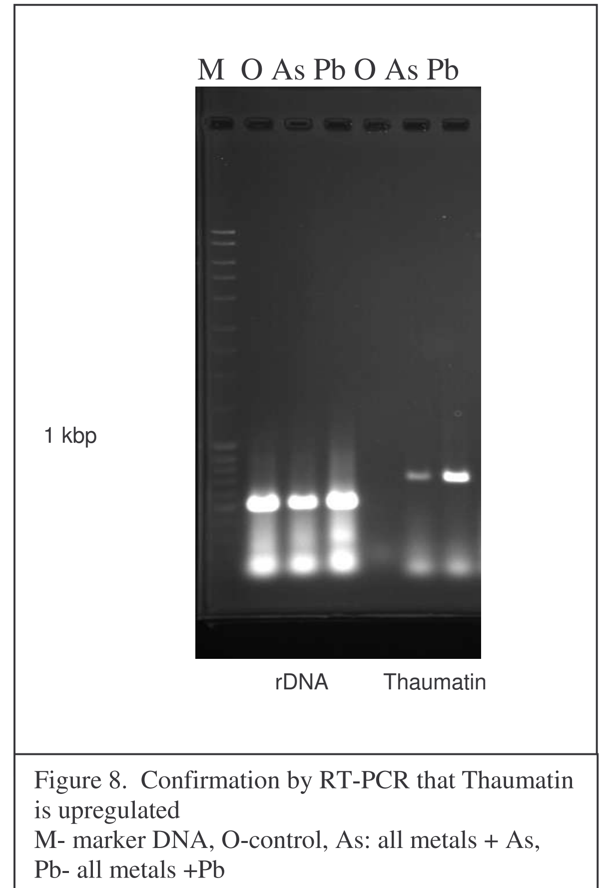


Figure 8. Confirmation by RT-PCR that Thaumatin is upregulated
M- marker DNA, O-control, As: all metals + As, Pb- all metals +Pb

Figure 7. Two-D gel leaves of plants (a) control and (b) treated with Cd, Cr, Ni and Pb. Note the extra proteins 1-chitinase and 2 - thaumatin