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Investigating heavy metal accumulation and oxidative stress tolerance of non-accumulators and hyperaccumulators in the Brassicaceae plant family

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INVESTIGATING HEAVY METAL ACCUMULATION AND OXIDATIVE STRESS TOLERANCE OF NON-ACCUMULATORS AND HYPERACCUMULATORS IN THE *BRASSICACEAE* PLANT FAMILY

A Thesis

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ABSTRACT

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Physiological and enzymatic analysis indicated that the non-accumulator *A. thaliana* showed an oxidative stress response in all assays except for protein carbonylation. Other non-accumulator plant species showed a significant difference in oxidative stress response in the TBARS assay; however, for some assays no stress response was evident. The hyperaccumulator plant species showed no significant difference in oxidative stress as indicated by the all of the assays with the exception of *T. montanum* var. *montanum* which showed an oxidative stress response in the SOD assay. Basal catalase enzyme activity was notably higher in the hyperaccumulators *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense* as compared to the non-accumulator *A. thaliana*. Results of the oxidative stress assays were also compared to the levels of nickel present in the various plant species. These experiments show that hyperaccumulators may have additional mechanisms that allow resistance to oxidative stress more effectively than non-accumulators.

DEDICATION

To my parents, the sacrifices you have made to provide your children with the education and experiences you dreamed for us to have is the drive that finished this thesis. For my sisters, who gave me the push to always finish what I have started, made me laugh, and allowed me to cry along the way. To my better half Aroldo, for making me a better person striving for things I thought was beyond my reach.

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CHAPTER I

INTRODUCTION

The presence of oxygen in living organisms leads to the production of free radicals, also known as reactive oxygen intermediates (ROI) (Dalton *et al*., 1999). These reactive oxygen intermediates are considered to be by-products of aerobic metabolism and could lead to cell damage and death if their over-accumulation occurs in plant cells (Mittler, 2002). Certain heavy metals react with oxygen and water to produce very aggressive oxygen radical species that lead to oxidation of cellular components (Mithöfer *et al*., 2004). Transition metals such as Cu, Fe, Ni, Zn, and Cd can mediate a redox reaction with oxygen and water to form harmful free radicals.

Plants possess mechanisms to respond to oxidative stress such as enzymes and small molecules (i.e. glutathione) to interact with ROI to neutralize or reduce the oxygen radicals and protect the plant cells from harmful effects (Mittler, 2002). There exist certain species of plants (hyperaccumulators) that accumulate high concentrations of heavy metals without the damaging physiological effects caused by oxidative stress when compared to other plants (non-accumulators). Millions of years of perfervid selective pressure of heavy metals loading in plants from serpentine soils, communities of plants have evolved biological mechanisms to resist or detoxify heavy metals in their tissues. This has resulted in the evolution of hyperaccumulators, or metallophytes (Antonovics *et al.,* 1971; Wild and Bradshaw, 1977; Shaw, 1990; Whiting *et al.,* 2004). The introduction of heavy metals as a consequence of the industrial revolution into the

environment has also caused the evolution of pseudometallophytes which are plants that have developed the ability to tolerate heavy metals in soil. (Whiting *et al.,* 2004). In native soil, hyperaccumulators accumulate more than 100 μg g^{-1} nickel, 10,000 μg g^{-1} zinc or manganese, 1000 μg g^{-1} cobalt or copper, and 100 μg g^{-1} cadmium (Brooks *et al.,* 1977; Reeves, 1988; Baker and Brooks, 1989; Baker *et al.,* 2000). According to Brooks *et al.* (1977), the rising interest in Ni hyperaccumulators could possibly be due to its importance in mineral exploration and to aid in solving metal tolerance problems concerning plant biochemistry and physiology. The investigation of the biological characteristics of these hyperaccumulator plant species is being explored in order to improve phytoremediation techniques.

Hyperaccumulators, also known as metallophytes, have evolved mechanisms to tolerate, resist, or even thrive in heavy metal rich environments in comparison to non-accumulators, as these plants do not have the ability to tolerate high concentrations of heavy metals (Whiting *et al.,* 2004). One possible adaptive mechanism that hyperaccumulators employ is to increase cellular tolerance to oxidative stress induced by these heavy metals via extracellular and intracellular processes (Hall, 2002). Nickel can be accumulated to high concentrations *in planta* by about 300 of the approximately 450 plant species of known hyperaccumulators, of which the majority are in the *Brassicaceae* plant family (Boularbah *et al.*, 2006).

Heavy metal treatment (Ni Acetate) of non-accumulator and hyper-accumulator plants can be performed, and the resulting oxidative stress induced in the plants can be analyzed and compared via various stress assays.

The goals of this thesis are to study the physiological response to oxidative stress in the *Brassicaceae* plant family and provide a basis to search for which genes may be involved in the oxidative stress responses of plants to heavy metals. Under controlled environmental conditions

two tubs for each species are set up with one tub as the control and the other tub treated with 100 μM Ni Acetate for of 6 days, 8 days, and 12 days. This Ni treatment can cause the production of ROI, which leads to oxidation of proteins and lipids as well as the induction of antioxidant enzymes. Biochemical assays, utilized as physiological stress indicators, and enzymatic assays to measure enzyme activity in response to stress, will be used to detect and quantify heavy metal induced oxidative stress in the plants.

Statement of the problem

Of increasing environmental concern is soil contamination with heavy metals (Taiz and Zeiger, 2002). In excess, heavy metals (e.g. Zn, Cu, Co, Ni, Hg, Pb, Cd, Ag and Cr) can be severely toxic to ecosystems. Anthropogenic activities (i.e. battery manufacturing, mining, pesticides) produce wastes that result in the contamination of soil with high amounts of heavy metals and these affect plants and organisms in the environment (Boularbah *et al.*, 2006). Studies elucidating an organism's response to heavy metal stressors can be helpful to reduce the potential harmful effects of heavy metals in the environment.

A biological means of removing heavy metal contaminants from soil or water using plants is called phytoremediation. The plants are used as "solar-driven pumps" extracting heavy metals from the environment and stored in the vacuole (Yang *et al.*, 2005). Hence, the storage of heavy metals in the vacuoles ultimately depletes from the high heavy metal concentrations in the soil and decreases the effects of heavy metal toxicity to the environment. Although hyperaccumulator plants species have been studied for their heavy metal tolerance and importance for use in phytoremediation, these plants are still threatened by anthropogenic effects such as environmental toxins produced by industrial development (Whiting *et al.*, 2004).

Therefore, these hyperaccumulators species are considered rare endangered organisms and plant conservation actions must be implemented. However, despite the threat of the destruction of the environment in which these hyperaccumulator plants are native to, the importance of their use in phytoremediation has made them a valuable tool for further studies on the ecological impacts, genetic characterization, and growth habits to aid in the search for the most efficient phytoremediation plants and techniques.

Because hyperaccumulating plants can take up very high heavy metal concentrations, certain plant characteristics are still necessary for phytoremediation to be more efficient. Hyperaccumulators are not fast-growing and have a small biomass which decreases the potential for effectiveness of metal extraction and limits the proper use of agronomic practices (i.e. mechanical harvest) (Yang *et al.,* 2005).

Plants that would be ideal for phytoremediation would have the following criteria that would result in a successful employment of the plants in remediation techniques. These plants must: (a) be fast growing, (b) have high biomass to uptake more heavy metals without showing effects of toxicity in a short period of time, (c) have an extensive root system in order to maximize volume of soil remediated, (d) be easy to harvest so that techniques applied for fast harvest (i.e. mechanical) will also aid in a quick turnover, (e) tolerate and accumulate a range of desired heavy metals (Yang *et al.,* 2005). In addition, plants must also be adapted to various soil types and climates which may be present at phytoremediation sites.

Conceptual Framework

Non-accumulator (*Brassica juncea, Arabidopsis thaliana, Streptanthus farnsworthianus*) and hyper-accumulator (*Thlaspi montanum var. siskiyouense, Thlaspi montanum var. montanum,*

Streptanthus polygaloides) seeds were surface sterilized and germinated in magenta boxes. The seeds of each species were then grown in hydroponics tubs until reaching maturity. Control and 100 μM Ni Acetate-treated plants were assayed for oxidative stress enzyme induction (Superoxide Dismutase [SOD], Catalase [CAT], Glutathione Reductase- [GR]). Also, the cellular effects of oxidative stress were assayed via lipid peroxidation (Thiobarbituric Acid Reactive Substances assay [TBARS]) and Protein carbonylation [Pcarb]). Nickel was chosen due to the fact that it is the most common metal hyperaccumulated amongst the *Brassicaceae* plant species (Reeves and Baker, 2000).

The goal of this thesis is to find how non-accumulator and hyper-accumulator plants differ in their response to oxidative stress when subjected to heavy metal treatment. These assays will help determine oxidative stress levels in each species by comparing control and 100 μM Ni-treated leaves in plants grown in hydroponics.

Research Questions and Hypothesis

The objective of this study was to compare the physiological and oxidative stress response, upon exposure to 100 μM nickel acetate, between heavy metal tolerant hyperaccumulator plant species and heavy metal sensitive non-accumulator plant species of the *Brassicaceae* family. This study evaluated the stress response of 3 non-accumulator and 3 hyperaccumulator *Brassicaceae* plant species using biochemical and enzymatic assays as biomarkers of Ni-induced oxidative stress.

Thiobarbituric Acid Reactive Substances (TBARS) and Protein Carbonylation assays were used to determine lipid peroxidation and protein carbonylation resulting from the oxidation of lipids and protein by oxygen radicals. The enzymatic activity of anti-oxidative enzymes

(SOD, CAT and GR) involved in detoxification of oxygen radicals induced by Ni treatment also was a parameter used to study the reaction of the plants to Ni-induced oxidative stress.

The Student's t-test was used to compare stress response parameters between the control and 100 μM nickel-treated plants of each species. Metal analysis was also performed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) to measure metal concentrations in the plant leaves.

The hypothesis is that in comparison to non-accumulators, hyperaccumulators contain novel or enhanced mechanisms for heavy metal accumulation and tolerance in general. Specifically, are hyperaccumulator plants better at tolerating heavy metal-induced oxidative stress as compared to non-accumulator plants? And if so, what factors are involved in this heavy metal tolerance?

Significance of the Problem

Using these oxidative stress assays to compare Ni-induced oxidative stress in each plant species to their corresponding controls, the results can be used to give some insight of the intracellular mechanisms of heavy metal tolerance in plants.

The non-accumulator plant, *A. thaliana* has been a popular research subject in the plant sciences due to its short lifecycle, ease of growth in the laboratory, its sequenced genome and the availability of a wide variety of biochemical and molecular techniques that can be used to study the physiology of this plant (Peer *et al.,* 2003). Hyperaccumulator plants in the same family as *A. thaliana,* are a good comparison for the Ni-induced oxidative stress response. These plants have also been analyzed for similar gene sequence encoding for the anti-oxidative stress enzymes used in this study. Therefore, comparison of the results of the assays can provide useful information as

to which of the anti-oxidant enzyme genes can be used as potential candidates for gene transfer. The results to the studies will then help to produce transgenic plants more tolerant to heavy metals that can be used in phytoremediation.

CHAPTER II

MATERIALS AND METHODS

Seed Preparation for Growth, Development, and Treatment

Plants used in Study

Non-accumulator plant species *B. juncea* seeds were obtained from the United States Department of Agriculture (USDA) seed bank. *A. thaliana* (var. *columbia*) seeds were obtained from LEHLE seeds (Round Rock, TX). *S. farnsworthianus* seeds were collected in the Sierra Nevada Mountains of California by Ron Ratko of Northwest Native Seed (Seattle, WA).

Hyperaccumulator plant species: *T. montanum* var. *siskiyouense* seeds were collected the Siskiyou Mountains in Josephine County, Oregon and were provided by Ron Ratko of Northwest Native Seed (Seattle, WA). *T. montanum* var. *montanum* seeds were collected in the Wasatch Mountains of Utah by Ron Ratko of Northwest Native Seed (Seattle, WA). *S. polygaloides* seeds collected in the Sierra Nevada Mountains of California were provided by Robert Boyd from Auburn University.

Seed Sterilization

Seeds were surface sterilized with 1 mL 70% Ethanol/0.02% Triton X-100 for 3 min. for non-accumulators (6 minutes for hyperaccumulator seeds). Following ethanol wash, the non-

accumulator seeds were washed with 1 mL 50% bleach /0.02% Triton X-100 for 5 min. (10 min. for hyperaccumulator seeds). Both types of seeds were then washed 10 times with 1 mL sterile distilled water per wash. For removal of excess water, seeds were placed on #3 filter paper disks, sterilized by washing with 100% ethanol or sterilized via autoclaving. Seeds were then placed onto ½ X MS (Murashige and Skoog) media in magenta boxes using sterile tweezers and sterile toothpicks. For seed vernalization, magenta boxes containing *S. polygaloides* and *T. montanum* var. *siskiyouense* seeds were wrapped in foil and placed at 4°C for seven days, while *A. thaliana* seeds were refrigerated for two days at 4°C. Other plant species were not cold treated and seed germination was performed by placing the magenta boxes in a dark drawer for five days. Afterwards, the magenta boxes were unwrapped and placed under 9 hr. light cycle (125 μEinsteins light intensity) in a plant growth room at 23^{o}C and 60% RH for seed germination to occur.

Hydroponics

After sprouting 2 true leaves, the magenta boxes were opened for 2 days to allow the plants in the magenta box to acclimate to the humidity conditions present in the plant room. For further plant growth and development, the plants were transferred to hydroponic tubs containing 8 liters of 0.1 X Hoaglands nutrient solution. This solution is composed of 200 mL of 4 X Hoaglands solution, 40 ml of 40 mM FeDTPA, and 7760 mL distilled water. The nutrient solution was modified from the recipe from Hoagland and Arnon (1950) and use of FeDTPA as suggested by Misra and Sharma (2006). Each species was grown hydroponically in two tubs painted black with the rims covered with foil to prevent the entrance of light into the solution, which then minimizes the growth of algae. A rectangular Styrofoam pad with 16 holes was fitted

for each tub into which plants were inserted. A plastic microcentrifuge tube, cut at the 0.1 ml mark, containing a polyester plug to hold the seedling in place were placed in the foam pad holes for the plant root to penetrate through and be exposed to the hydroponics solution so that the plant leaves were not in contact with the hydroponics solution. Air was bubbled into the hydroponics solution and prevented anoxia using aerators and airstones.

Metal Treatment

After 2 to 3 months of growth in hydroponics solution, 2 mL of a 400 mM Nickel Acetate solution (100 μM Ni final concentration in hydroponics solution) was added to one tub of each plant species while the other tub had no Ni added and was kept as the control. The treatment period was 12 days. Each experimental treatment was composed of 3 replicate tubs for each species. The plants were flash frozen in liquid nitrogen in a sterile mortar, ground into a fine powder with a sterile pestle, and placed in 14 mL plastic tubes for storage at -80°C until analyzed.

Biochemical and Enzymatic Assays

Thiobarbituric acid reactive substances

As described by De Vos *et al*. (1989), the assay measures the lipid peroxides that are produced when lipids react with oxygen radicals. Two ml screw-cap microfuge tubes were hexane washed and dried before using the tubes for the assay. Fifty mg plant tissue was ground with a hexane-washed pestle in 250 μL of TBARS grinding buffer. Grinding buffer (20 mL) which contained 50 mM Tris-MES (pH 7.1), 2% (w/v) SDS, and 0.01% (v/v) β-mercaptoethanol

was used. To the ground sample, 2 µL 1% butylated hydroxytoluene (BHT) and 700 µL of 0.8% thiobarbituric acid (TBA)/10% trichloroacetic acid (TCA) were added. The samples were re-suspended by vortexing, followed by 15 min. incubation at 95°C in a heat block with loosened caps. At the end of the incubation period, the caps were tightened, samples were vortexed, and caps loosened for another 15 min. of incubation at 95°C in a heat block. After second incubation, 800 µL of n-butanol was added to each sample, the tube recapped, vortexed, and centrifuged for 10 min. at 14,000 x g. Three layers are present after centrifugation: a top aqueous layer of a brown color, a middle layer of plant debris, and a bottom layer organic phase with the TBARS present in the n-butanol layer. The organic layer samples (700 µL) were then transferred to 1.5 mL plastic cuvettes (Fisher Scientific, Pittsburg, PA)and absorbance read at 600 nm (nonspecific background absorbance) and 532 nm with a spectrophotometer (Bio-Rad Laboratories, SmartSpec3000, Hercules, CA), using 700 µL of n-butanol as blank. To quantify lipid peroxides in units of pmol/mg tissue for each plant sample, the following equation was used: $[((A_{532} - A_{600}) / 156000) * 0.0008] * 10^{12}$.

Protein Carbonylation

The Protein carbonylation (PCarb) assay indicates oxidative damage in plants caused by free radicals and is quantified in units of µg carbonyls/µg protein as described by Adams *et al.* (2001) with modifications. Fifty mg of frozen plant tissue samples were ground in 600 µL of Homogenization buffer which contained 0.29 M Sucrose, 25 mM HEPES, 20 mM EDTA, 0.5% (w/v) PVP, pH 8.5. Just before use of the buffer for sample preparation, 300 µL of 3 mM Dithiothreitol and 20 µL 0.1 M phenyl methyl sulfonyl fluoride (PMSF) were added to the buffer. The samples were centrifuged for 3 min. at 14,000 xg. Twenty µL of supernatant was

saved and used for protein analysis. The remainder of the supernatant was transferred to a new tube and 300 μL of 10% Trichloroacetic acid (TCA) was added to each sample followed by inverting the tube. Samples were incubated on ice (4ºC) for 10 min. prior to centrifugation at 4ºC at 14, 000 xg for 3 min. The supernatant was discarded and the pellet was re-suspended in 500 μL of 10 mM 2,4-dinotrophenylhydrazine (DNPH)/2M Hydrochloric acid (HCl). The pellets were re-suspended thoroughly and incubated at room temperature (22^{o}C) for one hour. The samples were then precipitated with 250 μL of 24% TCA by inverting tube to mix and incubated for 10 min. at room temperature. The samples were centrifuged at 14,000 xg for 3 min. after which the supernate was discarded. The pellet was washed with 500 μL of ethanol-ethyl acetate (1:1 v/v) for the removal excess of DNPH from the pellet. The samples were left to stand for 10 min. at room temperature followed by a 5 min. centrifugation at 14,000 xg. The supernatant is discarded and the steps from the ethanol-ethyl acetate wash were repeated three more times to fully remove excess DNPH from pellet. One mL of 6M Guanidine HCl/2mM Potassium Phosphate was added to each sample and the tube was inverted to mix the solution with the pellet. The samples were placed in a 60ºC water bath and incubated for at least 10 min. or until the pellets were dissolved. To remove any remaining solid particles in suspension, the samples were centrifuged for 5 min. at 14,000 xg. The supernatant was then transferred to a 1.4 mL quartz cuvette and absorbance was read at 360 nm with a spectrophotometer (Bio-Rad Laboratories, SmartSpec3000, Hercules, CA). To quantify protein carbonyls as pmol carbonyls/μg protein, the following equation was used: $((A_{360} / 22000) * 0.0006) * 10^{12}$.

Protein Analysis

For each assay (excluding TBARS) a total protein assay was performed after biochemical analysis. A protein assay kit from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) based on the Bradford protein assay (Bradford, 1976) was used to determine the protein concentration. Plastic 1.5 ml cuvettes were filled with 800 µL sterile distilled water, 200 µL BioRad protein assay reagent, and 20 µL of the sample. A 20 µL blank sample used for each assay was composed of the sample isolation buffer (with no PVP) used in the particular enzymatic assay. The samples were allowed to stand at room temperature for 15 min. prior to measuring the samples at an absorbance of 595 nm using a (BioRad SmartSpec3000) spectrophotometer (BioRad, Hercules, CA). The µg/mL of protein in the samples was determined using the following equation: (A_{595} + 0.0106) / 0.5472) /1000.

Superoxide Dismutase

To determine SOD enzyme activity, the protocol was followed described by Dojindo SOD Assay Kit-WST manual (Dojindo city state). Modifications included the 1:4 dilutions of samples with homogenization buffer without PVP due to the high amount of SOD activity in experimental plants. Homogenization buffer was composed of Phosphate buffer saline (PBS buffer) containing 0.024% Potassium phosphate (KH_2PO_4), 0.8% Sodium Chloride (NaCl), 0.02% Potassium Chloride (KCl), 0.14% Sodium Hydrogen Phosphate (Na_2HPO_4) 1 mg/mL PVP, pH 7.4. Fifty mg of frozen plant tissue samples were ground with 250 µL PBS buffer with PVP using a microfuge tube and motorized pestle. After grinding, 750 µL PBS (no PVP) was added to each plant sample to dilute the sample 1:4. The samples were spun for 3 min at 14,000 xg to remove plant debris. The supernatants were removed and placed in a new microfuge tube

and these samples were used to measure SOD enzyme activity. Enzyme activity in the samples was determined in mU SOD/µg protein by using the following method. As per kit instructions: For protein analysis, 20 µl of supernatant was set aside, and 20 µl of sample, 200 µl of WST working solution, and 20 µl of enzyme working solution were combined together in a single well of a 96 well microplate per sample. The microplate was incubated at 37^{o}C for 20 min. The microplate was then read in a microplate reader (Bio-Rad Laboratories, model 680, Hercules, CA) at 450 nm. From the A450 values the % Inhibition of enzyme activity was calculated. $\{[(A_{Blank1} - A_{Blank3}) - (A_{Sample} - A_{Blank2}) / (A_{Blank1} - A_{Blank3})\}$ x 100. This % SOD activity inhibition was then compared to a standard curve (generated as per kit instructions) and the equation % Inhibition = 14.65(Ln (SOD Units)) + 59.15 was used to calculate SOD activity in the sample.

Catalase

The Amplex Red Catalase (CAT) assay (Molecular Probes Inc., Eugene, OR) measured the activity the enzyme catalase in µU CAT/µg protein. The protocol followed was from Molecular Probes Amplex® Red Catalase Assay Kit (A22180) with no modifications. The assay used PBS buffer 0.02% Potassium phosphate (KH_2PO_4), 0.8% Sodium Chloride (NaCl), 0.002% Potassium Chloride (KCl), 0.14% Sodium Hydrogen Phosphate (Na_2HPO_4) 1 mg/mL PVP, pH 7.4. Fifty mg of frozen plant tissue samples were ground with 250 µL PBS buffer + PVP with a plastic pestle. To separate plant debris from supernatant, the samples were centrifuged for 5 min at 14000 x g in a 4ºC centrifuge. For protein analysis 20 µL of sample was set aside and, 25 µL of sample was added onto a single well of a 96 well microplate then placed on ice with a plastic saran wrap under microplate. Enzyme activity in the samples was determined in µU CAT/µg protein by using the following method. As per kit instructions 25 µl of sample and 25 µl of 40

μM H_2O_2 solution was added to a single well of a 96 well microplate per sample and incubated at room temperature for 30 min. Then, 50 μL of 100 μM Amplex Red/0.4 U/mL HRP was added to the well and then the microplate was wrapped in foil to prevent exposure to light, and incubated for 30 min. at 37ºC prior to reading with a microplate reader (Bio-Rad Laboratories, model 680, Hercules, CA) at 570 nm. Catalase enzyme activity was calculated according to the standard curve (generated as per kit instructions): A570 = -0.0691 (Ln (CAT Units)) + 0.5511. The mU of CAT obtained from the equation was multiplied by 1000 to obtain μU of CAT per sample.

Glutathione Reductase

The Glutathione Reductase (GR) assay (Sigma, Saint Louis, MO) measured the GR enzyme activity in μU GR/μg protein. Using the Glutathione Reductase Assay Kit from Sigma (Saint Louis, MO), the colorimetric protocol used was modified to function in a microplate reader at 415 nm instead of 412 nm absorbance. The GR assay grinding buffer contains 100mM KPO_4/1mM EDTA at a pH of 7.5 with PVP (1 mg / mL). Fifty mg of plant tissue sample were ground with GR assay buffer + PVP then centrifuged for 5 min. at 14000 xg at 4ºC. For protein analysis, 20 μL of supernatant was set aside and 20 μL of supernatant was placed in a single well of a 96 well microplate per sample. Added to each sample were 100 μL of 2mM oxidized glutathione, 50 μL of 3mM DNTB, and 20 μL of GR assay buffer. The samples with reagents were transferred to wells containing 10 μL NADPH, to start the reaction. The microplate required reading as soon as the reactions started at 415 nm using a microplate reader (Bio-Rad Laboratories, model 680, Hercules, CA). The kinetic reading of each sample was plotted on a graph and a linear equation obtained to determine the value of ΔOD/sec. The value was then incorporated into the following equation to obtain the GR activity in mU/mL: (ΔOD/sec) =

0.00003 (GR Units) + 0.0005. The mU of GR obtained per sample was multiplied by 1000 to gain the final GR enzyme activity in μU GR.

Nickel Analysis

The frozen, ground plant samples were analyzed for Ni content using ICP-AES. Triplicate freeze-dried tissue samples were weighed prior to digestion with 5 mL concentrated nitric acid, which was then reduced to a small volume (< 1 mL) by heating with a hot plate. The plant samples were then cooled and diluted with 25 mL distilled water. Fifteen mL of the digested plant samples were transferred into a 15 mL conical tube prior to running the samples in the ICP machine. ICP-AES Machine operation was performed by the UTPA analytical lab. The mg/L Ni in each sample was multiplied by 0.025 L and 1000, and then divided by the weight of the sample in grams to obtain the Ni concentration in ppm.

CHAPTER III

RESULTS

Biochemical Assays

Lipid peroxidation

The Thiobarbituric Acid Reactive Substances (TBARS) assay quantified the production of lipid peroxides due to oxygen radicals induced by nickel toxicity.

After 12 days of Ni exposure, there was a significant difference ($P \leq 0.05$) between Ni-treated plants versus the control plants in the non-accumulator species *A. thaliana, B. juncea, S. farnsworthianus:* ($p = <0.001$, $p = 0.01$, $p = <0.001$; respectively) (Fig. 1). No significant difference between control and Ni-treated plants was observed in the hyperaccumulator plant species *T. montanum* var. *montanum, T. montanum* var. *siskiyouense,* and *S. polygaloides* ($p = 0.40$, $p = 0.47$, $p = 0.22$; respectively).

Protein Carbonylation

The Protein Carbonylation (PCarb) assay quantified the levels of protein carbonyls produced under Ni induced oxidative stress.

After 12 days of nickel exposure no significant difference ($P \leq 0.05$) was evident between the control and Ni-treated plants for all plant species: *A. thaliana* ($p = 0.07$), *B. juncea*

(p = 0.15), *S. farnsworthianus* (p = 0.31), *T. montanum* var. *montanum* (p = 0.82), *T. montanum* var. *siskiyouense* (p = 0.62), *S. polygaloides* (p = 0.47) (Fig. 2).

Enzymatic Assays

Superoxide Dismutase

As a response to oxidative stress induced by the treatment with the heavy metal Ni, there should be a predicted increase of superoxide dismutase (SOD) enzyme activity in all non-accumulator plant species for detoxification of oxygen radicals from heavy metal exposure.

There is a significant increase ($P \leq 0.05$) of SOD activity in non-accumulator plant species *A. thaliana* and *B. juncea* (p = <0.001 and p = <0.001, respectively) between the control and Ni treatments (Fig. 3). The other non-accumulator plant species *S. farnsworthianus* showed no significant difference with regard to SOD enzyme activity between control and Ni-treated plants (p = 0.34). The hyperaccumulator *T. montanum* var. *montanum* also showed a significant increase in SOD enzyme activity in the Ni-treated plants, p = 0.04 as compared to control plants. The other hyperaccumulator plant species *T. montanum* var. *siskiyouense* (p = 0.74) and *S. polygaloides* (p = 0.08) showed no significant difference in SOD enzyme activity between control and Ni-treated plants.

Catalase

It is also predicted that there should also be an increase of catalase (CAT) enzyme activity in the non-accumulator plant species, treated with Ni, in response to oxidative stress via detoxification of hydrogen peroxide production by SOD due to Ni-induced oxygen radical formation.

The non-accumulator *A. thaliana* showed a significantly higher level ($P \leq 0.05$) of CAT enzyme activity in the control plants versus the Ni-treated plants (p = <0.001) (Fig. 4). The other non-accumulators *B. juncea* and *S. farnsworthianus* did not show a significant difference between control and Ni-treated plants with regard to CAT activity (p = 0.34 and p = 0.12, respectively). However, it should be noted that *B. juncea* showed a higher level of CAT enzyme activity in the control plants as compared to the Ni treated plants, similar to the trend seen in *A. thaliana*. The hyperaccumulators *T. montanum* var. *montanum, T. montanum* var. *siskiyouense,* and *S. polygaloides* all showed no significant difference in CAT activity between control and Ni-treated plants (p = 0.16, p = 0.56, p = 0.63 respectively).

Glutathione Reductase

The glutathione reductase (GR) enzyme should also show a predicted increase in all non-accumulator plant species, treated with Ni, due to the increased need to reduce the oxidized glutathione molecule after reaction with oxygen radicals.

Non-accumulators *A. thaliana* and *B. juncea* showed a significant increase ($P \leq 0.05$) of GR enzyme activity in Ni-treated plants as compared to the control plants (p = <0.001 and p = 0.01, respectively) (Fig. 5). The non-accumulator *S. farnsworthianus* along with all three hyperaccumulator species *T. montanum* var. *montanum, T. montanum* var. *siskiyouense, S. polygaloides* showed no significant difference between control and Ni-treated plants (p = 0.08, p = 0.46, p = 0.23, p = 0.55 respectively).

Nickel Analysis

The control plants of the non-accumulator plant species varied in Ni concentration from undetectable levels to 64.65 ppm (Table 1). The control plants of the hyperaccumulator plant species varied in Ni concentration from undetectable levels to 121.23 ppm. The treated plants of the non-accumulator plant species varied in Ni concentration from 658.84 ppm to 871.36 ppm. The treated plants of the hyperaccumulator plant species varied in Ni concentration from 1474.46 ppm to 4521.55 ppm. There was a higher concentration of Ni in the Ni-treated plants of all species. However, the hyperaccumulator plant species possessed a markedly higher level of Ni (3.56-fold difference on average compared to non-accumulators) in the plant tissues in comparison to the non-accumulator plant species.

Table 1. The average Ni content in *Brassicaceae* plant species before and after 100 μM Ni-treatment, using ICP-MS. Units represented in parts per million (ppm). *ND = Not Detected. Detection limit of ICP-AES for Ni is 0.05 ppm.

Species	Control	100 μM Nickel Treated
A. thaliana	*ND	658.84 ± 32.16
B. juncea	64.65 ± 27.07	871.36 ± 110.37
S. farnsworthianus	*ND	694.49 ± 226.22
T. montanum var. *montanum*	121.23 ± 4.96	1928.29 ± 654.50
T. montanum var. *siskiyouense*	*ND	4521.55 ± 340.46
S. polygaloides	*ND	1474.46 ± 319.86

Table 2. Biomass of plant species relative to *A. thaliana* biomass and relative to Ni-treatment compared to control biomass. *A. thaliana* biomass set to equal 1 for comparison purposes.

Species	Control	100 μM Nickel Treated (Relative to control)
A. thaliana	1	0.28
B. juncea	2.57	0.49
S. farnsworthianus	1.5	0.20
T. montanum var. *montanum*	0.63	0.84
T. montanum var. *siskiyouense*	0.36	1.91
S. polygaloides	0.96	0.56

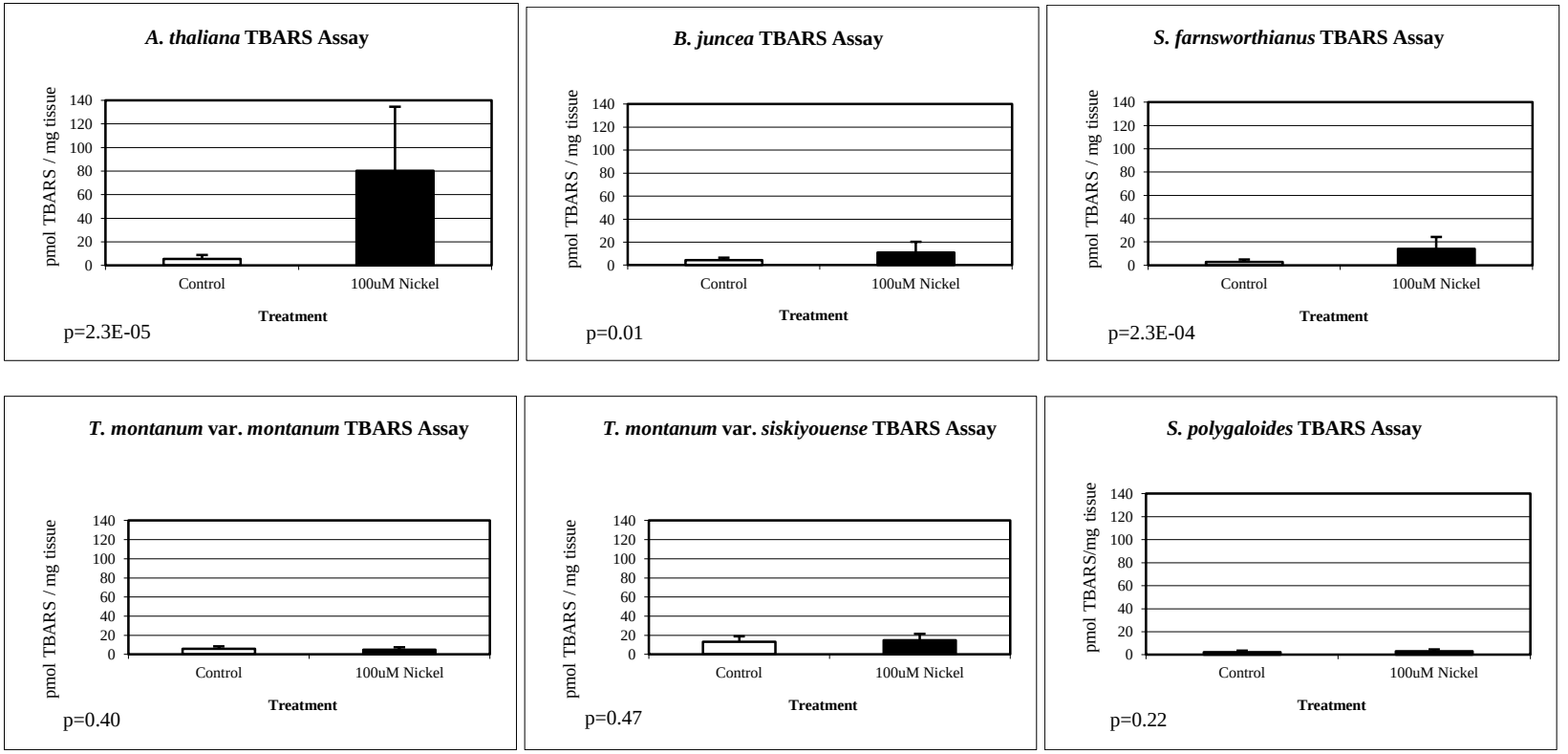

Fig. 1. TBARS values (pmol/mg tissue) of each *Brassicaceae* species. Top 3 graphs represent the non-accumulator plant species and bottom 3 graphs represent the hyperaccumulator plant species. n = 15.

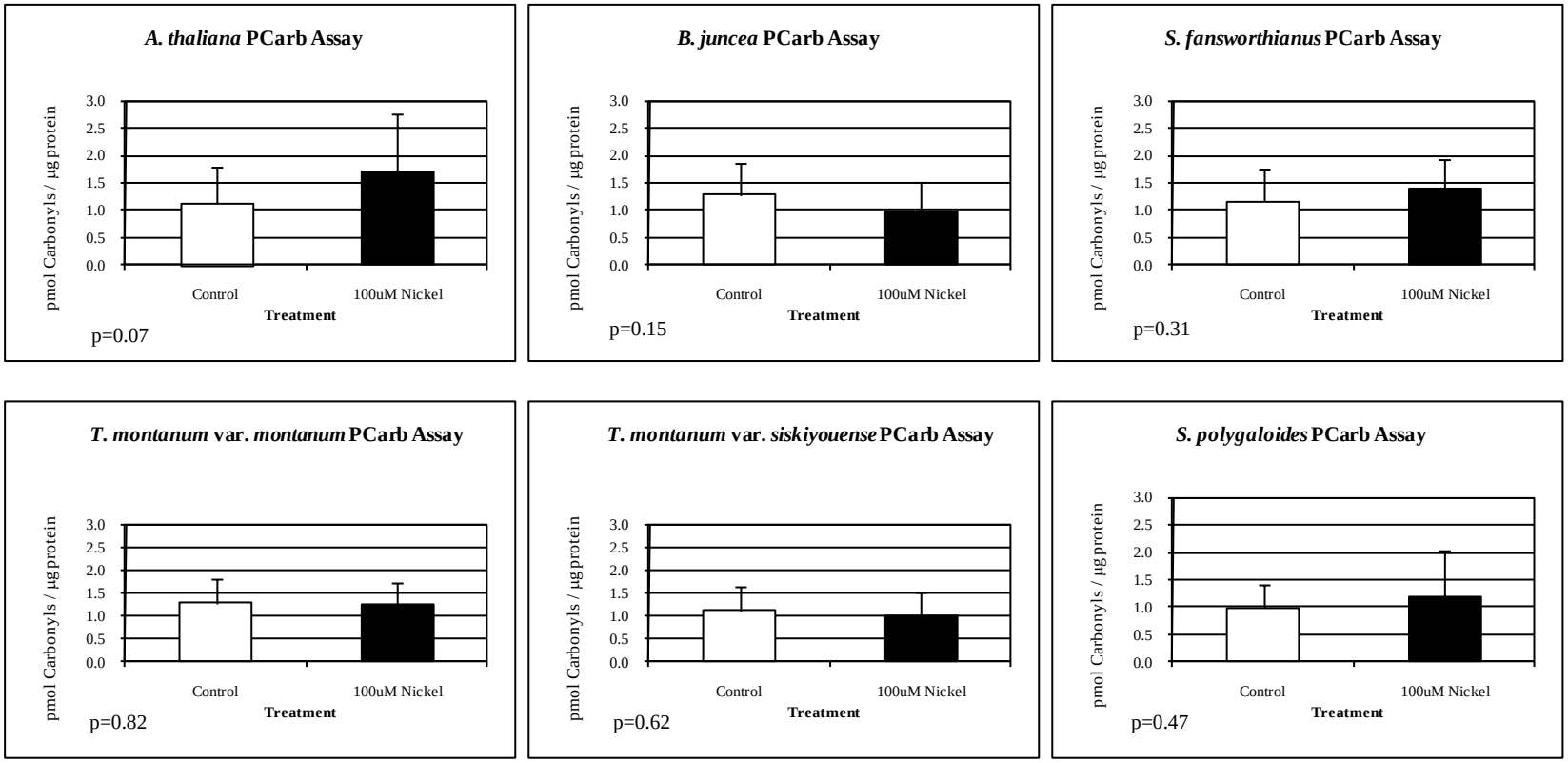

Fig. 2. PCarb values (pmol/mg tissue) of each *Brassicaceae* species. The graph scales are similar for the comparison of the levels of protein carbonyls in pmol Carbonyls/µg protein between species. Top 3 graphs represent the non-accumulator plant species and bottom 3 graphs represent the hyperaccumulator plant species. n = 15.

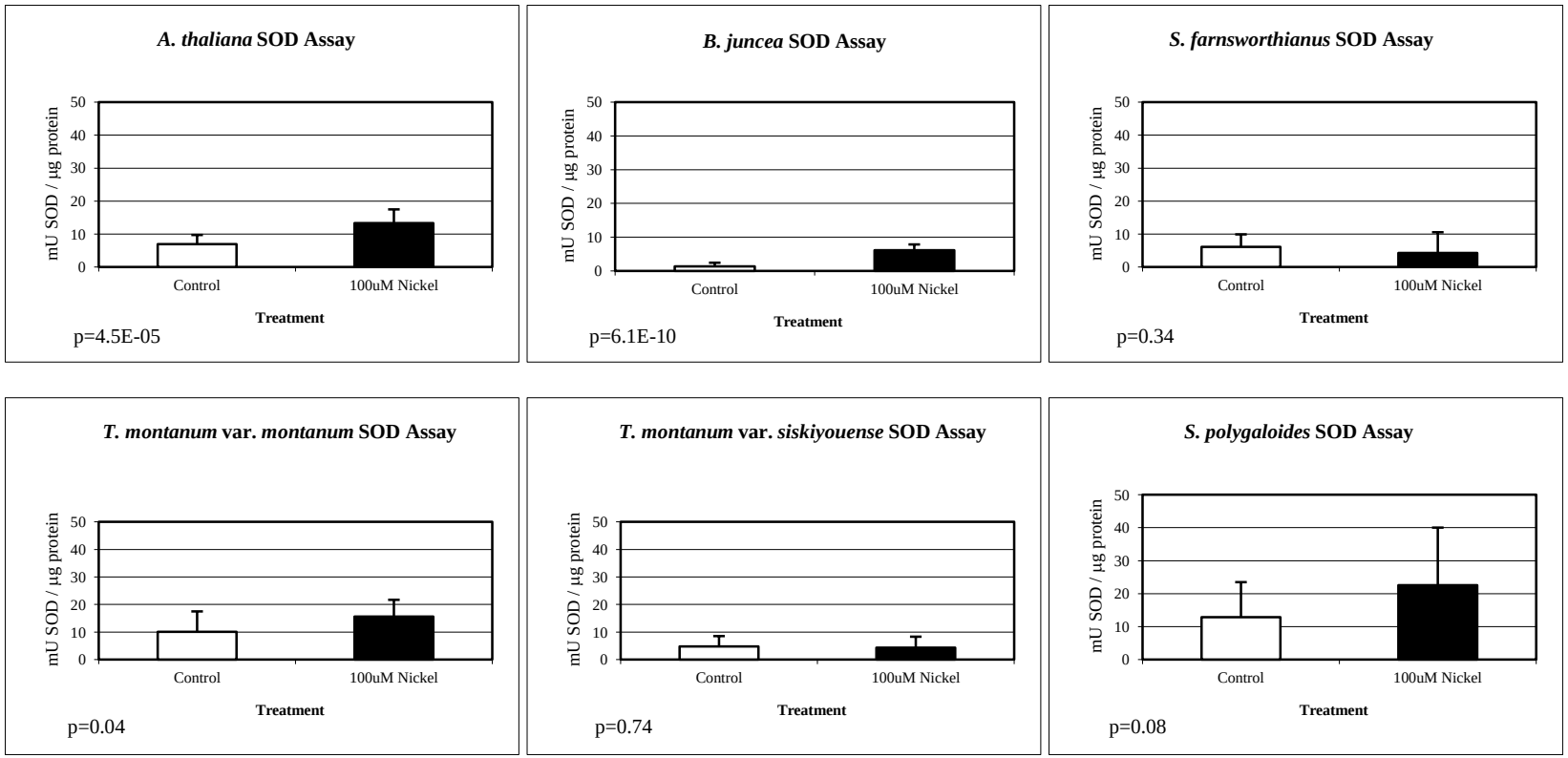

Fig. 3. SOD activity of each *Brassicaceae* species. The graph scales are similar for the comparison of the enzyme activity in mU SOD/µg protein between species. Top 3 graphs represent the non-accumulator plant species and bottom 3 graphs represent the hyperaccumulator plant species. n = 15.

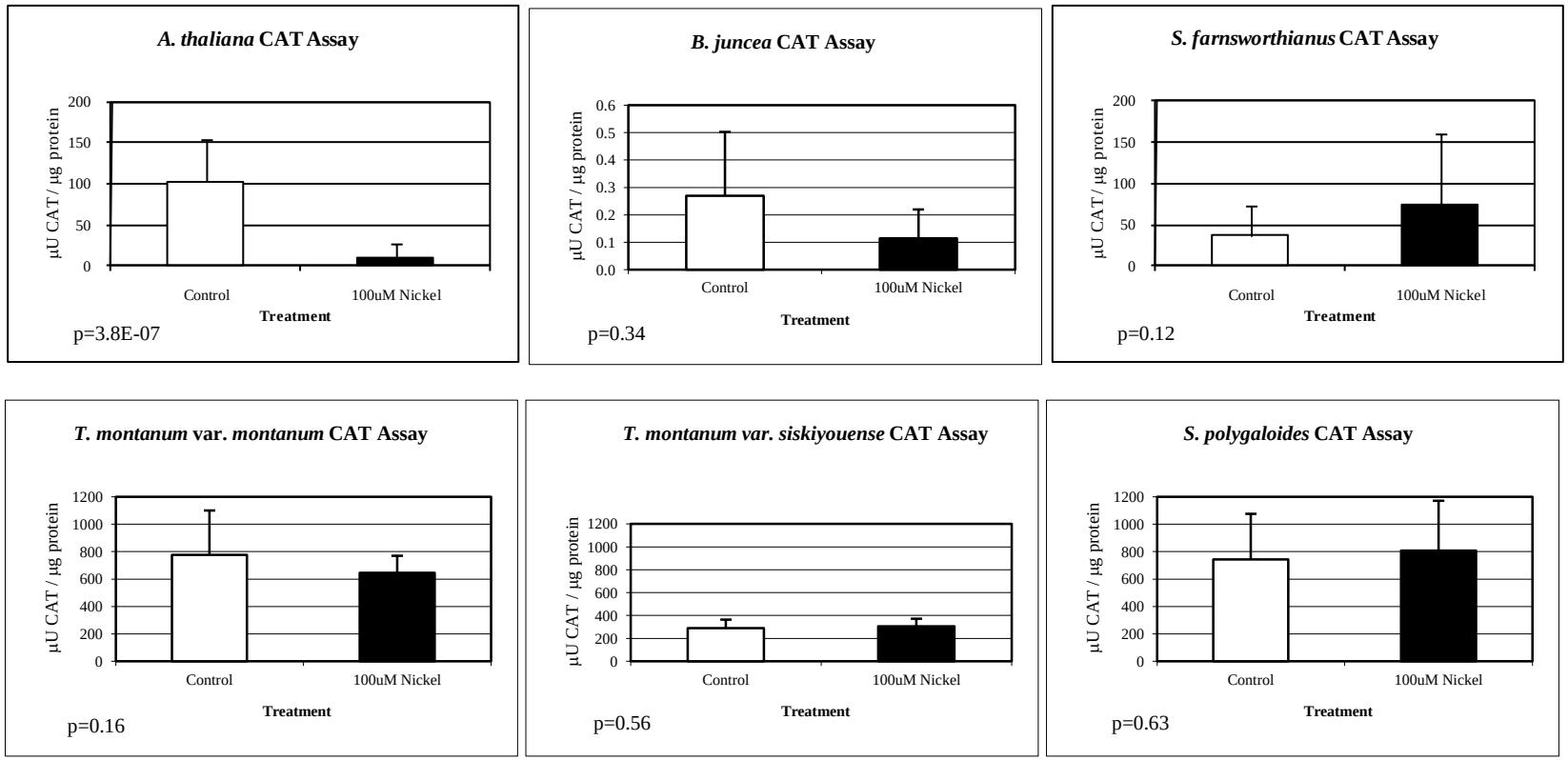

Fig. 4. CAT activity of each *Brassicaceae* species. The graph scales are not similar to show differences of the enzyme activity in µU CAT/µg protein between species. Top 3 graphs represent the non-accumulator plant species and bottom 3 graphs represent the hyperaccumulator plant species. n = 15.

Note: *B. juncea* n = 13 Ni-treated samples.

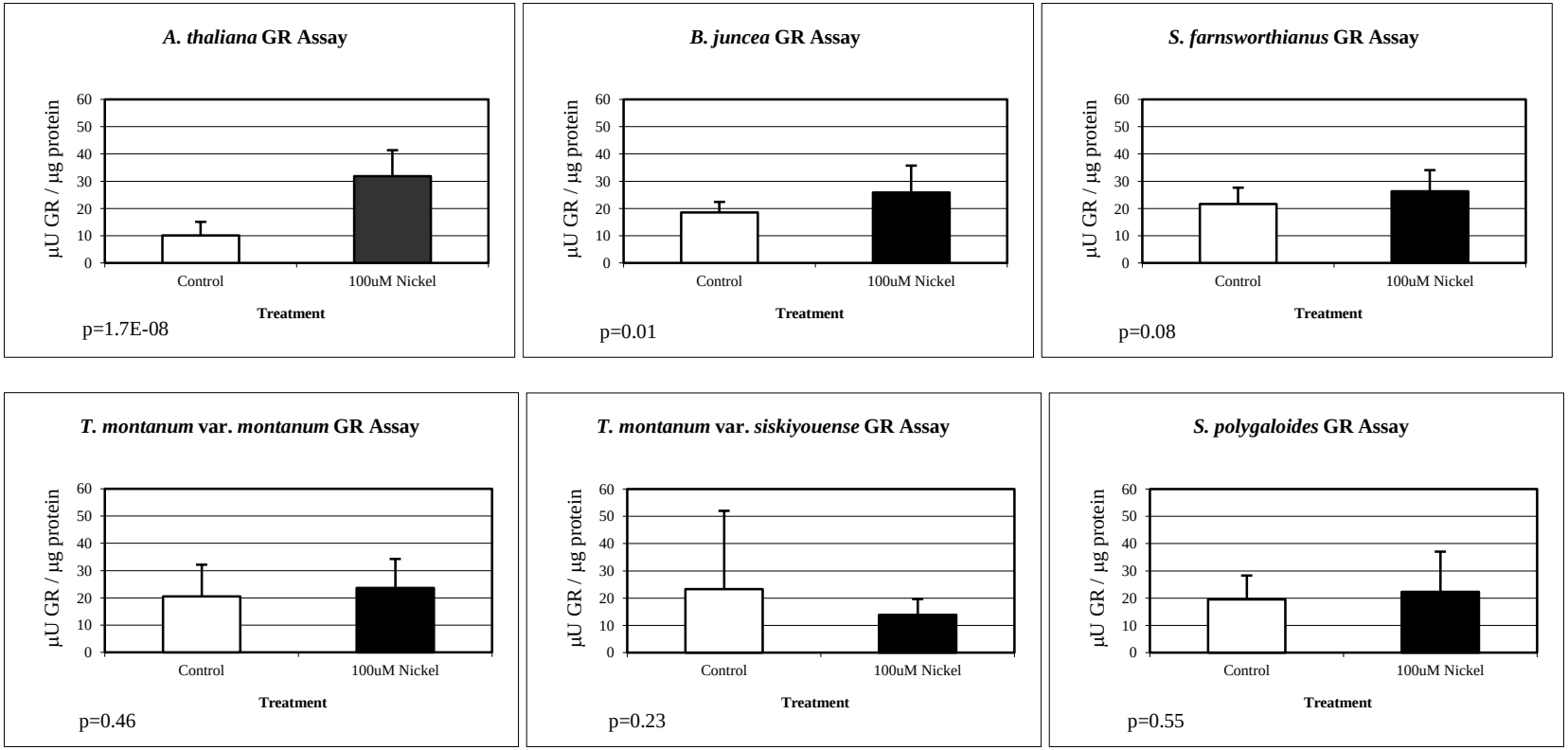

Fig. 5. GR activity of each *Brassicaceae* species. The graph scales are similar for the comparison of the enzyme activity in µU GR/µg protein between species. Top 3 graphs represent the non-accumulator plant species and bottom 3 graphs represent the hyperaccumulator plant species. n = 15.

Note: *T. montanum* var. *montanum* n = 13 control samples; *S. polygaloides* n = 14

CHAPTER IV

DISCUSSION

Experimental Assays

Results of all the assays (with the exception to PCarb assay) showed that the non-accumulator *A. thaliana* was the first plant species to significantly exhibit oxidative stress upon exposure to the heavy metal Ni for 12 days. This allowed this specific species of the *Brassicaceae* family to be used as a baseline of comparison to the other *Brassicaceae* species in the experiment. Also, *A. thaliana* with its sequenced genome and status as a model plant with abundant research resources makes it an ideal plant for comparison with other plants in this study.

Lipid peroxidation was first bio-indicator of Ni-induced oxidative stress as seen in non-accumulator plant species. These plants had a significantly higher amount of lipid peroxidation in Ni-treated plants compared to the control plants (*A. thaliana,* $p = <0.001$; *B. juncea,* $p = 0.01$; *S. farnsworthianus,* $p = <0.001$). Control plants of the non-accumulator plant species *A. thaliana, B. juncea,* and *S. farnsworthianus* contained similar levels of lipid peroxides with amounts of 5.45 ± 3.25, 4.46 ± 2.11, 2.73 ± 2.22 pmol of TBARS/mg frozen plant tissue, respectively. The hyperaccumulator plant species showed no significant differences between control and Ni-treated plants as expected presumably due to the metal tolerant mechanisms hyperaccumulator plants possess. The hyperaccumulators *T. montanum* var. *montanum, T. montanum* var. *siskiyouense,*

and *S. polygaloides* contained lipid peroxidation levels ranging between 5.07 ± 2.48 to 14.78 ± 6.58 pmol TBARS/ mg frozen plant tissue, with a slightly higher, but not significant, level in the nickel treated plants. The range in the amounts of TBARS found between non-accumulator and hyperaccumulator plant species were comparable in the control plants.

The PCarb assay revealed all tested plant species did not have a significant difference between the control and Ni-treated plants. The plants did not exhibit a detectible increase in oxidative damage to cellular proteins in the Ni-treated plants as compared to the controls. The levels of protein carbonyls of the non-accumulator control plants ranged from 1.133 ± 0.66 pmol/μg protein to 1.28 ± 0.57 pmol/μg protein. The hyperaccumulator plants species contained protein carbonyl levels ranging from 1.11 ± 0.55 and 1.28 ± 0.53 pmol/μg protein in the control plants. The levels of protein carbonyls in the non-accumulator and hyperaccumulator plants are within the same range (Fig.2) showing that the plants had a similar response to the Ni-induced oxidation of proteins. Although PCarb assay may be useful to determine basal level of protein oxidation in plants, it may not be the best bio-indicator of heavy metal-induced oxidative stress for the species used in this study.

Antioxidative enzymes and antioxidant molecules aid in the detoxification of heavy metals in plants via the removal of oxygen radicals formed by heavy metals. The Superoxide Dismutase enzyme is involved in the dismutation of superoxide anion radicals via hydrogen peroxide formation (Srivastaval *et al.*, 2005). Superoxide Dismutase is the enzyme that first acts on oxygen radicals. These radicals are produced in any cell compartment where the activation of oxygen radicals occur, i.e. where an electron transport chain is present such as in chloroplasts, mitochondria, peroxisomes, and the cytosol (Takahashi and Asada, 1983; Elstner, 1991; Grene, 2002). Glutathione reductase is an enzyme responsible for reducing oxidized glutathione, back to

a reduced state using NADPH (Connell and Mullet, 1986). Increasing the expression of the amount of GR enzyme has been shown to enhance oxidative stress resistance (Arisi *et al.,* 1998; Zhu *et al.,* 1999; Grene, 2002).

The non-accumulators *A. thaliana* and *B. juncea* showed a significantly higher SOD and GR enzyme activity in the Ni-treated plants as compared to controls. However, the non-accumulator *S. farnsworthianus* did not show a significant difference between control and Ni-treated plants in these enzymatic assays, which indicates that this particular plant species may show characteristics intermediate to a non-accumulator and hyperaccumulator plant species. As shown in table 1 this appears to be the case. The hyperaccumulators *T. montanum* var. *siskiyouense and S. polygaloides* also showed metal tolerance characteristics with the exception of *T. montanum* var. *montanum* having a significantly higher SOD enzyme activity in Ni-treated plants with 15.57 $\pm$ 6.07 mU SOD/μg protein, compared to control plants with 10.13 $\pm$ 7.32 mU SOD/μg protein ($p=0.04$). This difference in control and Ni-treated plants of *T. montanum* var. *montanum* species indicates that SOD enzyme activity was the first indicator for oxidative stress in this hyperaccumulator species showing a significant difference between control and Ni treated plants. This was the only significant difference in any enzyme activity amongst the hyperaccumulator plant species.

Catalase is a universal enzyme that converts hydrogen peroxide, which is mostly produced by SOD, into water and molecular oxygen (Asada, 1992; Lin and Kao, 2000; Srivastaval *et al.*, 2005). In this study, the CAT assay gave interesting results. *A. thaliana* was the only species showing a significant difference between control and Ni-treated plants with regard to the reduction of CAT activity as the control plants had a higher CAT enzyme activity with 103.34 $\pm$ 51.85 μU CAT/μg protein, in comparison to the Ni-treated plants with 10.02 $\pm$

17.81 μU CAT/μg protein. This could indicate that the CAT enzyme activity was inhibited by the presence of Ni in the plants. The non-accumulators *B. juncea* and *S. farnsworthianus* showed no significant difference between the Ni-treated plants and control plants with respect to CAT activity. However, *B. juncea* showed a much lower inherent level of CAT enzyme activity compared to *A. thaliana* and *S. farnsworthianus*. The lower catalase activity in the Ni-treated plants of *A. thaliana* and *B. juncea* could be explained by the results in a study performed by Kono and Fridovich. According to Kono and Fridovich (1982) the flux of oxygen radicals inhibited catalase activity and the inhibition could be relieved by superoxide dismutase. There was an even greater difference of CAT enzyme activity between non-accumulators and the hyperaccumulator plant species in the study. With the CAT enzyme activity ranging from 289.41 $\pm$ 73.65 μU CAT/μg protein to 776.09 $\pm$ 324.18 μU CAT/μg protein, hyperaccumulators still exhibited an exceptionally higher CAT enzyme activity as compared to the non-accumulator plants which ranged in CAT activity between 0.11 $\pm$ 0.11 and 103.34 $\pm$ 51.85 μU CAT/μg protein. The CAT gene may not be expressed at high constitutive levels in non-accumulator species and may be expressed at an even lower level in *B. juncea* due to the fact that its CAT enzyme levels were at least 100-fold less than the expression in *A. thaliana* and *S. farnsworthianus*. When comparing the control plants of the hyperaccumulator species with the control plants of *A. thaliana*, there was at least a 3-fold difference in the amount of CAT enzyme activity in hyperaccumulators. This suggests that the hyperaccumulators may have a higher level of constitutive expression of the CAT gene and this results in a higher CAT enzyme activity. This may be one of the mechanisms that explains the higher the metal tolerance in hyperaccumulators. The CAT enzyme basal activity results in this study were similar to the difference in CAT expression in the study performed by Srivastaval *et al.* (2005), where it was

found that a significantly higher CAT enzyme activity was present in the Arsenic (As) hyperaccumulator *Pteris vittata* than the non-accumulators (*Pteris ensiformis, Nephrolepis exaltata*) suggesting removal of hydrogen peroxide and other toxic peroxides mediated by CAT as an essential mechanism to tolerate arsenic toxicity.

The plant species were treated with a Ni concentration of 100 μM Ni-acetate or approximately 5.8 ppm Ni in 1/10X Hoaglands solution for 12 days. ICP-AES was used to determine the Ni concentration in the leaves after 12 days of treatment. The plants were found to have typical Ni concentrations in the leaves as compared to the range of metal concentrations as found in the literature for plants exposed to Ni (Kramer *et al.,* 1997). The hyperaccumulator plant species possessed on average 3.56 times more Ni in the plant leaves in comparison to the non-accumulator plant species, with little or no sign of physiological damage. This indicates that the hyperaccumulator plants can withstand more Ni induced oxidative stress that the non-accumulator plants at similar or greater Ni concentrations in the leaves.

Conclusions

The results show that the TBARS assay as the first assay to indicate Ni-induced oxidative damage and therefore the best bio-indicator of oxidative stress. PCarb assay was not a useful indicator of Ni-induced oxidative damage with all plant species under examination showing no significant difference in protein carbonylation levels between Ni-treated plants with their respective control plants ($P \leq 0.05$). Enzymatic assays showed an insight of the roles of anti-oxidative enzymes and molecules in the presence of nickel in plants. The results indicate a higher catalase enzyme activity in hyperaccumulator plant species compared to the non-accumulators, suggesting the enzyme serving as a defense against Ni-induced oxidative stress. The SOD and

GR assay results showed significantly increased enzyme activity in the Ni-treated plants of non-accumulators *A. thaliana* and *B. juncea*, indicating heavy metal toxicity in these species via oxidative stress. However, *S. farnsworthianus* did not show a significant increase in SOD and GR enzyme activity with Ni-treated plants presenting *S. farnsworthianus* as a metal tolerant non-accumulator plant species. According to Srivastaval *et al.* (2005), CAT and SOD along with ascorbic peroxidase (APX) and glutathione peroxidase (GPX) are considered as key enzymes in the mechanisms involved in anti-oxidative defenses, and determine oxygen radical and hydrogen peroxides cellular concentration. These enzymes may not act as directly as phytochelatins and vacuolar compartmentalization but show similar results as studies in heavy metal-induced oxidative stress as representations of a secondary defense mechanism for metal tolerance. The CAT assay indicated that removal of hydrogen peroxide is possibly an essential mechanism for increase Ni tolerance. The examination of APX and GPX activity may also be helpful in understanding the relationship of SOD and CAT activity in response to Ni-induced oxidative stress. An assay of APX and GPX may be important due to Mittler's (2002) findings in which APX, GPX, and CAT belong to three different hydrogen peroxide-scavenging enzyme classes. APX and GPX may contribute along with CAT in reducing the amount of hydrogen peroxide in the cells. Therefore, both should be included in oxidative stress studies.

The principle that hyperaccumulators can uptake and tolerate higher heavy metal concentrations compared to non-accumulators is the basis of phytoremediation. Two problems in developing a practical phytoremediation plant are that hyperaccumulator plants are slow growing and have a small biomass. These two problems need to be overcome to develop a plant that could be used for phytoremediation.

Plant biomass plays a role in heavy metal accumulation and metal tolerance. For example, non-accumulator *B. juncea* was more effective at removing zinc (Zn) from soil, via phytoextraction, compared to a known hyperaccumulator of Zn *Thlaspi caerulescens*, due to *B. juncea* producing 10 times more biomass (Ebbs *et al.*, 1997; Padmavathiamma and Li, 2007). This illustrates the importance of a plant's biomass in metal accumulation, but the results of *B. juncea* and *S. farnsworthianus* in this study demonstrate other players present in the plant system that enable the metal tolerance of *S. farnsworthianus* despite its biomass. Therefore, the effect of the role a plant's biomass plays in metal tolerance and accumulation needs further investigation.

This study shows a tentative relationship between plant biomass and heavy metal tolerance. The biomass of the non-accumulator and hyperaccumulator species in this study was noted and compared with *A. thaliana* (arbitrarily set to a biomass value of 1) to gauge the relative biomass of the plants to see if this difference had any effect as represented in Table 2. The non-accumulator *B. juncea* (2.57-fold relative biomass to *A. thaliana*) showed a Ni sensitivity level closer to *A. thaliana* however it was not as sensitive as *A. thaliana* to Ni treatment in that the CAT assay showed no significant difference. A possible reason for *B. juncea* not showing a significant difference in this assay may be the greater biomass of this plant species and this may allow it to absorb more Ni before showing stress. The non-accumulator *S. farnsworthianus*, (1.5 relative biomass to *A. thaliana*) showed a Ni sensitivity level intermediate between other non-accumulator and the hyperaccumulator plant species with only the TBARS assay showing a significant difference. This may be due to the larger biomass of this plant, but it is more likely other tolerance mechanisms may be responsible.

In comparison, the hyperaccumulator plant species had less or near the same relative biomass as *A. thaliana* (*T. montanum* var. *montanum*, 0.63*; T. montanum* var. *siskiyouense*, 0.36;

S. polygaloides, 0.96) but unlike *A thaliana*, showed no significant difference in any of the oxidative stress assays with the exception of *T. montanum* var. *montanum* which has a significant difference in the SOD assay. The hyperaccumulators also lost less relative biomass compared to the controls upon Ni treatment than the non-accumulators. Also in two cases (*T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense*) the hyperaccumulators actually increased in biomass relative to the controls. These results show that a plant's size may not be as important as a plant's heavy metal accumulation and tolerance characteristics.

To prevent environmental pollution, areas contaminated by heavy metals can be remediated physically, chemically, and/or biologically (McEldowney *et al.,* 1993; Whiting *et al.,* 2004; Padmavathiamma and Li, 2007). However, chemical and physical treatments render soils unsuitable for further plant growth and the destruction of biodiversity, according to Padmavathiamma and Li (2007). Hence, phytoremediation provides a natural and aesthetically pleasing solution to this problem and at a lower cost than the other more expensive and destructive techniques.

To engineer the ideal plant for phytoremediation, biochemical and genetic studies must be performed, such as bioavailability and acquisition, metal trafficking and homeostasis, detoxification and sequestration, in which the latter is of interest in this study (Whiting *et al.*, 2004). Due to problems in finding an ideal plant for phytoremediation, research to resolve this problem has increased. The goal of this thesis was to determine levels of oxidative stress response to Ni-induced oxidative stress in non-accumulator and hyperaccumulator plant species of the *Brassicaceae* family. And also to understand the factors influencing the metal tolerance of hyperaccumulators utilizing assays to find potential bio-indicators of oxidative stress. This study shows that preliminary identification of Ni-induced oxidative stress response by the plants in the

laboratory can be used to develop future oxidative stress response assays that would detect heavy metal pollution in the environment. Among the candidates would be biochemical and molecular assays such as those determining levels of the enzyme activity and/or gene expression of SOD, CAT, and GR, or finding other genes to modify high biomass non-accumulator plant species to produce the ideal plant for phytoremediation via genetic engineering.

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APPENDIX

APPENDIX

DEFINITION OF TERMS

Whiting *et al*. (2004), Antonovics *et al.* (1971), Wild and Bradshaw (1977), and Shaw (1990) defines the term hyperaccumulators as follows:

Hyperaccumulators: Also known as metallophytes, are species that have evolved through years of genetic pressure to contain biological mechanisms to tolerate, resist, or thrive on metalliferous soils that are toxic to other plants (non-accumulators), and are typically endemic to the native heavy metal rich soils.

Yang *et al.* (2005) defines the term phytoremediation as follows:

Phytoremediation: The use of plants (hyperaccumulators) to cleanup soils and water contaminated with pollutants such as heavy metals due to the plants acting as solar-driven pumps, extracting certain heavy metals from the environment.

BIOGRAPHICAL SKETCH

Fritzie Joy Into, attended South Texas High School for Health Professions in 1998 and obtained her High School Diploma in 2002. Afterwards, she was accepted to The University of Texas-Pan American where she majored in Biology and earned her Bachelors Degree of Science in Fall 2007. During the years as an undergraduate, she worked as a research and laboratory assistant in molecular biology under Dr. Michael Persans, which guided her into following a higher level of education in Biology with a Masters Degree. Therefore, following her graduation she was soon accepted to the graduate program in Spring 2008 for the Department of Biology in her old alma mater. In her three years as a Masters of Science candidate, she was able to teach Biology I and II laboratory to undergraduate students, mentor and teach summer research program candidates in her plant molecular biology research, and still managed to fulfill her responsibilities in the graduate program. After defending her thesis in December 2010, focused on plant molecular biology, she earned her Masters Degree of Science in Biology. Her permanent place of residence is 4005 Arroyo Vista Court in the city of Harlingen, TX preparing for the next chapter in her life with her new degree.