

ZINC AND CADMIUM TOLERANCE IN SALIX: A SEARCH FOR THE  
ROLE OF POLYPHENOLS

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## ABSTRACT

The enhanced metal accumulation and tolerance properties found in the *Salix* species have generated interest in using these plants in phytoremediation applications. However, little is known about the mechanisms behind these traits. Many aspects await elucidation, such as the molecular basis of metal uptake, tolerance and accumulation.

In this investigation an attempt is made to shed light on a possible ligand involved in metal sequestration in willow plants. Polyphenols, abundant in willows, are examined as potential candidates. Already known for their roles in plant growth, development and defense, these compounds might be a part of the metal tolerance mechanism.

In the first phase of the investigation, the potential role of polyphenols as ligands is explored by confirming their ability to chelate metals, namely Zn and Cd. Modeling exercises with representative phenolic moieties indicated that molecules with vicinal oxygens are more suitable chelators due to their ability to complex metal cations in a bidentate manner. A combination of acid titrations and infrared spectral analysis confirmed the ability of a representative polyphenol, tannic acid, to bind Zn and Cd over a physiologically relevant pH range.

In the second phase of the investigation, the effect of metal exposure on phenolic levels in plants is explored; the hypothesis being that if phenols are a part of the mechanism that confers enhanced metal tolerance in *Salix*, metal exposure will induce an upregulation of these compounds as part of a defensive response.

A total of 20 plants from three willow species, *S. alba*, *S. viminalis* and S-301, were subjected to one of two Zn and Cd treatments in a hydroponic setting for a period of two weeks. Leaf tissue was then harvested for analysis.

Metal levels were determined via a methanol extraction of fresh leaf tissue and analysis with atomic absorption spectroscopy (AAS). Colorimetric methods were performed on dried leaf tissue to ascertain gross phenolics as well as levels of condensed tannins and leucoanthocyanins.

Results showed that metal levels increased in plants as exposure concentrations increased but metal exposure appeared not to have a significant effect on the phenolic status of plants. However, these results do not necessarily disprove the above hypothesis. Small sample sizes as well as the considerable variations known to occur both between and within species could have obscured metal-induced changes in phenolic levels. Therefore, further investigation is warranted. More plants should be exposed to metals for longer periods of time with metal and phenol status assessed along the way. Moreover, assays should be employed to assess the levels of phenolics such as salicylates and hydrolyzable tannins.

Pursuing additional avenues of investigation aside from phenolic quantification would also be sensible. A combination of size exclusion chromatography techniques coupled with UV-VIS or other detectors and X-ray absorption spectroscopy could go a long way to solving the mystery of which plant constituents are responsible for enhanced metal tolerance.

In summary, while this examination did not yield conclusive results it provides a solid foundation for further investigation. The next steps briefly outlined above will determine if polyphenols involved in metal tolerance. If they are ruled out, the focus can shift to other compounds, such as organic acids. However, if it is shown that polyphenols do figure into the process then science will be one step closer to sorting out the metal tolerance mechanism in *Salix*.

## BIOGRAPHICAL SKETCH

I am not someone who sits still well. Nor am I someone who will ever be satisfied with the conventional. Instead of accepting what life hands me, I prefer to create my own destiny. Hand me lemons and I will make the best damn lemonade you have ever had- and I don't even like lemons.

These past few years have seen me wandering about the planet as I try to sort out my place in this world and capture the solace that comes from that. In the course of that journey, I have discovered that campaigning and crusading for a safer, cleaner planet is work that fulfills me in a way that nothing else does.

Several years into my first job I decided to go back to school. By studying Environmental Toxicology, I was trying to become a better advocate for the environment. However, I underestimated how hard it would be to take a hiatus from work; leaving my job meant stepping out of the game for a while and watching from the sidelines. The experience was just shy of torture but now that I am back on the field, I see how my time away is paying off. I may be a treehugger but I know what I am talking about.

It has taken almost four years to finish this degree. But with this thesis representing the final crossing of t's and dotting of i's for my Masters of Science from Cornell University, I can now say that I am really done and can now fully and completely get back to saving the planet.

## ACKNOWLEDGMENTS

Anyone who has ever engaged in a project such as this knows you can't do it alone. It took the wisdom, support and guidance of many to deliver what has been written in these pages. And for that, I owe many a gratitude of thanks.

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## CHAPTER 1

### REVIEW CHAPTER

#### *Introduction*

Plants are adapted to a wide range of environmental conditions that include drought, temperature extremes, flooding, salinity, nutrient imbalances and deficiencies in soils as well as exposure to toxic compounds. In this investigation, the interaction between plants and heavy metals is of greatest interest, as metal concentrations exist in excess of plant requirement in some soils as a result of anthropogenic activities such as mining, manufacturing, energy production and agriculture, potentially becoming toxic to plants (Wieshammer et al., 2007). The extent to which enhanced metal levels adversely affect plant growth largely depends upon soil characteristics, as they control the bioavailable fraction of the metals in soils.

In the plant kingdom, metals can be divided into two distinct classes- essential and non-essential. Essential metals are micronutrients that have structural and catalytic roles in proteins involved in metabolism and development. Zinc (Zn), iron (Fe), and copper (Cu), for example, are required for healthy plant growth and development (Haydon and Cobbett, 2007). On the other hand, non-essential metals, such as cadmium (Cd), lead (Pb), and nickel (Ni), serve no biological function and can be toxic to plants at very low any does.

The phytotoxic effects of metals are numerous and stem from their ability to disrupt cellular processes. Excessive concentrations of any metal generates harmful reactive oxygen species; non-essential metals displace endogenous metal co-factors causing enzyme malfunction or inactivation; and toxicity of the certain metals is owed often times to their reactivity with sulfur (S) and nitrogen (N) atoms in amino acid side chains.

### ***Mechanisms of Tolerance***

In plants, homeostatic mechanisms are responsible for maintaining concentrations of metals within physiologically acceptable limits. They manage the transportation, delivery and distribution of metals throughout the plant. Beyond homeostatic-mediated responses, plant exposure to elevated and potentially toxic metal levels elicits in one of three reactions: unrestricted uptake, exclusion or accumulation.

Unrestricted metal uptake is essentially a non-reaction that allows as much metal into a plant as is available for uptake. This usually results in phytotoxicity and death. An exclusion response can involve the initial uptake and subsequent efflux of metals out of cells or the exudation of compounds that complex with metals thereby preventing entry into root cells. In *Arabidopsis*, for example,  $\text{Pb}^{2+}$  toxicity is mediated by the ATP-binding cassette transporter, AtPDR12, which utilizes ATP to actively pump out  $\text{Pb}^{2+}$  that has accumulated in root cells (Lee et al., 2005). In certain strains of wheat, Aluminum III stems from an Al-stimulated release of organic acids that complex with Al, rendering it unavailable for uptake (Delhaize et al., 1993). These methods keep metal levels in excluding plants low and permit survival on soils with high metal concentrations.

In contrast to the above, accumulation allows metal uptake and mitigates toxicity by chelating and/or sequestering metals once they are in the plant cells. This prevents their interference with metabolic processes. Chelating agents are plant and metal specific, but include metallothioneins, phytochelatins, histidine and glutathione. On the extreme end of the accumulation spectrum are hyperaccumulators- plants capable of amassing exceedingly high metal concentrations without showing visible symptoms to suggest metal toxicity.

The term “hyperaccumulator” was introduced by Brooks et al. in 1977 in reference to plants that accumulate more than 1 mg of Ni per gram of dry weight in their shoots under natural conditions. To date, more than 400 species of hyperaccumulators have been identified (McGrath and Zhao, 2003). This trait is generally observed with Ni, Zn, Co and Se, although accumulators of Mn, Cd, As and Pb have been also characterized (Clemens, 2001). Perhaps the best-known hyperaccumulator is *Thlaspi caerulescens*. Certain ecotypes of this plant can have respective concentrations of Zn and Cd of 30,000 and 1,000 ppm, without exhibiting signs of toxicity. Compare this to non-accumulating plants, where Zn is generally toxic between 300-500 ppm and Cd has been shown to cause harm at levels as low as 20 ppm (Boominathan and Doran, 2002).

The physiological processes responsible for hyperaccumulation are largely unknown. Current research efforts to elucidate the mechanisms concerned are being driven primarily by the potential to use hyperaccumulating plants to clean-up metal contaminated soils.

### ***Plants as Remediators***

An abundance of metal-contaminated soils exist in the United States, a testament to anthropogenic activities that regard land as an expendable and disposable commodity. The legacy of this myopic view of the lithosphere has resulted in the designation of more than 30,000 sites for hazardous waste treatment services by the United States Environmental Protection Agency’s Comprehensive Environmental Response Compensation Liability Information System (Raskin, et al., 2000). The need to protect public health and the environment drives the efforts to restore the safety and integrity of such sites.

Current soil remediation practices rely on a variety of technologies such as excavation and landfilling, chemical washing and mechanical/pyrometallurgical

separation. These techniques are efficient but are expensive (Gardea-Torresdey et al., 2004) and often times destroy soil structure, leaving it biologically inactive (McGrath, 2001). The search for less costly and more desirable soil clean-up methods has put the spotlight on plants with metal accumulating properties.

The use of plants to clean up contaminated soils is referred to broadly as phytoremediation (Salt et al., 1998). Phytoextraction is a specific type of phytoremediation where plants remove metals from soils and concentrate them in their aboveground parts. This practice is relatively new and still emerging in many respects, but field trials have demonstrated its feasibility and potential for wider application.

The advantages of phytoextraction are many. Economics perhaps provides the most compelling case for its further development. Excavating and landfilling contaminated soils can cost upwards of \$400 per cubic meter while phytoextraction of similarly contaminated soils costs about one-tenth that amount. Other advantages include minimal disturbance of the natural environment is minimal and soil structure and integrity is preserved.

However, several downsides to phytoremediation exist. The first is the length of time needed for clean-up: traditional techniques require 6-9 months whereas plant driven methods can take years. In some instances, plant-based remediation of less polluted land may be achievable in a timeframe of 18-60 months (Raskin, et al., 2000). However, this is likely to be the exception rather than the rule. As an example, Felix (1997) calculated in his experiment that *Salix viminalis* would need 77 years to lower soil Cd concentrations to acceptable levels (Pulford and Watson, 2003) .

Another limitation is that phytoextraction might not work for all metals. For instance, Pb is notoriously difficult to phytoremediate; its strong affinity for the organic and mineral components limiting both solubility and bioavailability (Hettiarachci and Pierzynski, 2004). Issues such as these are not insurmountable but

might limit the effectiveness of plant-based techniques. Chelators can be employed to enhance metal solubility and combining phytoremediation with other techniques could also help (Raskin, et al., 2000).

Plants best suited for phytoextraction are high biomass producers that are easy to handle, have established cultivation practices and are genetically characterized (Raskin, et al., 2000). Unfortunately, this combination of traits is rarely found in hyperaccumulators. Plants like *Thlaspi caerulescens* are excellent metal extractors but have low biomass yields and slow growth rates (McGrath and Zhao, 2003). To overcome this, scientists are exploring ways to exploit the impressive accumulation properties of this plant by transferring these traits to faster growing, higher biomass producing plant species (Lasat, 2002). It is not certain if that will be feasible, however, as multiple traits are required for metal accumulation and tolerance. In the meantime, certain tree species are being looked at as possible alternatives (Wieshammer et al., 2007). While no commercially important trees are known to hyperaccumulate, they hold promise as a “low-cost sustainable and ecologically sound solution to the remediation of heavy metal contaminated land, especially when it is uneconomic to use other treatments or there is no time pressure on the reuse of the land” (Pulford and Watson, 2003).

One genus that has garnered significant interest from the scientific community is *Salix*. This genus of plants includes the ever-popular weeping willow, as well as 350 other species. It is one of the most taxonomically and ecologically diverse genera in the Northern Hemisphere (Punshon and Dickinson, 1997). Willow trees are easy to propagate, metal tolerant, perennial crops that possess extensive root systems and high evapotranspiration rates that aid in the stabilization of pollutants (Mertens et al., 2006). They possess the combination of enhanced metal accumulation and tolerance

traits, fast growth rates and high biomass production necessary for phytoextraction (Vervaeke et al., 2003).

Any disadvantages associated with using lower metal accumulators such as willow is more than offset by the sheer volume of biomass they produce- willow trees can actually remove more metals from soils than hyperaccumulating plants even though they accumulate at lower levels. For example, *T. caerulescens* yields 2.5 t/ha/yr of dry shoot matter (McGrath and Zhao, 2003) but the average yield for willows is between 10-15 t/ha/yr (Pulford and Watson, 2003). For Cd, high biomass production in willow can mean metal removal rates that are five times higher than of hyperaccumulators such as *T. caerulescens* and *Alyssum morale* (Pulford and Watson, 2003).

An ancillary benefit of using willows as remediators is that they hold promise as a renewable energy crop (Pulford et al., 2002, Keoleian and Volk, 2005). Harvested willow biomass from contaminated land can subsequently be burned for energy production, resulting in a two-birds-with-one-stone approach that makes the economics of phytoremediation even more attractive (Mirck et al., 2005, Tlustoš et al., 2006). Harvesting metal from combusted plant tissue for resale could provide additional revenue streams.

### ***Avenues of Investigation***

The discovery of hyperaccumulators revealed that plants have the genetic potential to clean up contaminated soils (Lasat, M., 2002). Harnessing that potential is now contingent upon the determination of how accumulation works and identifying the genes involved in the process. Beginning with the translocation of the metal cation from the soil solution into the root cell, continuing to vascular loading and unloading and ending with the sequestration of a metal-ligand complex in a vacuole or trichome, the specifics of accumulation await elaboration. In recent years, science has been able

to settle certain key pieces of the puzzle and bring us closer to understanding the whole story.

More specifically, research has been able to shed light on the role of transporters in metal hyperaccumulation as well as ligands responsible for binding to and shuttling metals from the apoplast to the cytoplasm and across the tonoplast into vacuoles. Unfortunately, as is often the case with science, there is no one-size-fits-all pathway for hyperaccumulation. Evidence suggests that the biochemical mechanisms behind hyperaccumulation are both species and metal specific (Lasat, M., 2002). To complicate matters even further, it appears that metal-ligand speciation is dynamic, varying not only between species but during developmental stages and within plants as metals are shuttled between tissues and subcellular compartments. A quick survey of the current state of knowledge surrounding the biochemistry of *T. caerulescens* with respect to Zn hyperaccumulation is illustrative of this.

A constitutive upregulation of a ZIP family transport protein, ZNT1, is implicated as the first link in the accumulation chain. Hyperaccumulation is correlated to a stimulation of Zn influx into root cells (Pence et al., 2000). In non-accumulating species, such as *T. arvense*, root cell vacuolar sequestration prevents Zn translocation to the shoots but this mechanism is disabled in *T. caerulescens* (Lasat, 2000). Zinc is ultimately sequestered in the leaf cell vacuoles but metal speciation as well as the tonoplast transporters involved have eluded identification. With regard to ligands, S and P compounds have been all but ruled out (Kupper et al., 1999). X-ray absorption spectrometry (XAS) and extended X-ray absorption fine structure (EXFAS) analysis have revealed citrate as the primary ligand for Zn followed by histidine. However, it is believed that the Zn-citrate complexes are relevant only for vacuolar sequestration while histidine is involved in chelating Zn in developing and older leaf tissues (Haydon and Cobbett, 2007).



Ultimately, unraveling the pathways involved in metal accumulation will allow for identification and isolation of the genes responsible for the hyperaccumulation (Lasat, M., 2002). Characterization efforts for plants such as *T. caerulescens* are well underway, although a great deal of work remains. But what is the current state of knowledge for metal accumulation in willow? Even if this plant is not a hyperaccumulator in a technical sense, it clearly meets the biomass criterion necessary for phytoextraction. A better understanding of the physiology and biochemistry behind its metal accumulation would allow for additional augmentation of these traits.

### ***Willows***

Research has shown that willow could be most useful in the removal of Cd and Zn from moderately contaminated soils (Vysloužilová et al., 2003, Mertens, et al., 2006, Dos Santos Utmazian et al., 2007). However, the success of *Salix* plants as phytoextractors depends on more than just their ability to pull metals out of the soil. Biomass production and metal partitioning are equally important factors (Riddell-Black, 1994).

Much of the research to-date has focused on screening various willow species for metal uptake, tolerance and accumulation to determine which plants might be useful for remediating polluted sites (Pulford et al., 2002). A range of approaches has been taken in this regard that include field trials, greenhouse pot experiments and hydroponic tests (Dos Santos Utmazian et al., 2007).

The body of knowledge amassed, while not extensive, does reveal that there is broad divergence amongst plants in the *Salix* genus and not all tree species would be suitable for phytoextraction (Landberg and Greger, 1996). Considerable variation exists both between and within species but the factors responsible remain unclear. It could be due, in part, to the widely divergent conditions under which willow species have been examined in different experiments. Evidence indicates that any number of

factors from plant age, growing conditions and experiment length to metal exposure levels and combinations, soil conditions and pre-treatments can influence a study's outcomes (Landberg and Greger, 1996, Landberg and Greger, 2002, Dos Santos Utmazian et al., 2007).

However, these sorts of dissimilarities cannot explain away the significant differences found within clones in the same experiment. For example, a study by Landberg and Greger (1996) revealed that the Cd concentrations in collected plant material for clones of *S. viminalis* and *S. daphnioides* varied by a factor of up to 25. In this experiment, ranges for metal accumulation within species were greater than between species. A similar investigation found that the Cd uptake capacity of 70 *Salix* genotypes differed as much as 43 times between clones with the highest and lowest values (Greger 1999). Even further, metal uptake and accumulation does not appear to be related to tolerance- resistance to heavy metal toxicity seems to be clone or hybrid specific.

The paucity of data regarding the mechanisms responsible for the enhanced metal acquisition and tolerance traits found in some willow plants means that efforts to explain these results are little more than educated guesses. These knowledge gaps hinder the realization of the full potential of *Salix* plants as part of an effective soil remediation strategy (Punshon and Dickinson, 1997).

### ***Pieces of the puzzle***

To contribute to the existing body of knowledge, this investigation will attempt to identify potential molecular mechanisms that play a role in enhanced metal uptake and tolerance in *Salix* species. As noted above, any number of starting places exist to determine the molecular basis of metal uptake, tolerance and accumulation- all three things must be operational in a plant if it is to be adequate as a phytoextractor. That means a mechanism for enhanced uptake of metals via root cells, enhanced root-to-

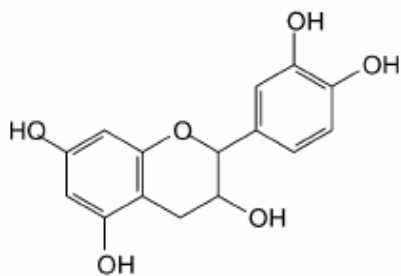
shoot transport via reduced sequestration in root vacuoles or increased xylem loading and enhanced tolerance via internal detoxification which likely involves some form of metal complexation and/or sequestration.

For this study, internal metal detoxification is the primary focus, more specifically, an attempt to shed light on a possible ligand involved in metal sequestration in willow plants. Research related to metal distribution in willows shows that a large proportion of metals end up in the aerial portions of the plant. A study by Vysloužilová et al (2003) on willow clones revealed that Cd and Zn are accumulated in higher concentrations in the leaves of plants than the twigs. Work undertaken by Hammer et al. (2003) and specifically examining Cd found that while the leaves from a field test involving *Salix viminalis* constituted 15-19% of total plant biomass, they contained 34-37% of the total amount of Cd extracted.

While the eventual cellular repository for metals in willow leaves remains a mystery, Mertens et al. (2006) showed that foliar Cd and Zn concentrations are well correlated; this points to a similar translocation mechanism for these elements and possible comparable sequestration fates in the leaves. It is already known that in many accumulating plants, such as *T. caerulescens*, Zn and Cd are sequestered in the vacuoles away from vital cell components and processes. For example, Ma et al. (2005) exposed *T. caerulescens* to Zn and Cd in a hydroponic setting and found that 91% of total Cd and 77% of total Zn was translocated to the protoplasts of mesophyll tissue. Of that, all of the Cd in the protoplasts was compartmentalized in vacuoles while 90% of Zn was similarly localized. Vacuoles could also serve as the final metal repositories in willow leaves.

Although any numbers of molecules are likely candidates as a metal ligand in leaves, one group of compounds that warrant attention are polyphenols, a class of secondary plant constituents well known for their antioxidant activity (Larson, 1988;

Bors and Michel, 2002). Polyphenols are synthesized by all higher plants (Levin, D., 1971) and include pharmacologically active compounds such as flavonoids, tannins and glycosides (Weber and Koniecznyński, 2003) as well as anthocyanins, which function as pollinator attractants (Levin, D., 1971) and are responsible for coloration in wines and musts (Salinas et al., 2004). They display tremendous structural heterogeneity, but are based on a six-carbon aromatic ring (Levin, D., 1971). A representative molecule, catechin, is shown below (Figure 1).



**Figure 1: Catechin**

Polyphenols have multiple biological tasks. They are important for normal plant growth and development but also serve a defensive role, protecting against infection and injury (Kähkönen et al., 1999). For example, plant phenolics act as feeding deterrents, growth inhibitors and are toxic to herbivorous insects (Lindroth et al., 1988; Hakulinen and Julkunen-Tiitto, 2000; Nyman and Julkunen-Tiitto, 2000). More relevant to this investigation is the metal chelation potential for this class of compounds (Kähkönen et al., 1999). Anthocyanins and tannins are known to complex metals such as Fe, Cu, Al and Mg (Esparza et al., 2005) and the ability of flavonoids to chelate metals has been widely reported in the literature (Hider et al., 2001; Bodini et al., 2001). Esparza et al. (2005) demonstrated Zn and Cu complexation with flavonoid ligands catechin, quercetin and rutin and there is evidence of Zn binding with hydroxyflavones (Lapouge et al., 2006). Additionally, Zn-polyphenol binding in medicinal plant extracts has been documented by Weber and Koniecznyński (2003).

Polyphenols occur in great abundance in willows, with the total phenolic content in most species exceeding 5% (Nyman and Julkunen-Tiitto, 2000). On the extreme end, it can be even greater, as is the case with *S. phylicifolia*. Total phenolic content in this species is more than 15% of dry weight. (Julkunen-Tiitto, 1986). Phenolic composition is highly species-specific; however, substantial differences occur even within a species and plant parts (Julkunen-Tiitto, 1989).

Given all of the above, it is reasonable to postulate that metal tolerance in *Salix* may depend partly on willow phenolic levels and composition. Phenols could serve as ligands that simply bind any excess metals within willow leaves to prevent interference with metabolic processes, and they could also function as one of a variety of complexing agents responsible for shuttling metals from the cytoplasm to vacuole. This investigation proposes to examine a possible association between plant phenolics and metal tolerance in *Salix*. To achieve this, a preliminary study will first confirm the metal chelation potential of representative phenolic compounds found in willow. Secondly, experiments will be run on several willow species known to tolerate higher metal levels to determine what effect, if any, metal exposure has on phenol levels in willow leaves. The hypothesis is that if phenols are a part of the mechanism that confers enhanced metal tolerance in *Salix*, metal exposure may induce an upregulation of these compounds as part of a defensive response.

## CHAPTER 2

### PRELIMINARY ANALYSIS OF CHELATION BY POLYPHENOLS

#### *Introduction*

Metal-phenolic complexation could serve as a basis for heavy metal tolerance in willow trees. However, before this hypothesis can be explored the affinity of phenols for various metal cations should be established. Of interest to this investigation is how well polyphenols complex with zinc and cadmium cations at physiologically relevant pHs.

The relationship between polyphenol complexation for a range of metals has been widely reported in the literature. As a class, they tend to be good metal complexing agents. Anthocyanins and tannins bind readily to metals such as Fe, Cu, Al and Mg (Ross et al., 2000, Salinas et al., 2004, Esparza et al., 2005, Castañeda-Ovando et al., 2009) and flavonoids have been shown to bind to a number of metal cations (Pletta, P 1988, Birjees Bukhari, S. et al., 2009, Castañeda-Ovando et al., 2009). The consequences of these interactions are themselves interesting avenues of investigation. It is known, for example, that metal-tannin binding alters wine properties such as taste and color (Esparza et al., 2005), while metal binding in medicinal plants is thought to influence the pharmacological effects of natural drugs (Weber and Koniecznyński, 2003).

Metal cation binding occurs through the phenolic oxygen atoms attached to the aromatic rings of polyphenolic compounds. Even though the pKa value of most phenols is in the region of 9.0-10.0, certain metal cations displace phenolic protons at much lower pH levels (Hider et al., 2001). Nevertheless, it is the structure of polyphenols that ultimately determines their effectiveness as complexing agents as well as the number of metal cations they can bind. Binding ratios vary depending on

the molecules involved. As an example, a study by Fernandez et al. (2002) showed binding ratios of 1:1, 1:2, 2:2 and 2:3 for metal-flavonoid complexes and established that the preferred binding site for flavones and the flavanone naringenin was at the 5-hydroxyl and 4-oxo groups.

For binding configurations, bidentate ligands are more effective complexers than monodentate ligands (Hider et al., 2001). Therefore, phenolics possessing the structural elements necessary to form bidentate complexes with metals would be expected to be good metal chelators. The catechol moiety with its vicinal hydroxy groups, found in many polyphenols, lends itself well to bidentate binding. Hence, catechol is predicted to be a better chelator than salicylate.

Polyphenols are already known to have an affinity for Zn cations (Bodini et al., 2001, Weber and Koniecznyński, 2003, Salinas et al., 2004, Esparza et al., 2005, Lapouge et al., 2006). Le Nest et al. (2004a, 2004b) demonstrated Zn<sup>2+</sup> chelation by quercetin in solutions buffered at pH 7, reporting the most probable binding site to be the catechol moiety. In work done by Esparza et al. (2005), binding ratios for Zn with catechin, quercetin and rutin were found to be 1:1, 1:2 and 1:3, respectively. Quercetin was shown to be linking two metal atoms through its catechol and 4-oxo-5-hydroxyl moieties, while rutin was able to chelate a third metal cation in the 3-rutinoside.

The binding affinity between Cd and phenols is perhaps less well characterized. While studies have shown, for example, that salicylic acid pretreatment confers protection against Cd toxicity in barley (Metwally et al., 2003) and soybean plants, (Drazic and Mihailovic, 2005), metal chelation is not considered the reason for this effect. Rather than concentrating on Cd-phenol interactions, much of the research has instead focused on phytochelatin-mediated detoxification, a major mechanism for Cd resistance in plants (Cobbett and Goldsbrough, 2002, Mendoza-Cózatl et al.,

2008). For that reason, the following chapter explores metal-phenolic interactions and provides confirmation of the binding relationship of the metal cations Zn and Cd with phenolic compounds representative of those found in willow trees.

### ***Materials and Methods***

#### **Initial modeling**

Binding of Zn and Cd with representative phenolic moieties, catechol and salicylate, was calculated using the CHEAQS<sup>1</sup> modeling program. This exercise was performed to demonstrate metal cation binding with different phenolic structures. Catechol possesses vicinal hydroxy groups on its aromatic ring while salicylate does not (see Figure 2). If bidentate binding is the manner in which Zn and Cd species complex with polyphenols, it was expected that catechol would be a more effective chelator.



**Figure 2: Catechol (left) and Salicylate (right)**

Results of this preliminary analysis were used to select a polyphenol compound for a titration assay evaluating binding efficiencies for Zn and Cd. In the end, tannic acid was chosen as the model polyphenol compound as it is structurally representative of many of the polyphenols found in willow plants.

#### **Titration assay**

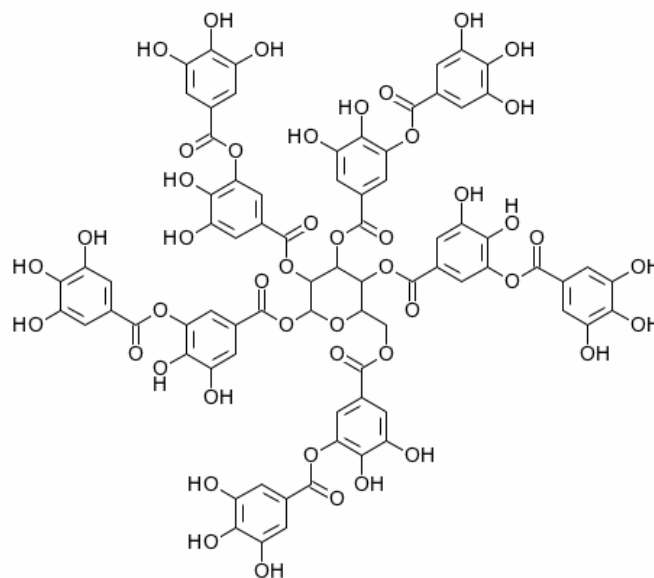
To assess the binding efficiencies of Zn and Cd with tannic acid, a classical pH titration was performed. This involves titrating, with base, solutions of tannic acid,

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<sup>1</sup> CHEAQS is a free Windows program for calculating equilibria in aquatic systems. Available at: <http://home.tiscali.nl/cheaqs/index.html>



with and without the metal present, and recording the change in pH with increasing base addition. The data are then graphed and analyzed.



**Figure 3: Structure for Tannic Acid,**  
**C<sub>76</sub>H<sub>52</sub>O<sub>46</sub>**

The shift in the pH of the titration curve for tannic acid with the metals present indicates binding. In the case of phenolic complexation with cations such as Zn<sup>2+</sup> and Cd<sup>2+</sup>, binding is presumed to be largely bidentate, with two oxygens on adjacent carbons binding one metal ion; the implication being that for every bound metal ion, two hydrogens are displaced or  $M^{2+} + LH_2 \longrightarrow ML + H_2$ .

The pH meter used for this assay was the Orion 2 STAR pH benchtop meter by Thermo Electric Corporation. After each titration, the data was collected and graphed. Results are presented below.

**Control solution titration curve:** The control solution was 0.01 M tannic acid in 0.01 M KNO<sub>3</sub>, whose pH was adjusted initially to 3 by adding approximately 0.5 mL of 0.01 M HNO<sub>3</sub> prior to commencement of the titration. The solution was titrated with a 0.1M KOH solution in 0.5 mL increments and the pH recorded after each

addition of base. This process was continued until the pH of the control solution reached 10.

***Zn titration curve:*** The Zn solution consisted of tannic acid (0.01 M) with 0.005 M  $\text{ZnSO}_4$  added. It was prepared with an approximate 2:1 ratio of potential bidentate binding site to moles of metal. The pH was adjusted to 3 by adding 0.1M KOH prior to commencing the titration, as the Zn addition had lowered the initial pH. The solution was titrated in the same manner as described for the control solution above. An additional solution containing only Zn (a 0.23 M  $\text{ZnSO}_4$ ) was also prepared and titrated in the manner described above.

***Cd titration curve:*** The Cd solution consisted of tannic acid (0.01 M) with 0.005 M  $\text{CdNO}_3$ . It was prepared with an approximate 2:1 ratio of potential bidentate binding site to moles of metal. The pH was adjusted to 3 by adding 0.1M KOH prior to commencing the titration. The solution was titrated in the same manner as described above.

### **Spectral analysis**

To confirm and examine the nature of binding for Zn and Cd with tannic acid, spectral analysis was performed on freeze-dried samples of the control (tannic acid) solution and the solutions containing Zn and Cd. This was done after they had been titrated to a pH of 7 in the manner described above. Freeze drying was achieved by transferring approximately 15 mL of each solution to separate containers, covering and freeze-drying over the course of a week. Spectral analysis was subsequently performed as detailed below.

***Infrared analysis:*** Freeze-dried materials of each solution were separately analyzed via infrared spectroscopy. Approximately 50 mg of sample was ground into a KBr matrix and analyzed, with the spectra from the control (tannic acid) solution

serving as the baseline. The IR spectrophotometer utilized was a Mattson Galaxy 5020 system.

***NMR analysis:*** Of all the NMR techniques, solid state NMR was perhaps best suited to analyze freeze-dried material. However, the necessary equipment was not available so an attempt was made to analyze the samples with solution state NMR. Freeze-dried material was mixed with methanol d-4 to dissolve and prepare the samples for analysis. Total volume for each prepared sample was 0.6 mL. In the end, the attempt to analyze the samples via solution state NMR was unsuccessful. The spectra obtained from each sample revealed nothing- as if the samples run were blanks. As a result, no useable spectral data were obtained. One possible explanation is that the tannic acid aggregated with freeze-drying and was not truly in solution when the analysis was conducted.

## ***Results and Analysis***

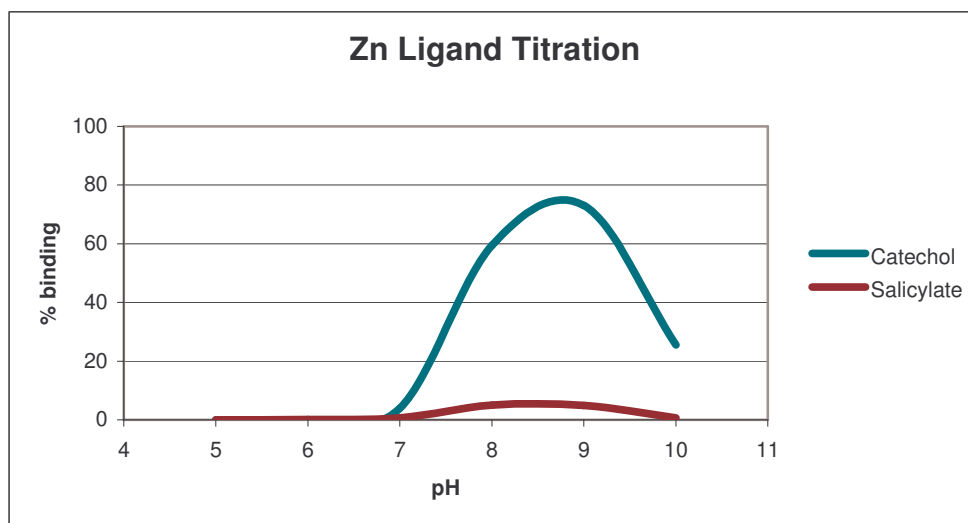
### **Modeling**

The results of the CHEAQS modeling analysis are summarized in the tables and graphs below. They reveal a pronounced difference in metal binding levels between the phenol moieties, favoring metal complexation by catechol. As postulated above, it is likely that this is due to the vicinal hydroxyl groups, which bind metal cations in a bidentate fashion. Such binding is not possible with salicylate, and the carboxylate group is a weaker ligand for the metal than the phenolate.

The CHEAQS modeling program predicted that complexation levels would be highest for catechol at a pH of 9 and 10 for Zn and Cd, respectively.<sup>2</sup> Comparing the two metal cations, it calculated that catechol would bind more readily to Zn than Cd regardless of the pH.

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<sup>2</sup> The metal and ligand concentrations in the CHEAQS modeling exercises were 0.001 M.



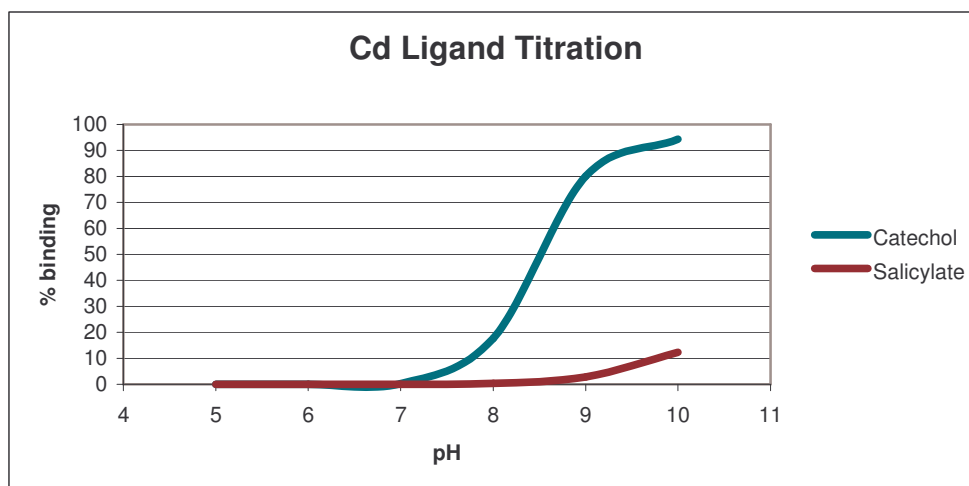
**Figure 4: Zn ligand titration. Predicted binding levels for zinc with catechol and salicylate over a pH range of 5 to 10.**

**Table 1: Percent binding levels for Zn with ligands**

Zn-catechol complexation		Zn-salicylate complexation	
pH	% binding	pH	% binding
5	0	5	0.01
6	0.04	6	0.06
7	3.89	7	0.63
8	59.48	8	4.98
9	73.08	9	4.96
10	25.53	10	0.61

**Table 2: Percent binding levels for Cd with ligands**

Cd-catechol complexation		Cd-salicylate complexation	
pH	% binding	pH	% binding
5	0	5	0
6	0	6	0
7	0.27	7	0.03
8	17.68	8	0.32
9	80.1	9	2.86
10	94.38	10	12.29



**Figure 5: Cd ligand titration. Predicted binding levels for cadmium with catechol and salicylate over a pH range of 5 to 10.**

Given these results, it seemed most logical to evaluate the metal binding abilities of a polyphenol compound with catechol moieties. Tannic acid, which is structurally representative of many polyphenols found in willow plants, has abundant vicinal hydroxyl groups that could serve as potential binding sites for metal cations. As such, tannic acid was selected as the modeled compound to further investigate the binding of Zn and Cd by polyphenol compounds.

### **Titration assay**

The results of the titration assay clearly show the binding of tannic acid with both metal cations (see Figures 6 and 7), as indicated by the shift in pH between the tannic acid solution and tannic acid with Zn and Cd present. Metal cations displace  $H^+$  ions, thereby requiring the addition of greater levels of base in order to raise the pH of the solution. The data show that both metals displaced a similar amount of H atoms at the various pH levels. A greater shift in the curve for the excess added Zn shows that higher the metal level, the more base is needed to counteract the release of  $H^+$  by metal cations. Table 3 summarizes the additional release of  $H^+$ , measured in micromoles ( $\mu$ moles) per liter of base, for the metal solutions.

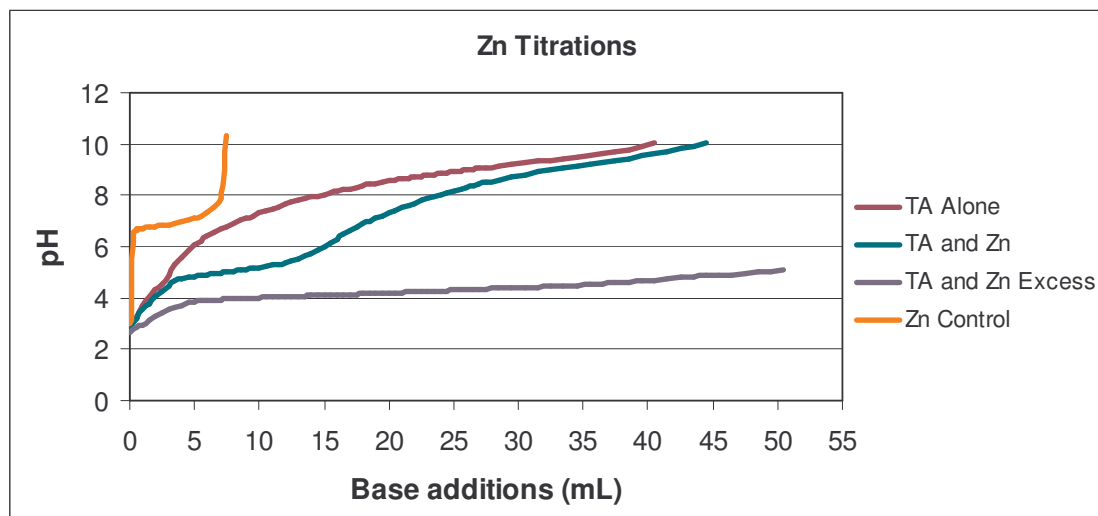


Figure 6: Zn-tannic acid titrations

Table 3:  $\mu\text{moles of H released/liter of base}$

Metal solution	$\mu\text{moles H/liter of base}$		
	pH5	pH6	pH7
Zn Standard	450	1000	1000
Zn Excess	4550	7450	16300
Cd Standard	100	900	900

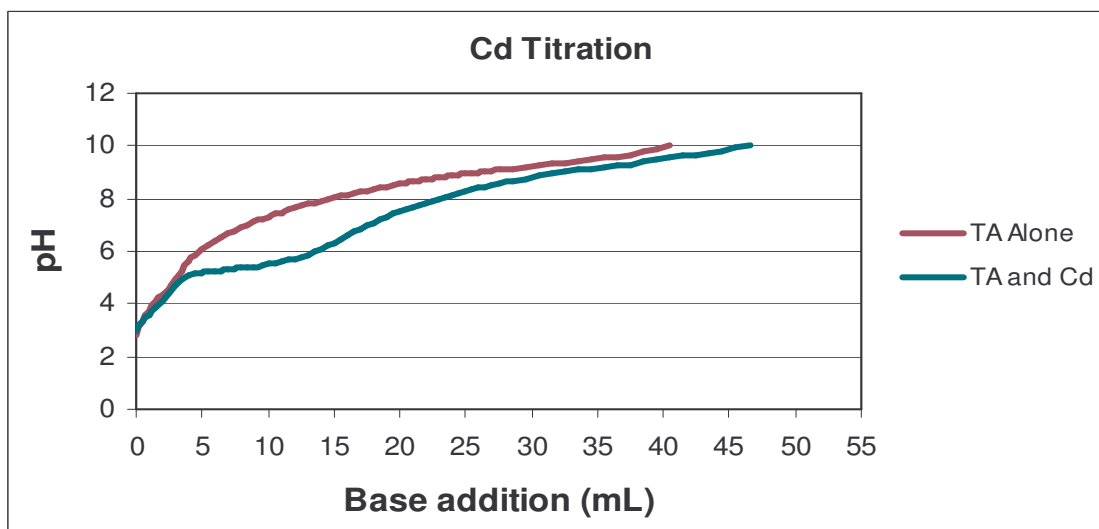
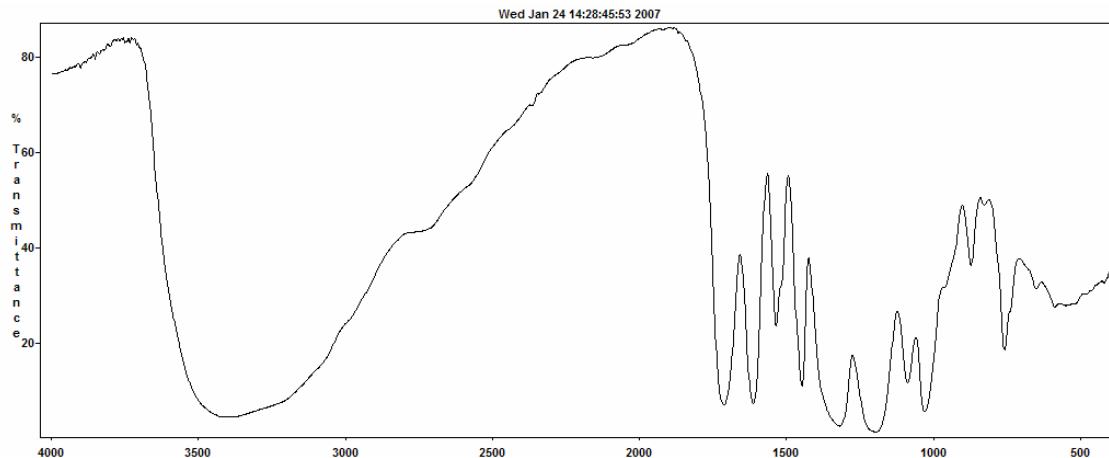


Figure 7: Cd-tannic acid titration

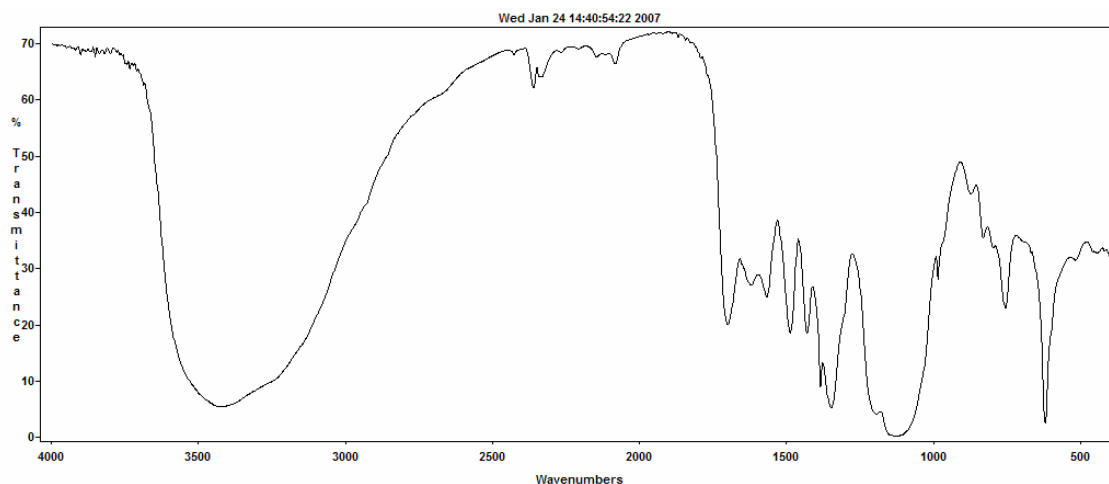
**Infrared Analysis:** Spectra of tannic acid, both uncomplexed and complexed to Zn and Cd at pH 7, were compiled and analyzed (see Figures 8, 9 and 10). In

comparison to the tannic acid spectrum, a somewhat diminished -OH peak (around  $3500\text{ cm}^{-1}$ ) is apparent in the Zn-tannic acid spectra. The same peak is much more dramatically decreased in the Cd-tannic acid spectra. Also, the two strong peaks around  $1300$  and  $1200\text{ cm}^{-1}$  in tannic acid, attributable to phenolic -OH deformation and C-O stretch, are strongly perturbed in the metal-tannic acid spectra. This gives further evidence that tannic acid is binding the metal cations by displacing  $\text{H}^+$  from the phenolic groups. The differences in the -OH peaks between the metal spectra could be due to metal hydroxide formation, as zinc is more likely to form hydroxides than cadmium because of the lower solubility of  $\text{Zn}(\text{OH})_2$ .

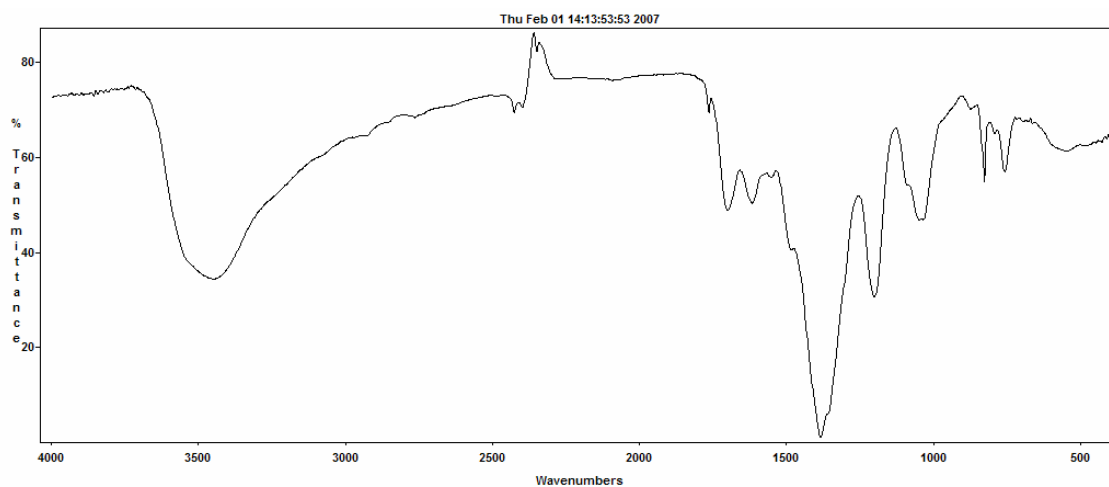
Another peak of interest is carboxylic acid, located at around  $1700\text{ cm}^{-1}$ . It is quite clearly present in the tannic acid spectrum, less pronounced in the Zn-tannic acid spectrum and even more diminished in the Cd-tannic acid spectrum. While tannic acid itself does not have carboxylic acid groups, the commercial tannic acid used in this experiment is comprised of a heterogeneous mixture of compounds that are likely to possess carboxylic acid side groups. Regardless, these results lend credence to the supposition that tannic acid can and does chelate metal species in vitro.



**Figure 8: IR Spectrum of tannic acid. A pronounced -OH peak is visible around  $3500$ .**



**Figure 9: IR Spectrum of Zn complexed with tannic acid.**



**Figure 10: IR Spectrum of Cd complexed with tannic acid.**

### ***Conclusion***

The CHEAQS modeling analysis showed that ligands with catechol (biphenolic) moieties are likely to be strong chelators for Zn and Cd cations over a physiologically relevant pH range, with carboxylate functional groups being less effective. In practice, the titration assay confirmed phenolic affinity for both metal cations. The degree to which  $H^+$  was displaced by both metals was almost identical, with  $Cd^{2+}$  displacing slightly less micromoles of  $H^+$  than  $Zn^{2+}$ . In IR spectral analysis, binding of metal cations to tannic acid was evident through diminishment of peaks for



–OH and carboxylic acid vibrations. It should be noted, however, that CHEAQS predicted complexation of  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  to only become important at pH levels greater than 7 and the titration data and infrared spectra indicated significant metal ion complexation to be occurring at much lower pH levels than this. The discrepancy can be attributed to the ‘cage effect’ of more than two ligands cooperatively binding metal cations in a chelate involving multidentate bonding.

These results point to the potential of tannic acid to serve as a metal chelator and perhaps explain metal tolerance in willow trees. Based on this preliminary analysis, it is reasonable to proceed with plant-based experiments to determine if heavy metal exposure impacts the level of polyphenols in selected willow species.

It is however, important to note that what the above modeling exercises and assays do not directly show is the exact nature of the binding between tannic acid and the metal cations. To elucidate the precise binding configuration as well as the binding ratios, additional experiments would need to be performed. Further theoretical modeling studies could be conducted and their results confirmed with methods that include the use of differential pulse anodic stripping voltammetry (Esparza et al., 2005), UV-vis (Lapouge et al., 2006), Job’s method (continual variation method) (Bukhari et al., 2009) and electrospray mass spectrometry (Fernandez et al., 2002).

## CHAPTER 3

### PHENOLIC ANALYSIS OF WILLOW LEAF TISSUE

#### ***Introduction***

The phytochemistry involved in metal transport and storage varies considerably depending on the metal and plant species involved. For plants with enhanced Zn and Cd tolerance, it was initially postulated that sulfur rich compounds, such as phytochelatins, were responsible for binding and shuttling cations into plant cell vacuoles. It is already known that phytochelatins are responsible for Cd resistance in many plants (Cobbett and Goldsbrough, 2002, Mendoza-Cózatl et al., 2008). However, in the case of hyperaccumulator *Thalpi caerulescens* at least one study indicates that S-containing compounds, such as phytochelatins, are unlikely to serve that role (Kupper et al., 1999).

This might also be the situation with *Salix*. Landberg and Greger (2004) failed to detect phytochelatins when clones of *S. viminalis* were exposed to Zn and Cd. A preliminary analysis for this project also failed to detect phytochelatins in any of the metal-exposed willow plants (data not shown).<sup>3</sup> Considering this, it seems reasonable to investigate the role that other compounds might play in the enhanced metal tolerance found in willow species.

As previously discussed, polyphenols present an interesting case: a broad range of polyphenolic compounds are present in willow trees in more or less species specific arrays. They are an abundant, varied class of compounds that play a role in plant growth and development and function as defensive agents, protecting against infection and injury (Kähkönen et al., 1999). What's more, they are known as

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<sup>3</sup> See Appendix A for further details on the phytochelatin assay.

effective metal chelators- this characteristic having been confirmed in the previous chapter (Hider et al., 2001, Bodini et al., 2001, Esparza et al., 2005).

To be examined here is what impact metal exposure has on polyphenolic levels in willows. Given the diversity within this class of compounds, it might very well be the case that only a certain sub-group contributes to enhanced metal tolerance; accounting for that as part of the experimental design means looking at overall phenolic levels but also assessing changes in different sub-groups. As such, additional analysis will be performed to assess the levels of flavonols, specifically leucoanthocyanins and condensed tannins. These sorts of compounds, such as quercetin, rutin and (+)-catechin, possess the catechol moieties that lend themselves well to bidentate binding.

If polyphenols are part of the mechanism that confers enhanced metal tolerance in *Salix*, the hypothesis is that their levels will rise in response to elevated plant tissue levels of Zn and Cd. Should this hypothesis be confirmed, it could serve as a basis for further investigation into how exactly polyphenols prevent the harmful effects of otherwise toxic metals levels.

## ***Materials and methods***

### **Plant material**

Three species of willow- *S. alba* (herein 'Pseudo'), *S. purpurea* (herein 'Hotel') and the hybrid species S-301(*S. eriocephala* x *S. exigua*) (herein '301') - were chosen based on a preliminary screening of willow cultivars for Zn tolerance and potential for Zn phytoaccumulation. A total of 20 cuttings were taken and rooted in the lab for two weeks. They were then transferred to a hydroponic set up in a climate-controlled greenhouse. Plants were potted 1-3 plants per square plastic pot in a non-soil medium (perlite) and then placed in one of three troughs. Each trough received a different solution continuously fed from large plastic containers via OK-PVC tubing and an

electric pump. This set up was based on the home-made version of the multiple reservoir method as detailed by Watson et al. (2003).

### **Treatments**

Plants were divided into three groups- control, half-metal treatment and full-metal treatment. Metal concentrations in the growing medium for exposed groups were based on work done by Watson et al. (2003). The treatment solutions were as follows:

Control-  $\frac{1}{4}$  strength Hoagland's nutrient solution;

Half-metal- 100  $\mu$ moles Zn and 5  $\mu$ moles Cd and  $\frac{1}{4}$  strength Hoagland's nutrient solution; and

Full metal- 200  $\mu$ moles Zn and 10  $\mu$ moles Cd and  $\frac{1}{4}$  strength Hoagland's nutrient solution.

These solutions were kept in large plastic containers and replenished on an as-needed basis. Metal treatment commenced 7 days after the initial plant set up and continued for a period of 14 days. After that time, plant tissue was harvested and analyzed. The harvesting consisted of cutting the shoots from each plant and allowing them to air dry in the lab. Leaves were then manually removed from stems and prepared for further analysis.

### **Leaf metal extraction and quantification**

An acid extraction method was used to release metals from fresh leaf tissue into solution. Preliminary studies had shown that this method extracts around 70% and 85% of Zn and Cd in the willow leaves, respectively. These extraction efficiencies are assumed here in the estimations of tissue metal concentrations.

Approximately 0.25 grams of freshly ground tissue was placed into an Erlenmeyer flask with 50 mL of 1M HNO<sub>3</sub>, covered and agitated for one hour. The solution was then processed through filter paper, Whatman No. 42, and analyzed for metal content with AAS.

The AAS equipment used was a Buck Scientific 210AA Model 210VCG Atomic Absorption Spectrophotometer with acetylene flame. Zn and Cd standards at concentrations of 0.10, 0.25, 0.50 and 1 ppm provided a standard curve for comparison. The matrix of the standards was matched with that of the extract solutions to avoid matrix errors as well as over or underestimation of metal levels.

The analysis for Zn was performed by taking 1 mL of the extract solution and diluting to 5 mL with 1M HNO<sub>3</sub> so that the metal levels were in the range of standard solutions used to calibrate the AAS. For the Cd analysis, this dilution step was not required. Solutions analyzed for Zn content had an overall dilution factor of 1000, while solutions analyzed for Cd content had a dilution factor of 200.

#### **Phenol extraction and determination**

To extract phenolic constituents, dried leaf tissue was mixed with pure methanol at a 1:20 ratio in an Erlenmeyer flask, covered and agitated for one hour at room temperature. After extraction, the solution was processed through filter paper, Whatman No. 42, and analyzed with a spectrophotometer after performing several colorimetric assays. The spectrophotometer used was a Perkin-Elmer Hitachi 200.

***Total Phenolic Determination:*** Gross polyphenol levels were determined using the Prussian Blue assay developed by Price and Butler (1977). The principle behind this assay is the reduction of ferric iron to ferrous iron by phenolics that results in a ferricyanide-ferrous color complex known as Prussian Blue. For this assay, 1 mL samples of plant extract were pipetted into plastic test tubes, after which 2 mL of 0.008 M FeCl<sub>3</sub> in 0.008 N HCl and 10 mL of 0.0015 M K<sub>3</sub>Fe(CN)<sub>6</sub> were added. Absorbance at 720 nm was read on the spectrophotometer 30 seconds after adding the final reagent. Each plant sample was analyzed in triplicate with an average taken of the readings in the final analysis. The background (blank) determination was made by preparing a NaCl solution sample in the same manner and subtracting the absorbance

from the sample readings. The standard curve was prepared by adding the reagents to 1, 0.10, 0.05 and 0.01 mM solutions of tannic acid. Results are reported in tannic acid equivalents (mmoles) per ml of leaf extract.

***Condensed Tannin Determination:*** The procedure for condensed tannins measurements followed the method outlined by Julkunen-Tiitto (1985). For this assay, 50-250  $\mu$ L samples of plant extract were pipetted into plastic test tubes wrapped in aluminum foil. Three milliliters of the reagent, 4% vanillin (w/v) in methanol (Broadhurst and Jones, 1978) was added to each tube and then shaken for about 10 seconds. Then 1.5 mL of concentrated HCl was added and the tubes were shaken once more. Samples were allowed to stand for 20 minutes at room temperature, after which absorbance readings were taken at 500 nm. Each plant tissue was analyzed in triplicate and the average of the readings reported in the final analysis.<sup>4</sup> A background (blank) determination was made by preparing a sample without the vanillin and subtracting the absorbance from the sample readings. The standard curve was prepared by adding the reagents to 1, 0.10, 0.05 and 0.01 mM solutions of (+)-catechin, and measuring the absorbance by the same method. Results are reported in catechin equivalents (mmoles) per ml of leaf extract.

***Leucoanthocyanin Determination:*** The procedure for leucoanthocyanin measurements followed the method outlined by Julkunen-Tiitto (1985). For this assay, 100  $\mu$ L samples of plant extract were pipetted into glass test tubes and 95:5 butanol:concentrated HCl (Bate-Smith, 1981) was added to bring the volume up to 4 mL. The samples were shaken for 2 hours at 95-98°C to allow for hydrolysis, cooled in the dark to room temperature and adjusted back to 4 mL with butanol-concentrated HCl. Absorbance readings were taken at 550 nm. Each plant tissue was analyzed in

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<sup>4</sup> Except in the case of Hotel plants 3 and 20. Due to a shortage of plant material, the assay for plant 3 was only run once and for plant 20 it was run twice.

triplicate and the average of the readings was reported in the final analysis.<sup>5</sup> The background (blank) determination was made by preparing a sample with the reagent but not hydrolyzing it, and measuring absorbance in the same manner. The standard curve was prepared using 1, 0.10, 0.05 and 0.01 mM solutions of cyanidin in place of tissue extracts. Results are reported in cyanidin equivalents (mmoles) per ml of leaf extract .

### ***Results and analysis***

#### **Overall plant health and biomass**

A total of 20 plants from three willow species were included in the willow hydroponic experiment, with at least one plant from each species in each treatment group (see Table 4). After two weeks of Zn and Cd exposure, most plants were relatively healthy. Biomass production was not impaired by metal exposure in 301 or Hotel but was in Pseudo. Of all the plant species, Hotel was the lowest biomass producer in each group. Also worth noting is that in the full metal treatment, all 3 species exhibited signs of metal stress. Yellow coloration was visible on leaves of each plant, presumably evidence of chlorosis.

#### **Metal accumulation**

Before examining the impact that metal exposure might have on phenol levels, leaf metal accumulation levels were determined. This was done using an acid extraction to remove metals from ground leaf tissue and then analyzing the solutions by AAS.

Unsurprisingly, the higher metal concentrations plants were exposed to, the greater their concentrations were in plant tissue. Plant tissue metal concentrations varied both within treatment groups and between individual species; no one plant species consistently accumulated high metal concentrations in comparison to others.

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<sup>5</sup> Except in the case of Hotel plants 3 and 20. Due to a shortage of plant material, the assay for plant 3 was only run once and for plant 20 it was run twice.

Hotel was the best accumulator in the half metal treatment but a 301 plant in the full metal treatment had the highest metal levels in the experiment (see Table 5).

On the individual plant level, the greatest intra-species variation for plants in the same treatment occurred amongst Pseudo plants 7 and 8. As Table 6 shows, the metal levels for these plants differed by 34% for zinc and 71% for cadmium. No explanation for this is readily available as these genetically identical plants were exposed to the same metal solution under the same conditions. However, variations such as these with willow plants are not uncommon as previously explained (see Chapter 1). It is beyond this scope of this investigation to speculate the reasons as to why this may be so.

**Table 4: Leaf biomass by plant and treatment group**

Treatment group	Species-Plant No.	Leaf biomass (g)
Control	301-1	8.4
	301-2	7.41
	Hotel-3	3.55
	Pseudo-4	18.9
	Pseudo-5	16.85
	Pseudo-18	6.96
Half	301-17	6.2
	301-19	6.92
	Pseudo-15	26.7
	Pseudo-16	17.2
	Hotel-20	4.8
	301-11	8.72
Full	301-12	11.64
	301-13	4.75
	Pseudo-6	5.94
	Pseudo-7	6.71
	Pseudo-8	9.28
	Pseudo-9	1.73
	Pseudo-10	4.5
	Hotel-14	5.62



**Table 5: Average metal accumulation (ppm)**

Treatment group	Species	Zn conc.	Cd conc.
<b>Control</b>	301	28.4	bd*
	Hotel	4.05	bd
	Pseudo	25.7	bd
<b>Half</b>	301	134	66.4
	Hotel	235	84.2
	Pseudo	177	60.7
<b>Full</b>	301	612	138
	Hotel	365	104
	Pseudo	507	129

\*bd= below AA detection limit

**Table 6: Individual plant metal levels- Pseudo**

Treatment	Species	Zn Conc.	Cd Conc.
<b>Full</b>	<b>Psuedo-7</b>	<b>361</b>	<b>52.8</b>
	<b>Pseudo-8</b>	<b>544</b>	<b>185</b>

### Plant phenolic levels

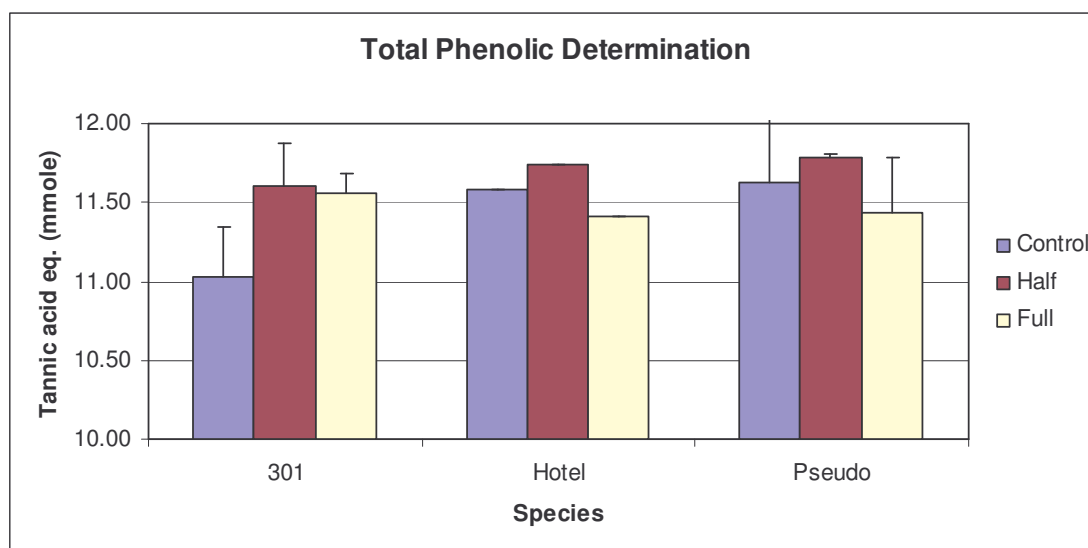
The assays performed in this investigation offer a “snapshot” of the polyphenolic levels for each plant at the time of harvest. In doing so, they provide evidence for whether or not metal exposure and uptake in willows yields any change in the amounts of these compounds in plant leaf tissue.

The assay assessing total phenolics, which broadly takes account of oxidizable substrates, shows virtually no difference between plant species and treatments. A statistical analysis of the results confirms that the treatment and species differences were not significant (For 301: ANOVA, df=6, F-value=4,209, p=0.104; For Pseudo: ANOVA, df=9, F-value= 0.494, p=0.630).<sup>6</sup> However, given that there are a great many phenolic constituents in willow; changes in the levels of specific types of polyphenols could easily be masked by a non-specific evaluation of total phenols such as this.

<sup>6</sup> A statistical analysis for Hotel was not performed as the n for the control and each treatment was 1.

It is for that reason that two additional colorimetric tests were performed. Both sought to measure a sub-group of polyphenols called flavanoids. The first assay, condensed tannin determination, quantified the levels of (+)-catechin-like polyphenols while the second assay, leucoanthocyanin determination, focused on levels of cyanidin-like compounds. Results are presented below.

**Condensed Tannin Determination.** The results of this assay show no discernable trend across treatments. For 301, cultivar levels of condensed tannins were lower in the half and full metal treatments in comparison to the control group.



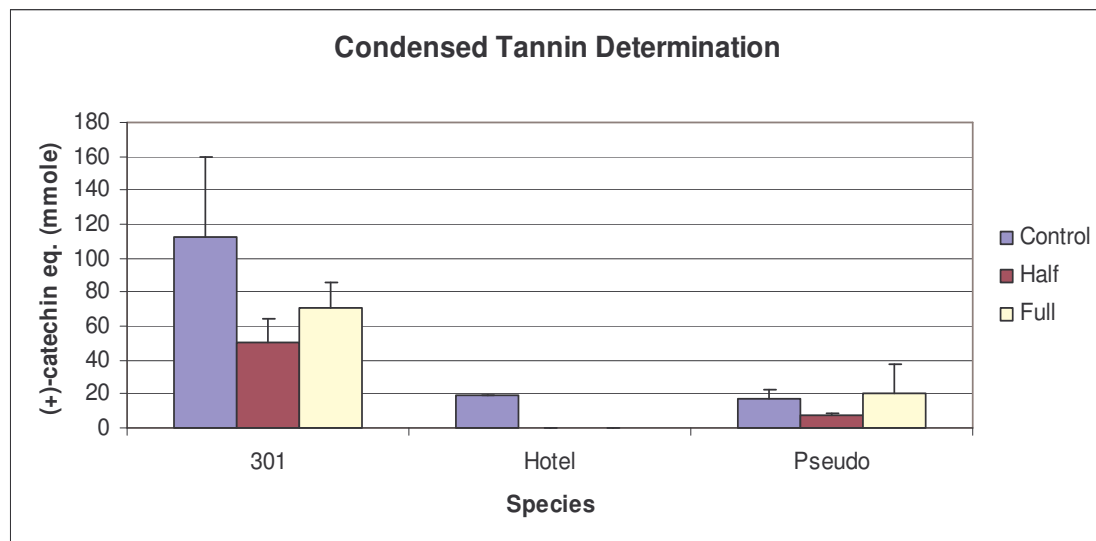
**Figure 11: Average levels of phenols in plant extracts (mmoles/mL) for all metal treatments (control, half and full metal) and *Salix* cultivars.**

However, interpreting these results in light of the levels found in 301 control plants should be done with caution. Although the average levels in the control are higher than in either the half or full metal treatments, this difference may not in fact be significant.

There was a wide variation in levels of condensed tannins for the two 301 plants in the control treatment. Plant 301-1 registered the highest levels of (+)-catechin

equivalents, 146 mmole, of any plant in the experiment. Plant 302-2 had a substantially lower level of 79.5 mmole. While experimental error might be a factor here, it is interesting to note that plant 301-1 also contained much higher levels of foliar Zn, Cd and leucoanthocyanins than its control group counterpart (as detailed below). The reason for substantial differences between the replicate plants 301-1 and 301-2 is unclear, but the variability necessitates careful interpretation of data in light of this fact.

Amongst Pseudo plants, the results for condensed tannins are similar to those found for 301. Condensed tannins decline by about 53% in the half metal treatment but are actually slightly higher than control levels in the full metal treatment. With Hotel, the scenario is different. Condensed tannin levels dropping from an average of 18.77 moles to almost zero in the half metal treatment, a fall in levels of 99%. They levels are much the same in the full metal treatment.



**Figure 12: Average level of (+)-catechin equivalents per mL of willow foliar extract**

For the Pseudo cultivar, the pattern for condensed tannins in response to metals is similar to those found for 301. Condensed tannins decline by about 53% in the half

metal treatment but are actually slightly higher than control levels in the full metal treatment. For Hotel, the pattern is different, with condensed tannin levels dropping from an average of 18.8 mmoles to almost zero in the half and full metal treatment, a decrease of about 99%.

**Table 7: Average levels of condensed tannins (mmoles)/mL**

Treatment	Species	Average
Control	301	112.76
	Pseudo	17.06
	Hotel	18.77
Half	301	50.71
	Pseudo	7.95
	Hotel	0.32
Full	301	71.19
	Pseudo	20.09
	Hotel	0.14

From these initial results, no uniform trend for the different cultivars emerges to show if zinc and cadmium exposure tends to change condensed tannin production. For the Hotel cultivar, it does seem reasonable to conclude that the heavy metal exposure suppressed levels of condensed tannins. However, the same conclusion cannot necessarily be drawn for 301 or Pseudo. In the case of 301, the situation is confounded by the seemingly aberrant performance of 301-1. For both cultivars, it could be that lower metal levels suppress (+)-catechin-like polyphenols through some sort of mechanism that is not triggered at higher metal exposure levels. This would explain why there appears to be a drop in condensed tannins at the half but not the full metal treatment. Nevertheless, the differences seen here could also be due merely to the wide variation in biological responses found among willow species, cultivars and individual plants in many experiments conducted previously (see Chapter 1).

A statistical analysis of this assay's results shows that the variations in condensed tannin levels for each species at the half and full metal treatments are not significant (For 301: ANOVA, df=5, F-value=2.299, p=0.248; For Pseudo: ANOVA, df=9, F-value=0.620, p=0.565). But given the small sample size, additional testing on a larger number of plants over a longer time frame is warranted in order to better determine if metal exposure does in fact elicit any changes in condensed tannin levels.

***Leucoanthocyanin determination.*** As with the condensed tannins analysis, there is no discernable trend across metal treatments for leucoanthocyanins, as shown in Table 8 and Figure 13. For 301, plants in the half and full metal treatments saw respective declines in their levels of cyanidin-like compounds of 43% and 31%. In the case of Pseudo, levels of cyanidin-like compounds started low, around 3 mmoles, and stayed low. Levels in the half metal treatment were about the same as in the control, and lower in the full metal treatment.

**Table 8: Average levels of leucoanthocyanins (mmoles)/mL of plant leaf extract**

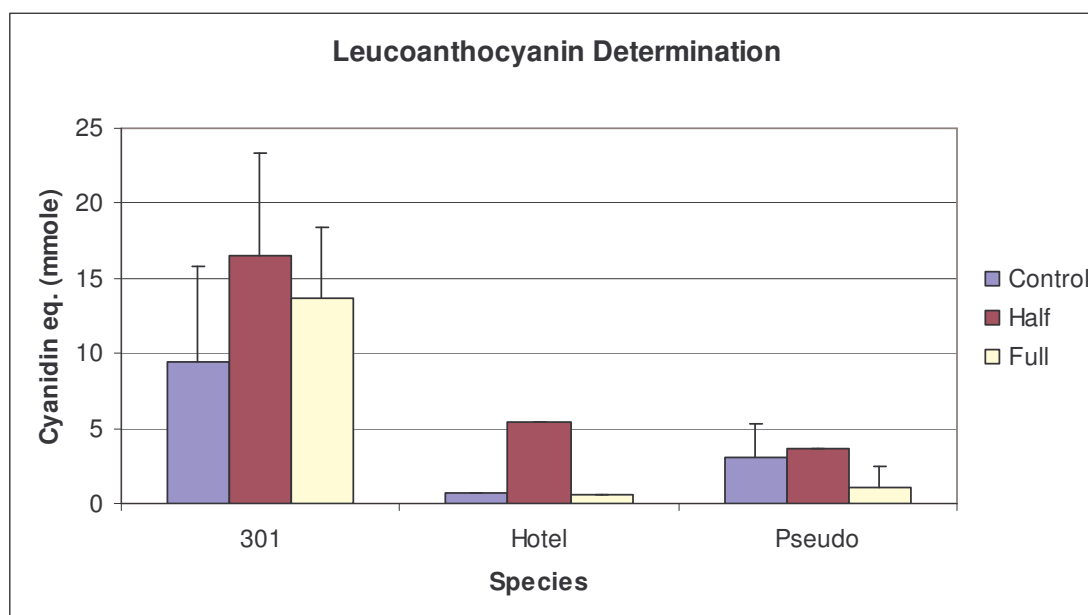
Treatment	Species	Average
Control	301	9.39
	Pseudo	3.11
	Hotel	0.72
Half	301	16.6
	Pseudo	3.61
	Hotel	5.39
Full	301	13.7
	Pseudo	1.08
	Hotel	0.54

For Hotel, the lack of replicates may have given the appearance of a large increase in leucoanthocyanins levels in the half metal treatment. The assay was performed in duplicate on the one Hotel plant in the half metal treatment, but the values obtained for the

two readings were very different. The first yielded an absorbance of 0.124, which translates into a cyanidin equivalent of 0.00 mmols. The second yielded an absorbance of 0.81, which equals a cyanidin equivalent value of 10.8 mmols. This suggests that there was an error in the assay, and one of these two readings is invalid. A lack of sufficient leaf tissue prevented retesting.

As with the other assays, the differences among the metal treatments are not significant. (For 301: ANOVA,  $df=6$ ,  $F\text{-value}=0.785$ ,  $p=0.516$ ; For Pseudo: ANOVA,  $df=9$ ,  $F\text{-value}=2.511$ ,  $p=0.151$ ).<sup>7</sup> But again, given the small sample size, additional testing is prudent before making definitive conclusions.

**Individual Plants.** Given the wide variation found in individual willow plants, aggregate analyses, such as the ones performed above, risk overlooking trends occurring at the plant specific level. A review of the data at the individual plant level indicates that this is not the case here (see Appendix B). There is no apparent correlation between metal and phenolic levels for individual willow plants.



**Figure 13: Average level of leucoanthocyanin equivalents per mL of willow foliar extract**

<sup>7</sup> A statistical analysis for Hotel could not be performed as the  $n$  for the control and treatments was 1.

## ***Conclusion***

The hypothesis being tested here is whether or not metal exposure elicits a change in polyphenol levels. The results from this initial round of experiments are inconclusive. Statistical analysis showed no significant change in these polyphenols in response to higher tissue Cd and Zn. It is possible that small sample sizes in combination with the considerable variations known to occur both between and within species obscured metal-induced changes in phenolic levels. Therefore, further investigation is warranted. A larger sample size of plants should be exposed to metals for longer periods of time with their metal and phenol status assessed over time, rather than at a single time point. Moreover, assays should be employed to assess the levels of other classes of phenolics such as salicylates and hydrolyzable tannins.

Should another round of experiments yield results similar to the ones obtained in this investigation, it would not mean that polyphenols are not part of the metal tolerance mechanism in *Salix*. It could very well be that the already high levels of phenolics found under natural conditions in willow plant tissue form a ready pool of defensive agents, and that their detoxification role does not require further augmentation. It should also be pointed out that willows are not hyperaccumulators, and toxic effects of Zn are seen at foliar concentrations around 1000 mg/kg.

Pursuing additional avenues of investigation aside from phenolic quantification would also be sensible. Size exclusion chromatography techniques coupled with UV-VIS or other detectors afford the opportunity to characterize metal species and shed light on which compounds are serving as ligands (Weber and Koniecznyński, 2003). *In situ* techniques, such as X-ray absorption spectroscopy, would provide information on the coordination chemistry of metal complexes (Gardea-Torresdey et al., 2004). These techniques in combination could go a long way to solving the mystery of which plant constituents are behind enhanced metal tolerance.

In summary, while this examination did not yield conclusive about the role of polyphenols in metal detoxification, results it provides a solid foundation for further investigation. The next steps briefly outlined above will determine if polyphenols involved in metal tolerance. If they are ruled out, the focus can shift to other compounds, such as organic acids. However, if it is shown that polyphenols do figure into the process, then science will be one step closer to sorting out the metal tolerance mechanism in *Salix*.



## APPENDIX A

### PHYTOCHELATIN DETERMINATION

A modified Bradford assay with subsequent HPLC and UV-VIS analysis was run on several samples of fresh willow tissue to determine if metal exposure in willows triggered the production of phytochelatin. The results of the assay showed no phytochelatin in any of the plant samples examined, regardless of the treatment group. While this could lend further evidence that phytochelatin is not involved in metal tolerance in *Salix*, these results should only be considered preliminary, as an adequate control was not used to confirm the robustness of the experimental design. A plant such as *Arabidopsis* would be sufficient in this respect as it is known to synthesize phytochelatin.

Detailed below are the steps used to perform the initial analysis. In order to confirm the results obtained from this assay, the experiment should be re-run with a proper control to verify the results and confirm that Zn and Cd exposure does not lead to phytochelatin synthesis in the species examined. Additionally, running this assay at different time points once metal exposure has commenced would further substantiate that this class of compounds is not involved.

#### **Protein extraction and analysis**

The procedure for protein extraction used is based on the method outlined by Jones et al. (1989). For protein extraction, 100 mg of fresh leaf tissue in liquid nitrogen is ground with a mortar and pestle. The N<sub>2</sub> is allowed to evaporate and then a ratio 1 mL of 0.1 N NaOH per 10 to 100 mg of leaf tissue is added and mixed with a mortar and pestle. Only about 25% of the initial volume of NaOH is used for grinding with the remaining volume saved to wash the plant material into a centrifuge tube. Once in the

centrifuge tube, samples are agitated for 3 seconds on a vortex mixer and left to extract for 30 minutes at room temperature.

Samples are then agitated for another 3 seconds and centrifuged for 5 minute at high speed (>5000g) on a bench centrifuge. The supernatant is decanted and agitated for 3 seconds more. Then 5  $\mu\text{L}$  of the extraction is mixed with 795  $\mu\text{L}$  of 0.1N NaOH. Added to that is 200  $\mu\text{L}$  of the 1:4 diluted Bradford dye reagent, prepared according to the Bio-Rad Laboratories Manual (Bio-Rad Laboratories, 1985), and modified with 3 mg/mL soluble polyvinylpyrrolidone (PVP) (MW ~ 40,000). Samples are agitated and left to sit for 5 minutes, after which time they are ready for HPLC and UV-VIS analysis.

**HPLC analysis:** An aliquot of each sample is taken and placed in centrifuge tubes. To that, 20  $\mu\text{L}$  of 50% 5-sulfosalicylic acid is added to precipitate out proteins and leave in solution only those non-protein peptides, among which are low molecular weight thiols such as phytochelatins. Varying amounts of 0.1 N NaOH are also added so that final volumes of each sample are 150  $\mu\text{L}$ . Samples are iced for 5 minutes and then centrifuged for 10 minutes at 13.2 rpm at 4°C. The supernatant is transferred to a clean centrifuge tube prior to injection into the C18 RP-HPLC column (Econosphere C18, 150 x 4.6 mm reverse phase column (Alltech)) for analysis. Acetonitrile with 0.05% phosphoric acid is used to cleanse the column between each analysis.

**UV-VIS analysis:** The HPLC analysis deposits a fractioned solution into 20 separate HPLC tubes. The fractions are collected and 500  $\mu\text{L}$  of 0.8 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 250 mM potassium phosphate buffer, pH 7.6 is added to each tube. After five minutes, readings are taken at 412 nm. The background determination is made by taking a sample of 800  $\mu\text{L}$  0.1 N NaOH, adding 200  $\mu\text{L}$  Bradford dye reagent with PVP and measuring the absorbance. A calibration curve is prepared by using bovine serum albumin (BSA) as the standard

protein. The BSA is prepared at a concentration of 1  $\mu\text{g}/\mu\text{L}$  and 2, 4, 8 and 10  $\mu\text{L}$  solutions are prepared. To each, 200  $\mu\text{L}$  of the Bradford dye reagent are added along with 0.1 N NaOH so that the final volume for each sample is 1000  $\mu\text{L}$ . To that the DTNB reagent is added and readings are taken after five minutes.

***Preparation of 250 mM potassium phosphate buffer, pH 7.6:*** Dissolve 34 grams of  $\text{KH}_2\text{PO}_4$  (potassium phosphate monobasic, MW 136.09) in 1000 mL of de-ionized water. Adjust the pH with  $\text{K}_2\text{HPO}_4$  (potassium phosphate dibasic anhydrous, MW 174.18).

## APPENDIX B

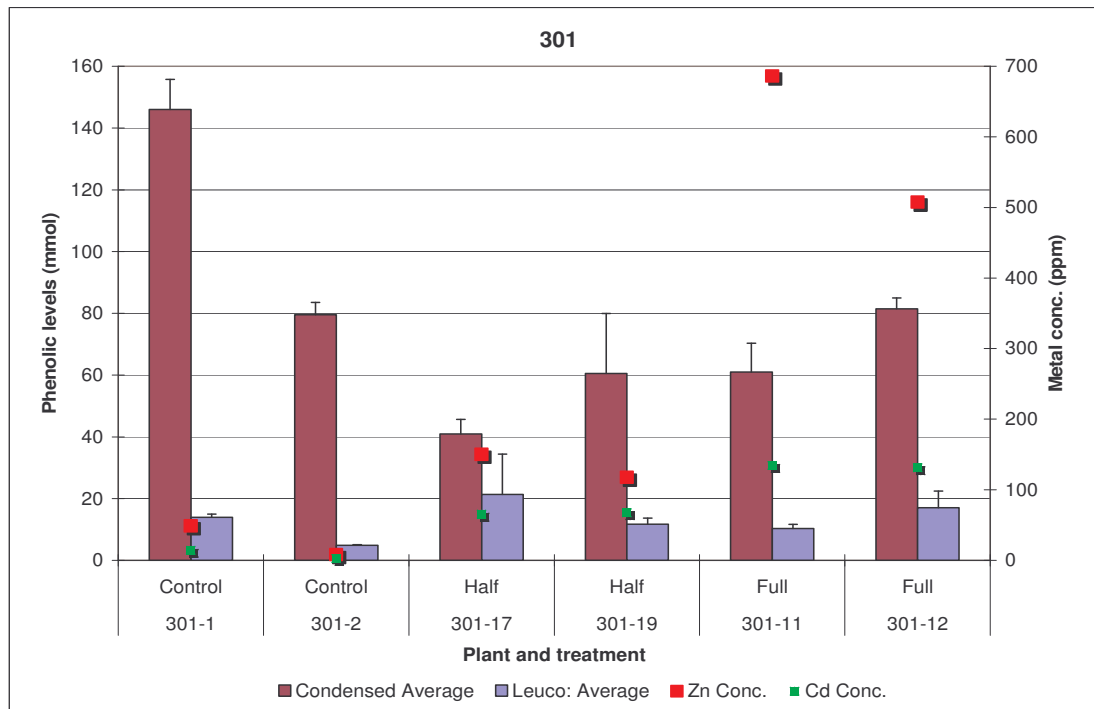
### INDIVIDUAL PLANT DATA

This appendix provides a summary of the experimental results obtained for individual plants. Data from the metal extractions as well as the condensed tannin and leucoanthocyanins assays has been provided. As these results show, there is no apparent correlation between plant metal and phenolic levels.

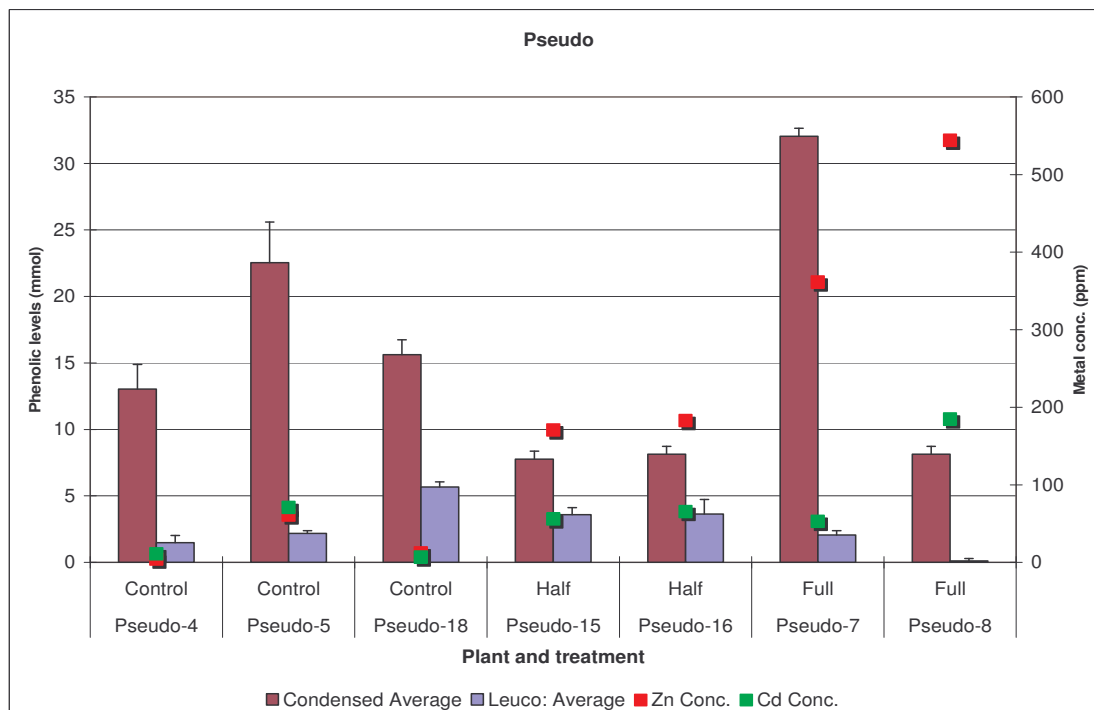
**Table 9: Individual plant metal, condensed tannin and leucoanthocyanins levels**

Treat- ment	Species- Plant No.	Condensed Tannin Average	St Dev	Leucoantho -cyanin Average	St Dev	Zn Conc.	Cd Conc.
Control	301-1	145.99	9.722	13.92	1.11	48.71	bd*
	301-2	79.53	3.99	4.85	0.244	8.11	bd
	Hotel-3	18.77	0	0.72	0	4.05	19.35
	Pseudo-4	13.03	1.85	1.48	0.546	4.05	10.98
	Pseudo-5	22.54	3.05	2.17	0.214	60.89	70.63
	Pseudo-18	15.62	1.12	5.68	0.376	12.17	6.79
Half	Pseudo-15	7.76	0.61	3.58	0.537	170.52	55.98
	Pseudo-16	8.14	0.58	3.64	1.09	182.7	65.4
	301-17	40.92	4.78	21.38	12.98	150.22	65.4
	301-19	60.49	19.47	11.71	1.97	117.74	67.49
	Hotel-20	0.32	0.31	5.39	7.63	235.48	84.23
Full	Pseudo-7	32.04	0.605	2.06	0.317	361.35	52.84
	Pseudo-8	8.141	0.583	0.109	0.189	544.05	184.7
	301-11	60.966	9.364	10.33	1.32	686.16	134.47
	301-12	81.414	3.577	17.03	5.4	507.51	131.33
	Hotel-14	0.138	0.782	0.54	0.725	365.41	104.12

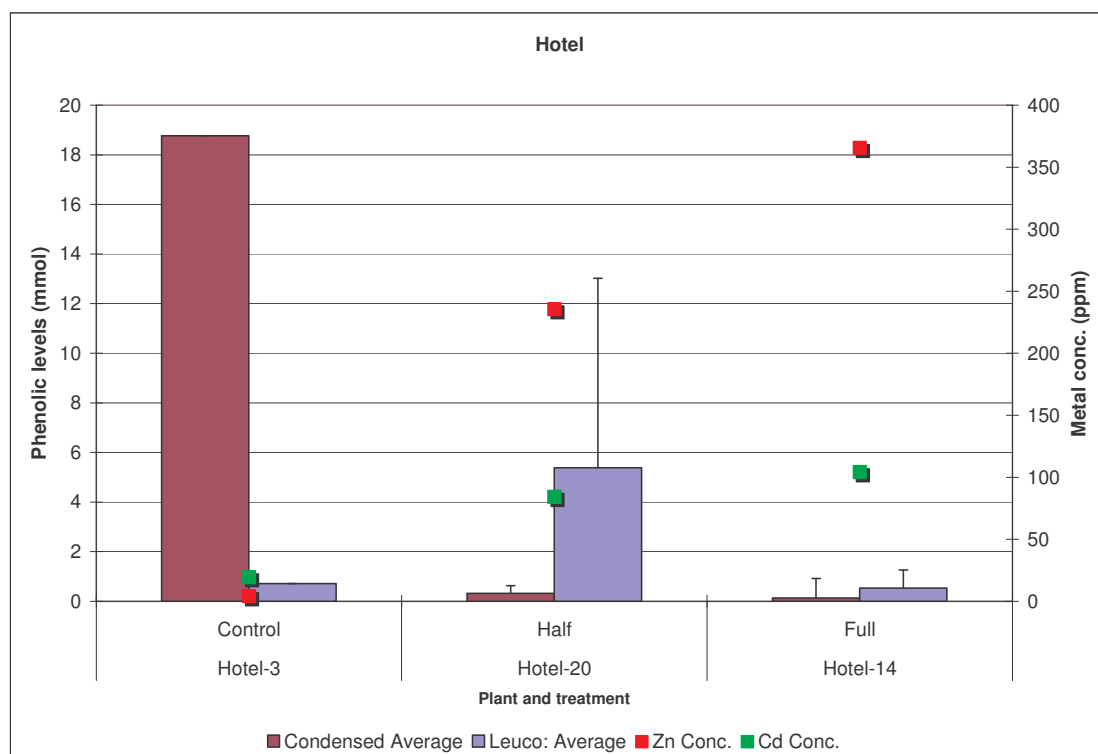
\*bd= below AA detection limit.



**Figure 14: Individual plant data for 301. Levels of Zn, Cd, condensed tannins and leucoanthocyanins in individual 301 plants across treatment groups**



**Figure 15: Individual plant data for Pseudo. Levels of Zn, Cd, condensed tannins and leucoanthocyanins in individual Pseudo plants across treatment groups**



**Figure 16: Individual plant data for Hotel. Levels of Zn, Cd, condensed tannins and leucoanthocyanins in individual Hotel plants across treatment groups**

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