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Tolerance and Accumulation of Copper, Lead and Zinc by Ceratopteris richardii

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TOLERANCE AND ACCUMULATION OF COPPER, LEAD AND ZINC BY CERATOPTERIS RICHARDII

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Tolerance and Accumulation of Copper, Lead and Zinc by *Ceratopteris richardii*

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Abstract

Environmental contamination has increased rapidly in recent years. There is a need to develop cheap and environmentally friendly remediation methods. Numerous studies have evaluated the ability plants to remove contaminants from the environment. The present study evaluated the ability of *Ceratopteris richardii* gametophytes and sporophytes to tolerate copper, lead and zinc and for the sporophytes to accumulate the three metals in their shoots. Spores were sown in plant medium treated with varying concentrations of Cu, Pb and Zn. Fifteen-day-old sporophytes were transferred into medium mixed with varying concentrations of the three metals. *Ceratopteris richardii* tolerated and accumulated each of the three metals differently. Pb was tolerated at higher levels than the other two in both the gametophytes and sporophytes but was accumulated the least. Cu was the least tolerated in both gametophytes and sporophytes but was accumulated at significant levels. *Ceratopteris richardii* tolerated Zn at moderate levels in both gametophytes and sporophytes but was accumulated at higher levels than Cu and Pb by the sporophytes.

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Dedication

To my late father Joseph Cheruiyot Rotich, whose love and dedication comforts and inspires me everyday and everywhere I go. This thesis would not be here if it was not for his confidence in me.

Introduction

Contamination with heavy metals in the environment has increased alarmingly in recent years. This increase could be caused by several factors including population, urbanization, agriculture and industrialization. Elevated levels of heavy metals in the environment can be toxic to both animals and plants, depending on the levels of the heavy metals and tolerance levels of the individual plant or animal. However, the methods to control heavy metals or remediate already contaminated sites are not being developed at the same rate as the contamination (Cunningham *et al.* 1996).

Aquatic and terrestrial environments are both directly and indirectly affected by heavy metal p ollutants. Once deposited on terrestrial or aquatic environments, heavy metals can be transported into streams, rivers, lakes and other water reservoirs via surface runoff, infiltration or as aerosols. Over time, heavy metals in rivers, streams and other aquatic systems can eventually settle in the sediments within the system (Milenkovic *et al.* 2005).

Cleaning up a contaminated site can be extremely costly and damaging to an ecosystem. The cost and negative impacts that can be caused by present methods of remediation have prompted the need for cheaper and more environmentally friendly methods, such as bioremediation and phytoremediation. The cost for phytoremediation per acre of soil at 50 cm in depth ranges from

$60,000 to $100,000, compared to $400,000 to remediate a contaminated site by conventional engineering techniques (Zynda 2001).

For the above reasons, phytoremediation has received a lot of attention recently from researchers, politicians and economists all over the world (Cunningham *et al.* 1996). Phytoremediation uses any of the following processes, individually or in combination, to remove contaminants from the environment: phytoextraction, phytotransformation, phytodegradation and rhizofiltration. In phytoextraction, the contaminants are accumulated in the plants' biomass. Phytotransformation and phytodegradation both involve the uptake, storage and degradation of the contaminants. In rhizofiltration, the contaminants are absorbed from the soil and accumulated in the plant roots (Peuke and Rennenberg 2005).

Numerous studies have indicated that some plants can accumulate elevated levels of metals from soil or water in their roots, stems or leaves (Ali *et al.* 2003). These plants seem to not only tolerate the elevated levels of heavy metals but also to accumulate them in their tissues. For example, in Lusaka, Zambia, researchers found that the grass *Streochlaena cameronii* can tolerate high levels of Cu, Pb and Zn and accumulate the metals in the roots and above-ground tissues (Reilly and Reilly 1973). However, a study on the accumulation of cadmium, chromium, copper, nickel, and zinc by the water fern *Azolla filiculoides* indicated that there is a difference in the amount of heavy metals accumulated in the shoot and the roots depending on the mobility of the metal ions. Specifically,

there were higher concentrations of Cr, Cu and Ni than Cd and Zn in the roots (Sela *et al.* 1989).

A plant's status as accumulator or hyperaccumulator depends on the concentration levels of metal that can accumulate in their above-ground tissue, but minimum concentrations for each status differ between metals (Table 1). Plants that can accumulate metals to levels that exceed the amounts needed for normal nutrition are considered accumulators. Hyperaccumulator plants have unusually high concentrations of metals in their tissues (Reeves and Baker 2000).

Table 1. Minimum values for status as normal, accumulator and hyperaccumulator for 8 metals commonly accumulated by plants. Levels are expressed as ppm (µg/g) of dry mass of plant tissue (Reeves and Baker 2000).

Metal	Level Accumulated (ppm)		
	Normal	Accumulator	Hyperaccumulator
Cd	0.1 to 3	20	100
Co	0.03 to 2	20	100
Cr	0.2 to 5	50	100
Cu	5 to 25	100	1000
Mn	20 to 400	2000	10000
Ni	1 to 10	100	1000
Pb	0.1 to 5	100	1000
Zn	20 to 400	2000	10000

Accumulation of heavy metals can have negative effects on plant growth, development and reproduction. Therefore, accumulators and hyperaccumulators

have developed mechanisms to protect themselves from the toxicity of heavy metal ions. These mechanisms include antioxidant enzymes and other antioxidant substances (Ali *et al.* 2003), phytochelation and compartmentalization of absorbed metals (Cobbet 2000, Jonak *et al.* 2004).

Aquatic and semi-aquatic plants, such as the water fern and water hyacinth, have shown a higher tolerance for and accumulation of heavy metals compared to terrestrial plants. The amounts of Cd, Cu, Ni, and Zn that were accumulated by *A. filiculoides* were higher than those accumulated by soybean, maize, wheat, oat, and rice (Sela *et al.* 1989). The water hyacinth *Eichhornia crassipes*, can accumulate up to 500 parts per million of Cd, Pb and Hg. These results suggest that aquatic plants might be more effective in phytoremediation than terrestrial plants (Prasad and Freitas 2003, Sela *et al.* 1989).

My research assessed the utility of the fern *Ceratopteris richardii* (*C-richardii*) for phytoremediation of heavy metal-contaminated habitats. *Ceratopteris richardii* is a homosporous fern that is adapted to aquatic and semi-aquatic environments such as rivers, wetlands, marshes, ponds and swamps. *Ceratopteris richardii* is distributed in parts of North and West Africa, Madagascar, South America, the Caribbean and North America. In North America, isolated populations have been found in Louisiana and Florida (Lloyd 1974). It is a vascular plant that exhibits a life cycle with independent haploid

(gametophyte) and diploid (sporophyte) phases (Hickok 1987, Nakazato *et al.* 2006).

Ceratopteris richardii is an ideal model system for studying basic problems in plant biology (Hickok *et al.* 1995). It has a short life cycle of approximately 4 months, and it is easy to culture and maintain in the laboratory. The haploid phase of its life cycle lends itself to isolation of interesting new mutants in a single generation after mutagenesis. Therefore, mutants that show altered ability to accumulate metals could be isolated by screening of gametophytes. Mutants that accumulate higher than normal amounts of a metal could be used directly in phytoremediation. Mutants that accumulate less than normal amounts could be used to identify genes that are involved in metal accumulation. Knowledge of these genes could be used to genetically engineer *C. richardii* and other plants for use in phytoremediation. However, little is known about the ability of *C. richardii* sporophytes and gametophytes to accumulate various metals.

My research addressed the following questions with regard to the ability of *C. richardii* gametophytes and sporophytes to accumulate Cu, Pb and Zn in their shoots:

1. What is the highest concentration of each heavy metal (Pb, Cu and Zn) on which *C. richardii* spores can germinate and develop for 12 days without any signs of growth defects?

2. What is the highest concentration of each metal that 15-day-old *C. richardii* sporophytes can withstand for 15 days without any signs of growth defects?
3. What is the concentration of each metal accumulated in the above-ground biomass of sporophytes after 15 days of growth in the presence of the metals?
4. Is there a relationship between concentration of each metal in the growth medium and the amount of metal taken up by sporophytes?

Methods

Ceratopteris richardii gametophytes and sporophytes were produced according to standard methods described in the C-FERN Manual (Warne and Hickok 1997). Wild-type (strain RNWT1) spores and *C. richardii* medium were purchased from Carolina Biological Supply Company. Spores were disinfected and sown on *C. richardii* medium in 60-mm plates, and plates were incubated at 28 ± 2°C under a 15-watt fluorescent lamp. After 12 days, the gametophytes were watered to induce fertilization and incubated for an additional 15 days to obtain sporophytes.

Sporophytes were transferred to plates containing various concentrations of Pb, Cu or Zn. There were 6 plates per treatment with 15 plants per plate. The metals used were in the forms of lead nitrate [$Pb(NO_3)_2$], cupric nitrate [$Cu(NO_3)_2 \cdot 2½H_2O$], and zinc nitrate [$Zn(NO_3)_2 \cdot 6H_2O$]. Concentrations varied for each experiment and are specified below. The experiments were monitored in the growth chamber for 15 days. Each day, the plants' health and growth were assessed by noting the color of the leaves, the number of leaves and the length of stems. After 15 days, shoots were harvested and washed in distilled water and oven-dried at 90°C for 24 hours (Quereshi et al. 1985). The samples were digested and analyzed following the digestion protocol EPA 3050B (US EPA 1996). Determination of metal concentrations in the samples was performed at Auburn University's soil lab using Atomic Absorption Spectroscopy.

To find the maximum concentration of Pb, Cu and Zn on which *C. richardii* spores can germinate and develop, spores were sown on basic *C. richardii* medium containing 0, 5, 10, 15, 25, 50, 100, 200, 500 and 1000 ppm of Pb, Cu or Zn. There were 6 plates per concentration per metal. The plates were incubated under standard conditions for 12 days. Normal germination and growth were indicated by the presence of both males and hermaphrodites of normal size and color compared to controls grown on regular *C. richardii* medium.

To find the highest concentration of Pb, Cu and Zn that are not toxic to sporophytes, sporophytes were exposed to 0, 5, 10, 15, 25, 50, 100, 200, 500 and 1000 ppm of Pb, Cu or Zn. Each experimental group had 6 plates with 15 plants per plate. The growth of sporophytes was monitored for 15 days. The sporophytes were compared to controls and differences in color and size were noted.

To compare the concentrations of Pb, Cu and Zn accumulated in the above-ground tissue of *C.richardii*, sporophytes were exposed separately to each of the three metals at the highest concentration that allows normal growth. After 15 days of growth, plants were harvested and prepared for analysis as described above. Each treatment was replicated 6 times with 15 plants per replication.

In order to compare the uptake of Pb, Cu and Zn at different concentrations, sporophytes were grown at low, medium and high concentrations of each metal. The actual concentrations were determined from the results of the

experiments described above. Low concentration for each metal was 1/10 of the highest concentration at which the plants could grow without any signs of stress, and medium concentration was 1/2 of the highest concentration. After 15 days the plants were harvested and prepared for analysis as described above. The mean concentration of metals in the above-ground biomass was determined, and the mean amounts accumulated at different concentrations were compared. The significance of mean differences was tested using Analysis Of Variance (ANOVA) followed up with the Tukey Honestly Significant Difference test to locate mean differences (Lowry 2005).

Results

In order to find the maximum concentration of Pb, Cu and Zn on which *C. richardii* spores can germinate and develop, spores were sown in basic C-FERN medium containing various concentrations of the three metals and incubated under standard conditions for 12 days. Normal germination and growth were indicated by the presence of both males and hermaphrodites of normal size and color compared to controls.

Table 2 shows that as the concentration of Cu in the medium increased, both spore germination and gametophyte growth were affected. In the presence of 5 ppm of Cu, spores germinated but gametophytes appeared smaller than normal gametophytes and were yellow-brown instead of the normal green color. Spores were not able to germinate in medium with concentrations of Cu higher than 10 ppm.

Table 2. The germination of spores and growth of gametophytes in the presence of different concentrations of Cu.

Cu Concentration (ppm)	Comments
0	Spore germination and normal growth
5	Spore germination and affected growth
10	No germination
15	No germination
25	No germination
50	No germination
100	No germination
200	No germination
500	No germination
1000	No germination

Table 3 shows the effects of different concentrations of Zn in the medium on spore germination and the growth of gametophytes. Spores germinated and developed normally with concentrations up to 5 ppm. Between 10 ppm and 25 ppm, spores germinated but growth and development were affected. In the presence of 10 ppm and 15 ppm, gametophytes were yellow instead of green. In the presence of 25 ppm, the gametophytes were brown and smaller than controls. Spores were not able to germinate in medium with 50 ppm Zn and higher.

Table 3. The germination of spores and the growth of gametophytes in the presence of different concentrations of Zn.

Zn Concentration (ppm)	Comments
0	Spore germination and normal growth
5	Spore germination and normal growth
10	Spore germination and slightly affected growth
15	Spore germination and affected growth
25	Spore germination and affected growth
50	No germination
100	No germination
200	No germination
500	No germination
1000	No germination

Table 4 shows the effects of varying concentrations of Pb on spore germination and development of gametophytes. Spores germinated and gametophytes developed normally in concentrations from 0 ppm to 100 ppm.

However, with 200 ppm of Pb in the medium, spores germinated but gametophytes were brown and smaller than normal gametophytes. With 500 ppm and 1000 ppm, spores did not germinate.

Table 4. Germination of spores and the growth of gametophytes in the presence of different concentrations of Pb.

Pb Concentration (ppm)	Comments
0	Spore germination and normal growth
5	Spore germination and normal growth
10	Spore germination and normal growth
15	Spore germination and normal growth
25	Spore germination and normal growth
50	Spore germination and normal growth
100	Spore germination and normal growth
200	Spore germination and affected growth
500	No germination
1000	No germination

In order to find the highest concentrations of Pb, Cu and Zn that are not toxic to sporophytes, 15-day-old sporophytes were exposed to various concentrations of Pb, Cu or Zn. Growth and development of sporophytes were monitored for an additional 15 days, and any abnormalities were noted.

Table 5 shows the effects of different concentrations of Cu on the growth and development of sporophytes. In the presence of 5 ppm and 10 ppm Cu, sporophytes developed normally with extensive root systems and green leaves. Sporophytes grew in the presence of 15 and 25 ppm, but their development was affected. Their roots were not as extensive as those at lower concentrations, and

their leaves were brown. The sporophytes grown in medium containing 50 ppm Cu and higher died within 2 days after transfer.

Table 5. Growth and development of sporophytes in the presence of different concentrations of Cu.

Cu Concentration (ppm)	Comments
0	Normal growth
5	Normal growth
10	Normal growth
15	Growth
25	Growth
50	No Growth
100	No Growth
200	No Growth
500	No Growth
1000	No Growth

Table 6 shows the effects of different concentrations of Zn on the growth and development of sporophytes. Sporophytes transferred to medium with up to 15 ppm of Zn exhibited normal growth and development, while sporophytes transferred to medium containing 25 ppm had brown leaves and small roots that barely penetrated the surface of the medium. Sporophytes transferred to medium containing more than 50 ppm of Zn died within 2 days after transfer.

Table 6. Growth and development of sporophytes in the presence of different concentrations of Zn.

Zn Concentration (ppm)	Comments
0	Normal growth
5	Normal growth
10	Normal growth
15	Normal growth
25	Slightly affected growth
50	No Growth
100	No Growth
200	No Growth
500	No Growth
1000	No Growth

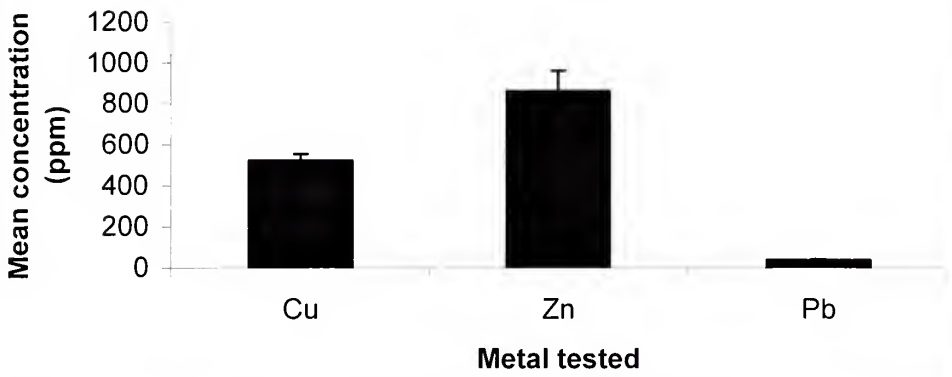
Table 7 shows the effects of different concentrations of Pb on the growth and development of 15-day-old sporophytes. Sporophytes that were transferred into medium with concentrations up to 100 ppm showed normal growth. Interestingly, sporophytes transferred to 200 ppm Pb showed enhanced growth including extensive roots and deeper green leaves than control sporophytes. In the presence of 500 ppm, sporophytes were yellow and smaller than controls, and the roots were smaller and barely broke the surface of the medium. Sporophytes transferred to medium containing 1000 ppm Pb died within 2 days after transfer.

Table 7. Growth and development of sporophytes in the presence of different concentrations of Pb in the medium.

Pb Concentration (ppm)	Comments
0	Normal growth
5	Normal growth
10	Normal growth
15	Normal growth
25	Normal growth
50	Normal growth
100	Normal growth
200	Enhanced growth
500	Affected Growth
1000	No growth

In order to compare the concentrations of Pb, Cu and Zn accumulated in the above-ground tissue of *C. richardii*, sporophytes were exposed separately to each of the three metals at 10 ppm, which was the highest concentration that allowed normal growth for all the metals. After 15 days of growth, stems and the leaves were harvested and prepared as described for analysis using Atomic Absorption Spectroscopy. Figure 1 shows that sporophytes accumulate different amounts of the various metals. Zn was accumulated at the highest level, followed by Cu, and Pb was accumulated at the lowest level. ANOVA ($F=141.36$, $p < 0.0001$) and Tukey HSD indicated that the mean accumulations of all three metals differed significantly from each other.

Figure 1. The mean concentration of Cu, Zn and Pb accumulated into the above-ground biomass of *C.richardii* sporophytes exposed to 10 ppm of each metal. Error bars indicate standard deviations.



In order to compare the uptake of each metal at different concentrations, sporophytes were grown at low, medium and high concentrations of Cu, Zn, and Pb. The actual concentrations were determined from the results of the second experiment described above. The concentrations that were used for Cu and Zn were 10, 15 and 25 ppm, and for Pb they were 10, 200 and 500 ppm. After 15 days, the plants were harvested and prepared for analysis as described in Methods.

Figure 2 shows the accumulation of Cu by sporophytes at 10, 15 and 25 ppm. Plants exposed to 10 and 15 ppm did not differ greatly in their accumulation, but those that were exposed to 25 ppm showed a higher accumulation compared to the plants that were exposed to 10 and 15 ppm.

ANOVA ($F=223.15$, $p < 0.0001$) indicated that there was a significant difference in accumulation levels between plants exposed to different concentrations of Cu. Tukey HSD showed that there was a significant difference in uptake between sporophytes exposed to 10 and 25 ppm and between 15 and 25 ppm. However, there was no significant difference between sporophytes exposed to 10 ppm and 15 ppm.

Figure 2. Accumulation of Cu by sporophytes of *C.richardii* at different concentrations. Error bars indicate standard deviations.

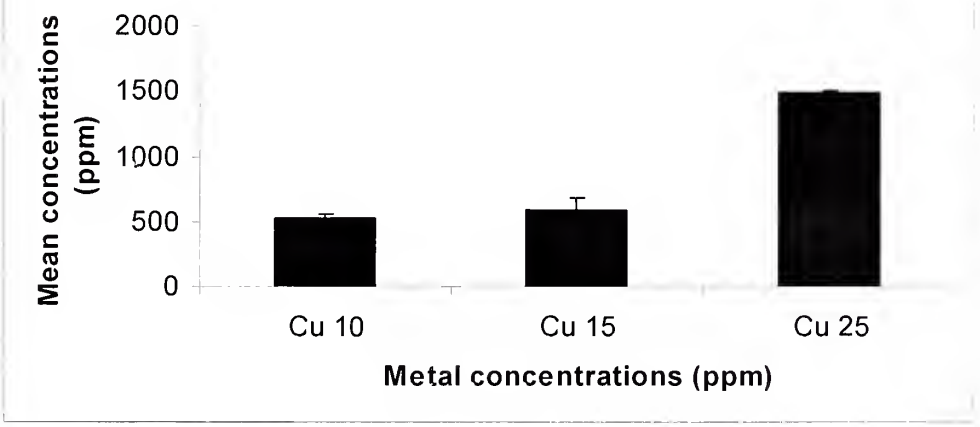


Figure 3 shows the accumulation of Zn by sporophytes at 10, 15 and 25 ppm. The amount accumulated increases steadily up to 15 ppm in the medium and then appears to level off at 25 ppm. Accumulation in plants exposed to 15 ppm differs from those exposed to 10, while those exposed to 25 ppm did not appear to differ greatly from those exposed to 15 ppm. However, ANOVA

(F=91.27, p = 0.0001) and Tukey HSD indicated that all three mean accumulation levels were significantly different from each other.

Figure 3. Accumulation of Zn by sporophytes of *C.richardii* at different concentrations. Error bars indicate standard deviations.

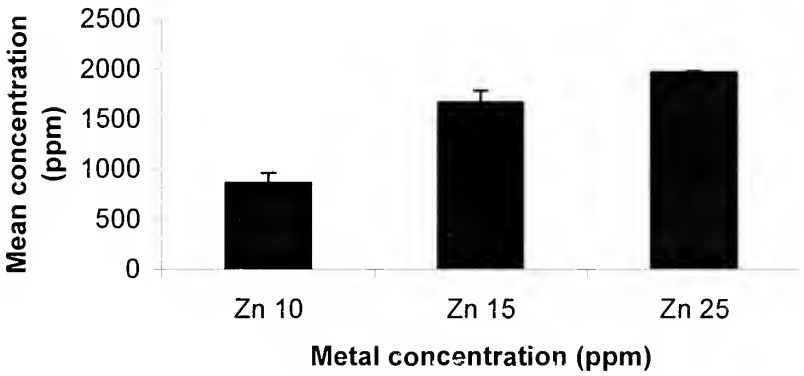
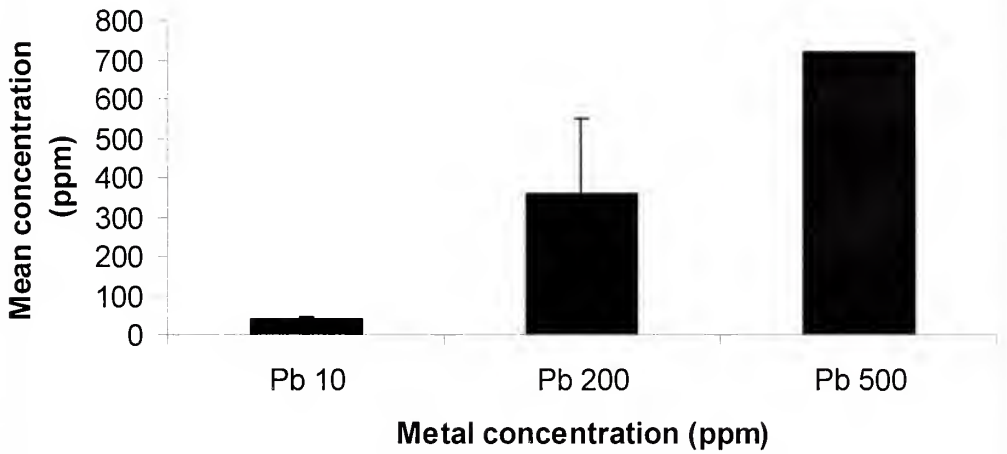


Figure 4 shows the accumulation of Pb by sporophytes at 10, 200 and 500 ppm. The amount of Pb accumulated by sporophytes increases steadily up to 500 ppm in the medium. ANOVA (F=11.88, p = 0.02) and Tukey HSD indicated that there was a significant difference in uptake levels between plants exposed to 10 and 200 ppm. The error bar is absent in the sporophytes exposed to 500 ppm because there was only one sample analyzed. Sporophytes exposed to 500 ppm grew poorly and had an unexpectedly small biomass. Therefore, biomasses from

all repetitions were combined to create enough for an analysis sample.

Figure 4. Accumulation of Pb by sporophytes of *C.richardii* at different concentrations. Error bars indicate standard deviations.



Discussion

Studies show that heavy metals in the environment can stress plants and, in extreme cases, completely inhibit a plant's growth and development (Baker 1987). A similar pattern was observed in this study with gametophytes and sporophytes of the fern *C. richardii*. Spore response to the presence of metals in their growth medium was significant and measurable. Spores sown in medium treated with Cu were affected at as low as 5 ppm, but were affected only at higher concentrations of Zn and Pb (25 ppm and 100 ppm respectively).

Ceratopteris richardii sporophytes in this study tolerated higher levels of Cu and Pb than gametophytes. With Zn, tolerance by sporophytes was only slightly higher than gametophytes. Sporophytes grown in medium containing Cu were able to tolerate concentrations as high as 25 ppm compared to 5 ppm in gametophytes. Sporophytes tolerated as high as 500 ppm Pb compared to 200 ppm for gametophytes. As for Zn, gamtetophytes showed affected growth at as low as 15 ppm while sporophytes showed normal growth at 15 ppm but affected growth at 25 ppm (Tables 3 and 6).

With all three of the metals tested, sporophytes grown in higher concentrations had brown leaves instead of green. Also, sporophytes that were grown in medium containing 25 ppm of Zn had unusually short roots. Previous studies show that increased levels of metals present in the environment influence both shoot and root growth (Baker 1987, Brune *et al.* 1994, Rengel 2000, Peng

and Yang 2005). Gametophytes and sporophytes grown in medium containing higher levels of all three metals lacked the normal green color. Previous research shows that heavy metals can reduce the biosynthesis of chlorophyll and, in some cases, inhibit it completely (Ali *et al.* 2003).

Metal accumulation in *C.richardii* generally increased in concert with increasing concentrations in the medium. This result is consistent with results of numerous studies showing that as concentrations of metals increase in the environment the levels measured in plant tissues also increase (Tilstone and Macnair 1997, Basile *et al.* 2001, Peng and Yang 2005, Rengel 2000).

Sporophytes that were treated with 25 ppm Zn, 25 ppm Cu and 500 ppm Pb had shorter roots that barely penetrated the surface of the medium. Even so, these plants survived for at least 15 days. Reduced root development might be a normal response to high levels of metals in the environment, allowing the plants to avoid absorbing the metals.

Ceratopteris richardii responded to each of the three metals in the medium differently in terms of tolerance and accumulation. Although it showed the highest tolerance to Pb, it actually accumulated Pb to the lowest level. Cu, which was the least tolerated in both gametophytes and sporophytes, was accumulated to significant levels. *Ceratopteris richardii* accumulated Zn at the highest level of the three metals and was moderately tolerant of Zn in both gametophytes and sporophytes. In a previous study, Livett *et al.* (1979) analyzed Pb, Cu and Zn in

the British blanket peat, and found that Zn was accumulated at higher levels than the other two metals.

The fact that Zn and Cu were found at higher levels in the plants' tissues could be because both Cu and Zn are micronutrients. Therefore, plant roots are equipped to extract them from the growth medium and move them further up and down the plant tissue, but they do not have a mechanism to extract and transport Pb (Basile *et al.* 2001).

The ability of a plant to tolerate elevated concentrations of metals is not necessarily an indicator of their ability to hyperaccumulate metals. Some plants have developed ways to tolerate and survive in soils with elevated levels of metals (Lasat 2002). A plant has to be able to accumulate levels greater than 100 times the normal levels found in plant tissue and not be affected by the metal in order to be considered a hyperaccumulator (Lasat 2002). It is important to note that all three metals were measured in this study at levels higher than accumulator status based on the table by Reeves and Baker (2000), and Cu was measured above the hyperaccumulator levels.

Although the results of this study are promising, more extensive analysis on the abilities of *C. richardii* as a phytoremediation candidate is needed. For example, tolerance and accumulation should be measured in soil and water in addition to artificial growth medium. Also, concentrations in shoots, roots and

fronds should be measured separately to determine how metals are transported and distributed within the plant.

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