Qualitative study of the effects of Cadmium ion on the morphology, physiology and protein profile of Lolium perenne (Perennial Ryegrass) and Trifolium hybridum (Alsike Clover)

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BIOL 448

June 1, 2012

[Academic work for this project was completed on 2010/April 30]

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UBC SEEDS RESEARCH PROJECT

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Abstract

Roadside plants are exposed to heavy metal ions every year in the Lower Mainland. On an average rainy day, roadside groundwater may contain potentially toxic cadmium concentrations as well as other heavy metals. Does this pose severe problems for plant growth and survival? The current study investigated the physiological symptoms of Ryegrass (*L. perenne*) and Clover (*T. hybridum*) in response to cadmium stress. Using plate culturing and hydroponic growth medium, we found that both plant species in high cadmium concentration (1000μM) experienced 1) reduction in the morphological development of seedling and young plants, and 2) reduction in metabolism (e.g. photosynthesis rate). These findings shed light on the tolerance limits of roadside plants and their sustainable growth in heavy-metal prone environments.

Introduction

One of the recourses of industrialization is the effect of chemical leaching on the environment. Mining processes can be a major source of mineral leaching, which not only can significantly affect the chemical and structural composition of the environment impacted but also the animals and plants that reside there(Laws, 2000). Mining can very often produce environmental toxins such as cadmium (Cd^{2+}), iron (Fe), zinc (Zn^{2+}), lead (Pb^{2+}) and copper (Cu^{2+} or Cu^{4+}) at a concentration suspected to be intolerable to many species (Laws, 2000; EPA 2000). In humans, Cd^{2+} is not easily degraded, long-term exposure to which can cause kidney to malfunction, adversely affect calcium metabolism and bone formation, e.g. osteoporosis (Laws, 2000). For the present study, the focus was to determine at *different concentrations* of Cd^{2+} and *different lengths of exposure* whether certain plant species commonly grown on the BC roadside exhibit 1) tolerance reflected in morphology, transpiration and photosynthesis rates, and to identify 2) proteins that may be highly expressed as a function of toxin tolerance. It was thought that due to the rarity of cadmium in the natural environment, organisms would not be generally adapted to high Cd^{2+} concentrations, e.g. no more than 1-10μM (EPA, 2000). However, a study on tumbleweed Cd^{2+} tolerance by de la Rosa et al (2005) led to the hypothesis that some plants may be fairly resistant even at higher concentrations. The hope is that if tolerant species are found, they can be favoured to grow at industrial sites. Moreover, species that tolerate internally high amounts of cadmium have the potential to act as a buffering zone to prevent chemical leaching to nearby watersheds (Laws, 2000). Therefore, the potential exists for some plants to play important roles in bioremediations especially considering the growing demand for zinc, which when mined yields releases cadmium as a byproduct that accounts for most of the cadmium emitted to date (Laws, 2000; EPA 2000).

Methods and material

I. Plant species selection (see Figure 1)

Seeds of thirteen (13) common roadside grown plant species near BC highways were incubated in separate petri dishes (n=15) on filter paper soaked in 1.5 mL of water or various diluted concentrations of cadmium in the form of cadmium chloride. After sealing in the moisture with parafilm, one set of the seeds were incubated in the dark and another identical set in the light at constant room temperature for 2-3 days, the normal time found for germination of these seeds. The morphology (colour, texture) of the seedlings was noted, in addition to the roots and shoot lengths of each seedling being measured using a computer tracing program. The results for each petri dish were averaged from the specimen that germinated. With the water treatment as a control, the seedlings that showed little/no change at high concentrations of cadmium were then selected for the next step.

II. From the step I, it was determined that Clover and Ryegrass were the most tolerant among the eleven other commonly grown plant species along BC highways. It was now to determine at what concentration and the length of exposure to cadmium do seedlings become intolerant along with the accompanying morphological and physiological characteristics they exhibit.

Plant seedlings (n>3) that germinated successfully at high Cd^{2+} concentration were transferred from the petri dishes to small, short boxes filled with either diluted nutrient solutions (see lab book for a full recipe) or nutrient solutions mixed with various concentrations of Cd^{2+}. Since hydroponic growth of Ryegrass (started before Clover) revealed no visible effect from exposure to Cd^{2+} concentration below 1M, it was assumed that 10M Cd^{2+} would be a reasonable minimum concentration for Clover. Plastic netting with Styrofoam edges was used to support the seedlings upright while their roots were exposed to the medium solution. All media were aerated using a handmade bubbling device with a valve controlled air source (see Figure 2). The incubation continued as long as the seedlings sustained a healthy appearance. Leaf samples were frozen for protein analysis when changes were observed and when possible, photosynthesis and transpiration rates were taken with the LiCOR machine as measures of the overall physiological performance of each replicate. Technically speaking, LiCOR measures and records the CO_2 concentration and water loss, which were later converted to photosynthesis rate and transpiration rate with a computer program.

III. Protein analysis

With the frozen leaf sample from step II, standard SDS gel electrophoresis were performed to detect if major protein groups (e.g. RUBISCO/ATP Synthase and Light Harvesting complexes) increase or decrease with higher cadmium concentration. These groups can give us an indication of whether cadmium inhibits or stimulates the major biochemical functions of the plants. With proteins migrated onto the SDS gel, Western blotting using the heat shock protein group Dehydrin (AgriSara ™ enzyme) at a ratio of 1:5000 (vol/vol protein versus 5-10 mL of milk protein blotting solution) was performed as a biochemical indicator of stress. It is important to note that the biochemical cues do not usually explain all macroscopic changes because there are many, interacting microscopic processes.

Limitations to the methods

It was difficult to keep the conditions for hydroponic growth consistent throughout the experiment, especially considering the unwanted growth of algae in the nutrient solution and the periodic evaporation of medium through the atmosphere via aeration. These difficulties could have led to non-negligible nutrient deficiencies, confounding the results from both the controls and the treatments.

For the Western Blot, the samples were only assayed with Dehydrin though there could be other proteins up- or down-regulated as a result of cadmium stress. The rationale for testing with Dehydrin was that this family of proteins has been linked to stress symptoms and heavy metal detoxification in plants (Zhang et al, 2006). Jonak et al (2004) have demonstrated the upregulation of certain mitogen-activated protein kinases aiding in stress response in alfalfa exposed to high [Cd^{2+}]. However, another reason for studying

Dehydrin is that it is generally less characterized than other protein groups known for their roles in plant metabolism.

Due to time constraints, the hydroponics experiment was not able to be replicated. Roots, shoots and metabolic rates sampled at each sampling time were based on one replicate from each treatment. The results from the current study were therefore qualitative only and not statistically robust.

Results & Discussion

Perennial Ryegrass (*Lolium perenne*)

Morphology

After 2 days of hydroponic growth with various concentrations of cadmium, the Ryegrass seemed to exhibit no changes in root or shoot length relative to the control (Fig. 3). On the 8th day, however, plants treated at 1000 uM began to show stunted growth (Fig. 3), yellowing of the shoots and lack of turgor as compared to the control and to other treatments at lower Cd^{2+} concentrations. These symptoms are typical of plants under heavy metal stress, which indicates an interference with the normal physiological functions (Zhu et al, 2005)

Physiology

Photosynthesis rates – on day 2, there was a ten-fold decrease in photosynthesis rate from the control for the treatment at 1000 uM Cd^{2+} (Fig. 4). A similar decreasing pattern was expected on day 7 but this was not reflected in the graph. We suspected that the aeration mechanism in the hydroponics was not consistent throughout the experiment and thus occasional lack of O_2 in the medium could have induced stress on some of the plants, including the control, resulting in photosynthesis rates generally being low on day 7 compared to day 2. Decrease in photosynthesis was expected because plants under ionic stress tend to produce fewer stomata due to inhibition of guard cell precursors (Zhu et al, 2005), leading to decreased intake of CO_2, the substrate for photosynthesis.

Transpiration rates – on day 2, there was a two-three fold increase in transpiration in the 1-100uM Cd^{2+} treatments compared to the control (Fig. 5). Similarly on day 7, there was a two-fold increase in transpiration rate at 0.1uM and an even larger increase (five fold) at 1000uM. This finding is contrary to that of Leita et al (1995), who cited attributed to lower transpiration in soybean plants to stomatal closure. However, this may be due to different plant used since Zhu et al (2005) found larger stomatal aperture in *Brassica juncea*, which if found to be the case in this experiment, would explain in part the heightened transpiration in Ryegrass.

Protein analysis

For all but one treatment (1000 μM), it was possible to detect distinct, near-uniform bands for Rubisco and ATP synthase and light-harvesting complexes (LHC) I & II on the stained gel (Fig. 6). This could explain the lack of morphological changes in treatments at Cd^{2+} concentrations under 1000 μM, but generally do not reflect the changes in physiology, i.e. decrease in photosynthesis and rise in transpiration with increasing cadmium concentrations, the exception being the “smeared” or degraded gel pattern of plants that became increasingly stressed by high Cd^{2+} as the treatments continued (i.e. undergoing proteolysis). However, the lack of biochemical signals may instead suggest that cadmium does not directly antagonize physiological functions directly, but rather more directly through the closure of stomata, which lead indirectly to the observed physiological changes

(Zhu et al, 2005). Dehydrin was present in various amounts in the controls and treatments (Fig. 7), which indicated that experimental conditions in general were not properly controlled (e.g. through unequally sustained air pressure and algal growth on nutrient solution past day 2) and could have resulted in plants functioning out of their normal range.

Alsike Clover (*Trifolium hybridum*)

Morphology

It was possible to visualize toxic effects of cadmium in the 1000uM Cd^{2+} treated clover seedlings on day 2. Notably, the plants showed stunted growth, yellowing and crispy texture on most of its leaves as well as lack of turgor throughout the plant (Figs. 8 and 12). By the 7th day, the 100uM treatment had also begun to exhibit the same effects (Fig. 8). Compared to Ryegrass, Clover appeared to be more vulnerable to the heavy metal. This could be partially due to the large surface area of clover leaves which makes the plant more susceptible to functional decline which leads to more visible signs of stress.

Physiology

Photosynthesis rates – a 100% drop in photosynthesis from the control treatment in 1000uM Cd^{2+} treated clover on day 2 was found (Fig. 9). Though the data are not shown here, the decreasing trend showed signs of continuation on day 7.

Transpiration rates - there was a slight to negligible decline in transpiration of all treatments on day 2 (Fig. 10) and cadmium seem to have resulted in more toxic effects on Clover as more time passed (day 7 data incomplete, not shown).

Altogether, the rates for Clover were much lower than those of the Ryegrass, which is counter-intuitive since the large surface area should favour higher metabolic rates. Aside from potentially stressful conditions caused by the algae in the Clover hydroponics, we suspected there may have been errors in calibration of the LiCOR, which tended to underestimate rates as the CO_2 filters became congested over time. This would have caused an overall lack of physiological stress signal expressed. This is another important reason that the experiment needs to be replicated in the future to confirm the current findings.

Protein analysis

Unlike the Ryegrass, even though there was no appreciable difference in protein patterns between the control and the treatments from 0.1-100μM Cd^{2+} in both days 2 and , the 1000 uM treatment appeared to be affected since the gel lane had a smear pattern that is usually accompanied by protein degradation of a dying plant, which corresponds to morphological symptoms of stress described earlier. While the general protein pattern did not indicate a difference between the control and a stressed cadmium treatment, Dehydrin was found to be highly expressed in the 1000μM treatment, suggesting that part of the plant's stress response can be traced to the upregulation of Dehydrin. On day 7, however, only a smear of Dehydrin was found in the 100μM sample, possibly resulting from proteolysis.

Synthesis

It appears that Ryegrass (*L. perenne*) is generally very tolerant of high Cd^{2+} concentrations, only showing major signs of stress at 1000μM, a concentration that is two to four magnitudes higher than what is generally found at industrial sites (Peralta-Videa et al, 2009; EPA, 2000). Clover (*T. hybridum*) exhibited fairly high tolerance to cadmium, though not as much as *L. perenne* in terms of morphology and photosynthesis. The differences in stress responses, especially in morphology between the two plants may be attributed to differential uptake by leaves (de la Rosa, 2005), possibly more controlled in

Ryegrass than it is in Clover. Or, as was mentioned earlier, the morphology of the plants could also be a factor by relating our results to Haghiri (1973) where the relative sensitivity of soybean (a trifoliate) to Cd^{2+} was found to be higher than in wheat (a grass species). *T. hybridum* appears to be resistant over extended periods of time at 10uM Cd^{2+}but not at higher concentrations (Fig. 12). *T. hybridum*'s response to cadmium is also time and concentration dependent, which was reflected in the presence of Dehydrin. Where Dehydrin was not detected (due to absence of expression or to proteolysis) in Ryegrass, it was in Clover, suggesting that Dehydrin can play a significant in mediating the stress response of these plants to cadmium. Among the very few studies available examining the relationship between Dehydrin and heavy-metal stress, Zhang et al (2005) and Xu et al (2008) have proposed that the found Dehydrin-like proteins (two specific proteins cloned by the authors) seek out oxidative derivatives harmful to the plant that is under the stress of heavy metals. Kruger et al (2002, cited in Xu et al, 2008), however, found heavy-metal binding Dehydrins. There appears to be diverse but important roles of Dehydrin in heavy-metal tolerance of plants.

Figures

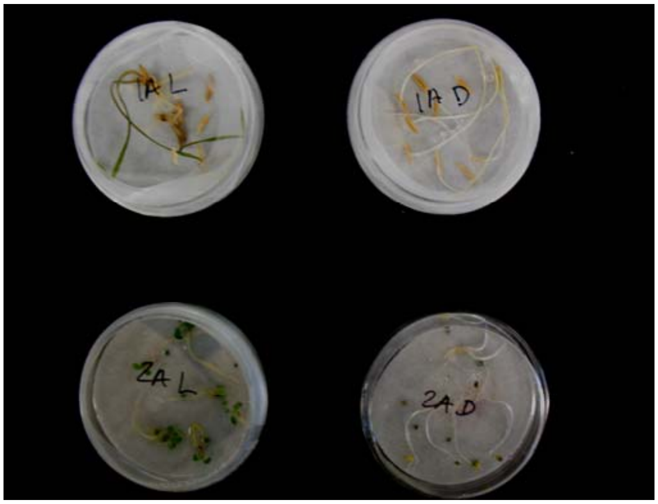

Figure 1. Plate culturing experiment with Ryegrass (above) and Clover (below) seedlings incubated in various concentrations of Cd^{2+} in light and dark conditions.

Figure 2. Hydroponics setup of Clover seedlings grown at various concentrations of Cd^{2+}.

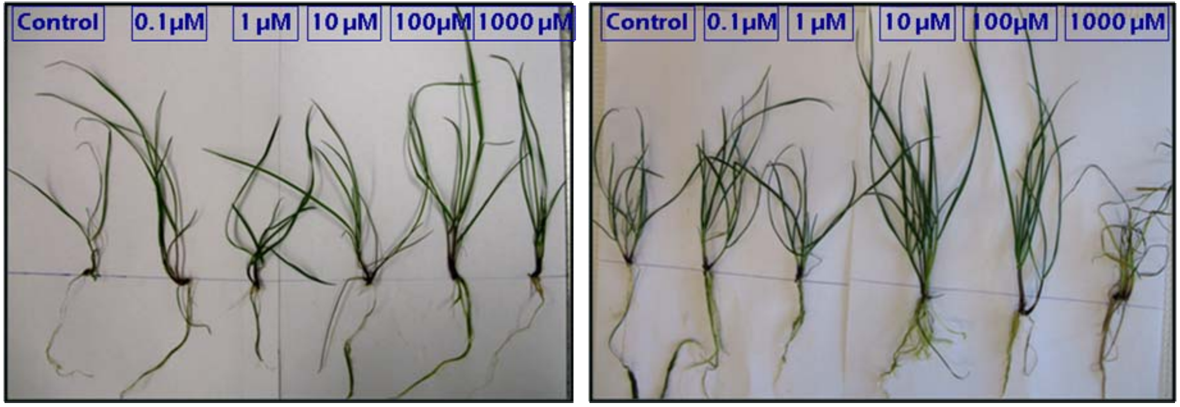

Figure 3. Ryegrass morphology (roots and shoots) subject to 2 days (left) and 8 days (right) of cadmium treatments (concentrations indicated above).

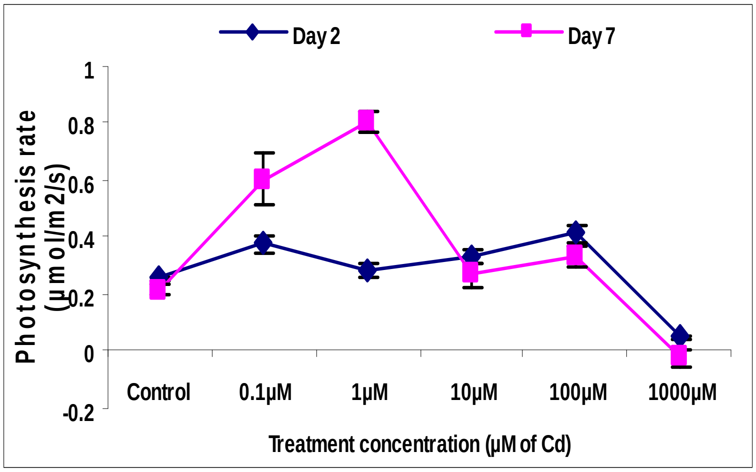

Figure 4. Photosynthesis rates of Ryegrass treated at various concentrations on days 2 and 7.

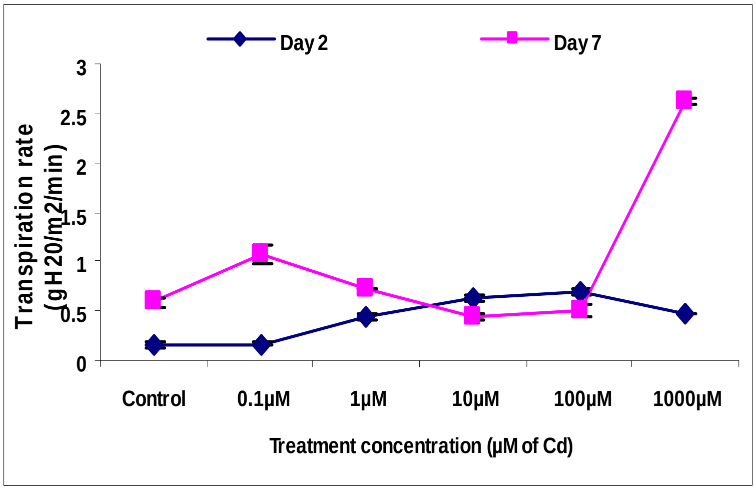

Figure 5. Transpiration rates of Ryegrass treated at various concentrations on days 2 and 7.

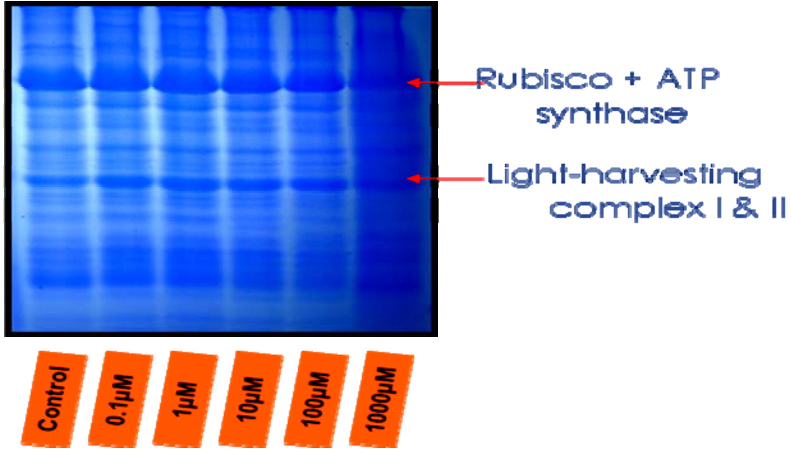

Figure 6. Day 8 Ryegrass SDS electrophoresis gel showing degradative protein profile at 1000µM Cd^{2+}

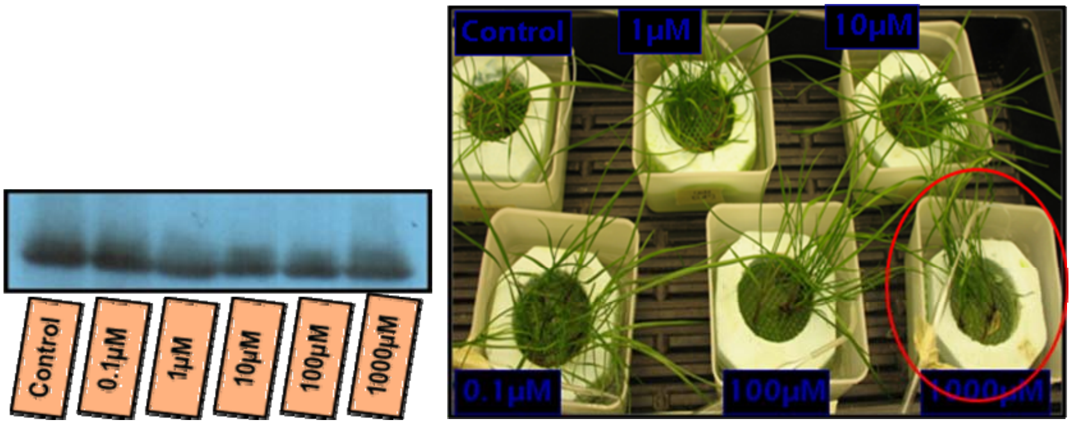

Figure 7. Day 2 Ryegrass Dehydrin profile (Western blotting) at various Cd^{2+} concentrations and accompanying morphology.

Figure 8. Clover morphology (roots and shoots) subject to 2 days (left) and 7 days (right) of cadmium treatments (concentrations indicated above).

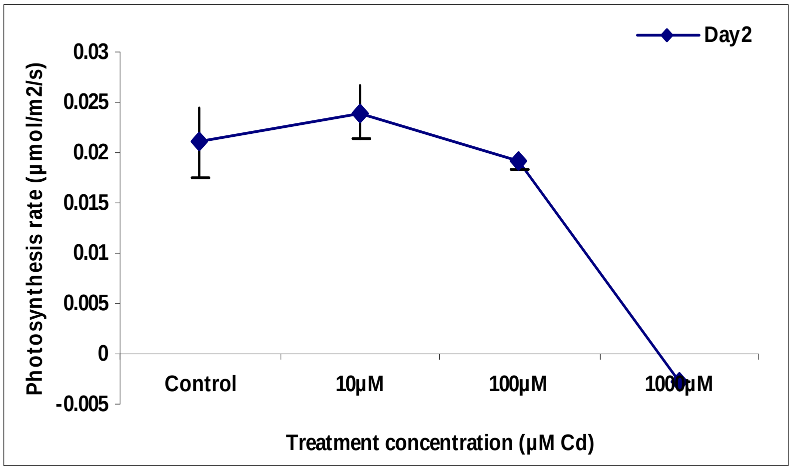

Figure 9. Photosynthesis rate of Clover treated at various concentrations on day 2; day 7 data not available.

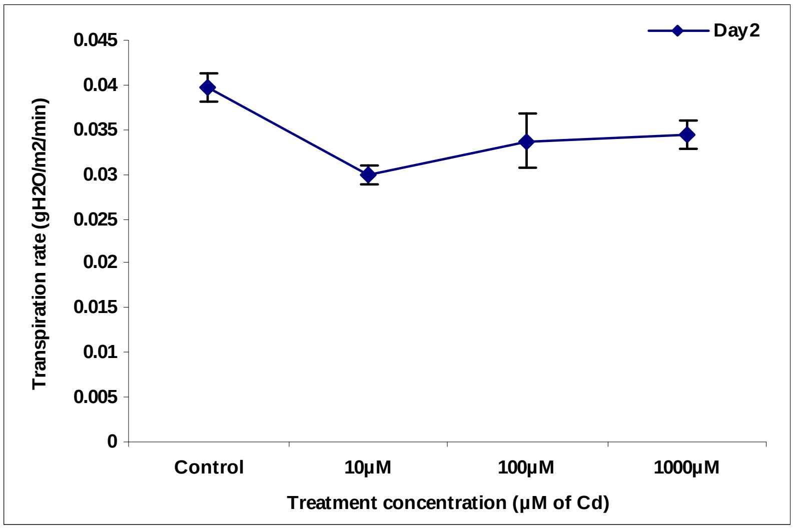

Figure 10. Transpiration rate of Clover treated at various concentrations on day 2; day 7 data not available.

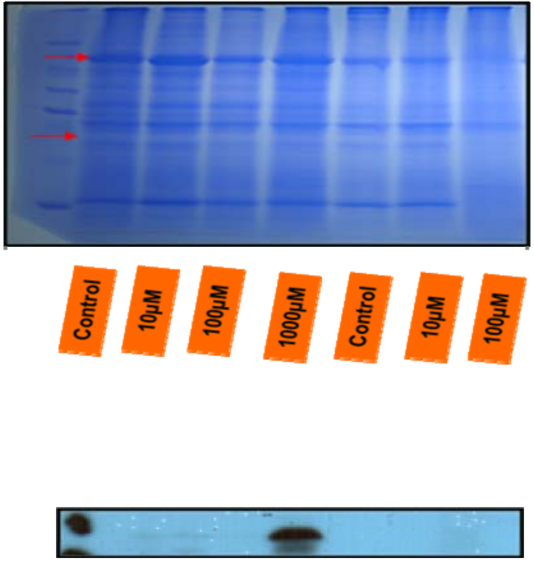

Figure 11. Top: Day 2 and 7 SDS electrophoresis gel (left to right) of Clover treated at various concentrations of Cd^{2+}. Arrows indicate protein groups specified in Figure #. Bottom: accompanying Dehydrin profile.

Figure 12. Clover hydroponics revealing morphological differences between different treatments of Cd^{2+} (control at top left, [Cd^{2+}] increase is seen counterclockwise.

Conclusions and future direction

The highest concentrations detected in both Clover and Ryegrass in this study are more elevated than the typical Cd^{2+} levels reported at industrial sites, usually not more than the 10μM range (Peralta-Videa, 2009; EPA, 2000), and near highways (usually 0.04-0.1μM). Considering the results of this study, it is reasonably likely that both Clover and Ryegrass have the ability to germinate and survive for some time at higher than usual concentration. It is worth noting that the highest concentrations of heavy metal usually occur in stormy seasons when the mineral leachates are the most easily flushed out intothe surrounding biological environment but this is expected to last a few days (Laws, 2000). In the case of Clover, tolerance to high but short-lived flushes of Cd^{2+} seems quite possible. All in all, heavy metal resistance demonstrates in both Clover and Ryegrass would make them competitive in Cd^{2+}-prone environment in addition to their being fast germinating species.

It is still difficult to extrapolate our result to the natural soil environment where many factors can impact cadmium toxicity. For instance, it is possible that cadmium ions (positively charged) are adsorbed by the negatively charged soil particles, which makes Cd^{2+} more bioavailable to plants. In addition, some studies have found cadmium to be co-transported with iron and zinc under certain circumstances, which leads to increased Cd^{2+} uptake (Peralta-Videa et al, 2009; de la Rosa et al, 2005). This study did *not* investigate whether cadmium absorption by Clover and Ryegrass roots was indeed happening and if so, whether either/both plants have a threshold for its intake. The strong tolerance of Ryegrass to high Cd^{2+} concentration suggest that cadmium may be taken up only past a certain concentration or it may have a very high threshold of accumulation. By contrast, Clover exhibits limited tolerance to cadmium, suggesting a Cd^{2+} is readily metabolized. The possibilities of Cd^{2+} binding and compartmentalization cannot be ruled out either. These considerations can be helpful for future studies, which may be interested in establishing whether certain plant species as particularly strong absorbants for cadmium while being resistant to it, thus capable of acting as buffer zones to prevent heavy metal runoffs to watersheds. From the viewpoint of physiological studies, it would be insightful to determine the *fate* of the cadmium ion once in the plant, i.e. whether it is transported to different organs, stored or metabolized. A relevant implication of this is that studies have shown Cd^{2+} to be selectively accumulated in different plant organs, which while not phytotoxic at this level, can be bioaccumulated or pose significant health risks to humans and mammals (Peralta-Videa et al, 2009, EPA 2000; Haghiri, 1973).

Acknowledgments

I would like to thank Dr. Santokh Singh for advice and support throughout this project, and Mr. Jarnail Mahroke and for technical help. Thank you to Brenda Sawada from UBC SEEDS for coordinating this project, and to Kelly Coulson for supplying seeds of various grasses.

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