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Chemically Induced Phytoextraction of Lead (Pb) Contaminated Soil by Switchgrass (Panicum virgatum L.)

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Chemically Induced Phytoextraction of Lead (Pb) Contaminated Soil by, Switchgrass (*Panicum virgatum L.*)

A Thesis Presented in Partial Fulfillment of Requirements for the Master of Science in Integrative Biology for the Department of Ecology, Evolution, and Organismal Biology

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Abstract

Soils contaminated with lead (Pb) from human activities including industrial byproducts create an environmental and human health risk. Phytoextraction through chemical acquisition of Pb in soils offer a potentially cost effective and ecologically driven approach to remediation. Switchgrass (*Panicum virgatum* L.) is known to have a tolerance for a wide range of heavy metals including Pb. Switchgrass is also able to grow in a wide range of climates. Soil chemistry manipulation, using chelates and plant growth promoters could provide an effective field method for phytoremediation using switchgrass. In this study chemically enhanced phytoextraction using the soil-fungicide Infuse (active ingredient propiconazole), the chelator nitrilotriacetic acid (NTA), and the phytohormones salicylic acid (SA) and benzylaminopurine (BAP) were tested on switchgrass grown in Pb contaminated soil from a former superfund site in Cedartown, GA. The soil chemical manipulation aimed at increasing the bioavailability and uptake of Pb by switchgrass. Previously, the chelate ethylenediaminetetraacetic acid (EDTA) was found to be effective in increasing the bioavailability of Pb in soils but the main problem with its use was a long persistent time in soils. The chelator NTA is a derivative of EDTA which has been found to persist for a much shorter length of time in soils comparatively and in this study, is tested as an alternative to EDTA.

Switchgrass was planted in 5L pots containing Pb-contaminated soil and grown in the Kennesaw State University Research Greenhouse. Pots were given 200 ml of nutrient solution twice a week for six months. On 49 days after planting (dap), foliar application of the phytohormones salicylic acid and benzylaminopurine were sprayed on target plants twice a week. On 170 dap, Infuse solution was given to target plants. On 184 dap, the NTA solution was

given to target plants twice a week for a one-month period. The NTA solution was brought to pH 5.5 using citric acid. Increased soil acidity would increase the bioavailability of Pb. The NTA solution was applied alongside the soil fungicide Infuse for suppression of symbiotic arbuscular mycorrhizal fungi with the aim of increasing Pb uptake. On 226 dap, plants were harvested. Acid digested plant samples were analyzed for Pb using an ICP-OES instrument at the Chemistry Department of Kennesaw State University.

Soil application of NTA and Infuse significantly increases the concentration of Pb in the foliage of plants in all treatments over Control plants and the standard Infuse treatment. Application of the soil fungicide Infuse containing resulted in a significantly higher concentration of Pb in plants foliage compared to Control plants. The difference in Pb concentration of plants foliage among plants in different NTA treatments was insignificant but the highest average Pb concentration was found in the NTA treatment without foliar phytohormone application. This suggests that the phytohormones SA and BAP could play a role in a resistance to heavy metal toxicity. Biomass increases were insignificant between all treatments. This indicated that under the NTA application, the phytohormones did not have a great effect on biomass among treatments.

The NTA application demonstrated that using this chelator will significantly increase translocation and concentration of the heavy metal Pb in switchgrass above ground biomass. The application of NTA alongside the soil fungicide Infuse can provide the tools for a viable technique for phytoremediation of Pb contaminated soils without potentially harmful effects of long-term persistence.

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Abbreviations

Calculations

PE	Phytoextraction Efficiency

Chemical Names

BAP	Benzylaminopurine (Benzyl Adenine)
EDTA	Ethylenediaminetetraacetic acid
NTA	Nitrilotriacetic acid
SA	Salicylic Acid

Elements

Al	Aluminum
C	Carbon
N	Nitrogen
Pb	Lead
Zn	Zink

General

AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of variance
CDC	U.S. Centers for Disease Control
DI water	Deionized water
DM	Dry Mass
EPA	U.S. Environmental Protection Agency
ICP-OES	Inductively coupled plasma - optical emission spectrometry
LSD	Least Significant Difference
ROS	Reactive Oxygen Species

Treatment groups

BAP	Benzyladenine+Infuse+NTA
I	Infuse
IN	Infuse+NTA
SA	Salicylic acid+Infuse+NTA

Introduction

Lead Contamination and Health Effects

Industrialization has brought upon several pollutants that are harmful to human health [Hu, et al. 2013; Järup, 2002]. In particular, adverse health effects of lead (Pb) exposure include for example: poor muscle coordination, nerve damage, hearing and vision impairment, organ damage and developmental disorders [Mason, 2014]. Lead toxicity is especially harmful for children since their blood brain barrier is not yet developed allowing for increased neurological disruption [Järup, 2002]. Currently there is no blood lead level known that is not considered harmful to human health and it is predicted that reduction in Pb exposure could result in improved healthcare costs [Järup, 2002; Paulson, 2019].

The wide environmental dispersal of Pb contamination today was mainly from Petrol emissions. In addition, Pb contamination is further spread away from contamination sources like mining sites, smelting sites and old industrial sites [Hovsepyan and Greipsson, 2004; Järup, 2002; Zhuang, et al 2009; Li, et al. 2020]. After the 1970's, Pb contamination has decreased in developed countries especially with the introduction of unleaded petrol but leaded petrol contributed to about 50% of initial Pb emissions worldwide [Järup, 2002]. Highly contaminated sites near pollution sources are expensive to remediate for beneficial land uses [BenDor, 2011]. The average background level of Pb in uncontaminated urban area soils is typically 10 mg kg^{-1} [BenDor, 2011]. Modern Pb removal methods, such as excavation and soil washing are usually considered too expensive commercially compared to the rise in land value that comes with the remediation [BenDor, 2011]. *Ex-situ* methods usually cause a high level of disturbance when removing contaminants and could lead to secondary pollution from runoff [Park, 2010].

Current Remediation Methods for Contaminated Soils

Soil has a critical function in the environment and heavy metal contamination can reduce biodiversity of microorganisms which in turn can affect soil productivity [Fiorentino, et al. 2018]. Traditional, engineering methods of soil remediation has involved: soil washing, soil leaching, or excavation and removal [Khodadoust, et al. 2005]. Soil washing with water is usually not efficient and stronger selective chemicals are needed [Wuana, 2011]. However, due to the use of chemicals, important nutrients for plants will be washed away [Wuana, 2011]. Moreover, the high cost (30-100 USD per m^3) of engineering methods of soil remediation impedes widespread uses [Gleeson, 2007; Cunningham, et al. 1995].

Switchgrass as a Target of Phytoextraction

Phytoremediation has emerged as an environmentally friendly and cost-effective alternative to current engineering remediation practices [Lasat, M. 2002, Gleeson, A. 2007]. The ideal plant for phytoremediation application would preferably have certain characteristics. A promising plant should be a hyper accumulator of heavy metals in the aboveground tissues and be a fast-growing plant with high biomass yield [Arora, et al. 2016]. Unfortunately, most heavy metal hyper accumulating plants do not produce high biomass [Oh, et al. 2013]. On the other hand, switchgrass (*Panicum virgatum* L) produces high biomass but is not classified as a hyper accumulator of heavy metals [Shrestha, et al. 2019]. However, switchgrass is found to be resistant to heavy metal toxicity [Arora, et al. 2016]. Furthermore, the ability of switchgrass to accumulate Pb in its tissues given various soil chemical manipulations were examined in this study.

Switchgrass is particularly attractive for use in phytoremediation since it can survive in a wide range of climates and studies have shown improved metal uptake through chemical addition [Gleeson, 2017; Arora, et al. 2016; Hovespyan and Greipsson, 2004]. Switchgrass has demonstrated exceptional ability to grow on marginal land which could be attributed to a recent discovery of a nitrogen (N_2) fixing bacteria (*Flavobacterium nitrogenifigens sp. nov.*) found within the grass's rhizosphere [Kämpfer, 2015]. In addition, switchgrass is able to efficiently use soil nitrogen to build optimal biomass although chemical fertilizers usually increase the biomass of switchgrass under agricultural conditions [Bahulikar, et al. 2014]. Addition of chemical fertilizers can have adverse effects on the environment through eutrophication of groundwater [Bahulikar, et al 2014]. Switchgrass has been found to contain expression of many nitrogen fixing bacterial genes which should aid in the production of high amount of biomass in marginal lands with low soil nutrients [Zhuang, et al. 2013; Shrestha, et al. 2019; Bahulikar, et al. 2014]. This makes switchgrass potentially capable of sustainable growth and therefore suitable for phytoremediation of Pb contaminated sites [Gleeson, 2017, Beavers, 2016]. Altogether, the characteristics of switchgrass make it a great candidate for phytoremediation resulting in reduction of Pb from contaminated soils over a period of several years [Gleeson, 2007; Beavers 2016; Arora, et al. 2016].

Phytoextraction by Switchgrass as a Second-Generation Bioenergy Crop

Phytoextraction has been found to be useful in remediating contaminated soils. Adding on the economic incentives the use of bioenergy crops in phytoextraction has shown some promises (Balsamo, et al. 2015). A second-generation bioenergy crop is defined as a non-food producing crop that can be used and produced sustainably for biofuel [Antizar, et al. 2008]. The UN definition of a second-generation bioenergy crop is similar but adds that second-generation biofuels are made from ligno-cellulosic biomass feedstock using advanced technological processes [Antizar, et al. 2008]. Switchgrass is classified as a second-generation bioenergy crop due to its short growth time relative to its biomass production and its capability to be sustainably grown [Sanderson and Paul, 2008]. Switchgrass is a low input high yield bioenergy crop that takes 3 years to reach full maturity and can produce large amounts of above ground biomass with minimal agricultural inputs [Pogrzeba, et al. 2019; Daverdin, et al. 2014]. Switchgrass also has a low soil nutrient removal rate for its high biomass yield and since switchgrass is a hardy plant that can live in a wide range of environments, it is a good candidate to treat heavy metal contaminated sites while being grown as a second-generation bioenergy crop [Sanderson and Paul, 2008; Gnomes, 2012; Van Ginneken, et al. 2007]. Studies have found that under adverse conditions, switchgrass will grow and translocate Pb to above ground biomass [Gleeson, 2007; Beavers, 2016; Aderholt, et al. 2017].

Metal Uptake in Grasses and Fe homeostasis

Grasses take up actively various metals in the soil as macronutrients, this includes iron (Fe), copper (Cu) and zinc (Zn) [Watt, et al. 2006]. Most heavy metal contaminants are thought to be inadvertently taken up by plants through cation channels since common plant nutrients like Ca, Fe, and Mg cations have the same number of valence electrons heavy metals like Pb [Tangahu, et al. 2011]. The uptake and translocation of heavy metals to above ground biomass in plants is strongly dependent on the pH of the soil. Metals are much more likely to be taken up by plants when soil pH is below 6.0 to 5.5 [Roy, et al. 2005, Fuentes, et al. 2018]. When soils are below pH 5.2 aluminum ions (Al^{3+}) starts to dissolve into solutions replacing calcium (Ca) and magnesium (Mg) which may become phytotoxic to plants [Chaney and Baklanov, 2017]. When soils are lower than pH 5.0, plants may suffer irreversible physiological damages [Roy, et al. 2005].

When metals bind to the soil clay particles they are less bioavailable to the plants [Roy, et al. 2005]. Metals have been shown to be more readily available in hydrated environments where movement is easier for elements [Fritioff and Maria, 2003]. Most plants perform oxidation reduction reactions with root secretions to create an environment where bioavailability is increased, so soil mobility is key for increased heavy metal uptake outside of the root zone [Tangahu, et al. 2011]. The ability of plants to acquire metals is dependent on the root zone. For a plant to even take up nutrients the molecule must be inside the rhizosphere which is an area of about 2-3 cm around the root where nutrients are taken up by root hairs [Watt, et al. 2006]. Factors effecting the uptake of nutrients within the rhizosphere are root diameter, root elongation, and the ability of the root to move through the soil [Watt, et al. 2006].

Iron (Fe) is a necessary element for processes that carry out electron transfer functions or enable chemical transitions [Connorton, et al. 2017]. In plants when there is an excess of heavy metals Fe homeostasis is disturbed and chlorosis is observed in the foliage [Lešková, et al. 2017]. An excess of heavy metals in the soil can out compete the uptake and transport of Fe by the Iron-Regulated Transporter (IRT1) [Lešková et, al. 2017]. Heavy metals like Zn and Pb share a similar chemical structure and behavior to Fe and can be transported by IRT1 [Lešková, et al. 2017]. In addition, grasses excrete siderophores, iron-chelating compounds that improve bioavailability and translocation of Fe [Connorton, et al. 2017]. In grasses these siderophores are transported into the plant through the oligopeptide transporter YS1, but studies show that grasses have an IRT1 or IRT1 homolog that transports Fe as well [Connorton, et al. 2017].

Chelation and Phytoextraction

Lead (Pb) in highly contaminated soils may not always be bioavailable to plants [Roy, et al. 2005]. Usually, increased mobility of the Pb in the soil results in higher amount of Pb in plants [Johnson, 2014]. In soils with pH of greater than or equal to pH 5.0 and with at least 5% organic matter, atmospheric deposited Pb is retained in the upper 2-5 cm of undisturbed soil [EPA 1986]. This allows for the switchgrass roots to get to the target depth at full growth. For the switchgrass to acquire Pb, first it must become mobile in the soil. Lead can be mobilized in the soil by the addition of heat, oxidation, or a change in pH [Filgueiras, et al. 2002]. For this mobilization, heating the dirt or oxidizing it can be detrimental to the soil and expensive, but lowering the pH through chemical manipulation of the soil is more practical in a remedial application. Chelation of Pb in soils by combining the natural processes of switchgrass with the manipulation of soil chemistry using organic or aminopolycarboxylic acids has been shown to increases the bioavailability of Pb [Beavers, 2016; Perry, et al., 2012; Aderholt, et al. 2017]. The chelator ethylenediaminetetraacetic acid (EDTA) was used in these studies. This chelator is found to be effective in increasing the bioavailability of Pb. Concerns with EDTA included Pb making its way into the ground water, the long persistence time of EDTA, and its resistance to breakdown by soil microbes [Bucheli & Egli, 2001; De Araújo et al. 2010].

NTA (Nitrilotriacetic acid) is a derivative of EDTA and has been shown to have similar effects. The NTA is seen to be a possible alternative since NTA is more readily biodegradable in soil compared to EDTA and is more economically available due to its commercial mass use as phosphate free detergents [Naghipour, et al., 2016, Elliott and Brown 1989]. Environmental persistence is the primary reason natural acids like citric acids being tested in lieu of EDTA and that is why a solution of NTA and citric acid together is promising [De Araújo, et al., 2009, Fuentes, et al., 2018] In the Naghipour, et al, (2016) study phytoextraction efficiency increased as pH was lowered and with the substitution of NTA increased heavy metal bioavailability. Though EDTA has had more effective results in most treatments, NTA persists for less time in soil due to its biodegradability and still produces significant increases in Pb concentration [Hu, et al. 2017].

Arbuscular Mycorrhizal Fungi (AMF) in Phytoextraction of Metals

Plant soil interactions go beyond the soil chemistry and plant excretions. AMF form symbiotic relationships with about 80% of land plant species, and AMF are found to improve plant biomass and tolerance to abiotic stress [Wang, 2017]. Symbiotic relationship of plants with AMF benefits the plant by creating a site of chemical exchange influencing nutrient exchange including metals like Fe and Zn. In exchange for these micro-nutrients the plant will provide the AMF with sugars from its photosynthetic processes [Bonfante and Marc-André, 2010]. AMF also play a role in heavy metal toxicity by impacting the interactions between metals and roots [Khan, 2005; Yang et al. 2016]. Heavy metal exposure creates oxidative stress for the AMF, this is from formation of reactive oxygen species (ROS), resulting in differential gene expression in extra radical mycelia responsible for encoding the proteins potentially involved in heavy metal tolerance [Hildebrandt, et al. 2007; Upadhyaya, et, al. 2010]. To offset ROS's AMF can produce antioxidant enzymes to alleviate heavy metal stress [Sarkar, 2018]

For phytoextraction purposes, the effects of AMF and Pb uptake in switchgrass have been studied. Hovsepyan and Greipsson, (2004) examined responses of corn (*Zea mays* L.)

where suppressing the AMF through the use of soil fungicide (benomyl) resulted in improved uptake and translocation of Pb into the plant's foliage. Iron is essential in photosynthesis and Pb can interfere and disrupt photosynthesis in plants, but increased Fe concentration in plants can offset the disruption of photosynthesis from Pb [Tamayo, et al. 2018,]. Physiological responses of heavy metal contamination include chlorosis and stunted growth from reduced uptake of Fe from competition of other heavy metals such as Pb and Cd [Lešková, et al. 2017]. When plants grown in contaminated soils show chlorosis it is likely that Fe is being out competed by Pb [Tamayo, et al. 2018; Lešková, et al. 2017]. Recently, AMF have been found to play a critical role in Fe acquisition and could possibly aid plants to overcome heavy metal toxicity [Tamayo, et al. 2018]. Studies indicate that improved uptake of Pb can be achieved with the application of benomyl before the addition of the chelate EDTA rather than with simultaneous applications [Perry, et al. 2012]. Previous studies with the fungicide benomyl showed reduced AMF colonization in root cells and subsequently increased uptake of Pb with in switchgrass [Aderholt, et al. 2017].

Growth Promotion of Switchgrass with Benzyladenine and Salicylic Acid

This study examined the efficacy of the phytohormone Benzyladenine (BAP) in phytoextraction. Benzyladenine is a cytokine that stimulates cell division in plants and promotes growth [ACS 2016]. Cytokines take part in several stage of plant development and growth processes. Using BAP with switchgrass could potentially provide an increase in biomass and increase Pb concentration in the above ground biomass. BAP was reported to stimulate stomatal opening in *Commelina benghalensis* and *Tridax procumhen* [Tanaka, 2006]. If BAP stimulates stomatal opening in switchgrass, this may increase transpiration while likely increasing Pb uptake and translocation in the plant.

Salicylic acid (SA) is another plant hormone. It is a hydroxybenzoic acid ($C_7H_6O_3$) and was first found in willow trees as a derivative of naphthalene [ACS 2015]. The role of SA is to regulate plant response during abiotic stress while playing a crucial role in the regulation of physiological and biochemical processes of plants throughout their lifespan [Vicente and Plasencia, 2011]. SA has been shown to promote plant growth and was found to reduce the inhibitors of nitrogen reductase activity in some plants [Zottini, 2007]. Reduced nitrogen reductase inhibitors should result in increased growth of switchgrass. SA also plays a role in stress tolerance of plants and could promote growth of plants in Pb contaminated soils [Horváth, 2007]. The important role of SA in ameliorating heavy metal stress in plants was recently reviewed (Sharma et al. 2020).

Aims and Hypotheses

Past studies examined the chelator EDTA and its effects on Pb uptake by *panicum virgatum* (Switch grass) with soil-chemical application in tandem with plant hormones. As previously stated, the chelator EDTA persists in soils longer than preferred and the chemical's derivative NTA has been found to not persist in the soil as long. The Hovespan and Greipsson, (2004) study showed that the suppression of AMF by the fungicide binomial increased metal uptake. The fungicide Infuse will be tested to see if the application will significantly increase Pb uptake in switchgrass. The aims of this study are to test the application of the EDTA derivative NTA, a chelator, on the plant *Panicum virgatum* (switchgrass) since NTA could act as more environmentally friendly replacement for EDTA. Along with the soil chemical application of NTA, foliar application of the plant hormones salicylic acid and Benzylaminopurin will be tested to access if the application will increase plant biomass even under the effects of chelators. The hypothesis of this study is the soil chemical application of NTA will significantly increase the Pb translocation in switchgrass, the hormones will significantly increase the plant biomass, and combined will provide insight into a viable method for the phytoremediation of Pb contaminated areas.

Material and Methods

Greenhouse Experiment

This study evaluated chemically enhanced phytoextraction technique where switchgrass was subjected to foliar application of growth hormones and given soil fungicide and chelate solution. This was to test and access if there would be a significant increase in uptake of Pb using the fallowing experimental method. Switchgrass was subjected to the following treatments: (1) Control (CO), (2) Infuse (I), (3) Infuse & NTA (IN), (4) Salicylic acid (SA) + Infuse & NTA, (5) 6-Benzyladenine (BAP) + Infuse & NTA. Pots (5 L) were filled with Pb contaminated soil (3800 ppm Pb) obtained from a former Superfund site in Cedartown, GA. The "Alamo" variety of switchgrass seeds were obtained from the University of Georgia. Pots were placed in a randomized block design on the greenhouse bench, filled with Cedartown soil, and 25 seeds of Alamo switchgrass were mixed into the top 2 cm of surface soil on the 1st dap (June 1^{st,} 2019) which was marked as day of planting (dap). Pots were re-randomized weekly on the greenhouse bench to eliminate potential differences due to light and temperature variation within the greenhouse. Plants were grown under controlled environmental conditions in the Science Greenhouse at Kennesaw State University, Kennesaw, GA. Pots were watered with 200 mL of nutrient solution twice a week until the Infuse and NTA treatments started. The watering schedule and volume was chosen to eliminate runoff through the drain holes in the pot. Foliar application of the plant growth hormones salicylic acid (SA) and 6-Benzyladenine (BAP) began on 49th dap (July 20th) and was applied twice a week. The soil fungicide, propiconazole (trade name Infuse®) was used (2 ppm) in order to suppress symbiotic arbuscular mycorrhizal fungi (AMF) starting 170th dap (December 13th). On the 184th dap application of the chelate NTA (nitrilotriacetic acid), was started (200 mL, 5 mM solution kg^{-1} soil) and continued twice a week until plants were harvested on 226th dap (January 13th). Final plant height was measured after harvesting. Then the number of dead or yellowing (chlorosis) leaves and the number of live leaves were recorded.

Soil

The soil used for this experiment was collected from a former Superfund site in Cedartown GA. Tough the land had been cleared by the EPA highly contaminated soil with Pb was still found. The soil was prepared prior to planting by sieving and removal of large debris. After removal of large debris, the soil was thoroughly mixed to create a homogenous mixture and ensure uniform Pb concentration in all treatments.

BAP (Benzyladenine) Foliar Application

The BAP solution used for foliar application was 1uM. To make the solution. The foliar application of BAP started 39th dap. The BAP solution was created by was dissolving .0252 g of BAP powder in serially diluted DI water to the desired concentration of 1uM. Foliar application of BAP was performed once a week for the greenhouse experiment. Foliar application of BAP was performed in the greenhouse with a spray bottle. Comparison of plants in different treatments with or without BAP application was used to determine the effect of foliar hormone application on shoot length, biomass, and Pb.

Salicylic acid (SA) Foliar Application

Foliar application of a 150 ppm aqueous solution of salicylic acid was applied once a week, with the same method as the BAP solution. Salicylic acid solution was made by mixing 150 mg salicylic acid powder in 1000 mL of water in a beaker. Comparison of plants in different treatments with or without SA application was used to determine the effect of foliar hormone application on shoot length, biomass, and Pb uptake concentrations of plant tissues.

Infuse Application

Suppression of AMF has previously been shown to increase the Pb uptake of plants. Infuse is a demethylation inhibiting fungicide, that binds to a dimethyl enzyme and inhibits cell growth [Infuse SDS]. Application of Infuse was performed in a 2 mg L^{-1} solution and began 6 months after planting. Application of Infuse was at previously stated concentration with 100 mL of water per each pot of target treatment. Infuse was added before chelating agent when applied on the same day. Infuse can last up to a month in soils, but for bio amplification Infuse was applied twice a week along with the NTA solution. Switchgrass has been targeted for biofuel production on marginal lands and the long-term use of Infuse on surrounding areas must be further tested.

Application of the Chelating Agent NTA

The chelation agent NTA was applied to the plants after fungicide (Infuse) application and during the SA and/or BAP treatments. A 5mM NTA kg^{-1} soil solution was chosen. The NTA solution (5 mM kg^{-1}) was made by dissolving 2.47 g NTA powder in a 2000 ml beaker. The NTA powder was mixed in 1000 ml of DI water and NaOH solution (10 N) was carefully added until the NTA powder was dissolved. The 10N solution of NaOH solution was made by dissolving 20g of NaOH pellets in 10 mL of water. The NaOH was added to the NTA solution till fully dissolved. The solution was then brought to 1800 ml where the adjustment of the pH began. The NTA solution was about pH 8.0. The NTA solutions was adjusted to the desired pH 6.0 for optimal soil acidification. To lower the solution's pH, citric acid was carefully added until the desired solution pH 6.0. Twice a week, plants were treated with 200 ml of the NTA solution until severe chlorosis was observed in plants. The acidified NTA solution reduced the soil pH which in turn allows for the Pb in the soil to be more bioavailable.

Harvest and Analysis

After switchgrass plants formed flower heads they were measures for final shoot length. The entire plant was harvested carefully with roots and shoots collected and carefully rinsed. Each plant sample was carefully observed with number of leaves, the number of dead/ yellow, and the number of live leaves recorded. After rinsing plants were separated at the basis of roots with precession in order to not cross contaminate samples. The switchgrass was then placed carefully in paper bags, labeled and put in an oven at 65^{o}C for 48 hours. After drying the plants were placed on a scale and weighed for DM.

Acid Digestion and Chemical Analysis

The dried plant material was acid digested prior to chemical analysis by the ICP-OES at the Kennesaw State University Chemistry Department as described by Perry, et al. (2012). Plant tissue (0.5 g) was digested in Fisher Scientific® ACS grade 38% HCl (10.0 mL) and Fisher Scientific® ACS grade 70% HNO_3 (10.0 mL) in Environmental Express® 100.0 mL plastic digestion tubes, allowed to sit at room temperature for 24 hours, then refluxed at 95°C in an Environmental Express® HotBlock system for 50 minutes. Samples were allowed to cool at room temperature and the volume was brought to 100.0 mL with trace-metal grade distilled water. Diluted samples were vacuum filtered prior to ICP-OES analysis. Digested shoot and root samples were analyzed for Pb using an ICP-OES instrument at the Chemistry Department of Kennesaw State University.

Data Analysis

The performance of the switchgrass phytoextraction of Pb from the soil was measured by difference in Pb concentration between treatments and the phytoextraction efficiency. Phytoextraction efficiency (PE), of each treatment, is calculated by taking the average concentration Pb in the live leaves for the treatment and multiplying it by the average DM of the treatment switchgrass. The PE was calculated for to represent an average variable which could accurately compare the efficiency of Pb concentration and uptake per g of switchgrass DM.

DM was recorded in excel. The DM was averaged to compare differences between treatments especially between the phytohormone treatments. Longest leaf, number of leaves, dead and live leaves, and the dead leaf to life leaf ratio was all calculated with averages by pot (3-4 plants per pot and 4 pots per treatment) then averaged by treatment. Standard deviation was then calculated per treatment.

Data was analyzed using one-way analysis of variance (ANOVA) followed by post hoc Fisher's test for Least Significant Difference (LSD) using Excel ver 15.33. Statistical significance was accepted at the level of $p < 0.05$.

Results

Longest leaf

There were no significant differences between treatments for the average value of longest leaf (figure1). Plants treated with SA had on average longest leaf of 0.93m (Figure1). Plants treated with IN had on average longest leaf of .929m (figure1). Plants treated with I had on average longest leaf of .925 (figure1). Lastly plants treated with BAP had on average longest leaf of .872m (figure 1).

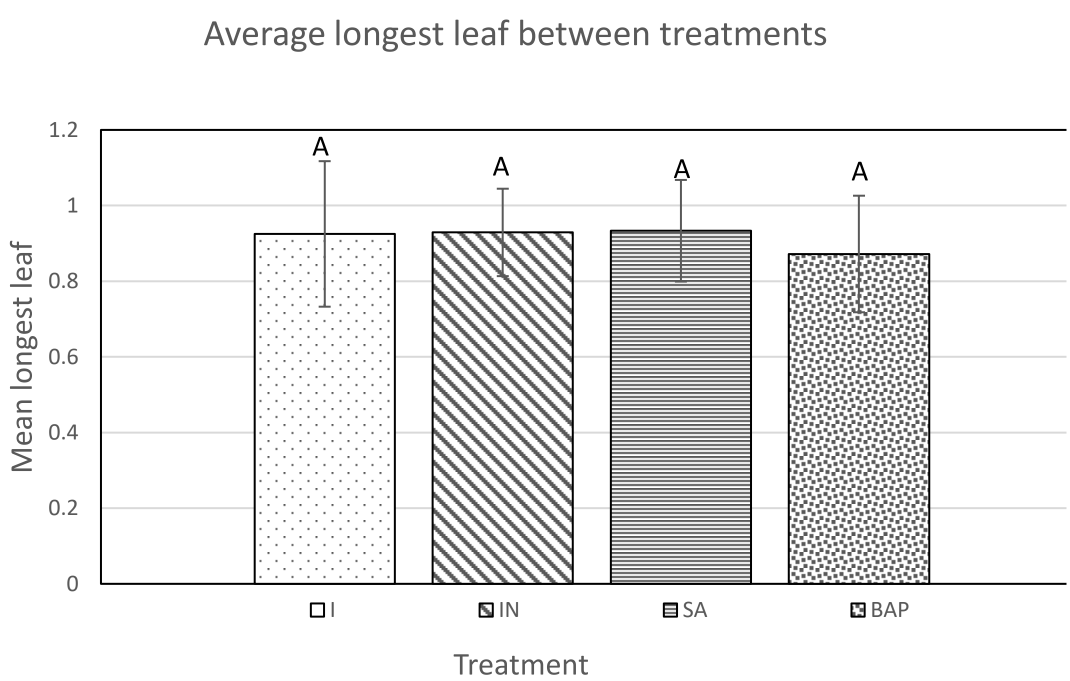

Figure 1. Average Length of Longest Leaf (m) (±SD) of *Panicum virgatum* at final harvest. Treatments labeled: I = Infuse, IN = Infuse/NTA, BAP = Benzyladenine+Infuse+NTA, SA =

Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

Above Ground Biomass

Plant Dry mass (DM) did not differ significantly between all treatments. The highest DM (8.78g) was found in plants treated with I (figure 2). Plants treated with BAP had an average DM of 6.90 g (figure 2). Plants treated with IN had an average DM of 6.50 g (figure 2). Plants treated with SA had the lowest average recorded DM of 5.80g (figure 2).

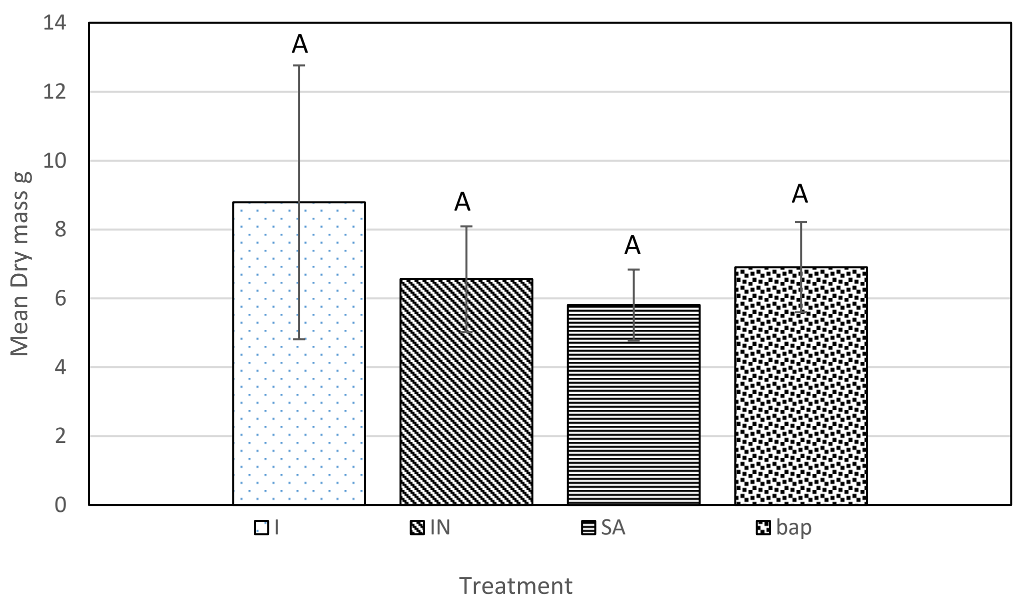

Figure 2. Average Foliage Dry Mass (g) (±SD) of *Panicum virgatum* at final harvest. Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

Observed Chlorosis

All plants displayed chlorosis of leaves from the negative effects of the chelating agent NTA that was observed in harvest plants (figure 2). Plants treated with I had significantly lower proportion of chlorotic leaves compared to plants in other treatments (figure 4). Plants in all treatments involving NTA showed significantly higher proportion of chlorotic leaves compared to plants treated with I (figure 4).

A: **B:** **C:**E

Figure 3. Examples of normal, deformed, and chlorotic leaves from (IN) treatment. A: (left) Chlorotic leaves showing display of Pb toxicity; B: (middle) Deformed leaf from Pb contamination; C: (right) Normal green leaf.

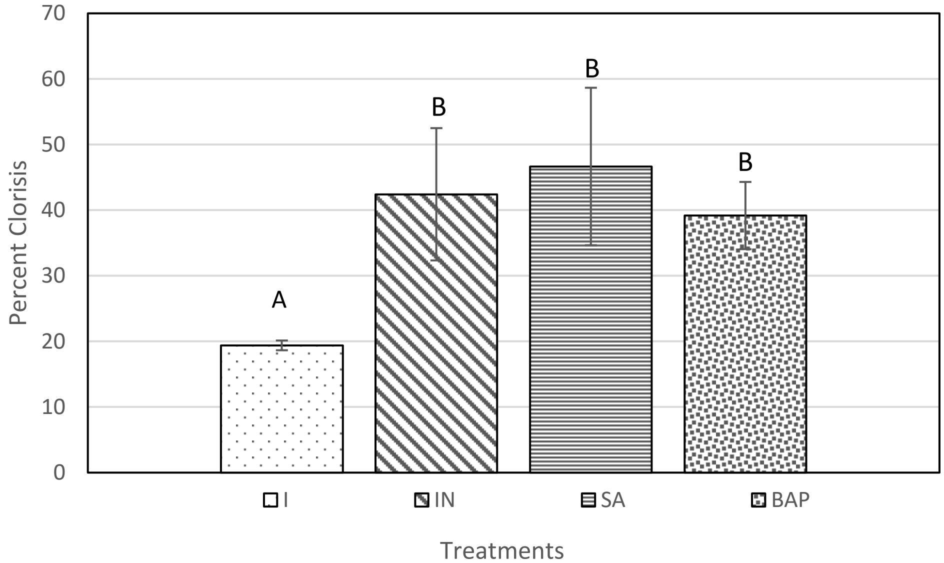

Figure 4. Average percent chlorosis (±SD) of *Panicum virgatum* at time of harvest. Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

Lead (Pb) Concentration

Analysis of Pb concentration in above ground biomass revealed that there were significant differences between treatments. Control plants had significantly lower Pb concentrations in the foliage compared to plants in the following treatments I,IN,BAP, and SA (Figure 5) ($p<.001$). Plants in the following treatments IN, BAP, and SA had a significantly higher Pb concentration than plants in the I treatment (Figure 5) ($p<.001$). Plants treated with IN had the highest average Pb concentration of 996 mg kg^{-1} (Figure 5), this value was 3820% higher than the ones of Control plants (figure 6), this value was 500 % higher than the ones of plants treated with I (figure 7). Plants treated with BAP had an average Pb concentration of 858 mg kg^{-1} (figure 5), this value was 3270 % higher than the ones of Control plants (figure 6) and 417% higher than the ones of plants treated with I (figure 7). Plants treated with SA had an average Pb concentration of

738 mg kg^{-1} (figure 5), this value was 2980 % higher than ones of Control plants (figure 6) and 372 % higher than ones of plants treated with I (figure 7). Plants treated with I had an average Pb concentration of 165.84 mg kg^{-1} (figure 5), this value was 552 % higher than the ones of Control plants (figure 6).

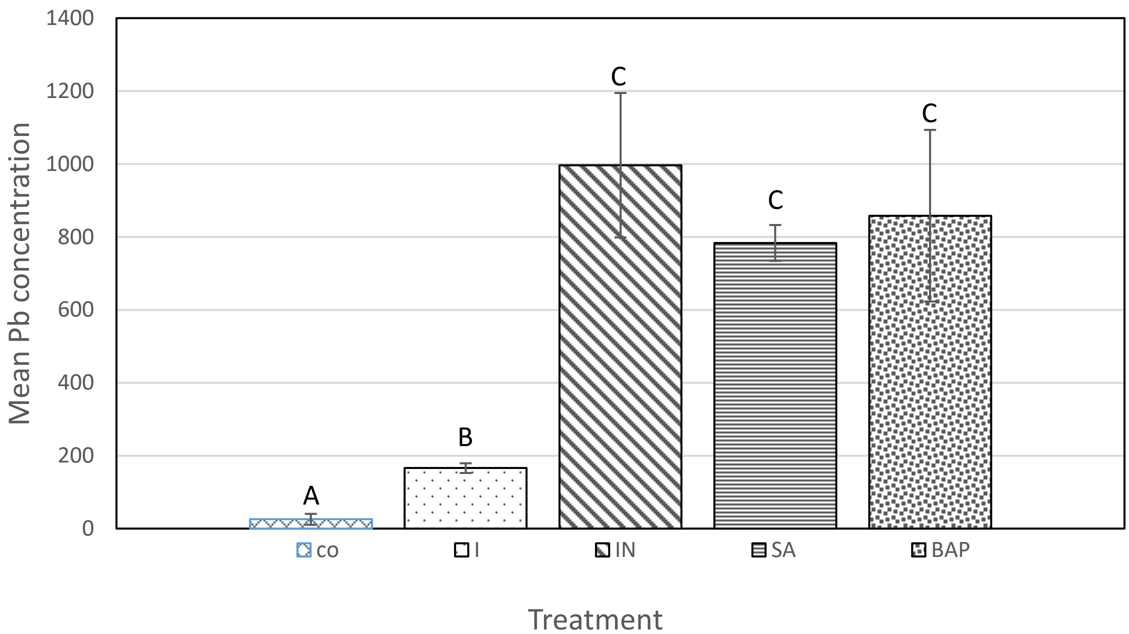

Figure 5. Average Pb concentration (mg kg^{-1}) (±SD) of *Panicum virgatum* at final harvest. Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

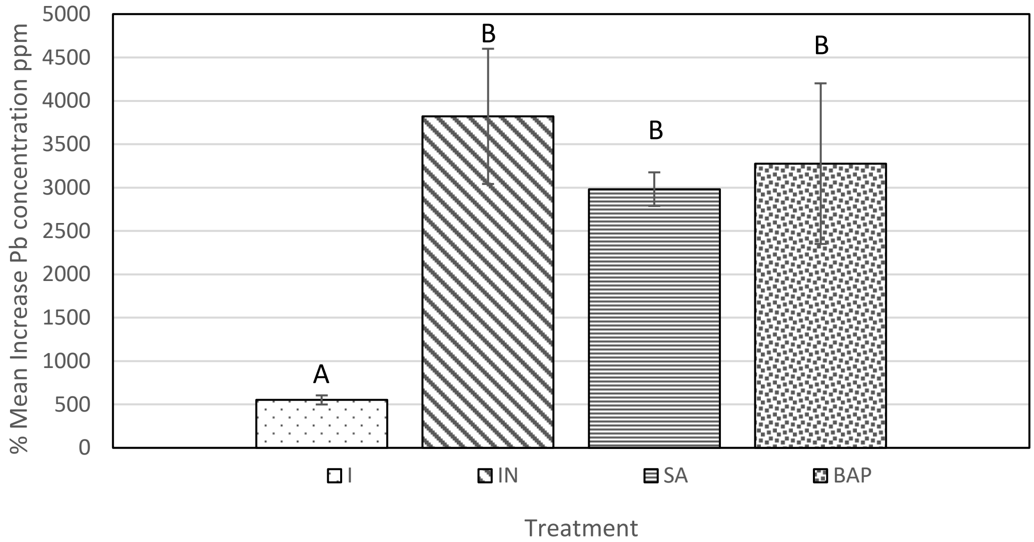

Figure 6. Percent increase concentration ppm (±SD) over controls of *Panicum virgatum.* Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

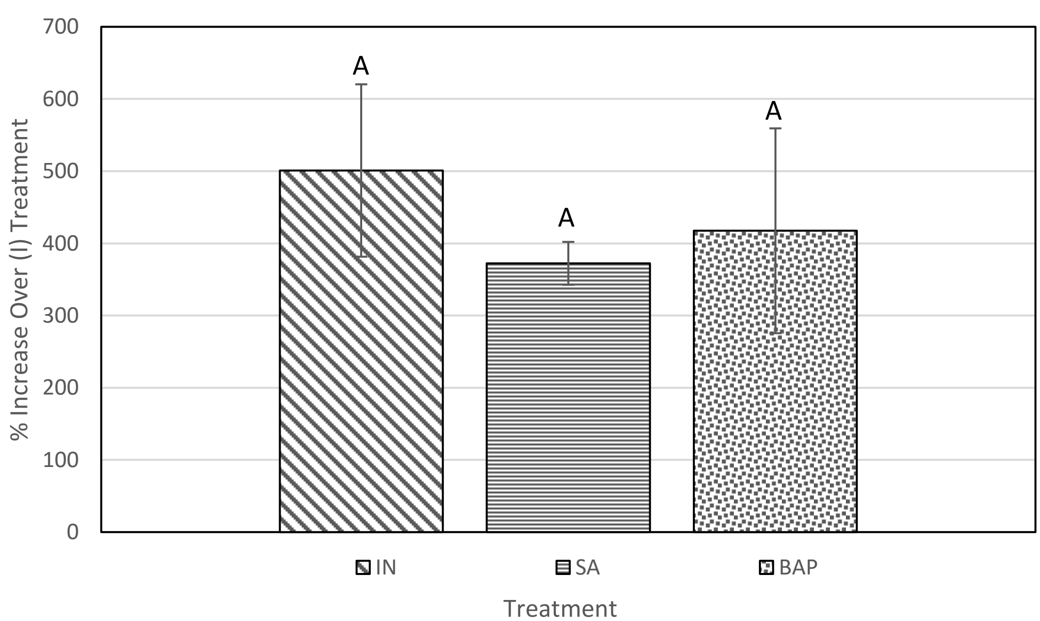

Figure 7. Percent increase concentration ppm (±SD) over (IN) treatment *Panicum virgatum.* Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

Phytoextraction Efficiency

Phytoextraction efficiency (PE) calculations were carried out to determine a relative extraction value to compare between treatments. The PE was calculated by multiplying the mean live leaf number of the treatment by the mean DM of the treatment to obtain a PE value. Control plants had the significantly ($p<.001$) lowest PE value compared to plants of other treatments (figure 8). Plants treated with I had significantly ($p <.001$) lower value of PE compared to plants in treated with NTA (i.e. IN, SA, BAP) (figure 8). The highest PE value of 6590 was found in plants treated with IN (figure 8), this value was 83,300 % higher the PE value of Control plants (figure 9). Plants treated with BAP had a PE value of 5,920 (figure 8), this value was 75,600 % higher than the PE value of Control plants (figure 9). Plants treated with SA had a PE value of 4540 and this value was a 58,000 % higher than the PE value of Control plants (figure 9). Plants treated with I had a PE value of 145 (figure 8), this value was an 18,500% increase over control PE (figure 9).

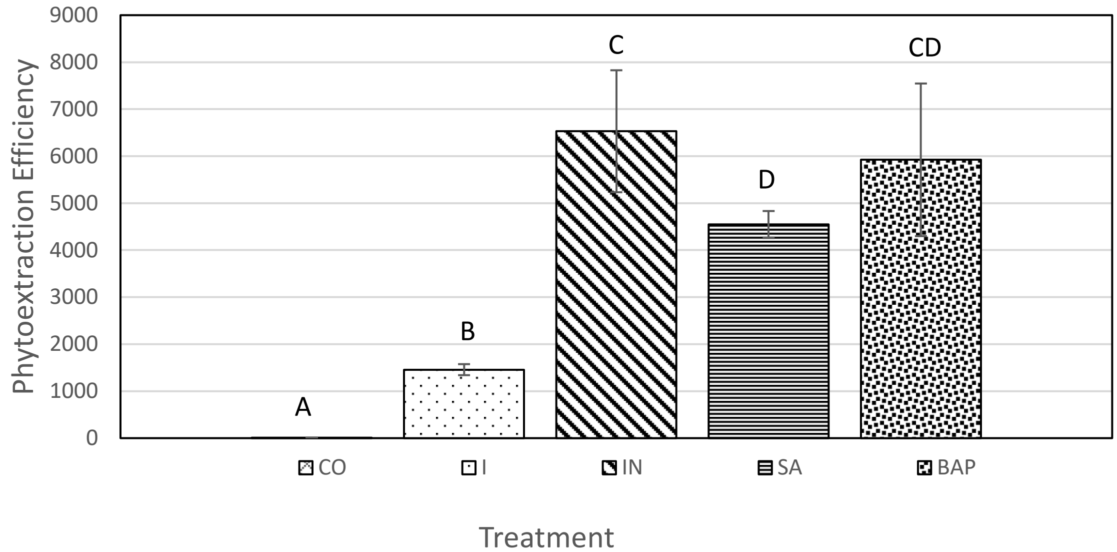

Figure 8. Phytoextraction efficiency (±SD) of *Panicum virgatum* at time of harvest. Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

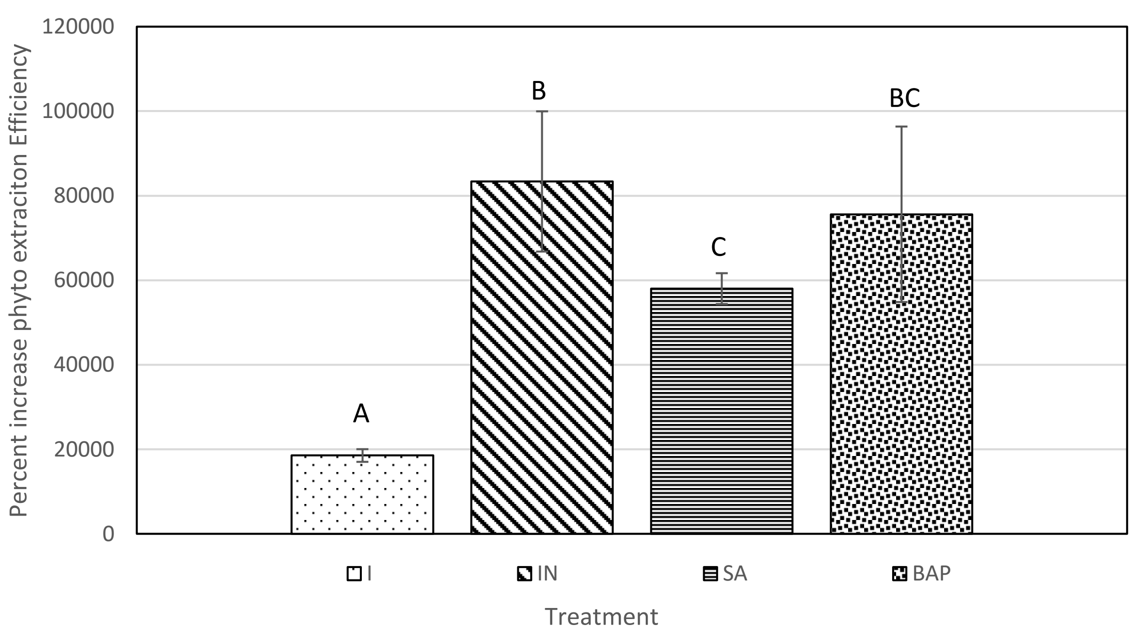

Figure 9. Percent increase in efficency (±SD) of *Panicum virgatum* at time of harvest. Treatments labeled: I = Infuse, IN = Infuse+NTA, BAP = Benzyladenine+Infuse+NTA, SA = Salicylic acid+Infuse+NTA. Means for columns with same letter are not significantly different (H_a>.05).

Discussion

Phytohormones, Biomass Production, and Pb Uptake

One aim of this study was to determine if different phytohormones affected overall biomass and growth of plants. Results are consistent with previous a study on switchgrass revealing the longest leaf does not coincide with having the greatest DM [Aderholt, et al. 2017]. However, the phytohormone application was not only standalone and applied along with the chelate NTA instead of EDTA as in previous experiments [Aderholt, et al. 2017]. The results also suggested that the longest leaf measurements are not a strong indicator for the index of phytoextraction efficiency (PE) or foliage Pb concentration (Figures 1, 5, & 8). The IN treatment had the highest PE index (6590). The BAP and SA treatments were both lower in PE compared to the IN treatment. The SA treatment did have a significantly different PE compared to the IN treatment though the Pb concentration was not significantly different in this study. This suggests that certain phytohormones can have a negative effect on Pb uptake and plant health. This is contradictory to a preliminary study by Greipsson, et al [un published] where SA application significantly increased DM. However, in this study NTA was not used in tandem with SA. The preliminary study involved small grow-cone pots with single plants used short term over 3 months. This thesis study was conducted in large pots with multiple plants long term allowing for variation in treatments that could if been the key variable in the variations between these results.

The DM was inconsistent with Pb concentration since SA treatments had a lower average Pb concentration than both BAP and IN treatments, but also had a lower DM (Figures 1&5). The BAP application was more effective than SA application at promoting increase in biomass in this study and also was found to be effective in previous studies [Aderholt, et al. 2017]. However, in previous studies BAP was not tested under NTA application or the increased concentration of chelators and fungicides [Beavers, 2016; Aderholt, et al. 2017]. Previous studies have shown that switchgrass var. 'Alamo' can generate 17,800 kg of harvestable DM per hectare (ha) [Smith, et al. 2015]. Switchgrass, var. 'EG 1101', can generate 32,000 kg ha^{-1} [Smith, et al. 2015]. The cost of switchgrass biomass generation is estimated to be \$8-9 ton^{-1}, this is lower than other biomass producing plant candidates [McLaughlin & Kszos, 2005].

Although the BAP treatment did not have the greatest Pb concentration it did have the highest Pb concentration between the phytohormone treatments (Figure 5). The BAP treatment did not show a significant difference in dry mass compared to the SA treatment, but was noticeably higher in the DM compared to SA treatment and slightly higher than the IN treatment (Figure 1). Previous study found that the phytohormone application could be a key part of a chemically enhanced phytoextraction with increased biomass, though a possible minor reduction in Pb uptake from metal toxicity mediation by phytohormones is possible [Wani, et al. 2016]. This would be consistent with the reduced Pb levels in both the BAP and SA treatments. Studies also show that if a concentration of phytohormones is too high there could be adverse effects on plant health [Shaddad, et al. 1989; Varshney, et al. 2012]. This could possible explain why even though the SA treatment had a lower Pb concentration if SA concentrations were too high DM could be affected. Another explanation touched back on the preliminary experiment being a short term experiment. This could suggest that the phytohormenes BAP and SA cound be only beneficial during ewarly months of growth.

The phytoextraction efficiency (PE) between the phytohormone treatments were not significantly but were noticeable. The IN treatment had the highest PE (6590). The BAP and SA treatments had lower PE index compared to the IN treatment. The SA treatment did have a significantly smaller PE index compared to the IN treatment though the Pb concentration was not significantly different in this study. This suggests that certain phytohormones can have a negative effect on Pb uptake, while at the same time, negatively affecting plant health.

Switchgrass is currently being studied as a possible bioenergy crop due to its ability to provide large amounts of biomass production per hectare [Smith, et al. 2015]. The BAP treatment contained NTA, but all dry masses between treatments were insignificant. This reinforces the use of BAP as a strong candidate for phytoextraction and bioenergy production since there was a noticeable difference in increased DM over the SA treatment and slight increase over DM of the IN treatment. This is consistent with previous studies where BAP did yield significantly higher DM in switchgrass grown in Pb contaminated soils, but SA was not compared [Aderhold, et al. 2017].

NTA and phytoextraction.

Plants treated with NTA (ie IN, BAP, and SA) had the highest concentration of Pb, these results are consistent with the findings of a previous study [Hu, et al. 2017]. Phytohormones were not used though in the study of Hu, et al. (2017). The SA and BAP treatments had lower Pb concentrations than the IN treatment (Figure 5). Phytohormones have been shown to alleviate metal stress and activate processes to reduce or mediate metal toxicity [Wani, et al. 2016; Perveen, et al. 2011]. This could explanation though NTA was used, phytohormone treatments had lower Pb concentrations. Increased metal concentration is correlated with reduction in biomass and plant health [Perveen, et al. 2011]. The IN treatment had the least average DM compared to all treatments, this is consistent with increased metal concentration being correlated to a reduction in biomass. However, the SA treatment had the lowest average Pb concentration of the NTA treatments, but also had a lower DM.

The results on the efficiency of the chelate NTA are promising since the chelate EDTA persist longer in soils [Bucheli & Egli, 2001; De Araújo, et al. 2010]. The NTA treatments in switchgrass provided significant increases in Pb concentration and did not reduce Pb uptake compared to the I treatment. In this study NTA did not apparently reduce Pb translocation like EDTA did in the Aderholt et, al. (2017) study. However, in that study citric acid was applied in combination with EDTA. The NTA solution in this study used citric acid to balance pH, but sodium hydroxide was also used to balance out the pH between 5.0-6.0. However, in the Aderholt, et al. (2017) study soil pH was kept at a range of 4.0-4.5 for EDTA application which is well within the range of Al^{3+} solubility and could have contributed to the recorded reduced uptake in Pb within switchgrass.

Effects of treatments on plant health

The effects of heavy metal toxicity were observed in all plants subjected to chemical treatments. These results are consistent with previous studies [Johnson, 2015, Perry, et al. 2012]. Significantly higher levels of chlorosis and necrosis was observed in NTA treatments compared to the I treatment. Between the three NTA treatments SA displayed the highest percent chlorosis and level of dead leaves at 49%. This elevated level of chlorosis even though the SA treatment had the lowest average concentration Pb in the foliage is of interest. Based on this study SA did not protect plants from heavy metal toxicity but studies have shown that phytohormones including SA should reduce the effected form heavy metal toxicity by activating the antioxidative system and indirectly decreasing the uptake of Pb [Wani, et al. 2016]. This would aid in understanding the reduced uptake of Pb within the SA treatments, but not the reduced biomass from heavy metal toxicity that would be expected to be alleviated by SA. It is possible that increased concentrations of phytohormones can affect plants negatively and maybe the SA concentration chosen in this study was too high. Alternatively SA could only be beneficial for growth in young plants and remain only for regulatory control in older plants.

Infuse and its effect on switchgrass

The I treatment had the highest DM but also contained the highest standard deviation. Though the I treatment did not have the highest average Pb concentration compared to the NTA treatments, the I treatment still had a large increase in Pb concentration compared to Control plants. This is consistent with the past research on AMF [Aderholt, et al. 2017, Hovsepyan & Greipsson, 2005]. These studies used the soil fungicide benomyl instead of Infuse, but both yielded significant increases in foliage Pb concentration. The difference between NTA treatments and the I treatment may also lie in pH. The NTA treatments were subjected to a solution with a pH of 5.0-6.0 and the I treatment in Aderholt, et al. (2017) was not. Metals are much more likely to be taken up within plants when soil pH is below 6.0 to 5.5 [Roy, et al. 2005, Fuentes, et al. 2018]. This could be a deciding factor in economic phytoextraction where a fungicide treatment with a purposely lowered pH solution is monitored and tested for future research. Then followed by research into chelate treatments in the realm of bioenergy verses reduction in biomass by chelate treatment beyond EDTA and NTA.

The PE index was substantially higher in all treatments compared to Control plants. The I treatment had significantly lower PE index, but still had a much larger PE index than the control treatments. The PE index of the I treatment was significantly larger than control plants, 18,500% larger, though significantly smaller than the NTA treatments. Though benomyl was used these results are consistent with results from previous studies by Aderholt, et al. (2017) for application of soil fungicide. Treatment with the use of NTA and Infuse have not been used together on switchgrass in previous published studies.

The AMF protects plant from heavy metal toxicity and in this study the effects were demonstrated. Chlorosis was observed in the I treatment but was significantly less than NTA treatments (Figure 4). Reductions in AMF allow for protections by AMF to be reduced and Pb uptake to be increased [Vosatka, et al. 2006]. The results show evidence of chlorosis and elevated Pb over controls. While no AMF colonization of plant roots was accessed in this study evidence was seen of reduce plant health from Infuse application. Previous studies indicate that benomyl has many plant pathogenic fungi that are resistant to it, this study used Infuse and root pathogenic fungi colonization from a lack of AMF was not analyzed, but could be another reason for the observed reduction in plant health for the I treatment [Jung, et al. 1992; Yarden & Katan, 1993].

Effect of Combined phytoextraction using NTA, Infuse and phytohormones

This study was conducted to determine the effects of Infuse, chelate (NTA), and the phytohormones salicylic acid (SA) and Benzylaminopurin (BAP) on the phytoextraction efficiency and uptake of Pb of switchgrass. Plants in all treatments obtained higher concentrations than control plants. Overall combined treatments did have significantly higher Pb concentration over I treatment and control plants. The phytohormones treatments were inconclusive about general phytohormone application and their interactions with NTA since SA treatment had a lower Pb concentrating and PE index while also having the lowest DM. The BAP treatment had the highest DM of the three NTA treatments but though insignificantly different from the I treatment, still had a noticeably lower DM comparably to the I treatment. These results are similar to the study by Aderholt, et al. (2017) where phytohormones in combination of chelates had little effect on dry mass of plants. Chemical combinations of phytohormones, chelates, and soil fungicides are being suggested for future optimization [Calonne, et al. 2012; Aderholt, et al. 2017].

Recent study has suggested that biomass from phytoextraction projects utilizing switchgrass could potentially be used in bioenergy applications for production of ethanol [Balsamo, et al. 2015]. This would mean the extraction of heavy metals from switchgrass biomass for recycling and using the harvested biomass for energy production mainly in the form of ethanol. Switchgrass has been identified as a potential candidate for combined phytoextraction and biomass production practices because of the ability to accumulate high concentrations of Pb in foliage combined with large biomass generation per harvest [Chen, et al. 2012]. In this process harvested foliage is digested and the metal extracted for recycling [Balsamo, et al. 2015]. The harvested biomass can be reused for commercial ethanol production [Balsamo, et al. 2015]. By using the recycled biomass from switchgrass for energy production there could possibly be reduce costs associated with remediation by commercializing bioenergy crop after Pb extraction. Switchgrass production for bioenergy could also be produced on marginal or contaminated lands that is currently not suitable for agricultural uses because ability to handle many soil types and conditions. The extracted Pb could then be recycled for commercial applications and the biomass for ethanol production [Sanderson, et al. 1996].

Future Research Directions

Future research is needed to optimize phytohormone concentration for optimal growth under heavy metal conditions while maintaining the optimal increase in Pb uptake by switchgrass. Foliar application of BAP has continuously improved plant yield in multiple studies but, should be refined further in dosage application and concentration [Aderholt, et al. 2017 Matteo, et al. 2015], Foliar application of SA should be further studied since growth of switchgrass in another study was significantly improved. There could however be differences in growth responses of switchgrass to phytohormone applications in short-term studies compared to long-term studies as the one reported on in this thesis. Also, interaction of phytohormone application on the efficiency of the chelating agent NTA deserves further study. Furthermore, foliar application of commercial products that contain SA could be examined. These include: “Sentinel” that contains SA and silicon (19%), “Recover RX 3-18-18” that contains 0.5% SA and is used for foliar spray application on grasses, “Triple 12+ Liquid Fertilizer” that contains SA.

Phytoextraction would benefit from further studies on combined application of chelating agents and surfactants. The surfactant alkyl polyglucoside (APG) when combined with NTA improves absorption amounts of Pb (9.7-fold) in root interior in *Scirpus triqueter* [Hu, et al. 2017]. In another study the combined application of APG and NTA promoted the accumulation and translocation of Pb (BF/TF, 0.44/0.61) [Hu, et al. 2017].These results indicated that the combined application of APG and NTA could be promising for improved efficiency of the chemical agent NTA. However, different dosage and ratios of APG and NTA for phytoremediation needs to be further examined [Hu, et al. 2017, Liu, et al. 2020]. APG application and optimal concentrations of it can be looked into for future research.

Pathogenic fungal resistance of Infuse should be considered for future research. Infuse has been found to effect pathogenic fungi that are currently not resistant to benomyl [Calonne, et al. 2012]. Propiconazole (Infuse) is effective at reducing AMF colonization, however its performance when combined with chelates warrants further study [Calonne, et al. 2012].

Conclusion

Phytoextraction of Pb contaminated soils by switchgrass that is chemically enhanced with chelates, Infuse, and phytohormones have promise in the future research of phytoextraction and the bioenergy industry. The chelate EDTA is known to have a long persistence time in soil and can potentially make its way into surrounding areas through runoff and groundwater mobilization. A study was performee on the EDTA derivative NTA to test if its application would yield significant increases in Pb concentration in switchgrass. Plants treated with the combined application of NTA, phytohormones, and the soil fungicide Infuse yielded significantly higher Pb concentration in the foliage compared to Control plants and plants treated with Infuse while producing a slightly reduced biomass, but insignificant between treatments. The difference between the SA and BAP treatments in above ground biomass provides information for the bioenergy industry. Plants treated with the phytohormone BAP produced the highest dry mass of the NTA treatments while plants treated with the phytohormone SA had the lowest one. This outcome of increase in biomass from BAP application has been seen in past studies and suggests this is the optimal phytohormone for maximizing dry mass and could be reliable for the bioenergy industry. The phytohormone SA based on past evidence should have allowed for increased biomass and metal toxicity mitigation, but in this study remained unseen and maybe even of hindered biomass production under soil fungicide and chelate conditions.

Infuse and NTA alone produced the highest Pb concentration and phytoextraction efficiency index. Over the SA and BAP treatment it was the most effective in phytoextraction, but did not yield significantly different results from the phytohormone treatments. This indicates that even under chelate conditions, where increased Pb would decrease plant health and decrease biomass, phytoextraction of contaminated soils would not be significantly affected for biomass production and bioenergy uses. However, SA treatment under chelate and fungicide conditions was noticeably lower and would not be recommended under the results of this study, but SA in general is well known to increase biomass.

Application of Infuse produced significant increases in Pb phytoextraction, but is defiantly inferior to NTA application. Significantly less chlorosis was observed and phytoextraction efficiency was significantly lower than NTA treatment, but higher than controls in the Infuse treatment.

Integration

This study integrated the field of biology and chemistry. The integration of soil chemistry, plant physiology, and metal uptake were examined in the pursuit of a phytoextraction method to produce the optimal uptake and translocation of Pb within switchgrass. The chemistry of heavy metals and interactions with the rhizosphere were studied to create a combined method using an organism and manipulating the environment around its roots to optimize uptake of the target element Pb. From this research, the chelating agent NTA was accessed as a strong candidate for phytoextraction while being a more environmentally friendly chemical due to its documented shorter persistence time in soil compared to EDTA. Manipulation of the pH of the chelating agent was employed to ensure optimal Pb bioavailability in soil and uptake of plants while observing plant physiology to make shore plants do not die before optimal uptake has been achieved.

Soil microbe interactions were influenced through the application of the soil fungicide Infuse. AMF play a key role in plant pathogen and regulation of toxic metals such as Pb. Application of Infuse resulted in increased uptake of Pb mainly due to suppression of AMF by the soil fungicide. Translocation of Pb was likely also effected by the suppression of AMF.

The impact of two phytohormones (BAP and SA) on the growth of switchgrass and potential application in the bioenergy industry was studied. Furthermore, the combined application of phytohormones and chelating agent (NTA) in order to optimize Pb phytoextraction which could contribute to the creation of a holistic and economic approach to the problem of heavy metal contamination in soils. Chemistry, industry, and biology were taken into account to create an ecologically centered and practical study to study the phytoremediation of heavy metal contaminated soils.

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