Phytoextraction of Lead from Contaminated Urban Soils by Panicum virgatum Enhanced with Phytohormone, EDTA, Benomyl, and Citric Acid Application

Matthew Aderholt
Kennesaw State University, maderho1@students.kennesaw.edu

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Phytoextraction of Lead from Contaminated Urban Soils by
Panicum virgatum Enhanced with Phytohormone, EDTA,
Benomyl, and Citric Acid Application

By
Matthew Aderholt

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

Kennesaw State University
1000 Chastain Road
Kennesaw, GA 30144
May 2015

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Dr. Sigurdur Greipsson, Thesis Chair
Associate Professor of Biology
Kennesaw State University

____________________________
Dr. Dale Lynn Vogelien
Professor of Biology
Kennesaw State University

____________________________
Dr. Marina Koether
Professor of Chemistry & Biochemistry
Kennesaw State University
Abstract

Lead (Pb) contamination in soil represents a threat to human health and cleanup efforts often require expensive or environmentally disruptive techniques. Phytoextraction has gained attention as a possible alternative to traditional remediation methods because of lower cost and minimal soil disruption. The North American native switchgrass (*Panicum virgatum* L.) was examined due to its ability to grow across a variety of environmental conditions and climates. Switchgrass was chemically enhanced with applications of the soil-fungicide benomyl, chelates (EDTA and citric acid), and phytohormones for phytoextraction of Pb from contaminated urban soils in Atlanta, GA.

Chemical manipulation was conducted on urban contaminated soil collected from downtown Atlanta to increase Pb bioavailability and uptake into above ground harvestable biomass of switchgrass. Although EDTA is often regarded as the most effective chelate, its use has some problems due to long persistence time in soil and possible Pb mobilization into groundwater. Citric acid has been proposed as a possible alternative because of its short persistence time and lower risk of groundwater contamination; however no consensus exists on its ability to generate similar phytoextraction efficiency to EDTA.

Exogenous phytohormone application was also examined to determine the effect on switchgrass biomass and Pb uptake. Three phytohormones were studied: indole-3-acetic acid (IAA), benzylaminopurine (BAP), and Gibberellic Acid (GA$_3$). Each phytohormone was tested in multiple concentrations to determine effects on switchgrass growth. In the first study, BAP (1.0 μM) was found to generate a 48% increase in biomass compared to Control plants.
Chemical application of citric acid, EDTA, benomyl, and phytohormone BAP were tested separately and in combination in a pot experiment in an environmentally controlled greenhouse to determine the efficacy of phytoremediation by switchgrass. Application of EDTA increased Pb concentrations in plant roots, but had no significant effect on Pb translocation into foliage. Application of citric acid without supplemental EDTA application was found to produce shoot Pb concentrations no different than those of plants treated with EDTA alone. This indicated that there was no benefit to translocation of Pb into foliage from EDTA application alone, and that citric acid without EDTA was able to achieve similar shoot Pb concentrations. Application of BAP was found to significantly increase root Pb concentration when combined with EDTA, citric acid, and benomyl (E+B+C+H), but did not result in increased translocation when combined with benomyl and citric acid (B+C+H). All treatments resulted in greater total Pb phytoextraction compared to Control plants. Total Pb phytoextraction was greatest in plants treated with combined chemical application of B+C and B+C+H. Plants treated with E+B+C and E+B+C+H resulted in less total Pb extracted due to lower biomass. Plants treated with single chemical application of E, B, C or H phytoextracted more Pb than Control plants, but were all inferior to plants treated with B+C or B+C+H.

Application of benomyl demonstrated the ability of broad-spectrum soil fungicide to reduce AMF colonization and consequently increase Pb uptake. Application of benomyl was also shown to dramatically increase levels of pathogenic fungi infection as compared to Control plants.
Acknowledgements

I would like to thank first my supervisor, Dr. Sigurdur Greipsson, for his incredible knowledge, patience, and guidance throughout this entire experience. Many thanks to my other committee members Dr. Dale Lynn Vogelien and Dr. Marina Koether for all of the knowledge and expertise they provided, and to Dr. Koether for the use of her lab and equipment which made this project possible. I would also like to thank Dr. Joel McNeal for his assistance in providing research plants and arranging for space within the greenhouse. To all of the professors and faculty members who have added to my education and learning while here at KSU through classroom instruction, lab techniques, or practical knowledge of teaching biology, I would like to thank Dr. Joseph Dirnberger, Dr. Sigurdur Greipsson, Dr. Thomas McElroy, Dr. William Ensign, Dr. Antonio Golubski, Dr. Scott Nowak, Dr. Scott Reese, Dr. Joel McNeal, Joy Brookshire, and many others.

Many thanks to my fellow lab members Deayne Johnson, Dan Pingel, Adrianna Beavers, Collin Carlson, Ben Brandstetter, and all members of the Greipsson lab for their invaluable assistance over the last two years. Special thanks to Adrianna Beavers for her help with data collection, plant harvest & processing, and acid digestion. To all of my fellow graduate students in the MSIB program, thank you for all of the smiles, laughter, and friendship that made this whole experience so much more fun. Most of all, I would like to thank my friends and family for your love, encouragement, and laughter that kept me going. You will never know how much I appreciate your support.
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List of Abbreviations

Calculations:
| BF | Bioaccumulation Factor |
| PR | Phytoextraction Ratio   |
| TF | Translocation Factor   |

Chemical Names:
| BAP | Benzylaminopurine (Benzyl Adenine) |
| EDDS | Ethylenediamine-\(N,N'\)-disuccinic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| GA\(_3\) | Gibberellic Acid\(_3\) |
| IAA | Indole-3-Acetic acid |

Elements:
| Al | Aluminum |
| C | Carbon |
| Cd | Cadmium |
| N | Nitrogen |
| Ni | Nickel |
| O | Oxygen |
| P | Phosphorus |
| Pb | Lead |

General:
| AA | Atomic Absorption Spectrophotometry |
| 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 |
| LSD | Least Significant Difference |
| ROS | Reactive Oxygen Species |
## Treatment Groups:

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Introduction

Lead (Pb) Contamination in Soil

Lead (Pb) has been commonly used in many industrial applications and as tetra ethyl Pb ((CH₃CH₂)₄Pb), a former additive in automotive gasoline and current additive to some aviation fuel, making it one of the most widespread heavy metal pollutants in soil (Lagerwerff & Specht, 1970; Gulson, et al. 1995; Mielke & Reagan, 1998). Airborne Pb emitted from smelting or recycling facilities or from burning tetra ethyl Pb containing fuel is typically deposited in topsoil (Centers for Disease Control & Prevention, 2010; Greipsson, et al. 2013). The Centers for Disease Control and Prevention (CDC) has stated that soil containing more than 50 ppm of Pb poses a risk to human health through possible ingestion, uptake by garden plants, and transfer to indoor spaces (Malcoe, et al. 2002; CDC, 2010). The average background level of Pb in uncontaminated urban area soils is typically 10 mg kg⁻¹, however contaminated areas can have significantly higher Pb concentrations without close proximity to the contamination source (CDC, 2010; Greipsson, et al. 2013). High concentrations of Pb soil contamination persist in many urban areas that carried out Pb smelting or recycling, resulting in soil Pb levels in excess of 200 ppm (Ragains, et al. 1977; Ettler et al. 2004; CDC, 2010; Greipsson, et al. 2013). Soil contamination from smelting and recycling applications is not confined to industrial areas; many residential and suburban areas are also contaminated with unacceptable Pb levels (Deocampo, et al. 2012; Solt, et al. 2015). The United States Environmental Protection Agency (EPA) estimates that 207,000 Pb contaminated sites including millions of hectares exist that require remediation in the United States (EPA, 1997).
Health effects of Pb exposure include poor muscle coordination, nerve damage, hearing and vision impairment, organ damage, developmental disorders, and many other disorders (Needleman & Bellinger, 1991; Lanphear, et al., 1998; CDC, 2010). Although all humans are susceptible, children under the age of seven are most at risk to the effects of Pb exposure (Reagan & Silbergeld, 1990; Lanphear, et al., 1998). The CDC estimates that as many as 11.7 million children may be exposed to unacceptable levels of Pb in soil in the United States (CDC 2010). Studies indicate that blood Pb levels below the CDC 10 μg dL\(^{-1}\) standard results in reduced IQ and attention deficit-related behaviors, in addition to physiological effects (Bellinger et al. 2003). Studies also indicate low level Pb exposure results in decreased cognitive and developmental test scores (Banks, et al. 1996). Studies on wildlife have shown that Pb exposure in Salvelinus fontinalus (brook trout) indicate long term exposure over multiple generations may result in severe spinal deformities and may contribute to development of scoliosis (Holcombe, et al. 1976; Barbeito, et al. 2010).

**Traditional Remediation Methods**

Traditional methods of soil cleanup include soil washing, leaching, and excavation (Martin & Ruby, 2004). Soil washing is an environmentally damaging process which requires soil excavation and chemical washing to remove Pb (McGrath and Zhao, 2003). This process is expensive, requires major soil disturbance, resulting in lower productivity after treatment (McGrath and Zhao, 2003; Leung, et al. 2013). Most essential nutrients necessary for plant growth are washed away along with the Pb contamination and beneficial microorganisms present in the soil are destroyed (Huang, et al. 1997, Leung, et al. 2013). Soil excavation requires the complete removal of contaminated soil for disposal in a landfill (Mulligan, et al.
Excavation and landfill disposal is expensive, requires significant disturbance to the environment, and only moves the contamination from one site to another and therefore does not remediate Pb concentration of the soil (McGrath and Zhao, 2003). Traditional methods of Pb cleanup are on average ten times more expensive than phytoremediation strategies (McGrath and Zhao, 2003).

**Phytoextraction**

Phytoextraction is a possible alternative to traditional soil cleanup techniques for moderately contaminated sites because of lower cost and much lower degree of soil disruption (Cunningham & Berti, 2000). Phytoextraction utilizes plants to extract pollutants such as metals and chemical pollutants from soil by translocation of the pollutant into harvestable plant tissue (Cunningham & Berti, 2000; Peer, *et al.* 2006). Soil microbes such as arbuscular mycorrhizal fungi (AMF) can influence pollutant uptake through secretions into the rhizosphere which can bind pollutants to soil particles preventing uptake, up-regulation of plant phytochelatin expression, and down regulation of plant genes responsible for metal uptake (Leyval, *et al.* 1997; Vosatka, *et al.* 2006; Hildebrandt, *et al.* 2007; Xu, *et al.* 2014). Phytoextraction can be applied at any site where soil and climatic conditions are suitable for growth of plants (Pilon-Smits, 2005).

Chelating agents such as Ethylenediaminetetraacetic acid (EDTA) (C$_{10}$H$_{16}$N$_{2}$O$_{8}$), Ethylenediamine-$N,N'$-disuccinic acid (EDDS) (C$_{10}$H$_{16}$N$_{2}$O$_{8}$), or natural acids are commonly applied to increase bioavailability of Pb in the soil for absorption by plant roots (Huang, *et al.* 1997). The plant progresses through the growing season extracting Pb from the soil and
depositing Pb into stem and leaf tissue by translocation through vascular xylem (Wu et al. 1999; Peer, et al. 2006). Metal enters the plant through the root symplast by crossing the plasma membrane of root endodermal cells or through root apoplast (Wu et al. 1999; Peer, et al. 2006). Metal is thought to be transported through the Casparian strip into the xylem by the plasma-membrane pump ZNT1, cation channels, and nonspecific metal transporters (Wu et al. 1999; Peer, et al. 2006). Metal is then transported to leaf tissue by flow of xylem sap (Wu et al. 1999; Peer, et al. 2006). Lead (Pb) ions are thought to be transported by phytochelatins through xylem sap flow and stored in the vacuole of foliage cells to limit toxicity and interference with cellular processes such as photosynthesis (Peer, et al. 2006). In phytoextraction, biomass produced is harvested and disposed or recycled (Garbisu & Alkorta, 2001; Sas-Nowosielska, et al. 2004). Methods of recycling Pb include processing by acid digestion or through incineration of foliage and extraction of accumulated metals (Garbisu & Alkorta, 2001; Sas-Nowosielska, et al. 2004; Balsamo, et al. 2015).

Benefits of phytoextraction over traditional soil washing or excavation techniques include lower cost and reduced environmental impact through decreased soil disturbance (McGrath and Zhao, 2003). Phytoextraction of contaminants can be carried out at any site that is amenable to plant growth, reducing the need for transportation of large and difficult to move machinery to remote or inaccessible sites (McGrath and Zhao, 2003; Pilon-Smits, 2005). Lower cost and lower levels of disturbance also make phytoextraction more publically appealing than traditional remediation methods (Cunningham & Berti, 2000; Leung, et al. 2013). Phytoextraction, unlike engineering remediation methods, does not result in lower soil productivity due to loss of essential nutrients or natural soil microorganisms (Yoon et al., 2006).
Phytoextraction by plants alone is not yet feasible on an industrial or economic scale because of slow uptake and incorporation of Pb into plant tissues (Huang et al., 1997; Van Nevel, et al., 2007). Phytoextraction is often a lengthy process requiring years to cleanup a contaminated site to an acceptable level based on current treatment strategies. Remediation of soil with 1000 ppm Pb could take up to 30 years to cleanup to an acceptable level (Huang et al., 1997). Slow Pb uptake is caused by many factors including low solubility of Pb in soil for uptake by plants and presence of symbiotic soil microbes such as arbuscular mycorrhizal (AMF) (Leung, et al. 2013).

Some plant species are known to hyperaccumulate high levels of metal into harvestable tissue, but generally suffer from low biomass production, slow growth, and require specific environmental conditions for growth making them unsuitable for phytoextraction on an industrial scale (McGrath and Zhao, 2003; Mackova, et al. 2006; McGrath, et al. 2007). Alternative to hyperaccumulators include the use of high biomass plants including corn, alfalfa, and grasses producing large amounts of foliage in one growing season and suitable for planting across many ecozones (Mackova, et al. 2006). High biomass plants take up significantly less Pb per gram of plant tissue than hyper-accumulator species but are utilized because of high biomass and fast growth essential for successful soil remediation (Mackova, et al. 2006). Phytoextraction capability of fast growing and high biomass plants can be optimized through addition of chemical additives to alter soil chemistry and increases temporarily Pb mobility (Huang et al., 1997).
Study Plant: *Panicum virgatum*

This study investigated the phytoextraction potential of *Panicum virgatum* (var. Alamo) (switchgrass) as a possible candidate for use in Pb soil remediation. Switchgrass is a perennial grass native to North America that grows in a wide variety of climates and tolerates numerous soil types (Pimentel & Patzek, 2005). Switchgrass is currently used for studies in the biofuels industry due to rapid growth and high biomass production (Pimentel & Patzek, 2005, Smith, *et al.* 2015). Previous studies indicate that the “Alamo” variety is capable of generating 17,800 kg ha\(^{-1}\) of harvestable biomass. Another variety of switchgrass (EG 1101) can generate 32,000 kg ha\(^{-1}\) (Smith, *et al.* 2015). The cost of switchgrass biomass production is estimated at $8-9 ton\(^{-1}\), this value is much lower than for other high biomass crops (McLaughlin & Kszos, 2005). Another advantage of switchgrass as a phytoextraction plant at contaminated sites in North America is that it would eliminate the use of invasive plant species. Lead (Pb) uptake ability of switchgrass enhanced with citric acid (C\(_6\)H\(_8\)O\(_7\)), EDTA, benomyl, and exogenous phytohormone application has not been previously studied.

**Soil Chemistry and Use of EDTA**

Lead (Pb) in soil is often in the form of Pb-carbonates or Pb-sulfates that are largely immobile and thus unavailable for phytoextraction (Zimdahl & Skogerboe, 1977; Huang *et al.*, 1997). Ion exchange interactions with soil particles, particularly clay, result in the immobility of Pb to leach through the soil column (Zimdahl & Skogerboe, 1977). Because of poor Pb solubility in soil, the highest levels of Pb contamination are typically found in the top 10 cm of topsoil (CDC 2010). Mobility of Pb is also affected by presence of cations and anions in the soil column,
water and mineral content, and drainage (Zimdahl & Skogerboe, 1977). The addition of chelating agents such as EDTA, EDDS, or organic acids increases solubility and bioavailability by forming soluble Pb complexes (Wu et al. 1999; Peer, et al. 2006).

The use of EDTA as an effective chelating agent by increasing the availability of Pb for uptake in many plant species has been established by several studies (Wu et al. 1999; Hovsepyan & Greipsson, 2005; Lopez et al., 2005; Peer, et al. 2006; Perry, et al., 2012). EDTA increases bioavailability of Pb by forming soluble EDTA-Pb complexes (Peer, et al. 2006). However, EDTA has met resistance to field application at contaminated sites due to potential problems associated with its use (de Araujo, et al. 2010). Problems include possible mobilization of Pb and other heavy metals into ground water, long persistence time of several years in the soil, and inability to be broken down by soil microbes (Bucheli-Witschel & Egli. 2001; de Araujo, et al. 2010). Long persistence times and possible groundwater contamination has led to concern over its potential use in phytoextraction (Oviedo & Rodriguez, 2003). Application of EDTA has also been observed to negatively affect plant growth and biomass (Meers, et al. 2010). Application of EDTA can also induce severe oxidative stress in plants resulting in further declines in plant health (Geebelen, et al. 2002).
Alternative Chelates & Citric Acid

Because of concerns associated with synthetical EDTA application, natural acids have been considered as a replacement chelate in phytoextraction. Studies have indicated that soils with low pH values may experience more leaching of Pb than soils with high pH (Greipsson, et al. 2013). One study by Dong, et al. (1999) indicated that ideal soil pH is between 4.0-4.5 for maximum Pb mobility. Citric acid has been proposed as a possible alternative to EDTA because it is readily broken down by soil microbes, has a short persistence time of approximately one week in soil, and has therefore lower risk of groundwater contamination than EDTA (Freitas et al. 2013). Previous studies using citric acid indicated that Pb solubility in soil samples was drastically reduced 7 days after citric acid application (Freitas et al. 2013). Repeated applications are necessary to keep Pb available for plant uptake over an extended period of time (Freitas et al. 2013). Soil acidification has been observed to increase plant nutrient availability by increasing solubility of essential micronutrients present in the soil (Kidd & Proctor, 2001). Citric acid application can also have adverse effects by inducing hydrogen toxicity resulting in chlorosis and necrosis of plants (Kidd & Proctor, 2001). Lowering soil pH may also solubilize toxic levels of other metals present in the soil besides Pb (Kochian, 1995; Geebelen, et al. 2002). Decreasing soil pH has been observed to drastically increase levels of soluble aluminum (Al) resulting in Al toxicity in plants used for phytoextraction (Kochian 1995).

Use of citric acid as a replacement for EDTA has experienced limited success in previous studies (Chen, et al., 2003; Cui, et al., 2004; Sinhal, et al., 2010). Research by Cui, et al. (2004) indicated that soil acidification by citric acid alone is not feasible for effective phytoextraction in
*Quamolit pennata, Antirrhinum majus,* and *Celosia cristata pyramidalis* and citric acid must be supplemented with EDTA to achieve effective phytoextraction. However, Freitas *et al.* (2013) indicated that citric acid can be effectively used as a chelate without supplemental application of EDTA in a field study utilizing *Zea mays* and *Chrysopogon zizanoides*. The effect of citric acid on Pb uptake of switchgrass with and without supplemental applications of EDTA has not been previously studied.

**Arbuscular Mycorrhizal Fungi**

Arbuscular mycorrhizal fungi (AMF) present in the soil affect phytoextraction of Pb from contaminated soils by blocking metal uptake into plant roots (Vosatka, *et al.* 2006). AMF form symbiotic relationships with over 85% of land plants, providing mineral nutrients such as nitrogen (N) and phosphorus (P) to host plants in exchange for carbon (C) compounds (Reynolds, *et al.*, 2003; van der Heijden, *et al.*, 2015). Macronutrients and C compounds are exchanged through formation of fungal arbuscules within the cytoplasm of cortical cells of plant root (Reynolds, *et al.*, 2003). In addition, AMF form external hyphae that act as root extensions, effectively increasing root surface area for increased water and nutrient absorption from surrounding soil (Leyval, *et al.* 1997). AMF act as a barrier to plant phytoextraction of Pb, preventing uptake into harvestable foliage (Leyval, *et al.* 1997; Hildebrandt, *et al.* 2007; Xu, *et al.* 2014). Heavy metal exposure results in oxidative stress in AMF from formation of reactive oxygen species (ROS), resulting in differential gene expression in extraradical mycelia responsible for encoding for proteins potentially involved in heavy metal tolerance (Hildebrandt, *et al.* 2007; Upadhyaya, *et al.* 2010). These proteins are thought to be
responsible for managing heavy metal induced oxidative stress in AMF by depositing heavy metals into the fungal cell wall or granules within the cytoplasm (Hildebrandt, et al. 2007). AMF also up-regulate expression of plant genes encoding for phytochelatin synthase (PCS1) which is responsible for phytochelatin production in response to heavy metal exposure (Xu, et al. 2014). Phytochelatins are thought to be critical for transporting and sequestering Pb ions into plant cell vacuoles to prevent interference with cellular processes (Xu, et al. 2014). AMF also protect plant hosts through down-regulation of plant genes encoding for metal transporters MT2 & Nramp1, preventing metal transportation from root cells into vascular xylem (Ouziad, et al. 2005; Hildebrandt, et al. 2007).

AMF also benefit plants through suppression of pathogenic fungi infection through secretion of exudates and pathogenic fungi gene regulation in addition to supplying host plants with P (Rovira 1969; Hildebrandt, et al. 2007; Ismail, et al. 2011). Species of AMF in the Glomeraceae family possess protection ability of host roots from pathogenic infection while species in Gigasporaceae lack host root protection ability (Sikes 2010). AMF inhibit pathogenic fungal colonization through modulation of mycotoxin gene regulation (Ismail, et al. 2011). Increases in pathogenic fungi infection have been observed to negatively affect plant growth and reduce fecundity even in asymptomatic plants (Newsham, et al. 1994).

Previous studies have indicated that improved Pb uptake and translocation occurs after the application of the fungicide benomyl (Hovsepyan & Greipsson, 2004). Application of benomyl prevents the formation of microtubules in fungal structures, preventing AMF colonization of plant roots (Paul, et al. 1989; Hovsepyan & Greipsson, 2004; Perry, et al. 2012).
Studies indicate that improved uptake of Pb can be achieved by benomyl application before the addition of the chelate EDTA rather than with simultaneous applications (Perry, et al. 2012). Treatment with benomyl application has been demonstrated to be effective at controlling colonization of AMF and was utilized in this study. Application of benomyl also results in negative effects such as decreased P uptake of plants, which may result in decreased biomass production or chlorosis (Perry, et al. 2012; Johnson, et al. 2015). A preliminary study by Greipsson, et al. (unpublished) using Laser-Ablation Inductively Coupled Plasma Mass-Spectrometry (LA-ICP-MS) has shown that benomyl and EDTA treatments resulted in about 50% reduction of P concentration in leaf tips (0.5 cm) of ryegrass (Lolium perenne L.).

**Phytohormones & Remediation**

Benomyl and EDTA applications negatively affect plant growth, resulting in lower plant biomass as compared to controls (Perry, et al. 2012; Greipsson, et al. unpublished). Effective phytoextraction is typically achieved with high biomass plants such as corn, switchgrass, and alfalfa (Huang et al., 1997; Shen, et al. 2002; Hernández-Allica, et al. 2008). Phytohormones affect plant biomass and stress response related to metal toxicity in plants and can result in alleviation of toxic effects including low biomass related to heavy metal uptake (Sayed, 1999; Tassi, et al. 2008). Exogenous applications of phytohormones can result in increased biomass, longer plant lifespan, and increased Pb uptake by plants (Sayed, 1999; Tassi, et al. 2008). Effects of exogenous phytohormone application on switchgrass shoot length, biomass production, and Pb uptake is not well known and no consensus exists on efficacy of exogenous phytohormone applications or proper application concentrations. This study examined the efficacy of three
phytohormones: indole-3-acetic acid (IAA), benzyl aminopurine (BAP), and gibberellic acid (GA$_3$) tested at multiple concentrations to determine effects on plant growth and Pb translocation from roots to shoot.

**Indole-3-Acetic Acid (IAA)**

IAA is the most abundant of the auxin class of hormones and functions to induce cell elongation and division and is critical for root development (Pasternak, et al. 2002; Weyens, et al. 2009). IAA is widely used in the bioenergy industry and in phytoextraction studies for maximizing plant biomass (Pasternak, et al. 2002). Successes in increasing biomass in smaller plants has been achieved using IAA (Yuan, et al. 2008; Fässler, et al. 2010; Hadi, et al. 2010; Du, R., et al. 2011; Cabello-Conejo, et al. 2013). Previous studies have shown that IAA also reduces the observed toxic effects of Pb absorption in plants (Fassler, et al. 2010; Tassi, et al. 2008). A study by Lopez et al.(2005) using IAA and EDTA showed that IAA increased phytoextraction of Pb into harvestable biomass by 2800% over control plants and 600% over plants treated with EDTA alone.

**Benzylaminopurine (BAP)**

BAP is a cytokinin which promotes cell division, growth, and differentiation in shoots and roots, bud development and growth, leaf expansion, and chlorophyll synthesis (Barker & Tagu. 2000; Tassi, et al. 2008; Burris, et al. 2009). Cytokinins can also indirectly affect stomatal opening which could result in increased rates of transpiration and Pb uptake (Barker & Tagu. 2000; Tassi, et al. 2008). Studies indicate that BAP has experienced previous success in increasing biomass in grasses (Burris, et al. 2009).
Gibberellic Acid (GA$_3$)

GA$_3$ belongs to the gibberellin class of phytohormones and is responsible for seed germination, stem elongation, and senescence (Weyens, et al. 2009). GA$_3$ is known to be involved in plant extension, especially in the stem region which could have resulted in increased shoot length and biomass (Weyens, et al. 2009). GA$_3$ has experienced previous success at increasing biomass in perennial grasses and application through increases in stem length (Weyens, et al. 2009). Gibberellins function to control root development which could contribute to increased Pb uptake from the soil (Weyens, et al. 2009).

Research Questions

While previous studies examining phytoextraction using switchgrass have been performed, the effect of soil acidification and exogenous phytohormone application remains unknown (Johnson, et al. 2015). This study compared application of citric acid with and without EDTA. EDTA forms soluble EDTA-Pb complexes and is known to be an effective chelating agent. In addition, EDTA application represented the best treatment strategy currently known (Wu et al. 1999; Hovsepyan & Greipsson, 2005; Lopez et al., 2005; Peer, et al. 2006; Perry, et al., 2012;). The use of EDTA in some treatment groups was regarded as necessary due to past poor performance in uptake of Pb by plants treated with citric acid alone (Chen, et al., 2003; Cui, et al., 2004; Sinhal, et al., 2010). One aim of this study was to examine utilization of citric acid with and without supplemental application of EDTA, which could eliminate the use of EDTA from future switchgrass studies and alleviate the negative consequences associated with its use. Another novel feasibility study was performed that involved the investigating the effect of
exogenous phytohormone applications on switchgrass shoot length, biomass production, and Pb uptake. Successful use of switchgrass combined with soil acidification and exogenous phytohormone application could provide a feasible alternative to expensive and disruptive traditional remediation methods in the future.
Materials & Methods

Experiment 1: Effect of Exogenous Hormone Application on Switchgrass Biomass Production

A study was performed to examine the effects of three plant growth regulating hormones (IAA, GA₃, and BAP) on switchgrass shoot length and biomass production. The results from this study were used to determine an effective concentration of phytohormone application to achieve increased shoot length and biomass. Switchgrass seeds were planted in 15.0 cm diameter plastic pots with 1.2 kg commercially available nursery potting soil and treated with concentrations of IAA, GA₃, or BAP between 1.0 -100.0 μM to determine an effective phytohormone and concentration level that generated the largest biomass production. Previous experiments with other perennial grasses utilized hormone concentrations between 1.0 -100.0 μM; however, no consensus on optimal phytohormone concentration exists (Lopez, et al., 2005; Fässler, et al. 2010; Hadi, et al. 2010). Multiple concentrations were needed to identify a concentration that significantly affected switchgrass shoot length and biomass as compared to controls. Concentrations tested included: low (1.0 μM), medium (10.0 μM), and high (100.0 μM).

Pots were divided into ten treatments with each one receiving a different phytohormone application (Table 1). The first treatment received no phytohormone and was allowed to grow under identical conditions as other treatments to function as a control. Experimental plants were grouped according to concentration and hormone to be tested (Table 1). Pots were treated in seven day intervals with 2.5 mL of 100.0 μM, 10.0 μM, or 1.0 μM concentrations of IAA, GA₃, or BAP for a total of five treatments over 35 days. Phytohormone
solutions for foliar application were prepared by adding phytohormone to 10.0 mL of 85% of ethanol for IAA and GA$_3$ and 10.0 mL dilute NaOH (0.01 M) for BAP to create 0.1M phytohormone stock solutions. Stock solutions were serially diluted in water to desired concentrations for foliar application. Each phytohormone solution (2.5 mL) was sprayed on to the leaf surface at each treatment interval. Plant height was recorded in 7 day intervals, starting before the first phytohormone application.

All plants received 150 mL of water twice weekly and were grown in a climatically controlled greenhouse at Kennesaw State University, Kennesaw, GA. Pots were arranged in the greenhouse on a test bench in a randomized block design with five replicate blocks. Pots were re-randomized weekly within each block to eliminate differences in growth due to environmental conditions that could potentially exist within the greenhouse. All plants were grown under the same conditions except for varying phytohormone applications. Eight weeks after initial phytohormone application, final plant height was measured prior to harvesting. Plant shoots were harvested and dried in an oven at 65°C for 48 hours and the dry mass (DM) was recorded. Information derived from this preliminary experiment was used to determine the proper type of phytohormone and concentration level to be used to optimize biomass production in the second experiment that examined the combined effect of EDTA, benomyl, and citric acid on Pb phytoextraction by switchgrass.
<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IAA: 1.0 µM</td>
</tr>
<tr>
<td>2</td>
<td>IAA: 10.0 µM</td>
</tr>
<tr>
<td>3</td>
<td>IAA: 100.0 µM</td>
</tr>
<tr>
<td>4</td>
<td>GA3: 1.0 µM</td>
</tr>
<tr>
<td>5</td>
<td>GA3: 10.0 µM</td>
</tr>
<tr>
<td>6</td>
<td>GA3: 100.0 µM</td>
</tr>
<tr>
<td>7</td>
<td>BAP: 1.0 µM</td>
</tr>
<tr>
<td>8</td>
<td>BAP: 10.0 µM</td>
</tr>
<tr>
<td>9</td>
<td>BAP: 100.0 µM</td>
</tr>
<tr>
<td>10</td>
<td>None (Control)</td>
</tr>
</tbody>
</table>

**Table 1:** Plant experimental phytohormone treatments and concentration levels that were applied during experiment 1.
Experiment 2: Phytoextraction by Switchgrass Enhanced with EDTA, Benomyl, and Phytohormones

Soil Collection

This study utilized Pb-contaminated soil from an urban site in downtown Atlanta (Deocampo, et al. 2012) (Figure 1). Contaminated soil was collected from a residential area known to contain unacceptable levels of Pb in the soil as described in Deocampo, et al. (2012). The top 10 cm of soil that typically contains the highest Pb concentrations was collected (CDC 2010). Soil was prepared prior to planting by sieving to remove large debris. After debris removal, the soil was extensively mixed to create a homogenous mixture and ensure uniform Pb concentration across all treatments. Each pot contained 1200.0 g of prepared Pb contaminated topsoil.
Figure 1: Satellite image (A) and map (B) of Pb contaminated soil site from downtown Atlanta, GA as indicated by Deocampo, et al. (2012). Collection location indicated by red arrows. Images courtesy Google Earth (2015) & Google Maps (2015).
Soil Testing

Collected soil was tested to determine Pb concentration by acid digestion and analysis with ICP-AES at the University of Georgia Soil Sciences Lab in Athens, GA. Results revealed that collected soil contained 108 mg kg\(^{-1}\) of Pb, more than twice the CDC (2010) threshold of 50 mg kg\(^{-1}\) Pb for soils to be deemed uncontaminated. A preliminary study was performed to determine the proper amount of citric acid needed to achieve pH 4.0-4.5 (Dong, et al. 1999). Soil (100.0 g) was placed in beakers and treated with varying amounts (25.0 mmol kg\(^{-1}\) soil, 40.0 mmol kg\(^{-1}\) soil, 50.0 mmol kg\(^{-1}\) soil, 75.0 mmol kg\(^{-1}\) soil, and 100.0 mmol kg\(^{-1}\) soil) of citric acid (Sigma Laboratory Grade). Soil was mixed with 150.0 mL DI water and stirred to create a homogenous mixture. Initial pH readings were taken in the lab at 23°C using a pH meter standardized with pH 4.0, 7.0, and 11.0 standards. Citric acid was added and mixed to the soil slurry to create a homogenous mixture. Readings were taken after citric acid application and every two days for a duration of 8 days to determine the required application interval.

Experimental Design & Treatments

Fractional factorial experimental design was used where each chemical was tested separately and selected combinations of chemical manipulations were also tested (Table 2). Five replicates were used for each treatment. Control plants did not receive chemical manipulation during the experiment. Each chemical was tested separately: EDTA (E), benomyl (B), citric acid (C), and BAP (H). In addition, combinations of chemical applications were tested: benomyl and citric acid (B+C); benomyl, citric acid, and BAP (B+C+H); EDTA, benomyl, & citric acid (E+B+C); EDTA, benomyl, citric acid, and BAP (E+B+C+H). In total, 45 pots were used in all
treatments. Pots were put in a randomized block design on the greenhouse bench. Pots were randomly re-randomized weekly on the greenhouse bench to eliminate potential differences due to light and temperature variation within the greenhouse.

The “Alamo” variety of switchgrass seeds were obtained from the University of Georgia Plant Biology department and planted. Plants were watered twice weekly with 150 mL DI water and shoot length was measured in seven day intervals. This watering schedule and volume was chosen to eliminate runoff through the drain holes in the pot so that no Pb was lost during water applications.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>1 (Control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benomyl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td>Citric Acid</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>BAP</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Nine treatments were used in experiment 2 consisting of single chemical applications and selected combinations of chemical applications used in fractional factorial design. + signs indicate chemicals each received
Phytohormone Application

Benzylaminopurine (BAP) at 1.0 µM was selected as the optimal phytohormone and concentration level that yielded greatest plant height and biomass to apply to plants in Pb contaminated soil. Two treatment groups of switchgrass plants were used to examine the effect of phytohormones on biomass production and Pb uptake (Table 2). BAP was dissolved in dilute NaOH (0.01 M) and serially diluted in DI water to the desired concentration. Application of hormone solution began 21 days after germination in the early stages of plant growth to ensure optimal effect (Table 3). Comparison of plants in different treatments with or without BAP application was used to determine the effect of exogenous hormone application on shoot length, biomass, and Pb uptake.

Benomyl Application

Benomyl applications began immediately after phytohormone application (Table 3). Benomyl powder (20 mg kg⁻¹ soil) was applied to the soil surface and watered in as described in Perry, et al. (2012) prior to addition of chelating agents. Benomyl powder was mixed into 50.0 mL DI H₂O and vortexed to dissolve clumps and proper absorption into the soil rhizosphere. Research by Perry, et al. (2012) indicated that better uptake of Pb was achieved with benomyl application followed by chelate rather than simultaneous applications of both chemicals.
**Soil Acidification & Chelate Application**

Soil acidification by addition of citric acid began one week after benomyl application (Table 3). Since citric acid readily breaks down in soil, repeated applications on a weekly basis were necessary to keep the soil in the target range of 4.0-4.5 (Freitas et al. 2013). Soil pH was verified using a pH meter. Citric acid (40.0 mmol kg\(^{-1}\) soil) was mixed in 100 mL DI water and applied to test pots. Control plants were given only DI H\(_2\)O. Some plants were additionally given supplemental application of EDTA to determine if citric acid without supplemental EDTA application could attain similar uptake. Na-EDTA solution (1.0 mmol kg\(^{-1}\) soil) was applied 21 days after treatment with benomyl (Hovsepyan & Greipsson, 2005; Perry, et al. 2012).
<table>
<thead>
<tr>
<th>Event or Chemical Treatment</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed Germination</td>
<td>14</td>
</tr>
<tr>
<td>BAP (2.5 mL 1.0 μM)</td>
<td>35</td>
</tr>
<tr>
<td>BAP (2.5 mL 1.0 μM)</td>
<td>42</td>
</tr>
<tr>
<td>BAP (2.5 mL 1.0 μM)</td>
<td>49</td>
</tr>
<tr>
<td>BAP (2.5 mL 1.0 μM)</td>
<td>56</td>
</tr>
<tr>
<td>BAP (2.5 mL 1.0 μM)</td>
<td>63</td>
</tr>
<tr>
<td>Benomyl (20 mg kg$^{-1}$ soil)</td>
<td>70</td>
</tr>
<tr>
<td>Citric Acid (40.0 mmol kg$^{-1}$ soil)</td>
<td>77</td>
</tr>
<tr>
<td>EDTA (1.0 mmol kg$^{-1}$ soil)</td>
<td>79</td>
</tr>
<tr>
<td>Citric Acid (40.0 mmol kg$^{-1}$ soil)</td>
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<td>Citric Acid (40.0 mmol kg$^{-1}$ soil)</td>
<td>91</td>
</tr>
<tr>
<td>Citric Acid (40.0 mmol kg$^{-1}$ soil)</td>
<td>98</td>
</tr>
<tr>
<td>Plant Harvest</td>
<td>105</td>
</tr>
</tbody>
</table>

**Table 3**: Timeline of seed germination, chemical applications, and harvest. Days of application are calculated from days after planting (DAP)
Plant Harvest & Analysis

After plant chlorosis and necrosis appeared, all switchgrass plants were measured for final shoot length. The entire plant was removed from the pot and roots carefully rinsed to remove soil. Root samples from each of the test groups were randomly divided. Half of the roots samples were stored in 70% ethanol in a refrigerator at 4°C for later evaluation of AMF colonization. The remaining roots were dried in an oven at 65˚C for 48 hours and DM was recorded prior to acid digestion. Shoots and roots DM was recorded prior to processing for acid digestion. Shoots and roots were ground to a powder using a coffee grinder to ensure complete digestion and placed into Environmental Express® 100.0 mL plastic digestion tubes for storage.

Root Staining for AMF Observation

Root were prepared for AMF evaluation following the procedure described in Perry, et al. (2012) and Phillips and Hayman (1970). Samples were gently washed to remove soil and placed in 10% KOH solution and heated in a water bath at 90˚C for 30 minutes to clear roots of cell nuclei and organelles. Cleared roots were rinsed in tap water 5 times and placed in 2.5% HCl for 30 minutes at room temperature for acidification. Roots were stained in 0.05% Trypan blue for 15 minutes at 90˚C. Roots were destained in glycerol acidified with 2.5% HCl (acidic glycerol) and stored in a refrigerator until AMF observation.

Roots samples were cut into 1.0 cm lengths and placed on microslides for AMF observation. Twenty root samples were observed for each treatment group under 400x magnification using a compound light microscope. Presence of fungal structures, such as hyphae, arbuscules, and vesicles, were counted to determine the percentage of AMF
colonization in plant roots of different treatments as described in Johnson-Green, et al. (1995). Pathogenic fungal structures were also noted if present in root samples. Pathogenic fungi were differentiated from AMF by presence of characteristic haustoria and conidia structures characteristic of parasitic fungi (Freeman & Katan, 1997; Jumpponen & Trappe, 1998; Addy, et al., 2005)

**Acid Digestion and Analysis**

The dried plant material was acid digested for Atomic Absorption (AA) Spectrophotometer analysis at 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% HNO₃ (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 approximately 50 minutes. Samples were allowed to cool and the volume was brought to 100.0 mL with trace-metal grade distilled water. Diluted samples were vacuum filtered prior to AA analysis. Digested shoot and root samples were analyzed for Pb using a flame Atomic Absorption Spectrophotometer (Varian model SpectrAA 220) at the Chemistry Department of Kennesaw State University.

**Data Analysis:**

The performance of switchgrass phytoextraction of Pb from the soil was measured by the dry biomass produced by the plant and by the amount of Pb translocated to the harvestable tissue, known as the bioaccumulation factor. Bioaccumulation factor is defined as a ratio of Pb concentration of the harvestable tissue to Pb concentration of the soil (McGrath and Zhao,
The bioaccumulation factor was calculated for each treatment group. Successful phytoextraction strategies have a bioaccumulation factor greater than one (Sun et al., 2009).

Translocation factor was calculated to examine translocation of Pb from roots into harvestable above-ground tissues. The translocation factor was a ratio of Pb concentration in the harvestable biomass to Pb concentration present in the roots, revealing the efficiency of switchgrass at Pb translocation. Phytoextraction ratio (PR) was calculated as defined in Perry, et al. (2012) where the capacity of harvestable plant biomass is a ratio of dry plant biomass and volume of contaminated soil.

Data was analyzed using one-way analysis of variance (ANOVA) followed by post hoc Fisher’s test for Least Significant Difference (LSD) using SPSS v.22. Statistical significance was accepted at the level of p < 0.05.
Results

Experiment 1: Effect of Exogenous Phytohormone Application on Switchgrass Shoot Length and Biomass Production

Shoot Length

Phytohormone applications resulted in significant differences in longest shoot length among plants in different treatments (Figure 2). Plants treated with IAA (1.0 μM), BAP, (1.0 μM), and GA₃ (1.0 μM) resulted in significantly longer mean shoot length compared to plants in other treatments (p< 0.01) (Figure 2). Plants treated with 1.0 μM BAP had the largest increase in longest mean shoot length of 75.1 cm a 10% increase over Control plants (Figure 2). Other phytohormone treatments did not result in significant changes in shoot length compared to Control plants (Figure 2).
Figure 2: Average shoot length (cm) (±SD) of Panicum virgatum as measured by longest leaf length after 8 weeks of growth following applications of phytohormone indole-3-acetic acid (IAA), benzylaminopurine (BAP), or gibberellic acid₃ (GA₃). Means for columns with same letters are not significantly different (α = 0.05). ( * = p< 0.05) ( ** = p<0.01).
Dry Biomass

Plants in all treatments were dried in an oven and the DM was recorded. Plants treated with BAP (1.0 µM) and GA₃ (1.0 µM) had significantly higher DM than plants in other treatments (p<0.001) (Figure 3). Plants treated with BAP (1.0 µM) had the largest increase in DM over Control plants with mean DM of 0.5 g, a 48% increase over Control plants (Figure 3). Plants treated with GA₃ showed DM of 0.47 g a 38% increase compared to Control plants (Figure 3). Other phytohormone concentrations tested did not result in significant increases in switchgrass DM production compared to Control plants (Figure 3).

Figure 3: Mean dry biomass (g) after 8 weeks of growth following applications of phytohormone indole-3-acetic acid (IAA), benzylaminopurine (BAP), or gibberellic acid (GA₃). Columns with same letters are not significantly different (α = 0.05). (* = p<0.05) ( ** = p<0.01).
Observed Negative Effects of Phytohormone Application

Some negative effects of phytohormone treatment were observed at some of the applied concentrations (i.e. 100.0 µM and 10.0 µM for BAP and GA₃). Negative effects observed included increased plant mortality, deformed appearance with bends in the stem at the node, necrosis, and limp/weak stems. Applications of BAP (100.0 µM & 10.0 µM) and all concentrations of GA₃ resulted in significantly increased rates of mortality over the treatment period compared to other treatment plants (p< 0.001) (Figure 4). Plants treated with GA₃ (100.0 µM) experienced the highest rate of mortality, with 23% of plants dying during the weeks of foliar phytohormone application or shortly after the treatment (Figure 4). On the other hand, applications of IAA (all concentrations) and BAP (1.0 µM) did not experience mortality rates significantly higher than Control plants (α = 0.05) (Figure 4).

Other negative effects of phytohormone application were observed in plants treated with BAP (100.0 µM) and GA₃ (100.0 µM). Plants treated with BAP (100.0 µM) experienced elevated levels of necrosis. However, many plants appeared to recover after final foliar BAP application. Treatment with GA₃ (100.0 µM) resulted in a deformed appearance resulting in plants with a thin, stretched appearance, weak/limp stems that could not support the mass of the upper leaves without drooping, and prominent bends at nodes not present in controls (Figure 5).
Figure 4: Mortality rate among plants in different phytohormone treatment with applications of indole-3-acetic acid (IAA), benzylaminopurine (BAP), or gibberellic acid$_3$ (GA$_3$). Columns with same letter are not significantly different ($\alpha = 0.05$) (*= p<0.05) (**= p<0.01) (***= p<0.001)
A: Plants treated with GA$_3$ (100.0 µM) exhibiting thin, weak, and drooping foliage, with arrows pointing to prominent bends at nodes not present in Control plants.

B: Control plants with straight, upright, and robust stems.

Figure 5: (A) Plants treated with GA$_3$ (100.0 µM) exhibiting thin, weak, and drooping foliage, with arrows pointing to prominent bends at nodes not present in Control plants. (B) Control plants with straight, upright, and robust stems.
Experiment 2: Examining Phytoextraction by Switchgrass Enhanced with EDTA, Benomyl, and Phytohormones

Soil Testing

Preliminary soil testing with citric acid was carried out to determine proper concentrations for application to soil collected from the site in downtown Atlanta. Initial pH of collected soil was 6.4. Varying concentrations of citric acid were added to Pb contaminated soil to determine the minimum concentration required to achieve pH 4.0-4.5. Readings of soil pH were recorded every two days for an eight day period (Figure 6). The results indicated that 40.0 mmol kg\(^{-1}\) soil of citric acid was required to achieve and maintain pH 4.0-4.5 between weekly reapplication intervals (Figure 6). Application of a low concentration (25.0 mmol) did not result in maintenance of the required pH for the seven day reapplication interval. Higher concentrations resulted in pH readings below 4.0, outside of the optimum range.
Figure 6: Soil pH recordings before and after application of varying concentrations of citric acid. Citric acid was applied on Day 1 and monitored over an 8 day period with a pH meter.
**Shoot Length**

Weekly measurements of longest shoot length revealed differences in growth among plants in different treatments (Figure 7). Final measurements were taken of longest shoot length for each plant prior to harvest at week 11. Plants treated with BAP (1.0 µM) had the significantly longest mean shoot length of 84.0 cm compared to plants in other treatments (p<0.001) (Figure 7). Plants treated with EDTA had a mean shoot length of 68.3 cm, plants treated with benomyl had a mean shoot length of 70.08 cm, and plants treated with citric acid had a mean shoot length of 67.06 cm (Figure 7). Plants treated with EDTA, benomyl, and citric acid (E+B+C) had a significantly shorter mean shoot length of 60.41 cm, (p<0.01) (Figure 7). The application of BAP (1.0 µM) along with EDTA, benomyl, and citric acid (E+B+C+H) resulted in significantly increased mean shoot length over E+B+C, similar to control plants (p<0.01) (Figure 7). Mean shoot length for plants treated with benomyl and citric acid (B+C) and benomyl, citric acid, and BAP (B+C+H) were not significantly different from control plants (Figure 7).
Figure 7: Average shoot length (cm) (±SD) of Panicum virgatum at time of harvest. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05) (*= p<0.05) (**= p<0.01) (***= p<0.001).
Foliage Biomass

Measurements of foliage DM were recorded after harvest, prior to acid digestion (Figure 8). Application of BAP 1.0 μM resulted in a significantly increased shoot DM with a mean of 0.78 g (p<0.001) (Figure 8). Treatment with EDTA negatively affected plant DM production, reducing mean DM to 0.47 g, significantly less than other treatments (p< 0.01) (Figure 8). Treatment with benomyl or citric acid had no significant effect on DM of plants (Figure 8). Plants treated with EDTA, benomyl, and citric acid (E+B+C) experienced significantly decreased DM compared to other treatments with a mean of 0.49 g (p<0.01)(Figure 8). Application of BAP, EDTA, benomyl, and citric acid (E+B+C+H) resulted in a significant increase in DM over E+B+C treatments with a mean of 0.59 g (p<0.01)(Figure 8). DM of plants treated with benomyl and citric acid (B+C and benomyl, citric acid, and BAP (B+C+H) were not significantly different from other treatments.
Figure 8: Average foliage DM (g) (±SD) of *Panicum virgatum* at time of harvest. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different ($\alpha = 0.05$) (*= p<0.05) (**= p<0.01) (***= p<0.001).
Root Biomass

Root DM varied significantly among plants in different treatments (Figure 9). Control plants and plants treated with BAP (1.0 µM) (H) had the highest mean DM compared to plants in other treatments (Figure 9). Plants treated with EDTA, benomyl, or citric acid showed significantly lower root DM compared to Control plants (Figure 9) (p<0.05). Plants treated with combined chemical application of B+C, B+C+H, E+B+C, and E+B+C+H had significantly lowest root DM compared to plants in other treatments (p<0.001).

Figure 9: Average root DM (g) (±SD) of Panicum virgatum at time of harvest. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05) (*= p<0.05) (**= p<0.01) (***= p<0.001).
Observation of Chlorosis and Necrosis

Numerous negative effects of chelate application including chlorosis and necrosis were observed in plants in different treatments but not generally present in Control plants (Figures 10 & 11). Plants treated with EDTA (E), benomyl (B), or BAP (H) did not exhibit significantly elevated levels of chlorosis and necrosis compared to Control plants (Figure 12). Plants treated with citric acid resulted in significantly elevated level of chlorotic and necrotic leaves compared to Control plants (Figure 12). Plants treated with combined chemical application of B+C, B+C+H, E+B+C, or E+B+C+H all showed significantly elevated levels of chlorosis and necrosis compared to other treatments (Figure 12). Plants treated with B+C, B+C+H, E+B+C, or E+B+C+H also experienced severe purple discoloration of the leaves not present in other treatments (Figures 10 & 11).
Figure 10: Comparison of *Panicum virgatum* plant appearance. A: (left) Control plants exhibiting minimal chlorosis, B: (center) characteristic plant appearance with treatment with E, B, or C exhibiting elevated levels of chlorosis, C: (right) characteristic appearance of plants treated with combined chemical application of B+C, B+C+H, E+B+C, or E+B+C+H.
Figure 11: Detail of normal and discolored *Panicum virgatum* leaves. A: (left) Control plant with normal green leaf; B: (center) chlorotic leaf characteristic of plants treated with E, B, or C; C: (right) severely discolored purple leaf characteristic of plants treated with combined chemical application of B+C, B+C+H, E+B+C, or E+B+C+H.
Figure 12: Average chlorotic/necrotic leaf count (±SD) of *Panicum virgatum* at time of harvest. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05).
**Lead (Pb) Analysis**

Analysis of Pb concentration in plant shoots revealed significant differences in Pb uptake into harvestable biomass and total amount of Pb extracted for plants in different treatments. Treatments of combined chemical applications of B+C, B+C+H, E+B+C, and E+B+C+H showed significantly increased shoot Pb concentrations compared to plants in other treatments (Figure 13). Plants treated with B+C+H showed the highest mean shoot concentration of 38.0 mg kg\(^{-1}\) (Figure 13), a value 955% increase over Control plants (Figure 14). Plants treated with B+C showed a mean Pb shoot concentration of 36.8 mg kg\(^{-1}\) Pb, a value 922% increase over Control plants (Figure 14). Plants treated with E+B+C+H showed a mean shoot Pb concentration of 35.2 mg kg\(^{-1}\) Pb, a value 877% increase over Control plants (Figure 14). Plants treated with E+B+C showed a mean concentration of 34.8 mg kg\(^{-1}\) Pb, a value 866% increase over Control plants (Figures 13 & 14). Treatments of citric acid or BAP showed significantly increased Pb uptake compared to other treatments with citric acid producing a mean shoot Pb concentration of 27.2 mg kg\(^{-1}\), a value 655% increase in Pb concentration over Control plants (Figures 13 & 14). Benzylaminipurine (BAP) with mean of 25.2 mg kg\(^{-1}\) Pb, a value 600% increase over Control plants (Figures 13 &14). EDTA also produced significant differences in shoot Pb concentration with EDTA having a mean foliage concentration of 18.6 mg kg\(^{-1}\) Pb, a value 417% increase over Control plants(Figures 13 & 14) Benomyl had a mean foliage concentration of 16.2 mg kg\(^{-1}\) Pb, a value 350% increase (Figures 13 & 14). Control plants had significantly lowest shoot Pb concentration (Figure 13).

Total foliage dry biomass was multiplied with Pb concentrations to give the total Pb phytoextracted for plants in each treatment (Figure 15). Plants in all treatments phytoextracted
significantly more Pb from the soil than Control plants (Figure 15). Plants treated with B+C and B+C+H yielded the largest mean amount of phytoextracted Pb compared to plants in other treatments (Figure 15). Plants treated with E+B+C+H yielded significantly more phytoextracted Pb than plants treated with E+B+C (Figure 15). While shoot Pb concentrations were not significantly different between plants treated with E+B+C and E+B+C+H, plants treated with E+B+C+H had greater DM with BAP application than plants treated with E+B+C, resulting in a greater total amount of Pb extracted (Figure 15).

Analysis of plant roots for Pb concentration revealed significant differences among treatments (Figure 16). Plants treated with combined chemical application of E+B+C and E+B+C+H, had significantly higher root Pb concentrations than plants in other treatments (p<0.01) (Figure 16). Plants treated with E+B+C+H resulted in a Pb concentration of 64.4 mg kg⁻¹ (Figure 16). Plants treated with E+B+C showed a mean root Pb concentration of 57.6 mg kg⁻¹ (Figure 16). Plants treated with B+C and B+C+H showed higher Pb root concentrations than Control plants (p< 0.01), but were not significantly different than plants treated with E, B, C, or H (Figure 16). Addition of BAP did not produce any significant increases in root Pb concentration in plants treated with B+C+H as compared to B+C (Figure 16). Plants treated with E, B, C, or H showed significantly higher concentrations of Pb in roots compared to Control plants (p< 0.01) (Figure 16).
Figure 13: Average Pb concentration (mg kg\(^{-1}\)) (±SD) of *Panicum virgatum* foliage. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (\(\alpha = 0.05\)) (* = p<0.05) (**) = p<0.01) (***) = p<0.001).
Figure 14: Average increase in foliage Pb concentration (%) (±SD) of Panicum virgatum above Control plants for each treatment. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05) (* = p<0.05) (** = p<0.01) (*** = p<0.001),
Figure 15: Average total phytoextracted Pb (mg) (±SD) in foliage of Panicum virgatum. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05) (*= p<0.05) (**= p<0.01) (***= p<0.001).
Figure 16: Average Pb concentration (mg kg\(^{-1}\)) (±SD) in roots of Panicum virgatum. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (\(\alpha = 0.05\)) (*= \(p<0.05\)) (**= \(p<0.01\)) (**= \(p<0.001\)).
Figure 17: Average percent (%) increase (±SD) in Pb root concentrations over control plants. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different (α = 0.05) (*= p<0.05) (**= p<0.01) (***= p<0.001).
**Remediation Efficiency Calculations**

Bioaccumulation, translocation, and phytoextraction ratio calculations were carried out to determine the efficiency of the remediation strategy. Bioaccumulation factor (BF) measures the ability of switchgrass to accumulate Pb efficiently. Suitable phytoextraction strategies should generate BF values above 1 (Sun, et al. 2009). While all treatments improved BF compared to control plants, no treatment achieved a BF greater than 1 (Table 4). The highest BF was found in plants treated with the combined chemical application of B+C+H showing a BF of 0.35. Plants treated with the combined chemical application of B+C, B+C+H, E+B+C, and E+B+C+H showed the highest BF values compared to plants in other treatments (p< 0.05) (Table 4). Plants treated with single chemical application of E, B, C, and H showed significantly higher BF values compared to Control plants (p< 0.05) (Table 4).

Translocation factor (TF) was calculated to determine the ability of switchgrass in various treatments to translocate Pb from roots into harvestable foliage (Table 4). All treatments dramatically increased Pb translocation compared to Control plants. In particular, combined chemical treatment of B+C and B+C+H had the highest TF values of all treatments with 0.86 and 0.85, respectively. Chemical treatment with single application of citric acid (C) and BAP (H) performed better than single application of EDTA (E) and benomyl (B) (p< 0.05) (Table 4).

Phytoextraction ratio (PR) was calculated for each treatment to determine the percentage of Pb removed from the soil by each treatment in a single harvest (Table 4). Plants treated with combined chemical application of B+C and B+C+H had the highest PR values with 0.18% of Pb extracted (Table 4). Application of EDTA and benomyl produced PR values in plants
significantly higher than Control plants with 0.07% and 0.08% of Pb extracted, respectively (p<0.05)(Table 4). Plants treated with citric, hormone, B+C, B+C+H, E+B+C, and E+B+C+H extracted more Pb than plants treated with EDTA, benomyl, and Control plants (p<0.05) (Table 4).

<table>
<thead>
<tr>
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<th>Bioaccumulation Factor</th>
<th>Translocation Factor</th>
<th>Phytoextraction Ratio*</th>
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<td>0.02 (C)</td>
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<td>0.45 (C)</td>
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Table 4: Comparison of bioaccumulation factor (BF), translocation factor (TF), and phytoextraction ratio (PR) for Panicum virgatum across treatments. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Groups with same letter are not significantly different (α = 0.05). *Phytoextraction Ratio gives the total percentage of Pb removed per harvest.
Arbuscular Mycorrhizal Fungi Observation

Plant roots in each treatment were processed and stained for AMF detection as described in Phillips & Hayman (1970) to verify colonization and number of fungal structures (e.g. hyphae, arbuscules, and vesicles). Control plants had the highest total AMF colonization of 92% (Figure 18). Chemical treatment with EDTA, citric acid, and BAP did not result in significant reductions of total AMF colonization compared to Control plants (Figure 18). Application of benomyl significantly reduced AM fungal colonization to 59% compared to 91% in roots of Control plants (p< 0.001) (Figure 18). Plants treated with combined chemical application of B+C, B+C+H, E+B+C, and E+B+C+H had similar level of total AMF colonization compared to plants treated with benomyl; 67% for E+B+C, 62% for E+B+C+H, 66% for B+C, and 57% for B+C+H (Figure 18). All plants treated with benomyl alone or combined application of benomyl with other chemicals did not have significantly different root AMF colonization (Figure 18).

It was also observed that treatments with benomyl and other chemicals affected the presence of fungal structures hyphae, arbuscules, and vesicles (Figures 19, 20, & 21). Plants treated with benomyl had the largest reduction in arbuscules (47%) compared to 84% of Control plant roots (Figure 19). Benomyl application also reduced the amount of vesicles (30%) compared to 59% in Control plants (Figure 19). Plants treated with EDTA did not reduce the total root AMF colonization, but significantly decreased the colonization of arbuscules and vesicles present (p<0.01) (Figure 19). Application of EDTA also resulted in lower colonization of vesicles (45%) compared to 59% in Control plants (p< 0.001) (Figure 19).

Plants treated with citric acid and BAP did not show significant lower number of hyphae, arbuscules, or vesicles compared to Control plants (Figure 19). Plants treated with combined
chemical application of B+C, B+C+H, E+B+C, and E+B+C+H did not show lower numbers of individual fungal structures as compared to plants treated with benomyl alone (Figure 19).

**Figure 18:** Average percentage (%) of total arbuscular mycorrhizal fungi colonization of roots of *Panicum virgatum*. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Means for columns with same letter are not significantly different ($\alpha = 0.05$) (*= p<0.05) (**= p<0.01) (** *= p<0.001).
Figure 19: Average percentage (%) of root samples colonized by Arbuscular Mycorrhizal fungi structures hyphae, arbuscules, and vesicles. (Treatment labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Significance groups for separate structures are indicated by single letters (A or B) for hyphae, double letters (AA, BB, or CC) for arbuscules, and triple letters (AAA, BBB, or CCC) for vesicles. Means for columns with same letter(s) are not significantly different (α = 0.05).
Figure 20: Arrow pointing to cluster of AMF structures including arbuscules, vesicle, and hyphae in Trypan blue stained root.

Figure 21: Arrows indicating presence of arbuscular mycorrhizal fungi arbuscules (A) and hyphae (B) stained with Trypan blue with individual plant cell walls visible.
Pathogenic Fungi Observation

Arbuscular mycorrhizal fungi (AMF) staining and observation also revealed substantial levels of pathogenic fungi infection in some treatments through observation of parasitic fungi structures (haustoria and conidia) and septa in fungal cell wall (Figures 22 & 23). All plants treated with benomyl, whether with benomyl alone or in combination with other chemicals, showed elevated levels of pathogenic fungi infection (Figure 23). Treating plants with benomyl raised pathogenic fungi infection to 38%; and even higher when plants were treated with E+B+C (49%); when treated with E+B+C+H (41%); when treated with B+C (31%) and B+C+H (43%) (Figure 23). Three percent of samples from Control plants showed evidence of pathogenic fungal infection through observation of fungal structures such as haustoria and conidia (Figure 22). Single chemical application of EDTA, citric acid, and BAP did not result in significantly elevated levels of pathogenic fungi colonization compared to Control plants (Figure 23).
**Figure 22**: Comparison of pathogenic fungi with haustoria & conidia (A & B) and AMF hyphae (C) stained with Trypan blue viewed under 400x magnification.
Figure 23: Pathogenic fungi infection percentages (%) of *Panicum virgatum* across treatments. (Treatments labeled: E = EDTA, B = Benomyl, C = Citric Acid, H = Hormone BAP). Columns with same letter are not significantly different ($\alpha = 0.05$) ($p<0.01$).
Discussion

Effects of Phytohormones on Switchgrass Growth

The first study was conducted to determine the type of phytohormone and concentration level effective at significantly increasing switchgrass biomass production. The results are consistent with previous studies that indicated that plant biomass production could be maximized through exogenous phytohormone application (Fernandez, et al. 2009; Jin, et al. 2011; Wuddineh, et al. 2014). However, no consensus existed on proper phytohormone use or concentration for application on switchgrass. The results indicated that switchgrass DM can be maximized through application of BAP (1.0 µM) and GA3 (1.0 µM)(Figure 3). The data also demonstrated that an increase in overall shoot length does not necessarily indicate an increase in DM or have an advantage to phytoextraction strategies as indicated by results of plants treated with IAA (1.0 µM)(Figures 2 & 3). These results could be critical in future phytoextraction and bioenergy projects, such as biofuel production, as an effective phytohormone and concentration level resulting in a 48% increase in DM over control plants was identified in this study.

Previous studies have shown that switchgrass var. ‘alamo’ is capable of generating 17,800 kg of harvestable DM per hectare (ha) (Smith, et al. 2015). Another variety of 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, which is much lower than other high biomass crops (McLaughlin & Kszos, 2005). Significant increases in biomass production through exogenous phytohormone application could have implications in future bioenergy
production and phytoextraction projects by increasing uptake of metals through maximizing switchgrass biomass. The 48% gain in DM in plants treated with BAP compared to Control plants could make switchgrass production for bioenergy applications more feasible and cost-effective. A 48% increase in DM yield through BAP application could potentially produce 26,300 kg ha\(^{-1}\) for Alamo and 47,800 kg ha\(^{-1}\) for biofuel production.

The results of this study also confirmed the possible adverse effects of high concentrations of phytohormones on plant health and mortality that has already been documented in other studies (Shaddad, et al. 1989; Varshney, et al. 2012). Concentrations over 1.0 µM were ineffective at increasing either shoot length or biomass and significantly increased observations of abnormal stem appearance in plants treated with GA\(_3\), leaf burn in BAP, and premature plant death in GA\(_3\) and BAP. Switchgrass is currently being studied as a possible bioenergy crop due to its ability to provide large amounts of biomass per hectare (Smith, et al. 2015). Results of this study suggest that DM could be increased by as much 48% with application of phytohormone BAP (1.0 µM).

**Phytoextraction of Lead by Switchgrass**

One objective of this study was to examine the efficiency of switchgrass in Pb phytoextraction and the use of EDTA as a chelating agent. Plants in all treatments accumulated more Pb into harvestable tissues than Control plants. Plants treated with combined application of benomyl and EDTA (B+C, B+C+H, E+B+C, and E+B+C+H) yielded the largest amount of Pb-phytoextraction, this was consistent with findings of a previous study (Perry, et al. 2012). In this respect, single chemical applications of E, B, C, or H were all inferior to combined chemical
application of B+C, B+C+H, E+B+C, or E+B+C+H. Plants treated with combined chemical application of B+C and B+C+H showed higher foliage Pb concentrations compared to plants treated with E+B+C and E+B+C+H; these results are consistent with previous studies which indicated that citric acid could achieve sufficient Pb uptake (Frietas, et al. 2013). Increased Pb uptake by plants treated with citric acid could have resulted from the high initial pH of the collected soil (pH 6.8) which would have resulted in very low Pb solubility (Dong, et al. 1999). Previous studies have suggested that Pb solubility in soil is generally highest between pH 4.0-4.5 (Dong, et al. 1999). A previous study by Johnson, et al. (2015) using switchgrass found that treatments of EDTA and benomyl did not produce shoot Pb- concentrations significantly higher than control plants. The results of the current study indicate that citric acid and benomyl application resulted in increased uptake and was achieved without the addition of EDTA. Supplemental application of EDTA to plants receiving citric acid and benomyl (E+B+C) resulted in decreased shoot Pb- concentrations as compared to plants treated with B+C.

Application of EDTA was found to be effective at increasing Pb concentration in plant roots compared to plants that did not receive EDTA (B+C and B+C+H). This strongly suggests that EDTA does increase Pb solubility and this finding is consistent with the result of previous phytoextraction studies (Johnson, et al. 2015; Perry, et al. 2012). However, greater root Pb-concentrations did not translate into greater translocation into harvestable foliage or a greater amount of Pb-phytoextraction. This result implicate substantial reduction in the use of EDTA by demonstrating that citric acid is capable of achieving similar levels of Pb-uptake into harvestable foliage of switchgrass.
EDTA has met resistance to its use due to negative effects associated with application including long persistence time in the soil environment, potential mobilization of Pb into groundwater, and negative effects on plant growth (Oviedo & Rodriguez, 2003; de Araujo, et al. 2010; Meers, et al. 2010). The results indicated that single applications of EDTA application do significantly reduce switchgrass shoot and root biomass contrary to the findings of Johnson, et al. (2015). Treatments including EDTA, benomyl, citric acid, and BAP (E+B+C+H) were not significantly different in inducing accumulation of Pb into harvestable tissues as compared to other treatments, particularly B+C or B+C+H. This indicates that there was no benefit of combining EDTA application with citric acid at increasing uptake into harvestable foliage biomass in this study. Treatment with citric acid combined with benomyl (B+C) generated Pb uptake into harvestable tissues greater than when plants were additionally treated with EDTA (E+B+C), which could eliminate its use and negative effects on biomass from future phytoextraction projects.

Bioaccumulation factors (BF) were far below 1 for all treatments. Previous research suggests that plants should have a BF of one or greater to be considered viable for efficient phytoextraction (McGrath and Zhao 2003; Sun, et al. 2009). Greater bioaccumulation is needed to achieve sufficient uptake and reduce the time required for remediation to be viable (McGrath and Zhao 2003). Increases in biomass can increase total Pb extracted, reducing the time required for phytoextraction projects. Another approach involves uptake into harvestable foliage. Research by Balsamo, et al. (2015) indicates that switchgrass is capable of accumulating high concentrations of Pb in the foliage efficiently at greater soil Pb concentrations than were used in this study.
Translocation factor (TF) varied widely among plants in different treatments. Plants in all treatments achieved significant improvement in TF compared to Control plants. The significantly highest TF (0.86) was observed in plants treated with B+C and plants treated with B+C+H (TF 0.85). Application of citric acid (C) and BAP (H) significantly increased Pb-translocation compared to Control plants. Benomyl (B) and EDTA (E) also increased Pb-translocation as compared to Control plants, but were significantly lower than treatment with citric acid (C) and BAP (H). Plants treated with citric acid or BAP alone showed significantly higher Pb-translocation compared to plants treated with E+B+C and E+B+C+H. This result suggests that EDTA negatively affected Pb translocation from roots to shoots. These results are consistent with findings by Jean, et al. (2009), in which EDTA was found to alter plant physiology and affect TF of nickel (Ni) and chromium (Cd) in Datura inoxia Mill. (thorn-apple).

Phytoextraction ratio (PR) represents efficiency of the treatments by giving the total percentage of Pb removed from the soil per harvest. PR values were far below one percent indicating the tested treatment strategies would take decades to reduce Pb soil concentrations to an acceptable level. With rapid growth of switchgrass enhanced with BAP, it may be possible to maximize growth and achieve multiple harvests per year, multiplying the percentage Pb removed per year and shortening cleanup time required at a contaminated site.

Effects of Phytohormones on Lead Phytoextraction

Application of the phytohormone BAP was found to significantly increase shoot Pb-concentrations compared to Control plants. This result is consistent with previous research by Lopez, et al. (2005) which demonstrated that exogenous phytohormone application can
increase Pb uptake into harvestable foliage. However, when combined with other chelates and benomyl, BAP had no effect on Pb uptake into foliage. This result is contrary to the findings of Lopez, et al. (2005) in which phytohromone application resulted in greater shoot Pb when combined with chelate application. Application of BAP was found to have a significant 11.6% increase in Pb concentrations in the roots of plants treated with E+B+C+H as compared to plants treated with E+B+C. BAP is known to indirectly affect stomatal opening which could have increased phytotranspiration and thus Pb absorption into roots; however, this effect was not observed and did not translate into increased Pb translocation into harvestable foliage (Barker & Tagu. 2000; Tassi, et al. 2008).

In addition to the direct effects on Pb uptake, BAP application also resulted in difference in root and shoot DM. Benzylaminopurine (BAP) increased shoot DM by 16% as compared to control plants. Plants treated with E+B+C+H had significantly higher shoot length and DM compared to plants treated with E+B+C. While EDTA application resulted in decreased shoot length and DM, treatment with BAP rescued shoot length and DM to levels similar to Control plants. Application of BAP in combination with benomyl and chelates (B+C+H & E+B+C+H) had no effect on root DM. BAP was also observed to have no effect on levels of chlorosis and necrosis across treatments indicating that chlorosis and necrosis was likely cause by chelate application as indicated by previous studies (Kochian, 1995; Johnson, et al. 2015).

Exogenous phytohormone application has also been reported to alter mycorrhizal symbioses (Pozo, et al. 2014). These symbioses can alter and improve plant response to external stimuli such as plant stress resulting from environmental conditions (Pozo, et al. 2014).
Phytohormone application resulted in no significant differences in levels of AMF colonization with BAP application as compared to Control plants. Application of BAP also had no effect on pathogenic fungi infection rates indicating no alteration of AMF activity in protecting host plant roots from pathogenic infection.

Effects of Chelate Application on Plant Health

Many negative effects of chelate application were observed in tests plants and results are consistent with findings of plant stress and metal toxicity in previous studies (Johnson, et al. 2015; Kochian, 1995; Perry, et al. 2012). Elevated levels of chlorosis and necrosis were observed in plants treated with citric acid whether in single applications or combined with EDTA, benomyl, and BAP (E+B+H). Plants treated with citric acid (C) were observed to have increased levels of chlorosis as compared to control plants with the appearance of yellow leaves. Plants treated with combined chemical application of B+C, B+C+H, E+B+C, or E+B+C+H resulted in severe discoloration with an abnormal purple color and the highest levels of chlorotic and necrotic leaves. Previous research indicated this purple discoloration can be caused by multiple factors including metal toxicity and severe phosphorus (P) deficiency (Johnson, et al. 2015; Kochian, 1995). A preliminary study by Greipsson, et al. (unpublished) using Laser-Ablation Inductively Coupled Plasma Mass-Spectrometry (LA-ICP-MS) has shown that benomyl and EDTA treatments resulted in about 50% reduction P in leaf tips (0.5 cm) suggesting that P deficiency could have been responsible for the severe discoloration observed.

Metal toxicity has been observed to result in increased levels of chlorosis and necrosis through interference in chlorophyll synthesis (Foy, et al. 1978; Kochian, 1995). Plant cells often
sequester Pb into cell vacuoles to limit interference in cellular processes such as photosynthesis (Morel, et al. 2009). Increasing Pb concentrations in shoots may be responsible for metal toxicity and foliage discoloration (Kochian, 1995). Nonspecific iron (Fe) transporters within plant cells also result in increased Pb concentrations when Pb is inadvertently transported into cellular locations instead of Fe (Vert, et al. 2001; Guo, et al. 2015).

Metal toxicity from excessive aluminum (Al) uptake has also been observed to cause similar effects in other plant species, resulting in severe chlorosis, necrosis, and discoloration (Kochian, 1995). High levels of Al present in the soil can be solubilized in soils below pH 5.5 (Kochian, 1995). Soil testing at the University of Georgia Soil Sciences lab revealed high levels of Al present in the contaminated soil (21,850 mg kg⁻¹). Addition of citric acid could have solubilized Al resulting in foliage discoloration. Future studies should include Al analysis of plant tissue to determine if Al toxicity contributes to severe foliage discoloration.

Purple discoloration of the leaves could also have been caused by severe nutrient deficiency (Bouma, et al. 1966). Arbuscular mycorrhizal fungi (AMF) form mutualistic relationships with 85% of land plants including perennial grasses such as switchgrass by supplying plants with up to 90% of scarce essential nutrients such as phosphorus in exchange for C compounds (Reynolds, et al., 2003; van der Heijden, et al., 2015). Benomyl decreased levels of AMF colonization by inhibiting microtubule formation (Oakley & Morris, 1980). This would have decreased the amount of phosphorus received by switchgrass from the AMF. Future studies may consider foliar P application to increase available P to the plant which could result in greater plant biomass. Benomyl application alone cannot explain elevated levels of
chlorosis and necrosis present in plants treated with B+C, B+C+H, E+B+C, or E+B+C+H since plants treated with a single application of benomyl (B) did not exhibit significantly elevated rates of chlorosis and necrosis.

Plants treated with citric acid (C) experienced elevated levels of chlorosis and necrosis as compared to control plants and treatments E, B, and H. This suggests that citric acid may have contributed to elevated levels of chlorosis and necrosis through possible solubilization of toxic levels of Al as described in Kochian, et al. (1995). However, the appearance of the plant leaves being chlorotic with citric acid (C) treatment compared to purple leaves when treated with B+C, B+C+H, E+B+C, and E+B+C+H may indicate a phosphorus deficiency due to benomyl application. Foliar applications of P may reduce chlorosis of plants in future phytoextraction studies and field applications.

Effects of Benomyl on Fungal Infection

The results showed reduced AMF colonization AMF after application of benomyl and the consequent increase in Pb uptake are consistent with findings in previous research AMF (Hovsepyan & Greipsson, 2005; Perry, et al. 2012). Reduced AMF activity allows for increased uptake of Pb due to elimination of the ability of AMF to protect the plant host from uptake of toxic metals (Leyval, et al. 1997; Vosatka, et al. 2006). The result showed decrease in AMF colonization in plants receiving treatments of benomyl in combination with other chemicals (B+C, B+C+H, E+B+C, E+B+C+H) and in plants treated with single applications of benomyl as well.
Reduction in AMF colonization also appeared to eliminate the ability of the mutualistic symbiotic fungi to protect the switchgrass host plant from infection with pathogenic fungi. Similar reduction in AMF protection for plant pathogens was described in Azcón-Aguilar, et al. (2002). Elevated levels of pathogenic fungi infection were observed in all plant roots that received benomyl application, indicating AM fungi were protecting host roots from infection through secretion of exudates as described in Azcón-Aguilar (2002). Upon application of benomyl, this ability appeared to be significantly reduced, resulting in increased pathogenic fungi infection in switchgrass roots. The results suggest that benomyl affected symbiotic AMF and pathogenic fungi species differently. While colonization of beneficial fungi was reduced, pathogenic infection rates were increased upon benomyl application, suggesting that benomyl is not as effective at controlling pathogenic fungi species. Previous studies confirm that benomyl resistance is widespread across many pathogenic fungal species (Goettel, et al. 1990; Jung, et al. 1992; Yarden & Katan, 1993; EPA, 2002; Yan & Dickan, 1996). Benomyl was withdrawn from production in the United States in 1991 mainly due to the appearance of many resistant pathogenic fungi species (EPA, 2002). The existence of benomyl resistant fungi species could explain the results of higher pathogenic fungi infection rates in plant roots that received benomyl application.

Recent research 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 technique involves extraction of heavy metals from the biomass for recycling and use of 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). 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). The use of recycled biomass for energy production could also reduce costs associated with remediation by commercializing the produced biomass after Pb extraction. Commercial switchgrass production could also be produced on marginal or contaminated land not suitable for agricultural use because of its tolerance of many soil types and conditions. The extracted Pb could then be sold for commercial applications and the biomass for ethanol production (Sanderson, et al. 1996).

**Future Research Directions**

Further study is needed in identifying optimal hormone concentrations for maximizing switchgrass biomass production and Pb uptake. A greater range of concentrations of phytohormones must be examined since no consensus exists on optimal concentrations or proper timing of application for phytoextraction and bioenergy applications. Studies are needed to examine the number of successive phytohormone applications to increase biomass production. Future study should focus further on the effect of GA$_3$ on Pb translocation into harvestable biomass. GA$_3$ has been observed to affect metal uptake in other plant species, resulting in increased accumulation of cadmium (Cd) into harvestable tissues (Hadi, et al. 2014). Furthermore, GA$_3$ is also known to affect Fe uptake which could result in increased Pb translocation due to nonspecific Fe transporters (Vert, et al. 2001; Guo, et al. 2015).
Further investigation into the viability of citric acid as a chelating agent for Pb is needed to determine if sufficient uptake can be achieved. A better understanding is needed of how soil pH and acid application affect Pb solubility in Ultisol soil types and translocation into foliage. The results indicated that EDTA application alone does increase Pb absorption into roots, but the benefits are negated by no increase in Pb translocation into foliage. Therefore, more research is needed on strategies to maximize translocation of Pb into foliage. One possible method of increasing Pb uptake could be through exogenous application of nitric oxide (NO) donors (Yu, et al. 2012). Exogenous application of NO donors is proposed to act as signal in plants to increase iron (Fe) uptake from the roots into foliage (Yu, et al. 2012). Since Fe transporters on root cells are nonspecific, signaling for Fe uptake could result in inadvertent increase in translocation of Pb into foliage (Vert, et al. 2001; Guo, et al. 2015).

More study is needed in the effects of citric acid and chelation of other metals toxic to plants. The cause of observed poor plant health, appearance of severe chlorosis and necrosis, and abnormal purple coloration needs to be explored further and could result from multiple factors such as nutrient deficiency of nitrogen (N) or phosphorus (P) or from metal poisoning. Commonly used agricultural practices such as supplementing plants with commercially available fertilizer (even through foliar application) could alleviate poor plant health, especially upon elimination of AMF.

Other acids have also been suggested as possible alternatives to citric acid which may result in greater translocation into harvestable tissues. Picolinic acid (C₅H₄N) has been shown to be effective at chelation and translocation of cadmium (Cd) (Simon, et al. 2003). It is thought
that picolinic acid forms soluble Pb complexes similar to citric acid, but performance at increasing Pb solubility and uptake needs to be studied further (Simon, et al. 2003).

Uptake of toxic levels of aluminum (Al) could explain stunted growth and abnormal purple leaf color observed upon citric acid application (Kochian, 1995). One possibility for remediation is delayed citric acid application after plants have achieved a larger size and larger biomass which could result in greater Pb phytoextraction. Improvements to plant health are needed to increase biomass yield and to keep plants alive if multiple harvests per year are to be viable.

More study is also needed to better understand the role of soil microbes and their effect on plant health and efficiency of phytoextraction. Since benomyl reduces AMF colonization, but is ineffective at controlling resistant pathogenic species, a new method of controlling fungi is needed. Another fungicide, propiconazole is one possible alternative to benomyl to which pathogenic fungi are currently not resistant (Calonne, et al. 2012). Propiconazole is effective at reducing AMF colonization, however its performance when combined with chelates warrants further study (Calonne, et al. 2012).
**Conclusion**

Phytoextraction of Pb by switchgrass chemically enhanced with chelates, benomyl, and phytohormone has many implications in future research for both phytoextraction and bioenergy industry. Given the long persistence time of EDTA in soil and potential mobilization of Pb into groundwater, a study was conducted to determine if citric acid application could achieve similar effects as EDTA. Plants treated with citric acid and benomyl (C+B) had similar Pb uptake as plants treated with EDTA alone. The results indicated that switchgrass did not translocate more Pb into foliage when supplemented with EDTA and thus EDTA provided no extra benefit to total Pb phytoextracted from soil and translocation into foliage.

Plants treated with exogenous phytohormone application showed dramatic increase in DM when applied in low concentrations; no benefit in Pb translocation into foliage was however found. Exogenous BAP application resulted in significant gains in switchgrass DM which could have implications for bioenergy industry and phytoextraction projects. Greater biomass production is most likely cost effective in commercial bioenergy production and viable for phytoextraction strategies. All treatments resulted in greater total phytoextraction compared to Control plants. Total phytoextraction was greatest in plants treated with combined application of B+C and B+C+H. However, plants treated with combined application of E+B+C and E+B+C+H resulted in less total Pb extracted due to low biomass. Plants treated with single chemical application of E, B, C or H extracted more Pb than Control plants, but were all inferior to treatments of combined application of B+C or B+C+H.
Application of the fungicide benomyl was found to reduce AMF colonization as reported by other studies. However, benomyl also resulted in increased levels of pathogenic fungi infection because of widespread benomyl resistance.
Integration

This study integrated techniques from multiple fields of biology and chemistry, combining alterations of soil chemistry, manipulation of soil microbes, and foliar application of phytohormones in a phytoextraction treatment regimen. Information on Pb solubility and heavy metal chelation was used to determine a treatment strategy that attempted to maximize Pb solubility for greatest phytoextraction. Solubility of Pb was increased through applications of chemical chelating agents EDTA and citric acid to form soluble Pb complexes and lowered soil pH.

Mutualistic plant-fungal interactions were manipulated through application of the fungicide benomyl. Information from previous molecular studies elucidated the ability of AMF to prevent Pb uptake through secretion of exudates and gene regulation. Previous studies also provided information on the ability of AMF to protect host plants from pathogenic fungal infection. Benomyl application resulted in decreased AMF colonization and increased uptake of Pb into harvestable foliage. Information from previous molecular studies also revealed the role of AMF in Pb translocation, sequestration, and plant tolerance of heavy metals.

This study also included aspects of plant physiology and bioenergy through foliar applications of phytohormones and analysis of the resulting physiological effects of maximizing biomass production. Previous studies were used to identify possible phytohormones and concentrations to maximize foliages biomass. Plant physiology was also examined through the study of Pb translocation from roots to foliage.
References


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