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Phytoextraction of lead (Pb) contaminated soil by switchgrass (*Panicum virgatum* L): Impact of BAP and NTA applications

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The accumulation of Pb deposits in soil is a growing global concern. Soil remediation options include phytoextraction that involves the use of plants and associated soil microorganism. Switchgrass (*Panicum virgatum* L.), a second-generation bioenergy crop was used in this study due to its ability to produce high biomass and grow in metal polluted soils. Plants were grown in Pb-contaminated soil (5,802.5 mg kg^{-1}) in an environmentally controlled greenhouse. Plants were treated with exogenous application of the plant growth regulator (PGR) benzylaminopurine (BAP) or complete foliar nutrient solution (Triple-12®) twice a week until harvested. Plants also received the soil fungicide propiconazole (Infuse™) that was followed by the soil chelate nitrilotriacetic acid (NTA). Two concentrations of NTA were compared (5 mM and 10 mM) and combined application of NTA (10 mM) + APG (alkyl polyglucoside). Soil fungicide (propiconazole) was used to arrest arbuscular mycorrhizal fungi (AMF) activities in the roots of switchgrass in order to enhance Pb-phytoextraction. Lead (Pb) was measured in dry plant materials using an ICP-OES. Phytoextraction by switchgrass was significantly improved by dual soil applications of 10 mM NTA, APG and foliar applications of BAP which resulted in the greatestaverage Pb concentration of 5,942 mg kg^{-1}. The average dry mass of plants and the average value for total phytoextracted Pb (mg) per pot were significantly greatestfor plants treated with 10 mM NTA, APG and BAP. Also, plants treated with NTA and BAP showed average bioconcentration factor of 1.02. The results suggested that chemically enhanced phytoextraction significantly improved biomass production of switchgrass and at the same time increased phytoextracted Pb which is important for phytoremediation and bioenergy industry.

1 Introduction

Accumulation of lead (Pb) in soil is a growing concern due to the human health implications and associated environmental damage (Anyanwu et al., 2018; Eiró et al., 2021). Lead contamination in soils is derived from various sources, including past use of leaded gasoline, smelting and mining and impurities in pesticides and chemical fertilizers (Zhuang et al., 2006; De Araújo and Do Nascimento 2010; Greipsson et al., 2013; Wu et al., 2016; González-Chávez et al., 2019; Alengebawy et al., 2021; Mufalo et al., 2021). Lead is persistent in the topsoil, but Pb exposure can occur if soil become re-suspended (Laidlaw and Taylor, 2011; Mielke et al., 2016). Levels of Pb-contamination in soil and blood lead level (BLL) in children are strongly correlated (Laidlaw and Taylor 2011). Currently there is no safe level of Pb exposure for humans (Eiró et al., 2021; McFarland et al., 2022). Neurological dysfunctions have been associated with children who had blood Pb levels of 10 mg/L or less (Canfield et al., 2003; Skerfving et al., 2015; McFarland et al., 2022). Even, long-term exposure to low-level Pb is associated with neurological damages which includes cognitive dysfunction in children (Canfield et al., 2003; Martinez-Finley et al., 2012; Paulson and Brown, 2019), along with Alzheimer's (Bakulski et al., 2012; Fathabadi et al., 2018) and Parkinson's diseases (Chen et al., 2017).

The removal of Pb-contaminants from soil can be accomplished through phytoextraction, an emerging cost-effective heavy metal remediation technique (Garbisu and Alkorta 2001; Greipsson 2011; Kafle et al., 2022). Phytoextraction has the potential to remove significant amounts of heavy metal contaminants from soil and restore the impacted soil for future uses (Huang et al., 1997; Greipsson et al., 2022b). The effectiveness of phytoextraction is mainly a function of a plant's biomass production and the level of contaminants in the harvested biomass. Phytoextraction involves the use of plants and their associated soil microbes to remove heavy metals from contaminated soils (Greipsson 2011; Yan et al., 2020; Kafle et al., 2022). Phytoextraction usually requires acidification or chelation of soil which further facilitates the uptake of Pb (Aderholt et al., 2017; Chen et al., 2022). Soil chelation may be improved based on the activation efficiency of the chelating agent used, but that is widely dependent on specific heavy metal interactions within soil (Evangelou et al., 2007; Liu et al., 2020). Ethylenediaminetetraacetic acid (EDTA) was previously identified as a successful chelator in several phytoremediation studies but has raised environmental concerns related to its limited biodegradability (Elliott and Brown 1986; Oviedo and Rodríguez 2003; Greipsson 2011; Johnson et al., 2015; Aderholt et al., 2017). To limit potential environmental impacts, biodegradable chemicals other than EDTA including nitrilotriacetic acid (NTA) and natural chelates like citric acid

have been tested in phytoremediation studies as well (Freitas et al., 2013; Aderholt et al., 2017; Hart et al., 2022).

The use of plant growth regulators (PGR) have been used to increase plants biomass production in phytoextraction (Chen et al., 2022). Application of the plant growth regulator benzylaminopurine (BAP) on switchgrass was found to significantly increase biomass and shoot Pb concentrations (Aderholt et al., 2017; Hart et al., 2022). The BAP is a cytokine stimulator that has been shown to encourage plant bud growth and further stimulate multiple root growth *via* cell division (Bouré et al., 2019). Also, application of foliar nutrients has positively impacted growth of plants used in phytoremediation (Meers and Tack 2004).

Soil microorganisms play a critical role in the survival of plants on metal contaminated soils (Wang 2017). Arbuscular mycorrhizal fungi (AMF) are commonly found in roots of plants growing in metal contaminated soils and their presence limits Pb uptake of plants (Leung et al., 2013; Xu et al., 2014; Cabral et al., 2015; Cabral et al., 2015; Wang 2017; Sarkar et al., 2018). During phytoextraction it is important to allow plants to grow and attain great biomass, but metal uptake should be facilitated before harvest (Greipsson 2011). Application of soil fungicide can arrest AMF activities and therefore enhance Pb-phytoextraction (Hovsepyan and Greipsson, 2004; Perry et al., 2012; Hart et al., 2022). Previous studies showed that applications of the soil-fungicide benomyl improved phytoextraction of Pb (Perry et al., 2012). Another soil-fungicide, propiconazole was found to reduce AMF colonization in roots and improve Pb phytoextraction by switchgrass (Calonne et al., 2012; Aderholt et al., 2017).

Switchgrass, a second-generation bioenergy crop was used in this phytoextraction study. Switchgrass is a potential candidate for combined phytoextraction and biomass production for biofuel production (Chen et al., 2012; Balsamo et al., 2015). Furthermore, the use of switchgrass biomass for biofuel production could potentially reduce costs associated with phytoextraction. The cultivation of biofuel crops on polluted soils may improve energy security and aid in mitigating climate change (Qin et al., 2015). In addition, the cultivation of bioenergy crops on polluted soils may reduce the need for using primary agricultural lands for biofuel production.

The main aim this study was to examine the efficacy of Pb phytoextraction by switchgrass through the combined addition of chelating agent (NTA) and APG, PGR and a complete foliar nutrient solution.

2 Materials and methods

2.1 Soil

Soil was collected from a highly contaminated former Superfund site in Cedartown, Georgia, United States. This site

was used as a Pb smelting operation from 1874 to 1980. The Georgia Piedmont soils are generally clay-rich acidic ultisols with low base cation saturation (Rose 1998). The mineral compositions reflect the generally granitic or gneissic parent materials of the Georgia Piedmont that have weathered to produce soils rich in quartz, feldspar, mica, Fe-oxyhydroxides, kaolinite, and illite (Franklin et al., 2007). Soil samples were randomly selected from the site and were homogenously mixed prior to planting to ensure uniform heavy metal concentrations across experimental treatments. The soil was left unsterilized in order to maintain the indigenous soil microbiota; however, debris larger than 0.5 cm were removed by hand. Chemical analysis (by the Soil, Plant, and Water Laboratory of the College of Agricultural & Environmental Sciences, University of Georgia, 2,400 College Station Road, Athens, GA 30602) demonstrated that the soil contained high concentrations of Pb (5,802.5 mg kg^{-1}), iron (Fe) (12,316 mg kg^{-1}) and aluminum (Al) (5,362 mg kg^{-1}). The soil was slightly acidic with pH of 5.7. The soil was left unsterilized in order to maintain the indigenous soil microbiota; however, debris larger than 0.5 cm were removed. In addition, eleven species of arbuscular mycorrhizal fungi (AMF) were identified in the soil from Cedartown using DNA metabarcoding a high-throughput, taxonomic identification of micro-community assemblages within a soil sample.

2.2 Plant species

Switchgrass (*Panicum virgatum* L.) a perennial C$_4$ grass has several characteristics coveted for phytoremediation such as high yield biomass and tolerance to heavy metals toxicity (Chen et al., 2012; Fike at al. 2017). Also, switchgrass is one of the most extensively studied plant species for ligno-cellulosic feedstock production which can be processed in biofuel (ethanol) production (Antizar-Ladislao and Turrion-Gomez, 2008; Sanderson and Adler, 2008). Switchgrass is utilized as a second-generation biofuel grass because it consistently yields acceptable harvestable biomass, has large ethanol conversion potential, and can grow under diverse environmental conditions (Hernández-Allica et al., 2008; Gomes, 2012; Boyer et al., 2013; Min et al., 2017). The Alamo cultivar of switchgrass shows low phytotoxicity to Pb when grown in contaminated soils making it suitable for Pb phytoextraction (Johnson et al., 2015).

2.3 Plant growth conditions

Switchgrass seeds were germinated in a sand culture and seedlings were transplanted into 5 L pots (18.6 cm deep and 19 cm diameter) containing the Pb-contaminated soil. Pots were filled with 5,000 g of contaminated soil. The pots were placed on wire-topped greenhouse benches with individual plastic saucers placed under each pot to prevent soil loss and cross contamination. Plants were grown under environmentally

TABLE 1 Experimental design for control and treatment groups. Chemical application is indicated with X and absence of chemical application is indicated with 0.

Treatments	NTA + APG	Infuse	Triple-12	Promalin
1. Control	0	0	0	0
2. NTA	X	X	0	0
3. NTA + Nu	X	X	X	0
4. NTA + BAP	X	X	0	X

controlled conditions in the Kennesaw State University Research Greenhouse. Plants were supplemented with white, fluorescent overhead light (10,000 Lux) for approximately 16 h per day. Temperature (average 25°C) and humidity was monitored and maintained at a near-constant level. Pots were randomized every 7 days and watered three times weekly with appropriate solutions.

2.4 Experimental setup

Four replicated pots served as the control and did not receive any chemical treatment throughout the duration of the experiment. Four replicated pots were used in each of the following treatments: 1) control, 2) NTA (NTA + APG and Infuse™), 3) NTA + Nu (NTA + APG and Infuse™ and Triple-12®), 4) NTA + BAP [NTA + APG and Infuse and Promalin® (Table 1)3.].

A complete soil-nutrient solution containing: 332 mg L^{-1} nitrogen (N), 111 mg L^{-1} phosphorous (P) and 222 mg L^{-1} potassium (K) was supplied to all plants twice a week until 64 days after planting (dap). All plants were watered with 200 ml of water three times per week until 80 dap. After 80 dap, plants in treatments with foliar applications were sprayed three times per week. The Triple-12® foliar nutrient solution was used by mixing 5 ml Triple-12® into 1L H$_2$0. The Triple-12® foliar nutrient solution contained 12% N, 2% P and 12% K. The foliar BAP solution was made by using Promalin® solution. The soil fungicide Infuse™ (propiconazole) was applied after 152 dap. The soil-fungicide was given to all plants for two weeks for suppression of symbiotic arbuscular mycorrhizal fungi. After 166 dap the 10 mM NTA + APG solution was applied to treatments 2, 3, and 4 three times per week until plants were harvested at 179 dap. The NTA (10 mmol kg^{-1} soil) solution was made by mixing powdered NTA with an aqueous 10 M NaOH solution. The Triton X-100 (2%) was added to the NTA + APG solution in 100 ml/1 L increments (Hu et al., 2017).

2.5 Harvesting

Plants in all treatments and control plants were grown until fully mature. Plants were harvested following appearance of

chlorosis and/or heavy metal toxicity and the presence of purple leaf tips. The longest leaf, most affected leaves (visible chlorosis) and remaining leaves were collected from mature plants in all treatments. Harvested plant leaves were placed in paper bags, labeled, and dried in an oven at 65°C for 48 h prior to acid digestion.

2.6 Acid digestion and chemical analysis

Dried plant samples were weighed, placed in 100 ml disposable acid digestion tubes (Environmental Express®, Inc.) following a modified EPA method 3,050 B and digested in a combined solution of 38% HCL (10.0 ml) and 70% HNO$_3$ (10.0 ml) (U.S. EPA, 1996). Plant samples were capped and placed in the solution for 24 h until were mostly digested. Digestion tubes were then placed in the Hotblock Express System™ and refluxed for 55 min at 90°C–95°C (U.S. EPA, 1996). Digestion tubes were allowed to cool to room temperature. Cooled digestion tubes had deionized water added to them up to the 100 ml line and were vacuum filtered through 0.45-micron filters. Following filtration each sample was pipetted into 50 ml centrifuge tubes stored in a refrigerator until chemical analysis using inductively coupled plasma mass spectrometry (ICP-OES: Perkin Elmer Avio 200). Metals Al, Fe and Pb concentrations were identified in each sample collected and tested in triplicate against each standard. In addition, the bioconcentration factor (BCF) was calculated as the proportion of Pb concentration in plant's foliage (C_{plant}) to the Pb concentration in the bulk soil (C_{soil}). The following equation was used: BCF = C_{plant}/C_{soil}.

2.7 Statistical analysis

Concentrations of Al, Pb and Fe were calculated and statistically analyzed through Analysis of Variance (ANOVA), average and standard deviation calculations, and Fisher's least significant difference (LSD) for post-hoc analyses. Excel's data analysis package along with appropriate R studio's packages were used to perform the statistical tests (RStudio Team, 2021). The main statistical method used for analysis was one-way ANOVA through Excel. Data reorganization, determination of ANOVA type and data variance were verified in the R studio (RStudio Team, 2021). The ANOVA tables were calculated to identify interactions between variables. Fisher's (LSD) was used to determine the significance of p-values. Statistically significant p-values were accepted at $p < 0.05$ and 5% (LSD) confidence levels, $\alpha = 0.05$.

3 Results

Chlorosis of leaves was first observed following NTA applications after 170 dap. During the continued
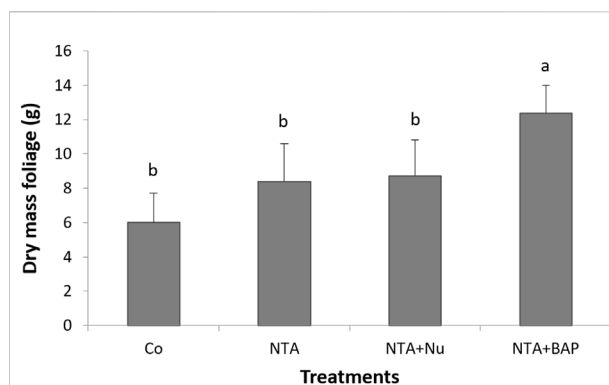

FIGURE 1
Average dry mass (g) (+SD) of *Panicum virgatum* per pot after final harvest. Means for columns with same letters are not significantly different (α = 0.05). (Treatments: NTA—10mM NTA, Nu—foliar nutrients application of Triple-12®, BAP- Promalin® foliar application).
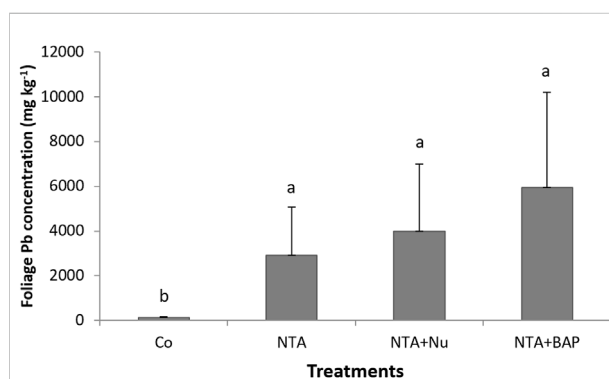

FIGURE 2
Average Pb concentration (mg kg^{-1}) (+SD) of *Panicum virgatum* in different treatments. Treatments: NTA—10 mM NTA, Nu—foliar nutrients application of Triple-12®, BAP- Promalin® foliar application Means for columns with same letters are not significantly different (α = 0.05).

applications of NTA, the tips of plant leaves turned yellow and eventually purple as the Pb toxicity progressed. The average dry mass of the foliage was significantly greatest for plants treated with 10 mM NTA and BAP (Figure 1). The dry mass of the foliage was doubled by the foliar BAP application compared to control plants (Figure 1). Phytoextraction of Pb was improved by treating the Pb contaminated soil with 10 mM NTA instead of 5 mM NTA. Plants treated with 5 mM NTA and BAP had an average Pb concentration of 858 mg kg^{-1} in the plant's foliage. This was improved further when plants were treated with 10 mM NTA alone resulting in an average Pb concentration of 2,914 (mg kg^{-1}) Figure 2). Plants treated with 10 mM NTA and BAP showed an average Pb concentration of 5,942 mg kg^{-1} the greatest one across all
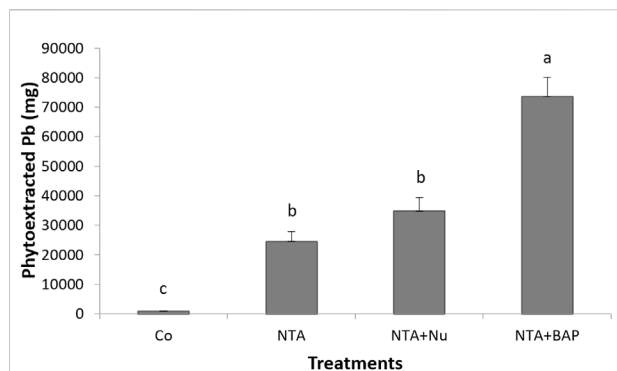
FIGURE 3
Phytoextracted Pb (g) (+SD) per pot. Treatments:
NTA—10 mM NTA, Nu—foliar nutrients application of Triple-12®,
BAP- Promalin® foliar application Means for columns with same
letters are not significantly different (α = 0.05).

FIGURE 5
Average Fe concentration (mg kg⁻¹) (+SD) of *Panicum
virgatum* in different treatments. Treatments: NTA—10 mM NTA,
Nu—foliar nutrients application of Triple-12®, BAP- Promalin®
foliar application. Means for columns with same letters are
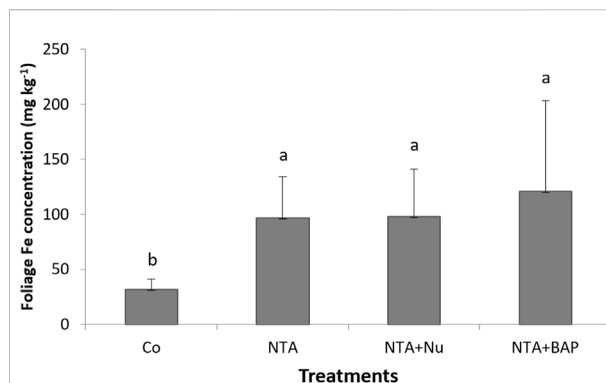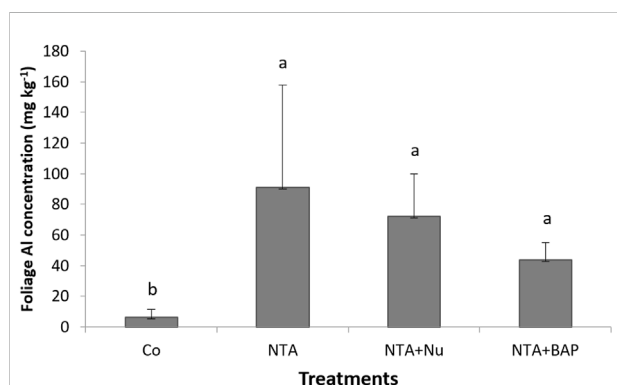not significantly different (α = 0.05).

FIGURE 4
Average Al concentration (mg kg⁻¹) (+SD) of *Panicum
virgatum* in different treatments. Treatments: NTA—10 mM NTA,
Nu—foliar nutrients application of Triple-12®, BAP- Promalin®
foliar application Means for columns with same letters are not
significantly different (α = 0.05).

treatments (Figure 2). High variation in Pb concentration in plants was observed and one plant in the 10 mM NTA and BAP treatment showed the greatest Pb concentration of 10,820 mg kg⁻¹ (Figure 2). The average value for phytoextracted Pb (mg) per pot was significantly greatest for plants treated with 10 mM NTA and BAP (Figure 3). Whereas control plants showed the lowest average value for phytoextracted Pb per pot (Figure 3). The average value for phytoextracted Pb was more than doubled by foliar application of BAP (NTA + BAP) compared to NTA application alone (Figure 3). Other metals showed different trends in metal accumulation in plant's foliage. Plants treated with BAP showed lower average concentration of Al in plant's foliage than plants

treated with foliar nutrients or just 10 mM NTA alone, although significant differences were not established (Figure 4). However, control plants showed significantly the lowest average concentration of Al (Figure 4). Plants treated with BAP showed greater average concentration of Fe in plant's foliage than plants treated with foliar nutrients or just 10 mM NTA alone, although significant differences were not established (Figure 5). However, control plants showed significantly the lowest average concentration of Fe (Figure 5). The bioconcentration factor was greatest for plants treated with NTA and BAP (1.03), suggesting great phytoextraction efficiency.

4 Discussion

This study was conducted to determine if phytoextraction of Pb-contaminated soil by switchgrass can be improved by soil applications of a chelate (10 mM NTA) and APG (Triton X-100), soil-fungicide (Infuse™) and foliar applications of a PGR (BAP). The average value for phytoextracted Pb (mg) per pot was significantly highest for plants treated with 10 mM NTA, APG and BAP. Also, an average bioconcentration factor of 1.03 was greatest for plants treated with NTA and BAP. This value suggests a great phytoextraction efficiency for the NTA and BAP treatment. This study showed that the average Pb concentrations in the foliage of plants could be ranked by treatments as following: NTA (5 mM) (858 mg kg⁻¹ Pb) < NTA (10 mM) (2,914 mg kg⁻¹ Pb) < NTA (10 mM) and foliar application of the complete nutrient solution (3,987 mg kg⁻¹ Pb) < NTA (10 mM) + APG and foliar application of BAP (5,942 mg kg⁻¹). The results are consistent with previous studies that indicated that Pb-phytoextraction by

switchgrass could be optimized by the use of phytohormones and varying chelating agents (Aderholt et al., 2017; Greipsson et al., 2022a). Previous studies demonstrated that application of EDTA with the addition of phytohormone BAP and citric acid showed more Pb uptake efficacy compared to EDTA alone (Johnson et al., 2015; Aderholt et al., 2017). Furthermore, exogenous applications of PGR have been associated with an increase in phytoextraction efficiency (Beavers et al., 2021). The combined applications of PGRs, chelating agents, and soil-fungicide to suppress AMF activities yielded higher levels of Pb in the phytoextraction processes (Beavers et al., 2021; Hart et al., 2022). A more effective phytoremediation process could help reclaim contaminated soils in a shorter time period and would limit heavy metal exposure to individuals in sensitive populations (Filippelli and Laidlaw 2010; Fasinu and Orisakwe 2013; Nissim and Labreccque 2021).

The effects of PGRs on plant growth and Pb translocation was previously outlined (Aderholt et al., 2017). PGRs like BAP have increased growth of switchgrass and improved phytoextraction (Hart et al., 2022). Previous studies have shown that exogenous applications of PGRs were associated with an increase in Pb-phytoextraction (Greipsson et al., 2022a). Also, previous study has shown that phytoextraction processes were improved with combined foliar applications of SNP (nitric oxide donor) and GA3 (gibberellic acid) (Beavers et al., 2021). A recent study suggested that combined applications of SA alongside the PGR DA-6 (diethyl aminoethyl hexanoate) was associated with an increase in Pb uptake (Greipsson et al., 2022a).

Using second-generation bioenergy crop such as switchgrass for phytoremediation of contaminated soils has shown great potentials (Shen et al., 2002; Van Ginneken et al., 2007; Hernández-Allica et al., 2008; Chen et al., 2012; Oh et al., 2013; Balsamo et al., 2015; Min et al., 2017; Quarshie et al., 2021). This is largely due to the plants amount of harvestable biomass, ethanol conversion potential and its ability to be grown in diverse climates as a remediator (Hernández-Allica et al., 2008; Boyer et al., 2013; Min et al., 2017). Planting contaminated sites with biofuel crops and maintaining them as phytoextraction sites could potentially remediate Pb soil contamination, with the long-term goal of reclaiming the marginal land for future uses. Cultivation of bioenergy crops on marginal lands may reduce the need for using primary agricultural lands for biofuel production and might therefore improve energy security and aid in mitigating climate change (Qin et al., 2012; Qin et al., 2015).

Optimizing phytoextraction efficiency of bioenergy crops has significant implications for bioenergy production (Balsamo et al., 2015). This study showed that foliar application of BAP improved the biomass of switchgrass. Improving biomass of plants used in phytoextraction is an important task. The Alamo cultivar of switchgrass used in this study can produce 17,800 kg ha^{-1} of harvestable dry mass (Smith, Allen and Barney 2015). Also, biomass production of switchgrass is economical compared to the production of other high biomass crops (McLaughlin and Kszos 2005).

As demand for remediation work in brownfield sites grows, so does its association with phytoremediation as these sites have high potentials for reclamation (Nissim and Labreccque 2021). Successful removal of Pb from contaminated soils may benefit more sensitive human populations who are at risk of Pb exposure. Often contaminated farm sites lack access to additional viable soil and remediation is a clear path to continued vegetation growth or utilization of sites (Yan et al., 2020). There is a growing need for additional advances in effective phytoremediation practices to sustain the environment in affected cities (Anyanwu et al., 2018; Lee et al., 2021). Continued phytoremediation research on the reclamation of brownfield sites and contaminated farmland may benefit the environment and associated redevelopment projects (Mielke et al., 2016; Anyanwu et al., 2018). Further study is required to identify the effects of increased concentrations of additional chemical chelates for switchgrass used in phytoextraction. Future experiments can specifically explore increased NTA applications in combination with APG to determine if the additional concentrations continue to be positively associated with Pb uptake. Continued research focusing on the benefits of APGs, PGRs and chemical chelate applications within phytoremediation could broaden areas of interest with current knowledge gaps.

5 Conclusion

Phytoextraction of Pb-contaminated soil by switchgrass that was chemically enhanced with chelates, APG, soil fungicide and PGR has many implications for both phytoextraction and bioenergy industry. This study demonstrated that switchgrass treated with foliar application of a PGR (BAP) resulted in increased dry mass production of switchgrass which is important in both phytoextraction and bioenergy industry as well. Also, phytoextraction was improved using a chelate (NTA) and APG. The use of NTA has been emphasized in phytoextraction due to the long persistence time of EDTA in soil and potential mobilization of Pb into groundwater. Total phytoextraction was greatest in plants treated with combined application of soil applications of a chelate (10 mM NTA) and APG (Triton X-100), soil-fungicide (Infuse™) and foliar applications of a PGR (BAP). These findings strongly suggest that phytoextraction can be improved through judicious chemical application and at the same time enhancing switchgrass biomass production.

Data availability statement

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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