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Effects of Exogenous Application of Plant Growth Regulators (SNP and GA3) on Phytoextraction by Switchgrass (*Panicum Virgatum* L.) Grown in Lead (Pb) Contaminated Soil

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Abstract: Soil lead (Pb) contamination is a major environmental and public health risk. Switchgrass (*Panicum virgatum*), a second-generation biofuel crop, is potentially useful for the long-term phytoremediation and phytoextraction of Pb contaminated soils. We evaluated the efficacy of a coordinated foliar application of plant growth regulators and soil fungicide and a chelator in order to optimize phytoextraction. Plants were grown in soil culture under controlled conditions. First, three exogenous nitric oxide (NO) donors were evaluated at multiple concentrations: (1) S-nitroso-N-acetylpenicillamine (SNAP); (2) sodium nitroprusside (SNP); and (3) S-nitrosoglutathione (GSNO). Second, the effect of SNP (0.5 µM) was examined further with the model chelate EDTA and the soil fungicide propicanazole. Third, a combined foliar application of SNP and gibberellic acid (GA3) was examined with EDTA and propicanazole. The soil application of propiconazole (a broad-spectrum fungicides) reduced AMF colonization and allowed greater Pb phytoextraction. The foliar application of SNP resulted in similar concentrations of Pb (roots and foliage) to plants that were challenged with chelates and soil fungicides. The combined foliar application of SNP and GA3 resulted in significantly greater average Pb concentration (243 mg kg^{-1}) in plant foliage in comparison to control plants (182 mg kg^{-1}) and plants treated with GA3 alone (202 mg kg^{-1}). The combined foliar application of SNP and GA3 resulted in the greatest phytoextraction efficiency and could therefore potentially improve phytoextraction by switchgrass grown in Pb contaminated soils.

Keywords: bioenergy-crop; gibberellic acid; lead (Pb); nitric oxide; phytoextraction; switchgrass (*Panicum virgatum* L.)

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1. Introduction

Anthropogenic lead (Pb) soil contamination is mainly derived from industrial sources such as mining, battery recycling, agricultural pesticides, and from tetraethyllead ($(CH_3CH_2)_4Pb$), an additive formerly found in automotive fuels [1–5]. Airborne Pb from the smelting and burning of tetraethyllead containing fuel falls out of the atmosphere and deposits in topsoil [1]. Even residential areas have been found to be contaminated with high levels of soil Pb contamination [6–8]. It is estimated that 207,000 Pb contaminated sites, comprising millions of hectares, exist throughout the United States [9].

Environmental Pb contamination is a recognized global health problem; long-term low-level Pb exposure can result in neurological dysfunctions [10–15]. Even low (50 mg kg^{-1}) concentrations of Pb in soil is correlated with increased risk of elevated blood lead level (BLL) in humans [16,17]. Children may additionally acquire prenatal neurological damage due to epigenetic effects from Pb accumulation in parents or grandparents [18]. Currently, there is no blood Pb level known that is not considered harmful to human health [19]. Measures have been put in place to reduce human exposure to environmental Pb contamination

including site closing, ground covers, and in extreme cases, soil removal [20]. Although these measures may reduce interactions with contaminated soils, they still leave the harmful contaminant in the environment [20]. Levels of Pb contaminated soil have previously been correlated to Pb levels in tissues of domesticated animals and wildlife [21–24].

The removal of contaminants from soil can be employed through phytoextraction, an emerging heavy metal remediation technique [25]. In phytoextraction, the contaminant is absorbed and sequestered within plant shoots, resulting in a low degree of soil disruption [26]. The process of phytoextraction of Pb contaminated soil typically begins by soil acidification and chelation [27]. Lead ions are positively charged, causing them to bind to particles in the topsoil and resist leaching into deeper soil horizons [28,29]. Several studies have found that the synthetic chelator ethylenediaminetetraacetic acid (EDTA) is a highly effective Pb-chelation agent that forms soluble Pb complexes in the soil [26,30,31]. The chelator EDTA has been used extensively in phytoremediation studies [32].

Soil microorganisms such as arbuscular mycorrhizal fungi (AMF) form symbiotic relationship with plants, facilitate nutrient uptake, and prevent the uptake of harmful elements, such as Pb [33–36]. Switchgrass benefits from AMF symbiosis through macro-nutrient homeostasis [37]. The AMF act as a barrier against Pb uptake of plants and are commonly found associated with roots of plants growing on contaminated soils [35,38,39]. In most circumstances, plants that have symbiosis with AMF exhibit low Pb in the foliage. While AMF benefits the plant when growing on contaminated soil, it is disadvantageous to phytoextraction efforts [33,39,40]. To counteract the protecting effect of the AMF, contaminated soils must be treated with a broad-spectrum fungicide such as propiconazole to inhibit the function of the AMF [41]. Studies have shown that another soil fungicide, benomyl treatment, especially prior to EDTA application, improved Pb uptake and translocation [42,43].

An exogenous application of plant growth regulators has been shown to increase phytoextraction efficiency [44,45]. Nitric oxide (NO) is an important cellular signaling molecule in plants and appears to play a role in plant iron (Fe) maintenance and stress response signaling [44,46,47]. It has been suggested that NO contributes to iron homeostasis in two ways; first, as a reducing agent to change iron from Fe^{3+} to Fe^{2+}, and secondly, by the formation of dinitrosyl-iron complexes (DNICs) which may facilitate iron transport through cellular membranes [48,49]. Nitric oxide can be exogenously applied to have a similar effect as endogenous NO [50]. Nitric oxide donor molecules have been shown to significantly reduce initial heavy metal toxicity in plants as well as increase metal uptake [47,51–53].

The plant growth regulator gibberellic acid (GA3) has several physiological effects on plants including growth stimulation [54]. The exogenous application of GA3 was found to enhance phytoextraction by ryegrass (*Lolium perenne*) [55]. The optimal concentration of GA3 was found to be 1 µM to increase growth of ryegrass and increase the proportion of Pb in the cell wall [55]. Higher doses (100 µM) of GA3 had adverse effects on plants and Pb phytoextraction [55]. Moreover, the foliar application of GA3 was found to counteract the negative effect of EDTA on the growth of maize (*Zea mays*) [45].

Switchgrass (*Panicum virgatum* L.) is a C_4 perennial grass adapted to a broad range of climates, topography, and soil conditions throughout North America [56,57]. As a perennial grass, switchgrass may be harvested more than once in a growing period, and it will continue to grow for up to 10 years [58]. Another attribute contributing to the selection of switchgrass is its high tolerance for Pb in soils [59] and high biomass production. Previous studies have estimated that switchgrass var. "Alamo" is capable of generating 17,800 kg of harvestable tissue per hectare (ha) [60]. Switchgrass is regarded as a second-generation biofuel crop and its biomass could be used in advanced biofuel production [61,62]. The phytoremediation of contaminated soils using second-generation bioenergy crops such as switchgrass has great potential [63–67]. The cultivation of biofuel crops on marginal lands may improve energy security and aid in mitigating climate change [68]. In addition, the cultivation of bioenergy crops on marginal lands may reduce the need for using primary agricultural lands for biofuel production. The cost of this biomass production is estimated to be much lower than that of other high biomass yield crops [69]. The switchgrass cultivar

"Alamo" (AP13) originating from Live Oak County, Texas, is particularly well acclimated for use in Georgia [70]. Derived from the "Alamo" cultivar, EG 1101, a high biomass yield isolate, was provide by the University of Georgia and used in this study.

This study examined phytoextraction efficiency of switchgrass in the following ways: (1) effect of different exogenous NO donors; (2) effect of foliar application of SNP along with chelate (EDTA) and soil fungicide (propicanazole); (3) effect of combined foliar application of SNP and the plant hormone gibberellic acid (GA3) along with chelate and soil fungicide.

2. Materials and Methods

2.1. Soil

Plants were grown in soil that was collected from a brownfield in downtown Atlanta, Georgia, USA. Atlanta soils are generally clay-rich acidic ultisols with low base cation saturation [71]. 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 [72]. This soil was tested using ICP-AES for elemental content at the University of Georgia, Stable Isotope Ecology lab, Athens, Georgia. Soil testing revealed that the soil contained 108 mg kg^{-1} of Pb, 7765 mg kg^{-1} of iron (Fe) and 21,850 mg kg^{-1} aluminum (Al). The soil pH was 5.5. The soil was spiked to 350 mg kg^{-1} Pb using a standard Pb spiking solution ($Pb(NO_3)_2$) at a concentration of 1000 mg kg^{-1} that had been diluted to the necessary concentration with DI H_2O [62,73]. After the soil was spiked, it was mixed to homogenize Pb distribution.

2.2. Plant Growth Conditions

Plants were grown under controlled environmental conditions in the Science Greenhouse at Kennesaw State University (KSU), Kennesaw, GA, USA, at an average temperature of 22.9 °C (30.6 °C max and 15.6 °C min). The soil was left unsterilized in order to maintain the indigenous soil microbiota; however, debris larger than 0.5 cm were removed by hand. Pots were filled with 5000 g of contaminated soil and planted with approximately 30 seeds of switchgrass at a depth of 0.25 cm to allow for maximum germination [57]. Seedlings were thinned to three per pot. The pots were placed on wire-topped greenhouse benches with individual plastic saucers placed under each pot to prevent soil loss and cross contamination. Natural light varied over time but not across treatments with the sun availability as per the greenhouse conditions and supplemented with 14 h of artificial cool-white-fluorescent overhead light (10,000 Lux) each day.

2.3. Selection of Exogenous NO Donor

Three exogenous NO donors were tested: (1) sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5NO]$), which has shown promises in phytoextraction applications in studies using *Arabidopsis thaliana* by providing a protection against Pb uptake [52]; (2) S-nitroso-N-acetylpenicillamine (SNAP) ($C_7H_{12}N_2O_4S$), which may be active in upregulating cell division in plants, potentially causing the uptake of excess soil contaminants [74]; and (3) S-nitrosoglutathione (GSNO) ($C_{10}H_{16}N_4O_7S$), which has shown to be active in Pb stressed plants, alleviating heavy metal oxidation and stress [75,76]. The effect of three exogenous NO donor molecules (SNP, SNAP, GSNO) were tested in three different concentrations (0.1 µM, 0.2 µM, 0.5 µM) on switchgrass.

2.4. Phytoextraction by Switchgrass Enhanced by Coordinated Application of SNP, Chelate and Soil Fungicide

Seeds of P. virgatum were sown into 12 pots that were filled with contaminated soil (5 kg). The pots were randomly divided into three treatments: (1) control, (2) EDTA + propicanazole (EP); (3) EDTA + propicanazole + SNP (EPS). Three plants were grown in each pot that were arranged in a complete randomized block design, with re-randomization every seven days. Plants were given DI water (100 mL) twice a week until soil chemical exposure began. Phytoextraction was induced by a coordinated foliar application of SNP

and soil application of soil fungicide and chelate. The soil fungicide propiconazole (trade name Infuse®) is a short-lived fungal suppressant and is usually required in multiple applications [41]. It was prepared in a 2 mg L^{-1} solution with DI H_2O and applied as a soil drench at 20, 40, 60, and 80 days after planting (dap) [41]. The model chelate EDTA was applied as 1.0 mmol kg^{-1} soil [43]. The EDTA application occurred at 91 dap. This application difference was determined from findings of a previous study, suggesting that applying EDTA chelator after AMF suppression by soil fungicide resulted in more efficient Pb uptake than simultaneous application [43]. The EDTA treatments were prepared using granular EDTA mixed with 90 mL DI H_2O; the solution was then vortexed and applied to pots in appropriate treatments. Of the three exogenous NO donor being tested, SNP (0.5 μM) was selected for this experiment due to several factors, including efficacy and potential costs associated with its use in large-scale field applications. Crystallized SNP was dissolved in DI H_2O to a concentration of 0.5 μM and applied as a 20.0 mL foliar leaf spray at 100, 110, and 120 dap. At 135 dap, the plants showed slight yellowing (chlorosis) of leaves and all plants were harvested. The plants were removed from the pots and rinsed with DI H_2O to remove soil traces. Root samples were divided and three root samples from each pot were stored in 70% ethanol at 5 °C for later AMF staining. The remaining roots and shoots were dried for 48 h in an oven at 65 °C. Once the plant tissues were dried, dry mass (DM) was recorded for each sample prior to acid digestion.

2.5. Phytoextraction by Switchgrass Enhanced by Coordinated Application of SNP, GA3, Chelate and Soil Fungicide

Seeds of *P. virgatum* were sown into 40 pots that were filled with contaminated soil (5 kg). The pots were randomly divided into four treatments: (1) control, (2) SNP (0.5 μM), (3) GA3 (1.0 μM), (4) SNP and GA3 (0.5 μM and 1.0 μM, respectively). Three plants were grown in each pot that were arranged in a complete randomized block design, with re-randomization every seven days. The plants were given DI water (100 mL) twice a week until soil chemical exposure began. Phytoextraction was induced by a coordinated foliar application of SNP and soil application of soil fungicide and chelate. At 100, 110, 120 dap, SNP (0.5 μM) was exogenously applied on plant's foliage in treatments 2 and 4 (5 mL of SNP was exogenously applied to each pot). At the same time, the plant hormone gibberellic acid GA3 (1.0 μM) was applied exogenously on plants in treatment 3 (10 mL was applied to each pot) and to plants in treatment 4 where combined application of SNP and GA3, was applied as well. The plants were given the soil fungicide propiconazole at 20, 40, 60 and 80 dap as described above. This was followed by EDTA application at 90 dap as described above. All plants were harvested at 140 dap and treated as described above.

2.6. Acid Digestion & Chemical Analysis of Plant Samples

Dried plant material was digested using the HotBlock digestion system (Environmental Express®, Inc., Charleston, SC, USA). Dried plant tissues (1.0 g) were digested in 38% HCl (10.0 mL) and 70% HNO_3 (10.0 mL) in Environmental Express® 100.0 mL plastic digestion tubes following a modified EPA Method 3050B. The tubes were capped and rested at room temperature for 24 h, then refluxed at 95 °C in an Environmental Express® HotBlock system for 55 min. Samples were capped, allowed to cool for another 24 h period, and had their volume brought to 50 mL using trace-metal grade DI H_2O before being vacuum filtered. The filtered samples were chemically analyzed using the Varian SpectraAA 220 FAAS in the Department of Chemistry and Biochemistry at KSU.

2.7. Trypan Blue Staining of Roots for AMF Assessment

Preserved root samples from both experiments were cleared and stained for AMF evaluation [43,77]. The samples were placed in 10% KOH solution and heated in a water bath at 90 °C for 30 min to clear roots of non-chitinous cellular structures. Cleared roots were rinsed in DI H_2O five times and placed in 2.5% HCl for 30 min at room temperature for acidification. The roots were stained in 0.05% trypan blue for 15 min at 90 °C, and then

de-stained in glycerol acidified with 2.5% HCl for 2 h to remove excess trypan blue and stored in acidic glycerol in a 5 °C refrigerator prior to AMF assessment.

Cleared and stained root samples from both experiments were evaluated for AMF colonization using the root-segment method [78]. The root specimen (n = 150) pieces (1 cm) per treatment were mounted on microscope slides and observed under a bright field microscope at 200× and 400× magnifications. AMF root colonization was calculated as the number of root segments colonized by AMF divided by the total number of root segments examined [78]. Colonization percentages were established by counting the occurrence of different fungal structures: hyphae, arbuscles, and vesicles for each treatment.

2.8. Statistical Analysis and Remediation Calculation

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by post hoc Fisher's Least Significant Difference (LSD) using IBM SPSS Statistics 27. Additionally, a relationship between categorical treatments and foliage Pb concentrations was calculated by regression analysis. Statistical significance was accepted at the level of $p < 0.05$.

Bioaccumulation factor (BCF) measures the ability of the plant to accumulate Pb from the soil and is defined as the direct ratio of Pb in the total harvestable plant tissues to Pb in the soil [79,80]. A BCF of ≥ 1.0 is considered to be a successful phytoextraction [80].

3. Results

Coordinated chemical treatments showed dramatic increases in Pb concentrations in plants. Plants treated with propiconazole and EDTA had a significantly higher average Pb concentration (172 mg kg^{-1}) in their foliage compared to control plants (12.4 mg kg^{-1}) (Figure 1). Plants treated with propiconazole and EDTA and SNP showed a high value in the foliage (176 mg kg^{-1}), a 1330% increase in shoot Pb concentration compared to control plants (Figure 1). Similarly, Pb concentrations in roots of plants treated with propiconazole and EDTA (1070 mg kg^{-1}) and with the addition of SNP (822 mg kg^{-1}) were significantly higher compared to control plants (97.4 mg kg^{-1}) (Figure 2). The foliar application of SNP was not found to increase the Pb concentration in the foliage or roots (Figures 1 and 2). The effectiveness of the combined chemical application was further demonstrated by the use of the bioaccumulation factor. Plants treated with propiconazole and EDTA and with the addition of SNP had significantly higher bioaccumulation factor compared to control plants (Figure 3). The foliar application of SNP did not increase the bioaccumulation factor (Figure 3). The soil application of propiconazole had a dramatic impact on total AMF colonization in roots (Figure 4). The soil application of propiconazole significantly reduced AMF hyphae colonization to 48% compared to 92.5% in the roots of control plants (Figure 4). It was also observed that the soil application of propiconazole negatively affected AMF fungal structures such as arbuscules. The suppression of AMF activity through the application of propiconazole resulted in greater Pb accumulation in the foliage. Combined foliar application of GA3 and SNP increased Pb concentrations in the foliage. Plants treated with a foliar application of GA3 and SNP (243 mg kg^{-1}) had significantly higher Pb concentrations in the foliage compared to control plants (182 mg kg^{-1}) and plants that received GA3 alone (202 mg kg^{-1}) (Figure 5). Plants that received a foliar application of SNP alone and plants that received GA3 and SNP did not differ significantly in Pb concentration in the foliage (Figure 5). The Pb concentration in leaves increased stepwise between treatments (Figure 5). A linear regression of treatment categories (see Figure 5) vs. foliage Pb concentrations showed a strong relationship (R^2 = 0.97) between the average Pb concentration in leaves and treatments.

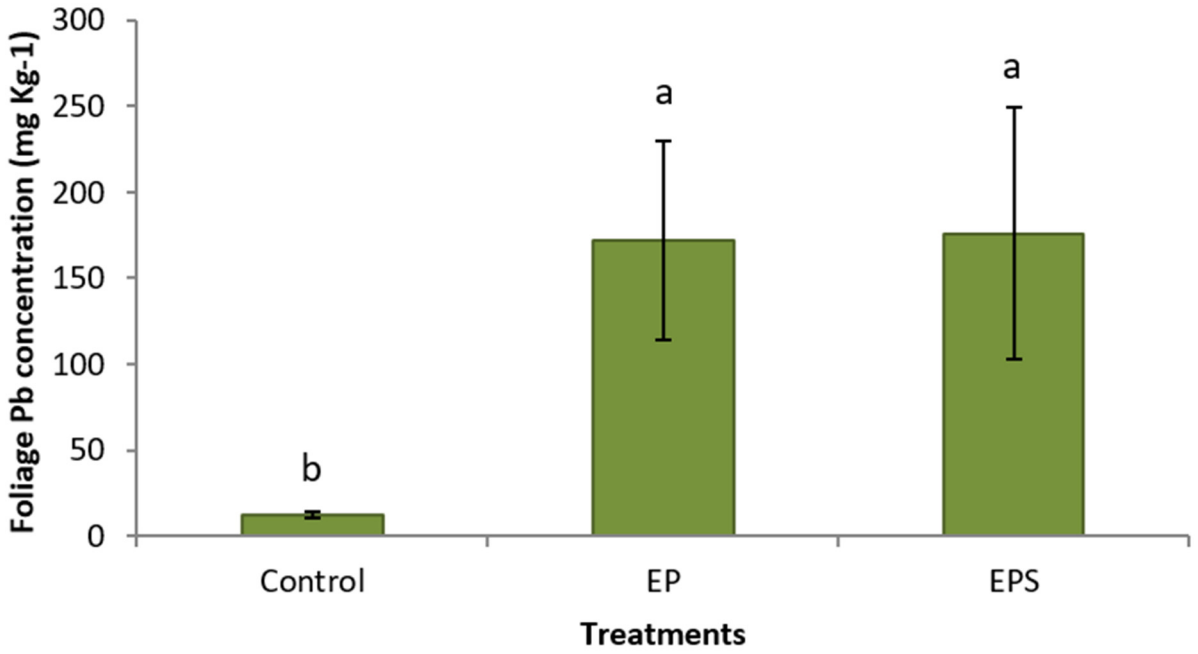

Figure 1. Mean (+/− SD) foliage Pb concentration (mg kg^{-1}) of *Panicum virgatum* at time of harvest. Means for columns designated with the same letter are not statistically significantly different ($\alpha = 0.05$). (Treatments: E—EDTA, P—propiconazole, S—SNP).

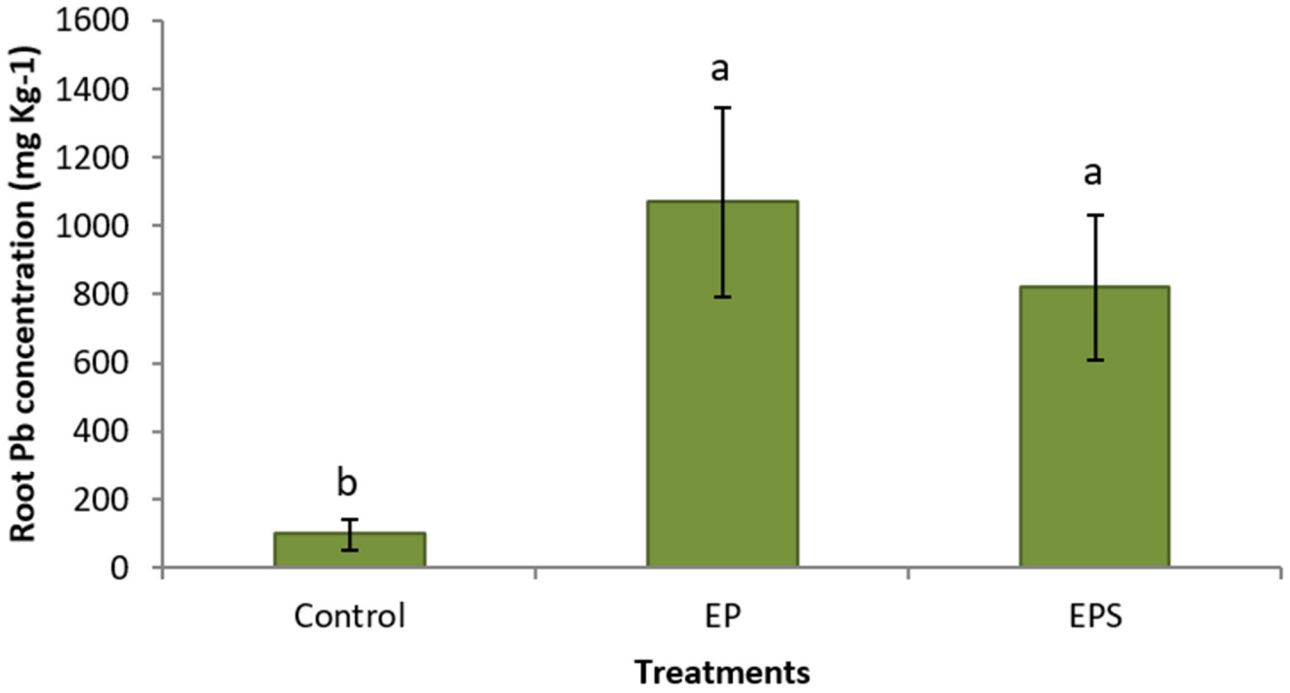

Figure 2. Mean (+/− SD) root Pb concentration (mg kg^{-1}) of *Panicum virgatum* at time of harvest. Means for columns designated with the same letter are not statistically significantly different ($\alpha = 0.05$). (Treatments: E—EDTA, P—propiconazole, S—SNP).

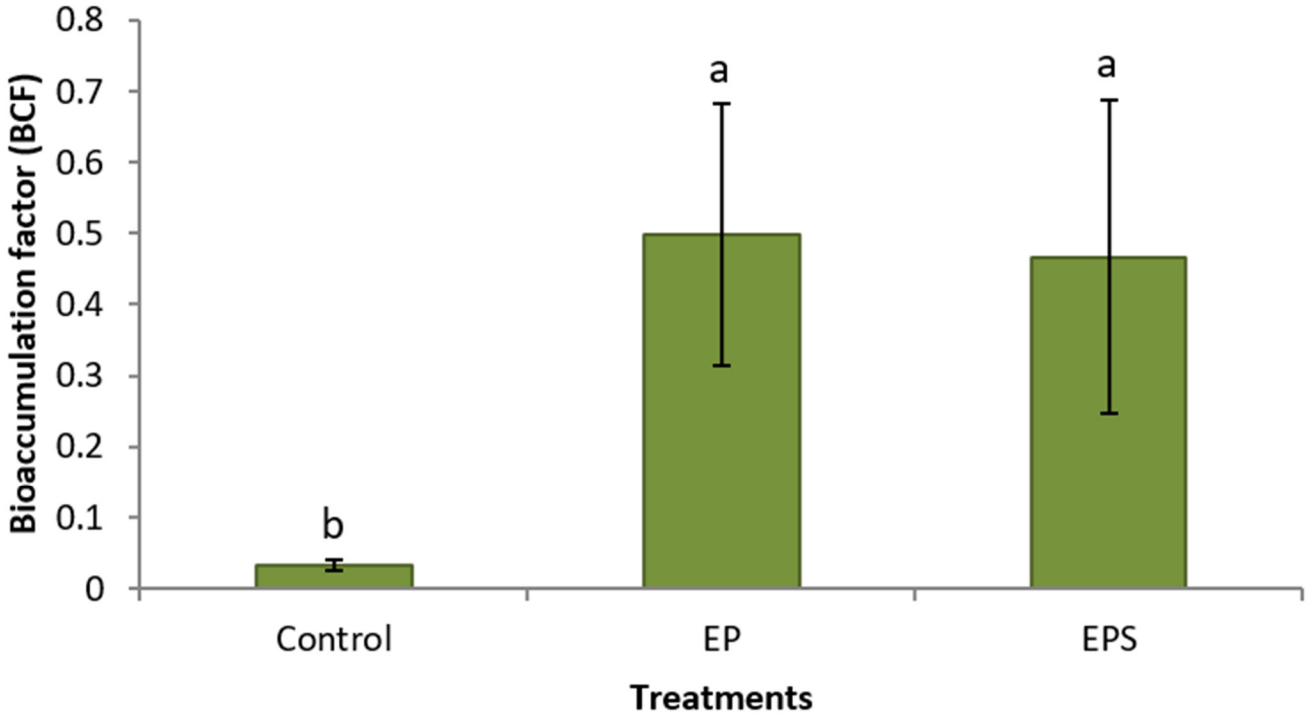

Figure 3. Mean (+/− SD) bioaccumulation factor (BCF) of *Panicum virgatum*. Means for columns with same letter not statistically significant ($\alpha = 0.05$). (Treatments: E—EDTA, P—propiconazole, S—SNP).

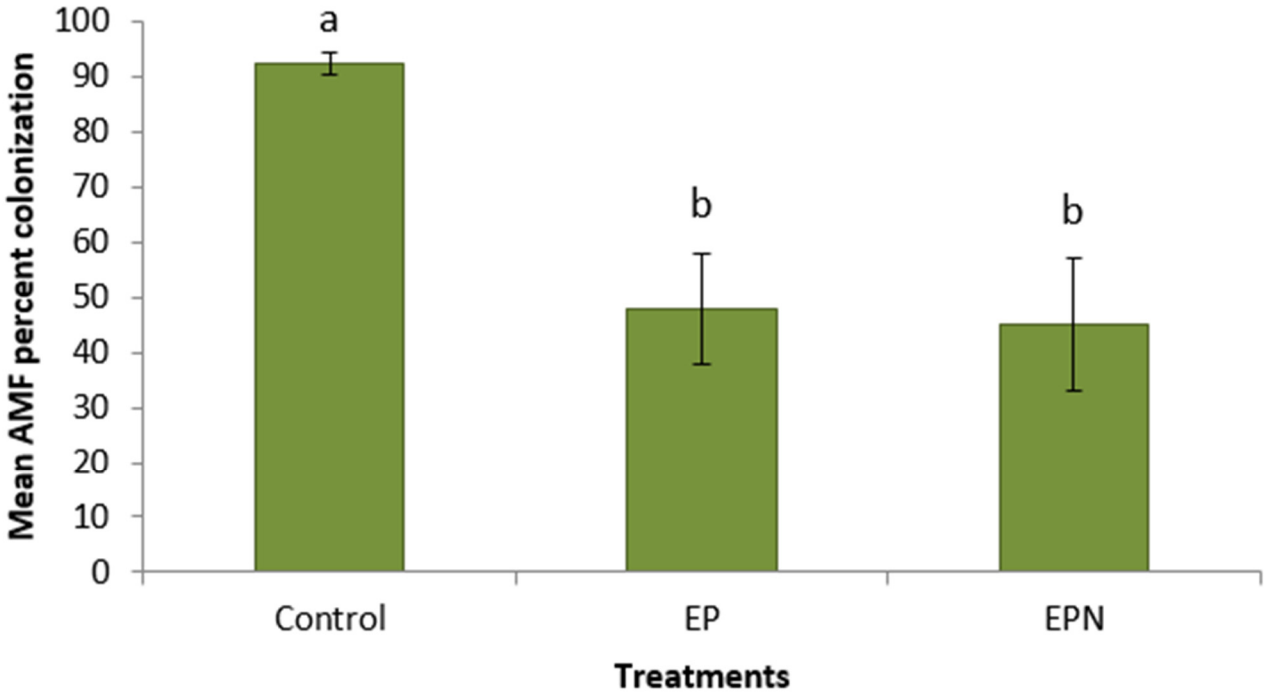

Figure 4. Average (+/− SD) percentage (%) of total arbuscular mycorrhizal fungi colonization in *P. virgatum* roots at time of harvest. Means for columns with same letter not statistically significantly different ($\alpha = 0.05$). (Treatments: E—EDTA, P—propiconazole, N—SNP).

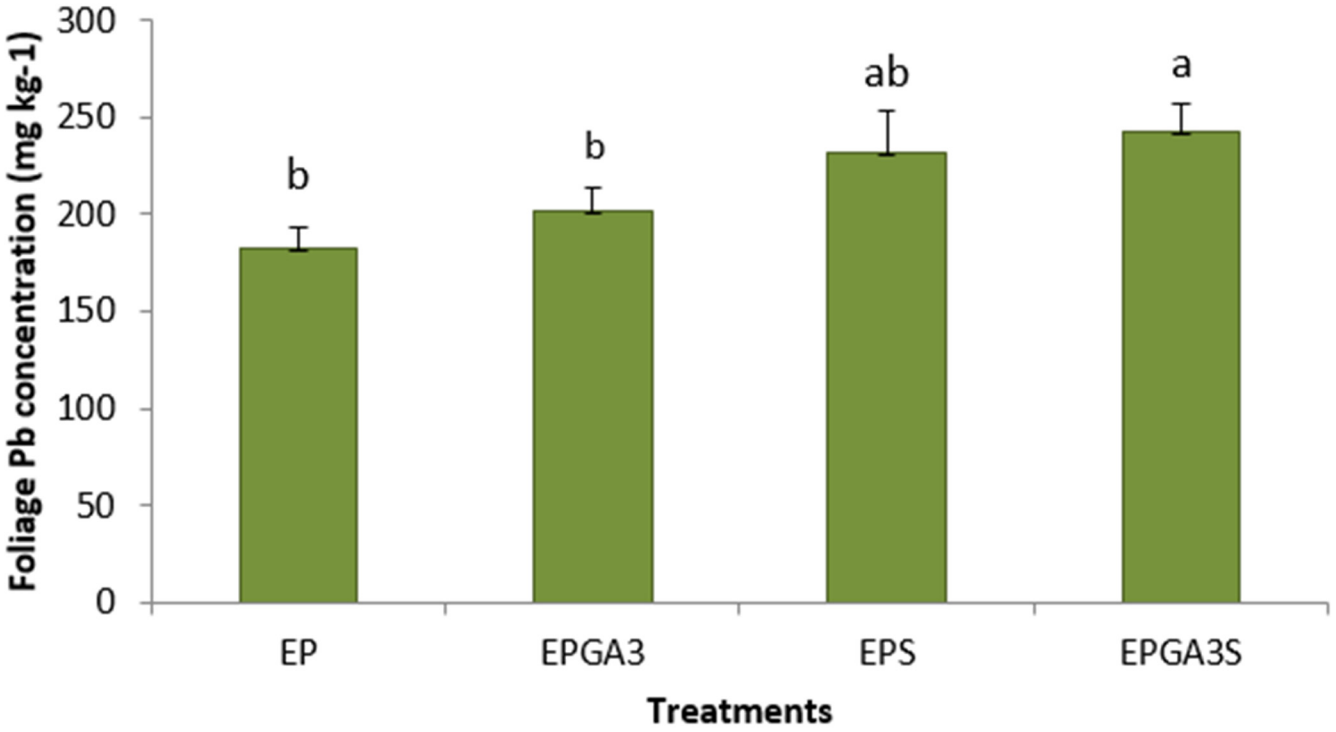

Figure 5. Mean (+/− SD) foliage Pb concentration (mg kg^{-1}) of Panicum virgatum at time of harvest. Means for columns designated with the same letter are not statistically significantly different ($\alpha = 0.05$). (Treatments: E—EDTA, P—propiconazole, gibberellic acid—GA3, S—SNP).

4. Discussion

The objective of this study was to examine the impact of exogenous NO donor on plants used in phytoextraction. A foliar application of the NO donor resulted in a significantly higher concentration of Pb in foliage compared to control plants. However, the foliar application of the NO donor SNP in addition to the model chelate agent EDTA and propiconazole fungicide treatments did not affect Pb uptake of plants. Similarly, it was found that SNP application did not have an effect on heavy metal accumulation in bean plants [11]. It was previously suggested that NO plays an important role in maintaining Fe homeostasis and may ameliorate the negative effects of Fe stress in plants [48,81]. This study found no statistically significant differences in shoot and root Fe concentration between SNP treated plants or the control plants. These results are consistent with other study findings showing that although exogenous NO donors can be used to simulate the function of endogenous NO, the application of exogenous NO donors may not change the amount of Fe absorbed into roots or translocated into shoots [48,49]. Overall, the foliar NO donor SNP (0.5 μM) application resulted in a dry mass effect in plants that was not statistically significant compared to the control plants and other treatments that did not receive SNP application. Previous studies have shown that the application of SNP may accelerate switchgrass seed germination, but not plant growth [52,82,83]. Further research into the appropriate timing and application method of exogenous NO donors may suggest more beneficial methods to optimize switchgrass growth in a phytoextraction context, but the results of this study suggest that exogenous NO donors, specifically SNP, applied as foliar spray did not stimulate switchgrass growth or Pb uptake, a finding consistent with [83].

The foliar application of SNP and the plant growth regulator GA3 resulted in significantly higher Pb concentration in the foliage compared to control plants and plants treated with GA3 alone. Although GA3 foliar application did not result in an improved biomass of plants, phytoextraction was improved. Similarly, the foliar application of GA3 was found to enhance phytoextraction by ryegrass (*Lolium perenne*) [55]. An optimal concentration of GA3 was found to be critical for improving phytoextraction by ryegrass [55]. The foliar

application of GA3 was not found to improve any toxic effects on switchgrass although GA3 was previously found to counteract the negative effect of EDTA on the growth of maize (*Zea mays*) [45].

The phytoextraction of Pb by switchgrass enhanced with chemical applications has many implications in future research for both the phytoremediation and bioenergy industries. This study showed that EDTA treatments increased Pb accumulation by plants and this finding agrees with previous studies [43,84,85]. However, EDTA is persistent in soil and may mobilize Pb and other metals through the soil column and into ground water, thus increasing the risk of human exposure [86–89]. In addition to its soil effect, EDTA has been observed to negatively impact plant health and reduce biomass [90,91]. Due to these issues, natural acids and other chelates with shorter soil persistence are being studied as alternatives to the synthetic chelator EDTA. A previous study suggests that combined citric acid and soil fungicide application could achieve similar results to EDTA application [85]. Furthermore, the soil application of nitrilotriacetic acid (NTA) and alkyl polyglucoside (APG) has shown improved Pb uptake of plants compared to EDTA [92].

The application of propiconazole demonstrated the ability of this soil fungicides to reduce AMF root colonization. The symbiotic association of AMF and switchgrass provides a barrier against phytoextraction, so fungal suppressants must be applied during phytoextraction. This study showed that plants treated with the fungicide propiconazole exhibited significant decreases in mean AMF percent colonization compared to the control plants. These trends were conserved when observing AMF colonization by fungal structure as well; all propiconazole treatments resulted in significantly reduced vesicle and arbuscle percentages, a finding consistent with [41].

Plants treated with exogenous NO donor showed no growth effects. Optimizing plant biomass is important for maximizing Pb soil remediations and also has applications in the bioenergy industry. While the results of this study showed no significant difference between the control plants and plants treated with exogenous NO donors, no clear consensus on the appropriate timing of exogenous NO donor application exists. This lack of consensus suggests further study into the possible timing of exogenous NO donor applications may be necessary to determine if timing is critical for the production of greater harvestable biomass.

5. Conclusions

This study demonstrated that the phytoextraction of Pb by switchgrass was enhanced by a combined foliar application of the exogenous nitric oxide donor SNP and GA3. These results could aid in the phytoextraction of Pb contaminated soils and bioenergy production. The merits of Pb phytoextraction extend beyond the removal of harmful heavy metals. Switchgrass biomass is currently harvested as a lingo-cellulosic biofuel feedstock. Optimizing biomass production and the Pb uptake of plants has significant implications for phytoextraction and bioenergy production. Cultivating biofuel crops such as switchgrass on contaminated sites for phytoextraction could potentially remediate Pb soil contamination, with the long-term goal of reclaiming the land for future uses.

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