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Ecological restoration of lead/zinc/copper mine tailings: Phytomanagement and amendment strategies to enhance substrate functionality and biomass production

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ECOLOGICAL RESTORATION OF LEAD/ZINC/COPPER MINE TAILINGS: PHYTOMANAGEMENT AND AMENDMENT STRATEGIES TO ENHANCE SUBSTRATE FUNCTIONALITY AND BIOMASS PRODUCTION

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ABSTRACT

The extreme physiochemical characteristics of mine tailings inhibit microbial processes and natural plant growth. Consequently, vast and numerous tailings sites remain barren for decades and highly susceptible to wind and water erosion. Phytostabilization is a cost-effective and ecologically productive remediation approach; however, tailings revegetation is generally challenging and must often be assisted with appropriate soil amendments. Amendments applied individually in greenhouse studies discussed herein revealed notable improvement in bioenergy crops growth only with biosolids treatments. Recalcitrant carbon amendments (biochar and humus) showed notable impact only on tailings physichochemical and hydraulic properties. Nevertheless, biosolids may not support sustained vegetation due to their nutrient lability and rapid decomposition. Therefore, strategies to sustain phytostabilization were evaluated by co-applying biosolids with recalcitrant carbon or biological amendments to synergistically ameliorate tailings characteristics while supporting sustainable growth to stimulate soil formation. Co-applying with biochar exhibited efficient nutrient release while concurrently reducing metal availability and uptake. Co-applying with mycorrhizal fungi further improved biomass production, increased organic matter input, and reduced metal bioavailability and uptake. To non-destructively assess plant health, a rapid screening approach was also developed utilizing computer vision and imaging techniques. A wide range of native species was also screened for potential to revegetate mine tailings for greater ecosystem benefit and utilizing the developed approach greatly facilitated quantification of plant responses to phytomanagement strategies for mine-impacted sites.

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1. INTRODUCTION

1.1. ENVIRONMENTAL IMPACTS OF MINE TAILINGS

Mining and smelting activities are generally accompanied by a continuous generation of byproducts and waste materials (including mine tailings). Historic mining activities have left behind a legacy of many abandoned mine sites. Mine tailings, in particular, owing to their unfavorable physicochemical and biological properties, are generally devoid of vegetation and have poor soil structure. Thus, soil microbial processes and plant growth are generally inhibited, and the tailings surfaces remain exposed and highly susceptible to aeolian dispersion and water erosion, which has conveyed metal-enriched tailings particles to tens of hectares of additional soils and water streams (Mendez and Maier, 2008). Tailings transport and distribution have adversely impacted both local and regional terrestrial and aquatic ecosystems (Allan, 1995), as increased levels of heavy metals have been reported in aquatic habitats which correlated with increased metal accumulation and decline in diversity and richness of macroinvertebrates including midges, crayfish, amphipods and mussels (Allert et al., 2009; Johnson et al., 2016; Poulton et al., 2010). Similarly, decreases in fish biodiversity, population size, and reproductivity along with metal accumulation within tissues have been documented in mining-impacted areas compared to reference streams. Elevated metals exposure also resulted in increasing the risk of metals transfer to higher trophic levels, which is evident by increased concentrations of Pb, Zn, and Cd in blood, liver and kidney of birds from mining areas (EPA, 2015; Johnson et al., 2016). Elevated blood lead levels were also found in children residing in towns nearby mining areas compared to

reference towns, although trophic-transfer was not shown as a direct cause (Hai et al., 2018). In addition, significantly higher rates of stroke, chronic kidney problems, hypertension, heart disease, skin cancer and anemia were found among residents, along with higher death rates (Johnson et al., 2016). Thus, economic and effective approaches are in great demand to remediate, stabilize, and ecologically reclaim these vast and numerous mine tailings sites in order to mitigate the continuing transport of tailings to the surrounding environments and to benefit public health.

1.2. REMEDIATION OPTIONS FOR MINE TAILINGS

Conventional remediation technologies that are commonly used include physical and chemical methods. Physical methods entail waste excavation and dumping or covering the tailings surface with a cap of clay or topsoil. Chemical treatments, on the other hand, involve either using organic chemical reagents to remove metals through soil leaching/extraction or resinous agents to solidify the tailings material and provide a temporal stabilization (Tordoff et al. 2000). These methods are unfortunately cost prohibitive especially for large-scale tailings, which are often abandoned and without financial resources for full stewardship. Moreover, these techniques may add more disturbance to the environment (Wang et al. 2017). Borrowed soils, for instance, are often not replaced, causing further ecological impacts in the area. Alternatively, phytotechnologies are cost-effective and sustainable remediation techniques to achieve long-term stabilization while concurrently stimulating soil ecological revitalization. Among them, aided phytostabilization which aims at using excluder plant species, their associated microorganisms, and appropriate soil amendments to create a self-sustaining

biological cap that can reduce the risk of contaminant spread to the surrounding environments (Kidd et al., 2015; Mendez and Maier, 2008). In the past 10 years, phytotechnologies have evolved into a more comprehensive concept, called ‘phytomanagement’, which entails sustainable ecological restoration of contaminated soils while concurrently offering ecological benefit and producing a valuable biomass that is resulting in a financial return (Evangelou and Deram, 2014). Therefore, this new concept targets specific plant species that are tolerant to metal toxicity with a high phytostabilization efficiency, while producing high biomass that is economically valuable (Kidd et al., 2015; Mench et al., 2010; Vangronsveld et al., 2009). Candidate plant species for this concept can be fast growing species and short rotation coppice such as willow, poplar, miscanthus, etc. (Guittonny and Lortie, 2017; Wang et al., 2021). While the phytomanagement concept has been successfully practiced for metal-contaminated soils, its efficacy for multi-metal mine tailings remains to be explored due to the multidimensional deficiencies generally associated with the latter.

1.3. ROLE OF AMENDMENTS IN TAILINGS PHYTOMANAGEMENT

Mine tailings revegetation is generally very challenging, as the extreme characteristics noted often prohibit natural plant growth and sites remain barren for decades. Therefore, when choosing revegetation as a remediation option, besides selection of candidate plant species, a successful phytomanagement strategy must also entail a careful selection of appropriate soil amendment or a combination of amendments that can ameliorate the severe physicochemical and biological properties of tailings while concurrently attenuating metal toxicity. Many soil amendments have been evaluated for

potential to ameliorate the unfavorable characteristics of mine tailings, including but not limited to, inorganic amendments such as lime, zeolite, apatite, bentonite, Fe-oxides, etc; and organic and rich carbon amendments such as yard wastes, wood processing byproducts, compost, manure, biochar (BC), municipal biosolids (BS), etc. Among these amendments, BS have shown promise to revegetate mine tailings owing to their rich organic matter (OM) and nutrient content.

1.3.1. Biosolids-Aided Phytostabilization. Compared to other soil amendments, the superiority of BS for mine tailings revegetation is largely derived from their high OM content (up to 50%), which serves as a reservoir for nutrient supply and substrate for microbiota in addition to other benefits such as improving tailing's structure, increasing water holding capacity (WHC) and metal binding through sorption and complexation mechanisms (Baghaie et al., 2011; Wijesekara et al., 2016). Biosolids also enhance tailings revegetation by improving soil fertility through altering soil physical- (i.e., porosity, bulk density, and aggregation); biological- (i.e., microbial diversity and activity); and chemical-properties (i.e., pH and cation exchange capacity (CEC)) (Gardner et al., 2010). Moreover, BS contain a wide range of nutrients and OM needed to supply macronutrients NPK and micronutrients, particularly Fe which is often deficient in alkaline tailings associated with carbonate-bearing minerals such as in this study (Jaynes and Zartman, 2005; Wijesekara et al., 2016). However, most BS application studies on mine tailings have been conducted on acidic mines with limited studies involved alkaline mines (Brofas et al., 2000; Jones et al., 2011). Using BS for tailings treatment also offer beneficial use of the high BS quantity generated (projected to be 17.5 x 10^7 tons per year by 2050) (Wijesekara et al., 2016).

Nevertheless, BS also have some limitations in achieving successful ecological restoration of mine tailings. First of all, due to the severity of tailings degradation, higher BS application rates are needed to support a sustained vegetation, yet tailings sites are usually located in remote areas, and the limited availability and high transport cost may limit high BS application rates particularly for large-scale sites. Also, due to high nutrient availability especially at initial stages, BS may attract invasive species thus not supporting diverse plant growth for true ecological productivity. In addition, the effectiveness of BS as an amendment is limited by the rapid decomposition of the OM in BS. A key driver in OM decomposition is C/N ratio. Residues with C/N ratio below 20-25 decompose more quickly compared to those with higher C/N ratios, and up to 75% of residue can be decomposed within the first 10 weeks after application when C/N ratios were as low as 11, such as BS (Reichel et al., 2018). At initial stages, BS also provide excess nutrients, which are often lost via leaching or volatilization. Moreover, the high content of OM in BS at initial stages may provide high sorption capacity to immobilize toxic metals, which can be re-released as OM diminishing due to rapid BS decomposition, hence posing metal toxicity risk which may affect revegetation success. Rapid decomposition also means losing other benefits of improved structure, physical and biological properties. Given the fact that mine tailings are extremely degraded, such condition, if it happened before reaching a point of self-sustainment, most likely leads to a revegetation failure. Therefore, when dealing with revegetation of multi-metal mine tailings, a pressing need exists for more research to explore potential phytomanagement strategies that can prolong the BS benefits, increasing their effectiveness as a sustainable amendment while optimizing the application rates needed.

1.3.2. Pyrolyzed/Humified Waste Materials as Recalcitrant Amendments. Compared to raw waste materials, pyrolyzed and humified products such as BC and humic substances (HS) are characterized by high C:N ratio and highly recalcitrant carbon with unique physical, chemical, and biological properties (Joseph et al., 2010; Stevenson, 1994). For instance, the high pore volume and surface area with abundant, diverse functional groups makes BC rich in both CEC and anion exchange capacity (AEC) with unique sorption characteristics. Studies have shown that BC mechanisms govern metal immobilization and nutrient retention are mainly through i) direct adsorption via electrostatic interactions, ion exchange, complexation, or physical adsorption and precipitation; and ii) indirect improvement to the adsorption capacity of the soil through changes in physicochemical and biochemical properties of the soil induced by BC addition (Wang et al., 2021). In addition, there is increasing evidence that BC may induce negative priming effect on both native soil OM and newly added OM via various mechanisms such as: sorptive protection of labile/dissolved OM, inhibition of microbial activity, and net N-immobilization (DeCiucies et al., 2018; Dodor et al., 2018). Thus, BC may contribute to slowing OM decomposition, and ultimately promoting C-sequestration (Wang et al., 2016). C-sequestration could be of a particular interest in mine tailings reclamation efforts to accelerate C-input into tailings. Positive effects of BC application on mine tailings have been reported. However, such studies have mostly focused on BC impact on tailings physicochemical, hydraulic, and/or geochemical properties (i.e., mainly metal immobilization) (Beauchemin et al., 2015; Fellet et al., 2011; Fellet et al., 2014), and often ignored the primary goal which is sustained vegetation that is necessary to gradually restore the degraded ecosystem. Unfortunately, given the extreme

characteristics of mine tailings, this goal cannot be achieved by applying such amendments alone despite their unique sorptive and recalcitrance characteristics and potential for increased C-input. Thus, research is needed to investigate phytomanagement strategies that can effectively employ these unique amendments in the revegetation efforts of mine tailings to increase the phytorestoration success.

1.3.3. Mycorrhizal Fungi-Aided Phytostabilization. Mycorrhizal fungi (MF) play a vital role in ecosystems, especially in ecosystem variability, diversity, and function. In a symbiotic relationship with plants, MF play important roles in regulating plant-soil as well as plant-plant interactions, thus contributing significantly to ecosystem biodiversity and development. Inclusion of MF during mine lands reclamation, in particular, may promote diverse ecosystem services such as enhanced plant nutrition, sustained growth, an increase in plant tolerance to biotic and abiotic stress of toxic metals, and soil aggregate formation and stability (Teixeira et al., 2017). Development of lateral fine roots has been also linked to MF colonization, the turnover of fine root biomass is considered to be a primary contributor to soil OM (Vogt et al., 1986). The MF can also contribute to soil OM input through the turnover of mycorrhizal hyphae and external mycelium (Godbold et al., 2006). The extraradical hyphae in prairie soil, for example, have been assessed to be as high as 28 m cm^3 with an annual hyphal turnover of 26% (Solaiman, 2014). Thus, the benefits of the symbiotic relationship between most plants with MF can be employed during mine tailings restoration efforts to contribute to enhanced phytomanagement strategies. The potential role of MF in improving growth and enhancing plant tolerance in mine tailings revegetation has been demonstrated (Madejón et al., 2012; Madejón et al., 2010; Solís-Domínguez et al., 2011); however,

most of the studies have mainly focused on plant response parameters such as increased aboveground biomass, reduced metal uptake, and activation of leaf antioxidant enzymes (Riaz et al., 2021; Wang, 2017). Furthermore, most studies investigated the MF impact on soil characteristics involved metal-contaminated soils. Such effects cannot be generalized on mine tailings giving the severity of degradation for the latter, which has been also found to dramatically hinder MF development and their root colonization efficiency, thus limiting the provision of associated ecosystem services (Wang, 2017). Therefore, ameliorative phytomanagement strategies are needed to facilitate the development of MF-plant root symbiosis, hence improving their functional efficiency, besides understanding their role in improving tailings characteristics and promoting soil development.

1.4. IMPORTANCE OF AMENDMENTS CO-APPLICATION AND SYNERGISM

Owing to the extreme characteristics of multi-metal mine tailings, singular amendment application may not be sufficient to achieve sustained vegetation. However, the superior ameliorative effect of BS compared to other amendments along with their large availability make them the primary amendment to consider when aiming to revegetate highly degraded systems such as mine tailings. Nevertheless, co-application with other amendments might be more effective to enhance and sustain the BS benefits or overcome other issues which may arise from the BS or cannot be improved by BS alone. Co-application with carbon-rich waste byproducts such as BC and HS may adjust C/N ratio, which plays a key role in divergence of microorganisms enzymes activities and succession, thus affecting humification and mineralization rates and changing pathways

of carbon and nitrogen cycles within an ecosystem (Dodor et al., 2018; Riaz et al., 2017; Shanmugam et al., 2021). Due to their high sorption capacities, these waste byproducts may retain labile organic compounds and readily available/mineralizable nutrients, therefore increase stability and reduce potential nutrient leaching and preserve them for prolonged efficient release to support sustained vegetation biomass (Page-Dumroese et al., 2018). Co-application of BS with BC, in particular, may maximize the phytomanagement efforts due to the unique and complementary properties of both amendments. The recalcitrant nature of BC and the induced negative priming on already existing OM or freshly added OM (Weng et al., 2017) are of a paramount interest which means the sorptive characteristics for both metals and nutrients are sustained longer. Biochar addition significantly suppressed decomposition of native OM and added OM represented by corncob residues (Riaz et al., 2017), and in another study co-application with BC slowed OM decomposition of cattle manure (Dodor et al., 2018). Research examining the synergistic effect of co-application of BC and BS is notably lacking for phytomanagement of multi-metal mine tailings. A recent study investigated the benefits of combining BC and sewage sludge on immobilization of metals in mining soils, however, was a relatively short-term study (4-weeks) and only focused on metals status while ignoring other interrelated properties (Penido et al., 2019). Long-term impacts have not been investigated or studied for plant growth and tailings stabilization.

Similarly, inclusion of MF in BS-assisted phytomanagement of mine tailings can be more beneficial than applying BS alone, due to the diverse ecosystem services found to be provided by the former. In addition to their well-known role in enhancing nutrient uptake especially P in deficient soils, MF may influence soil C storage and aggregate

formation and stabilization. Researchers have hypothesized that MF may lead to aggregate-protected OM and reduction in carbon decomposition rates (Sosa-Hernández et al., 2019). Such effect is of great importance in mine tailings revegetation and sustaining amendment benefits especially with BS application. According to Lehmann et al. (2017); Rillig and Mummey (2006), mechanisms for aggregate formation and stabilization can be summarized as follows: 1) biophysical: entanglement of soil particles and aggregates through MF hyphae or altered root architecture, particles realignment through physical force/penetration, and altered water regimes; 2) biochemical: release of fungal products from living or decomposing mycelium such as glomalin-related soil proteins, polysaccharides, glycoproteins, hydrophobins and other extracellular compounds; and 3) biological: modification of microbial communities in the mycorrhizosphere. Promoting soil aggregate stability and structure improvement can be a strategy to physically protecting soil OM within the aggregates against decomposition (Rabbi et al., 2016). Likewise, inducing higher development of fine roots can be a strategy to increasing the belowground litter inputs to soil OM and promoting aggregate formation and stabilization.

1.5. IMAGE-BASED PHENOTYPING FOR CANDIDATE SPECIES SELECTION

The success of tailings revegetation and return to ecological function is also highly dependent on plant species selection for revegetation potential. Mine tailings are typically very heterogenous across the numerous sites, numbering in the thousands, thus revegetation strategies including plant selection are not able to be prescriptive across the various types of sites and climates. Greenhouse studies may greatly direct revegetation

efforts by screening combinations of plant species and amendment strategies for specific tailings types (Gil-Loaiza et al., 2016), however this is hindered by costly and time-consuming chemical analyses and plant vitality and growth assessment. To improve the efficacy of screening studies that aid in potential restoration success, new, non-destructive, high-throughput, and cost-effective screening approaches are desirable to facilitate wide-range screening efforts.

Abiotic environmental stresses such as heavy metals toxicity and nutrient deficiency generally induce biochemical and physiological disorders and adversely impact plant metabolism (Barceló and Poschenrieder, 1990; Nagajyoti et al., 2010). The over-production of reactive oxygen species (ROS) is typically the response of plant cells to environmental stresses. The imbalance of ROS level generally promotes the impairments of cell membranes and lipid peroxidation and eventually causes oxidative damage to plant cells (Mittler, 2002). Oxidative stress generally causes impairment of chlorophyll synthesis and damage to the chloroplast membranes with a marked reduction in chloroplast density (Baryla et al., 2001; Viehweger, 2014), resulting in a decline in chlorophyll pigment as well as production of other pigments such as carotenoids and anthocyanins which act as nonenzymic antioxidants against metal stress (Baek et al., 2012; Hermle et al., 2007). Imbalance of ROS consequently leading to chlorosis and necrosis symptoms which typically manifest in plant leaves (Panwar et al., 2016; Stambulska et al., 2018) and lead to longer-term visible injuries and phytotoxic responses such as reduced growth rates, decreased biomass, leaf chlorosis/necrosis, etc. (Yadav, 2010). While visual observation can clearly note plant response to abiotic stress (i.e., vigor and health), quantifying the response from visual inspection is more subjective and

not reproducible nor capable to differentiation in stress responses. As such, visually determining robust differences between different species in response to environmental stress or responses of a certain species to different treatments is reliant on the observer. Nevertheless, employing image-based phenotyping and computer visualization techniques may greatly facilitate evaluating plant and amendment selection by determining statistically robust plant responses that can measure plant health and vigor throughout developmental stages and not just in destructive testing at the end of experiments.

1.6. GOALS AND OBJECTIVES OF THIS STUDY

The overall goal of this research is to advance abilities in revegetation and ecorestoration of mine tailings and blighted lands by better understanding the role of soil amendments, amendment combinations and the interaction with candidate plant species. The specific goal is to improve the knowledge of how to optimize the benefits of targeted rich organic and recalcitrant carbon amendments through amendment co-application and interaction with beneficial mycorrhizal fungi to chemically and biologically assist the phytostabilization of Pb/Zn/Cu mine tailings using bioenergy crops and native prairie species. To reach this specific goal, the following objectives were developed.

- **Objective 1:** Investigate the impact of singular application of rich organic biosolids and recalcitrant carbon amendments such as biochar and humic substances as well as biological amendment i.e., mycorrhizal fungi on tailings physicochemical and hydraulic properties and biomass production.

- Hypothesis: Tailings physicochemical and hydraulic properties will be significantly improved by the application of organic and rich carbon amendments. Nevertheless, enhanced plant growth responses will be largely influenced by the application of biosolids owing to their rich organic and readily available macronutrient content compared to the other amendments that are evaluated.

- **Objective 2:** Assess the potential of promoting synergistic benefit of tailings phytomanagement through co-application of biosolids and recalcitrant carbon amendments such as biochar and humic substances and their impact on biophysicochemical properties and reduced heavy metal bioavailability.
  - Hypothesis: Combining biosolids with recalcitrant carbon amendments which are characterized by high cation and anion exchange capacities, high surface area, and diverse functional groups, may further enhance the benefits of biosolids. Co-application may lower the decomposition rates of organic matter in biosolids, provide increased and prolonged metal sorption, and simultaneously stimulate retention and immobilization of excess nutrients particularly N and P, thus sustain slow and efficient release of nutrients for agronomic benefit.
- **Objective 3:** Determine the potential benefit for production of a variety of bioenergy crops through co-application of biosolids and recalcitrant carbon amendments such as biochar and humic substances.
  - Hypothesis: Combining biosolids with recalcitrant carbon amendments will further influence plant growth responses to mirror changes in the

biophysicochemical and geochemical properties compared to biosolids alone. However, the biochar induced nutrient immobilization may counteract its benefit of reduced metal bioavailability resulting in inhibition or no effect on biomass production for various bioenergy crops.

- **Objective 4:** Quantify the interactive benefits of biosolids application and mycorrhizal fungi inoculation on growth of bioenergy crops and tailings properties.
    - Hypothesis: In extremely degraded ecosystems such as mine tailings, ability of mycorrhizal fungi to thrive is also strongly hindered. Biosolids can facilitate the development of efficient mycorrhizal fungi symbiosis. In addition to their role in altering root morphology and architecture, mycorrhizal fungi may directly influence biochemical and geochemical processes through their extended hyphae and the secretion of mycelium-based products. Therefore, mycorrhizal fungi may contribute to organic matter input into the tailings system, hence modifying biophysicochemical properties and reducing heavy metal bioavailability. Mycorrhizal fungi may further improve plant growth responses by enhancing nutrient acquisition and tolerance to metal phytotoxicity.
- **Objective 5:** Develop a plant phenotyping assessment approach utilizing computer vision and image-based analysis to enable early detection and quantification of plant tolerance and response to abiotic stress at low cost and high throughput.
    - Hypothesis: Current assessment of plant vigor, growth and viability often involves destructive sampling, time-consuming manual methods, and costly chemical analyses, making it infeasible to evaluate transient and time-series

responses or screen a wide range of species or soil amendment strategies. Developing a non-destructive approach that relies on the visual symptoms generally result as a response to abiotic stress can greatly improve phytomanagement efforts of mine lands by facilitating the screening of a broader pallet of plants and amendment strategies, which helps in setting a site-specific strategy that ensures successful revegetation before scaling up.

- **Objective 6:** Identify native and prairie species with great potential for ecologically beneficial phytostabilization of Pb/Zn/Cu mine tailings.
    - Hypothesis: widely varying plant species will show differential responses to the abiotic stress and to the amendment strategies. Leguminous, tree, and grass species may exhibit differential fitness and tolerance, and the combinations will provide varying ecosystem services in field applications. Image-based phenotyping can greatly facilitate plant species screening by quantifying statistically robust morphological and color differences in response to nutrient deficiency and metal phytotoxicity.

PAPER

I. AMENDMENT-ASSISTED REVEGTATION OF MINE TAILINGS: IMPROVEMENT OF TAILINGS QUALITY AND BIOMASS PRODUCTION

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ABSTRACT

Mining activities have left a legacy of large metals-containing tailings impoundments. After mine closure, reclamation of mine wastes can be achieved by restoration of a vegetation cover. This study investigated the impact of biochar (BC), biosolids (BS), humic substances (HS), and mycorrhizal fungi (MF) for improving mine tailings fertility and hydraulic properties, thus supporting plant establishment, tailings revegetation and enabling growth of energy crops. We conducted a series of pot trials by growing willow, poplar, and miscanthus in Pb/Zn/Cu mine tailings untreated or amended with two rates of amendments (considered as low or high input). Biosolids resulted in the most significant changes in tailings properties, neutralizing pH and increasing organic carbon, nutrient concentrations, cation exchange capacity, water retention, and saturated hydraulic conductivity. The greatest increase in energy crops production was also observed in BS treatments enabling the financial viability of mine reclamation. Although BC resulted in significant improvements in tailings fertility and hydraulic properties, its

impact on biomass was less pronounced, most likely due to its lower N and P concentrations. Increases in willow and miscanthus biomass were observed in HS and MF treatments in spite of their lower nutrient content. A pot experiment is underway to assess the synergistic effect of combining BS with BC, HS, or MF.

1. INTRODUCTION

Mining activities generate large quantities of solid waste products, typically characterized by elevated metal concentrations, extreme pH, low organic matter (OM), nutrient deficiency, and low water retention (Wang et al. 2017). In such marginal lands, soil microbial processes and plant growth are generally inhibited (Mendez and Maier 2008; Shu et al. 2002). Conventional remediation approaches involve covering tailings with topsoil, gravel, or clay capping. These are not cost effective solutions for large-scale areas regarding the quantities of soil capping needed (Karaca et al. 2018). Revegetation is an environmentally friendly, low-cost option to achieve long-term reclamation of tailings. The implementation of a vegetation cover can be aided by the selection and incorporation of relevant soil amendments (Mendez and Maier 2008). Soil amendments and vegetation cover must sustainably improve soil biophysical and chemical properties such as OM and nutrient contents and restore the cascade of biological processes and functions which in turn promotes ecosystem services (Mench et al. 2010; Bolan et al. 2014).

Biosolids (BS) generated by municipal wastewater treatment facilities are one of the most used amendments for mine revegetation. Large quantities of BS are generated in wastewater treatment plants as a byproduct. Recycling of BS as fertilizer could be a good

management practice to restore mine waste fertility and improve substrate physical (i.e. porosity, bulk density, aggregation, and water holding capacity (WHC)) (Silveira et al. 2003); biological (i.e. microbial activity) (Mingorance et al. 2014); and chemical properties (i.e. pH and cation exchange capacity (CEC)) (Gardner et al. 2010). Moreover, BS contain nutrients and OM to support plant growth, including macronutrients NPK (Wijesekara et al. 2016) and micronutrients, particularly Fe, which is often deficient in alkaline soils (Jaynes and Zartman 2005). Nevertheless, the excess amount of nutrients present in BS along with pathogens, emerging contaminants, and odor emissions have raised environmental and public health concerns associated with land application of BS (Lu et al. 2012). Consequently, these concerns have led to the formulation of regulations for proper treatment practices to generate quality-controlled BS; in addition to proper management practices and precautionary actions during BS land application, followed by continuous monitoring to ensure long-term sustainability and environmental security (Wijesekara et al. 2016). Many studies investigated BS application on acidic mine wastes (Sydnor and Redente 2002; Mingorance et al. 2014); however, only a few involved alkaline mines (Brofas et al. 2000; Jones et al. 2011).

Biochar (BC) is the product of thermal degradation of organic materials in the absence of oxygen (pyrolysis) (Lehmann et al. 2011). Raw feedstock and pyrolysis conditions strongly affect BC characteristics (Ippolito et al. 2012). In general, pyrolysing woody biomass at high temperature results in BC with recalcitrant carbon that possesses high surface area and specific adsorption properties (i.e. high nutrient retention and metal complexation) (Kloss et al. 2012). Biochar effects on soils depend on many parameters such as biochar properties (i.e. raw material, initial carbon content, pyrolysis temperature

and sorption capacity) and characteristics of soils to be remediated (i.e. pH, soil texture, and OM content) (Park et al. 2011). Improved soil fertility and plant yields were demonstrated following BC amendment, and attributed to increases in soil pH, nutrient availability, CEC, water retention, and soil microbial activity (Chan et al. 2007; Spokas et al. 2012). In some cases, negative impacts associated with BC application were reported and attributed to reduced availability of water and nutrient due to retention/immobilization by BC and reduction of N mineralization rate caused by increasing C/N ratio (Kelly et al. 2015). Furthermore, contaminants such as polycyclic aromatic hydrocarbons (PAHs), dioxins, and furans are likely formed during the pyrolysis process of BC and may induce acute toxicity to soil microorganisms if present at high concentrations (Ding et al. 2016).

Humic substances (HS) are heterogeneous mixtures of high molecular weight organic compounds formed through humification of OM by soil microorganisms (Stevenson 1994). Due to their molecular complexity and interactions with soil mineral phases, these substances are very stable against microbial decomposition and last in soil for thousands of years (Stevenson 1994; Trevisan et al. 2010). The great diversity of functional groups on HS surfaces enables them to interact with soil minerals and metal ions through ion exchange, chelation, and oxidation–reduction reactions (Kochany and Smith 2002). Humic substances play an important role in increasing plant nutrient availability, especially Fe under alkaline pH, and also increase WHC, CEC, pH buffering capacity, and enhance microbiological activity (Khaled and Fawy 2011; Pettit 2004). Metals toxicity is also mitigated by complexing with HS rendering them less (phyto)available (Klučáková 2015). Additionally, HS improve plant growth through

physiological and metabolic effects by changing root architecture and hormone-like activities (Trevisan et al. 2010; Canellas and Olivares 2014).

Mycorrhizal fungi (MF) are one of the most important rhizosphere microorganisms that colonize most plants. This symbiosis greatly influences nutrient and metal uptake, and ultimately plant growth and health. Under limited water and nutrient availability, the extended fungus hyphae help plants to acquire water (Colombo et al. 2017) and nutrients, specifically P (Smith et al. 2003). Mycorrhizal fungi can also enhance plant tolerance to metal stress through excretion of binding agents to detoxify toxic metals (Eisenman and Casadevall 2012; French 2017). Furthermore, MF hyphae combined with plant root morphological alterations can further promote stability and improve soil structure (Leifheit 2014).

Combining land remediation with post-processed biomass to energy conversion or high value bio-products is an option to enhance the financial viability of mine revegetation (Jiang et al. 2015). Potential candidates must be fast-growing, deep-rooted, tolerant to excess metal concentrations with low root-to-shoot metal transfer, and able to stabilize metals in the root zone. Candidates should also produce dense root and valuable aboveground biomass, preferably perennial and sustainable, which is pivotal for preventing wind and water erosion (Mendez and Maier 2008). Among energy crops, short rotation coppice (willow and poplar) and perennial grass (miscanthus) are great choices for mine revegetation due to their high metal tolerance and great biomass production with low nutrient requirements (Karp and Shield 2008; El Kasmioui and Ceulemans 2012). These characteristics make them used as suitable species in short rotation coppices (SRC) for production of valuable feedstock for bioenergy productions, albeit miscanthus

invasiveness is a matter of debate in some part of the United States (Smith and Barney 2014). These species with proven bioenergy, biofuel, or timber value could enhance the economic viability of mine reclamation.

This study aimed to evaluate the potential for BS, BC, HS, and MF to support revegetation of Pb/Zn/Cu mine tailings with biomass crops. The objectives were to investigate amendments impacts on tailings fertility, hydraulic properties (e.g., water retention and saturated hydraulic conductivity (Ks)), and biomass production of willow, poplar, and miscanthus.

2. MATERIAL AND METHODS

2.1. TAILINGS, AMENDMENTS AND TAILINGS TREATMENTS

The tailings were collected from a Pb/Zn/Cu mine tailings impoundment covering ~110 ha area in Viburnum, MO, US (37°42'07.1"N 91°06'22.3"W), with a long history of mining activity (1960 – 2004). In 2011, tailings revegetation by miscanthus (*Miscanthus × giganteus*) was investigated in a 4.45 ha pilot-scale trial. However, the limited substrate fertility resulted in poor survival and plant growth, leading to this study. Tailings were collected in the 0 – 30 cm substrate layer, air dried, passed through a 2-mm sieve, and thoroughly homogenized. They were characterized by very fine texture and particle size and consisted of 40% sand, 50% silt, and 10% clay (Soil and Plant Testing Laboratory, University of Missouri, Columbia, MO) (Table 1).

Table 1. Characterization of the physicochemical parameters of mine tailings and amendments.

Parameter	**Mine tailings (MT)**	**Background levels[a]**	**Biochar (BC)**	**Biosolids (BS)**	**Humic substances (HS)**
pH (H_2O)	7.8	> 6.1[b]	8.6	6.4	4.1
EC (mS cm^{-1})	0.4	-	0.1	-	-
Total P (mg kg^{-1})	87.1	-	420	14200	380
NH_4–N (mg kg^{-1})	1.7	-	-	8220	-
NO_3–N (mg kg^{-1})	0.5	-	-	69.1	-
Total N (mg kg^{-1})	600	-	3180	66500	9800
Pb (mg kg^{-1})	3553	20[a]	23.3	31.5	-
Zn (mg kg^{-1})	966	49[a]	-	735	-
Cu (mg kg^{-1})	479	13[a]	-	522	-
Cd (mg kg^{-1})	13.7	<1[a]	0.2	-	-
Ni (mg kg^{-1})	70.7	14[a]	59.6	22.4	-
Cr (mg kg^{-1})	11.5	54[a]	9.2	24.6	-
TC	7.3		82.6	-	-
OM%[c]	0.6		-	57.6	58.9
CEC (cmol(+) kg^{-1}	3.6		8.1	41.3	62.7

[a] Background concentrations for metals in Missouri soils (Tidball 1984)
[b] Summary of soil fertility status in Missouri (Nathan et al. 2007)
[c] Loss on Ignition

Biochar used was a commercial product derived from pine chips (Range Fuels Company, Soperton, GA). The BC had an alkaline pH (Table 1). The ash content was 4.8%, the Brunauer-Emmett-Teller (BET) surface area was 310 m^2 g^{-1}, pores volume (<20 Å) as measured by CO2 was 0.12 cm^3 g^{-1}, and WHC was 72% and 259% on a wet and dry basis respectively (Cool Planet Company, Camarillo, CA). The aerobically/anaerobically digested BS were obtained from the Southeast Wastewater Treatment Plant, Rolla, MO, dried to approximately 37% solid content, and thoroughly mixed and homogenized prior to use. Metal concentrations of the BS meet U.S. Environmental Protection Agency Class B standards for land application (Table 1). Humic substances and mycorrhizal fungi were commercial products provided by Soil

Secrets, LLC., Los Lunas, NM. The MF inoculum was a sand-based blend with abundant spores of endomycorrhizae (i.e. vascular arbuscular mycorrhizae) at a minimum 1.45 million viable spores of *Glomus intradices* per pound, and ectomycorrhizae species (MycoMaxima Professional). The HS amendment was a granular form of cultured humus concentrate containing humic acids, fulvic acids, and humin fractions, and some beneficial microorganisms (TerraPro).

Four tailings amendments: BC, BS, HS, and MF were tested in this study. Amendment trials were conducted in a greenhouse located at Missouri University of Science and Technology, Rolla, MO. Each amendment was mixed thoroughly with air dried tailings at two rates (considered as low and high). Untreated and amended tailings (% DW/DW) were potted in plastic pots (1.35 kg in 2.2 L cylindrical pots) resulting in the following nine treatments (in twelve replicates for each treatment):

(1) Untreated Pb/Zn/Cu mine tailings (MT)
(2) Tailings + 3% BC (BCL)
(3) Tailings + 5% BC (BCH)
(4) Tailings + 1.7% BS (BSL)
(5) Tailings + 5% BS (BSH)
(6) Tailings + 0.15% HS (HSL)
(7) Tailings + 0.3% HS (HSH)
(8) Tailings + 10 g pot^{-1} MF (MFL)
(9) Tailings + 30 g pot^{-1} MF (MFH)

All pots were watered with deionized water for a 2-week period, allowing amendments to react with the tailings and for microbial communities to develop. For each treatment, three out of twelve replicates were incubated and watered under greenhouse conditions over four months for chemical and hydraulic properties analysis of the substrate.

2.2. SUBSTRATE CHEMICAL AND HYDRAULIC PROPERTIES ANALYSIS

After four months, the incubated tailings (n=3 for each treatment) were air dried, crushed to pass through a 2-mm sieve, homogenized thoroughly, and stored for substrate analysis. Substrate pH was measured in MΩ water at 1:2.5 substrate/solution ratio (Orion 370, Thermo Fisher Scientific, Inc.). Total carbon (TC) and total nitrogen (TN) were measured using elemental analyzer (Perkin Elmer 2400 CHN analyzer). Samples were analyzed for organic carbon (OC) using Walkley-Black method (Page 1982). The Olsen P method was used for extracting available P (Pierzynski 2000). The P extracts were then treated with (~200 mg) charcoal, filtered, acidified to 2%, and analyzed using inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer, Avio 200) (Soltanpour et al. 1982). For extractable cations, the samples were centrifuged, filtered, acidified, and analyzed for Ca, Mg, K, and Na by ICP-OES. After supernatant collection, the substrates retained in the tubes were used for CEC measurements. Substrate water retention characteristics were assessed by measuring water content (WC) at -0.1, -0.3, and -15 bar matric potential using 5 and 15 bar pressure plate apparatuses, respectively (Soil Moisture Equipment Crop, model#1600F1 and 1500F2). Based on the tailings fine textural properties, field capacity (FC) represents water content measured at -0.3 bar (Joseph 2010). Plant available water content (AWC) was calculated from the difference between FC and permanent wilting point (WP) (Kirkham 2014). Saturated hydraulic conductivity (Ks) was measured by falling head permeability test (ASTM 2016) using permeameter cells (7.62 cm i.d. by 15.75 cm height).

2.3. PLANT CULTIVATION

Stem cuttings of laurel leaf willow (*Salix pentandra*) and hybrid poplar DN34 (*Populus deltoids* × *Populus nigra, DN34*) (roughly 15 cm long) were collected from a nearby park (Schuman Park, Rolla, MO), and pre-rooted in water prior to planting. In each pot, three uniform pre-rooted cuttings of willow, poplar, or two rhizomes of miscanthus (*Miscanthus* × *giganteus*) were transplanted in each of the three replicates (n=3 pseudo-replicates per pot for willow and poplar and n=2 for miscanthus) and cultivated for four months in a greenhouse. The pots were placed in shallow trays and watered three times a week to maintain 70% WHC. After a 4-month growth period, willow and poplar were harvested by separating new branches, leaves, and roots from the old cuttings, whereas miscanthus was collected by separating the shoots and roots from the rhizomes. The miscanthus cultivated in the MF treatment did not grow and were removed from the study. All plant samples were rinsed with tap and deionized water, and subsequently oven dried at 60 °C for 72 h. The dry matter was then weighed to determine the aboveground and root biomass.

2.4. STATISTICAL ANALYSIS

Influence of treatments on chemical composition of the substrate, water retention, saturated hydraulic conductivity, and plant biomass were tested using one-way analysis of variance (ANOVA). Normality and homoscedasticity of residuals were met for all tests. When significant differences occurred between treatments, multiple comparisons of mean values were made using post-hoc Tukey HSD tests. Differences were considered statistically significant at $p < 0.05$. Principal component analysis (PCA) was conducted

for pH, TC, OC, TN, CEC, and Ca, Mg, K, Na and Olsen P concentrations in the tailings. Spearman's correlations were used to test the relation between the (1) field capacity and the plant available water content and (2) organic carbon and the water content. All statistical analyses were performed using R software (version 3.0.3, Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS AND DISCUSSION

3.1. SUBSTRATE QUALITY MODIFICATIONS

3.1.1. Chemical Changes. The untreated tailings had a slightly alkaline pH equal to 7.8±0.04 (Table 2). Changes in tailings pH were a reflection of the initial pH of the amendment added (Table 1). The addition of alkaline BC significantly increased tailings pH i.e. 8.1±0.02 (BCL) and 8.2±0.03 (BCH), whereas other amendments (BS, HS, and MF) significantly decreased the pH in the 7.1 (HSH) – 7.5 (BSL) range, compared to the untreated tailings. As expected, untreated tailings exhibited low CEC, characteristic of sandy and/or low OM substrate (i.e. less than 5 cmol(+) kg^{-1}) (Table 2). With the exception of MFL and HSL, adding amendments significantly increased CEC of tailings. All cations, except for Na, followed a similar trend. Their availability significantly increased in all organic amendment treatments (i.e. BC, BS and HS), with the greatest concentrations measured in BS treatments (Table 2).

Table 2. Chemical composition of the substrate after 4-months.

Treatments		(cmol(+) kg^{-1})	(%)	(%)	(%)	Nutrients (mg kg^{-1})				
	pH	**CEC**	**TC**	**OC**	**TN**	**Olsen P**	**Ca**	**Mg**	**K**	**Na**
MT	7.8±0.04b	3.2±0.5c	7±0.6d	0.5±0.07g	0.06±0.01e	3±0.3f	425±23f	237±14e	21±1.4g	32±2.7c
Biochar										
Low	8.1±0.02a	4.8±0.2b	11±0.1ab	1±0.07d	0.08±0.01cde	4.5±0.9cde	495±19de	292±15cd	31±1.1d	33±3.1c
High	8.2±0.03a	5.2±0.3b	13±1.4a	1.2±0.05b	0.1±0.012c	5.8±0.5c	559±20bc	328±18bc	37±1.9c	38±1.6c
Biosolids										
Low	7.5±0.01c	5.3±0.1b	11±0.8ab	1±0.06c	0.18±0.046b	25.7±1.4b	595±3b	378±24b	53±1.8b	52±3.7b
High	7.2±0.07fg	7.6±0.6a	13±0.4a	2±0.01a	0.4±0.023a	52.7±4.4a	871±43a	540±23a	137±1.2a	98±7.5a
Humic substances										
Low	7.3±0.01ef	4.5±0.1bc	9±0.2cd	0.8±0.04ef	0.09±0cd	3.6±0.6def	479±19de	271±6de	27±1.3def	34±1.5c
High	7.1±0.00g	4.8±0.5b	10±0.4bc	1±0.05cd	0.11±0.006c	5.1±0.3cd	525±15cd	313±16cd	30±0.5de	36±2.2c
Mycorrhizal fungi										
Low	7.4±0.03cd	4.1±0.9bc	8±0.8cd	0.7±0.03f	0.07±0.006de	3.3±0.3ef	452±10ef	236±25e	23±1.6fg	32±2.8c
High	7.3±0.04de	4.2±0.7bc	10±0.4bc	0.9±0.05de	0.06±0.006e	3.5±0.5ef	464±24def	235±9e	25±4efg	31±0.8c
Background levels of Missouri soils	> 6.1[a]	5-25[b]	1.30[c]	1.25[c]	-	-	3300[c]	2600[c]	56-110[a]	5300[c]

Mean value ± SD for each treatment (n=3).Values with different letters differ significantly (one way ANOVA, p-value<0.05).

[a] Nathan et al. 2007

[b] Scrivner and Cooper 1985

[c] Tidball 1984

Due to the high carbonate content in the tailings, elemental analysis showed a high TC concentration compared to the background value 1.3% in Missouri soils according to Tidball (1984). Increased TC were measured following low and high application of BS and BC but only at high application for HS and MF. All treatments resulted in a significant increase in OC compared with the untreated tailings (0.5±0.07%), with the greatest increase in OC (~3-fold) observed in BSH treatment (Table 2). Organic carbon is the main constituent (~58%) of soil OM (Bianchi et al. 2008), thus increases in OC of amended tailings are attributed to the high content of OM in the amendments added (Table 2). All amendments, except MF and BCL, significantly increased TN concentration in tailings, with the greatest increase (more than 5-fold) observed in BSH treatment. The available P concentration in tailings was low (Table 2). Similarly, to OC and TN, the substantial increase in Olsen P (~17-fold) was observed in BS treatment at high application rate. For BC and HS amendments, increases in Olsen P were measured only at the high application rate (Table 2).

The X canonical weights of the Principal Component Analysis (PCA) accounting for pH, TC, OC, TN, CEC, Ca, Mg, K, Na, and Olsen P concentrations in the tailings explained 93% of the total variance (Figure 1). The first axis (81%) separated organic amendments, especially the BS amended tailings at high and low application rate, characterized by high CEC and nutrients concentration, from MT and MF treatments with low values for these parameters. The second axis (12%) was characterized by the BC treatment, driven by high substrate pH and TC concentration.

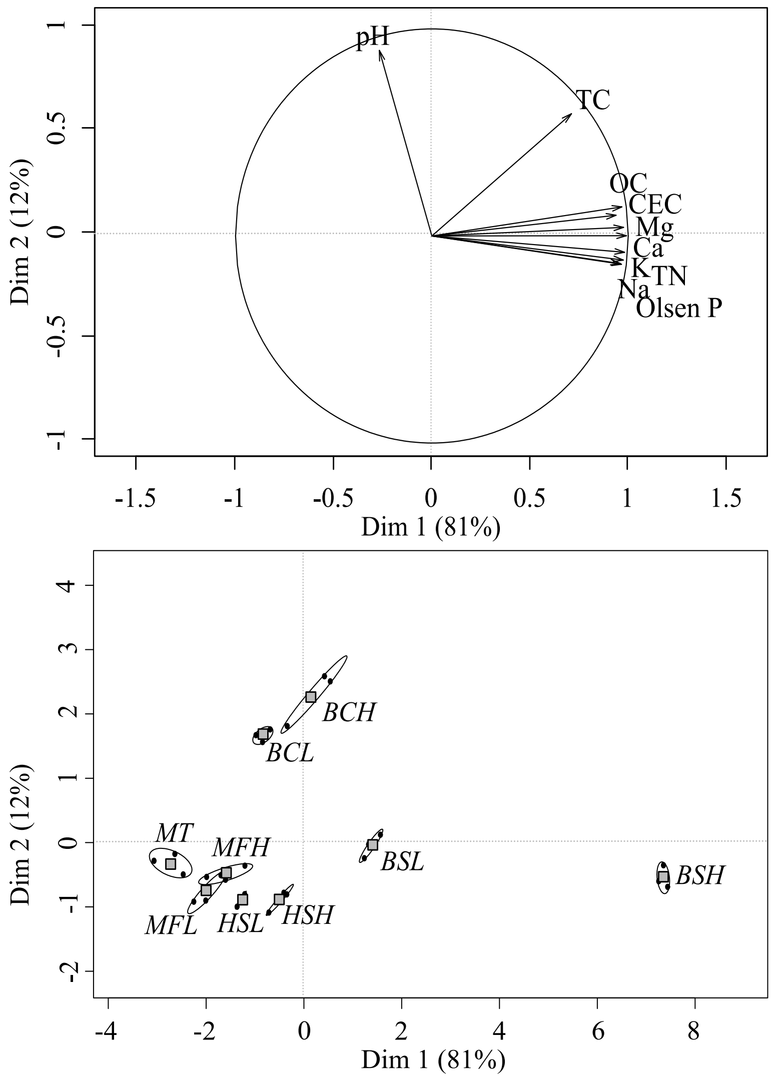

Figure 1. Principal Component Analysis (PCA) on pH, TC, OC, TN, CEC, Ca, Mg, K, Na, and Olsen P concentrations in the tailings (MT) treated with different amendments at low and high application rates: biochar low (BCL) and high (BCH), biosolids low (BSL) and high (BSH), humic substances low (HSL) and high (HSH), mycorrhizal fungi low (MFL) and high (MFH).

The improvement in nutrient status resulted from amending tailings with organic amendments (e.g., BC, BS, and HS). It can be explained by the direct supply of nutrients from these amendments. Several benefits were associated with increasing substrate OM, including enhancement of WHC and CEC (Carter and Stewart 1996), and improvement

of substrate structure and aggregation. Furthermore, by changing pH and increasing CEC, organic amendments enhance nutrient availability (e.g., enhance retention and reduce leaching of nutrients). The improvement in tailings fertility resulting from BS addition were previously attributed to their high concentration of readily available nutrients, particularly N, P, and (Jones et al. 2011). In addition, the high OM concentration in BS resulted in a great increase in CEC, along with decreasing tailings pH and increased exchangeable cation availability (Wijesekara et al. 2016). Brofas et al. (2000) investigated the impact of sewage sludge addition at seven rates on calcareous sandy bauxite mine spoils properties. The improvement was enhanced at high application rate (120 dt ha^{-1}) and characterized by a decrease in substrate pH and increase in N, Olsen P, OM, CEC and exchangeable cations. In agreement with these results, similar effects (e.g., enhanced TN, TC, CEC, and available K and P) following BS amendments were found in alkaline copper mine tailings (Gardner et al. 2010), or (i.e. improved OC, TN, extractable P, and exchangeable Mg and K) in alkaline bauxite sand residue (Jones et al. 2011). Similar benefits in soil chemical properties were observed in a slightly acidic Zn/Pb/Cd contaminated soil amended with four application rates (0, 1.5, 3, and 5%) of sugar cane straw-derived BC (Puga et al. 2015). Biochar positively influenced substrate macronutrients and exchangeable cations and proportionally increased substrate OM for benefits of revegetation. Benefits are also observed in alkaline soils as addition of orchard prune residues-derived BC to alkaline clay mine tailings increased P, K, and CEC when tailings were amended with 5% and 10% BC (Fellet et al. 2011). The increases in soil CEC following BC application is attributed to carboxylic functional groups present on the surfaces of BC particles themselves and to carboxylic groups associated with OM

adsorbed on BC surfaces, which contribute to the more negative charge density in the BC amended soils (Liang et al. 2006). In addition, the high surface area and porosity of BC may have a positive impact on soil CEC (Spokas et al. 2012). Similar impacts of BC application on nutrient availability and soil fertility on different soil types were reported (Chan et al. 2007; Laird et al. 2010).

3.1.2. Water Retention. The tailings exhibited poor substrate structure and hydraulic and aeration properties caused by very fine texture, low OM, and composition of 40% sand, 50% silt, and 10% clay. The tailings had 32.6% volumetric water content (WC) at -0.1 bar matric potential (Figure 2a). Increasing matric potential to -0.3 bar (FC) and -15 bar (WP) dropped WC of tailings to 13.9% and 2.7%, respectively. Adding BC and BS significantly increased tailings WC for all tested matric potentials, except for WP in BCL treatment. No improvement in water retention was observed in MF treatments, while HS only increased FC at high rate. Increased FC following organic amendment addition was correlated with increase in plant AWC (Spearman's correlations, R^2=0.97). Compared with untreated tailings, adding HS, BC, and BS resulted in a 23%, 67%, and 72% increase in plant AWC at low addition rate, and 49%, 79%, and 99% at high rate addition, respectively (Figure 2b).

Positive significant correlations between OC and WC were observed in this study, (Spearman's correlations: R^2=0.74 for FC, and R^2=0.71 for WP). The increase in water retention from OM amendments can be attributed to the direct effect of organic particles to adsorb water on their surfaces and improve soil structure, pore size distribution, and soil aggregates (Tsadilas et al. 2005). Other studies reported soil water retention improvement following rich OM amendments (Price and Voroney 2007; Herath et al.

2013). The improvement in tailings capacity for water retention and water infiltration is a key player to sustain plant growth during drying periods, in addition to increasing nutrient fluxes.

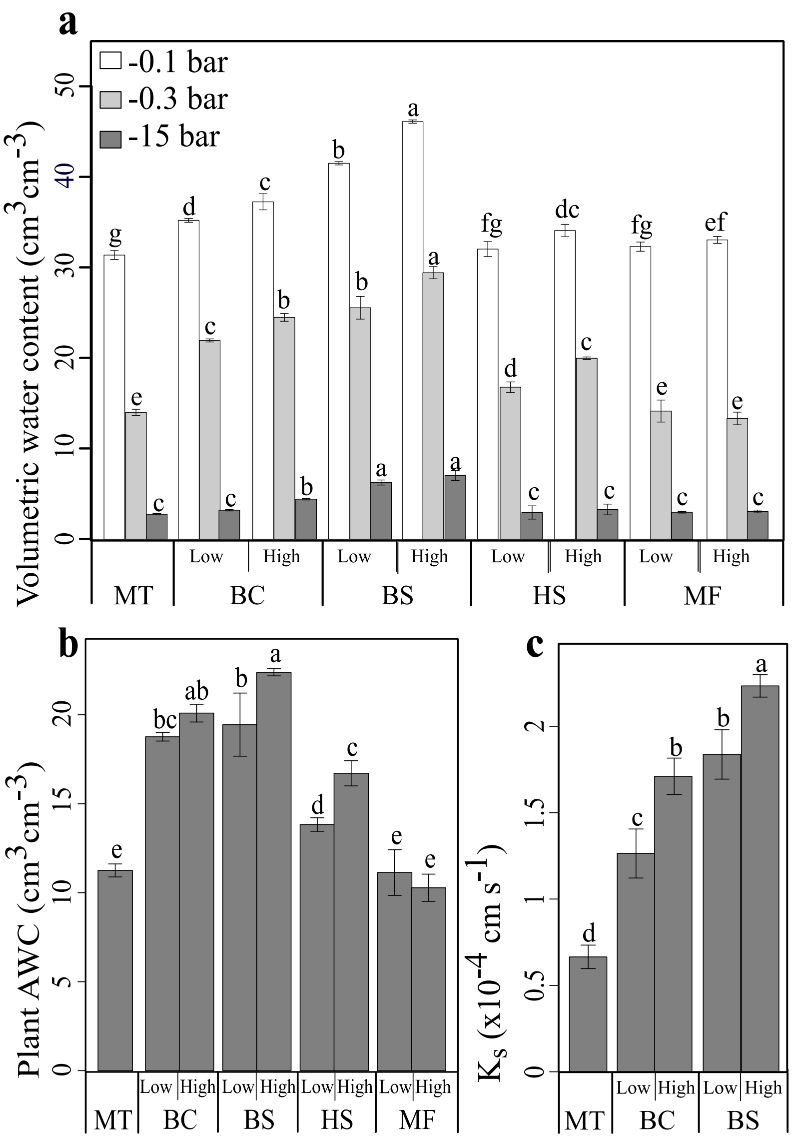

Figure 2. Volumetric water content (a) plant available water content (AWC) (b) and saturated hydraulic conductivity (Ks) (c) on mine tailings (MT) treated with biochar (BC), biosolids (BS), humic substances (HS), and mycorrhizal fungi (MF) at low and high application rates. Values are means ± SD per treatment (n=3). Values with different letters differ significantly (one way ANOVA, p-value < 0.05).

3.1.3. Saturated Hydraulic Conductivity (Ks). Untreated tailings exhibited low permeability, Ks (cm $s^{-1} \times 10^{-4}$), equal to 0.67±0.07 (Figure 2c), limiting water and aeration for plant roots, as well as low infiltration that can aggravate surface flow and water erosion. Adding amendments significantly increased Ks to: 1.26±0.14 and 1.84±0.14 for BCL and BSL and 1.71±0.10 and 2.23±0.07 for BCH and BSH, respectively (Figure 2c). The increase in Ks was primarily due to the OM increase, as a positive significant correlation between OC and Ks was observed (R^2=0.83). Impacts on soil physical properties, such as decreases in bulk density and increased porosity and macro and micro-aggregates, have been reported as a result of organic amendment addition (Jones et al. 2010; Asensio et al. 2013). As OM can induce soil aggregate formation through some binding agents like organomineral complexes, fungal hyphae, and polysaccharides (Tisdall and Oades 1982; Leifheit 2014), the increase in Ks was likely attributed to the improved macro-aggregate stability and macro-pores distribution (Price and Voroney 2007). The beneficial effect of BS on Ks agreed with several previous studies, BS addition to a clay loam soil (50 dt ha^{-1}) doubled the soil infiltration rate under field conditions (Tsadilas et al. 2005). Biosolids added to a calcareous silty clay loam soil also resulted in Ks increases (Zare et al. 2010). Divergent responses of Ks to BC application were observed in previous studies. In an alfisol and andisol silt loam soil amended with corn stover-derived BC at a rate of 10 t ha^{-1}, increased Ks was measured in the 41% (andisol) – 139% (alfisol) range, compared to the untreated soils (Herath et al. 2013). Three-fold increases in Ks were reported in loamy sand soil following BC additions (Eibisch et al. 2015). On the contrary, no impact on Ks was reported when fine, loamy soil was amended with mixed hardwood BC (Laird et al.

2010). Also, a decrease in soil Ks was documented following BC addition to sandy soil (Barnes et al. 2014) and sandy loam soil (Igalavithana et al. 2017). The variations in Ks response to BC addition may be explained by the variable properties of different types of BC and different substrates used in these studies. Using two types of BC pyrolyzed at the same temperature, woodchip BC resulted in Ks values higher than those with dairy manure BC (Lei and Zhang 2013). The authors attributed this to more macro-pores being formed when woodchip BC was added.

3.2. PLANT BIOMASS

The biomass production of the three species showed a similar response when grown in BS treatments, whereas it varied among the three species with the other amendments (Figure 3).

3.2.1. Effect of Biosolids. Biosolids had the greatest influence on plant growth, significantly increasing willow, poplar, and miscanthus aboveground and root biomass at the high application rate (Figure 3). Only poplar biomass did not significantly increase following BSL treatment. Compared with the untreated tailings, BSH resulted in increased total biomass of dicots (willow and poplar) 2.9-fold and 3.8-fold, respectively, and 8.4-fold in the monocot (miscanthus) (Figure 3). In BS treatments, nutrients were not limiting factors as they were readily available in BS, particularly macronutrients NPK, in addition to the indirect supply resulting from decomposition of OM present in the BS (Table 2). The changes in tailings quality induced by BS addition, such as pH reduction and CEC improvement, could have enhanced micronutrient availability and retention, particularly Fe which is deficient in these tailings (Lombard et al. 2011).

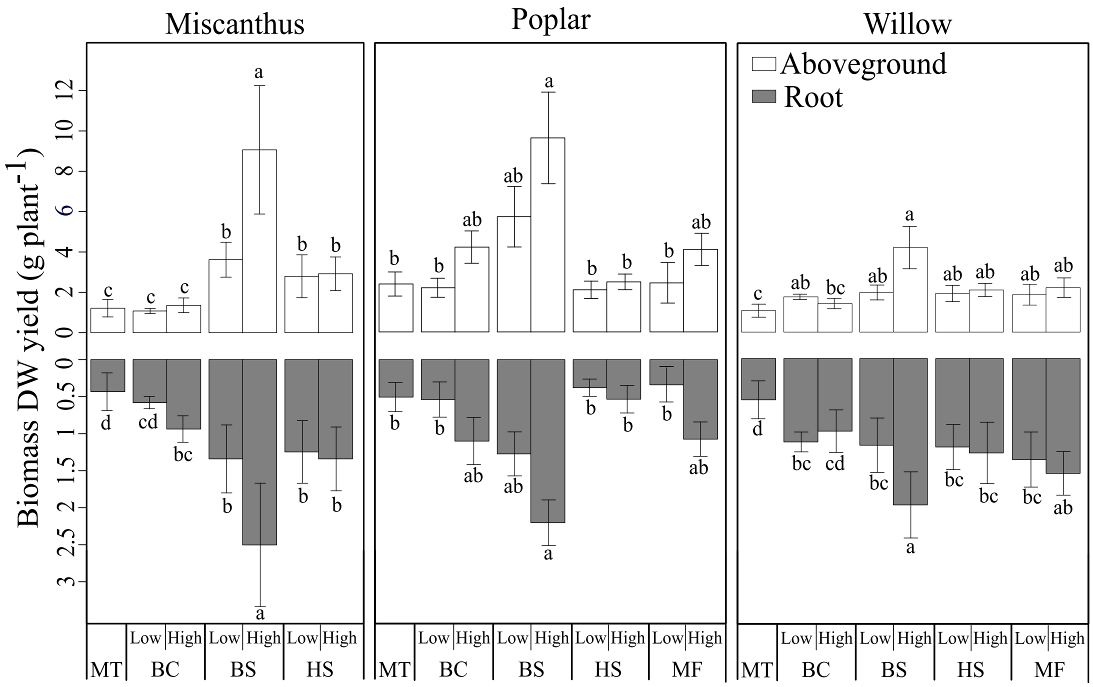

Figure 3. Shoot and root DW yields (g DW plant^{-1}) of willow, poplar and miscanthus grown on mine tailings (MT) treated with biochar (BC), biosolids (BS), humic substances (HS), and mycorrhizal fungi (MF) at low and high application rates. Values are means ± SD per treatment (For willow and poplar: MT n=34, and BC, BS, HS, MF, n=9; for miscanthus: MT n=6 and BC, BS, HS, n=6, with some losses due to mortality). Values with different letters differ significantly (one way ANOVA or Kruskal-Wallis test, p-value < 0.05).

3.2.2. Effect of Biochar. Similarly, biochar addition resulted in significant increases in willow aboveground and root biomass at the low application rate (81% increase in total biomass) (Figure 3). However, increases in root and aboveground biomass in the BCH treatment were not significant. Biochar amendment had no significant effect on poplar and miscanthus aboveground biomass, but significantly increased miscanthus root biomass (Figure 3). Although BC addition induced great improvement in most tailings properties, biomass increases in BC treatments was less pronounced. Macronutrient availability such as N and P was most likely the limiting

factor in these treatments (Table 2). Biochar application tends to increase C/N ratio, thus slowing the decomposition of OM matter present in soil and lowering organic N and P transformation to plant-available forms. Chan et al. (2007) observed no significant effect on radish biomass when BC applied in the absence of N fertilizer even at high rate (100 t ha^{-1}); however, a considerable increase in biomass was observed when N fertilizer added and a significant BC × N fertilizer interaction was found, suggesting that BC improved N fertilizer use efficiency.

3.2.3. Effect of Mycorrhizal Fungi and Humic Substances. Humic substances application significantly increased both aboveground and root biomass of willow and miscanthus but had no significant impact on poplar (Figure 3). The HSH resulted in 85% and 99% increases in total biomass of willow and miscanthus, respectively (Figure 3). Amending tailings with MF resulted in a significant increase in willow aboveground and root biomass with 128% (MFL) and 156% (MFH) increase in total biomass. Mycorrhizal fungi treatments did not significantly increase poplar root and aboveground biomass (Figure 3). Although nutrient concentrations were low in tailings amended with HSL and MFL compared to BC treatment (Table 2), the increase in plant biomass was comparable to BC, and in some cases even higher. This positive impact might be partially explained by the effect of these amendments on plant physiology and metabolism. In addition to their role in improving soil fertility, HS can alter plant root architecture as they induce root hair formation and lateral root development, thus increasing root surface area and subsequently improving nutrient and water acquisition (Trevisan et al. 2010; Canellas and Olivares 2014). Furthermore, HS can chelate and mobilize certain elements, such as Fe, which is deficient in these tailings (Chen et al. 2004). Similarly, the fine hyphae that are

typically extended from root-colonizing MF could have enhanced plant growth by increasing root surface absorbing area. These hyphae are also able to explore places that are not accessible to plant roots, providing plants with more water and nutrients, especially P which is deficient in these tailings (Smith et al. 2003). Moreover, the plant growth improvements observed might also be ascribed to the positive effect MF has on increasing plant tolerance to trace elements, potentially due to detoxification by binding metals with chitin and melanin that are present in cell walls of MF (Eisenman and Casadevall 2012; French 2017).

4. CONCLUSIONS

Significant improvements of tailings fertility, hydraulic properties, and plant productivity were observed for BC, BS, and HS. The improvement was the greatest with BS addition. Adding BC positively impacted most of tailings fertility properties and greatly enhanced water retention and Ks. However, its impact on biomass production was less pronounced, likely due to limited N and P content. In spite of the low application rate, HS improved tailings properties and enhanced willow and miscanthus productivity. Mycorrhizal fungi positively improved plant growth; however, its impacts on tailings properties were not significant. Findings from this study could serve as a starting point for investigating synergistic effects of combined amendments of BC, HS, and MF with BS. A greenhouse pot experiment is underway.

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II. PHYTOMANAGEMENT OF LEAD/ZINC/COPPER TAILINGS USING BIOSOLIDS-BIOCHAR OR -HUMUS COMBINATIONS: ENHANCEMENT OF BIOENERGY CROP PRODUCTION, SUBSTRATE FUNCTIONALITY AND ECOSYSTEM SERVICES

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ABSTRACT

The extreme physiochemical characteristics of mine tailings generally prohibit microbial processes and natural plant growth. Consequently, vast and numerous tailings sites remain barren for decades and highly susceptible to windblown dust and water erosion. Amendment-assisted phytostabilization is a cost-effective and ecologically productive approach to mitigate the potential transport of the residual metal content. Due to the contrasting and complementary characteristics of biosolids (BS) and biochar (BC), co-application might be more efficient than individually applied. Studies considering BS and BC co-application for multi-metal tailings revegetation are scarce. As tailings revegetation is a multidimensional issue, clearly notable demand exists for a study that provides a comprehensive understanding on the co-application impact on interrelated properties of physicochemical, biological, mineral nitrogen availability, metal immobilization, water-soil interactions, and the impacts on plant cultivation and biomass

production. This 8-month greenhouse study aimed at investigating the efficacy of co-application strategies targeting BS and carbon-rich amendments (i.e., BC or humic substances (HS)) to phytomanage a slightly alkaline Pb/Zn/Cu tailings with bioenergy crops (poplar, willow, and miscanthus). A complementary assessment linking revegetation effectiveness to ecosystem services (ES) provision was also included. Owing to their rich nutrient and organic matter contents, BS had the most pronounced influence on most of the measured properties including physicochemical, enzyme activities, NH_4^+-N and NO_3^--N availability, immobilization of Zn, Cu, and Cd, and biomass production. Co-applying with BC exhibited efficient nutrient release and was more effective than BS alone in reducing metal bioavailability and uptake particularly Pb. Poplar and willow exhibited more superior phytostabilization efficiency compared to miscanthus which caused acidification-induced metal mobilization, yet BC and BS co-application was effective in ameliorating this effect. Enhancement of ES and substrate quality index mirrored the positive effect of amendment co-application and plant cultivation. Co-applying HS with BS resulted in improved *nutrient cycling* while BC enhanced *water purification* and *contamination control* services.

1. INTRODUCTION

Mining activities have left an important legacy of abandoned and disturbed lands with large area accounting for tailings deposit (Karaca et al., 2018). Mine tailings are the materials remaining after extraction and beneficiation of ores. In general, they are characterized by low water retention capacity, deficiencies in nutrients and organic matter

(OM), limited cohesion, unfavorable texture and structure and high concentrations of metal(oid) (Wang et al., 2017). Even if they suffer from mechanical, chemical, physical and biological limitations (Li and Huang, 2015) they are accepted as soils and defined as Technosols by the World Reference Base for Soil Resources (IUSS, 2006): "*soils dominated by human-made material, whose properties and pedogenesis are dominated by their technical origin*". Mine tailings unfavorable characteristics make them unsuitable substrate that limits the development of plants, animal, and microorganisms (Wang et al., 2017).

Phytotechnologies are sustainable remediation techniques that can be carried out *in situ* to ameliorate these properties. Among them, (aided) phytostabilization which aims at using plants, their associated microorganisms and soil amendments to retain contaminant linkages through the environmental compartments and the food chain (Kidd et al., 2015). The development of a vegetative cover can provide a range of benefits, such as controlling soil erosion and particle dispersion, limiting water run-off and/or leaching and creating a habitat for soil micro and macrofauna (Mendez and Maier, 2008). An adequate plants and waste materials selection, based on the needs of the impaired technosols, could accelerate soil formation, improve its ecological functions which ultimately enhance ecosystem services (ES) and will help the mine area to return to a productive and healthy status (Mench et al., 2010).

In the past decade, phytotechnologies have evolved into a more comprehensive concept 'the phytomanagement'. This new sustainable concept encompasses the ecological rehabilitation of contaminated soils with the production of valuable biomass resulting in a financial return (Evangelou and Deram, 2014). The selected species for the

phytomanagement of contaminated sites has to be tolerant to soil contamination, characterized by an excluder phenotype (i.e., phytostabilization potential) and has an economical value (Kidd et al., 2015; Mench et al., 2010; Vangronsveld et al., 2009). Fast growing species such as short rotation coppice (willow and poplar) and perennial grass (miscanthus) are great choices for tailings revegetation and biomass production due to their low nutrient requirements, tolerance to excess metal, high biomass production rate, and their dense deep-rooted system which can prevent wind and water erosion (Al-Lami et al., 2019; Guittonny and Lortie, 2017; Wang et al., 2021a). According to Li and Huang (2015), considering fast growing species over other plant species is of great importance especially at the early stages of phytostabilization due to the fast input of OM which is essential to buildup biocapacity of tailings.

Using appropriate waste materials rich in OM as an amendment can contribute to reverse the severe degradation of mine tailings through: increasing nutrient status, OM content and water holding capacity (WHC); improving soil structure, quality and fertility; and immobilizing toxic metal(oids) (Kumpiene et al., 2008). Among these materials, biosolids (BS), owing to their rich OM content and a wide range of readily available nutrients, have shown promise to ameliorate multiple characteristics of technosols and thus enhancing tailings revegetation (Al-Lami et al., 2021; Al-Lami et al., 2019; Gardner et al., 2010; Harris et al., 2021; Silveira et al., 2003; Wijesekara et al., 2016). An increased number of studies have also reported the BS ability to provide metal(oids) binding through sorption and complexation processes, thus inducing metal immobilization in tailings (Brofas et al., 2000; Ciarkowska et al., 2017; Jones et al., 2011; Mingorance et al., 2014). However, due to the severe degradation of mine tailings, higher

BS application rates are generally needed to support sustained vegetation (Brown and Henry, 2001). As such, this may cause some side effects of potential input of contaminants both organic and inorganic which may represent an immediate threat to the surrounding environments through nutrient lability, notably N and P (Lu et al., 2012). Rapid decomposition of OM in BS is another issue accounting for possible revegetation failure as their overall supporting benefits and metal immobilization efficacy may dramatically decline before the established vegetation and soil microbiota reach a self-sustaining point that is necessary for ecosystem diversity and productivity (Nason et al., 2013). Moreover, transport cost may prohibit the high BS application rates particularly for large scale tailings since these sites are usually located in remote areas. Thus, when dealing with tailings reclamation, strategies that target co-application of amendments can be more efficient than applying BS alone (Asemaninejad et al., 2021; Brown et al., 2003), in particular incorporation of carbon-rich and recalcitrant waste byproducts.

Biochar (BC) is the product of thermal degradation of organic materials in the absence of oxygen (i.e., pyrolysis) (Lehmann et al., 2011). Biochar is characterized by highly recalcitrant carbon with unique physical, chemical, and biological properties (Joseph et al., 2010). The high pore volume and surface area with abundant and diverse functional groups makes BC rich in both cation and anion exchange capacity (CEC and AEC) with unique sorption characteristics (Paz-Ferreiro et al., 2014). Thus, BC has been used in soil reclamation and remediation efforts of different toxic metals (Wang et al., 2021b; Wang et al., 2018; Zhang et al., 2013). Similarly, Humic substances (HS) is another carbon-rich and highly recalcitrant amendment. Humic Substances are a heterogeneous mixture of organic compounds formed during the humification of OM by

soil microorganisms (Stevenson, 1994). Humic substances can increase plant nutrient availability, WHC, CEC, pH buffering capacity, and microbiological activity (Khaled and Fawy, 2011; Pettit, 2004). In contaminated soils, it may help mitigate metals (phyto)toxicity by complexing them with organic ligands such as humic and fulvic acids (Kochany and Smith, 2002). Additionally, due to their molecular complexity and interactions with soil mineral phases, these HS materials are very stable against microbial decomposition and thus can improve long-term C sequestration in soil (Stevenson, 1994; Trevisan et al., 2010).

Despite the unique sorption characteristics of BC and HS, applying these amendments singularly unfortunately is not sufficient to support adequate plant growth on mine tailings due to deficiency in essential nutrients (Al-Lami et al., 2019; Fellet et al., 2014; Szczerski et al., 2013). Most of studies in literature have focused on the impact of such amendments, particularly BC, on tailings physicochemical and geochemical properties, mainly metal immobilization, while ignoring the primary goal which is the enhancement of a sustained vegetation that is necessary to gradually restore the degraded ecosystem of tailings and sustain soil properties restoration (Beauchemin et al., 2015; Fellet et al., 2011). While literature has shown that amending tailings with BC will inevitably improve physicochemical properties and reduce metal bio(availability), strategies to effectively employ this unique amendment to achieve the primary goal of enhanced tailings revegetation remain to be explored. One recommended strategy can be through co-applying or co-composting such amendments with organic- and nutrient-rich amendment such as BS owing to their contrasting and complementary characteristics (Antonangelo et al., 2021). Co-application of BS with carbon-rich amendment such as

HS or BC may adjust C/N ratio which plays a key role in divergence of microbes or their enzyme activities thus reducing OM decomposition rates (Dodor et al., 2018; Riaz et al., 2017; Shanmugam et al., 2021). Incorporating with BC may also contribute to increasing carbon sequestration through negative priming effect on BS-OM and newly added biomass due to its high sorption capacity and lowering the microbial mineralization rates (DeCiucies et al., 2018; Han Weng et al., 2017). Furthermore, the excess nutrients associated with high BS application rates especially at early stages can be adsorbed on BC surfaces or assimilated into microbial biomass (Page-Dumroese et al., 2018). However, there is a dearth of research examining the synergistic effect of co-application of BS and BC for revegetation of multi-metal mine tailings. Penido et al. (2019) investigated the benefits of combining BC and sewage sludge on immobilization of metals in mining soils, yet their study was a relatively short-term (4-weeks) and only focused on metals while ignoring biophysicochemical and nutrients status. Thus, clearly notable demand exists for a relatively longer-term study to evaluate the efficacy of this approach to phytostabilize multi-metal tailings and provide a comprehensive understanding on its impact on biophysicochemical properties, mineral nitrogen availability, metal immobilization, and the interaction with plant species cultivation.

Phytostabilization of tailings with bioenergy crops assisted with organic waste byproducts is a potential option to produce a valuable biomass while enhancing tailings functionality and ES. Therefore, we investigated the impact of application of BS, alone or combined with BC or HS to a slightly alkaline Pb/Zn/Cu mine tailings, unplanted or cultivated with either poplar, willow or miscanthus. This was tested through: (1)

metal(loid)s and nutrient bioavailability; (2) biomass production; (3) shoot metal(loid)s uptake; and (4) tailings functionality and ES.

2. MATERIAL AND METHODS

2.1. MINE TAILINGS, BIOSOLIDS (BS), BIOCHAR (BC), AND HUMIC SUBSTANCES (HS)

The mine tailings were sampled from a Pb/Zn/Cu mine tailings impoundment, located at Viburnum MO, US (37°42’07.1"N; 91°06’22.3"W). The tailings impoundment (~110 ha) was used continuously as an opencast discharge for disposing of mine wastes for about 41 years. For more details on the tailings impoundment refer to Al-Lami et al. (2019). Tailings were sampled in the 0–30 cm layer and transported to the greenhouse, where they were air-dried, crushed and sieved to 2-mm, and manually homogenized.

The aerobically/anaerobically digested BS were obtained from the Southeast Wastewater Treatment Plant, Rolla, MO. They were air dried to approximately 40% solid content, thoroughly mixed and homogenized. These BS meet U.S. EPA Class B standards for land application, more characteristics are presented in Table 1. The BC was a commercial product derived from pine chips (Range Fuels Company, Soperton, GA). This BC characterized by alkaline pH equal to 8.6 and high surface area 310 m^2 g^{-1} and pore volume 0.12 cm^3 g^{-1} with high carbon content (Table 1). More characteristics on this BC can be found in Al-Lami et al. (2019). Humic substances were a granular form of cultured humus concentrate containing humic acids, fulvic acids, and humin fractions, and some beneficial microorganisms, and characterized by acidic pH and high OM and

CEC (Table 1). This amendment was a commercial product provided by Soil Secrets, LLC., Los Lunas, NM.

Table 1. Physicochemical characterization and main properties of mine tailings and amendments.

Parameter	**Mine tailings (Unt)**	**Background levels**[a]	**Biosolids (BS)**	**Biochar (BC)**	**Humic substances (HS)**
pH (H_2O)	7.8	> 6.1[b]	6.4	8.6	4.1
EC (mS cm^{-1})	0.4	-	-	0.1	-
CEC ($cmol^{+}$ kg^{-1})	3.6	5-25[c]	41.3	8.1	62.7
OM (%)[d]	0.6	-	57.6	-	58.9
Total C (%)	7.3	1.3	-	82.6	-
Organic C (g kg^{-1})	0.5	-			
Total N (mg kg^{-1})	600	-	66500	3180	9800
NH_4^{+}–N (mg kg^{-1})	1.7	-	8220	-	-
NO_3^{-}–N (mg kg^{-1})	0.5	-	69.1	-	-
Total P (mg kg^{-1})	87.1	-	14200	420	380
P-Olsen (mg kg^{-1})	3.0	-	-	-	-
Elements (mg kg^{-1})					
Pb	3553	20	31.5	23.3	-
Zn	966	49	735	-	-
Cu	479	13	522	-	-
Cd	13.7	<1	-	0.2	-
Ni	70.7	14	22.4	59.6	-
Cr	11.5	54	24.6	9.2	-

[a] Background concentration for metals in Missouri soils (Tidball 1984)

[b] Summary of soil fertility status in Missouri (Nathan et al. 2007)

[c] Scrivner and Cooper 1985

[d] Loss on Ignition (LOI).

2.2. POT EXPERIMENT

Cylindrical plastic pots were filled with 3.5 kg of untreated tailings or mixed substrate (i.e., treated tailings). The primary amendment was BS at low 3% or high 5% application rate, which is equivalent to about 60 and 100 dry ton per hectare, respectively. Substrates were prepared by treating tailings with BS alone or a blend of BS plus BC or HS, thus resulting in seven different treatments as listed below:

1. Untreated mine tailings (Unt)
2. Tailings + 3% biosolids (LBS)
3. Tailings + 3% biosolids + 3% biochar (LBS+BC)
4. Tailings + 3% biosolids + 0.3% humic substances (LBS+HS)
5. Tailings + 5% biosolids (HBS)
6. Tailings + 5% biosolids + 3% biochar (HBS+BC)
7. Tailings + 5% biosolids + 0.3% humic substances (HBS+HS)

The amendment application rates were based on our previous study Al-Lami et al. (2019). To ensure homogeneity, thorough mixing of tailings and amendments was done individually for each pot. Six replicates were prepared for each treatment. Prepared pots were arranged in a randomized block design under controlled conditions in a greenhouse located at Missouri University of Science and Technology, Rolla, MO. Before planting, amendments were allowed to react for a 4-week period with the tailings.

Three biomass crops were cultivated in this study: miscanthus (*Miscanthus x giganteus*), laurel leaf willow (*Salix pentandra*), and hybrid poplar DN34 (*Populus deltoids_Populus nigra*, DN34). Miscanthus rhizomes were collected from a 4.45 ha pilot-scale trial implemented in 2011 in the tailings impoundment in Viburnum, MO,

USA, and planted directly in the prepared pots. Whereas, willow and poplar cuttings (roughly 20 cm long) were collected in a park (Rolla, MO, USA), and pre-rooted with tap water prior to planting. Six replicates were prepared for each treatment and plant species. In addition, another set of replicated pots was included in the experiment to serve as references of unplanted treatments. Plants were cultivated under controlled conditions in a greenhouse. Planted and unplanted pots were regularly watered, and shallow trays were placed underneath each pot to capture any leachate.

2.3. PLANT TISSUE AND SUBSTRATE SAMPLE COLLECTION

After an 8-month growth period, plants were harvested and the leaves, stems and roots of willow and poplar were collected by separating new branches from the old cuttings, whereas miscanthus was collected by separating the shoots and roots from the rhizomes. Two different samples of plant leaves were collected representing upper (i.e., new) and lower (i.e., old) growth. Plant samples were then extensively washed with tap water to remove any adhering substrate particles, and subsequently rinsed with deionized water thrice. Sampled materials were placed in paper bags and oven dried at 60 °C until constant weight, and then weighed to determine the shoot and root dry weight (DW) yields.

For microbial enzyme activity measurements, fresh rhizosphere and bulk substrate samples were collected. The rhizosphere sample was collected by vigorously shaking the root system to collect substrate particles closely adhering to the roots (Luster et al., 2006). Another homogenous subsample was collected to represent a bulk substrate.

Subsamples were also collected, air-dried, passed through a 2-mm sieve, and stored for physicochemical and metal(loid) determination.

2.4. PLANT TISSUE DIGESTION AND IONOME ANALYSIS

Leaf samples were ground to < 1 mm particle size, and subsamples of 0.5 g were hot block digested with 5 mL of concentrated HNO_3 and 3 mL of 30% H_2O_2 (Huang and Schulte, 1985). A certified reference material (WEPAL IPE-126; Zea mays) was also routinely included during the digestion. Digested samples were analyzed for Cd, Cu, Fe, Mn, Ni, Pb, and Zn using ICP-MS (Perkin Elmer, NexION 300/350), and Ca, K, Mg, and P using ICP-OES (Perkin Elmer, Avio 200). In addition, subsamples of homogeneous plant material were analyzed for total N using CHN elemental analyzer (Perkin Elmer 2400 CHN analyzer).

2.5. SUBSTRATE PHYSICOCHEMICAL AND BIOLOGICAL ANALYSIS

2.5.1. Physicochemical Parameters Determination. Substrate pH and EC were measured in a substrate/MΩ water (1:2.5; *w/v*) suspension using digital meters (Orion 370, Thermo Fisher Scientific, Inc; VWR, 61161-362, respectively). Elemental analyzer was used to determine total carbon (TC) and total nitrogen (TN) in substrate samples. Organic carbon (OC) was determined using dichromate wet oxidation Walkley-Black method (Page, 1982). Olsen P was extracted using 0.5 M $NaHCO_3$, pH 8.5 solution according to Pierzynski (2000), extracts were then treated with charcoal, filtered, acidified and analyzed using ICP-OES (Pierzynski, 2000). Substrate NH_4^+-N and NO_3^--N were extracted using 2 M KCl and analyzed by flow injection autoanalyzer (Soil and

Plant Testing Laboratory, University of Missouri Extension, Columbia, MO). Mineral N was calculated by adding extractable NH_4^+-N and NO_3^--N according to Keeney et al. (1983). For available K determination, 0.1 M $BaCl_2$ extracting solution was used, compulsive exchange method was then followed for CEC determination (Gillman and Sumpter, 1986).

2.5.2. Heavy Metal Bioavailability Determination. The readily available fraction of heavy metals was extracted with 1 M NH_4NO_3 solution at 1:2.5 (*w/v*) ratio according to Gryschko et al. (2005). The samples were mechanically shaken for 2 h, supernatant solution was then filtered through 0.45 µm and the filtrate was acidified before analysis for Cu, Mn, Ni, Pb, and Zn using ICP-OES while Cd was analyzed using GFAA (Perkin Elmer AAnalyst 600).

For all the measured analytes, high purity reagents and standards were used, and quality control (QC) protocols were followed. More details on QC procedures along with the metal(loid)s detection limits can be found in Al-Lami et al. (2021).

2.5.3. Microbial Enzyme Activity Measurement. Dehydrogenase enzyme activity (DHA) in the fresh bulk and rhizosphere substrate samples was determined colorimetrically by the method of Casida Jr et al. (1964). Briefly, 2,3,5-triphenyltetrazoliumchloride (TTC) solution was added as an electron acceptor and samples were incubated for 24 h at 37 °C. At the end of the incubation period, the triphenyl formazan (TPF) produced by the reduction of TTC was extracted with methanol and the intensity of coloration in the extracts was measured spectrophotometrically at 485 nm wavelength (Varian Cary 50 Scanning UV/Vis Spectrophotometer). The DHA

activity was expressed as milligrams of TPF per kilogram dry soil per 24 h (mg TPF kg^{-1} $24h^{-1}$).

2.6. ECOSYSTEM SERVICES (ES) AND SUBSTRATE QUALITY INDEX (SQI) CALCULATION

Data on physicochemical and biological parameters along with heavy metal bioavailability were used to determine the provision of ES according to Burges et al. (2016) who proposed to group such parameters in higher-level categories (i.e., ecosystem attributes) using the treated-soil quality index (T-SQI) proposed by Mijangos et al. (2010), and represented by the following equation:

$$ES = 10^{\log m + \frac{\sum_{i=1}^{n}(\log n_i - \log m)}{n}}$$

where *m* is the control (i.e., mean value of untreated mine tailings, set to 100%) and *n* corresponds to the measured value for each parameter as a percentage of the control. This T-SQI is suitable for those cases where soil is intentionally treated to enhance soil quality indicators, as in the present study. Moreover, unlike other indices (Bloem et al., 2005), evaluation of the treatment effects using this index is not limited to the magnitude of the change but also takes into consideration its direction whether increased or decreased (Epelde et al., 2014b). Using this index and following Burges et al. (2016), substrate physicochemical and biological parameters along with heavy metal bioavailability were grouped within relevant ES as follows: (1) primary production: above-ground plant biomass; (2) carbon storage: TC, OC, and biomass; (3) contamination control: NH_4NO_3-extractable metal concentrations and metal accumulation in plant tissue ; (4) fertility maintenance: pH, OC, CEC, TN, Olsen P, available K, nitrate, ammonium, and mineral

N; (5) water purification: NH_4NO_3-extractable metal concentrations; and (6) nutrient cycling: bulk and rhizosphere DHA. Finally, the overall substrate quality index (SQI) was calculated by taking the arithmetic mean of these six ES.

2.7. STATISTICAL ANALYSIS

Influence of treatments on substrate physicochemical properties, plant DW yields, leaves ionome, microbial enzyme activity, ES and SQI were tested using one-way analysis of variance (ANOVA). All data were checked for normality and homoscedasticity. Data not meeting these assumptions were normalized by log transformation. Multiple comparisons of mean values were made using post-hoc Tukey HSD tests. Differences were considered statistically significant at $p < 0.05$. In addition, a principal component analysis (PCA) was conducted on substrate properties and plant biomass to assess the multivariate effect of tested treatments and the correlations between variables. To evaluate the effect of different crop cultivation compared to unplanted substrates, a PCA-biplot was also generated. All statistical analyses were performed using R software (version 3.6.2).

3. RESULTS AND DISCUSSION

3.1. TAILING'S CHARACTERISTICS, IMPORTANCE OF AMENDMENT CO-APPLICATION AND MULTIVARIATE ASSOCIATIONS

The tailings studied here are considered typical of many other mine impoundments in the region (Johnson et al., 2016). These tailings consisted of 40% sand, 50% silt, and 10% clay (Al-Lami et al., 2019), and characterized by very fine texture, low

OM and nutrient content, high concentrations of heavy metals notably for Pb, Zn, Cu, and Cd with a slightly basic pH 7.8, as shown in Table 1. Due to their extreme biophysichochemical characteristics, these tailings have not supported natural vegetation and remained barren for decades. A previous greenhouse study on these tailings, where BS, BC, or HS was applied singularly for potential to improve tailings characteristics and support biomass crop production (Al-Lami et al., 2019), guided the design of this study, choice of amendments and treatment combinations. In Al-Lami et al. (2019), the most pronounced impact on tailings physicochemical and hydraulic properties along with biomass production was observed under BS treatments compared to BC or HS which also positively impacted tailings characteristics yet to lesser extent, and their impact on biomass was negligible particularly BC, mainly due to macronutrient deficiency. Nevertheless, BS alone may not support sustained vegetation due to their nutrient lability and rapid decomposition as mentioned above (Brown et al., 2003; Nason et al., 2013). As such, BS was considered as a primary amendment in this study and role of co-applying with BC and HS as additives to enhance BS effectiveness and increase success of revegetation efforts was outlined and extensively discussed in the subsequent sections.

To assess the relationships between the large number of substrate parameters in the seven tested treatments and biomass production for each three cultivated species, PCA plots were generated as shown in Figure 1. With miscanthus cultivation, the first two axes of the PCA together explained 84.2% of the total variance. The first axis (58.4%) opposed the untreated tailings, with high ratio of C/N and NH_4NO_3-extractable Zn, Cu, and Cd, to other treated tailings substrates which positively correlated to a cluster of physicochemical properties, nutrient availability, enzyme activity (noted as DHA_{bulk}),

and biomass. The second axis (25.8%) separated combined treatments of BS and BC, which characterized by high pH and low NH_4NO_3-extractable Pb, Ni, and Mn, from other BS treatments (Figure 1a). Poplar and willow cultivation exhibited similar trend. The first axis (61.4% and 59.2%, poplar and willow, respectively) separated the untreated tailings, with high ratio of C/N and NH_4NO_3-extractable Zn, Cu, and Cd, from other treated substrates which positively correlated to a cluster of physicochemical properties, nutrient availability, and biomass. The second axis (20% and 24.3%, poplar and willow, respectively) opposed BS and BC combined treatments, with high pH and low enzyme activity, low extractable nitrate, and low NH_4NO_3-extractable Pb, Ni, and Mn, to the other singular BS treatments or BS and HS combinations (Figures 1b and c). Overall, PCA results indicated that BS amendment application played a principal role in enhancing the physichochemical and biological properties of the tailings and providing essential nutrients while inducing metal immobilization particularly Zn, Cd, and Cu. On the other hand, BC amendment as an additive played a principal role in carbon sequestration and inducing further immobilization for all tested metals notably Mn, Ni, and Pb.

3.2. EFFECTS OF TREATMENTS AND PLANT CULTIVATION ON PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

3.2.1. Physicochemical Parameters. The untreated mine tailings exhibited extremely poor characteristics for all tested physicochemical properties **(**Table 2). After 8 months, the untreated and unplanted tailings had a slightly alkaline pH (7.68 ± 0.13). Biosolids treatment significantly decreased pH to 7.45 ± 0.06 and 7.38 ± 0.06 under low and high application rates, respectively.

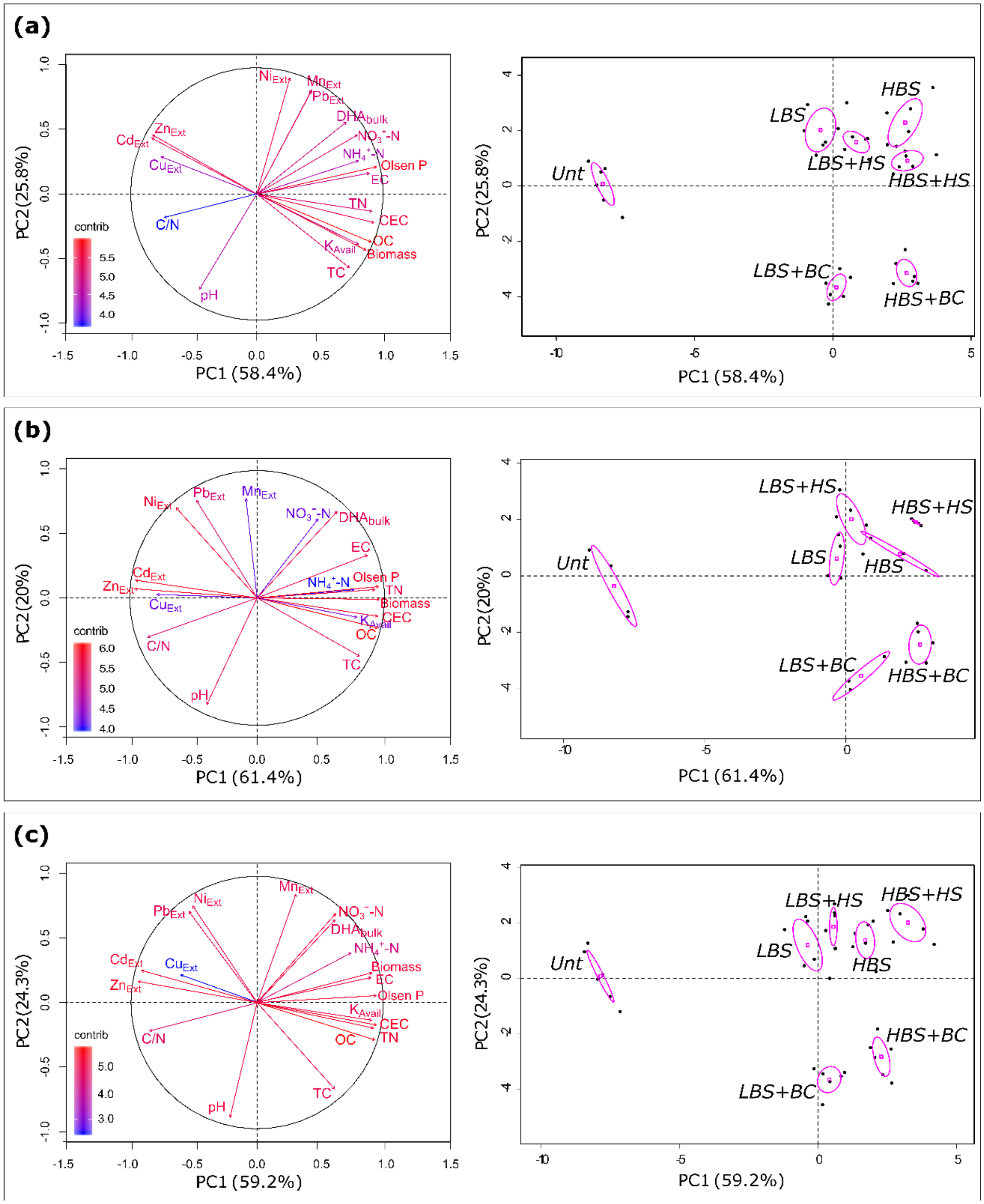

Figure 1. Principal component analysis (PCA) on plant biomass and substrate properties after 8-months of plant cultivation of (a) miscanthus, (b) poplar, and (c) willow in: mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Parameters included in the PCA are: plant biomass, physicochemical properties (pH, EC, CEC, OC, TC, TN, C/N, NH_4^+-N, NO_3^--N, Olsen P, available K), NH_4NO_3-extractable metals (Pb, Zn, Cu, Cd, Mn, and Ni), and biological enzyme activity in bulk substrate (DHA_{bulk}).

The BS-induced pH decrease was likely due to OM decomposition and N mineralization processes which are associated with the formation of organic acids and release of H^+ ions (Hossain et al., 2011). Further reduction in pH was observed when BS was applied in combination with HS, however the reduction was statistically insignificant. In contrary, significant increases in pH were observed in BS and BC combinations compared to BS alone, and the resulting pH was comparable or higher than pH of untreated tailings when BC blended with the lower rate of BS (Table 2). The BC-induced pH increases were attributed to the liming effect of the hardwood BC due to its high inherent pH and contents of base cations and calcium carbonates (Shetty and Prakash, 2020). After 8 months of plant growth, the trend in treatment effect on pH as induced by amendment addition was similar irrespective of cultivated species. However, compared to uncultivated substrates, miscanthus cultivation induced a significant acidification effect whereas poplar and willow induced alkalinization, albeit this effect was insignificant (Table 2). The contrasting effect of species cultivation on pH could be attributed to the complex rhizosphere processes driven by plant-soil interactions including the release of root exudates with different composition and ionic forms (Hinsinger et al., 2003). Consequently, leading to acidification through release of H^+ for increased nutrient uptake as observed in miscanthus species, or alkalinization through release of OH^- as a defense mechanism to alleviate metal toxicity as observed in poplar (Blossfeld et al., 2010). Similar acidification effect by *Miscanthus x giganteus* cultivated on slightly alkaline multi-metal contaminated soil was reported by Nsanganwimana et al. (2021). Whereas, Foulon et al. (2016) reported an increase in pH (7.22 ± 0.08 to 7.4 ±

0.00) as an effect of poplar cultivation used for phytomanagement of multi-metal contaminated soil.

Except for TC, the enhancement in other parameters (i.e., EC, CEC, OC, TC, TN, Olsen P, and available K) was largely affected by the BS addition and proportionally increased with increasing the application rate of BS (Table 2). Ciarkowska et al. (2017) reported similar improvements in physicochemical parameters of slightly alkaline multi-metal tailings after 3-years of sewage sludge treatment. Irrespective of plant cultivation, the addition of BC or HS with the BS resulted in further improvement in CEC and OC, although the effect on OC was statistically significant ($P < 0.05$) only with BC addition **(**Table 2). Regarding influence of plant cultivation, miscanthus surprisingly induced a significant reduction in OC compared to unplanted substrates except in treatment when BC added with the lower rate of BS. These results contradict those reported by Al Souki et al. (2017) where miscanthus cultivation resulted in a remarkable increase in soil OC and CEC, however their experimental setting was longer (1-year) and involved metal contaminated soil without biosolids addition or any other amendment. A possible explanation could be the positive priming effect on BS-OM induced by miscanthus root exudates. In a 6-year study where land use changed from C3 grassland to C4 miscanthus, Zatta et al. (2014) used stable carbon isotopes to provide an evidence for positive microbial priming effect stimulated by fresh belowground miscanthus biomass leading to faster decomposition of native OC, however C4 miscanthus carbon eventually replaced the initial C3 grassland carbon.

No significant effect of additives HS or BC was observed on TN indicating that this parameter is solely influenced by the input from the BS application. However, the

highest levels of TN were measured in unplanted substrates and plant cultivation significantly decreased TN concentrations in the following order: miscanthus < willow < poplar (Table 2). Similar trend was observed with Olsen P and available K with a few exceptions where additive BC, especially when added to the lower rate BS, which resulted in a decrease in Olsen P and an increase in available K. The untreated tailings exhibited high content of TC which is mainly representing inorganic carbon fractions associated with the carbonate-bearing minerals in these tailings (Seeger et al., 2008). Biosolids application also increased TC in amended tailings, but the notable effect on this parameter was mainly associated with the BC additive application which is likely an effect of the carbon-rich hardwood BC used in this study (Tables 1 and 2).

3.2.2. Dehydrogenase Enzyme Activity (DHA). Microorganisms are an integral component of all soil ecosystems which is involved in many important biogeochemical processes associated with: OM mineralization, humus formation, nutrient conversion and cycling, toxicity detoxification, etc. (Bertrand et al., 2015). Enzymatic activities may serve as important ecological indictors of soil quality, specifically the status of microbial community and its functional diversity. Among others, dehydrogenase is an oxidoreductase enzyme and can serve as an important biological indicator of overall soil microbial activity since they are intracellular enzymes associated with all living microbial cells (Wolińska and Stępniewska, 2012). The untreated tailings exhibited extremely low DHA in both bulk and rhizosphere substrates regardless of plant cultivation (Figure 2), this further indicates that the high content of TC measured in these tailings (Table 1) is inert and mainly associated with inorganic carbon that does not support microbial growth.

Table 2. Main properties and physicochemical parameters of substrates 8-months after amendment and plant cultivation.

		μS cm^{-1}	cmol kg^{-1}	%			mg kg^{-1}	
	pH	EC	CEC	OC	TC	TN	Olsen P	Available K
Miscanthus								
Unt	7.49 ± 0.09abB	389 ± 44eA	2.9 ± 0.2dA	0.44 ± 0.07dA	8.5 ± 0.6dA	0.03 ± 0.01dB	2.3 ± 0.2dC	12 ± 1dB
LBS	7.21 ± 0.06cB	777 ± 98dA	5.0 ± 0.3cA	1.37 ± 0.11cB	11.2 ± 0.4cA	0.15 ± 0.03cC	31.7 ± 2.6bcC	19 ± 2cC
LBS+BC	7.60 ± 0.13aB	738 ± 71dA	6.0 ± 0.4bA	1.94 ± 0.12bB	14.9 ± 0.5aA	0.15 ± 0.02cB	27.4 ± 3.6cC	23 ± 2aC
LBS+HS	7.17 ± 0.11cB	804 ± 62cdA	5.9 ± 0.4bA	1.50 ± 0.09cB	12.1 ± 1.0bcA	0.13 ± 0.03cC	35.0 ± 2.5bC	19 ± 2bcD
HBS	7.10 ± 0.09cB	1060 ± 103abA	6.1 ± 0.3bB	1.82 ± 0.06bB	12.5 ± 0.8bcA	0.20 ± 0.03abC	43.6 ± 4.7aC	22 ± 1abC
HBS+BC	7.42 ± 0.12bB	941 ± 56bcA	7.1 ± 0.3aB	2.28 ± 0.18aB	15.2 ± 0.7aA	0.22 ± 0.03aC	35.6 ± 2.6bC	24 ± 1aC
HBS+HS	7.07 ± 0.08cB	1128 ± 80aA	7.0 ± 0.2aA	1.97 ± 0.12bB	12.6 ± 1.2bA	0.19 ± 0.02bB	45.3 ± 2.7aC	20 ± 1bcC
Poplar								
Unt	7.80 ± 0.07abA	464 ± 36dA	3.0 ± 0.3cA	0.45 ± 0.03dA	8.6 ± 1.1dA	0.03 ± 0.01dB	2.7 ± 0.4cBC	14 ± 2bB
LBS	7.58 ± 0.10bcdA	894 ± 89bcA	5.3 ± 0.4dA	1.67 ± 0.10cA	12.7 ± 0.9cA	0.21 ± 0.04cAB	41.3 ± 2.7bB	22 ± 2aBC
LBS+BC	7.89 ± 0.16aA	817 ± 97cA	6.8 ± 0.6abcA	2.12 ± 0.20bA	15.7 ± 1.7abA	0.22 ± 0.08bcAB	38.4 ± 3.0bAB	25 ± 3aC
LBS+HS	7.48 ± 0.10cdA	953 ± 118bcA	6.0 ± 0.3cdA	1.58 ± 0.09cB	12.6 ± 1.0cA	0.22 ± 0.03cB	36.4 ± 3.7bB	25 ± 2aC
HBS	7.50 ± 0.13cdA	1101 ± 65abA	6.5 ± 0.5bcAB	2.08 ± 0.17bA	13.1 ± 1.5bcA	0.30 ± 0.09aAB	53.6 ± 4.6aB	26 ± 4aC
HBS+BC	7.70 ± 0.11abcA	1001 ± 72bcA	7.5 ± 0.3aA	2.49 ± 0.11aA	15.9 ± 1.0aA	0.29 ± 0.03abB	51.1 ± 2.1aB	28± 2aC
HBS+HS	7.42 ± 0.10dA	1206 ± 120aA	7.0 ± 0.2abA	2.17 ± 0.09bA	13.0 ± 0.6cA	0.32 ± 0.05aA	54.0 ± 4.8aB	23 ± 4aC
Willow								
Unt	7.70 ± 0.07aA	447 ± 71dA	3.0 ± 0.2cA	0.47 ± 0.04dA	8.5 ± 0.4cA	0.02 ± 0.01cB	2.9 ± 0.4cB	15 ± 1dB
LBS	7.50 ± 0.10bA	862 ± 72cA	5.1 ± 0.3dA	1.53 ± 0.09cA	11.7 ± 1.2bA	0.18 ± 0.02cdBC	37.1 ± 3.1bB	27 ± 3cB
LBS+BC	7.87 ± 0.11aA	802 ± 75cA	6.4 ± 0.4bA	2.10 ± 0.08bAB	15.5 ± 0.9aA	0.21 ± 0.02bcAB	34.2 ± 3.0bB	34 ± 3abB
LBS+HS	7.45 ± 0.06bA	928 ± 86bcA	5.9 ± 0.2cA	1.61 ± 0.10cAB	11.4 ± 0.8bA	0.18 ± 0.02cdB	34.0 ± 1.2bB	31 ± 4bcB
HBS	7.45 ± 0.08bA	1115 ± 106aA	6.4 ± 0.3bAB	2.03 ± 0.16bA	12.0 ± 0.9bA	0.23 ± 0.04abBC	55.6 ± 3.1aB	33 ± 3abB
HBS+BC	7.76 ± 0.13aA	1034 ± 95abA	7.3 ± 0.2aAB	2.43 ± 0.09aAB	16.2 ± 0.8aA	0.26 ± 0.02aBC	52.7 ± 2.3aB	36 ± 2aB
HBS+HS	7.48 ± 0.15bA	1168 ± 103aA	7.2 ± 0.4aA	2.22 ± 0.17bA	11.5 ± 1.7bA	0.22 ± 0.04bcB	54.3 ± 3.6aB	36 ± 4aB
Unplanted								
Unt	7.68 ± 0.13aA	411 ± 38eA	2.8 ± 0.2dA	0.47 ± 0.03dA	8.2 ± 0.9cA	0.05 ± 0.01cA	3.6 ± 0.5eA	22 ± 4eA
LBS	7.45 ± 0.06bA	733 ± 87cdA	5.6 ± 0.2cA	1.62 ± 0.09cA	12.3 ± 0.6bA	0.24 ± 0.02bA	52.0 ± 4.6cA	66 ±10dA
LBS+BC	7.76 ± 0.07aAB	696 ± 46dA	6.6 ± 0.3bA	2.18 ± 0.16bAB	15.9 ± 1.2aA	0.27 ± 0.04bA	45.5 ± 2.8dA	90 ± 4cA
LBS+HS	7.36 ± 0.11bcA	791 ± 63cdA	6.5 ± 0.3bA	1.78 ± 0.08cA	13.0 ± 1.0bA	0.29 ± 0.03bA	54.0 ± 2.6cA	70 ± 5dA
HBS	7.38 ± 0.06bcA	948 ± 119abA	6.8 ± 0.4abA	2.12 ± 0.13bA	13.1 ± 0.8bA	0.37 ± 0.03aA	66.6 ± 1.4abA	115 ± 7bA
HBS+BC	7.61 ± 0.09aAB	861 ± 92bcA	7.6 ± 0.2aA	2.57 ± 0.09aA	16.5 ± 0.7aA	0.41 ± 0.03aA	63.1 ± 4.5bA	139 ± 10aA
HBS+HS	7.30 ± 0.07cA	1025 ± 69aA	7.5 ± 0.2aA	2.31 ± 0.04abA	12.5 ± 1.5bA	0.39 ± 0.06aA	69.6 ± 5.2aA	126 ± 4bA

Treatments as follows: untreated mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Mean values ± SD per treatment (n=6). Different lowercase letters indicate significant differences between treatments (one-way ANOVA, p -value < 0.05), whereas different uppercase letters indicate significant differences between plant cultivation within the same treatment (one-way ANOVA, p -value < 0.05).

Similar observation was reported by Siebielec et al. (2018) in reclaimed Zn/Pb smelter waste. Treatments, largely as an effect of BS application, dramatically activated bulk and rhizosphere DHA likely due to high content of soluble and easily degradable organic carbon (Kussainova et al., 2013). Combining HS with BS further stimulated microbial activity and significantly ($P < 0.05$) increased DHA compared to BS alone. This effect could be linked to the beneficial microorganisms present in the HS used in this study. Although HS have been widely reported to positively impact soil microbial composition and activity through changing soil physichochemical properties and providing a source of carbon as a substrate and for colonization due to their large molecular size (Goel and Dhingra, 2021).

In contrast, combining BC with BS exhibited inhibition effect and significantly ($P < 0.05$) decreased bulk and rhizosphere DHA, nevertheless activity still remarkably higher than that for untreated tailings. This effect could be attributed to the adsorption of easily degradable carbon substrate and nutrient on BC surfaces rendering them less available for microbial utilization (Elzobair et al., 2016). The multivariate associations presented in Figure 1 showed that DHA strongly negatively correlated with C/N and pH, conversely DHA positively correlated with nutrient availability particularly NO_3^--N. Thus, the BC-induced increase in substrate C/N ratio and pH (Table 1) and decrease in NO_3^--N availability (Figure 3) could be other mechanisms to explain the negative impact of BC on microbial activity observed in this study. Indeed, the C/N ratio was found to be significantly negatively related to the content of DHA (Sawicka et al., 2020), and NO_3^--N was the most significant predictor of soil enzyme activities (Xu et al., 2015). Furthermore, optimal pH values for DHA were reported between 6.5-7.2 (Sinha et al.,

2009). The impact of BC on microbial activity observed in this study could imply enhanced OC use efficiency through BC-induced negative priming on BS-OM, thus decelerating its decomposition rate, and ultimately prolonging BS benefits which is of great importance to enhance ecorestoration effectiveness through supporting sustained vegetation.

In general, rhizospheric DHA was higher than that measured in bulk substrate of the respective treatment and plant species (Figure 2), indicating the role of root exudates to stimulate microbial activity in the immediate vicinity (Bais et al., 2006). Regarding the influence of plant cultivation, the lowest microbial activity was observed in unplanted substrate as measured by bulk DHA in 8-month incubated substrate (Figure 2a), whilst cultivation stimulated much higher bulk and rhizosphere DHA notably under miscanthus and willow growth and to lesser extent with poplar (Figure 2 a and b). This variation in microbial activity as a response to species cultivation is again linked to the wide variety of compounds excreted by plant roots to regulate soil microbial structure and activity to cope with environmental stresses (Shukla et al., 2011).

3.2.3. Extractable Mineral Nitrogen. The effect of treatments and plant cultivation on mineral N species (mainly NH_4^+-N and NO_3^--N) is presented in Figure 3. In general, BS addition resulted in a significant increase in NH_4^+-N content compared to untreated tailings (Figure 3a). Combined application of BC or HS with BS had no significant effect on NH_4^+-N except for treatment when BC combined with the higher rate of BS where a decrease in NH_4^+-N content observed under willow cultivation. In contrary, both factors (i.e., treatment and plant cultivation) induced a significant effect on the content of NO_3^--N (Figure 3b).

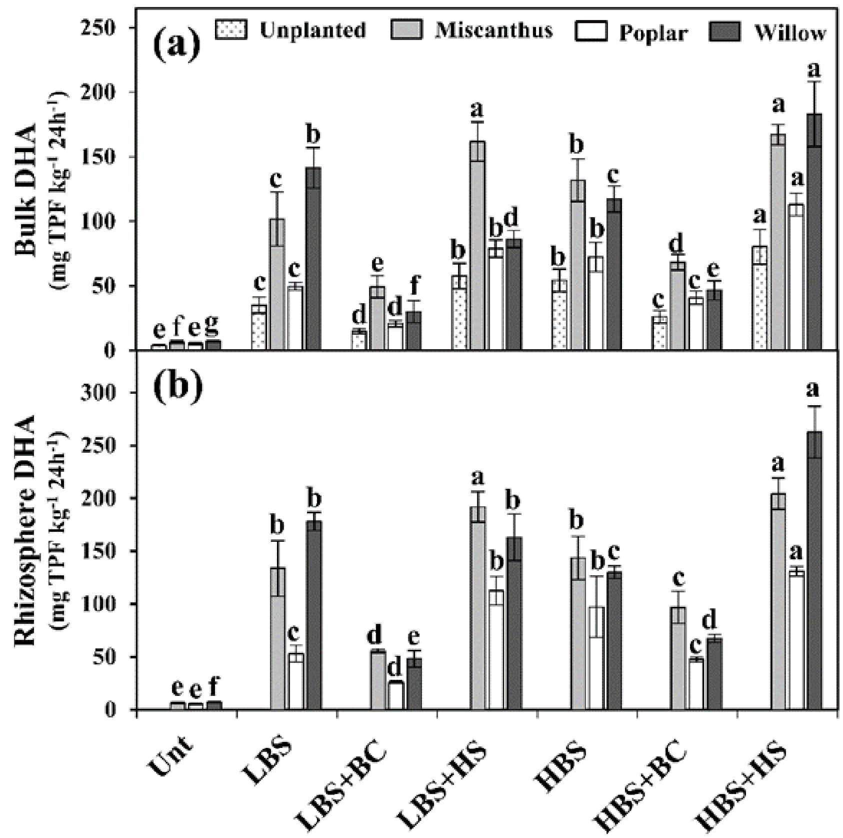

Figure 2. Dehydrogenase enzyme activity of planted or unplanted substrate after 8-months: (a) bulk substrate and (b) rhizosphere substrate. Treatments as follows: tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Values are means ± SD per treatment: (n=6). Within a plant species, different letters above the bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

Irrespective of plant cultivation, BS application generally resulted in an increase in NO_3^--N compared to the untreated tailings, whereas BC additive resulted in decreased NO_3^--N contents compared to BS alone. The highest NO_3^--N contents were observed in unplanted incubated substrates and peaked (i.e., 42.4 ± 5.4 mg kg^{-1}) at the higher application rate of BS, however NO_3^--N was significantly reduced by 63% and 51% when BC added in combination with low and high BS rates, respectively (Figure 3b).

Remarkable reduction in NO_3^--N was observed under plant cultivation compared to unplanted substrates and miscanthus cultivation induced the lowest NO_3^--N availability, which is likely as an effect of plant uptake. Mineral N, which is the measurement of plant available N and accounts for both NH_4^+-N and NO_3^--N, unsurprisingly mirrored the pattern observed for NO_3^--N since the latter is the predominant N fraction observed in this study.

The availability behavior of N species observed in this study could be largely attributed to the C/N ratio and microbial enzyme activity as supported by multivariate correlations presented in Figure 1. Extractable N species correlated negatively with C/N ratio and positively with DHA. The negative correlation was stronger between C/N and NH_4^+-N than that with NO_3^--N indicating the strong effect of C/N ratio in decreasing OM mineralization, and subsequently transformation to NH_4^+-N. Similar observation was reported by Sawicka et al. (2020), confirming the role of C/N in driving humification and mineralization rates and affecting pathways of C and N cycles within an ecosystem (Brown et al., 2003; Riaz et al., 2017). The positive correlation, on the other hand, was stronger between DHA and NO_3^--N than that with NH_4^+-N indicating that nitrification processes were strongly affected by inhibition in microbial activity. This could explain in part the observed impact of BC on reduced NO_3^--N extractability. Biochar was also found to reduce N availability through surface sorption mechanisms (Fidel et al., 2018). Likewise, Knowles et al. (2011) found BC addition to decrease N availability and significantly mitigate NO_3^--N leaching associated with BS application to uncontaminated soil.

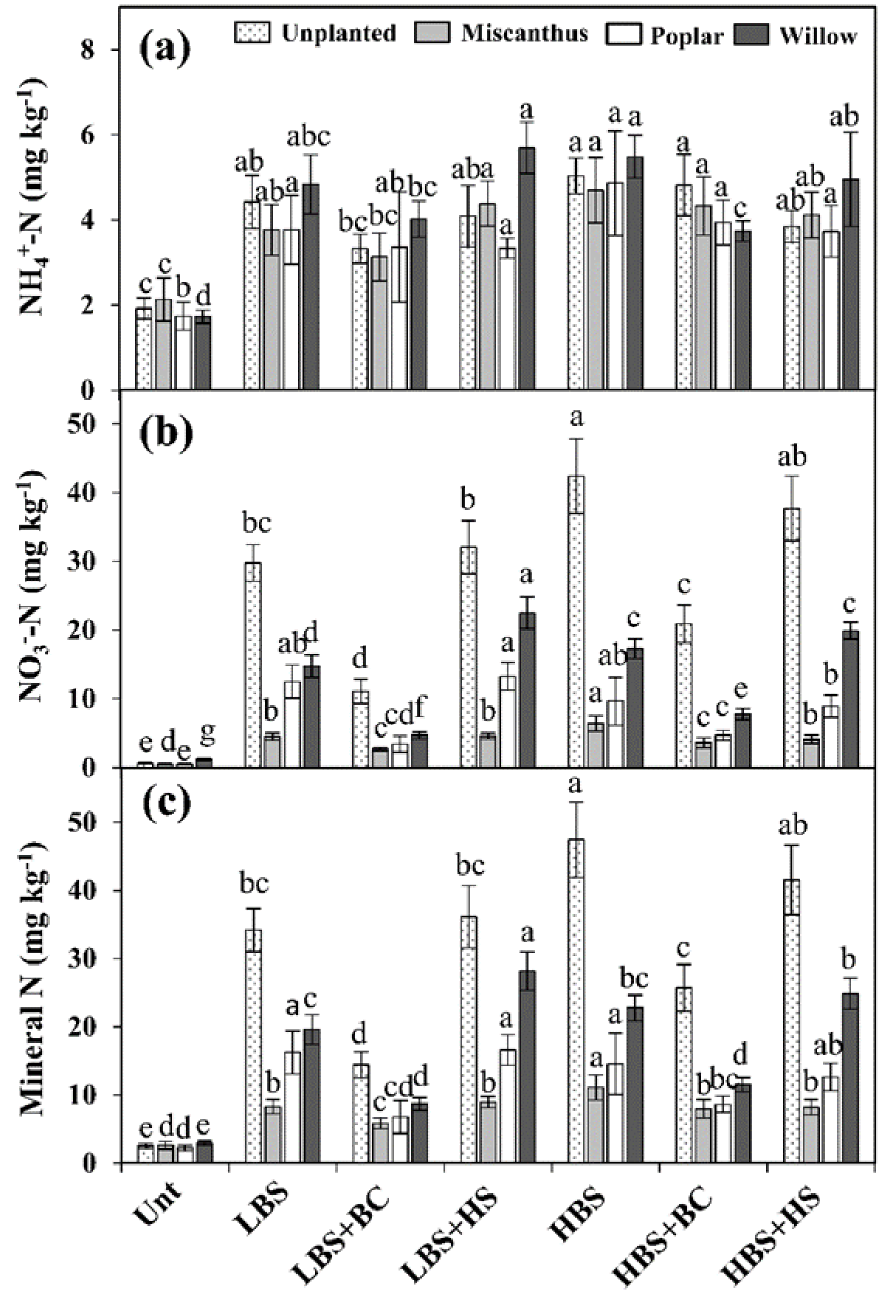

Figure 3. Extractable mineral nitrogen fractions of planted or unplanted substrate after 8-months: (a) ammonium, (b) nitrate, and (c) total mineral nitrogen. Treatments as follows: tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Values are means ± SD per treatment: (n=6). Within a plant species, different letters above the bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

3.2.4. Heavy Metals Bioavailability. The bioavailability of heavy metals was assessed based on extraction with 1 M NH_4NO_3 solution. The NH_4NO_3-extractable fraction represents the readily soluble and plant available metal content and was found be much better predictor for plants and ecotoxicological responses than DTPA- or other strong solutions-extractable fractions (Kumpiene et al., 2014). The concentrations of NH_4NO_3-extractable Pb, Zn, Cu, Cd, Ni, and Mn are reported in (Figure 4). In 8-months incubated untreated and unplanted tailings, low Pb, Cd, Ni, and Mn availability was observed with average concentrations of 1.82, 0.24, 2.76, and 2.84 in mg kg^{-1}, respectively (Figure 4 a, d-f), whereas availability of Zn and Cu was high with average concentrations of 42.7 and 39.8 in mg kg^{-1}, respectively (Figure 4 b and c). This variation in availability is likely related to the total metal concentration, and its form and fraction distribution within the tailing’s material. Lead being inherently immobile metal, which forms $PbCO_3$ at pH > 6 exhibited low bioavailability despite its high total concentration in these tailings (Table 1 and Figure 4 a). Examining the distribution and metals phytoavailability in mine tailings, Yuan et al. (2018) also found Zn and Cu to be more bioavailable than other metals, as they do not generally exist in mineral crystal and are mainly adsorbed on the surfaces of clay minerals and OM. Other authors have also concluded that lability of Zn and Cu is mainly controlled by total concentrations regardless of the mine tailings minerology (Li and Huang, 2015).

The metals responded differently to the amendment application. In unplanted substrate, effect of BS application was more pronounced on Zn and Cd with a significant reduction of 79 and 65%, respectively, whereas less reduction of about 20, 30, and 25% was observed in Pb, Cu, and Ni, respectively, under high BS treatment compared to

untreated tailings (Figure 4 a-e). On the other hand, BS application increased extractable Mn concentration by 29 and 25% at the low and high application rate, respectively (Figure 4 f). Ciarkowska et al. (2017) reported similar effect during phytostabilization of slightly alkaline Zn/Pb floatation tailings where sewage sludge was effective in reducing $CaCl_2$-extractable concentrations of Zn and Cd but not Pb. Except for Cu, combining BC with BS further enhanced metal immobilization and reduced extractable concentrations under high BS treatment compared to untreated tailings by 88, 82, 40, 40, and 21% for Zn, Cd, Pb, Ni, and Mn, respectively (Figure 4). These results in agreement with those reported by Penido et al. (2019), where co-application of BC and sewage sludge to mining soils resulted in the greatest reduction in Pb, Zn, and Cd bioavailability compared to sewage sludge alone.

Regarding effect of plant cultivation, except for Cu, miscanthus surprisingly induced a substantial metal mobilization and under treatments of BS or BS and HS blend, bioavailability of Pb, Ni, and Mn surpassed that observed in untreated tailings, however Zn and Cd concentrations were still lower than those in untreated tailings. In contrast, metal immobilization was induced by poplar cultivation with Pb concentrations significantly lower than those in unplanted substrates (Figure 4). This contrasting effect of plant species on metal bioavailability can be explained in part by the different composition of root exudates. Miscanthus roots, for instance, were reported to exude various organic acids such as proprionic, succinic, and citric acids as dominant molecules which may induce rhizosphere acidification, as observed in this study (Table 2), and thus increasing metal mobility through dissolution/desorption processes or formation of soluble metal complexes (Nsanganwimana et al., 2014).

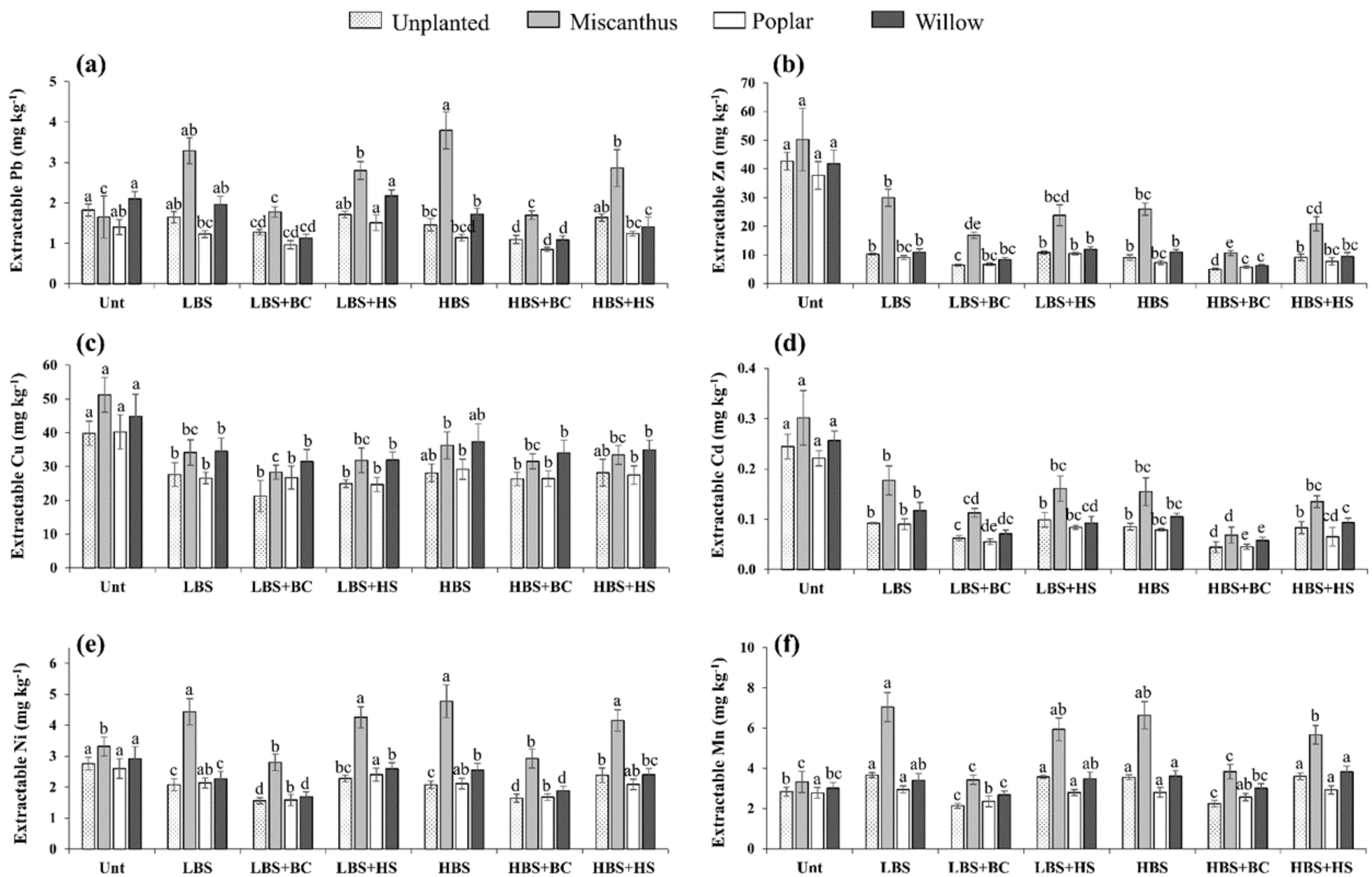

Figure 4. Ammonium nitrate extractable metals of planted or unplanted substrate after 8-months: (a) Pb, (b) Zn, (c) Cu, (d) Cd, (e) Ni, and (f) Mn. Treatments as follows: tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Values are means ± SD per treatment: (n=6). Within a plant species, different letters above the bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

The multivariate correlations presented in Figure 1 showed that OC and CEC played the most significant role in immobilizing Zn, Cd, and Cu in this study. Olsen P also contributed to reducing bioavailability of these metals, however its effect was less pronounced under miscanthus cultivation. These observations indicate that BS amendment played the most significant role in metal immobilization notably for Zn, Cd, and Cu. The BS-induced metal immobilization is attributed largely to their OM input. In addition to altering soil physichochemical properties, OM contains insoluble organic ligands and diverse functional groups such as, -COOH and -OH (Torrecillas et al., 2013), thus facilitating metal complexation, adsorption, and specific binding processes (Guo et al., 2006). Furthermore, metal immobilization was found to be also significantly affected by inorganic fractions of BS, such as phosphates and easily reducible Fe and Mn (Baghaie et al., 2011). Interestingly, BS-induced acidification (Table 2) effect on metal bioavailability was overshadowed by the dominant immobilization mechanisms provided by BS. Also, Pb, Cu, Ni are known to have great binding affinity for dissolved organic ligands (McLean and Bledsoe, 1992), thus presence of dissolved OM particularly in fresh BS may have counteracted the effect of retention mechanisms resulting in their different bioavailability behavior as observed in this study. The BC-induced further immobilization observed in this study could be partly attributed to the liming effect of the additive BC which was more clear under miscanthus cultivation by providing buffering capacity to the induced acidification (Table 2, Figures 1 and 4). Biochar used in this study characterized by high surface area and pore volume, thus may have also induced direct metal immobilization through electrostatic interactions, complexation with surface functional groups, and precipitation or co-precipitation with its mineral phases (Wang et

al., 2018). The other possible indirect mechanism could be the potential negative priming effect of BC on BS-OM as discussed in Section 3.2.2, which could have resulted in lower decomposition rate thus prolonging the retention mechanisms associated with the BS-OM, nevertheless this mechanism warrants further investigation.

3.3. EFFECTS OF TREATMENTS ON PLANT CHARACTERISTICS

3.3.1. Plant Nutritional Status and Metal Uptake. Concentration of nutrients in plant shoots along with common values reported in literature for healthy plants are presented in Table 3 and Figures S1-S3. In untreated tailings, the three cultivated plants were deficient in N, P, K, and Mn with one exception for the latter which was sufficient in willow. Irrespective of plant species, N and P concentrations significantly increased under BS application as shown in Table 3, albeit no significant effect of increasing the BS application rate or combining with BC and HS additives was observed. Regardless of amendment addition, K concentrations were far below the common values reported in healthy plants, even in BS treated tailings. Others also found BS application is not enough to correct K deficiency during mine tailing's revegetation (Al-Lami et al., 2021; Ciarkowska et al., 2017; Harris et al., 2021). Calcium concentrations were within the common range. In contrast, Mg concentrations were above the common ranges which is likely a reflection to the mineralogy of these tailings that is dominated by dolomite (Johnson et al., 2016). Combining HS with BS resulted in increased Fe concentrations yet not significant in all cases (Table 3). Humic substances may act as chelators to form soluble Fe/P-HS complexes or biostimulants and hormones thus affecting root morphology and physiology and regulating membrane activities related to Fe and P

acquisition (Zanin et al., 2019). This effect is important as Fe and P availability is very low under the alkaline conditions of this study. Nevertheless, HS affected Fe uptake but not P and was only statistically significant under willow cultivation. As shown in Table 3, irrespective of treatment, the three plants accumulated Fe concentrations within the common range found in healthy plants despite the low Fe availability (not detected by ICP analysis) under the alkaline conditions of these tailings. This is likely attributed to the different Fe-acquisition strategies evolved by plants to cope with Fe deficiency such as Fe-chelate reductase or Fe complexing with phytosiderophores (Barker and Pilbeam, 2015). Biosolids application increased Mn concentrations in miscanthus and willow but had no effect on poplar. Combining BC with BS resulted in decreased Mn concentrations with most pronounced effect in miscanthus and willow (Table 3). Regarding nutrient status of mature and new leaves within each plant, lower N, P, and K concentrations were found in older leaves especially K which was likely the cause for the deficiency symptoms observed that mainly manifested in older leaves (Figures S1-S3) (Panwar et al., 2016).

Table 3 and Figures S4-S6 present treatment effect on foliar accumulation of Pb, Zn, Cu, Cd, and Ni. Except for Zn in willow, concentrations were generally far below values set for maximum tolerable levels for cattle which are represented by Domestic Animal Toxicity Limit (DATL): 100, 500, 40, 10, and 100 in mg kg^{-1} for Pb, Zn, Cu, Cd, and Ni, respectively (NRC, 2005). This indicates a minimal risk that these toxic metals enter the food chain through sheltered animals. Unlike miscanthus and willow, lower Pb concentrations were found in poplar ranging from lowest 1.1 ± 0.5 mg kg^{-1} under combined BC and BS treatment to highest 2.0 ± 0.2 mg kg^{-1} under combined HS and BS

treatment (Table 3). When combined with BS, BC was more effective than HS in significantly decreasing Pb uptake by miscanthus and willow. Similarly, compared to BS alone, combining BC with BS induced further reduction in foliar Zn in poplar and willow, albeit Zn concentrations in miscanthus were generally very low (Table 3). Cadmium concentrations were below detection limit in miscanthus and generally lower in poplar than in willow with reduced uptake as a response to BC addition (Table 3). Copper and Ni followed a similar trend which are mainly affected by BS application and to lesser extent by increasing the BS application rate or BC and HS additives. This variation in metal accumulation is explained by the different strategies and mechanisms plants use to cope with metal toxicity (Yang et al., 2014). Metal accumulation within plant tissue is also a species dependent, poplar and willow for example, are known to accumulate Pb and Cu mainly in their roots while translocating Zn and Cd to their shoots and accumulation in their wood is generally low (Pilipović et al., 2019). Plant uptake as a treatment effect to some degree mirrored the metal bioavailability behavior measured by NH_4NO_3 extraction in the respective substrates (Figure 4). Regarding metal accumulation in old and new leaves within each plant, higher lead concentrations were found in old leaves especially in miscanthus and willow compared to new leaves (Figures S4-S6).

3.3.2. Plant Biomass. Figure 5 and Table S1 show the impact of amendment application on above- and below-ground biomass. Unsurprisingly, untreated tailings exhibited very poor plant growth which stunted within the first two months of the study with severe chlorosis and necrosis symptoms and yielded very low biomass.

Table 3. Shoot ionome of poplar, willow and miscanthus after 8-months of plant growth.

	Elements (mg kg^{-1})							(g kg^{-1})				
	Pb	Zn	Cu	Cd	Ni	Mn	Fe	N	P	K	Ca	Mg
Miscanthus												
Unt	13.5±0.4a	61±4a	10.3±0.6a	<d.l.	5.8±0.0a	24±4b	51±4d	4.7±1.1b	0.23±0.03d	6.0±1.0a	5.0±0.2b	5.7±0.2a
LBS	8.3±0.9b	32±3b	2.8±0.4c	<d.l.	3.9±1.6ab	52±12a	72±4bc	8.0±1.4a	0.93±0.05c	2.7±0.3bc	8.3±0.2a	7.2±0.3a
LBS+BC	4.4±0.7cd	23±4b	2.6±0.4c	<d.l.	2.6±0.5b	26±1b	62±4c	7.9±1.3a	1.31±0.10a	1.7±0.0c	9.0±1.5a	8.2±1.5a
LBS+HS	8.4±1.1b	29±6b	4.3±0.9b	<d.l.	2.7±0.8b	58±15a	88±4a	8.2±1.6a	0.97±0.03bc	5.1±1.4a	9.3±0.9a	7.4±1.3a
HBS	6.6±0.7bc	29±2b	3.4±0.5bc	<d.l.	2.8±1.2b	58±9a	76±8b	8.0±0.9a	1.01±0.05bc	4.1±0.3ab	9.2±0.9a	7.3±0.5a
HBS+BC	3.0±0.2d	27±4b	2.5±0.1c	<d.l.	2.8±0.8b	47±2a	80±10ab	8.3±1.0a	1.21±0.04a	2.8±0.5bc	8.4±0.3a	6.6±0.8a
HBS+HS	6.3±1.0bc	28±6b	3.4±0.5bc	<d.l.	3.1±0.2b	54±16a	71±4bc	8.5±1.2a	1.04±0.09b	4.3±0.5ab	9.6±1.7a	8.2±1.4a
Poplar												
Unt	1.9±0.3a	231±11a	10.5±1.5a	0.53±0.08a	15.8±1.5a	35±1ab	26±15b	8.4±1.0b	0.92±0.29b	2.8±0.8a	11.0±1.4a	13.6±0.5ab
LBS	1.4±0.3a	104±3c	6.0±0.7bc	0.36±0.13abc	2.0±0.4c	31±5ab	94±11a	12.0±1.3a	1.38±0.27ab	2.6±0.4a	9.7±0.9a	10.3±1.5b
LBS+BC	1.2±0.5a	71±14d	5.6±0.9bc	0.18±0.04c	2.0±0.1c	25±4b	103±11a	12.1±2.0a	1.56±0.2a	2.6±0.3a	11.4±0.7a	13.9±1.7ab
LBS+HS	2.0±0.2a	134±4b	8.6±0.7a	0.37±0.13abc	3.3±0.6b	34±3ab	144±32a	12.5±1.1a	1.55±0.37ab	3.4±0.3a	15.0±5.4a	14.7±1.4a
HBS	1.2±0.5a	131±15b	5.9±0.4bc	0.5±0.1ab	2.4±0.5bc	39±9a	99±16a	13.2±2.2a	1.47±0.04ab	2.4±0.2a	9.6±1.8a	10.8±1.5b
HBS+BC	1.1±0.5a	67±17d	3.8±0.3c	0.24±0.04bc	1.0±0.2d	37±4ab	122±33a	12.6±1.4a	1.91±0.16a	2.7±0.1a	12.8±2.1a	14.6±1.4a
HBS+HS	1.5±0.7a	142±33b	6.2±0.7b	0.44±0.12abc	2.2±0.2c	28±3ab	139±19a	13.5±1.6a	1.5±0.06ab	3.1±0.2a	11.6±0.5a	13.0±0.3ab
Willow												
Unt	7.8±2.1a	1114±72a	15.8±3.8a	1.25±0.03b	50.7±12.8a	223±9bc	44±7e	9.4±1.2b	0.65±0.09c	3.4±0.4a	24.0±1.3ab	26.0±1.7a
LBS	5.9±1.0ab	504±60bc	10.0±0.5b	2.13±0.12a	9.9±1.5b	443±13a	63±3cd	13.3±1.9a	1.84±0.12b	2.0±0.1b	27.9±2.3a	18.4±1.6bc
LBS+BC	2.5±1.0c	215±56d	7.3±1.2b	0.36±0.09d	5.8±1.3b	168±31c	58±4de	13.4±1.6a	1.93±0.06ab	3.0±0.6bc	18.5±2.0b	14.9±1.1c
LBS+HS	4.4±1.6bc	541±71bc	8.7±0.5b	1.23±0.24b	7.2±0.9b	360±130abc	95±6ab	13.5±1.4a	2.02±0.08ab	2.3±0.1b	25.8±2.04a	18.6±1.8bc
HBS	6.6±0.2ab	624±33b	8.1±0.8b	1.11±0.13bc	6.2±1.7b	409±60ab	74±9cd	13.7±2.0a	2.01±0.2ab	2.1±0.3b	29.0±2.9a	22.6±2.7ab
HBS+BC	5.3±0.6abc	368±54c	7.4±0.1b	0.76±0.03c	6.2±0.7b	241±27bc	80±3bc	13.3±1.4a	1.92±0.22ab	3.5±0.5a	24.4±3.6ab	19.8±2.9bc
HBS+HS	8.2±0.7a	580±223bc	10.0±1.0b	0.74±0.19cd	4.7±0.4b	470±104a	100±10a	13.9±1.1a	2.33±0.29a	3.5±0.5a	24.0±0.9ab	17.1±0.9c
Common Values[a,b]	-	-	3-20	-	0.1-6	50-500	20-300	10-60	1-10	20-50	1-50	1-5
DATL[c]	100	500	40	10	100	2000	500	-	-	-	-	-

Treatments as follows: untreated mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Mean values ± SD per treatment (n=6). Within a plant species, different letters indicate significant differences between treatments (one-way ANOVA, p -value < 0.05). <d.l. = below detection limit.
[a] Tremel-Schaub and Feix (2005) [b] Barker and Pilbeam (2015) [c] Domestic animal toxicity limit (DATL) i.e., the maximum tolerable level for cattle according to NRC (2005).

In general, BS addition dramatically improved plant growth and resulting biomass was proportionally increased with increasing the BS application rate. This great effect is attributed to the nutrient supply, mainly macronutrients NPK, in addition to the BS-induced metal toxicity alleviation particularly Zn, Cu, and Cd as supported by the strong correlations illustrated in Figure 1. These particular metals presented the most mobile fraction compared to the other metals as shown in Figure 4, and their phytotoxicity have been widely reported to induce much greater growth inhibition compared to that caused by Pb phytotoxicity for example (Fargašová, 2004).

As shown in Figure 5, plant responded differently to the amendment co-application. Willow growth was unaffected by addition of BC in combination with BS; however, HS additive significantly promoted its growth with about 15- and 23-fold biomass increase when combined with low and high BS rate, respectively, compared to untreated tailings (Figure 5 and Table S1). This is in line with the increased Fe and P and decreased Cd uptake in willow caused by HS additive (Table 3). Miscanthus growth, on the other hand, was notably influenced by BC and BS combination with about 2-fold increase in biomass compared to that produced under BS alone, likewise for HS and BS combination yet the effect was to lesser extent. Poplar yielded the highest biomass compared to miscanthus and willow and was mainly affected by BS amendment, however, HS and BC additive further enhanced its growth when combined with the high BS rate (Figure 5 and Table S1). The positive impact of BC additive on miscanthus biomass can be explained by the remarkable effect of BC to significantly reduce metal bioavailability under this species (Figure 4). Furthermore, BC inhibition effect on microbial activity and N mineralization was less under miscanthus cultivation compared

to poplar and willow indicating that BC did not greatly limit mineral N supply under miscanthus growth (Figure 3). Yet, the remarkable BC-induced N-immobilization under poplar and willow may have cancelled out its positive effect on metal immobilization, thus leading to no further increase in biomass.

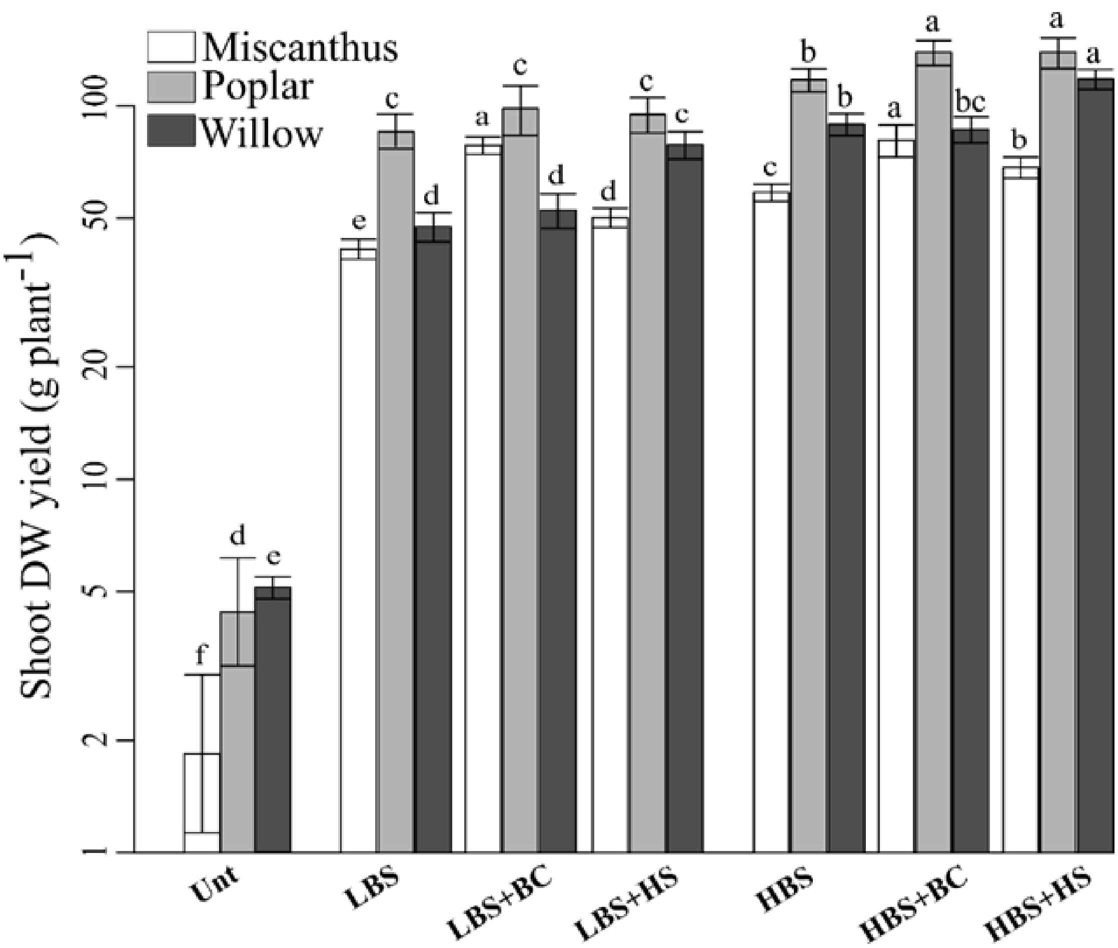

Figure 5. Aboveground biomass of miscanthus, poplar, and willow grown in the following substrates: mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Values are means ± SD per treatment: (n=6). Within a plant species, different letters above bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

3.4. PHYTOMANAGEMENT POTENTIAL OF CULTIVATED CROPS, AND ROLE OF AMENDMENT CO-APPLICATION

To compare the impact of species cultivation on physicochemical, biological, and metal bioavailability in untreated or treated tailings after 8-month growth, a PCA biplot was generated. As shown in Figure 6, the first two axes of the PCA together explained 74.4% of the total variance. The first axis explained 49.5% of the total variance and was positively correlated with a cluster of physicochemical properties and available nutrients, and negatively correlated with NH_4NO_3-extractable Zn, Cu, and Cd. The second axis explained 24.9% of the total variance and was driven by pH, enzyme activity and NH_4NO_3-extractable Pb, Ni, and Mn (Figure 6).

Regardless of cultivation or the species tested, a cluster of untreated tailings was observed which was positively correlated with NH_4NO_3-extractable Zn, Cu, and Cd and negatively correlated with major physicochemical and biological properties. This indicates the extremely poor characteristics of these tailings and implies the limited role of plants to modify tailings characteristics with no amendment applied. Miscanthus cultivation, in singular or BS and HS combined treatments, formed a separate cluster from unplanted or poplar and willow substrates and was mainly driven by low pH, high enzyme activity and NH_4NO_3-extractable Pb, Ni, and Mn. Combing BC with BS, however, shifted miscanthus substrates to the other cluster of unplanted, poplar, and willow which formed a cluster that is negatively correlated with metal availability (Figure 6). This illustrates the remarkable impact of BC as an additive to mitigate the negative issues associated with miscanthus cultivation. This further stress the importance of choosing cautious management strategies that target selection of candidate plant species

and appropriate amendment or amendment combination to enhance revegetation effectiveness and increase ecorestoration success.

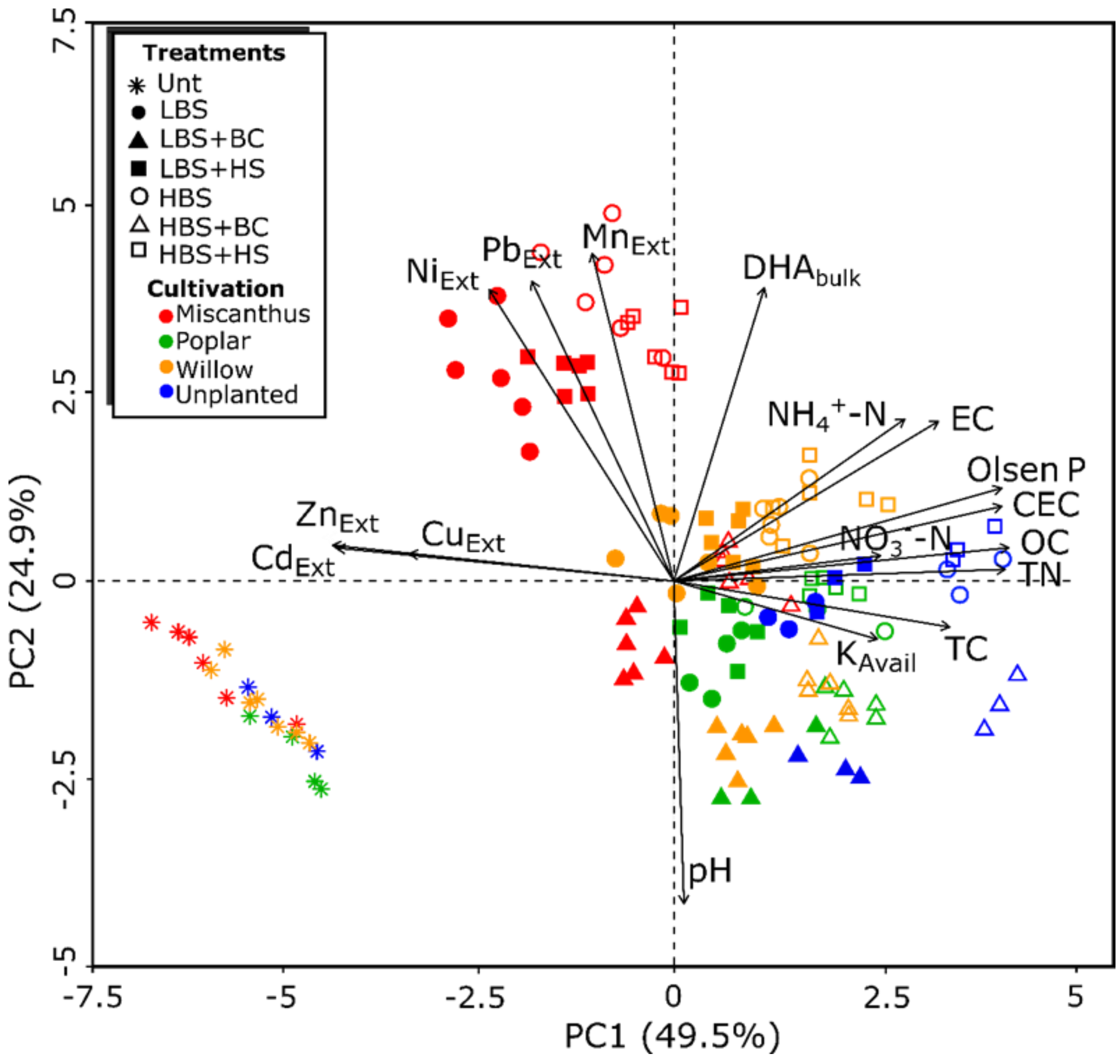

Figure 6. Principal component analysis (PCA) biplot on substrate properties after 8-months of unplanted mine tailings or planted with miscanthus, poplar, and willow in: mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Parameters included in the PCA are: physicochemical properties (pH, EC, CEC, OC, TC, TN, NH_4^+-N, NO_3^--N, Olsen P, available K), NH_4NO_3-extractable metals (Pb, Zn, Cu, Cd, Mn, and Ni), and biological enzyme activity in bulk substrate (DHA_{bulk}).

3.5. EVALUATION THROUGH ECOSYSTEM SERVICES AND SUBSTRATE QUALITY INDEX

Besides minimizing metal bioavailability, tailing's revegetation efforts must also consider stimulation and restoration of soil-functioning processes. Due to complexity of the tailing's ecosystem most of the measured properties are interlinked and usually difficult to interpret, as such, the impact of a phytomanagement strategy on tailings quality cannot be assessed by individual properties. To facilitate interpretation and more meaningful assessment of tailings restoration efficacy, measured properties can be grouped into higher-level categories of ecological attributes and an integrated soil quality index can be calculated (Burges et al., 2016; Mukhopadhyay et al., 2016).

The overall effect of amendment application and plant cultivation on ES is presented in Table 4 and Figure 7. For all the three species tested, amendment application had a substantial positive effect on all ES compared to untreated tailings, yet the predominant impact on most services was mainly a result of BS application (Figure 7). According to Trimmer et al. (2019), BS are explicitly connected with ES as they can enhance multiple services when applied as a soil amendment owing to their rich nutrient and OM contents. Other authors stressed the need to study the land application of BS within the context of ES to investigate the connection between the observed impact and ES, and most importantly to optimize their provision (Toffey and Brown, 2020).

Compared to singular BS application, *nutrient cycling* increased in HS and BS combined treatments, whereas co-application of BC and BS decreased this service, particularly under poplar and willow cultivation. *Carbon storage*, *contamination control*, and *water purification* were highest in BC and BS combined treatments with remarkable increase compared to BS alone, notably for the latter two services (Figure 7), and

particularly under miscanthus cultivation (Figure 7a). This further confirms the positive impact of the management strategy of BC and BS co-application.

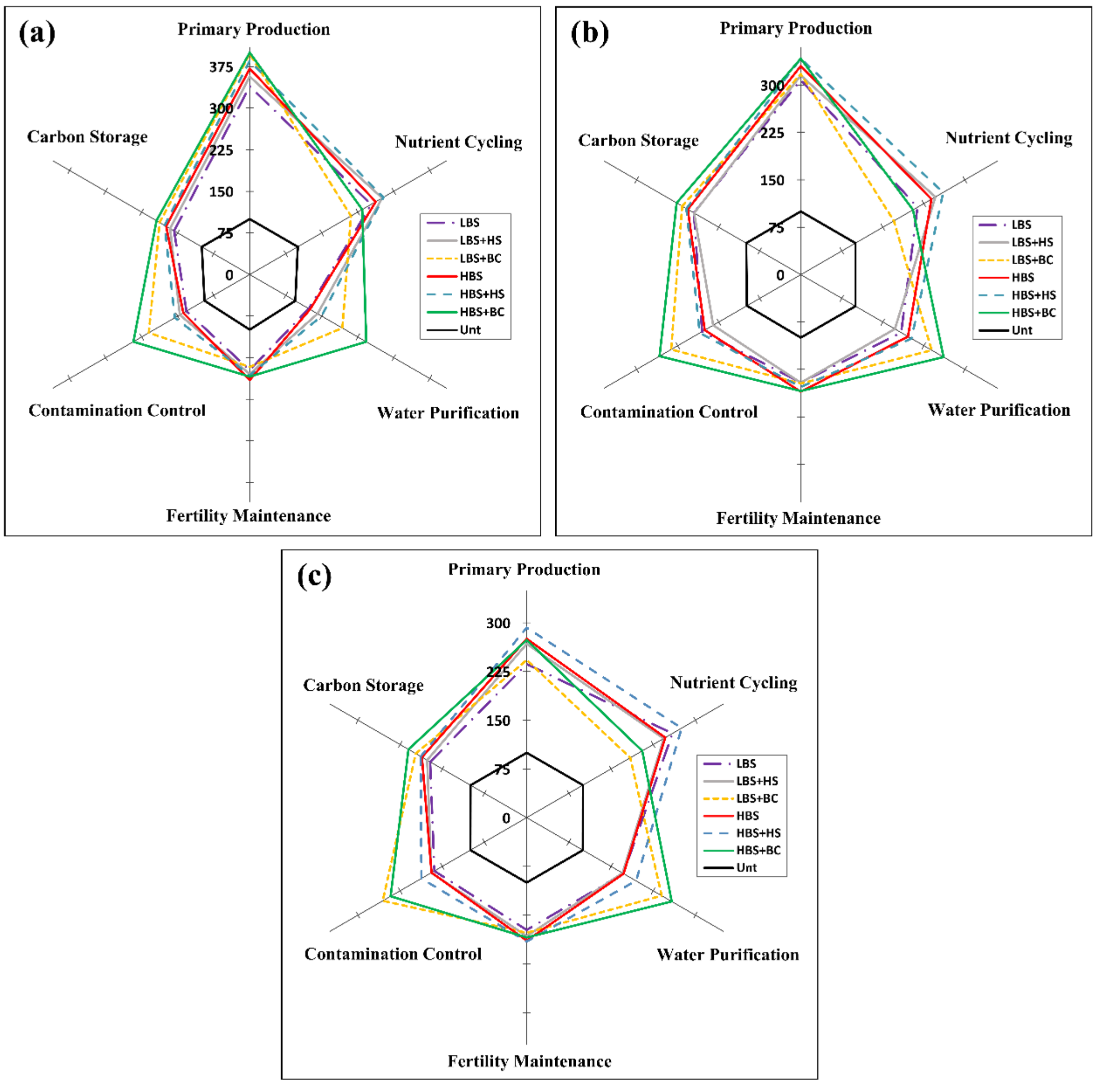

Figure 7. Sunray plot of ecosystem services provision after 8-months of amendment and plant cultivation of (a) miscanthus, (b) poplar, and (c) willow in: un-amended mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). A value of 100 corresponds to the mean value obtained for each ecosystem service in the Unt (i.e., control treatment).

Table 4. Effect of treatments on ecosystem services and the substrate quality index (SQI) after 8-months of plant cultivation. Values are expressed as a percentage of the un-amended planted controls.

	Primary production	Nutrient cycling	Water purification	Fertility maintenance	Contamination control	Carbon storage	SQI
Miscanthus							
Unt	100 ± 17fA	100 ± 7eA	95 ± 10eA	99 ± 6dA	94 ± 8cA	100 ± 3fA	99 ± 4fA
LBS	339 ± 6eA	253 ± 9bA	123 ± 13dB	172 ± 5bcA	132 ± 28bcB	158 ± 3eC	196 ± 6eA
LBS+BC	398 ± 5abA	211 ± 6dA	193 ± 10bB	167 ± 5cB	210 ± 35aB	188 ± 2bC	228 ± 6bA
LBS+HS	357 ± 5dA	276 ± 4aA	141 ± 9cdB	179 ± 4abA	145 ± 25bB	166 ± 6dC	211 ± 6cdA
HBS	371 ± 5cA	262 ± 7bA	126 ± 13dC	190 ± 8aA	138 ± 22bB	174 ± 4cC	210 ± 3dAB
HBS+BC	400 ± 9aA	234 ± 6cA	243 ± 14aB	184 ± 7aA	242 ± 48aA	194 ± 4aC	250 ± 10aA
HBS+HS	385 ± 6bA	278 ± 3aA	150 ± 10cB	182 ± 6abB	156 ± 20bB	177 ± 6cC	221 ± 4bcB
Poplar							
Unt	100 ± 23cA	100 ± 8eA	100 ± 11dA	100 ± 4cA	98 ± 8cA	100 ± 10dA	100 ± 7dA
LBS	308 ± 7bB	213 ± 5cB	184 ± 14bcA	171 ± 5bA	180 ± 15bA	195 ± 4cA	209 ± 6cA
LBS+BC	318 ± 10abB	170 ± 4dB	239 ± 6aA	172 ± 12abAB	237 ± 22aAB	217 ± 4abA	226 ± 3bA
LBS+HS	315 ± 8bB	246 ± 6bC	172 ± 10cA	171 ± 4bB	160 ± 15bAB	195 ± 6cA	210 ± 6cA
HBS	330 ± 5abB	239 ± 12bB	196 ± 13bcA	185 ± 7aA	176 ± 13bA	207 ± 10bcA	222 ± 7bcA
HBS+BC	342 ± 5aB	205 ± 4cB	261 ± 12aA	184 ± 3aA	258 ± 19aA	227 ± 6aA	246 ± 6aA
HBS+HS	342 ± 6aB	260 ± 3aB	202 ± 8bA	178 ± 5abB	185 ± 15bA	209 ± 2bA	229 ± 2bA
Willow							
Unt	100 ± 4dA	100 ± 5fA	100 ± 10dA	100 ± 3eA	100 ± 9cA	100 ± 3eA	100 ± 4eA
LBS	237 ± 5cC	259 ± 3bA	172 ± 19cA	173 ± 5dA	164 ± 24bAB	172 ± 6dB	196 ± 7dA
LBS+BC	243 ± 7cC	184 ± 10eB	240 ± 11aA	177 ± 4dA	256 ± 25aA	197 ± 3bB	216 ± 7bA
LBS+HS	268 ± 5bC	244 ± 5cB	170 ± 5cA	182 ± 5cA	170 ± 8bA	177 ± 4dB	202 ± 3cdB
HBS	275 ± 4bC	246 ± 3cB	172 ± 7cB	189 ± 4abA	169 ± 10bA	186 ± 6cB	206 ± 4cB
HBS+BC	273 ± 5bC	206 ± 5dB	258 ± 15aAB	184 ± 2bcA	242 ± 22aA	210 ± 4aB	229 ± 6aB
HBS+HS	293 ± 4aC	276 ± 6aA	194 ± 12bA	191 ± 5aA	187 ± 14bA	188 ± 9cB	221 ± 6bAB

Treatments as follows: untreated mine tailings (Unt), treated with biosolids at low (LBS) or high (HBS) application rates alone or in combination with biochar (BC) or humic substances (HS). Mean values ± SD per treatment (n=6). Within a plant species, different lowercase letters indicate significant differences between treatments (one-way ANOVA, p-value < 0.05), whereas different uppercase letters indicate significant differences between plant cultivation within the same treatment (one-way ANOVA, p-value < 0.05).

The ES results also confirm that assisted phytomanagement using poplar and willow is more superior for phytostabilization efficiency than miscanthus due to their supporting to higher *contamination control*, and *water purification* services (Table 4). Finally, according to the overall SQI, co-application of additives HS or BC with BS had a significant positive effect on the overall soil quality, notably with BC addition (Table 4).

Interestingly, the interpretation based on ES assessment approach mirrored those observed directly from individual substrate properties. Nevertheless, by grouping these properties into higher-level categories of ES, this approach is useful to provide information that is easier to interpret (i.e., the more the better) (Burges et al., 2016). According to Epelde et al. (2014a), this assessment approach is especially useful for long-term monitoring programs as it is stable against changes in techniques, equipment, methods, interests, etc. Furthermore, incorporating the concept of ES provision into the consideration of the tailings phytomanagement strategy may provide the best information for optimizing the provision of targeted ES through the manageable properties especially when trade-offs are considered in the decision-making.

4. CONCLUSIONS

The stimulation of soil-functioning processes on multi-metal tailings entails cautious selection of proper phytomanagement strategies that target candidate plant species and appropriate soil amendments. In this study, the role of BS alone or co-applied with BC or HS was investigated. The most predominant impact on tailings parameters and plant growth responses was observed as an influence of BS addition owing to their

rich OM and nutrient contents. Biosolids significantly improved all physicochemical properties and dehydrogenase enzyme activities, increased mineral nitrogen availability, and induced metal immobilization particularly Zn, Cu, and Cd, nevertheless BS were less effective in reducing Pb bioavailability. Biomass production was also observed to be largely influenced by BS addition which reflected the positive impact on nutrient uptake notably macronutrient NPK. Nevertheless, BS may not support sustained vegetation growth and vitality due to BS nutrient lability and rapid decomposition. Co-applying BC with BS resulted in a lower enzyme activity and mineral nitrogen availability, which could imply an induced negative priming effect on BS-OM thus supporting efficient nutrient release and prolonging BS associated benefits. Co-applying BC also induced further metal immobilization and was more effective in reducing Pb bioavailability than BS alone. Co-applying HS with BS increased enzyme activity and Fe uptake. Superior phytostabilization efficiency was associated with poplar and willow cultivation but not miscanthus which induced metal mobilization due to acidification, however, this effect was counteracted by co-applied BC. These results highlight the importance of co-applying carbon-rich amendments in particular BC with BS and the suitability of bioenergy crops particularly poplar and willow for tailings phytostabilization and concurrent biomass production. The evaluation through ES and SQI mirrored the positive effect of amendment co-application and plant cultivation. Co-applying HS with BS resulted in improved *nutrient cycling* while BC enhanced *water purification* and *contamination control* services. The long-term effectiveness of the phytomanagement strategies studied here should be evaluated in a pilot-scale experiment under field conditions.

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III. INTERACTIVE EFFECT OF BIOSOLIDS APPLICATION AND INOCULATION WITH MYCORRHIZAL FUNGI ON LEAD/ZINC/COPPER TAILINGS PHYTOSTABILIZATION AND BIOENERGY CROP PRODUCTION

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ABSTRACT

The symbiotic relationship between most plants and mycorrhizal fungi (MF) may greatly contribute to restoration of mine tailings due to the provision of several ecosystem services which have been linked to MF-plant interactions. However, the development of a functional MF-plant root colonization is also severely hindered by the extreme characteristics of these degraded systems. Nevertheless, adding appropriate soil amendment with proven amelioration potential such as biosolids (BS) may facilitate beneficial and efficient MF-plant interactions. Thus, the aim of this greenhouse study was to determine the interactive effect of co-application of BS and inoculation with MF on tailings physicochemical and biological properties, metal bioavailability, and biomass production of poplar, willow, and miscanthus grown in a slightly alkaline Pb/Zn/Cu mine tailings. Compared to BS alone, MF inoculation significantly increased P uptake, reduced Pb, Zn, and Cu leaf accumulation, and increased biomass production for the three cultivated species with 2-fold increase in miscanthus biomass compared to non-

inoculated BS application. Inoculation also induced significant positive effects on tailings substrates represented by increased organic carbon (OC) input, enhanced microbial activity, and induced metal immobilization. These multiple and interrelated positive impacts induced by MF inoculation further support the important role MF-plant interactions play in stimulation of soil functioning and formation processes. This is particularly important in phytostabilization of mine tailings which implies acceleration of site ecorestoration.

1. INTRODUCTION

Long-term opencast mining activities have left a legacy of disturbed sites with a large quantity of waste materials such as mine tailings (Karaca et al., 2018). Mine tailings contain high levels of toxic metals, especially cadmium, copper, lead, and zinc that are usually present together in many minerals in the Midwest of USA (Johnson et al., 2016). If stable vegetation cover had not developed, particles originated from tailings deposit are spread out in the surrounding environment of the mining area through wind erosion or water run-off and/or leaching (Padoan et al., 2021). This increases the occurrence of contaminants in the ecosystems and cause deleterious impact on soils and water streams. On barren tailings deposit, the self-establishment of vegetation cover is unlikely due to unfavorable edaphic conditions (Mendez and Maier, 2008). Mine tailings are mechanically, physically, chemically and biologically deficient (Li and Huang, 2015). They are characterized by extreme pH, low water retention capacity, limited cohesion, high compaction and low porosity, deficiencies in nutrients and organic matter (OM), and

high concentrations of toxic metals (Wang et al., 2017). These unfavorable characteristics generally prevent natural plant growth on mine tailings. Therefore, it is necessary to take an action to assist the reclamation of tailings deposits and assure the long-term self-sustainability of these systems.

Among remediation techniques for metal-contaminated substrates, aided phytostabilization which combines soil amendment and metal excluder plants and their associated microorganisms to minimize contaminant linkages through the environmental compartments and the food chain (Kidd et al., 2015). Soil amendment and vegetation cover must fulfill the objectives of (1) improving tailings physicochemical properties such as OM and nutrient contents (2) reducing contaminant linkages, and (3) improving substrate ecological processes and functions that ultimately enhance ecosystem services (Mench et al., 2010). Selection of relevant plant species, play a key role in the successful establishment of a vegetation cover (Kidd et al., 2015). Fast growing species such as poplars, willow, or miscanthus were suggested for tailings revegetation due to their high biomass production, low nutrient requirements, tolerance to excess metals and their dense deep-rooted system which can physically stabilize the tailings thus preventing wind and water erosion (Guittonny and Lortie, 2017).

Biosolids (BS) are byproduct generated in large quantities by municipal wastewater treatment facilities (Wijesekara et al., 2016). They are the superior amendments for mine tailings revegetation due to their ability to (1) improve soil fertility and plant yields (2) form immobilized complexes between organic ligands and toxic metals, (3) improve soil structure, cation exchange capacity (CEC), pH and water retention (Harris et al., 2021; Silveira et al., 2003; Wijesekara et al., 2016). An increased

number of studies have reported the BS ability to immobilize metals in tailings, thus limiting their phytotoxicity (Brofas et al., 2000; Jones et al., 2011; Mingorance et al., 2014). Reclaiming tailings with BS would be a good waste management practice if environmental and public health concerns were not associated with its recycling (Lu et al., 2012). The high quantity of nutrients, besides toxic metals or emerging contaminants in BS may make it a hazardous waste material when applied on the field. However, a careful dosage of BS may limit the risk associated with its application on tailings. As suggested by (Madejón et al., 2012), combining BS with mycorrhizal fungi can be more efficient than BS alone for the reclamation and revegetation of acidic mine tailings. It allowed to optimize the dosages applied on arsenical sulphidic mine tailings as low amounts of BS inoculated with mycorrhizal fungi produced the same positive response on tailings reclamation than at higher application rate of non-inoculated BS (Madejón et al., 2012).

Mycorrhizal fungi (MF) are rhizospheric microorganisms that establish mutual symbiosis with most of higher plants. The extended fungus hyphae increase physical and chemical connection between substrates and plant roots that benefit the host plants and improve substrate properties (Colombo et al., 2017). This symbiosis greatly influences plant growth by providing nutrients and water supply and plays vital roles in the global element cycling. Moreover, MF may greatly improve soil structure and stabilization by binding particles into stable aggregates that resist erosion (Leifheit, 2014). On metal-contaminated soils, mycorrhizal symbiosis may greatly enhance survival of host plants by mediating the interactions between the plant and the metals (French, 2017). Mycorrhizal fungi may contribute to metal detoxification by several mechanisms such as: (1)

extracellular precipitation and complexation with glomalin, molecules that are exuded in the soil, (2) biosorption in the hyphal cell walls, and (3) intracellular compartmentation in the vacuole or chelation into the cytoplasm (Solís-Domínguez et al., 2011). All these processes provide a metal excluder barrier for the plant.

The mine tailings used in this study were obtained from the Viburnum mine site located in the Southeast Missouri (Al-Lami et al., 2019). They are slightly basic and contain high levels of Cu, Pb, and Zn (479, 3553, and 966 mg kg^{-1}, respectively). In these tailings, even though inoculation with MF has shown to induce a positive impact on growth of bioenergy crops when no other soil ameliorant added, this effect was not very significant. It has been hypothesized that a beneficial MF and plant root symbiosis is also significantly hindered under severe soil characteristics (Wang, 2017). Thus, when considering MF in mine tailings ecorestoration, applying an appropriate amendment can facilitate a beneficial and functional symbiosis with plant roots which may greatly maximize the restoration efforts. In these tailings, applying BS showed a positive impact on plants growth and tailings physicochemical properties but also highly increased nutrient availability and potential leachability (Al-Lami et al., 2021; Al-Lami et al., 2019).

Giving the unique benefits and provision of several ecosystem services linked to each (Koziol and Bever, 2019; Koziol et al., 2020; Toffey and Brown, 2020), combining mycorrhizal fungi inoculation with BS amendment application may be the key player for a secure and successful reclamation of these tailings. Thus, this study aimed at assessing the effect of interaction between mycorrhizal fungi and the host plant on phytostabilization of a slightly alkaline Pb/Zn/Cu mine tailings assisted with BS as an

ameliorative amendment. The novelty of this study was to quantify the synergistic influence of this combined treatment on: (1) Tailings key physicochemical properties; (2) Metal bioavailability; (3) Biomass production of bioenergy crops (poplar, willow, and miscanthus); (4) Metal and nutrient uptake; and (5) Microbial enzyme activity.

2. MATERIAL AND METHODS

2.1. TAILINGS, BIOSOLIDS AND MYCORRHIZAL FUNGI

The mine tailings were collected from a Pb/Zn/Cu mine tailings impoundment located in Viburnum MO, US (37°42’07.1"N, 91°06’22.3"W). These tailings are characterized by very poor soil properties represented by very fine texture consisted of 40% sand, 50% silt, and 10% clay, extremely low contents of OM and essential nutrients, and a slightly basic pH of 7.8, along with high contents of toxic metals notably for Pb, Zn, Cu, and Cd (Table 1). Thus, due to the abovementioned severe characteristics, these tailings have not supported a natural vegetation.

The aerobically/anaerobically digested BS were obtained from the Southeast Wastewater Treatment Plant, Rolla, MO, dried to approximately 40% solid content and thoroughly mixed and homogenized prior to use. The commercial mycorrhizal fungi inoculum (Soil Secrets, LLC., Los Lunas, NM, USA) used in this study was a concentrated blend with abundant spores of endomycorrhizae (i.e., vascular arbuscular mycorrhizae) of *Glomus intraradices* and ectomycorrhizae species (MycoMaxima Professional).

Table 1. Mine tailings and biosolids properties.

Parameter	**Mine tailings (Unt)**	**Background levels**[a]	**Biosolids (BS)**
pH (H_2O)	7.8	> 6.1[b]	6.4
EC (mS cm^{-1})	0.4	-	-
CEC ($cmol^+$ kg^{-1})	3.6	5-25[c]	41.3
OM (%)[d]	0.6	-	57.6
Total N (mg kg^{-1})	600	-	66500
NH_4^+–N (mg kg^{-1})	1.7	-	8220
NO_3^-–N (mg kg^{-1})	0.5	-	69.1
Total P (mg kg^{-1})	87.1	-	14200
P-Olsen (mg kg^{-1})	3.0	-	-
Elements (mg kg^{-1})			
Pb	3553	20	31.5
Zn	966	49	735
Cu	479	13	522
Cd	13.7	<1	-
Ni	70.7	14	22.4

[a] Background concentrations for metals in Missouri soils (Tidball 1984)
[b] Summary of soil fertility status in Missouri (Nathan et al. 2007)
[c] Scrivner and Cooper 1985
[d] Loss on Ignition (LOI)

2.2. TAILINGS TREATMENTS

The tailings were air-dried, sieved to 2-mm, and manually homogenized. Biosolids were manually homogenized before mixing with the tailings. The tailings were mixed by rotation in a plastic flask with 3 or 5% (*w/w*) of BS, alone or in combination with the mycorrhizal fungi inocula, resulting in 5 different treatments (in 6 replicates):

1. Untreated mine tailings (Unt)
2. Tailings + 3% biosolids (LB)
3. Tailings + 3% biosolids + 5 g pot^{-1} mycorrhizal fungi (LB + M)
4. Tailings + 5% biosolids (HB)
5. Tailings + 5% biosolids + 5 g pot^{-1} mycorrhizal fungi (HB + M)

Treatments were packed into plastic pots (3.5 kg in cylindrical pots), and placed under controlled conditions in a greenhouse, with a bottom cup to avoid any leaching. They were watered and incubated during a 4-week period to allow BS to react with the tailings and microbial communities to develop.

2.3. PLANT CULTIVATION AND SAMPLE COLLECTION

Stem cuttings of laurel leaf willow (*Salix pentandra*) and hybrid poplar DN34 (*Populus deltoids_Populus nigra*, DN34) (approximately 20 cm long) were collected in a park (Rolla, MO, USA). Rhizomes of miscanthus (*Miscanthus x giganteus*) were sampled from a pilot-scale trial which was implemented in the tailings impoundment in Viburnum, MO, USA. Stem cuttings were pre-rooted in individual pots filled with tap water for 1-month in a greenhouse prior planting, whereas miscanthus rhizomes were planted directly. For each treatment, one standardized plant of either willow, poplar, or rhizome of miscanthus was planted in pots and cultivated in a greenhouse during 8-months growth period. Pots were arranged in a fully randomized block design and regularly watered. After 8-month growth period, the leaves, stems and roots of willow and poplar were harvested by separating new branches, leaves, and roots from the old cuttings. Miscanthus was collected by separating the shoots and roots from the rhizomes. All harvested biomass was thoroughly washed with tap water and a final rinse of deionized water, oven dried at 60 °C to constant weight for 72 h and weighed for determining the aboveground and belowground dry weight (DW) yields.

At harvest, fresh rhizosphere and bulk substrate samples were collected for microbial enzyme activity determination. The root systems were vigorously shaken to

collect adhering substrate particles for rhizosphere sample collection (Luster et al., 2006). Another homogenous subsample was also collected for bulk substrate samples, and subsamples were then air-dried, sieved, and stored for physicochemical and metal determination. Random segments of fresh fine lateral roots for each plant species were also collected, during the harvest, for root mycorrhizal colonization observations. The collected fine roots were carefully washed to remove any adhering substrate particles then stored in plant fixative until clearing and staining. The fixative, formaldehyde alcohol acetic acid (FAA), consisted of 10% formaldehyde, 50% alcohol, 5% acetic acid, and 35% water (Daykin and Hussey, 1985).

2.4. SUBSTRATE, PLANT AND MYCORRHIZAL FUNGI ANALYSIS

Substrate pH was measured in a substrate/MΩ water (1:2.5; *w/v*) suspension using a digital meter (Orion 370, Thermo Fisher Scientific, Inc). Elemental analyzer was used to determine total nitrogen (TN) in substrate samples (Perkin Elmer 2400 CHN analyzer). Organic carbon (OC) was determined using wet oxidation method (Page, 1982). Olsen P was extracted using 0.5 M $NaHCO_3$, pH 8.5 solution according to Pierzynski (2000) and analyzed using ICP-OES (Pierzynski, 2000). The extractable fraction of heavy metals was measured using 1 M NH_4NO_3 solution at 1:2.5 (*w/v*) ratio according to Gryschko et al. (2005). The samples were then analyzed for Cu, Pb, and Zn using ICP-OES (Perkin Elmer, Avio 200). Dehydrogenase enzyme activity (DHA) in the fresh bulk and rhizosphere substrate samples was determined using the colorimetric method by Casida Jr et al. (1964). Briefly, samples were incubated in 2,3,5-triphenyltetrazoliumchloride (TTC) solution for 24 h at 37 °C. The triphenyl formazan (TPF) formed at the end of the

incubation period was extracted with methanol. The color intensity was then measured spectrophotometrically at 485 nm wavelength (Varian Cary 50 Scanning UV/Vis Spectrophotometer). The DHA activity was expressed as milligrams of TPF per kilogram dry soil per 24 h (mg TPF kg^{-1} $24h^{-1}$).

Plant tissue digestion was performed using a hot block system. Leaf samples were ground to < 1 mm particle size, and subsamples of 0.5 g were digested with 5 mL of concentrated HNO_3 and 3 mL of 30% H_2O_2 (Huang and Schulte, 1985). A certified reference material (WEPAL IPE-126; Zea mays) as well as spike samples were also routinely included. Digested samples were analyzed for Cd, Cu, Fe, Mn, Ni, Pb, and Zn using ICP-MS (Perkin Elmer, NexION 300/350), and Ca, K, Mg, and P using ICP-OES. In addition, CHN elemental analyzer was used to measure total N in plant samples.

For mycorrhizal root colonization observations, the FAA stored root segments were cut to 1-cm. The root samples were then cleared with 10% KOH for 10 min at 90 °C, followed by bleaching in alkaline hydrogen peroxide at room temperature for 30 min, acidification in 1% HCl at room temperature for 1 h, and then staining with 0.05% trypan blue at 90 °C for 10 min (Phillips and Hayman, 1970). The stained roots were then observed under the microscope using phase contrast microscopy (Olympus CKX41).

2.5. STATISTICAL ANALYSIS

Influence of treatments on physicochemical composition of the substrate, plant biomass, leaves ionome and microbial activity was tested using one-way analysis of variance (ANOVA). Normality and homoscedasticity of residuals were met for all tests. When significant differences occurred between treatments, multiple comparisons of mean

values were made using post-hoc Tukey HSD tests. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using R software (version 3.6.2)

3. RESULTS

3.1. BIOMASS PRODUCTION

The untreated tailings inhibited the growth of biomass crops which were negatively affected by the extreme characteristics of tailings and exhibited severe metal toxicity and nutrient deficiency symptoms with very low production for both above- and below-ground biomass as shown in Figure 1. The most pronounced positive effect on biomass for the three cultivated species was a result of BS application with biomass increased proportionally to the increasing BS application rate. However, a positive interaction between BS application and inoculation with the MF further improved plant growth and increased biomass production yet the increase was more notable with miscanthus than with poplar and willow. For example, the interaction of the low rate of BS with MF resulted in 10, 28, and 105% increase in biomass of poplar, willow, and miscanthus as compared to non-inoculated BS as shown in Figure 1. Interaction with the higher rate of BS also resulted in further increase of 23, 16, and 92% in biomass of poplar, willow, and miscanthus as compared to non-mycorrhizal plants grown in non-inoculated BS treatments.

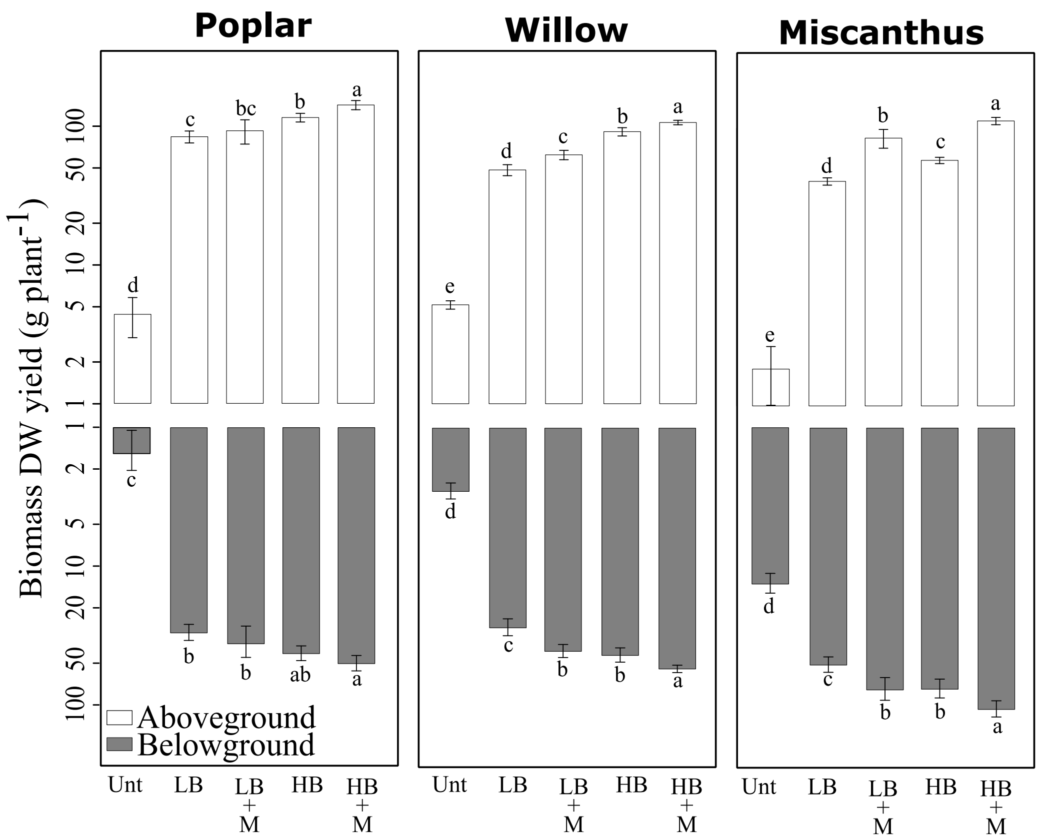

Figure 1. Shoot and root DW yields (g DW plant^{-1}) of poplar, willow and miscanthus grown in mine tailings (Unt) treated with biosolids at low (LB) or high (HB) application rates non-inoculated or inoculated with mycorrhiza fungi (M). Mean values per treatment (n=6). Values with different letters differ significantly (one-way ANOVA, p value <0.05).

3.2. MYCORRHIZAL ROOT COLONIZATION

Biosolids facilitated an effective mycorrhizal infection of plant roots for the three cultivated species. All mycorrhizal structures were observed colonizing plant roots including: arbuscules, vesicles, intraradical and extraradical hyphae as shown in Figure 2. While all these structures were observed colonizing the roots of each species tested, colonization in miscanthus was associated with high density of arbuscules compared to poplar and willow which in contrast exhibited high colonization density of vesicles.

Further, the frequency of extraradical hyphae was evenly observed among the cultivated species. Nevertheless, the intraradical hyphae were mostly associated with poplar.

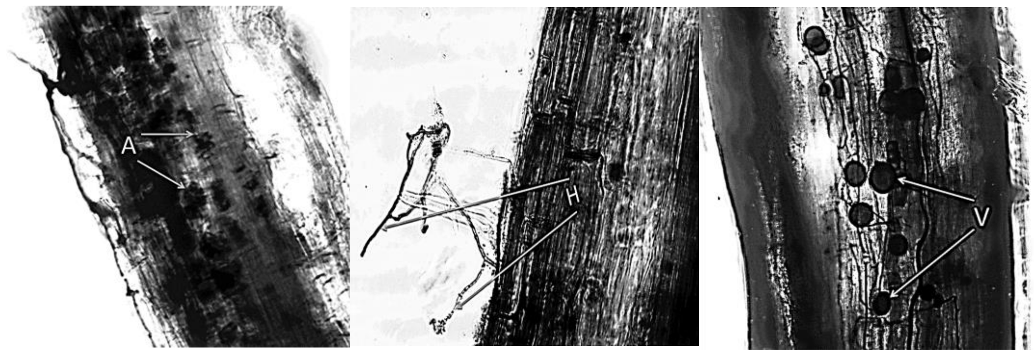

Figure 2. Arbuscular mycorrhizal fungi structures colonizing plant roots after 8-month growth in biosolids treated tailings and inoculated with endo- and ectomycorrhizal fungi: Arbuscule (A) in miscanthus, hyphae (H) in willow, and vesicles (V) in poplar.

3.3. PLANT NUTRITIONAL STATUS

The concentrations of macronutrients (N, P, K, Ca, and Mg) and micronutrients (Fe and Mn) in plant leaves are shown in Table 2. Nitrogen nutrition was mainly associated with BS treatment and was within the normal ranges reported in healthy plant i.e., 10-60 g kg^{-1} (Barker and Pilbeam, 2015), yet very close to the lower end. Miscanthus N nutrition, on the other hand, was below the normal range. While P was also mainly affected by BS application, further enhancement in P plant nutrition was observed as an impact of interaction with MF inoculation and this was more notable in miscanthus resulting in 31 and 55% increase in P uptake compared to non-inoculated BS treatments at low and high application rates, respectively.

Table 2. Leaves ionome of poplar, willow and miscanthus after 8-months of plant growth.

Treatments	Elements (mg kg^{-1})				(g kg^{-1})				
	Cd	Ni	Mn	Fe	Ca	Mg	N	P	K
Poplar									
Unt	0.53±0.08a	15.83±1.47a	35±1a	26±15b	11±1a	14±1a	8.4±1.0c	0.92±0.29b	2.79±0.76a
LB	0.36±0.13ab	1.97±0.36b	31±5a	94±11a	10±1a	10±2a	12.0±1.3b	1.38±0.28ab	2.64±0.41a
LB+M	0.12±0.09b	1.50±1.20b	33±8a	153±39a	12±2a	14±2a	13.1±1.7a	1.54±0.19a	2.25±0.44a
HB	0.50±0.09a	2.39±0.44b	39±8a	99±16a	10±2a	11±1a	13.2±2.2a	1.47±0.04ab	2.44±0.22a
HB+M	0.32±0.06ab	1.67±0.28b	35±3a	125±20a	12±1a	13±1a	13.9±2.2a	1.76±0.12a	3.42±0.27a
Willow									
Unt	1.25±0.03b	50.7±12.77a	223±9b	44±7c	24±1a	26±2a	9.4±1.2 b	0.65±0.09d	3.39±0.42ab
LB	2.13±0.12a	9.90±1.50b	443±13a	63±3bc	28±2a	18±2bc	13.3±1.9a	1.84±0.12c	1.98±0.09c
LB+M	0.58±0.04c	3.40±0.70c	179±17bc	70±6b	25±3a	18±1c	13.9±1.8a	2.63±0.07a	2.16±0.04bc
HB	1.11±0.13b	6.10±1.70b	409±60a	74±9b	29±3a	23±3ab	13.7±2.0a	2.01±0.20bc	2.12±0.34bc
HB+M	0.49±0.12c	2.40±0.11c	112±23c	101±11a	27±1a	17±1c	13.8±1.9a	2.33±0.21ab	3.63±0.95a
Miscanthus									
Unt	<dl	5.82±0.02a	24.28±3.59b	51±4c	5.01±0.17b	5.68±0.24a	4.7±1.1b	0.23±0.03d	6.01±1.03a
LB	<dl	3.89±1.56a	52±11.52ab	72±4b	8.28±0.18ab	7.19±0.34a	8.0±1.3a	0.93±0.05c	2.7±0.33b
LB+M	<dl	3.43±0.66a	43.03±16.93ab	88±12ab	9.06±2.74a	7.96±1.82a	8.8±1.6a	1.22±0.04b	3.07±0.16b
HB	<dl	2.78±1.17a	58.21±9.24a	76±8ab	9.17±0.9a	7.33±0.5a	8.0±0.9a	1.01±0.05c	4.06±0.3b
HB+M	<dl	3.50±2.25a	47.79±15.2ab	93±7a	8.85±1.03a	7.28±1.4a	9.2±1.7a	1.56±0.14a	3.1±0.42b
Common Values [a,b]	-	0.1-6	50-500	20-300	1-50	1-5	10-60	1-10	20-50
DATL [c]	10	100	2000	500	-	-	-	-	-

Treatments as follows: untreated mine tailings (Unt), treated with biosolids at low (LB) or high (HB) application rates non-inoculated or inoculated with mycorrhizal fungi (M).
Mean values ± SD per treatment (n=6). Different lowercase letters indicate significant differences between treatments (one-way ANOVA, p-value < 0.05). <d.l. = below detection limit.
[a] Tremel-Schaub and Feix (2005) [b] Barker and Pilbeam (2015) [c] Domestic animal toxicity limit (DATL) i.e., the maximum tolerable level for cattle according to NRC (2005).

Other macronutrients i.e., K, Ca, and Mg were less affected by tailings treatment, K concentrations were far below the common ranges exhibiting deficiency symptoms, whereas Mg concentrations were higher than that reported in normal plants.

Micronutrients Fe and Mn exhibited different behavior to tailings treatment. Biosolids application enhanced Fe nutrition and interaction with MF further increased Fe uptake yet not statistically significant. Manganese, on the other hand, was not affected by tailings treatment, except in willow where BS application resulted in high Mn accumulation which was significantly alleviated by MF interaction with BS as shown in Table 2.

3.4. PLANT METAL UPTAKE

Figure 3 presents Cu, Pb, and Zn metal accumulation in leaves of poplar, willow, and miscanthus. Irrespective of plant species, BS application significantly decreased Cu accumulation in leaves, with no significant further reduction as an effect of MF inoculation. Nevertheless, Cu uptake by all the three species was generally low. Willow and miscanthus accumulated higher Pb concentration compared to poplar. While BS application significantly reduced Pb uptake in miscanthus it had no significant effect on poplar and willow. However, remarkable decrease in Pb concentrations was observed as an interactive effect of BS with MF in the three cultivated species. The MF inoculation, for example, induced 62 and 29% reduction in Pb uptake by poplar compared to non-inoculated low and high BS application rates, respectively (Figure 3). Biosolids application significantly reduced Zn uptake in the three cultivated species and further reduction was observed as an interactive effect of BS and MF particularly in poplar and

willow which accumulated higher concentrations of this element. Similarly, MF inoculation was also more effective than applying BS alone in reducing Cd and Ni uptake in willow which tended to accumulate higher concentrations of these metals as shown in Table 2.

3.5. SUBSTRATE ANALYSIS

The interactive effect of BS application and MF inoculation along with plant cultivation impacted tailings physicochemical and biological properties and significantly affected metal bioavailability. As shown in Table 3, BS application induced pH reduction, however, no significant effect on pH observed as a response to MF inoculation. Total nitrogen and Olsen P were unsurprisingly found to be mainly affected by the BS application and their concentrations increased proportionally with increasing the BS application rate. Interestingly, significant impact of MF inoculation was observed on OC which offsets the miscanthus-induced mineralization on OC compared to unplanted substrates (Table 2). More importantly, the MF inoculation induced increase in OC in poplar and willow surpassed that observed in the respective unplanted substrates indicating their interactive effect for potential OC input which is of great importance especially in mine tailing’s restoration. The interactive effect of MF and BS was also evident in further improving biological properties of the tailings as measured by enzyme activities in the bulk and rhizosphere samples as shown in Figure 4.

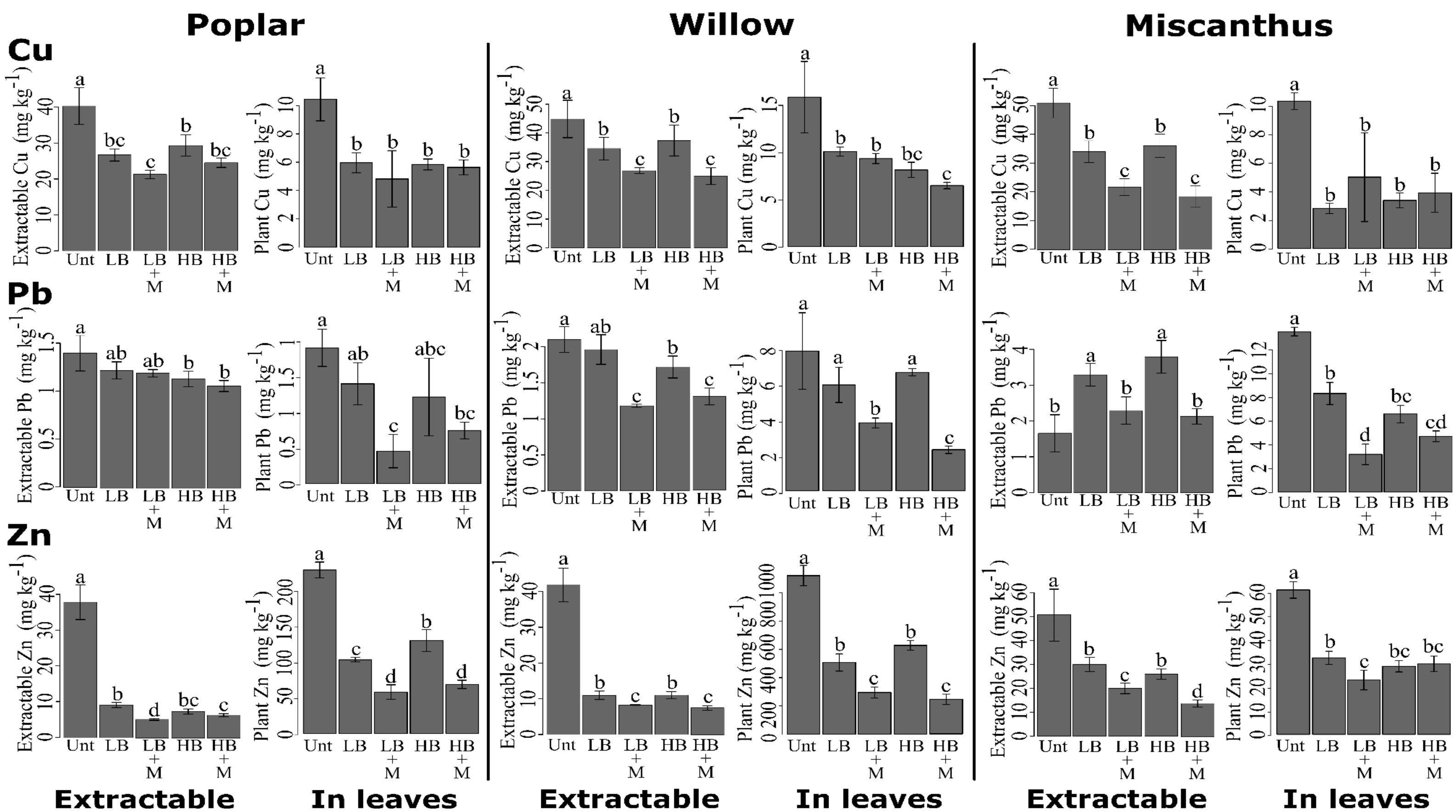

Figure 3. Copper, Pb and Zn concentrations (mg kg^{-1}), extractable (1 M NH_4NO_3) and in the leaves of poplar, willow and miscanthus grown in mine tailings (Unt) treated with biosolids at low (LB) or high (HB) application rates non-inoculated or inoculated with mycorrhiza fungi (M). Mean values per treatment (n=6). Values with different letters differ significantly (one-way ANOVA, p value <0.05).

The interactive effect of BS application and MF on metal bioavailability is presented in Figure 3. Biosolids application was more effective in reducing Cu and Zn extractable fractions particularly Zn, however, BS were less effective in immobilizing Pb and even induced higher extractability under miscanthus cultivation. Biosolids application and inoculation with MF was found to have a great positive effect in reducing bioavailability of the three tested metals as shown in Figure 3. The notable effect of MF inoculation on Pb immobilization under willow and miscanthus is very important as BS alone was not effective in reducing availability of this toxic metal.

4. DISCUSSION

In degraded soil systems such as mine tailings, MF development is also strongly hindered. According to Wang (2017), in order for a MF-plant symbiotic relationship to develop it has to be beneficial for both and if a plant is extremely stressed due to the severe soil characteristics such as in mine tailings it cannot provide the MF with their needs to proliferate particularly at initial stages. In our previous study Al-Lami et al. (2019), when the same species cultivated here inoculated with MF alone and evaluated for potential biomass production and impact on tailings characteristics, a limited effect was observed. In the current study, ameliorating tailings characteristics with BS facilitated the development of a beneficial and interactive relationship between MF and all the three plant species tested, resulting in a great improvement in plant growth and a synergistic effect on tailings properties. Other studies have also found amelioration of mine tailings characteristics with organic-rich amendments facilitates a beneficial MF-

plant interactions. Madejón et al. (2012) found that application of BS to acidic arsenical sulphidic mine tailings increased both ecto- and endo-mycorrhizal colonization in roots. The same authors reported a beneficial relationship between BS and arbuscular MF during revegetation of acidic sulphidic gold mine tailings (Madejón et al., 2010). Compost application was also found to facilitate a positive interaction with MF during phytostabilization of acidic Pb/Zn mine tailings (Solís-Domínguez et al., 2011), or during revegetation of acidic Cu mine tailings (Pérez et al., 2021).

The positive impact of MF inoculation on biomass production observed in this study can be attributed to several mechanisms. Among them, increased nutrient acquisition particularly P which is often deficient under alkaline conditions such as the case of this study (Wahid et al., 2019). This is supported by the great increase in miscanthus biomass in line with the increase in P concentrations in leaves (Table 2). The improved nutrient acquisition is not limited to P, and the increased microbial activity observed in this study (Figure 4) implies the provision of nutrient cycling ecosystem service by MF, thus facilitating nutrient uptake by host plant (Barea et al., 2005). Another mechanism by which MF may have enhanced plant growth can be attributed to the induced metal immobilization thus limiting bioavailability and phytotoxicity (Janeeshma and Puthur, 2020). This mechanism was probably more effective under miscanthus than poplar and willow as cultivation of the former induced substrate acidification (Table 3) and subsequently metal mobilization as shown in Figure 3. This could further explain the superior interactive impact observed between BS and MF on miscanthus growth compared to poplar and willow, which yielded up to 2-fold biomass increase compared to BS alone.

Table 3. Main physicochemical properties of substrate 8-months after amendment and plant cultivation.

Treatments	pH	OC (%)	TN (%)	Olsen P (mg kg^{-1})
Poplar				
Unt	7.80±0.07a	0.45±0.03d	0.03±0.01c	2.71±0.44d
LB	7.58±0.10b	1.67±0.10c	0.21±0.04b	41.32±2.68c
LB+M	7.63±0.15ab	2.18±0.14b	0.17±0.06b	43.71±4.27c
HB	7.50±0.13b	2.08±0.17b	0.30±0.09a	53.56±4.64b
HB+M	7.57±0.12b	2.55±0.13a	0.27±0.10a	62.02±5.49a
Willow				
Unt	7.70±0.07a	0.47±0.04d	0.02±0.01b	2.94±0.43c
LB	7.50±0.10b	1.53± 0.09c	0.18±0.02a	37.13±3.14b
LB+M	7.50±0.15b	2.09± 0.13b	0.16±0.01a	37.37±2.58b
HB	7.45±0.08b	2.03± 0.16b	0.23±0.04a	55.58±3.06a
HB+M	7.50±0.07b	2.46± 0.11a	0.20±0.03a	56.29±4.02a
Miscanthus				
Unt	7.49±0.09a	0.44±0.07d	0.03±0.01c	2.30±0.21c
LB	7.21±0.06b	1.37±0.11c	0.15±0.03b	31.75±2.58b
LB+M	7.20±0.09bc	1.98±0.16b	0.13±0.03b	28.40±1.73b
HB	7.10±0.09d	1.82±0.06b	0.20±0.03a	43.60±4.71a
HB+M	7.14±0.04cd	2.21±0.20a	0.18±0.02a	38.29±4.33a
Unplanted				
Unt	7.68±0.13a	0.47±0.03c	0.05±0.01c	3.58±0.49c
LB	7.45±0.06b	1.62±0.09b	0.24±0.02b	52.02±4.63b
LB+M	7.40±0.09b	1.78±0.11b	0.27±0.04b	47.48±5.92b
HB	7.38±0.06b	2.12±0.13a	0.37±0.03a	66.63±1.44a
HB+M	7.35±0.13b	2.25±0.12a	0.36±0.04a	66.35±4.98a

Treatments as follows: untreated mine tailings (Unt), treated with biosolids at low (LB) or high (HB) application rates non-inoculated or inoculated with mycorrhizal fungi (M). Mean values ± SD per treatment (n=6). Different lowercase letters indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

Mycorrhizal fungi have been shown to influence metal-plant and metal-soil interactions in many ways, including but not limited to, inducing soil physicochemical changes in the soil by altering root exudation system or the input of OC such as the effect observed in this study (Table 2) which can significantly induce metal retention rendering

them less toxic (Kaur and Garg, 2018). Mycorrhizal fungi may also directly affect metal availability by sequestration within their vesicles, arbuscules, intraradical and extraradical hypha, in addition to the release of mycelium products such as polysaccharides and glomalin related soil proteins which may act as metal chelators, thus inducing metal complexation and rendering them less bioavailable (Janeeshma and Puthur, 2020).

The increased OC content observed in this study (Table 3) as an interactive effect of BS and MF with plant species is of great importance especially in the reclamation efforts of extremely degraded ecosystems such as mine tailings. And more importantly this OC input was associated with increased microbial activity (Figure 4) which implies the stimulation of soil functioning and development processes. The potential mechanisms by which MF facilitate OC input can be through direct input of mycelium products or indirect input through modification of plant root morphology and architectural. Development of extended and lateral fine roots has been linked to MF colonization, the turnover of fine root biomass is considered a great contributor to soil OM (Vogt et al., 1986). Thus, the extended development of fine roots can be a strategy to increasing the belowground litter inputs to soil OM within larger volume of tailings profile. As mentioned above, the MF can also contribute to soil OM input through the turnover of mycorrhizal hyphae and external mycelium (Godbold et al., 2006). Thus, the benefits of the mutual relationship between most plants and MF, should be employed during mine tailings restoration and revegetation efforts to stimulate soil functioning and formation processes.

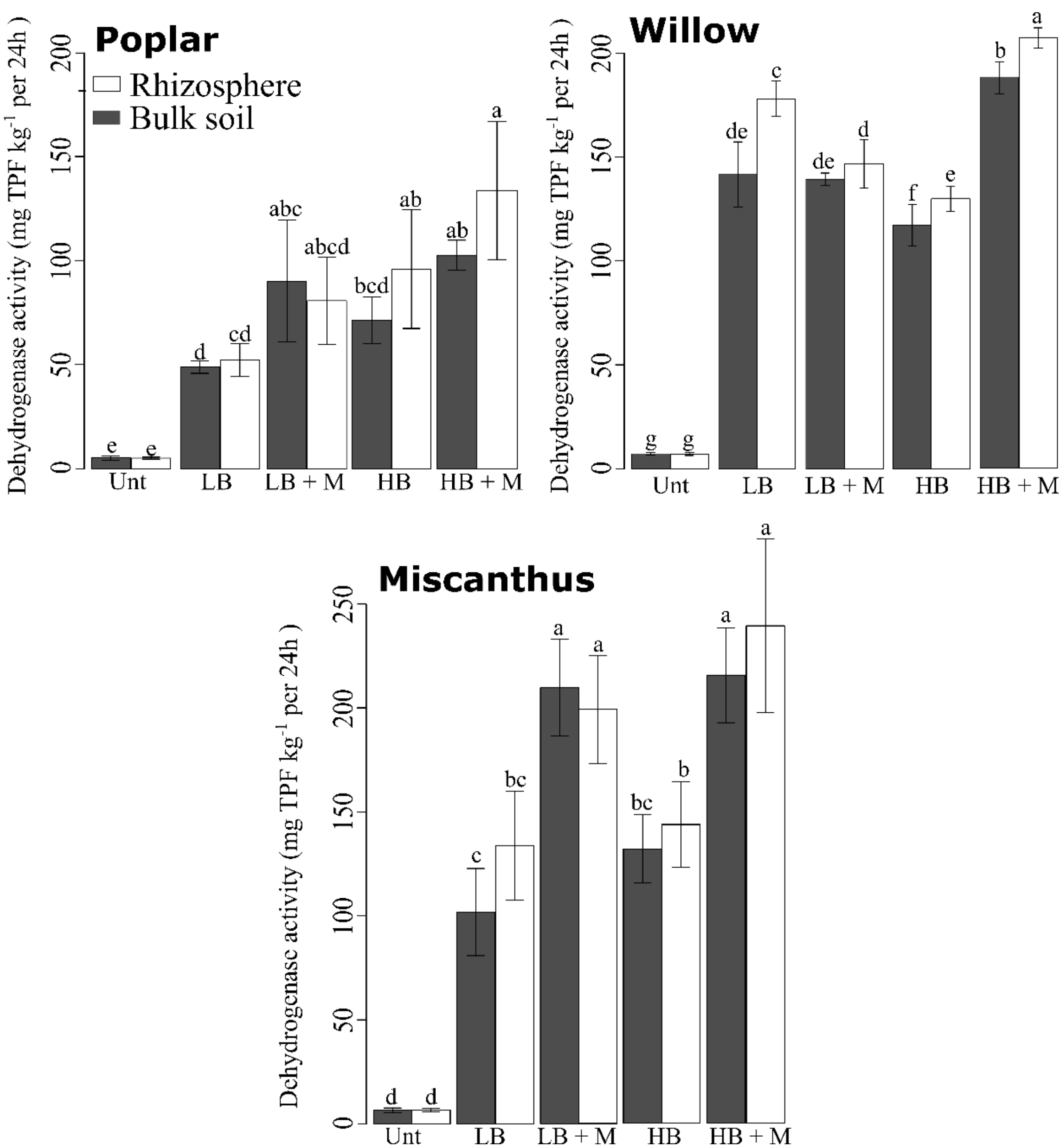

Figure 4. Microbial dehydrogenase enzyme activity (DHA) in (mg TPF kg^{-1} $24h^{-1}$) of the bulk soil (grey bars) and the rhizosphere (white bars) of poplar, willow and miscanthus grown in mine tailings (Unt) treated with biosolids at low (LB) or high (HB) application rates non-inoculated or inoculated with mycorrhiza fungi (M). Mean values per treatment (n=6). Values with different letters differ significantly (one-way ANOVA, p value <0.05).

5. CONCLUSIONS

Biosolids application facilitated a functional and efficient MF-plant root colonization which resulted in several interrelated positive impacts on plants responses and mine tailings properties. Compared to BS alone, inoculation with MF further enhanced plant growth and significantly increased biomass production for the three cultivated species. However, MF inoculation had a superior effect on miscanthus biomass production with an increase of 2-fold compared to BS alone. The MF inoculation further enhanced P uptake compared to BS alone with the greatest effect also observed in miscanthus, and reduced metal accumulation in plant leaves for the three cultivated species. In addition, the interactive effect of MF inoculation and BS application was also observed on tailings substrates which represented by significant increases in OC input and further reduction in metal extractability particularly Pb as BS alone was not effective in reducing this metal bioavailability. Inoculation of MF and BS also further enhanced microbial activity measured by DHA. This effect along with the other observed benefits especially the enhanced OC input implies the great role MF-plant interactions played in stimulation of soil formation processes that is particularly important in mine tailings ecorestoration efforts.

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IV. HIGH THROUGHPUT SCREENING OF NATIVE SPECIES FOR TAILINGS ECO-RESTORATION USING NOVEL COMPUTER VISUALIZATION FOR PLANT PHENOTYPING

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ABSTRACT

Historical hard-rock mine activities have resulted in nearly half a million mining-impacted sites scattered around the US. Compared to conventional remediation, (aided) phytostabilization is generally cost-effective and ecologically productive approach, particularly for large-scale sites. Native species act to maintain higher local biodiversity, providing a foundation for natural ecological succession. Due to heterogeneity of mine waste, revegetation strategies are inconsistent in approach, and to avoid failure scenarios, greenhouse screening studies can identify candidate plants and amendment strategies before scaling up. This greenhouse study aimed to concurrently screen a variety of native species for their potential to revegetate Cu/Pb/Zn mine tailings and develop a high throughput and non-destructive approach utilizing computer vision and image-based phenotyping technologies to quantify plant responses. A total number of 34 species were screened in this study, which included: 5 trees, 8 grasses, and 21 forbs and legumes. Most of the species tested were Missouri native and prairie species. Plants were non-destructively imaged, and 15 shape and color phenotypic attributes were extracted

utilizing computer vision techniques of PlantCV. Compared to reference soil, all species tested were negatively impacted by the tailings' characteristics, with lowest tolerance generally observed in tree species. However, significant improvement in plant growth and tolerance generally observed with biosolids addition with biomass surpassing reference soil for most legumes. Accumulation of Cu, Pb, and Zn was below Domestic Animal Toxicity Limits in most species. Statistically robust differences in species responses were observed using phenotypic data, such as area, height, width, color, and 9 other morphological attributes. Correlations with destructive data indicated that area displayed the greatest positive correlation with biomass and color the greatest negative correlation with shoot metals. Computer visualization greatly increased the phenotypic data and offers a breakthrough in rapid, high throughput data collection to project site-specific phytostabilization strategies to efficiently restore mine-impacted sites.

1. INTRODUCTION

Historic mining activities have left a legacy of mining-impacted sites. Within the US, hard-rock mining has resulted in approximately half a million such sites scattered around the country (Lyon et al., 1993). Mine tailings, particularly due to their unfavorable physical, chemical, and biological properties, are generally not vegetation supportive and have poor soil structure and fertility. As a result, the tailings surface remains highly susceptible to aeolian dispersion and water erosion (Hayes et al., 2014; Mendez and Maier, 2008). Conventional remediation technologies include physical treatments that entail covering the tailings surface with clay or topsoil cap or chemical

treatments that involve using resinous agents that form a crust over the tailings and provide temporary stabilization (Tordoff et al., 2000). While the estimated cost for treating 1 m^3 of tailings using the above-mentioned techniques varies, it can reach up to US$450 using chemical treatments, making them unsuitable for large-scale sites due to the high projected cost (Mendez and Maier, 2008). Moreover, these techniques may add more disturbance to the environment (Wang et al., 2017). Approaches to target establishing vegetation, called phytostabilization, offer an alternative environmentally- and eco-friendly remediation approaches to reduce the remediation cost to US$0.40-26 per m^3 (Henry et al., 2013; Mendez and Maier, 2008).

In phytostabilization, a vegetation cover is planted directly into the tailings to create a self-sustaining biological cap that acts to provide long-term stabilization of tailings and reduces the risk of contaminant spread to the surrounding environments. The plant canopy decreases air dispersal by reducing wind speed just above the tailings surface, while root development provides structural support and stability against both wind and water erosion and decreases leaching to the groundwater (Ciarkowska et al., 2017; Wang et al., 2017). In phytostabilization, the goal is also to minimize plant uptake and any aboveground metal accumulation. Plant roots may still sequester significant amounts of metals by accumulation within root tissue or adsorption onto the root surface (Cunningham et al., 1995). In addition, plants provide phytocatalyzed immobilization of metals within the root zone, such as for instance, bio-precipitation driven by plant root exudates or metal chelating substances excreted by the associated rhizosphere microorganisms (Ayangbenro and Babalola, 2017; Gil-Loaiza et al., 2016).

Plant growth on mine tailings is generally hindered by their unfavorable characteristics and severity of disturbance. Thus, phytostabilization must often be assisted with an ameliorative approach that attempts, through the addition of a proper soil amendment, to achieve optimum conditions to sustain plant growth, particularly at the initiative stages until reaching a state of self-sustaining (Mendez and Maier, 2008; Tordoff et al., 2000). Biosolids (BS), one of the most widely used amendments, have proven to enhance plant growth on mine tailings by improving their physicochemical and hydraulic properties and stimulating microbial growth and activity (Al-Lami et al., 2019; Alcantara et al., 2015; Ciarkowska et al., 2017; Ploughe et al., 2020).

While the primary goal of phytostabilization is to reduce metal bioavailability and mobility, recently, there is a growing interest in a more ecologically conscious phytostabilization approach that also aims to promote land reclamation and ecosystem functions restoration (Beale and Boyce, 2020; Burges et al., 2018; Swab et al., 2017). Such an approach relies on the selection of native and/or indigenous species that adapt readily to the local environment and act to maintain higher local biodiversity and provide a foundation for natural ecological succession (Mendez et al., 2007; Swab et al., 2017). In the Midwestern US, native prairie grasses and forbs can be a great choice to achieve the abovementioned reclamation goals owing to their unique characteristics and role in soil-building processes. Generally, prairie species are well-adapted to the reoccurring local drought and have high tolerance to salt and low nutrient conditions (Miller, 1998). They have deeply grown and extensive root systems that typically form symbiotic relationships with rhizosphere beneficial microorganisms, such as mycorrhizal fungi, rhizobia, and rhizobacteria, leading to higher rates of soil carbon input, nitrogen fixation and nutrient

cycling (Block et al., 2020; Guzman et al., 2016). Therefore, revegetation with a mix of prairie grasses and forbs can significantly improve the physicochemical and biological properties of mine tailings while maintaining long-term stabilization (Block et al., 2020; Chandrasoma et al., 2016; Swab et al., 2017; Zhang et al., 2020). These pioneer plants are attractive throughout the year, providing preferred habitats for diverse wildlife and promoting colonization of other native species that are less adaptive, thus maintaining functional diversity and integrating the site into the local ecosystem (Beale and Boyce, 2020; Miller, 1998; Swab et al., 2017).

Due to heterogeneity of mine tailings, no universal revegetation strategy exists. For a given site, with unique characteristics, a specific strategy should be designed depending on the tailings' characteristics, type, concentration, and extent of metals. Therefore, to avoid failure scenarios, greenhouse screening studies can identify candidate plants and proper amendments to aid in establishing the vegetation cover before scaling up to the field. Gil-Loaiza et al. (2016) demonstrated that 60-day preliminary greenhouse studies guided a successful phytostabilization in field trials, and only a single compost application sustained growth of native species over four years and promoted soil development of acidic mine tailings. Nevertheless, the assessment of greenhouse studies often involves destructive sampling, time-consuming, and costly chemical analysis, making it infeasible to screen a wide range of phytostabilization options. Hence, development of cost-effective and high throughput assessment techniques will significantly benefit efficient screening and successful full-scale application.

Abiotic and biotic environmental stresses generally induce biochemical and physiological disorders and adversely impact plant metabolism (Barceló and

Poschenrieder, 1990; Nagajyoti et al., 2010). Consequently, leading to longer-term visible injuries and phytotoxic responses such as reduced growth rates, decreased biomass, tissues deformation, leaf chlorosis/necrosis, and eventually plant death (Yadav, 2010). Phenotyping morphological and physiological traits of plants can reveal specific information regarding their responses to environmental stress. Image-based high throughput phenotyping can dramatically facilitate phenotyping efforts through fast and non-destructive measurements (Fahlgren et al., 2015). Depending on the targeted traits, a variety of imaging techniques have been used in plant phenotyping. Visible light (RGB) imaging has been used for measuring plant morphological traits such as area, height, width, number of leaves, and color. Whereas, hyperspectral, thermal, near-infrared (NIR), and fluorescent imaging have been used for assessing abiotic and biotic stresses (Armonienė et al., 2018; Li et al., 2020). Image-based phenotyping is a valuable approach to measure plant traits; however, some issues have arisen such as problems with segmentation of plant material from the background or inaccurate estimation of plant growth based on area measurements as affected by overlapped leaves, leaf curling and twisting, especially with one-view imaging (Humplík et al., 2015; Tovar et al., 2018). While commercial phenotyping platforms are powerful tools to collect consistent data, they are generally cost prohibitive for wide spread application (Tovar et al., 2018). In this study, we sought to develop a rapid, non-destructive, cost-effective, and high throughput biomonitoring and screening approach utilizing computer visualization and image-based phenotyping technologies to quantify plant responses to abiotic stress (i.e., metal toxicity and nutrient deficiency) through the analysis of morphological- and color-based stress indicators. Morphological- and color traits are extracted from the image using Plant

Computer Vision (PlantCV) (Fahlgren et al., 2015), PlantCV is an open-source image processing software for plant phenotyping to quantify statistically robust differences among plants in response to environmental factors. Transient responses to abiotic stress can also be efficiently measured as the method is non-destructive and rapid. To the best of our knowledge, this study presents the first application of computer visualization for biomonitoring of tailings treatment and concurrent eco-restoration with a wide variety of prairie species. This novel approach can greatly improve phytostabilization efforts of mine lands by facilitating the screening of a broader pallet of plants and amendment strategies, which helps in setting a site-specific strategy that ensures successful revegetation before scaling up.

The objectives of the present study were to: 1) improve the abiotic tailings deposit conditions by using BS from neighboring lagoons as an amendment option to allow the sustainable growth of native species; 2) screen a large number of native prairie and indigenous species to identify those with great potential for ecologically conscious aided phytostabilization; and 3) develop a new assessment approach utilizing computer vision and image-based analysis to enable early detection and quantification of plant tolerance and response to metal stress at low cost and high throughput.

2. MATERIALS AND METHODS

2.1. TAILINGS, BIOSOLIDS, AND REFERENCE SOIL

The tailings used in this study were collected from The New Viburnum Tailings Impoundment, which belongs to The Viburnum Mine/Mill Facility #28, and located in

Viburnum, MO, US (37°42’09.0"N, 91°06’18.4"W). This facility has a long history of lead mining and was in operation for about 41 years. Mine waste disposal at tailings impoundment continued until 2000. The tailings were collected from the top layer (0-30cm), transported to the greenhouse, air dried, crushed, sieved to 2mm, and thoroughly mixed. The tailings characterized by elevated concentrations of metals, particularly Cu, Pb, and Zn, with a slightly basic pH (Table S1). Detailed description and characterization of the tailings can be found in Al-Lami et al. (2019). Biosolids used in this study were collected from Viburnum city sanitary lagoons, which are located at a distance of 1 km North-West of the tailings impoundment (37°42’36.8"N, 91°07’04.6"W). Biosolids have been accumulated in these lagoons for about 40 years and are located very close to the tailings impoundment, these sludge characterized by high concentrations of metals, particularly Cu, Pb, and Zn (Table S1). The collected sludge transported to the greenhouse, dewatered, and dried to about 35% solids content before using it as an amendment. Uncontaminated soil was also included in this experiment to serve as a reference soil. The reference soil was collected from an undisturbed site with similar forecrop history, which is located at a distance of 9 km North-East of the tailings impoundment (37°46’22.6"N, 91°03’26.6"W). More characteristics of the reference soil are presented in (Table S1).

2.2. PLANT SELECTION AND TREATMENT PREPARATION

Plant species were selected based on a verity of beneficial characteristics, including adaptive and tolerance, the potential for building soil profile through carbon sequestration and nitrogen fixation, as well as the unique ecological characteristics of

these species that typically attract diverse wildlife thus promoting ecosystem restoration. As listed in Table S2, a total number of 34 plant species were tested in this study, which included: 5 trees, 8 grasses, and 21 forbs and legumes. With the exception of 3 introduced legume species, all plants tested were Missouri native and prairie species. Some of these species were identified as indigenous to the mine tailings site (Table S2). These species have shown the ability to establish as juvenile plants initially, but not yet effectively colonize the site.

Plant seeds and dormant cuttings were obtained from local nurseries. Poplar and willow were planted from pre-rooted cuttings. All other plants were started from seeds with different seed treatments prior to sowing, including cold stratification, scarification, and soaking in warm water. Seeds of each of the legume species were also inoculated with specific rhizobium bacteria just before planting. Plant screening in this study was achieved by conducting two greenhouse experiments, as described in the following.

2.2.1. Pre-Screening Experiment. The pre-screening phase aimed to screen all grasses and forbs/legumes species listed in (Table S2) for germination, early development, and viability when grown in tailings or BS-treated tailings. Seeds were germinated in plastic trays (~0.5 L cell size). Each cell was filled with ~400 g of untreated tailings or tailings treated with 3% BS (equivalent to 60 dry ton per hectare). Germinated seeds were allowed to grow for ~5 weeks under controlled greenhouse conditions of 20/27 °C night/day average temperature, around 80% relative humidity with 16 h photoperiod and watered 3 times a week to maintain about 70% of field capacity. A qualitative assessment of the viability of each species was then done using the following criteria (Table S3): seed germination rate and early survival were assessed

visually, and scores ranging from 0- no germination or followed by a quick death of seedlings to 3- very good germination; the cover-abundance scale using Braun-Blanquet (1964) method with scores ranging from 1- individuals with low surface cover (<5) to 5- individuals with high surface cover (75-100%); visual assessment of overall plant health was also recorded observing chlorosis/necrosis and seedlings vigor. Along with this, 20 young seedlings (1 week old) were transplanted to each pot to assess species survival rates after ~5 weeks growth period.

2.2.2. Screening Experiment. The screening experiment was conducted after concluding the pre-screening phase. This experiment aimed to screen the most promising species based on performance in the pre-screening phase, for their potential for revegetation and enhanced restoration of Cu/Pb/Zn mine tailings. As indicated in Table S2: a total number of 17 species (5 trees, 4 grasses, and 8 forbs/legumes) were selected to grow in this phase of the study. Tree cuttings and pre-treated seeds were planted in 2.2 L cylindrical plastic pots filled with ~1.35 kg of untreated tailings, tailings treated with 3% BS, or uncontaminated reference soil. Five replicates were used for each species. To examine the bioavailability of metals and nutrients, a set of five pots for each treatment was used for pore water extraction after incubation for one month and maintaining water availability to 70% of field capacity. Fifty milliliters of pore water were collected from each pot using inserted Rhizon MOM moister samplers (Eijkelkamp Agrisearch Equipment, The Netherlands).

2.3. IMAGE ACQUISITION AND DERIVED MEASUREMENTS

2.3.1. Image Acquisition. On week 15 of growth, six of the plant species growing in the screening experiment were non-destructively imaged (*D. illinoensis, D. purpurea, C. fasciculate, A. fruticosa, M. officinalis, and P. deltoids*). Imaging was conducted by manually loading individual plants into a constructed hexagon-shaped chamber (Figure S1), which was adapted from Appendix 4 of Tovar et al. (2018). The hexagon chamber (143.5cm height and 70cm of each hexagonal side) was constructed from polyvinyl chloride (PVC) pipes framing and covered with white fabrics. Three Raspberry Pi cameras were affixed to the chamber and set up to simultaneously imaging one top-view and two side-views approximately 90° angle apart of each plant loaded to the chamber. A color-checker card was affixed to the chamber wall next to the plant as a color reference and correction. Image acquisition was obtained by transferring captured images as .png files from the three Raspberry Pis to a connected laptop computer. A python script was written to automate the process of capturing the images and performing post-processing. All three Raspberry Pis capture an image simultaneously and send the raw data to a networked laptop where various post-processing steps are performed automatically.

2.3.2. Plant Segmentation Using Machine Learning. To facilitate automatic plant segmentation, a novel technique utilizing simple linear iterative clustering (SLIC) and multiple instance convolutional neural network was developed. This method uses the SLIC algorithm to group similar pixels together to form superpixels. The superpixels are distributed into two bags; one bag labeled positive and the other negative. The positive bag can contain both plant pixels and background pixels. The negative bag contains only background pixels. Once the bags were generated, they were fed into a modified

convolutional neural network to label each superpixel as either plant or background. An example of the SLIC algorithm performed on the dataset in various stages of the pipeline can be seen in Figure 1.

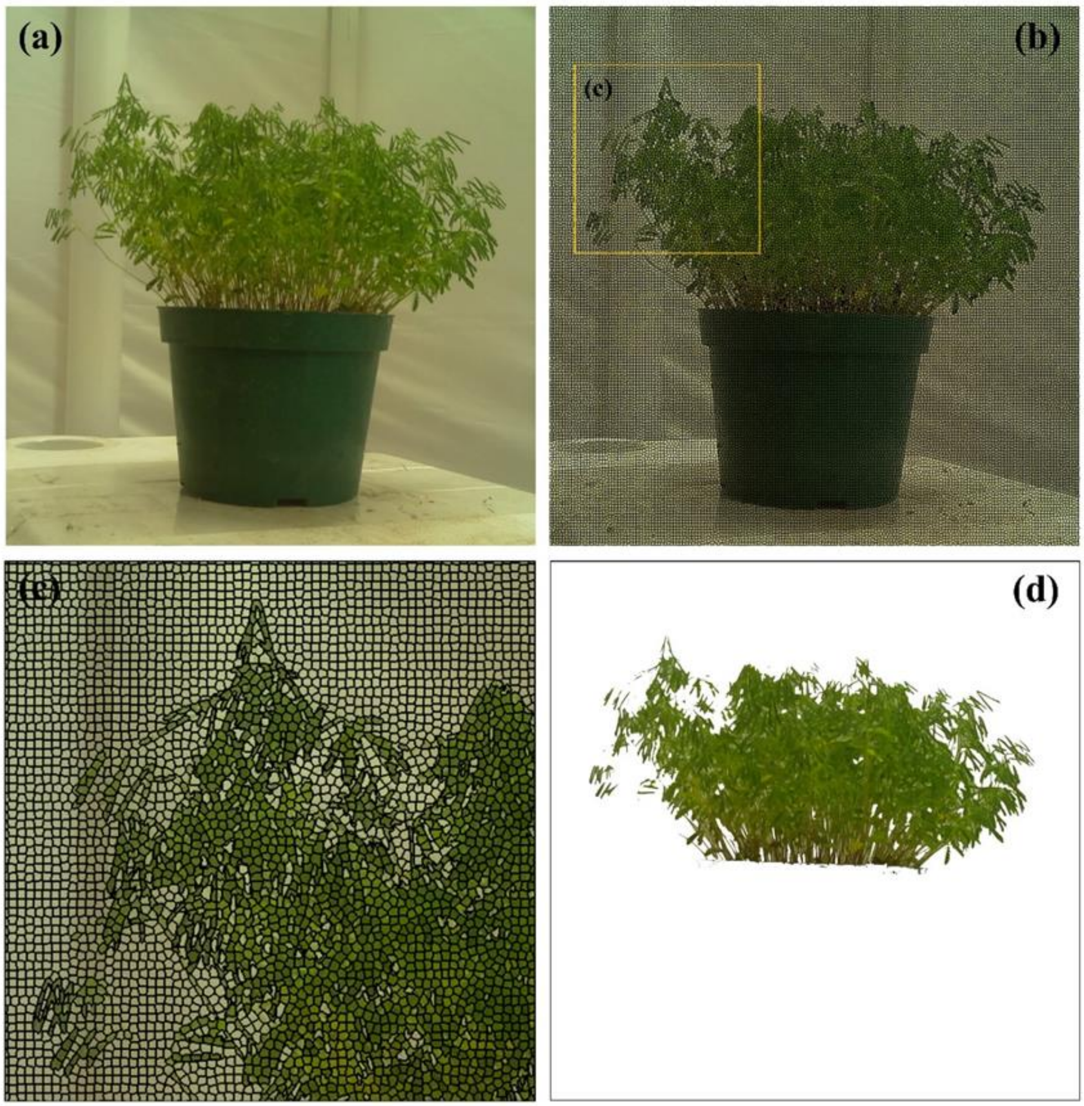

Figure 1. An example of image segmentation using machine learning: (a) Raw image, (b) Generation of simple linear iterative clustering, (c) Close up of generated clusters, and (d) Multiple instances learning segmentation applied to clusters.

The training set was generated using PlantCV segmentation pipeline. Binary thresholds were applied to various color channels in the image to segment vegetation from background. Bitwise operations combined all the binary thresholds to produce a single mask containing just vegetation. The superpixels were iterated through each superpixel and a bitwise add operation was performed with the PlantCV generated mask. If the operation generated a 1 value, then that superpixel was classified as positive and likewise.

2.3.3. Image Processing and Trait Extraction Using PlantCV. The raw images and the binary masks containing just plant pixels were imported into Python. The raw images were represented as a NumPy array of (x, y, c) where x, y are the coordinates of each pixel and c being the channel. The binary mask can also be represented as a numb array of (x, y); no channel information is required as the mask is a binary grayscale image. To extract a quantitative trait from a given image (Figure 2a and b), the binary mask is used to represent the shape of the plant in question (Figure 2c). The first step in extracting shape information is to generate contours of the binary mask. Contours can be explained simply as a curve joining all the continuous points (along the boundary), having similar intensity (https://docs.opencv.org/master/d4/d73/tutorial_py_contours_begin.html). The contours generated are stored as a list with the same dimensions as the binary mask array. This list of contours creates a hierarchy of contours which describes nested features. The contour in interest is the child of the region of interested parent contour. Once the desired contour and all of its child contours are found, the convex hull operation is applied. The convex hull generates the shape of a group of contours into a tight-fitting convex boundary. With

this convex hull, attributes such as area, height, width, perimeter are calculated from the resulting shape of the convex hull.

PlantCV v3.0.dev2 (Fahlgren et al., 2015; Gehan et al., 2017), an open-source image processing software coded in Python and targeted for plant phenotyping (https://plantcv.danforthcenter.org/), performs the steps above automatically, lowering the barrier to entry for imaged based phenotyping. A pipeline was then developed using PlantCV for the side-view and top-view images separately to measure 12 morphological attributes such as area, height, width, perimeter, convex-hull properties, and other parameters (Figure 2d). These measurements are presented in more detail in Enders et al. (2019). Color traits were extracted using the final masked plant material, and for each identified plant pixel, color intensity was recorded by PlantCV for RGB (red, green, blue) channels, HSV (hue, saturation, value), and LAB (lightness, green magenta, blue-yellow) (Figure 2e). Data files were then created that contain shape and color acquired information (Figure 2f). Outliers were defined as measurements that fall above and below ± 1.5 times the interquartile range.

2.4. BIOMASS AND TOLERANCE INDEX DETERMINATION

On week 16 of growth, developed shoots were harvested. Shoots were then washed with tap water, followed by washing with 0.1% HCl solution to remove any external deposits, and then thoroughly rinsed with deionized water thrice (Oliva and Raitio, 2003; Usman et al., 2019). Samples were oven-dried at 60 °C for 72 h, and subsequently weighed for aboveground biomass determination.

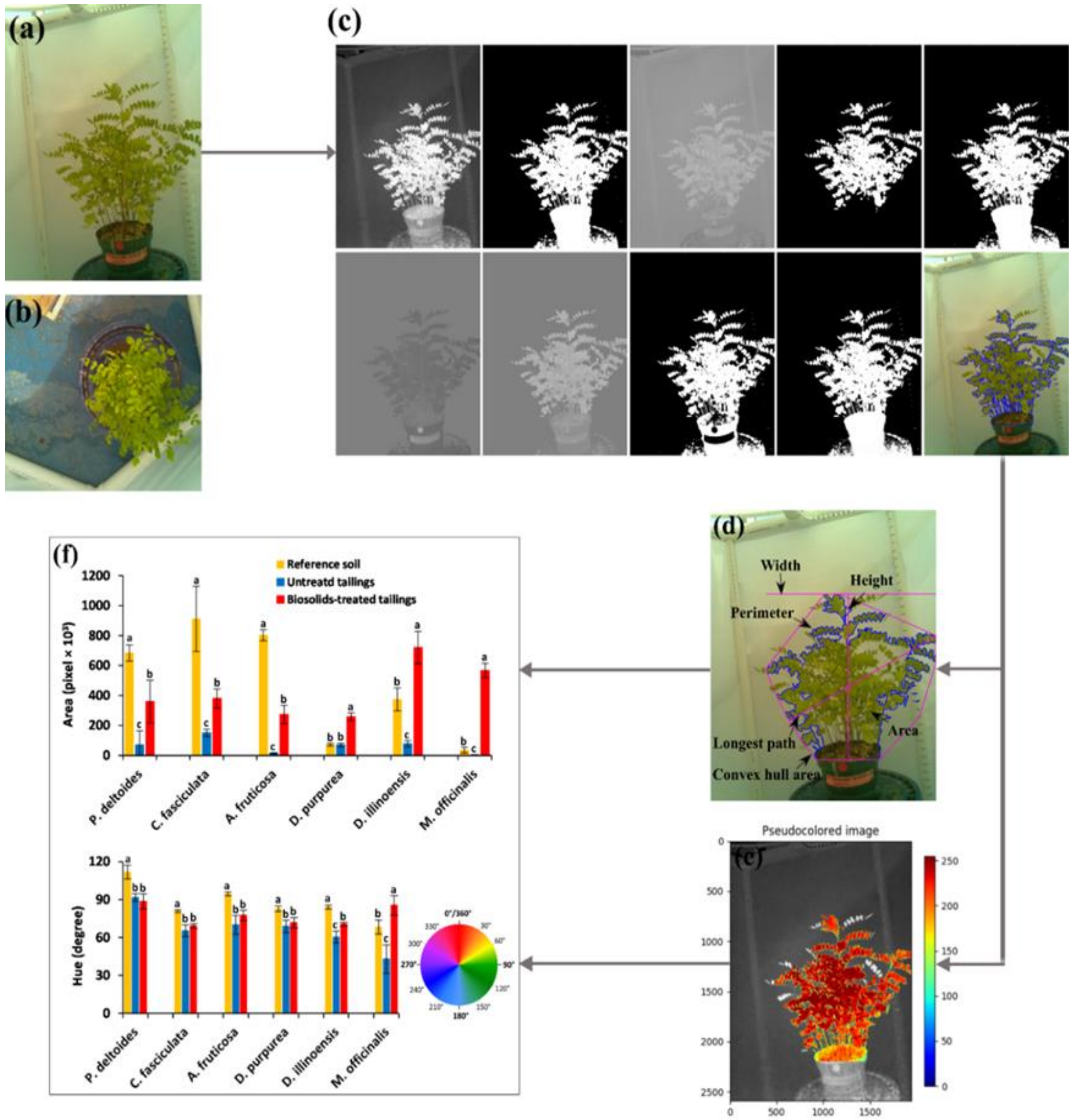

Figure 2. An example of image analysis and trait extraction using PlantCV (species A. *fruticosa* from biosolids-treated tailings on week 15 is shown): (a) Side view and (b) Top view example original raw.png images taken in the imaging chamber, (c) Various mask generation to isolate plant material from the background, (d) Morphological traits extraction, (e) Pseudo color based on hue values to ease in the detection of color variation, and (f) An example of data extracted: morphological trait (area in pixel) and color trait (hue in degree).

Plant's ability to tolerate metal stress was assessed by calculating its tolerance index (Ti) using the following equation (Meyer et al., 2016; Wang et al., 2016; Wilkins, 1978):

$$T_i(\%) = \frac{Biomass_T}{Biomass_R} \times 100\%$$

where $Biomass_T$ is the plant biomass when grown on untreated tailings or BS-treated tailings and $Biomass_R$ is the plant biomass when grown on unpolluted reference soil.

2.5. PORE WATER AND PLANT TISSUE CHEMICAL ANALYSIS

Aliquot of 15 ml of the collected pore water was acidified to 2%, and concentrations of Ca, Cu, Fe, K, Mg, Mn, P, Pb, and Zn in the acidified samples were determined using ICP-OES (Perkin Elmer, Avio 200). Measurements of pH and EC were performed on fresh samples using digital meters (Orion 370, Thermo Fisher Scientific, Inc; VWR, 61161-362, respectively). Immediately following pore water collection, dissolved organic carbon (DOC) and total nitrogen (N) were determined using TOC analyzer (Shimadzu© TOC 5000 A carbon analyzer).

Shoot samples were ground to < 1 mm particle size, and subsamples of 0.5 g were placed in polypropylene digestion vessels. Hot block digestion was then carried out by adding 5 ml of concentrated HNO_3 to each vessel and gradually heated to 60 °C for 0.5 h, followed by addition of 3 ml of 30% H_2O_2 and raising the temperature to 120 °C until sample volume reduced to ~2 ml (Huang and Schulte, 1985). Ultrapure MΩ water was added to each vessel to bring sample volume to 20 ml. Concentrations of Ca, Cu, Fe, K, Mg, Mn, P, Pb, and Zn in the digested samples were then determined using ICP-OES.

The method detection limit was determined using the linear regression method (Shrivastava and Gupta, 2011), and limits for Cu, Pb, and Zn (in mg L^{-1}) were 0.045, 0.068, and 0.04; respectively. For quality control, blanks, duplicates, and spikes were included in each batch tested. In addition, a certified reference material (WEPAL IPE-126; Zea mays) was also routinely included. Recoveries for spikes and reference material were all within the acceptable range of 80 to 120%, and the relative percent differences of duplicates were < 5%. All test utensils were soaked in a 3% HNO_3 solution for at least 24 h and subsequently rinsed with MΩ water thrice before use. High purity standards and trace metal grade reagents were used in all analytics.

2.6. STATISTICAL ANALYSIS

Treatment effects on plant biomass, shoot elemental concentrations, as well as image-based morphological and color traits, were tested using a one-way analysis of variance (ANOVA). Normality and homoscedasticity of residuals were met for all tests. When significant differences occurred between treatments, multiple comparisons of mean values were made using post-hoc Tukey HSD tests. Differences were considered statistically significant at $p<0.05$. Correlations between destructively measured parameters and image-based traits were assessed using simple linear correlation (Pearson's r). Non-destructive PlantCV data (i.e., image-based morphological and color traits: area, convex hull area, width, height, perimeter, longest path, ellipse major axis, ellipse minor axis, solidity, hue circular mean, and hue circular median) were included in the principal component analysis (PCA), and biplots were generated. All statistical analyses were performed using R software (version 3.6.2).

3. RESULTS AND DISCUSSION

3.1. PHYSICOCHEMICAL PROPERTIES OF PORE WATER

Treating tailings with BS decreased pH of the pore water to 7.3±0.0, compared to untreated tailings which had a pH of 7.6±0.0 (Table S4). Whereas the reference soil pH was 6.7±0.1, which is more optimum for plant growth. Biosolids addition resulted in a double increase in EC compared to untreated tailings, and the lower EC was measured in the reference soil (Table S4). While very low DOC observed in untreated tailings, the addition of BS resulted in a significant increase in DOC. Similar effect was observed with macronutrients: N, P, K, Ca, and Mg, notably for N, which reflects the high content of OM and nutrients in the BS added (Tables S1 and S4). Macronutrient concentrations in BS-treated tailings were significantly higher than reference soil except for K. Improvements in tailings fertility with BS treatment were also observed in other studies (Al-Lami et al., 2019; Ciarkowska et al., 2017; Gardner et al., 2010).

As shown in Table S4, metals were not detected in the reference soil. Compared to untreated tailings, BS addition resulted in a significant increase in concentrations of Cu, Pb, and Zn in extracted pore water (Table S4). This effect of BS in contrast with our previous study where BS significantly decreased ammonium nitrate extracted metals particularly Zn and Cu (unpublished data). However, BS used in this study were collected from lagoons neighboring to the tailings impoundment and characterized by elevated metal content as indicated in Table S1, whereas BS used in our previous study contained very low metal content.

3.2. GERMINATION, EARLY DEVELOPMENT, AND VIABILITY IN THE PRE-SCREENING EXPERIMENT

Qualitative assessment of the 29 species tested in the pre-screening experiment under untreated tailings or BS-treated tailings such as germination, cover-abundance, and early survival is shown in Table S5. Most species screened in this phase of the study were negatively affected by the tailings. They exhibited symptoms of nutrient deficiency and metal toxicity, such as stunted growth and chlorosis/necrosis. Biosolids amendment enhanced growth of most species, which was evident by improving germination and cover-abundance and vigorous growth with less chlorosis/necrosis compared to untreated tailings. The positive effect on germination and early growth could be attributed to the improvement in physical and biological properties as well as nutrient content (Table S4) (Al-Lami et al., 2019; Gardner et al., 2010; Madejón et al., 2012). Grasses performed better than forbs in terms of germination, cover-abundance, and early survival; however, symptoms of abiotic stress were more clear in grasses compared to most of the screened forbs. Surprisingly, poor performance was observed with species identified indigenous to the site. Species with most promising performance in this pre-screening phase (i.e., top 4 grasses and 8 forbs) were selected to be screened and studied along with 5 trees in another screening experiment for a complete growing season (Table S2).

3.3. PLANT GROWTH AND PERFORMANCE IN THE SCREENING EXPERIMENT

3.3.1 Copper, Pb, and Zn Concentrations in Shoots. Plant aboveground tissues were analyzed for metals to assess tolerance and phytostabilization potential of the species tested. All the selected species from the listed in Table S2 were examined except

for three species P. *americana*, C. lanceolate, and L. capitate, which exhibited low growth or mortality over the 16 week growth period and not included in further analyses. In general, plants exhibited different patterns in terms of metal uptake when grown on untreated and BS-treated tailings, and their uptake was low under unpolluted reference soil (Figure 3). Shoot metal concentrations generally did not exceed Domestic Animal Toxicity Limit (DATL), which represents maximum tolerable levels for cattle: 40, 100, and 500 mg kg^{-1} for Cu, Pb, and Zn, respectively (NRC, 2005). This indicates a limited risk for the fauna that could be sheltered in the future restored site with minimal risk to the food chain, even though exposure through soil ingestion during grazing will also have to be investigated. Only some exceptions of Cu with S. *nutans* species under both untreated and BS-treated tailings, and A. *gerardi*i and E. *virginicus* under untreated tailings (Figure 3). The elevated Cu concentrations in these grass species could be linked to the low iron availability generally associated with alkaline tailings. Studies showed an interaction between Cu and Fe nutrition, and an increase in leaf Cu concentrations was observed under low Fe supply (Waters and Armbrust, 2013). In response to Fe deficiency, grasses tend to release phytosiderophores that chelate Fe(III) and may also complex with Cu, thus increasing its uptake (Barker and Pilbeam, 2015; Boiteau et al., 2018; Reichman and D.R, 2005). In addition, plants generally have different metal accumulation strategies, which explains the differences in metal concentrations within their tissues (Turnau et al., 2010; Yang et al., 2014). When comparing shoot metal accumulation under mine tailings phytostabilization, buffalo grass accumulated up to 51.2±20.1 mg kg^{-1} Cu concentrations, which were 4-5 fold higher than quail bush shrub planted in the same study (Gil-Loaiza et al., 2016).

Lead uptake was undetected in S. *exigua* under the three treatments and in all species under reference soil treatment (Figure 3a). Significantly higher Zn concentrations ($P < 0.05$) were detected in all species under untreated and BS-treated tailings compared to reference soil (Figure 3b), whereas Cu concentrations were generally higher under untreated and BS-treated tailings albeit not statistically significant for all species (Figure 3c). Plants responded differently to treating tailings with BS. Compared to untreated tailings, BS addition significantly ($P < 0.05$) increased Cu, Pb, and Zn concentrations in tree and grass species, with some exceptions where its effect was not statistically significant. In contrast, BS significantly decreased concentrations of Cu, Pb, and Zn in Forbs species, with only one exception where an increase in Zn concentration in D. *purpurea* was observed (Figure 3). As indicated in Table S4, BS addition resulted in increase in metal concentration in pore water; thus, the decrease in metal concentrations in forbs (legumes) under BS-treated tailings is less likely to be a treatment effect, especially the opposite trend was shown in tree and grass species (Figure 3). The decrease in metal uptake in leguminous species could be linked to a potential effect of rhizobium symbiosis. However, rhizobium species differ in their tolerance and response to metal toxicity and nutrient deficiency (Beck and Munns, 1985; Hao et al., 2014).

3.3.2. Nutritional Status of Plant Tissue. Concentrations of nutrients in aboveground tissue were generally below the common range documented for plant tissue except for Ca and Mg (Table 1). Tissue Ca concentrations varied between species and between treatments, yet all were within the common values in plants (i.e., 1-50 g kg^{-1}), with highest concentrations been detected under BS-treated tailings (Tremel-Schaub and Feix, 2005).

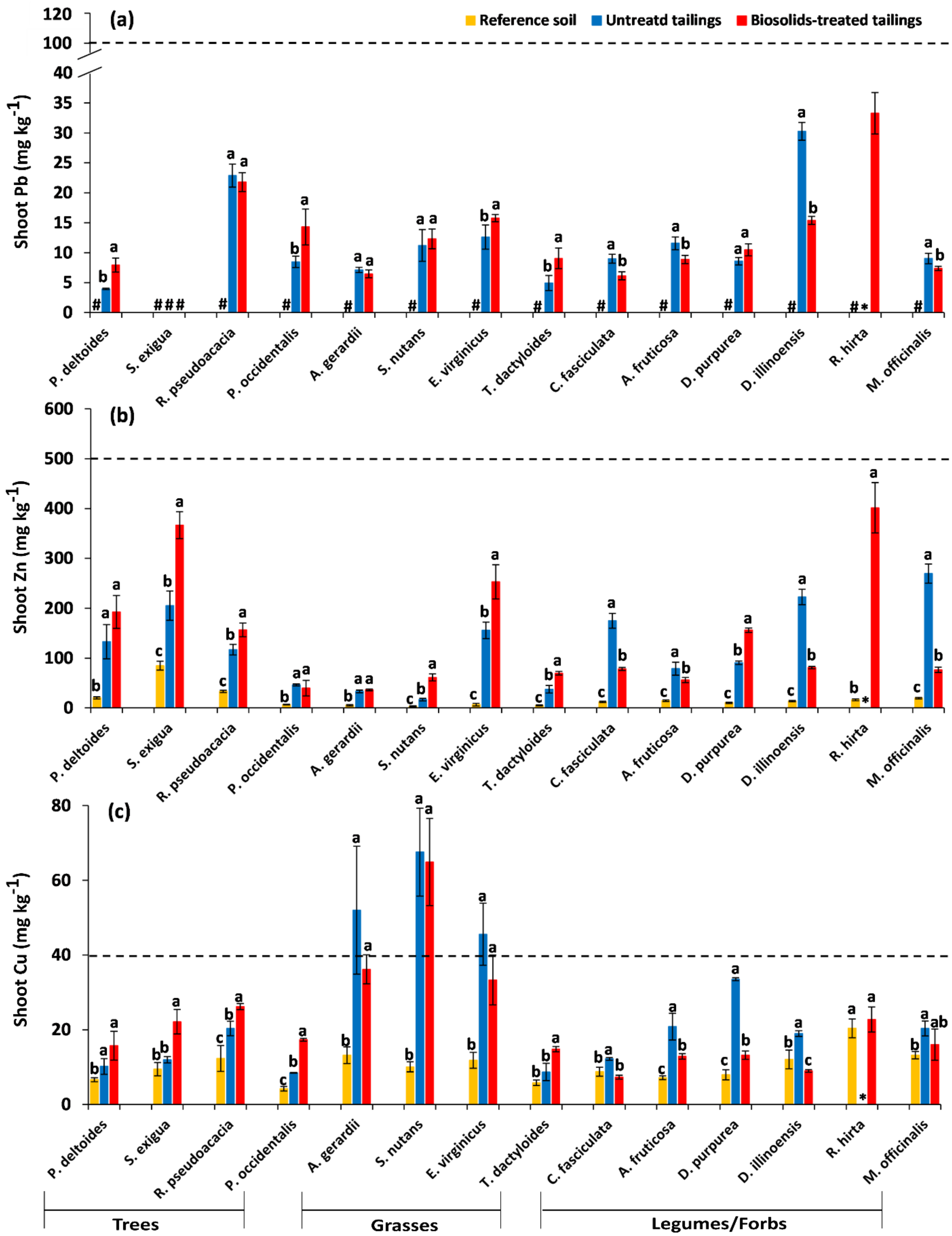

Figure 3. Metals concentrations (mg kg^{-1}) in plant shoots grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings: (a) Pb, (b) Zn, and (c) Cu. Values presented are means ± SD (n=5). Different letters above bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05). *Seeds did not germinate or germination followed by quick mortality; # Measurements were below method quantification limit. Dash lines represent Domestic Animal Toxicity Limit (DATL) i.e., the maximum tolerable level for cattle (NRC, 2005).

Whereas Mg concentrations in all screened species were above the common range (i.e., 1-5 g kg^{-1}) when grown under untreated tailings or BS-treated tailings (Barker and Pilbeam, 2015). The high concentrations of Ca and Mg reflect the mineralogy of these tailings as the bulk of the rocks are predominantly dolomite (Table S4) (Johnson et al., 2016). In most of the species, concentrations of K and P were below the common values documented in plant tissues (i.e., 20-50 g K and 1-10 g P kg^{-1}). Although K concentrations in some species under reference soil were significantly ($P < 0.05$) higher than the two tailings treatments, yet these concentrations are still below the optimal values (Table 1). Phosphorus concentrations were less than the normal range in most of the species under the three treatments; however, under BS-treated tailings P concentrations were generally equal or higher than concentrations measured under reference soil, which reflects the high P content generally found in BS (Tables S1 and S4). Other studies reported K and P deficiency as a common issue associated with Pb/Zn mine tailings revegetation (Chiu et al., 2006; Turnau et al., 2010). Micronutrients Mn and Fe were generally within the common values reported in plant tissues (i.e., 50-500 mg Mn and 20-300 mg Fe kg^{-1}) under reference soil treatment; however, under untreated tailings and BS-treated tailings, Mn concentrations were lower than this range in most species whereas Fe concentrations were lower only in some species (Table 1). Manganese and Fe deficiency is most prevalent at high pH conditions; tailings in this study were calcareous with slightly alkaline pH (Tables S1 and S4). To cope with Fe deficiency, plants evolved iron-acquisition strategies such as: Fe(III)-chelate reductase which is employed by dicots to reduce Fe(III) to Fe(II) and take up the released Fe(II)

ions, or phytosiderophores which are released mostly by grasses to chelate Fe(III) ions and take up the phytosiderophore-Fe(III) complex (Barker and Pilbeam, 2015).

3.3.3. Plant Biomass and Tolerance Index. The highest biomass was produced under reference soil treatment in all grasses and tree species, except for legume tree *R. pseudoacacia*. However, leguminous forb species had a different pattern with biomass been produced mostly under BS-treated tailings in *D. purpurea, D. illinoensis, and M. officinalis; whereas C. fasciculate* and *A. fruticose* produced significantly higher biomass under reference soil (Figure 4a). The lowest biomass was generally produced under untreated tailings (Figure 4a). No growth of non-leguminous forb species R. *hirta* was recorded in untreated tailings and low growth was observed under both reference soil and BS-treated tailings. Nutrient requirements for plants are generally in the order of woody species < grasses < forbs/legumes, this could explain the pattern of biomass production observed in this study (Barker and Pilbeam, 2015).

Tolerance index of 50% of optimum growth (i.e. under no metal toxicity) is the desired minimum biomass production for plants growing under metal toxicity (González et al., 2017). Under untreated tailings, Ti was lower than 50%, except for *D. purpurea* legume with Ti reached 87% (Figure 4b). Treating tailings with BS increased Ti in all species. However, the great increase was observed in leguminous species with the following species surpassing 100% Ti (*M. officinalis* > *D. purpurea* > *D. illinoensis* > *R. pseudoacacia*), which could possibly indicate a potential role of rhizobium symbiosis in alleviation of metal toxicity (Hao et al., 2014).

Table 1. Shoot nutrient concentrations (RS = reference soil, MT = untreated tailings, BS = biosolids-treated tailings).

Species	Treatments	Nutrients (g kg^{-1})				(mg kg^{-1})	
		Ca	Mg	K	P	Mn	Fe
P. deltoides	RS	10.9±0.7 b	4.8±0.2 b	8.2±0.7 a	0.52±0.03 a	104.8±21.7 a	33.2±6.0 a
	MT	6.1±1.1 c	8.8±0.8 a	5.6±0.2 b	0.50±0.15 a	29.7±26.7 b	30.5±3.3 a
	BS	12.6±1.0 a	8.1±0.6 a	5.5±0.3 b	0.57±0.05 a	37.7±5.5 b	13.8±2.2 b
S. exigua	RS	18.9±1.2 b	6. 1±0.4 b	7.2±0.4 a	0.77±0.07 a	70.8±2.5 a	17.4±0.7 a
	MT	14.6±1.5 c	14.7±0.9 a	3.6±1.0 b	0.42±0.04 c	22.4±8.3 b	17.0±4.5
	BS	23.7±1.4 a	15.1±0.5 a	4.4±1.0 b	0.67±0.03 b	21.2±1.5 b	12.5±1.2 b
R. pseudoacacia	RS	24.4±0.3 a	8.6±0.2 b	6.1±0.2 b	2.07±0.01 a	208.6±7.7 a	74.6±15.3
	MT	7.1±0.4 b	19.2±4.1 a	14.6±3.1 a	0.56±0.05 b	19.5±1.9 c	48.6±9.9 b
	BS	25.5±2.3 a	15.8±1.0 a	7.0±1.1 b	1.99±0.23 a	53.7±3.8 b	28.5±2.4 b
P. occidentalis	RS	6.2±0.6 a	3.1±0.4 b	4.2±0.2 b	0.54±0.08 b	70.3±18.0 a	109.4±9.9
	MT	4.0±0.8 b	6.4±2.2 a	9.0±0.1a	0.36±0.01 b	37.4±1.7 b	76.1±7.2 a
	BS	5.0±0.9 ab	4.3±0.7 ab	11.9±3.1 a	1.85±0.74 a	22.6±1.0 b	90.9±39.1
A. gerardii	RS	3.4±1.2 b	4.3±0.6 b	15.5±2.2 a	1.07±0.24 a	65.2±8.2 a	47.3±4.4 a
	MT	4.8±0.2 b	9.4±0.7 a	5.6±0.7 b	0.60±0.08 b	10.0±0.8 c	23.4±4.0 c
	BS	18.2±1.8 a	8.5±0.5 a	3.7±0.4 b	0.57±0.02 b	22.3±1.8 b	32.2±2.1 b
S. nutans	RS	3.3±0.4 b	3.7±0.5 b	12.8±1.6 a	0.96±0.28 a	116.4±23.5 a	56.5±5.7 a
	MT	5.5±0.5 b	7.5±0.6 a	4.2±0.4 b	0.52±0.08 b	8.1±1.4 c	30.8±7.9 b
	BS	34.2±4.9 a	8.3±1.2 a	1.5±0.2 c	0.76±0.08 ab	36.9±4.2 b	68.±9.7 a
E. virginicus	RS	4.8±0.4 b	4.0±0.1 c	18.8±1.3 a	1.18±0.26 a	52.3±5.2 a	44.4±3.4 a
	MT	5.6±0.6 b	11.5±0.7 a	9.1±1.4 b	0.63±0.07 c	12.8±0.5 c	24.0±2.1 b
	BS	11.7±1.8 a	7.7±1.7 b	9.0±1.8 b	0.90±0.05 b	21.7±4.0 b	21.1±1.2 b
T. dactyloides	RS	2.0±0.2 b	2.4±0.3 c	10.4±1.1 b	0.38±0.03 c	35.7±6.7 a	29.4±4.8 a
	MT	1.9±0.2 b	4.8±0.5 b	15.2±2.2 a	0.57±0.04 b	9.6±0.7 c	13.9±1.6 b
	BS	4.5±0.4 a	6.0±0.8 a	16.3±3.1 a	0.67±0.04 a	21.2±1.8 b	17.9±1.6 b
C. fasciculata	RS	9.3±0.7 b	4.4±0.3 c	7.5±0.6 b	0.60±0.06 a	83.4±8.7 a	35.7±2.0 a
	MT	10.1±0.9 ab	18.5±0.4 a	21.9±4.2 a	0.38±0.03 b	80.3±8.5 a	14.4±0.9 b
	BS	11.0±0.8 a	12.1±1.0 b	7.3±0.3 b	0.38±0.02 b	61.2±3.4 b	33.6±1.1 a
A. fruticosa	RS	13.1±0.4 b	3.9±0.2 c	7.9±0.5 b	0.42±0.02 b	221.9±30.4 a	42.8±3.5 a
	MT	8.8±1.2 c	9.5±1.7 a	19.4±3.6 a	0.31±0.05 c	29.8±3.5 b	10.5±1.2 c
	BS	16.5±1.5 a	7.3±0.5 b	7.4±0.7 b	0.56±0.02 a	34.1±3.5 b	19.7±1.6 b
D. purpurea	RS	19.9±1.8 b	10.3±0.7 b	8.0±0.7 b	0.48±0.03 b	277.0±31.0 a	89.1±4.5 a
	MT	13.2±0.5 c	23.0±1.0 a	10.9±0.4 a	0.34±0.01 c	32.0±1.5 b	29.4±4.5 b
	BS	44.1±3.1 a	22.3±0.6 a	5.9±0.5 c	1.39±0.11 a	42.4±3.2 b	17.4±0.9 c
D. illinoensis	RS	25.6±2.5 a	9.0±0.5 b	5.7±0.5 a	0.44±0.03 a	279.4±13.8 a	180.9±9.7
	MT	17.7±0.9 b	8.2±0.8 b	2.3±0.2 b	0.43±0.01 a	54.9±7.9 b	24.9±0.7 b
	BS	23.7±1.9 a	11.4±1.3 a	2.6±0.6 b	0.42±0.01 a	32.3±3.6 c	31.9±7.9 b
R. hirta	RS	38.6±5.2 a	16.6±1.4 b	25.9±1.8 a	0.77±0.02 a	323.2±79.8 a	192.0±39.5
	MT*						
	BS	44.8±6.5 a	28.5±4.2 a	7.7±1.3 b	0.72±0.08 a	63.3±7.4 b	12.4±1.4 b
M. officinalis	RS	11.2±1.1 b	7.8±0.3 c	12.9±0.5 a	0.41±0.02 c	187.5±25.8 a	81.0±3.3 a
	MT	14.7±1.5 b	13.0±1.1 b	2.7±0.5 b	0.54±0.04 b	23.3±9.2 b	11.4±1.7 c
	BS	28.2±2.6 a	19.0±2.1 a	3.8±1.0 b	0.88±0.06 a	28.5±3.0 b	32.3±3.5 b
Common		1-50	1.0-5.0	20-50	1.0-10	50-500	20-300

Values are mean ± SD, n = 5; data were evaluated using one-way ANOVA with post-hoc analysis; Within a plant species, treatments with the same letter are not statistically different ($p<0.05$); *Seeds did not germinate or germination followed by quick mortality; †Tremel-Schaub and Feix (2005)

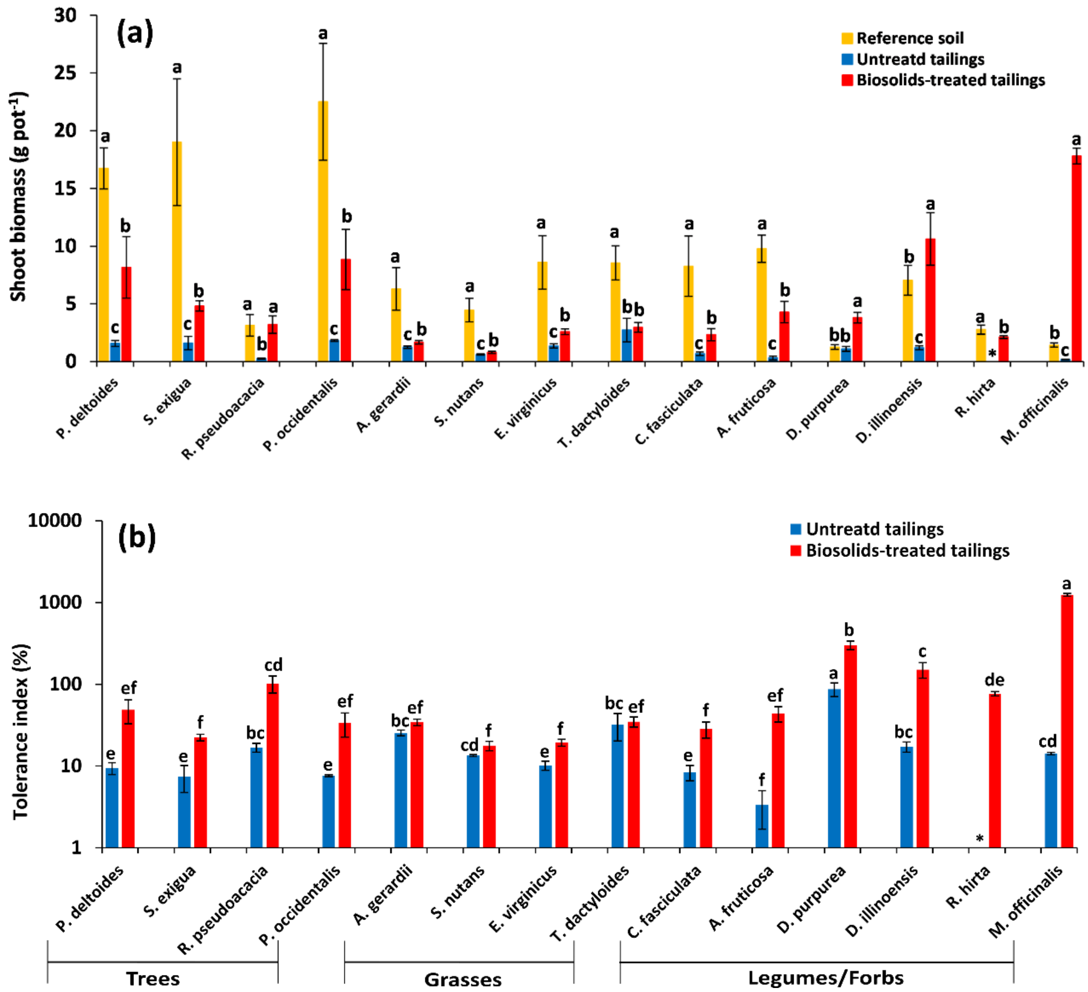

Figure 4. Growth performance of selected native prairie and indigenous species: (a) Shoot biomass (g pot^{-1}), (b) Tolerance index (%) of plant species grown on untreated tailings or biosolids-treated tailings in comparison to their growth in unpolluted reference soil. Values presented are means ± SD (n=5). Different letters above bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05). *Seeds did not germinate, or germination followed by quick mortality.

3.4. PLANT RESPONSES TO ABIOTIC STRESS USING IMAGE-BASED METHODS

3.4.1. PlantCV and Image-Based Screening. When exposed to abiotic stress such as nutrient deficiency or metal phytotoxicity, plants usually undergo alteration in biochemical and physiological processes (Barce and Poschenrieder, 1990; Nagajyoti et

al., 2010). This stress-induced alternation can be visually observed through longer-term visible injuries and phytotoxic responses such as reduced growth rates, inhibition of growth, tissues deformation, leaf chlorosis/necrosis, reduced levels of photosynthetic pigments, and consequently plant death. (Baryla et al., 2001; Panwar et al., 2016; Sun et al., 2018; Yadav, 2010). While visual observation can clearly show plant response to abiotic stress (i.e., vigor and health), it is impossible to quantify the response from just visual inspection. As such, it is not feasible to visually determine robust differences between different species in response to environmental stress or responses of a certain species to different treatments. Thus, this part of the study aimed to develop an approach that could identify and quantify stress-induced symptoms much earlier and more precisely than human eyes could recognize. To achieve this goal, we utilized image-based and computer vision techniques to facilitate statistically robust quantification of plant response. The non-destructively collected images were processed by automatically segmenting plant material using PlantCV platform. Segmenting plants automatically reduces time consuming masking of plants, but it often requires qualified personnel. However, automatic plant segmentation brings its own challenges in the form of intrinsic and extrinsic issues. Examples of extrinsic issues can include illumination changes where binary thresholds no longer be valid to mask out the plant. Intrinsic issues can include ambiguous plant background boundaries and complex backgrounds. To solve the issues arisen with automatic plant segmentation, a novel technique utilizing a superpixel-based machine learning algorithm was used to segment plant material, the segmented image then passed to PlantCV where plant phenotypes parameters are measured and stored as described above (see sections 2.3.2 and 2.3.3, Figures. 1and 2). From a given image, we

were able to extract a total of fifteen morphological and color phenotypes simultaneously (Table S6). The whole process of image acquisition and trait extraction took less than 30 sec per plant.

While using machine learning to automatically segment plants solved many of the challenges faced, few intrinsic issues remain. With the current setup, occluded leaves are not properly segmented. This leads to variance in plant leaf surface area measurements. To solve this issue, depth information needed to be included. Methods for achieving depth information can include stereoscopic camera placement or active sensors such as light detection and ranging (LiDAR). Another potential limitation with the machine learning algorithm introduced, is that convolution neural network requires a large dataset. With the current dataset size, it is possible that the back gradient loss function locked onto a local minimum, preventing further improvement or overfitting may have occurred. With continued use with this approach and methodology, larger more varied datasets can help improve the machine learning model for this arrangement and similar research.

3.4.2. Impact of Abiotic Stress on Plant Morphology and Leaf Color. Effects of abiotic stress on plant phenotypic responses were assessed by statistically analyzing the quantitatively measured morphological and color traits listed in Table S6 such as area, hull area, width, height, perimeter, longest path, ellipse major axis, ellipse minor axis, solidity, and hue intensities. All extracted traits were generally influenced by abiotic stress and exhibited differences between treatments with some exceptions (Table S6, Figure 5). Among the twelve measured morphological traits, area measurements based on pixel numbers showed a similar pattern to that observed with biomass for all of the imaged species, with statistically significant differences ($P < 0.05$) between the three

treatments (i.e., unpolluted reference soil, untreated tailings, and BS-treated tailings) (Table S6, Figures. 4a and 5a,). Other studies also found the area to be the plant morphological trait that exhibited the most significant treatment effect (Neilson et al., 2015; Veley et al., 2017). Other morphological features such as convex hull area, width, and height also displayed significant treatment effect, however not for all species. In contrast, solidity was the morphological trait that displayed the least treatment effect in this study (Table S6).

To assess plant health based on color quantification, we used PlantCV to extract hue values, which measures color based on pixels found at each degree of 360° hue circle. As shown in Figure 5b, the hue colors of most importance are gradients of yellow and green with corresponding peaks of 60° and 120°, respectively. The highest hue colors were generally observed under unpolluted reference soil treatment with values in degree ranging from 80.8°±1.1 to 111.7°±5.4, except for *M. officinalis* species with a value of 68.2°±5.5 (Table S6, Figure 5b). Untreated tailings treatment induced a green to yellow shift in hue color in all species indicating severe chlorosis/necrosis reaching the yellow peak in some cases. Among the six species tested, the significant impact of BS addition was limited to *D. illinoensis and M. officinalis* species with a color shift from yellow to green, exceeding reference soil and consistent with the marked impact on biomass for the later species (Table S6, Figures 4a and 5b). These observations attributed to the adverse effects on plant metabolism induced by nutrient deficiency and metal toxicity. The over production of reactive oxygen species (ROS) is typically the response of plant cells to stresses, and ROS eventually causes oxidative damage to plant cells (Mittler, 2002). Oxidative stress generally causes impairment of chlorophyll synthesis and damaging to

the chloroplast membranes with a marked reduction in chloroplast density (Baryla et al., 2001; Viehweger, 2014), consequently leading to declining in pigment and development of chlorosis and necrosis symptoms which manifests in plant leaves and overall size reduction (Panwar et al., 2016; Stambulska et al., 2018). Others also used hue value to measure leaf color indices to assess chlorosis/necrosis induced by abiotic and biotic stress (Veley et al., 2017; Zheng et al., 2019). While this area of research is rapidly growing, the literature examining the use of image-based and computer visualization to quantify plant responses to abiotic stresses is limited to drought, cold, heat, and nitrogen deficiency stress (Enders et al., 2019; Kumar et al., 2020; Shakoor et al., 2019; Tovar et al., 2019; Veley et al., 2017).

3.4.3. Relationships Between Imaged Traits and Destructively Measured Parameters. Correlations between destructively measured parameters and image-based morphological and color traits for each of the six species are shown in Figures 6 and S2-S6. In general, all the non-destructively imaged traits for shape and color attributes correlated positively with destructively measured plant biomass, however morphological traits generally exhibited stronger correlations with biomass compared to color traits. Furthermore, the area measurement was the morphological trait that exhibited the greatest positive correlations with biomass for all six species (R^2 ranged from 0.95-0.99) for legumes and R^2=0.89 for poplar tree. Other morphological traits such as convex hull area, width, height, perimeter, and longest path also showed high positive correlations with plant biomass, except for solidity which was the morphological trait that displayed the least positive correlation with biomass in most cases (Figures 6 and S2-S6).

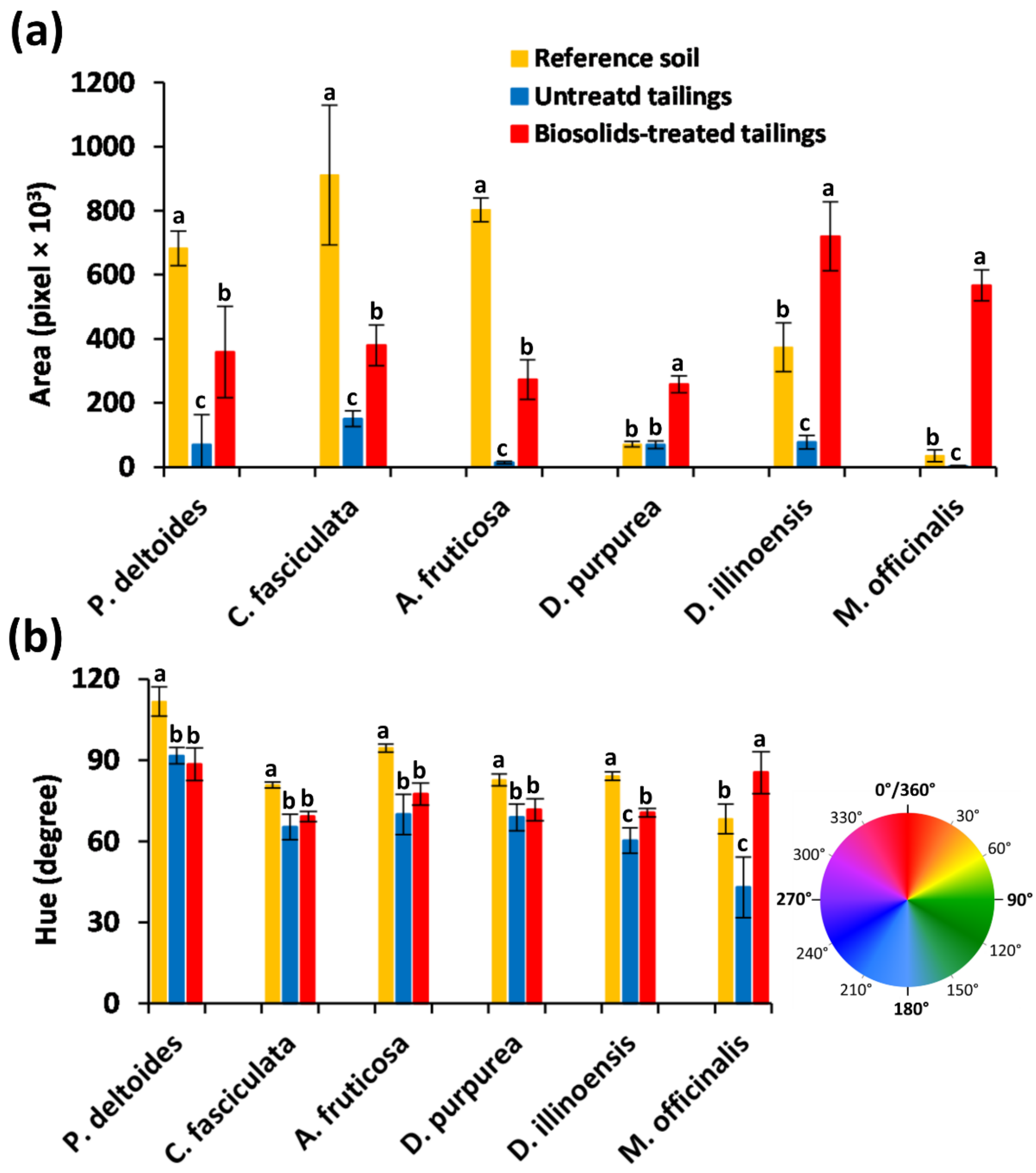

Figure 5. Image-based plant growth response of selected native and indigenous species at week 15 of growth in: unpolluted reference soil, untreated tailings, or biosolids-treated tailings: (a) Area (pixels) and (b) Color trait represented by average hue value (degree). Values presented are means ± SD (n=5). Different letters above bars indicate significant differences between treatments (one-way ANOVA, p-value < 0.05). The color chart represents the RGB coordinates corresponding to the hue value (degree).

These correlations also indicate that biomass estimation based on shape phenotypes, particularly area, is more robust with forb plant species compared to tree biomass crop. Results in this study are consistent with others who found image-based area measurement to be highly correlated with biomass for several species and can be used as a proxy for biomass estimation (Fahlgren et al., 2015; Shakoor et al., 2019; Veley et al., 2017). While color traits measured by hue degree also exhibited positive correlations with biomass for most of the species, these traits displayed stronger negative correlations with Cu, Pb, and Zn concentrations accumulated within plant shoots. In most of the species tested, Pb and Zn seem to have the strongest negative correlations with hue color values, while the least negative correlations were generally observed with Cu (Figures 6 and S2-S6). These results also show strong positive correlations between color traits and Fe and Mn concentrations accumulated within plant shoots. Contrary to color traits, no clear trend was observed for correlations between shape phenotypic traits and metal accumulation within plant shoots. While studies have shown a wide range of morphological and physiological symptoms associated with phytotoxicity of Cu, Pb, and Zn and deficiency of Fe and Mn, results in this study reveal that abiotic metal stress symptoms are greatly associated with color phenotypic attributes and to a lesser extent with plant shape attributes.

These elements, except for Pb, are essential for plant metabolism and play a pivotal role in the structure of enzymes and proteins, however above certain concentrations they turn into toxins inhibiting photosynthesis, chlorophyll biosynthesis, cell respiration and nitrogen metabolism, which collectively impair plant development and cause irreversible damage via induced oxidative stress (Ashfaque et al., 2016;

Emamverdian et al., 2015). This is also true for essential elements when bioavailable concentrations are below plant requirements such as Fe and Mn deficiency due to the high pH in this study (Santos et al., 2019). The symptoms of plant response to Cu, Pb, and Zn toxicity or Fe and Mn deficiency stress can be visually observed through morphological alteration such as leaf-shape damage, reduced leaf area and number of leaves, reduced shoot length, and collectively decreased plant yield, in addition to leaf discoloration including chlorosis and/or necrosis (Reichman, 2002; Santos et al., 2019; Zhao et al., 2011). Leaf discoloration is of a particular interest as it is the result of chlorophyll pigments degradation induced by oxidative stress, as well as the production of carotenoids and anthocyanins pigments which are believed to act as non-enzymic antioxidants to protect plant against environmental stresses (Neill et al., 2002). Studying leaf responsiveness of poplar and willow to Cd, Cu, Pb, and Zn stress, Hermle et al. (2007) observed leaf injury symptoms represented by whitish adaxial stippling scattered across the leaf blade followed by brown necrotic expanding spots due to stress-induced carotenoids pigments production. Similarly, investigating the response of A. *thaliana* seedlings to metal stress of increasing concentrations of Cu, Pb, or Zn, Baek et al. (2012) observed a proportional decrease in chlorophyll content accompanied by increasing of carotenoids and anthocyanins contents as a response to metal stress and they concluded that A. *thaliana* used anthocyanins as main protectants against Cu stress while carotenoids against Pb stress. In addition, studies have shown that toxic metals may interfere with the uptake of essential nutrients particularly Fe and Mn resulting in metal-induced Fe deficiency chlorosis-like symptoms (Printz et al., 2016; Rout and Das, 2009). Results in this study showed strong negative correlations between Fe and Mn with Cu,

Pb, and Zn accumulation in plant shoots (Figures 6 and S2-S6). All the above-mentioned could explain the trend of correlations observed in this study between color traits with other plant destructively and non-destructively measured parameters and confirm the importance of color phenotyping in assessing plant health against abiotic stress besides shape phenotyping.

3.4.4. Principle Component Analysis Based on Non-Destructive Phenotypes. We used principal component analysis (PCA) on the image-based parameters alone to investigate the capability of this non-destructive approach (i.e., imaging and PlantCV analysis) to identify the effects of separate treatments. The PCA for each of the six species revealed that morphological and color traits could be used to separate all three treatments tested in the experiment (i.e., unpolluted reference soil, untreated tailings, or BS-treated tailings) (Table S7 and Figure 7). With the exception of *M. officinalis* species, PCA indicated that separation of the reference soil treatment was mainly driven by color traits (i.e., hue color values). This can be attributed to the effect of oxidative metal stress on chlorophyll content and plant health in the tailings' treatments, as discussed in the section above and shown in Figure 5b. Whereas PCA showed that both morphological and color traits influence separation between untreated and BS-treated tailings treatments but for most species strongly driven by morphological traits and area measurements once again displayed the most significant treatment effect (Table S7 and Figure 7).

3.4.5. Ecological Significance. The robustness of this non-destructive approach in determining statistically significant differences is of a particular interest in identifying phenotypic traits and intraspecific variation within metal tolerant populations that

evolved through natural selection in response to high metal content and hostile environments of mine lands (O'Dell and Rajakaruna, 2011).

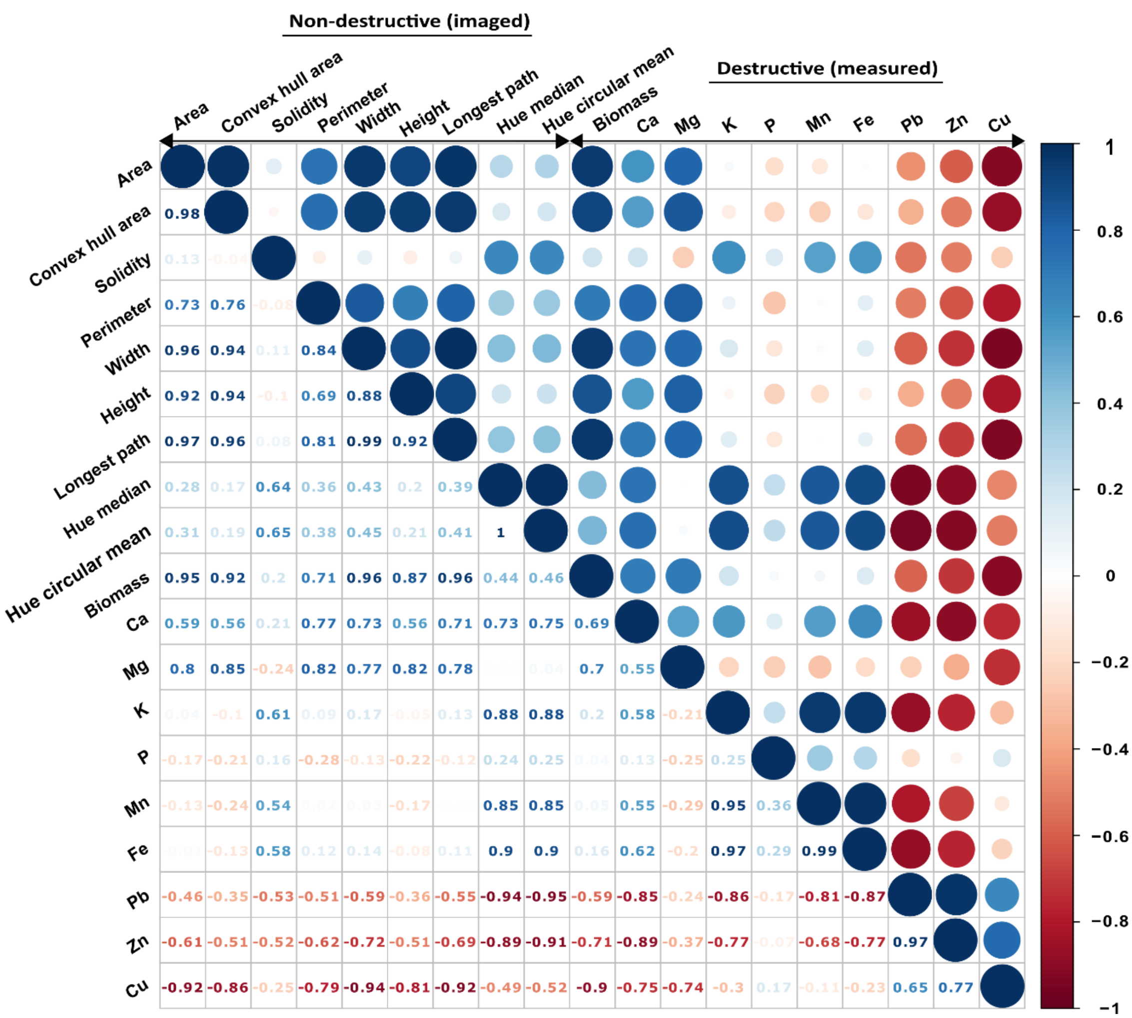

Figure 6. Correlation between non-destructive image-based traits and destructively measured parameters for plant species example *D. illinoensis* grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles. Correlations for other species are presented in the supplementary material (Figures S2-S6).

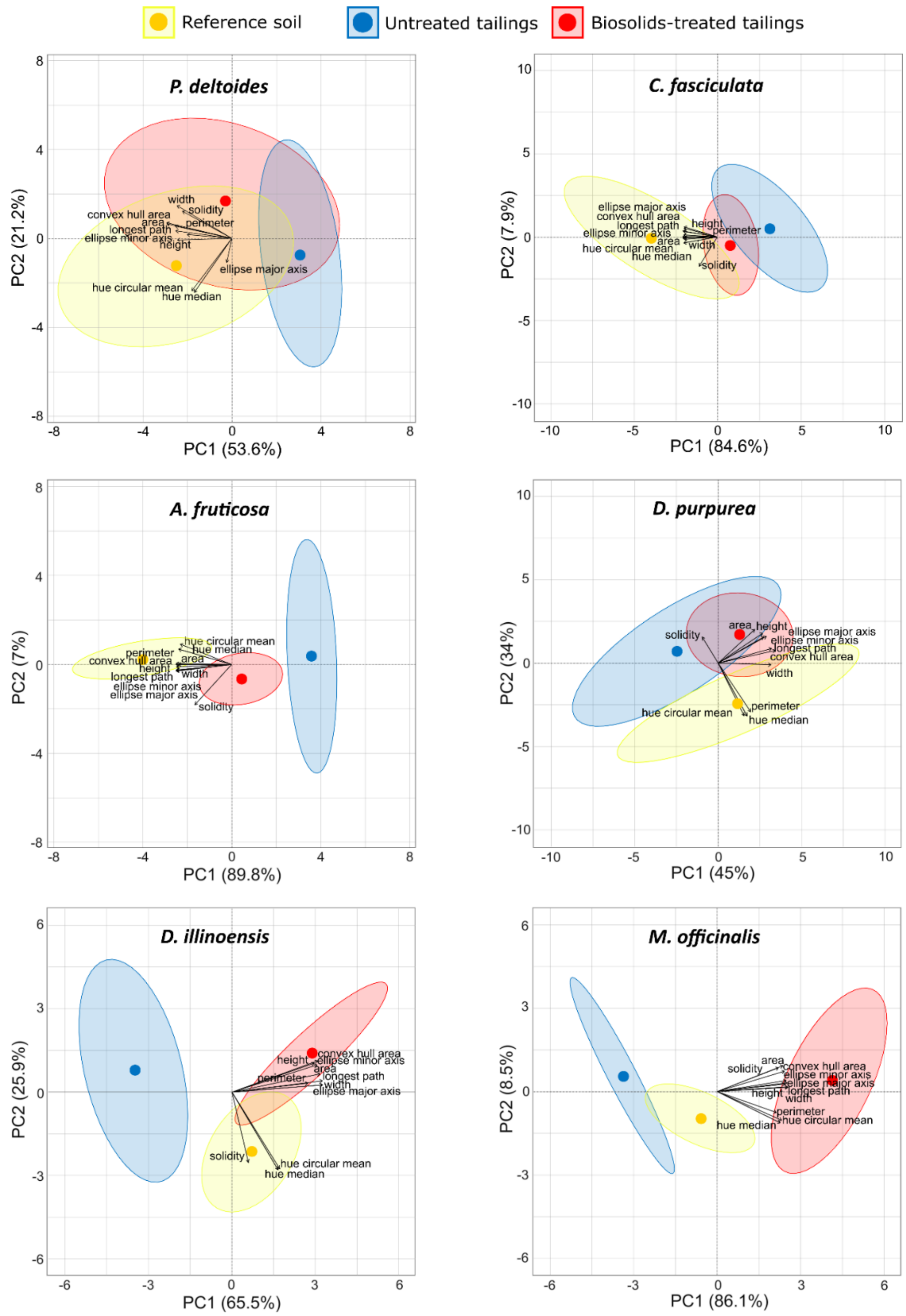

Figure 7. PCA biplots showing separation between treatments derived by non-destructive image-based traits. Species imaged at week 15 of growth in: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, followed by image analysis by PlantCV. The morphological traits included in the PCA are as follows: area, convex hull area, solidity, height, width, perimeter, longest path, ellipse minor axis, and ellipse major axis; while hue circular mean and hue circuar median included as color traits.

Identifying such species, cultivars, or ecotypes can greatly benefit mine lands reclamation and ecological restoration as these species can provide the foundation for ecosystem structure and function and ultimately biodiversity. Surveys and screening studies can identify plant ecotypes and traits associated with tolerance and adaptation (Santos et al., 2017). A recent study by Gastauer et al. (2020) showed that leaf functional traits can guide native species selection for successful mine land rehabilitation and ecological restoration. However, such studies are usually restricted by costly analyses and destructive testing. The image-based screening approach proposed in this study can facilitate wide-range of screening experiments to identify responsive plant traits non-destructively and statistically study their transient and temporal responses.

4. CONCLUSIONS

Compared to reference soil, most species were negatively affected by the tailings and exhibited symptoms of nutrient deficiency and metal toxicity such as stunted growth and chlorosis/necrosis. However, significant improvement in plant growth was observed with BS addition despite the substantial metal content in the BS used in this study. Positive plant phenotypic response is most likely a reflection of the improvement of the tailings physical and chemical properties and increased nutrient content. Although BS increased metal shoot accumulation in some cases compared to untreated tailings, metal concentrations did not exceed DATL except for Cu in one grass species. Legumes performed better than trees and grasses under BS treatment with reduced metal accumulation and improved vigor in most cases. In general, numerous native plants tested

in this study are candidates for tailings phytostabilization. Incorporating metal-containing BS from neighboring lagoons is a win-win strategy to concurrently dispose of the large quantities of the metal-contaminated sludge while ameliorating the tailings properties with minimal transport cost. Plant morphological and color traits extracted from the images utilizing computer vision techniques showed robust differences between species in response to metal and nutrient stress. Analyzing non-destructive PlantCV data clearly showed differential phenotypic response between three treatments included in this study. Plant area showed the highest correlation with biomass, which can be used as a proxy measurement for biomass and for overall plant vigor. Color traits showed a significant negative correlation with metal content across the species screened, indicating a rapid and temporal method to assess plant health related to metal content. Through this screening tool, we report a cost-effective and time-efficient robust approach to identify abiotic stress-tolerant plants and amendment strategies, which can significantly benefit selection of species for revegetation of various mine sites. Findings from this study served as a starting point for ongoing work investigating image-based plant phenotyping for non-destructive testing. Such non-destructive methods can be applied temporally to detect responses to abiotic stress across developmental plant stages and elucidate relationships for both bioavailability of nutrients and metals in wide range of chemical and biological amendment strategies.

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SECTION

2. CONCLUSIONS AND RECOMMENDATIONS

2.1. CONCLUSIONS

Due to the high metal phytotoxicity and extremely poor soil characteristics, Pb/Zn/Cu mine tailings with no addition of soil amendment did not support plant growth regardless of the species tested. However, positive impacts were observed when chemical and biological amendments considered in the tailings revegetation, yet to varying extents and generally not effective in correcting the multidimensional deficiencies in tailings particularly when singularly applied. For example, singular application of biochar and humic substances in this study significantly improved tailings physicochemical properties and water holding capacity but their impact on biomass production was limited by the nutrient deficiencies and lack of organic matter. Similarly, impact of singular application of biological amendment mycorrhizal fungi was also limited. Biosolids, on the other hand, were the amendment that exhibited the most superior impact on physicochemical and hydraulic properties while resulting in remarkably higher biomass production. The factors driving the benefits of biosolids compared to the other tested amendments were found to be the rich nutrients and OM contents. The problem, sustainability of biosolids is questionable due to their nutrient lability and high decomposition rates compared to the other amendments i.e., biochar and humic substances. Thus, phytomanagement strategies that can sustain biosolids benefits and enhance the tailings revegetation effectiveness

were also studied through co-application with the other amendments owing to their unique and complementary characteristics.

Co-application of biosolids with the other amendments i.e., chemical (biochar and humic substances) or biological (mycorrhizae fungi) showed synergistic effects with potential to support more sustained tailings revegetation and restoration. Nevertheless, the superior impact on biomass production and tailings physicochemical, biological, and geochemical properties was also mainly affected by the biosolids application, which is again attributed to their rich nutrient and organic matter content. Co-application with biochar induced further immobilization of metals particularly Pb which was less affected or even mobilized by the biosolids application, depending on the interaction with plant species. The effect of co-applying biochar on metal immobilization was remarkable under miscanthus cultivation because this species induced high organic matter mineralization besides an acidification effect which resulted in a significant increase in metal bioavailability, nevertheless, this effect was counteracted by the co-applied BC. Moreover, co-applying biosolids with biochar increased C/N ratio, thus resulted in a lower enzyme activity and mineral nitrogen availability, which could indicate an induced negative priming effect on organic matter in biosolids thus supporting efficient nutrient release and prolonging the biosolids associated benefits. Whereas, co-applying humic substances with biosolids significantly increased microbial enzyme activity and Fe uptake, which was deficient in these alkaline and calcareous tailings.

On the other hand, co-application of biosolids with mycorrhiza fungi exhibited a great interactive effect compared to biosolids alone. Biosolids facilitated the development of a beneficial plant-mycorrhizal fungi relationship by serving as a soil ameliorant and

correcting the tailings severe characteristics that inhibit mycorrhizal colonization on plant roots. The co-application of mycorrhizal fungi and biosolids resulted in further improvement in growth of poplar, willow, and miscanthus. Compared to biosolids alone, this effect was more superior on miscanthus resulting in a 2-fold increase in biomass production compared to biosolids applied with no mycorrhizal inoculation. Mycorrhizal inoculation resulted in further reduction in metal accumulation (i.e., Pb, Zn, Cu, Cd, and Ni) in plants aboveground tissues and facilitated more P uptake which is also deficient in these tailings. The beneficial interactive effect of biosolids co-application with mycorrhizal fungi was not limited to plants, but also further improved tailings characteristics by reducing metal bioavailability and increasing microbial activity and most importantly inducing organic carbon input into tailings which implies their role in stimulation of soil formation processes.

This study also showed that the efficiency of aided phytostabilization strongly depends on the plant species selected. Superior phytostabilization efficiency was associated with poplar and willow cultivation but not miscanthus which induced higher organic carbon mineralization and metal mobilization due to an acidification effect. Also, screening a large number of native and prairie plant species for their potential to revegetate these tailings in biosolids aided phytostabilization showed a superior performance for nitrogen fixing leguminous species compared to the other tested plants of trees and grasses. Screening of plant species or amendment strategies for potential tailings phytostabilization is generally hindered by the costly and time-consuming analyses. In addition, mine tailings are typically very heterogenous making selection is highly site specific. Therefore, a non-destructive and cost-effective screening approach

was also developed in this study relying on image-based phenotyping and computer vision techniques. This approach greatly facilitated plant screening efforts and provided statistically robust plant responses to the abiotic stress associated with tailings with morphological traits particularly area positively and strongly correlated with biomass whereas color traits exhibited strong negative correlations with metal uptake.

These results highlight the importance of phytomanagement strategies that target candidate plant species selection and amendment co-application. Such as co-applying biosolids with other complementary amendments in particular biochar and mycorrhizal fungi, and the suitability of bioenergy crops particularly poplar and willow for tailings phytostabilization and concurrent biomass production, or leguminous native prairie species for tailings phytostabilization and concurrent ecological restoration.

2.2. RECOMMENDATIONS

Assessment of amendment impact on tailings revegetation and their interaction with plant species has been unfortunately often done in relatively short-term experiments. Impact of co-application of biosolids with biochar or mycorrhizae fungi and the interaction with candidate plant species is likely to exhibit different behaviors with time due to their potential in influencing soil carbon sequestration. Thus, clearly notable demand exists for long-term experiments that target phytomanagement strategies using co-application of these amendments with specific plant species particularly leguminous native prairie species due to their high fixation capacity for both nitrogen and carbon which are extremely deficient in tailings. Thus, the following research should be investigated:

1. Assess the long-term effectiveness of co-application of recalcitrant biochar with biosolids on temporal bioavailability behavior of heavy metals and nutrients as well as tailings geochemical properties and how it is different from co-application with raw non pyrolyzed waste byproducts.
2. Quantify the organic matter input induced by long-term co-application of biosolids with biochar or with mycorrhizal fungi and how it is affected by the interaction with different species particularly nitrogen fixing and native prairie species.
3. Investigate the functionality of different mycorrhizae fungi species and their relationship with compatible plant species, for example mycorrhizal species isolated from prairie soils to be used for aided phytostabilization with prairie plant species.
4. Determine the role of co-application of biosolids and biochar along with mycorrhizal fungi inoculation to stimulate a beneficial symbiotic relationship with plants thus maximizing the phytostabilization outcomes.
5. Assess the distribution of mycorrhizal fungi within and below the amended layer and impact of mycorrhizal fungi on soil development indicators in phytostabilization of tailings. Mycorrhizae fungi may affect formation and stabilization of tailings aggregates through biophysical and biochemical processes in addition to their role in altering root morphology and architecture. However, the ecosystem services provided by mycorrhiza fungi likely depend on the extent of the mycorrhizosphere (i.e., volume of tailings occupied by mycorrhizal hyphae as well as their abundance).

APPENDIX A.

SUPPORTING INFORMATION: II. PHYTOMANAGEMENT OF LEAD/ZINC/COPPER TAILINGS USING BIOSOLIDS-BIOCHAR OR -HUMUS COMBINATIONS: ENHANCEMENT OF BIOENERGY CROP PRODUCTION, SUBSTRATE FUNCTIONALITY AND ECOSYSTEM SERVICES

Table S1. Belowground root and rhizome dry weight yield in grams of miscanthus and root of poplar and willow.

Treatment	Miscanthus		Poplar	Willow
	Rhizome	Root	Root	Root
Unt	12±2d	1±0.2e	2±0.5c	3±0.4e
LB	33±5c	14±2.5d	29±3.8b	26±3.6d
LB+BC	43±7.4abc	21±4bc	33±4.4b	37±4.9cd
LB+HS	35±6.9bc	26±4.9cd	32±6.3b	34±9.2bc
HB	46±6.3ab	24±4.4bc	41±4.9a	42±4.9bc
HB+BC	55±8.9a	31±4.1a	46±4.9a	53±4.6ab
HB+HS	49±10.4a	40±5b	47±5.7a	49±4.7a

Mean values ± SD per treatment (n=6) for miscanthus and willow, (n=5) for poplar with some losses due to mortality. Different letters indicate significant differences between treatments (one-way ANOVA, p-value < 0.05).

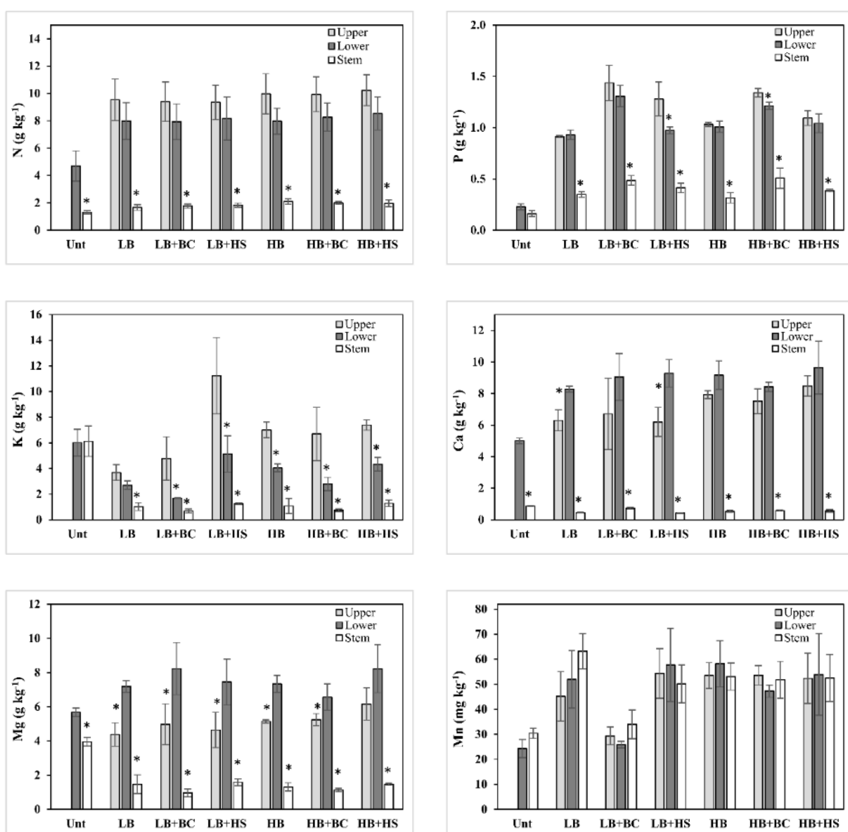

Figure S1. Nutrient accumulation in upper (new growth) and lower (old growth) leaves and stems of miscanthus.

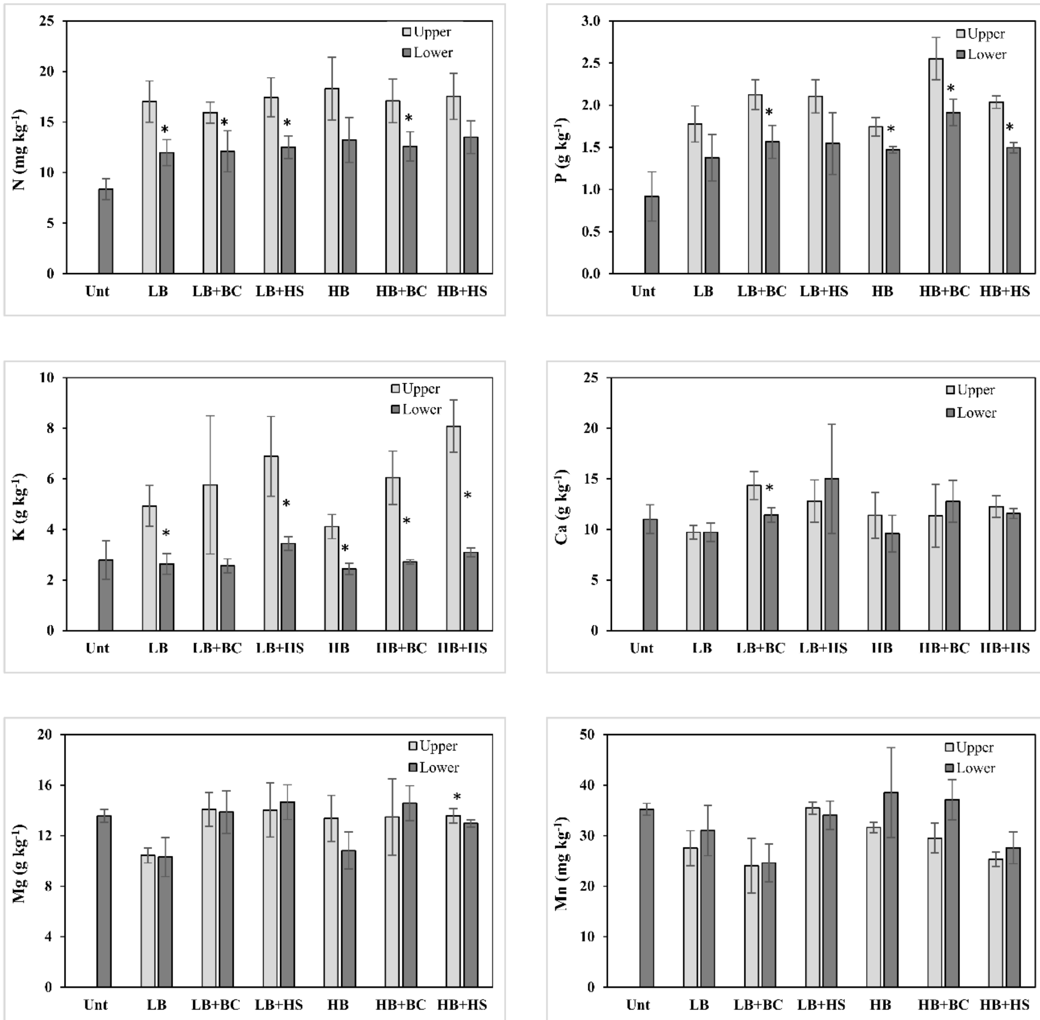

Figure S2. Nutrient accumulation in upper (new growth) and lower (old growth) leaves of poplar.

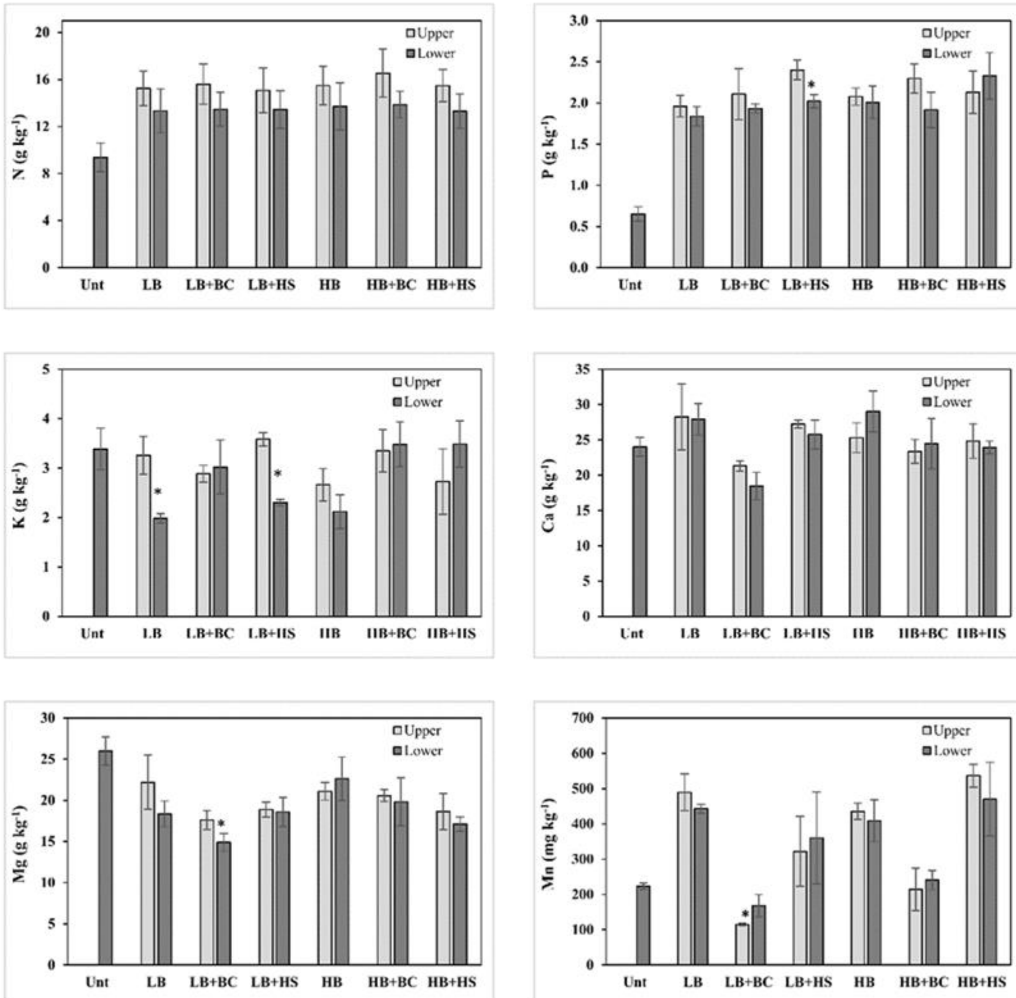

Figure S3. Nutrient accumulation in upper (new growth) and lower (old growth) leaves of willow.

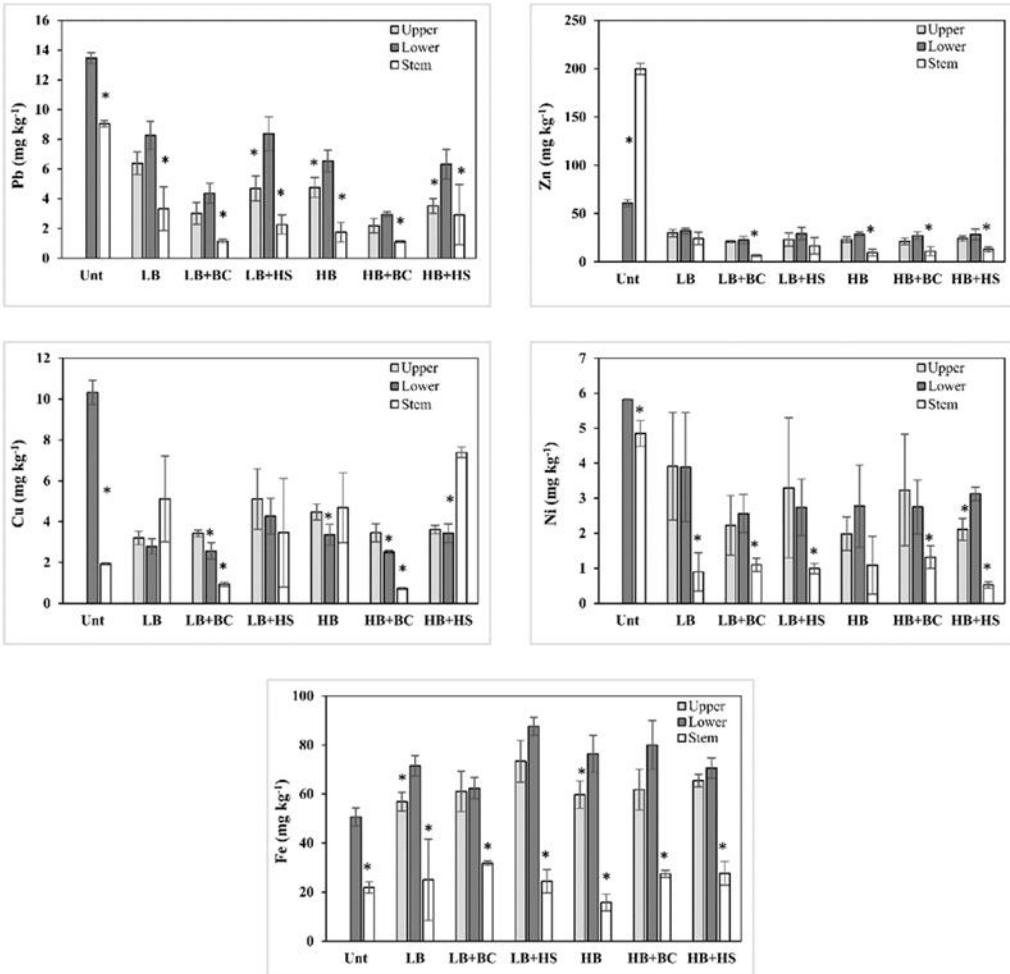

Figure S4. Metal accumulation in upper (new growth) and lower (old growth) leaves and stems of miscanthus.

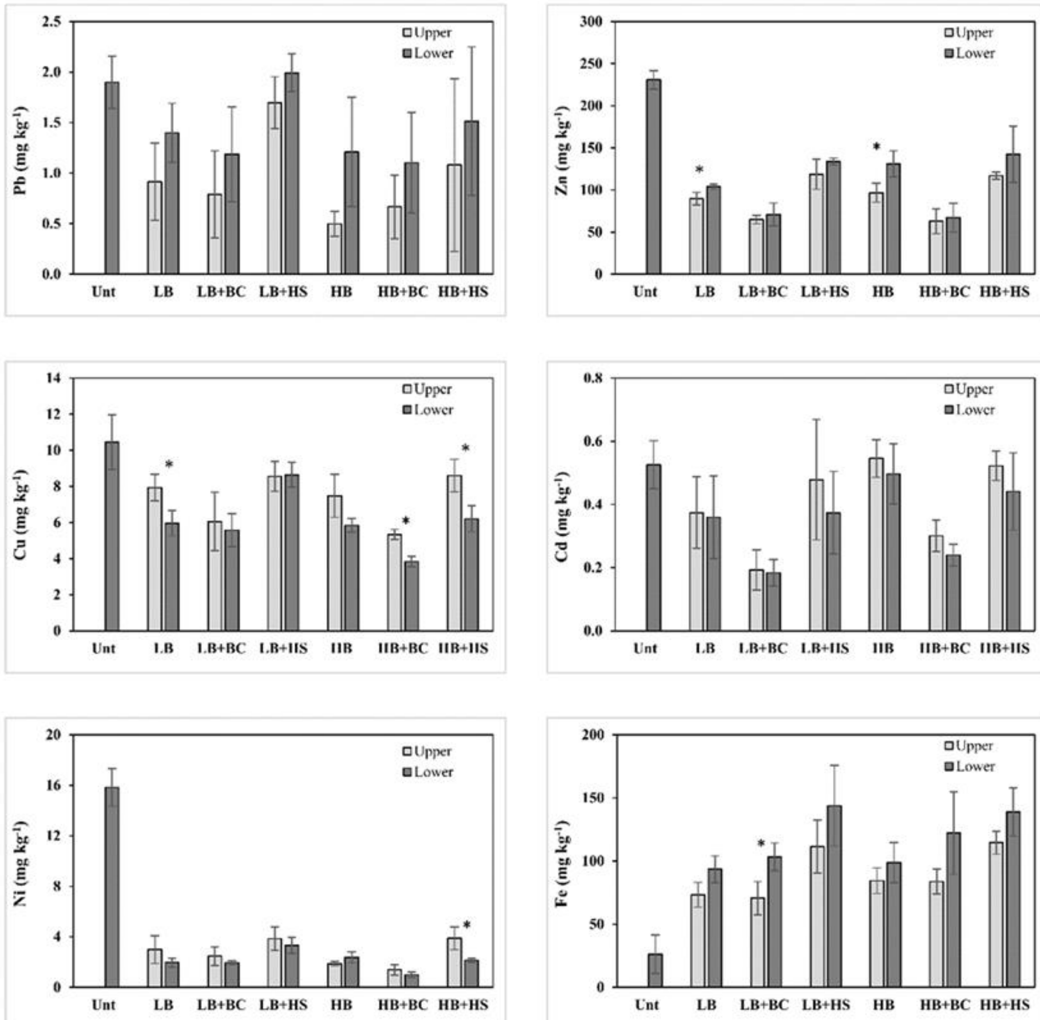

Figure S5. Metal accumulation in upper (new growth) and lower (old growth) leaves of poplar.

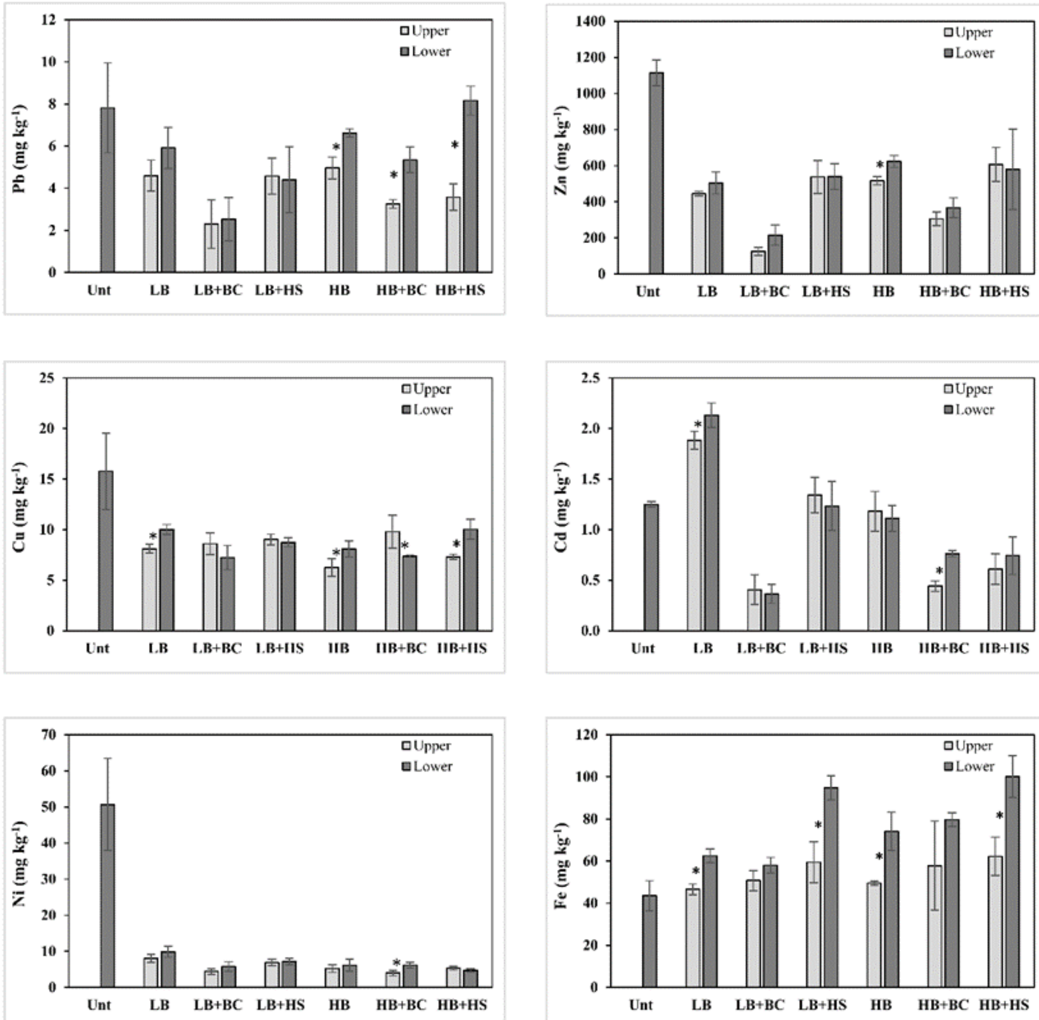

Figure S6. Metal accumulation in upper (new growth) and lower (old growth) leaves of willow.

APPENDIX B.

SUPPORTING INFORMATION: IV. HIGH THROUGHPUT SCREENING OF NATIVE SPECIES FOR TAILINGS ECO-RESTORATION USING NOVEL COMPUTER VISUALIZATION FOR PLANT PHENOTYPING

Table S1. Characterization of the physico-chemical parameters of mine tailings (MT), biosolids (BS), and unpolluted reference soil (RS).

Parameter*	**Mine tailings (MT)**	**Unpolluted reference soil (RS)**	**Biosolids (BS)**	**Background levels**
pH (H_2O)	7.6	6.8	6.89	> 6.1[†]
EC (mS cm^{-1})	1.2	0.8	4.28	-
CEC (cmol(+) kg^{-1})	3.7	9.8	21.6	5-25[‡]
OM (%)	0.2	1.8	42.4	-
Total N (g kg^{-1})	0.6	1.4	28.3	-
Total P (g kg^{-1})	0.09	0.12	9.4	-
Total K (g kg^{-1})	0.4	1.9	2.2	
NH_4–N (mg kg^{-1})	0.45	5.4	1.29	-
NO_3–N (mg kg^{-1})	3.3	0.6	167	-
Available P (mg kg^{-1})	3	5	935	-
Available K (mg kg^{-1})	31	120	169	-
Metals (mg kg^{-1})				-
Cd	11.8	-	20	<1[ⱡ]
Cu	451	9.2	1253	13[ⱡ]
Ni	56.7	-	226	14[ⱡ]
Pb	3744	25.9	3102	20[ⱡ]
Zn	998	24.5	2223	49[ⱡ]

*Analysis performed by Soil and Plant Testing Laboratory, University of Missouri, Columbia, MO).
†Summary of soil fertility status in Missouri (Nathan et al., 2007).
‡Scrivner and Cooper 1985.
ⱡBackground concentrations for metals in Missouri soils (Tisdall and Oades, 1982).

Table S2. List of native and site indigenous species tested in this study.

Type	**Scientific name**	**Common name**
Trees	*Populus deltoids*†*	Cotton wood
	Salix exigua†*	Sandbar willow
	Robinia pseudoacacia †*	Black locust
	Platanus occidentalis†*	Sycamore
	Prunus americana†*	American plum
Grasses	*Andropogon scoparius*	Little bluestem
	*Andropogon gerardii**	Big bluestem
	Panicum virgatum	Switchgrass
	*Sorghastrum nutans**	Yellow indian grass
	Chasmanthium latifolium	Broad-leaf wood-oat
	*Elymus virginicus**	Virginia wildrye
	Spartina pectinata	Prairie cordgrass
	*Tripsacum dactyloides**	Eastern gamagrass
Forbs and legumes	*Asclepias syriaca*	Common milkweed
	*Desmanthus illinoensis**	Illinois bundleflower
	*Rudbeckia hirta**	Black-eyed-susan
	Strophostyles leiosperma	Silk-seed wildbean
	Verbena hastata	Swamp verbena
	Symphyotrichum novae-angliae	New England aster
	Dalea purpurea *	Purple prairie clover
	*Coreopsis lanceolata**	Lanceleaf coreopsis
	Penstemon digitalis	Foxglove beardtongue
	Echinacea pallida	Pale coneflower
	Liatris pycnostachya	Thick-spike gayfeather
	*Lespedeza capitata**	Rounded head lespedeza
	*Chamaecrista fasciculata**	Prairie partridge pea
	*Amorpha fruticosa**	Desert false indigo
	Solidago missouriensis	Prairie goldenrod
	Oenothera biennis†	Evening primrose
	Erigeron annuus†	Annual fleabane
	Symphyotrichum pilosum†	Hairy aster
	Lespedeza thunbergii†‡	Bush cover
	Melilotus officinalis†‡*	Yellow sweet clover
	Kummerowia stipulacea†‡	Korean lespedeza

†Species identified indigenous to The New Viburnum Tailings Impoundment, Viburnum, MO,

‡Introduced legume species.

*Species selected to be moved to the screening experiment.

Table S3. Evaluation criteria used in the prescreening experiment.

Parameter	**Measurement**
Germination	Scale 0-3 (0 = no germination or germination followed by quick death; 1 = low germination; 2 = good germination; 3 = very good germination)
Cover abundance	Scale 1-5 (1 = low cover < 5% of the surface; 2 = covering 5 to 25% of the surface; 3 = covering 25 to 50% of the surface; 4 = covering 50 to 75% of the surface; 5 = covering > 75% of the surface)
Early survival	Scale 0-1 (the number of seedlings still viable after 1-month growth divided by the initial number of transplanted seedlings (i.e., 20 seedlings))

Table S4. Comparison of physico-chemical parameters of soil pore water extracted from treatments after one month of incubation (RS = reference soil, MT = untreated tailings, BS = biosolids treated tailings).

Treatments		(mS cm^{-1})	(mg L^{-1})	Nutrients (mg L^{-1})					Metals (µg L^{-1})		
	pH	EC	DOC	N	P	K	Ca	Mg	Pb	Zn	Cu
RS	6.7±0.1 c	0.9±0.1 c	21.6±3.1 b	30.4±4.2 b	0.26±0.04 b	50 ±10 a	204±22 b	119±20 c	<d.l.	<d.l.	<d.l.
MT	7.6±0.0 a	1.8±0.2 b	2.7± 0.5 c	11.3±2.1 c	0.17±0.02 c	13±2 c	78±8 c	164±21 b	149±5 b	820±91 b	107±12 b
BS	7.3±0.0 b	3.6±0.2 a	34.7±3.8 a	213.6±17.5 a	0.46±0.03 a	21±1 b	317±42 a	263±18 a	206±35 a	1504±154 a	303±28 a

Values are mean ± SD, n = 5; data were evaluated using one-way ANOVA with post-hoc analysis; For each parameter, treatments with different letters differ significantly ($p<0.05$); d.l. = method detection limit.

Table S5. Cover abundance, germination, and early survival of species tested in the pre-screening experiment (MT = untreated tailings, BS = biosolids-treated tailings).

Species	Germination		Cover abundance		Early survival	
	MT	BS	MT	BS	MT	BS
A. scoparius	1	1	2	2	0.9	0.8
A. gerardii	3	3	5	5	0.95	0.8
P. virgatum	3	2	5	4	1	0.8
S. nutans	3	3	5	5	0.85	0.7
C. latifolium	1	2	2	3	0.4	0.45
E. virginicus	3	3	4	4	1	1
S. pectinata	3	3	3	4	0.9	0.75
T. dactyloides	1	2	1	2	1	1
A. syriaca	2	3	4	5	0.45	0.65
D. illinoensis	3	3	5	5	0.85	0.95
R. hirta	3	3	5	5	0.65	0.7
S. leiosperma	1	1	1	1	0.15	0.25
V. hastata	0	3	1	5	0.35	0.7
S. novae-angliae	3	3	5	5	0.55	0.65
D. purpurea	1	3	1	5	0.85	0.9
C. lanceolata	3	3	5	5	0.65	0.85
P. digitalis	1	1	1	2	0.65	0.7
E. pallida	2	2	3	3	0.35	0.4
L. pycnostachya	1	1	2	2	0.65	0.3
L. capitata	3	3	5	5	0.6	0.7
C. fasciculate	1	1	1	2	0.85	0.9
A. fruticosa	3	3	5	5	0.9	1
S. missouriensis	0	0	1	1	0.5	0.05
O. biennis†	0	1	1	1	-	-
E. annuus†	-	-	-	-	0.05	0.15
S. pilosum†	0	1	1	1	-	-
L. thunbergii†‡	-	-	-	-	-	-
M. officinalis†‡	1	3	1	5	0.35	1
K. stipulacea†‡	0	0	1	1	-	-

†Species identified indigenous to The New Viburnum Tailings Impoundment, Viburnum, MO, US.
‡Introduced legume species.

Table S6. Image-based phenotypic parameters extracted by PlantCV (RS = reference soil, MT = untreated tailings, BS = biosolids-treated tailings).

Species	Treatments	Morphological traits									Color traits (hue)	
		(pixels) × 10^5		(pixels) × 10^3							(degree)	
		Area	Convex hull area	Width	Height	Perimeter	Longest path	Ellipse major axis	Ellipse minor axis	Solidity	Circular mean	Circular median
P. deltoides	RS	6.11±1.50 a	17.16±2.82 a	1.21±0.23 a	1.76±0.06 a	47.3±16.9 a	12.6±0.6 a	1.54±0.10 a	1.05±0.18 a	0.35±0.05 a	111.7±5.4 a	111.0±8.2 a
	MT	0.70±0.94 c	4.60±2.03 b	0.65±0.24 b	1.31±0.22 b	13.0±4.2 b	9.3±1.4 b	1.60±0.92 a	0.73±0.37 a	0.16±0.15 b	91.7±3.0 b	92.0±3.3 b
	BS	3.59±1.43 b	14.65±3.75 a	1.26±0.15 a	1.67±0.23 ab	39.9±6.8 a	12.6±1.8 a	1.37±0.24 a	0.92±0.18 a	0.32±0.09 a	88.5±6.0 b	87.2±6.7 b
C. fasciculata	RS	9.12±2.18 a	19.12±2.18 a	1.71±0.18 a	1.57±0.28 a	77.6±23.2 a	12.1±1.4 a	1.47±0.22 a	1.18±0.19 a	0.48±0.05 a	80.8±1.1 a	82.4±0.9 a
	MT	1.51±0.25 c	5.53±1.68 c	0.85±0.17 c	0.90±0.15 b	22.2±9.3 b	7.2±1.7 b	0.85±0.22 b	0.55±0.10 c	0.35±0.07 a	65.3±4.7 b	64.0±3.2 b
	BS	3.80±0.63 b	8.47±0.85 b	1.20±0.15 b	1.01±0.12 b	40.4±13.7 ab	8.6±0.8 b	1.01±0.06 b	0.81±0.07 b	0.45±0.08 a	69.1±1.9 b	71.4±0.9 b
A. fruticosa	RS	8.03±0.37 a	13.77±1.96 a	1.42±0.15 a	1.29±0.12 a	80.2±25.6 a	10.2±1.1 a	1.26±0.18 a	1.02±0.03 a	0.63±0.02 a	94.5±1.4 a	98.0±1.4 a
	MT	0.13±0.04 c	0.55±0.22 c	0.37±0.11 c	0.21±0.05 c	3.9±2.0 c	2.5±0.6 c	0.34±0.07 c	0.19±0.03 c	0.37±0.18 a	69.9±7.5 b	69.6±5.9 b
	BS	2.73±0.62 b	4.95±1.38 b	0.90±0.09 b	0.70±0.14 b	25.7±10.0 b	6.3±0.6 b	0.78±0.06 b	0.58±0.12 b	0.61±0.04 a	77.6±4.1 b	78.6±3.6 b
D. purpurea	RS	0.71±0.08 b	4.44±1.57 ab	0.91±0.14 a	0.75±0.26 a	82.8±10.3 a	6.3±0.8 a	0.79±0.26 a	0.48±0.14 a	0.22±0.04 b	82.7±2.2 a	83.0±2.4 a
	MT	0.70±0.11 b	2.26±0.85 b	0.58±0.03 b	0.69±0.28 a	7.3±2.1 c	5.5±1.2 a	0.72±0.24 a	0.40±0.12 a	0.36±0.20 ab	68.8±4.9 b	67.2±4.1 b
	BS	2.59±0.26 a	5.59±0.60 a	1.00±0.11 a	0.89±0.05 a	23.6±2.1 b	6.5±0.4 a	0.92±0.05 a	0.58±0.13 a	0.47±0.07 a	71.8±4.1 b	70.4±3.0 b
D. illinoensis	RS	3.74±0.76 b	6.14±0.81 b	1.31±0.16 b	0.64±0.02 b	81.7±34.6 a	8.8±0.9 b	1.12±0.14 b	0.54±0.03 b	0.61±0.11 a	84.1±1.6 a	84.8±1.1 a
	MT	0.78±0.21 c	1.66±0.60 c	0.60±0.02 c	0.39±0.20 c	21.3±4.5 b	4.3±0.7 c	0.50±0.02 c	0.28±0.05 c	0.48±0.10 a	60.3±4.7 c	60.8±5.8 c
	BS	7.20±1.08 a	14.34±2.45 a	1.78±0.15 a	1.01±0.16 a	122.2±38.0 a	12.3±1.4 a	1.56±0.12 a	0.93±0.17 a	0.50±0.02 a	70.6±1.6 b	71.1±2.1 b
M. officinalis	RS	0.36±0.18 b	2.93±0.80 b	0.79±0.10 b	0.55±0.11 b	63.8±23.6 a	5.2±0.8 b	0.62±0.08 b	0.38±0.05 b	0.12±0.04 b	68.2±5.5 b	68.4±6.4 b
	MT	0.02±0.02 c	0.18±0.09 c	0.32±0.09 c	0.09±0.03 c	2.5±1.2 b	2.0±0.6 c	0.29±0.09 c	0.09±0.03 c	0.10±0.10 b	42.9±11.2 c	43.2±14.0 c
	BS	5.67±0.48 a	14.25±4.03 a	1.79±0.09 a	1.09±0.29 a	79.7±11.2 a	12.0±1.0 a	1.57±0.14 a	0.99±0.20 a	0.45±0.05 a	85.4±7.7 a	84.8±7.0 a

Values are mean ± SD, n = 5; data were evaluated using one-way ANOVA with post -hoc analysis; Within a plant species, treatments with the same letter are not statistically different ($p<0.05$).

Table S7. The quality of representation of variables of the principal components (i.e., Cos2) for the imaged species shown in PCA biplots in Figure 7.

Traits	*P. deltoides*		*C. fasciculata*		*A. fruticosa*		*D. purpurea*		*D. illinoensis*		*M. officinalis*	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Area	0.91	0.05	0.96	0.00	0.98	0.00	0.36	0.30	0.92	0.04	0.89	0.10
Convex hull area	0.92	0.05	0.94	0.03	0.98	0.00	0.87	0.04	0.87	0.12	0.92	0.05
Solidity	0.53	0.16	0.27	0.69	0.43	0.52	0.07	0.18	0.03	0.59	0.78	0.08
Height	0.64	0.00	0.87	0.08	0.97	0.00	0.53	0.24	0.80	0.10	0.90	0.00
Width	0.65	0.23	0.87	0.00	0.97	0.01	0.74	0.00	0.98	0.01	0.99	0.00
Perimeter	0.69	0.03	0.89	0.00	0.90	0.00	0.28	0.64	0.68	0.01	0.66	0.17
Longest path	0.68	0.01	0.90	0.02	0.98	0.01	0.76	0.06	0.97	0.02	0.98	0.00
Ellipse minor axis	0.43	0.00	0.94	0.00	0.95	0.00	0.62	0.19	0.97	0.01	0.98	0.01
Ellipse major axis	0.01	0.13	0.87	0.06	0.98	0.01	0.56	0.18	0.25	0.72	0.92	0.05
Hue circular mean	0.35	0.60	0.86	0.00	0.84	0.13	0.18	0.74	0.27	0.70	0.77	0.19
Hue circular median	0.31	0.64	0.89	0.03	0.90	0.08	0.22	0.75	0.25	0.72	0.73	0.22

Morphological traits: area, convex hull area, solidity, height, width, perimeter, longest path, ellipse minor axis, ellipse major axis; Color traits: hue circ ular mean, hue circular mean.

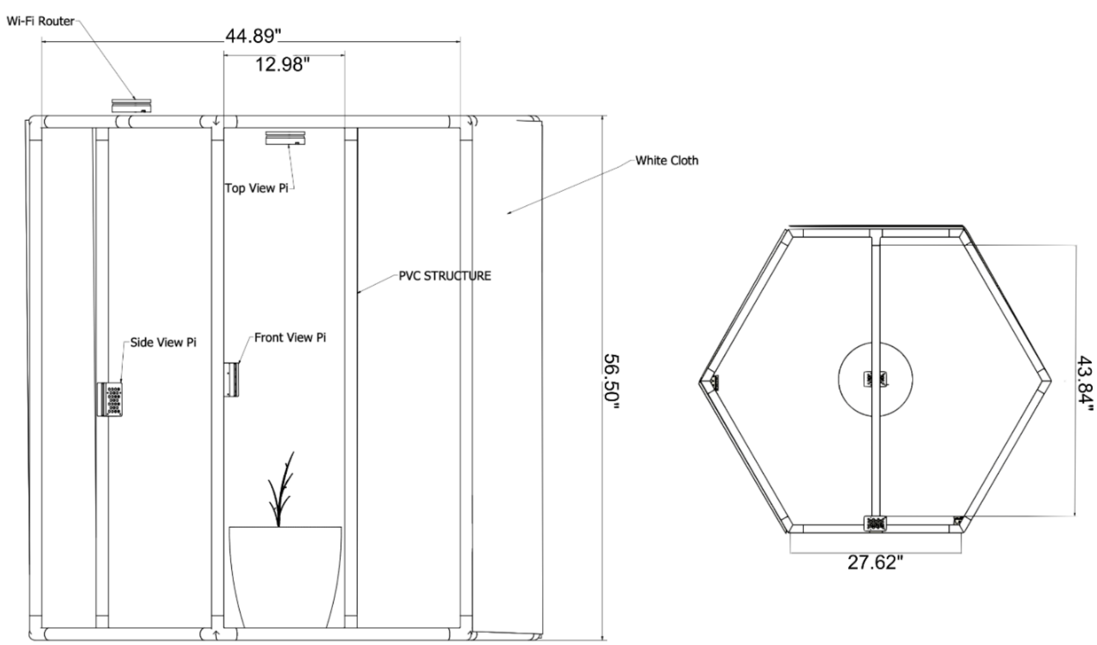

Figure S1. Schematic of hexagon imaging system with the multi-image Raspberry Pi cameras: top view (right) and side view (left).

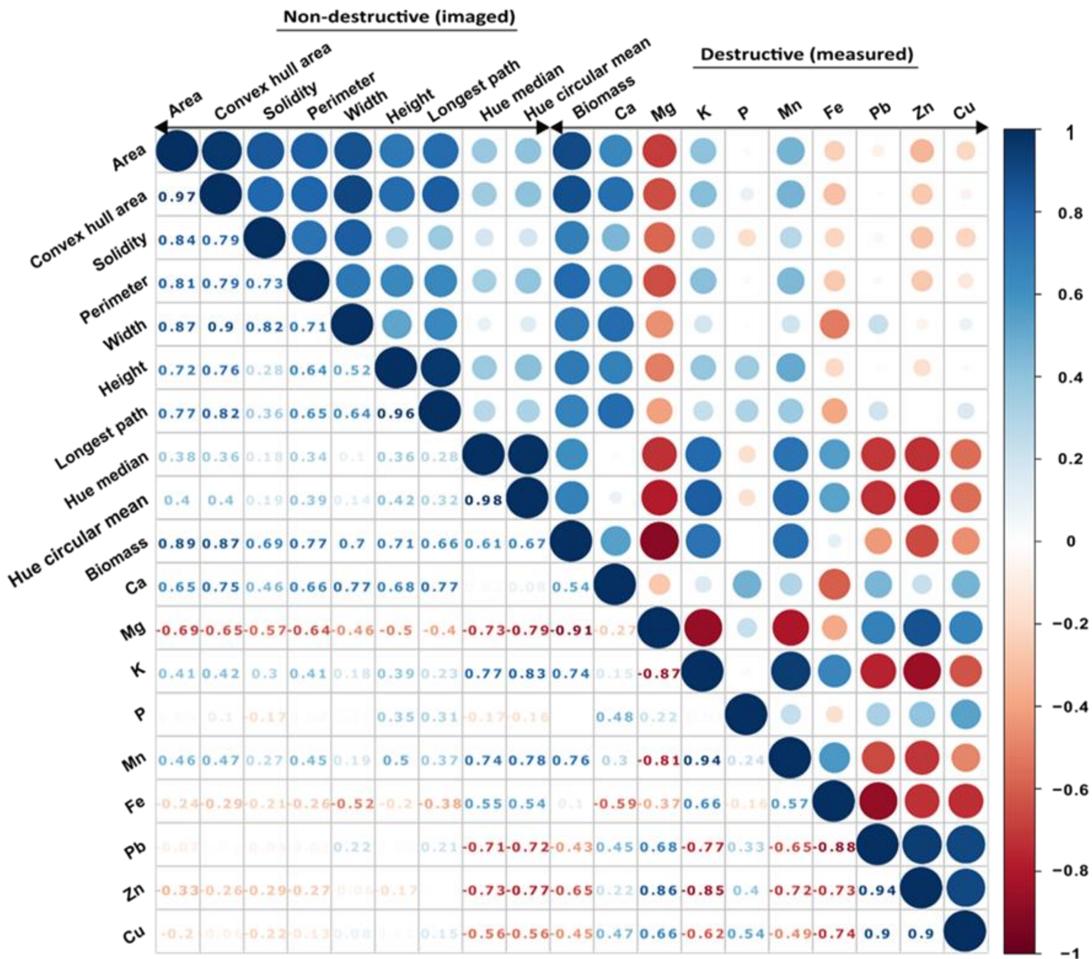

Figure S2. Correlation between non-destructive image-based traits and destructively measured parameters for plant species P. deltoids grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles.

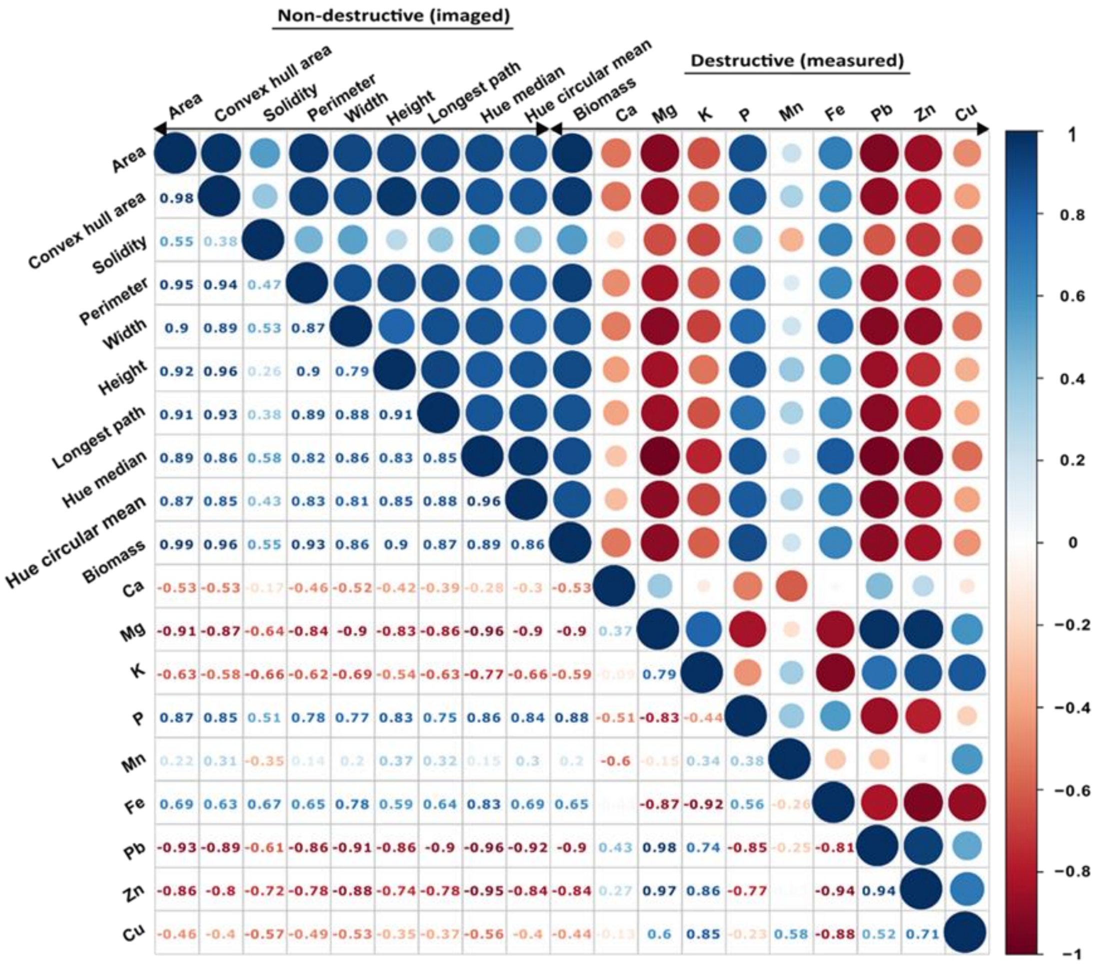

Figure S3. Correlation between non-destructive image-based traits and destructively measured parameters for plant species *C. fasciculata* grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles.

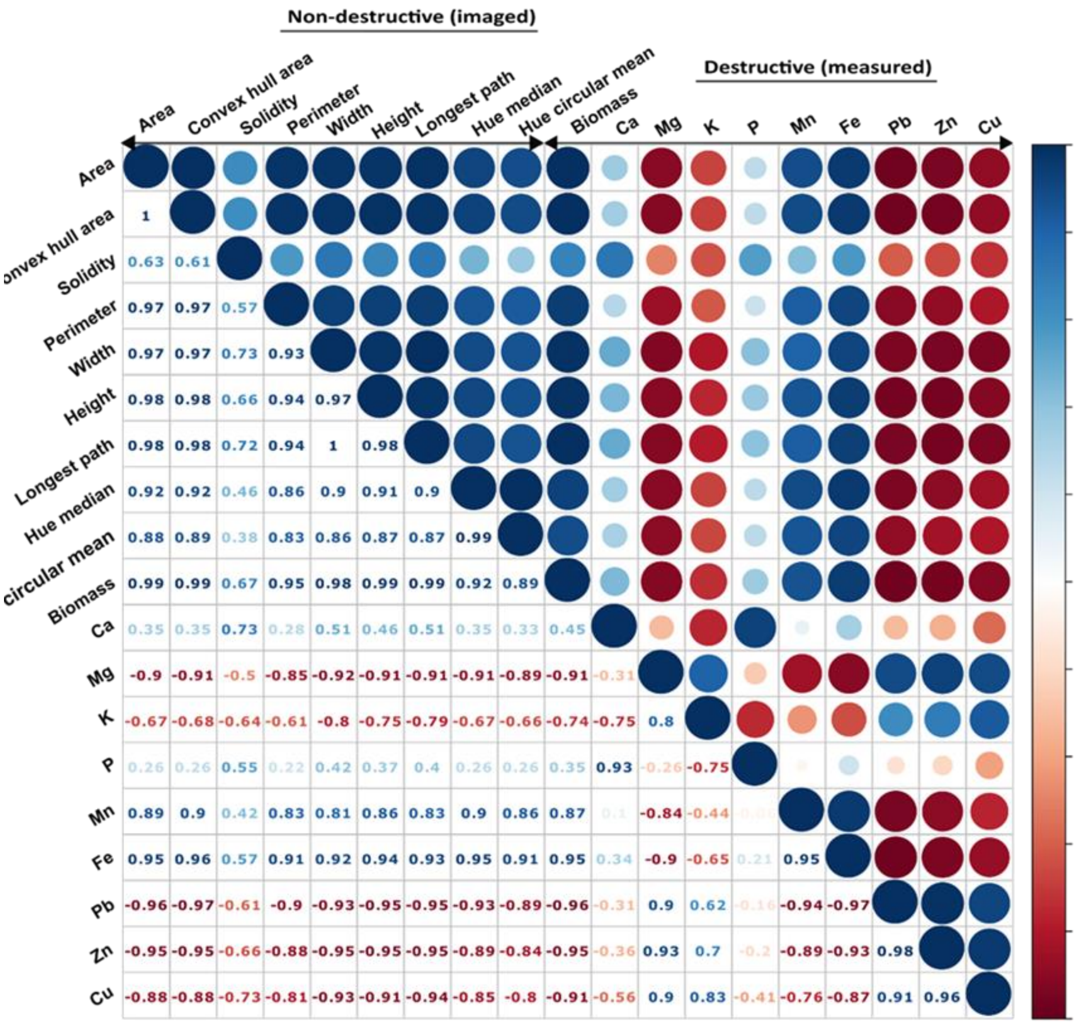

Figure S4. Correlation between non-destructive image-based traits and destructively measured parameters for plant species A. fruticosa grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles.

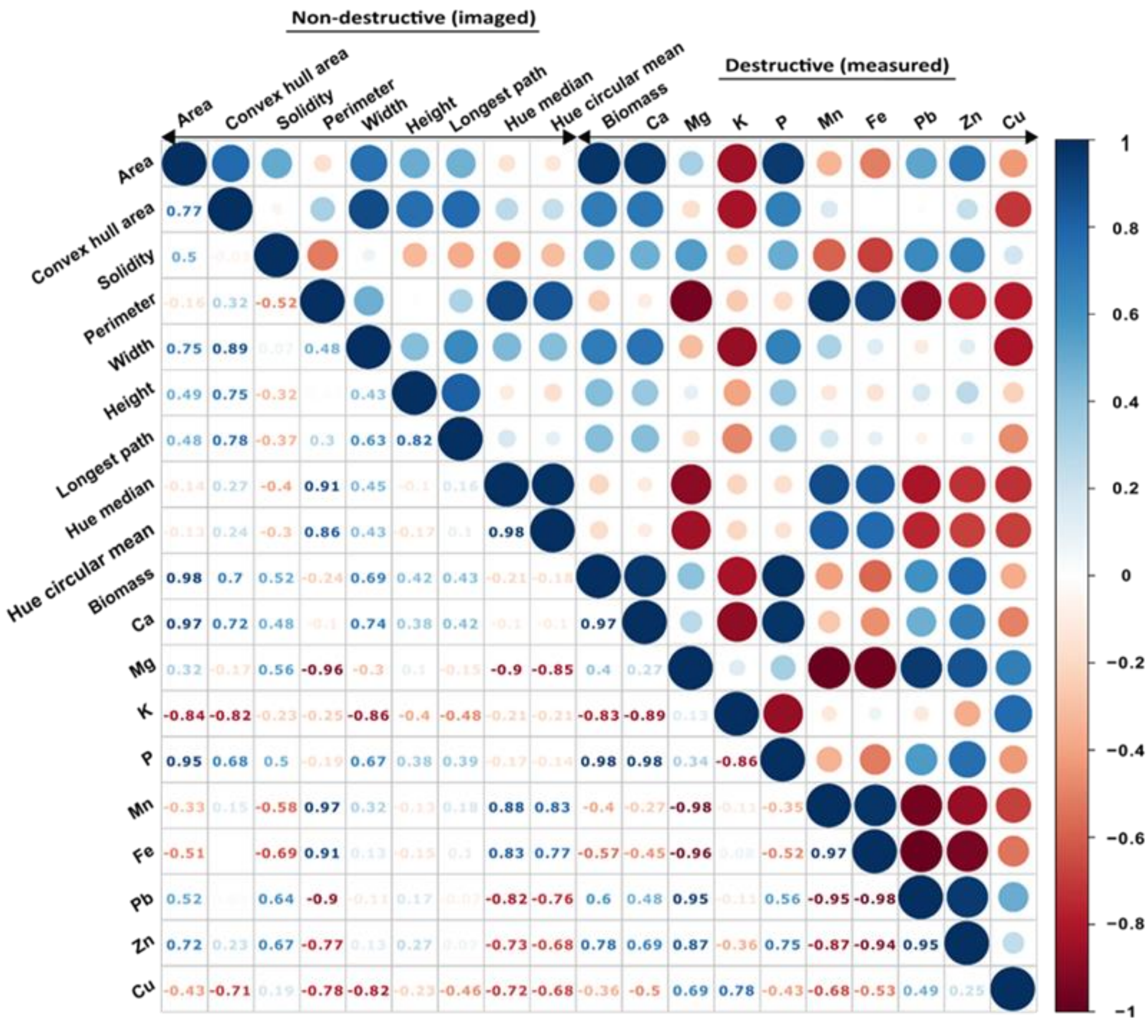

Figure S5. Correlation between non-destructive image-based traits and destructively measured parameters for plant species D. purpurea grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles.

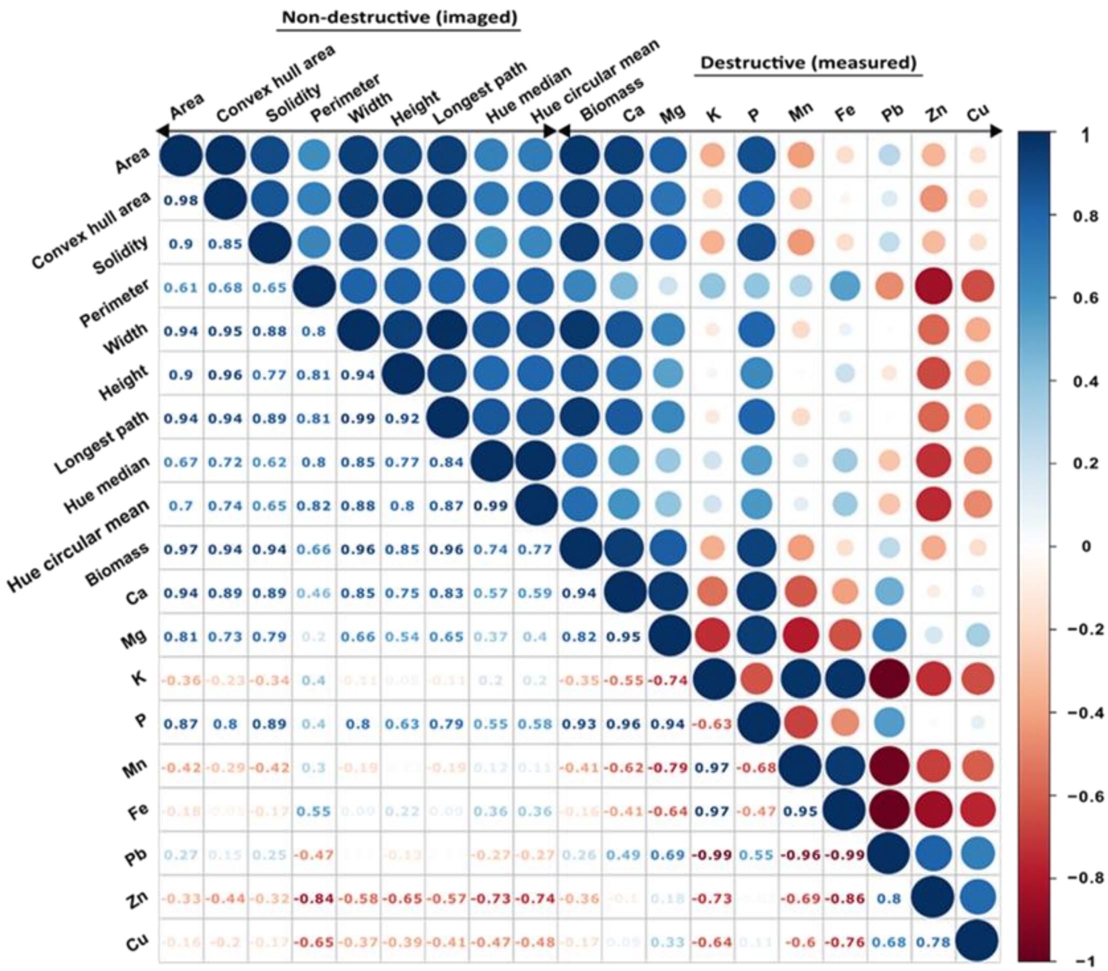

Figure S6. Correlation between non-destructive image-based traits and destructively measured parameters for plant species M. officinalis grown on: unpolluted reference soil, untreated tailings, or biosolids-treated tailings, imaged at week 15 and analyzed by PlantCV. Blue color represents positive correlations on scale 0 to 1; while red color represents negative correlations on scale 0 to -1. The correlation coefficient for each relation is shown in the corresponding box below the colored circles.

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