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# Arbuscular Mycorrhizal Fungi and Exogenous Glutathione Mitigate Coal Fly Ash (CFA)-Induced Phytotoxicity in CFA-contaminated Soil

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**Abstract**

Coal fly ash (CFA) makes a bulk of the coal combustion wastes generated from coal-fired power plants. There are several environmental mishaps due to coal ash spills around the world and in the United States. Management of CFA-polluted sites has proven inefficient resulting in soil infiltration, leaching, and phytotoxicity. This study assessed the mitigation strategies for CFA-induced phytotoxicity using biological [arbuscular mycorrhizal fungi (AMF)] and chemical [exogenous glutathione (GSH)] agents. Indices of phytotoxicity include seed germination, plant morphometrics, lipid peroxidation and genomic double-stranded DNA (dsDNA) in switchgrass plant (*Panicum virgatum*). Experiments include laboratory screening (0, 5, 10, 15 and 20% w/w CFA/soil) and greenhouse pot study (0, 7.5 and 15% w/w CFA/soil) culturing switchgrass plant in Armour silt loam soil co-applied with AMF (*Rhizophagus clarus*) and GSH. Experiments showed that CFA exposure caused a concentration-dependent increase in seed germination. 10% CFA increased seedling growth while 15 and 20% CFA decreased seedling growth and induced leaf chlorosis. Furthermore, CFA (7.5 and 15%) in the 90-d pot study significantly ($p < 0.05$) impaired plant growth, induced lipid peroxidation and reduced genomic dsDNA. However, the incorporation of AMF or GSH enhanced seed germination, plant growth, and/or genomic dsDNA, reduced lipid peroxidation and prevented leaf chlorosis in CFA-exposed switchgrass plant. This study demonstrates that AMF and GSH have the potential to mitigate CFA-induced phytotoxicity. These biological and chemical strategies could be further harnessed for efficient utilization of switchgrass plant in the phytoremediation of CFA contaminated soil environment while simultaneously limiting CFA-induced phytotoxicity.

**Keywords:** Management strategies; CFA-soil contamination; CFA-induced phytotoxicity; Arbuscular mycorrhizal fungi; Exogenous glutathione.

**Abbreviations:** CFA, coal fly ash; GSH, reduced glutathione; AMF, arbuscular mycorrhizal fungi; dsDNA, double-stranded deoxyribonucleic acid; ASL, armour silt loam; PTE, potentially toxic element; ROS, reactive oxygen species; HSP, heat shock protein; PBMN, peripheral blood mononuclear; pH, potential hydrogen; TVA, Tennessee valley authority; Gly, glyoxalase; MG, methylglyoxal; GST, glutathione-S-transferase; GR, glutathione reductase; GPX, glutathione peroxidase; GSSG, glutathione disulfide; AsA, ascorbic acid; $H_2O_2$, hydrogen peroxide; PC, phytochelatin; FW, fresh weight; MDA, malondialdehyde; MC, moisture content; FL, foliage length; FN, foliage number; RN, root number; TE, tris ethylenediaminetetraacetate; EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloric acid; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TBA thiobarbituric acid; ANOVA, analysis of variance; SE, standard error; LSD, least significant difference; Ap, plowed surface horizon A; FW, fresh weight; LC, leaf chlorosis; SG, stunted growth; LP, lipid peroxidation.

## 1. Introduction

Coal fly ash (CFA)-induced toxicities have been reported in several studies with limited information on the potential mitigation strategies. These toxicities include oxidative stress induction in peripheral blood mononuclear (PBMN) cells (Dwivedi et al., 2012), cytotoxicity in the channel catfish ovary cell (Medunić et al., 2016), growth inhibition in Lemna (*Lemna minor* L.) (Radić et al., 2018), and oxidative DNA damage in Chang liver cell (Sambandam et al., 2015). Phytotoxicity effects that are due to CFA-soil contamination and exposure have been attributed to its constituent potentially toxic elements (PTEs), e.g. Cd, Cr, Pb, and As (Radić et al., 2018; Singh et al., 2016), most of which are present in coal ash spill sites requiring remediation.

There had been incidences of large coal ash spills around the world, and in the United States with widespread environmental and economic impact. Amongst the popular coal ash spills in the United States was the spill by Tennessee Valley Authority (TVA) Kingston Fossil Plant, Roane County, Tennessee in 2008. The spill resulted in the release of ~5.4 million cubic yards of coal into Swan Pond Embayment, Emory River channel and ~300 acres outside of the plant (USEPA, 2016). More recently, in 2014, was the Duke Energy coal ash spill which released ~39,000 tons of coal ash from its Dan River Steam Station into the Dan River in Eden, North Carolina (USEPA, 2017). Coal ash spills result in environmental contamination and degradation that pose threats to the health and survival of living organisms including plants and animals inhabiting such fly ash contaminated sites. The PTEs in CFA such as heavy metals may generate reactive oxygen species (ROS) in exposed organisms (plants and animals) that may attack and disrupt cellular macromolecules including deoxyribonucleic acids, lipids, and protein (Awoyemi and Dzantor, 2017a).

Various studies concerning the environmental impact of CFA ranged from soil and water (surface and groundwater) contamination, to impairment of organisms' growth and function (including the productivity and yield of plant crops). For instance, Roy and Joy (2011) conducted short-term laboratory and field studies on the dose-based effect of CFA on chemical and microbial properties of laterite cropland soil. They mixed sandy loam soil with farmyard manure (10% w/w) and amended with fly ash at 5%, 10%, 20%, 40% w/w (50–400 $t\ ha^{-1}$). The study showed temporary inhibition of bacteria, fungi and actinomycetes populations at 5% and 10% CFA doses, but 20% and 40% were harmful coupled with a decline in soil enzymes at higher doses (Roy and Joy, 2011). In another study by Raja et al. (2014), $\geq 0.5\ g\ m^{-2}\ day^{-1}$ CFA significantly reduced the photosynthesis, stomatal conductance, transpiration and albedo in rice crops. At higher rates of CFA deposition, all growth and yield parameters were significantly influenced, and a significant reduction in

grain yield was recorded, compared to the control treatments when 0.5, 1.0 and 1.5 g $m^{-2}$ $day^{-1}$ CFA was dusted (Raja et al., 2014). Moreover, CFA have been reported to reduce soil enzymatic activities (e.g. dehydrogenase, acid phosphatase, β-glucosidase and urease), induce lipid peroxidation in plant crops, augment sterility, impair plant morphology and growth (Singh et al., 2016).

Arbuscular mycorrhizal fungi (AMF) (Firmin et al., 2015) and exogenous reduced glutathione (GSH) (Hossain et al., 2012) have been suggested to play important roles in modulating metal-induced toxicity and oxidative stress in plants (Awoyemi and Dzantor, 2017a). Therefore, the main objective of this current study was to assess the protective roles of AMF (*Rhizophagus clarus*) and exogenous GSH in mitigating CFA-induced phytotoxicity in switchgrass (*Panicum virgatum*), a bioenergy crop plant. Furthermore, to determine the optimal concentration of the CFA that AMF and GSH co-application would be most efficient for mitigating CFA-induced phytotoxicity. The indices of toxicity that were assessed include seed germination, seedling growth, plant morphometrics, genomic double-stranded deoxyribonucleic acid (dsDNA), chlorosis, and lipid peroxidation.

Acute and chronic phytotoxicity studies were conducted using soil-on-agar technique under laboratory-controlled condition, and deep-pot experiment modified to allow for infiltration, in a controlled greenhouse. This study is important because coal fly ash has been more recently considered as a resource for soil amendment, besides its potential to contaminate the environment with PTEs. Therefore, providing data on alternative management strategies for CFA-soil contamination and determining the concentration at which CFA can be co-applied effectively with AMF and/or GSH would serve as protective measures in mitigating CFA-induced phytotoxicity.

## 2. Materials and Methods

2.1. Experimental design for seed germination and seedling growth

Laboratory screening of switchgrass seeds germination and seedling growth was conducted using 20% volume/volume (v/v) soil-on-agar technique (Voigt et al., 1997) in 50 mL conical centrifuge tubes (Falcon$^{TM}$, Fisher Scientific, Hampton, NH). Armor silt loam (ASL) soil was treated with five concentrations of CFA, 0 (control), 5, 10, 15 and 20% weight/weight (w/w) CFA/soil in four replicates. To separate aliquots of the CFA/soil treatments, 3% w/w propagating mixture of arbuscular mycorrhizal fungi (AMF), *R. clarus,* WV234 (INVAM, Morgantown, WV, USA) was inoculated. A total of four sterilized seeds of switchgrass (Star Seeds Inc., Osborne, Kansas) was cultured in each tube of the CFA treatment (in four replicates) for 5 d. To another portion of the similar CFA/soil/AMF treatments, a total of four 5-d old seedlings initially germinated in Petri plates was transplanted and allowed to grow for 21 d. The number of seedlings that survived the 21-d exposure period was used to determine seedling growth (%), while percentage seed germination was calculated as expressed in Eq. 1.

$$\%\ seed\ germination = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds\ planted}\ x\ 100 \quad \text{.....(1)}$$

To determine the optimum AMF concentration that will efficiently support switchgrass seedling growth, 20% v/v media-on-agar technique was used. Treatments include 0 and 20% w/w CFA in ASL soil. The experiment was conducted in 50 mL tubes and replicated with soilless (1:1:1 of peat moss: vermiculite: sand) media for comparison. To separate treatments of the CFA/soil and CFA/soilless media, 0, 1, 3 and 5% w/w propagating mixture of the AMF, *R. clarus* were inoculated. A total of four 5-d old seedlings of switchgrass was transplanted in each tube of the CFA treatment (in five replicates) in the presence and absence of AMF for 28 d. Seedling height was measured at days 21 and 28. The properties of the ASL soil and the CFA used in this study are described in Table 1 (see supplementary data). The ASL soil is a fine-silty, mixed, thermic Ultic Hapludalf collected

from A horizon with dark brown (10 yr 3/3) color, ranged from 0 to 6 inches in thickness and belongs to the subgroup typical pedon Ap, i.e. plowed surface horizon A.

### 2.2. Experimental design for greenhouse mesocosm study

The pot experiment was conducted according to the methods described by Awoyemi and Dzantor (2017a, 2017b). Briefly, the ASL soil was sieved through 2 mm sieve, and ~1.5 kg was transferred into 6-inch deep standard nursery pots, treated with 0, 7.5 and 15% w/w CFA. The second portion of each CFA treatment was inoculated with 3% w/w AMF, *R. clarus* and a third portion was treated with 10% 0.65 mM GSH solution (Acros Organics, NJ, USA) to make a total of nine treatments in four replicates. To each treatment, four 5-week old switchgrass seedlings which were initially cultured in germination trays containing potting mix (Farfard #2 mix) were transplanted, and plant growth was monitored for a period of 90 d. The greenhouse-controlled conditions were the temperature of 24 °C at daytime, 26 °C at night, and humidity was 80%. At 90 d, plants were harvested, thoroughly washed with tap water and fresh weight (FW) was determined using analytical balance (Mettler Toledo, Scientific Instruments, Columbus, OH). Other plant morphometrics determined include moisture content (MC), foliage length (FL), foliage number (FN), and root number (RN).

### 2.3. Measurement of genomic dsDNA

Assay for genomic double strand DNA concentration (dsDNA) was carried out according to the methods of Edwards et al. (1991). Freshly weighed (25 mg) plant leaves were pulverized with 200 µL of tris ethylenediaminetetraacetate (TE) buffer [10 mM Tris HCl + 0.1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.5] to form slurry. The slurry was mixed 400 µL lysis solution [200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS)] in 1.5 mL microcentrifuge tube, and incubated at 65 °C

for 5 min. After incubation, 600 µL of chloroform was immediately added, gently emulsified by inversion (3-5 times) and centrifuged at 10000 rpm (~9400 x g) for 2 min. The upper aqueous phase containing DNA was transferred into a new 2 mL microcentrifuge tube, and 800 µL of isopropyl alcohol was added, mixed gently by several inversions, incubated for 1 h at -20 °C and centrifuged at 10000 rpm for 2 min. The supernatant was discarded, and DNA pellet was dissolved in 100 µL of NaCl solution by gentle vortexing. 300 µL of 70% cold ethanol was added to precipitate DNA (10 min at -20 °C), centrifuged at 10000 rpm for 4 min, and ethanol was discarded. The DNA pellet was washed with 70% cold ethanol and dissolved in 100 µL of sterile deionized water by gentle vortexing. Absorbance was read at 260 nm and 280 nm using a high-performance hybrid multi-mode microplate reader (Synergy$^{TM}$ H1, BioTek Instruments Inc., Winooski, Vermont, USA). The genomic dsDNA concentration was expressed as ng $\mu L^{-1}$ and DNA purity expressed as $A_{260}/A_{280}$.

## 2.4. Measurement of lipid peroxidation

Assay for lipid peroxidation was conducted according to the method of Buege and Aust (1978) with minor modifications as described by Awoyemi and Dzantor (2017a). Briefly, 100 mg freshly weighed plant samples were homogenized with 2 mL of 50% ethanol, in a pre-chilled mortar. The homogenates were centrifuged at 10000 x g and 4 °C for 10 min to obtain extracts. 100 µL of the plant tissue extract was added with 100 µL of 8.1% SDS solution and 4 mL of trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-HCl reagent [15% (w/v) TCA, 0.375% (w/v) TBA and 0.25 N HCl]. The contents were incubated at 95 °C for 60 min, cooled and centrifuged at 1600 x g for 10 min at 4 °C. Absorbance was read at 535 nm using a microplate reader, and lipid peroxidation was expressed in µM malondialdehyde (MDA) $g^{-1}$ fresh weight (FW).

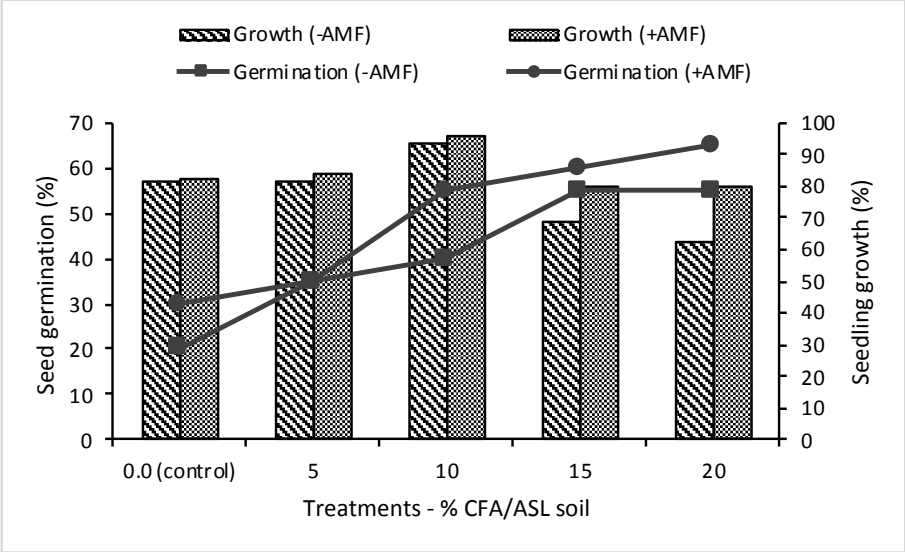
### 2.5. Statistical analysis

The results obtained were expressed as mean ± standard error (SE) and presented in the form of descriptive graphs created with Microsoft Excel v16.17 (2018). The data were subjected to one-way and two-way analysis of variance (ANOVA). Where statistical significance occurred with the ANOVA at $p < 0.05$, post-hoc analysis was performed using Duncan's multiple range and Fisher's LSD tests to separate the means. The statistical tools used for inferential analysis include IBM SPSS v20.0 (2016), Microsoft Excel v16.17 (2018), and JMP Pro v14.0 (2018).

## 3. Results

### 3.1. Switchgrass seed germination and growth of transplanted seedlings in CFA contaminated ASL soil co-applied with AMF

The results of seed germination and seedling growth shown in Fig. 1 indicates that seed germination increased (by 15-35%) in a concentration-dependent manner with CFA application. Furthermore, there was an increase in seedling growth (by up to 12.5%) in 10% CFA contaminated soil compared to control, while at 15% and 20% CFA, there was a decline in the seedling growth by 12.5% and 18.75%, respectively. However, AMF co-application enhanced both seed germination and seedling growth with increased concentrations of CFA. The AMF-induced seed germination enhancement ranged from 10% in control soil to 45% in 20% CFA contaminated soil while AMF-induced seedling growth enhancement ranged from 1.25% in control soil to 17% in 20% CFA contaminated soil.




210

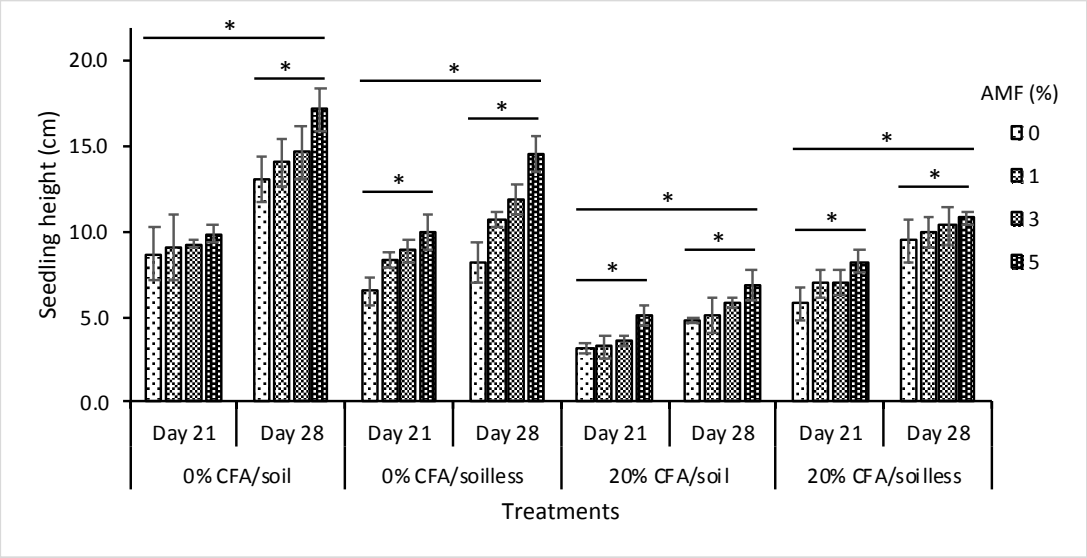
211 Fig. 1. Seed germination and seedling growth (%) in CFA contaminated ASL soil co-applied
212 with AMF. The number of seedlings that survived the 21-d exposure period was used to
213 determine seedling growth (%).

214

215 3.2. Switchgrass seedling growth in CFA/soil and CFA/soilless media co-applied with
216 AMF

217 The height of the switchgrass seedlings cultivated on CFA/soil and CFA/soilless
218 media significantly increased ($p < 0.05$) temporally between 21 d and 28 d (Fig. 2). The CFA
219 at 20% significantly impaired the seedling height, compared to the control. The seedling
220 height impairment was significantly higher in CFA/soil than the CFA/soilless media.
221 However, there was a concentration-dependent increase in seedling height in CFA/soil and
222 CFA/soilless media with AMF inoculum (Fig. 2). The 20% CFA caused leaf chlorosis which
223 was higher in ASL soil than the soilless media (Plate 1, see supplementary data).

224




225

226 Fig. 2. Height (cm) of switchgrass seedlings cultivated in CFA contaminated ASL soil and
227 soilless (1:1:1 of peat moss: vermiculite: sand) media. The CFA/soil and CFA/soilless media
228 were co-applied with AMF at varying concentrations. Data with asterisks (*) are significantly
229 different temporally and/or between treatments ($p < 0.05$, two-way ANOVA).

230

231 3.3. Fresh weight and moisture content of switchgrass plant cultivated in CFA
232 contaminated ASL soil co-applied separately with AMF and GSH

233 There was a significant ($p < 0.05$) concentration-dependent decrease in fresh weight
234 of switchgrass plant exposed to the CFA (Fig. 3). However, in the presence of co-applied
235 AMF, fresh weight of switchgrass was significantly enhanced while this was not the case
236 with co-applied GSH which had a noticeable but non-significant impact in enhancing the
237 plant weight. The co-application with AMF or GSH were prevented the plant against CFA-
238 induced leaf chlorosis. Moisture content in switchgrass plant ranged from a minimum of
239 ~52% in 15% CFA to a maximum of ~73% in 7.5% CFA co-applied with AMF. The
240 moisture content was higher with AMF or GSH co-application than with CFA alone at 7.5%
241 and 15% (Fig. 3).

242

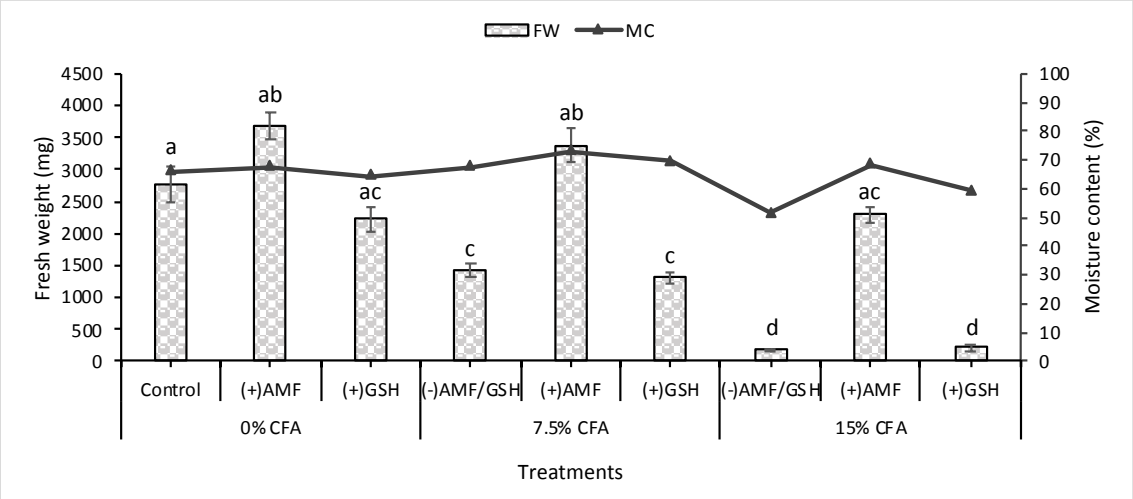



Fig. 3. Fresh weight (mg) and moisture content (%) of switchgrass plant cultivated in CFA contaminated ASL soil co-applied with AMF or GSH. Legend: FW – fresh weight, MC – moisture content. Data with different alphabets are significantly different between treatments ($p < 0.05$, one-way ANOVA, Duncan multiple range and Fisher's LSD tests).

### 3.4. Morphometrics (foliage length, foliage number, and root number) of switchgrass plant cultivated in CFA contaminated ASL soil co-applied separately with AMF and GSH

The measured plant morphometrics including foliage length, numbers of foliage and root of the switchgrass plant were decreased with increased concentration CFA exposure (Fig. 4) The quality of the plant morphometrics was enhanced in CFA-exposed switchgrass plants cultivated in ASL soil co-applied with AMF. The co-applied GSH had a noticeable but non-significant impact in mitigating the CFA-induced impairment of switchgrass plant morphometrics (Fig. 4). CFA-induced leaf chlorosis was mitigated in switchgrass plant cultivated in CFA contaminated ASL soil co-applied with AMF or GSH (Plate 2, see supplementary data).

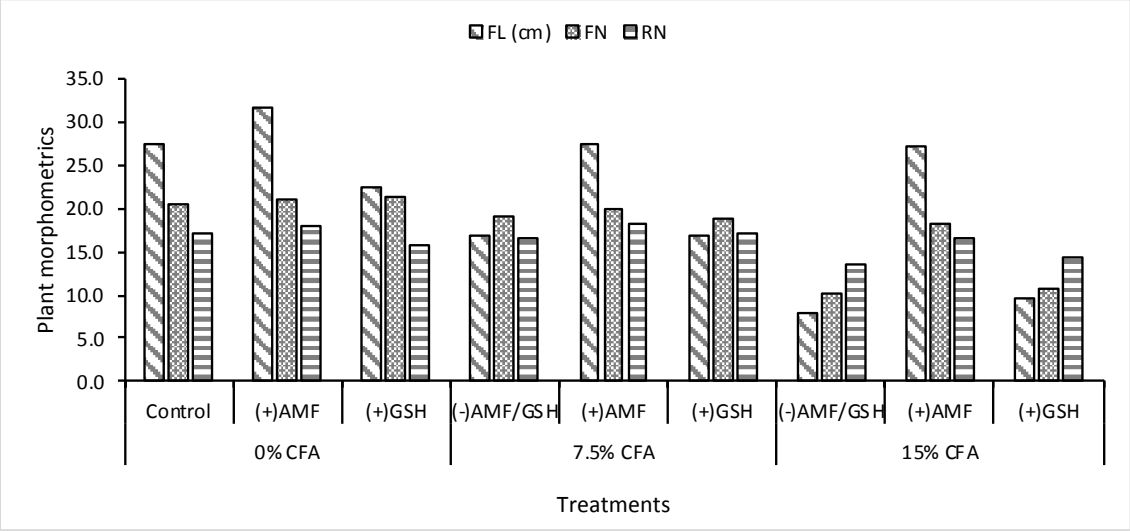
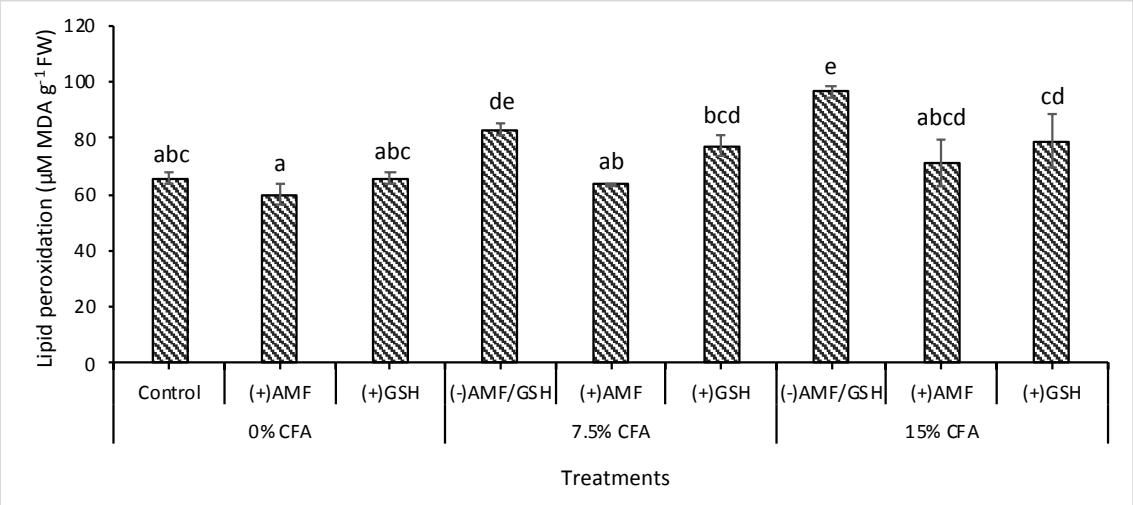



Fig. 4. Morphometrics of switchgrass plant cultivated in CFA contaminated ASL soil co-applied with AMF or GSH. Legend: FL - foliage length, FN – foliage number, and RN – root number.

### 3.5. Lipid peroxidation and total genomic dsDNA in switchgrass plant cultivated in CFA contaminated ASL soil co-applied separately with AMF and GSH

Lipid peroxidation was significantly ($p < 0.05$) higher in switchgrass plants exposed to CFA compared to the control (Fig. 5). However, co-application with AMF significantly reduced lipid peroxidation in switchgrass plants exposed to 7.5 and 15% CFA while co-application with GSH significantly reduced lipid peroxidation only in switchgrass plant exposed to 15% CFA (Fig. 5).

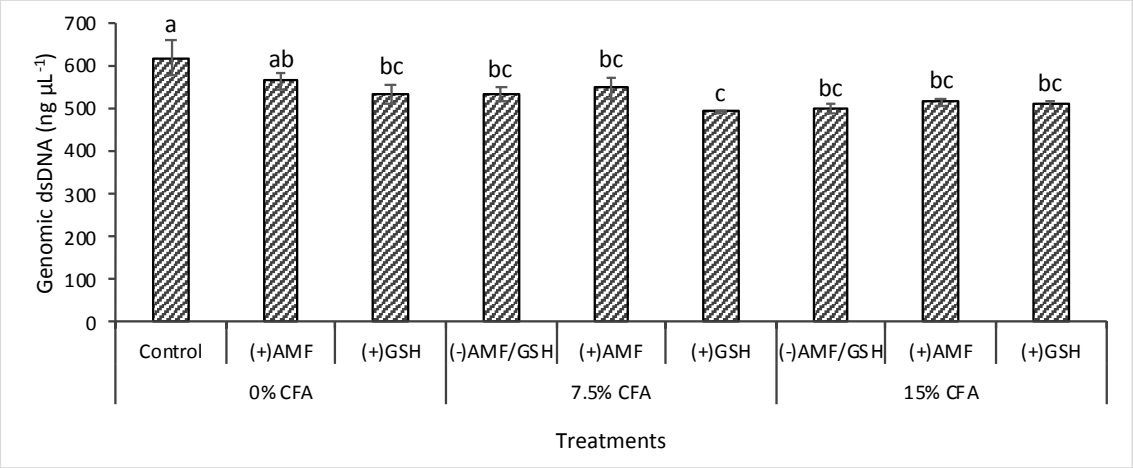
Genomic dsDNA was significantly ($p < 0.05$) decreased in switchgrass plants exposed to CFA compared to the control (Fig. 6). However, co-application with AMF enhanced concentrations of dsDNA in switchgrass plants exposed to 7.5 and 15% CFA while co-application with GSH enhanced concentrations of dsDNA in switchgrass plant exposed to 15% CFA (Fig. 6).




278

279 Fig. 5. Lipid peroxidation (µM MDA $g^{-1}$ FW) in switchgrass plant cultivated in CFA
280 contaminated ASL soil co-applied with AMF or GSH. Data with different alphabets are
281 significantly different between treatments ($p < 0.05$, one-way ANOVA, Duncan multiple
282 range and Fisher's LSD tests).

283




284

285 Fig. 6. Genomic dsDNA (ng $\mu L^{-1}$) in switchgrass plant cultivated in CFA contaminated ASL
286 soil co-applied with AMF or GSH. Data with different alphabets are significantly different
287 between treatments ($p < 0.05$, one-way ANOVA, Duncan multiple range and Fisher's LSD
288 tests).

289

## 4. Discussion

Environmental contamination from coal ash have continued to adversely impact resources including water, soil, plants, and animals, either direct from mining activities and indirectly from accidental spills or leaching from storage sites (Carlson and Adriano, 1993; Gajić et al., 2018). However, there are studies that have continued to explore the potential for utilization of CFA as a soil amendment for plant cultivation but are limited by the phytotoxic impact of the CFA (Adriano and Weber, 2001). Hence, we investigated the potential of AMF (*R. clarus*) and exogenous GSH for mitigating CFA-induced phytotoxicity.

In this study, the germination of the switchgrass seeds was enhanced with CFA exposure (concentration dependent) when compared to the control ASL soil that contained no CFA. The percentage increase in seed germination relative to control are 15%, 20%, 35% and 35%, respectively, in 5%, 10%, 15% and 20% CFA/ASL soils. The higher rates of CFA-induced seed germination have been reported in other studies as described in the review article by Yunusa et al. (2012). Previous field experiments carried out by Swamy et al. (2010) revealed that fly ash applied to soil at the rate of 5 ton/hectare increased germination, shoot height, leaf number, root number, root length, number of bulbs, peroxidase activity and cell division process. Our study showed that the growth of the transplanted switchgrass seedlings in CFA contaminated ASL soil compared to the control increased with 10% CFA while there was a decline in seedling growth at 15% and 20% CFA. Additionally, the phytotoxic effects of CFA were higher in the ASL soil than in the soilless media indicating that besides CFA type, properties and concentrations, the media type may account for variations in the CFA-induced phytotoxicity and/or beneficial agricultural effects.

Results from several studies have revealed that coal ash application to soil increased crop biomass and yields (He et al., 2017; Schönegger et al., 2018). The enhancement of seed germination and seedling growth induced by lower concentrations of CFA may be due to its

315 desirable agricultural properties including mineral elements composition and water holding
316 capacity (Carlson and Adriano, 1993; Tsadilas, 2014). For instance, Dash et al. (2015)
317 reported a favorable increase in the growth of *Capsicum annuum* cultivated in 5% fly ash
318 amended soil. However, the potentially toxic elements (PTEs) in CFA including boron and
319 heavy metals (Awoyemi and Adeleke, 2017) may account for CFA-induced phytotoxicity
320 and impaired seedling growth at higher concentrations of CFA exposure. Higher
321 concentrations of CFA have resulted in impaired growth of rice (*Oryza sativa* L.) exposed to
322 50% CFA (Singh et al., 2016) and substantially lowered the germination counts of turfgrass
323 (Adriano and Weber, 2001). In a study conducted by Bilski et al. (2011), the concentrations
324 of CFA in growth media higher than 40% were not able to sustain seedling growth after
325 initial germination, for canola (*Brassica campestris*), rapeseed (*Brassica napus*), alfalfa
326 (*Medicago sativa*), and perennial ryegrass (*Lolium perenne*). From our study, a concentration
327 of up to 20% CFA having alkaline and liming property in the ASL soil (Awoyemi and
328 Dzantor, 2017a, 2017b) would be adequate to enhance of seed germination while only up to
329 10% would support seedling growth and survival.

330 CFA-induced phytotoxicity in switchgrass plants as shown in this study was
331 concentration dependent. CFA reduced plant growth, foliage length, fresh weight, and
332 moisture content. Similarly, CFA reduced the concentrations of intact genomic dsDNA,
333 caused leaf chlorosis, and increased lipid peroxidation in CFA-exposed switchgrass plants.
334 This is in contrast with the CFA-induced enhancement of crop productivity reported in
335 several studies (Pandey et al., 2009). Dash et al. (2015) reported that the application of fly
336 ash up to 5% favors the growth of *C. annuum* while at concentrations beyond 10%, growth
337 was impaired due to the accumulation of heavy metals present in fly ash. A significant
338 decrease in the productivity of rice crops exposed to 50% CFA coupled with a significant
339 increase in lipid peroxidation was reported by Singh et al. (2016). The phytotoxic effects

induced by CFA may be attributed to its constituent PTEs (Adriano and Weber, 2001; Radić et al., 2018). Additionally, CFA-induced reduction of genomic dsDNA in switchgrass plants may be due to DNA damage associated potentially with the oxidative attack by PTEs in the CFA (Dwivedi et al., 2012; Sambandam et al., 2015).

Soil inoculation with AMF can protect plants against metal induced toxicity (Firmin et al., 2015) and oxidative stress (Awoyemi and Dzantor, 2017a). The protective mechanisms exhibited by AMF include: binding metals to cell wall, organic matter or mycelium and sequestering them in their vacuole or other organelles (Hall, 2002; Huang et al., 2005); releasing heat shock protein (HSP) and GSH (Hildebrandt et al., 2007); allocation plasticity, proteome changes, and metabolic shift (Aloui et al., 2011); increased uptake of macronutrients e.g. phosphorus, nitrogen, and sulphur (de Andrade and da Silveira, 2008); phytostabilization of potentially toxic trace element-polluted soils by sequestration (Garg and Chandel, 2011); increasing root and shoot growth (Mohammadi et al., 2011); changing mycorrhizosphere pH (Bano and Ashfaq, 2013; Shivakumar et al., 2011); increasing the activities of antioxidant enzymes (Awoyemi and Dzantor, 2017a); decrease in lipid peroxidation and electrolyte leakage (Garg and Aggarwal, 2012).

Reduced glutathione (GSH) is a low molecular weight tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) which plays a key role as a non-enzymatic antioxidant in plant defense system against environmental stressors (Hossain et al., 2010). It functions in the antioxidant defense and glyoxalase (Gly) systems by directly and indirectly controlling ROS, methylglyoxal (MG) and their reaction products (Hossain et al., 2012). Studies have shown that in addition to detoxification, complexation, chelation, and compartmentalization of metals, GSH by itself and its metabolizing enzymes notably glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR), Gly I and Gly II, protect against ROS- and MG-induced damage (Hossain et al., 2012). GSH functions with ascorbic acid

(AsA) via the AsA-GSH cycle to control $H_2O_2$ (Foyer and Noctor, 2005) and it is synthesized into phytochelatin (PC) which complexes metals (Blum et al., 2007). GSH-glutathione disulfide (GSSG) redox couple buffers cellular homeostasis and control signaling systems including the activation of genes that encodes GSH and AsA related enzymes (Gill et al., 2013). The extent to which AMF and GSH can moderate CFA-induced phytotoxicity depends of several factors including the concentration of GSH or AMF, species of AMF, plant type, the prevailing rhizosphere or plant conditions (Emamverdian et al., 2015).

The results of this current study showed that the co-application of CFA-contaminated ASL soil with AMF or GSH played notable roles in mitigating the CFA-induced phytotoxicity in switchgrass plant. The AMF, *R. clarus* used in this study enhanced plant growth, increased foliage and root number, reduced lipid peroxidation, prevented leaf chlorosis, and enhanced the concentration of intact genomic dsDNA. Similarly, exogenous GSH application mitigated CFA-induced phytotoxicity. However, the mitigation potential of GSH compared to AMF was limited by the CFA concentration. This requires further studies to identify the optimum GSH concentration that will be most effective for mitigating CFA-induced phytotoxicity at varying exposure concentrations to CFA. There are several studies that have reported the potential of AMF (Firmin et al., 2015; Garg and Singh, 2018) and/or exogenous GSH (Chen et al., 2010; Wei et al., 2010) to mitigate phytotoxicity induced by exposures to PTEs in single exposure bioassays. However, in reality, the environment is exposed to a mixture of contaminants. Therefore, assessing the mitigation potential of AMF and GSH against the phytotoxicity induced by a contaminant mixture such as CFA at environmentally-relevant concentrations make this study very significant.

## 5. Conclusion

This study investigated biological (arbuscular mycorrhizal fungi, *R. clarus*) and chemical (exogenous glutathione) methods to mitigate and/or manage coal fly ash-induced phytotoxicity in coal fly ash contaminated soil. Results showed a concentration-dependent increase in phytotoxicity of coal fly ash against switchgrass plant impairing plant growth, inducing chlorosis and lipid peroxidation. However, co-application with *R. clarus* mitigated the coal fly ash-induced phytotoxicity, enhanced plant growth and prevented lipid peroxidation and chlorosis. Co-application of *R. clarus* (3-5%) with coal fly ash (up to 15%) in Armour silt loam soil is recommended for efficient mitigation of phytotoxicity. Whereas, the phytotoxicity mitigation potential of exogenously applied glutathione was limited by the concentration of the coal fly ash. Further studies are required to optimize these biological and chemical phytotoxicity mitigation strategies for use in the management and phytoremediation of coal fly ash polluted environments. Also, assessing the joint effects of *R. clarus* and glutathione in mitigating coal fly ash-induced phytotoxicity may be necessary to empirically determine if these two agents have synergistic effects.

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**References**

Adriano, D.C., Weber, J.T., 2001. Influence of Fly Ash on Soil Physical Properties and Turfgrass Establishment. J. Environ. Qual. 30, 596–601. https://doi.org/10.2134/jeq2001.302596x

414 Aloui, A., Recorbet, G., Robert, F., Schoefs, B., Bertrand, M., Henry, C., Gianinazzi-
415 Pearson, V., Dumas-Gaudot, E., Aschi-Smiti, S., 2011. Arbuscular mycorrhizal
416 symbiosis elicits shoot proteome changes that are modified during cadmium stress
417 alleviation in *Medicago truncatula*. BMC Plant Biol. 11, 75.
418 https://doi.org/10.1186/1471-2229-11-75
419 Awoyemi, O.M., Adeleke, E.O., 2017. Bioethanol Production from Switchgrass Grown on
420 Coal Fly Ash-amended Soil. World J. Agric. Res. 5, 147–155.
421 https://doi.org/10.12691/wjar-5-3-4
422 Awoyemi, O.M., Dzantor, E.K., 2017a. Toxicity of coal fly ash (CFA) and toxicological
423 response of switchgrass in mycorrhiza-mediated CFA-soil admixtures. Ecotoxicol.
424 Environ. Saf. 144, 438–444. https://doi.org/10.1016/j.ecoenv.2017.06.059
425 Awoyemi, O.M., Dzantor, E.K., 2017b. Fate and Impacts of Priority Pollutant Metals in Coal
426 Fly Ash-Soil-Switchgrass Plant Mesocosms. Coal Combust. Gasif. Prod. 9, 42–51.
427 https://doi.org/https10.4177/CCGP-D-14-00004.1
428 Bano, S.A., Ashfaq, D., 2013. Role of mycorrhiza to reduce heavy metal stress. Nat. Sci. 05,
429 16–20. https://doi.org/10.4236/ns.2013.512A003
430 Basu, M., Pande, M., Bhadoria, P.B.S., Mahapatra, S.C., 2009. Potential fly-ash utilization in
431 agriculture: A global review. Prog. Nat. Sci. 19, 1173–1186.
432 https://doi.org/10.1016/j.pnsc.2008.12.006
433 Bilski, J., McLean, K., McLean, E., Soumaila, F., Lander, M., 2011. Environmental health
434 aspects of coal ash phytoremediation by selected crops. Int. J. Environ. Sci. 1, 2028–
435 2036. https://doi.org/10.1186/s40945-017-0033-9.Using
436 Blum, R., Beck, A., Korte, A., Stengel, A., Letzel, T., Lendzian, K., Grill, E., 2007. Function
437 of phytochelatin synthase in catabolism of glutathione conjugates. Plant J. 49, 740–749.
438 https://doi.org/10.1111/j.1365-313X.2006.02993.x

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation, in: Methods in Enzymology. pp. 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Carlson, C.L., Adriano, D.C., 1993. Environmental Impacts of Coal Combustion Residues. J. Environ. Qual. 22, 227–247. https://doi.org/10.2134/jeq1993.00472425002200020002x

Chen, F., Wang, F., Wu, F., Mao, W., Zhang, G., Zhou, M., 2010. Modulation of exogenous glutathione in antioxidant defense system against Cd stress in the two barley genotypes differing in Cd tolerance. Plant Physiol. Biochem. 48, 663–672. https://doi.org/10.1016/j.plaphy.2010.05.001

Dash, A., Pradhan, A., Das, S., Mohanty, S., 2015. Fly ash as a potential source of soil amendment in agriculture and a component of integrated plant nutrient supply system. J. Ind. Pollut. Control 31, 249–257. https://doi.org/10.1016/j.fuel.2004.10.019

de Andrade, S.A.L., da Silveira, A.P.D., 2008. Mycorrhiza influence on maize development under Cd stress and P supply. Brazilian J. Plant Physiol. 20, 39–50. https://doi.org/10.1590/S1677-04202008000100005

Dwivedi, S., Saquib, Q., Al-Khedhairy, A.A., Ali, A.-Y.S., Musarrat, J., 2012. Characterization of coal fly ash nanoparticles and induced oxidative DNA damage in human peripheral blood mononuclear cells. Sci. Total Environ. 437, 331–338. https://doi.org/10.1016/j.scitotenv.2012.08.004

Edwards, K., Johnstone, C., Thompson, C., 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19, 1349–1349. https://doi.org/10.1093/nar/19.6.1349

Emamverdian, A., Ding, Y., Mokhberdoran, F., Xie, Y., 2015. Heavy Metal Stress and Some Mechanisms of Plant Defense Response. Sci. World J. 2015, 1–18. https://doi.org/10.1155/2015/756120

Firmin, S., Labidi, S., Fontaine, J., Laruelle, F., Tisserant, B., Nsanganwimana, F., Pourrut,

B., Dalpé, Y., Grandmougin, A., Douay, F., Shirali, P., Verdin, A., Lounès-Hadj Sahraoui, A., 2015. Arbuscular mycorrhizal fungal inoculation protects *Miscanthus giganteus* against trace element toxicity in a highly metal-contaminated site. Sci. Total Environ. 527–528, 91–99. https://doi.org/10.1016/j.scitotenv.2015.04.116

Foyer, C.H., Noctor, G., 2005. Redox Homeostasis and Antioxidant Signaling: A Metabolic Interface between Stress Perception and Physiological Responses. Plant Cell 17, 1866–1875. https://doi.org/10.1105/tpc.105.033589

Gajić, G., Mitrović, M., Pavlović, P., 2018. "Ecorestoration of fly ash deposits by native plant species at thermal power stations (Serbia)," in: Pandey, V.C., Bauddh, K. (Eds.), Phytomanagement of Polluted Sites: Market Opportunities in Sustainable Phytoremediation. Elsevier, pp. 113–168.

Garg, N., Aggarwal, N., 2012. Effect of mycorrhizal inoculations on heavy metal uptake and stress alleviation of *Cajanus cajan* (L.) Millsp. genotypes grown in cadmium and lead contaminated soils. Plant Growth Regul. 66, 9–26. https://doi.org/10.1007/s10725-011-9624-8

Garg, N., Chandel, S., 2011. Arbuscular Mycorrhizal Networks: Process and Functions, in: Sustainable Agriculture Volume 2. Springer Netherlands, Dordrecht, pp. 907–930. https://doi.org/10.1007/978-94-007-0394-0_40

Garg, N., Singh, S., 2018. Arbuscular Mycorrhiza Rhizophagus irregularis and Silicon Modulate Growth, Proline Biosynthesis and Yield in *Cajanus cajan* L. Millsp. (pigeonpea) Genotypes Under Cadmium and Zinc Stress. J. Plant Growth Regul. 37, 46–63. https://doi.org/10.1007/s00344-017-9708-4

Gill, S.S., Anjum, N.A., Hasanuzzaman, M., Gill, R., Trivedi, D.K., Ahmad, I., Pereira, E., Tuteja, N., 2013. Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. Plant Physiol. Biochem. 70, 204–212.

https://doi.org/10.1016/j.plaphy.2013.05.032

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11. https://doi.org/10.1093/jxb/53.366.1

He, H., Dong, Z., Peng, Q., Wang, X., Fan, C., Zhang, X., 2017. Impacts of coal fly ash on plant growth and accumulation of essential nutrients and trace elements by alfalfa (*Medicago sativa*) grown in a loessial soil. J. Environ. Manage. 197, 428–439. https://doi.org/10.1016/j.jenvman.2017.04.028

Hildebrandt, U., Regvar, M., Bothe, H., 2007. Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68, 139–146. https://doi.org/10.1016/j.phytochem.2006.09.023

Hossain, M.A., Hasanuzzaman, M., Fujita, M., 2010. Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. Physiol. Mol. Biol. Plants 16, 259–272. https://doi.org/10.1007/s12298-010-0028-4

Hossain, M.A., Piyatida, P., da Silva, J. a. T., Fujita, M., 2012. Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. J. Bot. 2012, 1–37. https://doi.org/10.1155/2012/872875

Huang, Y., Tao, S., Chen, Y., 2005. The role of arbuscular mycorrhiza on change of heavy metal speciation in rhizosphere of maize in wastewater irrigated agriculture soil. J. Environ. Sci. (China) 17, 276–80.

Kumar, A., Vajpayee, P., Ali, M.B., Tripathi, R.D., Singh, N., Rai, U.N., Singh, S.N., 2002. Biochemical Responses of Cassia siamea Lamk. Grown on Coal Combustion Residue (Fly ash). Bull. Environ. Contam. Toxicol. 68, 675–683. https://doi.org/10.1007/s001280307

514 Medunić, G., Ahel, M., Mihalić, I.B., Srček, V.G., Kopjar, N., Fiket, Ž., Bituh, T., Mikac, I.,
515 2016. Toxic airborne S, PAH, and trace element legacy of the superhigh-organic-sulphur
516 Raša coal combustion: Cytotoxicity and genotoxicity assessment of soil and ash. Sci.
517 Total Environ. 566–567, 306–319. https://doi.org/10.1016/j.scitotenv.2016.05.096
518 Mohammadi, K., Khalesro, S., Sohrabi, Y., Heidari, G., 2011. A review: beneficial effects of
519 the mycorrhizal fungi for plant growth. J. Appl. Environ. Biol. Sci. 1, 310–319.
520 Pandey, V.C., Abhilash, P.C., Singh, N., 2009. The Indian perspective of utilizing fly ash in
521 phytoremediation, phytomanagement and biomass production. J. Environ. Manage. 90,
522 2943–2958. https://doi.org/10.1016/j.jenvman.2009.05.001
523 Radić, S., Medunić, G., Kuharić, Ž., Roje, V., Maldini, K., Vujčić, V., Krivohlavek, A.,
524 2018. The effect of hazardous pollutants from coal combustion activity: Phytotoxicity
525 assessment of aqueous soil extracts. Chemosphere 199, 191–200.
526 https://doi.org/10.1016/j.chemosphere.2018.02.008
527 Raja, R., Nayak, A.K., Rao, K.S., Puree, C., Shahid, M., Panda, B.B., Kumar, A., Tripathi,
528 R., Bhattacharyya, P., Baig, M.J., Lal, B., Mohanty, S., Gautam, P., 2014. Effect of Fly
529 Ash Deposition on Photosynthesis, Growth and Yield of Rice. Bull. Environ. Contam.
530 Toxicol. 93, 106–112. https://doi.org/10.1007/s00128-014-1282-x
531 Roy, G., Joy, V.C., 2011. Dose-related effect of fly ash on edaphic properties in laterite
532 cropland soil. Ecotoxicol. Environ. Saf. 74, 769–775.
533 https://doi.org/10.1016/j.ecoenv.2010.10.041
534 Sambandam, B., Devasena, T., Islam, V.I.H., Prakhya, B.M., 2015. Characterization of coal
535 fly ash nanoparticles and their induced in vitro cellular toxicity and oxidative DNA
536 damage in different cell lines. Indian J. Exp. Biol. 53, 585–93.
537 Schönegger, D., Gómez-Brandón, M., Mazzier, T., Insam, H., Hermanns, R., Leijenhorst, E.,
538 Bardelli, T., Fernández-Delgado Juárez, M., 2018. Phosphorus fertilising potential of fly

ash and effects on soil microbiota and crop. Resour. Conserv. Recycl. 134, 262–270. https://doi.org/10.1016/j.resconrec.2018.03.018

Shivakumar, C.K., Hemavani, C., Thippeswamy, B., Krishnappa, M., 2011. Effect of inoculation with arbuscularmycorrhizal fungi on green gram grown in soil containing heavy metal zinc. J. Exp. Sci. 2, 17–21.

Singh, P.K., Tripathi, P., Dwivedi, S., Awasthi, S., Shri, M., Chakrabarty, D., Tripathi, R.D., 2016. Fly-ash augmented soil enhances heavy metal accumulation and phytotoxicity in rice (*Oryza sativa* L.); A concern for fly-ash amendments in agriculture sector. Plant Growth Regul. 78, 21–30. https://doi.org/10.1007/s10725-015-0070-x

Swamy, T., Dash, N., Nahak, G., Deo, B., Sahu, R., 2010. Effect of Coal Fly Ash on Growth, Biochemistry, Cytology and Heavy Metal Content of *Allium cepa* L. New York Sci. Journal, 3(5), 10-16. 3, 10–16.

Tsadilas, C.D., 2014. Agricultural use of fly ash: Expected benefits and consequences. WACAU-2014, Israel International Workshop on Agricultural Coal Ash Uses, 27 – 29 May 2014. http://coal-ash.co.il/sadna14/Tsadilas_FlyAshUse.pdf. Accessed 25th November, 2017.

[USEPA] United States Environmental Protection Agency, 2017. EPA's Response to the Duke Energy Coal Ash Spill in Eden, NC. https://www.epa.gov/dukeenergy-coalash. Accessed December 27 2018.

[USEPA] United States Environmental Protection Agency, 2016. EPA Response to Kingston TVA Coal Ash Spill. https://www.epa.gov/tn/epa-response-kingston-tva-coal-ash- spill. Accessed December 27 2018.

Voigt, P.W., Morris, D.R., Godwin, H.W., 1997. A Soil-on-Agar Method to Evaluate Acid-Soil Resistance in White Clover. Crop Sci. 37, 1493. https://doi.org/10.2135/cropsci1997.0011183X003700050013x

564 Wei, S., Ma, L.Q., Saha, U., Mathews, S., Sundaram, S., Rathinasabapathi, B., Zhou, Q.,
565 2010. Sulfate and glutathione enhanced arsenic accumulation by arsenic
566 hyperaccumulator *Pteris vittata* L. Environ. Pollut. 158, 1530–1535.
567 https://doi.org/10.1016/j.envpol.2009.12.024
568 Yunusa, I.A.M., Loganathan, P., Nissanka, S.P., Manoharan, V., Burchett, M.D., Skilbeck,
569 C.G., Eamus, D., 2012. Application of coal fly ash in agriculture: A strategic
570 perspective. Crit. Rev. Environ. Sci. Technol.
571 https://doi.org/10.1080/10643389.2010.520236

## Graphical Abstract