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Research article

C_3 and C_4 plants as potential phytoremediation and bioenergy crops for stabilization of crude oil and heavy metal co-contaminated soils-response of antioxidative enzymes

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Abstract: Metal accumulation in 15 (C_3 and C_4) plants growing on crude oil spill laden soil and the responses of antioxidative enzymes were examined. In this study, the synergistic effect of four different metals was examined to find out the antioxidative stress responses. Plants were collected from their natural habitat (crude oil spill laden soil) during the rainy season at the vegetative stage (before flowering) and analyzed for shoot metal concentrations and activities of catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA). The shoot metal concentrations (mg kg^{-1}) of all the individual metals (Mn, Co, Cd, and Zn) were found in different concentration. All the metal accumulating plants, CAT and SOD activities were found to be high in comparison to the control plants. The highest SOD activity was found in *Cynodon dactylon* (47 μg^{-1} FW) whereas the lowest was found in *Fimbristylis dichotoma* (13 μg^{-1} FW). The SOD activity increased considerably in all the metal accumulating plants, and the increase ranges 13–47 μg^{-1} FW. Catalase activity was also found to be high (2–18 μg^{-1} FW) in all the grass and sedges, of which the highest was recorded in *Echinochloa colonna* (18 μg^{-1} FW) and lowest in *Arundo donax* (2 μg^{-1} FW). The significant decrease in MDA activity (between 1–0.04 nmol g^{-1} FW) in the leaves of all metal accumulating plants, suggested metals in soil induced oxidative damage. The antioxidant responses among the species grown in a contaminated site displayed higher levels of activity in all the enzymes compared to no-polluted plants. Therefore, it can be assumed that the heavy metal uptake and bio-productivity (the coordinated manifestation of the efficiency that operates at various molecular and cellular level of these species) is sustained through antioxidative defense system in the examined plants.

Keywords: C_3 and C_4 plants - Metals - Crude oil - Antioxidative stress - Bioenergy.

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INTRODUCTION

The plants have a certain potential cellular mechanism through which they defend too many abiotic stresses. These include exposures to heavy metals (inorganics) and hydrocarbons (organics). The heavy metal stress induces free radicals which damage different plant macromolecules inside the plant body (Fu & Huang 2001). Plant accumulation of metals from contaminated media (soil, air, water) induce the overproduction of reactive oxygen species (ROS); these ROS (superoxide anion (O^{-}_2), hydroxyl radical ($^{\bullet}OH$), as well as nonradical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$),) which is harmful for plant metabolism, growth and development. The overproduction of ROS is accountable for oxidative damage on plant macromolecules that includes lipids, proteins and nucleic acids as substantiated by research evidence (Yadav 2010, Pospíšil *et al.* 2017). Furthermore, the presence of organic compounds in soil is also detrimental for plants growth causing a damaging effect on their metabolism (Sarma *et al.* 2017). Thus, the investigated plants (grass and sedges) exhibit the combined stress tolerance of organics and inorganics since they are adopted in crude oil and heavy metal co-contaminated soil.

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These grass and sedges can accumulate metals and hydrocarbons from polluted fields through a cluster of the process that helps the restoration of such degraded soil (Fig. 1). One of the important criteria for these plants is that they generate the high amount of biomass that could have a potential of application *viz.*, a) direct combustion for bioenergy b) enzymatic digestion of the biomass for making a second-generation biofuel (Sarma *et al.* 2017). Therefore, the growth and adaptation of such plants in polluted fields not only help the phytoextraction of metals and degradation of hydrocarbon but their harvested biomass could be a profitable business for making the lignocellulosic product (Sarma & Lee 2018).

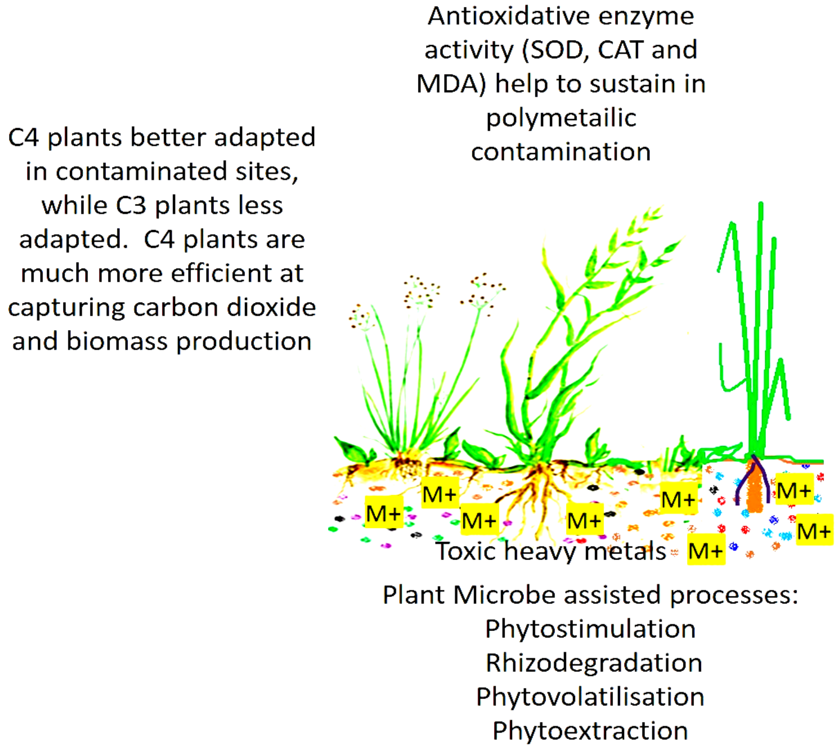

Figure 1. Grasses and sedges accumulate metals and degrades hydrocarbon from soil through a variety of biogeochemical process.

Table 1. Phytoremediation and energy crops for possible application in filed.

Plant species	Family	Pollutant remediation aspects
Arundo donax L.	Poaceae	Phytoremediation of soil contaminated with Cd, As and Ni (Papazoglou *et al.* 2005, Mirza *et al.* 2005)
Axonopus compressus (Sw.) P. Beauv.	Poaceae	Phytoaccumulator of Cd (Sao *et al.* 2007)
Cannabis sativa L.	Cannabaceae	Phytoremediation of soil contaminated with Cd, Cu, Pb and Zn, Cd, Cr and Ni and other heavy metals (Kos *et al.* 2002, Arru *et al.* 2014)
Cynara cardunculus L.	Asteraceae	Phytoremediation of soil contaminated with Cd and as (Ge *et al.* 2012)
Cynodon dactylon (L.) Pers.	Poaceae	Phytoextractors of Cr (Sampanpanish *et al.* 2010)
Cyperus esculentus L.	Cyperaceae	Phytoaccumulator of Fe (Chandra & Yadav 2011)
Cyperus rotundus L.	Cyperaceae	Phytoaccumulator of Cd (Kos *et al.* 2002)
Dactyloctenium aegyptium (L.) Wild.	Poaceae	Phytoaccumulator of heavy metals (Irshad *et al.* 2015)
Echinochloa colonum (L.) Link	Poaceae	Phytoextraction of Cr, Cd, P (Subhashini & Swamy 2015)
Eucalyptus sp.	Myrtaceae	Phytoremediation of soil contaminated with As and other heavy metals and bioaccumulation of Pb, Zn and Cr (Zhuang *et al.* 2007, Li *et al.* 2011)
Fimbristylis dichotoma (L.) Vahl	Cyperaceae	Phytoextraction of Cu, Ni (Das & Maiti 2007)
Hibiscus cannabinus L.	Malvaceae	Phytoremediation of soil contaminated with As, Fe, Pb and Cd (Ho *et al.* 2008, Meera & Agamuth 2012)
Jatropha curcas L.	Euphorbiaceae	Phytoremediation of Al, Fe, Cr, Mn, Ar, Zn, Cd and Pb (Pandey *et al.* 2012)

Leersia hexandra Swartz.	Poaceae	Phytoextraction of Cr (Liu *et al.* 2011)
Linum usitatissimum L.	Linaceae	Phytoremediation of soil contaminated with Cd, Ni and other heavy metals (Vrbova *et al.* 2013)
Miscanthus sp.	Poaceae	Phytoextraction of As, Sn, Cd, Cr, Cu, Ni, Pb, Zn and Al (Pidlisnyuk *et al.* 2014)
Paspalum conjugatum Berg.	Poaceae	Phytoaccumulator of Pb (Paz-Alberto 2007).
Phalaris arundinacea L.	Poaceae	Phytoremediation of soil contaminated with different heavy metals and trace metal (Kacprzak *et al.* 2014, Polechonska *et al.* 2014)
Poa annua L.	Poaceae	Phytoaccumulator of Zn, Pb, Cd (Barbafieri *et al.* 2011)
Populus sp.	Salicaceae	Phytoremediation of Cd, Cr, Cu, Se, Pb and Zn (Pilon-Smits *et al.* 1998, Robinson *et al.* 2000, Sebastiani *et al.* 2004, Chen *et al.* 2015)
Ricinus communis L.	Euphorbiaceae	Phytoremediation of Cd, Pb, Zn, As (Meloa *et al.* 2009, Costa *et al.* 2012, Olivares *et al.* 2013, Kiran *et al.* 2017)
Salix sp.	Salicaceae	Phytoremediation of Cd and Zn and Bioaccumulation of Cd, Cr, Cu, Ni, Pb and Zn (Meers *et al.* 2007, Miller *et al.* 2011)

The C_3 and C_4 (biomass crops) having phytoremediation potentials were listed in the table (Table 1) showing amazing phytoextraction of wide-ranging metals in their biomass. The C_4 plants are more tolerant and better adapted in contaminated sites, unlike C_3 plants; the C_4 plants possess a bundle sheath cells (a special type of cell around the vascular bundle), while C_3 plants do not possess these cells. In addition to such anatomical difference, the cells of C_4 plants, produces a special type of enzyme phosphoenolpyruvate carboxykinase (PEPCK) that plays a key role in the C_4 cycle. Therefore, the C_4 plants are much more efficient at capturing carbon dioxide for photosynthesis which helps more biomass production. In addition to that C_4 plants are better for application in phytoremediation and biomass production reported in the current literature. However, it is also emphasized that some C_3 plants have been explored as an ideal candidate for metal remediation and biomass production (Zhuo *et al.* 2017).

Plant cells are able to respond to elevated levels of reactive oxygen species (ROS) by activating their antioxidative defense systems (Noctor *et al.* 2017). To protect the lipids, proteins and nucleic acids (cells macromolecules) and counteract oxidative damage, plant activated endogenous antioxidants defense system. Therefore, the expression of antioxidant enzymes (in C_3 and C_4 plants) suggested as more reliable and sensitive indicators of exposure to the abiotic stress including metals (Yadav 2010). The antioxidative system includes metabolites GSH, ascorbate and the enzymes ascorbate peroxidase (APX), glutathione reductase (GR), monode hydro ascorbate reductase (MDHAR), superoxide dismutases (SOD), catalases (CAT) and peroxidases (PODs) are involved in detoxification of O_2^-, and H_2O_2 respectively; thereby, preventing the formation of OH^- radicals (Noctor *et al.* 1998).

The oxidative stress induced by chromium was tolerated by many plants through the hyperactivity of their antioxidant defense system (Yadav 2010). Related experiments confirmed that chromium was inhibited by superoxide generation and mitochondrial electron transport and can seriously interrupt normal metabolism through oxidative damage to cellular components in higher plants (Liu *et al.* 2011), but data remain scanty in case of herbs and shrubs. The Lead is recalcitrant in the environment and can cause damage to biota (Zeng *et al.* 2017). Some plants accumulate Pb, although it is not necessary for growth and development. It was established that Pb accumulation reduced the total chlorophyll and carotenoids content and cause oxidative damage in plants. However, unlike Pb and Cr, the synergistic effect of Mn, Co, Cd, and Zn in C_3 and C_4 plants that cause oxidative damage by increasing lipid peroxidation still remain scanty. Therefore, in this study, the synergistic effect of four different metals was examined to find out the antioxidative stress responses in 15 biomass crop (C_3 and C_4) that can be used for stabilization of crude oil spill laden soils.

MATERIALS AND METHODS

In this study, 15 species that were grown in the metals and hydrocarbon contaminated habitat were collected from crude oil drilling sites of Borhola, Assam (Table 2). The sample collection site is a hydrocarbon-rich agro-ecosystem, located in the north-eastern region of India with an area of 5 km^2, which is surrounded by tea garden (Sharma *et al.* 2018). The species were identified using flora of India. The total of 45 individuals (n=3) were taken to the laboratory, washed with distilled water and stored in the refrigerator before analysis. Leaves (0.2 g) were taken from the -20°C refrigerator, ground in a mortar and pestle. The crude extracts of each part were homogenized in 3 mL 50 mM potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 8000 rpm for 20 min at 4°C. The supernatant (*i.e.*, the enzyme extract) was used for determinations of enzyme activities *viz*. superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA).

Table 2. The grass and sedges collected from crude oil and heavy metal co-contaminated soils for analysis.

Species	Family
Arundo donax L.	Poaceae
Axonopus compressus (Sw.) P. Beauv.	Poaceae
Cynodon dactylon (L.) Pers.	Poaceae
Cyperus esculentus L.	Cyperaceae
Cyperus rotundus L.	Cyperaceae
Dactyloctenium aegyptium (L.) Willd.	Poaceae
Digitaria sanguinalis (L.) Scop.	Poaceae
Echinochloa colona (L.) Link	Poaceae
Fimbristylis dichotoma (L.) Vahl	Cyperaceae
Imperata cylindrica (L.) Raeusch.	Poaceae
Kyllinga brevifolia Rottb.	Cyperaceae
Leersia hexandra Sw.	Poaceae
Paspalum conjugatum P.J.Bergius	Poaceae
Poa annua L.	Poaceae
Saccharum spontaneum L.	Poaceae

A SOD assay was performed according to the method of Giannopolitis & Ries (1977) with slight modifications. Briefly, 3 mL reaction mixtures contain 0.3 mL of each: 750 µmol L^{-1} nitro blue tetrazolium, 20 µmol L^{-1} riboflavin, 130 mmol L^{-1} methionine, and 100 µmol L^{-1} EDTA Na, 1.5 mL of 50 mmol L^{-1} phosphate buffer (pH 7.8), 0.25 mL of deionized water, and 0.05 mL of enzyme extract. The test tubes were placed under light for 20 min, and the absorbance of the reaction mixture was measured at 560 nm. A reaction solution placed in the dark was used as the control. The enzyme activity (one unit) was well defined such as the amount of the enzyme that resulted in inhibition (50%) of the rate of nitro blue tetrazolium (NBT) reduction.

A CAT assay was modified from the method of Brennan & Frenkel (1977). An enzyme extract (0.1 mL) was added to mixture solution of 1 mL 0.3% H_2O_2 and 1.9 mL 50 mmol L^{-1} phosphate buffer (pH 7.0) to initiate the reaction. The activity of CAT was measured by determining the rate of change of H_2O_2 absorbance at 240 nm. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 1% absorbance reduction. Lipid peroxides were measured by the thiobarbituric acid test for MDA according to Heath & Packer (1968). Supernatant (1.5 mL) was added to 2.5 mL of 20% trifluoroacetic acid containing 0.5% thiobarbituric acid. The mixture was kept at 100°C in a water bath for 20 min, then cooled quickly in an ice bath followed by centrifugation at 10,000 rpm for 10 min. The absorbance of the resulting solutions was determined using UV-Vis at 450, 532 and 600 nm.

In this study SOD, CAT and MDA were also analyzed in the same species organically grown in the experimental garden of the institute. There are numerous studies on the impact of individual environmental factors on plants but few have tried to explore their combined effect on antioxidant defense system in plants. The present study seeks to address this particular aspect that has hitherto remained unexplored, by looking at a combination of hydrocarbons and heavy metals impact on plant antioxidant defense system.

The oven dried samples (0.03 g) were then put inside a heating flask and heated in a mixture of 4mL HNO_3 (70%, Merck India, Mumbai) and 1 mL $HClO_4$ (30%) and digested at 140°C for 2 hours. The digested plant samples were treated with 30 mL water and 2.5 g H_3BO_3 and filtered through Whatman no.1 filter paper and analyzed for metal concentration using atomic absorption spectrophotometer.

To assess analytical precision and quality control, the Trace CERT certified standards (Sigma Aldrich) reference material, reagent blanks and duplicates of every third sample were applied. The limit of detection (LOD) and limit of quantification (LOQ) of HMs were carried out by recovery experiments. The results showed a recovery of metals in the range of 90.6–98.5 %. The data obtained were then analyzed using statistical software.

RESULTS AND DISCUSSION

In this study, four metals *viz*. Mn, Co, Cd, and Zn were analyzed in plants to correlate it with their antioxidative responses. The results illustrated that individual metal and species exhibited dissimilar trends of accumulation (Table 3). The test species continue to survive in extreme polymetallic environment. The considerable accumulation of Co was recorded in *C. esculentus* (16.46), *D. sanguinalis* (15.34), *E. colona* (13.36), and I. *cylindrical* (12.36). The limited uptake of Cd was seen only two species within the range of 2–8 mg kg^{-1} which are *C. esculentus* (8.67) and *K. brevifolia* (2.60). Again, in this study, the exceptional ability to uptake moderately high concurrent ternary metals (Mn, Co and Cd) in *C. esculentus* were recorded and for (Mn, Co, and Zn) uptake potential was shown by in *E. colona*. The Mn (mg kg^{-1}) accumulations were recorded within the range of (0.09–26.33) in all the species. The species which exhibited maximum accumulation of Mn were presented in chronological orders from highest to lowest: *L. hexandra* (26.33), *E. colona* (21.25), *C. dactylon* (14.5), *C. esculentus* (11.40), and *S. Spontaneum* (7.52). Similarly, highest accumulation of zinc found in *D. sanguinalis* (893.45) while lest uptake was recorded in *E. colona* (26.15). Metal contents in aerial parts of different plant samples showed significant differences ($P<0.01$).

Table 3. Accumulation of heavy metals (mg kg^{-1}) in 15 bioenergy crops collected from crude oil exploration site, Assam.

Plants	Family	Common name	Mn	Co	Cd	Zn
Arundo donax L. [C_3]	Poaceae	Giant reed	0.46	0.43	2.6	162.15
Axonopus compressus (Sw.) P. Beauv. [C_4]	Poaceae	American carpet grass	0.51	0.57	0.6	340.25
Cynodon dactylon (L) Pers. [C_4]	Poaceae	Bermuda grass	14.56	0.63	0.6	306.95
Cyperus esculentus Linn [C_4]	Cyperaceae	Nut sedge	16.41	16.5	8.7	784.15
Cyperus rotundus L. [C_4]	Cyperaceae	Nutgrass	0.21	0.53	0.4	127.75
Dactyloctenium aegyptium (L.) Wild. [C_4]	Poaceae	Egyptian crowfoot grass	0.46	0.52	0.3	148.65
Digitaria sanguinalis (L.) Scop [C_4]	Poaceae	Hairy crabgrass	0.1	15.3	0.6	893.45
Echinochloa colona (L.) Link [C_4]	Poaceae	Jungle rice	21.25	13.4	0.3	26.15
Fimbristylis dichotoma (L.) Vahl [C_4]	Cyperaceae	Tall fringe rush	0.07	0.64	1.6	167.75
Imperata cylindrica (L.) Raeusch. [C_4]	Poaceae	Japanese bloodgrass	0.4	12.4	0.4	235.75
Kyllinga brevifolia Rottb. [C_4]	Cyperaceae	Green kyllinga	0.46	35	2.6	82.95
Leersia hexandra Swartz. [C_3]	Poaceae	Clubhead cutgrass	26.33	0.63	0.6	152.15
Paspalum conjugatum P.J. Bergius. [C_4]	Poaceae	Buffalo grass	0.41	3.37	1.6	115.55
Poa annua L. [C_3]	Poaceae	Annual bluegrass	0.34	12.4	0.6	212.15
Saccharum spontaneum L. [C_4]	Poaceae	Wild sugarcane	7.52	0.63	1.3	93.55

Note: Value are mean of ± standard deviation of (n=3).

The antioxidant stress responses among the species grown in a contaminated site displayed higher levels of activity in all the enzymes compared to plants grown in an experimental garden (normal) (Figs. 2–4). This is because of abiotic stress produced by hydrocarbon and metals. Plants under stress from individual environmental factors may produce antioxidative enzymes SOD and CAT that directly or indirectly detoxify reactive oxygen species (ROS). Plants respond to changes in the environment by changing their antioxidative systems. Environmental factors such as salinity, drought and elevated levels of metals/metalloids may regulate antioxidant defense systems in plants; this is the reason that we recorded higher leaf SOD and CAT activities among the examined species (Panda *et al.* 2017). The SOD was highest in the leaves of *C. dactylon* (47µg^{-1} FW) and lowest in *F. dichotoma* (13 µg^{-1} FW). All the energy crops populating the study site showed increments of SOD within the range of 13–47 µg^{-1} FW. Elevated SOD activities are beneficial for the protection of plants against oxidative stresses (Schwanz *et al.* 1996). Similarly, CAT activities were (µg^{-1} FW) within the range of 2–18 of which is even higher in comparison to control species. This is routinely used as an index of lipid peroxidation caused by heavy metals (HMs) toxicity. Previous studies have shown the respective consequences of salinity and HMs stress on the MDA level (Neto *et al.* 2006, Zhang *et al.* 2007) but, as stated earlier, little research has been conducted on the combined effects of hydrocarbons (HCs) and multiple metals. Therefore, in this study, there was a significant decrease in MDA activities (between 1–0.04 nmol g^{-1} FW) in the leaves of all the energy crops in the contaminated site; suggesting significant alleviation of heavy metals induced oxidative damage in them.

The application of high biomass yielding plants for the remediation of metals and hydrocarbon contaminated soil is a groundbreaking low-cost strategy to derive added profits (*i.e.* biomass for bioenergy) from such remediation approaches. There are a wide variety of grasses and sedges that have been explored for concomitant phyto-management of polymetallic sites, besides obtaining beneficial phytoproduct such as second-generation energy, biodiesel, bioethanol, wood, fiber, biochar, charcoal, and bioplastics during the remediation process. Recently, the production of bioenergy from biomass is getting international attention as it assumed as a clean and a multipurpose alternate source of energy. Nevertheless, there are several challenges to be achieved before the large volume commercial production of bioenergy from a contaminated site (Tripathi *et al.* 2016).

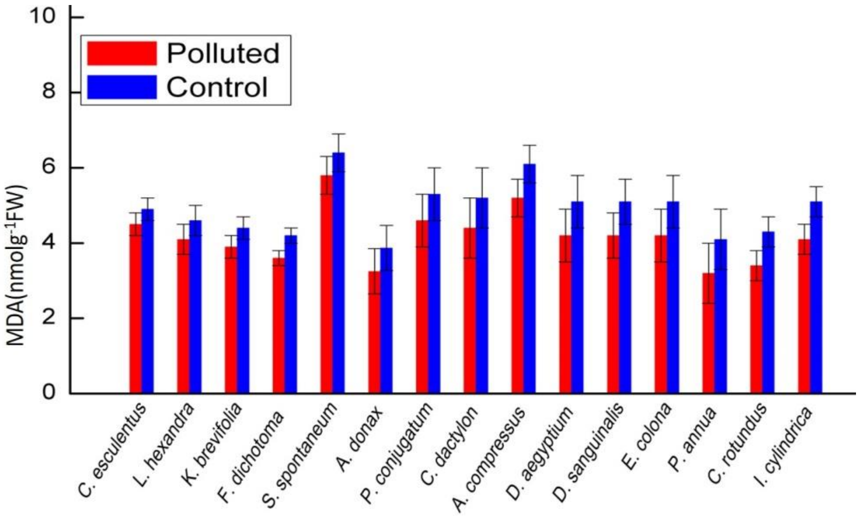

Figure 2. Malondialdehyde (MDA) activity of selected grasses and sedges in polluted and control sites.

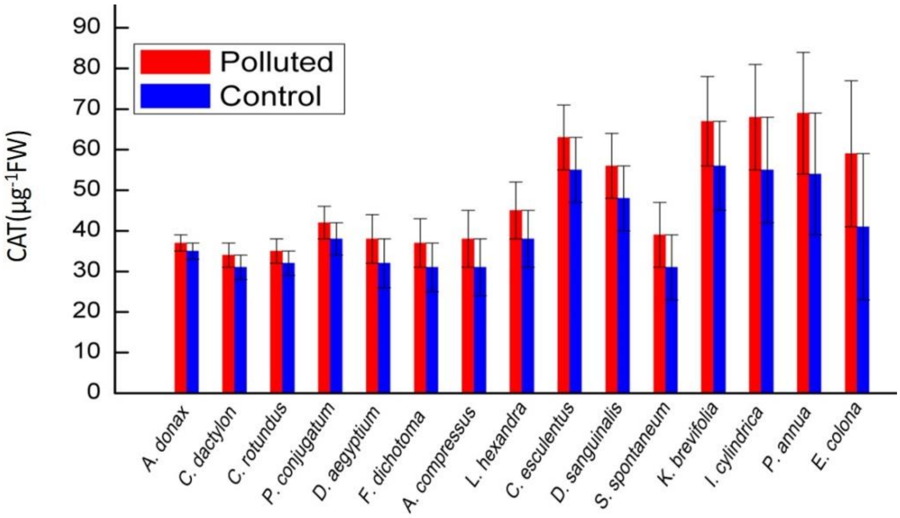

Figure 3. Catalase (CAT) activity of selected grasses and sedges in polluted and control sites.

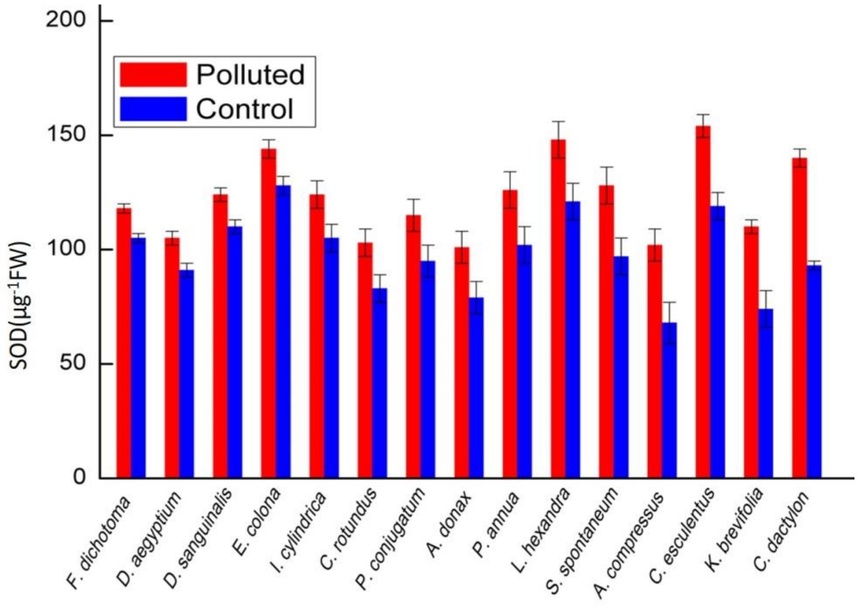

Figure 4. Super oxide dismutase (SOD) activity of selected grasses and sedges in polluted and control sites.

CONCLUSIONS

The antioxidative stress responses of 15 C_3 and C_4 plants grown in the heavy environmental metal-rich crude oil spill laden soils has been examined. All the plants examined were found to accumulate metals (Mn, Co, Cd, and Zn) in their biomass. The antioxidant responses among the species grown in a contaminated site displayed higher levels of activity in all the enzymes compared to nopolluted plants. Therefore, it can be assumed that the heavy metal uptake and bioproductivity is sustained through antioxidative defense system in the examined plants. The C_4 plants were better adapted in contaminated sites, whereas C_3 plants were less adapted as we recorded only 3 species of C_3 plants in the examined site. This is because C_4 plants are much more efficient at capturing carbon dioxide and biomass production and more resilient by chance, not the choice. However, the study established that the synergistic effect of four different metals (Mn, Co, Cd, and Zn) and how they created a negative effect of antioxidative stress responses for some biomass crops, although they are tolerant to heavy metals.

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