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Crude oil induces plant growth and antioxidant production in *Leersia hexandra* Sw.

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Abstract: The potential of *Leersia hexandra* grass in phytoremediation and natural attenuation of three groups of bacteria in soil contaminated with crude oil was evaluated for 180 days. The quantities of new shoots, root and aerial biomass were evaluated; changes in antioxidant concentrations in leaf and root caused by abiotic stress; population densities of *Azotobacter*, *Azospirillum* and *Pseudomonas*; and microbial respiration. The experimental data showed oil-induced increases of 315% and 196% in new shoots and root phytomass, respectively, and a 44% decrease in leaf + stem phytomass. The enzymatic defence in the grass leaf was manifested by higher concentrations of hydrogen peroxide, phenylalanine ammonium lyase and total flavonoids; the increases fluctuated from 35% to 52%. The response in the root was positive in catalase (16%), and in ammonium phenylalanine lyase, it increased 275% due to the effect of crude oil. The group of indigenous *Azotobacter* bacteria were tolerant to crude oil exposure, both in the phytoremediation process and in natural attenuation; the population densities varied from 212 to 438 × 10^3 colony-forming units (CFUs); they are greater than 49% to 106% compared to densities in control soil. *Azospirillum* spp. and *Pseudomonas* spp. recorded population abiotic stress. The grass activates enzymatic and plant defence, complementing microbial respiration in response to adaptation to crude oil.

Keywords: adaptation; hydrocarbon stress; phytotoxic effect; resistance; tolerance; root biomass; total phenols

Crude oil (CO) contains aliphatic, aromatic and polar hydrocarbons or resins, as well as asphaltenes. Hydrocarbons are hydrophobic, recalcitrant, persistent, potentially carcinogenic, mutagenic and toxic to living beings (Logeshwaran et al. 2018). They also induce physical, chemical (Devatha et al. 2019) and biological changes in the soil. The evolutionary process of various wetland plant species has involved a process of adaptation to stress factors. The list of such plants is long, *Leersia hexandra* Sw. (Orocio-Carrillo et al. 2019), *Phragmites australis* (Cav.) Trin. ex Steud. and *Typha domingensis* Pers. (Afzal et al. 2019). These plants have developed physiological and biochemical mechanisms that provide them with tolerance and resistance to various types of stress by inducing regulatory responses, homeostatic cellular

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restoration, or reducing phytotoxic effects (Mickelbart et al. 2015). Most studies of the effect of plant exposure to CO examine the relationship between dose and vegetative growth and biomass production. Only a few studies focus on the relationship between reactive oxygen species (ROS), antioxidants, growth, and production. H_2O_2 is a ROS species with a dual function inside plants. At low concentrations, it acts as a signal for acclimation and triggers tolerance to biotic and abiotic stress (Laloi et al. 2004). At high concentrations, H_2O_2 induces programmed cell death, also called apoptosis (Dat et al. 2000). Cell death can occur as a result of the role played by H_2O_2 in physicochemical reactions that convert high-molecular-weight acids into lipid peroxide and degrade cell wall proteins and nucleic acids (Sachdev et al. 2021). Plant resistance to abiotic stress manifests in its foliage and root morphology, as well as in cell metabolism, including the synthesis of H_2O_2 and antioxidant molecules (Siqueira-Soores et al. 2013). The main factors related to CO responsible for the self-regulation of phytotoxic processes, resistance and plant adaptation require further study.

Plant biomarkers derived from oxidative stress include the non-enzymatic antioxidants ascorbic acid/ascorbate, total phenols (TP) and total flavonoid (TFV), as enzymatic antioxidants such as catalase (CAT), mainly accumulated in peroxisome and mitochondria (Yap et al. 2021). Another antioxidant is phenylalanine ammonia lyase (PAL), synthesised from chorismate by the action of dehydratase and aminotransferase enzymes (Chen et al. 2006). Antioxidants counteract oxidative stress by protonating H_2O_2 in the electron transport chain during photorespiration (Halliwell 2006). Phytoremediation technology for the recovery of soil contaminated with CO is based on the use of plants that have adapted to contaminated soils and that produce biochemical biomarkers (ROS and antioxidants) in response to stress (Agathokleous and Calabrese 2020, Fan et al. 2021). They also form fibrous, extensive roots and support free-living and symbiotic microorganisms associated with the rhizosphere that oxidise and reduce petroleum hydrocarbons (Rivera-Cruz et al. 2016, Zuzolo et al. 2021).

Recent studies in oil regions in western India show significant advances in the knowledge of the biochemical and physiological responses of two species of the sedge Cyperus and the medicinal herb *Laucus aspera* (Willd.) Link (Boruah et al. 2020, Chakravarty and Deka 2021, Kalita et al. 2022). The botanical family Ciperacea is the most abundant in the studied site; it stands out that *Cyperus brevifolius* (Rottb.) Endl. ex Hassk. developed enzymatic defence induced by the stress of oil accumulated in the soil (Chakravarty and Deka 2021). The outstanding findings in plant defence indicate increased dehydrogenase, urease, alkaline phosphatase, catalase and amylase in contaminated soil where the *C. brevifolius* plant grew for 60 days. These authors also report that a decrease in peroxidase and polyphenol oxidase activities occurred in samples of the same soil. In the case of the Lamiaceae *L. aspera*, the authors (Kalita et al. 2022) determined the potential for phytoremediation and enzymatic defence developed to face the stress derived from exposure to crude oil. The results show enzymatic and biochemical plasticity evidenced by significant increases in the enzymes dehydrogenase, urease, alkaline phosphatase, catalase, amylase and cellulose in contaminated soil.

L. hexandra is a hydrophytic grass that grows in oil-contaminated wetlands in southeastern Mexico. This anthropogenic environmental impact of the oil industry began in the 1940s. It was characterised by the accumulation of drilling sludge in soil and lagoon bodies, so chronic oil and sludge spills persist today (González 1995). The grass has developed stress tolerance mechanisms that manifest in the hormetic response of its growth rate in the increase of its plant biomass and crude protein content (Orocio-Carrillo et al. 2019). The grass evaluated also has a rhizosphere colonised by hydrocarbonoclastic bacteria and fungi (González-Moscoso et al. 2019). The adaptation of these microorganisms to the presence of crude oil suggests the presence of genes related to hydrocarbon degradation similar to those found in the bacteria *Pusillimonas*, *Dietzia*, *Bradyrhizobium*, *Azospirillum*, and *Azotobacter* (Arias-Trinidad et al. 2019, Wang et al. 2021). When associated with the roots of *L. hexandra*, these bacteria stimulate the removal of total petroleum hydrocarbons (TPH) from the soil (Orocio-Carrillo et al. 2019). This study aims to increase our knowledge of why this grass, when exposed to CO under stress conditions, increases its growth rate and biomass production while removing CO from the soil. These questions can be answered by studying the synthesis of ROS and the antioxidants that regulate metabolism under stress conditions. The objectives of the present study were: (a) to determine the effect of CO on the biomass production of *L. hexandra* and formation of biochemical stress

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biomarkers (H_2O_2) and antioxidants (non-enzymatic and enzymatic) in leaves and roots; (b) analyse the effect of the grass rhizosphere and its rhizobacteria on the elimination of TPH and the formation of root antioxidants; (c) to propose a biological technology for the recovery of soil contaminated with CO based on a native grass of the Mexican humid tropics adapted to the stress induced by CO.

MATERIAL AND METHODS

Soil characteristics and seedling cultivation. Uncontaminated soil (Gleysol) was collected in Ejido Blasillo 4th Section, Huimanguillo, in the state of Tabasco (18°05'08.4"N, –93°56'50"W). The soil was collected in May 2020 from a single point on the surface horizon (0–30 cm), was dried, sieved (5 mm mesh), and analysed to determine its properties before being mixed with different concentrations of fresh oil for the experimental bioassay. The properties of uncontaminated soil were as follows: sandy clay loam texture (Bouyoucos 1962); moderately acid pH (5.5); very high content of organic carbon (9.98%) (Walkley and Black 1934); high content total nitrogen (0.66%) (micro Kjeldhal, Page et al. 1982), high content of sulfate (45 mg/kg) (Etchevers 1992); and high content phosphate (85 mg/kg) (Olsen and Sommers 1982). Cultivation of seedlings was similar to the procedure used by Orocio-Carrillo et al. (2019). Plants 19.5 ± 2.3 cm tall were produced 30 days after planting and were used for the experiment.

Soil contamination and experimental design. CO was medium crude petroleum with an American Petroleum Institute of 10.8°, specific gravity of 0.84 g/cm^3, 56.4, 23.7 and 14% of aliphatic, aromatic and polar (resin and asphaltene) fractions, respectively. The CO was obtained from the oil field "Cinco Presidentes" in La Venta, Tabasco, Mexico (18°12'11.8"N, –94°08'37.8"W) (Orocio-Carrillo et al. 2019). The experiment was carried out under a completely randomised design and 4 × 2 factorial arrangement: four concentrations of oil [0 (control), 30, 60 and 90 g/kg DW (dry weight)] and two technologies, phytoremediation (PH) with *Lerrsia hexandra* and natural attenuation (NA), native microorganisms. The present experiment is part of a line of research that started 10 years ago; throughout that period, the treatments evaluated have transitioned from studies of the soil to plant organs and their enzymatic defences to stress induced by crude oil. Eight treatments with six repetitions were maintained in a micro-tunnel for 180 days (August 2020 to January 2021) with an average temperature of 29 ± 6 °C, relative humidity between 85% and 96% and humidity at a field capacity of 32 ± 5%. The parameters evaluated in PH treatments were plant growth (number of YP), production of aerial dry matter (ADM) and root dry matter (RDM); hydrogen peroxide in the leaf of YP (H_2O_2L) and root (H_2O_2R), ascorbic acid/ascorbate in leaf (AAL) and root (AAR), total phenols in leaf (TPL) and root (TPR), total flavonoids in leaf (TFVL) and root (TFVR), catalase in leaf (CATL) and root (CATR), and phenylalanine ammonia-lyase in leaf (PALL) and root (PALR). The parameters evaluated in PH and NA treatments were microbial respiration (CO_2), population (colony-forming unit (CFU)/g) of *Azospirillum* (AZP), *Azotobacter* (AZT) and *Pseudomonas* (PSE) groups, and total petroleum hydrocarbons (TPH) removal.

Formation of new plants and production of phytomass. These variables include the growth of new plants from the plant initially planted on day 1, counted on day 180; regarding phytomass, we refer to the root and aerial biomass (stems + leaves) also harvested on day 180 of the experimental cycle. The dry matter of stems, leaves and roots were dried in an oven at 60 °C for 72 h and weighed on a digital scale with a precision of 0.01 g.

Biochemical analysis in leave and root. Fresh leaves and roots were stored at –80 °C until use. The samples were lyophilised (lyophiliser, Yamato Scientific Co. Ltd., Model D401, Santa Clara, USA). For H_2O_2 (µmol/g DW) compound determination, 25 mg of lyophilised tissue and 1 mL of trichloroacetic acid 0.1% were quantified in a UV-Vis spectrophotometer (Spectrophotometer UNICO UV2150, Dayton, USA) with a wavelength of 390 nm (Velikova et al. 2000). The acid/ascorbate (AA) (mg/g DW) compound was determined with the colourimetric method using 2.6 dichlorophenol, 1 g of lyophilised tissue and 1 mL of metaphosphoric acid (1%) (Hung and Yeng 2002). TP (mg/g DW) was determined using the Folin-Ciocalteu reagent in 0.2 g of lyophilised tissue (Cumplido-Nájera et al. 2019). TFV (mg/g DW) was determined according to the method of Arvouet-Grand et al. (1994), 100 mg of lyophilised tissue extracting with reagent-grade methanol and quantifying with a methanolic solution (2%) of aluminium trichloride. The reading was taken at 415 nm in a UV-Vis spectrophotometer. For antioxidant enzyme compound determination, 100 mg of lyophilised tissue and 20 mg of polyvinylpyrrolidone were weighed. After this, 1.5 mL of phosphate buffer with a pH of

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7–7.2 (0.1 mol/L) was added, and the mixture was then subjected to micro-centrifugation at 12 000 rpm for 10 min at 4 °C. The supernatant was filtered with a nylon membrane. With this extract, the antioxidant capacity in hydrophilic compounds was determined. CAT (EC 1.11.1.6) (UTP1, where U is equal to the mmol equivalent of H_2O_2 consumed per mL/min) was quantified using the method of Dhindsa et al. (1981). PAL (EC 4.3.1.5) was determined according to the method of Sykłowska-Baranek et al. (2015).

Population densities of rhizobacteria. The effects of the four oil levels and the two soil remediation options were determined by counting viable cells from four groups of microorganisms established in specific culture media (Gonzalez-Moscoso et al. 2019). AZP bacteria were seeded in a congo Rojo agar culture medium (Döbereiner et al. 1966). AZT bacteria in Asby agar culture medium (Döbereiner et al. 1966), and PSE bacteria in cetrimide + glycerol agar culture medium (Garrity et al. 2005). The cultures were incubated at 28 °C for 72 h. The bacteria colonies were counted and expressed as CFU per gram of soil. Microbial respiration was measured by CO_2 emission after incubation using alkaline solution and titration with the method described by Stotzky (1965).

Analysis of total petroleum hydrocarbons. The determination of TPH in soil samples was carried out by gravimetry. Soil samples were collected on days 1 and 180 after sowing the grass, and the oil was extracted for 8 h in soxhlet equipment (USEPA 1996) with analytical grade dichloromethane. The amount of TPH was calculated by the weight difference between the quantity extracted on day 1 and the quantity corresponding to day 180, and the difference was multiplied by 100 (Gonzalez-Moscoso et al. 2019).

Statistical analysis of data. Unifactorial ANOVA was used to analyse the data of several YP, ADM and RDM, as well as H_2O_2, AA, TF, TFV, CAT and PAL in the leaf of the YP and root according to the CO concentration. Bifactorial ANOVA was used to analyse the data of CFU of rhizobacteria (AZS, AZT, and PSE), CO_2 and TPH removal according to technologies and CO concentration. Data homogeneity was verified. The differences between variables were analysed using Duncan's separation of means ($P \leq 0.05$). Bivariate Pearson correlation was used to analyse all the variables in the four PH treatments. All statistical analyses were performed using SAS v.8.01 (SAS 2005).

RESULTS AND DISCUSSION

Effect of crude oil on the growth of *Leersia hexandra*. Table 1 shows the quantities of young plants and the dry phytomass of the aerial part (leaf and stem) and the root. It stands out that incorporating CO into the soil was associated with significant differences (Duncan $P < 0.05$) between mean values in YP and RDM but not in ADM (dried stems and leaves). The oil induced a hormetic effect on the number of YP and production of RDM. Exposure to 90 g of CO promoted an increase of up to 76% in the number of new plants compared to the control soil. The results of RDM increased up to 66% in the most contaminated soil (90 g/kg of CO) compared to the control soil. Unlike the roots, the aerial dry biomass of grass decreased up to 44% with the highest dose of CO (Table 1).

Table 1. Quantities of young plants, aerial dry matter and root dry matter of *Leerxia hexandra* exposed to crude oil (CO) for 180 days

CO (g/kg DW)	Young plants		Dry matter			
	quantity	(%)	aerial (g)	(%)	root (g)	(%)
0	26 ± 3.2		148 ± 2.7*		27 ± 1.1	
30	51 ± 2	+49	118 ± 10	–20	62 ± 4.4	+24
60	70 ± 10.2	+63	99 ± 18[b]	–33	74 ± 3.4	+63
90	108 ± 14*	+76	83 ± 14	–44	80 ± 15.4*	+66
No oil	26		148*		27	
Oil	76*	+66	100	–32	72*	+62
Coefficient of variation	7.3		11.27		5.82	

The symbol % (+) represents an increase and % (–) a decrease in young plants and vegetable dry matter concerning the values of the control treatment (0 g/kg crude oil). *Treatment means with a greater statistical difference (Duncan, $P \leq 0.05$, $n = 6$); DW – dry weight

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Table 2. Changes in catalase (CAT), phenylalanine ammonium lyase (PAL) and hydrogen peroxide (H_2O_2) contents in the leaf and root of *Leersia hexandra* exposed to crude oil (CO) for 180 days

CO (g/kg DW)	CAT (U/g protein)	(%)	PAL (U/g protein)	(%)	H_2O_2 (µmol/g DW)	(%)
Leaf						
0	169 ± 21		12 ± 35*		2.97 ± 0.54	
30	182 ± 40*	+17	10 ± 0.57	–16.6	3.8 ± 0.18	+22
60	169 ± 31	0	10.9 ± 1.2	–9	4.3 ± 0.48*	+31
90	118 ± 16	–30	10.3 ± 0.98	–14	4.4 ± 0.46*	+32
Root						
0	63 ± 10.7		0.8 ± 0.13		0.62 ± 0.03*	
30	110 ± 26*	+42.7	2 ± 0.25	+60	0.57 ± 0.02	–8
60	91 ± 13.5	+30.7	2.4 ± 0.21	+66	0.53 ± 0.01	–16
90	73 ± 9.8	+13.7	3 ± 0.38*	+73	0.49 ± 0.009	–21

The symbol % (+) represents an increase and % (–) a decrease in CAT, PAL and H_2O_2, in leaf and root, concerning the values of the control treatment (0 g/kg crude oil). *Treatment mean with greater statistical difference (Duncan, $P \leq 0.05$, $n = 6$); DW – dry weight

Effect of crude oil on the production of hydrogen peroxide and antioxidants. Table 2 shows the contents of CAT, PAL and H_2O_2 in the leaf and root of *L. hexandra* exposed to four different doses of CO. The mean concentrations of these three substances in leaf and root showed statistically significant differences ($P \leq 0.05$). The H_2O_2 content in leaf YP increased up to 32% in plants exposed for 180 days to 90 g/kg of CO. The same concentration of CO induced a phytotoxic process in the root, and the content of H_2O_2 decreased by 21%, from 0.62 to 0.49 µmol/g (Table 2). The AA in the leaf varied from 2.3 to 2.5 mg and showed a similar variation in the root, from 2.3 to 2.4 mg. All treatments produced similar results in both plant organs (Duncan, $P \leq 0.05$) (Table 3). In the case of TP, exposure to CO induced a hormetic response in the leaf of YP subjected to the three oil treatments. The highest concentration was 12 ± 0.86 mg of TP from 90 g/kg of CO, 34% higher than the treatment without CO (Table 3). The opposite effect occurred in the root, where the same concentrations inhibited 56% of the TP biomarker. Regarding the production of TFV in leaf and root, it increased in contaminated soils, showing statistically significant differences (Table 3). The concentration of TFVL of plants exposed to 90 g of CO was 2 181 mg/100 g.

Table 3. Changes in ascorbic acid (AA), total phenols (TP) and total flavonoids (TFV) contents in leaf and root of *Leersia hexandra* exposed to crude oil (CO) for 180 days

CO (g/kg DW)	AA (mg/g DW)	(%)	TP (mg/g DW)	(%)	TFV (mg/100 g DW)	(%)
Leaf						
0	2.3 ± 0.04*		7.9 ± 1.3		1 617 ± 62	
30	2.5 ± 0.08*	+0.8	8.4 ± 0.34	+6	2 044 ± 13	+21
60	2.4 ± 0.01*	+0.4	8.9 ± 0.27	+11	1 851 ± 86	+13
90	2.4 ± 0.03*	+0.4	12 ± 0.86*	+34	2 181 ± 23*	+26
Root						
0	2.4 ± 0.02*		1.23 ± 0.04*		236 ± 6.9	
30	2.3 ± 0.01*	–0.4	0.75 ± 0.12	–39	352 ± 15*	+33
60	2.4 ± 0.03*	0	0.71 ± 0.10	–42	239 ± 5.4	+1.3
90	2.4 ± 0.04*	0	0.54 ± 0.06	–56	251 ± 0.22	+5.9

The symbol % (+) represents an increase and % (–) a decrease in AA, TP and TFV, in leaf and root, with respect to the values of the control treatment (0 g/kg crude oil). *Treatment mean with greater statistical difference (Duncan $P < 0.05$; $n = 6$); DW – dry weight

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Table 4. Changes in population of *Azospirillum* (AZP), *Azotobacter* (AZT) and *Pseudomonas* (PSE), and microbial respiration (MR) in soil with crude oil (CO) on day 180 after phytoremediation (PH) and natural attenuation (NA)

Technology/CO (g/kg)	AZP (10^3 CFU/g DW)	(%)	AZT (10^3 CFU/g DW)	(%)	PSE (10 CFU/g DW)	(%)	MR (mg CO_2/kg DW)	(%)
PH								
0	84*		212		94*		12	
30	67	−20	371	+43	57	−60.6	19	+37
60	5	−94	481*	+56	33	−64.8	38	+68
90	21	−75	438*	+52	73	−22	48*	+75
NA								
0	84		244		314*	−49	9.6	
30	95*	12	328	+32	160	−85	13	+26
60	70	−17	370	+34	46	−75	28	+66
90	41	−51	364	+33	77		29	+67
PH	44^B		375^A		64^B		29^A	
NA	72^A		326^A		149^A		20^B	

The symbol % (+) represents an increase and % (–) a decrease in the population densities of the three groups of bacteria and microbial respiration concerning the values of the control treatment (0 g/kg crude oil). *Treatment mean with greater statistical difference (Duncan, $P \leq 0.05$, $n = 6$); CFU – colony-forming unit; DW – dry weight

CO increased the amount of TFVL by 26% compared to the control. In root, the concentration of FV was 33% higher (325 mg) in plants exposed to 30 g of CO compared to the control. The concentration of the antioxidant enzyme CATL and CATR showed statistically significant differences (Duncan $P \leq 0.05$) when the plants were exposed to a CO concentration of 30 g (Table 2). The CAT increased by 17% and 42.7% in leaf and root, respectively. The concentration of the PAL enzyme in the root showed a significant increase in the presence of oil. The three evaluated doses promoted the production of PAL. The highest concentration reached was 3.0 ± 0.38 U/g of protein, 73% more than obtained with the control soil (Table 2). In the leaf, the production of PAL was inhibited by CO.

Crude oil and rhizobacteria technology. The two technologies, PH and NA, used to recover soils contaminated with up to 90 g/kg of CO showed significant differences in the population of three groups of plant growth-regulating bacteria and microbial respiration (Table 4). The AZP bacteria were generally more abundant in NA than in PH. In NA, the highest population (95 × 10^3 CFU) was recorded in soil with 30 g of CO, 12% higher than in the control soil. AZT bacteria reached the highest population with PH

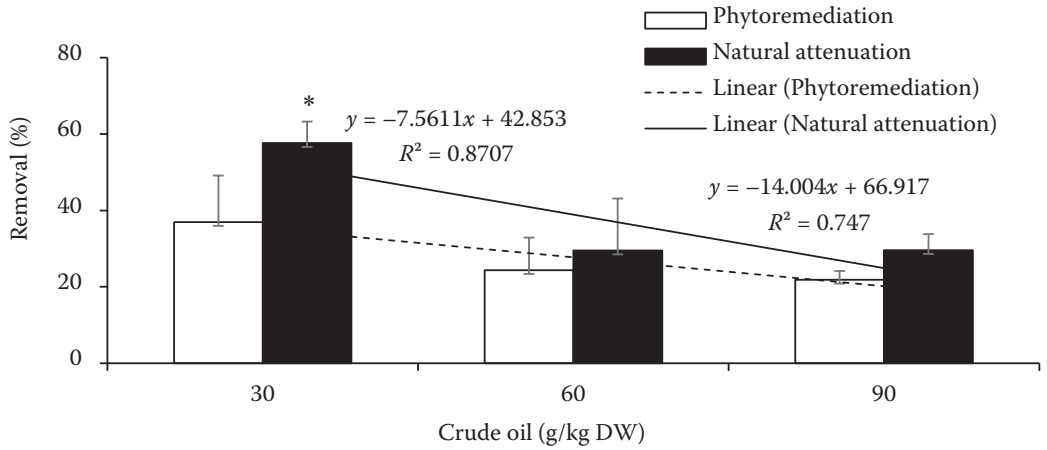

Figure 1. Removal of total petroleum hydrocarbons induced by phytoremediation and natural attenuation (Duncan $P \leq 0.05$, $n = 6$); DW – dry weight

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in the presence of 60 and 90 g of CO, ranging from 438 to 481 × 10^3 CFU, 52% to 56% higher than in the control soil, respectively. The PSE bacteria formed less CFU than the two other bacteria groups, in the order of 10^1 CFU, two units less. CO inhibited this group of bacteria, both in PH and NA. Microbial respiration increased up to 75% due to the effect of PH and 67% to NA (Table 4).

Crude oil removal according to technology. The data of CO removal for PH and NA on day 180 showed an inversely proportional trend to the level of soil contamination (Figure 1). Highest removal was 57.6% in soil with 30 g/kg of CO subjected to NA; on the other hand, the lowest was 22% with PH in soil with 90 g/kg of crude oil.

Hydrogen peroxide and antioxidants in *Leersia hexandra* leaf. Studying oxidative stress and the antioxidant response induced by hydrocarbons and inorganic elements of CO in plants is a complex affair. The effects of CO contamination on living beings have been studied mainly by considering how CO affects the soil. CO causes changes in temperature, hydrogen potential, electrical conductivity, water availability, and nutrient availability. It also favours the accumulation of heavy metals and toxic hydrocarbon metabolites derived from degradation and mineralisation (Devatha et al. 2019). The results of the present study indicate a high concentration of H_2O_2L in YP in the presence of CO and a highly significant positive relationship between them (0.791**, Table 5). The increase in H_2O_2 agrees with the results of Ahmad et al. (2020), but there is no agreement on the H_2O_2L × YP relationship. In the cv. Krona of rye (*Secale cereale*), H_2O_2 increases with 12% of oil, but RDM and ADM decrease (Skrypnik et al. 2021). Ahmad et al. (2020) reported that 5% and 10% concentrations of hydrocarbons inhibit leaf biomass production in *Brassica oleracea* but increase the concentration of H_2O_2 in the leaf.

Excessive accumulation of H_2O_2 in the grass leaf is a biomarker of oxidative stress. There is evidence that plant metabolism regulates physiological processes of senescence by eliminating the production of H_2O_2 at the right place and time (Hasanuzzaman et al. 2014) since high levels of ROS have a deleterious effect on plasma membranes (Kaya et al. 2019). The positive relationship (0.812** and 0.678**, Table 5) between the concentrations of TPL and TFVL and the concentration of CO indicates that these substances can serve as stress biomarkers at high concentrations of CO in the soil. However, CATL increases

Table 5. Pearson correlation values between crude oil, plant, antioxidants, phytoremediation and bacteria

	YP	RDM	H_2O_2L	TPL	TFVL	PALL	H_2O_2R	TPR	TFVR	PALR	REM	AZP	AZT	PSE	MR
CO	0.970**	0.882**	0.778**	0.812**	0.678**	ns	−0.937**	−0.876**	ns	0.864**	ns	−0.977**	0.850**	ns	0.995**
YP		0.831**	0.791**	0.845**	0.706**	ns	−0.911**	−0.848**	ns	0.882**	ns	−0.977**	0.777**	ns	0.972**
RDM			0.838**	0.652**	0.768**	ns	−0.831**	−0.897**	ns	0.709**	0.601*	−0.809**	0.927**	−0.608*	0.857**
H_2O_2L				0.575*	0.718**	ns	−0.728**	−0.732**	ns	0.764**	ns	−0.724**	0.837**	−0.575**	0.750**
TPL					0.625**	ns	−0.801**	−0.651**	ns	0.807**	ns	−0.890**	0.509*	ns	0.843**
TFVL						−0.610*	0.718**	−0.738**	ns	0.693**	0.579*	−0.693**	0.589*	ns	0.676**
PALL							ns	ns	−0.742**	ns	−0.578*	ns	ns	ns	ns
H_2O_2R								−0.732**	ns	0.764**	ns	−0.764**	0.837**	−0.575*	0.750**
TPR									ns	−0.595*	−0.741**	0.824**	−0.868**	0.593*	−0.852**
TFVR										ns	0.676**	ns	ns	ns	ns
PALR											ns	−0.891**	0.622*	ns	0.878**
REM												ns	0.592*	−0.609*	ns
AZP													−0.738**	ns	−0.985**
AZT														−0.773**	0.802**
PSE															ns

*$P \leq 0.05$; **$P \leq 0.01$; ns – no significance. CO – crude oil; YP – young plants; RDM – root dry matter; H_2O_2L – hydrogen peroxide in leaf; TPL – total phenols in leaf; TFVL – total flavonoids in leaf; PALL – phenylalanine ammonia lyase in leaf; H_2O_2R – hydrogen peroxide in root; TPR – total phenols in root; TFVR – total flavonoids in leaf; PALR – phenylalanine ammonia lyase in root; REM – removal of crude oil; AZP – *Azospirillum* spp.; AZT – *Azotobacter* spp.; PSE – *Pseudomonas* spp.; MR – microbial respiration

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only at low doses of CO (Table 2). TPL and TFVL contribute to stress resistance and interact with many enzymes, growth regulators, and antioxidants in the plant, although the latter is still under debate (Treutter 2010). These compounds also mediate ROS homeostasis in vacuoles, acting mainly as antioxidants (Agati et al. 2012). The results of the present study indicate an increase in TP in the leaves of the grass *L. hexandra* due to the effect of CO. Similar results were reported in Krona and Valdai rye with 12% of CO (Skrypnik et al. 2021) and in *C. leucanthemum* with 7.5% CO (Noori et al. 2012). TFVL also increase in the presence of CO, similar to the behaviour of phenanthrene in *Arabidopsis thaliana* L. (Fini et al. 2011) and *Chrysanthemum leucanthemum* with 7.5% to 10% of CO (Noori et al. 2012). The regulation of redox homeostasis in plants under polluted conditions is based on the activity of low molecular weight molecules of the enzymatic and non-enzymatic antioxidant systems (Skrypnik et al. 2021). Some authors assume that the enzymatic system provides the metabolic process that provides the most effective protection against damage caused by ROS (Quan et al. 2008). In the present study, the positive correlation of TPL (0.575*) and TFVL (0.718**) with H_2O_2L (Table 5) confirmed that the enzymatic system may serve as a self-regulatory mechanism for the removal of H_2O_2 in YPL. However, Agati et al. (2012) indicate that the correlation between oxidative stress, ROS generation, and ROS removal activity by TP and TFV remains a controversial subject because the interaction between antioxidant enzymes, substrate (flavonoid), and H_2O_2 occurs only after the rupture of the tonoplast membrane. In the present study, the highly significant positive relationship between YP and TPL (0.845**) and TFVL (0.706**) (Table 5) suggests that these non-enzymatic antioxidants are stress signalers that contribute to YP growth. These compounds cause changes in the levels of 3-indoleacetic acid and the biosynthesis of LEA (late embryogenic abundant) proteins that bind to cell membranes and proteins of cell reproductive organelles (Matilla 2008).

In YPL, CAT was more sensitive than TP and TFV. The production of this enzyme was stimulated only at low doses of CO, showing a negative relationship with CO (–0.530*, Table 5). These results are similar to those Al-Hawas et al. (2012) reported in *Simmondsia chinensis*. In that case, the concentration of CATL increased in plants exposed to 1% CO but decreased when exposed to doses of 2% and 3%. In *A. thaliana*, CAT behaves in a similar way when the plant is exposed to phenanthrene; in *Kandelia candle* L., when exposed to any of the 16 polycyclic aromatic hydrocarbons (Liu et al. 2014); in *Lolium perenne* L. to phenol-polycyclic aromatic hydrocarbons (Malicka et al. 2021); and in *Cynara cardunculus* var. *altilis*, when exposed to 100 mmol NaCl (Docimo et al. 2020). The inhibition of CAT in YPL exposed to CO indicates that the presence of oil disrupts cellular synthesis, which directly induces the accumulation of H_2O_2 in the leaf, considering that this antioxidant is directly involved in converting H_2O_2 into water and oxygen under stressful conditions that cause ROS to accumulate (Glorieux and Calderón 2017).

Hydrogen peroxide and antioxidants in *Leersia hexandra* root. Plant roots adapt to different soil conditions. In *L. hexandra* roots, the amount of H_2O_2 synthesised decreases according to the CO concentration (–0.937**, Table 5). Similar behaviour is observed in the *Glycine max* root exposed to NaCl (Neves et al. 2010). An opposite behaviour is observed in the roots of *G. max* exposed to Cd (Finger-Teixeira et al. 2010) and in *Rumex dentatus* L., *Euphorbia helioscopia* L., *Cannabis sativa* L. and *Parthenium hysterophorus* L. exposed to lead (Pb) and chromium (Cr) (Ullah et al. 2019). These recent findings show variation in the synthesis of H_2O_2 in the roots of different plants when exposed to stress. Each type of plant regulates the synthesis of H_2O_2 differently. H_2O_2 is a signal molecule involved in acclimation signalling that triggers stress tolerance (Gill and Tuteja 2010). The low concentration of H_2O_2 in the root of grass exposed to CO suggests that it is a stress-signalling molecule. Still, this concentration is sufficient for promoting plant growth and for preserving the permeability of the cellular channels that conduct calcium and potassium for cellular homeostasis (Foreman et al. 2003), cell cycle regulation (Mittler et al. 2004), growth and development (Foreman et al. 2003) and the timely translation of genetic signals (Quan et al. 2008).

The synthesis of root antioxidants as stress biomarkers behaved differently than YPL. In the roots, CO negatively affected TPR (–0.876**) and positively PALR (0.864**). The behaviour of TPR when the grass is subjected to oil-induced stress is similar to that of *Lens culinaris* cv. Krak. The tub was exposed to 0.5 mmol Cu^{2+} (Janas et al. 2009). The opposite behaviour is observed in *C. leucanthemum* exposed to 10% CO (Noori et al. 2012). The significant negative relationship TPR × RDM (–0.897**, Table 5) suggests

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that TP possibly promotes increased root growth by controlling the endogenous levels of 3-indoleacetic acid that the plant requires for its growth (Engels et al. 2012). In the present study, *L. hexandra* regulates the physiological balance of the roots under stress based on a significant and positive relationship between TPR × H_2O_2 (0.817**, Table 5). The CAT in the root shows no relationship with H_2O_2R and RDM but increased in roots exposed to 30, 60 and 90 g/kg of oil compared to the control. This behaviour is different than that observed in the roots of *Lactuca sativa* L., *Cichorium endivia* L., *Apium graveolens* L., *Petroselinum crispum*, (Mill.) Fuss, and *Solanum melongena* L. but similar to the behaviour observed in *Lycopersicon esculentum* L. exposed to 3-amino-1,2,4-triazole (Chioti and Zervoudakis 2017). Since CAT has been reported to be synthesised in the mitochondria (Willekens et al. 1995) and H_2O_2 is generated during mitochondria respiration (Giorgio et al. 2007), the physiological role of this isoenzyme in the roots of *L. hexandra* could be to promote the reduction of H_2O_2 by oxidising it and obtaining water and oxygen, as indicated by Glorieux and Calderón (2017). An outstanding result of the present study is the increase in the concentration of PAL at the grassroots. This enzyme's highly significant positive relationship with CO (0.864**) and RDM (0.709**, Table 5) indicates the protective role it plays in the roots and as a stress biomarker. Similar behaviour has been observed in the roots of *G. max* exposed to Cd (Finger-Teixeira et al. 2010). An opposite behaviour has been observed in *G. max* and *Zea mays* L. exposed to dopamine (Siqueira-Soares et al. 2013) in *G. max* and *Beta vulgaris* L. treated with mannan oligosaccharide for the control of *Meloidogyne javanica* (Treub) nematodes, where PAL is an indicator of stress resistance (Débia et al. 2021). PAL biosynthesis has been reported to increase in pea seedlings when ethylene was applied to the plants (Hyodo and Yang 1971), which suggests that ethylene is present in soil derived from the degradation of linear petroleum hydrocarbons (Fessenden and Fessenden 1983), which in contact with the roots of *L. hexandra* induce root growth. PAL synthesis is important for plant defence and growth due to its role in the synthesis of the secondary metabolite phenylpropanoid, which plays a crucial role in the biosynthesis of monolignols, salicylic acid, phytoalexin, and flavonoids (Deng and Lu 2017, Odukoya et al. 2019).

The presence of CO in the soil promotes the growth of YP and the production of RDM, H_2O_2, TP and TFV in leaves. The production of CAT and PAL enzymes also increases in *L. hexandra* roots, but H_2O_2 and TP decrease in roots, even though root production increases. The root system regulates stress by synthesising low concentrations of H_2O_2 but high concentrations of CAT and PAL enzymes, maintaining increased RDM production. The increased growth of roots in soil with oil provides the *L. hexandra* grass with more nutrients and increases the production of YP or tillers. The grassroots downregulate the synthesis of H_2O_2 to avoid cell damage in balance with the production of non-enzymatic antioxidants, TP and TFV in the leaf. The root system of the grass associated with AZT spp. induces the removal of 37, 24 and 22% of TPH in soil with 30, 60 and 90 g of oil, respectively. *L. hexandra* is proposed for use as a remediation technology in soil contaminated with up to 90 g/kg oil due to its capacity to adapt to stress conditions, manifested by regulating the synthesis of H_2O_2 and antioxidants in the leaf and root during the removal of TPH in clay soil in the Mexican humid tropics. Our results and published research have favourable perspectives for the recovery/decontamination of soils affected by crude oil spills.

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