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Physiological and microbiological hormesis in sedge Eleocharis palustris induced by crude oil in phytoremediation of flooded clay soil

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Abstract

Soil contamination with petroleum hydrocarbons affects plants and rhizospheric microorganisms. A nine-month experiment was set up to study the effect over time of oil on plant height, new plant production, plant matter production, and the population of rhizospheric microorganisms in the sedge *Eleocharis palustris*. The removal of total petroleum hydrocarbons from the soil was also evaluated. The results showed that plants responded differently depending on age and petroleum concentration. Concentration of 60 g of petroleum per kg of soil induced a significant increase in height (weeks 28 to 38) and new plant production (weeks 13 to 38). At the end of the evaluation period, a high petroleum concentration induced a significant increase in the number of primary roots, root and aerial dry matter, population of microalgae, total fungi, actinomycetes, *Pseudomonas* spp and hydrocarbonoclastic bacteria. Physiological and microbiological hormetic index values ≥1 showed a linear response to petroleum concentration in plant height and new plant production from weeks 3 and 2 to 38, respectively. A similar response curve was found for the number of primary roots, root and aerial dry matter, microalgae, total fungi, actinomycetes, *Pseudomonas*, and hydrocarbonoclastic bacteria (week 38). After 38 weeks of exposure to petroleum-contaminated soil, the rhizosphere of *E. palustris* showed petroleum removal percentages of 71, 72, 56, 42, 33 and 23% in soil that initially contained 3, 15, 30, 45, 60 and 75 g oil, respectively. The increase in petroleum removal was significantly and negatively correlated with the populations of microalgae, actinomycetes, *Pseudomonas* bacteria and hydrocarbonoclast bacteria.

Introduction

A long-standing problem in the tropical wetlands is the contamination of Gleysol soils with hydrocarbons associated with the extraction and transportation of crude oil (CO). Due to the flooded conditions of these soils, the underground pipelines are frequently damaged, which can lead to the accidental release of oil into the environment (Rivera-Cruz 2011; Rodríguez-Rodríguez et al. 2016). CO is made up mainly of total petroleum hydrocarbons (TPH) as well as saturated hydrocarbons, aromatic compounds, asphaltenes, and resins. These groups of hydrocarbons and their epoxides are toxic, mutagenic, and/or carcinogenic, and cause damage both to microorganisms and higher organisms, including humans (Winquist et al. 2014; Moubasher et al. 2015). In wetland Gleysols, the presence of CO induces structural changes in soil, air, and water, altering their original physicochemical properties (Caravaca and Roldán 2003; Iturbide et al. 2007). The biological balance of oil-contaminated soil changes according to the living organisms that inhabit it. Soil contamination with hydrocarbons can even have a positive effect on the biology of the organisms that inhabit historically contaminated environments (González-Moscoso et al. 2017; Orocio-Carrillo et al. 2019; Alanbary et al. 2019; Rodríguez-Uribe et al. 2021).

The positive effect of CO on plants and various beneficial microorganisms has been studied by considering the relationship between oil quantity, composition, and degree of affectation (Al-Mutairi et al. 2008). The use of physiological and microbiological bioindicators to measure the toxicity and hormesis of CO hydrocarbons in plants and microorganisms has several advantages, compared to the use of physicochemical indicators since the first have greater sensitivity, flexibility, and higher response speed

than the latter (Dutka et al. 1991; Pérez et al. 2008; Pentreath et al. 2015). The physiological response of plants and microorganisms can be expressed as an absolute numerical value that indicates the harm and/or benefits they derive from exposure to the pollutant. This value depends on the type of soil and pollutant (Chaîneau et al. 2003; Pérez et al. 2008; Calabrese and Blain 2009). Indicators of hormesis show the typical dose-response relationship for low and high doses that have a stimulating effect on living beings (Calabrese 2013). Hormesis is an evolutionary response of organisms that had to adapt to stressful conditions, has implications for ecology, toxicology, risk assessment, and other disciplines (Agathokleous and Calabrese 2020a; Calabrese and Agathokleous 2020). Hormesis plays a crucial role in the control and remediation of soil contamination (Agathokleous and Calabrese 2020b; Fan et al. 2021).

The response variables of plants and soil and rhizosphere microorganisms to stress caused by CO contamination, according to the reports by USEPA (2002), Vasquez-Luna et al. (2011), and Agathokleous et al. (2020), are growth, root density, accumulation of aerial and root matter, and population densities of microorganisms. Tropical wetlands are home to "C3" plants of the Cyperaceae family, which have short growth cycles, accumulation of aerial plant matter, and abundant roots. These properties are due to their high photosynthetic and transpiration rates, which give these plants the cellular capacity to synthesize sugars, proteins, and high polysaccharides (Mommer and Visser 2005; Salisbury and Ross 1999). Cyperaceae plants have a fibrous root system that holds onto the flooded soil to form the rhizosphere. The roots release organic and inorganic exudates into the rhizosphere that act as inducers of the growth of microalgae, actinomycetes, fungi, and TPH-degrading bacteria (Cao et al. 2012; Tang et al. 2011; Neumann and Römheld 2012). Microalgae play an important role in the rhizosphere by capturing inorganic carbon through photosynthesis. The metabolism of these autotrophic microorganisms releases carbonic acid into the soil, increasing its acidity (Pepper and Gerry 2015). Fungi, actinomycetes, and bacteria with heterotrophic metabolism oxidize and/or reduce carbon of biogenic origin or from petroleum in soil and sediment. They also provide NH_3, $H_2PO_4^{-2}$, and SO_4^{-2} to plants and microorganisms (Madigan et al. 2015; Smith et al. 2015).

Various plants adapted to petroleum-induced stress inhabit tropical and subtropical wetlands. Some of these plants are *Typha latifolia* (Omari et al. 2003), *Scirpus grossus* (Al-Baldawi et al. 2015), *Ludwigia octovalvis* (Almansoory et al. 2017), *Scirpus mucronatus*, *Cyperus laxus*, *Cyperus esculentus*, and *L. peploides* (Almansoory et al. 2021). The roots of these plants host nitrogen-fixing bacteria, *Pseudomonas* spp, and *Rhodococcus* spp., which show increased growth when exposed to hydrocarbon stress, compared to soil without roots (Vásquez-Luna et al. 2011; Rodríguez-Uribe et al. 2021). In Gleysol, petroleum hydrocarbons are absorbed into organic matter and sediment. This slows down the metabolization and biodegradation of said hydrocarbons (Madigan et al. 2015). During the anaerobic biodegradation of TPH, fungi, actinomycetes, and bacteria use H_2, SO_4^{2-}, Fe^{3-}, and NO_{3-} as electron donors (Maier and Gentry 2015). In these environments, the rhizosphere is reported to stimulate the flow of oxygen, organic molecules, nutrients, and facultative anaerobic microorganisms. Microorganisms release enzymes that oxidize high molecular weight hydrocarbons and arrange them, in the aqueous

phase, in the form of polycyclic hydrocarbons that are dissolved or absorbed in solubilized organic matter (Cunningham et al. 1996; Brady and Weil 2008).

In recent decades, different public and scientific organizations have taken an interest in the decontamination of wetland soils polluted with CO. The use of phytoremediation with native plants has been one of the preferred alternatives since it has several advantages, including economic ones (Calabrese and Agathokleousalabre 2021). Phytoremediation under anaerobic conditions is slower than under aerobic conditions but it has the potential to reduce the harmful impact of petroleum pollution by mineralizing hydrocarbons (Lawniczak et al., 2020). The following native wetland plants have shown the ability to remove TPH from flooded soils contaminated with oil, gasoline, or diesel: *L. octovalvis* (Alanbary et al. 2019), *Spartina alterniflora* (Fleeger et al. 2015), and *S. grossus* (Al-Baldawi et al. 2015).

Eleocharis palustris is a Cyperaceae that grows in large wetland areas used for livestock production in the humid Mexican tropics. This plant grows underground storage organs such as basal bulbs and tubers. In stressful environments (soil contaminated with CO), young plants regenerate from these storage organs (Daehler 1998; Rivera-Cruz et al. 2016). The physiological and microbiological response that allows *E. palustris* to survive in Gleysol soils contaminated with CO is still unknown. The present work aimed to identify indicator variables of physiological and microbiological hormesis index in response to oil-induced stress. Together with the ability of the rhizosphere of *E. palustris* to remove TPH, these variables could serve to develop a biological technology to remediate Gleysol soil from contaminated wetlands in the Mexican humid tropics.

Materials And Methods

Collection of soil and rhizomes

Uncontaminated soil (250 kg) was collected in January 2020 from a site on the surface horizon (0-30 cm) of a Gleysol located at Paso and Playa village, Huimanguillo municipality, Tabasco, México (17° 57' 41" N and 92° 21' 38" W) at 17 masl. Soil was dried, ground and sieved (5 mm in diameter) before subjecting it to analysis to determine the properties of a composite sample. The collected soil was then mixed with different concentrations of fresh oil to establish the experiment. Soil properties were as follows: clay texture (12 % sand, 51 % clay, and 37 % silt), SOC 3.5 %, TPH 1024 mg/kg, pH_{H2O} 6.4. electrical conductivity 0.3 dS/m, exchangeable cation capacity 9.6 Cmol/kg, NO_3 55.5 ppm, available P 30.1 ppm, Ca 3.7 meq/100 g, Mg 2.2 meq/100g, K 0.4 meq/100 g, and extractable sodium 27% (Rodríguez-Uribe et al. 2021). *E. palustris* rhizomes were collected from wetland soil contaminated with weathered oil in José Narciso Rovirosa village, southeast of the “La Venta” gas processing complex, state Tabasco state (18° 04' 39.8" N and 94° 02' 39.4" W). E. *palustris* had been exposed to chronic contamination as a result of crude oil spills (Rivera-Cruz et al. 2016). Rhizomes were cut with a stem length of 5 cm for use in the experimental bioassay.

Contaminated soil and experimental design

CO was obtained from the Ogarrio 2 oil battery in Huimanguillo, Tabasco state (18° 02' 12" N and 93° 55' 40" W). Oil is of medium grade, Isthmus type, 1.8 sulfur content, with an American Petroleum Institute gravity of 32-33 degree. CO composition was saturated hydrocarbons (61.2%), aromatic hydrocarbons (24.8%), and asphaltenes and resins (13.4%). Experiment 1 (Exp-1), which evaluated the growth of *E. palustris*, was conducted under a completely randomized design using a 7x37 factorial arrangement. Experiment included seven oil concentrations (0, 3, 15, 30, 45, 60 and 75 g/kg dry weight) and 37 exposure times (2 to 38 weeks after transplantation). Experiment 2 (Exp-2), evaluated the production of plant material, the population densities of microorganisms, and the phytoremediation of TPH, was conducted under a random design using a 7x2 factorial arrangement and oil concentrations similar to those used in Exp-1. Two remediation techniques were evaluated: phytoremediation (PR) with *E. palustris* and natural attenuation (NA), in both experiments four repetitions per treatment were used. OC were used because showed that *E. palustris* tolerates 76 g of OC Rivera-Cruz et al. 2016). During 38 weeks the height plant (PH) was measured and the new plants (NP) of each experimental unit were also counted. Root and aerial biomass, microbial population densities, and oil removal were determined at week 38. Vegetable variables studied were the number of primary roots (NPR), root dry matter (RDM), and aerial dry matter (ADM). Population densities of microorganisms counted were 14 treatments with PR and NA. Microorganisms evaluated were microalgae (Mi) cells (mL^{-1}), heterotrophic fungi (HF), total actinomycetes (ACT), *Pseudomonas* spp (Ps), and hydrocarbonoclastic bacteria (HB) (CFU g^{-1} soil or dry rhizosphere). Removal of TPH (%), organic carbon (%), total nitrogen (%) and sulfate (mg/kg) from the soil and rhizosphere of *E. palustris* were also quantified.

All treatments were established in a greenhouse at an average temperature of 34 °C in soil permanently saturated with water (5 cm subsurface water level). Initial oil concentrations were 0, 3, 15, 30, 45, 60, 75 mg/kg soil. Each oil concentration level was manually homogenized. TPH concentration was determined by extraction for 8 h using soxhlet equipment (USEPA 1996) with analytical grade dichloromethane. Experimental unit consisted of a container (16 height x 40 length x 28 cm width) with 9000 g of dry soil, an *E. palustris* plant for PR, and no plant for NA.

Plant growth and production

PH and NP were evaluated in 28 experimental units during 38 consecutive weeks, height was measured from the base of the stem to the apex using a ruler graduated in cm, and NP was determined directly based on the number of plants that emerged from the base of the main plant and from the young rhizomes. At week 38, the NPR that emerged from the base of the stem was counted. The ADM (stems, flowers, and fruits) and RDM were determined after drying the fresh material at 65 °C for 72 h. The dry material was weighed on a semi-analytical balance (Ohaus, Scout Pro SP202) with 0.01 gram readability.

Population densities of microorganisms

Population densities (CFU mL^{-1}) of Mi was determined in soil and rhizosphere samples collected at the end of week 38, by direct microscopic cell count in 0.1 mL of dilution 10^1 in a Neubauer chamber (Madigan et al. 2015). Populations (CFU g^{-1} dry weight) of HF, HB, PsB, and ACT were determined with the viable count technique and serial dilution using specific culture media (Madigan et al. 2015). Potato dextrose agar (Baker®) was used for HF, arginine-glycerol-salt agar for ACT (Johnson and Curl 1972), cetrimide + glycerol for PsB (Garrity et al. 2005), 20 g agar-agar, 1.0 L distilled water, 100 µL of crude oil, and mineral culture medium (0.2 g NH_3Cl, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.1 g KCl, 0.56 g $Na_2FeEDTA$, 0.025 g $Na_2MoO_4.2H_2O$, 0.025 g yeast extract, 0.5 g sodium lactate, 0.4 g $MgSO_4.7H_2O$, 0.12 $CaCl_2.2H_2O$, 0.1 mL of biotin + aminobenzoic acid) for HB (González-Moscoso et al. 2019).

Organic carbon, nitrogen, and sulfate

Organic carbon was determined in the soil after PR and NA using the Walkley and Black (1934) technique, TN through micro-Kjeldahl technique after digestion with H_2SO_4 (Page et al. 1982), assimilable sulfate (SO_4^{-2}) was determined in a buffer solution (K_2SO_2, 2N glacial acetic acid, and distilled H_2O) and a standard solution [$Ca(H_2PO_4)2.H_2O$, 2N glacial acetic acid, and distilled H_2O]. Sulfate quantification was performed by the turbidimetric method in a spectrophotometer at 670 nm (Etchevers 1992).

Removal of total petroleum hydrocarbons

Crude oil removal was evaluated based on the percentage of TPH removed from the soil (NA) and the rhizosphere of *E. palustris* (PR). Simple soil samples, from each experimental unit, were collected on day 1 of week 1 and also on the last day of week 38. TPH extraction was carried out, with analytical dichloromethane, for eight hours in soxhlet equipment (USEPA 1996). The amount of TPH removed was calculated by gravimetry based on the amounts extracted on day 1 with respect to the value of week 38 (González-Moscoso et al. 2019).

Statistical analysis

The normal distribution of the data was assessed using the Shapiro-Wilk test, while the homogeneity of the data was evaluated with the Bartlett test. An analysis of variance was also applied, and Duncan's test was used to compare the means ($p \leq 0.05$). All tests were run using the statistical program SAS v.9.4 (SAS, 2005).

Hormesis indexes

Physiological hormesis index (PHI) and the microbiological hormesis index (MHI) were calculated for each variable *(x)*. To measure the effect of the treatments, the PHI (*x*) and MHI (*x*) of each variable were compared with those of the control (0 g/kg TPH). The PHI *(x)* and MHI (*x*) were obtained by dividing the values corresponding to the treatments with crude oil between the values corresponding to the control treatment. This allowed us to evaluate the biological response to contaminated soil. Total physiological hormesis index (TPHI) was calculated as the sum of all the PHI (*x*), while the total microbiological

hormesis index (TMHI) was calculated as the sum of all the MHI (*x*) divided by the total of the variables under study, whose value represents the response of plants (PHI) and rhizospheric microorganisms (MHI) to the contaminant as a function of all the physiological and/or microbiological variables under study, as well as the dry plant material. The following equations were used:

$$\text{PHI}(x) \text{ and } \text{MHI}(x) = \text{TcoR1}/\text{TcR1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

$$\text{TPHI}\ (x) = \sum_{i=1}^{n} \left(\frac{PHI(x)}{n}\right) \ldots\ldots\ldots\ldots\ldots\text{i=1, 2, 3}\ldots\ldots\text{t} \quad (2)$$

$$\text{TMHI}\ (x) = \sum_{i=1}^{n} \left(\frac{MHI(x)}{n}\right) \ldots\ldots\ldots\ldots\ldots\text{i=1, 2, 3}\ldots\ldots\text{t} \quad (3)$$

Where:

PHI(*x*): physiological hormesis index for variable *x*, and MHI (*x*): microbiological hormesis index for variable *x*.

Tco: crude oil concentration

Tc: control

R1: repetition 1….n

N = number of variables

$TPHI_{(x)}$ = ∑ (PHIPH + PHINP + PHINPR + PHIRDM + PHIADM)/n

Where:

PHIPH: physiological hormesis index of the plant height variable, NP: new plant, NPR: number of primary roots, RDM: root dry matter, ADM: aerial dry matter, n=number of variables. The total microbiological hormesis index (TMHI) represents the sum of the MHIMi *(x)* of mL^{-1} of microalgae cells, the CFU g^{-1} of fungi, actinomycetes, *Pseudomonas* bacteria, and hydrocarbonoclastic bacteria in the rhizosphere. It was calculated using the following formula TMHI = ∑ (MHIMa + MHTF + MHIACT + MHIPsB + MHIHB)/n

Results

Plant height and new plants

Analysis of variance of the PH and NP data of *E. palustris* showed highly significant statistical differences between the crude oil treatments from week 2 of the experimental cycle and was maintained for the next 36 weeks. In treatments without and with CO, the logarithmic phase of PH occurred between weeks 2 and 8; the linear phase between weeks 9 and 29, and the senescence or asymptotic phase and senescence, or asymptotic phase, started at week 30 and ended at week 38 (Fig. 1A). Values of PH were

higher in the treatments with CO. The highest plant height (94 to 95 cm) was recorded during weeks 30 to 38, it occurred in the plant exposed to 60 g / kg of CO, on the other hand, the lowest height (63 cm) in the same period corresponded to the plant established in soil without oil. It is interesting that the control plant had the lowest growth during the entire experimental cycle with respect to the six oil treatments. The percentage difference in height between the control plant and the largest, corresponding to the one that grew in soil with 60 g of oil, varied from 7.6% according to the measurement made at week 10, increased to 53% in it 23, and decreased slightly to 51% the last week, the 38. Data show that the *E. palustris* plant was tolerant to oil exposure during the 38 weeks, equivalent to nine months.

As in the PH, the increase of NP also showed means with highly significant differences and correlation values close to 1.0 (Fig. 1B). The growing trend of plant formation shows that this biological process of repopulating areas contaminated with up to 75 g of oil will not yet diminish. During the 38 weeks the vegetative multiplication of new organisms, typical of hydrophytic individuals belonging to the botanical family Cyperaceae, maintained a logarithmic trend in all the evaluated treatments, only the plant exposed to 60 mg of oil decreased the vegetative slope from week 36 (Fig. 1B). As in the altitude, the number of seedlings was higher in soils contaminated with the six doses of crude oil. During weeks 36 to 38, 297, 298 and 299 new plants were counted in soil contaminated with 60 g of oil. These data show the stabilization of vegetative growth, only one seedling increased per week. The smallest amounts were formed in the control soil, the data are 185, 189 and 193 plants in weeks 36 to 38. The increase was four seedlings per week, which also suggests unfavorable restrictions for the plant. The amount of NP was more sensitive than the height. The effect of oil caused a greater increase in plants during the 38 weeks. Again the 60 g dose promoted a greater number of new plants compared to the control treatment, the comparative results were 36% in week 10, increased to 79% in week 30 and decreased to 55% in week 38 (Fig. 1B).

Physiological hormesis indexes for plant height and number of new plants

The analysis of variance of the physiological hormesis index for PH (PHIPH) and NP (PHINP) of *E. palustris* showed statistically significant differences ($p \leq 0.05$) between the 222 treatments depending on time (37 times) and CO concentration. Plant height decreased during the first weeks of exposure to the six CO concentrations. The PHIPH <1 varied with the CO per week: weeks 2 to 10 with 3 g; weeks 3 and 4 with 15 g; week 2 with 30 g; weeks 2 to 5 with 60 g; weeks 2, 3,7 and 9 with 75 g. The PHIPH ≥1 after 10 week indicated an increase in PH when exposed to CO (Fig. 2A). The PHIPH increased with time and CO. The ranges of the indices between weeks 11 and 38 were 1.05 to 1.18 (3 g), 1.03 to 1.30 (15 g), 1.07 to 1.47 (30 g), 1.04 to 1.49 (45 g), 1.04 to 1.52 (60 g), and 1.02 at 1.35 (75 g). The highest PHIPH (1.50 to 1.52) was recorded between 33 and 38 weeks of exposure to 60 g of CO, which means an increase of 50 to 52% in PH, compared to the control plant.

The PHINP values <1 were recorded at different times with four petroleum concentrations. Between week 2 and 12 with 3 g of CO, from week 4 to 8 with 15 g, week 2 to 8 with 30 g, and from 2 to 6 with 75 g. PHINP ≥1 predominated in 176 the 222 treatments according to time and CO. According to the CO,

different stress response values were obtained during 2 to 38 weeks. A CO concentration of 3 g was associated with indices values between 1.01 and 1.12; 15 g with 1.02 to 1.21; 30 g with 1.14 to 1.78; 45 g with 1.03 to 1.68; 60 g with 1.21 to 1.78; 75 g with 1.06 to 1.53. The highest PHINP (1.75 to 1.78) was recorded at weeks 13, 21, 22, 23, 24, and 25 of exposure to 60 g CO (Fig. 2B). These values indicated that the presence of CO stimulated the multiplication of *E. palustris* in Gleysol soil contaminated with petroleum.

Physiological hormesis indexes for number of roots and dry matter accumulation

The analysis of variance of the PHI for NPR, RDM, and ADM of *E. palustris* showed highly significant differences ($p \leq 0.05$) between the treatments at 38 weeks. The PHINR, PHIRDM, and PHIADM were ≥1 at 38 weeks with the six CO concentrations. The treatment with 60 g showed the highest PHI for NPR and ADM, while the treatment with 75 g showed the highest PHI for RDM (Fig. 3), an increase of 138, 38, and 45% compared to the control, respectively. The highest value of the number of roots (298), ADM (13 g), and RDM (42.7 g) were recorded in the treatments with 60 and 75 g of CO, which indicates that these concentrations are not toxic for this species. The lowest value of NPR (192), ADM (9.5 g), and RDM (99g) were recorded in the control treatment. The general effect of CO on the growth and production of *E. palustris* was positive. The values of the TPHI were ≥1. The highest (1.63) was obtained with 60 g of CO, compared with 1.08, 1.23, 138, 1.31, and 1.46, which were obtained with 3, 15, 30, 45, and 75 g/k, respectively (Fig. 3). These values show that *E. palustris* has a positive stress response when growing in flooded soils contaminated with CO.

Microbiological hormesis indexes for rhizospheric microorganisms

The analysis of variance of the MHI for Mi, TF, ACT, PsB, and HB in the rhizosphere of *E. palustris* showed significant differences ($p \leq 0.05$) between the concentrations of 3, 15, 30, 45, 60 and 75 g of CO. The MHIMi, MHTF, MHIACT, MHIPsB, and MHIHB increased with the concentration of CO. The five microbial groups showed hormetic index values ≥1 (Figs. 4A and 4B). The microalgae population showed MHI values of 3.15 and 3.26 as a result of exposure to 60 and 75 g of CO, respectively; equals increase of 71.4 and 69.3%, compared to the control. The populations of TF and ACT showed MHI values greater than 3.1 and 1.4 in 60 g of CO, an increase of 67.9 and 73.5%, compared to the control, respectively. The population densities of PsB and HB increased by 83.3 and 99.4% in soil with 75 g of CO, compared to the control, with MHI values of 6 and 172.8, respectively. These results indicate that each of the five microbial groups adapted to and was stimulated by the presence of CO in the rhizosphere of *E. palustris* growing in flooded soil. This behavior was confirmed by the TMHI values ≥1 (2.7, 7.1, 5.9, 30, 31.8, and 37) in soil with 3, 15, 30, 45, 60, and 75 g of CO, respectively (Fig. 4B). The rhizosphere of *E. palustris* is a microcosm that, when exposed to CO, is capable of sustaining the growth of the five microbial groups evaluated in the present study.

Population densities of microorganisms according to remediation technology

The mean population of Mi, ACT, TF, *Pseudomonas* spp, and HB showed significant statistical differences (Duncan $p \leq 0.05$) as a result of the combined effect of CO, NA, and PR (Table 1). The treatment with 75 g of CO induced the highest populations of Mi ($94x10^3$ cell mL^{-1}), *Pseudomonas* spp ($64x10^2$ CFU g^{-1} rs), and HB (1885×10^2 CFU g^{-1} rs) in the rhizosphere of *E. palustris*, while the highest populations of ACT ($507x10^3$ CFU g^{-1}) and TF ($22x10^2$ CFU g^{-1}) were recorded in the treatments with 60 and 15 g of CO, respectively. These results suggest a positive effect of PR as a result of the stimulation effect that the presence of CO had on four microbial groups in the rhizosphere of *E. palustris* growing in Gleysol soil containing 60 and 75 g of CO. Population densities of TF were stimulated by the presence of CO only in soil with 15 g of CO (Table 1). Compared to NA, PR induced an increase of 53, 59, 58, 37, and 52% in the population of Mi, ACT, TF, *Pseudomonas* spp, and HB, respectively (Table 1).

Removal of total petroleum hydrocarbons

There were statistically significant differences (Duncan $\leq p0.05$) in the removal of TPH between treatments at 38 weeks. The highest percentage of removal (85%) was obtained in soil with 3 g of CO treated with NA, 16% higher compared to PR. The lowest percentage of removal (23%) was obtained in soil with 75 g treated with PR (Fig. 5A). PR removed 71, 72, 56, 42, 33, and 23% of TPH in soil with 3, 15, 30, 45, 60, and 75 g of CO, respectively. PR was 16, 3, 24, 23, 41, and 38% less efficient than NA in the decontamination of Gleysol soil contaminated with CO. In soil containing between 3 and 75 g of CO, PR removed 21.7% less TPH than NA (Fig. 5B). The removal of crude oil registered negative correlations between concentration and microorganism: Mi = -0.341**, ACT=- 0.265**, PsB = -0.624**, and HCB = -0.775**. The ratio between organic carbon and total nitrogen and sulfate was negative (SOC/TN= -0.409** and SOC/SO_4^{-2}= -0.466) (Table 2). These results suggest that the SOC of biogenic origin and the SOC derived from petroleum were mineralized at the same time as the TPH were degraded. This process made TN and sulfate aerobically available to plants and microorganisms.

Discussion

TPH accumulated in Gleysol contain complex recalcitrant chemical structures with low water solubility. They remain for a long time absorbed in the soil and organic matter (Pravecek et al. 2005; Jacques et al. 2007). TPH change the organic, macro and micronutrient carbon content (Caravaca and Roldán 2003; Shukry et al. 2013) of the soil and its biological balance, they also reduce or increase plant growth according to the plant species (Shirdam et al. 2008; Gbadebo and Adenuga 2012; González-Moscoso et al. 2019; Orocio-Carrillo et al. 2020).

The results of the present study indicate that *E. palustris* adapts to grow in artificial wetland soil containing between 3 and 75 g/kg of CO. The variables NP and PH showed a linear trend from 2 to 37 weeks. *E. palustris* has three growth phases, a logarithmic one, a linear one, and a senescent one (Figs. 1A and 1B). These phases are similar to those of plants that grow in environments without stress, as reported by Salisbury and Ros (1994); Hausen (2006); Ogle et al. (2012). Rivera-Cruz et al. (2016)

reported that *E. palustris* covers large areas of wetland soil. It adapts to unfavorable conditions induced by man in soil and water.

Hormesis is a positive response of some living organisms to soil contamination (Calabrese 2013). This is shown by the results of the present study, with PHI values ≥1 for the PH and NP of *E. palustris* at the 37 measurement times. The 60 g TPH dose increased PH and NP by 34 and 43.8% respectively. Similar results were reported by Cedergreen et al. (2007), who found a hermetic response of 13% in the aquatic plant *Lemna minor* when exposed to herbicide. Orocio-Carrillo et al. (2019) reported a hermetic response of 7.4% in the stolon length of *Leerxia hexandra* when exposed to 7.9 g TPH.

The increased growth of *E. palustris* when exposed to crude oil in wetland clay soil shows its sustainability under stress. Cabrese and Blain (2009) reported that a hormetic response of the physiological variables of plants is crucial for the sustainability of the associated ecosystem. According to Jia et al. (2015) and Azcón-Bieto et al. (2013), the hormetic response of the stems and new plants of *E. palustris* is due to the improved photosynthetic activity of the adapted plant, which stimulates plant development. Fu and Huang (2001) explained that the mechanisms of the antioxidant system in the aerial shoots of these plants evolved together with their aerobic metabolism to counteract oxidative stress and avoid generating reactive oxygen species (ROS), which can induce cell death. ROS such as superoxide dismutase (SOD) convert O_2 to H_2O_2, and Catalase (CAT) and peroxidase trigger the conversion of H_2O_2 and O_2 (Jeffrey 2002).

The root support system of plants under stress due to contamination with CO shows morphological characteristics of adaptation and hormesis. Root hormesis influences root length, number of roots, surface area, and/or root volume, improving water balance and nutrient access (Arduini et al., 1996, Jia et al., 2015). The NPR and RDM of *E. palustris* increase in soils containing between 3 and 75 g of CO, with PHI values ≥1. According to Lynch et al. (2012), the vigorous growth of primary and secondary roots and the production of root dry matter are observable primary adaptations to stress. Exposure of *L. hexandra* to doses of 238 g/kg of CO increased the production of root dry matter by 444%, compared to the control, generating a J-shaped dose-response curve (Orocio-Carrillo et al. 2019).

The hormetic response of the roots of *E. palustris* at 38 week, when exposed to CO, is possibly influenced by the high content of organic carbon that characterizes Gleysol contaminated with petroleum. Segura (2008) indicated that the aromatic cytokinin 6-amino purine stimulates cell division. This hormone comes from the degradation of organic material and plant synthesis. Erichert and Fernandez (2012) reported that cytokinin synthesis is activated in plants by stress. The meristem at the base of the root activates genetic signals for the biosynthesis of higher amounts of phytohormones and cytokinins. Likewise, according to Escaso et al. (2010), auxins are hormones that are activated under stress conditions and promote cell expansion and division in the apical meristem of young plants, as well as root elongation.

The rise of the radical system can also be explained by plant-microbe interactions. The results of the present study indicate positive relationship between RDM and Mi (0.926**), TF (0.633**), PsB (0.457**),

and HCB (0.395**) (Tables 2). Rhizospheric microorganisms are plant growth promoters that influence root growth by providing nutrients and phytohormones, and inhibiting pathogens (Dutta and Podile 2010; Marschner 2012). They provide resistance to biotic and abiotic stress due to their ability to solubilize phosphate, produce 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), biosurfactants, phytohormones, and exopolysaccharides, fix biological nitrogen, produce siderophore and volatile compounds, and express various stress-related genes (Ratogi et al. 2021).

The PHIADM values of *E. palustris* were ≥1 when exposed to 3, 15, 30, 45, 60, and 75 g of CO in artificial wetland soil. This response differs from that of other plants that inhabit the wetland. González-Moscoso et al. (2017) reported that in Gleysol with 90 g/kg of CO, the ADM of *L. hexandra* decreased by 16% compared to the control. Alambary et al. (2019) reported that the dry matter of *L. octovalvis* decreased when growing on drilling mud with 14700 mg/kg of TPH. Almansoory et al. (2020) reported that the dry matter of *L. octovalvis* increased by 66%, compared to the control, after 42 days of exposure to 2 g/kg of gasoline. The toxicity response of foliage to the presence of petroleum has been explained by the alteration of stomatal conductance, which regulates the rate of diffusion of carbon dioxide in the leaves for photosynthesis, and by loss of water through transpiration (Rebetze et al. 2000).

We assumed that the increase in ADM of *E. palustris* was due to the positive relationship between RDM (0.976**) and rhizospheric microbial activity that was found in the present study. This is because the density of roots and long root hairs is an important factor in the absorption of nutrients supplied by diffusion. There is a linear relationship between root density and the absorption rate of nutrients (Marschner and Rengel 2015). The growth of the root system increases the number of sites that can be colonized by microorganisms that provide nutrients for the cellular metabolism of the plant (Neumann and Römheld 2015). In this regard, ADM is positively related to the population of Mi (0.638**), TF (0.628 **), ACT (0.801 **), PsB (0.353 *) and HCB (0.351 *) (Table 2).

Under anaerobic conditions and stress an CO, roots the plant wetland habitats provide the rhizosphere oxygen, exudates rich in amino acids, organic acids, and carbohydrates (Visser et al. 2000; Dagher et al. 2019). Microalgae provide nitrogen and sulfate, while fungi provide water-soluble carbon. Actinomycetes and bacteria help in the fixation of Nitrogen, the degradation of carbon of biogenic and petroleum origin, and the mineralization of essential nutrients (Pepper and Gentry 2015). In petroleum-contaminated soil, this contributes to the mineralization of organic carbon, making it less toxic and providing sulfate for cellular metabolism. There is positive relationship of ADM*SOC/SO_4^{-2} = 0.371** (Table 2).

In an environment flooded, the rhizosphere of *E. palustris* is associated with low oxygen concentration and high CO_2 content (Mitsch and Gosselink 2015). The rhizosphere of *E. palustris* was colonized by a large population of Mi, ACT, HF, *Pseudomonas* spp, and HB during 38 week when exposed to 60 and 75 g of TPH, compared to soil without plants. Similar results were reported by Rivera-Cruz et al. (2016) for N-fixing bacteria. They found 94 x10^2 CFU g^{-1} in the rhizosphere of *E. palustris* contaminated with 92 g of TPH. González-Moscoso et al. (2019) reported an increase in CFU of HB (121x10^4) in the rhizosphere of *L. hexandra* growing in soil with 76 g of TPH. Rodríguez Uribe et al. (2021) reported a higher population

of *Pseudomonas* spp and *Rhodococcus* spp in rhizosphere of *E. palustris* exposed to 51 g of TPH, compared to soil without plants. George et al. (2012) indicated that these microbial groups are facultative anaerobes that use electron acceptors for respiration of compounds other than O_2 such as H, NO_3, Mn^2, Fe^2, and H_2S, and for reducing CO_2 to CH_4, which allows them to colonize these environments.

According to Maier and Gentry (2015), the establishment and colonization of microorganisms in soil with TPH is explained by the induction of genetic changes in them. These include genetic mutations or transferences that generate new metabolic capacities. The petroleum-induced stimulation of these five microbial groups associated with *E. palustris* was confirmed in the present study by the TMHI values ≥1 that were obtained when the plants were exposed to 3, 15, 30, 45, 60, and 75 g/kg of CO (Fig. 4). These values indicate that CO induced an increase in the population of these microbial populations associated with the rhizosphere of *E. palustris*. This phenomenon is probably associated with the surface of the roots of wetland plants since the root surface provides a habitat for microorganisms thanks to the physicochemical properties of the roots, their secretion of oxygen and exudates (Tordera et al. 2020), their physiological development, and supply of nutrients (Marschner, 2012).

The efficiency shown in the present study by NA and PR in the elimination of total petroleum hydrocarbons under anaerobic conditions contrasts with the results of other studies carried in which the removal of TPH required more than one years (Rivera-Cruz et al. 2016; Afza et al. 2019). The 21.7% higher efficiency of NA compared to PR when using *E. palustris* for the removal of TPH in the wetland coincides with the results of González-Moscoso et al. (2019), who reported that NA was 15.8% more efficient than PR in the removal of TPH after nine months. Removal efficiency depends on several processes: photooxidation, evaporation, volatilization, and biodegradation by autochthonous microorganisms (Abu and Diki 2008).

In the wetlands, the water surface hosts autochthonous microorganisms that have been shown to degrade hydrocarbons to a greater percentage than biostimulation (Fodelianakis et al. 2015). Carbon oxidation by microorganisms has been shown to be associated with the availability of nitrogen and sulfate (Brady and Weil 2008). The present study found a negative relationship between SOC/TN and SOC/SO_4^{-2} (Table 2). NA induced by microbial activity (Gerba and Pepper 2015) in wetland soil was not favored by Mi, ACT, *Pseudomonas* spp, and HB (Tables 1 and 2). It is possible that other microbial groups that mineralize nitrogen and sulfate were also established in the same wetland soil favoring the degradation of CO.

Phytoremediation removed only 49.6% of the TPH found in Gleysol contaminated with 3-75 g of petroleum at 38 week. Various authors have reported that PR is a non-destructive technology in wetland that requires a long time to decontaminate soil, depending on the concentration of crude oil (Marcek et al. 2000; Lee et al. 2015; Shehzadi et al. 2015; Policarpo et al. 2020). Different wetland plants have shown to be able to remove TPH from soil: *Scirpus grossus* removed 66.6% of TPH in soil contaminated with 0.25% diesel-water; *Typha domingensis*, *Phragmites australis*, *Leptochloa fusca*, and *Brachiaria mutica* removed 99.13% of hydrocarbons in wetland soil contaminated with 319 mg/L of petroleum at month 18

(Almansoory et al. 2017; Alanbary et al. 2019). These authors argue that the use of PR in contaminated wetlands stabilizes the petroleum through the influence of the abundant root systems, retaining and enveloping hydrocarbons, which in turn favors the establishment of microorganisms with CO metabolic capacity that can use carbon of biogenic and crude oil (Atlas and Bartha 2002; Cai et al. 2010; Rivera-Cruz et al. 2016).

Conclusions

Eleocharis palustris cyperaceae, a plant native to wetlands, was exposed to crude oil contamination for 38 weeks. A hormetic effect was observed in all the physiological variables evaluated from week 2 to 38.

The production and microorganisms evaluated at week 38 showed a hormesis index ≥1 to oil.

Plant height and new plant production showed an increase at most of the 37 evaluation time points, with statistically significant differences due to the effect of petroleum concentration. The number of primary roots, root dry matter and aerial dry matter increased up to 138, 38 and 45% in plants exposed to 75 g of crude oil compared to the control. These results were associated with high concentrations of petroleum, indicating a hormetic effect. The same trend was observed in the rhizospheric population of microorganisms, with 75 g of petroleum yielding hormetic index values ≥1 for microalgae, total fungi, actinomycetes, *Pseudomonas* bacteria, and hydrocarbonoclastic bacteria. The amount of microalgae, *Pseudomonas* bacteria and hydrocarbonoclastic bacteria increased by 69.3, 83.3 and 99.4% when the rhizosphere of *E. palustris* was exposed to 75 g of oil, while the amount of total fungi and actinomycetes increased by 67.9 and 73.5% when exposed to 60 g of oil. The population of the five microbial groups under study increased in the rhizospheric system of *E. palustris* used for phytoremediation.

The removal of petroleum was greater in soil subjected to natural attenuation. In soil contaminated with 3 g of petroleum, phytoremediation showed a negative relationship between TPH removal and petroleum concentration. At week 38, the effect of phytoremediation on petroleum removal was lower than with natural attenuation. The correlation between the removal of total petroleum hydrocarbons and the population of microalgae, actinomycetes, *Pseudomonas* bacteria, and hydrocarbonoclastic bacteria was highly significant and negative.

The hormetic effect induced by petroleum in the physiological and microbiological variables of the rhizosphere of *E. palustris*, as well as the removal of TPH stimulated by it, support the potential of this Cyperaceae as a biological technology for the remediation of contaminated wetlands in the Mexican humid tropics.

Declarations

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Compliance with ethical standards

Conflict of interest.The authors declare that they have no conflict of interest.

Ethical approval.This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Population densities of five microbial groups isolated in week 38 from soils contaminated with seven concentrations of crude oil treated with two technologies during May 2020 to January 2021.

Crude oil (g/kg)/ Technology	Microalgae	Actinomycetes	Fungi	*Pseudomonas* spp	Hydrocarbonoclastic bacteria
	(10^3 cell mL^{-1})	(10^3 CFU g^{-1})		(10^2 CFU g^{-1})	
Natural attenuation					
0	26fgh	492abc	11b	17c	53c
3	18fgh	451c	4df	11c	59c
15	15h	18g	9bc	11c	20c
30	40de	40g	8cd	16c	20c
45	48cd	204c	6de	13c	150c
60	33ef	105f	4fg	24b	864b
75	30def	33g	2g	15c	1426ab
Phytoremediation					
0	23gh	372d	10b	11c	10c
3	73b	492abc	8cd	13c	74c
15	73b	475abc	22a*	14c	32c
30	60bc	401d	12cd	16c	253c
45	55c	462cd	10c	26b	1572a*
60	69b	507a*	12b	23c	1629a*
75	94a*	475abc	13b	64a*	1885a*
Natural attenuation	30B	187B	5B	15B	370B
Phytoremediation	64A*	455A*	12A*	24A*	779A*

*Different letters in columns are statistically different (Duncan $p \leq 0.05$, n = 9, a>b).

Table 2. Correlation coefficients between total petroleum hydrocarbons, soil variables and plant variables.

	REM	SOC	SOC/TN	SOC/SO	RDM	ADM	Mi	TF	ACT	PsB	HB
CO	-.814**	.827**	.610**	-.752**	ns	Ns	.266*	ns	-.272*	.609**	.768**
PR	-.334*	.311*	ns	-.460**	.973**	-.976**	.636**	.612**	.822**	.368*	.320*
REM		-.813**	-.409**	-.466**	-.400**	-.369**	-.341*	ns	-.265*	-.624**	-.791**
SOC			.617**	.470**	.389**	.342*	.409**	ns	ns	.743**	.775**
SOC/TN				-.472**	ns	ns	ns	ns	-.346*	.658**	.413**
SOC/SO					.362*	.371**	ns	ns	.474**	-.319*	-.438**
RDM						.976**	.926**	.633**	.807**	.457**	.395**
ADM							.638**	.628**	.801**	.353*	.351*
Mi								.448**	.465**	.410**	.359*
TF									.461**	ns	ns
ACT										ns	ns
PsB											.671**

CO crude oil, PR phytoremediation, REM removal TPH, SOC soil organic carbon, TN total nitrogen, SO sulphate, RDM root dry matter, ADM aerial dry matter, Mi microalgae, TF total fungi, ACT actinomycetes, PsB *Pseudomonas* bacteria, HB hydrocarbonoclastic bacteria.

*Correlation with significant statistical difference ($p \leq 0.05$).

**Correlation with highly significant statistical difference ($p \leq 0.01$).

Figures

Figure 1

Weekly average height of plant (A) and new plants (B) of *Eleocharis palustris* exposed to seven concentrations of crude oil during May 2020 to January 2021. *Treatment means with statistical significant differences (Duncan $p \leq 0.05$, n = 4, a> b).

Figure 2

Weekly values of the Physiological Hormesis Index (PHI) due to the effect of exposure to seven concentrations of crude oil on the growth of Eleocharis palustris. Plant height (A) and new plants (B). *Treatment means with statistical significant differences (Duncan $p \leq 0.05$, n = 4, a> b). PHI ≥ 1 indicates hormesis.

Figure 3

Physiological hormesis index for number of primary roots (NPR), root dry matter (RDM), aerial dry matter (ADM), and total (TPHI) of Eleocharis palustris exposed to seven concentrations of crude oil during May 2020 to January 2021. *Different letters in columns of the same color indicate significant differences at the 5% level according to the LSD test (Duncan $p \leq 0.05$, n = 4, a>b).

Figure 4

Microbiological hormesis index for populations of microalgae (Mi), fungi (F), actinomycetes (ACT), and Pseudomonas spp (PsB) (A), and hydrocarbonoclastic bacteria (HB) (B). Total microbiological hormetic index (TMHI) (B) for the rhizosphere of Eleocharis palustris. exposed to seven concentrations of crude oil during May 2020 to January 2021. *Different letters in columns of the same color indicate significant differences at the 5% level according to the LSD test (Duncan $p \leq 0.05$, n = 4, a>b).

Figure 5

Removal of total petroleum hydrocarbons from soil contaminated during May 2020 to January 2021. A) Effect of crude oil concentration, and B) Technology type effect. *Different letters in columns of the same

color indicate significant differences at the 5% level according to the LSD test (Duncan $p \leq 0.05$, n = 4, a>b).