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Phytoremediation of soils contaminated with crude and weathered oil using two rice varieties (*Oryza sativa* L.)

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ABSTRACT

Objective: To evaluate the potential for soil phytoremediation with new and weathered hydrocarbons with two rice varieties (*Oryza sativa* L.).

Materials and Methods: The assessed treatments were 150 mg kg^{-1} (control soil), 30,000; 60,000 and 90,000 mg kg^{-1} of new oil and 79,457 mg kg^{-1} of weathered oil 1 and 42,000 of mg kg^{-1} of weathered oil 2; they were established in a completely randomized design with 6×3 factorial, with four repetitions each. The evaluated variables were populations of total bacteria (colony forming units CFU per grams of dry soil), free-living nitrogen-fixing bacteria (CFU), total fungi (CFU), and total dry biomass (g). Total bacteria and fungi were quantified at the beginning of the experiment at 90 and 145 days.

Results: The highest total petroleum hydrocarbons (HTP) degradation was 73 and 72% in the 79,457 and 42,000 mg kg^{-1} concentrations of weathered HTP 1 and 2, in the rhizosphere of rice silver line 21. The total dry biomass reported significant differences ($p \leq 0.05$), evidencing a lower effect in the 60,000 mg kg^{-1} concentration in new oil, which caused a 33% reduction compared to the control.

Results/Conclusions: The rice variety line 21 has a greater potential to phytoremediate soils contaminated with crude and weathered oil in field conditions in tropical areas.

Keywords: Microorganism, Biodegradation, Petroleum hydrocarbons, Rice

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INTRODUCTION

Organic pollutants such as total petroleum hydrocarbons (TPHs) are currently one of the most far-reaching pollutants in terrestrial and aquatic ecosystems and for human health (Wu *et al.*, 2017; Yu *et al.*, 2018a; Haider *et al.*, 2021; Zuzolo *et al.*, 2021). Therefore, the remediation of soils contaminated with TPHs is a global challenge; consequently, in recent years various technologies have been developed to solve this problem (Ossai *et al.*, 2020). Physical and chemical methods can offer quick and fast solutions for decontamination, but require a large amount of material, heavy tools, and labor (Hussain *et al.*, 2019; Xia *et al.*, 2020). However, these methods tend to be costly and harmful to the environment (Alkani

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et al., 2020). Biological measures have recently been preferred over chemical and physical techniques for soil remediation, due to their low cost and their ability to prevent pollutants accumulation (Bonnier *et al.*, 1980; El-Nawawy *et al.*, 1987). Research has shown that some plants are capable of remediating crude oil-contaminated soils by phytoremediation (Haider *et al.*, 2021; Ostovar *et al.*, 2021; Zuzolo *et al.*, 2021). Phytoremediation involves using plants to remove, transfer, stabilize, and / or degrade contaminants from soil, sediments, and water (Xie *et al.*, 2017; Rajaei & Seyedi 2018; Nero *et al.*, 2021). Phytoremediation is a low-cost passive method with the potential to treat toxic organic and inorganic contaminants (Jeelani *et al.*, 2017; Omara *et al.*, 2019). The objective of this research was to assess and compare the potential of two varieties of rice (*Oryza sativa* L.) to phytoremediate soils contaminated with hydrocarbons from new and weathered oil.

MATERIALS AND METHODS

Study area and soil collection

Soils were collected from the surface horizon (0-30 cm), in two sites with the same pedogenetic characteristics (Gleysols). Site one, control soil (150 mg kg^{-1} biogenic origin hydrocarbons) was collected at the Arroyo Hondo - Santa Teresa ejido, Coatzacoalcos - Cárdenas highway, Tabasco, Mexico (17° 59' 52.45" N - 93° 24' 56.58" W). Site 2, located at ejido José Narciso Rovirosa, two km southeast of the La Venta Gas Processing Complex, Huimanguillo, State of Tabasco, Mexico (18° 04' 54" N - 94° 02' 31" W). Site two is an area affected by hydrocarbon spills from 1980 to 2005, due to the pipeline's ruptures. At the time of sampling, a 79,457 mg kg^{-1} of concentration was found, dry base (weathered oil 1). In 2004 it was restored with physical-chemical processes. At the end of the restoration process, the TPHs concentrations were quantified by gravimetry, and concentrations of 42,000 mg kg^{-1} of TPHs (weathered oil 2) were recorded (Rivera-Cruz *et al.*, 2016; Arias-Trinidad *et al.*, 2017). In each one of the sites, 200 kg^{-1} of soil was collected from the surface horizon (0-30 cm) using a punctual sampling. All physical and chemical parameters were determined following the methods on the norm NOM-021-RECNAT (2002) (Table 1).

Total oil hydrocarbon analysis

The TPHs content in the collected samples was extracted with soxhlet equipment using 99.5% purity dichlomethane solvent (Sigma-Aldrich®), following the EPA 3540B method (United States Environmental Protection Agency [USEPA], 1994). The pH of the

Table 1. Physical and chemical characteristics of the three evaluated soils.

Suelo	**pH %**	**M**	**N**	**P**	**K**	**CIC Cmol$^{(+)}$ kg^{-1}**	**Arcilla**	**Limo**	**Arena**	**Textura**
		mg kg^{-1}					**%**			
Suelo 1	6.3	5.6	0.44	27.90	0.43	42.14	61	29	10	Arcillosa
Suelo 2	4.2	25.8	ND	3.58	0.35	43.50	48	33	19	Franco
Suelo 3	4.2	6.0	0.25	7.60	0.19	20.59	31	19	50	Migajón-arenosa

M=Organic Matter, N=inorganic nitrogen, P=phosphorus, K=potassium, CIC=Cation exchange capacity, ND=Not determined.

samples was adjusted to 2.0 with concentrated HCl and subsequently dried with $MgSO_4$. The solvent was then evaporated using a rotary evaporator, the extract was quantified by gravimetry (g kg^{-1}) in a semi-analytical balance (Sartorius, Analytic Model AC 210S, Illinois, USA) following the NMX-AA-134-SCFI-2006 method (DOF, 2006).

Experiment Establishment

The seeds of *Oryza sativa* L. (Criollo canelo variety and line 21) were collected at the municipality of Comalcalco, Tabasco, Mexico and in the INIFAP Huimanguillo Experimental Field (Jiménez, 2003), which showed 95% germination. The initial contractions of crude and weathered oil were 150 (hydrocarbons of biogenic origin), 30,000, 60,000, 90,000 mg kg^{-1} and 79,457 and 42,000 mg kg^{-1} dry base TPHs. The experimental units were set in glass containers 18 cm high and 14 cm in diameter with 1400 g of soil and one *O. sativa* plant per container (15 cm high and 18 days after emergence). The experimental units were irrigated with distilled water to maintain humidity at 80% field capacity. Sowing was carried out under similar controlled conditions for 145 days. The mean annual temperature was 26 °C and the mean annual precipitation was 2,200 mm (CONAGUA, 2014).

Microorganisms evaluation

The populations of total batteries (TB), free-living nitrogen fixers (FLNF), and total fungi (TF), were evaluated at the beginning (day 1), 90, and 145 days after the experiment was established. These bacterial groups were assessed due to their importance in the recycling of nutrients and for their contribution to the degradation of hydrocarbons. The microbiological analysis was done with the plate dilution and counting method (Madigan *et al.*, 2009), using specific culture media for total bacteria (nutritive agar, Baker®), the FLNF were quantified using the medium proposed by Rennie (1981) and for total fungus, the Potato Dextrose Agar medium (PDA, Baker®) was used.

Growth variables evaluation

After 145 days, the plants were evaluated and harvested; the height of the plant was measured (the height in cm from the base of the stem to the apex of the youngest leaf, leaf zero), the radical, aerial, and total dry biomass. The leaves, stems, and roots were dried in an oven (FELISA, Model 242-A, Mexico City) at 65 °C for 72 h until a constant weight was obtained to determine their total dry biomass.

Experimental design and statistical analysis

The experiment had a 6×3 factorial design (six concentrations and three types of hydrocarbons), resulting in 18 treatments with four repetitions each, a total of 72 experimental units distributed in a completely randomized experimental design. The considered factors were the presence of fuel oil in the soil (0, 30,000, 60,000, 90,000 mg kg^{-1} TPHs, 79,457 mg kg^{-1} TPHs 1, and 42,000 mg kg^{-1} TPHs 2) with and without established plant species (Creole canelovarietie and line 21 rice). The data were subjected to an analysis of variance and an LSD type mean comparison test (Tukey $p \leq 0.05$).

RESULTS AND DISCUSSION

Effect on the plant total dry biomass

The present concentrations of fresh and weathered hydrocarbon in the soil significantly reduced the growth and development of the two varieties of O. sativa (Criollo canelo rice and Line 21 rice). The height of the plant was statistically different ($p < 0.05$) between treatments (Figure 1A and 1B). The highest height of the two O. sativa species was obtained in the control soil (150 mg kg-1); on the contrary, the weathered soil 2 with the 90,000 mg kg-1 concentration of new TPH, showed the greatest reduction (23 and 26 %) in both varieties (Figure 1A and 1B).

The aerial, root, and total dry biomass showed significant statistical differences ($p \leq 0.05$) between treatments (Figure 2). In the case of Criollo canelo cultivar, the highest root dry biomass (12 and 10 g) was recorded in the control and the 60,000 mg kg^{-1} concentration; on the contrary, the 42,000 mg kg^{-1} concentration of weathered 2 evidenced the greatest reduction in the root biomass (50%) (Figure 2A). Regard the rice line 21 established in the

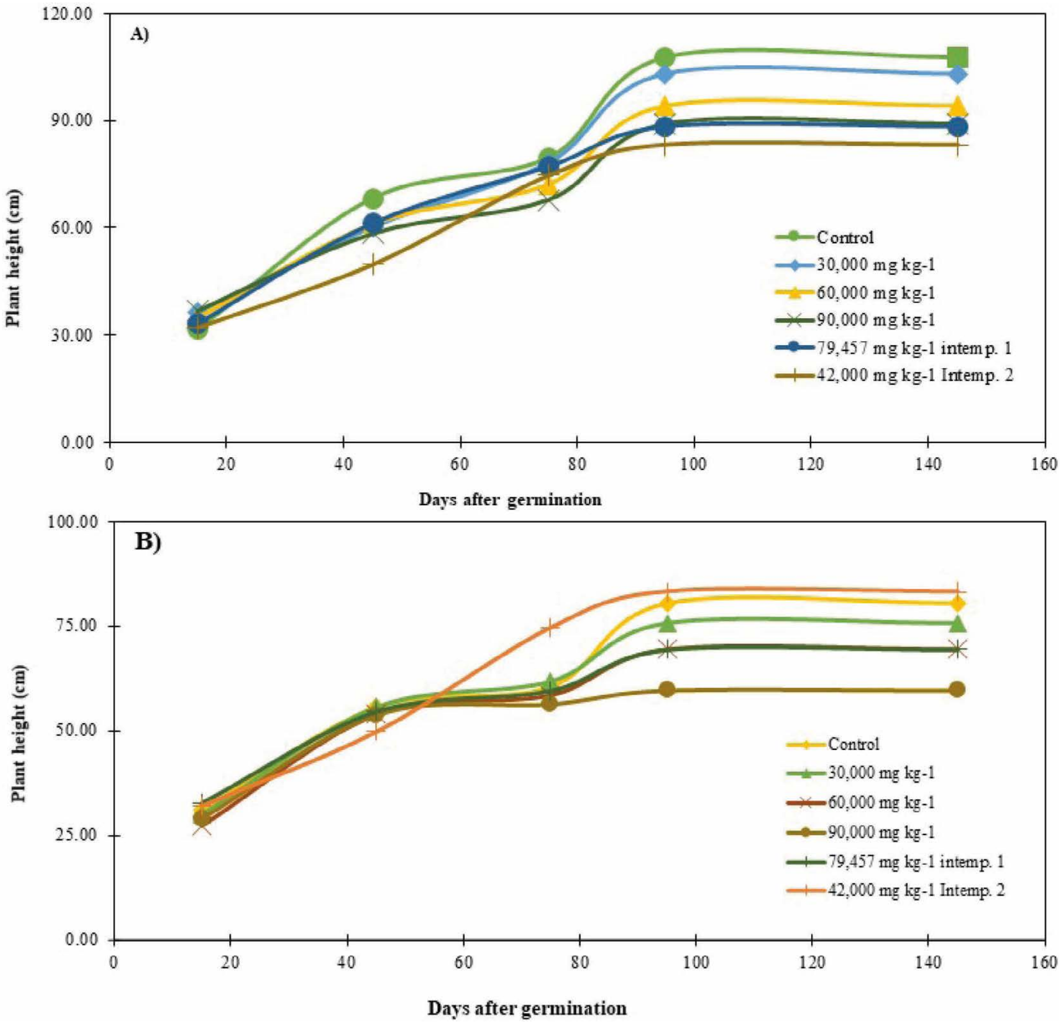

Figure 1. Height of the Criollo canelo rice plant and rice line 21 through time in different concentrations of crude and weathered oil.

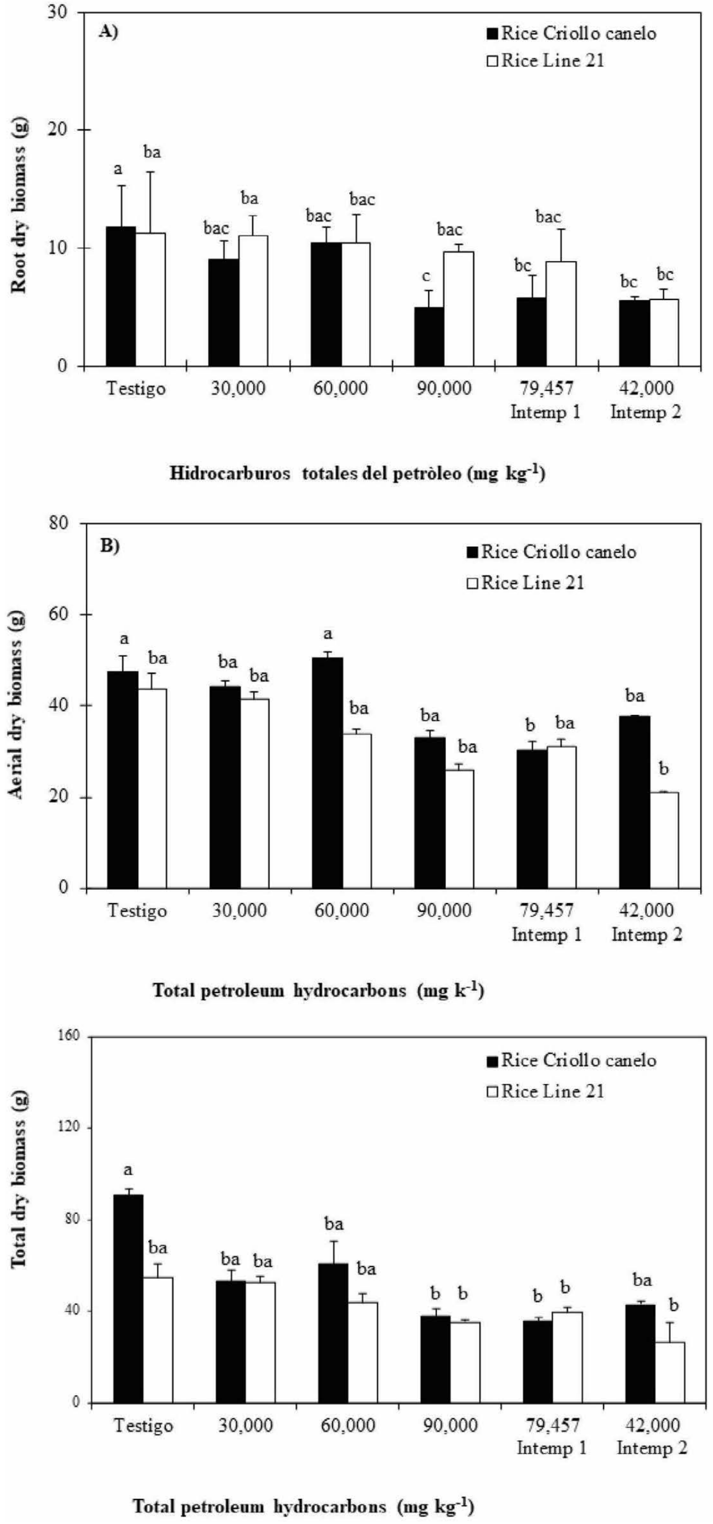

Figure 2. Effect of the concentration on the aerial dry biomass, root and total dry biomass of Criollo canelo and line 21 rice (*Oryza sativa* L.) through time in the different concentrations of crude and weathered oil. Bars with the same letters are statistically equal ($p \leq 0.05$).

control soil and the 30,000 mg kg^{-1} concentration showed the highest radical biomass (11g); on the contrary, when increasing the concentrations of petroleum hydrocarbon, the dry biomass decreased in the 2, 7, 12 and 16% compared to the control and at the lowest concentration (Figure 2B). The aerial dry biomass showed similar results to the root dry biomass which shows the tendency to increase the concentration in both rice varieties (Figure 2B). The highest total dry biomass was obtained in the Criollo canelo in the control soil and the 60,000 mg kg^{-1} concentration new TPH (91 and 63 g); compared to the rest of the treatments, which showed a maximum 33% reduction in the 30,000 mg kg^{-1} concentration of new TPH (Figure 2C). Regard line 21 rice, showed a trend like that presented by the Criollo canelo, reporting the highest total dry biomass in the control soil (55 g). On the contrary, a 42,000 mg kg^{-1} concentration of weathered TPH reduced the total dry biomass by 51% (Figure 2C).

In general, the negative effect of TPH and TPH 1, observed in the physiological variables (height, and root, aerial and total dry biomass) is attributed to the inhibition caused by the increase in the different concentrations of hydrocarbons in the soil, which suppress the nutrient's availability (Cartmill *et al.*, 2014; Ruley *et al.*, 2019; Omara *et al.*, 2020) and delay the water absorption by plants (Wang *et al.*, 2013; Ruley *et al.*, 2020; Deebika *et al.*, 2021). Likewise, oil hydrocarbons alter the physical properties of soils such as permeability, in addition to suffocating roots, especially in fine-textured (clay) or shallow soils, affecting plant growth (Akinwumi *et al.*, 2014; Grifoni *et al.*, 2020; Ostovar *et al.*, 2021). Reynoso-Cuevas *et al.* (2008) observed root damage and root length decrease in different species as the hydrocarbon's concentration increased.

Total bacterial populations in soil with new and weathered oil

The total bacterial population shows that the total hydrocarbons of crude and weathered oil (TPH) did not cause an effect at the beginning (day 1), nor at 90 days ($p \leq 0.085$, $p \leq 0.192$). On the contrary, at 145 days highly significant differences were observed ($p < 0.05$) (Figure 3). At the beginning of the experiment (time zero), the 60,0000 and 90,000 mg kg^{-1} concentrations stimulated the highest TB population in the rhizosphere of the Criollo canelo rice followed by that in line 21 rice (2.33×10^{-3} and 1.85×10^{-3} CFU), for the control without plants (Figure 3A). However, at 90 days, the maximum TB populations (1.2×10^{8} to 4.5×10^{9} CFU g^{-1}) were recorded at the 30,000 mg kg^{-1} concentration (Figure 1B) in the rhizosphere of Criollo canelo rice (Figure 3B). After 145 days, TB populations decrease two orders of magnitude (2.98×10^{6}, 1.3×10^{6} and 5.9×10^{6} CFU g^{-1}) both in the soil without plants and in the two plant species (Figure 3C).

The populations of Free-living Atmospheric N Fixing bacteria (FLNFB) showed highly significant differences between treatments ($p \leq 0.05$). At time zero, the interaction of line 21 rice and the 30,000 mg kg^{-1} and 79,457 concentrations of weathered 1 stimulated the largest population of FLNFB (9.52×10^{2}, and 9.20×10^{2} CFU), compared to the soil without plants and to the Criollo canelo rice (Figure 3 D). At 90 days after the experiment was established, the interaction in the rhizosphere of line 21 rice and the 42,000 Weathered 2 concentration stimulated the maximum population of FLNFB (4.36×10^{8} CFU) (Figure

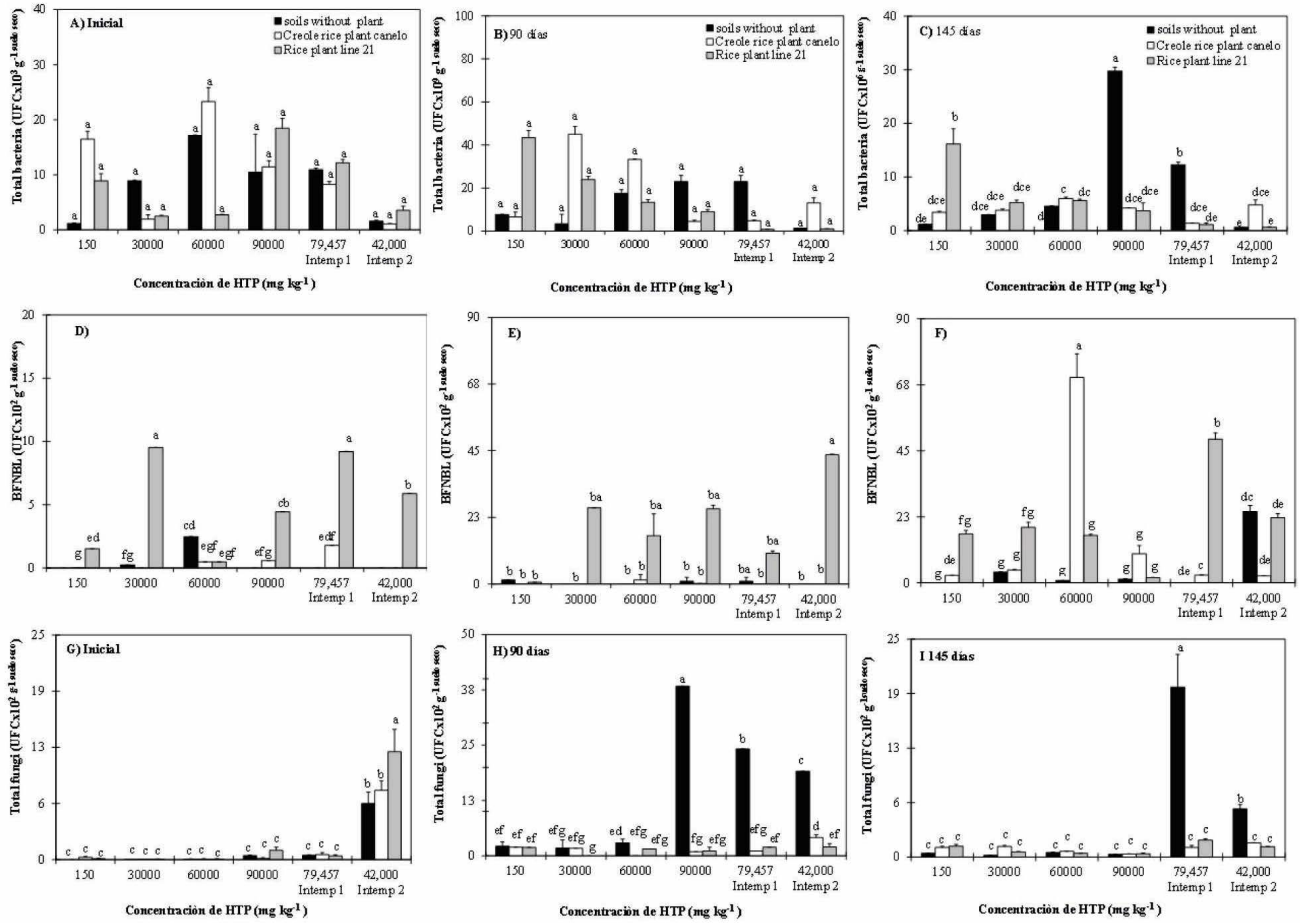

Figure 3. Kinetics of bacterial and total fungal populations in soil without plant, in soil with Criollo canelo rice plant and in soil with rice plant line 21 exposed for 145 days to soils with different concentrations of new, weathered 1 and weathered 2 crude oils (restored soil). Columns with the same letter within each time are statistically equal ($p \leq 0.05$).

3E). Population which decreased two orders of magnitude by days145 in the soil without plants as in the soil with plants (Figure 3F).

For the total fungi, significant differences ($p \leq 0.001$) between treatments were presented (Time zero, 90 and 145) (Figure G, H and I). The 42,000 weathered TPH 2 concentration presented the highest population of HT (1.2×10^3) in rhizospheric soil of line 21 rice plants on day zero (Figure 3A). On the contrary, at 90 and 145 days, the soil without plants showed the highest fungi population in the 90,000 mg kg^{-1} and 42,000 weathered 2 concentrations compared to the soil with plants (2×10^3 and 6×10^2) (Figure 3H and I).

Overall, the obtained results in the research suggest that fresh and weathered hydrocarbons presented a selective effect on soil microorganisms, favoring those with the ability to degrade or use petroleum hydrocarbons as a carbon source and energy (Freedman, 1989; Alexander, 1994; Alarcón *et al.*, 2019). In this regard, Rovina and McDougall (1967), Soleimani *et al.* (2001), and White *et al.* (2006) mention that plants, in general, can promote microbial activity through the release of organic compounds in the radical systems (amino acids, organic acids, sugars, enzymes and carbohydrates). In this sense, Bordoloi *et al.* (2012), Cartmill *et al.* (2014), and Xie *et al.* (2017) indicate that Pomacea, due to their dense root systems stimulates the bacterial populations involved in degrading petroleum hydrocarbons (González-Moscoso *et al.*, 2019). On the contrary, Rodhes and Hendricks (1990) mention that high concentrations of oil inhibit the growth of populations and the diversity of microbial communities. Chikere *et al.* (2009) indicate that the reduction of bacterial populations is an adaptive response to oil hydrocarbons due to their hydrophobic properties, which reduce the ability of plants and microorganisms to absorb water and nutrients from the soil (Alarcón *et al.*, 2019).

Effect of non-rhizospheric and rhizospheric soil on the degradation of new or weathered crude oil

The degradation of TPH in absolute terms of oil in non-rhizospheric soil (soil without plant), rhizospheric soil 1 (soil with Criollo canelo rice plants), and rhizospheric soil 2 (soil with line 21 rice plants), reported significant statistical differences ($p \leq 0.5$) (Figure 4).

Regardless of the treatments, the TPHs concentrations reduced during the 145 days, the results indicate that the highest TPHs degradation occurred in rhizospheric soils (Figure 4). The rhizospheric soil 2 presented the highest (73, 72 and 69%) degradation percentage of new and weathered crude TPH in the 30,000 mg kg^{-1} new crude TPH concentrations, 42,000 weathered TPH 2 and 79,457 from weathered TPH 1 treatment. It was followed by non-rhizospheric soil that reported degradation of 50 and 47% of the contents of 79,457 of weathered TPH 1 and 42,000 mg kg^{-1} of weathered TPH 2. On the contrary, rhizospheric soil 1 evidenced a maximum degradation of 40 and 39% equivalent to 1.9 times less than the recorded percentage in rhizospheric soil 2 and 1.2 times less than non-rhizospheric soils.

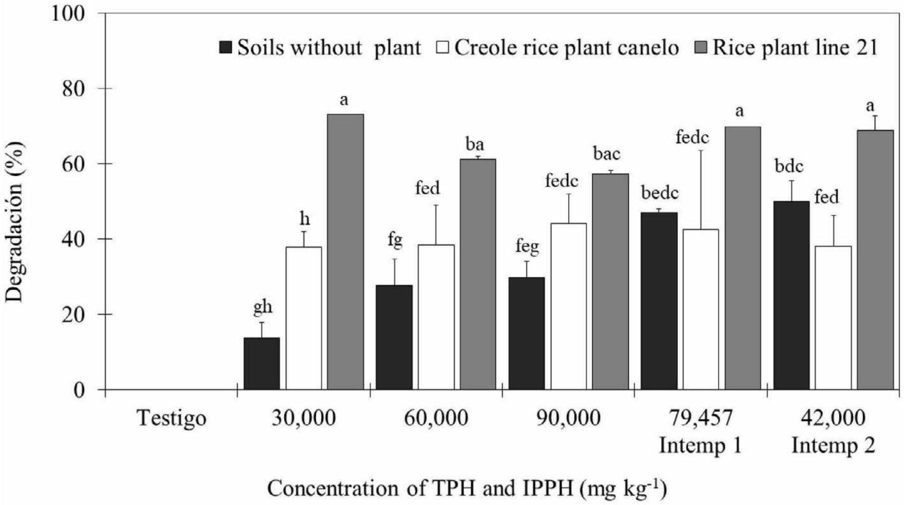

Figure 4. Degradation percentage of oil in non-rhizospheric and rhizospheric soils with *Oryza sativa* variety Criollo canelo and line 21 at 145 days. Columns with the same letter are not significantly different ($p \leq 0.05$).

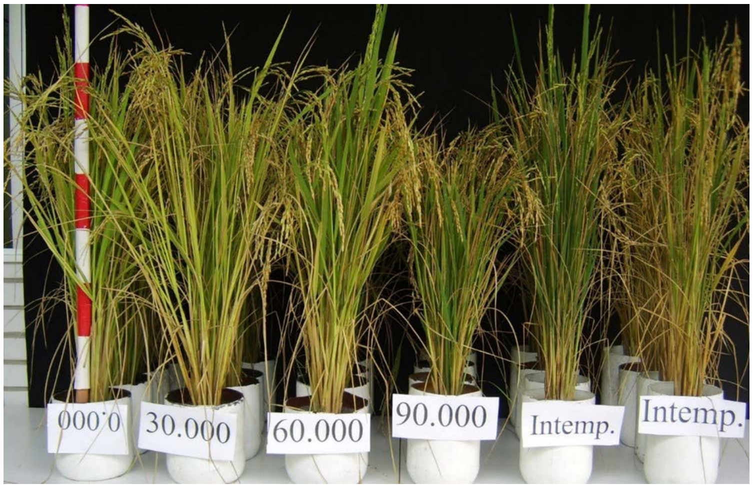

Figura 5. Rice plants (*Oryza sativa* L.) used in the experimentation.

Likewise, biodegradation of fuel oil was stimulated by line 21 rice plants (*O. stiiva* L.), which contributed by decreasing the total oil hydrocarbons proportion by 73%. Likewise, the soils without a plant (non-rhizospheric soil) showed a similar behavior until reaching a maximum 50% degradation, greater than the rhizospheric soil of the Criollo canelo rice plants, which showed a maximum degradation of 40% each.

Overall, the best results were obtained in rhizospheric soil 2 in the 79,457 concentration of weathered TPH 1. These results suggest that plant's presence increases the degradation of TPH by increasing exudates and microorganisms (bacteria and fungi) of the soil (Jeelani *et al.*, 2017; Zhang *et al.*, 2021). In this regard, Zozulo *et al.* (2020) found an 87% TPH biodegradation due to the effect of the rhizosphere in Pome plants and 89% due to the effects of Fabaceae. For their part, Kenday *et al.* (2018) found greater TPHs degradation in rhizospheric soils with respect to non-rhizospheric soils suggesting that the presence of plants in contaminated soil significantly improved TPHs elimination (Oleszczuk *et al.*, 2019: Košnář *et al.*, 2020).

CONCLUSIONS

Rice line 21 variety could be a sustainable alternative to phytoremediate soils contaminated with crude oil and weathered. The rhizospheric system of rice plants stimulated the largest population of total bacteria and fungi. The highest biodegradation of hydrocarbons occurred in the rhizospheric soil of line 21 rice plants.

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