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Plant root exudates drive changes in rhizobacterial gene expression: effects on hydrocarbon degradation and plant growth-promoting rhizobacteria traits in rhizoremediation

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Abstract

Purpose Rhizoremediation leverages plant–microbe interactions to restore hydrocarbon-contaminated soils. However, the molecular mechanisms that mediate these interactions remain poorly understood. This study aimed to investigate how tropical grasses and their root exudates modulate rhizobacterial gene expression related to hydrocarbon degradation and plant growth promotion, providing functional insights to optimize rhizoremediation strategies.

Materials and methods A greenhouse pot experiment was conducted using petroleum-contaminated soil planted with *Brachiaria decumbens* and *Megathyrsus maximus*. Total petroleum hydrocarbon (TPH) removal and soil physicochemical parameters were evaluated after 3 months. Rhizospheric soil was analyzed for expression of six functional genes (alkB, alkB2, P450, rhlA, nifH, and gcd) by qRT-PCR. A complementary microcosm assay was performed to assess gene expression responses to individual and mixed root exudate analogs (sucrose, citric acid, oxalic acid, glutamic acid, flavanone, isoflavone) at days 7 and 15. Statistical comparisons were conducted using ANOVA or non-parametric equivalents, and integrative multivariate analyses were applied to explore associations between gene expression and soil quality indicators.

Results and discussion *Megathyrsus maximus* showed the highest TPH removal (41.47%) and improved soil properties, including pH, porosity, cation exchange capacity, and nutrient availability. Gene expression analyses revealed upregulation of alkB2 and P450 in vegetated soils, while alkB remained unchanged. Expression of PGPR-related genes nifH and gcd was also enhanced in planted treatments. In the microcosm assay, sucrose and the compound mix induced the highest alkB2 and P450 expression at day 7, with expression declining by day 15, suggesting transient microbial activation by labile exudates. Principal component analysis showed strong associations between gene expression, improved soil parameters, and vegetation. These findings highlight species-specific and compound-specific modulation of microbial functional activity in rhizoremediation contexts.

Conclusions Tropical grasses stimulate microbial gene expression linked to both hydrocarbon degradation and nutrient cycling, enhancing soil restoration. *M. maximus* outperformed *B. decumbens* in TPH removal and nitrogen cycling. The microcosm assay demonstrated that specific exudate compounds can transiently enhance microbial degradation potential. Overall, the study provides mechanistic evidence supporting the use of plant–microbe associations and exudate-informed biostimulation to improve the effectiveness of rhizoremediation in petroleum-contaminated soils.

Keywords Rhizoremediation · Gene expression · Rhizobacteria · Hydrocarbons · Root exudates

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1 Introduction

Soil contamination by petroleum hydrocarbons is a global environmental concern due to their persistence and their adverse impact on soil fertility, elemental balance, biogeochemical cycles, and the risk of secondary pollution of groundwater and air. These compounds also threaten soil microbial communities and human health (Hu et al. 2013; Galitskaya et al. 2021). Total petroleum hydrocarbons (TPH) comprise a complex mixture of aliphatic and aromatic hydrocarbons derived from crude oil (Hoang et al. 2021). While aliphatic hydrocarbons constitute up to 90% of TPH (Kostecki and Calabrese 1991), most research on hydrocarbon-contaminated soil remediation has focused on the degradation of aromatic compounds and has overlooked the substantial role of aliphatic hydrocarbons in terrestrial contamination (Stroud et al. 2007). Given their chemical stability and lower solubility in water, aliphatic hydrocarbons can persist in soil matrices for extended periods due to their limited microbial accessibility, and their degradation efficiency is reduced.

Rhizoremediation has emerged as a sustainable and cost-effective strategy that uses plant-microbe interactions to enhance hydrocarbon degradation in contaminated soils (Correa-García et al. 2018; Kiamarsi et al. 2020). This approach is based on the rhizosphere effect, in which root exudates influence microbial communities by modifying soil conditions and stimulating metabolic pathways that are involved in hydrocarbon degradation (Nie et al. 2010). Unlike conventional bioremediation techniques, rhizoremediation not only enhances pollutant degradation, but also contributes to soil restoration by improving physicochemical properties such as nutrient availability, porosity, and microbial diversity.

Root exudates contain a diverse array of organic compounds, including sugars, amino acids, phenolics, and organic acids, which modulate microbial metabolism and gene expression (Hartmann et al. 2009; Rohrbacher and St-Arnaud 2016). These exudates play dual roles in rhizoremediation: enhancing hydrocarbon bioavailability by acting as biosurfactants and regulating microbial gene expression, particularly genes encoding hydrocarbon-degrading enzymes (Martin et al. 2014). Studies have demonstrated that specific root exudate compounds can upregulate the genes *alkB*, *alkB2*, *P450*, and *rhlA*, which play central roles in the microbial catabolism of hydrocarbons (Iqbal et al. 2019). However, the extent to which different root exudate components influence these gene-expression patterns remains unclear.

Beyond their role in hydrocarbon degradation, root exudates also stimulate the activity of plant growth-promoting rhizobacteria (PGPR), which enhances soil fertility and plant health by facilitating nitrogen fixation (*nifH* gene) and phosphate solubilization (*gcd* gene) (Rohrbacher and St-Arnaud 2016). The interplay between hydrocarbon-degrading bacteria and PGPR under rhizoremediation conditions remains poorly understood, particularly in regard to the dynamics of rhizospheric microbial communities and the regulation of functional genes. Grass such as *Brachiaria decumbens* and *Megathyrsus maximus* have been identified as promising candidates for rhizoremediation due to their extensive root systems and ability to tolerate hydrocarbon-contaminated soils (Gaskin and Bentham 2010; Asemoloye et al. 2017). However, there is limited knowledge on how their root exudates influence microbial hydrocarbon degradation pathways and PGPR activity at the molecular level. Most studies have focused on microbial abundance rather than gene expression, which has left a gap in understanding of the functional responses of rhizosphere microbial communities to plant-derived compounds.

The aim of this study was to investigate the effect of *B. decumbens* and *M. maximus* on rhizobacterial gene expression in relation to hydrocarbon degradation and PGPR traits. We assessed changes in the expression of *alkB*, *alkB2*, *P450*, and *rhlA*, which are involved in hydrocarbon degradation, as well as *nifH* and *gcd*, which are associated with nitrogen fixation and phosphate solubilization, respectively. Greenhouse pot assays were conducted using hydrocarbon-contaminated soils, along with microcosm experiments with selected root exudate compounds. By integrating gene expression analysis with measurements of soil physicochemical properties, this study provides insights into the molecular mechanisms underlying rhizoremediation and contributes to the optimization of plant–microbe interactions for enhanced hydrocarbon degradation.

2 Materials and methods

2.1 Pot experiment

A total of 200 kg of soil with historical petroleum hydrocarbon contamination was collected from the eastern region of the department of Antioquia, Colombia. Samples were taken from the upper 50 cm of the soil profile, transported to the laboratory, air-dried, sieved (2-mm mesh), and homogenized. The initial physicochemical characteristics were analyzed prior to the experiment. An assay was conducted under greenhouse conditions for 3 months at temperatures of 21–24 °C and 65–71% relative humidity.

Polypropylene pots (1.5-kg capacity) were each filled with homogenized contaminated soil. Certified seeds of *B.*

decumbens and *M. maximus* were obtained from a commercial distributor and sown directly into the soil (1 g per pot). Five replicate pots were prepared for each treatment (one per plant species), along with five unplanted control pots. All pots were arranged randomly within the greenhouse and irrigated with 400 mL of tap water three times per week throughout the experimental period.

2.2 Microcosm experiment

Microcosm assays were designed to evaluate gene expression responses to individual root exudate compounds under controlled conditions. Soil samples (50 g dry weight equivalent) were placed in sterile Magenta® culture boxes. Based on the composition of grass root exudates reported in previous studies (Lu et al. 2017; Wang et al. 2021), six representative compounds were selected: sucrose, citric acid, oxalic acid, glutamic acid, flavanone, and isoflavone.

Each compound was added individually to the soil at a final concentration equivalent to 30 mg TOC (total organic carbon) kg^{-1} soil. This concentration was selected to represent a conservative, low-to-moderate rhizosphere carbon input. Rhizodeposition is widely recognized as a substantial and variable plant-derived carbon flux to soil, with magnitudes commonly discussed at the order of tens to approximately 100 mg C kg^{-1} soil, depending on plant species, soil properties, and temporal scale (Kuzyakov and Domanski 2000; Jones et al. 2004; Nguyen, 2009). In addition, soil microcosm studies commonly apply defined low-molecular-weight compounds representative of root exudates at comparable or higher concentrations to simulate rhizosphere substrate inputs and assess microbial functional responses (Eilers et al. 2010; Shi et al. 2011). The selected dose was deliberately kept moderate to avoid unrealistically high carbon enrichment and potential priming effects (Kuzyakov et al. 2000; Blagodatskaya and Kuzyakov 2008).

An additional treatment combining all six compounds (mix) and a control without added compounds were also included. Each treatment was performed in triplicate, resulting in eight experimental conditions. All microcosms were incubated in the dark at 28 °C and 50% relative humidity for 15 days. Soil moisture was maintained at 60% field capacity using sterile deionized water. Samples were collected on days 1, 7, and 15 for RNA extraction and TPH quantification.

2.3 TPH determination

TPH was quantified by Soxhlet extraction with gravimetric determination according to U.S. EPA Method 3540 C (US EPA 2019), a standardized procedure widely used for the determination of petroleum hydrocarbons in soils and sediments within physicochemical soil analysis frameworks. Briefly, 10 g of moist soil were extracted with hexane for 4 h in a Soxhlet apparatus. The extract was mixed with 3 g of activated silica for 5 min, filtered through a 150-mm Whatman filter, and concentrated using a rotary evaporator at 75 °C. Residues were further dried at 103 °C for 1 h and then placed in a desiccator until constant weight was achieved. The recovery efficiency was assessed by spiking uncontaminated soil with analytical-grade hexadecane (Merck, Darmstadt, Germany, Batch S7495033 809).

2.4 Soil physicochemical analyses

Soil moisture content was determined gravimetrically by drying samples at 105 °C for 24 h. Soil pH was measured according to ASTM D4972-19 using a suspension containing a 1:5 ratio of soil to deionized water (w/v). Electrical conductivity was determined according to the method used by (He et al. 2012). Soil porosity was calculated according to (Vomocil 1965), and available phosphorus (AP) was measured using (Bray and Kurtz 1945). Cation exchange capacity (CEC) was determined with ammonium acetate (Chapman 1965)]. Soil organic carbon (SOC) and organic matter (SOM) were determined according to (Walkley 1946), and ammoniacal nitrogen (AN) was quantified using methods 4500 A and B from the APHA standard procedures. All analyses were performed in triplicate.

2.5 RNA extraction and qRT-PCR gene analysis

Total RNA was extracted from 1 g of soil using the E.Z.N.A.® Soil RNA Mini Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. RNA concentration and purity were evaluated spectrophotometrically. Complementary DNA (cDNA) was synthesized using SuperScript™ IV Reverse Transcriptase (Thermo Scientific, USA) with random hexamer primers using a thermal program of 10 min at 23 °C, 50 °C, and 80 °C sequentially.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a Bio-Rad CFX96 thermal cycler in 20-µL reaction volumes to assess the relative expression of the target genes *alkB*, *alkB2*, *P450*, *rhlA*, *nifH*, and *gcd*. The *gyrB* gene was used as a housekeeping reference. The primers and thermal cycling conditions are presented in Table 1.

PCR efficiency was calculated from standard curves constructed using 10-fold serial dilutions of target gene fragments. Relative expression ratios were determined by

Table 1 Primer sequences and real-time PCR conditions

Gene	Primer	Sequence 5'−3'	PCR parameters	References
gyrB	*gyrB-F*	CGCAAGGACCTGAAAGACAC	40 cycles at 95 °C for 10 s, 50 °C for 20 s and 72°for 25 s	Galisa et al. 2012
	gyrB-R	GACCAGGAAGATCTCGGTCTC		
alkB	*alkB-F*	AAC TAC MTC GAR CAY TAC GG-3	45 cycles at 84 °C for 20 s, 50 °C for 30 s and 72° for 40 s	Powell et al. 2006
	alkB-R	TGA MGA TGT GGT YRC TGT TCC		
P450	*P450F*	CGCATGCTAGCCTCACTG	45 cycles at 95 °C for 45 s, 58° for 60 s and 72 °C for 60 s	Denaro et al. 2010
	P450 R	GCCATATCTGCCGCGTCATC		
rhlA	*rhlaF*	AACGAGACCGTCGGCAAATA	40 cycles at 95 °C for 10 s and 56° for 30 s	Wang et al. 2014
	rhlarR	AAATGCACGTGGCTCTGGAT		
nifH	*nifH-F*	TGYGAYCCIAAIGCIGA	45 cycles at 94 °C for 30 s, 51° for 60 s and 72 °C for 60 s	Pogoreutz et al. 2017
	nifH-R	TCIGGIGARATGATGGC		
gcd	*gcd-F*	CGGCGTCATCCGGG-SITIYRAYRT	35 cycles at 95 °C for 60 s, 60° for 60 s and 72 °C for 45 s	Bergkemper et al. 2016
	gcd-R	GGGCATGTCCAT-GTCCCAIADRTCRTG		

Pfaffl's method (Pfaffl 2001). All reactions were conducted in triplicate.

2.6 Statistical analysis

Relative gene-expression data were analyzed using the Pfaffl method, and statistical comparisons were performed using R software (version 3.6.1, R Core Team, 2019). Differences among treatments were evaluated using one-way analysis of variance (ANOVA) when assumptions of normality and homoscedasticity were met, which were verified by Shapiro–Wilk and Levene's tests, respectively. When assumptions were violated, the non-parametric Kruskal–Wallis test was applied. Post-hoc comparisons were conducted using Tukey's honestly significant difference (HSD) test or Dunn's test as appropriate. A significance level of $p < 0.05$ was used throughout.

3 Results

3.1 Pot experiment

3.1.1 TPH removal

The initial average TPH concentration was 19,472.34 mg/kg across all experimental units at month 0. After 3 months of treatment under greenhouse conditions, differential removal efficiency was observed among treatments. The *M. maximus* treatment achieved the highest average TPH removal with a mean reduction of 41.47%, followed by *B. decumbens* with 21.14% (Fig. 1). In contrast, the unplanted control exhibited a negative removal rate of 30.11 ± 10.65%, indicating a net accumulation of hydrocarbons during the experimental period. These differences were statistically significant (Kruskal–Wallis, $p < 0.001$), and post-hoc comparisons confirmed that both vegetated treatments significantly outperformed the control ($p < 0.01$). *M. maximus* showed superior performance compared to *B. decumbens* ($p < 0.05$).

3.1.2 Soil physicochemical analyses

The soil parameter results are shown in Fig. 2. Both *B. decumbens* and *M. maximus* increased soil pH compared to the unplanted control with average values of 6.94 and 6.74, respectively versus 5.38 in the control. Organic carbon (OC) and organic matter (OM) content were markedly lower in planted treatments. *B. decumbens* showed 8.08% OC and 13.72% OM, and *M. maximus* showed 6.13% OC and 12.67% OM, in contrast to 14.65% OC and 19.06% OM for the control. CEC was also higher in the vegetated soils (41.21 meq/100 g for *B. decumbens* and 39.00 meq/100 g for *M. maximus*) than in the control (21.23 ± 3.38 meq/100 g).

Ammoniacal nitrogen was lowest for *M. maximus* (4.21 mg/kg), followed by *B. decumbens* (6.22 mg/kg), while it was highest for the control (10.20 mg/kg), which demonstrates distinct nitrogen dynamics among treatments. Available phosphorus was significantly increased in the planted soils with means of 7.68 mg/kg for *B. decumbens* and 6.51 mg/kg for *M. maximus*, while 2.54 mg/kg was observed in the control. Similarly, porosity was markedly improved by plant treatments (49.0% for *B. decumbens* and 51.8% for *M. maximus*), while the control had a lower average of 32.2%.

Post-hoc analyses confirmed that these differences were statistically significant between the vegetated treatments and the control for all variables mentioned, although no significant differences were observed between *B. decumbens* and *M. maximus*. Significant differences were observed among treatments for most soil physicochemical parameters at the end of the greenhouse assay. The Kruskal–Wallis test

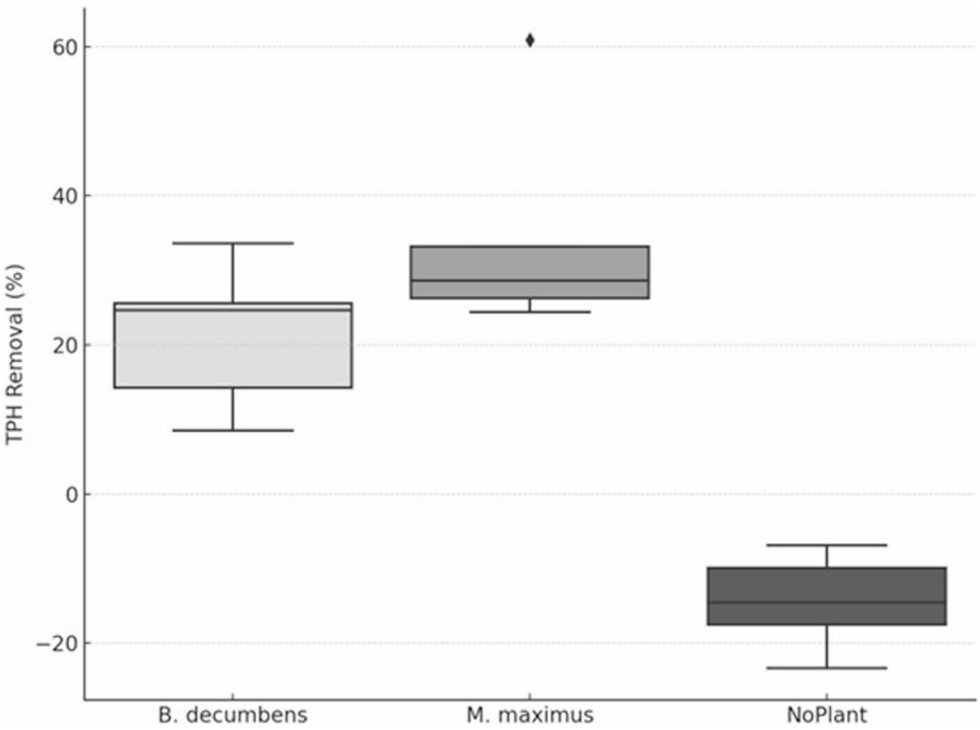

Fig. 1 Total petroleum hydrocarbon (TPH) removal efficiency by treatment after three months of the pot experiment. Differences among treatments were evaluated using the Kruskal–Wallis test followed by Dunn's post-hoc test. Statistical significance was set at $p < 0.05$

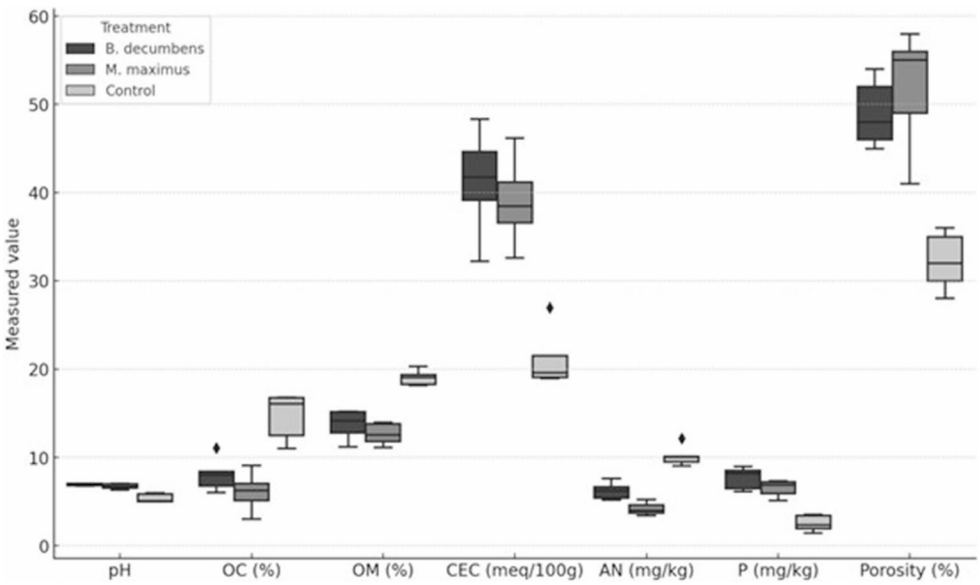

Fig. 2 Comparison of soil physicochemical parameters in vegetated (*B. decumbens and M. maximus*) and unplanted treatments after three months of rhizoremediation. Differences among treatments were assessed using the Kruskal–Wallis test ($p < 0.05$). Variables showing significant differences among treatments are described in the Results section

indicated that pH, OC, OM, CEC, AN, available phosphorus P, and porosity all differed significantly between treatments ($p < 0.05$), whereas electrical conductivity did not.

3.2 Gene expression

3.2.1 Pot experiment

Figure 3 shows the relative expression levels of *alkB*, *alkB2*, *P450*, *rhlA*, *nifH*, and *gcd* across the three experimental treatments. According to the Kruskal–Wallis test, expression levels of *alkB2*, *P450*, *rhlA*, *nifH*, and *gcd* differed significantly among treatments ($p < 0.01$), whereas *alkB* did not show statistically significant variation ($p = 0.059$). The *alkB2* gene, which encodes an alkane monooxygenase involved in terminal oxidation of alkanes, showed the highest expression in *B. decumbens* (1.80), followed by *M. maximus* (1.66) and then the control (1.25). Post-hoc comparisons confirmed significant differences between *B. decumbens* and both *M. maximus* and the control ($p < 0.05$).

The *P450* gene encodes a cytochrome P450 monooxygenase that is involved in the oxidation of hydrophobic

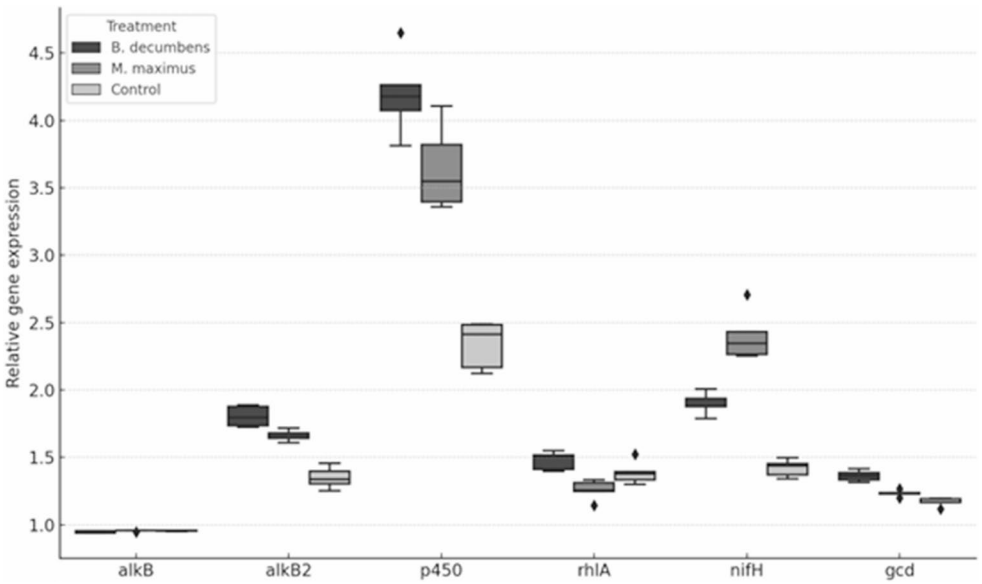

Fig. 3 Relative gene expression (fold change) of hydrocarbon-degrading and plant growth-promoting rhizobacteria (PGPR)-related genes under different plant treatments. Differences among treatments were analyzed using the Kruskal–Wallis test followed by Dunn's post-hoc comparisons when appropriate. Statistical significance was considered at $p < 0.05$

hydrocarbons. Both vegetated treatments showed elevated *P450* expression levels relative to the control (*B. decumbens*: 4.20, *M. maximus*: 3.65, control: 2.07), with no significant difference between the two plant species. The *rhlA* gene is responsible for the synthesis of the rhamnolipid precursor 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA). This gene was also significantly upregulated in *B. decumbens* (1.48) compared to *M. maximus* (1.26) and the control (1.25), suggesting species-specific enhancement of biosurfactant production pathways.

Genes associated with PGPR traits responded differently between treatments. The *nifH* gene, a marker for nitrogenase activity and potential nitrogen fixation, exhibited its highest expression with *M. maximus* (2.40), followed by *B. decumbens* (1.90) and the control (1.33). In contrast, the *gcd* gene, which encodes glucose dehydrogenase involved in phosphate solubilization, had highest expression in *B. decumbens* (1.36), followed by *M. maximus* (1.23) and the control (1.07).

3.2.2 Integration of gene expression and soil properties

To evaluate the integrated response of microbial functional activity and soil quality to plant-based treatments, a principal component analysis (PCA) was conducted using gene expression data (*alkB*, *alkB2*, *P450*, *rhlA*, *nifH*, *gcd*) and soil physicochemical parameters (pH, organic carbon, OM, cation exchange capacity, ammoniacal nitrogen, phosphorus, and porosity). The first two principal components accounted for 85.8% of the total variance, with PC1 explaining 71.3% and PC2 14.5%. The PCA biplot (Fig. 4) revealed clear clustering of samples according to treatment. Replicates from *B. decumbens* and *M. maximus* were located on the left side of PC1 distinctly separated from the unplanted control, which clustered on the right. Moreover, *B. decumbens* and *M. maximus* samples showed partial separation along PC2, suggesting species-specific effects on soil and microbial dynamics.

Vectors representing *alkB2*, *P450*, *gcd*, *nifH*, pH, cation exchange capacity, available phosphorus, and porosity were oriented toward the vegetated treatments, indicating strong association with plant-mediated soil restoration and microbial stimulation. In contrast, ammoniacal nitrogen, OM, and OC were negatively associated with these samples and aligned with the unplanted control, which reflects lower degradation and soil improvement in the absence of vegetation. These multivariate patterns support the hypothesis that grass-mediated rhizoremediation induces coordinated shifts in microbial function and soil quality and couples hydrocarbon degradation with nutrient cycling. The convergence of gene expression and soil quality vectors supports a functionally integrated response driven by root–microbe interactions in contaminated soils.

Correlation analysis revealed robust associations between the expression levels of functional genes and key soil physicochemical parameters. The hydrocarbon degradation genes *alkB2* and *P450* and the plant growth-promoting genes *gcd* and *nifH* exhibited strong positive correlations with available phosphorus ($r = 0.97$, 0.97, 0.93, and 0.82, respectively), cation exchange capacity ($r > 0.78$), and porosity ($r > 0.79$). This indicates that improved soil structure and nutrient status were associated with elevated microbial gene activity. In contrast, the same genes showed strong negative correlations with OC and OM ($r < -0.83$), suggesting an association between gene expression and the mineralization of recalcitrant organic fractions.

Notably, *nifH* and *gcd* expression displayed pronounced negative correlations with ammoniacal nitrogen ($r = -0.87$ and -0.85, respectively), which is consistent with enhanced nitrogen assimilation and microbial nutrient cycling in

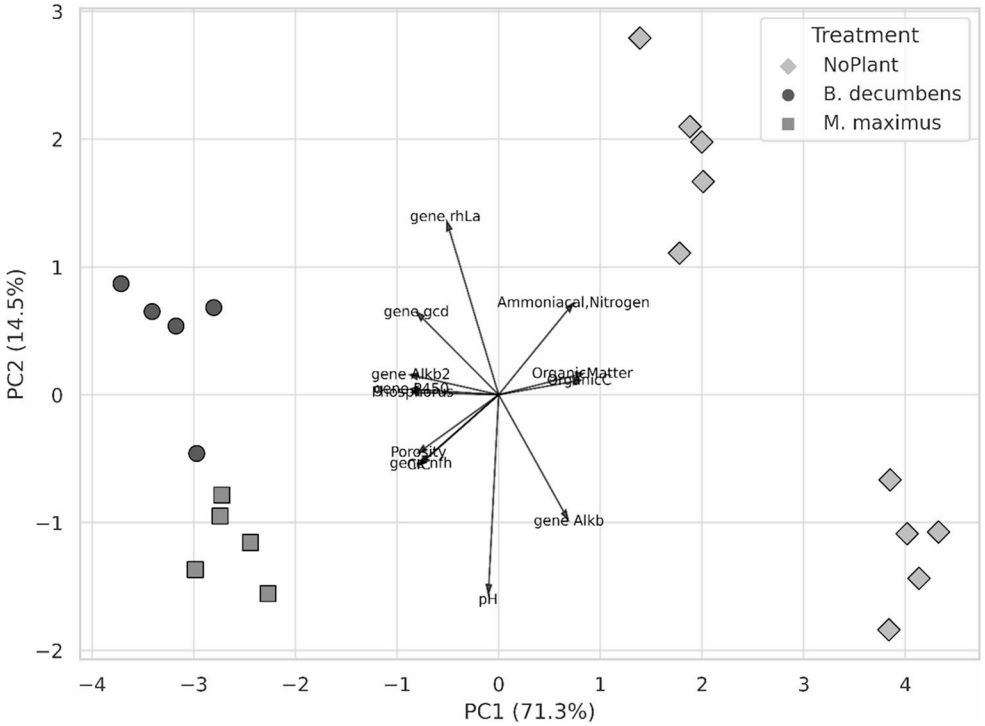

Fig. 4 PCA biplot of soil physicochemical parameters and microbial gene-expression profiles in planted and unplanted treatments

Table 2 PHC removal percentage in microcosm experiment

Treatment	Removal percentage
Saccharose	16.5
Citric acid	13.1
Oxalic acid	15.0
Glutamic acid	14.2
Flavanone	13.4
Isoflavone	10.1
Mix	22.8
Control	5.2

vegetated treatments. These trends support the hypothesis that plant-induced changes in the rhizosphere environment promote not only hydrocarbon degradation, but also microbial functions involved in nutrient transformation. The tight coupling observed between hydrocarbon-degrading genes (*alkB2*, *P450*) and PGPR-associated genes (*gcd*, *nifH*) supports the view that rhizoremediation elicits a coordinated microbial response. This functional integration likely contributes to both pollutant removal and the partial restoration of soil fertility, which highlights the dual ecological roles of rhizosphere microbial communities in plant-mediated bioremediation.

3.3 Microcosm experiment

3.3.1 TPH removal

Descriptive analysis revealed moderate levels of TPH removal across all treatments in the microcosm assay (Table 2). The highest removal was observed in the synthetic compound mix (22.8%), followed by saccharose (16.5%), oxalic acid (15.0%), and glutamic acid (14.2%). The lowest removal efficiencies were recorded for the control (5.2%) and isoflavone (10.1%). These values indicate that individual exudate components can stimulate hydrocarbon removal to varying extents, with synergistic effects evident in the compound mix.

3.3.2 Gene expression

In terms of gene expression, both *alkB2* and *P450* exhibited higher average relative expression on day 7 (1.50 and 1.72, respectively) than on day 15 (1.17 and 1.41, respectively), suggesting early activation of hydrocarbon degradation pathways (Fig. 5). This trend was consistent across most treatments and indicates that microbial response to root exudate compounds was most pronounced in the first week of incubation. For *alkB2*, the overall average expression decreased from 1.50 ± 0.34 on day 7 to 1.17 ± 0.22 on day 15. Similarly, *P450* expression declined from an average of 1.72 ± 0.33 to 1.41 ± 0.24 over the same period. This trend suggests that microbial activation in response to root exudate compounds was most pronounced early in the incubation period, possibly due to the immediate availability and metabolism of labile carbon substrates.

Wilcoxon signed-rank tests confirmed that the decrease in *alkB2* expression from day 7 to day 15 was statistically significant in most treatments ($p = 0.0039$), whereas the

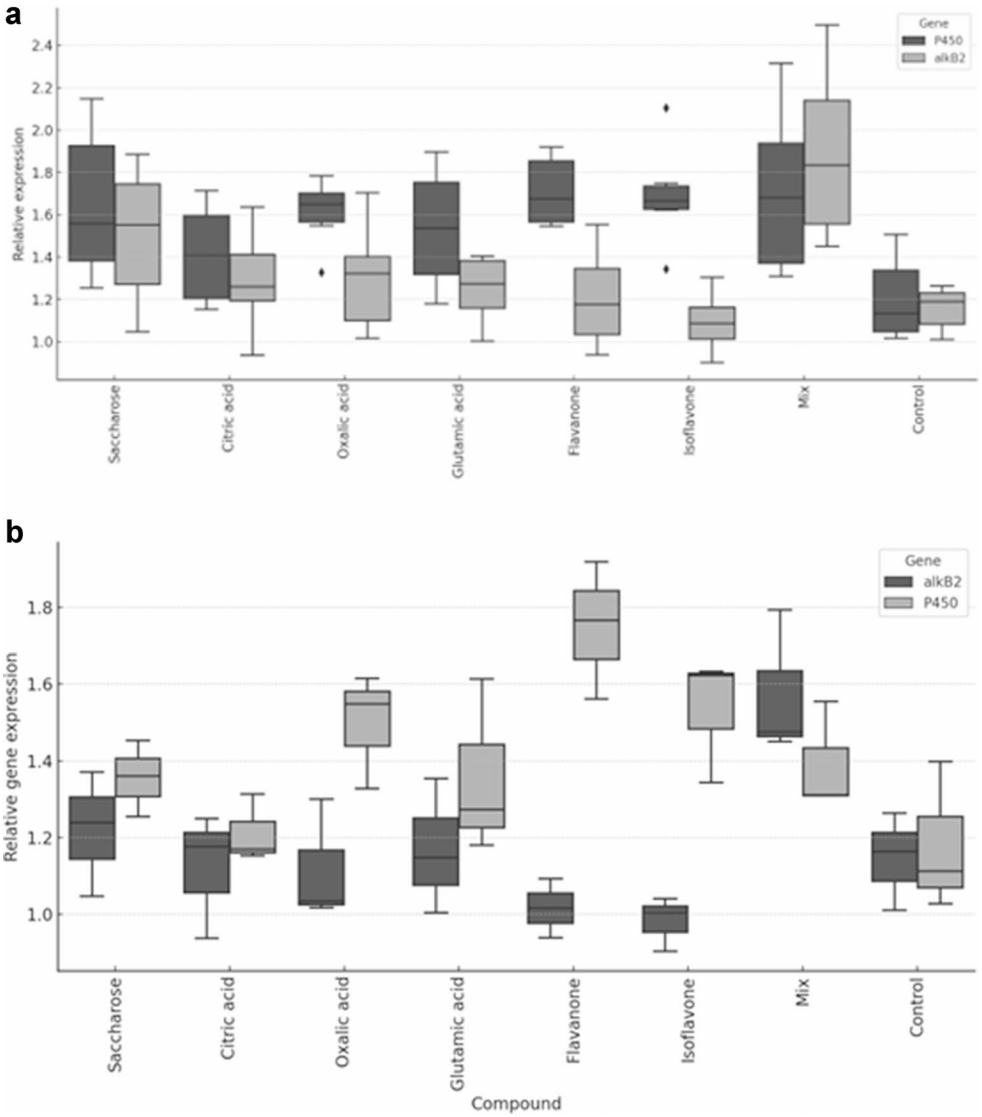

Fig. 5 Effect of root exudate analogue compounds on the relative expression (fold change) of hydrocarbon-degrading genes in soil microcosms: (**a**) day 7 and (**b**) day 15. Differences among treatments were evaluated using the Kruskal–Wallis test followed by Dunn's post-hoc test. Temporal differences between day 7 and day 15 were assessed using the Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$

control showed no significant change ($p = 0.426$). For *P450*, the reduction in expression over time was significant in several treatments ($p < 0.05$), but not in the control or flavanone treatment ($p = 0.055$), which highlights the compound-specific temporal responses. On day 15, the Kruskal–Wallis tests revealed significant differences in gene expression among treatments for both *alkB2* ($H = 38.36$, $p < 0.001$) and *P450* ($H = 42.39$, $p < 0.001$). Post-hoc comparisons indicated that the compound mix induced the highest expression of both genes, which significantly differed from several individual compounds and the control. Specifically, for *alkB2*, the mix differed from flavanone, saccharose, and citric acid, while for *P450*, it differed from flavanone and the control.

Correlation analyses to evaluate the association between gene expression and TPH removal revealed strong positive relationships, particularly on day 7. For *alkB2*, Spearman's correlation coefficient was $\rho = 0.905$ ($p = 0.002$), while for *P450*, Spearman's ρ was 0.762 ($p = 0.028$). These findings confirm that early expression levels of *alkB2* and to a lesser extent *P450* are reliable indicators of hydrocarbon biodegradation efficiency. Overall, these results suggest that the stimulatory effects of specific root exudate compounds on microbial gene expression are time-sensitive and play an important role in modulating the functional response of rhizosphere microbial communities during rhizoremediation.

4 Discussion

4.1 Hydrocarbon removal in pot experiments

The enhanced removal of TPH observed in the vegetated treatments, particularly with *M. Maximus*, confirms the key role of plant–microbe interactions in rhizoremediation under greenhouse conditions. The significantly higher removal efficiency (41.47%) compared to the unplanted

control supports the idea that rhizosphere processes mediated by grasses can accelerate the degradation of hydrocarbons in aged contaminated soils. This finding is consistent with studies showing that the establishment of certain Poaceae species can stimulate hydrocarbon-degrading microbial communities through mechanisms such as root exudation and rhizosphere oxygenation (Zuzolo et al. 2021; Hoang et al. 2021).

The apparent accumulation of hydrocarbons in the unplanted control contrasts with the consistent degradation observed in the planted treatments. Similar phenomena have been reported in historically contaminated soils, where desorption of aged hydrocarbon fractions from soil aggregates into more bioavailable forms occurs due to rehydration or shifts to microaerophilic conditions (Chen et al. 2024). These results underscore the limitations of relying solely on natural attenuation in such environments and support the potential of phytoremediation as an effective biostimulation strategy. The observed differences in remediation efficiency between plant species may be explained by variations in root architecture, exudate profiles, and associated microbial consortia (Banks et al. 2003; Yuan-Wen et al. 2003; Iffis et al. 2017).

4.2 Restoration of soil physicochemical properties through rhizoremediation

The observed improvements in key soil physicochemical parameters, including pH, porosity, CEC, and available phosphorus, underscore the potential of grass-mediated rhizoremediation to stimulate microbial hydrocarbon degradation and contribute to broader soil restoration. Both *B. decumbens* and *M. maximus* were associated with increased pH and decreased levels of OM and ammoniacal nitrogen compared to the unplanted control, indicating enhanced biogeochemical cycling in the rhizosphere. Soil acidification is a common consequence of chronic hydrocarbon contamination and is often driven by microbial metabolic by-products and the accumulation of organic acids (Chan-Quijano et al. 2020). In this context, the pH increase observed in the vegetated treatments suggests the establishment of a more favorable chemical environment for microbial activity and nutrient mobilization. These effects are consistent with studies indicating that rhizosphere alkalization may be mediated by plant uptake of cations and rhizobacteria-driven nitrification processes (Chowdhury et al. 2017; Sieradzki et al. 2023).

The significant reductions in OM and OC in planted treatments, particularly with *M. maximus*, point to the activation of microbial mineralization pathways. This may be attributed to the synergistic influence of root exudates and rhizodeposition in enhancing microbial turnover of both labile and recalcitrant carbon pools (Rohrbacher and St-Arnaud 2016). The concurrent increases in porosity and CEC further support this interpretation: as microbial activity and root proliferation restructure the soil matrix, physical properties that are favorable for aeration and ion exchange are progressively restored (Hoang et al. 2021).

A key observation is the inverse relationship between ammoniacal nitrogen levels and parameters such as pH and phosphorus availability. Elevated ammoniacal nitrogen concentrations in the unplanted control likely reflect limited microbial immobilization and inefficient nitrogen cycling. In contrast, the planted treatments exhibited a redistribution of nitrogen forms, which is potentially driven by increased microbial assimilation, enhanced nitrification, or plant uptake. These processes are commonly associated with improved nitrogen dynamics in rhizoremediated systems (Zhang et al. 2023).

4.3 Functional gene expression in rhizosphere communities: metabolic activation and plant specificity

The analysis of microbial gene expression under greenhouse conditions demonstrated that both *B. decumbens* and *M. maximus* influenced the activity of key functional genes associated with hydrocarbon degradation and plant-growth promotion. The expression of *alkB*, which is typically linked to short-chain alkane monooxygenase activity, did not vary significantly among treatments. However, vegetated soils showed significant upregulation of *alkB2* and *P450*, which encode enzymes with broader substrate specificity and greater affinity for long-chain hydrocarbons. This suggests that rhizosphere microbial communities selectively responded to plant-derived stimuli by activating catabolic pathways that are more suited to the prevailing hydrocarbon profile.

The upregulation of *alkB2* and *P450* aligns with previous findings (Wang and Shao 2013), which showed that alkane-degradation genes are differentially induced depending on the alkane chain length and environmental context. In the present study, *alkB2* expression was highest in the treatment with *B. decumbens*, while both grasses stimulated significantly higher *P450* expression compared to the unplanted control. These patterns suggest that although both species enhance microbial hydrocarbon metabolism, *B. decumbens* may exert a stronger selective influence on specific alkane-oxidizing populations, which may potentially be due to variations in root exudate chemistry or root spatial distribution (Rohrbacher and St-Arnaud 2016).

The expression of *rhlA*, a gene involved in rhamnolipid biosynthesis, was also elevated in the *B. decumbens* rhizosphere, indicating activation of microbial biosurfactant

production (Déziel et al. 2003). This is particularly important in light of the role of rhamnolipids in increasing hydrocarbon bioavailability, which facilitates microbial uptake and degradation (Campos-García et al. 1998). The fact that biosurfactant synthesis is not consistently activated across rhizoremediation systems underscores the relevance of plant-specific microbial recruitment strategies (Zhao et al. 2015; Arisah et al. 2024).

Beyond hydrocarbon degradation, both plant species stimulated the expression of *nifH* and *gcd*, which are linked to nitrogen fixation and phosphate solubilization, respectively. The strongest *nifH* expression occurred in the *M. maximus* rhizosphere, indicating activation of diazotrophic microbial populations that are capable of contributing nitrogen inputs under nutrient-limited conditions (Rolando et al. 2023; Sun et al. 2023; Liao et al. 2024; Wang et al. 2025). In contrast, *gcd* expression was more pronounced with *B. decumbens*, which supports the hypothesis that distinct exudate profiles can favor phosphate-solubilizing microbial communities (Montes-Montes et al. 2024). This differentiation aligns with research showing that plant species establish distinct rhizosphere niches that selectively enrich microbial taxa with complementary nutrient-cycling functions (Pathan et al. 2018; Liu et al. 2025).

4.4 Multivariate integration of microbial functional traits and soil recovery indicators

The integration of microbial gene expression data with soil physicochemical parameters via PCA provided a comprehensive view of the rhizosphere's response to phytoremediation treatments. There was clear separation between vegetated and non-vegetated samples along PC1, which explained over 55% of the total variance. This highlights the coordinated shifts in microbial metabolic activity and soil conditions driven by plant presence (Chen et al. 2017; Jian et al. 2022). This result supports the concept of rhizosphere priming, in which plant-derived carbon inputs selectively enrich microbial taxa with specialized catabolic and nutrient-cycling functions (Liu et al. 2020; Li et al. 2023).

The strong alignment of *alkB2*, *P450*, *gcd*, and *nifH* vectors with vegetated samples reflects a tightly coupled microbial response associated with improvements in key soil properties, particularly pH, phosphorus availability, cation exchange capacity, and porosity (Wu et al. 2021; Zhao et al. 2022; Zhi et al. 2023). These parameters are widely recognized as proxies for soil recovery after contamination, and their co-variation with functional gene expression supports the use of molecular markers as early indicators of remediation progress (Wang et al. 2023). Importantly, the joint association of hydrocarbon-degradation genes (*alkB2*, *P450*) with plant growth-promoting genes (*nifH*, *gcd*) suggests that the microbial communities fostered by grasses contribute not only to pollutant removal, but also ecosystem multifunctionality (Shimazu et al. 2010; Pacwa-Płociniczak et al. 2024). This supports the view that successful rhizoremediation requires the integration of biogeochemical functions beyond degradation, including nutrient cycling, microbial resilience, and stress mitigation (Zhi et al. 2023; Gao et al. 2024).

In contrast, the unplanted control showed a distinct cluster and was associated with elevated ammoniacal nitrogen and OM content. These two parameters negatively correlate with microbial activity and are indicative of impaired nitrogen cycling and limited OM turnover in the absence of rhizosphere effects (Li and Bengtson 2022). These trends support the idea that plant–microbe interactions reconfigure soil biochemical networks in ways that support both contaminant attenuation and ecological recovery (Henneron et al. 2020; Yang et al. 2022; Sieradzki et al. 2023).

4.5 Functional activation by root exudate compounds under microcosm conditions

The microcosm experiment revealed a dynamic and time-sensitive activation of hydrocarbon-degrading genes (*alkB2* and *P450*) in response to individual and combined root exudate analogs. Contrary to the sustained increase expected under continuous stimulation, peak gene expression was observed on day 7, followed by a general decline by day 15 across most treatments. This temporal trend likely reflects the rapid microbial uptake and metabolism of labile carbon substrates contained in the exudate mixtures (Kuiper et al. 2002; Liao et al. 2021) and suggests that initial exposure triggers a transient spike in microbial catabolic activity, which decreases as depletion or assimilation of key inducers occurs (Rohrbacher and St-Arnaud 2016).

The early and pronounced upregulation of *alkB2*, which encodes an integral membrane monooxygenase involved in terminal alkane oxidation, correlated strongly with TPH removal efficiency ($\rho = 0.905$, $p = 0.002$). This correlation supports its value as a molecular marker for alkane degradation potential in soil systems. This observation aligns with studies linking *alkB2* expression to pollutant mineralization and supports its use as a functional indicator of in situ biodegradation processes.

Although *P450* was also upregulated at day 7, its correlation with TPH removal was lower ($\rho = 0.762$), potentially due to its broader substrate range encompassing both aliphatic and aromatic hydrocarbons. This is consistent with evidence that cytochrome *P450* enzymes act on a diverse array of endogenous and xenobiotic compounds, including fatty acids, alkanes, and polycyclic aromatic hydrocarbons (Pedrini et al. 2013). The differential responsiveness

of *alkB2* and *P450* illustrates the complexity of microbial degradation networks, where distinct pathways are selectively activated depending on compound specificity, environmental stimuli, and microbial regulation (Schottlender et al. 2024; Fatunla et al. 2024).

Among treatments, the synthetic mixture of saccharose, citric acid, oxalic acid, glutamic acid, flavanone, and isoflavone consistently induced the highest expression of degradation genes and the greatest TPH removal. This suggests potential synergistic effects among chemically diverse root exudate analogs. This finding is supported by research showing that mixtures of carbon sources can enhance microbial functional diversity and sustain catabolic redundancy, even under conditions of reduced microbial richness (Pang et al. 2025).

Additionally, the mixture selectively enriched hydrocarbon-degrading populations such as *Pseudomonas* and *Arthrobacter*, which is consistent with earlier reports on polycyclic aromatic hydrocarbon-contaminated soils. In contrast, individual compounds like flavanone and isoflavone produced weaker or inconsistent gene responses, which was likely due to lower bioavailability or selective recruitment of non-degrading microbial taxa (Rentz et al. 2004). These results emphasize that compound composition and compatibility with microbial metabolism are important determinants of biostimulant efficacy.

The statistical analyses revealed significant differences in gene expression across treatments, which highlight the compound-specific nature of rhizosphere signaling. Sugars and organic acids such as saccharose, citric acid, and glutamic acid are known to increase hydrocarbon-degrading bacterial activity by providing assimilable substrates and inducing catabolic pathways (Wang et al. 2024). In contrast, flavonoids like flavanone and isoflavone may play more regulatory or selective roles of modulating quorum sensing or recruiting PGPR without directly enhancing degradation activity (Rentz et al. 2004). These findings underscore the importance of compound identity and compatibility with microbial metabolism in shaping rhizosphere function and determining biostimulant efficacy in rhizoremediation.

5 Conclusions

This study has demonstrated that rhizoremediation using tropical grasses can simultaneously enhance hydrocarbon degradation and promote soil restoration via plant–microbe interactions. Under controlled greenhouse conditions, *M. maximus* and *B. decumbens* significantly improved key soil physicochemical parameters and stimulated the expression of microbial genes involved in both hydrocarbon catabolism (*alkB2*, *P450*) and plant growth promotion (*nifH*, *gcd*). The superior performance of *M. maximus* in TPH removal and nitrogen cycling suggests species-specific functional effects that warrant further investigation.

The integration of microbial gene-expression profiles with soil quality indicators through multivariate analysis revealed strong functional coupling between microbial activity and environmental recovery. In addition, microcosm experiments confirmed that individual root exudate compounds selectively modulate catabolic gene expression, with *alkB2* emerging as a robust early molecular marker for assessing biodegradation efficacy. The correlation observed between gene expression and TPH removal supports the use of functional gene markers as tools for monitoring and optimizing rhizoremediation performance.

Overall, this study provides mechanistic evidence of the microbial and biochemical processes driving plant-assisted bioremediation. Furthermore, it highlights the importance of combining gene expression analysis with physicochemical indicators to evaluate and refine remediation strategies. These findings contribute to a growing body of knowledge that supports the development of biologically informed sustainable approaches for restoring petroleum-contaminated soils.

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Declarations

Competing interests The authors declare that they have no financial or non-financial competing interests that are directly or indirectly related to the work submitted for publication. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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