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RESEARCH ARTICLE

RHIZODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) BY BACILLUS CEREUS CPOU13

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ABSTRACT

We conducted pot-culture experiments to study the rhizodegradation of three PAHs compounds namely phenanthrene, anthracene and pyrene with blackgram plants. PAHs compounds were accurately weighed then added to experimental soils *viz*., rhizosphere and non-rhizosphere sets and made the final concentration of PAHs 600ppm. Fresh cultures of PAHs degrading bacteria strain, B. cereus CPOU13 added to the soil and made final concentration of the strain 3.3×10^4 CFU, then the experiment was conducted for 90days. The PAHs compounds were extracted finally from soil samples after the 90days and their concentrations were determined using HPLC methods. High degradation of the PAHs was observed in the rhizosphere soil treatments over the non-rhizosphere soils. The strain from rhizosphere and non-rhizosphere soil treatments recorded high degradation when compared to natural microflora in non-autoclaved soils. Inrespect to the phenanthrene the strain recorded 83.03% of degradation in autoclaved rhizosphere soils. Anthracene was degraded up to 76.10% in the treatment of autoclaved rhizosphere soil by the strain and it was slightly decreased in non-autoclaved rhizosphere soil. The strain degraded pyrene up to 82.1% in autoclaved rhizosphere soil and its low degradation observed in non-rhizosphere soil treatments. During this 90days of experiment population of the bacteria was enumerated in the treatment soils at regular time intervals *viz*., 0, 15, 30, 60 and 90days using serial dilution technique. Population of the bacteria was increased more in rhizosphere treatments over the non-rhizosphere treatments and it gradually increased from the 0th day and reached at maximum by 60days. Afterwards, slight decrease was observed in bacterial population. Hence this study determined that the PAHs degradation under rhizodegradation perhaps associated with the increasing population of PAHs degrading strain and its increased biological activities.

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INTRODUCTION

The extensive use of fossil fuel based products in our modern society and industrial production of chemicals has resulted contamination of soils with polycyclic aromatic hydrocarbons (PAHs) that have carcinogenic and mutagenic properties. Increasing accumulation of PAHs in the surrounding environment is threatening human health and the economy. As the PAHs categorized as priority environmental pollutants by United States Environmental Protection Agency (USEPA) and European Union (EU) fast amelioration of these compounds from soil has became an important task and ways to clean up the environments attracted the attention of the scientific world. The PAHs contaminated sites disfavour the growth of plants and living organisms, and only those plants or microorganisms that can tolerate the toxic effect of PAHs may seldom grow in that environment. Hence, plants associated remediation systems have great potential for cleaning up root accessible soils (rhizosphere) contaminated with PAHs.This process is plant growth dependent and influenced by various biotic and abiotic factors in soil environment. Rhizodegradation of PAHs by bioaugmenting potent PAHs degrading micro organisms to soils systems may be effective than other classical methods that commonly applied for soil remediation systems. In view of this significance, we have investigated the rhizodegradation of PAHs by bioaugmenting one potent PAHs degrading bacteria namely *Bacillus cereus* CPOU13 that previously isolated from PAHs prone industrial area with blackgram [*Vignamungo* (L.) Hepper] plants using pot-culture methods. For this we have categorized the treatments soils into different rhizosphere and non-rhizosphereas autoclaved and non-autoclaved sets for effective screening of PAHs degradation.Wehave assessed the rhizodegradation of PAHs after 90days of experiment period

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and the population of bacteria at respective time intervals and compared them for non-rhizosphere soils.

MATERIALS AND METHODS

POT CULTURE STUDIES

Soil used for pot culture was collected from Botanical Garden, Osmania University campus, Hyderabad, Telangana State. The soil had no previous history of PAHs contamination. The soil was air-dried at room temperature (28-31°C) for at least 24h to constant weight before use. Nitrogen concentration was estimated by the method of Subbaiah and Asija (1956). Phosphorus concentration was estimated by the method of Olsen *et al*. (1954) and potassium concentration was estimated by the procedure of Muhr *et al*. (1965). Phenanthrene (Sigma 99% purity), anthracene (Sigma 99% purity) and pyrene (Sigma 99% purity) were accurately weighed and dissolved in acetone separately. Each PAH solution was transferred to a glass sprayer and spiked onto the experimental soil and made the final concentration of 600mg/kg soil (600ppm). The soil was mixed thoroughly and equally distributed. Spiked soil was air-dried at room temperature(28-30°C) for more than 24h or until the smell of acetone was disappeared. Soil used for autoclaved treatments were autoclaved at 121°C for 15min before PAHs spiking and the soil used for non-autoclaved treatment soil was directly used without autoclaving.

One milliliter 7-day cultures of *B. cereus* CPOU13 grown in LB broth were transferred separately to 50ml nutrient broth and incubated for 24h at 30°C on a rotary shaker at 150rpm speed. One ml of the bacterial culture was suspended in 9ml of nutrient broth and mixed with soil to a final concentration of 3.3×10^4 CFU of the strain *B. cereus* CPOU13 per gram dried soil. Bacterial enumeration was done by viable plate counting after serial dilution (Chouychai *et al*. 2009).

Experimental Design and Analytical Method

The method for evaluating rhizodegradation of PAHs by the strain *B. cereus* CPOU13 was adopted from Chouychai *et al*. (2009). In the present study, the treatments were categorized and set to following seven types:

I. Non-rhizosphere soils
(a) Autoclaved soil(ACS) - Control
(b) Non-autoclaved soil(NACS)
(c) *B. cereus* CPOU13 in autoclaved soil
(d) *B. cereus* CPOU13 in non-autoclaved soil
II. Rhizosphere soils
(e) Plant in non-autoclaved soil
(f) Plant and *B. cereus* CPOU13 in non-autoclaved soil
(g) Plant and *B. cereus* CPOU13 in autoclaved soil

Three replicates were maintained per treatment. The soil used for autoclaved treatments was autoclaved at 121°C for 15min for three times in three days.

Extraction of Pahs from pot Culture Soils

The method for PAHs extraction from soil was adopted from Yuan *et al*.(2000). Two grams of soil from each pot was collected after the 90days and placed separately in 50ml test tubes. 5ml of n-hexane was added to each test tube prior to being shaken with a rotary shaker for 24hrs at 160rpm. Then layer of n-hexane was collected and aqueous layer was further extracted with additional n-hexane and adding anhydrous Na_2SO_4 for complete moisture removal. The step was repeated for 3-4 times. Extracts were centrifuged at 12,000g for 10min and filtered through 0.2µm filters. Finally, extracts were concentrated using vacuum evaporator under low pressure conditions. The remnants of sample were dissolved in 3ml of HPLC grade acetonitrile and stored at 4°C until the HPLC analysis. HPLC studies were conducted as described in previous sections. Unknown concentrations of phenanthrene, anthracene and pyrene in the soil samples were determined using standard chromatograms.

Statistical Analysis

All the experiments in the study were performed in triplicates. Mean and standard deviation of triplicate in independent experiments were calculated. Mean values were compared with the values of LSD (least significant difference) to found significance at 0.01 and 0.05 probabilities. LSD values were calculated using a software STAR (Statistical Tools for Agricultural Research) made by CRIDA(Central Research Institute for DrylandAgriculture, Hyderabad, Telangana State).

HPLC Analysis

Determination of PAHs compounds degradationwas studied with a reverse phase HPLC (SHIMADZU, model RF-10AXL). The instrument consists of dual pump system and connected with UV detector (SPD-20A). Instrument was equipped with column C18 (250mm×4.6mm, 5A° particle size) of Phenomenex Co. Mobile phase was consisted of 75% acetonitrile and 25% of de-ionized water. Detector was set at 250nm and mobile phase was maintained at flow rate of 0.8ml/min in isocratic mode. 20µl of sample was injected into HPLC with a HPLC injector (Rheodine injector) that prior filtered with 0.22µm syringe filters. Data of each peak on HPLC chromatogram was analyzed using chromatography software 'LC Solutions'.

Total bacterial population in pot culture soils

The plate count method was followed to enumerate total bacterial population for pot culture soil treatments and this was done as described by Kim *et al*. (2007). One gram of soil from each treatment was added to 9ml of sterilized water and mixed vigorously. After settling, 1ml of supernatant was transferred to another test tube containing 9ml of sterilized water to achieve a dilution of 10^{-2}. Other samples were treated in the same manner. A serial dilution technique subsequently yielded five additional test tubes with the dilutions from 10^{-3} to 10^{-7}, each with three replicate samples. For dilutions of 10^{-3} to 10^{-7} spread on Tryptic soy agar plates and incubated at room temperature (25°C) for 10days. Total bacterial colony forming units (CFUs) were counted and means were taken for the studies.

RESULTS

Rhizodegradation of polycyclic aromatic hydrocarbons

Effect of selected the strain *B. cereus* CPOU13 upon the rhizodegradation of phenanthrene, anthracene and pyrene in soils amended with PAHs was studied in pot culture with blackgram. After growing the crop for 90days soil samples

from each treatment were collected and studied using HPLC and residual concentrations of phenanthrene, anthracene and pyrene in respective soils were quantified (Fig. 1). Based on the concentrations of respective PAHs remained in soil rhizosphere samples, rhizodegradation and their percentages were determined using standard HPLC chromatograms.

Extraction efficiency of phenanthrene, anthracene and pyrene from pot culture soil samples

Extraction efficiency of PAHs from soils is important in decoding PAHs degradation after time periods. In view of this, the soil samples from pot culture were extracted with acetonitrile and assayed for determining extraction efficiency using HPLC. The results are presented in Table 1.

Soil Analysis

Composition of soil and the nutrients play an important role in supporting microbial activity and plant growth. In a view of this, concentrations of macronutrients like nitrogen, phosphorus and pottassium were estimated and the results are presented in Table 2.

Effect of *B. cereus* CPOU13 on rhizodegradation of phenanthrene, anthracene and pyrene

The strain, *B. cereus* CPOU13 showed an enhancement effect on rhizodegradation of phenanthrene, anthracene and pyrene over the control in pot culture with blackgram (Fig. 1 and 2).

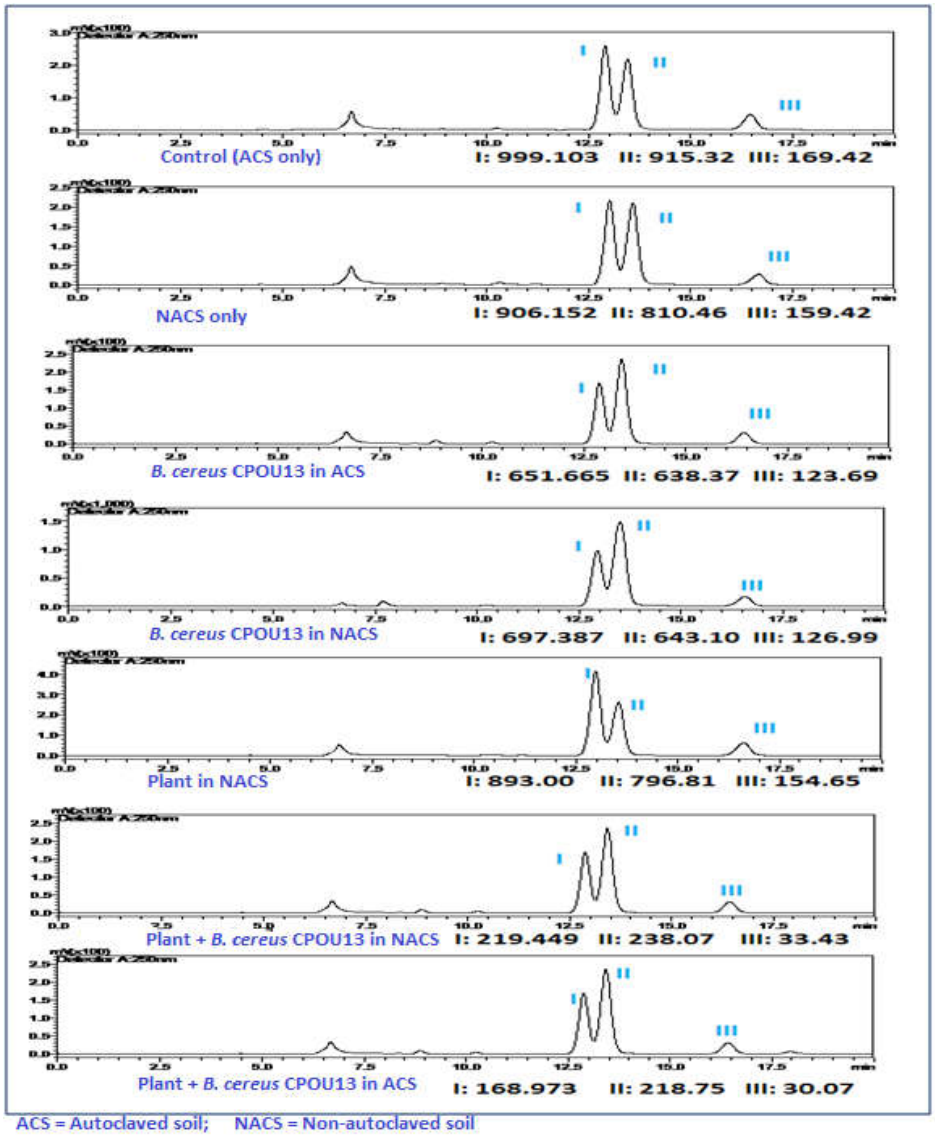

Fig. 1. HPLC chromatograms showing the effect of *B. cereus* CPOU13 on rhizodegradation of PAHs in pot culture(I= Phenanthrene; II= Anthracene; III= Pyrene)

Table 1. Extraction efficiency of PAHs from the soils

PAH Name	Amount of PAH added(ppm)	Extracted concentration(ppm)	Extraction efficiency(%)
Phenanthrene	200	119.31 ± 1.00	59.65
Anthracene	200	141.19 ± 0.91	70.59
Pyrene	200	51.39 ± 1.01	25.69

Table 2. Concentrations of N, P and K in the soils of pot culture

Element	Concentration(kg/ha soil)
Nitrogen	112-120
Pottassium	120-150
Phosphorus	35-40
pH	7

The results are presented in Table 3. High degradation of test PAHs was observed in the rhizosphere soil treatments than the

non-rhizosphere soils. *B. cereus* CPOU13 from rhizosphere and non-rhizosphere soils recorded high degradation than the natural microflora in non-autoclaved soils. In autoclaved rhizosphere soils the maximum degradation of phenanthrene (83.03%) by *B. cereus* CPOU13 was observed. The strain also showed moderate degradation(30-40%) in autoclaved and non-autoclaved non-rhizosphere soils. The strain, *B. cereus* CPOU13 degraded anthracene up to 76.10% in the treatment of autoclaved rhizosphere soil and it was slightly decreased in non-autoclaved rhizosphere soil.Degradation of anthracene was moderate(30-40%) in autoclaved and non-autoclaved non-rhizosphere soils. Degradation of pyrene by *B. cereus* CPOU13 reached up to 82.1% in autoclaved rhizosphere soil.Low degradation of pyrene(15-30%) was observed in autoclaved and non-autoclaved non-rhizosphere soils.

augmentation with *B.cereus* CPOU13 in soils amended with phenanthrene, anthracene for 90days. Soil samples were collected from each treatment at regular time intervals *viz*., 0, 15, 30, 60 and 90days and total bacterial population was estimated as colony forming units (CFUs) on nutrient agar medium using serial dilution method. Further, results were analyzed with two-way ANOVA to found significance at 0.05 and 0.01 probabilities. Augmentation of *B.cereus* CPOU13 to PAHs contaminated soils showed a prominent effect on bacterial population in pot culture studies (Fig. 3 and Table 4). Population of bacteria was increased more in rhizosphere soils over non-rhizosphere soils. Bacterial population gradually increased from the 0thday and reached at maximum by 60days. Afterwards, slight decrease was observed in bacterial population.

Table 3. Effect of *B. cereus* CPOU13 on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture

Sl. No.	Treatment		Phenanthrene		Anthracene		Pyrene	
			Quantity (µg/g soil)	Degradation (%)	Quantity (µg/g soil)	Degradation (%)	Quantity (µg/g soil)	Degradation (%)
1	Control (Autoclaved soil)	Non-rhizosphere soils	119.31	0	141.19	0	51.39	0
2	Non-autoclaved soil		108.21	9.3	125.10	11.4	48.24	6.11
3	*B. cereus* CPOU13 in Autoclaved soil		77.82	34.8	98.47	30.26	37.52	26.98
4	*B. cereus* CPOU13 in non-autoclaved soil		83.28	30.21	99.20	29.74	38.69	24.71
5	Plant in non-autoclaved soil	Rhizosphere soils	106.64	10.62	122.91	12.95	47.91	8.72
6	Plant + *B. cereus* CPOU13 in non-autoclaved soil		26.21	78.03	36.74	73.98	10.12	80.31
7	Plant + *B. cereus* CPOU13 in autoclaved soil		31.49	73.61	41.40	70.68	15.20	70.42

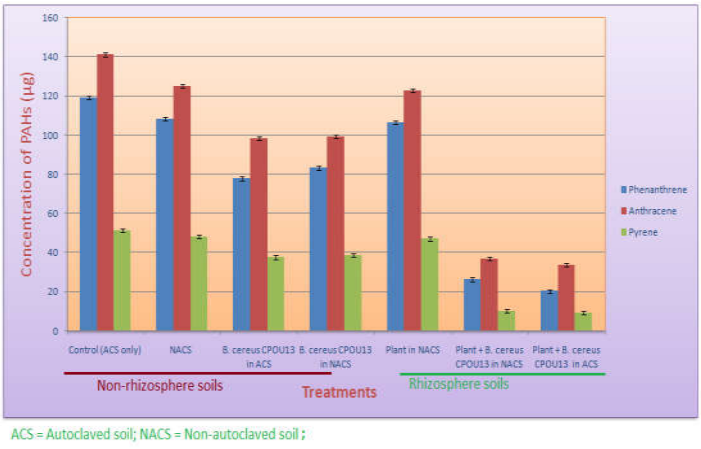

Fig. 2. Effect of *B. cereus* CPOU13 on rhizodegradation of phenanthrene, anthracene and pyrene in pot culture after 90days (Error bars represent standard deviation of three replicates)

Table 4. Total bacterial populationin *B. cereus* CPOU13 augmented blackgram soils in pot culture

S. No.	Bacterial population($\times 10^4$ CFU/g soil)						
	Treatment		0th day	15days	30days	60days	90days
1	Control(Autoclaved soil)	Non-rhizosphere soils	0	0	0	0	0
2	Non-autoclaved soil		73	76	76	79	76
3	*B. cereus* CPOU13 in autoclaved soil		3.3	5.6	7.3	7.3	7.0
4	*B. cereus* CPOU13 in non-autoclaved soil		166	200	200	200	160
5	Plant in non-autoclaved soil	Rhizosphere soils	73	86	93	130	100
6	Plant + *B. cereus* CPOU13 in non-autoclaved soil		160	230	300	400	360
7	Plant + *B. cereus* CPOU13 in autoclaved soil		3.3	80	100	300	300

Total bacterial populationin *b.cereus* CPOU13 augmented soils during rhizodegradation

Population of the bacteria in rhizosphere is of great importance in plant growth promotion and PAHs rhizodegradation. In view of this, population of bacteria was enumerated after

Highest bacterial population was observed in non-autoclaved rhizosphere soils while minimum bacterial population was noticed in *B. cereus* CPOU13 in non- autoclaved rhizosphere soil. Statistically the mean differences between treatments and time intervals are significant at 0.05 probability.

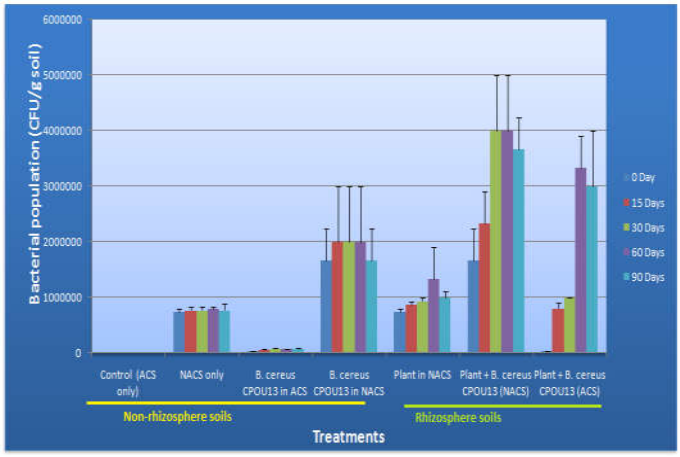

Fig. 3. Total bacterial population in *B. cereus* CPOU13 augmented rhizosphere and non-rhizosphere soils during rhizodegradation of PAHs in pot culture (Error bars represent standard deviation of three replacates)

DISCUSSION

Rhizosphere is a nutrient enriched microecological niche recognized for intensified root exudates, nutrients, abundant microbial population and their activities (Liste and prutz, 2006; Al-Abdulla *et al*. 2006). As this region accommodate huge microbial density it encompasses great diversity and catalyze several biochemical reactions including degradation of toxic pollutants like PAHs (Golubev *et al*. 2011; Tejeda-Agradano *et al*. 2013). More extentthe mutual association between plants and bacteria increase the efficiency of rhizodegradation of PAHs (Kuiper *et al*. 2004; Chaudhry *et al*. 2005). The bioaugmented bacillus strain efficiently degraded three PAHs namely phenanthrene, anthracene and pyrene. Interestingly, PAHs degradation was more in rhizosphere soils over the non-rhizosphere soils. Similarly, addition of strains, *Pseudomonas putida* UW3, *Azospirillumbrasilense* Cd and *Enterobacter cloacae* degraded creosote (PAHs mixture) up to 45% in rhizosphere soils of *Festucaarabinacea* in four months was reported (Huang *et al*. 2004). In autoclaved rhizosphere soils phenanthrene degradation occurred about to 83.03% by the influence of strain vise versathe strain recorded moderate level of PAHs degradation (30-40%) in autoclaved and non-autoclaved non-rhizosphere soils. The anthracene degrded up to 76.10% in the treatment of autoclaved rhizosphere soil and it was slightly decreased in non-autoclaved rhizosphere soil.Degradation of anthracene was moderate in autoclaved and non-autoclaved non-rhizosphere soils. Degradation of pyrene by the strain reached up to 82.1% in autoclaved rhizosphere soil. However,low degradation of pyrene(15-30%) was observed in autoclaved and non-autoclaved non-rhizosphere soils. This dismilar degradation of PAHs compounds by the strain may be due to difference in avilabilty, structural complexes of PAHs and substrate specific enzymes during degradation process (Teng *et al*. 2010; Baneshi *et al*. 2014).

Survival and division of the bio-augmented PAHs degrading bacteria in soil is crucial for start and proceeding of PAHs degradation in green house and field experiments (Radzi *et al*. 2015). High level of competence exists in rhizosphere because of diversified microorganisms. Sometimes this competition may limit survival and growth of introduced PAHs degrading bacteria (Child *et al*. 2007). In the present study, the strain recorded significant PAHs compounds degradation by the survival and division of the strain in pot culture studies. Its population increase was more in rhizosphere soils compared to non-rhizosphere soils. Increased in bacterial population reached maximum by 60days and afterwards, the population was decreased slightly. Comparatively, the highest bacterial population was observed in non-autoclaved rhizosphere soils while the lowest bacterial population was noticed in *B. cereus* CPOU13 non- autoclaved rhizosphere soil. In effective PAHs remediation systems, population of PAHs degraders drastically increase (Tejeda-Agredano *et al*. 2013) and become ultimate forces for rhizodegradation (Parrish *et al*. 2005). Diab and Sandouka (2010) reported significant increase in rhizosphere soils while non-significant increase in non-rhizosphere soils. The similar results published by Leigh *et al*. (2002), Ling and Gao, (2004). In the present study, we also observed slight increase in bacterial population non-rhizosphere soils was may be due to watering, aeration, nutrients availability and optimum growth conditions in green house.

Conclusions

Thepresent study paves ways to determine the effect of bioaugmentedbacteria and naturally soil inhabitant bacterial population for their abilities towards PAHs degradation. Perhaps the determined PAHs degradation in the treatment soils is accomplished by the ability of the selected strain to degrade PAHs compounds and its successful survival and divisions during the study period. Yet this type of studies needs more efforts in near future for clarification of many counters that arise during natural and induced rhizoremediation systems.

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