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Effect of Zinc Sulfate and Salicylic Acid on Biological Degradation of Phenanthrene in the Cd Polluted Soil under Sorghum Cultivation Inoculated with *Pseudomonas Putida*

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KEYWORDS

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ABSTRACT: Co-contamination of soils with heavy metals or petroleum hydrocarbons is one of the important environmental problems. This study was done to evaluate the effect of $ZnSO_4$ and salicylic acid (SA) on biological degradation of phenanthrene in the Cd polluted soil under sorghum cultivation inoculated with *Pseudomonas putida* (*P.putida*). Treatments were consisted of applying $ZnSO_4$ (0 and 40 kg/ha), SA foliar application (0 and 1.5 mmol/lit), Cd polluted soil (0, 5 and 10 mg Cd/kg soil) and soil pollution with phenanthrene at the rates of 0, 3 and 6% (W/W) in three replicate in the presence of *P. putida*. Plant in this experiment was sorghum. At the end of this experiment, plant was harvested and the plant Cd concentration was measured using atomic absorption spectroscopy. On the other hand, the degradation of phenanthrene (%) in the soil and soil microbial respiration via evaluated CO_2 were measured. Based on the results of this study, applying 40 kg/ha $ZnSO_4$ significantly decreased the plant Cd concentration by 14.3 %. In addition, a significant increasing by 15.4 % in degradation of phenanthrene in soil was also observed when the soil received 40 kg/ha. The similar results were also observed for SA foliar application.Soil application of $ZnSO_4$, the presence of *P. putida* and foliar application of salicylic acid can increase plant resistance to abiotic stresses and thereby have significant effect on biological degradation of phenanthrene. However, the role of plant type on degradation of phenanthrene cannot be ignored.

INTRODUCTION

Total petroleum hydrocarbons (TPHs) referred to a large family of different chemical compounds that origin from crude oil. In different petroleum products, TPH compounds are known as the initial component which is mainly released due to the petroleum product spills either on land or water [1]. The physic-chemical characteristics of some TPH compounds and petroleum products containing TPHs are well described and can be applied to determine the fate of TPH transport fractions after releasing it to the environment [2].

Human activities via agriculture or industrial products have considerable effect on the spill of hydrocarbons. Despite the main role of petroleum as a source of energy and its significant contribution in development of economic and social situation of a country, it has recognized as a most important kinds of organic pollutant which is released in

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environment through the main leakage of underground storage tanks and accidental spills during transportation and disposal. Total petroleum hydrocarbons in soil have a negative influence on human health and plant growth [3].

Simultaneous contamination of soil with TPHs and heavy metals is a common problem around the world [4, 5]. According to the US EPA's national priority list (NPL) about 40% of dangerous waste places are co-contaminated with organic (TPHs, PAHs and PCBs) and heavy metal (As, Cd, Cr, Cu, Pb, Hg, Ni and Zn) contaminants [6, 7]. Soil pollution with heavy metals and organic pollutants threaten human health, as well as plants and animals. TPHs are recognized as a major organic pollutants, especially in recent years [8, 9].

Overall, many different methods, such as physical, chemical degradation, photodegradation, have been developed for remediation of polluted soil. However, most of these technologies are not suitable for remediation of petroleum hydrocarbon polluted soil [10]. For example, some of these methods produced the new compounds (daughter compounds) which are more hazardous for the environment than the initial compounds (parent compounds) [11].

The use of some plants (phytoremediation method) to remove the pollutants from soil is a common approach to detoxify the contaminated environments. However, the combination of organic and inorganic pollutants may reduce the efficiency of phytoremediation through their interaction with each other and/or plants and rhizosphere. In this way, using methods to increase plant resistance to abiotic stress may help enhance phytoremediation efficiency. In this regard, Guo et al. reported that SA might improve plant defense against Cd toxicity. However, they only pointed to the role of SA on Cd toxicity. On the other hand, biological methods are recognized as the best environmental friendly approach for remediating soils contaminated with hydrocarbon and heavy metal. In this method, the capacity of indigenous microorganisms in the soil is used to convert the hydrocarbons and heavy metals into harmless substances [13].

Biological remediation, a technology that uses the microorganisms or plants for removing or detoxifying organic and inorganic xenobiotic compounds from the soil, is a green technology to solve the environmental degradation problem. This approach benefits from the microbial enzymatic activities to transform or degrade the pollutants in the soil. Compared to other methods, this is an effective remediation method; since it is a natural process and it usually does not provide toxic by-products. Instead, it provides a permanent solution to eliminate or reduce pollutants from the soil through completely mineralizing the contaminants in the soil. Briefly, the advantages of biological remediation method over other remediation methods are reduction of contaminants transferring to another place by distracting them in place, reduction of workers' exposure to the pollutants, enhancing the public health, as well as the significant reduction in the period of the remediation process [15, 16].

Pantsyrnaya et al. studied the role of *Pseudomonas putida (P.putida)* in biodegradation of phenanthrene and found that it had an effective contribution in degradation of phenanthrene [17]. Han et al. investigated the biodegradation of phenanthrene by *P. putida* and mentioned the effective roles of temperature and pH of experimental condition on degradation of phenanthrene. However, in this study which was done in vitro the effect of soil chemical agents such as heavy metal pollutant and its effect on the *P. putida* activity have not been studied [18].

It is necessary to mention that plant growth condition and plant tolerance to abiotic stress such as heavy metal contamination can influence on biodegradation of petroleum hydrocarbons [19]. Therefore, using a suitable strategy to improve plant resistance against abiotic stress such as enhancing the biomass production of plants can help to elevate the petroleum hydrocarbons biodegradation. In this way, the results of studies showed that Zn also participates in antioxidant enzymes defense system and it can increase the plant resistance to abiotic stresses [20, 21]. Nevertheless, the interaction effects of abiotic stresses and their role in biological degradation have not been considered. Thus, this research was carried out to investigate the effect of zinc sulfate ($ZnSO_4$) and salicylic acid (SA) on biological degradation of phenanthrene in the Cd polluted soil under sorghum cultivation.

MATERIALS AND METHODS

To investigate the effect of $ZnSO_4$ and SA on biological degradation of phenanthrene in the Cd polluted soil a non-saline soil with the low organic carbon was selected. Selected physic-chemical properties of studied soil are shown in Table 1.

Table 1. Selected physic-chemical properties of studied soil.

Characteristic	Unit	Amount
Sand	%	55
Silt	%	30
Clay	%	15
pH	----	6.9
EC	dS/m	1.2
OC	%	0.1
Total Pb	mg/kg	ND*
Total Zn	mg/kg	ND
Total Cd	mg/kg	ND

*ND: Not detectable by atomic absorption spectroscopy

This research was done as a factorial experiment in the layout of randomized completely block design at three replications. Treatments (72 treatments in three replicate) consisted of Cd pollution at the rates of 0 (Cd0), 5 (Cd5), and 10 (Cd10) mg Cd/kg soil, soil pollution to phenanthrene at the rates of 0 (P_0), 3 (P_3) and 6 (P_6) % (W/W). Foliar application of SA at the rates of 0 (SA_0) and 1.5 ($SA_{1.5}$) mmol/lit was done two weeks after sorghum (Pegah cv.) seedling growth in the presence (*P.putida* (+)) and absence of *P.putida (P.putida* (-)) and finally application of $ZnSO_4$ as a Zn source at the rate of 0 (Zn_0) and 40 (Zn_{40}) kg/ha.

Soil was polluted with Cd at the mentioned levels and incubated for two weeks to equilibrium. Then, the soil was treated with $ZnSO_4$ at the rates of 0 and 40 kg/ha and two weeks incubated. During this time the soil was wet and dried to equilibrium. After that, the soil was polluted with phenanthrene at the rates of 0, 3 and 6% (W/W) and two weeks incubated. The 5 kg pots were filled with the treated soil. Thereafter, half of the sorghum seeds were inoculated with a bacterial suspension as described by Planchamp et al (2015) [22], and then planted in the experimental pots.

Two weeks after planting, foliar application of SA was applied. After 8 weeks, plants harvested and plant Cd concentration was measured using atomic absorption spectroscopy (Perkin Elmer model 3030). The degradation of phenanthrene in the soils were extracted from 30 g soil subsamples by Soxhlet using a 1:1 (v/v) dichloromethane and n-hexane (150 ml) mixture for 24 h and concentration of residual phenanthrene in soil samples were determined in soil extracts using gas-chromatography (GC) according to the Besalatpour et al. [23]. The basal soil microbial respiration was measured as evolved CO2 [24]. For this purpose, 3 replicate soil samples of each treatment were incubated for three days at 26°C in 250-ml glass containers closed with rubber stoppers. The evolving CO_2 was trapped in NaOH solution and the excess in alkali was then titrated with HCl.

The statistical analysis was done using SAS V. 9.1. The least significant difference (LSD) test was used to determine the differences between the means. The 95 percentage (P=0.05) probability value was considered to determining the significant difference

RESULTS AND DISCUSSION

The interaction effects of SA foliar application, Cd concentration, $ZnSO_4$ application and soil pollution to phenanthrene on soil Zn concentration was significant (P=0.05).

The greatest soil Cd concentration has belonged to the soil without receiving Zn sources with the highest level of soil pollution to Cd that maybe related to the interaction effects of Cd and Zn (Table 2), while the lowest that was observed to the Cd-polluted soil (5 mg Cd/kg soil) with the highest apparition rate of $ZnSO_4$. Soil Cd concentration in non-polluted soil was not detectable by AAS.

Table 2. Effect of treatments on soil Cd concentration (mg/kg).

Phenanthrene (%)	**$ZnSO_4$ (kg/ha)**	***Pseudomonas putida* (+)**						***Pseudomonas putida* (-)**					
		$Cd0SA_0$	**$Cd0S_AA_{1.5}$**	**$Cd5SA_0$**	**$Cd5SA_{1.5}$**	**$Cd10SA_0$**	**$Cd10SA_{1.5}$**	**$Cd0SA_0$**	**$S_1SA_{1.5}$**	**$Cd5SA_0$**	**$Cd5SA_{1.5}$**	**$Cd10SA_0$**	**$Cd10SA_{1.5}$**
P0	0	ND*	ND	4.01u	4.30r	8.15k	8.31j	ND	ND	3.51x	3.92v	8.14k	8.51h
	40	ND	ND	3.71w	4.22s	8.00m	8.12l	ND	ND	3.44y	3.72w	8.00m	8.31j
P3	0	ND	ND	4.22s	4.42q	8.42i	9.00b	ND	ND	3.71w	4.12t	8.31j	8.72e
	40	ND	ND	4.02u	4.31r	8.31j	8.65f	ND	ND	3.50x	4.00u	8.12l	8.42i
P6	0	ND	ND	4.51p	4.84n	9.00b	9.17a	ND	ND	3.92v	4.3r1	8.72e	8.92c
	40	ND	ND	4.42q	4.72o	8.71e	8.88d	ND	ND	3.71w	4.01u	8.54g	8.71e

*ND: Not detectable by AAS, **means with the similar letters are not significant (P=0.05),

Soil Cd concentration in non-polluted soil was not detectable by atomic absorption spectroscopy (AAS). Based on the results of this study, applying 40 kg/ha $ZnSO_4$ significantly decreased the soil Cd availability by 12.3%. Ueno et al. investigated the interactions between Cd and Zn in relation to their hyper-accumulation in *Thlaspi caerulescens* and concluded that increasing soil Zn concentration had adverse effect on soil Cd concentration that is a positive point in environmental studies [25].

SA foliar application significantly increased the soil Cd concentration that maybe related to the role of SA application on increasing plant biomass (Table 2) and thereby enhance plant root exudate. Increasing root exudate can facilitate the availability of Cd in the soil. Luo et al. investigated the variation of root exudates from the Hyperaccumulator *Sedum alfredii* under cadmium stress and concluded that root exudates can react with soil heavy metal ions and increase soil metal solubility, mobility, and phytoavailability [26]. However, plant type and the compound of root exudate have different effect on soil heavy metal availability. Root exudates from a plant are plant metabolites that are released to root surfaces or into the rhizosphere to enhance plant nutrient uptake or copy with environment stresses. They are generally classified into two types, namely, high molecular weight (HMW) and low molecular weight (LMW) materials. In this way Pinto et al. studied the effect of Cd on root exudates of sorghum and maize plants and concluded that the metal-binding capabilities of root exudates may be an important mechanism for stabilizing metals in soil, as, they reported that malate and citrate should contribute to tolerance mechanisms of these plants, reducing deleterious effects of free Cd on root growth [27]. Increasing soil Cd availability was also observed in the presences of *P. putida* ,as, the results of this study showed that the greatest soil Cd availability was belonged to the plant inoculated *with P.putida.* The greater soil Cd availability in the presence of *P. putida* may be attributed to its role in decreasing soil pH [28]. Increasing soil heavy metals availability with decreasing soil pH is mentioned by researchers [29, 30].

The greatest root Cd availability was observed in the Cd polluted soil (10 mg Cd/kg soil) polluted with 6% (W/W) phenanthrene, while the lowest that has belonged to the Cd polluted soil (5 mg Cd/kg soil) without receiving any petroleum hydrocarbons (Table 3).

Table 3. Effect of treatments on root Cd concentration (mg/kg).

Phenanthrene (%)	$ZnSO_4$ (kg/ha)	*Pseudomonas putida* (+)						*Pseudomonas putida* (-)					
		$Cd0SA_0$	$Cd0S_AA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$	$Cd0SA_0$	$S_1SA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$
P0	0	ND	ND**	5.1u	6.0pq	12h	12.5g	ND	ND	4.7v	5.6st	11.0j	11.4i
	40	ND	ND	4.8v	5.5t	11.4i	12.0h	ND	ND	4.2w	5.0u	10.4k	10.9j
P3	0	ND	ND	5.9qr	6.3n	13.0f	13.5e	ND	ND	5.5t	5.9qr	12.5g	13.1f
	40	ND	ND	5.5t	6.0pq	12.6g	13.1f	ND	ND	5.0u	5.6st	12.0h	12.6g
P6	0	ND	ND	6.2no	6.9l	14.3bc	14.6a	ND	ND	5.8r	6.2no	13.8d	14.2c
	40	ND	ND	6.1op	6.5m	13.8d	14.4b	ND	ND	5.4t	6.0pq	13.5e	13.9d

ND: Not detectable by AAS,**means with the similar letters are not significant (P=0.05), *ND: Not detectable by AAS.

Root Cd concentration in non-polluted soil was not detectable by AAS. The remarkable point is that with increasing soil pollution to phenanthrene, the soil microbial activities was increased indicating that soil contaminated have adapted with petroleum compounds and used it as a carbon source [24]. Increasing soil microbial respiration (Table 4) with increasing soil pollution to phenanthrene confirms our results clearly. However, the greater level of soil petroleum hydrocarbons have adverse effect on soil microbial activates due to its toxicity.

Table 4. Effect of treatments on soil microbial respiration (mg C-CO_2/kg soil).

Phenanthrene (%)	$ZnSO_4$ (kg/ha)	*Pseudomonas putida* (+)						*Pseudomonas putida* (-)					
		$Cd0SA_0$	$Cd0S_AA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$	$Cd0SA_0$	$S_1SA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$
P0	0	8.21b'	8.41z*	8.00e'	8.11d'	7.77h'	7.91f'	8.00e'	8.11d'	7.76h'	7.60i'	7.50j'	7.60i'
	40	8.29a'	8.52y	8.11d'	8.19c'	7.89g'	8.00e'	8.21b'	8.30a'	7.89g'	8.00e'	7.77h'	7.91f'
P3	0	14.75j	14.81i	14.50m	14.71k	14.00s	14.21q	14.50m	14.72k	13.12w	14.11r	13.00x	14.03s
	40	14.83i	15.00e	14.66l	14.83i	14.11r	14.38o	14.70k	14.75j	14.40n	14.52m	14.00s	14.11r
P6	0	15.11d	15.31b	14.8i3	15.1d1	14.70k	14.91g	15.00e	15.22c	13.85t	14.66l	13.44v	13.58u
	40	14.21q	15.42a	14.96f	15.22c	14.88h	15.11d	15.11d	15.30b	14.66l	14.40n	14.22q	14.30p

*Means with the similar letters are not significant (P=0.05).

Increasing soil microbial respiration due to increasing carbon sources can affect soil Cd availability that may be related to the role of soil microorganism in decreasing soil pH. Although the results of this study showed that increasing soil pollution to phenanthrene can help the microbial activities in Cd polluted soil, but the greater level of soil Cd may adverse effect on soil microorganism activity that is needed to investigate in the future studies.

SA foliar application had significant effect on increasing root Cd concentration, as, the greatest root Cd concentration has belonged to the plant receiving SA foliar application (Table 3). Applying 1.5 mmol/lit SA significantly increased the root Cd concentration by 8.4 % that can be related to the role of SA foliar application on increasing plant resistance to abiotic stress (enhance plant biomass) and thereby increasing root Cd concentration. Kohli et al. investigated the role of SA in plants tolerance to heavy metal stress and concluded that application of optimal concentrations of SA can enhance plants tolerance to heavy metal stress that is similar to our results [31]. On the other hand, application of 40 kg/ha $ZnSO_4$ significantly decreased the Cd root concentration, as, the greatest root Cd concentration was observed in the soil without receiving any Zn sources. The similar results were observed for Cd shoot concentration (Table 5).

Table 5. Effect of treatments on shoot Cd concentration (mg/kg).

Phenanthrene (%)	$ZnSO_4$ (kg/ha)	***Pseudomonas putida* (+)**						***Pseudomonas putida* (-)**					
		$Cd0SA_0$	$Cd0S_AA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$	$Cd0SA_0$	$S_1SA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$
P0	0	ND	ND	3.5wx**	4.1rs	8.0i	8.3h	ND	ND	3.0y	3.7uv	6.5m	7.0l
	40	ND	ND	3.1x	3.7uv	7.5j	8.0i	ND	ND	2.8z	3.2x	6.3n	6.6m
P3	0	ND	ND	4.0st	4.5p	9.0f	9.7b	ND	ND	3.6vw	4.0st	7.3k	9.0f
	40	ND	ND	3.8uv	4.2qr	8.5g	9.4d	ND	ND	3.2x	3.5wx	7.0l	7.6j
P6	0	ND	ND	4.5p	4.9o	9.5c	10.1a	ND	ND	3.9tu	4.5p	8.0i	9.5c
	40	ND	ND	4.2qr	4.5p	9.2e	9.7b	ND	ND	3.6vw	4.3q	7.6j	9.0f

*ND: Not detectable by AAS, **means with the similar letters are not significant (P=0.05),

The greatest degradation of phenanthrene in soil was observed in the non Cd –polluted soil in the presence of *P. putida*, while the lowest that was in the absence of *P.putida* (Table 6).

Based on the results of this study, the presence of *P.putida* significantly increased the phenanthrene degradation in the Cd-polluted soil (5 mg cd/ kg soil) by 14.6 %. However, with increasing soil Cd concentration the phenanthrene degradation in soil was decreased. Pantsyrnaya et al. investigated biodegradation of phenanthrene by *P. putida* and concluded that increasing soil pollution to phenanthrene has adverse effect on its degradation due to decreasing soil microbial activity. However, they did not considered the soil chemical properties such as soil heavy metals [17]. The important point of our research is that soil contamination with phenanthrene up to 6 (W/W) did not have any effect on reducing the activity of Putida bacteria, indicating that the bacteria used this contamination as carbon source.

Table 6. Effect of treatments on phenanthrene degradation in soil (%)

Phenanthrene (%)	$ZnSO_4$ (kg/ha)	***Pseudomonas putida* (+)**						***Pseudomonas putida* (-)**					
		$Cd0SA_0$	$Cd0S_AA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$	$Cd0SA_0$	$S_1SA_{1.5}$	$Cd5SA_0$	$Cd5SA_{1.5}$	$Cd10SA_0$	$Cd10SA_{1.5}$
P0	0	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
	40	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
P3	0	66.4g	70.5c	63.1i	65.7h	57.4m	60.4k	60.1k	63.2i	57.4m	60.4k	50.1p	53.2o
	40	69.8d	72.1b	65.8h	68.1e	60.3k	63.1i	65.7h	67.3f	60.3k	63.2i	53.9o	55.9n
P6	0	70.8c	73.1b	67.3f	69.8d	60.4k	65.7h	65.4h	68.8e	60.5k	65.6h	55.3n	58.1l
	40	72.2b	74.6a	69.1d	72.1b	65.2h	68.1e	68.2e	72.1b	65.2h	69.8d	58.4l	61.2j

NM: Not measured, **means with the similar letters are not significant (P=0.05).

Application of $ZnSO_4$ had significant effect (P=0.05) on phenanthrene degradation in soil, as, the greatest degradation of soil was associated with the application of 40 kg/ha $ZnSO_4$. It can conclude that application of $ZnSO_4$ can reduce the soil Cd availability (the competitive effect) and thereby decrease its negative role on soil microbial activity. However, the positive and direct role of $ZnSO_4$ application on increasing soil microorganism activity cannot be ignored.

Raiesi et al. investigated the decomposability of some plant residues and their subsequent influence on soil microbial respiration and biomass, and enzyme activity and concluded that bacteria need a minimum amount of nutrients for their functions, and in many cases the nutrients in plant residues can help greatly to increase the activity of microorganisms [32] that is similar to our results. Karimian et al. was also showed the similar results [33]. On the other hand, the interaction effects of Zn and Cd and its role on the changes on soil microbial activity (soil microbial respiration) cannot be ignored. Accordingly, application of 40 kg/ha $ZnSO_4$ significantly increased the degradation of phenanthrene and soil microbial respiration in soil by 11.3 and 14.2 %. As mentioned before, using Zn sources as $ZnSO_4$ can enhance the plant resistance to abiotic stress (increasing plant biomass) and therefore increase root exudate.

Regardless of the amount of soil pollution to heavy metals or petroleum hydrocarbons, root exudates are a source of carbon and energy; they can provide good conditions for increasing microorganism activities. Based on the results of this study, applying 40 kg/ha $ZnSO_4$ significantly increased the plant biomass, soil microbial respiration and degradation of phenanthrene in soil by 10.3, 14.2 and 16.1 %, respectively.

There was a strong relationship between the phenanthrene degradation in soil and soil microbial respiration indicating that 71% degradation of phenanthrene in soil can be estimated by soil microbial respiration (Figure 1). However, the role of other soil chemical properties on phenanthrene degradation in soil cannot be ignored.

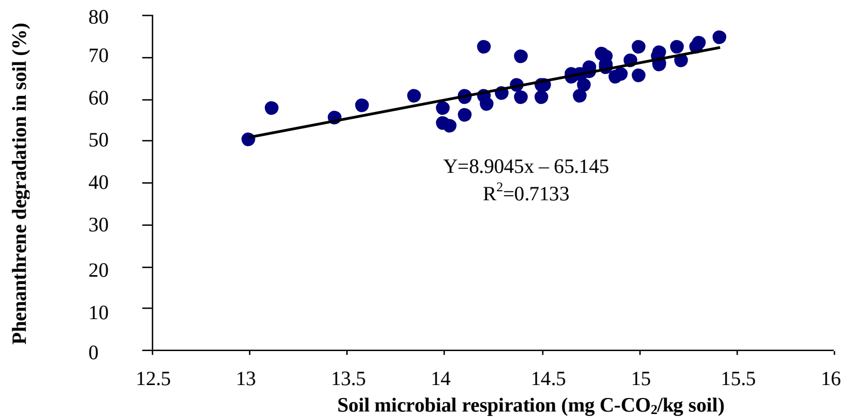

Figure 1. Regression equation between phenanthrene degradation in soils and soil microbial respiration.

CONCLUSIONS

The results of this study showed that application of SA and $ZnSO_4$ had significant effect on biological degradation of phenanthrene in soil. According this, the presence of *P. putida* had significant effect on increasing plant resistance to abiotic stress and thereby increased the degradation of phenanthrene in soil. A strong relationship (R^2=71) between phenanthrene degradation in soils and soil microbial respiration was observed. Based on the results of this study, increasing soil pollution to phenanthrene significantly increased the soil microbial respiration. However, the role of soil chemical properties on phenanthrene degradation in soils cannot be ignored. In addition, the interaction effects of heavy metals with petroleum hydrocarbions on degradation of phenanthrene in soil should be considered in the future studies.

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Conflicts of interest

There are no conflicts of interest.

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