Original Article

Additive Effect of Piriformospora Indica Fungus and Rhodococcus Erythropolis Bacteria on Bio-Remediation of Pyrene in a Pb-Polluted Soil Treated With Tire Rubber Ash

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Abstract

Background and Purpose: This research was conducted to evaluate the effect of *Piriformospora indica* fungus (P. indica) and *Rhodococcus erythropolis* (R. erythropolis) bacteria on bio-remediation of pyrene in a Pb-polluted soil that was treated with tire rubber ash.

Materials and Methods: Treatment consisted of applying tire rubber ash at the rates of 0 and 300 mg/kg soil, soil polluted with pyrene at the amount of 0 and 100 mg/kg soil, soil pollution with Pb (0, 400 and 800 mg/kg soil), and finally plant inoculated with P. indica fungus and R. erythropolis bacteria, and the plant used in this experiment was canola. After 60 days, plants were harvested and plant Pb and Zn concentration was measured using atomic absorption spectroscopy (AAS). The pyrene concentration in the soil samples were extracted by soxhlet using n-hexane and a 1:1 (v/v) dichloromethane during 24 h and measured according to the Besalatpour et al. (2011). The basal soil microbial respiration was measured as evolved CO_2.

Results: A significant increase (P=0.05) by 15.1% was observed in pyrene degradation in soil when plant inoculated with P. indica and R. erythropolis. However, soil pollution with Pb significantly decreased the pyrene degradation in the soil. At the same time, adding tire rubber ash to the soil significantly increased the plant biomass and pyrene degradation.

Conclusion: Plant inoculation with P. indica and R. erythropolis had an additive effect on pyrene degrading (bio-remediation) in soil that is an important factor in environmental studies. However, soil pollution with heavy metals showed an adverse effect on it.

Keywords: Pyrene; Lead; Bioremediation; Fungi; Polycyclic Aromatic Hydrocarbons

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1. Introduction

Simultaneous contamination of soil with petroleum hydrocarbon and heavy metals is a big environmental concern (1, 2). Petroleum hydrocarbons do not degrade naturally in soils due to their low water solubility and biological availability (1, 3). The presence of petroleum hydrocarbons in soils has effects on soil physic-chemical properties (4). On the other hand, petroleum hydrocarbons can reduce plant growth and activity of microorganisms by providing an impermeable layer at the soil surface (5). However, the most negative impact of them is their penetrative damage which is caused by the movement of pollutant into the fruit structure, and thereby injuring the fruits tissues and also threatening the human health (5, 6). Pyrene is one important component of petroleum hydrocarbons with the same structure as several carcinogenic polycyclic aromatic hydrocarbons, which are usually recognized in places polluted with crude oil, coal tar, and other complex mixtures of polycyclic aromatic hydrocarbons. It is one of the model substrates to investigate the metabolism of persistent polycyclic aromatic hydrocarbons (7). Petroleum hydrocarbons and heavy metals provide synergistic impact on soils which increases their toxicity for environment (3). Overall, different chemical and physical mechanisms can be used for remediation of contaminated soil (8). However, the application of these methods has some limitations as they are usually expensive and time consuming. In contrast, bioremediation is introduced as a suitable approach, because it is a cost-effectiveness method with a high removal efficiency of contaminants from the soil without any adverse effect on the environment (8, 9). Petroleum hydrocarbons are consumed by microbes to provide their required energy and nutrients, and they are finally neutralized or degraded to non-dangerous components (8). However, only some few strains of bacteria are able to grow in the petroleum hydrocarbon contaminated medias (8, 9). It has been revealed that the bacterial species of Staphylococcus, Streptococcus, Corynebacterium, Pseudomonas, and Acinetobacter are tolerant to heavy metals and petroleum hydrocarbons, and are able to degrade organic pollutants (10). In addition to this, some other soil microorganisms, such as Piriformospora indica (P. indica), can increase the plants resistance against biotic and abiotic stress by different mechanisms, such as increasing the nutrient uptake and improving the plant growth, activating plant antioxidant enzymes, increasing the synthetize of some plants hormones, and immobilizing pollutant via absorption or uptake (11). Additionally, this fungus can reduce the toxicity of pollutant in the edible parts of crop plants by reduction their translocation from roots to the aerial parts of plants (12). On the other hand, increasing the availability of some micronutrient, such as Zn in soil, especially in arid and semiarid regions, can reduce the Pb uptake by plant via competitive interaction of Zn and Pb (13), and also enhance the plant tolerant against pollutant (14).

It has been well-documented that tire rubber is a rich source of Zn (1–2 %), and might be used as a suitable and safe fertilizer (15, 16). Biodegradation of ground rubber in a Zn-deficient soils by soil microbes overtime increases the concentration of Zn and some other nutrients, such as calcium, iron, and sulfur (17). However, it is necessary to increase the realization of Zn from ground tire with some strategies, such as using some bacteria species which are capable in degradation of tire. The Rhodococcus erythropolis (R. erythropolis) has been recognized as a suitable and efficient species in degrading ground rubber and realizing the Zn from its structure (17). The notable point here is that, this species belongs to the Actinobacteria phylum which is able to survive in petroleum hydrocarbons contaminated soils. Considering this knowledge, the present research was conducted to investigate the pyrene degradation in soil and Pb uptake by canola (Brassica napus) as an important crop plant in Iran, as affected by the presence of P.indica and addition of ground rubber inoculated with R. erythropolis in a Pb polluted soil.

2. Materials and methods

To investigate the effect of the presence of P. indica fungus and R. erythropolis bacteria on bio-remediation of pyrene, a non-saline soil with low organic carbon was collected from the top 30 cm soil layer of a field at Pakal village, located 30 km to the west of Arak. This study was done as a factorial experiment (with 5 factors) in the layout of completely randomized block design. The treatment consisted of applying tire rubber ash at the rates of 0 and 300 mg/kg soil, soil polluted with Pb (at the rates of 0,400 and 800 mg Pb/kg soil), and pyrene spiked to the soil at the amount of 0 and 100 mg/kg soil in the presence of P. indica fungus and R. erythropolis bacteria. The selected soil physico-chemical properties are shown in Table 1.

Table 1. Some selected physicio-chemical properties of soil in this study

Characteristic	Unit	Amount
Soil texture	-----	Silty Loam
pH	------	7.0
EC	dS/m	0.8
Organic carbon	%	0.1
Cation exchange capacity	Cmol/kg soil	11.9
Available Pb	mg kg^{-1}	ND*
Available Cd	mg kg^{-1}	ND

***Before enrichment**

The soil was polluted with Pb at the rates of 0, 400 and 800 mg/kg soil, and incubated for two weeks to equilibrium. After that, the pyrene was spiked in to the soil at the amount of 0 and 100 mg/kg soil, and incubated for two weeks. Then, the polluted soil was treated with the bacterial (R. erythropolis) inoculated tire rubber ash and added to the 5 kg plastic pots. The inoculated bacteria in this study was capable of degrading tire rubber ash that was prepared according to the Khoshgoftarmanesh et al. methods (17). Plants used in this experiment were canola.

On the other hand, the fungal strain of P. indica used in this study was obtained from soil biology of water and soil research institute, and the P. indica inoculum in this experiments was prepared according to the Zamani et al., methods (18). Canola seedling were first surface sterilized in 15% H_2O_2 thoroughly washed in distilled water, and pre-germinated on moistened filter paper. After that, two canola seedlings were planted into each pot with five kg soil (14). After germination, two uniform sets of seedlings with radicles of about 1 cm length were selected for the experiment, one of which was inoculated with P. indica by immersion for 3 hours in inoculums (adjusted nearly to 2×10^6) under gentle shaking. The non-inoculated seedlings were dipped in sterilized distilled water containing Tween 0.02% (19). Then, the 5 kg pots were filled with the treated soils. The inoculated or non-inoculated seedling (10 seeds) was planted at a depth of 1cm in the uncontaminated top soil layer in the center of each pot, and irrigated to reach near field capacity (FC). After 70 days, plants were harvested and plant Pb and Zn was measured using atomic absorption spectroscopy (AAS) (20). In addition, the DTPA-extractable Pb (soil Pb availability) was measured using AAS which is the method described by Lindsay (21). Soil microbial respiration was determined by measuring the soils released CO_2 during 48 h of incubation (22). In addition, the pyrene concentration in the soil samples were extracted by soxhlet using n-hexane and a 1:1 (v/v) dichloromethane during 24 h, and then measured according to the Besalatpour et al. method (23).

Statistical analyses were calculated according to the ANOVA procedure. The statistical differences between the means were calculated by using the least significant difference (LSD) test. The 95 percentage (P=0.05) probability value was considered to determine the significant difference. By the mean, compliance of data dispersion with ANOVA model assumptions was verified using Shapiro-Wilcoxon's normality and Levene's equal variance tests. If necessary, the data were $\log_{10}$ or square root transformed.

3. *Results*

The greatest soil Pb availability belonged to the Pb-polluted soil (800 mg Pb/kg soil) that was polluted with 100 mg pyrene /kg soil, while the lowest was observed in the Pb polluted soil (400 mg Pb/kg soil) without any pyrene pollution (Table 2).

Table 2. The effect of tire rubber ash, Pb concentration, pyrene concentration, and the presence of P.indica and R.erythropolison on soil Pb availability (mg/kg)

Pyrne (mg/kg)	**tire Rubber ash (mg/kg)**	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Soil Pb (mg/kg soil)											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	ND*	80.3g**	95.3b	ND	70.2l	85.7e	ND	74.7j	90.7d	ND	65.2n	80.1g
	300	ND	77.4h	90.8d	ND	67.4m	80.3g	ND	71.7k	85.4e	ND	62.4o	74.7j
100	0	ND	85.2e	98.3a	ND	75.3i	90.5d	ND	80.2g	94.1c	ND	70.1l	85.1e
	300	ND	81.7f	95.4b	ND	70.1l	85.6e	ND	75.8i	90.6d	ND	65.3n	77.3h

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

The soil Pb availability in non-polluted soil was not detectable by AAS. Increasing soil pollution with pyrene significantly increased the soil Pb availability. For instance, increasing soil pollution with pyrene from 0 to 100 mg pyrene /kg soil significantly increased the soil Pb availability by 13.6% (Table 2).

Applying tire rubber ash had significant effect on the plant biomass (Table 3) and plant Zn concentration (Table 4), as the results of this study showed that applying 300 mg tire rubber ash/kg soil increased plant Zn concentration, and the plant biomass were grown in the soil polluted with 100 mg pyrene/kg soil by 8.3 and 14.6 %, respectively.

Table 3. The effect of tire rubber ash, Pb concentration, pyrene concentration, and the presence of P.indica and R.erythropolison on plant biomass (g/pot)

Pyrne (mg/kg)	**tire Rubber ash (mg/kg)**	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Soil Pb (mg/kg soil)											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	5.37b*	5.31d	5.20g	5.22f	5.18h	5.11j	5.31d	5.26e	5.16i	5.00m	4.86n	4.71q
	300	5.41a	5.35c	5.26e	5.26e	5.22f	5.15i	5.37b	5.31d	5.19h	5.11j	5.01m	4.82o
100	0	5.31d	5.23f	5.11j	5.15i	5.03l	4.87n	5.23f	5.19h	5.03l	4.77p	4.55r	4.33s
	300	5.38b	5.30d	5.15i	5.20g	5.15i	5.00m	5.30d	5.26e	5.07k	4.87n	4.76p	4.54r

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

Table 4. The effect of tire rubber ash, Pb concentration, pyrene concentration, and the presence of P.indica and R.erythropolison on plant Zn concentration (mg/kg)

Pyrne (mg/kg)	tire Rubber ash (mg/kg)	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Soil Pb (mg/kg soil)											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	12.8m*	12.6n	12.2o	11.4q	11.0s	10.5t	12.2o	11.8p	11.5q	11.0s	10.3u	10.0v
	300	30.2a	30.0b	29.4c	29.0d	27.8g	26.9h	29.5c	29.0d	28.4e	27.9g	26.4i	25.2k
100	0	12.6n	12.1o	11.8p	11.2r	10.2u	9.7w	11.8p	11.4q	11.1rs	10.3u	9.7w	9.2x
	300	30.0b	29.5c	29.1d	27.9g	26.8h	25.4j	29.1d	28.6e	28.0f	26.9h	25.4j	24.2l

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

The presence of P. indica or R. erythropolison had significant effect on increasing and decreasing plant biomass and plant Pb concentration (Table 5), respectively. Accordingly, the greatest plant biomass belonged to the treatments that simultaneously inoculated with P. indica and R. erythropolison. The important point of this study was that the synergistic effect of P. indica and R.erythropolison on increasing plant biomass was also significant. Based on the results of this study, a significant increase by 11.7, 13.6 and 15.2 % was observed in the biomass of plants grown in the Pb polluted soil (800 mg Pb/kg soil), when the plants were inoculated with P.Indica, R.erythropolison, and the co-inoculation of bacteria and fungi, respectively.

Table 5. The effect of tire rubber ash, Pb concentration, pyrene concentration and the presence of P.indica and R.erythropolison on plant Pb concentration (mg/kg)

Pyrne (mg/kg)	tire Rubber ash (mg/kg)	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Soil Pb (mg/kg soil)											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	ND*	30.3k**	40.2c	ND	25.4p	30.2k	ND	27.8n	34.2g	ND	22.9u	26.2o
	300	ND	28.7lm	39.1d	ND	23.1t	26.2o	ND	26.2o	33.1h	ND	20.1w	24.1s
100	0	ND	35.2e	45.8a	ND	27.9n	35.3e	ND	32.7i	40.1c	ND	24.9r	29.2l
	300	ND	34.7f	45.3b	ND	25.2q	32.1j	ND	30.2k	39.2d	ND	22.1v	26.1o

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

The greatest pyrene degradation in soil was related to the non-Pb polluted soil under cultivation of plant inoculated with P. indica and R.erythropolison. Increasing soil pollution with Pb had significantly decreased the pyrene degradation in soil (Table 6). As the results of this study showed, increasing soil Pb pollution from 400 to 800 mg Pb/kg soil significantly decreased the degradation of pyrene in soil by 10.9 %. On the other hand, adding tire rubber ash had significant effect on increasing degradation of pyrene in soils. Based on the findings of the current study, adding 300 mg /kg soil of tire rubber ash in the pyrene polluted soil significantly increased its degradation in soil by 22.4%. Soil microbial respiration (Table 7) also showed similar trend with the pyrene degradation in soil.

Table 6. The effect of tire rubber ash, Pb concentration, pyrene concentration, and the presence of P.indica and R.erythropolison on pyrene degradation in soil (%)

Pyrne (mg/kg)	tire Rubber ash (mg/kg)	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Soil Pb (mg/kg soil)											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	NM*	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
	300	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
100	0	67.1b**	65.3d	62.1f	60.5h	57.4k	53.5n	64.2e	61.6g	58.2j	55.5m	53.1n	50.8p
	300	69.3a	67.1b	64.6e	62.7f	59.3i	55.9m	66.4c	64.4e	60.7h	57.4k	56.7l	52.9o

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

Table 7. The effect of tire rubber ash, Pb concentration, pyrene concentration, and the presence of P.indica and R.erythropolison on soil microbial respiration (mgC-CO2/kg soil)

Pyrne (mg/kg)	tire Rubber ash (mg/kg)	R.erythropolison **(+)**						R.erythropolison **(-)**					
		P.indica (+)			P.indica (-)			P.indica (+)			P.indica (-)		
		Pb											
		0	400	800	0	400	800	0	400	800	0	400	800
0	0	14.2m*	13.7n	13.2p	13.0q	12.5r	12.0t	13.7n	13.1pq	12.4rs	12.3s	12.0t	11.6u
	300	14.8l	14.1m	13.8n	13.5o	13.0q	12.4rs	14.1m	13.7n	13.1pq	11.5u	12.5r	12.1t
100	0	20.7b	20.3cd	20.1e	19.8f	19.3h	19.0i	20.2d	19.8f	19.5g	19.5g	18.9j	18.5k
	300	20.9a	20.8ab	20.7b	20.3cd	19.7f	19.4gh	20.4c	20.1e	19.8f	20.0e	19.5g	18.9j

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

4. *Discussion*

According to the results of the present study, increasing soil pollution with pyrene significantly increased the soil Pb concentration that maybe related to the role of bacteria on decreasing soil pH (5). However, the competitive role of petroleum hydrocarbons and heavy metals for adsorption on soil exchangeable soil surfaces should not be ignored (24). Increasing soil Pb availability with decreasing pH was previously mentioned by researchers (14, 21). Hussain et al. studied the heavy metals accumulation in vegetables that was irrigated with treated wastewater in an experimental study, and concluded that decreasing soil pH had a significant effect on increasing soil Pb availability. However, the role of other soil chemical properties on the changes in the soil Pb availability should be considered (25).

As mention before, adding tire rubber ash significantly increased plant biomass and plant Zn concentration, and the competitive effect of Zn and Pb can be useful in decreasing the negative effect of heavy metal uptake by plant (26, 27). Taheri et al. investigated the kinetics of Zn release from rubber ash and ground tire rubber in a calcareous soil with a high pH as a Zn fertilizers, and concluded that ground rubber and rubber ash has a strong value as Zn organic fertilizer for Zn deficient soils (16). Rahimi et al. evaluated the soil quality index with applying Zn organic fertilizer and its relationship with grain Zn concentration of wheat plant, and concluded that applying 10 t/ha tire rubber ash can significantly affect increasing grain Zn concentration (28).

However, they did not consider the role of soil physico-chemical properties on the changes in soil quality factors. At the same time, Khoshgoftarmanesh et al. investigated the effect of tire rubber ash on increasing wheat Zn concentration, and concluded that soil application of 250 kg rubber tire ash/ha had significant effect on increasing and decreasing Zn and Cd concentration in grain wheat, respectively (29).

Today, Zn deficiency in semiarid and arid regions is a basic problem due to its high soil pH. Application of organic amendments, such as tire rubber ash, can increase the soil Zn availability that can be very effective in plant biomass (30, 31). It is noteworthy that fertilizer management and soil type have important influence on plant Zn uptake (32). As the results of the current study showed, soil pollution with heavy metals had negative effect on Zn uptake by plants. On the other hand, $ZnSO_4$ containing residues and by-products were used as a major source for correcting Zn deficiency. However, there was evidence indicating high Cd impurity and low Zn content of some of these Zn sources, which have been commonly marketed as commercial Zn fertilizers (16). Therefore, it is necessary to be careful in the use of fertilizers containing micro elements. Based on the results of our study, the concentration of Pb and Cd in tire rubber ash was below the detection limit of AAS (data was not shown). Therefore, tire rubber ash can be used as a Zn fertilizer even in industrial regions that are contaminated with heavy metals. However, microbial inoculation can help to release the nutrition elements (such as Zn) that are needed for the plant growth from organic amendments (such as tire rubber ash).

On the other hand, soil remediation seems necessary due to human induced soil pollution. Several in-situ and ex-situ physical, chemical, and biological methods have been used for soil pollution remediation. Among this, phytoremediation is a suitable way to remediate soil pollution. However, heavy metals have negative effects on plant biomass, and thereby decrease phytoremediation efficiency. Among these, plant inoculation can increase plant resistance to abiotic stresses. Mohd et al. reported that P. indica had increased the protection of host from heavy metals toxicity. According to their results, a significant increase by 1.7 times was observed in the plants biomass colonized with P. indica as compared to non-colonized plants (33). The important point of this study is that the additive effect of P. indica and R.erythropolison on increasing plant biomass was also significant, which can then lead to enhanced plant root exudate which is an important factor in increasing soil microbial population (Table 5). Meena et. al. stated that Co-inoculation of the P.indica with plant growth-promoting rhizobacteria can affect the microbial activity and the plant growth that is similar to our research (34).

Generally, heavy elements, such as Pb or Cd, have negative effects on the number, diversity, and microbial activity of soil microorganisms, and thereby, can decrease the release of their enzymes. However, soil chemical properties may also be affected by the toxicity of heavy metals. Li et al. reported that soil bulk density, water holding capacity, permeability and porosity depend on the concentration of heavy metals in soil (35). It is mentioned that the nutrient elements is needed for increasing soil microbial activities, and adding tire rubber ash can affect increasing soil Zn availability (29) that is an essential nutrient for the soil microbial activities, and thereby degradation of petroleum hydrocarbon in soil. Ponce-García et al. investigated the role of Zinc-Chelate on the green beans biomass and concluded that using Zn chelates has significant role in increasing plant biomass, and reported that the Zn source type had significant effect on increasing plant biomass, and thereby, changes in soil microbial activities (36), that is similar to our results. Increasing plant biomass can then help to increase plant root exudate that is needed for soil microbial activities (37).

The important point of this study was that although tire rubber ash is an important Zn source (38), Zn release from these compounds needed a long time, and microbial inoculation with specific bacteria decomposing polymer compounds, such as R. erythropolis, could help to increase Zn release. Khoshgoftarmanesh et al. introduced the R. erythropolis becyeria to speed the release of Zn from tire rubber, and used its product as a Zn fertilizer for corn and sunflower in a calcareous soil (17) that was similar to our results. Besed on the findings of our study, inoculation of tire rubber ash with R. erythropolis significantly increased the pyrene degradation and Plant Zn concentration, respectively. However, using P.indica speeds these processes.

5. Conclusion

Applying 300 mg tire rubber ash/kg soil significantly increased the pyrene degradation in Pb polluted soil. However, soil pollution with Pb significantly decreased the soil microbial respiration. Among them, the presence of R. erythropolis significantly increased the pyrene degradation in soil, and plant inoculation with P.indica had additive effect on it. Accordingly, the soil microbial respiration was increased with the simultaneous presence of P.indica and R. erythropolis. In addition, the presence of P. indica and R. erythropolis had significant effect on increasing plant biomass. According to the present research, it is necessary to investigate the role of other soil microorganisms on degradation of petroleum hydrocarbons in soil in future studies. By the mean, it is needed to consider the role of other co-contaminations on the changes in the population of petroleum hydrocarbons degrading bacteria.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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