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The Effect of Corn and White Clover Intercropping on Biodegradation of Diesel Oil in Arsenic Contaminated Soil in the Presence of *Piriformospora indica*

Amir Hossein Baghaie [a*] | **Amir Ghafar Jabari** [b] | **Roya Sattari** [c]

a. Department of Soil Science, Arak Branch, Islamic Azad University, Arak, Iran.
b. Department of Microbiology, Arak Branch, Islamic Azad University, Arak, Iran.
c. Department of Microbiology, Isfahan University of Technology, Isfahan, Iran.

***Corresponding author at:** Department of Soil Science, Arak Branch, Islamic Azad University, Arak, Iran.
Postal code: 38361-1-9131.
E-mail address: a-baghaie@iau-arak.ac.ir

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ABSTRACT

Background: This research was done to evaluate the effect of corn and white clover intercropping on bio-degradation of diesel oil in arsenic (As) contaminated soil in the presence of *Piriformospora indica (P.indica).*
Methods: Treatments included corn planting with three plant density of white clover (0, 20 and 30 seeds per pots) as an intercropping system, As polluted soil (0, 12 and 24 mg/kg soil) and diesel oil (0, 4 and 8 % (W/W) in the presence and absence of *P.indica* . After 10 weeks, plants were harvested and the soil and plant as concentration was measured using atomic absorption spectroscopy. In addition, the soil microbial activity and the percentage of diesel oil degradation were measured.
Results: The results of this study showed that cultivation of corn in intercropping system with white clover (1:20 seeds corn to white clover) relative to monoculture significantly ($P < 0.05$) increased the diesel oil degradation in soil by 14.6%. In addition, the soil microbial activity was increased by 13.4%. Plant inoculation with *P.indica* had additive effect on degradation of diesel oil in the soil.
Conclusion: Plant inoculation and intercropping system can effect on increasing the percentage of diesel oil degradation in As contaminated soil that is an important role in environmental studies.

1. Introduction

Petroleum hydrocarbons are combination of chemical mixtures that are all composed of carbon and hydrogen, so they are called hydrocarbons. These are chemically low soluble or even insoluble in water. Besides carbon and hydrogen which are required to form these compounds, nitrogen and sulfur may also exist in these compounds' structures. If these types of petroleum hydrocarbons are added to the environment, by their reaction with molecular oxygen, sulfur or nitrogen oxides and carbon monoxide are Produced and enter the atmosphere, thereby polluting atmospheric [1-3].

Polycyclic aromatic hydrocarbons (PAHs) compositions are composed by imperfect combustion of coal, oil, and gasoline and are utilized variously. For instance naphthalene is used in the production of insecticides, the dyeing industry, explosives, plastics, lubricants; anthracene is utilized in the production of insecticides, the dyeing industry and wood preservatives. Human can be affected differently from petroleum hydrocarbon contamination. some examples of getting contaminated to these oil derivatives at work or home, pesticides, drinking water pesticides, drinking water contaminated with total

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petroleum hydrocarbons (TPHs), working, at refineries in which oil is extracted or refined, residing in the areas nearby sites of oil extraction or refining, contact with TPHs contaminated soils as well as utilizing plants contaminated with these compounds or animals feeding on these plants. Likewise, different compounds of petroleum hydrocarbons can influence the human nervous system [4-6].

In many cases soils are contaminated with heavy metals, and contamination of petroleum hydrocarbons with heavy metals can endanger soil quality. Hence, we should seek significant strategies for reducing soil contamination with these compounds. Due to the growing costs and the relatively limited efficiency of regular chemical and physical approaches for remediation of contaminated soils, researchers make attempts to find new methods of soil remediation [7-10]. Thus, there is a need for an economy, sustainable and environmentally friendly way. One of the means embraced tremendously nowadays is phytoremediation. This technique is quite suitable for filtration of large areas of contamination as well as various pollutants in shallow sites, contaminated soils and water surface and subsurface. Actually, this is an innovative and modern approach in which plants play a key role in refining soil, sediments, and surface and groundwater contaminated with heavy metals, organic compounds, and radioactive materials [11-13].

Phytoremediation is presented as an environmentally-friendly way, with negligible, yet inexpensive, side effects on soil refining that is much more economical compared to conventional physical and chemical means to eliminate or reduce the concentration of hazardous pollutants from the soil [14-16]. However, decreasing plant biomass due to heavy metal toxicity can diminish the phytoremediation efficiency [17,18]. Accordingly, using intercropping system or plant inoculation with *Piriformospora indica (P.indica)* may affect the phytoremediation efficiency [19-21]. Though, they did not consider the simultaneous effect of heavy metal with petroleum hydrocarbons on plant phytoremediation efficiency. Thus, this research was conducted to evaluate the effect of corn and white clover intercropping on biodegradation of diesel oil in a arsenic (As) contaminated soil in the presence of *P.indica.*

2. Materials and Methods

Treatments included corn (*Z. mays L.Cv.* 704) planting (corn as a main crop and white clover as an intercrop) with three plant density of white clover (0, 20 and 30 seeds per pots) as an intercropping system and the simultaneous pollution of soil that was As (at the rates of 0, 12 and 24 mg/kg soil) and diesel oil (0, 4 and 8 % (W/W) in the presence and absence of *P.indica*. This research was done as a factorial experiment in the layout of randomized completely block design with three replications. Selected some physic-chemical properties are shown in Table 1.

Table 1: Selected Physicochemical Properties of Soil

Characteristic	Unit	Amount
Soil texture	-----	Silt loam
pH	------	7.1
EC	dS/m	1.2
Pb available	mg kg^{-1}	ND*
Cd available	mg kg^{-1}	ND
As available	mg kg^{-1}	ND
Organic carbon	%	0.2
$CaCo_3$	%	5

*ND: Not detectable by atomic absorption spectroscopy (AAS)

2.1. Soil Preparation and Plant Cultivation

To do this experiment, the soil was polluted with As at the mentioned rates and incubated for two weeks to equilibrium. After that, The As polluted soil was polluted with diesel oil (at the rates of 0.4 and 8% W/W) and incubated for two weeks to equilibrium.

Three days after seed germination on filter paper, the corn seedlings (as a main crop) and white clover (as an intercrop) were transferred into 5 kg plastic pots (two seedlings in each pot) as a monoculture and intercropping system and filled with the treated soil. Two fungal plugs of 10 mm in diameter were placed at a distance of 1 cm below the corn and white clover seedlings in the soil at sowing time. P. indica was obtained from the soil biology of water and soil research institute. P. indica was cultured in Petri dishes on a modified Kafer medium. During the plant growth, soil moisture was maintained at the field capacity (FC). After 10 weeks of plant growth, plant roots and stems were harvested, washed with tap water, rinsed with deionized water and oven-dried at 55°C for 72h. After that, the samples were digested with HCl 2 N and the As concentration in extracted solution was measured using atomic absorption spectroscopy (AAS) (PerkinElmer 3030; PerkinElmer, Wellesley, MA, USA) [22].

2.2. Chemical Analysis

The soil microbial respiration was measured according to the Besalatpour *et al.* (2011) method [23]. Accordingly, soil samples of different treatment were incubated for one week at 25°C in 250-ml glass containers closed with rubber stoppers. The CO_2 produced was trapped in NaOH solution and the excess in alkali was then titrated with HCl.

In addition, the total concentration of soil hydrocarbon (TPHS) were determined using the GC-MS with a Delsi DI 200 chromatograph equipped with a direct injection port and an FID detector at 340 °C aacording to the Besalatpour *et al.* (2011) [23]. The carrier gas was helium under 0.08 MPa and the column was a CP Sil 5 CB (Chrompack) capillary column (50 m by 0.32 mm, film thickness 0.25 μm).

2.3. Statistical Analysis

A completely block randomized design in three replications was used. The statistical analyses of data were performed using the ANOVA procedure by SAS V. 9.1 software. Differences between means were evaluated using the least significant difference (Duncan test). The 0.05 probability value was used to determine the significant difference.

3. Results and Discussion

The greatest soil As concentration belonged to the soil under cultivation of corn and white clover intercropping

(1:30 corn to white clover) that was polluted with the greatest level of diesel oil, while the lowest that was observed in the soil under corn monoculture system that was polluted with 12 mg As/kg soil and 4% (W/W) diesel oil (Table 2).

Increasing soil pollution to diesel oil from 0 to 4 % (W/W), increased the soil As concentration by 11.3% (Table 2). The results of the study of Mehrabadi *et al.* (2014) about the increasing soil and plant heavy metal concentration with increasing soil pollution to petroleum hydrocarbons confirm our results clearly [24]. In this regards, Askary *et al.* (2012) showed that simultaneous pollution of soils to heavy metals and petroleum pollutions had additive effect on increasing soil heavy metals availability [25].

In addition, intercropping system and its population density had significant effect on increasing soil As concentration. Accordingly, increasing white clover population density from 1:20 to 1:30 in intercropping system significantly (P < 0.05) increased the soil As concentration by 13.4%. Baghaie *et al.* (2018) investigated the heavy metal phytoremediation of corn and white clover cultivation in monoculture and intercropping system and concluded that intercropping system has positive effect on increasing Pb phytoremediation efficiency [26]. However, they did not consider the role of other soil pollutant such as petroleum hydrocarbons on the changes of heavy metals availability. Liu *et al.* (2013) investigated the heavy metal concentration in maize monoculture and intercropping with different legume species and reported that legumes caused a greater effect on heavy metal concentration in maize and peanut was a potential intercrop for enhancing heavy metal extraction from soil, that is similar to our results. Generally, Intercropping of maize with legumes is an alternative to maize monoculture and has a number of advantages compared to monoculture systems [27]. Li *et al.* (2010) studied the metal mobilization and production of short-chain organic acids by rhizosphere bacteria associated with a Cd/Zn hyper-accumulating plant and concluded that plant root exudate due to intercropping system had significant effect on increasing soil heavy metal availability [28].

In addition, corn inoculation with *P. indica* in monoculture and intercropping system had significantly (P< 0.05) increased the soil As concentration.

The results of this study showed that corn inoculation with *P.indica* in monoculture and intercropping system significantly (P < 0.05) increased the soil As convention in the soil that was polluted with 4% (W/W) diesel oil by 9.7% and 13.1%, respectively. Increasing soil As concentration maybe related to the role of *P.indica* fungus on increasing plant biomass and probably increasing the plant root exudate that help to increase soil heavy metal solubility Gill *et al.* (2016) investigated the role of *P.indica* on plant resistance to abiotic stress and concluded that plant inoculation with *P.indica* can decrease the negative effect of soil pollution and thereby increase the plant biomass. Accordingly increasing plant biomass may increase the plant root exudate that increases the solubility of soil As [29]. On the other hand, Wu *et al.* (2018) reported that *P. indica* could enhance phosphorous sorption by plants, thereby increasing the plant biomass by incrementing the acid phosphatase activities and organic acids in the plants, which was in turn associated with the higher plant resistance to abiotic stresses [30].

The greatest corn As concentration has belonged to the inoculated plants with *P.indica* that cultivated in the soil polluted with 24 mg As/kg soil and 8 %(W/W) diesel oil (Table 3), while the lowest that was measured in the plants grown in the As-polluted soil (12 mg As/kg soil) without any pollution with diesel oil. Increasing soil pollution to As significantly (P < 0.05) increased the plant As concentration in corn and white clover intercropping system that could be related to the increasing of soil As solubility. Increasing soil As solubility and thereby increase plant As concentration was reported by researchers. Mottaghi et al. (2015) reported that intercropping system is a useful method for heavy metal phytoremediation from contamination Soils [31]. Mansouri *et al.* (2017) investigated the effect of soil pollution to As on phosphorus, iron, zinc and manganese concentrations in soil and corn plant and concluded that increasing soil pollution to As significantly (P < 0.05) increased and decreased the As and nutrient elements, respectively [32]. However, they did not consider the role of other pollutant such as petroleum hydrocarbons on the changes heavy metal availability. The white clover As concentration showed the similar results (Table 4).

The presence of *P.indica* had significant (P < 0.05) effect on increasing corn and white clover As concentration. Based on the results of this study, the greatest plant As concentration was belonged to the inoculated plants with *P. indica.*

Table 2: Effects of corn and white clover intercropping, heavy metal and diesel oil on soil As concentration (mg/kg soil) in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	ND**	10.02v*	21.12n	ND	10.12u	21.51k
	4	ND	10.11u	21.34m	ND	10.26t	21.72i
	8	ND	10.24t	21.49l	ND	10.38s	21.85h
1:20	0	ND	10.18u	21.39m	ND	10.37s	22.01f
	4	ND	10.34s	21.53k	ND	10.54r	22.18e
	8	ND	10.57r	21.78i	ND	10.62q	22.44c
1:30	0	ND	10.39s	21.65j	ND	10.53r	22.31d
	4	ND	10.51r	21.78i	ND	10.87p	22.56b
	8	ND	10.84p	21.99g	ND	11.73o	22.78a

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

Table 3: Effects of corn and white clover intercropping, heavy metal and diesel oil on corn As concentration (mg/kg) in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	ND**	9.51w	21.56k	ND	9.77u	22.05h
	4	ND	9.65v	21.72j	ND	9.86t	22.11g
	8	ND	9.78u	21.97i	ND	9.93s	22.55c
1:20	0	ND	9.78u	22.00h	ND	10.04r	22.11g
	4	ND	9.82t	22.19g	ND	10.21p	22.24f
	8	ND	10.34	22.55c	ND	10.65m	22.78b
1:30	0	ND	10.11q	22.11g	ND	10.35o	22.39e
	4	ND	10.36o	22.32e	ND	10.44n	22.41d
	8	ND	10.67m	22.73b	ND	10.81l	23.89a

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

Table 4: Effects of corn and white clover intercropping, heavy metal and diesel oil on white clover As concentration (mg/kg) in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	ND**	8.74y	16.82m	ND	9.12v	17.31j
	4	ND	8.81x	16.95l	ND	9.55s	17.56i
	8	ND	9.08w	17.13k	ND	9.71t	17.87h
1:20	0	ND	9.01w	17.18k	ND	9.78t	19.11v
	4	ND	9.23u	17.37j	ND	9.87q	19.46e
	8	ND	9.38t	17.59i	ND	10.36o	19.83d
1:30	0	ND	31	17.38j	ND	10.12p	20.15c
	4	ND	9.34t	17.81h	ND	10.32o	20.74b
	8	ND	9.50s	18.28g	ND	10.65n	21.31a

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

Generally, inoculated plant with *P.indica* can enhance the plant biomass (Table 5) and plant resistance to abiotic stresses such as heavy metals or petroleum hydrocarbons. Increasing plant biomass can stimulate the soil microorganism activities that can increase soil heavy metals availability. However, the role on plant root exudate on low organic acid secretion and consequently decreasing soil pH cannot be ignored. Increasing soil heavy metal availability with redesigning soil pH is reported by researchers. On the other hand, applying white clover as an intercrop in corn cultivation had a significant effect on increasing plant phytoremediation efficiency. According to the results of Li *et al.* (2009) legumes can increase nitrogen fixation, which could help to increase the biomass and nutrient uptake of main crop (corn) and consequently increase its heavy metal phytoremediation efficiency [33]. On the other hand, the presence of white clover as an intercrop can provide the growth condition for soil microbial activity and thereby affect as phytoremediation efficiency. Nascimento *et al.* (2006) mentioned that low organic acids can affect on desorption of heavy metals from contamination soils and thereby increase heavy metal solubility. However, they concluded that increasing heavy metal solubility depends on the heavy metal type, as, the results of their studies showed that soil as solubility was not affected by low organic acids [34]. It is noteworthy that these researchers did not consider the simultaneous effect of soil contamination with heavy metals and petroleum hydrocarbons [34].

The greatest percentage of diesel oil degradation was observed in the soil with the highest level of diesel oil that was not polluted with As and under cultivation of corn and white clover intercropping system, while the lowest that was measured in the soil with the greatest and lowest level of As and diesel oil concentration, respectively (Table 6).

Increasing soil pollution with As significantly (P< 0.05) decreased the degradation of diesel oil in soil. Based on the results of this study, with increasing soil pollution with As from 0 to 12 mg As/kg soil, the degradation of diesel oil in soil was decreased by 13.9%. Xie *et al.* (2016) investigated the effect of heavy metals pollution on soil microbial diversity and plant genetic variation and reported that heavy metal pollution is a serious global environmental problem that alter the population of soil microbial activity [35]. Accordingly, decreasing soil microbial activity (Table 7) has negative effect on degradation of diesel oil in soil.

According to the results of some studies, petroleum hydrocarbons at low concentration can be introduces as a carbon sources for increasing soil microbial activities that can help to degrade the petroleum hydrocarbons.

Al-Hawash studied the microbial degradation of petroleum hydrocarbons in the environment and reported that petroleum hydrocarbons can be used as a carbon source for increasing soil microbial activates [36]. In this regard, Besalutpour *et al.* (2011) reported the similar results. Based on the results of their study petroleum hydrocarbon at the low concentration can be used as a carbon source for soil microbial activities [23].

The result of our study showed that increasing in heavy metal concentration has prevented the using of petroleum hydrocarbons as a carbon source by soil microorganisms and reduced the percentage of petroleum hydrocarbon degradation in soil. For instance, with increasing soil pollution with diesel oil from 0 to 4 and 8 % (W/W) the

Table 5: Effects of corn and white clover intercropping, heavy metal and diesel oil on corn plant biomass (g) in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	4.85e	4.77h	4.69l	4.81g	4.70k	4.65m
	4	4.76h	4.70k	4.64m	4.73i	4.64m	4.60o
	8	4.70k	4.63n	4.58p	4.69l	4.60o	4.56q
1:20	0	4.92c	4.84f	4.80g	4.87d	4.80g	4.74i
	4	4.88d	4.80	g4.75h	4.81g	4.73i	4.70k
	8	4.84f	4.75h	4.72j	4.77h	4.70k	4.65m
1:30	0	4.98a	4.89d	4.85e	4.92c	4.86e	4.80g
	4	4.95b	4.85e	4.84f	4.87d	4.80g	4.73i
	8	4.91c	4.80g	4.75h	4.81g	4.75h	4.70k

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

diesel oil degradation in non Pb-soil increased by 14.3 and 18.6%, respectively. However, with increasing soil As pollution from 0 to 12 and 24 mg As/kg soil, the degradation of diesel oil in soil decreased by 9.3 and 13.3%, respectively.

Plant inoculation with *P.indica* significantly (P < 0.05) increased the degradation rate of diesel oil in soil, as, the greatest soil degradation rate of diesel oil in soil was observed in the soil under cultivation of corn inoculated with *P.indica* in intercropping system.

The results of this research showed that plant inoculation with *P.indica* can diminish the negative effect of soil pollution with As on degradation of diesel oil in soil ,as, corn inoculation with *P.indica* significantly increased the degradation percentage of diesel oil in As-polluted soil (24 mg As/kg soil) by 13.3%. This increasing rate can be related to the role of *P.indica* on increasing plant resistance to abiotic stresses and thereby increase the soil microbial activity that help to degradation of diesel oil in soil. Increasing in corn biomass via decreasing AS- bio-transfer and increasing microbial activity in the soil under cultivation of plant inoculated with *P.indica* in monoculture and intercropping system confirm our results clearly. Mohd *et al.* (2017) reported that *P.indica* mediated protection of host from arsenic toxicity [37]. In addition, they suggested that *P. indica* protects the plants from As toxicity (that is similar to our results) via three mechanisms: by decreasing the As concentration in the plant, bio-transformation of the toxic arsenic salts into insoluble particulate matter and modulating the antioxidative system of the host cell [37].

Table 6: Effects of corn and white clover intercropping, heavy metal and diesel oil on the percentage of diesel oil degradation in soil in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	NC**	NC	NC	NC	NC	NC
	4	52.3n*	50.5p	45.6r	45.8r	44.1s	40.5t
	8	70.2f	67.5h	64.3j	66.5i	64.2j	62.1k
1:20	0	NC	NC	NC	NC	NC	NC
	4	54.6m	52.5n	51.30	50.2p	48.3q	45.5r
	8	73.5c	72.7d	71.7e	70.2f	68.4g	67.5h
1:30	0	NC	NC	NC	NC	NC	NC
	4	55.8l	54.3m	51.9o	54.2m	52.7n	51.6o
	8	75.8a	74.3b	72.6d	74.6b	73.1c	70.9f

*Data with the similar letters are not significant (P= 0.05), **NC: Not calculated

Table 7: Effects of corn and white clover intercropping, heavy metal and diesel oil on soil microorganism activity (mg-C-Co_2/kg soil) in soil in the presence and absence of *P.indica*

Corn to white clover ratio	Diesel oil (% W/W)	Soil As concentration (mg/kg soil)					
		P.indica (+)			*P.indica* (-)		
		0	12	24	0	12	24
1:0	0	9.27w*	9.00y	8.78z	9.03y	8.71z	8.47z
	4	11.39p	11.21q	11.00s	11.29q	11.16r	11.00s
	8	14.41	14.23g	14.01i	14.15h	14.00i	13.82j
1:20	0	9.47u	9.26w	9.11x	9.34v	9.19x	9.00y
	4	11.78m	11.56n	11.44o	11.55n	11.32p	11.11r
	8	14.71b	14.52d	14.27g	14.55d	14.32f	14.12h
1:30	0	9.53t	9.43u	9.22w	9.48u	9.29w	9.11x
	4	12.12k	12.00l	11.73m	11.78m	11.54n	11.32p
	8	14.84a	14.75b	14.62c	14.65c	14.55d	14.42e

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

4. Conclusion

The results of this study showed that corn and white clover intercropping system had significant effect ($P < 0.05$) on increasing the percentage of diesel oil degradation in soil. Accordingly, the presence of *P.indica* had significant effect ($P < 0.05$) on degradation of diesel oil in the soil. In addition, inoculation of corn with *P.indica* significantly ($P < 0.05$) decreased and increased the plant As concentration and plant biomass, respectively. Increasing the plant density of white clover as a intercrop significantly ($P < 0.05$) increased the As phytoremediation efficiency. However, soil physico-chemical condition can affect on the changes in heavy metal availability that is necessary to be considered in the future studies. Given that there is a problem of contamination of heavy metals and petroleum hydrocarbons in industrial areas of the country, it is necessary to study the simultaneous effect of other heavy metals and petroleum. In addition, it is necessary to consider the crop management in the polluted area.

Authors' Contributions

A.H.B., and A. G.J., designed the manuscript and have role in performing the experiment, data collection and data analysis; R.S., has help in biological processes of the experiment, all the authors read and approved the final manuscript.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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