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Biodegradation of 2,4-dichlorophenoxyacetic acid by bacteria with highly antibiotic-resistant pattern isolated from wheat field soils in Kurdistan, Iran

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Abstract Recently, there has been increasing interest to clean up the soils contaminated with herbicide. Our aim was to determine the bioremediation of 2,4-dichlorophenoxyacetic acid (2,4-D) from wheat fields which have a long history of herbicide in Sanandaj. Based on our literature survey, this study is the first report to isolate and identify antimicrobial resistant bacteria from polluted wheat field soils in Sanandaj which has the capacity to degrade 2,4-D. From 150 2,4-D-exposed soil samples, five different bacteria were isolated and identified based on biochemical tests and 16S ribosomal RNA (rRNA). *Pseudomonas* has been the most frequently isolated genus. By sequencing the 16S rRNA gene of the isolated bacteria, the strains were detected and identified as a member of the genus *Pseudomonas* sp, *Entrobacter* sp, *Bacillus* sp, *Seratia* sp, and *Staphylococcus* sp. The sequence of Sanandaj 1 isolate displayed 87% similarity with the 16S rRNA gene of a *Pseudomonas* sp (HE995788). Similarly, all the isolates were compared to standard strains based on 16S rRNA. Small amounts of 2,4-D could be transmitted to a depth of 10–20 cm; however, in the depth of 20–40 cm, we could not detect the 2,4-D. The isolates were resistant to various antibiotics particularly, penicillin, ampicillin, and amoxicillin.

Keywords Soil · Herbicide · *Pseudomonas* · Degradation · 2,4-D degradation · 16S rRNA

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Introduction

The usage of herbicide particularly 2,4-Dichlorophenoxyacetic acid (2,4-D) in farms has always been the environmental and health concerns because it has a very negative impact on water and soil quality and, more importantly, pose special problems for biological remediation (Ames and Hoyle 1999; Thill 2003; Botrè et al. 2009). Furthermore, as reported by others 2,4-D is a level II toxicity hormonal herbicide and also considered a carcinogenic agent which affects the liver, heart, and central nervous system (Prado and Airold 2001; Tatiane et al. 2007).

Removing herbicides to protect plants and water resources have thus become a great challenge. Therefore, removal or treatment of any toxic and hazardous compounds is very important, which can be done by various methods like physical, chemical, or biological processes (Ames and Hoyle 1999; Fontmorina et al. 2013). In other words, studies from all over the world

have suggested bioremediation as an option for the removal of this kind of herbicide (Getenga et al. 2004; Cycoń et al. 2011; Dehghani et al. 2013). Many researchers such as Silva, Han, and Cycoń have shown that different bacteria are able to degrade 2,4-D in soil (Silva et al. 2007; Cycoń et al. 2011; Han et al. 2014). These bacteria are known for the ability to utilize 2,4-D as sole carbon and energy sources, and the wide use of 2,4-D has prompted interest in its biodegradation (Tarao and Seto 2000; Bahig et al. 2008; Stibal et al. 2012). Although metabolic pathways for 2,4-D degradation have been available for many years, and genes encoding 2,4-D catabolism have been identified for several organisms (Han et al. 2014), we should consider that the fate, resistance, and durability of 2,4-D is dependent upon many environmental factors like soil pH, organic matter, clay, moisture, temperature, and oxygen concentration; all of which undergoes many processes such as runoff, adsorption–desorption, abiotic transformations, and photodecomposition (Cycoń et al. 2011; Stibal et al. 2012). Accordingly, these conditions are not the same in different places and certainly a key factor controlling the microbial degradation.

It is worth to note that scientists are trying to find out a variety of ways to degrade this herbicide. For example, Lee reported that 2,4-D is readily degraded and used as a sole source of carbon by various microorganisms which have been isolated from soil (Lee et al. 2005).

Moreover, this herbicide is one of the most commercially available herbicide in the world for controlling the weeds, particularly in Iran, which is easily available from local markets. This herbicide is used selectively to control the broadleaf weed by farmers in Iran. Based on available information, the annual consumption of 2,4-D is about 12,000 t per year in the country. More than 50,000 l of this amount are consumed annually in Kurdistan Province, because formers believe that it is highly selective and systemic; however, this herbicide is transported through the plant and gets gathered in the roots. Therefore, it may inhibit the growth of weeds (Zazouli et al. 2010; Ali et al. 2014). Although this herbicide has a short half-life in soil and aquatic environments, it is generally used widely and therefore it is a cause for concern, since it can be a potential threat to the environment and human health (Chinalia et al. 2007; Stibal et al. 2012). Therefore, the development of an efficient degradation process for this herbicide is extremely relevant and necessary. However, based on available literatures, no studies have been carried out on the bioremediation of 2,4-D polluted in wheat field soils in Iran, especially in the Kurdistan Province (one of the major center of wheat production).

For successful bioremediation of polluted soil with 2,4-D, it is necessary to find a way to remove or treat such a polluted soil, particularly by the microbes so that they can be productively exploited (Singh 2008; Stibal et al. 2012). Therefore, the application of native bacteria from contaminated soil is a practical strategy for the bioremediation of herbicide such as 2,4-D. Thus, the aim of this study was isolation, characterization, and identification of 2,4-D-degrading bacterial strains isolated from wheat field soil with a long history of 2,4-D use.

Materials and methods

Soil sample collection

One hundred fifty soil samples were taken from five different sites and studied the physicochemical properties and to isolate 2,4-D-degrading bacteria. All the soil samples were taken from the farms which are having a history of 7–8 years of 2,4-D applications. The soil samples were collected with a hand-driven soil auger in 0–10, 10–20, and 20–40 cm of soil depth at random location at each site at different times (including 7, 14, 21, 30, 60, 90, 120, 150, 180, and 210 days after application of herbicide) and stored at 4 °C until they were used.

Soil analysis

General physicochemical properties of soil were determined according to the standard procedure by the International Organization for Standardization (ISO) and summarized in Table 1. The reference of each method is presented in Table 1.

Isolation of 2,4-D-degrading bacteria by enrichment culture

Medium for isolation

Salt medium FTW (Herman and Frankenberger 1999) with the following composition was used: (g l^{-1} deionized water) K_2HPO_4 0.225, $KH_2PO_4$0.225, $(NH_4)_2SO_4$ 0.225, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.005, and $FeCl_2 \cdot 4H_2O$ 0.005, blended with 1 ml of Focht trace elements solution (Zakaria et al. 2010). The Focht trace

Table 1 General physicochemical characteristics of soil samples

Physicochemical characteristics	Site 1	Site 2	Site 3	Site 4	Site 5	Method of determination	Reference
pH	7.9	8.1	7.8	8.2	8.09	Measurement with glass electrode	ISO 10390:2005
EC (dS m^{-1})	0.57	0.58	0.6	0.43	0.64	Measurement with glass electrode	ISO 11265:1994
Organic carbon (%)	1.7	1.0	6.4	1.85	1.3	Oxidation in the presence of H2SO4	ISO 14235:1998
Total neutralizing value (%)	13.5	31.0	14.0	20.5	28.0	Titrimetric methods	EN 12945:2014 (E)
K (mg l^{-1})	208.1	77.52	365.6	374.8	95.6	Dissolution by alkaline fusion	ISO 14869-2:2002
P (mg l^{-1})	09.3	07.9	09.8	16.0	08.3	Dissolution with hydrofluoric and perchloric acids	ISO 14869-1:2001
Sand	35.08	33.07	34.08	32.08	34.08	Sedimentation and sieving method	ISO 11277:2009
Silt	28.0	46.0	47.0	29.0	29.0	Sedimentation and sieving method	ISO 11277:2009
Clay	36.90	20.1	18.92	38.92	36.92	Sedimentation and sieving method	ISO 11277:2009
Soil texture	Clay loam	Clay loam	loam	Clay loam	Clay loam	Sedimentation and sieving method	ISO 11277:2009

element solution contained the following (mg l^{-1}): $MnSO_4 \cdot H_2O$ 169, $ZnSO_4 \cdot 7H_2O$ 288, CuSO4·5H_2O 250, $NiSO_4 \cdot 6H_2O$ 26; $CoSO_4$ 28, and $Na_2MO_4 \cdot 2H_2O$ 24, pH = 7.2.

In order to remove impurities such as stones and plant materials, each soil sample was sieved through a 2-mm mesh sieve. Then, to a 250-ml conical flask containing 100 ml FTW media, 10 g of soil sample was added and it was incubated at 30 °C on a rotary shaker (ω = 150 rpm) for 2 days. Later, 5 ml aliquots were taken and enriched in order to isolate pure culture of bacteria and, finally, it was centrifuged at 3000 rpm for 5 min and the cell pellets were re-suspended in 2 ml sterile media. On agar plates, aliquots of the suspension were streaked. Under aerobic conditions at 30 °C, the inoculated plates were incubated and discrete colonies were isolated. Based on standard methods, for each isolate, the morphological, physiological, and biochemical tests were performed. Bergey's Manual of systematic bacteriology was used for taxonomically identification of the bacterial strains (Buchannan and Gibbson 1974).

Gas chromatography analysis

For the presence of 2,4-D residues, the soil samples were subjected to gas chromatography using acetone-hexane (20:80) mixture. Briefly, 40 ml methanol and acetic acid solution were added to 50 g of soil and acidified to pH 2, and then 20:80 acetone/diethyl ether was extracted and filtration was done and finally it was extracted with additional diethyl ether and concentrated by an evaporator. The residue was dissolved in 5 ml diethyl ether and subjected to GC-ECD for 2,4-D.

Extraction of 2,4-D residues

After the sample was treated three times to organic solvent with acetone-hexane (20:80) mixture, the extract was filtered and finally evaporated by rotary. The residues were dissolved in 5 ml hexane and stored at 4 °C until used.

GC-ECD

2,4-D residues were studied by Gas Chromatograph Model Varian-CP-3800 using an electron capture detector (ECD). Column cp-sil 8 (50 m × 0.25 mm internal diameter × 0.12 μm film thickness) was used for separation, and nitrogen was used as a carrier gas at a constant flow rate of 50 ml/min. The injector temperature was 285 °C, and the initial temperature was 100 °C (for 1 min) and then rose to 280 °C at the rate of 10 °C/min (for 10 min). Detector temperature was kept at 290 °C.

Genomic DNA isolation and sequencing of 16S rRNA gene

Bacterial DNA was extracted via boiling method. Primers PA-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and PA-R (5′-AAGGAGGTGATCCAGCCGCA-3′) were amplified for the 16S ribosomal RNA (rRNA) sequences of the bacterial strains (Zakaria et al. 2010). By using agarose gel electrophoresis (1% *w*/*v*) amplicons were analyzed. Gels were stained with 0.5 μg ml^{-1} GelRed for 20 min and then visualized with UV transillumination using the Syngene G Box Gel Documentation System. PCR products obtained from bacterial strains purified and sequenced (Seqlab, Sequence Laboratories Gottingen, Germany). The achieved sequences were submitted in the GenBank.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing for all the bacterial isolates to various antimicrobial agents was determined using a disc diffusion assay according to the Clinical Laboratory Standards Institute (Focht and Weaver 1994). The following antibiotics were used: gentamicin, nitrofurantoin, cotrimoxazol, amikacin, ampicillin, amoxicillin, tetracycline, and penicillin.

After the preparation of Mueller-Hinton medium, it was poured into 15 × 150 mm petri dishes at a depth of 5–6 mm. After inoculation of the target cultures, the plates were incubated at 37 °C, overnight. The sensitivity of isolates to each agent was evaluated by comparing the zone sizes to a standard interpretative chart as recommended.

Results

A total of 150 soil samples was taken from wheat field soils in Sanandaj during May to November, 2013. Five different bacteria were isolated from 2,4-dichlorophenoxyacetic acid-exposed soil samples. These bacterial isolates were purified and identified at the genus level by standard biochemical tests as described in Bergey's Manual of Systematic Bacteriology (Table 2). *Pseudomonas* was the most frequently isolated genus followed by *Staphylococcus* sp., *Entrobacter* sp., *Seratia* sp., and *Bacillus* sp.

By sequencing the 16S rRNA gene of the isolated bacteria comparing them with previously published 16S rRNA gene sequences, the strains were classified as a member of the genus *Pseudomonas* sp., *Entrobacter* sp., *Bacillus* sp., *Seratia* sp., and *Staphylococcus* spp., respectively. The sequence of Sanandaj 1 isolate displayed 87% similarity with the 16S rRNA gene of a *Pseudomonas* sp. (HE995788), strain Sanandaj 2 showed (91%) similarities with *Enterobacter* sp. (JQ398852.1), strain Sanandaj 3 showed (98%) similarities with *Bacillus* sp. (HM037905.1), strain Sanandaj 4 showed (98%) similarities with *Serratia* sp. (JX141319.1), and strain Sanandaj 5 showed 97%, the highest identity with *Staphylococcus* sp. (KF779127.1).

Table 2 The selected bacterial strains in the present study

Bacterial strain	Strains	Accession No.
Pseudomonas sp.	Sanandaj1	KJ747366
Entrobacter sp.	Sanandaj2	KJ747367
Bacillus sp.	Sanandaj3	KJ747368
Serratia sp.	Sanandaj4	KJ747369
Staphylococcus sp.	Sanandaj5	KJ747370

Nearly 1500 bp length of 16S rRNA from representative bacteria and five reference genuses were amplified by PCR amplification. All the bacteria produced a single band and the expected PCR products on agarose gel using 1000 bp marker were detected in all strains (Fig.1). After analysis, the sequences with chromas and EditSeq softwares, all sequences were submitted in NCBI (National Center for Biotechnology Information). GenBank with accession numbers are shown in Table 2. The antibiotic resistance pattern of the isolates to various antibiotics by disc diffusion method is shown in Table 3.

Fig. 2 and Table 4 demonstrate detection of 2 4-D in soils in different depths. In our study, we could not detect the residual 2,4-D in 20–60 cm depth. However, the residual 2,4-D in the depth of 0–10 cm was decreased linearly as time goes. 2,4-D did not penetrate to lower soil layers. Small amounts in some soils transited to a depth of 10–20 cm; however, in the depth of 20–40 cm, we could not detect the 2,4-D. The isolates particularly gram negative bacteria were resistant to penicillin.

Discussion

The dissipation of herbicide is an important aspect to be exploited, due to their contamination potential. The

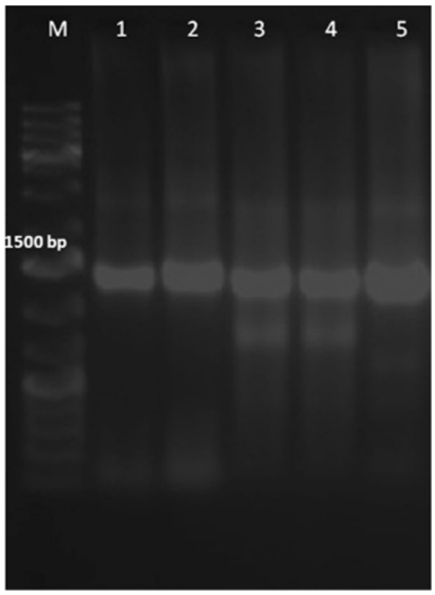

Fig. 1 Electrophoresis of PCR products of 16S rDNA gene for 1500 bp fragment in five isolated strains, the first well is 1Kb ladder (*M*) and the wells 1–6 introduced strain 1–5

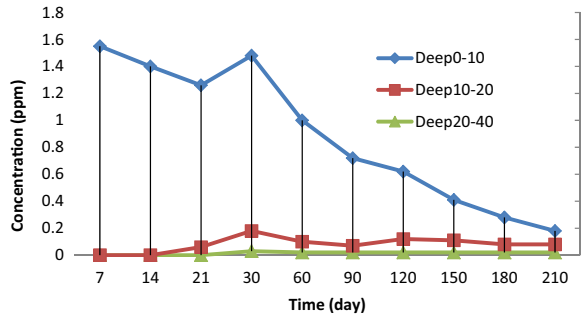

Fig. 2 GC analysis for detection of residual amount of the 2,4-D in site 3 at different depth (cm)

primary method for the dissipation of some herbicide like 2,4-D in soil is considered to be microbial degradation (Botrè et al. 2009). Five different bacterial isolates were the outcome of purification of mixed cultures from the 150 wheat field soil samples in Sanandaj. Isolates were isolated, identified, and characterized according to standard procedures. The most dominant genera of bacteria isolated from soil were *Pseudomonas* sp., *Staphylococcus* sp., *Entrobacter* sp., *Seratia* sp., and *Bacillus* sp. These results are in agreements with the data obtained by Showkat et al. (Showkat et al. 2014). However, other results have also been reported that *Bacillus* was the greatest in contaminated soil (Perreault et al. 2012). To our knowledge, this is the very first report on the ability of above mentioned bacteria to degrade 2,4-D in Sanandaj wheat fields.

As other researchers reported, the mechanism of 2,4-D degradation could be attributed to the mineralization rates of side chain- and ring-labeled 2,4-D. A wider spectrum of microbes may attack and metabolize the acid side chain, but the aromatic ring could be attacked by fewer microorganisms. Therefore, the proportion of 2,4-D degraders, which are able to attack the ring, may play a crucial role in creating the difference in degradation of side chain- and ring-labeled 2,4-D. By sequencing the 16S rRNA gene of the isolated bacteria, the strains were proved and classified as a member of the genus *Pseudomonas* sp., *Entrobacter* sp., *Bacillus* sp., *Seratia* sp., and *Staphylococcus* sp. As another researcher group also reported, by using 16s rRNA gene, we could find up to 97% similarity with standard strain which is consistent with others (Han et al. 2014).

As seen in Fig. 2 and Table 4, we could not detect the residual 2,4-D in 20–60 cm depth. However, the residual 2,4-D in the depth of 0–10 cm was decreased linearly as time goes and degradation was almost completed after 210 days; this shows that the 2,4-D has been

Table 3 Antibiotic resistance pattern of bacteria isolated from wheat field soils in Sanandaj

Antibiotic	*Pseudomonas* sp.	*Entrobacter* sp.	*Bacillus* sp.	*Staphylococcus* sp.	*Seratia* sp.
Gentamicin	S	S	S	S	S
Nitrofurantoin	S	S	S	S	S
Cotrimoxazol	R	S	S	I	S
Amikacin	I	S	I	I	S
Ampicillin	R	R	I	R	R
Amoxicillin	R	R	S	R	R
Tetracycline	R	R	S	R	I
Penicillin	R	R	S	R	R

Table 4 Residual amount of the 2,4-D in soil at days after application and different depths (cm)

Sampling site	Depth of sampling	2,4-D residual (ppm) in soil at days after application									
		7	14	21	30	60	90	120	150	180	210
Site 1	0–10	1.40	1.35	1.45	1.05	0.75	0.25	0.15	0.15	0.60	0.05
	10–20	–	–	0.08	0.20	0.15	0.15	0.10	0.08	0.05	–
	20–40	–	–	–	0.02	0.04	0.05	0.03	0.04	0.02	0.02
Site 2	0–10	1.50	1.35	1.20	1.25	0.80	0.65	0.45	0.35	0.20	0.15
	10–20	–	–	0.08	0.01	0.06	0.09	0.07	0.06	0.05	–
	20–40	–	–	–	0.02	0.02	0.02	0.02	0.02	–	–
Site 3	0–10	1.55	1.40	1.20	1.30	0.90	0.65	0.50	0.30	0.2	0.10
	10–20	–	–	0.06	0.15	0.08	0.05	0.10	0.09	0.06	0.06
	20–40	–	–	–	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Site 4	0–10	1.25	1.20	1.10	1.20	0.70	0.55	0.35	0.3	0.20	0.15
	10–20	–	–	0.02	0.04	0.09	0.01	0.04	0.02	0.07	0.04
	20–40	–	–	–	–	0.02	0.02	0.02	0.05	0.02	0.02
Site 5	0–10	1.30	1.25	1.15	1.10	0.80	0.65	0.40	0.25	0.15	0.10
	10–20	–	–	0.04	0.09	0.10	0.07	0.04	0.05	0.02	–
	20–40	–	–	–	0.02	0.02	0.02	0.02	0.02	–	–

metabolized or has undergone enzymatic degradation. As other scientists also reported that degradation 2,4-D is relatively slow with a half-life of 312 days (Dejonghe et al. 2000).

Bacteria isolated from toxic materials like herbicides demonstrate antimicrobial resistance; therefore, monitoring of antibiotic-resistant bacteria in soil can be used as an indicator of pollution (Arias et al. 2005). Based on this, we determined the antibiotic resistance pattern of the isolates to various antibiotics by disc diffusion method. Our study revealed that the isolated bacteria showed multiple resistances to antibiotics in particular to beta-lactam antibiotics; this resistance could be because of exposure of soil to chemicals like herbicide, pesticide, heavy metals, and antibiotics; perhaps isolated bacteria may acquire this resistant from other bacteria through genetic exchange.

Conclusion

Based on our literature survey, this study is the first report of isolation and identification of antimicrobial resistant bacteria from polluted wheat field soils in Sanandaj which has the capacity to degrade 2,4-D.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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