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BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND 4-CHLOROPHENOL IN CONTAMINATED SOILS BY *Pseudomonas fluorescens* strain HH

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ABSTRACT

Herbicides with 2,4-dichlorophenoxyacetic acid (2,4D) has been commonly used to control weeds and widely detected in environments. In this study, biodegradating activity of *Pseudomonas fluorescens* HH on 2,4D and 4-chlorophenol (4CP) in soil was carried out. The inoculation with *Pseudomonas fluorescens* HH in soils increased the degradation of 4CP and 2,4D by from 47.0% to 51.4% and from 38.4% to 47.4%, respectively, compared to the degradation by autochthonous microorganisms. *Pseudomonas fluorescens* HH could degrade well 2,4D and 4CP in various soils, but the most efficient chemical removal was observed when they were in the loamy soil. Moreover, the efficiency of chemical degradation was significantly affected by the moisture contents with the highest performance of degradation at 10 and 20% soil moisture. Also, the addition of nitrogen (N), phosphorous (P) and potassium (K) stimulated the dissipation rates. The determination of degradation pathway for 2,4D in *Pseudomonas fluorescens* HH indicated that 2,4-dichlorophenol (2,4DCP) and 4CP were formed as metabolites.

Keywords: *Pseudomonas fluorescens* HH, 2,4-dichlorophenoxyacetic acid, 4-chlorophenol, loamy soil, degradation.

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INTRODUCTION

Herbicides including 2,4D are commonly used to control weeds. Because of high agricultural application, 2,4D has been widely detected in environments. For example, the compound has been detected in groundwater (Williams et al., 1988; Kolpin et al., 2000), surface water (Frank and Logan, 1988), wastewater treatment plants (Hope et al., 2012), sediment (Konasewich et al., 1978; Klecka et al., 2010) and soil (Webber and Wang, 1995).

2,4D has been classified as a hormonal herbicide with level II by the World Health Organization (WHO). This chemical causes depression of the central nervous system and damage to the liver and kidneys of human and animals (Moody et al., 1992; Duffard et al., 1996; Mattsson et al., 1997; Charles et al., 2001; Kwangjick et al., 2001; Kim et al., 2005). While 2,4D acts as an active auxin at low concentrations, it causes changes of the normal pattern resulting in the death of plants at high concentrations (Harborne, 1988).

2,4D is moderately mobile in soils, and the mobility depends on soil characteristics (Ordaz-Guillen et al., 2014). 2,4D exists predominantly as an anion which is adsorbed to positively charged sites on the edges of clay particles in soil preventing its cellular uptake and biodegradation (McGhee et al., 1999). The degradation of 2,4D in soil has been investigated in various laboratories (Jacobsen & Pedersen, 1991; Bryant, 1992; Balajee & Mahadevan, 1993; Entry et al., 1996; Chang et al., 1998; Cycoń et al., 2011; Musarrat et al., 2000; Chang et al., 2016; Xia et al., 2017). However, the degradation of 2,4D in various soil with different physico-chemical properties has not been conducted extensively.

Although 2,4D and also 4CP may be remediated by physical and chemical methods, the degradation by microorganisms is a major process for cleaning up the compounds. The biotransformation of 2,4D usually produced chlorophenols as intermediates (Bryant 1992; Chang et al., 1998; Robles-González et al., 2006; Wu et al., 2010; Yang et al., 2017). Chlorophenols are suspected to be carcinogens and mutagens, so they are also listed as hazardous substances (WHO, 1989). The use in industries and agricultural herbicides resulted in serious chlorophenols contamination in soil (Nowak & Mrozik, 2018).

P. fluorescens HH which can aerobically utilize 2,4D as a sole carbon and energy source was isolated and its degradation ability in liquid medium was determined (Nguyen Thi Oanh et al., 2018). In this study, the chemical degradation of 2,4D and 4CP by *P. fluorescens* HH was investigated for various soil types with different components. Also, the effects of N, P, K and moisture content on the bioremediation of highly contaminated soils by *P. fluorescens* HH were examined.

MATERIALS AND METHODS

Bacteria used for chemical degradation

P. fluorescens HH isolated from soil can utilize 2,4D as the sole carbon (Nguyen Thi Oanh et al., 2018). The isolate has been deposited in the Culture Collection at the Center for Biochemical Analysis (Dong Thap University, Vietnam) under the deposition number DUCOANH2015-7C.

Degradation of 2,4D and 4CP in contaminated soils

The degradation of 2,4D and 4CP in soil was carried out according to the methods in a previous report (Duc, 2017) with slight modification. Soil samples were taken from a depth of 10–50 cm in some places in Dong Thap Province, Vietnam. Soil samples were then air-dried at room temperature (approximately 30^{o}C) until the weight became constant, then they were sieved through 2 mm mesh to remove large debris before assaying chemical components. The physical and chemical properties of each soil sample adjusted to unit dry soil weight are presented in table 1. The soil types were classified based on the Soil Survey Division Staff (USA). Before the experiments, the concentrations of 2,4D and 4CP which might contaminate soils by farmers were analyzed, but no such chemicals were detected in all soil samples.

Table 1. Physico-chemical characteristics of four dry soil samples

Soil texture	Loamy sand	Sandy loam	Sandy clay loam	Loamy soil
Granulometric properties (%)				
Coarse sand (> 0.2 mm)	7.5	5.4	5.5	0.7
Fine sand (0.2–0.02 mm)	77.4	65.5	33.7	34.4
Silt (0.02–0.002 mm)	7.7	13.1	25.5	40.4
Clay (< 0.002 mm)	7.9	16.0	35.3	24.5
Agrochemical properties				
pH	6.3	6.4	6.1	6.2
Total C (%)	1.3	2.7	3.5	4.4
Total N (%)	0.08	0.17	0.30	0.44
P_2O_5 (ppm)	33.7	55.6	34.4	28.8
K_2O (ppm)	6.6	30.1	18.8	8.4

200 g of each soil type were placed in a 500-mL glass jar covered with aluminum foil. The soil samples were spiked with 100 mg 2,4D or 4CP per 1.0 kg dry soil. Then, the soil samples were inoculated with the cell suspension of *P. fluorescens* HH to give an initial population of 10^6 cells/g dry soil. The jars were then incubated at room temperature (approximately 30^{o}C) in the dark. To determine chemical degradation in various soil types and to evaluate the effects of NKP on degradation, soil moisture was maintained at 20% of the water-holding capacity by sprinkling sterile water. For the experiments on the effects of the moisture content on substrate degradation, soil moisture was adjusted from 5% to 40%. The jars were manually shaken every 5-days to enhance soil O_2 availability. The controls without inoculation with *P. fluorescens* HH were run in parallel. The bacterial inoculum was prepared by cultivation of *P. fluorescens* HH in LB medium for 12 hr. The culture was centrifuged for 5 min at 12,000 rpm, washed twice with phosphate buffer (50 mM, pH 7.0) and resuspended in sterile water.

To determine chemical degradation, chemicals were extracted from 5 g soil with 15 mL methanol (> 99%) twice (Cotterill 1980). The extract was concentrated and filtered through a 0.22-µm syringe filter. The mean recovery of 2,4D from loamy sand, sandy loam, sandy clay loam and loam was 96.4%, 95.5%, 93.3 and 97.7%, respectively. 4CP recovered from these soils was 95.5%, 93.3%, 91.4 and 96.3%, respectively.

Effects of NPK on degradation of 2,4D and 4CP

The effects of NPK on degradation of 2,4D and 4CP were conducted according to the methods described by McGhee et al. (1999). Soil samples (200 g of each type) were placed in a 500-mL glass jar and amended with nitrogen (NH_4NO_3, 2.5 mg/g), phosphorus ($NaHPO_4.2H_2O$, 3.5 mg/g) and potassium (K_2CO_3, 4.5 mg/g) which are the same amount and ratio of N, P and K of the commercial combined NPK fertilizer. Samples were taken after 15 days of incubation to determine the degradation of chemical degradation.

Analytical methods

The 2,4D and 4CP concentrations were determined using HPLC equipped with a 4.6 mmU25 cm Ultrasphere C18 column (Beckman). The mobile phase was the mixture of methanol, water and acetic acid (40/57/3, v/v) which run at a flow rate of 1.0 mL/min. GC-MS with HP-5MS column (30 m × 0.25 mm × 0.25 mm; Agilent, Palo Alto, CA, USA) was used to determine metabolites of 2,4D degradation. The UV detection was at 283 nm. The process was carried out using an electron ionization (EI) mode (70 eV) with an Agilent gas chromatograph equipped with an

MS detector (5975C). Temperatures of the injection port and the detector were controlled at 250°C and 280°C, respectively. The temperatures of the program were held at 50°C for 7 min, raised 5°C per min to 280°C and finally held at this temperature for 5 min. During the operation process, Helium (1 mL/min) was used as the carrier gas. The HPLC and GC-MS results were compared with retention times and authentic standards of known compounds.

Statistical analysis

Data were calculated and shown as the mean ± one standard deviation from at least in triplicate experiments. The SPSS software program version 22.0 was used to analyze variance, and significant differences ($p < 0.05$) were calculated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Degradation of 2,4D and 4CP in various soils

The degradation of 2,4D and 4CP was carried out in various soil types which represent the soil types commonly used for cultivation in the Mekong Delta. The remediation rates and adaptation ability of *P. fluorescens* HH to different constituents were compared in those soil samples. The degradation of the substrates was carried out in sterile and non-sterile soils. Table 2 showed that the degradation rates of 2,4D in soils inoculated with bacteria were, regardless of the types of soil samples, significantly higher than those in soils without inoculation. The degradation rates of 4CP and 2,4D by *P. fluorescens* HH were from 47.0 to 51.4% and from 38.4% to 47.4% higher compared to the degradation in control by native microorganisms, respectively (table 2). Significantly higher amounts of 2,4D were degraded in non-sterile soils compared with in sterile soils illustrating that 2,4D and 4CP were also degraded by indigenous microorganisms, and *P. fluorescens* HH cooperated well with autochthonous microorganisms. The 2,4D degradation by indigenous microorganisms in soils was reported previously (Comeau et al., 1993; McGhee et al., 1999). The biotic and abiotic factors of soils affect the success of biodegradation. The survival and growth of inoculated bacteria play a key role in bioaugmentation. The physico-chemical environmental parameters of soils also strongly influence the mineralization process of organic contaminants.

Table 2. Degradation of 2,4D and 4CP in various soil types and the roles of inoculation of *P. fluorescens* HH on degradation. Soils were inoculated with 100 mg/kg of chemical substrates. Soil samples were incubated for 15 days

Soils	Substrates	Substrate degradation (%)*			
		Loamy sand	Sandy loam	Sandy clay loam	Loamy soil
None-inoculated soils					
Sterile soil	2,4D	4.8 ± 0.9^{aA}	5.5 ± 0.8^{aA}	7.8 ± 1.0^{aB}	5.5 ± 1.0^{aA}
	4CP	3.9 ± 0.5^{aA}	4.2 ± 0.6^{aA}	8.8 ± 1.1^{aC}	6.4 ± 1.2^{aB}
None-sterile soil	2,4D	10.3 ± 1.6^{aA}	10.2 ± 1.4^{aA}	14.5 ± 2.6^{aB}	17.8 ± 3.2^{bC}
	4CP	8.4 ± 1.8^{aA}	13.4 ± 1.7^{aB}	18.0 ± 2.2^{aC}	21.1 ± 3.5^{bC}
Soils inoculated with bacteria					
Sterile soil	2,4D	48.7 ± 5.9^{bA}	55.7 ± 6.0^{bAB}	60.7 ± 7.4^{bAB}	65.2 ± 8.2^{cC}
	4CP	55.5 ± 6.4^{bcA}	60.4 ± 6.9^{bcAB}	65.7 ± 7.5^{bcAB}	72.5 ± 7.0^{cdB}
None-sterile soil	2,4D	53.7 ± 6.2^{bcA}	65.0 ± 6.4^{cdAB}	71.4 ± 7.9^{cdB}	73.4 ± 6.2^{cdB}
	4CP	58.4 ± 6.5^{cA}	70.4 ± 7.9^{dAB}	77.0 ± 8.4^{dB}	80.7 ± 5.7^{dB}

Note: *Different capital superscript letters (A, B and C) and small superscript letters (a, b, c and d) indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively.

The soil texture and soil nutrients can affect the degradation rates. The degradation was effective in loamy soil, while it was low in loamy sand (table 2). The nutrients available in soils probably accounted for the degradation rates. The loam and sandy clay loam with higher carbon and nitrogen (table 1) resulted in higher degradation rates. Phenol degradation by *Pseudomonas* sp. JS150 was significantly faster in soils with higher organic matter content (Mrozik et al., 2011). Clay with fine grains has low permeability and retarded oxygen transport in the soil. However, the degradation rate in the sandy clay loam in this study was not low compared to the rates in other soil types. This probably is because sand grains in this soil enhanced the permeability. Related to this, the clay content in soil did not affect the degradation of 2,4D (Boivin et al., 2005).

Effects of NPK on 2,4D and 4CP degradation

To enhance crop yield, farmers not only use fertilizers, but also use herbicides. The main components of inorganic fertilizers are N, P and K. The degradation of 2,4D and 4CP with the supplementation of these nutrients shown in table 3 was higher than those in soils without supplementation of nutrients presented shown in table 2. Nutrients may be needed to manipulate soil conditions to enhance inoculum survival, proliferation and activities of microorganisms (Greer & Shelton, 1992). Nevertheless, the degradation of 2,4D and 4CP was not complete in this study. 2,4D may be undergone the adsorption and/or reactions with clays and humics in soil reducing bioavailability to microorganisms (Ogram et al., 1985; Greer & Shelton, 1992; McGhee et al., 1999) probably resulting in incomplete biodegradation.

Table 3. The degradation of 2,4D and 4CP with the supplementation of NPK

Substrates	Substrate degradation (%)*			
	Loamy sand	Sandy loam	Sandy clay loam	Loamy soil
None-inoculated soils				
2,4D	17.7 ± 2.7^{aA}	20.3 ± 3.6^{aA}	22.3 ± 3.2^{aAB}	24.4 ± 5.5^{aB}
4CP	27.0 ± 3.8^{aA}	28.3 ± 3.8^{aA}	28.3 ± 3.8^{aA}	$33. \pm 3.7^{bA}$
Soils inoculated with bacteria				
2,4D	62.3 ± 7.4^{bA}	73.3 ± 7.2^{bAB}	80.3 ± 5.0^{bBC}	90.4 ± 3.0^{cC}
4CP	67.3 ± 7.3^{bA}	75.3 ± 7.4^{bAB}	85.0 ± 6.6^{bBC}	92.6 ± 2.2^{cC}

Note: *Different capital superscript letters (A, B and C) and small superscript letters (a, b, c and d) indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively.

Effects of soil moisture on the degradation of 2,4D and 4CP by *P. fluorescens* HH

The loamy soil which showed relatively effective degradation described above was used in this experiment. The optimum moisture value of soils affecting on biodegradation depends on pore size distribution and soil texture. In this experimental condition using loamy soil, the degradation rates of 2,4D and 4CP was highest at the 10 and 20% of moisture contents (Fig. 1). The degradation rates of 4CP and 2,4D in loamy soil with 40% moisture content was slightly lower than those in 10 and 20% moisture but statistically not different with each other. The low level of moisture content (5%) and excess water (more than 20%) decreased the degradation efficiency. The restriction of water content which resulted in low degradation might be due to the reduction of microbial activities and chemical diffusion. Meanwhile, the excess water in soil may interrupt oxygen

diffusion and produce an unwanted leachate resulting in the decrease of degradation (Schjønning et al., 2011). For 4CP degradation, Cho et al., (2000) reported that about 10 days are required to reach complete degradation by indigenous microorganisms at the initial concentration of 60 mg/kg in loamy sand with the optimal moisture contents of 10 and 15%. In another report, the inoculation with *Pseudomonas* sp. CF600 increased 4CP degradation in soil (Nowak & Mrozik, 2018).

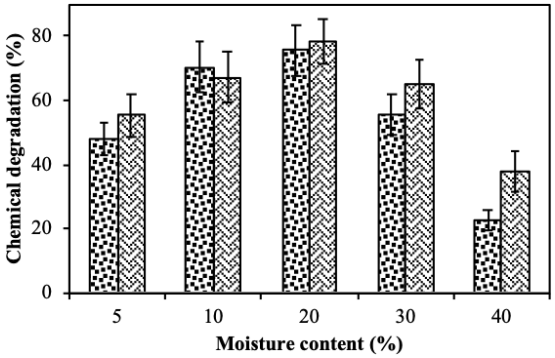

Figure 1. Effects of moisture content on degradation of 2,4D (▩) and 4CP (▨) in sterile loamy soil inoculated with *P. fluorescens* HH. Individual chemicals were supplemented at 100 mg/kg dry soil

Degradation pathways for 2,4D in *Pseudomonas fluorescens* HH

The degradation products of 2,4D in loamy soil were analyzed based on the results of HPLC and GC/MS profiles. During the transformation of 2,4D, a product was proposed to be 2,4DCP (*m/z* 162, 164, 98, 63 in GC/MS), suggesting that the side-chain removal was the first step of the process. Another metabolite with HPLC retention time of 14.2 min and *m/z* 128, 130, 64 in GC/MS analyses was identified to be 4CP. The concentrations of 4CP produced during the degradation of 2,4D were always higher than those of 2,4DCP (Fig. 2). 4CP is assumed to be *oxidized* further*; however,* other metabolites such as phenolic compounds were not detected in soil samples probably because their concentrations were so small or they were immediately transformed in the degradation process. From these results, the plausible complete degradation pathway for 2,4D is proposed in figure 3.

As for the supportive evidence, *P. cepacia* BRI6001 degraded 2,4D to produce 2,4DCP (Greer et al., 1990). Similarly, *Achromobacter* sp. LZ35 transformed 2,4D to 2,4DCP, although 4CP was not detected as the degradation product (Xia et al., 2017). In another study, 2,4D was transformed to 4CP by *Azotobacter* sp. SSB81 (Gauri et al., 2012).

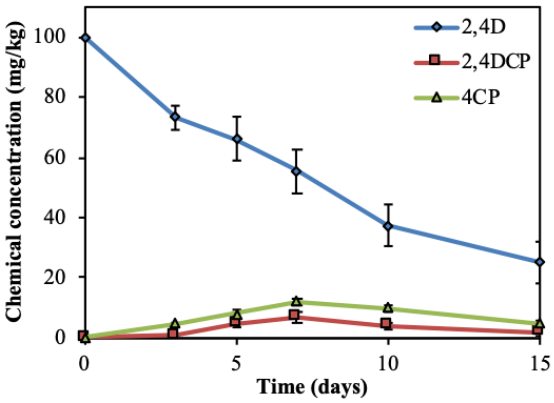

Figure 2. Degradation of 2,4D by *Pseudomonas fluorescens* HH in loamy soil and the formation of 2,4DCP and 4CP during the degradation

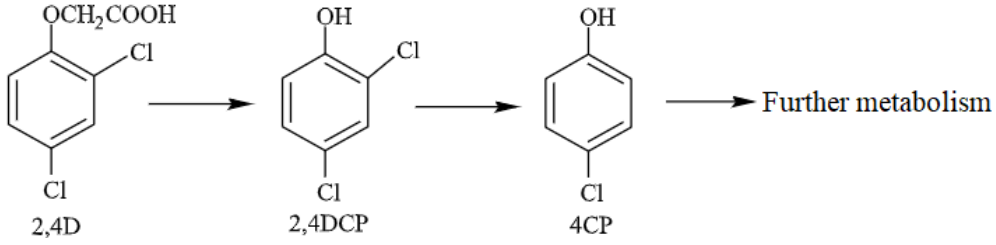

Figure 3. Proposed the degradation pathway for 2,4D in *Pseudomonas fluorescens* HH

CONCLUSION

P. fluorescens HH augmented degradation of 2,4D and 4CP in four soil types with different characteristics. The loamy soil was favorable for the degradation of 2,4D and 4CP. Soil conditions such as moisture and nutrients also affected the degradation of those chemicals by *P. fluorescens* HH. 2.4D is supposed to be degraded to 2,4DCP and then 4CP. This study provides knowledge about better conditions to augment biodegradation by *P. fluorescens* HH.

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