INCREASED DEGRADATION OF ACETOCHLOR IN SOIL BY MIXED CULTURE OF *P. fluorescens* KT3 and *B. subtilis* 2M6E

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Article history

Received: 25/12/2020; Received in revised form: 01/03/2021; Accepted: 05/4/2021

Abstract

The herbicide of acetochlor has been widely applied to control weeds in agricultural sector, but it is responsible for numerous environmental hazards. In the current study, we investigated the effects of the herbicide on bacteria and microfungi communities in soil. The research findings revealed that acetochlor used at 1.24 mg/kg inhibited the growth of both bacteria and microfungi. Moreover, the degradation half-life values were greater at higher acetochlor concentrations in soil, from 12.3 ± 1.2 days at the concentration of 1.0× to 24.5 ± 2.5 days at 2.0×. The augmentation of P. fluorescens KT3 and amendment with peat in soil increased the degradation rates. Besides, the cultivation of peanut enhanced degradation of the compound, and stimulated the growth of bacteria and microfungi. This study showed a process to enhance the remediation of acetochlor in soil by augmentation of P. fluorescens KT3 and cultivation of peanut.

Keywords: *Acetochlor, bacteria, microfungi, degradation, peanut.*

DOI: https://doi.org/10.52714/dthu.11.5.2022.981

Cite: Nguyen Thanh Hung, Tran Ngoc Chau, Nguyen Thi Thuy, and Ha Danh Duc. (2022). Increased degradation of acetochlor in soil by mixed culture of *P. fluorescens* KT3 and *B. subtilis* 2M6E. *Dong Thap University Journal of Science, 11*(5), 60-67.

TĂNG CƯỜNG PHÂN HỦY ACETOCHLOR TRONG ĐẤT BẰNG CÁC DÒNG VI KHUẨN *P. fluorescens* KT3 và *B. subtilis* 2M6E

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Lịch sử bài báo

Ngày nhận: 25/12/2020; Ngày nhận chỉnh sửa: 01/03/2021; Ngày duyệt đăng: 05/4/2021

Tóm tắt

Thuốc diệt cỏ acetochlor được sử dụng rộng rãi để kiểm soát cỏ dại trong nông nghiệp, cũng là tác nhân gây ô nhiễm môi trường. Trong bài báo này, chúng tôi đã khảo sát ảnh hưởng của thuốc diệt cỏ đối với hệ vi khuẩn và nấm trong đất. Kết quả nghiên cứu cho thấy, acetochlor được sử dụng ở mức 1,24 mg/kg ức chế sự phát triển của cả vi khuẩn và nấm. Thời gian bán hủy phân hủy dài hơn khi nồng độ acetochlor trong đất cao hơn, từ 12,3 ± 1,2 ngày ở nồng độ 1.0× đến 24.5 ± 2.5 ngày ở nồng độ 2.0×. Sự bổ sung P. fluorescens KT3 và than bùn trong đất làm tăng tốc độ phân hủy hợp chất này. Ngoài ra, việc trồng đậu phộng giúp tăng sự phân hủy này, đồng thời kích thích sự phát triển của hệ vi khuẩn và vi nấm trong đất. Nghiên cứu này cho thấy việc bổ sung vi khuẩn P. fluorescens KT3 kếp hợp với trồng lạc (đậu phộng) giúp đẩy nhanh tốc độ phân hủy acetochlor trong đất.

Từ khóa: *Acetochlor, vi khuẩn, nấm, phân hủy, đậu phộng.*

1. Introduction

Acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-acetamide) is a chloroacetamide herbicide widely used in farming. However, the compound has been found to accumulate in both soil and water, resulting in environmental hazards (Lengyel and Földényi, 2003). It has been known to act as an endocrine disruptor (Crump *et al*., 2002; Li *et al*., 2009), a genotoxic agent (Hill *et al*., 1997) and a mutagen of male rat germ cells (Ashby *et al*., 1997). Moreover, this herbicide has been classified as a carcinogen by the US Environmental Protection Agency (EPA) (Xiao *et al*., 2006).

Acetochlor is quite persistent in the natural environment (Jablonkai, 2000; Oliveira *et al*., 2013). Biodegradation is considered as the major way to remediate the compound. Its half-life (TD_{50}) values in soil are affected by a variety of factors, including physicochemical properties of soil and environmental conditions (Taylor *et al*., 2005; Oliveira *et al*., 2013). Moreover, the presence of degrading microorganisms and the number and activity of microbial degrading population also play important role in herbicide degradation (Vanni *et al*., 2006).

In the Mekong Delta, rice and peanut have been cultivated over a large surface area and the rotation of rice with peanut has been promoted. A previous study showed that peanut cultivation resulted in the increase of bensulfuron-methyl degradation in soil (Ha and Nguyen, 2020). Knowledge of degradation process for an herbicide is essential in understanding its potential for application and remediation. Even though the natural degradation of acetochlor in soil has been documented, no study on bioaugmentation to increase the process has been reported. Moreover, only a few of studies on herbicide degradation have been carried out in Viet Nam (Ha Danh Duc *et al*., 2020). In our previous report, the cooperation of two bacterial strains isolated from soil, *P. fluorescens* KT3 and *B. subtilis* 2M6E, effectively degraded the compound (Ha and Nguyen, 2020). This study determined acetochlor degradation by indigenous microorganisms compared to the degradation with the augmentation of *P. fluorescens* KT3 and *B. subtilis* 2M6E, and stimulated by cultivation of peanut (*Arachis hypogaea* L.).

2. Materials and methods

2.1. Soil collection and natural degradation of acetochlor in soil

Soil samples were procured from several rice-field sites in Cao Lanh District, Dong Thap Province,Vietnam. Soil was transported to the lab within a day. The soil samples were mixed, pulverized, and eventually sieved through 2.0 mm mesh to eliminate large debris. The soil components were determined according to method of American Public Health Association (APHA, 2012) and shown in Table 1. Subsequently, 1.0 kg soil was transferred to a plastic container (length×width×depth of 15×25×20 cm).

Acetochlor (>98%) was diluted in absolute ethanol at 0.1 M and used as a stock solution. The herbicide was added into the soil at 800 g/ha as the standard dose to control weeds, given 0.62 mg/kg dried soil (1.0×). The degradation was also carried out at 1.5× (0.93 mg/kg) and 2.0× (1.24 mg/kg) in soil. Distilled water was added to 40% of the soil water-holding capacity and then mixed thoroughly. The soil containers were placed in a greenhouse and incubated for one month. Sterilized water was regularly sprinkled to keep moisture contents of 40% during the incubation. Soil samples were collected at interval times to determine the remaining acetochlor and numbers of bacteria and microfungi.

2.2. Augmentation of bacteria and addition of canetrash and peat to soil

The mineral salt medium with the components described in a previous study (Duc and Oanh, 2019) supplemented with 100 mg/L of acetochlor and 1.0 g/L of ammonium sulfate was used to culture bacteria. After incubating for 30 hours at room temperature (~30^{o}C) in the medium, bacteria were collected by centrifugation at 10.000 rpm for 5 min. Cell bullets were rinsed with sterilized saline (0.85% NaCl) twice. Bacteria were then suspended in the mineral salt medium to give 108 colonies forming units (CFUs) per mL (resting cells).

Canetrash collected from a sugarcane field in Tra Vinh Province after harvesting several days. The canetrash was dried using a Memmert oven (Germany) at 80oC for two days. Dried canetrash was then ground using a grain-mil (VCCI Company, Vietnam). The ground canetrash with diameter < 0.5 mm was used

for bacteria immobilization. Peat collected from Maren, Thanh Hoa district, Long An Province was also used. The components of canetrash and peat are shown in Table 1.

In this experiment, *P. fluorescens* KT3 and *B. subtilis* 2M6E isolated from soil (Duc and Oanh, 2019) were used to augment the degradation process. *B. subtilis* 2M6E did not degrade acetochlor, but it degraded 2-methyl-6-ethylaniline (a metabolite of acetochlor degradation) resulting in enhancement of the degradation process.

The resting cells of individual strains were mixed with ground canetrash or dried peat to obtain 0.25×10^8 CFUs/g dried weigh in total. Canetrash and peat with bacteria were mixed with soil to give final bacterial numbers of 10^6 CFUs/g soil (dry weight basis). For the augmentation of both *P. fluorescens* KT3 and *B. subtilis* 2M6E, the numbers of each strain were the same. Acetochlor was added at 1.24 mg/kg (2.0×) into soil. Sterilized water was sprinkled on soil and mixed thoroughly to give 40% of the soil water-holding capacity. The soil containers were placed in a greenhouse and incubated for one month.

At the second cycle, no augmentation of bacteria and amendment with canetrash or peat was conducted. Only acetochlor was supplemented at 2.0× and the degradation by indigenous soil microorganisms was carried out for one month.

Table 1. Physicochemical properties of the dried soil, canetrash and peat

	Units	Soil	Canetrash	Peat
Silt	%	31.6 ± 3.3	-	-
Sand	%	40.4 ± 5.3	-	-
Clay	%	28.0 ± 4.1	-	-
Total organic carbon	%	3.6 ± 0.5	58.4 ± 4.4	18.5 ± 1.7
Total N	%	0.16 ± 0.04	3.4 ± 0.3	1.1 ± 0.1
P_2O_5	ppm	30.8 ± 3.4	0.055 ± 0.00	< 0.001
K_2O	ppm	6.3 ± 0.6	< 0.001	0.032 ± 0.00
pH		6.4 ± 0.1	4.4 ± 0.1	6.7 ± 0.1

2.3. Peanut cultivation

Peanuts (*Arachis hypogaea* L.) of a cultivar named GV10, a widely cultivated variety, were used in this experiment. Seeds were surface-disinfected in sodium hypochlorite solution (0.5%) for 5 min, followed by rinsing thrice in sterile distilled water. The seeds were pregerminated for 24 h at room temperature by placing them in petri dishes on wet paper towels and incubating in darkness. Thereafter, two peanut seeds were sown in each plastic container.

The containers were placed in a greenhouse and the experiment was carried out during the dry season, having an average temperature of about 30°C and relative humidity of 70-75%. The soil moisture was maintained by sprinkling sterile water daily. After one month, the plants were harvested, and soil was used to analyze bacteria abundance and acetochlor remaining.

2.4. Determination of chemical concentrations and enumeration of bacteria and microfungi in soil

Acetochlor in soil was extracted with an equal volume of hexane solvent three times. A 5g soil sample was added to a 50 ml-centrifuge tube containing 10 mL of hexane. The mixture was shaken for 30 min at 250 rpm on a rotary shaker. The sample was then centrifuged and the supernatant was decanted, evaporated to dryness under nitrogen gas. The residues were dissolved in acetonitrile. The recovery of acetochlor from the soil was 93.7%.

The concentrations of acetichlor were analyzed using a reverse phase of high performance liquid chromatography (HPLC) equipped with a UV detector (240 nm). The separation was performed at 40°C on C18 HPLC column (5 µm, 250 mm×4.6 mm; Hyperclone, Phenomenex, USA). A 7:3 (v/v)

ratio acetonitrile: ultrapure water mixture served as the mobile phase at a flow rate of 1 mL/min.

Populations of bacteria and microfungi were enumerated and expressed as number of CFUs/g soil. Soil samples were serial diluted and placed on agar medium of mineral salt medium supplemented with glucose (1.0 g/L) and ammonium sulfate (1.0 g/L). For fungal enumeration, the medium was added with streptomycin (30 mg).

2.5. Statistical analysis

All obtained data from at least three experiment replicates are shown as the mean ± standard deviation. Significant differences among means were statistically analyzed using one-way Duncan's test ($p < 0.05$) in SPSS program version 22.0.

3. Results and discussion

3.1. Natural degradation of acetochlor in soil

The degradation of acetochlor in soil at different concentrations is shown in Figure 1. The increase of chemical concentrations resulted in lower degradation percentages. More than 80% of acetochlor at 1.0× was degraded, while only about 55% of the substrate at 2.0× was removed after 30 hours. However, the specific degradation rates were significantly higher at higher acetochlor concentrations, given 16.84 ± 0.42 μM/day, 21.86 ± 1.01 μM/day and 23.64 ± 1.54 μM/day at the concentrations of 1.0×, 1.5× and 2.0×, respectively. Acetochlor dissipation was no more than 15% in sterilized soil (control).

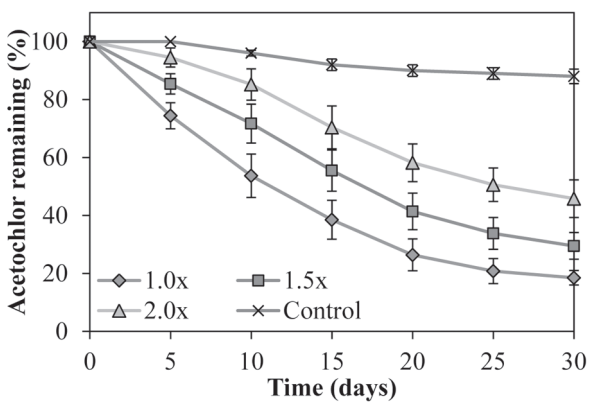

Figure 1. Acetochlor degradation in soil at 1.0× (0.62 mg/kg), 1.5× (0.93 mg/kg) and 2.0× (1.24 mg/kg) in soil. The degradation (at 1.0×) in control was run in parallel

DT_{50} values were significantly longer at higher concentrations, increasing almost twice from 1.0× to 2.0× (Table 2). The determination of DT_{50} values for acetochlor in soil has been carried out in previous studies. Thomas *et al*. (1999) showed that the value was 6.5 days. In another report, the values at 1.68 kg/ha were from 10.5 to 15.1 days (Kucharski *et al*., 2018). DT_{50} values also depended on the depth of soil layer, ranging from 6.51 to 13.9 days for surface soils, and from 20.3 to 26.7 days for subsurface soils (Oliveira *et al*., 2013). Moreover, the decrease of degradation rates in soil by indigenous at higher acetochlor were reported (Cai *et al*., 2007).

3.2. Effects of acetochlor on numerous bacteria and microfungi in soil

At the beginning, the numbers of bacteria and microfungi were the same. Bacteria always outnumbered microfungi. The abundance of bacteria and microfungi significantly increased at all treatments. The abundance of microbial organisms in control and in soil samples increased probably due to the favorable condition in this soil sample. Suitable moisture and dark incubation stimulated the growth of microorganisms. However, enumeration of both bacteria and microfungi in soil at 2.0× was significantly lower than other concentrations (Table 2). The toxicity of the herbicide inhibited the growth of soil microorganisms.

The effects of acetochlor on microorganisms varied at different previous reports, depending on soil components and experiment conditions. A previous report showed that the application of acetochlor had no significant positive or negative effects on the microbial populations (Hong *et al*., 2018). Another study presented that acetochlor at 50, 150 and 250 mg/kg stimulated fungal communities at day 7 after application, but after that the suppression effect occurred (Xin-Yu *et al*., 2010). However, Tyagi *et al*. (2018) showed that the effect of the herbicide on soil microbes was only temporary (Tyagi *et al*., 2018).

3.3. Acetochlor degradation in soil with the bioaugmentation of *P. fluorescens* KT3 and *B. subtilis* 2M6E

Acetochlor degradation in soil amended with ground canetrash was not statistically increased compared to unamended soil at the first cycle

(Figure 2). However, the amendment of peat mildly increased the degradation in soil with and without augmentation (Figure 2). The augmentation of only *P. fluorescens* KT3, and both *P. fluorescens* KT3 and *B. subtilis* 2M6E significantly enhanced the degradation performances. Even though the presence of *B. subtilis* 2M6E increased the acetochlor degradation by *P. fluorescens* KT3 in liquid media described in a previous report (Duc and Oanh, 2019), *B. subtilis* 2M6E did not stimulated the substrate degradation in soil in this work. This result indicated that *P. fluorescens* KT3 could adapt to new condition well; however, *B. subtilis* 2M6E might not grow well in soil.

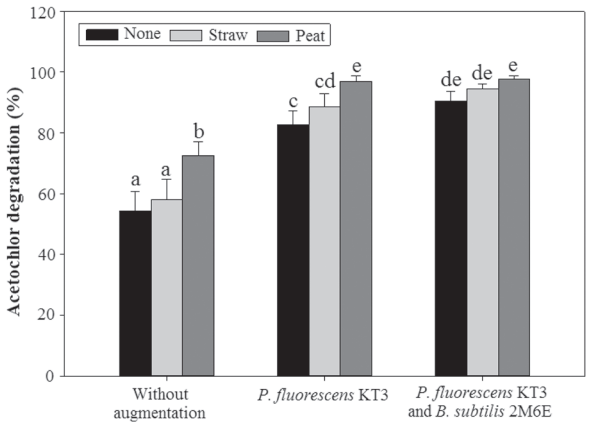

Figure 2. Acetochlor degradation at the first cycle in soil with and without bioaugmentation at 2.0× (1.24 mg/kg) for 30 days

At the second cycle, no ground canetrash, peat and bacteria were added into soil. However, acetochlor degradation rates in soil with bioaugmentation at the first cycle were significantly higher than those in unaugmented soil, increasing acetochlor degradation in soil by from 10.3% to 18.0% compared to the first cycle. More than 95% of the herbicide was dissipated in all augmented soil samples (Figure 3). The result proved that *P. fluorescens* KT3 could survive and work well for a long time in soil. Moreover, the degradation rates at the second cycle in soil without augmentation were higher than those at the first cycle from 8.1% to 15.8%. Native microorganisms became adapted to the herbicide, and showed better degradation performance at the repeated time.

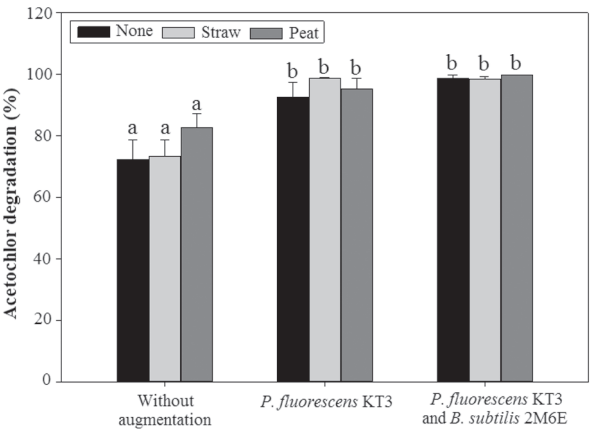

Figure 3. Acetochlor degradation at the second cycle in soil with and without bioaugmentation at 2.0× (1.24 mg/kg) for 30 days

The degradation percentages in soil with and without ground canetrash and peat amendment were not statistically different at the repeated cycle (Figure 3). Nutrients in peat were probably consumed by microorganisms at the first cycle, and did not generate degradation at the second one.

3.4. Effects of peanut cultivation on acetochlor degradation in soil

Although the peat amendment increased acetochlor degradation in soil without augmentation as described above, the phenomenon was not found in soil cultivated with peanut. Because the amendment of *B. subtilis* 2M6E did not increase degradation performance, the bacterial strain was not used in this experiment. Table 3 shows that the augmentation of *P. fluorescens* KT3 also increased the degradation. For soil without canetrash and peat, the cultivation with peanut increased the degradation compared with controls (without peanut shown in Figure 2) by from 16% to 23% after 30 days. However, the addition of canetrash and peat only increased no more than 10% in comparison with none cultivated treatments. This is probably because the degradation performances were more than 90% and reach threshold level. Similarly, a previous study reported that peanut cultivation enhanced the degradation of bensulfuron-methyl in soil (Ha and Nguyen, 2020). Root exudates were indicated to stimulate the remediation (Yu *et al.*, 2005).

Peanut cultivation also increased the abundance of bacteria and microfungi in soil. The numbers of

bacteria and microfungi in cultivated soil without augmentation shown in table 3 [$(3.8 \pm 0.40)\times10^6$ CFUs/g and $(6.0 \pm 0.51)\times10^3$ CFUs/g, respectively] were almost twice as many as the numbers in uncultivated soil shown in table 2 [$(1.9 \pm 0.20)\times10^6$ CFUs/g and $(2.7 \pm 0.23)\times10^6$ CFUs/g, respectively]. This result indicated that peanut favored the growth of microorganisms in soil. The quantities of bacteria and microfungi in augmented and unaugmented soil samples, with and without amendment of ground canetrash and peat were not statistically different (Table 3).

Table 2. Abundance of bacteria and microfungi in soil samples without bacteria augmentation and peanut cultivation

Acetochlor	At the beginning		After 30 days		DT_{50} (days)
	Bacteria ($\times10^6$ CFUs/g dry soil)	Fungi ($\times10^3$ CFUs/g dry soil)	Bacteria ($\times10^6$ CFUs/g dry soil)	Fungi ($\times10^3$ CFUs/g dry soil)	
0.0×	0.7 ± 0.06	0.4 ± 0.02	2.7 ± 0.21^b	3.5 ± 0.40^b	-
1.0×	0.7 ± 0.06	0.4 ± 0.02	3.2 ± 0.28^{bc}	5.1 ± 0.50^c	12.3 ± 1.2^a
1.5×	0.7 ± 0.06	0.4 ± 0.02	2.5 ± 0.21^b	4.1 ± 0.32^{bc}	17.0 ± 1.9^b
2.0×	0.7 ± 0.06	0.4 ± 0.02	1.9 ± 0.20^a	2.7 ± 0.23^a	24.5 ± 2.5^c

Notes: Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column. Data are means of the results from at least three individual experiments, and mean values and standard deviations are shown.

Table 3. Acetochlor degradation and abundance of bacteria and microfungi in soil planted with peanut. Data were numerated after 30 days of peanut seedlings in soil supplemented with 2.0× (1.24 mg/kg) acetochlor

	Without augmentation			Augmentation with *P. fluorescens* KT3		
	None	Canetrash	Peat	Free cells	Mixed with canetrash	Mixed with peat
Acetochlor degradation (%)	77.2 ± 6.5^a	78.2 ± 5.5^a	88.5 ± 4.7^b	92.6 ± 4.7^c	98.6 ± 4.4^c	95.2 ± 3.4^c
Bacteria ($\times10^6$ CFUs/g dry soil)	3.8 ± 0.40^a	4.1 ± 0.33^a	4.4 ± 0.31a	4.3 ± 0.42^a	4.5 ± 0.50^a	4.8 ± 0.46^a
Microfungi ($\times10^3$ CFUs/g dry soil)	6.0 ± 0.51^a	6.6 ± 0.55^a	6.0 ± 0.65^a	6.3 ± 0.66^a	7.1 ± 0.70^a	6.2 ± 0.61^a

Notes: Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a line. Data are means of the results from at least three individual experiments, and mean values and standard deviations are shown.

4. Conclusion

The addition of acetochlor at 1.24 mg/kg inhibited the growth of bacteria and microfungi in soil. The augmentation of *P. fluorescens* KT3 increased acetochlor degradation and reduced the inhibition. Moreover, the amendment with peat in soil enhanced the degradation rate. In addition, the cultivation of peanut also augmented the herbicide dissipation and favored the growth of bacteria and microfungi in soil. The results in this study proved that *P. fluorescens* KT3 effectively degraded acetochlor in soil, which should be further study for application.

Acknowledgements

This study was supported by Dong Thap University. Authors thank all who have provided supports./.

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