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EFFECTS OF AUGMENTATION WITH *Bacillus* sp. DT1 ON CARBONFURAN DEGRADATION AND BACTERIAL COMMUNITY IN SOIL

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ABSTRACT

Carbofuran is frequently applied to crop fields to combat insects globally causing serious environmental pollution. In this study, the enhanced degradation of carbofuran in soil by augmentation with *Bacillus* sp. DT1 was determined. The results showed that the augmentation with the bacterial strain increased the degradation by almost 30% compared to attenuation after 30 days at an initial concentration of 10 mg/kg dry soil. The half-life values of the insecticide were 41.6 and 17.9 days for attenuation and augmentation, respectively. Moreover, the augmentation with *Bacillus* sp. DT1 significantly increased sequence numbers of 16 S rRNA in soil compared to the initial stage. However, α-diversity indices, i.e., values of OTUs, ACE, Chao1 and Shannon showed no statistical differences in soil with and without the insecticide addition, with and without augmentation after 30 days. These results showed that carbofuran did not inhibit or stimulate the growth of soil bacteria at 10 mg/kg dry soil.

Keywords: Carbofuran, *Bacillus* sp. DT1, degradation, augmentation, α-diversity indices.

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INTRODUCTION

Carbamates are insecticides which act through the inhibition of the enzyme acetylcholinesterase, thus affecting chemical synapses (Colovic et al., 2013). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad-spectrum carbamate, which is applied to control insect pests in a range of agricultural and horticultural crops. The compound is of high mobility in soil (Farahani et al., 2008), and its residues have been detected in soils and water sources (Otieno et al., 2010). Carbofuran exhibits high toxicity on aquatic life and has been highly regulated or even banned in several countries (Gupta, 1994; Lewis et al., 2016). Carbofuran has been also banned in Vietnam.

Biodegradation is considered to be effective and environmentally friendly in treating this chemical. There are quite a number of microbial isolates which can degrade carbofuran such as *Arthrobacter, Pseudomonas, Bacillus, Actinomyces* (Ambrosoli et al., 1996), *Pseudomonas* sp. (Bano & Musarrat, 2004), *Sphingomonas* sp. SB5 (Ryeol et al., 2006), *Novosphingobium* sp. (Nguyen et al. 2014), *Burkholderia cepacia* (Plangklang & Reungsang 2009), and *Cupriavidus* sp. (Gupta et al., 2019).

Since carbofuran is frequently applied to the crop fields to combat the insects, it becomes imperative to evaluate their effects on soil microorganisms as well as their rate of dissipation in soil. After application, a large portion of insecticides accumulates in the soil, which may affect the populations and biological activity of soil microbial directly. Some studies on the effects of carbofuran on soil microorganisms have been reported (Das et al., 2005; Zhou et al., 2012; Castro-Gutiérrez et al., 2017). However, the reports on the effects of the insecticide on the diversity of bacterial communities are scarce.

In our previous report, *Bacillus* sp. DT1 isolated from soil showed effective degradation of carbofuran in liquid media and soil (Duc, 2022). In this study, the enhancement of carbofuran degradation in soil by augmentation with *Bacillus* sp. DT1 and the effects of the insecticide on bacterial community structure are determined.

MATERIALS AND METHODS

Soil collection

The samples of the soil exposed to carbofuran were collected from a field in Thap Muoi district, Dong Thap province, Vietnam, in which rice was cultivated in the rotation with corn and other crops. Carbofuran had been extensively applied to control pests before, but it was banned in Viet Nam recently. The samples were collected at a depth not exceeding 25 cm. All soil samples were transported to the lab within a day. The soil samples were mixed, pulverized, and sieved through a 2 mm mesh to eliminate large debris, and the physicochemical properties of the soil samples were determined. The dry soil sample contained 21.0% clay, 33.5% silt and 41.8% sand. Other main compositions of the soil included 2.02 g/kg total organic carbon, 0.34 g/kg total nitrogen, 1.5 g/kg total phosphorus, 0.8 g/kg potassium, and pH value of 6.4. Subsequently, soil samples of 500 g each were transferred to a plastic container (length × width × depth of 15 × 25 × 20 cm).

Bacteria and culture media

The carbofuran-degrading bacterial strain, *Bacillus* sp. strain DT1 (GenBank accession N_0. MH109504.1), was used in this study. The mineral medium (MM) with the components described in a previous study (Duc, 2022) was used to cultivate bacteria. The medium was added with carbofuran (120 mg/L), glucose (1.0 g/L) and ammonium sulfate (1.0 g/L). Bacteria were cultured at room temperature (about 30 ^oC) at 150 rpm for 20 hours. The culture was centrifuged at 10.000 rpm for 5 min to collect bacteria. Bacteria were rinsed twice with the MM medium and resuspended in this medium to give a solution with approximately 10^9 CFU/mL. The condensed bacteria were used in soil augmentation.

Soil augmentation

The soil sample was added with sterile water to 40% soil moisture. The moisture content was determined using the oven-drying method. The moisture content was calculated from the sample weighed before and after drying at 80 ^{o}C for two days. The cell suspension was added to the soil to give 10^6 CFUs of *Bacillus* sp. strain DT1 per mg dry soil. Carbofuran was also added at 10 mg/kg dry soil. Each soil sample was mixed thoroughly after adding sterile distilled water to 40% of the soil water holding capacity. The containers were capped with a plastic cover and incubated in the dark at room temperature (30 ^{o}C) in the lab. The soil was sterilized at 121 ^{o}C for 15 min served as a control. After one month, the residual carbofuran and the diversity and relative abundance of the bacterial community in the soil samples were analyzed.

Diversity and relative abundance of soil bacterial community

Soil samples collected at the beginning and after 30 days of incubation were used in this experiment. 1.0 g of soil collected from every single experiment was diluted with sterile distilled water (1:10; w/v), from which bacterial DNA was isolated using the UltraCleanTM Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). The relative abundance of the bacterial community in soil was determined through Illumina MiSeq sequencing of 16 S rRNA genes as described in a previous study (Oanh & Duc, 2021). The relative abundances of the bacterial species in the soil slurries were determined by sequencing 16 S rRNA genes using an Illumina MiSeq bench-top sequencer. The universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the 16S rRNA genes at the V3-V4 region. The PCR amplifications were performed in 20 µl reaction volume using TransGen AP221-02: TransStart Fastpfu DNA polymerase. All amplifications were performed in quadruplicate. The initial denaturation step of 3 min duration at 95 ^{o}C was followed by 27 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C, and 45 s at 72 ^{o}C. The final extension was performed for 10 min at 72 ^{o}C. All PCR products were purified by AxyPrep DNA Gel Extraction Kit (AXYGEN, USA). TruSeq™ DNA Sample Prep Kit (Illumina, USA) was used to generate the sequencing libraries. The raw sequencing reads were quality-controlled using the Trimmomatic tool (version 0.39). Paired-end reads were merged using FLASH tohol. Sequences were grouped into operational taxonomic units (OTUs) based on 97% identity assigned using the Ribosomal Database Project (RDP) classifier. Rarefaction and α-diversity indices, i.e., abundance-based coverage estimators (ACE), Chao1, Simpson, Shannon, were calculated using Mothur software. The treatments included soil without carbofuran, soil added with carbofuran, soil added with carbofuran and augmentation with *Bacillus* sp. DT1. Each treatment was conducted in three replicates. A soil sample was collected from each individual replicate to determine the relative abundance of the soil bacterial community. The soil that did not add the pesticide served as the control sample.

Determination of carbofuran concentration in soil

Soil samples were collected every 5-day period. Carbofuran in 3 g fresh soil (about 40% soil moisture) was extracted twice with 7 mL of acetone/ethyl acetate (1/1) for 5 min. The extracts were filtered using 0.22 µm filters and concentrated. The concentrated extracts were used to determine carbofuran and its metabolites. Concentrations of carbofuran and its degradation metabolites were measured using an HPLC system. The degradation metabolites were determined using HPLC. The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of LC 20AD pumps, SIL-20A autosampler, and an SPDM20A photodiode array (PDA) detector. Shimadzu Shim-Pack XR-ODS column was used to separate carbofuran metabolites. The mobile phase (acetonitrile and water 30:70) was pumped isocratically at a rate of 0.5 mL/L, and a 5 µl sample was injected into

the HPLC system. The column oven temperature was maintained at 40 °C, and the detection was performed at a wavelength of 250 nm.

Statistical analysis

All data obtained from three replicate experiments are presented as mean ± standard deviation. In addition, Duncan's multiple range test ($p < 0.05$) in SPSS 22.0, was used to analyze significant differences among means.

RESULTS AND DISCUSSION

Carbofuran degradation in soil

The degradation of carbofuran in soil without augmentation, with augmentation and sterile soil was compared (Fig. 1). After 30 days, the concentration of carbofuran was reduced by about 15% in sterilized soil, indicating that the insecticide was absorbed into soil components, or was naturally removed by physical and chemical processes. 51.4% carbofuran was dissipated in soil without augmentation (the attenuation process), which indicated that indigenous microorganisms played a key role in the degradation. The augmentation with *Bacillus* sp. DT1 increased the degradation by almost 30% compared to attenuation. The half-life (TD_{50}) for attenuation and augmentation treatments were 41.6 and 17.9 days, respectively. This result showed that *Bacillus* sp. DT1 could adapt to the soil and degrade carbofuran.

In a previous report, the augmentation with *Bacillus* sp. DT1 increased carbofuran degradation in soil (Duc, 2022). In this context, about 32.8 ± 6.0% carbofuran has remained in soil without herbicide history, and 19.7 ± 3.9% carbofuran has remained in soil with herbicide history (Duc, 2022). Similarly, Trabue et al. (2001) showed that the increase in soil degradation of carbofuran was caused by repeated application of the pesticide. In another study, the half-life of the carbofuran for soil augmented with *Burkholderia cepacia* PCL3 was 13–17 days, while data for soil attenuation was 75 days (Plangklang & Reungsang, 2009).

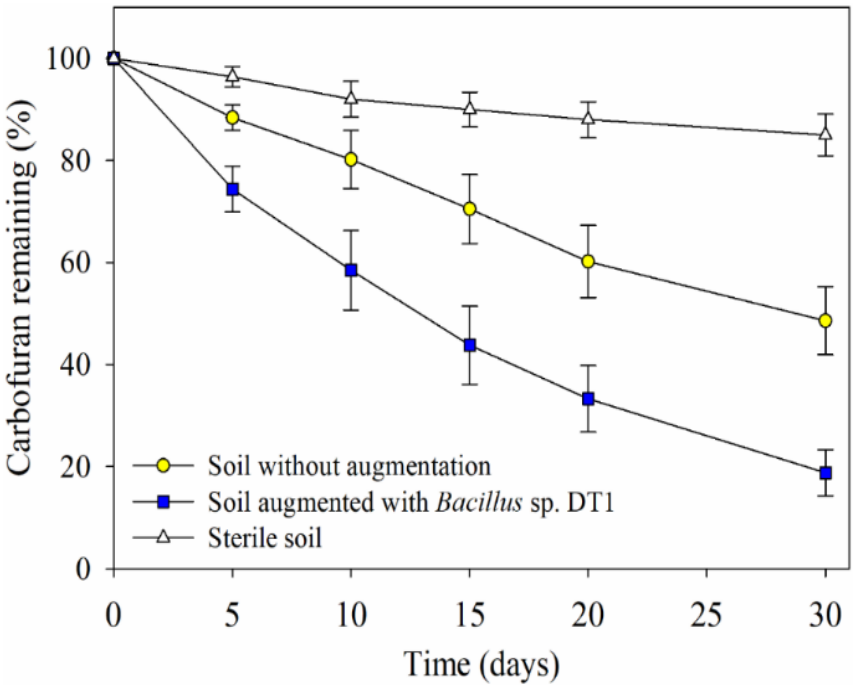

Figure 1. Carbofuran degradation in soil with and without *Bacillus* sp. DT1 augmentation. Carbofuran was added at 10 mg/kg dry soil. Sterile soil served as the abiotic control

Effects of carbofuran on bacterial community structure in soil

After 30 days of incubation, the shifts of bacterial community structure were observed in soil without carbofuran (control), soil with carbofuran and soil with carbofuran and augmentation. The changes occurred in levels of phylum and genus levels and alpha diversity analysis.

At the phylum level, Proteobacteria was dominant in all treatments with 43.1%, and its relative abundance was not statistically changed in all treatments over 30 days (Table 1). Other phyla, i.e., Firmicutes, Bacteroidetes, Acidobacteria, Nitrospirae and Chloroflexi were relatively stable during the incubation. On the other hand, Actinobacteria was the second abundance at the beginning with 13.6% which slightly increased to 14.3% in the soil without carbofuran, 15.9% in soil added with carbofuran and without augmentation, and 16.4% in the soil in soil added with carbofuran and with augmentation. The relative abundance of Cyanothece significantly decreased in all herbicide-supplemented samples.

At the genus level, all genera with relative abundances higher than 1% of the initial sample are shown in Table 2. *Enterobacter, Comamonas, Aeromonas, Arthrobacter, Acrobacter, Pseudomonas, Achromobacter* and *Rhizobium* abundant initially did not statistically shift during incubation. The relative abundance of *Bacillus* was 4.6% at the beginning, which slightly increased to 5.1% in soil without carbofuran and 5.2% in herbicide-supplemented soil, and slightly increased to 6.1% in herbicide-supplemented, augmented soil. The increase in relative abundance and higher abundant level of *Bacillus* in augmented soil indicate the growth of *Bacillus* sp. DT1 in soil. The relative abundances of both *Agromyces* and *Paracoccus* increased, while *Cyanothece* exhibited a reduction during the incubation in all treatments.

Alpha diversity (a measure of microbiome diversity in a single sample) was measured using the Chao1, ACE, Shannon and Simpson indices in the non-sterile slurries of the sediment and the soil. The Operational Taxonomic Units (OTUs) were classified at 97% similarity. After 30 days, the bacterial sequences of 16 S rRNA were significantly increased compared to the initial soil; however, the sequence numbers in all soil samples after 30 days were not statistically different (Table 1). Moreover, other α-diversity indices, i.e., values of OTUs, ACE, Chao1 and Shannon showed no statistical differences in all treatments (Table 3). These results suggested that carbofuran at 10 mg/kg did not cause negative effects on the bacterial community.

Table 1. Relative abundance of bacterial communities at phylum level in soil samples with and without carbofuran (10 mg/kg dry soil) and *Bacillus* sp. DT1. Data in each column are obtained from three single treatments

Phylum	Original soil	After 30 days		
		Soil without carbofuran	Soil with carbofuran	Carbofuran and augmentation with *Bacillus* sp. DT1
Proteobacteria	43.1 ± 3.33	44.7 ± 4.46	46.1 ± 5.06	46.1 ± 5.12
Actinobacteria	13.6 ± 1.54	14.3 ± 1.52	15.9 ± 1.55	16.4 ± 1.56
Firmicutes	7.9 ± 0.53	9.6 ± 1.03	9.1 ± 1.04	9.8 ± 1.11
Bacteroidetes	8.0 ± 0.61	9.1 ± 0.85	8.4 ± 0.77	6.9 ± 0.55
Acidobacteria	3.0 ± 0.04	0.7 ± 0.01	1.1 ± 0.14	1.8 ± 0.14
Nitrospirae	1.1 ± 0.01	2.0 ± 0.41	1.1 ± 0.03	1.3 ± 0.11
Chloroflexi	1.0 ± 0.00	1.6 ± 0.22	0.8 ± 0.11	0.7 ± 0.10
Cyanothece	4.4 ± 0.23	3.5 ± 3.34	2.0 ± 0.17	2.0 ± 0.18
Others	11.1 ± 0.76	9.1 ± 1.07	9.1 ± 1.04	9.2 ± 1.01
Unclassified	6.8 ± 3.33	5.4 ± 0.64	6.4 ± 0.55	5.8 ± 0.64

Table 2. Relative abundance of bacterial communities at the genus level in soil samples with and without carbofuran (10 mg/kg dry soil) and *Bacillus* sp. DT1. Data in each column are obtained from three single treatments

Genera	Original soil	After 30 days		
		Soil without carbofuran	Soil with carbofuran	Carbofuran and augmentation with *Bacillus* sp. DT1
Enterobacter	5.6 ± 0.02	5.0 ± 0.04	5.7 ± 0.06	4.8 ± 0.04
Comamonas	5.2 ± 0.03	5.3 ± 0.05	4.8 ± 0.04	5.2 ± 0.05
Aeromonas	5.0 ± 0.03	5.3 ± 0.05	5.2 ± 0.02	5.0 ± 0.04
Arthrobacter	5.0 ± 0.03	4.6 ± 0.04	5.3 ± 0.06	5.0 ± 0.05
Acrobacter	5.0 ± 0.02	5.4 ± 0.06	4.7 ± 0.04	5.3 ± 0.05
Pseudomonas	4.8 ± 0.02	5.0 ± 0.05	5.5 ± 0.05	5.3 ± 0.04
Bacillus	4.6 ± 0.03	5.6 ± 0.06	5.5 ± 0.05	7.1 ± 0.07
Achromobacter	4.5 ± 0.03	4.4 ± 0.04	5.0 ± 0.05	4.4 ± 0.04
Rhizobium	4.5 ± 0.03	5.0 ± 0.05	5.3 ± 0.02	5.0 ± 0.05
Cyanothece	4.4 ± 0.03	3.5 ± 0.04	2.0 ± 0.02	2.3 ± 0.02
Cavicella	4.2 ± 0.02	2.2 ± 0.01	2.7 ± 0.02	4.0 ± 0.04
Flavobacteriia	4.0 ± 0.03	4.4 ± 0.04	3.0 ± 0.02	3.3 ± 0.03
Mycobacterium	3.8 ± 0.02	3.0 ± 0.03	4.0 ± 0.04	4.4 ± 0.04
Streptococcus	3.3 ± 0.03	4.0 ± 0.03	3.6 ± 0.03	2.7 ± 0.02
Rhodococcus	3.0 ± 0.01	0.7 ± 0.01	1.1 ± 0.01	1.8 ± 0.02
Hydrotalea	3.0 ± 0.01	2.7 ± 0.03	3.0 ± 0.03	2.4 ± 0.02
Agromyces	2.8 ± 0.02	4.0 ± 0.04	3.3 ± 0.03	4.0 ± 0.04
Acinetobacter	2.3 ± 0.02	3.1 ± 0.02	3.1 ± 0.03	3.1 ± 0.02
Paracoccus	2.0 ± 0.01	4.0 ± 0.03	4.1 ± 0.04	4.0 ± 0.04
Gordonia	2.0 ± 0.01	2.7 ± 0.02	3.3 ± 0.03	2.0 ± 0.02
Nitrospira	1.1 ± 0.01	2.0 ± 0.01	1.1 ± 0.01	1.3 ± 0.01
Anaerolinea	1.0 ± 0.01	1.6 ± 0.01	0.8 ± 0.01	1.0 ± 0.01
Saprospiria	1.0 ± 0.01	2.0 ± 0.02	2.4 ± 0.02	1.6 ± 0.02
Others	11.1 ± 0.05	9.1 ± 0.08	9.1 ± 0.08	9.2 ± 0.10
Unclassified	6.8 ± 0.04	5.4 ± 0.06	6.4 ± 0.06	5.8 ± 0.06

Table 3. Diversity and richness of bacterial community in soil with and without supplementation with carbofuran and augmentation with *Bacillus* sp. DT1. Data in each column are obtained from three separate experiments

Indices	At the beginning	After 30 days		
		Control	Carbofuran	
			Without augmentation	With augmentation
Sequences	39875 ± 845.4^{a}	47182 ± 4559.5^{ab}	44520 ± 4497.8^{ab}	48267 ± 4900.0^{b}
OTUs	1450 ± 85.5^{a}	1522 ± 112.2^{a}	1484 ± 131.0^{a}	1557 ± 151.5^{a}
ACE	2101 ± 71.1^{a}	2144 ± 220.4^{a}	2120 ± 205.5^{a}	2324 ± 245.5^{a}
Chao1	2059 ± 65.5^{a}	2055 ± 171.8^{a}	2092 ± 189.4^{a}	2242 ± 235.5^{a}
Simpson	3.52 ± 0.02^{a}	3.60 ± 0.33^{a}	3.57 ± 0.38^{a}	3.57 ± 0.36^{a}
Shannon	0.033 ± 0.001^{a}	0.032 ± 0.002^{a}	0.034 ± 0.002^{a}	0.035 ± 0.002^{a}

Some previous studies described the effects of carbofuran on soil microorganisms. The addition of carbofuran at 25 mg/kg into soil stimulated the growth of fungi and slightly inhibited soil bacteria (Ingham & Coleman, 1984). The application of the pesticide at 1.0 kg/ha induced the growth and development of bacteria, actinomycetes, fungi, N_2-fixing bacteria and phosphate solubilizing microorganisms (Das et al., 2005). The addition of 1.0 mg carbofuran into 1.0 kg soil markedly stimulated the populations and activity of soil methane-producing bacteria, soil anti-nitrifying bacteria, soil nitrogen-fixing bacteria, and soil sulfate-reducing bacteria in purple paddy rice soils (Zhou et al., 2012). However, higher carbofuran concentrations (10 and 50 mg/kg significantly inhibited the populations and activity of the above bacteria (Zhou et al., 2012). Carbofuran caused only modest changes in microbial community patterns for both bacteria and fungi (Castro-Gutiérrez et al., 2017). In this study, carbofuran added at 10 mg/g dry soil did not significantly inhibit or stimulate the soil bacterial community.

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.

CONCLUSION

The augmentation with *Bacillus* sp. DT1 into soil significantly increased carbofuran degradation. Moreover, the augmentation of the bacterial isolate significantly increased the relative abundance of the 16S rRNA sequence after 30 days compared to the initial stage. However, Chao1, ACE, Shannon and Simpson indices are not statistically different among soil with and without the insecticide, with and without augmentation. The addition of carbofuran at 10 mg/kg dry soil did not cause negative effects on the bacterial community under aerobic conditions.

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