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ENSIFFER SP. CNN3 - AN INDIGENOUS BACTERIAL STRAIN IN VIET NAM HAS PROPERTIES OF PLANT GROWTH PROMOTION AND ORGANOPHOSPHORUS DEGRADATION

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Abstract. Organophosphorus pesticides (OPPs) comprise the most effective and widely used insecticides in agriculture worldwide and in Viet Nam. However, the overuse of OPPs may result in severe health and environmental problems, such as the accumulation in food and underground water, leading to DNA mutations in living organisms, OPP poisoning in human and alteration in terrestrial and aquatic living communities. In recent years, the biodegradation of OPPs by microorganisms have gained attentions from scientists for bioremediation of organophosphorus residues. In this study, an indigenous effective OPP-degrading bacterial strain has been isolated from soil samples of contaminated tea farming site in Northern Viet Nam with chlorpyrifos degradability and plant growth promoting characteristics. The bacterial isolate was determined to belong to genus *Ensifer* (syn. *Sinorhizobium*); exhibiting remarkable chlorpyrifos degradability in liquid culture and in test soil with 94.75 % and 76.27 % of substrate removal after 14 days of inoculation, respectively. Besides, *Ensifer* sp. CNN3 appeared as a promising growth promoting bacteria with IAA excretion and phosphate solubilizing properties. The results open a prospect of applying the dual-effective bacterial strain in agriculture practices either to reduce the use of chemical fertilizer or to remediate OPP contaminated soils.

Keywords: Ensifer, chlorpyrifos, organophosphorus, pesticide, bioremediation.

Classification numbers: 1.2.1, 1.3.4.

1. INTRODUCTION

Since pesticides and chemical fertilizers play a pivotal role in modern agriculture for controlling pests and maintaining food security, these artificial chemicals have been introduced to almost all areas of the world in recent century. Among diverse groups of chemical pesticides, organophosphates (OPs) had been known with wide spectrum effects and relatively fast

degradation rates, and thus had been considered to be safe and appropriate for agricultural application [1]. As a result, organophosphorus pesticides (OPPs) have become the most widely used product for plant protection. However, OPPs toxicity has been mentioned as earlier as 1980s [2]. Recent researches showed negative effects of OPPs to the environment, including the accumulation in soil, underground water, non-target terrestrial and aquatic species, and impact on human health, such as endocrine disrupts, breast carcinoma and mutation [1]. There have been increasing reported poisoning cases and deaths concerned over the residual OPPs [3]. The toxicity of the most widely used OPPs, i.e. chlorpyrifos, malathion and parathion, was claimed to arise from their inhibition of the neuroenzyme acetylcholinesterase by phosphorylation, both at the synapse of neurons and in the plasma, in exposed organisms [4]. Once being applied to soil, the OPPs accumulate and deposit in soils, sediments and underground water for up to eight years depending on biological, chemical and physical processes [5]. With thousands tons of annually applied OPPs, the remediation of these chemicals in contaminated sites is an imperative need.

The degradation of organophosphorus compounds by numerous *in situ* and *ex situ* methods such as chemical and physiological treatments has been studied, but most of them were considered not applicable in larger scales with low concentrations of OPP fates [6]. Bioremediation of residual OPPs by indigenous microorganisms was proposed to be the most sustainable method regarding effectiveness and compatibility to natural conditions. A great number of microorganisms, including fungi, algae and bacteria have been known to be able to degrade OPPs in both liquid and solid conditions [3, 7]. Microbial degraders belonging to genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Flavobacterium*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Serratia*, *Stenotrophomonas*, *Burkholderia*, *Athrobacter*, *Plesiomonas*, etc. have been proven to efficiently transform organophosphorus substrates [6]. More importantly, the use of such species in the bioremediation of OPP contaminated soils has achieved encouraging results [3, 8].

Beside pesticide degrading property, several soil bacteria were known as a source of beneficial metabolites enhancing plant growth and have been commonly applied to promote plants and improve crop yield [9, 10]. The application of such microorganisms in crop production has thus been considered an approach to a sustainable, environmentally-friendly agriculture [11].

With the knowledge of the biotransformation of OPPs and microbial plant growth promotion, we conducted the present study aiming to isolate and screen indigenous microbial candidates with OPP degrading property for remediation of contaminated agricultural farming sites in Viet Nam.

2. MATERIALS AND METHODS

2.1. Materials

Soil samples were collected from tea (*Camellia sinensis*) farming sites with a history of organophosphorus pesticide chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] applications in Dai-Tu, Thai-Nguyen province (21°36’77”N, 105°39’ 20”E) and stored at 7 ^{o}C before being studied in laboratory.

2.2. Methods

Enrichment and isolation of bacterial strains

Prior to the enrichment, 30 g of soil samples were added to 100 mL Herman-Frankenberger Medium [HFM, which was composed of (in g/L): K_2HPO_4 0.225; KH_2PO_4 0.225; $(NH_4)_2SO_4$ 0.225; $MgSO_4.7H_2O$ 0.05; $CaCO_3$ 0.005; and $FeCl_2.4H_2O$, 0.005 blended with 1 mL of trace elements solution (composition in mg/L: $MnSO_4.H_2O$ – 169; $ZnSO_4.7H_2O$ – 288; $CuSO_4.5H_2O$ – 250; $NiSO_4.6H_2O$ – 26; $CoSO_4$ – 28; and $Na_2MoO_4.2H_2O$ – 24)] [12] and agitated (220 rpm, 25 ^oC) for 15 hours. The mixture was then settled for 15 mins to allow separation of soil particles and supernatant.

Aliquots of the supernatant (5 mL each) were used as input for enrichment by adding to Erlenmeyer flasks, each containing 45 mL of mineral salt medium (MSM) supplemented 0.05 g/L chlorpyrifos (Pestanal, Merck, Germany) as the sole carbon source. The MSM was composed of (in g/L): K_2HPO_4 – 1.8, NH_4CI – 4.0, $MgSO_4.7H_2O$ – 0.5, NaCl – 0.1, $FeSO_4.7H_2O$ – 0.1, and 1 mL of trace elements solution [12]. Its final pH was adjusted to 7. The culture was incubated at 30 °C on a rotary shaker at 220 rpm. After 14 days, 0.1 mL aliquots of enrichment cultures were pipetted and spread on MSM agar plates (basal MSM added with 2 % agar) containing 200 ppm of chlorpyrifos to isolate chlorpyrifos degrading bacterial strains. Discrete bacterial colonies were further purified by streaking on fresh agar plates and isolates were stored in slants at 4 ^oC.

Determination of pesticide degradation

Bacterial isolates exhibiting ability to grow in the presence of pesticide substrate were inoculated into 100 mL Erlenmeyer flasks containing fresh MSM broth containing 300 and 1000 ppm chlorpyrifos, respectively. The culture was orbitally shaken at 220 rpm (30 °C) and sampled every two days during the incubation period. The bacterial densities in culture broths were measured at 620 nm using Tecan's infinite spectrophotometer (TECAN, Switzerland).

For measurement of chlorpyrifos content in cultures, 5 mL vials were centrifuged at 10,000×g for 10 min to remove bacterial cells. The collected supernatant was filtered and used as input for determination in Agilent's Gas Chromatography/Mass Spectrometer (GC/MS) (USA) following US EPA's method 8270D [13]. In general, samples were introduced into the system by injecting with a narrow-bore fused-silica capillary column. The GC column (30 m × 0.25 mm ID, 1 µm film thickness silicone-coated) was temperature-programmed to separate the analytes, which were then detected with the connected MS. Identification of chlorpyrifos was accomplished by comparing its mass spectrum with the electron impact spectra of standard.

Determination of plant growth promoting (PGP) traits

Indole acetic acid (IAA) production

Indole acetic acid (IAA) producing bacterial isolates was determined by microplate method as described by Sarwar & Kremer [14]. Bacterial cultures were grown for 72 h on Luria-Bertani medium [composed of (g/L) $Ca_3(PO_4)_2$ – 5.0, glucose – 10.0, $(NH_4)_2SO_4$ – 0.5, KCl – 0.2, $MgSO_4.7H_2O$ – 0.1, trace of $MnSO_4$ and $FeSO_4$, yeast extract – 0.5, and agar – 15.0, pH = 7.0] at 30 °C, followed by centrifugation at 3000 rpm for 10 min and filtration (Whatman filter paper Grade 1) to remove cells. The culture filtrate (150 µl) was transferred into microplate and mixed with 100 µl of Salkowski reagent ($FeCl_3$ 0.5M in perchloric acid 35 %, 1:50 v/v) then incubated 30 min at room temperature. The microplate was measured at 530 nm with the help of Tecan's infinite spectrophotometer. Concentration of IAA produced by bacterial isolates was calculated by regression analysis (Excel, Microsoft Office, USA) using a pre-determined calibration curve established with IAA standards (Sigma-Aldrich, USA) in the range from 10 to 200 µg/mL. Sterile Luria-Bertani medium served as negative control in the test.

Phosphate solubilizing assay

Qualitative analysis of solubilization of tricalcium phosphate in liquid NBRI-BPB medium containing bromophenol blue (BPB) was performed as described by Mehta & Nauriyal [15]. The NBRI-BPB medium contained (g/L) glucose - 10, $Ca_3(PO_4)_2$ - 5; $MgCl_2$. $6H_2O$ - 5; $MgSO_4.7H_2O$ - 0.25; KCl - 0.2, $(NH_4)_2SO_4$ - 0.1, and BPB - 0.025, pH = 7.0. The method was developed basing on the decolorization of BPB by phosphate solubilizing bacteria. The level of decolorized cultures was determined by measuring absorbance at 600 nm by spectrometer (TECAN, Switzerland).

Molecular and biochemical characterization

The selected isolate was biochemically characterized by Gram's reaction, carbohydrate fermentation (utilization of carbon sources), oxidase, catalase and urease test, H_2S production using API®20NE Microbial Identification Kit (bioMerieux, France). It was also identified by 16S rRNA gene sequence analysis after its 16S rRNA gene fragment was amplified by colony polymerase chain reaction (PCR) using the primers Pr16F (5'-AGAGTTTGATCCTGGCTCA-3') and Pr16R (5'-TACGGTTACCTTGTTACCGACTT-3') [16] and sequenced on ABI PRISM® 3100 Avant Genetic Analyzer System (Applied Biosystems, USA). The generated 16S rRNA gene sequence was analyzed by comparing it to those in GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/) using BLASTn tool. Phylogenetic tree was constructed using MEGA X software [17].

In vitro soil application test

Soil substrate was collected from tea farming site and its characteristics were determined at Soils and Fertilizers Research Institute (SFRI) with following parameters: organic matter, 2.13 %; Total N, 0.12 %; Clay, 3.52 %; Silt, 59.76 %; Coarse sand, 17.28; Fine sand, 19.44 %; pH_2O, 3.20. The soil substrate was air dried, blended, passed through 2 mm sieves and placed in sterilized 100 mL chambers. Chlorpyrifos was added to each 50 g soil portion at concentration of 400 ppm, and the mixture was stirred well for evenly distribution. The soil mixture was then inoculated with test bacteria in MSM solution (OD~0.6) (5 mL for each soil chamber) and incubated at 30 ^oC for 20 days. The residual chlorpyrifos in soil samples over time (3-day intervals) were determined by GC/MS following US EPA method 354 ^oC [18].

3. RESULTS AND DISCUSSION

Isolation of chlorpyrifos degrading bacterial strains

At the end of the enrichment period, the optical density at 620 nm (OD_{620}) of culture reached 0.95. After being incubated on MSM agar plates supplemented with 200 ppm of chlorpyrifos for 48 h, bacterial colonies were observed. Four represented colonial morphology types, namely CNN1, CNN2, CNN3 and CNN4, were noticed and the respective colonies selected to purify. Among them, CNN3 was capable of growing on MSM agar plates with chlorpyrifos at concentration of 2000 ppm and thus was chosen to be characterized and further studied.

Pesticide reducing property in liquid

The growth of bacterial strain CNN3 determined through OD_{620} and the degradation kinetic of chlorpyrifos in liquid MSM medium in dependence to culture time are presented in Fig. 1.

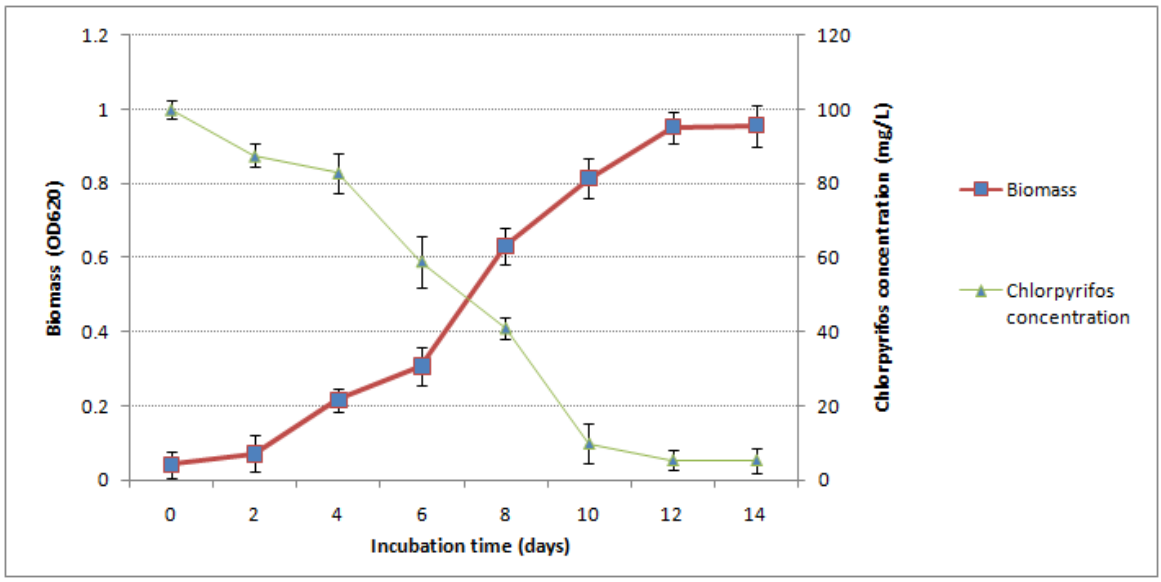

Figure 1. The growth and chlorpyrifos degradation of bacterial strain CNN3.

Figure 1 indicated significant differences in growth kinetics of CNN3 in liquid culture within 14 days. The bacterial culture attained a maximum OD_{620} after 10 days (Fig. 1). The results showed that after 3, 7 and 14 days of incubation, 17.11 %, 59.03 % and 94.75 % of the initial insecticide dose were degraded by bacterial strain CNN3, respectively.

Plant growth promoting property

The chlorpyrifos degrader CNN3 was further investigated for its *in vitro* plant growth promoting traits, including IAA excretion and phosphate solubilization. While IAA is a well-known plant-growth regulator in the initial processes of root formation and elongation [9], phosphate-solubilizing trait plays an important role in improving the availability of phosphorus to plants and increasing the crop yield [15]. The amounts of *in vitro* IAA produced and solubilized phosphate by the bacterial strain CNN3 were much significant higher than that by negative control (Table 1). The plant growth promoter *Bacillus megaterium* strain INN1 provided by Microbial Collection of Institute of Natural Products Chemistry (INPC-VAST) was used as positive control for both IAA producer and phosphate solubilizer.

Table 1. Plant-growth-promoting traits of bacterial strain CNN3.

Test samples	**IAA production (ppm)**	**Phosphate solubilization (mg/mL)**
Negative control	0	0
Positive control	84.62 ± 3.90	68.59 ± 4.15
Strain CNN3	54.77 ± 5.31	26.14 ± 2.58

The results showed in Figure 1 and Table 1 suggested the potential of applying bacterial strain CNN3 in bioremediation of contaminated culture soils. In the present investigation, biological characterization and the *in vitro* OP degrading bioactivity of the bacterium in soil were additionally studied.

Morphological, biochemical and molecular characterization

The bacterial isolate CNN3 was characterized on the basic of its morphological, biochemical characteristics and 16S rRNA gene sequence. On Luria-Bertani agar plate, colonies of CNN3 appeared in circular form, 0.5 - 1 mm in diameter, slimy moist, whitish and produce no obvious pigment (Fig. 2A). Under microscope (×100), the bacterium appeared with singly rod shape, 0.3 - 0.7 × 1.0 - 2.0 µm (Fig. 2B).

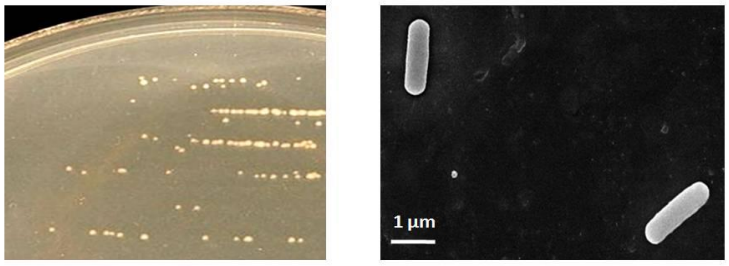

Figure 2. Colony morphology on LB agar (image A) and microscopic morphology (image B) of bacterial strain CNN3 (×100).

Employing API's identification tests, biochemical profile of strain CNN3 was determined. As described in Table 2, CNN3 was Gram-negative, aerobic, non-motile and non-microaerophile. The bacterium performed to be able in utilizing various carbon sources such as arabinose, xylose, glucose, galactose and sucrose.

Table 2. Physiological characteristics of strain CNN3.

Characteristics	**Strain CNN3** *		**Characteristics**	**Strain CNN3** *
Gram	-		Utilization of carbon sources	
Aerobic	+		*Arabinose*	+
Microaerophile	-		*Xylose*	+
Motility	-		*Glucose*	+
Enzyme production			*Fructose*	-
Catalase	+		*Galactose*	+
Oxidase	+		*Sucrose*	+
Urease	+		*Starch*	-
			Optimal temperature	28 ÷ 32 ^{o}C
			Optimal pH	6.0 ÷ 7.0
H_2S production	-			

*Symbols + and – indicate positive and negative for a characteristic, respectively.

The partial 16S rRNA of bacterial strain CNN3 was sequenced and deposited in the GenBank with accession number MT893347. The gene was analyzed and aligned with corresponding genes in GenBank to create the phylogenetic tree as described in Figure 3. The isolate CNN3 has the most identity to *Ensifer adhearens* strain H1 (Accession: LC076288.1) (with 98.21 % similarity in 16S rRNA gene sequences), then to *Ensifer* sp. strain PZS_S05 (Accession: KY992904.1, identity of 98.11 %) and *Ensifer adhaerens* strain PZG_S11 (Accession: KY660602.1, identity of 98.11 %). The result was consistent with aforementioned

morphological and biochemical properties of the bacterial strain to support the fact that CNN3 is a bacterium of genus *Ensifer* (syn. *Sinorhizobium*).

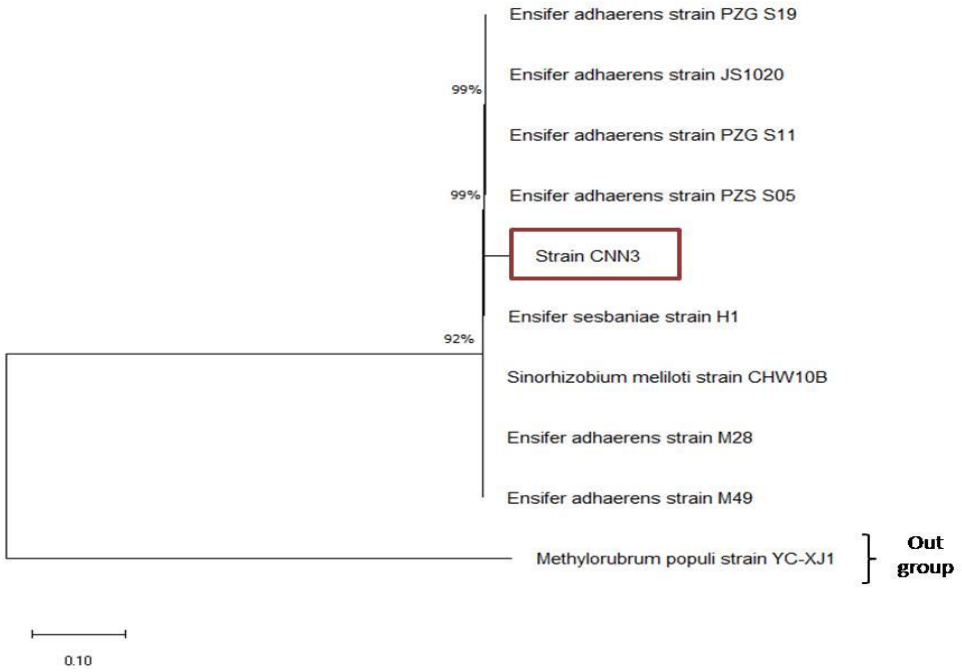

Figure 3. Phylogenetic tree constructed from 16S partial rRNA gene sequences of strain CNN3 and related organisms in GenBank by MEGA X sofware (ClustalW alignment, maximum likelihood method, bootstraps 100). *Methylorubum populi* strain YC-XJ1 served as the out group.

In general, CNN3 shared a number of similar traits to the described species *E. adhaerens* [19], a well studied plant growth promoting rhizobacteria with neonicotinoid insecticide thiamethoxam biodegradability [20] as well as heavy metal bioaccumulating potential [21]. However, extensive determination such as whole-genome sequencing or GC content is necessary to fully claim CNN3 a strain of *E. adhaerens*.

Degradation of chlorpyrifos by Ensifer sp. CNN3 in soil

The biodegradation of chlorpyrifos by *Ensifer* sp. CNN3 in broth culture was evidenced, however, its OPP degradability in soil environment have not been confirmed. In another experiment, the ability of *Ensifer* sp. CNN3 to degrade chlorpyrifos in soil condition was studied. Chlorpyrifos in *in vitro* inoculated and uninoculated soil samples was measured within 15 days under controlled parameters (25 ^{o}C, direct light protection). Results demonstrated in Figure 4 revealed a reduction of chlorpyrifos level in experimental inoculated soil over time.

Notably, the decrease of OPP in uninoculated soil samples was also recorded, but with a less transformation rate than in inoculated ones. *Ensifer* sp. CNN3 appeared to be an effective chlorpyrifos degrader with 76.27 % of removal at day 15, in comparison to 32.41 % in uninoculated control (Fig. 4). The data provided convincing evidences for effective degradation of chlorpyrifos both in liquid and in soil conditions by our indigenous bacterial strain.

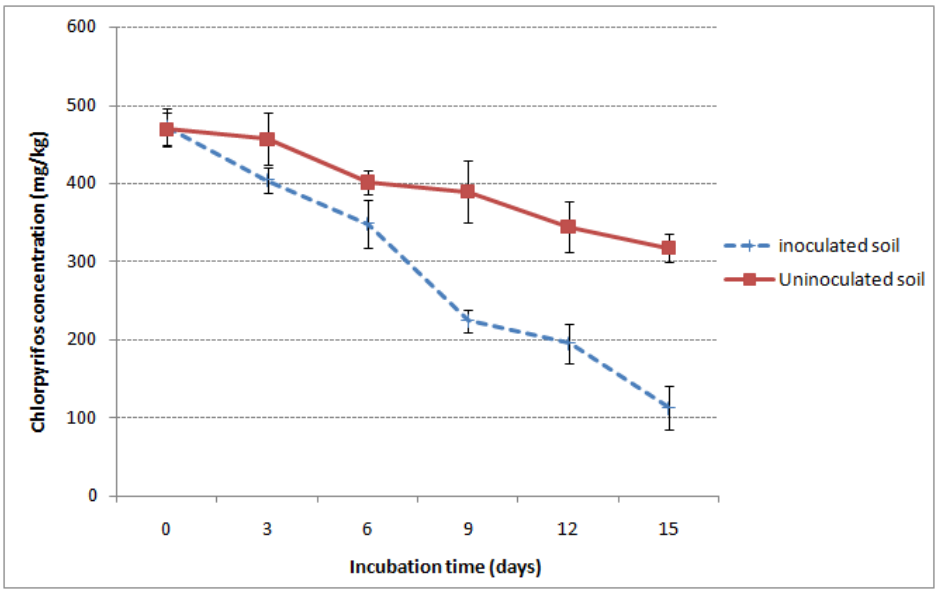

Figure 4. Chlorpyrifos degradation in *Ensifer* sp. CNN3 inoculated and uninoculated soil samples in *in vitro* test.

As mentioned, several bacteria of genus *Ensifer* have been identified with multipurpose applicability. In 2016, *E. adhaerens* was applied in biosorption and biodegradation of polychlorinated biphenyls from contaminated water [22]. More recently, *E. adhaerens* was claimed to retain plant growth promoting activities, biosorption ability and chromium reducing potential [21]. To the best of our knowledge, the ability of *Ensifer* sp. in degradation of organophosphorus pesticide substrates has not been referenced earlier.

The biotransformation of chlorpyrifos by bacteria was believed to include a hydrolysis to diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP), and stepwise a dechlorination of TCP to pentanoic acid [23]. While the first step has long been known to be catalyzed by organophosphorus hydrolase (OPH) [24], the later phase is considered more complicated with two distinct proposed hypotheses, namely reductive and oxidative dechlorination [23]. Possibly, the degrading mechanisms of *Ensifer* sp. CNN3 for chlorpyrifos may involve not only the transformation of chlorpyrifos to TCP, but also a dechlorinating process, which may share similarities to that previously reported mechanism of *E. adhearens* in degradation of chlorinated aromatic substrates PCBs [22]. Therefore, further studies are advisable to uncover the underlying mechanism of *Ensifer* sp. CNN3 in biodegradation of chlorpyrifos.

4. CONCLUSIONS

In the present study, an *Ensifer* strain with plant growth promoting traits and biodegradability of organophosphorus pesticide chlorpyrifos was reported for the first time. The bacterial strain *Ensifer* sp. CNN3 was isolated from a chlorpyrifos contaminated tea farming site in Viet Nam, and displayed its high potential for use as an effective bacterial strain for enhancing crop production and treatment of residual OPPs in contaminated agricultural soils.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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