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Microbial degradation of second generation Neonicotinoid: Thiamethoxam in clay loam soils

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Abstract

Thiamethoxam is a second-generation neonicotinoid poses risk to non-target organism and have potential of leaching to contaminate underground water. Biodegradation of thiamethoxam by microbial species is a biological way to reduce the contaminant. In the present paper, persistence of thiamethoxam in clay loam soil was studied for 56 days in laboratory conditions. *Bacillus aerophilus* strain IMBL 4.1 bacterium isolated from field soil and used to spike the soil sample containing different concentration (25, 50 and 100 mg kg⁻¹) of thiamethoxam. *Bacillus aerophilus* strain IMBL 4.1 resulted in the active biodegradation of thiamethoxam. Half-life ($t_{1/2}$) and the correlation coefficient (R^2) were studied and none of value was found more than 0.99 (R^2 values) so, total thiamethoxam residues did not follow the first-order kinetics neither in control nor in amended samples. From the study, it was demonstrated that *B. aerophilus* has potential for biodegradation of thiamethoxam in clay loam soils.

Keywords: Biodegradation, thiamethoxam, *Bacillus aerophilus*, clay loam soil

Introduction

Pesticide is synthetic chemicals used against pest to protect the crop, to enhance the crop yield and full fill the growing demand of crop. The class of synthetic organic insecticide was designed in the '1980s, which established new key components in insect control programs worldwide. It hold registrations in more than 120 countries accounted for about 24% of the total world insecticides market in 2007 [1] and uses in 64 countries. Neonicotinoids are one of these pesticides, being highly systemic with long-term persistence but with relatively low toxicity to the environment and mammals [2]. Neonicotinoids also has new mode of action, affecting the nicotinic acetylcholine receptor (nAChR) present on insect cell membrane and leads to the paralysis and ultimately death of insect by accumulating the neurotransmitter acetylcholine and blocking the nicotinic neuronal pathways [3].

Thiamethoxam, is a second-generation chlorothiazolylmethyl neonicotinoid discovered and developed by Ciba Crop Protection in 1996 (Syngenta Crop Protection). It has been marketed as Cruiser® for seed and Actara® for foliar treatment since 1998; these brands have offered effective broad-spectrum pest protection on numerous crops around the world [4]. Thiamethoxam is active against chewing and sucking insects that attacks various crops [5], it is nitromethylene derived neonicotinoid hydrolytically more labile at pH 9 (half-life a few days) and very stable at pH 7 (at RT, half-life 200-300 days) and at pH 5 (at room temperature, RT; half-life >1year [6]. Soil DT₅₀ is 51 days. However, low soil sorption and high leaching capability are its characteristic properties; make it potential contaminants of surface and underground waters [7]. European Food Safety Authority (EFSA) conducted new studies on the use of clothianidin, imidacloprid and thiamethoxam and concluded that its uses pose high risk on non-target pests such bees, which help in pollination. In view of risk assessment European Union partial, ban these substances in May 2013.

As only less than 0.1% of applied pesticides reach the target organism, more than 99.9% of the used pesticides make its way into the environment adversely affect the public health and beneficial biota through contamination of soil, water and atmosphere of the ecosystem [8-9]. In bioremediation method, selected microbes use to degrade the specific pesticides *in situ*.

As it also offers the most attractive and cost effective remediation approach, this study was undertaken to develop bioremediation strategy to dissipate thiamethoxam from fortified soil cultures for future exploitation in contaminated soil and water environments in future. The Present studies were undertaken biodegradation of thiamethoxam in clay loam soil.

Materials and Methods

Source of microbial culture for degradation of thiamethoxam

For the purpose of degradation of thiamethoxam in soil pure bacterial culture of *B. aerophilus* strain IMBL 4.1 was used throughout the present study. Bacterial culture was preserved at 4°C and taxonomically identified by BLAST tool in the Insect Molecular Biology laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana, India. It was isolated from sugarcane field soil in Gurdaspur district of Punjab, India, [10] for the degradation purpose of phorate. Previous studies concluded that *B. aerophilus* strain IMBL 4.1 has potential to degrade the synthetic organic pesticide. To check the broad spectrum of pesticide degradation by bacterial specie in soil, we used *B. aerophilus* strain IMBL 4.1 for degradation of thiamethoxam.

Bacteriological grade media and Reagent used

Two freshly prepared media: General-purpose media (Luria Bertani) [11] and minimal media (mineral salt medium) [12] were used throughout the study to maintain growth of microbe. Reagents used such as acetone, acetonitrile, activated charcoal, sodium sulfate were analytical grade, including technical grade standard of thiamethoxam. Sigma-Aldrich, India supplied the thiamethoxam standard and claimed 99.7% purity.

Source of Clay Loam soil

The topsoil of Northern India plains is very good for Rabi and kharif crops as texture varies from sand to clay. The greater part of soil is being light loam, porous in texture and naturally fertile. The sample of clay loam soil was used for the study, taken randomly from the field of PAU, Ludhiana, Punjab have Cation Exchange Capacity (CEC) that hold and then release cation to the soil water that have been lost through leaching or plant uptake. The sample of clay loam soil was collected (0 to 15 cm depth of soil) in gunny bags from the field area of PAU, Ludhiana, Punjab and brought to the Pesticide Residue Analysis Laboratory, Entomology Department of Punjab Agricultural University, Ludhiana for further analysis.

Preparation of soil samples

The clay loam soil was used during the experiment to observe the persistence of Thiamethoxam, an insecticide. Soil sample first cleaned to remove the unwanted particles, aggregates of soils destroyed with gloves wearing hands, finally clay loam soil air-dried and sieved. Before preceding the experiment, the physical characteristics of soil were determined. Autoclaving of soil sample was done in cleaned beaker covering with aluminum foil to sterilized the soil sample so, no natural microbes were present responsible for degradation of pesticides.

Soil amended with microbes and thiamethoxam

Sterilized clay loam soil sample weighed about 25g and filled

in palstic cup. Prepared working solution of thiamethoxam from the stock solution so that the clay loam soil sample were fortified with thiamethoxam @ three concentration 25, 50 and 100 mg kg⁻¹. Above, soil samples were spiked with 250µl microbial cells mass of *B. aerophilus* strain IMBL 4.1 except the controls of each concentration. Each treatment was replicated thrice to get two group: sterile soil (insecticide+microbe) and control soil (insecticide+without microbe). Then, covered these fortified soil samples with aluminum foil to reduce the chance of contamination. The whole experiment conducted in laminar-air flow under sterilized conditions and fortified soil sample incubated at 25±2 °C. Sampling was done at regular interval of 7 days gap up-to 56 days along with control samples after inoculation with isolated microbes. The each sample was moistening with water at 7days interval.

Residue analysis

QuEChERS was a method used to determine the thiamethoxam residues followed by weighing of 15 g soil sample from each respective concentration. Sample took from the above-incubated 50 g soil into a 50 mL centrifuge tube. About 30 mL acetonitrile and 10 mL water were poured to these centrifuge tubes and homogenized for 2-3 min at 14,000 rpm using high-speed homogenizer (Heidolph Silent Crusher-M®). After this, these subjected to phase separation. In phase separation, an acetonitrile layer was obtained after adding 10±0.1 g sodium chloride (NaCl) to homogenized sample and centrifugation at 2,500 rpm for 3 min. Cleanup the acetonitrile layer through Dispersive solid phase extraction (DSPE) by transferring an aliquot of 15ml over 10g sodium sulfate in a test tube. Again, an aliquot of 6 ml acetonitrile was taken in a test tube containing 0.15±0.01 g PSA sorbent, 0.90±0.01 g anhydrous MgSO₄ and 0.05±0.01g graphitic carbon black. The content was vortexed for thoroughly mixing. Finally, centrifuged at 2,500 rpm for 1 min. Separate the layer and evaporate about 4 mL aliquot of this acetonitrile to dryness using low volume evaporator at 35°C. Add acetone to made final volume 2mL. Analysis of thiamethoxam done by GLC equipped with ⁶³Ni ECD (Electron Capture Detector).

Degradation kinetics of total thiamethoxam residues in soil

The degradation kinetics was determined to calculate the half-life value of thiamethoxam in clay loam soil. A graph plotted between the residue concentrations against time. The maximum squares of correlation coefficients were found use to determine the equations of best-fit curves.

Results and Discussion

Twelve bacterial species were previously isolated using soil sample taken from the sugarcane field through enrichment method on the MSM media. 16S rDNA nucleotide-sequence homology with Gen Bank database was used to identify these species by our Laboratory, which proved to have ability to degrade phorate- a pesticide [13]. In another 15 days experiment, which was conducted [14] in the same laboratory, showed the bacterial species capability for degrading thiamethoxam in MSMT liquid culture. *Bacillus aerophilus* strain IMBL 4.1 reduced 45.28% thiamethoxam (50-µg ml⁻¹) as bacterial species used neonicotinoid as carbon and nitrogen source for growth depending weather microbe is catabolic or cometabolic, in its metabolic activity [15]. Hence, this strain further selected for study, carried on soil amended with *Bacillus aerophilus* + thiamethoxam to optimizing their

bioremediation potential in agricultural soils.

In the present investigations, recovery experiment was carried out at different levels to establish the reliability and validity of the analytical method and to know the efficiency of extraction and clean-up procedures. Samples of clay loam soils (15 g) were spiked with thiamethoxam standard at different levels viz. 0.01, 0.05, 0.10 and 0.50 mg kg⁻¹. These samples were extracted, cleaned and analyzed, following the method already described. The control samples from untreated plots and reagent blanks were also processed in the same way to find out the interferences, if any, due to the substrate and reagents, respectively. The mean recoveries of thiamethoxam were found to be 88.52 - 96.27 per cent (Table 1). The average recovery values from the fortified samples were found to be more than 85 per cent. Therefore, the results have been presented as such without applying any correction factor. The precision of the method was determined by repeatability studies and expressed by RSD values (Relative standard deviation). The RSD for repeatability, ranged from 3.19 to 4.34 per cent for thiamethoxam for different spiking levels as shown in Table 1.

The samples of clay loam soil, fortified with different concentration of thiamethoxam at 25, 50 and 100 mg kg⁻¹ along with 45 × 10⁷ *B. aerophilus* microbe cells were analyzed after 7 day at regular time interval upto 56 days after initiation of this experiment. The maximum residues of thiamethoxam were found to be 17.84, 34.56 and 78.61 mg kg⁻¹ in soil collected at 7 days after the application of thiamethoxam @ 25, 50 and 100 mg kg⁻¹, respectively. These residues were degraded to 11.15, 19.99 and 48.61 mg kg⁻¹, respectively within 21 days. In this study, residue of thiamethoxam in amended and unamended soils were found to be 0.62, 1.13, 3.88 mg kg⁻¹ and 7.12, 16.21, 29.54 mg kg⁻¹, in 56 days, respectively. The residue of the thiamethoxam gradually decreases in amended and unamended soil with no visual metabolites of thiamethoxam. As the incubation period preceded the degradation of thiamethoxam high in amended soil than unamended. These results show that bacterium *Bacillus aerophilus* strain IMBL 4.1 has a great role in the bioremediation of thiamethoxam-contaminated soils (Fig.1). Persistence of Thiamethoxam was found to be very less in amended soil than the unamended soil, it indicate the role of microbes in amended soil to degrade the thiamethoxam whereas thiamethoxam was found to be persist more than 56 days in unamended soil. Microbes accelerate the degradation rate of thiamethoxam and help to clean the environment by mean of biological way.

Table 2 showed the statistical data on regression analysis and half-life for the dissipation of thiamethoxam on clay loam soil amended with *Bacillus aerophilus*. Regression analysis estimate the relationships among variables whereas half-life

($t_{1/2}$) or DT₅₀ find out the time taken for disappearance of given material from its initial concentration to half of its value. So half-life ($t_{1/2}$) or DT₅₀ of pesticide represent the degradation of pesticide to 50 per cent of its initial concentration i.e. persistence of an insecticide to its half-life. Based upon the data in Table 2, the relative thiamethoxam degradation potential of the bacterial specie was interpreted in terms of half-life ($t_{1/2}$) period of thiamethoxam, in the presence of growth of specific bacterial specie (Table 2). Half-life ($t_{1/2}$) of total thiamethoxam were observed to be 11.15, 11.58 and 12.54 days following spiking of thiamethoxam @ 25, 50 and 100 mg kg⁻¹, respectively in clay loam soil amended with bacteria. In unamended soil, the value were found to be 33.44, 37.63 and 37.63 days following application of thiamethoxam @ 25, 50 and 100 mg kg⁻¹ (Table 2). Table 2 also showed data on correlation coefficient (R²), the R² values were 0.973, 0.976, 0.949 for control sample and 0.912, 0.933, 0.951 for amended sample containing thiamethoxam @ 25, 50 and 100 mg kg⁻¹, respectively. None of value was more than 0.99 (R² values) so, total thiamethoxam residues did not follow the first-order kinetics neither in control nor in amended samples (Table 2). For the sample studied, exponential relations were found to apply, corresponding to first order rate equation. Confirmation of the first order kinetics was further made graphically from the linearity of the plots of log C against time (Fig. 2).

In present study, the added microbes influenced the biodegradation of thiamethoxam in sterile clay loam soil. Degradation of thiamethoxam in amended soil was high as compared to the unamended soil, kept as control. It concluded that *Bacillus aerophilus* has ability to degrade the thiamethoxam in soil under suitable conditions. There are many studies done by different researchers on microbial degradation of thiamethoxam and found different species such as *Ensifer adhaerens* [16], *Pseudomonas* sp. [17] and *Bacillus amyloliquefaciens* IN937a, *B. pumilus* SE34 and *B. subtilis* FZB24 [18], which evident that respected bacterial species have ability to degrade thiamethoxam.

Table 1: Recovery studies of thiamethoxam on clay loam soil

Fortification Level	^a Recovery %	^b SD	^c RSD
0.01	88.52	3.84	4.34
0.05	92.84	2.96	3.19
0.10	96.27	3.69	3.83
0.50	89.58	3.53	3.94

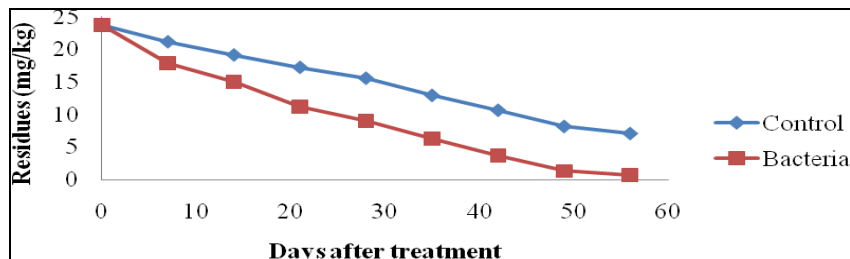
^aNumber of replicates at each level (n=3) all made under the same conditions on the same day

^bSD= Standard deviation

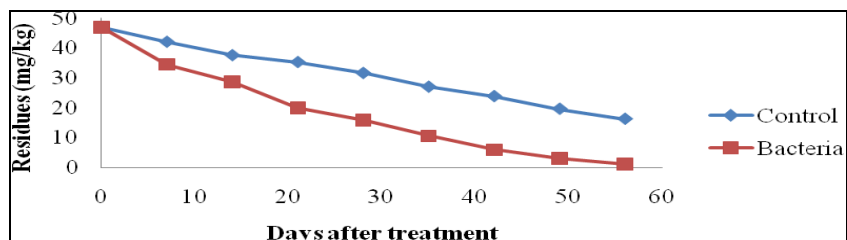
^cRSD= Relative standard deviation

Table 2: Statistical data on regression analysis and half-life for the dissipation of thiamethoxam on clay loam soil amended with *Bacillus aerophilus*.

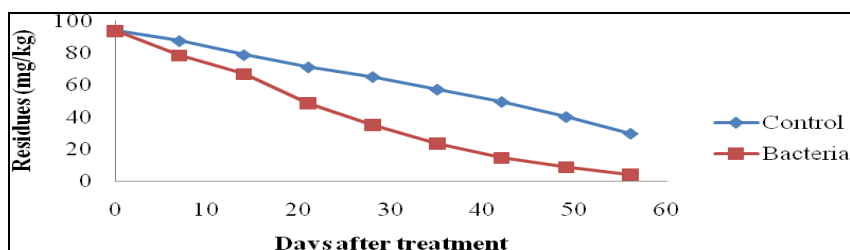
Bacteria	Dose (mg kg ⁻¹)	Regression equation (Y)	Half-life (Days)	Correlation coefficient (R ²)
Control	25	-0.009x+3.414	33.44	0.973
	50	-0.008x+3.693	37.63	0.976
	100	-0.008x+4.012	37.63	0.949
<i>Bacillus aerophilus</i> strain IMBL 4.1	25	-0.027+3.535	11.15	0.912
	50	-0.026x+3.809	11.58	0.933
	100	-0.024x+4.114	12.54	0.951



(a)



(b)



(c)

Fig 1: Residues of thiamethoxam (mg kg^{-1}) in clay loam soils fortified @ (a) 25, (b) 50 and (c) 100 mg kg^{-1} and amended with *Bacillus aerophilus*.

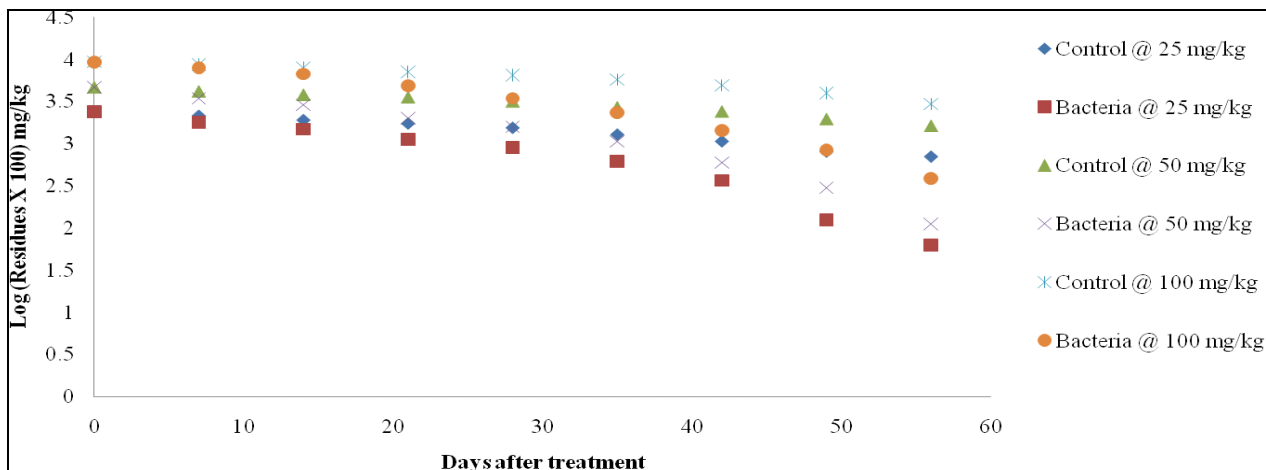


Fig 2: Semi-logarithm graph showing dissipation kinetics of thiamethoxam residues on clay loam soil.

Conclusion

As the demand of pesticide increases to enhance the productivity, the need of biodegradation by bacterial species provides the great approach to reduce the contamination of soil. Present study, concluded that many soil bacteria have ability to degrade pesticide on broad spectrum. Further research on this field provides species that are more valuable. Although, knowledge of biochemical, physiology, genetics and effect of environment on desired microorganism may enhance the biodegradation of the different pesticide in less time.

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