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Biodegradation of Profenofos by *Bacillus subtilis* Isolated from Grapevines (*Vitis vinifera*)

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ABSTRACT: The biodegradation of profenofos, an organophosphorus insecticide, by four *Bacillus subtilis* strains, namely, DR-39, CS-126, TL-171, and TS-204, isolated from grapevines or grape rhizosphere was studied in liquid culture, on grape berries, and in vineyard soil. Each of the four *B. subtilis* strains enhanced the degradation of profenofos in all three matrices. Degradation rate constants were best obtained by first + first-order kinetics module. In nutrient broth spiked with 5 μg/mL profenofos, inoculation with *B. subtilis* strains DR-39, CS-126, TL-171, and TS-204 reduced the half-life (DT_{50}) of profenofos to 4.03, 3.57, 2.87, and 2.53 days, respectively, from the DT_{50} = 12.90 days observed in the uninoculated control. In Thompson Seedless grapes sprayed with profenofos at a field dose of 1250 mL ai/ha, the DT_{50} values were 1.07, 1.00, 2.13, and 2.20 days in grapes inoculated with *B. subtilis* strains DR-39, CS-126, TL-171, and TS-204, respectively, as compared to 2.20 days in uninoculated grapes. These four *B. subtilis* strains also enhanced the degradation of profenofos in autoclaved soil (DT_{50} = 5.93, 7.47, 6.00, and 4.37 days) and in nonautoclaved soil (DT_{50} = 0.87, 2.00, 2.07, and 2.43 days) amended with 5 μg/g profenofos from the half-lives of 17.37 and 14.37 days in respective uninoculated soils. Growth dynamic studies indicated that all four *B. subtilis* strains were able to establish and proliferate on berries and soil equally well in the presence or absence of profenofos. Degradation product 4-bromo-2-chlorophenol was identified by GC-MS. Strain DR-39 was most effective in the natural environments of grape and soil.

KEYWORDS: *degradation kinetics, GC-MS, LC-MS/MS, grape, soil, 4-bromo-2-chlorophenol, grapevine, rhizosphere*

INTRODUCTION

Grape is commercially cultivated in the tropical wet and dry or the arid and semiarid climatic regions of peninsular India where the weather is conducive to support insect pest attacks on grapevines during both the vegetative and fruiting seasons. Frequent pesticide applications are necessary to control the different pests and prevent qualitative and quantitative losses. The management of pesticide residues has become a major concern for the stakeholders of the grape industry.

In India, a residue monitoring program is implemented for monitoring of 175 chemicals in grapes, which include recommended, restricted use, and banned pesticides appearing from direct or indirect sources.[1] One such chemical being monitored is the broad spectrum organophosphorus insecticide profenofos and its metabolite, 4-bromo-2-chlorophenol. Although profenofos has no label claim for grapes, it is registered and marketed in India for the control of mealybugs in cotton.[2,3] Profenofos and its metabolite were sometimes detected in grapes[4] at limits above the EU MRL of 0.01 mg/kg,[1] implying that it has entered the vineyard through direct or indirect sources.

The higher than MRL levels of pesticide residues on fruits indicate the need for a safe, convenient, and economically feasible method of in situ detoxification. Microbial degradation of pesticides has been recognized as a potential solution for elimination of residues. Naturally occurring bacterial isolates capable of metabolizing organophosphorus compounds have received considerable attention as they provide the possibility of both environmentally friendly and in situ detoxification[5,6] and can act in a range of environmental conditions.[7] Two bacterial species, namely, *Pseudomonas putida* and *Burkholderia gladioli*, were found capable of degrading profenofos.[8] Although no reports on degradation of profenofos by *Bacillus* species are available, they are considered good candidates for biodegradation of pesticides[9−11] including organophosphorous pesticides.[12−14] *Bacillus* species are producers of phosphotriesterases and carboxylesterases, which are implicated in the enzymatic hydrolysis of organophosphorus compounds.[15,16]

Most of the biodegradation studies so far were conducted in vitro or in soil or aquatic environments,[17−19] conditions that would be supportive of bacterial growth and activity. In contrast, aerial environments are considered to be harsher and prone to more drastic fluctuations in physical and chemical properties of the phyllosphere. However, recently biodegradation of organophosphorus pesticides on phyllosphere utilizing epiphytic bacteria has been reported,[20] suggesting that the selected, introduced bacteria would be able to establish and proliferate and be biologically active on aerial surfaces.

Usually the contaminated sites are the preferred locations to search for microorganisms that will have the ability to degrade that particular toxicant. However, we have taken a different approach. In grapes, powdery mildew infections can occur on bunches near the end of the growing season, and frequently residues of those pesticides, which are used for its control, are detected above MRL values. A strain possessed of the unique

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ability to suppress powdery mildew and degrade pesticide residues would be useful in Indian viticulture.

In ongoing studies on the biological control of powdery mildew, we have identified endophytic and nonendophytic *Bacillus* strains that have the ability to minimize powdery mildew infections on grapes (unpublished data). In this research paper we report on the ability of four of these strains to degrade the insecticide profenofos in vitro, on grape berries, and in vineyard soil.

MATERIALS AND METHODS

Chemicals and Reagents. Residue analysis grade ethyl acetate (Thomas Baker, Mumbai, India), primary−secondary amine (PSA, 40 μm, Agilent Technologies, Bangalore, India), and graphitized carbon black (GCB) (United Chemical Technology, Bristol, PA, USA) were procured for analysis. Anhydrous sodium sulfate (analytical reagent grade), HPLC grade acetonitrile, methanol, and water were purchased from Merck (Mumbai, India).

Profenofos, Standards, and Matrices. The commercially available formulation of profenofos (Curacron 50EC, Syngenta India Ltd., Mumbai, India) containing 50% active ingredient was used for the study. The certified reference standard of profenofos (92% purity) and its metabolite, 4-bromo-2-chlorophenol, were purchased from Dr. Ehrenstorfer GmbH, Germany. Biodegradation studies were done in the three important matrices, in vitro in liquid culture media, on grape berries, and in vineyard soil. For in vitro studies, nutrient broth (M001) was procured from HiMedia (Mumbai, India), and the medium was prepared per the manufacturer's instructions. The degradation on grapes was studied in the field on table grape cv. Thompson Seedless at the research vineyard of this Centre, located in the city of Pune (latitude, 18.31 N; longitude, 73.55 E), India. The canopy architecture was Y-shaped having row-to-row spacing of 10 ft and vine-to-vine spacing of 6 ft. The vines were 2 years old with uniform canopy and free from infestation of insect pests, diseases, or any disorder. Soil sample was collected from an uncultivated area of this Centre during the summer season. It was air-dried, ground, and passed through a mesh sieve of 2 mm pore size. Soil organic carbon, cation exchange capacity, water-holding capacity, and other physicochemical parameters were analyzed.[21] Half of the soil sample was sterilized by autoclaving on three consecutive days at 121 °C for 60 min; the other half was used as such.

***Bacillus subtilis* Strains, Media, and Inoculum Preparation.** Four *B. subtilis* strains, namely, DR-39, CS-126, TL-171, and TS-204, obtained from the culture collection of this Centre were used for the studies. Analysis of 16s rRNA sequence of these four strains had shown 96−99% homology with *B. subtilis* accessions NR 027552.1 and NR 024931.1 available in the NCBI database. The media used were nutrient broth (NB) (M001) and nutrient agar (NA) (M002) procured from HiMedia (Mumbai, India) and prepared as per the manufacturer's instructions. All cultures were maintained on NA at 4 °C until required. Inoculum was prepared in 100 mL of sterilized NB inoculated with a loop full of culture taken from a 24-h-old colony on NA plate. The flasks were incubated on an orbital shaker at 28 °C and 150 rpm for 72 h. The cells were harvested by centrifugation at 2795*g* for 5 min, washed twice with sterile distilled water, and then resuspended in sterile distilled water to give a final count of about 1×10^8 CFU/mL.

Method Validation. *Linearity.* The stock solutions (1000 mg/kg, w/w) of profenofos were prepared in ethyl acetate for GC-MS and in methanol for LC-MS/MS. Stock solutions were stored at −20 °C. An intermediate standard solution (5 mg/kg) was prepared by serial dilution with the ethyl acetate and methanol. The calibration standards (five calibration points) ranging from 0.0025 to 0.5 mg/kg were prepared by successive dilutions of the intermediate standard and analyzed by both systems. The calibration curves for profenofos were obtained by plotting the peak area against the concentration of the calibration standard.

Accuracy and Precision. The recovery experiments were carried out on fresh untreated grapes and NB medium by fortifying the samples (10 g/mL) in triplicate with profenofos separately at three concentration levels of 0.025, 0.05, and 0.5, mg/kg/L. The recovery experiments were also carried out on air-dried soil by fortifying the samples in triplicate with profenofos separately at three concentration levels, that is, 0.01, 0.05, and 0.5 mg/kg.

Degradation Kinetics of Profenofos. *Liquid Culture.* Erlenmeyer flasks containing 150 mL of sterile NB were inoculated separately with 100 μL of inoculum of the four *B. subtilis* strains and spiked with profenofos at 5 μg/mL of medium. Flasks were incubated on an orbital shaker (Orbitex, Scigenics Biotech, India) at 28 °C and 150 rpm in the dark. Uninoculated NB spiked and unspiked with profenofos were maintained as controls. All treatments were replicated three times. Separate flasks were maintained for residue analysis and for bacterial counts. Ten milliliter aliquots were removed aseptically after 0, 1, 3, 5, 7, 10, and 15 days of incubation for profenofos residue analysis and count enumeration.

Grape. Profenofos was applied as foliar spray at 1250 mL ai/ha (1000 L water) during March 2012. After 1 h, the vines were sprayed with a suspension of any one of the four strains to completely cover bunches and foliage. Vines sprayed with profenofos but not with any *B. subtilis* strain were maintained as control. Each treatment was replicated three times in randomized blocks; each replicate consisted of four vines. The *B. subtilis* treatments were repeated on the fifth day, after sampling. The weather parameters in the vineyard were monitored. The grape samples were collected at random from each replicate of the treated and control plots separately at regular time intervals on 0 (1 h after application of *B. subtilis* inoculum), 1, 3, 5, 7, and 9 days after spray. Bunches hidden inside the canopy were not included in the samples. The grape berries were separated from the pedicels and directly processed for analysis without any washing or other pretreatment. From each replicate, 1 kg of sample was processed for residue analysis and 25 berries were processed for count enumeration.

Soil. Both sterilized and nonsterilized soil samples (50 g) were placed in presterilized 100 mL Erlenmeyer flasks. They were spiked with 5 μg/g of profenofos and inoculated with one of the four *B. subtilis* strains separately to give a count of about 1×10^8 CFU/g dry soil. Flasks were incubated at 28 ± 2 °C in an environment chamber (Binder KBW 400) maintaining 60% water-holding capacity. Controls consist of uninoculated sterilized and nonsterilized soils spiked with the same amount of profenofos. Samples were collected at 0 day (1 h after inoculation) and subsequently at 1, 3, 5, 7, 10, 15, 20, 25, 30, and 35 days after inoculation and processed for residue analysis and count enumeration.

Extraction of Profenofos Residue. *Liquid Culture and Grapes.* The "quick, easy, cheap, effective, rugged, and safe" (QuEChERS) method was followed for extraction of residues from the liquid culture in NB.[22] Ten milliliters of acetonitrile, 4 g of $MgSO_4$, and 1 g of NaCl were added to 10 mL aliquots for separation of aqueous and organic layers. The samples were vortexed for 2 min and centrifuged for 5 min at 1006.2*g*. For extraction from grapes, the entire 1 kg berry sample was crushed thoroughly in a blender. Approximately 200 g of the crushed sample was further homogenized. A 10 g homogenized sample was extracted with ethyl acetate (10 mL) followed by anhydrous sodium sulfate (10 g). The sample was homogenized for 2 min at 15000 rpm and followed by centrifugation at 447*g* for 5 min.[23]

Soil. The soil samples (50 g) were extracted at room temperature on a mechanical shaker with 100 mL of 80% methanol. The extracts were separated from soil solids by centrifugation at 1788.8*g* followed by decantation and filtration. The extract was evaporated to complete dryness on a low-volume concentrator at 35 °C. The residue was dissolved in 5 mL of methanol and cleaned by dispersive solid phase extraction with a mixture of 200 mg of C_{18} and 40 mg of PSA before analysis.

Determination. *GC-MS Analysis.* One milliliter of the sample extract from NB or grapes was cleaned with 25 mg of the cleanup agent PSA by centrifugation at 8385*g* for 5 min. The clear supernatant was filtered through a 0.2 μm PTFE membrane filter and used for GC-

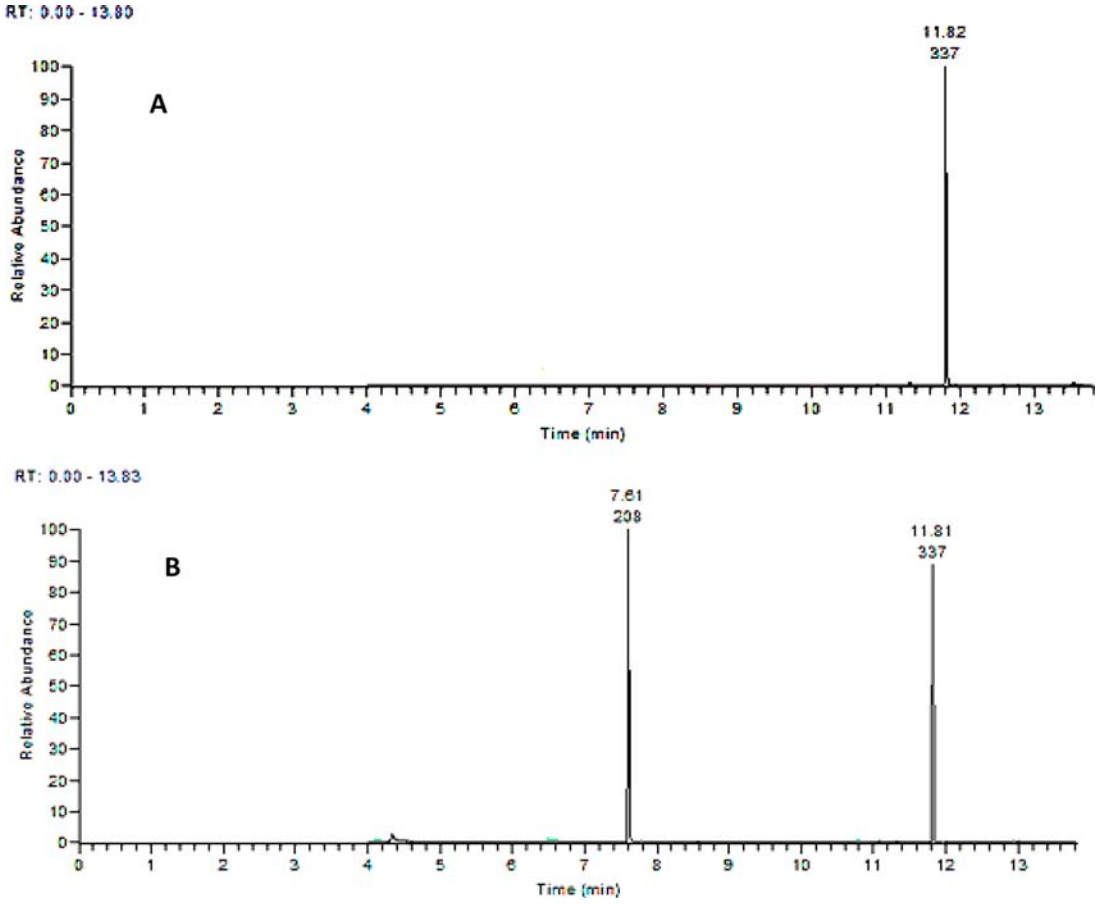

Figure 1. GC-MS chromatograms for profenofos (A) and its metabolite 4-bromo-2-chlorophenol (B).

MS injection.[23] GC-MS analysis was carried out on an ion trap GC-MS (ITQ 900; Thermo Electron, Waltham, MA, USA) with electron impact (EI) ionization, operated at full-scan mode. Samples of 2 μL were injected into a splitless injector connected to a VF-5MS capillary column (30 m × 0.25 mm i.d., 0.25 mm). The oven temperature was programmed with an initial temperature of 100 °C, held for 1 min, increased to 285 °C at 25 °C/min, held for 1 min, and finally increased to 285 °C at 5 °C/min and held for 14 min. Profenofos eluted at the retention time (RT) of 8.79 min and 4-bromo 2-chlrophenol at 4.20 min.

LC-MS/MS Analysis. The analysis was carried out on a Waters Alliance 2695 HPLC system connected to a Quattro Premier mass spectrometer (Waters India Pvt. Ltd., Bangalore, India) equipped with an electrospray ionization (ESI) probe. A 10 μL aliquot was injected through an autosampler. A Purospher STAR RP18e column (50 mm × 2.1 mm, 3 μm) with a flow rate of 0.4 mL/min was used. The mobile phase was composed of (A) methanol/water (20:80, v/v) with 5 mM ammonium formate and (B) methanol/water (90:10, v/v) with 5 mM ammonium formate; the gradient was as follows: 0−0.25 min, 85% A phase; 0.25−3 min, 85−2% A phase; 3−8.5 min, 2% A phase; 8.5−9 min, 2−85% A phase; and 9−12 min, 85% A phase. Estimation was performed in positive mode by multiple reaction monitoring (MRM). The mass transitions are 373 → 303 and 373 → 97 with a scan time 0.02 ms. The dissolvation temperature was 400 °C.

Degradation Rate Kinetics and Data Analysis. The degradation kinetics of profenofos in all three matrices, namely, liquid culture medium, grapes, and soil, was determined by nonlinear regression analysis with the following mathematical expression:

$$\text{first + first-order model: } [A]_t = [A]_1 \exp(-k_1 t) + [A]_2 \exp(-k_2 t)$$

$[A]_t$ is the concentration (mg/kg grapes) of A at time t (days) and $[A]_1$ and $[A]_2$ are the initial concentrations of A at time 0 degraded through one first-order and another first-order process. k_1 and k_2 are the degradation rate constants for 1 and 2 per day (days^{-1}). The half-life denoted DT_{50} (degradation time 50%) in which the profenofos concentrations were reduced by 50% was obtained from the nonlinear equation obtained from the first + first-order models. The equation parameters were calculated by use of a commercially available program Table Curve 2D (V 5.01).

Enumeration of Bacteria Counts. *Bacillus* populations were determined at each time interval in each of the experiments using a serial dilution plating method. For liquid culture studies a 1 mL aliquot was taken from each flask. For analysis of populations on grapes, 25 berries from each replicate were transferred into sterile Erlenmeyer flasks containing 100 mL of sterile distilled water and shaken at 100 rpm for 30 min. For analysis of the populations in soil, 1 g soil samples[11] were suspended in 9 mL of sterile distilled water in 50 mL Erlenmeyer flasks and shaken at 100 rpm for 1 h. An aliquot (1 mL suspension) was withdrawn from each flask, and 10-fold dilutions were made; 0.1 mL aliquots from each dilution were spread on NA plates and the counts recorded after 24 h of incubation at 28 °C. Dilutions in which the total counts were from 30 to 300 colonies per plate were used for counting,[24] except in those instances where the counts even at ×1 were <30. The microbial counts were converted to the base 10 logarithm of the number of colony-forming units per milliliter per berry or per gram soil.

RESULTS AND DISCUSSION

Method Validation. The linearity of the calibration curve was established in the range from 0.0025 to 0.5 mg/kg with correlation coefficient (R^2) of the calibration curve >0.99. For matrix calibration, too, the R^2 was >0.99 for all matrices, that is,

liquid culture, grapes, and soil. The recovery experiment was conducted at 0.01, 0.05, and 0.5 mg/kg for all matrices. The average percent recovery of profenofos for GC-MS (n = 6) was 85.5 ± 2% from liquid culture matrix, 89.5 ± 5% from grape berry matrix, and for LC-MS/MS was 84.2 ± 4.8% from soil matrix, and 88 ± 5% for liquid culture matrix.

Degradation Kinetics of Profenofos. *Liquid Culture.* GC-MS analysis showed profenofos to be disappearing with simultaneous appearance of 4-bromo-2-chlorophenol metabolite in all spiked samples (Figure 1). Simultaneously the same samples were analyzed by LC-MS/MS in MRM-EPI survey scan triggered with information-dependent acquisition (IDA). In the IDA experiment, by using the intensity criteria of 1000 cps, it was possible to scan the EPI spectra at a collision energy of 35 eV (collision energy spread = 15 eV). By the LC-MS/MS analysis additional peaks at 3.56 min for *O*-(4-bromophenyl) *O*-ethyl hydrogen phosphorothioate and at 8.17 min for *O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-(3-hydroxypropyl) phosphorothioate of minor metabolites of profenofos were detected. During the first 5 days, there was a very slow decline in the profenofos concentration in the uninoculated control, whereas the decline was steep in spiked NB inoculated with any of the four *B. subtilis* strains (Figure 2). Profenofos concentration

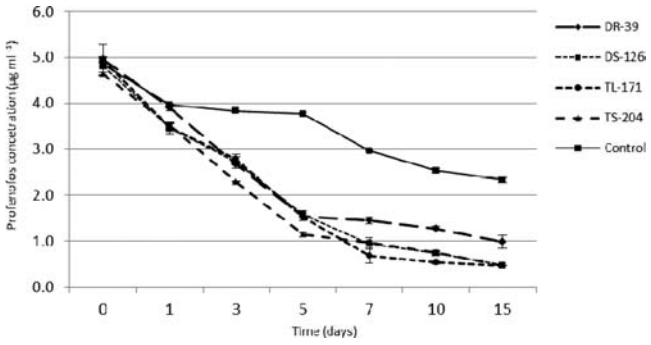

Figure 2. Profenofos disappearance dynamics in nutrient broth spiked with 5 μg/mL profenofos by four *B. subtilis* strains, namely, DR-39, CS-126, TL-171, and TS-204, at 28 °C in the dark as monitored by GC-MS with electron impact ionization at full scan mode. Each value is the mean of three replicates with error bars indicating standard deviation from the mean.

continued to decline further, although not so sharply, and at the end of 15 days, it was detected at <0.5 μg/mL in NB inoculated with strains CS-126, TL-171, and TS-204 as compared to 0.99 μg/mL in NB inoculated with strain DR-39 and 2.33 μg/mL in uninoculated spiked control. Thus, the observed data showed two compartment degradation patterns; the first compartment represented the initial 5 days, and the second compartment represented degradation up to 15 days. Results indicate that all four *B. subtilis* strains were able to degrade profenofos even when other carbon sources were available in the medium, to the extent of 90% (TS-204, TL-171, CS-126) or 79% (DR-39) as compared to 52% degradation observed in the uninoculated control. The kinetics for in vitro profenofos degradation showed that the half-life was minimized from 12.90 day in the uninoculated spiked control to <4.03 days by the *B. subtilis* strains (Table 1).

The growth dynamics of the four *B. subtilis* strains, namely, DR-39, CS-126, TL-171, and TS-204, were the same in medium spiked or unspiked with 5 μL/mL profenofos (Figure 3), indicating that neither was profenofos toxic to the *B. subtilis* strains nor did it enhance the growth of the strains. Furthermore, all four strains exhibited almost the same growth pattern over the 15 day incubation period. After inoculation, the average viable count was 5.54 ± 0.02 $\log_{10}$ CFU/mL at day 0. Thereafter, the strains showed exponential phase of growth, and the number of colonies reached a maximum of 8.21 ± 0.05 $\log_{10}$ CFU/mL on day 1. Subsequently, there was strainwise slight differences in growth pattern, but after 3 days of incubation all strains showed decreases in populations reaching 5.11 ± 0.05 $\log_{10}$ CFU/mL on day 15.

Grapes. GC-MS analysis showed the disappearance, and the degradation kinetics of profenofos on grapes are shown in Figure 4A and Table 2. The degradation pattern in all of the treatments including control (uninoculated) confirms the two-compartment nonlinear dissipation of profenofos in grapes under the field conditions.[25] A steep dissipation was observed up to the third day representing the first compartment, after which the dissipation occurred slowly up to 9 days. The comparative DT_{50} and R^2 values of first- and first + first-order kinetics (Table 2) signify the latter with better R^2 values and closeness to the real-time values obtained in field conditions. After spray application at 125 μg/mL ai, the average initial deposit of profenofos was found to be 3.92 mg/kg of grapes. After 9 days of application, the residue on grapes in control was 1.20 mg/kg (68.2% degradation), whereas in strains DR-39 and CS-126 the residues had decreased to 0.41 mg/kg (89.4% degradation) and 0.45 mg/kg (87.6% degradation), respectively. Strains TL-171 and TS-204 were not as efficient as they degraded profenofos to 0.93 mg/kg (74.2% degradation) and 1.05 mg/kg (72.8% degradation), respectively. The degradation kinetics showed low half-lives for strains DR-39 (1.07 day) and CS-126 (1.0 day) as compared to the half-life of 2.20 days in control.

Table 1. Degradation Kinetics of Profenofos in Liquid Culture[a]

	B. subtilis DR-39		*B. subtilis* CS-126		*B. subtilis* TL-171		*B. subtilis* TS-204		uninoculated spiked control	
parameter	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics
A_1 (mg/kg)	4.12	3.57	4.34	3.74	4.49	4.29	4.53	4.14	4.53	3.62
K_1 ($days^{-1}$)	0.15	0.23	0.19	0.27	0.22	0.73	0.23	0.32	0.05	0.09
A_2 (mg/kg)	NA	1.00	NA	0.70	NA	1.00	NA	0.67	NA	1.00
K_2 ($days^{-1}$)	NA	0.70	NA	0.67	NA	0.27	NA	0.51	NA	1.34
R^2	0.94	0.97	0.98	0.97	0.98	0.98	0.98	0.98	0.98	0.99
DT_{50}	4.83	4.03	3.17	3.57	3.20	2.87	3.07	2.53	13.90	12.90

[a] K_1, K_2, first and second degradation rate constants; R^2, regression coefficient; DT_{50}, half-life period; NA, not applicable.

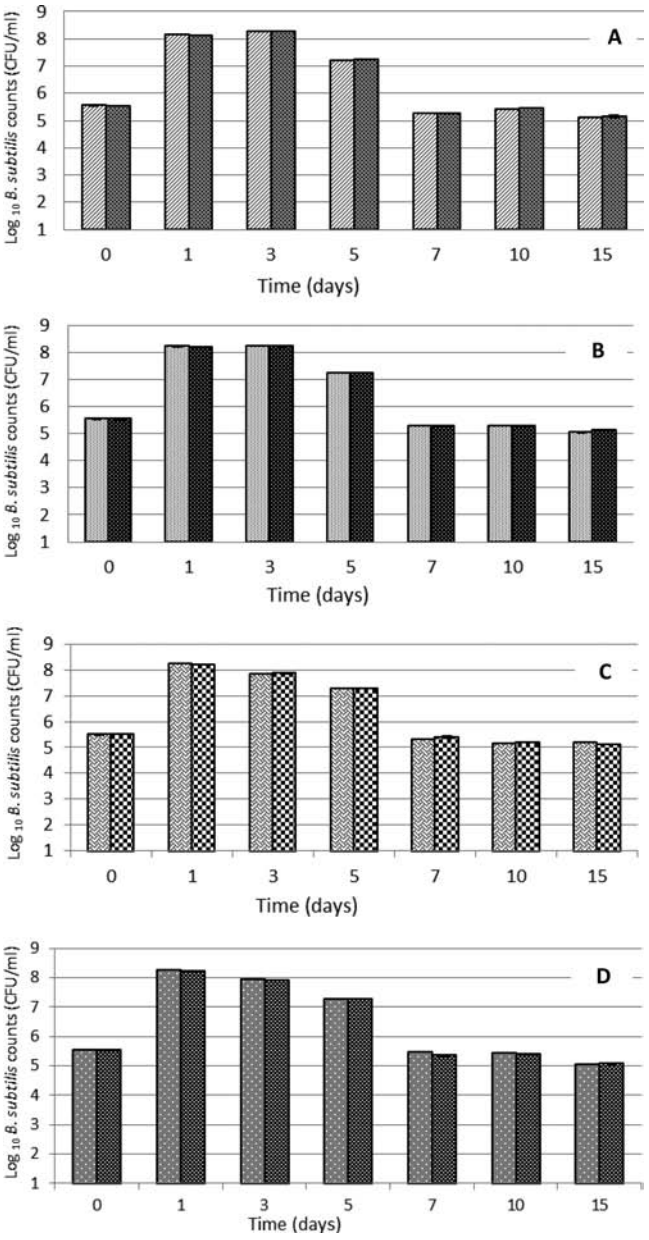

Figure 3. Growth dynamics of *B. subtilis* strains DR-39 (A), CS-126 (B), TL-171 (C), and TS-204 (D) in nutrient broth incubated at 28 °C and 150 rpm in the dark in the absence (left) and presence (right) of 5 μg/mL profenofos.

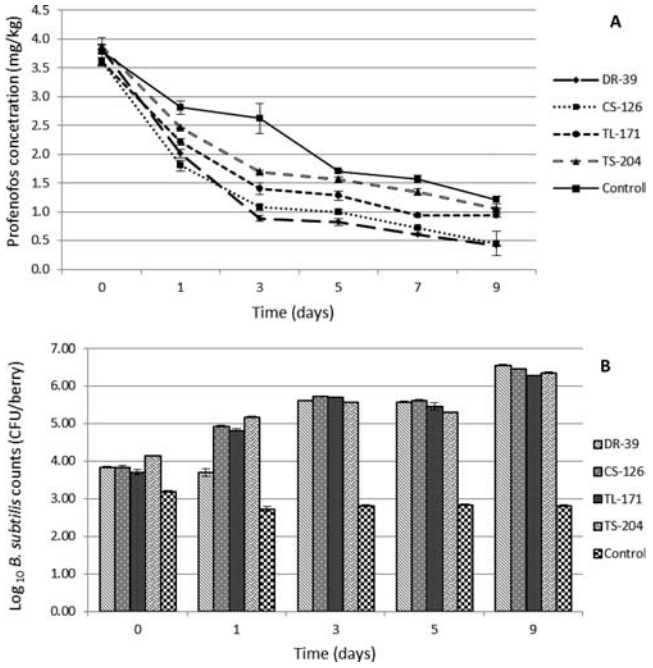

Figure 4. Profenofos concentration (A) and CFU counts (B) on a time frame of 0−9 days after spray, on grapes sprayed with 125 μg/mL profenofos and inoculated with *B. subtilis* strains DR-39, CS-126, TL-171, and TS-204 and uninoculated control.

The half-life observed by us in grapes was slightly longer than the half-lives of 1.84, 1.74, and 1.96 days reported for sweet pepper, hot pepper, and eggplant, respectively,[26] and much lower than the half-life of 5.47 days reported for rice plant.[27] These differences in half-lives could be due to the microbial load on the different commodities at the time of experiment as well as the differences in physical and chemical environments, all of which contribute to the degradation process.

The biodegradation study was conducted in a natural field environment where the average maximum and minimum temperatures were 35 and 11.7 °C, relative humidity was 73%, and there was no rainfall during the period of study. Furthermore, a native dynamic bacterial microflora was expected to be present on the berry surface.[28] This bacterial microflora on grape berries was estimated to be 3.20 $\log_{10}$ CFU/berry at the initiation of the experiment, and it showed a slight decrease in population during the period of study (Figure 4B).

After inoculation, the $\log_{10}$ CFU counts/berry were 3.84, 3.85, 3.71, and 4.15 in *B. subtilis* strains DR-39, CS-126, TL-171, and TS-204, respectively, on day 0. Thereafter, the four strains showed exponential phase of growth, and the corresponding $\log_{10}$ CFU/berry were 5.61, 5.72, 5.70, and 5.57 on day 3 after inoculation. This conformed to the period when maximum degradation of profenofos was observed. On day 9, the counts had increased to 6.55, 6.46, 6.29, and 6.36 $\log_{10}$ CFU/berry in strains DR-39, CS-126, TL-171, and TS-204, respectively, due to the repeat *B. subtilis* application made on day 5. This was done to ensure a stable population as in a preliminary study it was observed that the *B. subtilis* populations started decreasing after 5 days.

Quantitative analysis indicated that the applied *B. subtilis* strains could establish and survive on the grape berries and contribute significantly to the biodegradation of profenofos under natural conditions. The grape berry surface is a rich source of exuded nutrients, which at the postveraison stage consist mainly of glucose, fructose, and potassium.[29] The sugars act as easily assimilable carbon sources, and potassium increases the surface pH; both these factors would be able to support and stimulate the applied bacterial growth[10,7] and enhance the biodegradation. Contrary to their in vitro efficiency, the *B. subtilis* strains CS-126 and DR-39 were more efficient in degrading profenofos on grape berries as compared to strains TS-204 and TL-171. This may be due to their better proliferation on the berry surface as evident by the higher $\log_{10}$ CFU values on the days 5 and 9. The nutrients present in the environment also affect the efficiency of bacteria to degrade a pesticide,[10] and it is possible that strains CS-126 and DR-39 preferred to utilize the easily available nutrient sources from the

Table 2. Degradation Kinetics of Profenofos on Grape Berries[a]

	B. subtilis DR-39		*B. subtilis* CS-126		*B. subtilis* TL-171		*B. subtilis* TS-204		uninoculated sprayed control	
parameter	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics
A_1 (mg/kg)	3.57	2.65	3.19	1.96	3.19	1.98	3.39	1.38	3.57	5.45
K_1 ($days^{-1}$)	1.40	1.06	0.29	2.58	0.20	0.74	0.16	1.67	0.13	0.43
A_2 (mg/kg)	NA	0.10	NA	0.12	NA	0.04	NA	0.13	NA	0.67
K_2 ($days^{-1}$)	NA	1.18	NA	1.65	NA	1.30	NA	2.48	NA	1.10
R^2	0.97	0.99	0.96	0.98	0.98	0.99	0.98	0.99	0.98	0.99
DT_{50} (days)	1.57	1.07	2.37	1.00	3.53	2.13	4.33	2.20	5.57	2.20

[a] K_1, K_2, first and second degradation rate constants; R^2, regression coefficient; DT_{50}, half-life period; NA, not applicable.

Table 3. Physicochemical Properties of Soil Used in This Study

property	value	property	value	property	value
max water-holding capacity	73.26%	total P	3 ppm	Cl^-	8.88 ppm
organic carbon	0.81%	Ca^{2+}	11449 ppm	Zn^{2+}	1.28 ppm
EC	0.155 ds/m	Mg^{2+}	4480 ppm	Cu^{2+}	3.25 ppm
pH	7.66	K^+	200 ppm	Fe^{2+}	4.87 ppm
total N	156.8 kg/ha	Na^+	225 ppm	Mn^+	4.80 ppm

Table 4. Degradation Kinetics of Profenofos in Autoclaved Soil[a]

	B. subtilis DR-39		*B. subtilis* CS-126		*B. subtilis* TL-171		*B. subtilis* TS-204		uninoculated spiked control	
parameter	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics
A_1 (mg/kg)	3.99	1.19	4.19	2.00	3.84	1.60	4.78	2.40	3.48	2.19
K_1 ($days^{-1}$)	0.09	1.47	0.08	0.07	0.07	0.94	0.16	0.16	0.01	0.04
A_2 (mg/kg)	NA	0.07	NA	0.09	NA	0.07	NA	0.16	NA	0.04
K^2 ($days^{-1}$)	NA	3.57	NA	1.46	NA	2.79	NA	2.39	NA	2.22
R^2	0.94	0.95	0.94	0.95	0.93	0.92	0.96	0.96	0.92	0.94
DT_{50}	8.17	5.93	8.77	7.47	8.83	6.00	4.30	4.37	17.43	17.37

[a] K_1, K_2 degradation rate constants; R^2, regression coefficient; DT_{50}, half-life period; NA, not applicable.

Table 5. Degradation Kinetics of Profenofos in Nonautoclaved Soil[a]

	B. subtilis DR-39		*B. subtilis* CS-126		*B. subtilis* TL-171		*B. subtilis* TS-204		uninoculated spiked control	
parameter	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics	1st-order kinetics	1st + 1st-order kinetics
A_1 (mg/kg)	3.84	2.48	3.80	2.67	3.85	2.51	0.49	2.26	3.99	3.13
K_1 ($days^{-1}$)	0.22	2.75	0.19	1.25	0.17	0.77	2.32	0.49	0.04	0.05
A_2 (mg/kg)	NA	0.09	NA	0.10	NA	0.09	NA	0.15	NA	0.05
K_2 ($days^{-1}$)	NA	2.04	NA	2.24	NA	2.07	NA	2.32	NA	1.85
R^2	0.91	0.95	0.96	0.97	0.96	0.97	0.97	0.98	0.97	0.98
DT_{50}	3.10	0.87	3.67	2.00	4.07	2.07	2.43	2.43	15.87	14.37

[a] K_1, K_2, degradation rate constants; R^2, regression coefficient; DT_{50}, half-life period; NA, not applicable.

culture broth rather than the pesticide and, hence, their degradation efficiency was not fully exploited.

Soil. The soil used in this study was black clay soil with 0.81% carbon, 7.66 pH, and other physicochemical properties as given in Table 3. LC-MS/MS analysis showed the addition of *B. subtilis* strains to autoclaved soil resulted in a more rapid rate of profenofos degradation than that in uninoculated autoclaved soil. In uninoculated spiked control, the half-life period was 17.37 days (Table 4). All four *B. subtilis* strains were able to significantly enhance the degradation process, as evident by half-life periods of 4.37−7.47 days. Bacterial species are also known to proliferate in an alkaline environment, and their toxicant-degrading abilities are also higher in soils with near-neutral to alkaline pH.[30] Although the soil used in this experiment did not have a high carbon content, bacteria are known to degrade pesticides in soils with as low as 0.3% carbon content.[7] Among the four *B. subtilis* strains, TS-204 was the most efficient followed by DR-39 and CS-126.

In nonautoclaved soil, too, inoculation with the four *B. subtilis* strains sharply enhanced the rate of degradation as seen by the lower DT_{50} values of 0.87 day in DR-39 to 2.43 days in TS-204, as compared to the DT_{50} of 14.37 days in the uninoculated control (Table 5). Furthermore, these DT_{50} values in the nonautoclaved soil samples were lower than those observed in the respective autoclaved soil samples, indicating that the natural soil microflora was also able to

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degrade profenofos, although its contribution to the degradation process was not substantial (Tables 4 and 5). Earlier, Malghani et al.[8] had also observed faster degradation of profenofos in nonsterilized soil than in sterilized soil.

The degradation kinetics in all of the soil treatments followed first + first-order rate kinetics owing to the best R^2 values and realistic DT_{50} as compared to the first-order kinetic model (Tables 4 and 5). A trend of almost 4 times slower degradation of pesticides in the soil, as compared to that on the plant surface, has been observed in a number of chemicals.[31] However, in our studies, the half-lives of profenofos in soil (DT_{50} = 0.87−2.43 days) were almost on par with that on the grape berry (DT_{50} = 1.00−2.20 days).

It is interesting to note that in culture medium and autoclaved soil, maximum degradation of profenofos occurred in treatments with strain TS-204, but it showed least degradation in nonautoclaved soil and on grapes as compared to the other three strains. This indicates that the profenofos-degrading ability of strain TS-204 is reduced in the presence of the native microflora present on the berry surface and in the soil. On the other hand, strain DR-39 showed high degradation of profenofos on grapes and nonautoclaved soil, indicating that it would be able to compete with the native microbial population and retain its biodegrading efficiency in natural environments. This behavioral difference is interesting in view of the original source of the strains; strain DR-39 was originally isolated from rhizosphere soil and thus is expected to have better competitive saprophytic ability than strain TS-204, which was endophytic in nature. Furthermore, even though DR-39 was a rhizosphere strain, it could establish successfully on the grape fructosphere.

The recoveries of the added *B. subtilis* strains after 24 h of inoculation were 5.64, 5.53, 5.55, and 5.44 $\log_{10}$ CFU/g soil in DR-39, CS-126, TL-171, and TS-204, respectively (Figure 5).

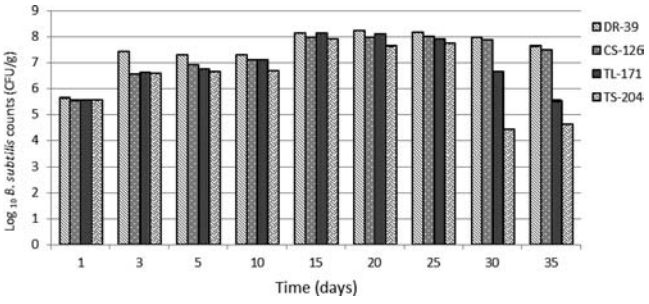

Figure 5. $\log_{10}$ CFU counts on a time frame of 1−35 days of *B. subtilis* strains DR-39, CS-126, TL-171, and TS-204 in autoclaved soil amended with 5 μg/mL profenofos and incubated at 28 °C in the dark in an environment chamber and maintained at 60% water-holding capacity.

Thereafter, the strains showed exponential phase of growth, which continued until 15 days in strains CS-126, TL-171, and TS-204, whereas strain DR-39 continued to grow until 20 days. At peak growth, they reached counts of 8.24, 7.99, 8.13, and 7.89 $\log_{10}$ CFU/g soil in DR-39, CS-126, TL-171, and TS-204, respectively. *Bacillus* spp. are known to reach such high populations in soil.[32,33] At 30 and 35 days a decline in populations of strains TS-204 and TL-171 was observed, even though they had more initial counts, whereas in the other two strains the decline in numbers was not as marked.

Our observed half-life of profenofos was 14.37 days in vineyard soil used in this study, whereas it was reported to be 3.75 days in soil of rice fields.[27] Profenofos degradation is affected by the pH of the environment; it is faster in alkaline than in acidic environments. The half-lives of profenofos in pH 5, 7, and 9 are reported to be 104−108, 24−62, and 0.33 days, respectively.[34] It was about 20 days in water of pH 8.0,[35] and the pH of the soil used in this study was 7.66. Apart from soil pH, the microbial diversity as well as the physicochemical properties of the soil would also affect the degradation.

These findings may be relevant to other fruit and vegetable crops, too, where use of profenofos is considered to be impractical because of the high waiting periods.[36]

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