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Biodegradation of nitenpyram (neonicotinoid insecticide) by endophytic bacterium, *Bacillus thuringiensis* strain NIT-2, isolated from neonicotinoid-treated plant samples

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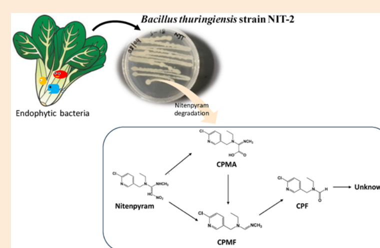
S ***Supplementary material***



Nitenpyram (neonicotinoid insecticide) is commonly used for crop protection from pests. Currently, due to its widespread use, the nitenpyram accumulation in the environment is anticipated to be high. Hence, the removal of nitenpyram residue from the environment is essential. However, the biodegradation of nitenpyram by endophytes is still unreported. Therefore, we aimed to isolate and identify a bacterial strain capable of degrading nitenpyram. We isolated approximately 300 endophytic strains from *Brassica rapa* var. *perviridis* that had been exposed to different neonicotinoid insecticides. After 14 days of incubation, a bacterial strain, NIT-2, with nitenpyram degradation capability (approximately 65%) was found. *Via* 16S rRNA gene sequencing, the strain was identified as *Bacillus thuringiensis*. In addition, metabolites, 2-[*N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid, *N*-(6-chloro-3-pyridilmethyl)-*N*-ethyl-*N*-methylformamidine (CPMF), and *N*-(6-chloro-3-pyridilmethyl)-*N*-ethylformamide (CPF), were identified during the degradation. Moreover, CPMF and CPF were further degraded 71% and 18%, respectively by NIT-2. Thus, *B. thuringiensis* strain NIT-2 is the first reported endophytic bacterium capable of degrading nitenpyram.

Keywords: nitenpyram, *Brassica rapa*, *Bacillus thuringiensis* strain NIT-2, biodegradation.

Introduction

Neonicotinoids are insecticides with structures similar to nicotine and are used to control pests, especially sap-feeding insects, such as aphids and root-feeding grubs.[1)] These insecticides have been widely used over the last two decades owing to their low toxicity to mammals and other animals.[2)] Although neonicotinoids were first introduced in 1991, most were marketed extensively from 1995 to 2002. Neonicotinoids are the most popular insecticides, representing almost one-fourth of the insecticide market in the world.[3)] A key advantage of using neonicotinoids is that they are plant systemic. As small molecules with high water solubility, neonicotinoids dissolve rapidly in water and are absorbed by plants very quickly.[4)] Depending on plant uptake, neonicotinoids are distributed *via* xylem throughout plant tissues and persist for a long time.[5,6)] For instance, the active residue of neonicotinoids was found for up to 1 year in woody plants.[4)] Therefore, the rising neonicotinoid concentration in plants after harvesting the target vegetable is becoming an emerging concern. In the USA, neonicotinoid concentration ranged from 0.1–100.7 $ng\,g^{-1}$ for fruits; 0.4–13.7 $ng\,g^{-1}$ for vegetables; and 0.1–0.5 $ng\,g^{-1}$ for honey.[7)] Japan, one of the largest users of neonicotinoids, is also experiencing high neonicotinoids concentration in foods and beverages.[8)] Takamoto *et al.* 2018 reported 1.44–4.94 $ng\,mL^{-1}$ neonicotinoids concentration in Japanese green tea.[9)] Meanwhile, Japan Endocrine-disruptor Prevention Action set 0.5–3 $\mu g\ g^{-1}$ as the maximum neonicotinoid residue level for fruits and vegetables, which was 1.7–25 and 3–500 times higher than that of the United States and Eu-

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ropean Union, respectively.[10] Although high temperatures and precipitation influence the use of these pesticides, the key factor behind this might be that Japan uses more neonicotinoid insecticides than the US and Europe, making the reduction difficult.[10] Considering these circumstances, these insecticides may have detrimental effects on non-target communities such as honeybees,[11,12] bees[12] butterflies, flies, aquatic vertebrates and invertebrates, and even humans.[13,14]

Neonicotinoid insecticides are classified into three major subgroups depending on their pharmacophore characteristics: *N*-nitroguanidines, *N*-cyanoamidines, and nitromethylenes. Nitenpyram ((*E*)-1-*N′*-[(6-chloropyridin-3-yl)methyl]-1-*N′*-ethyl-1-*N*-methyl-2-nitroethene-1,1-diamine) belongs to the nitromethylene subgroup and is one of the most commonly used neonicotinoids since 1995.[3] Nitenpyram is mostly used in crops, such as cotton, corn, paddies, and different vegetables.[15] Recently, the use of this insecticide has increased due to its efficiency in controlling pests, especially during rice production.[16] Like other neonicotinoids, nitenpyram is highly water soluble ($500\,mg\,L^{-1}$) because of the presence of polar groups; therefore, it has lower persistence in the soil (half-life: 1–15 days), depending on the soil quality and weather conditions.[17] Because of its high water-solubility, this insecticide can move very quickly with runoff and seepage water, polluting both surface and ground water.[18] Moreover, plants can take up this insecticide at a much higher rate due to its systemic characteristics.

Endophytes are microorganisms that live symbiotically within plant tissues without causing any significant adverse effects on host plants.[19] Endophytes are needed for ecological investigations, such as biocontrol, bioremediation, and adaptation to extreme conditions. For instance, endophytes develop resistance to the toxic effects of pesticides and its metabolites.[20] Some endophytes originate from the soil through the root system. Once the roots are established in the soil, an abundance of microorganisms is formed around the rhizosphere zone, and biofilms are created depending on the surrounding environmental conditions.[20] From there, microorganisms penetrate the plant tissues and create an endophytic community. Compared to bulk soil or rhizosphere soil, fewer microorganisms can be found in the endospheric zone of the host plant, decreasing the competition among the microbes.[21–23] Moreover, host plants always harbor groups of microbes, which can provide them protection and sustainability under any stress condition.[24] Hence, locally adapted microbes may be potential sources for the remediation of pollutants. Therefore, endophytic bacteria have attracted scientific attention for the microbial remediation of pollutants in the environment.

Bioremediation techniques based on studies on host plants and their endophytic bacteria may overcome the problem of nitenpyram accumulation in the environment.[24,25] Moreover, primary metabolite degradation is necessary to produce less harmful products. Thus, endophytic bacteria with the ability to degrade both nitenpyram and its metabolites can be considered as the most efficient nitenpyram-degrading microbes. Although several studies have investigated the biodegradation of different pesticides by endophytic bacteria,[26–28] the biodegradation of nitenpyram by endophytes has not yet been reported. However, some locally adapted bacteria and fungi that can degrade nitenpyram have been reported.[29–31] As endophytes have less competition and include a profusion of pesticide-degrading microorganisms under pesticide-driven conditions, nitenpyram-degrading microorganisms may be isolated from the endophytic zone under neonicotinoid-exposed conditions. Therefore, our study aimed to isolate and characterize a neonicotinoid-adapted endophytic bacterium that could be used as a potential degrader of nitenpyram insecticides and its known metabolites and investigate the growth response of the nitenpyram-degrading bacterium that utilizes nitenpyram as its sole carbon or nitrogen source. Overall, this study focused on the use of pure bacterial cultures for nitenpyram degradation.

Materials and methods

1. Chemicals and materials

Analytical high performance liquid chromatography (HPLC) grade nitenpyram standard (99.0% purity) was used as nitenpyram reference. HPLC grade standard 2-[*N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA; 98.0% purity); (*N*-(6-chloro-3-pyridilmethyl)-*N*-ethyl-*N′*-methylformamidine (CPMF; 95.6% purity); and *N*-(6-chloro-3-pyridilmethyl)-*N*-ethylformamide (CPF; 99.3% purity) were used as the known metabolite references. All the chemicals were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC grade acetonitrile (99.8% purity) and acetone (99.8% purity) were purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan). To prepare the standard solution, nitenpyram was dissolved in acetone. CPMA, CPMF, and CPF were dissolved in sterilized deionized water (Nacalai Tesque, Inc., Kyoto, Japan) to obtain a $500\,mg\,L^{-1}$ stock solution.

Potato dextrose agar (PDA; $4.0\,g\,L^{-1}$ potato extract, $20.0\,g\,L^{-1}$ dextrose, $15.0\,g\,L^{-1}$ agar; Oxoid Ltd., Basingstoke, UK) media was used to isolate endophytic bacteria from the plant samples. Potato dextrose broth (PDB; $4.0\,g\,L^{-1}$ potato starch, $20\,g\,L^{-1}$ dextrose; Becton, Dickison and company, Le Pont-de-Claix, France) media was used to run degradation tests using pure bacteria. Both media were sterilized at 121°C for 15 min before bacterial inoculation. The mineral salt (MS) media was prepared to confirm whether nitenpyram was used as a carbon (C) or nitrogen (N) source during degradation. The MS media contained 1.2 g $Na_2HPO_4.12H_2O$ and 0.5 g KH_2PO_4 per liter. The mixture was autoclaved (121°C for 15 min) and then supplemented with 20 mg $MgSO_4.7H_2O$ and 10 mL metal trace element solution per liter. The metal trace element solution contained EDTA (500 mg); $FeSO_4.7H_2O$ (200 mg); $ZnSO_4.7H_2O$ (10 mg); $MnSO_4.H_2O$ (5 mg); H_3BO_3 (30 mg); $CoSO_4.7H_2O$ (24 mg); $CuSO_4.5H_2O$ (5 mg); $NiSO_4.H_2O$ (5 mg); Na_2MoO_4 (5 mg); and $Ca(OH)_2$ (50 mg) in 1 L of sterilized distilled water (pH 7.2). Glucose ($5\,g\,L^{-1}$) and NH_4NO_3 ($5\,mg\,L^{-1}$) were added to the MS media as C and N sources, respectively, during the growth

of bacteria in the media; however, when nitenpyram was monitored as the sole C or N source, glucose or NH_4NO_3, respectively, was removed from the MS media. Glycerol and sodium chloride (Nacalai Tesque, Inc, Kyoto, Japan) were used to prepare glycerol stock. All chemicals were of the highest quality.

2. *Isolation of endophytic bacterial strains*

For isolation of endophytic bacterial strains, *Brassica rapa* var. *perviridis* (Komatsuna) was selected as the target plant. For the cultivation of Komatsuna, pot experiments were conducted where a Gray Lowland soil was used as the growth medium. In 13 June 2022, soil was collected from University of Yamanashi, Japan research farm area (N35.604073, E138.578506), which was characterized by gray–brown color with well-drained conditions. 300 g soil sample was taken in nine Neubauer pots (size, 500 mL) where three different neonicotinoids (imidacloprid, acetamiprid, and dinotefuran) were applied separately just before seedling transplantation of Komatsuna. The application rate was 0.4 mg kg^{-1} (active ingredient) for imidacloprid, 0.6 mg kg^{-1} (active ingredient) for acetamiprid, and 0.6 mg kg^{-1} (active ingredient) for dinotefuran and soil drenching technique was followed during the application of neonicotinoids. For growing Komatsuna, seeds were collected from Atariya Holdings Co. Ltd., Chiba, Japan, which were surface sterilized by 70% ethanol for 1 min and 1% sodium hypochlorite (NaOCl) for 2 min. Afterward, the seeds were washed and rinsed with sterilized distilled water (SDW) five times. Subsequently, the sterilized seeds were kept on the wet petri dishes for germination at room temperature. Finally, at 29 June 2022, the germinated seeds were transferred to the Neubauer pots filled with neonicotinoid-treated soil. Since the growth of Komatsuna was conducted in the climate room set at 25±2°C, the soil water content was always maintained 50% of its water holding capacity. To isolate a nitenpyram-degrading strain, Komatsuna plants grown in different neonicotinoid-treated conditions were harvested at a matured stage. First, whole plants were washed with running water, and leaves were removed aseptically. Subsequently, the bulk samples were cut into 2–3 mm discs using aseptic scissors. The whole sample surface was sterilized by adding 70% ethanol for 1 min and 1% sodium hypochlorite for 2 min. The discs were washed and rinsed at least five times with sterilized distilled water. Finally, half of the sterilized samples were placed on agar media to check the success of sterilization and ensure the absence of any microbial colonies. Thereafter, 0.5 g discs were crushed with 4.5 mL standardized distilled water using a sterilized mortar and pestle. An aliquot of 50 μL was spread on the PDA media (pH 7) and replicated three times using a disposable spreader. The plates were incubated for 3–5 days at 25°C for the appearance of distinct colonies. Subsequently, each colony was streaked, and a pure culture was prepared. Small colonies were also counted during pure colony preparation. All colonies were kept in 20% glycerol stock (at 0.8% NaCl w/v) and stored at −80°C.

3. *Nitenpyram biodegradation assays and metabolite detection*

300 isolated endophytic bacterial strains were cultured in 3 mL sterilized PDB medium in sterilized 20 mL glass tubes with rubber corks (sterilized at 121°C for 15 min). Initially, nitenpyram was added from the stock solution (500 mg L^{-1} in acetone) to all the tubes at a final concentration of 5 mg L^{-1}. The tubes without bacterial strain inoculation were used as controls. All samples were analyzed in triplicate. Finally, all glass tubes were incubated at 25°C and 140 rotation per minute (rpm) for 14 days. To determine the degradation status, nitenpyram concentrations were measured using HPLC (Shimadzu Corporation, Kyoto, Japan). For the extraction of nitenpyram, 3 mL acetonitrile was mixed with each culture fluid (1 : 1, v/v) and the samples were homogenized by vortexing. The samples were centrifuged at 13,000 rpm for 15 min, and the supernatants were used to measure the concentration of nitenpyram using HPLC (Shimadzu Corporation). Degradation efficiency by applying different nitenpyram concentrations (1, 2.5, 5, and 10 mg L^{-1}) was also evaluated by following the same experimental conditions. To identify and validate the metabolites, the extracted 7-day grown PDB culture was assessed using the standard retention curve of known metabolites (CPMA, CPMF, and CPF)[32] using liquid chromatography-mass spectrophotometry (LC-MS/MS).

4. *Characterization of degradable strain*

In liquid media, except for strain NIT-2, none of the other strains reduced the concentration of nitenpyram from the initial concentration. Based on this degradation efficiency, endophytic bacterial strain, NIT-2, was designated as the most efficient nitenpyram-degradable strain. After that, the nitenpyram-degradable strain, NIT-2 was identified and characterized using 16S rRNA gene sequencing. The primers, 341F (5′-CCTACG GGAGGCAGCAG-3′) and 1541R (5′-AAGGAGGTGATCCAG CC-3′), were used to directly amplify the 16S rRNA gene sequence. The isolate name was endorsed by comparing the proportional similarities (>97%) in the reference list of NCBI BLAST search results (https://blast.ncbi.nlm). All the sequence data of strain NIT-2 were submitted to the DNA Data Bank of Japan (DDBJ) to obtain accession numbers. A phylogenetic tree of strain NIT-2 was constructed based on the neighbor-joining method using Mega version 11 after repeated alignment of the sequencing data with CLUSTAL W, provided by DDBJ (http://www.ddbj.nig.ac.jp). The topology of the phylogenetic tree was expressed using bootstrap values as percentages (presented at each branch) of 1000 replicates.

5. *Time–course degradation by strain NIT-2*

Two batches (three replicates each) of strain NIT-2 were grown in sterilized 3 mL PDB medium (pH 7) at 25°C with 140 rpm. Nitenpyram was added at 5 mg L^{-1} as initial concentration. Uninoculated PDB was used as the control for each batch. The inoculated strain in PDB without nitenpyram was also grown in parallel to evaluate the growth of bacteria in samples inoculated with nitenpyram. To measure bacterial growth, the optical density

(OD) of the strain was recorded on 0, 3, 7, 14, 21, and 28 days at 600 nm absorbance using UV–vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The concentration of the remaining nitenpyram was measured on 0, 3, 7, 14, 21, and 28 days using HPLC (Shimadzu Corporation), following the same extraction technique described in the nitenpyram biodegradation assay section. Nitenpyram concentration and bacterial growth were plotted against time (days) to construct a time–course degradation curve. Time–course degradation was performed in MS medium to test whether strain NIT-2 could use nitenpyram as a carbon or nitrogen source. Four batches (three replicates each) of strain NIT-2 were applied to 3 mL MS media (1st and 2nd batch: with glucose and without NH_4NO_3; 3rd and 4th batch: with NH_4NO_3 and without glucose) under the same conditions along with the same initial nitenpyram concentration in PDB medium. Uninoculated MS medium was used as the control in each batch. Data on bacterial growth and nitenpyram concentrations were recorded using the same method described above.

6. *Metabolite degradation assay*

To identify the downstream metabolites, the degradation of known metabolites (CPMF and CPF) was carried out in PDB medium (pH 7). Strain NIT-2 was inoculated in 3 mL PDB medium, which was supplemented with 10 mg L^{-1} CPMF and CPF separately in 20 mL sterilized glass tubes with rubber stoppers. A control was used to evaluate the performance of strain NIT-2 during metabolite degradation. Three replicates were performed for each degradation process. Subsequently, the tubes were incubated at 25°C and 150 rpm for 14 days, and the samples were tested by extracting the metabolites with acetonitrile (1 : 1, v/v). The resting metabolite concentration was also measured using HPLC (Shimadzu Corporation) under the defined conditions, as described in the previous section.

7. *Analytical methodology of chemicals*

The concentration of nitenpyram, was examined using HPLC (Shimadzu Corporation) equipped with UV detector and an InertSustain PFP 5 μm (4.6×250 mm) column (GL Sciences Inc., Tokyo, Japan). The mobile phase composition was 25 : 75 acetonitrile/0.1% aqueous trifluoroacetic acid with 1 mL min^{-1} flow rate. A wavelength of 270 nm was selected to measure the column elution. Thereafter, 25 μL samples were injected under the previous described condition to observe the retention time (t_R). The t_R of nitenpyram was 6.9 min. Nitenpyram metabolites were identified using LC-MS/MS. The LC-MS/MS analysis was performed using an Aquity UPLC H Class (Waters Corp., Milford, MA, USA) coupled with a Xevo TQD (Waters Corp.) equipped with an electrospray ionization (ESI) source. The parameters used for MS under ESI positive ion mode were: 0.5 kV capillary voltage, 500°C desolvation temperature, 150°C source temperature, 1000 L h^{-1} desolvation gas (nitrogen gas), and argon gas as the collision gas. For MS detection of nitenpyram and its metabolites, the following multiple reaction monitoring mode was used: precursor ion m/z=271.01 and product ion m/z=98.95 for nitenpyram (cone voltage=28 V; collision energy=26.0 V); precursor ion m/z=256.18 and product ion m/z=126.12 for CPMA (cone voltage=20 V; collision energy=25.0 V); precursor ion m/z=211.96 and product ion m/z=125.95 for CPMF (cone voltage=30 V; collision energy=22.0 V); and precursor ion m/z=198.92 and product ion m/z=125.71 for CPF (cone voltage=22 V; collision energy=18.0 V). Analytical separation of nitenpyram and its metabolites was performed on a Discovery HS F5-3 (150×2.1 mm, 3 μm particle size; Supelco, Bellefonte, PA, USA) at 40°C. The linear gradient elution profile, consisting of solvent A (0.1% acetic acid in methanol) and solvent B (0.1% acetic acid), was as follows: from 5% solvent A, linear change to 65% solvent A over 40 min.

During the degradation test of metabolites, CPMF and CPF concentrations were analyzed using HPLC under the same conditions applied during nitenpyram concentration measurement. The t_R of CPMF and CPF was at 4.7 and 9.3 min, respectively. A standard curve was drawn for each chemical to calibrate the concentrations of nitenpyram and metabolites. The recovery efficiency was evaluated at concentrations of 1, 2.5, 5, 10, and 25 mg L^{-1} of each standard in the PDB medium (pH 7). We found good recovery rates ranging from 94–99% for nitenpyram and 92–95% for CPMF and CPF.

8. *Statistical analysis*

Treatments means were analyzed and compared using student *t*-test at $p<0.05$ through the SPSS software version-16 (https://spss.software.informer.com/16.0/).

Results

1. *Isolation and selection of degradable strain*

Approximately 300 endophytic strains were isolated from *B. rapa* plant samples after 3–5 days of incubation in PDA media at pH 7. Among these, one potential strain (NIT-2) showed 65.16% degradation of nitenpyram from the initial concentration (5 mg L^{-1}) after 14-day incubation in PDB media at pH 7. Therefore, strain NIT-2 was selected primarily based on its ability to degrade nitenpyram. Of note, except for strain NIT-2, none of the other strains showed significant degradation of nitenpyram. However, the 16S rRNA gene sequences were used to identify the selected strain (1047 bp). A BLAST search of the selected sequence and comparison with other related sequences available in the DNA Data Bank indicated a high degree of similarity to *Bacillus thuringiensis* (100%). The nucleotide sequence data were submitted to DNA Data Bank of Japan with accession number LC777281 (available at http://getentry.ddbj.nig.ac.jp/). To construct a phylogenetic tree, the 16S rRNA gene of strain NIT-2 was associated with other representative *Bacillus* strains. Figure 1 shows a phylogenetic dendrogram of the strain NIT-2, indicating a close relationship between strain NIT-2 and *B. thuringiensis*. Consequently, NIT-2 was selected as the preferred candidate for nitenpyram degradation, and further degradation tests were performed using this strain. Subsequently, no degradation was observed in the uninoculated samples in PDB medium.

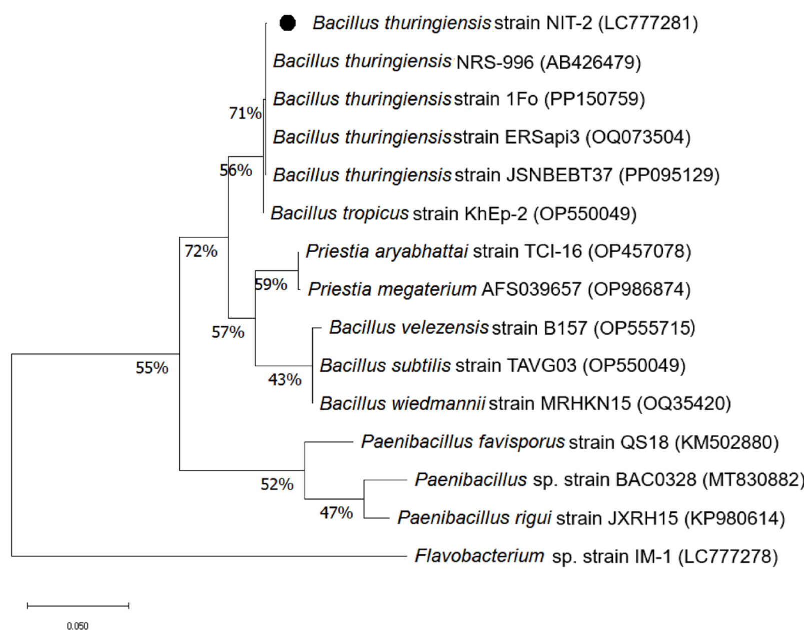

Fig. 1. Phylogenic tree of the 16S rRNA gene sequence of strain NIT-2 with similar types of *Bacillus* species based on the neighbor-joining method. The boost up values are given at each branching points (expressed as percentage of 1000 replications).

2. Degradation efficiency of NIT-2

Figure 2 shows the degradation efficiency of NIT-2 at different nitenpyram concentrations, depicting that the maximum nitenpyram degradation was obtained at 5 mg L^{-1} concentration (65.16%) with the highest bacterial density (1.725 at $OD_{600\,nm}$). However, compared to other nitenpyram initial concentrations ranging from 1 to 5 mg L^{-1}, the bacterial density and degradation efficiency were found to be lower when 10 mg L^{-1} concentration was added. At 10 mg L^{-1} concentration, the degradation rate decreased to 52.34%, with the lowest bacterial density (1.254 at $OD_{600\,nm}$). Thus, nitenpyram concentration at 10 mg L^{-1} showed the most detrimental effects on bacterial growth, which may slow down the degradation process. Therefore, considering the pattern of result, 5 mg L^{-1} was chosen as the optical condition for all subsequent experiments.

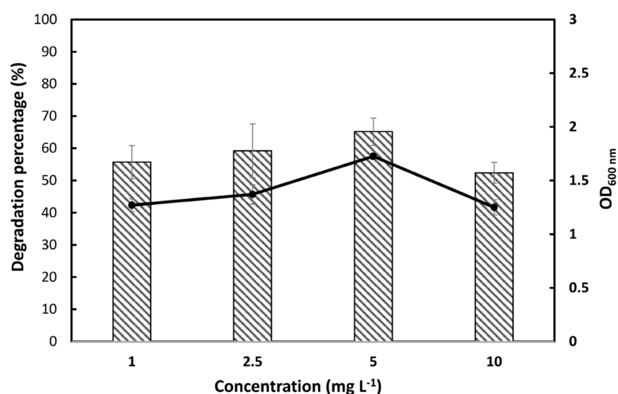

Fig. 2. Influence of different initial concentration of nitenpyram on strain NIT-2 growth and their degradation at 14 days of incubation. Here, line graph represents the growth of bacterial strain NIT-2 and bar graph indicates the degradation percentage of nitenpyram under different initial concentrations. Error bar indicates mean±replications (n=3).

3. Time-course degradation of nitenpyram by strain NIT-2

Considerable degradation of nitenpyram was observed after 7 days of incubation, along with an increase in bacterial density compared to those in the control (Fig. 3A). Approximately 26.20% degradation was observed on day 7, whereas no significant degradation was observed on day 3 (Fig. 3A). As the incubation days progressed, the rate of degradation increased. At 21 days of incubation, 96.63% degradation was observed with only 0.17±0.06 mg L^{-1} nitenpyram remaining in the medium. Finally, on day 28, almost all the nitenpyram was biodegraded, while the control showed a nitenpyram concentration of 4.91± 0.05 mg L^{-1}.

During nitenpyram biodegradation, bacterial density ($OD_{600\,nm}$) was higher in PDB medium containing nitenpyram than in PDB medium without nitenpyram after day-14 (Fig. 3A). As the degradation progressed faster from day 7, the growth rate of bacterial strain NIT-2 also increased. At day-14, the highest bacterial density was observed (1.725 at $OD_{600\,nm}$) when the nitenpyram concentration decreased sharply. From day-14, bacterial density remained consistent until day 21, when the maximum degradation of nitenpyram was recorded. After 21 days, bacterial density decreased until day-28 of incubation in the nitenpyram-supplemented medium. In the medium without nitenpyram, the density of the bacterial strain NIT-2 was the highest after 7 days of incubation, remained constant until 14 days, and decreased thereafter until day 28. In MS media, similar findings were observed, and the $OD_{600\,nm}$ revealed continuous incremental bacterial density until day-14 (Fig. 3B).

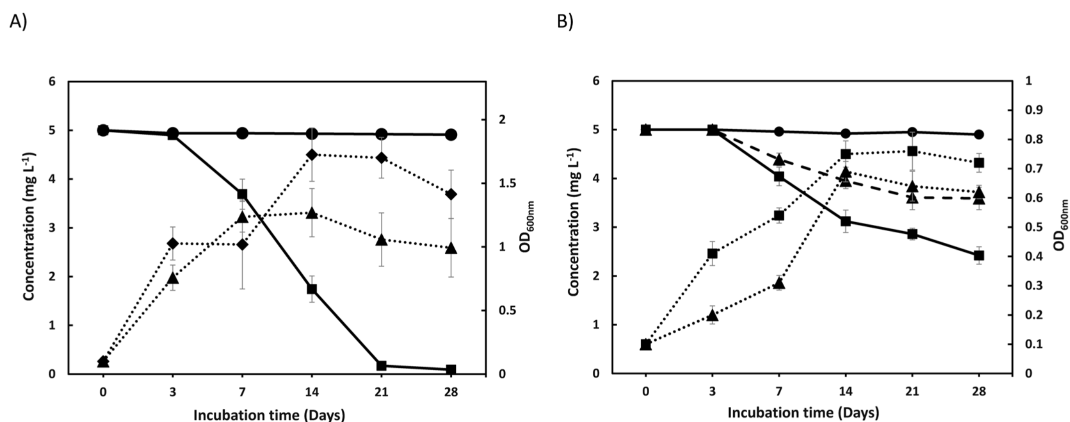

Fig. 3. Time course degradation of nitenpyram by strain NIT-2 in A) PDB media (–●– indicates the concentration of nitenpyram in control sample; –■– indicates concentration of nitenpyram at inoculated media; ···▲··· indicates bacterial growth without nitenpyram; ···◆··· indicates bacterial growth with nitenpyram) and B) Mineral Salt (MS) media (–●– indicates the concentration of nitenpyram in control sample; - -▲- - indicates nitenpyram used as C source; –■– indicates nitenpyram used as N source; ···▲··· indicates bacterial growth as C source; ···■··· indicates bacterial growth as N source). Data were taken at 3, 7, 14, 21, and 28 days consecutively. Error bar indicates mean±replications (n=3).

In contrast, HPLC analysis revealed a continuous decrease in nitenpyram concentration in the MS medium (Fig. 3B). Here, maximum degradation occurred between day-7 to day-14. During this period, the bacterial density increased from 0.31 to 0.69 and from 0.54 to 0.76 when nitenpyram was used as C and N source, respectively (Fig. 3B). However, nitenpyram degradation, accompanied by bacterial growth, was higher when strain NIT-2 used nitenpyram as the N source (Fig. 3B).

4. Metabolite identification and degradation

Several peaks, which were not observed in the control treatments, were observed in the HPLC due to nitenpyram degradation in the liquid media. To identify the metabolites, a 7-day culture of strain NIT-2 was analyzed using LC-MS/MS, and three metabolites were detected (Fig. 4A). The identified metabolites were compared with the standards of known metabolites as CPMA, CPMF, and CPF (Fig. S1), confirming that CPMA (major fragment ions at m/z=104, 146, 171, and 256); CPMF (major fragment ions at m/z=147, 155, 211, and 214);

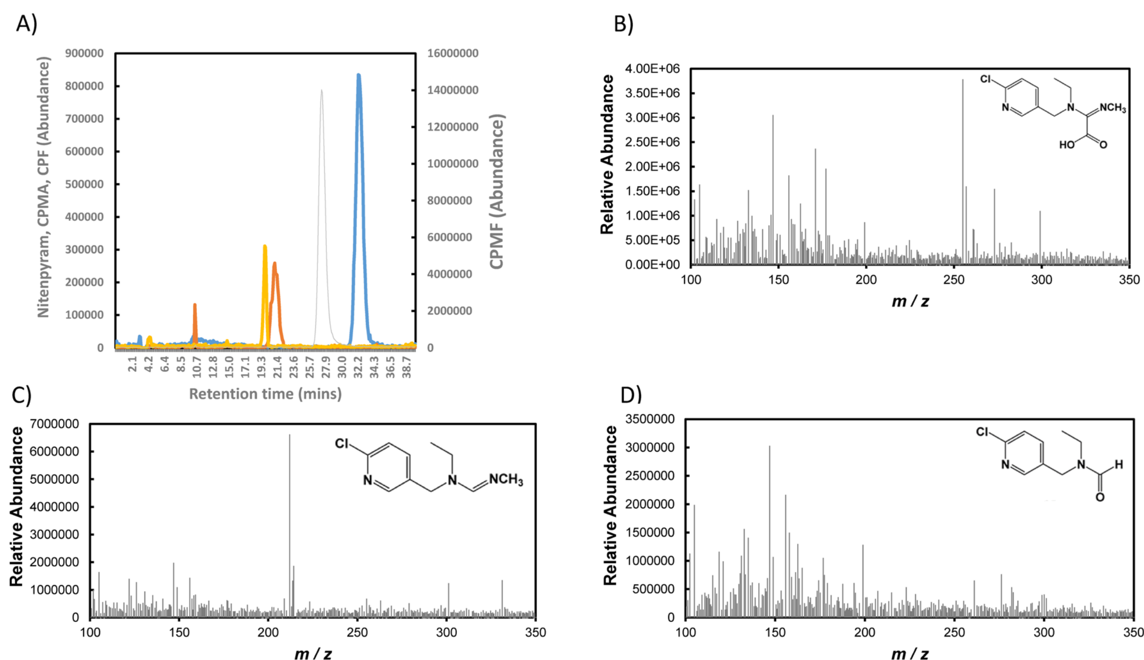

Fig. 4. Several metabolites of nitenpyram were detected by LCMS-MS after 7 days incubation by using strain NIT-2. A) Retention time (t_R) of nitenpyram and its metabolites CPMA, CPMF, CPF (— indicates t_R of nitenpyram; — indicates t_R of CPMA; — indicates t_R of CPF; and — indicates t_R of CPMF) along with mass spectra of B) CPMA C) CPMF D) CPF.

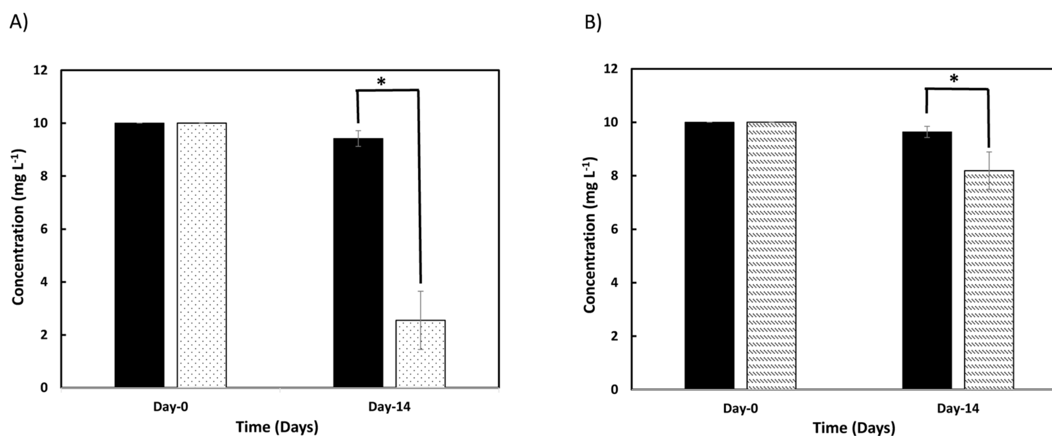

Fig. 5. Degradation of metabolites A) CPMF and B) CPF by strain NIT-2 after 14 days incubation. Dark color represents the concentration of each metabolite in control sample and light color represents the degradation percentage of each metabolite. Error bar represents mean±replications ($n=3$). *indicates the significant differences between treatments by following the student *t*-test ($p<0.05$).

and CPF (major fragment ions at $m/z=104$, 132, 156, and 198) (Fig. 4B-D) were formed after nitenpyram degradation. We further observed the degradation of known metabolites by strain NIT-2, and significant degradation was observed for both CPMF (71.79%) and CPF (18.14%) after 14 days of incubation (Fig. 5A and B) along with bacterial density ($OD_{600\,nm}$) 1.271 for CPMF and 1.229 for CPF.

Discussion

Our study aimed to isolate and characterize endophytic bacteria that could potentially degrade nitenpyram. It is noteworthy that among 300 isolated endophytic bacterial strains from *Brassica rapa* plants only *Bacillus thuringiensis* strain NIT-2 was able to degrade the significant amount of nitenpyram. Correspondingly, previous studies reported that *B. thuringiensis* could degrade various pesticides such as cyhalothrin,[33] chlorpyrifos, triazophos, dimethoate,[34] and neonicotinoid-like imidocloprid.[35] Therefore, *B. thuringiensis* has a significant potential to degrade different pesticides and organic contaminants. However, none of the endophytic bacteria as well as strains of *B. thuringiensis* that can degrade nitenpyram has not been reported yet. Thus, *B. thuringiensis* strain NIT-2 is a novel endophytic bacterium, which can degrade nitenpyram in PDB and MS media. To the best of our knowledge, this study is the first to describe the potential bacterial biodegradation pathways of nitenpyram by the novel endophytic bacterium, *B. thuringiensis* strain NIT-2.

Biodegradation is an efficient and economical method for transforming harmful chemicals into harmless derivatives.[36] Biodegradation process removes several pesticide residues from the environment. In our study, we observed 98.18% nitenpyram removal using strain NIT-2 in PDB media after 28 days of incubation. *Aspergillus* sp., a fungal strain acquired from commercial formulation biotechnology, degraded 92.9% nitenpyram.[37] Resting cells of the actinomycete *Rhodococcus ruber* CGMCC 17550 degraded 98.37% of nitenpyram.[29] *Phanerochaete sordida* YK-624, a white-rot fungal strain, degraded nitenpyram after 5 days of incubation in PDB media when P450 cytochrome was added into the media,[31] indicating that cytochrome P450 promotes nitenpyram biodegradation. However, under non-lignolytic conditions, only 20% nitenpyram degradation was observed.[31] Consistently, we found almost complete nitenpyram degradation without any enzyme addition in PDB media.

Nitenpyram degradation increased when bacterial density increased (Fig. 3). We observed the highest bacterial density under nitenpyram-treated conditions owing to the induction of pesticide biodegradation (Fig. 3A). The bacterial density was sharply higher after 14 days of incubation, when maximum biodegradation was observed. A similar trend was also observed in MS media, where we determined whether nitenpyram could be used as a C or N source. In MS medium, we supplied glucose and ammonium nitrate separately as co-substrates for the growth of strain NIT-2. Dai *et al.* 2021 applied glucose, fructose, and pyruvate as co-substrates during nitenpyram degradation, accelerating the degradation slightly.[29] In our experiment, we observed higher degradation rate when nitenpyram and glucose were used as the nitrogen and carbon sources, respectively, in the MS media (Fig. 3B). However, definite degradation was also observed when nitenpyram and ammonium nitrate were used as the carbon and nitrogen sources, respectively (Fig. 3B), indicating that strain NIT-2 can use nitenpyram as the sole carbon or nitrogen source. Moreover, we observed a higher bacterial growth and nitenpyram degradation in PDB medium than in MS medium, suggesting that nitenpyram degradation by strain NIT-2 depended on microbial growth and that strain NIT-2 used this pesticide as the sole carbon or nitrogen source for growth. Wang *et al.* 2023 reported similar nitenpyram biodegradation by *Ochrobacterium* sp. strain DF-1.[30] They found the highest nitenpyram degradation when *Ochrobacterium* sp. bacterial optical density ($OD_{600\,nm}$) reached the highest and when nitenpyram was the carbon or nitrogen source.[30] Similarly, actinomycetes *R. ruber* CGMCC 17550 could degrade nitenpyram after 75 hr of incubation, while the OD extended to 2.2 (the

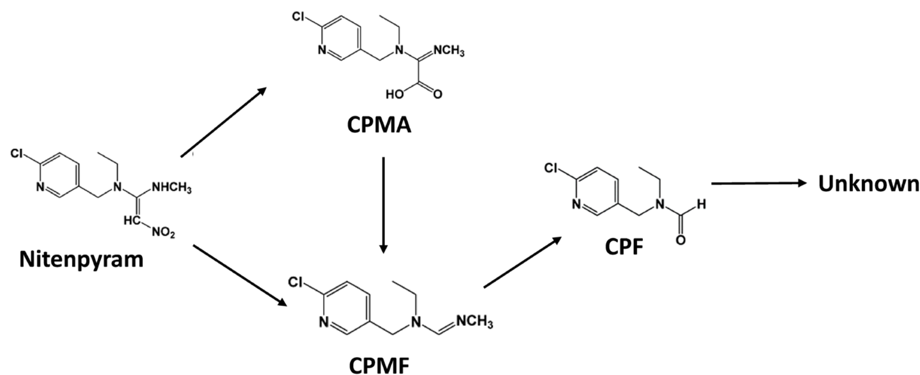

Fig. 6. Possible degradation pathways of nitenpyram by strain NIT-2.

highest density).[29] The microbial degradation of pesticides is usually governed by enzymatic reactions that occur inside microbial organisms.[38]

Generally, pesticides are used as nutrient substrates for microbial growth and are broken down into smaller compounds, such as CO_2 and H_2O, which are non/less toxic to the environment. Therefore, pesticide biodegradation is accelerated by the growth of degradable microbes.[39] This may explain why the nitenpyram degradation rate was associated with bacterial growth. However, pesticides produce different metabolites during biodegradation, which are also necessary for the analysis of biodegradation efficiency. In this study, we described a partial metabolic degradation pathway of nitenpyram by strain NIT-2 based on the degradable products (Fig. 6). Strain NIT-2 degraded nitenpyram to form CPMA, CPMF, and CPF. Other unknown metabolites, which were difficult to identify because of their lower intensity in the liquid media, were also formed after the initial metabolite degradation. The identification of metabolic pathways is not only necessary for environmental safety but also important for understanding microbial performance during biodegradation processes. To identify the metabolic pathways of nitenpyram degradation, the nitro groups of nitenpyram were reduced to form the metabolites CPMA and CPMF. The CPMF had higher intensity in the liquid media than CPMA (Fig. 4A). This is because CPMA is very unstable in liquid media and is converted to CPMF *via* decarboxylation. Previous studies on nitenpyram biodegradation have identified different pathways for the production of intermediate metabolites. The fungus *P. sordida* YK-624 degrades nitenpyram to produce the metabolite (*E*)-*N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-*N*0-hydroxy acetimidamide (CPMHA) in the presence of cytochrome P450.[31] During the degradation process, the nitro group of nitenpyram is reduced by the enzyme cytochrome P450, followed by denitrogenation or deamination to form CPMHA. In our study, we did not identify the presence of CPMHA. Moreover, during the identification of metabolites produced by actinomycetes, the hydroxylation pathway by *R. ruber* CGMCC 17550 was observed.[29] Wang *et al.* 2023 reported nitenpyram biodegradation by bacterium *Ochrobactrum* sp. strain DF-1 through the removal of nitro group (*N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-*N*-methylethene-1,1-diamine) followed by demethylation (*N*-((6-chloropyridin-3-yl)methyl)-*N*-ethylethene-1,1-diamine) and redox reaction *N*-((6-chloropyridin-3-yl) methyl)ethanamine).[30] These reports indicate that the loss of nitro groups is the primary requirement for nitenpyram degradation. Consistently we observed that the nitro group of nitenpyram was reduced to form CPMA and CPMF. Unlike the study by Wang *et al.* (2019),[31] we did not use any enzymes. Strain NIT-2 probably produces enzymes that promote nitenpyram degradation. Therefore, we confirmed the presence of CPMA and CPMF as the first metabolites produced after the breakdown of nitenpyram strain NIT-2 (Fig. 6). Immediately, we also detected CPF as another metabolite during the degradation process using LC-MS/MS. CPF is typically formed by the oxidation of CPMF. A previous report supported the conversion of CPMF into CPF.[32] Therefore, we ran known metabolite (CPMF and CPF) degradation tests in PDB medium (pH 7) to identify downstream metabolites. We did not run the CPMA degradation test because of the complexity of extraction from media. Tsumura *et al.* 1998 observed only 9.0% recovery of CPMA, where 80.4% of CPMA was converted to CPMF in a water/acetone (20 : 80) mixture.[40] However, the growth of strain NIT-2 was not suppressed by the addition of metabolites, instead strain NIT-2 showed significant metabolite degradation (Fig. 5). Therefore, NIT-2 may utilize these metabolites as growth substrates. In CPMF biodegradation analysis, we observed a distinct peak at the CPF retention time in HPLC after 14 days of incubation. Therefore, CPF can be considered a secondary metabolite of CPMF degradation (Fig. 6). For CPF degradation, we obtained several HPLC peaks at different retention times, which were absent in the control sample. However, they were difficult to identify under the current analytical conditions because of their low intensity in the medium (Fig. 6).

Our study identified some metabolites produced during nitenpyram degradation by pure bacterium. The subsequent steps in nitenpyram biodegradation by strain NIT-2 require further investigation. Considering its degradation potential, it is undeniable that strain NIT-2 can degrade nitenpyram and its metabolites, and is a novel observation that has not yet been reported.

Conclusion

In this study, we demonstrated that the endophytic bacterium *B. thuringiensis* strain NIT-2 can degrade both nitenpyram and its metabolites. Although further investigation is required to identify downstream metabolites after CPMF and CPF degradation, this study reveals that pure endophytic bacterium has the potential to degrade both nitenpyram and its metabolites. For a deeper understanding, cloning of molecularly degradable genes is suggested. The removal of nitenpyram and other neonicotinoids from the environment is necessary to ensure food safety. Thus, biodegradation by *B. thuringiensis* strain NIT-2 could be an effective and eco-friendly approach for the removal of neonicotinoid residues from the environment.

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Conflicts of interest

The authors have no conflict of interest.

Electronic supplementary materials

The online version of this article contains supplementary material (Fig. S1), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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