Biodegradation of imidacloprid by an isolated soil microorganism

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Imidacloprid (1-[(6-chloro-3-pyridinyl)-methyl]-\textit{N}-nitro-2-imidazolidinimine), a chloronicotinyl insecticide used to control biting and sucking insects, is very persistent in the soil with a half-life often greater than 100 days. Although a few soil metabolites have been reported in the literature, there are no reports of imidacloprid-degrading soil microorganisms. Our objectives were to discover, isolate, and characterize microorganisms capable of degrading imidacloprid in soil. Two soil-free stable enrichment cultures in N-limited media were obtained that degraded 19 mg L\textsuperscript{-1} (43\%) and 11 mg L\textsuperscript{-1} (16\%) of the applied imidacloprid, and produced about 19 mg L\textsuperscript{-1} 6-chloronicotinic acid in three weeks. Enrichment media without microorganisms had no loss of imidacloprid. Strain PC-21, obtained from the enrichment cultures, degraded 37\% to 58\% of 25 mg L\textsuperscript{-1} imidacloprid in tryptic soy broth containing 1 g L\textsuperscript{-1} succinate and D-glucose at 27°C incubation over a period of three weeks. Trace amounts of NO\textsubscript{3}/NO\textsubscript{2} were produced and six metabolites were characterized by high performance liquid chromatography (HPLC) using \textsuperscript{14}C-methylene-imidacloprid and liquid chromatograph-electrospray-mass spectrometer (LC-MS). Two of the metabolites were identified as imidacloprid-guanidine and imidacloprid-urea by HPLC standards and LC-MS. During the experiment, 6-chloronicotinic acid was not produced. Less than 1\% of the applied \textsuperscript{14}C was incorporated into the microbial biomass and no \textsuperscript{14}CO\textsubscript{2} was detected. Strain PC-21, identified as a species of \textit{Leifsonia} by PCR amplification of a 500 bp sequence of 16s rRNA, cometabolized imidacloprid.

Keywords: Biodegradation; imidacloprid; insecticide; metabolites; \textit{Leifsonia}; soil microorganism.

Introduction

Imidacloprid is a soil or plant-applied insecticide used in a wide variety of crops. Imidacloprid field dissipation rates are widely variable, and in some cases have been shown to degrade slowly in soil with half-lives exceeding 180 days in non-vegetated soil.\textsuperscript{[1,2]} The factors governing the dissipation of imidacloprid in soil are not clearly understood. Liu et al.\textsuperscript{[3]} reported that imidacloprid was stable in water at neutral and acidic pH and slowly degraded in alkaline solutions. Vegetation increased the rate of dissipation of imidacloprid, yielding a range of half-lives from 42 to 129 days.\textsuperscript{[1,4]} This effect of vegetation and the identification of a few metabolites may suggest a microbially mediated degradation process that is enhanced through the rhizosphere effect.

Biodegradation of pesticides is controlled by the bioavailability of the pesticide to a pesticide-degrading microorganism and the activity of the microorganism. Bioavailability may limit the biodegradation of imidacloprid and its metabolites in soil, resulting in the long half-lives observed. Imidacloprid and its metabolites become increasingly strongly bound to soil over time.\textsuperscript{[5,6]} Sorption of imidacloprid and its metabolites, tend to increase with increasing soil organic carbon (OC) content. As organic compounds age in soil, bioavailability has been shown to be reduced and degradation limited.\textsuperscript{[7]} The strong sorption of aged imidacloprid residues in soil may make biodegradation of the aged imidacloprid residues difficult.

Numerous imidacloprid metabolites have been proposed in degradation pathways.\textsuperscript{[8,9]} Possible microbial metabolites reported in soil metabolism studies include imidacloprid-guanidine, imidacloprid-guanidine-olefin, and imidacloprid-urea (Fig. 1). Although the biodegradation of various N-containing heterocycles, including pyridine and nicotine, by isolated microorganisms has been described,\textsuperscript{[10,11]} direct evidence of microbial degradation of imidacloprid has not been reported. Our
Fig. 1. Structure of imidacloprid and its metabolites, imidacloprid-guanidine-olefin, imidacloprid-guanidine, and imidacloprid-urea.

objectives were to isolate, and characterize microorganisms with the ability to degrade imidacloprid.

Materials and Methods

Chemicals

Analytical standards of imidacloprid (1-[(6-chloro-3-pyridinyl)-methyl-N-nitro-2-imidazolidinimine) (96.9% pure), as well as three metabolites, imidacloprid-guanidine (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-1H-imidazol-2-amine), imidacloprid-guanidine-olefin (1-[(6-chloro-3-pyridinyl)methyl]-1H-imidazol-2-amine), and imidacloprid-urea (1-[(6-chloro-3-pyridinyl)-methyl-2-imidazolidinone) were obtained from Bayer Corporation, Stillwell, KS (Fig. 1). Analytical grade (99% pure) 6-chloronicotinic acid was purchased from Aldrich Company. Two radio-labeled forms of imidacloprid were also obtained from Bayer Corporation: 14C-methylene-imidacloprid (4.64 MBq mg⁻¹, 99.9% pure) and 14C-4,5-imidazolidin-imidacloprid (4.59 MBq mg⁻¹, 98% pure).

Soils

Agricultural soils from Indiana (Drummer silty clay loam) and California (Exeter sandy loam) were sampled at surface and subsurface depths by Bayer Corporation at field sites near Oxford, IN and Fresno, CA. The Drummer soil has never had imidacloprid applied to it and no pesticide had been applied in the year prior to collection. The Exeter soil also had no prior exposure to imidacloprid and had no pesticide application history for five years prior to its collection. Drummer (IN) and Exeter (CA) surface soils, (0–15 cm) and subsurface soils, (15-76 and 46-61 cm, respectively) were collected from the field site with hand shovels, placed in water-impermeable bags and shipped to the National Soil Tilth Laboratory, Ames, IA. Soil organic carbon ranged from 0.11% in the Exeter subsurface soil to 4.49% in the Drummer surface soil. Total nitrogen ranged from 0.02% in the Exeter subsurface soil to 0.36% in the Drummer surface soil. Total soil microbial biomass C ranged from 10.2 µg C g⁻¹ in Exeter subsurface soil to 704.7 µg C g⁻¹ in Drummer surface soil. The Monona soil (pH 5.3, 1.63% OC, and 0.16% N) was collected from a farm near Treynor, IA that had a long history of triazine and chloroacetamide herbicide use, but no history of imidacloprid use. The Bravo soil was from an agrichemical dealership site and had been exposed to many herbicides and insecticides. This soil was a loamy sand with a pH of 6.5, 2.4% OC and 0.05% total N. Soils were stored in a 7°C incubator before the study.

Total soil carbon and soil nitrogen were determined using a Carlo-Erba NA 1500 NCS elemental analyzer (Haake Buchler Instruments, Paterson, NJ) and total soil microbial biomass was measured by the fumigation-extraction method and then analyzing the extracts using a Dohrmann DC-180 carbon analyzer (Rosemount Analytical Services, Santa Clara, CA).

Enrichment cultures

The four soils were used to create enrichment cultures for isolation of imidacloprid-degrading microorganisms. Three versions of Kaufman and Kearney's minimal salts media (MSM) were prepared. Fifty mL C-limited MSM with 83 mg L⁻¹ imidacloprid as the sole carbon source, 50 mL N-limited MSM with imidacloprid as the sole nitrogen source and sodium citrate and sucrose added as supplemental carbon sources, or 50 mL MSM broth, which contained all components plus sodium citrate, sucrose, and imidacloprid were inoculated with 2 g of soil to enrich for imidacloprid-degrading microorganisms. Cultures were maintained on the shaker at 100 rpm and 27°C. Subcultures were made monthly in order to obtain soil-free enrichments. Aliquots of the cultures were periodically removed from the shaker flasks for HPLC analysis.

HPLC & LC-MS analysis

Periodic analyses of imidacloprid concentrations were accomplished using a Waters HPLC (Division of Millipore, Milford, MA), which had a Reverse Phase C-18 (RP18) Symmetry Shield column (Waters-Millipore) (3.9 mm × 150 cm) and an ultra violet (UV) detector. Operating
conditions were: 30 min gradient of acetonitrile (ACN) and acidiﬁed (pH 3) ultrapure water [0 min 20%/80% ACN:H2O; (7 min) 22%/78% ACN:H2O; (14 min) 30%70% ACN:H2O; (21 min) 40%/60% ACN:H2O; (23-30 min) 20%/80% ACN:H2O], injection volume of 25 μL, at 0.6 mL min⁻¹, and UV detection at wavelengths of 220, 247, and 270 nm. Media samples were ﬁltered (0.2 μm) and diluted by half with methanol prior to analysis.

Analytical grade imidacloprid and imidacloprid-guanidine, imidacloprid-guanidine-olefin, and imidacloprid-urea metabolites, were used as standards, as well as technical grade 6-chloronicotinic acid. The retention times for imidacloprid and its metabolites are as follows: imidacloprid-guanidine and imidacloprid-guanidine-olefin co-elute at 1.65 min; imidacloprid-urea, 6.4 min; imidacloprid, 11.1 min; and 6-chloronicotinic acid, 18.2 min.

Radiochromatography (RC) was performed with the HPLC system previously described coupled to a Radiomatic detector (Packard Instrument Company, Downers Grove, IL) equipped with a ﬂow cell of either 2.5 mL or 0.5 mL. Ultima-Flo M scintillation cocktail was used at a rate of 1.8 mL min⁻¹ and a standard curve was generated using differing amounts of ¹⁴C-methylene-imidacloprid as standards. Retention times were increased by 0.3 to 0.4 min compared to UV detection times.

Strain PC-21 acetonitrile extracts were also analyzed on a liquid chromatograph-electrospray-mass spectrometer (LC-MS) (Waters Micromass ZMD coupled to a Waters Alliance 2690 HPLC, Milford, MA) operating in positive mode under the following conditions: Zorbax C-8 column (2.1 mm x 15 cm), 10 μL injection, ﬂow rate 0.2 mL min⁻¹, and a gradient of acetonitrile and formic acid/water [0 min 85%/15% ACN:1% formic acid/H2O; (3 min) 70%/30% ACN:1% formic acid/H2O; (6 min) 60%/40% ACN:1% formic acid/H2O; (12 min) 50%/50% ACN:1% formic acid/H2O; (15 min) 20%/80% ACN:1% formic acid/H2O; (20 min) 85%/15% ACN:1% formic acid/H2O].

Isolation and characterization of microorganisms

Two mixed enrichment cultures from the Monona soil that showed losses of imidacloprid during incubation, NTN-13 and NTN-16, were spread-plated using dilutions of 10⁻¹ to 10⁻⁶ on N-limited agar plates containing 36 mg kg⁻¹ imidacloprid and either streptomycin or cyclohexamide. After a one-week incubation, the plates were screened for colonies that visually appeared different from one another. In total, 29 colonies (PC-1 through PC-29) were transferred onto new agar plates with imidacloprid as the sole N source. After about three weeks, PC strains 1-26 (bacterial isolates) were put into 25 mL of tryptic soy broth (TSB) and PC strains 27-29 (fungal isolates) were put into 25 mL Capex Dox broth containing 25 mg L⁻¹ imidacloprid. After three days growth, each of the 29 isolates was centrifuged in HDPE tubes for 10 min at 6700 x g. The supernatant was poured off, and the isolates were re-suspended in sterile phosphate buffer for a total volume of 10 mL. A 2-mL sample of each isolate was inoculated into N-limited MSM, C-limited MSM, and TSB (described previously) all containing 30 mg L⁻¹ of imidacloprid in 25 mL total. Non-inoculated controls were also made by inoculating 2 mL of phosphate buffer into each of the media-filled ﬂasks. All samples were wrapped in aluminum foil and placed on a shaker operated at 27°C and 100 rpm. The three non-inoculated controls were analyzed for imidacloprid concentration. After seven days of incubation, the 29 cultures were extracted by adding 25 mL of methanol to the ﬂasks. The samples were then sonicated for six min. each at 50% duty cycle and centrifuged at 6700 x g. Four mL aliquots were ﬁltered through a 0.2 μm ﬁlter and analyzed on the HPLC.

Using ¹⁴C-methylene-imidacloprid to verify whether or not imidacloprid degradation was truly occurring, a second experiment was performed using the only three PC strains that showed possible imidacloprid degradation. In this experiment the three strains (PC-17, PC-21, and PC-27) were inoculated into 25 mL TSB broth with 25 mg L⁻¹ imidacloprid and incubated on the shaker at 27°C and 100 rpm and were covered with aluminum foil. After three weeks time the cell growth was terminated by adding 25 mL of methanol to the cultures. Samples were sonicated, centrifuged, and the pelleted cells were removed and air dried. The ¹⁴C content in the dried pellets (biomass) was determined by using a Harvey Biological Oxidizer. Imidacloprid in the sonicated culture supernatants was extracted by liquid-liquid partitioning done three times per sample with a total of 200 mL methylene chloride. The partitioned organic fraction of the samples was evaporated to dryness on a rotary evaporator and then redissolved in 4 mL of acetonitrile. The acetonitrile samples were analyzed by HPLC.

These experiments showed removal of imidacloprid and production of imidacloprid-guanidine by strain PC-21, indicating that it was capable of degrading imidacloprid. A more detailed experiment was performed to investigate imidacloprid metabolite production by strain PC-21. In this experiment, 2 mL of strain PC-21 were inoculated into 50 mL of either 10% TSB or full strength TSB. Cells were grown in TSB containing 25 mg L⁻¹ imidacloprid for three to five days. Cells were then harvested by centrifuging at 6700 x g to wash off the media, then resuspending the cells in phosphate buffer. This step was repeated two more times, each time pouring off the supernatant and then resuspending the cells in a phosphate buffer for a ﬁnal concentration of 2.5 x 10⁷ cells mL⁻¹.

Concentrated strain PC-21 (in phosphate buffer) was added to six replicates (three labeled with ¹⁴C) for each of three time points. Sample ﬂasks were covered with aluminum foil and placed on a shaker operating at 100 rpm and 27°C. Four-mL aliquots of the media were removed at day 0, 3, 7, 14, and 21 and ﬁltered through 0.2 μm ﬁlters for HPLC analysis. Growth was terminated in the culture ﬂasks
Fig. 2. Biodegradation of imidacloprid by strain PC-21 in 10% and full-strength tryptic soy broth (TSB) over 21 days.

at day 7, 14 or 21 by adding 50 mL of methanol to the flasks. Cultures were also sampled for NO$_3^{-}$/NO$_2^{-}$ (Lachat Instruments, Zellwegger Analytics, Inc, Milwaukee, WI). The $^{14}$C-methylene-imidacloprid samples were then sonicated, centrifuged, and the microbial pellet was removed. Liquid-liquid partitioning was performed as described above, and the methylene chloride extracts were evaporated to dryness and redissolved in 4 mL of acetonitrile. Cell-free acetonitrile extracts were analyzed by HPLC with radioactivity quantification for metabolite determination. Radioactivity in the aqueous fraction following methylene chloride partitioning was measured by liquid scintillation spectroscopy (LSC) to determine radioactivity. Production of $^{14}$CO$_2$ was measured in cultures spiked with $^{14}$C-4,5-imidazolidin-imidacloprid by trapping the CO$_2$ in 10 mL of 0.5 M NaOH. A mass balance was calculated.

Strain PC-21 was viewed under the scanning electron microscope (SEM). The cells were placed on a copper grid covered with Fomvar film for support and then stained with a negative stain of 1% phosphotungstic acid (pH 6). Excess media and stain were wicked away with a filter paper. Grids were viewed at 20,000X to 25,000X magnification.

Results and discussion

Two soil-free enrichment cultures, NTN-13 and NTN-16, were obtained from the Monona soil that degraded imidacloprid after several months of exposure to the insecticide. Cultures with consistent ability to degrade imidacloprid were not obtained from the other soils. In these enrichment cultures, imidacloprid concentrations decreased by about 43% and 16%, respectively, from initial concentrations of 44 and 70 mg L$^{-1}$ in a period of three weeks. These two cultures also produced about 19 mg L$^{-1}$ 6-chloronicotinic acid. Further subcultures were able to degrade imidacloprid slightly faster with approximately 44% remaining after eight days. Cell-free control cultures showed no imidacloprid loss.

Samples of the NTN-13 and NTN-16 enrichments were plated on N-limited agar containing imidacloprid. Twenty-nine pure colonies selected from these plates were tested for imidacloprid degradation, but only strain PC-21 consistently degraded imidacloprid.

PC-21 was not able to degrade imidacloprid in 10% TSB, but was able to degrade 37% to 58% of imidacloprid in the full strength TSB (Fig. 2). No degradation was observed in the uninoculated controls. Strain PC-21 grew slightly in full-strength TSB, increasing from $1.86 \times 10^8$ cells mL$^{-1}$ to $2.42 \times 10^8$ cells mL$^{-1}$ during the first four days, then decreasing to $2.75 \times 10^4$ cells mL$^{-1}$ by day 21.

Strain PC-21 grown in TSB consistently produced six metabolites from imidacloprid as determined by HPLC-RC and HPLC-MS (Table 1). Imidacloprid, imidacloprid-guanidine and imidacloprid-urea was detected by both HPLC-RC and HPLC-MS; $^{14}$C at HPLC retention times matching those of their respective standards, and LC/ESI/MS operating in positive ion mode producing molecular ions [M+H]$^+$ with a mass-to-charge ratio (m/z) consistent with imidacloprid (256.2), imidacloprid-guanidine (211.3), and imidacloprid urea (212.3). Possible metabolites for which standards were available, imidacloprid-guanidine-olefin and 3-chloropicolinic acid, were not detected by either HPLC-RC or HPLC-MS methods; although the LC/ESI/MS would have to have been operating in negative ion mode to observe 3-chloropicolinic acid. Although 6-chloronicotinic acid was seen in the mixed culture enrichments using HPLC-UV, it was not identified as any of these metabolites and does not appear to be produced by strain PC-21.
Biodegradation of imidacloprid

Table 1. Characterization of imidacloprid and metabolites HPLC-MS and HPLC-RC.

<table>
<thead>
<tr>
<th>Chemical [M+H]+</th>
<th>Retention time (min)</th>
<th>LC-MS</th>
<th>HPLC-RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidacloprid-guanidine (211.3)</td>
<td>2.3</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>imidacloprid-urea (212.3)</td>
<td>5.9</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Unknown-A</td>
<td>—</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Unknown-B (211.3)</td>
<td>5.4</td>
<td>4.8-5.2</td>
<td></td>
</tr>
<tr>
<td>Unknown-C (211.3)</td>
<td>5.3</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Unknown-D (235.3)</td>
<td>2.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Imidacloprid (256.2)</td>
<td>6.8</td>
<td>11.3</td>
<td></td>
</tr>
</tbody>
</table>

*ZorbaxTM C8 column. SymmetryTM C18 column.

Three metabolites were detected by HPLC-RC with retention times at 2.9, 5.0, and 5.9 min, which did not match retention times of available standards of metabolites (Table 1). Three additional metabolites were also observed using HPLC-MS, two of whose retention times could be matched with the metabolite retention times obtained using HPLC-RC (Table 1). Unknowns B and C exhibited molecular ions [M+H]+ consistent with imidacloprid-guanidine, but daughter fragments (m/z = 136.1) were not consistent with imidacloprid guanidine. No degradation and consequently no metabolites were produced in the non-inoculated controls.

Table 2 shows a 14C mass balance of imidacloprid and the six metabolites produced by strain PC-21. The initial imidacloprid degradation was rapid, only 36% of initially applied chemical remained after 7 d, and remained constant at 21 d. Imidacloprid-guanidine increased from 14% of applied 14C after a 7-d incubation to 20% after 21 d. Imidacloprid-guanidine was produced along with the constinant production of NO3-/NO2-. Due to the analytical procedure (cadmium reduction of nitrate to nitrite with colorimetric detection) used, it was not possible to determine whether NO3- or NO2- was produced. However, it seems more likely that NO2- is produced since this would not require an oxidation step. No NO3-/NO2- was produced in the non-inoculated controls. This indicates that strain PC-21 may be cleaving off the NO3- group on the end of the imidazolidin ring of imidacloprid to produce imidacloprid-guanidine. After a 7-d incubation, three unknown metabolites comprised 50% of the applied 14C. The amount of unknown-B remained relatively constant during the 21-d incubation. Unknown-A was present at day 7 (19%) and then decreased to below the detection limit of the HPLC by day 21. In contrast, unknown-C which was also present at day 7 (15%), but increased to 32%. The increase corresponded the decrease in unknown-A. This may indicate that strain PC-21 produces unknown-A, which is then transformed to unknown-C. The microbial biomass contained 1% of the applied 14C after 21 days of incubation (Table 2). Strain PC-21 incubated with 14C-4,5-imidazolidin-imidacloprid under similar conditions produced no 14CO2 (Table 2).

Colonies of strain PC-21 are typically yellow or white, circular, convex, opaque, gram positive, obligately aerobic, and catalase positive. Light microscopy and observation under a scanning electron microscope (SEM) showed that this strain was rod-shaped, and motile with a single, long, polar flagella. Further identification of strain PC-21 was obtained by MIDI Labs (Newark, DE) using polymerase chain reaction (PCR) to amplify a 500 bp sequence of 16S rRNA. The amplified rRNA sequence was matched to known bacterial sequences. Sequence comparison with Clavibacter michiganensis showed a 94% match. Strain PC-21 matched most closely to Leifsonia poae at 98% similarity. Clavibacter xyli has been reclassified as Leifsonia xyli, indicating that strain PC-21 is likely in the genus Leifsonia, although the species is uncertain. In general Leifsonia are non-sporforming, rod-shaped, obligate aerobes that test catalase-positive, and are usually motile,[14] which is consistent with PC-21 belonging to the genus Leifsonia. Extensive details of Leifsonia genetic and molecular biology traits can be found in Evtushenko et al.[14]

Leifsonia forms a coherent phylogenetic cluster attached to the branch of Agromyces spp. The genus Agromyces is classified as branched, slender, filamentous cells, which are gram positive, nonmotile, catalase-negative, and oxidase-negative. Agromyces spp. are considered to be fragmenting actinomycetes with diaminobutyric acid (DAB) in their cell walls.[15] Leifsonia also contains DAB. Agromyces are also closely related phylogenetically to Clavibacter spp., one of which has recently been reclassified as a Leifsonia sp.

Strain PC-21 degrades imidacloprid cometabolically. Cometabolism is the transformation of a compound (imidacloprid) to intermediates without the organism (PC-21) deriving any energy, carbon, or nutrients from the compound.
Evidence to support the cometabolic activities of strain PC-21 include the inability to produce $^{14}$C$_2$O$_2$, the production of stable metabolites, the inability to maintain and increase cell populations, and the inability to use imidacloprid as its sole carbon source. It is still unclear, however, whether or not strain PC-21 is able to use imidacloprid as its sole nitrogen source. Trace amounts of NO$_3^-$/NO$_2^-$ are produced as strain PC-21 degrades imidacloprid, presumably cleaving the NO$_2$ group off of imidacloprid. The NO$_2^-$/NO$_3^-$ is apparently consumed over time by strain PC-21 resulting in the low NO$_3^-$/NO$_2^-$ concentrations.

The degradation of imidacloprid by *Leifsonia* strain PC-21 is the first report of imidacloprid degradation by an isolated microorganism. This bacterium's transformation of imidacloprid may give us insight on the degradation process in soil. Imidacloprid is very slowly mineralized to CO$_2$ in soil which is consistent with our results and the conclusion that cometabolism is the dominant process. Degradation of imidacloprid-guanidine or imidacoprid-urea may proceed by breaking apart the imidazolidin ring at its two adjacent carbons or perhaps the methylene group is oxidatively cleaved producing 6-chloronicotinic acid and the imidazo-lidin ring as separate compounds.

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**References**


