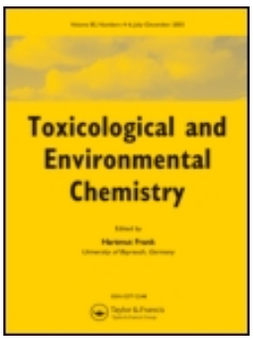

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Biodegradation of phenylurea herbicide diuron by microorganisms from long-term-treated sugarcane-cultivated soils in Kenya

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The phenylurea herbicide diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] is widely used alone or in a broad range of herbicide formulations. Its degradation in sugarcane-cultivated soils which have been impacted by the herbicide through repeated applications was studied. Liquid culture experiments with diuron as the only carbon source led to the isolation of different bacterial strains capable of degrading diuron. The bacterial species belonging to the genera *Bacillus*, *Vagococcus*, and *Burkholderia*, identified through biochemical and molecular characterization, degraded diuron to different extents. The isolated *Bacillus cereus*, *Vagococcus fluvialis*, *Burkholderia ambifaria*, and *Bacillus* spp1 degraded diuron by 21%, 25%, 22%, and 19% of the initially applied concentration of 40 mg L^{-1}, respectively, after 35 days of incubation in liquid culture media. Small amounts of 3,4-dichloroaniline and the de-methylated metabolite *N*-(3,4-dichlorophenyl)-*N*-methylurea were detected in liquid culture media. The combination of *V. fluvialis* and *B. ambifaria* showed an enhanced degradation of up to 30% of the initially applied concentration of 40 mg L^{-1}. Degradation by pure isolates was low (18–25%) compared to the capacities of diuron degradation shown by the bacterial communities (58–74%). This study showed the presence of diuron degraders in sugarcane-cultivated soils impacted by diuron due to repeated applications.

Keywords: diuron; degradation; metabolites; bacterial isolates; liquid cultures

Introduction

The phenylurea herbicides are an important group of pesticides that are used predominantly in either the pre- or post-emergence treatment of cotton, fruit, cereal, or other agricultural crops. One of the most widely used members of this group, diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea], is employed principally for the control of vegetation in crop and non-crop areas, including irrigation and drainage ditches. It is also used in many parts of the world as a broad-spectrum herbicide in urban and industrial areas, as an active ingredient (ai) in antifouling boat paints, and in algaecide formulations used in fountains and aquaculture. As a consequence, diuron has been detected in lakes, rivers, and groundwater (Eriksson et al. 2007; Green and Young 2006; Thurman, Bastian, and Mollhagen 2000), in marine waters and sediments (Thomas, McHugh, and Waldock

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2002), and in rain collected at urban and rural sites (Scheyer et al. 2007). Besides diuron itself, the main metabolites 3,4-dichloroaniline (3,4-DCA), *N*-(3,4-dichlorophenyl)urea (DCPU), and *N*-(3,4-dichlorophenyl)-*N*-methylurea (DCPMU) have been detected in natural waters (Claver et al. 2006; Green and Young 2006; Lapworth and Gooddy 2006; Thomas, McHugh, and Waldock 2002). These metabolites have been reported to be more harmful to nontarget organisms than diuron itself (Giacomazzi and Cochet 2004; Moncada 2004; Tixier et al. 2002). Diuron is a non-ionic compound with moderate water solubility of 22–42 mg L^{-1} at 20°C. Its hydrolysis rate is negligible at neutral pH but increases under strongly acidic or alkaline conditions (Spencer 1982). It is stable to oxidation and breakdown, persisting in soils for a full season or longer (Ashton 1982). It has a logarithmic octanol–water partition coefficient of 2.6, which is considered low to moderate. It is adsorbed to soils to some degree, with a moderate soil–water partition coefficient of 485 (Hamaker 1975). Diuron has the unfortunate combination of being both mobile and slowly degraded in the environment. The low tendency of diuron to sorb to soil, combined with its relatively high water solubility, is the likely explanation for its potential for leaching into underlying or nearby surface waters observed in several recent field experiments (Gooddy, Chilton, and Harrison 2002; Guzzella et al. 2006).

Several microorganisms that degrade diuron have been isolated (Walker and Cullington 1999; Widehem et al. 2000), but the degradation by these microorganisms resulted in the formation of the metabolite 3,4-DCA. Further degradation of 3,4-DCA by fungi was observed by Tixier et al. (2002) and Widehem et al. (2000) who isolated the *Arthrobacter* sp. N2 as a diuron-degrader from the soil. The isoproturon-degrading bacterium, *Sphingomonas* sp., was also able to mineralize diuron and chlorotoluron which contain a dimethylurea side chain such as isoproturon, but not linuron which contains a methoxy-methyl side chain (Sørensen, Christian, and Aamand 2001). Studies by Dellamatrice and Monteiro (2004) isolated a consortium of three bacteria, *Acinetobacter johnsonii* and two *Bacillus* spp., in a medium containing diuron as the only carbon source. Only *A. johnsonii* was able to grow alone in medium with diuron as the only carbon source. Bulcke et al. (2000) evaluated soil-applied diuron degradation in a pear orchard planted on loam soil after treatment with 3 kg ai ha^{-1}. In plots treated with diuron for 12 years, the soil half-life was 37 days. In other plots that had not been previously treated, the rate of diuron soil dissipation was 2.2 times lower, indicating moderately enhanced biodegradation in the previously treated plots. Bogaerts et al. (2000) studied the microbial degradation and ecotoxicology of diuron to investigate its fate after application to soils. Quantitative biodegradation assays were executed with fungal strains, showing that diuron was degraded but not entirely mineralized.

In Kenya, diuron is used in sugarcane farming, among other applications, under the trade name Kermex™. The herbicide has been in use for the last 15 years. The sugarcane farming region falls under a major catchment area of four major rivers that drain into Lake Victoria. Consequently, diuron residues and its metabolites have been detected in these rivers as well as in Lake Victoria.

Materials and methods

Chemicals

Diuron and its metabolites reference standards were purchased from Sigma Aldrich through Kobian Company (Nairobi, Kenya). HPLC grade solvents, $NaNO_3$, KH_2PO_4,

KCl, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, and $ZnSO_4$ were purchased from Kobian Company (Nairobi, Kenya) and used as obtained.

Soil

The soil samples were collected from the top 10-cm layer of sugarcane-cultivated fields 1120 and 9090 located at 34°34′00″ to 34°51′30″E and 0°23′00″ to 0°37′30″N; $F10_C$ located at 34°50′49″ to 35°35′41″E and 0°4′55″ to 0°20′11″N; F_1 and F_9 located at 34°49′07″ to 34°49′27″E and 0°02′10″ to 0°02′12″S. The soil texture ranged from clay, clay loam, and sandy loam to sandy clay loam; the soil pH ranged from 4.6 to 6.3. The nitrogen (%N) content was low, ranging from 0.05% to 0.22%. Carbon content ranged from 0.8% to 1.7%. The amount of phosphorus ranged from 2.2 to 8.2 mg kg^{-1} while that of sulfur ranged from 78 to 334 mg kg^{-1}.

Isolation of diuron-degrading bacteria by enrichment techniques

Mineral salt medium (MSM) for diuron liquid culture experiments was prepared by dissolving 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 mg each of $FeSO_4$ and $ZnSO_4$ in 1 L of de-ionized and distilled water (Dellamatrice and Monteiro 2004). The medium was sterilized by autoclaving at 121°C for 1 h. Aliquots of 200 µL of diuron from the stock solution (100 mg/10 mL of methanol) were transferred to sterile 100 mL Erlenmeyer flasks and the methanol was left to evaporate in a sterile flow bank. Aliquots of 49 mL of the sterile MSM were added to the flasks and stirred to dissolve the diuron resulting in a final concentration of 40 mg L^{-1} diuron. An aliquot of 1 g of soil was dissolved in 9 mL of 0.85% NaCl in de-ionized distilled sterile water and vortexed for 2 min. The mixture was diluted to 10^{-3} in 10-fold dilution steps. Dilutions of the soil samples (aliquot of 1 mL) were used for inoculating the 49 mL MSM with diuron as a substrate in 100 mL Erlenmeyer flask. The liquid cultures were incubated in a shaking water bath incubator (MRC laboratory equipment model BT-350) at 25 ± 1°C in the dark with shaking at 100 rpm. Aliquots of 3 mL were withdrawn periodically at a 2-day interval for the first 4 days, at 3–4-day interval for the next 26 days, and at 5-day interval thereafter to measure the optical density. Growth was determined by monitoring turbidity at optical density (OD_{600}) using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK). The same aliquot was used for HPLC analysis (LC-10AT VP Shimadzu). A plot of optical density and degradation expressed as % of diuron remaining against time was obtained.

Liquid cultures which showed enhanced degradation of diuron were further subjected to liquid culture enrichment experiments. Fresh diuron liquid culture medium was prepared and sterilized. Aliquots of 49 mL of MSM were transferred to sterile 100 mL flasks in a sterile hood and inoculated with 1 mL aliquots of the selected previous liquid cultures with enhanced diuron degradation capacity. A series of new liquid cultures were prepared from time to time to enrich the liquid cultures with diuron degraders by using inoculants from the previous liquid culture. A fully enriched mixed liquid culture was attained after the 5th enrichment (Mostafa and Heilling 2003). From the 5th enrichment of the mixed liquid culture, an aliquot of 100 µL was taken and diluted to 1 mL with fresh MSM. Serial dilutions were made up to the 3 rd dilution in sterile Eppendorf tubes. MSM with diuron as a substrate with the same composition as the one used for liquid culture experiments was prepared with 15 g of agar added to 1000 mL of the MSM solution and

then autoclaved. Aliquots of the medium were poured on the gar plates and left to cool. From the 1st and 3 rd dilutions, 100 µL aliquots were taken and spread on the agar plates in duplicates and the plates were incubated at 30°C. The individual colonies were separately streaked on another set of agar plates and incubated at 30°C. A total of 15 isolates were obtained from the MSM on the agar plate amended with 40 mg L^{-1} of diuron.

Degradation of diuron by the isolates

The 15 colonies were individually suspended as pellets in liquid culture medium amended with 40 mg L^{-1} diuron and incubated for 35 days. The MSM medium without inoculums with diuron as a substrate and an inoculated MSM medium without diuron as a substrate were used as controls. Turbidity and concentration of diuron and its metabolites DCA and DCPMU were periodically measured after every 3 days.

Analysis of diuron and metabolites

An aliquot of 3 mL withdrawn from the liquid cultures was centrifuged at 5000 rpm for 10 min at 25°C. The supernatant was extracted using agitation with 2 mL of ethyl acetate. This was repeated thrice followed by a final washing with ethyl acetate. The amount of diuron and its metabolites, DCA and DCPMU, were then determined using high-performance liquid chromatography (HPLC, LC-10AT VP Shimadzu) equipped with an UV-Visible detector (SPD-10 A VP Shimadzu) set at 254 nm. Separation was done isocratically on a reversed phase C-18 column 125 × 4 mm, 5 µm (Shimadzu) using acetonitrile : water (70 : 30) at a flow rate of 1 mL min^{-1} and an injection volume of 20 µL. Peak areas of standard solutions of the known concentrations of diuron and the metabolites were used for the preparation of standard curves. The curves were used to determine the concentration of diuron, DCA, and DCPMU in the liquid cultures and in the control.

Characterization of isolates

Colony morphology was determined using dissecting microscope. Biochemical tests included tests on the ability to utilize the following compounds as the sole source of carbon: glucose, arabinose, sucrose, lactose, D-mannitol, D-galactose, D-mannose, dextrose, and lactose. Other biochemical tests included nitrate reduction, citrate utilization, catalase test, H_2S production, indole test, motility test, and hydrolysis of urea (Cappuccino and Sherman 2002). The identity of the isolates was further confirmed through molecular characterization. Total bacterial DNA was extracted according to procedures described by Schmidt, DeLong, and Pace (1991), purified, and used as a template for the amplification of 16 S rRNA gene. Total DNA samples extracted from the isolates were detected using gel electrophoresis. Gel documentation was done using the Gel Logic 200 Imaging System (Sambrook, Fritsch, and Maniatis 1989). The 16 S rRNA gene sequences were PCR-amplified using bacterial primer pair 27 F forward 5′-GAG TTT G(AC)T CCT GGC TCA G-3′ and 1492 R reverse, 5′-TAC GG(CT) TAC CTT GTT ACG ACT T-3′ (Eurofins MWG GmbH). Amplification was performed using a model 9700 Fast Thermal Cycler from Applied Biosystems. PCR products were digested with MspI restriction enzyme (Promega Corporation Madison, USA). The PCR amplicons

from the isolates were excised from the gel and purified using quickClean 5 M gel Extraction kit (GenScript Corporation, 120 Centennial Ave, Piscataway, NJ 08854) according to the manufacturer's instructions. The sequencing of the 16 S rRNA genes was done at BECA (Biosciences Eastern and Central Africa) in ILRI (International Livestock Research Institute). Sequencing was conducted with BigDye^{TM} Terminator technology. The sequencing products were run on ABI 3130 Genetic Analyzers. The forward and backward 16 S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas software package (www.technelysium.com.au). They were then aligned using Bioedit sequence alignment editor software package (Hall 1999) to provide full sequences of about 1500 nucleotide bases. The sequences were compared with sequences in the public databases with the BLAST search program on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16 S rRNA gene sequences. The ARB database software package (Ludwig and Strunk 1996) was used to align and identify the closely related bacterial 16 S rRNA gene sequences. The 16 S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce Phylogenetic tree using MEGA software package (www.megasoftware.net).

Results and discussion

The liquid enrichment cultures inoculated with soils from different fields degraded diuron to different extents whereby the degradation was directly proportional to the growth of the strains in the cultures with average lag phases of 2–3 days. The liquid enrichment culture inoculated with soil from field $F10_C$ degraded diuron up to 74% of the initially applied concentration of 40 mg L^{-1}. In all cases, diuron degradation was quantifiable (4–6% of applied concentration) after 4 days in enrichment cultures inoculated with soils from fields F1, 9090, F_9, and 1120 degrading diuron by 68%, 58%, 72%, and 64% of the initially applied concentration. Degradation rates of diuron in the different liquid culture media varied from 0.63 to 0.71 mg L^{-1} per day. The medium inoculated with soil from field F_{10C} reached its highest cell mass of 0.26 at OD_{600} on the 37th day of incubation at a degradation rate of 0.79 mg L^{-1} per day. This was similar to that of liquid culture media inoculated with soils from fields F_9 and F_1 in which cell mass density of 0.25 at OD_{600} was attained at a degradation rate of 0.71 mg L^{-1} per day. Enrichment cultures of soil from fields 9090 and 1120 reached their highest cell mass of 0.21 at OD_{600} after 42 and 40 days incubation period, respectively. The degradation rate was 0.63 and 0.67 mg L^{-1} per day, respectively (Figure 1).

A total of 15 strains were isolated on MSM agar plates and transferred to liquid culture to test their diuron degrading capacities. Four strains, ISL 2, ISL 3, ISL 4, and ISL 6 were individually able to degrade diuron. The amount of diuron degradation was quantifiable (2–3% of applied concentration) after the fourth day of incubation for all the isolates. There was no much variation in the degradation of diuron by the individual isolates, except for the combination of ISL 3 and ISL 4 whereby there was an enhanced diuron degradation of 30.2%. Isolate ISL 2 which was obtained from soils in field 1120 degraded diuron by 21% of the initial concentration (Figure 2) over a period of 37 days with a degradation rate of 0.25 μg d^{-1} compared to 63.8% diuron degradation for the community in the mixed liquid culture before isolation. From the herbicide residue analysis of diuron and its metabolites, the soils in field 1120 had 0.6 μg g^{-1} diuron and 1.84 μg g^{-1} DCMPU residues. Diuron degradation by ISL 2 resulted in the formation of 0.7 % DCA, 7.4%

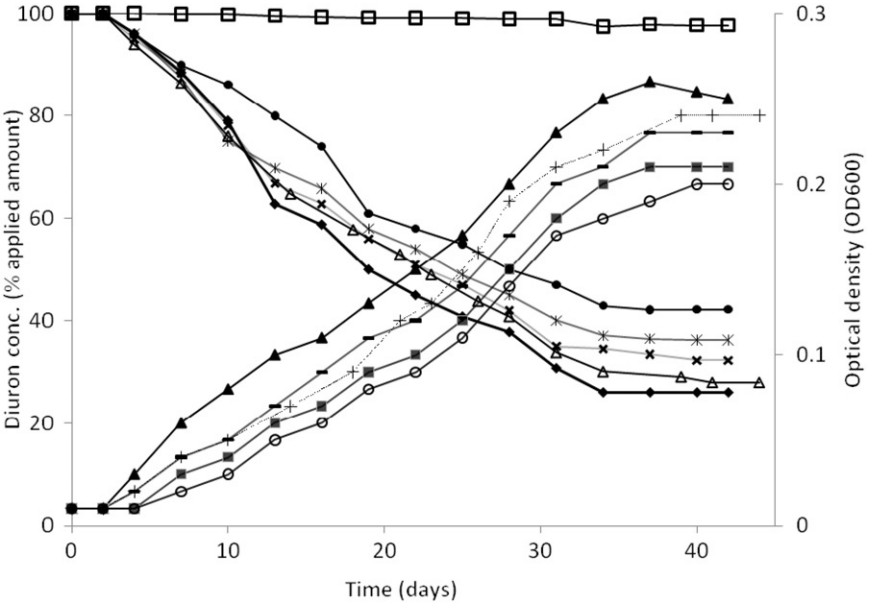

Figure 1. Bacterial growth and diuron degradation curves in liquid cultures inoculated with soils from fields F_{10C}, F_9, F_1, 1120, and 9090. The symbols represent: control, diuron degradation, and growth curve for enrichment cultures from sample $F10_C$; diuron degradation and growth curve for enrichment cultures from sample F_9; diuron degradation and growth curve for enrichment cultures from sample F_1; diuron degradation and growth curve for enrichment cultures from sample 1120; diuron degradation and growth curve for enrichment cultures from sample 9090.

DCPMU, and 12% of an unidentified metabolite at Rt. 0.98. ISL 3 degraded diuron up to 24.8% of the applied amount over a period of 36 days (Figure 2) with a 0.31 $\mu g\,d^{-1}$ degradation rate. This isolate was obtained from the liquid enrichment culture inoculated with soil from field F_9 that had diuron herbicide residues of 1.5 $\mu g\,g^{-1}$, and DCPMU metabolite of 3.3 $\mu g\,g^{-1}$. From the degradation of diuron by ISL 3, 0.6% DCA, 11% DCPMU, and two unidentified metabolites at Rt. 0.98 and 1.6 constituting 0.5% and 12%, respectively, were formed. Isolate ISL 4 that was obtained from the liquid enrichment culture inoculated with soil from field F_1 degraded diuron by 22% of the applied amount (Figure 3) over a period of 36 days with a degradation rate of 0.26 $\mu g\,d^{-1}$. The isolate degraded diuron into its metabolites, DCPMU 11.3%, and two unidentified metabolites at Rt. 0.98 and 1.6 with 10% and 0.35 %, respectively. No DCA was detected upon herbicide residue analysis of the supernatants of the isolate cultures. Soil from field F_1 had DCPMU residues of 5.29 $\mu g\,g^{-1}$ and no diuron residues had been detected upon herbicide residue analysis. Isolate ISL 6 degraded diuron by 18.8% of the applied amount over a period of 36 days with a degradation rate of 0.24 $\mu g\,d^{-1}$ (Figure 2). This isolate had been obtained from the liquid culture medium inoculated with soil from field 9090. Diuron and DCPMU residues were detected in the soils at concentrations of 2.5 and 3.7 $mg\,kg^{-1}$, respectively. Diuron degradation by ISL 6 resulted in the formation of DCA (0.8%), DCPMU (8.9%), and two unidentified metabolites at Rt. 1.6 and 0.96 constituting 0.4 and 8%, respectively.

The combination of the isolates ISL 3 and ISL 4 resulted in an increase in diuron degradation. Diuron was degraded up to 30% of the applied amount (Figure 3) over a

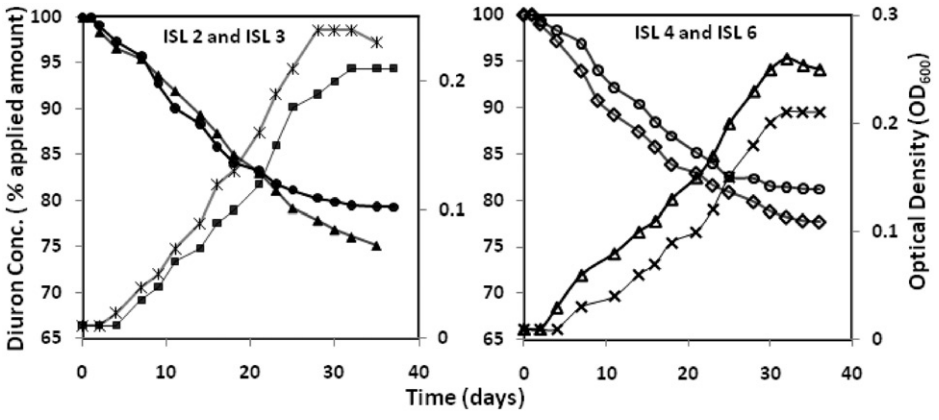

Figure 2. Bacterial growth and diuron degradation curves in liquid cultures by four isolates ISL 2, ISL 3, ISL 4, and ISL 6. The symbols represent: degradation and growth curve for ISL 2; degradation and growth curve for ISL 3; degradation and growth curve for ISL 4; degradation and growth curve for ISL 6.

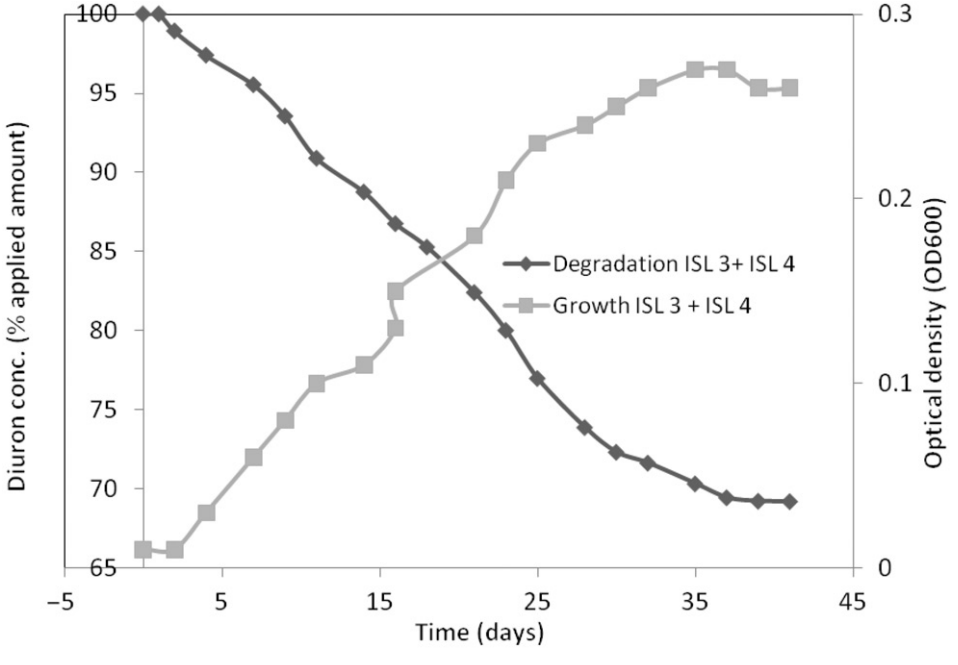

Figure 3. Bacterial growth and diuron degradation curves in liquid culture with combined isolates ISL 3 and ISL 4.

period of 41 days. Only two metabolites were detected, DCA (0.1%) and an unidentified metabolite (29%) at R_t of 0.97.

All the isolates utilized sucrose, glucose, fructose, and dextrose. ISL 4 utilized all the tested sugars. ISL 2 could not utilize galactose, ISL 3 could not utilize arabinose, whereas ISL 6 could not utilize mannose (Table 1).

Table 1. Utilization of sugars by isolates ISL 2, ISL 3, ISL 4, and ISL 6.

	Isolates			
Metabolic versatility tests	ISL 2	ISL 3	ISL 4	ISL 6
Lactose	+	+	+	+
Sucrose	+	+	+	+
Glucose	+	+	+	+
Mannose	+	+	+	−
Arabinose	+	−	+	+
D-Mannitol	+	+	+	+
D-Galactose	-	+	+	+
Maltose	+	+	+	+
Fructose	+	+	+	+
Dextrose	+	+	+	+

Note: + positive reaction, − negative reaction.

The ability of the isolate to excrete intracellular enzymes was tested through hydrogen sulphide production, nitrate reduction, catalase reactions, urease, methyl red, voges-proskauer, and citrate utilization. All the isolates in diuron degradation were motile and showed positive results in citrate utilization. All the four isolates gave negative results in VP, MR, indole tests, and H_2S production (Table 2).

The isolated strains were further characterized by molecular techniques through comparative sequence analysis of the 16 S rRNA coding genes. Phylogenetic analysis showed that isolate ISL 2 was a member of the genus *Bacillus*. This was supported by the clustering pattern on the phylogenetic tree which indicated that the isolate clustered with *Bacillus*. This clustering pattern was supported by high bootstrap values of between 63% and 99% (Figure 4). The identity was further confirmed after blasting the results which showed that the isolate had 16 S rRNA gene sequence similarity of 95% to *Bacillus cereus* DQ989214. Similarly, the phylogenetic analysis of 16 S rRNA gene sequences of isolate ISL 6 showed that the isolate clustered with the genus *Bacillus*. This clustering was supported by high bootstrap values of between 63% and 100%. The isolate had 16 S rRNA gene sequence similarity of 97% to *Bacillus* spp1 FJ899758 and 97% to *Bacillus pumilus* FJ263042. *Bacillus cereus* is Gram-positive, facultative aerobic spore-former whose cells are large rods in short to long chains and whose spores do not swell the sporangium. They are widely distributed in the soil, dust, and air. The isolated *B. cereus* strain and *Bacillus* spp1 could individually grow in a medium containing diuron as the substrate. In a study by Dellamatrice and Monteiro (2004), a consortium of three bacteria, *Acinetobacter johnsonii* and two *Bacillus* spp. was isolated in the medium containing diuron as the only carbon source This study reports the capability of *Bacillus* sp. to degrade diuron in the Kenyan soils under tropical conditions for the first time. Moreover, *Bacillus* sp. have been reported as capable of degrading other pesticides such as mesotrione, a triketone herbicide, (Batisson et al. 2009) and DDT (Mwangi et al. 2009).

Phylogenetic analysis showed that ISL 3 was a member of the genus *Vagococcus*. This was supported by the clustering pattern on the phylogenetic tree (Figure 4) which indicated that the isolate clustered with *Vagococcus*. This clustering pattern was supported by high bootstrap values of between 88% and 100%. ISL 3 had 16 S rRNA gene sequence similarity of 95% to *Vagococcus fluvialis* GQ337040 and a similarity of 95% to an

Table 2. Biochemical characterization of isolates from diuron degradation.

	Isolates			
Biochemical tests	ISL 2	ISL 3	ISL 4	ISL6
Cell type shape	rod	Cocci	rod	Rod
Gram stain	+	+	−	−
Colony color	Cream white	Cream white	Cream white	Cream white
Characteristics shape	Round	Circular entire margin	Round	Round
Elevation	Raised	Raised	Raised	Raised
surface	Smooth	Smooth	Smooth	Smooth
Citrate utilization	+	+	+	+
MR test	−	−	−	−
VP test	−	+	−	−
Urease test	−	−	−	−
Nitrate reduction	+	−	+	−
Motility at 37°C	+	+	+	+
Starch hydrolysis	−	−	−	−
H_2S production	−	−	−	−
Casein hydrolysis	+	−	+	+
Catalyse test	+	−	+	+
Oxidase	+	−	+	−
Indole	−	−	−	−

Note: + positive reaction, − negative reaction.

Uncultured Enterococcus, AY080979. *Vagococcus fluvialis* sp. is a species of Gram-positive catalase-negative cocci, related to the genera *Enterococcus* and *Carnobacterium* (Pot et al. 1994). The phylogenetic analysis of 16 S rRNA gene sequences of isolate ISL 4 showed that the isolate clustered with the genus *Burkholderia*. This clustering was supported by high bootstrap values of between 68% and 100%. The isolate had 16 S rRNA gene sequence similarity of 93% to *Burkholderia ambifaria* CP000440. The genus *Burkholderia* consists of some 35 bacterial species, most of which are soil saprophytes and phytopathogens that occupy a wide range of environmental niches. Several of these species, referred to as the *Burkholderia cepacia complex* (Bcc), are characterized by unusually high inter-species DNA–DNA hybridization values. *Burkholderia ambifaria* is generally found as the dominant *B. cepacia* complex species in natural environments where it is often associated with plant roots (Coenye et al. 2001).

In the current study, *B. cereus*, *V. fluvialis*, *B. ambifaria*, and a *Bacillus* spp. isolated from soils with a history of diuron applications degraded diuron as indicated by the presence of its metabolites DCA and DCPMU. In previous studies, enhanced degradation was observed in agricultural soil samples previously exposed to diuron, indicating that microbial adaptation to diuron degradation was possible in soil (Dellamatrice and Monteiro 2004; Piutti et al. 2003; Rouchaud et al. 2000). Several diuron-degrading bacteria have been isolated from different agricultural soils (Dellamatrice and Monteiro 2004; El-Deeb et al. 2000; Rouchaud et al. 2000; Sørensen, Ronen, and Aamand 2001) and river waters (Batisson et al. 2007). Furthermore, it has been shown that different fungi either obtained from various culture collections or freshly isolated from agricultural soils are capable of degrading diuron (Castillo et al. 2006; Fratila-Apachitei et al. 1999; Khadrani et al. 1999; Weinberger and Bollag, 1972). However, majority of the isolated strains degraded diuron to potentially harmful metabolites, such as 3,4-DCA. A study by

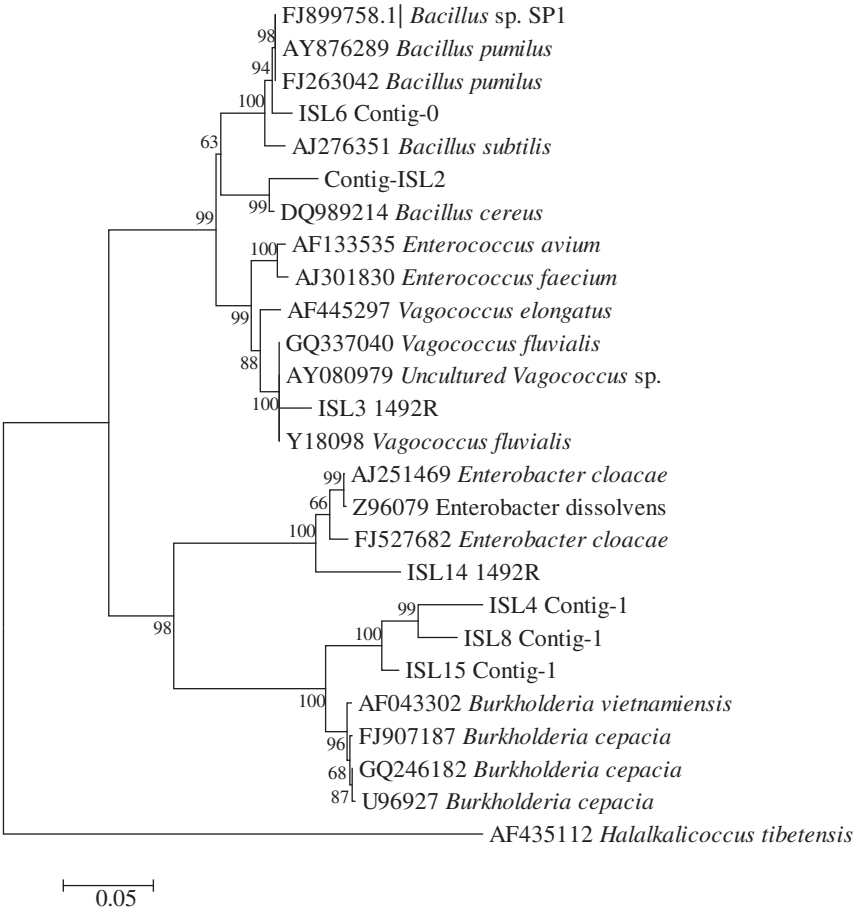

Figure 4. The scale bar indicates approximately 5% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The out group 16S rRNA gene sequencing of *Halalkalicoccus tibetensis* AF435112 was used as an out group.

Sørensen, Christian, and Aamand (2008) identified the first known bacterium, *Variovorax* sp., capable of mineralizing diuron and representatives of both the *N*,*N*-dimethyl- and *N*-methoxy-*N*-methyl-substituted phenylurea herbicides in a pure culture when supplemented with appropriate growth substrates.

In this study, the transformation by pure isolates was very low (18–25%) and slow at an average degradation rate of 0.33 μg diuron per day over a period of 35 days compared with the rate of diuron degradation by the communities before the isolation of pure bacterial strains from the liquid enrichment cultures. The communities degraded diuron up to 54–74% of applied concentration with an average rate of 0.71 μg diuron per day over a 42 days period. Complete mineralization by the isolated strains was not verified. The combination of *V. fluvialis* and *B. ambifaria* showed an increased degradation of diuron up to 30.2% of the applied amount, which could be a synergistic effect of the two strains. This clearly suggests that the complete degradation of diuron in these soils is carried out by a consortium and that none of the isolated strains is able to completely mineralize diuron. The combination of the strains *V. fluvialis* and *B. ambifaria* modified

the metabolites formation as exemplified by the detection of only two metabolites, DCA and unidentified metabolite at Rt. 0.97.

The transformation of diuron to DCPMU by *B. cereus*, and formation of a small amount of DCA suggest that the route to its degradation is not the direct conversion to DCA which is more toxic than diuron. Similarly, the transformation of diuron to its metabolites (DCPMU 11.3% and two unidentified metabolites) by *B. ambifaria* indicated that the route to its degradation was not the direct formation of DCA, as no DCA was detected in the supernatant.

Conclusion

This study showed that there are microorganisms that have adapted to diuron in tropical soils after repeated exposure to the herbicide. Moreover, the enhanced degradation by the enrichment cultures suggests that a consortium can be obtained in diuron degradation, or even a single efficient degrader may be isolated in these soils.

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