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Isolation of Diuron-degrading Bacteria from Treated Soil

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ABSTRACT

Studies were carried out on the isolation of diuron-degrading bacteria from treated soil. The mineralization of ^{14}C-diuron in soil following a three-year application and in soil without previous application were 68.95 µg.100 g^{-1} and 24.16 µg.100 g^{-1}, respectively, after a 64-day incubation period. In the first soil there was a significant increase in the number of bacteria, from 3.3 x 10^{6} to 1.9 x 10^{8}. The microbial biomass did not change, however, a significant ^{14}C-residue of diuron was found in the microbial biomass. A consortium of three bacteria, Acinetobacter johnsonii and two Bacillus spp., was isolated in 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.

Key words: Biodegradation, biomass, enhanced degradation, diuron, *Acinetobacter*, bioremediation

INTRODUCTION

Studies conducted by Cullington and Walker (1999) and Rouchaud et al. (2000) revealed that repeated applications favored the degradation of diuron in soil. According to Dalton et al. (1966), the degradation of diuron begins with the removal of N-methyl groups. Loss of the first methyl group reduces the herbicidal activity to half that of diuron, and loss of the second methyl group eliminates herbicidal activity. Decomposition is followed by the removal of the urea group, which results in the formation of 3,4-dichloroaniline, amonia and CO_2**.** Musumeci et al. (1995) reported 3,4-dichlorophenylmethylurea and 3,4-dichlorophenylurea, as products of degradation of diuron formed by hydrolysis of one or two methyl groups from the urea group.

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 3,4-dichloroaniline. Further degradation of 3,4-dichloroaniline by fungi was observed by Widehem et al. (2000) and Tixier et al. (2002) who isolated from soil the *Arthrobacter* sp. N2 as a diuron-degrader. 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 (Sorensen et al., 2001).

Acinetobacter strains with the ability to metabolise glyphosate as well as aminomethylphosphonate (Chung et al., 1996), diuron (Roque et al., 1998) and diclofop-methyl (Smithgrenier et al., 1996), when added in mineral medium as the only carbon source, have been isolated from heavily polluted streams. *Acinetobacter* has also been found to degrade several other products like PCBs (Brunner et al., 1985; Furukawa et al., 1983) and phenols (Martins et al., 1997; Hoyle et al., 1995).

The objective of this study was to evaluate the mineralization of ^{14}C-diuron in a Brazilian soil

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with and without any application, and to isolate the microorganisms responsible for that.

MATERIAL AND METHODS

Chemicals

^{14}C-U-ring diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], which had 96% radiochemical purity and 2.43 MBq.mg^{-1} activity, and nonradioactive diuron of 96.4% purity were used.

Soil

The soil samples were collected from the top 10 cm of a site cultivated with citrus with a three-year history of diuron use and from a nearby site that had never been treated with diuron, where weeds were controlled mechanically. Samples were collected in September 1998, from the Horticulture farm of ESALQ, Piracicaba, SP, Brazil. The soil had 78% sand, 16% clay, 18 g.dm^3 organic matter and a pH of 4.3. The soils were sieved (2 mm) and the moisture was maintained at 75% of field capacity.

Soil incubation

The labelled diuron was dissolved in acetone together with nonlabelled diuron to give a concentration of 4 μg.g^{-1} and a radioactivity of 51 kBq.100^{-1} g. Only one diuron application was made and the radioactivity was measured after the application by combustion of the soil samples. Samples of 100 g of soil without history of diuron application (control) and with three-year history of application were placed in 300 ml Erlenmeyer flasks, in four repetitions. The doses were determined by the specific activity and by HPLC analysis of the soil extracts. The $^{14}CO_2$ was measured using the Anderson method (Anderson, 1990) at 7, 14, 21, 28, 42 and 64 days.

Microbial Evaluation

The number and microbial biomass were determined at zero time and after 64 days of incubation. Bacteria, fungi and actinomycetes were counted by the spread-plate technique. The media used were nutrient agar, Martin agar and peptone medium. The soil microbial biomass was determined using a fumigation-extraction technique (Vance et al., 1996). For ^{14}C-constituents measurements, aliquots of soil extracts were analysed using a Liquid Scintillation Spectrometer (LSS) (Packard Tri-Carb 1.600 TR).

Residues determination

Soil samples of 25 g were extracted using agitation with 25 ml of 50% dichloromethane (v/v) for 30 min at 120 rev.min^{-1}. The liquid phase was separated by centrifugation at 13000 rev.min^{-1}. The extraction was repeated three times and the radioactivity of the combined supernatant was measured in LSS (Hassink et al., 1994).The soil residues were air-dried overnight and aliquots of 1 g were analysed for residual radioactivity by combustion analysis, using five repetitions for each treatment, in a Biological Oxidiser (OX500 Harvey Instruments), and the radioactivity was measured by LSS.

Metabolites

The metabolites were determined by TLC. Dichloromethane solution extracted from soil for residue analyses was applied in Silica Gel plates $60F_{254}$ (Merck) that were developed using dichloromethane/methanol (95:5). The radioactivity was measured using a Berthold TLC analyser.

Isolation of diuron-degraders

The soil with history of diuron application (10g) was suspended in 90 ml of minimal medium ($NaNO_3$ 6 g, KH_2PO_4 1.5 g, KC1.0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4$ and $ZnSO_4$ 0.0001 g, 1000 ml H_2O, pH 6.8). After stirring during 15 min, an aliquot (1 ml) was inoculated into 125 ml Erlenmeyer flasks containing 20 ml of the same medium with diuron at a concentration of 4 μg ml^{-1} as the only carbon source. After incubating for 15 days at 27º C, aliquots of 1 ml were transferred to fresh medium. The cultures were transferred four times over a two-month period. The microorganisms were isolated after vortexing in Tween 80 solution 0.1% and streaking in solid medium. Gram reaction and morphology were determined. The purified strains were tested in medium with diuron or 3,4-dichloroaniline as the sole carbon source. The isolated strains were identified through philogenetic analysis of the rDNA 16S sequences, by Fundação André Tosello, Campinas, SP, Brazil.

RESULTS AND DISCUSSION

Diuron degradation was low in the sample without diuron application, however in the sample with three-year diuron application there was an increase, about seven times, in the degradation of diuron. A lag phase occurred indicating that a period of adaptation was required (Fig 1). Soil samples without diuron application mineralised 15.41 µg.100 g^{-1} after 28 days and 24.16 µg.100 g^{-1} after 64 days of incubation. Soil with diuron application mineralised 36.63 µg.100 g^{-1} and 68.95 µg.100 g^{-1} of the total amount of diuron over the same period of time. The mineralization half-life of diuron was calculated to be 444 days in soil without application and 77 days for soil with application.

After incubating for 64 days, the soil samples without diuron application (control) showed small variation in the number of bacteria, but the number of fungi increased (Table 1). The microbial biomass was the same after the treatment (Table 2). In the soil with diuron application, there was a significant increase in the number of bacteria, from 3.3 x 10^6 to 1.9 x 10^8 after the incubation period (Table 1), showing that this group might be the responsible for degradation of diuron in this soil. The microbial biomass also did not change after 64 days (Table 2), but significant ^{14}C-residue of diuron was found in the microbial biomass, which might have a role in metabolism and growth. These results are in accordance with the $^{14}CO_2$ production, confirming increase in the mineralization of diuron in the sample with diuron application.

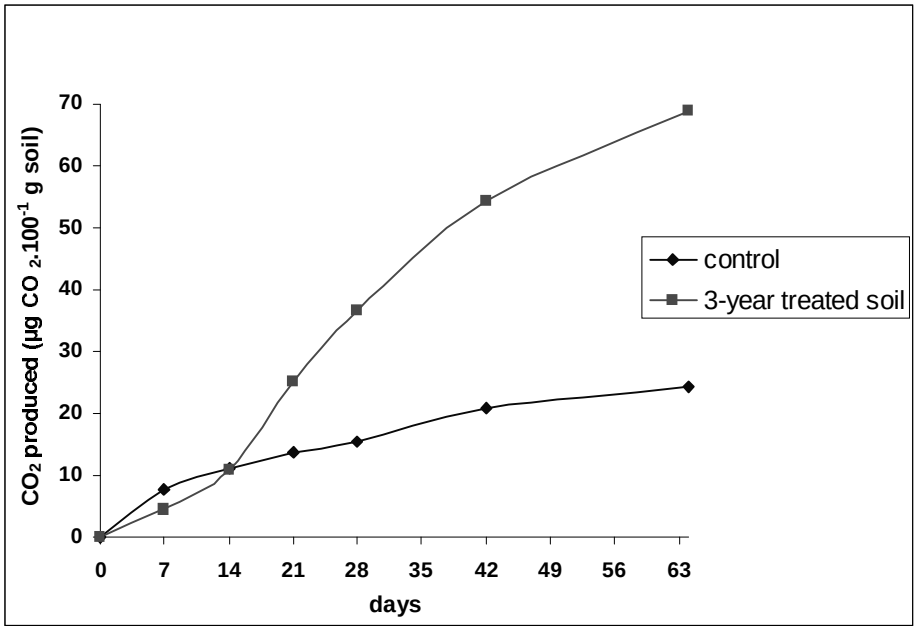

Figure 1 - Production of $^{14}CO_2$ from soil. Control are soil samples with no-history compared with soil samples with three years of diuron-history

Table 1 - Number of microorganisms (UFC. g^{-1}) for zero and 64 days of incubation

Treatment	UFC. g^{-1} soil		
	Fungi (x 10^3)	Bacteria (x 10^6)	Actinomycetes(x 10^5)
	Day 0		
Control	10	10	15
Soil treated with diuron	9	3.3	10
	Day 64		
Control	39	8.5	8.1
Soil treated with diuron	66	190	15

Table 2 - Microbial Biomass and ^{14}C-residues of diuron incorporated to microbial biomass for zero and after 64 days of incubation

Treatment	Biomass-C total	^{14}C-residues incorporated
	μg .g^{-1} soil	μg.100 g^{-1} soil
	Day 0	
Control	153.08 a	-
Soil treated with diuron	167.63 a	-
	Day 64	
Control	166.81 a	2.34
Soil treated with diuron	162.05 a	17.52

a: values within the same column followed by the same letter are not different at the 5% level by Tukey's Test

The extractable ^{14}C-residues were higher than the unextractable residues in soil without diuron application. In the soil with diuron application, the extractable residues were lower than the unextractable residues showing that the extractable residues were the preferential fraction degraded. These results also showed that the improvements in diuron mineralization were due mainly to the presence of microorganism adapted to diuron.

Only one metabolite was found for both treatments. The values for Rfs were 0.33 for the metabolite and 0.53 for the diuron. The metabolite Rf was different from the standard Rf of 3,4-dichloroaniline. The soil sample without diuron application had higher amounts of this metabolite (25.69%) when compared to the sample with diuron (9.97%), which indicated that although the mineralization have been 3.93% in this soil, 25.69% of diuron was degraded to this unknown metabolite. This suggests that diuron in this sample was degraded by the action of more than one specie of soil microorganism.

A consortium of three kinds of bacteria were isolated in medium containing diuron as the only carbon source. One strain was a gram-negative cocci (strain 1) and the others were gram-positive rods. The rods were separated based on colony morphology: one colony was white opaque with expansive growth that formed branches (strain 2) and the other was white hyaline (strain 3). Strain 1 formed fluorescent blue colony.

Only strain 1 was able to grow alone in the medium containing diuron as the sole carbon source. The others strains were not able to grow without the presence of strain 1, thus suggesting some kind of association, possibly symbiotic. All three bacteria were not able to grow in medium with 3,4-dichloaniline as the only carbon source, thus showing that this metabolite was not part of the pathway of diuron degradation for these bacteria.

Strain 1 was identified as *A. johnsonii*, strain 2 as *Bacilus amyloliquefaciens* or *B. vallismortis* (these two strains could not be differentiated by the 16S rDNA sequence), and the strain 3 as *B. subtilis*.

CONCLUSIONS

The mineralization of diuron was higher in a soil with treatment (enhanced degradation) than in soil without any application. Only one bacterium isolated from this soil, *A. johnsonii*, was capable of using diuron as the sole carbon source. The extractable fraction underwent most of the degradation. The metabolite 3,4-dichloroaniline was not pathway of diuron degradation by this microorganism.

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RESUMO

A degradação do herbicida diuron foi estudada em solo com e sem histórico de aplicação obtendo-se aproximadamente degradação sete vezes maior no solo com histórico de aplicação, após 64 dias de incubação. Houve um aumento no número de bactérias no solo com histórico de aplicação de 3.3 x 10 ^{6} para 1.9 x 10^{8} UFC. g^{-1} solo. Não houve aumento na biomassa após a incubação, porém foi

encontrado significante resíduo de ^{14}C-diuron na biomassa. Um consórcio de três bactérias foi isolado, *Acinetobacter johnsonii* e duas espécies de *Bacillus* sp., em meio contendo diuron como única fonte de carbono. Somente *A. johnsonnii* foi capaz de crescer sozinha em meio contendo diuron como única fonte de carbono.

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