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Degradation of Iprodione by a Soil *Arthrobacter*-Like Strain

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A bacterial strain able to transform iprodione was isolated from a fast iprodione-degrading soil by enrichment procedures. Transformation was detected through 3,5-dichloroaniline production as measured by a rapid colorimetric method. The strain, MA6, was tentatively identified as an *Arthrobacter* sp. When it was incubated with MA6 in a minimum mineral medium (pH 6.5), iprodione (8.8 μmol/liter) was transformed into two major metabolites that were identified by high-performance liquid chromatography analysis: 3,5-dichlorophenylcarboximide (metabolite 1) and (3,5-dichlorophenylurea)acetic acid (metabolite 2), which was produced after ring cleavage of the former product. These products were synthesized in the laboratory and compared with metabolites 1 and 2 which were formed during iprodione degradation. Small quantities of 3,5-dichloroaniline also appeared in the bacterial culture but did not substantially increase between the first and second days of incubation. In contrast, in the sterile control medium, iprodione was spontaneously transformed into hydantoic acid and an iprodione isomer. Chemical and biological transformations of iprodione seem to occur through two different pathways. One biological degradation pathway is proposed.

Agronomists periodically observe the failure of soil-applied pesticides to control targeted pests and disease. Pesticide failure could be due to acquired resistance of the organisms; however, in many cases it is known to be the result of a highly limited persistence of the active ingredient (13).

Except for some recalcitrant, insoluble chlorinated hydrocarbons (1), almost every pesticide is more or less rapidly degraded after it has been incorporated into the soil. Physicochemical reactions and microbial effects are involved. When microbial degradation is the main process, repeated applications of a chemical to the soil frequently lead to enrichment of the degrading populations, which is followed by accelerated disappearance of the chemical. The first description of microbial enrichment in response to pesticide treatments was reported by Audus (2) for 2,4-dichlorophenoxyacetic acid. We are now concerned with herbicides, insecticides, and fungicides currently used in crop protection (15).

The cyclic imide fungicides iprodione [3-(3,5-dichlorophenyl)*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] and vinclozolin have been used against several soil-borne pathogens. They provide very effective control of lettuce collar rot (*Sclerotinia minor* Jagger) and onion white rot (*Sclerotium cepivorum* Berk). However, an enhanced degradation of both fungicides has been observed in France and in the United Kingdom (9, 18) and more recently in New Zealand (14). Field studies and laboratory data have confirmed that the phenomenon is due to enhanced microbial degradation of the fungicides (11). *Pseudomonas* and *Bacillus* strains utilizing vinclozolin as the sole source of carbon and energy were isolated from Russian soils; however, iprodione was not studied (6), and only unstable mixed enrichment cultures were isolated as microbial agents of iprodione degradation (7).

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Walker (17) demonstrated that 3,5-dichloroaniline (3,5-D) was a degradation product of iprodione and vinclozolin and appeared concomitantly with the disappearance of these fungicides. He described a test, based on colorimetric characterization of 3,5-D, for identifying soils in which rapid degradation of iprodione and vinclozolin occurs. It is possible to investigate great numbers of samples with this test. Thirty-three soils from England were examined by Walker (17), and 46 soils from Southern France were examined by Martin et al. (10), confirming the relationship between accelerated biodegradation and intensity of 3,5-D formation. We used Walker's test for the isolation of a bacterial strain able to degrade iprodione. We studied the ability of this strain to transform iprodione without additional C and N sources in the incubation medium. Some metabolites were isolated and identified by comparison with synthesized compounds. On the basis of these results, an iprodione metabolism pathway in this isolate is proposed.

MATERIALS AND METHODS

Chemicals. Iprodione and its isomer isopropyl-3-*N*-(3,5-dichlorophenyl)2,4-dioxoimidazolidine-1-carboxamide (compound I) were gifts from Rhône-Poulenc Agrochimie (Lyon, France). 3,5-D was purchased from Aldrich Chemical (St. Quentin Fallavier, France). 3-Isopropyl carbamoyl-(3,5-dichlorophenyl)-5-hydantoic acid (compound II) was obtained by chemical synthesis according to the method described by Belafdal et al. (3).

Media used for bacterial isolation. The mineral salts solution had the following composition in grams per liter: $MgSO_4 \cdot 7H_2O$, 0.2; and K_2HPO_4, 0.5. The pH was adjusted to 6.5 by the addition of 0.1 N HCl (iprodione is more stable in acidic solution), and the medium was autoclaved at 121°C for 20 min. The iprodione mineral medium (IMM) contained base mineral salts solution, supplemented with biotin (150 μg/liter), which was sterilized by filtration (Sartorius Minisart NML; pore size, 0.2 μm), and iprodione (50 mg/liter), which was solubilized in acetone and sterilized by filtration (FG 0.2-μm-pore-size filter; Millipore). Three different agar (15 g of Noble agar per liter; Difco) media were used: (i) IMM; (ii) mannitol-rich medium (0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 1 g of mannitol, 150 μg of biotin, 50 mg of iprodione per liter [pH 6.5]); and (iii) soil extract medium (12) (1 liter; 150 μg of biotin per liter and 50 mg of iprodione per liter [pH 6.5]) Filtration-sterilized biotin and iprodione were added to the autoclaved medium.

Enrichment procedures, isolation, and bacterial characterization. Soil sam-

ples were collected in 1990 from the top 20 cm of a 24-m^2 loamy sand field at the Ecole Nationale Supérieure Agronomique in Montpellier, France. The organic matter content of the field was 2% (pH 6.8). This soil, with no history of iprodione treatment, was used for isolation of iprodione-degrading bacteria by the following enrichment procedure. A 100-ml volume of sterile water containing 0.25 mg of iprodione (commercial formulation, Rovral; Rhône-Poulenc) was added to 25 g of fresh soil (20% moisture) in a 120-ml flask. The flask was then shaken and incubated at 28°C for 14 days. Every 2 days, 1 ml containing 0.25 mg of iprodione was added. The soil suspension was then diluted 10-fold from 10^{-1} to 10^{-6}. One milliliter of each diluted soil suspension was transferred to a tube containing 9 ml of IMM in which iprodione was the sole source of carbon and nitrogen. Soil suspension tubes were incubated at 28°C and after 4 days were tested for the presence of 3,5-D by the colorimetric method on the basis of the production of a diazo color complex, which has been described previously (17). One milliliter of each positive tube (i.e., positive for the presence of 3,5-D) was transferred into a new liquid IMM tube. This procedure was repeated seven times. Then, 0.1 ml from each tube from 10^{-1} to 10^{-6} issued from the seventh enrichment procedure was plated in duplicate on the three different agar media described above. Petri dishes were incubated at 28°C. After 4 days, 100 colonies were randomly removed from each of the three media and transferred into tubes containing IMM, which were tested for their capacity to degrade iprodione. Ten iprodione-degrading isolates were obtained, and one was selected for further study. This strain, which was designated MA6, was characterized by standard procedures (5, 8) and by fatty acid analysis (4). The cultures were maintained at 4°C on IMM agar.

Iprodione chemical transformation study. To monitor the chemical transformation of iprodione, we distributed the basal medium supplemented with 150 μg of biotin per liter and iprodione (30 μmol/liter), sterilized by filtration on 0.2-μm-pore-size filters, by 0.5-ml parts into 10 1.5-ml sterile tubes. The tubes were then incubated at 28°C with shaking. The tubes were periodically sampled, and 0.5 ml of acetonitrile was added. The analysis was performed by high-performance liquid chromatography (HPLC).

Fungicide and metabolite degradation study. One milliliter of a bacterial culture suspension of MA6 grown on IMM was inoculated into a sterile Erlenmeyer flask (100 ml) containing 19 ml of basal mineral medium supplemented with 150 μg of biotin per liter and with either iprodione (8.8 μmol/liter) or its metabolites (hereafter designated metabolite 1 and metabolite 2 [45 μmol/liter]). Iprodione or metabolites were added as sole carbon and energy sources and were dissolved directly in the medium. After magnetic stirring, the culture medium was sterilized by filtration on 0.2-μm-pore-size filters. A control experiment with *Arthrobacter* cells that had been killed by steam sterilization was carried out under the same conditions. The cultures were then incubated at 28°C for 8 days with shaking at 200 rpm. Aliquots (0.5 ml) of the culture were periodically sampled, and 0.5 ml of acetonitrile was added. The samples were analyzed by HPLC.

Analytical methods. Compound analysis was routinely monitored by HPLC. HPLC analyses were performed on an ultrabase column (UB 255; 5-μm C_8 column) and a UV (235-nm wavelength) detector (Shimadzu SPD 2A). The mobile phase consisted of acetonitrile-water-acetic acid (70/30/0.5 [vol/vol/vol]). The flow rate was set at 1 ml/min. The metabolites were identified by comparison of retention times and spectra to those of authentic reference compounds. The melting points were measured on an electrothermal apparatus (IA 9100). Infrared spectra were recorded on a Perkin-Elmer 1600 series Fourier-transform infrared spectrometer. ^1H nuclear magnetic resonance (NMR) spectra of purified metabolites were recorded on a JEOL EX 400 spectrometer (400 MHz).

Enrichment and metabolite isolation. Iprodione has low water solubility (13 mg/liter); therefore, to increase the contents of metabolites in the culture medium, we added iprodione (6 μmol) in sterilized acetone solution (20 mg/ml) at 0, 24, 96, 168, 192, and 240 h in 200 ml of mineral medium inoculated with an MA6 iprodione-degrading culture. Aliquots were periodically sampled and analyzed by HPLC. After 11 days of incubation, 100 ml of the culture medium was chromatographed through a C_{18} column (SepPak; Waters). Under these conditions, *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine (metabolite 1) was completely fixed on the column and only a small amount of metabolite 2 was fixed. The filtrate contained only (3,5-dichlorophenylurea)acetic acid (metabolite 2). The filtrate was applied to a C_{18} column by 10-ml fractions, which allowed metabolite 2 fixation. Metabolite 2 was eluted with 2 ml of acetonitrile. The combined 2-ml fractions were evaporated to dryness, taken up in a small volume of dimethyl sulfoxide (DMSO), and analyzed by ^1H NMR spectroscopy and HPLC.

Synthesis of metabolites 1 and 2. (i) Metabolite 2 preparation. Twenty milliliters of a solution containing phosgene and toluene (20%) was added dropwise to 2.4 g (0.02 M) of 3,5-D in 10 ml of dry toluene at reflux. The mixture was refluxed for 1 h. The flask was flushed with nitrogen to evaporate the toluene. The residue was used without any purification for the ensuing second reaction. The reaction product was added dropwise to 1.5 g (0.02 M) of glycine in 10 ml of NaOH (0.75 M) solution. The mixture was heated to 30°C and stirred for 90 min. It was filtered on a Büchner funnel, and the solution was acidified at 0°C. The solid precipitate was collected by filtration (1.9 g, 42%) and has a melting point of 199 to 200°C. Infrared, νNH, 3,384 cm^{-1}; νCO, 1,704 cm^{-1}; νCONH, 1,651 cm^{-1}. ^1H NMR (DMSO d6) δ 3.8 (2H, d, CH_2), 6.6 (2H, s, NH), 7 (1H, s, arom), 7.4 (2H, s, arom), and 9.3 (1H, s, CO_2H) ppm. ^{13}C NMR ($CDCl_3$) δ 41.5, 115.8, 120.4, 134.2, 143, 155, and 172.1 ppm.

(ii) Metabolite 1 preparation. Ten milliliters of a solution of metabolite 2 (0.35 g) in hydrochloric acid (2.4 N) was refluxed with stirring for 2 h. After cooling, the solid precipitate was collected by filtration (0.2 g, 60%) and has a melting point of 198 to 199°C. Infrared, νNH, 3,454 cm^{-1}, 3,248 cm^{-1}; νCO, 1,782 cm^{-1}, 1,770 cm^{-1}, 1,722 cm^{-1}. ^1H NMR (DMSO d6) δ 4.05 (2H, s, CH_2), 7.5 (2H, s, arom), 7.65 (1H, s, arom), and 8.5 (1H, s, NH) ppm. ^{13}C NMR ($CDCl_3$) δ 46.06, 125.1, 127.2, 133.7, 134.6, 155.6, and 170.7 ppm.

RESULTS

Isolation and characterization of iprodione-degrading microorganisms. After the enrichment procedure with IMM, the final (seventh) solutions were plated on the three different agar media. A total of 10 colonies able to produce 3,5-D were obtained only from the IMM agar medium. One isolate, which was named MA6, was used in further experiments. The MA6 strain was tentatively identified by J. D. Janse (Wageningen, The Netherlands) and the Germe Company (Marseille, France) as an *Arthrobacter* sp. according to its morphological and biochemical characteristics. This strain was an aerobic, motile, and non-spore-forming gram-positive bacterium which showed typical *Arthrobacter* pleomorphism, as described by Keddie and Jones (7). The cells were irregular rods (0.5 to 1.5 μm) but eventually became coccoid forms. The results for the esculin hydrolysis test and the oxidase test were positive and negative, respectively. No acids were produced from the sugars tested (glucose, arabinose, lactose, glycerol, fructose, sorbitol, galactose, and starch) or from citrate. The catalase test gave a negative result. The fatty acid analysis did not allow identification of the MA6 strain.

Iprodione transformation in a noninoculated medium. The iprodione concentration in sterile mineral medium at pH 6.5 decreased from 30 to approximately 4 μmol per liter after 7 days (Fig. 1); the half-life was 72 h. Two simultaneously formed products were identified by HPLC analysis: hydantoic acid (compound II), which was reaching its maximum concentration, and the iprodione isomer (compound I), which had a steadily increasing concentration. After 192 h, iprodione was almost completely transformed into isomer I. In a first experiment, we had noted that isomer I was not very soluble in water and was adsorbed on the walls of the Erlenmeyer flask. To allow complete dissolution of transformation products, a specific procedure was used for this experiment. Similar results were obtained with control experiments with killed *Arthrobacter* cells (data not shown).

Iprodione metabolism by the MA6 strain. When iprodione was used as the sole carbon and nitrogen source for MA6, the fungicide was rapidly transformed and the half-life was 10 h (Fig. 2). Simultaneously, large quantities of metabolite 1 appeared. Approximately 1 mol of metabolite formed for every mole of iprodione that disappeared. An increase in metabolite 2 in the medium was observed. A slight increase in the concentration of 3,5-D was also detected in the culture medium by HPLC (Fig. 2). Different experiments to show the growth of *Arthrobacter* cells were unsuccessful. The very low solubility of iprodione does not allow growth sufficient for significant results.

Enrichment of metabolites. To produce larger amounts of metabolites in the culture medium, we periodically added iprodione in acetonic solution. The metabolite accumulation is depicted in Fig. 3. After a slight lag, the concentration of metabolite 1 increased for as long as 192 h and then decreased. The concentration of metabolite 2 increased throughout the experiment, and it was the major product formed. The concentration of 3,5-D increased at a lower rate in the medium. After 268 h, the relative proportions of metabolite 1, metabo-

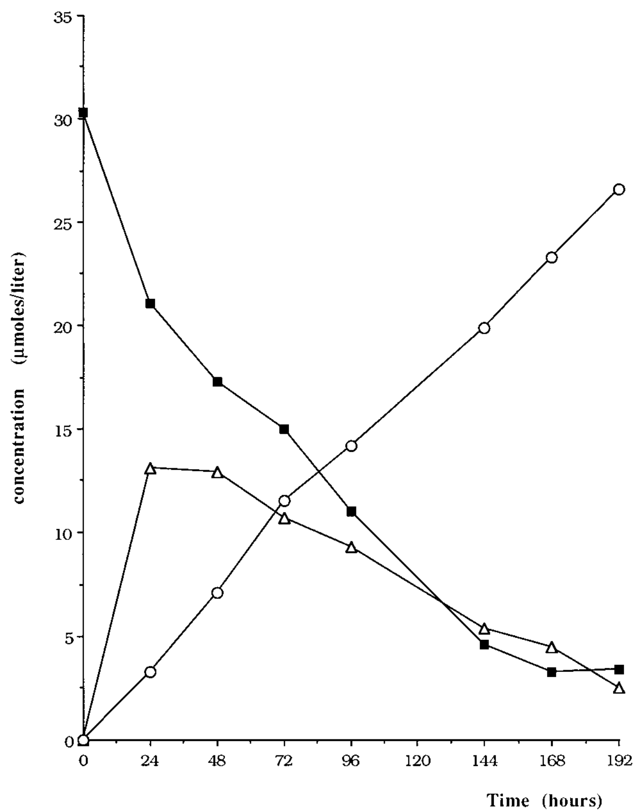

FIG. 1. Iprodione (30 µmol/liter) transformation in an uninoculated mineral medium (pH 6.5) at 28°C. Transformation was measured by HPLC analysis. ■, iprodione; ○, iprodione isomer (compound I); △, hydantoic acid (compound II).

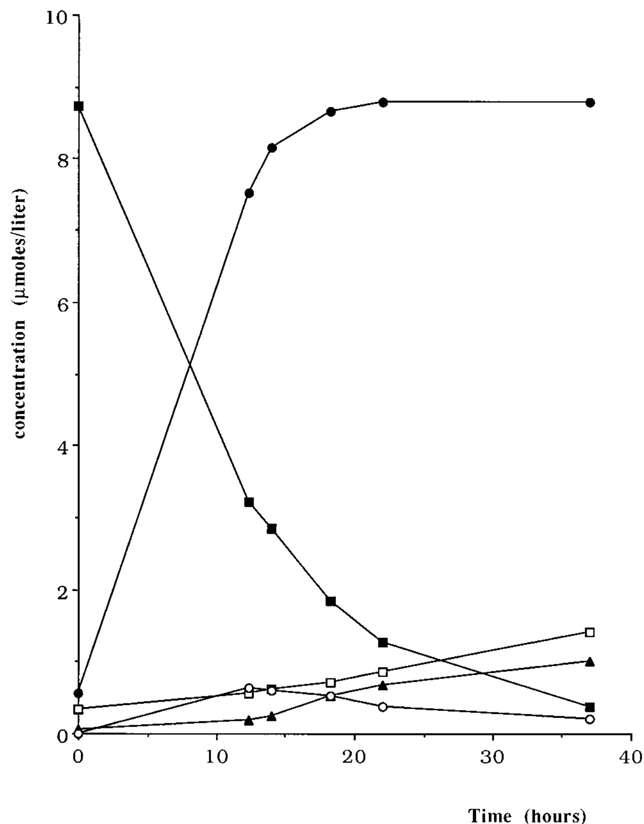

FIG. 2. Iprodione (8.8 µmol/liter) transformation by strain MA6 cultivated in minimal mineral medium (pH 6.5) at 28°C. ■, iprodione; ○, iprodione isomer (compound I); ●, metabolite 1; □, metabolite 2; ▲, 3,5-D. Traces of metabolites 1 and 2 and of 3,5-D at the beginning of the experiment were contributed by the inoculum.

lite 2, and 3,5-D were 14.5, 80.6, and 4.8%, respectively (Fig. 3).

Metabolite identification. After 11 days of culture, metabolite 2 produced by MA6 from iprodione was isolated by chromatography and analyzed by ^{1}H NMR. The spectrum data, (DMSO d6) δ 3.6 (2H, d), 6.6 (1H, s), 6.8 (1H, s), 7.4 (2H, s), and 9.4(2H,s) ppm, corresponded to those of (3,5-dichlorophenylurea)acetic acid. For comparison, this compound was synthesized according to the scheme shown in Fig. 4A. Metabolite 2 comigrated with the prepared compound during HPLC analysis and gave an identical ^{1}H NMR spectrum. The (3,5-dichlorophenylurea)acetic acid was cyclized in acidic medium to give *N*-(3,5-dichlorophenyl)2,4-dioxoimidazolidine (Fig. 4B). This product comigrated with metabolite 1 during HPLC analysis. Metabolites 1 and 2 were identical to the two synthesized compounds.

Transformation of metabolites 1 and 2. Metabolite 1 or metabolite 2 was incubated in a culture of strain MA6 or in an uninoculated control medium for 8 days. When metabolite 1 was used as the substrate, its concentration decreased and the concentration of metabolite 2 increased under both sterile and inoculated conditions. By contrast, no transformation of metabolite 2 was observed during the 8-day incubation period. No 3,5-D was detected in any of these experiments (data not shown).

DISCUSSION

No pure bacterial culture able to metabolize iprodione has been isolated until the present study. All attempts to isolate pure iprodione-degrading strains had failed, and only mixed enrichment cultures of microorganisms were readily obtained from soils showing enhanced iprodione degradation (7). We succeeded in isolating a pure strain, tentatively identified as an *Arthrobacter* sp. This strain was able to transform iprodione in medium containing iprodione as its sole source of carbon and nitrogen. There was evidence of a biological transformation by the appearance of at least three metabolites: the two newly identified metabolites 1 and 2 and 3,5-D. They were not detected in the sterile controls. The metabolite 2 concentration increased as the metabolite 1 concentration decreased, suggesting a possible transformation of metabolite 1 into metabolite 2. Walker (16) reported a two-stage degradation of iprodione; the final metabolite was 3,5-D, but the intermediate metabolite was not identified. In contrast, only the iprodione isomer and hydantoic acid were identified in the noninoculated medium. This is in agreement with results showing that iprodione was physicochemically converted into a major cyclic form (compound I) and an open form (compound II) (3). A scheme of the biological degradation of iprodione is proposed in Fig. 5. First, the urea function of iprodione is broken, producing a 3,5-dichlorophenyl dicarboximide (metabolite 1). Metabolite 1 is then converted, by chemical hydrolysis, into (3,5-dichlorophenylurea)acetic acid (metabolite 2). These metabolites had never been described in studies on iprodione degradation in soil. However, the nonidentified product reported by Walker (16) from soil treated by iprodione might well be our metabolite 1. Indeed, metabolite 1 has approximately the same R_f value as the R_f value of the degradation

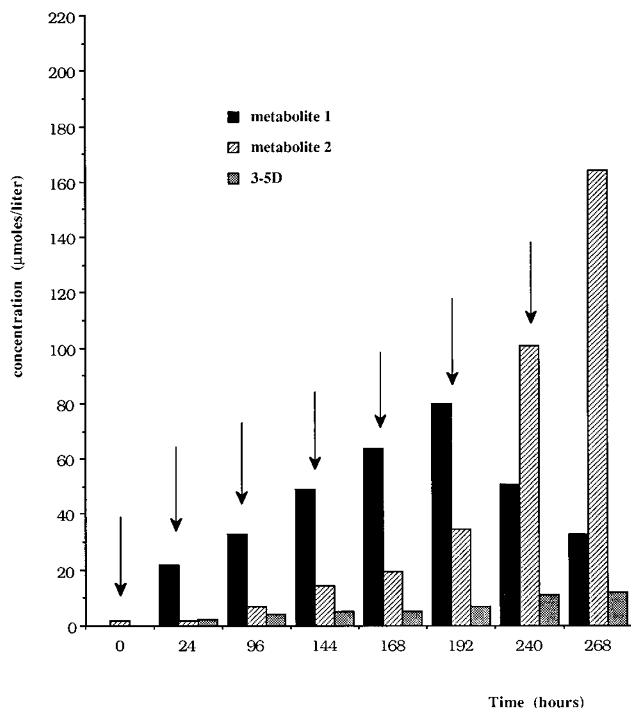

FIG. 3. Concentrations of the different iprodione metabolites in 200 ml of an MA6 strain culture, incubated at 28°C. Samples were analyzed by HPLC. Immediately after each sampling (0, 24, 96, 144, 168, 192, and 240 h), 6 μmol of iprodione in acetonic solution was added to the culture to obtain metabolite enrichment (arrows).

product observed by Walker during the early stages of iprodione degradation. Metabolite 2 did not migrate (unpublished results).

Strain MA6 was isolated on the basis of its ability to produce 3,5-D from iprodione in the medium. Metabolites 1 and 2 were formed through the major pathway of iprodione degradation by this strain. Metabolites 1 and 2 apparently did not produce 3,5-D by microbial degradation when they were incorporated

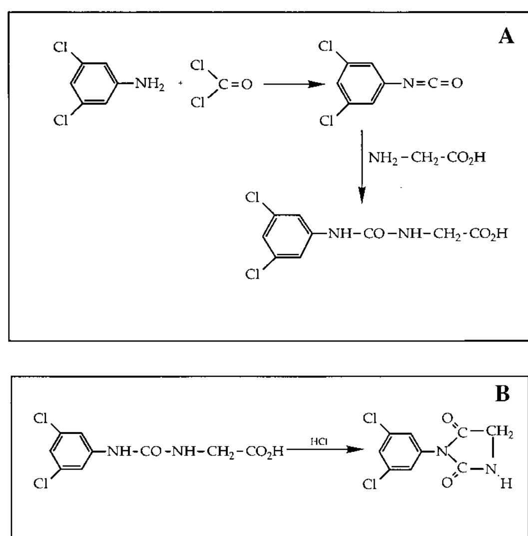

FIG. 4. Chemical synthesis of metabolites 2 (A) and 1 (B).

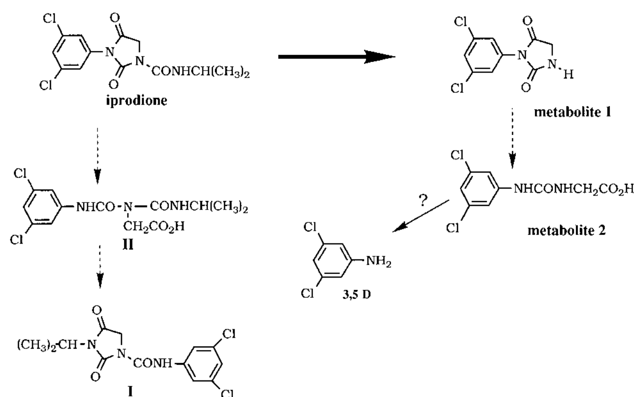

FIG. 5. Proposed pathways for iprodione transformation. Dashed arrows, chemical pathway; solid arrow, pathway proposed for strain MA6.

in the place of iprodione into the culture medium. Moreover, 3,5-D was not detected in the uninoculated medium when it contained either iprodione or metabolite 1 or 2. 3,5-D formation could occur through a minor pathway of microbial iprodione degradation.

Another dicarboximide fungicide, vinclozolin, could be degraded by *Pseudomonas fluorescens* and *Bacillus cereus*, and a small amount of 3,5-D was also detected in the bacterial suspension. Two pathways of vinclozolin biodegradation have been proposed. The mechanism of 3,5-D formation has not yet been clearly established (6). However, cross-degradation between iprodione and vinclozolin apparently does not occur (10, 16). Indeed, strain MA6 could not use vinclozolin as a substrate (unpublished result).

Our results confirmed that enhanced iprodione degradation in soil occurs through microbial pathways and that bacteria are clearly involved, as already suggested by Walker and Welch (19) and Martin et al. (11).

The slow growth of MA6 in vitro contrasted with its very fast iprodione metabolism (Fig. 2). It is now essential to know whether this isolate is able to multiply in iprodione-enriched soil. With this objective, the population kinetics of MA6 are now being studied with specific fluorescent antibodies.

We obtained isolation by an enrichment method based on 3,5-D formation. If 3,5-D and metabolites 1 and 2 are elaborated according to two different pathways, it is possible that some bacteria decompose iprodione without forming 3,5-D. If this is the case, such bacteria could have been discarded when we conducted our colorimetric test. New procedures should thus be considered, whereby the formation of metabolite 1 or 2 would be measured or the disappearance of iprodione would be assessed directly. Isolation and study of other degrading species should provide further information on iprodione degradation pathways.

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