Chemical and Biological Transformation of the Fungicide Vinclozolin

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Two mixed bacterial cultures were isolated from a French soil adapted to the dicarboximide fungicide vinclozolin. The vinclozolin was transformed by the mixed bacterial cultures according to two degradation pathways: (a) the formation of 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid and then 3,5-dichloroaniline or (b) the formation of 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide and then 3,5-dichloroaniline. The structure of 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid was unambiguously established by ^{1}H and ^{13}C 2-D NMR analysis. A bacterial strain isolated from soil that degrades this compound was identified as a strain of *Corynebacterium* sp. Attempts to obtain pure vinclozolin-degrading strains via 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide were unsuccessful.

Keywords: *Vinclozolin; fungicide-degrading bacteria; chemical transformation*

INTRODUCTION

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione] is marketed by BASF under the trade name Ronilan. This cyclic imide fungicide is used to control diseases of grapevine, fruit trees, and vegetable crops caused by *Botritys* spp., *Sclerotinia* spp., and *Monilinia* spp.

A loss of efficacy of vinclozolin after several treatments has been demonstrated in studies performed in several countries over the past 10 years. For example, such findings have been observed in France by Martin et al. (1991), in the United Kingdom by Walker et al. (1986), Walker (1987), and Mitchell and Cain (1996), and in New Zealand by Slade et al. (1992). This phenomenom was related by these authors to the enhanced microbial degradation of the fungicide. Furthermore, with the exception of very acidic soils (pH <5), the degradation rate of vinclozolin was found to increase according to the number of applications of the pesticide (Walker, 1987)

Melkebeke et al. (1986), Szeto et al. (1989), and Villedieu et al. (1994) have observed that the rate of chemical transformation of vinclozolin in liquid medium was related to the pH of the medium and that the fungicide was more stable in acidic buffer. Transformation rates of vinclozolin reported by these authors were similar, but the transformation patterns were different. Szeto et al. (1989) reported the breakdown of the amide bond, whereas Clark (1983), Melkebeke et al. (1986), Pirisi et al. (1986), and Villedieu et al. (1994) suggested the breakdown of ester function.

Head et al. (1988), Golovleva et al. (1991), and Cain and Mitchell (1996) described the isolation of vinclozolin-degrading strains of bacteria from soils exhibiting enhanced degradation of the fungicide. Five bacterial strains capable of degrading vinclozolin were isolated. However, these results need to be viewed with caution because, in liquid medium without the bacterial strain, the transformation rates of vinclozolin reported in these studies were not in agreement with the chemical hydrolysis rate at the same pH observed by Clark (1983), Melkebeke et al. (1986), Szeto et al. (1989), and Villedieu et al. (1994).

The experiments described in this paper were designed to investigate both the chemical hydrolysis of vinclozolin and the microbial degradation of the fungicide by mixed and pure bacterial cultures isolated from vinclozolin-degrading soil.

MATERIALS AND METHODS

Vinclozolin and Its Degradation Products. Vinclozolin (**1**) [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione] in the form of commercial wettable powder formulation (Ronilan 500 g kg^{-1} of active ingredient) was purchased from BASF (Limburgerhof, Germany). The active ingredient was extracted from Ronilan according to the method of Villedieu et al. (1994). 2-[[(3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid (**2**) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (**4**) were synthesized according to the method of Clark (1983). Analytical grade 3,5-dichloroaniline (**5**) was purchased from Aldrich Chemical (Saint Quentin Fallavier, France). Structures of vinclozolin and its metabolites are shown in Figure 1.

NMR Techniques. The NMR spectra of compounds were measured on a JEOL EX400 spectrometer at 100 MHz for ^{13}C and 400 MHz for ^{1}H. The long-range $^{1}H-^{13}C$ correlation was measured with a COLOC procedure (Kessler et al., 1984).

Analytical Methods. Concentrations of vinclozolin and its degradation products were determined by HPLC. HPLC analyses were performed on a system with a Beckman pump and a Shimadzu SPD 2A UV detector (235 nm wavelength). The operating parameters were as follows: column, Ultrabase UB 235-5 μm C8; mobile phase, acetonitrile/water/trifluoroacetic acid (65:35:0.1) (v/v/v), delivered at a flow rate of 1 mL min^{-1}. All compounds studied were quantified using external standards.

Transformation of Vinclozolin or Butenoic Acid in Aqueous Buffer pH 4.5. Portions (0.5 mL) of vinclozolin or butenoic acid solution at 1 mg mL^{-1} in methanol were thoroughly mixed with 50 mL of aqueous buffer solution, pH 4.5. This buffer was prepared with a mixture of sodium acetate (0.01 M) and acetic acid (0.01 M) and was filter-sterilized with a 0.22 μm filter. These flasks were incubated

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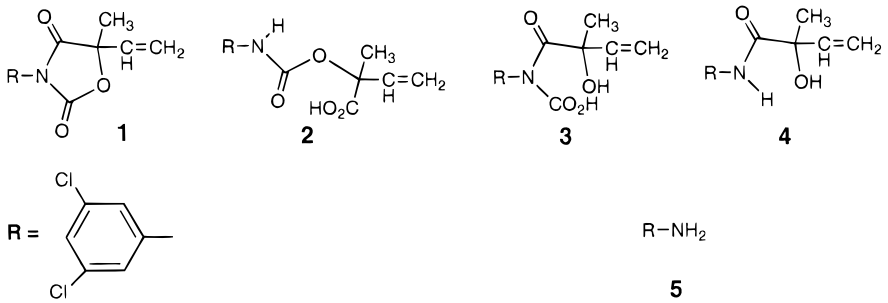


Figure 1. Structures of vinclozolin (**1**) and its metabolites: 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid (**2**); carbamic acid (**3**); 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (**4**); 3,5-dichloroaniline (**5**).

at 35 °C in an oven. Duplicate samples were periodically removed from each flask and analyzed by HPLC.

Soil Adaptation. Soil sample (Saint Nazaire) was collected from a depth of 0−15 cm in the south of France. The soil properties have been previously described (Vega et al., 1992). This soil (pH 6.4) had no previous history of dicarboximide fungicide treatment. In the laboratory, 1 kg of this soil was treated with an aqueous suspension of Ronilan to give a final concentration of 10 mg kg^{-1} [active ingredient (ai)] of dry soil with 20% soil moisture. The soil was then incubated at 28 °C. When 50% of vinclozolin was degraded, an identical fungicide treatment was repeated up to six times.

Extraction. After each treatment to adaptation, soil samples (20 g) were removed and extracted by shaking for1 h with 20 mL of acetonitrile/acetic acid (99:1). After decantation, the supernatant was centrifuged for 2 min, to remove the solid matter, and analyzed by HPLC.

Culture Media. Cultures were carried out in a mineral medium (MM) containing 50 mL of the Winogradsky stock salts solution (a) and 1 mL of trace elements stock solution (b) adjusted to 1 L with distilled water. The mineral medium was adjusted to pH 6.5 and sterilized by autoclaving for 20 min at 121 °C. When required, this mineral medium was solidified by the addition of agar (15 g L^{-1}).

(a) The composition of the Winogradsky stock salts solution was as follows: K_2HPO_4, 5 g; $MgSO_4$, 2.5 g; NaCl, 2.5 g; $Fe_2(SO_4)_3$, 50 mg in 1 L of distilled water.

(b) The composition of the trace elements stock solution was as follows: K_2MoO_4, 50 mg; $NaBO_2 \cdot 4H_2O$, 50 mg; $FeCl_3$, 3 mg; $Co(NO_3)_2 \cdot 6H_2O$, 50 mg; $CdSO_4 \cdot 8H_2O$, 50 mg; $CuSO_4 \cdot 5H_2O$, 50 mg; $ZnSO_4 \cdot 7H_2O$, 50 mg; $MnSO_4 \cdot 5H_2O$, 50 mg in 1 L of distilled water. These two solutions, (a) and (b), were sterilized at 121 °C for 20 min before storage.

Acetone solutions of vinclozolin or butenoic acid (5 g L^{-1}) were filter-sterilized (FG 0.22 μm pore size filter, Millipore, Saint Quentin Yvelines, France) and added to cooled and sterile MM to obtain a final concentration of 10−70 μmol L^{-1}.

To isolate colonies, nutrient agar (Difco, Detroit, MI) was prepared.

Isolation of Vinclozolin-Degrading Microorganisms (Mixed Cultures). A vinclozolin-degrading soil (2.5 g) was added to MM (pH 6.5, 50 mL) supplemented with vinclozolin (70 μmol L^{-1}) in an Erlenmeyer flask. This soil suspension was incubated at 30 °C in a rotary shaker at 200 rpm. Samples of the culture (0.5 mL) were removed at regular intervals, diluted with acetonitrile (0.5 mL), and centrifuged briefly to remove solid matter before injection into the chromatographic column for analysis. When 50% of vinclozolin was degraded, 5 mL of these soil suspensions was subcultured in 45 mL of fresh MM with vinclozolin as the sole source of carbon and nitrogen. After ∼10 h of incubation, 100 μL of this suspension was spreaded on plates of MM plus agar (15 g L^{-1}) or on plates of nutrient broth, both supplemented with vinclozolin (50 mg L^{-1}). The plates were incubated at 30 °C. After several days of incubation, all of the cells on the plate surface were scraped and dispersed in 5 mL of mineral medium. These mixed cultures will be used as inocula.

Isolates were purified by restreaking single colonies onto plates of MM plus agar supplemented with vinclozolin (50 mg L^{-1}).

Growth was monitored by measuring the optical density at 550 nm.

Vinclozolin Degradation by Two Mixed Bacterial Cultures. All of the mixed cultures obtained by scraping the surface of the plates were subcultured in 30 mL of MM, pH 6.5, supplemented with vinclozolin (20 μmol L^{-1}) in Erlenmeyer flasks. After incubation at 30 °C in a rotary shaker at 200 rpm, 1 mL of these cultures was diluted in MM (10 mL) supplemented with vinclozolin (20 μmol L^{-1}) and incubated under the same conditions as previously described. A control experiment with noninoculated mineral medium was also carried out. Samples (0.5 mL) of both bacterial cultures and noninoculated medium were periodically removed, diluted with acetonitrile (0.5 mL), and centrifuged briefly to remove solid matter before injection. The concentration of vinclozolin and its metabolites was monitored by HPLC.

Vinclozolin and Compound 2 Degradation by an Isolated Pure Bacteria Strain. Microorganisms from a single colony were harvested from MM agar plates and inoculated in 30 mL of MM, pH 6.5, supplemented with vinclozolin or compound **2** (10 μmol L^{-1}). Incubations, the control experiments, and HPLC analyses were carried out as previously described.

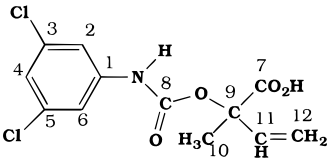
RESULTS AND DISCUSSION

Structure of Compound Obtained by Basic Hydrolysis of Vinclozolin. A compound **X** was obtained by synthesis according to the experimental method of Clark (1983). This compound had the same physicochemical characteristics (mp, mass spectrum, ^1H NMR) as those reported by Clark (1983), with the formula proposed by Clark being the carbamic acid **3**. It is known that the chemical stability of this type of compound is generally low, such that spontaneous decarboxylation usually occurs. However, we observed that the compound **X** was stable and easily isolated.

A second structure of a vinclozolin transformation product, compound **2**, was described by Szeto et al. (1989) and Golovleva et al. (1991), although the method of synthesis and the physicochemical characteristics were not reported.

The ^1H and ^{13}C NMR spectra of compound **X** were measured, and the assignment of the different protons and carbons was established (Figure 2). Long-range $^1H-^{13}C$ correlation from CH_3 and C_7 and N−H and C_2 (C_6) of the compound were in accordance with the structure of **2**. The chemical shifts of the two acidic hydrogens were 10.2 and 13.05 ppm, corresponding to N−H and CO_2H functions, respectively, and thus confirming the structure of compound **X** to be the same as that of **2**.

Hydrolysis of Vinclozolin and Compound 2 at pH 4.5. The chemical transformation of both vinclozolin and compound **2** was studied in a pH 4.5 buffer under the same conditions as those described by Szeto et al.



δ ppm (DMSO d6)

	$H_{2,6}$	H_4	CH_3	CH	CH_2	NH	CO_2H		
	7.5	7.2	1.6	6.2	5.32	10.2	13.1		
C_1	$C_{2,6}$	$C_{3,5}$	C_4	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}
141.4	116.2	134.2	121.8	171.9	151.7	79.7	22.8	137.4	115.8

Figure 2. ^1H and ^{13}C NMR chemical shifts of compound **X**, which are the same as for compound **2**.

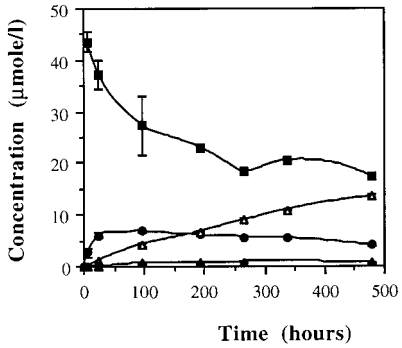


Figure 3. Vinclozolin transformation in aqueous buffer at pH 4.5. Mean values and standard deviations are given for vinclozolin (■), compound **2** (●), compound **4** (△), and 3,5-dichloroaniline **5** (▲).

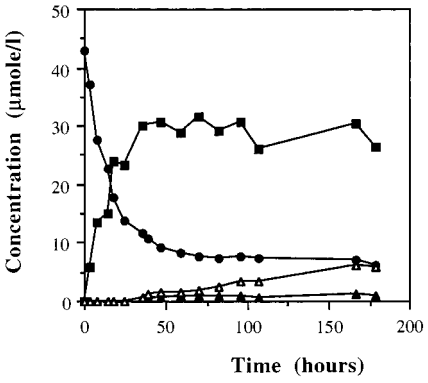


Figure 4. Compound **2** transformation in aqueous buffer at pH 4.5. Data are the average of duplicate measurements for vinclozolin (■), compound **2** (●), compound **4** (△), and 3,5-dichloroaniline **5** (▲).

(1989). Vinclozolin was transformed into **2**, while at the same time, **4** was also rapidly formed (Figure 3). Under the same incubation conditions, compound **2** was rapidly transformed into vinclozolin and **4** (Figure 4). Compound **2** showed the same reactivity as the compound obtained by Szeto et al. (1989). Our findings suggest that the transformation pathway of vinclozolin proposed by Clark (1983), Pirisi et al. (1986), and Villedieu et al. (1994) was not supported in this instance: the carbamic acid **3** was never isolated in these experiments, and the structure of the isolated intermediate was **2**.

Soil Adaptation. The transformation rate of vinclozolin in soil from Saint Nazaire increased after repeated applications of the fungicide, such that the half-life of vinclozolin was 22 days after the first treatment and 2.15 h after the sixth treatment. The compounds formed in untreated and treated soils were different: **2** and **5** were formed in untreated soil (Figure 5a), whereas **4** and **5** were formed in treated soil (Figure 5b). The quantities of **5** and **4** found at the beginning of the curve in Figure 5b correspond to residues of the degradation products that had accumulated during the soil adaptation.

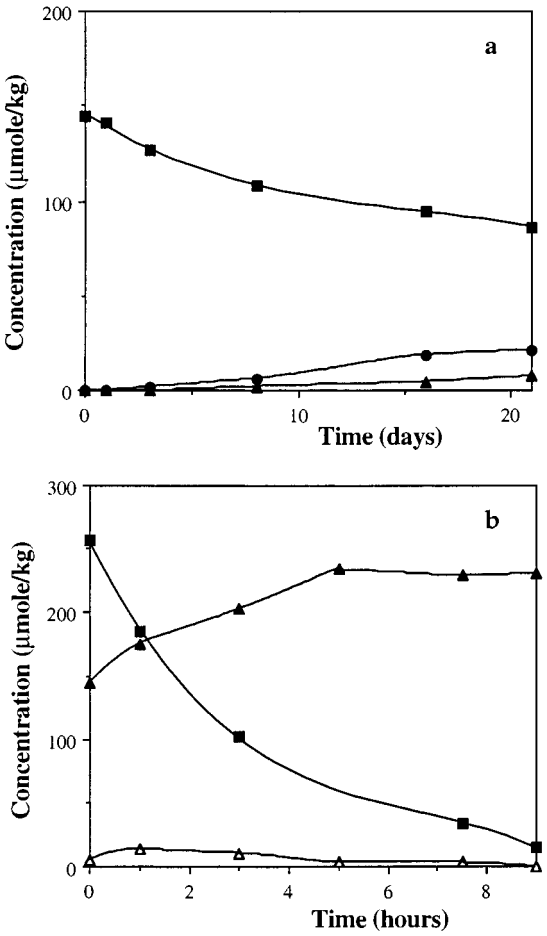


Figure 5. Vinclozolin degradation in nonadapted soil (a) and in adapted soil (b): vinclozolin (■), compound **2** (●), compound **4** (△), and 3,5-dichloroaniline **5** (▲).

Enrichment and Isolation of Vinclozolin-Degrading Microorganisms. Mixed enrichment cultures of microorganisms that were able to degrade vinclozolin and showed enhanced degradation of the fungicide were obtained from the Saint Nazaire soil. After plating on mineral salt medium plus agar or on nutrient medium plus agar, two different mixed bacterial cultures, named MA and MB, respectively, were obtained.

Three bacterial strains were isolated from MA, but only one of these isolates was able to grow on and degrade vinclozolin in liquid mineral medium with the fungicide as the sole source of carbon and nitrogen. This strain was grown aerobically, forming a yellow, smooth, circular colony. It was Gram-positive, motile, and rod-shaped and was tentatively identified as *Corynebacterium* sp. by the Institut Pasteur (Lyon, France).

All attempts to isolate vinclozolin-degrading pure strains from MB failed.

Vinclozolin Degradation by the Mixed Bacterial Cultures, MA and MB. In mineral medium, pH 6.5, with vinclozolin as the sole source of carbon and nitrogen, the mixed bacterial culture MA degraded the fungicide into **2** and **5** (Figure 6). The transformation rate of vinclozolin was greater in the inoculated medium

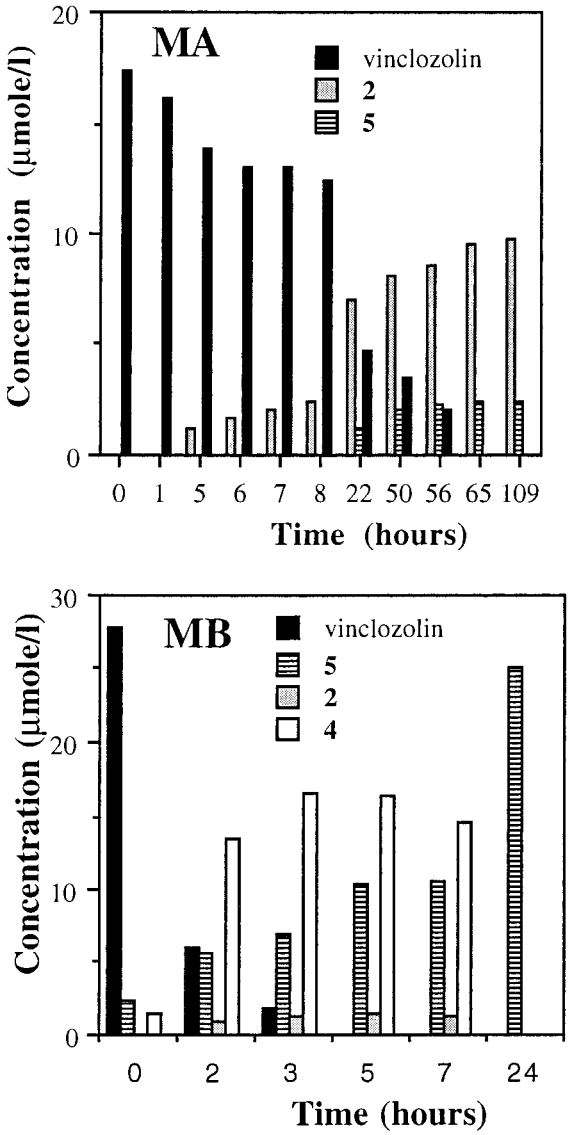


Figure 6. Vinclozolin degradation by mixed cultures MA and MB in pH 6.5 mineral medium with incubation at 30 °C in a rotary shaker at 200 rpm.

(half-life = 15 h) than in the uninoculated one (half-life = 40 h). After incubation, the final pH of the culture remained unchanged.

Under the same culture conditions, the mixed bacterial culture MB degraded vinclozolin more rapidly than MA, as a measured half-life of 6 h was found in the presence of MB. Moreover, the degradation pathway was different, with **4** and **5** being formed (Figure 6). A small amount of **2** corresponding to the chemical hydrolysis was detected.

Vinclozolin Degradation by *Corynebacterium* sp. When the *Corynebacterium* sp. strain was inoculated in mineral medium supplemented with vinclozolin, the transformation rate of vinclozolin was only slightly increased (Figure 7). Nevertheless, the products formed were different from those obtained by chemical hydrolysis; the breakdown of vinclozolin led to the formation of **2**, which rapidly disappeared. **5** was formed in large amounts.

In mineral medium supplemented with vinclozolin, a slight increase in the bacterial population was observed. After 48 h of incubation, the turbidity of the

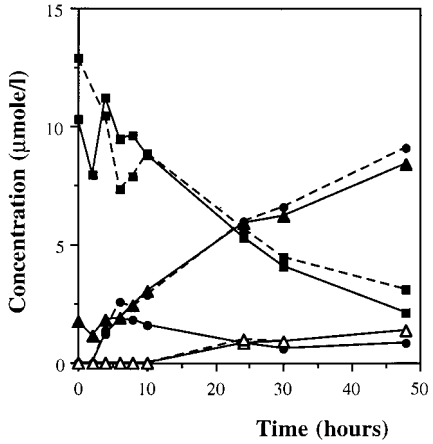


Figure 7. Vinclozolin degradation in pH 6.5 mineral medium at 30 °C inoculated with *Corynebacterium* sp. (full line) and without strain (broken line): vinclozolin (■), compound **2** (●), compound **4** (△), and 3,5-dichloroaniline **5** (▲).

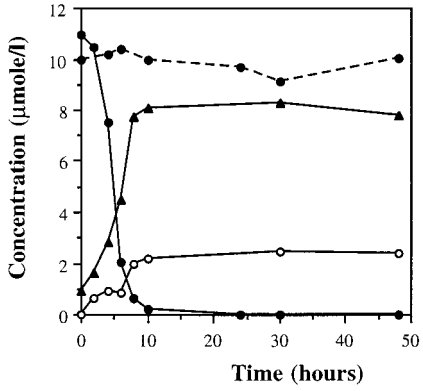


Figure 8. Compound **2** degradation in pH 6.5 mineral medium at 30 °C inoculated with *Corynebacterium* sp. (full line) and without strain (broken line): compound **2** (●), 3,5-dichloroaniline **5** (▲), and unidentified compound **A** (○).

Corynebacterium sp. culture was 3 times higher with the OD increasing from 0.018 to 0.06.

Compound 2 Degradation by *Corynebacterium* sp. Compound **2** was chemically stable in the mineral medium (Figure 8). In the same medium inoculated with *Corynebacterium* sp., **2** was very rapidly degraded. Two metabolites were formed: **5** and an unidentified compound (compound **A**). A small quantity of this compound was isolated, but the data obtained by NMR and MS did not permit the unambiguous determination of its chemical structure. However, we noticed that the **5** ring was present in this molecule. We postulated that the molecular absorption coefficients of compounds **A** and **5** were similar. This allowed an evaluation of the concentration of compound **A** by measuring and comparing the UV absorption of this compound to the UV absorption of a solution of **5** at known concentrations.

These two results (Figures 7 and 8) were consistent with the hypothesis that the slight increase in the degradation rate of vinclozolin in the liquid *Corynebacterium* sp. culture might be related to the changing chemical equilibrium between vinclozolin and **2**.

CONCLUSION

The mechanism of the vinclozolin transformation seems more complex than the one of other dicarboximide fungicides, such as iprodione (Athiel et al., 1995; Mercadier et al., 1997). Indeed, two different bonds of

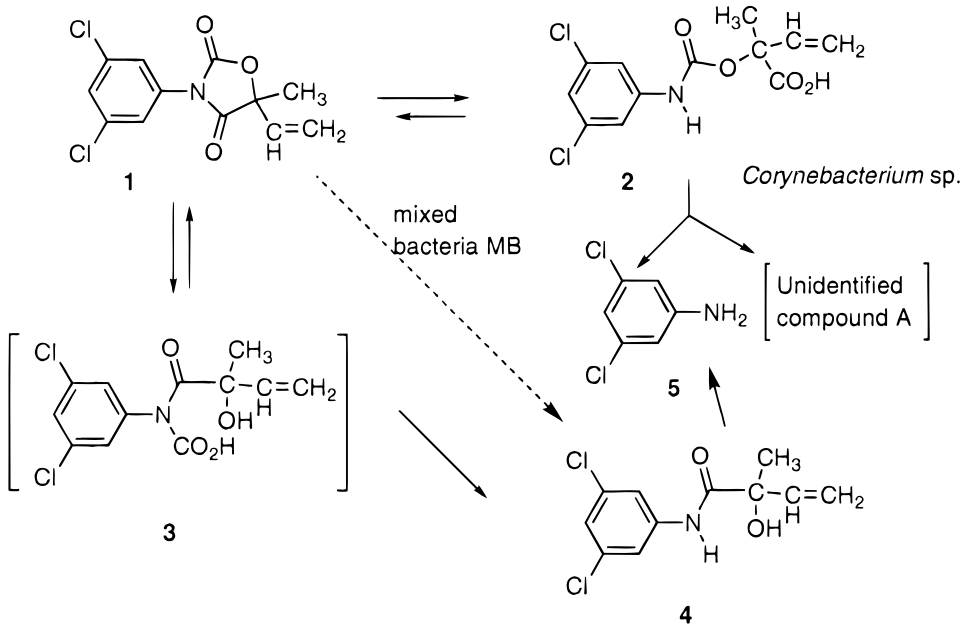


Figure 9. Suggested pathways for chemical and biological vinclozolin transformation.

the 2,4-oxazolidinedione cycle of vinclozolin may be broken. A scheme of the transformation pathway of vinclozolin is proposed in Figure 9.

We were able to show that vinclozolin was converted by chemical hydrolysis into an open form, the butenoic acid derivative **2**, and that the fungicide was in chemical equilibrium with this intermediate. Vinclozolin was also transformed into a carbamic acid derivative, **3**. This unstable intermediate **3** was then converted into an enanilide, **4**. These two chemical transformation pathways were observed by monitoring biological degradation. However, there was evidence of a biological transformation due to the appearance of the 3,5-dichloroaniline, this being compound **5**. Indeed, the intermediate compounds **2** and **4** might then be metabolized into **5**.

We succeeded in isolating two mixed bacterial cultures, MA and MB, involved in the vinclozolin degradation, with two degradation patterns observed. In media inoculated with MA, vinclozolin was converted into **2**, which was then degraded into **5**. In the presence of MB, the degradation of vinclozolin produced **4**, which was then converted into **5**.

A pure bacterial strain was isolated from the mixed culture MA and tentatively identified as *Corynebacterium* sp. All attempts to show that this isolate degraded vinclozolin failed. However, we demonstrated that *Corynebacterium* sp. was able to degrade **2** into **5** and into an unidentified compound **A**. Several authors have described the degradation of vinclozolin by pure isolated strains. Golovleva et al. (1991), for example, showed that the isolates were involved in the degradation of vinclozolin and that the compounds **2** and **4** were produced. However, the incubation time (10 days) and the metabolites formed were more in agreement with a chemical transformation. Cain and Mitchell (1996) detected only one metabolite, this being 3,5-dichloroaniline (**5**), with no other intermediate reported. Head at al. (1988) demonstrated that vinclozolin was first degraded to an unidentified metabolite by a mixed bacterial culture, with this metabolite then leading to the formation of 3,5-dichloroaniline (**5**). The diversity of results reported in the literature may be related to the chemical instability of vinclozolin and to the diversity of the isolated microorganisms.

In this study, we reported the isolation of mixed and pure cultures involved in the degradation of vinclozolin in liquid medium. Are these microorganisms responsible for the enhanced degradation of vinclozolin in adapted soils? The addition of degrading microorganisms to soils can increase pesticide transformation rates (Barles et al., 1979; Edgehill and Finn, 1983; Milhomme et al., 1989; Cork and Krueger, 1991). The transformation rate of vinclozolin was not increased by the addition of *Corynebacterium* sp. to untreated Saint Nazaire soil (data not shown), although 3,5-dichloroaniline (**5**) was the only metabolite detected in the soil. On the other hand, the transformation of vinclozolin in untreated soil led to the formation of both **2** and **5** (results not shown). This result suggests that this strain is not responsible for the enhanced degradation of vinclozolin in adapted soils, although it was able to degrade compound **2** into **5**. No similar experiment was carried out with the mixed culture MB. Nevertheless, in liquid medium inoculated with MB, vinclozolin was converted into **4** and **5**. We noticed that this transformation pathway was similar to that observed in the Saint Nazaire adapted soil. These findings suggest that microorganisms of the mixed culture MB might be involved in the vinclozolin degradation in soils and might also be responsible for the enhanced degradation of the fungicide in the Saint Nazaire adapted soil.

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