Pure Culture Studies of *Erwinia carotovora* with 3, 5-Diiodo-4-Hydroxybenzonitrile

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Interactions of ioxynil (3, 5-diiodo-4-hydroxybenzonitrile) with a pure culture of *Erwinia carotovora* grown in a glucose-simple salts medium were studied. Growth of *E. carotovora* was inhibited by ioxynil and, to a lesser extent, by its acid form at 25 and 50 μg/ml. Growth was not inhibited by the amide or ester forms of ioxynil or p-hydroxybenzonitrile at the same concentrations. *E. carotovora* could be trained to grow in 50 µg or higher concentrations of ioxynil per ml by serial transfers of the organism through increasing ioxynil concentrations. No degradation or detoxification of ioxynil was detected. Toxicity tests indicated that, in the adapted culture, cell-free supernatant fluid remained toxic to a nonadapted culture. Adaptation of *E. carotovora* resulted in a lengthened lag phase, a decreased growth rate, and very few adverse effects on the total population. The adapted resistant culture retained this characteristic only when ioxynil was present. Adaptation was demonstrated to be a physiological variation, not a selection of a mutant or of preexisting resistant cells. Ioxynil slightly stimulated the respiration rate of *E. carotovora* and moderately inhibited that of an adapted culture. Because the respiration rate of an adapted culture in the absence of ioxynil surpassed that of a parent culture still in the presence of ioxynil, a competition of two alternate routes of electron transport is implied. These data support the conclusion that an alternate growth mechanism is involved in the adaptation mechanism.

Previous research has been focused on identifying those microorganisms responsible for degrading specific groups of herbicides; less attention has been given to the impact of these herbicides on the soil microbial population (5). Most soil microorganisms are not concerned with the utilization of the herbicide as substrate (1), but many of these may be affected directly or indirectly by the herbicide in the soil. Therefore, consideration of herbicide effects on the majority of soil microorganisms is essential and important in considering effects on the total soil microbial population. Earlier investigations indicated that no herbicide tested at recommended field doses has more than a transitory effect on the total soil microbial population (1, 2, 7–9). However, repeated applications of the same herbicide for several years may result in population changes and possibly may alter basic metabolic processes of the organism (3).

The growth responses of specific soil bacteria vary with the types of herbicide added to the growth medium; these responses include stimulation and inhibition, either complete or partial (4). Where growth of the bacterium was not completely inhibited by a herbicide, long lag phases followed by growth were observed (4). This response has been interpreted to involve either adaptation of the bacterium to the herbicide or mutational selection. Studies on the interaction of a specific microorganism and a specific herbicide may reveal the mechanism of those responses and lead to a better understanding of interaction between herbicides and soil microorganisms. This study examined the interactions of ioxynil (3, 5-diiodo, 4-hydroxybenzonitrile) and *Erwinia carotovora* and the mechanisms involved.

**MATERIALS AND METHODS**

Test chemicals. Analytical grade ioxynil, 3, 5-diiodo-4-hydroxybenzamide, 3, 5-diiodo-4-hydroxybenzoic acid, and 3, 5-diiodo-4-octanoyloxybenzonitrile were provided in crystalline form by Amchem Products, Inc.; p-hydroxybenzonitrile was obtained from...
Eastman Kodak Co. Herbicides and reference compounds (10 mg/ml) were prepared in redistilled acetone with a final acetone concentration not exceeding 0.5% (vol/vol) in the test medium.

**Organism.** *E. carotovora* was obtained from Carolina Biological Supply (no. 15-5045), Burlington, N.C. Stock cultures were maintained on Trypticase soy agar (Difco) slants for 18 to 24 h at 30°C and then were stored at 0 to 3°C. The culture was transferred every 3 months; Gram stains were performed periodically.

**Cultivation.** Growth conditions followed the method of Breazeale and Camper (4). Hereafter the glucose-simple salts medium will be referred to as GSSM and the glucose-simple salts medium containingioxynil at 50 μg/ml as GSSIM. Adaptation of *E. carotovora* to ioxynil was accomplished by sequential transfer of the parent culture through GSSM containing 10, 25, 40, and 50 μg of ioxynil per ml after growth had reached the early stationary phase at each preceding concentration. Control flasks received the same quantity of acetone in GSSM.

**Viable count.** Viable cells were estimated, as colony forming units, by tenfold serial dilution of a 1-ml sample, after which a 0.1-ml sample was plated on nutrient agar (Difco). Cultures were sampled at 0-, 0.5-, 1-, 2-, 4-, and 10-h intervals.

**Transfer test.** The full grown culture in GSSIM (adapted culture) was subcultured three times and transferred into GSSM and into GSSIM separately. After growing in GSSM, the culture was then transferred into GSSIM and into GSSIM separately. After subsequent growth in GSSM, the culture was then transferred a third time into GSSIM and GSSM. The culture, originally transferred into GSSM and then into GSSIM, was now transferred into both GSSM and GSSIM. Tenfold serial dilutions were prepared from both nonadapted and adapted cultures. A 0.1-ml portion of each dilution and of the original culture was plated on the nutrient agar with and without 50 μg of ioxynil per ml. The plates were incubated at 30°C and were examined for colony appearance for 7 days. After transfer and subsequent growth of each single colony isolate in GSSM into late log phase, samples were removed and treated in the transfer test as described.

**Extraction.** Two methods were used to extract ioxynil and possible degradation products from the bacterial cultures. Both adapted and nonadapted cultures were centrifuged at 10,000 × g for 30 min (Sorvall RC2-B). Cellular pellets were washed once with the equivalent amounts of 0.05 M phosphate buffer (pH 7.0). The supernatant fluids, cell washes, and cellular residues were separately extracted twice with purified ether or ethyl acetate. Cellular residues were then extracted with acetone. The extracts of each portion were evaporated to dryness and redissolved in 1 ml of ether, and appropriate samples of the ether extracts were subjected to thin-layer chromatographic analysis. A second extraction method involved acidification of the culture to pH 1.0 with 12 N HCl, followed by extraction, twice with ether or ethyl acetate. The upper organic layer was then treated as described above. The two methods yielded the same results.

**Thin-layer chromatography.** For thin-layer chromatography (TLC), glass plates (20 by 20 cm) were coated with a 250-μm layer of silica gel containing fluorescent reagent (MN-Silica Gel G/UV, from Macherey, Nagel & Co., Germany). The plates were developed with a mixture of 89% chloroform, 10% ethanol, and 1% acetic acid (vol/vol/vol). After development, the chromatograms were examined under an ultraviolet (UV) light; UV absorbing compounds appeared as purple spots on a green background.

**14C tracer experiments.** 14C ring-labeled ioxynil was obtained from Amchem Products, Inc. (specific activity 1.08 mCi/mmol). Air passed through 2 N KOH solution was introduced into a culture flask containing GSSM with 14C-ioxynil and an adapted culture. A control flask contained only GSSM with 14C-ioxynil. The 14CO2 evolved was trapped in the saturated barium hydroxide solution, and volatile radioactive organic compounds were trapped in an ethyl acetate solution. Extraction and analysis of degradation products were performed by methods mentioned above. Developed thin-layer chromatograms were divided into grids of 1-inch (2.54 cm) squares, or smaller if necessary; the silica gel was then transferred to scintillation vials according to the grids so that any degradation compounds, including those not visible under UV light, could be traced and quantitated. Precipitates in the CO2 trap was filtered and dried under reduced pressure and then placed in vials. The solution in the organic traps was evaporated to dryness; the residue was redissolved in ether or ethylacetate, followed by TLC and scintillation counting. Scintillation vials contained 15 ml of counting fluid composed of 5.5 g of Permablend 2,5-diphenyloxazole, 91%: dimethyl 1,4-bis-(5-phenyloxazolyl)benzene, 9%; (Packard) and 25 g of thioropic gel powder (or Cab-O-Sil, Packard) per liter of toluene. Radioactivity was measured with an automatic liquid scintillation counter (Packard Tri-Carb model 3320).

**Toxicity tests.** Cell-free supernatant fluids from both adapted and nonadapted cultures were prepared by membrane filtration of 24-h cultures under reduced pressure. The membrane had a mean pore diameter of 0.22 μm (Millipore Corp., Bedford, Mass.). Each supernatant solution had been depleted of glucose content during a previous growth period; thus, glucose (0.1%) was added to certain growth flasks. Each supernatant medium was inoculated with either an adapted or a nonadapted culture, and growth was monitored as described.

**Respiration measurement.** O2 uptake and CO2 evolution of both adapted and nonadapted cell suspensions (final cell concentration of 1 mg [dry weight] per ml) in the presence and absence of 50 μg of ioxynil per ml were measured manometrically with a Warburg respirometer (B. Braun-Melsungen; Bronwill Scientific, Rochester, N.Y.). A 2.5-ml portion of a cell suspension containing either ioxynil (final concentration of 50 μg/ml) or the same amount of acetone was placed in the compartment of a 15-ml Warburg flask. A 0.1-ml portion of a 60 mM glucose solution was placed in the side arm, and 0.2 ml of 20% KOH solution was placed in the central well. A small,
pleated filter paper was also put in the control well for better absorption of CO₂. After equilibration of the flask contents at 30°C, the manometer taps were closed, and glucose was tipped into the main compartment. Manometers were then read at 10-min intervals for 1 h. All procedures described in this section were repeated three times.

RESULTS

Growth. Growth of E. carotovora was inhibited by ioxynil at 25 and 50 μg/ml concentrations (Fig. 1A, curves a and b) and, to a lesser extent, by the acid form of ioxynil (Fig. 1B, curves a and b). Growth was not inhibited by the ester form of ioxynil(Fig. 1A, curves c and d), the amide form of ioxynil (Fig. 1B, curves c and d), or p-hydroxybenzonitrile at 25 and 50 μg/ml (curve not shown). The viable cell count remained constant during the 10-h inhibitory period (Fig. 2); thus, no bacteriocidal effect of ioxynil on E. carotovora was observed.

Adaptation and nature of adaptation. Sequential transfers of a fully grown culture stepwise through GSSM containing 10, 25, 40, and 50 μg of ioxynil per ml produced an adapted culture that would grow readily on GSSIM, but at a slower growth rate than on GSSM (0.3117 mg [dry weight] per h versus 0.3639 mg [dry weight] per h). The nonadapted culture did not grow when transferred to GSSIM. The slower growth rate of the adapted culture continued to be observed after several transfers or subcultures onto GSSIM (Fig. 3, 4, curve b). When the adapted culture was transferred back into GSSM, it resumed the growth characteristic of a normal or nonadapted culture (Fig. 3, 4, curve c). After having grown in GSSM, the adapted culture was inhibited in GSSIM (Fig. 4A, curve a). The adapted culture, when transferred and

![Fig. 1. Growth of E. carotovora (absorbancy at 600 nm) versus time: (A) in the presence of ioxynil (50 μg/ml, curve a; 25 μg/ml, curve b) and the octanoyl ester of ioxynil (50 μg/ml, curve c; 25 μg/ml, curve d); and (B) in the presence of the acid derivative of ioxynil (50 μg/ml, curve a; 25 μg/ml, curve b) and the amide derivative of ioxynil (50 μg/ml, curve c; 25 μg/ml, curve d).](http://aem.asm.org/)

![Fig. 2. Growth of E. carotovora (absorbancy at 600 nm and viable cells per ml) with time in the presence and absence of 50 μg of ioxynil per ml.](http://aem.asm.org/)

![Fig. 3. Nature of adaptation of E. carotovora to ioxynil. Growth of nonadapted culture in GSSIM, curve a; growth of adapted culture in GSSIM, curve b; and growth of adapted culture in GSSM, curve c.](http://aem.asm.org/)

![Fig. 4. Nature of adaptation of E. carotovora to ioxynil. (A) Growth of adapted culture after two transfers in GSSM, curve c; two transfers in GSSM, curve b; and one transfer through GSSM and then into GSSIM, curve c; (B) growth of adapted culture after three transfers in GSSM, curve c; three transfers in GSSM, curve b; and two transfers through GSSM into GSSIM, curve a.](http://aem.asm.org/)
grown in GSSM twice, was inhibited to a greater extent when transferred onto GSSM (Fig. 4B, curve a) and finally responded as a nonadapted culture.

Colonies appeared only from adapted cultures on the nutrient agar withioxynil at 50 μg/ml. Growth characteristics of a culture developed from a colony were the same as the whole culture in the transfer tests (Fig. 3, 4).

Results suggested that a physiological variation, rather than a mutation, occurred in the adapted cell.

Detection of degradation products. Analyses of adapted cultures grown for 1, 2, 3, or 7 days in GSSIM revealed primarily a compound with a retardation factor (Rf) value equivalent to that of ioxynil and a minor component with an Rf value equivalent to that of an ester form (Table 1). The minor component was also found in the control flask. Table 2 shows the recovery and distribution of radioactivity after a 24-h incubation of ioxynil with an adapted culture of E. carotovora. No radioactive CO2 or volatile radioactive organic compounds were found. No radioactive areas were found on TLC plates other than those equivalent to ioxynil and its ester form. The ester form accounted for only a trace amount of radioactivity (less than 2%).

The recovered ioxynil appeared as one spot when co-chromatographed with standard ioxynil. The recovered, purified oxynil from TLC plates remained toxic to the growth of a nonadapted culture. These data suggest that the recovered ioxynil was the same as the parent ioxynil.

Toxicity tests. No growth was observed in any of the culture filtrates of GSSM or GSSIM without added glucose (Table 3). The adapted culture exhibited normal growth in culture filtrates of GSSM with 0.1% glucose or with 50 μg of ioxynil per ml and 0.1% glucose. The nonadapted culture exhibited normal growth after 0.1% glucose was added to the cell-free filtrate of GSSM but was inhibited in GSSIM culture filtrate with added glucose. This indicated that the GSSIM culture filtrate was still toxic to a nonadapted culture.

Respiration measurements. Respiratory activity of the nonadapted culture on GSSM or of the adapted culture on GSSIM was not drastically affected by the addition of 50 μg of ioxynil per ml (Fig. 5). These data represent an average of three experiments with two flasks per treatment. Ioxynil (50 μg/ml) slightly stimulated the respiration of a nonadapted culture (Fig. 5A). Little difference was observed with the adapted culture in the presence or absence of 50 μg of ioxynil per ml, but the adapted culture showed an inhibited respiration rate as compared with the nonadapted culture (Fig. 5B). The respiration rate of the adapted culture in the absence of 50 μg of ioxynil per ml surpassed that of the normal culture; however, a marked reduction in respiration occurred in the presence of 50 μg of ioxynil per ml.

Table 1. Rf values of reference standards and culture extracts

| Compounds                        | Rf (±0.2)* | Standard | Extracted from culture |
|----------------------------------|------------|----------|------------------------|
| Ioxynil                          | 0.70       | 0.70     |                        |
| α-Hydroxybenzonitrile            | 0.71       |          |                        |
| 3,5-Diiodo-4-hydroxybenzamide    | 0.50       |          |                        |
| 3,5-Diiodo-4-hydroxybenzoic acid| 0.60       |          |                        |
| 3,5-Diiodo-4-octanoyloxybenzonitrile| 0.83   | 0.83     |                        |

* Rf was calculated from glass plates coated with silica gel and developed in chloroform, ethanol, and acetic acid (98: 10: 1%, vol/vol/vol).

Table 2. Distribution of 14C activity after 24-h of incubation of adapted culture of E. carotovora with 50 μg of ioxynil per ml

| Treatment       | Location       | Count/ min* | Recovery (%) |
|-----------------|----------------|-------------|--------------|
| Without bacteria (control) | KOH trap       | 36          | 0            |
|                  | Organic trap   | 33          | 0            |
|                  | Culture flask  | 35,357      | 90           |
| With bacteria (experimental)  | KOH trap      | 35          | 0            |
|                  | Organic trap   | 36          | 0            |
|                  | Culture flask  | 33,441      | 87           |

* Total initial 14C activity calculated = 38,720 count/min; background = 30 to 40 count/min.

Table 3. Toxicity test of the culture supernatant fluids from cultures grown for 24 h

| Flask no. | Culture filtrate* | Glucose added (%) | Ioxynil added (μg/ml) | Culture inoculated* | Results |
|-----------|-------------------|-------------------|-----------------------|---------------------|---------|
| 1         | (0)               | 0                 | 0                     | NAC                 | -       |
| 2         | (0)               | 0.1               | 0                     | AC                  | +       |
| 3         | (0)               | 0.1               | 0                     | AC                  | -       |
| 4         | (0)               | 0.1               | 50                    | NAC                 | -       |
| 5         | (0)               | 0.1               | 50                    | AC                  | +       |
| 6         | (50)              | 0                 | 0                     | NAC                 | -       |
| 7         | (50)              | 0                 | 0                     | AC                  | -       |
| 8         | (50)              | 0.1               | 0                     | NAC                 | -       |
| 9         | (50)              | 0.1               | 0                     | AC                  | +       |
| 10        | (50)              | 0.1               | 50                    | AC                  | +       |

* (0), Cell filtrate of normal or nonadapted culture having grown for 24 h (GSSM); (50), cell filtrate of 50 μg/ml of ioxynil-adapted culture having grown for 24 h (GSSIM).

* NAC, Nonadapted culture; AC, adapted culture.

- , No growth; +, growth.
same culture in the presence of 50 μg of ioxynil per ml.

DISCUSSION

The development of resistant cells of *E. carotovora* was a physiological adaptation rather than a selection of preadapted or resistant mutants. This conclusion was based on the following observations: (i) no resistant cells were isolated on the ioxynil agar; (ii) the adapted culture remained adapted only in the presence of ioxynil (GSSIM); (iii) colonies isolated from adapted cultures exhibited the same growth responses as the whole culture; and (iv) ioxynil was bacteriostatic to *E. carotovora*. Results from fluctuation tests support this conclusion; i.e., no resistant colonies developed in the presence of 50 μg of ioxynil per ml and, hence, no difference in variation between sources of inoculations developed (unpublished data).

No ¹⁴CO₂ or volatile ¹⁴C products were detected from cultures in the presence of ring-labeled ioxynil, which suggested that the ring structure of ioxynil remained intact. Analysis of the adapted culture supernatants revealed no degradation products. The extracted and purified ioxynil co-chromatographed exactly with ioxynil and inhibited the growth of a nonadapted culture. Comparison of growth responses with p-hydroxybenzonitrile indicated that deiodination had not occurred. A majority of the initial radioactivity was recovered, which also suggested that most of the ioxynil remained unchanged in the culture medium. Toxicity tests of the adapted culture cell-free supernatant fluid indicated that the ioxynil remaining in the medium was still toxic to a nonadapted culture. Thus, degradation or detoxification of ioxynil was not involved in the adaptation mechanism reported herein. Previous studies have demonstrated degradation of ioxynil to its amide and acid form in soils (12).

The question remains: how could an inhibited cell start to grow in an environment where the toxic stress is still present? Factors other than induced degradative enzymes must be considered. Ioxynil slightly stimulated the respiration rate of a nonadapted culture of *E. carotovora*. Adaptation of *E. carotovora* resulted in a lengthened lag phase, a decreased growth rate, and very few adverse effects on the total population. These effects are similar to those elicited by sulphonamide to *Aerobacter aerogenes*, which was explained by the mechanism of an "alternative growth mechanism" (6). A similar mechanism seems to be involved in the adaptation mechanism reported herein.

Ioxynil slightly stimulated the respiration rate of a nonadapted culture of *E. carotovora*, as measured by O₂ uptake and CO₂ evolution. This suggests that ioxynil might also uncouple oxidative phosphorylation in *E. carotovora*. Ioxynil acts as a phosphorylation uncoupler in certain plant tissues (10, 11). Little difference was observed with the adapted culture of *E. carotovora* in the presence and absence of ioxynil, but adapted cultures showed an inhibited respiration rate as compared with nonadapted cultures. This phenomenon could be explained by an alternate growth mechanism in which some part of the respiratory chain had been inhibited resulting in a functional change and a reduced rate of electron flow (a less efficient electron transport). That the respiration rate of an adapted culture in the absence of ioxynil surpassed that of the culture still in the presence of ioxynil implies a competition of two alternate routes of electron transport. These data support the conclusion that an alternate growth mechanism may be involved in the adaptation phenomenon.

Recently, Cullimore (5) presented a theoretical consideration of some of the possible interactions between herbicides and soil microbial population. Our results may expand one of his categories; i.e., a herbicide is nondegradable and inhibitory, but not lethal, to some fraction of the population, thus reducing its size. After this reduction in cell numbers, there would be a gradual recovery of the resistant cells by alternate growth mechanisms. In the end, when the population has stabilized, it would be composed entirely of organisms tolerant to the herbicide. The herbicide may be decreased by a variety of
ways; however, the resistant cells may revert to normal cells after toxic stresses are released. Because microorganisms possess tremendous adaptation ability by alternate growth mechanisms (6), conceivably this fraction of microorganisms might be quite widespread in the soil and constitute an essential fraction of the population. Reviews (1, 2, 7–9) indicate that most herbicides used at recommended field rates do not significantly change soil microbial populations. In many cases, this is probably because some microorganisms are able to decompose the herbicide. More probably, alternate growth mechanisms maintain the genetic pool of the population, some of the organisms would wait until detoxification to resume growth, and some may start to grow even in the toxic environment by alternate routes.

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