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Stimulation of Autotrophic Ammonium Oxidation in Rice Rhizosphere Soil by the Insecticide Carbofuran

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The application of the insecticide carbofuran (technical or formulated) to rice rhizosphere soil suspensions at 10 and 100 ppm (μg/ml) of active ingredient distinctly stimulated the autotrophic oxidation of ammonium. Evidence suggested that *Nitrosomonas* sp. was enriched in the presence of carbofuran. Formulated carbofuran (Furadan 3G) exhibited a more pronounced stimulation of ammonium oxidation than that exhibited by technical-grade (99.5%) carbofuran, a result which was attributed to the $CaCO_3$ present in the formulation.

Nitrification is the biological conversion of nitrogen in either inorganic or organic compounds from a reduced state to a more oxidized state (7). A flooded rice field, although predominantly anaerobic, supports aerobic transformations such as nitrification in the thin surface layer and rice rhizosphere, primarily because of the unique ability of the rice plant to transport oxygen from the foliage to the root region (1, 15). But in an ecosystem with aerobic and anaerobic interface in close proximity, as in a wetland rice field, considerable loss of nitrogen in gaseous forms (N_2 and N_2O) can occur through a nitrification-denitrification sequence (5, 18). In a two-step autotrophic nitrification process, ammonium is sequentially oxidized to nitrite by *Nitrosomonas* sp. and related bacteria and then is oxidized to nitrate by *Nitrobacter* sp. and related bacteria. The autotrophic bacteria are particularly sensitive to the organics and other environmental and ecological factors (7). Contrary to the inhibitory or innocuous effects of a myriad of pesticides on autotrophic nitrification (12, 17), the present study reports a clear stimulation of autotrophic ammonium oxidation in a suspension of rice rhizosphere soil by the insecticide carbofuran.

MATERIALS AND METHODS

Rice rhizosphere soil suspension. Rice plants (Kalinga II) at flowering stage were uprooted from a wetland rice field (from the Experimental Farm of Central Rice Research Institute, Cuttack, India), and the roots were carefully washed with water to remove the large soil aggregates. The soil still adhering to the roots after washing (rhizosphere soil) was suspended (26) by shaking the roots in sterile distilled water, and the suspension was used as the rhizosphere soil suspension in all of the studies.

Effect of pesticides on nitrification. A rhizosphere soil suspension (100 ml) contained in 500-ml Erlenmeyer flasks was treated with ammonium sulfate (10 mg) alone or in combination with commercial formulations (see Table 1) of either carbofuran, carbaryl, hexachlorocyclohexane (HCH), or benomyl at active ingredient concentrations of 10 and 100 ppm (μg/ml). After being incubated for 40 days at room temperature, triplicate samples were filtered, and the ammonium, nitrite, and nitrate in the clear filtrate were assayed. The dry weight of the soil present in the suspension was also determined.

In another experiment, the rhizosphere soil suspension (100 ml) was treated with technical (FMC Corp., Middleport, N.Y.) or formulated carbofuran (0 or 100 ppm of active ingredient) plus ammonium sulfate (10 mg) as in the above experiments. Carbofuran was added directly to the suspension. To determine autotrophic versus heterotrophic nitrification, we treated another set of the rhizosphere soil suspensions, with and without carbofuran, with a filter-sterilized (Millipore filter, 0.45 μm) aqueous solution (10 ppm) of nitrapyrin (Dow Chemical Co., Midland, Mich.), a specific inhibitor of autotrophic nitrification (23). After 30 days of incubation, the remaining ammonium and the nitrate formed in the suspension were analyzed.

To find out whether the stimulation of ammonium oxidation by formulated carbofuran was due to $CaCO_3$ present in the formulation we first measured the amount of $CaCO_3$ in the formulation by an indirect method of reacting 1 g of Furadan with 10 ml of 0.1 N HCl. The acid remaining after the reaction was back titrated with 0.1 N NaOH. The amount of $CaCO_3$, derived from the formula of 1 ml of 0.1 N HCl = 0.1 meq of $CaCO_3$ (13), in the formulated carbofuran was 3.65%. Subsequently, to test the effect of added $CaCO_3$, 100-ml portions of rhizosphere soil suspension supplemented with ammonium sulfate were amended with $CaCO_3$ at 12 and 120 ppm, equivalent to the level of $CaCO_3$ present in 10 and 100 ppm of active ingredient in formulated carbofuran. Likewise, nitrification in a suspension which was similarly treated with 10 and 100 ppm (active ingredient) of formulated carbofuran, but without $CaCO_3$, was examined simultaneously. The ammonium, nitrite, and nitrate in all of the samples were assayed after 30 days of incubation at room temperature.

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TABLE 1. Effect of commercial formulations of selected rice pesticides on nitrification by the rice rhizosphere soil suspension[a]

Treatment	Concn (ppm)	Concn in rhizosphere soil at 40 days (mg of N/g)[b]		
		NH_4^+-N	NO_2^--N	NO_3^--N
Control		6.6 ± 0.70	0	1.4 ± 0.04
Carbofuran	10	2.6 ± 0.40**	0	5.9 ± 0.60**
(Furadan, 3 G)[c]	100	0.7 ± 0.02**	4.4 ± 0.30***	2.5 ± 0.30*
Carbaryl	10	6.9 ± 0.63	0	0.9 ± 0.07***
(Sevin, 50 WP)[d]	100	7.5 ± 0.43	0	0.3 ± 0.04***
HCH	10	7.4 ± 0.74	0	0.2 ± 0.02***
(Hexamar, 5 G)[e]	100	8.4 ± 0.22	0	0.1 ± 0.02***
Benomyl	10	7.4 ± 0.74	0	0.8 ± 0.04***
(Benlate, 50 WP)[f]	100	7.8 ± 0.82	0	0.2 ± 0.01***

[a] Results are means ± the standard error of three replicate samples.
[b] Difference from control is significant at $P = 0.05$,* $P = 0.01$,** and $P = 0.001$*** by the Student t test.
[c] Rallis (India) Ltd., Bombay, India. G, Granules.
[d] Union Carbide (India) Ltd., Bombay, India. WP, Wettable powder.
[e] Bharat Pulverizing Co., Bhopal, India. G, Granules.
[f] E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. WP, Wettable powder.

Stimulation of autotrophic ammonium oxidation by carbofuran. A specific mineral salts medium (20) for ammonium-oxidizing autotrophs (*Nitrosomonas* medium) was supplemented with 0 or 100 ppm of technical carbofuran. Fifty-milliliter portions of medium, both carbofuran amended and unamended, was inoculated with 1 ml of rhizosphere soil suspension that had been previously exposed for 40 days to formulated carbofuran (0 or 100 ppm) plus ammonium sulfate. After 20 days of incubation, the nitrite formed in the medium was estimated directly. The medium was also assayed for the amount of unoxidized ammonium remaining and the nitrate, if any.

Analyses. In the experiments with rhizosphere soil suspension, the ammonium, nitrite, and nitrate were analyzed directly after filtering the suspension; whereas in *Nitrosomonas* medium they were assayed directly. Ammonium was analyzed by nesslerization (13), nitrite was analyzed by diazotization (2), and nitrate was analyzed by a phenol disulfonic acid method (4). The variation within triplicate estimations of each sample in all of the experiments was always less than 5%.

RESULTS AND DISCUSSION

The addition of formulated HCH, carbaryl, and benomyl to a rice rhizosphere soil suspension inhibited the oxidation of ammonium to some extent at 10 ppm and inhibited oxidation almost completely at 100 ppm of active ingredient (Table 1); in contrast, formulated carbofuran accelerated the oxidation of ammonium by fivefold over that of the untreated control. Moreover, the oxidation of ammonium increased with the concentration of carbofuran, with concomitant formation of (essentially) nitrate at 10 ppm and nitrite plus nitrate at 100 ppm. The accumulation of nitrite at 100 ppm of carbofuran is attributed to the inhibitory action of the insecticide on autotrophic nitrite oxidation at higher concentrations (20). Technical carbofuran also stimulated nitrification, but to a lesser extent than formulated carbofuran did (Table 2). Nitrification in the rhizosphere soil suspension with and without carbofuran was due to autotrophs, because no oxidation occurred in the presence of 10 ppm of nitrapyrin, a selective inhibitor of ammonium-oxidizing autotrophs (23). There are reports of inhibition of nitrification by HCH (8, 21), carbaryl (3), and benomyl (9, 19); but a striking stimulation of nitrification by a pesticide, as noticed with carbofuran in this study, is probably new to the literature.

TABLE 2. Nitrification in rice rhizosphere soil suspension amended with formulated (Furadan) or technical carbofuran in combination with nitrapyrin[a]

Formulation of carbofuran	Concn (ppm)	Concn in rhizosphere soil at 30 days (mg of N/g)[b]		
		NH_4^+-N	NO_2^--N	NO_3^--N
Control		7.2 ± 0.8	0	0.9 ± 0.05
Furadan	10	4.0 ± 0.7*	0	2.1 ± 0.09***
Furadan + nitrapyrin	10 + 10	8.3 ± 0.6**	0	0
Technical carbofuran	10	4.8 ± 0.3*	0	1.5 ± 0.17*
Technical carbofuran + nitrapyrin	10 + 10	8.6 ± 0.5**	0	0

[a] Results are means ± the standard error of three replicate samples.
[b] Difference from control is significant at $P = 0.05$,* $P = 0.01$,** and $P = 0.001$*** by the Student t test.

TABLE 3. Effect of $CaCO_3$ alone or in combination with technical carbofuran as compared with that of formulated carbofuran (Furadan) on nitrification in rice rhizosphere soil suspension[a]

Treatment	Concn (ppm)	Concn in rhizosphere soil at 30 days (mg of N/g)[b]		
		NH_4^+-N	NO_2^--N	NO_3^--N
Control		6.9 ± 0.4	0	1.2 ± 0.04
Furadan	10	4.3 ± 0.5*	0	3.0 ± 0.06***
	100	0.9 ± 0.1***	5.1 ± 0.33***	1.8 ± 0.03***
$CaCO_3$	12[c]	6.6 ± 0.5	0	2.0 ± 0.09**
	120[d]	3.8 ± 0.4**	0	3.2 ± 0.10***
Technical carbofuran	10	6.2 ± 0.8	0	2.2 ± 0.03***
(99.5%)	100	6.5 ± 0.6	0	1.9 ± 0.01***
$CaCO_3$ + technical carbofuran	12 + 10	4.5 ± 0.2**	0	3.2 ± 0.08***
$CaCO_3$ + technical carbofuran	120 + 100	3.9 ± 0.6*	3.3 ± 0.24***	0.6 ± 0.01***

[a] Results are means ± the standard error of three replicate samples.

[b] Difference from control is significant at $P = 0.05$,* $P = 0.01$,** and $P = 0.001$*** by the Student t test.

[c] Equivalent to the amount of $CaCO_3$ present in 10 ppm of formulated carbofuran (Furadan).

[d] Equivalent to the amount of $CaCO_3$ present in 100 ppm of formulated carbofuran (Furadan).

Besides containing conventional inert filler materials, commercial formulations of certain pesticides may contain small amounts of $CaCO_3$ (22). We found that the formulated carbofuran (Furadan 3G) contained 3.65% $CaCO_3$. There is evidence in support of stimulation of nitrification by $CaCO_3$ added to acid soils (11); thus, the effect of $CaCO_3$ (equivalent to the amount present in the formulation) alone or in combination with technical carbofuran was examined. Nitrification, in terms of nitrate formed, proceeded more rapidly in samples amended with $CaCO_3$ than in unamended samples (Table 3); but the increase in the nitrate or nitrite plus nitrate formed was always greater in samples amended with formulated carbofuran. Nitrite accumulated when technical carbofuran (100 ppm) and $CaCO_3$ (120 ppm) were applied in combination (not singly), but to a lesser extent than when formulated carbofuran was applied. It was interesting that nitrification rates with formulated carbofuran alone and $CaCO_3$ plus technical carbofuran were almost comparable. Thus, the more pronounced ammonium oxidation with formulated rather than technical carbofuran is indeed due to the $CaCO_3$ present in the formulation.

The rhizosphere soil suspension previously exposed to the insecticide showed a remarkable ability to oxidize ammonium in *Nitrosomonas* medium. The amount of nitrite formed from the ammonium at 20 days was only 0.01 μg of NO_2-N per ml in the medium receiving an inoculum of rhizosphere soil suspension previously not exposed to carbofuran. In contrast, significant oxidation of ammonium to nitrite occurred in both carbofuran-amended (145 μg of NO_2-N per ml) and unamended (94 μg of NO_2-N per ml) *Nitrosomonas* medium receiving the inoculum previously exposed to 100 ppm of formulated carbofuran; the inoculum previously exposed to formulated carbofuran was more effective in oxidizing the ammonium than the inoculum previously exposed to technical carbofuran was.

A pure ammonium-oxidizing autotrophic bacterial culture was isolated from the *Nitrosomonas* medium inoculated with rhizosphere soil suspension previously exposed to 100 ppm of formulated carbofuran. Electron micrographs of the ammonium-oxidizing autotroph showed typical pairs of stout, short, and nonflagellate rods (about 1 μm long and 0.75 μm wide). These rods were not pointed at one end as are rods of *Nitrosomonas europaea* (25). Thus, the ammonium-oxidizing autotrophic bacterium present in *Nitrosomonas* medium showing high nitrification activity was certainly a *Nitrosomonas* sp., but probably not *N. europaea*.

Although carbofuran distinctly stimulated the autotrophic oxidation of ammonium in rhizosphere soil suspension, this stimulation was not always expressed; for instance, it was not always expressed in the presence of other pesticides. In fact, according to our earlier study (21), carbofuran when applied to a soil in combination with HCH or with benomyl synergistically increased the inhibitory effect of HCH or of benomyl on nitrification. Such interactions between pesticides in a combination are not uncommon.

Stimulation of a strict and sensitive chemoautotroph *Nitrosomonas* sp. in the rhizosphere soil by the pesticide carbofuran in the present study is probably unknown in the literature. There are reports of significant stimulation of autotrophic nitrification by heterotrophs in a mixed culture (6, 10, 24). It is interesting that nitrification by an estuarine *Nitrosomonas* sp. increased by 150 and 50% in the presence of the heterotrophs

Nocardia atlantica and *Pseudomonas* sp., respectively (14), although the heterotrophs were not capable of oxidizing ammonium in pure cultures. Whether the stimulation of ammonium oxidation in carbofuran-supplemented rhizosphere soil suspension is due to such interaction is not clear. Our findings may have some ecological significance in terms of the overall loss of nitrogen by denitrification and possible emission of N_2O, a pollutant of great concern (16), particularly in areas of intensive use of ammoniacal fertilizers and carbofuran and possibly related pesticides. The population of *Nitrosomonas* sp. in most of the soils is never high, and consequently, isolation of this autotroph is accomplished with great difficulty. Based on our observations, selective enrichment of a *Nitrosomonas* sp. in the soil upon the addition of carbofuran may assist in the isolation of this difficult, but important, group of organisms with greater ease than hitherto possible.

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